# Klärung der molekularen Mechanismen der apoptotischen Wirkung des Myrtucommulons aus *Myrtus communis*

# Elucidation of molecular mechanisms of apoptosis induction by myrtucommulone from *Myrtus communis*

Dissertation

der Fakultät für Chemie und Pharmazie
der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

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vorgelegt von
**Dagmar Blaesius**

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# Contents

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# Abbreviations

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| | |
|---|---|
| -/- | deficient |
| 5-HETE | 5-hydroxy eicosatetraenoic acid |
| 5-LO | 5-lipoxygenase |
| AA | arachidonic acid |
| AML | acute myeloid leukaemia |
| ANT | adenine nucleotide transporter |
| Casp | caspase, cysteine aspartyl specific protease |
| CD | cluster of differentiation |
| Chel | chelerythrine |
| CHX | cycloheximide |
| CNK | connector enhancer of KSR |
| COX | cyclooxygenase |
| CrK | creatin kinase |
| CsA | cyclosporine A |
| CypD | cyclophilin D |
| DAG | diacylglycerol |
| Dauno | daunorubicin |
| DMSO | dimethyl sulfoxide |
| $EC_{50}$ | half maximal effective concentration |
| ERK | extracellular signal-regulated kinase |
| FADD | FAS-associated death domain |
| FAS | death receptor, also called Apo1 or CD95 |
| FASN | fatty acid synthase |
| FCCP | carbonyl cyanide-p-trifluoromethoxyphenylhydrazone |
| FL | filter (flow cytometry) |
| fMLP | N-formylmethionyl-leucyl-phenylalanine |
| FPR | formyl peptide receptor |
| GEF | guanine nucleotide exchange factor |
| GMP-PNP | guanosine 5´-[β,γ-imido]triphosphate |
| GPCR | G protein coupled receptor |
| Grb2 | growth factor receptor bound protein 2 |

| | |
|---|---|
| HK | hexokinase |
| $IC_{50}$ | half maximal inhibitory concentration |
| IFN | interferone |
| IL | interleukin |
| JNK/SAPK | c-Jun N-terminal kinase/stress-activated protein kinase |
| Jvec | MOCK-transfected Jurkat cells |
| KSR | kinase suppressor of Ras |
| LDL | low density lipoprotein |
| $LTB_4$ | leukotriene B4 |
| MAC | mitochondrial apoptosis-induced channel |
| MAPK | mitogen-activated protein kinase |
| MARCKS | myristoylated alanine-rich protein kinase C substrate |
| MC | myrtucommulone A |
| MEK | MAP kinase/ Erk kinase, MAPKK, MAP2K |
| MEKK | MAP kinase/ Erk kinase kinase, MAPKKK, MAP3K |
| mPGES | microsomal prostaglandin E2 synthase |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| NSAIDs | non-steroidal anti-inflammatory drugs |
| PARP | poly ADP-ribose polymerase |
| PBMC | peripheral blood mononuclear cells |
| PBR | peripheral benzodiazepine receptor |
| PBS | phosphate buffered saline |
| PGC | PBS +0.1% glucose + 1 mM $CaCl_2$ |
| $PGE_2$ | prostaglandin E2 |
| PI | propidium iodide |
| PKB | protein kinase B (also called Akt) |
| PKC | protein kinase C |
| PMA | phorbol 12-myristate 13-acetate |
| PMNL | polymorphonuclear cells |

| | |
|---|---|
| PMSF | phenylmethanesulfonylfluoride |
| pNPP | para-nitrophenylphosphate |
| PS | phosphatidylserine |
| PTP | permeability transition pore |
| PTPase | protein tyrosine phosphatase |
| QVD | QV-D-Oph, pan-caspase inhibitor |
| Raf | rat fibrosarcoma or rapidly growing fibrosarcoma |
| Ras | rat sarcoma |
| ROS | reactive oxygen species |
| RT | room temperature |
| RTK | receptor tyrosine kinase |
| S.E. | standard error |
| SDS | sodium dodecylsulfate |
| SDS-b | SDS-PAGE sample loading buffer |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| Shc | src homology 2 domain containing transforming protein |
| Sos | son of sevenless |
| Src | sarcoma |
| Stauro | staurosporine |
| STI | soybean trypsin inhibitor |
| TCA | trichloroacetic acid |
| VDAC | voltage dependent anion channel |
| veh | vehicle (DMSO) |
| wt | wild type |
| $\Delta\Psi_m$ | mitochondrial membrane potential |

# Introduction

# Introduction

## *1 Myrtus communis* and myrtucommulone

*Myrtus communis* L., also called `True Myrtle´, belongs to the family of the *Myrtaceae* (Rosopsida, Rosidae, Myrtales, Myrtaceae, *Myrtus*).

To this family also the eucalypt tree, guava and allspice (myrtle pepper) are assigned. The myrtle is an evergreen bush which grows mainly in mediterranean areas such as Sardinia and Corsica. The plant has small lanceolate leaves and delicate white, flavoursome flowers which develop into darkly coloured berries.

***Fig. Myrtle (Myrtus communis)***

Aside from its use in mediterranean cuisine (as substitute for pepper and bay leaves, to make liqueur), myrtle was described by the greek erudite Pedanios Dioskurides in his `materia medica´ as treatment of minor burns, eschar, inflammation of the skin or the eyes, ulcer and excess flow of sweat.

The dried leaves contain up to 0.5% oily components with 23% limonene, 20% linalool, 51.3% α-pinene, 34.7% 1,8-cineole, myrtenol, myrtenol acetate, 0.12% myrtucommulone A (MC) and others [1, 2].

There are only a few publications concerning the effect of myrtle extracts, most of them stating MC to have analgesic [3], antibacterial [4, 5], antihyperglycaemic [6-8], antioxidant [9] and antigenotoxic [10] properties.

In his review about aspirin and analgesic treatments, Levesque reports that Assyrians and Egypts used a preparation of myrtle leaves for the alleviation of joint pains [3], another example for the use of myrtle in traditional medicine.

More scientifically funded are studies of Al-Saimary et al.. They tested aqueous extracts of myrtle leaves on inhibition of bacterial growth in *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the predominant pathogens found in burn

infections. Interestingly, the effects of myrtle extracts on diameter of growth inhibition zones by agar diffusion method lay within the limits of some antibiotics (penicillin, tetracyclin, gentamycin and others) which were tested alongside (5-20 mg/ml) [4].

Bonjar et al. were also trying to give the use of folkloric medicine a scientifically funded background. They were screening the antibacterial activity of methanolic plant extracts (*Myrtus communis* seeds as one of them) on gram-negative and gram-positive bacteria in general and found that these myrtle extracts are highly active (minimal inhibitory concentration of 0.62 mg/ml) against *Staphylococcus aureus* (inflammation of the skin and lung), *Bacillus cereus* (intoxication of aliments) and *Bacillus bronchiseptica* (infection of the respiratory system) [5].

Aside from these antibacterial effects of myrtle extracts, it could be shown by Onal et al. that one fraction of myrtle extracts could inhibit α-glucosidase activity in vitro with an $IC_{50}$ of 38 μg/ml. Alpha glucosidase inhibitors are well established as treatment in patients with type II diabetes [8]. And act by delaying the absorbance of carbohydrates in the intestine. Furthermore, this possible therapeutic benefit was supported by in vivo studies of myrtle oil in alloxan-diabetic rabbits by Sepici et al. [7]. Again, the incitement for this investigation came from (this time Turkish) folk medicine. Here, leaves from *Myrtus communis* and the volatile oil of the leaves are both used to lower blood glucose levels in type 2 diabetic patients. Sepici et al. could show that myrtle oil (administered in 50 and 100 mg/kg per day) significantly lowered blood glucose by 51% in alloxan-diabetic rabbits but not in normoglycaemic animals without affecting insulin levels in the blood. The authors postulated that inhibition of the α-glucosidase in the small intestinal mucosa, induction of glukokinase and higher rates of glycogenesis are the reasons for the reduction in blood glucose levels [7].

Hayder et al. used myrtle extracts obtained by different extraction methods and screened their ability to block Aflatoxin B1- (AFB1) and Nifuroxazide-induced SOS response in *Escherichia coli* PQ37. The SOS response in bacteria is a DNA repair system thought responsible for the evolvement of antibiotic resistance. The methanol and aqueous myrtle extracts showed the highest inhibition of SOS response induced by AFB1. Aside from these antigenotoxic effects, some of the myrtle extracts showed free-radical scavenging activity against the DPPH (1,1-diphenyl-2-picrylhydrazyl radical [10].

The antioxidant effect by myrtle extracts was also investigated by Romani and coworkers. Inspired by the anti-inflammatory uses of myrtle in folk medicine they prepared liquid-liquid extracts of myrtle leaves with different solvents. Hydroalcoholic extracts, ethylacetate and aqueous extracts had the highest antioxidant activities (0.36 μM, 2.27 μM and 2.88 μM respectively) in copper ion induced oxidation of human low density lipoprotein (LDL), apparently due to the presence of galloyl derivatives [9].

All this research so far could only hint to the part or the parts of myrtle extract which are responsible for the effects observed.

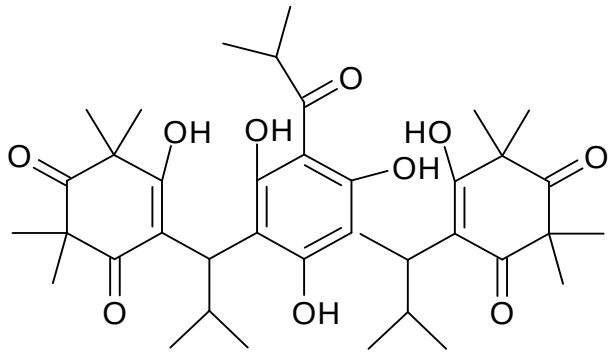



***Fig. myrtucommulone A (MC)***

In 1974, a non-prenylated acylphloroglucinol named myrtucommulone A (MC) (see Fig.) and its antimicrobial properties against gram-positive bacteria (*S. aureus, B. subtilis, Streptococcus faecalis* and others) was described for the first time by Rotstein et al. [11]. His aim was to isolate active compounds from antibacterial plant extracts and test them in growth inhibition zone tests for the ability to affect bacterial growth. Bacteria were seeded on agar plates, the tested compounds were administered to a paper disk and this in turn applied to the agar plates. 80 μg MC per disk had the same effect on growth of the gram-positive bacteria tested as 10 U penicillin or streptomycin per disk. Rotsteins work is concluded by the remark that `phenolic compounds are more active against gram-positive than gram-negative bacteria´ and that `the presence of organic matter such as milk or serum effectively reduces their activity´. In vivo infections in mice induced by intraperitoneal injection of *S. aureus* were not affected by application of MC as a paste [11]. Another model (acute inflammation) was tested by Rossi and coworkers who could show just recently that MC, when injected intraperitoneally, could significantly reduce paw oedema and pleurisy induced by carrageenan [12]. Paw oedema were reduced dose-dependently (0.5, 1.5 and 4.5

mg/kg i.p.) by MC. For the effect of MC in the model of pleurisy the authors show that 4 h after carrageenan injection in the pleurisy model, MC reduced: 1) the exudate volume and leukocyte numbers; 2) lung injury (histological analysis) and neutrophil infiltration (myeloperoxidase activity); 3) the lung intercellular adhesion molecule-1 and P-selectin immunohistochemical localization; 4) the cytokine levels (tumor necrosis factor-α and interleukin-1 β) in the pleural exudate and their immunohistochemical localization in the lung; 5) the leukotriene $B_4$, but not prostaglandin $E_2$, levels in the pleural exudates; and 6) lung peroxidation (thiobarbituric acid-reactant substance) and nitrotyrosine and poly (ADP-ribose) immunostaining.

Appendino et al. published in 2002 [1] the testing of MC on antibacterial properties in multidrug resistant *Staphylococcus aureus* strains (MDR). They showed that MC could inhibit growth in strains with efflux mechanisms of resistance for macrolides, tetracyclines and fluoroquinolones (minimal inhibitory concentration (MIC) = 0.5 - 2 μg/ml). For comparison: in the strains of tetracycline and fluoroquinolone resistance, the antibiotic tetracycline had a MIC of 256 μg/ml and 32 μg/ml respectively.

Furthermore, it was published that MC has antioxidative properties [13] and might be (at least partly) responsible for the antioxidative effect of myrtle extracts as described by Romani [9]. It was observed that MC could protect linoleic acid in vitro against auto-oxidation and oxidation mediated by $FeCl_3$ and EDTA.
The effect of myrtle extracts on LDL oxidation could be confirmed with isolated MC by Rosa et al. [14]. MC and the truncated form of MC, semi-myrtucommulone (S-MC), showed strong antioxidant activity in vitro towards thermal, solvent-free degradation of cholesterol. Pre-treatment with MC could preserve LDL from oxidative damage induced by copper ions and could also protect polyunsaturated fatty acids and cholesterol from oxidation. This might be an important effect of MC when used as treatment in cardiovascular diseases.

Many data were obtained about the anti-inflammatory properties of hyperforin from St Johns worth (*Hypericum perforatum*), a prenylated acylphloroglucinol and a structure analogue of MC [15-17]. In regard to the anti-inflammatory effect

of MC and its molecular targets, it has been shown that MC can directly inhibit cyclooxygenase-1 (COX-1) in platelet homogenates and intact platelets ($IC_{50}$ = 17 μM) and can inhibit 5-lipoxygenase (5-LO) in granulocytes (PMNL) ($IC_{50}$ = 1.8 μM) after stimulation with arachidonic acid and ionomycin. This inhibition of 5-LO was not competitive. Furthermore, the signaling of G protein coupled receptors (GPCRs) activated by fMLP leading to the release of $Ca^{2+}$ from intracellular stores, to the formation of reactive oxygen species (ROS) and to elastase release from granules was prevented by MC ($IC_{50}$ = 0.55 μM, $IC_{50}$ = 0.9 μM and $IC_{50}$ = 0.24 μM). However, the release of $Ca^{2+}$ and elastase and the formation of ROS induced by ionomycin were not or only partly affected (ROS production with 25% rest activity) probably by circumventing GPCR signaling using ionomycin. In the light of these effects, MC was suggested as potent anti-inflammatory agent with possible therapeutic benefit in treatment of inflammation related diseases [18].

The findings of the work done by Dr. C. Feißt et al. will be further explored in this thesis, trying to give a new perspective of the effect of MC on GPCR signaling concerning apoptosis and cancer.

Concerning GPCRs, another work was published by Carroll et al. [19] showing that MC A and structural homologues of MC isolated from the seeds of *Corymbia scabrida*, possess significant competitive affinity to the thyrotropin-releasing hormone receptor-2 (TRHR2) in rat HEK2935 cells with an $IC_{50}$ value of 39 μM for the tested TRH. Carroll et al. also observed an interesting property of MC A by $^{1}$H-NMR. The phenolic protons and the adjacent carbonyl oxygens of the molecule obviously form strong hydrogen bonds which prevent the forming of hydrogen bonds with the solvents used, making the compound non-polar. Furthermore, on treatment with acid, MC A underwent cyclisation and dehydration giving a symmetrical pentacyclic compound.

Koeberle et al. could prove in a cell free assay that MC inhibits isolated microsomal prostaglandin $E_2$ synthase-1 (mPGES-1) with an $IC_{50}$ of 1 μM in a reversible fashion. MC could inhibit $PGE_2$ formation in whole A549 cells (human lung carcinoma epithelial cells) and in human whole blood in low micromolar concentrations ($IC_{50}$ = 30 μM and $IC_{50}$ > 30 μM respectively). Interestingly, no effect on cyclooxygenase 2 (COX-2) up to 30 μM MC in either A549 cells or on the isolated enzyme could be detected. Cellular and cell-free COX-1 was inhibited

by MC as shown before [18] with an $IC_{50}$ > 15 μM. This higher inhibitory specifity of MC towards $PGE_2$ synthesis by mPGES-1 gives many perspectives in treating inflammation and fever, since common treatment often targets the entire prostaglandin biosynthesis by inhibition of COX-1 and COX-2 (aspirin, diclofenac, ibuprofen, celecoxib) leading to side effects like gastrointestinal ulceration and/or heart attacks [20].

All research on MC done so far attempted to explain the effects of myrtle extracts in traditional medicine on inflammation, diabetes and bacterial infections as well as investigating the role of the myrtle constituent MC A in this respect.
Indeed, it was suggested that MC might be responsible for almost all effects of myrtle extracts observed so far. However, plant extracts might have effects related to the content of different active substances and sometimes the concerted action of multiple compounds could be responsible for the observed `outcome´.

Recently, more and more attention has been focussed on mechanisms leading from inflammation to development of cancer.
During studies concerning the regulation of the 5-lipoxygenase [21], another enzyme important in inflammation, it was found that acylphloroglucinols and especially MC could induce apoptosis in cancer cell lines [22]. According to these findings, the aim of the present investigation was to elucidate the exact mechanism of the pro-apoptotic effect of MC on apoptosis and also to find a possible connection between the effects on signal transduction [18] and apoptosis induction.

# 2 Cancer

During the last decades, cancer is gaining more and more in importance especially in developed countries. According to the WHO, cancer causes 13% of all human deaths (2006).

The term cancer refers to a group of diseases affecting all parts of the body. It develops from one single abnormal cell which shows uncontrolled growth, invasiveness and in some cases metastasising properties. The reasons for the development of this disease are manifold but apart from the patient's genetic disposition, many external substances called carcinogens are initiators of this cell degeneration. Among carcinogens, radiation (UV and ionising), tobacco smoke, aflatoxin and infections by certain viruses (e.g. hepatitis B, human papilloma Virus, HIV) are well recognised.

The most diffuse types of cancer are lung, stomach, colorectal, liver and breast/prostate cancer. By taking care of a healthy lifestyle and decreasing the exposition to risk factors, about 30% of all cancers could be prevented.

Cancers are named after the first degenerated cell they are derived from. Carcinomas are cancers of epithelial origin (breast, lung, prostate and colon cancer), sarcomas are tumors derived from connective tissues, lymphomas and leukemias from hematopoietic cells and blastomas are tumors of immature/embryonic tissue.

## 2.1 *Leukaemia*

Leukaemia is the cancer derived from and found in the blood and bone marrow, manifesting itself in abnormal proliferation of white blood cells (leukocytes). The first classification into subtypes gives the classes of acute and chronic leukemia. The acute form consists of the rapid uncontrolled proliferation of immature blood cells which determines a reduced ability of the bone marrow to give healthy mature blood cells. If not treated directly, the abnormal cells enter the blood circulation and spread themselves over the whole body. This type of leukaemia is mostly found in children. The chronic form of leukaemia includes the excessive but slow proliferation of mature blood cells which progresses over years. This form is mainly found in elder people.

A different classification takes into consideration which kinds of cells are affected. In the lymphoblastic forms the progenitors of lymphocytes are affected and in the myeloid form the type of marrow cell developing into red blood cells or erythrocytes. Therefore one can distinguish four different types of leukemia: acute lymphoblastic leukaemia (ALL), chronic lymphoblastic leukaemia (CLL), acute myeloid leukaemia (AML), and chronic myeloid leukaemia (CML).

The causes for developing leukemia are diverse. As in other types of cancer, cancerogenes like radiation, virus infections (human T-lymphotropic virus), chemicals like benzene and hair dyes as well as the use of tobacco can be a reason.

Treatment of leukemia involves the use of interferons, chemotherapeutics (often in combination therapy as to avoid disease resistance), radiation therapy and bone marrow transplant. Interferon α (IFNα) is a (glyko-) protein released by leukocytes, monocytes and fibroblasts upon infection of a cell by a virus. When IFNα binds to its specific receptor on the surface of the infected cell, it activates Jak/Stat kinases which bind to the interferon stimulated response element (ISRE) leading to activation of specific genes. This helps to reduce the abnormal cell proliferation and makes degenerated cells more susceptible for the removal by the immune system. This property is used as treatment in newly diagnosed patients who are not candidates for bone marrow transplants. The most common treatment in AML is the combination of three days intravenous administration of DNA intercalating anthracyclines (doxorubicin, daunorubicin) and seven days treatment with the bone marrow suppressor Arabinosyl-Cytosin. As third chemotherapeutic, 6-thioguanine can be added which is incorporated into RNA and DNA and leads subsequently to cell cycle arrest and apoptosis. Tretinoin (Vesanoid®), the all - trans retinoic acid (ATRA) (the acid form of vitamin A) is another possibility to treat AML. It is given orally and causes immature blood cells to differentiate and eventually die.

All these treatments cause severe side effects ranging from cardiac damage, gastrointestinal damage, nausea, vomiting, fatigue, bleeding, fever, chills to hyperleukocytosis, weight gain, edema and pleural effusion (ATRA syndrome). The five year relative survival rate has quadrupled during the last 50 years and is for AML 21.3% in total and 55.2% for children under 15 years [Leukemia & Lymphoma society].

Of course, research is advancing to either circumvent possible disease resistance to common drugs [23, 24], but also to find new drugs and therapies with improved mode of action and less side effects [25-27]. In the present investigations, data supporting the use of MC as an excellent candidate to treat leukaemia will be provided.

## 2.2 *Cancer and inflammation*

It has been observed that chronic inflammation caused by e.g. *Heliobacter pylori* (ulcer), by the immune disorder ulcerative colitis or by the Hepatitis B virus, dramatically increases the risk of developing stomach, colon or liver cancer. On the other hand, patients who regularly take medication such as non-steroidal anti-inflammatory drugs (NSAIDs) have a lower risk of developing cancer [28]. Research focussed on finding a reason for the connection of chronic inflammation and cancer, and two possible ways are thought to be critical: the activity of transcription factor nuclear factor kappa-light chain enhancer of activated B-cells (NFκB) [29] and the action of inflammatory active immune cells [30].

Immune cells are attracted to infected cells by cytokines and other chemoattractants. When reaching the infected or degenerated cell they release reactive oxygen species (ROS), cytokines and growth factors in order to further stimulate immune responses and to eliminate pathogens and the infected cell. However, these released factors seem to rather stimulate tumour growth and, even worse, stimulate tumour migration.

NFκB is overactive in some cancer types, has antiapoptotic properties [29] and functions as a tumour promotor in inflammation-associated cancer [31]. Upon stimulation with growth factors, cytokines (TNFα, ILβ), bacterial (e.g. lipopolysaccharide (LPS)) or viral antigens NFκB is activated and can regulate the expression of the COX-2 gene which encodes an enzyme needed for the production of proinflammatory, tumour-inducing prostaglandin $E_2$ ($PGE_2$) [32] and which can induce blood vessel growth towards tumour cells [33, 34]. Thus, cytokines and growth factors released by immune cells together with activation of NFκB in cancer cells may act concertedly to promote inflammation, tumour growth and migration and to inhibit apoptosis.

Another theory on this topic has been recently developed. It was observed that the amount of myeloid derived suppressor cells (MDSC) was significantly increased

in tumour tissue, spleen and peripheral blood of tumour bearing mice or cancer patients [35, 36] and that T-cell activity in these patients was impaired. MDSC are thought to be immature myeloid cells but recently it was found that they have a phenotype similar to granulocytes [37]. They normally suppress antibody producing B-cells, T-helper cells (CD4+) and T-killer cells (CD8+). Their task is to control inflammation and prevent an overactivation of immune responses. MDSCs prevent for instance binding of the antigen to the T-cell receptors [36] by the involvement of ROS and peroxynitrite. The vascular endothelial growth factor (VEGF) released by tumour cells can trigger accumulation of MDSC in tumour tissues and MSDCs in turn release VEGF thus further stimulating tumour growth [38]. Also IL1β and the overexpression or constitutive expression of COX-2, leading to elevated amounts of $PGE_2$, are thought to induce the accumulation and activation of MDSCs [39-41] since MDSCs express the $PGE_2$ receptor E-prostanoid 2 (EP2). Obviously, MDSCs/granulocytes also influence the amounts of the T-cell activator L-arginine by release of arginase 1 [42] probably from granules [37]. Arginase hydrolyses L-arginine to ornithine and urea. By this depletion of arginine, T-cell proliferation and cytokine production is inhibited and the expression of the T-cell receptor CD3-zeta chain is reduced [43, 44].

The important immunosuppressant task of MDSCs in acute inflammation becomes threatening when, during chronic inflammation or cancer, the offending agent is not removed. Then the inactivation of T-cells by MSDCs/granulocytes leads to further tumour growth and facilitates tumour migration. The beneficial effect of therapeutics like COX- and mPGES-inhibitors or anti-inflammatory drugs in general (Celecoxib, aspirin, indomethacin) as well as agents that interfere with arginase activity (Viagra, Cialis) on progression and invasiveness of tumours is well established by now. MC, as anti-inflammatory [12] and, as this study will prove, anti-cancer compound [45], is well worth being (and already was) tested in this respect [46].

## 2.3 *Metabolism of cancer cells*

Over 80 years ago, Warburg published his idea about altered metabolism in cancer cells which is hence known as the Warburg effect. He examined healthy and cancerogenous cells from the human liver and observed elevated glucose

uptake and an increased rate of glycolysis in cancer cells compared to non-transformed cells [47]. He postulated that the effect he saw was due to changes or to damage of mitochondrial oxidative phosphorylation rendering glycolysis the only source for ATP synthesis. Furthermore, he observed a connection between glycolitic ATP production and aggressiveness of cancer cells [48]. He suggested that the impaired function of mitochondria is even the cause for cancer development and that cancer cells might be eliminated by inhibition of oxidative phosphorylation. This would decrease mitochondrial function below a critical point important for cell survival, whereas normal cells could still compensate this loss of mitochondrial function. Cancer cells obviously have still functioning oxidative phosphorylation and do produce ATP by ATPase activity (although only decreased levels) [49].

More recent work on this topic showed that tumor hypoxia and the hypoxia inducible factor (HIF) led to altered glucose metabolism. Due to high amounts of energy needed and the local vasculature being unable to supply enough oxygen to the cancer cell, cells become hypoxic and change their main source of energy production from mitochondrial respiration to glycolysis activated by HIF. HIF itself influences the activity and the expression of enzymes such as pyruvate dehydrogenase [50, 51], lactate dehydrogenase and others [52]. Additionally, it inhibits mitochondrial biogenesis and respiration by repression of c-Myc activity. The transcription factor c-Myc leads also to altered gene expression and activity of metabolic enzymes [53].

Aside from HIF, the loss of p53, overactivation of protein kinase B (PKB/Akt), overexpression of lactate dehydrogenase (LDH) genes and altered lipid metabolism are thought to be the main changes in metabolism of cancer cells or are changes which might even lead to the development of cancerogenous properties in a cell.

PKB/Akt is a serine threonine kinase playing a pivotal role in survival, proliferation and metabolism [54, 55]. Its activation is triggered by insulin and various growth factors and involves phosphatidylinositol-3-dependent kinase (PI3K). It induces aerobic glycolysis [56], is responsible for glucose transporter (GLUT) translocation to the plasma membrane [57] and stimulates mitochondria-associated hexokinase (HK) activity [58]. HK-1 and -2 are located on mitochondrial membranes, are part of the permeability transition pore and, when

active, suppress apoptosis and maintain the mitochondrial membrane potential ($\Delta\Psi_{m,}$ see section below).

P53 is the major tumor suppressor in the cell and is mutated in many cancer types. It protects the genome by detecting DNA-damage, inducing cell cycle arrest and triggers DNA-repair or, if damage is irreparable, induces apoptosis. In non-transformed cells p53 is activated by an elevated AMP/ATP ratio and functions as metabolic checkpoint [59]. Elevated AMP/ATP levels indicate energy depletion and eventually lead to cell cycle arrest. Energy depletion can also lead to the activation of the AMP activated protein kinase (AMPK) which induces catabolic pathways which includes increased glucose uptake, fatty acid oxidation and stimulation of mitochondrial metabolism [60]. Tp53 induced glycolysis and apoptosis regulator (TIGAR) is activated by p53 and might regulate production of ROS [61]. It leads to decreased levels of fructose-2,6-bisphosphate, a positive regulator of glycolysis, thus shifting the flux of the early substrate of glycolysis glucose-6-phosphate towards the pentose phosphate pathway (PPP). Due to increased NADPH concentrations by the increased activity of the PPP, the amount of reduced glutathione (GSH) rises and is able to scavenge reactive oxygen species (ROS). In many cancer types, p53 is mutated or deleted (see section HL60) resulting in decreased TIGER expression, which stimulates glycolysis and ROS production, leads to low levels of NADPH and subsequently leads to activation of PKB/Akt [62]. In p53 -deficient cells, the p53-dependent expression of the cytochrome oxidase 2 (SCO2) is lost and therefore impairs mitochondrial respiration [63].

The lactate dehydrogenase catalyses the conversion of pyruvate to lactate under hypoxic conditions. Hypoxia averts metabolisation of pyruvate in the citric acid cycle since oxygen is needed for mitochondrial endoxidation. LDH expression is induced by c-Myc activity and helps cancer cells to survive under hypoxic conditions [64].

In many cancer cell types, the lipid metabolism is shifted towards high rates of de novo fatty acid synthesis probably to support the increased need for membrane biosynthesis in highly proliferating cancer cells. This aim is achieved by overexpression of the fatty acid synthesis (FASN) [65-67].

All metabolic changes observed so far occur to support the energy needs of the highly proliferating cancer cell and rendering glycolysis as only source of ATP production.

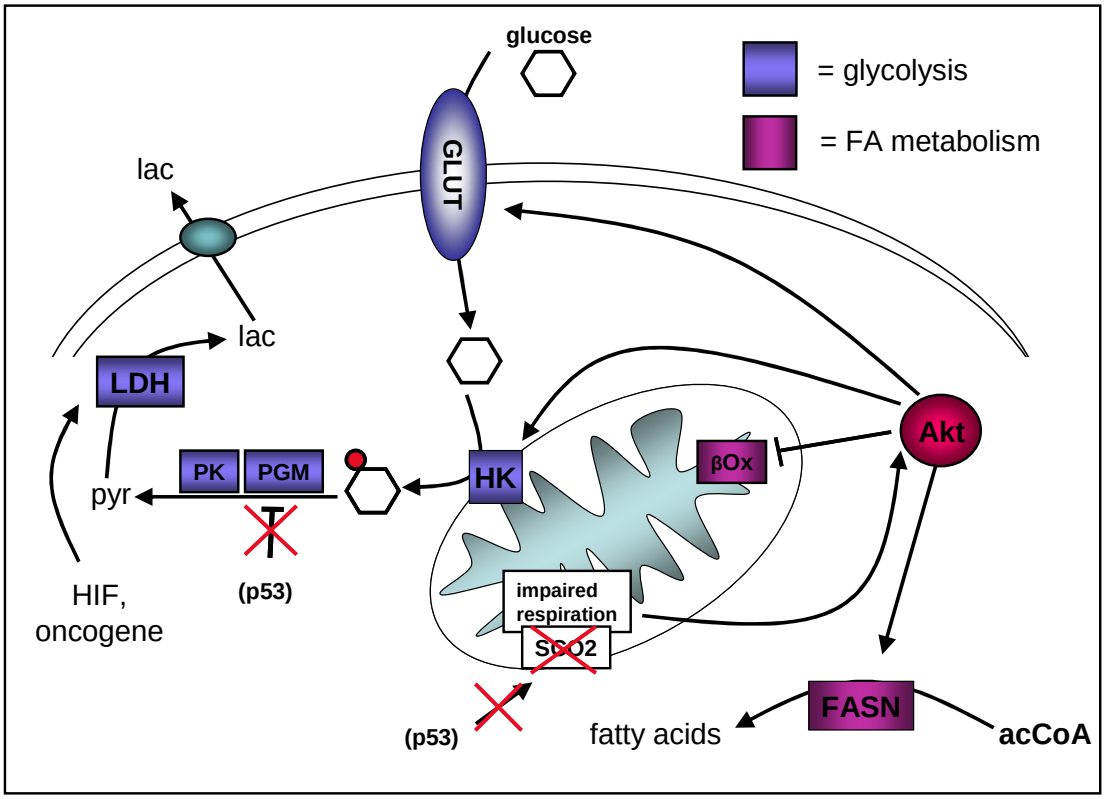



***Fig. Metabolism of cancer cells [68] (description see text).*** *GLUT = glucose transporter, lac = lactate, pyr = pyruvate, LDH = lactate dehydrogenase, PK = pyruvate kinase, PGM = phosphoglucomutase, HIF = hypoxia inducible factor, HK = hexokinase, FASN = fatty acid synthase, AcCoA = Acetyl-Coenzyme A, βOx = beta oxidation, SCO2 = cytochrome oxidase 2, FA = fatty acid.*

# 3 Apoptosis

Apoptosis is a term derived from the greek απόπτόσισ (`apo´ = from and `ptosis´ = fall) [69], referring to the falling of petals from a flower, since one event of apoptosis is the formation of apoptotic bodies making the cell look like a flower losing its petals. Apoptosis is the so-called programmed cell death, a mechanism to control cell proliferation or triggered in response to irreparable (DNA-) damage to the cell. It takes part in fetal development [70] as well as for example in elimination of self-reactive lymphocytes and downregulation of lymphocytes after immune response [71, 72]. Non functional apoptosis results in neurodegenerative diseases (Parkinson´s, Alzheimer´s) and hypothropy (when it is exaggerated) or in cancer (when it results insufficient). In contrast to necrosis which is always involving physical damage to the cell and leads to the release of cell debris into the surrounding matrix, causing an inflammatory response, apoptosis is the well organised `clean´ death of a cell.

Caspases are cysteine proteases that cleave after aspartate and are responsible for the morphological changes recognised as apoptosis [73]. The morphological changes include: losing of cell-cell contact, inhibition of translation, condensation of cells and organelles, condensation of chromatin, DNA fragmentation, fragmentation of the nucleus, loss of asymmetry of membrane lipid composition, constriction of small membrane enclosed cell fragments known as apoptotic bodies which then are eliminated by phagocytosis. These events finally result in necrosis when phagocytotic cells are not able to cope with high rates of apoptosis or in the case of total absence of phagocytotic cells (in cell culture) [74]. However, under normal conditions, apoptosis occurs without leakage of cell contents and without inflammatory response. Apoptosis is about 20 times faster than mitosis and it is therefore almost impossible to detect apoptotic cells in vivo. The signals leading to apoptosis can be manifold: starvation, damage beyond repair from radiation or toxic chemicals, infection by a virus etc. Then the cell itself decides to undergo apoptosis or this decision is made by a cell of the immune system. Therefore one differentiates between two ways of apoptosis induction.

## 3.1 *Pathways of apoptosis induction*

There are two main ways of apoptosis induction: the extrinsic way and the intrinsic way. The extrinsic way of apoptosis induction involves the activation of a death receptor (DR). They are tumor necrosis factor receptors (TNF R) or tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL). Fas is the best known of these receptors concerning apoptosis [75]. It is also referred to as CD95 or Apo-1. Upon binding of the Fas ligand, which is for example expressed on an activated T-cell, the receptor trimerises and forms the death inducing signaling complex (DISC) with the Fas associated death domain (FADD) [76] and caspase 8 or 10 involving hexa-α-helical death domains (DD) and death effector domains (DED). This formation of DISC is necessary for cleavage of the zymogen procaspase 8 to the active form [77]. The initiator caspase 8 then activates the effector downstream caspase 3 in a proteolytic cascade. On the other hand the intrinsic way involves cytochrome c release from mitochondria induced by various stimuli such as oxidative stress and treatment with cytotoxic drugs. Not only cytochrome c is released but also a great variety of other proteins such as AIF (apoptosis inducing factor) [78] and EndoG (endonuclease G), both DNases and Smac/Diablo (second mitochondria derived activator of caspases), eliminating the inhibitory effect of IAPs (see section caspases) [79]. The exact mechanism or the initial reason for protein release from mitochondria is still elusive and probably involves loss of mitochondrial membrane potential ($\Delta\Psi_m$), the permeability transition pore and pore formation by Bcl-2 proteins (see section below). Cytochrome c binds to the apoptotic protease-activating factor 1 (Apaf1). This triggers the formation of the apoptosome consisting of seven cytochrome c, seven dATP and seven procaspase 9 molecules [80, 81]. Upon apoptosome formation caspase 9 becomes active and can then in turn activate caspase 3.

## 3.2 *Caspases*

As mentioned above, caspases are cysteine aspartyl specific proteases responsible for the biochemical changes within the cell observed during apoptosis. In humans, 14 caspases have been identified so far [82] being grouped according to their amino acid sequence or their protease specificities. On the base of their function, they are often referred to as initiator caspases, standing far upstream the signaling cascade, or effector caspases being downstream close to the substrate targets [73].

The targets of effector caspases include protein kinases and other signal transducing proteins, proteins of the matrix and the cytoskeleton, chromatin modifying proteins or DNA repair proteins (poly ADP-ribose polymerase, PARP) and inhibitory subunits of endonucleases (inhibitor of caspase activated DNase, ICAD)[73, 83].

From a structural point of view, initiator caspases have a large N-terminal prodomain containing either death effector domains (DED) (e.g. caspase 8 and 10) , or caspase recruitment domains (CARD) (caspase 2 and 9). Effector caspases (e.g., caspase 3, 6 and 7) have a significant shorter N-residue and they depend on activation by initiator caspases. During activation, the zymogen is cleaved to a small (10 kDa) and a larger (20 kDa) fragment and usually forms a heterotetramer from two small and two big subunits to become active [83]. In the case of caspase 8, the weak protease activity of the uncleaved zymogen (around 1% of the fully active form) leads to trans-processing of two caspase 8 molecules when brought together (induced proximity model) [84].

This simple model of caspase activation and apoptosis induction is far from complete. For example, it has been recently shown that caspase 3 and 7 are not only effector caspases, but they are able to act early in apoptosis inducing mitochondrial events like cytochrome c release [85]. Other findings state that caspase 8 can be activated by caspase 9 or 3 implying the function of an amplification loop [86, 87].

The family of IAP (inhibitor of apoptosis) proteins are a family of apoptosis repressors [88, 89]. Most of them have been shown to bind and inactivate caspases, mainly the initiator caspase 9 and the effector caspases 3 and 7 [90-92] although they also have a role in controlling protein turnover [93].

## 3.3 *Bcl-2 proteins*

The mitochondrial way of apoptosis induction is mainly regulated by the concerted action of pro- and anti-apoptotic Bcl-2 proteins. This is a class of proteins mostly found associated to mitochondria. Bcl-2 (from B-cell lymphoma) was the first oncogene discovered able to delay entry from $G_0$ to S-phase [94]. All Bcl-2 proteins share so-called Bcl-2 homology domains (BH1-4) [95] important

for binding to each other and for the function of the proteins. Bcl-2 proteins are able to form homo- and heterodimers eventually leading to the formation of pores within the mitochondrial membrane [96, 97]. The decision of pro- or anti-apoptotic is made by the relative ratio of the different Bcl-2 family members. Bcl-2 and Bcl-Xl are antiapoptotic, **B**cl-2 **a**ssociated **X** protein (Bax), **B**cl-2 **a**ntagonist/**k**iller (Bak), Bad, Bim and **B**H3 **i**nteracting **d**omain death agonist (Bid) are some members of the pro-apoptotic part of the family. Bid and Bad (BH3 only proteins) miss the C-terminal transmembrane domain and are found in the cytoplasm.

