Vestec, 22nd June 2017

Reviewer's report of PhD Thesis entitled "Screening Cyanobacteria for apoptosis induction in human cancer cell limes: discovery of novel compound – Nocuolin A" submitted by Katerina Voracova, MSc.

The Thesis composed and today also presented by Katerina Voracova are concisely but distinctly and well understandably written and summarize her research endeavour in past couple of years of her PhD studies. Besides interesting especially unpublished data, the Thesis also touch the other less appealing but intrinsic side of research as dealing with not so positive data coming in this case from large-scale screening and fractionation of natural products (here extracts from cyanobacteria) in a hunt for novel and effective anticancer compound(s).

Formally, the Thesis is divided into introductory chapter composed of a brief overview of natural products universe and cyanobacterial secondary metabolites and more detailed part on regulated cell death. Then major aims of the Thesis together with the overview of published data from three attached articles are presented, followed by a chapter of unpublished data that are supported by a table, four figures and list of references. The last section contains three published papers together with their supplementary data.

Three attached papers form the fundaments of the Thesis deal mainly with the screening and fractionization of cyanobaterial extracts for pro-apoptotic activities of some of their components. First one published in the Journal of Applied Physiology deals methodical approaches, the other one published in Ecotoxicology and Enviromental Safety with a large scale, though unsuccessful screening for a pro-apoptotic compound. The third paper published in PLOS One analyses cancer cell death-inducing activity of another cyanobacterial metabolite Nocuolin A (NoA), which was identified in a different screening of cyanobacterium Nostoc sp. CCAP 1453/38. The data published in this paper suggest that this heterocyclic, likely highly hydrophobic metabolite triggers caspase-dependent apoptosis in HeLa and other cancer cells. These findings were then extended in the unpublished data section pointing to Nocuolin A as mitochondrial damaging agent triggering also extensive autophagy. These properties of Nocuolin A might be worthy of further investigation, which is however as I suspect is a bit limited by its availability (i.e. low yield from cyanobacteria extraction) and would thus likely require its chemical synthesis.

My questions, comments and suggestions are largely though not exclusively related to unpublished data:

1. In the screening for cells death-inducing compounds from cyanobacteria you used combination of time-lapse microscopy, caspase-3/7GLO and MTT assays. Each of them has its pitfalls and advantages so my question is did you consider some other approach e.g. in HTS screenings quite often used Cell Titer GLO viability assay (ATP concentration)? At IMG AS CR there is now established chemical biology HTS screening facility and thus might be helpful to get in contact with them.
2. The MMT viability assay performed with mouse fibroblasts deficient in certain apoptosis or necroptosis modulators/effectors treated with NoA and various inhibitors (Table 2) pointed to both NoA-triggered apoptotic and possibly also to necroptotic signalling (RIP3 KO) and strangely also to seemingly pro-survival role of Bax and Bak proteins. Does NoA triggers RIP1-

independent necroptosis (could be checked e.g. using MLKL inhibitor necrosulfonamide) and how could proapoptotic Bax/Bak proteins enhance survival of NoA-treated fibroblasts?

3. Treatment of HeLa and likely also of other tumour cells with nocuolin A longer than 2 hrs resulted in irreversible cell death triggering effects (Figure 5) possibly connected with mitochondria (Figure 6) and/or enhanced autophagy (Figures 6 and 7). Did you or do you plan to look in this phenomenon deeper (ROS production, respiration, mitochondrial membrane potential, release of cytochrome c etc.)?
4. The data in Figure 4 presenting increased mRNA expression of some pro-apoptotic (and also anti-apoptotic) genes, partly p53 transcriptional targets (p21, Puma, Noxa, Fas) point to some stress response, which could be especially at later time points a secondary one to a primary NoA-induced cellular damage. Did you check when during the time course of NoA treatment occurs to damage of chromosomal DNA?
5. Some notes to presented data. Were data in Fig. 4 reproduced? As presented w/o any deviation they were obtained max. from measurements. Data in Fig.4 possess so large standard deviations that their statistical significance is questionable. Either it will be necessary to obtain more data and/or use different statistical approach for their presentation.
6. In the discussion section the statement that cancer cells rely in ATP production mainly on glycolysis is not entirely true. A number of cancer cells do have very efficient mitochondrial respiration for the ATP synthesis and mitochondrial respiration is required for their growth.

