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Paclitaxel–albumin interaction in view of molecular engineering of polymer–drug conjugates*

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Abstract: The interaction of water-soluble polymer conjugates of the anticancer agent paclitaxel and albumin as model protein has been investigated using fluorescence spectroscopy and NMR. Drugs and drug conjugates can enter the hydrophobic core of albumin; the kinetics of the interaction with the fluorophore, however, differs. Given the information about the steric situation of the formed complexes, some aspects of molecular engineering of the drug are discussed.

Keywords: paclitaxel; polymer–drug conjugates; protein interaction; fluorescence quenching; NOESY; molecular engineering.

INTRODUCTION

Interactions of pharmaceutical drugs with serum constituents are an important issue in drug delivery. Specific interactions and competition for docking sites on transport proteins can significantly influence the behavior of a drug in serum liquid. Albumins such as human serum albumin (HSA) and bovine serum albumin (BSA) play an important role in the transportation of lipophilic substances in serum where they are the most important carrier molecules. 60 % of the blood proteins is albumin, 30–40 % of which is present in the intravascular space. These albumins are proteins with a molar mass of about 66 000 g/mol. The number of amino acids and their exact sequence depend on the particular species. For example, there are 585 amino acids in BSA, which include 2 tryptophans, trp 134 near the surface of the protein in a more polar environment, and trp 214 in the hydrophobic so-called Sudlow site I, subdomain IIA, which comprises the loops 4–5 (lys 199 to ala 291), see Fig. 1.

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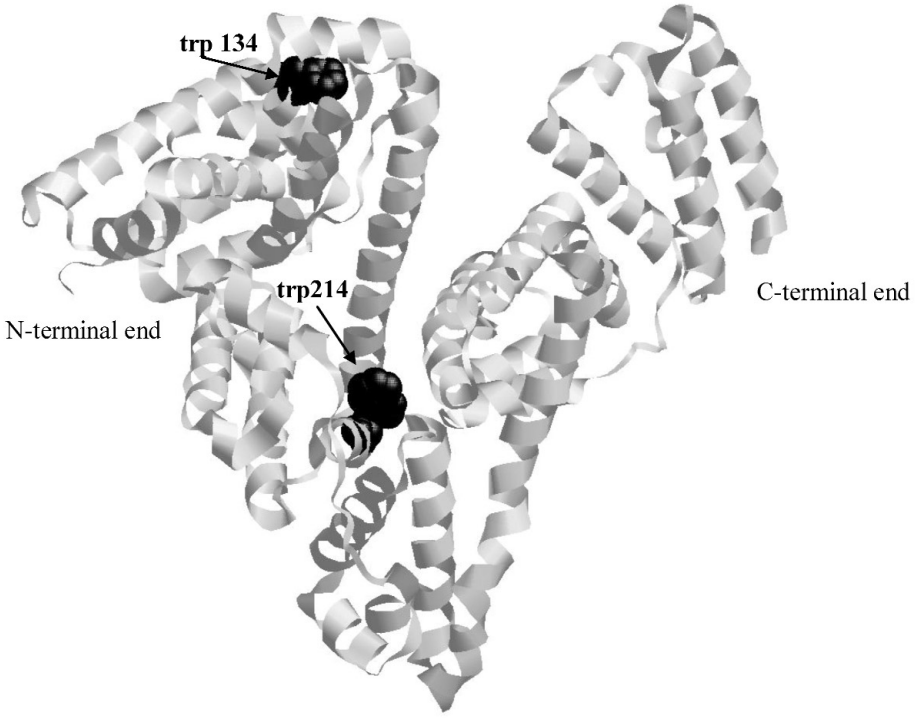

Fig. 1 BSA with the fluorescent amino acid trp134 close to the protein surface in a more polar environment and trp214 in docking site Sudlow I, subdomain IIa. trp shows intrinsic fluorescence around 345 nm after excitation with light of 290 nm. Other fluorescent amino acids such as phenylalanine or tyrosin require excitation at shorter wavelength and do not interfere. Protein structure from PDB 1AO6.

Tryptophane shows an intrinsic fluorescence when exposed to light with the excitation wavelength of 290 nm. The emission wavelength depends on the polarity of the environment of the fluorophore. The maximum is localized at 354 nm in an aqueous environment, and it is hypsochromically shifted when the environment becomes less polar. Quencher molecules can promote nonradiative decay processes of an excited state when the distance between the excited molecule and the quencher is less than 15 Å. This effect can be used to test if a substance can enter Sudlow site I. Certain substances, for example warfarin (see Fig. 2), can form a complex with trp that shows a strong, so-called extrinsic, fluorescence, Fig. 3. The warfarin-trp complex can be excited with light of 335 nm wave length, the emission wavelength is around 380 nm. Measurement of the concentration and temperature dependence of fluorescence lifetime or of the fluorescence intensity can be used to investigate the interaction between albumin and a substrate.