Bid is a target for the `extrinsic´ caspase 8 and after cleavage through the caspase to the truncated form (tBid), it reveals a Bcl2 homology domain (BH3) for dimerisation and a hydrophobic core thought to be important for membrane association on mitochondria [98, 99]. Upon its cleavage, it translocates to mitochondria and it leads to apoptosis induction by inducing oligomerisation of Bax and permeabilisation of the mitochondrial outer membrane (also called mitochondrial apoptosis inducing channel, MAC) [100]. Bcl-2 can antagonise this event by binding Bax [97]. Due to its function, Bid contributes to a possible crosstalk of the extrinsic and intrinsic pathway.

Bad has been shown to be phosphorylated by protein kinase B/Akt, protein kinase A and c-Raf/Raf 1 [101]. This phosphorylation inactivates the protein and prevents dimerisation and antagonising Bcl-2 or Bcl-Xl [102].

Bcl-2 proteins take part in inducing cytochrome c release from mitochondria and thus apoptosome formation and caspase 9 activation. If the formation of MAC is the main reason for cytochrome c release, or if it only leads to mitochondrial swelling and membrane rupture is still discussed [103].

The overexpression of Bcl-2 in haemapoietic cell lines leads to increased insensitivity in these cells to many cytotoxic substances [104-108]. In this study, stable transfectants of Jurkat cells overexpressing Bcl-2 and the Jurkat vector control cells were used (a gift from H. Walczak (Heidelberg, Germany)) to evaluate if they show similar resistance to MC induced cytotoxicity.

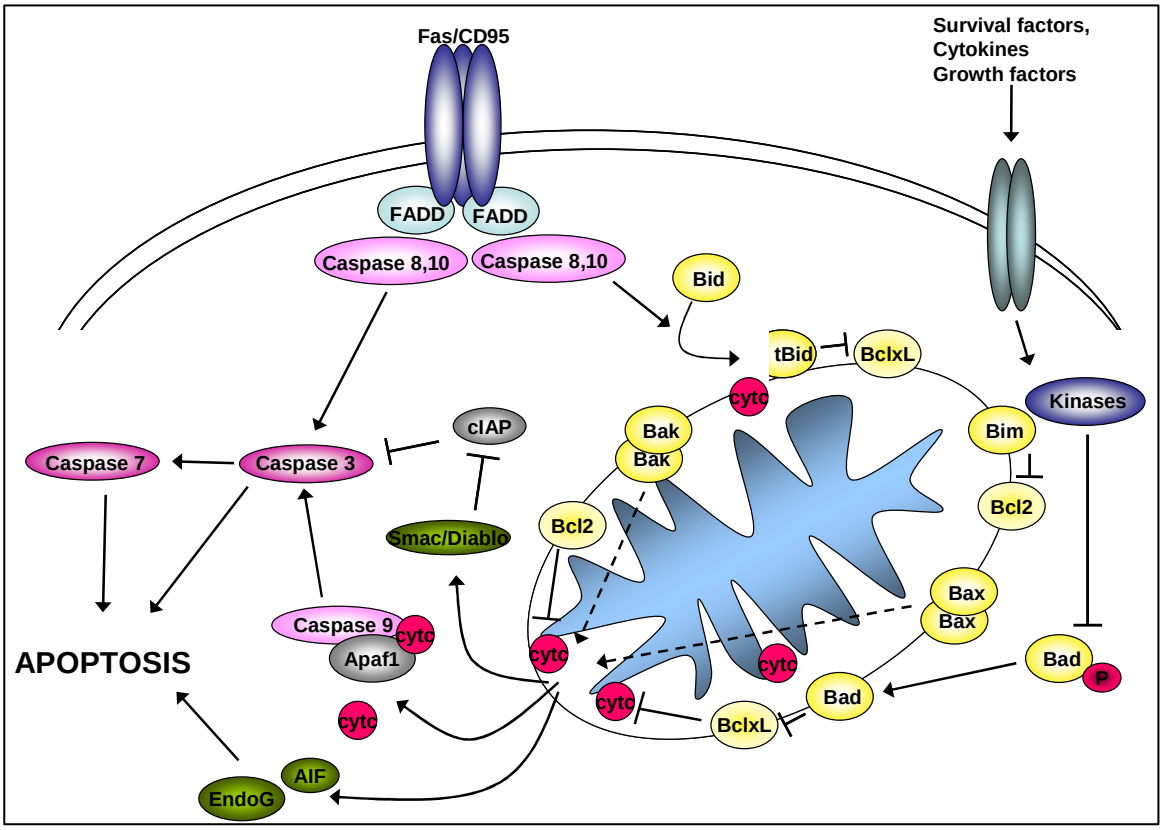



*Fig. Overview: apoptosis pathways (for further details see text).*

## 3.4 *Mitochondria and the permeability transition pore*

Mitochondria not only play an important role for energy metabolism, $Ca^{2+}$ homeostasis and ROS production of the cell (also important for the regulation of physiological processes), but they are key regulators of cell death pathways (see chapter 3.1).

During apoptosis, mitochondrial membrane integrity is abolished by multiple mechanisms and the permeabilisation of the outer mitochondrial membrane is thought to be a crucial event during early apoptosis [109] leading to cytochrome c release and activation of caspases (see chapter 3.1).

Aside from the concerted action of propapoptotic Bcl-2 proteins on the mitochondrial outer membrane (see chapter 3.3), another model for membrane permeabilisation in mitochondria has been proposed: the permeability transition pore (PTP) [110, 111] (see Fig.).

Permeability transition is an event leading to the permeability of the inner mitochondrial membrane to molecules up to 1500 Da. This is achieved by the opening of a dynamically regulated pore, the PTP. In the fully open state it probably has a pore diameter of 3 nm [112]. This pore was shown to be voltage-dependent and cyclosporin A (CsA) sensitive and had high conductance. It consists of many different proteins which, under normal conditions, have their own important functions in the cell, but under conditions favouring apoptosis act together to mediate mitochondrial cell death. Constituents are in/on the outer mitochondrial membrane: the voltage dependent anion channel (VDAC), the peripheral type benzodiazepine receptor, Hexokinase and Bcl-2 proteins. In/on the inner membrane the adenine nucleotide transporter (ANT), creatine kinase and cyclophillin D (the target of CsA) can be found. Several modulators can increase or decrease the open probability of the PTP (summarised in Fig. The permeability transition pore) leading to an decrease in the membrane potential ($\Delta\Psi_m$), loss of the proton gradient important for ATP-synthesis by ATP-Synthase and general equilibration of ion gradients and other molecules with less than 1500 Da. Especially this equilibration of ions leads to mitochondrial swelling by influx of water, causing mechanical membrane disruption which then could be responsible for release of larger molecules from mitochondria (e.g. cytochrome c).

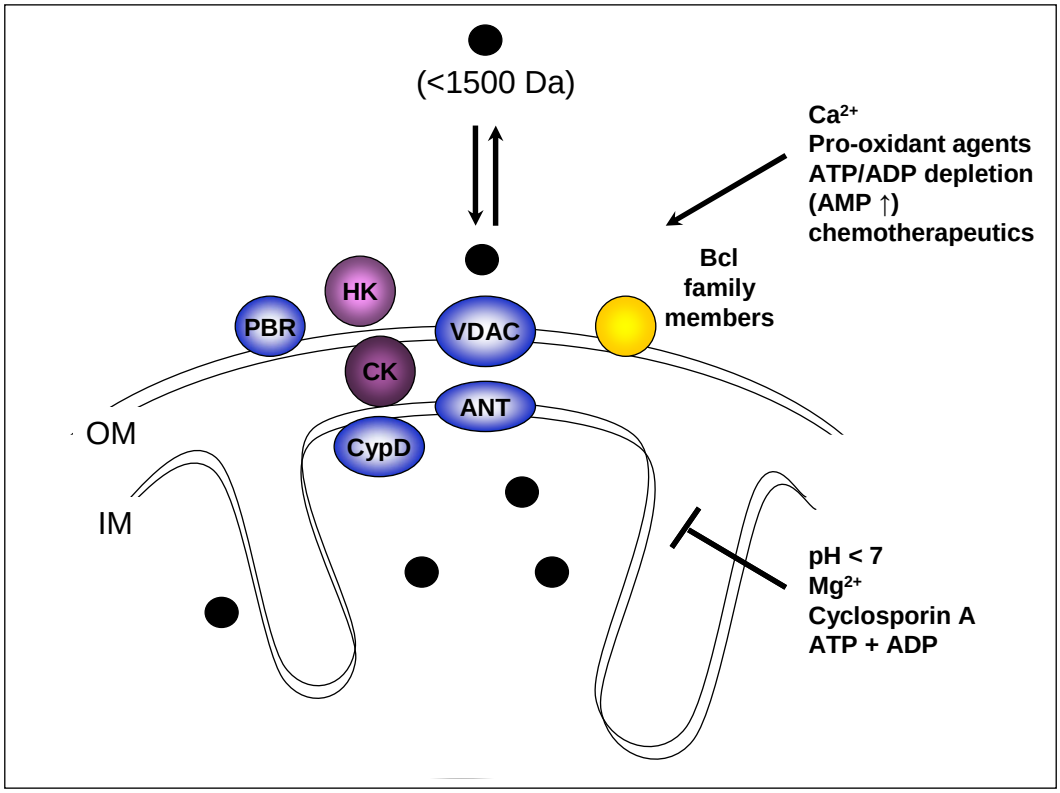



***Fig. The permeability transition pore [111].*** *PBR = peripheral benzodiazepine receptor, HK = Hexokinase, CK = Creatinkinase, VDAC = voltage dependent anion channel, ANT = adenine nucleotide transporter, CypD = cyclophillin D, OM = outer membrane, IM = inner membrane.*

# 4 Cell cycle

One prominent defect in tumour cells is the deregulation of the cell cycle leading to uncontrolled proliferation. Many cell cycle regulators are altered in cancer cells, in most cases resulting in a deregulation of phase G1 progression where the decision between proliferation and quiescence is made [113].

In 1951, the cell cycle was first described by Howard and Pele. It consists of several phases which are controlled by many regulatory proteins. The most important of them are cyclins, cyclin-dependent kinases (Cdks) and the tumour suppressors p53 and pRb. The cell cycle checkpoint or tumour suppressor protein p53 is one component of the cell cycle which is altered in cancer cells [114], also for example in HL60 cells (see section below). It has an N-terminal transcriptional activation domain and a dimerisation domain at the C-terminus. Its function is to arrest cells in G1 phase after genotoxic damage in order to allow DNA repair or, if DNA damage is beyond repair, to induce apoptosis. The antiproliferating effect is mediated by the expression of the Cdk inhibitor p21 [115]. Another regulator of the cell cycle is the retinoblastoma protein pRb [116]. Although its function is still not totally clarified, its phosphorylation plays a role [117]. pRb acts as a master of regulation of transcription [118] and interactions between p53 and pRb are possible [119]. It is thought that pRb is first hypophosphorylated and inhibits transcriptional activity of the transcription factor E2F. Upon phosphorylation (probably cyclin/cdk dependent) some hours before DNA synthesis is first detectable, phosphorylated pRb releases E2F and G1 progression to S-phase can take place [120-122]. The interaction between pRb and E2F also has a regulatory effect on genes of apoptosis regulation.

Cyclins are key regulators of cell cycle progression. They are 56kDa proteins and regulatory subunits of the Cdk-complexes. These complexes activate their targets by phosphorylation rendering effects like cell cycle progression in general (see Fig. 1), chromatin remodelling and microtubule formation. Their expression is also regulated by the NFκB family of transcription factors [123].

In the present study, an effect of MC on the cell cycle was at first excluded since HL60, promyeloid leukemian cells, have no or only fragments of p53 (see section below). However, this topic was then further explored since MC reduced the cell

number of Jurkat T-lymphocytes deficient in caspase 9, although apoptosis induction was abolished.

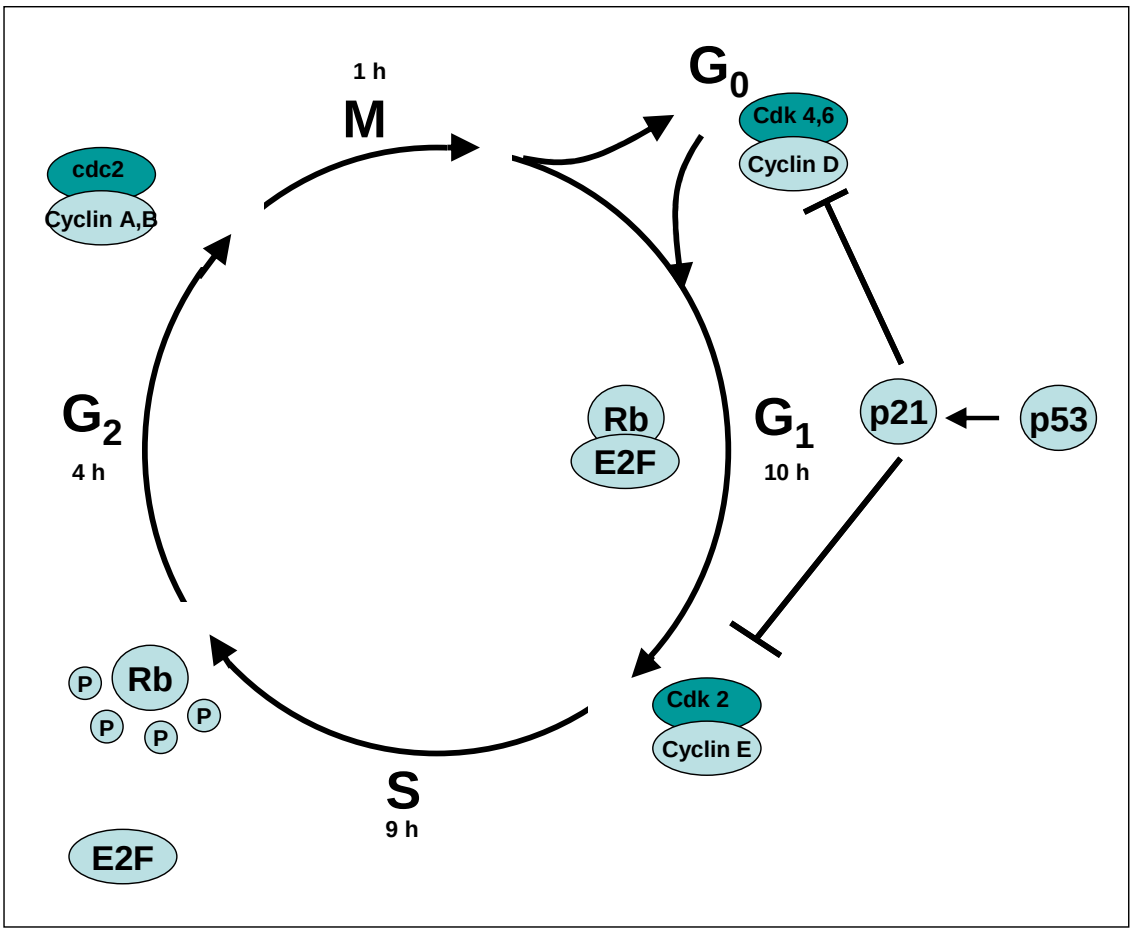



***Fig. Cell cycle control [124](explanation see text).*** *Cdk = cyclin dependent kinase, Rb = retinoblastoma protein, E2F = transcription factor.*

# 5 Cells

## 5.1 *HL60*

The HL60 cell line was established from the blood of a female patient with acute myeloid leukaemia (AML) in 1977 [125]. These cells are mainly promyelocytes with a block of differentiation but are able to differentiate upon varying stimuli into granulocytes (DMSO, actinomycin D, retinoic acid) or monocytes/macrophages (phorbol esters, vitamin $D_3$, sodium butyrate) [126, 127]. After isolation from the patient, cells were transferred into the appropriate medium leading to the development of a growth-factor independent immortal cell line growing in suspension with a doubling time of 20 to 45 hours. Over the years, intensive studies were done with these cells leading to the findings that the p53 gene is largely deleted [128], the gene of the small G protein N (neuroblastoma)-Ras is mutated in codon 61 [129] and the gene for the transcription factor c-Myc is amplified up to 30-fold [130]. All these properties make HL60 cells the ideal candidate to study the mechanism of differentation of stem cells and to use them in the study of MAPK pathways. In this study we used HL60 mainly for two reasons. First, we tried to find out the targets of MC for apoptosis induction in leukaemian cells and HL60 are useful as a leukaemian cell line, since, due to the lack in p53, they allow to exclude this pathway of apoptosis induction. Second, HL60 are also useful for a direct comparison with healthy granulocytes.

## 5.2 *MM6*

Mono-Mac-6 (MM6) are monocytic cells isolated in 1985 from the blood of a 64 year old man with acute monocytic leukemia (AML). They are round cells growing in suspension or sometimes loosely adhering, have a doubling time of around 48 h and about 1-5% of the cells are multi-nuclear giant cells. MM6 were shown to have the same phenotype as mature monocytes [131]. In this study, MM6 were used to test the apoptosis-inducing properties of MC. As control cell line for MM6 we used peripheral blood mononuclear cells (PBMC) from the blood of healthy donors.

## 5.3 *Jurkat T-lymphocytes*

Jurkat T-lymphocytes were established in 1977 from the blood of a 14 year old boy with acute lymphoblastic leukemia (ALL) [132]. They represent an immortalised cell line for the study of acute T cell leukemia and T cell signaling. They are round cells growing in suspension with a doubling time of around 25 to 35 hours. In the present investigation, differently modified Jurkat cells were used to study the apoptosis inducing effect by MC concerning the intrinsic and extrinsic pathway.

## 5.4 *Neutrophilic granulocytes*

Neutrophilic granulocytes constitute 50-60% of total blood cell number in the peripheral blood. They are the first line of defense against pathogens or exogenous `non-body´ substances intruding into the organism [133]. Usually, neutrophils are contained in the circulating blood. Upon inflammatory events, chemoattractants lead to the invasion of neutrophils into the area of inflamed tissue. This invasion starts with low affinity contact of neutrophils to the endothelium (rolling), sticking to the blood vessel wall (mediated by integrins, CD11/CD18 and ICAMs, intercellular adhesion molecules) and then migration through cell-cell junctions into subendothelial space. When reaching the site of inflammation, they start the production of ROS (by membrane associated NADPH-oxidase) [134] or NO (by NO-synthase) [135] and release the contents of their granules. The granules can be divided into azurophilic (primary), specific (secondary) and gelatinase granules (tertiary) [136]. These contain mostly proteolytic enzymes (catepsins, lysozyme, gelatinase) but also receptors and surface markers (fMLP receptor, CD 11). By these mechanisms, pathogens can be eliminated within only hours, so they are a hallmark of acute immune response.

In the present work polymorphonuclear leukocytes (PMNL) represented an suitable system to test signaling pathways affected by MC and to compare these cells to the leukemian cell line HL60. We obtained neutrophils together with other granulocytes in the PMNL fraction of buffy coats prepared from human blood. Since the life span of neutrophil granulocytes is only about 12 hours, they were not used for experiments concerning apoptosis or for other experiments requiring longer stimulation/incubation times.

## 5.5 *Mononuclear cells (PBMC)*

Apart from the granulocyte fraction isolated from buffy coats, also the lymphocytic/monocytic fraction termed peripheral blood mononuclear cells (PBMC) was used in this work. This fraction contains lymphocytes (T- and B-lymphocytes, constituting about 20% of the white blood cells) as well as natural killer (NK) cells and monocytes/macrophages. They all have a defined nucleus and are the part of the immune system especially involved in the adaptive immune response (T- and B-cells).

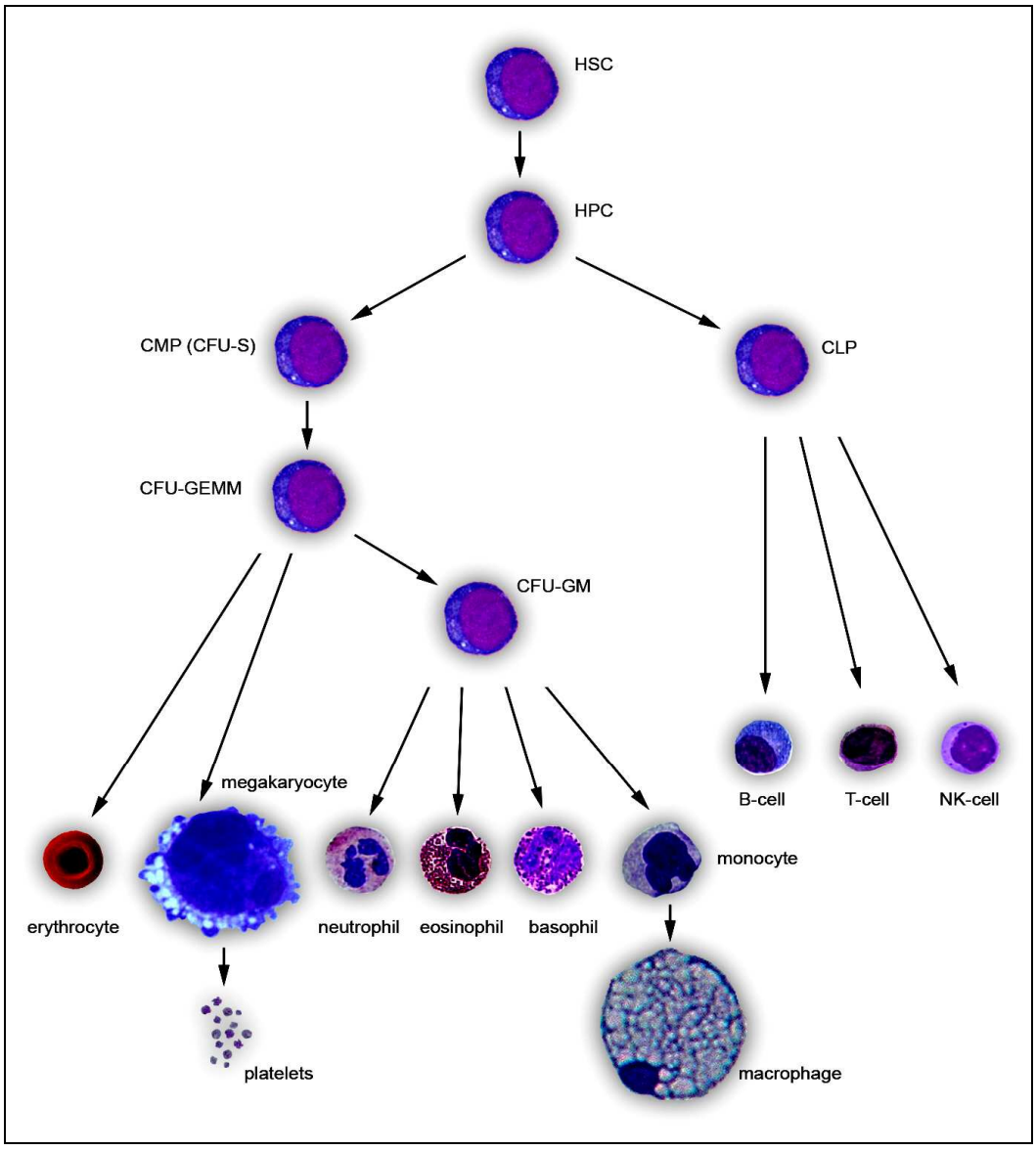



***Fig. Overview: haematopoiesis [137].*** *HSC = haematopoietic stem cell, HPC = haematopoietic progenitor cell , CLP = common lymphoid progenitor, CMP = common myeloid progenitor, CFU-GEMM = colony forming unit – granulocytes erythrocytes monocytes megakaryocytes, CFU-GM = colony forming unit-granulocytes monocytes.*

# 6 G protein coupled receptor pathways

One large group of agonist-activated receptors of the cell is the class of the G protein coupled receptors (GPCRs). They are membrane-spanning heptahelical structures with three extracellular and three intracellular loops [138, 139]. The N-terminus is located outside the cell, the C-terminus inside. Upon binding of an agonist, the receptor changes its conformation and this leads subsequently to an activation of an associated G protein, a small trimeric protein with intrinsic GTPase activity. For this activation mainly the second and third intracellular loops are responsible [139]. In the inactivated state, the α subunit of a G protein binds GDP. Upon activation GDP is exchanged in a rate limiting step by GTP [140, 141]. The α/β/γ-complex of the G protein disassembles and α or β/γ subunits then effect different targets such as phospholipases (PLCβ, PLD), adenylate cyclase (AC) and many others. One prototype receptor in human myeloid cells is, for example, the formyl peptide receptors (FPRs) with associated $G_i$ proteins whose β/γ subunits target PLC-β [142]. The inactivation of the G protein is achieved by the action of the GTPase activity of the α subunit [140, 143]. GTP is degraded to GDP and inorganic phosphate and the α subunit reassembles with the β/γ subunit.

## 6.1 *Downstream targets of the FPR*

Human neutrophils and HL60 possess FPRs, LTB4 and PAF receptors. Neutrophilic granulocytes are specialised on the destruction of microorganisms. They possess cellular mechanisms capable to react to pathogen intruders by detecting, moving towards them and killing them, e.g. by ROS and protease release. These events are triggered by substances that bind to GPCRs and since their binding results in chemotaxis they are also referred to as chemoattractants [144, 138]. The small peptide fMLP derived from bacteria or produced endogenously in mitochondria, for example, induces $G_i$-dependent (PT-sensitive) [145, 146] $Ca^{2+}$ release from intracellular stores, activation of NADPH oxidase leading to ROS formation [147], azurophilic granule release [148] and regulation of selected $K^+$-channels [149]. The release of granule contents includes cathepsin G, proteinase-3 and elastase. All these serine proteases play a role in degradation of the extracellular matrix or digesting pathogens at the sites of inflammated

tissue. Aside from involving $G_i$-proteins it is also stated that $G_q$-proteins might play a role in these events [150, 151].

Phospholipase C-β (PLC-β) lies downstream of the FPR. It is activated either by the β/γ subunit of $G_i$ [152, 142] or the α subunit of $G_q$ [153]. This PL leads to cleavage of phosphatidyl inositol bisphosphate ($PIP_2$) to diacyl glycerol (DAG) and inositol trisphosphate ($IP_3$), that triggers for $Ca^{2+}$ release [154]. PLC activation is one of the earliest events after fMLP stimulation [155]. DAG, a phorbol ester is able to activate PKC together with $Ca^{2+}$. PKC is probably responsible for ROS formation and influences the cytoskeleton by myristoylated alanine-rich C kinase substrate (MARCKS) [156, 157]. It furthermore activates Ras or Raf directly, thus, signaling towards the MAPK pathway [158].

$LTB_4$ and PAF receptors are thought to act in the same manner as FPRs in differentiated HL60 and PMNL. When activated, they lead to increased $[Ca^{2+}]_i$ but are not or less able to cause azurophilic granule release and ROS formation [159, 160]. $LTB_4$ acts on the LT receptors BLT1 and 2 [161] and PAF on the PAF receptor. Both receptors are coupled to $G_i$ and $G_q$ proteins.

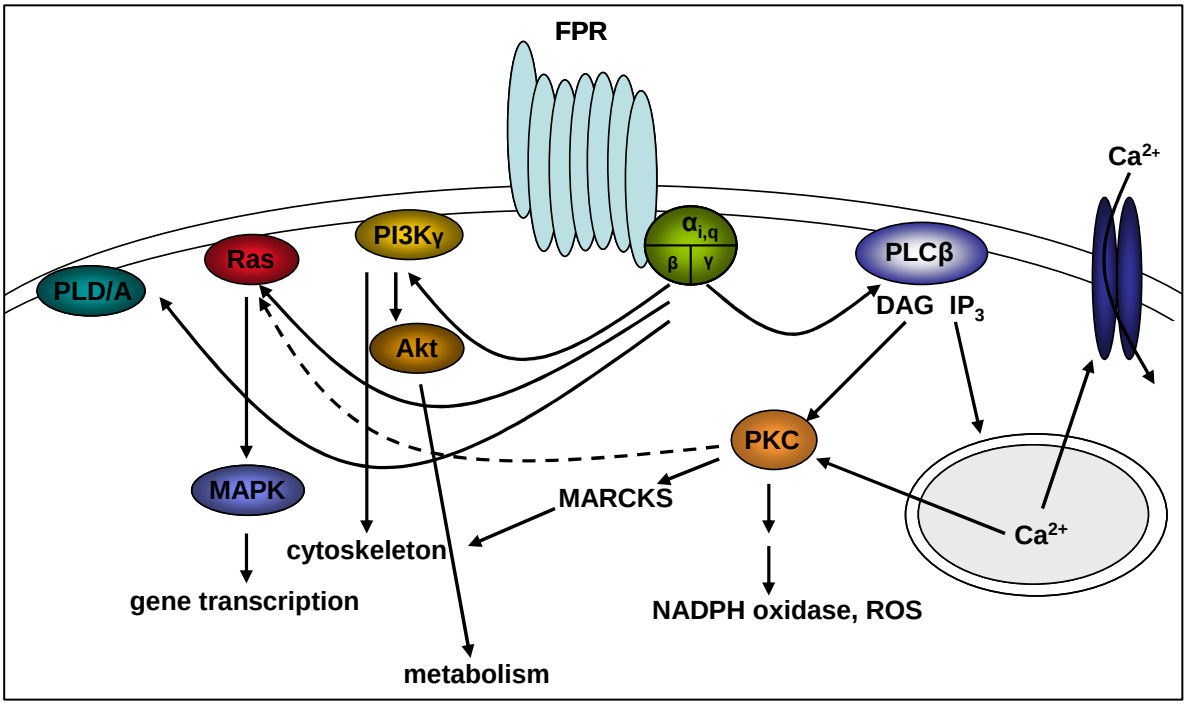



*Fig 6. Downstream targets of the fMLP receptor FPR.*

# 7 The MAPK pathways

The family of mitogen activated protein kinases (MAPK), also sometimes referred to as microtubule associated protein kinases, are serine/threonine specific kinases responding to extracellular stimuli, leading the signal on towards gene expression, cell proliferation and survival. There are three main groups of MAPK: a) the extracellular signal-regulated kinases 1 and 2 (ERK 1/2) being normally activated by growth factors and regulate growth, proliferation and differentiation [162], b) c-Jun N-terminal kinases (JNKs), also termed stress-activated protein kinases (SAPK) [163] and c) p38 MAPK [164]. JNK and p38 MAPK are stimulated by stress factors such as heat, osmotic shock, cytokines and radiation and having an influence on differentiation and survival/apoptosis.

Activation of the MAPK pathway usually involves receptor tyrosine kinases. These receptors are autophosphorylated before other kinases and adaptor proteins can bind the phosphorylated tyrosine residues or proline-rich domains via the src homology domains 2 and 3 (SH2, SH3), respectively [165]. The next component downstream is the growth factor receptor bound protein 2 (Grb2) which transfers the signal to the guanine nucleotide exchange factor (GEF) son of sevenless (Sos). This exchange factor is responsible for activation of a member of the Ras family of proteins. Ras can transmit the signal by phosphorylation of a MAPKKK which is then signaling downstream to a MAPKK that phosphorylates (and thus activates) MAPK itself.

The Ras (**ra**t **s**arcoma) oncogenes are a family of small G proteins. Their genes were first identified in rats by Harvey [166] and Kirsten [167] (K-ras, H-ras and N-ras). There are more than one hundred proteins in the Ras superfamily. In the present study, an antibody was used detecting total protein amounts of K-Ras, H-Ras and N-Ras, the Ras isoforms signaling towards gene expression concerning cell proliferation. Mutation of ras, resulting in impaired GTPase activity or in guanine nucleotide exchange, can lead to increased Ras activity being the reason for many carcinogenic cell transformations (see also HL60). Downstream of Ras there is the serine/threonine-specific kinase Raf (**ra**t **f**ibrosarcoma or **ra**pidly growing **f**ibrosarcoma). C-Raf or Raf 1 is similar in sequence to A-Raf and B-Raf but Raf 1 is the Raf isoforms signaling from Ras towards MEK 1/2 [168]. Aside from Raf 1, MEK 1/2 (MAPKK/MAP2K) is phosphorylated by a MAPKKK

(MAP3K/MEKK). Upon activation, MEK1/2 then in turn phosphorylates/activates ERK 1/2 [169].

Recent findings report that there are two scaffolding proteins involved in downstream processing of the signal from Ras towards ERK: kinase supressor of Ras (KSR) and connector enhancer of KSR (CNK) [170]. The theory bases on the assembly of a complex at the membrane consisting of Ras, c-Raf, KSR and CNK leading together to phosphorylation of MEK 1/2 and subsequently ERK [171, 172](see Fig. Complex formation model for MEK and ERK activation).

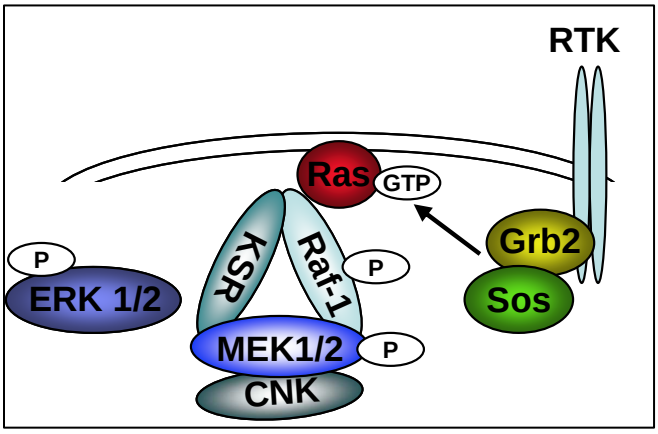



***Fig. Complex formation model for MEK and ERK activation [171, 172]***

## 7.1 *Activation of the Ras/Raf pathway by fMLP*

The small G protein Ras is one possible target of the FPR-associated Gi protein. Intensive studies have been conducted on the connection between the classical GPCR pathways and the MAPK pathways [173, 174, 158]. It was found, that fMLP induces Raf, MEK-1 and ERK1/2-activation after 30 sec and that this activation reached a maximum after 2-3 min. Interestingly, pertussis toxin, a classical $G_{\alpha i}$ inhibitor, reduced fMLP induced Raf activation, but only to around 65 to 80%. This means that there must exist at least two possible ways of activating Raf. Another study stated Raf being activated by PKC [175]. Nonetheless, this pathway is not used when stimulating neutrophils with fMLP. Raf activation in fMLP stimulated neutrophils was not affected when PKC was inhibited by 2 μM GF109203x for 0.5 to 5 min, while PMA activation of Raf could be inhibited by GF. Aside from a direct interaction of $G_i$ with Ras, there is the possibility of the involvement of tyrosine kinases which are usually activated by RTKs such as the EGF receptor. They transfer the signal from the GPCR towards the Ras/Raf pathway [176-178]. This process is called transactivation and is an excellent example for the intertwined activation events of signaling cascades. As example, Worthen et al. showed that fMLP-induced Raf activation can be inhibited by the tyrosine kinase inhibitor herbimycin A (thought to be most specific for Src-kinases) and thus, that activation of Raf by fMLP is dependent on tyrosine kinases. Recently, MEKK1, another upstream kinase of the MAPK pathway, has been identified [179] and might present an additional way of activating MEK and ERK. Taken together, in fMLP-induced Ras/Raf activation the direct interaction of $G_{\alpha i}$ with Ras is the fastest and most prominent event leading to ERK activation. Activation of Raf by PMA-stimulated PKC is also possible, albeit of no importance in fMLP-stimulated neutrophils.

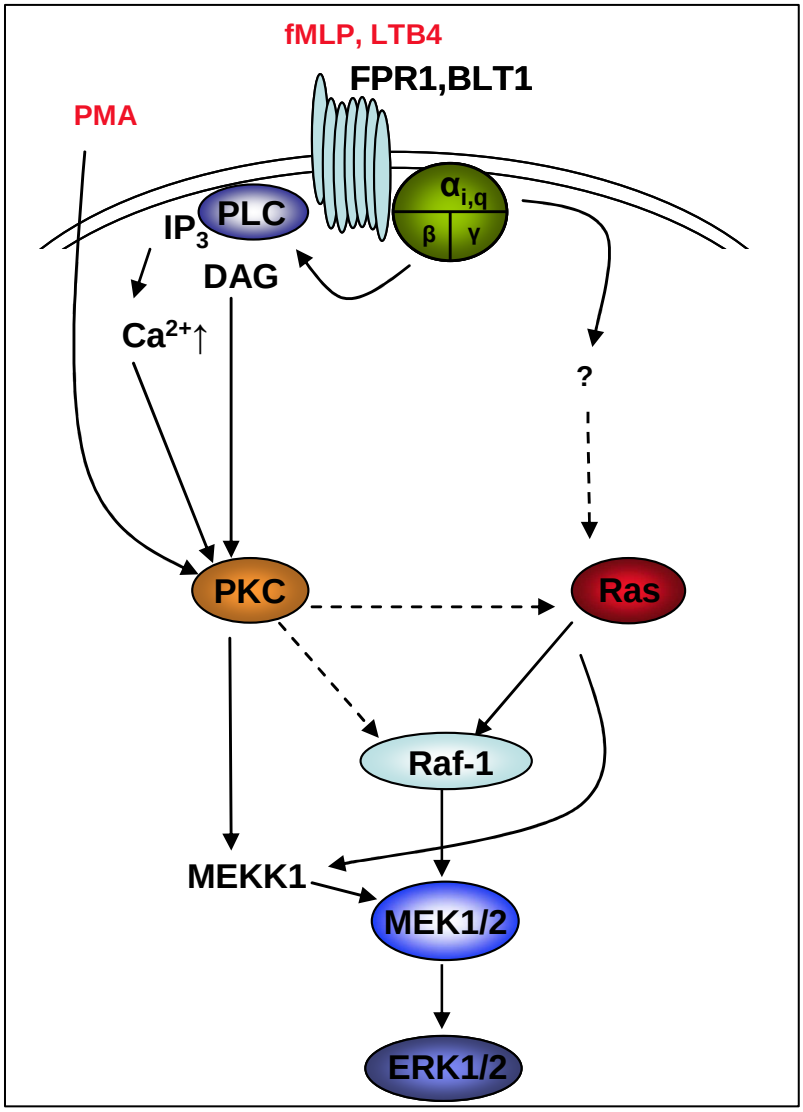



***Fig. Pathways of ERK-activation by fMLP and PMA [158].***

# 8 Aim of the study

It was shown that myrtle extracts and MC have strong anti-inflammatory effects [180, 18]. In the course of the studies concerning MC and its effects on inflammation it was found that MC can activate caspases [22]. Since nowadays the connection between inflammation and cancer is strongly emerging, the research was continued in the direction of apoptosis induction by MC in cancer cells. Initial experiments in cancer cells showed that MC indeed reduces cell viability most efficiently in cells of leukaemian origin and that untransformed control cells (PBMC) are hardly susceptible to MC [22].

The aim of the present study was to identify and to characterise the exact mechanisms of apoptosis induction by MC. This was no easy task since many details about apoptosis and how it is induced are still missing or not totally understood.

To put the cart before the horse, this work begins with explaining the late effects of apoptosis like caspase cleavage, DNA fragmentation, cytochrome c release and the differentiation between intrinsic and extrinsic way of apoptosis induction. Then it was the aim to explain the imminent events preceding these effects.