In conclusion I do consider Katerina Voracova's Thesis very well suitable for her Thesis defence and upon I believe successful presentation and defence of the Thesis do recommend granting her PhD title.

.....

RNDr. Ladislav Anděra, CSc.

Vestec, 22nd June 2017

Reviewer's report of PhD Thesis entitled "Screening Cyanobacteria for apoptosis induction in human cancer cell limes: discovery of novel compound – Nocuolin A" submitted by Katerina Voracova, MSc.

The Thesis composed and today also presented by Katerina Voracova are concisely but distinctly and well understandably written and summarize her research endeavour in past couple of years of her PhD studies. Besides interesting especially unpublished data, the Thesis also touch the other less appealing but intrinsic side of research as dealing with not so positive data coming in this case from large-scale screening and fractionation of natural products (here extracts from cyanobacteria) in a hunt for novel and effective anticancer compound(s).

Formally, the Thesis is divided into introductory chapter composed of a brief overview of natural products universe and cyanobacterial secondary metabolites and more detailed part on regulated cell death. Then major aims of the Thesis together with the overview of published data from three attached articles are presented, followed by a chapter of unpublished data that are supported by a table, four figures and list of references. The last section contains three published papers together with their supplementary data.

Three attached papers form the fundaments of the Thesis deal mainly with the screening and fractionization of cyanobaterial extracts for pro-apoptotic activities of some of their components. First one published in the Journal of Applied Physiology deals methodical approaches, the other one published in Ecotoxicology and Enviromental Safety with a large scale, though unsuccessful screening for a pro-apoptotic compound. The third paper published in PLOS One analyses cancer cell death-inducing activity of another cyanobacterial metabolite Nocuolin A (NoA), which was identified in a different screening of cyanobacterium Nostoc sp. CCAP 1453/38. The data published in this paper suggest that this heterocyclic, likely highly hydrophobic metabolite triggers caspase-dependent apoptosis in HeLa and other cancer cells. These findings were then extended in the unpublished data section pointing to Nocuolin A as mitochondrial damaging agent triggering also extensive autophagy. These properties of Nocuolin A might be worthy of further investigation, which is however as I suspect is a bit limited by its availability (i.e. low yield from cyanobacteria extraction) and would thus likely require its chemical synthesis.

My questions, comments and suggestions are largely though not exclusively related to unpublished data:

1. In the screening for cells death-inducing compounds from cyanobacteria you used combination of time-lapse microscopy, caspase-3/7GLO and MTT assays. Each of them has its pitfalls and advantages so my question is did you consider some other approach e.g. in HTS screenings quite often used Cell Titer GLO viability assay (ATP concentration)? At IMG AS CR there is now established chemical biology HTS screening facility and thus might be helpful to get in contact with them.
2. The MMT viability assay performed with mouse fibroblasts deficient in certain apoptosis or necroptosis modulators/effectors treated with NoA and various inhibitors (Table 2) pointed to both NoA-triggered apoptotic and possibly also to necroptotic signalling (RIP3 KO) and strangely also to seemingly pro-survival role of Bax and Bak proteins. Does NoA triggers RIP1-

independent necroptosis (could be checked e.g. using MLKL inhibitor necrosulfonamide) and how could proapoptotic Bax/Bak proteins enhance survival of NoA-treated fibroblasts?

