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Secondary metabolites from terrestrial cyanobacteria

Ph.D. Thesis

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Annotation

This project was mainly focused on isolation, structure elucidation and bioactivity assessment of a novel compound isolated from cyanobacterium *Nostoc* sp. Purified peptide was analyzed using structure elucidation techniques (nuclear magnetic resonance, high resolution mass spectrometry) and underwent several cell-based assays to reveal and characterize its anti-inflammatory potential. Within the project, development of a high throughput screening for anti-inflammatory compounds produced by cyanobacteria, using AlphaLISA™ cell-based technology was also presented and discussed. Several aspects of cyanobacterial cytotoxicity were researched as well, including large scale screening of cyanobacterial extracts and purified fractions.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, ...

Aleksandra Kapuścik

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Přírodovědecká fakulta
Faculty of Science

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Finally, I would like to thank all people from the Department of Phototrophic Microorganisms, CAS in Třeboň, for always great time together and very good atmosphere at work.

List of papers and author's contribution

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

I. Pflüger, M., **Kapuścik, A.**, Lucas, R., Koppensteiner, A., Katzlinger, M., Jokela, J., Eger, A., Jacobi, N., Wiesner, C., Hofmann, E., Onder, K., Kopecký, J., Schütt, W., Hundsberger, H. (2013). A Combined Impedance and AlphaLISA^{TM} Based Approach to identify anti-inflammatory and barrier-protective compounds in human endothelium. Journal of Biomolecular Screening 18, 67–74 (IF = 2.207)

Aleksandra Kapuścik was responsible for preparation of cyanobacterial sample set (extracts and purified fractions), participated in method development for anti-inflammatory screening of cyanobacterial samples as well as writing a part of the manuscript concerning isolation of bioactive compounds from cyanobacteria.

II. **Kapuścik, A.**, Hrouzek, P., Kuzma, M., Bártová, S., Novák, P., Jokela, J., Pflüger, M., Eger, A., Hundsberger, H., Kopecký, J. (2013). Novel Aeruginosin-865 from *Nostoc* sp. as a Potent Anti-inflammatory Agent. ChemBioChem 14, 2329 – 2337 (IF = 3.740)

Aleksandra Kapuścik was responsible for isolation and purification of aeruginosin-865, participated in anti-inflammatory testing (AlphaLISA^{TM} assay), interpreted the data and wrote the manuscript.

III. Hrouzek, P., **Kapuścik, A.**, Voráčová, K., Vacek, J., Pajchlová, J., Kosina, P., Voloshko, L., Ventura, S., Kopecký, J. (2014). Evaluation of Cyanobacterial Cytotoxicity by Using *in Vitro* models. A Comparative Study (2014). Manuscript

Aleksandra Kapuścik shared the equal contribution with Pavel Hrouzek – responsibility for preparation and MTT-cytotoxicity testing of about 100 cyanobacterial strains, interpretation of the data, participation in preparation and revision of the manuscript.

Contents

I Introduction

Cyanobactria are the most successful group of microorganisms on earth. Oxygen-producing cyanobacteria billions years ago contributed significantly in creating an early atmosphere on our planet, using water as the source for electrons in photosynthesis, which enabled evolution of aerobic metabolism and eukariotic photosynthesis (Steward and Falconer, 2008; Knoll *at al*., 2008). Cyanobacteria have remained important global producers of carbon and nitrogen, widely spread in marine, freshwater and terrestial ecosystems, in different climatic regions and habitats from deserts, hot springs and salt lakes, from temperate climatic zones to polar circles (Ward and Castenholz, 2000).

Cyanobacteria occur as unicellular or colonial species, creating filaments or biofilms, living independently or in symbiosis (Rai *et al*., 2002). Based on morphology, cyanobacteria are traditionally classified into five groups: *Chlorococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales* and *Stigonematales*. Ongoing phylognetic studies constantly contribute new aspects to the developement of this classification (Knoll *at al*., 2008).

Research into these organisms was mainly motivated by seasonal cyanobacterial blooms, which can be harmful for other organisms including humans, as well as by the desire to isolate and characterize cyanobacterial metabolites responsible for their toxicity. On one side the rapid growth of cyanobacteria in water reservoirs constitutes an ecological problem (Palus *et al*., 2007; Wiegand and Pflugmacher., 2005). Cyanobacterial toxins are classified into several groups – neurotoxins, hepatotoxins, cytotoxins and dermatoxins (Sivonen and Börner, 2008). One of the first hepatotoxic cyanobacterial compounds whose mechanism of action has been fully described, are cyclic peptides: microcystins. Chronic intoxication by low doses of microcystin through, for example, consumption of contaminated water may lead to destruction of liver cells and trigger apoptotic processes (Campos *et al*., 2010; Wiegand and Pflugmacher, 2005) as well as promote tumor growth. (Humpage *et al*., 2000; Nishiwaki-Matsushima *et al*., 1992).

On the other side numerous compounds produced by cyanobacteria have been identified as pharmacologically important products (Tan, 2007; Amador *et al*., 2003). In this context the most prominent cyanobacterial metabolites, dolastatins 10 and 15, must be mentioned. Isolated from *Lyngbya* and *Symplococa* (Luesch *et al*., 2001), the dolastatins interact with tubulin resulting in alternation of microtubule function, inhibition of cell proliferation thus leading to apoptotic cell death (Amador *et al*., 2003; Haldar *et al*., 1998). The most effective dolastatin 10, a linear peptide, has been chosen for phase I clinical trials as a promissing antitumor agent. Another example of potent anticancer agent is anti-microtubule alkaloid curacin A isolated from *Lyngbya majuscula* (Gerwick *et al*., 1994). Nowadays

synthetic analogues of Curacin A undergo preclinical trials (Tan, 2007). Similar activity based on microtubule assembly inhibition has been found in cryptophicin 1. Unfortunately, due to its high toxicity and strong side effects it was withdrawn from clinical studies (Rohr, 2006).

Much of the data presently available suggests that in future cyanobacterial metabolites can play an important role as drug candidates or as drug scaffolds.

I.1 Secondary metabolites

Cyanobacteria are known producers of an impressive number of secondary metabolites with diverse chemical structures. To mention only some of the major chemical groups, there are about 600 peptides or peptidic metabolites (Welker and von Dörhen, 2006a) in various taxa, more than 300 nitrogen containing compounds only in marine cyanobacteria (Tan, 2007) and many others belonging to polyketides, isoprenoids, proteins, aromatic- and nonaromatic compounds (van Vagoner *et al*., 2007). The greatest number of cyanobacterial metabolites has been found in *Oscillatoriales* and *Nostocales*, whereas only few in *Pleurocapsales*, which can be caused by a limited availability of strains and problems with their cultivation (Welker and von Dörhen, 2006a). The reason of such a great diversity are specific biosynthetic routes occurring in cyanobacteria, utilizing polyketide synthases (PKS) or non-ribosomal peptide synthetase (NRPS) as well as the hybrid pathway.

I.1.1 Biosynthesis

Both enzymes involved in biosynthesis of secondary metabolites in cyanobacteria show modular structure organization, where each module is responsible for adding and processing one unit to a nascent chain. Each module contains several enzymatic domains, which catalize a specific chemical transformation of a new unit, so the cyanobacterial enzymatic machinery seems to work as an assembly line, where each newly synthetized unit can be freely put together with another one (van Vagoner *et al*., 2007). Considering also subsequent possible modifications of synthesized molecules, it must be assumed that the cyanobacterial metabolism has an almost infinite potency of various compounds production.

The typical polyketide synthase (PKS) module contains following domains: acyl carrier protein, β-keto acyl synthase, acyl transferase, keto reducrase, dehydratase and enoyl reductase (van Vagoner *et al*., 2007). The reaction starts when a substrate, acetate or more complex carboxyl compound is loaded onto an acyl carrier protein, then it is moved and processed along the enzymatic module, undergoing usually several extension reactions before release from PKS by thioesterase, resulting in a linear or cyclic compound.

The concept of domain composition within the NRPS module is similar and it consists of a thiolation domain or peptidyl carrier protein, playing the same role as the acyl carrier protein in PKS module, condensation domain, adenylation domain, heterocyclization domain and tioesterase which terminates the biosynthetic process (Welker and von Dörhen, 2006a). The final structure of the synthesized molecule depends also on presence of other domains integrated in the NRPS module like oxidases, reductases, methylases and on many other associated enzymes.

Biosynthesis of cyanobacterial peptides has been an absorbing topic for the last two decades and there are still many questions to answer. The interesting fact is that both biosynthetic pathways can be mixed within one organism, which means that PKS and NRPS domains can occur within one module or both module types can coexist. A good example for this is the microcystin synthesis pathway (Christiansen *et al*., 2003). For some types of peptides, e.g. microviridins, also ribosomal origin has been confirmed (van Vagoner *et al*., 2007; Hemscheidt, 2012)

I.1.2 Peptides

The most important group of cyanobacterial secondary metabolites are peptides. Cyanobacreria produce a vast number of structurally different types of peptides classified into several groups. However, because of the huge diversity of structures and some inaccuracy in peptides nomenclature, this task is rather difficult. Moreover, there are still many peptides which cannot be assigned to any major known peptide class (Welker and von Dörhen, 2006a).

