The role of mitogen-activated protein kinases in the regulation of apoptosis

Dissertation
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Abbreviations

\( \alpha \)  anti
ATP / dATP  adenosine 5'-triphosphate / desoxyadenosine 5'-triphosphate
cAMP  adenosine 3':5'-cyclic monophosphate
cdk  cyclin-dependent kinase
cDNA  complementary DNA
CMV  cytomegalo virus
CREB  cAMP-responsive element binding protein
DAPI  4',6'-diamidine-2'-phenylindole
DISC  death-inducing signalling complex
dn  dominant negative
DNA  deoxyribonucleic acid
dNTP  deoxynucleoside 5'-triphosphate
ECL  enhanced chemiluminescence
EF  elongation factor
EGF  epidermal growth factor
EGFP  enhanced green fluorescent protein
ERK  extracellular signal-regulated kinase
ES  embryonic stem cell
FACS  fluorescence-assisted cell sorting
FADD  Fas-associated protein with death domain
FAK  focal adhesion kinase
FBS  fetal bovine serum
g  acceleration due to gravity
GDP / GTP  guanosine 5'-diphosphate / guanosine 5'-triphosphate
GFP  green fluorescent protein
HRP  horseradish peroxidase
hTERT  human telomerase reverse transcriptase
IAP  inhibitor of apoptosis protein
IP  immunoprecipitation
IPTG  isopropyl \( \beta \)-D-thiogalactopyranoside
JNK  Jun N-terminal kinase
MAP kinase/MAPK  mitogen-activated protein kinase
MEF  mouse embryonic fibroblast
MEK  MAPK/ERK kinase
MEKK  MEK kinase
MKK  MAPK/ERK kinase
NF\( \kappa \)B  nuclear factor \( \kappa \)B
ON  oligonucleotide
ONC  overnight culture
ONPG  o-nitrophenyl \( \beta \)-D-galactopyranoside
PAGE  polyacrylamide gel electrophoresis
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P-ERK</td>
<td>phosphorylated ERK</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphatidylinositol triphosphate kinase</td>
</tr>
<tr>
<td>P-JNK</td>
<td>phosphorylated JNK</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B (Akt)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>P-MKK</td>
<td>phosphorylated MKK4</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>P-p38</td>
<td>phosphorylated p38</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RBD</td>
<td>Ras-binding domain</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor associated protein with death domain</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl β-D-galactopyranoside</td>
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1 Introduction

1.1 Apoptosis

Each day, approximately 50 to 70 billion of cells in the average adult human die by apoptosis. This cell death is necessary to make room for the billions of new cells the body generates in the same time. Over a period of a year, the flux of newly produced cells and those eradicated by apoptosis equals nearly our entire body weight. Despite this, the concept that cell death by apoptosis is a controlled cellular decision and an important mechanism for the maintenance of tissue homeostasis in adults is only about 20 years old [1].

1.1.1 Activation of the apoptotic machinery

Originally, the definition of apoptosis was by morphological criteria. Cells undergoing apoptosis exhibit characteristic changes including cytoplasmatic shrinkage, chromatin condensation, membrane blebbing without the loss of membrane integrity, and finally the fragmentation into membrane-enclosed apoptotic bodies [1, 2]. At the cell surface, apoptotic cells externalise phosphatidylserine, which together with other co-factors promotes the recognition and removal of the dead cells by macrophages and other phagocytes [3, 4]. On molecular level, apoptosis is accompanied by the degradation of chromosomal DNA into oligonucleosomal fragments [5, 6] and by the proteolytic cleavage of specific proteins [7-9]. This cleavage is catalysed by a specialised family of cysteine-dependent aspartate-directed proteases termed caspases, whose activation is the major biochemical event that defines apoptosis.

By 1999, 14 mammalian caspases had been described; seven of those have been shown to be involved in the regulation and execution of apoptosis (reviewed in [10, 11]). All apoptotic caspases exist in healthy cells as inactive zymogens (procaspases), which have a very low intrinsic enzymatic activity. The fully active enzymes are heterotetramers composed of two identical small and large subunits [12], which are produced from procaspases by caspase-mediated cleavage. Apoptotic caspases can be divided into two subgroups: the initiator caspases 2, 8, 9 and 10 are activated by self-processing and subsequently set off a proteolytic cascade by cleaving and activating the effector or executioner caspases 3, 6 and 7 [13]. As by today, two major pathways leading to the activation of caspases have been identified (Fig. 1.1): the mitochondrial pathway and the DISC.
The mitochondrial pathway is initiated by the release of cytochrome C from the mitochondria into the cytosol (Fig. 1.1 right) [14]. There, it can form multimeric complexes with Apaf-1, this process requires energy in form of ATP or dATP [15]. The complex then recruits procaspase-9 in an approximate 1:1 ratio to Apaf-1 and procaspase-9 becomes activated by proteolysis through autocatalysis [15]. Active caspase-9 is subsequently released from the complex to cleave and activate the downstream effector caspases 3, 6 and 7. The other pathway that leads to caspase activation is initiated by cell surface death receptors like Fas (Apo-1/CD95), TNFR or TRAIL receptors and their specific ligands (Fig. 1.1 left, reviewed in [16]). Binding of their respective ligands induces trimerisation of the receptors, which leads to the recruitment of adaptor molecules like FADD (Fas-associating protein with death domain) or TRADD (TNF-receptor associated death domain) (reviewed in [17]). The complex of death receptor, ligand and adaptor molecules, which is termed the death-inducing signalling complex (DISC), is providing binding sites for the upstream procaspases 2, 8 or 10 (reviewed in [18]). This results in an oligomerisation of procaspases at the DISC, which allows the proteolytical self-activation by their own low intrinsic enzymatic activities [19]. In type I cells caspase-8 now activates the effector caspases directly. In type II cells, the level of caspase-8 is not sufficient to effectively
activate downstream caspases [20]. In these cells, caspase-8 cleaves and activates Bid, a pro-apoptotic member of the Bcl-2 family, that can then promote the release of cytochrome C and thereby induce the caspase cascade via the mitochondrial pathway (Fig. 1.1) [21, 22].

1.1.2 The consequences of caspase activation

Caspases are selective enzymes. Though some of their substrates might be “innocent bystanders” that happen to contain a caspase cleavage site by chance, in a number of cases cleavage by caspases has been shown to inactivate proteins that normally promote survival or to process inactive precursor proteins into death-promoting enzymes (reviewed in [10]).

First of all, caspases are their own targets. As mentioned earlier, caspases are generated from inactive zymogens by proteolysis. With the exception of granzyme B [23], the only mammalian enzymes that can activate caspases are caspases themselves.

Moreover, caspases trigger the disassembly of the nucleus. The cleavage of chromosomal DNA into oligonucleosomal fragments is mediated by the nuclease CAD/DFF40 [24, 25]. CAD is normally sequestered in an inactive state by interaction with the inhibitory protein ICAP/DFF45. Cleavage of ICAD by caspase-3 releases the active nuclease [26, 27]. Structural proteins of the nucleus, including lamins, are also cleaved during apoptosis (reviewed in [28]).

Caspases also disrupt cytoskeleton-associated survival pathways (reviewed in [29]). Cells monitor the attachment of the cytoskeleton through focal adhesions to the integrins and the extracellular matrix. Cytoskeletal structures are disrupted during apoptosis by cleavage of the structural proteins actin, α-fodrin, paxillin and cytokeratin 18 [10]. Moreover, the cleavage products of gelsolin and p120GAP actively promote disruption of the actin cytoskeleton, cell rounding and detachment from the matrix [30-32].

In addition to this, protein kinases involved in survival signalling are targeted by caspases. FAK, PKB (Akt) and Raf-1 are cleaved and inactivated [9, 33]. Moreover, protein phosphatase 2A (PP2A), a MAP kinase phosphatase crucial for the regulation of cell growth and division [34], is activated by caspase-3 [35].
Finally, caspases activate death-promoting signal transduction pathways. They support the activation of cyclin dependent kinases (cdk) by three different mechanisms [10]: they cleave the inhibitory kinase WEE1, they inactivate the cyclosome that triggers the degradation of cyclins and they degrade the cdk inhibitors p21 and p27. However, the consequences of cdk activation on apoptosis are still unknown. Moreover, certain PKC family members become cleaved during apoptosis. Expression of the activated fragments of PKCδ and PKCζ can induce apoptosis, suggesting that these fragments might mediate some of the effects of caspase activation [36, 37]. At last, caspases can cleave and constitutively activate MEKK1 [38-40], p21-activated kinase 2 (PAK2) [41, 42] and Mst1 [43, 44], which are all upstream kinases of the JNK pathway. The constitutively active forms of these kinases have been shown to be pro-apoptotic while their kinase-dead counterparts can inhibit apoptosis.

Taken together, caspases promote the activation of the JNK pathway by constitutively activating upstream kinases, whereas as discussed above the ERK pathway is disrupted through the cleavage its upstream kinase Raf-1 and the activation of phosphatases like PP2A.

1.1.3 **The regulation of apoptosis**

One of the key players in the regulation of apoptosis is the Bcl-2 family of proteins, which controls the release of cytochrome C from the mitochondria (reviewed in [45, 46]). Currently, 19 mammalian family members have been discovered [46], they include both pro- as well as anti-apoptotic proteins. One of their characteristics is their ability to form homo- or heterodimers, suggesting a neutralizing competition between pro- and anti-apoptotic family members. Indeed, the ratio between these two partially determines a cell’s susceptibility to apoptotic signals [47]. In healthy cells anti-apoptotic Bcl-2 proteins are mainly integral membrane proteins at the mitochondria, the endoplasmatic reticulum and the nuclear envelope. In contrast, pro-apoptotic members mostly localise to the cytosol or the cytoskeleton. Following an apoptotic signal, they undergo conformational changes that allow them to integrate into membranes, especially the mitochondrial membrane. In most cases it is still unclear how exactly apoptotic signals are transmitted to the pro-apoptotic Bcl-2 proteins; phosphorylation, proteolytic processing and transcriptional regulation are involved. For instance, phosphorylated Bad is sequestered in the cytosol by association with 14-3-3 proteins. Deprivation of survival signals leads to the inactivation of PKB (Akt), the main Bad kinase, and unphosphorylated Bad localises to the mitochondrial membrane (Fig. 1.1) [48, 49]. The pro-apoptotic, cytosolic Bid is a substrate of caspase-8, which is activated
by death receptors (see before and Fig. 1.1). Truncated Bid (tBid) translocates to the mitochondria and inserts into the outer membrane [21, 22]. Another family member, Bim is associated to microtubules via the dynein light chain in the absence of death signals and released to translocate to the mitochondria upon growth factor deprivation or UV irradiation [50]. How in detail pro-apoptotic family members mediate the release of cytochrome C from the mitochondria is not fully understood yet. One model suggests, that pro-apoptotic Bcl-2 proteins regulate a permeability transition pore in the mitochondrial membrane. According to another model, some pro-apoptotic family members like Bax and Bak can themselves form multimeric pores. Indeed, it has recently been shown that *in vitro* recombinant Bax can form pores in liposomes that are permeable for cytochrome C [51]. In both cases, the release of cytochrome C from the mitochondria is a consequence of the accumulation of multimeric complexes composed of pro-apoptotic family members in the mitochondrial membrane. Anti-apoptotic family members block this by sequestering pro-apoptotic members from these complexes [52].

Bel-2 proteins play an important role in the regulation of caspase activation, but caspases can also be controlled downstream of their proteolytic processing by a group of proteins termed IAPs (inhibitor of apoptosis proteins). By 1999, five mammalian IAPs had been discovered (reviewed in [11]). They inhibit apoptosis by binding to activated caspases, thereby blocking their enzymatic function and probably targeting them for degradation (reviewed in [53]). Another class of caspase inhibitors are the FLIPs (FLICE inhibitory proteins). These proteins have homology to procaspase-8 and 10 but do not possess any detectable enzymatic activity. Their mechanism of action is not completely understood yet but they are thought to interfere with the recruitment of initiator procaspases to the DISC during death receptor-mediated apoptosis.

### 1.1.4 Apoptosis and cancer

Cancer is a disease, which reflects defects in the regulation of signalling pathways that control cell proliferation and homeostasis. Tumorigenesis in humans is a multi-step process of subsequent genetic alterations (reviewed in [54]). These alterations lead to the malignant, transformed phenotype, which is defined by self-sufficiency in growth signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis as well as insensitivity to anti-growth signals and evasion of apoptosis (reviewed in [55]).

The expansion of a tumour cell population is not only determined by the rate of cell proliferation but also by the rate of cell death. Indeed, a number of oncogenes have been
shown to have pro-apoptotic activities [56]. Examples for this are c-Myc, the transcription factor E2F, the viral oncogene E1A, loss of the tumour suppressor Rb (reviewed in [57]) and also the v-Jun oncogene [58]. The mechanism by which oncogenes can induce apoptosis is still focus of intensive research. The dual signalling model suggests that growth and apoptosis are coupled processes and that activation of cell proliferation necessarily primes a cell for apoptosis unless appropriate survival signals are present (reviewed in [59]). According to this model, proliferation itself is an apoptotic stimulus and deregulated proliferation results in apoptosis. Though cancer cells have found a way of escaping to respond to this stimulus, the deregulation of proliferation still sensitises them to a wide range of mechanistically different triggers of apoptosis, including death receptor mediated pathways, interferons and hypoxia, and also protein synthesis inhibitors, nutrient deprivation and DNA damage. The latter mechanism is targeted by DNA-damaging chemotherapeutic drugs. Chemotherapeutic drugs act by inducing apoptosis in cancer cells (reviewed in [60, 61]). Chemotherapeutic drugs do exhibit cytotoxic effects on non-transformed cells as well, but since the deregulation of proliferation renders cancer cells hypersensitive to apoptosis, non-transformed cells can survive therapeutic concentrations of these anti-cancer drugs [57].

1.2 MAP kinase pathways

Mitogen-activated protein (MAP) kinase pathways are signal-transducing protein phosphorylation cascades, which become activated in response to a remarkably diverse range of stimuli including growth factors, cytokines, irradiation and osmotic stress (reviewed in [62, 63]). The core component of each MAP kinase pathway is a module of three kinases, that are activated by each other in a series of subsequent phosphorylations. By today, four major MAP kinase pathways have been described in mammals (Fig. 1.2).

1.2.1 Major mammalian MAP kinase pathways

The best characterised MAP kinase signalling pathway is the ERK pathway. The main stimuli of this pathway are growth and differentiation factors, which lead to the activation of the small GTP-binding protein Ras. Ras is activated by nucleotide exchange factors that stimulate the release of Ras-bound GDP and thereby promote its exchange for GTP. Ras in its GTP-bound form binds to a range of effector molecules including PI3-kinase, Ral-GDS and Raf kinases (reviewed in [64, 65]).
The binding to Ras-GTP at the plasma membrane allows the activation of Raf kinases, which is a multi-step process involving several protein phosphorylation and dephosphorylation events (reviewed in [66]). To date, the only known substrate of Raf kinases is MAPK/ERK kinase (MEK). MEK1 and MEK2 are activated by Raf and subsequently phosphorylate and activate ERK1 and ERK2. Other activators of MEK are MEK kinases (MEKK) 1 to 3, but little is known about their regulation (reviewed in [67]). The substrates of the ERKs include cytosolic proteins like S6 kinase (p90Rsk), phospholipase A2 and microtubule-associated proteins [68]. Moreover, ERKs are able to phosphorylate upstream components of the Ras/ERK signalling pathway, including the EGF receptor, the exchange factor Sos, Raf and MEK, leading to their inactivation. This is thought to provide a negative feedback loop [63]. Activated ERK can also translocate to the nucleus and phosphorylate different transcription factors including members of the AP-1 family (c-Jun, c-Fos, ATF-2), the TCF family (Elk-1, SAP1, SAP2), c-Myc and STATs (signal transducers and activators of transcription) [63, 68].

JNK was identified in 1994 as a new MAP kinase that was activated by cell stress and phosphorylated the N-terminal activating sites of c-Jun rather than the C-terminal inhibitory sites targeted by ERK [69, 70]. To date, three distinct JNK genes have been
discovered which can be differentially spliced, resulting in a total of 10 JNK isoforms [71]. JNKs are mainly activated by different forms of cell stress, including heat shock, DNA damage, oxidative and osmotic stress as well as by inflammatory cytokines [72]. Despite being the focus of intensive research, little is known about how cell stress triggers the initiation of the JNK signal transduction cascade. It has been shown that UV light and osmotic stress induce the multimerisation and clustering of cell surface receptors for EGF, TNFα and IL-1 [73]. This at least partially mediates JNK activation in response to these stimuli. But how UV irradiation and osmotic stress can lead to the activation of these receptors and if other stresses utilise the same pathways is still unknown. Unlike the ERK cascade, the upstream components of the JNK signalling pathway are less well defined. JNKs are activated by dual phosphorylation by MKK4 or MKK7 and a large number of MKK4/7 kinases have been identified. These include the MEKK family (MEKK1-4), Ask1, Tak1, Tpl2 and others (reviewed in [74]). Thus far, all JNK substrates identified are exclusively transcription factors, even though a large fraction of JNK remains cytoplasmatic after activation. JNK substrates include c-Jun, ATF-2, Elk and p53 (reviewed in [63]).

The p38 family of MAP kinases consists of four homologous proteins p38α-δ, which are activated by cellular stress and inflammatory cytokines, much the same stimuli that activate the JNK cascade. The direct upstream kinases of the p38 cascade are MKK3 and MKK6. Like for the JNK pathway, a number of MKK3/6 kinases have been found. These include the MEKKs 2 and 3, PAK and Ask1. All these kinases also activate JNK signalling and so far no specific activator of exclusively the p38 pathway has been identified. p38 substrates are cytoplasmatic proteins like MAP kinase activated protein (MAPKAP) kinase 2 and 3, which phosphorylate small heat shock proteins, and transcription factors like ATF-2, Elk1, Chop and Max [63].

The MAP kinase ERK5 (big MAP kinase, BMK) is activated by oxidative stress and hyperosmolarity but also by serum [75, 76]. Its direct upstream activator is MKK5, MEKK2 and MEKK3 have been implicated in the activation of MKK5 [77-79]. ERK5 activates the transcription factor MEF2, which induces the expression of c-Jun [76].

