A review of the doctoral dissertation by Katarzyna Ozdarska, MSc entitled:

"Synthesis of HDAC inhibitors and their biological assays (cytotoxicity, HDAC inhibition)"

The key to morphological and functional differentiation of cells in the human organism is establishing specific patterns of gene expression for which epigenetic modifications are co-responsible. Post-translational covalent modifications in histone N-termini play a crucial role in this respect.

The premises justifying the search for a relationship between epigenetic phenomena and various types of diseases include: evolutionary invariability of histone structures susceptible to epigenetic modifications, confirmed correlations of epigenetic changes with certain diseases, e.g. of the nervous system, or found in neurological conditions, such as Alzheimer's, Parkinson's or Huntington's disease, and the effectiveness of low molecular epigenetic modulators, as inhibitors of histone deacetylases (HDACi).

The zinc binding domain is common to most HDACs. Domains that differ from isoforms show substrate specificity and specificity in the mode of regulation. Mammalian histone deacetylases belong to classes I to IV. Understanding the processes which modulate gene expression and, consequently, indirectly influence the protein response, gave rise to the development of targeted therapies interfering with pathological processes at their very source.

The doctoral dissertation is closely related to this area of research and is based on a search for selective HDAC class I and II inhibitors as potential candidates for effective therapeutics in diseases caused by the abnormal HDAC levels.

The aim of the study was to: - design and synthesize selective HDACi towards HDAC class I and II, by modifying the structures of individual fragments, including the zinc binding domain of the known but non-selective HDACi; - evaluate the inhibitory activity of the newly synthesized compounds; - assess the cytotoxic activity of the selected compounds against four mammalian and human cell lines; - evaluate docking of selected newly synthesized HDACi to binding sites within HDAC (molecular modelling).

A total of 57 compounds were obtained in the study, including: sulfonylhydrazide, catechol and indolopyridazinone derivatives, with incorporation of the fluoro-analogues as zinc binding groups (used in order to increase selectivity against HDAC II). The yield of individual syntheses and the purity of compounds were determined by measuring melting points. The compounds were purified using HPLC. All derivatives were subjected to elemental analysis with NMR spectra. The mass spectra were recorded with Tandem Mass Spectroscopy using the ionization method by electrospray (ESI). All compounds were analyzed for their biological effects and HDAC1 inhibitor activity with the use of HDAC1 Fluorometric Drug Discovery Assay Kit and human recombinant HDAC enzymes. Some of the new compounds were evaluated for the inhibitory activity towards phosphodiesterases PDE4B and PDE7A, and compared in this respect with the known inhibitors. The inhibitory activity of the tested compounds against PDE was evaluated using the PDE-GloTM Assay Kit.

The selected compounds were assessed for their cytotoxic activity towards four human and mammalian cell lines: V79-4, Chinese hamster lung fibroblasts; HaCaT, human keratinocyte cell line; and two human neuroblastoma cell lines, SH-SY5Y and PC12, with the use of the popular viability tests, MTT and LDH.

In order to analyze binding of the ligands to the HDAC protein, molecular docking simulations were performed for some newly synthesized compounds using the Autodock Vina software.

The experiments, results, discussion and conclusions were described by the doctoral student in the form of dissertation.
The dissertation consists of 6 main parts: Introduction, Theoretical Part, Results and Discussion, Conclusion, Experimental Part and Bibliography. The work also includes Summary (in Polish), a list of tables, figures and diagrams as well as a list of abbreviations used in the text. A table of contents and a summary of individual parts in French were attached to the dissertation.
Diligence in the preparation of the work should be appreciated, both in terms of graphics (21 diagrams, 53 figures (!)), structure and edition, which with such a huge experimental material is especially praiseworthy. The logical layout and the scope of the theoretical part are noteworthy; this proves the extensive knowledge of the areas discussed. The description of molecular mechanisms (with a very good use of the literature) underlying the successive diseases fully justifies the purposefulness of conducting the research on HDACi as potential therapeutics as part of the studies. A description of the process of synthesis of 57 compounds in the chemical part of the work should be emphasized. The diagrams are constructed in a very simple and legible manner, which is a great achievement of the author given numerous syntheses and their complexity.

Introduction & Theoretical Part
The part is 57 pages long, it is divided into 6 chapters (Theoretical Part). One of them entitled "Objectives" describes the nature and purpose of undertaking subsequent syntheses. This part presents the main goals of the study, but it should be separated from the theoretical part and become an independent part of the dissertation.

The Theoretical Part was based on data obtained from the extensive literature review (193 items). The doctoral student included relatively recent research - 20% of the papers were published within the last 5 years.

In some parts of the Theoretical Part, there are no references to literature (point 2.3, page 32; point 3.4 - some fragments, page 37; points 4.4.1-4.4.2, page 60), there is also no information on the source of the content of the diagrams and tables, e.g. own diagram, or taken from (Table 1, page 23; Table 8, page 65; Scheme 3, page 33), also the lack of explanations of the table contents (Table 3, page 30).

