Polymeric Micelles and Dendritic Amphiphiles for the Anticancer Drug Sagopilone: Solubilization, Formulation Development, and Toxicity Assessment

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Selbstzufriedenheit ist der Sargdeckel jeden Fortschritts.

Philip Rosenthal

Für meine Familie

in Liebe und Dankbarkeit

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CHAPTER 1

INTRODUCTION

1. Solubilization with regard to anticancer drugs for parenteral application

1.1 Solubilization – some remarks

Successful drug development is a complex process from discovery and evaluation through pharmaceutical and clinical development to production and commercialization. Current drug discovery of new active pharmaceutical ingredients (APIs) displays an optimized selection process with regard to pharmacodynamic properties, mainly receptor/ target affinity and selectivity, using methods such as high throughput screening (HTS), combinatorial chemistry, and molecular genetics. However, the APIs selected by these methods are preferably lipophilic comprising poor to negligible water solubility. To date, approximately 40 % of the new drug compounds are considered poorly water-soluble [1]. In other words, "The more active a compound, the less water-soluble". Thus, solubilization represents one of the major challenges in drug development.

Solubilization is the process of making a compound soluble as well as enhancing its solubility using different techniques such as the use of cosolvents, complexing agents, or surfactants. It is a mandatory requirement to enable the therapeutic use of poorly water-soluble drugs.

Almost every phase of the drug development process is faced by solubility hurdles. At the very early stages of development poorly water-soluble compounds are usually dissolved in organic solvents such as dimethyl sulfoxide or ethanol and further diluted with an appropriate buffer to test their activity and efficacy *in vitro*. Subsequent *in vivo* testing both at discovery and preclinical stages requires early formulations [1]. At this phase, solutions and suspensions are the preferred dosage forms for the discovery as well as the preclinical leads whereas the latter may also be formulated using novel formulations such as nano-suspensions or lipid-based formulations [1]. Formulation development for use in clinical development represents the next hurdle, probably the most challenging one. Excipients used for this application have to be approved or generally regarded as safe by the authorities (GRAS status). Whereas oral

applicability offers a relatively broad range of approved excipients and dosage forms, parenteral application distinctly limits the choice of available solubilizers and the concentrations to be used.

According to the OECD Guideline for Testing of Chemicals No. 105 the term 'water solubility' is defined as the saturation mass concentration of a substance in water at a given temperature [2]. It is determined using a column elution or a flask method for compounds comprising a solubility below or above 0.1 g/L in a preliminary test, respectively. The results are expressed in mass of solute per volume of solution with the corresponding SI unit kg/m^3. However, the unit g/L depicts the most commonly used one in practice. According to the respective values of the water solubility, the compounds are classified in groups ranging from very soluble to practically insoluble. The European Pharmacopoeia (Ph. Eur.) defines seven descriptive terms with respect to solubility, as shown in Table 1 [3].

Table 1: Solubility terms according to Ph. Eur.[a]

Descriptive Term	Approximate Volume of Solvent in Millilitres per Gram of Solute			Approximate Solubility (g/L)
Very soluble	less than	1		> 1000
Freely soluble	from 1	to	10	100 – 1000
Soluble	from 10	to	30	33 – 100
Sparingly soluble	from 30	to	100	10 – 33
Slightly soluble	from 100	to	1000	1 – 10
Very slightly soluble	from 1000	to	10 000	0.1 – 1
Practically insoluble	more than	10 000		< 0.1

[a] Referred to a temperature between 15 and 25 °C, if not stated otherwise.

This stratified classification scheme allows a precise portrayal in terms of solubility characterization, but has not been implemented consistently in scientific publications, yet. Instead, the term "poorly water-soluble", which is not listed in the Ph. Eur., is mainly used for drug substances comprising water solubility issues in general. In the present work the term "poorly water-soluble drug" is used for drugs that exhibit a water solubility less than 1 g/L.

1.2 Solubilization principles used in parenteral formulations

The intravenous (i.v.) application of poorly water-soluble drugs requires the solubilization of the drug in an aqueous medium. The respective drug concentrations of the final formulations are often a multiple0 (up to 1000-fold) of the native solubility. In order to achieve this aim, various solubilization principles are used in the development of parenteral formulations including pH adjustment and the use of cosolvents, surface active agents, or complexing agents as well as the preparation of dispersed systems such as nano-suspensions, (micro)emulsions, and liposomes [4]. The resulting products are either ready-to-use formulations, infusion concentrates requiring further dilution, or lyophilizates intended for reconstitution and dilution prior to application [5].

Table 2 shows currently used solubilizing excipients and common concentrations in i.v. formulations. The present standard solubilization vehicles will be briefly introduced in the following sections.

Table 2: Currently used solubilizers in parenteral formulations for i.v. application

Solubilization Approach	Excipients	Concentration range[a] (%, w/v)	Maximum Potency[b] (%)
Cosolvents	Poly(ethylene glycol)	0.0005 - 65	
	PEG 300	n.a.	50 - 65
	PEG 400	n.a.	11.25 - 20.3
	Propylene glycol	4.6 - 60 (v/v)	30 - 82.04
	Alcohol	5.2 - 70 (v/v)	0.94 - 49
	Alcohol, dehydrated		0.03 - 80
	N,N-DMAc[c]	6.0	1.8
Surface Active Agents	Polyoxyl 35 castor oil	50 - 65	50 - 65
	Polysorbate 80	0.001 - 10	8 - 12.5
	Poloxamer 188	n.a.	0.22 - 0.6
Complexing Agents	HPβCD[d]	n.a.	n.a.
	SBEβCD[e]	n.a.	67.5
	γ-Cyclodextrin	n.a.	5

n.a. Not available/ not listed in the respective source.
[a-b] Values according to (a) Powell [6] and (b) FDA Inactive Ingredients Database [7]
[c] N,N-dimethylacetamide
[d-e] (d) Hydroxypropyl-*β*-cyclodextrin, (e) heptasubstituted sulfobutylether-*β*-cyclodextrin (Captisol®)

Cosolvent systems

The use of cosolvents is a simple and very common solubilization approach for parenteral formulation development. It allows for the formulation of compounds which are labile against hydrolysis due to the possibility to exclude water. Water-miscible cosolvents currently used in FDA-approved parenteral products include poly(ethylene glycol) (PEG), propylene glycol, ethanol, glycerine, and N,N-dimethylacetamide (DMAc) [4]. Acceptable dose levels are not defined, and a review of parenteral products reveals doses ranging from 10 to 100 % [4]. A selection of the latter derived from different sources is shown in Table 2. However, there are major concerns associated with their use including precipitation upon injection and the occurrence of adverse clinical effects ranging from moderate irritation to hemolysis and necrosis at the injection site [4]. Thus, the use of cosolvents has to be balanced from case to case depending on the total dose, the target group, and the duration of the therapy.

Besides the empirical determination of the drug solubility in cosolvent systems, there are several theoretical approaches using log-linear solubility relationships, polarity indexes, and solubility parameters [4]. The use of dielectric constants is the most common and straightforward one whereas the calculation of solubility parameters such as the Hildebrand parameter provides a more accurate method to estimate the solubility of a drug in a solvent [8, 9]. The latter has been developed to describe the enthalpy change on mixing of simple liquids with subsequent extension to polar solvents and drugs by the inclusion of dispersive, polar, and hydrogen bonding interaction forces [8]. However, it is less frequently used by formulation scientists.

Surfactants

According to DIN EN ISO 862, surface active agents (surfactants) are amphiphilic compounds possessing surface activity when dissolved in water by lowering the surface or interfacial tension through preferred adsorption at the liquid/vapour or other interfaces.

Upon a characteristic concentration, known as the critical micelle concentration (CMC), these compounds self-assemble into colloidal aggregates (micelles). Solubilization of poorly soluble drugs takes place by drug adsorption to or incorporation into surfactant micelles, and it presents a widely used formulation strategy [10]. Despite the existence of a wide variety of surfactants, only a small number is available for use in parenteral formulations including polysorbate 80, poloxamer 188, Cremophor® EL, Cremophor® RH 40, Emulphor EL 719®, polysorbate 20, 40 and 60 [4]. They are all PEG-based amphiphiles with varying hydrophobic structures and composition, and their choice is mainly a matter of empirical investigation. Parenteral drug products are often formulated as infusion concentrates allowing for the exclusion of water, which will be diluted prior to administration [10]. Disadvantages of surfactant-based formulations are their toxicity and low drug loading capacity [11]. They will be discussed in more detail in the respective sections below.

Polyoxyethylene castor oil derivatives, which are obtained from the reaction of either castor oil or hydrogenated castor oil with ethylene oxide, constitute complex mixtures of various hydrophilic and hydrophobic components [12]. Polyoxyl 35 castor oil (Cremophor® EL) and polyoxyl 40 hydrogenated castor oil (Cremophor® RH 40) are listed in the Ph. Eur. and are mainly used as solubilizers or emulsifying agents both in oral and parenteral formulations [12]. Cremophor® ELP is a commercially available, purified grade of Cremophor® EL containing lower amounts of water, potassium, and free fatty acids. It is primarily used in formulations of sensitive APIs since the higher purity of the excipient improves drug stability [13].

Polyoxyethylene sorbitan fatty acid ester (polysorbates) are partial fatty acid esters of sorbitol anhydride copolymerized with ethylene oxide. Polysorbate 20, 40, 60, and 80 are listed in the FDA Inactive Ingredients Guide [12]. Structurally, they are non-ionic surfactants containing 20 moles of ethylene oxide per mol sorbitan coupled to a specific fatty acid. However, they

rather comprise a composition of different compounds than a definite single one. They are widely used as emulsifying (1 – 15 %) and solubilizing (1 – 10 %) agents in oral and parenteral formulations [12].

Poloxamers (syn. Pluronic®) are non-ionic ABA triblock copolymers consisting of two poly(ethylene oxide) (PEO) blocks flanking a central poly(propylene oxide) (PPO) block. Among several poloxamer grades executed in the FDA Inactive Ingredients Database, poloxamer 188 (Pluronic® F68, BASF Corp., Germany) is the sole compound currently used in i.v. formulations [7].

Complexing agents

Complexation depicts another important solubilization principle in parenteral formulation development. A complex is a species of definite substrate (S) to ligand (L) stoichiometry (abbr. S_mL_n) that can be formed in an equilibrium process [14]. Inclusion complexes as well as molecular complexes between small molecules form the pharmaceutically used ones, and they are solely based on non-covalent interactions [14]. Cyclodextrins (CDs), cyclic oligosaccharides, are able to form inclusion complexes by incorporating the drug within their hydrophobic cavity. Parent *α*- and *β*-cyclodextrin have been associated with renal toxicity, probably due to their poor aqueous solubility, but hydrophilically modified derivatives as well as *γ*-cyclodextrin did not show nephritic damage and may be used for parenteral application [4]. Hydroxypropyl-*β*-cyclodextrin (HPβCD), a non-ionic *β*-cyclodextrin derivative, and sulfobutylether-*β*-cyclodextrin (SBEβCD), a polyanionic *β*-cyclodextrin derivative with sodium sulfonate salts separated from the lipophilic cavity by a butyl ether spacer group, represent the most commonly used CDs [14]. An evaluation of mono-, tetra-, and heptasubstituted SBEβCD has revealed SBE7-*β*-cyclodextrin (Captisol®, CyDex Pharmaceuticals, Inc., Lenexa, USA) to show the most desirable safety profile and drug carrier properties [15]. Relative to *β*-cyclodextrin, Captisol® provides a 50-fold increase

in the excipient water solubility while at the same time showing comparable or better complexation characteristics [15]. Although these agents are less toxic than most surfactants and cosolvents, they exhibit several disadvantages. For instance, they are not able to complex a wide variety of drugs determined by the fixed size of their internal cavity. Furthermore, solubility enhancement, especially of compounds comprising a low solubility, is very limited since most complexes are formed at a ratio of 1:1 necessitating very high amounts of excipients [14].

Liposomes

Liposomes are vesicular structures composed of one or multiple phospholipid bilayers surrounding a hydrophilic core. Thus, water-soluble as well as poorly soluble drugs may be formulated by drug incorporation into the core or within the lipophilic phase of the bilayer, respectively. They have been intensively studied within the last four decades and several liposomal products have already entered the market [16]. They may be therapeutically beneficial by altering the therapeutic index of drugs with drug loading, bilayer rigidity, and surface properties such as an additional PEG-shell (PEGylation) being important factors of influence [4]. One clear advantage of these systems is the low toxicity of the phospholipids used. Disadvantages of these systems include their complex manufacturing process as well as reported hypersensitivity reactions mainly caused by PEGylated liposomes [17].

1.3 Poorly soluble drugs in cancer therapy

Among the poorly soluble drugs used in current chemotherapy, the taxane drugs Paclitaxel and its semi-synthetic analogue Docetaxel are the most prominent examples. Their water solubility is reported to be 0.3 – 1 µg/mL and 5 – 6 µg/mL, respectively, necessitating the use of solubilizing vehicles [18, 19]. As summarized in Table 3, the standard formulations Taxol® (Paclitaxel) and Taxotere® (Docetaxel) contain high amounts of solubilizers and alcohol as a cosolvent. Both formulations are associated with some major concerns regarding stability and severe side effects [16, 20]. Additionally, Taxol® requires specific filter equipment and the use of non-plasticized solution containers and administration sets. The contribution of the particular excipients to these effects will be discussed in detail in the respective sections of this work.

Table 3: Composition and handling of Taxol® and Taxotere® [21, 22]

Drug Product	**Taxol®**	**Taxotere®**
Company	Bristol-Myers Squibb New York, USA	Sanofi-Aventis Paris, France
Product	Infusion concentrate	Single-dose infusion concentrate Singe-dose diluent
Predilution	No predilution	Mixing to form initial dilution
Composition	6 g/L Paclitaxel 527 g/L purified Cremophor® EL 49.7 % dehydrated alcohol, USP[a]	10 g/L Docetaxel 260 g/L polysorbate 80 9.75 % ethanol[b]
Preparation of Infusion Solution	Dilution with physiological saline (0.9 %) or glucose solution (5 %)	
Final Drug Conc.	0.3 – 1.2 g/L	0.3 – 0.9 g/L

[a] Concentration in (%, v/v)
[b] Concentration in (%, w/w)

Other examples of poorly soluble anticancer drugs intended for parenteral administration include the semisynthetic podophyllotoxin derivatives Etoposide (VePesid®) and Teniposide (Vumon®). They exhibit a solubility in water of approximately 100 µg/mL and 20 – 30 µg/mL, respectively [23, 24]. Parenteral formulations of these compounds contain

surfactants (polysorbate 80 or Cremophor® EL) in combination with cosolvents such as N,N-DMAc, benzyl alcohol, PEG 300, and alcohol [4].

Most of the new compounds for anticancer therapy are initially developed for intravenous use and entail solubility issues such as the epothilones [25]. They present a novel class of microtubule-stabilizing anticancer drugs. Originally, they are naturally occurring secondary metabolites produced by the myxobacterium *Sorangium cellulosum*, with epothilone A and B being the two major compounds [26]. Their mechanism of action is similar to Paclitaxel but they exhibit superior features relative to the latter. Besides their activity against various tumour types, they show low susceptibility to key tumour resistance mechanisms *in vitro*, and most importantly, *in vivo* [26]. Thus, they are effective in tumours resistant to Paclitaxel making them very likely to become successors to taxane therapy. Structurally, they are made up of a 16-membered macrolide (see Figure 1). A range of synthetic and semisynthetic analogues were developed to improve their therapeutic window and antitumour efficacy. Figure 1 shows the four epothilone derivatives which either have been approved or are under current clinical development (phase II / III).

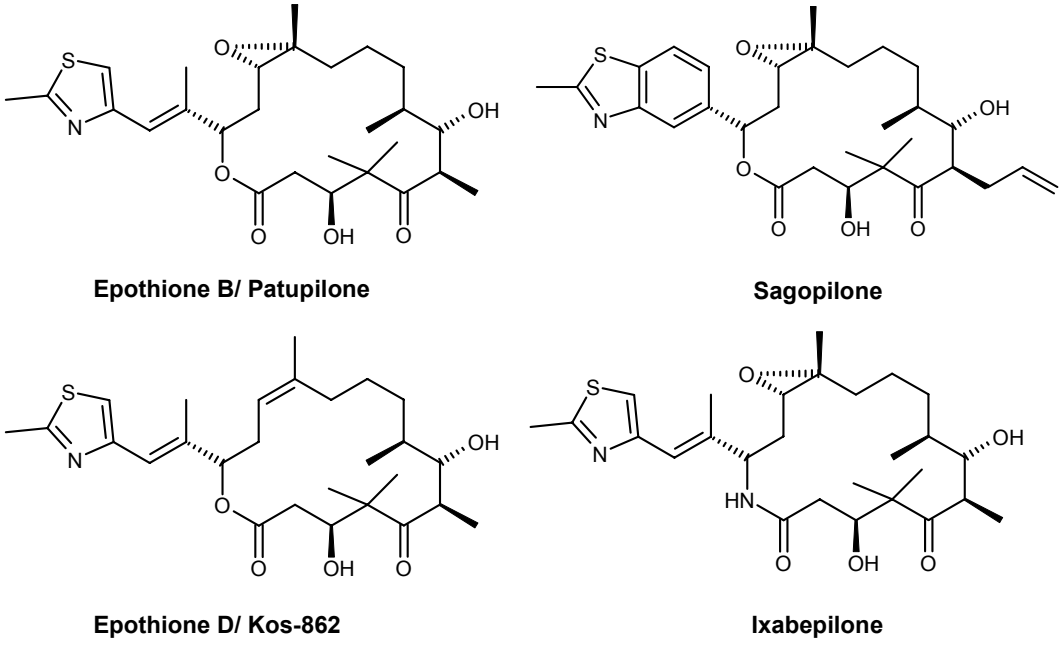


Figure 1: Structural formulas of naturally occurring epothilones and epothilone derivatives

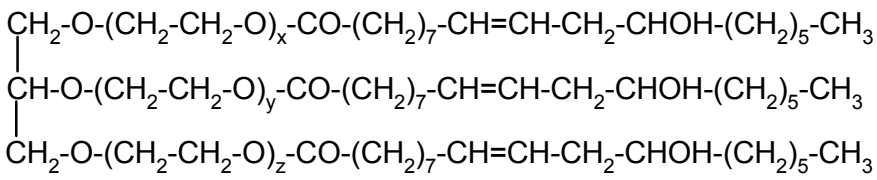
Ixabepilone (Ixempra®, Bristol-Myers Squibb, New York, USA), a lactam analogue of epothilone B, was the first epothilone approved by the FDA in 2007 for the treatment of locally advanced or metastatic breast cancer [27]. Patupilone (Novartis, Basel, Switzerland), the natural epothilone B, is currently undergoing phase III clinical trials for the treatment of ovarian cancer [26]. Sagopilone (Bayer Schering Pharma AG, Berlin, Germany), a synthetic analogue, was selected by a lead optimization program out of 350 epothilone derivatives due to its outstanding preclinical properties [28], and it is currently undergoing phase II clinical trials for the treatment of various types of cancer [29].

Although epothilones are more water-soluble than Paclitaxel their actual water solubility is still insufficient with values as low as 12 µg/mL for Sagopilone. Thus, solubilization is mandatory for the preparation of parenteral formulations containing epothilones posing a significant challenge for formulation development.

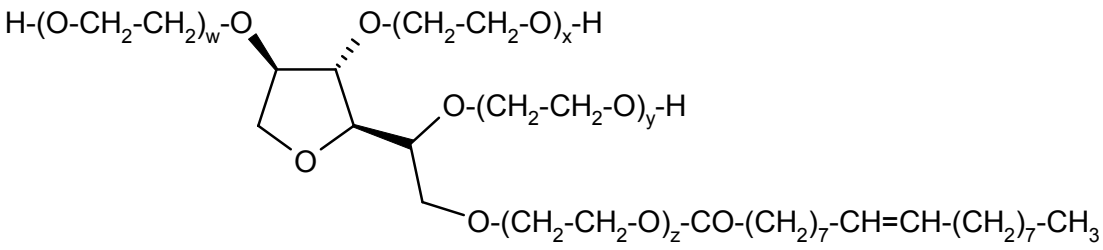
2. Limitations of current cancer therapy

2.1 Drawbacks of current solubilizers and implications for cancer therapy

The main limitations of current solubilizers are (a) insufficient drug solubilization and (b) pharmacological effects of the formulation vehicles. This will be discussed in more detail using the example of polyoxyl 35 castor oil (Cremophor® EL), present in Taxol®, and polysorbate 80, present in Taxotere®, as shown in Figure 2. The implications accompanied by these formulations have initiated extensive research to develop alternative solubilizers as well as alternative taxane formulations.



(A) (x + y + z ~ 35)



(B) (w + x + y + z ~ 20)

Figure 2: Structural formulas of (A) polyoxyl 35 castor oil and (B) polyorbate 80

Insufficient drug solubilization implies no or negligible solubilization capacity as well as instability issues both prior to and after dilution. Although surfactants are widely used for drug solubilization, only a few products can be considered as pure micellar systems [11]. Usually, the solubilization capacity is too low and the addition of cosolvents such as ethanol is inevitable. This, in turn, increases the risk of side effects [4]. The second aspect, formulation stability after dilution, may also implicate the addition of cosolvents

in micellar systems. One example in clinical practice is Taxotere®. As shown in Table 3 its infusion concentrate (Docetaxel in polysorbate 80) requires a predilution step using 13 % ethanol as a cosolvent. This is mandatory to prevent drug precipitation in the final infusion dilution. Furthermore, the final dilution has to be done in a way to obtain solutions comprising a Docetaxel concentration of no more than 0.9 g/L since higher concentrations may lead to drug precipitation [30].

Pharmacological effects of formulation vehicles are either due to intrinsic biological effects of the excipients and/ or an alteration of the drug disposition [20, 31]. Biological properties exhibiting clinical implications to a greater or lesser extent are acute hypersensitivity reactions (HSRs), peripheral neuropathy, dyslipidaemia, inhibition of P-glycoprotein activity, and intrinsic antitumour effects [20].

Acute hypersensitivity is characterized by allergic reactions such as dyspnoea, tachycardia, hypotension, angioedema, chest pain, and generalised urticaria already occurring after the first exposure. Taxol® therapy is a prominent example that causes HSRs in clinical practice [32]. Although patients are pre-medicated with corticosteroids and antihistamines by default, 40 % still suffer from minor reactions, and even 1.5 – 3 % develop major potentially life-threatening reactions [20]. Furthermore, the risk of HSRs is higher for the 1-h compared to the 3-h or 24-h infusions of Taxol® [25]. Various (pre)clinical studies point to Cremophor® EL to play a crucial role in the occurrence of HSRs [25], and Szebeni et al. suggested a complement activation related pseudo-allergy (CARPA) by Cremophor® EL as an important contribution to these reactions [33]. CARPA is characterized by complement activation and a subsequent histamine release. Pharmacokinetic (PK) studies of Cremophor® EL showed a linear, dose-independent, but schedule-dependent PK behaviour with a remarkably decreased clearance at the short infusion duration resulting in higher systemic exposure and, in turn, a higher risk for carrier-related side-effects [25].

