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REVIEW

of the Doctoral dissertation of Mr. Bac Viet Le

titled: **„TGFβ-dependent mechanism of the bone marrow-mediated resistance against PARP inhibitors in leukemias"**

made under the supervision of Dr. hab. Katarzyna Piwocka at the Nencki Institute of Experimental Biology of the Polish Academy of Sciences and Temple University Scholl of Medicine USA

This is an interesting work seeking for explanation of mechanisms behind the increased resistance of leukemic cells to the current therapeutic approach based on poly ADP-ribose polymerase inhibitor (PARPi)–induced synthetic lethality. Synthetic lethality using PARPi is a clinically approved cancer treatment, typically used in *BRCA1/2*–mutated breast and ovarian cancers. Its application for the treatment of blood cancer is an interesting clinical problem *per se*.

Understanding all components of the PARPi resistance process and the cellular response to therapy is not only cognitive, but also of great practical importance, as pharmacological enhancement or reduction of the response, or inhibition of cell death/degenerative processes is crucial to the success of the therapy of many diseases, including blood cancer.

Having an access to PubMed (National Library of Medicine) data of the Candidate, I noticed the co-authorship of 5 original high-impact papers. None of the publications is directly related to the doctorate. However, they were the basis and starting point for the research described in the dissertation. The authors found that expression of leukemic oncogenes leads to BRCA1/2 deficiencies, resulting in sensitivity to PARP inhibitors. Moreover, a specific oncogenic tyrosine kinase inhibitor FLT3 (ITD) induced the "BRCAness" phenotype, increasing the effectiveness of PARPi

inhibitors in acute myeloid leukemia (AML). However, despite the successful use of PARPi in BRCA1/2-deficient cancers, emerging resistance has been observed. The studies within the doctoral studies were focused on elucidating the precise role of bone-marrow-mediated resistance to PARP inhibitors in FLT3(ITD) and BRCA1/2 deficient leukemic cells and specimens from leukemia affected individuals of mouse and human origin.

The dissertation was prepared according to the traditional rules as a script-book composed basically of the Introduction, Aims, Materials and Methods, Results, Discussion, References and accompanying chapters. In connection with the AIMS, which are clearly and concisely formulated, a literature INTRODUCTION quite extensively presents the current state of knowledge in the field in question, the mechanism of presenting its principles, performance, known elements of molecular mechanism and clinical applicability. This section has been written with great competence and a good selection of information fairly within the frames of dissertation. A reader also receives carefully developed illustrative materials that helps to follow the line. Five of the twelve drawings are fully acceptable modifications to the originals published by other authors. The remaining drawings are original works by the author. I only miss a bit of a brief description of lymphoblastic leukemias, what causes them? I am also curious in why myeloid leukemias mainly occur in adults, and ALL and CLL in children? MATERIALS and METHODS seem to be adequately adjusted to meet the intended goals. Descriptions of the experimental conditions are accurate and detailed enough to be replicated in independent experiments of other researchers. The value of the presented new methods at work is very high and I rate it very highly. A strong technical point is the selection of techniques allowing to determine both cellular and molecular aspects of the title problem. The categorization of techniques comprises such classical attempts as clonogenic and cell viability assays, cell cycle analysis, DNA damage determination by neutral comet assay and double strand break assay, western blot, and *in vivo* experiments in mice. The techniques used are only partially listed. However, when PhD student can only operate with half of them, then any laboratory would be happy and proud to have such a talented researcher. I must add that all technical protocols were written with high precision and accuracy. RESULTS are presented on 42 pages. Huge work – long section. They represent a series of very carefully planned and conducted experiments. First, control experiments to confirm that inhibition with a specific FLT3 receptor inhibitor (IDT) silenced the signaling cascade leading to the repair of DNA double strand breaks (by acquiring the so-called BRCAness phenotype) and, consequently, sensitizing leukemia cells to PARP inhibitors, inducing synthetic lethality. For this

