Signaal transductie in celdood: Nieuwe inzichten door het gebruik van in de natuur voorkomende verbindingen en specifieke remmers

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Contents

Preface

It took plain old courage to be a chemotherapist in the 1960s and certainly the courage of the conviction that cancer would eventually succumb to drugs.

Vincent De Vita, National Cancer Institute investigator

Effective response to treatment is still a challenge in the clinical management of many types of tumors. As result of pharmacological intervention two different fates are observed for a cancer cell: survival or death. These two cell fates are intimately related to the overall response to treatment. Survival of a cancer cell to an aggressive treatment usually means inefficacy of a determined drug and cell death means that the cells were sensitive to the treatment. In the current literature many types of cell death are suggested. Of equal importance is the understanding of the mechanisms and signal cascades of survival. Crosstalk between multiple signal transduction pathways may organize an intricate survival network with overlapping functions that together provide strong survival signals to a dying cell. Importantly, these survival signals seem to be on the background of the classic mechanism of drug resistance which holds true for a lower efficacy of determined class of molecules or structurally unrelated ones, such as drug efflux, drug uptake, drug metabolism, DNA repair and impaired drug binding to its target. Importantly, different types of tumors present different aspects of surviving, as an example the building up of a drug resistant phenotype in hematological diseases is usually a long term process, resulting in an initial response to treatment. However, relapses occur after sometime, and not always remission is achieved with the same therapeutic strategy. The development or acquisition of drug resistance is a quite remarkable aspect of hematological malignancies. On another hand, this first response to pharmacological strategies is not really achieved in tumors such as, pancreatic cancer (PDAC). Less than 10% of patients treated with gemcitabine (first line of treatment for PDAC) present an objective response to this treatment. Many aspects of PDAC, such as poor vascularization, fibrosis and the upregulation of many important survival signal transduction pathways in these tumors, favor a development of an intrinsically resistant phenotype.

Chemoresistance and serious adverse effects related to 'conventional' chemotherapeutic drugs remain important issues. Even though it is fair to say that the combination of different classes of drugs became standard in clinical practice once combination of the so called 'cancer poisons' were very effective to treat acute childhood leukemia and advanced Hodgkin's disease in the 1960s and early 1970s. This fact overcame the prevailing pessimism about the ability of drugs to cure advanced cancers, and facilitated the use of these drugs as adjuvant therapy to improve the results of surgery/or radiation treatment. It is important to mention that 'conventional chemotherapy' is still first line in the treatment of most part of tumoral diseases and often used in combination with the recently developed targeted therapies.

However, these two important issues cited above combined with a better molecular understanding of the functioning of cancer cells prompted the search for the 'magic bullet' (or perhaps 'the magic target'): drugs, which combined, could potentially exhibit higher efficacy and less adverse effects; thus called 'targeted therapy'. Among these drugs, the most successful are the Abl inhibitors, this group of molecules changed the way of treating chronic myeloid leukemia. However, the high expectations with other groups of targeted therapies were not yet confirmed. Besides that these therapies are not free of adverse effects. As a result of this toxicity many of these molecules are usually used as single treatment. In addition, several publications have reported mechanisms of resistance to targeted therapies. Therefore, issues already existent remain to be solved also with the novel and 'cleaner drugs'.

Nevertheless, some important aspects must be taken into account in regard to targeted therapies. Firstly in the same tumors many signal transduction pathways are responsible for proliferation, survival and resistance to cell death. Thus, targeting a single molecule will not result in an effective response. Therefore, the right doses and combinations must be defined or these inhibitors must be improved at the molecular level in order to create molecules with a broader spectrum of targets. Suggesting that in this case the 'dirty drugs' may be a favorable approach. Importantly, aiming a certain target or group of targets should have broad consequences to the cancer cells. Secondly, the patients who can benefit from a specific targeted strategy must be clearly defined. Further understanding on which pathways must be targeted and a deeper molecular knowledge of how they crosstalk and overlap is needed. Furthermore, it is also important to establish whether an efficiently aimed target could really impact the functioning of cancer cells in such way that real clinical benefits are observed.

In this thesis, we focused on the study of cellular signaling in response to different pharmacological tools (specific and unspecific). We also generated comprehensive descriptions of different signal transduction pathways which play a role in the maintenance of the drug-resistant phenotype. In addition, some of the components of these signaling pathways were challenged and their effect on cell fate was determined.

Chapter 1

Insights in Hedgehog Signalling and its Pharmacological Manipulation

Karla C.S. Queiroz

Hedgehog Signalling Pathway

Together, the Hedgehog pathway, the Wnt pathway, the tyrosine kinase receptors and TGFβ signaling form a conserved morphogenetic coding system essentially conserved between the fruit fly and humans. It is fair to say that the hedgehog pathway remains the least well understood of these constituents of the morphogenetic code. The upstream activators of this pathway are the Hedgehog proteins (Hh), and at least three different isoforms have been described, Sonic, Indian and Dessert [1]. Sonic Hedgehog (Shh) is the most widely expressed during embryogenesis and Shh deficiency is embryonically lethal in mice. Indian Hedgehog (Ihh) is less widely expressed and Ihh-deficient mice survive up late gestation with skeletal and gut defects. Dessert Hedghog (Dhh) is expressed in peripheral nerves, male gonads and the endothelium of large vessels. Dhh-deficient mice are viable but present peripheral-nerve and male-fertility defects [3].

Several players are responsible to carry out Hhs' messages. As these proteins delivery their message on cell surface, it is clear the need of a receptor system to this pathway, but the Hedgehog signal receiving system is unusual. Two important players are on the cell surface, Patched (Ptc) and Smoothened (Smo) and both are transmembrane receptors. Here, Ptc plays a role as a negative regulator of Smo. Plausibly Ptc is an efflux pump, mediating excretion of molecules with the ability to inhibit Smo [1,4]. Khaliullina et al 2009, has shown that *Drosophila* Ptc recruits internalized lipoproteins to Ptc-positive endosomes an that its sterol-sensing domain regulates trafficking of both lipids and Smo from this compartment. Ptc seems to use lips derived form lipoproteins to destabilize Smo o the basolateral membrane. Thus, Smo degration is regulated by Ptc, this protein changes the lipid composition of endosomes through which Smo passes, and the presence of Hh on lipoproteins inhibits utilization of their lipids by Ptc. These Smo inhibitory molecules are almost certainly β-hydroxisteroids [5,6]. The pathway is activated when Hedgehog binds to Ptc and thus releases Smo from repression by Ptc. In this way, this seven-span transmenbrane protein is now able to transmit the Hedgehog signal into the cellular cytoplasm [7,8]. Interaction of Hedgehog with Ptc seems to require other cofactors, like plasma membrane heparan sulfates [6]. After de-repression Smo becomes multiply phosphorylated, which changes its cytoplasmic tail conformation and promotes dimerization. Smo acts through a complex constituted by kinesin-like protein Costal (Cos2), the Ser/Thr kinase Fused (Fu) and the transcription factor Cubitus interuptus (Ci, in Drosophilla) or Gli family (vertebrates) [9]. This culminates with Ci/Gli stabilization and its translocation to the nucleus, where Gli transcription factor modulate Hh target gene expression [10]. In the absence of Hh, Ptc seems to control the Smo levels at the plasma membrane. There is evidence that Smo cycles between the plasma membrane and an intracellular compartment, suggesting tha Ptc might prevent Smo accumulation by inducing Smo re-internalization and reducing the ability of Smo return to the plasma membrane or even by increasing its degradation. In this context it is important to note that the mechanism that allow specific Smo degradation but at the same time allowing Cos2 and Fu (which are physically associated to Smo) to escape such degradation it is not clear [10]. Although, this is also observed in other pathways, such as during NFκB activation [11]. In this situation, Cos2 exerts a crucial negative role on the Hh pathway, in absence of Hh this protein recruits Protein Kinase A (PKA), Casein Kinase I (CKI) and Glycogen Synthase Kinase 3 (GSK3) to hyperphosphorylate

Ci/Gli generating a recognition site for ubiquitin ligase complex and driving this protein to proteasomal degradation. As end product of proteasomal degradation the full-length of the transcriptional factor is converted in a shorter transcriptional repressor. Together with Cos2, the serine-threonine kinase fused (Fu) and the suppressor of fused (Sufu) regulate the status of Ci/Gli. Fu is a positive regulator of Hh signalling in Drosophilla, as in the absence of Hh protein this kinase reduces pathway activation [12]. Fu does not share any homology with other known kinase subfamilies and seems to function specifically in the Hh signalling pathway [10]. This kinase associates with Cos2 in a stoichiometric manner, and can also interact directly with Smo and Sufu. The phosphorylation of Cos2 and Sufu in presence of Hh protein is dependent on Fu kinase activation, however the direct action of this kinase on Cos2 and Sufu has not been shown yet. Sufu is a negative regulator of Hh pathway with a proposed role in nuclear-cytoplasmic shuttling othe Ci/Gli transcription factors [13]. Some studies have pointed human Sufu as a tumour suppressor gene based on a screen of 46 medulloblastoma tumours. Although, in another screen in 145 primitive neuroectodermal tumours, including 134 medulloblastoma and 11 medulloblastoma –derived cell lines, no mutations in Sufu were found [14], indicating that genetic alteration in this gene is a rare event in human medulloblastoma. Svärd et al, 2006 [15] have shown an important central regulatory role of Sufu in mammalian Hh signalling pathway by using gene targeting in mouse, a striking difference compared to Drosophilla. Interestingly, in this study was shown that Sufu knockouts are embryonic lethal and show strong similarities with Ptc-1 knockouts, and that Hh pathway was strongly activated in a ligand-independent manner in Sufu-/- cells. Although, important insight into the molecular details of Hh signalling has been gained, it still fair to say that many of the details remain obscure at best. For instance, Ser/Thr phosphatases have been proposed as potential important players in this intriguing network, but their actual function remains unknown. Therefore, the characterization of the communication among Hh signalling pathway components and the functionality of phosphorylation sites on them are important molecular aspects urgently need a better understanding. The current advent of peptide array technology may provide important answers here.

Synthesis, Secretion and Transport of Hedgehog proteins

Hhs are lipid-modified proteins, that are firstly made as a 45kDa precursor molecule, consisting of a C-terminal protease domain and an N-terminal signalling unit. The C-terminal endoprotease of Hh cleaves the precursor in an autocatalytic way to release the active signalling domain. After that, the C-terminal is covalentely modified by the addition of a cholesterol molecule. Finally, after cleavage the Hh is palmitoylated generating a 19kDa protein. Pepinsky et al, 1998 [16] has shown that in mammalian forms of Hhs lacking the cholesterol modification, the palmitoylation step is much less efficient. The function of the modification was investigated, and the lipid modification seemed to be important to localize the protein in specific places on the cell surface. Among the evidence supporting this observation is the fact that high concentrations of Hh protein overcome the lack of palmitoylation. In addition, the cysteine that is subject to this acylation on the Hh molecule can be substituted with various hydrophobic amino acids, and still retain some activity [17]. Considering the hydrophobic character of Hh proteins, the existence of a transporter system

was expected. Intracellular transport of Hh proteins are secreted by a 12 transmembrane domains protein called Dispatched [18]. This protein as well as Ptc are related to the resistance –nodulation division (RND) family of bacterial proton-driven pumps. This family of proteins uses a proton gradient to transport small lipophilic molecules across the lipidic bilayer. RND proteins present a signature domain, called sterol-sensing domain, also found in other proteins involved in sterol metabolism [6]. Different aspects are regulated by this domain in response to the sterol levels, including stability of proteins and alteration of membrane trafficking of transcription factors interfering with gene expression. Whether Hhs' secretion by dispatched is also dependent or regulated by sterol levels remains an open question.

Due the lipidic modification Hhs present many peculiarities and this protein can be found in cell culture supernatant forming complexe multimers having molecular weights between 158 and 4,000 kDa. Monomer-sized complexes do not signal as efficiently as multimeric complexes. The formation of the high molecular-weight multimer complexes depends on the lipid modification. Whether other proteins are in these hydrophobic Hh aggregates is not known [6].

Considering the Hhs secretion and the formation of high molecular-weight multimers many questions can still only be partially answered: How are they transported? How far can they travel? Some light on Hh transport was given by the study of the Eaton's group who showed that in Drosophilla, Hh protein is carried by lipoprotein particles (called lipophorins) present in this organism [19]. Whether the same can be applied for the mammalian taxon is not clear yet. In Drosophilla melanogaster this system seems highly relevant as the lipophorin appears necessary for facilitating a generation of Hh gradient through the tissue (imaginal disc) [19]. Recently, we have demonstrated the presence of Ihh in VLDL particles [20].

Another particularity of Hh signalling is that the competent protein is able to interact with heparan sulfate proteogycans (HSPGs), and the importance of this close relationship seems to be reflected in function as well. Lipid-modified Hh does not signal in tissue that can not synthesise heparan sulfate [21,22]. This interaction may also be involved in determining tissue affinity for Hhs. In this context it is interesting to note that the presence of heparan sulfate proteoglycans also favours the interaction of lipoproteins with their receptors, thus it is well possible that during evolution Hh carried by lipoproteins has taken advantage of a pre-existent mechanism to send its message to target cells. The Hh pathway clearly present an usual high level of molecular complexity. Furthermore, morphogenetic signals show a high level of mutual interaction (both positive and negative) adding further complexity to efforts directed at understanding the intricacies of Hh signal in the organism. However, uncovering of Hh's molecular secrets might permit us an efficient manipulation in a pharmacological level of this pathway for clinical purposes.

Functions of Hedgehog signalling

The hedgehog family of proteins are powerful morphogens mediating embryonic development. To this end, the Hh pathway orchestrates cell proliferation and differentiation in a diversity of patterning events (the Hh pathway regulates events ranging from embryonic segmentation and appendage development in insects, to neural tube differentiation in

vertebrates [1,2,22,23]. However, the Hh pathway assumes important morphogenetic postnatal roles as well. Important examples are tissue homeostasis, repair of damaged tissue and tumorigenesis. In this manner, the lack and the constitutive activation of Hh pathway brings serious pathological consequences; especially notable are its role played in human cancers, such as leukaemias, pancreas, prostate and colon cancer [1]. Also, activation of the Hh pathway seems to be related also with important aspects in cancer biology associated with treatment failure, including metastasis, angiogenesis and acquisition of resistance to chemotherapy. Therefore, inhibition of this pathway is often proposed as an interesting therapeutical approach to treat the cancerous process.

In addition, several groups have shown the role played by the Hh signals in the cardiovascular pathology. Pola and co-workers [2] showed that Shh is an indirect angiogenic agent able to up-regulate the expression of two different families of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and the angiopoetins (Ang). Shh also induced robust neovascularization of ischemic hind-limbs in aged mice. This group suggested that this morphogen has a potential therapeutic use in ischemic disorders [2]. Acordingly, several studies have demonstrated that activation of Hh signaling protects from ischemic damage and leads to functional improvements in different tissues. Interestingly, an Hh small molecule agonist decreased infarct size by 40% to 50% leading to improved behavior and body weight in a middle cerebral artery occlusion model [24]. In addition, it has been shown that treatment of diabetic mice with recombinant Shh protein not only promoted vascular growth in the skin and peripheral nervous tissue but also resulted in accelerated wound healing and improved peripheral nerve conduction velocity, two processes that are defective at baseline in diabetic mouse models [25,26]. These data implicate the Hh signaling pathway as na interesting target for therapeutic intervention to minimize ischemic damage and improve tissue function in conjunction with both macrovascular and microvascular disease. Considering the fact that the two most studied potential proangiogenic factors fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) have not performed adequately in the clinic. Shh appears as a novel candidate for therapeutic revascularization [27].

Hh signaling is also required for adult cardiac homeostasis and function, the acute removal of this signalling from the adult mouse heart resulted in loss of coronary vasculature, this event promoted tissue hypoxia, cardiomyocyte apoptosis, ventricular failure and lethality. Hence, Hh signalling seems important for maintaining proangiogenic signals essential to promote survival of the coronary vasculature. In addition, Hh signalling has been shown to play a role in the regulation of the cholesterol levels. Treatment of mice with the Hh-neutralising antibody 5E1 induced decreases in the plasma cholesterol levels, although increased atherosclerotic plaques were observed in this animals as well [28]. Nevertheless, the exact promise of therapeutic modulation of Hh signalling in dealing with cardiovascular disease remains open to debate [29].

Pharmacological Manipulation of Hegdehog Pathway

Viewing the wide array of diseases in which Hh signalling is involved, it can not come unexpected to the reader that pharmacological manipulation of Hh pathway activity is widely

pursued. Although the molecular basis and physiological importance of Hh pathway are not completely understood, some potential pharmacological tools have come forward. The most used experimental ways to inhibit Hh signaling are the neutralising anti-Hh 5E1 antibody and the plant steroidal alkaloid cyclopamine, a Smo inhibitor. Aditionally, inhibitors able to target different steps of the pathway have been developed. Together to the neutralising anti-Hh 5E1 antibody, robotnikinin is also able to neutralize Hh protein [30]. Similarly to cyclopamine the endogenous sterol derivative, Vitamin D3, has been described as negative regulator of Hh pathway [5]. In the same study, C3H/10T1/2 fibroblast supernatant in which Ptc expression was increased by transfection, also produced an inhibitory effect on Hh signalling. The production of this inhibitor was prevented by pravastatin and rescued by mevalonate, suggesting that this compound is also a sterol derivative [5]. In addition, Smo inhibitors such as GDC-0449, IPI-926, PF-04449913 and BMS-833923 are also used in clinical trials and their efficacy has been demonstrated in specific tumoral diseases, as an example GDC-0449 which successfully treated basal cell carcinoma and medulloblastoma. Although, Smo variants resistant to this inhibitor has been also described [31-33]. Another interesting target to be aimed in this pathway is the Gli family of transcription factors. Therefore, a few Gli inhibitors, such as Gant58, Gant61 and physallin have been developed and they have presented quite interesting results in preclinical studies [34,35]. Conversely, several different structural sterol-related molecules have been proposed to stimulate Hh signalling. Particularly, oxysterols regulate positively Hh signaling. Molecules, such as Colestherol, 20-, 22-, 24-, 25- hydroxycolestherol, and a small synthetic molecule SAG (Smoothened agonist), and purmorphamine apparently interfere positively with the activity of the Hh pathway [6, 36]. Although studies have suggested that these molecules are able to inhibit Ptc, it is still unexplained why these compounds also increase signalling in Patched-deficient cells. In this way, substantial gaps remain in the understanding about the mechanism of action of these molecules, hampering clinical application.

Summarizing, there is consensus that the Hh pathway is an interesting target to treat a variety of human disorders. These proteins are important in many different physiological processes especially for the maintenance and functioning of the cardiovascular system and in cancerous processes. With respect to the latter, targeting of this pathway might cause some cardiovascular side effects [27]. Development of therapeutic modulation of the Hh pathway awaits further understanding of the details regarding its mode of action in human physiology.

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Chapter 2

Hedgehog signaling maintains chemoresistance in myeloid leukemic cells

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Abstract

The development of resistance against chemotherapy remains one of the major challenges in the clinical management of leukemic disease. There is still limited insight into the molecular mechanisms that maintains the chemotherapy-resistant phenotype, despite the obvious clinical relevance that such knowledge would have. Here we show that the chemotherapy resistant phenotype of myeloid leukemia cells correlates with activation of the Hedgehog pathway, whereas in chemo-sensitive cells such activation is less pronounced. Importantly, the over-expression of Hh pathway components induces chemoprotection and inhibition of the pathway reverts chemoresistance of Lucena-1 cells, apparently by interfering with P-glycoprotein dependent drug resistance. Our data thus identify the Hh pathway as an essential component of multidrug resistance (MDR) myeloid leukemia and suggest that targeting the Hh pathway might be an interesting therapeutic avenue for overcoming MDR resistance in myeloid leukemia.

Introduction

Intrinsic and acquired multi-drug resistance (MDR) against chemotherapy remains a major challenge in the management of cancer in general and leukemia in particular. Several potential molecular/cellular mechanisms responsible for MDR have been elucidated. Alterations in DNA repair, defective regulation of apoptotic gene expression, enhanced intracellular drug detoxification and overexpression of membrane drug transport proteins (e.g. P-glycoprotein; P-gp) are all contributing mechanisms leading to MDR (Higgins, 2007). However, despite our increased understanding of MDR, current treatment options are still limited. This is at least to a certain extent due to the fact that the fundamental molecular events driving the MDR phenotype of leukemic cells remain obscure. The characterization of signaling pathways sustaining the MDR phenotype is therefore of utmost importance and such knowledge would be useful for designing rational novel therapies for MDR cancers.

The Hedgehog (Hh) signalling pathway is complex and entails two cellular receptors, i.e. patched-1 (Ptch-1) and smoothened (Smo). Under unligated conditions, Ptch1 represses Smo thereby silencing the Hh signaling pathway. Binding of the ligand (Sonic Hedgehog, Indian Hedgehog or Dessert Hedgehog) to Ptch-1 alleviates Ptch-1-mediated inhibition of Smo thereby initiating an intracellular signaling cascade leading to the activation and nuclear translocation of Gli transcription factors (Zhao et al., 2007; Lauth and Toftgård, 2007; Bijlsma et al., 2006; Dean, 1997).

Hh signaling, originally characterized as part of the morphogenetic code, is critical for growth and differentiation during embryogenesis (Zhao et al., 2007; Lauth and Toftgård, 2007; Bijlsma et al., 2006; Dean, 1997). However, Hh more recently emerged as a signaling system that remains active in adulthood where it mediates tissue regeneration and remodeling, hematopoietic homeostasis and T cell maturation but, as a downside, Hh has emerged as a critical mediator in various forms of oncogenesis. Interestingly, Hh signaling seems important in normal stem cell self-renewal, as abnormal Hh expression or deficient Ptch-1 activity leads to a premalignant stem cell displaying unrestrained local proliferation (Dean, 2005; Dierks et al., 2008). Moreover, the Hh pathway seems essential in the survival and expansion of Bcr-Abl+ leukemic stem cells (Dierks et al., 2008). In line with these findings, Zhao and colleagues recently showed that Hh signaling plays an important role in hematopoietic stem cell self renewal and in maintenance of cancer stem cells in leukemia, although the loss of Hh signaling through conditional deletion of Smo in the adult hematopoietic compartment had no apparent effect on adult hematopoiesis in mice (Hofmann et al., 2009), warranting further studies as to its actual role in the hematopoietic compartment. Together, these considerations prompt further investigation into the potential role of the Hh pathway in the pathophysiology of leukemic disease.

Various lines of evidence suggest that Hh might function in leukemia to promote chemoresistance. It has previously been shown that Hh signaling induces resistance to radiotherapy and a MDR phenotype in oesophageal adenocarcinomas (Sims-Mourtada et al., 2006). In apparent agreement, a significant upregulation of Hh and Gli-1 expression was observed in the majority of residual solid tumors after chemoradiotherapy, suggesting

that Hh signaling contributes to chemotherapy resistance in such tumors. Whether the Hh pathway plays a similar role in leukemia remains elusive and the current study therefore aimed at deciphering the potential role of Hh in chemotherapy resistance in leukemia. To this end, we compared Hh pathway activity in chemotherapy sensitive parental K562 cells and MDR resistant Lucena-1 daughter cells (Rumjanek et al., 2001). We established that Hh signaling maintains the chemoresistant phenotype in an apparently P-glycoprotein-dependent fashion and propose that Hh inhibition might be an attractive treatment strategy to revert (or prevent) chemoresistance in myeloid leukemia.

Material and methods

Compounds, antibodies, constructs and cell lines.

Vitamin D3, cyclopamine, mitoxantrone, daunorrubicin and vincristine were obtained from Sigma (St. Louis, MO). Gant61 was obtained from Alexis (Läufelfingen, Switzerland). Polyclonal antibodies against Gli1 and Ptch-1 were purchased from Cell Signaling (Beverly, MA). Antibodies against Smo, α-tubulin and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) whereas the monoclonal anti-P-glycoprotein antibody was obtained from Sigma (St. Louis, MO). 5E1 Shh-blocking antibody was obtained from the Developmental Hybridoma Bank (Iowa City, Iowa). K562, U-937, Jurkat, KG1a, ACHN and PC3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD), Lucena-1 cells were kindly donated by Prof. Vivan Rumjaneck, GLC4/Doxo cells were obtained from the oncology laboratory of the University of Groningen (the Netherlands). SmoM2-GFP in pMES was a generous gift of Dr Eberhart.

Cell culture and transfections

Cells were cultured in RPMI supplemented with non-essential amino acids and 10% fetal calf serum according to routine cell culture procedures. Transfections were performed using Amaxa® Cell Line Nucleofector® Kit V (VCA-1003) according to manufacturer's directions. Briefly, cells were transfected with SmoM2-GFP (5 μg DNA/1 $X10^6$ Cells) or GFP (2.5 μg DNA/1 $X10^6$ Cells) expression vectors. Next, the transfected cells were placed in RPMI 0.5% FCS for 16 hours, and subsequently the cells were treated with vincristine for 48 hours, and analyzed by FACS or MTT assays. For the reversion assays, cells were treated with mitoxantrone, doxorubicin, vincristine, or imatinib in combination with cyclopamine (10 μM), vitamin D3 (10 μM) or Gant61 (5 μM).

Cell viability assay

Cells ($3x10^4$) were seeded in flat-bottom 96 well plates treated with the indicated concentrations of drugs. During the last 2h, 0.5 mg/mL thiazolyl blue tetrazolium bromide (MTT) was added (Mosmann, 1983). After incubation, supernatant was discarded; cells were lysed in 100 μL 0.1N HCl in isopropanol and absorbance was measured at 570 nm in a Benchmark Plus Microplate Spectrophotometer (Bio-Rad, Hercules, CA).

Quantitative reverse transcriptase-PCR

After RNA isolation according to routine procedures, quantitative PCRs detecting the expression level of different proteins and GAPDH were performed. hP-gp-F GGC AAA GAA ATA AAG CGA CTG AA, hP-gp-R GGC TGT TGT CTC CAT AGG CAA T, hMRP1-F CTT CTG GAG GAA TTG GTT GTA TAG AAG, hMRP1-R GGT AGA CCC AGA CAA GGA TGT TAG A, SuFu-F CCTCCAGATCGTTGTGTCT, SuFu-R TCCGCATGTCAGTATCAGC, hGli1-F CAACTTGCCAGCTGAAGTCT, hGli1-R GATCCTGTATGCCTGTGGAGT, hGAPDH-F AAGGTGAAGGTCGGAGTCAAC, hGAPDH-R TGGAAGATGGTGATGGGATT. Standards consisted of dilutions of RNA from K562 and Lucena-1.

Nuclear extract preparation

Nuclear extracts were prepared according to routine procedures. Briefly, 2x107 cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 0.2 mL ice-cold cell extract buffer (10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethysulfonyl fluoride [PMSF]). Cells were kept on ice for 10 minutes to allow them to swell, mixed by vortex for 10 seconds, and centrifuged at 4° C at 14000g for 30 seconds. The supernatant was discarded, and the pellet was resuspended in 30 µL nuclear extraction buffer (20 mM HEPES-KOH [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF), placed on ice for 20 minutes, and centrifuged at 4° C at 14000g for 2 minutes. The remaining supernatant was used as nuclear extract in Western blotting assays.

Western blotting analysis

Cells ($2.5x10^7$) were lyzed in 200 µL lysis buffer (50 mM Tris–HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM O-Vanadate, 1 mM NaF, and protease inhibitors [1 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluoride-hydrochloride]) on ice for 2 h. Protein extracts were cleared by centrifugation, and the protein concentration was determined using the Lowry method (Hartree, 1972). An equal volume of 2x sodium dodecyl sulfide (SDS) gel loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added and samples were boiled for 10 minutes. Cell extracts were resolved by SDS-PAGE (12%) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in TBS/0.05% Tween 20 (TBST) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilutions. After washing in TBST, membranes were incubated with anti-rabbit, anti-goat and anti-mouse horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions in blocking buffer for 1 h. Blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland) on a LAS-3000 imaging system.

Statistical analysis

Unless otherwise indicated, all experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Data were analyzed by ANOVA. Western blots represent 3 independent experiments.

Results

Sensitivity to chemotherapeutic drugs of K562 and Lucena-1 cells

Studying therapy-induced drug resistance in vitro remains a challenge but Lucena-1 cells have previously been described as vincristine resistant leukemia cells derived from vincristine sensitive K562 parental cells (Rumjanek et al., 2001) (Figure 1) and indeed also in our hands the microtubule polymerization inhibitor vincristine, but also other therapeutically important drugs like the DNA intercalating mitoxantrone and doxorubicin efficiently kill K562 cells whereas these drugs only minimally affect Lucena-1 survival (Figure 1a). Chemoresistance of Lucena-1 was accompanied by upregulation of the drug transporters P-glycoprotein and MRP-1 (Figure 1b) whereas the P-glycoprotein inhibitor verapamil partially reverted drug resistance. We concluded that the comparison of K562 and Lucena-1 constitutes a valid model to study induced resistance for classical chemotherapy.

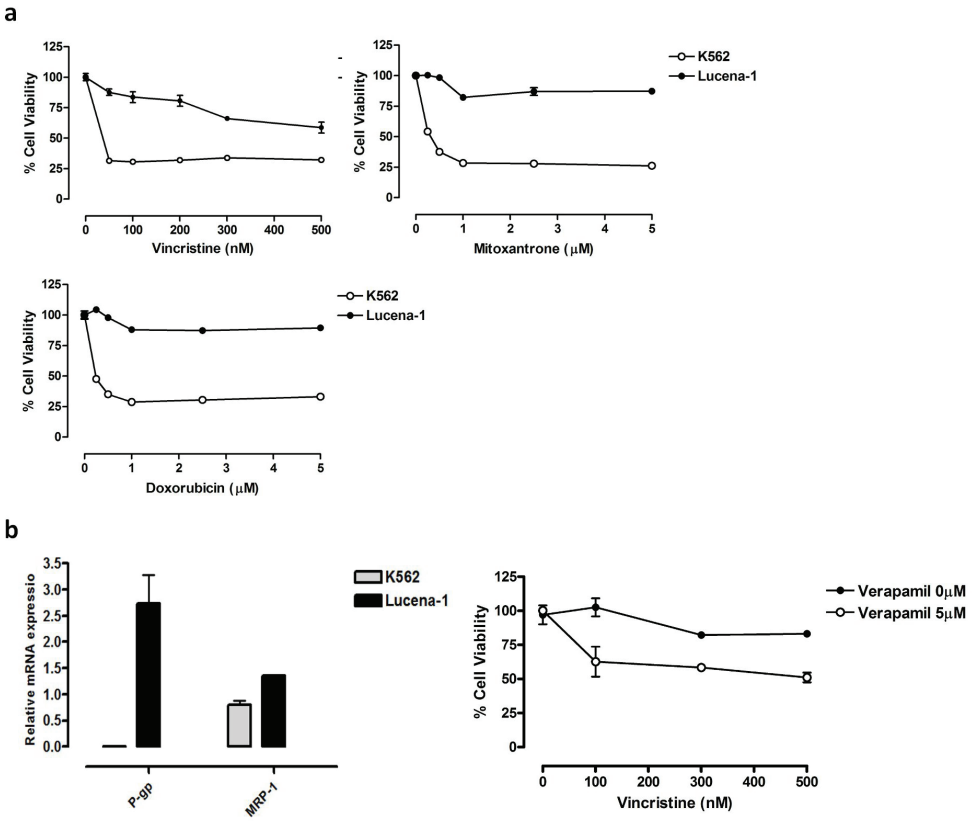

Figure 1. Cell viability of K562 and its multidrug-resistant counterpart Lucena-1 cells in the absence and presence of chemotherapeutic drugs. (a) K562 and Lucena-1 cells were treated with vincristine (0-500 nM), mitoxantrone (0-5 μM) and doxorubicin (0-5 μM) for 48 hours, and cell viability was assessed by MTT reduction assays. (b) Expression level of efflux pumps (P-gp and MRP-1) in K562 and Lucena-1 cells as determined by Q-pcr. (c) Lucena-1 cells were treated with vincristine in the absence or presence of the pump inhibitor verapamil (5 μM) and cell viability was assessed by MTT reduction assays. Shown are mean+/-SEM ($n \geq 3$).

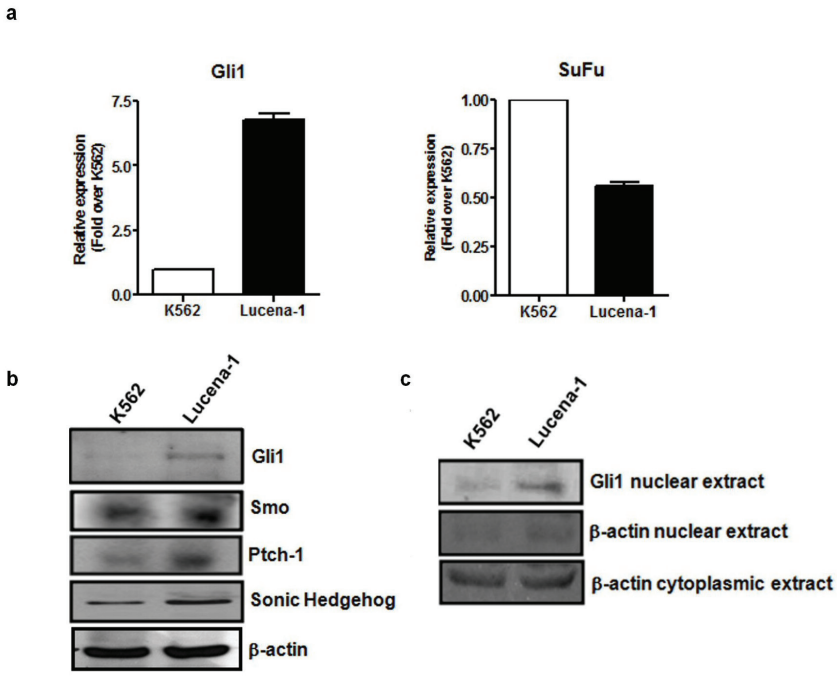

Figure 2. Hh signaling in K562 and its multidrug-resistant counterpart, Lucena-1. (a) K562 and Lucena-1 present differences in Hh pathway components. Shown are Gli1 and SuFu mRNA expression levels as determined by Q-PCR. (b) Expression levels of the Hh pathway components Gli1, Ptch-1, Smo and Shh on the protein level as determined by western blot analyses. (c) Gli1 protein levels in nuclear extracts of K562 and Lucena-1 as determined by western blotting.

Different sensitivity to chemotherapeutic drugs is determined by the Hh pathway

We decided to study whether activation of Hh signaling is a characteristic of chemoresistance under our experimental conditions. Importantly, as shown in Figure 2a, the bona-fide Hh target gene Gli1 displays 7-fold increased mRNA levels in Lucena-1 cells as compared to the chemosensitive parental line, whereas Sufu expression levels (which are suggested to be inversely associated with Hh signalling (Svärd et al., 2006), although this is not generally established) were two times reduced. On the protein level, we observed that Sonic Hedgehog was induced together with the classical Hh target genes Gli as well as Ptch-1 in the chemoresistant cells (Figure 2b). Finally, nuclear extracts showed the hall mark appearance of full length Gli1 in the nucleus in the chemoresistant cells but not in the chemosensitive cells (Figure 2c). Overall, these data show that the chemotherapy resistant phenotype is accompanied by a constitutively activated Hh signaling pathway.

Hh pathway maintains the multidrug resistant phenotype

To investigate the importance of Hh signaling for chemoresistance, K562 cells were transfected with SmoM2-GFP (a constitutive active Smo variant) or GFP overexpressing constructs. The transfections were effective (efficiency of approximately 40% (data not

shown)) and resulted in increased Gli1 and Ptch-1 expression levels (Figure 3a). As shown in Figure 3b, induction of Hh signaling by SmoM2 overexpression protected K562 leukemia cells from vincristine treatment. Furthermore, vincristine treatment increased the percentage of SmoM2-GFP positive cells but not that of GFP only transfected cells (Figure 3c) suggesting that Hh pathway activity might be an important component in the acquisition of resistance to classical chemotherapy in leukemia.

Pharmacological inhibition of Hh signaling reverts resistance in BCR-ABL+ cells

We observed that chemoresistance was accompanied by induction of Hh signaling and that activation of Hh signaling is sufficient for acquiring chemoresistance, but therapeutically it is more interesting to investigate to which extent the chemoresistant phenotype actually depends on activation of this pathway. Thus, we pharmacologically inhibited the Hh pathway in Lucena-1 cells and analyzed the effect on chemoresistance. As shown in Figure 4a, the Hh pathway inhibitors Cyclopamine (Chen et al., 2002) and Vitamin D3 (Bijlsma et al., 2006) indeed inhibited Hh pathway activity in Lucena-1 cells (using Ptch-1 levels as read out). Importantly, both Hh inhibitors did not affect cell survival per se in concentrations up to 10 µM (Figure 4b). The presence of the Hh inhibitors however substantially enhanced the sensitivity of Lucena-1 cells to vincristine to levels not markedly different from the parental line (IC50 approximately 10 nM in both cases; compare Figure 1 and Figure 4c). This effect was not restricted to microtubules system-targeted chemotherapy as also doxorubicin- or mitoxantrone-sensitivity was also restored to the levels of the parental cell line following inhibition of the Hh pathway.

To provide further proof for the notion that inhibition of the Hh pathway may revert chemoresistance, Lucena-1 cells were treated with vincristine in combination with Gant 61 (inhibitor of Gli1 transcriptional activity) (Lauth et al., 2007). As shown in Figure 4d, GANT61 efficiently inhibited Hh pathway activity and the inhibition of Gli1 transcriptional activity by GANT61 indeed reverses chemoresistance, suggesting that the increased level of Gli1 (Figure 2a) is important to maintain the resistant phenotype in Lucena-1 cells. To determine whether the effect of Hh inhibition is specific for MDR leukemia cells, the combinations

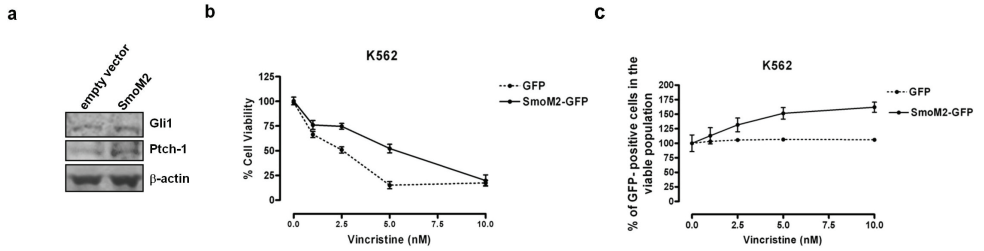

Figure 3. Over-expression of Hh pathway components is sufficient for chemo-protection of K562 cells. (a) K562 cells were transfected with SmoM2-GFP or GFP expression constructs after which Gli1 and Ptch-1 levels were determined by western blotting. (b) K562 cells transfected with SmoM2 are protected against vincristine induced cell death. (c) Vincristine treatment increased the percentage of SmoM2-GFP positive cells but not that of GFP only transfected cells. Shown are mean+/-SEM (n=3).