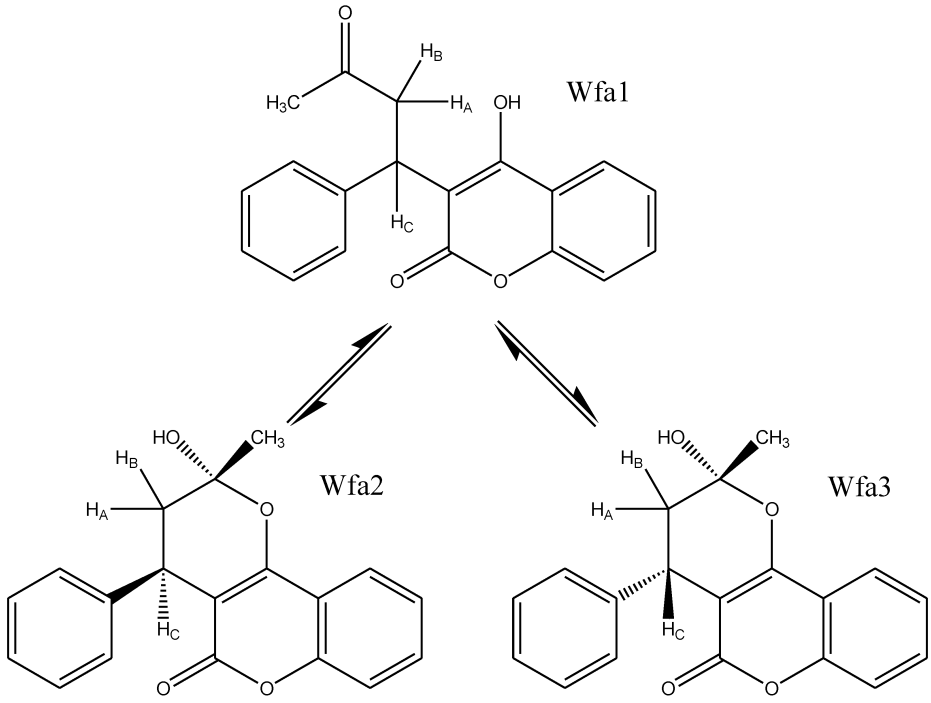

Fig. 2 Tautomeric forms of the warfarin molecule 4-hydoxy-3-(3-oxo-1-phenylbutyl)cumarin. The cyclic semiketal form shows a number of stereoisomers that are also observed by NMR spectroscopy. All three forms are present in solution with the cyclic forms preferred. Complex formation can shift the tautomeric equilibrium.

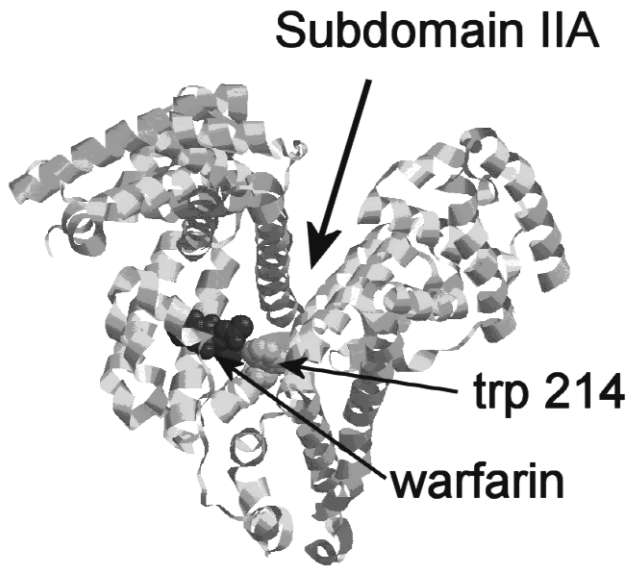

Fig. 3 Warfarin in subdomain IIa forming a fluorescent exciplex (λ_{ex}= 335 nm; $\lambda_{em} \cong$ 380 nm) with trp214. This so-called extrinsic fluorescence can be quenched by molecules coming closer than 15 Å and can therefore be used as a sensor for other molecules entering the docking site. Protein structure from PDB 1AO6.

Paclitaxel (or Taxol*) (M = 853.92 g/mol), Fig. 4, is a strong anticancer drug of the taxol family that can be extracted from the bark of the Asian yew tree (*Taxus brevifolia*) or other taxiceae. This tetracyclic taxan-type diterpen is lipophilic with only a poor water solubility (≈0.6 mmol/L) [1]. Paclitaxel is well known for its anticancer activity against a number of different types of cancer such as breast, lung, or ovarian cancer [2]. In recent years, it also drew attention in preventing restenosis after percutan transluminal angioplasty. Paclitaxel interferes in cell replication by stabilizing the microtubuli in the late G_2 phase of the mitosis so that the cells are unable to assemble the mitotic spindle [3].