Some of the issues raised in the Theoretical Part seem to be described unequally in terms of thoroughness and extensiveness, e.g. point 4.2.6 Psychiatric diseases or point 4.2.1 Parkinson's disease *versus* points from 4.4.1 to 4.4.4).

Results & Discussion
7 Sulfonylhydrazide derivatives
7.1 Synthesis of sulfhydrazide derivatives
The doctoral student describes the synthesis of a total of 36 compounds, with the yield of each of them (Table 10). Table 11 presents the inhibitory activity against HDAC1 for 39 compounds, without further comment, which somewhat distorts the clarity of the subsection.

There is also no justification for designing derivatives with phenyl substituents in meta- position, while, as the author indicates on page 83, most compounds with substituents at this position do not

show any activity against HDAC1 (it can be assumed that the PhD student refers to research conducted by other authors, although there is no relevant reference given). This is related to a more general remark regarding the entire subsection 7, i.e. in the descriptions of the syntheses of derivatives, the author did not emphasize the potential significance of the position for individual substituents, but focused merely on the inhibitory activity.

Discussing the results of the inhibitory activity against HDAC1, it would be appropriate to explain the meaning of the IC50 symbol, which is closely dependent on the type of test (in subsection 7.2 it is a measure of the inhibitory activity), especially since the same symbol is used in further parts of the paper dealing with biological tests, where it is a measure of the cytotoxic activity.

Inclined by the moderate inhibitory activity of all of the compounds towards HDAC1, the author very briefly summarized subsection 7.1. However, in the search for bioactive structures, any result, even mediocre, is very important as it is a prerequisite for designing further derivatives and avoiding modifications that are not significant for biological activity. In this context, there is a certain feeling of insufficiency when it comes to discussing the nonetheless diverse activity of sulfhydrazide derivatives towards HDAC1 (according to Table 10, the IC50 ranges from 77.3 to 13890 μM for individual derivatives), with reference to the differences in the structure of individual derivatives.

7.3 Cytotoxicity - MTT and LDH

In this subsection, the author made an attempt to assess safety of selected sulfhydrazide derivatives using cell viability tests, MTT and LDH, also by determining IC50 values. The derivatives were tested in a specific time schedule, each at 4 concentrations.

The work does not summarize the relationship between the modified structure of the compounds connected with the HDAC1 inhibitory activity, as well as an influence on the safety measured by the IC50 values determined using the MTT assay. It would also be advisable to relate the results of cytotoxic activity, i.e. IC50 values, to reference values, i.e. for a known inhibitor which is recognized as relatively safe. Although certain conclusions arise from tabular and graphic compilations, they have not been clearly articulated in the text. The description of the results of viability tests makes it difficult to deduce what the PhD student expects from derivatives: high or low cytotoxic activity.

The author did not explain why she used certain concentrations of the compounds to which the cells were exposed. The selection of normal hamster lung fibroblasts for viability tests was also not justified, especially since the selection of the remaining 3 cell lines for testing was thoroughly explained.

The doctoral student shows a certain freedom in choosing derivatives for the tests, resulting from the very moderate activity of sulfhydrazide derivatives as HDAC1 inhibitors, however, the compounds that showed the highest activity should be a selection criterion for the tests, these are the compounds shown in Table 11, e.g. 17f or 17h. This would allow to maintain consistency in the subsequent stages of a search for effective inhibitors and would give a greater chance of formulating conclusions on how to improve bioactive structures.

A remark also arises in relation to Tables 12 and 13. It seems that the PhD student used a mental shortcut when describing the content of these tables. The tables show the IC50 values determined during the exposure of cells to different concentrations of compounds over a specific time as shown in Figures 29-31 and 35, but they do not present (as reported) cell viability for the compounds at concentrations corresponding to the IC50 values.

The discussion on the results of the LDH test also contained some inaccuracies and generalizations, for instance the indication of the V79-4 cell line as more sensitive to the cytotoxic effect of the compounds than the HaCaT line, or SH-SY5Y (page 91), or the other way round, in the case of compound 11e, once it is more (verse 3, p. 91) and once less (verse 12, p. 91) cytotoxic than other derivatives. The lack of a cytotoxic effect for the majority of the tested compounds after 24-hour exposure of V79-4 cells (as was indicated by the author) points to the increased resistance not sensitivity compared to other cell lines. This feature of V79-4 is mostly confirmed by the MTT test (Table 12).
The confirmation of sulfhydrazide derivatives as showing "no toxicity" towards any of the tested lines: HaCaT, V79-4 and SH-SY5Y, up to the concentration of 80 µM, is also unsatisfactory, because according to the description of the experiments, the highest concentration was 40 or 50 µM depending on derivative, and moreover, this was only the case for the PC12 line.

The results of tests conducted on the PC12 line, which due to its functional similarity to neurons is the most important line for the evaluation of new compounds, are discussed at length and clearly.

