UNIVERZA V LJUBLJANI
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DIPLOMSKA NALOGA

UNIVERZITETNI ŠTUDIJ FARMACIJE

Ljubljana, 2014

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VREDNOTENJE PROAPOPTOTIČNEGA DELOVANJA SINTEZNIH ANALOGOV KLATRODINA NA HUMANIH CELICAH EMBRIONALNEGA KARCINOMA NTERA-2 KLON D1

EVALUATION OF PROAPOPTOTIC ACTIVITY OF SYNTHETIC CLATHRODIN ANALOGS IN HUMAN EMBRYONAL CARCINOMA CELLS NTERA-2 CLONE D1

Ljubljana, 2014

Diplomsko nalogo sem opravljala na Fakulteti za farmacijo v Strasbourgu v Franciji pod mentorstvom doc. dr. Tihomirja Tomašića, mag. farm. in somentorstvom dr. Christiana D. Mullerja. Eksperimentalno delo sem opravljala v celičnem laboratoriju dr. Christiana D. Mullerja v Strasbourgu.

ZAHVALA

Za pomoč pri načrtovanju in praktični izvedbi eksperimentov bi se rada zahvalila doc. dr. Tihomirju Tomašiću, dr. Christianu D. Mullerju, prof. dr. Danijelu Kiklju, izr. prof. dr. Luciji Peterlin Mašič in doc. dr. Nacetu Zidarju. Za podporo med izvajanjem in pisanjem diplomske naloge bi se rada zahvalila Dominiku Nabergoju ter svoji družini.

IZJAVA:

Izjavljam, da sem diplomsko delo izdelala samostojno pod mentorstvom doc. dr. Tihomirja Tomašića in somentorstvom dr. Christiana D. Mullerja.

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ABSTRACT

Marine sponges have been known to produce a wide variety of compounds possessing anticancer, anti-inflammatory, immunosuppressive, antiviral and antimicrobial activities. Sea sponge *Agelas clathrodes* has been recognized as a rich source of many compounds, including alkaloid clathrodin, which was used as a template compound for the design of our synthetic analogs. In our study we investigated potential proapoptotic activity of nine synthetic clathrodin analogs in NTERA-2 clone D1 cell line, derived from a patient with lung metastasis of teratocarcinoma that contain cancer stem cells named embryonal carcinoma cells.

Cancer stem cells are a small population of undifferentiated cells that have been identified in many tumors and possess ability to self-renew, differentiate and form tumor initiating and migrating cells. They display specific mechanisms that make them resistant to anticancer therapy. Conventional therapies act mostly on differentiated cancer cells, whereas many cancer stem cells remain undamaged and are therefore responsible for tumor relapse.

Microcapillary flow cytometry was used for monitoring of all cell-based assays. Preliminary screening using annexin V and propidium iodide as apoptotic markers revealed that all nine synthetic clathrodin analogs triggered cell death of NTERA-2 clone D1 cells after 24 h incubation at 50 μM. Furthermore, we showed for the five most active compounds that they induce cell death after 24 h incubation with EC_{50} values in the lower micromolar range. Among all compounds, compound **4** was selected for further mechanistic experiments, such as experiment with pan caspase inhibitor Z-VAD-fmk, MitoTracker assay, cell cycle assay and caspase-3/-7 assay all confirming induction of apoptosis on NTERA-2 clone D1 cell line by compound **4**. Moreover, results obtained for caspase-8/-9 activation indicate the extrinsic pathway of apoptosis for NTERA-2 clone D1 cells.

To sum up, synthetic clathrodin analogs represent new group of compounds that induce apoptosis in human embryonal carcinoma cell line NTERA-2 clone D1 and therefore represent interesting new starting points for the design of novel therapeutic agents against resistant cancer stem cells.

RAZŠIRJENI POVZETEK

Celična smrt je normalen fiziološki proces, ki poteka v vsakem večceličnem organizmu in omogoča odstranitev poškodovanih celic. Obstaja več različnih delitev celične smrti. Na podlagi biokemijskih značilnosti celično smrt delimo na ekstrinzično in intrinzično apoptozo, avtofagijo, regulirano nekrozo in mitotično katastrofo. Apoptoza je programirana celična smrt, za katero je značilna masivna aktivacija kaspaz, izguba mitohondrijskega transmembranskega potenciala, permeabilizacija zunanje membrane mitohondrija, fragmentacija DNA in translokacija fosfatidilserina na zunanjo stran celične membrane. Poleg značilnih biokemijskih sprememb pride v apoptotični celici tudi do morfoloških sprememb, ki se kažejo kot zmanjšanje volumna celice, kondenzacija kromatina, razpad jedra, nabrekanje celične membrane in tvorba apoptotičnih teles. V organizmu makrofagi pogoltnejo apoptotična telesa in s tem omogočijo odstranitev poškodovanih celic brez neželenega vnetnega odziva imunskega sistema. Apoptoza se lahko začne z vezavo liganda na receptor na zunanji površini celične membrane (ekstrinzična pot), ali pa z znotrajcelični signali, kot so oksidativni stres, poškodbe DNA in povečana koncentracija Ca^{2+} v celici, ki kvarno delujejo na membrano mitohondrija (intrinzična pot). Po aktivaciji ekstrinzične ali intrinzične poti se sproži val znotrajceličnih dogodkov, ki v vodijo do razpada celice. Medtem ko ekstrinzična pot apoptoze vedno poteka preko aktivacije kaspaz, lahko intrinzična pot poteče tudi brez njihove aktivacije.

Proces apoptoze je zelo natančno reguliran, saj lahko preveč ali premalo apoptoze privede do nastanka številnih bolezni, kot so rak, avtoimunske ter nevrodegenerativne bolezni. Rak je dolgo časa veljal za homogeno tkivo, sestavljeno iz hitro delečih se celic. Na podlagi te predpostavke so se razvile konvencionalne terapije zdravljenja raka, kot so kemoterapija in radioterapija, ki ciljajo ta tip celic. Danes vse več raziskav ugotavlja, da so tumorji zelo heterogena tkiva, ki se med seboj razlikujejo v morfologiji, izraženih membranskih markerjih, rasti in odzivu na terapijo. Model hierarhičnega razvoja tumorja predpostavlja obstoj rakavih matičnih celic, ki tvorijo jedro hierarhično organiziranega tumorja in se nahajajo v posebnem mikrookolju tumorja, imenovanem niša. Rakave matične celice imajo sposobnost samoobnavljanja, diferenciacije, tvorbe novih rakavih matičnih celic, novih tumorjev in metastaz ter predstavljajo glavni dejavnik odpornosti na terapijo. Obstaja več razlag, zakaj so rakave matične celice tako odporne: (*i*) Večina rakavih matičnih celic je spečih, v fazi G0 celičnega cikla, kar jih dela neobčutljive na terapijo s spojinami, ki

primarno prizadenejo DNA, saj le-te delujejo na hitro deleče se celice. (*ii*) Rakave matične celice imajo povečano število transporterjev, ki črpajo toksične substance iz celice. (*iii*) Rakave matične celice vsebujejo manjšo koncentracijo reaktivnih kisikovih zvrsti v primerjavi z ostalimi rakavimi celicami, kar zmanjša poškodbe bioloških molekul, s čimer se poveča celično preživetje. (*iv*) Rakave matične celice imajo močno aktiviran sistem, ki popravlja poškodbe DNA, nastale s citotoksičnimi substancami. (*v*) Rakave matične celice izražajo veliko anti-apoptotičnih proteinov, kar jih dela odporne na apoptozo. Zaradi vseh teh specialnih mehanizmov veliko rakavih matičnih celic preživi konvencionalno terapijo in kasneje povzroči ponoven nastanek tumorja. Danes se razvijajo novi pristopi za bolj ciljano zdravljenje, ki so usmerjeni v direktno ubijanje rakavih matičnih celic ali v njihovo diferenciacijo.

Morske spužve proizvajajo številne spojine, ki imajo protitumorno, protivnetno, imunosupresivno, protivirusno in protibakterijsko delovanje. Ena izmed morskih spužev je tudi *Agelas clathrodes*, ki proizvaja klatrodin, pirolimidazolni alkaloid, za katerega so ugotovili, da deluje citotoksično na živalskih celičnih linijah, je modulator napetostno odvisnih natrijevih kanalov in antagonist muskarinskih receptorjev. Molekula klatrodina ima lastnosti značilne za zdravilne učinkovine in ustreza pravilom Lipinskega, saj ima v svoji strukturi manj kot 5 donorjev in manj kot 5 akceptorjev vodikove vezi, molekulsko maso pod 500, manj kot 5 obročev in log P manjši od 5. Klatrodin tako predstavlja zanimivo spojino vodnico za nadaljnjo optimizacijo in sintezo analogov. Sintezni analogi klatrodina, ki so bili uporabljeni v tej diplomski nalogi, imajo naslednje modifikacije: v centralnem delu spojin je bila dvojna vez zamenjana s fenilnim obročem (rigidizacija), pirolni obroč je bil zamenjan z različno substituiranimi piroli in indoli, 2-aminoimidazolni obroč je bil pri večini spojin ohranjen in je v nekaterih primerih vseboval s *t*-butiloksikarbonilno ali metilno skupino, pri eni spojini pa je bil 2-aminoimidazolni obroč zamenjan z pirazolnim.

V diplomski nalogi smo testirali devet sinteznih analogov klatrodina in proučili njihovo sposobnost, da izzovejo celično smrt rakavih matičnih celic. Kot model smo uporabili humano celično linijo embrionalnega karcinoma NTERA-2 klon D1, ki je nastala z izolacijo in kloniranjem rakavih matičnih celic, ki so bile pridobljene iz pljučnih metastaz raka testisov. Glavna metoda, ki smo jo pri testiranju spojin uporabili, je bila pretočna citometrija.

Najprej smo celice NTERA-2 inkubirali s spojinami **1**-**9**, ki smo jih izbrali na podlagi predhodnih testiranj na celični liniji HepG2, v testni koncentraciji 50 μM. Rezultati po 24 h so pokazali, da vse spojine povzročajo celično smrt NTERA-2 celic. Spojine **2**, **3**, **4**, **5** in **9**, ki so izkazovale najvišjo stopnjo citotoksičnosti, smo uporabili v nadaljnjih testih, kjer smo spojinam določili EC_{50} vrednosti in ugotavljali morebiten pojav apoptotične populacije. EC_{50} vrednosti za spojine **2**, **3**, **4**, **5** in **9** so bile v nizkem mikromolarnem področju in so bile med seboj zelo primerljive (med 11 in 22 μM), pri čemer je spojina **4** izkazovala najmočnejšo jakost delovanja z najnižjo vrednostjo EC_{50}. Eksperimenti časovne odvisnosti delovanja spojin **2**, **4** in **9** so pokazali povečanje populacije apoptotičnih celic, medtem ko tega pojava nismo opazili pri spojinah **3** in **5**. Po 9 h inkubacije celic s spojinama **2** in **4** je bilo v apoptozi približno 50% celic, medtem ko je bilo v primeru spojine **9** v apoptozi približno 30% celic. Za spojino **3** nismo opazili hitrega porasta apoptotične populacije, smo pa opazili hiter porast mrtvih celic, saj je bilo po 9 h urah inkubacije mrtvih približno 80% celic. Spojina **5** je izmed vseh spojin delovala najpočasneje, ker je bilo po 9 h še 80% celic živih. Zanimivo je, da kljub podobnim EC_{50} vrednostim testiranih spojin, le-te sprožajo celično smrt različno hitro. Da smo ugotovili, ali spojine povzročajo celično smrt z aktivacijo kaspaz, smo izvedli test s širokospektralnim kaspaznim inhibitorjem Z-VAD-fmk ter spojinami **2**, **3**, **4**, **5** in **9** v 25 μM koncentraciji. Dobljeni rezultati so pokazali, da se je odstotek živih celic zvišal, ko so bile celice predhodno inkubirane z Z-VAD-fmk v primerjavi z netretiranimi celicami, kar nakazuje, da spojine sprožijo celično smrt preko aktivacije kazpaz. Na podlagi vseh pridobljenih rezultatov smo za nadaljnje testiranje mehanizma delovanja izbrali spojino **4**. Rezultati merjenja mitohondrijskega transmembranskega potenciala, analize celičnega cikla in aktivnosti kaspaz-3 in -7 so potrdili, da spojina **4** v 25 μM koncentraciji sproži apoptozo NTERA-2 celic. Pri inkubaciji NTERA-2 celic s spojino **4** v 25 μM koncentraciji smo opazili padec mitohondrijskega potenciala, analiza celičnega cikla pa je pokazala povečanje populacije apoptotičnih celic, saj se je s časom število celic v sub-G1 fazi celičnega cikla povečevalo. Uspeli smo dokazati tudi prisotnost aktiviranih kaspaz-3 in -7, kar predstavlja eno izmed najpomembnejših značilnosti apoptoze. Naredili smo še dodaten test z inhibitorjema kaspaz-8 in -9, ki je pokazal aktivacijo obeh kaspaz, pri čemer smo opazili, da se kaspaza-8 aktivira pred kaspazo-9, kar kaže na ekstrinzično pot apoptoze.