purpose, several murine and human cell models were tested. Finally, the results were confirmed *in vivo*, showing an extended survival of leukemia-bearing mice. The candidate further proved that the increase in synthetic lethality is due to the bone marrow microenvironment, more specifically, stromal cells-derived factors/cytokines. Ultimately, using a multi-faceted approach, the author finally manage to prove that the activated TGFβ1-TGFβR-SMAD3 signaling pathway is responsible for this process. The results confirmed that pharmacological inhibition of the TGFβR receptor can be used in the treatment of myeloid leukemias, making them more sensitive to synthetic lethality caused by PARP inhibitors. The graphs and pictures in this section convincingly documented the obtained results. The DISCUSSION section is well done and responds to the required explanation of possibilities and limitations of the study as well as confronts its own results and conclusions with the publications of other authors. It also allowed the PhD student to properly summarize the work and outline a proposal for further clinical application. The summary ending the thesis contains the most important conclusions from the doctoral dissertation being assessed here, collected in brief points, which allows the reader to clearly assess the content of the whole.

To summarize, the results obtained by the doctoral student are innovative and valuable, they bring significant findings in the field of research on the mechanism of resistance to PARP inhibitors in the bone marrow microenvironment of leukemia cells and affected individuals, objectively confronting them with the existing state of knowledge. Moreover, they promise to find an effective long-term therapeutic strategy for leukemias related to the activation of oncogenic tyrosine kinases. These results are already ready to be published in a high-quality article and will likely be submitted soon.

The dissertation consist of 153 pages. The book looks nicely in terms of esthetic impression. Concerning the formal aspects, there were hardly any typing or editing errors. This is not a common case. I also appreciate the linguistic correctness.

Detailed comments:

1. Page 12, Introduction: "Several oncogene-positive leukemias (*eg.* "AML1-ETO, BCR-ABL1, MLL-AF9), that carry deficiencies in BRCA1/2 proteins, have been **eliminated** by PARPi...". I think in this sentence it would be better to use the phrase ..."have been **cured**..." instead of „ have been **eliminated**...", because that sounds like saying that these leukemias were removed from the population. This statement is used for some infectious diseases and

vaccines (*e.g.* vaccines eliminated smallpox). However, in this case, unfortunately, this was not the case.

2. Page 20, Introduction: Base excision repair is not responsible for the repair of DNA double strand breaks, as stated in the sentence: "More importantly, deficiencies in DSB repair pathways (comprising homologous recombination, non-homologous end joining, base excision repair)...". While it is true that unrepaired oxidative DNA damage can lead to single strand breaks, which then turn into double strand breaks during replication. ".
3. Page 27, Introduction: "Among all types of DNA damage, the DNA double-strand break (DSB) **is the most** lethal type of DNA lesion to cells". This statement is not entirely true. Inter-strand cross-links (ICLs) are even more deadly, and DNA double strand breaks are an intermediate step generated during the repair of ICLs. I think it would be more correct to write " Among all types of DNA damage, the DNA double-strand break (DSB) **is one of the most** lethal types of DNA damage." ICLs are created, among others (including chemotherapeutic agents), as a result of cellular metabolism (attack of reactive aldehydes formed after ROS attack on cellular lipids).
4. Page 27, Introduction: I do not really understand what the term "chromosomal inactivation" means. Maybe it was about "chromosomal aberration"? Nor is it clear to me what the "constitution of oncogenes" means?
5. Page 30, Introduction: BER pathway repairs base damage derived from intracellular metabolic processes, including oxidation, alkylation and deamination, but also from extracellular sources, such as, for example, alkylating agents, including chemotherapeutic drugs.
6. Page 96, Results, Figure 4.27: It is not clear whether the statistical significance (*) relates to the difference between SB431542 + PARPi + TKi and Vehicle or SB431542 + PARPi + TKi and PARPi + Tki?
7. Page 108 and 109, Results: Figures 4.37 and 4.38 should be placed in the reverse order as described in the text.

A few critical comments listed above do not affect the substantive value of the work, which I rate very highly. In my opinion, the doctoral dissertation entitled "TGFβ-dependent mechanism of the bone marrow-mediated resistance against PARP inhibitors in leukemias" meets the conditions specified in Art. 187 of the Act of July 20, 2018 Law on Higher Education and Science (Journal of Laws of 2018, item 1668) and the Regulations of the Scientific Council of April 13, 2018, Annex no.

