Monitoring the Interaction of Albumin with a Paclitaxel-Polymer Conjugate: Complex with Warfarin as Local Probe

Prof. Dr. Jung-Il Jin, Department of Chemistry, Korea University, Seoul, Republic of Korea, on the occasion of his 65th birthday

Stefan Wunderlich,[1] *Manfred Zähres,*[1] *Byung-Wook Jo,*[2] *Michael Hess**[1,2]

Summary: We have used warfarin as a local probe to investigate the orientation of paclitaxel and water soluble polymer conjugates of paxlitaxel in albumin. The relative orientation of warfarin and paclitaxel in a 1:1 complex in solution was investigated by ^{1}H-NMR-spektroskopy (NOESY) and the results are used for the interpretation of the steric situation of paclitaxel respectively the polymer conjugate in albumin.

Keywords: interaction; NMR; proteins; warfarin; water-soluble drug conjugates

Introduction

The water-solubility of many drugs is a problem for their application. This problem of drug-delivery and drug-interaction can be solved by proper molecular engineering: the hydrophobic drug can, be incorporated in a vesicle or a micelle, a water-soluble drug derivative can be synthesized by forming a polymer-drug conjugate with a water-soluble polymer, such as poly(ethylene glycol) - PEG, with a self-immolating link to control the stability[1] or a link that is temperature of pH-sensitive. What ever enters a body is faced with transport phenomena in veins, across membranes etc. Modification on the molecular level can improve certain properties or block others so that intelligent molecular modelling can contribute significantly to the efficacy, circulation time or targeting properties so that the bioavailability can be influenced.

For several years we are focussing on the anti-cancer drug paclitaxel, a hydrophobic taxol that can be isolated from the bark of the pacific yew tree *taxus brevifolia* or other taxiceae. Paclitaxel is a tetracyclic taxan-type diterpen that interferes with the microtubuli formed during the late G_2 phase of mitosis by stabilizing the tubuli. A water-soluble derivative with controllable stability was synthesized by Jo[1] using a monofunctional PEG that is chemically bonded to the carbon C7 through a self-immolating ester structure, see Figure 1. The polymer-drug conjugate is therefore termed PP7.

The properties of PP7 in aqueous solution have been intensively investigated during the recent years.[2–10] Although there is no evidence for self-assembly of PP7 it was shown that there are significant interactions with blood constituents like albumin – human serum albumin (HSA) and bovine serum albumin (BSA). There is interaction with the polymer conjugate as well as with just the lipophilic paclitaxel molecule. Although the number of paclitaxel ligands differs in literature, it is sure that the Sudlow site I houses one paclitaxel molecule in Subdomain IIA near tryptophan trp214. Subdomain IIA ranges from

[1] Department of Physical Chemistry, University Duisburg-Essen, Campus Essen, D-45117 Essen, Germany
E-mail: michael.hess@uni-duisburg-essen.de
[2] Department of Polymer Science and Engineering, Chosun University, 375 Seosuk-dong, Dong-gu, GwangJu, 501-759, South Korea
E-mail: bwj@mail.chosun.ac.kr

