Mechanism of cell death induced by caspase activators PAC-1 and 1541 and tubulin inhibitor combretastatin A-4

Crosstalk between caspases, ROS and growth factors

By

Gulzeb Aziz

Thesis for the degree of Ph.D.

Department of Pharmaceutical Biosciences

School of Pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo

2011

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 1146

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Unipub.
The thesis is produced by Unipub merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

“Dedicated to my Mother, Mehmooda Akthar (1948-2011) for her endless efforts and unforgettable love for me. I will miss her always.”

Table of Contents

Acknowledgements

The work presented in this thesis was performed at the Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, from January 2008 to September 2011. Financial support from the University of Oslo (Quota program) is gratefully acknowledged.

First and foremost I would like to express my gratitude to my supervisor Professor Ragnhild E. Paulsen for scientific guidance, enthusiasm and continuous encouragement throughout the years. I would also like to thanks my co-supervisors Tor Gjøen and Trond Vidar Hansen for giving me encouragement and always taking time for discussions.

I am grateful to Mona Gaarder for all the practical help and valuable technical assistance she gave me and to all the members of the Neurobiology Group, especially Gro Haarklou Mathisen, Karen Bolding Deberbard, and Bjørn Oddvar Strøm for all the interesting discussions, help and support. I am also thankful to everyone at the ZEB building for making an enjoyable work environment.

During the entire process of completing this PhD I had constant support from family, both here and in Pakistan; I am especially thankful for the support of my cousin Aamer Shehzad, my mother, father and brothers. My mother, the sole source of love and prayers in my life, passed away on June 16, 2011. I would like to dedicate this PhD thesis to my lovely mother, may her soul rest in peace. I would also like to thank my lovely father Zulfiqar Hussain for his continuous encouragement during this difficult period of my life.

Finally, I am very thankful to my wife Zubaida for being a supportive and patient person during the fulfilment of this thesis. I am blessed to have you in my life.

Oslo, September 2011

Gulzeb Aziz

Abbreviations

ADP/ATP	Adenosine diphosphate/adenosine triphosphate
AIF	Apoptosis inducing factor
Asp	Aspartate
Bad	Bcl-associated death promoter
Bak	Bcl-2 family antagonist
Bax	Bcl-2-associated X-protein
Bcl-2	B-cell lymphoma 2
CGN	Cerebellar granule neuron
Cys	Cysteine
Cyt c	Cytochrome c
DHE	Dihydroethidium
DHR	Dihydrorhodamine 123
EGF	Epidermal growth factor
ERK/P-ERK	Extracellular signal-regulated kinase/phospho-ERK
H_2O_2	Hydrogen peroxide
MT Red	MitoTracker Red
$^{\cdot}NO$	Nitric oxide radical
$^{\cdot}O_2^-$	Superoxide anion radical
$^{\cdot}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PARP-1	Poly (ADP-ribose) polymerase 1
PLA_2	Phospholipase A_2
ROS	Reactive oxygen species

List of Papers

I. Procaspase-activating compound 1 induces a caspase-3-dependent cell death in cerebellar granule neurons. (2010). **Gulzeb Aziz**, Øyvind W. Akselsen, Trond V. Hansen and Ragnhild E. Paulsen. Toxicol Appl Pharmacol 247, 238-242.

II. Cell death induced by novel procaspase-3 activators can be reduced by growth factors. (2011). Karen A. Boldingh Debernard, **Gulzeb Aziz**, Annine Thomassen Gjesvik and Ragnhild E. Paulsen , BBRC 413, 364-369.

III. Combretastatin A-4 and structurally related 1,5-disubstituted 1,2,3-triazole analogues induce caspase-3 and ROS dependent cell death in PC12 cells. (2011). **Gulzeb Aziz,** Kristin Odlo, Trond V. Hansen, Ragnhild E. Paulsen and Gro H. Mathisen (Manuscript).

Aims of the present study

Caspases are a group of proteases that cleave intracellular substrates and thereby regulate the process of apoptosis. Growth factors can protect cells against apoptosis via the mitogen-activated protein kinase/extracellular-signal regulated kinase (MEK/ERK) signalling pathway. The production of reactive oxygen species can stimulate cytochrome c (cyt c) release and activate downstream caspase signalling pathways that lead to apoptosis. The focus of this work has been the interaction between epidermal growth factor (EGF) and caspase-3 as well as the crosstalk between ROS and caspase-3. These subjects were investigated using reported caspase-3 activators and apoptosis-inducing agents as experimental tools in chicken cerebellar granule neurons (CGN) and phaeocyhromocytoma (PC12) cells.

The specific aims of the present study are:

1. To investigate pro-caspase activating compound-1 (PAC-1) as a zinc chelator and caspase-3 activator in chicken CGN (Paper I).
2. To investigate the neurotoxicity of PAC-1 and proenzyme activator 1541 (1541) in chicken CGN and PC12 cells and establish these cells as model systems to study these compounds (Papers I and II).
3. To investigate the effects of growth factors such as EGF on the cell-death-inducing properties of PAC-1 and 1541 in PC12 and chicken CGN cells (Paper II).
4. To investigate the toxicity of combretastatin A-4 (CA-4) and triazole analogues in PC12 cells and to examine the crosstalk between caspase-3 activation and ROS production (Paper III).

1. Introduction

1.1 Cell death

Cell death is an integral part of development in all organisms (Penaloza *et al.*, 2006) and has begun even by the time the embryo consists of just eight cells. Many cells die during the processes of development, homeostasis and aging (Lockshin and Zakeri, 2007). Cell death can be exploited to kill tumours; chemotherapeutic agents, ɣ-irradiation, suicide genes and immunotherapy have been shown to induce apoptotic cell death. Although many pathways with unique mechanisms and morphologies can result in cell death, they can be grouped into three major types: apoptosis, necrosis and oncosis. In the 19th century, naturally occurring cell death was first described. The term apoptosis was introduced in 1972 (Kerr *et al.*, 1972), and the concept of physiological cell death was received with acclaim (Chimienti *et al.*, 2001). Cell death is an essential strategy to control of the dynamic balance in living systems (Vermeulen *et al.*, 2005). Complex multicellular organisms maintain a delicate balance between growth and cell death; perturbation of this balance can result in neoplasia or neurodegeneration (Crawford and Wells, 2011).

1.2 Apoptosis

Programmed cell death, or apoptosis, is a continuous, non-inflammatory physiological process and is an active field of biochemical research (Schultz and Harrington, 2003). Apoptosis is a crucial mechanism for organism survival and is functionally conserved in all higher eukaryotes. This important process is responsible for the removal of damaged or infected cells from the cellular population, which links apoptosis to the cell cycle, replication and DNA repair. Moreover, apoptosis is one of the main mechanisms governing accurate embryonic development and the maintenance of tissue homeostasis (Pecina-Slaus, 2009). Although

apoptosis is an essential process, it is also involved in a wide range of pathological conditions (Zimmermann *et al.*, 2001).

The deregulation of apoptosis is an important factor in pathological conditions such as cancer and autoimmune and neurodegenerative diseases. Apoptotic cells can be characterised by specific morphological and biochemical changes, including cell shrinkage, chromatin condensation, and the internucleosomal cleavage of genomic DNA. At the molecular level, apoptosis is tightly regulated and mainly orchestrated by the activation of an aspartate-specific cysteine protease (caspase) cascade (Zimmermann *et al.*, 2001). Two main pathways lead to caspase activation (Figure 1). The first of these pathways depends on mitochondrial participation (receptor-independent), and the second involves the interaction of a death receptor with its ligand.