This behaviour is possibly due to a saturation of serum esterase-mediated degradation of Cremophor® EL and correlates very well with the lower incidence of HSRs at a prolonged infusion duration [25]. Taxotere® and other therapies comprising polysorbate 80 exhibit HSRs as well. A comparative evaluation of non-haematological toxicities of patients treated with single agent regimens of either Taxol® or Taxotere® revealed a lower overall incidence of HSRs for the latter (15 %) compared to Taxol® (41 %) despite dexamethasone pre-medication in both groups [20].

Symptomatic peripheral neuropathy is another principal, clinically important adverse effect of formulations containing Cremophor® EL such as Taxol® and Sandimmune® (cyclosporine) after parenteral administration [20]. Electrophysiological investigations have provided evidence of both axonal degeneration and demyelinisation in patients with neuropathy after Taxol® therapy [20]. Cremophor is very likely to play an important causative role since radiolabelled Paclitaxel was not detected in peripheral nerve fibres of rats following systemic administration [34]. The appearance of peroxidation products of unsaturated fatty acids is suggested to cause neurotoxicity, but the precise mechanism has not been elucidated so far. Treatment with Taxotere® is associated with neuropathy as well. However, its incidence is much lower (49 %) compared to Taxol® (60 %), and, interestingly, etoposide formulated in polysorbate 80 causes no neurotoxicity at all [20].

Besides intrinsic biological properties, the drug disposition pattern may be modulated by the formulation vehicle. In particular, Cremophor® EL has been shown to alter the PK profiles of drugs after i.v. administration with a substantial increase in the systemic drug exposure and a concomitantly reduced systemic clearance [20]. Paclitaxel formulated in Cremophor® EL displays a nonlinear PK profile with clearance values decreasing substantially with an increase in the Taxol® dose. This effect has been linked to Cremophor® EL since it was not evident when Paclitaxel was formulated in other vehicles [20]. Henningsson et al. showed that

the nonlinear PK profile of Paclitaxel is predominantly due to nonlinear binding to Cremophor® EL [35]. Therefore, higher doses of Cremophor® EL lead to a disproportionate increase in the portion of Paclitaxel entrapped in the micelles and a decreased unbound drug fraction. Congruent with the described schedule-dependent Cremophor® EL clearance, systemic exposure to unbound Paclitaxel is also a function of the infusion duration. This has been confirmed in a higher area under the plasma concentration time curve (AUC) of unbound Paclitaxel after 3-h compared to 1-h infusions [36]. Thus, the advantage of a lower incidence of carrier-related side effects at the 3-h infusion schedule is diminished by more severe haematological toxicity due to the higher unbound fraction of Paclitaxel.

Although there are conflicting reports on the effects of polysorbate 80, the majority of the clinical studies has shown minimal alteration of the PK profile of agents formulated in polysorbate 80 [20]. This was attributed to the rapid degradation of polysorbate 80 by plasma esterases after i.v. application. However, nonlinear distribution pathways similar to Taxol® also exist in the case of Taxotere®, but they are not likely to have a significant impact at the dose level and administration schedule used in routine clinical practice [37]. The underlying mechanism is yet unclear, but it may also be related to the presence of surfactant micelles.

Overall, these examples point out clinical implications of currently used solubilizers both in their ability to cause severe side effects and to modulate drug disposition in an unfavourable manner, particularly obvious in current taxane therapy. Furthermore, it clearly indicates that pharmaceutical formulation is a seriously underestimated aspect of both anticancer drug development and drug therapy.

2.2 Drawbacks of current cancer therapy

Besides the limitations and serious side effects, which are mainly accompanied with the use of certain formulation vehicles, there are further limitations of current cancer therapy. Although small molecules such as Paclitaxel show a broad cytotoxicity against various cancer cells, not all malignancies are treatable *in vivo* due to insuperable obstacles faced on the drug's way to the target location. The main hurdles include the crossing of the blood-brain barrier and with it the access to the central nervous system, the inability to penetrate solid tumours entirely, especially their interior regions, and the overcoming of multi-drug resistance (MDR) [38]. Furthermore, current chemotherapy is non-specific and exhibits serious side effects such as haematological and skin toxicity known from taxane therapy. The epothilones show significant advantages compared to taxane therapy as shown in Section 1.4 addressing some of the hurdles such as MDR. Nevertheless, dosing of epothilones is limited due to the occurrence of peripheral neuropathy, a typical side effect of these compounds. It recently gave reason to the refusal of the marketing authorisation for the epothilone derivative Ixabepilone by the European Medicines Agency (EMEA) [39]. Thus, it constitutes a challenge for formulation development in addition to drug solubilization.

Overall, continuous research into novel formulations approaches as well as their applicability for various anticancer agents is one of the most important steps towards an improved chemotherapy besides the discovery of new targets and the development of novel drugs.

3. Novel formulations to address limitations

There are several novel approaches under current investigation to address both the limitations of current solubilizers and cancer therapy ranging from prodrug formation for i.v. and oral delivery (e.g. Docosahexanoic acid-Paclitaxel, Taxoprexin®, Protarga Inc.) up to polymer-drug-conjugates (e.g. Paclitaxel-poly(L-glutamic acid)-conjugate, Xyotax®) [40, 41]. Main focus of attention is the development of alternative taxane formulations devoid of Cremophor® EL. Whereas some of them such as Tocosol® (OncoGenex Pharmaceuticals), a vitamin E-emulsion, already failed the clinical primary endpoint relative to Taxol® [42], three nanobiotechnology-based products have been approved by the authorities in the last five years.

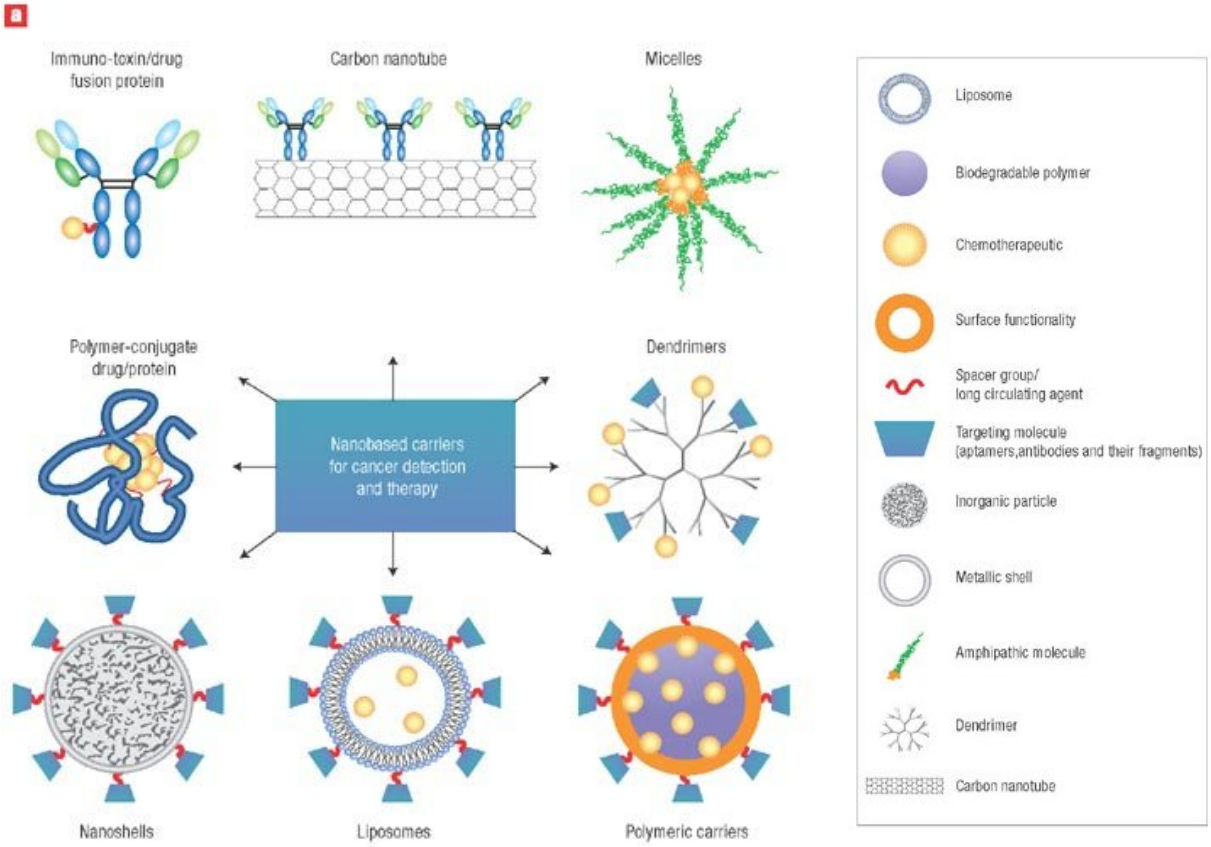


Figure 3: Types of Nanocarriers
Illustration of different types of nanocarriers used in cancer detection and therapy
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Nanotechnology for cancer therapy, also known as nanobiotechnology or nanomedicine, is one of the fastest growing areas of research in the fight against cancer with the aim of improving the therapeutic profile of anticancer drugs as well as establishing new therapeutic approaches [38, 43]. Nanotechnology-based drug delivery for cancer is mainly based on drug-loaded nanoparticles which are composed of a wide variety of materials such as polymers, lipids, dendrimers, and carbon as shown in Figure 3 [44].

They mainly function as drug carrier and drug delivery agents. The former represents their ability to accommodate a payload of drug molecules via chemical conjugation or physical entrapment. Poorly water-soluble drugs may be solubilized or encapsulated depicting an approach to make these compounds accessible to clinical application. Their function to specifically deliver drugs e.g. to solid tumours is based on passive or active targeting whereas the former relies solely on the natural biodistribution of the carrier itself [44].

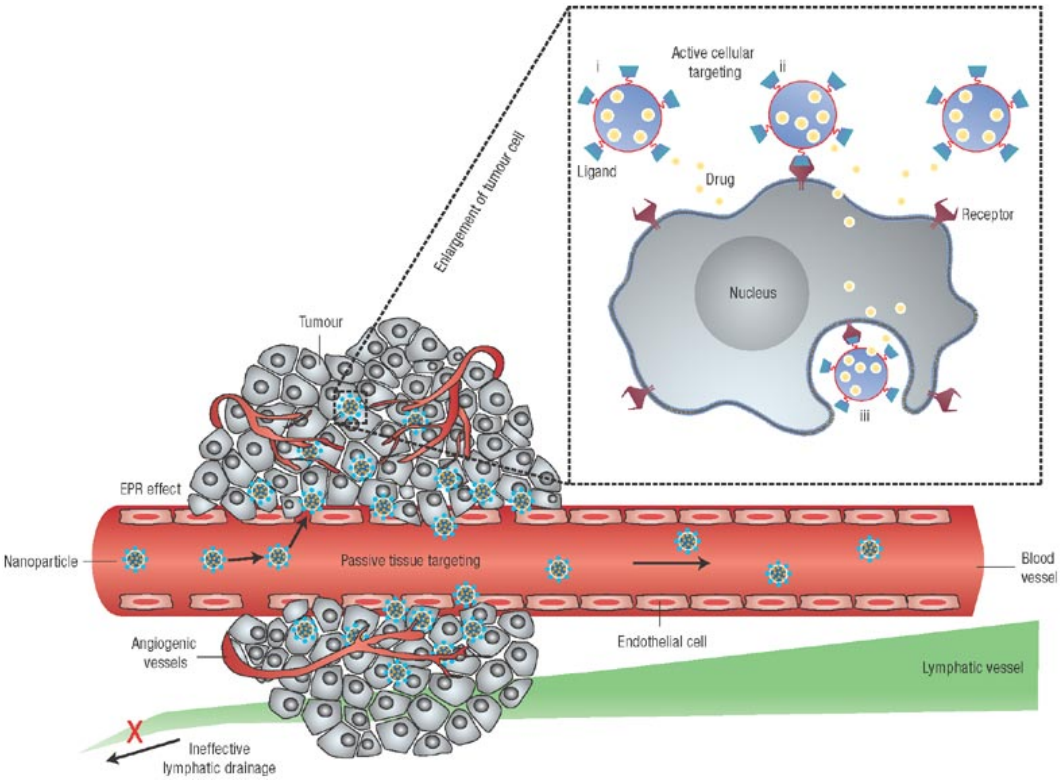

Figure 4: EPR-Effect
Illustration of the enhanced permeation and retention effect (EPR-Effect) within solid tumours (Reprinted by permission from Macmillan Publishers Ltd: *Nat. Nanotechnol.* (Vol. 2 (2007), 751-760), copyright (2007))

Passive targeting of nanocarriers in solid tumours is based on the enhanced permeability and retention (EPR) effect. It has been first described by Matsumura and Maeda in 1986 as a tumoritropic accumulation of smancs, a polymer-conjugated anticancer protein, and several other proteins [45]. This effect exploits two important characteristics of tumour biology, namely the high permeability of tumour blood vessels ("leaky vessels") due to rapid and defective angiogenesis and the dysfunctional lymphatic drainage. As shown in Figure 4 circulating nanocarriers may extravasate into tumour tissue via leaky vessels and accumulate due to ineffective lymphatic drainage. The threshold size was shown to be approximately 400 nm, whereas particles with sizes < 200 nm in diameter have been shown to be more effective [44]. This effect represents the basis of research and clinical therapy of nanotechnology-based cancer therapy. In contrast, low molecular weight micelles such as Cremophor® EL show a very low volume of distribution after i.v. application implying no extravasation either into normal nor tumour tissues supported by the fact that excipient levels were not detectable in the corresponding mice tissues [25].

The main characteristics of approved Paclitaxel-containing nano-formulations devoid of Cremophor® EL are summarized in Table 4. Abraxane®, albumin-bound nanoparticles of Paclitaxel, as well as Genexol®-PM and Nanoxel®, two polymeric micellar formulations, were specifically designed to avoid Cremophor® EL-related toxicities, to deliver higher amounts of Paclitaxel and, thus, increase its therapeutic efficacy. All of them have met the primary target to avoid HSRs. Thus, pre-medication is not necessary anymore. Furthermore, Abraxane® and Genexol®-PM have shown higher maximum tolerated doses (MTD) compared to Taxol®. More precisely, Genexol®-PM revealed a significantly increased MTD (390 mg/m^2) compared to Taxol® (200 mg/m^2) as well as Abraxane® [46]. Hence, Paclitaxel dosing could be increased to 300 mg/m^2, which is much higher than Taxol® (175 mg/m^2), and significant antitumour efficacy has been achieved in advanced malignancies [47, 48].

Table 4: Examples of nanobiotechnology-based formulations of Paclitaxel in clinical therapy

	Abraxane® ABI-007	**Genexol®-PM**	**Nanoxel®**
Company	Abraxis BioScience, USA	Samyang Pharmaceuticals, South Korea	Dabur Pharma, H.P., India
Approved	USA, Canada, Europe, India	Korea	India
Formulation	*nab*-Paclitaxel[a]	Polymeric micelles (PEG-*b*-PLA)[b]	Polymeric micelles (PVP-*b*-PNIPAM)[c]
Drug Product	Lyophilizate (Storage: 25 ± 2 °C)	Lyophilizate (Storage: 25 ± 2 °C)	Liquid formulation (Storage: 2-8 °C)
Key advantages compared to Taxol®	No HSR No pre-medication Increased MTD	No HSR No pre-medication Increased MTD, higher than Abraxane®	No HSR No pre-medication Infusion time: 1 hr instead of 3 hrs
Current clinical trials[d]	phase I/II (57); III (3)	phase I/II (4); III (1), IV (1)	phase I (1)

[a] Nanoparticle albumin-bound Paclitaxel
[b] PEG-*b*-Poly(D,L-lactide)
[c] Poly(vinyl pyrrolidone)-*b*-Poly(N-isopropyl acrylamide)
[d] active (recruiting and non-recruiting) clinical trials according to www.clinicaltrial.gov (access date: 29/01/2010) with the total number of the respective studies in parentheses

Consequently, polymeric micelles provide a promising formulation approach for poorly soluble anticancer drugs, and they will hopefully lead to the replacement of potentially toxic solubilizers currently used in parenteral formulations and safer therapies some day. Polymeric micelles as well as dendritic amphiphile micelles will be briefly introduced below.

3.1 Polymeric micelles for formulation of anticancer drugs

Over the past 20 years various polymeric micelles have been extensively studied as drug delivery systems for (a) solubilization and (b) passive tumour targeting, especially in cancer therapy [49-51]. Recent approaches use additional tumour-specific ligands for active tumour targeting and/ or the simultaneous encapsulation of imaging and therapeutic agents, the so-called "theranostics" [52]. Aspects of formulation as well as clinical parameters will be described below.

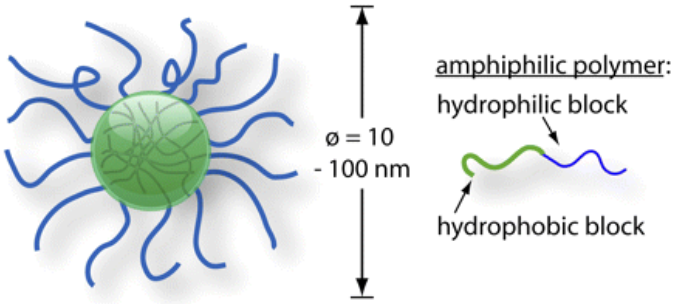

Figure 5: Polymeric micelles
Self-assembly of amphilic block copolymers into polymeric micelles
(Reprinted from *Exp. Biolog. Med.* (Vol. 234 (2009), 123-131))

Polymeric micelles are nanosized assemblies of amphiphilic block copolymers exhibiting an unique core-corona structure (see Figure 5). The hydrophilic corona is important to stabilize the micelles in an aqueous environment and to minimize clearance by the mononuclear phagocytic system (MPS) after systemic administration whereas the hydrophobic core functions as a drug reservoir [51]. In contrast to polymeric nanoparticles, the assembled polymers are in dynamic equilibrium with free unimers, and the particles are usually smaller (10 – 100 nm) displaying monodisperse size distributions [53].

The hydrophilic segment is usually composed of PEG or, alternatively, poly(N-vinyl-pyrrolidone) (PVP). The block copolymers mainly used can be classified into four categories, namely PEG-*b*-Poly(ester), PEG-*b*-Poly(amino acids), PEG-Phospholipids, and Pluronic® copolymers [54]. The former constitute a group of widely employed synthetic

polymers including poly(lactide) (PEG-*b*-PLA), as present in Genexol®-PM, poly(glycolide) (PEG-*b*-PGA), or poly(ε-caprolactone) (PEG-*b*-PCL) because of their biocompatibility, biodegradability, and their use in medical advices approved by the FDA [55].

A typical diblock copopolymer is composed of PEG at a molecular weight between 1000 and 12 000 g/mol and a hydrophobic block with a chain length equal to or less than the corresponding PEG length [55]. The selection of the corresponding blocks essentially influences the physico-chemical properties as well as the therapeutic efficacy. For instance, solubilization of hydrophobic drugs by physical entrapment is preferentially driven by the hydrophobic interactions between the drug and hydrophobic segments of the polymers. Thus, hydrophobicity plays a decisive role in the drug-loading process [56]. The preparation method is also crucial since inappropriate procedures may lead to supersaturated states with subsequent instability and drug precipitation during storage. Furthermore, the micelle morphology is reported to affect the solubilization capacity and biodistribution as shown for PEG-*b*-PCL worm-like micelles [57].

Compared to low molecular weight surfactants, major advantages of polymeric micelles include their high solubilization capacity without the need of additional solvents and low CMC values indicating their thermodynamic stability [53]. While liposomes and emulsions preferentially solubilize water-insoluble and fat-soluble drugs, respectively, polymeric micelles are suitable to solubilize both. Concerning product development, preparations free from water such as lyophilizates are needed to provide a storable form and prevent polymer degradation.

To date, solely Genexol®-PM and Nanoxel® have been approved by the authorities, whereas the latter is not under evaluation outside India [58]. Aside, there are several polymeric micelles in clinical trial evaluation for cancer therapy [55, 59]. For instance, SP1049C (Doxorubicin-loaded Pluronic® L61/ F127 micelles) has shown a chemosensitizing

effect against MDR cancers which is attributed to the Pluronic® composition [55]. NK911 (PEG-*b*-Poly(aspartic acid) micelles containing Doxorubicin both chemically conjugated and physically entrapped) and NK105 (Paclitaxel-loaded PEG-*b*-Poly(4-phenyl-1-butanoate aspartic acid) micelles) have been shown to act as true drug carriers exploiting an EPR-effect [55]. Both systems were specifically designed and modified for the particular drug since pure PEG-*b*-Poly(aspartic acid) micelles were not effective in terms of stable solubilization [55]. Hence, these polymers are not commercially available and/ or require chemical drug conjugation. This fact constitutes a disadvantage concerning the implementtation as novel standard solubilizers since versatile employment is an important prerequisite.

As described earlier, Genexol®-PM (Paclitaxel in PEG-*b*-PLA) significantly improved the MTD of Paclitaxel compared to Taxol® due to the biocompatibility and nontoxicity of PEG-*b*-PLA. Thus, an important requirement for novel excipients is met. PK-studies of Genexol®-PM in humans revealed a decreased plasma half life ($t_{1/2}$) and area under the concentration time curve (AUC) allowing two possible explanations [46, 55]. Either these micelles act as pure solubilizers rather than true drug carriers or the PK profile results from a shift of drug accumulation to tumour tissues as evidenced in the preclinical studies [60]. In fact, Burt et al. showed a rapid Paclitaxel dissociation from PEG-*b*-PLA micelles after i.v. administration suggesting a solely solubilizing function [55]. But finally, the absence or presence of an EPR-effect has not been shown to date. Moreover, these results highlight the discrepancy between the *in vivo* behaviour and current *in vitro* release assays suggesting a controlled release [53].

Other PEG-*b*-Poly(ester) such as PEG-*b*-PCL have shown promising preclinical results but have not been used in clinical evaluations, yet. For instance, solubilization of Hydroxy-camptothecin resulted in an extended half life and increased an AUC after systemic administration [61]. Biodistribution studies of radiolabeled PEG_{5000}-*b*-PCL_{5000} micelles have

shown a reasonable *in vivo* circulation half life comparable to other long-circulating colloidal particles (see Figure 6) [62]. Intact micelles were detectable in the central compartment even at doses that fall below the CMC upon injection revealing a prolonged circulation and a remarkably reduced uptake by liver, spleen, and kidney compared to the unimers.