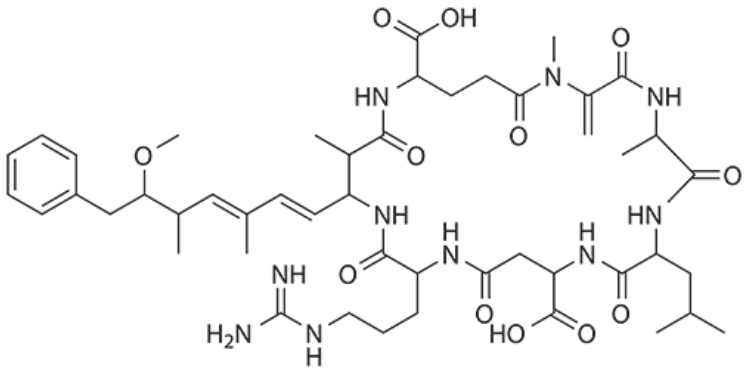
Cyanobacterial peptides consist of many different components belonging to amino acids, fatty acids and carbohydrates. Into one peptide, both proteino- and non-proteinogenic amino acids can be incorporated and in principle, each L-form can be transformed into D-form by a specific epimerase (van Vagoner *et al*., 2007). Very often characteristic amino acid derivatives are reported; for instance tyrosine derivative – hydroxy-phenyl lactic acid (Hpla) and arginine derivative – agmatine (Ersmark *et al*., 2008; reported also in this work), threonine derivative – amino-butenoic acid (Aba) (Kang *et al*., 2012) and many others (Welker and von Dörhen, 2006a). Many other common modifications can occur, like *N*,*O*-methylation, heterocyclization, hydroxylation, dehydratation, halogenation, whereas bromination has been observed mainly in marine habitats (Cabrita *et al*., 2010; Esquenazi *et al*., 2011). Some cyanopeptides include also simple sugars, glucose or xylose, and acyl moiety (Ishida *et al*., 2007; Shin *et al*., 1997), which significantly influences physical and chemical properties of the peptides.

All peptides classified within one family contain some conserved and variable positions in the chemical structure. Such conserved positions are usually occupied by characteristic amino acids or bond formations and usually decide about affiliation of a peptide to the certain family.

Only some peptide groups, significant for this work or generally prominent in cyanopeptides research, will be mentioned in this work.

I.1.2.A Microcystins

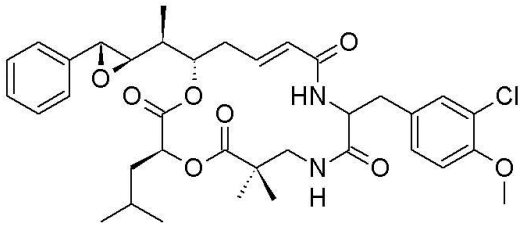
The best investigated group of cyanobacterial metabolites are hepatotoxic cyclic heptapeptides, microcystins, which are characterized by presence of unique C_{20} β-amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid). The biosynthesis of microcystins starts with forming this unit by NRPS/PKS hybride enzyme (Tillett *et al*., 2000). In chemical structure of microcystin also two highly variable positions, 2 and 4, occupied by different amino acids, are present. In order to regulate microcystins nomenclature, it was proposed to name the particular variants using one-letter amino acid code for substitution at those two most variable positions, so eg. microcystin-LR contains leucine (L) at position 2 and arginine (R) at position 4 (Carmichael *et al*., 1988).
The toxic effect of microcystins is caused by inhibition of protein phosphatases 1 and 2a, involved in many crucial cellular processes, e.g. cell division (van Wagoner *et al*., 2007; Campos and Vasconcelos, 2010). It was found that cyclic pentapeptides, nodularins that show similar bioactivity, also incorporate Adda moiety in the formation of their ring system, thus suggesting that Adda unit is crucial for the toxicity (Welker and von Dörhen, 2006a).



Microcystin-LR

I.1.2.B Cryptophycins

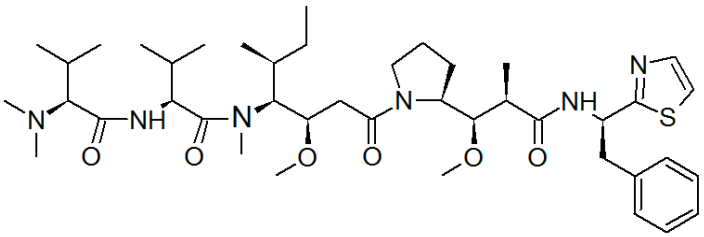
Cryptophycins are non-ribosomally synthesized cyclic depsipeptides. Cryptophicin 1 is a potent antitumor and antifungal agent, showing inhibition of microtubule polymerization in a concentration range of pM (van Wagoner *et al*., 2007; Sivonen and Börner, 2008). Unfortunately strong side effects caused by cryptophicin 1 as well as its analogues led to a stop of clinical trials (Rohr, 2006). Structure-activity relationship analysis revealed that for its bioactivity the intact macrolide ring structure, halogenated and methylated tyrosine moiety and epoxide ring are needed (van Wagoner *et al*., 2007).



Cryptophicin 1

I.1.2.C Dolastatins

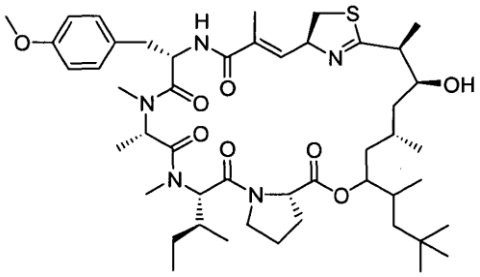
Dolastatins are another promising anticancer microtubule disruptor found in natural marine resources. First dolastatins were isolated from sea hare *Dolabella auricularia*, but recently several analogues have been found in cyanobacteria, giving an evidence of cyanobacterial origin of dolastatins (Luesch *et al*., 2002). Dolastatins show large structural diversity and are defined within several classes (van Wagoner *et al*., 2007). The most effective microtubule inhibitors, dolastatin 10, 15 and symplostatin 1, are linear peptides, characterized by high degree of *N*-methylation, which is essential for the bioactivity, and a C-terminal residue (van Wagoner *et al*., 2007). Nowadays several dolastatin analogues are under developement and undergo clinical tests (Amador *et al*., 2003; Tan, 2007).



Dolastatin 10

I.1.2.D Apratoxin A

Apratoxin A isolated from *Lyngbya majuscula* (Luesch *et al*., 2001) shows a novel anticancer mechanism of action. Structurally this compound is a cyclic depsipeptide synthesized on hybride PKS/NRPS pathway. Apratoxin A arrests cell cycle at the G1 phase and inhibits cell division, but for a long time its exact molecular target remained unknown (Tan, 2007). Recently it has been found that apratoxin A-mediated cell death involves reversible inhibition of the secretory pathway for several cancer associated receptors by perturbation of JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) pathway and interfering with cotranslational translocation (Liu *et al*., 2009), which results in deregulation of many tumorigenic traits like immunosupression or enhanced angiogenesis. Apratoxin A is a very potent cytotoxin, however *in vivo* it exhibits strong side effects. Due to this fact structure-activity relationship (SAR) studies have been taken up in order to develop new analogues and provide the basics for a second generation of apratoxins (Chen *et al*., 2011).



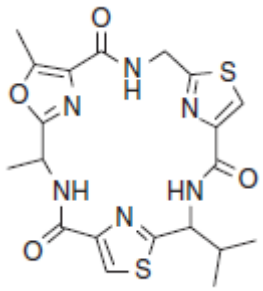
Apratoxin A

I.1.2.E Aeruginosins

An interesting group of cyanobacterial metabolites are aeruginosins, linear peptides, which have been reported as serine proteases inhibitors. Those relatively small molecules contain at the *N*-terminus a derivative of hydroxy-phenyl lactic acid (Hpla), a variable proteinogenic amino acid residue, 2-carboxy-6-hydroxyoctahydroindole (Choi) and arginine derivative side chain at the C-terminus (Ersmark *et al*., 2008). Biosynthesis of aeruginosines starts with activation of phenylpyruvate and its subsequent reduction to Hpla by hybride NRPS/PKS enzyme (Ishida *et al*., 2009). The basic molecule can undergo various modifications, for instance halogenation, sulphation (Ishida *et al*., 1999), glycosilation (Shin *et al*., 1997) or methylation (Welker and von Dörhen, 2006a). Recently aeruginosins have been an attractive research topic due to their inhibitory activity against trypsine-like serine proteases making them important targets in search for new anticoagulants (Matsuda *et al*., 1996).

I.1.2.F Cyclamides

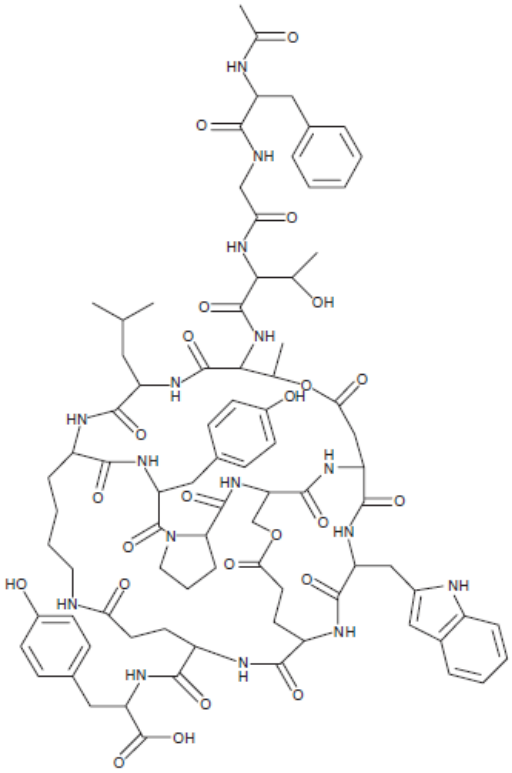
Cyclamides possess an unusual chemical structure. In typical peptides of this class the oxazole or tiazole rings, which are most likely formed by dehydration and reduction of native amino acids forming heterocycle, occur in alternation with unmodified amino acids to arrange a cyclic hexapeptide (Welker and von Dörhen, 2006a; van Vagoner *et al*., 2007). It is suggested that some cyclamide derivatives, patellamides, can be produced ribosomaly (Schmidt *et al*., 2005).