ABBREVIATIONS

7-AAD	7-Amino-actinomycin D
ABC transporters	ATP-binding cassette transporter proteins
AIF	Apoptosis-inducing factor
AnV-FITC	Annexin V-FITC
APAF1	Apoptotic protease activating factor 1
ATCC	American Type Culture Collection
Boc	*t*-Butyloxycarbonyl
CAD	Caspase-activated DNase
CSC	Cancer stem cell
CytC	Cytochrome C
EndoG	Endonuclease G
HtrA2	High temperature requirement protein A2
IAP	Inhibitor of apoptosis protein
IARC	International Agency for Research on Cancer
DD	Death domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium, culture medium
DMSO	Dimethyl sulfoxide
PBS	Dulbecco's phosphate buffered saline
EC	Embrional carcinoma
ES	Embryonic stem cells
FADD	Fas-associated protein with a death domain
FAM	Carboxyfluorescein
FasL	Fas ligand
FasR	Fas receptor
FBS	Fetal bovine serum
FLICA	Fluorochrome-labeled inhibitor of caspases
FSC	Forward-scattered light
MOMP	Mitochondrial outer membrane permeabilization
MTP	Mitochondrial transmembrane potential
NCCD	Nomenclature Committee on Cell Death

NT2/D1	NTERA-2 cell line clone D1
PI	Propidium iodide
PS	Phosphatidylserine
PTPC	Permeability transition pore complex
ROS	Reactive oxygen species
SCC	Side-scattered light
Smac/Diablo	Direct IAP-binding protein with low pI
tBID	Mitochondrion-permeabilizing fragment
SR	Sulforhodamine
TNFα	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TRAIL	TNFα-related apoptosis-inducing ligand
TRAILR	TRAIL receptor
Z-VAD-fmk	Carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone

1 INTRODUCTION

1.1 CANCER

Cancer is one of the main health problems in Europe and all around the world. The International Agency for Research on Cancer reported approximately 3.45 million new cases of cancer and more than 1.75 million deaths due to cancer in Europe in 2012, where lung, colorectal, breast and stomach cancers were the most fatal types (1). Cancer, also termed malignant tumor and neoplasm, can occur everywhere in the body and refers to complex diseases arising from cells with damaged and not repaired DNA. It consists of rapidly and uncontrollably dividing cells that form lumps or mass of tissue named tumor. Cancer cells do not form tumors in the case of leukemia, where cancer cells affect blood and blood forming organs (2). Treatment of cancer depends on many factors, such as type of cancer, size and its dissemination in the body. Cancers that are found in one location and are not spread in the body are usually removed by surgery. Besides surgery there are also other options to treat the cancer such as chemotherapy, radiotherapy, hyperthermia therapy and immunotherapy (3).

1.2 TESTICULAR CANCER

Testicular cancers represent 1% of all cancers and are the most common malignancies in men aged between 20 and 39 years (4). There are many evidences that the majority of testicular cancers arise from germ cells (5). Testicular cancers, usually classified into seminomas and nonseminomas, are very heterogeneous group of malignancies since more than 50% of cancers include more than one histological types, such as embryonal carcinoma, seminoma, teratoma, yolk sac tumor, polyembrioma and choriocarcinoma (6). Testicular cancers as well as their metastasis are curable cancers with survival rate of more than 90%. Early-stage cancers are usually removed by surgery, while more advanced forms are cured by additional surgeries, radiotherapy or cisplatin based chemotherapy (7,8). Teratocarcinomas are malignant testicular tumors consisting of somatic tissues in various stages of differentiation and population of cancer stem cells named embryonal carcinoma (EC) cells (9,10). Many studies on human- and animal-derived cells propose ECs as malignant equivalents of normal embryonic stem (ES) cells since both types of cells are pluripotent, have ability to self-renew and differentiate, and express some similar markers,

such as transcription factor OCT4, which is down-regulated during differentiation (11,12). Due to similarities between ECs and ESs, cell lines that consist of ECs, such as TERA-2 and NTERA-2 are often used in research of molecular mechanisms controlling differentiation of cells in humans (13,14). Due to high survival rate, knowledge about testicular cancer molecular biology could help to improve understanding and treatment of other cancers.

1.3 CANCER STEM CELLS

In the past, cancer was considered as a biologically homogeneous tissue consisting of rapidly dividing cells. Conventional therapies, such as chemotherapy or radiotherapy, were designed and established to act on highly proliferative cells. Nowadays, there is more and more evidence that solid and leukemic tumors are characterized by great morphological heterogeneity, markers on the cell surface, proliferation kinetics and therapy response (15).

Hierarchy or cancer stem cell (CSC) model defines tumor as a well-organized system with hierarchical structure similar to the hierarchy in normal tissue development, with CSCs placed on top (15–17). According to this theory, CSCs are located in a special place inside the bulk of the tumor called "niche", where they maintain their stem-cell state (18,19). CSCs, initially identified in the leukemic and testicular germ cell tumors, represent a small population of cells in the tumor with ability to self-renew, differentiate and cause formation of new tumors when transplanted into immune-compromised mice. CSCs are highly resistant to conventional anti-cancer therapy. CSCs of some tumors are also responsible for metastasis formation, but the mechanisms through which CSCs promote metastasis are still unclear (10,20).

There are many possible explanations why CSCs are so resistant to conventional anti-cancer therapies. Firstly, CSCs are most of the time quiescent in G0 phase of the cell cycle, which makes them insensitive to DNA-damaging drugs that act mostly on the rapidly dividing cells (20). Secondly, up-regulation of ATP-binding cassette (ABC) transporter proteins, which work as efflux pumps, protects CSCs from toxic substances since these are pumped out of the cells (21). Next, in comparison to non-stem cancer cells, CSCs contain lower concentrations of reactive oxygen species (ROS), which reduces damage of biological molecules and consequently, increases CSCs survival (22). Furthermore, CSCs

have proven enhanced DNA repairing system that promotes DNA repair after treatment by cytotoxic agents, and finally, it was also observed that CSCs express many anti-apoptotic proteins like BCL2 family, leading to resistance to apoptosis (20).

Typically, as shown in Figure 1, multidrug resistant CSCs, that survived anti-cancer therapy, are in the majority of cases, responsible for tumor relapse. Based on all new evidence concerning cancer recurrence mechanism, new approaches with more specific targeting of CSCs have been designed to provide better clinical outcomes after therapy. CSCs can be eliminated by destruction or by inducing their differentiation to create cells more sensitive to the conventional therapy. The main targets in CSCs specific therapy are CSCs markers, ABC transporters, niche-microenvironment, CSCs signaling pathways and microRNA based targeting (23,24). However, further research is needed for better understanding of CSCs and CSCs targeting drugs.

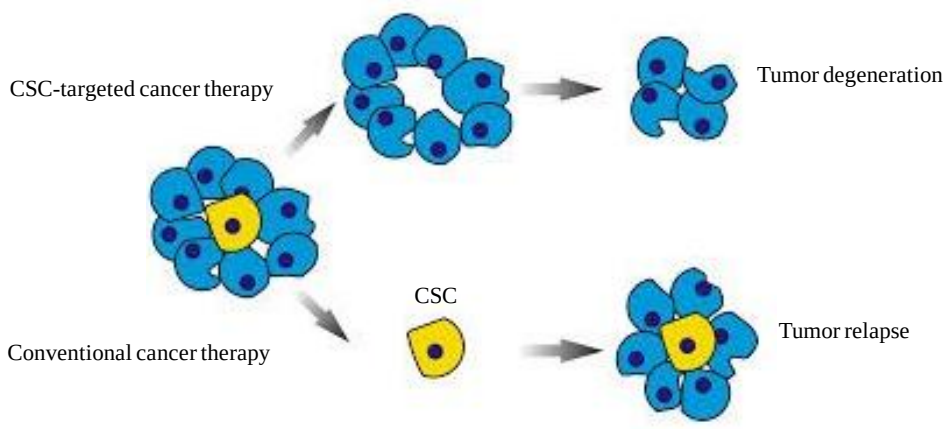


Figure 1: CSC specific and conventional cancer therapy. (Adapted from reference (25)).

1.4 APOPTOSIS

According to The Nomenclature Committee on Cell Death (NCCD), cell death is today classified into intrinsic apoptosis, extrinsic apoptosis, autophagic cell death, regulated necrosis and cell death by mitotic catastrophe. Moreover, intrinsic apoptosis is subdivided in the caspase-dependent or caspase-independent cell death, while extrinsic apoptosis is always caspase-dependent (26). Besides NCCD recommendations on cell death several others classifications exist, which are based on morphological, enzymological, functional or immunological criteria (27).

Apoptosis is a form of programmed cell death that normally occurs during development, ageing and daily maintenance of cell populations in multicellular organisms. Process of apoptosis is precisely regulated because too much or too little apoptosis leads to pathological conditions, such as cancer, developmental defects, autoimmune and neurodegenerative diseases (28). The term “apoptosis” comes from Greek word, meaning “dropping off or falling off” leaves from trees and was initially used in 1972 by John Kerr and his colleagues to describe this interesting biological phenomenon (29).

1.4.1 HALLMARKS OF APOPTOSIS

Cells undergoing apoptosis are characterized by specific morphological features, such as cell shrinkage, chromatin condensation (pyknosis), nuclear fragmentation (kariorrhexis), blebbing, formation of apoptotic bodies in the process called “budding” and finally, phagocytosis of apoptotic bodies by surrounding cells, usually macrophages. Ingestion of apoptotic bodies *in vivo* and their degradation within phagosomes prevents secondary necrosis and inflammatory response (28,29). Besides alterations in morphology, apoptotic cells also exhibit several unique biochemical features including caspase activation, DNA fragmentation, expression of phosphatidylserine (PS) on the outer leaflet of cell membrane and changes in the mitochondrial membrane permeability (28,30). Caspases are cysteinylaspartate-specific proteases, which represent an important group of enzymes involved in the process of apoptosis. They are produced in the cells as proenzymes and after their activation they trigger irreversible caspase cascades finally ending in a cellular breakdown (28,31). To date, 12 different caspases have been identified in humans. They are divided into three groups, namely **inflammatory** (caspases-1, -4, -5 and -12), **effector** (caspases-3, -6 and -7) and **initiator** caspases (caspases-2, -8, -9 and -10) (32). Fragmentation of DNA is the second biochemical hallmark of cells undergoing apoptosis. Endonucleases, activated by effector caspases-3, cleave DNA in specific places to form typical oligonucleosomal DNA fragments consisting of multiples of 180 base pairs (30). Then, PS, normally situated on the inner leaflet of the plasma membrane, is translocated to the cell surface by specific transporter proteins during the process of apoptosis. Expression of PS on the outer leaflet of the plasma membrane is considered as an early apoptotic marker and represents the “eat-me” signal for macrophages and surrounding cells (33). Apoptosis also affects mitochondria since mitochondrial outer membrane is permeabilized

during the process of apoptosis, resulting in a loss of mitochondrial transmembrane potential (MTP), release of toxic substances from the mitochondrial intermembrane space, inhibition of the respiratory chain and overproduction of the reactive oxygen species (ROS) (26).

1.4.2 MECHANISM OF APOPTOSIS

Apoptosis is a complex, sophisticated and well-regulated process, which occurs via extrinsic or intrinsic mechanistic pathway. Extrinsic apoptosis is always caspase-dependent, while intrinsic apoptosis can be either caspase-dependent or caspase-independent process. Briefly, extrinsic pathway starts after stimulation of transmembrane death receptors, while intrinsic pathway is triggered through the mitochondria by various non-receptor stimuli that produce intracellular signals. Both pathways end in a common execution pathway, which leads to characteristic hallmarks of apoptosis (28,31). Schematic representation of extrinsic and intrinsic pathways of apoptosis is displayed in Figure 2.

