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VREDNOTENJE PROTIVNETNEGA IN PROAPOPTOTIČNEGA DELOVANJA SINTEZNIH ANALOGOV KLATRODINA NA HUMANIH MONOCITNIH LEVKEMIČNIH CELICAH THP-1

EVALUATION OF ANTI-INFLAMMATORY AND PROAPOPTOTIC ACTIVITIES OF SYNTHETIC CLATHRODIN ANALOGUES IN HUMAN THP-1 MONOCYTIC LEUKEMIA CELLS

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Diplomsko nalogo sem opravljal na Fakulteti za farmacijo v Strasbourgu v Franciji pod mentorstvom prof. dr. Danijela Kiklja, mag. farm. in somentorstvom dr. Christiana D. Mullerja. Eksperimentalno delo sem opravljal v celičnem laboratoriju dr. Christiana D. Mullerja v Strasbourgu.

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ABSTRACT

Apoptosis, an important physiological mechanism for elimination of cells without producing inflammatory cytokines, represents a preferred form of cell death of cancer cells since there is no inflammatory response after all.
Chronic inflammation represents serious problem for organism since it can promote development of various inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, atherosclerosis and many others. Furthermore, chronic inflammation and development of cancer are also significantly related.

Clathrodin, a marine alkaloid isolated from sea sponge of genus *Agelas*, was used as a scaffold for design and chemical synthesis of analogues of clathrodin class. Nine synthetic analogues of clathrodin were assayed in THP-1 human monocytic leukemia cells in order to determine their potential proapoptotic and anti-inflammatory activity. Microcapillary flow cytometry was used for monitoring all biological assays.

We demonstrate here that some of the tested synthetic analogues of clathrodin induce cell death of THP-1 cells with half maximal effective concentration in the micromolar range after 24 h. Time-course experiment shows rapid and efficient induction of cell death in THP-1 cells since after only 7 h incubation majority of the cells were already dead. In addition, experiment with pan caspase inhibitor corroborates caspase-dependent cell death of THP-1 cells for the selected compound. Moreover, we confirm by an implementation of annexin V-FITC/propidium iodide apoptosis assay, cell cycle analysis and caspases assays that selected synthetic analogue of clathrodin induces extrinsic pathway of apoptosis in THP-1 cells.

We also describe here that some of the synthetic analogues of clathrodin possess anti-inflammatory activity since they could inhibit secretion of tumour necrosis factor α, one of the most important pro-inflammatory cytokines involved in the process of inflammation, with half maximal inhibitory concentration in the micromolar range.
To sum up, the tested synthetic analogues of clathrodin may represent putative new anticancer as well as new anti-inflammatory agents to be further studied.

RAZŠIRJENI POVZETEK

Celično smrt lahko na podlagi biokemičnih značilnosti razdelimo v štiri glavne skupine - apoptozo, regulirano nekrozo, avtofagijo in z mitozo povezano celično smrt. John Kerr s sodelavci je apoptozo kot obliko programirane celične smrti pomembno za eliminacijo poškodovanih celic brez posledičnih vnetnih procesov prvič opisal leta 1972. Beseda apoptoza izhaja iz grškega jezika in dobesedno pomeni »odpadanje listov z dreves«. Pri apoptozi gre za fiziološki proces, ki se normalno odvija v zdravem tkivu in uravnava celične populacije ter predstavlja ravno nasproten proces mitozi oziroma celični delitvi. Če proces uravnavanja apoptoze ne deluje pravilno, lahko pride do pretirane ali pa do zmanjšane apoptoze. Pretirana apoptoza lahko privede do razvoja avtoimunskih in nevrodegenerativnih bolezni, zmanjšana apoptoza pa lahko vodi do nastanka raka. Med procesom apoptoze se celice skrčijo, zmanjša se jim volumen, hkrati pa postanejo bolj okrogle oblike. Zaradi poškodb citoskeleta pride do značilnih sprememb v obliki membrane, pojavijo pa se tudi spremembe v celičnem jedru in sicer pride do kondenzacije kromatina in fragmentacije jedra ter dednega materiala. Apoptotične celice kasneje brez nastalih poškodb membrane razpadejo na manjše enote, ki jih imenujemo apoptotična telesca. Makrofagi prepoznajo apoptotična telesca preko »pojej-me« signalnih molekul na zunanji strani celične membrane ter jih nato fagocitirajo in razgradijo. Med procesom apoptoze namreč pride do prenosa fosfatidilserina z notranje strani celične membrane na zunanjo, kar predstavlja »pojej-me« signal za makrofage. Celice so na ta način odstranjene in razgrajene brez kasnejšega vnetnega procesa, ki je prisoten pri nekrotični celični smrti in lahko predstavlja dodaten stres za organizem. Apoptozo glede na mehanizem nastanka razdelimo na ekstrinzično in intrinzično apoptozo. Intrinzično apoptozo še naprej delimo na od kaspaz odvisno in neodvisno pot, ekstrinzična apoptoza pa je vedno od kaspaz odvisna pot. Kaspaze so serinske proteaze, ki so pomembne za izvršitev apoptotične celične smrti. Glede na funkcijo jih razdelimo v tri skupine, prva skupina so kaspaze, ki sodelujejo pri vnetnih procesih, v drugo spadajo izvršilne kaspaze in v tretjo skupino spadajo sprožilne kaspaze, ki so pomembne za aktivacijo izvršilnih kaspaz. Pri zdravljenju raka si želimo rakave celice uničiti na način, ki bi najmanj obremenjeval organizem, zato potrebujemo učinkovite in varne spojine, ki bi selektivno sprožile apoptozo rakavih celic, brez povzročitve vnetja.

Vnetje predstavlja normalen, fiziološki obrambni odziv telesa, ki ga sproži prirojeni imunski sistem ob poškodbi tkiva oziroma infekciji s patogenim mikroorganizmom. Glavna naloga vnetnega odziva je odstranitev patogena, zaščita zdravega tkiva z zajezitvijo infekcije in olajšanje celjenja poškodovanega tkiva. Ob poškodbi oziroma vdoru mikroorganizmov v organizem, makrofagi prepoznajo patogene preko zaznavanja posebnih molekul, ki jih patogeni vsebujejo na svojih celičnih membranah. Makrofagi fagocitirajo patogene, hkrati pa tudi izločajo različne signalne molekule kot so provnetni citokini in kemokini, ki uravnavajo vnetni odziv in povzročijo značilne znake vnetja na poškodovanem mestu kot so rdečina, oteklina, bolečina in vročina. Kemokini so proteini, ki spodbudijo kontrolirano premikanje efektorskih celic iz krvi na mesto poškodbe. Prihod nevtrofilcev in monocitov je pospešen z vazodilatacijo, povečano vaskularno permeabilnostjo in posledično hitrejšem pretoku krvi v okoliških krvnih žilah. Nevtrofilci so zadolženi za uničenje patogena s pomočjo izločanja citotoksičnih substanc kot so proteaze in reaktivne kisikove zvrsti, ki pa poškodujejo tudi zdravo okoliško tkivo. Proces vnetja se zavre, ko makrofagi zaznajo, da je patogen uničen in zato začnejo izločati lipoksine. Lipoksini zavrejo kemotakso nevtrofilcev in pospešijo prihod monocitov, ki so zadolženi za popravilo tkiv. Vnetni proces se s tem zaključi in regeneracija poškodovanega tkiva je omogočena. Vnetje je glede na trajanje procesa razdeljeno na akutno in kronično. Medtem ko je akutno vnetje fiziološki odziv organizma, pa kronično vnetje predstavlja resen problem, ker lahko pospeši razvoj kroničnih vnetnih bolezni kot so revmatoidni artritis, luskavica, ateroskleroza in kronična vnetna črevesna bolezen. Dokazana je tudi pozitivna povezava med kroničnimi vnetji in posledičnem nastankom in razvojem določenih vrst raka.

Morski organizmi, še posebej spužve, predstavljajo bogat in naraven vir novih in obetavnih biološko aktivnih spojin. Iz morskih spužv iz rodu *Agelas* so bili izolirani alkaloidi klatrodin, himenidin in oroidin, ki vsi izkazujejo relativno enostavno kemijsko strukturo z značilnim pirol-2-aminoimidazolnim skeletom. Za prej omenjene morske alkaloide in najrazličnejše njihove analoge je bil do sedaj ugotovljen širok spekter biološkega delovanja, saj delujejo kot nevrotoksini na natrijevih in kalcijevih kanalčkih in kot antagonisti muskarinskih receptorjev. Nekatere spojine imajo citotoksično delovanje na živalskih celičnih linijah, delujejo protimikrobno, zavirajo nastanek biofilma pri bakteriji *Pseudomonas aeruginosa* in predstavljajo modulatorje od napetosti odvisnih natrijevih

kanalčkov. V našem primeru je bil klatrodin uporabljen kot izhodišče za kemijsko sintezo analogov klatrodina. Devet sinteznih analogov klatrodina je bilo testiranih na humanih monocitnih levkemičnih celicah THP-1 z namenom določitve potencialnega proapoptotičnega in protivnetnega delovanja spojin. Vrednotenje bioloških testov je potekalo s pomočjo mikrokapilarnega pretočnega citometra.

Tekom raziskovalnega dela nam je uspelo pokazati, da nekateri izmed uporabljenih sinteznih analogov klatrodina povzročajo celično smrt THP-1 celic z EC_{50} vrednostmi v mikromolarnem območju po 24 urah. Testi kinetike so pokazali, da spojine hitro in učinkovito ubijajo THP-1 celice, saj je bila večina celic po 7 urah inkubacije že mrtvih. Na podlagi rezultatov eksperimenta z inhibitorjem kaspaz Z-VAD-fmk je bila za nadaljnje eksperimente izbrana spojina, pri kateri se je pokazala celična smrt THP-1 celic po od kaspaz odvisni poti. Izvedli smo različne teste za določitev mehanizma celične smrti za izbrano spojino. Analiza celičnega cikla je dokazala apoptotično smrt celic, saj smo uspeli določiti prisotnost celic z zmanjšano vsebnostjo dednega materiala, kar je posledica fragmentacije dednega materiala pri apoptozi. Izvedli smo tudi eksperiment s katerem smo določili prisotnost izvršilnih kaspaz, kar spet dokazuje apoptotično celično smrt THP-1 celic, hkrati pa smo preko določitve sprožilnih kaspaz nakazali kateri je glavni mehanizem apoptoze. S pomočjo vseh prej omenjenih testov za določanje mehanizma celične smrti nam je uspelo dokazati, da izbrana spojina povzroča ekstrinzično apoptozo THP-1 celic. Ugotovili smo tudi, da nekateri analogi klatrodina delujejo protivnetno na THP-1 celicah, saj so spojine z IC_{50} vrednostmi v mikromolarnem območju zavirale sproščanje tumorje nekrotizirajočega faktorja alfa, enega najpomembnejših citokinov udeleženih v procesu vnetja.

Tukaj opisani sintezni analogi klatrodina predstavljajo obetavne nove kandidate za protirakave in protivnetne učinkovine, ki bi jih bilo potrebno v nadaljevanju še bolj podrobno raziskati. V naslednjih eksperimentih bi lahko preučili morebitno selektivnost spojin za proapoptotično delovanje na normalnih človeških monocitih. Spojine bi lahko tudi testirali za proapoptotično delovanje na drugih humanih rakavih celičnih linijah ter na sferoidih in tako ovrednotili proapoptotično delovanje na tridimenzionalnih rakavih celičnih modelih. Zanimivo bi bilo tudi nadaljnje ovrednotenje vpliva spojin na izločanje ostalih provnetnih citokinov in določitev mehanizma protivnetnega delovanja.

ABBREVIATIONS

$\Delta\Psi_m$	Mitochondrial transmembrane potential
7-AAD	7-aminoactinomycin D
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor 1
ATCC	American type culture collection
ATP	Adenosine triphosphate
BOC	*t*-butyloxycarbonyl
Caspase	Cysteinyl aspartate-specific protease
dATP	Deoxyadenosine triphosphate
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EC_{50}	Half maximal effective concentration
EndoG	Endonuclease G
FasR	Fas receptor
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HtrA2	High temperature requirement protein A2
IAP	Inhibitors of apoptosis proteins
IC_{50}	Half maximal inhibitory concentration
LPS	Lipopolysaccharides
MOMP	Mitochondrial outer membrane permeabilization

NCCD	Nomenclature committee on cell death
PBMC	Peripheral blood mononucleated cell
PE	Phycoerythrin
PI	Propidium iodide
PS	Phosphatidylserine
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
SSC	Side scatter
TNFR	Tumor necrosis factor alpha receptor
TNF-α	Tumor necrosis factor alpha
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
Z-VAD-fmk	Carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone

1 INTRODUCTION

1.1 CELL DEATH AND APOPTOSIS

Cell death has been for a long time divided in different types according to morphological characteristics (1). Since such a classification became obsolete due to extensive research of molecular pathways and new findings, biochemical classification was recently proposed by Nomenclature Committee on Cell Death (NCCD). Thus, cell death can be divided into four major forms namely apoptosis, regulated necrosis, autophagy and cell death by mitotic catastrophe. To date, other atypical kinds of cell death like anoikis, entosis, parthanatos, pyroptosis, netosis and cornification are as well characterized (2).

The term apoptosis, describing a form of programmed cell death, was first established by John Kerr in 1972. Its Greek origin has the meaning of "leaves dropping of the tree". Kerr and colleagues were the first to describe this important biological mechanism for elimination of cells without producing inflammatory cytokines (3). More than 40 years after the first use of the word, apoptosis is morphologically and biochemically well described (4). Light and electron microscopy were extensively used to determine characteristic changes in morphology of apoptotic cells. During the early stage of apoptosis, the cell shrinks, becomes smaller and round-shaped. Chromatin condensation and membrane blebbing are observed and after certain time fragmentation of the nucleus occurs. Typical apoptotic bodies with intact cell membranes containing undamaged cell organelles are formed in the process called "budding" when the apoptotic cell is finally fragmented to numerous parts. Apoptotic bodies, also described as microparticles when present in the circulating blood, are finally phagocytized and digested by macrophages or surrounding cells (5). During apoptosis and subsequent phagocytosis cells are removed without causing any inflammatory reaction which could represent an additional stress for the organism (6). According to the biochemical classification of cell death, apoptosis can be divided due to different mechanistic pathways into extrinsic and intrinsic apoptosis (2). Figure 1 shows simplified scheme of apoptotic pathways.

