

**Treatment of a mantle cell lymphoma cell line with  
cannabinoids and cytostatics  
- effects on DNA synthesis and ceramide metabolism**

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## **Abstract**

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy with bad prognosis, which predominates in males with advanced age. However, studies of the endocannabinoid system and how it affects tumour behaviour provides the basis for designing innovative therapeutic strategies that could open new opportunities for treatment of patient with MCL. It has earlier been shown that the cannabinoid receptor ligand (R)-(+)-methanandamide (R-MA) induce cell death in MCL by accumulation of ceramide. Ceramide has a pro-apoptotic effect on the cell but could be metabolized by the enzymes glucosylceramide synthase (GCS) and sphingosine kinase 1 (SphK1) to molecules with pro-proliferative effect. Therefore, treatments with R-MA on Jeko-1 MCL cell line were performed in this study to determine interference in the proliferative behaviour as well as in the gene expression of the enzymes GCS and SphK1. In addition, treatments with chemotherapeutic substances, such as doxorubicin or cytarabine (Ara-C), and combinations of R-MA and chemotherapeutic substance, were performed for the same reason. Results showed that the proliferation behaviour of Jeko cells remained unaffected when treated with R-MA, in contrast to the decreased proliferative effects shown when treated with cytostatics or combinations of R-MA and cytostatics. Furthermore, a tendency for up-regulation of GCS and SphK1 expression was recognized when cells were treated with cytostatics or combination of cytostatics and R-MA, in contrast to cells treated with R-MA alone. Although, R-MA alone had a tendency for a small down-regulation of GCS expression, it contributed to a potential elevation of GCS expression when combined with Ara-C or doxorubicin. It is believed that the effect from up-regulated levels of the metabolizing enzymes GCS and SphK1 is balanced by, earlier observed, up-regulations of the ceramide synthesis enzymes.

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## **Introduction**

### ***Mantle cell lymphoma***

Mantle cell lymphoma (MCL) is a highly aggressive type of blood cancer that begins in a B-lymphocyte (white blood cell) and progresses usually to a lymph node or occasionally to other parts of the lymphatic system [1]. This tumour comprises 5-10% of all non-Hodgkin lymphomas, and predominantly affects elderly males [2]. Even though most patients respond well to initial chemotherapy, relapses are still common. It carries a poor prognosis with a median survival of approximately 3 years and only a few patients have been cured with current therapies [3].

MCL is characterized by the histological growth pattern that either can be nodular, spread out or more usually a tumour cell infiltration in the outer edge of a lymph node follicle, called the mantle zone [4]. An additional characteristic, which also is crucial for diagnosis, is the chromosomal alteration with the particular translocation t(11;14)(q13;q32) that leads to an overexpression of the cyclin D1 protein, a cell cycle regulator that is normally not expressed in B-cells. Cases that are cyclin D1 negative exist but these cases display substitutional up-regulated levels of cyclin D2 or D3 instead. The deregulated expression of cyclins D is accompanied by other genetic alterations in order to drive the tumour progression. For example, deletions in the 11q22-23 chromosome region where the encoding gene is located for a phosphoprotein kinase—a protein with a central role in the response to DNA damage in the cell, where its inactivation may contribute to genomic instability [5]. This implies that genetic alterations are involved in the pathogenesis in MCL and the understanding of their consequences is essential to new therapeutic strategies. Some of the therapies used to manage mantle cell lymphoma to date are radioimmunotherapy, proteasome inhibitors, stem cell transplantation and the conventional chemotherapy [1]. Since high doses of chemotherapy are often required and due to the fact that the risk group is elderly, who do not manage tough chemotherapies or stem cell transplantations, there is a need for less heavy treatments. Therefore, one of our groups' interests would be to decrease the chemotherapeutic drug concentration in treatment of MCL, by combining chemotherapeutic substances and cannabinoids—molecules targeting the endocannabinoid system to induce apoptosis in the tumour cells.

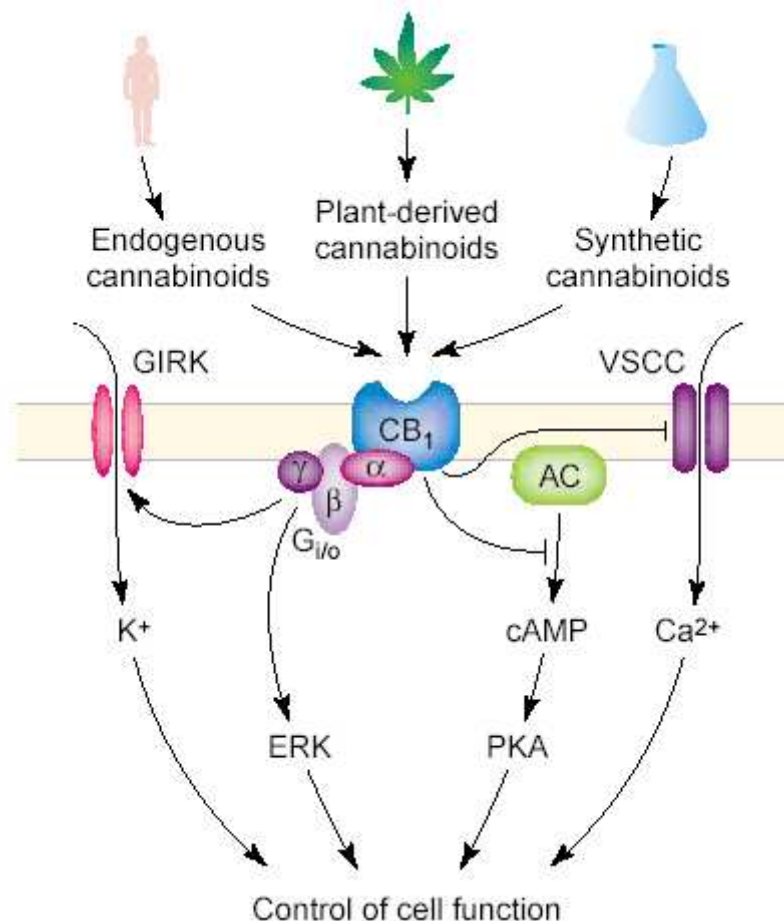
### ***Endocannabinoid system***

The endocannabinoid system (ECS) consists of cannabinoid receptors, their ligands that originate within the cell (endogenous), known as endocannabinoids, and of endocannabinoid anabolic and metabolic enzymes.

The endocannabinoids got their name as they first were identified as ligands to the same receptors as the primary components of cannabis, so-called cannabinoids. Cannabis, also known as marijuana or ganja, is the psychoactive product from the cannabis plant (*Cannabis sativa*) and due to attempts to understand the effect of  $\Delta^9$ -tetrahydrocannabinol (THC), the natural plant cannabinoid and the main psychotropic constituent of cannabis, led to the discovery of cannabinoid receptors (CB<sub>1</sub> and CB<sub>2</sub>) [6, 7]. The ECS is present in mammalian tissue and regulates several functions, usually with suppressive effects, in many of the body's organ systems [8]. However, interference with ECS can interestingly display anti-proliferative, pro-apoptotic effects on cancer stem cells, anti-angiogenic activity, reduced migration and anti-metastatic activity—all essential in cancer development [8-10].

