Application of Induced Circular Dichroism for Investigation of Drug-Macromolecule Complexes

PhD thesis

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1. Introduction

Molecules consisting of repetitive units of low molecular weight compounds (monomers) are called macromolecules. These include the naturally occurring nucleic acids and peptides/proteins, but plastics and macrocyclic molecules, such as cyclodextrins also belong in this category of substances.

Small molecules, such as pharmaceutical compounds might be capable of interacting with macromolecules. It can occur in the human body (protein binding of drugs, cytotoxic anticancer drugs, etc.), but these interactions can be exploited when choosing excipients in pharmaceutical technology.

These types of interactions are easily explored using circular dichroism spectroscopy. If a small achiral molecule, having a chromophore group interacts with a chiral macromolecule, its chromophore becomes chirally perturbed and an induced circular dichroism (ICD) sign appears in the absorption region of the chromophore. This ICD sign is selective to the complex formed. Its shape and intensity can be used to establish the mode of binding and characterise the strength of it.

2. Objectives

The objective of the doctoral work was to investigate the application of a special area of circular dichroism spectroscopy (CD), namely the induced circular dichroism spectroscopy (ICD). The ICD phenomenon is well known, however currently it is not used in either drug development or analysis, despite the fact that it can provide important information on interactions of drugs with different macromolecules or biopolymers.

The purpose was the exploration of DNA and protein interactions of drugs, as well as the investigation of complexation of cyclodextrins, which substances are widely used the pharmaceutical industry.

DNA binding experiments might serve as a basis for an *in vitro* genotoxicity pre-screening, while protein complexation might provide information on the rate of plasma protein binding for newly developed drug substances. In case of cyclodextrins the aim was to develop a fast and informative method for the pre-selection of the optimal compound.

3. Methods

Circular dichroism spectroscopy has been used for the determination of DNA and protein binding, as well as the investigation of cyclodextrin complexes. For DNA binding studies differential frequency saturation transfer difference NMR and CD melting experiments were also performed.

Preparation and purification of the pUC18 plasmid DNA was performed in the Department of Medical Biochemistry in accordance with the related protocol.

Calculation of the stability constants for cyclodextrin complexes and protein binding was performed by nonlinear parameter fitting using Origin 8.5 Pro software.

4. Results

4.1. DNA binding studies

In the Ph.D. work DNA binding of multiple small molecules has been investigated. For these experiments naturally occurring linear DNAs and plasmid DNA of bacterial origin - having circular and super helical structure - were used.

The first objective was to establish whether it is possible to use plasmid DNA for binding studies. To prove this, the CD spectra of all DNA types have been recorded and compared. Next, two small molecules with well characterised binding mode for linear DNAs have been chosen: the intercalating ethidium bromide and the minor groove binding berenil. Spectra of the interaction of these molecules with natural DNAs and plasmid DNA have also been recorded and compared. Based on the obtained results the conclusion is that plasmid DNA can be used for binding studies.

The investigated small molecules included the naturally occurring curcumin and 28 curcuminoids, aristolochic acid I, which is also a compound found in nature and sunitinib, a kinase inhibitor anticancer drug. Experiments on plasmid DNA were performed for aristolochic acid I and sunitinib, binding of curcumin and curcuminoids were only investigated for the natural chicken erythrocyte (ChE) DNA. In case of sunitinib, next to the common CD measurements, CD melting and NMR experiments were also performed.

For curcumin, the recorded CD spectra proved definite DNA binding, however out of the investigated curcuminoids, only 5 provided an ICD signal, for the rest the binding studies gave negative results.

When investigating aristolochic acid I, interaction with linear DNAs could not be detected, however the molecule can bind to plasmid DNA, as the DNA bands in the CD spectra showed significant changes. Presumably aristolochic acid I disrupts the super helical structure of the plasmid.

Sunitinib base has the ability to bind to both natural and plasmid DNA. In case of the former, detailed titrations could not be performed due to precipitation happening even at low ligand concentrations. In case of plasmid DNA, based on CD and NMR results, a two-step binding mode is proposed. This theory is supported by the concentration-dependent duality of the ICD sign and the binding mode index, calculated from the NMR result which was 1.1, a value that is on the margin of intercalation and minor groove binding.

4.2. Investigation of cyclodextrin complexes

In this doctoral work the complexation of 5 antifungal azoles with native and synthetic cyclodextrins has been investigated. The reason for choosing these substances as model compounds was their poor water solubility and some drugs already being on the market as CyD complexes. Out of the 5 azoles, one was a triazole and the other 4 were imidazole derivates. For the experiments three native (α-cyclodextrin–ACyD, β-cyclodextrin–BCyD, γ-cyclodextrin–GCyD) and 5 synthetic (carboxymethyl-β-cyclodextrin–CMBCyD, dimethyl-β-cyclodextrin–DMBCyD, trimethyl-β-cyclodextrin–TMBCyD, sulfobuthylether-β-cyclodextrin-SBBCyD) CyDs have been used.

