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Review Report Ph.D. Thesis of Kamila Buzun
entitled
"Molecular mechanism of anticancer activity of novel 4-thiazolidinone derivatives"

Context and scope of the thesis

Ms Kamila Buzun did her doctoral dissertation under two supervisors: prof. Anna Bielawska, head of the Department of Biotechnology of the Medical University in Białystok and prof. Roman Lesyk, head of the Department of Pharmaceutical, Organic and Bioorganic Chemistry of the Medical University of Lviv. The dissertation is interdisciplinary. It fits perfectly into the mainstream of medical chemistry research, namely the search for new, effective substances that may become drugs in the future. I can therefore conclude that the choice of the topic of the Ph.D. thesis is scientifically interesting, with great practical value.

The thesis is based on fourth papers, which were coauthored by the Ph.D. candidate and which were published in highly ranked scientific with a total IF = 21.311. I would like to emphasize that the Ph.D. candidate was in all cases the first author, which indicates his major contribution to these papers. The statements attached to the commentary indicate the dominant (51%) contribution of the PhD candidate to the performance of experimental research and the creation of manuscripts.

The commentary section includes the *List of abbreviations* used, *List of publications* constituting the basis of the doctoral dissertation, *Introduction, Aims of the work,* description of scientific research (*Materials and methods*, *Results*, *Discussion and Summary*), *Literature, Abstract* in English and Polish. The commentary was accompanied by statements of the co-authors. At the end, the PhD candidate placed the research achievements questionnaire.

In the *Introduction*, Ms Kamila Buzun gives a brief history of cancer treatment, describes 4-thiazolidinone derivatives as a group of chemical compounds with various biological activities,

including anticancer properties, characterizes the process of autophagy and apoptosis, and points to topoisomerases as potential molecular targets for anticancer drugs. I appreciate that the thesis includes an introductory chapter providing a broader context of the work. All information needed for the comprehensive evaluation of the scientific values of the here presented work is available and appropriately described. The material in this part of the work is based on an in-depth analysis of research works from recent years and, according to the Reviewer, constitutes a comprehensive introduction to the experimental part.

The Introduction section introduces the reader to the issues discussed in the dissertation and justifies the choice of both the subject and the scope research. The aim of the work was to obtain and evaluate the potential anticancer activity of a series of novel 5-[(Z,2Z)-2-chloro-3-(4-nitrophenyl) -2-propenylidene]-4-thiazolidinone derivatives.

As part of the presented research, 48 derivatives were obtained by synthesis, using as substrates the derivatives of 2,4-thiazolidinedione, 2-thioxo-4-thiazolidinone and (2Z)-2-chloro-3-(4-nitrophenyl)prop-2-enal (*Ciminalum*). The reactions were carried out by heating the substrates in acetic acid with the presence of sodium acetate for 3 hours. The structures of all obtained derivatives were confirmed using spectral methods: ^{1}H NMR, ^{13}C NMR, LCMS and elemental analysis. X-ray structure analysis was performed for one compound: 6-{5-[(Z,2Z) -2-chloro-3-(4-nitrophenyl)-2-propenylidene]-4-oxo-2-thioxothiazolidin-3-yl}hexanoic acid. Then, five of the obtained compounds were submitted for testing for antitumor activity on a panel of 60 tumor cell lines in NCI DTP (USA). The obtained results indicated significant antitumor activity of all compounds in relation to the leukemia cell lines, lung cancers, colon cancers, central nervous system, ovary, kidney, prostate, breast cancers and melanoma. The GI_{50}, TGI and LC_{50} parameters were determined for each cell line. Two of the tested compounds with 4-hydroxyphenyl and carboxyethyl substituents inhibited the growth of all tumor lines at submicromolar and micromolar concentrations. The obtained results allowed the researchers to determine the relationship between structure and biological activity. It has been found that the (2Z)-2-chloro-3-(4-nitrophenyl)prop-2-ene fragment at the C5 position of the thiazolidinone ring is necessary for antitumor activity. Moreover, when analyzing the structure of the active *Ciminalum*-thiazolidinone hybrids, it was noticed that the substituent in the 3-position of the 4-thiazolidinone ring is equally important. The derivatives containing carboxylic acids and the above-mentioned 4-hydroxyphenyl substituent were the most active. The lack of a substituent in

the 3-position or additional groups in the aldehyde-derived leads to the weakening of anticancer cytotoxicity.

The next stage of biological research was to determine the cytotoxic activity against the gastric (AGS), colorectal (DLD-1) cancer cell lines and the MCF-7 and MDA-MB-231 breast cancer lines. All tested compounds turned out to be active against the cell lines used in the research. Two compounds with carboxyethyl and carboxyphenyl substituents were characterized by the highest activity, for which the GI_{50} values against the breast cancer lines ranged from 0.95-1.74 μM. One of the most common side effects of both anticancer drugs and drug candidates is the lack of selectivity for tumor cell lines. Accordingly, studies of the newly obtained compounds were also carried out on normal human blood lymphocytes. The derivatives were characterized by low cytotoxicity and a broad therapeutic index for leukemia cell lines.