1.2.2 MAP kinase pathways and apoptosis

The first study implicating a role of MAP kinases in the regulation of apoptosis and survival was carried out in neuronal cells [80]. Xia and colleagues showed that in differentiated PC12 cells – a model system for sympathetic neurones [81, 82] – which are dependent on NGF for survival, JNK and p38 became activated during apoptosis.
upon NGF withdrawal. Expression of a constitutively active form of MEKK1, that activated JNK and p38 in these cells, could also trigger apoptosis. Apoptosis induced by either stimuli could be inhibited by the expression of dominant negative components of the JNK or p38 cascades. Moreover, NGF withdrawal resulted in a deactivation of ERK. This inactivation and apoptosis could be prevented by stimulation of the cells with forskolin (an inhibitor of cAMP phosphodiesterase and thereby an indirect activator of protein kinase A), growth factors like FGF or insulin or by the expression of a constitutively active form of MEK.

Later on, MEKK1 and JNK signalling have been shown to be involved in apoptosis regulation in non-neuronal cells. In HeLa cells a dominant negative form of the JNK activator MKK4 blocked apoptosis caused by oxidative stress, while inhibition of the ERK pathway enhanced cell death under these conditions [83]. In another human cancer cell line, A431 cells, sub-lethal expression levels of a constitutively active form of MEKK1 sensitised the cells towards apoptosis induced by a variety of stimuli [84].

From these and other studies [85, 86] a model emerged according to which the fate of the cell – survival or apoptosis – is determined by the balance of pro- and anti-apoptotic MAP kinase signalling pathways (Fig.1.3).

However, the role of MAP kinase signalling in apoptosis is controversial. A variety of reports suggest that, depending on cell type and stimulus, JNK activation is not involved in apoptosis induction or can even play a protective role. An example for this is Fas-mediated apoptosis. In neuroblastoma cells dominant negative components of the JNK pathway could inhibit apoptosis, which was also inhibited by dominant negative ERK [87]. MKK4-deficient thymocytes, which fail to activate JNK in response to Fas stimulation, have been shown to be more susceptible to apoptosis [88] while in Jurkat T cells blocking JNK activation had no effect on Fas-mediated apoptosis [89].

p38 has also been reported to protect cells from apoptosis under some conditions [90]. Moreover, it has been shown that ERK activity can promote apoptosis in response to certain chemotherapeutic agents [91] and that the effects are dependent on whether ERK is inhibited before or after the induction of apoptosis [92].
1.3 MEKK1

Mitogen-activated protein kinase kinase (MEK) kinase 1 (MEKK1) is a 195 kDa serine threonine protein kinase that specifically activates the JNK and the ERK pathways [93-95]. It had originally been cloned from a mouse cDNA library as a 73 kDa protein with homology to the yeast Ste11 and Byr2 genes [96] and subsequently been shown to associate with Ras and phosphorylate MEK in a Ras-dependent manner [96-98]. Later it has been revealed that full-length MEKK1 is a membrane-associated 195 kDa protein with a large regulatory, N-terminal domain [93] (Fig. 1.4).
Tritration experiments in vivo and in vitro have shown that MEKK1 preferentially phosphorylates the JNK activator MKK4 (SEK) compared to MEK [93, 99, 100]. Frequently MEKK1 overexpression has been reported to also induce the activation of p38 [93, 101, 102]. However, more recently, targeted gene disruption in different cell systems demonstrated that endogenous MEKK1 does not activate the p38 pathway [94, 95, 103],

MEKK1 becomes activated by a variety of stimuli, but the mechanisms of activation are widely unknown. As for most protein kinases, MEKK1 activity seems to be modulated by phosphorylation [104]. It has been reported that MEKK1 is phosphorylated at residues within the kinase domain [105], but since these sites are also targets of autophosphorylation it is still unclear whether they play a role in the regulation of MEKK1. A variety of upstream kinases and small G-proteins have been implicated in the activation of MEKK1. These include the Ras [106, 107] and Rho families [108, 109] as well as heterotrimeric G proteins [110, 111]. Furthermore, haematopoietic progenitor kinase 1 (HPK1), Nck interacting kinase (NIK) and GCK-like kinase (GLK) have been shown to associate with and phosphorylate MEKK1, which suggests they may be involved in the regulation of MEKK1 activity [112-114].

Another mechanism for MEKK1 activation is proteolytic processing by caspases. This cleavage at Asp874 is mediated by caspase-3 and has been reported to occur during anoikis (apoptosis of epithelia cells after detachment from the extracellular matrix), after Fas ligation, UV-C irradiation and stimulation with TNF\(\alpha\) [9, 38, 39, 115]. During anoikis, a cleavage-resistant form of MEKK1 could block apoptosis. Intriguingly, the activation of caspases and the cleavage of MEKK1 depended on MEKK1 kinase activity. This suggests that MEKK1 induces caspase activity, which then in turn leads to a further activation of MEKK1, providing a positive feedback loop for apoptotic

**Figure 1.4 Schematic illustration of MEKK1.** The large N-terminus of the protein contains regulatory PH domains that mediate interaction with membranes and a proline-rich region that can associate with SH3 domains.
signalling. Cleavage of MEKK at Asp874 not only activated the kinase but also caused
the redistribution of the protein from a particulate to a diffuse cytosolic localisation
[39]. A more recent study could show that targeting of the 91 kDa MEKK1 cleavage
fragment to the plasma membrane does reduce its potential to induce apoptosis [116].
Interestingly, while expression of full-length MEKK1 and the 91 kDa fragment as well
as their membrane-targeted counterparts induced similar levels of JNK activation, only
the full-length MEKK1 proteins were capable of significantly activating ERK in this
study.

The kinase domain of MEKK1 is highly pro-apoptotic. It has been shown to induce
apoptosis in PC12 cells, Swiss 3T3 cells as well as rat embryonic fibroblasts [80, 85],
and to sensitise A431 cells towards apoptosis induced by a variety of stimuli [84]. This
is dependent on MEKK1 kinase activity. While most studies link the apoptotic potential
of MEKK1 to its ability to activate the JNK pathway, some reports dispute the
correlation between MAP kinase activation and apoptosis induction by MEKK1 [85,
117]. Furthermore, MEKK1 is required for apoptosis following DNA damage and
detachment from the extracellular matrix [38, 40]. Conversely, full-length MEKK1 has
also been implicated in mediating anti-apoptotic responses. MEKK1-deficient
embryonic stem cells, which fail to activate JNK in response to microtubule-disrupting
drugs, are more susceptible towards apoptosis induced by these stimuli [94]. Cardiac
myocytes derived from these cells are highly sensitive to oxidative stress [103].
Contrary to this, in the chicken bursal B-cell line DT40 targeted disruption of the
MEKK1 gene renders the cells more resistant to apoptosis induced by microtubule-
disrupting agents, indicating that the function of MEKK1 signalling – pro- or anti-
apoptotic – does not only depend on the stimulus but also on the cell type [95].

In addition to activating the JNK pathway, MEKK1 has been reported to activate the
transcription factor NFκB by phosphorylating and activating both IκBα and IκBβ
kinases [118]. NFκB transcription factors are homo- or heterodimers of closely related
family members that are maintained in the cytoplasm via the inhibitory subunit IκB.
The phosphorylation of IκB by IκB kinases induces the proteolytic degradation of the
inhibitor protein, releasing NFκB to translocate to the nucleus where it can fulfil its
tasks as a transcriptional regulator. (See [119, 120] for recent reviews on NFκB function
and signalling). However, MEKK1 knockout mouse embryonic fibroblasts and mouse
embryonic stem cells do not show any alteration in the activation of NFκB in response
Introduction

- 19 -

to pro-inflammatory stimuli, suggesting that MEKK1 might be dispensable for the activation of NFκB [121, 122].

1.4 Aim of this study

We have investigated whether the targeted modulation of MAP kinase pathways could be employed to induce apoptosis in human cancer cells. To eliminate cell type specific effects we used a range of cell lines including breast, cervix and squamous carcinoma and neuroblastoma cells. To activate the JNK pathway we expressed a constitutively active form of human MEKK1 (Fig. 1.5). To block ERK activation we inhibited Raf signalling by either expression of the Ras-binding domain (RBD) or the catalytic fragment of protein kinase A (PKA) to inhibit Raf-1. For the simultaneous activation of the JNK pathway and inhibition of Ras signalling we cloned two RBD-MEKK1 fusion proteins. RBD3-MEKK consists of the minimal Ras-binding domain of Raf-1 fused N-terminally to the catalytic domain of MEKK1. A second fusion protein, RBD1-MEKK contains additional residues of the Raf-1 regulatory domain, which have been shown to modulate and increase the Ras-Raf interaction [123].

In addition, we have investigated the consequences of modulating the activity of MAP kinases on the apoptotic action of chemotherapeutic drugs. We used three different human cancer cell lines, that represent common cancers and different transforming principles. A431 is a squamous carcinoma cell line that over-expresses the EGF receptor [124] and due to this fact features a constitutive activation of receptor dependent signalling processes. HeLa is derived from a cervical carcinoma, which is infected with papilloma virus and expresses E6 and E7, viral proteins that neutralise the Rb and p53 tumour suppressor proteins [125, 126]. MCF7 is an estrogene responsive mammary carcinoma cell line that overexpresses the anti-apoptotic Bcl-2 protein [127]. The following compounds were used: Taxol, an agent that targets microtubules and is one of the few chemotherapeutic drugs effective against a wide range of solid tumours [128]. Etoposide, a well established chemotherapeutic drug that induces DNA damage by inhibiting topoisomerase II [129]. Ceramide is a synthetic lipid that has not found therapeutical application, but it is a potent apoptosis-inducing substance that has been described as a second messenger of TNF and other apoptosis-inducing stimuli [130].
Figure 1.5 Induction of apoptosis by modulation of MAP kinase pathways. The balance between the pathways can be tipped by activating pro-apoptotic pathways (left), by inactivating survival pathways (middle) or by combination of these two (right).
2 Materials and Methods

2.1 Molecular Biology Methods

2.1.1 Frequently used reagents in molecular biology methods

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBE buffer</td>
<td>89 mM Tris, 89 mM boric acid, 2.5 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>LB medium 10 g/l Peptone 140 (Invitrogen)</td>
</tr>
<tr>
<td></td>
<td>5 g/l yeast extract (Difco)</td>
</tr>
<tr>
<td></td>
<td>10 g/l NaCl</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris-HCl pH 8.0, 1 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>LB-agar 1.5% Agar in LB</td>
</tr>
</tbody>
</table>

2.1.2 Transformation of E. coli

Competent *E. coli* DH5α were purchased from Stratagene (library efficiency) or kindly provided by Dr. Reynaldo Garcia. Transformations were carried out with 2 µl of a ligation reaction exactly as described in the protocol provided by Stratagene except that 1.5 ml microcentrifuge tubes were used instead of Falcon tubes. For re-transformation of plasmids, 20 µl competent cells were transformed with 50 to 100 ng DNA. After the heat shock, 80 µl LB were added to the mix and the cells were directly spread on LB-agar plates containing an antibiotic (100 µg/ml ampicillin or 25 µg/ml kanamycin).

2.1.3 Preparation of glycerol stocks

Glycerol stocks allow the long-term storage of bacteria clones. 0.5 ml of a liquid overnight culture (ONC) were mixed with 0.5 ml sterile glycerol and immediately frozen at -80°C.

2.1.4 Small scale preparation of plasmid DNA (minipreps)

For restriction analysis, DNA from a 1.5 ml ONC was isolated according to the alkaline lysis method ([131], p. 1.6.1-1.6.2). In the final step, the nucleic acid pellets were dissolved in TE buffer containing 0.1 µg/ml RNaseA.

2.1.5 Preparation of plasmid DNA for the transfection of eukaryontic cells

For transfection of eukaryontic cells, DNA was purified using QIAGEN or QIAfilter Plasmid Maxi Kits (Qiagen) according to the manufacturer’s instructions. Briefly, DNA from 250 ml ONC was purified on one QIAGEN-tip 500. The DNA was resuspended in TE buffer and its concentration assayed photometrically using the approximation that an absorption of one at 260 nm equals 50 µg/ml DNA.
2.1.6 Preparation of plasmid DNA for sequencing
For sequencing, DNA was purified with QIAprep® Spin Miniprep Kits according to the manufacturer’s instructions.

2.1.7 Sequencing of DNA
The Big Dye Terminator Sequencing Kit was used according to the manufacturer’s instructions. Briefly, PCR reactions were carried out with 200 to 500 ng plasmid DNA and 3.2 pmol of primer in a final volume of 10 µl. Samples were then analysed by the Beatson Molecular Technology Services on an ABI373A sequencer.

2.1.8 Isolation of DNA fragments from agarose gels
To isolate DNA from agarose gels we used the Agarose Gel DNA Extraction Kit (Roche) or the QIAquick™ Gel Extraction Kit (Qiagen) according to the respective manual.

2.1.9 Ligation of DNA fragments
For ligation reactions, we used the Rapid DNA Ligation Kit (Roche) according to the supplied protocol. Briefly, 200 ng of DNA fragments were ligated for 5 to 20 min at RT with a ratio of vector to insert DNA of about 1:3 to 1:5. If the vector DNA had self-compatible ends, it was dephosphorylated before the ligation with shrimp alkaline phosphatase (Roche) according to the manufacturer’s instructions. Oligonucleotide adaptors were used in an approximately 1000-fold excess (for the preparation of oligonucleotide adaptors see 2.1.10). 2 µl of a ligation reaction were used for transformations.

2.1.10 Preparation of oligonucleotide adaptors
Equal molar amounts of oligonucleotides were mixed in TE buffer and boiled in a water bath for 5 min. To let the DNA hybridise, the mixture was then allowed to slowly cool down to RT in the water bath.

2.1.11 Restriction analysis of DNA
Restriction reactions were carried out according to Ausubel et al. ([131], p. 3.1.3, Alternate protocol 2) using the reaction buffers provided by the enzyme’s supplier. Eco47III was purchased from Roche; all other restriction endonucleases were from Invitrogen or New England Biolabs.
2.1.12 **DNA agarose gel electrophoresis**

DNA agarose gel electrophoresis was carried out according to standard protocols ([131], p. 2.5A.1-2.5A.9) using TBE buffer.

2.1.13 **Polymerase chain reaction (PCR)**

PCR reactions were carried out using the Ready Load Master Mix (Advanced Biotechnologies) according to the product data sheet. This mix contains all four dNTPs as well as a polymerase enzyme and a red dye. The PCR products can be directly loaded onto agarose gels without any further purification.

2.1.14 **PCR mediated site-directed mutagenesis**

We used site-directed mutagenesis to generate a kinase negative mutant of MEKK1 by exchanging the conserved lysine in the ATP-binding site for asparagine. Site-directed mutagenesis requires two rounds of PCR (Fig. 2.1).

![Figure 2.1 Schematic diagram showing the principle of PCR mediated site-directed mutagenesis.](image)

Firstly, two separate PCR reactions were carried out with outer primer 1 and mutation primer 3 (green) and outer primer 4 and mutation primer 2 (blue) respectively. The two PCR products were separated on an agarose gel, isolated, mixed, and subsequently amplified and extended by PCR with primers 1 and 4 (B). The main final product of this second PCR, the mutated DNA fragment, was isolated after purification by agarose gel electrophoresis (C).
2.2 Tissue culture

2.2.1 Frequently used buffers and reagents in tissue culture

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>137 mM NaCl</td>
<td>freezing medium</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
<td>5.5 ml growth medium</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>8.1 mM</td>
<td>2.5 ml FBS</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.5 mM</td>
<td>2 ml DMSO</td>
</tr>
</tbody>
</table>

2.2.2 Maintenance of mammalian cell lines

All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Table 2.1 shows in which media the different cell lines were cultured. All tissue culture reagents were purchased from Invitrogen, foetal bovine serum (FBS) was from Harlan Sera Labs.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>human squamous carcinoma</td>
<td>DMEM + 10% FBS</td>
</tr>
<tr>
<td>A2780</td>
<td>human ovarian carcinoma</td>
<td>RPMI + 10% FBS</td>
</tr>
<tr>
<td>CP70</td>
<td>cisplatin resistant derivate of A2780</td>
<td>RPMI + 10% FBS</td>
</tr>
<tr>
<td>GPenvAM12</td>
<td>NIH derived packaging cell line</td>
<td>DMEM + 10% FBS</td>
</tr>
<tr>
<td>HeLa</td>
<td>human cervix carcinoma</td>
<td>DMEM + 10% FBS</td>
</tr>
<tr>
<td>HIM</td>
<td>human fibroblast, p21-/-</td>
<td>HAM’s F10 + 15% FBS +8 mg/l ciproxin (Bayer)</td>
</tr>
<tr>
<td>HL60</td>
<td>human leukaemia</td>
<td>RPMI + 10% FBS</td>
</tr>
<tr>
<td>LAN5</td>
<td>human neuroblastoma</td>
<td>DMEM + 10% FBS</td>
</tr>
<tr>
<td>lox26</td>
<td>human fibroblasts, wild type</td>
<td>see HIM</td>
</tr>
<tr>
<td>MCF7</td>
<td>human breast carcinoma</td>
<td>RPMI + 10% FBS</td>
</tr>
<tr>
<td>p53-/-</td>
<td>human fibroblasts, p53-/-</td>
<td>see HIM</td>
</tr>
<tr>
<td>SKNSH</td>
<td>human neuroblastoma</td>
<td>DMEM + 10% FBS</td>
</tr>
</tbody>
</table>

2.2.3 Freezing and thawing of mammalian cells

Cells for freezing were grown in 175 cm² flasks to approximately 75% confluence. The cells were washed twice with PBS, trypsinised and resuspended in 10 ml growth medium. Cells were collected by centrifugation at 400xg for 5 min and resuspended in 2.5 ml growth medium. To this cell suspension, 2.5 ml ice-cold, freshly prepared
Materials & Methods

freezing medium were added drop by drop while mixing. The cells were then immediately aliquoted at 1 ml into cyrotubes. For a slow freezing process, the tubes were wrapped in cotton wool and left in a -80°C freezer overnight before they were transferred to liquid nitrogen.

To thaw frozen cells, the cyrotubes were placed in a 37°C water bath promptly after the removal from the liquid nitrogen. To remove the DMSO, the cell suspension was then diluted with 10 ml growth medium and spun down at 400xg for 5 min. Afterwards, the cells were resuspended in 5 ml growth medium and seeded into 25 cm² flasks. Alternatively, the diluted cell suspension was seeded into a small flask directly and the medium was changed as soon as the cells had attached (4 to 6 h).

2.2.4 Transfection of cells

All cell lines were tested for their susceptibility to transfection with a variety of commercially available transfection reagents following the manufacturers’ instructions. To find the best reagent and the optimal conditions for each cell line, we used the EGFP-C1 plasmid and checked the cells for green fluorescence and cytotoxic effects by fluorescence microscopy. The results summarised in Table 2.2 are given for one 35mm dish of cells.