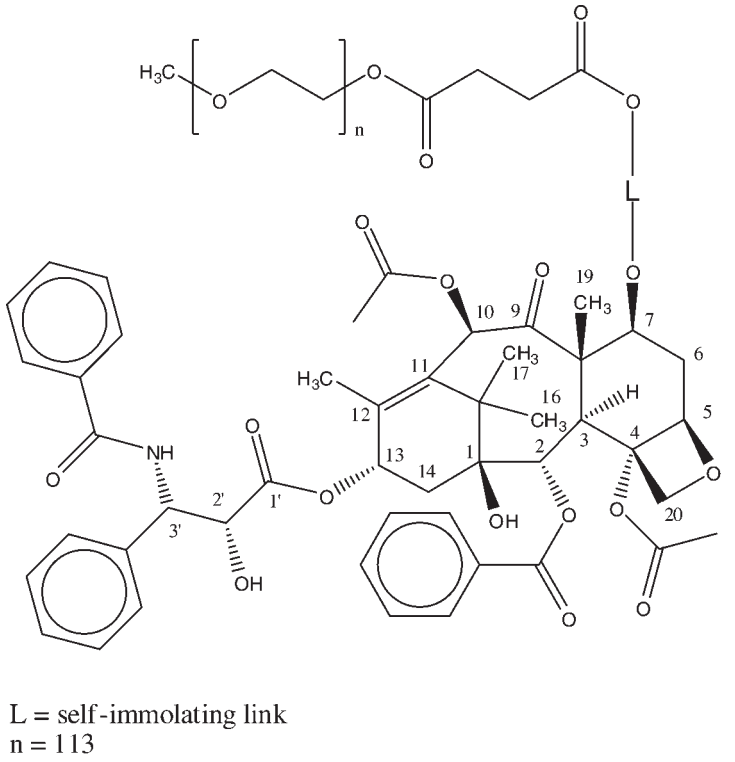

Figure 1.
Paclitaxel derivate PP7.

the amino acids 199 (lys) to 291 (ala) and forms a hydrophobic pocket inside the water-soluble protein.[11] Because of its chemical structure and morphology, albumin is known as one of the most important transport proteins. The amino acid trp in the core of the structure is an important fluorescence probe in the centre of BSA and HSA. BSA has a second trp that, in contrast, is located at number 134, close to the surface of the protein and it is believed that it is in a much more polar environment compared with trp214.

Studies of the intrinsic fluorescence of trp214 revealed that the paclitaxel molecule enters the hydrophobic pocket, coming closer to trp214 than 15 Å because the intrinsic fluorescence of the trp around 240 nm (excitation wave length $\lambda = 195$ nm) is effectively quenched by paclitaxel. It was also shown, that PP7 forms a complex with HSA and BSA, and a tail-anchor-model was derived for this specific hydrophobic interaction[2] of the bulky paclitaxel molecule with its long PEG-tail in the quite small but rather mobile albumin. Literature describes also more than one paclitaxel ligand on albumin,[3] but these are not considered here. There is evidence that the PEG-chain – a molar mass of 5,000 g/mol equivalent to about 114 repetitive units – is wrapped around the hydrophobic drug in aqueous solution. Proteins in solution coil in the same way with a hydrophobic core and a hydrophilic surface. That means that the complex-formation of PP7 and albumin requires some de-threading of the polymer coil. Albumin can also show certain esterase-activities but there is no evidence for that in the case of PP7.

There is an albumin-warfarin complex that shows a strong extrinsic fluorescence around 380 nm (excitation wave length $\lambda = 235$ nm) that can be used to monitor the near environment of the complex. It was observed that this fluorescence is quenched

by paclitaxel as well as by PP7. This gives a chance to gain information about the orientation of the paclitaxel residue in the hydrophobic pocket of albumin and has raised the idea to investigate which groups of warfarin and paclitaxel interact with each other in the albumin subdomain IIA, or that come sufficiently close to each other. Consequently, we have investigated the warfarin-paclitaxel system in solution and found that indeed there is a strong 1:1 warfarin-paclitaxel complex formed in aqueous solution.[2] It appears reasonable to assume that the relative orientation of warfarin and paclitaxel in solution is comparable to the orientation that is assumed inside albumin. In order to investigate this preferred relative orientation of the two molecules in the complex we have conducted a number of NMR-experiments such as NOESY and ROESY[12] which in principle can provide such information.

Materials and Methods

The NMR-spectra were measured on a BRUKER DRX-500 with a 5 mm broad band probe. The experimental conditions are summarized in Table 1.