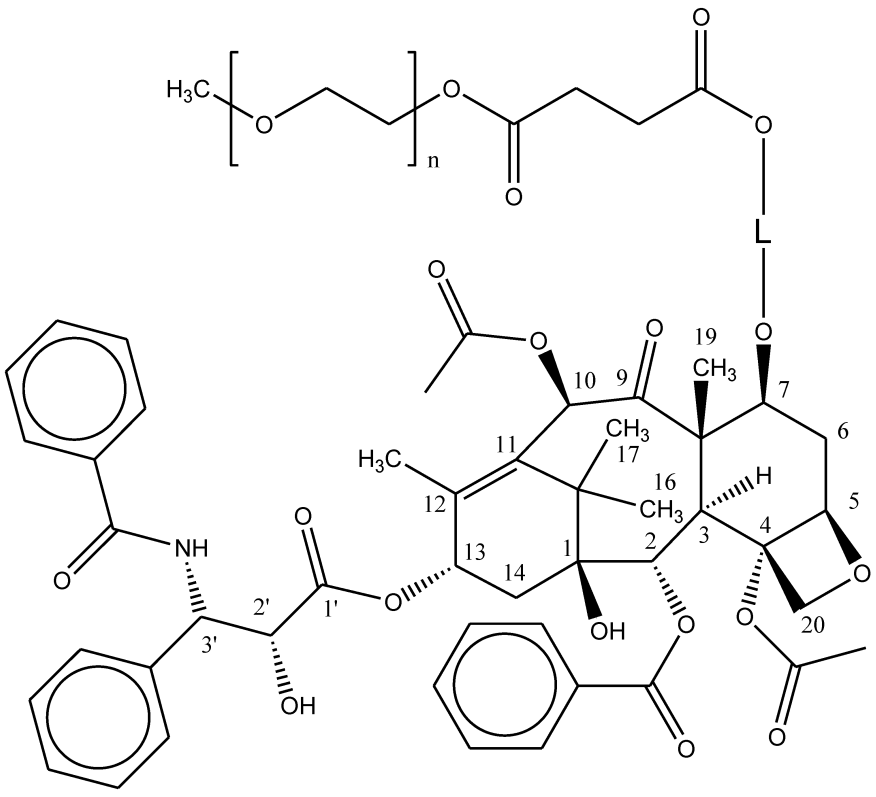

Fig. 4 The water-soluble polymer–paclitaxel conjugate PP7. The self-immolating linker, a succinic acid derivative, controls the stability of the conjugate in solution. The solubilizing PEG is coupled to the OH-function at paclitaxel C7. Another possibility is to couple at C2' while the hydroxyl group on C1 is difficult to access for steric reasons.

A number of different concepts have been applied to improve the water-solubility of paclitaxel [4–6]. The formation of a polymer–drug conjugate with a hydrophilic polymer, e.g., poly(ethylene glycol) (PEG), is one of them. Jo [7] has synthesized pegylated paclitaxel via a self-immolating link to control the stability of the conjugate, see Fig. 4. This is an important feature since the drug is inactive as a

*Taxol is also a registered trade name of Bristol-Myers-Squibb, New York, NY.

conjugate. The polymer chain, molar mass typically around 5000 g/mol equivalent to a degree of polymerization $P \cong 113$, can be attached to the paclitaxel molecule at position C2' or C7. The corresponding conjugates are in the following text called PP2' and PP7, respectively. This type of a polymer-modified drug is also termed "prodrug", which means a "harmless substance, which undergoes a reaction inside the body to liberate the active drug" [8] or "predrug" [9] to indicate that the drug is not yet active.

Interaction with blood serum constituents and competition for docking sites on transport proteins are, therefore, important issues in drug application, and we have studied the behavior of the drug conjugate in solution for many years [9–17].

INTERACTIONS BETWEEN THE PRODRUG AND ALBUMIN

Since albumin is the first major protein a substance is intravascularly faced with, we have recently started to investigate possible interactions of paclitaxel, PP7 and PP2' with albumin as a model protein. One reason for the investigation is to gain information about ways to engineer the drug for improved drug delivery and targeting, for example, by the "enhanced permeability and retardation effect" (EPR) [18–22].

From earlier investigations, it was already known that PP7 and PP2' show no significant surface activity and aggregation [12] in aqueous solution, although this was expected because of their amphiphilic structure. The conjugates obtain a core-shell conformation with the PEG chain coiling around the hydrophobic drug molecule showing behavior in solution that is different from the behavior of the pure polymer in solution. The molecules show a highly dynamic chain conformation forming an oblate or prolate coil around the drug. This causes differences in shape and hence in hydrodynamic behavior of the prodrug compared with the free polymer chain. Phase transitions of the drug are no longer observed, and the behavior of the polymer chain is dominant [14].