<table>
<thead>
<tr>
<th>cell line</th>
<th>transfection reagent</th>
<th>µl reagent</th>
<th>µg DNA</th>
<th>time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>FuGENE™ (Roche)</td>
<td>10</td>
<td>2</td>
<td>overnight</td>
</tr>
<tr>
<td>A2780</td>
<td>LipfectAMINE™ (Invitrogen)</td>
<td>4</td>
<td>1</td>
<td>5 h</td>
</tr>
<tr>
<td>CP70</td>
<td>LipfectAMINE™</td>
<td>4</td>
<td>1</td>
<td>5 h</td>
</tr>
<tr>
<td>GPenvAM12</td>
<td>Effectene™ (Qiagen)</td>
<td>5</td>
<td>1</td>
<td>overnight</td>
</tr>
<tr>
<td>HeLa</td>
<td>LipfectAMINE™</td>
<td>6</td>
<td>2</td>
<td>3-5 h</td>
</tr>
<tr>
<td>LAN5</td>
<td>LipfectAMINE™</td>
<td>4</td>
<td>1</td>
<td>5 h</td>
</tr>
<tr>
<td>lox26</td>
<td>retroviral infection</td>
<td></td>
<td></td>
<td>overnight</td>
</tr>
<tr>
<td>MCF7</td>
<td>FuGENE™</td>
<td>3</td>
<td>0.6</td>
<td>overnight</td>
</tr>
<tr>
<td></td>
<td>Effectene™</td>
<td>5</td>
<td>0.5-0.6</td>
<td>overnight</td>
</tr>
</tbody>
</table>
Table 2.2 continued

<table>
<thead>
<tr>
<th>cell line</th>
<th>transfection reagent</th>
<th>µl reagent</th>
<th>µg DNA</th>
<th>time</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53-/-</td>
<td>retroviral infection</td>
<td></td>
<td></td>
<td>overnight</td>
</tr>
<tr>
<td>SKNSH</td>
<td>LipfectAMINE™</td>
<td>4</td>
<td>1</td>
<td>5 h</td>
</tr>
</tbody>
</table>

### 2.2.5 Generation of retrovirus stocks

For safety reasons, the retroviruses that are used for gene transfer experiments in the laboratory are replication-incompetent. They lack the structural genes *gag*, *pol* and *env*, which are necessary for virus particle formation and replication (see [131], chapter 9.9 for an extensive overview of gene transfer by retroviral infection). These genes are supplied by packaging cell lines. Introduction of retroviral vectors that contain the RNA packaging signal Ψ⁺ into these cell lines leads to the production of infectious but replication-incompetent virus. We used the packaging cell line GPenvAM12 [132], which possesses an amphotropic *env* gene. Thus, amphotropic virus is produced that can infect a broad host range including human, mouse, rodent, dog, cat, mink and chicken. Because the retroviruses we generated were able to infect human cell lines but did not contain hazardous transgenes, they are classified as genetically modified organisms of the biological safety category 2. All experiments were therefore carried out in a category 2 laboratory taking appropriate safety precautions.

To generate virus, stocks GPenvAM12 cells were transfected with the retroviral plasmids as described in 2.2.4. After a change of medium the cells were incubated for a further two days after which the retrovirus containing medium was collected and cleared from debris and detached cells by centrifugation at 1000xg for 10 min. The virus stocks were then immediately used for infections.

### 2.2.6 Retroviral infections

Cells were seeded to approximately 50% confluency one day before retroviral infections. Then, the medium was replaced by the virus stock supplemented with 10 µg/ml polybrene (Sigma), a polycation that neutralises the negative surface charge of the cells and thereby increases infection rates [133]. About 1.5 ml of virus stock was used for one 35 mm dish of cells. After incubation overnight, the virus stock was replaced by normal growth medium.
2.2.7 Colony formation assay

To monitor the clonogenic survival of transfected or infected cells, the cells were seeded in 1:4, 1:12 and 1:40 dilutions one or two days after transfection/infection. One day later, the medium was supplemented with the appropriate antibiotic. The concentrations used are given in Table 2.1 The medium was changed every 3 to 4 days until the surviving cells formed colonies (two to four weeks). The cells were then fixed in 50% methanol in PBS followed by 100% methanol for 10 minutes each and stained with Gurr® (BDH Laboratory Supplies). Excess staining solution was washed away with water.

Table 2.3 Antibiotics for the selection of mammalian cells

<table>
<thead>
<tr>
<th>resistance gene</th>
<th>antibiotic</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>Genetecin (G418) sulphate (Invitrogen)</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Puromycin</td>
<td>Puromycin (Sigma)</td>
<td>0.25-0.5 µg/ml</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>Zeocin® (Invitrogen)</td>
<td>400 µg/ml</td>
</tr>
</tbody>
</table>

2.2.8 Clonogenic assay

To assay the long-term survival of drug treated cells, clonogenic assays were carried out on growing cells. Before treatment with the apoptosis-inducing agents, cells were pre-incubated for 0.5 to 1 h with 40 µM PD98059, 5 µM SB203580 or, as a control, DMSO to a final concentration of 0.2%. After treatment with taxol, etoposide or ceramide, cells were washed twice with PBS and incubated in normal growth medium in the presence of inhibitors or DMSO for a further 6 to 20 h. After this, the cells were trypsinised and seeded in 1:20, 1:60 and 1:200 dilutions in normal growth medium. Colonies formed within one to two weeks and were stained as described in 2.2.7.

2.3 Protein and cell biology methods

2.3.1 Frequently used buffers in protein and cell biology

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Buffer Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>25 mM Tris 200 mM glycine</td>
</tr>
<tr>
<td>TBS</td>
<td>10 mM Tris-HCl pH 8.0 150 mM NaCl</td>
</tr>
<tr>
<td>electrophoresis</td>
<td>0.1% SDS in TG</td>
</tr>
<tr>
<td>buffer</td>
<td></td>
</tr>
<tr>
<td>TBST</td>
<td>0.1% Tween 20 in TBS</td>
</tr>
</tbody>
</table>
5x protein sample buffer
50% glycerol
2.5% SDS
250 mM Tris-HCl pH 6.8
5 mM EDTA
100 mM DTT
0.1 mg/ml Pyronin Y

2.3.2 Preparation of cell lysates for SDS-PAGE and Western Blot

Cells were washed twice with ice-cold PBS and lysed in 20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1% Triton X-100, 0.05% SDS, 10 mM NaF, 10 mM β-Glycerophosphate, 0.5 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin for 30 min on ice. After centrifugation for 10 min at 21000xg the protein content of the lysates was determined using a BCA assay kit (Pierce) according to the manufacturer’s instructions. After their protein content was equalized with lysis buffer, lysates were mixed with ¼ volume of 5x protein sample buffer, boiled for 5 min and then kept on ice or stored at -20°C.

2.3.3 Immunoprecipitation

For Immunoprecipitations, cells were washed twice with ice-cold PBS and lysed in a low-detergent lysis buffer containing 20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 0.1% Triton X-100, 10 mM NaF, 10 mM β-Glycerophosphate, 0.5 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin. Lysates were cleared and equalised for protein content as described in 2.3.2. Precipitations were carried out by adding the antibody together with 15 µl protein A (Roche) (for rabbit antibodies) or protein G agarose (Roche) (for mouse, rat or sheep antibodies) and incubating for 4 h or overnight at 4°C on an end-over-end mixer. The amounts of antibody used for precipitations from one 35 mm dish of cells are given in Table 2.5. The agarose beads were collected by centrifugation for 1 min at 5000xg at 4°C and washed three times with 500 µl lysis buffer without inhibitors. The precipitates were then boiled in 30 to 50 µl 1x protein sample buffer or used in kinase assays (see 2.3.7-8).

2.3.4 SDS polyacrylamide gel electrophoresis (PAGE) and Western blot

SDS-PAGE and Western blotting were carried out according to standard protocols. Briefly, SDS-Page was carried out as illustrated in Ausubel et al. ([131], p. 10.2A4-10.2A.9) with the protein sample buffer given in 2.3.1. We blotted as described in [131], p. 10.8.1-10.8.4, except that we did not equilibrate the gels in blotting buffer beforehand. We used a tank transfer system, methanol-free TG buffer and PVDF membranes.
2.3.5 Immunodetection of proteins on Western blots
Blots were blocked in 5% non-fat dry milk or 5% BSA in TBST for 30 to 60 min at RT. For phosphorylation specific antibodies, blots were blocked exclusively with BSA. The blocking reagent was removed by washing twice with TBST for 5 min. Afterwards, blots were incubated in primary antibody overnight at 4°C with gentle shaking. Blots were then washed three times for 10 min each with TBST and incubated in secondary antibody for about 1 h at RT. After washing six times for 5 min each in TBST, blots were ready for the detection reaction. We used horse radish peroxidase (HRP) conjugated secondary antibodies and detected these with either BM Chemiluminescence Blotting Substrate (POD) (Roche) or ECL™ (Amersham) according to the respective product data sheets. The concentration of the antibodies used are given in Table 2.5 and Table 2.6. Primary antibodies were diluted in TBST supplemented with 5% BSA and 0.1% NaN₃, secondary antibodies were diluted in TBST.

2.3.6 Reprobing of Western blots
To reprobe the blots, bound antibodies were removed by "stripping". This was done by shaking the blot in 1% SDS, 0.2 M glycine pH 2.0 for 1 to 1.5 h at RT. For the phospho-ERK antibody, the stripping buffer was supplemented with 1% DTT.

2.3.7 MEKK1 kinase assay
MEKK1 activity was assayed as described in [99] except that no radioactive ATP was used but phosphorylated MKK4 was detected using a phosphorylation-specific antibody. Briefly, precipitates from 2.3.3 were washed once in MEKK1 assay buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM DTT). Kinase reactions were carried out in 50 µl assay buffer supplemented with 15 µM ATP and 0.5 µg GST-MKK4 as a substrate for 30 min at 30°C. GST-MKK4 was a generous gift from Dr. Dario Alessi. Reactions were stopped by the addition of 12.5 µl 5x sample buffer. After SDS-PAGE and Western blotting MKK4-phosphorylation was analysed by probing with anti-phospho-MKK4 antibody.

2.3.8 MAPKAP kinase 2 assay
MAPKAP kinase 2 activity was assayed according to Scheid et al. [134]. Briefly, precipitates from 2.3.3 were washed once with MAPKAP kinase assay buffer (20 mM Hepes, 20 mM MgCl₂). Kinase reactions were then carried out in 30 µl assay buffer supplemented with 50 µM ATP and 0.185 MBq (5 µCi) [γ-³²P]ATP using 3 µg Hsp25 (Sigma) as a substrate for 30 min at 30°C. Reactions were stopped by the addition of
8 µl 5x sample buffer. After SDS-PAGE and Western blotting the phosphorylation of Hsp25 was monitored by autoradiography.

2.3.9 JNK kinase assay
JNK activity was monitored by a so-called "pull-down" assay using the SAPK/JNK Kinase Assay Kit from Cell Signaling following the manufacturer’s instructions. In this assay, the JNK is not precipitated using antibodies but "pulled down" with its substrate, GST-c-Jun, immobilised on glutathione agarose beads. As an alternative to using the substrate supplied with the kit, GST-c-Jun (1-79) fusion protein beads were produced from E. coli as described in 2.3.10. c-Jun phosphorylation was then monitored using the phospho-c-Jun (Ser63) antibody from the Assay Kit.

2.3.10 Preparation of GST-c-Jun 1-79
10 ml of an ONC of E. coli containing the expression vector pEGX-cJun1-79 were diluted to 500 ml LB and grown at 37°C for 2 h. After that, expression of GST-c-Jun was induced by the addition of IPTG to a final concentration of 1 mM. Cells were grown for another 3 to 5 h at 37°C with shaking (150 to 200 rpm). The bacteria suspension was then centrifuged for 5 min at 3000xg. Cell pellets were stored at -80°C or used immediately. The bacteria were lysed in 10 ml BugBuster™ Protein Extraction Reagent (Novagen) supplemented with 1 mM PMSF and 10 µg/ml aprotinin for 15 min at RT. Lysates were cleared by centrifugation at 10000xg for 20 min at 4°C. To the supernatant, 500 µl of glutathione sepharose (Pharmacia) equilibrated in BugBuster™ were added and the mixture was incubated at 4°C for 3 to 5 h on a rotation mixer. The beads were spun down for 5 min at 10000xg and washed three times with cold JNK assay lysis buffer (1% Triton X-100 in TBS). Finally, the beads were resuspended in 500 µl lysis buffer containing 50% glycerol and stored at -20°C. We used 20 µl per sample of these beads in the JNK assay.

2.3.11 β-Galactosidase assay of cell lysates using ONPG as a substrate
To assay β-galactosidase activity, cells grown in 35 mm dishes were washed twice with PBS and then lysed in 100 µl of a detergent-free, hypotonic phosphate buffer (9.3 mM Na₂HPO₄, 3.9 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF) by three to four freeze-thaw cycles on dry ice and in a 37°C water bath. The crude lysates were centrifuged for 10 min at 21000xg and 4°C. The protein content was determined using a BCA assay kit (Pierce). Equal amounts of protein (maximal 30 µl lysate) were diluted with lysis buffer in a flat-bottom 96-well
Materials & Methods

microtiter plate to a final volume of 150 µl. After the addition of 30 µl o-nitrophenyl β-D-galactopyranoside (ONPG, Sigma) (4 mg/ml in lysis buffer) plates were incubated at 37°C. The enzyme hydrolyses ONPG and thereby generates the yellow o-nitrophenylate anion. The incubation period needed for colour development varied between about 30 min and 2 h. Reactions were stopped by addition of 100 µl 1M Na₂CO₃ and the absorption was read at 405 nm in an MRXII plate reader (Dynex Technologies).

2.3.12 β-Galactosidase staining with X-gal
X-gal (5-Br-4-Cl-3-indolyl β-D-galactopyranosid) was used to stain β-galactosidase expressing cells in tissue culture dishes. β-Galactosidase-catalysed cleavage of X-gal generates the insoluble, blue dye 5-Br-4-Cl-3-indolol. As a result, β-galactosidase expressing cells can be distinguished from non-expressing cells in the microscope. To stain the cells, they were first washed twice with PBS and then fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min at RT. Cells were washed twice with PBS and stained with 1 m/ml X-Gal (Sigma) in PBS supplemented with 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆] and 20 mM MgCl₂ at 37°C until the blue stain was readily visible under the microscope. The Fe³⁺/Fe²⁺ redox system accelerates the precipitation of indolol and thus reduces diffusion.

2.3.13 Cell cycle analysis
For cell cycle analysis, cells were grown in 35 mm dishes. The medium containing detached cells was collected in a 15 ml conical tube and the cells washed once with PBS. The cells were then trypsinised, resuspended in 2ml growth medium and this suspension was combined with the supernatant from the same sample. After centrifugation for 5 min at 400xg, the cells were washed once in ice-cold PBS and resuspended in 100 µl PBS. After the addition of 900 µl 70% ethanol cells were incubated on ice for 1 h or overnight. Cells were then sedimented by centrifugation for 5 min at 400xg and incubated in 300 µl PBS containing 250 µg/ml RNaseA and 10 µg/ml propidium iodide (Sigma) for 30 min at RT prior to FACS (fluorescence assisted cell sorting) analysis on a Becton Dickinson FACScan. The FACS data was analysed using the ModFit LT software package (Verity Software House Inc.). This is shown exemplary for growing, untreated HeLa cells in figure 2.2. The software identifies the G1 and G2/M peaks in the cell cycle profile and models them with gaussian curves (red). Based on these peaks, a rectangular component (blue striped) is fitted to model the S-phase.
Figure 2.2 Analysis of PI histograms with ModFit LT. First, the cell population to be analysed is selected manually (A, insert). The program then fits the histogram (A) by modelling two gaussian components (red) to the G1 and G2/M peaks and a rectangular component to the S-phase (blue stripes). Based on this, it calculates the distribution of the cells to the different phases of the cell cycle (B).

2.3.14 Cell viability assay

For cell viability assays, cells were seeded in 96-well flat bottom microtiter plates at a density of 1000 cells per well. Cells were pre-incubated for 0.5 to 1 h with 40 \( \mu \)M PD98059, 5 \( \mu \)M SB203580 or, as a control, DMSO to a final concentration of 0.2% and then treated with 50 nM taxol, 40 \( \mu \)g/ml etoposide or 20 \( \mu \)M ceramide overnight in the presence of the inhibitors. Afterwards, cells were washed twice in PBS and incubated in normal growth medium for 6 h before cell viability assays were carried out using Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer’s instructions.

2.3.15 Annexin V binding assay

Annexin V is a \( \text{Ca}^{2+} \)-dependent phospholipid-binding protein with a high affinity for phosphatidylserine [135]. Phosphatidylserine resides in the inner layer of the plasma membrane of healthy cells but translocates to the outer layer in the early stages of apoptosis [136, 137]. Hence, Annexin V can be used as a probe for phosphatidylserine to detect apoptotic cells. For the Annexin V-binding assay, detached and adherent cells were harvested as described in 2.3.13. We used the Annexin-V-FLUOS reagent (Roche) and followed the protocol provided by the manufacturer. Alternatively, we used Annexin V-PE (Pharmingen) following the same protocol except that 40 \( \mu \)l of Annexin V-PE and 5 \( \mu \)l 7-Aminoactinomycine D (7-AAD, 1 \( \mu \)g/\( \mu \)l, Sigma)) were used to prepare the staining solution.
2.3.16 Nuclear staining with 4',6'-diamidine-2'-phenylindole (DAPI)

One of the hallmarks of apoptosis is the condensation and fragmentation of chromatin in the nucleus of apoptotic cells. DAPI, a fluorescent dye that binds selectively to DNA, has been widely used to stain nuclei for the microscopic analysis of apoptosis. To examine nuclear morphology, the cells grown in 8-well plastic chamberslides were washed once with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at RT. Care has to be taken not to disturb the cells, because during apoptosis the cells become less adherent and are easily detached. After briefly washing with PBS the slides are mounted on coverslips using Vectashield mounting medium (Vector Laboratories) supplemented with 0.5 µg/ml DAPI. The slides were then sealed with nail polish and analysed by fluorescence microscopy. Pictures were taken with a digital imaging system (Princeton IPLab spectrum software). Overlays of pictures were created with the Confocal Assistant™ software package.