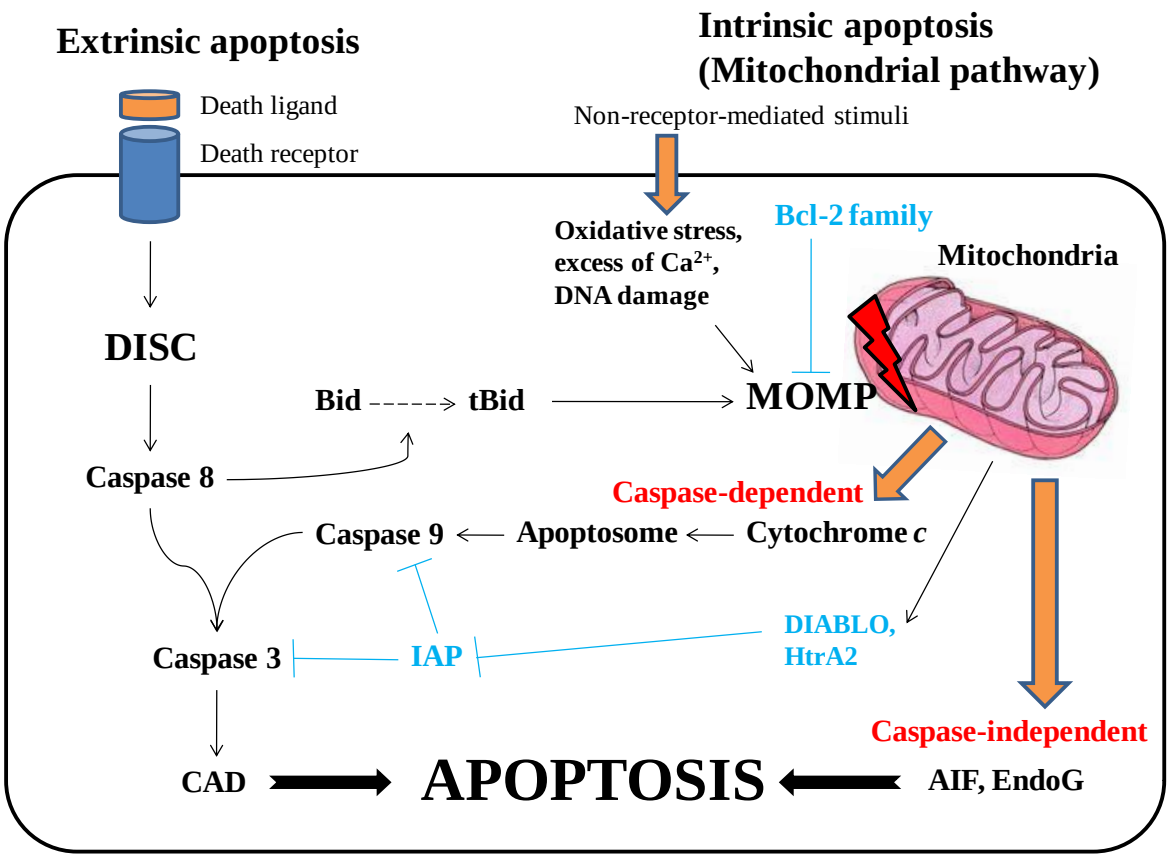


Figure 1: Graphical presentation of an extrinsic and an intrinsic apoptosis (adapted from reference (7)).

1.1.1 EXTRINSIC APOPTOSIS

Extrinsic apoptosis, a caspase-dependent cell death, is initiated by binding of extracellular triggering molecules to the death receptors (TRAIL-R, TNFR and FasR) all members of the transmembrane TNFR protein family. Death receptors got their name because they all include intracellular death domains (80 amino acids) responsible for transmission of extracellular stimuli from membrane to the inner signaling pathways. When the proper ligand binds to a death receptor, death-inducing signaling complex (DISC) is formed due to involvement of adaptor proteins. In this process, caspase 8 is activated which subsequently activates caspase 3 and triggers final execution of apoptotic cell death (8). Extrinsic apoptosis is potentially coupled with intrinsic apoptosis since caspase 8 activation can also cleave and activate pro-apoptotic protein Bid and cause permeabilization of mitochondrial outer membrane which in turn activates caspase 9 and later on also caspase 3 (6).

1.1.2 INTRINSIC APOPTOSIS

Intrinsic apoptosis, also called mitochondrial apoptosis, is further subdivided into caspase-independent and caspase-dependent intrinsic apoptosis. In both cases, non-receptor-mediated stimuli initiate intracellular signals such as excess of cytosolic Ca^{2+}, oxidative stress, DNA damage and others, finally resulting in the emergence of pores in the outer membrane of mitochondria. Beginning of mitochondrial outer membrane permeabilization (MOMP) is regulated by proapoptotic (Bid, Bax, Bad, Bak, Bim, Bik, Blk, Bcl-10) and antiapoptotic (Bcl-2, Bcl-x, Bcl-w, Bcl-XS, Bcl-XL, BAG) members of Bcl-2 protein family (9). When proapoptotic proteins dominate over antiapoptotic ones, MOMP occurs and results in a) inhibition of ATP synthesis due to irreversible loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), b) excessive production of reactive oxygen species (ROS) due to respiratory chain inhibition and c) release of two important groups of toxic proteins from intermediate space of mitochondrial membrane to cytosol (10). High temperature requirement protein A2 (HtrA2), direct IAP binding protein with low pI (DIABLO) and cytochrome *c* are members of the first group, whereas apoptosis inducing factor (AIF) and endonuclease G (EndoG) represent the second group of toxic proteins. The first group is responsible for activation of caspase-dependent intrinsic pathway since apoptosome is formed when cytochrome *c* binds to apoptotic protease activating factor 1 (Apaf-1), dATP and procaspase 9. In this process, caspase 9 is activated which leads to activation of caspase 3. Proteins HtrA2 and DIABLO facilitate intrinsic apoptosis by suppressing activity of apoptosis inhibitor proteins (IAP) (5). On the other hand, a second group of toxic proteins induce caspase-independent intrinsic apoptosis since EndoG and AIF cause direct nuclear condensation and DNA fragmentation without activating caspases (11,12).

1.1.3 CASPASES AND ACCOMPLISHMENT OF APOPTOSIS

Caspases, i.e. cysteinyl aspartate-specific proteases, represent the most important protein family engaged in the execution of cell death during the process of apoptosis. Caspases of human origin can be divided into three groups, according to substrate specificity and phylogenetic origin. The first group includes inflammatory caspases (caspase 1, 4, 5, 12), the second group consists of apoptotic effector caspases (caspase 3, 6, 7), which are important for terminal execution of cell death and the third group comprises apoptotic initiator caspases (caspase 2, 8, 9, 10), which are needed for triggering the apoptotic

pathway and activation of effector caspases. Apoptotic initiator and effector caspases are important for the execution of apoptosis, whereas inflammatory caspases are involved in the process of inflammation. Caspases are found in cytosol as pro-enzymes and are activated in the process of cascade signaling. Effector caspases cleave many substrates such as poly ADP-ribose polymerase, cytokeratins, alpha fodrin and many others (13). Activation of caspase 3, one of the most significant apoptotic effector caspases, leads to final execution of cell death since activated caspase 3 activates caspase-activated DNase (CAD) which causes fragmentation of DNA and chromatin condensation (14). Furthermore, caspase 3 is also responsible for reorganization of cytoskeleton and formation of apoptotic bodies (15). All mentioned activities of caspases contribute to incidence of typical morphologic and biochemical characteristics of apoptosis like chromatin condensation, degradation of nucleus and cytoskeleton, DNA fragmentation, protein cross-linking, emergence of apoptotic bodies and externalization of phosphatidylserine (PS) on outer layer of the cell membrane (4).

1.1.4 DEREGULATED APOPTOSIS AND CANCER

Apoptosis represents crucial genetically determined biological process which normally occurs in healthy tissue but can also be found in pathological conditions (16). It is significantly involved in regulation of cell populations in normal tissues since it is the opposite process of mitosis. Pathological conditions such as cancer, autoimmune (autoimmune deficiency syndrome) and neurodegenerative diseases (amyotrophic lateral sclerosis, Alzheimer's, Huntington's, and Parkinson's disease) can develop after deregulation of apoptosis, due to excess or deficiency of cell removal (5). Cancer often develops due to uncontrolled growth of cells resistant to apoptotic signals since reduced apoptosis represents a major role in carcinogenesis (17). Suppression of caspases, insufficient death receptor signaling and decreased expression of proapoptotic proteins as well as increased expression of antiapoptotic proteins, are the main reasons for deregulated apoptosis which can eventually lead to formation of tumours (18). Furthermore, the tumour suppressor protein p53, also called "guardian of the genome" for its important role in regulation of induction of apoptosis, is found mutated or absent in more than 50% of human cancers (19).

1.2 INFLAMMATION

Inflammation is a physiological defence mechanism triggered by the innate immune system after physical damage or irritation by noxious agents or infection caused by pathogens. The main purpose of the inflammatory response is to eliminate pathogens and to protect healthy tissue by preventing further infection, finally enabling rapid healing of the injured tissue (20). Pathogens express specific pathogen-associated molecular patterns which enable macrophages to identify and induce phagocytosis of invading microorganisms and secretion of pro-inflammatory cytokines and chemokines. Cytokines are immune signaling molecules important for regulation of inflammation and are responsible for occurrence of characteristic symptoms such as heat, swelling, redness, pain and potential loss of function, whereas chemokines are proteins that induce controlled movement called chemotaxis of effector cells from blood to the site of infection or injury. Histamine, prostaglandins and leukotriens secreted from tissue-resident and activated macrophages are responsible for vasodilatation, increased vascular permeability and consequent increased blood flow in the surrounding blood vessels, which enables better and faster recruitment of neutrophils and monocytes. Mixture of cytotoxic substances including proteases and ROS, which destroy pathogens but also affects normal cells, are released from neutrophils in the process called degranulation. When pathogen is destroyed, activated macrophages start to secrete lipoxins, which stop recruitment of neutrophils and promotes arrival of monocytes needed for tissue repair. Inflammation process ends by induction of apoptosis of T lymphocytes and regeneration of damaged tissue is enabled (21).

1.2.1 CHRONIC INFLAMMATION AND CANCER

Inflammation can be divided to acute and chronic inflammation. Acute inflammation is the normal, physiological response, whereas chronic inflammation represents a long time inflammation with bad consequences for the organism (e.g., rheumatoid arthritis, inflammatory bowel disease, psoriasis, atherosclerosis) (22). Chronic inflammation and development of cancer are related since a) patients suffering from chronic inflammatory diseases have higher chances of developing cancer, b) tumours usually develop in the parts of the organism exposed to chronic inflammation, c) effector cells create inflammatory microenvironment near to the tumour and secrete cytokines promoting invasion and

migration of tumour cells, d) risk of developing specific cancers is significantly reduced in patients with prescribed long-term non-steroidal anti-inflammatory drug therapy (23,24).

1.2.2 TUMOUR NECROSIS FACTOR ALPHA

Tumour necrosis factor α (TNF-α) is the most important pro-inflammatory cytokine involved in the process of inflammation. However, TNF-α also participates in other biological processes such as apoptosis, cell proliferation and differentiation (25). It got its name since it could induce necrosis of tumours due to high cytotoxic activity (26). TNF-α secreted from activated macrophages enhances vasodilatation and swelling, facilitates transition of leukocytes through endothelium and the process of blood coagulation, promotes production of ROS and activates fever (27). Due to significant involvement of TNF-α in the process of acute and chronic inflammation, a possibility of TNF-α inactivation after capture by humanized monoclonal antibodies or soluble TNF-α receptors was recognized as a new way to treat chronic inflammatory diseases. There are three TNF-α blockers on the market, namely adalimumab, infliximab and etanercept to treat chronic inflammatory diseases, especially rheumatoid arthritis and inflammatory bowel disease (28). Anti-TNF-α therapy has been in use for almost two decades and some adverse effects have been reported. Higher incidence of infections and malignant diseases, especially potential development of lymphomas, are the main problems (29). While TNF-α at high doses causes tumour necrosis, scientists figured out that low amount of TNF-α secreted in tumour microenvironment in fact encourages development and growth of tumours. TNF-α participates in tumour initiation, proliferation, invasion, angiogenesis and metastasis (30). Experiments with murine cancer models and knockout mice unable to produce TNF-α, showed high resistance to carcinogenesis of many cancers such as skin, liver and intestinal cancers. Furthermore, it was shown that development of metastasis is promoted by TNF-α and that metastatic lesions were significantly reduced when TNF-α blockers were added. Increased TNF-α concentration in blood was related to advanced stage of disease and poor survival of patients suffering from prostate, hematopoietic, pancreatic or metastatic breast cancer (22). Due to all activities of TNF-α regarding tumour initiation and promotion, some scientists believe that previously mentioned TNF-α blockers have a potential to be used against specific cancers as well. However, there is a need for new and safer anti-TNF-α drugs, considering the high prices and serious adverse effects of known TNF-α blockers (31).

1.3 FLOW CYTOMETRY

Flow cytometry is a light-based technique which provides rapid quantitative measurements and analysis of multiple features such as size, granularity and fluorescence of an individual cell or particle (32). Three main systems namely fluidics, optics and electronics are essential for flow cytometry. The first system is responsible for the alignment of the cells in a flow in front of the laser beam, the second is the illumination of the cells by the laser light and gathering scattered light signals (forward and side) and fluorescence emission with lenses, beam splitters and optical filters in front of detectors. The third system consists of light detectors in charge of transforming light signals into electric ones. Size of the cell is determined by forward collection lens which collects forward scattered light (FSC) along the same axis as the focused laser beam, whereas granularity (internal complexity) of the cell is determined by side collection lens positioned at 90 degrees to the laser beam (SSC). Fluorescence can be detected from the same position as SSC and separation of the different wavelengths is done by dichroic and emission filters (33). A laser is the most important part of the flow cytometer since it provides focused light beam, which illuminates the cells and excites the fluorochromes. Different lasers producing light in the UV or visible spectrum can be used, but usually laser which generates 488 nm (blue light), 515 nm (green light) or 630 nm (red light) are the most common ones (34).

Briefly, cells in suspension are collected from the sample vial, transported to the flow cell where cells are aligned due to fluidics focalization. Individual cells travel through focused laser beam and laser light is scattered by them. If the cells are as well labelled with fluorescent molecules, light with longer wavelength is emitted after excitation. A complex system of optical lenses, beam splitters and filters leads fluorescent and scattered light to sensitive photomultipliers where light is converted to an electric signal later digitized to be analyzed by computer software. Schematic diagram of a flow cytometer is displayed in Figure 2.

