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Screening Cyanobacteria for Apoptosis Induction in Human Cancer Cell Lines: Discovery of a Novel Compound – Nocuolin A

Ph.D. Thesis

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Annotation

Cancer-related diseases are mostly associated with reduced or inappropriate cell death. This thesis focuses on secondary cyanobacterial metabolites which induce apoptosis in human cancer cells *in vitro* and thus may serve as potential drug hits. Screening and selection of active natural extracts clearly precede activity-guided isolation of a bioactive compound itself. Summarizing the results of phenotypic screening of cyanobacterial extracts for inducers of apoptosis, I show that adjustment of measurement the activity of key apoptotic enzymes, caspases, per cell significantly enlarges the pool of detected hits. This could be of particular importance, since this correction is relevant for complex natural extracts as well as chemical libraries of pure compounds, and moreover applicable all the way from small-sized screens to high-throughput ones. Further, I investigated the apoptosis inducing activity of nocuolin A (NoA) – a new cyanobacterial compound isolated and described by our group. NoA shows remarkable characteristics regarding its structure (1,2,3-oxadiazine heterocycle), biosynthetic origin and also its biological activity. It induces caspase-dependent apoptosis and shows potency against a panel of nine human cancer cell lines, which makes NoA a pharmaceutically interesting compound. I also bring the first insights into elucidation of its mode of action in cancer cells *in vitro*.

Declaration [in Czech]

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České Budějovice, 29. 5. 2017

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Kateřina Voráčová

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Voráčová, K.**, Paichlová, J., Vicková, K., Hrouzek, P. (2017) Screening of cyanobacterial extracts for apoptotic inducers: a combined approach of caspase-3/7 homogeneous assay and time-lapse microscopy. **Journal of Applied Phycology** (doi:10.1007/s10811-017-1122-6)
Kateřina Voráčová performed and evaluated microscopy experiments; and contributed to evaluation and interpretation of the rest of data. She also wrote the manuscript.

II. **Voráčová, K.**, Hájek, J., Mareš, J., Urajová, P., Kuzma, M., Cheel, J., Villunger, A., Kapuscik, A., Bally, M., Novák, P., Kabeláč, M., Krumschnabel, G., Lukeš, M., Voloshko, L., Kopecký, J., Hrouzek, P. (2017) The cyanobacterial metabolite nocuolin A is a natural oxadiazine that triggers apoptosis in human cancer cells. **PLOS ONE** 12(3): e0172850. (doi:10.1371/journal.pone.0172850)
Kateřina Voráčová performed all experiments concerning the bioactivity of nocuolin A, including interpretation of these data. She participated in writing and editing of the manuscript.

III. Hrouzek, P., Kapuscik, A., Vacek, J., **Voráčová, K.**, Paichlová, J., Kosina, P., Voloshko, L., Ventura, S., Kopecký, J. (2016) Cytotoxicity evaluation of large cyanobacterial strain set using selected human and murine *in vitro* cell models. **Ecotoxicology and Environmental Safety** 124:177-185. (doi:10.1016/j.ecoenv.2015.10.020)
Kateřina Voráčová performed and evaluated the activity-guided fractionation of selected cyanobacterial strains. She also partly contributed to the writing and revision of the manuscript.

Co-author agreement

Pavel Hrouzek, the supervisor of this Ph.D. thesis and the corresponding author of all presented papers, fully acknowledges the contribution of Kateřina Voráčová as described above.

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Pavel Hrouzek

CONTENT

PREFACE

Bringing new advances in cancer treatment is the driving engine for many laboratories worldwide. Our research group is interested in one of the tools in basic cancer research, which is novel compounds of natural origin that induce apoptosis and can therefore bring new drug leads as well as molecular tools to study the role of key cellular components in the cell death pathways. An undisputed source of novel bioactive compounds are natural products, produced in our case by cyanobacteria, an ancient group of prokaryotes capable of oxygenic photosynthesis. Although the objectives seem straightforward there are many pitfalls related to this type of research to handle and its outcomes tend to be highly uncertain. In order to identify compounds from cyanobacteria that induce apoptosis in cancer cells I participated in two screening studies (Paper III and I). The pilot screen of a large dataset of cyanobacterial extracts was aimed at general cytotoxicity of cyanobacteria in connection with possible risks to human health. Even though Paper III only partly considered my topic, the screen itself served us the first round of extract selection and in fact the initial screen proved to be crucial for my subsequent research (since it led to the identification of the compound of our interest). Moving on towards cancer cell biology, in the second study I described a phenotypic screen of selected cyanobacterial extracts, which we performed in order to find those with apoptosis-inducing activity. The main purpose of this paper was to present data on assays and methods used in cancer cell biology and drug discovery, together with their drawbacks, which are often overlooked. Secondly, we provide amendments that deal with some of the drawbacks, and give suggestions for an optimal design of screening using natural extracts.

Luckily for me, the initial screening led to the identification of a completely novel cyanobacterial metabolite, nocuolin A (NoA), and I was given the opportunity to evaluate its pro-apoptotic properties (Paper II and unpublished results). A new compound of unknown mode and mechanism of action is a challenge, since there are multiple pathways of cell death and the effector molecules or organelles can have either well-established unique role(s), or their functions can greatly overlap. In Paper II I present NoA as an apoptosis-inducing compound and further, in the chapter of unpublished results and its following discussion, I speculate on its mode of action within a cancer cell in vitro. Currently, our knowledge of the biological activity of NoA resembles a puzzle with many parts still missing, so we cannot see the whole picture yet. However, some of my data indicate that NoA-induced cell death involves mitochondria in an uncommon way.

All in all, in the present thesis I attempt to merge drug discovery using cyanobacterial secondary metabolites and cancer cell biology, putting emphasis on regulated cell death pathways. I tried to embody these topics in the Introduction chapter. The thesis includes three publications: the ecotoxicological study (Paper III), the phenotypic screening linking cyanobacterial extracts and apoptosis (Paper I), and lastly the publication describing a novel compound with apoptosis-inducing activity (Paper II).

List of Abbreviations

ACD, accidental cell death
AE, auristatin E
AIF, apoptosis inducing factor
APAF1, apoptotic peptidase (protease) activating factor 1; contains caspase recruitment domain (CARD) and an ATPase domain
ATP, adenosine triphosphate
BCL-2, B-cell lymphoma 2 protein family
Bcl-2, B-cell lymphoma 2; the first identified member of BCL-2 family
Bid, BH3-interacting domain death agonist
BIRC, baculoviral IAP repeat-containing
BH domain, BCL-2 homology domain
BH3-only proteins, subclass of BCL2 family members with BH3 domain only
BNIP3L, BCL2/adenovirus E1B interacting protein 3-like
Casp, caspase(s); specific proteases,
CD30, cell membrane protein of the TNFR family
cFLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; It binds to FADD and/or caspase-8 (-10), which in turn prevents DISC formation.
cIAP, cellular IAP
Da, dalton
DAI, DNA-dependent activator of interferon regulatory factors
DAMPs, damage-associated molecular patterns/also known as danger-associated molecular patterns
dATP, deoxyribonucleotide ATP
DD, death domain
DED, death effector domain
DISC, death-inducing signaling complex
DKO, double knockout
DR, death receptor
EndoG, endonuclease G
ER, endoplasmic reticulum
FADD, FAS-associated death domain (FAS-associated protein via a death domain)
FLICE, FADD-like IL- 1β-converting enzyme
GFP-LC3, LC3 containing GFP (green fluorescence protein) tag
HACE1, HECT domain and ankyrin repeat-containing E3 ligase 1
HDAC, histone deacetylase
HTRA2, high temperature requirement protein A2
HTS, high throughput screening
IAPs, inhibitors of apoptosis
IC_{50}, inhibitory concentration
KO, knockout
LC3, microtubule-associated protein light chain 3
MEFs, mouse embryonic fibroblasts
MLKL, Mixed lineage kinase domain-like
MMAE, monomethylauristatin E
MOAP-1, modulator of apoptosis 1
MOMP, mitochondrial outer membrane permeabilization
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI-60, refers to Human tumour screen of 60 cell lines at the National Cancer Institute
Nec1, necrostatin 1; Ripk1 inhibitor
NK, natural killer cell
NKT, natural killer T cell
NoA, nocuolin A
NP, natural product
NRPS, non-ribosomal peptide synthetase
OPA1, optic atrophy protein; mitochondrial dynamin like GTPase
PARP-1, Poly(ADP-ribose) polymerase 1
PKS, polyketide synthetase
PS, phosphatidylserine
PTPC, permeability transition pore complex
Q-VD-OPh (QVD), Quinoline-Val-Asp-Difluorophenoxymethylketone; pan-caspase inhibitor
RCD, regulated cell death
RHIM, RIP homotypic interaction motif
RIPK, receptor-interacting protein kinase
ROS, reactive oxygen species
RT-MLPA, Reverse Transcriptase Multiplex Ligation-dependent Probe Amplification assay
Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI
tBid, truncated Bid
TNFR, tumour necrosis factor receptor
TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β
XIAP, X-link inhibitor of apoptosis
Z-VAD-FMK, Z-Val-Ala-Asp-Fluoromethyl Ketone; pan-caspase inhibitor
WT, wild type
3MA, 3-methyl adenine; inhibits early stages of autophagosome biogenesis (via inhibition of the class I phosphoinositide 3-kinase [PI3K])

CHAPTER I.

INTRODUCTION

CHAPTER I

NATURAL PRODUCTS

Many reviews highlight the significance of natural products (NPs) in drug discovery and development (Harvey, 2008; Harvey et al., 2015; Koehn and Carter, 2005; Mayer et al., 2010; Paterson and Anderson, 2005). Last several decades have seen an extensive investigation of both plant and animal secondary metabolite products, as well as the golden era of the field of microbial antibiotics (Newman and Cragg, 2012). Nonetheless, drug databases with thousands of compounds of different origin (the synthetic, and the natural with their analogues), and their use in high throughput screening (HTS) approaches and/or the involvement of in silico drug discovery and genome mining approaches are the trend nowadays. These alternatives have been applied to seek for novel pharmaceutics, with the argument that the NP screening in laboratory experiments is expensive, uncertain and not bringing sufficiently specific hits and should no longer be prioritized. Despite these drawbacks, screening for potential pharmaceutical hits in natural sources still brings novel important structures. The problems and current trends are discussed in many reviews or rather opinion articles (Amirkia and Heinrich, 2015; Bachmann et al., 2014; Ganesan, 2008; Harvey et al., 2015; Hughes et al., 2011; Li and Vederas, 2009).

NP screening is a pipeline with a long history. It might seem rather old-fashioned, in view of the fact that humans have used natural medicine since ancient times. So what makes natural (secondary) metabolites interesting and valuable in drug design? The main asset of NPs is their enormous structural diversity, the core value with which all expert scientists agree. Actually, the novelty in the structure and complex scaffolds, together with the efficiency of secondary metabolites in the interaction with various biological targets predetermine them to be valuable leads. Indeed, natural chemicals have primarily evolved to react as ligand-receptor partners. Such a long co-evolution led to the existence of compounds with scaffolds desirable for drug design (Carlson, 2010; Rodrigues et al., 2016). This was also reflected in turning the attention more into the microbial world – especially to microbes themselves and their interactions with the “host whence the microorganism was isolated” as a new potential field for active metabolites (Newman and Cragg, 2012).