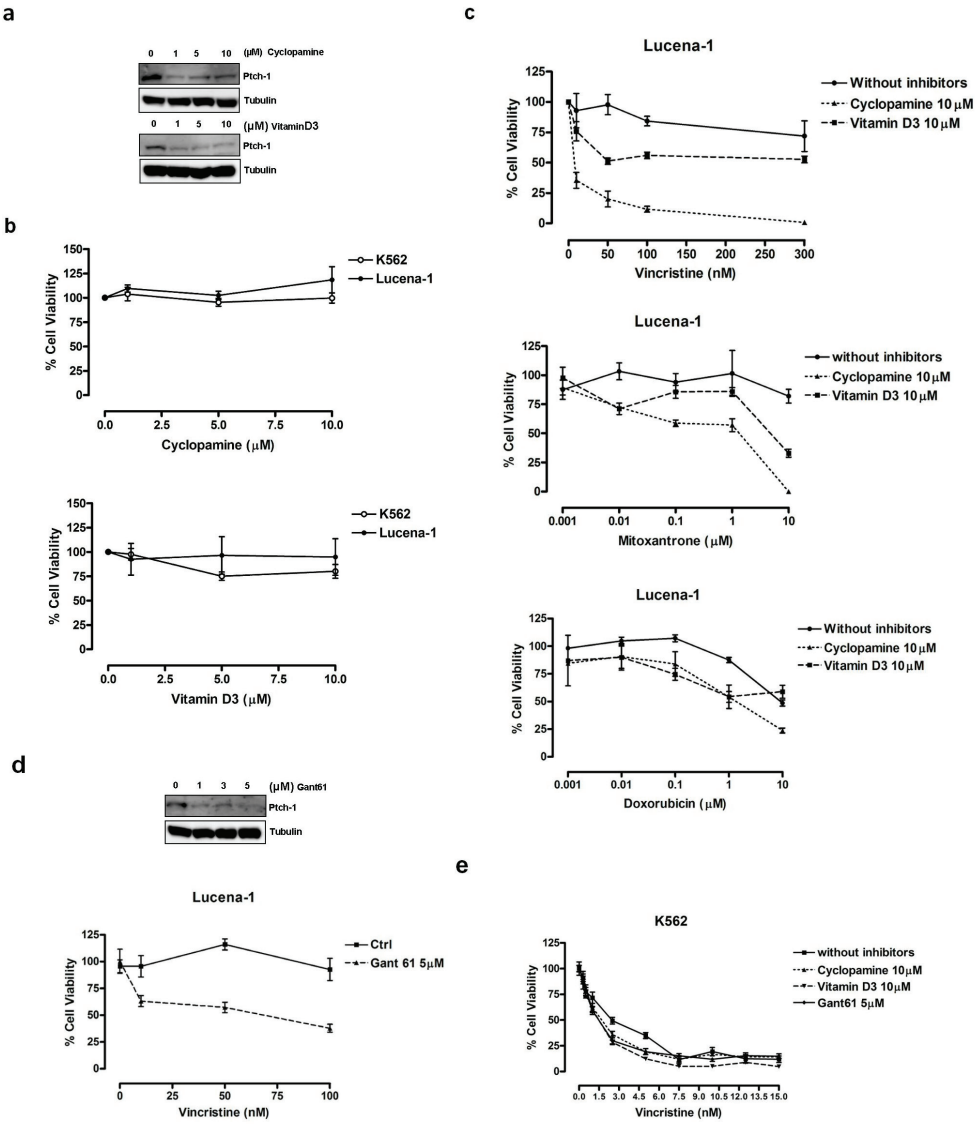

Figure 4. Hh signaling inhibitors counteract chemoresistance of Lucena-1 cells. (a) Ptch-1 expresion with and without cyclopamine or vitamin D3 treatment. (b) Cyclopamine and/or vitamin D3 treatment (0-10 μM for 48 hours) does not affect the viability of K562 and Lucena-1 cells as assessed by MTT reduction assays. (c) Both cyclopamine and vitamin D3 sensitize Lucena-1 cells for chemotherapeutic drugs. Lucena-1 cells were treated with increasing concentrations vincristine, mitoxantrone or Doxorubicin in the presence of 10 μM cyclopamine or vitamin D3 for 48 hours. (d) Treatment of Lucena-1 cells with the Gli-1 inhibitor Gant61 inhibits Ptch-1 levels and reverts chemoresistance to vincristine. (e) Hh pathway inhibitors do not sensitize K562 cells for vincristine. K562 cells were treated with increasing concentrations vincristine, in the presence of cyclopamine, vitamin D3 or Gant61 for 48 hours. Shown are mean+/-SEM ($n \geq 3$).

mentioned above (Hh pathway inhibitors plus vincristine) were also tested in K562 cells. As shown in Figure 4e, the Hh inhibitors hardly effected vincristine induced cell death (at least in comparison to the effect observed in Lucena-1 cells (Figure 4c) suggesting that the chemosensitisation induced by Hh pathway inhibitors is specific for MDR cells. Overall, it seems that pharmacological inhibition of the Hh pathway might constitute an interesting opportunity for chemosensitisation in the management of MDR myeloid leukemic disease.

Myeloid-specificity of chemosensitisation via inhibition of Hh signaling

To address the specificity of the effects observed, a more or less random panel of leukemia cells and solid cancers was exposed to pharmacological Hh inhibition and effects of this treatment on chemosensitivity were assessed. As evident from Figure 5, neither solid cancers nor T cell leukemia showed much evidence for such chemosensitization. Although these results show that alleviation of therapy resistance employing Hh inhibitors is restricted to the myeloid compartment, they provide also good evidence that effects seen are specific and cannot be explained from an off-action of the pharmacological inhibitors involved.

Pharmacological inhibition of Hh signalling interferes with P-glycoprotein expression

Subsequently, we were interested as to whether we could dissect the point of interaction of Hh signaling with classical cellular physiology of the leukemic cell. As Hh seems to affect resistance of MDR leukemia cells, we hypothesized that the targeting the Hh pathway might reduce P-glycoprotein levels. As shown in Figure 6, all Hh inhibitors indeed reduced expression levels of P-glycoprotein although to a different extent.

Discussion

Intrinsic and acquired MDR against chemotherapy remains one of the major problems in the management of leukemic disease (Sims-Mourtada et al., 2006; Martelli et al., 2003; O'Hare et al., 2006; Diehl et al., 2007; Löwenberg, 2007). The majority of leukemia patients (75%) of 60 years or younger do present an initial complete remission following chemotherapy. However, 60% of these responders will ultimately present disease relapse, usually occurring within in the first 2 years after the start of treatment (Löwenberg, 2007). Despite our increased understanding of MDR and the elucidation of several underlying mechanisms (Higgins, 2007; Dean, 2005; Kroemer and Pouyssegur, 2008), treatment options are still limited. The characterization of signaling pathways sustaining MDR is thus essential for designing rational novel therapies for MDR leukemia. Here we show that the chemotherapy resistant phenotype of myeloid leukemia cells correlates with activation of Hh signaling, that overexpression of Hh pathway components induces chemoprotection and that inhibition of the pathway reverts chemoresistance of Lucena-1 cells. Our data thus identify the Hh pathway as an essential component of MDR leukemia and suggest that targeting the Hh pathway might be an interesting therapeutic avenue for overcoming MDR resistance in myeloid leukemia. These data provide a mechanistic explanation as to the previous observation that inhibition of Hh pathway in Kasumi-1, -3 and TF1 myeloid cell

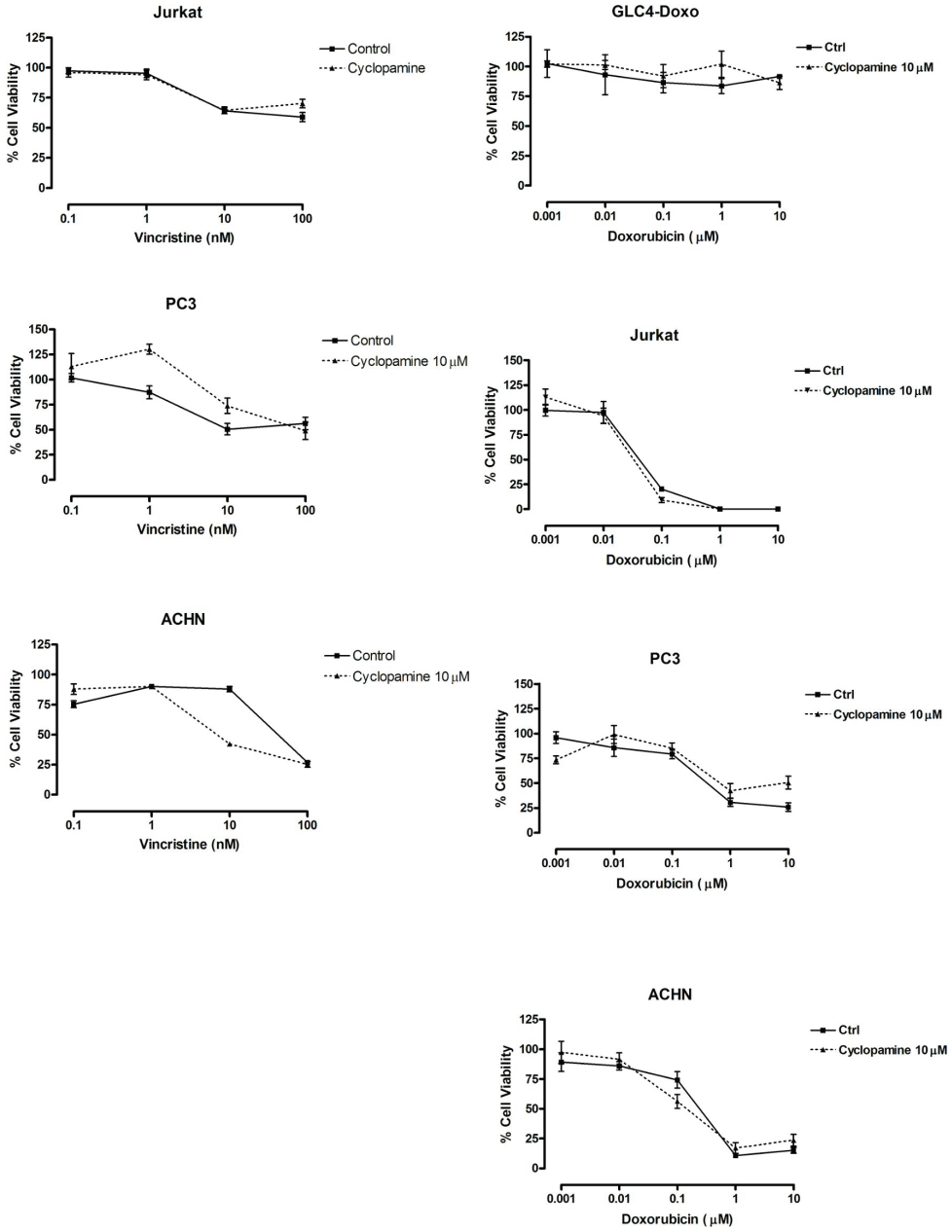

Figure 5. Cyclopamine-induced sensitization is not a general mechanism in cancer cells. Jurkat, ACHN, PC3 and GLC4-Doxo cells were treated with cyclopamine (10μM) in combination with different concentrations of vincristine or doxorubicin and cell viability was evaluated by MTT reduction assays. Shown is the mean+/-SEM (n=3).

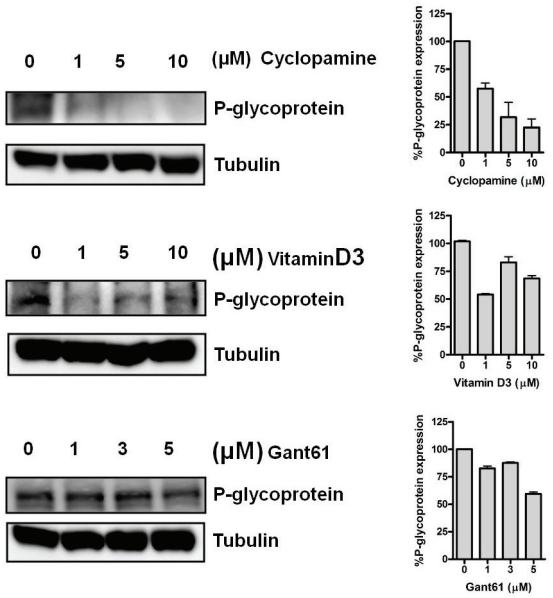

Figure 6. Hh pathway inhibitors sensitize Lucena-1 cells dependent on P-glycoprotein expression. P-glycoprotein expression in Lucena-1 cells was analyzed after 24 hours of treatment with cyclopamine, vitamin D3, and Gant61. Shown on the left is a representative experiment, whereas the quantification of three independent experiments is shown on the right (mean+/-SEM).

lines increased sensitivity to suboptimal doses of the antimetabolite cytarabine (Kobune et al., 2009). We propose that activation of the Hh pathway is both sufficient and essential for resistance to classical chemotherapy in myeloid leukemia.

The mechanism of action of Hh inhibitors is probably dependent on its effect on P-glycoprotein expression levels (as shown in Figure 6). This immediately explains that the chemosensitisation induced by Hh inhibitors in combination with vincristine is only observed in Lucena-1 cells and is not (very) effective in K562 or GLC4-doxo cells. These latter cells express MRP-1 but not P-glycoprotein and sensitization can consequently not be induced in these cells by Hh inhibitors that target P-glycoprotein. Overall these data indicate an important role of the Hh pathway in the maintenance of the MDR phenotype in myeloid leukemias.

Our findings might be relevant for the treatment of MDR leukemia but in addition our data may be relevant for the early detection of resistance. Indeed, the expression of sentinel markers (e.g. Ptch-1) of activation of the Hh pathway in peripheral blood myeloid leukemia cells may be an indication that a patient is developing chemoresistance and is possibly a useful predictor of treatment failure, necessitating the switch to alternative therapy. Further studies are obviously essential to address the validity of these latter points.

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Chapter 3

Assessing the efficacy of the Hedgehog pathway inhibitor vitamin D3 in a murine xenograft model for pancreatic cancer

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Abstract

The developmental Hedgehog (Hh) pathway has been shown to cause malignancies in the adult organism, specifically in the proximal gastrointestinal tract. Previous studies have used the Hh-inhibitory alkaloid cyclopamine to treat Hh-dependent tumor growth. The present study aimed to determine the efficacy and specificity of the recently discovered endogenous inhibitor of the Hh pathway, vitamin D3, on inhibition of pancreatic adenocarcinoma cell growth in vitro and in vivo. Vitamin D3 was found to inhibit cell growth specifically through inactivation of Smo and the downstream Hh pathway, rather than activation of the vitamin D3 receptor. However, in in vivo models vitamin D3 was not found to be effective against tumor cell growth.

Introduction

Hedgehog (Hh) proteins are involved in a plethora of patterning processes in the developing organism and most of the research effort in the field has focused on the roles and mechanisms of Hh proteins in prenatal development [1]. This effort is at least partly driven by the unusual and complex signal transduction of Hh proteins (reviewed in refs. 1 and 2). For instance, the Hh signal is relayed through the interaction between two receptors, patched-1 (Ptch1) and smoothened (Smo). In the absence of Hh, Ptch1 actively inhibits Smo and the downstream pathway is inactive. Binding of Hh to Ptch1 alleviates Ptch-mediated inhibition of Smo and the pathway becomes active. Another peculiarity is the synthesis of the Hh ligand itself; after translation, Hh is autocatalytically cleaved and a cholesterol group is added to the newly exposed C-terminus. Following sterolation, a palmitoyl group is added at the other terminus and despite these highly hydrophobic modifications, the protein is secreted.

The role of Hh proteins in the adult organism is attracting an increasing amount of research effort, and Hh proteins have been shown to be involved in for instance gastrointestinal homeostasis [3] tissue salvage following ischemia [4] and T-cell maturation [5]. An important downside of improperly sustained Hh activity in adult organisms is its causative role in carcinogenesis in, among others, skin [6], prostate [7], and the upper gastrointestinal tract[8]. The underlying causes for excessive pathway activity in these diseases vary from activating mutations in Smo rendering it unresponsive to endogenous inhibition by Ptch1 to inactivating mutations in Ptch1 paralyzing its inhibitory action on Smo[9]. Some tumors, however, do not depend on mutations in pathway components but in disproportionate Hh production, and such Hh expression is most associated with tumors of the upper gastrointestinal tract [8].

Very few effective treatment strategies are available for tumors of the proximal gastrointestinal tract and the relatively high incidence (10 in every 100,000 persons for pancreatic cancer each year in Western countries) necessitates research into novel therapeutic options. As the alkaloid cyclopamine has been found to be a potent inhibitor of Smo and its downstream signaling, it has seen extensive use as a tool in Hh research and inhibiting the Hh pathway in cancer cells of the upper gastrointestinal tract with cyclopamine has proven to be a successful way of reducing cancer cell viability [8, 10-12]. Although data from mouse models suggest cyclopamine might be a promising therapeutic option in the treatment of these cancers, confirmation by clinical application has yet to be achieved.

We recently identified the naturally occurring inhibitor of Smo, vitamin D3. Using an in vitro model, we showed that Ptch1 translocates vitamin D3 to the extracellular space, after which it binds to Smo and inhibits Hh pathway activity [13]. Because of its widespread clinical use, the proper dosage and side-effects of vitamin D3 are well known (e.g. hypercalcaemia) [14]. This makes vitamin D3 an attractive therapeutic option for the treatment of Hh-dependent tumors, as it is less likely to cause unexpected side effects compared to the relatively novel class of Smo antagonists. The higher Smo-inhibitory potential of vitamin D3 compared to cyclopamine also implies a better efficacy in inhibiting Hh-dependent cell growth [15].

There is a strong body of evidence for anti-tumor activity by vitamin D3. This ranges from tumor models in which action of vitamin D3 through the vitamin D receptor (VDR) has been established, to epidemiological studies correlating low vitamin D3 levels in patients with tumor incidence and poor outcome [16-18]. Here, we estimated the efficacy of vitamin

D3 in inhibiting growth of pancreatic adenocarcinoma tumor cells that are known to rely on excessive Hh expression. This dependency might be direct by acting on the tumor cells [11,19] or indirect by acting on the stromal cells that subsequently secrete survival- and growth factors that support tumor cell growth [20,21]. We confirmed the inhibitory action of vitamin D3 on Smo and showed that in sensitive in vitro models, vitamin D3 inhibits tumor cell growth. Also, we verified that this vitamin D3-induced cytotoxicity was mediated through inactivation of Smo and the downstream Hh pathway. However, in in vivo models as well as in vitro models that are less geared towards drug sensitivity, vitamin D3 was relatively ineffective against tumor cell growth, apparently precluding its therapeutic application in the treatment of Hh-dependent malignancies.

Material and methods

Compounds and constructs

Cyclopamine (Biomol, Plymouth Meeting, PA), AY-9944 (Calbiochem, San Diego, CA), vitamin D3 (cholecalciferol; Sigma, St Louis, MO), recombinant N-terminal Shh (rShh; R&D Systems, Minneapolis, MN), gemcitabine (Eli Lilly, Houten, Netherlands), 5-FU (TEVA Pharma, Mijdrecht, Netherlands) were commercially obtained. SmoM2-pRK7 was obtained from Genentech (South San Francisco, CA). The Gli1 cDNA in pcDNA1 was from Dr. Ruiz i Altaba. The δ51-LucII Gli-reporter was from Dr. Sasaki.

Cell culture and transfections

Hs766T, BxPC3, MIA PaCa-2, PANC-1, HepG2, mouse embryonic fibroblasts (MEFs), Shh-LIGHT II cells (ATCC, Manassas, VA) were cultured in DMEM (Cambrex, East Rutherford, NJ) containing 10% FCS. Shh-LIGHT II cells were grown in medium supplemented with neomycin (400 μg/mL) and zeocin (150 μg/mL). MEFs from Ptch1+/+ and Ptch1-/- mice were from Dr. Scott.36 Smo-/- MEFs were provided by Dr. Taipale [37]. Cells from VDR deficient mice were from Dr. Li[20]. 10.7 cells were provided by Dr. Iacobuzio-Donahue [11].
Transfections were performed using Effectene according to manufacturer's directions. For reporter assays, cells were transfected in 12-well cell culture plates. Per well, 1 μg DNA was used. For MTT assays, cells were transfected in 6-well plates (2 μg DNA per well) and after 16h, cells were transferred to 96-well plates (see below).

MTT reduction cell viability assay

Cells ($5x10^6$) were seeded in flat-bottom 96 well plates in DMEM containing 0.5% or 10% FCS and treated with the indicated concentrations of cyclopamine or vitamin D3 for 3d as described previously[23] Cells were stimulated either 2h or 1d after seeding. 0.5 mg/mL thiazolyl blue tetrazolium bromide (MTT) was added, absorbance was measured at 570 nm.

Western blotting

Cells were lysed and subjected to SDS-PAGE as described before15 using goat polyclonal anti-Smo C-17 (1:1000 dilution in 3% BSA/TBST) and anti-beta-actin I-19 (1:2000 dilution in 3% BSA/TBST) (both Santa Cruz Biotechnology, Santa Cruz, CA) After 1h incubation in

1:1000 of the appropriate HRP-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark), blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland) on a LAS-3000 imaging system.

Reporter assay

Cells grown to 70% confluence in a 12-well plate were transfected as described above with the Gli-reporter construct and a CMV-driven Renilla Luciferase control, and after 16h, stimulated with the indicated compounds. Subsequently, cells were lysed and luciferase activity was assayed as described before [15]. Each Firefly luciferase value was corrected for its co-transfected CMV driven Renilla luciferase standard to correct for transfection efficiency or dilution effects.

Murine orthotopic pancreatic adenocarcinoma model

NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were bred at the animal care facility of the Academic Medical Center, kept under specific pathogen-free conditions according to regulations of the experimental animal facility and ethical committee of the University of Amsterdam (Animal Ethical Committee (DEC) protocol reference number DIX101180). At 6-9 weeks of age, pancreatic adenocarcinoma 10.7 cells ($1x10^6$ in 200 µL of growth factor depleted Matrigel) were orthotopically xenografted as previously described.11 Vitamin D3 (dissolved in corn oil) was injected i.p. twice weekly at 5 µg/animal. Body weights were monitored twice weekly. Mortality and excessive primary tumor size (mice were sacrificed if tumor size >250 mm3) was monitored daily.

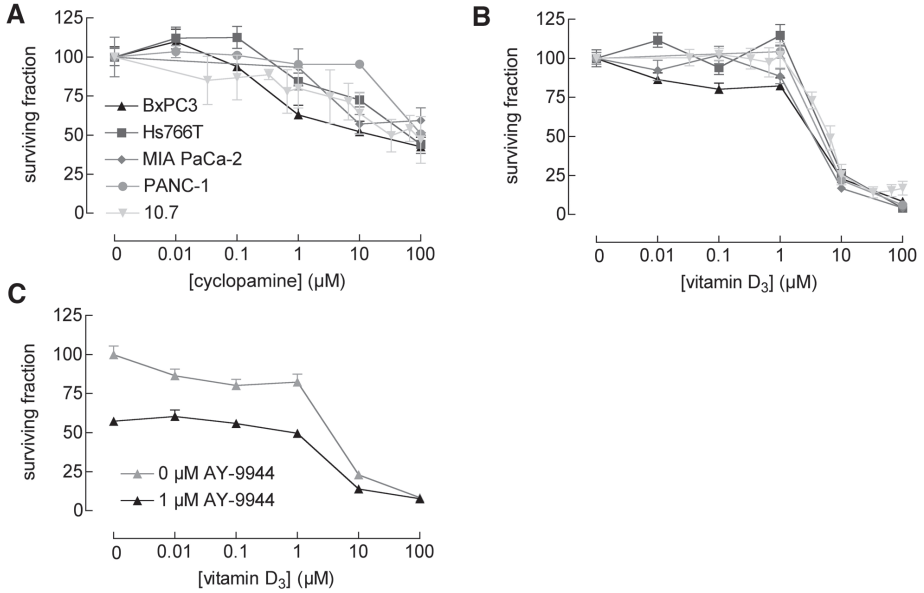

Figure 1. Vitamin D3 inhibits pancreatic adenocarcinoma cell growth in a sensitive model for cell growth inhibition. (A) Indicated cells were seeded in 0.5% FCS and exposed to the Smo inhibitor cyclopamine after 2h. After 3d, cell viability was assessed by MTT reduction. Data are expressed as percentage of control stimulated cells (0 µM; mean±SEM, n=8). (B) As for Fig. 1A, using vitamin D3. (C) BxPC3 cells were treated with vitamin D3 in the presence or absence of AY-9944.

Chick chorioallantoic membrane (CAM) preparation and grafting of tumor cells

Fertilized chicken eggs (Ideal Poultry, Cameron, TX) were incubated laterally in a humidified rocking incubator at 37.8°C. After 3d, eggs were opened and the CAM was exposed. At day 10 of incubation, a nylon ring (ID 6 mm) was placed on the CAM and $2.5x10^5$ BxPC3 cells in 50 µL growth factor depleted Matrigel were brought into the ring [31]. Cells were grown for 14 days, and treated with vitamin D3 twice during that period in 50 µL PBS at the indicated concentrations. After 14 days, tumor-bearing CAMs were weighed.

Irradiation

Cells were seeded in 96-well plates and irradiated with γ-rays from a 137Cs (137mBa) source at a dose rate of approximately 0.53 Gy/min. Subsequently, MTT assays were performed as described above.

Results

Vitamin D3 inhibits growth in freshly seeded pancreatic adenocarcinoma cells

Cell lines known to be dependent on Hh expression were used as a model system to examine the effect of vitamin D3 on Hh dependent tumor cell growth. These cell lines included the pancreatic adenocarcinoma cell lines Hs766T, BxPC3, MIA PaCa-2, PANC-1 and 10.7 that have previously been shown to have an elevated Hh pathway activity and subsequently increased cell growth, apparently caused by an excessive endogenous production of Hh [8,22]. For these experiments, cells were stimulated directly after seeding, as described previously [23]. Also, serum concentration in the medium was low (0.5% FCS). This setup greatly enhances sensitivity of cells to therapeutic agents, as the cells have just attached and are not yet proliferating, but it remains debatable how clinically relevant this model is. Cell growth was reduced when exposed to increasing concentrations of cyclopamine but even very high doses of cyclopamine only reduced growth by about 50-60% (Fig. 1A).

In comparison, vitamin D3 was more effective (Fig. 1B), showing dose-response curves with a high slope factor. The highest dose of vitamin D3 reduced growth (almost) completely. Although these data show that cyclopamine and vitamin D3 are both cytotoxic to pancreatic cancer cells in moderately high doses they do not necessarily prove that vitamin D3 acts via a similar pathway as cyclopamine. From the panel of cells tested, the BxPC3 cell line seemed most responsive to Hh pathway inhibition in our hands, and many previous studies have used this cell line for studying Hh pathway inhibitors as well (e.g. refs. 8, 11 and 24). Consequently, we performed further mechanistic studies with BxPC3 cells. To assess to what extent endogenously synthesized pro-vitamin D3 could inhibit cell growth, we used the 7-dehydrocholesterol reductase (7-DHCR) inhibitor AY-9944 [25]. Inhibition of 7-DHCR causes stacking of 7-dehydrocholesterol, the precursor of vitamin D3, effectively increasing the endogenous levels of vitamin D3. The addition of AY-9944 to BxPC3 cells indeed inhibited cell growth but it did not enhance the inhibitory effect of exogenously added vitamin D3 (Fig. 1C). Thus in this model, both exogenously added and endogenously produced pro-vitamin D3 are toxic to cells that depend on an excessively activated Hh pathway.

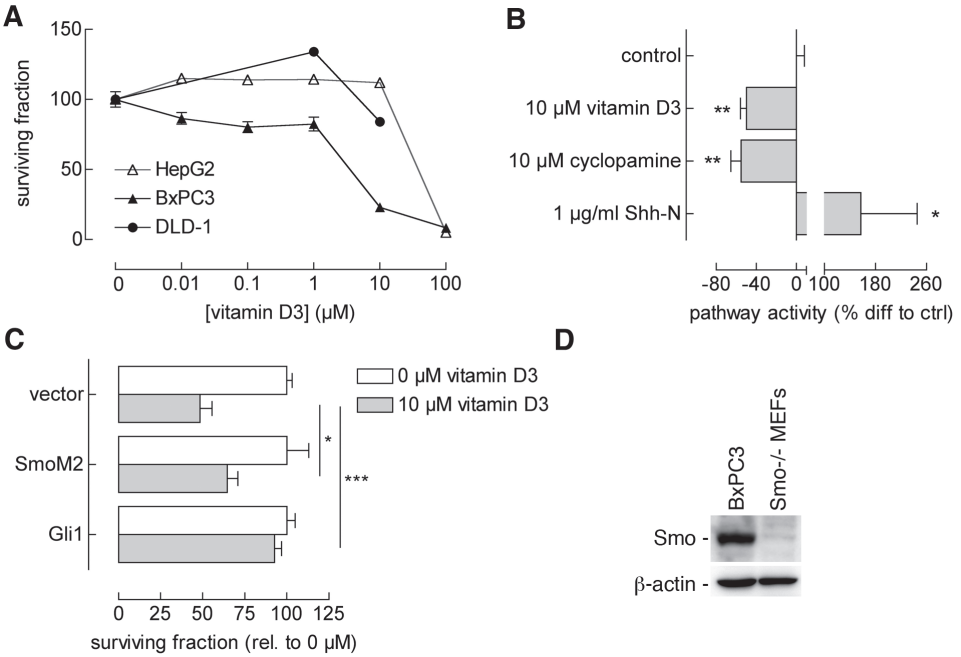

Figure 2. Vitamin D3 inhibits pancreatic adenocarcinoma cell growth by Hh pathway inactivation in a sensitive model for cell growth inhibition. (A) HepG2 and DLD-1 cells were treated with vitamin D3 as for the cell lines in Fig. 1. For comparison, BxPC3 data from Fig. 1 are plotted alongside. (B) BxPC3 cells were transfected with a Gli-luciferase reporter construct. 16h after transfection, cells were reseeded in 0.5%FCS and after 2h, stimulated with vitamin D3, cyclopamine, or rShh for another 24h. Luciferase activity is expressed as percentage in-/decrease over control stimulated cells (mean±SEM, n=3); **p>0.01; *p>0.05. (C) BxPC3 cells were transfected with vector DNA, SmoM2 mutant or the Gli1. 16h after transfection, medium was refreshed and cells were subsequently treated with 10μM vitamin D3 as for Fig. 1. Shown is mean±SEM, n=8; ***p>0.005; *p>0.05. D. BxPC3 cells and Smo-/- MEFs were lysed and subjected to Western blot analysis using antibodies against Smo and beta-actin.

Vitamin D3 inhibits pancreatic adenocarcinoma cell growth by Hh pathway inactivation

To determine if vitamin D3-induced cell growth inhibition acts through the Hh pathway rather than by a more general, aspecific cytotoxic effect, we treated freshly seeded HepG2 hepatocarcinoma cells in low serum medium with increasing doses of vitamin D3 (Fig. 2A). These HepG2 cells are not thought to rely on an excessively activated Hh pathway for their growth [26], and these cells were clearly less sensitive to vitamin D3 than the BxPC3 cells. Similarly, DLD-1 colon cancer cells, known to be independent of Hh pathway activation, were less sensitive to vitamin D3 treatment, suggesting at least some specificity of vitamin D3 for cells dependent on an active Hh signaling pathway.

As we previously showed vitamin D3 to inhibit Hh pathway activity, we aimed to confirm this effect of vitamin D3 in the pancreatic adenocarcinoma cells. To this end, BxPC3 cells were transiently transfected with a reporter construct sensitive to the glioma associated (Gli) transcription factors for the Hh pathway (Gli δ51-LucII) [27], and pathway activity was

assessed following vitamin D3 treatment (Fig. 2B). A strong decrease in pathway activity could be seen following treatment with vitamin D3 proving it to be able to counteract the active Hh pathway in these cells. Using the benchmark inhibitor cyclopamine, we observed an inhibition of similar magnitude. Surprisingly, we also observed substantial pathway activation following stimulation with recombinant N-terminal Shh. This suggests that although the BxPC3 cells are able to support their own growth by excessive Shh production, endogenous Shh production is not enough to maximally stimulate the pathway. These data also show that the pancreatic cancer cells are sensitive to Hh pathway stimulation and inhibition, in contrast to the recently reported insensitivity of these cells to their own Hh ligands [20].

As the cytotoxic effects of vitamin D3 on cancer cells are mediated through several mechanisms, nearly all of which are dependent on the VDR, we aimed to confirm that at least part of the observed reduction of cell viability is mediated through inactivation of Smo and the downstream transcription factors. To this end, BxPC3 cells were transfected with the Ptch1-insensitive Smo mutant SmoM [2,28] or the downstream transcription factor Gli1.29 Subsequently, the freshly re-seeded transfected cells were exposed to vitamin D3 and cell viability was assessed (Fig. 2C). The BxPC3 cells were rendered less sensitive to vitamin D3 when transfected with SmoM2, and almost completely insensitive when transfected with Gli1, confirming that at least a considerable part of the cytotoxic action of vitamin D3 is mediated through inactivation of Smo and the downstream pathway.

Previously, BxPC3 cells were reported not to express Smo[20], explaining the lack of sensitivity to cyclopamine found in some studies, and casting doubt on the results obtained in other studies that did show sensitivity to Smo inhibition. However, BxPC3 cells express Smo at a level detectable by traditional protein analysis (Fig. 2D). As expected, Smo protein was not detected in Smo-/- cells, confirming antibody specificity.

Vitamin D3 acts on Smoothened

A genetic approach to establishing the specificity of vitamin D3 action on Smo is shown in Fig. 3A. MEFs from mice genetically deficient for various receptors were transiently transfected with the Gli-reporter construct and treated with vitamin D3. Smo negative cells did not show a reduction in Hh pathway activity following vitamin D3 treatment, whereas wild type cells showed a negative Hh pathway response of a magnitude described previously [13]. In cells lacking Ptch1, inhibition of pathway activity was also observed after vitamin D3 treatment, proving that vitamin D3 acts by binding to and inhibiting Smo, as opposed to acting through Ptch1 to inhibit the Hh pathway.

For the Wnt pathway, the VDR is known to be required for the effects of vitamin D3 on the pathway [30]. To exclude a requirement for the VDR in mediating the Hh-inhibitory effect of vitamin D3, we determined Hh pathway activity in cells hetero- or homozygous for the VDR. VDR+/- and VDR-/- MEFs still showed a strong Hh-inhibitory response to vitamin D3, excluding the VDR to be important for Hh pathway inhibition by vitamin D3. When these cells were used in MTT assays, and exposed to increasing concentrations of vitamin D3 as for Fig. 1 (Fig. 3B) or cyclopamine (Fig. 3C), a similar sensitivity was found for both cell types. This suggests that the VDR is not important for the observed cytotoxicity by vitamin D3, and that the observed effects are most likely mediated through Smo.

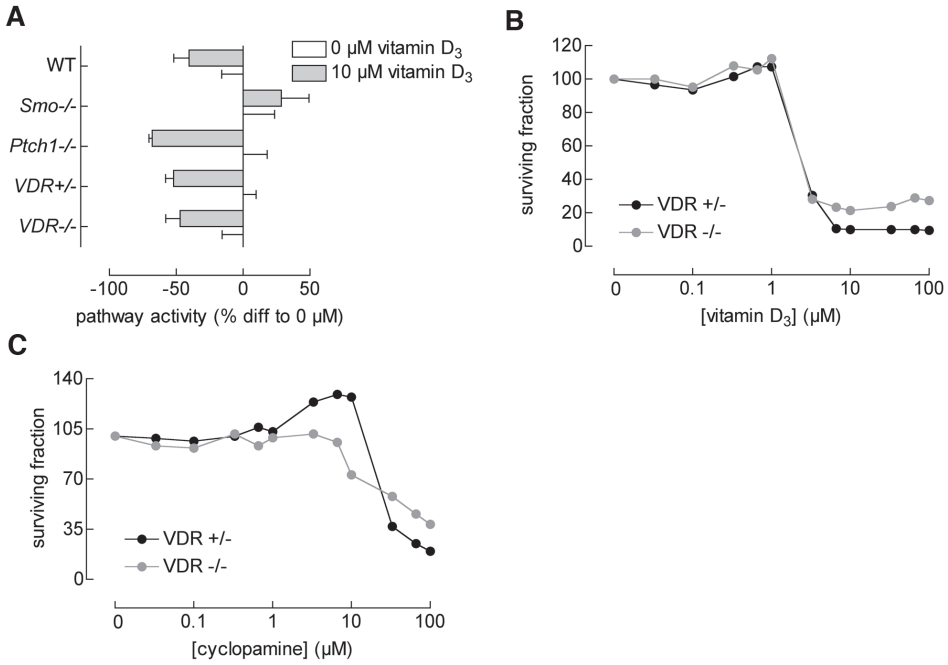

Figure 3. Vitamin D3 does not inhibit Hh pathway activity through the VDR. (A) MEFs of indicated genotypes were transfected with Gli-luciferase reporter construct. 16h after transfection, medium was refreshed and cells were stimulated with vitamin D3 for 16h. Following lysis, luciferase activity was determined and expressed as for Fig. 2B (mean±SEM, n=6). (B-C) VDR+/- MEFs were treated with vitamin D3 or cyclopamine as for Fig. 1, and cell viability was determined.

Vitamin D3 is ineffective in in vivo xenograft models for pancreatic adenocarcinoma

Encouraged by the results obtained in the in vitro models, we assessed the efficacy of vitamin D3 in an orthotopic xenograft model for pancreatic adenocarcinoma.11 Pancreatic adenocarcinoma 10.7 cells were injected into the pancreas of immunodeficient mice and subsequently, these mice were injected with high doses of vitamin D3 twice weekly. Approximately 4 weeks after tumor cell injection, we started observing mortality in both the control and vitamin D3 treated group (Fig. 4A). No statistically significant difference was observed between the groups. In some animals, the tumor size reached about 250 mm3 (Fig. 4B, determined post mortem), which was above the predefined humane end-point of the model and these remaining mice were sacrificed at day 34 (Fig. 4A). Thus, although the dose of vitamin D3 administered was high, no decrease in mortality could be observed. Metastases to distal organs were similar in both groups.

Glucose (Fig. 4C) and amylase levels (Fig. 4D) (markers of pancreatic injury) were respectively in- and decreased to similar levels in both the control- and vitamin D3 treated animals as compared to normal (no tumor) levels, showing no effect of vitamin D3. Decreased blood amylase levels are indicative of permanent damage rather than injury to the amylase-producing cells in the pancreas. Ca2+ levels were below the detection limit in both groups, indicating that the vitamin D3 treated animals were not hypercalcemic. Overall, these data

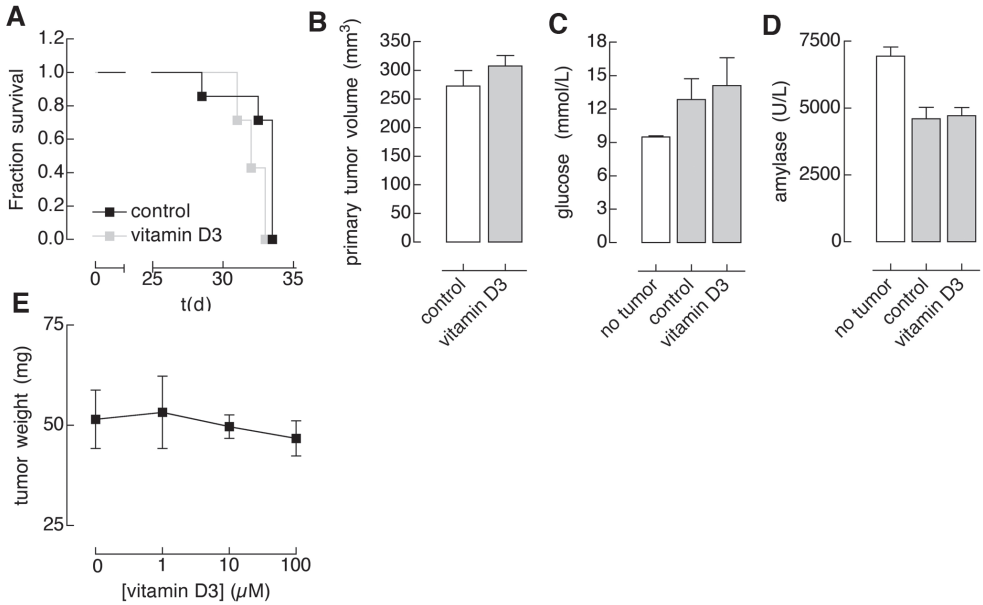

Figure 4. Vitamin D3 does not inhibit pancreatic adenocarcinoma cell growth in vivo. (A) The pancreas of immunodeficient mice was injected with 1x106 10.7 cells. Mice were injected with 5 µg vitamin D3 or control twice weekly for 34 days, and mortality was monitored (n=7 in each group). (B) After 34 days, remaining mice were sacrificed, tumors were excised and measured. (C-D) Glucose and amylase levels in plasma from the animals from both groups and from non-tumor bearing animals was determined. (E) CAM of 10 day-old fertilized chicken eggs were xenografted with 2.5x105 BxPC3 and allowed to grow for an additional 14 days. Tumors were treated with indicated concentrations of vitamin D3 once weekly. After 14d, tumors were isolated from the CAM and weighed (n=4).

suggest that vitamin D3 treatment did not reduce pancreas cancer growth, which seems to contradict our findings in vitro (Fig. 1 and 2), and suggest that vitamin D3 is not a very promising therapeutic option, especially given the inefficacy of a rather high dose.
Another model that yields tumors in a host organism is the CAM xenograft model [31]. In this model, vasculature under the CAM supports the growth of tumor tissue, thus making the fertilized egg act as stromal tissue. From the pancreatic adenocarcinoma cells, BxPC3 cells were most successful in establishing a tumor-like structure and these were used to test the effect of vitamin D3 on tumor growth. However, no decrease in tumor weight could be observed (Fig. 4E), again confirming that although vitamin D3 seemed effective against Hh-dependent tumor cell growth in vitro, it is not effective in in vivo models.

Vitamin D3 is unable to inhibit growth in proliferating tumor cells

To explain the results obtained in vivo, we evaluated the efficacy of vitamin D3 in in vitro models that are less artificially geared towards drug sensitivity than those used for Fig. 1 and -2. In this model, cells are allowed to adhere and proliferate 1 day before stimulation. Arguably, this mimics the clinical situation more, in which drugs are always administered after tumor diagnosis.

