Paclitaxel-Warfarin Interaction in Solution[#]

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Summary: Interactions of a water-soluble paclitaxel-polymer conjugate were investigated in aqueous buffer solutions and in the solid state in the presence of model proteins that are abundant in blood serum (bovine serum albumin and human serum albumin). Studies of the intrinsic fluorescence of albumins and extrinsic fluorescence of the albumin/warfarin complex and MALDI-TOF spectra proved significant interactions of the drug conjugate with the model proteins and warfarin.

Keywords: interaction; proteins; warfarin; water-soluble paclitaxel

Introduction

Paclitaxel or taxol (Taxol® by Bristol-Myers-Squibb) is an anti-tumour diterpen that can be extracted from the bark of the Pacific yew tree, *taxus brevifolia*. It belongs to the group of spindle poisons and interacts in mitosis by promoting the formation of the microtubule from tubulin thus inhibiting disassembly so that mitosis is stopped. Mechanism and sites of interaction of paclitaxel with tubulin have been subject of detailed investigations, e.g. [1,2]. Because of its low water-solubility [3,4] a polymer conjugate has been synthesised by Jo [5] that connects the drug with the hydrophilic poly(ethylene glycole) (PEG) through a self-immolating link that controls the hydrolytic stability of the prodrug, see Fig. 1.

Properties of this conjugate in aqueous solution have been subject of recent publications [6–10], and also interaction with blood serum constituents has been identified [11]. In particular interactions of paclitaxel, respectively the prodrug PP7, Fig. 1, with human serum albumin (HSA) and bovine serum albumin (BSA) have been studied using the intrinsic fluorescence of the amino acid tryptophan (trp) at position 214 in HSA (214 and 134 in BSA) and the extrinsic fluorescence of the warfarin-trp (214) complex of the albumins. Position 214 is situated in one of the primary docking sites for rather apolar, bulky ligands in a hydrophobic pocket, see Fig. 2.

Examples of typical ligands are salicylate, warfarin or bilirubin. While there are six to eight binding sites for fatty acids, rather randomly distributed over the molecule, see for example Goodsell [12], there are two major binding sites for all kinds of drugs, metabolids etc. according to the studies of Sudlow [13]:

Site I : in subdomain IIA, loops 4-5, lys 199 to ala 291
Site II : in subdomain IIIA, loops 7-8, pro 384 to ser 489

Site II is known to catalyze hydrolysis of some esters, however, we could show that the self-immolating link of PP7 is not affected in aqueous buffer solutions of pH 7.4 in the presence of BSA or HSA in the absence of other proteins [14].

Many different types of ligands interact with Site I, among them many drugs, and the fact that the fluorescing trp 214 is located in this hydrophobic pocket makes this site attractive for fluorescence studies.

[#]Prof. Wibren S. Veeman, Department of Physical Chemistry, University Duisburg-Essen, Germany, on the occasion of his 65th birthday

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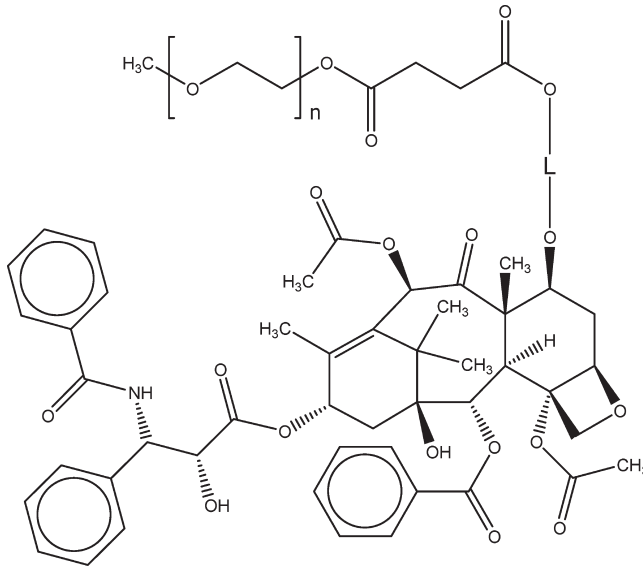

Figure 1.
Prodrug PP7, a watersoluble PEG-paclitaxel conjugate. L is a self-immolating link

Investgation of the paclitaxel-albumin complex shows that more than one paclitaxel molecule serves as a ligand on albumin [15–17]. Obviously, the same is true for the polymer conjugate PP7 with its flexible, hydrophilic polymer coil of PEG. Even in the gas phase of a MALDI-TOF experiment these aggregates can be observed [18].