Although I have highlighted the undisputed role of NPs in the medicine development, naturally there are some pitfalls to deal with. Usually it is the complexity of crude natural extracts and often also a limited supply of desired samples or compounds. Since the initial natural extracts are mixtures of literally hundreds of compounds, the crude extracts may mask interesting compounds due to their low concentration in the sample or a coeffect with other compounds; also, mixtures are unfavourable for HTS. The natural samples, isolated fractions or purified compounds are then very often limited in their amount and sometimes impossible to recollect. These constraints hinder structure elucidation and further evaluation

of biological activity and make NP screening a challenging path with uncertain outcome (Li and Vederas, 2009).

Overall medical care has gone a long way, and cancer treatment has advanced rapidly in the last 100 years or so. Novel approaches such as immunotherapy, targeted therapy, personalized medicine and advances in drug delivery systems have entered medical treatment for some types of cancer and brought great results for patients. Surely we can expect another forward steps to come. So can new compounds bring benefits to medical treatment? Shall we stop searching for novel natural metabolites as a drug leads? The answer is certainly no. In the reality of current treatment, chemotherapy together with surgery and radiation therapy are the most used approaches to fight cancer and for some people the standard chemotherapy may be the only treatment they receive. There are many effective anti-cancer agents available, but some are associated with a high level of toxicity and severe side effects. Cancer cells can also develop resistance to certain therapeutics (Housman et al., 2014). For these reasons the needs for novel cancer therapeutics may be seen as an area with unmet demands and new therapeutics can significantly improve some cancers treatment.

As examples of important NP discoveries I briefly discuss the plant alkaloid taxol (Wani et al., 1971) and the cyanobacterial peptide dolastatin 10 (Pettit et al., 1987). For both it took roughly 30 years to be approved as anticancer drugs. This might illustrate some difficulties in NP-drug discovery pipeline. This long path from discovery to the market had different reasons for these two NPs. Taxol faced problems mainly at the stage of establishing commercial production. In the case of dolastatin 10, despite its high potency in vitro, the desired activity in vivo was only achieved and modulated through development of a dolastatin derivative conjugated to an antibody to reach specificity to cancer cells. Lastly, I introduce one promising agent - aurilide, mined from marine environment only a few years back (Suenaga et al., 1996).

Taxol (paclitaxel), a plant-derived compound from a *Taxus* (yew) species, is a very successful drug used to treat several cancer types (ovarian, breast, lung, and others). It is a cytostatic agent targeting tubulin (suppresses the dynamic of microtubules), thus eventually causing disruption of cell division, leading to a cell cycle arrest. Taxol was structurally defined by Wani and colleagues in 1971 (Wani et al., 1971); but it took over 30 years, since it gained the first marketing approval from the U.S. Food and Drug Administration in 90s. The very long development time for this drug was a consequence of limited supply from the original source (the bark of the yew tree) and difficulties with establishing the synthetic commercial production (optimal yield and cost). In the end, the industrial production was established and two different approaches are mainly used. One is the Taxol-cell-culture technology, which involves a direct synthesis of the chiral compounds. The other approach uses a semi-synthetic route from precursors available from the needles of various yew species (as a renewable resource) (Croteau et al., 2006).

The **dolastatins and aurilide** were both isolated from the marine mollusc *Dolabella auricularia* (sea hare); only later the original source organism was recognized as a cyanobacterium. Indeed, the sea hare seems to be a source of surprising metabolites, even though very often of prokaryotic origin either from its diet or symbiotic microorganisms (Harrigan and Goetz, 2002; Yamada et al., 2010). In the case of dolastatins the cyanobacterial origin was confirmed as the analogues had been found in strains of the cyanobacterial species *Lyngbya majuscula* and *Symploca hydnoides* (Harrigan et al., 1998; Luesch et al., 2002a; Luesch et al., 2001; Luesch et al., 2002b; Pettit et al., 1989). For aurilides, it is very likely to be the same case as for dolastatins, since the analogues aurilide B and C have also been isolated and described from *L*. *majuscula*, and their complex structure resembles microbial secondary metabolites (Han et al., 2006).

The first **dolastatin** variants were purified, characterized and patented by the Pettit group in the early 70s and showed up to be very effective microtubule-binding agents. The most potent compounds, dolastatins 10 and 15, a related compound symplostatin, and their analogues underwent further investigation and evaluation studies. For example, the average IC50 value for dolastatin 10 was 120 pM (range 1 pM – 100 nM) according to NCI-60 (Human tumour screen of 60 cell lines at the National Cancer Institute) (Greenberger and Loganzo, 2008). Showing such potency, the compounds (dolastatin 10, its analogues: TZT-1027 [synonyms: auristatin PE, soblidotin] and LU103793, and an analogue of dolastatin 15: ILX651) went through several clinical investigations. These compounds were tested as a single agent and entered into phase I and II trials, but mostly failed to exhibit desired anticancer activity *in vivo* (Greenberger and Loganzo, 2008; Hoffman et al., 2003; Krug et al., 2000; Madden et al., 2000; Marks et al., 2003; Mita et al., 2006; Patel et al., 2006; Vaishampayan et al., 2000). Concurrently, some synthetic analogues of dolastatin 10, e.g. auristatin E (AE) and monomethylauristatin E (MMAE); were conjugated to antibody to ensure particular tumour selectivity (Doronina et al., 2003). The antibody-drug conjugates are stable in extracellular fluids and relatively non-toxic. After the binding of the conjugated antibody to its cancer cell surface antigen, the conjugate is internalized into the cell, where the toxic agent (e.g. MMAE) is released, thus only killing the cancer cell. So far, the most successful analogue of dolastatin 10 is MMAE linked to defined antibody. In this case the antibody target (developed by Seattle Genetics in collaboration with Takeda Pharmaceutical Company), CD30, is a defining marker of Hodgkin lymphoma. ADCETRIS® (brentuximab vedotin) is a trade name for the antibody-MMAE conjugate drug. Recently it was approved for relapsed Hodgkin lymphoma and relapsed systemic anaplastic large cell lymphoma in more than 60 countries, including the U.S., Canada, Japan and members of the European Union.

Aurilide was originally isolated as a minor constituent of a *Dolabella auricularia* extract in 1996 (Suenaga et al., 1996). The in vitro cytotoxicity evaluation of the pure aurilide against cancer cells was not possible at that time due to the small amount obtained in the original study (0.5 mg of aurilide was isolated from 262 kg of sea hare tissues [wet weight]).

However, organic synthesis via enantioselective total synthesis (a form of synthesis which results in the production of more than one isomer) confirmed the aurilide structure. The synthetic compound was employed to determine the cytotoxicity against human cell line HeLa S3 (IC50 – 0.011 µg/mL) (Mutou et al., 1997). Cyanobacterial origin of this compound was confirmed by isolating aurilide analogues, the aurilides B and C, from *L*. *majuscula* (Han et al., 2006). The authors speculated that aurilides were likely produced by nonribosomal peptide synthetases and polyketide synthetases and their structural variations might be the result of substrate multispecificity of adenylation domains. Aurilide B proved to be 4-fold more toxic to NCIH460 human lung tumour and neuro-2a mouse neuroblastoma cells than aurilide C. Furthermore, aurilide B toxicity was evaluated in the NCI-60 panel resulting in the observation of potent inhibition in leukemia, renal and prostate cancer lines and in the hollow fibre assay (Han et al., 2006). Subsequent investigation of synthesis approaches towards artificial aurilides led to as many as eight analogues being tested for their structure-toxicity relationship (Suenaga et al., 2008). Recent studies revealed that aurilide binds to prohibitin 1, which activates the proteolytic processing of optic atrophy 1 protein (OPA1) in mitochondria, resulting in mitochondria-induced apoptosis. Aurilide thus also represents a prospective tool as a lead compound to investigate the role of prohibitin (and OPA1) in inducing apoptosis and, additionally, it offers a potential novel anticancer drug target (Sato et al., 2011).

INTRODUCTION TO CYANOBACTERIAL SECONDARY METABOLITES

Cyanobacteria (blue green algae) are prokaryotic organisms capable of producing large amounts of diverse organic compounds, which are collectively called secondary metabolites. The role and function of these compounds for their producers have challenged the researchers for many decades and still have not been completely solved. Cyanobacteria produce a wide range of small organic molecules ranging in their molecular mass mainly between 500 and 2000 Da. Based on their structure they can be classified as peptides, polyketides, alkaloids, isoprenoids (including carotenoids, terpenoids, and steroids), aromatic, and heterocyclic compounds (Van Wagoner et al., 2007).

The majority of cyanobacterial secondary metabolites are synthesized by the activity of highly flexible membrane-associated biosynthetases. Namely, these are non-ribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS). Such systems are capable of building various structures including highly modified peptides with exotic moieties. These biosynthetic complexes can be further combined into large assembly lines (NRPS/PKS) to produce hybrid compounds, thus enlarging the chemical diversity even more. In the case of non-ribosomal synthetases, each biosynthetic step is performed by a protein module that operates without a ribonucleic acid template, so the synthesis is driven by the organization of the modules. On a lower structural level, the modules consist of the so-called domains, which are responsible for the sequential selection, activation, and condensation of precursors into the nascent product. The NRPS and PKS enzymes both operate in a similar fashion, namely with respect to the sequential addition of activated precursors to the nascent peptide or polyketide chain, respectively. In the case of the NRPS module, the precursor (amino acids and related carboxyl-containing compounds) is recognized and activated by the adenylation domain and further passes to the thiolation domain, which facilitates its delivery to the condensation domain. The condensation domain catalyzes the formation of the peptide bond between the activated residue and the nascent peptide. Thus each biosynthetic module needs to contain at least the adenylation, thiolation and condensation domain. The final structure of the metabolite mirrors the organization of the modules within the membrane. Analogously to the NRPS module system, in the case of PKS the acetyl-CoA is activated by an acyl transferase, then passes to the acyl-carrying protein and a new bond is formed by the ketosynthetase domain. Thus the new carbon chain is elongated by two carbons. Aside from the core domains indispensable for these machineries, additional modification steps can be implemented into the pathway. For NRPS various tailoring domains such as methyltransferase, epimerase, aminotransferase, and monooxygenase are known to participate in the process. In the case of PKS, the growing polyketide chain can be further modified by ketoreductases, dehydrogenases and enoyl reductases to generate a fully saturated carbon chain (Welker and von Dohren, 2006).

Taken together, the high structural diversity of cyanobacterial secondary metabolites, which predisposes them as interesting pharmaceuticals (although the unusual chemical moieties do not have to form the pharmacophore of the molecule), is driven by the plasticity and modularity of the above-mentioned biosynthetases, particularly:

- The variability of adenylation domains. More than 100 adenylation domains are currently described that are able to activate various precursor molecules, sometimes very different in their chemical structure from proteinogenic amino acids.
- Tailoring domains
- The combination of NRPS/PKS modules with a plethora of other enzymes.