It is already known from fluorescence- and UV-absorbance studies and electrophoresis and dialyse experiments that paclitaxel forms a complex with albumin in solution, however, the exact number of ligands to one protein molecule, the equilibrium constants, and the question whether the interaction is specific or unspecific are still under debate [15–17,23–26]. Although it is known that albumin can show some enzymatic activity, no degradation effects on the polymer–drug conjugates were observed.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) experiments [15] have shown that PP7 forms a complex with albumin that contains at least three PP7 molecules. Given the fact that the complexes were detectable in the MALDI-TOF experiment proves the high stability of the complex and makes corresponding structures in solution highly probable. As pointed out above, warfarin forms a strongly fluorescent exciplex with trp214 in the hydrophobic pocket of Sudlow site I and offers an indirect way to investigate the steric situation of this most prominent albumin–paclitaxel interaction. The addition of paclitaxel quenches this fluorescence, while at the same time the intrinsic trp214 fluorescence increases again. Quenching of the fluorescence means that the fluorophore and the quenching molecule have to come at least as close as 15 Å. The increasing intrinsic trp214 fluorescence on the other hand shows that the interaction of trp and warfarin is weakened. Their distance increases when paclitaxel enters the core of albumin. The same effect is observed when PP7 or PP2' is added to the warfarin-albumin complex. The kinetic, however, is a different one as will be shown below.

The quenching of a fluorophore can be visualized by a Stern–Vollmer plot [27], where the reciprocal relative fluorescence intensity $\frac{I_0}{I([Q])}$ with I_0 the intensity of the emitted light in absence of quencher. $I([Q])$ is the fluorescence intensity in the presence of the quencher concentration $[Q]$. In simple cases, the Stern–Vollmer plots show a linear concentration dependence, Fig. 5. An increase of the slope of the function with temperature indicates a kinetic (diffusion-controlled) quenching process with a more effective quenching because of an increasing number of collisions between substrate and quencher, while a slope decreasing with the temperature indicates a static quenching mechanism where

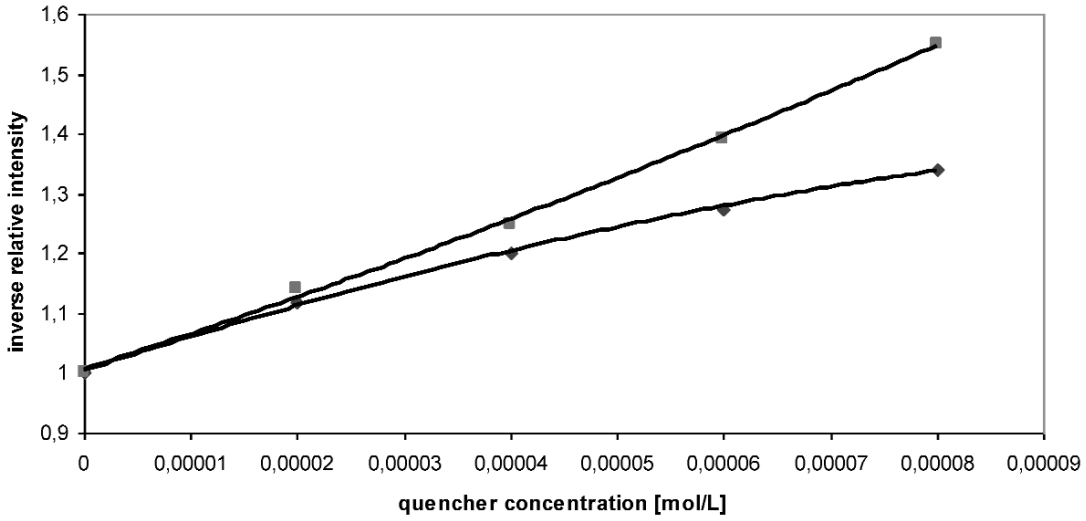

Fig. 5 Stern–Vollmer plot of the quenching of the trp-warfarin exciplex of albumin caused by paclitaxel (upper curve) and the prodrug PP7 (lower curve) after Hess et al. [15], solvent: Hank's solution *pH* 7.0. The curvature of the functions indicates a dynamic mechanism with some static contributions for the quenching by paclitaxel and a complex mechanism for the quenching by PP7. The temperature-dependent Stern–Vollmer plot identifies the quenching by the prodrug as a rather static process, see text and Fig. 6.

