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Master thesis

Apoptosis-inducing effects of curcumin derivatives in human leukaemia cells

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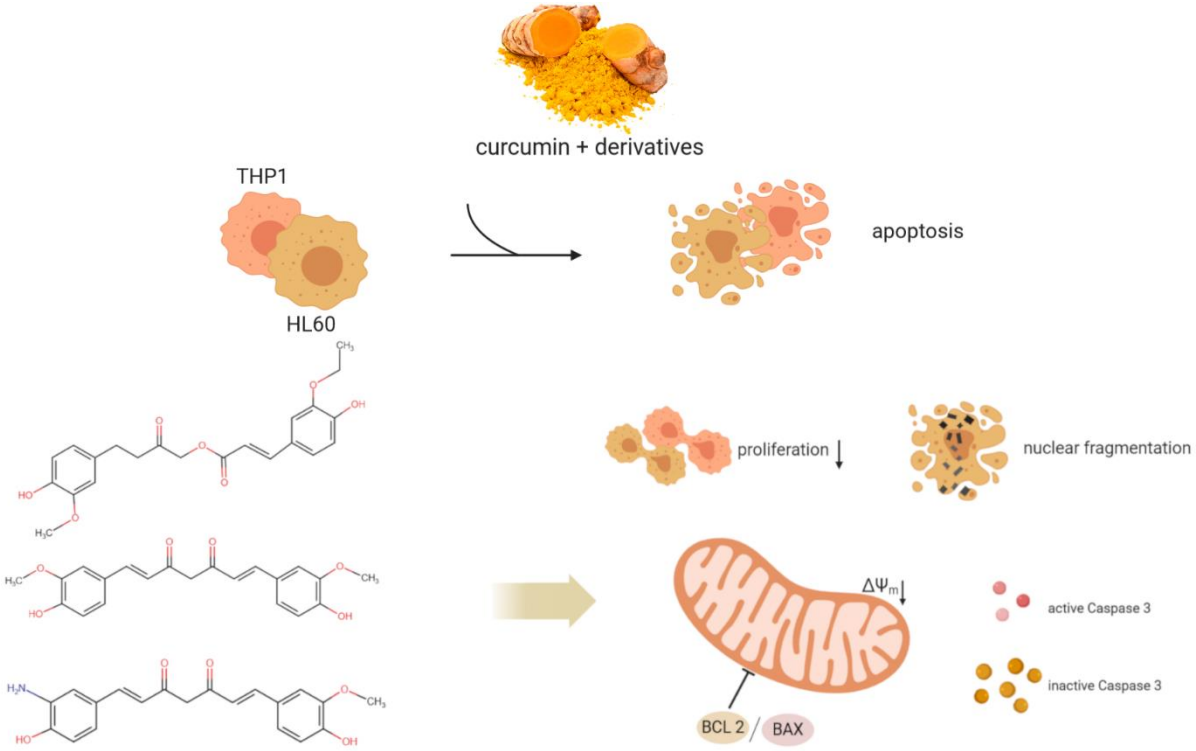
Graphical Abstract



Kurzfassung

Es wird angenommen dass Curcumin Apoptose auslösen kann. Allerdings hat Curcumin aufgrund seiner schlechten Wasserlöslichkeit und chemischen Instabilität im menschlichen Verdauungstrakt eine geringe Bioverfügbarkeit. Deshalb wurde Curcumin im Vergleich mit einigen seiner Strukturderivaten, die eine bessere Stabilität oder Löslichkeit aufweisen, auf Apoptose-auslösende Effekte in 2 unterschiedlichen Leukämie Krebszelllinien untersucht. Ein Einfluss auf den metabolischen Stoffwechsel der THP1 Zelllinie zeigte sich erst ab höheren Konzentrationen von Curcumin, Aminocurcumin und Calebin-A, während die HL 60 Zellen schon bei geringeren Konzentrationen sichtbare Effekte aufwiesen. Mittels Flow Cytometry konnte nur bei THP1 Zellen ein bemerkbarer Anstieg der DNA-Fragmentation im Vergleich zur unbehandelten Kontrolle festgestellt werden. Bei den HL 60 Zellen war dies nicht möglich, jedoch konnte auch in diesen Zellen eine erhöhte DNA-Fragmentierung mittels Fluoreszenzmikroskopie beobachtet werden. Die mikroskopische Untersuchung zeigte bei ansteigender Konzentration von Curcumin, Aminocurcumin und Calebin-A eine Zunahme an Zelltrümmern und eine Reduktion der Zellzahl. Es konnte auch eine Reduktion des Membranpotentials festgestellt werden, wodurch die Freisetzung von Cytochrom c ins Cytosol induziert und folglich über eine Signalkaskade Apoptose ausgelöst wird. Der Western Blot lieferte aber keinen eindeutigen Beleg für einen Apoptose-induzierenden Effekt der getesteten Substanzen. Generell zeigen die Ergebnisse einen Einfluss der Curcumin-Derivate auf die Viabilität der Zellen, jedoch konnte deren Apoptose-auslösende Aktivität nicht eindeutig bestätigt werden.

Abstract

Apoptosis inducing effects of curcumin were already demonstrated, but due to its poor water solubility and chemical instability in the human gastrointestinal tract a low bioavailability is resulting. Therefore, curcumin was compared to some of its derivatives, which show either a better stability or water solubility, with respect to apoptosis inducing effects in two different human leukaemia cancer cell lines. Curcumin, aminocurcumin and calebin-A reduced the metabolic rate of the cells in a concentration-dependent manner. The drugs showed an effect on THP 1 cells only at relatively high concentrations, whereas lower concentrations showed already an effect on HL 60 cells. Flow cytometry showed only an increase in DNA-fragmentation for the THP 1 cells. However, visual inspection of curcumin-treated HL 60 cells by fluorescence microscopy revealed also an increase in DNA-fragmentation for this cell line. Furthermore, the microscopic analysis of cells treated with increasing concentrations of curcumin, aminocurcumin and calebin-A showed an increase in cell debris and a reduction in cell number. The membrane potential was also reduced, which induces cytochrome c release into the cytosol and therefore enables a signal cascade resulting in apoptosis. However, the apoptosis inducing effects of the tested substances could not be confirmed by Western blotting analysis. The findings indicate that the treatment of THP 1 and HL 60 cells with curcumin derivatives causes a reduction in cell numbers and increased cell death, but apoptosis inducing effects could not be clearly confirmed.

摘要

研究指出薑黃素 (Curcumin)具有誘導細胞凋亡的功效，然而因薑黃素在腸道中具有較差的水溶性及化學不穩定性使其具有較低的生物利用率。因此本研究將比較薑黃素及其兩種不同衍生物(aminocurcumin 和 calebin-A)其與薑黃素相較之下可能具有較佳化學穩定性亦或是較佳水溶性並探討其對於不同人類白血球癌細胞株是否可以誘導細胞凋亡而具有抑制癌細胞生長的功效。結果顯示薑黃素、aminocurcumin 和 calebin-A 可以減少細胞生長代謝且具有劑量效應。此三種藥品在高劑量下對 THP 1 細胞才有作用而在 HL 60 細胞則在低劑量即顯示出效果。利用流式細胞儀及螢光顯微鏡檢測樣品對於人類白血球癌細胞生長的影響。流式細胞儀結果顯示樣品在 THP 1 細胞可以增加 DNA 片段，而在螢光顯微鏡結果顯示薑黃素在 HL 60 細胞可以增加 DNA 片段且薑黃素、aminocurcumin 和 calebin-A 隨著濃度的增加其細胞碎片與減少細胞數目。除此之外，樣品可以減少細胞膜電位，可能藉由 cytochrome c 釋放到細胞質並引發一連串效應而誘導癌細胞產生細胞凋亡。西方墨點法初步結果顯示藥品可以改變細胞凋亡相關蛋白質的表現。本研究發現薑黃素及其兩種不同衍生物(aminocurcumin 和 calebin-A)可以減少人類白血球癌細胞株(THP 1 和 HL 60)的細胞數目及誘導細胞凋亡，但相關的機制仍需要進一步探討

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Abbreviations

Bax	B cell leukaemia/lymphoma-2 associated X protein
Bcl-2	Beta-cell lymphoma protein 2
CalA	Calebin A
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
CUR	Curcumin
EGTA	Ethyleneglycol bis(2-aminoethyl ether)-N,N,N',N' tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HHC	Hexahydrocurcumin
MTT	3-(4,5-dimethylthiazole-2-yl)2,5-biphenyl tetrazolium bromide
NCUR	Aminocurcumin
NTHC	Aminotetrahydrocurcumin
PBS	Phosphate buffered saline
THC	Tetrahydrocurcumin

1. Aim

The aim of this master's thesis is to investigate and discuss the apoptosis inducing effects of curcumin and its derivatives on the human promyelocytic leukaemia sensitive HL60 cell line and the acute monocytic leukaemia THP-1 cell line and compare the efficiency of the different derivatives with curcumin.

2. Introduction

Cancer is a current health problem due to be one of the most frequent causes of death in the world. Most cancer types are caused by dysregulation of more than 500 different genes, therefore drugs that target multiple gene products are needed for cancer treatment. Considering the cancer treatments which are available nowadays, like chemotherapy where a high percentage of healthy cells are affected as well, an approach for treatments without losing healthy cells or causing any other adverse health effects is needed. It is widely considered that secondary metabolites, like alkaloids, terpenoids, flavonoids and tannins, which are produced by plants have biologic effects. Therefore many researchers focus on finding a cancer treatment within herbal sources. (Kooti et al., 2017; Kunnumakkara et al., 2008)

The focus of this thesis is a chemical extracted from the tropical plant Curcuma longa L., also known as turmeric, part of the ginger family, Zingiberaceae and native to south and southeast tropical Asia. In those regions it has been already used for centuries traditional medicine, like Ayurveda medicine or Chinese medicine, for the treatment of various diseases. Curcuma is also used as herb for Asian cuisine or dyeing agent. (Ammon & Wahl, 1991; Li, 2011)

The yellow coloured turmeric powder consists of various curcuminoids, such as curcumin, a diferuloylmethane which is the main curcuminoid with around 77 %, demethoxycurcumin with 17 %, bisdemethoxycurcumin with 3 %. (Shanbhag, 2017)

2.1. Curcumin

Curcumin is known for many diverse and beneficial health properties: anti-arthritic, anti-infectious, anti-inflammatory, anti-oxidative, chemopreventive, chemotherapeutic chemosensitizing, hepatoprotective, and thrombosupressive. Cellular proliferation, invasion and metastasis can be inhibited by curcumin by supressing multiple signalling pathways. (Shanbhag, 2017)

Curcumin is also widely used in food industry as food additive for colouring. The European Food Safety Authority (EFSA) approves an ADI of 3 mg/kg bw/day based on the NOAEL of 250-320 mg/kg bw/day. The United States Food and Drug Administration (FDA) states that curcumin has a low toxicity and is generally regarded as safe (GRAS) (European Food Safety Authority, 2010, Zheng et al., 2018)

The IUPAC name of curcumin is (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione), but it is also known as diferuloyl methane. The chemical structure (Figure 1) shows three specific characteristics: two aromatic ring systems containing o-methoxy phenolic groups, connected with each other by a 7 carbon atom chain, consisting of an α,β-unsaturated β-diketone moiety. (Priyadarsini, 2014).

Due to keto-enol tautomerism and cis-trans tautomerism, α,β-unsaturated β-diketones can exist in nine different forms. Various studies suggest, that the enolic form is more stable, than the keto form. (Nelson et al., 2017)

Due to its possibility to interact with numerous proteins, which are involved in cellular signalling process, Curcumin is potent to maintain progress and control of diseases. (Indira Priyadarsini, 2013)

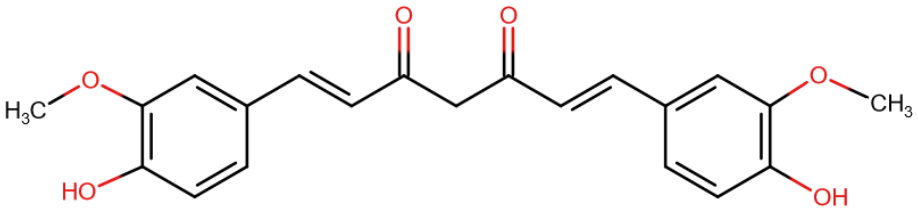

Figure 1 structure of curcumin – Ketoform

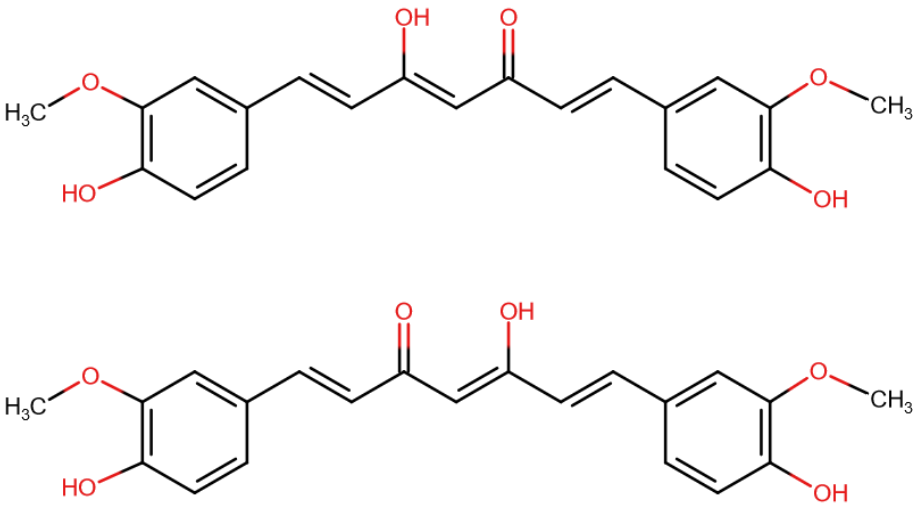

Figure 2 structure of curcumin - Enolform

Curcumin can interact with a diverse range of molecular targets, including enzymes, inflammatory mediators, transcription factors and protein kinases. Furthermore it can impact various signalling pathways. (Kunnumakkara et al., 2017)

2.2. Aminocurcumin (NCUR)

Amino curcumin is a bright red powder with the IUPAC name (1E,6E)-1-(3-amino-4-hydroxy-phenyl)-7-(4-hydroxy-3-methoxy-phenyl)hepta-1,6-diene-3,5-dione.

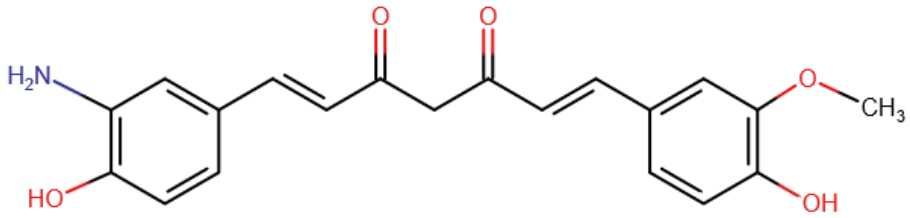

Figure 3 Structure of aminocurcumin

The features of the structure are the same as curcumin, except of one methoxy groups which was substituted with an amino group.