All NMR-spectra were recorded in $CDCl_3$ if not stated otherwise. Warfarin was obtained from Aldrich, paclitaxel from Brystol-Myers Squibb, USA. To obtain water soluble PP7, paclitaxel had been coupled to α-hydro-ω-methyl-poly(ethylene oxide) (PEO) with a self-immolating succinic acid spacer (Figure 1), as described elsewhere.[1] A highly uniform PEO of the molar mass $Mw = 5{,}000$ g/mol (PEOS 5,000) was used to create the desired water solubility. The dispersity of the molar mass was 1.05 for all polymers, the succinate derivative, PEOS, and the hydrophilized drug PP7.

Results and Discussion

It was shown earlier that paclitaxel as well as PP7 can enter the hydrophobic pocket of albumin and interact with the warfarin-trp214 complex in subdomain IIA by fluorescence quenching. The detailed quenching mechanism –dynamic or static– is presently under investigation. Now, when there is such a strong interaction inside albumin there is a high probability that there is also a paclitaxel-warfarin interaction in aqueous solution. The formation of a strong 1:1 paclitaxel-warfarin complex was verified by uv-spectroscopy that showed an isosbestic point and an equilibrium constant $K = (75{,}000 \pm 1{,}000)$ mol/L, the same was found for PP7 with an equilibrium constant $K = (28{,}000 \pm 1{,}400)$ mol/L[2] (both in buffered aqueous solution). Given the strength of the interaction it is even plausible to assume that the warfarin molecule inside albumin attracts the same paclitaxel sites as in an outside complex. That should have consequences for the accessibility of a drug-polymer conjugate since the attached polymer could exactly block the interaction sites and prevent the drug from entering site IIA. For example a conjugate with substitution at carbon C2' and C7 could be a candidate.

NMR-spectroscopy that reflects the close local chemical environment of nuclei

Table 1.
Experimental NMR conditions.

	1D-NMR	2D-NMR NOESY	2D-NMR ROESY
Pulse Angle [°]	15	90	90
Data	64K	1K, 256 t1 inc.	1K, 256 t1 inc.
Transients	128	32	32
Recycle Delay [s]	3	4	4
AQ-Time [s]	5.5	0.102	0.102
Apodization	no	cosine bell	cosine bell
Mixing time [s]	no	0.6	0.6
Spin-lock time [s]	no	no	0.3

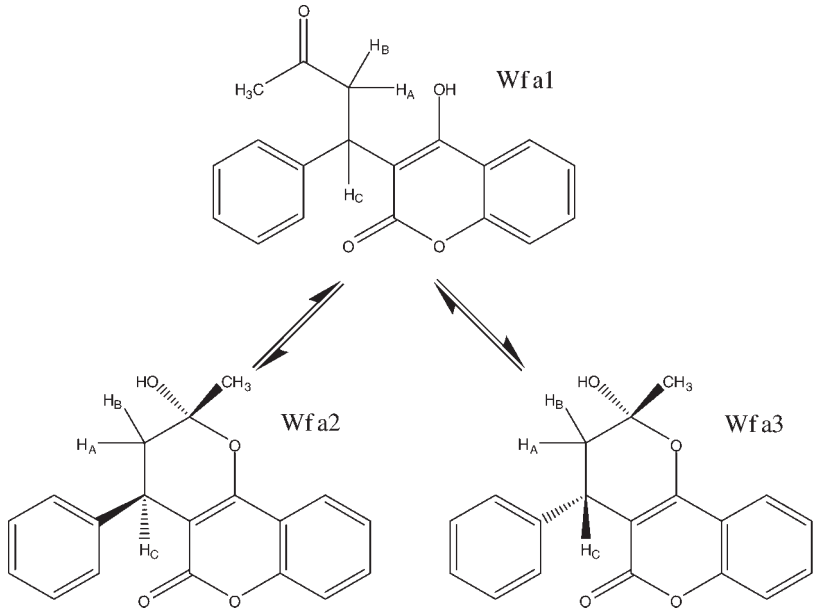

Figure 2.
Isomers of warfarin those are present in $CDCl_3$ solution.[13] The open-chain keto-form of warfarin (Wfa1) and the two cyclic hemiketals of the diastereomers of warfarin (Wfa2 and Wfa3).