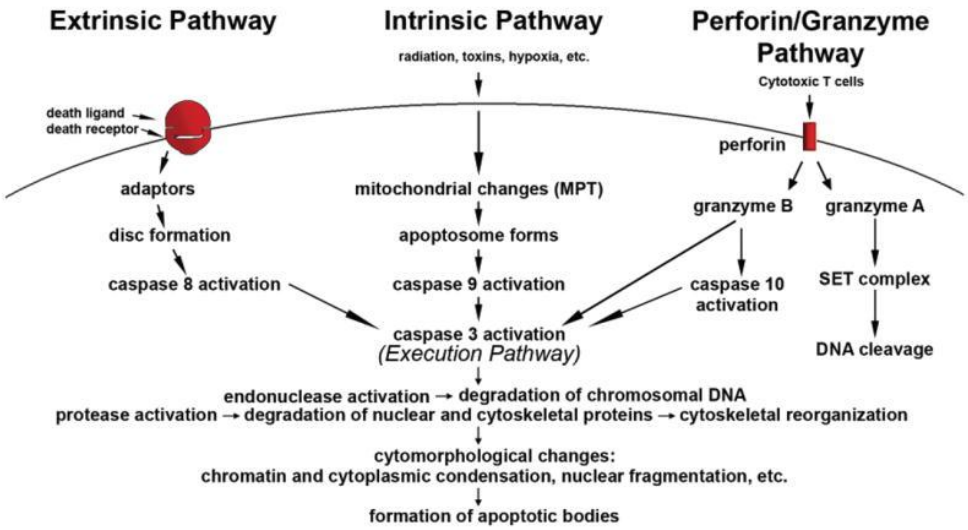

Fig 1. Schematic representation of apoptotic events. Apoptosis is induced by an extrinsic, intrinsic or perforin/granzyme pathway. Each pathway requires specific signals to trigger an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, or 10), which in turn activates the executioner caspase-3. However, granzyme A functions in a caspase-independent fashion. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, the formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages. *Figure and legend from* (Elmore, 2007).

1.3 Caspases

Caspases are a family of intracellular proteases involved in inflammation and are also crucial mediators of programmed cell death (Porter and Janicke, 1999). Once activated by a specific stimulus, caspases perform limited proteolysis of downstream substrates to trigger a cascade of events that culminates in specific biological responses (Pop and Salvesen, 2009). Caspase zymogens are single chain proteins with N-terminal prodomains preceding the conserved catalytic domains. Two zymogens are cleaved to create two small and two large subunits that assemble into heterotetramers (Figure 2).

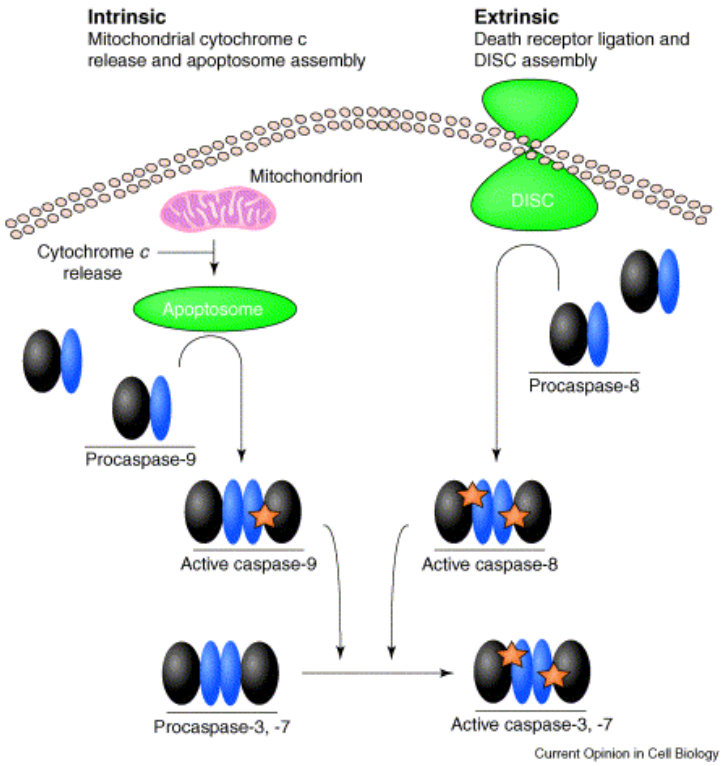

Fig 2. Schematic overview of the apoptotic pathways. Engagement of the extrinsic or intrinsic death pathways leads to the activation of the initiator caspases by dimerisation at multiprotein complexes. In the extrinsic pathway, the DISC is the site of activation for caspase-8 and, at least in humans, caspase-10. The active sites are represented by orange stars. Stimulation of the intrinsic pathway leads to activation of caspase-9 at the apoptosome. Caspase-9 is shown as having one active site as seen in its crystal structure. However, the number of active sites in vivo is unknown. Following activation, the initiator caspases then cleave and activate the executioner caspases-3 and -7. *Figure and legend from* (Boatright and Salvesen, 2003).

1.3.1 Classification

Caspases are classified into two main groups: apoptotic (caspase-2, -3, -6, -7, -8, -9, and -10) and pro-inflammatory (caspase-1, -4, -5, -11, -12 and -13). The apoptotic caspases are further categorised as initiators (caspase-2, -8, -9 and -10) or effectors (caspase-3, -6 and -7) (Pop and Salvesen, 2009).

1.3.2 Mechanism of activation

Caspases are synthesised as inert zymogens that are subsequently activated by internal or external apoptotic stimuli. Once these stimuli occur, cells activate initiator caspases that proteolytically cleave and activate effector caspases. Once active, this second set of caspases is capable of cleaving a large number of substrates that ultimately lead to cell death. Procaspases contain an N-terminal prodomain and sequences encoding the large (p20) and small (p10) subunits of procaspase (Kurokawa and Kornbluth, 2009) (Figure 3). Caspases specifically recognise a tetrapeptide sequence on their substrate with an absolute requirement of an Asp residue (Vermeulen *et al.*, 2005). The two major apoptotic pathways can be differentiated by the cyt c release from the mitochondria. In the intrinsic pathway, cyt c is released from the mitochondrial intermembrane spaces prior to caspase activation, and in the external pathway, it may be released after the activation of effector caspases by the activation of death receptors (Lossi and Gambino, 2008).

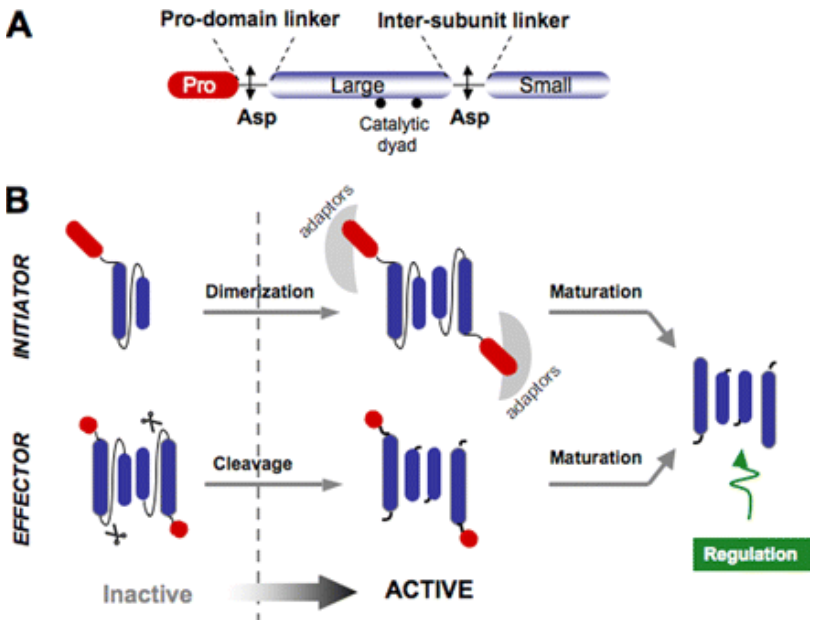

Fig 3. Caspase organisation. A, a prodomain precedes the catalytic domain, which is composed of two covalently linked subunits. Sites for (auto) proteolysis at Asp residues are indicated. *B*, activation mechanisms. Initiators are monomers that activate by prodomain-mediated dimerisation. Executioners are dimers that activate by cleavage of intersubunit linkers. Following activation, additional proteolytic events mature the caspases to more stable forms that are prone to regulation. *Figure and legend from* (Pop and Salvesen, 2009).