2.3. Tetrahydrocurcumin (THC)

The IPUAC name of tetrahydrocurcumin is 1,7-bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-dione

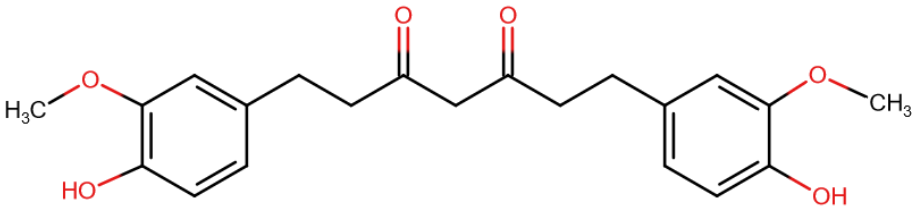

Figure 4: Structure of Tetrahydrocurcumin – Ketoform

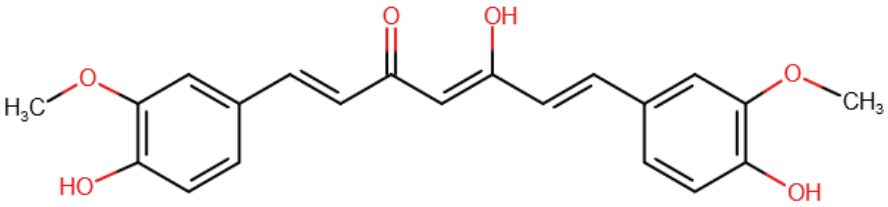

Figure 5 Structure of Tetrahydrocurcumin - Enolform

Tetrahydrocurcumin is a major (hydrogenated) metabolite of curcumin. Tetrahydrocurcumin is structurally very similar to curcumin, expect that it lacks the double bond in the hydrocarbon chain. The antioxidant activity is higher compared to curcumin and it has as well a low water solubility. (Plyduang et al., 2014)

THC is able to scavenge free radicals, inhibition of lipid peroxidation and formation of hydroperoxides in order to protect cells against oxidative stress. (Somparn et al., 2007) The induction of autophagic cell death in human leukaemia HL-60 cells through modulation of phosphatidylinositol 3-kinase/protein kinase B and mitogen-activated protein kinase signalling was reported by (J. C. Wu et al., 2011)

2.4. Aminotetrahydrocurcumin (NTHC)

4-Aminotetrahydrocurcumin,a novel active molecule with the IUPAC name 4-amino-1,7-bis(4-hydroxy-3-methoxy-phenyl)heptane-3,5-dione, has shown already cytotoxic ability against human colon adenocarcinoma cell lines (HT-29) (Plyduang et al., 2014)

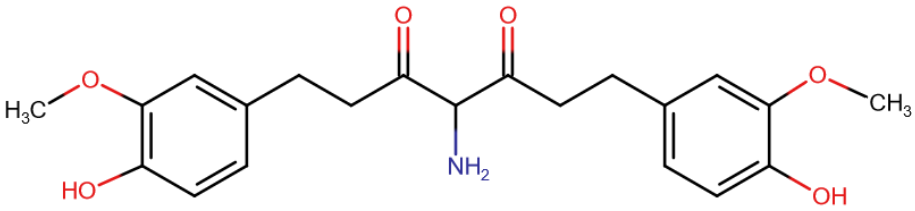

Figure 6 structure of aminotetrahydrocurcumin

2.5. Hexahydrocurcumin

Hexahydrocurcumin (IPUAC: 5-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3-on) – HHC

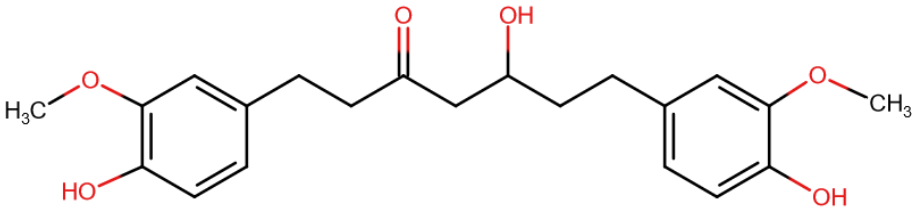

Figure 7 structure of Hexahydrocurcumin

Hexahydrocurcumin (HHC) is one of curcumin's major metabolites. (Zhao et al., 2015) It is mentioned to have a similar bioactivity, such as anti-inflammatory, antioxidant and antitumor properties, like curcumin by being tested in *in vitro* and *in vivo* studies. The structure has, like curcumin and other metabolites, phenolic groups or diketo moieties. The higher stability at pH 7.4 is explained with no double bonds present. It is mentioned, that HHC has the capability of scavenging free radicals. (Huang et al., 2018)

2.6. Calebin A

Calebin A, [(E)-4-(4-hydroxy-3-methoxyphenyl)-2-oxobut-3-enyl] (E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate), is a novel curcuminoid, which was isolated and identified from *Curcuma longa L*. by Kim. The yellow pale powder is reported to possess potent biological activities but low bioavailability, rapid metabolic elimination and caused recrystallization *in vivo*. Compared to other curcuminoids, the 1,3 – diketonic structure is missing, but calebin A possesses a ferulic acid ester bond. Toxicity tests suggest a "No Observed Adverse Effect Level" (NOAEL) with 100 mg/ kg/ day, which makes it safe for human consumption. (Kim & Kim, 2001; Majeed et al., 2015; Oliveira et al., 2015; M.-H. Pan et al., 2017)

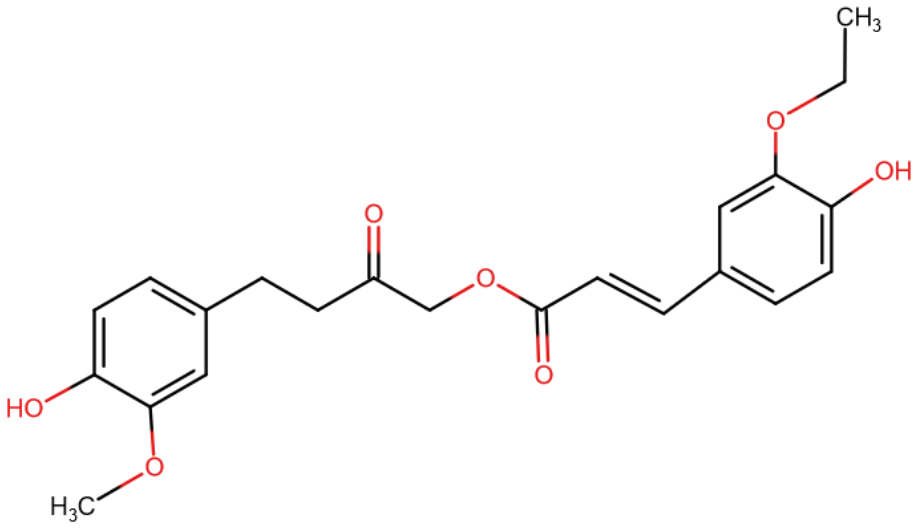

Figure 8 Structure of Calebin A

2.7. Cancer

In cancer development many complex factors are involved. Those factors are leading to uncontrolled spreading and growth of cancer cells throughout the body, called metastasis.

In order to survive cells transformed and acquired many different qualities including self-sufficiency in growth signals, insensitivity to anti-growth signals, circumvention of apoptosis, limitless replicative potential, senescence, altered immunity, ability of tissue invasion and sustained angiogenesis are some of them.

Telomerase, which repairs the telomere in cells, plays a major role in the cell replication process. It is expressed by a variety of different cancer cells resulting in no loss of DNA from the telomere. In the human body the expression of this enzyme is limited to stem cells and germinative cells.

In cancer cells the apoptotic controls are almost always affected, so they typically overexpress the anti-apoptotic Bcl-2 family proteins that introduce resistance to apoptosis induced by conventional chemotherapeutic agents. Bcl-2 family proteins can locate on mitochondria and endoplasmic reticulum (ER). (Cree, 2011a; Keller et al., 2009; M. H. Pan et al., 2008)

2.7.1. Human Leukaemia

Leukaemia is characterised by disorders such as proliferation of stem cells. Depending on which step in the maturation process is disrupted leukaemia can be classified into acute or chronic leukaemia and further divided into acute lymphoid leukaemia (ALL), which is more frequent in children and characterized by high numbers of immature lymphocytes. Acute myeloid leukaemia (AML), which occurs in high rates in adults and shows an increased proliferation of immature myeloid cells. Chronic myeloid leukaemia (CML) develops slowly and early and mild symptoms can be unnoticed.

Acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML) show telomerase activity, which results in no DNA loss from the telomere during replication. (Keller et al., 2009; Silva et al., 2019)

2.8. Apoptosis

Referred to as programmed cell death or suicide, apoptosis is a gene-directed form of cell death, where within the cell a mechanism is activated. (Figure 9) This mechanism is involved in the control of cell numbers within tissues or in the immune system. Apoptosis leads to DNA fragmentation, cytoplasm shrinkage, membrane blebbing, chromatin condensation, formation of DNA ladder with multiple fragments caused by internucleosomal DNA cleavage and in the end cell death, without cell lysis. Apoptosis is only affecting the cell, where the 'suicide' machinery has been activated, so neighbouring cells will not be damaged causing no inflammation or other sequelae. Apoptosis can be differentiated, by the mechanisms used, in an intrinsic or mitochondrial and extrinsic or death receptor pathway and is mediated by a balance of pro- and anti-apoptotic factors. (Cree, 2011a; Su et al., 2015)

Other forms of cell death, such as autophagy, necrosis and oncosis, can be distinguished from apoptosis by the involvement of a various number of intercellular signalling pathways, including members of caspase family of cysteine proteases. (M. H. Pan et al., 2008)

The mitochondrial pathway of apoptosis is regulated by the expression of pro-apoptotic and anti-apoptotic members of the Bcl-2 family members, where the ratio between those proteins is mainly affecting if the cell will undergo apoptosis or not. (Cheng et al., 2005)

The pro-apoptotic member of the Bcl-2 family, B cell leukaemia/lymphoma-2 associated X protein (Bax), is present in many cell types. This cytosolic protein is located in the mitochondria and reacts to apoptotic stimuli with participation in cytochrome c release. (Brady & Gil-Gómez, 1998)

The anti-apoptotic member of the Bcl-2 family, B-cell lymphoma 2 (Bcl-2), is present on the outer mitochondrial membrane and on some other membranes in other cell types. It prevents the mitochondria from cytochrome c release into the cytosol by regulating the mitochondrial membrane permeabilization. (Su et al., 2015)

The death receptor pathway is initiated by ligation to a transmembrane death receptor (Fas, TNF receptor) and activating caspase-8 which can activate caspase-3. (Pan et al., 2008)

Caspase-3, an aspartate-specific cysteine peptidase, is a downstream mediator in the apoptotic pathway. Caspase-3 cleaves multiple substrates including poly-(ADP-ribose) polymerase (PARP), which is leading to DNA fragmentation. Active Caspase-3 contains two p17 and two p12 subunits. (Porter & Jänicke, 1999)

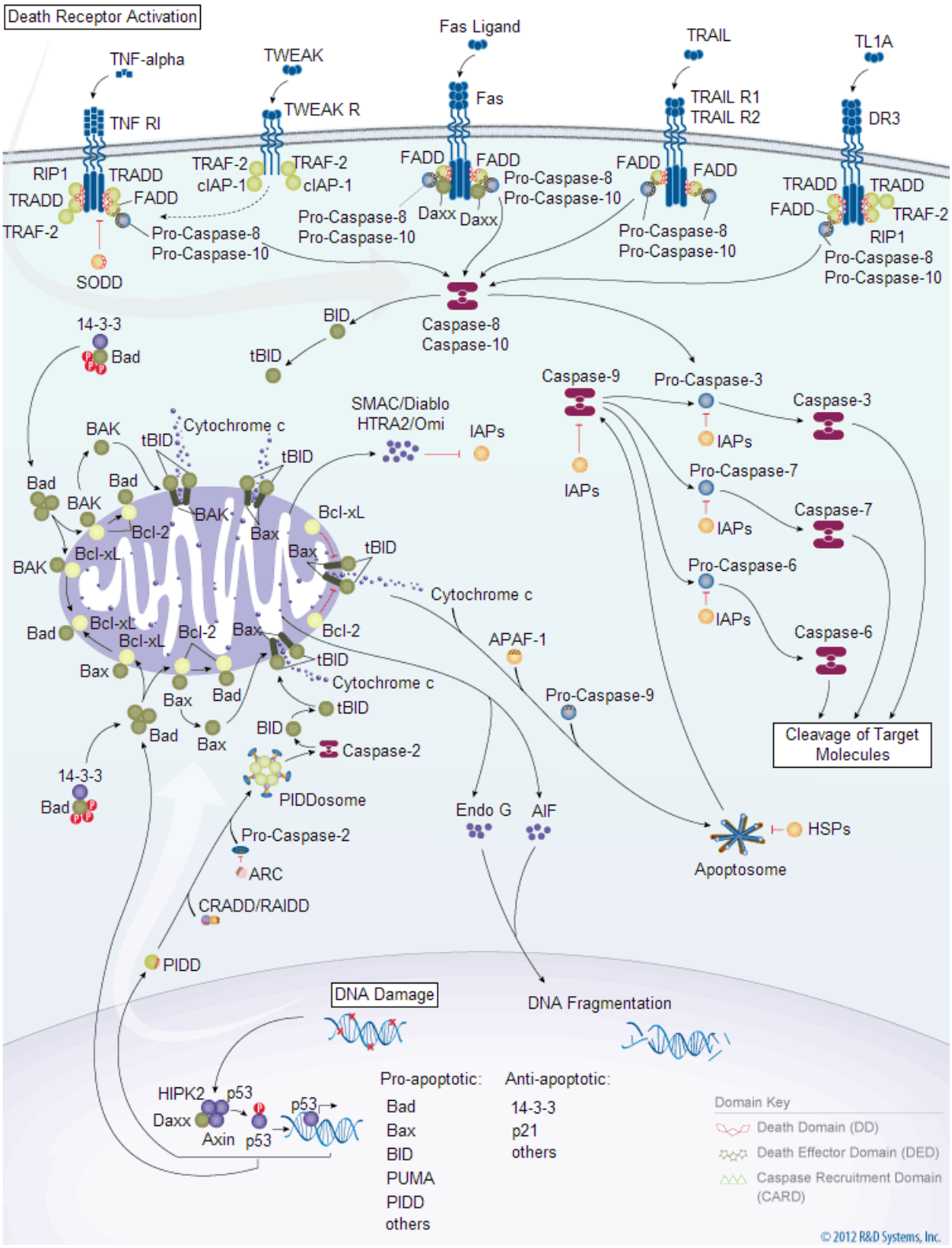

Figure 9 apoptosis signaling pathway (R&D Systems, 2012)

The process of cell replication and division to daughter cells is known as cell cycle. During the M-phase the cell is dividing. So called checkpoints (Figure 10) within this circle are passed before a cell can go from one to the next phase. When cells reduce their rate of protein synthesis and are not actively proliferating, they are arrested in the G_1 phase. In the S phase the cell is replicating DNA. In G_2 the cell is preparing for mitosis. Additionally there can be a Sub-G1 is referred to DNA damage and apoptotic cells. To characterize in which phase the cells are flow cytometry can be used. Nuclear DNA can be visualised with fluorescent dye where the DNA content can be determined. (El-Aouar Filho et al., 2017; Liou et al., 2016; Roche et al., 2011)

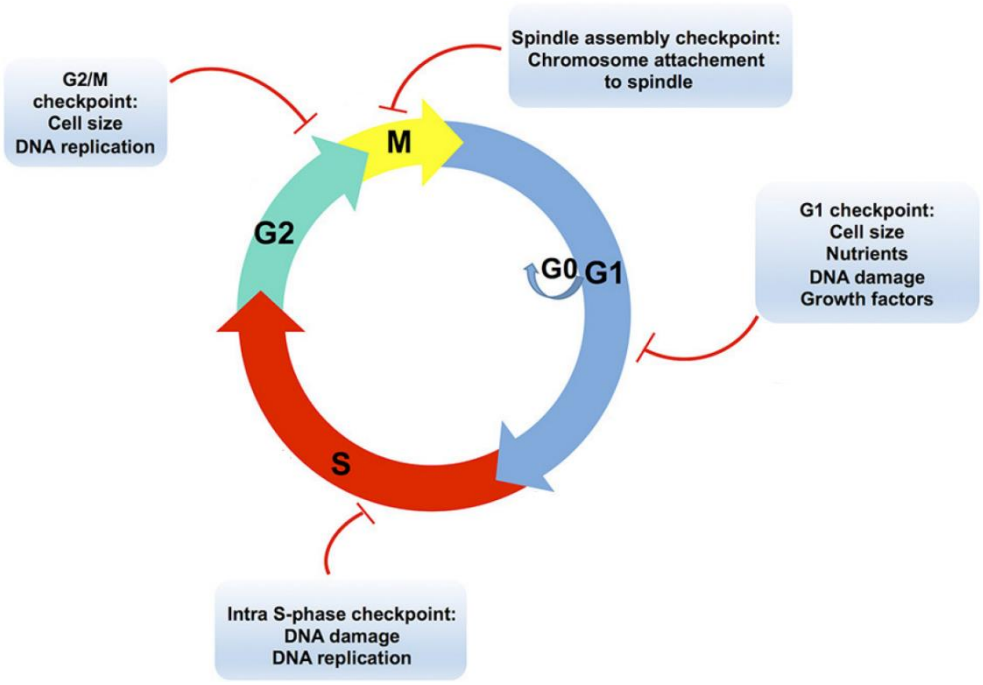

Figure 10 cell cycle (El-Aouar Filho et al., 2017)

3. Materials and Methods

3.1. Cell culture

3.1.1. Cell line HL 60

HL 60 cells were originally gained from a 36 year old women with acute promyelocytic leukaemia (PML) in 1977. PML is characterised by an accumulation of immature white blood cells (promyelocytes) in the bone marrow. Mutations in the gene code for tumour suppression causes uncontrolled proliferation of white blood cells.