Nostocyclamide

I.1.2.G Microviridins

Other peptides with unique structures and ribosomal origin are multicyclic depsipeptides, microviridins, the largest known cyanobacterial peptides. All amino acids in microviridins have been found in L- configuration, so it is presumed that they are synthesized ribosomally and the multicyclic structure occurs as posttranslational modification (Welker and von Dörhen, 2006a; Hemscheidt, 2012).



Microviridin B

I.1.3 Alkaloids

Cyanobacteria also synthesize a large spectrum of alkaloids, from simple linear and cyclic forms to more complicated hybrid structures. Although it is known that for alkaloids production cyanobactera utilize the same hybride PKS/NRPS enzymatic machinery, in comparison to peptides, knowledge about alkaloids biosynthesis is very limited and it often comes from earlier studies on dinoflagellates or actinomycetes where related compounds were

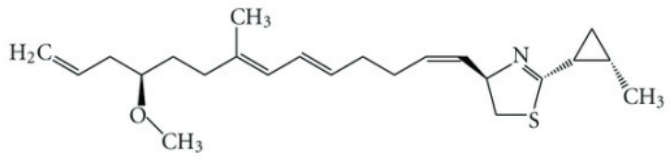
initially found (van Wagoner *et al*., 2007; Wiese *et al*., 2010). Regarding bioactivity and unique chemical structure, several cyanobacterial alkaloids should be mentioned here.

I.1.3.A BMAA

The very simple linear alkaloid, β-(methylamino) alanine (BMAA), produced by many cyanobacterial genera, was found to be a strong neurotoxin and was associated to the significantly high occurrence of neurodegenerative diseases like Parkinson's dementia complex and amyotrophic lateral sclerosis among Guam inhabitants (Cox *et al*., 2003). Permanent exposition to low doses of BMAA, due to diet, results incorporation of the toxin into proteins and then its slow release by protein's degradation (Murch *et al*., 2004). On the other hand, some neurotoxins interacting with eucaryotic voltage-gated sodium channels, like kalkitoxin isolated from *Lyngbya* (Tan, 2007), are postulated to be exquisitely potent in nanomolar range as analgesics and neuroprotectants (Blumenthal and Seibertm, 2003).

I.1.3.B Curacins

Curacins are small alkaloids isolated from *Lyngbya*, containing tiazoline and cyclopropyl ring. Curacins are strong cytotoxins, binding to colchicines-binding site on tubulin and inhibiting microtubule polymerization (Blokhin *et al*., 1995). Because of these unique features, in order to find the most potent anticancer analogue, curacin A was submitted to numerous SAR studies (Verdiert-Pinard *et al*., 1998; Blokhin *et al*., 1995), revealing that for interaction with colchicines-binding site a presence of thiazoline ring is necessary.

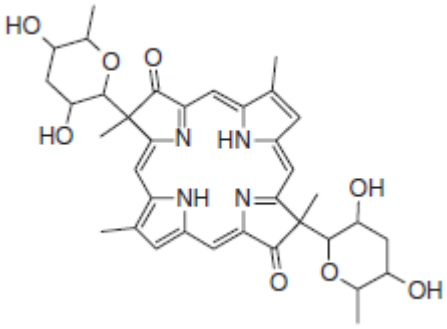


Curacin A

I.1.3.C Tolyporphins

Tolyporphins are heme containing glycoporphirins, isolated from *Tolypotrix*, are glycosylated derivatives from initially photosynthetic pigments (van Vagoner *et al*., 2007). Those compounds are tumor-photosensitizing agents, much more effective than commercially used derivatives of hematoporphirins (Morliere *et al*., 1998). It was also proved that

tolyporphins are able to reverse the multidrug resistance phenotype of various tumor cells and enhance the accumulation of vinblastine in some types of cancer cells (Smith *et al*., 1994).



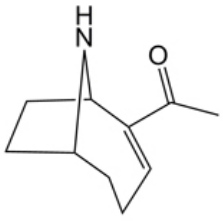
Tolyporphin D

I.1.3.D Saxitoxins

Saxitoxins (STX) and its analogues are unique neurotoxic guanidine-containing alkaloids, causing the paralytic shellfish poisoning (PSP) syndrome, which afflicts human health and impacts coastal shellfish economies worldwide (Wiese *et al*., 2010). Saxitoxins block sodium channels in nerve cells and due to their ability to accumulate in marine invertebrates, can cause human poisoning (Lopes *et al*., 2014). It is suggested that saxitoxins can play a huge role in pharmacy as anesthesia-inducing agents and it has been shown that as liposome formulation, STX can be an effective therapy in severe joint pain and localized pain (Chorny and Levy, 2009).

I.1.3.E Anatoxin-A

Anatoxin-A is a small tropane-related neurotoxic alkaloid acting at the neuromuscular junction by persisting stimulation and blocking further electrical transmission (van Apeldoorn *et al*., 2007), an agonist of nicotinic acetylocholine receptor ion channels, causing a rapid death by respiratory arrest (Sivonen and Börner, 2008). So far anatoxin-A has not been subjected to any drug development program or SAR studies.



Anatoxin-A

I.2 Selected aspects of cyanobacterial bioactivity

I.2.1 Cytotoxicity

Cyanobacterial cytotoxicity can be considered in two aspects. As it has been mentioned in the previous section, many cyanobacterial toxins can serve as potential drug candidates or drug scaffolds. This aspect of cyanobacterial toxicity has been subject of extensive studies for many years (Tan, 2007). It has been estimated that about 30% of cyanobacterial strains produce various cytotoxic metabolites (Hrouzek *et al*., 2011; Costa *et al*., 2013). About two third of them belong to peptides and within this group, cyclic depsipeptides represent the majority (Tomek, 2010). The potential number of cyanotoxins is, however, difficult to estimate. Given that many valuable cytotoxic compounds were found in still unexplored marine sources, mainly the genera *Lyngbya* (Gerwick *et al*., 1994; Luesch *et al*., 2002; Yoo and Gerwick, 1995) and *Symploca* (Horgen *et al*., 2002; Luesch *et al*., 2001) and considering the almost unlimited possibilities of the cyanobacterial NRPS/PKS apparatus (Welker *et al*., 2006b), it makes this group of organisms an almost infinite source of pharmaceutically potent compounds.

Yet, cyanobacterial toxic metabolites cause permanent problems, since cyanobacterial water blooms are a permanent problem in water management, agriculture and recreation areas in most of the countries, thus also influencing human health (van Apeldoorn *et al*., 2007; Wiegand and Pfugmacher, 2005; Zanchett and Oliveire-Filcho, 2013; Bellém *et al*., 2013). Considering that only a small amount of cyanobacterial toxins are known so far (Welker and von Dörhen, 2006a) and it must be assumed that there is almost unlimited potential of producing new cytotoxic compounds, it is very difficult to properly assess the risks resulting from the presence of cyanotoxins in water. It is known that usually one cyanobacterial strain produces more than one structurally different secondary metabolite and moreover, natural communities consist with a rich collection of strains. This leads to the conclusion that natural habitats of cyanobacteria, e.g. water reservoirs, contain a complex mixture of various potentially toxic compounds. Moreover, the compounds present in water environment may interact between themselves, exerting synergistic or antagonistic toxic effects to living organisms (Leão *et al*., 2010). The potential complexity of a cyanobacterial sample is one of the reasons why the appropriate assessment of cytotoxicity is problematic. Other complications arise from the interpretation of cytotoxicity itself and its extrapolation from *in vitro* models into real *in vivo* situations as well as the estimation of risks to human health. Those aspects of cyanobacterial cytotoxicity have been studied in detail in the presented thesis (Chapter VI).

I.2.2 Anti-inflammatory activity

Although cyanobacterial cytotoxicity is well known and deeply studied, the knowledge about anti-inflammatory potency is still relatively recent and unexplored, although anti-inflammatory activity has been verified for some cyanobacterial extracts (Rasool *et al*., 2006; Wiesner *et al*., 2007). Several cyanobacterial compounds are reported to be anti-inflammatory agents, however none of them belong to peptides and usually no mechanism of action is determined (Prinsep *et al*., 1996; Malloy *et al*., 2011). Although the knowledge about anti-inflammatory cyanobacterial compounds is still very limited, several relatively well described examples should be mentioned here. In general, cyanobacterial anti-inflammatory compounds are involved in regulation of innate immunological response acting on MyD88 (Myeloid differentiation primary response gene 88)-dependent or -independent pathways, leading to regulation of nuclear factor-κB (NF-κB) transcription and subsequently transcription of inflammatory cytokines (Akira and Takeda, 2004; Monako *et al*., 2004).