3. Treatment of HeLa and likely also of other tumour cells with nocuolin A longer than 2 hrs resulted in irreversible cell death triggering effects (Figure 5) possibly connected with mitochondria (Figure 6) and/or enhanced autophagy (Figures 6 and 7). Did you or do you plan to look in this phenomenon deeper (ROS production, respiration, mitochondrial membrane potential, release of cytochrome c etc.)?
4. The data in Figure 4 presenting increased mRNA expression of some pro-apoptotic (and also anti-apoptotic) genes, partly p53 transcriptional targets (p21, Puma, Noxa, Fas) point to some stress response, which could be especially at later time points a secondary one to a primary NoA-induced cellular damage. Did you check when during the time course of NoA treatment occurs to damage of chromosomal DNA?
5. Some notes to presented data. Were data in Fig. 4 reproduced? As presented w/o any deviation they were obtained max. from measurements. Data in Fig.4 possess so large standard deviations that their statistical significance is questionable. Either it will be necessary to obtain more data and/or use different statistical approach for their presentation.
6. In the discussion section the statement that cancer cells rely in ATP production mainly on glycolysis is not entirely true. A number of cancer cells do have very efficient mitochondrial respiration for the ATP synthesis and mitochondrial respiration is required for their growth.

In conclusion I do consider Katerina Voracova's Thesis very well suitable for her Thesis defence and upon I believe successful presentation and defence of the Thesis do recommend granting her PhD title.

.....

RNDr. Ladislav Anděra, CSc.

MARTIN-LUTHER-UNIVERSITÄT
HALLE-WITTENBERG

Faculty of Natural Science I -
Biological Science

Institute of Pharmacy

RG Biogenic Drugs

Prof. Dr. Timo Niedermeyer

Martin-Luther-University Halle-Wittenberg, 06099 Halle (Saale), Germany

Prof M. Oborník
Head of the Committee for PhD studies

University of South Bohemia in
České Budějovice

Your ref: Our ref: Date:

03th July 2017

Review of the PhD thesis of Ms Kateřina Voráčová

Dear Prof Oborník,

With great interest, I have read the thesis Ms Kateřina Voráčová has submitted.

Cyanobakterien are nowadays acknowledged as a valuable source for novel secondary metabolites with intriguing bioactivities. Especially cytotoxic compounds are often isolated from these organisms. As these toxic metabolites can potentially be used as lead structures in anticancer drug development, studying their chemistry as well as their pharmacodynamics is of great interest and value for the drug discovery community.

Ms Voráčová has contributed to this effort by screening a cyanobacteria extract library for apoptosis inducers, and by studying the biological activity of the novel cyanobacterial metabolite Nocuolin A, that could be isolated and identified subsequent to her bioactivity screening.

The thesis is a cumulative thesis, based on three manuscripts that have already been accepted and published in international peer-reviewed journals, two of them with Ms Voráčová as lead author. Her contributions have clearly been stated and justify her position as first author.
After a good introduction into natural products in general and cyanobacterial metabolites in particular she gives an overview over the current state of our current understanding about apoptosis. She then summarized both her published and her unpublished research and offers a conclusion. The concise literature reference section is followed by reprints of her publications.

Address:
Hoher Weg 8
06120 Halle (Saale)
Germany

Tel (0345) 55-25765
(0345) 55-25101

Fax (0345) 55-27407

Email:
timo.niedermeyer@pharmazie.uni-halle.de

Office:
kathrin.kunth@pharmazie.uni-halle.de

Internet:
www.pharmazie.uni-halle.de

The thesis is well written, although the order in which Ms Voráčová presents her publications is slightly confusing. Minor mistakes and inaccuracies (e.g. the IC_{50} is the half maximal inhibitory concentration, it is polyketide synthases instead of synthetase, BAX/BAK or Bax/Bak, etc.) do not significantly compromise her work.

As already stated, Ms Voráčová presents three published publications; this is adequate for six years PhD studies. Her written thesis as a whole is also an adequate work.

Thus I recommend to **accept** the thesis and proceed with the oral defense.

Sincerely yours,

Prof. Dr. Timo Niedermeyer

PIOTR RZYMSKI

POZNAN UNIVERSITY OF MEDICAL SCIENCES

DEPARTMENT OF ENVIRONMENTAL MEDICINE

Medical Biology Center
Rokietnicka 8, 60-806 Poznań
Poland

Phone: + 48 61 854-76-04
Fax: + 48 61 854-76-04
E-mail: rzymskipiotr@ump.edu.pl

The review report of the Ph.D. Thesis

„SCREENING OF CYANOBACTERIA FOR APOPTOSIS INDUCTION IN HUMAN CANCER CELL LINES: DISCOVERY OF A NOVEL COMPOUND NOCUOLIN A"

by Mgr. Kateřina Voráčová

(supervisor: RNDr. Pavel Hrouzek, Ph.D.)

prepared at Institute of Microbiology, Academy of Sciences of the Czech Republic, University of South Bohemia, České Budějovice, Czech Republic

Mgr. Kateřina Voráčová submitted the doctoral thesis dealing with pro-apoptotic activity of cyanobactial metabolites, and discovery of specific compound, named nocuolin A that was evidenced to reveal a potential to induce apoptosis in human cell lines.