2.4 Materials

2.4.1 Plasmids

Table 2.4  Complete overview of plasmids used in this studies

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<td>Invitrogen</td>
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<td>human MEKK1 in pCR®2.1 TOPO</td>
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<td>Clontech</td>
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2.4.2 Oligonucleotides

All oligonucleotides (ON) were synthesised by the Beatson Molecular Technology Services except for the T7-primer, which was purchased from New England Biolabs. ONs were dissolved in TE buffer and their concentration determined using a photospectrometer and the approximation that an absorption of 1 at 260 nm equals 37 µg/ml single-stranded DNA.

PCR primers for the amplification of human MEKK1

For MEKK1: 5’-AGGTCGACGACTATGCGTCTGAAGCTTTCAAAAGTGCAAGAGAAGATG-3’
Rev MEKK1: 5’-CTACCATGTTGTACGGAAGACAAGATCTCCTGGCCGGAGCTC-3’

Primers for sequencing reactions

M13 forward 5’-GTAAAACGACGGCCAGT-3’
mekk-rev 5’-TCATTAGGATGTTGG-3’
mekk-for 5’-CCCAGCATCCTGATG-3’
T7 promoter primer 5’-TAATACGACTCACTATAGGG-3’

Primers for site-directed mutagenesis (the mutation is underlined)

KN primer 1 5’-GGTCGACGACTATGCG-3’
KN primer 2 5’-TTGCCCTTCTCGAGG-3’
KN primer 3 5’-ATGGCTGTTAACCAGGTGACTATGTCAGAAAC-3’
KN primer 4 5’-GTCACCTGGTTAACAGCCATTAAAGTTCCAGTTC-3’

Oligonucleotides for adaptors

RaMe1: 5’-TCGACGACGATGCGTCGTAAGCTTGT-3’
RaMe2: 5’-AGCTACAAGCTTACGACGCATCGTCG-3’

2.4.3 Antibodies

Table 2.5 List of primary antibodies used in this study

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<td>Bcl-2</td>
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<td>β-Galactosidase</td>
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### Table 2.6 Horseradish peroxidase conjugated secondary antibodies

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2.4.4 *Molecular weight markers*

protein: Prestained SDS Molecular Weight Standard Mixture SDS 7-B (Sigma)

DNA: DNA Molecular Weight Marker X, 0.07-12.2 kb (Roche)

2.4.5 *Radiochemicals*

Redivue $[^\gamma]{\text{32P}}$ATP triethylammonium salt (>185 TBq/mmol, 370MBq/ml) was purchased from Amersham.

2.4.6 *Apoptosis-inducers and kinase inhibitors*

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<td>20 $\mu$M</td>
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</tr>
</tbody>
</table>

2.4.7 *Other reagents*

The suppliers of key reagents have been mentioned throughout this chapter. All other chemicals and reagents were purchased from Fisher Scientific, Melford Laboratories Ltd, and Sigma.
3 Results

3.1 Cloning

3.1.1 Cloning of the MEKK1 kinase domain

The kinase domain of human MEKK1 was amplified by PCR from a human tonsil cDNA library and cloned into pCR®2.1-TOPO®. The primers used are described in 2.4.2 and have been designed by W. Kolch based on the published rat cDNA sequence.

Four of the resulting clones were sequenced; two of those did not contain any frame shift mutations (data not shown). The clone with the higher homology to the published rat cDNA sequence ([93], GenBank accession no. U48596), clone1.2, was used for all further experiments. Figure 3.1 shows the protein sequence of clone1.2 in alignment to the rat protein and the since then published human sequence ([141], GenBank accession no. AF042838).

Differences between the sequences are highlighted in red. Clone1.2 is identical to the published human sequence apart from one amino acid and the human forms differ from the rat protein in three amino acids. Also marked (blue) is the location of an internal HindIII site, that is occurring only in the human sequences and has been used for further cloning. The conserved lysine in the ATP-binding site, that was changed to an asparagine to generate a kinase negative mutant of the protein, is marked in yellow.

Figure 3.1. The sequence of clone 1.2 in alignment to human and rat MEKK1. Sequences were aligned with Clustalw (www.ebi.ac.uk/clustalw). Differences between the sequences are highlighted in red, the conserved lysine in the ATP-binding site is shown in yellow. The location of a HindIII site that has been used for subcloning is highlighted in blue.
3.1.2 Cloning of MEKK1 expression plasmids

For expression in eukaryotic cells, the MEKK1 kinase domain was subcloned into pcDNA3. Thereto, the cDNA fragment was retrieved from clone1.2 by restriction with HindIII and XbaI and inserted into the corresponding sites in the vector (figure 3.2).

Figure 3.2 Mammalian expression plasmids for MEKK1. The HindIII/XbaI DNA fragment from clone1.2 (top) was cloned into the corresponding sites of pcDNA3 (middle left) and EGFP-C1 (middle right) resulting in the expression plasmids pc3MEKK1 for the MEKK1 kinase domain (lower left) and EGFP-MEKK1 for the fusion protein (lower right).
We also generated an EGFP-MEKK1 fusion protein, in which the kinase domain is fused C-terminally to the enhanced green fluorescent protein (EGFP), by inserting the same cDNA fragment into the EGFP-C1 vector (figure 3.2).

### 3.1.3 Cloning of RBD-MEKK1 fusion proteins

The RBD-MEKK1 fusion proteins consist of the Ras-binding domain (RBD) of Raf-1 fused N-terminally to the MEKK1 kinase domain. To generate these proteins, we used the expression plasmid pEFmycRaf, which encodes a Myc-tagged human Raf-1 protein under the control of the elongation factor 1α (EF) promoter. We used existing restriction sites in the regulatory domain of Raf-1 to obtain two fusion proteins which differ in the length of their RBD part. RBD1-MEKK contains amino acids 1-257 and RBD3-MEKK amino acids 1-148 of Raf-1, this corresponds to internal SalI and HindIII restriction sites respectively. For the generation of RBD1-MEKK, the RBD-coding SalI fragment of pEFmycRaf was ligated with the MEKK1-coding HindIII fragment of clone 1.2 using an oligonucleotide adaptor as shown in figure 3.3.

**Figure 3.3 Cloning of the RDB-MEKK fusion proteins.** A SalI fragment encoding the RBD and a HindIII fragment encoding for MEKK1 were ligated using an oligonucleotide adaptor (red). The resulting fusion protein RBD1-MEKK was used to generate a shorter fusion protein, RBD3-MEKK, by removal of an internal HindIII fragment.
This adaptor was designed to bring the DNA fragments into the same translational frame and disrupts the MEKK1 HindIII site. It also contains another HindIII site to allow the easy generation of the shorter fusion protein RBD3-MEKK by removing an internal HindIII fragment as illustrated in figure 3.3. These fusion proteins were then subcloned into pcDNA3. Thereto, the DNA was retrieved from the pEF-plasmids by limited digestion with MsiI and XbaI. An expression plasmid for the Ras-binding domain alone, pc3RBD, was derived from pc3RBD1-MEKK by removal of the internal SalI/XbaI fragment, thereby deleting the MEKK kinase domain.

3.1.4 Cloning of retroviral vectors

Because the human fibroblast cell lines that we used as a model system for non-cancer cells could not be transfected with commercially available transfection reagents, we had to deliver the genes by retroviral infection. Thereto, we used the retroviral expression vectors pBABE-Puro and pBABE-Bleo, which carry a puromycin and bleomycin resistance gene respectively and allow the expression of transgenes under the control of the long terminal repeat (LTR) promoter sequence.

The cloning strategies for the vector pBABE-Puro are summarized in figure 3.4. Briefly, the EcoRI fragment from clone1.2 and the EcoRI/SalI fragment from pc3RM1 were inserted into the corresponding sites of pBABE-Puro to produce expression vectors for MEKK1 and the RBD respectively. To generate an expression construct for the RBD1-MEKK fusion protein we isolated an EcoRI/BglII fragment from pc3RM1 along with a BglII/SalI fragment from pEGFP-MEKK1 and ligated them with pBABE-Puro digested with EcoRI/SalI. The DNA fragment coding for the EGFP-MEKK1 fusion protein was retrieved by restriction with Eco47III and SalI and cloned into the SnaBI/SalI site of the vector. A plasmid encoding for EGFP was derived from this by removing the internal XhoI fragment.

The vector pBABE-Bleo is identical to pBABE-Puro except for the resistance marker. All cloning was done as described above.
Figure 3.4 Subcloning in the retroviral expression system pBabe. The MEKK1 kinase domain, the RBD, RBD1-MEKK, EGFP-MEKK1 and EGFP were cloned into the retroviral vector pBabe-Puro as described in the text. The restriction sites used for cloning are highlighted in red and green.
3.2 Targeting MAPK kinase pathways to kill cancer cells

3.2.1 MEKK1 proteins phosphorylate GST-MKK4 in vitro

In order to test if the human MEKK1 cDNA we cloned encodes a biologically active protein, we performed an in vitro kinase assay using the physiological MEKK1 target MKK4 fused to glutathione S-transferase (GST) as a substrate. We transfected EGFP-MEKK1 and RBD1-MEKK into A431 cells and one day after transfection the MEKK1 proteins were precipitated using either anti-GFP or anti-MEKK1 antibodies. As a negative control, we used EGFP-transfected cells. For a positive control we used cells transfected with a constitutively active form of murine MEKK1 (ΔMEKK). As shown in figure 3.5, we could not detect endogenous MEKK1 activity in EGFP-transfected cells with this assay (compare lanes 1 and 2 with lane 3). ΔMEKK phosphorylated GST-MKK4 (compare lanes 3 and 7). EGFP-MEKK1 also showed a high kinase activity. The activity of the EGFP fusion proteins was greatly reduced, if the protein was precipitated with the anti-MEKK1 antibody compared to the anti-GFP antibody, even though both antibodies precipitated the protein with similar efficiency (lanes 4 and 5).

This was not surprising, considering that the anti-MEKK1 antibody is directed against the kinase domain and therefore can be expected to sterically inhibit the interaction between MEKK1 and its substrate GST-MKK4. Still, the antibody does only reduce, not abolish the kinase activity. The RBD fusion protein RBD1-MEKK also
phosphorylates GST-MKK4 in vitro, though its activity seems to be lower than EGFP-MEKK1 (compare lanes 5 and 6). This will be explored further in 3.2.7. The basal level of phosphate incorporation into GST-MKK4 is relatively high (lanes 1-3). This is most probably due to autophosphorylation, as the GST-MKK4 protein used in this assay is itself catalytically active and MKK4 has been shown to undergo autophosphorylation [142].

3.2.2 Expression of MEKK1 activates co-transfected MAP kinases in vivo

Next we investigated whether and which MAP kinase pathways became activated by MEKK1 in vivo. For this purpose, we transfected EGFP-MEKK1 or EGFP as a control together with HA-tagged JNK1, ERK2 or p38 in A431 cells and analysed the phosphorylation pattern of the recombinant MAP kinases one day after transfection. As shown in figure 3.6, we could detect activated MAP kinases using phosphorylation-specific antibodies only when EGFP-MEKK1 was co-expressed. EGFP-MEKK1 led to the activation of all three co-transfected MAP kinases HA-JNK, HA-p38 and HA-ERK.

Like EGFP-MEKK1, the RBD fusion protein RBD1-MEKK also induced the activation of all three MAP kinases JNK, ERK and p38 (data not shown and 3.2.10). This was
surprising, because the purpose of the RBD fusion was to design a protein that would simultaneously activate the JNK and inhibit the ERK pathway. That the RBD1-MEKK protein still activates ERK indicates that – regarding ERK activation – the kinase activity of MEKK1 compensates for the inhibitory effect of the RBD.

3.2.3 The mechanism of MAP kinase activation by MEKK1 is intracellular

We were interested to see if MEKK1 was capable of activating all three MAP kinase cascades directly or whether autocrine or paracrine mechanisms were involved. To test this possibility we performed a co-culture experiment whose set-up is summarised in figure 3.7.

MCF7 cells were transfected with a mix of HA-tagged MAP kinases or with EGFP-MEKK1. One day after transfection MAP kinase and EGFP-MEKK transfected cells were trypsinised, mixed and cultured together for another two days. Then the transgene MAP kinases were analysed for phosphorylation. As a negative control, we cultured MAP kinase transfected cells with EGFP transfected cells. For a positive control, we co-transfected EGFP-MEKK1 with the HA-MAPK mix (see Fig. 3.7). Figure 3.8 shows the phosphorylation of HA-ERK, HA-JNK and HA-p38 induced by co-transfection of EGFP-MEKK1 (lane 3). In cells that express only the HA-tagged MAP kinases phosphorylation is not detectable (lane 1). No JNK phosphorylation became apparent if

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**Figure 3.7 Experimental set-up of co-culture experiments.** For co-cultures, cells were transfected with either EGFP-MEKK1 or Ha-tagged MAPK (left). One day later, the transfected cells were detached by trypsinisation, mixed and cultured together for another two days. For co-transfections (right) both plasmids were transfected together. This results in the expression of two or more transgenes within one cell.
these cells were cultured together with EGFP-MEKK1 expressing cells, even after prolonged exposure. For ERK and p38 we could detect a slight activation in EGFP-MEKK1 co-cultured cells. This activation was small compared to the one achieved by co-transfection and could only be detected upon prolonged exposure. Similar results were obtained in HeLa cells, which were co-cultured for one day (data not shown). Taken together, these data suggest that the activation of MAP kinases by MEKK1 is predominantly intracellular for the ERK and the p38 pathway and might be exclusively intracellular with regard to the JNK pathway.

**3.2.4 MEKK1 reduces survival of human cancer cell lines**

To monitor the fate of MEKK1 overexpressing human cancer cells we transiently transfected the EGFP-MEKK1 fusion protein or EGFP as a control into MCF7, HeLa and A431 cells. We then determined the survival rate of the transfected cells over a period of four days by flow cytometry. Dead cells were eliminated from the analysis by counterstaining with propidium iodide (PI), a DNA-binding fluorescent dye that cannot permeate the membrane of living, healthy and apoptotic cells. The principle of this assay is outlined in figure 3.9. First, debris and aggregates are excluded from the analysis (Fig. 3.9A). Then, the population of PI negative cells is selected (Fig. 3.9B) and analysed for EGFP fluorescence (Fig. 3.9C and D).

Intriguingly, no population of PI and GFP positive, i.e. dead and transfected, cells can be detected in this assay, even at later time points (data not shown). This is most probably due to the EGFP leaking out of the cell after the loss of membrane integrity and has been described before [143].
Results

Multiple Document Interface for Flow Cytometry
WinMDI Version 2.8 - Windows 3.95/DOS 7.10
Thu Dec 13 16:45:14 2001
Gates: R1*R2
Total Events 16853 Gated Events 13413   79.59%

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Figure 3.9 Experimental set-up of short-term survival monitoring by FACS analysis. A. A dot plot of the side scatter (SSC-Height) versus the forward scatter (FSC-Height) of the cells allows to select the population of single cells (polygon) from debris and aggregates. B. Living, i.e. PI-negative cells are selected (rectangle) and analysed for EGFP expression (C and D). C1, C2, C3. Histogram showing the expression of EGFP in single, living cells on day 1, 2 and 3 after transfection respectively. The displayed markers have been set so that untransfected cells are in region M1 while M2 covers the EGFP-positive cells. D. Statistical analysis of C1, the percentage of transfected cells is shown in red.

HeLa and A431 cells expressing EGFP-MEKK1 died rapidly: already two days after transfection, the number of EGFP-MEKK1 expressing cells was greatly reduced compared to EGFP expressing cells and four days after transfection less than 10% of the cells were still alive (Fig. 3.10). This was due to apoptosis of the EGFP-MEKK1 expressing cells. HeLa cells expressing EGFP-MEKK1 showed extensive DNA condensation and nuclear fragmentation, a hallmark of apoptosis, two days after transfection (Fig. 3.11A-C). The nuclear morphology of EGFP expressing cells was not distinguishable from untransfected cells (Fig. 3.11D-F).
For MCF7 cells on the first two days after transfection the amount of MEKK1 expressing cells was not significantly different from the vector controls (Fig. 3.10). After two days however, the rate of MEKK1 expressing cells decreased compared to the control cells.
3.2.5 MEKK1 reduces clonogenic survival of human cancer cell lines

To investigate the long-term effects of activated MEKK1, the RBD, and the RBD-MEKK1 fusion proteins on human cancer cell lines we performed clonogenic assays (Fig. 3.12). We found that an EGFP-MEKK1 fusion protein almost completely abolished colony formation compared to the EGFP control plasmid in five of the seven cancer cell lines tested and reduced the numbers of colonies in A431 and SKNSH cells, indicating that expression of the kinase domain of MEKK1 inhibits cell growth and/or survival. The MEKK1 kinase domain alone gave the same results like the EGFP fusion protein (data not shown). Interestingly, even though the onset of apoptosis was delayed in MCF7 cells as shown earlier in 3.2.4, EGFP-MEKK1 was still capable of efficiently abrogating clonogenic survival of these cells.

Figure 3.11 MEKK1 induces apoptosis. HeLa cells were transfected with EGFP-MEKK1 (A-C) or EGFP (D-F). Two days after transfection, cells were fixed, stained with DAPI and examined for EGFP-expression (A, D) and nuclear morphology (B, E). For a better overview, some transfected cells are marked by white arrows. C, F. Overlay of A and B or D and E respectively.
Moreover, while most of the colonies formed from EGFP transfected cells showed expression of EGFP judged by fluorescence microscopy, the colonies derived from EGFP-MEKK1 transfected cells were not expressing detectable amounts of fusion protein (data not shown).

Expression of the RBD alone only had a moderate effect. The two RBD-MEKK1 fusion proteins had similar properties compared to each other and a stronger effect than the RBD, but were less potent in colony reduction than the MEKK1 kinase domain alone (data not shown) or EGFP-MEKK1.

We also tested whether protein kinase A had an effect on colony formation. PKA can phosphorylate Raf-1 and thereby inhibit Raf-1 activation as well as Raf-1 kinase
activity directly [144, 145]. Expression of the catalytic subunit of PKA, which is constitutively active, did not result in a reduction of colony formation (data not shown).

3.2.6 The reduction in colony formation by MEKK1 is dependent on kinase activity

We next tested if the effects of MEKK1 on long time survival were dependent on its kinase activity. For that purpose, we generated a kinase negative form of the MEKK1 kinase domain by site-directed mutagenesis. Most commonly, catalytically inactive forms of protein kinases are created by mutating a conserved lysine in the ATP-binding site of the protein, which is essential for its catalytic function [146]. The conserved lysine is highlighted in yellow in figure 3.1. We changed this amino acid to asparagine, because this mutation results in an HpaI restriction site on the DNA level and thereby can easily be detected by restriction analysis. We constructed an EGFP fusion protein, EGFP-MEKK-KN and initially tested its catalytic activity in an in vitro and an in vivo assay. The mutant protein did not phosphorylate GST-MKK4 in vitro (Fig 3.13) and did not induce the phosphorylation of co-transfected MAP kinases in vivo (Fig. 3.14).