Malyngamide F acetate, isolated from *Lyngbya majuscula*, a known producer of valuable secondary metabolites, has been proven to be a selective inhibitor of the MyD88-dependent pathway, decreasing transcription of interleukins: IL-1β, IL-6 and IL-10 (Villa *et al*., 2010), regulating inflammatory response. Another interesting anti-inflammatory compound has been isolated from freshwater cyanobacterium *Oscillatoria Planktortix* (Macagno *et al*., 2006). However, the structure has not been fully determined and it was assumed to be a lipopolysaccharide (LPS)-like compound, acting as an antagonist of bacterial LPS. This cyanobacterial molecule was shown to inhibit bacterial LPS-stimulated MyD88-dependent and -independent inflammatory pathways, inhibiting proinflammatory cytokines production, e.g. tumor necrosis factor (TNF). Another interesting example is scytomemin, isolated from *Stigonema* sp. This phenolic compound acts as a nontoxic antiproliferative agent, having no effect on nonproliferating cells (Stevenson *et al*., 2002). Scytonemin targets a special class of serine/treonine kinases involved in up-regulation of TNF-α production in monocytes, regulating the inflammatory response (Hu *et al*., 2013). Another anti-inflammatory and antioxidative mechanism shows biliprotein found in *Spirulina platensis*, C-phycocyanin (Reddy *et al*., 1998, 2000, 2003). C-phycocyanin is a selective inhibitor of cyclooxygenase-2 and lipid peroxidation as well as effective scavenger of free radicals.

Anti-inflammatory processes underlie many of human diseases and a huge demand of new anti-inflammatory drugs exists on the market. Anti-inflammatory activity is often tested in *in vivo* assays, however in order to effectively search for new anti-inflammatory agents, high throughput assay platform is needed. In this project, development and evaluation of *in vitro* high throughput approach, the AlphaLISA™ assays for the quantification of pro-inflammatory cytokines and adhesion molecules is presented (Chapter IV).

Application of above mentioned assay and fluorescent staining allowed us to detect and characterize a novel anti-inflammatory cyanobacterial peptide, aeruginosin-865, isolated from terrestrial *Nostoc* sp. strain. (Chapter V).

II Aims of the project

- Developing a high throughput platform for screening of anti-inflammatory compounds produced by cyanobacteria
  - preparation of a cyanobacterial sample set for screening (crude extracts and purified fractions)
  - setup and validation of the *in vitro* screening method

- Isolation and characterization of a new cyanobacterial compound
  - developing an efficient extraction procedure and an HPLC purification process
  - determination of the 2D and 3D structure of the new congener using high resolution MS and NMR techniques
  - characterization of its bioactivity and evaluation of its pharmacological potential using cell-based assays

- Cyanobacterial toxicity and its effect on human health – attempt of general characterization and risk assessment
  - carrying out a large scale screening of cyanobacterial extracts using two different endpoints
  - assessment of potential risks by comparison with effects on human hepatocytes
  - discussion on the nature of cyanobacterial toxicity

III Material and Methods

III.1 Cyanobacterial strains

About 120 cyanobacterial strains, mainly of soil origin (*Nostoc, Desmonostoc, Leptolyngbya, Phormidium, Cylindrospermum, Anabaena, Tolypothrix, Wollea, Synechocystis, Oscillatoria, Calotrix, Fisherella*), collected from natural habitats and housed in the Institute of Microbiology CAS in Trebon, were investigated within this project. The complete table of strains is enclosed in the supplementary material in Chapter VI.

III.2 Cultivation of cyanobacterial strains

All cyanobacterial strains used in this research work were cultivated under standard conditions, in 350 mL glass tubes on liquid Allen-Aron medium (Allen and Arnon, 1955), bubbled with 2% CO_2-enriched air at constant temperature of 28°C, under 50 W/m^2 continuous illumination (Philips, Osram Dulux L, 55W/12-950). Usually after 5-7 days of cultivation, depending on the strain, the culture was harvested by centrifugation (Hettich Universal 320) at 4500 rpm in 10 min, stored at -70˚C and lyophilized (Leybold-Heraeos lyophilizator Lyovac GT 3). The lyophilized biomass was used subsequently for extraction.

In order to obtain sufficient amount of biomass for isolation of aeruginosin-865 (Aer-865, see Chapter V), mass cultivation in 100 L glass panel under the same conditions was performed. After several days, when the culture reached high density (optical estimation), the biomass was harvested, centrifuged, froze and lyophilized as described before.

III.3 Analytical methods

III.3.1 Standard extraction

For preparation of crude extract samples for cytotoxicity and anti-inflammatory screening, a standard extraction procedure was developed. Lyophilized cyanobacterial biomass (200 mg) was extracted with ca. 6 mL of 70% methanol with mortar and pestle, with addition of sea sand, in order to break the cell wall more effectively. The extracts were transferred into 10 mL glass tubes and left for 1 h under light protection. Subsequently the extracts were centrifuged (15 min/4500 rpm, Hettich Universal 320) and the supernatants transferred into 50 mL heart-shape flasks and evaporated to dryness under reduced pressure at

40ºC (Heidolph Rotavac Senco evaporator or Büchi Rotavapor R-114 evaporator). The dry residues were dissolved in 1 mL of 100% MeOH (200 mg biomass/mL) using ultrasonic bath, transferred into 1.5 mL Eppendorf tubes and centrifuged in a minispin Eppendorf centrifuge (5 min/10000 rpm). The supernatants were transferred into 1.5 mL glass vials, analyzed on HPLC-MS and stored at -20˚C prior further testing.

III.3.2 Extraction and purification of aeruginosin-865 and nostopeptolides A1 and A3

The freeze-dried biomass of *Nostoc* sp. Lukešova 30/93 (4 g) was extracted twice with 50% MeOH (200 mL) by mortar and pestle for 1 h at room temperature. The extract was centrifuged at 4500 rpm for 15 min and the supernatant transferred into 1000 mL separatory funnel. Then liquid-liquid partitioning by addition of EtOAc and H_2O in the final ratio 1:1:4 (MeOH-EtOAc-H_2O) was performed. When the two phases separated, the upper one, containing pigments, was discarded; the lower one was collected and evaporated at 40°C under vacuum. The dry residue was dissolved in 20% MeOH (600 mL) and concentrated on C8 HLB Cartridges (60 mg, Waters Oasis®), keeping loading capacity of one cartridge below 0.7 g biomass per cartridge. Fraction of Aer-865 bounded to the stationary phase was eluted by 50% MeOH (3 mL); fraction of nostopeptolides was eluted by 70% MeOH (4 mL) and subjected to the subsequent purification processes.

III.3.2.A Preparative HPLC – aeruginosin-865

Concentrated fraction (1.3 g biomass/mL) was injected to the semi-preparative HPLC column (Reprosil 100 C18, 250 x 8 mm, 5 μm, dr. Maisch GmbH) and eluted by MeOH/H_2O gradient (30-45% MeOH for 5 min, 45% MeOH for 5 min, 45-55% MeOH for 25 min, 55-100% MeOH for 15 min, 100% MeOH for 5 min) with flow rate of 2.6 mL/min. Detection was performed both by UV-Vis detector in 210 nm (Knauer) and mass spectrometer (HP 1100 MSD SL-Ion Trap, Agilent) by splitting of 5 vol% of eluent coming out from the column, which allowed continuous monitoring and precise collection of eluted fractions. The retention time of Aer-865 was 19.5 min. Subsequently the sample was dissolved in 30% MeCN (2 mL), repurified on phenyl semi-preparative HPLC column (Reprosil 100 Phenyl, 250 x 8 mm, 5 μm, Watrex) by using MeCN/H_2O gradient (30% MeCN for 10 min, 30-40% MeCN in 10 min, 40-100% MeCN in 2 min, 100% MeCN in 3 min) with flow rate 2.0 mL/min. The retention time of Aer-865 was 7.2 min. The purity of Aer-865 was verified by HPLC analysis (Chapter III.3.3) and subjected to HiRes MS and NMR experiments (Chapter III.3.3.A and B).

III.3.2.B Preparative HPLC – nostopeptolide A1 and A3

Concentrated fraction of nostopeptolide A1 and A3 (0.5 mL, 1.0 g biomass/mL) was injected to the semi-preparative HPLC column (Reprosil 100 C18, 250 x 8 mm, 5 μm, dr. Maisch GmbH) and eluted by MeOH/H_2O gradient (50-80% MeOH for 50 min, 80-100% MeOH for 1 min, 100% MeOH for 6 min) with a flow rate of 2.6 mL/min. Detection of samples were performed as described above for aeruginosin-865 (Chapter III.3.2.A). Retention times of nostopeptolide A1 and A3 were 36.8 and 39.1 min, respectively. Subsequently, Nostopeptolide A1 was subjected to another purification step. The sample (0.5 mL, 1 mg/mL) was injected to the semi-preparative HPLC column (Reprosil 100 Phenyl, 250 x 8 mm, 5 μm, Watrex) and eluted with the flow rate 2.0 mL/min by MeCN/H_2O gradient (40-45% MeCN in 5 min, isocratic elution with 45% MeCN for 5 min, 45-60% MeCN for 10 min, 60-100% MeCN for 2 min and 100% MeCN for 5 min). The detection of eluted compounds was performed as described above. The retention time of nostopeptolide A1 was 11.4 min. Collected fractions were evaporated under vacuum and used in the analytical LC-MS experiments (Chapter III.3.3) and in the bioassays (Chapter III.4.1 and 2).