## Cannabinoid receptors

Mammalian tissues express at least two cannabinoid receptors, CB<sub>1</sub> and CB<sub>2</sub>, both composed of seven transmembrane domains and are part of a G-protein superfamily. The distinction between them is their distribution in the mammalian tissue, among other things. Whereas the CB<sub>1</sub> receptor is found predominantly in the central nervous system (CNS), with a suppressive effect on the neurotransmission, CB<sub>2</sub> is mainly found in the immune system, with a suppressive effect in cytokine release [6, 8, 11, 12]. The receptors also participate in the regulation of cell survival [11]. Both receptors principally signal intracellularly through the inhibitory G<sub>i/o</sub> proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinases (MAPK) [8, 12]. The pathway for the particular cannabinoid receptor CB<sub>1</sub> can be displayed by inhibition of adenylate cyclase, causing lower levels of cAMP in the cell and decreased activity of protein kinase A that, in turn, leads to activation of potassium channels. Direct cause of the G<sub>i/o</sub> activation by CB<sub>1</sub> is calcium channel inhibition and potassium channel activation [12]. Furthermore, the CB<sub>1</sub> receptor activates the extracellular signal-regulated kinase (ERK) cascade [11] (see figure 1). CB<sub>2</sub> has a more variable modulation of ion channels [6].



*Figure 1.* Stimulation of CB<sub>1</sub> precedes its control of cell functions via several different means. By the G-protein coupled signalling via CB<sub>1</sub> alters the activity of G-protein-activated inwardly rectifying K<sup>+</sup> channels (GIRK) and voltage sensitive Ca<sup>2+</sup> channels (VSCC). In addition, activation of mitogen-activated protein kinase ERK cascade and the inhibition of adenylate cyclase would also provide control of the cell functions. Illustration taken from Guzman, M., I. Galve-Roperh, and C. Sanchez, *Ceramide: a new second messenger of cannabinoid action*. Trends Pharmacol Sci, 2001. 22(1): p. 19-22.

There is data [13-15] suggesting that stimulation of cannabinoid receptors could be a novel approach to treating MCL considering overexpression of these two receptors in this particular disease compared with normal tissue. Stimulation of both the receptors induces decreased viability, growth suppression and cell death by apoptosis in MCL cells, in contrast to normal B-cells [16]. Normal B-cells would be spared due to the inconsiderable expression of the CB<sub>1</sub> and the requirement of the ligation of both CB<sub>1</sub> and CB<sub>2</sub> to cause the desirable effects [17].

## Cannabinoid receptor ligands

Natural as well as endogenous and synthetic cannabinoids are proper candidates for ligation to cannabinoid receptors. The “classical” cannabinoid,  $\Delta^9$ -tetrahydrocannabinol (THC) was recognized as a potential anti-cancer agent already in 1975 [8]. Around twenty years after this announcement were the first endocannabinoids, arachidonoyl-ethanolamide (anandamide) and 2-arachidonoyl-glycerol (2-AG), discovered with the promising suppressing effects in ongoing release of chemical messengers. Thereafter, the anandamide analog (R)-(+)-methanandamide (R-MA) was synthesized. It is selective for CB<sub>1</sub> and metabolically more stable, probably because of the presence of the methyl group attached to 1' carbon [18] (see figure 2).

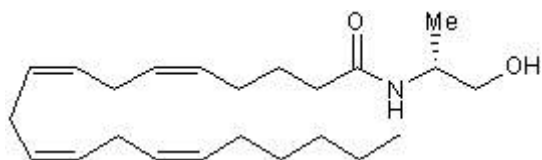


Figure 2. Molecular structure of (R)-(+)-methanandamide (C<sub>23</sub>H<sub>39</sub>NO<sub>2</sub>). Drawing taken from <http://www.tocris.com/dispprod.php?ItemId=2050>

The R-MA affinity for CB<sub>1</sub> is four times greater than that of anandamide [19] which is one of several important features as a potential anti-cancer therapy agent. R-MA has been found to induce programmed cell death in mantle cell lymphoma cell lines by direct binding to cannabinoid receptors consequently affecting signalling events and cellular pathways. Ligation of R-MA to both CB<sub>1</sub> and CB<sub>2</sub> is required to mediate apoptosis via accumulation of ceramide, phosphorylation of P38, loss of mitochondrial membrane potential, cytochrome c release, and caspase activation [10, 17] (see figure 3).

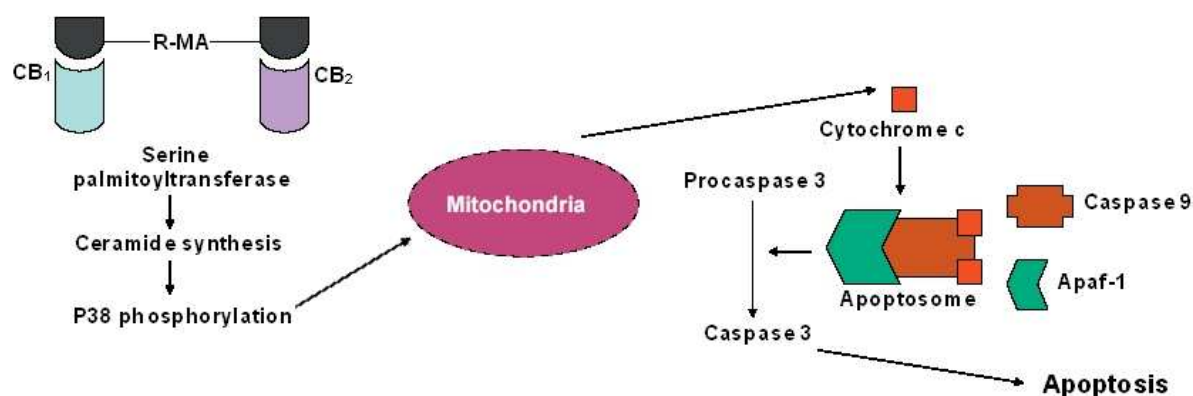


Figure 3. Stimulation to both the receptors by R-MA contributes to a pathway with apoptosis as the terminal effect. This final behaviour is established briefly due to de novo ceramide synthesis, P38 phosphorylation, depolarization of the mitochondrial membrane, and caspase activation. Illustration modified from: Rieder, S.A., et al., *Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression*. Immunobiology, 2009. Article in press.