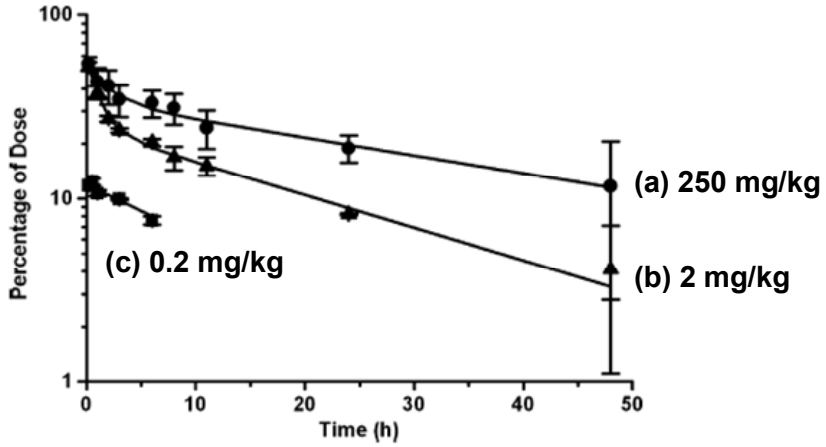

Figure 6: Plasma clearance of mPEG$_{5000}$-*b*-PCL$_{5000}$
Plasma clearance in Balb/C mice following i.v. injection of polymeric micelles at doses resulting in plasma concentrations (a) above and (b) below the CMC compared to (c) injection of polymer unimers (Reprinted from *Eur. J. Pharm. Biopharm.* (Vol. 65 (2007), 309-319))

Thus, dissociation of polymeric micelles does not stringently take place after parenteral administration and dilution below the CMC, and, in particular, PEG-*b*-PCL micelles may function as real drug carriers.

Overall, these various results highlight the importance of polymer-drug compatibility and core stability. Among others the latter is determined by the core crystallinity, which has to be balanced concerning solubilization, stability, and degradation demands.

In spite of their versatile potential especially for drug solubilization, the implementation of block copolymers such as PEG-*b*-PCL in the formulation workaday life has not been fulfilled yet. Further investigations are needed using various drugs to harness the full potential of the currently available polymers besides tailor-made polymer design. As a result, a well-established structure-solubilization relationship together with the implementation of theoretical approaches supporting the polymer selection would be helpful.

3.2 Dendrimers and dendritic amphiphiles: Novel solubilizers

Dendrimers and dendritic architectures have attracted great interest in drug solubilization and delivery throughout the last years [63, 64]. Unimolecular dendritic nanocarriers based on polyglycerol (PG), 3rd to 5th generation, or PEGylated poly(amido amine) (PAMAM) and poly(ethylene imine) (PEI) have been shown efficient solubilization of Paclitaxel and other small molecules [63]. Chemical modification of the core using hydrophobic moieties presents a promising approach to increase drug solubilization by non covalent interactions (Figure 7). However, this approach is limited to a certain degree determined by the threshold of the carrier water solubility.

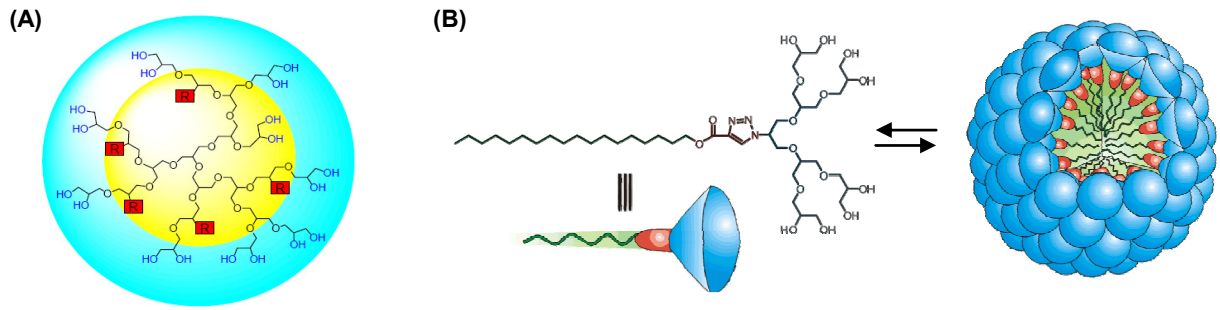

Figure 7: Polyglycerol-based Nanocarriers
(A) Core-functionalized polyglycerol (PG) and (B) dendritic PG-based amphiphile micelles

For instance, p-phenylbenzyl-functionalized polyglycerol has shown a 47-fold solubility enhancement for Nimodipine [65]. Interestingly, the molar drug-polymer ratio was found to be 1:12, and further investigations confirmed a supramolecular polymer assembly instead of unimolecular drug solubilization. Preliminary investigations using a similarly core-functionalized polyglycerol (M_r 13 900 g/mol, DF_{Core} 40 %) revealed a merely slight solubility enhancement for Sagopilone (unpublished data).

However, dendritic polyglycerol-based amphiphiles forming well-defined micelles have not been evaluated so far and may be superior in terms of drug solubilization. Furthermore, the use of PG headgroups instead of PEG may provide benefits with regard to biocompatibility.

4. Objectives of this work

In the present work, amphiphilic PEG-*b*-Polyester and novel dendritic amphiphiles are studied as drug delivery systems for Sagopilone, a poorly water-soluble anticancer drug. The aim is to develop and investigate novel alternative formulations allowing i.v. administration of Sagopilone without the need of potentially allergenic solubilizers such as Cremophor® EL or organic solvents.

Intravenous application of poorly soluble anticancer drugs is mainly accompanied by serious side effects caused by currently used solubilizers as well as drug related adverse effects such as peripheral neuropathy. The latter is known to essentially limit the dosing of Sagopilone. To address these limitations, alternative formulation approaches using polymeric micelles and dendritic amphiphile micelles are investigated.

Besides sufficient solubilization, safety and toxicity testing represent the main hurdles of the development and approval of novel excipients. Whereas PEG-*b*-Polyester have already proved non-toxicity in clinical or preclinical applications, polyglycerol (PG)-based dendritic amphiphiles represent novel solubilizers at the very early stages.

In **Chapter 2**, PEG-*b*-PLA and PEG-*b*-PCL block copolymers will be investigated systematically in terms of Sagopilone solubilization with the aim to identify suitable polymers for parenteral delivery and to assess the predictive power of solubility parameters. It is hypothesized that the copolymer type and composition as well as the method employed for the preparation influence the extent of solubilization, the physicochemical stability, and the micelle morphology. Thus, a set of polymers with hydrophobic/hydrophilic-ratios (w/w) varying from 0.3 to 1.3 will be examined using both sonication and film formation. The apparent solid-state solubility of Sagopilone in the block copolymers will be investigated by means of thermal analysis. It is hypothesized that the latter correlates with the loading

capacity of the corresponding block copolymer micelles, thereby providing an approach for stability prediction.

Clinical application of the resulting polymeric micellar formulations requires stability both prior to and after dilution. Concerning product development non-aqueous formulation approaches are needed to prevent drug and polymer degradation by hydrolysis as well as drug crystallization during storage. Additionally, their rapid and complete reconstitution later on has to be ensured. Thus, a comparative evaluation of the polymers identified in the screening process in Chapter 2 will be performed addressing these points, which will be described in **Chapter 3**. Drug loaded polymeric films of PEG-*b*-PLA, an intermediate in the micelle preparation procedure, will be investigated as a novel formulation approach in addition to lyophilization of polymeric micelles. Furthermore, the *in vitro* and *in vivo* toxicity will be studied in mice to assess the vehicle compatibility and to determine the maximum tolerated dose (MTD) for future studies in tumour models.

The suitability of novel dendritic glycerol-based amphiphiles as solubilizing vehicles will be studied and described in **Chapter 4**. Novel solubilizers are desired both in preclinical as well as in clinical testing. In contrast to currently used solubilizers, these amphiphiles do not contain PEG. Furthermore, they exhibit a definite composition instead of the multicomponent mixtures such as Cremophor® EL. Various amphiphiles comprising identical non-ionic dendritic polyglycerol headgroups but various core structures will be investigated. It is hypothesized that the core hydrophobicity will have an impact on the Sagopilone solubilization, formulation stability, and cytotoxicity. Their potential will be assessed in direct comparison to standard solubilizers used in parenteral formulations nowadays.

References

1. P. Li, L. Zhao. Developing early formulations: Practice and perspective. *Int. J. Pharm.* 341 (2007) 1-19.

2. Organisation for economic co-operation and development. OECD Guideline for the testing of chemicals No. 105 - Water solubility. (1995).

3. Council of Europe. European Pharmacopoeia 6.5. Straßbourg (2009).

4. S. Sweetana, M.J. Akers. Solubility principles and practices for parenteral drug dosage form development. *PDA J. Pharm. Sci. Technol.* 50 (1996) 330-342.

5. J. Swarbrick. Encyclopedia of Pharmaceutical Technology. Informa Healthcare, New York (2007).

6. M.F. Powell, T. Ngueyen, L. Baloian. Compendium of excipients for parenteral formulations. *PDA J. Pharm. Sci. Technol.* 52 (1998) 238-311.

7. FDA/ Center for Drug Evaluation and Research. Inactive Ingredients Database. *http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm* (accessed 26/01/2010).

8. A.F.M. Barton. CRC handbook of solubility parameters and other cohesion parameters. CRC Press LLC, Boca Raton (1991).

9. J.S. Trivedi, M.L. Wells. Solubilization Using CoSolvent Approach. In: R. Liu (Ed.). Water-Insoluble Drug Formulation. Interpharm Press, Englewood (2000) 141-168.

10. R. Liu, N. Sadrzadeh, P.P. Constantinides. Micellization and Drug Solubility Enhancement. In: R. Liu (Ed.). Water-Insoluble Drug Formulation. Interpharm Press, Englewood (2000) 213-354.

11. M.J. Lawrence. Surfactant systems: their use in drug delivery. *Chem. Soc. Rev.* 23 (1994) 417-424.

12. R.C. Rowe, P.J. Sheskey, P.J. Weller. Handbook of pharmaceutical excipients. Pharmaceutical Press and American Pharmaceutical Association, Washington (2003).

13. BASF Corporation. Technical information: Cremophor® ELP. www.pharma-solutions.basf.com (accessed 22/01/2010).

14. W.-Q. Tong. Applications of Complexation in the Formulation of Insoluble Compounds. In: R. Liu (Ed.). Water-Insoluble Drug Formulation. Interpharm Press, Englewood (2000) 111-140.

15. Cydex Pharmaceuticals Incorporation. Captisol. http://cydexpharma.com/whatiscaptisol.html (accessed 22/01/2010).

16. R. Liu, J.B. Cannon, Y. Li. Liposomes In Solubilization. In: R. Liu (Ed.). Water-Insoluble Drug Formulation. Interpharm Press, Englewood (2000) 355-404.

17. A. Chanan-Khan, J. Szebeni, S. Savay, L. Liebes, N.M. Rafique, C.R. Alving, F.M. Muggia. Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil®): possible role in hypersensitivity reactions. *Ann. Oncol.* 14 (2003) 1430-1437.

18. R.T. Liggins, W.L. Hunter, H.M. Burt. Solid-state characterization of paclitaxel. *J. Pharm. Sci.* 86 (1997) 1458-1463.

19. L. Huynh, J. Grant, J.C. Leroux, P. Delmas, C. Allen. Predicting the Solubility of the Anti-Cancer Agent Docetaxel in Small Molecule Excipients using Computational Methods. *Pharm. Res.* 25 (2008) 147-157.

20. A.J. ten Tije, J. Verweij, W.J. Loos, A. Sparreboom. Pharmacological effects of formulation vehicles: implications for cancer chemotherapy. *Clin. Pharmacokinet.* 42 (2003) 665-685.

21. Bristol-Myers Squibb. Label Information Taxol® approved by FDA. http://www.accessdata.fda.gov (accessed 22/01/2010).

22. Sanofi-Aventis. Label Information Taxotere® approved by FDA. http://www.accessdata.fda.gov (accessed 22/01/2010).

23. G. Bykadi, M.A. Kaplan, R.G. Corrao, E.J. Bara, I. Ullah, S.N. Agharkar. Etoposide solutions. U.S. Patent Specification US4927638 (1990).

24. J. Nemec. Epipodophyllotoxinquinone glucoside derivatives, method of production and use. U.S. Patent Specification US4609644 (1986).

25. H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. *Eur. J. Cancer* 37 (2001) 1590-1598.

26. H. Reichenbach, G. Höfle. Discovery and development of the epothilones: A novel class of antineoplastic drugs. *Drugs R. D.* 9 (2008) 1-10.

27. K.-H. Altman, G. Höfle, R. Müller, J. Mulzer, K. Prantz. The epothilones: An outstanding family of anti-tumor agents, from soil to clinic. In: A. D. Kinghorn, H. Falk, J. Kobayashi (Ed.). Progress in the chemistry of organic natural products. Springer, Wien (2009).

28. U. Klar, B. Buchmann, W. Schwede, W. Skuballa, J. Hoffmann, R.B. Lichtner. Total synthesis and antitumor activity of ZK-EPO: the first fully synthetic epothilone in clinical development. *Angew. Chem. Int. Edit.* 45 (2006) 7942-7948.

29. U.S.National Institutes of Health, Clinical Trials of Sagopilone, http://clinicaltrials.gov/ct2/results?term=Sagopilone (accessed 03/08/2009).

30. J.-S. Kim, N.H. Kim, N.K. Lee, J.Y. Lee, W.J. Jang, J.G. Oh, Y.-J. Lee, W.S. Kim, J.-H. Sung, K.A. Um. Stable pharmaceutical composition containing docetaxel and a method of manufacturing the same. Patent Application US20090163574 A1 (2009).

31. T.R. Buggins, P.A. Dickinson, G. Taylor. The effects of pharmaceutical excipients on drug disposition. *Adv. Drug Deliv. Rev.* 59 (2007) 1482-1503.

32. R.B. Weiss, R.C. Donehower, P.H. Wiernik, T. Ohnuma, R.J. Gralla, D.L. Trump, J.R. Baker, Jr., D.A. Van Echo, D.D. Von Hoff, B. Leyland-Jones. Hypersensitivity reactions from taxol. *J. Clin. Oncol.* 8 (1990) 1263-1268.

33. J. Szebeni, F.M. Muggia, C.R. Alving. Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. *J. Natl. Cancer Inst.* 90 (1998) 300-306.

34. G.J. Lesser, S.A. Grossman, S. Eller, E.K. Rowinsky. The distribution of systemically administered 3H-paclitaxel in rats: a quantitative autoradiographic study. *Cancer Chemother. Pharmacol.* 37 (1995) 173-178.

35. A. Henningsson, M.O. Karlsson, L. Viganò, L. Gianni, J. Verweij, A. Sparreboom. Mechanism-based pharmacokinetic model for paclitaxel. *J. Clin. Oncol.* 19 (2001) 4065-4073.

36. H. Gelderblom, K. Mross, A.J. ten Tije, D. Behringer, S. Mielke, D.M. van Zomeren, J. Verweij, A. Sparreboom. Comparative pharmacokinetics of unbound paclitaxel during 1- and 3-hour infusions. *J. Clin. Oncol.* 20 (2002) 574-581.

37. H.L. McLeod, C.M. Kearns, J.G. Kuhn, R. Bruno. Evaluation of the linearity of docetaxel pharmacokinetics. *Cancer Chemother. Pharmacol.* 42 (1998) 155-159.

38. M.M. Amiji. Nanotechnology for Cancer Therapy. CRC Press, Hoboken (2007).

39. European Medicines Agency. Doc.Ref. EMEA/602569/2008 - Questions and answers on recommendation for the refusal of the marketing authorisation for Ixempra. http://www.emea.europa.eu/pdfs/human/opinion/IxempraQ&A_60256908en.pdf (accessed 10/09/2009).

40. G.F.V. Ismael, D.D. Rosa, M.S. Mano, A. Awada. Novel cytotoxic drugs: Old challenges, new solutions. *Cancer Treat. Rev.* 34 (2008) 81-91.

41. C. Li, S. Wallace. Polymer-drug conjugates: Recent development in clinical oncology. *Adv. Drug Deliv. Rev.* 60 (2008) 886-898.

42. U.S. National Institutes of Health. Comparison of Safety and Efficacy of TOCOSOL® Paclitaxel Versus Taxol® for Treatment of Metastatic Breast Cancer. http://clinicaltrials.gov (accessed 22/01/2010).

43. K.K. Jain. Nanotechnology-based drug delivery for cancer. *Technol. Cancer Res. Treat.* 4 (2005) 407-416.

44. D. Peer, J.M. Karp, S. Hong, O.C. Farokhzad, R. Margalit, R. Langer. Nanocarriers as an emerging platform for cancer therapy. *Nat. Nanotechnol.* 2 (2007) 751-760.

45. Y. Matsumura, H. Maeda. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* 46 (1986) 6387-6392.

46. T.Y. Kim, D.W. Kim, J.Y. Chung, S.G. Shin, S.C. Kim, D.S. Heo, N.K. Kim, Y.J. Bang. Phase I and pharmacokinetic study of Genexol-PM, a Cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced malignancies. *Clin. Cancer Res.* 10 (2004) 3708-3716.

47. D.W. Kim, S.Y. Kim, H.K. Kim, S.W. Kim, S.W. Shin, J.S. Kim, K. Park, M.Y. Lee, D.S. Heo. Multicenter phase II trial of Genexol-PM, a novel Cremophor-free, polymeric micelle formulation of paclitaxel, with cisplatin in patients with advanced non-small-cell lung cancer. *Ann. Oncol.* 18 (2007) 2009-2014.

48. K.S. Lee, H.C. Chung, S.A. Im, Y.H. Park, C.S. Kim, S.B. Kim, S.Y. Rha, M.Y. Lee, J. Ro. Multicenter phase II trial of Genexol-PM, a Cremophor-free, polymeric micelle formulation of paclitaxel, in patients with metastatic breast cancer. *Breast Cancer Res. Treat.* 108 (2008) 241-250.

49. Y. Bae, K. Kataoka. Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. *Adv. Drug Deliv. Rev.* 61 (2009) 768-784.

50. V.P. Torchilin. Micellar nanocarriers: pharmaceutical perspectives. *Pharm. Res.* 24 (2007) 1-16.

51. H.M. Aliabadi, A. Lavasanifar. Polymeric micelles for drug delivery. *Expert Opin. Drug Deliv.* 3 (2006) 139-162.

52. E. Blanco, C.W. Kessinger, B.D. Sumer, J. Gao. Multifunctional micellar nanomedicine for cancer therapy. *Exp. Biol. Med.* 234 (2009) 123-131.

53. G. Gaucher, R.H. Marchessault, J.C. Leroux. Polyester-based micelles and nanoparticles for the parenteral delivery of taxanes. *J. Control. Release* 143 (2010) 2-12.

54. G.S. Kwon. Polymeric micelles for delivery of poorly water-soluble compounds. *Crit. Rev. Ther. Drug Carr. Syst.* 20 (2003) 357-403.

55. H. Lee, P.L. Soo, J. Liu, M. Butler, C. Allen. Polymeric Micelles for Formulation of Anti-Cancer Drugs. In: M.M.Amiji (Ed.). Nanotechnology for Cancer Therapy. CRC Press, Hoboken (2007) 317-355.

56. X. Shuai, H. Ai, N. Nasongkla, S. Kim, J. Gao. Micellar carriers based on block copolymers of poly(ε-caprolactone) and poly(ethylene glycol) for doxorubicin delivery. *J. Control. Release* 98 (2004) 415-426.

57. Y. Geng, P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko, D.E. Discher. Shape effects of filaments versus spherical particles in flow and drug delivery. *Nat. Nanotechnol.* 2 (2007) 249-255.

58. U.S.National Institutes of Health. Clinical Trials of Nanoxel. http://www.clinicaltrial.gov/ct2/results?term=Genexol-PM (accessed 29/01/2010).

59. H.M. Aliabadi, M. Shahin, D.R. Brocks, A. Lavasanifar. Disposition of drugs in block copolymer micelle delivery systems: from discovery to recovery. *Clin. Pharmacokinet.* 47 (2008) 619-634.

60. S.C. Kim, D.W. Kim, Y.H. Shim, J.S. Bang, H.S. Oh, S.W. Kim, M.H. Seo. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *J. Control. Release* 72 (2001) 191-202.

61. B. Shi, C. Fang, M.X. You, Y. Zhang, S. Fu, Y. Pei. Stealth MePEG-PCL micelles: effects of polymer composition on micelle physicochemical characteristics, in vitro drug release, in vivo pharmacokinetics in rats and biodistribution in S180 tumor bearing mice. *Colloid Polym. Sci.* 283 (2005) 954-967.

62. J. Liu, F. Zeng, C. Allen. In vivo fate of unimers and micelles of a poly(ethylene glycol)-block-poly(caprolactone) copolymer in mice following intravenous administration. *Eur. J. Pharm. Biopharm.* 65 (2007) 309-319.

63. R. Haag, F. Kratz. Polymer Therapeutics: Concepts and Applications. *Angew. Chem. - Int. Edit.* 45 (2006) 1198-1215.

64. S. Svenson, A.S. Chauhan. Dendrimers for enhanced drug solubilization. *Nanomedicine* 3 (2008) 679-702.

65. H. Türk. Dendritische, wasserlösliche Kern-Schale-Nanopartikel für Anwendungen in der Wirkstoffsolubilisierung. Dissertation. Fachbereich Chemie der Universität Dortmund (2006).

CHAPTER 2

SOLUBILIZATION OF SAGOPILONE, A POORLY WATER-SOLUBLE ANTICANCER DRUG, USING POLYMERIC MICELLES FOR PARENTERAL DELIVERY

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Abstract

Polymeric micelles were studied as a drug delivery system for Sagopilone, a poorly water-soluble anticancer drug, for passive tumour targeting. Poly(ethylene glycol)-*b*-poly(lactide) (PEG-*b*-PLA) and poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG-*b*-PCL) were investigated to identify suitable copolymers and to assess the predictive value of solubility parameters. The impact of the copolymer composition (different hydrophobic/hydrophilic-ratios (w/w) from 0.3 to 1.3) and the preparation method (sonication; film formation) on the solubilization efficiency, size characteristics, and micelle stability were studied. Thermal analysis was used to determine the apparent solid-state solubility. PEG_{2000}-*b*-PLA_{2200}, PEG_{2000}-*b*-PCL_{2600}, and PEG_{5000}-*b*-PCL_{5000} were identified as the most suitable delivery systems for Sagopilone. They exhibited efficient solubilization (≥ 70 %) yielding small (< 100 nm), monodisperse, and spherical micelles. (80 ± 12), (93 ± 0.4), and (96 ± 6) % of the drug still remained solubilized after 24 h, respectively. Calculated solubility parameters were not predictive since they showed a reversed order of preference relative to experimental data. High solubilization after film hydration was accompanied with a 'supersaturation'. The reason for this well-known effect and the solubilization of Sagopilone within the block copolymer was elucidated by the evidence of glass solutions exceeding the solubilization capacity of the corresponding micelles. Overall, micellar drug delivery systems for Sagopilone were identified offering the potential for an improved therapy.