The doubling time of HL 60 cells is about 36 to 48 hours and they proliferate continuously in a RPMI medium. (Ramachandran et al., 2014)

3.1.2. Cell line THP-1

THP-1 cells were originally gained from an one year old male infant with acute monocytic leukaemia. The doubling time is about 19 to 50 hours.

After every two to three days the cell suspension was sub cultured in fresh RPMI 1640 media.

To avoid contamination of the cell line and to assure personnel's and environment's safety, every working step was conducted in a laminar flow hood to maintain sterile criteria. Before transferring the instruments and materials into the laminar they and the surface were disinfected with ethanol (v/v 70%). To avoid contamination after usage, the surface was disinfected and the UV-lamp was switched on.

3.1.3. Media preparation

The RPMI 1640 media was prepared under sterile conditions using sterile membrane filtration. Streptomycin and penicillin were added with a final concentration of 100 µg/mL streptomycin and 100 U/mL penicillin.

RPMI 1640 was from gibco® by life technologies™ powder; [+] L-glutamine, [-] sodium bicarbonate; add: 2.0 g/L $NaHCO_3$; LOT 1930248; exp.: 2020-08-30

Streptomycin and penicillin were from Corning – Antibiotic antimycotic solution, 100X, LOT 30004130, exp.: 2019-12-01

3.1.4. Thawing of the HL-60 cell line

The cells (vial nr. 2-2; 13; HL-60; 105/11/24) were stored in liquid containing Dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS). For defrosting the vial was placed in a water bath at 37°C and afterwards centrifuged for 5 min with 10x1000 rpm at room temperature. The DMSO containing supernatant was removed and the remaining cell pellet resolved in 1 mL RPMI 1640 The cell suspension was transferred into a small petri dish containing 5 mL RPMI.

3.1.5. Cryopreservation

To secure cells of a low passage number and to create a working cell bank for future use, HL-60 cells were frozen and stored in a liquid nitrogen tank.

A cell pellet was dissolved in 10% DMSO in FBS. The vial was stored for one week at -79 °C before it was transferred into a liquid nitrogen tank.

3.1. DNA – staining – apoptotic bodies

Cells were seeded at a density of 5 x 10^5 cells/mL treated with drugs and incubated at 37 °C for 12 h. 5 µL of suspended cells were mixed on a slide with an equal volume of acridine orange solution (10 µg/mL in PBS). Green fluorescence was detected between 500 and 525 nm by using an Olympus microscope (CK40, Olympus America, Inc., Lake Success, NY).

3.2. Cell proliferation assay

3.2.1. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay is a homogenous cell viability assay used for 96-well plates. Only viable cells with active metabolisms can convert MTT into a purple coloured formazan product. The absorbance can be measured in a plate reader spectrophotometer at 570 nm. Factors which can affect the signals are the incubation time, the number of viable cells and their metabolic activity.
(Strovel et al., 2004)

Cells were seeded at a density of 2 x 10^5 cells/ mL into 96-well plates, treated with the drugs in different concentrations. Control cells were treated with DMSO to gain a final concentration of 0,05 % (v/v). After an incubation time of 48 h 0,02 % MTT solution was added to well, incubated for 60 minutes at 37°C and centrifuged for 10 min. The supernatant was discarded and the MTT-formazan crystals, formed by viable cells, were dissolved in 50 µL DMSO. The absorbance was measured at a wavelength of 570 nm.

3.3. Apoptotic ratio assay

3.3.1. Flow cytometry

Before the flow cytometry was conducted, the cell suspension was harvested at a cell density of 10^6 cells / mL. A 24 well plate was used, filled with 1 mL cell-suspension and treated with the desired drug and concentration. All treatments were done as triplicate. After 48 h of incubation at 37°C and 5% CO_2 atmosphere, the cell-drug suspension was transferred into a 1.5 mL Eppendorf tube and centrifuged at 3000 rpm at 4°C for 10 min. The cell pellet was washed twice with 1 mL PBS and centrifuged at 3000 rpm at 4°C for 10 min. For the cell fixing the pellet was dissolved in 200 µL PBS and 800 µL EtOH (99%) stored overnight at -20 °C and centrifuged at 3000 rpm at 4°C for 10 min. The cell pellet was washed twice with 1 mL PBS and centrifuged at 3000 rpm at 4°C for 10 min, before it was dissolved in hyptertonic buffer (0.5% TritonX + 0.5 µg/mL RNAse [10 mg/mL in 1x PBS]) and incubated at 37°C for 15 min. For cell staining 1 µL Propidium iodide [1 mg/mL] was added. The solution was gently mixed, incubated at 37°C for 15 min and afterwards centrifuged at 3000 rpm, room temperature for 10 min. 600 µL were discarded and the remaining 400 µL were filtered through a mesh into a new Eppendorf tube, which lid was cut off and put into the flow cytometry machine.

(CytoFLEX - Beckman Coulter). CytExpert (Version 2.0.0.153 Beckman Coulter) was used to quantify the cell cycle stages.

3.4. DNA-extraction and electrophoresis analysis

HL60 cells were seeded at 2 x10^6 cells / mL, treated with the drugs in different concentrations and incubated at 37°C with 5% CO_2 atmosphere for 48 h. Afterwards the cells were transferred to 2 mL Eppendorf vials and centrifuged (T = 4°C, t = 5 min, 2500 rpm). The cell pellet was washed with phosphate-buffered saline (PBS), centrifuged, resolved in gold lysis buffer and stored in a hear block at 100°C for 10 min.

Gold lysis buffer ingredients

10 % glycerol, 1% Triton X-100, 1 mM EGTA, 10 mM NaF, 1 mM Sodium pyrophosphate, 20 mM Tris (pH 7.9), 100 µM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, 1 mM Sodiumorthovanadate (Na_3VO_4)

3.4.1. Protein concentration

The protein concentration was determined with Bio-Rad protein assay (CA 94547, Bio-Rad Laboratories, Hercules, Munich, Germany) using a 96 well plate. 200 µL BIORAD® was added to each well and bovine serum albumin (BSA) was added to get the following concentrations: 20, 16, 12, 8, 4, 0 µg/mL. For the samples 0,4 µL drug where added to 200 µL BIORAD®. After 15 min incubation in the dark, the absorbance was measured at 595 nm using a plate reader.

3.5. Western blot analysis

3.5.1. Protein Extraction

A density of 2x10^6 cells per wells was tried to be achieved, in order to collect enough protein for the Western blot analysis. 6 well plates were filled with 2 mL cell suspension, treated with the desired drug and drug concentration and incubated for 48 h at 37°C and 5% CO_2 atmosphere. The 2 mL cell-drug suspension was transferred into an Eppendorf tube and centrifuged at 2500 rpm and 4°C for 5 min. The cell pellet was washed twice with 1 mL PBS and centrifuged at 3000 rpm and 4°C for 5min. The cells were disrupted dependend on their pellet size in 50 – 100 µL gold lysis buffer, incubated for 30 min on ice and afterwards centrifuged at 14000 rpm and 4°C for 15 min. 100 µL supernatant were transferred into a new Eppendorf tube and treated with 25 µL 5x sample buffer, incubated in a heating block at 100°C for 10 min. For storage in between the experiments, the proteins were frozen at -20 °C.

3.5.2. Bradford – Protein concentration determination

In order to load the SDS-PAGE with 50 µg protein the concentration was determined, using Bradford. A 96 well plate was filled with 200 µL BIORAD and treated with 0.4, 0.8, 1.2, 1.6, 2.0 µL BSA [2 mg/mL] and 0.4 µL sample. Incubated for 15 min in the dark and the absorption was measured with a 96-well plate reader at 595 nm.

3.5.3. Electrophoresis SDS-PAGE

Depending on the proteins, which wanted to be detected by antibodies in the Western Blot analysis, the resolving geld and stacking gel were prepared, transferred to the electrophoresis-gadget, filled with buffer and loaded with 50 µg protein sample and 2x protein marker. The running conditions for the stacking gel were 100 V for 45 – 60 min and for the resolving gel 120 V 60 – 90 min. If higher voltage (140 V) was used, due to time problems, the gadget was put into the fridge at 4°C.

The samples (50 µg of protein) of total cell lysates or cytosolic were size fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis

3.5.4. Blotting

After the electrophoresis the buffer was discarded and the gel was taken out, the stacking gel was removed and the resolving gel was put into the 'transferring gadget' in the following order: black side – sponge – filter – gel – membrane – sponge – grey side. The gadget was put inside a tank and filled up with transfer buffer (MeOH : 5x transfer buffer : ddH_2O = 1:1:3) The blotting conditions were 450 mA at 4°C for 60-90 min.

The proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (PVDF; Millipore Corp., Bedford, MA).

The membranes were blocked using a blocking solution (20 mM Tris–HCl pH 7.4, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide). (Liu et al., 2011)

The membrane was then further incubated with specific antibodies, at appropriate dilutions, using blocking solutions such as anti- Bcl-2, anti-Bcl-XL, anti-Bax, anti-GADD153, anti-b-actin (Santa Cruz Biotechnology), anti-PARP (UBI, Inc., Lake Placid, NY), anti-Bid (Transduction Laboratory, Lexington, KY), and anti-DFF45/inhibitor of caspase-activated DNase (ICAD) antibody (MBL, Naka-Ku, Nagoya, Japan) at room temperature for 1 h. The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated with horse- radish peroxidase (Transduction Laboratories, Lexington, KY) and detection was achieved by measuring the chemiluminescence of the blotting agents (ECL, Amersham Corp., Arlington Heights, IL), by exposure of the filters to Kodak X-Omat films.

3.5.5. Membrane blocking and antibody treatment

The membrane was placed in a small plastic box, around 20 mL blocking solution (BSA) were poured in and placed on an orbital shaker for 30 – 45 min. The membrane was washed twice with TBS and put on the orbital shaker for 30 min. The membrane was cut according to the antibodies, which were chosen before and the small pieces were put into the antibody solution for overnight incubation on an orbital shaker in the fridge. Membrane washing with TPBS was conducted twice, before the treatment with the secondary antibody, which was either anti-mouse or anti-rabbit, depending on the before used primary antibody, incubated for 1h on the orbital shaker and afterwards washed twice with TPBS.

For visualitation a ChemiDoc™ imaging system was used. Before themachine could be used, a warm up time of 30 min had to be considered. 1 mL of reagent ECL A and 1 mL of reagent

ECL B were mixed and poured on the membrane. The target protein level was measured based on the chemiluminescent activity.

3.6. Analysis of Mitochondrial Transmembrane potential

The disruption of the outer mitochondrial membrane and the contribution to the release of cytochrome c is caused by a decrease of mitochondrial membrane potential $\Delta\Psi_m$, which is an indicator of apoptosis. (Cheng et al., 2005; Ly et al., 2003)

Flow cytometry was used to monitor the mitochondrial transmembrane potential flow: HL-60 cells were exposed 12 h and 24 h to 25 µM and 50 µM curcumin, aminocurcumin and calebin-A, then 40 nM 3,3'-dihexylocacarbocyanine [DiOC6(3)] (Molecular Probes, Eugene, OR) was added. After cell staining for 15 min at 37°C fluorescence was measured. (M. H. Pan et al., 2001)

4. Results and discussion

4.1. Cell pictures

In the following pictures in 200x magnification after 48 h of treatment the cell morphology is displayed, as well the difference between the different treatments, which had been applied to both cell lines.

The blast-like cells in the control display the following morphology: round and slightly oval shaped cells with large rounded nuclei. The control picture are in accordance with the ones displayed by (Chen et al., 2008).