the nonfluorescent complex becomes less stable with increasing temperature, see Fig. 6. In cases where both mechanisms are active, a positively (upwards) curved function is observed. A downwards curved behavior indicates a more complex mechanism, again see Fig. 5. Due to the instrumental facilities presently available, it was only possible to measure the relative intensities but not the more informative lifetime τ of the excited states. A sketch of a simple static respectively dynamic quenching process is given in Fig. 7. The dynamic quenching is diffusion-controlled and given by

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_{SV}\left(T,\eta\right)\cdot\left[Q\right] \tag{1}$$

with the intensity of the unquenched fluorescence $I_{0,}$ the intensity of fluorescence in the presence of a quencher with the concentration $[Q]$. The Stern–Vollmer constant K_{SV}, which is a function of the temperature T and the viscosity η of the solution, is given by the kinetic expression

$$K_{SV} = K_D = k_{diff} \cdot \tau_0 \tag{2}$$

with the temperature and viscosity dependent diffusion coefficient k_{diff} and the lifetime of the excited state τ_0 in absence of a quencher, τ is the actual lifetime of the excited state.

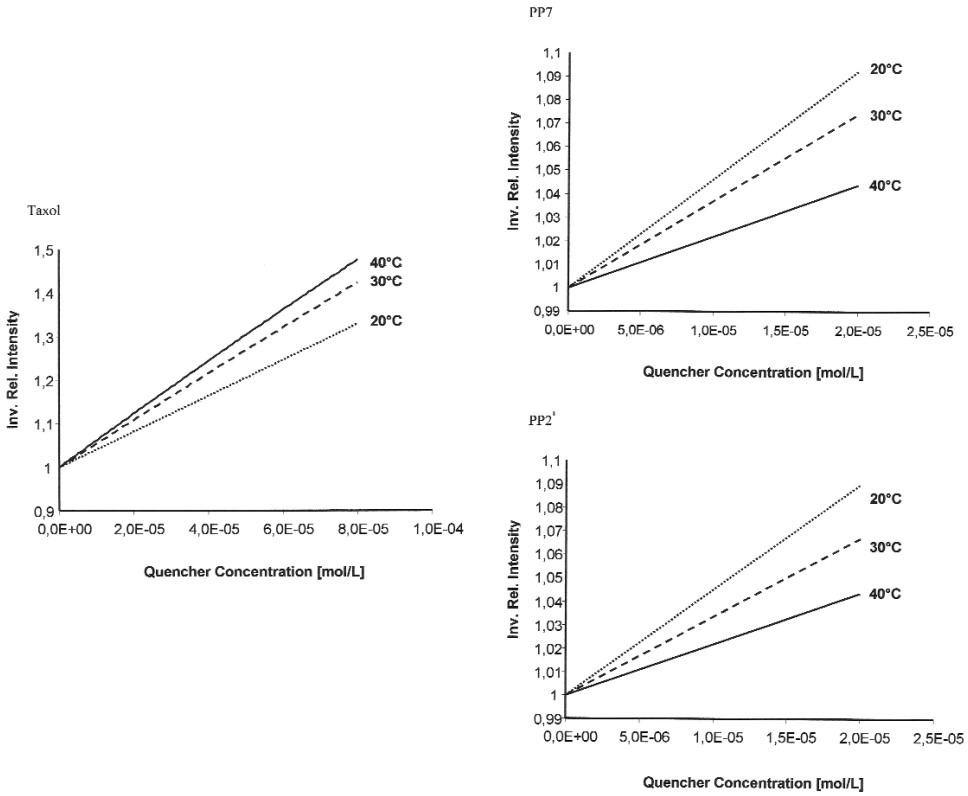

Fig. 6 Initial slopes of Stern–Vollmer plots of the quenching of the extrinsic fluorescence of the albumin-warfarin exciplex after addition of paclitaxel (top left), the prodrug PP7 (top right), and the prodrug PP2' (bottom right), solvent Hank's solution, pH 7.4. A slope increasing with the temperature indicates a dynamic quenching process where a higher collision rate causes a more effective quenching. When there is a negative temperature coefficient as found with both of the prodrugs PP7 and PP2', a static process is indicated because the equilibrium constant of the nonfluorescent complex formed by the quencher and the fluorophore becomes smaller with increasing temperature and the complex is less stable.

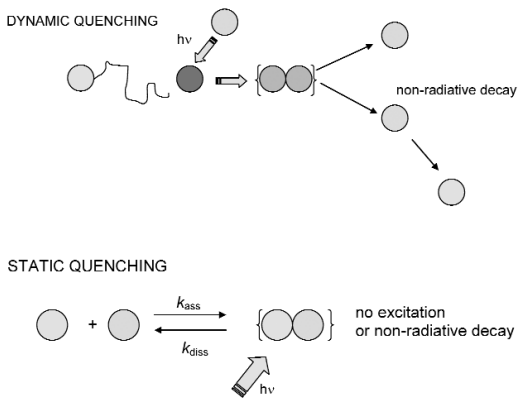

Fig. 7 In the **dynamic quenching** process (top) the quencher diffuses toward an excited molecule and forms an exciplex that is subjected to nonradiative decay. The process is controlled by the diffusion process. In a **static quenching** process (bottom), a fluorophore forms a complex with a quencher and this complex does not form an excited state upon irradiation. The corresponding equations are given in the text.