1. Introduction

To date many potent drugs entering the developmental stage were selected from high throughput screening and passed through numerous pharmacodynamic evaluations *in vitro* as well as *in vivo.* These drug candidates frequently show poor or negligible water solubility.

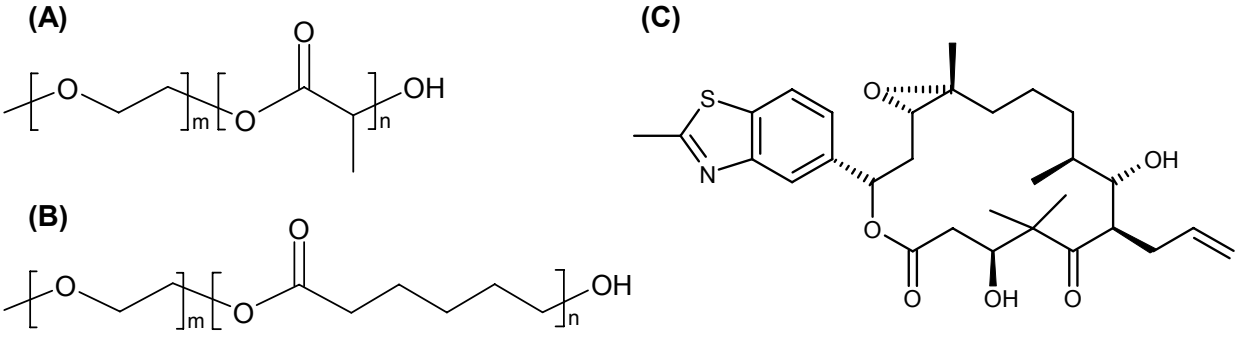

Figure 1: Structural formulas of (A) PEG-*b*-PLA, (B) PEG-*b*-PCL and (C) Sagopilone

Sagopilone (Fig.1) is a novel, potent derivative belonging to the group of epothilones, a new class of microtubule-stabilizing agents [1, 2]. It is currently undergoing Phase II clinical trials for the treatment of various types of cancer [3]. The parenteral administration of Sagopilone poses a challenge to formulation development due to its poor solubility in water (12 µg/mL). Furthermore, dosing of Sagopilone is limited due to the occurrence of peripheral neuropathy, a typical side effect of epothilones. Thus, the requirements of an ideal drug delivery system comprise (a) efficient and stable solubilization of the drug, (b) accumulation of the drug in tumour tissue, and (c) a reduction of drug related adverse effects at non-tumour sites.

Solubilizers currently used for parenteral administration like Cremophor® EL or polysorbate 80 have been implicated in clinically important adverse effects like hypersensitivity reactions and a highly increased systemic drug exposure along with a reduced cellular uptake [4]. Among different approaches polymeric micelles were extensively studied and reviewed as drug delivery systems for the solubilization of hydrophobic drugs [5-9] exhibiting no or marginal carrier-associated side effects after intravenous injection [10, 11].

Examples of anticancer drugs used for solubilization are Paclitaxel [12], Doxorubicin [13] and Camptothecin [14]. Furthermore, they offer the potential to alter the pharmacokinetic behaviour of anticancer drugs after parenteral administration achieving long circulation times and enhanced permeation and retention of micelles in solid tumours (EPR-effect) [15]. Thus, they may provide a promising approach for more efficient and patient-friendly cancer therapy [5]. To date, approximately seven formulations based on this concept have already entered clinical trials [16]. The amphiphilic copolymers used mostly contain poly(ethylene glycol) (PEG) as the hydrophilic block and can be classified into four categories according to the nature of the hydrophobic block, namely PEG-*b*-Poly(amino acids), PEG-*b*-Poly(ester), PEG-Phospholipids and Pluronics® [17]. The formulation of Paclitaxel in PEG-*b*-Poly(lactide) (PEG-*b*-PLA) micelles, e.g. in Genexol®-PM, is an impressive example for polymeric micelles as solubilization vehicles although clear evidence of an EPR-effect has not been provided to date. The key advantage of this formulation is a significantly increased maximum tolerated dose (MTD) in humans compared to Taxol® and the absence of carrier-related side effects [10, 18-20]. PEG-*b*-Poly(ε-caprolactone) polymers (PEG-*b*-PCL) exhibit very promising pharmacokinetics in terms of a drastically decreased clearance and consequential prolonged circulation times in preclinical studies [21, 22] but have not been evaluated in clinical trials so far. Toxicity testing of PEG-*b*-PCL micelles revealed their biocompatibility in terms of low cytotoxicity and no acute toxicity after i.v. application *in vivo* [21, 23]. Apart from the polymer structure also other factors such as the micelle size and morphology were reported to affect circulation times and biodistribution after parenteral administration [24]. Among the numerous literature, no reports appeared comparing PEG-*b*-PLA and PEG-*b*-PCL primarily with regard to their (a) solubilization capacity and (b) their effect on the pharmacokinetics of cytostatic drugs especially epothilones.

Thus, the aim of the present study was to investigate the solubilization of Sagopilone systematically using various PEG-*b*-PLA and PEG-*b*-PCL polymers (Fig.1). Solubility parameters were calculated to assess their predictive value. The hydrophobic/hydrophilic-ratio (w/w) of the block copolymers was varied in a range from 0.3 to 1.3 to define the optimum polymer composition in terms of the formation of monodisperse, spherical micelles and efficient, stable drug loading. Furthermore, two different preparation methods were applied and compared to each other. Using the film method a solid film is formed after complete removal of the organic solvent from a single-phase system und subsequent drying. This film could be suitable for storage, and the polymeric micelles are formed spontaneously upon film redispersion. However, 'supersaturation' with subsequent precipitation of the drug needs to be taken into account [6]. For comparison, direct dissolution was employed using sonication. Thermal analysis was performed to study the drug-polymer compatibility and the apparent solid-state solubility of Sagopilone within the block copolymers.

Since the micellar morphology was reported to affect the biodistribution *in vivo* the selected candidates were investigated by transmission electron microscopy to identify micelle size and morphology.

2. Materials and methods

2.1 Materials

Sagopilone was obtained from Bayer Schering Pharma AG (Berlin, Germany). The block copolymers poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG_{2000}-*b*-PCL_{500}, PEG_{2000}-*b*-PCL_{1400}, PEG_{2000}-*b*-PCL_{2600} and PEG_{5000}-*b*-PCL_{1600}, PEG_{5000}-*b*-PCL_{3600}, PEG_{5000}-*b*-PCL_{5000}), poly(ethylene glycol)-*b*-poly(D,L-lactide) (PEG_{2000}-*b*-PLA_{1200}, PEG_{2000}-*b*-PLA_{2200}) and poly(ethylene glycol)-*b*-poly(L-lactide) (PEG_{5000}-*b*-$PLLA_{2400}$, PEG_{5000}-b-$PLLA_{6000}$) were purchased from Polymer Source Inc. (Dorval, Canada). For the definition of the abbreviations used for these polymers see Table 1 as well as Section 3.2. All other ingredients were obtained in analytical quality.

2.2 Solubility parameter calculation

Solubility parameters of Sagopilone and different polymers were obtained using Hansen's approach [25]. It assumes that the total solubility parameter (δ), almost equal to the Hildebrand parameter, arises from dispersive (δ_d), permanent dipole-dipole interactions (δ_p), and hydrogen bonding forces (δ_h) according to Equation (1).

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \tag{1}$$

Calculation of the solubility parameters was done on the basis of the group contribution method by Hoy using the Solubility Parameter Software provided by Computer Chemistry Consultancy (Singen, Germany). Furthermore the difference of the three-dimensional solubility parameters between Sagopilone and various polymers (Δδ) were calculated (see Equation (2)).

$$\Delta\delta = \sqrt{(\delta_{d1} - \delta_{d2})^2 + (\delta_{p1} - \delta_{p2})^2 + (\delta_{h1} - \delta_{h2})^2} \tag{2}$$

2.3 Acid value determination

The Acid value is defined as the number that expresses, in milligrams, the quantity of potassium hydroxide required to neutralize the free acids present in 1 g of the substance [26]. It was determined by anhydrous titration according to method A of the European standard EN ISO 2114 with minor changes regarding the solvent due to the polymer solubility. Briefly, the polymer was dissolved in acetonitrile, phenolphthalein solution was added and the solution was titrated with potassium hydroxide solution (0.01 M) using a GP-Titrino 736 (Metrohm AG, Switzerland) equipped with a Photometer 662 (Metrohm AG, Switzerland) measuring the transmission of light at 570 nm. Data was analysed with TiNet Software 2.4. All measurements were performed in triplicate.

2.4 Preparation of polymeric micelles

Two different preparation methods were carried out to prepare loaded as well as unloaded polymeric micelles. (A) The sonication method consisted of the following steps, weighing of the polymer (30 mg) and Sagopilone (3.0 mg) into a screw-top glass vial, addition of 3.0 mL phosphate buffer (pH 7.4) and subsequent sonication using a Sonoplus HD2070 (Bandelin electronic, Berlin, Germany) at 100 % power in an unpulsed mode for 10 minutes. (B) The film formation method was performed as follows. Block copolymer (30 mg) and Sagopilone (3.0 mg) were weighed into a round-bottomed flask and dissolved in 3 mL acetonitrile. The solvent was evaporated under reduced pressure at room temperature with subsequent drying at 0.1 mbar for 1 h. The resulting film was redispersed with 3.0 mL phosphate buffer (0.05 M, pH 7.4) under shaking without additional heating or sonication. Empty micelles were prepared according to the same protocol in the absence of Sagopilone. The initial drug-polymer ratio was 1:10 for the drug-loaded samples and the resulting polymer concentration was kept uniformly at 10 g/L for all samples allowing comparisons between unloaded and loaded samples. Blanks were prepared without the addition of the polymer.

The samples were filtered using a syringe filter (Millex®-GV 0.22 µm, Millipore, USA) and the resulting micellar dispersions were used for further analysis.

2.5 Characterization of micelle size and size distribution

The micelle sizes and size distributions were determined by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK). Briefly the principle is based on the measurement of the backscattered light fluctuations at an angle of 173° and the calculation of an autocorrelation function. The samples were measured undiluted at 25 °C adjusted to the temperature for 1 minute prior to the measurement. The autocorrelation functions were analysed using the DTS v5.1 software provided by Malvern and the hydrodynamic diameter of the micelles (d_H) and their size distribution (PDI – polydispersity index) was calculated. Measurements were done in triplicate with 15 to 20 runs each and the calculated mean values were used.

2.6 Determination of the Sagopilone drug loading

The Sagopilone content of the micellar dispersions was determined by high performance liquid chromatography (HPLC) using an Agilent 1100 Series chromatography system (Agilent Technologies, Santa Clara, USA) consisting of a quaternary pump, an auto-injector, a column heater at 25 °C and a UV-detector. Two Chromolith® Performance RP-18e columns (100 x 4.6 mm, Merck, Germany) were used and a gradient was run from ACN/ water (25/75 v/v) to ACN/ water (45/55 v/v) in 10 min. followed by isocratic elution for 15 min. at a flow rate of 1 mL/min. Samples were diluted 5 – 10 times with ACN/ water (50/50 v/v) prior to analysis. The injection volume of the samples was 10 µL and Sagopilone was detected at a wavelength of 220 nm. The data was analysed using Empower™ 2 software (Waters Corporation, Milford, USA) and the amount of Sagopilone was determined by an external standard calibration.

The solubilization efficiency (SE) of Sagopilone was calculated according to Equation (3).

$$SE(\%) = \frac{mass\ \ of\ \ Sagopilone\ \ loaded\ \ in\ \ mg}{mass\ \ of\ \ Sagopilone\ \ fed\ \ in\ \ mg} \times 100\% \quad (3)$$

2.7 Differential scanning calorimetry (DSC)

DSC measurements were carried out on a DSC822e (Mettler Toledo, Switzerland) at a heating rate of 20 K/min using dry nitrogen purge gas. The samples were first heated to 100 °C, subsequently cooled to -100 °C with liquid nitrogen and heated again to 180 °C. Polymeric films with varying Sagopilone weight fractions prepared by the film formation method were measured using aluminium sample pans. At least three individual samples were prepared with three individual measurements per sample and the data was analysed with STARe Software 9.10. The thermograms were normalized to the sample weight. The DIN midpoint of the slope change of the heat flow plot of the second heating scan was considered as the glass transition temperature (T_g). The melting (T_m) and crystallization (T_c) temperatures were taken as the maximum of the endothermic and the minimum of the exothermic peaks, respectively. Furthermore, the heat capacity change (Δcp) at the T_g has been determined for the drug and the polymers.

2.8 Cryogenic transmission electron microscopy (cryoTEM)

Samples were prepared for cryoTEM analysis by preserving in a thin layer of vitreous ice supported on C-Flat holey carbon films (Protochips, Inc.) on 400 mesh copper grids. Grids were cleaned in a Solarus plasma cleaner (10 seconds, 25 % O_2 , 75 % Ar) immediately prior to vitrification using an FEI Vitrobot (4ºC, 95 % RH). Vitrified grids were transferred into the electron microscope using a cryoholder (Gatan, Inc.) that maintains the temperature of the grid below -170 ºC. Microscopy was performed using a Tecnai Spirit transmission electron microscope (FEI Co.) equipped with a 4k x 4k CCD camera. Images were acquired at

nominal magnifications of 52,000x (0.21nm/ pixel) and 21,000x (0.50nm/ pixel) using the Leginon data acquisition software [27] at a nominal underfocus of -6 µm (21,000x) and -3 µm (52,000x) with electron doses of 10-15 ($e^-/Å^2$). For shape investigations images were acquired at zero tilt (0°) as well as at a high tilt angle (55°). The alignment and classification process was done with the XMIPP processing package using the Kernel Probability Density Estimator Self-Organizing Map classification method as described in the literature [28, 29]. Briefly, algorithms in this package align the selected particles and sort them into self-similar groups of classes. Afterwards the class average diameters were measured.

2.9 Statistics

Data were recorded as mean ± standard deviation. All experiments were done at least in triplicate as specified in the Section 3. Means were analysed for statistical significance using unpaired student's t-test. Differences were considered significant at *p*-values < 0.05. Linear regression analysis was processed using SigmaPlot 8.0 (Systat Software Inc., San Jose, CA).

3. Results

3.1 Solubility parameters

Following the theory that two solvents are miscible, when the difference in their solubility parameters is small enough, the theoretical drug-polymer compatibility was estimated. The total solubility parameters (δ) of Sagopilone, poly(lactide) and poly(ε-caprolactone) were calculated to be 23.78, 21.78 and 20.64 $MPa^{1/2}$, respectively, resulting from dipole-dipole interactions, dispersive and hydrogen bonding forces.

Concerning the partial solubility parameters, PLA and PCL exhibit similar differences in their hydrogen bonding interactions to Sagopilone with $\Delta\delta_h$ of 0.71 and 1.00 $MPa^{1/2}$, respectively, but a distinct difference in the others. PCL reveals a high portion of dispersive/ van der Waals interactions with Sagopilone compared to PLA with $\Delta\delta_d$ of 1.11 and 2.97 $MPa^{1/2}$, respectively. In contrast, permanent dipole-dipole interactions seem to be the major interaction forces between Sagopilone and PLA pursuant to $\Delta\delta_p$ of 0.25 $MPa^{1/2}$ compared to 3.72 $MPa^{1/2}$ for PCL.

According to the lower difference in the total solubility parameter to Sagopilone, poly(lactide) (Δδ: 3.06 $MPa^{1/2}$) seems to be superior to poly(ε-caprolactone) (Δδ: 4.01 $MPa^{1/2}$) suggesting a better compatibility. This gives rise to the expectation that PEG-*b*-PLA micelles exhibit higher solubilization of Sagopilone and stability compared to PEG-*b*-PCL micelles.

3.2 Characterization of block copolymers

A set of 10 commercially available block copolymers was used in this study. These can be divided in two groups, namely (1) PEG-*b*-PCL and (2) PEG-*b*-PLA, which can be further subdivided according to the molecular weight of PEG at (a) 2000 and (b) 5000 Da. They were abbreviated as P2CL, P5CL, P2LA and P5LLA, respectively, as shown in Table 1. Within the particular groups the molecular weight of the hydrophobic blocks was varied.

The hydrophobic/hydrophilic-ratio, which is the quotient of the molecular weight of the hydrophobic and the hydrophilic block and stated in parentheses for the particular polymer, was varied in a range from 0.3 to 1.3 to ensure the formation of star-type micelles with a typical core-corona structure [30].

Table 1: Characteristics of PEG-*b*-PCL and PEG-*b*-PLA block copolymers

PEG-*b*-PCL[a]	*M*[b] (g/mol)	PDI[c]	AV[d] (mg/g)	PEG-*b*-PLA[a]	*M*[b] (g/mol)	PDI[c]	AV[d] (mg/g)
P2CL(0.3)	2000-500	1.08	7.4±0.7				
P2CL(0.7)	2000-1400	1.20	4.9±0.2	**P2LA(0.6)**	2000-1200	1.13	31.9±0.8
P2CL(1.3)	2000-2600	1.15	9.1±0.6	**P2LA(1.1)**	2000-2200	1.13	5.2±0.4
P5CL(0.3)	5000-1600	1.07	8.4±0.2				
P5CL(0.7)	5000-3600	1.10	5.6±0.4	**P5LLA(0.5)**	5000-2400	1.04	7.1±0.2
P5CL(1.0)	5000-5000	1.06	7.2±0.5	**P5LLA(1.2)**	5000-6000	1.04	23.2±1.0

[a] Polymer terminology, with number of hydrophobic-hydrophilic-ratio (w/w) in parentheses
[b] Values according to Polymer Data Sheets, determined by ^{1}H NMR
[c] Values according to Polymer Data Sheets, dermined by SEC
[d] Acid value (n=3)

The block copolymers investigated were characterized by the supplier using size exclusion chromatography (SEC), ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) and differential scanning calorimetry (DSC). To establish an additional quality control for these excipients, not listed in the pharmacopoeia yet, acid values of the polymers were determined. An anhydrous titration according to the European standard (EN ISO 2114:2000) was used to determine the free carboxyl groups in the form of free acids or homopolymers and anhydrides present in the material. The PEG-*b*-PCL polymers exhibited low acid values (4.5 – 9.5 mg KOH per g polymer) displaying neither a dependency on the PEG-content, the PCL/PEG-ratio nor the polydispersity of the polymers (see Table 1). For comparison, the limit values of standard excipients like polysorbates, polyoxyethylene castor oil derivatives, and sucrose ester are in a range of 2.0 – 6.0 mg KOH per gram raw material [26].

In contrast, the acid values of PEG-*b*-PLA were in a broader range (4 – 32 mg KOH per gram polymer) with remarkable high values of 32 and 23 mg KOH per gram polymer for P2LA(0.6) and the P5LLA(1.2), respectively.

3.3 Micelle preparation and characterization

The film formation and sonication method were investigated with regard to method applicability and solubilization efficiency. The results were compared to each other and correlated with the polymer properties.

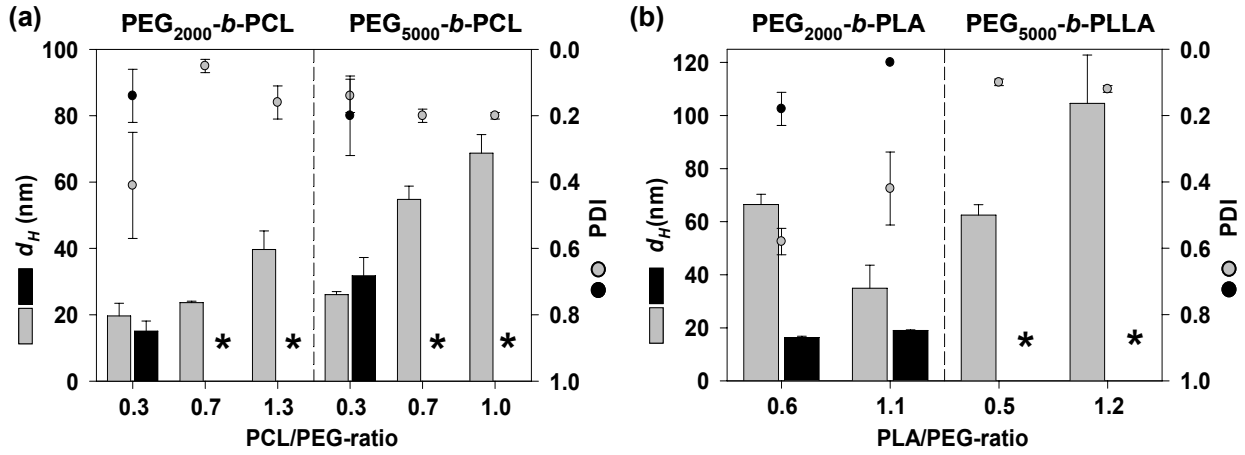

Figure 2: Characteristics of polymeric micelles prepared by different preparation methods
Hydrodynamic diameter d_H (bars) and polydispersity index PDI (dots) of unloaded polymeric micelles prepared by either sonication (▭/○) or film formation (■/●) as a function of the hydrophobic/hydrophilic-ratio of the block copolymers; * film redispersion not possible (n=3-4)

The PCL/PEG-ratio had a stake in the applicability of the preparation method as well as the micelle sizes as shown in Figure 2. At a constant molecular weight of PEG increasing PCL block lengths led to increasing micelle sizes in an almost linear manner (P2CL with $R^2 = 0.94$; P5CL with $R^2 = 0.99$) using the sonication method (Fig. 2). Independent of the molecular weight of PEG the PCL/PEG-ratio defined the redispersion behaviour of the polymeric films. A PCL/PEG-ratio of 0.3 allowed complete film redispersion (Fig. 2) and the corresponding polymeric films were clear with observable spherulites. In contrast, the films at higher PCL/PEG-ratios were turbid and not redispersible. Comparing P2LA and P5LLA revealed a different behaviour (Fig. 2). P2LA polymers with a PLA/PEG-ratio of 0.6 and 1.1

resulted in small, monodisperse micelles after film redispersion, which are almost equal in size at 16 nm and 19 nm, respectively. In contrast, sonication was not feasible to form monodisperse micelles for these polymers, indicated by PDI values in the range of 0.4 – 0.6 (Fig. 2). Using the same methods, the behaviour of P5LLA polymers was vice versa. The polymeric films were turbid and not redispersible. On the other hand, sonication led to the formation of monodisperse (PDI: 0.10 and 0.12), comparatively large (63 nm and 105 nm) micelles of P5LLA(0.5) and P5LLA(1.2), as shown in Figure 2.