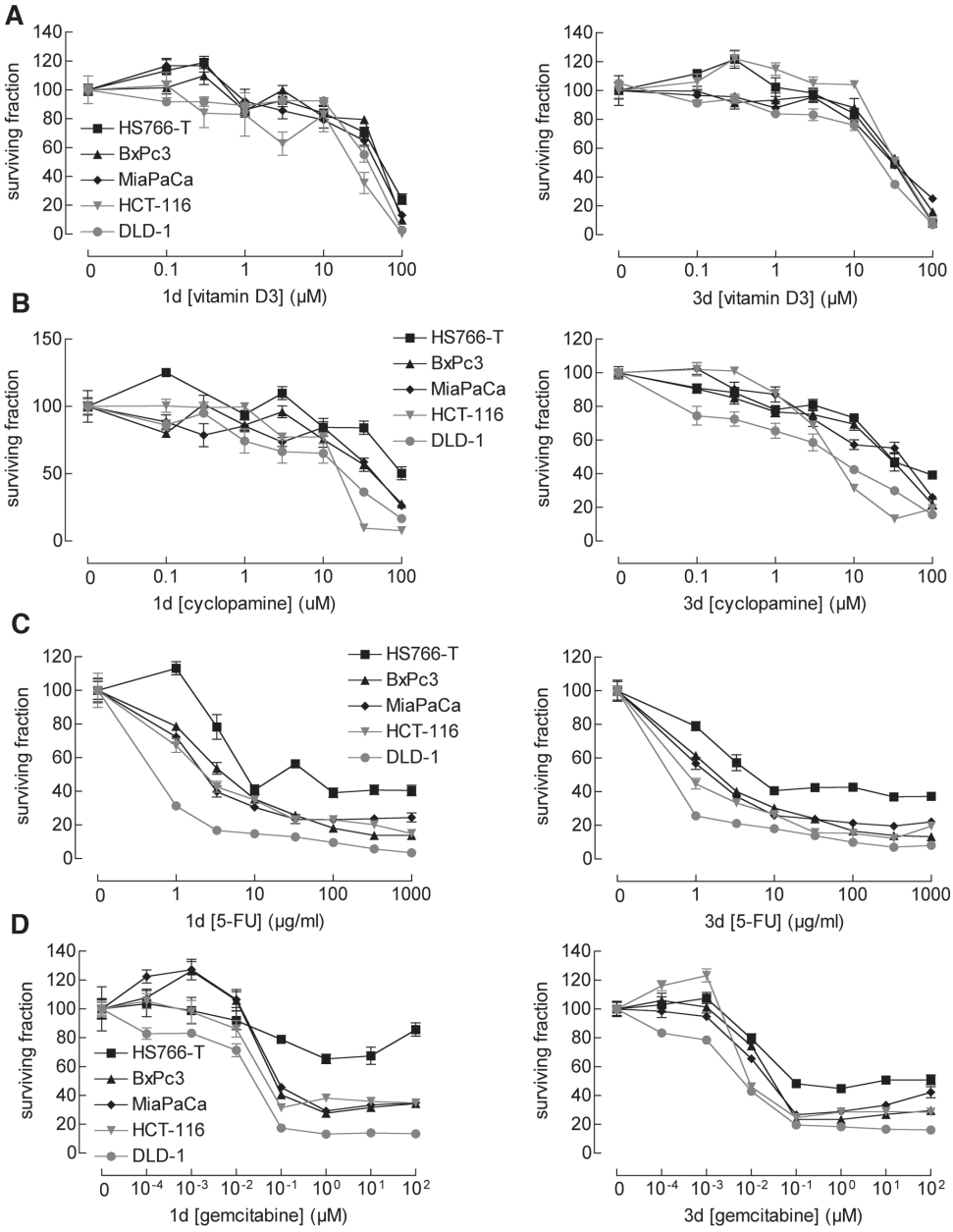

Figure 5. Vitamin D3 does not inhibit pancreatic adenocarcinoma cell growth in adherent and proliferating cells. (A) Indicated pancreatic adenocarcinoma cells and HCT-116 and DLD-1 colon cancer cells in 10% FCS were seeded in 96-well plates and after allowing growth for 1d or 3d, exposed to vitamin D3. 24h later, cell viability was assessed by MTT reduction. Data are expressed as percentage of control stimulated cells (0 μM; mean±SEM, n=8). (B) As for 5A, using cyclopamine. (C) As for 5A, using 5-FU. (D) As for 5A, using gemcitabine.

Additionally, the culture medium contains a higher concentration of serum (10% FCS) in these experiments, which is known to confer some growth and/or survival benefit.
Using these models, vitamin D3 is effective against tumor cell growth at very high concentrations (Fig. 5A). Moreover, we found no increased sensitivity to vitamin D3 for cells that are thought to be dependent on excessively activated Hh signaling (Hs766-T, BxPC3, MIA PaCa-2) compared to those that are not (HCT-116, DLD-1). Likewise, cyclopamine only inhibited tumor cell growth at high concentrations (Fig. 5B) and no difference in sensitivity was found between Hh-dependent and –independent cell types. Interestingly, the efficacy of cyclopamine was higher in this model than the one used for Fig. 1. Together these results indicate that although vitamin D3 and cyclopamine are potent inhibitors of the Hh pathway, and have been described to have anti-tumor effects, they do not inhibit tumor cell growth in most of the tumor models shown here.
As a positive control, we evaluated the cell growth-inhibitory effect of the traditional cytostatic nucleoside analogues fluorouracil (5-FU) and gemcitabine. 5-FU strongly inhibited cell growth already after 1d of treatment (Fig. 5C) and this effect was enhanced following longer treatment. Similarly, gemcitabine was a potent cell growth inhibitor after 1 or 3d (Fig. 5D). These results indicate that in this specific in vitro model for tumor cell growth, intervention is possible and that had vitamin D3 and cyclopamine been truly effective anti-tumor agents, an effect on MTT reduction should have been observed.

Vitamin D3 does not synergize with nucleoside analogues or irradiation

As treatment with vitamin D3 alone was not effective against tumor cell growth, we assessed if vitamin D3 might act synergistically with 5-FU or gemcitabine. To this end, proliferating cells in 10% FCS were treated with increasing concentrations of 5-FU with either 0 or 33 μM vitamin D3 for 3d (Fig. 6A). 5-FU was effective against tumor cell growth at relatively low concentrations and this could be enhanced by the addition of 33μM vitamin D3 for most cell types, although this effect was not synergistic. The same effect was found when using gemcitabine (Fig. 6B). In addition to not acting synergistically, for most cell types tested, the effect was also not additive, again consolidating the inadequacy of vitamin D3 as an anti-tumor agent.
A similar set of experiments addressing a possible synergy between treatment strategies was performed using irradiation in conjunction with vitamin D3. Pancreatic carcinoma cell lines (Fig. 6C-E) were treated with increasing concentrations of vitamin D3, and these cells were subsequently exposed to various doses of γ-radiation. After 3d, MTT reduction assays were performed and again, no synergistic effect of vitamin D3 on irradiation was observed, although the tumor cell inhibitory effect was additive. These somewhat diminutive effects of vitamin D3, even in conjunction with very strong anti-tumor treatments (high concentrations of nucleoside analogues or doses of radiation) lead us to conclude that although vitamin D3 exerts a strong Hh-inhibitory effect, it does not appear to be a very promising anti-tumor agent by itself or as an adjuvans for Hh-dependent malignancies.

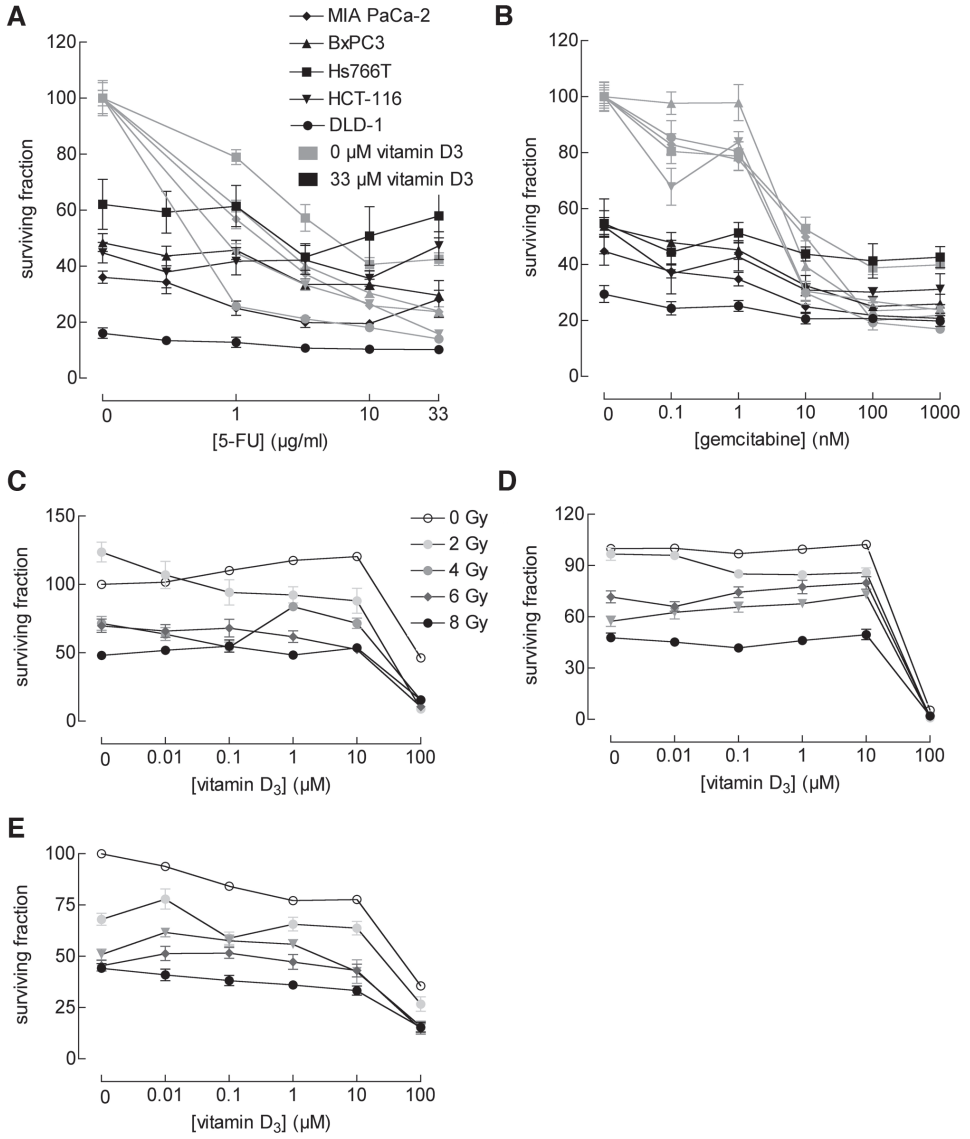

Figure 6. Vitamin D3 does not act synergistically with nucleoside analogues or radiation to inhibit pancreatic adenocarcinoma cell growth. (A) Indicated pancreatic adenocarcinoma cells and HCT-116 and DLD-1 colon cancer cells in 10% FCS were seeded and after allowing growth for 1d, exposed to either 0 (grey lines) or 33 µM (black lines) vitamin D3 in addition to 5-FU. After 3d, cell viability was assessed by MTT reduction. Data are expressed as percentage of 0 µM vitamin D3 / 0 µg/ml 5-FU. (B) As for 6A, using increasing concentrations of gemcitabine. (C-E) PANC-1, BxPC3 and 10.7 cells were treated with vitamin D3 and subsequently exposed to different doses of γ-radiation.

Discussion

Anti-tumor activity of vitamin D3 has been shown extensively in tumor models and in epidemiological studies. In tumor models, several mechanisms, like for instance inhibition of IL-8 induced angiogenesis, and activation of the Akt/mTOR pathway, have been shown to mediate the inhibitory effect. Nearly all of these effects are mediated through the VDR, although alternative means of perceiving vitamin D3 are available to a cell [16]. We previously identified vitamin D3 as a strong inhibitor of the Hh pathway, and we hypothesized that vitamin D3 would exert an inhibitory effect on those cancer cells that are critically dependent on an excessively activated Hh pathway, by inactivation of Smo rather than activation of the VDR.

Perhaps the most notorious of these Hh-dependent tumors are malignancies of the upper gastrointestinal tract, which rely on Hh signaling for their growth. Pancreatic cancer is a prime example of such an Hh-dependent tumor and it was this malignancy that we addressed in this study. In cell-based models that were geared towards showing drug sensitivity, vitamin D3 could inhibit proliferation of pancreas cancer cells through inhibition of the Hh pathway, and the cytotoxic activity of vitamin D3 was mediated through inhibition of Smo rather than activation of the VDR. Tumor cell types that were known not to critically rely on an excessively activated Hh pathway (hepato-, and coloncarcinoma cells) were not inhibited in their growth by vitamin D3, consolidating its effect to be mediated through the Hh pathway. Also, vitamin D3 showed a stronger cytotoxic effect than the benchmark Hh pathway inhibitor cyclopamine in the drug-sensitive models.

When we turned to in vivo models for pancreatic adenocarcinoma in mice and chick embryos, vitamin D3 showed no anti-tumor activity, even at a dose that had previously been described to be high but sub-lethal [32,33]. We are confident that had vitamin D3 harbored any considerable anti-tumor activity, it should have yielded a beneficial effect in our experimental set-up. It should be noted in this regard that despite the considerable amount of evidence for a beneficial role of vitamin D3 in pancreatic cancer, a fairly recent epidemiological study showed a detrimental correlation between vitamin D3 levels and the incidence of pancreatic cancer [34].

The lack of efficacy in vivo was confirmed in cell-based models that were much less geared towards drug sensitivity than the model that showed Hh-specific cytotoxicity of vitamin D3. Drug-sensitive models are obviously useful for mechanistic studies as they will show a large effect at relatively low concentrations of the drug of interest, but our in vivo studies show that they are not necessarily relevant to the clinical potential of the drug tested. To determine the cytotoxic capacity of a given compound, it is arguably better to choose an in vitro model that will actually offer some resistance to the cells from environmental clues, by for instance allowing adherence or providing a higher serum concentration. In these cell-based models, we were unable to find a synergistic effect of vitamin D3 when used in conjunction with nucleoside analogue cytostatics as well as γ-irradiation, casting further doubt on the anti-tumor potential of vitamin D3. In analogy to this approach, it was recently shown that Smo inhibition improved the delivery of gemcitabine to the tumor in a genetic model for pancreatic adenocarcinoma thereby greatly increasing the efficacy of gemcitabine [21]. Moreover, a recent study showed a strict requirement for combined mTOR/Smo/gemcitabine treatment for successful elimination of the cancer progenitor cell

pool in pancreatic cancer [35].
Another explanation for the lack of efficacy in vivo, besides the abovementioned need for combined therapy, might be the recently described paracrine Shh signaling model for the tumors of the upper GI tract [20]. These tumors overexpress Shh (as was previously known), but do not respond to, or rely on this Shh themselves. Rather, the Shh ligand is secreted and signals to the stromal cells, which in return supply the tumor cells with survival or growth factors. This paracrine mode of action might explain why our in vitro models were unsuccessful, as no stromal compartment is present in these models to respond to the Hh pathway inhibition, but the in vivo models should have shown an effect as both the tumor as well as the stromal cells are exposed to the Hh-inhibitory action of vitamin D3.
Concluding, we established that although vitamin D3 strongly inhibits the Hh pathway in tumor cells that are dependent on this pathway, it did not effectively inhibit their growth in vivo and in some in vitro models. These results were somewhat unexpected and could mean that vitamin D3 as single treatment strategy will not yield a therapeutic option for Hh-dependent malignancies.

Acknowledgements

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Chapter 4

Canonical Hedgehog signaling is essential for proangiogenic responses in endothelial cells

Comment on: Chinchilla P, et al. Cell Cycle 2010; 9:570–9

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Cell Cycle, 2010

Hedgehog (Hh) proteins constitute a highly conserved family of intercellular signaling molecules that are fundamental regulators of embryonic development as illustrated by dramatic embryonic malformations seen in humans and mice with perturbed Hh signal transduction [1]. The last decade it has become evident that the Hh pathway also remains active in the post-embryonic period and has for instance been found to play an essential role in maintaining tissue integrity and in tissue revascularization after ischemic stress [2,3]. The ligation of Hh ligand with its cellular receptor Patched (Ptch) alleviates the inhibitory action of Ptch on the associated receptor Smoothened (Smo) leading to the dissociation of a "Gli-inhibitor" complex. Subsequently, nuclear translocation of Gli transcription factors induces expression of, among others, angiogenic genes like vascular endothelial growth factor (VEGF) and the angiopoietins (Ang)-1 and 2.2 Interestingly however, endothelial cells are considered to be unresponsive to Hh directly [4,5]. Rather, adjacent mesenchymal cells are thought to translate the presence of Hh ligand in signaling molecules that act on the endothelium.

In a recent paper published in Cell Cycle, Chinchilla and coworkers elegantly show that endothelial cells do respond to Hh although not by inducing the canonical Gli-dependent pathway6 Instead, Hh induces Smo-dependent actin stress fiber formation in a Gi-protein and Rac1-dependent manner without inducing a transcriptional response. This non-canonical Hh pathway, previously shown to induce lamellipodia formation and consequent chemotaxis towards Hh in fibroblasts [7] and to induce the projection of neurites in neuralized embryonic stem cells8, induced tubulogenesis of endothelial cells suggesting that Hh controls angiogenesis by targeting endothelial cells to prime the initial steps of angiogenesis and mesenchymal cells to subsequent vessel maturation. Noteworthy, Chinchilla and coworkers strongly suggest that tubulogenesis of endothelial cells is Gli-independent although they do not directly target Gli to prove or refute this hypothesis.

Interestingly, we have recently obtained data that strongly suggest a Gli-dependence for

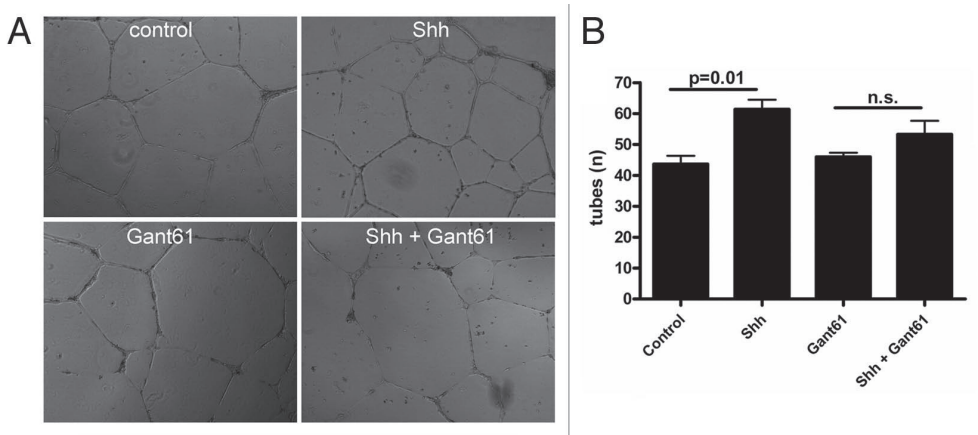

Figure 1. Gli1 inhibition by Gant61 reduces Shh-induced endothelial tubulogenesis. HUVECs ($4.5x10^4$ cells per well) were plated on growth factor reduced matrigel in a 24-well tissue culture plate in the absence (control) or presence of Shh, Gant61 or Shh/Gant61. After 24 hours stimulation, the number of tubes was measured in 5 independent fields at a 4x magnification. (A) Representative pictures of the different conditions. (B) Quantitative representation of the results depicted in panel A. Shown is the number of tubes+/-SEM (n=3). * $p<0.05$, ns: not significant.

tubulogenesis in response to Hh. We studied the effect on tubulogenesis in endothelial cells treated with GANT61, an inhibitor that interferes with Gli1 DNA binding and subsequently tumor cell proliferation both in vitro as well as in an in vivo xenograft model [9]. Both human umbilical vein endothelial cells (HUVECs) as well as immortalized 2H11 murine endothelial cells form proper endothelial tube like structures in response to Sonic Hedgehog (Figure 1 for HUVECs). Importantly, the addition of 5 μM GANT61 largely diminished Shh-induced tube formation showing that Gli1 plays an important role in Shh-induced tubulogenesis of endothelial cells. The role of the canonical Hh pathway, or at least that of the Gli family of transcription factors, in tubulogenesis and angiogenesis is therefore even more complex as anticipated. It seems however that, as already suggested by Dr Kanda and coworkers in 2003 [10], Hh induces tubulogenesis of endothelial cells through a combination of rapid Gi-dependent signaling and Gli1 mediated transcriptionally regulated pathways.

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Chapter 5

Human Plasma Very Low Density Lipoprotein Carries Indian Hedgehog

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Abstract

Hedgehog is one of the major morphogens and fulfils critical functions in both the development and maintenance of the vasculature. Hedgehog is highly hydrophobic and its diffusion toward target tissues remains only partly understood. In Drosophila, hedgehog transport via lipophorins is relevant for development, but neither the presence nor a function for a mammalian Hedgehog carried by human plasma lipoproteins has been established. We investigated the presence of Hedgehog on lipoprotein particles and determined its importance
for maintaining the endothelium. LTQ-Orbitrap XL analysis of defined plasma lipoproteins revealed that Indian Hedgehog (Ihh) is present in the human very low density lipoprotein (VLDL) fraction but not in other plasma lipoprotein fractions (low density lipoprotein (LDL) and high density lipoprotein (HDL)). Using the same approach, neither Sonic Hedgehog nor Desert Hedgehog could be detected in plasma lipoprotein fractions. Most likely, primary white adipocytes are the source of Ihh loading on VLDL as both transcriptome as well as immunofluorescence analysis showed high expression of Ihh in these cells. Additionally, we show that the endothelial compartment is most likely to be affected by the presence of Ihh on VLDL. Indeed, VLDL increased survival of primary endothelial cells, suggesting that Ihh transport by VLDL is important for maintaining the human endothelium. In conclusion, our study shows that VLDL carries Ihh throughout the body in mammals and Hedgehog signaling by human plasma VLDL particles may affect blood vessel pathophysiology. A combination of three state-of-the-art technologies, proteomics, genomics, and confocal microscopy, appeared to be a powerful tool for analyzing plasma lipoprotein-associated proteins.

Introduction

The Hedgehog (Hh) family of morphogenetic proteins consists of three isoforms, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), and is of pivotal importance for both embryonic developments as well as for the maintenance of adult vasculature [1]. In mice, genetically deficient for either Ihh or for the essential Hh signaling receptor Smoothened (SMO), embryonic yolk sack vascular development is either severely disturbed or completely absent [2]. In adult organisms, Hh is a potent inducer of vascularization[3] and Hh is both required and sufficient for angiogenesis following ischemia in experimental animals in vivo[4-6]. Finally, very recently it was shown that Hh signaling is essential for maintenance of the adult coronary vasculature in mice [7]. However, despite its obvious importance for cardiovascular physiology, many of the molecular mechanisms by which activation of Hh signaling in the vasculature is mediated remain obscure[8]. Hhs are highly unusual proteins in that they undergo autocatalytic proteolysis and are both sterolated and palmitoylated, resulting in a highly hydrophobic protein [9-11]. Following delivery to target cells, Hh binds to its transmembrane receptor Patched (Ptch1) [12]. Under Hh-unligated conditions, Ptch1 keeps a second transmembrane receptor called Smo inactive, in mammals probably by pumping a vitamin D3-like 3-hydroxysteroid [13,14] also a highly hydrophobic compound. Following binding of Hh to Ptch1, this inhibition is relieved and Smo subsequently initiates signaling through the family of Gli transcription factors [15]. Various mechanisms have been proposed as to how the hydrophobic Hhs may exert their effects over longer distances. One of the factors certainly involved is the formation of multimeric complexes, in which the hydrophobic parts of Hh are in the center of the complex, thus allowing diffusion through an aqueous medium [16]. In addition, Pana`kova` et al. described that in Drosophila Hh is carried by lipophorin (particles that are scaffolded by apolipoproteins and comprise a phospholipid monolayer surrounding a core of esterified cholesterol and triglyceride for transport through the body), and in larvae with reduced levels of this lipoprotein, Hh accumulates near its site of production resulting in developmental abnormalities [17]. However, whether a similar mechanism and function for the distribution of Hh proteins is present in vertebrates as well remains unknown. The above-mentioned considerations prompted us to investigate whether lipoproteins might be carriers of the Hh proteins in human blood and if so, whether such lipoprotein-carried Hh would be relevant for the endothelial compartment. Using tandem MS LTQ-Orbitrap XL, we were able to show that functional Ihh is carried by VLDL but not LDL and HDL and our data suggest that this VLDL-carried Ihh is relevant for maintaining endothelial cells.

Material and methods

Collection of Plasma, Isolation of Lipoprotein Fractions, and Delipidation of Lipoproteins

Blood was collected from fasted healthy volunteers after informed consent by venipuncture into tubes containing Na-citrate as anticoagulant (this study was approved by the Medical Ethical Committee of the University Medical Center Groningen under number,

METc2007.081). Subsequently, plasma was isolated by centrifugation at 1500× g for 15 min at 4 °C and stored at -80 °C for further analysis. To isolate plasma lipoproteins, salt gradient combined with ultracentrifugation was performed according to Kleinfeld et al [18] with some modifications as described previously. The lipoprotein fractions were then delipidated with two volumes of ice cold solution of diethyl ether: methanol (1: 3) [19]. The suspension was incubated for 15 min at room temperature followed by 45 min on ice. Subsequently, the mixture was centrifuged twice at 4000 rpm for 45 min at -5 °C. The supernatant was discarded, nd the pellets were dried and solubilized in appropriate buffers and detergents.

High Performance Gel Chromatography (HPGC) and LC-MS/MS Analysis

Collection of plasma lipoproteins from blood of consenting healthy volunteers has been described [3,19]. To control purity of the lipoprotein fractions, cholesterol profile was determined using HPGC using a Superose 6 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) according to routine procedures [19]. From the delipidated lipoproteins, a Dithiothreitol-reduced 1.5 mg protein fraction was loaded on a 20 × 20 cm 12.5% SDS-PAGE. Each lane was divided into 40 slices. After iodoacetamide alkylation, gel slices were exposed to tryptic in-gel digestion. Peptides were extracted and analyzed by advanced nanoLC-MS/MS (LTQ-Orbitrap-XL) on an Ultimate 3000 system (Dionex, Amsterdam, The Netherlands) and infused into the mass spectrometer via a dynamic nanospray probe. The automated gain control (AGC) was 5×10^5 charges and 1×10^4 charges for MS/MS at the linear ion trap analyzer. DDA cycle consisted of the survey scan within m/z 300-1600 at the Orbitrap analyzer with target mass resolution of 60 000 (fwhm, full width at half-maximum at m/z 400) followed by MS/MS fragmentation of the five most intense precursor ions under the relative collision energy of 35% in the linear trap. Ion selection threshold for triggering MS/MS experiments was 500 counts and analyzed according to stringent HUPO criteria.

Data Analysis

Protein identification was performed using the Turbo-SEQUEST algorithm in the BioWorks 3.1 software (Thermo Electron) and Uniprot database was applied to analyze the data (Swiss Institute of Bioinformatics (Prot database release 57, March 2009, 428 650 entries)). MS/MS spectra were searched with a mass tolerance of 2 ppm. Fixed and variable modifications were considered (Cys as carbamidomethylation (alkylation with iodoacetamide after cystine reduction results in the covalent addition of a carbamidomethyl group (57.07 Da) and prevents the formation of disulfide bonds) and Met as oxidized methionine, respectively), allowing one trypsin missed cleavage site and a precursor ion mass tolerance of 2 ppm and a mass tolerance of 0.8 amu (ion trap) for MS/MS fragment were used. The following HUPO SEQUEST criteria were selected for high confidence peptide identification: 1- charge state versus cross-correlation number (XCorr) > 1.9 for singly charged ions, XCorr >2.7 for doubly charged ions, and XCorr >3.70 for triply charged ions, 2- delta Cn 0.1, 3- peptide probability 0.001, 4- RsP 4, 5- final score (sf) 0.85) and 6- number of identified peptides [2].

Illumina BeadArray (mRNA Array)

For Illumina microarray analysis, total RNA was extracted from human primary subcutaneous adipocytes cultures. The quality and concentration of the RNA was determined by

Bioanalyser using the Agilent RNA 6000 Nano kit (Agilent, Amstelveen, The Netherlands). Illumina Total Prep RNA Amplification Kit was applied to amplify and label the RNA, according to instructions provided by Applied Biosystems (Applied Biosystems, Nieuwerkerk ad IJssel, The Netherlands). cDNA was made from total RNA and the concentrations were determined using Nanodrop technology. Biotinylated cRNA was made using the Illumina RNA Amplification Kit (Ambion, Inc., Austin, TX). Hybridization to the Sentrix HumanRef-8 V2 Expression BeadChip array (Illumina, Inc., San Diego, CA, USA), washing and scanning were done according to the Illumina Bead Station 500 manual (revision C). One BeadChip was used and the slide was scanned immediately. First line quality check, background correction and quantile normalization of the data was done with Bead studio Expression module v 3.2.7. Statistics and gene lists were generated using Gene spring GX 7.3.1 (Agilent).

Real Time PCR Analysis

Qiagen Rneasy lipid mini kit (Qiagen, GmbH) was used to isolate RNA from human primary subcutaneous adipocytes according to the manufacturer recommendations. The RNA was solved in 30 µL Rnase-free water and stored at -80 °C. Integrity of RNA and concentration was checked using a Nanodrop spectrophotometer (Isogen, Lifescience). For the cDNA synthesis 1 µg RNA was used with an end volume of 20 µL. Reverse transcription was performed with Quantitect Reverse Transcription kit (Qiagen GmbH). Relative Real-time PCR was performed on the AB Prism 7900HT Sequence detector (Applied Biosystems, U.K.) with a primer concentration of 900 nM, probe concentration of 250 nM and the input of cDNA was 25 ng. The PCR profile consisted of 10 min at 95 °C, followed by 40 cycles with heating of 95 °C for 15 s and cooling to 60 °C for 1 min.

Pathway Analysis of Ihh

KEGG software (http://www. genome.jp/kegg/), http://david.abcc.ncifcrf.gov/content.jsp?file)about_us.html, and STRING (http://string.embl.de/) were applied to gain more insight into hedgehog signaling pathway regarding Ihh protein.

Confocal Microscopy

Human primary subcutaneous adipocytes (from Promo Cell group Ltd., Switzerland) were cultured in six well plates and subsequently they were incubated with an antibody against: Indian hedgehog (Ihh; mAb (rabbit) Abcam (wet slides), USA). Bound antibodies were detected with an Alexa 562-coupled goat antirabbit antibody. Lipids and nuclei were stained with FITC and Topro, respectively. As control, adipocyte slides were not incubated with the primary antibodies and the rest of the procedure remained unchanged. Specific antibody binding was visualized using a high resolution Leica SP2 AOBS Confocal laser scan microscope (CLSM) (Leica Microsystems Nederland BV, The Netherlands), and images were obtained with Leica confocal software. Cell Culture and Treatments. Human Primary Coronary Artery Endothelial Cells (HPCAEC) were purchased from Lonza (Basel, Switzerland). The cells were maintained as suggested by the provider. Briefly, the cells were grown in EGM-2MV medium supplemented with 5% FCS, growth factors, hydrocortisone and gentamicine, at 37 °C in a 5% CO2 humidified atmosphere. For the viability assays, HPCAEC cells were treated with hedgehog pathway inhibitors, cyclopamine (5-15 µM) and Vitamin D3 (5-15 µM) for 24-72h. The cells were also treated with the purified human lipoproteins, VLDL, LDL and HDL. At

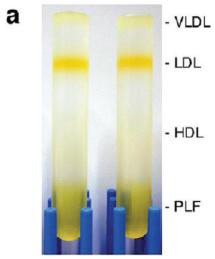
each time point, the viability of the cells was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), reduction assay,20 and cell counting with trypan blue.

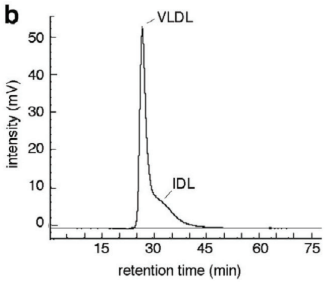
Results

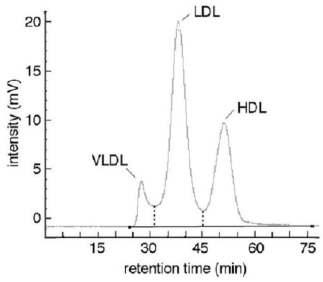
Human Indian Hedgehog is Carried by VLDL

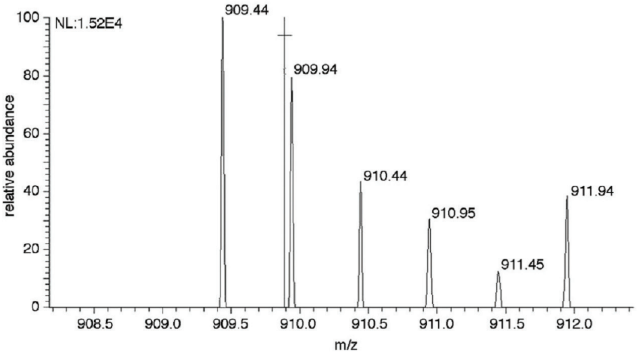
To investigate whether plasma lipoproteins or one of these lipoproteins are an in vivo carrier of Hh, the major Plasma lipoprotein fractions were collected using one-step salt gradient ultracentrifugation (Figure 1a), after which the purity of the lipoprotein fractions (VLDL, LDL, and HDL) was confirmed using high performance gel chromatography (HPGC) (Figure 1b and c) [19]. Subsequently these plasma lipoprotein fractions were trypsinized and the resulting proteolytic fraction was subjected to MS/MS analysis on a LTQ-Orbitrap XT mass spectrometer. Although analysis of neither HDL nor LDL showed the presence of Ihh, Shh, or Dhh, a strong Ihh signature was observed in the VLDL fraction (Figure 1d-g). The other Hh's were not detected in the VLDL lipoprotein fraction, demonstrating the specificity of this assay. In this context, it is important to note the technical complexity of working with purified VLDL fractions. Plasma lipoprotein VLDL consists mostly of triglyceride (TG, 60%), cholesterol (20%), phospholipids (15%), and only 5% proteins. Furthermore, of the protein fraction, approximately 90-95% constitutes apoB and other apoliproteins. However, Ihh was repeatedly and unambiguously identified as the major constituent of the remaining proteins in our VLDL fraction (Figure 1d-g) and thus represents a bona fide component of normal circulating VLDL. Hence like insect lipophorins, the mammalian VLDL carries a defined Hh ligand. Illumina BeadArray mRNA Profiling Reveals Expression of Hh Pathway Genes in adipocytes.

Next page: Figure 1. Identification of Ihh as a major hedgehog constituent of human VLDL particles. (a) Major human plasma lipoprotein fractions after one-step salt gradient ultracentrifugation. After that, the collected lipoprotein fractions were subjected to HPGC and compared to total pooled plasma. (b) VLDL fraction yielded a single peak with a retention time of 25 min. (c) HPGC elution of total plasma VLDL lipoprotein reveals identical elution properties, which confirms that the fraction contains only VLDL. LDL and HDL fractions isolated by the same method showed single peaks eluting at 33 and 43 min (data not shown), which were identical to those determined for total plasma (c) and commercial LDL and HDL lipoprotein, respectively (not shown). (d) Doubly charged precursor ion scan mass spectra of Ihh tryptic digest resulted in an m/z difference of 0.5 m/z. The MS/MS product ion spectrum (f, lowest panel) obtained from the fragmentation of this precursor ion at m/z 909.44 (d) with b-ions (red peaks) and y-ions (blue peaks) corresponding to the sequence EEGGFHPLGMS (e) coming from the tryptic Ihh peptide LLLEEGGFHPLGMSGAG. The sequence coverage was 4.5% with an extreme high peptide probability (3.2×10^{13}). The red and blue peaks in ms/ms spectra corresponding to b- and y-ions as depicted in (e). Accuracy of peptide masses was expressed as delta mass value (amu) as indicated in the y-as and amino acid sequence in x-as (f, upper panel). Protein identification was performed using the Turbo-SEQUEST algorithm in the BioWorks 3.1 software (Thermo Electron) and human database (Swiss Institute of Bioinformatics, Geneva, Switzerland). MS/MS spectra were searched with a mass tolerance of 2 ppm and the following HUPO SEQUEST criteria were selected for high confidence peptide identification: 1- charge state versus cross-correlation number (XCorr) > 1.9 for singly charged ions, XCorr >2.7 for doubly charged ions, and XCorr >3.70 for triply charged ions, 2- delta Cn 0.1, 3- peptide probability 0.001, 4- RsP 4, 5- final score (sf 0.85) and 6-identification of two peptides as shown in (g)









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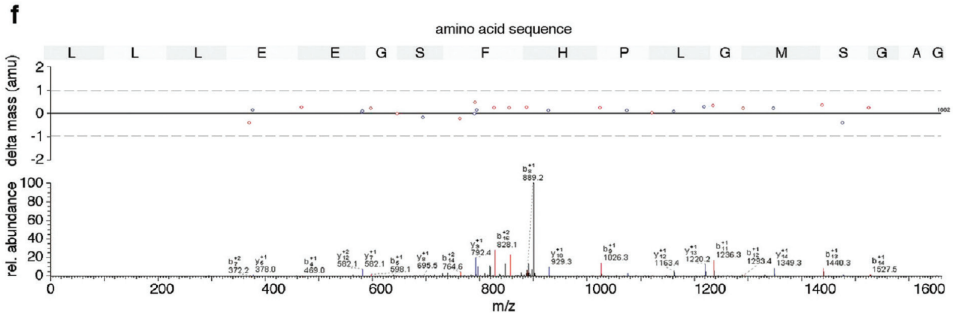
2	L	227.18	1704.79
3	L	340.26	1591.71
4	E	469.30	1478.62
5	E	598.34	1349.58
6	G	655.37	1220.54
7	S	742.40	1163.51
8	F	889.47	1076.48
9	H	1026.53	929.41
10	P	1123.58	792.36
11	L	1236.66	695.30
12	G	1293.68	582.22
13	M	1440.72	525.20
14	S	1527.75	378.16
15	G	1584.75	291.13
16	A	1655.81	234.11
17	G	1712.83	163.07



g

peptide	P(prob)	XC	ΔCn	sf	RSp	MW	accession	peptide (hits)
Human Indian hedgehog protein precursor	3.2e-13	10.2	4.4	0.85	4.4	45222.4	Q14623	2(2000)

To check whether liver express Ihh, human liver- (surplus material from donor transplantation procedures) derived RNA subjected to RT-PCR. In contrast to Shh, which is abundantly expressed in the liver [21] three independent RT-PCR analyses of human liver clearly showed that Ihh was not detectable or present at trace amounts (data not shown). In apparent agreement with a white adipocyte source for VLDL-carried Ihh, Illumina Beadarray analysis demonstrated substantial amounts of Ihh mRNA, although also some Shh mRNA and Dhh mRNA was detected in these white adipocytes (Figure 2a). Furthermore, human primary preadipocytes also contained substantial amounts of Ihh mRNA, suggesting that expression of Ihh seems to be a fundamental property of this cell type. RT-PCR analysis of human white adipocytes confirmed the strong expression of Ihh mRNA in these cells (Figure 2a inset). Moreover, the Illumina BeadArray mRNA profiling of adipocytes demonstrates high levels of Hh pathway target genes like Gli1, Ptch1, Dispatched-1 (Disp1) (Figure 2a), in apparent agreement with the presence of Ihh signaling in these cells. In addition, the functional integration and prediction of protein interactions involved in human Hh signaling pathway genes expressed in human white adipocytes were analyzed using STRING (http://string.embl.de/) with the high confidence setting (Figure 2b and c). The occurrence of a strong interaction between Hh signaling pathway components in human adipocytes confirmed that activation of this pathway is a bona fide property of human adipocytes. Besides, white adipocytes are known not to be involved in loading LDL and HDL particles (in contrast to the liver), as is also evident from the absence of a significant triglyceride constituent in LDL and HDL, and these lipoprotein particles also do not display detectable levels of Ihh. Ihh Protein Is Present in Human White Adipocytes. To confirm the presence of Ihh in white adipocytes, we next performed confocal microscopy analysis employing Ihh-specific antibodies. As shown in Figure 3, Ihh protein is omnipresent in white adipocytes (Figure 3c). Interestingly, the overlay presented in Figure 3d (depicting nuclei in blue, fat organelles in green and Ihh in red) shows that the Ihh protein was located mainly on the surface of the plasma membrane of the white adipocytes. These data thus suggest that white adipocytes are likely to be the source for the loading of Ihh on VLDL molecules. VLDL-Carried Ihh Maintains the Endothelial Compartment. In efforts to define the biological activity of VLDL-carried Ihh, we investigated the endothelium as a possible target. The vascular network is widely recognized to be targeted by Hh proteins as these morphogens constitute an important angiogenic signal following ischemic stress. Upon isolation of human healthy vessels, it appeared that these vessels show constitutively high Hh signal transduction activity (data not shown), as judged from the expression of the bona fide Hh targets Ptch1 and Gli1 in normal human vessels. Importantly, treatment of HPCAEC cells with Smo inhibitors, cyclopamine and Vitamin D3 for 24-72 h reduced cell viability, suggesting that Hh pathway activity is important to maintain HPCAEC viability (Figure 4a and b). Interestingly, HDL and LDL treatment (100 µg/mL of human purified plasma lipoproteins-associated proteins for 24 h) had no effect on cell viability. However, when VLDL was added to HPCAEC cultures, it enhanced endothelial cell survival substantially (Figure 4c). Thus, VLDL-carried Ihh seems both necessary as well as sufficient for the maintenance of endothelial integrity. These data correspond well with a recent report showing that Hh signaling in general is important for vascular integrity in at least the murine coronary circulation [7].