1 (as amended from 06/12/2019) (Stwierdzam, że rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z 2018 r. poz. 1668) oraz Regulaminu Rady Naukowej z 13.04.2018 r. załącznik nr. 1 (ze zm. z 06.12.2019 r.). Therefore, having an undoubtful positive opinion about the dissertation, I am applying to the Scientific Council of the Nencki Institute of Experimental Biology of the Polish Academy of Sciences to admit Mr. Bac Viet Le to further steps envisaged in the doctoral procedure.

Due to the high scientific value of the doctoral dissertation and its application potential, I would like to recommend this work for distinction.

Elżbieta Speina

Dr hab. Elżbieta Speina

Review of the PhD thesis of Bac Viet Le titled "TGFβ-dependent mechanism of the bone marrow-mediated resistance against PARP inhibitors in leukemias"

The PhD thesis was completed at the Laboratory of Cytometry, Nencki Institute of Experimental Biology Polish Academy of Sciences and Temple University School of Medicine, USA, under the supervision of dr hab. Katarzyna Piwocka, Professor of the Nencki Institute. The hypothesis tested in the dissertation was based on the previous reports of this international group showing that some tyrosine kinases inhibitors used in the targeted therapy of leukemia can induce deficiencies in DSB repair proteins involved in BRCA-mediated homologous recombination (HR) and DNA-dependent protein kinase-mediated nonhomologous end-joining (D-NHEJ). This opened up the possibility of co-treatment with poly(ADP)ribose polymerase (PARP) inhibitors, which nowadays are used in the clinic for BRCA-mutant cancers. Hypothetically, additional inhibition of PARP1-mediated DNA repair pathways (which serve as a backup for DNA repair) causing abundant accumulation of toxic DSBs might result in enhanced elimination of cancer cells through the mechanism of synthetic lethality. In the dissertation, an inhibitor of mutated FLT3 tyrosine kinase (Quizartinib/AC220) was tested for its ability to trigger deficiencies in DSB repair pathways and sensitize FLT3-mutated leukemia cells to PARP inhibitors. It is reasonable since internal tandem duplications of FLT3 (FLT3-ITD) are the most common mutations associated with acute myeloid leukemia (AML) and are a prognostic indicator associated with adverse disease outcome. Also, quizartinib (AC220) had good results in phase II clinical trial for refractory AML patients with FLT3 mutations. Noteworthy, resistance to PARPi is a frequent problem in the clinic. Hence, the dissertation also verified the hypothesis that bone marrow microenvironment (BMM) induces PARPi resistance in BRCA1/2 deficient leukemias. Furthermore, the molecular mechanism responsible for the BMM-mediated resistance to PARPi in leukemic cells *in vitro* and *in vivo* was investigated.

The **Introduction** section is strictly related to the topic of the dissertation. It starts from a general description of leukemia, focuses on leukemia-related oncogenic tyrosine kinases and their inhibitors, presents the concept of synthetic lethality, DNA double-strand break repair pathways, and the role of PARP1 in DNA repair. The current understanding of the role of bone marrow microenvironment in the acquisition of resistance to PARP inhibitors in leukemia is also described. The Introduction is logically organized, comprehensive, and very well written (based on 250 cited papers).

The **Materials and Methods** section starts from the list of cell lines used in the study (a large number of relevant models was involved). Human and mouse, established and primary, wild type or genetically modified cell lines of different origins are characterized and culture conditions are

