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Review of the doctoral dissertation of M.Sc. Ying Zhou entitled: "Equilibrium constants determination for anthracycline-DNA interactions: from aqueous solution to single cell study"

Increasingly full understanding of the human genome creates a unique opportunity to selectively block certain genes, including the genes responsible for neoplastic transformations and uncontrolled growth of neoplastic cells. It is believed that the most effective way to calm the activity of undesirable genes is to prevent their expression as early as possible by blocking the promoter sequences. Later regulations, done by e.g. blocking or degrading mRNA, may be much less effective. The selective blocking of the genes involved in the basic processes of the cell division by the specific ligands binding to them will stop the cell division, which is particularly important not only in anticancer but also in antiviral therapy.

From the pharmacological point of view, the key element allowing to understand the mechanism of the action of an anticancer or antiviral drug is the assessment of the nature of its interaction with DNA. The search for compounds-drugs is usually associated with lengthy and costly research. So, no wonder that the development of new research methods that will economically and simply verify the properties of biologically active substances is at the center of interest of a number of research laboratories. Ying Zhou's doctoral dissertation, entitled ″Equilibrium constants determination for anthracycline-DNA interactions: from aqueous solution to single cell study″ and performed at the Institute of Physical Chemistry of the Polish Academy of Sciences under the supervision of prof. dr hab. Robert Hołyst, aims at examining the usefulness of the proposed complementary research techniques for identification of the way of interaction of various forms of DNA with a number of compounds – anthracycline derivatives. Therefore, it perfectly fits the current research trends.

Selection of the topic, scope and purpose of the work, and the construction of the dissertation

DNA is the pharmacological target for many drugs that are currently in use or in clinical trials. Its complexation with drugs makes it possible to influence cell function by modulating gene expression or by interfering with replication. Many strategies have been developed to use the known structure of DNA for specific drug binding. One approach is to use small molecules that are able to recognize and bind to specific areas unique to DNA. Designing new drugs is a complex task that requires multi-stage and costly research. There is no single experimental technique that can fully determine how a given compound interacts with nucleic acids, so many approaches have been developed to explain drug-DNA interactions. Successful drug design requires a sufficiently clear and detailed picture of drug-DNA interactions. M.Sc. Ying Zhou undertook the problem of determination of the equilibrium constants of the anthracycline-DNA complexes in solution and in a single cell. In the research, the PhD student used various forms of DNA and four anthracycline derivatives: doxoribicin, daunorubicin, epirubicin and idarubicin.

The doctoral dissertation has a classic layout, as it begins with an introduction describing the literature data on the structure of anthracyclines and the mechanism of their interactions with DNA. Next, the PhD student moved to the thermodynamic parameters of the ligand-DNA complex formation process and the factors determining the affinity of partners and the driving force of this interaction. The basic thermodynamic parameter determined for a ligand / DNA complex is the equilibrium constant of such a complex. Its value is most often determined using the spectroscopic methods. Thus, the last fragment of the literature section describes the basics of the analytical methods used in the work.

The next part in the theses is experimental. It begins with a description of the research on the self-aggregation of the studied anthracycline derivatives. Chapters 3 and 4 constitute the main part of the work. They describe the individual research projects carried out by the PhD student. The thesis ends with: summary of the results, conclusions, perspectives for future research related to the kinetics of anthracycline-DNA interactions, and a list of the cited literature (including 137 items).

The information given in the literature part of the work is very concise and sometimes too general. It is a pity that the PhD student did not attempt a more accurate description of the interactions of selected compounds with various forms of DNA. The dissertation is written in the correct language and the number of typos, mental shortcuts and linguistic errors is small.