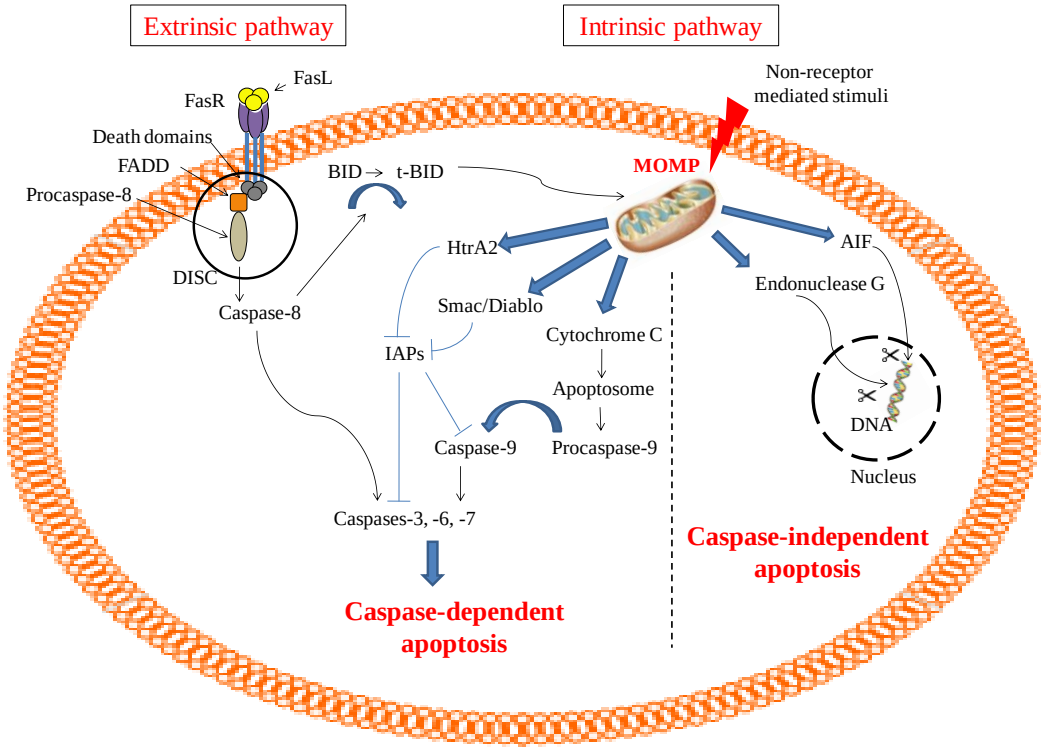


Figure 2: Schematic representation of extrinsic and intrinsic pathways of apoptosis. (Adapted from reference (26)).

1.4.2.1 EXTRINSIC PATHWAY

Extrinsic pathway starts when proapoptotic ligands, such as Fas ligand (FasL), tumor necrosis factor alpha (TNFα) and TNFα-related apoptosis-inducing ligand (TRAIL) bind to their specific death receptors including Fas receptor (FasR), tumor necrosis factor receptor 1 (TNFR1) and TRAIL receptor (TRAILR) (26). Death receptors are homotrimeric transmembrane proteins that belong to the tumor necrosis factor receptor gene superfamily, all containing characteristic cytoplasmic death domains (DD). Events that occur from ligand binding to final cell death in an extrinsic apoptosis can be illustrated by FasL/FasR model (Figure 2) (28). After binding of FasL to FasR, intracellular DD forms a death-inducing signaling complex (DISC) by binding Fas-associated protein with a death domain (FADD) and procaspase-8 (or -10). DISC initiates activation of procaspase-8 (or -10) (26,28). There are two possibilities that can occur after activation of caspase-8. The first option is that caspase-8 activates effector caspase-3 with no effect on mitochondria, while in the second case caspase-8 mediates formation of mitochondrion-permeabilizing fragments (tBID), which bind to mitochondria and activate the intrinsic pathway of apoptosis (26).

1.4.2.2 INTRINSIC PATHWAY

Intrinsic pathway starts inside the cell in mitochondrial membrane and is initiated by various intracellular stimuli such as an oxidative stress, DNA damage and cytosolic Ca^{2+} overload (26). Events occurring in mitochondrial membrane are controlled by Bcl-2 family of proteins, which consist of both anti-apoptotic and proapoptotic proteins (28). BAX and BAK proteins are proapoptotic, pore-forming proteins responsible for mitochondrial outer membrane permeabilization (MOMP). MOMP can also be triggered by opening of the permeability transition pore complex (PTPC), located in the inner mitochondrial membrane (26). MOMP results in the loss of MTP, inhibition of the respiratory chain, overproduction of ROS and release of toxic substances from the mitochondrial intermembrane space, including cytochrome C (CytC), apoptosis-inducing factor (AIF), endonuclease G (EndoG), direct IAP-binding protein with low pI (Smac/Diablo) and high temperature requirement protein A2 (HtrA2) (26). CytC together with the apoptotic protease activating factor 1 (APAF1) and dATP forms apoptosome, which then activates procaspase-9. Afterwards, caspase-9 activates caspase-3, which starts the execution phase

of apoptosis (26). Smac/Diablo and HtrA2 possess inhibitory activity on the apoptosis inhibitor (IAP) family, thus facilitating caspase-9, -3 and -7 activation (31). CytC, Smac/Diablo and HtrA2 activate caspase-dependent intrinsic apoptosis, while AIF and EndoG translocate directly to the nucleus to induce DNA fragmentation in a caspase-independent manner (26).

1.4.2.3 EXECUTION PATHWAY

Execution pathway begins by activation of effector caspases-3 (-6,-7) that cleave cellular substrates and proteins of the cytoskeleton and the nucleus. Caspase-3 also specifically activates caspase-activated DNase (CAD), leading to DNA fragmentation and chromatin condensation (34). Besides effector caspases there are also enzymes, such as AIF and EndoG that cause cellular breakdown in a caspase-independent manner. These last events cause morphological and biochemical changes in apoptotic cells resulting in an exposure of PS on the outer leaflet of cell membrane, blebbing, formation of apoptotic bodies and finally, engulfment by nearby cells.

1.4.3 DETECTION OF APOPTOSIS

Many methods have been designed to enable detection of apoptosis and distinguish it from other modes of cell death. Apoptotic cells exhibit specific morphological and biochemical features that can be measured in different assays. Transmission electron microscopy is the gold standard in research of apoptosis since images obtained can reveal certain ultrastructural morphological features of apoptosis, such as nuclear fragmentation and membrane blebbing. Besides morphological changes, biochemical features, such as activation of caspases, DNA fragmentation, cleavage of certain proteins such, as Bid or PARP, PS exposure, ROS production and MMP, can be also measured using different techniques such as flow cytometry, western blot and spectrophotometry (35).

1.5 SYNTHETIC ANALOGS OF CLATHRODIN

Marine sponges have been known to produce a wide variety of compounds with interesting biological activities, such as anticancer, anti-inflammatory, immunosuppressive, antiviral and antimicrobial activity (36,37). Substances derived from sponges are mostly derivatives

of amino acids, macrolides, saponins, sterols or terpenoids, and contain carbon skeleton rich in nitrogen or halogen (38). *Agelasidae*, *Hymeniacidonidae*, *Dyctionellidae* and *Axinellidae* families of sponges produce many compounds, such as pyrrole-imidazole alkaloids, which can possess relatively simple chemical structures like clathrodin, hymenidin, and oroidin or very complex structures, like stylissadine A (Figure 3) (39). Clathrodin contains 2-aminoimidazole ring, which can play an important role in interactions with different proteins, since it can establish π-π, cation-π and hydrogen bond interactions (40). To date, several activities of clathrodin have been reported including cytotoxicity against Chinese hamster ovary cells (CHO-K1), modulating the voltage-gated sodium channels and anti-muscarinic activity (41–43). Since chemical structure of clathrodin is very simple, it represents interesting lead compound for the design of novel analogs. It was shown that several clathrodin analogs possessed modulatory activity on different voltage-gated sodium channel isoforms and also inhibit the growth of some bacterial and fungal strains (44–46). 9 synthetic analogs of clathrodin were used in this research work. Analogs contained three main modifications in the chemical structure. Among several structural changes, double bond in the central part of the molecule was rigidified by replacement with the phenyl ring. In the eastern part of the molecule pyrrole ring was replaced by substituted pyrrole or indole moiety, whereas the western part contained 5-membered *N*-heterocycles. Chemical structure of clathrodin and and its structural modifications are presented in Figure 3.

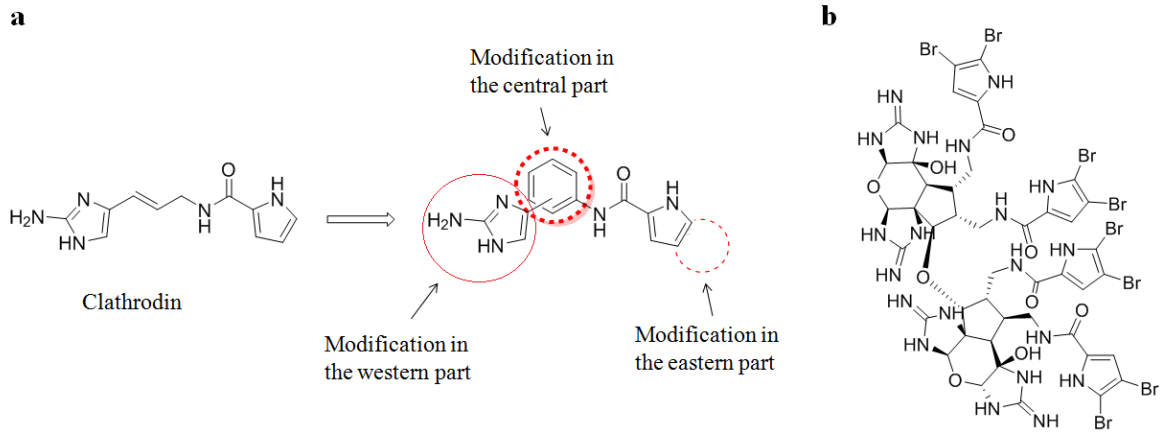


Figure 3: a) Structure of the lead compound clathrodin and its structural modifications; b) Structure of stylissadine A.

1.6 FLOW CYTOMETRY

Flow cytometry is a light-based method that enables simultaneous, multiparametric analysis of physical and chemical parameters of the cells. Cellular parameters including cell size, internal complexity and fluorescence intensity are measured when cells one by one pass in front of a laser beam. The main advantage of the flow cytometry compared to other cell-based monitoring methods is that each cell is evaluated individually and large amount of cells are collected and analyzed in a very short time. Modern cytometers can collect up to 100 000 cells per second and determine up to 20 parameters for each cell. Some flow cytometers can also be used for cell sorting since they can isolate cell population with specific characteristics (47). There are many fields where flow cytometry is applied. Besides routine use in the medical diagnostic laboratories, flow cytometry is today a standard tool for basic research in microbiology, cell biology and pharmaceutical sciences, especially for the purpose of drug discovery screening (48,49).

Flow cytometer consists of three main components: fluidics system, optics system and electronics-computer system as shown in Figure 4. The center of fluidic system represents flow chamber or flow cell, where sample of cells is injected into the laminar flow of the sheath fluid. In the process of hydrodynamic focusing faster sheath fluid forces cells in core stream to form single cell suspension, which is then brought to the laser beam. In the moment, when cell reaches the interrogation point, laser light is scattered and if any fluorochromes are present in or on the cell, they fluoresce, which means that after fluorochromes are excited they emit light of longer wavelength. Scattered and fluorescent lights are trapped by two systems of lenses. First lenses are placed forward on the axis of the laser beam and they collect forward scattered light (FSC), while the second system of lenses is placed at 90° to the laser beam and collects side scattered light (SSC) and fluorescent light as well. Forward scattered light provides information about size of the cell, whereas side scattered light provides information about the cell granularity. Scattered and fluorescent lights are then filtered by the series of optic filters, such as dichroic mirrors, and are directed to suitable photodetectors where light is transformed to an electronic signal that enables computer analysis (50). Analyzed data from the flow cytometer is mainly presented as histograms or dot plots and contain various statistic like total cell count and percentage of cells. Histogram gives information only about one cell parameter, for example intensity of fluorescence of specific fluorochrome, while dot plot

depicts two cell parameters, such as FSC and SSC and enables identification of sub-populations within whole population. Two-parameter dot plots are often displayed in an alternative way with density plots or contour plots.

Argon-ion blue laser (488 nm) is the most commonly used laser in flow cytometers since it can excite majority of biologically important fluorochromes, such as fluorescein isothiocyanate (FITC) and propidium iodide (PI) (51). Both listed fluorochromes are commonly used in detection of apoptosis.