1.4 Mitochondrial-induced apoptosis

Mitochondria play a pivotal role in the cascade of events in cell death (Perkins *et al.*, 2009), and B-cell lymphoma 2 (Bcl-2) family proteins are particularly important in the mitochondrial pathway. These proteins are subdivided in two groups: anti-apoptotic and pro-apoptotic. The first group includes Bcl-2 and B-cell lymphoma-extra large (Bcl-XL), and the second group includes Bcl-2-associated X-protein (Bax), Bcl-2 family antagonist (Bak) and Bcl-associated death promoter (Bad). The mitochondrial pathway involves the upregulation and translocation of the pro-apoptotic proteins Bax and Bak to the mitochondrial outer membrane, and this translocation leads to conformational changes and the oligomerisation of the outer mitochondrial proteins that increase mitochondrial outer membrane permeability (Figure 4). This process results in the release of cyt c, AIF and Smac/DIABLO, which trigger caspase 9 activation, thereby activating effector caspase 3, which results in the cleavage of numerous protein substrates. Anti-apoptotic proteins such as Bcl-2 and Bcl-XL prevent the release of cyt c from the mitochondria, and Bcl-XL also prevents caspase 9 activation by interacting with APAF-1 (Fan *et al.*, 2005).

1.5 Reactive oxygen species

ROS is a collective term used for O_2-derived free radicals such as superoxide anions ($O_2^{\cdot -}$) and hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals as well as O_2-derived nonradical species such as hydrogen peroxide (H_2O_2). Mitochondria are a major source of intracellular ROS (Circu and Aw, 2010) that are generated via mitochondrial respiration during the reduction of molecular oxygen as well as by distinct enzymatic systems. ROS have been implicated in the regulation of diverse cellular functions including pathogen defence,

intracellular signalling, transcriptional activation, proliferation and apoptosis (Davis *et al.*, 2001). Many cytotoxic drugs increase intracellular oxidative stress, which results in apoptosis. The cellular response to cytotoxic agents may generate ROS in excess of the levels of thiol buffers and other antioxidants, which may activate mitochondria (Figure 4). ROS may also activate other signalling pathways, e.g., through the stress kinase cascade. Alternatively, cytotoxic agents may modulate the compartmentalisation of GSH. In this scenario, although ROS are not generated directly, the loss of nuclear GSH may activate downstream proteolytic caspases that result in cell death (Davis *et al.*, 2001).

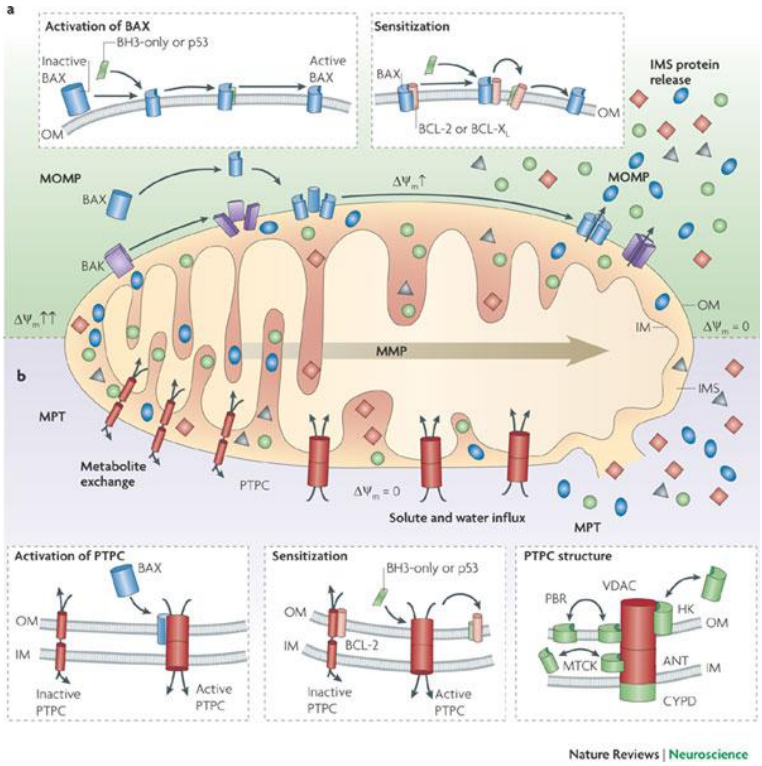

Figure 4. Mitochondrial membrane permeabilisation (MMP) can be generated by either of two distinct, but partially overlapping, molecular mechanisms. On the one hand, large channels formed by multidomain pro-apoptotic proteins from the BCL-2 family (for example, BCL-2-associated protein X (BAX) or BCL-2 antagonist/killer (BAK) might selectively promote mitochondrial outer membrane permeabilisation (MOMP) (**a**). On the other hand, cell death inducers such as Ca^{2+} overload and oxidative stress trigger MMP at the inner mitochondrial membrane (IM) by favouring the opening of the permeability transition pore complex (PTPC). This process is known as the mitochondrial permeability transition (MPT)(**b**). Anti-apoptotic proteins from the BCL-2 family (for example, BCL-2 and BCL-X_L) reportedly inhibit both MOMP and MPT. Similarly, the pro-apoptotic BCL-2 protein BAX mediates MOMP but can also bind to PTPC components such as adenine nucleotide translocase (ANT) and the voltage-dependent anion channel (VDAC) to favour MPT (inserts). BCL-2 homology domain 3 (BH3)-only proteins and p53 provide an additional, upstream level of control to MMP. Some BH3-only proteins (also known as activators; for example, BID and BIM (also known as BCL-2-like protein)) can directly interact with BAX and BAK, thereby triggering their pore-forming function. By contrast, so-called sensitisers (for example, BAD and HRK (also known as DP5)) promote MMP by disrupting the inhibitory interactions between anti-apoptotic BCL-2 proteins and BAX, BAK, activators or PTPC components. p53 might exert lethal functions on mitochondria by mimicking the activity of BH3-only proteins at multiple levels. *Figure and legend from* (Galluzzi et al., 2009).

1.6 Zinc deprivation

Zinc is essential for cell differentiation and proliferation, especially for DNA synthesis and mitosis (Beyersmann and Haase, 2001). Zinc is an integral part of many proenzymes in cells and may function as a cofactor. Zinc deficiency induces apoptosis though the intrinsic mitochondrial pathway, which can be triggered by the zinc-regulated enzyme caspase-3 and as a consequence of abnormal regulation of pro-survival signals (ERK1/2 and NF-Kappa B) in neurons (Adamo *et al.*, 2010) (Figure 5).

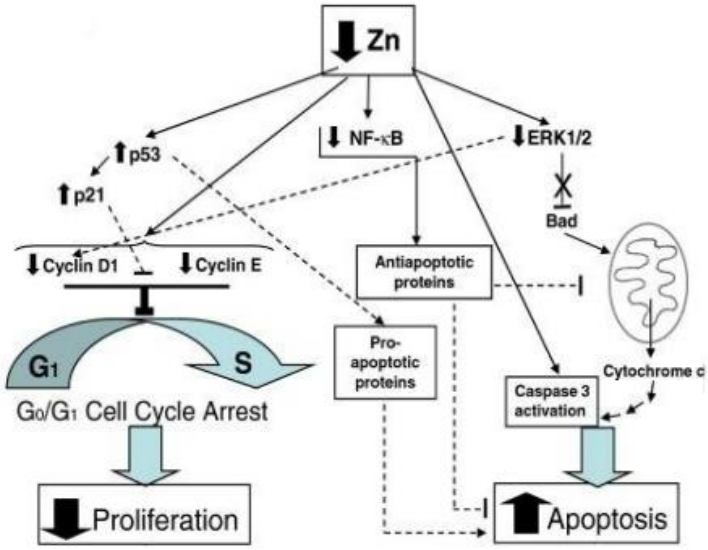

Figure 5. Proposed mechanisms for Zn-deficiency-induced decrease in cell proliferation and induction of apoptosis. *Solid or dotted lines* indicate proposed mechanisms leading to neuronal cell cycle arrest, decreased proliferation and induction of apoptosis as a consequence of a decreased Zn availability. *Figure and legend are modified from* (Adamo et al., 2010).