On the other hand it was important to consider previous data obtained by C. Feißt and colleagues which showed some short-term effects of MC in non-degenerated PMNL [18]. It was also important to understand these mechanisms of interference with signaling pathways in granulocytes and comparing them with the ones observed in the cancerogenous counterpart HL60. Here, we see the possible mechanistic fundament which then might lead to apoptosis induction in cancer cells. In the end, the study tries to give a theory why this interference renders apoptosis induction in cancer cells but not so in non-transformed cells, going deeply into detail about differences in signaling pathways and metabolic circumstances in non-transformed cells versus cancer cells.

The data presented in this work will show that MC has selective apoptotic effects on cancer cells and the respective mechanisms. Taking into account that MC also possesses potent anti-inflammatory effects (and the presumed link between inflammation and cancer), MC is proposed an efficient and selective drug with high therapeutical potential in leukaemia and inflammatory diseases, worth to be tested in pre-clinical animal studies for further development.

# Materials and Methods

# Materials and Methods

## 9 Materials

Myrtucommulone (MC) was isolated from myrtle leaves as described previously [1] and obtained from Dr. Appendino, Naples, Italy and Dr. J. Jauch, Saarbrücken, Germany. MC stocks were prepared at 15 mM in DMSO and kept in the dark at -20°C. Freezing-thawing cycles were kept to a minimum.

The following materials were obtained from:

**2´,7´-dichlorofluorescin diacetate** (DCFH-DA), SIGMA-Aldrich, Germany

**5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide** (JC-1), SIGMA-Aldrich, Germany

**5-bromo-4-chloro-3-indolylphosphate toluidine salt** (BCIP), Applichem

**5-HETE** (5-hydroxyeicosatetraenoic acid), Cayman Chemical, Tallinn, Estonia

**Arachidonic acid** (AA), Biomol, Hamburg, Germany

**β Actin-antibody**, Santa Cruz

**BWA4C** was a generous gift from Dr. L. G. Garland (Wellcome Research Laboratories)

**Carboynl cyanide 4-(trifluoromethoxy)phenylhydrazone** (FCCP), SIGMA-Aldrich, Germany

**Caspase 8 antibody (mouse)**, Prof. Wesselborg, University Hospital, Tübingen

**Chelerythrine**, SIGMA-Aldrich, Germany

**Cycloheximide** (CHX), SIGMA-Aldrich, Germany

**Cyclosporine A** (CsA), Calbiochem

**Daunorubicin**, Biomol Intern., USA

**Fas-Ligand**, Biozol, Eching, Germany

**GSH-Sepharose beads**, GE Healthcare, Freiburg, Germany

**Hybond$^{TM}$-C Extra nitrocellulose membrane**, GE Healthcare

**Ionomycin**, SIGMA-Aldrich, Germany

**Leukotriene $B_4$**, Biozol, Eching, Germany

**Leupeptin**, SIGMA-Aldrich, Germany

**N-formyl-methionyl-phenylalanine** (fMLP), SIGMA-Aldrich, Germany

**Nitro-blue tetrazolium chloride** (NBT), Roche

**peQGold Protein-Marker IV**, PeQLAB Biotechnology, Erlangen, Germany
**pGEX-Raf-ras binding domain**, Frankfurt
**Phenylarsine oxide** (PAO), SIGMA-Aldrich, Germany
**Phenylmethylsulfonylfluorid** (PMSF), SIGMA-Aldrich, Germany
**Phorbol 12-myristate 13-acetate** (PMA), Alexis Biochemicals, Lausen, Switzerland
**p-nitrophenyl phosphate** (pNpp), Applichem
**Primary antibodies**, New England Biolabs
**Propidium iodide** (PI), SIGMA-Aldrich, Germany
**phospho-tyrosine antibody**, Santa Cruz
**Q-VD-Oph (QVD)**, Calbiochem
**Roti-Nanoquant**, Roth, Heidelberg, Germany
**Secondary antibodies**, SIGMA-Aldrich, Germany
**Soybean trypsin inhibitor** (STI), SIGMA-Aldrich, Germany
**Staurosporine**, Calbiochem, Germany
**Thiazolyl blue tetrazolium bromide** (MTT), SIGMA-Aldrich, Germany
**Vybrant Apoptosis Kit #2**, Molecular Probes, Invitrogen

Chemicals not listed were obtained from SIGMA-Aldrich, Germany or Roche Diagnostics, Germany.

Cell culture and preparation materials were obtained from:
**RPMI 1640 medium**, PAA, Coelbe, Germany
**penicillin/streptomycin 100x** , PAA, Coelbe, Germany
**fetal calf serum (FCS)**, PAA, Coelbe, Germany
**1 M Hepes**, PAA, Coelbe, Germany
**MEM non-essential amino acids**, SIGMA-Aldrich, Germany
**Lymphocyte Separation medium**, PAA, Coelbe, Germany

Cell culture flasks, petridishes, 96-well, 12-well plates and other steril cell culture materials were obtained from Greiner Bio-One, Germany.

## 10 Cells

Jurkat-A3 cells, caspase-8-deficient Jurkat cells, and FADD-deficient Jurkat cells were provided by Dr. John Blenis, Boston, MA; caspase-9-deficient Jurkat cells and caspase-9-deficient Jurkat-caspase-9-retransfected cells were obtained from Prof. Dr. Klaus Schulze-Osthoff, Duesseldorf, Germany; HL-60 (acute promyelotic leukemia), Jurkat (acute T-cell leukemia) and Jurkat DD3 (acute T-cell leukemia, Fas-deficient), were obtained from the American Type Culture Collection (ATCC). Mono Mac 6 (MM6, acute monocytic leukemia) cells were a kind gift by Dr. H.W. Ziegler-Heitbrock, Muenchen, Germany. Jurkat vector cells and Jurkat Bcl 2-wt (Bcl2 overexpressing Jurkat cells) cells were obtained from the lab of Dr. Claus Belka, University Hospital, Tuebingen.

PBMC and PMNL were freshly isolated from leukocyte concentrates derived from human healthy donors obtained from the Blood Center at Tuebingen University Hospital. In brief, venous blood was taken from healthy adult volunteers and subjected to centrifugation at 4,000 × g for 20 min at 20°C for preparation of leukocyte concentrates. Cells were promptly isolated by dextran sedimentation and centrifugation on Lymphoprep cushions (PAA Laboratories). After washing in PBS pH 7.4, PBMC were resuspended in RPMI 1640 medium with supplements. PMNL were taken from the pellet and hypotonic lysis of remaining erythrocytes was performed as described [181].

All cells were cultured in respective media supplemented with 10% fetal calf serum (FCS, PAA, Coelbe, Germany), 100 µg/ml streptomycin and 100 units/ml penicillin and grown at 37°C in a 5% $CO_2$/ 95% air atmosphere. For harvesting, cells were centrifuged at 200 × g for 10 min at RT and finally resuspended as indicated.

# 11 Methods

## 11.1 *Cytotoxicity assay (trypan blue)*

Cells were incubated at $0.2 \times 10^6$ cells per ml in the appropriate medium with compounds or vehicle (DMSO) for the indicated times. Cells were harvested by centrifugation at 1200 rpm for 10 min at room temperature, washed with PBS and counted under a light microscope using a “Bürker” hemocytometer after addition of trypan blue (1:1, vol/vol; 0.2% trypan blue in 0.9% NaCl). Since trypan blue is a cell impermeable dye staining chromatin, dead cells appeared blue under the microscope, therefore, the cell viability could be determined by counting only viable cells.

## 11.2 *Cytotoxicity assay (MTT assay)*

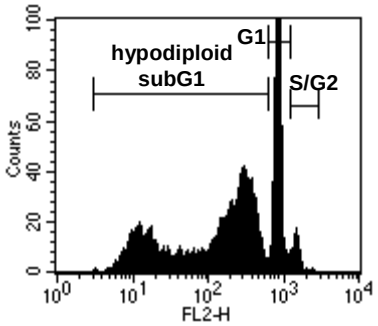
Cell viability was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [182] as described before [183]. In brief, cells were incubated at 37°C and 5% $CO_2$ atmosphere with the indicated compounds for the times indicated. DMSO was used as solvent for all compounds (never exceeding 0.5% (vol/vol)). Then, MTT reagent was added for a minimum of 3 hours or until untreated control cells clearly showed blue staining. Afterwards, 100 µl SDS lysis buffer (10% SDS in 20 mM HCl, pH 4.5) was added and cells were left on the shaker for 16-20 hours. Plates were read on a multiwell scanning spectrophotometer (Victor$^3$ plate reader, PerkinElmer) at a wavelength of 595 nm. Induction of cell death was determined as the relative reduction of the optical density compared to DMSO treated and EtOH treated cells.

The MTT assay is based on the reduction of the yellow tetrazolium bromide to a non-hydrolysable purple formazan. This reduction is accomplished by enzymes of mitochondria (succinate-dehydrogenases) and endoplasmatic reticulum and probably depends on NADH and NADPH. Therefore, only metabolic active, viable cells are able to reduce the MTT. However, it is important to note that reduction in cell viability measured by MTT assay can be due to decreased cell proliferation, increased apoptosis and/or necrosis or due to reduction in metabolic activity of cells.

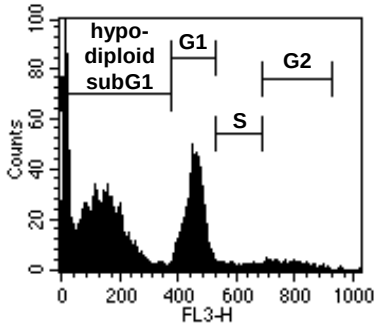
## 11.3 *Determination of DNA-fragmentation and cell cycle measurements*

According to the method developed by Nicoletti [184] nuclei of apoptotic cells were stained with propidium iodide to determine DNA-leakage taking place after DNA-fragmentation during apoptosis. In brief, $0.3 \times 10^6$ cells were incubated in the appropriate medium with DMSO, MC and controls in the amounts and for the times indicated. After incubation, cells were harvested by centrifugation at 4,200 g for 5 min at RT and washed once with PBS. The pellet was then resuspended in 100 µl hypotonic PI-lysis buffer (0.1% sodium citrate, 0.1% Triton X-100, 50 µg/ml propidium iodide) and incubated in the dark for 10 min at RT. Then, fluorescence intensity was measured using a FACScalibur (Becton Dickinson) flow cytometer (FL2) and was plotted logarithmically vs. cell counts.

To better evaluate cell numbers in G1, S and G2 phase, fluorescence intensities as measured in FL3 were plotted linear vs. cell counts. The fluorescence intensity of nuclei from non-apoptotic diploid cells was set to around $10^3$ in FL2 channel (585/42). The method is based on hypotonic rupture of cellular membranes to allow PI to enter the cells and the nuclei. Nuclei of apoptotic cells have lower fluorescence intensities due to the leakage of DNA-fragments during apoptosis, visualised as a hypodiploid peak (subG1).




***Histogram of mainly apoptotic nuclei(FL2).***




***Histogram of different cell cycle phases(FL3).***

## 11.4 *Measurement of cytochrome c release from mitochondria*

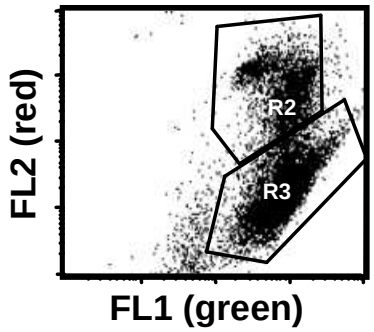
Cells were seeded at 4 × $10^6$/ml culture medium and incubated with the test compounds or vehicle (DMSO). After 24 hours cells were harvested, washed once in PBS and $10^7$ cells were resuspended in 200 µl PBS. In order to permeabilize cellular membranes, 600 µg/ml (300 µg/ml for Jurkat cells) digitonin was added and immediately vortexed for 10 seconds, incubated for another 30 seconds at RT and centrifuged at 20,000 × g at 4°C for 1 min. The supernatants (cytosolic fractions) were transferred to new tubes and mixed 1:1 (vol/vol) with 5% trichloroacetic acid. Precipitation of cytosolic proteins was performed at 4°C overnight. Proteins were pelleted by centrifugation at 20,000 × g at 4°C for 30 minutes and resuspended in 25 µl PBS. Aliquots of 5 µl were used for determination of protein concentration using Roti-Nanoquant (Roth, Karlsruhe, Germany). Equal amounts of protein were mixed 1:1 (vol/vol) with 2 × SDS/PAGE sample loading buffer and analyzed for cytochrome c by SDS-PAGE and Western Blot using an anti-cytochrome c antibody.

Digitonin dissolves cholesterol from the plasma membrane but leaves the membranes of intracellular organelles intact. By permeabilisation of the plasma membrane and subsequent centrifugation it is possible to separate cytosolic proteins from proteins contained in the organelles. This way, cytochrome c which is normally found within mitochondria should only be detected in the cytosol upon previous cytochrome c release due to apoptotic events.

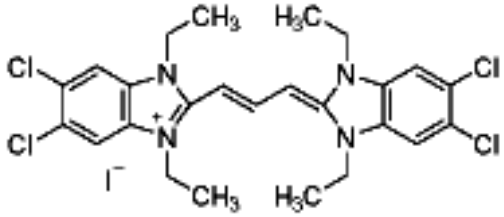
## 11.5 *Determination of the mitochondrial membrane potential ΔΨ*

Cells ($10^6$ per ml) were incubated in culture medium with solvent (DMSO), MC and controls as indicated. After incubation, 0.75 µg/ml JC-1 was added to the cells and incubated 15 min at 37°C. Then, cells were harvested, washed once with ice cold PBS and kept on ice until measuring red/orange against green fluorescence was analysed by flow cytometry, where JC-1 was excited at 488 nm. Red/orange fluorescence was detected through FL2 (575 nm) and green fluorescence through FL1 (530 nm). A log FL1 (x-axis) versus log FL2 (y-axis)

dot blot was created. The percentage of cells that lost $\Delta\Psi_m$ versus cells possessing $\Delta\Psi_m$ was calculated using the cellQuest software.




JC-1 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) is a substance which enters the cell and attaches to mitochondrial membranes. Depending on the membrane potential it either is a green fluorescent monomer (at low potentials) or forms red/orange fluorescent aggregates at higher potentials. The ratio of red/orange to green fluorescence is only dependent on the potential and not on the size or shape of mitochondria.




***5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1)***

## 11.6 *Annexin V / propidium iodide assay*

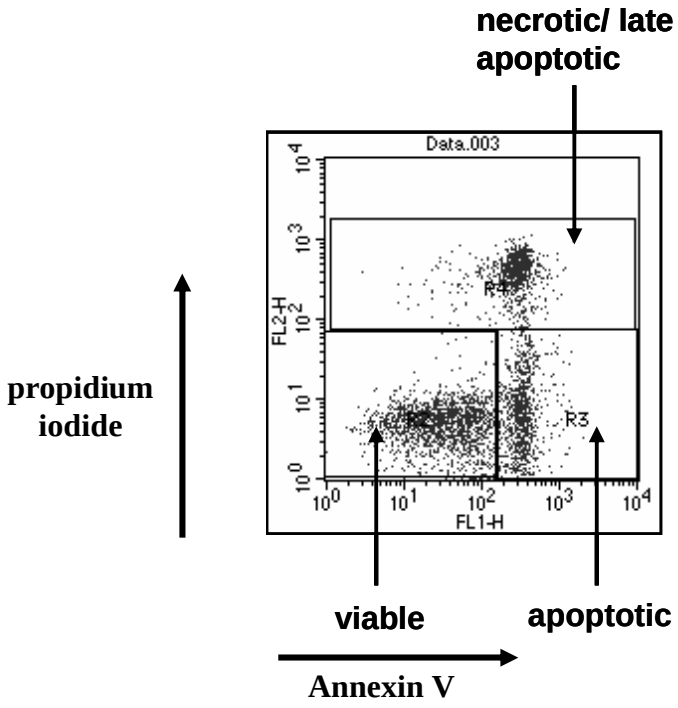
One event during early apoptosis is the externalization of the phospholipid phosphatidylserine (PS). Under normal conditions, PS is located to the inner layer of the plasma membrane. Upon apoptosis stimulation it is flipped to the outer membrane and functions as signal for macrophages to bind and ingest the apoptotic cell.

Annexin V, a coagulation protein that binds to PS $Ca^{2+}$-dependently, is an important modulator of phagocytosis of apoptotic cells [185]. This phenomenon has been used to develop a method using fluorophore-coupled Annexin V and another membrane impermeable dye such as propidium iodide (PI) staining DNA to discriminate between viable, apoptotic, necrotic and late apoptotic cells [186].

Not only externalised PS can be bound by Annexin V but also during late apoptosis and necrosis when cell membrans are disrupted, AnnexinV can enter the damaged cells and can bind PS from the inside. To distinguish between these two possibilities of Annexin V binding and therefore distinguish early apoptosis from late apoptosis and necrosis, also propidium iodide is used which only enters the

cell and stains DNA when membranes are disrupted. The Vybrant Apoptosis Kit #2 (Molecular Probes, Invitrogen) was used and instructions were as follows:

$0.2 \times 10^6$ cells per ml were incubated with DMSO, MC and controls in the appropriate medium for the times and in concentrations indicated. Then, about $4 \times 10^5$ cells were harvested, washed in cold PBS and resuspended in 100 µl 1 × annexin binding buffer. Then 3 µl annexinV-Alexa Fluor 488 and 1 µl PI working solution prepared as described in the Kit protocol were added and samples were measured as soon as possible in a FACScalibur flow cytometer (Becton Dickinson) (FL1 (530 nm) vs. FL3 (>575 nm)).




***Dot plot of AnnexinV-AlexaFluor 488/ propidium iodide stained cells.***

## 11.7 *SDS-PAGE and Western Blot*

Cells ($4 \times 10^6$) were resuspended in 50 µl PBS, mixed with the same volume of 2 × SDS/PAGE sample loading buffer (SDS-b) (20 mM Tris-HCl, pH 8, 2 mM EDTA, 5% (m/vol) SDS, 10% (vol/vol) β-mercaptoethanol), and boiled for 5 min at 95°C. Aliquots (20 µl) corresponding to equivalents of $0.8 \times 10^6$ cells were mixed with 4 µl glycerol/0.1% bromphenol blue (1:1, vol/vol) and proteins were separated by SDS-PAGE. After electroblotting to nitrocellulose membrane (GE Healthcare) and blocking with either 5% non-fat dry milk or 5% bovine serum albumin for 1 h at room temperature, membranes were washed and incubated with primary antibodies overnight at 4°C. The membranes were washed and incubated with 1:1000 dilution of alkaline phosphatase-conjugated immunoglobulin G for 3 hours at room temperature. After washing, proteins were visualized with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

## 11.8 *PTPase assay*

PTPase activity was measured as described previously [187]. In short, cells were set to $2 \times 10^6$ per ml in PBS + 0.1% glucose + 1 mM $CaCl_2$. DMSO, MC and phenylarsine oxide (PAO) were added to about $8 \times 10^5$ cells and samples were incubated for 30 min on ice. After centrifugation at 2,000 × g for 5 min and 4°C, pellet was carefully resuspended in 20 µl Tris/KCl/$MgCl_2$ (TKM) buffer + Triton X-100 (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 60 µg/ml STI, 10 µg/ml, 0.5% Triton X-100) and lysed for 15 min on ice. Cell lysates were obtained by centrifugation at 21,000 × g for 15 min. Since Triton disturbs the photometrical measurement of PTPase activity, the supernatant was diluted 1:10 in TKM buffer without Triton X-100. For each sample 100 µl was given into a 96 well plate and mixed with 20 µl 60 mM PTPase substrate p-nitrophenyl phosphate (p-NPP). Absorption was measured over the time at 405 nm in a Victor$^3$ plate reader (PerkinElmer).

## 11.9 *Ras-assay*

Ras activity was assessed by measuring the amounts of GTP-bound Ras using an affinity chromatographic method as described previously [188]. Briefly, $10^6$ PMNL or $5 \times 10^5$ HL60 cells were preincubated with DMSO, MC and controls for 30 min at 37°C and then stimulated as indicated. Then, cells were lysed by addition of 100 µl 2 × lysis buffer (50 mM Tris-HCl, pH 7.4, 10% glycerol, 1% NP-40, 2.5 mM $MgCl_2$, 200 mM NaCl, 60 µg/ml STI, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 10 min. Cellular debris was removed by centrifugation at 20,000 × g for 5 min, and supernatants were transferred to new tubes containing the Ras-binding domain of Raf-1 (Raf-1-RBD) coupled to GSH-Sepharose beads. An aliquot of each supernatant was reserved to determine total Ras expression by Western blot analysis. Following a 45 min incubation at 4 °C, the agarose beads were centrifuged at 2,500 × g for 1 min, the supernatant was discarded, and the agarose beads were washed three times in 1 x lysis buffer (see above). Bound proteins were eluted by the addition of 25 µl of SDS loading buffer, loaded onto an SDS-PAGE gel, and total amounts of Ras-GTP were quantified by Western blot.

The method is based on the fact that only the active GTP-bound form of Ras is able to bind the Raf-Ras binding domain (Raf-RBD). When immobilising this domain to Sepharose beads and bringing it together with cell lysates of different Ras-activation states, only Ras-GTP will bind to the beads and can be separated from the rest of the cellular proteins.

## 11.10 *Statistical analysis*

Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Bonferroni post-hoc tests. Where appropriate, student´s *t* test for paired and correlated samples was applied. A *p* value of <0.05 (*), <0.01 (**) or <0.001 (***) was considered significant.

# Results

# Results

## 12 Myrtucommulone induces apoptosis in various cancer cell lines

### 12.1 *MC induces cell death in different cancer cell lines*

Within the previous work of Dr. I. Tretiakova [22], the effect of MC on cell viability was assessed in various cancer cell lines and the respective $EC_{50}$ values were determined (Tab. 1). Cells were grown for 5 days in presence of MC in the corresponding cell culture medium. Then, the yellow water soluble dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) was added.

| **cell line** | **origin** | **cell death, $EC_{50}$ (µM)** |
|---|---|---|
| PC-3 | androgen-independent prostate carcinoma | 3.93 ± 0.54 |
| LNCaP | androgen-dependent prostate carcinoma | 8.86 ± 1.19 |
| KFR | rhabdomyosarcoma | 7.07 ± 1.05 |
| HL-60 | acute promyelotic leukaemia | 3.26 ± 0.51 |
| MM6 | acute monocytic leukaemia | 3.11 ± 0.66 |
| H9 | cutaneous T-cell lymphoma | 3.56 ± 0.50 |
| DLD-1 | colorectal adenocarcinoma | 8.75 ± 1.02 |
| JURKAT | acute T-cell leukaemia | 4.01 ± 0.52 |
| HFF | human foreskin fibroblasts | > 20 |
| PMNL | polymorphonuclear leukocytes | > 20 |
| PBMC | human peripheral blood mononuclear cells | > 50 |

***Tab. 1 $EC_{50}$ values of MC induced cell death in different cancer and non-cancer cell lines. (Data (except PMNL and PBMC) obtained by Dr. I. Tretiakova, Frankfurt)*** *0.3-0.5 × $10^6$ cells were incubated in a microtiter plate with vehicle and increasing amounts of MC for 5 days (24 h for PMNL) in the appropriate medium. Then, the dye MTT was added to the cells and incubated for another 3 h until the untreated control clearly showed blue staining. MTT lysis buffer was added and the plate was left shaking 16-20 h overnight at RT. The absorption was measured at 595 nm in a Victor™ plate reader. $EC_{50}$ values were determined by approximate analysis of the graphs obtained by MTT assay. Data are given as mean ± S.E.*

Viable, metabolic active cells could reduce the dye to its insoluble blue-purple formazan which can be measured photometrically at 595 nm. In this way, the number of viable cells could be quantified. This method is used mainly to determine the cytotoxic effects of substances of interest without allowing to differentiate between necrotic, apoptotic and antiproliferative effects.

In the cell lines analysed, the half maximal effective concentrations of MC was between 3 to 9 µM (Tab. 1). It is noticeable that especially cancer cells of leukaemian origin (HL60, MM6 and Jurkat cells) were most sensitive towards MC, with $EC_{50}$ values of 3 - 4 µM. This led to the decision to concentrate on evaluating the effect of MC on leukaemia cells and, in this respect, to test mainly HL60, MM6 and Jurkat cells.

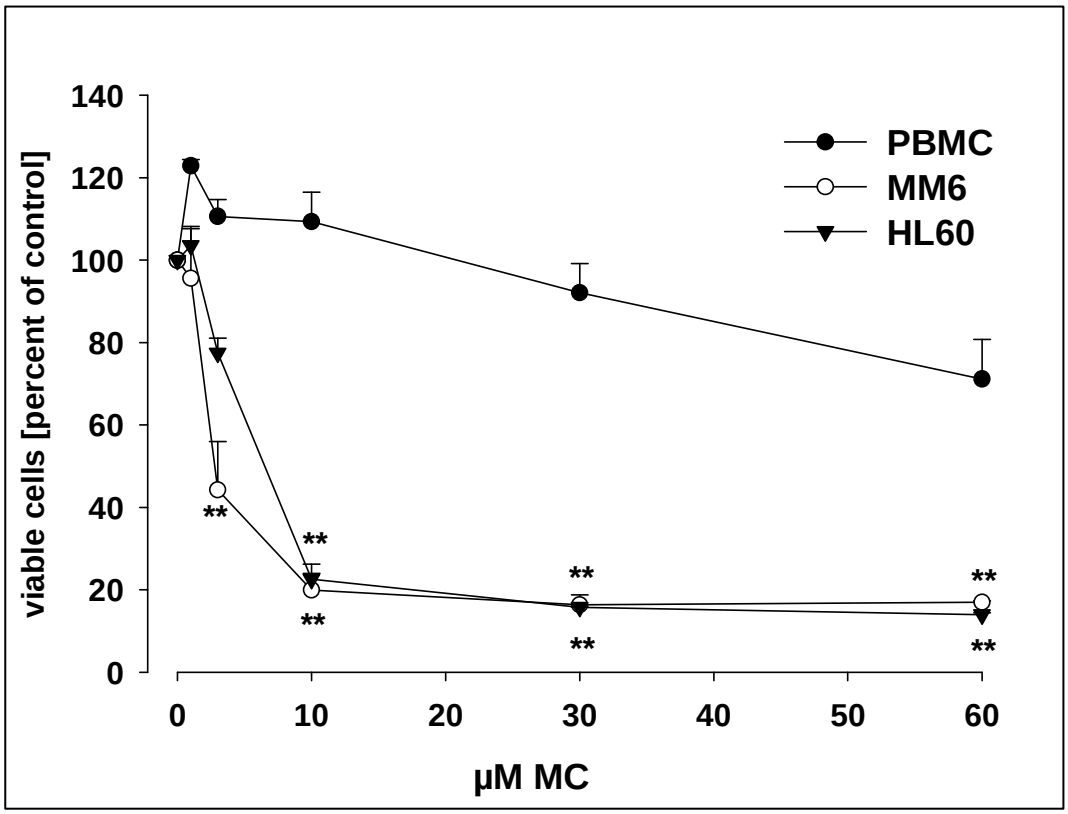



***Fig. 1 Cytotoxicity of MC in HL60 cells, MM6 cells and PBMC.*** *0.3-0.5 × $10^6$ cells were incubated in a microtiter plate with vehicle and increasing amounts of MC for 24 h in the appropriate medium. Then, the dye MTT was added to the cells and incubated for another 3 h until the untreated control clearly showed blue staining. MTT lysis buffer was added and the plate was left shaking 16-20 h overnight at RT. The absorption was measured at 595 nm in a Victor™ plate reader. Data are given as mean ± S.E. n=3-5 **p<0.01*

A strong difference in the potency of MC could be observed between cancer cells and primary blood cells where $EC_{50}$ values were higher than 20 or 50 µM.
When looking at the concentration-response curve of MC in HL60 cells, MM6 cells and PBMC the discrepancy of cytotoxicity in non-transformed cells compared to leukaemia cells is obvious (Tab. 1, Fig. 1).

## 12.2 *Induction of caspase-cleavage by MC in HL60 cells and MM6 cells*

Caspases are cysteinyl-aspartate-specific proteases which exist in the cell as inactive uncleaved procaspases and can be cleaved/activated quickly upon apoptosis signaling. Activation of caspases is essential for the programmed cell death. Concerning caspases, one differentiates between the so-called initiator caspases and the effector caspases. One of the main substrate of caspase 3 is poly-ADP-ribose-polymerase (PARP). In non-apoptotic cells, PARP (119 kDa) takes part in the DNA-repair. This is not necessary and even undesirable in apoptotic cells, therefore PARP is degraded. This degradation can be quantified by the 89 kDa fragment of PARP which is a classical marker of apoptosis [189].
The effect of MC on caspase cleavage in HL60 and MM6 cells was elucidated for further analysis of apoptosis induction. As control agent for apoptosis induction, cycloheximide (CHX), an inhibitor of protein biosynthesis, was used. Q-VD-Oph (QVD), a pan-caspase inhibitor, was used to assess if the induction of apoptosis by MC is caspase-dependent.
MC induced cleavage of the initiator caspases 2, 7 and 8 and of effector caspases 3 and 9 as well as PARP cleavage in a concentration-dependent manner starting at concentrations of 3-10 µM (Figs. 2 and 3). Caspase and PARP cleavage by MC could be inhibited by the pan caspase inhibitor QVD. CHX induced PARP cleavage and cleavage of caspase 8 as expected.

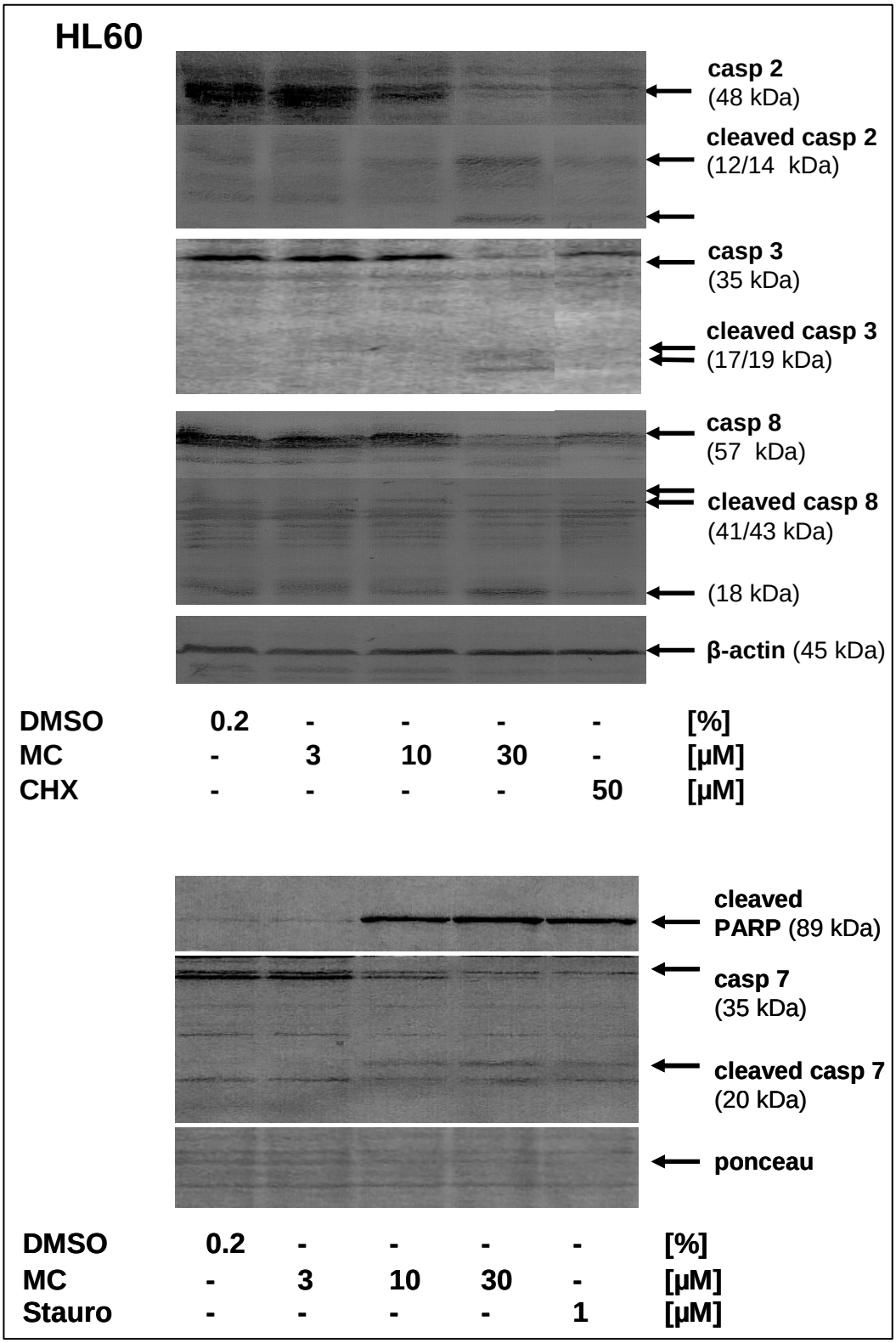



***Fig. 2 Caspase and PARP cleavage in HL60 cells.*** *0.2 × $10^6$/ ml HL60 cells were incubated for 16 h at 37 °C with DMSO, MC, cycloheximide (CHX) or staurosporine (Stauro) respectively. After centrifugation, the pellet was lysed with SDS-PAGE sample loading buffer (SDS-b) and analysed for caspases and PARP by SDS-PAGE and Western Blot. Data shown are representative for 4-5 independent experiments.*

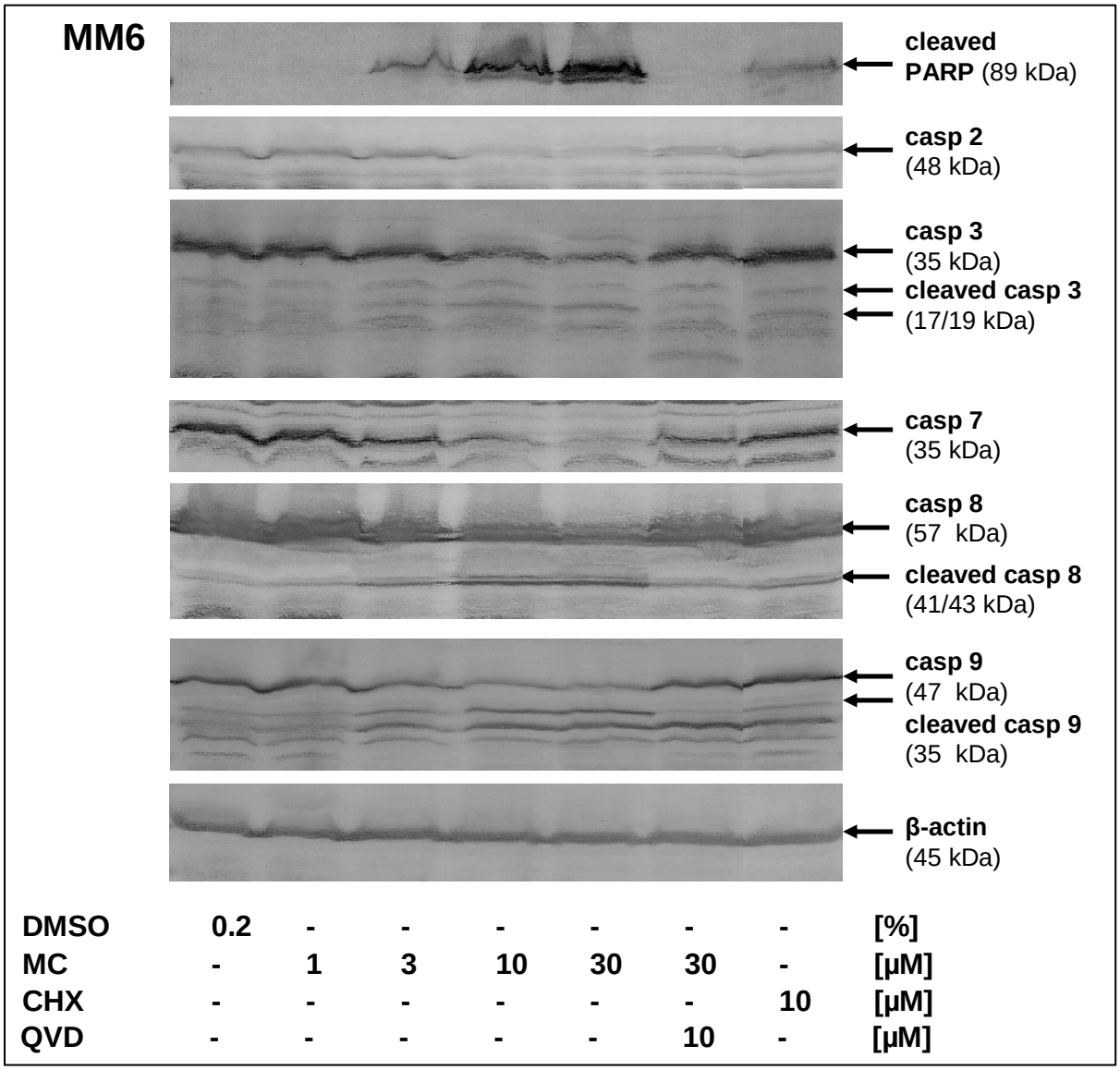



***Fig. 3 Caspase and PARP cleavage in MM6 cells.*** *0.2 × $10^6$/ml MM6 cells were incubated for 16 h at 37 °C with DMSO, MC, Q-VD-Oph (QVD) or cycloheximide (CHX), respectively. After centrifugation, the pellet was lysed with SDS-b and analysed for caspases and PARP by SDS-PAGE and Western Blot. Data shown are representative for 4-5 independent experiments.*

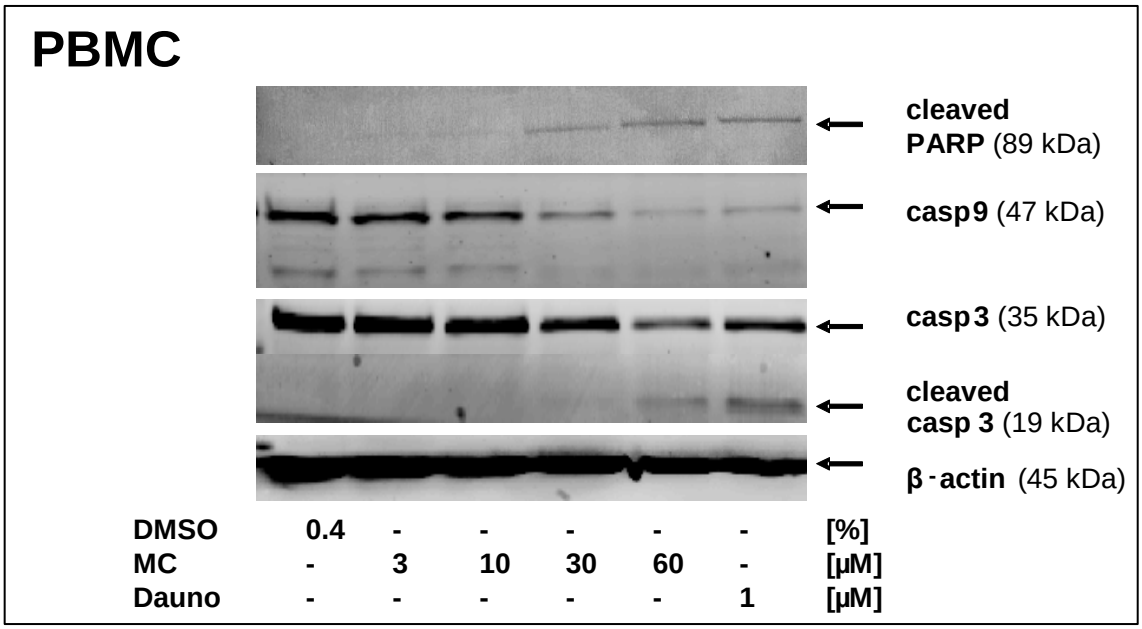



***Fig. 4 Caspase cleavage and PARP cleavage in PBMC.*** *PBMC were isolated from buffy coats as described in the materials and methods section. 0.5 × $10^6$/ml PBMC were incubated for 24 h at 37 °C with DMSO, MC or daunorubicin (Dauno). After centrifugation, the pellet was lysed with SDS-b and analysed for caspases and PARP by SDS-PAGE and Western Blot. Data shown are representative for 3-4 independent experiments.*

MC was also tested on PBMC for caspase and PARP cleavage. In these cells, a significant effect of MC on PARP, caspase 3 and caspase 9 was evident only at concentrations ≥30 µM (Fig. 4). 1 µM daunorubicin induced PARP cleavage as well as cleavage of caspase 3 and reduced the amounts of the uncleaved procaspase 9 in PBMC.