Paclitaxel as well as the albumins [19] are very flexible molecules – at least in parts. The diterpenoid structure (taxane) is quite rigid while the ester side chain that stretches out to the left in Fig. 1 is quite

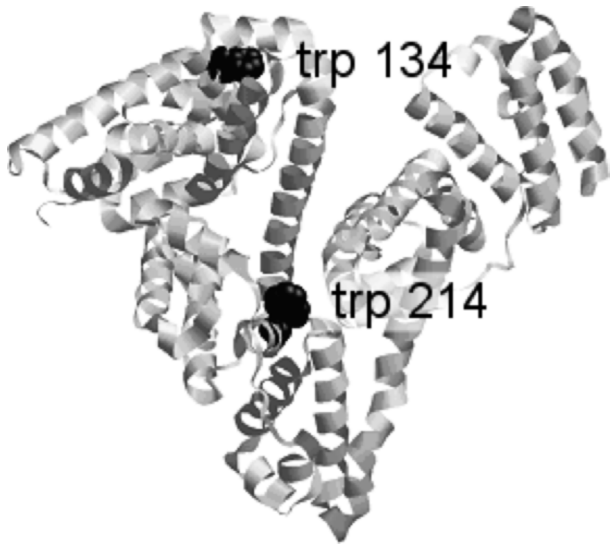

Figure 2.
The BSA molecule with trp 214 in the hydrophobic core of the molecule and a second trp at position 134 near the polar surface of the protein.

mobile and the two aromatic rings on C1′ and C3′ can change their orientation in a rather wide range. Depending on their chemical environment or ligand interaction they can obtain several favourite conformations in solution [20–24]. There is in particular the so-called ''hydrophobic collapse'' – a clustering of the 2 benzoate-, the 3′ phenyl- and the 4-acetyl group in the presence of water molecules [22]. This is apparently important when interactions with amphiphilic molecules such as albumins are discussed.

Warfarin, one of the well-known albumin ligands, exists in solution not only in its open keto form but also as hemi-ketal tautomers, Fig. 3.

It was experimentally shown that paclitaxel and the prodrug PP7 are able to enter Sudlow site I so that the intrinsic trp 214 fluorescence is quenched. The strong extrinsic fluorescence of the albumin trp 214-warfarin complex is also effectively quenched by paclitaxel and PP7. The orientation of warfarin near trp 214 in HSA as determined from the solid state and X-ray analysis is shown in Fig. 4 and Fig. 5.

The fluorescence quenching effect of paclitaxel is in both cases stronger compared with PP7 [26]. The Stern-Vollmer diagram shows a significant non-linearity indicating that also in solution complexes of albumin and PP7 respectively paclitaxel higher than 1:1 are existing and that the docking sites are not independent of each other. The fact that paclitaxel and its polymer conjugate interact with albumin makes it an important competitor in transportation of lipophilic substances in the blood and through membranes.

The entrance to the hydrophobic pocket of albumin is rather narrow and the paclitaxel molecule is rather bulky, see Fig. 6. In particular, the polymer conjugate PP7 is not able to enter the pocket completely. Therefore the question about the conformational arrangement of the principal complex arises.