1.7 MEK/ERK-signalling pathway

There is a controlled balance between cell death and survival that may be affected by the cell's external environment (Henson and Gibson, 2006). MAP kinases have a central role in signal transduction cascades triggered by extracellular substances such as hormones, neurotransmitters and growth factors that regulate various cellular processes (Seger and Krebs 1995). The MAP kinase cascade consists of several protein kinases that specifically phosphorylate and activate each other (Chuderland and Seger, 2005) (Figure 6). The MEK/ERK signalling pathway is a part of this cascade and plays important roles in proliferation, differentiation and survival. The MEK/ERK signalling pathway is affected by numerous factors such as serum nutritional content, growth factors and various types of stress (Seger and Krebs, 1995; Lewis *et al.*, 1998). The MEK/ERK-phosphorylation cascade starts with a ligand binding to a transmembrane tyrosine kinase receptor on the cell surface. This binding leads to the activation of the G-protein Ras, which recruits Raf kinase that phosphorylates and further activates MEK, which then activates ERK (Shaul and Seger, 2007). Activated ERK can interact with cytosolic substrates or translocate to the nucleus and regulate various transcription factors, kinases and cytoskeleton proteins with different interacting proteins (Lewis *et al.*, 1998; Shaul and Seger, 2007) (Figure 6). The duration of activation is also important; it has been shown in PC12 cells that nerve growth factor (NGF) promotes differentiation via strong and sustained ERK activation, whereas epidermal growth factor (EGF) promotes proliferation through strong and transient ERK activation (Nguyen *et al.*, 1993). ERK blocks caspase-9 activation by direct phosphorylation and also inhibits caspase-3 activation and apoptosis (Allan *et al.*, 2003). EGF acts as one of the many sensors

controlling the degree of apoptotic signalling within the cell (Henson and Gibson, 2006). EGF signalling enhances survival responses in cancer cells. When the EGF survival signalling pathway is targeted, cancer cells undergo apoptosis or become sensitive to radiation and chemotherapy.

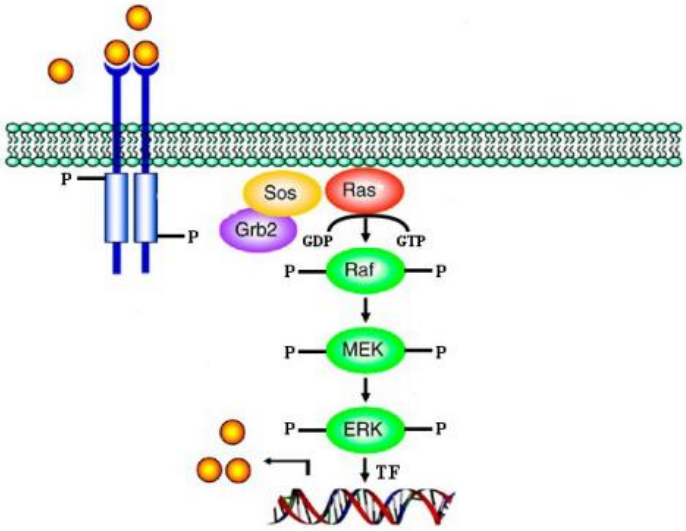

Figure 6. MEK/ERK-signalling pathway. Growth factors and other extracellular ligands bind to a transmembrane receptor that dimerises and phosphorylates tyrosine residues. SH2-domain-containing adaptor proteins such as Grb2 bind to the phosphorylated tyrosine residues and recruit SOS receiving Ras to convert GDP to GTP and become activated. Ras phosphorylates and activates Raf, which in turn phosphorylates and activates MEK, which then phosphorylates and activates ERK. ERK binds to and activates substrates in the cytosol and translocates to the nucleus, where it activates various transcription factors. *Figure and legend are modified from* (Seger and Krebs, 1995; Henson and Gibson, 2006).

1.8 Apoptosis-inducing compounds

There are many compounds that may initiate apoptosis via the intrinsic or extrinsic pathway in cancer cells and non-cancerous cells. Small molecules are under development to target the apoptotic cascade (Putt *et al.*, 2006). These molecules may be used as anticancer agents or tools to study caspase functions.

1.8.1 Caspase-3 activators

Caspase activation by small molecules such as PAC-1 and 1541 has emerged as a powerful anticancer strategy. These molecules are known to transform procaspase-3 (inactive zymogen) to active caspase-3 directly in cancerous cells (Putt *et al.*, 2006), which is a hallmark process of apoptosis.

1.8.2 PAC-1

PAC-1 was the first procaspase-3 activating compound to be discovered by high-throughput screening in 2006 (Putt *et al.*, 2006). It was originally thought that PAC-1 directly transformed procaspase-3 to caspase-3 in vitro and induced apoptosis in cancerous cells isolated from primary colon tumours in a manner directly proportional to the cellular concentration of procaspase-3 (Putt *et al.*, 2006). However, several mechanisms for the activation of caspase-3 by PAC-1 have since been proposed, including the sequestration of inhibitory zinc ions (Figure 7). Zinc is as an endogenous caspase-3 inhibitor and anti-apoptotic agent, which suggests a shared mode of action for certain zinc-chelating anti-cancer compounds (Peterson *et al.*, 2009a),(Peterson *et al.*, 2009b). PAC-1 binds to zinc, enzymatically activating procaspase-3 and allowing the subsequent activation of another molecule of procaspase-3 to form caspase-3 (Peterson *et al.*, 2009a). Zinc chelation is an

important mechanism to reduce cell proliferation and induce apoptosis (paper I). However, because PAC-1 is not able to fully activate procaspase-3, its potential as an anticancer drug may not be very promising (Wolan *et al.*, 2009).

PAC-1 was found to be neurotoxic in chicken CGN (Paper I) and dogs (Peterson *et al.*, 2010). The neurotoxic properties of PAC-1 raised challenges for its use as an anticancer drug and led to the development of S-PAC-1, which contains a polar sulfonamide on the benzyl ring of PAC-1. This compound is believed to have a similar procaspase-3 activating ability, but because it cannot cross the blood-brain barrier, it is not neurotoxic in mice or dogs (Peterson *et al.*, 2010).

The phenolic hydroxyl group of the PAC-1 molecule is important for caspase activation and zinc chelation. PAC-1 derivatives lacking this group do not activate procaspase-3 in vitro and do not induce death (Peterson *et al.*, 2009b) (paper I). It is also known that ortho-hydroxy N-acyl hydrazones, which are a part of the PAC-1 molecule, bind to metal ions and are essential for zinc binding. Any modification of this structure will result in compounds that are unable to bind zinc or activate caspase-3, which means they would be unable to induce cell death (Peterson *et al.*, 2009b). Conversely, other sites on PAC-1, such as the benzyl ring, could be altered without affecting either activity (Peterson *et al.*, 2009b).