Time course experiments for PARP cleavage and caspase 3 and 9 activation by MC in HL60 cells, showed that the PARP fragments and active subunits of caspases can first be seen after 9 to 12 hours of incubation with MC (Fig. 5B). Staurosporine induced PARP cleavage and caspase activation within 3 hours (Fig. 5C).

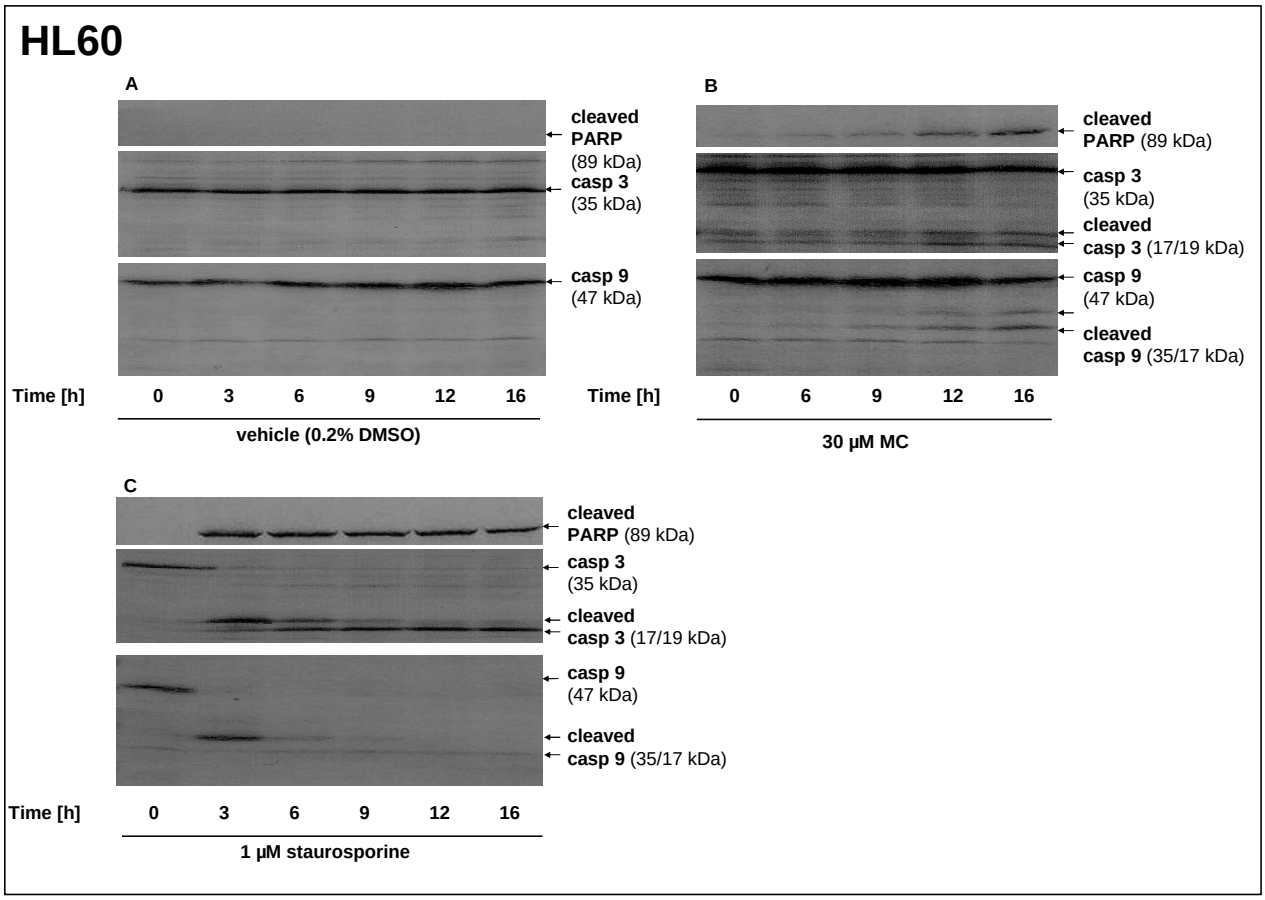



***Fig. 5 Time course of caspase and PARP cleavage in HL60 cells.*** *HL60 cells (0.2 × $10^6$ cells/ml) were incubated with* ***(A)*** *0.2% DMSO,* ***(B)*** *30 µM MC or* ***(C)*** *1 µM staurosporine for the indicated time periods at 37 °C. After centrifugation, the pellet was lysed with SDS-b and evaluated for caspases and PARP by SDS-PAGE and Western Blot. Data shown are representative for 3 independent experiments.*

## 12.3 *DNA fragmentation induced by MC in HL60 cells and MM6 cells*

An additional effect which can be observed during apoptosis is DNA fragmentation. To evaluate the effect of MC on DNA fragmentation, the DNA intercalating fluorophore propidium iodide was used.

Cells were incubated for 24 h with vehicle, MC or controls. Then, cells were harvested, washed once with PBS and carefully resuspended in 100 µl hypotonic propidium iodide lysis buffer. After 10 min incubation at RT, the number of apoptotic nuclei was measured in a flow cytometer (Fig. 6).

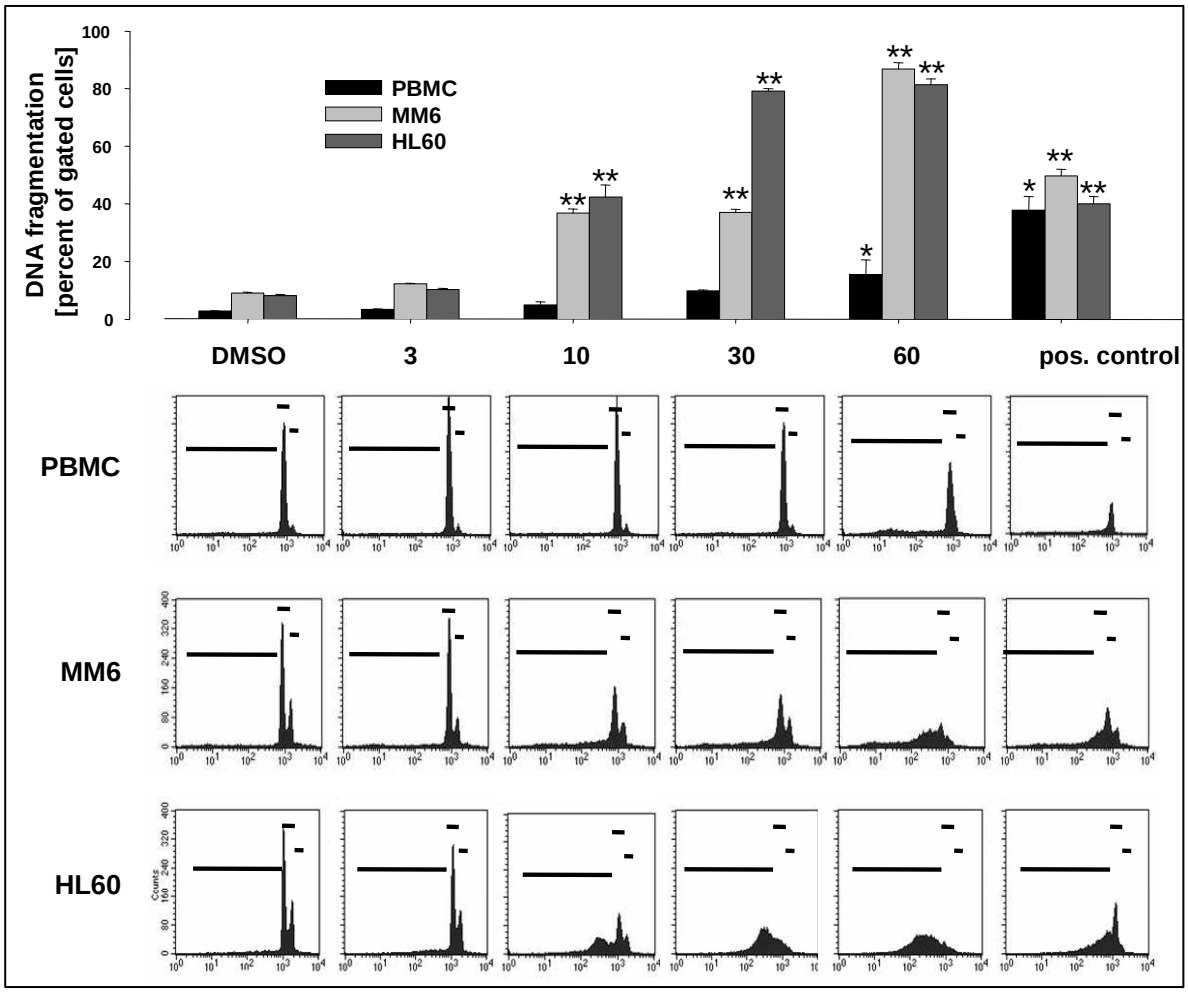



***Fig. 6 MC induces DNA fragmentation in HL60 cells and MM6 cells.*** *HL60 cells and MM6 cells (0.2 × $10^6$ cells/ml) and PBMC (0.5 × $10^6$ cells/m) were incubated with DMSO, with 3, 10, 30 and 60 µM MC or 3 µM staurosporine (PBMC), 1 µM staurosporine (MM6) or 50 µM CHX (HL60) for 24 h at 37 °C. Then, cells were harvested, washed in PBS and resuspended in 100 µl hypotonic propidium iodide buffer. After 10 min at RT, fluorescence intensities of cell nuclei were measured using a flow cytometer (FL2 585/42). Data are given as mean ± S.E. n = 2-5. *p<0.05, **p<0.01*

The amount of cells with lower fluorescence intensities (hypodiploid peak, subG1) was enhanced with increasing concentrations of MC. A significant effect of MC on DNA fragmentation was observed in HL60 cells and MM6 cells at concentrations of 10 µM. PBMC were less sensitive to MC and a significant although slight effect could be observed only at a concentration of 60 µM MC. Staurosporine induced DNA fragmentationin PBMC as well as in MM6 cells (at 3 µM and 1 µM respectively) and cycloheximide induced DNA fragmentation in HL60 cells as expected.

## 12.4 *MC leads to cytochrome c release in HL60 cells and MM6 cells*

Caspase cleavage and DNA fragmentation clearly indicated that MC-induced cytotoxicity in HL60 cells and MM6 cells is dependent on induction of apoptosis. The next step was to evaluate which mechanism is responsible for the effect of MC and if the intrinsic, mitochondrial pathway or the extrinsic pathway of apoptosis induction is involved.

The initial processes involving the mitochondrial state leading to apoptosis are: a) opening of the permeability transition pore (PTP), b) mitochondrial swelling, c) loss of mitochondrial membrane potential ($\Delta\Psi_m$), d) building of the mitochondrial apoptosis inducing channel (MAC) and e) release of mitochondrial proteins into the cytoplasm (cytochrome c, AIF, EndoG). However, the exact order of the occurence of each step is still unclear. Since DNA fragmentation might be dependent on release of mitochondrial proteins, and also activation of caspase 9 mainly depends on mitochondrial cytochrome c, the MC-induced release of cytochrome c from mitochondria was determined.

Cells were incubated for the times indicated with vehicle, MC or controls. After harvesting, the cells were treated with digitonin in order to permeabilise plasma membranes by dissolving cholesterol. This way, after centrifugation, the supernatant contains proteins of the cytoplasm, whereas mitochondria and other organelles remain intact. The release of cytochrome c can therefore be detected in the cytoplasm of the cells.

MC increased the amounts of cytochrome c in the cytoplasm of MM6 and HL60 cells after 24h incubation (Fig. 7 and 8). Cycloheximide also induced cytochrome c release in MM6 and HL60 cells as expected. The time-course showed that the cytochrome c release is induced by MC after 6 to 9 h and that it is not inhibited by QVD (Fig. 8). Therefore, MC-induced cytochrome c release is not caspase-dependent but rather an initial event in apoptosis induction.

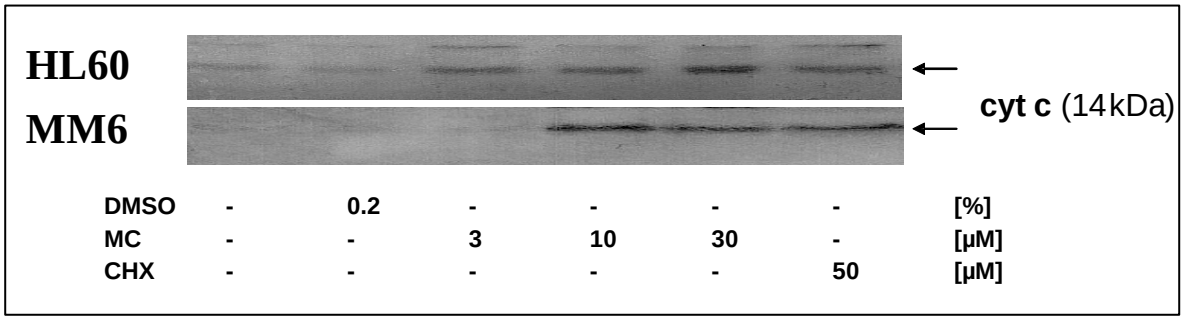



***Fig. 7 MC induces cytochrome c release in MM6 cells and HL60 cells.*** *2.5 × $10^6$cells/ml MM6 cells or HL60 cells were incubated for 24 h in the incubator with vehicle (DMSO), MC or cycloheximide (CHX) in the appropriate medium. After centrifugation, cells were treated with 600 µg/ml digitonin as described in materials and methods. Proteins in the supernatant were precipitated with 5% TCA overnight at 4°C. Total amount of protein was determined by Roti-Nanoquant and equal amounts of protein were loaded onto an SDS-PAGE gel. Data shown are representative for 4 independent experiments.*

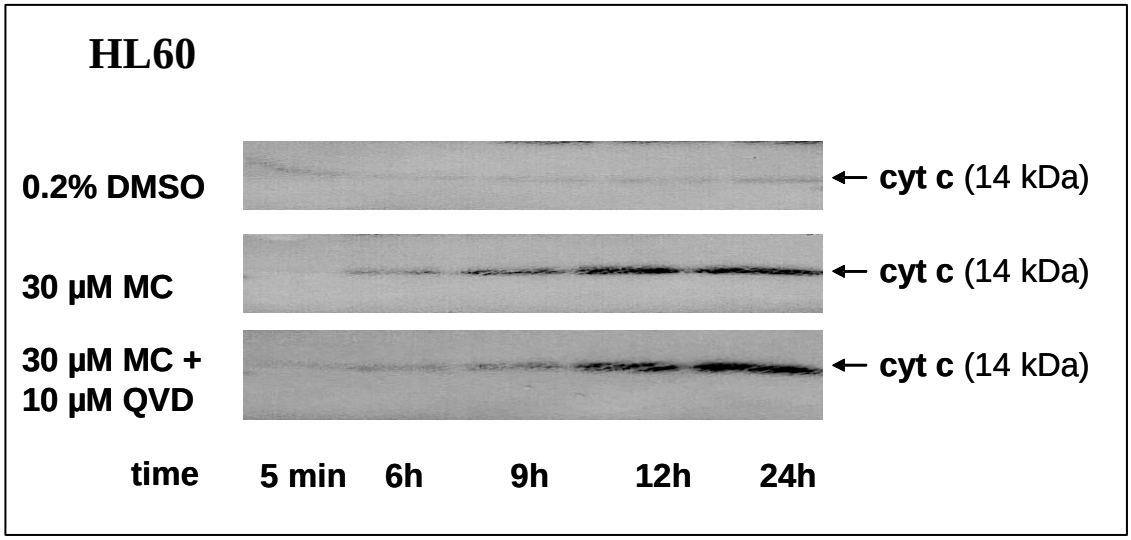



***Fig. 8 Time course of cytochrome c release induced by MC in HL60 cells.*** *2.5 × $10^6$ cells/ml HL60 cells were incubated for the times indicated with vehicle (DMSO), MC or MC + QVD respectively. After centrifugation, cells were treated with 600 µg/ml digitonin as described in materials and methods. Supernatant was conducted to protein precipitation with 5% TCA overnight at 4°C. Total amount of protein was determined by Roti-Nanoquant and equal amounts of protein were loaded onto an SDS-PAGE gel and afterwards analysed by Western Blot. Data shown are representative for 2 independent experiments.*

## 12.5 *MC causes loss of the mitochondrial membrane potential $\Delta\Psi_m$*

The intrinsic induction of apoptosis is characterised by permeabilisation of mitochondrial membranes and results in release of cytochrome c. MC is able to induce cytochrome c release. How is the induction of cytochrome c release by MC mediated?

Release of cytochrome c is related either to a more or less controlled formation of pores such as the PTP and MAC or to mitochondrial swelling and membrane disruption. After permeabilisation of mitochondrial membranes, the membrane potential $\Delta\Psi_m$ becomes instable which under physiological conditions is necessary to drive the ATP-Synthase. Normally the amount of $\Delta\Psi_m$ over the inner mitochondrial membrane is around -200 mV [190]. During membrane permeabilisation this potential is depleted.

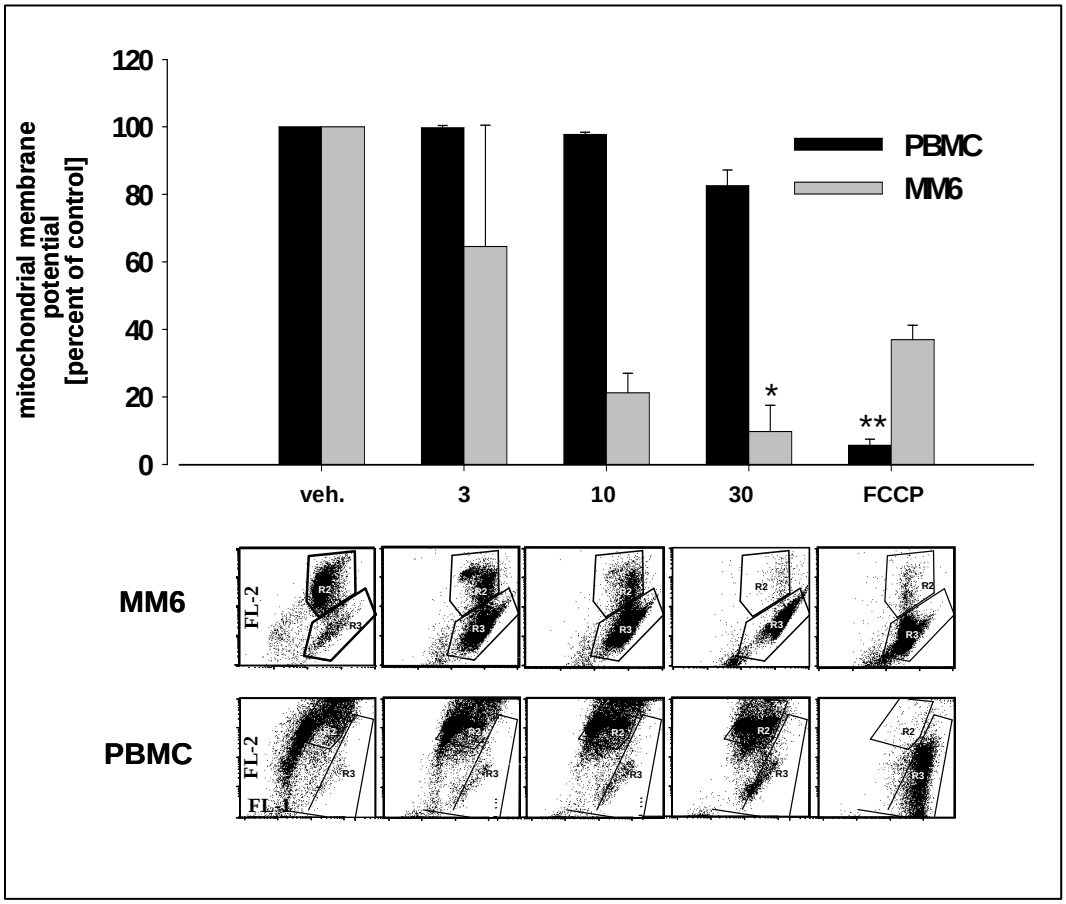



***Fig. 9 MC reduces the mitochondrial membrane potential in MM6 cells.*** *MM6 cells ($1 \times 10^6$/ml) were incubated for 24 h with DMSO (veh.) and MC. 1 µM FCCP was added directly to stained cells shortly before measuring. After incubation, cells were washed and then stained with 0.75 µg/ml JC-1 for 15 min at 37 °C. After additional washing in ice-cold PBS, green (FL1) and red/orange fluorescence (FL2) were measured in a flow cytometer immediately. Data are given as mean ± S.E. n = 2-5 *p<0.05, **p<0.01 or as representative of 2-5 independent experiments.*

MM6 cells and PBMC were treated with increasing concentrations of MC and the amount of red/green fluorescence was calculated as percentage of $\Delta\Psi_m$ in control cells (untreated cells). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) is a protonophor which can depolarise the plasma and the mitochondrial membranes and was used as control.

Incubation of cells with MC led to a loss of $\Delta\Psi_m$ starting at 3 µM in MM6 cells (and HL60 cells, data not shown) but not in PBMC (Fig. 9). A slight but not significant effect could be observed in non-transformed mononuclear cells only at 30 µM. Thus, the selectivity of MC for cancer cells starts at the level of mitochondria and involves loss of $\Delta\Psi_m$. FCCP reduced $\Delta\Psi_m$ in PBMC and MM6 cells as expected.

## 12.6 *Induction of cytochrome c release and loss of $\Delta\Psi_m$ by MC is unaffected by Bcl2 overexpression and independent of the opening of the permeability transition pore*

MC is able to induce cytochrome c release in HL60 cells and MM6 cells as well as loss of $\Delta\Psi_m$. Next, we determined how these events are triggered and if overexpression of the mitochondrial anti-apoptotic Bcl2 decreased apoptosis induction by MC. Stable transfectants of Jurkat cells overexpressing Bcl2 and the Jurkat mock-transfected (Jvec) cells were used.

MC reduced the number of viable cells independently of Bcl2 overexpression (Fig. 10). The potency of MC in Bcl2-overexpressing cells and Jurkat mock-transfected cells were similar to the one obtained in the Jurkat control cell line (4.01 µM, Tab. 1). As expected, CHX also led to reduced cell numbers.

Since reduced cell viability (Fig.10) could be simply due to a decrease in mitochondrial metabolism (and therefore decreased reduction of MTT) affected by Bcl2 overexpression, apoptosis induction by MC was checked by determination of PARP and caspase cleavage (Fig. 11). MC induced a prominent PARP and caspase 3 cleavage starting at 10 µM which was independent of the overexpression of Bcl2. 1 µM staurosporine also induced cleavage of PARP and caspase 3 as expected.

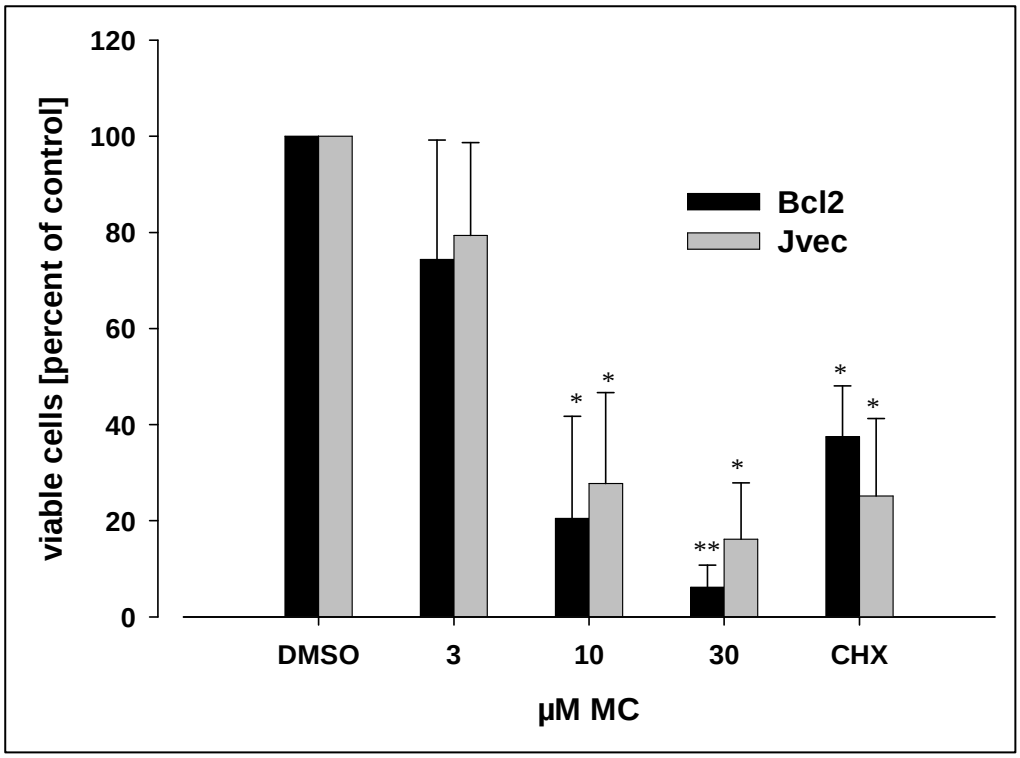



***Fig. 10 MC reduces cell numbers independently of Bcl2 overexpression.*** *3 × $10^5$ cells were incubated in a microtiter plate with vehicle, MC or 50 µM cycloheximide (CHX) for 24 h. Then, the dye MTT was added to the cells and incubated for another 3 h until the untreated control clearly showed blue staining. MTT lysis buffer was added and the plate was left shaking 16-20 h overnight at RT. The absorption was measured at 595 nm in a Victor™ plate reader. Data are given as mean ± S.E. n=3-5 *p<0.05, **p<0.01*

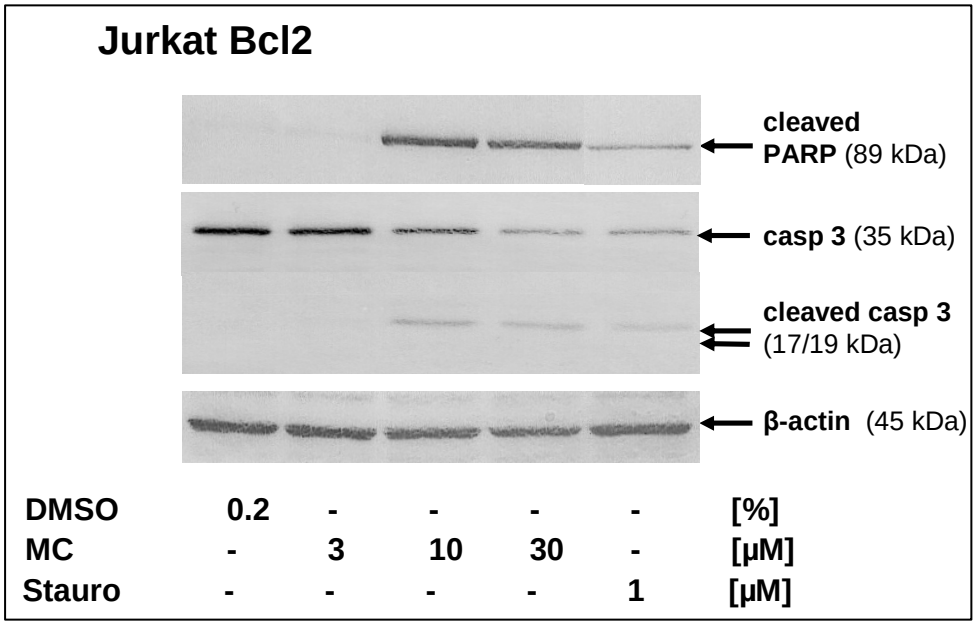



***Fig. 11 Caspase and PARP cleavage in Bcl2-overexpressing Jurkat cells.*** *0.2 × $10^6$/ ml Jurkat cells overexpressing Bcl2 were incubated for 16 h at 37 °C with DMSO, MC or staurosporine (Stauro) respectively. After centrifugation, the pellet was lysed with SDS-b and analysed for caspases and PARP by SDS-PAGE and Western Blot. Data shown are representative for 3 independent experiments.*

The loss of mitochondrial membrane potential $\Delta\Psi_m$ was significantly induced by MC in both cell types (Fig. 12). Also, MC-induced cytochrome c release in Bcl2-overexpressing Jurkat cells as well as in the mock-transfected Jurkat cells could be observed starting at 3 µM MC (Fig. 13). Interestingly, the chelerythrine-induced cytochrome c release, which is dependent on PTP and thus partly on Bcl2 proteins, is diminished in Bcl2-overexpressing Jurkat cells compared to the mock-transfected Jurkat cells (Fig. 13). Since Bcl2 overexpression did not abolish the effects of MC, it is reasonable to speculate that Bcl2 proteins are not involved in the induction of apoptosis by MC.

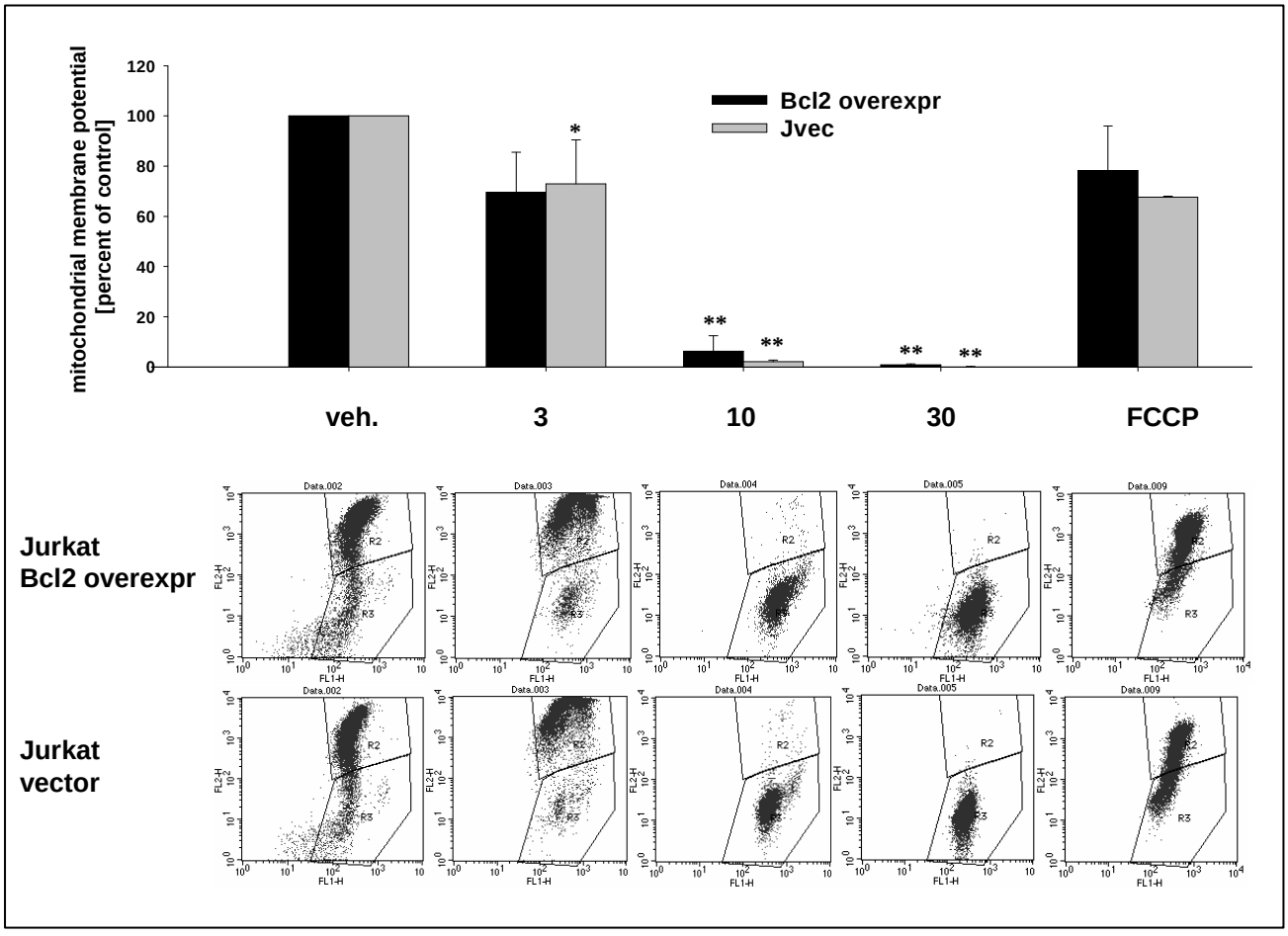



***Fig. 12 MC reduces the mitochondrial membrane potential in Bcl2-overexpressing Jurkat cells.*** *Jurkat cells overexpressing Bcl2 (Bcl2 overexpr) or MOCK-transfected cells (Jvec) were adjusted to 1× $10^6$ /ml and incubated for 24 h with vehicle and MC. 3 µM FCCP was added directly to stained cells shortly before measuring. After incubation, cells were washed and then stained with 0.75 µg/ml JC-1 for 15 min at 37 °C. After additional washing in ice-cold PBS, green (FL1) and red/orange fluorescence (FL2) were measured in a flow cytometer immediately. Data are given as mean ± S.E. n = 3-4 *p<0.05, **p<0.01 or are representative for 3-4 independent experiments.*

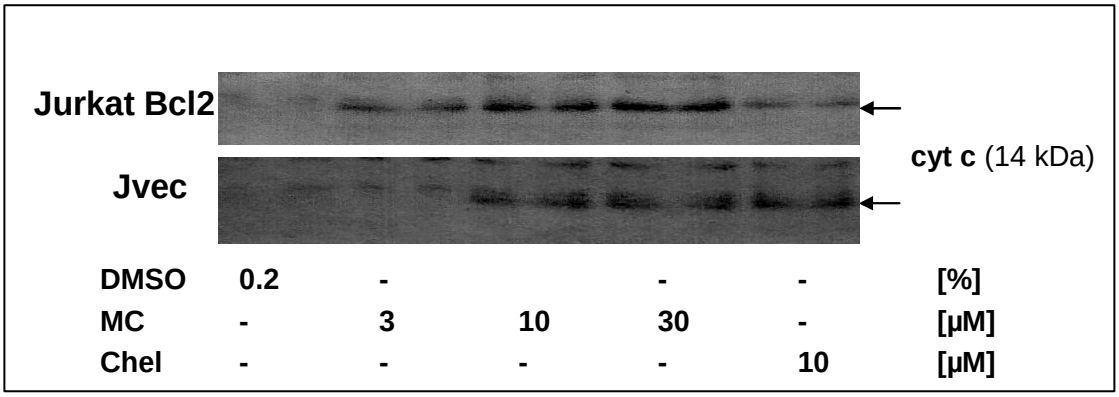



***Fig. 13 MC induces cytochrome c release in Bcl2-overexpressing Jurkat cells.*** *2.5 × $10^6$/ml Bcl2-overexpressing Jurkat cells (Jurkat Bcl2) or the MOCK-transfected Jurkat cells (Jvec) were incubated for 24 h at 37 °C with vehicle, MC or chelerythrine (Chel) in the appropriate medium. After centrifugation, cells were treated with 300 µg/ml digitonin as described in materials and methods. The supernatant was used for protein precipitation with 5% TCA overnight at 4°C. The total amount of protein was determined by Roti-Nanoquant and equal amounts of protein were loaded onto an SDS-PAGE gel and afterwards analysed by Western Blot. Data shown are representative for 3 independent experiments.*

To evaluate if the apoptosis induction by MC involves the PTP we used chelerythrine in combination with cyclosporin A (CsA). Chelerythrine was shown to be a potent PKC inhibitor [191] and leads to opening of the PTP by directly targeting mitochondria [192]. CsA is a substance isolated from the fungus *Tolypocladium inflatum GAMS*. It can block the opening of the PTP by binding to cyclophilin D which is also a part of the pore complex [193].

We tested the cytotoxic effect of chelerythrine in MM6 cells and PBMC and we analysed if it is possible to abolish the effect by CsA. Then, MC in combination with CsA was given to the cells and the effects were compared to the ones obtained by using chelerythrine (Fig. 14).

Again, MC reduced the number of viable MM6 cells starting from 3 µM and showed the first significant effect in PBMC only at 30 µM MC. At 10 µM, chelerythrine is cytotoxic for MM6 cells and PBMC reducing cell numbers to 10 - 20% of vehicle-treated cells. When adding 1 µM CsA together with chelerythrine, cytotoxicity could be reduced with high significance in MM6 cells and PBMC. When giving the same amount of CsA to cells treated with 10 µM MC, the effect of MC could not be reversed. From these results we can deduce that MC still shows cytotoxic effects although the opening of the PTP is blocked by CsA. Thus MC induces cytochrome c release and subsequently causes caspase 9 and caspase 3 activation independent of the PTP.