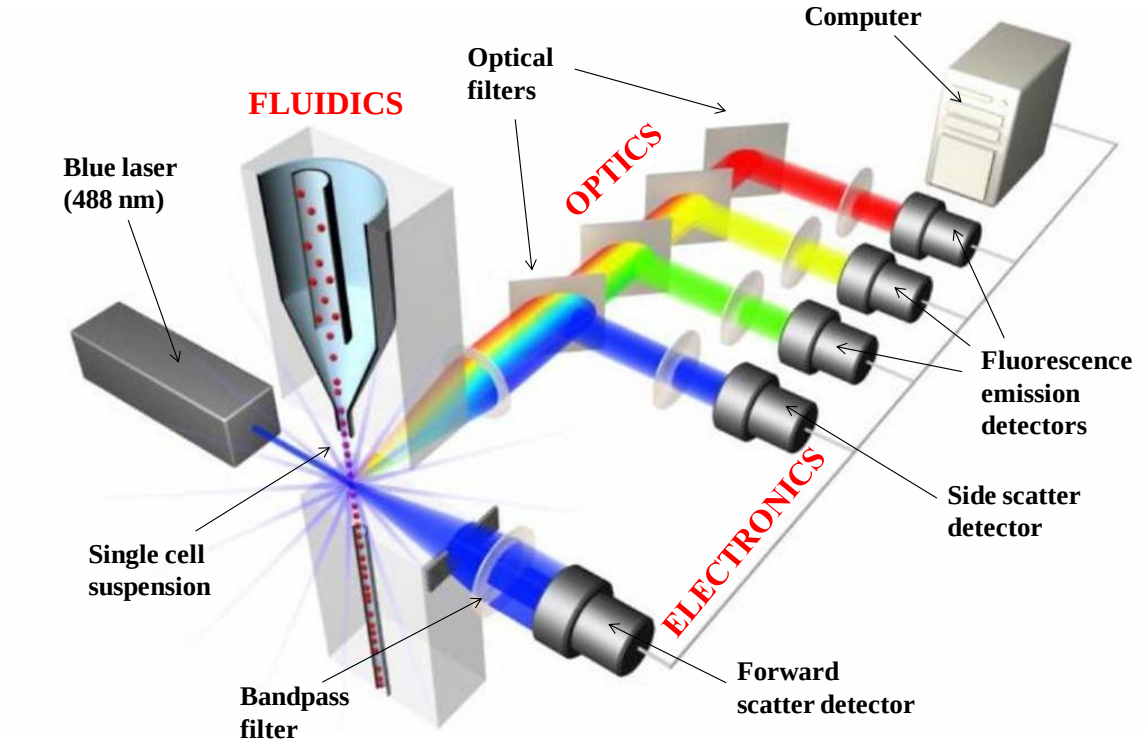


Figure 2: Schematic diagram of a flow cytometer (adapted from reference (35)).

Selection of the proper fluorochromes for a specific assay has to be done according to the wavelength of the laser light. For example, if flow cytometer is equipped with blue laser (488 nm), only fluorochromes excited with this light can be used. In that case, the fluorochrome emits light with longer wavelength due to a loss of energy (Stokes shift). The same laser light can excite various fluorochromes and emitted light of those can be monitored. Fluorochromes are usually conjugated to specific biological molecules such as proteins, monoclonal antibodies and others which can enable detection and discrimination of specific cell populations by binding to the characteristic chemical structures inside cell or on the cell membrane. All mentioned features of the fluorochromes enable multicolor and multiparametric analysis with a flow cytometer (36). Nowadays, modern bench-top flow cytometers represent rapid and efficient tool for multiparametric analysis of various cell properties since they can simultaneously detect fluorescence from more than six different fluorochromes at the same time, or from up to thirty when 3-4 lasers are used (37,38). Flow cytometry has become an important method for high-content screening of chemical libraries in order to find new lead compounds for various biological activities (39–42).

1.4 CLATHRODIN AND ITS SYNTHETIC ANALOGUES

Marine organisms, especially sponges, represent rich natural source of new promising compounds possessing interesting biological activities such as anticancer activity (43). Clathrodin, hymenidin and oroidin are marine alkaloids isolated from sea sponges of genus *Agelas* possessing a relatively simple chemical structure based on a pyrrole-2-aminoimidazole motif in which 2-aminoimidazole and pyrrole rings are linked through an unsaturated chain of 5 carbon atoms (Figure 3).

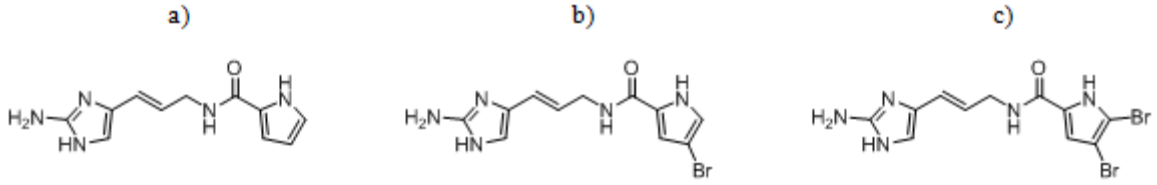


Figure 3: Chemical structure of a) clathrodin, b) hymenidin and c) oroidin.

Marine alkaloids clathrodin, hymenidin and oroidin showed interesting biological activities since they were recognized as a new potential sodium and calcium channel neurotoxins (44,45). Furthermore, they showed anti-muscarinic activity in rat brain membranes, cytotoxic activity in animal cell lines and also anti-biofilm activity against *Pseudomonas aeruginosa* (46–49). Chemical synthesis of clathrodin, hymenidin and oroidin was introduced due to their interesting biological activities and insufficient, time consuming isolation from sponges (50–52). Many synthetic analogues using clathrodin, hymenidin or oroidin as a scaffold were prepared and assayed for different biological activities. Recently, it was shown that synthetic analogues of clathrodin and oroidin class also possess antimicrobial activity and are promising state-dependent voltage-gated sodium channel modulators (53–56). For the purpose of this work, nine synthetic analogues of clathrodin, for which proapoptotic activity was previously observed on HepG2 cell line, were used with three main modifications in the chemical structure of the natural scaffold, including a) incorporation of the double bond in the central part of the compound into a benzene ring, b) introduction of additional ring in the eastern part of the molecule and c) methylation of amino group and/or introduction of *t*-butyloxycarbonyl (BOC) group in the western part of the molecule. Modifications in chemical structure of synthetic analogues compared to clathrodin are shown in Figure 4.

Figure 4: Chemical structure of clathrodin and presentation of main modifications introduced in synthetic analogues. Double bond in the central part was incorporated in benzene ring, additional ring was introduced in the eastern part and *N*-methylation or introduction of BOC group was performed in the western part of the molecule (R_1= H or BOC, R_2= H or CH_3).

2 AIMS

Nine synthetic analogues of clathrodin will be assayed in THP-1 human monocytic leukemia cells in order to determine their potential proapoptotic and/or anti-inflammatory activities. All assays will be performed and evaluated by microcapillary flow cytometry. Selection of compounds for further analysis will be based on the results of the primary screening.

Proapoptotic activity of synthetic analogues of clathrodin in THP-1 cells will be evaluated by using annexin V-FITC/propidium iodide apoptosis assay. Dose response curves, EC_{50} values and time-course experiments will be later determined for the most potent compounds. The most active compound will be selected for further mechanistic experiments in order to prove caspase-dependent cell death and induction of apoptosis and to identify if compound is inducing apoptosis through the extrinsic or intrinsic pathway.

Anti-inflammatory activity of nine synthetic analogues of clathrodin in THP-1 cells will be evaluated by TNF-α secretion assay. Dose response curves and IC_{50} values will be determined for the most potent compounds.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 REAGENTS

Table I: List of reagents used during research work and names of producers.

Reagent	**Producer**
10x Apoptosis wash buffer	Merck Millipore, USA
7-AAD reagent	Merck Millipore, USA
Annexin V-FITC	ImmunoTools, Germany
Caspase 3/7 kit for flow cytometry	Merck Millipore, USA
Caspase 8/9 kit for flow cytometry	Merck Millipore, USA
Celastrol	SurroMed, USA
DMSO	Sigma – Aldrich, USA
DPBS without Ca^{2+} and Mg^{2+}	Lonza, Belgium
Ethanol	Sigma – Aldrich, USA
FBS	Life Tehnologies, UK
LPS from Salmonella abortus equi	Sigma – Aldrich, USA
Pan caspase inhibitor Z-VAD-fmk	Promega, USA
Penicillin/streptomycin	Life Tehnologies, UK
PI solution 100 μg/mL	Miltenyi Biotec Inc., USA
PI/RNase staining solution	Life Technologies, USA
RPMI-1640 medium with GlutaMax™ supplement	Life Tehnologies, UK
TNF–α secretion assay, detection kit (PE)	Miltenyi Biotec Inc., USA
Trypan blue solution 0.4%	Sigma – Aldrich, USA

3.1.2 LABORATORY EQUIPMENT

Table II: List of laboratory equipment used during research work and names of producers.

Laboratory Equipment	**Producer**
12 – Well cell culture plates	Greiner Bio-One, Germany
24 – Well cell culture plates	Greiner Bio-One, Germany
96 – Well cell culture plates	Becton Dickinson and Co., USA
Automatic pipettes	Gilson, France
Celigo® S imaging cytometer	Brooks Automation, Inc., USA
Cell culture flasks (25 cm^2)	Greiner Bio-One, Germany
Cell freezing container (CoolCell® LX)	BioCision, USA
Centrifuge (Jouan C3-12)	Jouan S. A., France
Centrifuge tubes (15 mL and 50 mL)	TPP®, Switzerland
CO_2 incubator (Heracell 150)	Kendro, Germany
Cryogenic vials (2.0 mL)	Corning Inc., USA
Digital multichannel pipette (0.5-10 µL)	Eppendorf, Germany
Digital multichannel pipette (5-50 µL, 50-300 µL)	Labsystems, Finland
Digital weighing scale (Mettler AE240)	Mettler Toledo, Switzerland
Freezer (−18°C)	Liebherr, Switzerland
Inverted phase contrast microscope (Axiovert 25)	Carl Zeiss Microscopy, Germany
Liquid nitrogen storage container	Taylor-Wharton, USA
Microbiological safety cabinet	Heto-Holten A/S, Denmark
Microbiological safety cabinet	Jouan, France
Microcapillary flow cytometer (blue and red laser)	Guava Technologies, USA
Microcapillary flow cytometer (blue laser)	Guava Technologies, USA
Microscope eyepiece camera (Dino-Eye camera)	AnMo Electronics Corp., Taiwan
Microtubes (1.5mL)	Sarstedt AG &Co., Germany
Motorized pipette filler (Pipetboy)	Integra Biosciences, Switzerland
Pipette tips	Greiner Bio-One, Germany
Refrigerator (4°C)	Liebherr, Switzerland
Serological pipettes	Greiner Bio – One, Germany
Sterile reagent reservoirs (50 mL)	VWR International, USA

Tubes u-bottom (1.4 mL)	Micronic, Netherlands
Ultra-low temperature freezer (−80°C)	Liebherr, Switzerland
Ultrapure water systems	Merck Millipore, France
Ultrasound water bath (Branson 1210)	Branson Ultrasonic Corp., USA
Vortex mixer	Elektrocraft, India
Water bath huber	Bioblock Scientific, Germany

3.1.3 COMPUTER SOFTWARE

Table III: List of computer software used for evaluation and analysis of data and names of producers.

Computer software	**Producer**
Celigo® S software	Brooks Automation, Inc., USA
DinoXcope software for Mac	AnMo Electronics Corp., Taiwan
GuavaSoft software package 2.6 (InCyte™)	Merck Millipore, USA

3.1.4 COMPOUNDS

3.1.4.1 Chemical synthesis

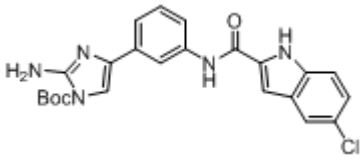
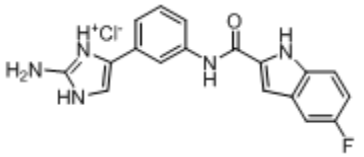
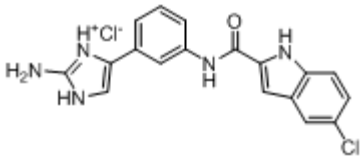
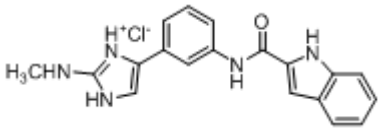
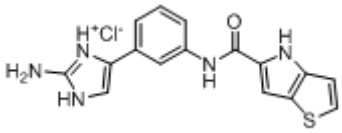
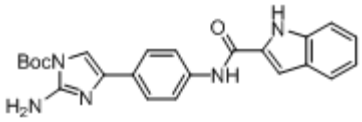
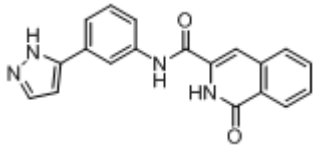
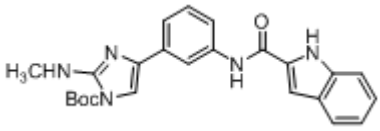
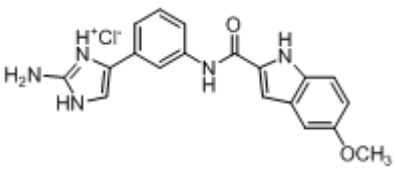
Compounds **1** to **9** for testing (Table IV) were synthesized by Assist. Prof. Dr. Nace Zidar, M. Pharm. in the frame of EU project MAREX at the Chair of Pharmaceutical Chemistry at the Faculty of Pharmacy, University of Ljubljana. All compounds were > 95% pure as determined by high-performance liquid chromatography.

3.1.4.2 Preparation and dilutions

Stock solution (10 mM) of each compound was prepared by dissolving solid compounds in dimethyl sulfoxide (DMSO). Compounds were very soluble in DMSO, thus clear and colourless solutions were obtained. Stock solutions were stored at 4°C. For the purposes of assays working solutions of compounds were freshly prepared in culture medium pre-warmed to 37°C. When needed different concentrations of compounds were prepared by cascade dilution in 1.4 mL tubes. Working solutions of compounds were well mixed before

each treatment. Final concentration of DMSO in working solutions used in biological assays never exceeded 0.6% v/v for apoptosis and 0.75% v/v for inflammation assays.