3.4 Solubilization of Sagopilone

The impact of the mechanism of micelle preparation on the solubilization efficiency (SE) of Sagopilone was investigated (Fig. 3). Target concentration was constant at 1 g/L corresponding to a solubilization efficiency of 100 %. The drug content as well as the physicochemical characteristics were determined after preparation and after storage at room temperature for 24 h.

P2CL and P5CL polymers showed similar results for the solubilization of Sagopilone, as shown in Figure 3a and b. Addition of Sagopilone did not alter the redispersion behaviour of the polymeric films. Hence, no solubilization was observed for the polymers with a PCL/PEG-ratio of 0.7 and higher using the film method. Solubilization efficiencies as high as (95 ± 6.8) %and (83 ± 1.2) % were achieved with P2CL(0.3) and P5CL(0.3), respectively, but a ‘supersaturation’ effect with subsequent precipitation of Sagopilone occurred (Fig. 3a and b). Using the sonication method the solubilization efficiency increased with the PCL/PEG-ratio in an almost linear manner within the group of P5CL ($R^2 = 0.98$) but without linear correlation for P2CL ($R^2 = 0.82$). The solubilization efficiency obtained with P2CL(0.3) and P5CL(0.3) of (15 ± 0.8) % and (16 ± 0.2) %, respectively, was similar to the drug content remaining after precipitation of the ‘supersaturated’ dispersions. The sonicated dispersions were stable for at least 24 h in contrast to dispersions prepared by film formation.

At the higher PCL/PEG-ratios of 0.7 and 1.0 – 1.3 solubilization efficiency of P2CL was higher than P5CL with (66 ± 5.4) % and (76 ± 1.6) % compared to (55 ± 1.1) % and (70 ± 4.2) %; respectively.

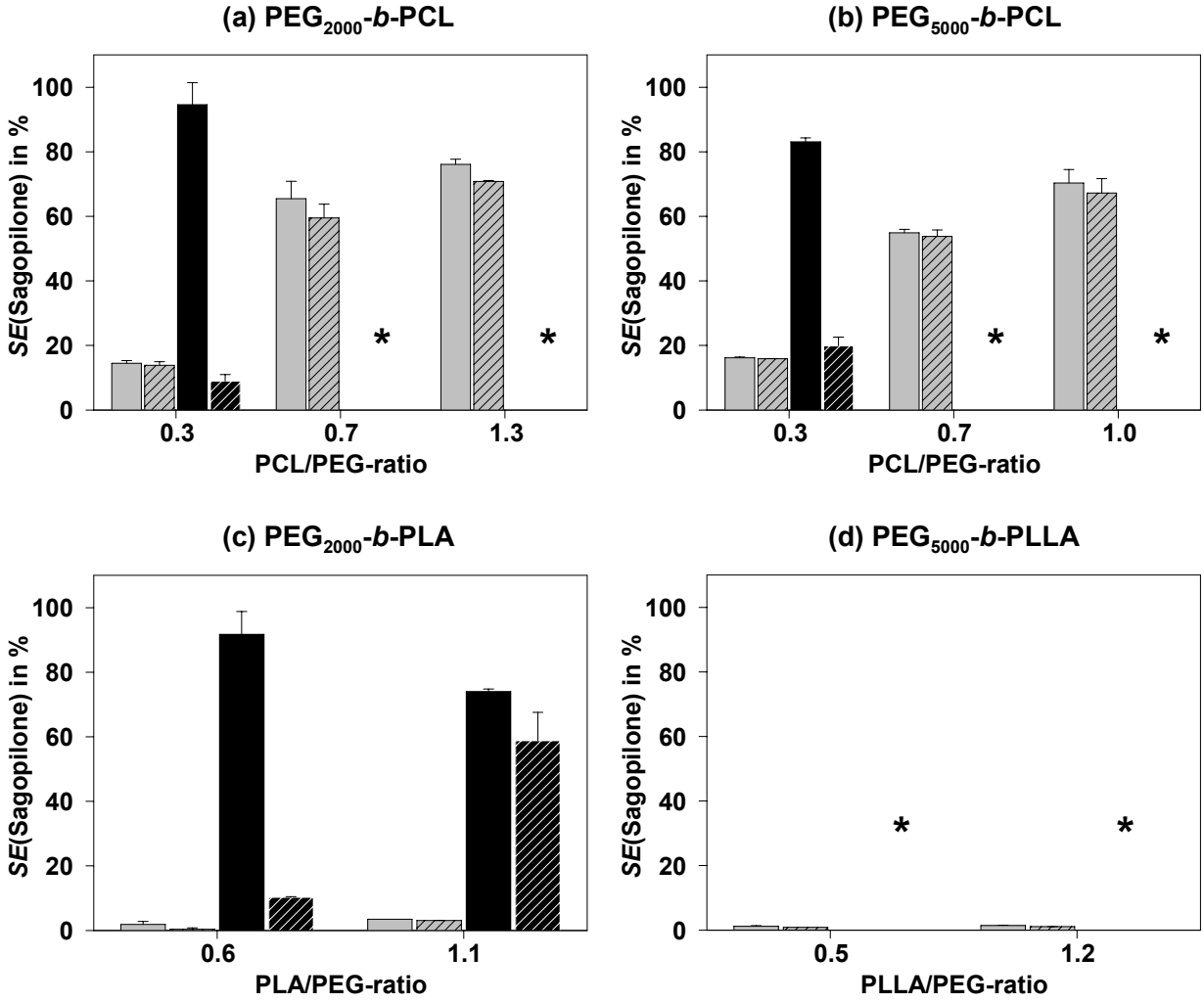

Figure 3: Solubilization of Sagopilone
Solubilization efficiency (SE) after preparation either by sonication (▭) or film formation (■) and after 24 h of storage at room temperature (▨ / ▩); c(polymer) of 10 g/L;
* film redispersion not possible. *(n*=3)

Interestingly, particle sizes of P2CL were not affected by drug loading in contrast to an increase in size for the P5CL micelles without a change in the size distribution (data not shown). In addition, the sonicated P5CL micelles still contained more than 95 % of initially loaded Sagopilone after 24 h compared to 91 % and 93 % within P2CL(0.7) and P2CL(1.3) micelles, respectively.

The P2LA polymers exhibited very low solubilization after sonication, namely (1.9 ± 0.9) % and (3.5 ± 0) % for P2LA(0.6) and P2LA(1.1), respectively (Fig. 3c). The application of the film formation method resulted in a high solubilization with (92 ± 7.1) % and (74 ± 0.8) %, respectively. These micellar dispersions showed the typical 'supersaturation' effect and 90 % and 20 % of the initially loaded Sagopilone precipitated within 24 h at a PLA/PEG-ratio of 0.6 and 1.1, respectively (Fig. 3c). Micelles of P2LA(0.6) (d_H = (16 ± 1) nm) remarkably increased after Sagopilone loading (d_H = (145 ± 17) nm). The P5LLA polymers showed almost no solubilization of Sagopilone after sonication (Fig. 3d) with solubilization efficiencies as low as (1.2 ± 0.2) % and (1.5 ± 0) % for P5LLA(0.5) and P5LLA(1.2), respectively, compared to (0.8 ± 0.02) % for the blank. Film formation was not applicable since drug-loaded polymeric films were not redispersible at all. As a result of this systematic screening, three polymers were selected as optimum materials for the solubilization of Sagopilone, namely P2LA(1.1), P2CL(1.3), and P5CL(1.0). As film formation was applied for P2LA(1.1), sonication was most suitable for P2CL(1.3) and P5CL(1.0). The well-known 'supersaturation' effect after film redispersion has been observed as well independent of the polymer used.

3.5 Thermal analysis

Thermal analysis was used to study the 'supersaturation' effect accompanying the film formation method more in detail, to provide an evidence of the solubility of Sagopilone within the amphiphilic block copolymers and to determine the apparent solid-state saturation solubility. Therefore, blank as well as drug-loaded polymeric films of P2CL and P2LA were analysed by DSC.

First, the thermodynamic transition points of the unloaded polymeric films, especially the glass transition temperatures of the hydrophobic blocks, were determined. As shown in Table 2 the polymeric films of P2CL(0.3) exhibited a glass transition as well as a melting.

That was in good correlation with the observation of spherulites within the films, which are spherical semi-crystalline regions inside non-branched linear polymers by definition. With regard to the value of the glass transition temperature (T_g) at (-66.3 ± 0.5) °C, the amorphous phase was composed of PCL. An increase of the PCL/PEG-ratio to 0.7 and 1.3 led to an increase in the glass transition temperature and distinct shoulders of the PEG melting peak.

Table 2: Thermal properties of blank polymeric films of PEG_{2000}-*b*-PCL and PEG_{2000}-*b*-PLA compared to $mPEG_{2000}$ and drug-loaded films of PEG_{2000}-*b*-PCL (*n*=3x3)

Films	Blank Polymeric Films					Sagopilone-loaded Films[a]			
	^1T_m (°C)	^2T_c (°C)	^3T_g (°C)	$^3\Delta cp$ ($J \cdot g^{-1} \cdot K^{-1}$)	^3T_m (°C)	T_g (°C)	^bT_g (°C)	^cT_g (°C)	$T_g/{}^cT_g$
P2CL (0.3)	49.1±1.2	5.4±16	**-66.3±0.5**	0.22±0.03	47.6±0.3	-50.6±1.8*	-84.0	-51.1	0.99
P2CL (0.7)	50.5±0.7	23.0±2.1	**-57.0±0.6**	0.29±0.03	48.1±0.2	-48.2±3.3*	-70.7	-46.0	1.05
P2CL (1.3)	50.2±0.3	18.7±6.5 11.4±7.9	**-52.3±0.2**	0.35±0.04	43.8±0.2 45.4±0.2 49.7±0.2	-46.0±1.8*	-64.2	-43.5	1.06
P2LA (1.1)	40.7±2.0	-	**-37.1±0.8**	-	38.7±0.3				
mPEG (2000 Da)	56.8±0.3	27.4±1.4	-	-	54.8±0.3				
Sago-pilone	-	-	**50.2±0.5**	0.33±0.04	-				

[1-3] Determined at first heating scan ([1]), at cooling cycle ([2]), and at second heating ([3])
[a] Sagopilone weight fraction of 0.09
[b] Theoretical T_g based on Fox Approach (see Equation (4))
[c] Theoretical T_g based on Couchman-Karasz equation (see Equation (5))
* Significant difference (p<0.05) in T_g compared to blank films

$$\frac{1}{T_g} = \frac{w_1}{T_{g,1}} + \frac{w_2}{T_{g,2}} \tag{4}$$

$$T_g = \frac{w_1 \cdot T_{g,1} + K_1 \cdot w_2 \cdot T_{g,2}}{w_1 + K_1 \cdot w_2};\ K_1 = \frac{\Delta cp_2}{\Delta cp_1} \tag{5}$$

Furthermore, the thermogram of P2CL(1.3) revealed two separate exothermic peaks during cooling indicating the formation of a third phase composed of crystalline PCL separately from PEG. This, again, correlated very well with the film turbidity of those polymers, in contrast

to P2CL(0.3). P2LA also exhibited a melting and a glass transition temperature as shown for P2LA(1.1) (Table 2). In contrast to P2CL, the measured T_g was an artefact due to the inhibition of the crystallization of the molten PEG phase during cooling at DSC measurement. The T_g of the PLA phase at approximately 38 °C could not be determined because of an overlap with the delayed crystallization and subsequent melting of PEG.

Hence, the apparent solid-state solubility of Sagopilone was investigated for P2CL based on the alteration of the glass transition of PCL. The T_g of drug-loaded polymeric films was determined and correlated to theoretical approaches. The films contained Sagopilone at a weight fraction equal to the solubilization experiments (Tab. 2). If Sagopilone functions as a plasticizer, T_g will be decreased as described by the Fox Approach (Equation (4), Tab. 2). In contrast, the formation of a glass solution is indicated by an increasing value of T_g compared to the blank according to the Couchman-Karasz equation (Equation (5), Tab. 2). The glass transition temperatures were significantly increased compared to the corresponding blanks, independent of the PCL/PEG-ratio (Tab. 2), and they correlated very well with the values calculated by the Couchman-Karasz equation. Hence, Sagopilone was solubilized within the glassy PCL region in terms of a glass solution. P2CL(0.3) showed the best correlation (deviation of 1.0 %) in comparison to the higher PCL/PEG-ratios. No alteration was observed for the other thermodynamic transitions (data not shown) underlying the phase separation nature of these films.

P2CL(0.3) was investigated further in order to determine the saturation solubility of Sagopilone within this polymer (Fig. 4, left). The measured T_g correlated very well with the Couchman-Karasz equation at Sagopilone weight fractions of 0.09 and lower. At higher weight fractions the T_g was constant at approximately -60 °C, which was remarkably lower than the predicted values by Couchman-Karasz but still elevated in comparison to the blank film. Moreover, a Sagopilone melting peak appeared at weight fractions of 0.5 and higher. The T_g observed at the higher drug loading of approximately -60 °C was similar to

the T_g of films comprising a Sagopilone weight fraction of 0.02 (Fig. 4, left). In addition, the micellar dispersions comprising a drug loading at this weight fraction (0.02) did not show the 'supersaturation' phenomenon as shown in the right graph of Figure 4, indicating a saturated loading.

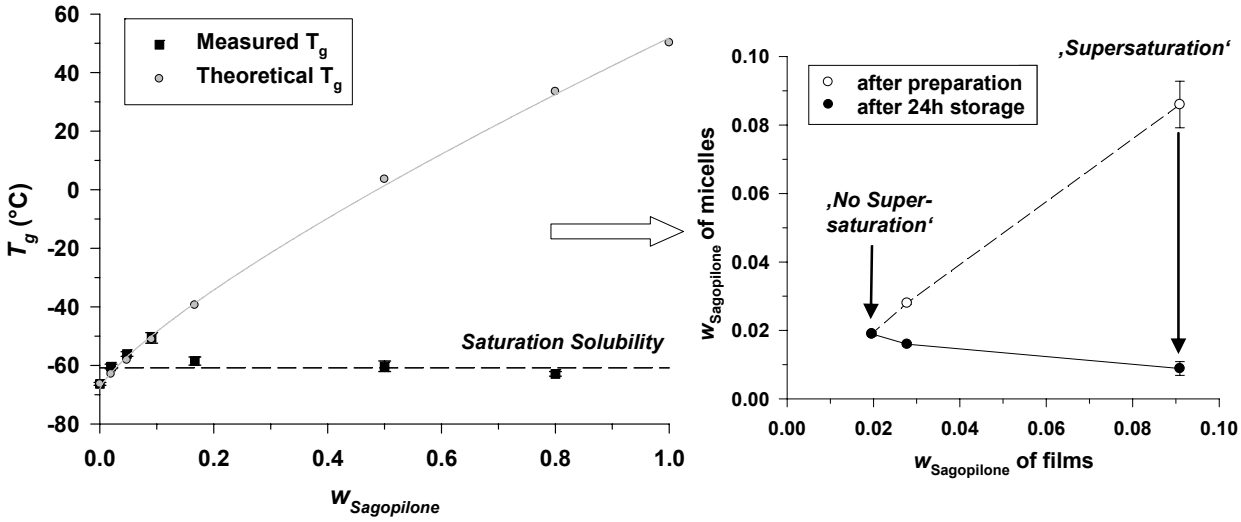

Figure 4: Apparent solid-state solubility of Sagopilone (--) in films composed of P2CL(0.3)
left: Measured and theoretical T_g according to Couchman-Karasz as a function of drug weight fraction (w) and determination of the apparent solid-state solubility (--); *right:* Drug weight fraction (w) of micellar dispersions after film redispersion and after 24 h at c(polymer) of 50 g/L. (n=5)

3.6 Characterization of micelle morphology

Morphology determination of the three selected micellar delivery systems was done by cryo transmission electron microscopy (cryoTEM) to preserve the three-dimensional structure of the micelles in their native hydrated state.

The images revealed that the micelles were spherical with a monodisperse distribution in the absence of larger aggregates independent of the polymer and preparation technique used (Fig. 5). The PCL-containing micelles exhibited a hexagonal arrangement with a high degree of order as seen for P5CL(1.0) in contrast to P2LA(1.1) micelles. The latter were randomly spaced apart.

Table 3: Comparison of particle characteristics obtained by DLS and cryoTEM of micellar dispersions

Sample	Polymer	f [a] (%)	Preparation Method	c (g/L)	DLS d_H (nm)	DLS PDI	cryoTEM size[b] (nm)	ΔSize[c] (nm)
A	P2LA(1.1)	47.6	Film formation	20	20.0	0.016	12.9	7.1
B	P2CL(1.3)	43.5	Sonication	20	32.6	0.145	16.9	15.7
C	P5CL(1.0)	50.0	Sonication	20	71.9	0.215	22.3	49.6

[a] Hydrophilic fraction f of the block copolymer (w/w)
[b] Median of measurement of 200 micelles (4 different images with 50 micelles each)
[c] Difference between hydrodynamic diameter (d_H) and size at cryoTEM

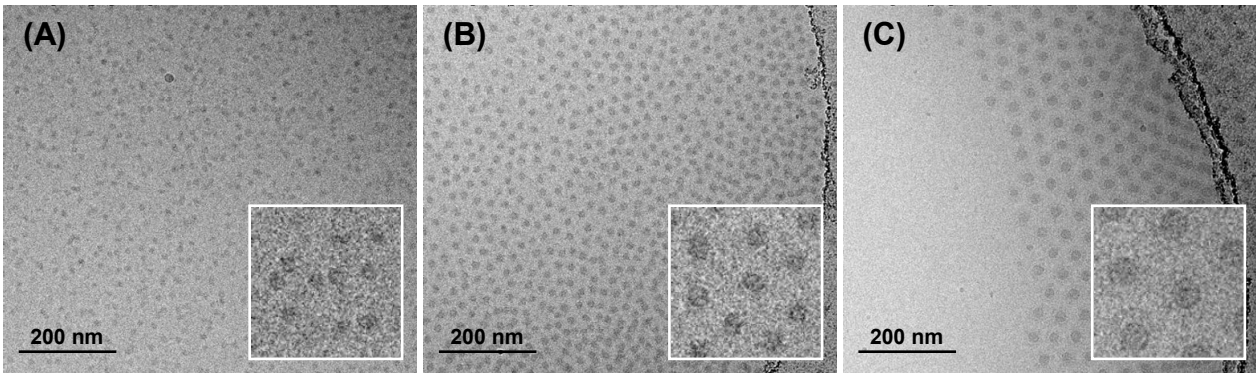

Figure 5: CryoTEM images
Images of polymeric micelles composed of (A) P2LA(1.1), (B) P2CL(1.3), and (C) P5CL(1.0) at a concentration of 20 g/L. The inset shows a small region (100 nm^2) of the image at a larger scale.

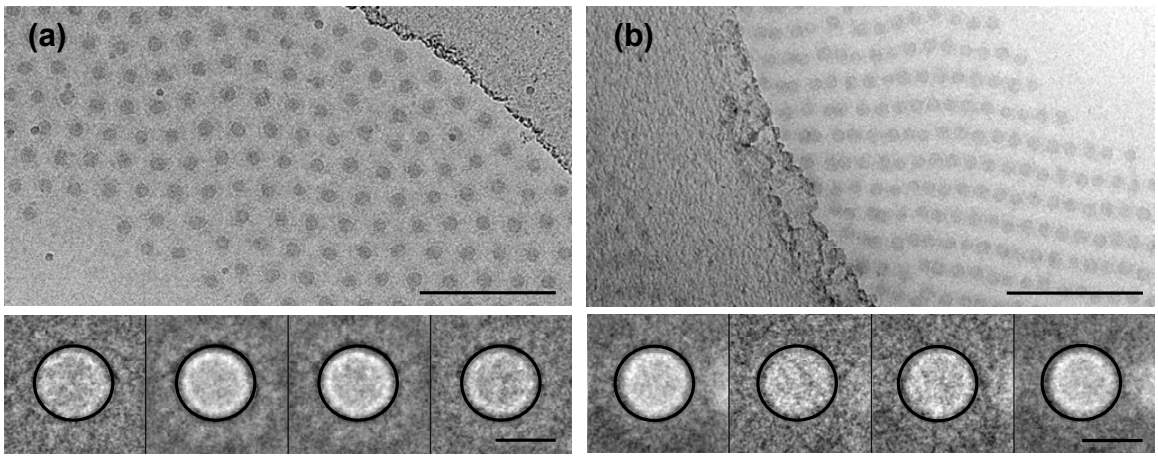

Figure 6: CryoTEM tilt study
Comparison of cryoTEM images of P5CL(1.0) micelles acquired at zero tilt (a) and at a high tilt angle of 55° (b) (bar = 200 nm). Underneath the respective class averages obtained by the described selection and classification process are represented (bar = 20 nm).

In addition, comparison of the values obtained by DLS and cryoTEM (Tab. 3) revealed a remarkable difference for the PCL-containing micelles although presumption for the size measurement by DLS, which is the existence of spherical particles, was fulfilled. This effect was pronounced most for P5CL(1.0).

A cryoTEM tilt study of P5CL(1.0) micelles was performed to clarify the exact morphology since spheres may be simulated by single imaging of cylinders in a topview. The images were taken at tilt angle of 0° and 55°. The micelles were proven to be spherical as shown in Figure 6. Approximately 300 particles per image were selected and classified automatically resulting in four classes, which were represented below the full images. They were almost equal in size at the respective measurement angle as well as between the different tilts (25.8, 25.3, 25.4, 24.7 nm at 0° vs. 25.2, 26.0, 25.6, 24.6 nm at 55°). This was additionally displayed by the overlay of a black circle of a fixed diameter of 26 nm.

4. Discussion

The method developed for acid value determination of amphiphilic block copolymers offers a fast and convenient test for purity assessment following standard tests of the European Pharmacopoeia. This is of high importance to their implementation as standardized excipients. Assuming the acid value is exclusively due to the cleavage of the polyester block, the emerging acids constitute a maximum of 2 % (w/w raw material) except for the outliers P2LA(0.6) and P5LLA(1.2). The latter may be due to the presence of possible by-products of the polymer synthesis such as poly(lactide) homopolymers and free lactic acid resulting from polymer degradation or cleavage of residual lactide present in the raw material. This fact has to be taken into account for result interpretation. Furthermore, future studies are needed to define threshold acid values for these novel excipients.