III.3.3 LCMS analysis

All extracts and fractions were analyzed on Agilent 1100 Series modular HPLC equipped with diode array detector (Agilent Technologies), connected to mass spectrometer HP 1100 MSD SL-Ion Trap (Agilent Technologies), on Zorbax XDB-C8 a column (4.6 × 150 mm, 5 μm, Agilent Technologies) at 30°C and eluted with flow rate of 0.6 mL/min by standard MeOH/H_2O gradient (30-100% MeOH for 30 min, 100% MeOH for 5 min), modified by 0.1% formic acid to improve ionization. The ESI-ion trap mass spectrometer and PDA detector were used to monitor the eluted compounds. The operating parameters of the mass spectrometer were as follows: the spray needle voltage was set at 4.5 kV, the drying temperature was 325^{o}C. As the nebulizing and drying gas nitrogen was used, with flow rate 50 and 10 mL/min, respectively. The ESI capillary voltage was 230 V and helium was used as auxiliary gas (15 mL/min).

In the MS/MS experiments for identification of nostopeptolides A1 and A3, selected precursor was targeted to 900 *m/z* and the collision energy 100% of 5 V was applied. All MS and MS/MS spectra were acquired in positive ion mode, in broad mass range 50-2200 *m/z* with scanning speed 13000 *m/z* per second.

III.3.4 Activity guided fractionation

Activity guided fractionation was performed in order to obtain semi-purified fractions of cyanobacterial metabolites from selected strains for testing of anti-inflammatory and cytotoxic bioactivity. Two approaches were used for the fractionation. First one was dependent on time-scale only, collecting fractions eg. every 10 or 5 min, disregarding selection of ions. The second approach favoured ions selection and each extract was fractionated individually. The details of fractionation of particular strains for cytotoxicity testing are included directly in the manuscript (Chapter VI). Hence the anti-inflammatory screening aimed strictly development and validation of the methodology, the fractionation details of each sample were omitted in the publication (Chapter IV).

The fractionation was performed on Agilent 1100 Series modular HPLC, using standard MeOH/H_2O gradient as described in the previous section (Chapter III.3.3). Each cyanobacterial extract (200 mg/mL) was injected five times (50 μL each time), so the total processed sample volume was 250 μL. The fractions were collected to the glass heart-shape flasks and evaporated to dryness under vacuum (Heidolph Rotavac Senco evaporator and Büchi Rotavapor R-114 evaporator). The dry residue of each flask was dissolved in 250 μL in 100% MeOH, transferred to 1.5 mL glass vials and stored at -20°C prior cytotoxicicty or anti-inflammatory testing.

III.3.5 Structure elucidation techniques

These techniques, including high-resolution mass spectrometry, NMR analysis, IR spectrometry and chiral subunits analysis, were applied in order to determine 2D and 3D structure of the novel cyanobacterial metabolite, Aer-865 (Chapter V).

III.3.5.A High resolution mass spectrometry

Accurate mass spectrometry was carried out in collaboration with Dr. Petr Novák from the Laboratory of Molecular Structure Characterization, Institute of Microbioloby, CAS, in Prague. The measurement was performed on APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and a Dual II ion source (Bruker Daltonics, Billerica, MA). Aer-865 was dissolved in MeOH/H_2O or D_3COD/D_2O (50:50) and analyzed by direct infusion. The analysis was carried out using ESI and the spectra were acquired in positive mode. The cell was opened for 1.3 msec, accumulation time was set at 0.2 s for MS experiment and 1.0 s for MS/MS experiment. For each sample one experiment, consisting of average of eight spectra, was collected. After MS experiment one MS/MS experiments was done from the most intensive ion. The isolation window was set to 4 a.m.u. and the collision

energy was kept at -16 V (positive mode). The acquisition data set size was set to 1M points with the mass range starting at *m/z* 150 a.m.u., resulting in a resolution of 100000 at *m/z* 400. The instrument was externally and internally calibrated using clusters of arginine, resulting in mass accuracy below 1 ppm. The acquired spectra were apodized with a square sine bell function and Fourier transformed with one zero-fill. The interpretation of mass spectra was done using DataAnalysis version 4.0 software package (Bruker Daltonics, Billerica, MA).

III.3.5.B NMR Experiments

NMR analysis was performed by Dr. Marek Kuzma in the the Laboratory of Molecular Structure Characterization, Institute of Microbioloby, CAS in Prague. NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer equipped with TCI CryoProbe (600.23 MHz for ^{1}H, 150.93 MHz for ^{13}C, Bruker Biospin GmbH, Rheinstetten, Germany) in DMSO-d_6 and CD_3OD at 303.1 K. The residual solvent signals were used as an internal standard (δ_H 2.500 ppm and δ_C 39.60 ppm for DMSO, δ_H 3.330 ppm and δ_C 49.30 ppm for CD_3OD). ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HSQC-TOCSY, *J*-resolved and ROESY spectra were measured using the standard manufacturer's software. The ^{1}H NMR spectrum was zero filled to 2-fold data points and multiplied by a window function (two parameter double-exponential Lorentz-Gauss function) before Fourier transformation to improve the resolution. The ^{13}C NMR spectrum was zero filled to 2-fold data points. Subsequently, the line broadening (1 Hz) was used to improve signal to noise ratio. Protons were assigned by COSY and TOCSY, and assignment was transferred to carbons by HSQC. The chemical shifts are given in the δ scale (ppm) and coupling constants are given in Hz. The digital resolution allowed us to present the proton and carbon chemical shifts to three and two decimal places, respectively, however in order to be in accordance with the usual standard NMR data notation, proton and carbon chemical shifts are presented with two and one decimal places, respectively. The proton chemical shift readouts from HSQC are reported to one decimal place and *J*-resolved readouts to two decimal places.

III.3.5.C Infrared spectrometry

IR spectra (650 to 4000 cm^{-1}) were recorded on a Nicolet IS10 spectrometer (Thermo Scientific) equipped with ATR module (ZnSe). Shallow amount of the sample dissolved in MeOH was poured over the surface of the crystal and after evaporating of solvent, firmly clamped against the crystal and the absorption spectrum was measured.

III.3.5.D Chiral subunits analysis

Hydrolysis, derivatization with Marfey reagent and HPLC-UV analysis of Aer-865 subunits were carried out with collaboration with Dr. Jouni Jokela from Department of Food and Environmental Science, University of Helsinki, Finland.

Marfey's method has been wildely used for structural characterization of peptides and detection of small quantities of D-amino acids, also in peptides isolated from microorganisms (Jokela *et al.*, 2010). Derivatization with chiral Marfey's reagent results in obtaining stable, UV-active L- and D-amino acids as diastereoisomers and allows to sepatare them in nanomolar range on a non-chiral columns with UV detection (Bhushan and Brückner, 2004).

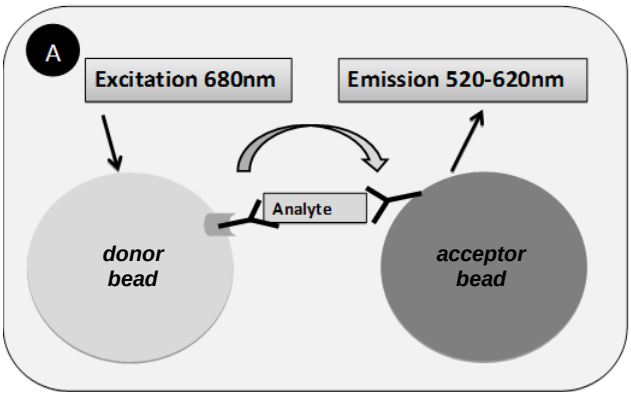
Aer-865 (0.1 mg) was hydrolyzed in 6N HCl (0.3 mL) at 110°C for 22h. Then the solvent was evaporated and the residue dissolved in water. An aliquot of 50 µL was subjected to reaction with Marfey's reagent, so 20 µL $NaHCO_3$ (1M) and 1% FDAA (Marfey's reagent – 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, Pierce) in 100 µL acetone were added. After incubation for 1h at 37°C, the reaction was terminated by adding 1N HCl (20 µL). The FDAA derivatives were purified by adding 800 µL water to the reaction mixture and then passing the solution through an equilibrated with 85% MeCN (1 mL) solid-phase extraction cartridge (StrataX Polymeric Sorbent, 30 mg, Phenomenex). The cartridge was washed with 400 µL 30% MeCN and subsequently 200 µL 60% MeCN. The FDAA derivatives were eluted with 100% MeCN (400 µL).

Reference compound, D-Hpla (D-hydroxyphenyllactic acid), was obtained by hydrolysis of the MeOH extract residue from *Microcystis viridis* NIES-102 (Matsuda et al., 1996). Both the reference compound and reference amino acid standards and D,L-*p*-Hpla (Sigma-Aldrich, USA) were derivatized as described before. FDAA derivatives of Aer-865 and reference substances were subjected to LC-UV analysis performed with a HP 1100 Series modular chromatograph containing diode array detector (Agilent Technologies), using Luna C18 column (150 x 4.6, 5µm, Phenomenex) at 40°C, with MeCN gradient. Retention times of amino/hydroxy acid standards (L-Ile, D-Ile, D-*allo*Ile, L-Leu, D-Leu, D,L-Hpla) and D-Hpla from *Microcystis viridis* NIES-102 were compared with amino acid and Hpla from the Aer-865 acid hydrolysate derivatised with Marfey reagent.

III.4 Cell-based assays

III.4.1 AlphaLISA™ assays for the quantification of pro-inflammatory cytokines and adhesion molecules

AlphaLISA™ (Amplified Luminescence Proximity Homogenous Assay) is a technique that applies a conjugation of specific antibodies with donor and acceptor beads. After binding a targeted analyte, the donor and acceptor beads reach close proximity (200 nm), which enables their interaction. Upon excitation at 680 nm, the photosensitizers inside the donor beads convert ambient oxygen to singlet oxygen state and when the donor and acceptor beads reach close proximity, the singlet oxygen molecule diffuse to acceptor beads, thereby resulting in emission at 520-620 nm. In our experiment specific antibodies against IL-8 (inerleukin-8) and ICAM-1 (intercellular adhesion molecule-1) were used, which enabled us to measure the level of investigated inflammatory mediators.