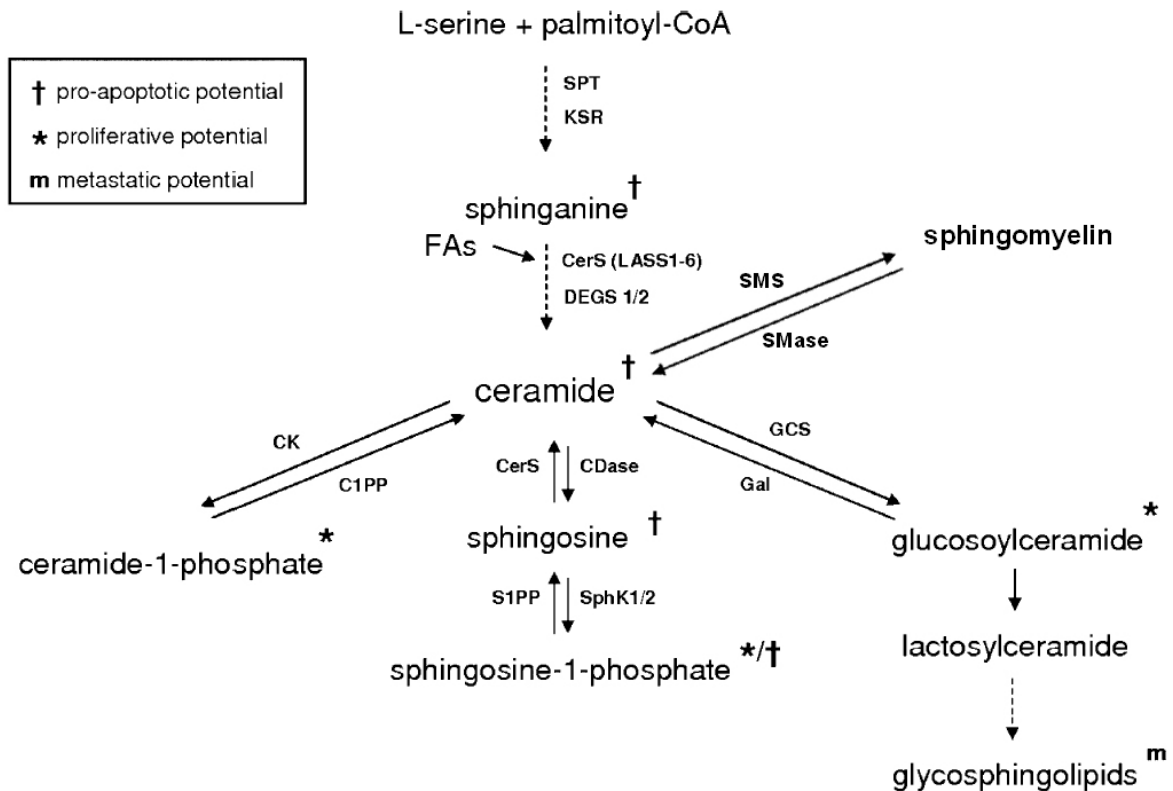
In view of the observed modulations in cell pathways, it may possibly be a therapeutic strategy to treat cancer types expressing cannabinoid receptors with ligands to the receptors. Cannabinoid action couples CB<sub>1</sub>, besides to G<sub>i/o</sub> proteins, to the generation of sphingolipid ceramide via two distinct pathways: sphingomyelin hydrolysis and ceramide synthesis de novo, described in the part sphingolipids [20].

### ***Sphingolipids***

Sphingolipids are structural components of biological membranes and composed, like phospholipids, of a polar head group and two nonpolar tails. The family sphingolipids is composed by variety of derivatives converted from the basic building block sphingosine. Some sphingolipids are bioactive molecules, chiefly ceramide, that are involved in the regulation of various cellular processes linked to cancer pathogenesis and therapy, including apoptosis, cell proliferation, cell migration, senescence, or inflammation [21].

Ceramide is the fundamental structural unit common to all sphingolipids. It works as a second messenger which actively participates in the initiation of cell death pathways [22]. A variety of stress factors associated with, for example, chemotherapeutic agents or cannabinoids promotes ceramide production through de novo synthesis or alternatively by sphingomyelin breakdown, in various of cells [14] (see figure 4). Several enzymes participates in the de novo synthesis, initially L-serine palmitoyl-CoA transferase (SPT) that condense L-serine and palmitoyl-CoA to form 3-ketosphinganine, at the endoplasmic reticulum. Followed by 3-ketosphinganine reductase (KSR) reducing 3-ketosphinganine to sphinganine which, in turn, acylates and oxidise to ceramide via dihydrocyamide by the enzymes (dihydro)ceramide synthases (CerS) and dihydroceramide desaturase (DEGS) respectively [21-24]. There are six species of ceramide synthases (CerS1-6) that provide varying chain length to ceramide, while those ceramides with C16 to C24 fatty acids are the most common [21, 22].

Several possible intracellular metabolic pathways for ceramide have also been proposed. Once ceramide is synthesized, it can be rapidly metabolized into sphingomyelin by sphingomyelin synthase (SMS), Ceramide-1-phosphate by ceramide kinase (CK), glucosylceramide by glucosylceramide synthase (GCS), or sphingosine by ceramidase (CDase) [14, 21, 23]. All these pathways are recognized as reversible and therefore also lead to ceramide formation, whereas hydrolysis of the complex lipid sphingomyelin by sphingomyelinase (SMase) was the mentioned alternative way to ceramide synthesis. Glucosylceramide and sphingosine can be further converted to complex glycosphingolipid (GSL) and sphingosine-1-phosphate (S1P), respectively [14]. Another important source of ceramide is provided by the breakdown of glycosphingolipids [23]. S1P conversion takes place through phosphorylation of sphingosine by one of two sphingosine kinases, type 1 (SphK1) or type 2 (SphK2) [23]. The product from SphK1 is S1P which has a proliferative potential like glucosylceramide and ceramide-1-phosphate. In contrast, SphK2 gives rise to a S1P form with a pro-apoptotic potential like the precursors sphingosine, ceramide and sphinganine (see figure 4) [21, 24].



*Figure 4.* Ceramide anabolism and metabolism pathways. Abbreviations: SPT, L-serine palmitoyl-CoA transferase; KSR, 3-ketosphinganine reductase; CerS, Ceramide synthase; FAs, fatty acids; DEGS, dihydroceramide desaturase; CK, ceramide kinase; C1PP, ceramide-1-phosphate phosphatase; CDase, ceramidase; S1PP, sphingosine-1-phosphate phosphatase; SphK1/2, sphingosine kinase type 1 / type 2; Gal, galactosidase; GCS, glucosylceramide synthase. Sketch modified from: Schiffmann, S., et al., *Ceramide synthases and ceramide levels are increased in breast cancer tissue*. *Carcinogenesis*, 2009. **30**(5): p. 745-52.

