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Report on the PhD thesis:

"Equilibrium constants determination for anthracycline-DNA interactions: from aqueous solution to single cell study"

by **M. Sc. Ying Zhou**

Anthracyclines: doxorubicin hydrochloride (DOX), daunorubucin hydrochloride (DNR) epirubucin hydrochloride (EPR) and idarubicin hydrochloride (IDR) are effective drugs used in cancer treatment. They bind to DNA and induce apoptosis of cancer cells. The binding strength is defined by the equilibrium constant K. The precise knowledge of this constant is important for proper determination of the doses used in the therapy. The K-values for anthracycline-DNA binding determined at the micromolar concentrations of reagents amount to 10^4-10^8 M^{-1} and might be distorted by anthracycline aggregation. The mechanism of the anthracycline-DNA reaction in a broad concentration range in solution and the effects of macromolecular crowding in cells are still under debate.

The main aim of this PhD thesis was to determine the anthracycline-DNA equilibrium constants in solution and in living cells down to nanomolar anthracycline concentrations. The studies were performed by means of fluorescence methods: fluorescence correlation spectroscopy (FCS) and single-molecule brightness (MB) analysis.

The studies presented in this thesis belong to the leading themes in the field of anti-cancer drug characterization and testing.

The content of the thesis closely corresponds to the title.

In Chapter 1 the structures of anthracyclines and mechanisms of anthracycline – DNA interactions are presented. The equilibrium constants are defined, different methods of their determination are discussed in terms of detection limits and their literature values are compared. Additionally, the effect of molecular crowding on the equilibrium constant in cells is discussed in terms of additional reactions with the local environment, in particular non-specific interactions, side reactions, partitioning between microenvironments and surface

interactions. Also literature data on equilibrium constants of different reactions in different crowded media are discussed.

In Chapter 1.4 fluorescence techniques used in this PhD thesis for equilibrium constant determination are presented. The theoretical basis and experimental setup of the fluorescence correlation spectroscopy (FCS) are discussed in detail and its application for the K-determination is presented. Next, the application of the single-molecule brightness (MB) analysis for determination of the reaction K-constant is discussed. In this part "confocal" instead of "confocal volume" is used incorrectly in several places. The application of the fluorescence intensity decay is discussed for reactions in which part of the fluorophores becomes immobile after the reaction, resulting in photobleaching. In Fig. 1.10 DOX ACFs are shown in aqueous solution and in a cell. Both ACFs are very noisy and the triplet decay cannot be seen. Why is the statistics of the data so bad? Additionally, the horizontal axis in the ACFs plot should be labeled "time" instead of "Correlation time". In Fig. 1.11 The time axis has no units.

Hers own results Ms. Ying Zhou has presented in Chapters 2-4.

First, in Chapter 2 the aggregation of anthracyclines, which may seriously distort the K-value measurement is studied using UV-VIS absorption and fluorescence spectra in the concentration range of 10 – 100 μM. It is concluded that no aggregation occurs in this concentration range because all the spectra are identical. There is, however, no evidence that at the lowest concentration of anthracyclines studied of 10 μM only monomers are present.

In Chapter 3 the equilibrium constants for anthracycline-DNA interactions are determined in aqueous solutions using FCS and MB analysis methods. For this study five linear DNAs of the length of 20 – 48502 bp and one circular DNA (2686 bp) were used (Table 3.1). The K-values obtained for DOX and circular and linear (2686 bp) DNAs amounted to about $1x10^6 – 3x10^6$ M^{-1} and were much lower than the expected values for the intercalation reaction of about 10^8 M^{-1}. This is explained by the 2-reaction model (intercalation + external aggregation). In this case the apparent K-value obtained from FCS describes a combined effect of the strong binding – intercalation and a weak binding – external aggregation ($K\sim10^5$ M^{-1}) and the two effects cannot be separated in this experiment. In order to confirm the 2 reaction model the MB analysis was performed based on different MB of DOX intercalated and externally aggregated to DNA. The MB of DOX and the ratio of MB(free DOX)/MB(complex) in each of the 2 reactions were determined (Fig. 3.6) for all 6 DNAs. While for external aggregation this ratio amounted to about 2.5 for all DNAs, the

change of MB in the intercalation process depended on the length and structure (linear/circular) of the DNA. In the latter case the ratio was increasing from about 5 for the 20 bp DNA to about 15 for the 48502 bp DNA and amounted to about 37 for the circular DNA. Thus, it was shown that the MB of intercalated DOX was decreasing with the length of the linear DNA. This is a very interesting result which was discussed in the thesis taking into account different mechanisms.

The fact that the MB of DOX is changing due to the change of environment (intercalation) is to be expected. Additional explanation is needed for the dependence of MB of intercalated DOX on the DNA length and structure.