Principle of AlphaLISA™ assay (Chapter IV)

Interleukin-8 is a chemokine acting mainly by attraction of neutrophils and monocytes toward the inflammation site. Intercellular adhesion molecule-1 is a transmembrane glycoprotein expressed by vascular endothelium and responsible for leukocyte transmigration across the endothelium into inflamed tissue (Yang *et al*., 2005). Excessive production of IL-8 and ICAM-1 is triggered by the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNF-α) (Yang et al., 2005; Vlahopoulos et al., 1999). Anti-inflammatory substances, such as piceatannol (PC) (Piotrowska *et al*., 2012) or SB203580 (SB) – a selective p38 mitogen activated protein kinase (p38 MAPK) inhibitor (Goldstein and Gabriel, 2005) inhibit the Nuclear Factor-κB (Nf-κB) pathway and down-regulate the level of inflammatory mediators (IL-8 and ICAM-1).

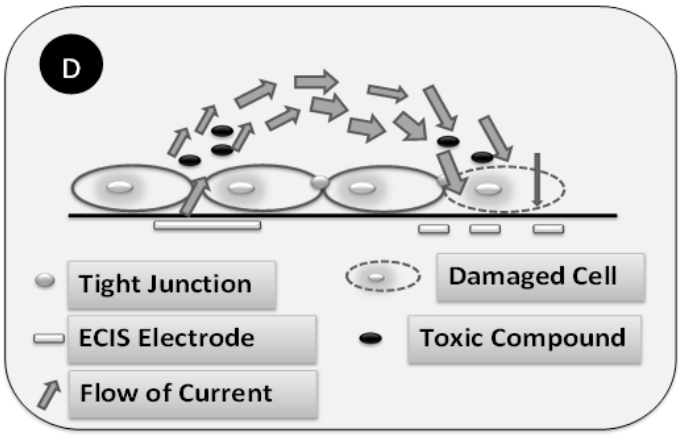
AlphaLISA™ (PerkinElmer) was performed on HL-MVECs (human lung microvascular endothelial cells, Provitro, Berlin, Germany). The cells were plated at a concentration $5x10^3$ cells per well in a white 384-well plate (BrandTech, Essex, CT) in endothelial medium (50 µL, GE Life Sciences; supplemented with 1% penicillin (10000 IU) /streptomycin (10000 µg/mL), 0% fetal calf serum (FCS)) and incubated at 37°C in an incubator with 5% CO_2 for 4 h. Subsequently the cells were pretreated for 30 min with Aer-865 (0.1 – 100 µg/mL in 2% DMSO) and stimulated with hTNF-α (0.1 ng/mL, NIBSC, Potters Bar, UK) for 18 h. As positive controls p38 MAPK inhibitor SB203580 (SB; 100 µm, Sigma-Aldrich) and piceatannol (PC; 30 µg/mL, Tocris Bioscience) were used.

By using the AlphaLISA™ ICAM-1 Immunoassay Research kit (PerkinElmer), HL-MVECs were grown in white 384-well plates and incubated with a mixture of biotinylated anti-ICAM-1 antibodies (1 nm), the AlphaLISA™ acceptor beads (10 µg/mL) and the Assay buffer, at room temperature for 1 h in darkness. Afterwards the AlphaLISA™ streptavidin donor beads solution was added (40 µg/mL) and the plate was incubated again for 30 min as previously. After incubation, the plate was read with the SpectraMax® Paradigm® Modular Multi-Mode Reader (Molecular Devices, Sunnyvale, CA) using AlphaScreen™ Detection Cartridge (excitation and emission wavelength at 680 and 570 nm, respectively).

For AlphaLISA™ IL-8 Immunoassay Research kit (PerkinElmer, USA) the medium was taken and mixed with the biotinylated anti-IL-8 antibody (1 nm) and AlphaLISA™ Acceptor beads (10 µg/mL). The further procedures of the assays were performed in the same way as with the AlphaLISA™ ICAM-1 Assay described above. The EC_{50} value was determined using linear regression analysis and defined as a sample concentration resulting in 50% reduction of inflammatory response.

III.4.2 Electrical Cell Substrate Impedance Sensing (ECIS)

In order to exclude false positive results of AlphaLISA™ assay, real-time cytotoxicity and barrier integrity of HL-MVECs was monitored by impedance measurement by Electrical Cell Substrate Impedance Sensing (ECIS). The ECIS technology can be used to analyze barrier integrity of cells forming monolayers (Wiesner *et al*., 2007). The heart of the measurement device is a specialized microplate that has 96 individual wells with ten 250 µm-diameter active gold electrodes and a much larger gold counter electrode deposited on the bottom of each well. Low amplitude of alternating current signal is applied across the pair of electrodes on which cells are plated. Each change of monolayer tightness and permeability is monitored by ECIS device, displaying a significant drop in impedance.



Principle of Electrical Cell Substrate Impedance Sensing assay (ECIS) (Chapter IV)

The experiments were performed on ECIS model 1600R (Applied Biophysics, Inc., Troy, NY). The ECIS plate (96W10E+, Applied Biophysics) was pre-coated with fibronectin (10 µg/mL, Sigma-Aldrich) and incubated for 1 h at 37°C in incubator with 5% CO_2. Afterwards HL-MVECs were plated in concentration $6x10^4$ per well in endothelial medium with FCS (200 µl, 1% penicillin/streptomycin, 10% FCS; PAA, Austria) and incubated overnight. The next day the medium was removed and fresh endothelial medium (200 µL) without FCS was added. The ECIS plate was measured with alternating current at 4 kHz and impedance (Ohm) was normalized. After 1 h the cells were preincubated with tested and control substances, subsequently stimulated with hTNF-α, as described for AlphaLISA™ assays, and impedance was measured again for 22 h. Six replicates were performed for each sample.

III.4.3 Confocal laser scanning microscopy for visualization of NF-κB translocation

Nuclear factor-κB is involved in transcription of many genes involved in cell survival, adhesion, inflammation, including IL-8 and ICAM-1 synthesis (Vlahopoulos *et al*., 1999; Ledebur and Parks, 1995). Under physiological conditions, NF-κB is a heterodimer composed of p65 and p50 subunits, bound to a specific inhibitor κB (IκB) which ensures its cytoplasmatic localization, due to masking a nuclear localization sequence. Stimulation with TNF-α turns on a signaling cascade, leading to phosphorylation and subsequent degradation of IκB and translocation of NF-κB complex (p65/p50) to the nucleus, which results in transcription of inflammatory mediators. Translocation of NF-κB is effectively suppressed by presence of SB inhibitor.

An eight-well chamber slide (BD Falcon 4108, BD Bioscience) was precoated with fibronectin (10 µg/mL) for 2 h, then human umbilical vein endothelial cells (HUVECs, #CRL-1730, ATCC) were seeded in concentration $4x10^4$ per well in endothelial medium

(PAA) and incubated overnight. The next day the medium was removed and the cells washed once with PBS (Life Technologies, USA). We used SB inhibitor (100 µm, Sigma-Aldrich) as a positive control, hTNF-α (0.1 ng/mL) for stimulating the cells and PBS as a blank sample. The cells were preincubated with Aer-865 (1, 10 or 100 µg/mL) or with SB inhibitor for 30 min, then the cells were stimulated with hTNF-α and incubated at 37°C for 1 h. Afterwards the cells were washed once with ice-cold PBS, fixed with 4% formaldehyde (Carl Roth) in PBS for 30 min, washed again (as above) and treated with 0.5% Triton X-100 (Sigma-Aldrich) for 10 min to permeabilize the cell membrane. After another washing step the cells were incubated with 50 mM NH_4Cl/0.1% Glycin solution for 30 min, to quench the autofluorescence and to block unreacted aldehyde groups in the cells. The chamber slide was blocked with BSA (0.1 % in PBS) for 1 h and then the mouse anti-human NF-κB p65 antibody (F-6, 2 µg/mL in PBS containing 0.1% BSA; sc-8008, SantaCruzBiotechnology, Santa Cruz, CA) was added to the cells for 3 h. Afterwards the cells were washed and incubated with the secondary antibody AlexaFluor 488 goat anti-mouse IgG (5 µg/mL, A11017, LifeTechnologies) as well as with Phalloidin AlexaFluor 568 (0.5 U/mL, A12380, LifeTechnologies) for 1 h. After staining and washing the cells, the chamber walls were removed and the wells dried at room temperature. The VectaShield mounting medium with DAPI (15 µL, Vector Laboratories, Peterborough, UK) was added to each well and the slide was covered by a large cover slip (LH25.1, Carl-Roth). A DMIRE2 microscope (Leica Microsystems, Wetzlar, Germany) equipped with Leica TCS SP 2 confocal laser was used to observe translocation of of NF-κB to the nucleus.