Among the family of sphingolipids, ceramide and sphingosine induce cell cycle arrest and apoptosis through modulation of protein kinases and other signalling pathways [23]. Sustained accumulation of these sphingolipids has been suggested as an effective method to regulate cancer cell growth. High levels of metabolizing enzymes such as GCS and SphK1 leads to a more general proliferative potential in a variety of tumour tissue [14, 21]. By interfering with the ceramide metabolism, cancer cell growth can be inhibited through accumulation of pro-apoptotic ceramide and concurrently decrease of sphingolipid levels associated with proliferative potential, such as glucosylceramide and S1P. Inhibition or down regulation of GCS and SphK1 also contributes to enhanced effects of therapeutic agents, such as cannabinoids and chemotherapeutics, in resistant cells [14, 25-28]. This is a fact due to chemotherapeutic drugs, such as doxorubicin and Cytarabine (Ara-C), moreover affect target cells by means of ceramide accumulation [26, 27, 29].

### ***Chemotherapeutic drugs***

Cytarabine and doxorubicin is two of the more common chemotherapy agents used in the treatment of non-Hodgkin lymphoma [30].

Cytarabine, also known as Ara-C, is a synthesized analogue to deoxycytidine (see figure 5). It inhibits the DNA-synthesis and holds a cytotoxic effect on the cells [31] by damaging the DNA. The inhibition appears due to inhibition of DNA polymerase as well as incorporation of



cytarabine into the genetic material. The cytotoxicity is highly specific for the S-phase of the cell cycle [32].

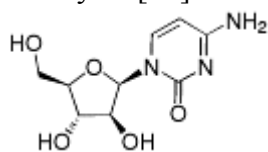


Figure 5. The structural formula of cytarabine ( $C_9H_{13}N_3O_5$ ).  
Drawing taken from [http://www.fass.se/LIF/produktfakta/substance\\_products.jsp?substanceId=IDE4POC7U9BT6VERT1](http://www.fass.se/LIF/produktfakta/substance_products.jsp?substanceId=IDE4POC7U9BT6VERT1)

Doxorubicin is a cytotoxic anthracycline antibiotic that has been isolated from cultures of *Streptomyces peucetius* var. *caesius* but nowadays it is semisynthesized from daunorubicin (see figure 6) [33]. The cytotoxic effect of doxorubicin is thought to be nucleotide base intercalation and cell membrane lipid binding activities. These actions result in the blockade of DNA and RNA synthesis and generation of free radicals that immediately damage the cell. Doxorubicin also inhibits DNA topoisomerase II, which is critical for unwinding and winding the DNA under the replication process. Although primarily killing cells undergoing DNA synthesis (S-phase), doxorubicin is not cell cycle specific [34, 35].

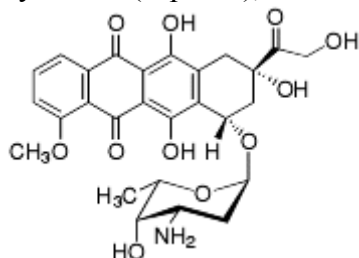


Figure 6. Molecular structure of doxorubicin ( $C_{27}H_{29}NO_{11}$ ).  
Drawing taken from [http://www.fass.se/LIF/produktfakta/substance\\_products.jsp?substanceId=IDE4POEPUAELMVERT1](http://www.fass.se/LIF/produktfakta/substance_products.jsp?substanceId=IDE4POEPUAELMVERT1)

There are studies suggesting that effects of sphingolipids in combination with doxorubicin enhance the therapeutic effects of the chemotherapeutic drug. This result is due to altering the composition of the cell membrane and therefore increases the cellular uptake of doxorubicin [22]. The combined effect enhance our groups interest to investigate whether chemotherapeutic substances, such as doxorubicin and cytarabine (Ara-C), or the cannabinoid (R)-(+)-methanandamide alone or in combination may have decreased proliferative effects in Jeko-1 MCL cell line.

## Aim

It has earlier been shown that the cannabinoid (R)-(+)-methanandamide (R-MA) can induce decreased viability in lymphoma cells, and that R-MA and cytostatics can decrease viability synergistically. Parts of the effect were attributed to apoptosis, but it is unclear if the decrease in viability is also caused by cell cycle arrest. This thesis work aims at investigating if R-MA and cytostatics alone or in combination induce decreased proliferation measured as incorporation of BrdU. Ceramides are bioactive lipids that mediate the induction of apoptosis after treatment with cannabinoids or cytostatics. The question will also be raised if the abovementioned treatments can up-regulate the levels of ceramide-metabolizing enzymes, which convert proapoptotic ceramide to growth-promoting sphingolipids. Levels of the

enzymes glucosylceramide synthase and sphingosine kinase 1 will be investigated using quantitative real time PCR.

## **Materials and methods**

### ***Cell culture***

The cell line used in this study was Jeko-1 derived from MCL patients. The Jeko-1 cell line carrying the t(11;14)(q13;q32) translocation [36] was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The cells were grown in RPMI 1640 culture medium (Invitrogen) containing 10% fetal bovine serum (FBS) and 50 mg/L gentamycin (Invitrogen), at 37°C and humidified atmosphere with 5% CO<sub>2</sub>. The cells reduplicate within 48 hours [37] and were therefore split twice a week to uphold a density range between  $(0,5 - 2) \times 10^6$  cells/mL. Cell density was estimated with a light microscope by cell counting in a Bürker chamber after addition of trypan blue exclusion dye (1:1 dilution).

### ***Experimental procedures***

All experiments were performed in serum free AIM medium (Invitrogen). Cells were washed with phosphate buffered saline (PBS) every time before they were suspended in AIM medium and prepared for treatment. The chemicals included in this study were (R)-(+)-methanandamide (R-MA) (Tocris bioscience) and the cytostatic chemotherapy agents Doxorubicin (Meda) and Cytarabine (Ara-C) (Pfizer). The cells in all experiments were treated with 100nM Ara-C or 200nM Doxorubicin or 10µM R-MA or combination of chemotherapeutic drug with R-MA. Jeko cell suspensions were added in small culture flasks with different concentrations such as 5 million cells/flask or 10 million cells/flask depending on the treatment. The higher concentrations of cells were used for treatment with a combination of chemicals and the lower for treatment with a single chemical or no added chemicals. The cells with the different treatments and the untreated controls were incubated for 24h in humidified atmosphere at 37°C for later RNA extraction followed by cDNA synthesis.

### ***RNA extraction***

Total RNA was extracted with RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. Isolated RNA was eluted from the columns with 30µl RNase free water. RNA quantification was done using a NanoDrop Spectrophotometer ND-1000 (Saveen Werner).

### ***cDNA synthesis***

First-strand cDNA synthesis was carried out with the Omniscript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's instructions. The extraction was performed to a total volume of 20µL cDNA, which required 2µg template RNA.