The next stage of the work was the designing and synthesis of a new derivative: 2-[5-[(Z,2Z)-2-chloro-3-(4-nitrophenyl)-2-propenylidene]-4-oxo-2-thioxothiazolidin-3-yl}-3-methylbutanoic acid. The structure and purity of the new derivative were confirmed by nuclear and carbon magnetic resonance spectroscopy, mass spectrometry and X-ray structure analysis. The obtained compound was subjected to expanded biological tests. Activity studies were carried out on two breast cancer cell lines (MCF-7 and MDA-MB-231) and the normal skin fibroblast cell line. The cytotoxicity assessment of the tested compounds was performed according to the Carmichael method, determining the viability of cells cultured in vitro with tetrazole salt (MTT). The studies used etoposide as the reference drug. In order to confirm that the tested compound inhibits cell proliferation, its influence on the process of DNA biosynthesis was assessed. The antiproliferative properties were determined by assessing the level of [^{3}H]thymidine incorporation into the DNA of the tested cells. The influence of the Les-3331 compound on the induction of the apoptosis process, the change of the mitochondrial membrane potential, and the expression of initiating caspases 8 and 9 was investigated. The selected compound significantly influenced the cell viability of both tested lines. The IC_{50} values were 5.02 μM for the MCF-7 line and 15.24 μM for the MDA-MB-231 line while for normal cells it was 28.52 μM.

The study of the effect of the derivative on the apoptosis process indicated a dose-dependent induction of apoptosis. Increased inhibition of [^{3}H]thymidine incorporation into DNA was observed, which proves the antiproliferative properties of the tested compound. The effect of the Les-3331 derivative on the autophagy process was tested in turn by checking the concentration

of LC3B, LC3A and Beclin-1. The new derivative inhibited the process of autophagy in both tumor lines.

An attempt was also made to determine the mechanism of antitumor action of the new derivative. *In silico* and enzymatic studies led to the conclusion that inhibition of topoisomerase II is a probable mechanism of antitumor activity of Les-3331 compound. The final stage of the study was to subject the new compound to ADME-Tox analysis. The research was carried out in cooperation with the research group of prof. Jadwiga Handzlik from the MC JU in Krakow. The possibility of penetrating biological membranes with the use of the PAMPA system, metabolic stability with the use of rat liver microsomes, the possibility of interaction of the new derivative with other drugs and mutagenicity were determined.

The research showed low passive permeability of the tested compound. Therefore, it can be assumed that other mechanisms are involved, e.g. active transport, in order to achieve the intracellular concentration necessary for the cytotoxic effect. The tested compound Les-331 showed moderate metabolic activity. The degradation of the 2-thioxo-4-thiazolidinone system was indicated as a probable metabolic pathway. In the AMES test, the derivative turned out to be safe at a low concentration, while in the higher concentration it showed a high risk of mutagenic activity. These results suggest possible interactions between Les-3331 and tumor cell DNA. The research conducted can and should be continued. In vivo tests would need to be performed, and the compound Les-3331 could also be tested on other tumor lines.

Summing up, I can say that the PhD student achieved her research goals. The methodology of the conducted research does not raise any objections. The analysis of all research results indicated that the compound Les-3331 is a potential anticancer drug with a multidirectional mechanism of action. The presented research contributes to our knowledge on structure and synthesis of 4-thiazolidinone derivatives as new anticancer agents, and brings new, important and original findings. It is an independent scientific output, fully meets the requirements for Ph.D. thesis.

Specific comments

I have only few questions, which should be answered during the Ph.D. thesis defense:

1. What led to the choice of compounds for research?
2. Was a reference drug used in the preliminary trials?

3. Was the Les-3331 compound designed based on *in silico* research or on the basis of SAR analysis?

4. Were there also attempts to synthesize and research compounds with carboxyalkyl substituents other than those presented in the dissertation?

Final evaluation statement

To sum up, the doctoral dissertation of M.Sc. Kamila Buzun, presented for assessment, entitled "Molecular mechanism of anticancer activity of novel 4-thiazolidinone derivatives" is characterized by significant advantages, which include methodology adequate to the tasks set, topicality and practical value of the obtained results as well as valuable discussion. Ph.D. thesis highlights very interesting results obtained in the framework of a consistent experimental work and well-designed objectives. I would like to emphasize the comprehensive interdisciplinary character of the Ph.D. thesis covering the research fields of medicinal chemistry, organic synthesis, pharmacology and biochemistry.

The other achievements of the PhD student deserve special attention as well. Ms Buzun is the co-author of three more articles in prestigious journals with a total impact factor of 12.377. Moreover, she took an active part in scientific conferences, both in Poland (2) and abroad (4). In addition, she completed an internship in ADME-Tox techniques at the Department of Drug Technology and Biotechnology of the Jagiellonian University Medical College in Krakow and a month-long internship at the Institute Gustave Roussy in Paris.

Ms Kamila Buzun's doctoral dissertation fully meets the requirements for this type of scientific dissertation, therefore I submit an application to the College of Pharmaceutical Sciences of the Medical University of Bialystok for the award of Kamila Buzun with the degree of doctor of pharmaceutical sciences.