The submitted thesis is based on the three papers published in JCR journals: Journal of Applied Phycology (IF=2.372), PlosOne (IF=3.057), and Ecotoxicology and Environmental Safety (IF=3.130). Mgr. Kateřina Voráčová is the first a of the former two, and fourth author of the third paper. The selection of these papers is, in my opinion, sufficient for doctoral thesis not only due to well-established scientific position of the abovementioned journals but mainly because each paper is a logical continuation of the previous one. The pilot paper deals with toxicological aspects of the large set of cyanobacterial extracts, second one links the activity of extracts with apoptosis inducement, and final paper presents the novel cyanobacterial metabolite from the group of oxadiazines, nocuolin A, that is proved to induce apoptosis in human cancer cell lines.

All three papers around which the thesis has been built were published recently: one was published in 2016 (and its online version appeared late 2015), and two other were published in 2017. Therefore it comes as no surprise that these papers are still not widely cited. According

to the Web of Science (WoS), study published in Ecotoxicology and Environmental Safety was cited once; the paper published in PlosOne was just recently indexed in WoS while the study published in Journal of Applied Phycology is yet to be indexed. According to Scopus database, the paper published in Ecotoxicology and Environmental Safety has one citation. However, if one considers the interdisciplinary scope of conducted investigations, and that they also include description of novel cyanobacterial metabolite with promising activities, it is highly expected that all three papers will increase the citation statistics soon.

The entire doctoral thesis consists of 128 pages. It is divided into five main chapters presenting the following: background and rationale for the conducted studies, specific aims of the study, summary to published results, presentation of the complementary unpublished results, and general conclusions that have been drawn. At the beginning, a helpful list of abbreviations is introduced while at the end of the thesis, a brief curriculum vitae of Mgr. Kateřina Voráčová is presented. The whole thesis is well and logically organized, presented in a clear and concise manner. In fact, it is enjoyable to read, specifically due to (*1*) very good English language; there are nearly no typing errors, (*2*) the tables (2) and figures (7) that enrich the main text; (*3*) supporting the background of thesis and discussion of the unpublished results with overall of 120 references encompassing papers published in repute peer-reviewed dealing with cell biology, environmental toxicology and cancer research.

Cyanobacteria are a very interesting and fascinating group of microorganisms which have attracted much attention over the decades due to several reasons: (*1*) The long-term evolution (at least 3.5 billion years) have triggered various adaptations and mechanisms that allowed them to occupy nearly every possible type of environment (including their contribution to human microbiome) and tolerate conditions known to be extreme, toxic or even, mostly inhabitable; (*2*) The blooms formed by cyanobacteria cause relevant ecological and economic consequences, and the incidence of these event have increased due to nutrient loading that originates from human activities; (*3*) The ability of some species (or strains) to produce toxins (some identified and some other yet to be identified) posing relevant risk to human and animal health; (*4*) The use of some species (e.g. *Arthrospira platensis*) as a source of food and animal feed; (*5*) The potential use of some species as a source of biofuels; (6) The potential of cyanobacteria as a source of compounds revealing anti-bacterial or anti-cancer activities. The submitted thesis deals with the latter aspect as it seeks for cyanobacterial metabolites to be potentially selected as anti-cancer agents. However the research on natural products has been

now largely reduced by pharmaceutical companies, seeking for the natural sources of anti-cancer drugs is still highly required due to several reasons: (*1*) It is estimated that that 23.6 million new cases of cancer will be recorded globally each year by 2030; (*2*) Various anti-cancer agents that are already in use reveal high toxicity against other human cells causing severe adverse effects in patients; (*3*) Some cancer cells can develop resistance to cytostatics. In conclusion, the doctoral thesis presented by Mgr. Kateřina Voráčová has a interdisciplinary character, fits into a very important field of drug research and has high application value (or at least it has a value to launch *in vivo* studies evaluating the use of nocuolin A as anti-cancer agent).