In contrast, the static quenching process does not depend on the viscosity of the solution and is given by

$$\frac{I_0}{I} = 1 + K_{SV}\left(T\right)\cdot\left[Q\right] \text{ and } \frac{\tau_0}{\tau} = 1 \tag{3a, b}$$

$$K_{SV} = K_s\left(T\right) = \frac{\left[PQ\right]}{\left[P\right]\cdot\left[Q\right]} \tag{4}$$

where K_s is the temperature-dependent equilibrium constant of the nonfluorescent complex as it is given by the law of mass action for dissociation.

A combined static and dynamic mechanism can be described by the nonlinear function

$$\frac{I_0}{I} = \left(1 + K_D\left[Q\right]\right)\left(1 + K_S\left[Q\right]\right) = \frac{\tau_0}{\tau}\left(1 + K_S\left[Q\right]\right) \tag{5}$$

Paclitaxel shows a diffusion-controlled dynamic quenching process with static parts at higher concentrations while both the prodrugs are static quenchers with a more complex behavior at higher quencher concentrations with downwards deviations from a linear behavior. This may be caused by the higher complexes found in the MALDI-TOF experiments mentioned above. The Stern–Vollmer equation is only valid as long as all quencher molecules are able to reach the site of the fluorophore. This might not be granted in the case of the polymer–drug conjugates. However, this problem is not accessible by a simple direct analysis, and more detailed investigations, in particular of the lifetime of the excited states, are required.

The Stern–Vollmer constant K_{SV} at room temperature is quite similar for both of the prodrugs but is fairly smaller for unmodified paclitaxel in Hank's solution (pH = 7.0)

K_{SV}(PP7) = (54 650 ± 2000)L/mol; K_{SV}(PP2') = (54 500 ± 2,000)L/mol; K_{SV}(paclitaxel) = (16 625 ± 1000)L/mol

It was also shown that paclitaxel does not replace warfarin in Sudlow site I. The fluorescence intensity of the exciplex is independent of the sequence of the addition of warfarin and paclitaxel to albumin; it is always warfarin that is closer to trp214. However, there are certainly additional docking sites for warfarin and paclitaxel on albumin that are not monitored by trp fluorescence.

The present results reveal the following facts:

i) Although paclitaxel is a quite bulky molecule, it can penetrate the highly flexible albumin and interact with the hydrophobic region around trp214. Its mobility inside albumin is still high, and the quenching is diffusion-controlled.

ii) Even the paclitaxel attached to the polymer chain can enter Sudlow site I coming as close to trp214, respectively, the warfarin-trp complex to cause significant fluorescence quenching by formation of a static, nonfluorescent complex and thereby most likely initiating further conformational changes of albumin. These conformational changes are clearly indicated by studies of the circular dichroism of prodrug-albumin solutions [28]. The paclitaxel molecule is already bulky, nevertheless, it can enter the hydrophobic core of albumin. In the case of the prodrug, however, this requires "dethreading" of the drug from the surrounding PEG chain (see Fig. 8) so that the polymer is now anchored to the protein and can interact with the chains of additional prodrug molecules through hydrogen bonds, resulting in the higher complexes that were observed in the MALDI-TOF experiments. This is possible because albumin [29] as well as PP7 [12,13] show a high conformational mobility. No such interaction was observed with pure PEG. The entropic disadvantage of the dethreading process has to be compensated either by enthalpic advantages or by

a release of water molecules during the formation of the albumin–prodrug complex (hydrophobic interaction). The interaction with subsequent prodrug molecules can explain the complex behavior of the concentration dependence of the quenching process.

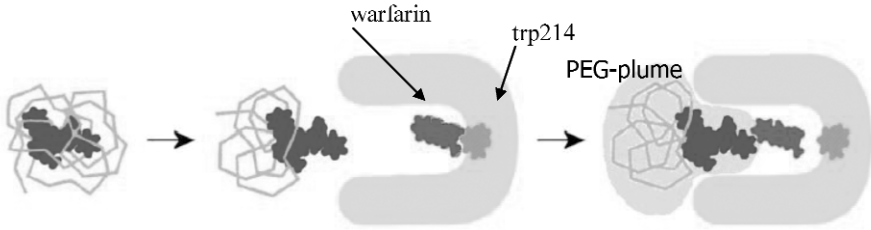

Fig. 8 Dethreading of the polymer coil from paclitaxel and interaction of the drug with warfarin so that the extrinsic fluorescence of the exciplex is quenched but the intrinsic fluorescence of trp214 increases. After Hess et al. [16].