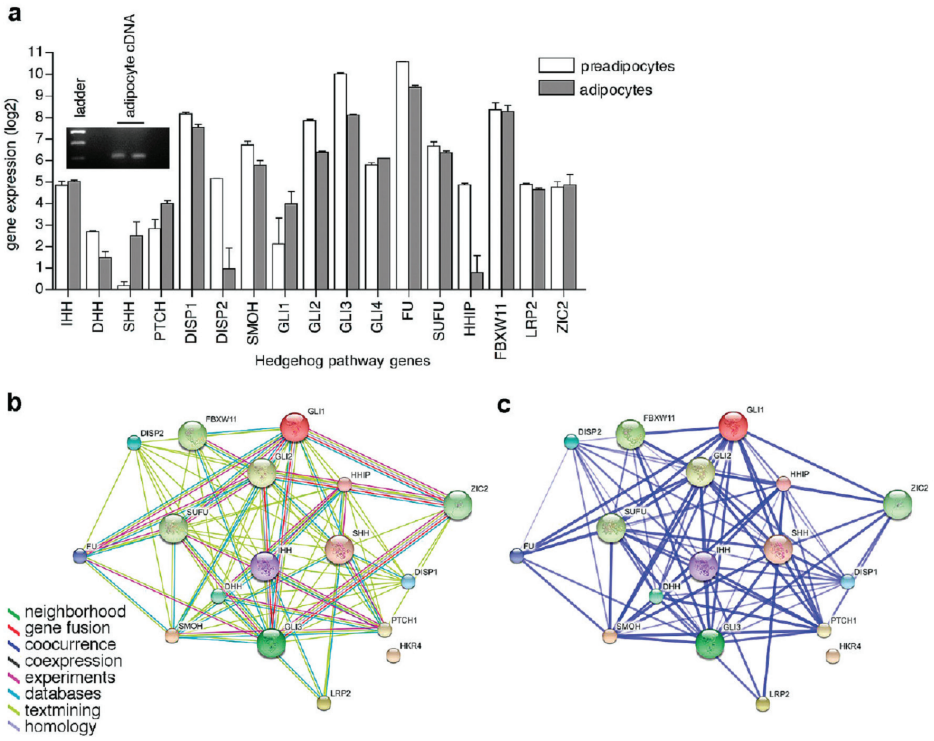


Figure 2. Hh pathway components are expressed in white adipocytes. (a) Illumina Gene profiling analysis exhibits the expression of complete Hh pathway components in white adipocytes. X-axis represents Ihh genes expressed by preadipocytes (white bars) and adipocytes (black bars). Gene expression was expressed as 2-log and shown on y-axis. RT-PCR products were run on 2% agarose gel. Two independent prepared human white adipocyte cDNAs showed that Ihh is very highly present in white adipocytes (a; inset). Ihh signaling genes interaction analysis was performed by string software (b and c). (b) Evidence view. The type of evidence for the association was indicated by the different line colors. (c) Confidence view of protein protein interaction and stronger associations are represented by thicker blue lines.

Discussion

Mammalian lipoproteins can roughly be subdivided in VLDL, LDL, and HDL molecules. Functionally and genetically, lipophorin is most related to VLDL (e.g., VLDL and lipophorins share the unique trait of being triglyceride-carrying lipoprotechnical teins), but lipophorin also substantially resembles LDL and HDL [19]. VLDL particles are synthesized in the liver, but loading with triglycerides takes place in the white adipocytes. The latter compartment also seems to be the source of Hh in VLDL molecules, as the VLDL-synthesizing liver parenchyma cells express only traces of Ihh [21,22]. Hh is a highly unusual intercellular messenger

[23,24] as the protein is both sterolated [25] and palmitoylated[9] and therefore particularly hydrophobic. For this reason, Hh's diffusion capacity is strongly limited in the aqueous medium that surrounds cells. Hh, however, has been shown to be capable of long-range signaling [23,24] and various models have been proposed to explain Hh distribution in tissue. First, the formation of Hh multimers [25] in which the hydrophobic groups of several Hh molecules are folded together so that the formed complex is soluble in an aqueous medium [26]. Alternatively, cells might transport Hh by several rounds of endo- and exocytosis. A third possible mechanism of longrange Hh transport relies on transportation of Hh on nodal vesicular parcels (NVP) [27]. In this model, the Hh-loaded NVPs are transported through the ventral node fluid by ciliary movement. An important breakthrough in this field was the study of Pana´kova´ et al. [17] who showed that in Drosophila Hh can be carried by lipoproteins. In the present study, we show that VLDL is the Hh carrying particle in analogy to the insect lipophorin. In retrospect this is expected, as the lipid composition of insect lipophorins is much more reminiscent of VLDL as compared to LDL and HDL. Hence a role for lipoproteins for carrying the Hh protein seems strongly conserved during evolution.
In the past it has already been suggested that Hh might be present on human lipoproteins[14] but with conventional techniques, this hypothesis could not yet be validated. In the current manuscript, we combine state of the art LTQOrbitrap XL technology with unique protein purification procedures and large-scale SDS-PAGE. First, we purified VLDL from human plasma using a modified salt gradient centrifugation step resulting in highly pure lipoprotein fractions[19]. Next, the VLDL fraction was delipidated thereby removing 80-90% of the VLDL associated (phospho)lipids allowing us to obtain a pure fraction of VLDL associated proteins. Due to the delipidation, low abundant hydrophobic proteins remain present in the protein fraction and not lost in the lipid fraction. Finally, the lipid free protein fraction was loaded on a 24 × 24 cm SDS-PAGE gel allowing us to load 1.5 mg of VLDL derived proteins (which would resemble the loading of 30 mg VLDL or 250 mL of blood). Only by using this novel approach, we were able to identify a low abundant protein as Ihh on VLDL.

The biological function for Ihh loading on VLDL may well lie in the support of the edothelial compartment. In the present study we show that endothelial cells require active Hh signaling for survival, at least during the in vitroconditions of our primary endothelial cultures, and we observed pro-survival effects from exogenous application of VLDL molecules. Such a role seems to be supported, at least in part by the current body of scientific literature. A recent report showed that Hh signaling in general is important for vascular integrity in at least the coronary circulation [7] Earlier, Kusano et al[6] demonstrated in an elegant experimental setup that intramyocardial gene transfer of Shh promoted recovery and preservation of left ventricular function in both acute and chronic myocardial ischemia by enhanced neovascularization and recruitment of bone marrow-derived endothelial progenitor cells[6] and this corresponded well with the observation that Hh mediates a profound upregulation of target genes involved in angiogenesis [4,5]. In cerebral ischemia, a positive role for Hh-mimicking molecules has also been shown in limiting the damage caused by artificial vessel occlusion in rats[28]. Together, these data strongly support the notion that Hh aids in the rescue of ischemic tissue, possibly through direct effects on endothelial physiology. Our study suggests that Hh reaches the endothelial compartment to exert its effects through VLDL. As Hh signaling is associated with neoangiogenesis and neoangiogenesis in turn is associated

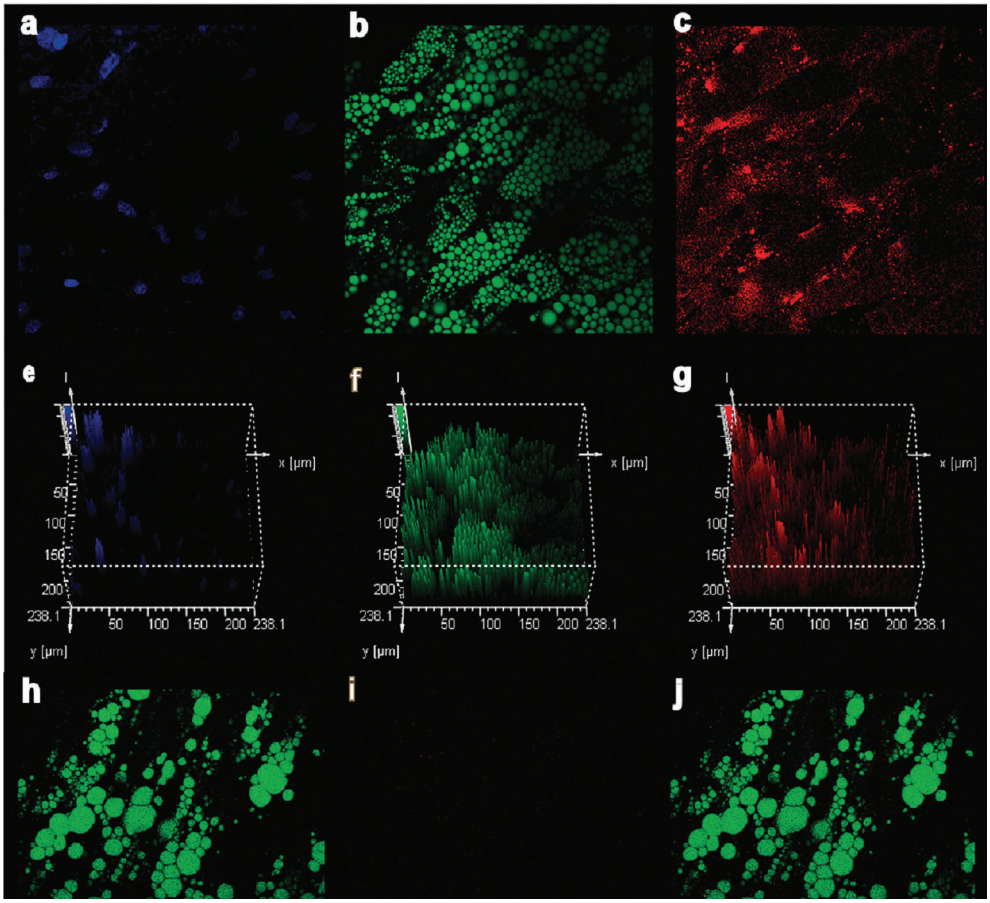


Figure 3. Ihh protein is localized on the surface of white adipocytes. From (a k), confocal microscopy nalysis was applied to localize Ihh protein in the white adipocytes. White adipocytes wet slides were tained with Topro to detect nuclei (A, blue color), fat organelle (lipids) were visualized with FITC (b) and adipocyte slide was incubated with Mab against human Ihh. Bound antibodies were detected with alexa 62 coupled goat antirabbit (c) and (d) exhibits an overlay of all three labeling (a, b, and c). Ihh protein was located on the surface of plasma membrane as depicted in Figure 2c. e, f, and g constitute a three-dimensional (3D) view of a, b, and c, respectively. In h and i, another white adipocytes wet slide was also stained only with FITC to detect fat organelle (h, as control for fluorescent contribution to background) and with alexa 562 coupled goat antirabbit (i; considered as negative control). This slide was not incubated with primary Mab against human Ihh. (j) Merged state. (k) 3-Dimensional view of (i). Fluorescent labeling was used for all detections. Original magnification used was 400 times.

with plaque instability and subsequent coronary events, it appears that lipoproteins are multiedged swords in human pathophysiology. VLDL carried Ihh might be important for maintaining endothelial integrity, but its pro-angiogenesis action might be deleterious once a plaque actually forms. Thus like understanding the interaction of Hh signaling with those of other morphogenetic signals, also the understanding of the interaction between Hh signaling and lipoprotein action on the endothelium may be essential for a better comprehension of the pathogenesis of cardiovascular disease. Regardless the actual

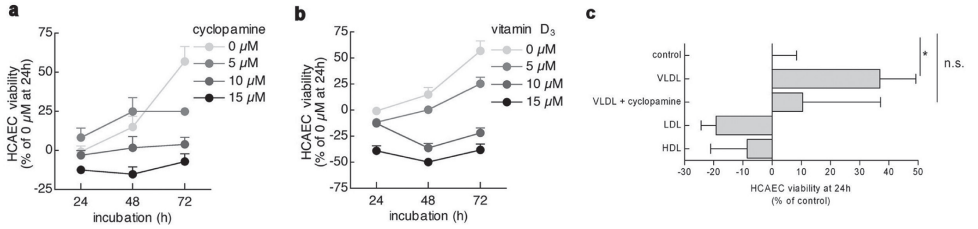


Figure 4. Maintenance of endothelial cell viability requires Hh signaling. Human primary coronary artery endothelial cells (HPCAEC) were treated with Hh pathway antagonist (cyclopamine 10 μM and Vitamin D3 10 μM) for 24 72 h or human purified main plasma lipoproteins, VLDL, LDL and HDL as well as a combination of VLDL and the Hh pathway antagonist cyclopamine 10 μM for 24 h. (a and b) Blockade of Hh signaling pathway with cyclopamine or vitamin D3 reduced viability of HPCAEC cells in culture as determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), reduction assays. (c) VLDL increased proliferation of HPCAEC cells, while HDL and LDL did not affect the viability of HPCAEC. HPCAEC cells were also treated with VLDL in combination with cyclopamine. Thereafter, cell viability was determined by cell counting in the presence of trypan blue.

(patho)physiological importance of Ihh carried on VLDL, the current manuscript shows that in analogy to Drosophila human lipoproteins do carry Ihh. Only by combining state of the art LTQ-Orbitrap XL technology with unique protein purification procedures and large-scale SDSPAGE, we were able to identify a low abundant protein as Ihh on VLDL.

Acknowledgements

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Chapter 6

Reversible phosphorylation in haematological malignancies: Potential role for protein tyrosine phosphatases in treatment?

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Abstract

Most aspects of leukocyte physiology are under the control of reversible tyrosine phosphorylation. It is clear that excessive phosphorylation of signal transduction elements is a pivotal element of many different pathologies including haematological malignancies and accordingly, strategies that target such phosphorylation have clinically been proven highly successful for treatment of multiple types of leukemias and lymphomas. Cellular phosphorylation status is dependent on the resultant activity of kinases and phosphatases. The cell biology of the former is nowwell understood; formost cellular phosphoproteinswe nowknowthe kinases responsible for their phosphorylation and we understand the principles of their aberrant activity in disease. With respect to phosphatases, however, our knowledge ismuch patchier. Although the sequences of whole genomes allow us to identify phosphatases using in silicomethodology, whereas transcription profiling allows us to understand how phosphatase expression is regulated during disease,most functional questions as to substrate specificity, dynamic regulation of phosphatase activity and potential for therapeutic intervention are still to a large degree open. Nevertheless, recent studies have allowed us tomakemeaningful statements on the role of tyrosine phosphatase activity in the threemajor signaling pathways that are commonly affected in leukemias, i.e. the Ras–Raf–ERK1/2, the Jak–STAT and the PI3K–PKB–mTOR pathways. Lessons learned from these pathways may well be applicable elsewhere in leukocyte biology as well.

Introduction

Haematological malignancies account for approximately 10% of cancer in adults and 40% of paediatric cancers [1]. Annually, more than 100,000 newcases of leukemia, lymphoma ormyeloma are diagnosed in the US alone, resulting in 60,000 deaths per year [2]. A host of genetic differences and molecular aberrations affecting blood cells at different stages of differentiation leads to a wide range of haematological tumors with different clinical manifestations. Many haematopoietic signaling pathways are regulated by protein and lipid phosphorylation, a process generally mediated by kinase activity. As proper regulation of phosphorylation events is critical for cellular homeostasis, dysregulation of activity of a single protein kinase can result in oncogenic transformation, characterized by increased cell division, growth, decreased differentiation and/or cell death [3]. Indeed, numerous protein kinases have welldefined, causative roles in human haematologicalmalignancies. Several of these fall into the class of receptor tyrosine kinases (RTK). For instance, activating mutations of the FLT3 RTK are found in 30% of acute myeloid leukemia (AML) patients and contribute to myeloid pathogenesis [4]. Others include activating pointmutations or the generation of activating fusion partners due to diverse translocations involving the platelet derived growth factor (PDGF) receptor, c-KIT or the fibroblast growth factor (FGF) receptor (reviewed in [5]). In addition, inappropriate activation or overexpression of non-receptor kinases have been reported in a wide range of haematological malignancies. Interestingly,most kinase deregulations observed in haematological cancers seem to affect one or more of three major signaling pathways: i) the Ras–Raf–MEK–extracellular signal regulated kinase (ERK1/2) pathway; ii) the Jak–signal transducer and activator of transcription (STAT) pathways; and iii) the phosphatidylinositol kinase (PI3K)–protein kinase B (PKB/AKT)–mammalian target of rapamycin (mTOR) pathway [6]. Aberrant expression or activity of kinases within these pathways themselves has been described independent of disturbed RTK signaling. The balance between activation and deactivation of signaling pathways is a delicate one, which is regulated not only through phosphorylation by kinases, but also through dephosphorylation events induced by a diverse range of phosphatases. Intuitively, by counter-balancing the activity of kinases, phosphatases should primarily act as tumor suppressors. However, novel regulatory actions of phosphatases in cancer are emerging, not only as negative modulators of kinase activity but also as causative agents in themselves [7]. Although the role of aberrant kinase activity and the potential for tyrosine and serine/threonine kinase inhibitors in the treatment of various leukemias have been investigated and reviewed extensively [8–11],the role of phosphatases in haematological malignancies has received less attention to date. In this review, we address the mechanisms by which some phosphatases may affect haematological malignancies and discuss their potential in therapeutic treatment. We explore general characteristics of those phosphatases, their role in the signaling pathways in the homeostatic cell and the result of their dysregulation in the progression or suppression of haematological malignancies.

Protein phosphatases

Two major families of protein phosphatases can be distinguished based on their substrate

Table 1 Classification of phosphatases.

	Family	Class	Sub-class (members)	Discussed in this review
PP	PTP	Class I (classic)	RPTP (21)	CD45
			NRPTP (17)	PTP1B, TC-PTP
				SHP-1, SHP-2
				HePTP
		Class I dual specificity phosphatase (DUSP)	MKPs (11)	DUSP-1/MKP-1, DUSP2/ PAC-1, DUSP16/MKP-7
			Atypical DUSPs (19)	
			Slingshots (3)	
			PRLs (3)	
			CDC14s (4)	
			Myotubularins (16)	
			PTENs (5)	PTEN
		Class II	LMWPTP (1)	LMWPTP
		Class III	CDC25 (3)	
		Asp-based PTPs	EyA (4)	
	STP	PPP	PP1	
			PP2A	
			PP2B	
			PP4	
			PP5	
			PP6	
			PP7	
		PPM	PP2B	
IP	IP4P			
	IP5P			SHIP-1, SHIP-2

PP: protein phosphatase; IP: inositol phosphatase; PTP: protein tyrosine phosphatase; STP: serine/threonine phosphatase. STPs are subdivided into monomeric Mg2+-dependent phosphatases (PPM) and a larger class of phosphatases (PPP) whose substrate specificity is determined by the formation multimeric holoenzyme complexes. PTPs are subdivided into Class I, II, III and Asp-based PTPs. Class I PTPs consists of RPTP: receptor PTP and NRPTP: nonreceptor PTPs. The IPs are classified based on the position of the inositol ring which they dephosphorylate; IP4P: inositol polyphosphate 4-phosphatase; IP5P: inositol polyphosphate 4-phosphatase.

specificity; the serine/threonine phosphatases (STP) and protein tyrosine phosphatases (PTP) (Table 1). Recently, a third class of protein histidine-phosphatases has emerged, but relatively little information is available onthis group of phosphatases (reviewed in [12]). Of the 148 human protein phosphatase genes recognized, only 30 encode for STP [12,13]. Despite there being only few STPs, more than 400 genes coding for serine/threonine kinases are identified, accounting for themajority of protein phosphorylation. This suggests a large degree of substrate-promiscuity of STPs. For this reason, although alterations in PPP family members have been described in (haematopoietic) cancer cells, and STPs appear to be viable cancer therapy targets [14,15] this group will not be further discussed in this review.
Of the 107 PTPs, only 81 are thought to be catalytically active and dephosphorylate phosphotyrosine residues, corresponding to 90 protein tyrosine kinases, of which around 80 are suggested to be catalytically active. This implies that there is a comparable substrate specificity of PTKs and PTPs, and an equal contribution in regulating tyrosine phosphorylation patterns. This is further suggested by the parallel tissue distribution of PTPs and PTKs, with haematopoietic tissues expressing the highest proportion of all PTPs [16].Within the PTPs, different classes are recognized based on amino acid sequence in the phosphatase

catalytic domains. Class I is by far the largest and can be subdivided into classical PTPs and dual specificity PTPs (DUSP), which aside from dephosphorylating both tyrosine and serine/threonine residues also recognize other substrates. The classical PTPs can be further separated into receptor PTPs (RPTP), and non-receptor PTPs (NRPTP),whereas the DUSPs are separated into 7 distinct categories (Table 1). Of these, the MKPs show specific activity towards mitogen-activated protein (MAP) kinase–ERK1/2 pathway, and are as such of particular interest in tumors where over-activation of this pathway is found [17]. Of particular interest is phosphatase and tensin homolog (PTEN), which is ordered under the DUSPs as it contains the cysteine-cased motif that classifies PTPs, [18] but seems to have a higher specificity for the 3 position of the inositol ring of the lipid phosphatidylinositol 3,4,5 phosphate (PtdIns (3,4,5)P3), and can therefore also be classified as a lipid phosphatase [19,20]. Lipid phosphatases generally counteract the role of the PI3K–PKB–mTOR pathway by dephosphorylating PIP3, and are classified by the position of the phosphate group they remove. There are 10 mammalian 5-inositol phosphatases known to date, two of which are the SH domain-containing inositol phosphatases, SHIP-1 and SHIP-2. In this review we will focus our attention on PTPs that counterac the three major signaling pathways commonly associated with haematological cancers, i.e. the Ras–Raf–ERK1/2, the Jak–STAT and the PI3K–PKB–mTOR pathways (Fig. 1). The biochemical properties o these phosphatases and their potential role in haematologica malignancies will be discussed.

JAK–STAT signaling

There are 4members of the Janus family of tyrosine kinases (JAKs); Jak1, Jak2, Jak3, and Tyk2. Cytokine stimulation of cells results in receptor dimerization, bringing their associated JAKs into close proximity to each other and allowing JAK cross-phosphorylation The subsequent activation of JAKs enables their phosphorylation of target proteins, which include the receptor itself and their major substrates; STATs. Cytoplasmatic STATs, of which 7 family members are known in mammals, are activated upon tyrosine phosphorylation which lead to their dimerization, nuclear translocation and regulation of target genes expression [21]. Mutations and translocations in the JAK genes (e.g. JAK2V617F) leading to constitutively active JAK proteins are associatedwith a variety of haematopoieticmalignancies including acute myeloid and lymphoblastic leukemias (JAK1 and JAK2), acute megakaryoblastic leukemia (JAK2 and JAK3), T-cell precursor acute lymphoblastic leukemia (JAK1), MPD and polycythemia vera (JAK2) [22–24]. Aberrant STAT activation has been observed in several leukemias as well (reviewed in [25]). Interestingly, STAT activation is not always linked to increased JAK activity in these diseases; the oncogenes Bcr–Ab and TEL–PDGFR, aswell as c-src have been described to activate STAT1 STAT3 or STAT5 independently of JAKs [26,27]. Careful modulation of the JAK/STAT pathway is essential for normal cell homeostasis, and can be regulated negatively or positively by protein phosphatases depending on the PTP involved, intensity levels of the pathways and cellular context. CD45, SHP-1, SHP-2, PTP1B, TC-PTP and LMWPTP [22,28,29] have all been demonstrated to modulate JAK/STAT pathways, and will be discussed below.

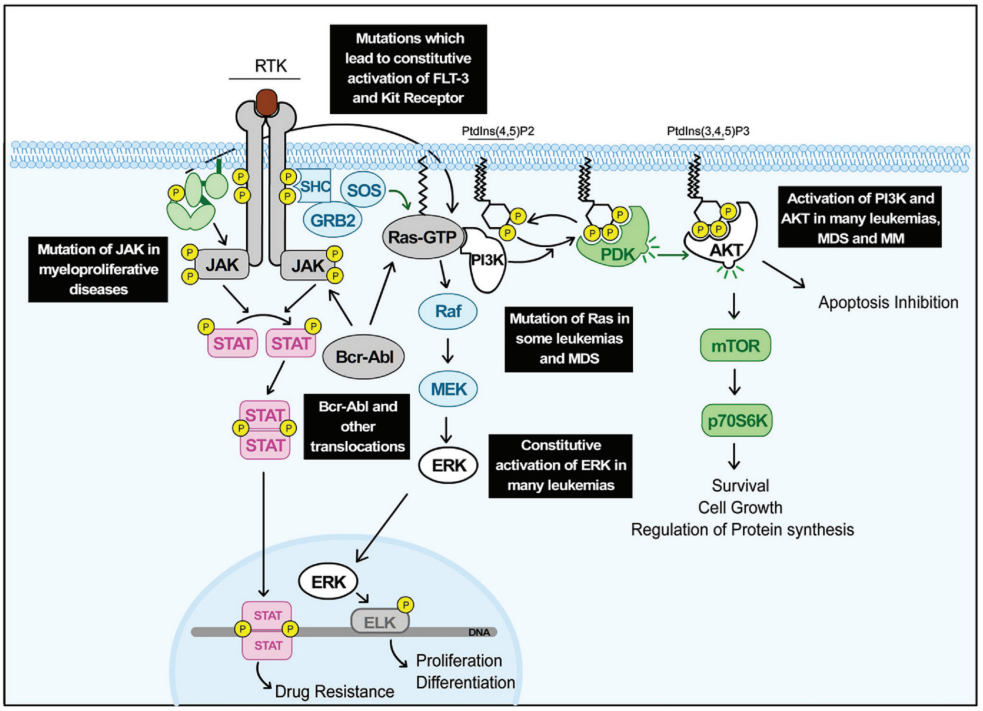

Figure 1. The threemajor pathways dysregulated in haematologicalmalignancies. The Janus kinase (JAK)–signal transducer and activator of transcription (STAT), Ras–Raf–MEK–extracellular signal regulated kinase (ERK) and phosphatidylinositol 3-OHkinase (PI3K)–protein kinase B (PKB/AKT)–mammalian target of rapamycin(mTOR) pathways are often deregulated in blood disease. The nature of their deregulation is indicated in black boxes. Proteins that are constitutively active as a result of genemutations are indicated in grey. RTK: receptor tyrosine kinase;MDS: myelodysplastic syndrome; AKT: protein kinase B (PKB/AKT); PDK: phosphoinositide-dependent protein kinase-1.

CD45

Characteristics and signaling

CD45 (otherwise known as leukocyte common antigen) is a transmembrane tyrosine phosphatase expressed onmost haematopoietic cells and their precursors except for mature erythrocytes and platelets' [30]. The intracellular tail of CD45 contains tandem repeats of two potential phosphatase domains, only one ofwhich seems to be active (for review see [31]). CD45 is a critical positive regulator of T-cell antigen receptor (TCR)- and B-cell antigen receptor (BCR)-mediated signaling required for the activation and development of lymphocytes. In humans, loss of CD45 in humans leads to severe combined immunodeficiency disease [32–34]. Cells isolated from CD45-deficientmice show decreased B-cell differentiation and accumulation of pro-B-cells, which correlates with hyperactivation of JAK1 and JAK3 upon cytokine stimulation [35]. The negative regulatory role of CD45 on JAK/STAT signaling originates either from direct association and dephosphorylation of JAK1 or JAK2, or from an alternative mechanism that involves the recruitment of the adaptor protein DOK-1 [36,37].

Loss of CD45 was also shown to increase interleukin (IL)-3-mediated JAK2 activation and proliferation inmast cell lines, and EPO-dependent formation of erythroid burst-forming units (BFU-E colonies) [36], suggesting that CD45 activity drives differentiation in favour of proliferation. Other proteins identified as molecular targets of CD45 are the Src family kinase (SFKs)members [38]. CD45 can dephosphorylate both the kinase domain and the COOH-terminal tyrosine SFKs (Fig. 2). Dephosphorylation of the positive regulatory site on some SFKs leading to their inactivation was shown for Hck and Lyn during integrin mediated adhesion in macrophages [39],p56lck kinase activity in developing thymocytes [40] and Lyn in B-cells [41]. However, dephosphorylation of COOH-terminal tyrosine, opens up the Src kinase to (auto)phosphorylation on activating residues. This appears to be the most commonly observed role for CD45 in haematopoietic cytokine signaling, providing a rationale for the positive regulatory function of CD45 in antigen receptor signaling [35], and might explain why activating CD45 mutations can result in lymphoproliferation and autoimmune disease [42]. Our group has shown that exclusion of CD45 from lipid raft signaling complexes prevents negative regulation of SFKs by CD45, providing a possible explanation for the dual positive and negative regulation of SFK activity by this phosphatase [43].

Role in malignancy

Different splice variants of CD45 are expressed in haematological cells during different stages of their differentiation. The low molecular weight isoform CD45RO seems to be associated with maturing blood cells, and is expressed on memory T-cells, maturing myelocytes and activated B-cells en route to becoming plasma cells [44,45].High molecular weight isoforms, CD45RAB, CD45RBC, CD45RABC and CD45RB, are variably expressed on naive T-cells and pre-B-cells [46]. Relatively little is known about the different roles of these isotypes, although differences in cellular functioning have been shown in T-cells recombinantly expressing different CD45 isoforms [47]. In haematolo- gicalmalignancy, isotype expression is often deregulated, aswas shown for AML [44,48] and Myeloma (MM) [49,50]. However, whether this reflects differences in signaling or variation in differentiation status of cells corresponding to FAB classification remains to be determined. CD45 deficiency in malignant T-cells was shown to result in resistance to apoptosis [51,52], whereas overexpression of CD45 in growth factor-dependent cell lines frequently causes growth arrest and/or cell death [30]. It would therefore stand to reason that tumor cells might down-regulate CD45 expression in order to escape apoptotic processes. However, although loss of CD45 expression has been reported in patients with Hodgkin's lymphoma and in N10% of patients with paediatric acute lymphoblastic leukemia (ALL), CD45 is still widely expressed in various types of haematological malignancies, and high expression levels of CD45 correlate with poor prognosis in AML and childhood ALL [53–56]. Interestingly, recent studies suggest that targeting CD45 with specific antibodies might improve survival of AML bearing mice by increasing chemotherapy uptake [57]. CD45 has been shown to play an important and complicated role in multiple myeloma. During normal B-cell lineage differentiation CD45 expression gradually declines, but is not lost, resulting in CD45+/dim plasma cells. In both normal and MM bone marrow CD45bright and CD45+/dim plasma cell fractions can be identified, although the CD45+/dim fraction is significantly bigger in MM patients [58], and seems to increase during progression of the disease tomoremalignant stages [59]. In 30% of patients, CD45 expression is completely annihilated on blood or bone marrow plasma

cells, representing end stage disease and correlatingwith poor prognosis [60,61]. As CD45 is a negative regulator of the JAK–STAT pathway, loss of CD45 expression might partly explain the constitutive activation of this pathway observed in MM patients [62]. Several studies have investigated the proliferative capacity of the different B- and plasma cell fractions in normal and MM bone marrow and blood, and have shown that the small CD45bright compartment has a significantly higher cell proliferation index than the CD45−/dimfractions [50,63]. CD45 expression plays a crucial role in determining signaling and proliferation of human myeloma cell responses to IL-6, insulin growth factor (IGF)-1 and other growth factors. It has repeatedly been shown that CD45+ MM cells are more responsive to IL-6 stimulation, leading to increased proliferation in this plasma cell fraction [64,65]. This effect is most likely due to an increased IL-6-induced Src kinase activation in CD45+ cells [66,67]. Interestingly, depending on cellular context, expression of CD45 can also make cells

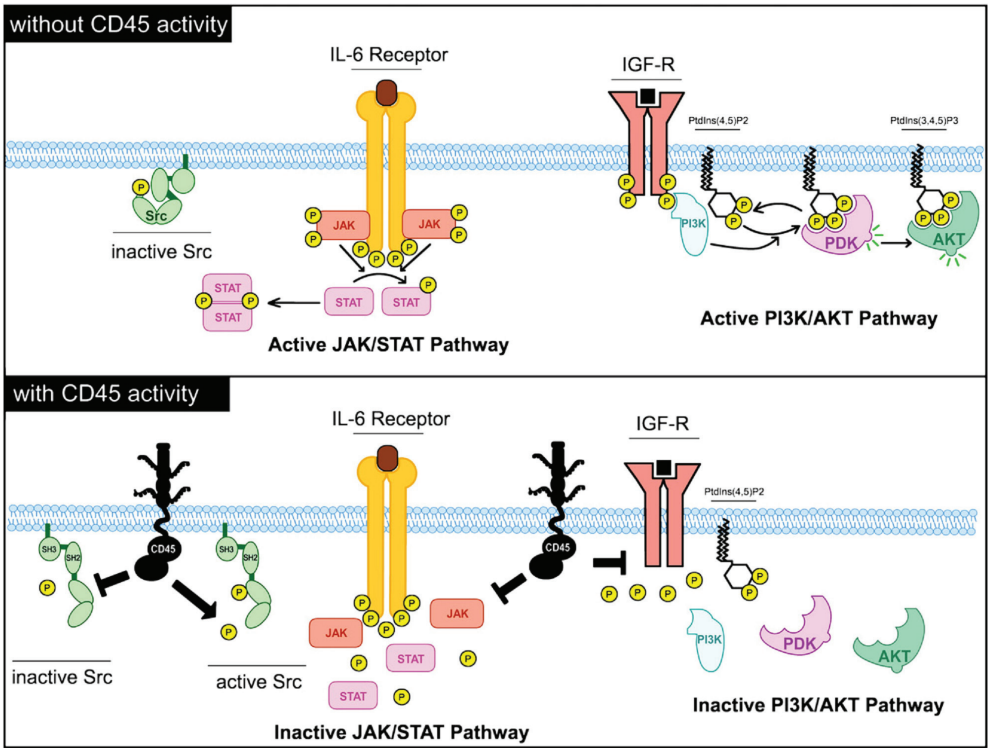

Fig. 2. CD45 signaling in haematopoietic cells. Upper panel: cytokine or growth factor (e.g. interleukin-6 [IL-6] or insulin like growth factor-1 [IGF-1]) stimulation results in dimerization of the corresponding receptor, bringing their associated JAKs into close proximity to each other and enabling STAT activation. In addition, PI3K activity leads to formation of PtdIns(3,4,5)P3, which subsequently activates PDK1 and PKB/AKT. Lower panel: one of the targets for CD45 is the Src family of kinases, represented here as Src. CD45 can dephosphorylate Src at its COOH-terminal tyrosine, allowing subsequent phosphorylation and activation by cytokine stimulation, providing a positive regulation of Src kinase activity. Alternatively, CD45 can dephosphorylate Scr at its positive regulatory sequence, leaving it inactive. In addition, CD45 can negatively regulate JAK/STAT and PI3K/PKB signaling, by dephosphorylating JAK and growth factor receptors, respectively.

more susceptible to apoptosis signals, which might be due to the dual role of CD45 in SFK activity or the decreased expression of the anti-apoptotic factor Bcl2 observed in CD45+ cells [58,68]. Another explanation for this phenomenon was presented by Descamps et al., who show that IGF-1-induced activation of the PI3K/PKB survival pathway is decreased in CD45+ cells, possibly as a direct result of IGF-R dephosphorylation by CD45 [69]. CD45 expressing MM cells appear to be of a more immature subtype compared to their CD45-negative counterparts as determined by co-expression of other cell surface markers [50,70]. In agreement with this, Matsui and co-workers showed that clonogenic capacity was contained within a CD45+ fraction of MM plasma cells, thus displaying potential progenitor cell characteristics [71]. In contrast, others showed that clonogenic potential was limited to CD45− MM cells [72]. This apparent discrepancy might be explained by differences in experimentalmodel potentially leading to the exposure of cells to different cytokines, as it has recently been demonstrated that IL-6 induced colony growth is mostly apparent in CD45+ MM cell lines, whereas IGF-1-induced colony formation was restricted to CD45− cells [73]. In summary, the role of CD45 in haematological malignancies is a complicated one; expression of CD45 is associated with poor prognosis in AML, possibly as a result of increased SFK activity, and CD45 might potentially be used as a target for treatment in AML. In contrast, the loss of CD45 expression is a poor prognostic factor in MM. The increased activity of the PI3K/PKB pathway in CD45− cells, in combination with the increased JAK–STAT activity observed in these patients, presents an opportunity for the use of kinase inhibitors of these pathways in MM treatment, whereas proliferation of CD45+ cells might be targeted by SFK or CD45 phosphatase inhibitors.

PTP1B

Characteristics and signaling

The ubiquitously expressed protein tyrosine phosphatase 1B (PTP1B) is located on the cytosolic face of the endoplasmic reticulum (ER) due to a hydrophobic sequence at its C-terminal end. PTP1B is involved in multiple normal and malignant signaling pathways [3] by regulating RTKs, including epidermal growth factor receptor (EGFR) [74], PDGFR [75], insulin receptor [76] and IGF-1 [77]. Dephosphor-ylation of RTKs by PTP1B occurs after endocytosis and translocation of the RTK to the ER, where after they are recycled to the cell membrane or directed to lysosomes [78]. PTP1B is thus able to dephosphorylate RTKs from different cell compartments, despite its localization to the ER. In addition to RTKs, PTP1B has other membrane-associated substrates: it can negatively regulate cytokine signaling through JAK2 and TYK2, either through dephosphorylation of STAT5a/b, or by direct association with the RTKs [79–81]. In addition, PTP1B has been shown to regulate integrin signaling through Src kinases [82]. Due to its negative regulation of multiple RTKs, PTP1B deficiency could lead to increased oncogenic signaling. Indeed, PTP1B deficient fibroblasts display increased IGF-1 receptor, EGFR, and PDGFR tyrosine phosphorylation [83]. However, Dubé and co-authors reported that PTP1B can also act as a positive regulator of Ras signaling downstream of RTKs through increased p120RasGAP (Ras GTPase-activating protein) expression and p62Dok (downstream of tyrosine kinase) phosphorylation [75], which partly explains why PTP1B knockoutmice do not display an increased incidence of tumors. In fact, overexpression of

PTP1B has been shown in a variety of (solid) tumors, including colon cancer cells in which it activates Src kinase signaling by decreasing its negative regulatory phosphorylation [84]. In addition, overexpression of PTP1B is enough to induce breast cancer in mammary gland cells, demonstrating a role for PTP1B as oncogene in its own right [85]. As a dual role of PTP1B is arising, the absence of its activity may either suppress or promote oncogenic transformation [75].

Role in malignancy

In humans, PTP1B is one of the negative regulators of cytokine induced JAK2–STAT3/5 pathways [79], and mice deficient for PTP1B showedhyperphosphorylation of JAK2 [86]. Genetic ablation of PTP1B in p53 null mice decreased survival rate and increased susceptibility towards the development of B lymphomas [87], although no correlation with JAK2/STAT5 activation was observed in this study. In addition, PTP1B can negatively modulate Bcr–Abl [88], a constitutively active fusion kinase expressed as a consequence of t(9;22), the so-called Philadelphia chromosome. This oncoprotein is found in 15–30% of adults with AML, and in nearly all CML patients, where it plays a major role in the development and progression of CML through chronic, accelerated, and blast phases [89,90]. Expression of Bcr–Abl results in constitutive activation of STAT5 and alterations in signal transduction and gene expression profiles that are associated with cytokine independence [91]. In addition to recognizing Bcr–Abl as a substrate, PTP1B disrupts the formation of a Bcr–Abl/Grb2 complex, thereby inhibiting signaling events and oncogenic transformation initiated by this oncoprotein [88]. Overexpression of PTP1B or inhibition of Bcr–Abl induced erythroid differentiation of CML cell line K562 and inhibition of PTP1B attenuates the induction of differentiation and apoptosis by STI571, a selective Bcr–Abl inhibitor, in CML cells [92].Thislatterstudy also showed that STI571-resistant ALL cells have a significantly reduced PTP1B activity, providing strong evidence that reduction of PTP1B expression and activity leads to resistance of Bcr–Abl-positive leukemia cells to STI571. However, increased PTP1B expression levels have been observed in activated B-cell-like diffuse large B-cell lymphomas. This was associated with a decreased IL4-mediated STAT6 dephosphorylation, which is independent of Jak1, but not JAK2 [93]. Thus, whereas decreased PTP1B may increase tumorigenicity by enhancing prolifer- ative JAK2/STAT5 signals, overexpression of PTP1B renders cancer cells unsusceptible to the anti-tumor effect of IL-4–STAT6 activation. The potential use of PTP1B in treatment of haematologicalmalignancy may therefore completely depend on its expression level, and the signaling pathways activated in these illnesses.

TC-PTP

Characteristics and signaling

TC-PTP (T-cell protein tyrosine phosphatase) is an intracellular enzyme encoded by Ptpn2. TC-PTP is mainly haematopoietic and alternative splicing of its gene allows expression of two distinct proteins, a p45 nuclear form called TC45 or TC-PTPa, and a p48 cytoplasmic form called TC48 or TC-PTP1b. TC-PTP−/− mice display defective haematopoiesis and immune function, characterized by anaemia and splenomegaly secondary to sequestration of

erythrocytes and accumulation of myeloid cells [86]. In addition, TC-PTP-null mice develop severe systemic inflammation, a phenotype with potential implications in oncogenesis. TC-PTP and PTP1B share over 70% amino acid sequence identity within their catalytic domains. However, while PTP1B is predominantly ER-localized, TC-PTP is largely nuclear. TC-PTP has been shown to control cytokine signaling events by its negative action on the JAK/STAT pathways [94]. Both TC-PTP and PTP1B selectively recognize a motif centered on the characteristic double tyrosine residues present in the JAK activation loop, but TC-PTP exhibits specificity for JAK1 and JAK3, while PTP1B interacts with JAK2 and TYK2. TC45/TC-PTP1B is responsible for selective JAK dephosphorylation and deactivation of nuclear STAT1, STAT3, and STAT5 [95,96]. TC-PTP was also shown to interact with TRAF2 downstream of the proinflammatory cytokine TNF. This interaction inactivates Src and suppresses MAPK signaling [97]. These results identify TC-PTP as a keymodulator of inflammatory signals as well as lymphocyte functions.

Role in malignancy

Despite its clear function in normal haematopoiesis, the role of TC-PTP in oncogenic transformation remains circumspect. Similar to PTP1B, a role for reduced TC-PTP in STI571 resistance of CML cells has been established [98]. However, as for PTP1B, increased nuclear TC-PTP expression, correlating with decreased STAT6 signaling was observed in activated B-cell like lymphomas compared to other types of B-cell lymphomas and may facilitate tumor survival [99]. Bourdeau and co-authors observed that TC-PTP-deficient bone marrow stromal cells fail to support normal B lymphopoiesis due to abnormally high secretion of interferon-γ, which reduced 2-fold the mitotic index of IL-7-stimulated TC-PTP−/− pre-B-cells. They noted a 40% increase in apoptosis of murine early pre-B leukemic cells cultured within a TC-PTP-deficient bone marrow stroma environment [94]. Moreover, they describe constitutive phosphorylation of STAT1 in TC-PTP−/−pre-B-cells, which may find practical application in the treatment of cancer. Unlike other STATs, STAT1 acts as a tumor suppressor (reviewed by [100]). Thus, reducing TC-PTP activity, thereby increasing STAT6 and STAT1 activity,may help reduce tumor burden and metastasis. TC-PTP-specific blocking agents might provide a useful pharmacologic approach toward this goal.