In all treatments at all concentrations a reduction in cell number, as well cell shrinkage and an increase in cell debris including cell fragments can be seen, especially for the 100 µM. A change in the cell shape from round and slightly oval to a blebbing membrane. Those are all characteristics for cell death. (Su et al., 2015)

Other researchers suggest colouring agents to make the cell compartments more visible, which makes a comparison with the results, which have been found, quite difficult. (Maniwa et al., 2015)

4.1.1. Curcumin

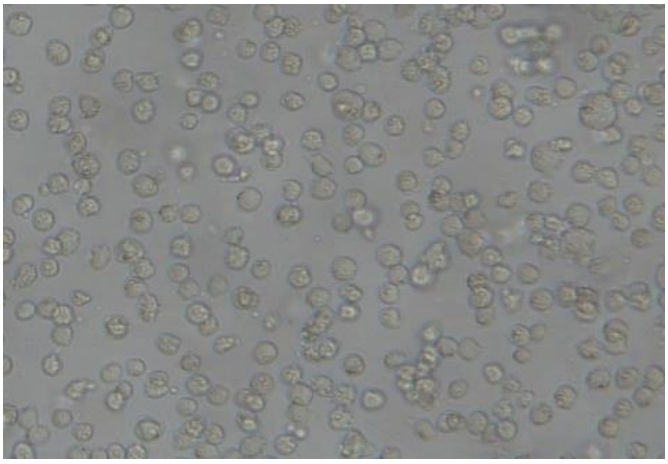

Figure 11 curcumin control - untreated HL 60 cells

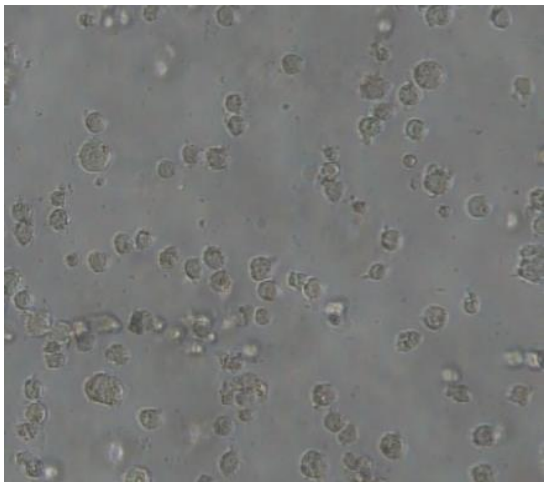

Figure 12 curcumin - 25 µM

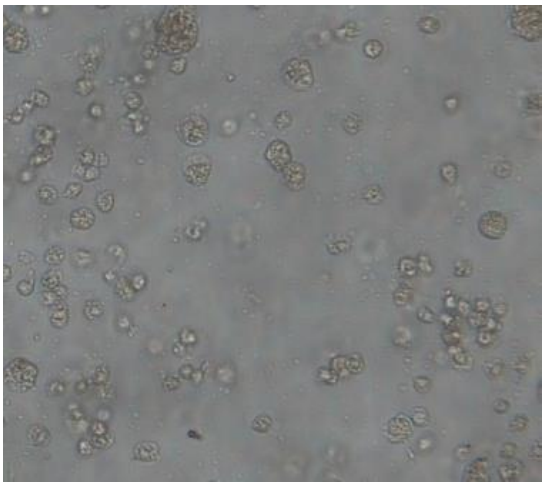

Figure 13 curcumin - 50 µM

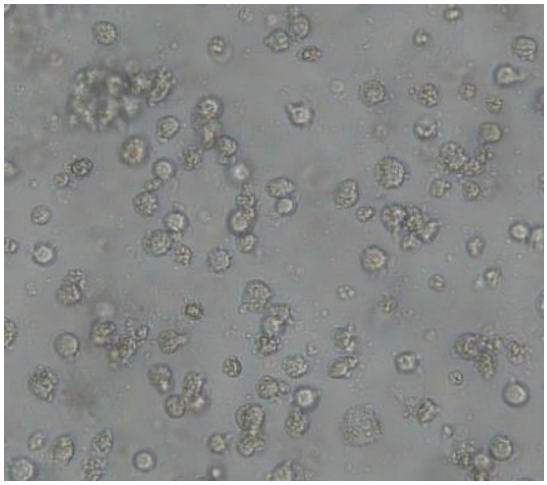

Figure 14 curcumin - 75 µM

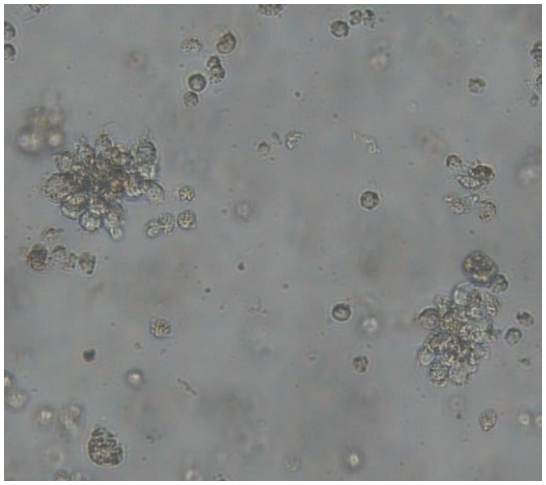

Figure 15 curcumin - 100 µM

4.1.1. Aminocurcumin

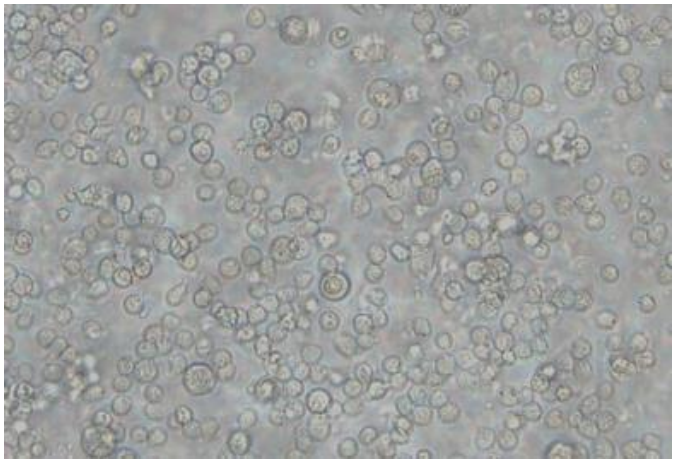

Figure 16 aminocurcumin control - untreated HL 60 cells

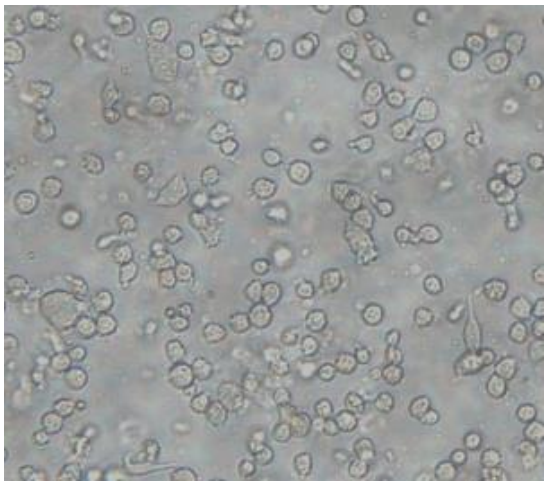

Figure 17 aminocurcumin - 50 µM

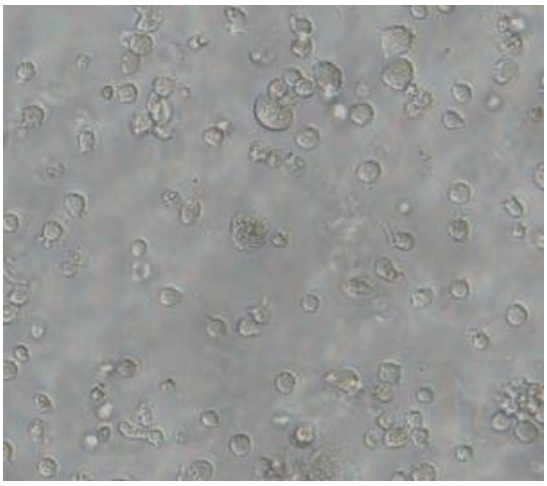

Figure 18 aminocurcumin - 25 µM

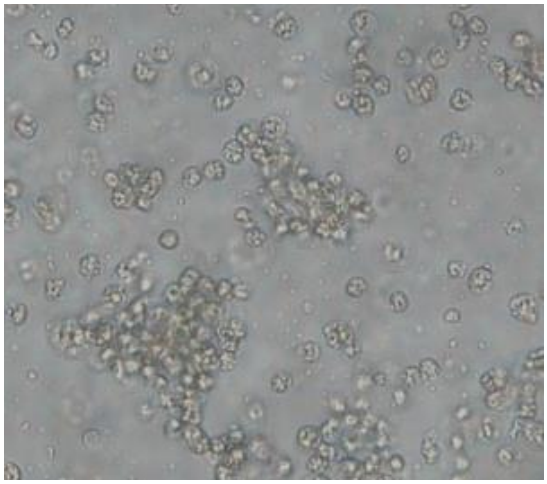

Figure 19 aminocurcumin - 75 µM

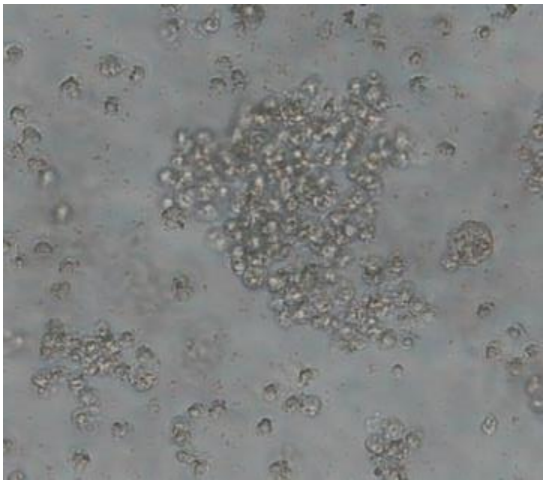

Figure 20 aminocurcumin - 100 µM

4.1.1. Calebin A

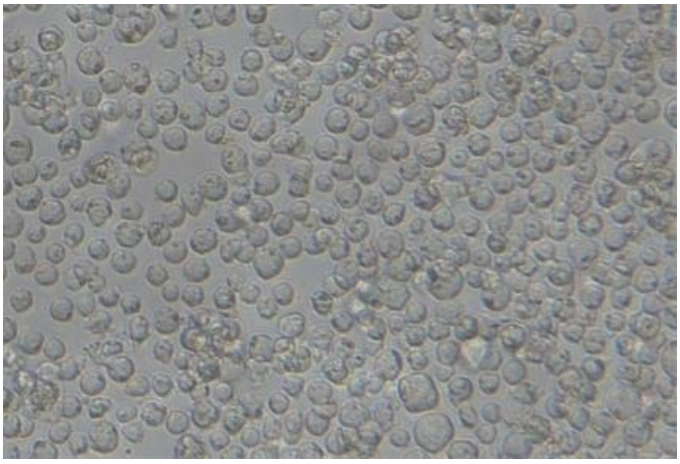

Figure 21 calebin A control - untreated HL 60 cells

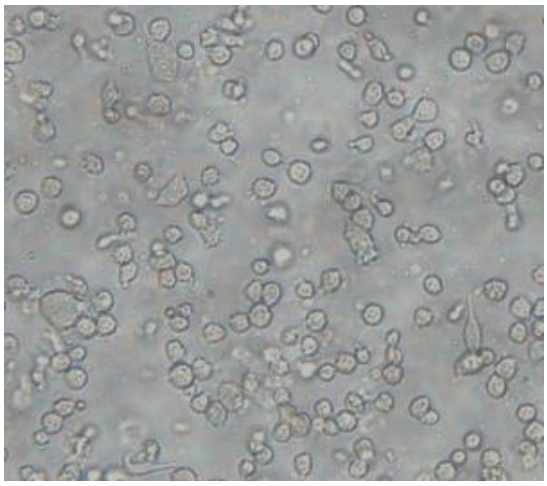

Figure 22 calebin A - 50 µM

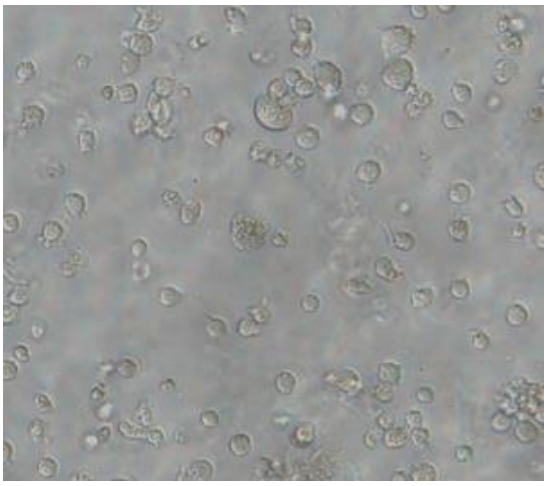

Figure 23 calebin A - 25 µM

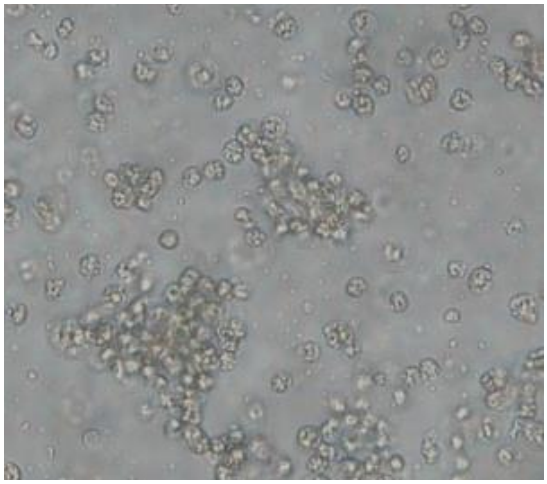

Figure 24 calebin A - 75 µM

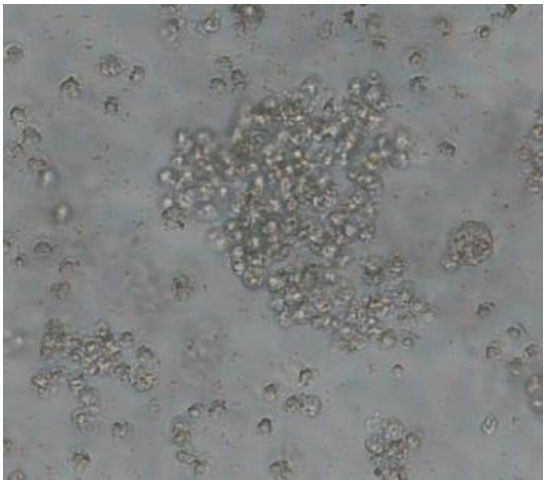

Figure 25 calebin A - 100 µM

4.1. DNA – staining – apoptotic bodies

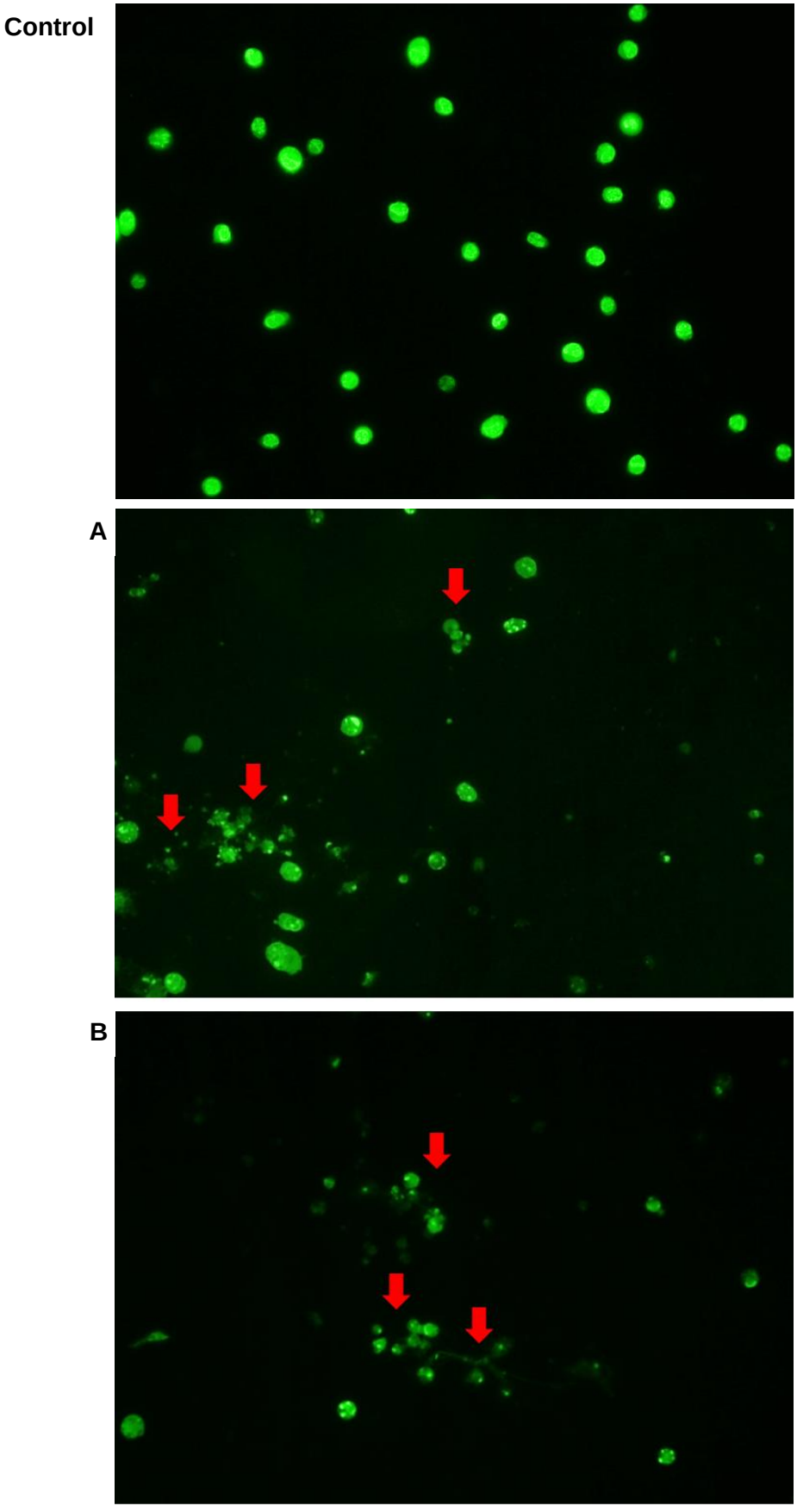

Figure 26 DNA staining
(control = untreated; A = 25 µM curcumin; B = 50 µM curcumin)