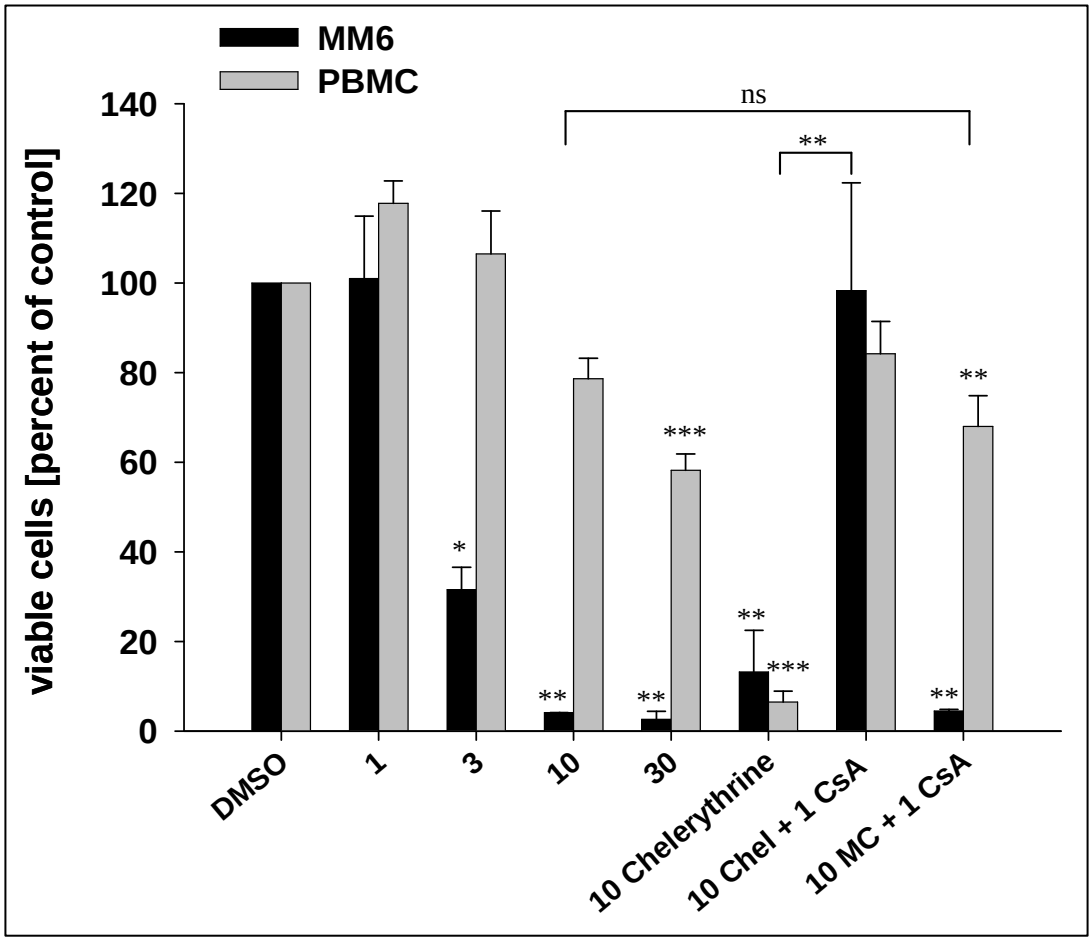



***Fig. 14 The effect of MC on cell viability is not reversed by the PTP inhibitor cyclosporine A.***
*$3 \times 10^5$ MM6 cells or $5 \times 10^5$ PBMC were incubated in a microtiter plate for 24 h with vehicle (DMSO), MC and/or 10 µM chelerythrine (Chel) and/or 1 µM cyclosporine A (CsA). Then, MTT was added to the cells and incubated for another 3 h until the untreated control clearly showed blue staining. MTT lysis buffer was added and the plate was left shaking 16-20 h overnight at RT. The absorption was measured at 595 nm in a Victor™ plate reader. Data are given as mean ± S.E. n=2-3 *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA, Bonferroni post-hoc).*

## 12.7 *Caspase 9 is essential for apoptosis induction by MC*

Induction of cytochrome c release and loss of $\Delta\Psi_m$ after MC incubation indicates that the intrinsic pathway of apoptosis induction is involved. To examine whether the effects of MC on cell viability are also due to activation of the extrinsic way of apoptosis induction, we examined if death receptors, the Fas-associated death domain (FADD) or caspase 8 are essential mediators of the effect of MC. As previously shown [45], MC could potently decrease cell viability (MTT assay) of the neuroblastoma cell line UKF-NB-3 which are resistant to typical death receptor stimuli [183, 194]. After addition of the death receptor ligands TNFα, Fas or TRAIL, no effect on cell viability could be observed in UKF-NB-3 although

they caused cell death at the same concentrations in Jurkat cells. This indicates that MC acts not likewise to classical death receptor stimuli.

Interestingly, MC caused cell death (trypan blue staining) and PARP cleavage in Jurkat cells deficient in FADD, and in the Jurkat cell line DD3, which is deficient in CD95-mediated signalling [195]. This indicates that the Fas-receptor and the associated FADD are not necessary for the effect of MC on cell viability and apoptosis (Fig. 15). In addition, Jurkat cells lacking caspase-8 which is the caspase downstream of the Fas receptor were susceptible to MC.

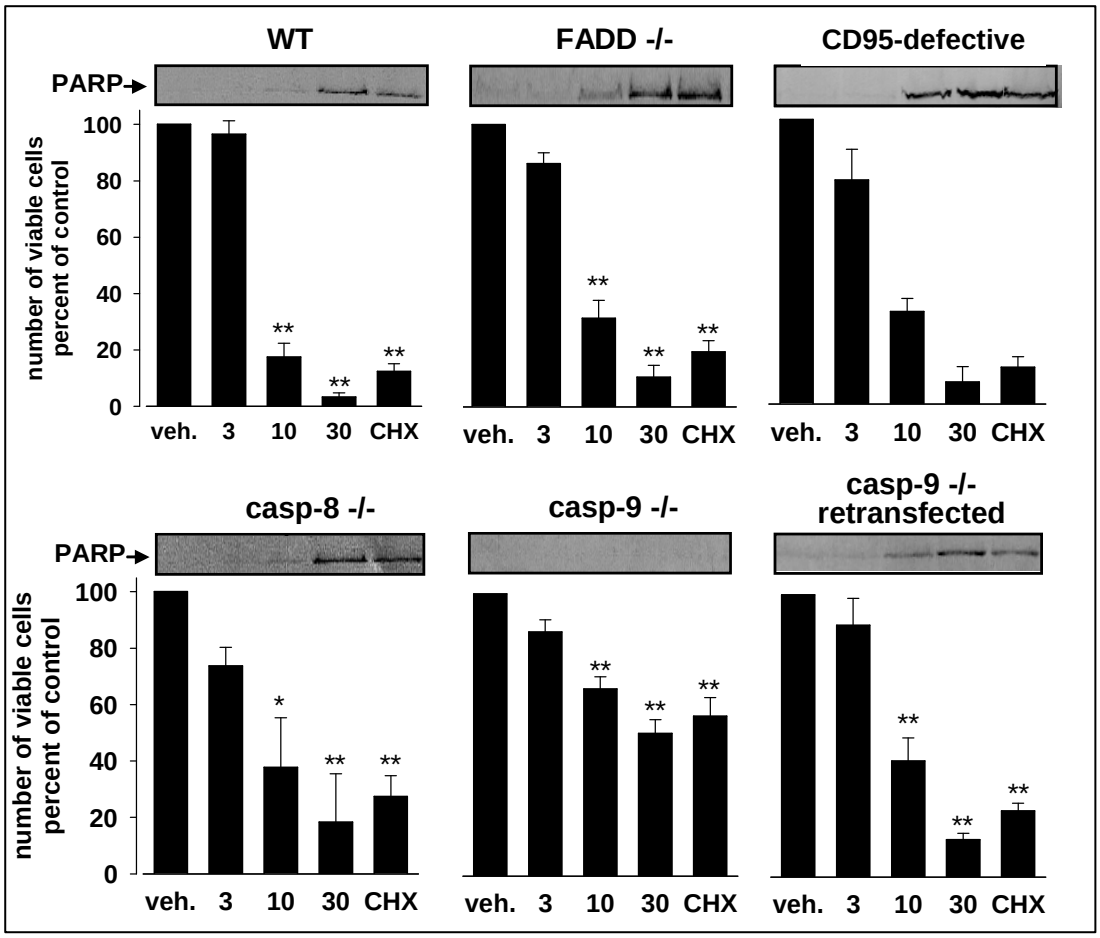



***Fig. 15 PARP cleavage and cell numbers in different Jurkat cells incubated with MC (Data from Jurkat CD95-defective obtained by I. Tretiakova, Frankfurt).*** *Normal Jurkat cells or Jurkat cell lines deficient in either FADD, CD95 signalling (CD95-defective), caspase 8, caspase 9 or caspase 9 and then retransfected with caspase 9 were incubated with 3, 10, and 30 µM MC, vehicle (DMSO) or 50 µM cycloheximide (CHX). Cells were harvested after 48 h and cell viability was determined by trypan blue staining. In the absence of test compound (vehicle-treated), the numbers of viable cells for each cell line increased within 48 h from 0.2 × $10^6$/ml to 0.70 × $10^6$/ml for normal Jurkat cells, 0.89 × $10^6$/ml for CD95-defective, 0.92 × $10^6$/ml for FADD$^{-/-}$, 0.58× $10^6$/ml for casp 8$^{-/-}$, 0.49 × $10^6$/ml for casp 9$^{-/-}$, and 0.81 × $10^6$/ml for casp 9$^{-/-}$ retransfected, respectively. For analysis of PARP cleavage, cells were harvested after 16 h, total cell lysates were prepared and analysed for processing of PARP (89 kDa) using SDS-PAGE and Western Blot. Results are representative for at least 3 independent experiments.* ± S.E. **p<0.05, **p<0.01*

Interestingly, Jurkat cells deficient in caspase 9 were partially resistant to the effects of MC on cell viability, and PARP cleavage was not apparent. Re-transfection of caspase $9^{-/-}$ cells with caspase 9 recovered the ability of MC to reduce cell viability and induce PARP cleavage. It should be noted that the reduced numbers of caspase $9^{-/-}$ Jurkat cells by MC (Fig. 15) is not due to reduced cell viability, confirmed by analysis of DNA fragmentation (Fig. 16). In caspase-$9^{-/-}$ Jurkat cells treated with 30 µM MC, no increase in the numbers of apoptotic nuclei was detectable, while in caspase 9-retransfected cells MC caused a clear leakage of fragmented DNA (Fig. 16). In Jurkat cells deficient in caspase 9 there was no cleavage of caspase 8 by MC detectable, while the Fas receptor ligand could still induce caspase 8 cleavage (Fig. 17) indicating a central and initial role of caspase 9 in apoptosis induction by MC.

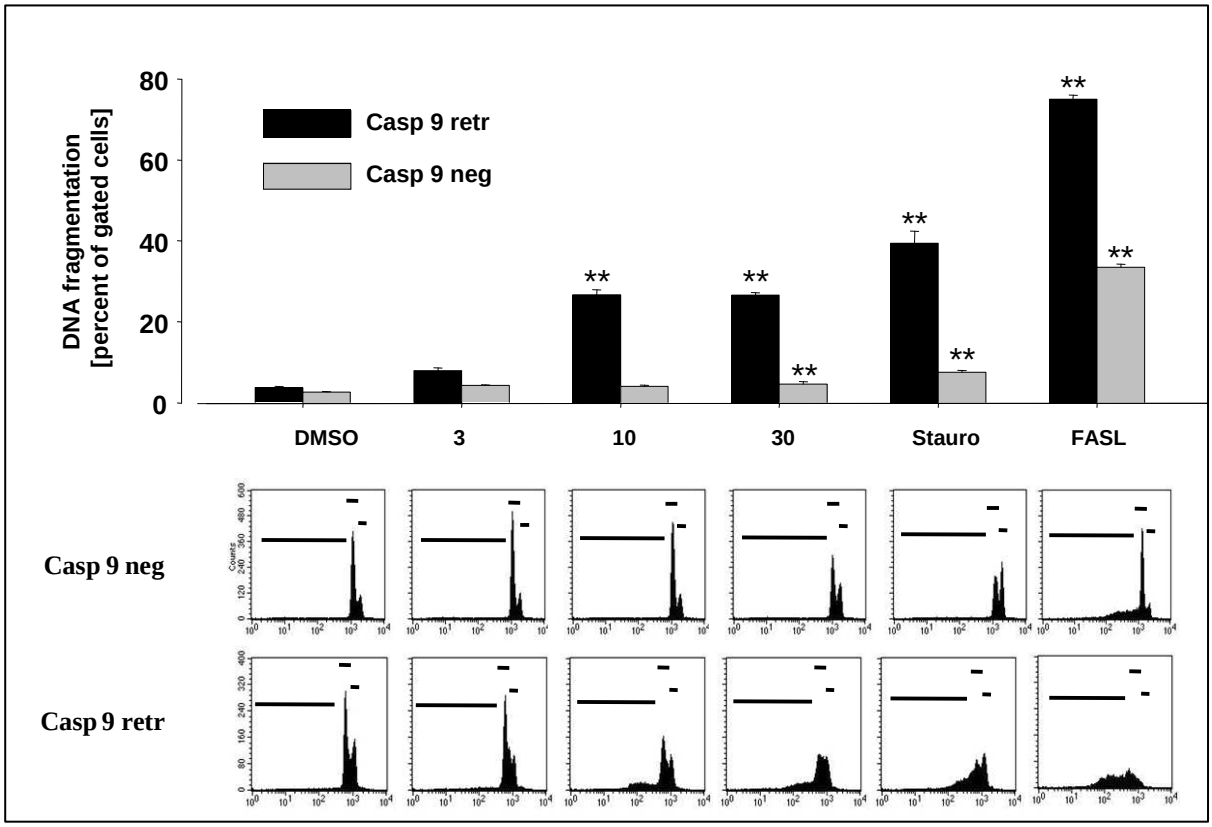



***Fig. 16 MC does not induce DNA fragmentation in Jurkat which are caspase 9-deficient.*** *Caspase 9-deficient Jurkat cells (Casp 9 neg) and caspase 9-deficient Jurkat cells again retransfected with caspase 9 (Casp 9 retr) (0.2 × $10^6$/ml) were incubated with DMSO, 3, 10 and 30 µM MC, 1 µM staurosporine (Stauro) and 100 ng/ml FAS ligand (FASL) for 24 h at 37 °C. Then, cells were harvested, washed in PBS and resuspended in 100 µl hypotonic propidium iodide buffer. After 10 min at RT, fluorescence intensities of cell nuclei were measured using a flow cytometer (FL2 585/42). Data are given as mean ± S.E. n = 3-6. *p<0.05, **p<0.01*

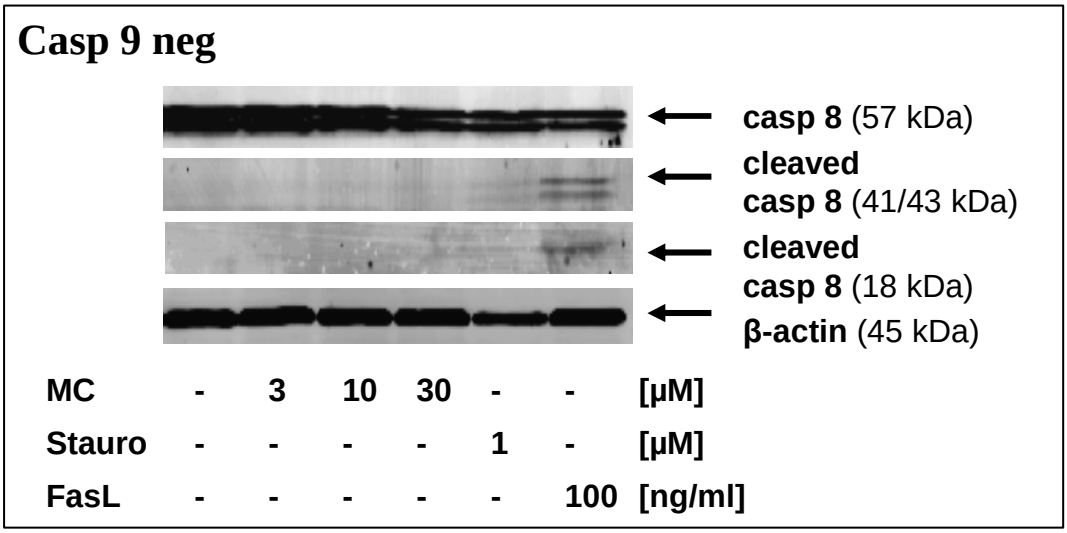



***Fig. 17 MC does not lead to caspase 8 cleavage in caspase 9-deficient Jurkat cells.*** *Jurkat cells deficient in caspase 9 were treated with the indicated compounds for 16 h and were then analysed for cleavage of caspase 8 by SDS-PAGE and Western Blot. Similar results were obtained in two additional experiments.*

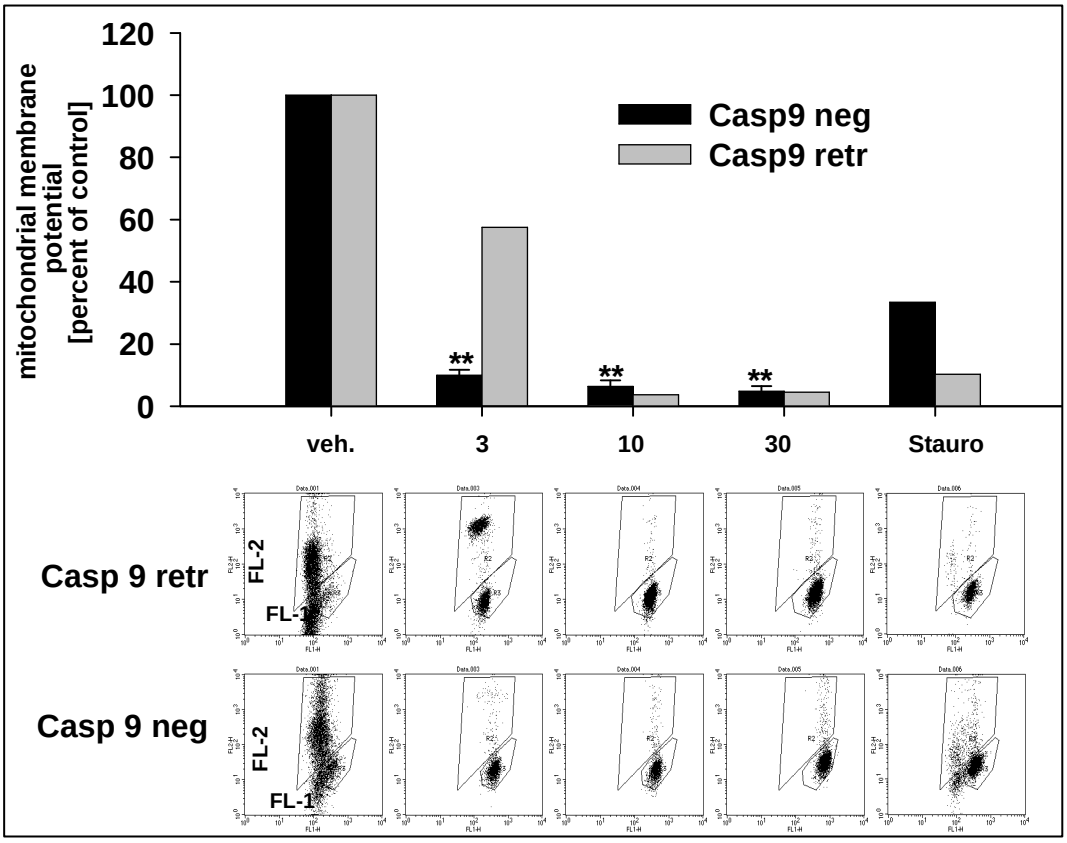



***Fig. 18 MC reduces the mitochondrial membrane potential in caspase 9-deficient Jurkat cells.*** *Jurkat cells deficient in caspase 9 (Casp9 neg) or deficient in caspase 9 and retransfected (Casp9 retr) with caspase 9 were adjusted to 1 × $10^6$ /ml and incubated for 24 h with DMSO (veh.), MC or 1 µM staurosporine (Stauro). After incubation, cells were washed and then stained with 0.75 µg/ml JC-1 for 15 min at 37 °C. After additional washing in ice-cold PBS, green (FL1) and red/orange fluorescence (FL2) were measured in a flow cytometer immediately. Data are given as mean ± S.E. n = 1-3 or are representative for 1-3 independent experiments (staurosporine and caspase 9-retransfected Jurkat cells: n = 1). **p<0.01*

As seen in Fig. 18, MC still induced loss of $\Delta\Psi_m$ in caspase 9-deficient cells comparable to caspase 9-retransfected Jurkat cells. In conclusion, the inability of MC to induce apoptosis in caspase 9-deficient cells is due to the lack of caspase 9 activity and not due to impairment of distal events, leading to caspase 9 activation.

## 12.8 *MC induces G2 phase arrest in caspase 9-deficient Jurkat cells*

In caspase 9-deficient Jurkat cells, the effect of MC on PARP cleavage could be abolished. Nevertheless, MC could still partially reduce cell numbers although not as prominent as in Jurkat wt or in caspase 9-retransfected cells. We checked on two possible reasons for this decrease in cell numbers. First, we tested if MC causes necrosis in caspase 9-deficient Jurkat cells and second we tested whether MC blocks cell proliferation.

One event during early apoptosis is the externalization of the phospholipid phosphatidylserine (PS). Under normal conditions, PS is located to the inner layer of the plasma membrane. Upon apoptosis stimulation it is flipped to the outer membrane and functions as signal for macrophages to bind and ingest the apoptotic cell. To evaluate whether reduction of cell viability by MC in caspase 9-deficient Jurkat cells is due to apoptotic or necrotic events, we analysed PS externalisation by Annexin V/ PI assay (Fig. 19).

After 16 hours exposure, MC and staurosporine both led to an increase of Annexin V-positive (= apoptotic) cells when caspase 9 is retransfected. In cells deficient in caspase 9, no effect could be seen (same effects after 6 or 20 hours respectively, data not shown). This excludes that MC causes necrosis in caspase 9-deficient Jurkat cells.

In caspase 9-retransfected Jurkat cells the amounts of double positive PI- and AnnexinV stained cells was quite high. Since in in vitro cell culture apoptotic cells are not phagocytised by cells of the immune system, apoptotic cells become secondary necrotic or late apoptotic. Here, similar to necrosis, cell membranes disintegrate and become permeable for PI, thus giving a positive signal for necrosis. In this respect, necrotic and secondary necrotic cells can not be separated by this method. Since in caspase 9-deficient Jurkat cells MC did not lead to

increased amounts of cells with Annexin V or propidium iodide staining at all, necrosis induction could be definitely excluded.

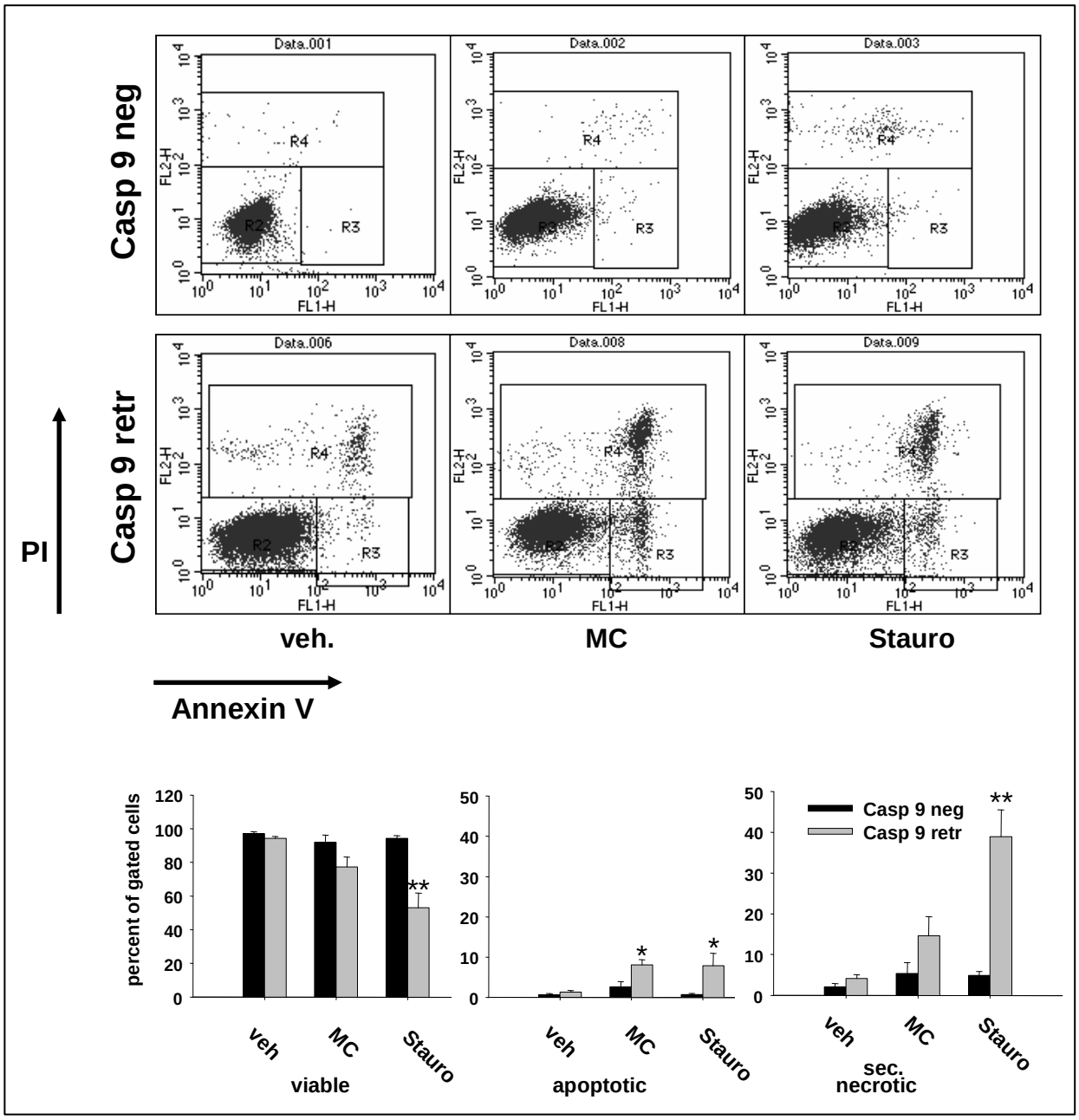



***Fig. 19 MC does not induce necrosis in caspase 9-deficient cells.*** *Cells (0.5 × $10^6$/ml) were incubated with vehicle (DMSO), 30 µM MC or 1 µM staurosporine (Stauro) for 16 h at 37 °C. Then, 0.25× $10^6$ cells were harvested, washed in ice-cold PBS and resuspended in 1 x annexin V binding buffer (Vybrant Apoptosis Kit #2, Molecular probes). After adding Alexa Fluor 488-Annexin V and PI, cells were analysed with a flow cytometer (FL1 vs. FL2) to give the amounts of viable, apoptotic and secondary (sec.) necrotic cells. Data are given as representatives (upper part) or given as mean ±S.E. n = 3. *p<0.05, **p< 0.01*

Second, we looked at the cell cycle distribution of caspase 9-deficient and caspase 9-retransfected Jurkat cells (Fig. 20). Cells were incubated for 24 hours with DMSO, MC or staurosporine (which is known to be a strong G2-phase arrestor).

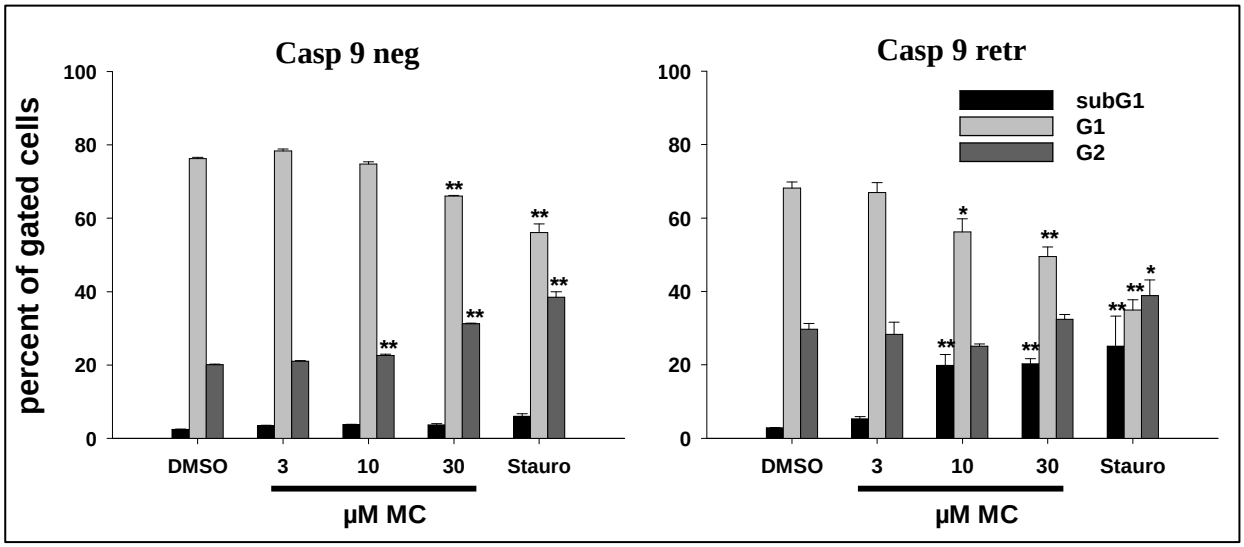



***Fig. 20 MC induces cell cycle arrest in caspase 9-deficient Jurkat cells.*** *Jurkat cells deficient in caspase 9 (Casp 9 neg) or deficient in caspase 9 and again retransfected with caspase 9 (Casp 9 retr)(0.2 × $10^6$/ml) were incubated overnight at 37 °C for 24 h with vehicle (DMSO), 3, 10 or 30 µM MC or 1 µM staurosporine (Stauro), respectively. Then, 30,000 cells were harvested, washed in PBS and carefully resuspended in hypotonic propidium iodide buffer. After 10 min incubation at RT, the fluorescence intensities of the cell nuclei were measured using a flow cytometer (FL3 vs. counts). Data are given as mean from 4-7 independent experiments. ±S.E. *p<0.05, **p<0.01*

MC led to a G2 phase cell cycle arrest in caspase 9-deficient Jurkat cells (Fig. 20, Casp 9 neg). Also, caspase 9-retransfected (Fig. 20) and Bcl2-overexpressing Jurkat cells (data not shown) were arrested in G2-phase. Since apoptotic events decreased total cell numbers and led to a prominent shift of the main host of the cells into subG1 phase, in caspase 9-retransfected Jurkat cells the cell cycle arrest was not as prominent as compared to caspase 9-deficient cells. Staurosporine led to an increase of cells in G2 phase after 24 hours as expected.

Although MC was not able to induce apoptosis in caspase 9-deficient Jurkat cells, the inhibition of the cell cycle at the G2 phase led to the decrease in numbers of viable cells (as observed in Fig. 15)

# 13 The effect of MC on signal transduction pathways in PMNL

Based on the results reported in the first part of this thesis, MC acts via the intrinsic pathway of apoptosis induction leading to loss of mitochondrial membrane potential and cytochrome c release and triggering caspase 9 activation. In caspase 9-deficient cells, no apoptosis could be induced by MC.

Next, we focussed on immediate events induced by MC. What are the early mechanisms for the effects that finally lead to apoptosis? Recent work about short term effects of MC by Dr. C. Feißt and colleagues showed that in PMNL MC inhibits fMLP-induced ROS production, leukocyte elastase release and $Ca^{2+}$ mobilisation from intracellular stores [18]. These events are closely related to the mechanism of (especially) G protein coupled receptor (GPCR) signaling. In order to identify the initial pathways affected by MC the following chapters will elucidate the influence of MC on cellular signaling pathways in PMNL and in HL60 cells.

## 13.1 *MC suppresses fMLP-induced phosphorylation of MAP Kinases and Akt/PKB.*

fMLP (N-formyl-methionyl leucyl-phenylalanine) is a small peptide derived from bacteria and acts as chemoattractant for polymorphonuclear leukocytes (PMNL) resulting in the release of proteases such as cathepsin G or leukocyte elastase. fMLP acts via the GPCR formyl peptide receptor (FPR) leading to activation of MAP Kinases, intracellular $Ca^{2+}$ release and ROS formation [196, 197]. It was shown previously that MC inhibits fMLP- and ionomycine-induced peroxide (ROS) formation in PMNL ($IC_{50}$= 0.8 µM) and could inhibit fMLP-evoked $Ca^{2+}$ release from intracellular stores ($IC_{50}$= 0.55 µM) [18].

Here, we evaluated activation of MAPK after incubation of freshly isolated human PMNL with vehicle (DMSO), MC and staurosporine and stimulation with fMLP (Fig. 21).

The fMLP-induced phosphorylation of ERK, p38 MAPK, JNK and Akt/PKB was abolished when cells were incubated with MC starting at a concentration of 3 µM. In contrast, the direct PKC substrate MARCKS was unaffected by MC (Fig. 21). 1 µM staurosporine did not affect ERK, JNK and p38 MAPK phosphorylation, had

only a slight effect on Akt/PKB phosphorylation but strongly decreased MARCKS phosphorylation.

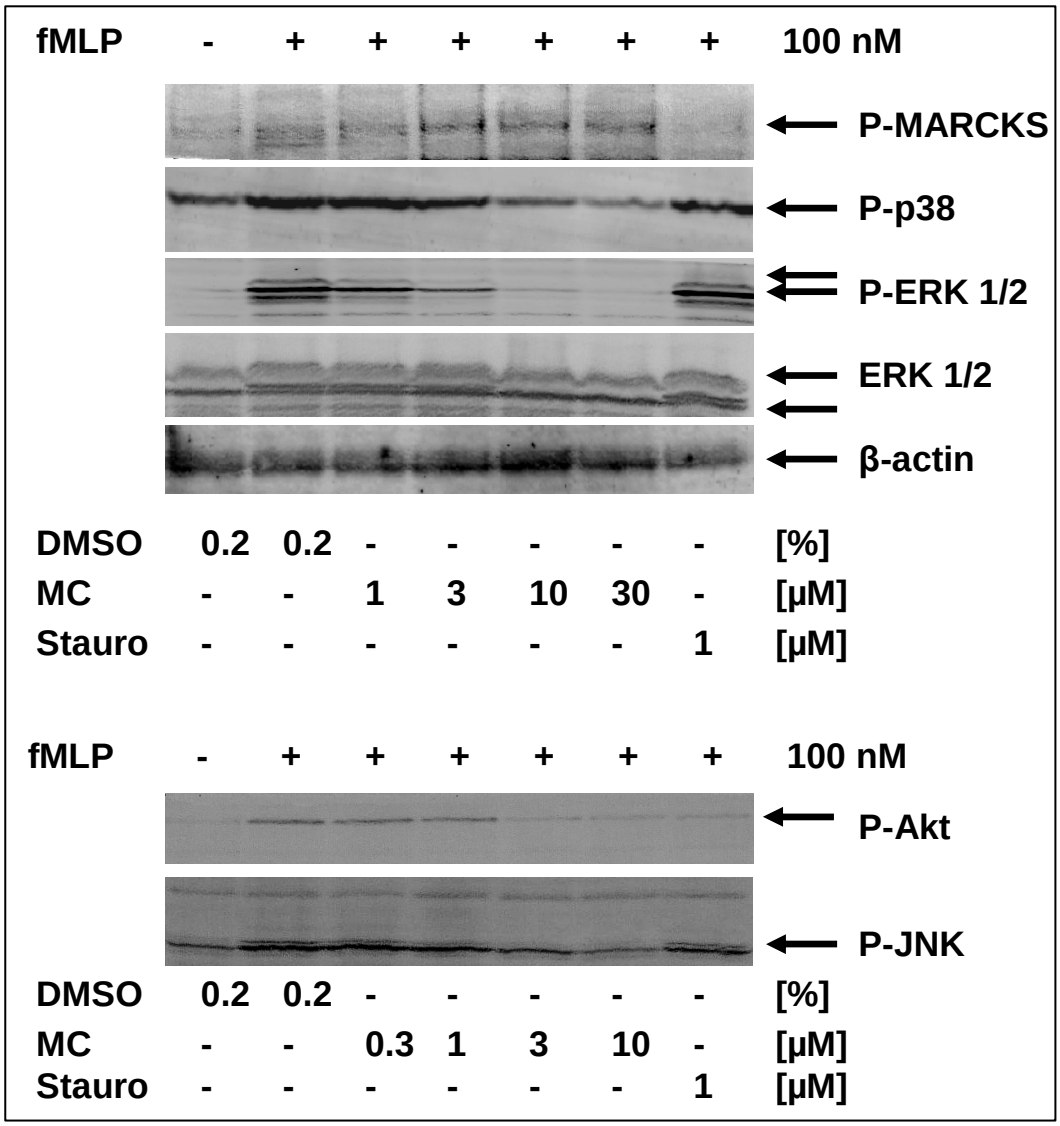



***Fig. 21 MC reduces phosphorylation of ERK, JNK/SAPK and p38 MAPK as well as Akt/PKB in fMLP-stimulated PMNL.*** *PMNL freshly isolated from the blood of healthy donors ($10^8$/ml) were resuspended in PBS + 0.1% glucose and 1 mM $CaCl_2$ (PGC). Cells were incubated for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro) before stimulation with 100 nM fMLP for 1.5 min. Samples were loaded to an SDS-PAGE gel and amounts of proteins were quantified by Western Blot. Data are given as representative for 3 independent experiments.*

## 13.2 *MC affects signaling of different GPCRs leading to decreased phosphorylation of MAPkinases and Akt/PKB.*

Leukotriene B4 ($LTB_4$) is a potent chemoattractant produced in leukocytes upon stimulation with inflammatory mediators. $LTB_4$ activates leukocytes and

neutrophils and in the latter type induces ROS formation and release of proteases [198, 199]. Signal transduction is primarily induced by binding of $LTB_4$ to its high-affinity receptor BLT1 which is also a GPCR.

To evaluate whether MC also affects signaling of other GPCRs than FPR, we checked the phosphorylation of Akt and ERK after stimulation of PMNL with $LTB_4$. Similar to the results in fMLP-stimulated PMNL, MC significantly reduced phosphorylation of ERK 1/2 without affecting the total amount of ERK. Phosphorylation of Akt/PKB was also slightly reduced (Fig. 22A). Staurosporine failed to reduce ERK phosphorylation but strongly decreased amounts of phospho-Akt/PKB as expected. We also checked the effect of MC on AA and 5-HETE signaling and similar results were obtained for MC (Fig. 22B and C). The 5-LO inhibitor BWA4C could decrease AA-induced ERK 1/2 phosphorylation but failed to decrease 5-HETE-induced ERK phosphorylation (Fig. 22B and C). This indicates that activation of the MAPK by AA is dependent on the conversion of AA by 5-LO.