SHP-1

Characteristics and signaling, role in malignancy

Src homology 2 domain-containing phosphotyrosine phosphatases (SHP)-1 are a subfamily of cytoplasmatic non-receptor PTPs. SHP-1 (also known as PTP1C or HCP — haematopoietic cell phosphatase) and SHP-2 (discussed below) are characterized by the presence of two N-terminal SH2 domains, a classic PTP domain and a C-terminal tail [101]. Their SH2 domainspermit associationwith phosphotyrosine fromproteins such as activated receptors or signaling molecules, triggering activation of the phosphatase domain and, subsequently, dephosphorylation of the substrate. SHP-1 is mainly expressed in haematopoietic cells although it is also present in epithelial and smooth muscle cells. Via its SH2 domains, SHP-1 is recruited to c-Kit, BCR, TCR and the receptors for FCγIII, IL-3 and erythropoietin (EPO),

thereby playing a role in the control of signaling cascades that couple growth factor receptors to haematopoietic cell differentiation [102,103]. Binding of SHP-1 to the EPO receptor results in dephosphorylation of the receptor-associated kinase JAK2 at position Tyr1008, thereby reducing its activity [28,101]. Mice deficient for SHP-1 display hyperphosphorylation of JAK1 and JAK2 following interferon-α (INFα), growth hormone (GH) or EPO treatment, resulting in important immunological and haematopoietic dysfunctions [104]. In humans, the Shp-1 gene is frequently altered in cancer cells, and various haematological malignancies, such as lymphomas, Myeloma and AML show silencing of Shp-1 by methyl- ation [105–107]. In addition, decreased SHP-1 mRNA and protein expression levels have been observed in diverse leukemic cell lines and paediatric AML [108,109]. SHP-1 may also affect leukemia development through its negative modulation of the Bcr–Abl pathway. Amin and co-authors [110] observed that SHP-1 levels are markedly decreased in advanced stage CML patients comparedwith those in chronic phase. However, neither mutation nor DNAmethylation was detected in the Shp-1 gene in CML cell lines or patient samples, suggesting that the decrease in SHP-1 in advanced stage CML patients is due to posttranscriptional modifications [110], and implying that a decrease in SHP-1 expression levels plays a role in the progression of CML. Together, these results suggest a potential role for SHP-1-inducing agents in cancer treatment. Interestingly, proof of concept of this hypothesis comes from experiments showing that restoration of SHP-1 expression in anaplastic large cell lymphoma using 5-aza-2′-deoxycitidine results in abrogation of constitutive JAK3–STAT3 signaling and increased drug sensitivity [111].

LMWPTP

Characteristics and signaling

Low molecular weight protein tyrosine phosphatases (LMWPTPs); also known as acid phosphatase locus 1 (ACP1), are a family of 18-kDa enzymes involved in cell growth regulation, cytoskeleton rearrangement and modulation of the immune response. This small molecule which interacts with growth factor receptors and proliferation signaling pathways, possesses some interesting biochemical and structural characteristics. LMWPTP activity is regulated by phosphorylation/dephosphorylation or oxidation of the protein. It possesses two phosphorylation sites, which seem to work as switches for different functions of the phosphatase. Bucciantini et al. have shown that SFK are able to phosphorylate both sites, triggering two different effects [112] Phosphorylation of tyrosine residue 131 increases the activity of LMWPTP 25-fold, whereas phosphorylation of tyrosine 132 leads to Grb2 recruitment. The second regulatory mechanism able to modify LMWPTP activity is the oxidation of the 12 and 17 cysteine residues, which renders the phosphatase inactive. However, the presence of an additional cysteine in the catalytic site (Cys17)makes LMWPTP able to undergo formation of an intramolecular S–S bridge. This event prevents the complete and irreversible oxidation of the catalytic cysteine (Cys12), enabling LMWPTP to rapidly recover its activity [113]. LMWPTP has been shown to be associated with molecules involved in cell growth and proliferation, such as PDGFR [114], JAK2 [115], STAT5 [29,116], Focal adhesion kinase (FAK) [117], Ephrin A2 receptors (Eph A2) [118] and β-catenin [119]. Although mostly regarded as negative regulator of kinase activity, a positive action

of LMWPTP in cell growth and proliferation signaling has been also described [115,120]. LMWPTP is an important regulator of STAT5 activity during megakaryocyte differentiation. Rigacci and co-workers suggested that the association of LMWPTP with STAT5 prevents phosphorylation of this transcription factor in the absence of a growth factor stimulus [29]. In contrast, LMWPTP oxidation mediates the sustained phase of JAK2 phosphorylation, which is required for the anti-apoptotic effects of IGF-I and serum on pancreatic cells [115]. This suggests that inhibition of LMWPTP leads to enhanced and sustained phosphorylation of the JAK/STAT pathway and suppression of apoptosis, and as such makes this PTP an interesting candidate in IGF-1-mediated MM cell survival. However, the oxidative switch of LMWPTP and its function is not completely understood, and it has also been observed that oxidation of this phosphatase is involved in Grb2 binding and increased ERK activation, implying that LMWPTP oxidation can directly trigger a prosurvival signal against the oxidant environment [120].

Role in malignancy

Increased expression levels of LMWPTP have been reported in several human tumors, such as neuroblastoma, breast and colon cancer. Analysis of patient survival indicates that higher LMWPTP levels are predictive of an unfavourable outcome, meaning that LMWPTP could confer tumor aggressiveness and its expression might be used as a marker in some types of cancer [121]. Interestingly, LMWPTP has been pointed out together with ZAP70 as predictors for treatment requirement in a case reported by Chen et al. [122]. These authors have shown that a patient with chronic lymphocytic leukemia presented a gradual loss of LMWPTP and ZAP70 as well as the active form of ZAP70 (phospho-ZAP70 tyr492) during continued chemotherapy. In addition, the return of these proteins to normal levels correlated with requirement for treatment [122]. Therefore, although LMWPTP has a negative regulatory function in JAK/STAT activation and hencewould appear to be tumor suppressive, its overexpression in many cancers suggests that this does not hold true for transformed cells, andmaymake this phosphatase a target for therapy in haematological malignancies. However, the regulation of its switches (phosphorylation and oxidation) and state (activated and inhibited) and their importance for the normal and cancer cell biology require further study.

Ras–Raf–MEK–ERK signaling

The Ras signaling pathway has been extensively studied and reviewed. Upon cytokine signaling, the adaptor protein Grb2, which is complexed to the guanine nucleotide exchange factor (GEF) Son of Sevenless (Sos), is recruited to activated receptors through its SH2 domain. The subsequent membrane localisation of this GEF allows activation of themembrane-bound small GTPase Ras by facilitating its ADP release and subsequent ATP binding. Ras activation results in a cascade ofmitogen-activated protein kinase (MAPK) activation,where MAPK kinase kinase (e.g., Raf) activates MAPK kinase (e.g., MEK1/2) which subsequently phosphorylates MAPK (e.g., extracellular signa regulated kinase; ERK1/2) [123]. MAPKs are subdivided into three subfamilies based on sequence similarity, differential regulation and substrate specificity: ERK1/2, c-Jun N-terminal kinase (JNK) (JNK 1, 2 and 3) and p38 (α, β,

δ and γ) [124]. Activating Rasmutations, predominantly investigated in leukemias andMDS, have been observed in a large cohort of studies (reviewed in [125]). Ras activity and function depends on its membrane localisation, a process facilitated by farnesylation of the protein, and farnesy transferase inhibitors have therefore been extensively tested in tumor cell biology. However, clinical phase II trialswith these inhibitorswere only marginally successful, and despite achieving clinically relevan positive results in some studies, this did not seem to be correlated to RAS mutation status of patients [126–128]. Downstream of Ras mutations in Raf and ERK1/2 have been observed in a number of haematological malignancies (reviewed in [129]). Increased phosphorylation of ERK1/2 is frequent in leukemia, and appears to play an important role in drug resistance [130]. In contrast, in low-risk MDS patients, a decreased ERK1/2 activity was observed in neutrophils and CD34+ progenitor cells, which correlated with decreased cel functionality [131–133]. As such, phosphatases interfering in MAPK signalingmay be of functional consequence in haematological disease and may provide a target for treatment. Some such phosphatases include SHP-2, HePTP, and theMAPK phosphatases (MKPs), and these will be further discussed below.

SHP-2

Characteristics and signaling

The protein tyrosine phosphatase SHP-2 is encoded by the PTPN11 gene (protein tyrosine phosphatase non-receptor-type 11) and localized on 12q24 [134]. SHP-2 is expressed ubiquitously, but particularly strongly in blood cells. In its basal state, SHP-2 activity is suppressed by intramolecular interactions between residues in the “backsideloop” of the N-SH2 domain (the side opposite the phosphotyrosyl peptide binding pocket) and the catalytic surface of the PTP domain [135]. Upon growth factor or cytokine stimulation, SHP-2 is recruited via its SH2 domains to phosphorylated tyrosine residues on RTKs (e.g. Kit-ligand, IL-3, EPO and granulocyte–monocyte colony-stimulating factor (GMCSF)) and/or scaffolding adaptors, such as insulin receptor substrate, fibroblast growth factor receptor substrate, or Grb2-associated binder (GAB) proteins [136]. Phosphotyrosyl peptide binding to the N-SH2 domain disrupts the auto-inhibitory interface; leading to exposure of the PTP domain and catalytic activation [7,135]. Activation of SHP-2 generally leads to activation of the Ras/Ras/ERK pathway, but its precise target(s) remains controversial. SHP-2 activates SFKs evoked by RTK and integrin signaling in fibroblasts, and SFKs in turn are required for sustained Ras activation on endomembranes [137]. Other authors suggest that SHP-2 ensures Ras signaling by dephosphorylating potential recruitment sites for p120RasGAP, a GTPase-activating protein that promotes Ras inactivation [138]. SHP-2 is essential for myeloid differentiation [83],and can negatively modulate c-Kit signaling by interacting with specific phosphotyrosine residues on this receptor [103]. Kit signaling is important in erythropoiesis, lymphopoiesis, megakaryopoiesis and mast cell development and function, and its expression and constitutive activation is found in for instance mast cell leukemia and AML[139].

Role in malignancy

SHP-2 was the first PTP implicated in leukemogenesis. Somatic gain of function mutations in

the PTPN11 gene are the most common cause of sporadic juvenile myelomonocytic leukemia (JMML), implicated in about 35% of cases [140]. In addition, PTPN11 mutations occur at lower incidence in othermyeloid neoplasms such AML (~5%), chronic myelomonocytic leukemia (CMML), and myelodysplastic syndrome (MDS) [140,141], as well as in the most common form of childhood leukemia, B-acute lymphoblastic leukemia (B-ALL) (~10%) [7,96]. Germline mutations in the SHP-2 gene occur in 50% of individuals with Nooman syndrome (NS), a developmental disorder with short stature, facial dysmorphia, skeletal anomalies and heart defects [141]. Children with this syndrome have a growth disorder as well as a predisposition to develop JMML [142]. Activating PTPN11 mutations negatively regulate the cellular response to growth hormone (GH) and explain the growth disorder observed in these patients. In vitro experiments demonstrated that SHP-2 associates directly with the GH receptor (GHR) in response to GH [143] and acts as a cytosolic phosphatase of STAT5, downregulating its activity [144]. In addition, a decreased STAT3 activation has been observed in NS/JMML cells with PTPN1 mutation [145]. As discussed above, SHP-2 is required for full activation of the Ras/Raf/Erk pathway and for multiple receptor-evoked functions, including cell proliferation, differentiation, and migration [101]. Indeed, gain of function mutations in PTPN11 have been shown to result in hyperactivation of ERK and protein kinase B activation [146,147]. Thus, the predisposition of the Noonan Syndrome patients to JMML is related to the activation of Ras/MAPK pathway by SHP-2 activation. It is important to emphasize that mutations in the RAS family members KRAS or NRAS genes, PTPN11 gene, or the RAS-GAP NF1 are observed in 65%–85% of JMML patients [148]. Konieczna and co-authors [134] found that constitutive activation of SHP-2 in mice cooperates with progression of myeloproliferative disorders. They showed that Interferon Consensus Sequence Binding Protein (ICSBP, also known as IRF8), an interferon-regulatory transcription factor, functions as a leukemia tumor suppressor only when phosphorylated. Co-expression of a constitutively active form of SHP-2 synergized with ICSBP haploinsufficiency to accelerate progression to AML, induce cytokine hypersensitivity and apoptosis resistance in vivo [134]. SHP-2 can also function as a positive factor in IL-6 signaling by stabilizing JAK2 or inducing Src kinase activation [149]. IL-6-induced JAK1,2–STAT3 signaling is one of the major growth signals in myeloma cells [62]. It has recently been shown that c-MET enhances IL-6-induced proliferation of MMcells through increased activation of SHP-2, suggesting a possible role of SHP-2 in MM pathology [150]. In conclusion, the evidence to date suggests that the role of SHP-2 in leukemogenesis is more prominent than its role in tumor suppression, implying a potential role for SHP-2 inhibitors as a molecular target of therapy. Interestingly, this seems to be corroborated by the finding that increased expression of SHP-2, which was correlated with enhanced FLT3 signaling in AML patients, was reduced by treatment of AML cells with a c-KIT/FLT3 kinase inhibitor [151].

HePTP

Characteristics and signaling, role in malignancy

Haematopoietic PTP (HePTP), also named leukocyte PTP (LC-PTP), is a NRPTP that contains a kinase interacting motif, allowing it to bind to ERK and the MAPK p38 [152]. This results in conformational changes in both the PTP and its substrate, leading to dephosphorylation

of the substrate, and subsequent phosphorylation of HePTP by the bound kinase [153]. In T-cells, HePTP functions as an early response gene, regulating phosphorylation state during IL-2-induced proliferation [154]. HePTP overexpression results in decreased ERK phosphorylation upon TCR signaling [155], whereas its reduction increased PMA-induced ERK1/2 activity in spleen cells from HePTP knockout mice [156]. In its capacity as a modulator of ERK1/2 activity and nuclear translocation, HePTP is also involved in megakaryocyte differentiation [157]. In addition, HePTP can be phosphorylated by protein kinase A in B-cells, where it inhibits p38 induction by beta-adrenergic receptor agonist signaling [158]. Expression of HePTP is limited to tissues of haematological origin such as thymus, spleen and all myeloid and lymphoid cells. As such, it is interesting to note that the HePTP gene is mapped next to another haematopoietic specific PTP, CD45, on chromosome 1q32 [159] and might therefore be generated by gene duplication of a common ancestral gene [160]. Deletions of this chromosomal region have been frequently found in B-cell lymphomas, which corresponds with a significantly decreased protein expression of HePTP observed in paediatric B-cell lymphoma cases [161,162]. In contrast, triplication of the HePTP gene and protein overexpression was observed inMDS and AML patients, suggesting a cell-type specific role for aberrant HePTP expression in haematological malignancies [163]. However, further studies will have to correlate changes in HePTP expression to downstream signaling strength in haematological malignancies, and determine whether this promising candidate phosphatase may be a potential target for treatment in haematological tumors.

MKPs

Characteristics and signaling

MAPKs transduce extracellular signals from environmental stress hormones, growth factors and cytokines which results in control of diverse physiological processes such as proliferation, differentiation, migration and apoptosis (reviewed in [17,164]). MAPK phosphatases (MKPs), a family of DUSPs, are able to inactivate MAPKs through dephosphorylation of tyrosine and/or threonine residues in the activation loop of MAPKs. Based on sequence similarity, substrate specificity, gene structure and subcellular localization the ten MKPs can be classified into three subfamilies. The first group is composed of nuclear MKPs which include DUSP1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2 and DUSP5/hVH-3. The second group of cytoplasmic MKPs comprises DUSP6/MKP-3, DUSP7/MKPX and DUSP9/MKP-4, and the final group consists of DUSP8/hVH-5, DUSP10/MKP-5 and DUSP16/MKP-7, which are found both in the cytoplasm and the nucleus. Abnormalities in MAPK pathways have been associated with a wide variety of human diseases, including cancer (reviewed in [17]). The role of ERK1/2 in haematological malignancies is well documented, whereas the involvement of the JNK and p38 MAPKs is more circumspect [129]. Activation of ERK is usually associated with cell proliferation and survival, whereas JNK and p38 contribute to cell apoptosis in response to stress [165]. The balance between MAPK and MKP activity directs cell fate to either survival or apoptosis. Negative regulators of ERK, such as MKP DUSP2/PAC1, are excellent candidate tumor suppressors. However, other MKPs, like MKP-1, have been associated with survival and tumorigenesis and are overexpressed in many types of cancer [165]. It is interesting that different DUSPs can have either a positive or negative regulatory role in ERK activation, as

increased ERK pathway activation is observed in 30 to 50% of AML cases, whereas some low-risk MDS patients show decreased ERK phosphorylation [132,166]. 4.3.2. Role in malignancy Recently, the DUSP16/MKP-7 was shown to be associated with leukemia. Altered subnuclear localization of the transcription factor Runx1, responsible for normal haematopoiesis, was linked to the etiology of AML by upregulating microRNA-24 (miR-24). miR-24 expression leads to inhibition of MKP-7 expression, consequently enhancing myeloid cell proliferation and blocking granulocyte differentiation by increasing p38 phosphorylation [167]. Although this may seem somewhat counterintuitive, as p38 activity has been associated with cell death and reduced tumorigenesis [168], p38 has also been shown to be detrimental for granulocyte development and others have demonstrated that decreasing constitutive p38 activation may restore normal haematopoiesis in malignant cells [169,170]. TheMKPDUSP2/PAC-1 is predominantly expressed in haematopoietic tissue, and acts as a negative regulator ofMAPK signaling [171–173].Loss of DUSP2/PAC-1 expression has been associated with elevated levels of ERK activation in acute leukemia, as Kim and co-authors showed that increased expression and activity of ERK and MEK in 17 of 26 cases (65.4%) indeed corresponded to downregulation of DUSP2/PAC-1 [174]. Thus, downregulation of MKPs can be responsible for the increased MAPK activation that is often described to contribute to haematological malignancy. Increasing these MKPs' activity for treatment of these diseases remains a challenge. However, enhanced MKP activity has also been linked to haematological illness. Constitutive overexpression of two transcripts related to DUSP2/PAC-1 was observed in large granular lymphocyte (LGL) leukemia [175]. LGL leukemia is a lymphoproliferative disorder originating either from mature T-cells or natural killer (NK) cells, and often associated with autoimmune disease (reviewed in [176]). The physiological role of DUSP2/PAC-1 in the immune system was described by Jeffrey et al. who showed that PAC-1-null mice display a decreased expression of proinflammatory mediators and cytokines such as IL-6, IL12α, cyclooxygenase-2 and IL-1β in response to LPS stimulation [173]. As the etiology of LGL leukemia is not completely elucidated, the discovery of DUSP2/PAC-1 overexpression in this disease has provided a focus in the search for a therapeutic target. Another MAPK phosphatase with a possible role in haematological cancer is the DUSP1/MKP-1, expression of which is induced by growth factors and stress signals in human cells [177].Initially,MKP-1was shown to dephosphorylate ERK and negatively modulate cell proliferation [178]. However, recent studies have described an anti-apoptotic effect ofMKP-1 by negativelymodulating JNKand p38 [179]. Inaddition, enhanced activity of the RAF/MEK/ERK pathway can be associatedwith increased mRNA levels of DUSP1/MKP-1 as shown in a study from Staber and co-workers, who demonstrated overexpression of MKP-1 accompanied by RAF/MEK/ERK pathway upregulation in bone marrow of relapsed AML patients compared to AML samples before high-dose chemotherapy [180]. This positive regulatory role for MKP-1 in cell survivalwas later confirmed by others, showing thatMKP-1 depletion in breast cancer induces apoptosis [165]. Together, these data indicate that MPKs can exert both positive and negative actions on leukemogenesis, and suggest that they might be viable targets for interference. Especially downregulation of MKP-1 would seem a promising avenue of investigation in cancer treatment. However, more research into the exact mechanisms of the different MKPs is clearly needed.

PI3K–PKB signaling

PI3K signaling is initiated upon growth factor or chemokine receptor activation, either through direct recruitment of the SH2 domain-containing p85 subunit of PI3K to the receptors or through binding of the kinase to active Ras GTPase. Once translocated from cytosol to plasma membrane, this lipid kinase phosphorylates membrane anchored phosphoinositides on the 3-position of the inositol ring. The main products formed are PtdIns(3,4)P2 and PtdIns(3,4,5)P3, which act as second messengers by recruiting molecules with a phospholipid binding (PH) domain to the membrane, where they are subsequently activated. One of these is protein kinase B (PKB, also known as Akt), which plays a major role in cell survival and phosphorylates, amongst others, mTOR, GSK3 and the forkhead transcription factors. Many tumors, including blood cancers, present with constitutive activation of the PI3K–PKB–mTOR pathway (reviewed in [181,182]). Although activating mutations in the PI3K gene (PIK3CA) and PKB gene (AKT E17K) have been described, their occurrence seems to be rare in haematological malignancies [183,184]. More often, constitutive PI3K signaling is thought to be a secondary effect: the presence of activating mutations in receptor tyrosine kinases or Ras, oncogenic translocation products Bcr–Abl and Tel–Abl, as well as autocrine cytokine signaling loops have all been cited as contributing factors [185]. Regardless of the activating mechanism, targeting this pathway with inhibitors of either PI3K/PKB ormTOR appears to be a promising approach in the treatment of leukemias (reviewed in [186]). Phosphatases counteracting the PI3K-initiated signaling cascade include PTEN, SHIP and SHIP-2. Their role in normal cell physiology and leukemic transformation will be discussed below.

PTEN

Characteristics and signaling

Phosphatase and tensin homolog (PTEN), located on 10q23.3, is a dual specificity phosphatase that has both lipid and protein phosphatase activity. PTEN classically converts PtdIns(3,4,5)P3 at the plasmamembrane to PtdIns(4,5)P2, thereby directly antagonizing the activity of PI3K [187]. Therefore, PTEN inactivation results in constitutive activation of the PI3K/PKB pathway and subsequent increases in protein synthesis, cell cycle progression, migration and survival [188]. Although several studies where PTEN was overexpressed or analysed in tumorigenic cell lines and tissues have shown that PTEN was mostly localized in the cytoplasm, studies in healthy tissues have suggested that PTEN is originally localized in the nucleus [189–191]. Loss of nuclear PTEN has now been observed in a variety of tumors, suggesting an association between a lack of nuclear PTEN and mitotic index [192–194]. This seems to be corroborated by the fact that the nuclear presence of PTEN is apparent in quiescent cells, but in actively dividing cells PTEN localizes mostly in the cytosol [195].

Role in malignancy

Deletion of Pten in mouse haematopoietic stem cells leads to a myeloproliferative disorder which is followed by acute T-ALL [196].In humans, many somatic mutations have been

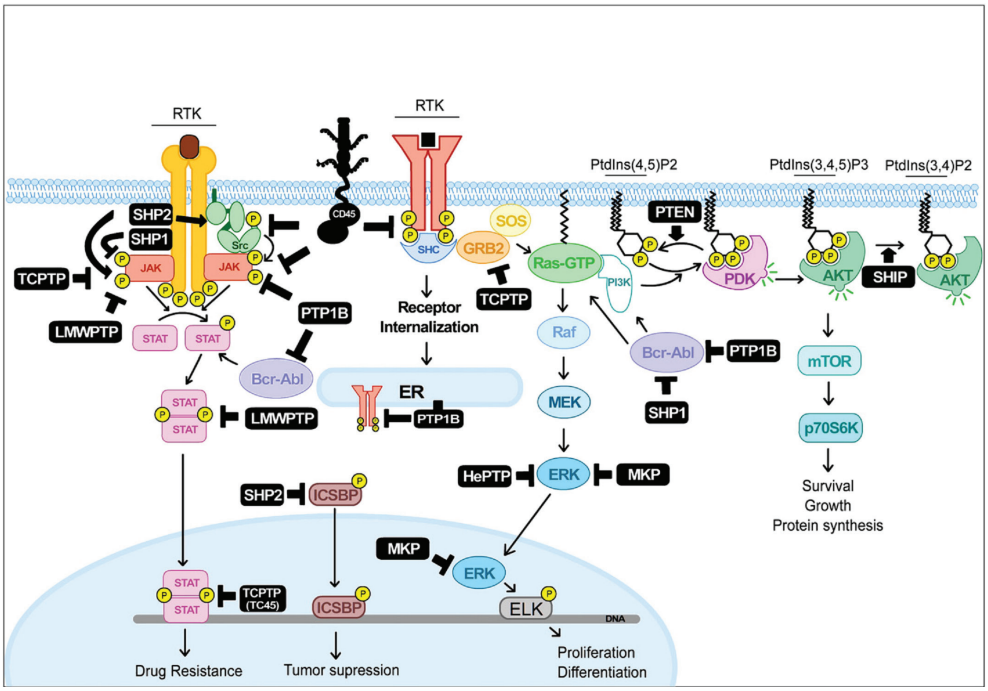

Figure 3. The role of phosphatases on the three major kinase pathways dysregulated in haematological malignancies. The JAK–STAT pathway can be upregulated by SHP-2 which stabilizes JAK2. On the other hand, LMWPTP, SHP-1, PTP1B and CD45 can negatively modulate JAKs. Inhibition of Bcr–Abl by PTP1B can also negatively modulate STAT signaling. In addition, CD45 is able to directly inactivate receptor tyrosine kinases (RTK) and inactivate Src, thus affecting JAK–STAT signaling. STATs can be dephosphorylated by LMWPTP in the cytosol and by TC-PTP in the nucleus. RTKs can be dephosphorylated and inactivated by TC-PTP in the cytosol face of endoplasmic reticulum (ER). In MAPK pathways, the main phosphatases involved are MAPK phosphatases (MKP). MKP can dephosphorylate ERK in the cytosol (DUSP16) or in the nucleus (DUSP2/PAC-1 and DUSP1/MKP-1). Another phosphatase able to negatively modulate ERK is HePTP. The major negative modulator of PI3K–PKB signaling is the lipid phosphatase PTEN. PTEN can dephosphorylate PtdIns(3,4,5) P3 to PtdIns(4,5)P2, thereby preventing PKB activation. PtdIns(3,4,5)P3 can also be dephosphorylated by SHIP at the position 5, although the ensuing product PtdIns(3,4)P2 has also been shown to be able to activate PKB.

described for the PTEN gene, although these occur in the minority of the tumors. Until recently, PTEN mutations had only been described sporadically in leukemia and lymphomas. However, Gutierrez and collaborators have now identified abnormalities in the PTEN gene with a frequency of 8.7% in T-cell acute lymphoblastic leukemia (T-ALL) patients, including homozygous and heterozygous deletions [197].These inactivating mutations carry a negative prognostic risk, related to a high risk of treatment failure [198]. This statement is in agreement with Palomero and co-workers who have reported the association of PTEN mutations and non-responsiveness to γ-secretase inhibitors (GSI). GSI target the NOTCH1 signaling pathway, which is present in the majority of T-ALL cases, and was shown to reduce PTEN expression via HES1. This in turn leads to a constitutive PKB activation which renders

Table 2 Involvement of phosphatases in normal and malignant signaling.

PTP	Normal function	Substrates	Nature of change	Consequences
CD45	Reduced JAK/STAT Reduced/increased Src	Src, JAK [38]	1. Deletion 2. Increased expression 3. Reduced expression	1. Severe combined immunodeficiency syndrome [33,34] 2. Poor prognosis in AML and ALL [53–56] 3. Increased IGF-1, decreased IL-6 signaling in MM [68,69]
PTP1B	Reduced RTK Reduced JAK/STAT	RTKs (such as EGFR) [74], PDGFR [75], insulin receptor [76], IGF-R [77], BCR–Abl [88], JAK2, TYK2, STAT5 [244–246]	1. Increased expression 2. Reduced expression	1. Decreased anti-tumorigenic STAT6 in DLBCL [93] 2. Role in CML resistance [92]
TC-PTP	Reduced JAK/STAT	JAK1, JAK3 [247], STAT1, STAT3, STAT5 [248,249]	1. Increased expression 2. Reduced expression	1. Decreased anti-tumorigenic STAT6 in DLBCL [93] 2. Role in CML resistance [98]
SHP-1	Reduced JAK/STAT	JAK, Kit [250,251]	1. Silencing by methylation 2. Decreased protein expression	1. Observed in leukemia, lymphoma, MM [105–107] 2. Observed in CML [110]
LMWPTP	Reduced JAK/STAT	JAK, STAT5 [252]	Increased expression	Unfavourable prognosis in solid tumors and leukemia [121,122]
SHP-2	Reduced GH signaling Enhanced Ras/MAPK Reduced JAK/STAT	JAK2, Src [149], ICSBP [134], Kit [255], STAT5 [256] GH receptor [143]	1. Constitutive active 2. Gain of function mutation	1. Myeloproliferative disease in mice [134] 2. Predisposition to JMML, growth disorder. Observed in AML, CMML, MDS, B-ALL [7,96,140–142]
HePTP	Proliferation pathway ERK1/2	p38, ERK1/2 [155]	1. Triplication of gene and overexpression of protein 2. Decreased expression	1. Observed in MDS and AML [163] 2. Observed in paediatric B-cell lymphoma [161,162]
DUSP1	Reduced MAPK pathways	ERK1/2, JNK and p38 [164]	Overexpression	Increased Ras–MAPK signaling in bone marrow from relapsed AML patients [180]
DUSP2	Reduced MAPK pathways	ERK1/2, JNK and p38 [164]	1. Loss of protein 2. Overexpression transcript	1. Increased ERK1/2 signaling in acute leukemia [174] 2. Observed in LGL leukemia, possible role in autoimmune disease [175]
DUSP16	Reduced MAPK pathways	ERK1/2, JNK and p38 [164]	Silencing by microRNA-24	Alters myeloid cell proliferation and differentiation [167]
PTEN	Reduced PI3K/PKB pathway	PtdIns(3,4,5)P_3 at position 3 [187]	1. Homozygous and heterozygous deletion 2. Hypermethylation 3. Decreased expression 4. Increased Expression 5. Silencing by miRNA-21 or miRNA-155 6. Inactivating phosphorylation	1. Associated with poor prognosis in T-ALL [196,197] 2. Deficient PTEN expression in JMML, increased imatinib resistance in AML [200,201] 3. Observed in AML and B-ALL [109] 4. Observed in monocytic leukemia and paediatric B-ALL [205] 5. Constitutive PKB activity in leukemia [203] 6. Correlates with poor prognosis in AML [207,208]
SHIP	Reduced PI3K/PKB pathway	PtdIns(3,4,5) P_3 at position 5 [20,187]	1. Inactivating mutation 2. Decreased expression	1. Observed in AML, leads to drug resistance [221] 2. Observed in ALL and CLL [224,225]

the cells resistant to GSI [199]. Loss of functional PTEN has been attributed to PTEN promotor hypermethylation in a number of cancers. Recently, Pten epigenetic mutations have been also described in JMML. Liu et al. suggested that DNA hypermethylation in the 5' upstream region of the PTEN gene was responsible for deficient PTEN gene expression in 77% of the patients analysed [200]. In addition, reduced PTEN expression in ALL due to promotor methylation resulted in imatinib resistance in ALL cell lines [201]. However, these data were disputed by Batz et al., who showed that hypermethylation of the PTEN upstream CpG island in JMML is infrequent and probably not a relevant epigeneticmechanism of disordered PTEN expression in leukemia [202]. Many reasons could account for this difference such as geographic variation or other heterogeneity between the two patient cohorts. Other regulatory mechanisms for PTEN expression have been described. For instance, downmodulation of PTEN expression, resulting in constitutive PKB activation in leukemia, has been attributed to the increased expression of oncogenic microRNA miR-21 and miR-155 in these tumor cells [203]. Antisense oligonucleotides targeting these miRNAs restored PTEN expression and enhanced apoptotic activity. Aberrant microRNA expression has been described in a number of malignancies, and strategies to target thesemiRNAs for the treatment of cancer are under investigation [204]. Low PTEN protein expression has been observed in AML, ALL, and paediatric B-ALL by flow cytometry, but surprisingly, increased expression was observed in monocytic leukemia [205]. In addition, using immunohistochemistry, Gauffiun et al. found protein overexpression of PTEN in paediatric ALL samples [109]. These conflicting data might be explained by Sylva and co-workers who reported increased PTEN protein levels in T-ALL cells, but found that this was correlated with decreased PTEN activity as a result of increased casein kinase 2 (CK2) activity and reactive oxygen species (ROS) production in these cells [206]. Inhibiting CK2 activity and ROS production restored PTEN activity, and reduced PI3K/PKB signaling in this study. PTEN is also inactivated through phosphorylation on serine/threonine residues at its C-terminus, and phosphorylated PTEN levels are associated with poor prognosis in AML [207,208].Ithasbeen suggested that resistance to the apoptosis inducing agent TRAIL was due to constitutive PTEN phosphorylation in ALL cell lines [209].As CK2 is the main kinase responsible for PTEN phosphorylation, there might be a potential role for CK2 inhibitors in the treatment of haematological malignancies presenting with PTEN phosphorylation. In fact, the use of CK2 as therapeutic target in AMLwas suggested after its expression was found to be an unfavourable prognostic factor in these leukemias, although unfortunately PTEN phosphorylation status was not investigated in this study [210].

SHIP-1/2

Characteristics and signaling

Although PTEN is classified as PTP, most of its actions in cancer are derived from its major role as a lipid phosphatase. Other lipid phosphatases have been described that are important in health and disease [20]. One of these is the SH2 domain-containing 5-inositol phosphatase, SHIP, which dephosphorylates inositol lipids at the 5D position of the inositol ring, hydrolysing PtdIns(3,4,5)P3 to PtdIns(3,4) P2. As such, SHIP has mostly been seen as negative regulator of PI3K activity. However, the SHIP-1 protein contains an N-terminal SH2 domain, a central conserved 5-phosphatase domain, two potential phosphotyrosine binding

domains, and a C-terminal proline rich SH3 binding region [19]. These different regions ensure that besides being a lipid phosphatase, SHIP also acts as an adaptor protein by binding to other molecules, such as Src kinase and the Ras activating protein Grb2 (reviewed by [211,212]). Therefore, studies into increased and eliminated SHIP expression on cellular effects need to be interpreted with care, as they might not only reflect the effect on the PtdIns(3,4)2 levels, but also take into account other SHIP functions. For instance, expression of an IGF-R mutant resulting in improper membrane localisation of SHIP-1 did not affect PKB activity, although IGF-induced ERK phosphorylation was downregulated, suggesting a PtdIns(3,4)P2-independent effect [213]. In addition, more and more evidence suggests that although PTEN is the major regulator of PI3K signaling by decreasing PtdIns(3,4,5)P3 levels, fine tuning of PI3K signaling is performed by SHIPs and not necessarily inhibitory. Although PtdIns (3,4,5)P3 is required, exogenous PtdIns(3,4)P2 enhances PKB phosphorylation, and is necessary for full PKB activation [214]. These results were further explained by Ma and colleagues, who showed that whereas PtdIns(3,4,5)P3 was responsible for Thr308 phosphorylation of PKB, PtdInd(3,4)P2 was required for Ser473 and hence membrane activity of PKB [215]. Nevertheless, the level of phosphorylation of PKB on Thr308, but not Ser473, was associated with poor prognosis in AML patients, suggesting that PtdIns(3,4,5) P3 is the major determinant of clinical outcome [216].

Role in malignancy

Multiple isoforms of SHIP-1 have been described, including a full length 145 kDa SHIP-1, a ~135 kDa SHIPbeta containing a deletion between the tyrosine binding motifs [217,218], a ~108 kDa SHIPdelta which lacks 167 nucleotides in the C-terminal region [219] and a stem cell specific 104 kDa isoform s-SHIP (also termed SIP-110 in humans) which lacks the SH2 domain [220–224]. SHIP-1 and SHIP-2 share 38% amino acid homology [225–227], but whereas SHIP-2 is ubiquitously expressed, SHIP-1 is predominantly found in cells of the haematopoietic lineage and bonemarrowmicroenvironment, with a higher expression in CD34+progenitor cells and T-cells and a lower expression in B-cells and granulocytes [228,229]. In addition, SHIP-1 rather than SHIP-2 is responsible for controlling PtdIns(3,4,5) P3 levels in haematopoietic cells [230]. SHIP−/− mice show increased haematopoietic stem cell numbers and proliferation, splenomegalomy and pulmonary macrophage infiltration [231]. Importantly, SHIP−/− mice display disturbed haematopoiesis as evidenced by decreased lymphopoiesis and increasedmyelopoiesis resulting inmyeloproliferative disease, suggesting that cell lineages are differentially affected by SHIP deletion [232].Luo and co-workers demonstrated the presence of a SHIP inactivating mutation in a patient with AML. Transduction of T-cell lines with SHIP harbouring this mutation resulted in increased cytokine-induced PKB phosphorylation and drug resistance [233].Thissamegrouplater reported the presence ofmultiplemutations in the coding region of the HIP gene INPP5D in a cohort of Chinese leukemia patients [234], although a similar study in a Caucasian population showed that codingregion SHIPmutationswere rare, and no differences in gene expression between AML and healthy controls were observed [235].Veryrecently, an American group reported decreased SHIP RNA and protein levels in almost all primary ALL samples studied, in conjunctionwith inactivating mutations and alternative splicing in the SHIP gene sequence [236]. Primary CLL samples do not contain SHIP-2, and variable expression of SHIP-1; decreased expression and tyrosine phosphorylation of SHIP-1 was observed in CLL

patients that are positive for the tyrosine kinase ZAP70, a poor prognostic indicator [237]. In addition tomutations in the SHIP gene itself, SHIP might also play a role in Bcr–Abl mediated malignancies, as SHIP expression is inhibited by this oncogenic fusion kinase, and SHIP-2 binds specifically to the SH3 domain of Abl. [238,239]. As activity of SHIPs is regulated by their protein levels, these studies imply a role for decreased SHIP activity in haematological malignancies [240]. Indeed, a SHIP agonist was identified, which increased SHIP activity, decreased PKB/AKT phosphorylation and was able to induce cytotoxicity in myeloma cells [241,242]. However, there is some controversy concerning the exact role of SHIP in tumor cell survival. A recently published report shows that loss of SHIP expression may increase leukemia incidence [243],whereas others have shown that SHIP deletion did not result in increased leukemogenesis but rather inhibited cell growth [244].Inaddition, whereas overexpression of SHIP-1 leads to apoptosis inmurinemyeloid cells, re-expression of SHIP in Jurkat T-cells, normally devoid of both SHIP and PTEN, leads to increased cell cycle time, but not apoptosis [245,246]. As PtdInd(3,4)P2 is capable of PKB/AKT activation, SHIP activity may actually contribute to PKB activation and hence lead to growth and survival of neoplastic cells. Indeed, besides PtdIns(3,4,5)P3, PtdIns(3,4)P2 levels are increased in leukemia cells and increased levels of PtdIns(3,4)P2 promote the transformation and tumorigenicity of mouse embryonic fibroblasts [247,248]. Furthermore, a recently identified specific SHIP-1 inhibitorwas able to reduce leukemic andMM cell growth in vitro, which could be rescued by the addition of endogenous PtdInd(3,4)P2 to cultures [249]. It is therefore very likely that PKB activity and subsequent cellular effects, such as survival, are very delicately regulated by the levels of PtdIns(3,4,5)P3 and PtdIns(3,4) P2,where disruption in either direction could lead to cell death. Its clear involvement in diversehaematologicalmalignancies and the availability of both agonists and antagonists make SHIP an interesting target candidate for treatment of blood disease.

Concluding remarks

Most research to date has focussed on the role of kinases in cancer cell biology, and as a consequence, kinase inhibitors play amajor role in novel therapeutic development. However, it is becoming increasingly clear that phosphatases are involved in numerous diseases, including haematological cancers, thus providing an additional focus of interest in drug development (Fig. 3, Table 2). In fact, several specific serine/threonine and tyrosine phosphatase inhibitors are under development, some of which have already been shown to be orally tolerable [14,250,251].Phosphatases are generally regarded as inhibitors of oncogenic signaling and hence tumor suppressors, which might account for the lack of attention they have received in haematological cancer treatment to date. Reduction of phosphatase activity is observed in a number of malignancies and seems difficult to overcome using targeted medicine. The challenge here would be to develop compounds that activate rather than inhibit phosphatases. For SHP-1 this has already been achieved; in a search for SH2-domain inhibitors, Park et al. discovered that SHP-1 is specifically activated by peptidyl aldehydes [252].However, this approach would not work in patients where reduced phosphatase activity is a result of an inactivating mutation or deletion. Gene therapy or protein delivery systems such as TAT fusion proteinswouldberequiredtohelpthese patients

[253]. Unfortunately, such technologies are still in infancy and safety issues make them unlikely to contribute substantially towards cancer treatment in the upcoming years. In some instances, i.e. PTEN and SHIP, it has been reported that reduced phosphatase expression in leukemic cellswas a result of aberrant miRNA expression [254]. As mentioned before, these miRNA are now being considered as targets for therapy [255]. Although downregulation of phosphatase activity often is apparent in haematological malignancy, there is also a growing body ofevidence suggesting that overexpression of phosphatases might contribute to the tumor pathology, as is the case with for instance SHP-2, LMWPTP, PTP1B and CD45. In these cases, specific inhibitors might find clinical applications. However, as not all patients exhibit increased phosphatase activity, the use of these inhibitors calls for personalised medicine. In addition, as some phosphatases might have both positive and negative regulatory functions, extensive research is needed to discover the long-termeffects of the use of these inhibitors. Due to their major role in cell growth, proliferation and survival protein and lipid phosphatases are of great potential interest in cancer treatment, and deserve a lotmore attention than they currently receive Further research into their precise role in normal and tumor cell biology might, in time, make them a more appreciated clinical target.