Figure 3.13 In vitro kinase activity of EGFP-MEKK-KN.
MCF7 cells were transfected with the indicated plasmids and harvested one day after transfection. EGFP-proteins were precipitated with an anti-GFP antibody and kinase assays were performed as described. Phosphorylated GST-MKK4 was detected using a phospho-specific antibody (lower panel), the upper panel shows the expression of EGFP-MEKK1 and EGFP-MEKK-KN by immunoblot analysis of the cell lysates.

Figure 3.14 In vivo activation of MAP kinases by EGFP-MEKK-KN.
Cos-1 cells were transfected with 0.5 µg HA-MAPK and increasing amounts (0-0.5 µg) of EGFP-MEKK-KN or EGFP-MEKK1 as a control. HA-MAPK were precipitated and analysed for phosphorylation two days after transfection.
Figure 3.15 illustrates, that EGFP-MEKK-KN did not significantly suppress colony formation in MCF7 cells while in the same experiment the wild-type protein EGFP-MEKK1 reduced the number of colonies compared to the EGFP vector control by more than 90%. Similar results were obtained in HeLa cells (Fig. 3.17). These data suggest that apoptosis induction by MEKK1 does indeed depend on kinase activity.

3.2.7 Oncogenic Ras reduces the kinase activity of RBD-MEKK1 fusion proteins

To find out why the RBD fusion proteins were less active in apoptosis induction we compared the kinase activity of the MEKK1 kinase domain, EGFP-MEKK1, and RBD-MEKK1 in an in vitro kinase assay using GST-MKK4 as a substrate. We found that the MEKK1 kinase domain alone and the EGFP-MEKK1 fusion protein had rather similar kinase activities (Fig. 3.16A). Therefore the N-terminal fusion of EGFP to the kinase domain did not inhibit its catalytic activity. In contrast, the kinase activity of RBD1-MEKK was greatly reduced compared to the EGFP-MEKK1 fusion protein (Fig. 3.16B) indicating that fusion to the RBD specifically inhibited the activity of the MEKK1 kinase domain. The RBD binds to Ras in its active, GTP-bound state with high affinity [147, 148]. To test if the reduced kinase activity of the RBD fusion protein was due to an interaction with Ras we performed co-transfection experiments using two activated Ras mutants. The S35 mutant has been described to be able to bind to Raf-1 but not significantly to Ral-GDS or phosphoinositide 3-kinase [149].
Figure 3.16 Fusion to the RBD decreases the in vitro kinase activity of MEKK1.
A. MEKK1 and EGFP-MEKK1 were precipitated from A431 cell lysates two days after transfection using an anti-MEKK1 antibody. The in vitro kinase activity of the immune complexes was determined using GST-MKK4 as a substrate. GST-MKK4 phosphorylation was assayed by immunoblotting with a phospho-specific antibody. B. A431 cells were transfected with decreasing amounts of EGFP-MEKK1 and RBD1-MEKK expression plasmid and in vitro kinase activity of MEKK1 proteins was assayed as described above. C. RBD1-MEKK and RBD(R89L)-MEKK were co-transfected in MCF7 cells with oncogenic Ras or vector controls. Two days after transfection the MEKK1 proteins were precipitated using an anti-Myc antibody and assayed for kinase activity.

We observed that co-expression of activated Ras reduced the in vitro kinase activity of RBD1-MEKK (Fig. 3.16C). To further establish that the decrease in kinase activity was due to Ras binding we used a mutant form of the RBD, RBD(R89L), that is unable to interact with Ras [150]. The RBD(R89L)-MEKK1 fusion protein was not affected by co-expressed Ras proteins (Fig. 3.16C). Taken together, these results show that the
decreased kinase activity of the RBD-MEK fusion proteins compared to the kinase domain alone is due to their interaction with Ras.

### 3.2.8 Effect of Bcl-2 on MEKK1-induced apoptosis

As shown in figure 3.10 MCF7 cells expressing MEKK1 survived longer than A431 or HeLa cells. MCF7 cells express high levels of the anti-apoptotic protein Bcl-2 compared to A431 and HeLa cells (see 3.4.2). To investigate whether MEKK1-induced apoptosis was delayed by Bcl-2 we used HeLa cells, in which endogenously expressed Bcl-2 is not detectable on Western blots (see 3.4.2). In addition to wild-type Bcl-2 we also tested the effect of a mutant form of Bcl-2 in which the three residues Thr69, Ser70, and Ser87 have been mutated to alanine. These sites become phosphorylated in response to microtubule-damaging agents. Ser70 is also phosphorylated during the G2/M phase of the cell cycle, presumably by JNK [139]. The triple mutant has been shown to result in a gain of the anti-apoptotic function of Bcl-2 in response to different stimuli [139]. We did not observe an increase in Bcl-2 phosphorylation (judged by gel-mobility shift) for either endogenous Bcl-2 in MCF-7 cells expressing EGFP-MEKK1 or in HeLa cells co-expressing EGFP-MEKK1 and wild-type Bcl-2 (data not shown).

When co-transfected with EGFP or the kinase negative EGFP-MEK-KN neither wild-type Bcl-2 nor the non-phosphorylatable triple mutant considerably affected the number of colonies in the colony forming assay (Fig. 3.17A and B). The small increase in the number of colonies with the kinase negative EGFP-MEKK-KN lies within the accuracy of the assay and is therefore not significant. The wild-type Bcl-2 or the mutant did not impede the reduction of colony formation by EGFP-MEKK1. In a short-term time course the wild-type and mutant Bcl-2 proteins did not prolong the survival time of EGFP-MEKK1 expressing HeLa cells (Fig. 3.17C). This suggests that the longer survival time of MCF7 cells compared to other cell lines is not due to their high levels of endogenous Bcl-2.
Figure 3.17 Bcl-2 does not prolong clonogenic or short-term survival of MEKK1 expressing HeLa cells. 

A. Colony forming assay of HeLa cells transfected with equal amounts of the indicated plasmids. B. Quantification of A, the number of colonies are shown relative to the vector (EGFP+pcDNA3) transfected cells, which has been set to 100%. Data shown is a representative of two independent experiments. C. HeLa cells were transfected with the specified plasmids in a 1:5 ratio. The survival of transfected cells was monitored over a period of three days. For clarity, the rate of transfection on day 1 has been set to 100%. Samples were analysed in duplicates. Data shown is a representative example of three independent experiments.
3.2.9 Lethal doses of MEKK1 expression activate JNK, ERK and p38

Knockout studies showed that endogenous, full-length MEKK1 is a specific activator of the JNK pathway, but can contribute to ERK activation in response to certain stimuli [94]. However, high overexpression of the active MEKK1 kinase domain can lead to the activation of all three MAP kinases JNK, ERK, and p38 [80, 100]. This is in accord with our own findings, that EGFP-MEKK1 causes the phosphorylation co-transfected JNK, ERK and p38. To test which MAP kinases become activated by apoptosis-inducing doses of MEKK1, we performed a titration experiment using the EGFP-MEKK1 fusion protein. We found that 30 ng of EGFP-MEKK1 expression plasmid (6.3% of total transfected DNA) were sufficient to reduce colony formation by about two third (Fig. 3.18A and B). The number of colonies further decreased with increasing amounts of EGFP-MEKK1. At 500 ng EGFP-MEKK1 (100% of transfected DNA) colony formation was virtually abolished. We then examined MAP kinase phosphorylation induced by these amounts of EGFP-MEKK1. MCF7 cells can be transfected with high efficiency; we routinely achieved transfection efficiencies above 50% (data not shown). Since MEKK1 caused the activation of MAP kinases through a predominantly intracellular mechanism (3.2.3), we forewent co-transfecting HA-tagged MAP kinases and instead analysed the phosphorylation status of the endogenous MAP kinases. All three MAP kinases JNK, ERK, and p38 became activated even with the lowest dose of EGFP-MEKK1 tested and could be detected using phosphorylation-specific antibodies (Fig. 3.18C). EGFP-MEKK1 did not detectably change the level of ERK or JNK expression. Interestingly, at the amounts of MEKK1 tested, JNK, ERK and p38 activation showed a similar dose dependence: for lower amounts of EGFP-MEKK1 the degree of MAP kinase activation increased in a dose dependent manner. Activation of MAP kinases reached a saturation with 150 ng EGFP-MEKK1 expression plasmid corresponding to 25% of total transfected DNA. At this dose, colony formation was not maximally suppressed yet, which suggests that MAP kinases might not be the only mediators of MEKK1-induced apoptosis. Similar results were obtained with A431 and HeLa cells (data not shown).
3.2.10 Low levels of MEKK1 expression preferentially activate JNK

The results from above suggest that apoptotic doses of the MEKK1 kinase domain lead to the activation of the three major MAP kinase families JNK, ERK and p38, even
though full-length MEKK1 at physiological levels of expression is a specific activator of the JNK and ERK pathways [94]. As overexpression of protein kinases can result in loss of substrate specificity, we were interested to see if low doses of EGFP-MEKK1 and the RBD-MEKK1 fusion proteins show specificity for the JNK pathway. In order to test this, we did titration experiments with sub-lethal doses of EGFP-MEKK1 and RBD1-MEKK in MCF7 cells. As shown in figure 3.19, transfection with as little as 7.5 ng expression plasmid (1.3% of transfected DNA) for EGFP-MEKK1 (Fig. 3.19A) or RBD1-MEKK (Fig. 3.19B) induced a low, but detectable increase in JNK phosphorylation. For ERK, 15 ng or 60 ng DNA are required to detect a change in phosphorylation status by EGFP-MEKK1 and RBD1-MEKK respectively.

![Figure 3.19 Low levels of MEKK1 expression activate JNK preferentially.](image)

Phosphorylated p38 could be detected with 7.5 ng EGFP-MEKK1 and 30 ng RBD1-MEKK. The amount of JNK, ERK and p38 was equal in all samples (data not shown). Because these data rely heavily on the (unknown) sensitivity of the different
phosphorylation-specific antibodies, they do not strictly prove that MEKK1 is a specific activator of the JNK pathway. Still, it seems likely that at very low doses of overexpression EGFP-MEKK1 and RBD1-MEKK lead to the activation of JNK preferentially.

3.2.11 Effect of dn-MKK4, MEK inhibitors and p38 inhibitor on MEKK1-induced apoptosis

Because apoptotic doses of MEKK1 expression activated JNK, ERK and p38 we were interested in the roles of the different MAP kinase families in MEKK1-induced apoptosis. We used a dominant negative (dn) mutant form of MKK4 (HA-SEK1-AL) to inhibit JNK activation by MEKK1 and the pharmacological inhibitors SB203580 and PD98059 to inhibit p38 and ERK signalling respectively. The inhibition of MEKK1-induced MAP kinase activity by this strategy in HeLa cells is demonstrated in figure 3.20. The first two panels illustrate the expression of the transfected proteins (for clarity, the expression of EGFP in the vector transfected controls is not shown). Co-transfection of HA-SEK1-AL slightly increased the amount of EGFP and EGFP-MEKK1 detected by Western blot. As FACS analysis revealed, this was due to an increased expression of these proteins rather than to an increase in transfection efficiency (data not shown). Still, co-transfection of HA-SEK1-AL almost completely abolished the activation of JNK by EGFP-MEKK1. The MEK inhibitor PD98059 prevented ERK activation by MEKK1 and, interestingly, also had a slight negative effect on JNK activation. This was not due to a decreased expression of EGFP-MEKK1 in these cells. The p38 inhibitor SB203580 did not interfere with the activation of JNK or ERK by EGFP-MEKK1. It also did not influence the phosphorylation of p38. This was expected, as the inhibitor targets p38 directly rather than p38 activation. To monitor p38 inhibition by SB203580 one has to look at the phosphorylation of p38 targets. The most commonly used indicator of p38 activity is MAPKAP kinase 2, a direct p38 substrate. As shown in figure 3.37C the concentration of SB203580 used here was sufficient to completely abolish activation of MAPKAP kinase 2 by anisomycin, a strong chemical inducer of p38 activity. None of the pharmacological inhibitors or the expression of dn MKK4 altered the expression levels of JNK or ERK.
Results

Intriguingly, co-expression of EGFP-MEKK1 and dominant negative MKK4 led to a slight but reproducible decrease in the level of IκB, while expression of EGFP-MEKK1 alone had no measurable effect. IκB is a cytosolic protein that binds the survival promoting transcription factor NFκB and prevents it from entering the nucleus, its physiological site of action [119]. Thereby, IκB inhibits NFκB function and a decrease in the IκB protein level normally corresponds to an increase in NFκB activity.

When we monitored the survival of EGFP-MEKK1 expressing HeLa cells over a period of three days we surprisingly found that inhibition of the JNK pathway did not have any measurable effect on cell survival (Fig. 3.21A). The MEK inhibitor PD98059 and the p38 inhibitor SB203580 also did not affect apoptosis (Fig. 3.21B). These data do not rule out an involvement of MAP kinases in MEKK1-induced apoptosis, but suggest that the induction of cell death by MEKK1 does not depend on the activation of a single MAP kinase cascade.
3.2.12 MEKK1-induced apoptosis is independent of c-Myc

One study of MEKK1-induced apoptosis in Swiss 3T3 cells and REF52 fibroblasts reports that apoptosis is not dependent on JNK or c-Jun activation and that MEKK1 induces transcription from a c-Myc responsive promoter independent of JNK [85]. This suggests that c-Myc might play a role in apoptosis induction by MEKK1. Thus, we tested if inhibition of c-Myc had an effect on MEKK1-induced apoptosis in HeLa cells. We used two strategies to inhibit c-Myc. Firstly, we overexpressed Mad, which will sequester c-Myc from transcriptionally active Max/Myc heterodimers. Secondly, we expressed c-Myc proteins lacking the N-terminal domain (c-Myc\(\Delta Br\)) or with an insertion in the DNA-binding domain (c-Myc\(\text{Ins373}\)), which have been described to act as dominant negatives [140]. As illustrated in figure 3.22, overexpression of Mad (Fig. 3.22A) or the dominant negative forms of c-Myc (Fig. 3.22B) had no effect on the
survival rate of MEKK1 expressing cells. Therefore, MEKK1 induced apoptosis independent of c-Myc.

![Graph A](image.png)

![Graph B](image.png)

**Figure 3.22 Survival of MEKK1 expressing cells is not prolonged by inhibition of c-Myc.** A. HeLa cells were transfected with EGFP or EGFP-MEKK1 and Mad or pcDNA3 as a vector control in a 1:5 ratio. Survival was monitored in triplicate samples. Transfection rates on day 1 have been set to 100%. B. HeLa cells were transfected with EGFP or EGFP-MEKK1 and dominant-negative forms of c-Myc or pRcCMV as a vector control in a 1:5 ratio. Survival was monitored in duplicate samples over a period of three days. For clarity, the transfection rate on day 1 has been set to 100%. Data shown is a representative of two independent experiments.

**3.2.13 MEKK1 proteins and the RBD reduce clonogenic survival of non-cancer cells**

We found only little differences in the response to MEKK1 overexpression between the human cancer cell lines used in this study, suggesting that killing by MEKK1 might be independent of the genetic background of the cancer cell. As a number of oncogenes renders cancer cells more susceptible to apoptosis [58, 151-153], we were interested to see if MEKK1 could induce apoptosis in non-transformed cells or if this was specific to
Results

cancer cells. Because primary human cells are not easily available and often difficult to transfect, we used telomerase-immortalised human fibroblasts as a model system for non-transformed cells. Transgenes can be introduced into these cells by retroviral gene transfer. We also used p53 and p21 knockout cell lines derived from these fibroblasts to investigate if the p53 status of a cell influences its susceptibility to apoptosis induced by MEKK1. We looked at the long-term viability of these cells by performing clonogenic assays. As shown in figure 3.23, the basal clonogenicity of these cells is relatively low. It is completely abolished by expression of the MEKK1 kinase domain and by the RBD1-MEKK fusion protein. The RBD alone reduces colony formation in these cell lines to a much greater extent than it did in the human cancer cell lines (see 3.2.5).

The retroviral constructs used in the experiments described above drive the expression of the transgene from the long terminal repeat (LTR) promoter region. The plasmids used for the colony assays with the human cancer cells express transgenes from the Cytomegalovirus (CMV) promoter. As illustrated in figure 3.24, the LTR is a considerably weaker promoter compared to the CMV promoter. Here, we transfected MCF7 cells with the CMV-controlled plasmids EGFP-C1 and EGFP-MEKK as well as with the LTR-driven, retroviral expression constructs Babe-EGFP and Babe-EMEKK1.
We harvested the cells two days after transfection. At this time point, no green fluorescence could be detected in cells transfected with the retroviral plasmids by fluorescence microscopy while expression from the CMV promoter was readily detectable already one day after transfection (data not shown). Immunoblot analysis revealed that the expression of the EGFP proteins from the CMV promoter is much higher than from the LTR promoter sequence (figure 3.24).

The light spots in the centre of the EGFP and the EGFP-MEKK1 signals in this figure appear because the signal intensity is so high, that the substrate for the ECL reaction has been used up in the middle of the bands. We wanted to confirm that the level of expression from the LTR was sufficient to reduce colony formation of human cancer cell lines. We tested this in A431, HeLa and MCF7 cells and found that, similar to the results in 3.2.5, the MEKK1 kinase domain alone and in fusion with the RBD reduced colony formation (Fig. 3.25). This effect was again most striking in the MCF7 cells. But the reduction of colony formation by MEKK1 proteins seemed to be less, when they were expressed from the LTR compared to the CMV promoter (compare Fig. 3.25 to Fig. 3.12). Different from 3.2.5, the RBD1-MEKK protein reduced colony formation as well as the kinase domain alone. The RBD, which had a low effect when expressed under control of the CMV promoter, had no effect when expressed from the LTR. Taken together, these results suggest that indeed the lower expression levels from the LTR compared to the CMV promoter reduce the potency of the transgenes in respect to their ability to reduce colony formation, but that the expression levels of the MEKK1 proteins are still sufficient to produce measurable effects.
Figure 3.25 The expression from retroviral constructs is sufficient to reduce clonogenic survival of human cancer cell lines. A431, HeLa and MCF7 cells were infected with the indicated retroviruses. Two days after infection, puromycin was added to the culture medium to a final concentration of 0.4 µg/ml. The resulting colonies were stained with Giemsa after 10 days. Infections were carried out in triplicates.
3.3 Use of β-Galactosidase as a marker for apoptosis

To monitor growth and survival of transfected cells we were looking for an enzymatic marker that – when co-transfected – could be easily assayed in a quantitative manner. One such marker is β-Galactosidase. The idea behind this assay is illustrated in figure 3.26. If β-Galactosidase is co-transfected with a protein that induces or sensitises cells to apoptosis, the ratio of transfected cells will decrease from the initial transfection rate and the Galactosidase activity in a cell lysate will decrease accordingly (Fig. 3.26A). If a co-transfected protein protects from apoptosis, the number of transfected cells will increase compared to untransfected cells (Fig. 3.26C). Thus, the ratio of transfected cells and the Galactosidase activity in a cell lysate will increase.