III.4.4 Cytotoxicity test

III.4.4.A Cell lines

The methyl-tetrazolium test (Mosmann, 1983) was performed on 6 different human and murine cell lines: adherent – HepG2 (human liver carcinoma), HeLa (human cervical cancer), Balb/c 3T3 murine fibroblasts, semi-adherent – Sp/2 (murine myeloma) and suspension cell line – Yac-1 (murine polymorphotic lymphoma) and primary hepatocytes culture. HepG2 cell line was kindly provided by Dr. H. Hundsberger (University of Applied Sciences in Krems, Austria). HeLa, Sp/2 and Yac-1 cell lines were purchased from the Institute of Parasitology (Biological Center of Academy of Sciences of the Czech Republic, České Budějovice) from Prof. Jan Kopecký. Murine fibroblasts Balb/c 3T3 was purchased from the European Collection of Cell Cultures (Salisbury, UK).

Human hepatocytes were isolated in the Department of Medical Chemistry and Biochemistry in Olomouc from the liver samples (n=3), obtained from multi-organ donors (women 61 and 62 years old and men 46 years old). The tissue acquisition protocol was

established in agreement to requirements of the Ethics Commission of the Faculty Hospital Olomouc. Acquired tissue were extensively washed with calcium-free 20 mM HEPES, pH 7.5, 120 mM NaCl, 5 mM KCl, 0.5% glucose at 37°C. Hepatocytes were isolated by two step collagenase perfusion technique described in detail by Pichard *et al*., 1990. At the end of collagenase perfusion the Glisson's capsula, covering the external surface of liver, was opened and the softened tissue was gently minced with scissors. The homogenate was suspended in 1 L of 0.025% collagenase solution (type IV, Sigma) and incubated 10 min at 37°C under gentle rotary shaking. Subsequently the hepatocytes were filtered through 250 and 150 μm gazue, isolated by low speed centrifugation (50×*g*) and washed three times with culture medium.

III.4.4.B MTT assay

Cells were plated in concentration $5x10^5$ cells/mL (200 μL, $3x10^4$ cells per well) in a transparent 96-well plate (Corning®Costar® cell culture plate 96-well, flat bottom, Sigma Aldrich) in a cultivation medium, one day before toxicity experiment. For HeLa cell line RPMI-1640 cultivation medium (Gibco Life Technologies), supplemented with 10% FBS (Gibco Life Technologies) and 1% penicillin/streptomycin (Gibco Life Technologies) was used. Sp/2 and Yac-1 cells were cultivated in RPMI-1640 medium (5% FBS, 1% penicillin/streptomycin). For HepG2 cells high glucose DMEM medium (10% FCS, 1% penicillin/streptomycin, Gibco Life Technologies) was used. Balb/c 3T3 cells were cultivated in DMEM medium supplemented with penicillin 100 U/ml, L-glutamine (2 mM), streptomycin (100 mg/L), 5% fetal calf serum, 5% new born calf serum. Human hepatocytes cultivated in Williams′ medium E and Ham's-F12 (1:1) culture medium containing 5% bovine serum, which after 12 h was changed for the serum free medium. All cell cultures were further incubated at 37°C in humidified incubator with 5% CO_2 for 24 h.

The cyanobacterial extracts (50 μL, 200 mg/mL) were transferred into 0.5 mL Eppendorf tubes, evaporated under nitrogen and dissolved in 50 μL MeOH/cultivation medium (20/80, v/v), obtaining exposure solutions. A possible pellet was discarded by centrifugation. The exposure solutions (10 μL) were used to treat the cells, each concentration in triplicate. As a blank control MeOH/cultivation medium (20/80) solution was used. After 24 h of incubation, 10 μL of MTT solution (4 mg/mL, Invitrogen) was added to the cell cultures and incubated for 4 h. Afterwards the plates were centrifuged (14 min/380×g), supernatant discarded and formazan crystals dissolved in 200 μL DMSO. The test and reference absorbance wavelenghts were read at 590 and 640 nm, respectively. The viability index was expressed as a ratio between the absorbance values of treated and control wells.

III.4.4.C Determination of IC_{50} values

The IC_{50} experiments were completed for 7 selected cyanobacterial strains (Chapter VI) and performed on 3 cell lines – HeLa, Sp/2 and Balb/c 3T3 fibroblasts. Desired volume of the crude cyanobacterial extract (200 mg/mL) was evaporated under nitrogen to dryness and dissolved in MeOH/cultivation medium (20/80, v/v) to prepare stock exposure solution of concentration 40 mg/mL. The stock solution was diluted and added to 200 μL of cells suspension, to obtain in the wells the biomass concentrations of 20, 10, 5, 1 and 0.1 mg/mL. The MTT assay was performed as described above (Chapter III.4.4.B).

III.4.4.D Time lapse microscopy

The fractions and crude extracts of selected strain *Nostoc* sp. (Chapter VI) were tested for the inhibition of the cell proliferation by time-lapse microscopy on Hela cell line. HeLa cells were plated as described for MTT assay on transparent 96-well plate. After addition of exposure solution, the plate was placed into Axio-Zeiss Observer Z.1 microscope equipped with cultivation chamber set at 37°C and supplied with 5% CO_2. One microscopy field was monitored per each well in 20 min interval for 24 h. After the experiment two subareas of the microscopy field were calculated (approx. 100 – 600 cells) at time points 0 and 24 h and the number of cell divisions was calculated. The inhibition of cell division was expressed as a ratio of number of treated and untreated cells in percent.

IV A Combined Impedance and AlphaLISA™ Based Approach to identify anti-inflammatory and barrier-protective compounds in human endothelium

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A Combined Impedance and AlphaLISA-Based Approach to Identify Anti-inflammatory and Barrier-Protective Compounds in Human Endothelium

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Abstract

Chronic inflammation is at least partially mediated by the chemokine-mediated attraction and by the adhesion molecule–directed binding of leukocytes to the activated endothelium. Therefore, it is therapeutically important to identify anti-inflammatory compounds able to control the interaction between leukocytes and the endothelial compartments of the micro- and macrocirculation. When testing novel drug candidates, it is, however, of the utmost importance to detect side effects, such as potential cytotoxic and barrier-disruptive activities. Indeed, minor changes in the endothelial monolayer integrity may increase the permeability of small blood vessels and capillaries, which, in extreme cases, can lead to edema development. Here, we describe the development of a high-throughput screening (HTS) platform, based on AlphaLISA technology, able to identify anti-inflammatory nontoxic natural or synthetic compounds capable of reducing tumor necrosis factor (TNF)–induced chemokine (interleukin [IL]–8) and adhesion molecule (ICAM-1) expression in human lung microvascular endothelial cells. Quantification of cell membrane–expressed ICAM-1 and of cell culture supernatant–associated levels of IL-8 was analyzed in HTS. In parallel, we monitored monolayer integrity and endothelial cell viability using the electrical cell substrate impedance sensing method. This platform allowed us to identify natural secondary metabolites from cyanobacteria, capable of reducing ICAM-1 and IL-8 levels in TNF-activated human microvascular endothelial cells in the absence of endothelial monolayer barrier disruption.

Keywords

inflammation, tumor necrosis factor (TNF), chemokine, AlphaLISA, high-throughput screening, cyanobacteria, ICAM-1, IL-8, electrical cell substrate impedances sensing (ECIS)

V Novel Aeruginosin-865 from Nostoc sp. as a Potent Anti-inflammatory Agent

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Novel Aeruginosin-865 from *Nostoc* sp. as a Potent Anti-inflammatory Agent

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Aeruginosin-865 (Aer-865), isolated from terrestrial cyanobacterium *Nostoc* sp. Lukešová 30/93, is the first aeruginosin-type peptide containing both a fatty acid and a carbohydrate moiety, and is the first aeruginosin to be found in the genus *Nostoc*. Mass spectrometry, chemical and spectroscopic analysis as well as one- and two-dimensional NMR and chiral HPLC analysis of Marfey derivatives were applied to determine the peptidic sequence: D-Hpla, D-Leu, 5-OH-Choi, Agma, with hexanoic and mannopyranosyl uronic acid moieties linked to Choi. We used an AlphaLISA assay to measure the levels of proinflammatory mediators IL-8 and ICAM-1 in hTNF-α-stimulated HLMVECs. Aer-865 showed significant reduction of both: with EC_{50} values of $(3.5 \pm 1.5)\ \mu g\ mL^{-1}$ ($(4.0 \pm 1.7)\ \mu M$) and $(50.0 \pm 13.4)\ \mu g\ mL^{-1}$ ($(57.8 \pm 15.5)\ \mu M$), respectively. Confocal laser scanning microscopy revealed that the anti-inflammatory effect of Aer-865 was directly associated with inhibition of NF-κB translocation to the nucleus. Moreover, Aer-865 did not show any cytotoxic effect.

VI Evaluation of Cyanobacterial Cytotoxicity by Using *in Vitro* models. A Comparative Study

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Manuscript (2014)

Evaluation of Cyanobacterial Cytotoxicity by Using In Vitro Models
A comparative Study

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Abstract: In the present study a selection of 120 cyanobacterial extracts was tested for its cytotoxicity to hepatic and non-hepatic human cell lines (HepG2 and HeLa) and three cell systems of rodent origin (Yac-1, Sp/2 and Balb/c 3T3 fibroblasts). Furthermore, a subset of the extracts was also tested for *in vitro* cytotoxicity against primary cultures of human hepatocytes as a reference of potential risks to humans. Roughly one third of cyanobacterial extracts tested caused cytotoxic effects in human cell lines and mice fibroblasts, showing a very high correlation coefficient. Significantly higher sensitivity to crude cyanobacterial extracts was found in murine cell lines Yac-1 and Sp/2. Expectedly, hepatic cancer line HepG2 exhibited highest correlations with primary cultures of human hepatocytes; however, the response of hepatocytes was also congruent with non-hepatic HeLa cells and murine fibroblast Balb/c 3T3. It was shown by comparison of cytotoxic effects on HepG2 and human hepatocytes, that specific liver injury was caused only by 6% of the tested extracts. In about 22% of the cases, the liver cells showed higher resistance to cyanobacterial samples in comparison to the other human cell lines. In selected extracts a presence of cytotoxic fraction was confirmed by activity guided HPLC fractionation and it was demonstrated that cyanobacterial cytotoxicity is caused by a specific cyanotoxin rather than a synergistic effect of many compounds. The presented data suggests generally low tissue specificity of most of cyanobacterial cytotoxins and opens a possibility to use cytotoxicity as a marker in monitoring of potential toxicity of complex cyanobacterial mixtures containing unknown compounds.