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Chapter 7

Molecular basis of chemotherapy resistance in myeloid leukemia cells

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Submitted

Abstract

Differences in cellular biochemistry between chemotherapy-resistant and sensitive leukaemia cells are still poorly understood, but knowledge of these differences may be of obvious use for designing improved therapy. This consideration prompted us to generate comprehensive descriptions of cellular kinase activities in a drug-resistant leukaemia cell line (Lucena-1) and its parental p210BCR-ABL-expressing erythroleukaemic CML cell line (K562). To this end, arrays of kinase peptide substrates were used, and the kinomic profile was validated by using conventional technology. The results provide a wealth of biochemical data on the molecular processes associated with the drug-resistant phenotype. Interestingly, the most prominent difference between Lucena-1 and K562 cells was a signalling pathway triggered by survival-associated receptor tyrosine kinase pathways feeding into the phosphatidylinositol-3-OH-kinase phosphate which, amongst others, produces the parallel activation of mTOR and p21Rac pathways. The importance of these pathways for the chemoresistant phenotype was investigated using pharmacological inhibitors and revealed that the mTOR pathway in part mediates chemoresistance. Overall, our findings pinpoint the mTOR pathway as a novel component in drug resistant leukaemia and provide a rationale for employing mTOR inhibitors in chemotherapy-resistant erythroleukaemia.

Introduction

Resistance to chemotherapy is a major drawback in the effective treatment of leukaemia. Although initially most leukaemias react favourably to chemotherapy, the ultimate treatment outcome of most adult acute myeloid leukaemias remains disappointing. The major problem here is the build-up of resistance against therapy. The processes that are associated with the acquisition of a therapy resistant phenotype remain only partially understood but involve overexpression of transporters (e.g. P-Glycoprotein), induction of autophagy, activation of the biotransformation enzymatic complex and an increase of intracellular vesicle trafficking [1,2]. Knowledge of the biochemical details associated with chemotherapy resistance may provide clues for rational design of novel therapeutic avenues. However, elucidating the specific characteristics of cellular physiology associated with the chemotherapy resistant phenotype is hampered by a lack of a priori knowledge of the signaling pathways actived or deactived in such cells. Hence, this problem is best approached in an unbiased comprehensive fashion. Over the last years, array and mass spectrometry technologies have enabled analysis of the transcriptome and proteome of biological systems. Although these technologies brought out significant knowledge about the molecular mechanisms that govern normal and pathological cellular responses towards diverse stimulus, an equally, if not more, important goal is to define the active kinases in these different conditions. Traditional genetic and biochemical approaches can certainly provide some of these answers, but for technical and practical reasons these are typically pursuing one gene or pathway at a time. Exitingly, however, extensive description of mammalian signaling cascades is possible through the sequential spotting of kinase substrates on a carrier. When such peptide arrays are incubated with cellular lysates and radioactive ATP, comprehensive descriptions of cellular signaling may be generated. Recently, important successes with this methodology were obtained including the description of lipopolysaccharide signaling in human peripheral blood monocytes [3], the identification of Fyn and Lck as early targets in glucocorticoid signaling [4] and the identification of the signaling pathways specifically active in Barrett's oesophagus as compared to the surrounding normal epithelium [5]. Hence, kinome profiling using peptide arrays offers the tantalizing possibility to study cellular signal transduction in a more or less comprehensive fashion. In the current study, we employ peptide microarrays to compare the kinome profile of the drug sensitive p210-BCR-ABL chronic myelogenous leukaemia cell line K562, and its resistant counterpart Lucena-1. Interestingly, the mTOR pathway is activated in Lucena-1 cells compared to K562 cells, and mTOR inhibition reverts chemoresistance of Lucena-1 cells. Hence, this study sheds light on the biology of chemotherapy resistance and provides a clear direction for strategies aimed at pharmacological intervention for chemotherapy resistant disease.

Material and methods

Cell line and Reagents

K562 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and the resistant cell line Lucena-1 was produced as described previously [6]. Polyclonal

antibodies against c-Raf (Ser338), ERK1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), JNK1/2 (Thr183/Tyr185), mTOR (Ser2448), S6 ribossomal kinase (Ser235/236), P70 S6 kinase (Thr389), PKB (Ser473), 4EBP1 (Thr70), paxillin (Tyr118), Src (Tyr416) and GSK3β (Ser9) were purchased from Cell Signalling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies, anti-sheep and anti-goat, were obtained from Dako (Glostrup, Denmark). Kinases inhibitors, SP600125 and SB203580, were purchased from Sigma (St. Louis, MO), U0126 was from Calbiochem (Darmstadt, Germany) and PP1 from BioMol (Exeter, UK). The stock solutions of these inhibitors were prepared in DMSO. Imatinib purchased from LC laboratories (Woburn, MA, US) and LY294002 from Alexis (Läufelfingen, Switzerland) was dissolved in methanol. Vincristine sulfate was purchased from Sigma (St. Louis, MO, US) and was dissolved in methanol.

Cell Culture

K562 and Lucena-1 cells were routinely grown in suspension in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 % heat-inactivated fetal bovine serum, at 37 °C in a 5 % CO_2 humidified atmosphere. The Lucena-1 culture medium was routinely supplemented with 60 nM Vincristine.

Pepchip analysis

Kinome arrays were performed essentially as described before [3-5]. In short, cells (2.5×10^5) were washed in PBS and lysed in a non-denaturing complete lysis buffer. The peptide arrays (Pepscan, Lelystad, The Netherlands), containing up to 1024 different kinase substrates in triplicate, were incubated with the cell lysates for 2 h in a humidified incubator at 37 ° C. Subsequently, the arrays were washed in 2 M NaCl, 1% Triton-X-100, PBS, 0.1% Tween and water, thereafter slides were exposed to a phospho-imaging screen for 24-72 h and scanned on a phospho-imager (Fuji, Stanford, USA). The level of incorporated radioactivity, which reflects the extent of phosphorylation, was quantified with specific array software (EisenLab ScanAlyze, version 2.50). Datasets from chips were then analyzed statistically using PepMatrix, as described by Milani et al. 2010 [7]. Basically, spot replications were scrutinized for consistency using two indexes: one being the standard deviation:average (SD/A) ratio and the other being the ratio between the average and the median (A/M) of all three replications for each chip. Parameters applied to the indexes were an SD/A < 50% and 80% < A/M < 120%. The fold change in phosphorylation between control and treated cells was assessed using Student's t-test, with $P < 0.05$ indicating significance. Distribution of shared events in K562 and Lucena-1 was visualized using Venny (Oliveros, J.C. (2007) VENNY http://bioinfogp.cnb.csic.es/tools/venny/index.html). Functional interaction networks were constructed using the Reactome FI plugin [8] for Cytoscape[9].

Western blot analysis

Western blot experiments were performed as described before [10]. In detail, cells (3×10^7) were lysed in 200 µL lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Tween 20, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na3VO4, 1 mM NaF and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl-fluorid-hydrochloride)] for 2 h on ice. Protein extracts were cleared by centrifugation and protein concentrations were determined using the Lowry method. An equal volume of 2X sodium

dodecyl sulphide (SDS) gel loading buffer [100 mM Tris–HCl (pH 6.8), 200 mM DTT, 4 % SDS, 0.1 % bromophenol blue and 20 % glycerol] was added to samples which were subsequently boiled for 5 min. Cell extracts were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to nitrocelulose membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)-Tween 20 (0.1 %) and incubated overnight at 4 °C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.1 %), membranes were incubated with anti-rabbit, anti-mouse, anti-sheep and anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:2000 dilutions, in blocking buffer for 1 h. Proteins were detected using enhanced chemiluminescence (ECL).

K562 and Lucena-1 treatment

The cells were plated at 1,5 x 10^5 cells/ml and treated with SP600125 (10 and 20 μM), SB203580 (10 and 20 μM), U0126 (10 and 20 μM), PP1 (200 and 500 nM), Imatinib (0,5 and 1 μM) or LY294002 (10 and 20 μM) for 24 hours. For drug resistance reversion assays, 5 x 104 cells/ml were plated and treated with SP600125 (5 μM), SB203580 (5 μM), U0126 (5 μM), PP1 (200 nM), LY294002 (5 μM) or Rapamycin (10, 20 and 50 nM) in the presence of vincristine (0-500nM) for 48 hours. After treatment, cell viability was assessed by trypan blue dye exclusion and MTT reduction assays as previously reported [11].

Rac activation assays

Activated Rac was precipitated using bacterial lysate containing glutathione S-transferase (GST)–cdc42/Rac-interactive binding (CRIB) domain of p21-activated kinse (PAK; GST-PAK-CRIB), as described previously12. Briefly, K562 and Lucena-1 were grown in 0.5% FCS. After 24 hours, 10^6 cells were stimulated with 1 μM vincristine for 30 sec, 2 min and 1h. As a positive control the cells were stimulated with 100ng/ml PMA for 5 min. After stimulation, cells were lysed for 10 min in lysis buffer [50 mM Tris, pH 7.4, 10% glycerol, 200 mM NaCl, 1% Nonidet P-40, 2 mM $MgCl_2$, 2 mM sodium orthovanadate, and protease inhibitors]. Lysates were cleared by centrifugation, and GST-PAK-CRIB protein, pre-coupled to glutathione-sepharose beads (Pharmacia, Uppsala, Sweden), was added for 30–45 min at 4°C. Beads were washed three times with 1x lysis buffer and boiled in Laemmli sample buffer. The bound proteins were separated by 15% SDS-PAGE and transferred to a nitrocelulose membranes (Biorad). Activated Rac was detected by probing the membrane with anti-Rac antibody and rabbit anti-mouse peroxidase-conjugated antibodies followed by enhanced chemiluminescence (ECL). Equal amounts of glutathione-sepharose beads were loaded in all samples as determined by Ponceau S staining of the membranes. Quantification of the amount of precipitated, active GTPase was performed by ImageJ software. Results are presented as normalized densitometry values [arbitrary units (AU)].

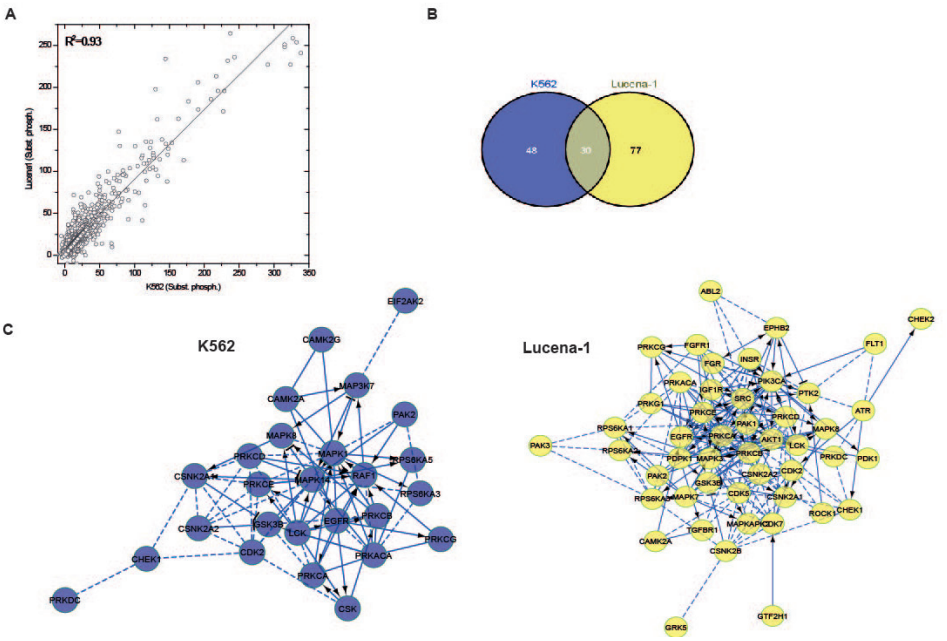
Statistical analysis

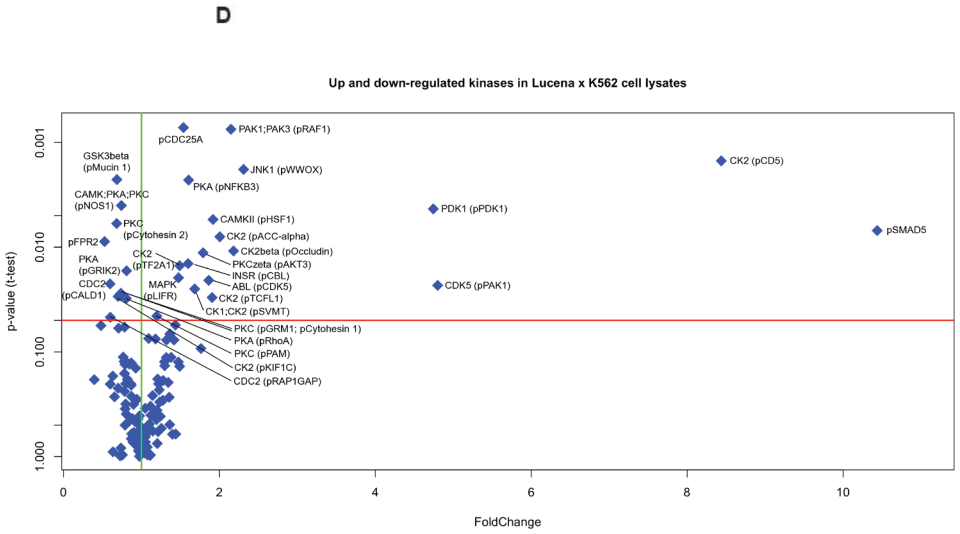
All experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Data from each assay were analyzed by ANOVA. Multiple comparisons among groups were performed with Turkey post hoc test. Differences were considered significant when the p value was less than 0.05.

Results

Kinome profiling of K562 cells and its multidrug-resistant counterpart Lucena-1

The changes in cellular biochemistry associated with acquisition of the multidrug resistant phenotype remain only partially understood but are important to ascertain in order to develop improved therapy. The erythroleukaemic cell line K562 and its derivative cell line Lucena-1 have been described to be a useful model for investigating the acquisition of multidrug resistance, the former being sensitive to a variety of therapeutically used agents, whereas the latter resists such treatment [10]. Hence, we studied kinase activity of K562 and Lucena-1 cells using kinase arrays displaying 1024 consensus peptides [3-5]. The quality of the profiles obtained was good as technical replicates showed an average Pearson product-moment correlation coefficient of 0.93 (figure 1A). Single data analysis of arrays with K562 cell lysates resulted in the statistically significant phosphorylation of 78 peptides, whereas lysates of Lucena-1 cells produced phosphorylation of 107 peptides. Of these two sets 30 peptides were similar (see Venn diagram figure 1B). Based on this analysis the prominent signalling network in K562 and Lucena-1 were built (figure 1C). Subsequent statistical analysis of signal intensity showed that the phosphorylation intensity of 30 peptides were significantly different when Lucena-1 and K562 cells were compared ($P < 0.05$) (figure 1D). In general all kinases with higher activities in Lucena-1 cells are involved in signalling pathways responsible for cellular proliferation and survival. A provisional signal transduction scheme interpreting these results and showing the apparent differences in cellular signalling associated with the acquisition of chemotherapy resistance is shown in





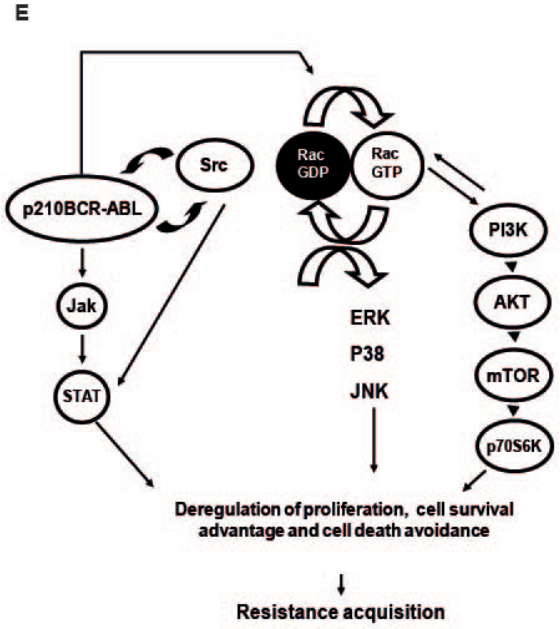


Figure 1. Comparison of the kinome profiling between K562 and Lucena-1 cells
(A) Scatter plot showing the correlation between the pepchip arrays for K562 and Lucena-1 cells in responding correlation R2 (B) Venn diagramm of statistically significant phosphorylated sports in K562 (blue) and Lucena-1 (yellow) (C) Signalling networks for K562 and Lucena-1 were buildusing cytoscape software and reactome repository as described in the methods (D) Comparison between statistically significant phosphorylated spots in L562 and in Lucena-1 cells are shown. This graph shows the correlation between fold and p-values for the statistically significant phosphorylated spots in both cell lines. (E) Provisional signal transduction scheme of active signaling pathways in Lucena-1 Cells.

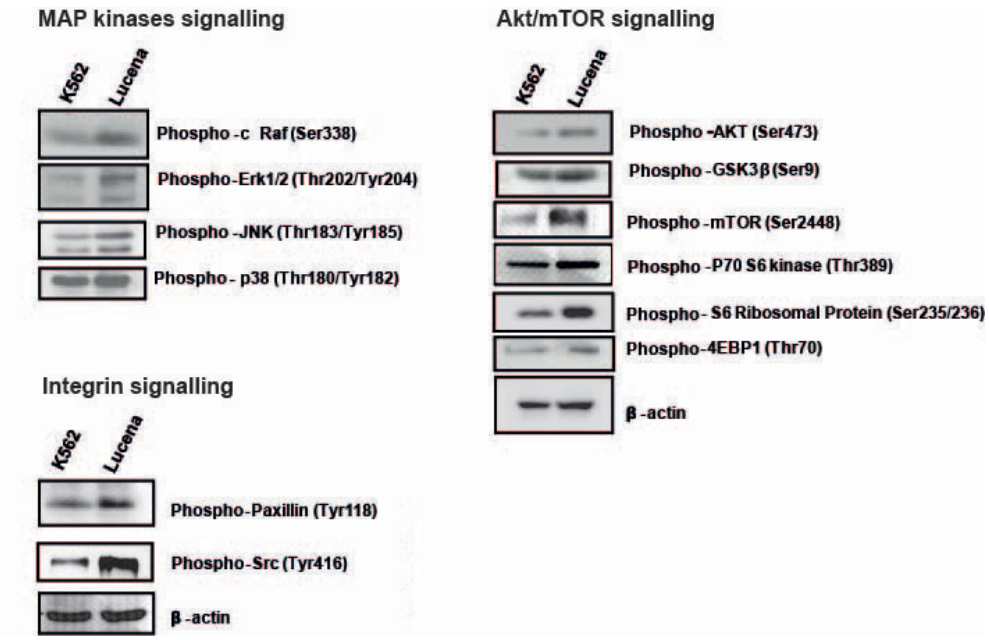


figure 2. Validation of the Kinome profiling
Lysates of K562 and Lucena-1 were submitted to western blotting and probed with anti phospho-site specific antibodies

Figure 1E. The most prominent features of this provisional scheme are increased activity of the mTOR pathway (as illustrated for instance by increased protein kinase B signalling and reduced GSK-3ß activity) and concomitantly the activation of p21Rac dependent signaling (as illustrated by increased downstream signaling, i.e. Pak1, Map kinases, Abl and insulin receptor). Hence, the transition in erythroleukaemia from a chemotherapy-sensitive to a chemotherapy resistant phenotype is accompanied by distinct changes in cellular biochemistry.

Validation of kinome profile results

Although kinome profiling is a robust and reproducible technology, western blot analysis was performed to validate the obtained peptide array data. As shown in figure 2, Lucena-1 cells exhibit increased activation of p21Rac targets in cytoskeletal remodelling (Src and Paxillin) as well as downstream targets of this GTPase including cRaf, ERK1/2 and Jun-N-terminal-kinase. In addition, a strong activation of mTOR kinase and downstream targets was observed. Thus the results obtained are in concordance with the data emerging from peptide array profiles and hence these results confirm that the transition from a chemotherapy-sensitive to a chemotherapy resistant phenotype in erythroleukaemia is accompanied by distinct set of changes in activation status of specific signal transduction pathways.

Maintenance of p21Rac activity in presence of vincristine

Support for the notion that increased activity of a pathway involving p21Rac and its downstream effectors may be implicated in resistance to chemotherapy was obtained from experiments in which activation of this GTPase was studied directly. Vincristine, a clinical chemotherapeuticum highly toxic to the parental K562 cells but not to the resistant Lucena-1 cells, effectively reduced GTP-loading of p21Rac in K562 cells (figure 3A). However, vincristine did not produce the same effect in Lucena-1 cells (figure 3A). Thus, the increased activity of the p21Rac-dependent signaling emerging from the peptide array profiles may well have important roles for maintaining the resistant phenotype. Challenging K562 cells

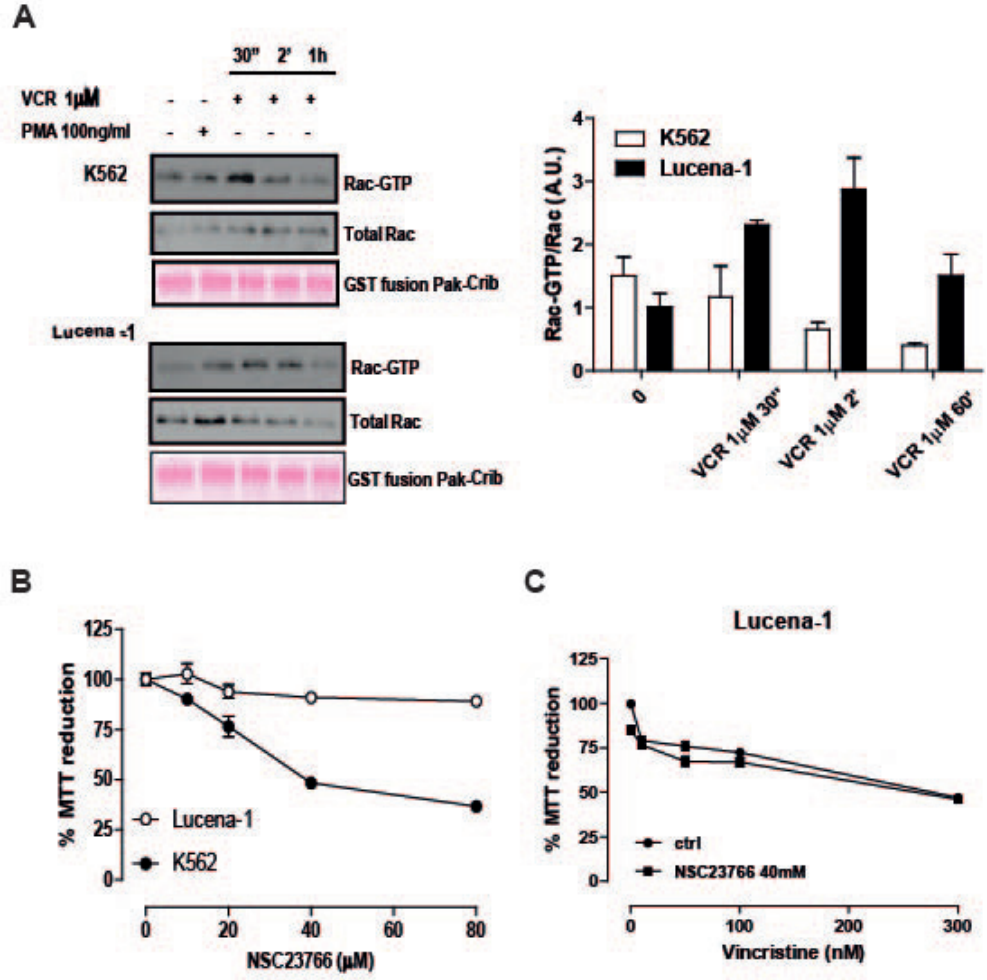


figure 3. p21rac activation in K562 and Lucena-1 in presence of Vincristine
(A) K562 and Lucena-1 were serum deprived for 24 hours, then stimulated with vincristine 1 μM (30 sec, 2 and 60 min). Cell lysates were prepared and incubated with GST beads pre-coupled with Pak-Crib fusion protein. After that, samples were analysed by western blotting. Western blotting images were analysed with ImageJ software. (B) K562 and Lucena-1 were treated with different concentrations of NSC23766 (rac inhibitor). In (C) Lucena-1 cells were treated with a combination of NSC23766 40 μM and vincristine. In B and D cell viability was determined by MTT reduction.

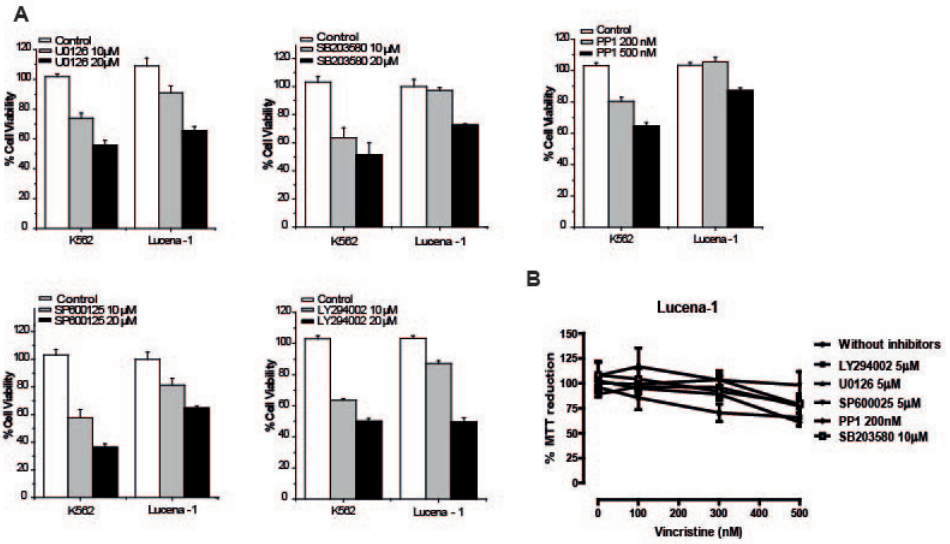


figure 4. Importance of different kinases for K562 and Lucena-1 viability
(A) cells were treated with kinase inhibitors for 24 hours, after this cell viability was determined. (B) Reversion of the Lucena-1 resistance in presence of JNK (SP600125), MAPKAP2(klopt dit? SB203580), Mek(U0126), PI3K (LY294002) and Src (PP1) kinase inhibitors. Lucena-1 cells were treated with vincristine 0-500 nM, with or without kinase inhibitors for 48 hours. After this cell viability was determined

with NSC23766, an inhibitor of rac GTP loading resulted in decreased viability and therefore a higher dependence on this event to survive (Figure 3B). However, treatment with NSC23766 did not decrease cell viability of Lucena-1 cells nor did it sensitize Lucena-1 cells to vincristine treatment (figure 3C). These data thus suggest that p21Rac is not the main player in chemoresistance of Lucena-1 cells. To confirm these findings, we also targeted downstream targets of the p21Rac pathway. As shown in figure 4A, targeting JNK (SP600125), MAPAPK2 (SB203580), Src (PP1) and Mek (U0126) reduces cell viability of both K562 and Lucena-1 cells when using these inhibitors in relatively high dose. More importantly, non of the kinase inhibitors did sensitize Lucena-1 cells towards vincristine (figure 4B) suggesting that the p21Rac pathway is indeed not the major pathway inducing drug resistance.

Rapamycin treatment sensitizes Lucena-1 cells to Vincristine treatment

Next to the p21Rac pathway, kinome profiling identified the mTOR pathway as a potential important pathway in drug resistance of Lucena-1 cells. To determine whether an activated mTOR pathway indeed contributes to drug resistance, Lucena-1 cells were treated with the well-known inhibitor rapamycin. As shown in figure 5A, Lucena-1 cells are sensitive to treatment with rapamycin pinpointing mTOR as an important component in this complex survival network present in Lucena-1 cells. Interestingly, rapamycin sensitizes Lucena-1 cells to vincristine treatment to some extent (figure 5B).

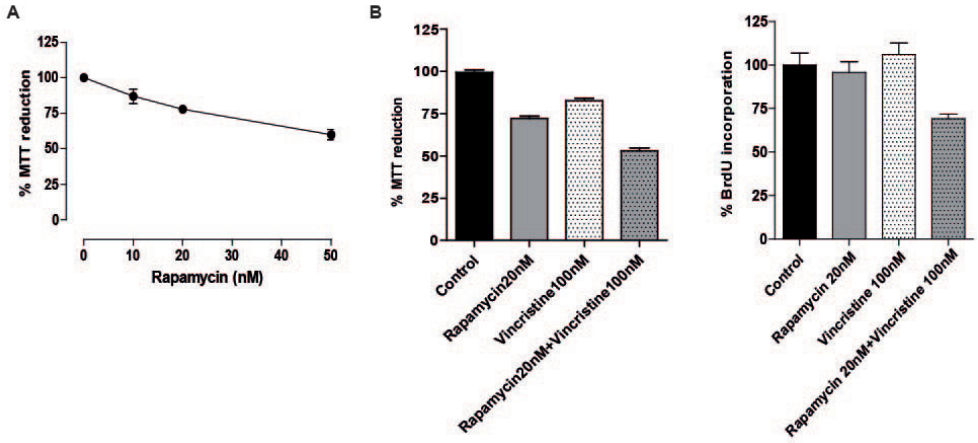


figure 5. Rapamycin enhances vincristine effect on Lucena-1 cell viability
(A) Lucena-1 cells were treated with rapamycin 0-50 nM for 24 hours, thereafter cell viability was measured by MTT. (B) Lucena-1 cells were treated with rapamycin 20nM in combination with vincristine 100nM for 48 hours. Cell viability and proliferation were determined by MTT reduction and Brdu incorporation, respectively.

Discussion

Among the major problems in dealing with leukaemic disease is the build-up of resistance against therapy [13-15]. This may include resistance associated with decreased drug accumulation in the cell, altered intracellular drug distribution, increased detoxification, diminished drug-target interaction, increased DNA repair, altered cell-cycle regulation, and uncoupling of the pathways linking cellular damage with programmed cell death [16,17]. In this context, increased expression of P-gp encoded by the mdr-1 gene is a well-characterized mechanism for chemoresistance in cancer cells. However, the biochemical mechanism behind drug resistance has still not been fully elucidated. Therefore, a better understanding of the mechanisms mediating resistance and novel targets for improved therapeutic options are required. In this paper, we profiled the kinome of K562 cells and its resistant counterpart Lucena-1 cells to potentially correlate the kinase activity with the resistant phenotype. The kinome profiling approach provided us with a global oversight of the alternation in kinase activities that accompanies the acquisition of multidrug resistance. A fairly coherent picture emerges in which the resistant phenotype exhibits more activity of survival-associated receptor associated tyrosine kinase signaling, promoting increased activity of the Akt/mTOR kinase pathway, diminished glycogen synthase kinase 3 activity, increased activation of p21 Rac (probably via phosphatidylinositol-3OH-kinase) leading to both cytoskeletal responses as well activation of the stress-activated kinase JNK. Measuring activity of these pathways using conventional technology confirmed these results and inhibition studies identified especially the mTOR kinase pathway as crucial for drug resistance. This kinase has been shown to be hyperactive in certain cancers, suggesting that the mTOR pathway is an attractive target for cancer therapy. Apparently activated mTOR provides certain tumor cells with a growth advantage by promoting protein synthesis [15]. The latter result is

important as it has been shown in clinical studies that mTOR inhibitors are safe, effectively inhibit this kinase in vivo and are associated with substantially reduced pro-inflammatory pathology [18-21]. Interestingly, rapamycin and novel rapalogs have already been used for the treatment of leukemia with rather disappointing results [22]. However, our data suggest that mTOR inhibitors should be administered together with routine chemotherapy.

It is important to note that the profiles obtained show that apart from mTOR many other kinase activities are different between resistant and sensitive cells. However, inhibitors of the kinases did not induce sensitivity suggesting these kinases are not essential for drug resistance. Alternatively, these kinases may still be important in drug resistance but due to redundancy in signaling pathways, single inhibitor treatment may not be the ideal strategy. Nevertheless, our identification of kinases as drugable mediators of resistance is of obvious importance for developing new therapies as well as biomarkers. Moreover, the identified kinases do contribute to the survival of the cells as high concentrations of the kinase inhibitors diminish viability of both K562 and Lucena-1 cells.

Overall, our findings suggest that mTOR driven signalling is a building block of the chemoresistant phenotype of Lucena-1 cells although mTOR inhibition does not completely revert chemoresistance. The exact mechanism by which mTOR contributes to resistance to chemotherapy remains obscure but may be related to the fact that inhibition of this kinase shifts the balance of important proteins regulating cell fate. Alternatively, mTOR inhibition by rapamycin is also suggested to decrease P-gp expression levels [23], although it is hard to believe that the rather subtle decrease in pump levels is the main mechanism by which mTOR inhibition induces sensitization of chemoresistant cells. Obviously, further research is required to fully understand the role mTOR in drug resistance and maybe more important to elucidate the cross talk between the different signal transduction pathways in drug resistance. Irrespective the actual mechanism by which mTOR contributes to drug resistance, our findings identify the mTOR pathway as an important component in drug resistant leukaemia and provide a rationale for employing mTOR inhibitors in chemotherapy-resistant erythroleukaemia.

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Chapter 8

Violaceinosis: an alternative mode of programmed cell death explaining the efficient chemoprevention by indole derivatives

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Submitted

Abstract

Programmed cell death (PCD) is essential for a plethora of physiological processes and is generally assumed to consist of 4 separate molecular modes of action: apoptosis, programmed necrosis (necroptosis), autophagy and pyroptosis, which are distinguished based on morphological and biochemical criteria. Developmental studies in genetically modified organisms indicate that additional mode(s) of PCD may exist as well, but these have not been characterized. Studying the cytotoxic effects of violacein, an antibiotic dihydroindolone synthesised by an Amazon river Chromobacterium, we observed that cell death induced in TF1 leukaemia cells is not mediated by the known PCD pathways. It is actually carried out by a biochemically and morphologically novel pathway involving inhibition of calpain and pro-inflammatory pathways concomitant with paradoxal activation of the canonical protein kinase B/mTOR and p42/p44MAP kinase signalling cassettes followed by an ultrastructurally highly characteristic cell death programme entailing amongst others endoplasmatic reticulum and Golgi linearization, nuclear mottening and cellular demise, a process which we have tentatively denominated as violaceinosis. We propose that violaceinosis is an alternative mode of cell death which may be important in explaining the action of cancer chemopreventive indolone derivatives.

Introduction

The physiology of metazoan organisms requires mechanisms that direct cellular death in a controlled fashion, a process collectively denominated as programmed cell death (PCD) [1]. During embryology and further development to sexual maturity, various structures like the pro-nephros and mesonephros have only transient functionality and are orderly disposed off when no longer necessary. Analogously, development of the immune system as well as termination of ongoing host-defence responses require the controlled elimination of a large number of effector cells [2]. Furthermore, aberrant compartment size regulation is implicated in many serious pathologies (most dramatically, maybe, in cancer), and prominently involves defects in programmed cell death [3]. Thus, the regulation of controlled cellular suicide represents one of the most fundamental issues in cell biology and the pursuit of determining the underlying mechanisms surpass those of purely academic interest, because of its ramifications in pathology. Nevertheless, despite tremendous progress in this field, the constituting components remain still unclear.

Cell-intrinsic programmed suicide is generally assumed to consist of four separate molecular modes of action: apoptosis, programmed necrosis (necroptosis), autophagy and pyroptosis, which are distinguished based on morphological and biochemical criteria [4]. Apoptosis is morphologically associated with cell shrinkage, membrane blebbing, and chromatin condensation, whereas biochemically it is characterised by the sequential activation of apoptotic caspases by cleavage of inactive proenzymes, yielding the cysteine-directed protease activity that drives the apoptotic cell death response [5]. Necroptosis is a deliberately-induced physiological variant of necrosis, normally an accidental and uncontrolled form of cell death lacking underlying signalling events. Morphologically distinctive features of necroptosis are cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space. Biochemically, it is characterised by TNFR-induced, TRADD-mediated RIP1 kinase activity. Many mediators are subsequently involved in the execution phase of necrotic cellular suicide, including reactive oxygen species, Ca_2+, calpains, cathepsins, phospholipases, and ceramide, finally producing PARP-1 supercleavage and necrotic cell death. Physiologically, the functioning of necroptosis lies in programmed cell death following ovulation, chondrocyte cell death associated with the longitudinal bone growth and intestinal cellular turnover [6]. A third mode of canonical PCD is autophagy. Biochemically, autophagic signaling acts through the shutdown of mTOR (mammalian target of rapamycin) kinase activity leading to the induction of autophagosome formation through a process that involves LC3 (Atg8) phosphatidylethanolamine lipidation, yielding LC3-II. The main morphological characteristic of autophagy is the engulfment and sequestering of cytoplasmic content in specialized double-membrane vesicles [7]. A last form of PCD is pyroptosis, which is a caspase-1-mediated cell death process and explicitly does not involve the apoptotic caspases, but is mediated by, amongst others, caspase 1-dependent nucleases. Pyroptosis is mainly associated with PCD following phagocyte infection of microbial pathogens, such as Salmonella or Legionella. Cells dying by pyroptosis have biochemical and morphological features of both apoptotic and necrotic cells, losing their mitochondrial membrane potential and plasma membrane integrity and releasing their cytoplasmic contents into the extracellular milieu. As in apoptosis, pyroptotic cells undergo

DNA fragmentation and nuclear condensation, but the oligonucleosomal fragmentation pattern characteristic of apoptosis is conspicuously lacking [8]. Thus different modes of cell death are biochemically and morphologically highly unique and if further modes of cell death do exist, it is to be expected that these would also be characterised by morphologically and biochemically highly distinctive features.

It is now generally recognised that different modes of PCD are intimately linked to the cancerous process, as apoptotic cell death is an important anti-neoplastic protective mechanism upon improper induction of cellular proliferation, and is also involved in cancer cell clearance by immunosurveillance, chemotherapy or radiotherapy [9]. Autophagy, which counteracts other forms of PCD including apoptosis, may play role in the escape from chemotherapy by transformed cells [10]. Necroptosis and pyroptosis are important for inducing cell death during inflammatory reactions, thereby preventing inflammation-associated cancer [4]. In addition, chemoprevention of cancer is also thought to be linked to PCD, inducing cellular suicide in pre-neoplastic lesions and thus halting development of full-blown cancer [11]. The mode of PCD involved in cancer chemoprevention is much less clear and may be different between types of chemopreventative agents and tumour cell types involved. Chemoprevention of colorectal cancer by NSAIDS [12], for instance, is most often linked to a pro-apoptotic response in the transformed compartment, but the robust chemoprevention of the same cancer type by the use of statins is linked to necroptosis [13]. We have recently presented evidence that chemoprevention induced by apigenin is linked to an autophagic response [10]. Generally speaking however the mode of PCD by many important cancer chemopreventative agents remains poorly characterised, prompting further research in this area.

One such chemopreventative compound is Violacein [3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1,3-dihydro-2H-indol-2-one], a purple-colored pigment-cum-antibioticum produced by Chromobacterium violaceum, a bacterium indigenous in certain parts of the Amazon river basin in Brazil [14]. The remarkable low incidence of immunoproliferative and autoimmune disease in areas habitated by C. violaceum led to the hypothesis that violacein counteracts uncontrolled proliferation in the human leukocyte compartment. This led to further investigations in this area and a broad spectrum of biological activities of the compound have been identified including strong anti-tumor activity [14-23], a property violacein shares with various other naturally occurring indolones [24], and violacein thus attracts wide-spread interest. The molecular mode of action, however, of indole derivatives-dependent chemoprevention in general and violacein in particular remains largely unclear.

In an effort to obtain further insight into the molecular mechanisms of violacein-mediated chemoprevention, we studied the effect of this compound on TF1 leukaemia cells. We observed induction of cellular suicide in these leukaemia cells, but subsequent studies to identify the mode of PCD responsible subsequently excluded apoptosis, necroptosis, pyroptosis and autophagy as important contributing factors. Morphological investigation as to the mode of cell death revealed a whole novel mode of cell death, involving endoplasmatic reticulum and Golgi linearization, and nuclear mottening. A biochemical

characterization involving kinome profiling using peptide arrays to yield comprehensive descriptions of cellular kinase activities revealed a calprotectin-based mechanism leading to cellular demise. Thus, our results demonstrate a novel mode of cell death involving both morphologically and biochemically unique properties. We propose that this novel form of cell death (tentatively called violaceinosis) mediates important aspects of indole-dependent chemoprevention.