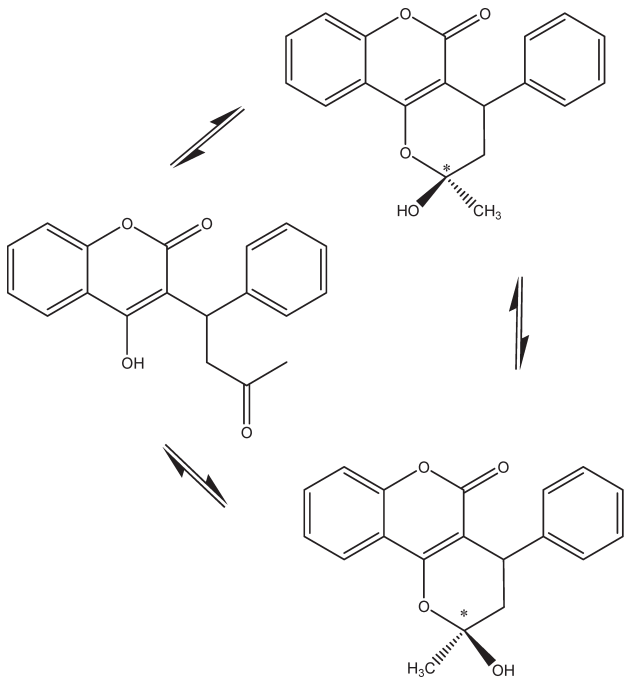

Figure 3.
Warfarin open isomer and cyclic tautomers. The equilibria depend on the nature of the solvent. The crystalline state consists of the cyclic tautomer [25].

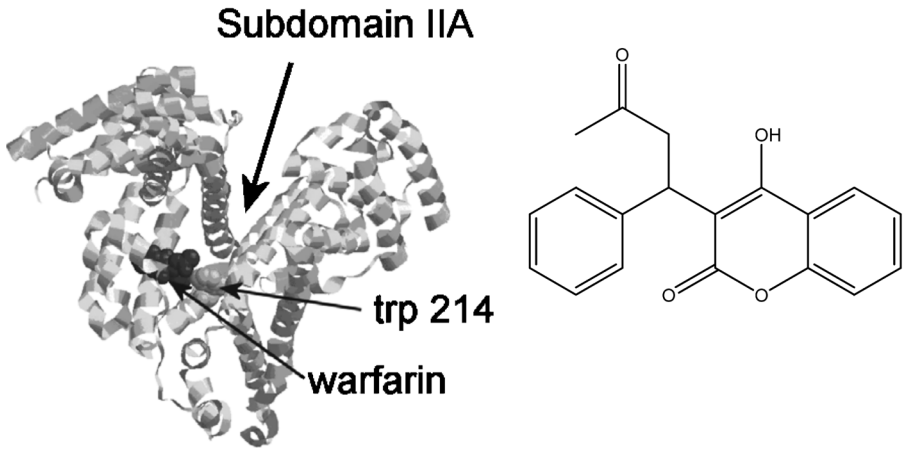

Figure 4.
The trp-warfarin complex, program RASMOL, after Polymer Data Bank, entries 1AO6, 1BJ5, 1BKE, 1BM0, 1E78, 1E7A, 1E7B, 1H9Z.

Materials and Methods

Paclitaxel had been coupled to α-hydro-ω-methyl-poly(ethylene oxide) (PEO) with a self-immolating succinic acid spacer (abbreviation of the PEO with the linker: PEOS) to obtain the water soluble product PP7 (Fig. 1), as described elsewhere [5,8–10,28]. A highly uniform PEO of the molar mass Mw = 5,000 g/mol (PEOS 5,000) was used

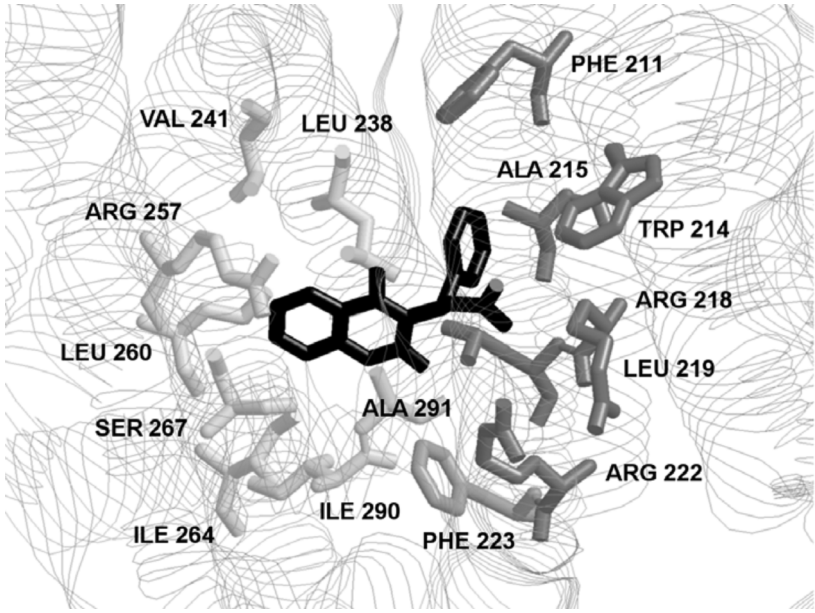

Figure 5.
Warfarin in the hydrophobic pocket of HSA: view from above into the pocket. Amino acids 211 to 291. The surrounding helices are visible. Warfarin is shown in the open keto configuration and appears to be accessible from the solution.