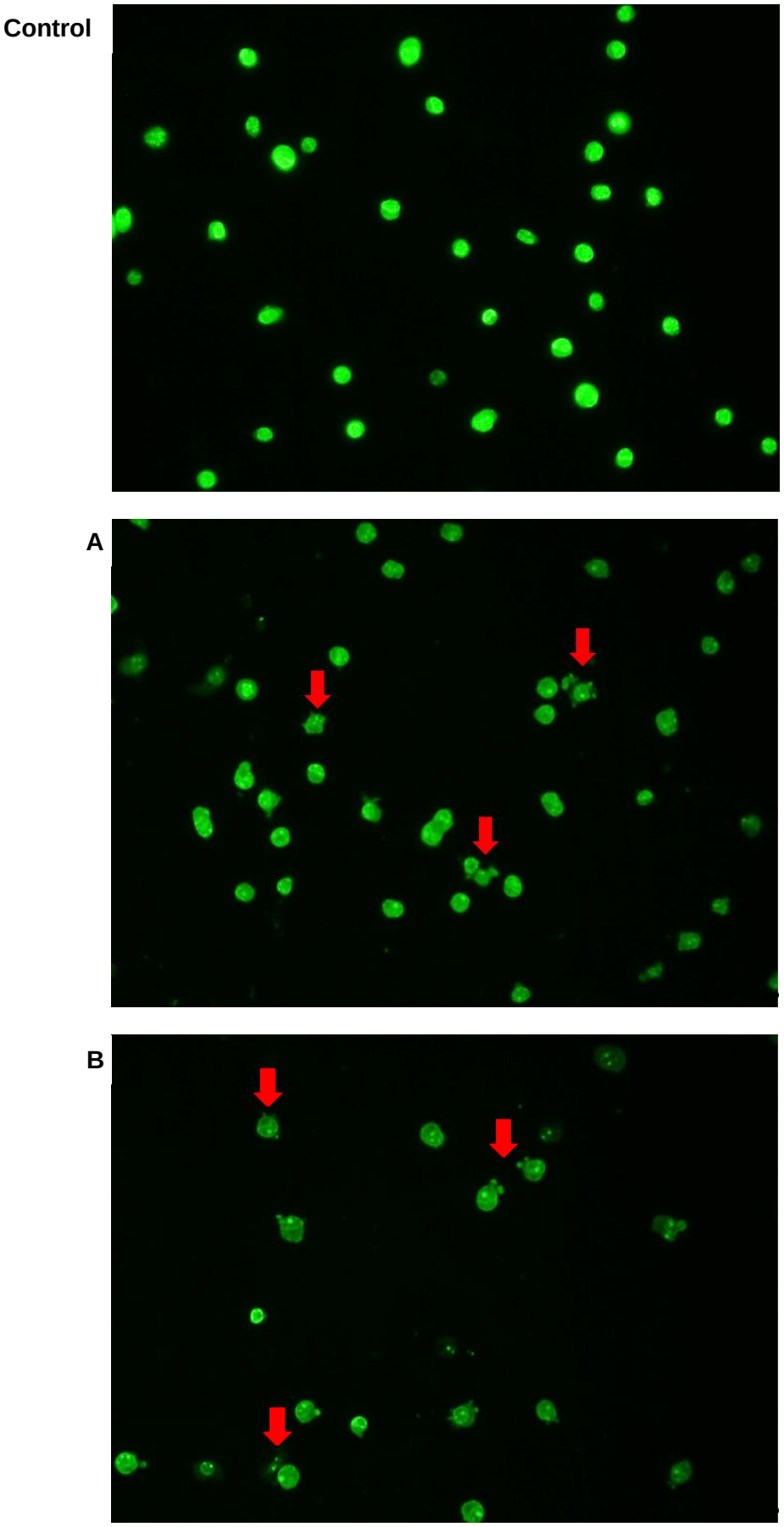

Figure 27 DNA staining
(control = untreated; A = 25 µM aminocurcumin; B = 50 µM aminocurcumin)

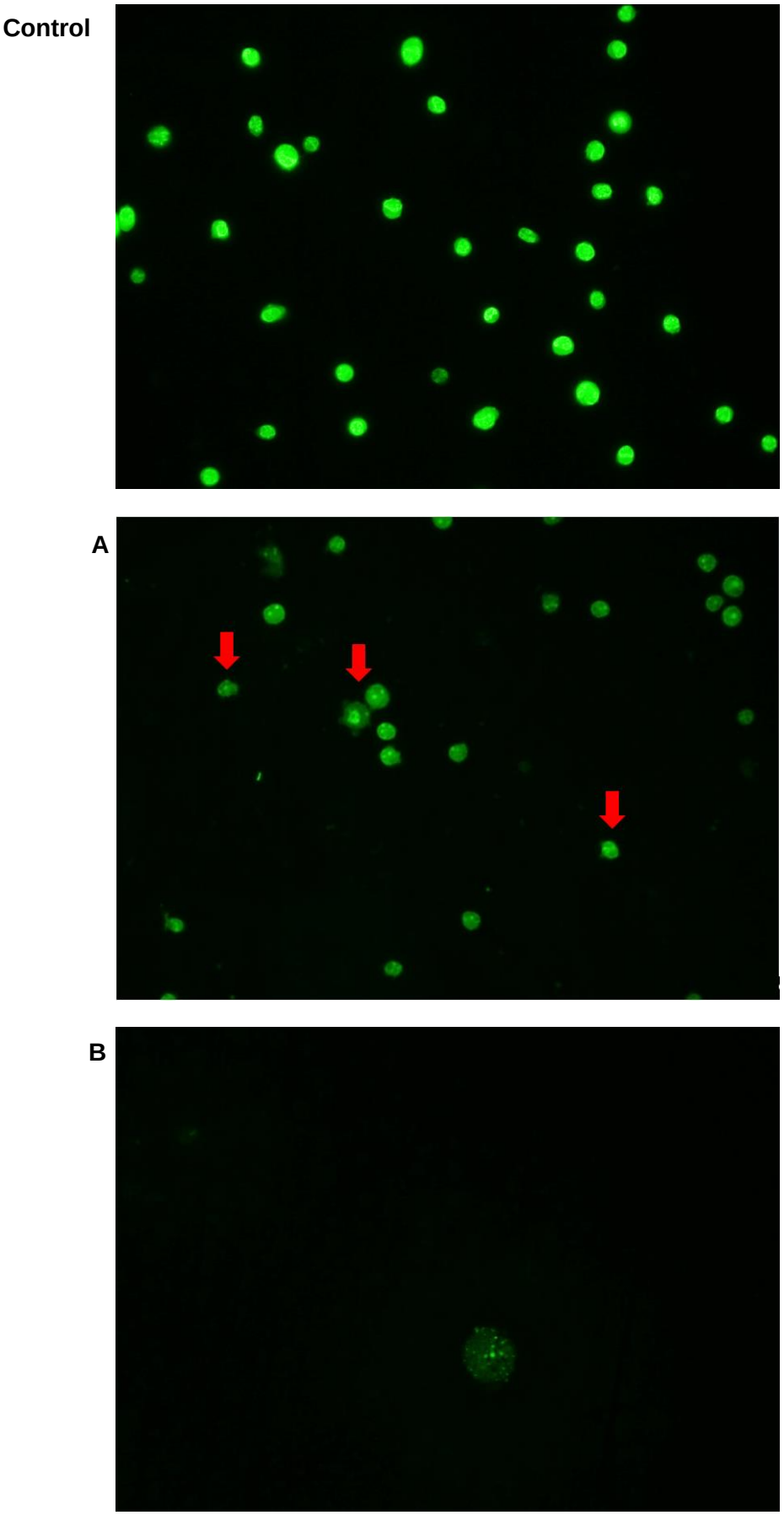

Figure 28 DNA staining
(control = untreated; A = 25 µM Calebin A; B = 50 µM calebin A)

All treatments show a visible difference showing typical morphologic futures of apoptosis, such as cell shrinkage and DNA-fragmentation compared to the control. Curcumin shows the biggest difference, there is a noticeable change in cell number. At the treatment of 50 µM calebin-A no cells could be detected.

Apoptotic cells can be distinguished from viable showing a homogeneously bright staining in nuclei, which is an indicative of induced apoptosis. (Liu et al., 2011)

Ly et al., 2003 describes that during apoptosis the nucleus becomes convoluted and is demerged in small fragments, which are encapsulated within apoptotic bodies. Additionally, due to cell breaking, cell fragments containing apoptotic bodies are spread around the cells. This is visible in Figure 26, Figure 27 and Figure 28.

All treatments, except 50 µM calebin-A, show apoptotic bodies, which are small bright shining dots within the cell. Therefore, apoptotic cell death can be assumed.

4.2. MTT assay

The cell viability was calculated as the ratio of with drug treated viable cells with to the untreated viable cells.

It is interesting, that both cell lines (Figure 29, Figure 30) show at the treatment's lowest concentration firstly an increase, before the cell proliferation is decreased.

4.2.1. HL60 cell line

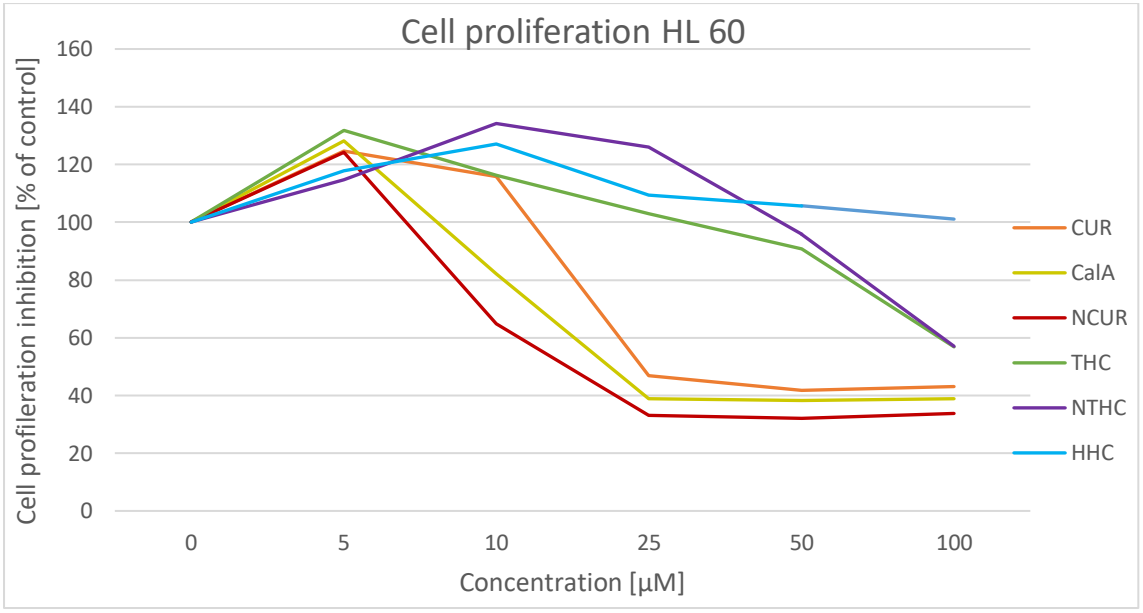

Figure 29 MTT assay result HL60 cell line

Figure 29 shows the result of the MTT assay, which was conducted. Interesting is that it seems to be that the cell proliferation increases at the treatment's lowest concentration. before it either increases, which is the case for NTHC and HHC or decreases, which applies for CUR, CalA, NCUR, THC.

Calebin-A and aminocurcumin seem to be more effective, even in lower concentrations, compared to curcumin. According these results, curcumin, calebin-A and aminocurcumin were selected to be further investigated using Western Blot and Flow Cytometry.

In previous studies has been observed already, that curcumin is able to reduce the cell proliferation of HL60 cells, which supports the decrease in cell proliferation.
(Kunnumakkara et al., 2008; L. X. Wu et al., 2003).

Compared with the results of Dikmen et al., 2011 the result complies with the decrease in general, but does not show an increase at concentrations 5 and 10 µM. A further look into the materials and methods section showed a small difference, because 0,04 M HCl in isopropanol was used instead of DMSO to dissolve MTT-formazan crystals.

It has been discussed that MTT reduction is a marker reflecting the viable cell metabolism and not the cell proliferation in specific. This would explain that the cell viability for low concentrations is not increasing but the metabolism is increasing due to cellular stress, caused by the treatments. (Strovel et al., 2004)

It is also suggested to not use the outer wells due to evaporation. Additionally some drugs can influence neighbouring wells, maybe this affected the experiment set up as well, due to usage of all 96-wells of the plate. (van Meerloo et al., 2011)

A mycoplasma infection, which can't be visually seen, also could be the reason for the increased proliferation. (Denecke et al., 1999)

Another possibility could be due to cell's adaption to cell culture genetic and phenotypic drifts of cell lines. (Cree, 2011b).