described. Remark to this part: it is not clear how FLT3(ITD)-positive BaF3 cells were obtained (probably via viral transduction but details are lacking), and if after clonal selection, individual clones were used or the mixture of clones. I appreciate the list of antibodies with all the details. Unfortunately, however, there is no information about the working concentrations of inhibitors, except the dose used in *in vivo* treatment of mice. I would also expect a list of all mouse strains used in experiments, more details about their genetic background, and the age of animals. I am not sure whether 200 Gy dose used to sub-lethally irradiate mice is quoted properly. In general, 7-11 Gy is a lethal dose (depending on the strain) while for immunosuppression in SCID mice, 0.5-2.5 Gy is recommended. This should be commented on. Other remarks to this section: some methods are described in sufficient detail (especially standard methods such as DNA cloning) but many details are generally missing or even appear to be wrong. For example, I have reservations about the composition of the buffers used for the nuclear extract preparation (very low salt concentration, i.e. 0.4 mM NaCl, would not allow the extraction of histones, which were assessed by western blot; e.g. in Fig. 4.2). Also, using 100 µg of RIPA protein extract from cell culture for the SDS-PAGE gel for western blot analysis might result in sample overloading. Plasmids used as reporters in the DNA double-strand break repair assay, as well as other plasmids used in the study, are not described in sufficient detail, and the source of pMIG-IRES-GFP is unknown. Furthermore, I can not find any methodology for cell death analysis by detection of Annexin-V-positive cells (shown in Fig. 4.4), doxorubicin treatment (shown in Fig. 4.7), splenocytes isolation, retroviral transduction. It would also be useful to clarify the principles of hematopoietic cell isolation and the selection of markers. Summarizing, this section enables an understanding of how the results were obtained in general (included Figures are very useful). However, based on the provided methods' description the experiments could not be successfully repeated.

In the first subsection of the **Results**, it was shown that selective inhibition of the FLT3(ITD) by AC220 in the FLT3(ITD)-positive leukemia cells (but not WT cells) affected DSB repair proteins level and activities of HR and D-NHEJ pathways. Thus, co-treatment with PARP inhibitors (to additionally block alternative non-homologous end-joining, B-NHEJ) resulted in increased cell death. The ability of AC220 to sensitize FLT3(ITD)-positive cells to PARP inhibitors was proven using several complementary methods in human and mouse established cell lines and primary cells isolated from the bone marrow of genetically modified mice as well as human primary cells from AML patients. Anti-leukemia effect of such combined treatment was also confirmed *in vivo* in mice engrafted with human FLT3(ITD)-positive leukemia from patients. Taken together, the presented data shows that inhibition of the oncogenic FLT3(ITD) receptor by FLT3 inhibitor triggers synthetic lethality induced by PARP inhibitors in the leukemia cells *in vitro* and *in vivo* due to rendering deficiencies of BRCA1, BRCA2, RAD51, and LIG4 in malignant cells.

The second subsection of the **Results** aimed to show that the bone marrow microenvironment is involved in the acquisition of resistance to PARPi-mediated synthetic lethality in leukemias. It was shown that BRCA-deficient leukemia cells were sensitive to olaparib (PARP inhibitor) in a dose-dependent manner but they became refractory when treated in co-culture with stromal cell lines (direct contact) in hypoxia. In clonogenic assay used in these experiments (Figs. 4.10-4.12), all cells were plated into methylcellulose (chapter 3.4.1), so the question is if/how stromal cells from co-culture contribute to the total number of colonies (the same question is for comet assay, Fig. 4.13). However, this issue was resolved by experiments with indirect co-culture and conditioned medium (Figs. 4.14-4.15). These experiments proved that direct contact between bone marrow stromal cells and leukemia cells is not necessary to induce the resistance to PARP inhibitors and the effect can be mediated by released cytokines. Comment to this part: one could be interested in how it was possible to obtain over 100% of quiescent cells (Fig. 4.15B).

The second variable whose influence should be excluded is the oxygen level differing in both culture conditions. This issue was partially investigated in the third subsection of the **Results** aimed to elucidate a molecular mechanism of the bone marrow microenvironment-dependent resistance to PARPi in leukemias. An increased TGFβR2 level in AML cells cultured in hypoxia alone (Fig. 4.18B) indicates/suggests that hypoxia may be critical for the PARPi resistance, not co-culture with stromal cells. Especially that it was observed by others (Wierenga et al., 2014) that CD34+ cells cultured under hypoxic conditions secreted high levels of latent TGFβ (maybe it should be also checked in the experimental system used here). However, no difference between normoxia and hypoxia in the sensitivity to PARPi olaparib was detected, at least in mouse AML-like FLT3(ITD); Tet2-/- cells cultured in the absence of bone marrow stromal cells (Fig. 4.21). Treatment of AML cells with recombinant TGFβ1 in hypoxia resulted in higher resistance to olaparib while targeting of TGFβ1 – TGFβR signaling pathway re-sensitized cells to olaparib. More importantly, such treatments are safe for Lin-CD34+ bone marrow cells from healthy donors. Further extensive *in vitro* and *in vivo* studies have shown that the TGFβ1 – TGFβR – SMAD3 signaling (but not TGFβR – miRNA-182 or TGFβR-mediated non-canonical protein kinase signaling), activated in the bone marrow niche, plays a role in the acquired resistance to PARPi-induced synthetic lethality in leukemias. At the same time, the involvement of the CXCL12-CXCR4 signaling pathway in this process was excluded. Importantly, the results showed that pharmacological inhibition of TGFβR is possible to sensitize CML/AML cells to PARP inhibitors.