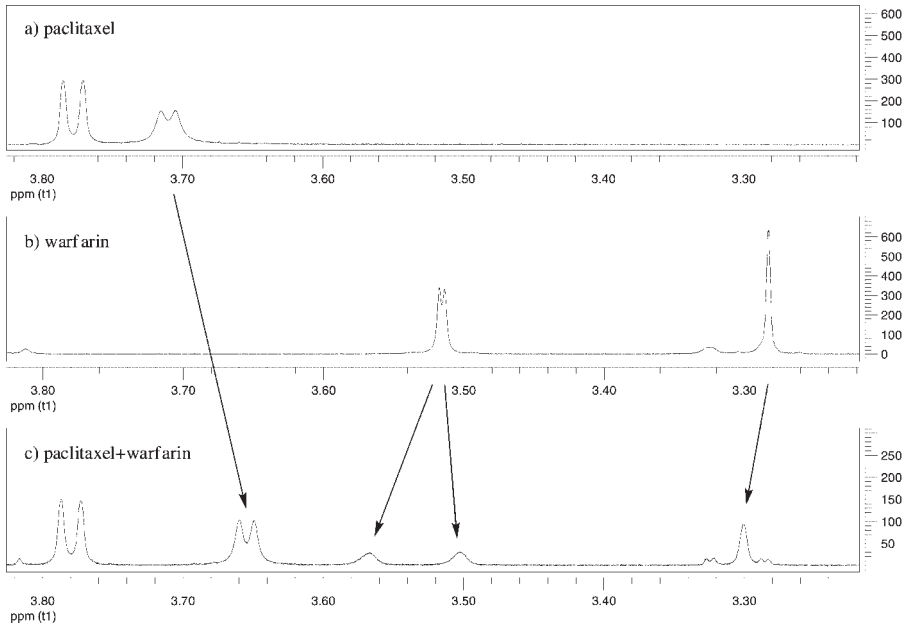

Figure 3.
^{1}H-NMR-spectra of a) pure paclitaxel b) pure warfarin, and c) a mixture of the samples a) and b) (1:1, molar). The paclitaxel C2′–OH-signal moves from 3.71 ppm to 3.66 ppm, the warfarin Wfa2 –OH-signal (see Figure 2) moves from 3.51 ppm to 3.54 ppm (centre) and the warfarin Wfa3 –OH-signal moves from 3.28 ppm to 3.3 ppm (centre).

appears to be a well-suited technique to tackle the problem. In the case of warfarin, different tautomers and stereoisomers (diastereoisomere) have to be considered, see Figure 2.[13]

Since we are ultimately interested in the orientation in the paclitaxel moiety in the apolar albumin environment and for sensitivity reasons we have performed the NMR-experiments in $CDCl_3$. The polarity of the solvent influences the tautomeric equilibrium of warfarin,[13] the presence of paclitaxel in solution does not affect the equilibrium significantly. ^{1}H-NMR-experiments revealed significant differences in chemical shift of the –O<u>H</u> protons between the individual components and the complex, which suggest the formation of a complex, see Figure 3. The resonance of the warfarin –O<u>H</u>s, Figure 3, are shifted to higher fields, while the paclitaxel –O<u>H</u> resonances experience a down-field shift in the mixture. The samples were carefully dried over a molecular sieve otherwise the resonances can broaden so much that they are almost undetectable.

The obtained ^{1}H-NOESY- and ^{1}H-ROESY 2D-spectra show a direct interaction of paclitaxel with each of the three warfarin tautomers. The spectra contain a set of NOE's for each of the tautomers and paclitaxel (see Figure 4), showing that there is an intermolecular interaction between these pairs of molecules with a distance between them of about 5 Å or less. Although the signals of the three warfarin tautomers can be clearly distinguished by their different chemical shifts, they all show the same correlations between them and paclitaxel.