![Diagram showing the assay process](image)

**Figure 3.26 Monitoring cell growth and survival by assaying β-Galactosidase activity.** Schematic overview. The protein X of interest is co-expressed with β-Galactosidase. If protein X induces apoptosis, the number transfected cells will decrease compared to untransfected cells. In a cell lysate, this will lead to a decrease of Galactosidase activity per mg protein (A). If protein X has no influence on apoptosis or growth and survival, the number of transfected cells should be equal to the vector control and the Galactosidase activity in the lysates should be the same (B). If protein X promotes growth, transfected cells should grow faster than untransfected and the Galactosidase activity per mg protein in lysates should increase accordingly (C).

First, we tested which amounts of a β-Galactosidase reporter gene resulted in a measurable enzyme activity in cell lysates. Thereafter, we transfected MCF7 cells with increasing amounts of pPGKβGal cells and harvested the cells two days after transfection. Lysates were then assayed for Galactosidase activity. Figure 3.27 shows,
that β-Galactosidase activity could be detected in 25 µl lysate from cells transfected with as little as 0.06 μg of reporter gene DNA or more. Moreover, the assay was in its linear range for all the amounts of DNA and lysate tested. For future experiments, 0.2 to 0.3 μg pPGKβGal were transfected and around 30 µl lysate were assayed.

We next wanted to see if co-transfection with EGFP-MEK1, RBD1-MEKK or the RBD alone would reduce the number of β-Galactosidase expressing cells and thereby lower the Galactosidase activity in cell lysates. In order to do so, we transfected these plasmids or EGFP as a control together with 0.2 μg pPGKβGal and harvested the cells after three days. As shown before (3.2.4), at this time the number of living, MEKK1 expressing MCF7 cells is reduced compared to control cells. Surprisingly we found a higher β-Galactosidase activity in lysates from cells co-expressing EGFP-MEK1, RBD1-MEKK and the RBD compared to EGFP co-expressing cells (Fig. 3.28).

In another experiment, in which we harvested the cells four days after transfection, we again found that co-expression of the MEKK1 kinase domain or EGFP-MEK1 increased the Galactosidase activity compared to the respective vector controls pcDNA3 or EGFP. RBD1-MEKK and the RBD had no effect in this experiment (Fig. 3.29).
One possible explanation for this could be, that the increased \( \beta \)-Galactosidase activity might be endogenous. Mammalian cells also express a Galactosidase, an increase of Galactosidase expression and activity has been shown to be associated with senescence [154]. Therefore, we tested if prolonged MEKK1 expression induces endogenous Galactosidase activity in MCF7 cells. However, we found no differences in the \( \beta \)-Galactosidase activity of lysates from EGFP or EGFP-MEKK1 expressing cells four days after transfection, suggesting MEKK1 expression does not induce endogenous Galactosidase (data not shown).

Another explanation for the increased \( \beta \)-Galactosidase activity in lysates from MEKK1 co-expressing cells could be, that MEKK1 might upregulate the expression of \( \beta \)-Galactosidase, which is under control of the murine phosphoglycerate kinase (PGK)
promoter. This would lead to an increase of the amount of β-Galactosidase in MEKK1 transfected cells. By assaying cell lysates, one averages enzyme activity over the whole cell population. In this situation, an increase of β-Galactosidase expression in MEKK1 co-expressing cells could compensate or even override a decrease in the amount of transfected cells. To test this hypothesis, we used MCF7 cells and harvested the cells one day after transfection. At this early time point, MEKK1 does not cause apoptosis of these cells yet (3.2.4), while a possible effect of MEKK1 activity on β-Galactosidase expression from the PGK promoter should already be detectable.

We found an increase of Galactosidase activity in lysates of cells co-expressing MEKK1 compared to the vector controls, supporting our hypothesis that MEKK1 upregulates transcription from the PGK promoter (Fig. 3.30). RBD1-MEKK caused a slight decrease in β-Galactosidase activity. Surprisingly, dominant negative MKK4 (SEK1-AL) also caused an increase in Galactosidase activity, suggesting that inhibition of JNK activation also increases PGK promoter activity.

The common feature of the proteins we co-transfected with β-Galactosidase is that they somehow interfere with JNK signalling. The major substrates of JNK are transcription factors. Hence, it is not surprising that co-expression of proteins like constitutively active MEKK1 or dominant negative MKK4 should modulate the transcription from the PGK (or any other) promoter. PGK is a housekeeping gene expressed constitutively in all somatic and premeiotic germ cells [155]. We were therefore looking for a promoter that is not responsive to JNK activity. A good candidate was the RSV promoter, as its activity seems to be independent of c-Jun N-terminal phosphorylation (D. Gillespie, personal communication). We consequently generated the reporter plasmid pRSVβGal that allows the expression of β-Galactosidase from the Rous sarcoma virus (RSV) promoter. We then co-transfected this reporter together with MEKK1, RBD1-MEKK
and the RBD into MCF7 cells and monitored $\beta$-Galactosidase activity in cell lysates over a period of four days. As a positive control, we used an oncogenic form of Ras (RasV12), which we expected to increase $\beta$-Galactosidase activity compared to control cells (see Fig. 3.26C). We found that co-expression of activated Ras strongly increased Galactosidase activity already one day after transfection (Fig. 3.31). Co-expression of the constitutively active kinase domain of MEKK1 also increased Galactosidase activity, though this effect became apparent only after day two post transfection. The $\beta$-Galactosidase activity of lysates from RBD and RBD1-MEKK co-expressing cells was slightly lower compared to control cells, but this effect was very small in comparison to the upregulation caused by RasV12 or MEKK1.

![Figure 3.31 Co-expression of $\beta$-Galactosidase from the RSV promoter.](image)

MCF7 cells were co-transfected with pRSV$\beta$Gal and the specified plasmids in a 1:5 ratio. Cells were lysed at the indicated time points and lysates equalized for protein content. Galactosidase activity was assayed in triplicate samples.

Taken together, it seems that with both the PGK and the RSV promoter $\beta$-Galactosidase activity measured in whole cell lysate is not a good indicator for the number of transfected, living cells. Therefore, we abandoned these experiments.
3.4 The role of MAP kinases in the action of chemotherapeutic drugs

While most chemotherapeutic drugs induce apoptosis and exhibit some selectivity for tumour cells, the mechanisms by which these drugs trigger cell death are largely unknown. It has emerged that they are capable of activating MAP kinases [156] but how MAP kinase activation is mediated and its consequences on the induction and execution of apoptosis are only poorly understood.

We have investigated the effects of the chemotherapeutic taxol and etoposide as well as the apoptosis-inducer ceramide on three different human cancer cell lines: the cervical carcinoma cell line HeLa, the squamous carcinoma cell line A431 that overexpresses the EGF receptor, and MCF7 cells, an estrogen responsive breast carcinoma cell line.

3.4.1 Induction of apoptosis 1: Cell morphology and Annexin V-binding

Firstly, we investigated the sensitivity of these cell lines towards taxol, etoposide and ceramide. Figure 3.32 column A shows the effects of these drugs on the morphology of HeLa cells. Taxol, a microtubule-disrupting agent which arrests the cells in the G2/M phase of the cell cycle ([157] and 3.4.5.C) led to extensive rounding and detachment of the cells. The topoisomerase II inhibitor etoposide caused intense membrane blebbing and the formation of so-called apoptotic bodies. Ceramide treated cells first shrank and later became round and detached. These differences in morphology were reflected in the light scattering properties of the cells during FACS analysis; the side scatter (SSC-Height, x-axis in Fig. 3.32B) indicates the granulation of a cell while the forward light scatter (FSC-Height, y-axis in Fig. 3.32B) reflects its size. For taxol, both values went slightly up, indicating an increase in size and granulation. Similar changes were observed with etoposide treated cells, though here the increase in granulation (SSC) was not as prominent. Ceramide treated cells, however, only showed very little changes in their light scattering properties. Though the three different drugs caused very distinct changes in the morphology they all induced apoptotic cell death. As shown in figure 3.32C, taxol, etoposide and ceramide-treated HeLa cells bound fluorescein-conjugated Annexin V. Annexin V is a Ca\(^{2+}\)-dependend phospholipid-binding protein with high affinity for phosphatidylserine (PS) [135]. PS is normally located at the inner surface of the plasma membrane and translocates to the outer layer during the early stages of apoptosis. Annexin V is widely used as a probe to detect PS exposure and hence apoptotic cells.
A431 cells showed very similar changes in morphology in response to apoptosis-inducing reagents compared to HeLa cells (Fig. 3.33).
In contrast to this, MCF7 cells were more robust to apoptosis induction. The cells shown in figure 3.34 have been treated with taxol, etoposide or ceramide for 40 hours, the HeLa and A431 cells have only been treated overnight. Still, hardly any morphological changes became apparent, only a slight rounding after treatment with taxol.

Figure 3.33 Drug sensitivity of A431 cells. A431 cells were treated and stained as described for figure 3.32. A. Phase contrast images of drug treated cells before trypsinisation. B. Light scattering properties. C. Annexin V-FITC staining.
Consequently, the light scattering properties of the drug-treated cells were similar to the control cells. Still, these cells were undergoing apoptosis: the ceramide and to a lower extend the taxol treated cells both show an increase in Annexin V-binding.

Figure 3.34 Drug sensitivity of MCF7 cells. MCF7 cells were treated with 50 nM taxol, 40 μg/ml etoposide or 20 μM ceramide for 40 h. Cells were then washed, detached by trypsinisation and stained with Annexin V-FITC as described in 2.3.15. A. Phase contrast images of drug treated cells before trypsinisation. B. Light scattering properties. C. Annexin V-FITC staining.
3.4.2 Expression of Bcl-2 and caspase-3

Because we were interested to see why MCF7 cells are more resistant to apoptosis compared to A431 and HeLa cells we looked at the expression levels of proteins known to be involved in the regulation and execution of apoptosis. We found, that our MCF7 cells displayed a strong overexpression of the anti-apoptotic protein Bcl-2 compared to the other cell lines (Fig. 3.35). High expression levels of Bcl-2 and other anti-apoptotic members of this protein family have been implicated in mediating resistance to apoptosis induced by different stimuli, including anti-cancer drugs [158-161].

Moreover, these cells did not express caspase-3, the main executioner protease of apoptosis (Fig. 3.35). This has been shown to be due to a 47-base pair deletion within exon 3 of the CASP-3 gene [162].

3.4.3 Induction of apoptosis 2: PARP cleavage

The lack of caspase-3 complicates the detection of apoptosis in MCF7 cells, as most assays probe for processes that are associated with the activation of this protease. One example for this is the cleavage of Poly-ADP-ribose polymerase (PARP). This 115 kDa protein is cleaved by caspase-3 directly and the appearance of the 85 kDa proteolytic fragment is one of the most widely used indicators for apoptosis. As illustrated in figure 3.36, PARP cleavage was readily apparent in A431 and HeLa cells in response treatment with taxol, etoposide or ceramide, and to growth factor withdrawal (serum starvation). In accordance to their lack of caspase-3, MCF7 cells did not display PARP cleavage, even tough they were sensitive to these agents (3.4.5.B-3.4.5.D).
3.4.4 Activation of MAPK cascades by apoptosis-inducing drugs

Even though taxol, etoposide and ceramide have very diverse intracellular targets, they all are able to induce apoptosis. The different mechanisms by which these drugs induce apoptosis and if and where these pathways converge are still unclear. MAP kinase cascades are good candidates, because chemotherapeutic drugs have been shown to have some bearing on MAP kinase signalling [156] and MAP kinases have been implicated in apoptosis regulation in certain cell types [80, 163, 164]. Consequently, we were interested to test the effects of taxol, etoposide and ceramide on MAP kinase activity in the three cell lines.

Phospho-specific antibodies were used to selectively stain the activated form of ERK. JNK activation could not be detected using commercially available phospho-specific antibodies and had to be monitored by kinase assays. Treatment with the drugs had no consequences on the expression levels of ERK, JNK and p38 (data not shown). Interestingly, the effects of the drugs on ERK and JNK activities were highly divergent, but mainly depending on the cell type rather than the type of drug (Fig. 3.37A). In A431 cells the ERK pathway is constitutively hyper-activated due to the overexpression of the EGF receptor. Nevertheless, all drugs enhanced ERK activation with delayed, but sustained kinetics. JNK activity was low, but also was induced by all agents. Because HeLa cells are very sensitive to apoptosis induced by taxol and ceramide we could monitor MAP kinase activation in response to these agents for only 24 and 18 hours respectively, compared to 36 hours for the other cell lines and etoposide treated HeLa cells. ERK activation by all agents was biphasic with a rapid, growth factor-like
induction after 15 minutes. The activity returned to almost baseline between 6-9 hours and then rose again at 12 hours. A similar biphasic activation pattern was seen for JNK activity in response to ceramide or etoposide. In contrast, taxol induced a slow, but steady increase of JNK activity. In MCF7 cells the activities of ERK and JNK exhibited only little response to drug treatment compared to A431 and HeLa cells. Only ceramide produced a clear biphasic ERK activation similar to that seen in HeLa cells. Taxol induced no initial activation of ERK, but a transient repression between 9-12 hours, which was followed by a slight hyper-activation. Taxol also induced a small biphasic JNK activation, whereas the other drugs caused a transient repression followed by a recovery of activity between 6-18 hours and another decline thereafter.

Similar to JNK, we could not detect any changes in the level of phosphorylated p38 with the commercially available phospho-specific antibodies, presumably because the changes in activity were below the threshold of detection. Therefore, the activity of MAPKAP kinase 2, a direct p38 substrate [165], was assayed instead (Fig. 3.37B). Only etoposide-induced MAPKAP kinase 2 activation in all three cell lines. The activation was transient in A431 and MCF7 cells reaching maxima at 6 to 12 and 6 hours respectively, while HeLa cells again showed a biphasic activation profile similar to the profile observed for the other MAP kinases. In order to verify that MAPKAP-2 activity reflects p38 activity, the p38 inhibitor SB203580 was employed (Fig. 3.37C). As a control anisomycin, a bona fide chemical inducer of p38 kinase activity was used. SB203580 inhibited both anisomycin- and etoposide-induced MAPKAP kinase 2 activation in a dose dependent manner validating the use of MAPKAP kinase 2 as a surrogate for measuring p38 activation.
Figure 3.37 Drug induced activation of MAPK pathways. A. Time course of ERK and JNK activation. Growing cells were treated with 50 nM taxol, 40 μg/ml etoposide or 20 μM ceramide in normal growth medium for the indicated time (in hours). Cells were lysed in JNK assay lysis buffer. Samples were normalised to equal protein contents and used to determine JNK activity by the phosphorylation of GST-cJun and ERK activation by immunoblotting with phospho-ERK (P-ERK) specific antibodies. B. Time course of MAPKAP kinase 2 activity. Cells were treated as in (A) and MAPKAP-2 activity was assayed. C. MAPKAP kinase 2 activity correlates with p38 activity. A431 cells were treated with 40 μg/ml etoposide for 7 h or 10 μg/ml anisomycin for 15 min in the presence of the indicated concentrations SB203580, and MAPKAP-2 activity was measured. Control cells were treated with the indicated concentrations of the inhibitor only.
3.4.5 **Relationships between MAPK activation and biological drug effects**

All drugs induced the activation of different MAP kinase pathways in a cell type specific manner. Chemotherapeutic drugs affect different cellular functions including cell cycle, cell viability and survival. To gain an insight into whether MAP kinase activation is required for any of these actions of we used pharmacological inhibitors of MEK and p38 and investigated their effect on apoptosis, cell viability, cell cycle and clonogenic survival.

### 3.4.5.A Apoptosis

First, we investigated whether the inhibition of MEK or p38 affects apoptosis induction. As a marker for apoptosis, PARP cleavage was used. In A431 cells the inhibitors had only small effects on PARP cleavage (Fig. 3.38), although they efficiently inhibited the respective kinases (Fig. 3.37C and data not shown). However, in HeLa cells the inhibition of p38 suppressed PARP cleavage, whereas the inhibition of ERK increased it. The latter observation supports the original reports that the ERK pathway prevents apoptosis while p38 promotes it [80]. However, the results also indicate that this phenomenon is cell type specific and cannot explain the action of the drugs in general.

**Figure 3.38 The effect of MEK and p38 inhibitors on drug-induced apoptosis.** A431 and HeLa cells were pre-treated for 1 h with 40 µM PD98059, 5 µM SB203580 or DMSO to a final concentration of 0.2%. The cells were then incubated overnight in the presence of 50 nM taxol, 40 µg/ml etoposide or 20 µM ceramide. Lysates were equalised for protein content and PARP cleavage was analysed by immunoblotting. Results shown are representatives of four independent experiments.

### 3.4.5.B Cell viability

In parallel, we assayed cell viability using the WST cell viability kit. This assay measures the metabolic function of the mitochondria and is widely used to assay cell proliferation and survival. All drugs reduced cell viability judged by mitochondrial metabolism (Fig. 3.39). Surprisingly, neither the inhibition of MEK or p38 affected the reduction of cell viability. In HeLa cells, p38 inhibition slightly enhanced viability in
response to etoposide and ceramide treatment, whereas MEK inhibition increased the viability of ceramide treated cells. In A431 cells, the MEK inhibitor modestly increased viability in control and taxol treated cells. These results are only in part consistent with the apoptosis assays presented in figure 3.38, suggesting that the drugs have multiple modes of action. Therefore, we further tested other biological parameters.

![Graph showing absorbance](image)

**Figure 3.39** The effect of MEK and p38 inhibitors on the drug-induced reduction of mitochondrial metabolic function. Cells were treated and analysed using the WST assay kit as described in 2.3.14. Samples were analysed in triplicates, and the data shown are representative of two or three independent experiments.