The existence of an isosbestic point in the UV spectrum at 258 nm (Fig. 9) indicates a 1:1 complex between warfarin and paclitaxel, respectively, with the prodrugs PP7 or PP2'. It is reasonable to assume that the interacting sites of the warfarin–paclitaxel complex are the same in the prodrug–warfarin complex, and that these strong interactions are also active when the drug or the prodrug enters Sudlow site I with a warfarin molecule already present in the trp214 exciplex. Therefore, we investigated the interaction between paclitaxel and warfarin in nuclear Overhauser enhancement spectroscopy (NOESY) and rotating frame Overhauser effect spectroscopy (ROESY) experiments [30].

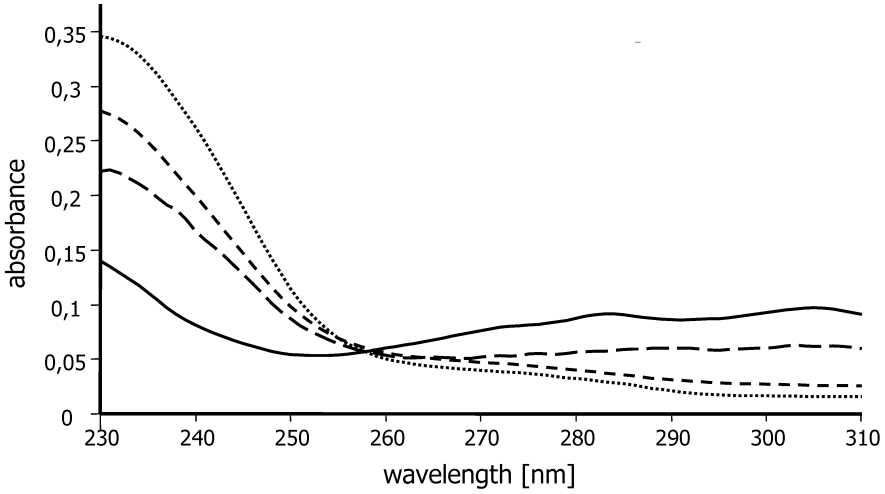

Fig. 9 UV-absorbance spectrum of pure warfarin 10^{-5} mol/L (solid line) and a sequence of mixtures of warfarin and paclitaxel (solvent $CHCl_3$) containing 0.1, 1.0, 2.0 mol/L paclitaxel, respectively. The isosbestic point at 258 nm indicates a 1:1 molar complex. After Hess et al. [16].

These 2D NMR experiments provide information about the interaction of nuclei through space as close as 5 Å. The results are displayed in Fig. 10. Experimental details are given in ref. [17]. Apparently, that part of the paclitaxel molecule with the interacting sites has to enter the hydrophobic albumin channel first and head-on. PEG chains connected to paclitaxel on C7 or C2' are still able to fold back from

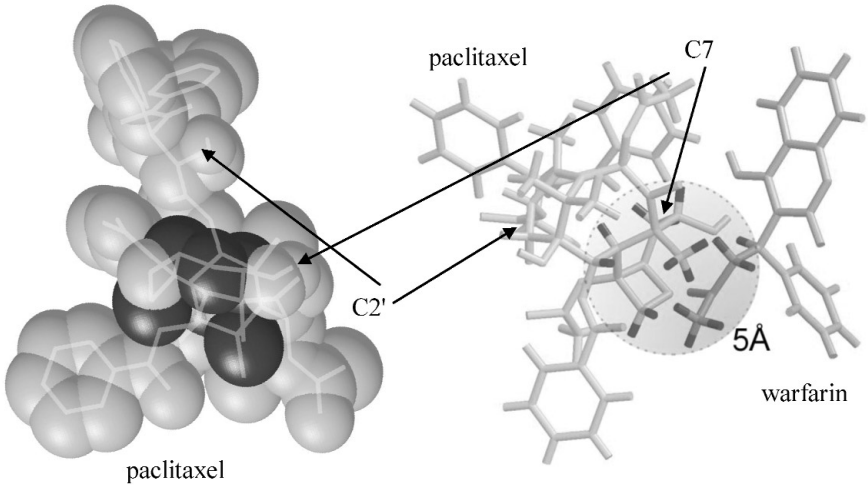

Fig. 10 Protons in paclitaxel and warfarin that come closer than 5 Å as determined by NMR NOESY experiments. A calotte model of paclitaxel with about the same orientation as in the complex is shown on the left. The positions C7 and C2' allow the PEG chain to "dethread" from the paclitaxel molecule and stay near the surface of albumin in the protein complex as shown in Fig. 8. It is reasonable to assume that the interacting sites of warfarin and paclitaxel are the same in solution and in the albumin complex.

the paclitaxel molecule after dethreading and remain on the surface of albumin, forming a plume. The drug acts as some kind of an anchor, and the complex with albumin is even stable under MALDI-TOF conditions. Complexes only with PEG (5000 g/mol) and albumin were not found.