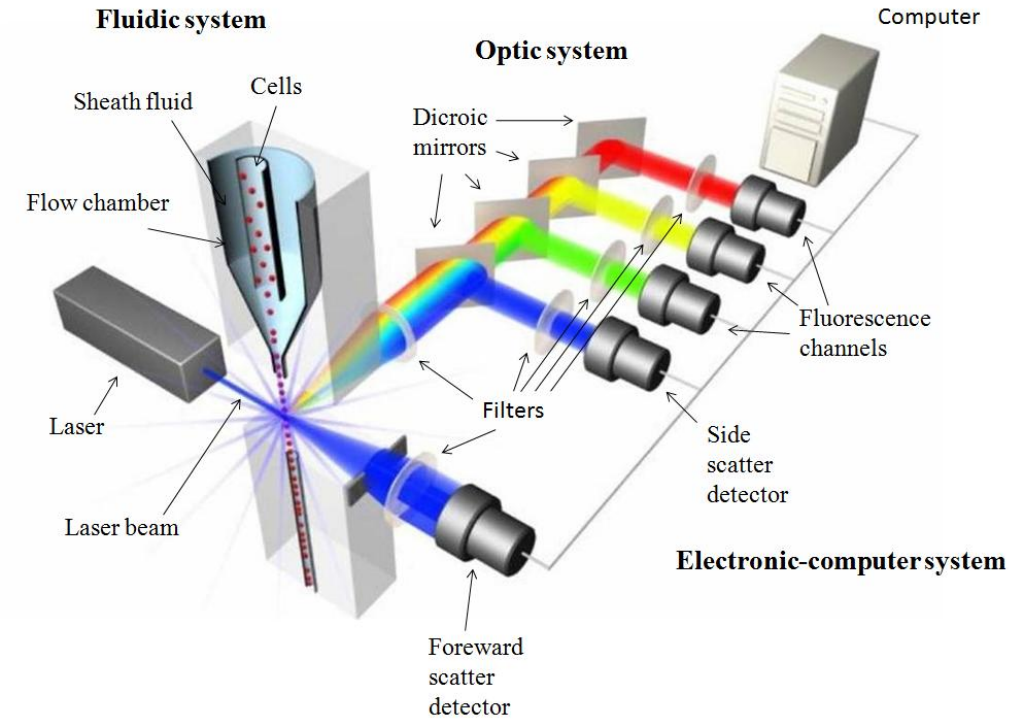


Figure 4: Main components of the flow cytometer. (Adapted from reference (52)).

2 AIMS

To date, some synthetic analogs of marine alkaloid clathrodin have been described for their antimicrobial activity and the ability to modulate voltage-gated sodium channels but their proapoptotic activity has not yet been evaluated.

Aim of our research work is to identify potential proapoptotic activity of 9 synthetic clathrodin analogs in NTERA-2 clone D1 cell line, derived from a patient with lung metastasis of a teratocarcinoma that contain cancer stem cells named embryonal carcinoma cells. We will try to figure out if synthetic analogs of clathrodin induce cell death of NTERA-2 cells, which will be used as a cancer stem cell model. If so, we will further try to determine their mechanism of action on cell death. All assays will be performed *in vitro* and readout will be done by microcapillary flow cytometry.

Initially, preliminary screening of 9 synthetic clathrodin analogs will be performed using annexin V-FITC/propidium iodide apoptotic assay and the most active compounds will be selected for further experiments. Dose-dependent and time-course experiments will be performed to determine EC_{50} values and velocity of the process, respectively. Assay with pan caspase inhibitor Z-VAD-fmk will be performed to define caspase-dependent or independent cell death.

The most promising compound will be used for mechanistic experiments. MitoTracker, cell cycle and caspases-3/-7 assays will then be performed in order to confirm apoptosis. Furthermore, caspase-8/-9 assays will be used to identify if compound is inducing apoptosis by the extrinsic or intrinsic pathway.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 REAGENTS

Table I: List with names of reagents used in research work and their manufacturer.

Reagents	**Manufacturer**
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
DMEM, high glucose	Sigma-Aldrich, USA
DMSO	Sigma-Aldrich, USA
Ethanol	Sigma-Aldrich, USA
FBS	Life Tehnologies, UK
L-glutamine	Lonza, Belgium
MEM non-essential amino acid solution	Sigma-Aldrich, USA
MitoTracker Red	Life Technologies, USA
PBS without Ca^{2+} and Mg^{2+}	Lonza, Belgium
Penicillin-streptomycin	Life Tehnologies, UK
PI/RNase staining solution	Life Technologies, USA
PI solution	MiltenyiBiotec Inc., USA
Trypan blue solution	Sigma-Aldrich, USA
Trypsin 10x	Sigma-Aldrich, USA
Z-VAD-fmk	Promega, USA

3.1.2 LABORATORY EQUIPMENT

Table II: List of laboratory equipment used in research work and its manufacturer.

Laboratory Equipment	**Manufacturer**
6-well cell culture plates	NUNC A/S, Denmark
12-well cell culture plates	Greiner Bio-One, Germany

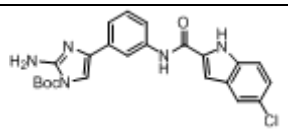
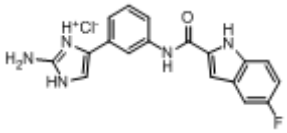
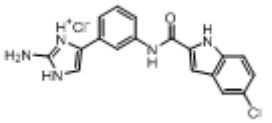
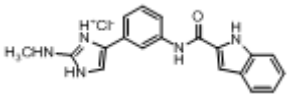
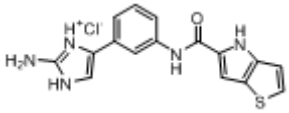
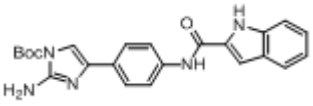
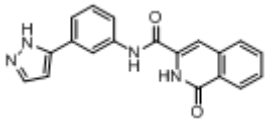
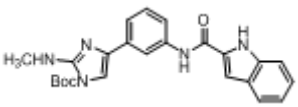
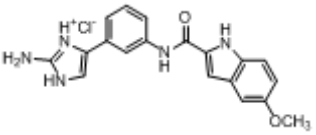
24-well cell culture plates	Greiner Bio-One, Germany
96-well cell culture plates	Becton Dickinson and Co., USA
Automatic pipettes	Gilson, France
Cell culture flask ($75cm^2$)	Corning Inc., USA
Cell freezing container	BioCision, USA
Centrifuge	Jouan S. A., France
Centrifuge tubes (15 mL and 50 mL)	TPP, 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 pipettes (5-50 µL, 50-300 µL)	Labsystems, Finland
Digital weighing scale	Mettler Toledo, Switzerland
Freezer (-18°C)	Liebherr, Switzerland
Inverted phase contrast microscope	Carl Zeiss Microscopy, Germany
Liquid nitrogen 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 (1.5 mL)	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
Water bath	Bioblock Scientific, Germany

3.1.3 COMPOUNDS

3.1.3.1 CHEMICAL SYNTHESIS

Compounds **1**-**9** (Table III), that were evaluated in this diploma thesis, were synthesized by Assist. Prof. Dr. Nace Zidar, Mr. Pharm. under the MAREX (7th Framework Programme) project at the Chair of Pharmaceutical Chemistry at the Faculty of Pharmacy, University of Ljubljana.

Table III: Labels and chemical structures of synthetic clathrodin analogs used for biological evaluation on NT2/D1 cell line.

Structure	Label of compound
	**1**
	**2**
	**3**
	**4**
	**5**
	**6**
	**7**
	**8**
	**9**

3.1.3.2 STOCK SOLUTIONS

Solid compounds with known mass and molecular weight were dissolved in pure dimethyl sulfoxide (DMSO) to obtain 10 mM final concentration. Stock solutions were stored in the dark at 4°C.

3.1.3.3 WORKING SOLUTIONS

Working solutions of the desired concentration were prepared by the diluting stock solution in DMEM high glucose growth medium. Working solutions were always freshly prepared and were used the same day. Final concentration of DMSO never exceeded more than 0.6% v/v in cellular assays.

3.1.4 CELL CULTURE

Human pluripotent testicular embryonal carcinoma cell line NTERA-2 clone D1 (NT2/D1), was obtained from the American Type Culture Collection (ATCC). NT2/D1 cells are adherent, epithelial-like and undifferentiated cells, with a doubling time of approximately 24 h. They have tendency to grow in tightly packed colonies and form monolayer of cells, where domes and floating vesicles may be created. To prevent differentiation of the NT2/D1 cells, they should be maintained at high density (53). Figure 5 present NT2/D1 cells in culture.

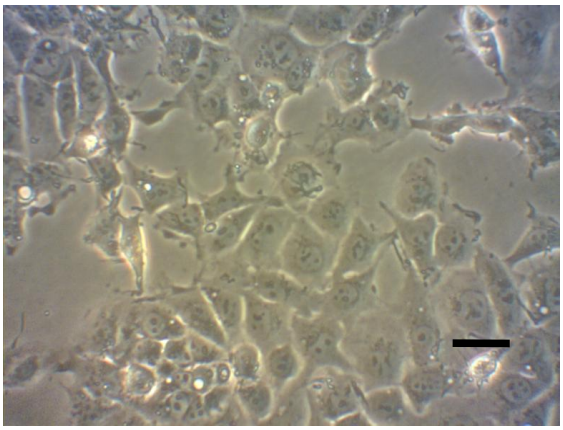


Figure 5: NT2/D1 cells under the inverted phase contrast microscope. Scale bar represents 20 μm.

3.1.4.1 MAINTENANCE OF CELL LINE

The NT2/D1 cell line was maintained in DMEM high glucose growth medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 0.5% (v/v) non-essential amino acid solution and 1% (v/v) penicillin-streptomycin. Cells were grown in a humidified incubator at 37°C (5% CO_2) and needed 2 days to be confluent. For all cell based assays we used only the cells in exponential growth phase.

3.1.4.2 SUBCULTURE OF ADHERENT CELL LINE

Firstly, old medium was removed, NT2/D1 cells were washed with 5 mL of PBS then 1 mL of trypsin 1× was added. After 5 min of incubation at 37°C, cells were diluted by 10-15 mL of pre-warmed medium (37°C) and transferred into the centrifuge tubes. Centrifugation was performed (150 g, 5 min), supernatant was discarded and pellet was resuspended in a suitable amount of the pre-warmed medium (37°C) and approximately 5×10^6 viable cells were finally seeded into new 75 cm^2 culture flasks.

3.1.4.3 RESUSCITATION OF FROZEN CELL LINE

Cryogenic vials of NT2/D1 cells were taken out of the liquid nitrogen container (-196°C) and were quickly thawed by heating the ampoules in a water bath (37°C) for 1-2 min. After that, cell suspension was transferred into the centrifuge tubes and 5 mL of pre-warmed medium was added to each tube. Cells were centrifuged (150 g, 5 min), supernatant was discarded and cell pellet was resuspended in 10-15 mL of fresh pre-warmed medium (37°C). Finally, cells were seeded into 75 cm^2 culture flasks and were cultivated at 37°C under 5% CO_2.

3.1.4.4 CRYOPRESERVATION OF CELL LINE

NT2/D1 cells were harvested at 80-90% confluency. Old medium was discarded, then cells were washed with 5 mL of PBS and 1 mL of trypsin 1× was added into 75 cm^2 culture flasks. After approximately 5 min of incubation (37°C) cells were diluted in 10-15 mL of pre-warmed medium. Cells were then transferred into sterile tube and were centrifuged (150 g, 5 min). Supernatant was discarded and pellet was resuspended in 1 mL of "freezing medium" (95% of culture medium, 5% of DMSO). Labeled cryogenic vials of cell

suspension ware placed at -80°C overnight and next day transferred to liquid nitrogen container (-196°C).