Small natural metabolites can bind to various molecules and possibly handle multiple targets. Table 1 highlights known cyanobacterial compounds or classes together with their possible targets (with respect to therapeutic uses). The information given in Table 1 is based on a review article from the authors Salvador-Reyes and Luesch (Salvador-Reyes and Luesch, 2015), which represents the most up to date review on active compounds from marine cyanobacteria; the marine environment is a well-established source of potential pharmaceutics (Gerwick and Moore, 2012; Mayer et al., 2010; Tan, 2007).

TARGET	CYANOBACTERIAL METABOLITES	ADDITIONAL INFORMATION
Actin Controls cytokinesis Maintains cell shape Cellular motion	Dolastatin 11 class related compounds: Majusculamide C, Lyngbystatins 1 and 3	Actin reorganization Cell cycle arrest
ER (Endoplasmic Reticulum) Production, folding, quality control and dispatch of proteins Lipid and steroid metabolism Detoxification function	Apratoxin A	Modulates receptor proteins and membrane-associated proteins in ER Inhibits glycosylation and signal peptide cleavage
HDACs (Histone deacetylases) Affect the chromatin structure Influence transcription factors and gene expression	Largazole Santacruzamate A Tanikolide dimer	Largazole inhibits class I of HDACs; antiproliferative effect *in vitro* and *in vivo*; modulates the invasiveness of breast cancer cells Santacruzamate A inhibits class I of HDACs Tanikolide dimer – SIRT inhibitor (noncanonical HDAC class III sirtuins)
Kinases Phosporylation of proteins Affect carcinogenesis	Bisebromoamide Scytonemin	Inhibit multiple targets (including kinases)
Metal chelation Modulates angiogenesis	Grassypeptolide Ascidiacyclamide Patellamides Lissoclinamides	Lissoclinamides were isolated from ascidians, but possibly of cyanobacterial origin
Prohibitins Mitochondrial membrane proteins Control mitochondrial integrity (fusion/fission)	Aurilides related compounds: Lagunamides and Kulokekahilides	Affect mitochondria through prohibitin 1 and OPA1 protein
Proteasome Protein degradation	Carmaphycins	Structural similarities with epoxomicin (proteasome inhibitor, drug lead of carfilzomib)
Tubulin Cell division Cytoskeleton Intracellular transport	Dolastatin 10 class (and its analogues) Curacins Cryptophycins	Bind via vinca domain or colchicine site of tubulin

Table 1. Selected cyanobacterial compounds based on a review article (Salvador-Reyes and Luesch, 2015) and their biological targets – suggested or validated.

CELL DEATH

CLASSIFICATION - CURRENT RECOMMENDATION

For many decades the classification of cell death was based solely on morphological evaluation. One of the first categorizations was proposed by Schweichel and Merker in 1973 (Schweichel and Merker, 1973). They performed a survey of cell death occurring in physiological and death-induced conditions of mainly rat and mouse samples. On the basis of manifestation of particular cytomorphological traits observed by electron microscopy, three cell death types were distinguished: type (1) featuring cytoplasm and nucleus condensation followed by fragmentation and uptake by neighbouring cells (apoptosis-like); type (2) featuring extensive cytoplasmic vacuolization (autophagy-like); and type (3) featuring cell structure disintegration, membrane fragmentation and cell break-down (necrosis-like). It is not surprising that classification based on morphological markers has dominated the field of cell death research for decades, especially since a light microscope used to be the conventional equipment in the laboratories worldwide. The rise of molecular methods has brought new insights into the biochemical mechanisms underlying the cell death process, as well as a need to revise the former classification, which apparently showed to be oversimplified and rather misleading.

In order to reflect new findings, the Nomenclature Committee on Cell Death, association of scientists and editors of the Cell Death and Differentiation journal, regularly formulates a set of recommendations regarding the distinct cell death modalities and appropriate terminology to be used in science communication concerning cell death. The first set of recommendations was published in 2005 (Kroemer et al., 2005), the following rounds came in 2009 (Kroemer et al., 2009), 2012 (Galluzzi et al., 2012) and the last in 2015 (Galluzzi et al., 2015). Throughout my thesis the text describing cell death and related topics is based mainly on vertebrate model organisms (and derived cancer cell lines) and follows recommendations of above-mentioned reviews.

REGULATED VS. ACCIDENTAL CELL DEATH

Essentially, cells can die either in an uncontrolled manner, or by triggering highly organized processes leading to their demolition. The first situation is referred to as accidental cell death (ACD, typical necrosis) the latter as regulated cell death (RCD) (Galluzzi et al., 2015). ACD follows after extreme physiological or mechanical circumstances to which cells are exposed and if there is no space to cope with such unwanted intervention. RCD is a genetically encoded and molecularly driven mechanism that can be activated in response to disturbances in extracellular or intracellular microenvironment or on the basis of a pre-

programmed cell-fate schedule (in that case, the cell death modality is usually termed Programmed cell death). Besides one well-known type of RCD, apoptosis, there are many other RCD pathways (Duprez et al., 2009; Galluzzi et al., 2012) that are mentioned and characterized later in this text.

During the process of dying, cells may release particular molecules called damage-associated molecular patterns (DAMPs). DAMPs are a heterogeneous group of molecules which promote the cytotoxic inflammatory or immune response. DAMPs are released outside the cell (or exposed on its surface) when the cell membrane is ruptured (Krysko et al., 2013). Initially only necrosis was associated with DAMPs release, and apoptosis-like cell deaths were considered as immunologically silent. It is now clear there are many RCD pathways, regardless of their morphology (apoptosis- or necrosis-like), which may induce an inflammatory or immune response (Galluzzi et al., 2015).

WHEN IS A CELL DEAD?

The proportion of dead cells in a population is one of the gold standard tests. Although this parameter seems straightforward, the number of dead cells (or inverse cell viability), obtained as a result of a biochemical assay, might be ambiguous and difficult to compare. So, when is a cell considered dead? The broadly accepted consensus is that the point of no return for a cell occurs when its cellular membrane has permanently lost its barrier function and/or the cell and its nucleus have gone through a complete breakdown into discrete bodies (Galluzzi et al., 2015). However, the reality is much more complex. This is especially true in the case of membrane permeabilization. Recently it has been shown that a cell can repair large membrane disruptions. Thus, even vast membrane damage (the so-called ‘leaky membranes’) does not ultimately have to lead to cell death (Dal Peraro and van der Goot, 2016). Similarly, the engulfment of a cell corpse by neighbouring cells or professional phagocytes used to be understood as the commitment of cell death (Galluzzi et al., 2012), yet it is also not an absolute point of no return (Galluzzi et al., 2015). Concerning the practical approach – quantifying dying and dead cells in population using a sole cell-death related parameter can be deceptive and even misleading; and employing multiple unrelated methods is recommended (Galluzzi et al., 2009). The biochemical criteria that are also closely related to cell death are namely: massive activation of caspases, exposure of phosphatidylserine (PS) residues, a loss of mitochondrial membrane potential, ATP depletion, and a complete permeabilization of the mitochondrial outer membrane. All of these markers are reversible in some cases and may even participate in pathways not related to cell death (Kroemer et al., 2009). For example, caspases are also activated in the context of non-lethal processes (Kuranaga, 2012; Miura, 2012) and the loss of mitochondrial membrane potential and PS exposure may not necessarily mean progression towards immediate cell death (de Graaf et al., 2004; Galluzzi et al., 2015; Yang et al., 2002).

CELL DEATH TYPES

APOPTOSIS

In 1972 scientists Kerr, Wyllie, and Currie defined and described the mechanism of controlled cell demolition, for which they proposed the name apoptosis (means "falling off" or "dropping off" in ancient Greek). In their original paper the emphasis was given on the morphological evaluation of distinctive features (e.g. forming of apoptotic bodies) in the context of both physiological and pathological conditions (Kerr et al., 1972).

Thanks to extensive research in the following years biochemical background of this process has been described to a large extent, as well as numerous signals leading to apoptosis triggering. Depending on the initiation of the signalling, there are two major apoptotic pathways recognized: Extrinsic and Intrinsic (Fig.1 and Fig.2). The first is mediated via membrane receptors and the latter upon intracellular stress, which may be caused by virtually hundreds of factors. Classical apoptosis deeply relies on the action of caspases (specific proteases) and proteins of BCL-2 family (B-cell lymphoma 2) (Elmore, 2007; Galluzzi et al., 2012; Taylor et al., 2008). BCL-2 proteins are the key factors playing role in both pro-apoptotic and anti-apoptotic signalling (Cory and Adams, 2002). The decision whether the cell initiates the apoptotic self-destruction process depends on the balance between pro- and anti-apoptotic BCL-2 members (see later chapter BCL-2 proteins). BCL-2 proteins act via their interaction with each other and/or other molecular partners. Collectively, actions of BCL-2 proteins result in altering the function of organelles; in the case of apoptosis these are most importantly mitochondria. Apart from its vital role for the cell, the mitochondrion plays also an important role in apoptotic signalling. The organelle functionality depends on the integrity of its double membrane. Its permeabilization marks an important checkpoint in the apoptotic signaling cascade. The mitochondrial membrane contains, besides many other membrane proteins, two pro-apototic BCL-2 members, Bax and Bak, which under specific circumstances form a pore. If the membrane integrity (e.g. through Bax/Bak pores or other actions) is altered, the so-called mitochondrial outer membrane permeabilization (MOMP) develops. The subsequently released molecules (e.g. cytochrome c) participate in the transduction of the signal towards the executive phase of apoptosis. In the executive phase, the coordinated disassembly of the cell is led by the effector caspases (which are caspase-3, -6, and -7; [Casp3/6/7]). The activation of effector caspases downstream of MOMP is a common route for apoptosis, irrespective of its triggers and upstream signalling (see intrinsic and extrinsic apoptosis as well). Broadly speaking, caspases carry out most of the proteolytic attack within a cell undergoing apoptosis. Caspases are endoproteases that gained their name due to their specific features: having cysteine in the active site and recognizing aspartate in the target protein (**c**ysteine-dependent **asp**artate-specific prote**ases**). They exist within the cell as inactive zymogens and conditionally (e.g. during apoptosis, but also inflammation) can become activated. There are many cellular proteins, which are cleaved by

caspases when the apoptotic process is ongoing. The prominent examples are poly(ADP-ribose) polymerase 1 (PARP-1) or components of cytoskeleton (actins and microtubulins) (Cohen, 1997; Taylor et al., 2008). PARP-1 is a nuclear enzyme which is engaged during DNA damage as an important factor for maintaining genomic integrity. The caspase activity can be blocked by action (binding) of proteins called IAPs (inhibitors of apoptosis) (Yang and Li, 2000).