Substantive evaluation of the work

The dissertation is experimental and is focused on difficult and demanding research objects, which are nucleic acids and the mechanisms of their interactions with rather small molecules such as anthracycline derivatives. Anthracycline derivatives, due to their anticancer properties, are a very attractive material for research. Unfortunately, their mechanism of interaction with DNA is not simple. Anthracyclines interact with DNA through intercalation and covalent bonds, but they also introduce changes to the structure of the nitrogen bases resulting from the interaction of the reactive oxygen species generated in the redox reactions with the participation of anthracyclines. Additionally, anthracyclines selfaggregate quite easily, especially in solutions of high concentration, which complicates the quantification of the interactions with DNA. Therefore, in the first place, the PhD student checked the tendency of the tested compounds to the process of self-aggregation in the concentration range of 10-100 μM. The research was performed using UV-vis and fluorescence spectroscopies. The obtained results allowed to state that in the studied concentration range, anthracycline derivatives did not aggregate. This chapter is important because the interactions between anthracycline molecules compete with the process of formation of a complex with DNA and / or take part in the formation of anthracycline-(anthracycline-DNA) complexes, which significantly complicates the quantification.

Another issue concerned the determination of the equilibrium constants of anthracycline-DNA complexes. The studies were performed using fluorescence correlation spectroscopy (FCS). Fluorescence Correlation Spectroscopy (FCS) is a non-invasive fluorescence technique that tracks the dynamics of single molecules through confocal microscopy. In conventional fluorescence spectroscopy, a relatively large sample volume (100 μl ÷ 1 ml) illuminated by excitation light provides us with the average fluorescence signal and, consequently, information about the entire molecular system. The sensitivity of the FCS method overcomes this limitation and provides non-averaged information on the test sample at the single molecule level. This method tracks the diffusion of fluorescently labeled molecules in a strictly defined volume illuminated by a focused excitation beam. Statistical analysis of the temporal correlation of fluorescence fluctuations provides information on the diffusion coefficient / diffusion time, indirectly on the particle size and concentration. On the basis of the performed tests, no influence of the DNA form on the determined values of the equilibrium constants was found. Moreover, taking into account the chemical structures of the studied anthracycline derivatives and, in fact, their high similarity to each other, the PhD student did not observe any significant differences in the determined K values. Being aware of the possible

interaction of anthracycline molecules with each other, the PhD student decided to check whether an anthracycline- (anthracycline-DNA) complex was also formed in the solution. To this end, she analyzed the brightness of single molecules (MB). The conducted research confirmed that the mechanism of anthracycline-DNA complex formation was two-step. The first step involved the intercalation of the compound molecule between the parallel base pairs. The second step concerned the interactions between the bound anthracycline molecule and the free molecule. Of course, both stages occurred simultaneously. The equilibrium constants of the intercalation complex formation reactions were definitely greater than the equilibrium constants of the anthracycline-(anthracycline-DNA) complexes. This difference strongly depended on the ionic strength of the solution.

The last part of the work concerned the analysis of the equilibrium constants of anthracycline-DNA complexes in a single cell. The HeLa cell line derived from cervical cancer cells and fibroblasts - connective tissue cells found in the dermis were used in the research. DNA molecules in the cell nucleus are bound to proteins, which allows them to condense. They achieve the highest packing level during the cell division. Therefore, such a form of DNA and the presence of other macromolecules at a significant concentration must have an impact on the determined values of the equilibrium constants of the complexes formed. They were 1-2 orders of magnitude smaller than those determined in the solution.

In my opinion the most important achievements of M.Sc. Ying Zhou are:

1) Development of original procedures for determination of the equilibrium constants of the formation of the anthracycline-DNA complex in very small volumes, which is difficult to achieve with other methods and gives the possibility of measurements on a physiologically relevant scale.

2) Analysis of the mechanism of interaction of selected anthracycline derivatives at the level of a single molecule.

3) Development of a method based on photon whitening of slow diffusion compounds for determination of the equilibrium constants of anthracycline-DNA complexes in single living cells.