3.1.4.5 CELL QUANTIFICATION

Cell concentration needs to be determined to have consistent cell-based assays with reproducible results. The cells were counted on a hemocytometer. The NT2/D1 cells were harvested as described before. Briefly, an old medium was removed, the cells were washed with PBS, trypsinized, centrifuged and resuspended in 5 mL of pre-warmed medium. To 60 µL of cell suspension the same amount of trypan blue solution was added to the microtube. A hemocytometer was filled with a homogeneous trypan blue stained cell suspension and observed under an inverted phase contrast microscope. Viable, bright cells were counted and cell concentrations were computed using Equation 1. Non-viable, blue stained cells were excluded from calculations.

$$C = 2 \times N \times 10^4 \text{ mL}^{-1} \qquad \textit{Equation 1}$$

C Concentration of the viable cells from cell suspension (live cells per mL)

N Number of live cells counted

3.2 METHODS

3.2.1 MICROCAPILLARY FLOW CYTOMETRY

Microcapillary flow cytometers (Guava EasyCyte) with blue (488 nm) and red (635 nm) lasers were used for the evaluation of all cell-based assays performed in our research. In comparison to traditional flow cytometers, the Guava microcapillary flow cytometers do not use sheath fluid and hydrodynamic focusing. Instead of that, cells are directly aspirated from the sample to the laser region which reduces sample volumes for analysis, enables direct absolute cell count and produces low waste (54). Guava microcapillary flow cytometers enable analysis of 96-well plates that makes them a convenient tool for high-throughput screening in drug discovery (55). Each cytometer is equipped by user friendly Guava InCyte software that allows many functions such as drag-and-drop gating of cell populations and determination of IC_{50}/EC_{50} curves (54).

3.2.2 ANNEXIN V-FITC / PROPIDIUM IODIDE APOPTOSIS ASSAY

Apoptosis assay was performed using annexin V-FITC (AnV-FITC) and propidium iodide (PI). In the early process of apoptosis asymmetry of the plasma membrane is disrupted and PS, which is under normal conditions placed on the inner leaflet, is translocated to the outer leaflet of the cell membrane (33). AnV-FITC binds to the exposed PS with high affinity and enables detection by flow cytometry (56). In the ongoing process of apoptosis cell membrane is damaged and PI, which does not stain live cells with intact membranes, enters the cell and binds to DNA and RNA. Simultaneous detection of AnV-FITC and PI by flow cytometry enables discrimination between live, apoptotic and dead cells (56).

In all experiments control samples were used to provide appropriate gating of cells. Celastrol, known to induce apoptosis in many different human cancer cell lines, was used as a positive control, while negative control contained untreated cells with the same concentration of DMSO as in treated samples (57,58).

3.2.2.1 PRIMARY SCREENING

Preliminary screening was done to determine if any of the 9 selected clathrodin analogs induces cell death of NT2/D1 cells. To perform the assay, NT2/D1 cells were seeded in 96-well plates at a density of 2.5-5 $\times 10^4$ cells/mL. Next day, cells were treated with different compounds at 50 μM final concentration. After 24 h of incubation cells were harvested, resuspended in 200 μL of culture medium, stained with 3 μL AnV-FITC and 3 μL PI and then incubated in the dark at room temperature for 10 min. Measurements were performed on microcapillary flow cytometer.

3.2.2.2 DETERMINATION OF EC_{50} VALUES

The EC_{50} value represents the concentration of the tested compound that induces half-maximal response and is widely used to evaluate activity of compounds *in vitro*. Generally speaking, lower EC_{50} value means higher potency of the compound (59). To determine EC_{50} values of compounds NT2/D1 cells were seeded in 96-well plates at a density of 2.5-5 $\times 10^4$ cells/mL the day before the assay was performed. Next day, the cells were treated with increasing concentrations of compound from 0.4 μM up to 60 μM. After 24 h incubation, cells were harvested, resuspended in 200 μL of culture medium, stained with 3

μL AnV-FITC and 3 μL PI and incubated in the dark at room temperature for 10 min. Measurements were performed on microcapillary flow cytometer.

3.2.2.3 TIME COURSE EXPERIMENT

In order to obtain information about time-dependent process of the cell death NT2/D1 cells were treated with the same concentration of compound at different times. NT2/D1 cells were seeded in 96-well plates at a density of 2.5-5 × 10^4 cells/mL. On the following day, the cells were treated with compound (25 μM) for different times (0, 1, 3, 5, 7 and 9 h). After incubation, cells were harvested, resuspended in 200 μL of culture medium, stained with 3 μL AnV-FITC and 3 μL PI and incubated in the dark at room temperature for 10 min. Measurements were performed on microcapillary flow cytometer.

3.2.2.4 ASSAY WITH Z-VAD-fmk

Z-VAD-fmk (Figure 6) is a cell-permeable, nonspecific caspase inhibitor that irreversibly binds to the catalytic site of almost all caspases and prevents caspase-dependent cell death (32,60). For the purpose of this assay, NT2/D1 cells were seeded in 12-well plates at a density of 2.5-5 × 10^4 cells/mL. The following day, one parallel of the cells was pretreated for 1 h with a pan caspase inhibitor Z-VAD-fmk (50 μM final) and the second parallel was not. Both parallels were then treated with the same compound at the same concentration (25 μM). After 4 h incubation, cells from both parallels were harvested, resuspended in 200 μL of culture medium, stained with 3 μL AnV-FITC and 3 μL PI and incubated in the dark at room temperature for 10 min. Measurements were performed on microcapillary flow cytometer.

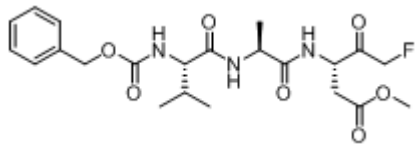


Figure 6: Chemical structure of pan caspase inhibitor Z-VAD-fmk.

3.2.3 MITOTRACKER ASSAY

During apoptosis, mitochondria are damaged and therefore their activity drops, which can be measured as a decrease in the MTP (61,62). MitoTracker probe is a cell-permeable dye that enters the cells and if the mitochondria are undamaged the probe is oxidized and binds to thiol group of proteins resulting in a fluorescent conjugate. Intensity of fluorescence can be measured by flow cytometry. NT2/D1 cells were seeded in 12-well plates at a density of 2.5-5 × 10^4 cells/mL. Next day, the cells were treated with compound (25 μM) for different times (0, 1, 3, 5, 7 and 9 h). After that, the cells were harvested and centrifuged (200 g, 5 min). Supernatant was discarded, pellet was resuspended in 1 mL of MitoTracker Red solution (500 nM final) and the cells were incubated at 37°C for 30 min. Cells were centrifuged in order to remove the reagent and pellet was resuspended in 200 μL of fresh medium. The samples were analyzed on the microcapillary flow cytometer. Non-treated cells with the same amount of DMSO as in wells with compounds were used as a negative control.

3.2.4 ANALYSIS OF CELL CYCLE

Lifespan of eukaryotic cells consists of cell growth, replication and finally, division to two daughter cells. It was noted that mitosis and apoptosis exhibit some similar morphological characteristics, such as rounding-up the cell, shrinkage, condensation of chromatin and membrane blebbing. In contrast to mitosis, where DNA is segregated, in the process of apoptosis DNA is degraded into fragments consisting of multiples of 180 bp (63). Flow cytometry represents one of the methods of choice to analyze DNA fragments using PI, as a fluorescent dye stoichiometrically binds to DNA and RNA. To remove unwanted PI-RNA intercalation pre-incubation with RNase is necessary (64,65). NT2/D1 cells were seeded in 6-well plates at a density of 5-10 × 10^5 cells/mL. Following day, the cells were treated with compound (25 μM) for different times (0, 3, 6, 9, 12 and 24 h). At a specific time, cells were harvested, washed with PBS and centrifuged (200 g, 5 min). Cell pellet was resuspended in 500 μL of PBS and fixed in 70% ethanol, on ice. Ethanol-suspended cells were kept at -20°C overnight. Next day, ethanol was thoroughly removed by centrifugation (400 g, 5 min). After that, cells were washed with PBS, centrifuged (400 g, 5 min) and stained with 500 μL FxCycle PI/RNase staining solution. After 30 min incubation in the dark at room temperature, samples were analyzed on the microcapillary

flow cytometer. Non-treated cells with the same amount of DMSO as in wells with compound were used as a negative control.

3.2.5 CASPASE-3/-7 AND -8/-9 ASSAY

Massive activation of caspases is one of the hallmarks of apoptotic cell death. The use of the fluorochrome-labeled inhibitors of caspases (FLICAs) enables detection of activated caspases in the cells undergoing apoptosis. The method is based on reversible or irreversible binding of small, cell permeable inhibitors that stoichiometrically (1:1) bind to the active site of caspases. To access appropriate fluorescent signal, unbound FLICAs have to be removed from the samples by washing buffer (60). In our research work Caspase-8 FAM/Caspase-9 SR kit and Caspase-3/-7 FAM kit with irreversible caspase inhibitors were used. In the first mentioned kit, inhibitor of caspase-8 was conjugated with carboxyfluorescein (FAM) and inhibitor of caspase-9 was conjugated with sulforhodamine (SR). The second kit contained FLICAs of caspases-3 and -7 which were labeled with FAM. 7-amino-actinomycin D (7-AAD) was added before analysis on microcapillary flow cytometer to exclude dead cells from analysis. The NT2/D1 cells were seeded in 12-well plates at a density of 2.5-5 × 10^4 cells/mL. Next day, the cells were treated with compound (25 μM) for different times (0, 3, 6, 9, 12 and 24 h). After certain time of incubation caspases assay was performed according to the manufacturer's instructions. The cells were harvested and 100 μL of cell suspension was transferred to 96-well plate, where 10 μL of specific caspase reagent working solution was added. After 1 h at 37°C in incubator, the cells were washed twice with 1× apoptosis wash buffer and were then centrifuged (300 g, 5 min). Finally, the cell pellet was resuspended in 200 μL of caspase 7-AAD working solution and was incubated at room temperature for 10 min before analysis on the microcapillary flow cytometer. Positive control that contained cells incubated with celastrol, and negative control that contained just the cells in the growth medium with the same amount of DMSO as in the test samples, were also used to provide appropriate gating of cells.

4 RESULTS

The NT2/D1 cells were treated with nine synthetic clathrodin analogs to evaluate their potential proapoptotic activity in NT2/D1 cell line, derived from a patient with lung metastasis of a teratocarcinoma that contain CSCs named embryonal carcinoma cells.

4.1 ANNEXIN V-FITC / PROPIDIUM IODIDE APOPTOSIS ASSAY

AnV-FITC/PI apoptotic assay was performed to detect and discriminate live, apoptotic and dead cells. For analysis, cells were presented as dots on FSC/SSC and AnV-FITC/PI two-color fluorescence dot plots as shown in Figure 7. FSC/SSC dot plot was used to sort cells based on their size and granularity. Threshold level was set and gates were introduced to FSC/SSC dot plot for each assay. Population of cells without debris was selected by appropriate gating and applied for AnV-FITC/PI two-color fluorescence dot plot. Fluorescence dot plot of AnV-FITC and PI can be divided into four quadrants, where the lower left quadrant contains live cells (AnV-FITC−, PI−), the lower right quadrant contains apoptotic cells (AnV-FITC V+, PI−) and the upper right quadrant contains dead cells (AnV-FITC+, PI+). Cells in the upper left quadrant are usually damaged cells (AnV-FITC−, PI+), which did not die by apoptosis and can occur due to handling with adherent cells.

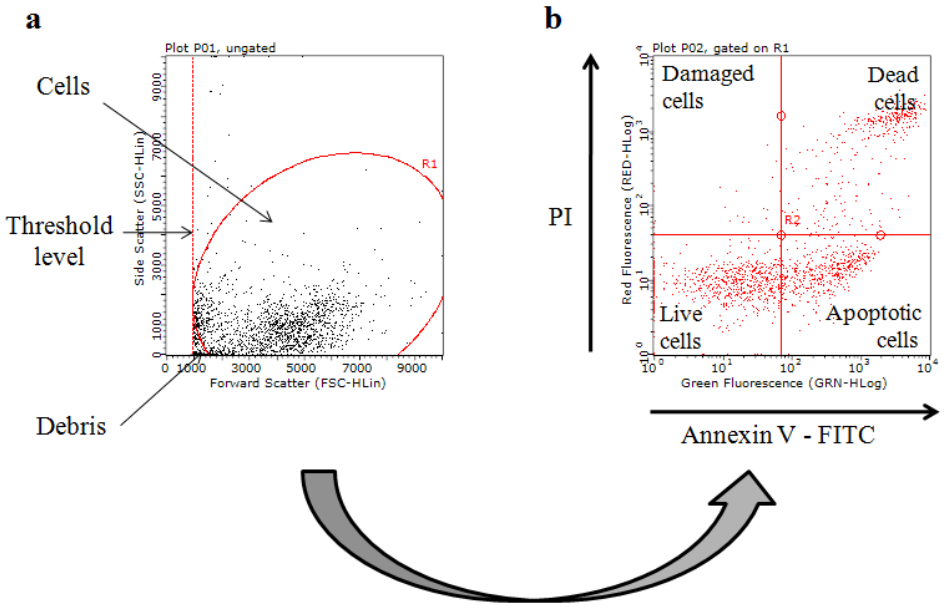


Figure 7: Representative dot plots of NT2/D1 cells obtained by analyzing cells with AnV-FITC/PI apoptotic assay. **a** The FSC/SSC dot plot showing threshold level and the R1 gate to remove debris. Selected R1 population is used for analysis in the two-color fluorescence dot plot. **b** The two-color fluorescence dot plot divided into four quadrants by R 2 gate to distinguish among live cells (AnV-FITC−, PI−) in the lower left quadrant, apoptotic cells (AnV-FITC V +, PI−) in the lower right quadrant and dead cells (AnV-FITC +, PI +) in the upper right quadrant. Cells in the upper left quarter are usually damaged cells (AnV-FITC−, PI +), which can occur due to handling with adherent cells.