In case of fluconazole, the only compound containing triazole ring, no ICD signal could be observed, thus the stability constants could not be calculated. The constants calculated for the other 4 substances (bifonazole-BIZ, clotrimazole-CLZ, miconazole-MIZ, tioconazole-TIZ) are summarised in Table 1.

Table 1: Logarithm of the calculated stability constants (M^{-1}) for the investigated antifungal azoles with selected cyclodextrins

	BIF	CLZ	MIZ	TIZ
ACyD	2,74	-	-	3,12
BCyD	3,40	2,65	2,70	3,18
GCyD	4,04	-	3,37	3,71
DMBCyD	4,21	3,23	3,68	3,86
TMBCyD	2,95	1,48	-	-
CMBCyD	3,40	2,91	2,96	2,85
HPBCyD	4,46	2,09	2,71	3,30
SBBCyD	4,72	3,47	3,18	3,97

4.3. Protein binding studies

Nimesulide is a non-steroidal anti-inflammatory drug, which has an acidic character as a result of its sulfonamide functional group. This property makes the molecule capable of binding to human serum albumin (HSA). The aim was to establish the binding constant based on the acquired ICD spectra. The results of three parallel measurements are summarised in Table 2.

Table 2: Calculated logK values (M^{-1}) of nimesulide-HSA complex (and their regression coefficients)

I.	4,63 (0,9971)
II.	4,75 (0,9959)
III.	4,71 (0,9990)
Average	**4,70±0,06**

5. Conclusions

In this Ph.D. work the binding of a number of small molecules to macromolecules has been investigated. The drugs or drug target candidate molecules were either achiral or used in a racemic form, while all of the macromolecules were chiral.

In case of cyclodextrins the ICD signal registered during the experiments is highly selective for the complexations. The registered sign gives more information compared to UV spectroscopy, while having the same sensitivity. Compared to NMR spectroscopy the amount of information that can be obtained is almost the same, however sensitivity of CD spectroscopy exceeds that of NMR. The selectivity of the ICD sign is highly advantageous when calculating stability constants. While in other methods the detected signal is usually a result of the mole-fraction-weighted average of the free ligand or guest molecule and the complex, the ICD sign originates selectively from the complex. This method might serve as a basis for a fast and informative technique, where a series of CyDs can be tried with a selected guest molecule and based on the acquired ICD sign, the

optimal one can be chosen for the purpose in question. 27 stability constants have been calculated using the ICD sign, which are summarised in Table 1. Some of the constants have not been published before, while others can be found in the literature, but have been determined by other methods. Most of these are in good correlation with the results published in the thesis, however some show significant differences. Multiple reasons might be responsible for these, such as the different degree of substitution of CyDs or changes in the experimental circumstances. Based on the results obtained with CyDs, a further objective is to investigate complexation of other polysaccharide type excipients.

The selectivity described above is valid for DNA and proteins as well if the CD sign of the macromolecules and the ICD originating from ligand binding does not overlap. It needs mentioning that changes in the CD of the macromolecules also imply ligand binding. Based on the results it can be established that CD spectroscopy is a selective method for investigating such interactions.

The experimental results show that plasmid DNA can be used for DNA binding studies, however due to its circular and super helical structure the registered spectra might show differences compared to experiments performed with linear DNAs.

DNA binding of curcumin has been compared to several curcuminoids. While curcumin showed definite interaction with DNA, only a few of the roughly 30 curcuminoids showed similar behaviour. Considering their structure and the shape of the ICD sign, curcumin binds to the minor groove of the DNA, while the curcuminoids interact with the sugar phosphate backbone of the molecule.

CD and NMR measurements proved that sunitinib base is capable of interacting with heterogenous, natural and bacterial plasmid DNA. In case of plasmid, the binding happens in two steps, first the super helical polynucleotide chain unwinds, the intercalation occurs.

It has been explored that aristolochic acid I does not bind to open chain, natural DNA, however it does interact with bacterial plasmid. This difference is the result of the plasmid's circular structure. This

phenomenon might serve as a basis for the development of drugs targeting the properties of bacteria connected to plasmids (e.g.: resistance).

Based on the above it can be said that the ICD signal is well usable for detecting DNA interactions in vitro, while also providing a way to determine the mode of binding. In case of sunitinib this binding might account for the side effects in association with oral dosage as it can influence the division of the enterocytes.

ICD spectroscopy can also be used to investigate protein binding of drugs. To validate this method, the binding of nimesulide to HSA has been investigated. If the chromophore of the ligand has absorption above the absorption region of proteins (λ>280 nm), then by analysing the ICD spectra the stability constant can be calculated in addition to establishing the binding. The method also gives opportunity for the quick investigation of drug binding to target proteins.

6. Bibliography

6.1. Publications related to the dissertation

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6.2. Other publications

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