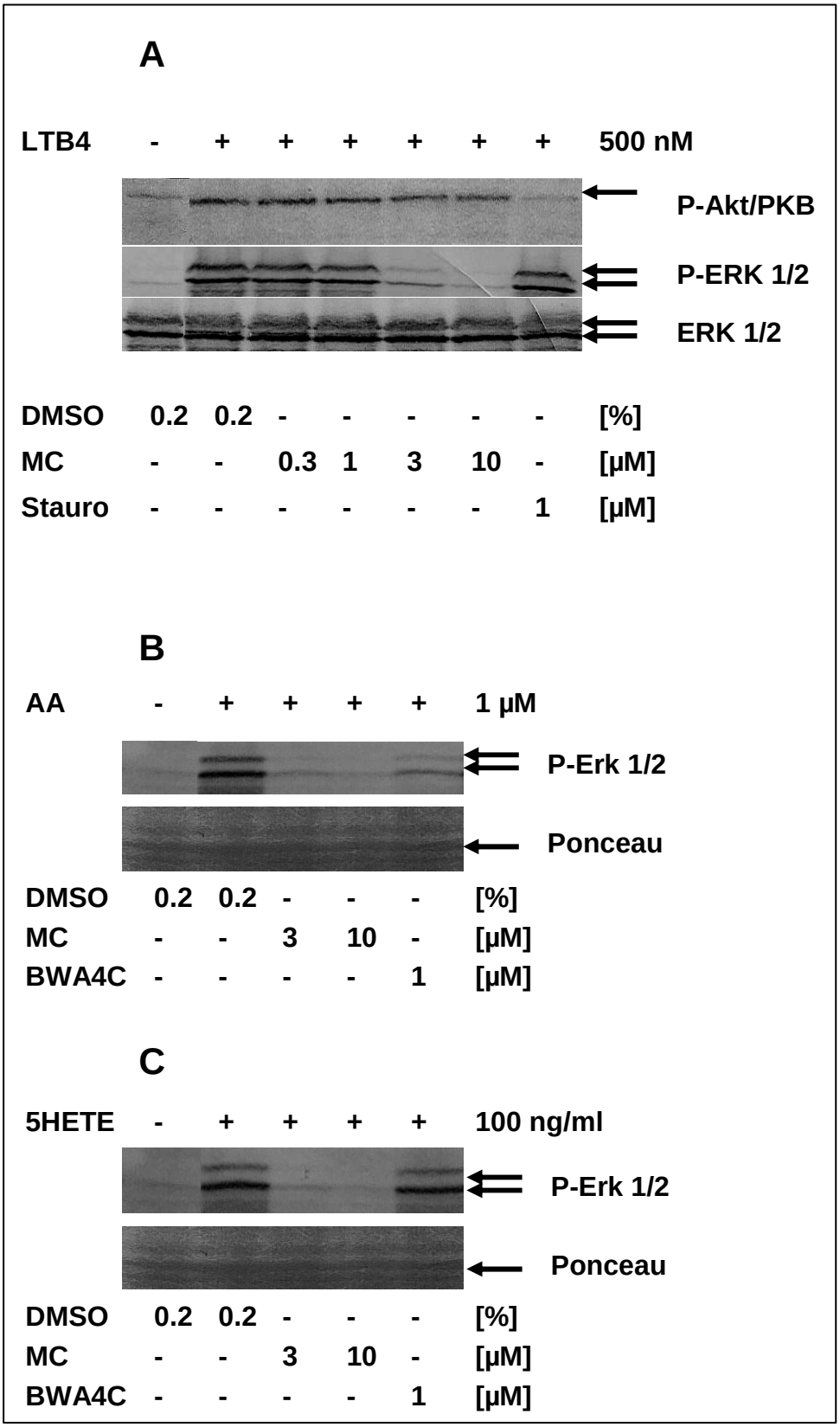



***Fig. 22 MC reduces phosphorylation of ERK and Akt/PKB in $LTB_4$-, AA- and 5-HETE-stimulated PMNL.*** *PMNL freshly isolated from the blood of healthy donors ($10^8$/ml) were incubated in PGC for 30 min at 37 °C with DMSO, MC and staurosporine (Stauro) before stimulation with 500 nM $LTB_4$ for 1 min **(A)**, 1µM arachadionic acid (AA) for 1.5 min **(B)** or 100 ng/ml 5-HETE for 1 min **(C)**. Samples were loaded to an SDS-PAGE gel and amounts of proteins were quantified using specific antibodies. Data shown are representative for 2-4 independent experiments.*

## 13.3 *MC leads to a slight decrease in PTPase activity in PMNL.*

So far, we could show that MC interferes with GPCR signaling leading to reduction of kinase phosphorylation/activation. Several reports described that GPCR-dependent activation of the Ras/Raf/MEK/ERK pathway involves cross talk to receptor tyrosine kinases (RTKs) [173, 174, 158, 200, 201]. Aside from protein kinases, protein tyrosine phosphatases (PTPases) have also an influence on MAP Kinase signaling by removing the phosphate from tyrosine residues of phosphorylated kinases such as Src-homology 2 containing (Shc), growth factor receptor bound protein 2 (Grb2), son of sevenless (Sos) and sarcoma (Src) family kinases. Sos is a guanine nucleotide exchange factor signaling from receptor tyrosine kinases to the small G protein Ras. We assumed that MC has an inhibitory influence on tyrosine kinases. This influence can derive from direct inhibition of the signaling cascade of tyrosine kinases directly or from activation of PTPases.

To elucidate if MC has an effect on tyrosine kinases (and therefore on phosphorylation of tyrosine residues) and an effect on the activity of PTPases, we tested PTPase activity in PMNL and assessed phosphorylation of tyrosine residues.

Suprisingly, MC rather reduced PTPase activity in PMNL (Fig. 23A) in a time dependent manner (Fig. 23B). Also an increase of tyrosine phosphorylation was observed in fMLP-stimulated PMNL pre-treated with MC, suggesting that MC does not reduce the activity of tyrosine kinases (Fig. 23C). Correspondingly, in unstimulated PMNL, MC led to an increase of phosphorylation of tyrosine residues (data not shown). Staurosporine inhibited the phosphorylation of tyrosine residues as expected (Fig. 23C). PAO, an inhibitor of PTPases, reduced PTPase activity and accordingly increased the phosphorylation of tyrosine residues (Fig. 23C) as expected.

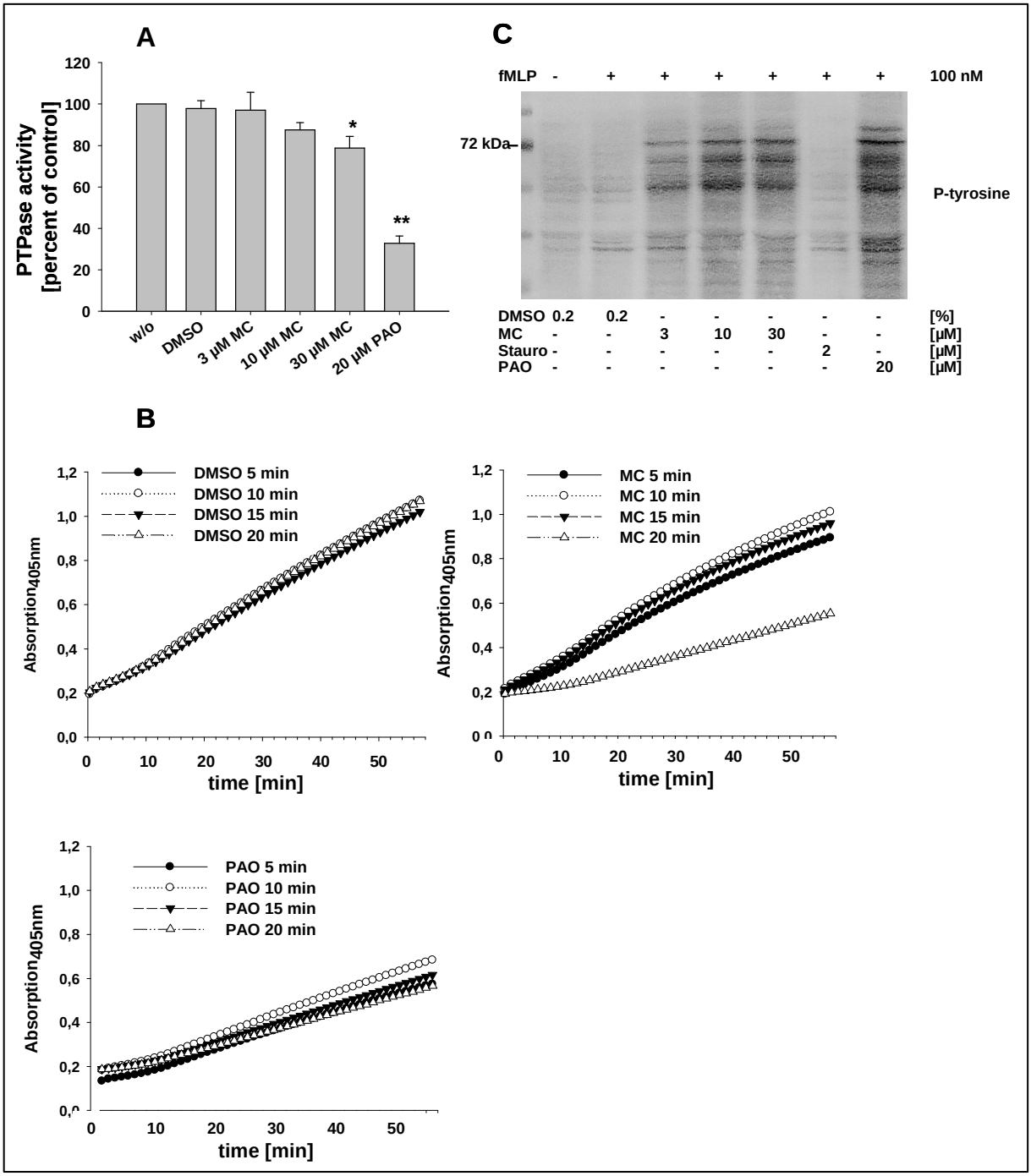



***Fig. 23 MC reduces PTPase activity in PMNL which leads to increased phosphorylation of tyrosine-residues.*** *PMNL were freshly isolated from the blood of healthy donors.* ***A) PTPase activity.*** *Cells ($8 \times 10^5$) were incubated in PGC for 30 min at 4 °C with DMSO, MC or 20 µM of the PTPase inhibitor phenylarsine oxide (PAO). After lysing the cells and centrifugation of the lysates, p-nitrophenyl phosphate (pNPP) was added to the supernatants and absorption at 405 nm was measured in a Victor™ plate reader. n = 3 ± S.E. *p<0.05, **p<0.01* ***B) Time course of PTPase activity.*** *Cells ($8 \times 10^5$) were incubated for the times indicated at 4 °C and then treated with DMSO, 10 µM MC or 20 µM PAO as described under A). Data are representative for 2 independent experiments.* ***C) Increase in phosphorylation of tyrosine residues.*** *$1 \times 10^7$ cells were incubated for 30 min at 37 °C with DMSO, MC, staurosporine (Stauro) or PAO before stimulation with 100 nM fMLP for 1.5 min. Incubation was stopped by adding SDS-b and putting the samples on ice. Data are representative for 3 independent experiments.*

## 13.4 *The effect of MC on MAP Kinase signaling is not due to a direct effect on PKC.*

Next, we concentrated on the classical signaling cascade from the fMLP-induced FPR1 receptor pathway involving G proteins, phospholipase C beta (PLCβ) and protein kinase C (PKC). Activation of PKC depends on binding of phorbol esters such as DAG or on binding of $Ca^{2+}$ respectively. PKC is able to activate Ras and c-Raf directly, though the exact mechanism is still unclear [175, 158].

To elucidate if PKC is directly affected by MC, freshly isolated PMNL were treated with DMSO, MC and staurosporine, stimulated with 100 nM PMA for 3 min (Fig. 24A) and phosphorylation of the direct PKC substrate MARCKS and of ERK was assessed. PMA, a phorbol ester, is able to enter the cells and target PKC directly, stimulating it and thus substitutes for DAG.

MC strongly decreased ERK phosphorylation starting from the concentration of 3 µM also after PMA stimulation. MARCKS phosphorylation was not affected by MC in fMLP-stimulated PMNL (Fig. 21) and also not in PMA-stimulated cells (Fig. 24A). Staurosporine, a kinase inhibitor, could decrease PMA-induced MARCKS and ERK phosphorylation as expected (Fig. 24 A).

To determine if the depletion of $Ca^{2+}$ from intracellular stores by MC affects PKC activity, we added ionomycin combined with PMA to cause influx of $Ca^{2+}$ from the extracellular medium.

Ionomycin led to a further increase in MARCKS and ERK phosphorylation. Nonetheless MC was still able to decrease the amount of ERK phosphorylation but had no obvious effect on MARCKS phosphorylation (Fig. 24B). As expected, staurosporine strongly decreased MARCKS phosphorylation and had only a slight effect on ERK phosphorylation.

Interestingly, MC had no direct inhibitory effect on PKC directly. In contrast, MC rather increased phosphorylation of the PKC substrate MARCKS (Fig. 21) in absence of PMA. Nonetheless, ERK phosphorylation is decreased. This narrows the number of possible targets for MC to the proteins located downstream of PKC and upstream of ERK which are e.g. Ras, Raf and MEK or alternative activation involving cross talk with other pathways (e.g. MEKK1).

Despite a complete suppression of fMLP-induced ERK phosphorylation by 10 - 30 µM MC, no complete inhibition was observed, when PMA was used as stimulus (compare Fig. 21 to Fig. 24). This might suggest that MC does not

completely abolish the PKC signal leading to ERK phosphorylation but could affect only selected pathways.

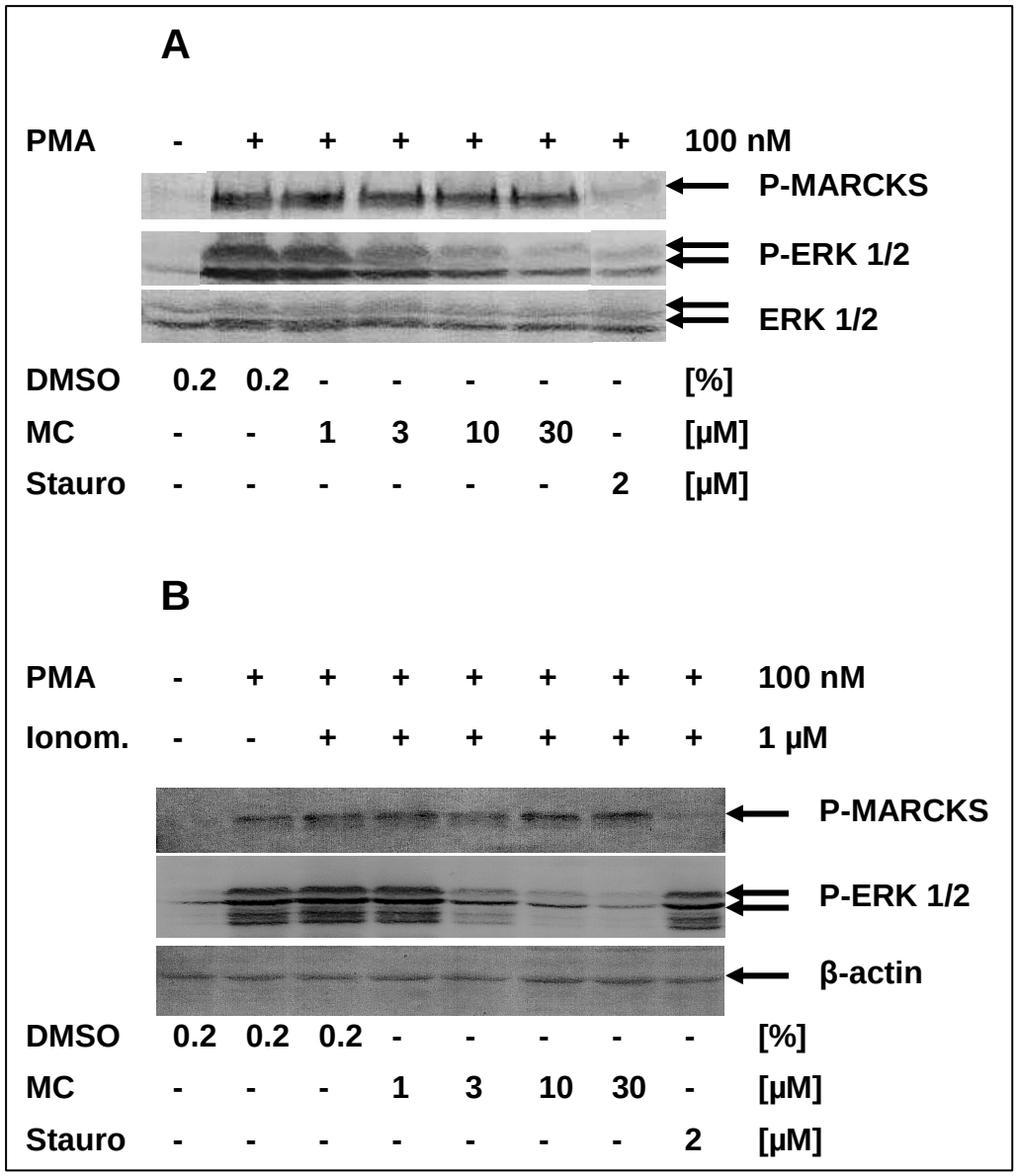



***Fig. 24 MC reduces ERK phosphorylation induced by PMA stimulation without affecting PKC directly.*** *PMNL freshly isolated from the blood of healthy donors were set to $10^8$ /ml in PGC.* ***A) ERK and MARCKS phosphorylation.*** *Cells were incubated for 30 min at 4 °C with DMSO, MC or staurosporine (Stauro). Then, cells were stimulated with 100 nM PMA for 3 min, incubation was stopped by adding ice-cold SDS-b and samples were analysed for phospho-ERK and phospho-MARCKS. Data are representative for 3 independent experiments.* ***B) ERK and MARCKS phosphorylation with PMA and ionomycin.*** *Cells were incubated as before and then stimulated with 100 nM PMA for 3 min and 1 µM ionomycin for 2 min. Data are representative for 3 independent experiments.*

## 13.5 *Time dependency of decreased phosphorylation of ERK and MEK by MC in PMA-stimulated PMNL*

Usually after activation of kinases, they become inactivated again after some time due to dephosphorylation events. To elucidate if MC shows a graduated effect on MARCKS phosphorylation compared to DMSO-treated cells and to see how MC affects phosphorylation states of MEK and ERK over the time, PMNL were stimulated with PMA for different times (0 to 30 min) and phosphorylation of kinases was detected by Western Blot (Fig. 25).

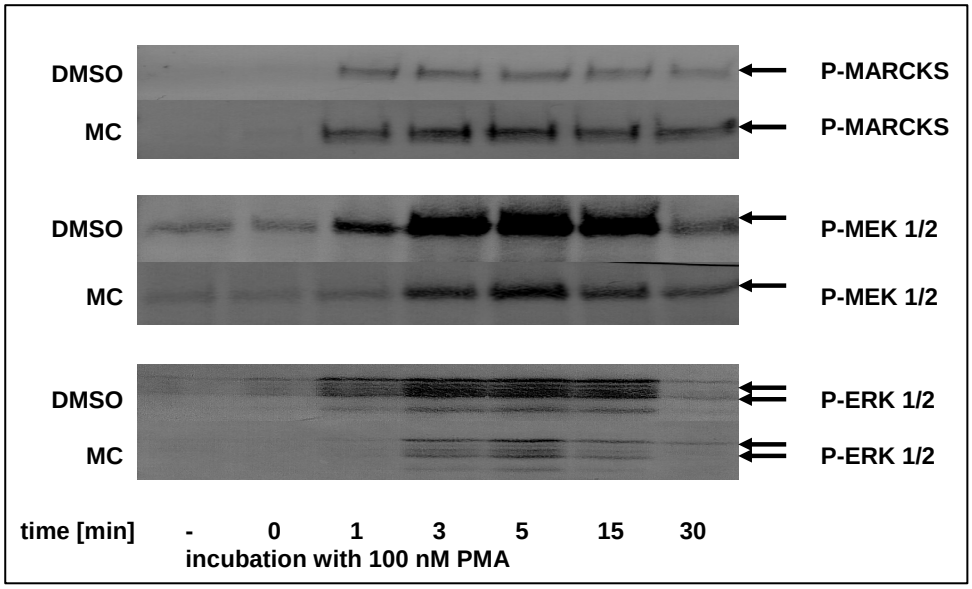



***Fig. 25 Time dependency of inhibition of ERK and MEK phosphorylation by MC.*** *PMNL ($10^8$/ml) freshly isolated from the blood of healthy donors were incubated in PGC for 30 min at 37 °C with 0.2% DMSO or 10 µM MC before stimulation with 100 nM PMA for the times indicated. Samples were loaded to an SDS-PAGE gel and amounts of phosphorylated proteins were quantified using specific antibodies. Data are representatives of 2 independent experiments.*

MC significantly reduced MEK and ERK phosphorylation in PMA-stimulated PMNL, but rather increased MARCKS phosphorylation compared to DMSO-treated cells. Interestingly, the activation of ERK and MEK was not totally inhibited. Phosphorylation of MEK and ERK is rather delayed in MC-treated cells compared to DMSO-treated cells and decrease in phosphorylation started earlier when cells were incubated with MC. No difference in the time to reach the onset of MARCKS phosphorylation between MC- and DMSO-treated cells could be observed.

## 13.6 *MC reduces the amount of active GTP-bound Ras in fMLP or PMA-stimulated PMNL.*

Ras is a small G protein with GTPase activity which is activated by the exchange of guanosine diphosphate with guanosine triphosphate. When Ras activates c-Raf, the subsequent protein of the cascade, c-Raf is recruited to the membrane and is able to transmit the signal by phosphorylation to MEK. Using a prenyl-residue, Ras is anchored to the inner side of the plasma membrane bringing it in close proximity to associated receptors and effector proteins.

Freshly isolated PMNL were treated with DMSO, MC and staurosporine. Cells were then stimulated with 100 nM fMLP for 1.5 min or with 100 nM PMA for 3 min and the amount of active Ras was analysed. In fMLP- and PMA-stimulated cells, MC significantly reduced the amount of the GTP-bound active form of Ras starting at concentrations of 0.3 to 1 µM (Fig. 26). While in fMLP-stimulated cells MC might inhibit Ras activation by a pathway not involving PKC (Fig. Pathways of ERK activation by fMLP and PMA), in PMA-stimulated PMNL, MC must target downstream of PKC in order to decrease Ras activation.

Staurosporine reduced the amount of GTP-bound Ras in PMA- and fMLP-stimulated cells as expected.

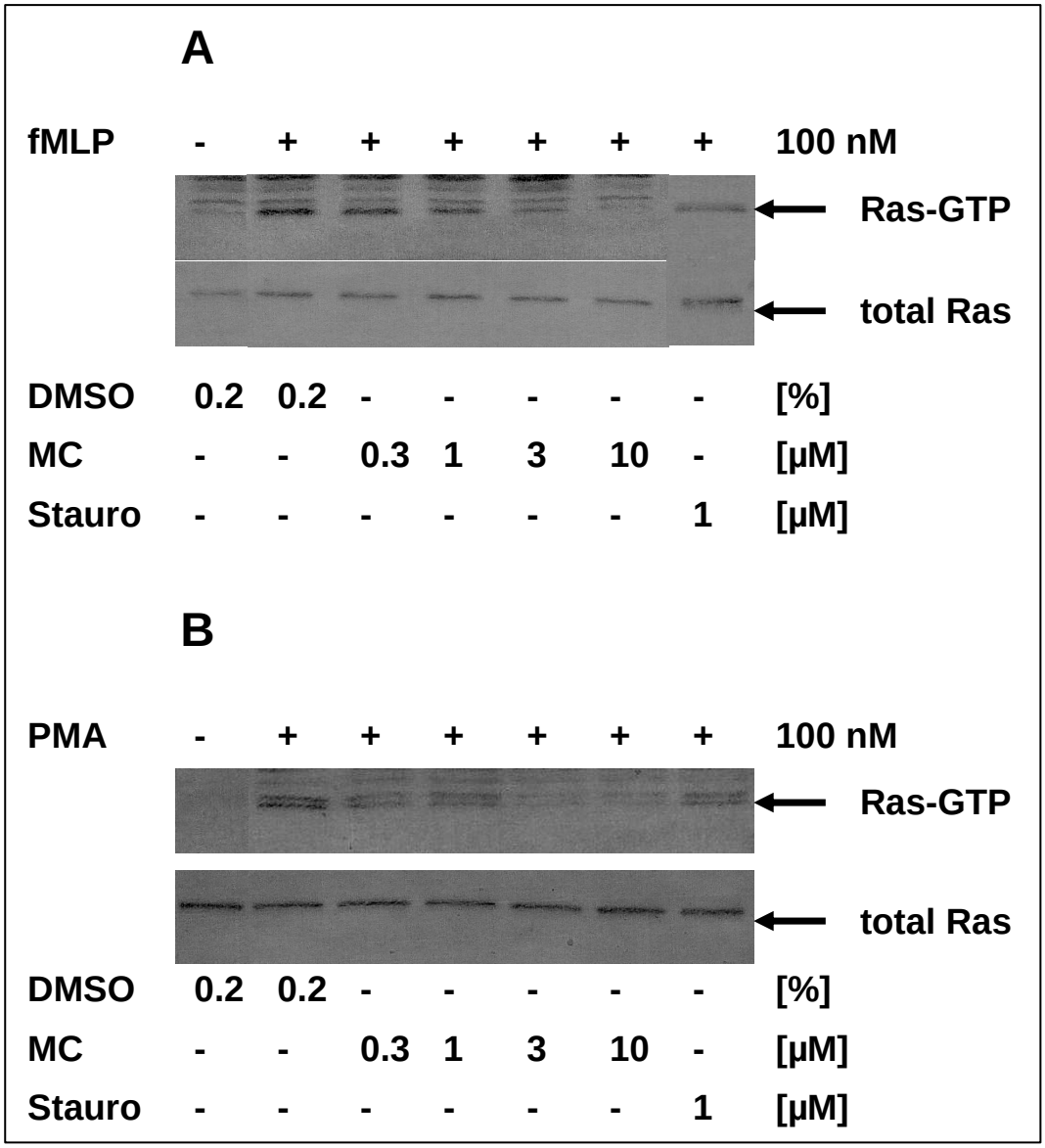



***Fig. 26 MC reduces the amount of GTP-Ras in PMA and fMLP-stimulated PMNL.*** *PMNL ($10^8$/ml) freshly isolated from the blood of healthy donors were incubated for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro) before stimulation with 100 nM fMLP for 1.5 min* ***(A)*** *or 100 nM PMA for 3 min* ***(B)****. After stopping the reaction, cells were lysed and lysates were transferred onto Raf-Ras binding domain (Raf-RBD) coupled Sepharose beads. 5 µl of lysates were directly subjected to SDS-PAGE and Western blot for detection of total amounts of Ras. Ras-GTP bound to the Sepharose beads was extracted from the lysates by centrifugation, several washing steps and addition of SDS-b. Samples were then loaded to an SDS-PAGE gel and amounts of Ras-GTP were quantified by Western Blot using a Ras-specific antibody. Data are representative for 3 independent experiments.*

## 13.7 *Phosphorylation of c-Raf (Raf 1) in PMA and fMLP-stimulated PMNL is diminished by MC.*

Downstream of the G protein Ras the protein kinase c-Raf (also termed Raf1) is located. Activation of c-Raf or signal transduction from Ras downstream to c-Raf, MEK and ERK is supposed to be accomplished by the formation of a complex of Ras, c-Raf, MEK and the two scaffolding proteins kinase supressor of Ras (KSR) and connector enhancer of KSR (CNK).

PKC, apart from activating Ras directly, is able to activate c-Raf and MEK. Since MC decreased the amount of active GTP-Ras in PMA-stimulated PMNL we tried to analyse the effect of MC on c-Raf phosphorylation in PMA-stimulated cells.

We observed a decrease in MEK phosphorylation after incubation with MC, according to the decrease in ERK phosphorylation in PMA- or fMLP-treated cells (Fig. 27). Regarding the phosphorylation state of the c-Raf isoform, we could see a decrease in phosphorylation of the Ser338 in fMLP- and PMA-stimulated cells which is thought to be phosphorylated by Ras and to lead to the activation of c-Raf. On the other hand, phosphorylations of the serine residues 289, 296 and 301, that are targeted by Src kinases, were not reduced but rather slightly stimulated by MC (data not shown), which is in accordance to the data concerning the slight increase in phosphorylation of tyrosine residues (see Fig. 23A and C). MC did not have an effect on phosphorylation of the similar site in B-Raf (Ser445) (Fig. 28). Staurosporine reduced the phosphorylation of c-Raf induced by fMLP or PMA as expected and reduced MEK phosphorylation in PMA-stimulated cells.

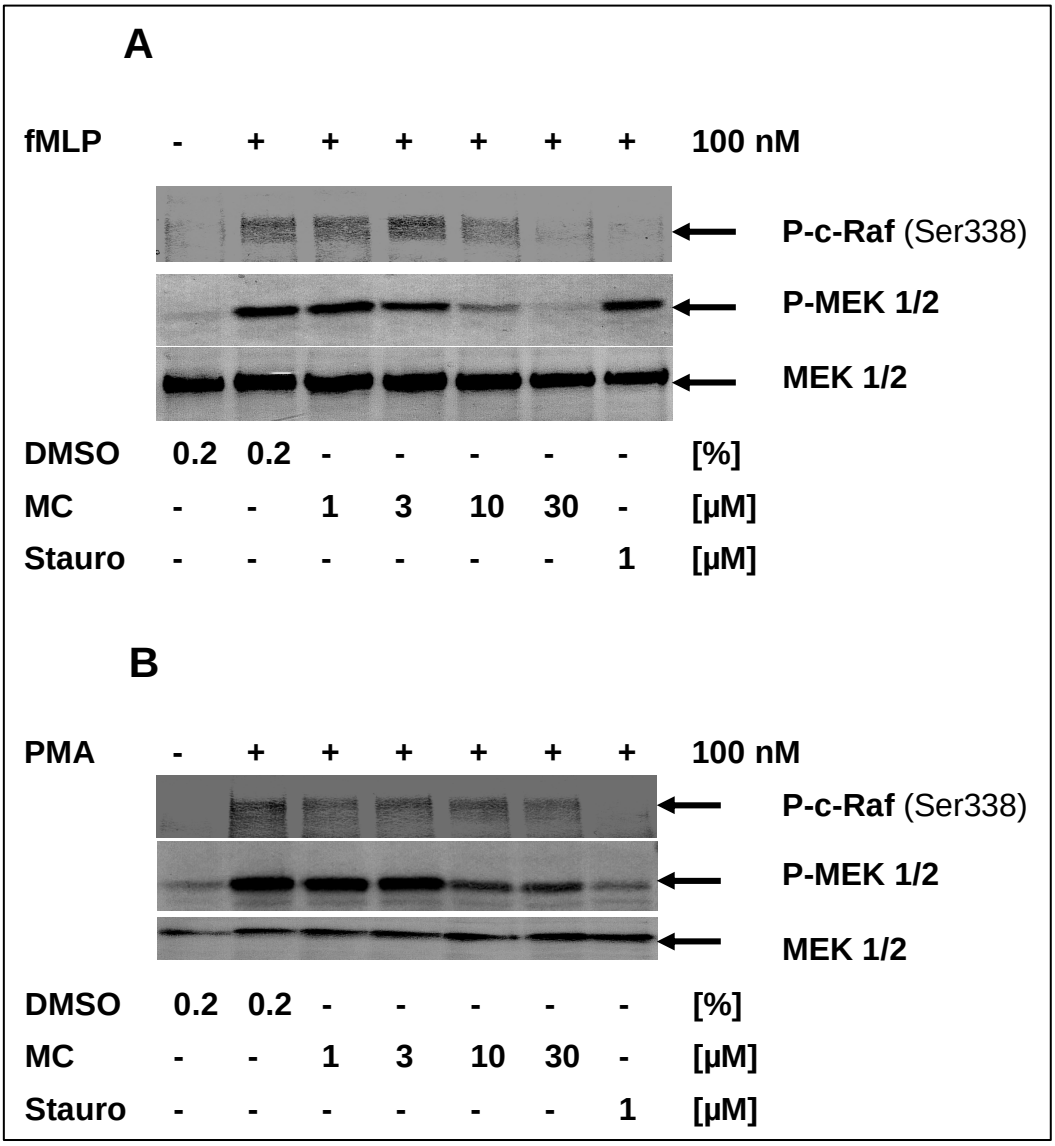



***Fig. 27 MC reduces phosphorylation of c-Raf and MEK 1/2 in fMLP- and PMA-stimulated PMNL.*** *PMNL ($10^8$/ml) freshly isolated from the blood of healthy donors were incubated in PGC for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro) before stimulation with 100 nM fMLP for 1.5 min or 100 nM PMA for 3 min. Samples were loaded to an SDS-PAGE gel and amounts of proteins were quantified by Western Blot using phospho-c-Raf (Ser338) and phospho-MEK 1/2 specific antibodies. Data are representative for 3 independent experiments.*

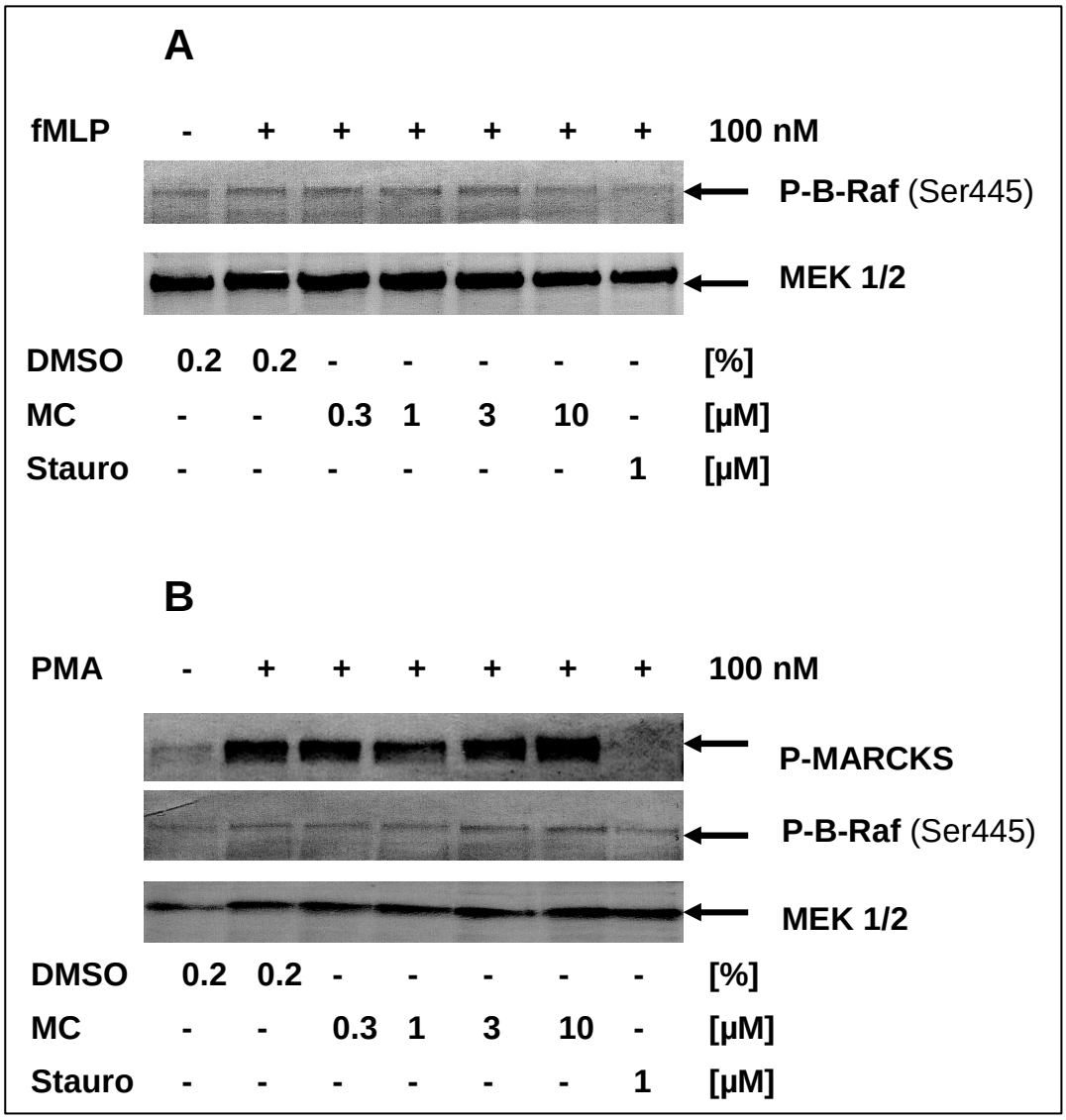



***Fig. 28 Phosphorylation of B-Raf in fMLP and PMA-stimulated PMNL is unaffected by MC.*** *PMNL freshly isolated from the blood of healthy donors were set to $10^8$/ml. Cells were incubated in PGC for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro) before stimulation with 100 nM fMLP for 1.5 min or PMA for 3 min. Incubation was stopped by adding SDS-b and putting the samples on ice. After cooking and sonification, samples were loaded to an SDS-PAGE gel and amounts of proteins were measured using phospho-B-Raf (Ser445) specific antibody. Data are representative for 3 independent experiments.*

# 14 Effects of MC on signal transduction in HL60 cells

In PMNL MC strongly inhibited phosphorylation of MAPK and several other targets (chapter 2) which probably are the mechanisms leading to the effects observed by others [18] including ROS-production, leukocyte elastase release and $Ca^{2+}$ mobilisation. Next, we analysed whether MC has the same effects in the acute myeloid leukaemia HL60 cells. One feature of HL60 cells is upregulation of N-Ras activity and of the whole downstream cascade towards ERK. We aimed to evaluate whether pro-apoptotic effects of MC are induced by targeting MAPK signaling in HL60 cells and whether this is the possible point of discrimination between non-transformed cells and degenerated cancer cells.

The ERK pathway has an important role in regulating differentiation and cell growth and several downstream transcription factors have been identified. The 90 kDa ribosomal S6 kinases p90RSK and Elk-1 are two examples. One is a key regulator of glucose homeostasis and cell size [202] the other, when overactivated, plays a role in intestinal cells after infection with *Helicobacter pylori.* Other MAPK pathways such as p38 MAPK or JNK are responsible for apoptosis and proliferation control.

Here we tested if MC is able to interfere with signaling pathways similarly to the effects in PMNL, irrespective of the upregulation of MAPK pathways in HL60 cells.

## 14.1 *The long term effect of MC on ERK phosphorylation in HL60 cells is caspase-dependent*

Along with the studies regarding the apoptosis inducing effect of MC, it was first tested if MC has long term effects (16 h) on ERK phosphorylation. ERK inhibition may in fact result in a reduction of BAD (inhibitory) phosphorylation which would enable it to translocate to the mitochondrial membrane and inhibit antiapoptotic Bcl2 proteins.

HL60 cells were incubated for 16 h with vehicle, MC, staurosporine and/or the pan caspase inhibitor QVD, and the amounts of cleaved PARP, phospho-

MARCKS, phospho-ERK 1/2, ERK 1/2 and beta-actin were quantified by SDS-PAGE (Fig. 29).

MC induced PARP cleavage as before indicating apoptosis induction. When caspases were inhibited by QVD, PARP cleavage by MC was abolished.

The phosphorylation of MARCKS and of ERK was clearly diminished when incubating the cells with MC or staurosporine. Adding QVD together with MC reversed the effect of MC, indicating that the decrease in ERK phosphorylation by MC is caspase-dependent.

Taken together with the fact that MC induces apoptosis independently of Bcl2 overexpression, as was shown above, it is highly unlikely that downregulation of ERK and therefore BAD phosphorylation is the reason for induction of the mitochondrial pathway of apoptosis.