4.1.1 PRIMARY SCREENING

NT2/D1 cells were incubated with compounds **1**-**9** (50 µM) for 24 h to identify their potential to induce cell death. As displayed in Figure 8, more than 90% dead cells (AnV-FITC+, PI+) were observed when the cells were treated with compounds **2**, **3**, **4**, **5** and **9**. Compounds **1**, **6**, **7** and **8** were less active, but they still induced 60% of dead cells in the case of compounds **6** and **7**, and more than 40% dead cells in the case of compounds **1** and **8**. Comparison between chemical structure and biologically activity of compounds revealed that less active compounds **1**, **6** and **8** contained Boc moiety in the structure whereas highly active compounds **2**, **3**, **4**, **5** and **9** did not. Compound **7** was an exception, it was less active and did not contain Boc group. Moreover, all five highly active compounds contained 2-aminoimidazole or 2-methylaminoimidazole moiety and

substituted pyrrole ring in their structures. Compounds **2**, **3**, **4**, **5** and **9** that induced more than 90% dead cells after 24 h treatment were chosen for further biological evaluation.

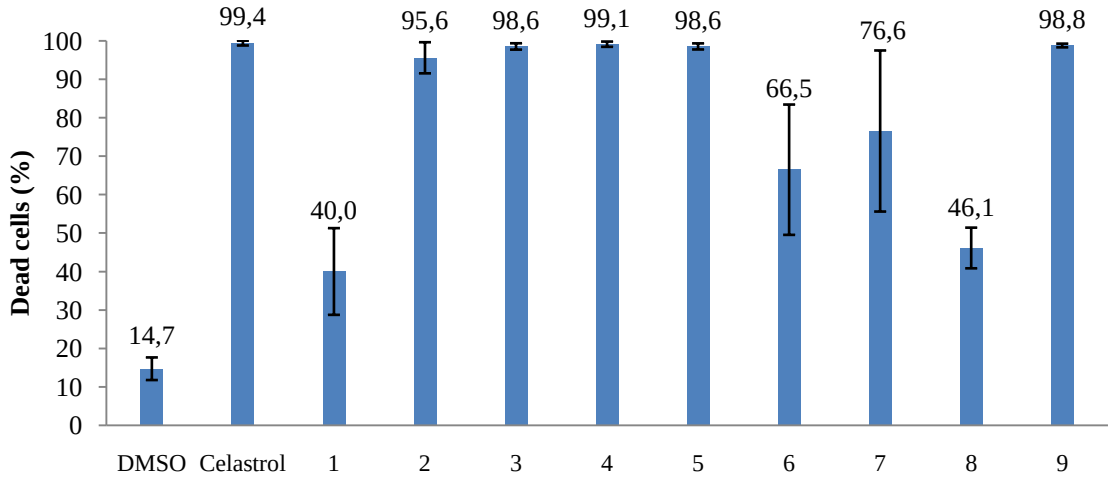


Figure 8: Percent of dead NT2/D1 cells (AnV-FITC+, PI+) after 24 h of incubation with compounds **1**–**9** at 50 µM concentration using AnV-FITC/PI apoptosis assay. N=3 in duplicates. 2000 cells per sample were analyzed.

4.1.2 DETERMINATION OF EC_{50} VALUES

EC_{50} values for compounds **2**, **3**, **4**, **5** and **9** for the dose-dependent treatment and 24 h of incubation were calculated by the Guava InCyte software. Dose response curves are displayed in Figure 9 and show activity of compounds in the lower micromolar range. Comparison of EC_{50} values among all compounds revealed similar potency of compounds against NT2/D1 cells. Among all tested compounds, 2-methylaminoimidazole-based compound **4** was the most potent with EC_{50} value of 11 µM. Compounds **2**, **3** and **9** (EC_{50} values from 13 to16 µM), which differ only on the substituent on the indole ring, had similar EC_{50} values as compound **4**, whereas compound **5** (EC_{50} 22 µM) was slightly less active.

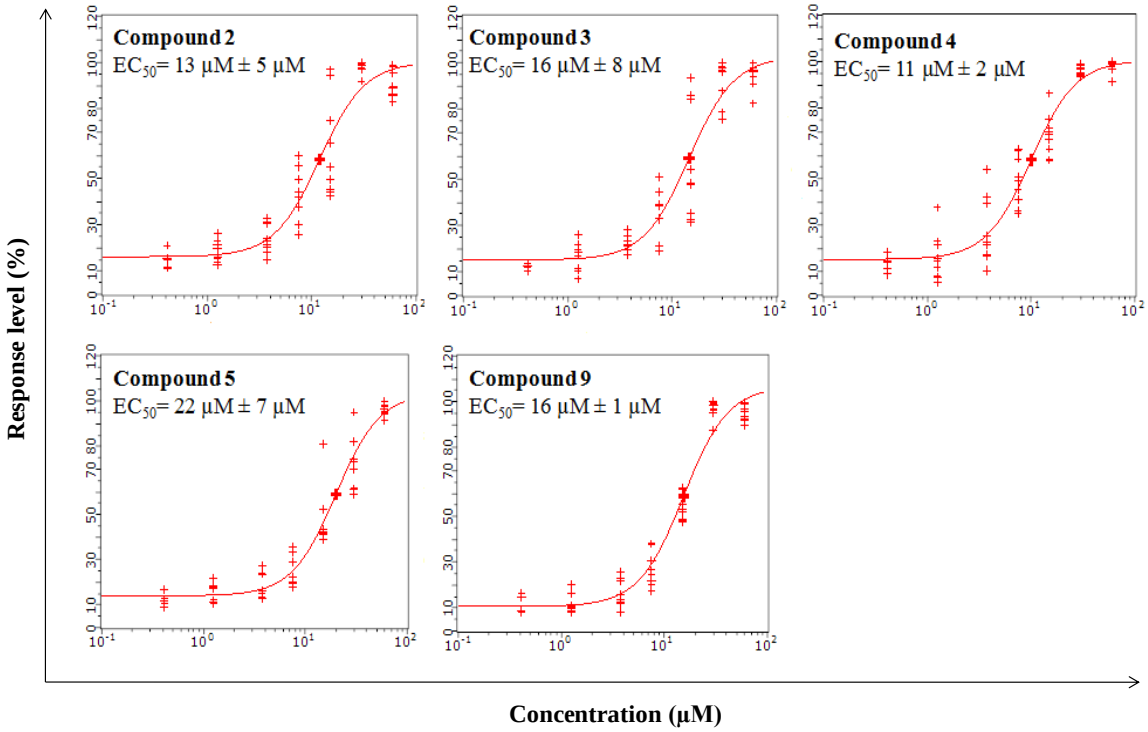


Figure 9: Dose response curves and EC_{50} values for compounds **2**, **3**, **4**, **5** and **9** in raising concentration range (0.4 µM to 60 µM) determined on NT2/D1 cell line after 24 h of incubation using AnV-FITC/PI apoptotic assay. N=3 in triplicates. 2000 cells per sample were analyzed.

4.1.3 TIME COURSE EXPERIMENT

NT2/D1 cells were treated with compounds **2**, **3**, **4**, **5** and **9** (25 µM) for different times (0, 1, 3, 5, and 9 h) in order to monitor time-dependent increase of apoptotic cell population. 25 µM concentration was chosen based on additional time-dependent experiments (data not shown) that revealed selected concentration as the most optimal. In all further experiments 25 µM concentration was used.

Results of time course experiments are displayed as graphs in Figure 10 showing changes in the percentage of live, apoptotic and dead cells during 9 h incubation of NT2/D1 cells with the tested compounds. The results showed steady increase of apoptotic cell population for compounds **2**, **4** and **9**. After 9 h of incubation, treatment of NT2/D1 cells with compounds **2** and **4** resulted in 48.6% and 50.1% cells in apoptosis, while compound **9** induced apoptosis in 30.0% of NT2/D1 cells. Although, we could not detect the significant increase in the number of apoptotic cells after treatment with compound **3**, we could still

observe fast increase in the number of dead cells, since after 9 h of treatment 83.0% cells were already dead. On the other hand, compound **5** showed slow induction of the cell death since 71.4% of the NT2/D1 cells remained live after 9 h of treatment. Figure 11 shows representative dot plots obtained during time-dependent treatment of NT2/D1 cells with compound **4**.

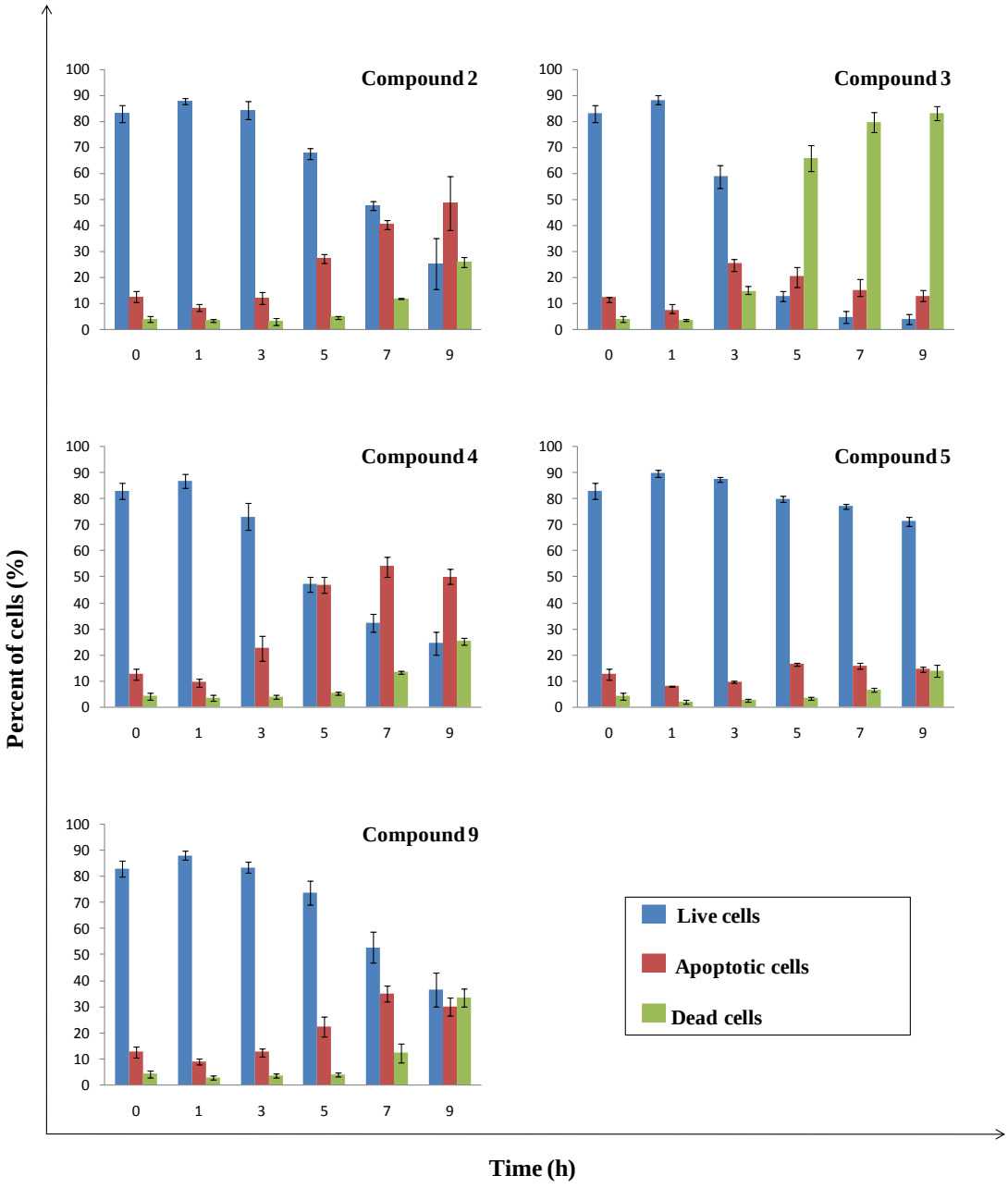


Figure 10: Percent of live, apoptotic and dead cells after incubation of NT2/D1 cells with compounds **2**, **3**, **4**, **5** and **9** (25 μM) for different times (0, 1, 3, 5, and 9 h) using AnV-FITC/PI apoptotic assay. N=1 in triplicates. 2000 cells per sample were analyzed.