Table IV: Chemical structures and molecular mass of synthetic analogues of marine alkaloid clathrodin.

Label of compound	Chemical structure	Molecular mass (g/mol)
1		451.91
2		371.80
3		388.25
4		367.83
5		359.83
6		417.46
7		330.35
8		431.49
9		383.83

3.1.5 CELL CULTURE

THP-1 human monocytic leukemia cell line is derived from peripheral blood of 1-year old child diagnosed with acute monocytic leukemia (57). Acute monocytic leukemia, a subtype M5 of acute myeloid leukemia, is a hematopoietic cancer which is characterized by excessive spread of myeloid blasts in the peripheral blood or bone marrow due to loss of normal differentiation ability and at least 80% of the leukemic cells of monocyte origin (58). THP-1 cell line was recognized as the first stable immortal leukemic cell line with monocytic properties in 1980. Cells show round, single-cell morphology with a diameter of 12-14 μm. They grow in suspension, since they do not attach to the surface of the cell culture flask. Stimulation of THP-1 cells with lipopolysaccharides (LPS) leads to production and release of inflammatory cytokines including TNF-α, which enables THP-1 cells as a suitable in vitro model for studying anti-inflammatory activity of compounds (59–61). For the purposes of this work the THP-1 (ATCC® TIB-202™) cell line was purchased from American Type Culture Collection (ATCC).

3.1.5.1 Culture medium

THP-1 cells were grown in RPMI 1640 medium with 2 mM L-alanyl-L-glutamine (GlutaMax™ supplement) additionally supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. Every 3-4 weeks currently used bottle of culture medium was replaced by new, freshly prepared culture medium. For the purposes of inflammation assays the same composition of culture medium was used, only FBS was prior heat-inactivated at 56°C for 30 minutes in order to inactivate complement proteins.

RPMI 1640 medium is suitable for human leukemic cells as it contains high amount of vitamins (biotin, PABA, vitamin B12), glutathione, inositol and choline which is not the case for other media. Because RPMI 1640 medium does not contain growth factors, proteins or lipids, supplementation of these substances is needed. L-alanyl-L-glutamine represents better substitute to L-glutamin as it is more stable in aqueous solution, degradation to toxic ammonia is reduced during storage or incubation which finally improves cell viability and growth of cells. FBS is the most important component of culture medium, since it represents vital source of vitamins, proteins (especially albumins) and growth factors. Combination of penicillin/streptomycin is added to culture medium in order to avoid potential bacterial contamination of cell cultures.

3.1.5.2 Subculture of suspension cell line

Manipulation of cell lines takes place in aseptic environment. All the reagents and laboratory equipment used when handling cells are sterile. Personal protective equipment such as laboratory coat, gloves and safety glasses must be worn all the time during the work with biological hazards such as human cancer cell lines. Working surface of microbiological safety cabinet, motorized pipette filler and automatic pipettes have to be cleaned with 70% ethanol before and after use.

Suspension cell lines should be divided when cells proliferate exponentially and before they achieve 90% confluency. THP-1 cells are grown in suspension as single cells and they do not form clumps, therefore subculture by addition of fresh medium was easy. Cell cultures were first assessed using inverted phase contrast microscope and confluency was estimated. Cell concentration was determined using cell quantification protocol. An appropriate amount of fresh culture medium pre-warmed to 37°C was added to reach a final cell concentration of 2 x 10^5 cells/mL. Cell suspension was then split to new cell culture flasks labelled with name of the cell line, date and passage number. Next subculture was done when cell concentration reached 8 x 10^5 cells/mL. Doubling time for THP-1 cells was approximately 26 hours, meaning that medium renewal was done every 2 to 3 days. ATCC recommendations are that concentration of THP-1 cells should not exceed 10^6 cells/mL. Cell culture flasks (25 cm^2) with THP-1 cells were kept in CO_2 incubator at fully humidified atmosphere at 37°C, 5% CO_2 and 95% air. Figure 5 shows photo of THP-1 cells before and after subculturing.

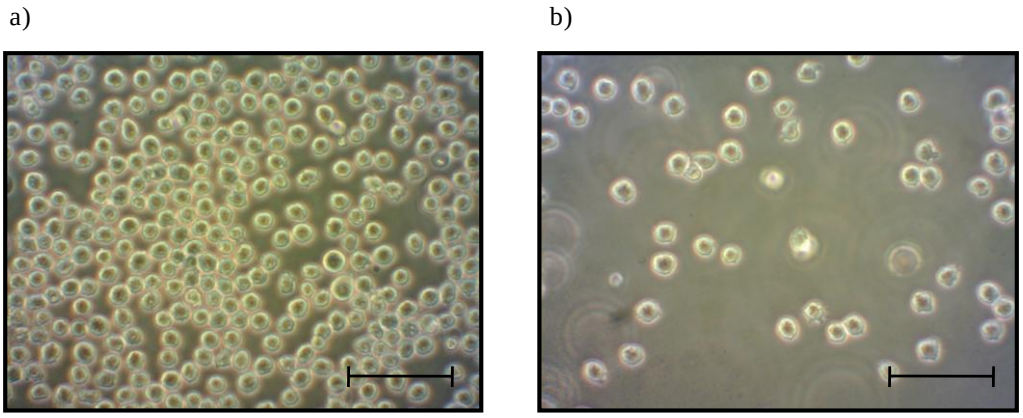


Figure 5: Photos of THP-1 cells using inverted phase contrast microscope (200x magnification) and microscope eyepiece camera a) before and b) after dilution. Scale bar represents 100 μm.

3.1.5.3 Cell quantification

For the majority of cell-based assays number of cells has to be determined to be sure that results obtained from these assays are not cell concentration dependent. Using a consistent number of cells in all assays gives results of better reproducibility. In our case, cells were counted under the microscope using a hemocytometer. First, hemocytometer and coverslip were rinsed with 70% ethanol and then dried for 5 minutes. Next, 100 µL of cell suspension was taken from the cell culture flask (25 cm^2) and then the same volume of trypan blue was added to the microtube. Trypan blue is a dye, which colours only dead cells, live cells with undamaged cell membranes do not uptake the dye. Good mixing with automatic pipette was performed and then both sides of the chamber were carefully filled up with cell suspension. Cells were viewed under an inverted phase contrast microscope and only live cells were counted. Cell concentration of live cells was finally computed using *equation 1*.

$$\mathbf{N = N' \times 2 \times 10^4 \ \ (\frac{cells}{mL})} \qquad \textit{Equation 1}$$

N cell concentration of live cells

N' average number of counted cells

2 dilution factor

10^4 correction factor converts 0.1 mm^3 to 1 mL

3.1.5.4 Recovery of frozen cells

THP-1 cell line is provided by ATCC in frozen state. Since thawing has to be performed fast to avoid damage of cell membranes in order to ensure maximum viability of the culture, frozen ampoule was transferred to 37°C water bath for 1-2 minutes. After thawing, cell suspension was collected in centrifuge tube and culture medium pre-warmed to 37°C was slowly added. Cryoprotectant (DMSO) was removed from the cell suspension by centrifugation at 150 g for 5 minutes. Supernatant was discarded and cell pellet was re-suspended in fresh culture medium pre-warmed to 37°C. Cell quantification protocol was performed to determine cell concentration of live cells and additional volume of fresh culture medium pre-warmed to 37°C was added to ensure proper cell concentration for

seeding the cells into the cell culture flasks (25 cm^2). Finally, cell culture flasks were transferred to CO_2 incubator.

3.1.5.5 Cryopreservation for ultra-low temperature storage of cell line

Cell lines are stored in liquid nitrogen storage containers. Vapour phase of liquid nitrogen provides ultra-low temperature environment (−196°C), which is the safest way for long-term storage of the cell lines. Freezing has to be performed slowly and it is advised to include a cryoprotectant such as DMSO or glycerol in the medium to avoid formation of water crystals that would damage the cells. "Freezing medium" for THP-1 cell line consisted of complete culture medium supplemented with 5% (v/v) DMSO. THP-1 cells were collected in centrifuge tube when cell concentration 8 x 10^5 cells/mL was reached. Cell concentration of live cells was determined using cell quantification protocol and minimum 90% cell viability was needed to proceed with next step. Centrifugation (150 g for 5 minutes) was performed, supernatant was discarded and cell pellet was re-suspended in "freezing medium" pre-warmed to 37°C. Cell concentration 2 x 10^6 cells/mL in "freezing medium" was prepared and 1 mL aliquots of cell suspension were transferred in cryogenic vials labelled with name of the cell line, date and passage number. Cryogenic vials were put in cell freezing container (CoolCell® LX), which provides slow controlled cooling rate of −1 to −3°C per minute, and were then transferred to ultra-low temperature freezer (−80°C) over night. Next day, cryogenic vials were stored in the liquid nitrogen container to ensure long term storage.

3.2 METHODS

3.2.1 MICROCAPILLARY FLOW CYTOMETRY

Microcapillary flow cytometry system (Guava® EasyCyte) with blue (488 nm) and red (642 nm) laser was used for acquisition and evaluation of all apoptosis and inflammation assays. Guava® EasyCyte cytometers represent a new generation of bench-top flow cytometers equipped with microcapillary flow cell, automated 96-well plate loader tray and powerful mixing paddle. Direct sampling by aspiration with no requirement of sheath fluid is possible by using patented microcapillary flow cell. GuavaSoft software package provides user-friendly acquisition and data analysis tool with a lot of useful properties such as possibility of drag-and-drop gating of cell populations, determination of dose response curves and heat mapping for faster graphical evaluation of complex results (37). Readouts were evaluated with GuavaSoft software (InCyte 2.6) and Microsoft Excel. Dose response curves and EC_{50}/IC_{50} values were plotted and computed using InCyte 2.6. All the charts and tables were made in Microsoft Excel. Dot plots and histograms were exported from InCyte 2.6.

3.2.2 APOPTOSIS ASSAYS

3.2.2.1 Annexin V-FITC / propidium iodide apoptosis assay

Annexin V-FITC / propidium iodide apoptosis assay is used to monitor apoptosis. Annexin V-FITC is a fluorescein-labelled lectin which binds to phosphatidylserine (PS) on cell membrane. Externalization of PS from inner to outer leaflet of the cell membrane represents an early indicator of apoptosis since it represents an “eat-me” signal for the phagocytic cells (62). Propidium iodide (PI) is a fluorescent intercalating agent, which binds to DNA. PI stains only dead cells with damaged cell membranes and is excluded from live cells (63). Combination of both dyes enables detection and discrimination of live, apoptotic and dead cells (64). In our case, positive and negative controls were included in all annexin V-FITC/PI apoptosis assays to ensure appropriate gating of before mentioned populations of cells. Celastrol (50 μM) was used as a positive control since it induces apoptosis in mostly all mammalian cancer cell lines (65–67). Untreated cells in culture medium with DMSO (50 μM) were used as a negative control.

3.2.2.1.1 Primary screening

Primary screening to define potential proapoptotic activity caused by compounds in THP-1 cells using annexin V-FITC and PI was performed. THP-1 cells were seeded in 96-well plate with cell concentration 1 x 10^5 cells/mL. To each well containing 180 μL of cells in culture medium, 20 μL of compound in culture medium was added using multichannel pipette to reach the final concentration (50 μM). 96-well plate was then incubated in CO_2 incubator for 24 h. Next day, annexin V-FITC (3 μL) and PI (3 μL) were added to each well and the plate was incubated at room temperature in the dark for 10 minutes. Finally, fluorescence measurements were performed using microcapillary flow cytometer.

3.2.2.1.2 Determination of dose response curves and EC_{50}

Half maximal effective concentration (EC_{50}) represents a concentration of compound which causes half of maximal biological response after a certain time period (68). In our case, EC_{50} represents the concentration, which induces apoptosis in 50% of all cells after 24 h incubation. THP-1 cells were seeded in 96-well plate with cell concentration 1 x 10^5 cells/mL. Increasing concentrations of compounds (from 0.412 to 60 μM) in culture medium were freshly prepared from stock solution prior to each experiment. To each well containing 180 μL of cells in culture medium, 20 μL of compound in culture medium was added. 96-well plates were then incubated in CO_2 incubator for 24 h. Next day, annexin V-FITC (3 μL) and PI (3 μL) were added as described before and fluorescence measurements were performed using microcapillary flow cytometer.

3.2.2.1.3 Time-course assay

Time-course experiments using annexin V-FITC/PI apoptosis assay were performed in order to study the kinetics of proapoptotic activity (69). THP-1 cells were seeded in 96-well plate with cell concentration 1 x 10^5 cells/mL. Cells were prepared and treated as described before with compounds at final concentration of 35 μM. THP-1 cells were incubated in CO_2 incubator for increasing times (1, 3, 5, 7 h). Then, annexin V-FITC (3 μL) and PI (3 μL) were added as described before and fluorescence measurements were performed using microcapillary flow cytometer.

3.2.2.1.4 Assay with pan caspase inhibitor Z-VAD-fmk

Z-VAD-fmk is a cell-permeable, nonspecific synthetic peptide inhibitor of the majority of caspases. Cell death is inhibited when cells are pre-treated and incubated with Z-VAD-fmk before treatment with compounds inducing apoptosis. Assay with Z-VAD-fmk enables us to determine potential caspase-dependent cell death (13,70). THP-1 cells were seeded in 96-well plate with cell concentration 1 x 10^5 cells/mL and were pre-treated with pan caspase inhibitor Z-VAD-fmk (final concentration 50 μM). Cells were incubated in CO_2 incubator for 1 h. To each well containing 180 μL of cells in culture medium, 20 μL of compound in culture medium was added to reach final concentration of 35 μM. THP-1 cells were treated and incubated in CO_2 incubator for increasing times (2, 3, 4, 7 h). Then, annexin V-FITC (3 μL) and PI (3 μL) were added as described before and fluorescence measurements were performed using microcapillary flow cytometer.