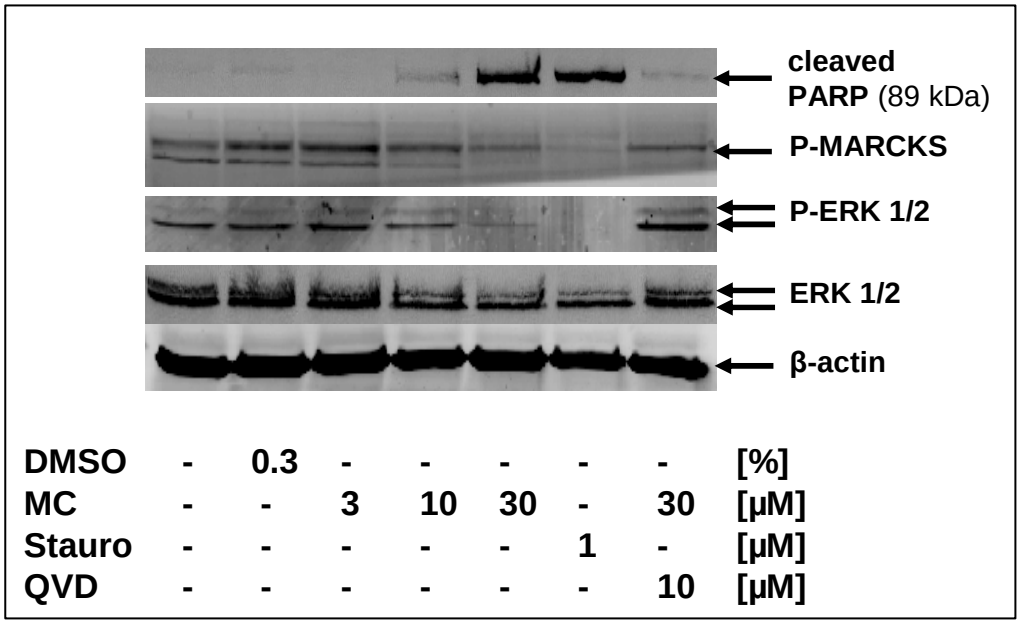



***Fig. 29 The decrease in ERK and MARCKS phosphorylation in HL60 cells after 16 h incubation with MC is caspase-dependent.*** *HL60 (0.2 × $10^6$ /ml in the appropriate medium) were incubated for 16 h at 37 °C with DMSO, MC, staurosporine (Stauro) and Q-VD-Oph (QVD) respectively. After centrifugation, the pellet was lysed with SDS-b and evaluated for amount of PARP, phospho-ERK, phospho-MARCKS, ERK and beta-actin by SDS-PAGE and Western Blot. Data shown are representative for 3 independent experiments.*

## 14.2 *MC decreases phosphorylation of p38 MAPK, ERK and Akt but not JNK/SAPK in HL60 cells*

The long term effect of MC seen on ERK phosphorylation is caspase-dependent and obviously only proximal to caspase activation and apoptotic disassembly of the cell. In order to investigate if the short term effects of MC observed in PMNL could be seen in HL60 cells as well, HL60 cells were incubated with vehicle, MC or staurosporine and stimulated with fMLP and PMA as done with PMNL (Fig. 30).

Interestingly, the basal phosphorylation state of the observed kinases could not be further elevated by fMLP or PMA. This is probably due to the upregulation of Ras activity not only activating the ERK cascade but also cross signaling to other kinase pathways. Nonetheless, MC showed a strong decrease in phosphorylation of ERK and p38 MAPK, a slight decrease in Akt phosphorylation but no effect on JNK/SAPK.

Phosphorylation of MARCKS was also unaffected by MC reflecting the results in PMNL and hinting that PKC is not involved. Staurosporine showed strong inhibition of MARCKS phosphorylation but not of ERK phosphorylation. This would indicate that in HL60 cells activation of ERK and PKC are mutually independent events.

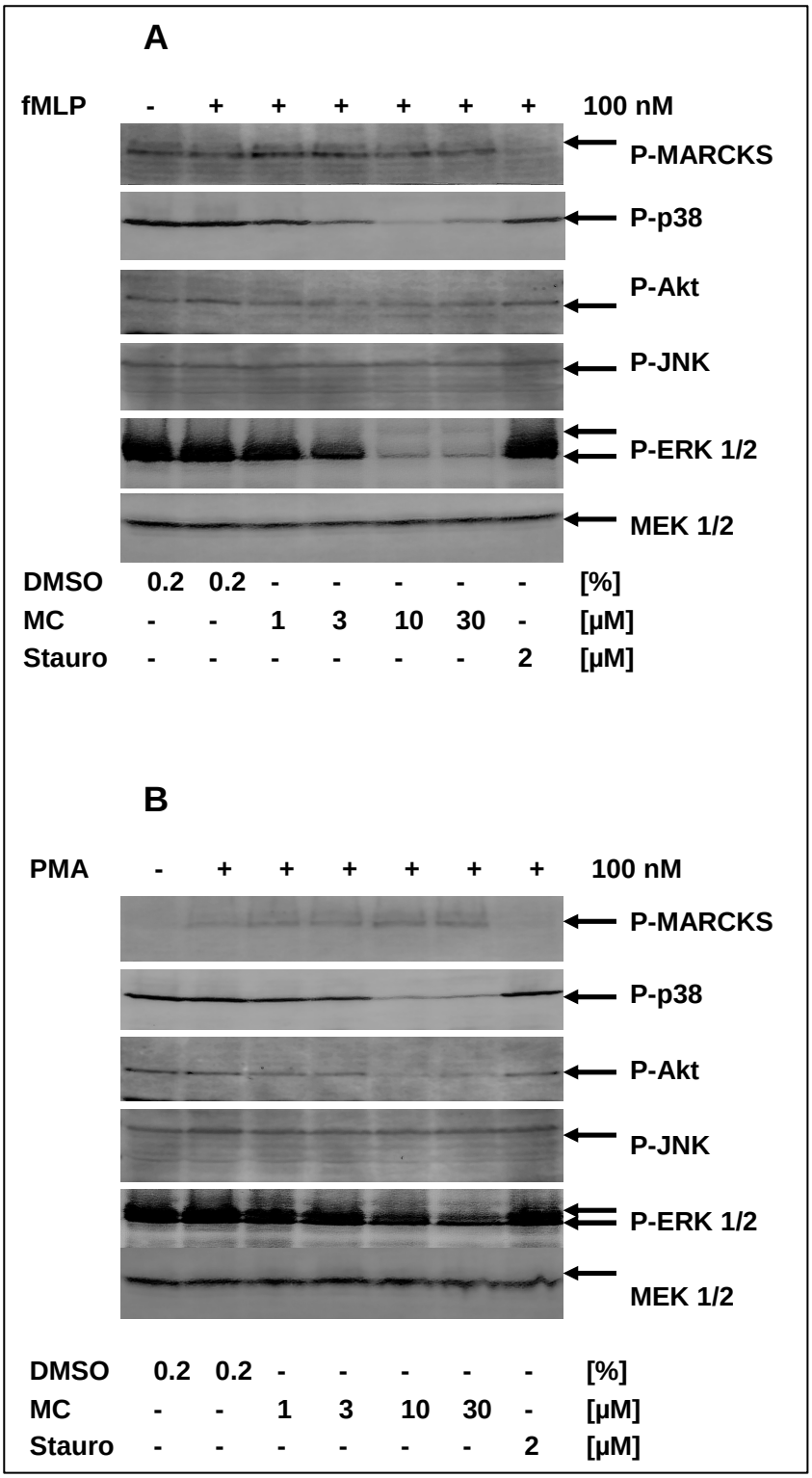



***Fig. 30 MC reduces phosphorylation of the MAP Kinases ERK, p38 MAPK as well as Akt/PKB in fMLP-stimulated HL60 cells.*** *HL60 cells ($5 \times 10^7$ /ml) were incubated in PGC for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro) before stimulation with 100 nM fMLP for 1.5 min* ***(A)*** *or 100 nM PMA for 3 min* ***(B)****. Incubation was stopped by adding SDS-b and putting the samples on ice. After cooking and sonification, samples were loaded to an SDS-PAGE gel and amounts of proteins were measured using specific antibodies. Data are representative for 2-5 independent experiments.*

## 14.3 *MC reduces ERK phosphorylation in unstimulated cells*

Although fMLP and PMA did not lead to increased phosphorylation states of the tested kinases in HL60 cells, MC reduced especially ERK and p38 MAPK phosphorylation.

As seen in Fig. 31, the decrease in ERK phosphorylation by MC can also be observed in unstimulated cells and is comparable to fMLP-treated HL60 cells. Independent of any external stimuli, MC can target and interrupt MAPK signaling which is upregulated in HL60 cells.

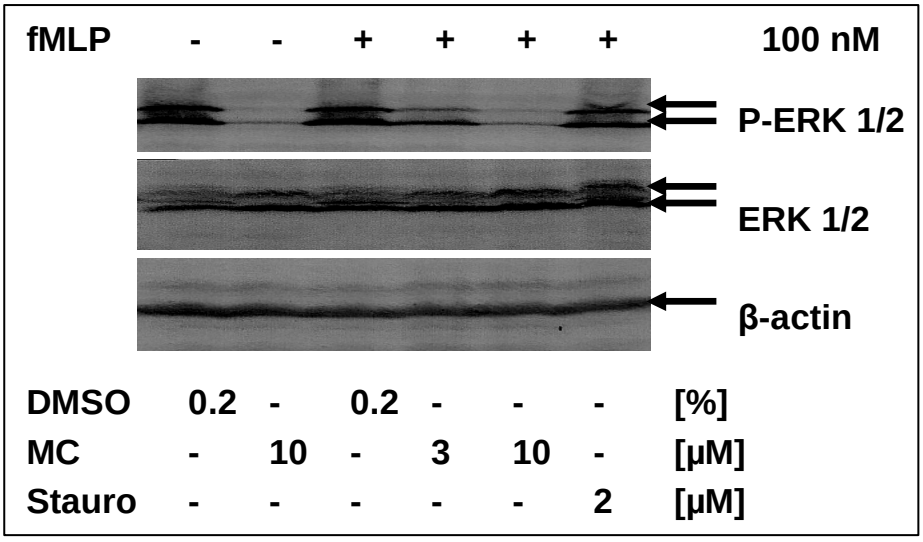



***Fig. 31 MC reduces phosphorylation of ERK in unstimulated HL60 cells.*** *HL60 cells ($5 \times 10^7$ cells/ml) were incubated in PGC for 30 min at 37 °C with DMSO, myrtucommulone (MC) or staurosporine (Stauro) before stimulation with 100 nM fMLP for 1.5 min. Incubation was stopped by adding SDS-b and putting the samples on ice. After cooking and sonification, samples were loaded to an SDS-PAGE gel and amounts of proteins were measured using specific antibodies. Data are representative for 3 independent experiments.*

## 14.4 *PTPase activity is not affected by MC in HL60 cells*

According to the results in PMNL on PTPase activity, we wanted to evaluate the effect of MC on PTPase activity in HL60 cells (Fig. 32). Instead of leading to an inhibition of PTPases as observed in PMNL, in HL60 cells, MC was not able to significantly affect PTPase activity (Fig. 32A). Interestingly, the effect of MC on the phosphorylation of tyrosine residues was also abolished (Fig. 32B). This indicates that the decrease in ERK phosphorylation after treatment with MC is seemingly not mediated by action on tyrosine kinases and phosphatases.

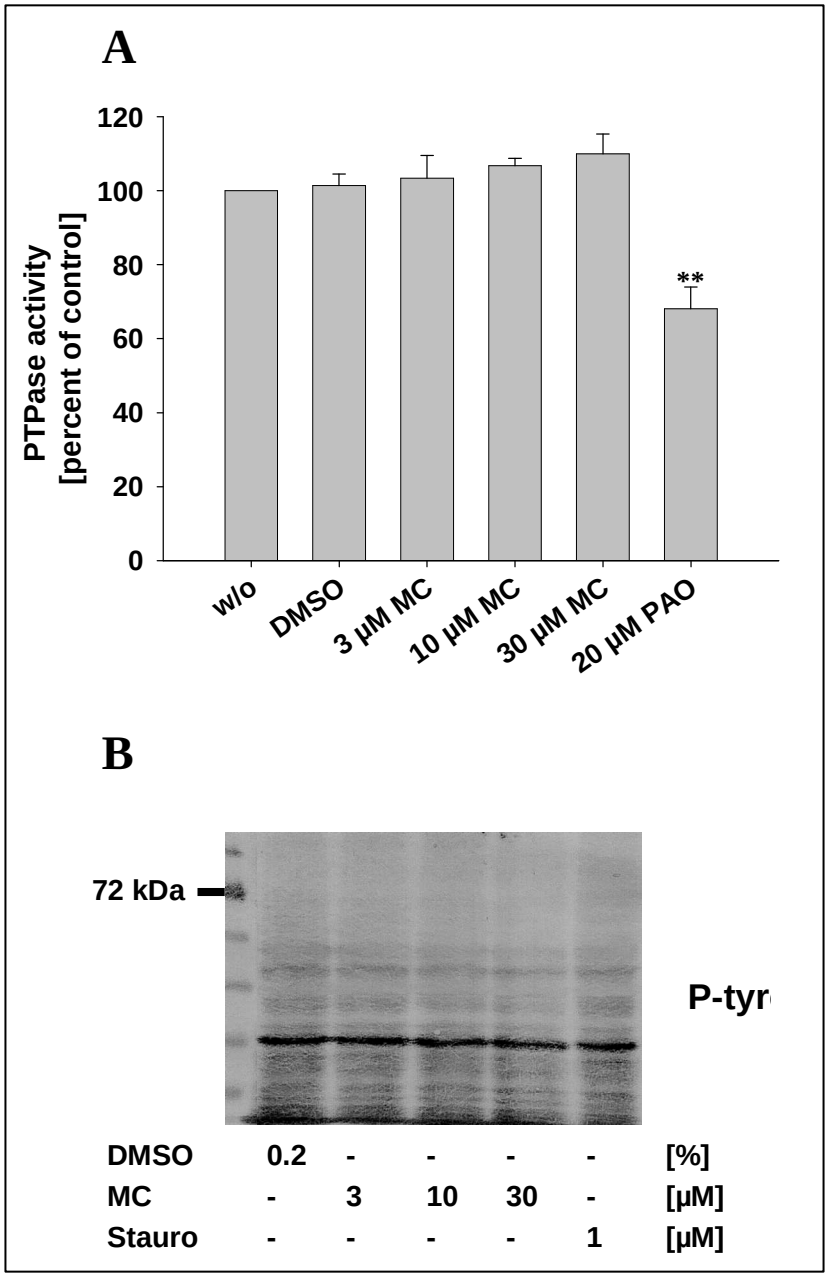



***Fig. 32 MC has no effect on PTPase activity in HL60 cells. A) PTPase activity.*** *Cells ($8 \times 10^5$) were incubated for 30 min at 4 °C with DMSO, MC or PAO. After lysing the cells and centrifugation of the lysates, p-nitrophenyl phosphate (pNPP) was added to the supernatants and absorption at 405 nm was measured in a Victor™ plate reader. n = 3 ± S.E. **p<0.01* ***B) Phosphorylation of tyrosine residues.*** *$5 \times 10^6$ cells were incubated for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro). Incubation was stopped by adding SDS-b and putting the samples on ice. Data are representative for 3 independent experiments.*

## 14.5 *MC fails to reduce the amount of GTP-bound Ras in HL60 cells*

ERK phosphorylation in HL60 cells is strongly reduced by MC. In PMNL this was at least partly due to inhibition of Ras activation and to a decrease in the

active GTP-bound form of Ras. We evaluated whether similar effects concerning Ras activation could be observed in HL60 cells. An effect on Ras activity by MC might be the reason for `normalised´ activity of signaling pathways and higher susceptibility to subsequent events (like apoptosis), since N-Ras is constitutively active in HL60 cells.

Surprisingly, no effect of MC on amounts of Ras-GTP in fMLP- or PMA-stimulated HL60 cells could be observed (Fig. 33). The kinase inhibitor staurosporine also failed to reduce the amounts of Ras-GTP, indicating high stability of the overactivated Ras-GTP complex in HL60 cells.

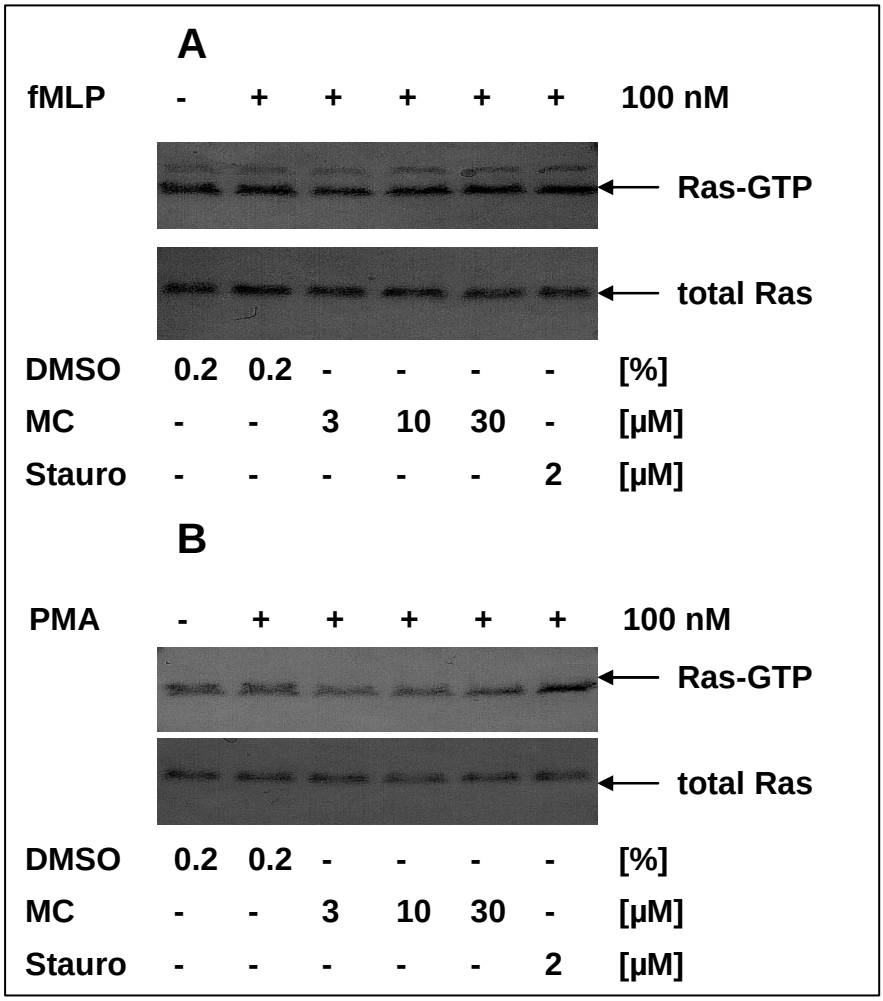



***Fig. 33 MC fails to reduce the amount of GTP-Ras in PMA and fMLP-stimulated HL60 cells.*** *HL60 cells ($5 \times 10^7$/ml) were incubated for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro) before stimulation with 100 nM fMLP for 1.5 min* ***(A)*** *or 100 nM PMA for 3 min* ***(B)****. After stopping the reaction, cells were lysed and lysates were transferred onto Raf-Ras binding domain (Raf-RBD)-coupled Sepharose beads. 5 µl of lysates were directly assigned to SDS-PAGE and Western blot for detection of total amounts of Ras. Ras-GTP bound to the Sepharose beads was extracted from the lysates by centrifugation, followed by several washing steps and addition of SDS-b. Samples were then loaded to an SDS-PAGE gel and amounts of Ras-GTP were quantified by Western Blot using a Ras-specific antibody. Data are representative for 3 independent experiments.*

## 14.6 *MC does not affect the Ras/Raf/MEK signaling cascade*

In HL60 cells, MC did not inhibit Ras activation although the phosphorylation of ERK was strongly reduced. To test wether MC might have an effect on the kinases downstream of Ras, we assessed the phosphorylation states of c-Raf and MEK 1/2 in HL60 cells after preincubation with MC and staurosporine and subsequent stimulation with fMLP and PMA (Fig. 34).

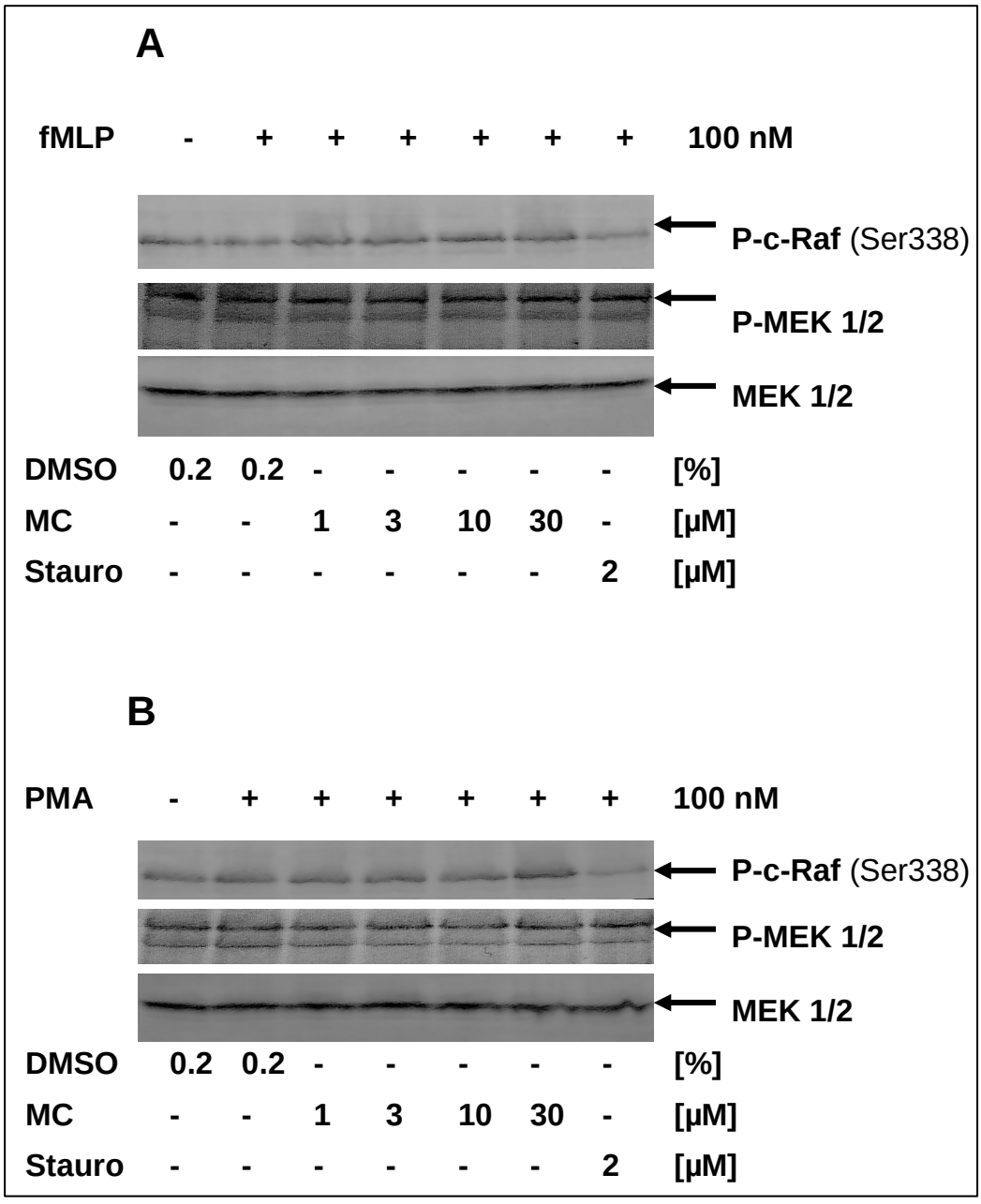



***Fig. 34 MC has no effect on phosphorylation of c-Raf and MEK 1/2 in HL60 cells.*** *HL60 cells ($5 \times 10^7$/ml) were incubated for 30 min at 37 °C with DMSO, MC or staurosporine (Stauro) before stimulation with 100 nM fMLPfor 1.5 min* ***(A)*** *or 100 nM PMA for 3 min* ***(B)****. Samples were loaded to an SDS-PAGE gel and amounts of proteins were quantified by Western Blot using phospho-C-Raf (Ser338) and phospho-MEK 1/2 specific antibodies. Data are representative for 3 independent experiments.*

No effect of MC on phosphorylation of c-Raf or MEK 1/2 could be observed. Again, fMLP or PMA could not further increase the amount of phosphorylation of c-Raf and MEK 1/2 indicating the maximal activation state of the signaling pathway in HL60 cells. Staurosporine was not able to reduce the amounts of phospho-MEK 1/2 but apparently had a slight effect on c-Raf phosphorylation. According to the effect of staurosporine on MARCKS phosphorylation (Fig. 30), staurosporine obviously still has an effect on PKC in HL60 cells leading to decreased MARCKS and c-Raf phosphorylation. Nonetheless, this decrease is not sufficient to reduce MEK phosphorylation.

The inhibition of ERK by MC in HL60 cells due to decreased phosphorylation is obviously not mediated by the Ras/Raf/MEK pathway. The influence of MC on ERK must therefore be caused by interference with another pathway upstream of ERK.

# Discussion

# Discussion

## 15 MC and apoptosis induction

Previous studies aiming to evaluate the physiological and biochemical effects of MC on whole cells as well as on isolated enzymes and molecules mainly concentrated on effects of MC in inflammation and bacterial infections and on antioxidant properties of the substance. Only little is known about the effects of MC on apoptosis induction and on cancer cells [22]. The anticancer drugs which nowadays are in use partly show severe side effects like gastrointestinal and cardiac damage, bleeding, fever etc.. They always bear the risk of inducing chemoresistance in cancer cells. The development of new drugs and the improvement of common treatment against cancer are in progress and there is great need to find new substances with selective cytotoxicity for cancer cells and marginal or no damaging effects on non-degenerated cells.

Indeed, MC could reduce cell viability in several cancer cell lines with $EC_{50}$ values between 3 and 9 μM, whereas non-transformed cells (PMNL, PBMC) were affected only at concentrations of > 20 μM and > 50 μM MC. Although plasma levels of MC after oral application of the substance are yet to be determined, hyperforin, a prenylated acylphloroglucinol with structural similarities to MC, was already tested in this respect. After three oral doses of 300 mg/kg of *Hypericum perforatum* extracts (correlating to 3 times 14.8 mg hyperforin) per day, plasma levels between 0.18 μM (steady state) and 0.28 μM (maximal concentration) hyperforin were reached in humans [203]. Assuming that MC shows similar bioavailability and gives comparable plasma concentrations, cytotoxicity for non-transformed cells should be evanescent, although the effects on cancer cells and on signal transduction pathways would perhaps not reach half maximal inhibition. Tests concerning bioavailability and different modes of administration of MC in rats to reach concentrations of optimal activity in cancer cells are recently conducted.

Apoptosis is a mechanism often deregulated in cancer cells. Pathophysiological properties of cancer cells include increased cell growth, proliferation, changes in

metabolism (to meet higher energy needs of the cells), as well as increased invasiveness and metastasising properties. Changes in degenerated cells include upregulation of signal transduction pathways, increased metabolism and reduced apoptotic signaling. This includes effects on e.g. p53, NFκB and PKB/Akt which also leads to altered gene expression usually worsening the situation [204]. Apoptosis induction in cancer cells is therefore one of the most important strategies for cancer therapy. In the present investigation the apoptosis inducing properties of MC were elucidated, showing that MC induces apoptosis involving the intrinsic pathway.

Since MC showed the strongest cytotoxic effects in leukaemic cells with $EC_{50}$ values of 3 - 4 µM, in this study the focus was placed on promyelocytes (HL60), pre-mature monocytic cells (MM6) and Jurkat cells. MC concentration-dependently induced cleavage (and therefore activation) of caspases 2, 3, 7, 8 and 9 in HL60 cells and MM6 cells, starting at concentrations of 3 to 10 µM. PARP, as main substrate of caspase 3, was also cleaved and inactivated suggesting apoptosis induction. Comparing the results to caspase cleavage in PBMC, it was obvious that tenfold higher concentrations of MC were needed to induce cleavage of PARP and caspase 3. Cycloheximide (CHX) is an inhibitor of protein biosynthesis by blocking translational elongation in eukaryotes. Furthermore, CHX exerts toxic effects leading to DNA damage. Its apoptosis inducing effects are probably due to decreased synthesis of short-lived death antagonists and to decreased glucose uptake [205]. CHX was used as positive control for cytotoxicity in HL60 and MM6 cells and showed PARP and caspase 8 cleavage as expected.

Daunorubicin is an anthracycline commonly used as chemotherapeutic drug. It slows the growth of cancer cells by DNA intercalation, thus blocking transcription. Furthermore, it can stimulate the production of superoxide anions [206, 207]. Daunorubicine exerts cytotoxic effects especially for rapid growing cells but has also severe side effects due to cytotoxicity if not quickly administered intravenously. In PBMC, 1 µM daunorubicin induced cleavage of PARP, caspase 9 and 3, showing its cell death inducing potency also in non-transformed PBMC.

One could argue that this selective effect of MC on cancer cells, especially concerning numbers of viable cells, could be due to the simple difference in

proliferation rates of the cells. This could be refuted by stimulation of PBMC with 5 µg/ml PHA-L for 24 hours, which significantly increased numbers of PBMC in the G2-phase (from 1.4 to 16% in PHA-L-stimulated cells, data not shown). MC-induced cytotoxicity in PHA-L-stimulated cells was not significantly increased and $EC_{50}$ values ranged around 20-30 µM (data not shown).

Regarding the time-course of caspase 3 and 9 as well as PARP cleavage in HL60 cells, MC-induced cleavage started after 9 hours preincubation of the cells. Staurosporine inhibits protein kinases by competitive binding of ATP binding sites [208, 209]. Although the exact mechanism of apoptosis induction by staurosporine is not totally understood, activation of caspase 2 might be involved. The staurosporine-induced caspase and PARP cleavage and apoptosis induction, as observed in Western Blot experiments as well as in DNA fragmentation assay and Annexin V/PI-assay (data not shown), started significantly earlier than the effects of MC after less than 3 hours preincubation with staurosporine. Such early apoptosis-inducing effects of staurosporine were observed before [209].

The release of proteins from mitochondria is a hallmark of early apoptosis. Endonucleases (EndoG and AIF) leading to DNA fragmentation, as well as cytochrome c, activating caspase 9, are released upon treatment with MC. DNA fragmentation could be detected in HL60 and MM6 cells by flow cytometry, after 24 hours incubation with MC. Significant effects could be observed starting from 10 µM MC in HL60 and MM6 cells whereas only 60 µM MC caused a significant effect in PBMC. Cytochrome c release was observed in HL60 cells and MM6 cells starting at 10 µM MC and in accordance with the time course of caspase and PARP cleavage (Fig. 5), the cytochrome c release started 6 to 9 hours after MC treatment. Furthermore, cytochrome c release by MC could not be inhibited by the pan caspase inhibitor QVD indicating that MC-induced cytochrome c release is caspase independent. This refutes the theory by our previous work where we assumed that caspase 8 activation precedes and even causes cytochrome c release by cleavage and activation of Bid to tBid [22]. Caspase 8 is probably rather activated in a feedback amplification loop by caspase 9 and caspase 3 [86, 87].

Concerning the preceding events that cause the release of cytochrome c and endonucleases from mitochondria, the effect of MC on the mitochondrial potential $\Delta\Psi_m$ was evaluated. After 24 hours incubation, MC reduced $\Delta\Psi_m$ in MM6 cells

starting at 3 µM but not in PBMC. Interestingly, investigating $\Delta\Psi_m$ after 30 min incubation with MC, the loss of $\Delta\Psi_m$ induced by MC in PBMC was equal to the loss of $\Delta\Psi_m$ in MM6 cells (data not shown). Obviously, MC affects $\Delta\Psi_m$ in PBMC. Since endoxidation and ATPase activity are not downregulated in PBMC as compared to cancer cells [53], the reversible reaction of ATPase, hydrolysing ATP to ADP and phosphate, probably reconstitutes $\Delta\Psi_m$ (uncoupling induced ATPase activity). Thus, it can be concluded that the selectivity of MC for cancer cells starts at the level of mitochondria and probably involves alterations in mitochondrial metabolic enzyme activities.

There is a strong debate regarding the order of membrane disrupting events (PTP, loss of $\Delta\Psi_m$, mitochondrial swelling, MAC) in mitochondria which then subsequently lead to cytochrome c release and apoptosis induction.

The MAC is formed upon oligomerisation of Bax (probably by involvement of tBid) and can be inhibited by the anti-apoptotic Bcl2. If the mechanism of mitochondrial apoptosis induction by MC involves opening of MAC, this should be inhibited by overexpression of Bcl2. However, MC still reduced cell viability, led to PARP and caspase 3 cleavage, induced loss of $\Delta\Psi_m$ and led to cytochrome c release in Bcl2-overexpressing Jurkat cells in the same manner as in MOCK-transfected Jurkat cells. Thus, MAC formation is not required for apoptosis induction by MC. The second possibility of mitochondrial membrane disruption tested, was the formation of the PTP. By interacting with one of the PTPs numerous components, MC might be able to induce PTP opening. Chelerythrine and cyclosporine A were used as control reagents. Chelerythrine is able to directly target mitochondria and open PTP, whereas cyclosporine A is able to bind cyclophillin D and inhibits opening of the pore. The effect of MC on cell viability was not affected by CsA and by inhibiting PTP opening, whereas chelerythrine caused cytotoxic effects on both, MM6 cells and PBMC, which could be totally reversed by inhibition of PTP with CsA. Thus, also PTP formation is obviously not involved in MC-induced apoptosis.

Is it possible that the intrinsic pathway of apoptosis induction is not the only mode of action involved in MC-induced apoptosis? Or does MC induce a caspase independent way of apoptosis? Both theories could be abandoned by testing MC in various Jurkat cell lines, deficient or defective in members of the extrinsic and intrinsic pathway. MC still reduced cell numbers and could induce PARP

cleavage regardless of deficiency in FADD, CD95 signaling or caspase 8 with comparable concentrations that are effective in wild type cells. Only in caspase 9-deficient cells PARP cleavage and DNA fragmentation by MC was totally abolished. Also caspase 8 was not cleaved in caspase 9-deficient cells, indicating that caspase 8 activation observed in HL60 and MM6 cells is dependent on caspase 9 rather than constituting the event leading to cytochrome c release and caspase 9 activation. Surprisingly, the numbers of viable cells in caspase 9-deficient cells are still reduced by MC. Untreated caspase 9-negative cells grew to a cell density of $0.49 \times 10^6$ cells/ml after 48 hours, whereas MC-treated cells only reached an amount slightly more than the starting concentration of $0.2 \times 10^6$ cells/ml. What could be the reason for this persistent effect on caspase 9-deficient cells irrespective of the apoptosis induction by MC?

Two possible reasons were checked. First, we tested if MC had necrosis-inducing effects and second, we analysed if the cell cycle was affected by MC. Neither 30 μM MC nor 1 μM staurosporine could induce apoptosis or necrosis after 16 hour (or 24 hours, data not shown) incubation in caspase 9-deficient Jurkat cells, tested in the Annexin V / propidium iodide assay. The MC- and staurosporine-induced phosphatidylserine externalisation could be detected in caspase 9-deficient cells retransfected with caspase 9. Hence, necrosis is obviously not the reason for the decrease in cell numbers of caspase 9-deficient cells by MC. The results concerning staurosporine contradict results obtained by another group who could show that staurosporine induces apoptosis in a catalytically inactive caspase 9 mutant cell line [209]. They stated that higher concentrations of staurosporine were needed in this mutant cell line than in normal cells to get similar apoptosis induction (60% apoptotic nuclei determined by DNA fragmentation assay after 24 hours incubation with 5 μM staurosporine in JM319). The staurosporine concentration used (1 μM) as control in this study might have been not sufficient to see an effect in the Annexin V /propidium iodide assay.

MC led to a slight inhibition of the cell cycle after 24 hours which caused increased numbers of cells in G2-phase in caspase 9-deficient and retransfected Jurkat cells. Staurosporine which is known to be a strong inducer of G2-phase arrest [210] shows a significant increase in cells located in G2-phase after 24 hours for both cell types. This implies that although apoptosis induction by MC

depends on caspase 9, MC has another effect on the cell which leads to cell cycle arrest.

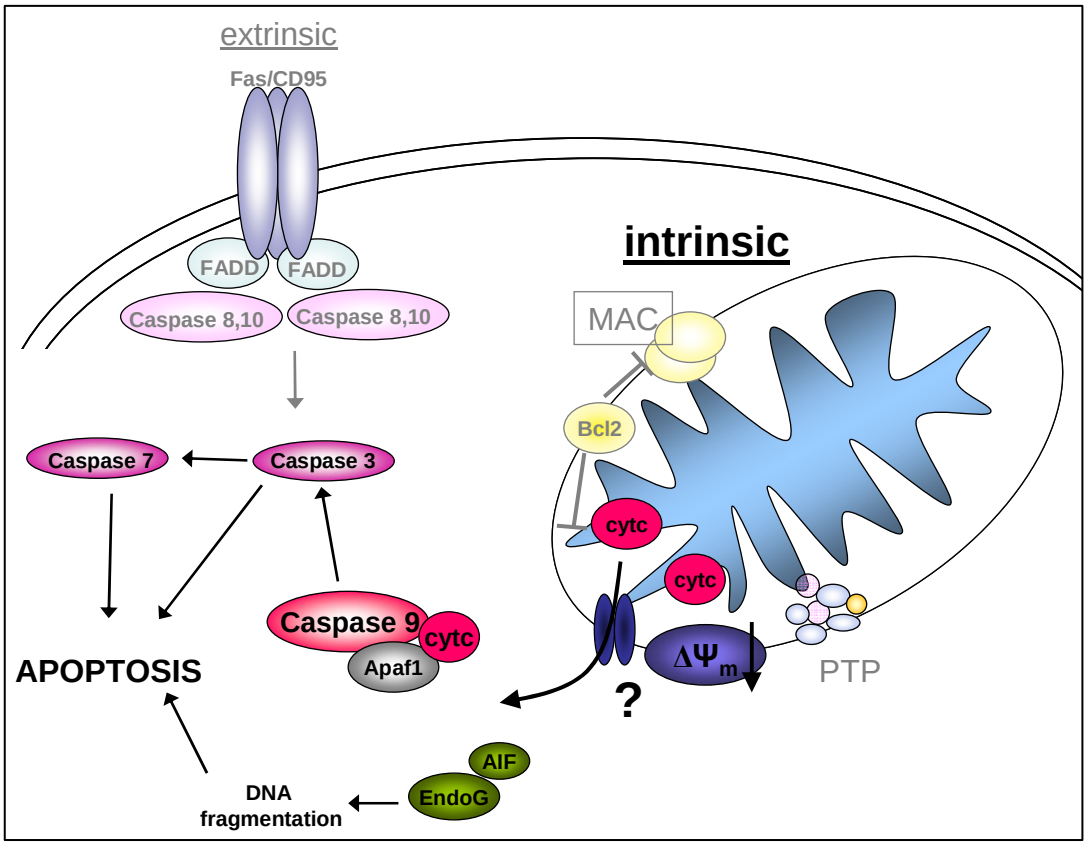



***Fig. Overview: Apoptosis induction by MC.*** *(parts of lower importance are drawn translucent) In the present investigation, it could be shown that MC induces apoptosis in cancer cells by inducing the intrinsic way of apoptosis. Caspase 9 is essential whereas CD95, FADD and Caspase 8 are of no importance for apoptosis induction by MC. Loss of $\Delta\Psi_m$ and cytochrome c release induced by MC is independent of Bcl2 proteins and opening of the PTP. (further explanations see text)*

Many questions still remain and could not be answered during this thesis. Why do PBMC restore $\Delta\Psi_m$ and cancer cells do not? Is this the main difference for apoptosis induction by MC leading to selectivity of MC for cancer cells? What effect of MC in the cell leads to the loss of $\Delta\Psi_m$ and cytochrome c release?