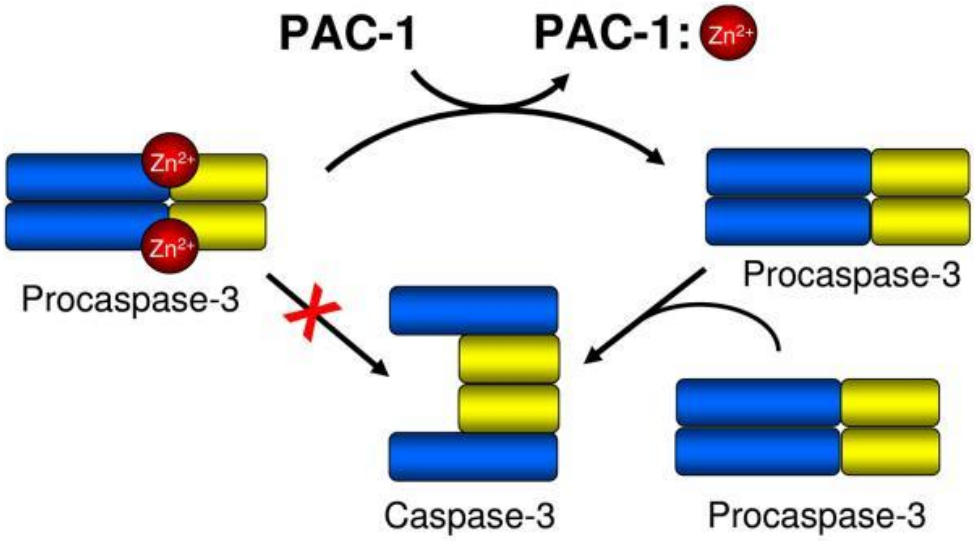

Figure 7. The proposed mechanism for PAC-1-induced activation of procaspase-3 *in vitro*. Zinc inhibits the catalytic activity of procaspase-3, PAC-1 binds tightly to zinc, and PAC-1 relieves the zinc-mediated inhibition of procaspase-3. This zinc sequestration enables procaspase-3 to function as an enzyme and proteolytically cleave another molecule of procaspase-3, converting it to caspase-3. *Figure is from* (Peterson *et al.*, 2009a).

1.8.3 Proenzyme activator 1541

After the discovery of PAC-1, another small-molecule activator of procaspase-3 was introduced in 2009 and considered as a direct activator (Figure 8). Proenzyme activator 1541 bypasses the upstream pro-apoptotic signalling pathway and induces rapid apoptosis in a variety of cell lines (Wolan *et al.*, 2009). When compared with granzyme B, which is a natural activator of procaspase-3, 1541 induced accelerated activation of caspase-3 to a level that was finally 70% that of granzyme within 2.5 hours. Granzyme B and 1541 both induced full proteolytic cleavage of procaspase-3 into large (17 kD) and small (12 kD) subunits of caspase-3. 1541 promoted the activation and self-processing of procaspase-3 and procaspase-6. 1541 did not activate procaspase-1 and procaspase-7 and was considered to be an excellent activator of procaspase-3 in a variety of cancer cells that are resistant to pro-apoptotic stimuli (Wolan *et al.*, 2009). When compared with PAC-1, 1541 was significantly better at activating procaspase-3 (Wolan *et al.*, 2009).

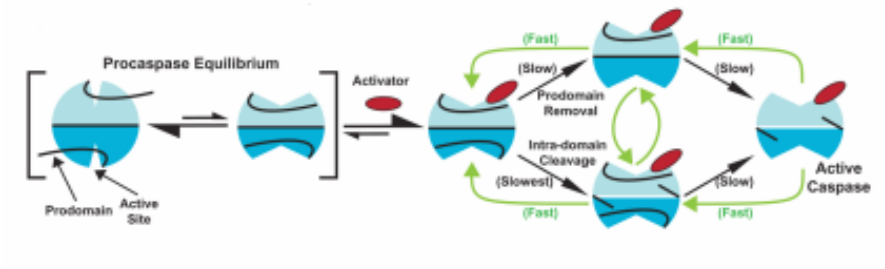

Figure 8. Proposed model for small-molecule-assisted procaspase self-activation. Procaspases are hypothesised to be in a dynamic equilibrium between an off-state (left) and on-state. Binding of a small-molecule activator shifts the equilibrium to an on-state (centre). This complex slowly undergoes autoproteolytic activation (black arrows) that accelerates with the increased production of the mature caspase (green arrows). This model also accounts for activation at low concentrations of the small molecule where the on-state is preferred, one active site is available in the dimer for processing and, at high concentrations, where both sites are saturated and can lead to inhibition. *Figure and legend are modified from* (Wolan et al., 2009).

1.8.4 CA-4 and analogues

The combretastatins are small natural products isolated from the bark of the African bush willow tree, *Combretum caffrum.* CA-4, which is a phenolic *cis*-stilbene natural product, has entered several clinical trials as a potential anticancer drug (Kanthou and Tozer, 2009). Whereas PAC-1 and 1541 are thought to activate caspases directly, CA-4 is an indirect caspase activator. Several of its analogues have been shown to have cytotoxic effects. Recently, certain 1,5-disubstituted 1,2,3-triazole analogues of CA-4 with the two aryl groups retained in the *cis*-configuration were reported to display potent cytotoxic activity against several human cancer cell lines (Odlo *et al.*, 2008) (Odlo *et al.*, 2010). A CA-4 analogue promotes apoptosis in chondrosarcoma cells by inducing mitochondrial dysfunction through a

caspase-9- and caspase-3-mediated mechanism (Liu *et al.*, 2011). CA-4 induces cell death in the H460 lung cancer cell line via activation of the mitochondrial signalling pathway (Mendez *et al.*, 2011). CA-4 and analogue 2 are potent tubulin inhibitors and are highly toxic to cancer cells. Little is known about analogue 3 as a tubulin inhibitor (Odlo *et al.*, 2010). ERK 1/2 were shown to protect against blebbing induced by CA-4-phosphate (CA-4-P), which was associated with decreased viability in human endothelial cells (Kanthou and Tozer, 2002).

In paper III, 1,5-disubsituted 1,2,3 triazole analogues of CA-4 were studied to shed light on their structure-activity relationships and pharmacological mechanisms. Analogue 2 has a *cis* configuration and a phenolic hydroxyl group, analogue 3 lacks a phenolic hydroxyl group and analogue 4 has a *trans* configuration.

2. Discussions of Methods

2.1 Primary cultures of chicken CGN and PC12 cells

The cerebellum is a prominent brain structure in most mammals, and most of its neurons are granule cells that are located in the deepest layer. CGN primary culture has been used for many years and is a recognised model for studying neuronal development, function and pathology (Henson and Gibson, 2006). Chicken CGN are an economical and simple alternative to mouse or rat CGN. In chicks, CGN are formed the last week before hatching by intense mitotic activity in the outer granule layer of cells followed by migration to the inner cell layer. Isolation of CGN on embryonic day 18 has been shown to provide a good yield. Chicken CGN develop excitotoxicity with ROS production and caspase-3 activation after 3 days in vitro and can be used as a model in studies of the mechanisms involved in apoptosis (Jacobs *et al.*, 2006) (Paper I and Paper II). PC12 cells are a neuroendocrine cell line derived from a phaeochromacytoma in the rat adrenal medulla (Trujillo, 2011). The growth of PC12 cells in serum-containing medium results in a round or angular morphology. At a high cell density, they become more rounded, and the cells clump together (Kanthou and Tozer, 2002). PC12 cells are a good model for studying signalling pathways because specific responses to differentiation, proliferation and survival can be studied independently, and there are few growth factors and hormones that the cells do not respond to. This cell line has been used to study the MEK/ERK-signalling pathway, and stimulation with EGF was shown to lead to proliferation (Vaudry *et al.*, 2002). These cells have properties similar to those of sympathetic neurons and form long projections upon NGF stimulation. They are a useful model to study the nervous system at the cellular and molecular levels (Trujillo, 2011). Several pharmacological and biochemical substances induce death in PC12 cells (Kanthou and Tozer, 2002), including PAC-1, which has been shown to induce cell death via procaspase-3 (Putt *et*

al., 2006). Therefore, PC12 cells can also be used as a model to study cell death mechanisms (Paper I, Paper II and Paper III).