3.2.2.2 Cell cycle assay

Cell cycle assay enables detection of apoptotic cells. DNA in apoptotic cells is heavily degraded to characteristic oligonucleosomal DNA fragments (180 bp and multiples of 180 bp). Apoptotic cells containing less DNA can be easily detected by flow cytometry using PI/RNase staining solution since PI is fluorescent dye, which binds stoichiometrically to DNA/RNA. Specific DNA staining is achieved by enzymatic removal of RNA by RNase (71). THP-1 cells were seeded in 12-well plate with cell concentration 1 x 10^6 cells/mL. To each well containing 1800 μL of cells in culture medium, 200 μL of compound in culture medium was added to reach final concentration of 35 μM. THP-1 cells were treated and incubated in CO_2 incubator for increasing times (3, 6, 9, 12 and 24 h). After incubation, approximately 10^6 cells were collected in centrifuge tubes, washed with DPBS and centrifuged at 200 g for 5 minutes. Pellets were re-suspended in 500 μL of DPBS and cells were fixed by addition of 4.5 mL of ice-cold 70% ethanol. Centrifuge tubes were then transferred to freezer (−18°C) over night. Following day, ethanol was removed by centrifugation at 400 g for 5 minutes, cells were washed with DPBS, again centrifuged at 400 g for 5 minutes and incubated with PI/RNase staining solution (0.5 mL per sample) at room temperature in the dark for 30 minutes. Finally, samples were transferred to 96-well plate and fluorescence measurements were performed using microcapillary flow cytometer.

3.2.2.3 Caspase 3/7 and 8/9 assay

THP-1 cells were seeded in 24-well plate with cell concentration 5 x 10^4 cells/mL. To each well containing 900 µL of cells in culture medium, 100 µL of compounds in culture medium was added to reach final concentration of 35 µM. THP-1 cells were treated and incubated in CO_2 incubator for increasing times (1, 3, 5, 7 and 9 h). Meanwhile, apoptosis wash buffer and caspase reagent working solution were prepared according to the manufacturer's instructions (72). After incubation, cells were collected in 1.5 mL microtubes, washed with DPBS and centrifuged at 200 g for 5 minutes, supernatant was discarded and pellet re-suspended in fresh culture medium. Samples were then transferred to 96-well plates. To each well containing 100 µL of cells in culture medium, 10 µL of specific caspase reagent working solution was added and plates were placed in CO_2 incubator for 1 h. Then, 100 µL of apoptosis wash buffer was added to each well and samples transferred to microtubes were centrifuged at 300 g for 5 minutes. Another washing step was performed and supernatants were discarded. 200 µL of previously prepared 7-AAD working solution or 200 µL of apoptosis wash buffer were added to appropriate microtubes. Samples from microtubes were transferred to 96-well plate and incubated at room temperature in the dark for 10 minutes. Finally, fluorescence measurements were performed using microcapillary flow cytometer.

3.2.3 INFLAMMATION ASSAYS

3.2.3.1 TNF-α secretion assay

TNF-α secretion assay is designed for detection of TNF-α secreting leukocytes. Kit includes a TNF-α catch reagent and a TNF-α detection antibody. Catch reagent consists of an anti-TNF-α monoclonal antibody (mouse IgG1) conjugated to cell surface specific monoclonal antibody (mouse IgG2a). Detection antibody represents anti-TNF-α monoclonal antibody (human IgG1) conjugated to fluorescent phycoerythrin (PE). Briefly, leukocytes are first stimulated by specific antigen which cause production and secretion of TNF-α. Catch reagent is attached to the cell surface of all leukocytes by his cell surface specific monoclonal antibody and secreted TNF-α is captured by the anti-TNF-α monoclonal antibody linked to the first antibody. Then, a detection antibody binds to the caught TNF-α which enables detection of TNF-α secreting cells by microcapillary flow cytometry. PI staining is used to exclude dead cells (73). Principle of the TNF-α secretion

assay is illustrated in Figure 6. In our case, THP-1 cells were stimulated by LPS and incubated with compounds for 4 h. Positive and negative controls were included in all TNF-α secretion assays to ensure appropriate gating. Celastrol (50 µM) was used as a positive control for cytokine inhibition since it inhibits pro-inflammatory cytokine secretion (TNF-α, IL-1β, IL-8, IL-6) in peripheral blood mononucleated cells (PBMC) in vitro (74,75). LPS-stimulated cells in culture medium without DMSO and compounds were used as a negative control.

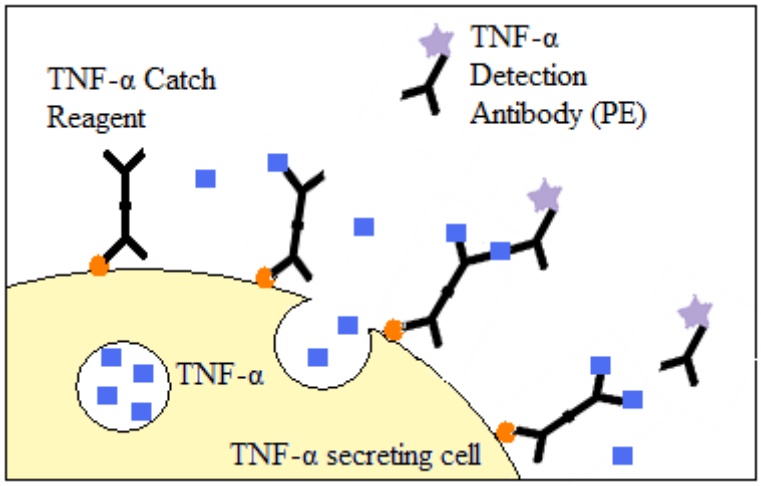


Figure 6: Graphical presentation of principle of TNF-α secretion assay (adapted from reference (73)).

3.2.3.1.1 Primary screening

Primary screening to explore potential anti-inflammatory activity induced by the tested compounds in THP-1 cells using TNF-α secretion kit (yellow fluorescence) and PI (red fluorescence) was performed. THP-1 cells were seeded in 96-well plates with cell concentration 3 x 10^5 cells/mL. To appropriate wells containing 180 µL of cells in culture medium, 20 µL of compound in culture medium was added using a multichannel pipette to reach final concentration of 50 µM and 2 µL of LPS was added to reach final concentration of 1 µg/mL. After treatment, 96-well plates were incubated in CO_2 incubator for 2 h. Then, 2 µL of catch reagent and 2 µL of detection antibody were added to each well and plates were incubated in CO_2 incubator for another 2 h. After the second incubation, samples were transferred to microtubes, and then centrifuged at 200 g for 5 minutes. Cells were washed twice with DPBS and the supernatant discarded. 200 µL of fresh culture medium and PI (3 µL) were added to each microtube. Samples were transferred to 96-well plate and

then incubated at room temperature in the dark for 10 minutes. Finally, fluorescence measurements were performed using microcapillary flow cytometer.

3.2.3.1.2 Determination of dose response curves and IC_{50} for TNF-α inhibition

Half maximal inhibitory concentration (IC_{50}) represents a concentration of compound which inhibits half of maximal biological response after a certain time period (68). In our case, IC_{50} represents concentration of compound which inhibits TNF-α secretion by 50% after 4 h incubation. THP-1 cells were seeded in 96-well plate with cell concentration 3 x 10^5 cells/mL. Increasing concentrations of compounds (from 6 to 75 μM) in culture medium were freshly prepared from stock solution prior to each experiment. To appropriate wells containing 180 μL of cells in culture medium, 20 μL of compound in culture medium was added using a multichannel pipette to reach specified final concentration of compound and 2 μL of LPS was added to reach final concentration of 1 μg/mL. After treatment, 96-well plates were incubated in CO_2 incubator for 2 h. Then, 2 μL of catch reagent and 2 μL of detection antibodies were added to each well and 96-well plate was incubated in CO_2 incubator for another 2 h. After the second incubation, samples were transferred to microtubes and centrifuged at 200 g for 5 minutes. Cells were washed twice with DPBS and supernatants were discarded. 200 μL of fresh culture medium and PI (3 μL) were added to each microtube. Samples from microtubes were transferred to 96-well plate and then incubated at room temperature in the dark for 10 minutes. Finally, fluorescence measurements were performed using microcapillary flow cytometer.

4 RESULTS AND DISCUSSION

Nine synthetic analogues of marine alkaloid clathrodin, for which proapoptotic activity was previously observed on HepG2 cell line, were assessed in THP-1 cells to determine their potential proapoptotic and anti-inflammatory activities.

4.1 APOPTOSIS ASSAYS

4.1.1 ANNEXIN V-FITC / PROPIDIUM IODIDE APOPTOSIS ASSAY

Readouts by flow cytometer need careful attention since appropriate gating has to be applied in order to ensure proper interpretation of the results. Debris is excluded from analysis by setting a threshold value and applying gates on SSC/FSC dot plots. SSC represents cell granularity and FSC represents cell size. Annexin V-FITC after excitation with blue laser emits green fluorescence, while PI emits red fluorescence. Combination of both dyes enables detection and discrimination of live, apoptotic and dead cells when proper gating is applied for red/green fluorescence dot plot. Live cells can be found in the lower left quadrant (annexin –, PI –), apoptotic cells in the lower right quadrant (annexin +, PI –) and dead cells in the upper right quadrant (annexin +, PI +). Example of a proper gating when using annexin V-FITC/PI apoptosis assay is shown in Figure 7.

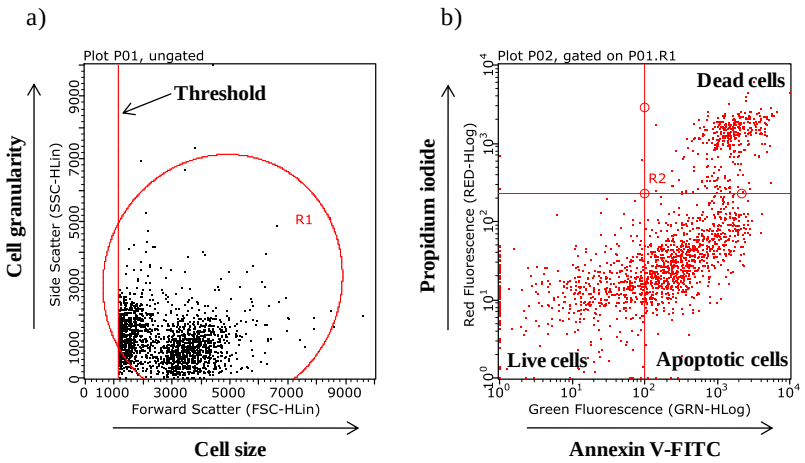


Figure 7: a) SSC/FSC dot plot with gates R1 excluding debris from cells, b) Red/green fluorescence dot plot with gates R2 dividing cells in populations of live, apoptotic and dead cells. Each dot represents one cell.

As already described, positive and negative controls are important to place the gates in the right position in order to be able to distinguish different populations of cells correctly as we are working in arbitrary units for the fluorescence values. Celastrol (50 µM) was used as a positive apoptotic control and untreated cells in culture medium with DMSO (50 µM) were used as a negative apoptotic control. For each experiment gating was adjusted according to the positive and negative controls. Figure 8 shows example of red/green fluorescence dot plots of positive and negative controls for annexin V-FITC/PI apoptosis assay.

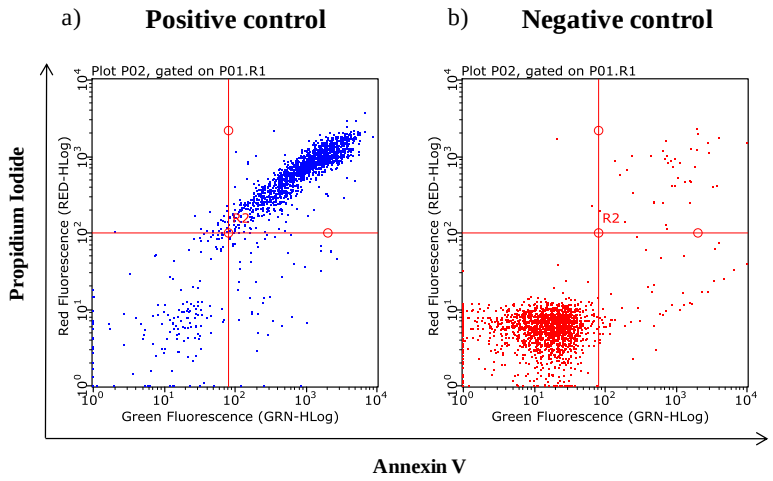


Figure 8: Red/green fluorescence dot plots of a) positive and b) negative apoptotic controls with applied gating. Positive control shows high red and green fluorescence of dead cells (annexin +, PI +), whereas negative control shows basal red and green fluorescence of live cells (annexin –, PI –).

4.1.1.1 Primary screening

Primary screening was performed in order to determine which compounds should be later on used in further experiments. THP-1 cells were seeded in 96-well plates and were incubated for 24 h with compounds **1** – **9** at 50 µM final concentration. Experiments were performed in duplicates and repeated three times. Positive control showed almost total induction of cell death since 99.2% of cells were dead by apoptosis, whereas negative control showed only 5.2% of dead cells. Compounds **1**, **5**, **6**, **7** and **8** caused cell death in less than 20% of THP-1 cells, while compounds **2**, **3**, **4** and **9** were selected for further evaluation since they induced cell death in more than 85% of cells after 24 h incubation. All selected compounds contained indole moiety in the eastern part of the molecule,

whereas BOC group was not present on the 2-aminoimidazole moiety in the western part. It seems that indole moiety is responsible for inducing cell death of THP-1 cells, when western part of the molecule does not contain BOC moiety. This can be illustrated by comparing percent of dead cells for compounds **1** and **3** (13.0% vs. 96.5%). Results of the primary screening are presented in Figure 9.

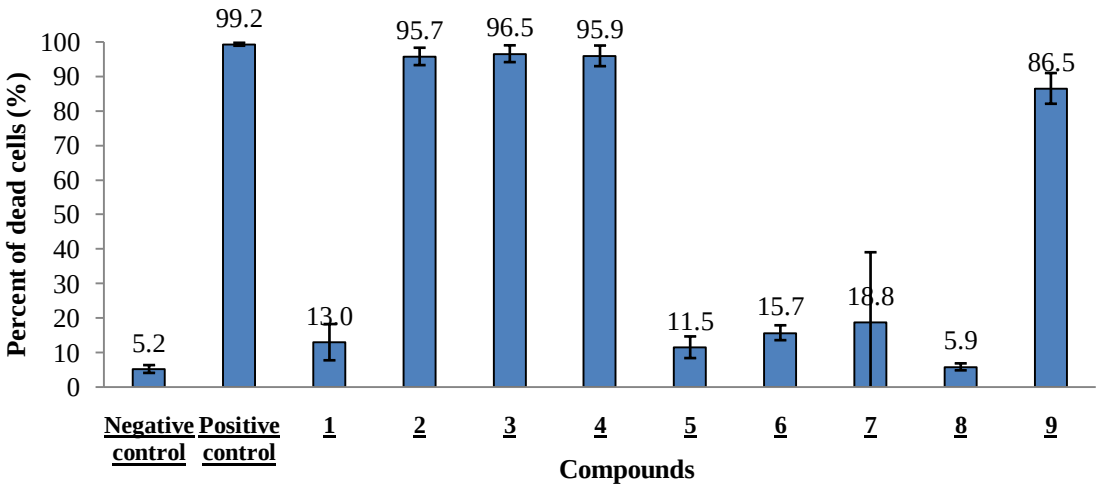


Figure 9: A primary screening of compounds **1** to **9** at 50 μM final concentration assayed in THP-1 cells for 24 h incubation (n=3 independent experiments in duplicates, 2000 events per sample were analyzed).