Apart from biochemical interactions orchestrated within the apoptotic cell, the cell morphology is largely altered as well. After all, morphological characteristics used to be the privileged descriptive features of apoptosis. This situation has dramatically changed and apoptotic morphology is now only one of more discriminating factors, yet it still should be taken into account. During apoptosis the cell becomes rounded and detaches from its neighbours and matrix. Then follows the dynamic release of apoptotic bodies, which is the defining feature of apoptotic morphology. These cell fragments are membrane-bound vesicles, which may contain parts of the intracellular matrix (including organelles and DNA). At this point the dying cell and the apoptotic vesicles still retain integrity and are cleared out by specialized cells (macrophages) or by neighbouring cells, preventing thus the inflammatory reaction (Galluzzi et al., 2012). Avoiding a large inflammatory reaction is a favoured approach in cancer therapy (Baig et al., 2016).

Apoptosis is widely regarded as one of the opposite mechanisms to cell division. It holds the key to balancing cell numbers and thereby the homeostasis of a multicellular organism. In both extremes, meaning either too little, or too much of cell death, homeostasis is disturbed and such an imbalance in cell number regulation results in pathologies such as neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer (Elmore, 2007).

Besides apoptosis, there are other cell death modes which have been characterized in more or less detail. Some of them are triggered only by unique signals (e.g. anoikis – activated by the loss of cell attachment) or occur only in specific cell types (e.g. cornification – epithelial cells). Here I list the main types followed with a short description for certain cases: necrosis, regulated necrosis, autophagy, cornification, mitotic catastrophe, anoikis, pyroptosis, Wallerian degradation, excitotoxicity, paraptosis, entosis (Galluzzi et al., 2012; Kroemer et al., 2009), and methuosis (Maltese and Overmeyer, 2014).

NECROSIS

When a cell suffers from non-physiological conditions, it most likely undergoes necrosis (in this case the ACD). The plasma membrane of necrotic cells ruptures and subsequently the cell loses its intracellular contents. The release of DAMPs into the extracellular space initiates an inflammatory reaction in the surrounding tissue (Galluzzi et al., 2015; Galluzzi et al., 2012).

REGULATED NECROSIS

A prototypic form of regulated necrosis is necroptosis, which was firstly thought to be an alternative cell death pathway in the situations when apoptosis is non-functional. Today it is apparent that necroptosis plays an important role in cell and tissue reaction, for example to pathogen infection. Necroptosis depends on activation and formation of necrosome – a protein complex in which a receptor-interacting protein kinase (Ripk) 3 is activated along canonical (mediated by Ripk1) or noncanonical pathways (mediated by DAI [DNA-dependent activator of interferon regulatory factors] or TRIF [Toll/IL-1 receptor domain-containing adaptor inducing IFN-β]). The activated Ripk3 then phosphorylates mixed lineage kinase domain-like protein (MLKL) and this stimulates its action further resulting in membrane rupture (Vanden Berghe et al., 2016), see also Figure 3.

PYROPTOSIS

This mode of cell death was described in macrophages infected by pathogens (*Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Bacillus anthracis* and so on), but could be triggered also by other stimuli (e.g. stroke, heart attack, cancer) (Bergsbaken et al., 2009). It involves the activation of Casp1 and an inevitable inflammatory response. Although pyroptotic macrophages may exhibit morphology typical of apoptosis (Labbe and Saleh, 2008), it is categorized as a type of regulated necrosis (since it also features necrosis-like traits: e.g. loss of plasma membrane integrity) (Vanden Berghe et al., 2016).

AUTOPHAGY

Autophagy is a lysosomal degradation pathway. It is an essential cellular mechanism acting under both physiological and pathological conditions. This pathway is often upregulated when a cell suffers from for example: nutrient starvation, growth factor deprivation, experiences oxidative stress; or needs to handle the clearance of damaged organelles and intracellular proteins. Autophagy is involved in important processes such as survival, differentiation, development, and homeostasis. The primary role of autophagy is presumed to compensate for the impaired energetic metabolism, through recycling of intracellular components (including organelles, damaged or non-functional proteins etc.), in order to allow cell survival. It is also used to eliminate intracellular pathogens (e.g. viruses and bacteria), thus being important for immunity response and inflammatory reaction. The main recognized types of autophagy are: macroautophagy (here referred to as autophagy), microautophagy and chaperone-mediated autophagy. The degradation process is performed by the so-called autophagosome organelles. The creation of an autophagosome starts *de novo* by formation of the isolation membrane, which elongates and culminates into the double-membrane organelle: the autophagosome. Autophagosomes sequester cellular

components and in the next stage they fuse with lysosomes to generate the autolysosome. Here the content is broken down by lysosomal enzymes. Regarding cell death processes, autophagy involvement can modulate cell death response to a great extent. It is difficult to generalize the role of autophagy in cancer and cell death, since its real contribution and role may be very complex. However, it is principally considered a cytoprotective response that damaged cells activate to survive; so its inhibition can therefore accelerate cell death. Under low-oxygen conditions occurring in the tumour microenvironment, autophagy can turn into mitophagy (degradation of the mitochondrial network) and thus block the amplification of the apoptotic signal, making the cancer cells more resistant to apoptosis. For the purposes of this thesis, it is worth to mention the microtubule-associated protein light chain 3 (LC3), which is known to accumulate on autophagosome membrane, and as such it is widely used as a marker of the presence of autophagosomes (Galluzzi et al., 2014; Levine and Kroemer, 2008; Levine et al., 2011; Mizushima, 2007; Mizushima et al., 2010).

APOPTOSIS

INTRINSIC APOPTOSIS

When the signal to initiate the apoptotic process is triggered inside the cell, in majority of cases caused by non-physiological conditions (in the intracellular or extracellular environment), the process is termed intrinsic apoptosis (Fig.2). Cellular stress is a very broad term and may cover DNA damage, cytosolic $Ca2^{+}$ overload, oxidative stress, accumulation of unfolded proteins and many others. The common hub, where all these stimuli are transformed into a death pathway, is mitochondria. As mentioned above, their function is essential for the cell, and so the development of the irreversible MOMP in the majority of mitochondria within a single cell usually has lethal consequences. MOMP can start at the outer mitochondrial membrane due to the pore-forming activity of Bax and Bak proteins, or this can originate at the inner mitochondrial membrane due to the opening of a multiprotein platform, the so-called permeability transition pore complex. MOMP is the breaking point in the intrinsic apoptotic cascade, as it immediately precedes the executional phase. The irreversible MOMP is accompanied with: the loss of mitochondrial membrane potential, respiratory chain inhibition (hence ROS production) and a release of distinctive components such as cytochrome c. The cytosolic cytochrome c together with the signalling proteins APAF1 (apoptotic peptidase [protease] activating factor 1) and dATP participate in the cell death execution by forming a protein complex called apoptosome. Consequently, within the apoptosome, Casp9 gets activated and induces activation of the proteolytic cascade via the effector caspases Casp3/7. Other proteins released from mitochondria, which play an important role in the apoptotic pathway, are: AIF (apoptosis inducing factor), endonuclease G, Smac/DIABLO (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI), and HTRA2 (high-temperature requirement protein A2). AIF and endonuclease G relocate into the nucleus, where they mediate DNA fragmentation independently of caspases. Smac/DIABLO and HTRA2 are IAP antagonists, so they indirectly boost caspase activity by inhibiting the anti-apoptotic function of several IAP family members (Galluzzi et al., 2012).

The contribution of regulatory proteins to the direct execution of the intrinsic apoptosis may vary under distinctive physiological, pathological and experimental scenarios. On top of that the situation is even more complex, since along with the propagation of the pro-death cascade, there are also pro-survival mechanisms, which are simultaneously engaged to allow cells to cope with stress conditions (Galluzzi et al., 2015; Galluzzi et al., 2012).

EXTRINSIC APOPTOSIS

The death signal leading to extrinsic apoptosis is transduced through specific transmembrane receptors. There are basically two ways how extrinsic apoptosis through membrane receptors may be initiated: by a ligand binding to a receptor; or, in the case of "dependence receptors", in the absence of their ligands (Galluzzi et al., 2012) (Fig.1).

Extrinsic apoptosis by ligand binding: Upon binding of a lethal ligand to a death receptor (DR), the extracellular part of the DR changes its conformation, which allows then another set of changes at the cytosolic part of the DR. These actions include the interaction of the DR intracellular domain with adaptor proteins and the assembly of a dynamic multiprotein complex at the cytosolic tail of the DR. This multiprotein platform is named the death-inducing signalling complex (DISC) and is assembled at the initiation phase of extrinsic apoptosis. Proteins that participate in this aggregate constitution include: Ripk1, FAS-associated protein with a death domain (FADD), multiple isoforms of c-FLIP (cellular FLICE-like inhibitory protein), cellular IAP (cIAP), E3 ubiquitin ligases and pro-Casp8 (or pro-Casp10). Within the fully assembled DISC the Casp8 (or 10) gets activated and can trigger the execution phase of apoptosis in a mitochondrion-independent or dependent manner. The executive Casp3/6/7 activation may be mediated by the Casp8 proteolytic cleavage directly (unconditional contribution of mitochondria, type I cells), or the executive caspases get activated only upon mitochondrial involvement (Galluzzi et al., 2012). In the latter case (type II cells), Casp8 first catalyses transition of the BH3-interacting domain death agonist (Bid) into mitochondrion-permeabilizing fragment (known as truncated Bid or tBid). The tBid protein mediates MOMP (Li et al., 1998), which subsequently leads to Casp3/6/7 maturation. The mitochondrial organelle, in particular the MOMP development, is the intersection point that may connect the extrinsic and BCL-2-regulated intrinsic pathways. Of note, cells that undergo the extrinsic caspase-dependent apoptosis in a mitochondrion-independent manner may still show signs of mitochondrial involvement, particularly the presence of tBid and MOMP, but these are in this case dispensable for the execution of apoptosis. The role of Casp10, a close homologue of Casp8, is still not fully understood. It is not clear, whether Casp10 can fully substitute Casp8 and what its actual contribution to the death signalling is (Galluzzi et al., 2012).

Death receptors are members of the tumour necrosis factor receptor (TNFR) superfamily. As membrane proteins, they consist of the extracellular, transmembrane and intracellular parts. The receptor specificity is defined by the tertiary structure of the extracellular region, which is responsible for the ligand binding. DRs display some level of homology (cysteine-rich domain) in the extracellular region and in the cytosolic part some DRs share a conserved sequence of roughly 80 residues, called the death domain (DD). The DD is an essential feature of DRs capable of death signalling, since it enables interaction with specific adaptor partners. Signalling through these receptors is complex; initially DRs were thought to be involved in the cytotoxicity responses only. However, DRs play a role in

processes unrelated to apoptosis as well, including the regulation of cell proliferation and differentiation, chemokine production, inflammatory responses and tumour-promoting activities (Galluzzi et al., 2012; Guicciardi and Gores, 2009). One example is the tumour necrosis factor receptor 1 (TNFR1) which can trigger apoptosis, necroptosis as well as survival (Christofferson and Yuan, 2010). TNF ligand binding to TNFR1 activates necroptosis only when both NF-κB and apoptosis (Casp8) are blocked. Recently, a key gatekeeper of TNFR1 mediated cell fate has been identified: HACE1 (HECT domain and ankyrin repeat-containing E3 ligase 1). In the absence of HACE1 (genetic manipulation) the survival pathway (NF-κB) and apoptosis are impaired, whereas necroptosis is not (Tortola et al., 2016). The most extensively studied DRs are: FAS, TRAIL-R1, TRAIL-R2, and TNFR1. The prototypic signalling to extrinsic apoptosis is for example through FAS receptor (Kaufmann et al., 2012).