### ***Quantitative real time PCR***

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) is used to amplify and simultaneously quantify a targeted DNA molecule. The main purpose is to either assess the

amount of DNA or RNA in the sample before the amplification begins or to describe the change in expression of the target gene relative to some reference group. This is possible due to the fluorescent dye that is present in the reaction and binds to every freshly formed DNA-fragment. As a consequence leads to an increase in fluorescence intensity, measured at each cycle, which also is proportional to the DNA concentration. Then, depending on the analysing method provide either the initial amount of the sequence of interest or the relative changes in gene expression [38]. In our case, the change in SphK1 expression and GCS expression relative to untreated control was of curiosity. Thus, primer designs, selection of an endogenous control, insurance of the efficiency similarity between target gene and endogenous control, and several optimizations were required steps in the evaluation of the experiment.

The  $\beta$ -actin gene was selected to be used as a loading control, a so called endogenous control. The primers used were designed using Beacon Design software (Premiere Biosoft International) and are listed in table 1. As all the genes of interest possessed several exons, it was possible to use intron spanning primer design that eliminates the risk of amplifying genomic DNA.

Table 1. Primers designed for amplifying the genes of interest.

<b>Target</b>	<b>Forward primer sequence 5'-3'</b>	<b>Reverse primer sequence 5'-3'</b>
$\beta$ -actin	AAAGACCTGTACGCCAACACA	AGTACTTGCGCTCAGGAGGA
GCS	TTCACGGGCTGCCTTACGTA	CCTGTCACACATTTGAAACCAGTT
SphK1	CTGGCAGCTTCCTCCTTGAACCAT	GTGCAGAGACAGCAGGTTC

The gene expression analysis was carried out using the qRT-PCR kit Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with fluorescein isothiocyanate (FITC) (Invitrogen). To monitor DNA synthesis SYBR Green was used as the fluorescent dye. It emits green light only when binding to double-stranded DNA. FITC with a final concentration of 10nM was used as reference dye to normalize the fluorescent reporter signal in SYBR Green reaction.

A master mix containing Supermix, FITC, Forward primer, Reverse primer, Template and RNase free water was prepared to a total volume of 80 $\mu$ L for each sample. Samples were loaded in triplicates of 25 $\mu$ L per well in a 96-well PCR plate (Abgene) and run on iCycler IQ (BioRad) Real-time PCR detection system. Primer concentration was optimized to 600nM for all primers and the annealing temperature, the temperature were the specific primers best bind to the complementary DNA strand, was optimized to 60°C (data not shown). The PCR program used for the reactions to proceed was 95°C for 10 min followed by 40 cycles, each cycle consisting 15 sec at 95°C and 1min at 60°C, and finally 80 cycles of 0,5°C temperature gradient, 10 sec for each cycle, starting from 55°C. The temperature gradient step was for constructing a melt curve, which is important for checking that the correct fragments were amplified.

Using the  $2^{-\Delta\Delta C_T}$  method, the data are presented as the fold change in gene expression normalized to  $\beta$ -actin (the endogenous reference gene) and relative to the untreated control [38]. After each run with the PCR machine threshold cycle ( $C_T$ ) values were obtained from amplification of SphK1, GCS and  $\beta$ -actin. Average  $C_T$  was calculated for each triplicate. Subsequently, the  $C_T$  value for the housekeeping gene was subtracted from the  $C_T$  values obtained from the gene of interest in order to calculate  $\Delta C_T$ . The  $\Delta\Delta C_T$  values were calculated by subtracting the  $\Delta C_T$  values for the pooled controls (untreated Jeko cells) from the  $\Delta C_T$  values for the samples. The relative fold increase (RFI) of SphK1 and GCS was finally

calculated by the equation  $2^{-\Delta\Delta CT}$ . To use this analysing method, the amplification efficiencies of the target and reference must be similar [39]. A validation experiment was performed to determine that the absolute value of the slope of serial cDNA dilutions versus  $\Delta C_T$  was less than 0,1 (see Results). The serial dilutions of cDNA from Jeko cells were: 1:1; 1:2; 1:4; 1:8; 1:16 and 1:32.

### ***Cell proliferation (BrdU)***

Cell proliferation was analyzed by using an assay measuring DNA synthesis. It is a technique based on the measurement of BrdU incorporated into newly synthesized DNA strands of actively proliferating cells. The pyrimidine analogue BrdU is incorporated in place of thymidine and allows detection by immunochemistry. Cell Proliferation ELISA, BrdU (colorimetric) from Roche Applied Science was used to quantify proliferating cells in the Jeko cell suspensions treated with 100nM Ara-C or 200nM doxorubicin or 10 $\mu$ M R-MA or combination of chemotherapeutic drug with R-MA, in the relation to the untreated Jeko cell suspension. The cells used, as controls and the ones cultured in the presence of chemicals, were seeded in triplicates of approximately  $6 \times 10^3$  cells per well in a 96-well tissue culture plate and pre-incubated in humidified atmosphere at 37°C for 48h. Subsequently, BrdU was added to respective well and the cells were reincubated for about 22h. Samples with no BrdU added were used as negative controls. Further steps in the method were carried out according to the manufacturer's instructions.

### ***Viability assay (XTT)***

Cell viability can be defined as the number of healthy cells in a sample. Cell viability was determined by an assay detecting the mitochondrial capacity to reduce XTT tetrazolium salt to formazan. The spectrophotometrically measured formazan dye is a result of the activity of mitochondrial enzymes in healthy living cells, a hallmark for cell viability. Cell Proliferation Kit II (XTT) from Roche Applied Science was used to investigate the viability of the cells. Approximately  $4,5 \times 10^4$  cells per well were seeded in a 96-well tissue culture plate. The cells treated differently and the untreated controls were pre-incubated in humidified atmosphere at 37°C for 72 hours before the addition of XTT to each well. After 4 hours of further incubation in humidified atmosphere at 37°C the absorbance was measured at 470nm with a microplate spectrophotometer (Powerwave, Biotek Instruments, Inc).