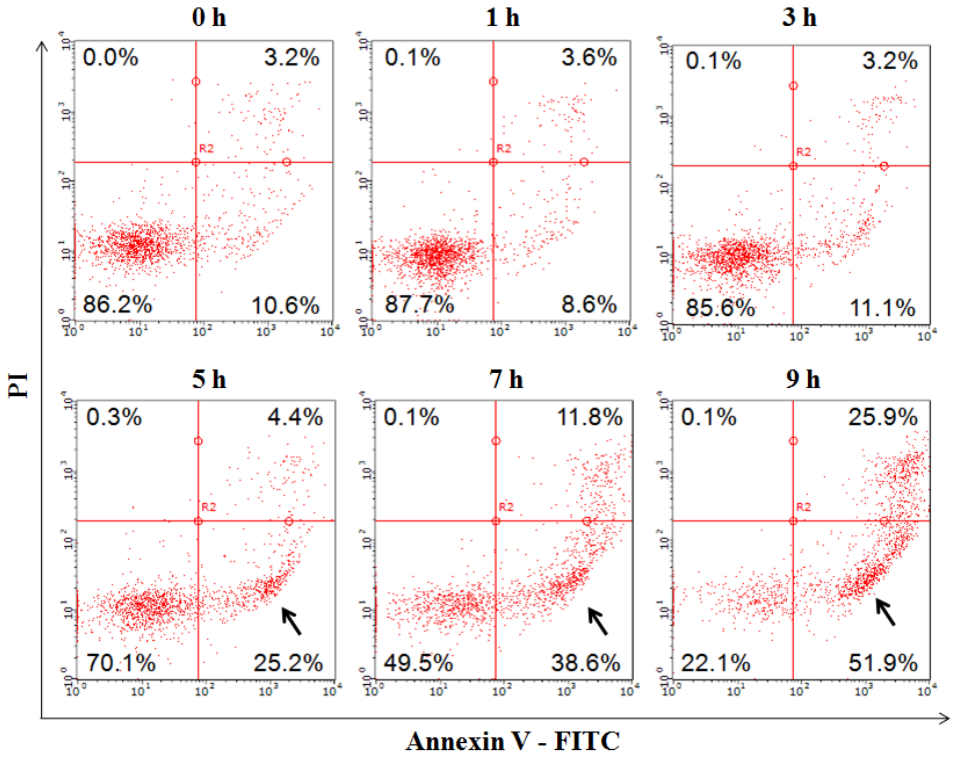


Figure 11: Representative dot plots obtained after time-dependent treatment (0, 1, 3, 5, 7 and 9 h) of NT2/D1 cells with compound **4**. The results showed steady increase of apoptotic cell population (black arrow) from 10.6% at 0 h to 51.9% at 9 h. 2000 cells per sample were analyzed.

4.1.4 ASSAY WITH Z-VAD-fmk

Assay with the cell-permeant pan caspase inhibitor Z-VAD-fmk was performed to obtain information whether compounds **2**, **3**, **4**, **5** and **9** induce cell death via caspase-dependent apoptosis pathway or not. NT2/D1 cells were incubated with compounds **2**, **3**, **4**, **5** and **9** (25 µM) for 4 h in the presence or absence of Z-VAD-fmk (50 µM). Results presented in Figure 12 show that the percent of live cells increased for all tested compounds when cells were pre-treated with Z-VAD-fmk compared to the not pre-treated conditions. Z-VAD-fmk irreversibly inhibits caspases and disables caspase-dependent cell death, which was reflected as an increase in the population of live NT2/D1 cells compared to the condition without the Z-VAD-fmk inhibitor.

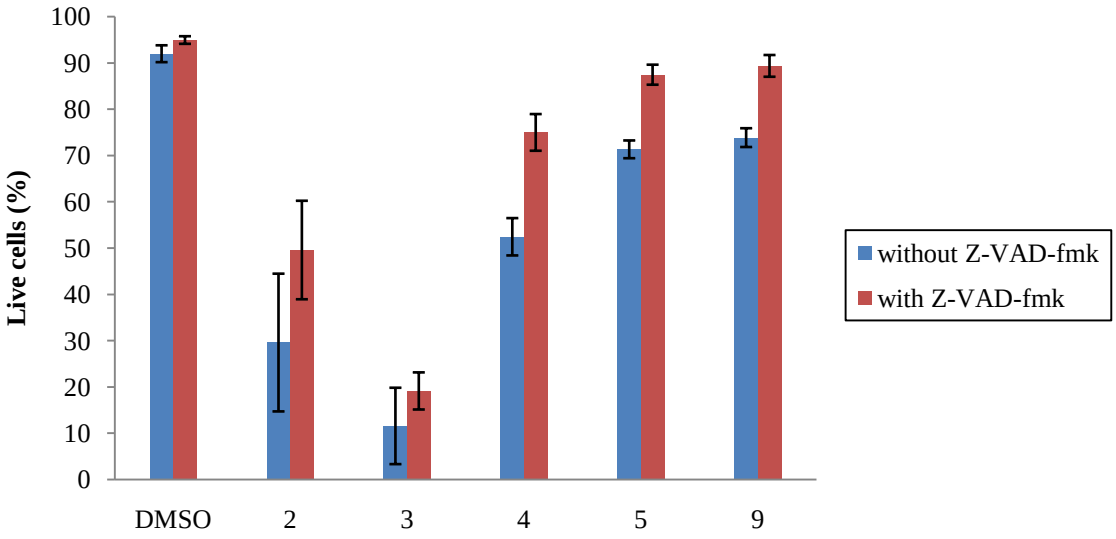


Figure 12: Percent of live NT2/D1 cells after 4 hours of incubation with compounds **2**, **3**, **4**, **5** and **9** (25 µM) without and with pan caspase inhibitor Z-VAD-fmk (50 µM). Percent of live NT2/D1 cells increased for all compounds when cells were pre-treated with Z-VAD-fmk compared to the not pre-treated cells. N=1 in triplicates. 2000 cells per sample were analyzed.

4.2 MITOTRACKER ASSAY

MitoTracker assay was performed to monitor active mitochondria after time-dependent treatment of NT2/D1 cells with compound **4** (25 µM). Graphs presented in Figure 13 show decrease of MTP since R1 population with lower MitoTracker Red fluorescence intensity increased from 5% to almost 50%. Damaged mitochondria and loss of MTP suggests that compound **4** acted on mitochondria.

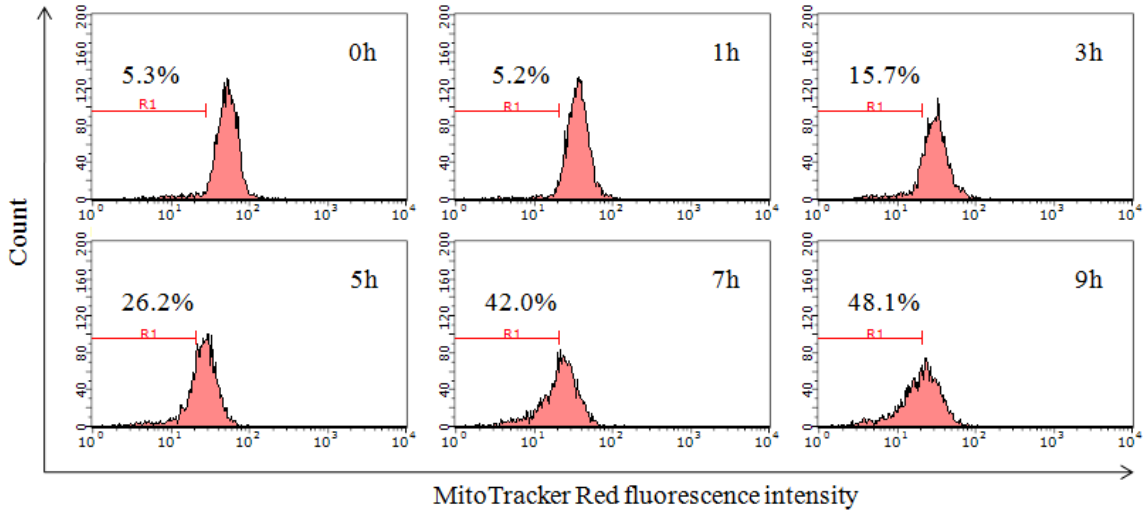


Figure 13: Measurements of the red fluorescence intensity of MitoTracker Red after time-dependent incubation (0, 1, 3, 5, 7 and 9 h) of NT2/D1 cells with compound **4** (25 µM). Graphs showed decrease of MTP since R1 population with lower MitoTracker Red fluorescence intensity increased. N=1. 3000 cells per sample were analyzed.

4.3 ANALYSIS OF CELL CYCLE

Cell cycle assay was performed to determine potential presence of fragmented DNA, which can be detected as a sub-G1 population below G0/G1 phase. Debris and aggregates were excluded from analysis by appropriate gating. Histograms of cell cycle displayed in Figure 14 show distribution of the cells in different phases of the cell cycle, where sub-G1 population represents the apoptotic cells, G0/G1 population represents cells, which are quiescent or grow, S population represents cells that duplicate DNA and G2/M population represents cells before final division with two identical strands of DNA.

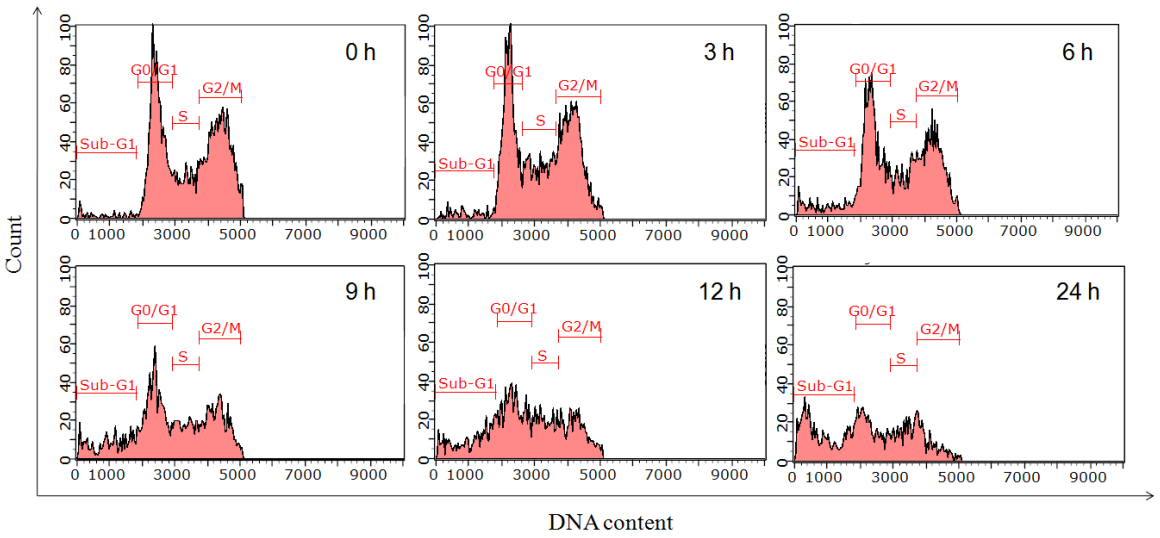


Figure 14: Cell cycle assay. Histograms of NT2/D1 cells incubated with compound **4** (25 µM) and for different times (0, 3, 6, 9, 12 and 24 h). Each histogram represents distribution of cells in sub-G1, G0/G1, S and G2/M phase. N=1. 5000 cells per sample were analyzed.

Results showed a time-dependent increase of sub-G1 population with a decrease in the G0/G1 and G2/M phase. Percent of cells in sub-G1 phase increased from 2.3% to 37.3% after 24 h incubation, confirming DNA fragmentation. Increase of the sub-G1 population confirms apoptotic cell death for compound **4**. Percent of cells in the sub-G1 population is presented in Table IV.