4.1.1.2 Determination of dose response curves and EC_{50}

Dose response curves and EC_{50} values for proapoptotic activity were determined for compounds **2**, **3**, **4** and **9**. THP-1 cells were seeded in 96-well plate and incubated with increasing concentrations (from 0.412 to 60 μM) of compounds for 24 h. Experiment was performed in triplicates and repeated three times. All four compounds showed similar EC_{50} values between 20 and 24 μM after 24 h incubation. Proapoptotic potency of the compounds was almost the same although they contained different substituent on indole moiety in the eastern part of the molecule. Dose response curves and EC_{50} values are displayed in Figure 10.

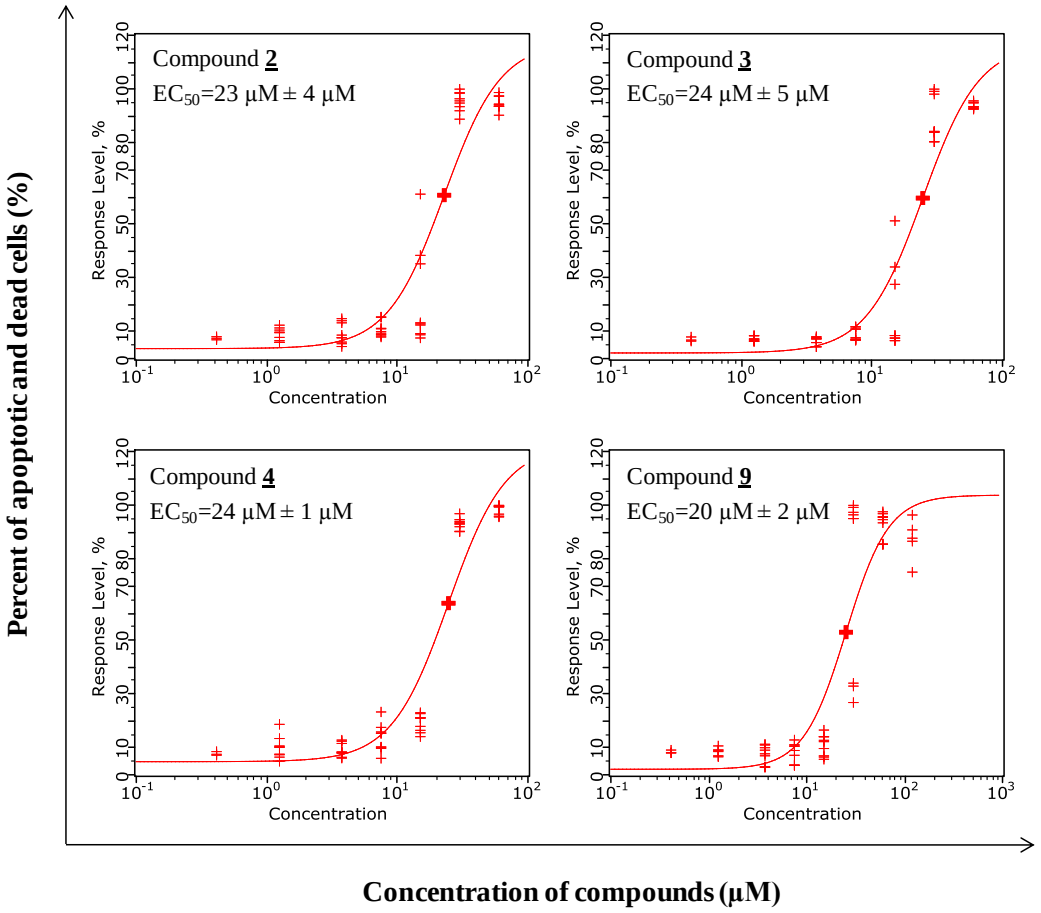


Figure 10: Dose response curves and EC_{50} values for proapoptotic activity of compounds **2**, **3**, **4** and **9** after 24 h incubation in THP-1 cells (n=3 independent experiments in triplicates, 2000 events per sample were analyzed).

4.1.1.3 Time-course assay

Time-course experiments using annexin V-FITC/PI apoptosis assay were performed to determine if information on kinetic of proapoptotic activity could be obtained. THP-1 cells were seeded in 96-well plate and were incubated for increasing times (1, 3, 5 and 7 h) with compounds **2**, **3**, **4** or **9** at 35 µM final concentration. Final concentration of 35 µM was chosen since the results of preliminary time-course experiments using 15, 25 and 35 µM concentrations of compounds showed that 35 µM was the most suitable for further testing (data not shown). Experiments were performed in triplicates. Although compounds showed similar EC_{50} values, results in time-course assay were different when tested at the same concentration. Compound **9**, containing methoxy substituent on indole moiety, showed the slowest proapoptotic kinetic among all compounds, considering that after 7 h incubation

approximately 80% of the cells remained alive. On the other hand, compound **3**, containing chloro substituent on indole moiety, revealed the fastest kinetic since 90% of the cells were dead already after 7 h incubation. Almost the same profile was shown for compounds **2** and **4** (50-60% of dead cells after 7 h incubation). However, time-dependent increase of number of apoptotic cells was not observed for compound **2**. Compound **2** contains fluoro substituent on indole moiety, whereas compound **4**, which contains methyl group on 2-aminoimidazole moiety, does not contain a substituent on indole moiety. Figure 11 shows the results of time-course experiment.

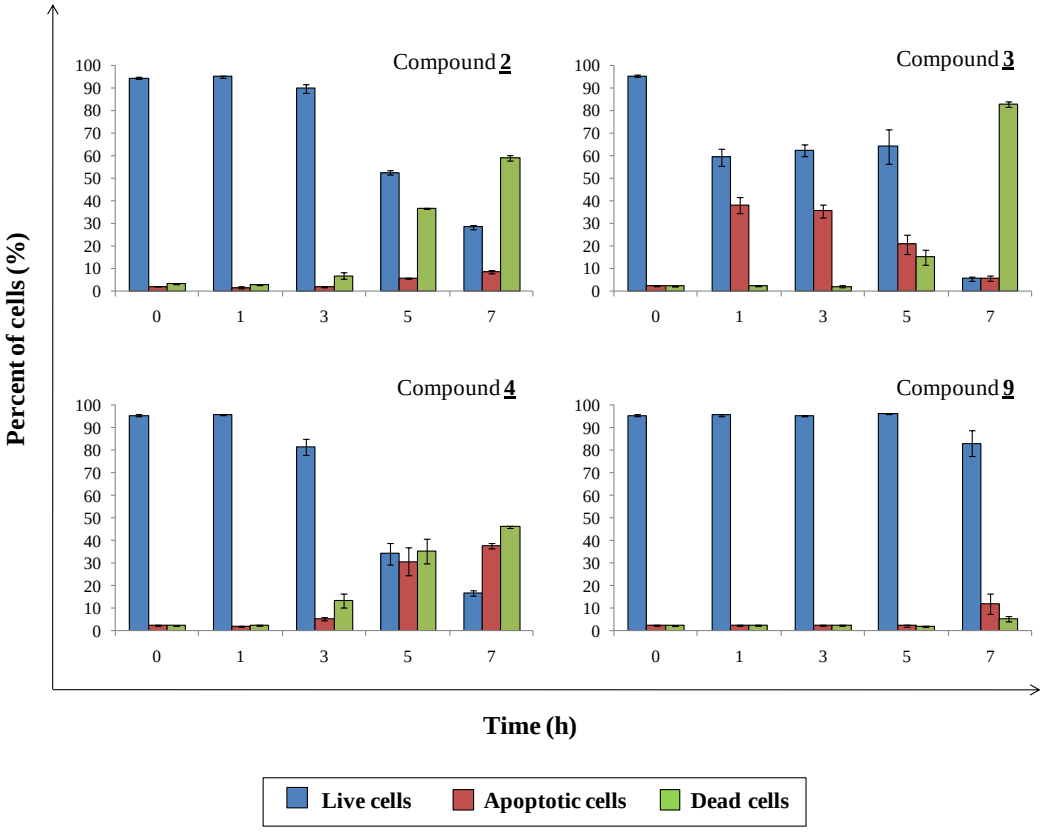


Figure 11: Time-course experiment for determining time-dependent proapoptotic activity of compounds **2**, **3**, **4** and **9** at 35µM concentration in THP-1 cells (n=1 in triplicates, 2000 events per sample were analyzed).

4.1.1.4 Assay with pan caspase inhibitor Z-VAD-fmk

An assay with pan caspase inhibitor Z-VAD-fmk was performed for the selection of compound for further mechanistic experiments since assay with Z-VAD-fmk enables to determine caspase-dependent cell death. THP-1 cells were seeded in 96-well plate in two

parallels and one parallel was pre-treated with pan caspase inhibitor Z-VAD-fmk for 1 h (final concentration of 50 µM). Both parallels were then incubated with compounds **2**, **3**, **4** or **9** at 35 µM concentration for 7 h. Experiment was performed in triplicates. Figure 12 shows how presence or absence of pan caspase inhibitor affected cell death induced by tested compounds.

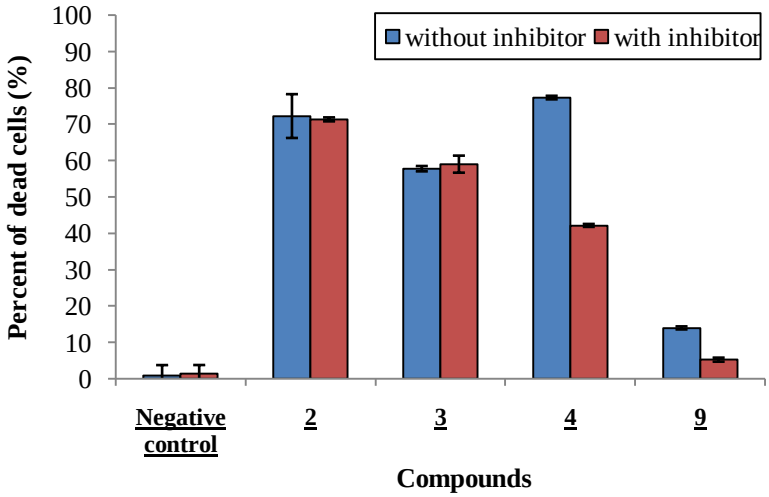


Figure 12: Comparison of percent of dead cells after 7 h incubation with compounds at 35 µM concentration with and without pan caspase inhibitor Z-VAD-fmk (50 µM) in THP-1 cells (n=1 in triplicates, 3000 events per sample were analyzed).

Inhibition of cell death in a parallel with added Z-VAD-fmk after 7 h incubation was only observed for compound **4** and **9**, indicating caspase-dependent cell death of THP-1 cells induced by these two compounds. For compound **4** percent of dead cells dropped from 77.3% to 42.2%, whereas for compound **9** percent of dead cells decreased from 14.0% to 5.2%. On the other hand, percent of dead cells remained nearly the same for compound **2** and **3**. Due to these results compound **4** was selected for further mechanistic experiments. Time-course experiment with Z-VAD-fmk for compound **4** was performed to evaluate time-dependent inhibition of cell death. THP-1 cells were seeded in 96-well plate in two parallels and one parallel was pre-treated with pan caspase inhibitor Z-VAD-fmk (final concentration of 50 µM) as before. Both parallels were then incubated with compound **4** at 35 µM concentration for increasing times (2, 3 and 4 h). Inhibition of cell death was still observed at all times of incubation. Percent of dead cells decreased from 89.4% to 17.2% comparing both conditions after 4 h incubation. These results indicate caspase-dependent

cell death induced by compound **4** in THP-1 cells since a substantial decrease of dead cells was observed. It is interesting that although pan caspase inhibitor could inhibit cell death of THP-1 cells, externalization of PS was not inhibited at all since time-dependent increase of percent of apoptotic cells (annexin +, PI –) was observed comparing increasing time points of parallel with inhibitor. These results indicate caspase-independent apoptotic externalization of PS. Similar caspase-independent exposure of PS during apoptosis of primary T lymphocytes was described by Ferraro-Peyret and colleagues (76). However, it has been recently shown that apoptotic PS exposure seems to be caspase-dependent process since activated caspase 3 and 7 activates scramblase Xkr8, protein responsible for PS externalization (77). Results of the time-course experiment using Z-VAD-fmk for compound **4** are displayed in Figure 13.

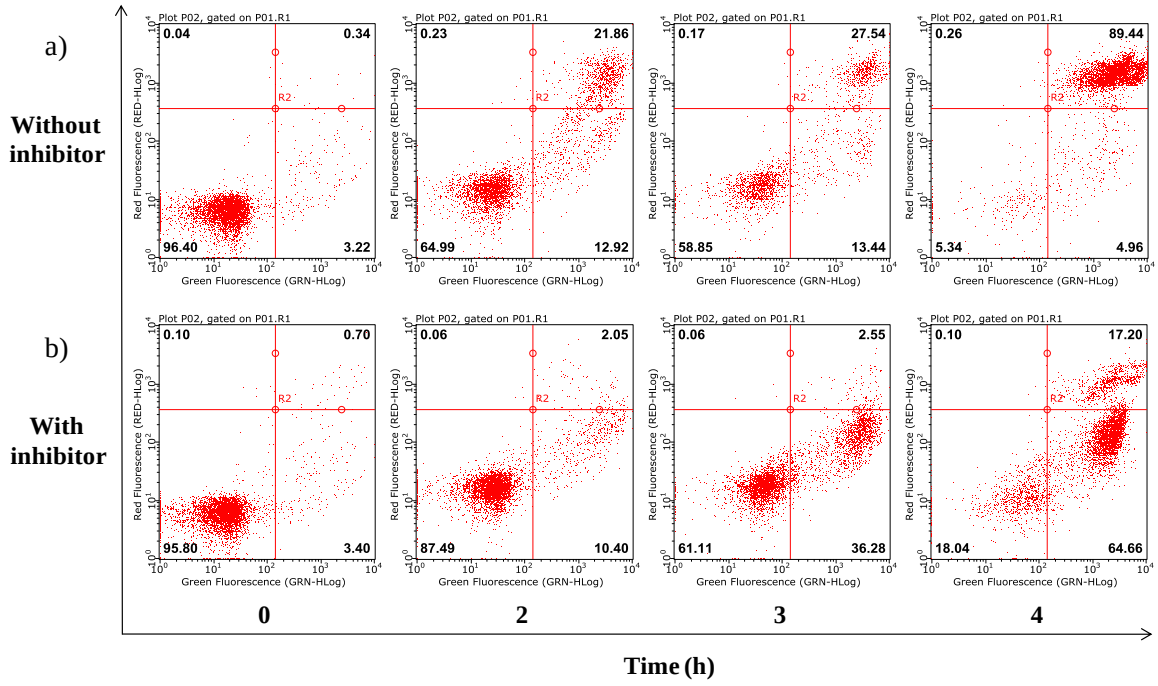


Figure 13: Time-course experiment a) without and b) with pan caspase inhibitor Z-VAD-fmk (50 µM) for increasing times of incubation for compound **4** at 35 µM concentration in THP-1 cells (n=1 in triplicates, 3000 events per sample were analyzed).