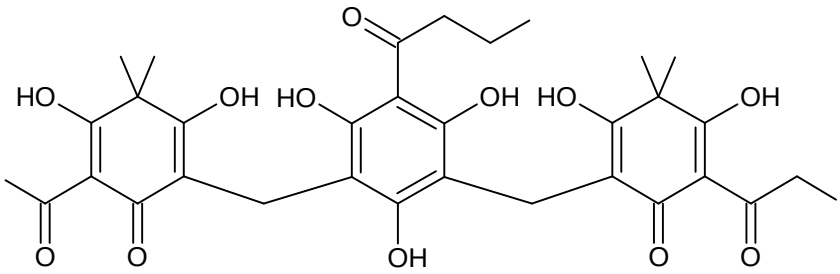
Common compounds used for treatment of cancer or applied in *in vitro* studies concerning apoptosis investigations, often induce apoptosis by the intrinsic pathway due to inhibition of protein biosynthesis (cycloheximide) and due to DNA-crosslinking, -intercalation and topoisomerase inhibition (cisplatinum, daunorubicin, etoposid). Apoptosis induction is then triggered, accompanied by cell cycle arrest and/or changed protein expression. The effect of MC seems to be more multifaceted. Apoptosis induction is not as rapid in onset as for the kinase

inhibitor staurosporine but takes more than 6 hours until effects on caspases and PARP cleavage can be seen. On the other hand, the loss of $\Delta\Psi_m$ induced by MC is a very rapid event occurring after 30 min without any selectivity for non-transformed cells or cancer cells. Instead of selectively triggering the opening of MAC or PTP, the effect of MC on mitochondria seems to be rather unspecific.

At a first glance, MC seems to be a quite hydrophilic and polar substance. The presence of two vinylog carboxylic acids and several hydroxy and carbonyl residues support this assumption. Some publications state however, that within the MC molecule strong hydrogen bonds are formed rendering the molecule more lipophilic [1, 19]. This raises the question, if the effect of MC might be an unspecific incorporation or adsorption in/onto membranes in general. MC may possibly adhere to plasma membranes or enter the cell and insert into mitochondrial membranes where it changes membrane properties and leads to membrane disruption. At least the non-prenylated acylphloroglucinol hyperforin has been shown to be able to modify neuronal membranes in vivo [211]. Hyperforin changed membrane fluidity as determined by fluorescence anisotropy of diphenylhexatriene. In order to evaluate the effect of hyperforin on synaptosomal uptake of norepinephrine, dopamine and serotonine it was observed that hyperforin does not bind to the respective transporters. It was rather postulated that hyperforin might either mechanically change the physical conformation of the lipid bilayer leading to a structural change in receptor and channel proteins or that hyperforin directly interacts with e.g. channel proteins leading to elevation of $[Na^+]_i$ and thus inhibiting the $Na^+$ gradient-dependent uptake of neurotransmitters.

Recently, investigations are conducted showing an interaction of MC with membranes isolated from platelets that are protein free by digestion with proteinase K (unpublished data by A. LeBlanc). Nevertheless, the effects of MC on isolated proteins such as 5-LO, mPGES-1 and COX-1 [46] [18] must be explained somehow. 5-LO as well as mPGES-1 and COX-1 activities all depend on binding of fatty acids (FA) such as arachidonic acid and derivatives. Maybe MC is able to interfere with FA-binding domains of certain enzymes. Another publication about acylphloroglucinols isolated from the fern *Dryopteris crassirhizoma* showed that filixic-acid-ABP being structurally related to MC (already described in [11]) shows an inhibition of the fatty acid synthase with an

$IC_{50}$ of 31.0 +/- 2.7 µM [212], an enzyme that synthesises palmitic acid from acetyl-CoA and malonyl-CoA.




***filixic acid-ABP***

This is of interest when taking into consideration that fatty acid synthase (FASN) inhibition can trigger apoptosis in MCF7, a human breast cancer cell line [213]. Zhou and colleagues could show that the FASN inhibitor C75 could induce apoptosis due to interference with phospholipid biosynthesis during S-phase. And indeed, filixic acids have been shown to possess tumor inhibitory activity [214].

Aside from its apoptosis inducing effects, MC obviously has another effect that is seemingly independent of caspase 9 and leads to an arrest of cell growth as seen in the experiments with caspase 9-deficient and retransfected Jurkat cells.

As it will also be discussed later on in the section concerning the effect of MC on signal transduction, MC might induce changes in protein expression leading to reduced metabolic rates in the cell such as lipid biosynthesis which is needed for proliferation especially in cancer cells.

When assessing the effect of MC on ROS production (data not shown), MM6 cells were incubated with MC for 3 and 6 hours and a decrease in ROS production was observed. ROS can be produced during oxidative phosphorylation in mitochondria (which is visible as slight and steady increase of the baseline in untreated cells). The effect of MC on ROS production could not be reversed by the addition of cycloheximide (and therefore inhibition of proteinbiosynthesis) suggesting that altered gene expression induced by MC is not the reason for this effect. Furthermore, MC could reduce the oxidant properties of $H_2O_2$ added after 3 or 6 hours incubation with MC (data not shown).

MC might influence the oxidative phosphorylation leading to decreased formation of reactive oxygen species and additionally shows anti-oxidant properties. It must be mentioned that the experiments in the present study concerning ROS

production were conducted in unstimulated MM6 cells rather than in fMLP-stimulated PMNL as performed by C. Feißt et al. [18]. Stimulation of granulocytes with fMLP leads to activation of the NADPH oxidase subunit p47 phox by a PKC dependent mechanism which is responsible for oxidative burst and ROS production in granulocytes for fighting intruding pathogens. Also here MC showed a significant effect.

# 16 MC and signal transduction pathways

## 16.1 *Effects of MC on signal transduction in PMNL*

As implicated from the previous chapter, no convincing explanations could be found for the early effects that initialise apoptosis induction by MC. Although some hints exist that membranes, membrane associated receptors and their activation of signal transduction cascades leading to changes in protein expression and activity might be involved.

The results of the loss of mitochondrial membrane potential after 30 min preincubation with MC showed that aside from the `late´ apoptotic events observed after hours of treatment, more rapid effects are induced by MC.
C. Feißt et al. demonstrated a decrease in ROS production, decreased elastase release and a reduction of $Ca^{2+}$ mobilisation from intracellular stores in fMLP-stimulated PMNL which was caused by MC treatment for only 10 min. These effects were obvious in non-transformed granulocytes. Is there a connection of these effects to cancer and induction of apoptosis? Can any selectivity between non-transformed PMNL and HL60 cells which were tested alongside in this study be observed?

Stimulation of granulocytes by chemoattractants such as LPS or fMLP is essential to evoke an acute inflammatory response and to destroy pathogens. Binding of these chemoattractants to the respective receptors initialises signal transduction events of high complexity which then elicit ROS production, degranulation, reassembly of the cytoskeleton and chemotaxis. Upon binding of fMLP to its receptor, the classical pathways involving G proteins, PLC, PKC and MAPK pathways are activated [158]. Upon fMLP stimulation and direct stimulation of PKC by the phorbolester PMA, we could indeed observe an increase of ERK phosphorylation in PMNL. Activation of these MAPK pathways plays an important role in inflammatory diseases and also in cancer. Additionally, many cancer cell lines exhibit constitutively overactivated MAPK cascades. Due to these reasons and due to the fact that MC affects $Ca^{2+}$ signaling upon fMLP

stimulation [18], the influence of MC on signal transduction and especially activation of MAPK pathways was an interesting and promising approach.

MC caused a strong inhibition of ERK phosphorylation in fMLP-stimulated PMNL, starting at a concentration of 3 μM. The p38 MAPK and JNK, as well as the kinase Akt/PKB were stimulated by fMLP and their activation could be inhibited by MC. Interestingly, staurosporine was not able to significantly inhibit phosphorylation of p38 MAPK, ERK and JNK. Only MARCKS and Akt/PKB phosphorylation was decreased by staurosporine. These results with MC indicate that the target for MC probably lies upstream of the mentioned kinase cascades. Staurosporine instead inhibits phospholipid and $Ca^{2+}$-dependent kinases. This might explain the selective effect of staurosporine on PKC (MARCKS) and Akt/PKB in the present experimental setup.

In view of our working hypothesis, the phosphorylation of MARCKS is an interesting point. MARCKS is a direct substrate of PKC which was thought to be involved in MAPK/ERK activation by fMLP [158], but obviously MC had no effect on its phosphorylation. This would mean that a) MC has no direct effect on PKC and b) MC has no effect on the upstream events leading to PKC activation after stimulation with fMLP. Both could be confirmed by stimulation of PKC with PMA and PMA + ionomycin (to exclude any effect of MC on $Ca^{2+}$ concentrations causes an effect on PKC activity). Again, MC significantly reduced ERK phosphorylation but did not affect the amounts of phospho-MARCKS. This is quite astonishing since ROS-formation upon fMLP stimulation observed by Feißt et al. depends on activation involving PKC and was obviously inhibited by MC [18]. Hence, MC must clearly affect downstream events of PKC which then lead to decreased ROS-formation by NADPH oxidase or there are other possibilities aside from PKC to activate ROS.

If MC does not affect upstream signaling of PKC then how is the PLC and $IP_3$-dependent release of $Ca^{2+}$ from intracellular stores inhibited by MC? MC could possibly exert direct effects on release of $IP_3$ from the plasma membrane or is able to affect membranes and membrane associated channels in $Ca^{2+}$ storing organelles.

It could be excluded that MC specifically inhibits the FPR receptor itself by testing MC in cells stimulated with other GPCR ligands. $LTB_4$- , AA- and 5-

HETE-increased ERK phosphorylation could also be inhibited by MC in similar manner as in fMLP-stimulated cells. AA is converted by 5-LO to 5-HpETE and further reduced to 5-HETE within the cell. 5-HETE then leads to ERK activation in a pertussis sensitive way [215]. This experiment should also prove that inhibition of AA-induced ERK activation by MC is not due to inhibition of the 5-LO. BWA4C, a selective inhibitor for 5-LO, could prevent 5-LO-induced ERK activation but was not able to affect the activation of the pathway by 5-HETE. MC could inhibit both, AA- and 5-HETE-induced ERK phosphorylation, indicating that MC is obviously affecting 5-HETE signaling but not its synthesis.

It was postulated that the activation of the MAPK pathway by fMLP functions via $G_i$ proteins leading either to activation of phospholipases, PKC and activation of Ras, Raf or MEK by PKC or by direct interaction of the G protein with Ras [158]. Furthermore, a cross activation of tyrosine kinases by G proteins which then would lead to activation of Ras by Sos, the classical GEF of RTK signaling, appeared possible. The latter seemed an attractive target since it has been published that phosphorylation of tyrosine residues plays a considerable role on programmed cell death. Whether this apoptosis induction is due to inhibition of tyrosine kinases [216, 217], or due to activation [218, 219] is still discussed and also may depend on cell types and cell states. To check the possibility if MC was able to interfere with cross-activation from GPCRs to tyrosine kinases, protein tyrosine phosphatase activity was determined and the phosphorylation state of tyrosine residues was checked. Rather than increasing PTPase activity in PMNL, MC slightly reduced PTPase activity concentration- and time-dependently resulting in increased phosphorylation of tyrosine residues. In view of the time dependency of inhibition of ERK and MEK phosphorylation by MC in PMA-stimulated cells, it is interesting that MC did not totally abolish phosphorylation but rather led to a delay in activation and an attenuation of activation/phosphorylation intensities. Again, phosphorylation of MARCKS was rather slightly increased by MC over the time period monitored. Hence, interaction of proteins downstream of PKC might be hindered but not totally inhibited by MC.

The downstream events of fMLP stimulation leading to ERK phosphorylation are thought to involve Ras and Raf [158]. The next logical step was to evaluate if Ras

activity stimulated by fMLP and by PMA was affected by MC. MC led to a reduction of Ras-GTP in PMNL stimulated with either stimuli starting at concentrations as low as 0.3 and 1 μM. Note that phosphorylation of c-Raf on serine residue 338 was diminished by MC although not as strongly as the effect on Ras. Phosphorylation of the activatory site of B-Raf (Ser 445) was not affected by MC. Thus, it can be stated that the effect of MC on the MAPK cascade in PMA- and fMLP-stimulated PMNL must be at the level of Ras activation. Either MC hinders activation of Ras by the receptor or the respective upstream signaling events or MC possibly has a stimulating effect on the intrinsic GTPase activity of Ras.

Phosphorylation of MEK1/2 is the subsequent event after Raf activation and was also inhibited by MC. Nevertheless, one interesting difference between fMLP- and PMA-stimulated PMNL was observed: phosphorylation of ERK 1/2, MEK 1/2 and c-Raf in PMA-stimulated PMNL is not as strongly affected by MC as in fMLP-stimulated cells. Phosphorylation of ERK and MEK in fMLP-stimulated PMNL is totally abolished with 30 μM MC whereas in PMA-stimulated cells a residual phosphorylation remains, concordant with the phosphorylation state in fMLP-treated cells after treatment with only 3 μM MC. This implies the involvement of more than one possible pathway leading from PKC to MEK and ERK activation, whereas only one pathway is affected by MC (see Fig. Possible pathways affected by MC in PMA- and fMLP-stimulated PMNL).

It was found before that indeed PKC activation is not necessary for Raf activation by fMLP [158] and that fMLP-induced ERK activation does not depend on PKCγ [220]. So PKC which was unaffected by MC is probably not involved in fMLP-stimulated MAPK signaling.

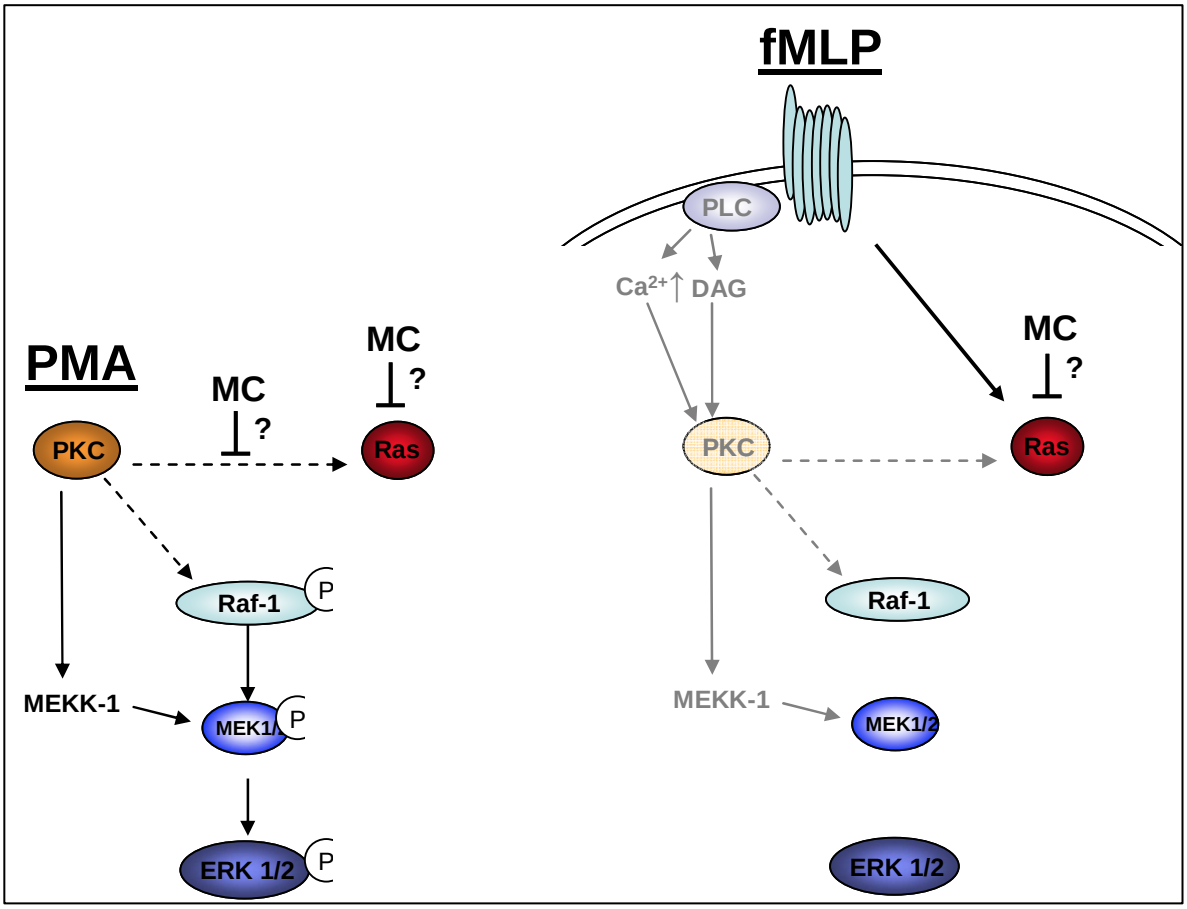



***Fig. Pathways possibly affected by MC in PMA- and fMLP-stimulated PMNL.*** *(parts of lower importance are drawn translucent) This figure tries to explain the differences of MC effects in PMA- or fMLP-treated cells.* ***PMA*** *activates PKC which in turn activates Ras, Raf or MEK. MC possibly inhibits initial activation or increases GTPase activity of Ras leading to decreased amounts of Ras-GTP. This way, signaling to Raf is abolished. Activation of Raf and MEK by PKC is not inhibited by MC, thus explaining the residual phosphorylation of Raf, MEK and ERK as observed in the present study (further explanations see text).* ***fMLP*** *activates the FPR1 leading to Ras activation. Here MC cannot inhibit the nucleotide exchange in Ras, but probably increases the GTPase activity rendering Ras inactive. Since PKC signaling plays no or only a minor role in MAPK activation (see text), the inactivation of Ras by MC leads to a total decrease in Raf, MEK and ERK phosphorylation.*

## 16.2 *Effects of MC on signal transduction in HL60 cells*

After elucidating the inhibitory effects on ERK phosphorylation by MC in PMNL the first question was if this could also be observed in HL60 cells, if these effects persisted over the time (16 hours) and if this led to decreased (inhibitory) phosphorylation of BAD, the proapoptotic Bcl2 protein. HL60 cells show massive overactivation of the Ras/Raf/MEK/ERK cascade due to a mutation of N-Ras which impairs the intrinsic GTPase activity of the small G protein [129, 221].

This leads to strong activation and phosphorylation of downstream targets. One of these targets is the proapoptotic Bcl2 protein BAD which is bound to 14-3-3 proteins and located in the cytosol when phosphorylated, being unable to induce apoptotic events on mitochondria [222]. If ERK phosphorylation is diminished by MC in HL60 cells also BAD phosphorylation should be reduced rendering the protein able to translocate to mitochondria and thus to induce apoptosis. It was clearly shown that Bcl2 overexpression did not prevent cytochrome c release, loss of $\Delta\Psi_m$ or apoptosis induction induced by MC excluding the participation of Bcl2 proteins to the apoptosis inducing effect. Regarding the long-term effect in HL60 cells, MC reduced ERK and MARCKS phosphorylation while inducing PARP cleavage and apoptosis. By inhibiting caspases with the pan caspase inhibitor QVD, this effect could be reversed. Long-term inhibition of ERK and MARCKS phosphorylation might therefore depend on caspase activity.

In short-term experiments, similar to the ones conducted in PMNL, neither fMLP nor PMA stimulation of HL60 cells gave a significant additional activating effect on the kinases analysed. Phosphorylation states, especially of ERK and p38 MAPK, were quite high. Nonetheless, MC showed a considerable decrease in phosphorylation of p38 MAPK, ERK and a slight decrease in PKB/Akt activation. JNK/SAPK and MARCKS were unaffected by the MC concentrations used. Interestingly, 2 μM staurosporine could inhibit phosphorylation of the PKC substrate MARCKS but did not show an effect on any other kinases.

If fMLP and PMA stimulation showed no increase in signaling events, how does MC influence signaling cascades? Unstimulated HL60 cells were pretreated with MC and it was observed that independently of any stimuli MC could decrease ERK phosphorylation. To further elucidate if tyrosine kinases and phosphatases were influenced by MC the effects of MC on PTPases in HL60 was assessed. There was no significant effect of MC observed in HL60 cells in this respect. Obviously, the effect of MC in HL60 cells hinders MAPK signaling independently of any receptors. Has MC a direct target in the MAPK cascade? We tested whether Ras-Raf or Raf-MEK interactions are targeted by MC directly. Suprisingly neither Ras, nor c-Raf and MEK 1/2 are inhibited by the concentrations of MC tested in HL60 cells. From this we can conclude that the influence on Ras, c-Raf and MEK 1/2 seen in PMNL is not observed in HL60

cells and therefore is negligible for the inhibitory effect of MC on ERK phosphorylation. When PMNL are treated with the non-hydrolysable GTP analogue GMP-PNP, incubated with MC and stimulated with fMLP, MC is no longer able to decrease amounts of Ras-GTP (data not shown) in PMNL. By treatment with GMP-PNP, Ras in PMNL is no longer able to process GTP to GDP by GTPase activity. This mimics the mutational change found in HL60 where GTPase activity of Ras is impaired. The results for MC in these cells imply that MC does not inhibit loading of Ras with GTP (induced by fMLP) but rather stimulates GTPase activity of Ras in PMNL. This process is not applicable to HL60 cells due to the Ras mutation. Nonetheless, ERK phosphorylation in HL60 is significantly reduced by MC although Ras seems unaffected. Even though Ras is affected in PMNL, this cannot be the sole reason for decreased ERK phosphorylation. To activate ERK, a complex consisting of Ras, Raf, MEK as well as the scaffolding proteins KSR and CNK is formed [171, 172]. Another possible mode of action of MC could be the prevention of transmitting the signal from the Ras/Raf/MEK complex towards ERK.

Although the mechanism by which MC reduces ERK phosphorylation could not totally be elucidated, it is clear that: a) MC probably does not affect the FPR directly since in unstimulated HL60 cells there was still a significant decrease in phospho-ERK, b) all pathways upstream of PKC ($G_{i\beta\gamma}$, PLC, DAG release) are unaffected since phospho-MARCKS is not reduced by MC in fMLP-stimulated cells, c) MC has an effect on fMLP-induced $Ca^{2+}$ release from intracellular stores, possibly by interacting with $IP_3$-receptors or by interacting with organelle membranes directly [18], d) since Ras, Raf and MEK 1/2 activity in HL60 cells is not influenced by MC, upstream events of this cascade (including MEKK1) are probably not involved in the inhibitory effect of MC on ERK, e) MC possibly inhibits transmission of the signal from the Ras/Raf/MEK complex towards ERK or f) MC might target ERK directly (see Fig. Comparison of MC effects in PMNL and HL60 cells).

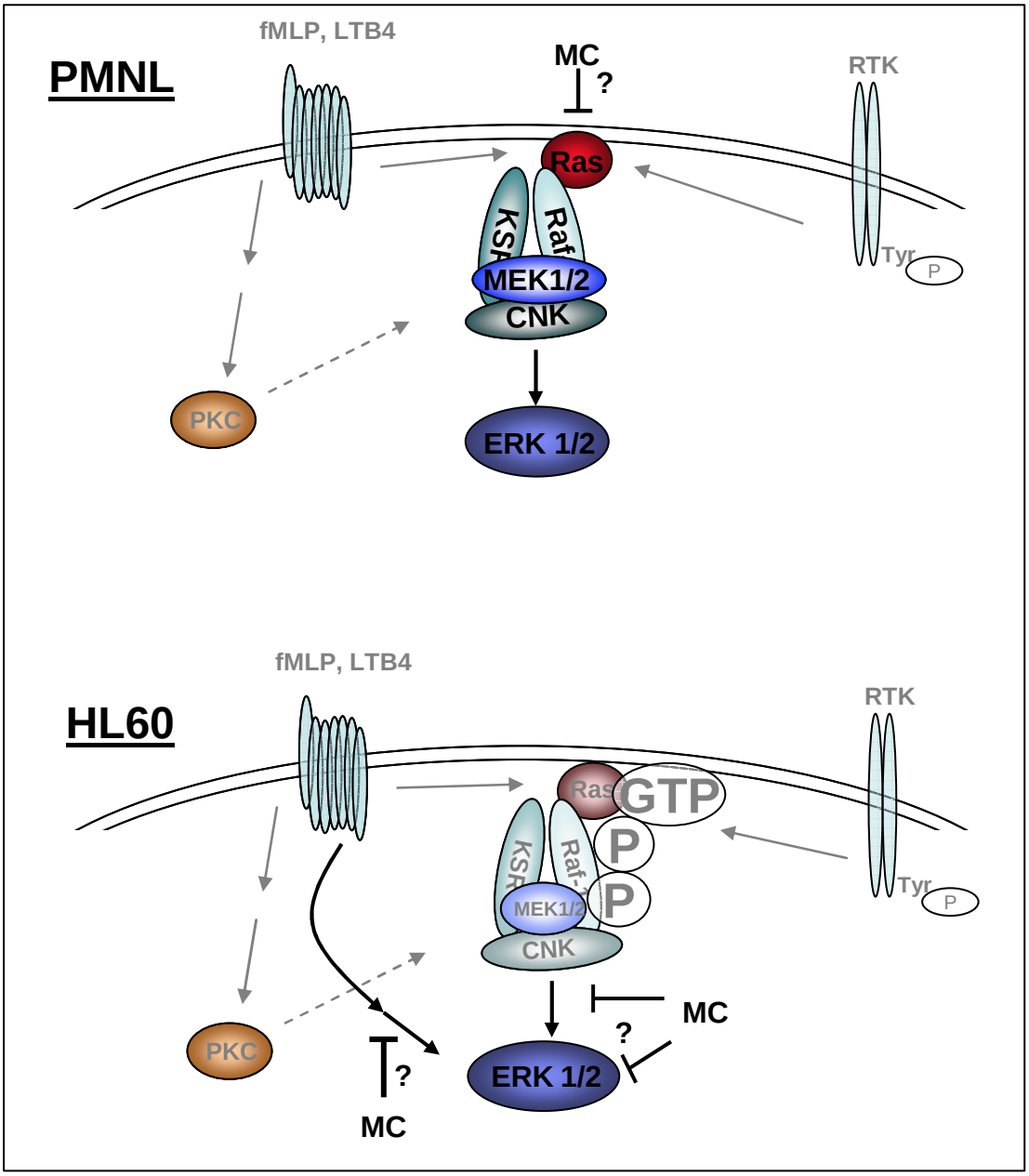



***Fig. Comparison of MC effects in PMNL and HL60 cells.*** *(parts of lower importance are drawn translucent) In* ***PMNL****, MC inhibited activation of Ras, Raf, MEK and ERK, independently of PKC or RTKs. Whereas in* ***HL60****, Ras, Raf and MEK seemed unaffected by MC although phosphorylation of ERK was abolished by MC as strongly as in PMNL. Here, MC probably inhibits signaling from the Ras/Raf/MEK complex towards ERK, inhibits ERK directly or affects an alternative pathway leading directly from the GPCR to ERK (further explanations see text).*

The activation of ERK can lead to manifold events within the cell. ERK alters gene expression by activation of transcription factors such as Elk-1, c-Myc and others [223] which lead to expression of growth factors [224, 225], anti-apoptotic proteins (e.g. Bcl-Xl) [226], proteins influencing cell cycle progression [227] and inhibition of the expression of pro-apoptotic genes [227, 228]. Furthermore, it was

shown that LPS-induced chemotaxis in neutrophils is regulated by the coordinated action of p38 MAPK and ERK 1/2 [229]. And indeed, it was found that MC could inhibit chemotaxis in PMNL induced by fMLP (unpublished data, A. Henkel, Tübingen).

ERK, aside from other signaling pathways (such as SAPK/JNK or PKB/Akt), can activate NFκB involving IκB kinase and IκB [230]. MC reduced phosphorylation of NFκB concentration-dependently in IL-1β- and TNFα-stimulated PBMC as well as in unstimulated cells (data not shown). Additionally, the transriptional activity of NFκB, as assayed by luciferase assay in chronic myeloid leukemia K562, was inhibited by MC with an $IC_{50}$ of 3.0 ± 0.3 μM (unpublished data, M.Schumacher, Luxembourg).

# 17 Conclusion

Stimulation of neutrophils with chemoattractants such as fMLP is critical to establishing an acute inflammatory response mediated by upregulation of signaling pathways leading to activation of e.g. ERK and NFκB. ERK was shown to directly phosphorylate and activate key enzymes of eicosanoid biosynthesis as for example 5-LO [231] or $cPLA_2$ [232] and thus stimulating production of further mediators of inflammation such as leukotrienes and prostaglandins. But also gene expression is upregulated by activation of these signaling pathways. NFκB is an important transcription factor e.g. for expression of genes for COX-2 and IL-6. By inhibiting signaling cascades, the events leading to innate immune response and the expression and release of further immune stimulative substances such as interleukins can be effectively inhibited. In many diseases the signals from chemoattractants leading to neutrophil responses are of considerable importance and inhibition of the signaling pathways has a beneficial effect. Our present data support that MC exerts potent anti-inflammatory properties by inhibiting activation of neutrophil signal transduction pathways.

Furthermore these pathways are also closely connected with the development of cancer. HL60 cells display cancerogenous properties by overactivation of the Ras/Raf/MEK/ERK cascade and elevated gene expression mediated by c-Myc, leading to increased cell proliferation. Overactivation of NFκB by stimulation of

macrophages or T-lymphocytes with IL, endotoxins like LPS and TNFα not only triggers inflammatory responses (IL-1, -6, -8 and TNFα release), leading to a positive stimulation feedback loop towards NFκB, but also, if downregulation of the NFκB activity fails, chronic inflammation and cancer can be the outcome [233]. By inhibiting signaling pathways that might initially lead to chronic inflammation and then, by inducing cellular and DNA damage, might promote cancer, MC might be a potential preventive drug for cancer.

Not only preventive effects of MC on cancer development can be reasoned. As could be shown in the present investigation, MC effectively and selectively induces apoptosis in cancer cells. It can be speculated that changes in gene expression due to the inhibition of signal pathways in cancer cells might lead to induction of apoptosis by MC.

Taken together, MC proves to have the properties to be an effective treatment for inflammation and cancer and although many questions about its exact mode of action still have to be resolved, it will be interesting to follow results concerning MC effects in vivo in animal and clinical trials.

# Summary

# 18 Summary

Extracts of the mediterranean plant myrtle (*myrtus communis*) have a long tradition in folk medicine as a remedy for the treatment of infections and inflammatory diseases. Over the past decade, many scientific publications could support and rationalise the therapeutic use of myrtle preparations. In search for single components responsible for the beneficial effects, myrtucommulone A (MC), a non-prenylated acylphloroglucinol, was isolated from myrtle leaves and was shown to have strong anti-inflammatory properties. Anti-inflammatory drugs often show beneficial effects on development or progression of cancer. Therefore, it was reasonable to test whether MC might induce apoptosis and to evaluate its potential for treatment of cancer and especially leukaemia.

## 18.1 *MC induces apoptosis in cancer cells*

In the course of this study it could be shown that MC proved effective in inducing apoptosis in several leukaemia cell lines with marginal cytotoxicity for non-degenerated blood cells. Aside from caspase cleavage, PARP cleavage and DNA fragmentation, loss of the mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome c release were the main features in MC-induced apoptosis.

Furthermore, it was observed that caspase 9 and the intrinsic way was essential for apoptosis induction by MC, whereas caspase 8 and other components of the extrinsic pathway could be excluded as relevant mediators. The cause for cytochrome c release and activation of caspase 9 was independent of Bcl2 proteins and of the opening of the mitochondrial apoptosis inducing channel or of the opening of the permeability transition pore. Aside from its apoptosis-inducing effect, MC reduced the numbers of viable cells in caspase 9-deficient Jurkat cells, although apoptosis was clearly abrogated. This effect was not related to necrosis but rather due to G2 phase arrest induced by MC. We conclude that MC may affect cell metabolism and signaling pathways, slowing growth of cancer cells compared to non-degenerated cells due to differences in activation of metabolic pathways and gene expression.

## 18.2 *MC inhibits signaling pathways*

To elucidate the effects of MC on signal transduction pathways in PMNL and to check if similar effects are obvious in cancer cells, the phosphorylation states of several kinases in PMNL and HL60 cells were assessed. MC concentration dependently inhibited phosphorylation of ERK, p38 MAPK and Akt/PKB in leukaemic HL60 promyelocytes and in non-degenerated human PMNL. Whereas MC reduced activation of Ras, Raf and MEK upstream of ERK in PMNL, this pathway was unaffected by MC in HL60 cells. The effects of MC were also independent of PKC, since phosphorylation of the direct PKC substrate MARCKS was not affected. Although in PMNL MC blocked kinase activation following simulation with fMLP, PMA and other stimuli, in HL60 cells no stimulation was needed to see a strong inhibitory effect by MC on ERK phosphorylation. This influence of MC on especially ERK and p38 MAPK implies consequences on NFκB transcriptional activity in K562 chronic myeloid leukaemia cells and on fMLP-induced chemotaxis in PMNL.

In conclusion, MC exhibits potent and manifold effects on signaling pathways involved in inflammation and apoptosis induction. Thus, MC has the potential as an effective and selective therapeutic to treat inflammation

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# 19 References


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# Appendix

# 20 Akademische Lehrer

Im Laufe meiner akademischen Ausbildung haben mich folgende akademischen Lehrer unterrichtet/betreut:

| | |
|---|---|
| Prof. Dr. E. Bade | Cell and Tumor Biology |
| PD Dr. C. Bechinger | Physik |
| Prof. Dr. Beste | Experimentalphysik |
| Prof. Dr. E. Bohl | Mathematik und Statistik |
| Prof. Dr. W. Boos | Mikrobiologie |
| Prof. Dr. P. Böger | Plant Physiology and Plants |
| Prof. Dr. D. Brdiczka | Biochemisrty and Energy Metabolism |
| Dr. R. Bretthauer | Zoologische Bestimmungsübungen |
| Prof. Dr. A. Bürkle | Molekulare Toxikologie |
| Prof. Dr. A. M. Cook | Mikrobiologie |
| PD Dr. M. Deicher | Physik |
| Prof. Dr. P Fisher | Mikrobiology |
| Prof. Dr. S. Ghisla | Biologische Chemie |
| PD Dr. T. Hartung | Medizin |
| PD Dr. Hellmann | Pflanzenbestimmungsübungen |
| Prof. Dr. H.W. Hofer | Enzyme Chemistry |
| PD Dr. J.H. Kleinschmidt | Membranbiophysik |
| Prof. Dr. R. Knippers | Genetik |
| Prof. Dr. P. Kronek | Bioanorganische Chemie |
| Prof. Dr. W. Kutsch | Neuroethology |
| Prof. Dr. D. Malchow | Entwicklungsbiologie |
| Prof. Dr. D. G. Müller | Botanik |
| Dr. W. Nagl | Statistik |
| Prof. Dr. D. Pette | Physiological Chemistry |
| Prof. Dr. H. Plattner | Zellbiologie |
| Prof. Dr. W. Rathmayer | Zoologie |
| Prof. Dr. B. Schink | Limnologie, Mikrobielle Ökologie |

| | |
|---|---|
| Prof. Dr. G. Stark | Membranbiophysik |
| Prof. Dr. C. Stürmer | Entwicklungsneurobiologie |
| Prof. Dr. V. Ullrich | Biochemistry |
| Prof. Dr. W. Welte | Biophysik und Strukturbiologie |
| Prof. Dr. O. Werz | Pharmazeutische Chemie |
| Prof. Dr. S. Wesselborg | Innere Medizin |

# 21 Curriculum vitae

## Dagmar Blaesius

**Persönliche Information**

Familienstand: ledig
Staatsangehörigkeit: deutsch
Geburtsdatum: 18.11.1977
Geburtsort: Saarbrücken
Eltern: Helga Blaesius (geb. Mühlberger), Lehrerin
Hans-Jürgen Blaesius, Polizeibeamter im Ruhestand

**Werdegang**

***1984 – 1988*** Grundschule Lasbach Quierschied

***1988 – 1997*** Theodor-Heuss-Gymnasium Sulzbach

- Abitur, Abschlußnote 1,6

***1997 – 1998*** Tätigkeit als Au Pair Memphis, USA

***1999*** Universität des Saarlandes Saarbrücken

***1999 – 2004*** Universität Konstanz Konstanz

- ***Januar 2002 – Juni 2002*** Forschungsaufenthalt, La Trobe University, Melbourne, Australien bei Prof. Dr. Paul Fisher

- Abschluß: **Diplomarbeit** über das Thema **„Die Rolle der 6-Phosphogluconat-Dehydrogenase sowie der Trehalose-6-Phosphat Hydrolase in der Regulation des Maltosesystems von *Escherichia coli*“,** Diplom Biologie Abschlußnote 1,5

***Oktober 2005 – voraussichtlich September 2009***
Eberhard-Karls-Universität Tübingen

- Abschluß: **Promotion** über das Thema **„Klärung der molekularen Mechanismen der apoptotischen Wirkung des Myrtucommulons aus *Myrtus communis“*** in der Arbeitsgruppe von Prof. Dr. O. Werz, Pharmazeutisches Institut

**Wissenschaftliche Referenzen**

**Lesmüller-Posterpreis** der Deutschen Pharmazeutischen Gesellschaft im Rahmen der DPhG-Jahrestagung in Marburg, 2006

**Vortrag DPhG Jahrestagung**, Erlangen, 2007

**Vortrag PSE conference** „Natural products in cancer therapy“, Neapel 2008

**Einladung** zum Vortrag bei Herrn Dr. Marc Diederich, Laboratoire de biologie moléculaire et cellulaire du cancer (LBMCC), Luxemburg

## Publikationen

**Schaloske RH**, **Blaesius D**, **Schlatterer C**, **Lusche DF**.; **Arachidonic acid is a chemoattractant for Dictyostelium discoideum cells.,** J Biosci. 2007 Dec;32(7):1281-9.

**Tretiakova I***, **Blaesius D***, **Maxia L**, **Wesselborg S**, **Schulze-Osthoff K**, **Cinatl J Jr**, **Michaelis M**, **Werz O**.; **Myrtucommulone from Myrtus communis induces apoptosis in cancer cells via the mitochondrial pathway involving caspase-9.,** Apoptosis. 2008 Jan;13(1):119-31.

(* contributed equally)

**spezielle Qualifikationen**

MS Office (Word, Excel, PowerPoint)

**Sprachkenntnisse**

Englisch (verhandlungssicher in Wort und Schrift)

Französisch

**Sonstige Interessen**

Joggen, Tanzen, Singen, Wandern, Reisen, Tennis, Klavier, Querflöte