The **Discussion** chapter summarizes the results obtained in the experimental part of the work in the context of the literature data (with the predominance of re-describing the results). The rationale for the experiments was also presented, including explanations of why certain potentially relevant

signaling pathways were not explored. This part of the dissertation proves the candidate's knowledge of the topic and scientific maturity.

In general, the organization of the dissertation is reader-friendly. However, due to the simplified description, many important details are not presented sufficiently (e.g. treatment conditions in many experiments). Moreover, figure legends are kept to a minimum, with a tendency to generalize the results, which could lead to confusion. Some examples:

1. The clonogenic assay is the primary method used for the estimation of cell survival. The percentage of colonies was calculated based on colony number of the untreated group considered as 100% (chapter 3.4.1). Whereas 50-125% of colonies are shown in groups described as untreated in Fig. 4.24. Thus, probably these groups are not untreated.
2. Involvement of FLT3(ITD) inhibition on the level of DSB repair proteins was investigated in mouse BaF3 cells as well as in primary FLT3(ITD)-positive leukemia cells which were also used for analyses of DSB repair proteins under inhibition of JAK1/2, PI3K, and RAF1. Whereas further functional studies (DSB repair activities of HR and D-NHEJ pathways, DSBs level analyses) were performed in different lines.

It has to be mentioned that some of the results included in the PhD thesis were obtained by collaborators (for example Fig. 4.28, 4. 34B), which is properly marked in the text. However, the methodology of these experiments is not described in the Materials and methods section. Nevertheless, it is a matter of debate whether these results are necessary for the dissertation.

Some minor inconsistencies/mistakes:

- Chapter 3.4.5: eBioscience Cell Proliferation Dye eFluor 670 (Invitrogen #65-0840) is used in the quiescent stem cell assay (it can be detected using a filter set equivalent to APC). This information is inconsistent with chapter 4.2.2 (page 82) where cell proliferation dye based on CFSE (with different excitation/emission spectra) is described.
- PALB2 – not shown in Fig. 4.1 as it is stated in the text.
- Why in Fig. 4.7B “colony number” instead of % of colonies is shown?
- Incorrect reference to chapter 4.2.6 on page 94 (no such number).
- Fig. 4.25 – incomplete the y-axis description.
- Fig. 4.35A – wrong description. I would not show this figure in the Results section. Additionally, since there are no plasmid maps, one cannot assess if the cloning was successful.
- Minor linguistic errors need to be corrected.

Summary

The assumptions and goals of the study were clearly defined. The planned experiments were adequate to resolve the scientific problem and performed properly. The summary of the results in nine points sufficiently reflects the content of the dissertation (more results were presented in the dissertation). The conclusions are correctly drawn and supported by the results. The PhD thesis proved the candidate's general theoretical knowledge in a discipline and the ability to independently conduct scientific work. Also, the subject of the PhD thesis is an original solution to a scientific problem. Therefore, the PhD thesis meets the conditions specified in Art. 187 of the Act of July 20, 2018, Law on Higher Education and Science (Journal of Laws of 2018, item 1668) and the Regulations of the Scientific Council of the Nencki Institute of Experimental Biology of April 13, 2018, Annex no. 1 (as amended from December 6, 2019).