The same 96-well plate used for the time-course assay with Z-VAD-fmk for compound **4** was also evaluated by cell imaging cytometry and images of the cells were made for both conditions. These results followed the same trend as results obtained by microcapillary

flow cytometry since obvious differences in percent of dead cells (red and green fluorescent cells) between parallels treated with or without Z-VAD-fmk were easily observed. These results again suggest caspase-dependent cell death of THP-1 cells. Figure 14 contains images of both conditions.

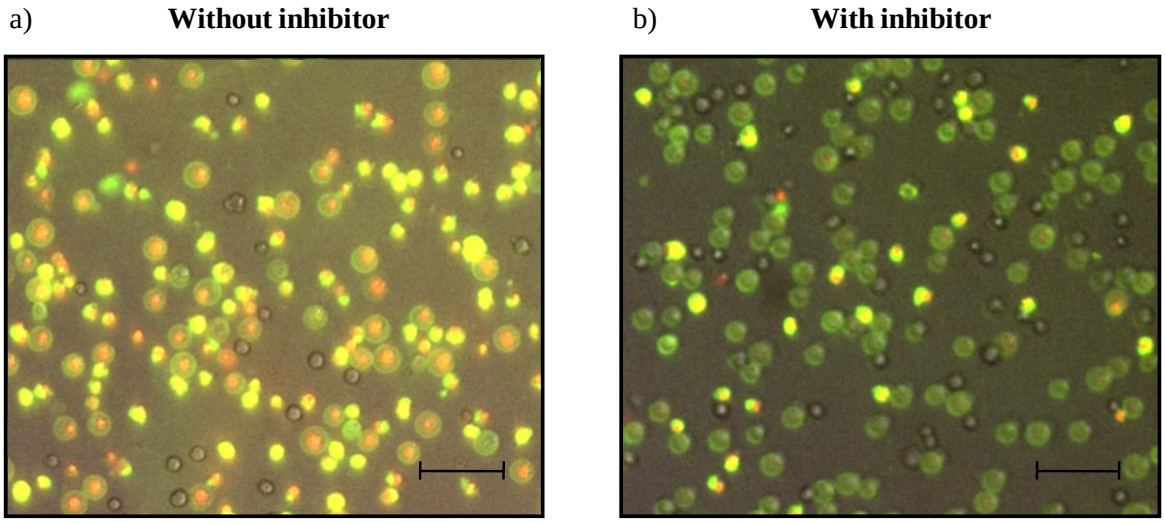


Figure 14: Images of THP-1 cells taken by Celigo® S imaging cytometry for a time-course experiment of compound **4** at 35 µM after 4 h incubation a) without and b) with pan caspase inhibitor Z-VAD-fmk (50 µM). Grey non-fluorescent cells are alive (annexin –, PI –), only green fluorescent cells are apoptotic (annexin +, PI –) and both, red and green fluorescent cells are dead (annexin +, PI +). Scale bar represents 100 µm.

4.1.2 CELL CYCLE ASSAY

Cell cycle can be divided in four important phases when a parent cell divides into two daughter cells. G1 is the first phase in which cells start to grow and prepare for DNA synthesis. DNA replication happens during the S phase. G2 phase is an intermediate phase between S phase and mitosis. In this phase, cells are growing and preparing for division. Cells in M phase stop growing and all the energy is available for the final cell division. After division, cells leave the cycle to enter the G0 phase where they rest and stop dividing (78). Phases of cell cycle and eventually apoptotic cells can be detected by evaluation of DNA content by microcapillary flow cytometry. Apoptotic cells contain less DNA per cell than in G0/G1 phase since during apoptosis cells undergo heavy DNA fragmentation. Small DNA fragments can be extracted from the cells after fixation and permeabilization

of the cell membranes allowing clear discrimination between apoptotic and live cells. Apoptotic cells are gathered left to the G0/G1 peak in a sub-G1 population (79). In our case, cell cycle assay was performed to evaluate DNA content of the cells and to detect apoptotic cells. THP-1 cells were incubated with compound **4** at 35 µM for increasing times (3, 6, 9, 12 and 24 h). Cells were fixed with 70% ethanol and then transferred to −18°C over night. The next day, ethanol was removed and cells were incubated with PI/RNase staining solution. Fluorescence measurements were performed using microcapillary flow cytometer. Debris and aggregates were excluded by applying proper gates. Figure 15 shows histograms of the cell cycle assay for increasing time points.

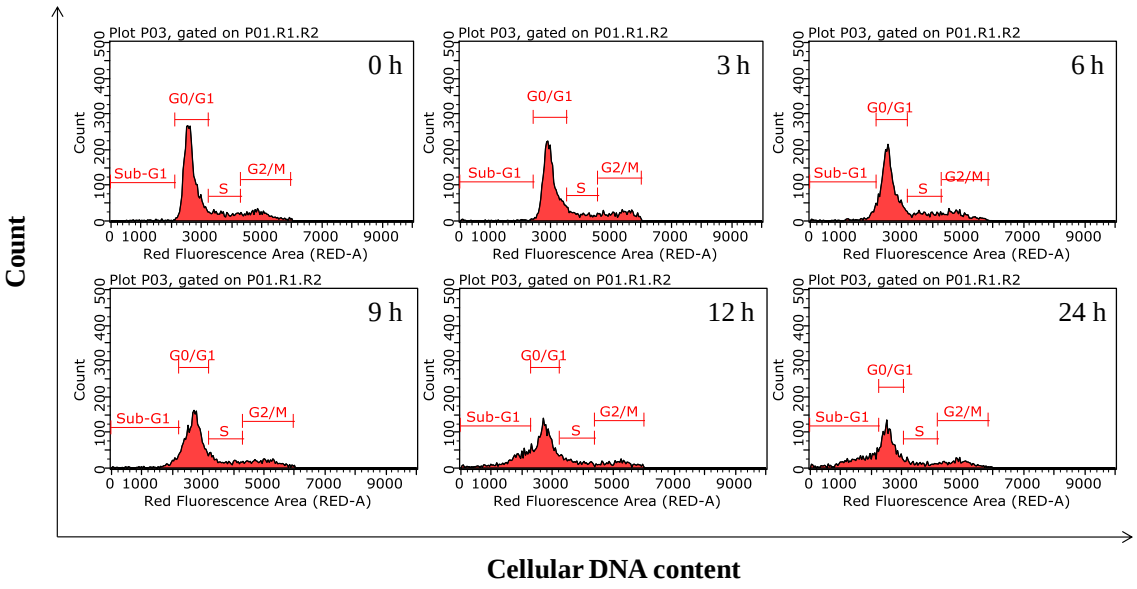


Figure 15: DNA content histograms of cell cycle assay for compound **4** at 35 µM concentration and increasing times of incubation in THP-1 cells (n=1, 5000 events per sample were analyzed).

Time-dependent increase of apoptotic cells (sub-G1 population) was observed confirming induction of apoptosis by compound **4** at 35 µM in THP-1 cells. Apoptotic cells increased from 1.8% to 29.8% after 24 h incubation mainly due to decrease of G1/G0 cells. Percent of cells in other phases remained almost the same during different times of incubation as it can be seen in Figure 16.

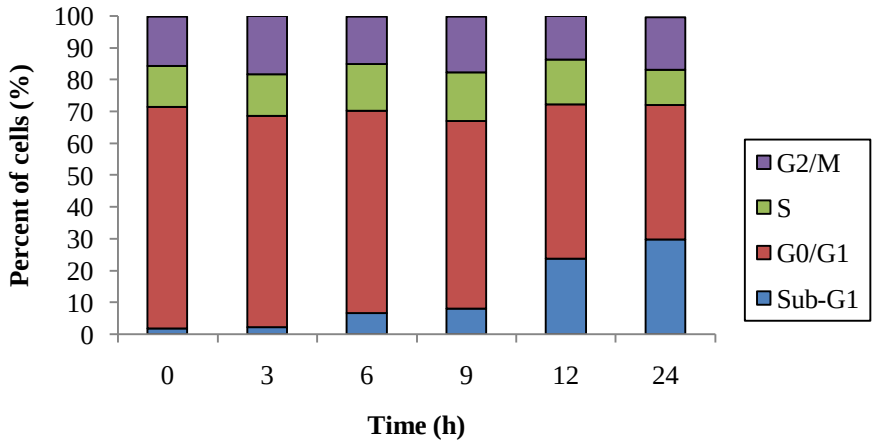


Figure 16: Distribution of cells between specific phases of cell cycle analysis for compound **4** at 35 µM concentration and increasing times of incubation in THP-1 cells (n=1, 5000 events per sample were analysed).

4.1.3 CASPASE 3/7 AND 8/9 ASSAY

THP-1 cells were incubated with compound **4** at 35 µM for increasing times (1, 3, 5, 7 and 9 h) and caspase 3/7 or caspase 8/9 activities were monitored. Dead cells were excluded using 7-AAD staining and activated caspases were detected only in live cells undergoing apoptosis. Caspase 3/7 kit was used to detect activated effector caspases and to confirm apoptosis, whereas caspase 8/9 assay was performed to determine extrinsic or intrinsic pathway of apoptosis. Figure 17 shows activation of caspases as a time-dependent increase of mean fluorescence intensity.

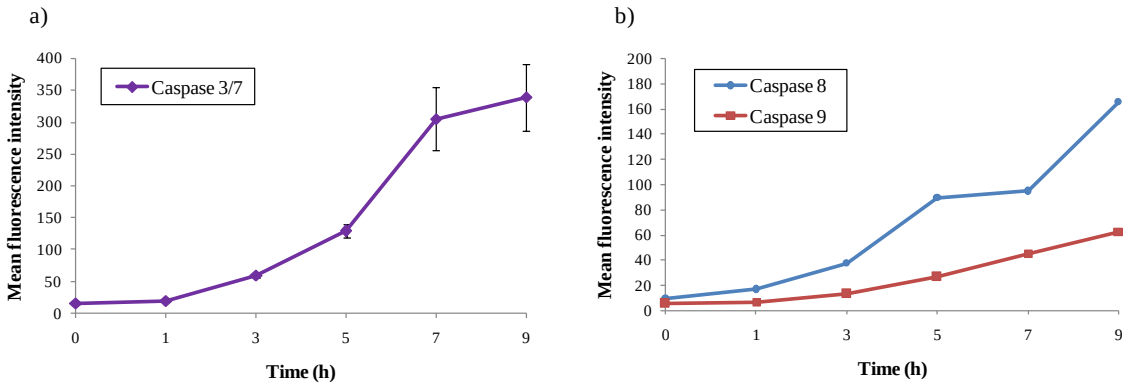


Figure 17: Time-dependent increase of mean fluorescence intensity demonstrating a) caspase 3/7 and b) caspase 8/9 activities (n=1 in duplicates, 2000 events per sample were analysed).

Time-dependent activation of caspase 3 and 7 was observed confirming apoptosis. These results followed the same trend as results of the cell cycle analysis. Furthermore, earlier caspase 8 activation prior to caspase 9 was observed suggesting the activation through extrinsic pathway. We assume that activation of caspase 8 led to later activation of caspase 9 by caspase 8–dependent activation of proapoptotic protein Bid (6). Process of apoptosis was amplified by activation of both apoptotic pathways resulting in a fast and efficient induction of apoptosis in THP-1 cells.

In summary, we have shown that compounds **2**, **3**, **4** and **9** induce cell death of THP-1 cells with similar EC_{50} values (around 20 μM). Results of the experiment with pan caspase inhibitor Z-VAD-fmk indicated caspase-dependent cell death of THP-1 cells induced by compounds **4** and **9**. Compound **4** was selected for further mechanistic experiments since additional time-course experiment with pan caspase inhibitor once again suggested induction of caspase-dependent cell death. Externalization of PS on outer leaflet of a cell membrane, internucleosomal DNA fragmentation and activation of caspases represent biochemical characteristics of apoptosis (4). All this characteristics are unique features of apoptosis that enable more clear discrimination between apoptosis and necrosis. During the process of necrosis, cells become bigger by swelling, plasma membrane is rapidly ruptured and toxic products are released outside the cell initiating inflammatory response causing additional damage and stress to surrounding cells. On the other hand, apoptosis does not cause inflammation since apoptotic cells are engulfed and digested by macrophages or surrounding cells (80). Apoptosis is a preferred form of cell death of cancer cells because of the absence of any inflammatory response (81). We have demonstrated by implementation of annexin V-FITC/PI apoptosis assay, cell cycle analysis and caspases 3/7 and 8/9 assays that compound **4** at 35 μM induces extrinsic pathway of apoptosis in THP-1 cells. Additional mechanistic experiments should be done to determine exactly to which death receptor (if any) synthetic analogues of clathrodin bind and trigger apoptosis. It would be also interesting to perform apoptosis assays in normal non-cancerous human peripheral blood mononuclear cells (PBMC) from healthy donors in order to determine potential selectivity of compounds against cancer cells. Synthetic analogues of clathrodin should also be tested for proapoptotic activity in other human cancer cell lines and maybe also on multicellular tumour spheroids to determine potential anticancer activity in 3D cell cultures (82).