The nature of the hydrophobic block, the preparation technique as well as the composition of the block copolymers has had a significant impact on the micelle formation and the solubilization of Sagopilone. Sonication was applicable to P2CL and P5CL exhibiting the same dependencies within the particular groups. In comparison with published data using a similar polymer, the micelles of P5CL(1.0) formed by sonication (69 nm, PDI 0.20) were smaller than micelles obtained by a co-solvent evaporation method (87.5 nm, PDI 0.198) [31]. But they were larger than those formed by solvent displacement with subsequent sonication (41.0 nm) [32]. Likewise, the sonicated micelles of P5CL(0.7) exhibited remarkably smaller sizes (55 nm, PDI 0.20) compared to the use of a co-solvent evaporation method (71.8 nm) [33]. The hydrophobic/hydrophilic-ratio was found be a crucial parameter for the applicability of the film formation for P2CL and P5CL. At values of 0.3 film redispersion was possible in contrast to the general statement that this preparation method is inappropriate for this kind of polymers [31]. These results support the published observations that the procedure of the micelle formation plays a significant role in determining the average

diameter and size distribution aside from the block copolymer molecular weight [31, 34]. Larger micelle sizes reported for the co-solvent evaporation method may be due to the precipitation driven micelle formation, in contrast to the self-assembly of amphiphilic block copolymers into micelles during direct dissolution using sonication. Comparison of the latter with micelles prepared by film formation, if applicable, revealed only small differences. This fact further corroborates the theory since film hydration is self-assembling driven as well. The application of identical procedures for PEG-*b*-PLA polymers revealed a distinct difference between P2LA and P5LLA due to the different stereoisomers of the lactic acid monomers. Film redispersion was only feasible if the hydrophobic block was formed of poly(D,L-lactide). In contrast, only P5LLA polymers containing a poly(L-lactide) block produced monodisperse micelles when sonicated. It is known that steric factors play an important role in chain flexibility, chain packing, and subsequent crystallization behaviour [35]. For this reason, P2LA micelles having an amorphous poly(D,L-lactide) core were almost similar in size (around 20 nm) independent from the MW of PLA. P5LLA micelles comprising a semi-crystalline poly(L-lactide) core were remarkably larger (63 and 105 nm) with a dependence on the PLA block length [36]. These findings are in good agreement with literature values [37-39].

Solubilization efficiency of P2CL and P5CL micelles prepared by sonication increased with the particular PCL/PEG-ratio with comparable results between the two groups. This is in contrast to previous observations reporting an increase in the drug loading e.g. of Paclitaxel with the PCL block length independent of the molecular weight of PEG [33]. However, the latter was observed for micelles prepared by co-solvent evaporation pointing out again the impact of the preparation method**.** Using the co-solvent evaporation method clustering of Sagopilone with the hydrophobic blocks is promoted since the drug and the polymer are dissolved prior to the micelle formation and encapsulation event. On the contrary, drug and polymer dissolution, micelle formation, and drug loading take place at the same time during

the sonication method displaying an additional loading hindrance due to the shield of the hydrophobic core by a PEG corona. This is very likely to be the reason for the higher solubilization of P2CL besides a partial degradation of the P5CL polymers. The latter can be excluded since it has not been observed in terms of higher acid values. Contrary to P2CL, the sizes of P5CL micelles increased after drug loading. This effect was not predictable since all possible alterations have been reported in the literature, namely an increase in size after solubilization of Cyclosporine A for P5CL(1.0) [31], no alteration in the size of Paclitaxel-loaded micelles of P5CL(0.8) [33] as well as a slight decrease in size of P5CL(1.0) micelles together with an increase of P2CL(1.0) micelles after Doxorubicin loading [32]. Possible explanations for this effect are the formation of a small amount of drug nanocrystals with diameters less than the pore size of the filter (0.22 μm), measurement artefacts due to the presence of dust, or an altered viscosity. However, the former have not been detected as a single size population at DLS and the corresponding blank samples exhibited negligible Sagopilone concentrations (0.8 ± 0.02 %). Further studies are needed to elucidate the effect of the drug used, the polymer structure and concentration, and, in particular, the measurement settings.

Very high solubilization values after film hydration, if feasible, and the subsequent precipitation of Sagopilone coincide with the described ‘supersaturation’ effect of polymeric micelles especially obtained by a film formation method [6, 40]. The same range of the effective solubilization after precipitation and after sonication without subsequent precipitation provides an indication of the loading capacity of the particular micelles. A big discrepancy was observed in the solubilization capacity of micelles comprising a poly(D,L-lactide)- and poly(L-lactide)-core. This is not in accordance with the theory that the intermolecular forces between the hydrophobic drug and the core-forming block of the polymer are the major criterion for the solubilization capacity. The fact that Sagopilone was not solubilized by poly(L-lactide) was not predictable and may be due to a high degree of core

crystallinity without molecular dispersion of Sagopilone. The high accommodation of Sagopilone into poly(D,L-lactide)-containing polymeric micelles indicates that the amorphous core structure is superior for drug solubilization. Using the film formation for P2LA a ‘supersaturation’ effect has been observed as well with a distinct lower amount of drug precipitation at the larger PLA/PEG-ratio. The instability of P2LA(0.6) may be additionally enlarged by hydrolytic degradation of the polymer as indicated by the high acid value. Consequently, the hydrophobic/hydrophilic-ratio was best at approximately 1 regarding solubilization efficiency and stability. Thus, P2LA(1.1), P2CL(1.3), and P5CL(1.0) were selected as delivery vehicles for further studies along with the appropriate method of preparation.

Correlation of the effects described with the calculated solubility parameters revealed that the latter were not predictive. According to the theoretical estimation PLA exhibits a better compatibility compared to PCL, whereas direct comparison of P2LA(1.1) and P2CL(1.3) micelles revealed similar to higher solubilization and, most notably, more stable drug loading of P2CL(1.3). This is in contrast to findings of Jubo et al. [41] demonstrating a good correlation between solubility parameters and drug formulation characteristics like drug loading for PEG-*b*-PLA and PEG-*b*-PCL micelles. As shown by the partial solubility parameters the portions of the different interaction forces between Sagopilone and PCL differ from those of PLA. Structurally, both polymers consist of a polyester backbone with possible hydrogen bonding between their carbonyl functions and free hydroxyl groups of Sagopilone. This is congruent with the similar difference in the particular hydrogen bonding parameter. Hence, the better compatibility of PEG-*b*-PCL is very likely to be due to the higher portion of dispersive/ van der Waals forces between Sagopilone and PCL as well as between PCL chains itself resulting in higher micelle stability. Further evidence of the non-correlation was shown by the inability of P5LLA to solubilize Sagopilone compared to sufficient solubilization of P5CL micelles. The distinct solubilization capacities of poly(L-lactide) and poly(D,L-lactide)

as core-forming blocks were not covered by this method since the solubility parameter does not distinguish between different stereoisomers. However, the difference in the stereochemistry of the lactic acid monomer results in different aggregation behaviour/ crystallinity of the resulting polymer, and this in turn highly affects the solubilization. Thus, the theoretical methods need to be adapted to accommodate this type of difference.

Thermal analysis revealed a highly phase-separated composition of the polymeric films. As shown for P2CL(0.3-1.3) the films consisted of crystalline PEG, amorphous PCL, and crystalline PCL. The formation of the latter was essentially influenced by the PCL/PEG-ratio instead of the mere PCL molecular weight and determined the film redispersion behaviour. This is in good correlation with findings that nanoscale confinement of normally semi-crystalline PCL within blends with 100 nm dispersed phases impedes the crystallization of PCL, yielding liquid-state PCL domains at room temperature [42]. Thermo-analytical investigations of drug-loaded films indicated the presence of glass solutions comprising Sagopilone and PCL besides crystalline regions of the film. The good correlation with the theoretical approach describing glass solutions (Couchman-Karasz approach) provided clear evidence that Sagopilone was molecularly dispersed in the amorphous PCL phase. Complete molecular dispersion of the drug was obtained with P2CL(0.3) indicated by a deviation of only 1.0 % from the theoretical value. This finding is in contrast to a described plasticizer effect of drugs like propranolol after loading into PCL nanoparticles indicated by a decrease of T_g [43]. The findings of this study provide an explanation of the repeatedly described ‘supersaturation’ effect of polymeric micellar dispersions after film hydration. Sagopilone was shown to be completely dispersed in the liquid-like PCL phase of P2CL(0.3) films at a maximum drug weight fraction of 0.09. This value was not consistent with the solubilization capacity of the micelles as observed by the ‘supersaturation’ effect. Further thermal analysis revealed a saturation solubility of the films at a weight fraction of 0.02. This value correlated well with the solubilization capacity of the corresponding

micelles indicated by the absence of any 'supersaturation' (Fig. 4). This is a very promising approach for the determination of the solubilization capacity and has to be proven for further drugs. To expand this approach to PEG-*b*-PLA the measurement parameters have to be optimized with respect to a reliable determination of the T_g of PLA.

Depending on the hydrophilic fraction *f* self-assembly of amphiphilic block copolymers leads to the formation of micelles with spherical (f > 50 %) or worm-like (f = 40-50 %) morphologies or vesicular structures called polymersomes (f = 25 – 40 %) [44]. Additionally, the preparation method has an impact on the formation of a specific morphology [45]. Thus, worm-like as well as spherical micelles may be expected for the three micellar dispersions selected comprising block copolymers with *f*-values ≤ 50 % using the particular preparation methods (Tab. 3). Particle analysis by cryoTEM provided clear evidence of spherical micelles independent of the type of the block copolymer. This was additionally confirmed in a cryoTEM tilt study to exclude misinterpretation of images of worm-like structures in a top view pretending spheres as well. Interestingly, an additional bright shell surrounding the particles was visualized after the addition of several pictures during the retrospective classification procedure. This shell was not detected at pictures of single measurements because they were taken with short times of electron beam to avoid liquidation of the vitrified sample. This finding provides a good explanation for the observed difference in the particle sizes obtained by DLS and cryoTEM (Tab. 3) since the latter describes the size of the core of the polymeric micelles. The size difference of approximately 50 nm as seen for P5CL(1.0) correlates very well with the theoretical thickness of the PEG-water-shell of 25 nm. The latter could be estimated by the constant particle interspaces of 50 nm observed within the hexagonal arrangement, the densest packing of spheres. The small difference in the core size between P5CL(1.0) and P2CL(1.3) (22 and 17 nm, respectively) is in good correlation with their almost similar solubilization capacity of Sagopilone. The remarkably larger hydrodynamic diameter of P5CL(1.0) (d_H = 71.9 nm) compared to P2CL(1.3) (d_H = 32.6 nm)

was due to the higher molecular weight of PEG. Furthermore, the distinct higher PDI-values of P2CL(1.3) and P5CL(1.0) micelles at DLS in spite of the spherical, monodisperse morphology imaged with cryoTEM may be due to the dense sphere packing and subsequent hindrance of the free Brownian-motion of the micelles. This theory is encouraged by the absence of such a long range order for P2LA(1.0) micelles along with a remarkably lower polydispersity at DLS. At least, the formation of larger aggregates, increasing the PDI value at DLS, has not been observed in any of the images taken.

5. Conclusion

The poorly water-soluble anticancer drug Sagopilone was sufficiently solubilized by PEG-*b*-PCL and PEG-*b*-Poly(D,L-lactide) micelles with an optimum hydrophobic/hydrophilic-ratio of approximately 1 regarding the solubilization efficiency and stability. No solubilization was observed for PEG-*b*-Poly(L-lactide). Sonication was most suitable for polymers with a high PCL/PEG-ratio (≥ 0.7). Film formation was superior for poly(D,L-lactide)-containing polymers and those comprising a PCL/PEG-ratio of 0.3 at the most. Drug loading into PEG-*b*-PCL micelles was superior to PEG-*b*-PLA due to the absence of a 'supersaturation' effect after sonication. Thermal analysis revealed the molecular dispersion of Sagopilone in the liquid-like PCL phase of the polymeric films in form of a glass solution. Contrary to previous publications, calculated solubility parameters were not suitable as predictive parameters. Against theoretical prediction, PCL was superior to PLA and the serious difference between the two stereoisomers of PLA in their ability to solubilize Sagopilone was disregarded by this approach. The three selected drug delivery systems composed of P2LA(1.1), P2CL(1.3), and P5CL(1.0) consist of small (< 100 nm), monodisperse, and spherical micelles with slightly different core sizes and distinct differences in their hydrodynamic shell. The impact of the core material as well as the PEG-shell at a constant Sagopilone loading and micelle morphology on the *in vitro* as well as *in vivo* behaviour is under further study with the aim to enhance the therapy of Sagopilone.

References

1. U. Klar, B. Buchmann, W. Schwede, W. Skuballa, J. Hoffmann, R.B. Lichtner. Total synthesis and antitumor activity of ZK-EPO: the first fully synthetic epothilone in clinical development. *Angew. Chem. Int. Edit.* 45 (2006) 7942-7948.

2. J. Hoffmann, I. Vitale, B. Buchmann, L. Galluzzi, W. Schwede, L. Senovilla, W. Skuballa, S. Vivet, R.B. Lichtner, J.M. Vicencio, T. Panaretakis, G. Siemeister, H. Lage, L. Nanty, S. Hammer, K. Mittelstaedt, S. Winsel, J. Eschenbrenner, M. Castedo, C. Demarche, U. Klar, G. Kroemer. Improved cellular pharmacokinetics and pharmacodynamics underlie the wide anticancer activity of Sagopilone. *Cancer Res.* 68 (2008) 5301-5308.

3. U.S. National Institutes of Health. Clinical Trials of Sagopilone. http://clinicaltrials.gov/ct2/results?term=Sagopilone (accessed 03/08/2009).

4. A.J. ten Tije, J. Verweij, W.J. Loos, A. Sparreboom. Pharmacological effects of formulation vehicles: implications for cancer chemotherapy. *Clin. Pharmacokinet.* 42 (2003) 665-685.

5. Y. Bae, K. Kataoka. Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. *Adv. Drug Deliv. Rev.* 61 (2009) 768-784.

6. V.P. Torchilin. Micellar nanocarriers: pharmaceutical perspectives. *Pharm. Res.* 24 (2007) 1-16.

7. N. Nishiyama, K. Kataoka. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. *Pharmacol. Ther.* 112 (2006) 630-648.

8. H.M. Aliabadi, A. Lavasanifar. Polymeric micelles for drug delivery. *Expert Opin. Drug Deliv.* 3 (2006) 139-162.

9. M.C. Jones, J.C. Leroux. Polymeric micelles - a new generation of colloidal drug carriers. *Eur. J. Pharm. Biopharm.* 48 (1999) 101-111.

10. T.Y. Kim, D.W. Kim, J.Y. Chung, S.G. Shin, S.C. Kim, D.S. Heo, N.K. Kim, Y.J. Bang. Phase I and pharmacokinetic study of Genexol-PM, a Cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced malignancies. *Clin. Cancer Res.* 10 (2004) 3708-3716.

11. T. Kawaguchi, T. Honda, M. Nishihara, T. Yamamoto, M. Yokoyama. Histological study on side effects and tumor targeting of a block copolymer micelle on rats. *J. Control. Release* 136 (2009) 240-246.

12. R.T. Liggins, H.M. Burt. Polyether-polyester diblock copolymers for the preparation of paclitaxel loaded polymeric micelle formulations. *Adv. Drug Deliv. Rev.* 54 (2002) 191-202.

13. K. Kataoka, T. Matsumoto, M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto, G.S. Kwon. Doxorubicin-loaded poly(ethylene glycol)-poly(β-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance. *J. Control. Release* 64 (2000) 143-153.

14. P. Opanasopit, M. Yokoyama, M. Watanabe, K. Kawano, Y. Maitani, T. Okano. Block copolymer design for camptothecin incorporation into polymeric micelles for passive tumor targeting. *Pharm. Res.* 21 (2004) 2001-2008.

15. H. Maeda, G.Y. Bharate, J. Daruwalla. Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. *Eur. J. Pharm. Biopharm.* 71 (2009) 409-419.

16. H.M. Aliabadi, M. Shahin, D.R. Brocks, A. Lavasanifar. Disposition of drugs in block copolymer micelle delivery systems: from discovery to recovery. *Clin. Pharmacokinet.* 47 (2008) 619-634.

17. G.S. Kwon. Polymeric micelles for delivery of poorly water-soluble compounds. *Crit. Rev. Ther. Drug Carr. Syst.* 20 (2003) 357-403.

18. S.C. Kim, D.W. Kim, Y.H. Shim, J.S. Bang, H.S. Oh, S.W. Kim, M.H. Seo. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *J. Control. Release* 72 (2001) 191-202.

19. D.W. Kim, S.Y. Kim, H.K. Kim, S.W. Kim, S.W. Shin, J.S. Kim, K. Park, M.Y. Lee, D.S. Heo. Multicenter phase II trial of Genexol-PM, a novel Cremophor-free, polymeric micelle formulation of paclitaxel, with cisplatin in patients with advanced non-small-cell lung cancer. *Ann. Oncol.* 18 (2007) 2009-2014.

20. K.S. Lee, H.C. Chung, S.A. Im, Y.H. Park, C.S. Kim, S.B. Kim, S.Y. Rha, M.Y. Lee, J. Ro. Multicenter phase II trial of Genexol-PM, a Cremophor-free, polymeric micelle formulation of paclitaxel, in patients with metastatic breast cancer. *Breast Cancer Res. Treat.* 108 (2008) 241-250.

21. B. Shi, C. Fang, M.X. You, Y. Zhang, S. Fu, Y. Pei. Stealth MePEG-PCL micelles: effects of polymer composition on micelle physicochemical characteristics, in vitro drug release, in vivo pharmacokinetics in rats and biodistribution in S180 tumor bearing mice. *Colloid Polym. Sci.* 283 (2005) 954-967.

22. J. Liu, F. Zeng, C. Allen. In vivo fate of unimers and micelles of a poly(ethylene glycol)-block-poly(caprolactone) copolymer in mice following intravenous administration. *Eur. J. Pharm. Biopharm.* 65 (2007) 309-319.

23. X. Wei, C. Gong, M. Gou, S. Fu, Q. Guo, S. Shi, F. Luo, G. Guo, L. Qiu, Z. Qian. Biodegradable poly(ε-caprolactone)-poly(ethylene glycol) copolymers as drug delivery system. *Int. J. Pharm.* 381 (2009) 1-18.

24. Y. Geng, P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko, D.E. Discher. Shape effects of filaments versus spherical particles in flow and drug delivery. *Nat. Nanotechnol.* 2 (2007) 249-255.

25. A.F.M. Barton. CRC Handbook of Solubility Parameters and Other Cohesion Parameters. CRC Press Taylor & Francis Group, Boca Raton (1991).

26. Council of Europe. European Pharmacopoeia 6.4. Straßbourg (2009).

27. C. Suloway, J. Pulokas, D. Fellmann, A. Cheng, F. Guerra, J. Quispe, S. Stagg, C.S. Potter, B. Carragher. Automated molecular microscopy: the new Leginon system. *J. Struct. Biol.* 151 (2005) 41-60.

28. A. Pascual-Montano, L.E. Donate, M. Valle, M. Bárcena, R.D. Pascual-Marqui, J.M. Carazo. A novel neural network technique for analysis and classification of EM single-particle images. *J. Struct. Biol.* 133 (2001) 233-245.

29. C.O.S. Sorzano, R. Marabini, J. Velázquez-Muriel, J.R. Bilbao-Castro, S.H.W. Scheres, J.M. Carazo, A. Pascual-Montano. XMIPP: a new generation of an open-source image processing package for electron microscopy. *J. Struct. Biol.* 148 (2004) 194-204.

30. C. Allen, D. Maysinger, A. Eisenberg. Nano-engineering block copolymer aggregates for drug delivery. *Colloid Surf. B-Biointerfaces* 16 (1999) 3-27.

31. H.M. Aliabadi, A. Mahmud, A.D. Sharifabadi, A. Lavasanifar. Micelles of methoxy poly(ethylene oxide)-b-poly(ε-caprolactone) as vehicles for the solubilization and controlled delivery of cyclosporine A. *J. Control. Release* 104 (2005) 301-311.

32. X. Shuai, H. Ai, N. Nasongkla, S. Kim, J. Gao. Micellar carriers based on block copolymers of poly(ε-caprolactone) and poly(ethylene glycol) for doxorubicin delivery. *J. Control. Release* 98 (2004) 415-426.

33. X. Shuai, T. Merdan, A.K. Schaper, F. Xi, T. Kissel. Core-cross-linked polymeric micelles as paclitaxel carriers. *Bioconjugate Chem.* 15 (2004) 441-448.

34. P. Vangeyte, S. Gautier, R. Jérôme. About the methods of preparation of poly(ethylene oxide)-b-poly(ε-caprolactone) nanoparticles in water: analysis by dynamic light scattering. *Colloid Surf. A-Physicochem. Eng. Asp.* 242 (2004) 203-211.

35. J.M.G. Cowie and V. Arrighi. Polymers: Chemistry and Physics of Modern Materials. CRC Press Taylor & Francis Group, Boca Raton (2008).

36. K.M. Huh, Y.W. Cho, K. Park. PLGA-PEG block copolymers for drug formulations. *Drug Deliv. Technol.* 3 (2003) 42-49.

37. J. Zhang, W. Jiang, X. Zhao, Y. Wang. Preparation and characterization of polymeric micelles from poly(D, L-lactide) and methoxypolyethylene glycol block copolymers as potential drug carriers. *Tsinghua Science & Technology* 12 (2007) 493-496.

38. K. Yasugi, Y. Nagasaki, M. Kato, K. Kataoka. Preparation and characterization of polymer micelles from poly(ethylene glycol)-poly(D,L-lactide) block copolymers as potential drug carrier. *J. Control. Release* 62 (1999) 89-100.

39. L. Jongpaiboonkit, Z. Zhou, X. Ni, Y.-Z. Wang, J. Li. Self-association and micelle formation of biodegradable poly(ethylene glycol)-poly(L-lactic acid) amphiphilic di-block co-polymers. *J. Biomater. Sci. Polym. Ed.* 17 (2006) 747-763.

40. M.G. Carstens, P.H.J.L.F. de Jong, C.F. van Nostrum, J. Kemmink, R. Verrijk, L.G.J. de Leede, D.J.A. Crommelin, W.E. Hennink. The effect of core composition in biodegradable oligomeric micelles as taxane formulations. *Eur. J. Pharm. Biopharm.* 68 (2008) 596-606.