### 3.4.5. C Cell cycle

All drugs have in common that cells feature high ERK and JNK activity around 18 hours after treatment. As shown in figure 3.40, taxol and ceramide cause cells to accumulate in the G2/M phase of the cell cycle at this time point with the exception of ceramide treated HeLa cells, which retained a normal cell cycle profile. We used the MEK inhibitor PD98059 and the p38 inhibitor SB203580 to investigate, whether MAP kinases play a role in G2/M arrest. The inhibitors failed to rescue drug-induced cell cycle blockades in A431 (Fig. 3.40A) or HeLa cells (Fig. 3.40B), indicating the arrest might be independent of ERK and JNK.
3.4.5.D Clonogenic survival

At last, we tested the effects of MAP kinase activation on the clonogenic survival of drug treated cancer cells. This assay is perhaps most important parameter for the effectiveness of a chemotherapeutic drug because it shows the long-term consequences of a pulse of drug treatment on cancer cell viability.

Cells were treated with drugs and MEK and p38 inhibitors as detailed in the figure 3.41. After that they were seeded into normal growth medium and the outgrowth of cell colonies was scored 10-14 days later. Figure 3.41 shows, that all drugs significantly reduced colony formation.

Figure 3.40 The effect of MEK and p38 inhibitors on drug-induced cell cycle blocks. Cells were treated (c: control, t: taxol, e: etoposide, C₆: ceramide) as in 3.4.5.A and cell cycle profiles were analysed as described in 2.3.13. A. A431 cells. B. HeLa cells. Data shown is representative of two independent experiments.
Figure 3.41 The effect of ERK and p38 inhibitors on drug-induced reduction of clonogenic survival. Cells were pre-treated with the inhibitors or DMSO as a control as described in 2.2.8. Apoptosis was stimulated with 50 nM Taxol for 3 h or 4 mg/ml etoposide for 1 h in all cell lines. A431 cells were treated with 20 \( \mu \)M C6-ceramide for 6 h, HeLa and MCF7 were treated overnight with the same concentration. Data shown are representative of five independent experiments.
Curiously, the MEK inhibitor PD98059 increased the number of colonies in taxol treated HeLa and MCF7 cells suggesting that MEK inhibition was protective. In contrast, in A431 and to a lesser degree in MCF7 cells both the MEK and p38 inhibitors decreased the colony yield in response to etoposide. Similar results were obtained with another MEK inhibitor, UO126 (data not shown).
4 Discussion

4.1 Targeting MAP kinase pathways to kill cancer cells

The present study demonstrates the ability of activated MEKK1 to induce apoptosis in human cancer cell lines. MEKK1 strongly inhibited colony formation in seven different human cancer cell lines, indicating that the ability of MEKK1 to induce apoptosis might be independent of the genetic background of the cancer cell.

4.1.1 Activation of MAP kinases by MEKK1

MEKK1 had originally been identified as a protein kinase that activates MEK in a Ras-dependent manner [96, 166]. Only later it has been shown to be a specific activator of the JNK and the p38 pathway [99, 100] and to be able to induce or sensitise cells to apoptosis. Most studies on the role of MEKK1 in the induction of MAP kinase activity and apoptosis have been carried out in overexpression systems. A more recent study with MEKK1 knockout mouse embryonic fibroblasts (MEFs) showed that MEKK1 is a specific activator of the JNK pathway in these cells [94]. It also contributed to ERK activation in response to certain stimuli, but the knockout MEFs did not show an impaired activation of p38 in response to any stimuli tested. These cells have been shown to be more sensitive to apoptosis in response to certain anticancer drugs including microtubule-disrupting agents, suggesting a protective role of the MEKK1-JNK pathway [103, 167]. But more recently, in a chicken B-cell line the disruption of the MEKK1 gene has been shown to have the opposite effect on the sensitivity of these cells towards microtubule-damaging drugs [95]. Therefore, MEKK1 signalling can, depending on the cell type, have opposing roles in apoptosis and survival in response to the same stimulus.

The apoptotic potential of MEKK1 has been reported to be dependent on MAP kinase, i.e. JNK activation [80]. It is not completely clear yet how the activation of JNK might induce apoptosis. Phosphorylation events mediated by JNK have been reported to inactivate Bcl-2 [139, 168] and to stabilise p53 [169, 170]. Another suggested mechanism is that JNK signalling leads to an increase in the expression of Fas-ligand and therefore can induce apoptosis via an autocrine or paracrine mechanism [171, 172]. However, we did not find any evidence that killing by MEKK1 was using autocrine or paracrine factors. Conditioned supernatants from EGFP-MEKK1 expressing MCF7 cells did not induce apoptosis of HeLa cells, which are susceptible to Fas-mediated apoptosis (data not shown). Moreover, MAP kinases have been shown to be
downstream signalling molecules of the TNF and Fas receptors, but the induction of MAP kinase activity by MEKK1 was predominantly intracellular.

Overexpression of the kinase domain of MEKK1 results in the loss of specificity and the activation of all three MAP kinases JNK, ERK and p38. Specificity for the JNK pathway can be restored by lowering the expression level of MEKK1 ([100] and our own observations). When we tested which MAP kinases become activated by lethal doses of MEKK1 expression, we found that only amounts of MEKK1, which were sufficient to induce activation of all three MAP kinases – JNK, ERK, and p38 – were capable of reducing colony formation. Therefore, activation of the allegedly anti-apoptotic ERK pathway by MEKK1 overexpression did not seem to counterbalance the supposedly pro-apoptotic effects of JNK and p38. This suggests that not the activation of the stress signalling MAP kinases JNK and p38 per se, but the general deregulation of MAP kinase activity by overexpression of the (unspecific) activator MEKK1 results in apoptosis. This is supported by our findings that neither the co-expression of a dominant negative mutant of MKK4, nor the p38 inhibitor SB203580 or the MEK inhibitor PD98059 impinged on apoptosis induced by MEKK1 overexpression. These mutants or drugs block the activation of a single pathway but do not restore the ability of the cell to regulate these pathways in a balanced way.

4.1.2 The role of Bcl-2 in MEKK1-induced apoptosis

The survival time of MEKK1 expressing cells differed with the cell line. MCF7 cells survived much longer than HeLa or A431 cells. More than 50% of MEKK1 expressing MCF7 cells were still alive four days after transfection compared to less than 10% of A431 or HeLa cells. MCF7 cells express high amounts of the anti-apoptotic protein Bcl-2. High expression levels of Bcl-2 and other anti-apoptotic members of this protein family have been implicated in mediating resistance to apoptosis induced by different stimuli, including anti-cancer drugs [158-161]. Moreover, these cells do not express caspase-3, the main executioner protease of apoptosis [162]. Still, MCF7 cells preserve the ability to undergo apoptosis [162, 173], which is consistent with our finding that in the colony forming assay MEKK1 was as potent in killing MCF7 cells as cell lines that had a much shorter survival time. The exact mechanism by which Bcl-2 protects cells from apoptosis is not fully understood. Several reports suggest that Bcl-2 and other anti-apoptotic Bcl-2 family proteins prevent the release of cytochrome C from the mitochondria into the cytosol [174-176] by sequestering pro-apoptotic members of the family [52]. As expatiated in 1.1.1, cytosolic cytochrome C leads to the activation of caspase-9 and thereby initiates the caspase cascade that executes apoptosis [177, 178].
However, our studies with HeLa cells suggest that the longer survival time of MCF-7 cells compared to other cell lines is not linked to their high level of Bcl-2 expression. The overexpression of either wild-type Bcl-2 or the non-phosphorylable triple mutant could not prevent the reduction of colony formation by EGFP-MEKK1 in HeLa cells, which express only very low levels of endogenous Bcl-2. This was not surprising because like any other anti-apoptotic protein Bcl-2 is more likely to delay than to prevent apoptosis. But co-expression of wild-type or mutant Bcl-2 also failed to promote the survival of EGFP-MEKK1 expressing HeLa cells in the short-term time course experiments, which were carried out on the first three days after transfection. Therefore, MEKK1 seems to act independently or downstream of Bcl-2.

The hypothesis that MEKK1 might act independently of Bcl-2 is supported by our finding that apoptosis was not delayed by the inhibition of JNK activation. The interactions of JNK signalling and Bcl-2 are still unclear, but v-Jun and JNK have been shown to sensitise cells towards apoptosis by amplifying the release of cytochrome C from the mitochondria [58, 179]. As discussed earlier, anti-apoptotic Bcl-2 family members prevent the release of Cytochrome C into the cytosol [174-176]. Taken together, these findings suggest that apoptotic JNK signalling and apoptosis regulation by Bcl-2 might converge somewhere on the mitochondrial pathway of apoptosis induction. Though JNK has been reported to phosphorylate and inactivate Bcl-2 [139] it is still unclear, if Bcl-2 is upstream or downstream of JNK in apoptosis induction. That neither inhibition of JNK activation nor Bcl-2 overexpression could delay cell death induced by MEKK1 suggests that MEKK1 at least partially induces apoptosis independent of the mitochondrial pathway of caspase activation. Still, apoptosis induction was dependent on MEKK1 kinase activity and seemed to be intracellular. We were therefore looking for other molecules, which might mediate MEKK1-induced apoptosis.

4.1.3 The role of c-Myc in MEKK1-induced apoptosis

There is one other report which suggests that MEKK1 can induce apoptosis independently of JNK activation. This study also reports that MEKK1 induces c-Myc dependent transcription autonomously of JNK [85]. The transcription factor c-Myc was the first oncogene that has been shown to promote not only proliferation but also apoptosis [151]. The mechanisms and transcriptional targets by which c-Myc accomplishes these seemingly diverse functions are not fully understood and focus of extensive research (reviewed in [180]). It has been reported that JNK can phosphorylate c-Myc and that this promotes Myc-dependent apoptosis [181, 182]. Contrary to this,
another report states that JNK signalling in response to TGF-β prevents Myc-induced apoptosis [183]. However, our own results suggest that MEKK1-induced apoptosis does not depend on JNK activation; a possible connection between MEKK1 signalling and c-Myc independent of JNK is to date unexplored. To test if MEKK1 induces apoptosis via a Myc-dependent pathway we inhibited c-Myc function by two different strategies. Firstly, by ectopic expression of dominant negative forms of c-Myc, that are incapable of DNA binding. Secondly, we overexpressed Mad, a protein that sequesters Max from Myc-Max heterodimers, the transcriptionally active form of c-Myc (see [184] for an extensive review on function and regulation of c-Myc). Both these strategies have successfully been employed to inhibit c-Myc function [140]. However, none of the c-Myc mutants nor overexpression of Mad could prolong the short-term survival of MEKK1 expressing HeLa cells. Therefore, apoptosis induction by MEKK1 does not seem to depend on c-Myc. Thus, it is unlikely that c-Myc is a downstream executioner of MEKK-1 in the apoptosis pathway.

4.1.4 Properties of the RDB-MEKK1 fusion proteins

Unexpectedly, the RBD-MEKK1 fusion proteins, which were designed to combine the apoptotic principles of JNK/p38 activation and inhibition of Ras signalling, were less potent than the MEKK1 kinase domain alone. As we could show, the fusion proteins had a reduced kinase activity in vitro, that was further inhibited by co-expression of oncogenic Ras. Because the EGFP fusion protein had a similar activity like the kinase domain alone, it is unlikely that the reduced activity of the RBD fusion proteins was a result of the fusion per se. Therefore, these data indicate that their lower apoptotic potential might be due to their interaction with Ras. Full-length MEKK1 has been reported to be membrane associated [93], where it is inactive. One mechanism of MEKK1 activation is cleavage by caspase-3 upon which the catalytically active kinase domain supposedly relocates to the cytoplasm [39]. Like many other kinases activation of MEKK1 is also regulated by phosphorylation [105, 111, 112, 166, 185]. This suggests that either MEKK1 is activated by kinases that reside in the cytosol or that it is kept in an inactive state at the plasma membrane. Several reports suggest that the subcellular localisation of MEKK1 activity is the crucial difference between stress signalling and apoptotic signalling (reviewed in [186]). Via their interaction with Ras the fusion proteins are targeted to the plasma membrane. Therefore, it is likely that the reduced kinase activity and the lower apoptotic potential of the fusion proteins are due to their targeting to the plasma membrane where they are sequestered in an inactive
state. Indeed, we found that in vivo the EGFP fusion protein was a more potent inducer of MAP kinase phosphorylation than the RBD-MEKK1 proteins.

As discussed earlier, overexpression of constitutively active MEKK1 results in a loss of substrate specificity and the activation of all three major MAP kinases JNK, ERK and p38. Consequently, overexpression of the RBD1-MEKK fusion protein, which had originally been designed to simultaneously activate the JNK and inhibit the Ras-ERK pathway, also led to the activation of ERK. The activity of the MEKK1 kinase domain compensated for the inhibitory effect of the RBD in this respect. The RBD binds to active, GTP-bound Ras with very high affinity [147, 148]. Therefore overexpression of the RBD sequesters active Ras from its physiological effector proteins like Raf and PI3-kinase (see [64, 187] for recent reviews on Ras signalling) and acts as a dominant negative mutant (reviewed in [66]). Hence, if the fusion protein did inhibit the activation of Ras effectors, including Raf, this would not be detectable by monitoring ERK activity because of the stimulating effect of the MEKK1 kinase domain on this pathway. Indeed, we could show that the reduced kinase activity of the RBD1-fusion proteins compared to the MEKK1 kinase domain alone was further inhibited by activated Ras. A mutant form of the fusion protein, RBD(R89L), that cannot interact with Ras [150, 188] was not affected by co-expression of oncogenic Ras. Taken together, this shows, that the RBD-MEKK fusion proteins do bind to activated Ras and therefore can be expected to inhibit Ras signalling. Still, the RBD-MEKK1 fusion proteins had a significantly lower apoptotic potential compared to the kinase domain alone. This suggests, that the activity of the MEKK1 kinase domain is the major player in apoptosis induction by the RBD-MEKK1 fusion proteins and that the RBD only plays an ancillary role. This is further supported by the comparatively small effect of the RBD alone in the clonogenic assays in the cancer cell lines.

4.1.5 Modulation of MAP kinase pathways in non-transformed cells

MEKK1 strongly suppressed the clonogenic survival of cancer cell lines as diverse as papilloma virus infected HeLa cells, estrogen responsive MCF7 breast carcinoma cells, EGF receptor overexpressing A431 cells, the squamous carcinoma cell lines A2780 and its cisplatin-resistant derivative CP70, as well as the neuroblastoma cell lines SKNSH and LAN5. This suggests that apoptosis induction by MEKK1 might be independent of the genetic changes that led to the transformed phenotype. Though cancer is a disease which includes a malfunction in the regulation of apoptosis, cancer cells still preserve the ability to undergo programmed cell death. Indeed, a variety of oncogenes like c-Myc, v-Jun, E1A and E2F sensitise cancer cells towards apoptosis [58, 151-153].
Therefore, it was interesting to see if MEKK1 was capable of inducing apoptosis of “normal”, i.e. non-transformed cells. Because primary human cells cannot be efficiently transfected and, more importantly, are not easily obtainable and difficult to culture, we used hTERT-immortalised human fibroblasts as a model system for non-transformed cells. It has been shown that ectopic expression of the telomerase catalytic subunit (human telomerase reverse transcriptase or hTERT) and subsequent activation of telomerase can allow postsenescent cells to proliferate beyond crisis rendering the cells immortal [189]. Still, primary human cells which have been immortalised with hTERT have an apparently normal, non-transformed phenotype [190, 191]. We found that the telomerase-immortalised fibroblasts were sensitive to MEKK1 as evidenced by a clear reduction of clonogenic survival. Therefore, MEKK1-induced killing was not specific to a transformed phenotype. Moreover, the survival of the telomerase-immortalised cells was also compromised by expression of the RBD, which had only little effect on the human cancer cell lines. This suggests that either the fibroblast cell lines are dependent on Ras signalling for growth and survival while the cancer cell lines are not, or that Ras-dependent survival signalling in the cancer cell lines is constitutively activated downstream of Ras and consequently cannot be inhibited by overexpression of the RBD.

In addition to wild-type immortalised fibroblasts we also tested cell lines which are deficient in p53 and p21 respectively. The p53 tumour suppressor gene is mutated in around half of all human cancers, making it probably the most frequently mutated gene during malignant transformation. The p53 protein is a transcription factor which mediates a variety of cellular processes including differentiation, senescence and DNA repair. Moreover, it can induce cell cycle arrest and apoptosis [192]. The exact mechanism by which p53 can induce apoptosis is unknown [193]. p53 has been shown to activate the expression of many genes that could be involved in the apoptotic response. Moreover, activities of p53 that are independent of transcription also play a role in the induction of apoptosis. One direct target of the p53 tumour suppressor protein is the cyclin dependent kinase (cdk) inhibitor p21Cip/Waf [194], and the deletion of this gene significantly reduces p53-induced cell cycle arrest [195]. p21 inhibits cell cycle progression in two ways: it can inhibit a variety of cyclin/cdk complexes. It also can impede DNA synthesis through PCNA (proliferating cell nuclear antigen) binding (see [196] for a review on cdk inhibitors). However, we did not find any protection from the reduction of clonogenic survival by MEKK1, the RBD or the fusion proteins in the p53 or p21 deficient cell lines. Taken together, these data indicate that apoptosis induction by MEKK1 is independent on the p53 status of the cell. This is
supported by our findings with the human cancer cell lines. MEKK1 suppressed clonogenic survival of MCF7, HeLa and A431 cells. These cell lines differ in their p53 status: MCF7 cells express normal p53 [197, 198]. In HeLa cells p53 is inactivated by the viral E6 protein [126], while A431 cells have a mutated p53 [199].

MAP kinases are involved in cell cycle regulation and different MAP kinases become transiently activated during different stages of the cell cycle (reviewed in [200]). Therefore, losing the ability to regulate MAP kinase activity in a co-ordinated fashion could result in a loss of cell cycle control, and finally apoptosis.

4.2 β-Galactosidase

By their nature proteins that induce growth arrest or apoptosis cannot be stably expressed in cells unless their expression is made inducible. It can be very time consuming to establish cell lines that express transgenes in an inducible way. Moreover, they are very often subject to substantial clonal variety. Hence, we decided to study the effect of MEKK1, the RBD and the fusion proteins in transiently transfected cells.