FAS-induced apoptosis. The FAS receptor is expressed in the majority of cell types throughout the body. It is of particular importance in the control of the immune system, where it functions as a guardian against autoimmunity and tumourigenesis. The broad tissue distribution of the FAS receptor is in contrast to the selected pool of cells that express the FAS ligand (FASL/CD95L). These cells are: activated-T cells, NKT cells (natural killer T-cells) and NK cells. FASL can be present either in a membrane-anchored form, or when cleaved and released, it may occur in a soluble trimeric form. Only the membrane-bound FASL can trigger apoptosis. The membrane-bound FASL activates the aggregation of the pre-assembled FAS trimers on the plasma membrane of the target cell. The induced conformation changes, through the FASL/FAS receptor aggregation, then promotes changes at the cytosolic part of the target cell, which include the formation of DISC. DISC, a multi-protein complex, is formed on the basis of interactions of several partners. Casp8 activation (within DISC) can be regulated positively or negatively by cFLIP (depending on concentration of cFLIP isoforms). Once Casp8 is fully enzymatically active, it leaves the DISC and can lyse its specific substrates to initiate the executive caspase cascade (also mentioned above). There are also protein factors acting downstream of the FAS-receptor signalling, one of the essential factors being XIAP (X-linked IAP). XIAP can bind to and inactivate certain effector caspases and to a lesser extent also Casp9.

Extrinsic apoptosis by dependence receptors: The signalling of dependence receptors relies on the presence or absence of their ligands as well; however, apoptosis by dependence receptors is initiated in the absence of the ligand. The signal is further transduced towards the assembly of the activating complex containing Casp9, and subsequently (direct or MOMP-dependent) to Casp3/6/7 activation and the execution cascade leading to cell death (Galluzzi et al., 2012).

BCL-2 PROTEIN FAMILY

This protein family was named after the first discovered member, the Bcl-2 protein. Its gene was found to exhibit altered expression as a consequence of chromosomal translocation in lymphocytic leukemia (Tsujimoto et al., 1985). BCL-2 family is a heterogeneous group of proteins with large implications regarding cell death and survival pathways. Members of this family contain one to four regions termed BCL-2 homology (BH) domains, designated as BH1 – BH4. Phylogenetic analyses based on the BH motif sequences indicate existence of a monophyletic group (possibly consisting of three clades: Bcl-2-like, Bax-like and Bid-like) sharing 3D structural folds, and a group of phylogenetically unrelated proteins exhibiting similarity in the BH3 motif only (Aouacheria et al., 2013). However, traditionally, BCL-2 members are categorized into three groups taking into consideration their function in the apoptotic pathway: the multidomain pro-apoptotic class (e.g. Bax, Bak and Bok), the multidomain anti-apoptotic class (e.g. Bcl-2, Bcl-XL, Bcl-W, Mcl-1, and A1/Bfl-1), and the BH3-only class (e.g. Noxa, Puma, Bid, Bim, Bmk and others). The role of particular members in the context of apoptosis is executed through a complex network of interactions. Essentially, BCL-2 proteins interact with each other or with other cellular components, which significantly influence the balance between death and survival mechanisms, and alter the function of mitochondria and ER. As mentioned in the previous sections, the well-known role of BCL-2 members is to engage the mitochondria to release factors participating in caspase activation (and thus apoptosis execution), via the employment of pro-apoptotic Bax and Bak to develop MOMP. The pro-apoptotic activity of Bax and Bak is counteracted by anti-apoptotic BCL-2 members. In turn, these anti-apoptotic BCL-2 proteins can be inhibited by the BH3-only class members, therefore BH3-only proteins can indirectly activate Bax and Bak. Moreover, some BH3-only proteins can bind to Bax and Bak, and thereby directly activate them (due to an induction of conformation changes). Essential for the function of BCL-2 proteins are post-translational modifications, which may be crucial for their 3D conformation and binding-associated activity, or target them for degradation via their ubiquitination, for example. Expression of BCL-2 proteins is very often altered in many cancer types. Generally, the genes encoding anti-apoptotic proteins are primarily upregulated and vice versa, the pro-apoptotic downregulated. However, these changes are often cancer-specific and more complex. For example, the expression of Bax, Bid, Puma and Noxa is influenced by the transcriptional factor p53, which can undergo loss of function in some cancer types (Yip and Reed, 2008). Since the discovery of Bcl-2, many members were identified, and available data on related proteins rapidly increased. BCL2DB (http://bcl2db.ibcp.fr) is a database, available since July 2013, designed to integrate data on

BCL-2 family members from different databases (Ensembl, Ensembl Genomes, European Nucleotide Archive and Protein Data Bank databases).

MULTIDOMAIN (BH1 - BH4) ANTI-APOPTOTIC (PRO-SURVIVAL) MEMBERS

Concerning apoptosis, the multidomain anti-apoptotic proteins (**Bcl-2**, **Bcl-XL**, **Mcl-1**, **Bcl-W** and **A1**) function mainly as guardians of mitochondrial membrane integrity. They prevent permeabilization of the mitochondrial outer membrane, thereby blocking apoptosis, either through direct interaction with Bax and Bak. Or alternatively, they bind to some BH3-only proteins (e.g. Bid or Bim), which also act as inducers of apoptotic signalling upstream of mitochondria, when not sequestered (Yip and Reed, 2008).

Targeting and neutralizing the anti-apoptotic proteins is one of the concepts for developing new therapeutics (together with developing direct activators of pro-apoptotic proteins). BH3 mimetics are small molecules mimicking BH3-only proteins and thereby are able to specifically bind to particular anti-apoptotic proteins and also to displace the BH3-only proteins (e.g. ABT199 and Bcl-2). This eventually leads to the induction of apoptosis (Billard, 2013). An alternative novel approach might be targeting the BH4 domain of Bcl-2, since it can turn Bcl-2 from anti-apoptotic to pro-apoptotic effector molecule (Cheng et al., 1997). Such a change together with the fact that Bcl-2 is commonly overexpressed in some cancers may be very attractive for therapy. A small molecule has been identified, BDA-366, which selectively binds the BH4 domain of Bcl-2. This induces a conformation change in Bcl-2 and a subsequent apoptosis induction *in vitro* and leads to growth inhibition of cancer in *in vivo* models (e.g. for human multiple myeloma xenografts in mice) (Deng et al., 2016).

MULTIDOMAIN (BH1 - BH4) PRO-APOPTOTIC MEMBERS

Bax and **Bak** are well-known pro-apoptotic BCL-2 proteins. Bak is inherently mitochondrial, whereas Bax is largely cytosolic. When activated they rearrange into pore-forming oligomers at the outer mitochondrial membrane. The activation stimuli may include a subset of activated BCL-2 proteins (Bid, Bim, and Puma) and the p53 protein, although the contribution of particular components can be cell type-specific and influenced by other factors (Zhang et al., 2016). The contribution of Bax and Bak to apoptosis induction can be surveyed with double knockout (DKO) Bax/Bak MEF (mouse embryonic fibroblast) cells, which proved to be effectively resistant to various stimuli of intrinsic apoptosis (Wang and Youle, 2012; Wei et al., 2001). Since mice deficient in Bax/Bak are viable (although with lower birth survival and display developmental defects), it is obvious that the apoptosis

pathway is very flexible and there are alternative mechanisms substituting the physiological function of Bax/Bak (Roset et al., 2007). In addition to the function of Bax/Bak regarding mitochondria, they may mediate apoptosis via localization in ER (Zong et al., 2003).

Bok is a pro-apoptotic protein located, similarly to Bax and Bak, in the mitochondrial membrane. Its function is not fully understood; however, a recent study indicates that under certain conditions when overexpressed Bok can facilitate cytochrome c release even in the absence of Bax and Bak (Einsele-Scholz et al., 2016). Just as Bax and Bak, Bok is probably present and active at the outer membrane of ER and Golgi, which implies a role of Bok in ER stress-induced apoptosis (Carpio et al., 2015; Llambi et al., 2016). Its overexpression leads to apoptosis accompanied by Bax/Bak oligomerization in several cell systems, and its downregulation causes lower sensitivity to cell death stimuli. In murine models Bok was shown to be widely expressed in reproductive tissues, colon, stomach, and also brain (Hsu et al., 1997; Ke et al., 2012). Interestingly, it is deleted in some cancer types (e.g. ovarian and breast) (Beroukhim et al., 2010), suggesting a potential role of Bok as a tumour suppressor.

BH3-ONLY MEMBERS

The main function of BH3-only proteins was mentioned above. To summarize, these members can uniformly antagonize the function of anti-apoptotic members (via binding through BH3). And some of them, namely Bid, Bim, Puma and possibly Noxa, can also directly activate Bax and Bak to stimulate their pro-apoptotic function (Hardwick and Soane, 2013; Chen et al., 2015). Their function is tightly regulated at transcriptional and post-translational level (Happo et al., 2012).

Puma and **Noxa** are known to be transcriptionally regulated through the p53 protein. Both are required for DNA damage-induced apoptosis. Interestingly, different DNA lesions may modulate target gene preferences of p53. The transcription of Puma is also upregulated in response to cytokine deprivation (Happo et al., 2012). Noxa is a negative regulator of Mcl-1, and thus might be an important determinant of cancer cells' sensitivity to some BH3 mimetics (Nakajima et al., 2014). Besides the involvement of Puma and Noxa in DNA-damage response, they contribute to ER stress pathway as well.

The **Bid** protein in its full-length form is inactive and expressed in most tissues. It is proteolytically cleaved by Casp8 into the active tBid, which contributes to the amplification of apoptosis signalling through mitochondria (Happo et al., 2012).

Bim cooperates with Puma to mediate apoptosis triggered by cytokine deprivation, exposure to DNA damaging agents, ER stressors and after a deregulated calcium flux in diverse cell types (Happo et al., 2012).

Apoptosis together with other regulated cell deaths are highly complex pathways, partly due to many interconnection points. In this thesis, I highlighted only small portion regarding the cancer- and apoptosis-related topics.