Therefore, I am requesting for Bac Viet Le to be admitted to the next stages of the procedure. In my opinion, the weakness of the thesis is the tendency to generalize the results and the deficiencies in the description of the research methodology. However, taking into account the wide scope of the research and its high quality (including complementarity of research models), the logical layout of the work, and the importance of the research problem, the possibility of distinction can be considered.

Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z 2018 r. poz. 1668) oraz Regulaminu Rady Naukowej Instytutu Biologii Doświadczalnej im. Marcelego Nenckiego z 13.04.2018 r. załącznik nr. 1 (ze zm. z 06.12.2019 r.).

Wiesława Widłak

prof. dr hab. Wiesława Widłak

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Gliwice, July 29, 2020

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MEDICAL UNIVERSITY OF WARSAW

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Review of the doctoral dissertation

Title: TGFβ-dependent mechanism of the bone marrow-mediated resistance against PARP inhibitors in leukemias
Author: Bac Viet LE, MSc
PhD Thesis Supervisor: Dr hab. Katarzyna Piwocka, Professor of the Nencki Institute

The leading topic of the doctoral dissertation by Mr. Bac Viet LE, MSc is centered around the applicability of poly ADP ribose polymerase inhibitors (PARPi) as anticancer compounds in hematological malignances of the leukemia type. Generally, PARPi can be applied for a range of indications, but their most acknowledged application occurs in cancers defective in function of such proteins as BRCA1 or BRCA2. While the potential application of PARPi in leukemias has been proposed previously, which is properly mentioned in the doctoral dissertation, this topic is very vital for the current biomedicine and the results regarding synthetic lethality of PARPi in leukemic cells presented by the PhD Candidate in his doctoral dissertation shed new light on several important aspects within this research area.

The doctoral dissertation has been prepared in the form of an extensive monograph comprising 153 pages. The dissertation is well organized in a typical layout for PhD theses in the biotechnology field, including 6 main chapters: 1- Introduction, 2- Aims, 3- Materials and Methods, 4- Results, 5- Discussion, 6- Summary and Conclusion, and also the title page, list of funding organizations, acknowledgement, list of abbreviations, abstracts in English and Polish, table of contents, ending with the list of 336 relevant references. The text of the main chapters is supported by 57 figures and 2 tables.

Chapter 1, Introduction, provides at the beginning a brief information concerning the general medical characteristics and classification of leukemias along with the epidemiological data regarding the incidence and mortality due to leukemias in Poland and United States of America. Furthermore, this chapter describes leukemia-related oncogenic tyrosine kinases (OTK) with special emphasis placed on FLT3(ITD) in acute myeloid leukemia (AML) and BCR-ABL1 in chronic myeloid leukemia (CML), and also shortly characterizes the inhibitors of both types of

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kinases, such as quizartinib for FLT3(ITD) and imatinib mesylate for BCR-ABL1. The Author describes the most common mechanisms of the resistance against OTK inhibitors in leukemias and introduces the concept of synthetic lethality that can be therapeutically exploited, and also is the basis for application of PARPi in cancers. In the continuation of the Chapter 1, the Author presents a comprehensive review of the cellular DNA break repair pathways, including the roles for PARP in the repair of double- and single-strand DNA breaks. Subsequently, the Author the characterizes compounds acting as PARP inhibitors, both the FDA-approved ones and the ones currently under development, and describes the modes of their actions and the most common mechanisms of resistance to PARPi. In line with this information, the Author presents the bone marrow microenvironmental (BMM) niche as a potentially novel contributor to resistance against PARPi in leukemic malignancies. Notably, this is a novel approach to the potential applicability of PARPi against leukemias, derived directly from the previous publications from the laboratory of Prof. Tomasz Skorski (Temple University School of Medicine, Philadelphia, PA, USA), who has been co-supervising the project described in the doctoral dissertation. Lastly, the Author introduces the CXCL12-CXCR4- and TGFβ-mediated signaling cascades as the points of interests in tackling the molecular mechanisms of the role of BMM in resistance to PARPi. Altogether, Chapter 1 is very interesting as a scientific review of the studied research area and properly introduces the reader to the topics of PARPi-induced synthetic lethality in FLT3(ITD)-positive leukemic cells and the TGFβ-mediated mechanisms of resistance against PARP inhibitors in leukemias, as analyzed further in the doctoral dissertation.