In all three cases the intermolecular interactions take place between the aliphatic groups of warfarin and the aliphatic part of paclitaxel. All three warfarin tautomers show interaction with the very same hydrogen atoms (at carbons C3, C5, C6, C7, C19 and C20, see Figure 1), all close together within the aliphatic taxane-substructure of paclitaxel. These atoms are protruding

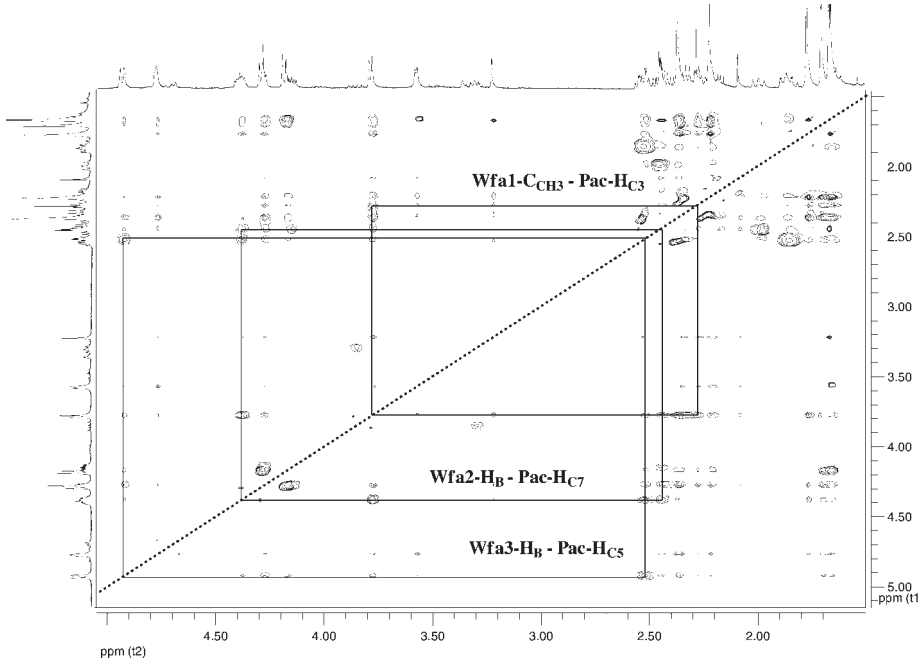

Figure 4.
NOESY-^{1}H-NMR-spectrum of a paclitaxel-warfarin mixture. Positive signals (exchange) are removed, only negative signals (NOE's) are shown. The positive diagonal of the spectrum is replaced by a dotted line. The Figure shows some intermolecular correlation examples between the warfarin tautomers and paclitaxel. Identification of the protons see Figure 1 and Figure 2.

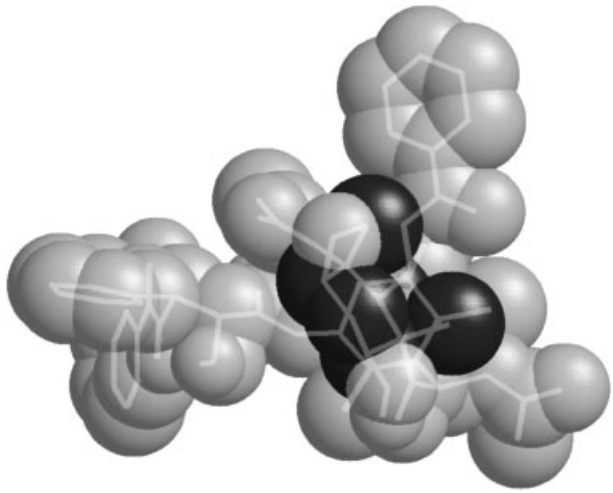

Figure 5.
Paclitaxel molecule. Sites interacting with warfarin are black.