2.2 Cell death

Cell death mechanisms have been investigated in PC12 cells and chicken CGNs. There are different advantages and disadvantages for each culture type. PC12 (dividing cells) and CGNs (post-mitotic cells) have similar levels of procaspase-3 (Paper I), which is one of the reasons for choosing these two models to study cell death. Interestingly, PAC-1 has similar EC_{50} values in the two cell types (paper I). PAC-1 induces caspase-3-dependent cell death in different cancer cell lines (Putt *et al.*, 2006).

Several methods are used to measure cell viability, each based on different principles, including cell morphology, membrane permeability and mitochondrial activity (Aras *et al.*, 2008). Cell death in CGN and PC12 cells was measured with the Trypan Blue exclusion assay. Live cells have intact cell membranes that are impermeable to Trypan Blue; dead cells have ruptured membranes that are permeable to the dye. The number of blue cells was counted manually. Cell death can also be measured by the LDH release and MTT assays. However, the trypan blue exclusion assay is more reliable because cell division does not influence the outcome, and it has the added advantage of providing morphological information about the cells.

Propidium iodide staining and subsequent fluorescent microscopy analysis would have contributed additional information about nuclear morphology.

2.3 Caspase-3 activation and detection

Several different methods are used to measure activated caspase-3 (Paroni and Brancolini, 2011). The detection of caspase activation is essential to investigate several biological processes and can be used as biochemical marker for apoptosis induced by diverse stimuli (Vaculova and Zhivotovsky, 2008). Caspase activation can be detected by measuring caspase activity, the release of cyt c, or cleaved caspases by western blot analysis (Krysko *et al.*, 2008). For these studies, a caspase-3 activity assay was used to measure cellular responses to the different apoptosis inducers PAC-1 and 1541 as well as CA-4 and its analogues. A specific caspase-3 substrate (Z-$DEVD_2$ Rhodamine 110) was used, and RFU values were measured by Bio assay readers. This method is advantageous because it is fast, and the RFU values are directly proportional to the activity inside the cells. Western blot analysis is a reliable method for detecting and characterizing specific proteins in complex extracts; however, quantification is more difficult with this method (Paper I) (Tovey *et al.*, 1987).

2.4 ROS

The vulnerability of some cells to oxidative signals is a therapeutic target for rationally designed anticancer agents. In addition to their well-characterised effects on cell division, many cytotoxic anticancer agents can induce oxidative stress by modulating levels of reactive oxygen species such as superoxide anion radical, hydrogen peroxide and hydroxyl radicals (Montero and Jassem, 2011). Thus, it is important to understand the roles of different ROS generated in response to diverse apoptosis-inducing stimuli. The ideal chemical ROS probe would be highly reactive at low concentration, specific, non-toxic, chemically well-

characterised and easy to load into the cells without unwanted diffusion, excretion or metabolism (Wardman, 2007).

2.4.1 Measurement of ROS production

ROS production in cells can be quantified by using ROS-specific probes that penetrate the cell membrane and generate a fluorescent signal upon oxidation. These studies employed dihydrorhodamine (DHR) and dihydroethidium (DHE) to measure ROS (Paper III). DHR is cell-permeant with a particular affinity for mitochondria. DHR is subsequently oxidised to fluorescent rhodamine 123, which is more specific for peroxynitrite (Wilhelm *et al.*, 2009). DHE undergoes a two-electron oxidation to form a DNA-binding fluorophore. In the case of mitochondrial generation of superoxide, DHE binds to mitochondrial DNA, and at higher concentrations, it also binds to nuclear DNA. DHE is oxidised into two fluorescent products, 2-hydroethidium, which is more specific to superoxide and ethidium (Wilhelm *et al.*, 2009). CA-4 and its triazole analogues increased intracellular oxidative stress in PC12 cells as measured by DHR and DHE fluorescence. CA-4 and its triazole analogue 2 oxidised both DHR and DHE, whereas analogue 3 only oxidised DHE, which indicates that CA-4 and its triazole analogues produce different types of ROS (paper III). MT Red CM-H_2XROS is cell-permeant, oxidised inside the mitochondria and can also be used to investigate mitochondrial ROS generation.

2.4.2. ROS scavenging

The synergistic ability of vitamins C and E to scavenge toxic ROS was studied (paper III). These scavengers reduced cell death induced by CA-4 and its analogues, which demonstrates that ROS are involved in cell death. DHE protected against CA-4 and triazole analogues 2 and 3, whereas DHR protected against CA-4 and analogue 2. These results show that vitamins as well as DHE and DHR alone or in different combinations can be used as ROS scavengers (paper III). This study also highlights the death mechanism involved.

2.5 Pharmacological inhibitors

Caspase-3 activation by PAC-1, CA-4 and triazole analogue 2 was detected with a caspase-3 substrate (Z-DEVD$_2$ Rhodamine 110), and A caspase-3 inhibitor was used as a negative control. In the cell death assay, the caspase-3 inhibitor was used to block caspase-mediated cell death by PAC-1 and 1541 as well as CA-4 and its triazole analogues 2 and 3. Most commercially available caspase substrates and inhibitors lack the specificity required to monitor individual activity; it is difficult to differentiate among caspase-3 -6 and -7 (Berger *et al.*, 2006; Pereira and Song, 2008). For this reason, an MEK inhibitor (U0126) was also used. The MEK kinases are unique in that they show very high specificity to ERK; in fact, ERK is the only substrate for MEK. Therefore, blocking MEK from phosphorylating ERK only affects ERK activation (Trujillo, 2011).

3. Discussion of Results

3.1 PAC-1, 1541, CA-4 and analogues induce cell death

Papers I and II showed that PAC-1 and 1541 induce cell death in chicken CGN and PC12 cells. These results are consistent with previous studies using different cancer cell lines (Putt *et al.*, 2006; Wolan *et al.*, 2009). PAC-1 was initially introduced as a direct activator of procaspase-3, and later an alternative mechanism was proposed that PAC-1 activates procaspase-3 into caspase-3 via the chelation of inhibitory zinc (Peterson *et al.*, 2009a). It was possible to protect against PAC-1 induced cell death in PC12 cells by adding 10 µM $ZnSO_4$ (unpublished), which supports a zinc-dependent mechanism. However, later studies showed that PAC-1 also induces cell death in MCF-7 cells that do not express procaspase-3 (Putt *et al.*, 2006; Denault *et al.*, 2007), which indicates additional mechanisms. Both EGF (paper II) and ROS scavenging (unpublished) yielded a small reduction in cell death. Low concentrations of PAC-1 increased toxicity in PC12 cells exposed for longer periods of time (paper I).

1541 was reported to be a more specific caspase-3 activator than PAC-1. It directly activates the catalytic domain of procaspase-3 and stabilises an active conformation of the enzyme (Wolan *et al.*, 2009; Zorn and Wells, 2010). However, 1541 showed results similar to those of PAC-1 in both chicken CGN and PC12 cells.

In paper III, CA-4 and its triazole analogues were studied in PC12 cells. CA-4 has been reported as a potent anticancer agent against a variety of human cancer cells including multidrug-resistant cancer cells (Nam, 2003). CA-4 and its triazole analogues 2 and 3 were toxic to PC12 cells at 1 µM and 10 µM. CA-4 and its triazole analogue 2 activated caspase-3 as shown in the caspase-3 activity assay. Similarly, treatment with a caspase-3 inhibitor

protected against CA-4- and analogue-2-induced cell death, whereas the effect of analogue 3 was not caspase-3-dependent. This finding provides evidence that CA-4 and its triazole analogue 2 are caspase activators. This caspase activation was linked to an ROS molecule that could be blocked by DHR (paper III).