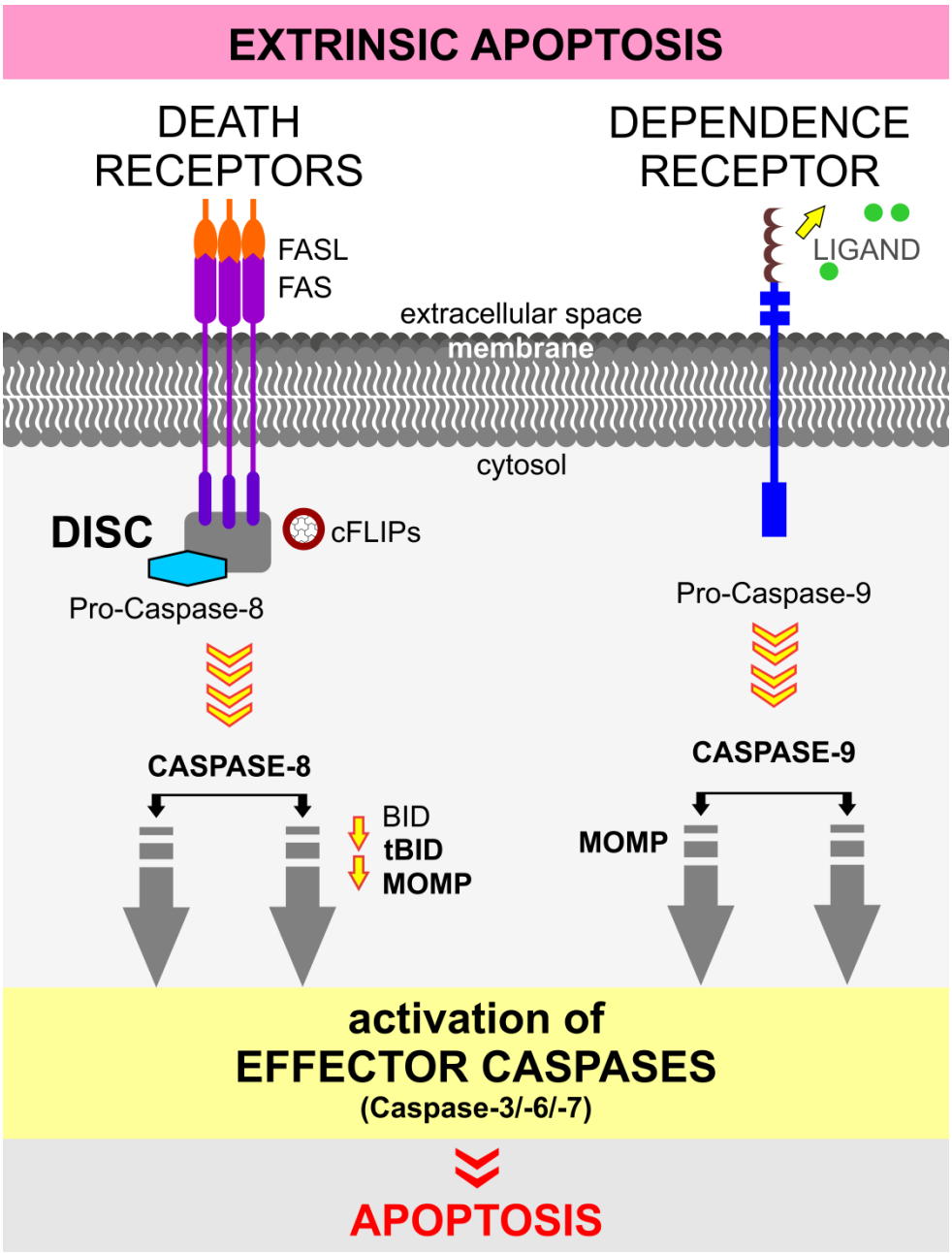

Figure 1. Simplified scheme of extrinsic apoptosis

The extrinsic apoptosis is triggered via membrane receptors; the signalling cascade is initiated by the action of their binding partners. The well-known participants of extrinsic apoptosis are: multiprotein complex DICS (death-inducing signaling complex), initiator caspase 8 or 9 and many others. The effector caspases (and so the execution of apoptosis) can be activated directly by the initiator caspases or throughout the involvement of mitochondria via development of MOMP (mitochondrial outer membrane permeabilization). BID – BH3-only protein; tBID (truncated BID); cFLIP – cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; FAS cell surface death receptor; FASL – FAS ligand. For details, please see Introduction (Apoptosis).

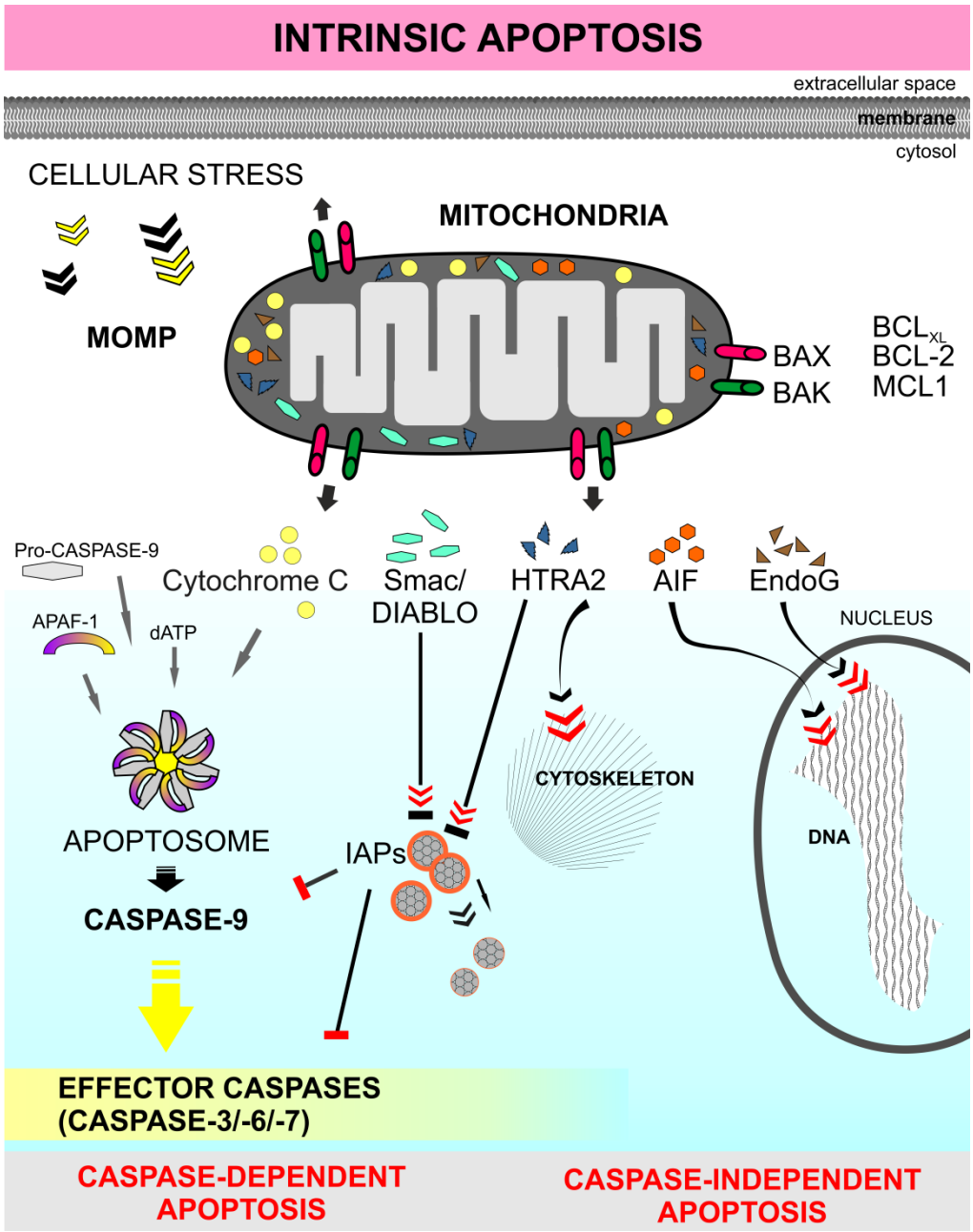

Figure 2. Simplified scheme of intrinsic apoptosis

Generally, the intrinsic apoptosis can be initiated due to different cellular stress. The target organelle, which is involved, is the mitochondrion. Mitochondria are also the intersection point between the extrinsic and intrinsic pathway. There are multiple components being activated or inactivated; for details, please see the Introduction (Apoptosis). BCL-2 family members: BAX, BAK, BCL_{XL}, BCL-2, MCL-1; AIF (apoptosis inducing factor); EndoG (endonuclease G); APAF-1 (apoptotic peptidase [protease] activating factor 1); HTRA2 (high temperature requirement protein A2); IAPs (inhibitors of apoptosis); MOMP (mitochondrial outer membrane permeabilization); Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI). For details, please see Introduction (Apoptosis).