Key words: cytotoxicity, cyanobacteria, human cell lines, extract fractionation, primary hepatocytes

VII Summary of results

In the present thesis the following results have been achieved:

- Developing a high throughput platform for screening of anti-inflammatory compounds produced by cyanobacteria
    - AlphaLISA^{TM}-based assay combined with Electrical Cell Substrate Impedance Sensing, for the detection of adhesion molecule ICAM-1 and chemokine IL-8 in endothelial cell line after stimulation with hTNF-α has been developed and validated.
    - Calculated Z-factor (0.70) indicates suitability of the assay for use in a full-scale, high-throughput screen. For cells stimulated with hTNF-α, 5-fold increase of IL-8 level was observed, whilst preincubation with p38 MAPK inhibitor SB203580 down-regulated the IL-8 to the basic level. The level of inflammatory mediators was proportional with concentration of hTNF-α. Measurement of ICAM-1 level in response to different concentrations of SB inhibitor revealed IC_{50} = 82 μM. All results were reproducible.
    - An anti-inflammatory substance isolated from *Nostoc* sp. Lukešová 30/93 was confirmed to be a mixture of nostopeptolide A1 and A3 by applying MS/MS analysis.
    - The mixture showed moderate activity, reducing by half the level of IL-8 in TNF-stimulated endothelial cells and ICAM-1 to physiological level. In the ECIS assay, nostopeptolides also showed a barrier protective effect on endothelial cells monolayer.

- Isolation and characterization of a new cyanobacterial compound
    - Based on NMR spectroscopy, MS and HPLC analysis of Marfey derivatives it has been assumed that the new peptide, aeruginosin-865, isolated from *Nostoc* sp. Lukešová 30/93 is made up of D-Hpla, D-Leu, 5-OH-Choi (2-carboxy-6-hydroxyoctahydroindol), substituted with HA (hexanoic acid) and ManA (mannuronic acid) moieties, and 4-amidinobutylamide (agmatine, Agma).
    - In AlphaLISA^{TM} assay, in human lung microvascular endothelial cells (HL-MVECs) stimulated with hTNF-α, Aer-865 was able to inhibit IL-8 and ICAM-1 production in a concentration-dependent manner with an EC_{50} of 3.5±1.5 μg/mL (4.0±1.7 μm) and 50.0±13.4 μg/mL (57.8±15.5 μM), respectively.

Moreover, Aer-865 in ECIS assay showed no effect on cell monolayer stability, indicating no cytotoxic effect.

- Anti-inflammatory effect of Aer-865 was associated, depending on concentration, with partial or total inhibition of NF-κB translocation into the nucleus in TNF-stimulated endothelial cells.

- Cyanobacterial toxicity and its effect on human health – attempt to general characterization and risk assessment

  - Study on cyanobacterial cytotoxicity showed that generally rodent cell lines (Sp/2, Yac-1 and Balb/c 3T3 fibroblasts) were more sensitive to cyanobacterial extracts, indicating 40-50% extracts as strongly cytotoxic. The human cell lines (HepG2, HeLa and primary hepatocytes) showed strong cytotoxic effects in response to about 20% of tested strains. Within the group of rodent cell lines, non-adherent (Yac-1) and semi-adherent (Sp/2) growth-type cells were the most sensitive.

  - Least square linear regression analysis showed the highest correlation in cytotoxicity discrimination for HeLa and HepG2 cell lines (R^2=70%), as well as Balb/c 3T3 fibroblasts with both HeLa and HepG2, resulting in high correlation coefficients R^2 =75% and R^2=66%, respectively. These results suggest low tissue specificity of cyanobacterial toxins.

  - Only 6% of tested extracts were specifically toxic to human hepatocytes, whereas about 22% showed significantly lower toxicity to primary liver cells in comparison to the other cell lines tested.

  - The fractions analysis showed that usually a cytotoxic effect of the extract was attributed to one fraction only, suggesting a presence of a specific toxic compound. However, in some cases the cytotoxic effect could not be attributed to the activity of only one particular fraction, but it was a result of an additive effect of several active fractions with lower individual inhibition values.

VIII Conclusions

Cyanobacterial secondary metabolites have been intensively studied for more than two decades, however, it has been demonstrated once again that the Nature is still an unexplored source of new ideas and solutions.

The novel cyanobacterial tetrapeptide, aeruginosin-865 is the first compound of its type found in a terrestrial *Nostoc* strain. The new aeruginosin possesses unique structural features not described within this group of peptides before, like the presence of uronic acid and fatty acid moiety. It is assumed that aeruginosin-865 can represent a type of evolutionary intermediate and it would be very interesting to study its biosynthesis gene cluster in order to verify this hypothesis. Moreover, bioactivity tests showed remarkable anti-inflammatory activity and no cytotoxic or barrier disruption effects, which makes this new compound an interesting immunomodulatory agent. It seems that finding a new source of aeruginosins resulted not only in revealing new possibilities of structure modifications, but also new bioactivity. Generally not much is known about cyanobacterial metabolites and their ability to influence the human immune system. Eventually, they might serve for the existing demand of new therapeutic approaches in treating immunological diseases. In this regard, cyanobacteria continue to be a rich source of new drug leads.

In order to successfully explore the natural resources, new methodology for screening of natural samples must be developed and validated. In this project we undertook the attempt to develop and establish a new methodology for *in vitro* anti-inflammatory and cytotoxicity screening.

The new anti-inflammatory assay presented here established a novel combination of assays, the AlphaLISA^{TM}-based screening and Electrical Cell Substrate Impedance Sensing, for identification and toxicity profiling of potentially anti-inflammatory compounds isolated from cyanobacteria, showing high through-put screening ability and capability of screening both weak and strong inhibitors by adapting the stimulation dose of the pro-inflammatory cytokine TNF. The combination of the two methods allows us to identify promising anti-inflammatory substances. Since the compounds are tested early and in real-time in pathologically relevant primary human microvascular cells, cytotoxic and barrier disintegrating side effects can be detected at an early phase of pre-clinical research.

Cyanobacterial toxicity, although deeply studied and discussed already, still causes problems with proper assessment of potential risks coming from contaminated water or accumulation of toxins by lower organisms, being a diet component. Dealing with different compounds present in natural sample containing cyanobacterial toxins, might hinder proper evaluation of the results – applied bioassay often do not show expected sensitivity and, on the

other hand, interactions between different mixture constituents or artifacts might provide false positive or negative results. It has been shown that using standard, easy-handled, adhesive cell lines highly correlated results can be obtained and the specific liver injury risk does not play an important role for cyanobacterial toxicity screening.

Standardization of extraction procedure and cell culture conditions as well as development and validation of screening panels based on *in vitro* testing, using different endpoints, together with chemical analyses, might be a solution of many ecotoxicological problems caused by cyanobacteria and a very useful tool for searching of pharmaceutically promising compounds.

IX Glossary

Aer-865	aeruginosin-865
Agma, agmatine	4-amidinobutylamide
AlphaLISA™	Amplified Luminescence Proximity Homogenous Assay
BSA	bovine serum albumin
Choi	2-carboxy-6-hydroxyoctahydroindol
COSY	correlation spectroscopy
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
EC_{50}	half maximal effective concentration
ECIS	electrical cell substrate impedance sensing
ESI	electrospray ionisation
EtOAc	ethyl acetate
FCS	fetal calf serum
FDAA	Marfey's reagent; 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide
HA	hexanoic acid
HeLa	cervical carcinoma cell line
HepG2	human liver carcinoma cell line
HiRes MS	high resolution mass spectrometry
HL-MVEC	human lung microvascular endothelial cell line
HMBC	heteronuclear multiple-bond correlation spectroscopy
Hpla	hydroxy-phenyl lactic acid
HPLC	high performance liquid chromatography
HSQC	heteronuclear single-quantum correlation spectroscopy
hTNF-α	human tumor necrosis factor-α
HUVEC	human umbilical vein endothelial cell line
IC_{50}	half maximal inhibitory concentration
ICAM-1	intercellular adhesion molecule-1
IL-8	interleukin-8
IR	infrared spectrometry
LC-MS	liquid chromatography coupled with mass spectrometry
ManA	mannuronic acid
MeCN	acetonitrile
MeOH	methanol
MS	mass spectrometry
MTT	methyl-tetrazolium test
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NMR	nuclear magnetic resonance
NPRS	non-ribosomal peptide synthetase

p38 MAPK	p38 mitogen activated protein kinase
PBS	phosphate buffered saline
PC	piceatannol
PDA	photodiode array detector
PKS	polyketide synthases
RPMI	Roswell Park Memorial Institute medium
SAR	structure-activity relationship
SB	p38 MAPK inhibitor SB203580
Sp/2	murine myeloma cell line
TOCSY	total correlation spectroscopy
Yac-1	murine polymorphotic lymphoma cell line

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