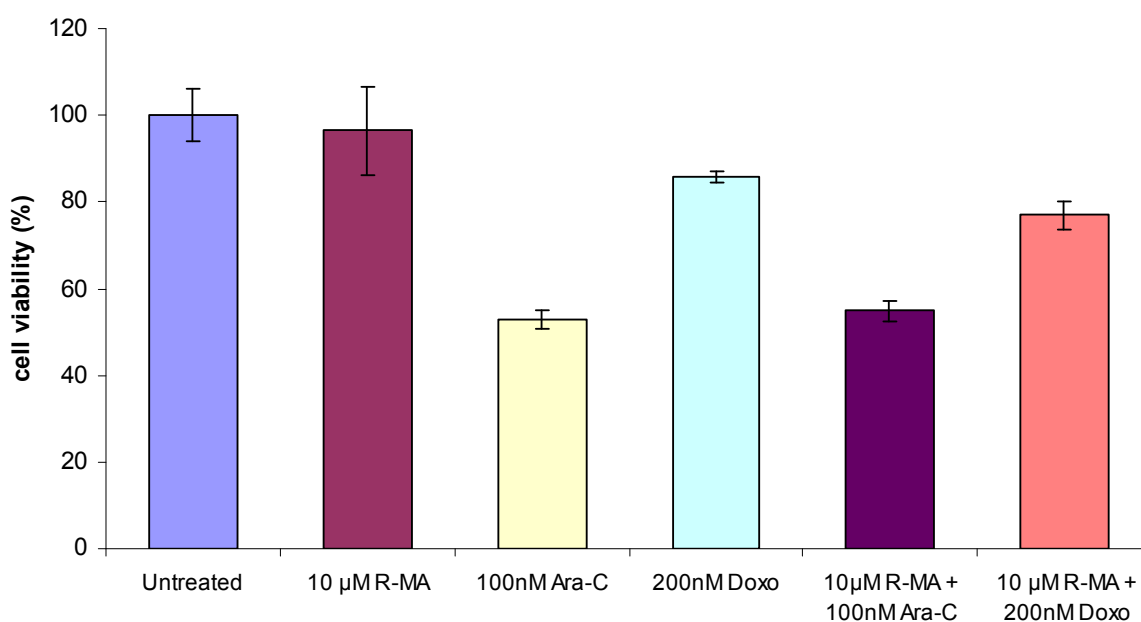
### ***Statistical analysis***

Statistical analysis was performed using the software Statistica (Statsoft AB). Experiments were repeated at least three times on the same batch of cells. Cell proliferation and gene expression was compared using Mann-Whitney U test between control and treated cells. Results were considered to be statistically significant with p-values smaller than 0,05.

## Results and discussion

### *Cell viability*

Earlier studies by our group (unpublished data) have demonstrated interesting effect of the cannabinoid (R)-(+)-methanandamide (R-MA) on the viability of lymphoma cells. It has been shown that R-MA can induce decreased viability and also that R-MA and cytostatics can decrease viability synergistically (unpublished data). To ensure the similarity of the effect of the chemicals on the Jeko cells, due to the purchasing of chemicals from different companies, a verification experiment has been done using the same method, XTT viability assay, as in earlier studies. Although the optimal parameters for the XTT viability assay were used, as the optimization was made earlier, some of the results did not exhibit as expected (see figure 7). No synergistic effect could be seen for the combined treatment with R-MA and Ara-C. A nominal synergistic effect can be seen for the combined treatment with the R-MA and Doxorubicin but still were far-off from the expected effect. The inconsistent results from the XTT viability test in present study made it difficult to draw any conclusions about the decreased viability between the treatments. However, the conflicting results could perhaps partly be explained by the insufficient repeats of the experiment as well as the state of the cells, instead of the alternative purchased chemicals.



*Figure 7.* Cell viability of Jeko cells in response to different treatments for 72 hours. Unfortunately synergistic effect as expected was not shown for the combined treatment with R-MA and Ara-C. The small synergistic effect from the combined treatment with R-MA and Doxorubicin did also not correlate with earlier studies. Statistical analyze was not possible due to the insufficient repeats of the experiment.

### *Cell proliferation optimization*

To obtain trustworthy results from the cell proliferation experiments, one is obligated to perform measurements in a region lying within the limits of the linearity range. That is when the cells have an exponential proliferating rate. The optimal initial cell amount per well was determined in a preliminary experiment using the BrdU enzyme-linked immunosorbent assay (ELISA). This was done by measuring the absorbance for several dilutions of initial cell

amount (see figure 8). In further experiments  $6 \times 10^3$  cells, a concentration within the linear range, were seeded in a volume of  $100\mu\text{L}$  AIM medium per well.

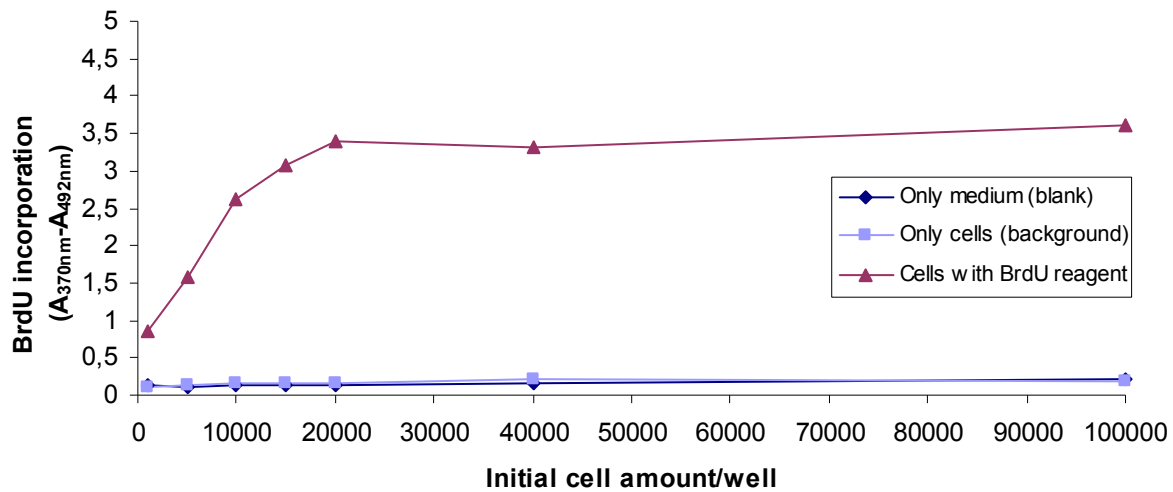


Figure 8. BrdU incorporation in various concentrations of Jeko cells presents a linear range between 1000 and 10000 in initial cell amount/well.

### Cell proliferation

Previous studies (unpublished data) showed that the decreased viability observed after treatment with R-MA and chemotherapeutics could partly be explained by induction of cell death. To look at cell proliferation activities after treating the Jeko cells with R-MA and cytostatics are therefore assumed to be an important complement to the earlier studies done by our group. The cell proliferation was detected 72h after treatment measuring the absorbance of cells labelled with BrdU. R-MA treated Jeko cells showed almost unaffected proliferation behaviour, in contrast to cells treated with doxorubicin, that partly inhibited proliferation, and Ara-C, that inhibited almost all proliferation activity (see figure 9). The treatment with combination of R-MA and Ara-C showed similar decrease of proliferation as Ara-C alone. However, treatment with the combination R-MA and doxorubicin resulted in a tiny difference relative to the treatment with doxorubicin alone. Fortunately, the negative control which indicates the unspecific bindings that could provide background noise turned out to be inconsiderable. With these results in concern, some conclusions whether growth suppression accounts for part of the decreased cell viability observed in treated Jeko cells can be made. The decreased cell viability after treatment with cytostatics, especially Ara-C, is likely to be a result of cell growth inhibition by incorporation into the DNA strand and inhibition of crucial enzymes that acts in the replication process, although cell death by apoptosis cannot be excluded.