The achievement of the Author of the reviewed dissertation, in addition to broadening and deepening the knowledge on the interactions of molecules with DNA, is also of technological importance, as it provides valuable guidance for the development of new drugs. The use of fluorescence correlation spectroscopy and single-molecule brightness analysis to quantify the anthracycline-DNA reaction seems to be competitive with other spectroscopic methods (SPR, UV-vis, CD, RLS, etc.) commonly used in this type of analysis, especially if it is about the measurements at the cellular level. The research material collected and presented

in the dissertation constitutes a significant and valuable contribution to the knowledge and the understanding of the mechanism of interaction of compounds with DNA. I rate the quality of the research carried out by the author of this dissertation very highly, nevertheless, usually, in each assessed work, various ambiguities and shortcomings can be found. After all, the superior role of the Reviewer is not to praise the work, but to point out the shortcomings to the author of the dissertation and to provoke her to further in-depth discussion. So, discharging this obligation, I propose a few points for the discussion in the public defense:

1) In chapter one, section 1.1.2. is devoted to the ways anthracyclines interact with DNA. It is a pity that the Author did not attempt to present a more in-depth review of the literature, pointing to the places particularly active in DNA in this type of interaction.
2) Table 1.1. contains a list of techniques along with the principle of operation and the level of the detection limit reached. It would be a much more valuable part of the work if the Author had given the limitations, advantages and disadvantages of the techniques mentioned. In this type of research, voltammetric techniques are also commonly used, but they are not mentioned at all.
3) The quantitative characterization of the affinity of anthracyclines for DNA is based on the determination of the value of the constant *K*. However, it should be remembered that this constant cannot be treated as a traditionally understood equilibrium constant, also known as the binding or association constant, due to the complexity of the reaction system composition and the coexistence of many forms of the substrates in the solution. Thus, the determined constants are conditional constants, because their values strictly depend on the measurement conditions, such as: pH, type and concentration of the buffer used, and ionic strength of the solution. I wonder if Author was able to find literature data obtained in identical or very similar conditions to the conditions she used?
4) The forms of DNA used in the research differed in structure (linear, circular) and in the number of base pairs. Did they also differ in the content of guanine-cytosine pairs? This information is very important. since anthracyclines prefer sites rich in guanine and cytosine.
5) Why were the studies of the susceptibility of anthracyclines to self-aggregation, their interactions with DNA in solution and with a single cell conducted in different buffers? The stability of the measurement conditions would enable a better comparison of the obtained results with each other.
6) There are several ways in which anthracyclines interact with DNA: intercalation, major or minor groove binding, covalent binding, non-specific binding outside the DNA strand, and a mixed mode of binding. Thus, the existence of two ranges of linearity in

Figure 3.5 can be explained not only by the two-step mechanism, it can also be a result of mixed bonding, e.g. intercalation and electrostatic interactions.

7) How did the Author define / confirm the way of interaction of the studied anthracyclines with DNA?
8) It is surprising that the *K*-value is not influenced by the form of DNA. How can this be explained?
9) Is binding of anthracyclines to DNA cooperative or anti-cooperative?
10) Why was only K_1 calculated for in vivo measurements?
11) Error bars appear in the graphs. What were the parameters of the statistical evaluation of the results (n, P, α)?
12) The Author claims that "To monitor the electrostatic binding, a sensitive method was required, because the MB change of anthracyclines was not as obvious as that in intercalation." So, which more sensitive method could be used in this case?
13) What did the author mean "...DOX changed 16.56 ± 10.70 folds…"? Is this an error or range?
14) I find the details of the preparation of the solutions superfluous.
15) The editorial side of the work leaves much to be desired.
16) I think that if the work is not a collective work, it should be written in the first or third person and not in the second.

Summary

In conclusion, I would like to state that the submitted doctoral dissertation of M.Sc. Ying Zhou fully meets the conditions set out in Art. 187 of the Act of July 20, 2018 on academic degrees and title as well as degrees and title in art (as amended). Therefore, I am requesting the High Council of Institute of Physical Chemistry, PAS to accept the thesis and allow the PhD student to enter further stages of the doctoral tract.

Sincerely,

Anna M. Nowicka

Prof. dr hab. Anna M. Nowicka