Material and methods

Reagents

Polyclonal antibodies against ERK1/2 (Thr202/Tyr204), MAPAPK2 (Thr222), p38 (Thr180/Tyr182), mTOR (Ser2448), PKB, phospho-PKB (Ser473), phospho-PDK1 (Ser241), phospho-Src (Tyr527), phospho-GSK3β (Ser9), LC3B, Beclin1, and Apoptosis inducer factor (AIF), were purchased from Cell Signaling Technology (Beverly, MA). Secondary anti-rabbit and anti-mouse peroxidase-conjugated antibodies were also obtained from Cell Signaling Technology (Beverly, MA). Antibodies against anti-MAP LC3 and cleaved-PARP and secondary anti-goat antibodies were purchased from Santa Cruz (St. Louis, MO). Anti-Fas receptor (Fas), anti-Fas ligand (FasL) were from Immunotech (Marseille, France). TNFα was obtained from R&D Systems. Violacein (3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1,3-dihydro-2H-indol-2-one) was extracted and purified as previously described (25).

Cell culture and Treatments

TF1 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were routinely grown in RPMI 1640 culture medium (Gibco) containing 10% fetal bovine serum, 2 mM L-glutamine, 5ng/ml GM-CSF, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified incubator with 5% CO2 in air.

Cell viability

Cell viability was assessed by trypan blue dye exclusion and MTT reduction assays as previously reported [13, 26].

Cell Cycle Analysis

TF1 cells were cultured for 24 hours at a density of 2 x 10^4 cells/ml in serum free RPMI. After 24 h of serum starvation cells were treated with violacein for 24 h, and subsequently cells were washed with PBS and resuspended in 200 µl of a sodium citrate dihydrate (1 g/L) solution, containing 50 µl Ribonuclease A 10 mg/ml (Fermentas), propidium iodide 0.02 mg/ml (Sigma) and Triton X-100 (Sigma) 0.1%. Next, the cells were incubated in the dark for 60 min at room temperature. The analysis was performed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The cells were analyzed in low speed and at least 10,000 events were analyzed per sample. The DNA content was evaluated using a FL2 detector in a linear scale. To eliminate cell aggregates, the cell population to be analyzed was selected from a bivariate histogram showing the area (FL2A) versus the width (FL2W) of the signal FL2. The analysis of cell percentage in the different phases of the cell cycle (G0/G1, S, and

G2/M) was performed using the ModFit LT software (BD Biosciences, San Jose, CA, USA).

Western blotting

Cells (3 x 10^7) were lysed in 200 µL cell lysis buffer (50 mM Tris [tris(hydroxymethyl) aminomethane]–HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM O-Vanadate, 1 mM NaF, and protease inhibitors [1 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluoride-hydrochloride]) for 2 h on ice. Protein extracts were cleared by centrifugation, and the protein concentration was determined using Lowry. An equal volume of 2x sodium dodecyl sulfide (SDS) gel loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added and the samples were boiled for 10 minutes. Cell extracts, corresponding to 3 x 10^5 cells, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)–Tween 20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-goat and anti-mouse horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions (in all Western blotting assays) in blocking buffer for 1 h. Detection was performed by using enhanced chemiluminescence (ECL).

Transmission electron microscopy

After incubated with violacein, the cells were fixed with 2.0% phosphate-buffered glutaraldehyde. The cells were then postfixed in 1% phosphate-buffered OsO4, and embedded in Spurr's resin. Thin sections (0.12 µm) were cut, double stained with UO2(CH3COO)2 (uranyl acetate) and Pb3C12H10O14 (lead citrate), and visualized with a Philips TECNA10 transmission electron microscope (TEM). Fifty cells from randomly chosen TEM fields were analyzed for each treatment or control [27].

Kinomic array

Kinome arrays were performed essentially as described before [28-30]. In short, cells were washed in PBS and lysed in a non-denaturing complete lysis buffer. The peptide arrays (Pepscan, Lelystad, The Netherlands), containing up to 1024 different kinase substrates in triplicate, were incubated with the cell lysates for 2 h in a humidified incubator at 37 °C. Subsequently, the arrays were washed in 2 M NaCl, 1% Triton-X-100, PBS, 0.1% Tween and water; thereafter slides were exposed to a phospho-imaging screen for 24-72 h and scanned on a phospho-imager (Fuji, Stanford, USA). The level of incorporated radioactivity, which reflects the extent of phosphorylation, was quantified with specific array software (EisenLab ScanAlyze, version 2.50). Datasets from chips were then analyzed statistically using PepMatrix, as described by Milani et al. 2010. Basically, spot replications were scrutinized for consistency using two indexes: one being the standard deviation:average (SD/A) ratio and the other being the ratio between the average and the median (A/M) of all three replications for each chip. Parameters applied to the indexes were an SD/A<50% and 80%<A/M<120%. The fold change in phosphorylation between control and treated cells was assessed using Student's t-test, with $P<0.05$ indicating significance.

Statistical evaluation:

The Western blots represent 3 independent experiments. Cell viability data were expressed as the means ± standard deviation of 3 independent experiments carried out in triplicates. Data from each assay were analyzed statistically by ANOVA. Multiple comparisons among group mean differences were checked with the Tukey test. Differences were considered significant when the p value was less than 0.05.

Results

Violacein has a unique capacity to induce PCD in TF1 leukaemia cells

The CD34+/c-Kit+/P-glycoprotein+/MRP1+ TF1 leukaemia progenitor cell line has been reported to be unusually resistant against PCD [31]. In apparent agreement, we observed that neither stimulation with high concentrations of Fas ligand, tumour necrosis factor-α (TNF-α) or strong chemotherapeutic agents like mitoxatrone for 24 hours affected TF1 survival to a large degree as assessed by MTT reduction (figure 1a). Accordingly, these stimuli neither produced evident Annexin V positivity in FACS analysis nor an obvious cell death phenotype in transmission electron microscopy (not shown). Importantly, however, these cells turned out to be highly sensitive to violacein, clear effects on MTT reduction already being evident in the low µM range after 24 h and a 2µM concentration of violacein was incompatible with TF1 cell survival after 72 h (figure 1b). Subsequent analysis showed that the cell death by violacein is not accompanied by loss of trypan blue exclusion (not shown), suggesting the effect of violacein is not due to direct toxic necrosis but that a specific cell death programme mediated cellular demise. In apparent agreement, analysis of TF1 cell DNA content shows that violacein-dependent cell death was accompanied by destruction of the genome (figure 1c). Hence we conclude that violacein induces a form of PCD in otherwise highly intrinsically PCD-resistant TF1 leukaemia progenitor cells.

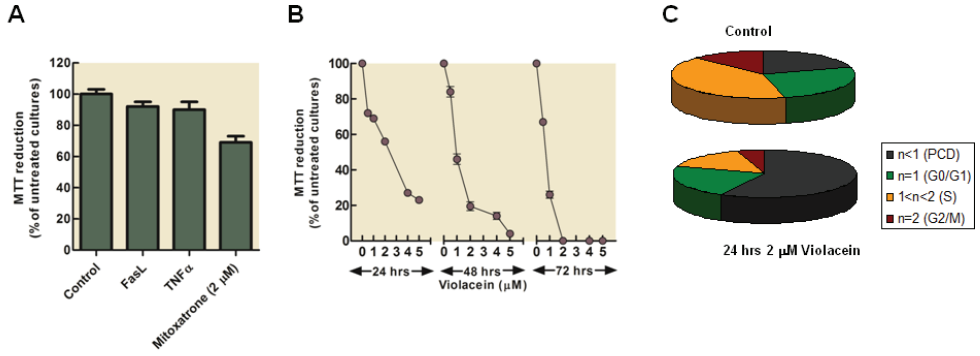


Figure 1. Induction of cell death by Violacein in intrinsically resistant TF1 myeloerythroid leukaemia cells. (A) TF1 cells are unusually resistant with respect to PCD. Cells were exposed for 24 hrs to FasL, TNFα and mitoxantrone. (B) TF1 cells were exposed to various concentrations of Violacein (X-axis) in the presence of 10% FCS and analyseed for cellular survival (as assayed by the capacity of cellular cultures to reduce MTT; Y-axis) 24 hrs, 48 hrs and 72 hrs later as indicated. (C) Analysis of cellular DNA content by FACS shows that Violacein-induced TF cell death is accompanied by breakdown of the genome.

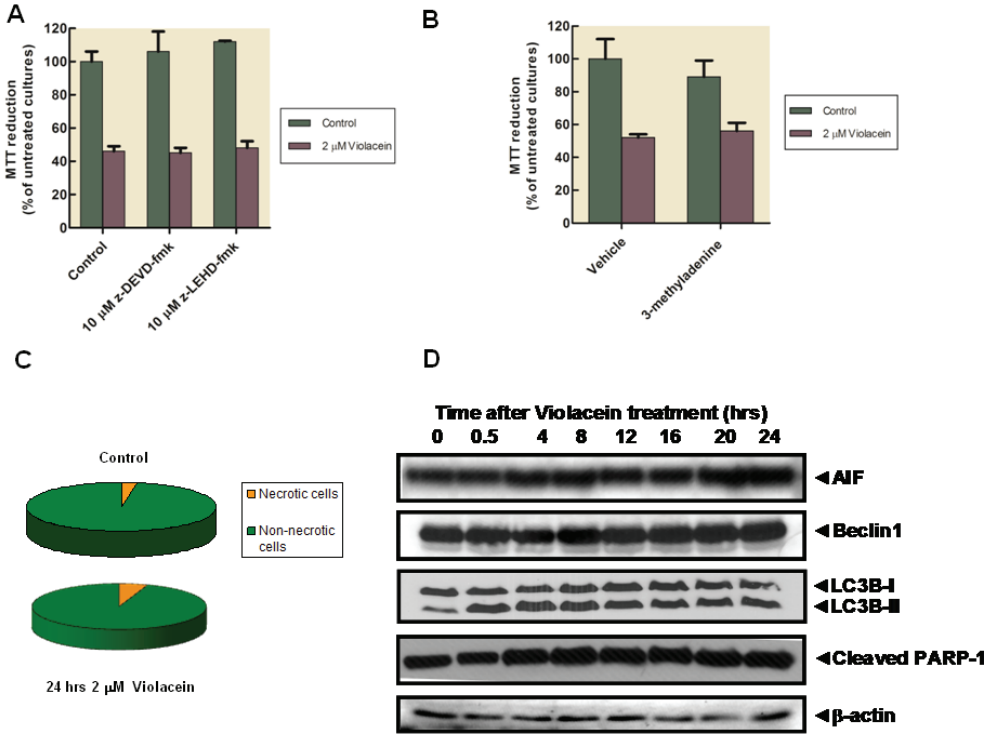


Figure 2. Induction of cell death by Violacein does not progress through canonical modes of programmed cell death (A) Cell death induced in TF1 cells by a 24 hrs treatment with 2 μM of Violacein is not sensitive to inhibitors of either pro-apoptotic or pro-inflammatory caspases. (B) The autophagosome inhibitor 3-methyladenine does not impair Violacein-induced cellular suicide in TF1 cells. (C) Apparent absence of a necrotic effect of Violacein in TF1 cells, as assayed by the ethidium bromide/acridine orange assay. (D) Western blot analysis of the release of apoptosis inducing factor (AIF), beclin-1 and autophagy-specific LC3B isoform levels excludes a major role of apoptosis and autophagy in Violacein effects in TF1 cells. Nevertheless, Violacein clearly increases levels of cleaved PARP and thus cell death induction by Violacein represents a mode of PCD

Violacein does not induce cellular suicide through the canonical PCD modes

Canonical PCD can either progress through apoptotic, necroptotic, pyroptotic or autophagic cell death(4). None of these canonical modes, however, seem involved in violacein effects on cellular survival in TF1 cells. Apoptosis and pyroptosis are excluded because of the lack of effect of inhibitors of pro-apoptotic caspases or pro-inflammatory caspases on violacein-dependent cell death (figure 2a). Also, the known class III PI3K inhibitor, 3-methyladenine (5mM 3-MA), which inhibits the formation of autophagosomes [32], has no effect on violacein-induced cell death (figure 2b). Furthermore, the apparent absence of a necrotic effect of violacein, as assayed by the ethidium bromide/acridine orange assay (figure 2c), is at bay with a major role for violacein on TF1 cell survival. Thus death of TF1 cells following application of violacein seems not to be mediated by one of the canonical PCD modalities. In apparent agreement, violacein treatment is accompanied by neither mitochondrial

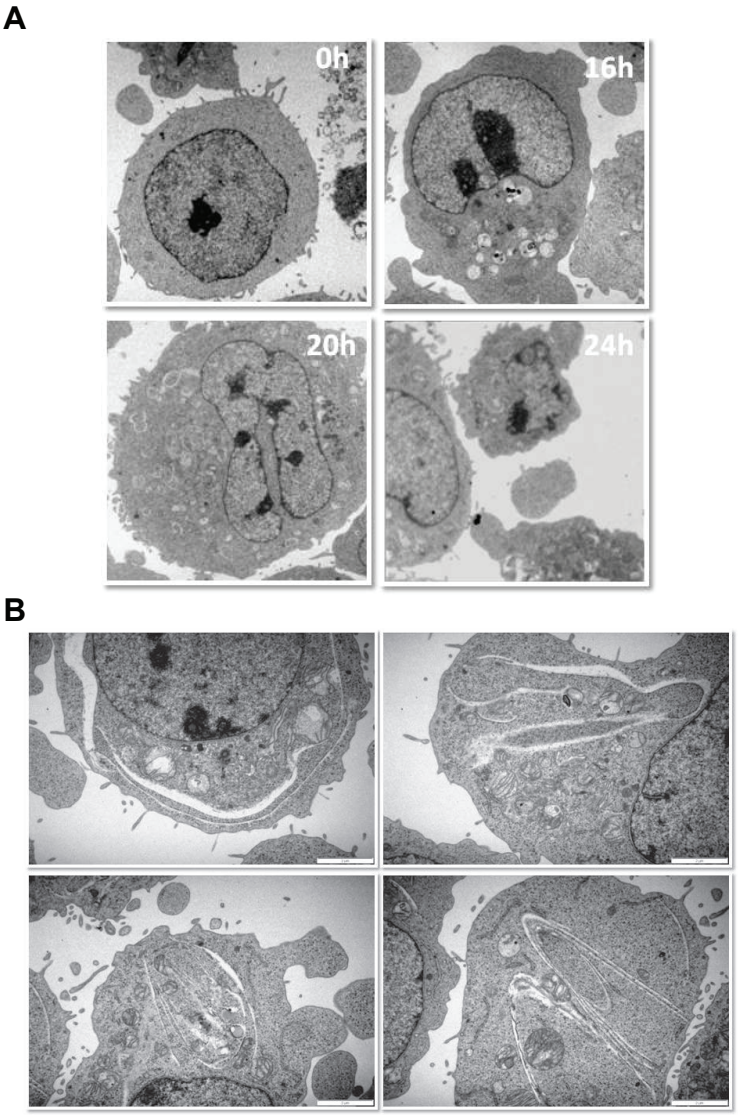


Figure 3. Induction of cell death by Violacein is associated with an ultrastructurally unique programme of cellular demise (A) Using transmission electron microscopy, the ultrastructural effects of Violacein-induced cell death were characterised at different time points following application of the indole derivative. No apparent morphological evidence for induction of either autophagia, apoptosis, necroptosis or pyroptosis is obtained, as for instance the integrity of the mitochondria and plasma membrane is maintained until the final stages of cellular suicide and no increase in the number of autophagosomes is seen. Highly distinctive of Violacein-induced cell death, however, is the endoplasmatic reticulum and Golgi linearization and at later time points nuclear mottening. (B) High magnification examples of endoplasmatic reticulum and Golgi linearization, characteristic for Violacein effects 20 hrs following application of the chemopreventive indole.

release of apoptosis inducing factor (AIF), increased expression of Beclin-1 nor formation of the autophagosome associated form of LC3-B (figure 2d), further excluding an important role of autophagy or apoptosis in violacein-mediated cell death. Nevertheless, violacein treatment clearly and substantially increased the levels of the enzymatically active cleaved PARP (figure 2d) and thus violacein-induced cell death does represent a specific intrinsic cell death programme.

Ultrastructural characterisation of Violacein effects in TF1 cells defines an alternative mode of cell death

To obtain further insight into the mechanisms underlying TF1 cell death following Violacein treatment, the ultrastructure of these cells was studied using transmission electron microscopy (figure 3a). No apparent morphological evidence for induction of either autophagy, apoptosis, necroptosis or pyroptosis was obtained, as for instance the integrity of the mitochondria and plasma membrane was maintained until the final stages of cellular suicide and no increase in the number of autophagosomes was seen. Highly distinctive of Violacein-induced cell death, however, is the endoplasmatic reticulum and Golgi linearization (see high power magnification pictures in figure 3b), and at later time points nuclear mottening, initiating the last phase before cellular demise becomes prominent. The highly unusual ultrastructural aspect of Violacein-treated TF1 cells strongly suggests that the induction of cell death by this compound progresses through a novel, hitherto undescribed, mode of PCD.

Biochemical characterisation of Violacein-induced PCD employing kinome profiling shows induction of anti-apoptotic and anti-autophagic signalling

The absence of a priori assumptions of the biochemical mechanisms that mediate the morphologically unique cell death programme induced by Violacein prompts for techniques that allow the generation of unbiased comprehensive descriptions of cellular signalling. One such technique is kinome profiling using peptide arrays and in the past we used this methodology successfully to unravel the signalling mechanisms mediating, amongst others chemoprevention by coxibs in colorectal cancer [33] or the non-genomic mechanisms employed by the glucocorticoid receptor [34] to limit white blood compartment expansion. For the present study, we generated kinome profiles using peptide arrays by incubating TF1 cell lysates obtained from cultures either left untreated or subjected to 2μM violacein treatment for respectively 30 min, 16 h and 24h. The arrays incorporated substantial amounts of radioactivity and the technical quality of the profiles was good as the average Pearson product moment obtained for the technical replicas ranged from 0.78 to 0.90 (see Suppl. Table 1). As expected from a compound inducing cell death, application of Violacein to cells caused major rearrangement of the cellular kinome, which progresses over time (figure 4a). Densitometric values for all substrates and the statistical significance of the results obtained compared to untreated cells for all time points are given in Suppl. Tables 2 through 4. In figure 4b graphs presenting a correlation between fold change and p-value for each time point compared to control are presented. Analysis of these profiles, however, produces little evidence for an involvement of canonical PCD pathways in violacein-induced

effects in TF1 cells. As it is also shown in figure 4c, the PKB/mTOR pathway, whose activation is associated with inhibition of both apoptotic and autophagic PCD modalities [35], is not inhibited by violacein treatment and if anything it is stimulated after treating TF1 cells with violacein. This notion is confirmed by experiments in which the activation status of signalling elements involved in this pathway was directly probed using Western blotting (figure 4c). Treatment with violacein produced a sustained upregulation of PDK1 and the key survival kinase PKB, which corresponds well with the increased enzymatic activity of PKB as seen in the kinome profiling (figure 4b). GSK3β is through phosphorylation negatively regulated by PKB and indeed concomitant with the increased PKB enzymatic activity, reduced kinase activity of GSK3β is detected, whereas also increased phosphorylation of GSK3β is detected, although the kinetics of these phosphorylations do not completely correspond to those of its upstream regulators of GSK3β kinase activity. mTOR is through its phosphorylation a positively regulated target of the survival signalling cassette as well and mediates, amongst others, inhibition of autophagy. We observed upregulation of its phosphorylation following Violacein treatment (figure 4c). Therefore stimulation of mTOR activity through PKB is stronger than the inhibitory influence exerted on this signalling element through AMPK, whose activation is also induced, at least at 16 hrs following Violacein application to TF1 cells (figure 4b). Hence, the absence of apparent apoptosis and autophagy following Violacein treatment of TF1 cells corresponds well to the biochemical profiles obtained under these conditions.

Signalling pathways associated with Violacein-dependent PCD

Subsequently we analysed our profiles to find clues as to signalling events possibly implicated in mediating the distinctive morphological and biochemical mode of cell death by Violacein. Striking in the context of a cell death response was the activation of kinase activity directed towards peptides implicated in the activation of the canonical p42/p44MAP kinase cascade (figure 4b), and Western blot analysis confirmed that such activity coincided with increased phosphorylation of p42/p44MAP kinase on Thr180/Tyr182 (figure 4c). Thus activation of canonical p42/p44MAP signalling is a prominent feature of Violacein effects. Also, the strong activation of death associated protein kinase, which has been associated with PCD associated with interferon γ withdrawal but is also known to inhibit apoptosis and whose role in PCD thus remains obscure [37], is an obvious candidate mediator in violacein action. In addition, the calpain L1 large subunit are significantly less phosphorylated in violacein-treated cells, an event associated with deactivation of this cysteine protease [38]. Importantly, defects in calpain functionality are associated with a variety of deficiencies, including lethality, dystrophy, and tumorigenesis, suggesting that a calpain-like system could be important to induce programmed cell death in these cells [39]. Hence, we investigated the capacity of calpeptin (an inhibitor of calpain enzymatic activity [40]) to mimic violacein effects on cell death and indeed we observed that such inhibition is almost equipotent to violacein in inducing cell death in TF1 cells (figure 4d), indicating that the calpain system may play a role in PCD by violacein. Thus, violacein-induced cell death is associated with a unique set of biochemical events and signalling induced by this indole derivative seems to represent a novel mode to provoke cellular suicide.

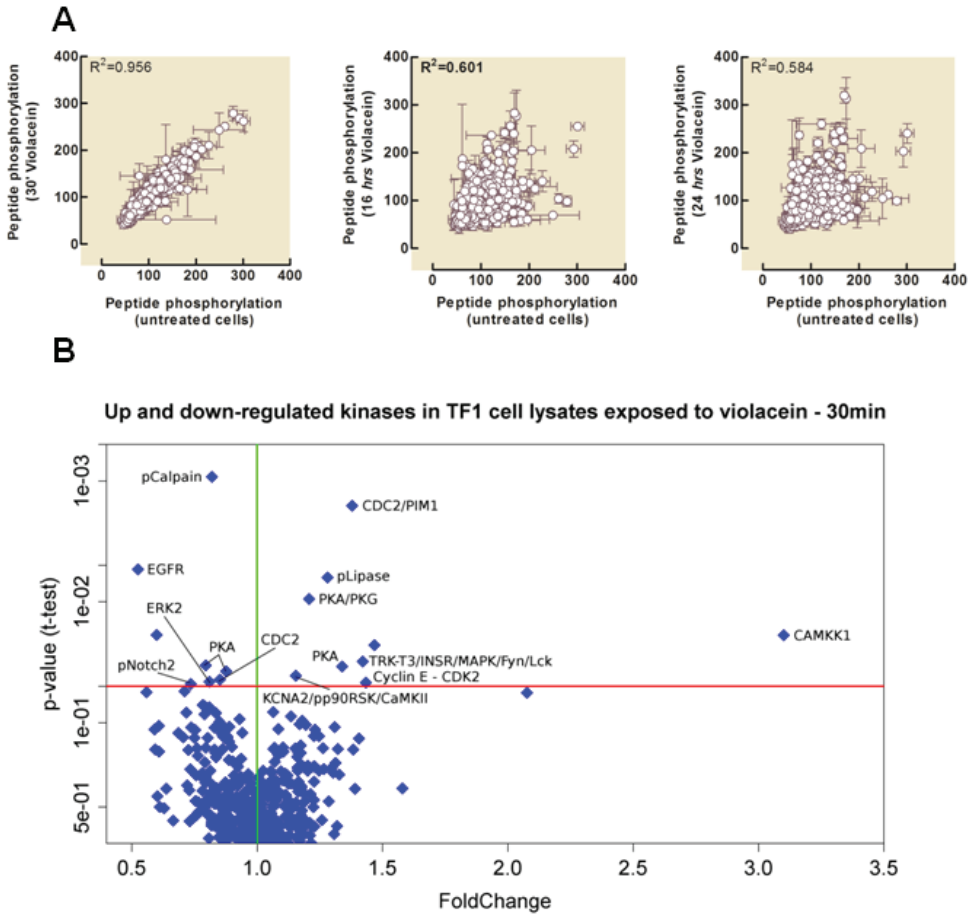
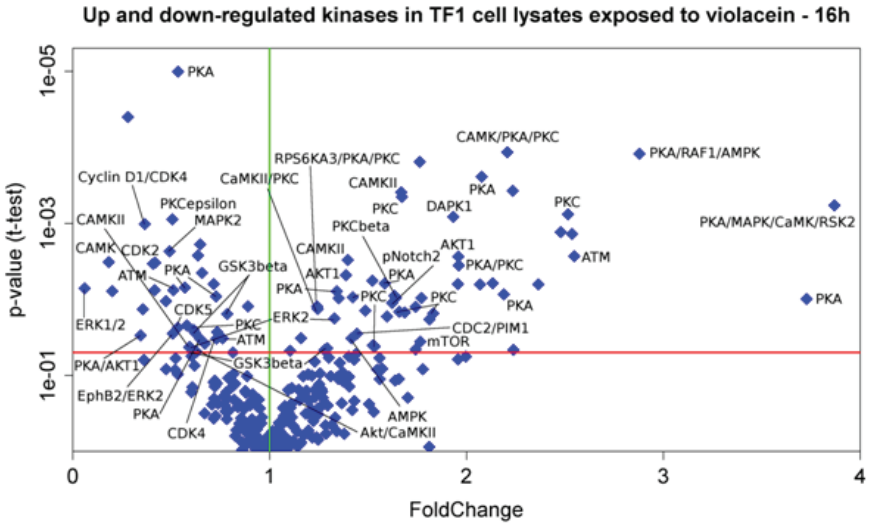


Figure 4. Induction of cell death by violacein is associated with a major rearrangement of cellular biochemistry (A) Plotting correlation between phosphorylation of specific peptide substrates shows that 30 minutes following Violacein treatment only minor changes in the cellular kinome are observed. More long-term treatment, however, causes major remodelling of the kinome. The value in the graph gives the Pearson product. (B) Comparison between statistically significant phosphorylated spots in TF1 cells treated with violacein 2μM in 0hrs time point compared to TF1 cells treated for 16hrs and 24hrs are shown. This graph shows the correlation between fold change and p-values for the statistically significant phosphorylated spots in the different time points of treatment with violacein. (C) The PKB-mediated survival cassette is not inhibited by 2μM violacein treatment in TF1 cells. Although Western Blot analysis of signalling intermediate phosphorylation does not perfectly correlate with the kinase enzymatic activity results in table 1, it is obvious that also phosphorylation status analysis does not provide evidence for diminished survival signalling following TF1 cell stimulation. Note that GSK3β activity is negatively regulated by its PKB-mediated phosphorylation. Inflammatory signalling, however, as analysed by p38MAP kinase or MAPKAP2 phosphorylation is negatively regulated by Violacein. (D) Next, Annexin/pi positive TF1 cells were also evaluated in presence of Calpeptin or Violacein treatment.

Figure 4 continued



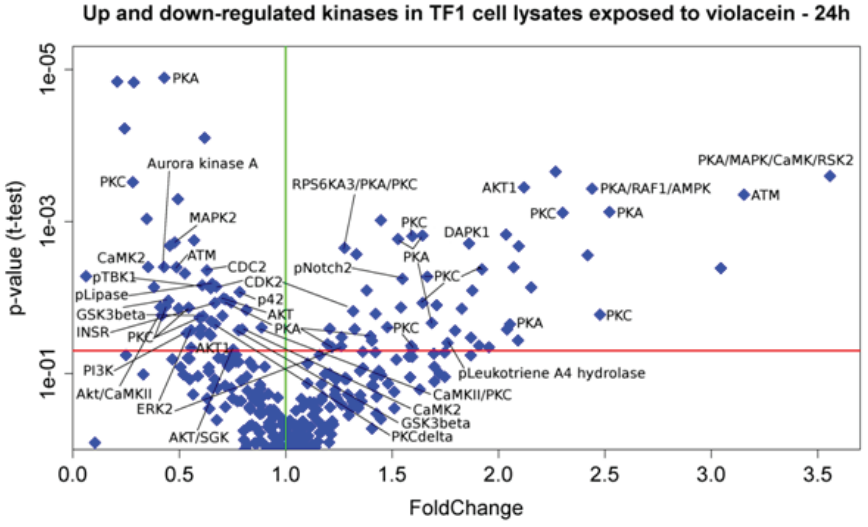
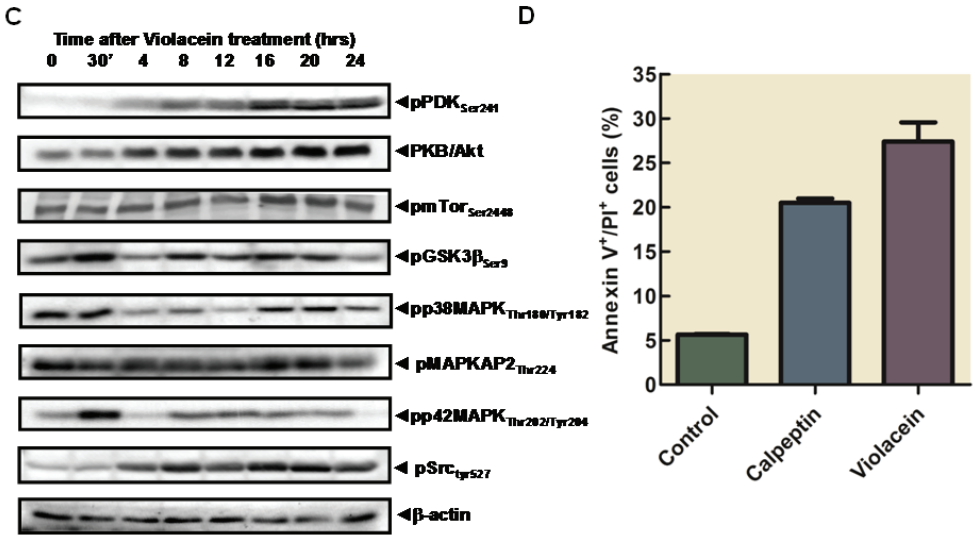


Figure 4 continued



Discussion

Violacein and related indolic compounds attract attention because of their presumed chemopreventive action, originally discovered from epidemiological studies in the Amazon basin [15]. Although clearly highly biologically active [18, 19], the mechanism by which violacein might interfere with the oncologic process has remained unclear. Nevertheless, it has become evident that despite the absence of induction of cell death in untransformed cells or low in vivo toxicity of the compound in humans and experimental animals [18, 19, 41], it is strongly cytotoxic towards a number of transformed cell types. In the present study we show its potency in the otherwise highly chemoresistant CD34+/c-Kit+/P-glycoprotein+/MRP1+ TF1 leukaemia progenitor cell line, suggesting that Violacein activates death pathways that are not associated with other chemotherapeutic agents, but which can be activated in cancer cells but less so in untransformed cell types. Such induction of cancer cell-specific cell death may well explain the chemopreventive action of compounds like violacein on human disease.

The induction of PARP cleavage and the resulting breakdown of the cellular genome as well as the absence of trypan blue incorporation during violacein-induced cell death indicates that violacein acts through a specific cellular suicide program. Studies of cellular biochemistry, ultrastructural morphology and inhibitors of apoptosis, autophagy and pyroptosis indicate that violacein acts through a molecular mechanism distinct from these known manifestations of cellular death. In apparent agreement, detailed ultrastructural characterisation of violacein effects on cellular morphology revealed a unique cascade of events involving first endoplasmatic reticulum and Golgi linearization and at later time points nuclear mottening. Biochemically, these morphological events were accompanied by highly distinctive effects on cellular signal transduction, involving paradoxal activation of PKB/mTOR and canonical MAP kinase signalling cassettes, inhibition of inflammatory signalling, a concomitant downregulation of the Calpain cysteine protease system followed by strong activation of the death-associated protein kinase DAPK1. These unique morphological and biochemical characteristics of Violacein-induced cell death lead us to propose that violacein effects on TF1 cells reveal a hitherto unrecognised novel form of PCD, which we tentatively propose to denominate as violaceinosis.

All currently described forms of cell death seem to a certain extent to be inhibitory to other forms of PCD [4] and violaceinosis does not appear to be an exception. Violacein effects are accompanied by strong activation of survival signalling through the PKB/mTOR cellular survival cassette, which counteracts both apoptosis as well autophagic cell death. It is tempting to speculate that DAPK1 activation is involved in this process as inhibition of pro-apoptotic caspase activation through PKB signalling is well-established [42]. Conversely, the potent anti-inflammatory signal transduction elicited by violacein corresponds well with a possible inhibitory effect on pyroptosis, which heavily depends on activation of the pro-inflammatory caspases. The exact biochemical details by which violacein-induced cell death progresses evidently require further analysis, but it is interesting to see that inhibition of calpain mimics important aspects of violaceinosis, suggesting that inhibition of calpain enzymatic activity may be an important factor in this process. In apparent agreement, genetic ablation of calpain enzymatic activity strongly increases the propensity to tumorigensis in

experimental animals. Hence, it is conceivable that violacein and related indole derivatives stimulate an endogenous tumour-suppressive process.

Disregarding the exact molecular details of violacein-stimulated signal transduction processes in normal physiology, we feel that the compound reveals an as yet unrecognised mode of cellular death. It is thus tempting to speculate that violaceinosis represents a last line of defence of the body against malignant transformation and that stimulation of this mechanism can explain the remarkable chemopreventive properties of violacein and related indolic compounds. Increased knowledge of the biochemical and physiological characteristics of this process may well prove exceedingly useful in the fight against cancerous disease.

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Chapter 9

A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours

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Abstract

Riboflavin is a potent photosensitizer as well as part of the vitamin B complex. Recently we demonstrated that the products generated by irradiation of riboflavin have potential as anti-leukaemic therapy. The possible action, however, of the riboflavin photoproducts in solid cancers has not been addressed. Hence, we investigated the effects of irradiated riboflavin on androgen-independent human prostate cancer cells (PC3), a known model for solid tumour cells with an exceptional resistance to therapy. Our results show that riboflavin photoproducts are cytotoxic to these cells in a FasL-Fas-dependent manner. Furthermore, irradiated riboflavin inhibited matrix-degrading proteases, caused downregulation of VEGF and upregulation of TIMP1 suggesting anti-metastatic potential. Together, these results show that the anti-neoplastic action of riboflavin photoproducts is not limited to haematological malignancies, warranting clinical studies in solid tumours.

Introduction

In recent years, vitamins and their derivatives are gaining prominence in anti-cancer strategies, especially in haematological cancers. For example all-trans retinoic acid (vitamin A) is now widely used for the treatment of myeloid leukaemia [1,2] and vitamin D is in clinical trial for this disease as well [3]. Very recently, we provided evidence that photoderivatives of riboflavin, a constituent of the vitamin B complex (vitamin B2), has strong activity in haematological malignancy as well [4]. The potential of such vitamin-derived products in solid tumours has, however, received much less attention, with the possible exception of vitamin D, which is highly promising in pancreatic cancer [5].

We showed earlier that in leukaemia, the photoproducts of riboflavin act through the activation of FasL-Fas-dependent signaling, which together with diminished protein kinase B-dependent survival signaling seems responsible for its anti-leukaemic action [4]. As FasL-Fas signaling should, in principle, also be anti-neoplastic in solid tumours we felt that these results prompted investigation into the possible usefulness of riboflavin photoproducts (Fig. 1a) in solid tumours. Hence, we decided to study the action of irradiated riboflavin in the PC3 prostate cancer cell culture system, which is generally considered to be a good experimental model for highly therapy resistant solid tumour cells [6]. The results show that irradiated riboflavin inhibits cell growth in this androgen-independent prostate cancer, apparently dependent on the induction of caspase-mediated cell death through activation of the FasL-Fas system. Furthermore, concomitantly activity of collagen matrix-degrading proteases was reduced, suggesting anti-metastatic potential. Together these results provide important support for commencing clinical testing of riboflavin photoproducts in terminal solid tumour cancer.

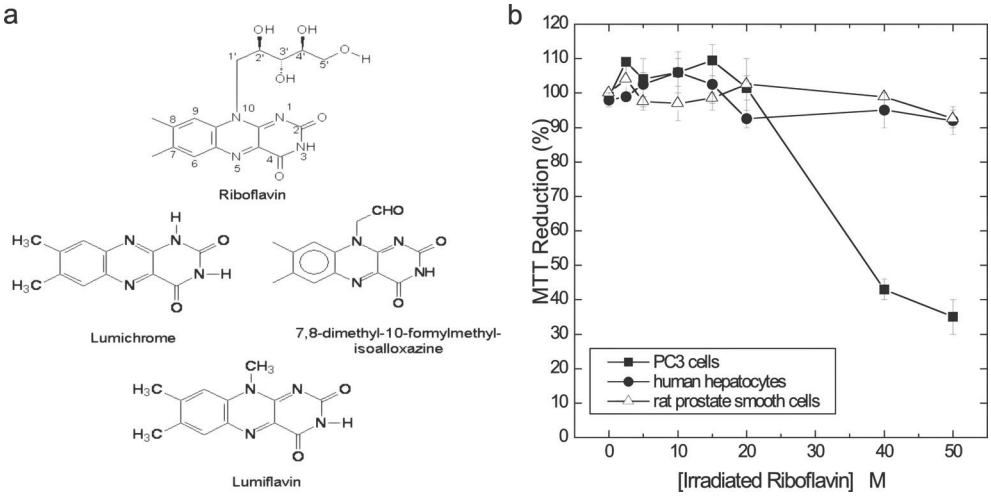

Figure 1: Irradiated riboflavin presents cell specific toxic effect.(a) Chemical structure of riboflavin and its photoproducts. (b) Cells were treated with different concentrations of irradiated riboflavin. The effect of photoproducts was evaluated by MTT reduction after treating the cells for 24 h. In the absence of irradiated riboflavin, cell viability was considered as 100%. The experiment was performed in a 24 wells plate. Results represent the means ± standard error of three experiments run in triplicate ($p < 0.05$).

Material and methods

Reagents

Polyclonal antibodies against anti-phospho-p38 mitogen-activated protein kinase (MAPK p38), anti-phospho-c-jun-NH2-terminal protein kinase 1/2 (JNK 1/2), PKB/Akt, phosphatidylinositol-3 kinase (PI3K), AIF, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-PP2A, TNF receptor 1 (TNRF1), Fas-associated death domain protein (FADD), Bcl2, Bax, c-IAP1, NFκBp65, IKKα, TIMP-1, VEGF and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fas neutralizing antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Riboflavin and Fumonisin B1 were from Sigma Chemical Co. (St. Louis, MO). Caspase 3 Colorimetric Assay Kit was obtained from R&D Systems (Minneapolis).

Cell lines and culture

PC3 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). PC3 and normal rat prostate smooth cells were routinely grown in RPMI 1640 medium (Sigma Chemical Co.) containing 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2 in air. Dexamethasone (1 µM) and humam insulin (1pM) were added to normal prostate smooth cell medium.

Human hepatocyte line (IHH) was a kind gift from Professor Didier Trono and Dr Tuan Nguyen. IHH cells were maintained at 37°C and 5% CO_2 in phenol red-free DMEM/F-12 medium (GIBCO-BRL) containing 1 µM dexamethasone, 1pM human insulin (Humalog, Lilly) and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin) with 10% fetal bovine serum.

Riboflavin irradiation

Solution of 250µM riboflavin in RPMI 1640 medium at the pH 7.4 (15 ml) was placed in a Petri dish and irradiated with UV light (253.5 nm) for 30 min; the lamp was placed 40cm from the riboflavin solution. The RPMI 1640 medium was irradiated in the same conditions and used as a control. After irradiation the percentage of riboflavin and its photoproducts was the following, as determined by mass spectrometry: 79% of riboflavin, 6.2% of lumichrome, and 14.8% composed of formylmethylflavin, lumiflavine, 2-ketoriboflavin and 4-ketoriboflavin.

Treatment of cells with irradiated riboflavin

Cells were incubated in 24-well plates until reach the semiconfluence and then treated with different concentrations of irradiated riboflavin (5 – 50 µM final concentrations) for 24 h. In order to investigate the mechanisms of riboflavin cytotoxicity, PC3 cells were also pre-treated with 5 µM of Fumonisin B1 or anti-Fas (10 µg/mL) for 30 min. Cell viability was assessed by the MTT reduction assay.

MTT reduction assay

The medium containing irradiated riboflavin was removed and 1 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (0.5 mg/mL of culture medium) was added to each well. After incubation for 4 h at 37 °C, the medium was removed and the formazan crystals solubilized in 1 mL of ethanol. The plate was shaken for 5 minutes on a plate shaker and the absorbance was measured at 570 nm [7].

Western blotting

Cells (3 x 10^7) were lysed in 200 µL cell lysis buffer (50 mM Tris [tris(hydroxymethyl) aminomethane]–HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM O-Vanadate, 1 mM NaF, and protease inhibitors [1 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluoride-hydrochloride]) for 2 h on ice. Protein extracts were cleared by centrifugation, and the protein concentration was determined using the Lowry method [8]. An equal volume of 2x sodium dodecyl sulfide (SDS) gel loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to samples and boiled for 10 minutes. Cell extracts, corresponding to 3 x 10^5 cells, were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2%) in Tris-buffered saline (TBS)–Tween 20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-goat or anti-mouse horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions (in all Western blotting assays) in blocking buffer for 1 h. Detection was performed by using enhanced chemiluminescence (ECL).

Caspase 3 activity assay

Caspase 3 activity was determined by the measurement of p-nitroaniline (pNA) released from the cleavage of caspase 3 substrate (Ac-DEVD-pNA) at 405 nm.