It is important to mention the great bulk of the results and variables assessed in the biological tests. Here, the outcomes can be interpreted from various points of view, e.g. comparing the cytotoxic activity of derivatives (only based on IC50 values, and this would probably be the simplest and most appropriate for these tests), analyzing the sensitivity of individual cell lines or individual compounds in terms of time required to generate the cytotoxic effect. It seems that the PhD student attempted to interpret the results in all aspects, which was very ambitious. However, such a large number of results is very difficult to present clearly and to draw relevant conclusions.
When discussing the biological activity of newly synthesized compounds, we may also attempt to compare the results of the MTT and LDH tests, because they confirm the same effect, but obtained through different intracellular phenomena.

The above remarks do not apply to the experiments themselves as they have been properly designed and conducted, but to the way of their description, and this in no way downgrades the undoubted value of the work.

8 Catechol series

8.1 - 8.2 Synthesis and docking of catechol derivatives

The PhD student describes the synthesis of 3 series of catechol derivatives, differing in the position of the hydroxyl group (2- or 4-) or the substituent in position -4- (difluoromethoxyl- or hydroxyl-). Table 14 shows the yield of synthesis of a total of 16 new compounds. Four compounds, coded 41, 26, 45, 54 were subjected to docking analysis based on structural analysis and the evaluation of the inhibitor activity towards HDAC1 and HDAC7. A known HDAC7 inhibitor, TMP269 was the reference compound.

8.3 Cytotoxicity

The cytotoxicity tests were performed using derivatives encoded: 25, 26, 32. The MTT test was carried out; using only SH-SY5Y cells. The MTT test seems sufficient to assess the biological activity at this stage of a search for new HDAC inhibitors. Figure 47 shows that derivatives of this group are incomparably less cytotoxic towards SH-SY5Y cells than sulfhydrazide derivatives. The catechol compounds did not show the expected activity towards HDAC1 and HDAC7.

Maintaining consistency and continuity in planning further stages of the project makes the assessment of the results easier and, above all, facilitates interpretation by the researcher. This is particularly important in a search for new compounds and seeking relationships between the structure of the molecule and its biological activity. Each result is important, because it allows to determine the importance of a given structure for the tested activity, and this is invaluable knowledge. Therefore, if derivatives 41, 26, 45 and 54 were selected for the molecular analysis, a similar order should be followed in biological tests.

Like for sulfhydrazide derivatives, the choice of compounds for biological tests of catechol derivatives was not explained. There was also no explanation for using only one cell line - SH-SY5Y in the tests. Although it is understandable from the PhD student's point of view in the absence of the activity towards the HDAC, the research description has certain rules which ought to be followed.

9 Indolopyridazinone series

9.1 Synthesis of indolopyridazinone derivatives

A search for selective HDAC7 inhibitors included the synthesis of the compounds, determination of the yield of the cyclic compounds as well as docking analysis of the selected derivative, 72.

There was no mentioning of the benefits of the syntheses performed in the context of further research.

The section Results & Discussion did not confront the study results with the outcomes obtained by other authors, especially in the context of changes made so far in the structure of the molecules of known HDAC inhibitors and the effects of these alternations on the activity.

Conclusion

The conclusions are quite general and therefore somewhat unfairly present the enormous work done by the PhD student. One would rather expect a clear indication of which direction in the design of new inhibitors should be followed and which ought to be avoided.

Summary of the review

The subject undertaken up by the PhD student focuses on the area which is extremely important for medicine and therapy, i.e. modification of the genetic response. Interference in the interactions between the transcriptionally active area and the surrounding chromatin is one of the highest levels of expertise currently achievable in medical science. Hence, it is a potential key to solving a number of therapeutic problems. Therefore, the work of Katarzyna Ozdarska, MSc., perfectly fits into contemporary scientific trends. The scope of the synthetic work (several dozen new compounds were obtained (!)), analytical work (all compounds were characterized in terms of their structure)

and the use of highly advanced methods to characterize derivatives (NMR, hybrid Tandem Mass Spectroscopy (Quadrupolar/time-off light)) should be appreciated, as well as a comprehensive approach to the problem at this stage of research, i.e. including chemical synthesis, enzymatic activities, docking of molecules and biological activities.

The PhD student's work closely combines chemical methods with the study of the biological material. Such a combination in the work of one author proves a comprehensive approach to the project, thoroughness and courage in describing and interpreting the results in such various fields.

The high quality of graphics and the way of presenting the results are also noteworthy, starting from complicated synthesis schemes, complex graphs with detailed data (e.g. graphs of cell viability including the three-time exposure of 6 derivatives, each in 4 concentrations), and high resolution photographs of cell cultures.

With such a large amount of information obtained during the experiments, their description was very difficult. This approach allowed to adopt different points of view in interpreting the results, especially when it comes to the biological part.

The comments on the description of the work included in the review may be used by the PhD student in the future as guidelines when describing further experiments. In no way do they diminish the undoubted value of the topic, its difficulty and performance of experiments.

The work meets the statutory requirements for doctoral dissertations. Therefore, I apply for admission of Katarzyna Ozdarska, MSc to further stages of the registration and conferment procedure for a doctoral degree.

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Warsaw, 29.10.2020