41. L. Jubo, Y. Xiao, C. Allen. Polymer-drug compatibility: a guide to the development of delivery systems for the anticancer agent, ellipticine. *J. Pharm. Sci.* 93 (2004) 132-143.

42. J. Kim, R.W. Sandoval, C.M. Dettmer, S.T. Nguyen, J.M. Torkelson. Compatibilized polymer blends with nanoscale or sub-micron dispersed phases achieved by hydrogen-bonding effects: Block copolymer vs blocky gradient copolymer addition. *Polymer* 49 (2008) 2686-2697.

43. N. Ubrich, P. Bouillot, C. Pellerin, M. Hoffman, P. Maincent. Preparation and characterization of propranolol hydrochloride nanoparticles: a comparative study. *J. Control. Release* 97 (2004) 291-300.

44. D.E. Discher, F. Ahmed. Polymersomes. *Annu. Rev. Biomed. Eng.* 8 (2006) 323-341.

45. S. Cai, K. Vijayan, D. Cheng, E.M. Lima, D.E. Discher. Micelles of different morphologies – advantages of worm-like filomicelles of PEO-PCL in paclitaxel delivery. *Pharm. Res.* 24 (2007) 2099-2109.

CHAPTER 3

POLYMERIC MICELLES FOR PARENTERAL DELIVERY OF SAGOPILONE: PHYSICOCHEMICAL CHARACTERIZATION, NOVEL FORMULATION APPROACHES AND THEIR TOXICITY ASSESSMENT *IN VITRO* AS WELL AS *IN VIVO*

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Abstract

Purpose: The block copolymers PEG_{2000}-*b*-PLA_{2200}, PEG_{2000}-*b*-PCL_{2600}, and PEG_{5000}-*b*-PCL_{5000} have been currently identified as optimal solubilizing agents for Sagopilone, a poorly water-soluble anticancer drug. In the present study, the stability, formulation feasibility, and *in vitro* as well as *in vivo* toxicity were evaluated.

Methods: Dispersion media, storage conditions, and dilutions were varied for stability assessment. The critical micelle concentration (CMC) was determined using a fluorescent probe technique. Furthermore, the toxicity was studied *in vitro* and *in vivo* using HeLa/MaTu cells and a nude mouse model, respectively.

Results: A drug-polymer-ratio as low as 1:20 (w/w) was sufficient to solubilize Sagopilone effectively and to obtain stable dispersions (24 h: drug content ≥ 95 %). Although the micelles exhibited a similar thermodynamic stability (CMC: 10^{-7} – 10^{-6} M), PEG-*b*-PCL micelles were kinetically more stable than PEG_{2000}-*b*-PLA_{2200} (24 h at 37 °C: drug content ≥ 90 % compared to 30 %, respectively). Lyophilization of PEG-*b*-PCL micelles and storage stability of solid drug-loaded PEG_{2000}-*b*-PLA_{2200} films (3 m, 6 °C: drug content of (95.6 ± 1.4) %) were demonstrated for the first time. The high antiproliferative activity has been maintained *in vitro* (IC_{50} < 1 nM). Carrier-associated side effects have not been observed *in vivo* and the maximum tolerated dose of micellar Sagopilone was determined to be 6 mg/kg.

Conclusion: The results of this study indicate that polymeric micelles, especially PEG-*b*-PCL micelles, offer excellent potential for further preclinical and clinical cancer studies using Sagopilone.

1. Introduction

Solubilization represents one of the major challenges in formulation development nowadays since approximately 40 % of the new compounds in drug discovery are poorly water-soluble [1]. This is of particular concern in the parenteral delivery field because the number of approved excipients is restricted. Furthermore, currently used solubilizers such as Cremophor® EL have been implicated in clinically important adverse effects and unfavourable alterations of the pharmacokinetics of drugs as shown for Paclitaxel [2].

Sagopilone (Fig. 1) is a novel poorly water-soluble anticancer drug belonging to the group of epothilones that is administered parenterally [3, 4]. The epothilones present a novel class of microtubule-stabilizing anticancer drugs originally occurring in *Sorangium cellulosum*. Their mechanism of action is similar to Paclitaxel but they exhibit superior features relative to the latter. Besides their activity against various tumour types, they show low susceptibility to key tumour resistance mechanisms *in vitro*, and most importantly, *in vivo* [5]. Thus, they are effective in tumours resistant to Paclitaxel making them very likely to become successors to taxane therapy. Sagopilone (Fig. 1) is a synthetic epothilone derivative which is currently under clinical trial evaluation [6]. Dosing of Sagopilone is limited due to the occurrence of peripheral neuropathy. This is a typical side effect of epothilones, which recently gave reason to the refusal of the marketing authorisation for the epothilone derivative Ixabepilone by the European Medicines Agency (EMEA) [7]. The agency concluded that the benefits in the treatment of breast cancer with Ixabepilone did not outweigh its risks due to neuropathy.

Thus, an optimal delivery system for this class of anticancer drugs requires (a) solubilization of the drug, (b) accumulation of the drug at the tumour site due to enhanced permeation and retention (EPR-effect) [8, 9], and (c) reduction of drug-related adverse effects at non-tumour sites. Among several approaches, polymeric micelles offer great potential to meet these

demands [10-13] with regard to solubilization [1, 14-16], vehicle safety after administration [17, 18], and passive tumour targeting [19, 20].

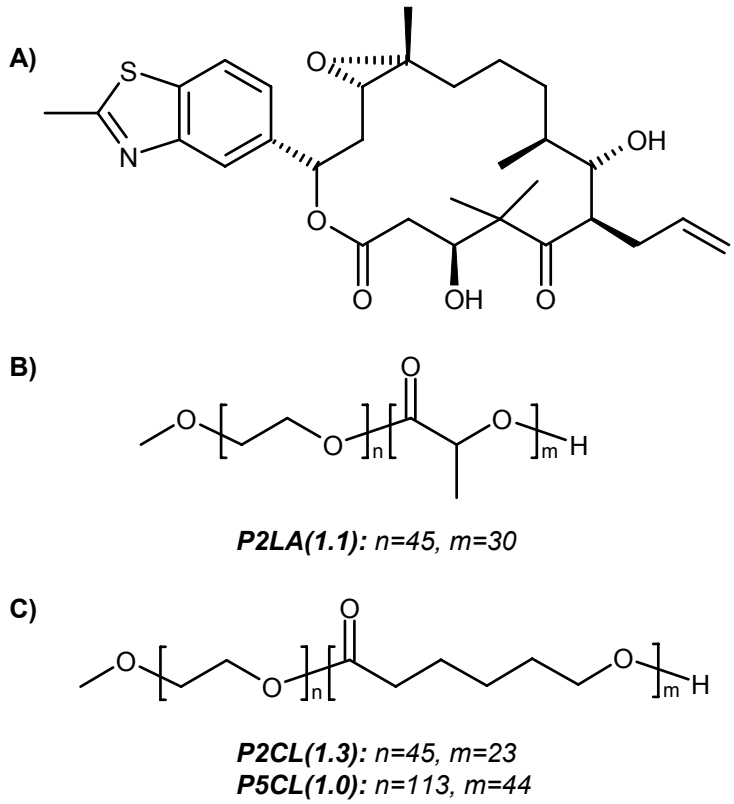

Figure 1: Structural formula of (A) Sagopilone, (B) PEG-*b*-PLA and (C) PEG-*b*-PCL

Until now, numerous publications have described various polymeric micellar systems with respect to solubilization and *in vivo* performance using different drugs and various animal models. For this reason, the results are difficult to compare. In our previous study, amphiphilic block copolymers composed of poly(ethylene glycol) (PEG) and a biodegradable polyester block of poly(lactide) (PEG-*b*-PLA) or poly(ε-caprolactone) (PEG-*b*-PCL) were investigated with regard to the solubilization of Sagopilone for parenteral delivery [21]. As a result, three polymers along with the appropriate method of preparation were selected as optimal solubilizing agents. The polymers used were: PEG_{2000}-*b*-PLA_{2200}, PEG_{2000}-*b*-PCL_{2600}, and PEG_{5000}-*b*-PCL_{5000} (Fig. 1) abbreviated as P2LA(1.1), P2CL(1.3), and P5CL(1.0), respectively, in which the number in parentheses details the hydrophobic/hydrophilic-ratio (w/w) of the block copolymer.

A critical point for formulation development is the stability of polymeric micelles [22]. They have to be stable both prior to clinical application and after intravenous (i.v.) administration since intact micelles are considered an important prerequisite for passive tumour targeting. The stability of polymeric micelles is often considered sufficient in general due to their low critical micelle concentration (CMC) values. However, this view disregards the kinetic stability, which may exhibit serious differences depending on the nature and state of the micellar core [1], especially important in the field of drug delivery. Thus, the selection of a core-forming block providing a high degree of kinetic stability in conjunction with a slow rate of disassembly is described as a strategy for the preparation of micelles that stay intact until reaching the tumour site [23] besides other approaches like core-crosslinking [24] or the chemical modification of the core-forming block [25, 26]. Examining a set of PEG-*b*-PCL polymers Liu et al. showed superior *in vitro* as well as *in vivo* stability of P5CL(1.0) [23]. A significant portion of the copolymer remained assembled as intact micelles even 24 h after administration of thermodynamically unstable micelles (2 mg/kg body weight), that would likely fall to concentrations below the CMC following distribution [23]. In the present work, a comparative study of the physicochemical stability of PCL- and PLA-containing micelles was performed assuming that PCL-containing cores exhibit a higher stability due to their nature (higher hydrophobicity) and state (semi-crystalline) compared to amorphous poly(D,L-lactide).

In addition, the applicability of polymeric micelles to clinical development requires stable formulations with sufficient shelf-life. Since the polymers used are sensitive to hydrolytic degradation, aqueous dispersions of the micelles are not suitable for ready-to-use formulations. This issue has been rarely addressed, especially for PEG-*b*-PCL micelles. With regard to the semi-crystalline nature of PCL, potential aggregation has to be taken into account during freeze-drying. With this in mind, the feasibility of lyophilization was studied

using different conditions to prevent crystallization of PCL and provide a storable formulation of PEG-*b*-PCL micelles. As an alternative to lyophilization, solid drug-loaded polymeric films of PEG-*b*-PLA were investigated as a novel approach for stabilizing parenteral formulations.

Following the physicochemical and formulation studies, the *in vitro* as well as the preclinical *in vivo* toxicity were studied to determine the safety profile of the carriers and the maximum tolerated dose (MTD) of the drug-loaded micelles for future *in vivo* tumour efficacy studies.

2. Materials and methods

2.1 Materials

Sagopilone was obtained from Bayer Schering Pharma AG (Berlin, Germany). The block copolymers poly(ethylene glycol)-*b*-poly(ε-caprolactone), namely PEG_{2000}-*b*-PCL_{2600} and PEG_{5000}-*b*-PCL_{5000} (abbr.: P2CL(1.3) and P5CL(1.0), respectively), and the poly(ethylene glycol)-*b*-poly(D,L-lactide) PEG_{2000}-*b*-PLA_{2200} (abbr.: P2LA(1.1)) were purchased from Polymer Source Inc. (Dorval, Canada). Pyrene, sucrose, trehalose, and mannitol were obtained from Merck KGaA (Darmstadt, Germany). Hydroxypropyl-*β*-cyclodextrin (abbr.: HPβCD) was purchased from Roquette (Lestrem, Fance). Polyvinylpyrrolidone (abbrev. PVP, Kollidon® 17PF, M_r = 7 000 – 11 000 g/mol) was purchased from BASF (Ludwigshafen, Germany). All other ingredients were obtained in analytical quality.

2.2 Micelle preparation and drug loading

Loading of Sagopilone within block copolymer micelles was done by the appropriate method of preparation as described previously [21]. In brief, sonication was used to prepare PEG-*b*-PCL micelles by simply weighing the polymer and Sagopilone, adding phosphate buffer (0.05 M, pH 7.4), and sonication for 10 min. Micelles composed of the PEG-*b*-PLA polymer P2LA(1.1) were prepared by a film formation method. The polymer and the drug were dissolved in acetonitrile, and the organic solvent was evaporated under reduced pressure at room temperature with subsequent drying at 0.1 mbar for 1 h. Micelle formation took place upon redispersion of the resulting film with phosphate buffer (0.05 M, pH 7.4) while shaking without additional heating or sonication. Unloaded micelles and blanks were prepared according to the same procedures in the absence of Sagopilone or the polymer, respectively. The resulting dispersions were sterilized by filtration through 0.22 µm syringe filters (Millex®-GV 0.22 µm, Millipore, USA).

2.3 Determination of drug content and micelle size

The final Sagopilone concentration present in the micelles was determined by reversed-phase high performance liquid chromatography (RP-HPLC) using two Chromolith® Performance RP-18e columns (100 x 4.6 mm, Merck, Germany) and an Agilent 1100 Series chromatography system (quaternary pump, auto-injector, column heater at 25 °C, and UV-detector) from Agilent Technologies (Santa Clara, USA). The method used has been described in detail previously [21].

The solubilization efficiency (SE) and the loading (% and mol/mol) of Sagopilone were calculated according to Equation (1), (2), and (3), respectively.

$$SE(\%) = \frac{mass\ \ of\ \ Sagopilone\ \ loaded\ \ in\ \ mg}{mass\ \ of\ \ Sagopilone\ \ fed\ \ in\ \ mg} \times 100\% \quad (1)$$

$$Loading(\%) \frac{m_{Sagopilone\ \ in\ \ mg}}{m_{polymer\ \ in\ \ mg}} \times 100\% \quad (2)$$

$$Loading(mol/mol) \frac{M_{Sagopilone\ \ in\ \ mol}}{M_{polymer\ \ in\ \ mol}} \quad (3)$$

The micelle sizes and size distributions were measured by Dynamic Light Scattering (DLS) at a scattering angle of 173° using a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK) with a temperature controller set at 25 °C. Autocorrelation functions were calculated and analysed using the DTS v5.1 software provided by Malvern. Measurements were done in triplicate with 15 to 20 runs each, and the calculated mean values of the hydrodynamic diameter (d_H) and the size distribution (PDI: polydispersity index) were used.

2.4 Determination of critical micelle concentration

The critical micelle concentration (CMC) of the amphiphilic block copolymers was determined by a fluorescent dye assay as reported previously [15]. In brief, excitation spectra of pyrene were obtained at a constant pyrene concentration of $6 \cdot 10^{-7}$ M in the presence of amphiphilic block copolymers at concentrations ranging from $1 \cdot 10^{-5}$ to 10 g/L using a Spex Fluorolog-2 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ). The ratio of the intensity at 338 to 333 nm was plotted against the concentration of the block copolymer on a logarithmic scale to determine the CMC.

2.5 Lyophilization

Different lyoprotectants were added to the micellar dispersions (c(polymer) = 20 g/L) at varying polymer-lyoprotectant weight ratios ranging from 1:0 to 1:20. Two millilitres of the dispersions were filled in 6R-glass vials fitted with 13-mm lyophilization stoppers. The samples were either frozen by immersion in liquid nitrogen (-196 °C) or at -45 °C over 4.5 h. They were lyophilized for 56.5 h at 0.09 – 0.01 mbar in a Genesis Super XL (VirTis, USA) with a condenser temperature of -60 °C. The resulting lyophilizates were redispersed by adding 2 mL water and subsequent shaking. When redispersion was complete, the drug content and the micellar characteristics were determined.

2.6 X-ray powder diffraction (XRPD)

Data collection was carried out in transmission mode on the automated STOE Powder Diffractometer STADI P using germanium-monochromatized CuKα_1-radiation (= 1.5406 Å). The X-ray tube with copper anode was operated at 40 kV and 30 mA. The 2Θ scans were performed using the small linear position sensitive detector with an angular resolution of 0.08° between 12°≤ 2Θ ≤ 23° (step width 0.1°). The samples were enclosed between two

polyacetate films. Data acquisition and evaluation was performed using Version 2.07 of the STOE WinX^{pow} software package.

2.7 *In vitro* cytotoxicity

For the *in vitro* cytotoxicity study, the human cervix carcinoma cell line HeLa/MaTu (Epo GmbH Berlin) was used. The cytotoxic activity was evaluated at five dilutions ranging from 10^{-6} to 200 µM Sagopilone using the crystal violet assay according to the standard method [27]. In brief, cells were harvested from exponential phase cultures growing in DMEM/HAMS F12 (Biochrom AG) medium supplemented with 2 mM L-Glutamine and 10 % fetal calf serum, counted and seeded onto 96-well plates with a density of 3000 cells per well. After a 24 h recovery at 37 °C in a humidified atmosphere with 5 % CO_2, the cells were incubated with 200 µL medium containing free Sagopilone or Sagopilone-loaded micelles. Each sample and concentration step was plated in octuplicate. Untreated (medium) and positive controls (Paclitaxel) were included as well. Following 4 days of exposure, the cells were treated with glutaraldehyde solution (10 %) for 15 min and washed three times. Afterwards, the viable cells were stained with crystal violet for 20 min, which was detected at 595 nm using a Tecan Sunrise Microplate reader after the addition of 10 % acetic acid. As a result, the inhibitory concentration IC_{50}, which is the concentration of Sagopilone producing 50 % inhibition of cell proliferation, was determined as a mean from three independent experiments.

2.8 *In vivo* toxicity

In vivo tolerability studies were performed in healthy female, adult NMRI: nu/nu mice (6-8 weeks of age, lack of mature T-lymphocytes, Taconic, 4623 Lille Skensved, Denmark). To determine the acute tolerability of the carriers, unloaded micellar dispersions were administered intravenously at a dose of 200 mg/kg to two animals per carrier type. Mice were monitored daily for acute reactions and variation in body weight over 1 week. In the absence

of toxic effects of the carriers, the maximum tolerated doses (MTD) of the drug-loaded dispersions were determined according to OECD guideline No. 425 for one drug. Therefore, groups of adult NMRI: nu/nu mice (female, 33.5 ± 2 g) with three animals per group received slow i.v. bolus injections of Sagopilone-loaded micellar dispersions (application volume: 0.2 mL per 20 g mouse body weight) at a dose of 6, 8, and 10 mg/kg. Mice were inspected daily for treatment-related toxicity. The body weight was determined daily, and changes in the body weight served as a parameter of toxicity. The MTD was defined as the dose where the median body weight loss does not exceed 15 % nor leads to remarkable changes in general behaviour or to death due to toxic side effects within 2 weeks after administration. Animals showing weight loss exceeding 20 % were sacrificed.

All animal experiments were conducted in accordance with Recommendations from the Declaration of Helsinki, the UKCCCR regulations for the welfare of animals and the German animal protection law, in addition to approval by local authorities.

2.9 Statistics

Data were recorded as mean ± standard deviation. All experiments were done at least in triplicate as specified in the results section. Means were analyzed for statistical significance using unpaired Student's *t*-test. Differences were considered significant at p-values < 0.05.

3. Results and discussion

3.1 Solubilization capacity and stability of micellar dispersions

Sagopilone was solubilized by polymeric micelles with the appropriate method of preparation for PLA- and PCL-containing block copolymers, using the film formation and sonication method, respectively. The aim was to reach the clinically relevant Sagopilone concentration of 1 g/L necessitating an 83-fold solubility enhancement compared to the solubility in water (12 µg/mL). Using a polymer concentration of 10 g/L resulted in comparable molar drug loading capacities of (0.57 ± 0) and (0.64 ± 0.01) mol Sagopilone per mol polymer for P2LA(1.1) and P2CL(1.3), respectively (Tab.1). This is distinctly lower compared to the loading capacity of the higher molecular weight polymer P5CL(1.0) at (1.29 ± 0.08) mol Sagopilone per mol polymer (Tab.1). Assuming that the drug was solubilized by the hydrophobic blocks within the micellar core, the corresponding loading capacities were almost equal at 14 % and 13 to 14 % (w/w hydrophobic block) for PLA and PCL, respectively. However, the block copolymer concentration was too low to reach the target concentration of Sagopilone.

Table 1: Solubilization of Sagopilone (*n*=3)

Sample	Polymer *c* in g/L	[a]Prep. Met.	Sagopilone		Loading of Sagopilone	
			c (mg/L)	SE (%)	(wt.%)	(mol/mol)
Blank	-	FF	56.7 ± 8.5	5.7	-	-
P2LA(1.1)	10	FF	739.8 ± 8.1	74.0	7.40 ± 0.08	0.571 ± 0.006
P2LA(1.1)	20	FF	997.2 ± 11.4	99.7	4.98 ± 0.24	0.385 ± 0.004
Blank	-	SO	8.3 ± 0.2	0.8	-	-
P2CL(1.3)	10	SO	761.3 ± 15.9	76.1	7.61 ± 0.16	0.644 ± 0.013
P2CL(1.3)	20	SO	996.8 ± 48.5	99.7	5.06 ± 0.16	0.422 ± 0.021
P5CL(1.0)	10	SO	703.1 ± 41.9	70.3	7.03 ± 0.42	1.293 ± 0.078
P5CL(1.0)	20	SO	1011.0 ± 31.8	101.1	4.99 ± 0.06	0.930 ± 0.029

[a] Preparation method (Prep. Met.), either film formation (FF) or sonication (SO)

Therefore, the amount of the polymers was increased to 20 g/L, resulting in micellar dispersions that contained Sagopilone at a satisfactory concentration of 1 g/L (Tab.1), equivalent to a Sagopilone loading of 5 % (w/w). The corresponding solubilization efficiency was 100 %, indicating the absence of any drug loss during the preparation for all polymers used.

The hydrodynamic diameters of the drug loaded micelles were (20.2 ± 0.1), (38.6 ± 0.9), and (68.4 ± 3.3) nm for P2LA(1.1), P2CL(1.3), and P5CL(1.0), respectively (Fig. 2A). PEG-*b*-PCL micelles exhibited a higher polydispersity (PDI: 0.13 – 0.21) compared to PEG-*b*-PLA micelles (PDI: 0.01 – 0.05), independent of the drug loading and polymer concentration (Fig. 2A).