This is explained by inefficient excitation of DOX molecules intercalated in the DNA due to the inhomogeneous distribution of the orientation of the DOX absorption dipole moment resulting from the DNA structure and a slower DNA rotational and translational diffusion. This causes a decrease of the number of intercalated DOX molecules with parallel orientation of the absorption dipole moment to the electric field vector of the exciting light. Perhaps other mechanisms should be also considered like: (i) bleaching of intercalated DOX molecules increasing with the slowing down of the DNA diffusion and DNA length, (ii) additional quenching of DOX fluorescence resulting from interaction with the intercalated neighboring DOX molecules.

The MB of externally aggregated DOX molecules does not depend on the DNA length and structure because in this case the DOX molecules can rotate with respect to DNA, thus, reaching a more isotropic distribution of orientation of the absorption dipole moments.

Equilibrium constants $K_1=(8.3\pm1.2)\text{x}10^7\ M^{-1}$ (intercalation) and $K_2=(2.0\pm0.5)\text{x}10^6\ M^{-1}$ (external aggregation) were determined for the interaction of DOX with all 6 different DNAs (Fig. 3.8) and were independent of the DNA length and structure. The discrepancy between the obtained K_1/K_2 ratio and the literature data was explained by the different ionic strength of the studied systems. Analogous reaction constants were also determined for the analogs of DOX and were very similar to those of DOX (Fig. 3.10). In the figure captions of Fig. 3.9 and 3.10 there is no information which DNA was used in the study.

In Chapter 4 the studies of the interaction of DOX with DNA in the nucleus of He-La and Fibroblast living cells by means of fluorescence decay and MB analysis are described. The obtained values of the equilibrium constants in the cells: $(1.5\pm0.9)\text{x}10^5\ M^{-1}$ – in He-La cells and $(1.7\pm1.1)\text{x}10^5\ M^{-1}$ in Fibroblast cells were similar and were 1-2 orders of magnitude smaller than in solution. This confirms the fact that the DOX molecules are equally efficient

in distraction of both the cancerous and healthy cells and for therapeutic applications selective targeted delivery systems are needed. Three mechanisms were proposed to explain the smaller K-values in cell nucleus: (i) hinderance by histones, (ii) effect of side reactions and (iii) ionic strength of the nucleus. The fact that the K-value in different cells of the same kind varied by 50% (Table 4.1) was explained by partitioning between microenvironments and heterogeneous distribution of DNA. It was also shown that the K-value measured in different locations in the cancer cells is not affected by their heterogeneity.

The results obtained in the PhD thesis are summarized in Conclusions (Chapter 5) and potential directions of further research are given in Outlook - Chapter 6.

Additional experimental details are given in Appendix B.

In my opinion the most important new achievements and conclusions of this PhD thesis are:

- determination of the equilibrium constants K for anthracycline – DNA interactions in the nanomolar concentration range using FCS,
- confirmation that the anthracycline – DNA interactions consist of 2 reactions: intercalation and external aggregation, explanation of the possible reaction mechanisms and determination of the corresponding K_1 and K_2 values,
- determination of the K-values for DOX-DNA interactions in the nucleus of living cells using MB and a newly developed method of fluorescence decay analysis.

All the new and very interesting results are critically discussed in the framework of available models and compared with literature. The obtained results are very important for better understanding of anthracycline – DNA interactions and the possibility to use anthrecyclines for anti-cancer treatment.

The thesis is well written in correct English with some minor errors. It contains a sufficient review of the literature in the field – 137 publications are listed in the Bibliography. The experimental methods and data analysis are well chosen and clearly described. The presentation of results is sufficient and clear. The results obtained by the author are clearly separated from literature data and compared with them, wherever possible.

In the course of her PhD studies Ms. Ying Zhou became an expert in FCS and MB studies. The description of the fluorescence methods corroborates the candidate's general theoretical knowledge in FCS and MB. As a graduate student she showed herself to be a very talented and hard working person with high experimental skills able to solve independently scientific problems. She has also demonstrated a high ability to work with others. She has published five publications in leading scientific journals. Two of them published in *The*

Analyst and *Phys Chem Chem Phys* are related to the PhD thesis and in one of the them (in *The Analyst*) she is the first author.

The scientific value of the presented PhD thesis is very high. The author obtained very interesting, new and important results. All this led to important conclusions concerning the nature of the anthracycline – DNA interactions and the reaction rate constants. I have no doubt that the new very interesting results obtained by the Candidate essentially broaden our knowledge on the anthracycline – DNA interactions and are very useful for the use of anthracyclines in the anti-cancer treatment.

I am fully convinced that the PhD thesis "Equilibrium constants determination for anthracycline-DNA interactions: from aqueous solution to single cell study" by M. Sc. Ying Zhou fulfills all the formal and scientific requirements (art. 187 ustawy z dnia 20 lipca 2018r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 ze zm.) and I recommend to proceed with the public oral defense of the thesis.

Poznań, June 14, 2021.

Prof. dr hab. Adam Patkowski