In Chapter 2, Aims, the Author, based on the available literature and previous observations from the Author's research group, describes the leading hypothesis of the presented project that "stromal cells–mediated BMM induces resistance to PARPi in BRCA1/2-deficient leukemias". To address this hypothesis, the Author proposes three main Objectives, which are, in brief: 1. Studies involving FLT3 inhibitor, 2. Verification of the role of bone marrow microenvironment in a model of stromal cells grown in hypoxia, and 3. Investigation on the molecular mechanism responsible for BMM-mediated resistance to PARPi in leukemia cells. All the Objectives are well justified in order to address the hypothesis of the project.

Chapter 3, Materials and Methods, contains a clear description of the research techniques used. In addition to standard cell biology techniques, such as cell culture of numerous malignant cell lines and primary cells of human or murine origin, the Author describes various models of cell co-culture or with the transfer of conditioned media, also in hypoxia, for reconstituting in vitro the conditions of bone marrow microenvironment. This is a modern wholistic approach allowing to obtain data more closely resembling the real-life conditions. The remaining laboratory techniques described in this chapter include, among others: clogenic assay, cell viability assay, neutral comet assay, double strand break repair assay, nucleotransfection, quiescent stem cell assay, flow cytometry analysis, cell cycle analysis, DNA cloning and SDS-PAGE followed by western blot, and also the use of genetically manipulated cells by CRISPR/Cas9-based technique. Moreover, the chapter describes advanced animal experimentation with in vivo models of the patient-derived malignant cell growth in immunocompromised mice and treatment with respective inhibitors. The Figures in Chapter 3 help the reader to comprehend the design of the in vitro co-culture models and the in vivo experiments. Notably, the Author suitably mentions the approvals from the respective committees for using both human primary cells and the animal experimentation. It must be

admitted that the number and variety of laboratory methods used by the Author in vitro and in vivo are remarkable.

Chapter 4, Results, is divided into 3 main sections, 4.1-4.3, each of them addressing the respective Objective 1-3, and then into numerous subsections listing the robust findings from the experiments carried out by the Author. The main results for each of the sections 4.1-4.3 are as follows:

4.1. The Author presents in vitro data indicating that the specific inhibition of FLT3(ITD) kinase by AC220 compound produces "BRCAness" phenotype in leukemic cells and synergizes with PARPi in induction of the synthetic lethality. The studies were conducted in several leukemic cell lines, expressing either FLT3(ITD) or FLT3(WT) receptor, and then confirmed in the human primary FTL3(ITD)-positive cells from the AML patient. Importantly, the Author claims that the combination of FLT3i and PARPi does not seem to affect normal (i.e., non-malignant) hematopoietic cells. Furthermore, the Author presents convincing in vivo data of amplification of the anti-leukemic effects of PARPi by FLT3i.

4.2. The results presented in this section are very important for understanding the biology of leukemia in general. The Author uses advanced cell culture models to address the question whether bone marrow microenvironment can induce the resistance of leukemic cells, that were initially sensitive to PAPRi. The results obtained clearly indicate that the presence of primary stromal cells in hypoxia can significantly increase the resistance of leukemic cells to PARPi, regardless of the category of the PARP inhibitor used, but not to FLT3i. The Author further explains this phenomenon as mediated by the soluble factor derived from the stromal cells, presumably the cytokine(s), which is the prelude to the results described in the subsequent section.

4.3. This section contains the most robust amount of experimental work. The Author firstly compares the effects of the CXCL12-CXCR4- versus TGFβ1-TGFβR-mediated signaling pathways on the resistance of leukemic cells to PARPi. The results of this research (with the use of genetic targeting, chemical inhibitor or externally added rTGFβ1) suggest that only the activation of the TGFβ1-TGFβR2-mediated pathway is to the large extent responsible for the increased resistance observed. These findings are supported by the in vivo models. Then, the Authors presents stepwise experiments aiming at explanation of the exact molecular pathway involved and identifies SMAD3 as a crucial protein in this system.

Chapter 5, Discussion, is extensive, interesting, substantively justified and supported by graphical schematics. Most of the results obtained in the assessed work are correctly interpreted and related to the current state of knowledge in the field.