The background of the thesis is divided into three subsections: one discusses the role of natural products in drug development, second introduces cyanobacteria and their metabolites, and third presents the types and pathways of cell death. Altogether these sections provide enough rationale for the conducted studies and introduce discuss all aspects significant to understand the meaning of results which were obtained. However, in my opinion, these sections, and specifically those dealing with natural products and cyanobacyerial metabolites are presented too briefly and as long as they convey most important information, I believe some extensions would be beneficial.

In the first part, number of pitfalls related to research on natural products as potential novel drugs are presented. These pitfalls have led to reduced the research investment in natural products over the last decade by some large players on pharmaceutical market although some European and Asian companies are still involved in such investigations. Because there are number of natural products that have been approved as drugs, Mgr. Kateřina Voráčová decided to present three compounds that reveal anti-cancer activity in more detail (paclitaxel, dolastatin and aurilide). It should be however stressed that since 1961 nine plant-derived compounds have been approved in the US for use as anti-cancer pharmaceuticals. It is obvious that anti-cancer therapies attract most of attention but there are other therapeutical areas in which natural products are already involved. I regret that these areas and compound groups were not summarized in some form, e.g. as a table. It would make a nice contribution to this already fine thesis.

In the second background section secondary metabolites produced by cyanobacteria are introduced. In this part, Mgr. Kateřina Voráčová provides an excellent and concise description of the general biosynthesis pathways of these compounds. This part is also supplemented with a helpful table presenting selected compounds isolated from marine cyanobacteria and their

potential or evidenced biological targets. The thesis obviously pays attention to potential therapeutical agents produced although being an ecotoxicologist I have to highlight that number of toxic metabolites are also synthesized by cyanobacteria (e.g. microcystins, cylindrospermopsin, anatoxin-a, saxitoxins, beta-methyl-L-alanine - BMAA etc.). Research on these compounds, their toxic activity and mechanism of action that still attract much attention can also lead to important discoveries, related surprisingly to potential development of therapeutical strategies (*vide* BMAA-induced neurodegeneration and its prevention with the use of L-serine). I have only one technical comment - I would prefer table captions to be presented classically, above the main item (instead of below the table) just as they are given in the published papers.

The last section of thesis background focuses on cell death. It clearly appears that this is truly the field Mgr. Kateřina Voráčová feels best at. She describes, in all important details, the characteristics of different types of cell death: necrosis, necroptosis, pyroptosis, autophagy and apoptosis. Then the intrinsic and extrinsic pathways of the latter are introduced, accompanied by self-made figures (which are a very fine contribution, thank you!). Finally, the proteins expressing pro- or anti-apoptotic function are described. Along with two previous chapters, the background fully supports the objectives of the conducted studies which were clearly defined by Mgr. Kateřina Voráčová.

As I have already mentioned, the presented papers are logically organized. The study published in Ecotoxicology and Environmental Safety provides a large data set on cytotoxic activities of over 100 crude extracts of terrestrial cyanobacteria species. The study used *in vitro* model and employed different human and rodent cell lines. To the best of my knowledge this is the broadest screening of this kind performed so far, very valuable for other researchers dealing with toxicity of cyanobacteria. The study highlights that terrestrial cyanobacteria (which are generally less investigated that they marine/freshwater relatives) contain powerful, biologically-active compounds - as reported as much as one-third of tested extracts revealed relevant cytotoxic effects in different cell lines. Apart from toxicological studies, the extracts found to be most active were also characterized with the use of activity-guided HPLC. This approach was a valuable contribution to the study because it revealed that the toxicity of extracts has to be attributed to the mixture of several compounds rather than just one toxic metabolite.