Zymographic analysis

The proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography as described by de Souza et al. [9]. After the treatment the viable cell number was determined, the culture medium was collected and stored at -20°C in the presence of 1 mM PMSF (phenyl-methyl sulphonyl fluoride-serine-protease enzyme inhibitor). Samples were diluted in non-reducing buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 1% SDS and 0.001% bromophenol blue). Volume of the samples loaded was proportional of the viable cells and resolved by SDS-polyacrylamide gel (10%) and 4% gelatin. Protein renaturation was performed in 2% Triton X-100 for 1 h followed by incubation with 50 mM Tris-HCl and 10 mM $CaCl_2$ (pH 7.4) at 37°C for 18 h. Gels were stained with 0.5% Comassie Blue G 250 for 30 minutes and then washed in a 30% methanol and 10% glacial acetic acid solution.

Statistical evaluation

Western blots represent 3 independent experiments. Quantitative analysis of the proteins was performed by volume densitometry after scanning of the film (data are presented as

the protein to β-actin ratio). Cell viability data were expressed as the means ± standard errors of 3 independent experiments carried out in triplicates. Data from each assay were analyzed statistically by ANOVA. Multiple comparisons among group mean differences were checked with the Tukey test. Differences were considered significant when the p value was less than 0.05.

Results

Irradiated riboflavin displays differential effects on cell viability

PC3 cells represent a fast growing cell type remarkably resistant to chemotherapeutic intervention. Also in our hands, cultures of PC3 cells expanded exponentially when grown in fetal calf serum containing medium. Importantly, however, irradiated riboflavin dose-dependently stopped PC3 growth and was even cytotoxic with an apparent IC50 of 20 – 30 μM (Fig. 1b). The toxic effect was strictly dependent on the presence of riboflavin photoproducts, since non-irradiated riboflavin had no influence on PC3 cell growth. Thus, the anti-neoplastic effect of riboflavin seems not limited to haematological malignancies, but also extends to therapy-resistant solid tumour cells, at least as judged from the PC3 model system. Importantly, normal cells such as human hepatocytes and rat prostate smooth cells were not sensitive to irradiated riboflavin (Fig. 1b).

Fas neutralising prevents the toxic effect of riboflavin photoproducts

In leukaemia, the anti-neoplastic effect of riboflavin seems dependent on the induction of FasL-Fas signalling. In order to test whether a similar situation also holds true for the PC3 model system, cultures were tested for riboflavin photoproducts cytotoxicity in the presence of a Fas-neutralising antibody or solvent control. It appeared that whereas control cultures responded to irradiated riboflavin with a strong inhibition of cell growth, cultures treated with a Fas-neutralising antibody were not affected by riboflavin photoproducts (Fig. 2a). Furthermore, concomitant with irradiated riboflavin-induced cytotoxicity, FADD levels were increased, in apparent agreement with the upregulation of the FasL-Fas signalling system as a consequence of the treatment with riboflavin. The latter responses to riboflavin photoproducts were specific to the FasL-Fas signalling system. TNFR1 as well as NFκB were downregulated which is in agreement with the upregulation of IKK alpha (Fig. 2b). The expression of other pro-apoptotic proteins was checked; the expression of AIF as well as Bax displayed a slight decrease after irradiated riboflavin treatment. Additionally, c-IAP1 level dropped when 40 and 50 μM irradiated riboflavin were used. Finally we controlled for the expression of other proteins involved in the Fas-induced cytotoxic response; their presence was readily detected. Furthermore, activation of caspases, a hallmark of Fas-induced cytotoxicity, was readily detected after stimulation of PC3 cells with irradiated riboflavin (Fig. 2c). We conclude that the cytotoxic response to riboflavin photoproducts in the PC3 model system is reminiscent to that in leukaemia cell, this action of riboflavin being specifically mediated via the induction of FasL-Fas signalling.

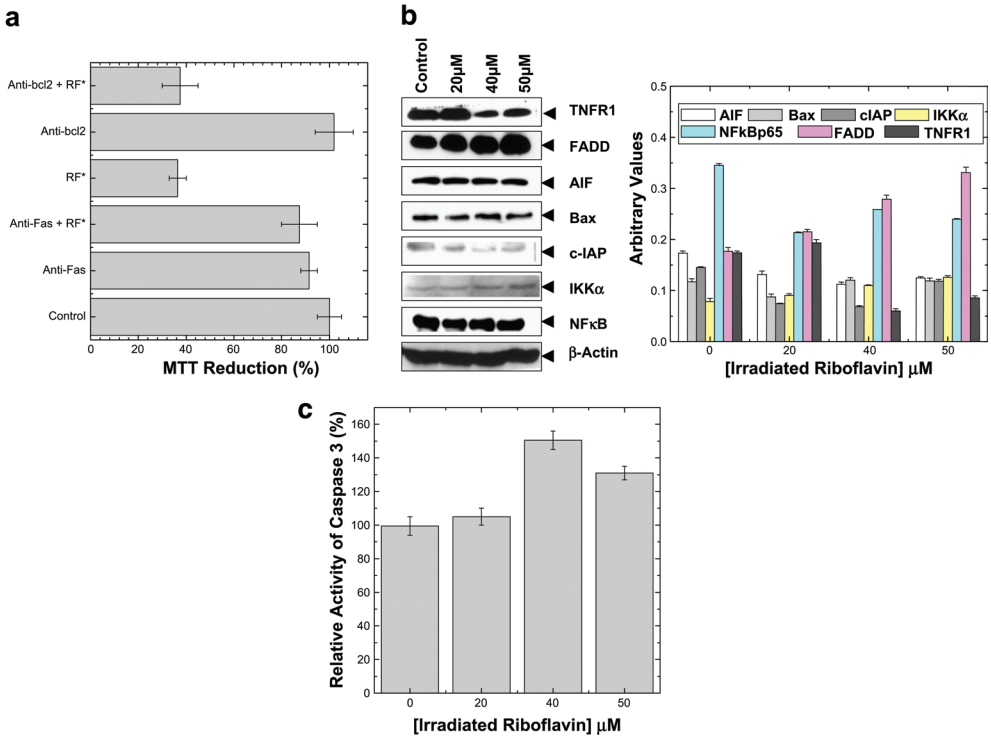

Figure 2. Apoptosis induction in prostate cancer cells by irradiated riboflavin. (a) Cells were incubated with Fas neutralising antibody (antimouse) or anti-Bcl2 (10 lg/mL) for 30 min and afterwards treated with 50 lM irradiated riboflavin (RF*) for 24 h. Viability was determined by the MTT reduction assay. (b) The expression of TNFR1 and FADD was evaluated by immunoblotting using extracts obtained from cells treated with irradiated riboflavin. (c) Colorimetric assay was performed after 24 h of treatment to determine caspases 3 activity. Results represent the means ± standard error of three experiments run in triplicate ($p < 0.05$). Anti-Bcl2 (anti-mouse) was used as control.

Riboflavin photoproducts action in the PC3 model system is highly similar to that action in leukaemia

In leukaemic cells, treatment with irradiated riboflavin is associated with a downregulation of survival signals through the PI3K/AKT signalling pathway. When the activity of this signalling system was tested in the PC3 model, we observed strong inhibition of AKT/PKB and of p110 PI3K protein expression levels, showing that as in leukaemic cells, irradiated riboflavin targets this signalling pathway. MAPKp38 was also inhibited by irradiated riboflavin treatment, while in regard to JNK1/2 was observed an activation at 40 μM irradiated riboflavin. Additionally, the protein serine/threonine phosphatase (PP2A) was inhibited by irradiated riboflavin, an effect seen in leukaemia as well (Fig. 3a). Further evidence that the effects induced by irradiated riboflavin in PC3 cells are similar to those seen in leukaemic cells was obtained in experiments using Fumonisin B1, a known inhibitor of the enzyme ceramide synthase involved in de novo synthesis of ceramide in the endoplasmic reticulum and mitochondria. This compound was previously shown to prevent the cytotoxic response

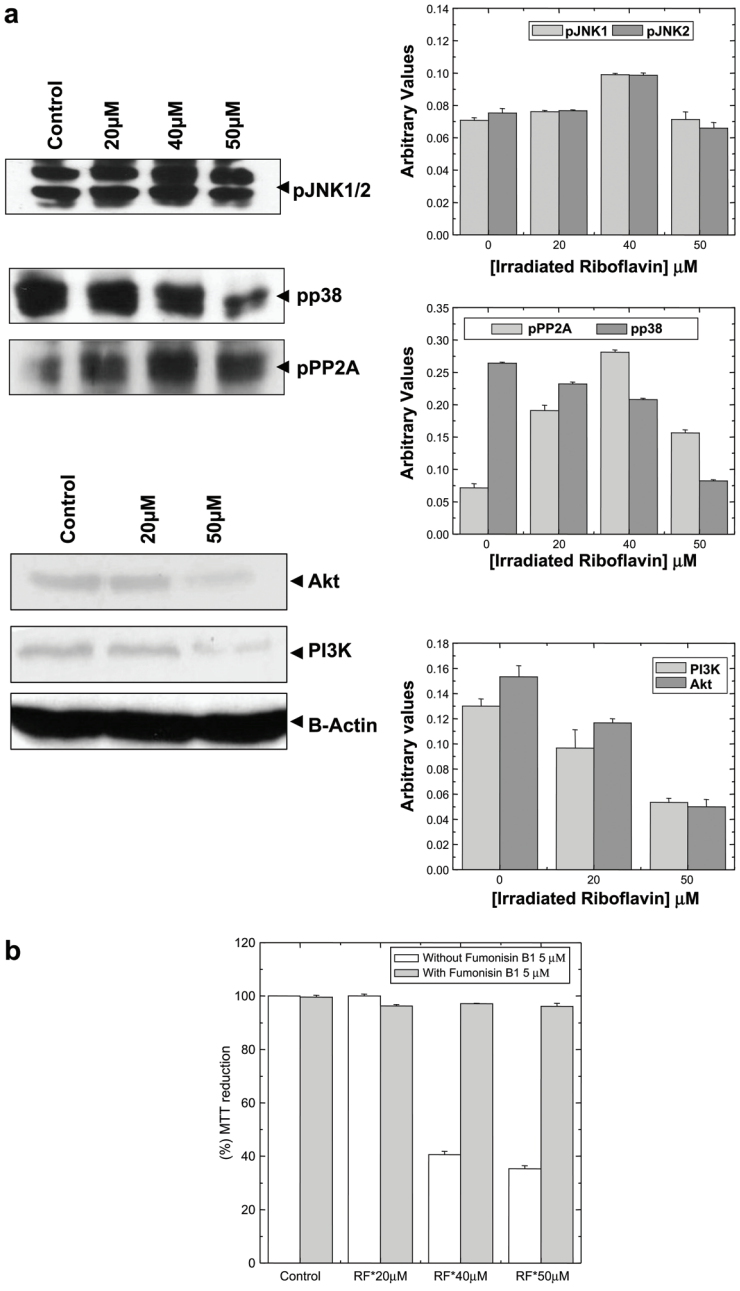

Figure 3. Irradiated riboflavin causes differential effect on the expression of proteins involved in cancer cell survival and death. (a) Cells were treated with irradiated riboflavin and immunoblotting analysis was performed to evaluate the expression levels of key proteins involved in cell survival and death. (b) Cells were incubated with 5 lM of Fumonisin B1 for 30 min and afterwards treated with irradiated riboflavin for 24 h. Viability was determined by the MTT reduction assay. RF*: irradiated riboflavin.

to riboflavin photoproducts in HL60 cells, a model system for leukaemia. When tested in the PC3 system, Fumonisin B1 potently prevented the toxic effect of irradiated riboflavin (Fig. 3b). Thus multiple aspects of irradiated riboflavin signalling in leukaemia are reflected in the PC3 model system as well.

Metalloproteinases activities, VEGF and TIMP1 expression are affected by irradiated riboflavin

An important difference between haematological malignancies and solid tumour cancers is that the latter depend on their capacity to degrade matrix proteins for successful dissemination through the body (metastasis), whereas in haematological malignancy this much less of an issue. Hence we were interested to analyse the capacity of riboflavin photoproducts to influence the activity of enzymes capable of breaking down matrix components. PC3 are reported to be highly metastatic and accordingly zymographic analysis revealed substantial activity of the metalloproteinases 2 and 9 with regard to a collagen substrate. Interestingly, treatment of PC3 cultures with riboflavin photoproducts substantially decreased the activity of these enzymes with concomitant upregulation of TIMP-1 (Fig. 4). In addition, we observed donwregulation of VEGF. Apparently, the induction of cytotoxicity is accompanied by a reduction of matrix degrading potential as well as inhibition of angiogenesis, suggesting that treatment with irradiated riboflavin might reduce metastatic potential in solid tumours.

Discussion

The clinical management of prostate cancer remains highly challenging and novel adjuvant strategies are urgently called for. In the present study, we provide compelling evidence that the anti-neoplastic effects of riboflavin photoproducts as observed in leukaemia cells are mirrored in the PC3 model system, not only with respect to a strong cytotoxic effect of this compound but also with respect to the underlying molecular mechanism, the induction of FasL-Fas signaling. The PC3 model system is well known to be unusually resistant to pharmacological intervention, thus these results agree well for possible expansion of clinical trials with irradiated riboflavin to solid tumour types [6]. The results obtained support the candidacy of these photoproducts as adjuvant agents in prostate cancer therapy, as toxic effects of the photoproducts on the cancer cells were observed at concentrations lower than 40 μM. This concentration is assumed to be non-toxic for nontransformed cells. Despite the property of riboflavin as a photosentitizer is documented in the literature, the effect of its photoproducts has not been addressed in detail, neither in vivo nor in vitro. Wollensak and co-authors have reported that riboflavin/UVA treatment leads to a dose-dependent keratinocyte damage [10]. In addition, the applicability of irradiated riboflavin has also already been suggested as an inactivator of pathogens of blood components [11,12].

The specificity of apoptosis being involved rather than necrosis or other type of cell death was highlighted by the activation of caspase 3, which effect was maximum when 40 μM irradiated riboflavin was used. This result was in agreement with the low level of c-IAP1 observed at 40 and 50 μM of riboflavin. However, there is a possibility of the occurrence of other type of cell death at the highest concentration of irradiated riboflavin used. Interestingly, AIF did not seem to be involved in the death mechanism triggered by irradiated

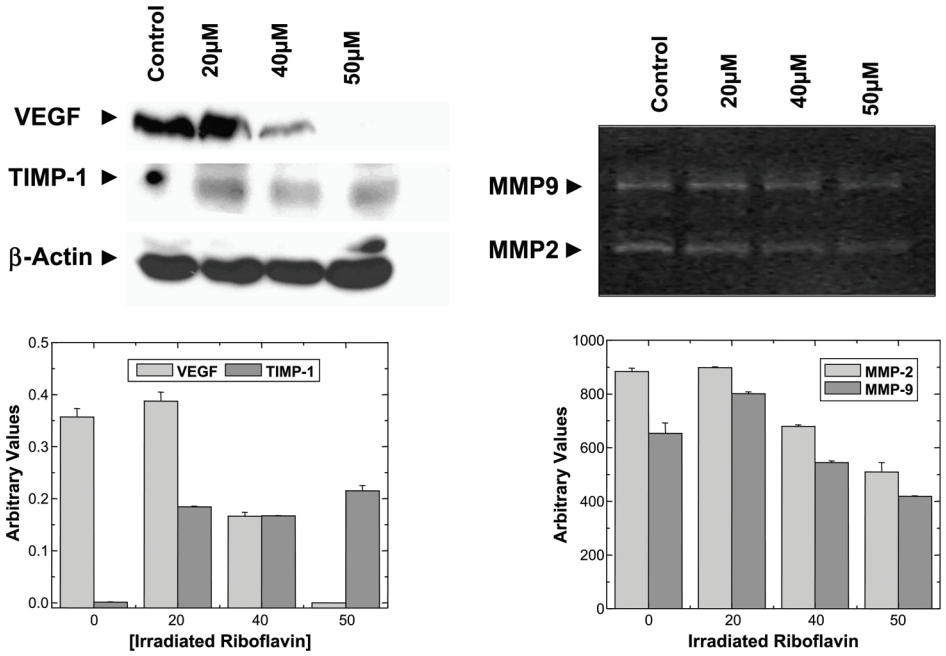

Figure 4. Effect of irradiated riboflavin in metalloproteinases 2 and 9 activities and expression of VEGF and TIMP1. The proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography in the culture medium of prostate cancer cells treated with different concentrations of irradiated riboflavin for 24 h. The loading medium volume on the gel was normalized by the viable cell number after treatment (control - 250,000; 20 μM RF* -181,500; 40 μM RF* -118,750; 50 μM RF* - 82,500 cell/mL, loading volume: 4.95, 6.8, 10.4 and 15 μL, respectively). VEGF and TIMP1 expressions were determined by Western blotting.

riboflavin, since the level of this protein remained unchanged.

This remarkable action of these photoproducts may well be explained by our observations that a multitude of relatively unlinked death pathways is targeted, which together may all be necessary to drive apoptosis in this system (inhibition of e.g. Fas or ceramide signalling is already sufficient to blunt the apoptotic process). Pre-treatment with anti-Fas neutralizing antibody resulted in complete inhibition of cell death, indicating that the extrinsic pathway is a major signaling response involved in the apoptotic process induced by riboflavin. This result is consistent with the upregulation of FADD observed in PC3 cells treated with riboflavin photoproducts. Despite the coexpression of both cell-surface proteins Fas and FasL and the constitutive secretion of biologically active soluble FasL, prostate cancer cells are resistant to Fas-mediated apoptosis [13-15]. However, there are some reports in the literature that demonstrated the reactivation of the apoptotic potential of autocrine Fas signal in these cells when PI3K is inhibited [15]. Our findings also indicate that inhibition of PI3K represents a pivotal role in the riboflavin-mediated PC3 death. Therefore, our data imply that PI3K inhibition induced in prostate cancer cells occurs through the action of FasL secreted by the cell, acting in an autocrine or paracrine fashion, and/or through the action of Fas/FasL interactions occurring on the cell surface.

Intriguingly, our results showed that the inhibition of ceramide synthase by Fumonisin B1 was able to counteract cell death provoked by irradiated riboflavin at all concentrations tested, suggesting that riboflavin photoproducts induced PC3 cell death is associated to the activation of Fas receptor induced via a ceramide-dependent pathway or vice-versa. In this respect, previous data from the literature have shown that ceramide as a second lipid messenger is involved in the reorganization of lipid rafts into ceramide-enriched platforms responsible for increasing Fas clustering and activation [7,16-22]. In contrast, ceramide release after Fas ligation has also been described [22]. In fact, an increase in ceramide synthesis in the mitochondria, which is under the control of ceramide synthase and ceramidase activities, seems to occur prior to the execution phase of apoptosis, and recent evidence suggests that this event is due, at least in part, to the ceramide ability to form protein-permeable channels in the outer mitochondrial membrane, thus favouring the release of cytochrome c and apoptogenic proteins such as Smac/DIABLO and HtrA2/Omi among others [16]. The fact that cells treated with irradiated riboflavin presented downregulation of TNFR1 as well as NFκB, suggests that this vitamin and its photoproducts display anti-inflammatory property. Recently, Bertollo and co-workers (2006) demonstrated that riboflavin inhibits early and prolonged inflammatory responses towards LPS by unknown mechanism [23].

The reversible phosphorylation of proteins, regulated by protein kinases and protein phosphatases, influences virtually all cellular functions and it is an essential mechanism in the control of cell proliferation, differentiation and transformation [24-26]. Riboflavin photoproducts induced a decrease in the phosphorylation level of both serine/threonine and tyrosine residues. The inhibition of the serine/treonine phosphatase PP2A observed after treatment is in agreement with other works showing an induction of apoptosis by PP2A inhibitors [4].

Especially encouraging is the observation that riboflavin photoproducts might suppress the metastatic potential of solid tumours which is supported by the following statements: a) inhibition of metalloproteinase-mediated degradation of the matrix component collagen as demonstrated by decreasing MMPs activities and upregulation of TIMP-1; b) downregulation of VEGF; and c) inhibition of MAPK p38. Because of MMPs extracellular matrix-degradation activities, certain aspects of these enzymes involvement in tumor metastasis, such as tumor-induced angiogenesis, tumor invasion, and establishment of metastatic foci at secondary site, have received extensive attention that resulted in an overwhelming amount of information in favour of critical roles of MMPs in these processes. In this context, physiological MMPs inhibitors, TIMPs, and natural products have been considered as important negative modulators of cancer cells invasion [27,28,29]. MAPK p38 plays a multitude of cellular functions, and recently Ye and Yuan (2007) reported that inhibition of p38 impaired the angiogenesis process [30]. Based on this observation, we can hypothesize that the inhibition of p38 by irradiated riboflavin could be also important to prevent PC3 cells metastasis.

The ultimate molecular basis for the strong effects of riboflavin photoproducts on various kinds of cancer cells remains somewhat enigmatic. The products generated are markedly more hydrophobic as compared to the parent compound riboflavin, due to the loss of the ribityl moiety of the molecule during the photodegradation. Thus, it is highly likely that they will either end up in the plasma membrane or enter the cytosol without the need for active take up by the cancer cell. Further studies, employing purification of interacting proteins using radio labeled parent compound may well be required to answer this question.

In any case the remarkably similar biochemical changes observed in PC3 and leukaemia cells suggest that a common upstream mediator is responsible for death in both cell types. Alternatively, specific interference with the build of specialized lipid plasma membrane domains, like lipid rafts, is a possibility as well. The present study has shown that riboflavin photoproducts are highly promising candidates for solid tumour cancer therapy and provide strong support for commencing small scale human phase I/II studies in terminal solid tumour cancer employing these compounds. Lastly, it is important to mention that our findings about the effect of irradiated riboflavin in tumor cells as well as others related to intact riboflavin pointed out the broad application of this vitamin in the cancer therapy field. For instance, there are some reports on the literature about the augment of tamoxifen efficacy against breast cancer cells, when this chemotherapic was combined with riboflavin. This property is based on the capacity of riboflavin in maintaining the cellular antioxidant response closer to normal level [31].

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Summarizing discussion

In the present thesis, molecular events mediating cell fate decisions were studied. To this end, several in vitro systems were challenged with pharmacological tools to understand cellular response to treatment. We were particularly interested in molecular events which can shift the balance of survival signals. The study of these events can provide important information in the understanding of effective response to chemotherapeutic drugs, however more importantly they can give insights in the underlying mechanism of lack of response to treatment. In this thesis, we studied the contribution of different signaling pathways, such as the Hedgehog (Hh) pathway and the Akt/mTOR pathway, for maintaining resistance to chemotherapeutic drugs. In addition, we determined whether natural compounds (violacein and riboflavin photoproducts) are able to decrease survival signals in tumor cells. Below the chapters are briefly summarized.

In the Chapter 1, we discuss interesting biological aspects of Hh signaling. First, we introduce the molecular aspects of Hh signaling, such as synthesis, secretion and transport. In addition, we discuss the pharmacological targeting of different players of this pathway.
In Chapter 2 we show that the chemotherapy resistant phenotype of myeloid leukemia cells correlates with activation of the Hedgehog pathway, whereas in chemo-sensitive cells such activation is less pronounced. Importantly, the over-expression of Hh pathway components induces chemoprotection and inhibition of the pathway reverts chemoresistance. Our data thus identified the Hh pathway as an essential component of multidrug resistance (MDR) myeloid leukemia and suggest that targeting the Hh pathway might be an interesting therapeutic avenue for overcoming MDR resistance in myeloid leukemia. Our findings may be relevant for the treatment of MDR leukemia but in addition our data may be also relevant for the early detection of resistance. Indeed, the expression of sentinel markers (e.g. Ptch-1) of activation of the Hh pathway in peripheral blood myeloid leukemia cells may be an indication that a patient is developing chemoresistance and is possibly a useful predictor of treatment failure, necessitating the switch to alternative therapy.

In Chapter 3 we studied the developmental Hh pathway in the pancreatic cancer setting because this signal transduction pathway has been shown to be implicated in malignancies in the adult organism, specifically in the proximal gastrointestinal tract. In the present study, we determined the efficacy and specificity of the recently discovered endogenous inhibitor of the Hh pathway, vitamin D3, on inhibition of pancreatic adenocarcinoma cell growth in vitro and in vivo. Vitamin D3 was found to inhibit cell growth specifically through inactivation of Smo and the downstream Hh pathway, rather than activation of the vitamin D3 receptor. However, in in vivo models vitamin D3 was not found to be effective against tumor cell growth. We thus established that although vitamin D3 strongly inhibits the Hh pathway in tumor cells that are dependent on this pathway, it did not effectively inhibit their growth in vivo. These results were somewhat unexpected and could mean that vitamin D3 as single treatment strategy will not yield a therapeutic option for Hh-dependent malignancies.

In Chapter 4, we comment on the role of canonical Hh signaling in endothelial cells tube formation. We show that both human umbilical vein endothelial cells (HUVECs) as well as immortalized 2H11 murine endothelial cells form proper endothelial tube like structures in response to Sonic Hedgehog. Importantly, the addition of 5 μM GANT61 largely diminished

Shh-induced tube formation showing that Gli1 plays an important role in Shh-induced tubulogenesis of endothelial cells. The role of the canonical Hh pathway or at least that of the Gli family of transcription factors, in tubulogenesis and angiogenesis is therefore even more complex as anticipated. We therefore agree with the idea, already suggested by Dr Kanda and coworkers, that Hh induces tubulogenesis of endothelial cells through a combination of rapid Gi-dependent signaling and Gli1 mediated transcriptionally regulated pathways.

In Chapter 5, we provide important insight on the transport of Hh proteins in humans and we show the presence of the Indian Hedgehog (Ihh) in the very low density lipoprotein (VLDL) fraction. As described previously in this thesis, Hh proteins are important morphogens and fulfill critical functions in both the development and maintenance of the vasculature. These proteins are highly hydrophobic and its diffusion toward target tissues remains only partly understood. In Drosophila, hedgehog transport via lipophorins plays a relevant role in development, but neither the presence nor a function for a mammalian Hh carried by human plasma lipoproteins has been established. To study this specific point, we investigated the presence of Hh on lipoprotein particles using LTQ-Orbitrap XL analysis of isolated plasma lipoproteins. These analysis revealed that Ihh is present in the human very low density lipoprotein (VLDL) fraction but not in other plasma lipoprotein fractions (low density lipoprotein (LDL) and high density lipoprotein (HDL). Using the same approach, neither Shh nor Dhh could be detected in plasma lipoprotein fractions. Additionally, we show that the endothelial compartment is most likely to be affected by the presence of Ihh on VLDL. In conclusion, our study shows that VLDL carries Ihh throughout the body in mammals and Hh signaling by human plasma VLDL particles may affect blood vessel pathophysiology.

In Chapter 6 we summarized the potential role of protein tyrosine phosphatases (PTPs) in hematological malignancies. As described in that chapter, PTPs are implicated in three major signaling pathways deregulated in hematological malignancies, JAK-STAT signaling, Ras-Raf-MEK-ERK signaling and PI3K-PKB signaling. Phosphatases are generally regarded as inhibitors of oncogenic signaling and hence tumor suppressors, which might account for the lack of attention they have received in haematological cancer treatment to date. However, there is also a growing body of evidence suggesting that overexpression of phosphatases might contribute to tumor pathology, and this is most prominent for phosphatases such as SHP-2, LMWPTP, PTP1B and CD45. In these cases, specific inhibitors might find clinical applications. However, as not all patients exhibit increased phosphatase activity, the use of these inhibitors calls for personalised medicine. In addition, as some phosphatases might have both positive and negative regulatory functions, extensive research is needed to discover the long-term effects of the use of these inhibitors. In this chapter, we suggest that this class of enzymes might be of relevance for the treatment of hematological malignancies. Further research into their precise role in normal and tumor cell biology might, in time, make them a more appreciated clinical target.

In Chapter 7 we have generated a comprehensive description of cellular kinase activities in the drug-resistant leukaemia cell line (Lucena-1) and the parental p210BCR-ABL-expressing erythroleukaemic CML cell line (K562). The results provide a wealth of biochemical data

on the molecular processes associated with the drug-resistant phenotype. An especially prominent molecular process identified was a signalling pathway triggered by survival-associated receptor tyrosine kinase pathways feeding into the phosphatidylinositol-3-OH-kinase phosphate which, amongst others, produces the activation of mTOR, p21Rac and MAP kinases. Measuring activity of these pathways using conventional technology seemed to confirm these results and inhibition studies identified especially the mTOR kinase pathway as crucial for drug resistance. The exact mechanism by which mTOR contributes to resistance to chemotherapy seems to be related to the fact that inhibition of this kinase shifts the balance of important proteins regulating cell fate decisions and decreases P-gp expression. Obviously, further research is required into this issue and investigation using expression arrays may shed light into the role of these different pathways in chemoresistance. Nevertheless, identification of kinases as drugable mediators of resistance is of obvious importance for developing new therapies as well as biomarkers.

In Chapter 8 we studied the cytotoxic effects of violacein in unusual resistant leukemia cells. We observed that the cell death induced in TF1 leukaemia cells is not mediated by the classical PCD pathways. It is actually carried out by a biochemically and morphologically novel pathway involving inhibition of calpain, Src and pro-inflammatory pathways concomitant with paradoxal activation of the canonical protein kinase B/mTOR and p42/p44MAP kinase signalling cassettes followed by an ultrastructurally highly characteristic cell death programme entailing amongst others endoplasmatic reticulum and Golgi linearization, nuclear mottening and finally nuclear globilusation and cellular demise. We propose that the effect induced by violacein is an alternative mode of cell death which may be important in explaining the action of cancer chemopreventive indolone derivatives. Disregarding the exact molecular details of Violacein-stimulated signal transduction processes in normal physiology, we feel that the compound reveals an as yet unrecognised mode of cellular death, which seems particularly prone to provoke cellular suicide in transformed cells resistant to other modes of PCD. Increased knowledge of the biochemical and physiological characteristics of this process may well prove useful in the fight against cancerous disease.
In Chapter 9 we investigated the effect of irradiated riboflavin on androgen-independent human prostate cancer cells (PC3), a known model for solid tumor cells with an exceptional resistance to therapy. Our results show that riboflavin photoproducts are cytotoxic to these cells in a FasL-Fas-dependent manner. Furthermore, irradiated riboflavin inhibited matrix-degrading proteases, caused downregulation of VEGF and upregulation of TIMP1 suggesting anti-metastatic potential.

The ultimate molecular basis for the strong effects of riboflavin photoproducts on various kinds of cancer cells remains somewhat enigmatic. The products generated are markedly more hydrophobic as compared to the parent compound riboflavin, due to the loss of the ribityl moiety of the molecule during the photodegradation. Thus, it is likely that they will either end up in the plasma membrane or enter the cytosol without the need for active take up by the cancer cell. In any case the remarkably similar biochemical changes observed in PC3 and leukaemia cells suggest that a common upstream mediator is responsible for death in both cell types. Alternatively, specific interference with the build of specialized lipid plasma membrane domains, like lipid rafts, is a possibility as well. The present study

has shown that riboflavin photoproducts are promising candidates for solid tumors therapy. Lastly, it is important to mention that our findings about the effect of irradiated riboflavin in tumor cells as well as others related to intact riboflavin pointed out the broad application of this vitamin in the cancer therapy field.

Together the presented results provide interesting insights on molecular events involved in the survival of chemoresistant cells and endothelial functioning.

Nederlandse samenvatting

In het onderliggende proefschrift hebben we de moleculaire processen betrokken bij het besluit van cellen om zich te delen of om te sterven bestudeerd. Hiertoe hebben we in verschillende systemen cellen blootgesteld aan specifieke farmacologische remmers van cellulaire signaaltransductiepaden om vervolgens te bestuderen hoe cellen hier op reageren. Onze nadruk bij deze experimenten lag op moleculaire processen betrokken bij de balans van zogenaamde “overlevingssignalen”. Het bestuderen van deze processen leidt tot belangrijke inzichten in de werking van chemotherapie maar, misschien nog belangrijker, geeft ook inzicht in het mechanisme hoe cellen “ontsnappen” aan celdood als gevolg van de chemotherapie (beter bekent als resistentie). Het hoofddoel van dit proefschrift was het bestuderen van het effect van farmacologische middelen die ingrijpen op de Hedgehog (Hh) en de Akt/mTOR signaleringsroute op resistentie voor chemotherapie. Daarnaast hebben we het effect van in de natuur voorkomende middelen (violacein en riboflavin) op tumorcelgroei bestudeerd. In detail, hebben we de volgende experimenten uitgevoerd:

In hoofdstuk 1 bediscussiëren we een aantal interessante aspecten van de Hh signaaltransductie route. Zo beschrijven we eerst de basale kenmerken van Hh (synthese, secretie en transport) waarna we het potentiële belang van verschillende farmacologische Hh remmers bespreken.

In hoofdstuk 2 laten we zien dat het chemotherapie resistente fenotype van myeloide leukemie cellen correleert met de activatie status van de Hh signaleringroute. Belangrijker, overexpressie van specifieke componenten van de Hh signaaltransductieroute leidt tot resistentie van leukemie cellen terwijl het remmen van de Hh signaaltransductieroute leukemie cellen gevoeliger maakt voor chemotherapie. Deze data tonen dus aan dat de Hh signaaltransductie route een essentieel onderdeel vormt van “multidrug resistente (MDR)” myeloide leukemie en de data suggereren dat aangrijpen in de Hh signaaltransductieroute een interessante klinische behandeling zou kunnen zijn bij patiënten met MDR leukemie. Daarnaast suggereren onze data ook dat de Hh signaaltransductieroute een rol kan spleen bij de vroege ontdekking van drug-resistentie. Zo zou de expressie van Patched (receptor in de Hh signaaltransductie route) in witte bloedcellen kunnen duiden op de ontwikkeling van drug-resistentie bij patiënten en zou al vroegtijdig kunnen worden overgeschakeld op een andere behandelstrategie.

In hoofdstuk 3 hebben we de rol van Hh signaaltransductie in pancreaskanker bestudeerd. Ten eerste hebben we het effect van een recent beschreven endogene Hh remmer (Vitamine D3) op de groei van pancreaskankercellen bestudeerd. Hierbij bleek dat Vitamine D3 de groei van pancreaskankercellen remt door specifiek in te grijpen in de Hh signaleringsroute en niet door eventuele effecten via de vitamine D3 receptor. Helaas bleek in een muismodel voor pancreaskanker dat vitamine D3 geen effect had op tumorgroei en/of metastase. Het lijkt er dan ook op dat (ondanks het gunstige effect in vitro) vitamine D3 niet geschikt is als monotherapie in de behandeling van pancreaskanker.

In hoofdstuk 4 bestuderen we de rol van Hh signalering in vaatvorming van endotheliale cellen. Zowel humane navelstreng endotheliale cellen als muizen tumor endotheliale cellen vormen vaatjes na behandeling met Hh. Dit vaatvormende effect van Hh werd grotendeels

teniet gedaan door de toediening van GANT61 (gli-1 remmer) wat aantoont dat Gli-1 essentieel is voor Hh-geïnduceerde vaatvorming.
In hoofdstuk 5 beschrijven we dat Indian Hh voorkomt op lipoproteïne partikels in plasma (specifiek op de very low density lipoprotein (VLDL) fractie) en dat Hh op deze manier over langere afstand getransporteerd kan worden. Dit is belangrijk omdat Hh eiwitten erg hydrofobe eiwitten zijn die niet tot nauwelijks instaat worden geacht zich door het lichaam te verplaatsen. Door gebruik te maken van een nieuwe techniek (LTQ-Orbitrap XL) waren we instaat om alle eiwitten in de verschillende lipoproteïne fracties in kaart te brengen. Hierbij bleek Indian Hh (maar niet SHh of DHh) voor te komen op VLDL maar niet op LDL of HDL. Verder laten we in dit hoofdstuk zien dat Indian Hh op VLDL directe effecten heeft op endotheliale cellen. Samenvattend, deze data tonen aan dat VLDL verantwoordelijk is voor het transport van Ihh door het lichaam en dat Ihh op VLDL partikels bloedvat pathophysiology beïnvloed.

In hoofdstuk 6 geven we een overzicht van de potentiële rol die “protein tyrosine phosphatases (PTPs)” zouden kunnen hebben in hematologische maligniteiten. PTPs zijn essentieel in drie verschillende signaaltransductieroutes waarvan bekent is dat ze verstoord zijn in hematologische maligniteiten, te weten JAK-STAT signalering, Ras-Raf-MEK-ERK signalering and PI3K-PKB signalering. PTPs worden over het algemeen beschouwd als remmers van oncogene signalering en dus als tumor-suppressors wat zou kunnen verklaren waarom PTPs weinig aandacht hebben gekregen in hematologische maligniteiten. Recente literatuur suggereert echter dat overexpressie van PTPs een schadelijke rol speelt in hematologische maligniteiten. Specifieke PTP remmers zouden dus van klinisch belang kunnen zijn maar aangezien er slechts bij een beperkte groep patiënten sprake is van PTP overexpressie is klinische toepasbaarheid niet evident en slechts mogelijk in een personalized-medicne benadering. Daarnaast hebben sommige PTPs ook een remmende werking op de ontwikkeling van de maligniteiten en er is dus nog veel onderzoek nodig voordat PTP remmers in de kliniek bruikbaar zijn.

In hoofdstuk 7 geven we een uitgebreide beschrijving van alle geactiveerde signaaltransductieroutes in drug-resistente leukemie cellen (Lucena-1) en in drug-gevoelige leukemie cellen (K562). De vergelijking van deze twee celtypes resulteerde in een overzicht van signaaltransductie routes mogelijk betrokken bij drug-restistentie. Het meest opvallende verschil tussen de resistente en gevoelige cellen was de activatie van de mTOR, p21Rac and MAP kinase signaleringsroutes in de resistente cellen. Vervolg experimenten lieten zien dat voornamelijk mTOR van belang is bij drug-resistentie. Het onderliggende mechanisme waarbij mTOR bijdraagt aan drug-restistentie is niet helemaal duidelijk maar er lijkt een belangrijke rol voor mTOR in het verlagen van de expressie van de PgP drug-efflux pomp. Vervolgonderzoek zal moeten aantonen of mTOR inderdaad klinisch relevant is bij resistentie tegen chemotherapie.

In hoofdstuk 8 hebben we het cytotoxische effect van violacein bestudeerd in resistente leukemie cellen. Hierbij bleek dat violacein geïnduceerde celdood niet loopt via de klassieke celdood mechanismes. Violacein bleek een nieuw celdood programma te induceren bestaande uit calpaine, Src en pro-inflammatoire signaleringsroutes in combinatie met

activatie van de proteïne kinase B/mTOR en p42/p44MAP kinase signaleringsroutes. Als gevolg van de activatie van deze signaleringsroutes zagen we onder andere linearisatie van het endoplasmatisch reticulum en het Golgi, “nuclear mottening” en “nuclear globilusation” met als gevolg het dood gaan van de cel. We hypothetiseren dat violacein een nieuwe manier van celdood initieert die vooral van belang lijkt in situaties waarbij getransformeerde cellen resistent zijn tegen de klassieke vormen van celdood. Vervolgonderzoek naar de exacte biochemische en fysiologische veranderingen in de cel is echter nodig om het potentiële klinische belang van deze nieuwe vorm van celdood te achterhalen.

In hoofdstuk 9 hebben we het effect van bestraald riboflavin op androgeen-onafhankelijke humane prostaat kankercellen (PC3) bestudeerd als modelsysteem voor solide tumoren met een enorme drug-resistentie. Het blijkt dat fotoproducten van riboflavin cytotoxisch zijn voor deze cellen in een FasL-Fas-afhankelijke mannier. Daarnaast bleken de fotoproducten van riboflavin te leiden tot verminderde activiteit van matrix-afbrekende proteases, verminderde expressie van VEGF en een toename van TIMP1 expressie suggererend dat de fotoproducten van riboflavin remmend op tumorcel metastase zouden kunnen werken. Deze data laten dus zien dat fotoproducten van riboflavin van belang bij de behandeling van solide tumoren zou kunnen zijn. Huidige dierexperimenten moeten echter aantonen of dit inderdaad zo is.

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PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Karla Cristiana de Souza Queiroz **Erasmus MC Department:** Gastroenterology and Hepatology	**PhD period:** May 2006 to May 2011 **Promotor:** Prof. dr. M.P. Peppelenbosch **Co-promotors:** Prof. dr. C.V. Ferrreira Dr. C.A. Spek

1. PhD training		
	Year	**Workload (hours/ECTS)**
General Courses		
Radioprotection Course 5B	2006	3 ECTS
Laboratory Animal Science (Article 9)	2008	4 ECTS
Drug Discovery Simulation	2011	2 ECTS
Presentations		
Young Scientist Program – 21st IUBMB & 12th Faomb international congress of Biochemistry and Molecular Biology, Shangai, China	2009	2 ECTS
Voorjaarsvergadering Nederlandse Vereniging voor Gastroenterologie (NVGE) 2011, Veldhoven	2011	2 ECTS
XXIII Congress of the International Society on Thrombosis and Haemostasis, Kyoto, Japan	2011	2 ECTS
International Conferences		
32nd FEBS Congress, Molecular Machines and their Dynamics in Fundamental Cellular Functions, Vienna, Austria	2007	3 ECTS
21st IUBMB & 12th Faomb international congress of Biochemistry and Molecular Biology, Shangai, China	2009	3 ECTS
Gastro 2009 UEGW/WCOG, London, United Kingdom	2009	3 ECTS
XXIII Congress of the International Society on Thrombosis and Haemostasis, Kyoto, Japan	2011	3 ECTS
2. Teaching activities		
Supervising Internship student		
Carolin Sieber, Molecular Medicine, University of Freiburg, Freiburg- Germany	2009	1.5 ECTS
Supervising Master's student		
Therese Liechtenstein, Biology, University of Amsterdam, Amsterdam	2011	3 ECTS