A disadvantage of this strategy is that apoptosis induced by the expression of a transgene in transiently transfected cells can be difficult to track. Firstly, some assays like colorimetric caspase assays or immunoblot analysis monitoring the cleavage of caspase substrates are not suitable, if – like in a transient transfection – only a subpopulation of the cells is expressing the transgene. These assays are carried out on cell lysates and thereby average over the whole cell population. If the rate of transfection is small, the background of untransfected cells might be too high to detect apoptosis in the transfected cells, especially as adherent cells commonly detach from the culture dish during apoptosis and are lost from the assay. Moreover, even though we routinely achieved transfection efficiencies of about 20 to 60%, most of these assays are unsuitable for MCF7 cells because they monitor the activation of caspase-3, which is not expressed in these cells. Other assays, like Annexin V-binding or TUNEL staining, can detect apoptosis on a single cell level. They require the co-transfection of a marker protein like EGFP to distinguish transfected cells.

We wanted to set up an assay, in which the transfection marker could also act as an indicator for the survival and growth of transiently transfected cells. Analogous to the
EGFP, which we used to monitor transfection rates by FASC analysis, we wanted to establish an easily detectable and quantifiable enzyme activity as a marker for the survival of transfected cells. β-Galactosidase activity in cell lysates can be assayed by straightforward colorimetric assays. Moreover, there was no endogenous enzyme activity detectable in the cancer cell lines we used and expression plasmids for β-Galactosidase were readily available.

However, we found that β-Galactosidase activity in cell lysates strongly depended on the co-transfected protein. This was not due to variations in the transfection rate: when we looked at β-Galactosidase activity on a single cell level by staining with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) we found differences in the intensity of the stain, but not in the overall number of stained cells (data not shown). This means, that the expression of β-Galactosidase from the phosphoglycerate kinase (PGK) promoter was influenced by the co-transfected protein. The murine PGK promoter contains five potential binding sites for Sp1 [199]. Sp1 is an ubiquitously expressed transcription factor required for the constitutive and inducible expression of a variety of genes. Little is known about the regulation of Sp1 activity, but proteins of the Rb family and G1-specific cyclins seem to be involved [201]. Sp1 is also phosphorylated by several kinases. Most of the phosphorylation events seem to enhance its transactivating properties [201].

The proteins we co-transfected impinge on MAP kinase pathways and the final target of all MAP kinase signalling cascades are transcription factors (reviewed in [202]). Therefore, it was not surprising that co-transfection of oncogenic Ras, constitutively active MEKK1 or dominant negative MKK4 could influence transcription from the murine PGK promoter, even though PGK is an ubiquitously expressed housekeeping gene [203]. We tried to find an eukaryontic promoter that would be independent of MAP kinase signalling. One candidate was the Rous sarcoma virus (RSV) long terminal repeat (LTR), because its activity was apparently unchanged by co-transfection of a mutant form of c-Jun, that cannot be phosphorylated by JNK (D. Gillespie, personal communication). However, we found that the transcriptional activity from the RSV LTR was also dependent on the co-transfected protein.

Similar observations were made in a different experiment when we tested if apoptosis induced by EGFP-MEKK1 could be inhibited by a dominant negative mutant form of MKK4. There, we found that co-transfection of the dominant negative MKK4 increased
Discussion

the amount of EGFP and EGFP-MEKK1 in cell lysates. FACS analysis clearly revealed, that this reflected an intracellular increase of the amount of protein and was not simply due to a variation in transfection efficiency (data not shown). In this experiment, EGFP and EGFP-MEKK1 were expressed under the control of the CMV promoter.

Since our educated guess, the RSV-LTR, failed to be MAP kinase independent and the study of promoter activity was not within the remit of our project, we abandoned the experiments with β-Galactosidase as a survival marker at this stage. β-Galactosidase co-transfection is often used as a convenient survival marker. However, as our experiments show, the use of β-Galactosidase is not without problems and must be carefully controlled.

4.3 The role of MAP kinases in the action of chemotherapeutic drugs

Many chemotherapeutic agents induce apoptosis in cancer cells (see [204-206] for recent reviews on this subject). The exact mechanisms by which they do so are mostly unknown, but they do activate MAP kinase pathways and modulation of these has been implicated to affect the apoptosis response [163, 205]. However, most studies dealing with the involvement of MAP kinases in the action of chemotherapeutic drugs were confined to one cell type or one particular class of drugs. The purpose of this part of our work was to re-examine these issues on a broader scale and to evaluate whether the modulation of MAP kinase pathways could be employed to enhance the effectiveness of chemotherapeutic drugs.

We tested the effect of three drugs from distinct classes on three different cancer cell lines: squamous carcinoma A431 cells, papillomavirus transformed HeLa cells and MCF7 breast cancer cells. The drugs we used were the microtubule-disrupting agent taxol, the topoisomerase II inhibitor etoposide and the apoptosis inducing lipid C6-Ceramide. We found that HeLa and A431 cells exhibited similar sensitivities towards these agents: both lines started to die around about 12 h after treatment as judged by morphological changes and 48 h after treatment virtually all cells were dead (data not shown). On the other hand, MCF7 cells were largely unaffected after 12 h of treatment except for a slight rounding with taxol. Only after 48 h of treatment the drugs began to show visible effects. As mentioned earlier, the high drug tolerance of MCF7
cells is consistent with their high expression levels of Bcl-2. Moreover, though one study reports the proteolytic activation of caspase-3 and subsequent cleavage of PARP in MCF7 cells [207], we could not detect any expression of caspase-3 by immunoblot analysis in our MCF7 cells. This is in accordance with most other reports in literature [162].

The original hypothesis on the involvement of MAP kinases in the regulation of survival versus apoptosis states that stress-activated MAP kinases such as JNK and p38 promote apoptosis, whereas mitogen-activated MAP kinases such as ERK are protective [80]. These early studies were carried out in PC12 cells, a rat pheochromocytoma cell line that is widely used as model system for neuronal differentiation [81, 82]. Their results stimulated further research whether MAP kinase pathways are involved in the cellular response to chemotherapeutic drugs.

Activation of the ERK pathway has most commonly been linked to cell proliferation and survival. It has been shown to stimulate proliferation by inducing the expression of cyclinD and hence activation of cyclin dependent kinases (cdk) [208, 209]. In addition, ERK may phosphorylate and inactivate the p27KIP cdk inhibitory protein [210, 211]. However, prolonged ERK signalling at high intensity has an opposing effect: it halts cell cycle progression by inducing the expression of cdk inhibitory proteins such as p21Cip/Waf and p27KIP [212]. ERK can also interfere with apoptosis in a more direct manner: it can prevent the activation of caspasers [213-215] and induce the expression of anti-apoptotic factors such as Mcl-1 [216] and IAPs, a family of small proteins that can bind to and inhibit caspasers [217]. Some of these functions may be indirect and due to its ability to activate the CREB and NFκB transcription factors that participate in major survival pathways [218, 219]. On the other hand, ERK also has been reported to stimulate apoptosis in T-cells by enhancing the expression of Fas ligand [220] and to signal apoptosis in response to ceramide by preventing the inactivation of the pro-apoptotic Bcl-2 family member BAD [221]. ERK activation also partially mediates the cytokine-induced apoptosis of pancreatic beta-cells [222].

The stress-activated kinases JNK and p38 at large have been associated with increasing apoptosis in response to external stress signals. It is difficult to distinguish if the activation of stress-activated MAP kinases are the cause or merely a consequence of apoptosis. The execution of programmed cell death features the activation of proteases and endonucleases, resulting in protein degradation and DNA strand breaks. These are stimuli that are known to activate stress signalling cascades. For instance, caspase-3, the
main executioner caspase of apoptosis, cleaves and thereby activates MEKK1 [39]. Consequently, the process of apoptosis itself will always trigger the activation of JNK, regardless of whether JNK signalling was involved in the induction of apoptosis in the first place or not. Despite great efforts, the mechanism by which JNK activation could transduce an apoptotic signal is still not completely clear. JNK was reported to stimulate the expression of Fas ligand [172, 223] and to phosphorylate and inactivate Bcl-2 [139]. Bcl-2 also has a role as a cell cycle inhibitor, and it has been reported that it needs to be phosphorylated and inactivated by JNK during physiological cell cycle progression [139]. This role of JNK may be important in regard to its requirement for cell proliferation in some cell types [224]. Curiously, the genetic removal of SEK-1, an immediate upstream activator kinase of JNK, promotes the apoptosis of hepatocytes [225] and T-cells [226], revealing a dual role of JNK in the regulation of cell survival. Interestingly, the genetic ablation of MEKK1, an upstream activator of MKK4, increases the sensitivity of murine embryonic fibroblasts to taxol [167], but protects chicken bursal B-cells from apoptosis in response to the same stimuli [95]. Therefore, the role of JNK in response to microtubule-disrupting agents seems to be cell type specific.

p38 has been isolated as the target of chemical inhibitors that interfered with the expression of pro-inflammatory cytokines including pro-apoptotic factors such as TNF [227]. Similar to JNK, p38 activation has been reported to mediate and enhance the apoptotic response to a variety of stimuli in different cell types. For instance, it has been shown to mediate apoptosis in response to taxol in breast cancer cells [228] and after growth factor withdrawal in PC12 cells [80, 229]. The mechanisms by which p38 promotes apoptosis are not fully understood. It has been reported to mediate the release of cytochrome C in response to UVB irradiation [230] and to induce the transcription of pro-apoptotic genes in PC12 cells [229]. Moreover, p38 has been described to phosphorylate p53 and mutation of the putative phosphorylation sites protected from apoptosis [231]. p38 can prevent cell cycle entry by downregulating the expression of cyclinD [209]. It also mediates a G2/M checkpoint by inactivating the Cdc25B phosphatase which is needed to initiate G2/M phase progression [232]. More recently, a number of reports stated that p38 activation is either of no consequence for apoptosis [83] or even could protect cells from apoptosis [233-235]. This suggests that – similar to ERK and JNK – the role of p38 in the regulation of apoptosis seems to be dependent on cell type and stimulus.
Taxol has been reported to activate JNK and p38 in a number of cell types [236]. However, the reports on its effects on ERK are controversial: some studies show an activation, other an inactivation of ERK. In the A431, HeLa and MCF7 cells used in this study ERK became activated after taxol treatment. However, when we assayed ERK activity in HL60 leukaemia cells we found it decreased in response to taxol (data not shown). One explanation for this discrepancy might be that taxol inhibits ERK in suspension cells (like HL60) but activates ERK in adherent cells (like A431, HeLa or MCF7). This hypothesis would be consistent with most other reports in literature about suspension [216, 237] and adherent cell lines [238-240] and might be related to the association of a pool of ERK with microtubules and the preferential activation of this pool by external signals in macrophages [241]. In our cell lines, the extent of ERK activation by taxol was modest, except in HeLa cells, and maximum activation occurred at the late time points between 18-36 hours. At these time points, taxol induces a pronounced G2/M arrest. As ERK is normally active during this phase [242, 243] the late ERK activation could be an indirect consequence of the cell cycle block. Consistent with this explanation the late activation of ERK by ceramide was stronger in A431 cells, where ceramide induced a G2/M block, than in HeLa where the cell cycle was not inhibited. Some reports suggest, that some of the effects of microtubule-disrupting drugs such as Bcl-2 phosphorylation and JNK activation normally occur transiently during the G2/M phase of the cell cycle [139, 244]. They conclude that activation of JNK and Bcl-2 phosphorylation induced by microtubule-disrupting drugs might be a consequence of the G2/M cell cycle block rather than a direct effect of the drugs and that the extension of these normally transient phenomena contributes to the induction of apoptosis. Interestingly, Fan et al. report that cells in the G2/M phase are more susceptible to a variety of apoptotic stimuli, supporting the hypothesis that cell proliferation results in a sensitisation to apoptosis.

A number of studies showed that taxol toxicity was diminished by the induction of high ERK activity and suggested that MEK inhibitors may enhance taxol effects in such cells [238-240]. This hypothesis is supported by our findings that MEK inhibition increases PARP cleavage induced by taxol and etoposide in HeLa and A431 cells. Both agents strongly induce ERK activity in these cells. Curiously, MEK inhibitors did not have corresponding effects on other parameters of cell viability and apoptosis we tested. In the WST assay, which measures the short-term metabolic integrity of mitochondria, MEK inhibition slightly improved the basal level and reduced taxol inflicted damage in
A431 cells and had no effect in HeLa cells. In the long-term clonogenic assay MEK inhibition reduced colony formation of etoposide treated A431 cells, but promoted clonogenic survival of HeLa cells. In this assay p38 inhibition also increased the etoposide toxicity in A431 cells. The influence of ERK signalling on taxol-induced apoptosis is a matter of controversy in literature. As mentioned above, we and a number of other laboratories reported a survival promoting function of ERK activity in response to taxol. There are however other reports stating that ERK activation in response to taxol promotes apoptosis and that cell death is decreased when ERK activation is inhibited [228]. At present there is no obvious explanation for these discrepancies, some of them might be due to different parameters that were monitored to assay apoptosis. But the overall experimental set-up also seems to be important. Yu et al. reported that MEK inhibition can promote, decrease or have no effect on taxol-induced apoptosis in the same cell line depending on whether the cells are treated with the inhibitor before, during or after taxol treatment [92].

The most striking aspect of our study is the almost complete lack of correlation between the activation of MAP kinase pathways and drug effects measured in different assays. The clonogenic assay is perhaps the most relevant, because it reveals the long-term consequences of a single application of drug. This most closely reflects a clinical treatment situation, where tumour cells are exposed to drugs in pulsed intervals rather than continuously. However, the clonogenic assay does not distinguish between different possible causes for a reduction of clones, which could be due to apoptosis, cell cycle delay or arrest, senescence, lower metabolic capacity or a combination thereof. Therefore, we used a number of short-term assays to evaluate these parameters individually. Chemotherapeutic drugs yielded clear and reproducible effects in any of these assays suggesting that they interfere with cell viability and proliferation on multiple levels. The manipulation of MAP kinase pathways alone or in combination with these drugs gave either no or rather diverse results. For instance, they had no effect on drug-induced cell cycle blocks, and diverse and small results in the WST assay. In the caspase activation assay (PARP cleavage), we observed the classical pattern of an increase in apoptosis after inhibition of the ERK pathway and a protection from apoptosis by inhibition of p38 in both HeLa and to a lesser extend also in A431 cells. However, in the clonogenic assay the effects of MAP kinase inhibition on drug treated cells were dependent on the drug and the cell line. MEK inhibition increased the clonogenic survival of taxol treated HeLa and MCF7 cells, the apparently diametrical effect than the one observed in PARP cleavage, but had no effect in A431 cells. There, MEK and p38 inhibition decreased survival after etoposide treatment. This effect of the
inhibitors on etoposide treated cells could also be observed in MCF7, but not in HeLa cells.

Thus, our results suggest that the original view of ERK promoting cell survival and JNK and p38 furthering apoptosis is too simplistic, and in this form cannot be exploited as a general tool to enhance chemotherapy. Rather, our results suggest that the potential benefits of MAPK inhibition are confined to the treatment of a subset of tumour cells with a particular class of drugs, and therefore need to be addressed individually. They also show that currently no single experimental assay suffices to define these criteria in vitro, and that a battery of assays must be employed.
5 Summary

MAP kinase pathways comprise a group of parallel protein phosphorylation cascades which are involved in signalling triggered by a variety of stimuli. Previous findings indicated that the ERK and the JNK/p38 pathways have opposing roles in regulating proliferation and survival or apoptosis, and that apoptosis can be promoted by blocking the ERK pathway or by activation of the JNK and p38 pathways.

In order to test this hypothesis and explore whether it can be exploited as a strategy for killing human cancer cells, we used gene transfer experiments with a range of cancer cell lines. We expressed the catalytic fragment of human MEKK1 to activate JNK and the Ras-binding domain (RBD) of Raf-1 to inhibit ERK. In addition, we designed several RBD-MEKK1 fusion proteins aiming to simultaneously activate the JNK and block the ERK pathway. We found that the MEKK1 proteins as well as the RBD alone could reduce colony formation in all cell lines. The survival time of MEKK1 expressing cells depended on the cell line and could not be prolonged by co-expression of the anti-apoptotic protein Bcl-2 or by inhibition of c-Myc. The RBD-MEKK1 fusion proteins were less effective in apoptosis induction than the MEKK1 kinase domain alone, probably due to a lower kinase activity of the RBD-MEKK1 proteins compared to MEKK1. Using mutant forms of Ras and Raf-1 we could show that the reduced kinase activity of RBD-MEKK1 fusion proteins was caused by binding to the Ras protein. The expression of lethal doses of MEKK1 resulted in a strong activation of all three major MAP kinase families JNK, ERK and p38. Blocking of these pathways by either co-expressing a dominant negative form of MKK4 or with inhibitors of MEK or p38 failed to inhibit apoptosis. This suggests that MEKK1 induces apoptosis by causing a general dysregulation of MAP kinase signalling rather than by the activation of a single pathway.

We also investigated the role of MAP kinase pathways in the cellular response to taxol, etoposide and ceramide in three different human cancer cell lines. Our results show that all drugs activate MAP kinase pathways, but to different extents and with distinct kinetics of activation. We used pharmacological inhibitors of the ERK and p38 pathways to assay the biological consequences of drug-induced MAP kinase activation and measured different short-term parameters including apoptosis, cell viability and cell cycle arrest as well as long-term clonogenic survival. We found that the modulation of MAP kinase pathways could enhance or decrease drug efficacies, but the effects were only detectable by assays measuring apoptosis and clonogenic survival. Moreover, the
effects were highly dependent on the drug and the cell line without any clear pattern of correlation emerging. These results suggest that the modulation of MAP kinase pathways to enhance the efficacy of chemotherapeutic drugs is of limited value unless it is tailored to a specific combination of drug and cancer.
6 Literature


Curriculum vitae

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Academic Development

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Publications

S. Boldt, U. Weidle and W. Kolch. Targeting MAP kinase pathways to kill cancer cells. Manuscript submitted for publication

S. Boldt, U. Weidle and W. Kolch. The role of MAPK pathways in the action of chemotherapeutic drugs. Manuscript submitted for publication


Conferences

Presentations

2002 Cell Signaling, Transcription and Translation as Therapeutic Targets, Luxembourg
Poster presentation “Induction of apoptosis in human cancer cell lines by MEKK1”

2001 11th International Conference on Second Messengers and Phosphoproteins, Melbourne, Australia
Poster presentation “The role of protein kinases in the regulation of apoptosis”

2000 British Society for Cell Biology Autumn Meeting, Edinburgh
Oral presentation and poster “The role of protein kinases in the induction of apoptosis”

Other conferences attended

2001 Beatson International Cancer Conference “Genomic Regulation and Cancer”

1999 NCI Symposium “Killing Cancer Cells”, Amsterdam, Netherlands
Beatson International Cancer Conference “Invasion and Metastasis”