4.2.2. THP-1 cell line

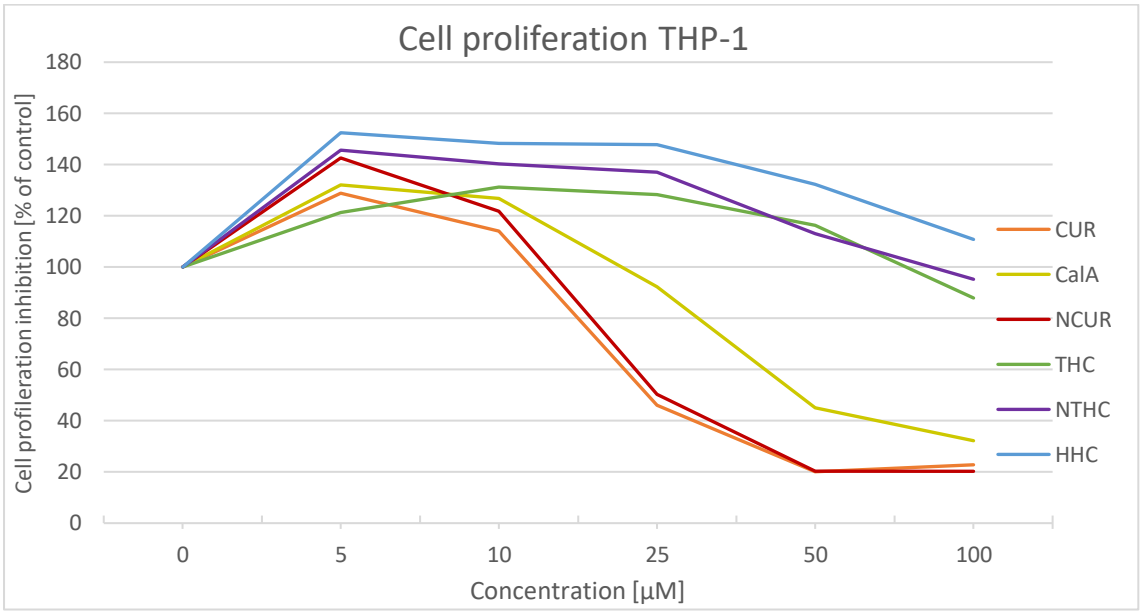

Figure 30 MTT assay result THP-1 cell line

Figure 30 shows the first result of the MTT assay for the THP-1 cell line. The same phenomenom as in Figure 29 already can be seen, appears here as well. The increase in cell proliferation at the lowest concentration of all treatments. The least effective substances are Hexahydrocurcumin, Aminotetrahydrocurcumin and Tetrahydrocurcumin. It seems, that they are increasing the cell proliferation until 50 µM, which made this three drugs not interesting for further investigation. Curcumin resulted in being the most effective substance in interaction with the cell proliferation. Aminocurcumin shows similar values and Calebin is not as effective as the other two. All three substances were included in further investigation using Flow Cytometry.

4.3. Flow Cytometry

4.3.1. HL60 cell line

4.3.1.1. Curcumin

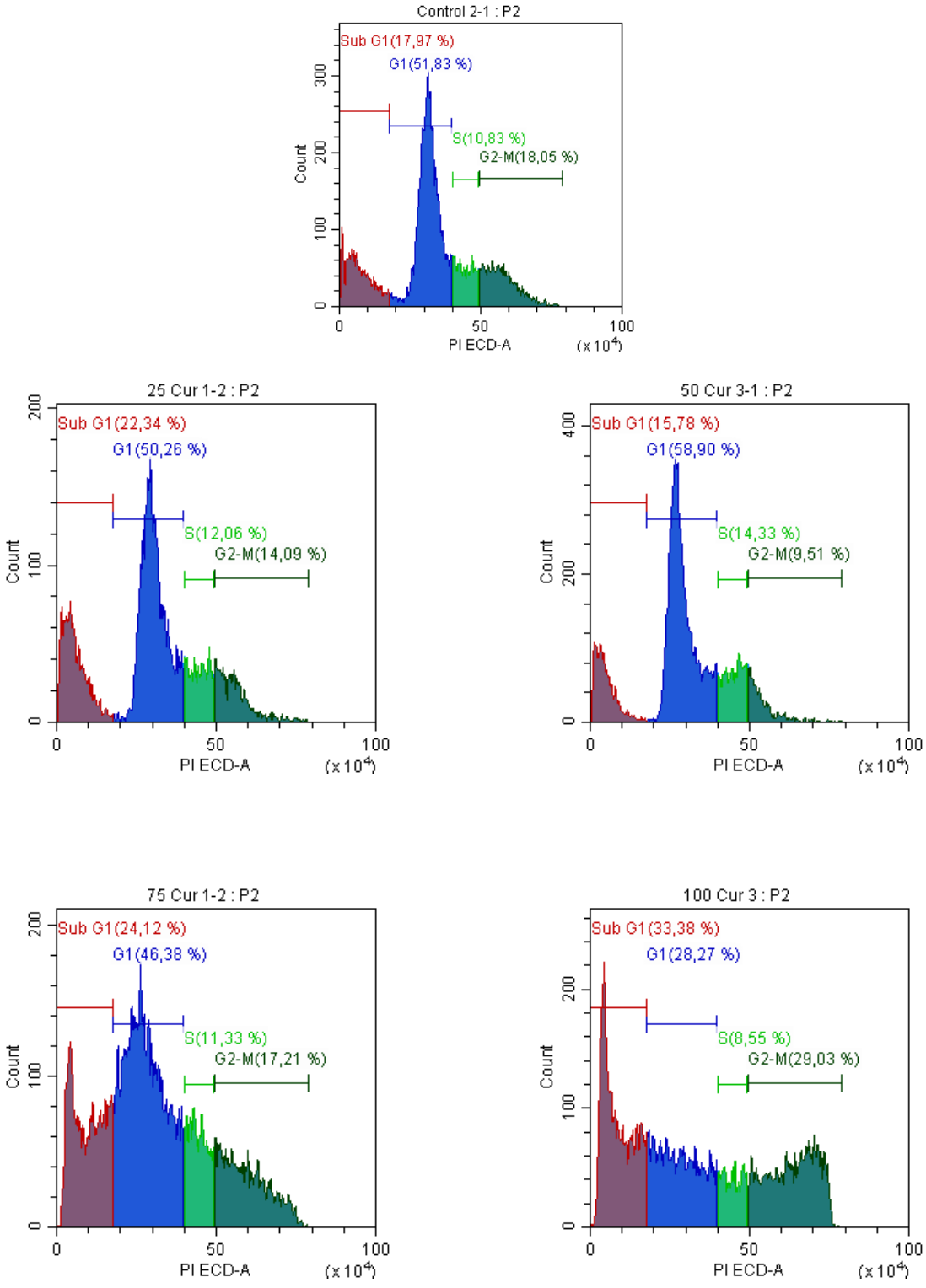

Figure 31 flow cytometry HL-60 cells with curcumin as treatment – the numbers indicate the concentration

4.3.1.1. Aminocurcumin

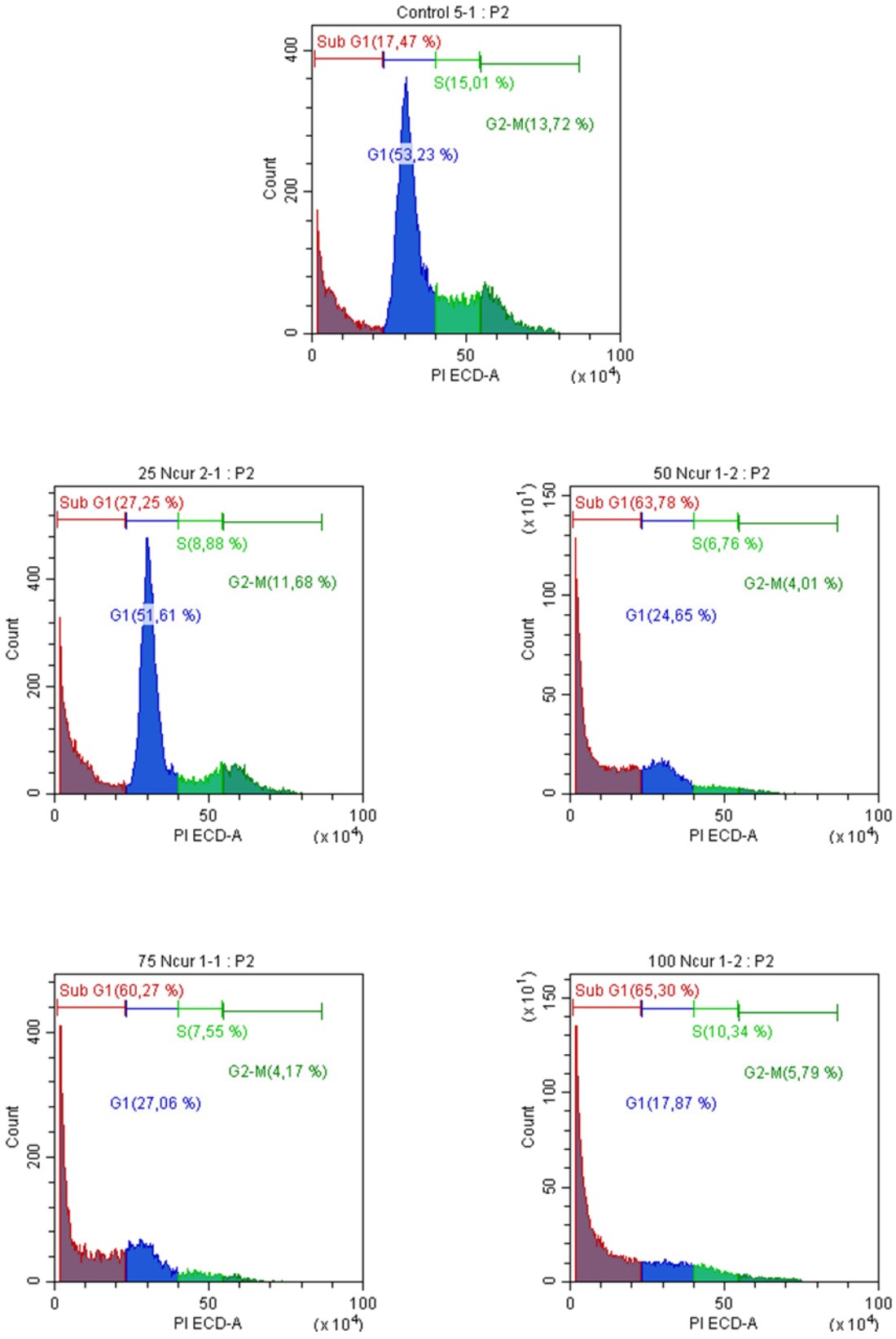

Figure 32 flow cytometry HL-60 cells with calebin-A as treatment – the numbers indicate the concentration

4.3.1.2. Calebin A

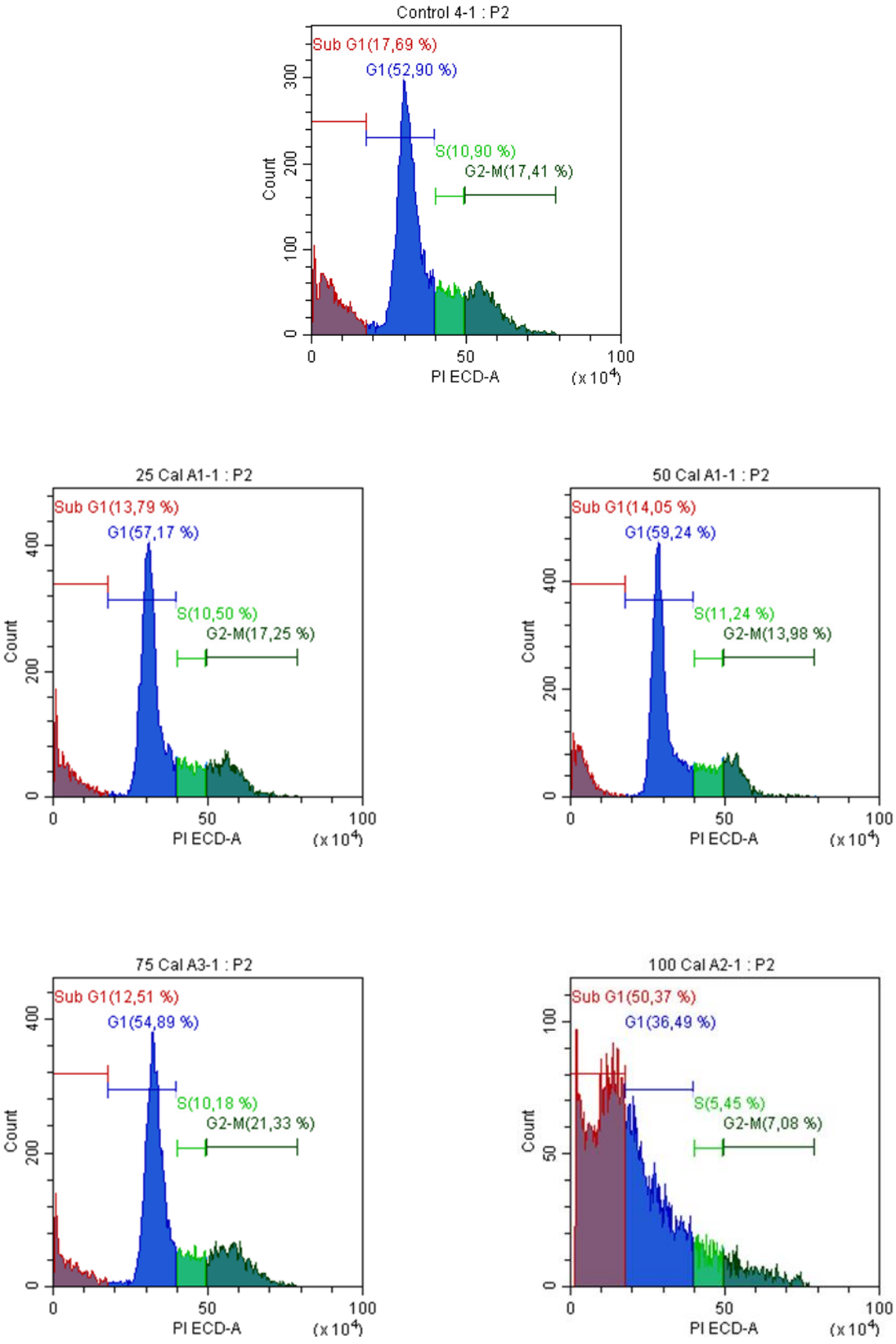

Figure 33 flow cytometry HL-60 cells with calebin-A as treatment – the numbers indicate the concentration

4.3.2. THP-1 cell line

4.3.2.1. Curcumin

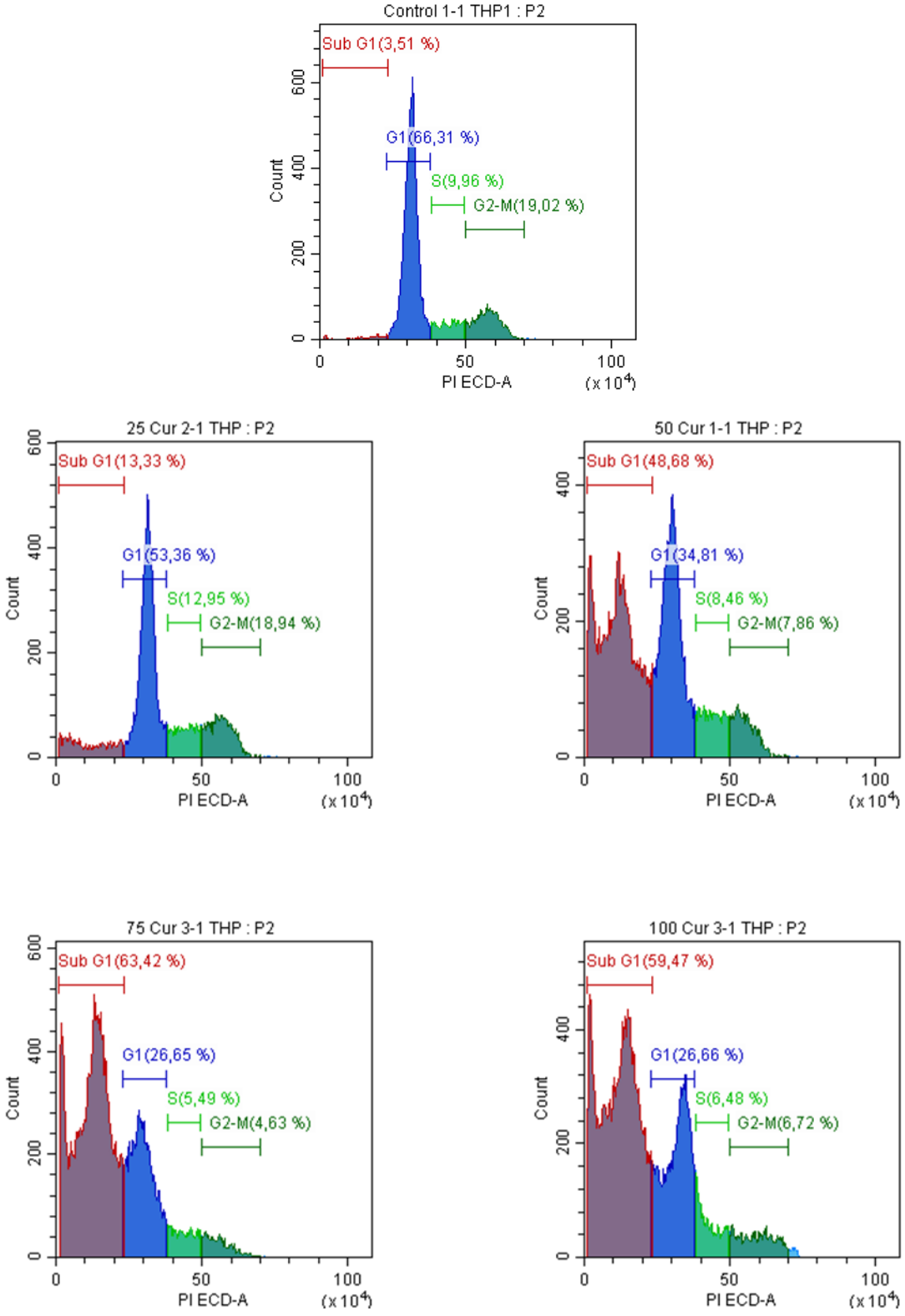

Figure 34 flow cytometry THP1 cells with curcumin as treatment – the numbers indicate the concentration