Paal and coworkers [31] have identified a secondary paclitaxel docking site in the cleft between Sudlow domains I and II at the entrance to the hydrophobic core of albumin. They locate the primary docking site at the interface of subdomains IIA and IIIA, near trp214, with the side group emanating from C13 (see Fig. 4) being buried in the drug binding site I in subdomain IIA so that the core of the molecule is more or less pointing upwards in the channel. With warfarin occupying site I and considering the paclitaxel–warfarin interactions concentrated in the taxane core part of paxlitaxel, the orientation of the drug appears to be inverted with the taxane core buried in the hydrophobic albumin channel and close to the interacting groups of the warfarin. In particular, this seems to be sterically preferred when paclitaxel is pegylated and the polymer chain requires additional space.

CONSEQUENCES FOR MOLECULAR ENGINEERING

The fact that paclitaxel as well as many other drugs compete with docking sites on transport proteins like albumin can be a disadvantage, in particular in multidrug therapies. Even the voluminous PEG chain does not prevent paclitaxel from entering the hydrophobic core of albumin. Albumin and the PEG chain are too flexible. One option to make it more difficult for paclitaxel to enter Sudlow site I might be to attach a second PEG chain and have a PP(2',7). This could even give rise to the formation of micellar structures of the polymer conjugates which were not observed in the monosubstituted drug. The formation of supramolecular structures that are no longer able to interact with Sudlow site I can also be accomplished with structures that provide a less flexible polymer chain at least in the vicinity of the drug. As an additional advantage besides avoiding competition for Sudlow site I, there is the high local density of the drug that is provided by nanoscopic superstructures and their possible efficiency in the EPR process. The first positive results have already been found introducing specific block copolymers as hydrophilizers.

Introduction of short and rigid hydrophilic chains can also contribute to the water-solubility of paclitaxel and prevent interaction with albumin at the same time and also with the option to self-assemble. It will probably not be an advantage in this sense to combine two paclitaxel molecules with a bifunctional PEG chain because there is still the chance of having the detreading mechanism of the drug on one or both ends of the polymer. Even two albumins could be coupled this way. Advantages and disadvantages are not yet clear and require further investigation. Again, the chance to find self-aggregation of these "dumbbell" molecules is given.

No interaction of PEG and albumin was found. With the paclitaxel "anchor", however, PEG appears to form some kind of a polymer plume at the entrance to Sudlow site I. Different types of subsequent interactions might be the consequence, such as the addition of additional ligands and a stronger binding to the protein as they were found in the MALDI-TOF experiment.

Finally, it is worth mentioning that fluorescence marking of the drug and the polymer chain provides the option to track the fate of the polymer–drug conjugate in normal and malign tissue and identify the site where it is degraded and which way the polymer and drug take.

CONCLUSION

The results of our experiments show that the prodrugs PP7 and PP2' are both able to penetrate albumin and dock in close to trp214 despite their long polymer substituent—a "dethreading" of the drug has to be assumed. There is no competition for the docking site with warfarin because the formation of the warfarin-trp exciplex is observed independent of the sequence of addition of warfarin and the prodrug to albumin. This is certainly not necessarily given for other drugs. However, the fact that the extrinsic fluorescence of the exciplex is quenched by the presence of the prodrug while the intrinsic fluorescence of trp214 increases at the same time, can be interpreted as the formation of a competitive paclitaxel–warfarin complex, where the distance warfarin–trp214 is increased. This competitive complex is of rather static nature in the case of one of the prodrugs, while a kinetic process is dominant for the unmodified paclitaxel. The formation of the PEG plume may enable the addition of further ligands as they were observed in the MALDI-TOF experiment, which were not observed with only PEG. All these processes are only possible because albumin is a highly flexible molecule, and also for the prodrug it could be shown by molecular dynamics calculations that the polymer coil shows a high conformational dynamic that facilitates the dethreading. These results can be used to develop new strategies to either hamper or reinforce protein–drug interaction.

One single polymer chain is obviously not able to prevent the prodrug from docking in at Sudlow site I. If this is not desired in order to minimalize competition with other substances for this docking site in multidrug therapies, a double substitution with PEG at C2' and C7 appears to be an option. This structure might also force this type of predrug to form micellar structures. Prodrug micelles are advantageous since they provide a high local concentration of the drug. If two paclitaxel molecules are combined with one (bifunctional) PEG chain, a formation of micellar structures is possible and interaction with albumin appears still to be possible, which opens many options for molecular engineering to optimize the efficiency of drug delivery.

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