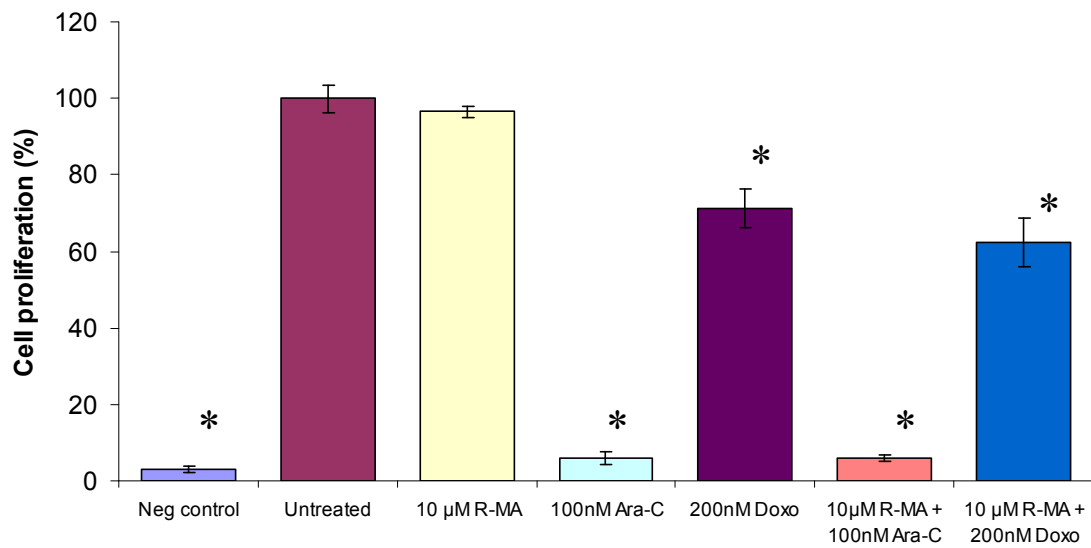


Figure 9. Jeko cells were incubated with AIM medium without serum and pretreated with vehicle or with 10μM R-MA, 100nM Ara-C, 200nM Doxorubicin or combinations, for 48h prior to incubation with BrdU for 22h. No BrdU was added to cells used as negative control. One representative of three individually performed experiments is shown and the error bars represent the standard deviation. \*,  $P < 0,05$ , treated cells compared with control cells (Mann-Whitney U Test).

### Efficiency test

The method providing the relative changes in gene expression was used to analyze the data from qRT-PCR. Using this method requires the amplification efficiency for the target genes and the housekeeping gene to be similar. The criterion was accomplished by constructing a graph demonstrating the  $\Delta C_T$  values for the serial cDNA dilutions, and then confirming that the gradient of the line was less than 0,1 (see figure 10). This was achieved using the primer concentration 600nM and the annealing temperature 60°C.

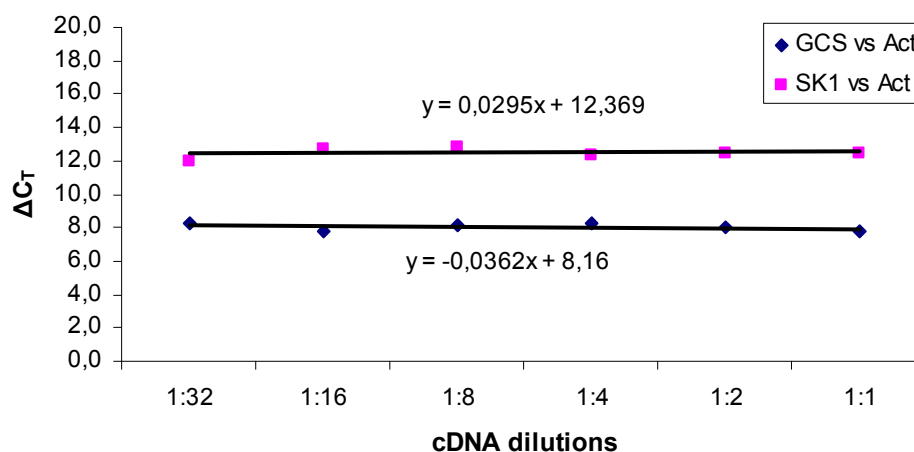
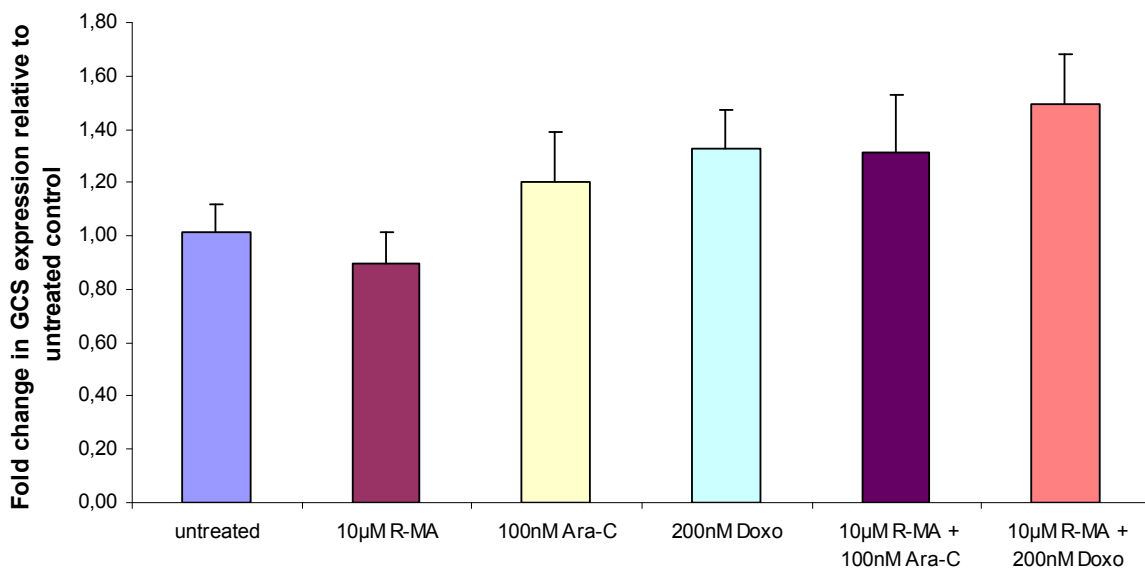


Figure 10. Graph demonstrating serial dilutions of input cDNA of a random sample against  $\Delta C_T$  values (GCS- $\beta$ -actin and SK1-  $\beta$ -actin). The slope of the lines were  $\ll 0,1$  which indicates similar amplification efficiency for GCS and SK1 in relation to  $\beta$ -actin, and with the current settings.