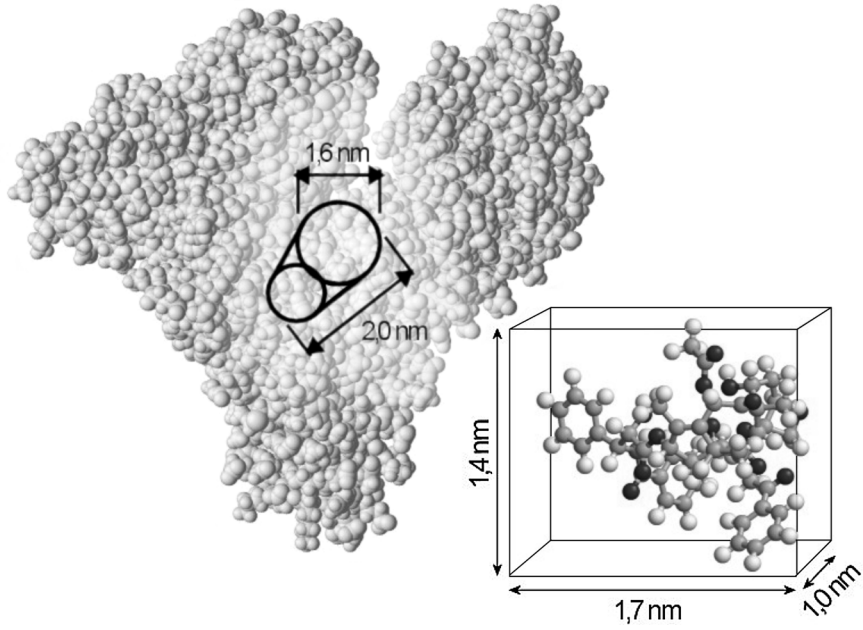

Figure 6.
The steric situation at the entrance of Site I and the approximate size of palitaxel. The conformations of the drug can vary depending on its chemical environment. In particular in mixed polar/apolar environment the "hydrophobic collapse" of the drug's conformation is observed. With the pure drug and with the drug conjugate PP7 concentration-dependent alterations of the CD-spectrum indicates conformational changes of albumins affecting the helical content [27].

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. UV-spectra were measured with a Perkin-Elmer UV-vis λ40, for the fluorescence measurements a Perkin-Elmer LS50B was used. All measurements were performed in buffer solution (modified Hank's solution) at pH 7.4 and room temperature.

Warfarin-Paclitaxel-Interaction

Since PP7 is able to quench the intrinsic fluorescence of trp 214 in albumins the paclitaxel part of PP7 has to come into interaction range with the excited trp 214 for energy transfer that means approximately 20 Å or less. The conformation of the prodrug in solution appears to be a deformed coil with core-shell structure hiding the lipophilic drug covered by the hydrophilic PEO that provides a polar surface [8–11,18,26]. Consequently, the complex formation inside Sudlow Site I can be assumed to consist of roughly two steps: a docking of the prodrug near the entrance of Site I, probably induced by hydrophobic interaction, followed by a "dethreading" of the drug from the coil and penetration into the pocket. Time and temperature dependent fluorescence measurements could reveal details of this process. As a result of this process the drug would be arranged inside the pocket like a stem of a mushroom (acting as an anchor) with the parasol of the "mushroom" (*i.e* the polymer coil) stabilizing the complex outside like a shield. The high flexibility of both the protein and the prodrug facilitate this process. The existence of albumin-PP7 complexes in the gas-phase support this model. The warfarin-albumin complex interacts with

paclitaxel and PP7 but the warfarin is not replaced by paclitaxel or PP7 because the warfarin-trp 214-interaction is observed independently of the sequence in which the drug and warfarin are added to albumin. Apparently, the warfarin-trp 214 energy transfer is weakened and this can be explained by an increase of the distance between trp and warfarin,. which can be caused by attractive forces of paclitaxel acting on warfarin as schematically shown in Fig. 7. Our model – Fig. 7 – is supported by the following consideration: The emission wave length of the maximum of the intrinsic fluorescence of trp 214 in albumin, as shown in Fig. 9, reflects the polarity of its environment within a radius of less than 2 nm. The intrinsic fluorescence of trp 214 is quenched by the presence of warfarin when the complex is formed. The maximum of the band is red-shifted (bathochromic shift) which indicates a more polar environment of trp 214. This more polar environment is provided by the polar groups of warfarin. Addition of paclitaxel (or PP7, not shown in Fig. 8) to the albumin-warfarin complex results in a competitive situation and apparently paclitaxel attracts warfarin.