4.3.2.2. Aminocurcumin

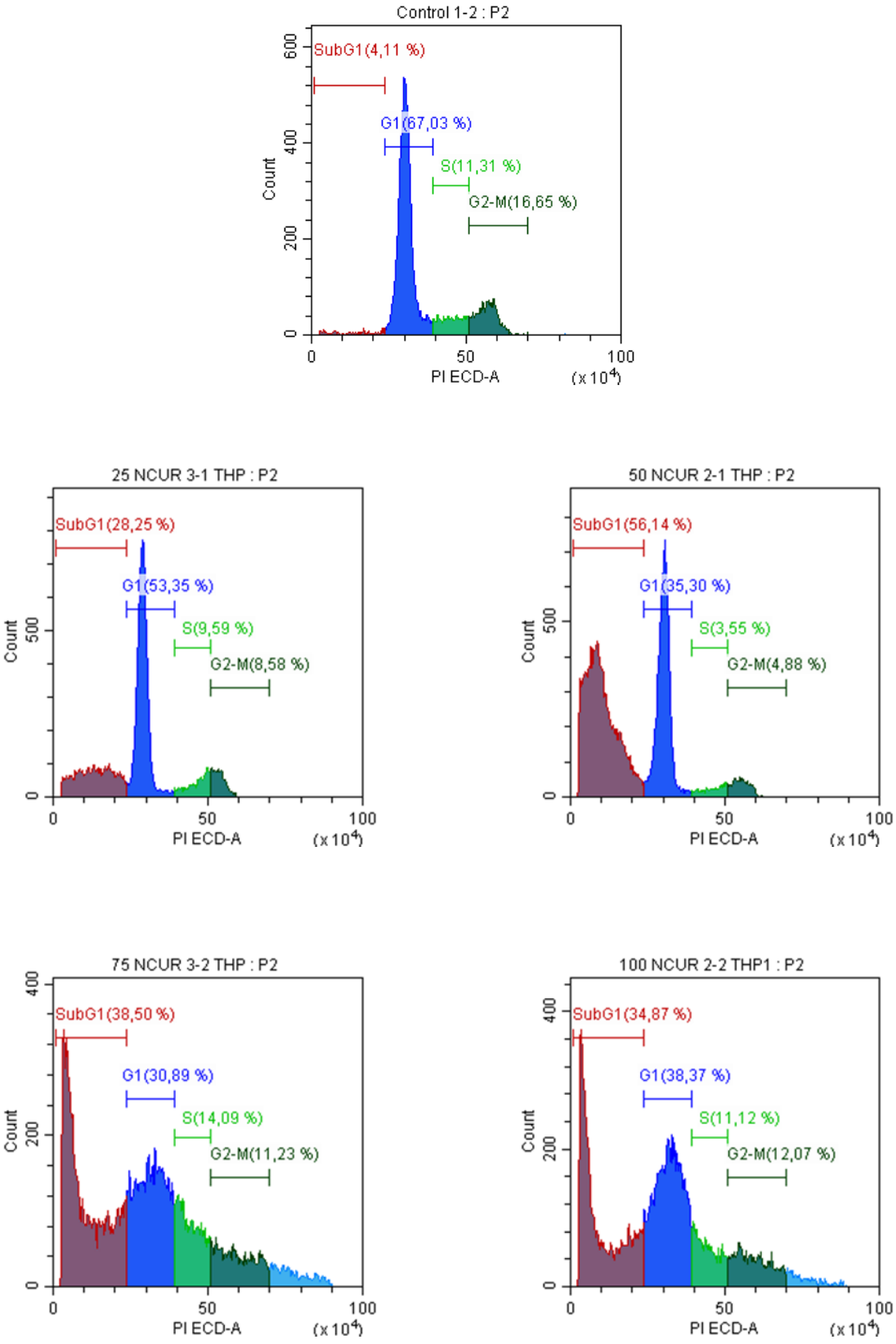

Figure 35 flow cytometry THP1 cells with aminocurcumin as treatment – the numbers indicate the concentration

4.3.2.3. Calebin-A

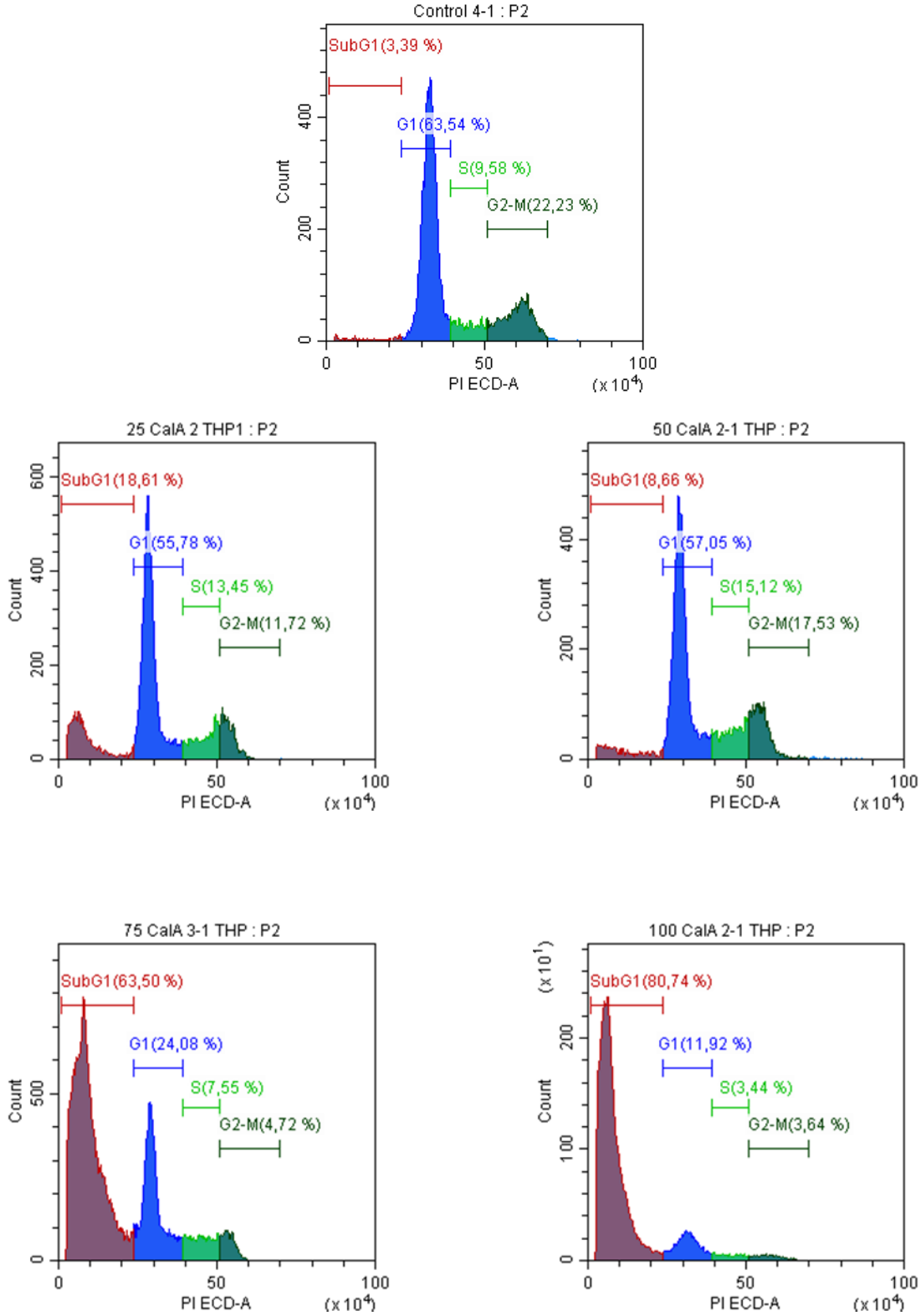

Figure 36 flow cytometry THP1 cells with calebin-A as treatment – the numbers indicate the concentration

The diagrams of the conducted Flow Cytometry for the HL60 cell line are shown in Figure 31, Figure 32, Figure 33 where unfortunately the control has already an enormous amount of cells in the Sub G1 phase. Therefore, it cannot be concluded, that the treatments trigger apoptosis and cause DNA-fragmentation. Possible reasons for this observation could be the long incubation of 48 h or that a contamination caused cell death or that the cell passage number was already too high. Clearly visible peaks for the Sub G1 phase using flow cytometry results, when leukaemia cells or other cancer cell lines have been treated with curcumin or calebin-A, have been shown already. (Dikmen et al., 2010; Kuttikrishnan et al., 2019; M. H. Pan et al., 2001; L. X. Wu et al., 2003; Yang et al., 2012)

The diagrams of the conducted Flow Cytometry for the THP1 cell line are shown in Figure 34, Figure 35 and Figure 36. Compared with the results from the HL60 cell line, the control always showed around 4% of cells in the Sub G1 area, therefore the comparison with all treatments, especially 75 µM and 100 µM curcumin and 50 µM, 75 µM and 100 µM aminocurcumin, an increase in the Sub G1 area is visible. According this data it can be suggested that all treatments can induce cell death.

A similar pattern of results, where curcumin after 24 h treatment increased the percentage of cells in the SubG1 cell cycle phase, was obtained by (Yang et al., 2012).

4.4. Membrane Potential

4.4.1. Curcumin

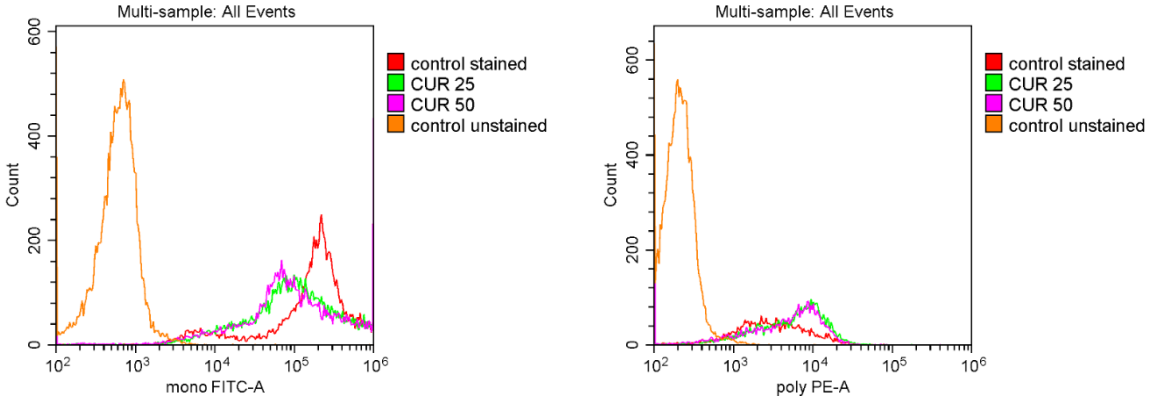

Figure 37 membrane potential 12 h after treatment with curcumin

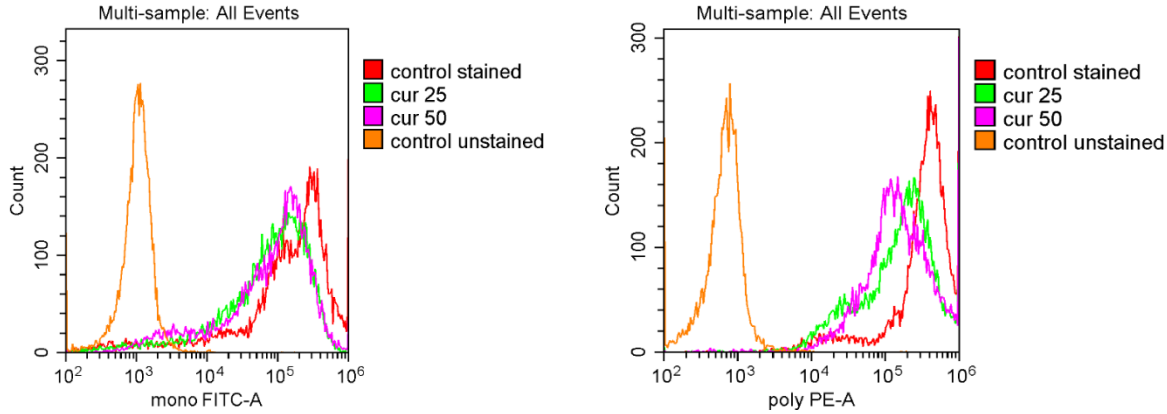

Figure 38 membrane potential 24 h after treatment with curcumin

In Figure 37 and Figure 38 a change in the membrane potential for the treatment with 25 µM and 50 µM curcumin is visible due to a peak shift to the left.

Similar results have been found for the treatment with curcumin in B-precursor acute lymphoblastic leukaemia, where a rapid reduction of mitochondrial membrane potential was mentioned. (Kuttikrishnan et al., 2019)

A change in membrane potential for HL60 cells undergoing apoptosis as a late occurrence in the apoptotic pathway was been reported already. (Ly et al., 2003).