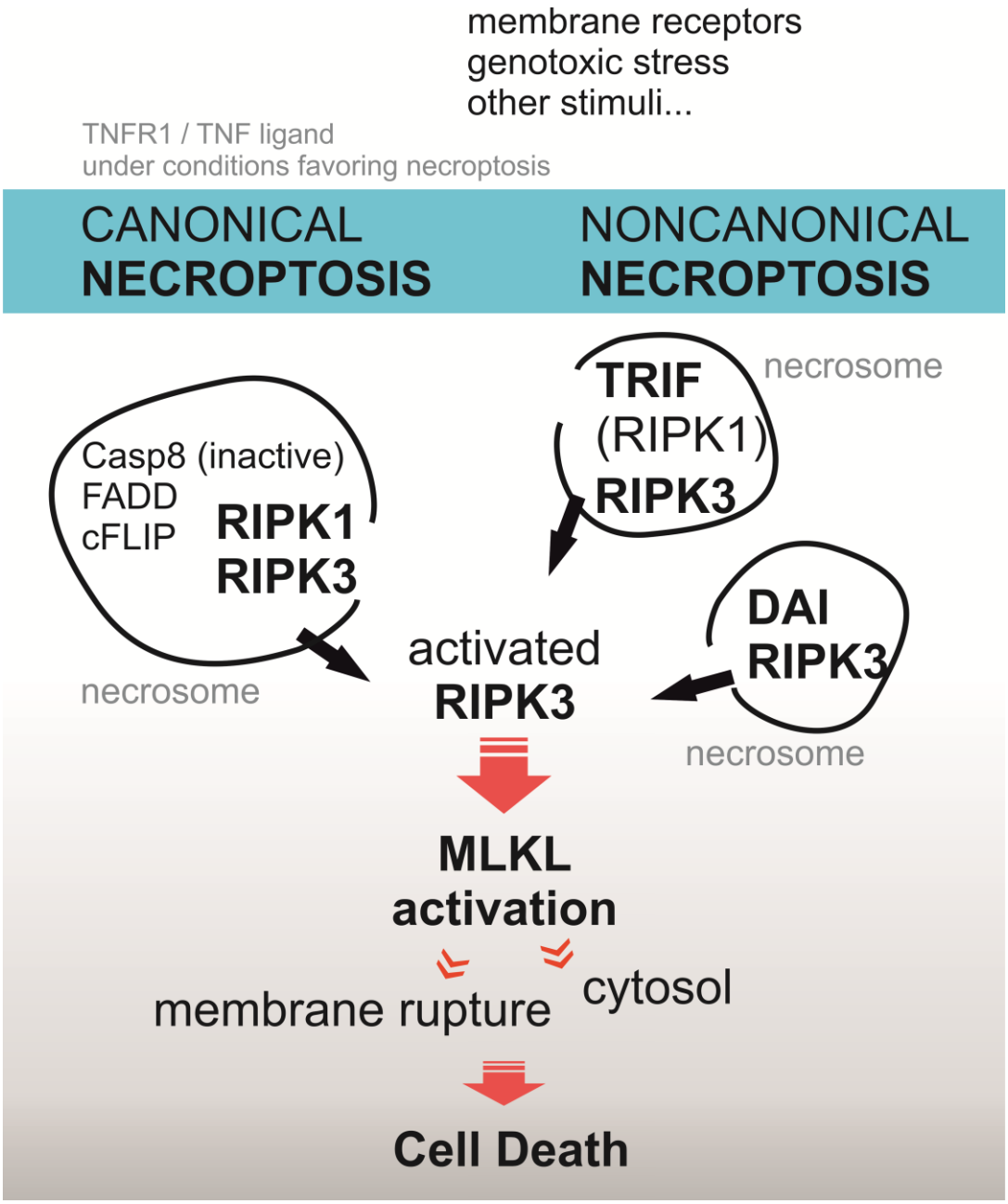

Figure 3. Simplified scheme of necroptosis

Necroptosis is form of cell death (more specifically, of regulated necrosis), which is tightly controlled by molecular factors and rather suppressed during normal development and cell homeostasis. As in passive necrosis, it results in a loss of membrane integrity and a subsequent inflammation reaction. Necroptosis is driven by the action of a protein complex called necrosome, in which an activated Ripk3 (receptor-interacting protein kinase) phosphorylates MLKL (Mixed lineage kinase domain-like), which then affects the cell membrane (possibly by direct binding to phospatidylinositol phosphates and/or indirectly, through the impact on sodium or calcium channels). Within the necrosome, binding to the partners via the RIP homotypic interaction motif (RHIM) is crucial for Ripk3 activation. So far there are only three RHIM-containing proteins reported: Ripk1 (responsible for activation of the canonical necrosome); DAI (DNA-dependent activator of interferon regulatory factors) and TRIF Toll/IL-1 receptor domain-containing adaptor inducing IFN-β), in this case responsible for noncanonical necrosomes.

AIMS OF Ph.D. THESIS

The objective of my PhD studies was to identify cyanobacterial metabolite(s), which induces apoptosis in human cancer cells *in vitro*. Here, I introduce the partial aims, which were needed to meet the main goal. These comprise the research in two fields: the screening studies of complex cyanobacterial extracts and the experiments with the pure compound.

Goals of the screening studies

- Find the optimal methodology for screening aiming at apoptosis inducers from complex cyanobacterial extracts
- Select cyanobacterial extracts for further work and perform activity-guided fractionation in order to find compounds responsible for the cytotoxic effect

Goals of apoptosis-inducing compound characterization

- Confirm the development of apoptosis in human cancer cells *in vitro* in response to the compound treatment
- Show the potency of the compound against several cancer cell lines
- Explore its mechanism of action

PUBLISHED RESULTS

SUMMARY TO PUBLISHED RESULTS

Cyanobacteria are a well-established source of potential pharmaceutics (Salvador-Reyes and Luesch, 2015). Their extracts contain a large pool of unknown secondary metabolites, some of which may, however, also possess attributes of risk to human health (Bláha et al., 2009). My colleagues and I tested over 100 samples of crude cyanobacterial extracts against standard cell line models to assess their cytotoxicity in general context (Paper III). We used cell lines of different origin to cover different cell types and both human and murine models. Obviously, cell lines cannot fully mimic the complexity of an organism or its organs; but there are studies showing good correlation between *in vivo* mouse models and mammalian cell lines *in vitro*, when crude cyanobacterial extracts were tested (Teneva et al., 2003; Teneva et al., 2013). Paper III brings evidences of a wide distribution of cytotoxic substances produced by cyanobacteria, which is in agreement with former studies (Cox et al., 2003; Faasen et al., 2012; Hrouzek et al., 2011; Jokela et al., 2012). It also confirms the prevailing general cytotoxic effect of crude extracts, as only a few extract samples demonstrated cell line specificity. Many researchers intend to develop bioassays that deal with cyanobacterial samples containing unknown constituents. Our results then contribute in showing how it is possible to monitor the cytotoxicity of cyanobacterial products with *in vitro* cell lines of different origin due to their general toxic effect. My contribution to the published part of the results, the activity-guided fractionation of selected extracts in HeLa cells, shows that the overall cytotoxic effect of an extract can be attributed to multiple constituents rather than to a single toxin; however, the latter is possible as well. Furthermore, toxic constituents within one sample can exhibit additive or other effects, which significantly alter the resulting impact of the whole extract.

In the field of drug discovery, using natural sources is one of well-established strategies. This is particularly thanks to their richness in production of chemically diverse compounds. Such a complexity of natural samples unfortunately complicates appropriate adjustment of screen methods and the interpretation of the data. We used cyanobacterial crude extracts as the starting material and as the valid bioactivity of an extract we set the induction of apoptosis in cancer cells *in vitro* (Paper I). Apoptosis is the main regulatory mechanism enabling the destruction of damaged or unwanted cells without causing severe or even any inflammatory response. Cancer cells commonly deregulate cell signaling pathways and escape cell death (including apoptosis); therefore compounds successfully inducing apoptosis are recognized as promising strategy in targeted cancer therapy (Baig et al., 2016). The choice of the pancreatic cell line PaTu 8902 in our screening was motivated by the difficulties with the treatment of pancreatic tumor. Additionally, our collaborator has established a mouse xenograft model, which might be beneficial for *in vivo* tests in the future. As the main result of Paper I I show that our combined approach of Caspase-3/7

homogenous luminescence assay and time-lapse microscopy (time-lapse microscopy was used in parallel to the caspase assay to monitor the cell counts in order to adjust the luminescence readouts) brings a larger number of effective hits for further work, some of which would otherwise be missed. Activation of executioner caspases 3 and 7 (Casp3/7) is one of the hallmarks of apoptosis. For the detection of activated caspases we employed the luminescence homogenous assay, since it is a selective and sensitive method, very suitable for screening (Hassig et al., 2014; Kepp et al., 2011). I demonstrate that the correction of caspase activity per cell is necessary in order to obtain more accurate results. In addition to caspases, we followed the induced changes in cellular morphology and measured cell viability with a conventional MTT assay. Cell viability is an important parameter often used in cell death research, even if the methods for viability assessment suffer from considerable drawbacks as described for example in (Galluzzi et al., 2009) and moreover they do not reflect cell death modality. Indeed, we obtained enhanced Casp3/7 activity in samples displaying low metabolic activity as well as in samples with viability comparable to or even higher than that of control cells. Our data thus clearly agreed with the notion that MTT assay could be misleading and that it is not a suitable method for finding apoptosis-inducing hits neither from pure compounds nor from complex natural samples. Apoptotic cells typically feature blebbing (Kerr et al., 1972). While this morphological alteration is one of the hallmarks of apoptosis, it is not unique to apoptosis and can accompany other types of cell death as well (Barros et al. 2003, Galluzzi et al. 2015). In support of this statement, we detected enhanced Casp3/7 activity in samples regardless of morphology.

Based on our overall experience from both screens and in comparison to (Hassig et al., 2014) we have learned that cellular morphology (despite its obvious limitations) can successfully serve as the first selection criterion and corrected caspase activity as the second criterion in a successful screening for apoptosis inducers from natural extracts.

Arguably the most valuable findings I have made during my PhD are based on a novel cyanobacterial compound identified by Pavel Hrouzek and Alexandra Kapuscik. This compound, which was given the name nocuolin A (NoA), turned out to be the lead compound for my work and one of the significant issues in our research group. NoA was found to have a unique structure containing a direct N-N-O linkage in the six-membered ring (1,2,3-oxadiazine), which is the first time such a compound has been described and isolated from nature. Moreover, its biosynthesis is secured by an unusual biosynthetase coded by the noc gene cluster. Even though the chemical and biosynthetic characteristics of NoA are both very interesting, these are only indirectly related to the topic of my thesis, as I focused mainly on exploring its pro-apoptotic properties.

The NoA metabolite was originally identified in a crude extract of Nostoc sp. CCAP 1453/38, which provoked the typical apoptotic morphology, and on this basis was chosen for further work. In Paper II I proved that NoA induces caspase-dependent apoptosis in HeLa cells. I detected typical apoptosis markers, namely: blebbing, an increase of sub-G1

population (cells with reduced content of DNA), activated effector caspases (using the homogenous Casp3/7 luminescence assay and Western blot) and obvious processing of a downstream substrate of caspases, PARP-1 (poly[ADP-ribose] polymerase 1). In fact, detection of the caspases either with Western blot or through the measurement of DEVD substrate cleavage showed very poor readouts (the increased Casp3/7 luminescence activity of treated cells was marked only when corrected per cell). Inhibition of caspases in NoA-treated cells, with conventional pan-caspase inhibitors (Q-VD-OPh and Z-VAD-FMK), reduced caspase activity showed as decrease of luminescence signal and as absence of PARP-1 cleavage fragment, both of which were then originally result of NoA action within a cell. The example of NoA suggests that assuming apoptosis primarily as a pathway with robust activation of caspases can be deceptive. The possible explanation can involve molecules which affect caspase activity, e.g. the family of internal caspase inhibitors – IAPs (inhibitors of apoptosis) (Yang and Li, 2000) and molecules which can substitute caspase function to some extent – such as calpains and cathepsins (McStay and Green, 2014; Momeni, 2011). Since the cell population is to some degree heterogeneous (in the sense of the ratios of molecules mentioned above; together with the fact that the cells are desynchronized in cell cycle) and regulated cell death is a dynamic process (Galluzzi et al., 2015), then the degree of response (e.g. measured as caspase activity) to a cell death stimulus can also be heterogeneous throughout the cell population as shown in (Morgan and Thorburn, 2001; Vorobjev and Barteneva, 2015), when not measured on a single cell level and continuously in time.

Further, I demonstrated NoA potency *in vitro* against nine human cancer cell lines with inhibitory concentration (IC_{50}) values ranging between 0.7 and 4.5 μM. Interestingly, the cell lines bearing a mutation in the tumor suppressor gene p53 were more sensitive to NoA-induced cell death. This was clearly apparent for two glioma cancer lines, U251 (mutated p53) and U87 (wild type p53), which were the most and the least sensitive ones, respectively, from the list. The actual role of p53 is complex. However, it is known that p53 is frequently mutated in cancers and that these mutations can contribute to their drug resistance (Bieging et al., 2014). NoA may thus offer a new scaffold for the development of drugs that have the potential to target tumor cells independently of their p53 status.

The experiments aiming to describe the precise activity of NoA were limited to a large extent by the small amount of the compound available. I confirmed the pure compound as an apoptosis inducer, showing the common apoptosis hallmarks. These are the first findings concerning NoA biological activity published so far. Paper II then establishes NoA the first representative of a novel class of bioactive secondary metabolites, based on its unique structure and the activity.

I.

Screening of cyanobacterial extracts for apoptotic inducers: a combined approach of caspase-3/7 homogeneous assay and time-lapse microscopy.

Voráčová, K., Paichlová, J., Vicková, K., Hrouzek, P.