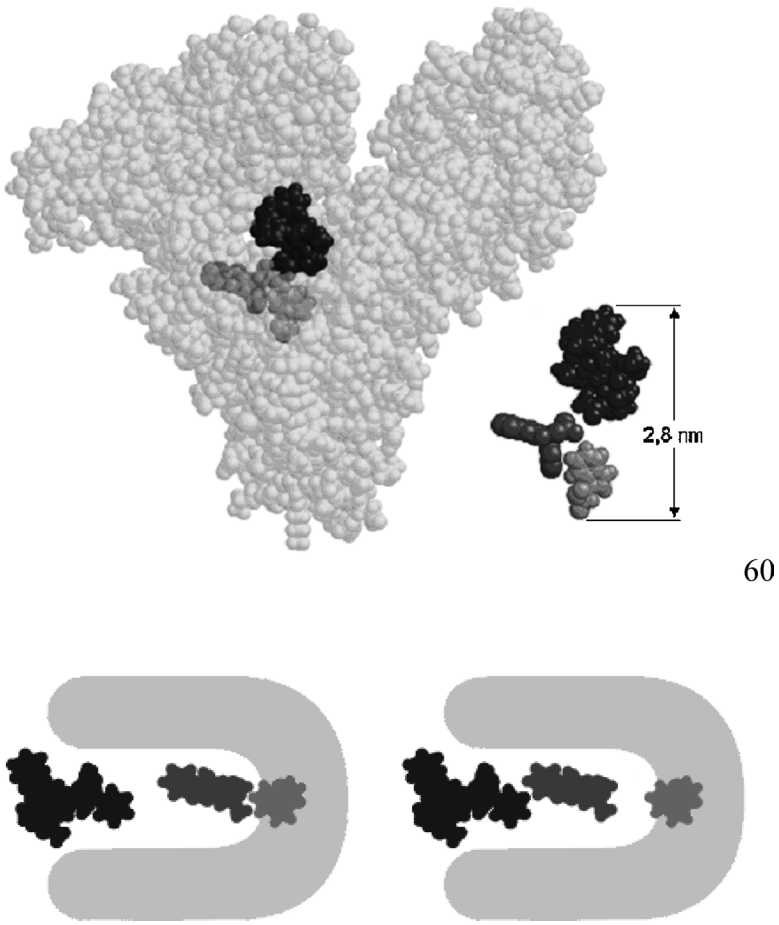

Figure 7.
Schematic representation of the interaction of paclitaxel with a warfarin molecule inside Sudlow Site I weakening the energy transfer between trp 214 and warfarin by attracting it. The process is facilitated by the mobility of the protein. The arrangement of the molecules above was not calculated but represent the best geometrical fit.