Chapter 6, Summary and conclusion, sums up the most important findings of the study and, as such, is very useful for the reader because of the high complexity of the research carried out in the project.

Specific comments/remarks:

- In reference to the results obtained in Section 4.1 the Author states that, as quoted "[AC220 + PARPi] therapy is totally capable for entering clinical demonstration as a precision medicine." While the Autor's enthusiasm is understandable and the results are

compelling, in my opinion some more safety tests should be carried out in preclinical settings before justifying the use of such combinations in clinical trials.

- The experiments described in subsection 4.2.1 referring to the impact of BMM on efficacy of PARPi-mediated synthetic lethality, are conducted in two parallel cell culture systems differing one from another by two factors at once – i.e., the presence of bone marrow stromal cells and oxygen concentration. While the results of comparison between these two models are very convincing, I would expect that these experiments should further benefit from adding two additional models each differing by only one factor, i.e., one growing the leukemic cells with bone marrow stromal cells in normoxia and another one growing the leukemic cells alone in hypoxia. Such design would allow to differentiate between the effects elicited on the model studied by each of the factors. Perhaps the results presented in Figures 4.18 and 4.21 respond to some extent to this question?
- If I understood correctly, the idea between reconstituting BMM models in vitro, as described in Pages 77-79, was that the human stromal cells (HS-5 or primary MSC, allogeneic or autologous) were co-cultured with human leukemic cells and the mouse stromal cells (OP-9 or primary autologous) were co-cultured with mouse leukemic cells. Such settings are, of course, reasonable from the biological point of view and scientifically sound. However, as the in vivo models relied on implantation of human leukemia cells into mouse, I wonder whether additional establishing of an artificial in vitro model of human leukemic cells grown with mouse stromal cells could be also of use, as it would be closer to the in vivo experimentation model used in the study?
- In Figure 4.17, primary stromal cells are mentioned, however it is unclear whether these cells are of human or mouse origin. Also, I would assume that the ELISA assay used in this experiment is cross-reactive to human/mouse TGFβ1, however, I could not find this information or a catalog number of the assay in the dissertation text.

The above-mentioned substantive comments, mainly of a marginal or polemical nature, do not affect the overall highly positive assessment of the presented doctoral dissertation.

Editorial comments:

- In sections 4.1 and 5.1, the FLT3 inhibitor is mostly referred to as "AC220", while in sections 4.2 and 4.3 and the other sections of the text as "quizartinib". Also, the duality in naming of this compound is stated several times within the text of dissertation. It would be advisable, for the sake of clarity, to maintain the same nomenclature for the same compound throughout the whole text, with explaining the alternative names just once at the first use.
- There are several minor typographical and grammatical errors in the text of dissertation, some examples are presented below:
    - Page 9 – "cell cultured conditions"
    - Page 43 – "Verification [of the?] role of bone marrow"
    - Page 43 – "Investigation [on?] the molecular mechanism"
    - Page 59 – "to demonstrate [the effect of the?] dominant negative mutant"
    - Page 102 – "these mechanisms includes"
    - Page 109 – "*BRC[A]1/2*"
    - Page 115 – "leukemias were also did not respond"

These errors are indeed minor and do not change the high quality of the dissertation text.

In summary, in his doctoral dissertation Mr. Bac Viet LE, MSc has undertaken a very interesting and meaningful research subject in the current biomedicine. The amount of work devoted to this project along with the high quality of the experiments conducted and obtained results, and also the clarity of their presentation are impressive.

Therefore, I confirm that the doctoral dissertation entitled “TGFβ-dependent mechanism of the bone marrow-mediated resistance against PARP inhibitors in leukemias” by Bac Viet LE, MSc meets the requirements for the doctoral thesis, as stated in Polish below:

Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z 2018 r. poz. 1668) oraz Regulaminu Rady Naukowej z 13.04.2018 r. załącznik nr. 1 (ze zm. z 06.12.2019 r.).

I, therefore, recommend proceeding to further stages of the doctoral process. Given the high quality of the presented PhD Thesis, I also entrust the issue of the distinction to the decision of the Scientific Council.

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