## Gene expression analysis of GCS and SphK1 in Jeko cells

It has been suggested that treatment with cytostatics, on various types of cancer, affect the gene expression of the enzymes glucosylceramide synthase (GCS) and sphingosine kinase 1 (SphK1) in the endocannabinoid system [25, 27, 40]. In order to obtain insight into the enzymes of the sphingolipid pathway that contribute to reduced levels of sphingolipids associated with pro-apoptotic effect, the mRNA levels of GCS and SphK1 were investigated using real-time qPCR. Although, ceramide kinase (CK) is as well a metabolic enzyme that converts ceramide to a sphingolipid associated with pro-apoptotic effect, it was left out from this particular study due to several reasons. The expression of the metabolizing enzymes GCS and SphK1 were compared between untreated Jeko cells and cells treated with R-MA, cytostatics or combinations. Results showed a tendency for up-regulation of GCS expression when cells were treated with cytostatics, in contrast to cells treated with R-MA alone (see figure 11). Although, R-MA alone had a tendency for a small down-regulation of GCS expression, it contributed to a potential elevation of GCS expression when combined with Ara-C or doxorubicin. The reason could be that the cells get more sensitive against the cytostatics when R-MA is present. Moreover, up-regulation of the enzyme GCS in response to doxorubicin correlates with results from other studies [27, 28].

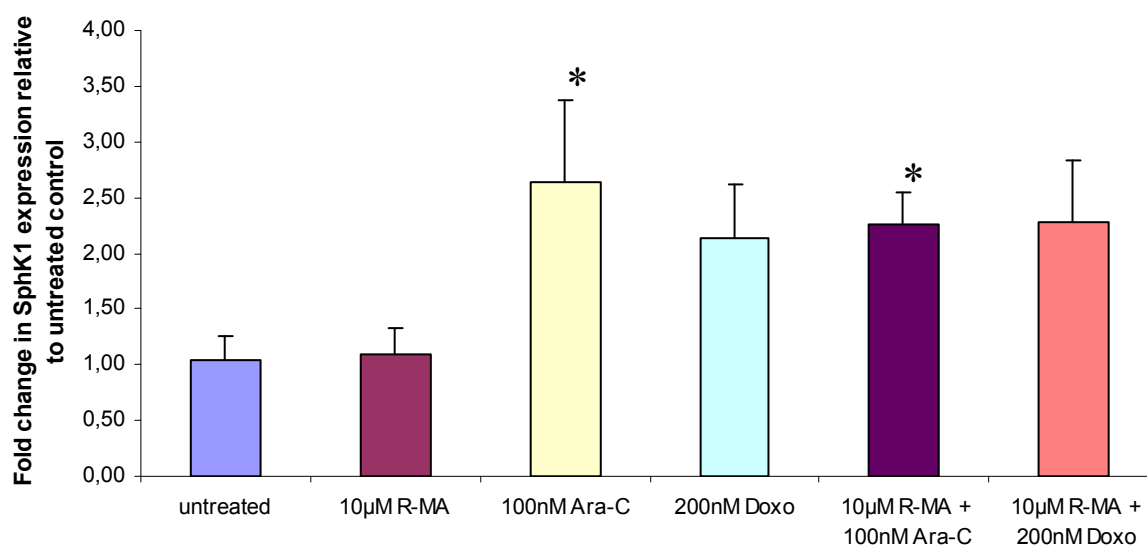


*Figure 11.* Jeko cells were incubated with AIM medium without serum and treated for 24h with vehicle or with 10µM R-MA, 100nM Ara-C, 200nM Doxorubicin or combinations. After treatment, levels of glucosylceramide synthase (GCS) were detected by qRT-PCR. Values represent relative fold increase (RFI) in the expression of GCS in treated samples compared to control samples. All samples were normalized to the reference gene  $\beta$ -actin. All treatments, except for the treatment with R-MA alone, gave rise to an up-regulation of GCS but none was significant. The error bars represent the standard error.

The enzyme that converts sphingosine to sphingosine-1-phosphate (S1P), sphingosine kinase 1 (SphK1), is a critical regulator for the cell fate. Whereas S1P stimulates growth and survival, sphingosine is usually pro-apoptotic. Earlier studies have shown that apoptotic cells may up-regulate the expression of SphK1 to produce and secrete S1P. It is also believed that S1P serves as a chemotactic factor to attract phagocytic cells, in order to prevent necrosis [25].



Cells treated with Ara-C or a combination of Ara-C and R-MA express significant increase in the expression of SphK1 relative to untreated cells (see figure 12). Treatment with doxorubicin and the combined treatment with R-MA and doxorubicin demonstrated similar tendency for up-regulation of SphK1. On the other hand, cells treated with R-MA alone remained unaffected regarding SphK1 expression. Regulations of SphK1 expression correlate negatively with cell viability (see figure 7 and figure 12), as treatments that contribute to lower cell viability also contribute to higher levels of SphK1. It is also assumed that a balance between the effect from the up-regulated levels of the metabolizing enzymes GCS and SphK1, and the effect from earlier observed up-regulations of the ceramide synthesis enzymes [14, 24, 26] take place. This assumption is due to the obvious decreased viability which is associated with ceramide accumulation, among other things.



*Figure 12.* Jeko cells were incubated with AIM medium without serum and treated for 24h with vehicle or with 10µM R-MA, 100nM Ara-C, 200nM Doxorubicin or combinations. After treatment, levels of sphingosine kinase 1 (SphK1) were detected by qRT-PCR. Values represent relative fold increase (RFI) for the expression of SphK1 in treated samples compared with control samples. All samples were normalized to the reference gene  $\beta$ -actin. \*,  $P < 0,05$ , treated cells compared with control cells (Mann-Whitney U Test). The error bars represent the standard error.

The earlier observed role of biologically active sphingolipids has suggested that one could enhance the cell death-promoting effects of R-MA by inhibiting the transformation of sphingolipids associated with pro-apoptotic effect into species with opposing effects. Therefore, modulating the levels of the metabolizing enzymes SphK1 and GCS by inhibitors or siRNA is thought to potentiate the cytotoxic response to the different treatments used in this study, especially the ones which contribute to higher levels of the metabolizing enzymes. In summary, a treatment with R-MA on Jeko cells had an inconsiderable effect on cell proliferation activity as well as expression of GCS or SphK1. However, treatment with the chemotherapeutic agents Ara-C or doxorubicin decreased cell proliferation activity. The same treatment induced the expression of the metabolizing enzymes GCS and SphK1, as well. Since both these chemotherapeutic agents moreover affect target cells by means of ceramide accumulation, it is assumed that the effect could enhance by inhibition of the enzymes GCS and SphK1.

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