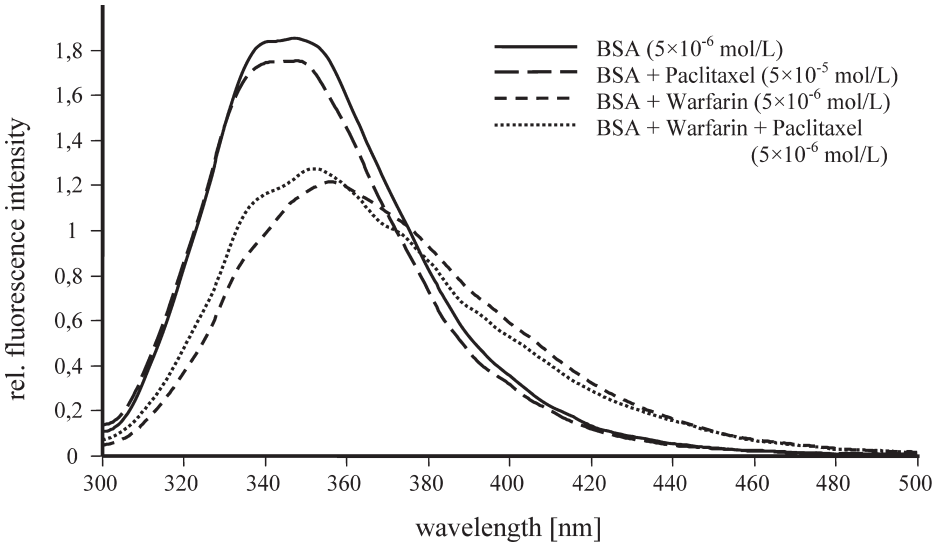

Figure 8.
Intrinsic fluorescence of trp ($\lambda_{ex} = 295$ nm) in the presence of warfarin and paclitaxel, explanation see text.

Retraction of warfarin from the trp 214 site of albumin results in a now less polar environment of trp 214; the experiment shows a hypsochromic shift of the maximum of the intrinsic trp-fluorescence down to approximately 350 nm as expected from the model in Fig. 7. Also the intrinsic fluorescence of trp 214 is slightly increased again. The polymer-drug conjugate PP7 shows the same effects.

To justify the model sketched in Fig. 7, interactions between paclitaxel and war-

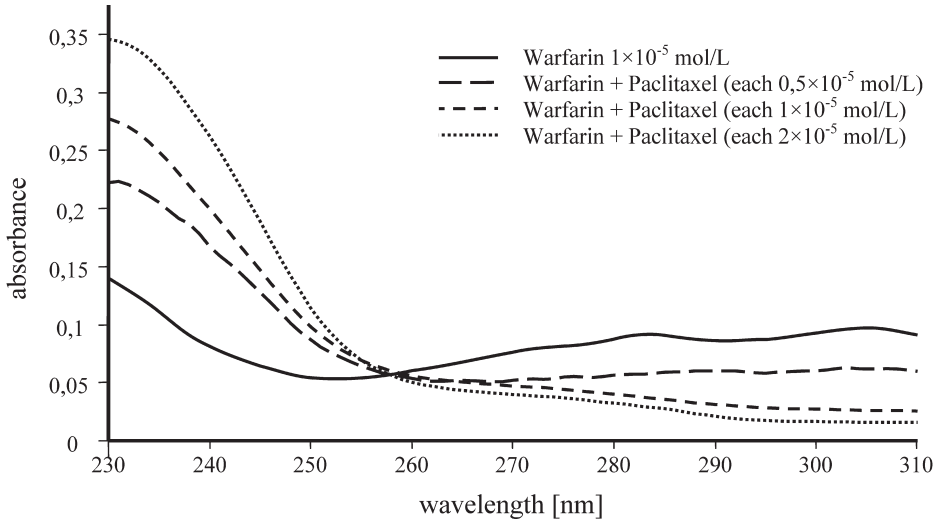

Figure 9.
UV-spectrum of different buffer solutions (pH 7.4) containing a constant amount of warfarin and different amounts of paclitaxel (as indicated in the legend). There is an isosbestic point at $\lambda = 258$ nm and a rank analysis of the absorbance matrix ***A*** gives a matrix rank $s = 1$. This clearly indicates a one-step equilibrium between two components [29].

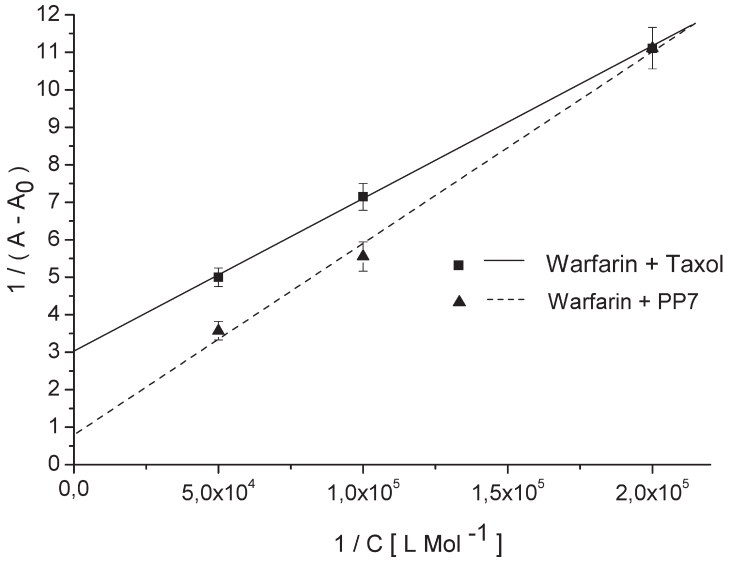

Figure 10.
Scatchard-plot (eq. 3) of the warfarin-PP7 respectively warfarin-paclitaxel system in aqueous buffer solution at pH 7.4. *A* is the UV absorbance.