4.2 INFLAMMATION ASSAYS

4.2.1 TNF-α SECRETION ASSAY

TNF-α secretion assay was performed to determine any potential anti-inflammatory activity of compounds. Combination of TNF-α secretion kit (yellow fluorescence) and PI staining (red fluorescence) was used to monitor only live TNF-α secreting cells. As previously described, appropriate gating was applied to ensure correct interpretation of the results. Debris was first excluded from analysis by setting a threshold value and applying gates on SSC/FSC dot plots. Staining with PI was important to exclude dead cells from further analysis as they could be assimilated to cells not secreting TNF-α. Gates for SSC/yellow fluorescence dot plot was created to evaluate percent of TNF-α secreting cells. Explanation of gating for TNF-α secretion assay is displayed in Figure 18.

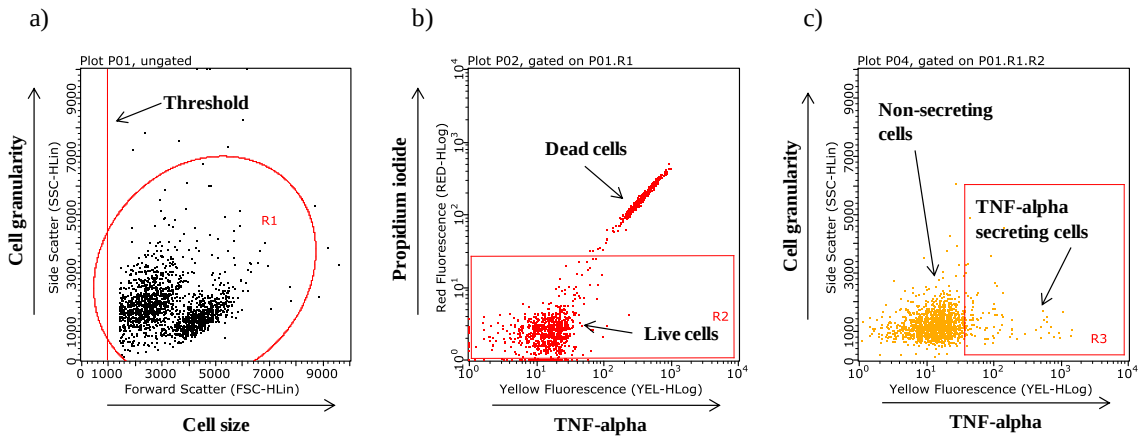


Figure 18: a) SSC/FSC dot plot with gates R1 excluding debris from cells, b) Red/yellow fluorescence dot plot with gates R2 excluding dead cells and c) SSC/yellow fluorescence dot plot with gates R3 dividing cells in populations of TNF-α secreting and non-secreting cells.

Celastrol (50 μM) was used as a positive control for cytokine inhibition and cells treated only by LPS without addition of DMSO were used as a reference control of full TNF secretion (negative control for cytokine inhibition). Untreated cells in culture medium with DMSO (50 μM) were also included to determine anti-inflammatory effect of DMSO, since it was shown that DMSO in low concentrations can possess anti-inflammatory activity (83,84). Gating was adjusted according to the positive and negative controls for each plate. Positive control showed almost total inhibition of TNF-α secretion since only 2.2% of THP-1 cells secreted TNF-α for cells stimulated by LPS. On the other hand, maximum

TNF-α secretion resulted in 45.0% of THP-1 cells secreting TNF-α after stimulation by LPS. Sample with untreated cells and DMSO (50 µM) confirmed that DMSO reduces inflammatory response since significant less LPS-stimulated THP-1 cells secreted TNF-α when compared to negative control (27.1% to 45.0%). Figure 19 displays SSC/yellow fluorescence dot plots of positive and negative controls and DMSO (50 µM) for TNF-α secretion assay.

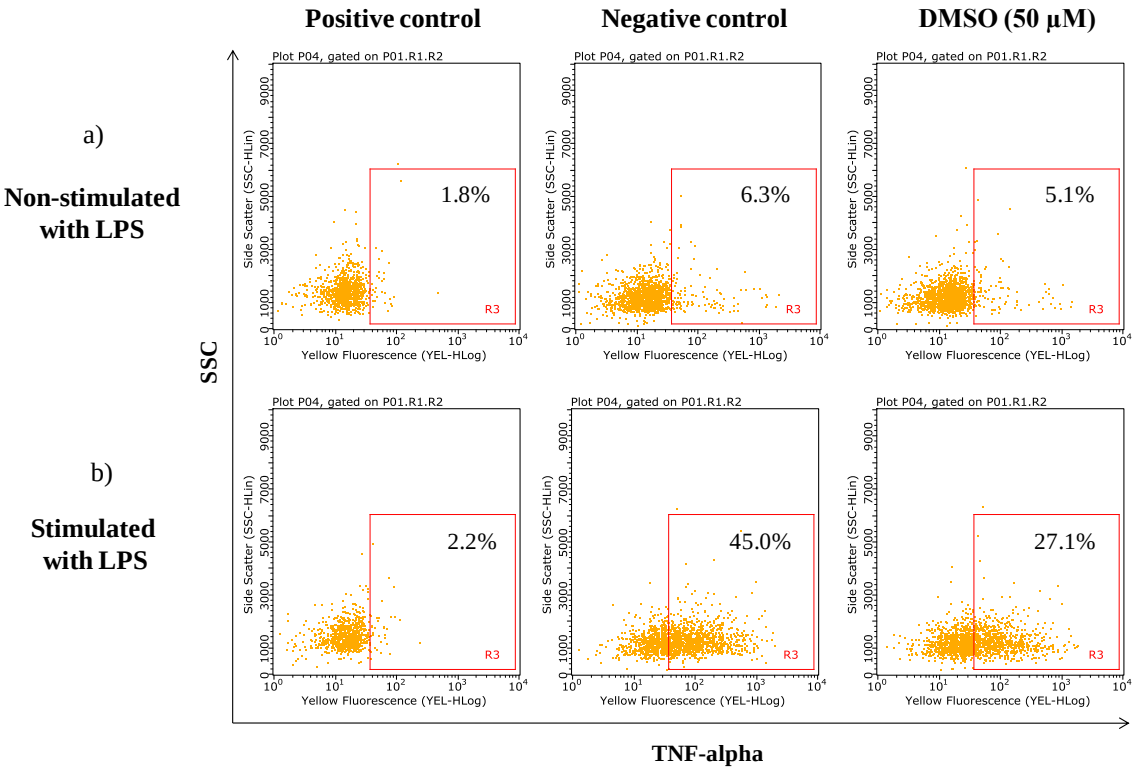


Figure 19: Example of SSC/yellow fluorescence dot plots of positive and negative controls and DMSO (50 µM) for a) non-stimulated and b) stimulated cells by LPS.

4.2.1.1 Primary screening

Primary screening was performed in order to evaluate potential anti-inflammatory activity of compounds. THP-1 cells were stimulated by LPS and incubated with compounds **1** – **9** at 50 µM final concentration for 4 h. Experiment was repeated three times. Positive control showed almost total inhibition of TNF-α secretion since just 2.6% of live cells secreted TNF-α, whereas negative control showed maximum TNF-α secretion since 46.0% of THP-1 cells secreted TNF-α. Results of positive and negative control confirmed that TNF-α secretion assay was well performed. Since DMSO possesses anti-inflammatory activity,

results of compounds have to be compared to the sample with DMSO (50 µM). Only compounds **5** and **9** showed potential inhibition of TNF-α secretion when compared to sample with untreated cells and DMSO (50 µM) since percent of live cells secreting TNF-α was 9.7% and 5.4% respectively. Compounds **1**, **4** and **7** did not show any difference when compared to sample with DMSO (50 µM), whereas compounds **2**, **3**, **6** and **8** showed even increased TNF-α secretion. Based on the results of primary screening compounds **5** and **9** were selected for further evaluation. Results of the primary screening are presented in Figure 20.

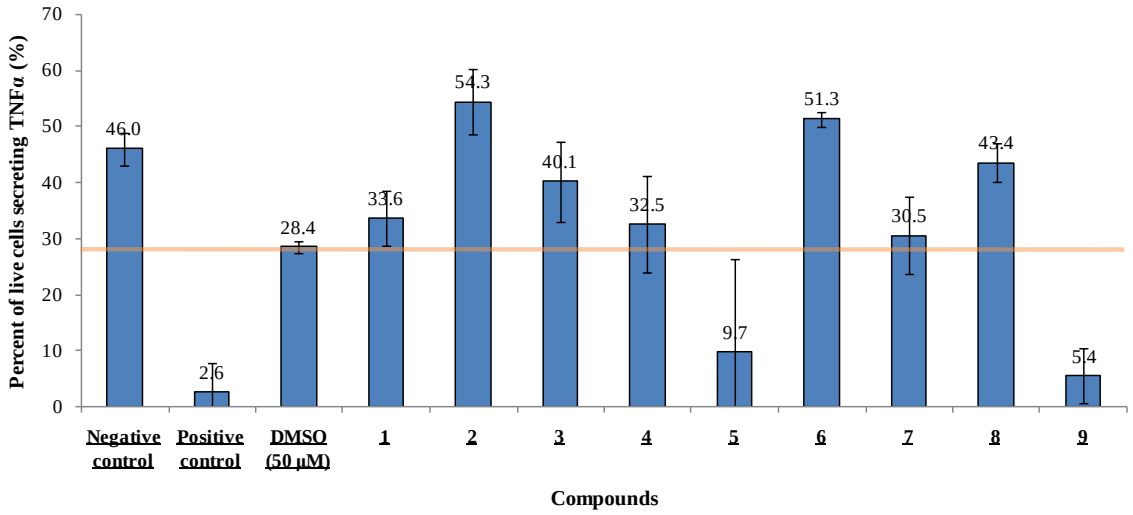


Figure 20: Primary screening of compounds **1** to **9** at 50 µM final concentration assayed on LPS-stimulated THP-1 cells after 4 h incubation (n=3 independent experiments, 2000 events per sample were analyzed).

4.2.1.2 Determination of dose response curves and IC_{50} for TNF-α inhibition

Dose response curves for IC_{50} determination of anti-inflammatory activity were computed for compounds **5** and **9**. THP-1 cells were stimulated by LPS and incubated with increasing concentrations (from 6.25 to 75 µM) of compounds for 4 h. The experiment was performed in duplicates. Compound **9** showed higher anti-inflammatory activity with a lower IC_{50} value (20 µM) compared to compound **5** with IC_{50} value of 52 µM. Dose response curves and IC_{50} values are displayed in Figure 21.

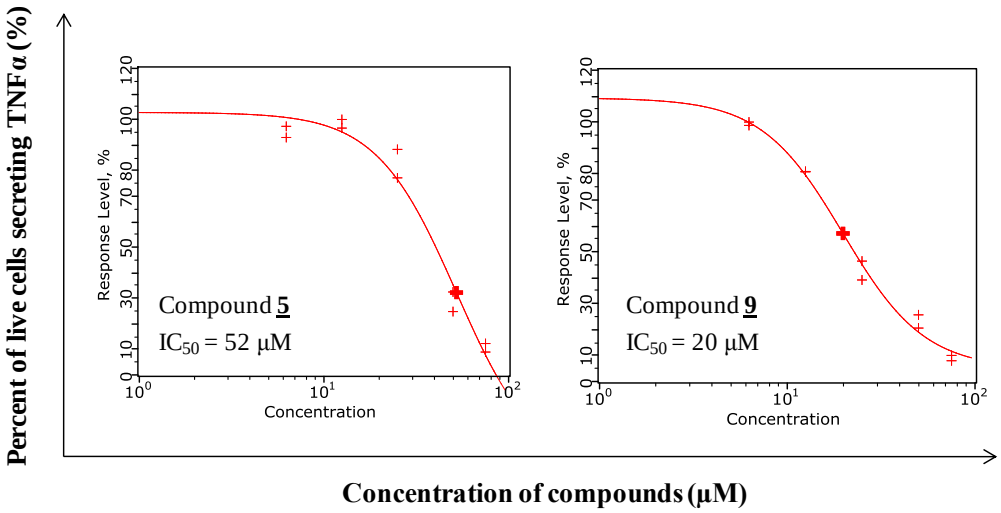


Figure 21: Dose response curves for anti-inflammatory activity of compounds **5** and **9** after 4 h incubation on LPS-stimulated THP-1 cells (n=1 in duplicates, 2000 events per sample were analyzed).

In summary, we have shown here that compounds **5** and **9** inhibit TNF-α secretion thus possessing anti-inflammatory activity. However, experiments for determination of dose response curves and IC_{50} values were preliminary and should be repeated. It would be interesting to follow the secretion of other pro-inflammatory cytokines such as interferon gamma (IFN-γ), interleukins 1, 12 and 18 (IL-1, IL-12 and IL-18). In addition, mechanistic experiments should be performed with compounds **5** and **9** to determine the pathway of inflammatory activity they inactivate. It is also interesting that compound **9** can induce apoptosis and also inhibits inflammatory response in THP-1 cells at the same time with similar EC_{50} and IC_{50} values around 20 µM. On the other hand, compound **5** did not show significant proapoptotic activity but possesses anti-inflammatory activity.

5 CONCLUSIONS

We demonstrated that some of the tested synthetic analogues of clathrodin possess proapoptotic and anti-inflammatory activities in THP-1 human monocytic leukemia cells.

Compounds **2**, **3**, **4** and **9** induce cell death of THP-1 cells with similar EC_{50} values (around 20 μM) after 24 h incubation. Time-course experiment with compounds **2**, **3** and **4** at 35 μM concentration showed fast and efficient induction of cell death in THP-1 cells since after only 7 h incubation between 50-90% of cells were already dead. Experiment with pan caspase inhibitor Z-VAD-fmk confirmed caspase-dependent cell death of THP-1 cells induced by compounds **4** and **9**. We confirmed by implementation of annexin V/PI apoptosis assay, cell cycle analysis and caspases 3/7 assays that compound **4** at 35 μM induces apoptosis in THP-1 cells. Furthermore, results of caspase 8/9 assays indicated extrinsic pathway of apoptosis induced by compound **4** in THP-1 cells.

We also found that compounds **5** and **9** inhibit TNF-α secretion and thus possess anti-inflammatory activity. Compound **9** showed higher anti-inflammatory activity with lower IC_{50} value (20 μM) compared to compound **5** with IC_{50} value of 52 μM.

To sum up, the tested synthetic analogues of clathrodin represent putative new anticancer and anti-inflammatory agents to be further studied.

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