The natural step forward was to screen cyanobacterial extracts for their potential pro-apoptotic activity, and this step was approached with another study published in Journal of Applied Phycology. Again, the study tested a large set of cyanobacterial extracts. The

pancreatic tumor cell line PaTu 8902 was employed as an *in vitro* experimental model while the potential apoptotic action of extracts was assessed by activity of caspase 3 and 7 (Casp3/7) detected using luminescence assay. As found number of extracts caused enhanced Casp3/7 activity. On the methodological note, authors also highlighted that the luminescence data has to be normalized to cell count which is quite an obvious observation, Similarly, when performing colorimetric or fluorometric evaluation of Casp3/7, one has to simultaneously determine protein content to avoid a bias. Importantly, the study reveals the greatest pro-apoptotic activity by an extract from *Nostoc* sp. that contained nocuolin A.

The third paper presents the chemical structure of nocuolin A, a novel cyanobacterial metabolite. It is identified based on coupled analyses employing NMR, HRMS and FTIR techniques and classified as a oxadiazine. This study opens a new set of questions to be assessed in future research such as those related to the potential production nocuolin A by other cyanobacteria, and the ecological role of this compound. Nevertheless, the presented study reports that nocuolin A cause a drop in ATP and reveal pro-apoptotic activity in various human cancer cell lines. However the observed effects were lower than those induced by taxol, the p53-mutated lines were found to be more sensitive which is a very promising finding. The study also provides information on molecular bases of nocuolin A biosynthesis. This is particularly valuable for future studies aiming on identification of this compound in other species/strains. All in all, I find this paper as an excellent summary of this doctoral thesis, a valuable contribution to the field of basic research on cyanobacteria and cancer studies. This paper is followed by yet unpublished studies, presented at the end of the doctoral thesis, and aimed to elucidate exact mechanism of action revealed by nocuolin A. Importantly, the rapid onset of permanent effects was reported and evidenced by changes in mitochondrial and cellular morphology, and wash-out experiment. The gene expression profile after exposure revealed changes in pro-apoptotic but also some anti-apoptotic genes. Interestingly, the potential of nocuolin A to induce autophagy was observed. These unpublished results are delivered and discussed logically and clearly. Mgr. Kateřina Voráčová approach them with appropriate caution by stressing limitations in interpretation and need for further studies.

I find the conducted studies and presented results of high quality. provide a very interesting, promising and inspiring results. I hope the studies on nocuolin A will be continued with the use of other models. I have however two questions that I would like Mgr. Kateřina Voráčová to address:

(*1*) The toxicity of extracts has been evaluated using MTT assay. Could you please comment on potential interference of this assay with some natural compounds? Would you recommend use of other assay to assess cytotoxicity in cell cultures?

(*2*) Is there any data on general toxicity of nocuolin A? As presented, it reveals a promising pro-apoptotic activity in cancer cells its potential toxicity in other cells would limit its further application.

Apart from doctoral thesis, I have reviewed other scientific achievements of Mgr. Kateřina Voráčová. After defending her MSc thesis on isolation of intact plastids of the *Chromera velia* in 2001 she has become a PhD student. She has attended total of six conferences, including international ones, at which she has presented posters and delivered oral presentation. She has also undertaken two research visits, one in BC Cancer Agency in Canada and second in Innsbruck Medical University in Austria. It is obvious that these visits had to influence her current interest dealing with discovering novel anti-cancer drugs on natural origin. It should also be stressed that Mgr. Kateřina Voráčová is co-author of one patent, and has mentored a student preparing a bachelor thesis. These achievements are fully sufficient for PhD candidate. I express my hope that Mgr. Kateřina Voráčová will continue her work as a researcher and expand it by involving herself in education and science popularization.

In my humble opinion, the reviewed doctoral thesis submitted by Mgr. Kateřina Voráčová fulfills all conditions required by doctoral theses. The thesis is ready to be defended, in front of respective committee. The presented data has interdisciplinary character, high application value and will likely inspire future studies on therapeutical applications of cyanobacterial metabolites. I highly recommend Mgr. Kateřina Voráčová for the PhD degree.

Poznań, June 15th, 2017

Dr hab. n. med.
Piotr Rzymski