farin in solution was examined. UV-spectroscopic studies in aqueous buffer solutions showed an isosbestic point indicating a complex between the two components, see Fig. 9.

There is a reversible equilibrium and not a chemical reaction such the formation of a semi- or full ketalization of the warfarin-carbonyl. The complex-formation was analyzed according to [29,30] using a Scatchard plot [31]. The general case is that there are m_i classes of docking sites ($i = 1, 2, 3, \ldots m$), each of these classes comprises a number of $n_{i,k}$ sites (of class m_i) with an intrinsic association constant K_m of class m_i. As long as these sites are independent of one another (no cooperation) and assumed that the association constants of one class are equal (which defines a class) there is [32]:

$$\nu = \sum_{i=1}^{m} \frac{n_i \cdot K_i \cdot [L]_{free}}{1 + K[L]_{free}} \tag{1}$$

If there is the simple case of only one class ($m = 1$) with n_i independent binding sites eq. 1 becomes eq. 2:

$$\nu = \frac{n_i \cdot K_i \cdot [L]_{free}}{1 + K[L]_{free}} \tag{2}$$

Eq. 2 can be linearized and in the present case setting ν and n_i for the present case equal to one, eq. 3 can be used to estimate the equilibrium constant of the paclitaxel-warfarin complex:

$$\frac{1}{\nu} = \frac{1}{n_i} + \frac{1}{n_i K_i} \cdot \frac{1}{[L]_{free}} \tag{3}$$

The equilibrium constant for the warfarin-paclitaxel complex is evaluated from Fig. 10:

$$K_{\text{paclitaxel}} = (75,000 \pm 1,000)\ \text{mol/L}$$

For the prodrug PP7 a significantly smaller equilibrium constant is evaluated:

$$K_{\text{PP7}} = (28,000 \pm 14,000)\ \text{mol/L}$$

The warfarin-albumin complex formation constant is given by Pinkerton [34] as:

$$K_{\text{alb}} = 3.3 \cdot 10^5\ \text{mol/L}$$

The paclitaxel-albumin complex is roughly given by several authors [17,35] as:

$$K_{\text{alb}} \approx 10^4\ \text{mol/L} \ldots 10^6\ \text{mol/L}$$

These results show that an interaction of paclitaxel as well as the prodrug PP7 with albumin-bound warfarin is likely and that the presence of any of them – although they are not able to replace warfarin – they can

at least weaken the warfarin trp 214 complex significantly.

First NMR-spectroscopic investigations indicate that the complex formation between warfarin and paclitaxel involves interactions near C2′, the cyclic ether structure near C20 of paclitaxel and the keto-group of warfarin, most probably with the free keto-form. A more detailed investigation is in preparation.

Conclusion

It could be shown that increased water solubility by a hydrophilic polymer conjugate does not necessarily hinder complex formation of a lipophilic drug with other constituents of blood serum, pharmaceuticals or other components present in living systems. This can be a disadvantage when a drug competes with a transport system in a living system such a transport protein or a membrane in particular in multi-drug therapies. It can be an advantage if it can be utilized to improve transport properties, aggregation to larger assemblies that show a higher local drug density, membrane – interaction, functionalization to provide thermolability or pH-sensitivity of a prodrug *etc*. The interaction of the components is not trivial and can also depend on the conformation or the existence of different tautomers that have to be considered.