3.2 CA-4 and analogue 2 induce caspase-3 and ROS-dependent cell death

CA-4 and its triazole analogue 2 induce cell death via caspase-3 activation and ROS production. The effect of CA-4 and its analogues on ROS production has previously not been well documented. DHE and DHR are specific probes used to detect superoxide and peroxynitrite, respectively (Wardman, 2007). Together with the results obtained from the slot blot analysis of the nitrotyrosination of proteins, which is a modification that is thought to result from the reaction of peroxynitrite with proteins (Ischiropoulos, 1998), the results in paper III support peroxynitrite formation following treatment with CA-4 and analogue 2. Results from ROS assays with MT Red CM-H_2XROS, which is oxidised to a fluorescent product inside the mitochondria, indicate that ROS are mainly produced in the mitochondria; these species trigger the downstream caspase cascade and cell death (unpublished).

3.3 Crosstalk between ROS production and caspase-3 cascade

ROS production may be primary or secondary to caspase-3 activation. Mitochondria-generated ROS play an important role in release of cyt c and other pro-apoptotic proteins that can trigger caspase activation and apoptosis (Ott *et al.*, 2007). Many anticancer agents function in a similar way, with effects specific to cancer cells (Orrenius *et al.*, 2007). To investigate the crosstalk between ROS and caspase cascades, we utilized different ROS probes, including DHE, DHR, MT Red CM-H_2XROS and a caspase-3 inhibitor. CA-4 and analogue 2 appear to produce toxic ROS that activate caspase-3 and cell death (Paper III).

However, the caspase-3 inhibitor reduced ROS, which suggests that signalling pathways are bidirectional.

3.4 Growth factors prevent cell death induced by PAC-1 and 1541

The results presented in Paper II show reduced PAC-1- or 1541-mediated cell death when PC12 cells are treated with EGF, and these results are further augmented by the addition of MEK inhibitor. It is known that EGF can protect against cell death through the activation of the MEK/ERK-signalling pathway, which regulates the transcription or function of apoptotic or anti-apoptotic proteins (Henson and Gibson, 2006). EGF also stimulates the proliferation of PC12 cells through the strong and transient activation of ERK (Sasagawa *et al.*, 2005). Similar results were obtained in chicken CGN. These results raise challenges to the use of direct caspase activators as anticancer therapeutic agents.

3.5 Crosstalk between the MEK/ERK signalling pathway and the caspase cascade

It has been shown that EGF reduces PAC-1-induced caspase-3 activity in PC12 cells. If PAC-1 is a direct activator of caspase- 3, then caspase-3 depends on upstream mechanisms even when it is activated directly. EGF may up-regulate IAP, which directly inhibits caspase-3 and other caspases (Deveraux *et al.*, 1999; Henson and Gibson, 2006). It is also known that EGF can down-regulate upstream caspase-9. A third possibility is that PAC-1 does not activate caspase-3 directly, which is supported by cell death in MCF-7 cells that do not express caspase-3 (Denault *et al.*, 2007). However, 1541-induced cell death was also reduced by EGF, which suggests a common mechanism.

4. Concluding remarks

The results presented in this thesis show that:

- Both PAC-1 and 1541 induce cell death in CGN and PC12 cells.
- PAC-1 is a zinc chelator in vitro and kills cells by a mechanism that is partly dependent on caspase-3. 1541 seems to have similar effects in cells but has not proven to be a more specific activator of caspase-3 than PAC-1. The crosstalk between the caspase cascade and the MEK/ERK-signalling pathway makes it difficult for the compounds to specifically interact with caspase-3.
- Agents such as PAC-1 and 1541 can be used to study apoptosis mechanisms in cell models of apoptosis regulation and as potential pharmacological treatments.
- CA-4 and its triazole analogues 2 and 3 are toxic to PC12 cells, and this toxicity is derived for ROS production and caspase-3 activation, which lead to cell death.
- PC12 and chicken CGN are useful models to study cell death induced by PAC-1, 1541 and CA-4 and its triazole analogues.

5. References

Adamo, A. M., Zago, M. P., Mackenzie, G. G., Aimo, L., Keen, C. L., Keenan, A., and Oteiza, P. I. (2010). The role of zinc in the modulation of neuronal proliferation and apoptosis. *Neurotox Res* **17**, 1-14.

Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S., and Clarke, P. R. (2003). Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. *Nat Cell Biol* **5**, 647-654.

Aras, M. A., Hartnett, K. A., and Aizenman, E. (2008). Assessment of cell viability in primary neuronal cultures. *Curr Protoc Neurosci* **Chapter 7**, Unit 7 18.

Berger, A. B., Sexton, K. B., and Bogyo, M. (2006). Commonly used caspase inhibitors designed based on substrate specificity profiles lack selectivity. *Cell Res* **16**, 961-963.

Beyersmann, D., and Haase, H. (2001). Functions of zinc in signaling, proliferation and differentiation of mammalian cells. *Biometals* **14**, 331-341.

Boatright, K. M., and Salvesen, G. S. (2003). Mechanisms of caspase activation. *Curr Opin Cell Biol* **15**, 725-731.

Chimienti, F., Seve, M., Richard, S., Mathieu, J., and Favier, A. (2001). Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. *Biochem Pharmacol* **62**, 51-62.

Chuderland, D., and Seger, R. (2005). Protein-protein interactions in the regulation of the extracellular signal-regulated kinase. *Mol Biotechnol* **29**, 57-74.

Circu, M. L., and Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* **48**, 749-762.

Crawford, E. D., and Wells, J. A. (2011). Caspase substrates and cellular remodeling. *Annu Rev Biochem* **80**, 1055-1087.

Davis, W., Jr., Ronai, Z., and Tew, K. D. (2001). Cellular thiols and reactive oxygen species in drug-induced apoptosis. *J Pharmacol Exp Ther* **296**, 1-6.

Denault, J. B., Drag, M., Salvesen, G. S., Alves, J., Heidt, A. B., Deveraux, Q., and Harris, J. L. (2007). Small molecules not direct activators of caspases. *Nat Chem Biol* **3**, 519; author reply 520.

Deveraux, Q. L., Stennicke, H. R., Salvesen, G. S., and Reed, J. C. (1999). Endogenous inhibitors of caspases. *J Clin Immunol* **19**, 388-398.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**, 495-516.

Fan, T. J., Han, L. H., Cong, R. S., and Liang, J. (2005). Caspase family proteases and apoptosis. *Acta Biochim Biophys Sin (Shanghai)* **37**, 719-727.

Galluzzi, L., Blomgren, K., and Kroemer, G. (2009). Mitochondrial membrane permeabilization in neuronal injury. *Nat Rev Neurosci* **10**, 481-494.

Henson, E. S., and Gibson, S. B. (2006). Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. *Cell Signal* **18**, 2089-2097.

Ischiropoulos, H. (1998). Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* **356**, 1-11.

Jacobs, C. M., Aden, P., Mathisen, G. H., Khuong, E., Gaarder, M., Loberg, E. M., Lomo, J., Maehlen, J., and Paulsen, R. E. (2006). Chicken cerebellar granule neurons rapidly develop excitotoxicity in culture. *J Neurosci Methods* **156**, 129-135.

Kanthou, C., and Tozer, G. M. (2002). The tumor vascular targeting agent combretastatin A-4-phosphate induces reorganization of the actin cytoskeleton and early membrane blebbing in human endothelial cells. *Blood* **99**, 2060-2069.

Kanthou, C., and Tozer, G. M. (2009). Microtubule depolymerizing vascular disrupting agents: novel therapeutic agents for oncology and other pathologies. *Int J Exp Pathol* **90**, 284-294.

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

Krysko, D. V., Vanden Berghe, T., Parthoens, E., D'Herde, K., and Vandenabeele, P. (2008). Methods for distinguishing apoptotic from necrotic cells and measuring their clearance. *Methods Enzymol* **442**, 307-341.