Journal of Applied Phycology (doi:10.1007/s10811-017-1122-6)

ABSTRACT

Cyanobacteria are well known to produce valuable secondary metabolites, including compounds with anticancer activity. An advantageous feature of candidate anticancer compounds is their ability to induce apoptosis. One possible approach to screen for apoptosis inducers is via the detection of activities of caspases 3 and 7 (Casp3/7), the key apoptotic enzymes. Pilot screening of natural samples may be intricate due to sample complexity. We tested cyanobacterial extracts for their ability to enhance Casp3/7 activity, inhibit proliferation, and cell metabolism in human pancreatic tumor cells PaTu 8902. The majority of extracts inhibited cell division, but this was only partly reflected by a concurrent MTT viability measurement. The time elapsed by the end of the measurement affects the cell number differently in treated and control cells. The resulting cell counts greatly influence the evaluation of the Casp3/7 assay, since we obtained substantially different results when evaluating primary luminescence data (3 hits) as opposed to when the actual cell number was taken into account (23 hits). Based on the fact that crude extracts manifest miscellaneous effect including cytostatic activity, it is necessary to couple Casp3/7 assay with cell count measurement. The crude extract of *Nostoc* sp. CCAP1453/38 and its active fraction (proapoptotic metabolite nocuolin A) were used to demonstrate the validity of our approach since its effects was detectable only when normalized to cell number. We demonstrate that the Casp3/7 luminescence assay is useful for apoptotic inducer screening from cyanobacterial extracts and present amendments which help deal with the drawbacks of the method.

II.

The cyanobacterial metabolite nocuolin A is a natural oxadiazine that triggers apoptosis in human cancer cells.

Voráčová, K., Hájek, J., Mareš, J., Urajová, P., Kuzma, M., Cheel, J., Villunger, A., Kapuscik, A., Bally, M., Novák, P., Kabeláč, M., Krumschnabel, G., Lukeš, M., Voloshko, L., Kopecký, J., Hrouzek, P.

PLOS ONE 12(3): e0172850. (doi:10.1371/journal.pone.0172850)

ABSTRACT

Oxadiazines are heterocyclic compounds containing N-N-O or N-N-C-O system within a six membered ring. These structures have been up to now exclusively prepared via organic synthesis. Here, we report the discovery of a natural oxadiazine nocuolin A (NoA) that has a unique structure based on 1,2,3-oxadiazine. We have identified this compound in three independent cyanobacterial strains of genera *Nostoc*, *Nodularia*, and *Anabaena* and recognized the putative gene clusters for NoA biosynthesis in their genomes. Its structure was characterized using a combination of NMR, HRMS and FTIR methods. The compound was first isolated as a positive hit during screening for apoptotic inducers in crude cyanobacterial extracts. We demonstrated that NoA-induced cell death has attributes of caspase-dependent apoptosis. Moreover, NoA exhibits a potent anti-proliferative activity (0.7–4.5 μM) against several human cancer lines, with p53-mutated cell lines being even more sensitive. Since cancers bearing p53 mutations are resistant to several conventional anti-cancer drugs, NoA may offer a new scaffold for the development of drugs that have the potential to target tumor cells independent of their p53 status. As no analogous type of compound was previously described in the nature, NoA establishes a novel class of bioactive secondary metabolites.

III.

Cytotoxicity evaluation of large cyanobacterial strain set using selected human and murine *in vitro* cell models.

Hrouzek, P., Kapuscik, A., Vacek, J., **Voráčová, K.**, Paichlová, J., Kosina, P., Voloshko, L., Ventura, S., Kopecký, J.

Ecotoxicology and Environmental Safety 124:177-185. (doi:10.1016/j.ecoenv.2015.10.020)

ABSTRACT

The production of cytotoxic molecules interfering with mammal cells is extensively reported in cyanobacteria. These compounds may have a use in pharmacological applications; however, their potential toxicity needs to be considered. We performed cytotoxicity tests of crude cyanobacterial extracts in six cell models in order to address the frequency of cyanobacterial cytotoxicity to human cells and the level of specificity to a particular cell line. A set of more than 100 cyanobacterial crude extracts isolated from soil habitats (mainly genera *Nostoc* and *Tolypothrix*) was tested by MTT test for *in vitro* toxicity on hepatic and non-hepatic human cell lines, HepG2 and HeLa, and three cell systems of rodent origin, Yac-1, Sp2 and Balb/c 3T3 fibroblasts. Furthermore, a subset of the extracts was assessed for cytotoxicity against primary cultures of human hepatocytes as a model for evaluating potential hepatotoxicity. Roughly one third of cyanobacterial extracts caused cytotoxic effects (i.e. viability < 75%) on human cell lines. Despite the sensitivity differences, high correlation coefficients among the inhibition values were obtained for particular cell systems. This suggests prevailing general cytotoxic effect of extracts and their constituents. The non-transformed immortalized fibroblasts (Balb/c 3T3) and hepatic cancer line HepG2 exhibited good correlations with primary cultures of human hepatocytes. The presence of cytotoxic fractions in strongly cytotoxic extracts was confirmed by an activity-guided HPLC fractionation, and it was demonstrated that cyanobacterial cytotoxicity is caused by a mixture of components with similar hydrophobic/hydrophilic properties. The data presented here could be used in further research into *in vitro* testing based on human models for the toxicological monitoring of complex cyanobacterial samples.

GENERAL CONCLUSION

In conclusion, the richness and complexity of natural extract samples is their main asset, but at the same time it makes their use in drug discovery so intricate. This is partly reflected in the two screening studies presented in this thesis. As I show in the first study (Paper III), activity-guided fractionation of selected extracts points out the fact that the overall cytotoxicity of a crude extract can result from multiple constituents. This also means that the target compound within an extract can be masked by other active compounds, or the activity of an extract is detected only due to the additive effects of compounds within, making it thus unsuitable for isolation. The next criterion to have in mind is the complexity of regulated cell death pathways. In connection with apoptosis detection, I show that caspase activity normalization per cell significantly improves the number of potential apoptosis-inducing extracts (Paper I). According to caspase-positive hits, I confirm that apoptotic morphology and the cellular viability are rather misleading when employed as a sole parameter for evaluation of the effect of an extract. Activity-guided fractionation and isolation is a necessary step in the identification of an active compound. I applied this approach to numerous selected extracts, but I did not succeed in isolation of any novel metabolite with stable production in the corresponding cyanobacterial strain, which brings us back to the uncertainty of using natural sources for drug discovery.

Finally, at the core of my thesis I evaluated the pro-apoptotic properties of a novel cyanobacterial compound – nocuolin A (NoA). NoA induces caspase-dependent apoptosis and displays IC50 values between 0.7 and 4.5 µM for a panel of nine human cancer cell lines (Paper II). Additionally, I bring the first results on NoA mode of action (albeit still far from a precise description), which can then serve as groundwork for further investigation.

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PUBLISHED ARTICLES

CURRICULUM VITAE

PERSONAL DETAILS

Name: Kateřina Voráčová
Nationality: Czech
Born: 27th March 1984, Czech Republic
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EDUCATION

2011–Present

PhD student of Molecular and Cell Biology and Genetics, Department of Molecular Biology, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic. Institute of Microbiology, Academy of Sciences of the Czech Republic. PhD thesis: *Screening Cyanobacteria for Apoptosis Induction in Human Cancer Cell Lines: Discovery of a Novel Compound —Nocuolin A*; supervisor: Pavel Hrouzek

2007–2011

MSc, Experimental Biology, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic. Master thesis: *Isolation of intact plastids of the alga Chromera velia and the treatment of the alga with rifampicin*. Laboratory of molecular taxonomy, supervisor: Miroslav Oborník

2004–2007

BSc, Biological Sciences, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic. Laboratory of molecular taxonomy. Bachelor thesis: *Evolution of selected enzymes of the shikimate pathway and the haem biosynthetic pathway in Rhodophyta*. Laboratory of molecular taxonomy, supervisor: Miroslav Oborník

EMPLOYMENT

2011–Present

Algatech – Institute of Microbiology, Academy of Sciences of the Czech Republic; previously as the laboratory technician (Roman Sobotka's group) and currently as the PhD. Student (Pavel Hrouzek' s group)

TEACHING

Mentoring a student at the University of South Bohemia:

Kateřina Vicková, Bachelor thesis: Cytotoxic effect of some cyanobacterial crude extracts and metabolites against selected human cancer cell lines, especially focusing on pancreatic cancer cell line PaTu (defended in 2015, Department of Medical Biology).

CONFERENCES

2015 Mitochondria, apoptosis and cancer (MAC 2015), EMBO workshop; Frankfurt (Germany), poster

2014 Trends in Natural Products Research; Olomouc (Czech Republic), oral presentation

2014 Cell Death Signaling in Cancer and the Immune System (Keystone Symposia); Sao Paulo (Brazil), poster

2013 Mechanism of the cell death: The command to die (ICDS); Fuengirola (Spain), poster

2012 20th Euroconference on Apoptosis; Rome (Italy), poster

2011 Conference on Impedance-Based Cellular Assays; Regensburg (Germany)

RESEARCH VISITS

2014 (3 months) – **Dr. Marcel Bally** Laboratory, BC Cancer Agency (Canada)

2013 (2 months) and 2012 (1 month) – **Prof. Andreas Villunger** Laboratory, Biocenter Innsbruck, Medical University (Austria)

PATENT

Patent CZ 305944

Institute of Microbiology, Academy of Sciences of the Czech Republic; Biology Centre Academy of Sciences of the Czech Republic; Saint-Petersburg State University, Russia
Hrouzek, P; Voráčová, K; Kuzma, M; Hájek, J; Urajová, P; Cheel Horna, J; Mareš, J; Lukešová, A; Voloshko, L; Pinevich, A; Kopecký, J; Villunger, A.
Isolated blue-green alga strain Nostoc sp. and isolated blue-green alga strain Nodularia, blue-green alga metabolite, process for preparing the blue-green alga metabolite and use of the blue-green alga metabolite as medicament.
http://hdl.handle.net/11104/0267690

PUBLICATIONS

Voráčová, K., Paichlová, J., Vicková, K., Hrouzek, P. (2017) Screening of cyanobacterial extracts for apoptotic inducers: a combined approach of caspase-3/7 homogeneous assay and time-lapse microscopy. Journal of Applied Phycology (doi:10.1007/s10811-017-1122-6)

Voráčová, K., Hájek, J., Mareš, J., Urajová, P., Kuzma, M., Cheel, J., Villunger, A., Kapuscik, A., Bally, M., Novák, P., Kabeláč, M., Krumschnabel, G., Lukeš, M., Voloshko, L., Kopecký, J., Hrouzek, P. (2017) The cyanobacterial metabolite nocuolin A is a natural oxadiazine that triggers apoptosis in human cancer cells. PLOS ONE 12(3): e0172850. (doi:10.1371/journal.pone.0172850)

Hrouzek, P., Kapuscik, A., Vacek, J., **Voráčová, K.**, Paichlová, J., Kosina, P., Voloshko, L., Ventura, S., Kopecký, J. (2016) Cytotoxicity evaluation of large cyanobacterial strain set using selected human and murine *in vitro* cell models. Ecotoxicology and Environmental Safety 124:177-185. (doi:10.1016/j.ecoenv.2015.10.020)