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[1] M. Malaloom, D. Crètien, E. Karsenti, J. H. K. Hørbe, *J. Cell Sci.* **1994**, *107*, 3127–3131
[2] P.B. Schiff, J. Fant, S. B. Horwitz, *Nature* **1979**, *277*, 667
[3] B.J. Tar, S. H. Yalkowsky, *J. Parent Sci.* **1987**, *41*, 31–36
[4] V. Farina, *The Chemistry and Pharmacology of Taxol and its Derivatives*, Elsevier, New York, 1995
[5] Jo B-W 2000 Korean Patent 2000-0019873; US-patent 6,703,417 B2, March **2004**
[6] M. Hess, J.-S. Sohn, S.-K. Choi, B.-W. Jo, *Macromol. Symp.* **2003**, *201*, 163
[7] M. Hess, M. Zähres, B.-W. Jo, *Macromol. Symp.* **2004**, *214*, 351–359
[8] J.-S. Sohn, S.-K. Choi, B.-W. Jo, M. Hess, *Mater. Res. Innovat.* **2005**, *9*(1), 85
[9] J.-S. Sohn, B.-W. Jo and M. Hess, K. Schwark, S. Dehne and M. Zähres *Macromol. Symp.* **2005**, *225*, 31
[10] J.-S. Sohn, S. K. Choi, B.-W. Jo, K. Schwark and M. Hess, *e-Polymers* **2005**, *007*, 1
[11] M. Hess*, B.-W. Jo, B. Wermeckes, S. Dehne, J.-S. Sohn, S. Wunderlich, M. Zähres Macromol. Symp. **2006**, *321*, 28
[12] D. S. Goodsell, Protein Data Bank entry ie71; http://www.rcsb.org/pbd/molecules/pbd37_1.html
[13] G. Sudlow, D. J. Birkett, D. N. Wade, *Mol. Pharmacol.* **1976**, *12*, 1052
[14] M. Hess, unpublished results
[15] L. Trynda-Lemiesz, M. Łuczkowski, *J. Inorg. Biochem.* **2004**, *98*, 1856
[16] L. Trynda-Lemiesz, *Bioorg. & Medical Chem.* **2004**, *12*, 3269
[17] M. Purcell, J. F. Neault, H. A. Tajmir-Riahi, *Biochim. Biophys. Acta*, **2000**, *1478*, 61
[18] B. Wermeckes, M. Hess*Stefanie Dehne, Byung-Wook Jo, Jung-Sun Sohn, *Mater. Res. Innov.* 2006 accepted
[19] T. Peters, *All about Albumin: Biochemistry, Genetics, and Medical Application*, Acad. PressOrlando, FL, 1966
[20] J. P. Snyder, N. Nevins, D. O. Cicero, J. Jansen, J. Am. Chem. Soc. **2000**, *122*, 724
[21] H. J. Williams, A. I. Scott, R. A. Dieden, C. S. Swindell, L. S. Chirilan, M. M. Franci, J. M. Heerding, N. E. Krauss, Tetrahedron **1993**, *49*, 6545
[22] D. G. Vander Velde, G. I. Georg, G. L. Grunewald, C. W. Gunn, L. A. Mitscher, J. Am. Chem. Soc. **1993**, *115*, 11650
[23] L. Gomez Paloma, R. Kiplin Guy, W. Wrasidlo, K. C. Nicolaou, Chemistry & Biology **1994**, *1*(2), 107
[24] J. Dubois, D. Guénard, F. Guéritte-Voegelein, N. Guedira, P. Potier, B. Gillet, J.-C. Beloeil, Tetrahedron **1993**, *49*, 6533
[25] E. J. Valente, E. C. Ligafelter, W. R. Porter, W. F. Trager, J. Med. Chem. **1977**, *20*(11), 1489
[26] S. Wunderlich, Diploma Thesis, Duisburg 2006
[27] M. Hess, V. Buss, unpublished results
[28] Jo, B.-W. and Kolon Inc., PCT/Kr01/00168
[29] J. Polster, H. Lachmann, *Spectrometric Titration*, Wiley, New York, 1989
[30] I. M. Klotz.D. L. Hunston, Arch. Biochem. Biophys. **1979**, *193*314–328
[31] K. Paál, J. Müller, L. Hegedûs, Biochem. **2001**, *268*, 2187
[32] G. Scatchard, Ann. NY Acad. Sci. **1949**, *51*, 660
[33] H. R. Kalbitzer, D. Stehlik, Z. Naturf. 1979, 34c, 757
[34] T. C. Pinkerton, K. A. Koeplinger, Anal. Chem. **1990**, *62*, 2114
[35] K. Paál, J. Müller, L. Hegedûs, Eur. J. Biochem. **2001**, *268*, 2187