from the bulk of the drug, so that this region is quite exposed to the solvent (Figure 5). This region is also located equidistantly from the two ends of the paclitaxel molecule, so in case of interaction within the pocket of BSA or HAS, warfarin could reach the preferred site at paclitaxel quite easily. This enables the complex formation of paclitaxel and warfarin independently of the orientation in with paclitaxel has entered the albumin pocket. Analysis of the fluorescence quenching behavior of PP7 and another similar paclitaxel prodrug PP2′ with the polymer chain attached to carbon C2' instead of C7 (compare Figure 1), supports this hypothesis. Depending on how the polymer chain attaches to opposite parts of the molecules the paclitaxel part of them should enter the albumin pocket in different directions. Despite of this, they are both able to quench the extrinsic fluorescence of the albumin-warfarin complex in a nearly identical way.

Conclusion

Paclitaxel and its polymer conjugate PP7 show strong interaction with warfarin in $CHCl_3$ solution and in the hydrophilic docking site IIA of albumin where warfarin forms a strongly fluorescent complex with trp214. It is reasonable to assume that the strongly interacting sites of the paclitaxel-warfarin complex in solution are the same sites of paclitaxel that interact with warfarin when it occupies the albumin site IIA, so that warfarin can act as a monitor or a reporter group.

Either are the warfarin sites free accessible to the penetrating paclitaxel or the paclitaxel molecule changes the warfarin orientation at site IIA. Both reactions would be able to cause the quenching of the extrinsic fluorescence at 380 nm of the warfarin–trp214 complex that was observed.[3] ^{1}H-NOESY and ^{1}H-ROESY experiments revealed that the interaction site of paclitaxel is localised near the aliphatic taxane substructure near carbons C3, C5, C6, C7, C19 and C20 (see Figure 1 and Figure 5) at one end of the molecule. Coupling of a PEO-chain to C7 does not prevent a penetration of the paclitaxel moiety into albumin with its taxan end first. This requires some dethreading of the paclitaxel surrounding polymercoil, have the drug acts as a kind of anchor. This decoiling of the polymer coil from paclitaxel may be best described as a mushroom

with the (drug) stem anchored inside the albumin and the cap spread on the surface of albumin.

Coupling of the polymer chain to C2′ seems not to change the situation significantly because C2′ is even farther away from the taxane end of paclitaxel and an even easier penetration of PP2' compared with PP7 is likely. Corresponding quantitative measurements of the extrinsic fluorescence are under the way. Investigation of the life-time of the warfarin-trp complex will give information about kinetics of the quenching process, whether it is static or dynamic. Since the orientation of paclitaxel entering the albumin seems to be clear, more thoughts can be spent on intelligent molecular engineering on the drug molecule to influence its interaction with albumin. Substitution at C2′ might be advantageous for a stronger interaction – this is presently investigated. To prevent interaction of paclitaxel and albumin a double substitution at C2′ and C7 might be advisable or a more rigid or bulky substituent such as a sugar or a cyclodextrin. The information about the warfarin-paclitaxel interaction sites might also be very useful to develop new complex-based drug transport systems instead of covalent by attachments of water–soluble groups to paclitaxel using comparable structures like those in warfarin. Furthermore, one might think about useful drug combination complexes[14] that combine several useful properties and which may be sensitive to pH-changes or temperature or magnetic effects. Interaction of paclitaxel and warfarin where already observed in human patients through changes in the international normalised ratio INR with paclitaxel administration.[15,16] It was concluded, that 95%–98% is bound to plasma proteins and replaces warfarin from protein binding sites. Our results show that the situation is probable more complex and in particular earlier fluorescence studies indicate[17] that warfarin it not necessarily replaced by paclitaxel but that they can form a strong complex even near to albumin's subdomain IIA.

Acknowledgements: We thank Prof. Dr. W. S. Veeman and Dipl.-Ing. H. Bandmann Duisbur-Essen for helpful discussions, and C. P. Lee, Cambridge, for support.

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