Kurokawa, M., and Kornbluth, S. (2009). Caspases and kinases in a death grip. *Cell* **138**, 838-854.

Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998). Signal transduction through MAP kinase cascades. *Adv Cancer Res* **74**, 49-139.

Liu, J. F., Fong, Y. C., Chang, K. W., Kuo, S. C., Chang, C. S., and Tang, C. H. (2011). FPTB, a novel CA-4 derivative, induces cell apoptosis of human chondrosarcoma cells through mitochondrial dysfunction and endoplasmic reticulum stress pathways. *J Cell Biochem* **112**, 453-462.

Lockshin, R. A., and Zakeri, Z. (2007). Cell death in health and disease. *J Cell Mol Med* **11**, 1214-1224.

Lossi, L., and Gambino, G. (2008). Apoptosis of the cerebellar neurons. *Histol Histopathol* **23**, 367-380.

Mendez, G., Policarpi, C., Cenciarelli, C., Tanzarella, C., and Antoccia, A. (2011). Role of Bim in apoptosis induced in H460 lung tumor cells by the spindle poison Combretastatin-A4. *Apoptosis* **16**, 940-949.

Montero, A. J., and Jassem, J. (2011). Cellular redox pathways as a therapeutic target in the treatment of cancer. *Drugs* **71**, 1385-1396.

Nam, N. H. (2003). Combretastatin A-4 analogues as antimitotic antitumor agents. *Curr Med Chem* **10**, 1697-1722.

Nguyen, T. T., Scimeca, J. C., Filloux, C., Peraldi, P., Carpentier, J. L., and Van Obberghen, E. (1993). Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. Distinct effects of the neurotrophic factor, nerve growth factor, and the mitogenic factor, epidermal growth factor. *J Biol Chem* **268**, 9803-9810.

Odlo, K., Fournier-Dit-Chabert, J., Ducki, S., Gani, O. A., Sylte, I., and Hansen, T. V. (2010). 1,2,3-triazole analogs of combretastatin A-4 as potential microtubule-binding agents. *Bioorg Med Chem* **18**, 6874-6885.

Odlo, K., Hentzen, J., dit Chabert, J. F., Ducki, S., Gani, O. A., Sylte, I., Skrede, M., Florenes, V. A., and Hansen, T. V. (2008). 1,5-Disubstituted 1,2,3-triazoles as cis-restricted analogues of combretastatin A-4: Synthesis, molecular modeling and evaluation as cytotoxic agents and inhibitors of tubulin. *Bioorg Med Chem* **16**, 4829-4838.

Orrenius, S., Gogvadze, V., and Zhivotovsky, B. (2007). Mitochondrial oxidative stress: implications for cell death. *Annu Rev Pharmacol Toxicol* **47**, 143-183.

Ott, M., Gogvadze, V., Orrenius, S., and Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis* **12**, 913-922.

Paroni, G., and Brancolini, C. (2011). Measurement of caspase activity: from cell populations to individual cells. *Methods Mol Biol* **740**, 65-79.

Pecina-Slaus, N. (2009). [Genetic and molecular insights into apoptosis]. *Acta Med Croatica* **63 Suppl 2**, 13-19.

Penaloza, C., Lin, L., Lockshin, R. A., and Zakeri, Z. (2006). Cell death in development: shaping the embryo. *Histochem Cell Biol* **126**, 149-158.

Pereira, N. A., and Song, Z. (2008). Some commonly used caspase substrates and inhibitors lack the specificity required to monitor individual caspase activity. *Biochem Biophys Res Commun* **377**, 873-877.

Perkins, G., Bossy-Wetzel, E., and Ellisman, M. H. (2009). New insights into mitochondrial structure during cell death. *Exp Neurol* **218**, 183-192.

Peterson, Q. P., Goode, D. R., West, D. C., Ramsey, K. N., Lee, J. J., and Hergenrother, P. J. (2009a). PAC-1 activates procaspase-3 in vitro through relief of zinc-mediated inhibition. *J Mol Biol* **388**, 144-158.

Peterson, Q. P., Hsu, D. C., Goode, D. R., Novotny, C. J., Totten, R. K., and Hergenrother, P. J. (2009b). Procaspase-3 activation as an anti-cancer strategy: structure-activity relationship of procaspase-activating compound 1 (PAC-1) and its cellular co-localization with caspase-3. *Journal of medicinal chemistry* **52**, 5721-5731.

Peterson, Q. P., Hsu, D. C., Novotny, C. J., West, D. C., Kim, D., Schmit, J. M., Dirikolu, L., Hergenrother, P. J., and Fan, T. M. (2010). Discovery and canine preclinical assessment of a nontoxic procaspase-3-activating compound. *Cancer Res* **70**, 7232-7241.

Pop, C., and Salvesen, G. S. (2009). Human caspases: activation, specificity, and regulation. *J Biol Chem* **284**, 21777-21781.

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

Putt, K. S., Chen, G. W., Pearson, J. M., Sandhorst, J. S., Hoagland, M. S., Kwon, J. T., Hwang, S. K., Jin, H., Churchwell, M. I., Cho, M. H., Doerge, D. R., Helferich, W. G., and Hergenrother, P. J. (2006). Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. *Nat Chem Biol* **2**, 543-550.

Sasagawa, S., Ozaki, Y., Fujita, K., and Kuroda, S. (2005). Prediction and validation of the distinct dynamics of transient and sustained ERK activation. *Nat Cell Biol* **7**, 365-373.

Schultz, D. R., and Harrington, W. J., Jr. (2003). Apoptosis: programmed cell death at a molecular level. *Semin Arthritis Rheum* **32**, 345-369.

Seger, R., and Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB J* **9**, 726-735.

Shaul, Y. D., and Seger, R. (2007). The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim Biophys Acta* **1773**, 1213-1226.

Slagsvold, H. H., Rosseland, C. M., Jacobs, C., Khuong, E., Kristoffersen, N., Gaarder, M., Fallgren, A. B., Huitfeldt, H. S., and Paulsen, R. E. (2003). High molecular weight DNA fragments are processed by caspase sensitive or caspase independent pathways in cultures of cerebellar granule neurons. *Brain Res* **984**, 111-121.

Tovey, E. R., Ford, S. A., and Baldo, B. A. (1987). Protein blotting on nitrocellulose: some important aspects of the resolution and detection of antigens in complex extracts. *J Biochem Biophys Methods* **14**, 1-17.

Trujillo, J. I. (2011). MEK inhibitors: a patent review 2008 - 2010. *Expert Opin Ther Pat* **21**, 1045-1069.

Vaculova, A., and Zhivotovsky, B. (2008). Caspases: determination of their activities in apoptotic cells. *Methods Enzymol* **442**, 157-181.

Vaudry, D., Stork, P. J., Lazarovici, P., and Eiden, L. E. (2002). Signaling pathways for PC12 cell differentiation: making the right connections. *Science* **296**, 1648-1649.

Vermeulen, K., Van Bockstaele, D. R., and Berneman, Z. N. (2005). Apoptosis: mechanisms and relevance in cancer. *Ann Hematol* **84**, 627-639.

Wardman, P. (2007). Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free Radic Biol Med* **43**, 995-1022.

Wilhelm, J., Vytasek, R., Ostadalova, I., and Vajner, L. (2009). Evaluation of different methods detecting intracellular generation of free radicals. *Mol Cell Biochem* **328**, 167-176.

Wolan, D. W., Zorn, J. A., Gray, D. C., and Wells, J. A. (2009). Small-molecule activators of a proenzyme. *Science* **326**, 853-858.

Zimmermann, K. C., Bonzon, C., and Green, D. R. (2001). The machinery of programmed cell death. *Pharmacol Ther* **92**, 57-70.

Zorn, J. A., and Wells, J. A. (2010). Turning enzymes ON with small molecules. *Nat Chem Biol* **6**, 179-188.

6. Papers I, II and III

I

II

III