4.4.2. Calebin A

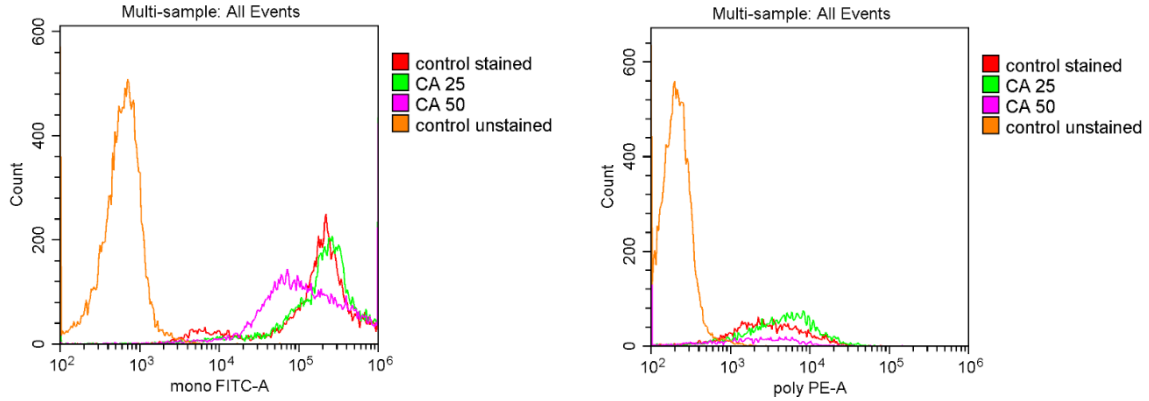

Figure 39 membrane potential 12 h after treatment with calebin A

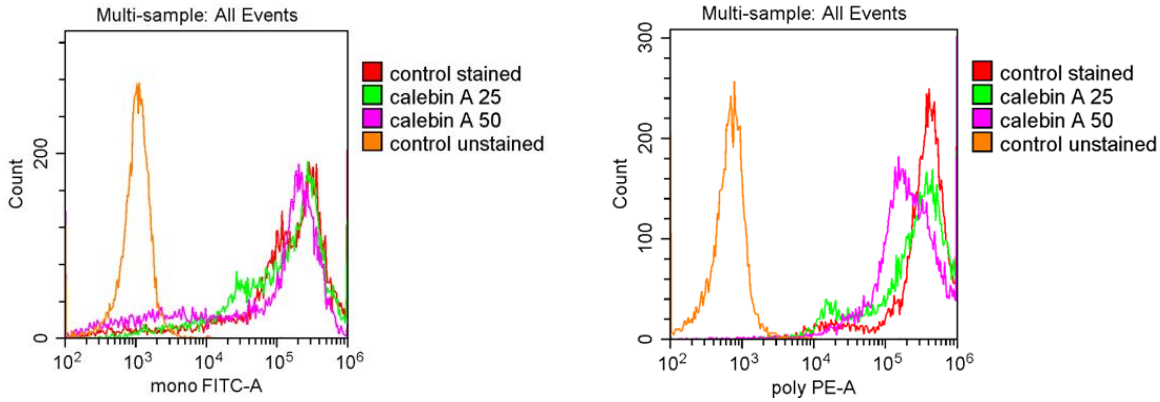

Figure 40 membrane potential 24 h after treatment with calebin A

In Figure 39 and Figure 40 the change of membrane potential is only visible for the treatment of 50 µM calebin A.

Comparable studies could not be found in the literature, hence the assumed loss of mitochondrial membrane potential still requires confirmation.

4.4.3. Aminocurcumin

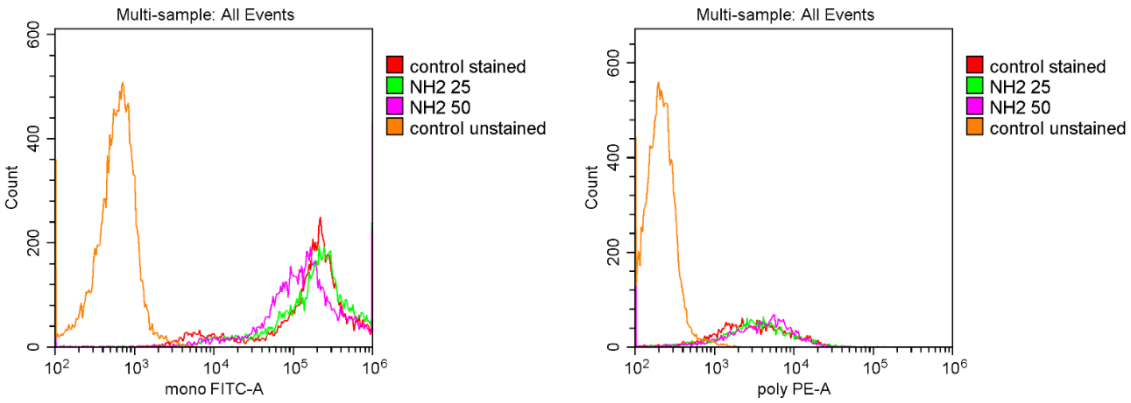

Figure 41 membrane potential 12 h after treatment with aminocurcumin

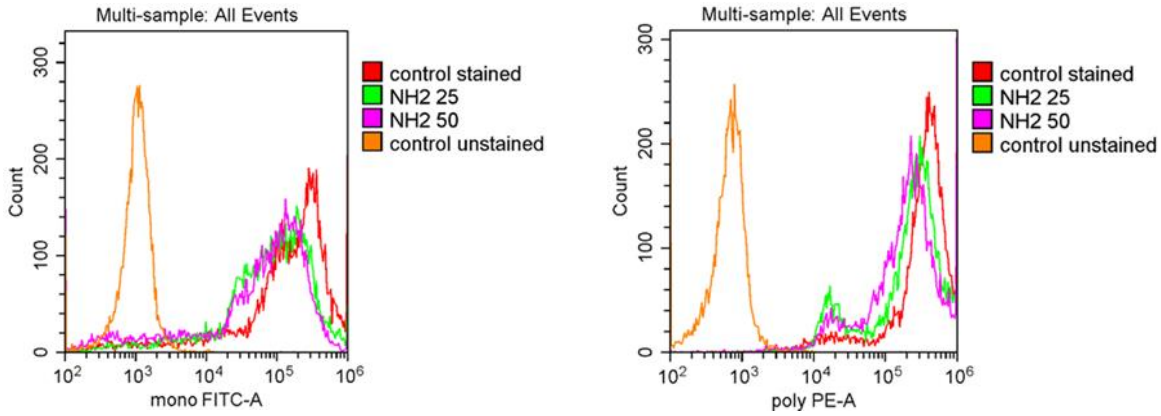

Figure 42 membrane potential 24 h after treatment with aminocurcumin

Figure 41 and Figure 42 show both a peak shift which indicates a change of membrane potential.

There are no comparable literature data available so far, therefore the assumption of a loss of mitochondrial membrane potential is still tentative.

4.5. Western Blot

4.5.1. HL60 cell line

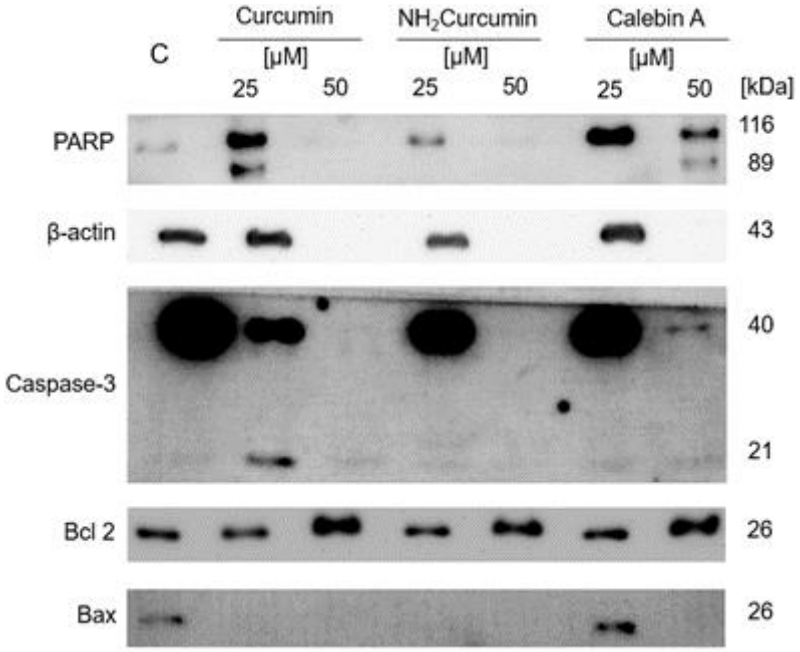

Figure 43 Western Blot HL60 cell line (C = control)

The Western Blot picture, shown in Figure 43, has no bands for PARP, β-actin, Caspase 3 and Bax in the lanes for the treatment of 50 µM curcumin and aminocurcumin. Bax shows only a band at the control lane C and treatment of 25 µM calebin A.

PARP shows only for the treatment of 25 µM curcumin and 50 µM calebin A the fragment at 89 kDa.

The loading control β-actin does not show any signal in any of 50 µM treatment lanes. This could be due to a not equal transfer of the proteins or the duration in combination with the high concentration of the treatment, that the protein activity was lowered or too many cells died, so that the protein concentration was not high enough to show any band.

Caspase-3 shows no band in all treatments for the 50 µM concentration. Either there is no band visible due to protein destruction, which would be compliant with no visible band at the loading control or the cleavage of caspase-3, which would be an indicator of the apoptotic pathway.

Bcl-2 appears to have an increase if the control and 25 µM are compared to the 50 µM treatment which is known to be a repressor of apoptosis. (Zimowska et al., 1997) found similar results for L1210 leukaemic cells. Their assumption is due to the exposure of oxidative stress the cell is naturally defending itself against apoptosis and cellular lipid peroxidation.

Bax only shows a visible band for the control and treatment of 25 µM calebin-A. It has been reported (Wood et al., 1998) that drug induced apoptotic cell death can cause Bax cleavage by capases and calpains and then is translocated to mitochondria. (Cheng et al., 2005) shows that Bax can produce small fragments, which was not the case in this experiment.

This could be due to the small protein size that during the blotting process the protein already detached from the membrane and got lost in the buffer because the time was too long.

The ratio of Bcl-2 to Bax within cells is important in how the cell responds to apoptotic stimuli. An Overexpression of Bax in T-Cells results in higher sensitivity to apoptotic stimuli like DNA damage. (Brady & Gil-Gómez, 1998)

5. Conclusion and outlook

The impact of curcumin, aminocurcumin and calebin-A on the reduced metabolic rate in both cell lines was clearly visible. The drugs seem to affect the HL60 cells already in a lower concentration compared to THP 1 cells. The induction of apoptosis with curcumin in monocytic leukaemia cells was already mentioned. (Yang et al., 2012)

Flow cytometry results assist the measured reduced metabolic rate where higher concentrations of the drug treatments lead to more DNA-fragmentation for THP-1 cells. The findings for HL 60 cells could not give a clear result, however fluorescence microscopy showed visually an increase in DNA-fragmentation, as well the microscopic analysis showed with increasing concentrations of curcumin, aminocurcumin and calebin-A an increase in cell debris and a reduction in cell number. The determination of the membrane potential showed a reduction, which induces cytochrome c release into the cytosol and therefore enabling a signal cascade resulting in apoptosis. Western Blot analysis didn't provide clear results to confirm the apoptosis inducing effect.

Overall the findings indicate a reduction in cell numbers and increased cell death, but apoptosis inducting effects cannot explicitly be confirmed. Therefore, more experiments would be needed to prove the assumption of apoptosis inducing effects.
Furthermore, there are several pathways, like MAKP or PI3K, in the regulation of apoptosis involved, therefore further research is necessary to investigate with which of those pathways the cell death can be associated with.

Due to confirmation of *in vivo* studies, that the anti-tumour activity of liposomal curcumin is similar to free curcumin, an approach of different delivery systems, like polymeric nanoparticle encapsulated formulations of curcumin, might be a possible solution to improve the bioavailability of curcumin and its derivatives. It has been reported that the encapsulation of Calebin-A into lipid microvesicles is already possible. (Indira Priyadarsini, 2013; M.-H. Pan et al., 2017; Tomeh et al., 2019)

Another possible approach could be electrochemotherapy for proliferation control, which was suggested by (Ramachandran et al., 2014), where cytotoxic drugs have direct access into the cytosol and could overcome the challenge of prospective drugs with poor water solubility.

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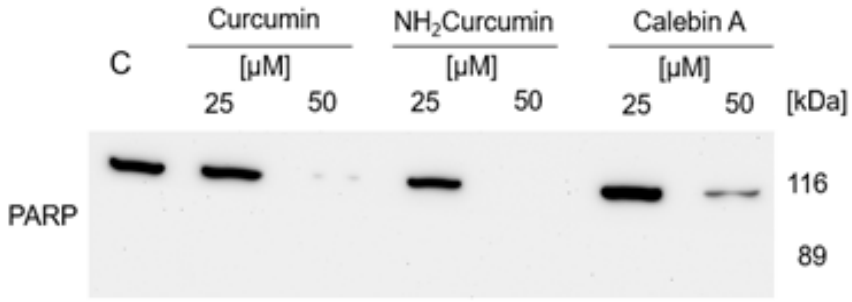
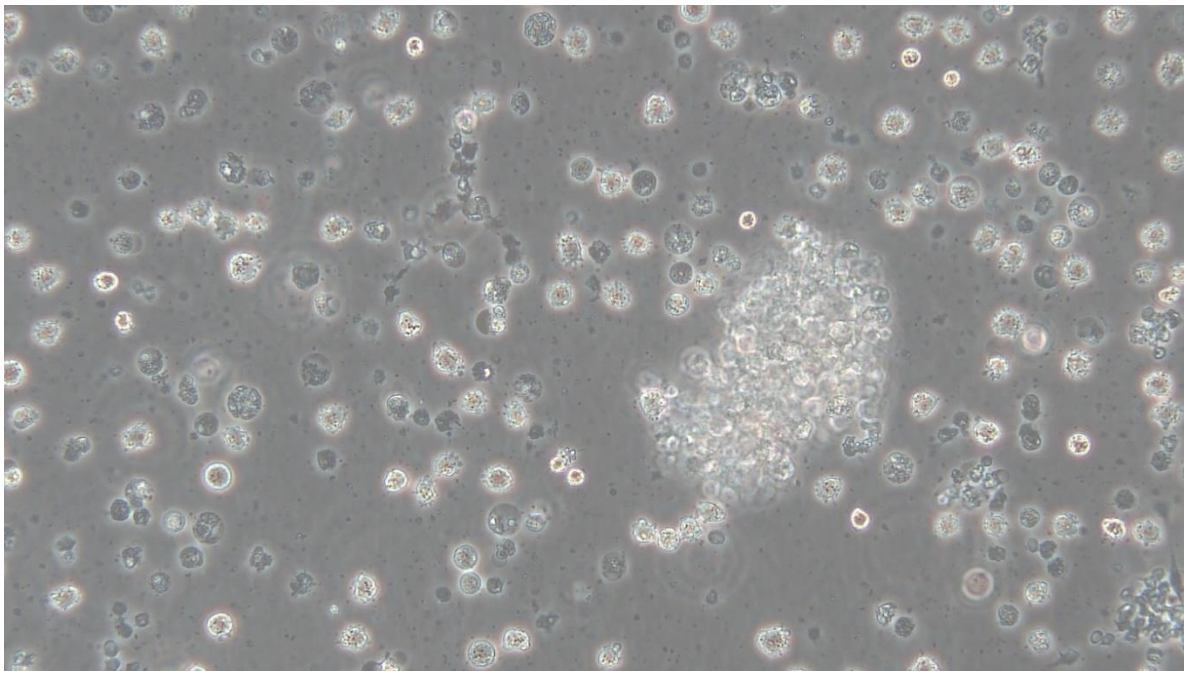
7. Appendix



Appendix 1 Western Blot HL60 cell line (C = control) – PARP



Appendix 2 HL 60 cells with a possible mycoplasma infection

I declare in lieu of oath that I wrote this thesis myself. All information derived from the work of others has been acknowledged and explicitly marked in this thesis, a list of references is included.

…… ……

Date (DD/MM/YYYY) Sophie Hollauf