Table IV: Increase in the percentage of NT2/D1 cells in the sub-G1 phase of the cell cycle measured after 0, 3, 6, 9, 12 and 24 h of treatment with compound **4** (25 µM).

Time (h)	Sub-G1 (%)
0	2,3
3	2,5
6	8,2
9	17,5
12	21,7
24	37,3

4.4 CASPASE-3/-7 AND -8/-9 ASSAY

Detection of the activated caspases is an important mechanistic assay to confirm apoptosis, since activation of caspases represents one of the hallmarks of apoptosis. In order to investigate whether group of the cysteine proteases is activated during the time-dependent treatment with compound **4** (25 μM), caspase-3/-7 and caspase-8/-9 assays were performed using FLICAs. 7-AAD was added to exclude dead cells from further analysis with appropriate gating. Results displayed in Figure 15 show time-dependent increase of the mean fluorescence intensity for activated caspase-3 and -7 confirming the apoptotic cell death of the NT2/D1 cells.

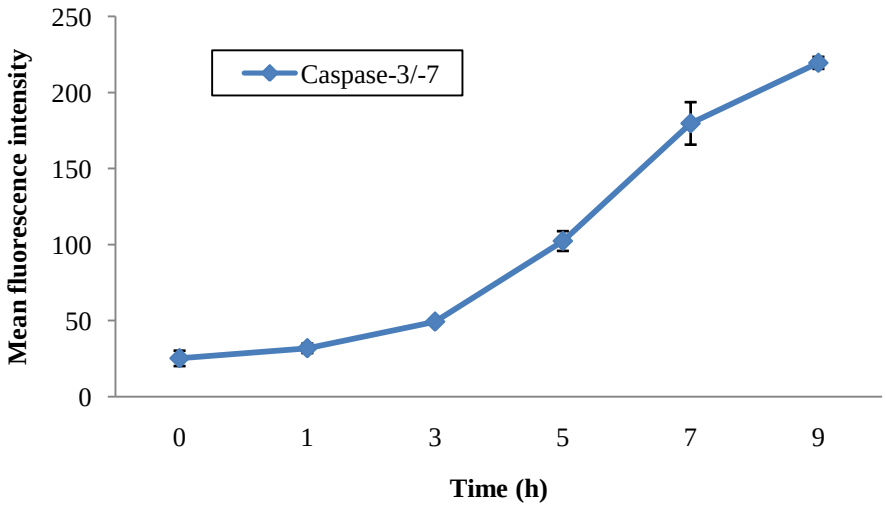


Figure 15: Increased mean fluorescence intensity after the incubation with caspase-3/-7 inhibitor. Cells were treated with compound **4** (25 μM) for different times (0, 1, 3, 5, 7 and 9 h). N=1 in triplicates. 3000 cells per sample were analyzed.

Time-dependent increase of the mean fluorescence intensity was also observed using caspase-8/-9 assay. Results in Figure 16 show activation of both caspases. Furthermore, we can assume that earlier caspase-8 activation compared to later caspase-9 activation indicates extrinsic pathway of apoptosis.

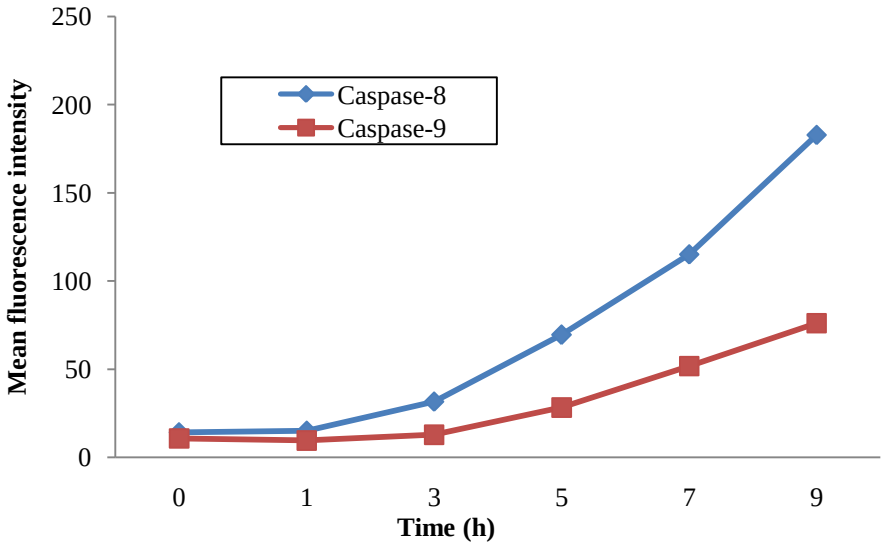


Figure 16: Mean fluorescence intensity after the incubation with caspase-8 and -9 inhibitors. Cells were treated with compound **4** (25 µM) for different times (0, 1, 3, 5, 7 and 9 h). N=1. 3000 cells per sample were analyzed.

5 DISCUSSION

Marine sponges have been shown to produce a wide variety of compounds with interesting biological activities, such as anticancer, anti-inflammatory, immunosuppressive, antiviral and antimicrobial activity (36,37). In our research we investigated proapoptotic activity of nine synthetic analogs of marine alkaloid clathrodin and their ability to trigger apoptosis in NT2/D1 cell line, derived from a patient with lung metastasis of a teratocarcinoma that contain cancer stem cells named embryonal carcinoma cells.

CSCs are a small population of undifferentiated cells that have been identified in many tumors and that possess the ability to self-renew, differentiate and form tumor initiating and migrating cells. They have specific mechanisms that make them resistant to the anticancer therapy. Conventional therapies act mostly on the differentiated cells, whereas many CSCs remain undamaged and are therefore responsible for cancer relapse. According to the all listed facts, new approaches with more specific targeting of CSCs are needed (10,20).

Initially, preliminary screening of nine synthetic analogs of clathrodin (Table III) was done using AnV-FITC/PI apoptotic assay to identify their potential to induce cell death on the NT2/D1 cells. Cells were treated with compounds **1**-**9**, for which apoptosis-inducing activity was previously observed on HepG2 cell line, at 50 μM concentration. Results after 24 h treatment of NT2/D1 cells with compounds **1**-**9** revealed that compounds **2**, **3**, **4**, **5** and **9** induced cell death in more than 90% of cells, compounds **6** and **7** showed more than 60% dead cells and compounds **1** and **8** only around 40% dead cells. Comparison between chemical structure and biologically activity of compounds revealed that less active compounds **1**, **6** and **8** contained Boc moiety in the structure whereas highly active compounds **2**, **3**, **4**, **5** and **9** did not. Compound **7** was an exception, it was less active and did not contain Boc protecting group. Moreover, all five highly active compounds contained 2-aminoimidazole or 2-methylaminoimidazole moiety and substituted pyrrole ring in their structures. Compounds **2**, **3**, **4**, **5** and **9** that caused cell death of more than 90% NT2/D1 cells were further evaluated to determine their potency and to monitor their time-dependent increase of the apoptotic cell population. Comparison of the EC_{50} values among compounds **2**, **3**, **4**, **5** and **9** showed comparable potencies, which were in the lower micromolar range (EC_{50} between 11-22 μM), whereas 2-methylaminoimidazole-based

compound **4** was found to be the most active with the lowest EC_{50} value of 11 μM. Compounds **2**, **3** and **9** (EC_{50} values from 13 to16 μM) differ only on the substituent on the indole ring whereas compound **5** (EC_{50} 22 μM) was slightly less active. Comparison of the kinetics revealed steady increase of the apoptotic cell population for compounds **2**, **4** and **9**, while compounds **3** and **5** did not show the same trend. After 9 h of incubation with compounds **2** and **4**, analysis showed 48.6% and 50.1% of cells in apoptosis, while compound **9** induced apoptosis in 30.0% NT2/D1 cells. Although, we could not detect significant increase in the number of apoptotic cells for compound **3**, we could still observe fast increase in the number of dead cells since after 9 h of treatment approximately 83.0% of the cells were already dead. On the other hand, compound **5** showed slow induction of the cell death since 71.4% of cells remained live after 9 h of treatment. It is interesting that although compounds **2**, **3**, **4**, **5** and **9** showed similar EC_{50} values for 24 h of incubation, time-dependent induction of cell death highly varies among them. To figure out whether compounds cause cell death through caspase-dependent or caspase-independent pathway, we performed an assay with the pan caspase inhibitor Z-VAD-fmk. Results obtained showed that the percent of live cells increased in the case of all compounds, when cells were pre-treated with Z-VAD-fmk compared to the not pre-treated condition, which suggests caspase-dependent cell death. Compound **4** was selected for further mechanistic studies based on all the results obtained. To confirm apoptosis as a cell death for compound **4**, measurements of the MTP, cell cycle analysis and caspase-3/-7 assay were also performed. Experiment with MitoTracker Red revealed significant decrease in the MTP suggesting that the apoptosis occurred via mitochondrial pathway. Results of the cell cycle analysis showed the time-dependent increase in the number of apoptotic cells since the sub-G1 population increased due to decrease in the number of cells in G0/G1 and G2/M phase. Caspase-3/-7 assay revealed activation of both effector caspases confirming apoptosis as a cell death. To determine whether compound **4** triggered caspase-dependent apoptosis via extrinsic or intrinsic pathway, assay with caspase-8/-9 was performed. Time-dependent increase of mean fluorescence intensity showed activation of both caspases. Furthermore, caspase-8 was activated earlier than caspase-9 suggesting extrinsic pathway of apoptosis (31).

To sum up, we showed that all nine synthetic clathrodin analogs cause cell death of NT2/D1 cells. For five compounds it was shown that they induced cell death in NT2/D1

cell line with the EC_{50} values in the lower micromolar range after 24 h of incubation. Among all tested compounds, compound **4** was selected for further mechanistic experiments such as MitoTracker assay, analysis of the cell cycle, caspase-3/-7 and caspase-8/-9 assay. Induction of apoptosis of NT2/D1 cells by compound **4** was proven by all these experiments. Moreover, we assume that compound **4** induces extrinsic pathway of apoptosis in the NT2/D1 cell line.

Synthetic analogs of clathrodin represent new group of compounds that induce apoptosis in NT2/D1 cell line and therefore they could represent interesting lead compounds in the discovery of new therapeutic agents active against resistant CSCs. To understand the mode of action of compounds **2**-**5** and **9** in more detail further research and additional experiments have to be performed. The next step could be to test these compounds on different human cancer stem cell lines, such as cell lines derived from brain tumors, pancreas cancer, breast cancer or lung cancer, and to compare the induction of apoptosis in different human cancer stem cell lines and between human cancer or non-cancer cell lines. These results would help us to determine the potential selectivity against cancer stem cell lines. It would also be interesting to perform additional mechanistic experiments to better define the exact mechanism of apoptosis induction. For example, we could try to determine to which death receptor these compounds bind and cause apoptosis. We could also assayed compounds for their ability to trigger apoptosis in culture of spheroids (3D culture) since spheroids better mimic tumors then cells grown as monolayer (2D culture) (66). Besides additional mechanistic experiments, synthetic analogs of clathrodin could also be modified to obtain compounds with improved potency.

6 CONCLUSION

In our research, we confirmed that nine synthetic clathrodin analogs induce cell death in NTERA-2 clone D1 cell line, derived from a patient with lung metastasis of a teratocarcinoma that contain cancer stem cells named embryonal carcinoma cells. More than 90% of NTERA-2 cells were dead after incubation with compounds **2**, **3**, **4**, **5** and **9** for 24 h. Dose-dependent experiment for compounds **2**, **3**, **4**, **5** and **9** revealed that all compounds had similar EC_{50} values in the lower micromolar range. Assay with the pan caspase inhibitor Z-VAD-fmk for these compounds showed an increase in the number of live cells when comparing the parallel with and without Z-VAD-fmk, which indicates induction of the cell death via caspase-dependent pathway. Among all compounds, compound **4** was selected for further mechanistic studies. Assay with MitoTracker was performed and significant decrease in MTP potential was observed. Determination of the sub-G1 population in the cell cycle analysis and detection of the activated effector caspases-3 and -7 confirmed induction of apoptosis. We also performed the caspase-8/-9 assay, which revealed activation of both initiator caspases with earlier activation of caspase-8 indicating extrinsic pathway of apoptosis. To conclude, synthetic analogues of clathrodin represent new group of compounds that induce apoptosis on cancer stem cell line NTERA-2 and therefore they could represent interesting lead compounds in the design of new therapeutic agents against resistant cancer stem cells. However, further optimization of the structure is necessary to obtain more potent compounds as well as screening on different cancer stem cell lines and additional assays to get more insight into the mode of action of these compounds are still needed.

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