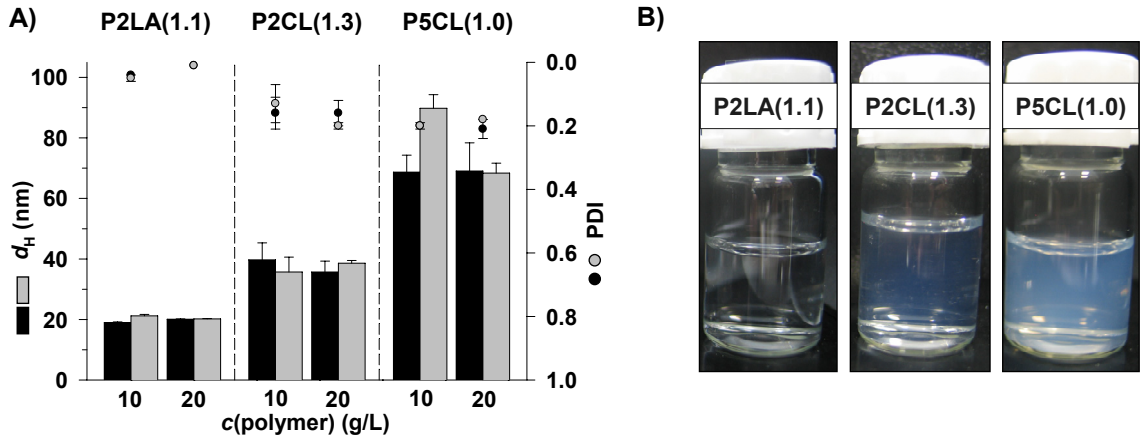

Figure 2: Size characteristics of micellar dispersions
(A) Particle Characteristics of unloaded (▬ /●) and Sagopilone-loaded (▭ /○) polymeric micelles as a function of the polymer concentration. (B) Appearance of Sagopilone-loaded micellar dispersions at a polymer concentration of 20 g/L. (*n*=3)

As previously shown in a cryoTEM study [21], the PEG-*b*-PCL micelles exhibited a uniform size distribution despite their higher PDI values, and aggregation was not observed. In contrast to the clear dispersions comprised of P2LA(1.1), unloaded as well as drug-loaded PCL-containing formulations showed a slight or intense white to pale blue opalescence (Fig. 2B), indicative of crystalline light scattering structures in the submicron size range. The particle sizes as well as the PDI did not differ between the unloaded and drug-loaded

micelles except for P5CL(1.0) at a concentration of 10 g/L (Fig. 2A). The exception revealed a significant increase in the micellar size ($p = 0.01$) while size distribution (PDI) did not change significantly ($p = 0.25$). This phenomenon may be due to the formation of a small amount of drug nanocrystals with diameters less than 0.22 µm. However, they may only account for a marginal proportion of the total number of particles, since they have not been detected as a single size population at DLS, and the corresponding blank samples exhibited negligible Sagopilone concentrations (Tab. 1).

The dispersions were stored at room temperature and their remaining drug content was determined after 24 h to assess their stability. As shown in Figure 3A, all dispersions were stable at a polymer concentration of 20 g/L while precipitation of the drug occurred at a P2LA(1.1)-concentration of 10 g/L. Thus, a further requirement for clinical development or processing, namely the stability of the dispersions for a specific time period, was met. The previously described 'supersaturation' effect of P2LA(1.1)-dispersions prepared by a film formation method was not observed at the higher polymer concentration. The lower Sagopilone loading (5 wt.%, Tab. 1) did not exceed the loading capacity of the P2LA(1.1)-micelles, circumventing a subsequent precipitation of excessive Sagopilone, and (97.2 ± 1.3) % of the drug still remained solubilized after 24 h (Fig. 3A). In addition, the time dependent behaviour of the P2LA(1.1)-micelles was monitored by DLS (Fig. 3B). The "supersaturated" dispersions revealed a slightly ascending PDI during the first 9 h (PDI: 0.05 – 0.11) with a subsequent sharp increase from 0.11 to 0.20 whereas the polydispersity of the micelles comprising a higher polymer concentration of 20 g/L did not change for at least 48 h (Fig. 3B). This points to the importance of determining the drug content at multiple time points in addition to a single measurement after preparation. The latter often leads to a misinterpretation of the micellar loading capacity especially if the film formation method is used due to the 'supersaturation' effect.

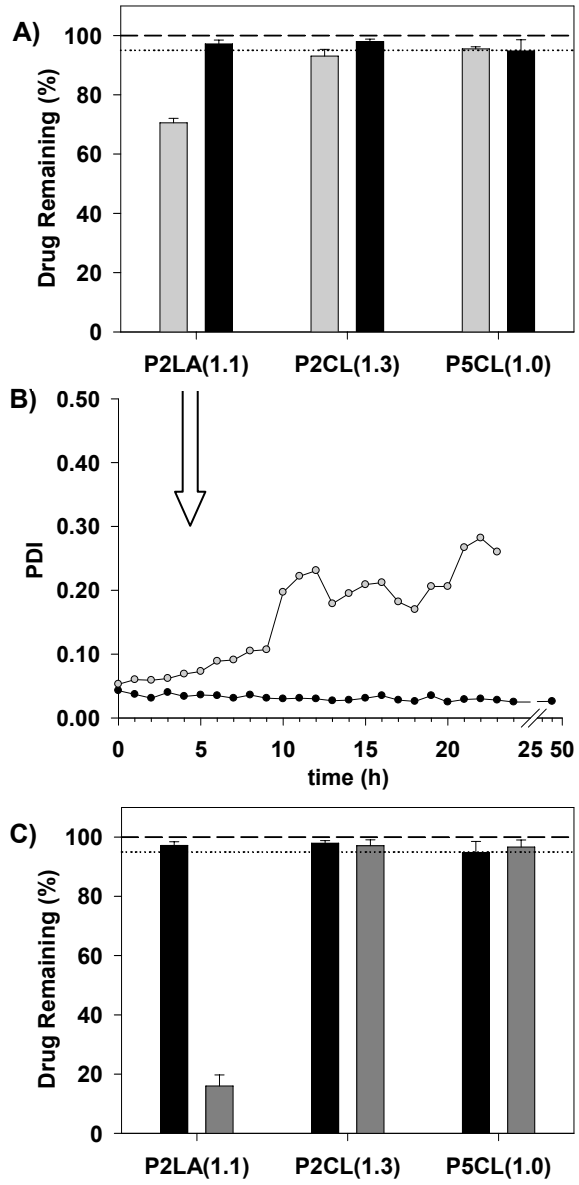

Figure 3: Stability of micellar dispersions
24h-Stability of Sagopilone-containing micellar dispersions (target conc. 1 g/L) at room temperature. (A) Remaining drug content after 24 h at a polymer concentration of 10 g/L (▭) and 20 g/L (▬) in phosphate buffer (0.05M, pH 7.4). (B) Absence of the 'supersaturation' effect of P2LA(1.1) at c(polymer) of 20 g/L (●) compared to 10 g/L (○) shown by a 24-h DLS measurement. (C) Remaining drug content after 24 h in dispersions comprising phosphate buffer (0.05M, pH 7.4) (▬) and phosphate buffer saline (0.05M, pH 7.4) (▬) at a polymer concentration of 20 g/L. (*n*=3)

The previously described dispersions were prepared in phosphate buffer (0.05 M, pH 7.4) as a dispersion medium. The higher concentration of sodium chloride present in the phosphate buffer saline (0.05 M, pH 7.4) remarkably decreased the stability of P2LA(1.1)-micelles as drug content dropped to (16.0 ± 3.8) % after 24 h (Fig. 3C). Conversely, the stability of the

PEG-*b*-PCL micelles was not affected after 24 h. Thus, phosphate buffer (0.05 M, pH 7.4) was used for further investigations.

3.2 Critical micelle concentration and stability upon dilution

Using a fluorescent probe technique, the critical micelle concentration (CMC) was determined as a thermodynamic parameter characterizing the micelles' stability during dissolution.

Table 2: CMC-Values (determined at 25 °C, *n=3*)

Polymer	CMC (µg/mL)	CMC (10^{-6} M)	ΔG^o (kJ/mol)
P2LA (1.1)	7.3 ± 1.9	1.7 ± 0.5	-32.9 ± 0.7
P2CL (1.3)	6.7 ± 2.7	1.5 ± 0.6	-33.5 ± 1.1
P5CL (1.0)	5.3 ± 3.2	0.5 ± 0.3	-36.1 ± 1.4

The three polymers tested exhibited a very low CMC on an order of magnitude of 10^{-7} to 10^{-6} M as shown in Table 2. Furthermore, the free energy ($\Delta G°$) of the micelle formation process was calculated according to Equation (4), where the CMC is expressed in units of mole fraction, R is the gas constant, and T is the absolute temperature of the system [28].

$$\Delta G^{\circ} = RT \ln(CMC) \tag{4}$$

The obtained $\Delta G°$ values were negative, independent from the polymers used, indicating a self-assembly process. Thus, the spherical nanoparticles detected at DLS (Fig. 2A) and visualized by cryoTEM [21] were proven to be thermodynamically stable, self-assembled micelles. Based on their CMC values, the micellar dispersions (c(polymer) = 20 g/L) of P2LA(1.1), P2CL(1.3), and P5CL(1.0) may be diluted by a factor of 2740, 2990, and 3780, respectively, to fall below the CMC. As previously stated, the micelles are not necessarily destroyed after dilution below the CMC, depending on their kinetic stability [1, 20]. By definition, unimers exist in equilibrium with polymeric micelles at concentrations above the CMC. The rate of the exchange of polymer unimers between the micelles as well as the dissociation defines the kinetic stability. It may occur rapidly, gradually or not at all,

depending on the state of the core (liquid-like, glassy or crystalline), whereas the latter are known as 'frozen' micelles [1].

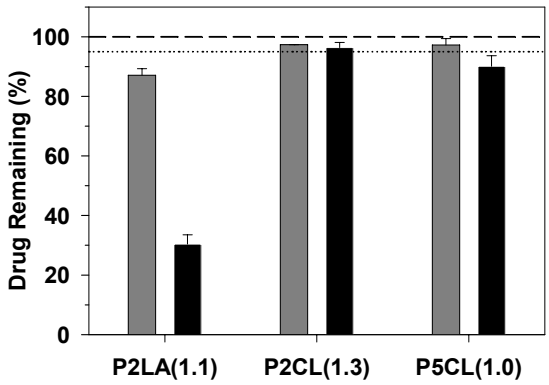

Figure 4: Stability upon dilution
Stability demonstrated by the remaining drug content of the dilutions after 24 h stored at 4 °C (▭) and 37 °C (▬). Dilution of micellar dispersions (1 g/L Sagopilone; 20 g/L polymer) in phosphate buffer (0.05 M, pH 7.4) with normal saline (0.9 %) in a ratio of 1:10 (v/v). (*n*=3)

To define the kinetic stability at concentrations above the CMC, dilution experiments were performed by mixing the micellar dispersions (1 g/L Sagopilone, 20 g/L polymer) with normal saline (0.9 %) in a ratio of 1:10. Subsequently, the dilutions were stored at 4 °C and 37 °C for 24 h and the remaining drug content was determined thereafter (Fig. 4). In contrast to P2LA(1.1), the PEG-*b*-PCL micelles exhibited a high stability upon dilution. (97.2 ± 2.2) % and (97.3 ± 0) % of the drug content remained solubilized after 24 h at 4 °C using P5CL(1.0) and P2CL(1.3), respectively, in contrast to (87.1 ± 2.2) % for P2LA(1.1). The superior stability of the PCL-containing micelles was even more obvious at 37 °C. At this temperature, more than 70 % of the initially solubilized drug substance precipitated in micellar dispersions of P2LA(1.1) whereas (96.4 ± 1.7) % and (90.1 ± 3.6) % of the drug remained solubilized in P2CL(1.3) and P5CL(1.0), respectively.

By summarizing the dilution (Fig. 4) and stability experiments in the presence of sodium chloride (Fig. 2C), a remarkable difference in the stability of PLA- and PCL-containing micelles was observed. P2LA(1.1) and P2CL(1.3) exhibited similar values in their CMC, but P2LA(1.1)-micelles were less stable, both in phosphate buffer saline and after dilution

with normal saline despite the fact that the polymer concentration was beyond the CMC for all polymers tested. Hence, PEG-*b*-PCL micelles exhibited a superior kinetic stability despite the similar thermodynamic stability parameters for the PLA- and PCL-containing polymers. This is most likely due to the semi-crystalline core of the PEG-*b*-PCL micelles. As the amorphous portion of PCL solubilizes Sagopilone [21], the partial crystallinity leads to "frozen" micelles with very slow exchange rates between unimers and micelles and consequently an increased kinetic stability. In contrast, P2LA(1.1)-micelles comprise glassy cores at ambient temperature, which are kinetically less stable due to the absence of crystalline structures and subsequent unhampered exchange between unimers and polymeric micelles. Consequently, these systems are more susceptible to having their equilibrium affected by a higher amount of sodium chloride towards a destabilized state. The kinetic stability was additionally decreased at 37 °C since the glass transition temperature of PLA (approximately 38 °C) was reached, resulting in (a) an increased fluidity and exchange rate of the liquid-like core and (b) a shift towards free unimers since the polymer is more soluble at higher temperatures. The observed superior stability of P5CL(1.0) at concentrations above the CMC coincides with the described superior kinetic stability and a slow rate of disassembly of P5CL(1.0) at concentrations below the CMC [23]. Thus, CMC values can be used to provide evidence of a self-assembly process, but all facets of stability have to be considered.

3.3 Formulation development

Using different types and amounts of lyoprotective agents, both blank and Sagopilone-containing dispersions (1 g/L Sagopilone, 20 g/L polymer) were freeze-dried as described in Table 3. Lyophilized P2LA(1.1)-micelles were completely redispersed even without the addition of lyoprotective agents. As shown in Figure 5a, the micelles did not change in size after lyophilization (prior: 20 nm; after: 19 – 22 nm) and this behaviour was not altered in the presence of Sagopilone (prior: 21 nm; after: 21 – 24 nm).

Table 3: Redispersion behaviour after lyophilization (*n*=3)

Lyo-protectant	Ratio[a]	Freezing[b]	P2LA(1.1)[c]		P2CL(1.3)[c]		P5CL(1.0)[c]	
			No drug	Sago. 1 g/L	No drug	Sago. 1 g/L	No drug	Sago. 1 g/L
-	-		✓	✓	> 1µm	> 1µm	> 1µm	> 1µm
Mannitol	1:1		✓	✓	> 1µm	> 1µm	> 1µm	> 1µm
Sucrose	1:1		✓	✓	> 1µm	> 1µm	> 1µm	> 1µm
Sucrose	1:20	x	-	-	> 1µm	> 1µm	✓	> 1µm
Trehalose	1:5	x	-	-	> 1µm	> 1µm	> 1µm	> 1µm
Trehalose	1:20	x	-	-	> 1µm	> 1µm	> 1µm	> 1µm
PVP	1:5	x	-	-	> 1µm	> 1µm	> 1µm	> 1µm
PVP	1:20	x	-	-	✓	✓	✓	✓
HPβCD	1:20	x	-	-	✓	✓	✓	✓

[a] Polymer-lyoprotectant ratio (w/w)
[b] Freezing by immersion with liquid nitrogen (-196 °C) prior to lyophilization
[c] *c*(polymer) = 20 g/L
✓ Complete redispersion possible

For PEG-*b*-PCL micelles, a lyoprotective agent such as polyvinylpyrrolidone (PVP) or Hydroxypropyl-*β*-cyclodextrin (HPβCD) was necessary to obtain complete redispersion of the unloaded as well as drug-loaded samples. In addition, the dispersions were frozen by immersion in liquid nitrogen prior to lyophilization to avoid potential sedimentation and aggregation of the micelles. Interestingly, redispersion of lyophilizates containing P5CL(1.0) and a sufficient amount of sucrose were completely redispersible, but their drug-loaded counterparts were not.

The addition of PVP or HPβCD to the micelle dispersions prior to lyophilization led to an alteration in the sizes measured by DLS as seen in Figure 5b and c. This is very likely to be due to a measurement artefact by the altered composition and viscosity of the dispersion medium affecting the micelle mobility, which in turn presents the basis for size calculations at DLS. A change in the micelle morphology is very unlikely since the polydispersity and size distributions did not change and precipitation phenomena were not detected. The altered sizes were used as comparative values in the assessment of the redispersion behaviour of the

corresponding lyophilizates. Micelles comprised of P5CL(1.0) revealed similar sizes prior to and after lyophilization (Fig. 5c), independent of the lyoprotective agent and drug loading. On the other hand, lyophilizates comprised of P2CL(1.3) and HPβCD exhibited a remarkable increase in their micellar size despite complete redispersion. This was also observed for the drug-loaded micelles of P2CL(1.3) using PVP as a lyoprotectant. Irrespective of the polymer used, the drug content did not change after redispersion (data not shown).

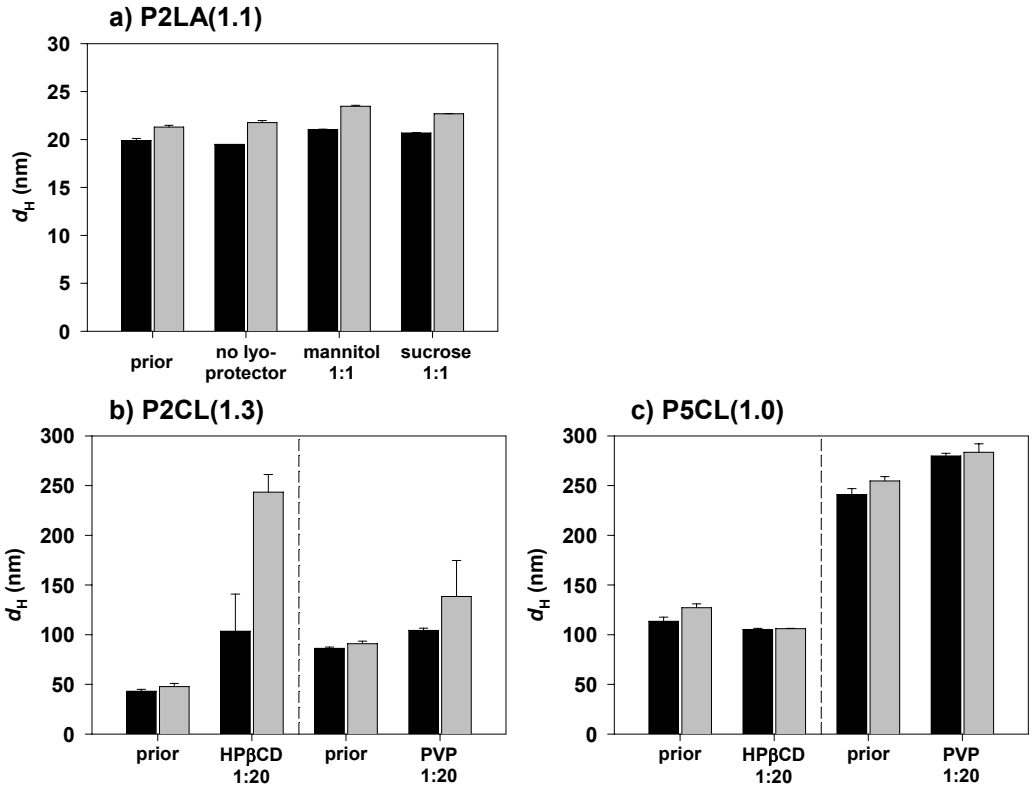

Figure 5: Lyophilization
Hydrodynamic diameter (d_H) of unloaded (▬) and Sagopilone-loaded (▭) micelles, prior to lyophilization and after redispersion of the lyophilizates, as a function of the lyoprotective agent added and its amount (polymer-lyoprotectant ratio (w/w)). The particle sizes of (b) and (c) prior to freeze-drying were measured in the presence of HPβCD or PVP. (n=3)

Overall, lyophilization was feasible using the specified conditions and can be considered a viable option for parenteral formulations. The results of P2LA(1.1) are in good agreement with the described freeze-drying of Paclitaxel-loaded PEG-*b*-PLA micelles [26]. To date, the preparation of freeze-dried PEG-*b*-PCL micelles for storage and redispersion later on has

not been addressed extensively. There are only few reports addressing refreezing that present storable forms for PEG-*b*-PCL micelles [29].

Application of the same conditions used for PEG-*b*-PLA was not feasible for the lyophilization of PEG-*b*-PCL micelles. Again, the different nature of the hydrophobic blocks had a great impact. The amorphous structure of PLA itself was superior to semi-crystalline PCL with regard to the redispersion behaviour and the need for lyoprotection. Embedding of the latter within a dense matrix of PVP or HPβCD preserved the micellar structures resulting in complete redispersion. Despite the large molecular weight of PEG and PCL, P5CL(1.0) was superior to P2CL(1.3) with regard to aggregation behaviour. This may be due to the higher PEG content of P2CL(1.3) (0.22 mmol PEG per g polymer) compared to P5CL(1.0) (0.1 mmol PEG per g polymer). Previous freeze-drying studies of PEG-*b*-PLA nanoparticles [30] showed a clear relationship between the amount of grafted PEG and the degree of aggregation because of the formation of stable PEG crystallized bridges between neighbouring particles. This may also be due to an efficient shielding of the PCL core by longer PEG chains and subsequent prevention of the formation of PCL-aggregates. Optimization of the procedure as well as an elaborative elucidation of the change in the particle sizes will be the focus of future studies.

As an alternative to freeze-drying, solid polymeric films for redispersion prior to clinical application were investigated. The films were composed of P2LA(1.1) and Sagopilone at a drug loading of 5 % (w/w) to avoid ‘supersaturation’ phenomena after redispersion.

At a storage temperature of 6 °C, no crystallization of Sagopilone was observed as shown in the XRPD pattern (Fig. 7D, blue diffractogram). The observed peak at 19 2Θ corresponded to the crystalline PEG phase of the films, which was present in the blank films of P2LA(1.1) as well (Fig. 7C). Redispersion by the use of simple shaking by hand was easy and complete resulting in micellar dispersions with a mean drug content of (96 ± 1) % (Fig. 6, blue bars).

Precipitation did not occur afterwards, and the dispersions still exhibited (97 ± 2) % in drug content after 12-h storage at 2 to 8 °C (Fig. 6).

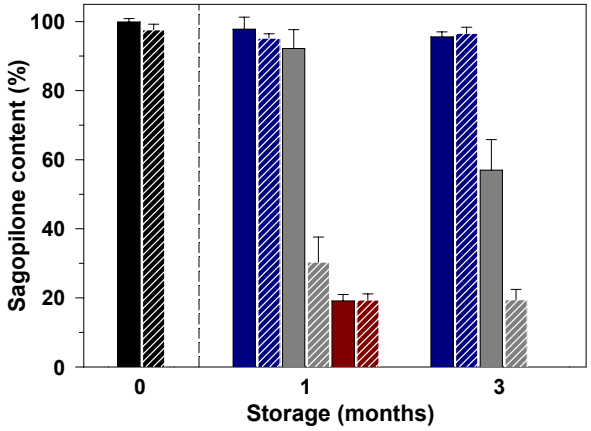

Figure 6: Film stability
Stability of Sagopilone-loaded P2LA(1.1) films as a function of the storage time and temperature (6 °C: ▬, 25 °C: ▬, 40 °C: ▬) represented by the mean Sagopilone content of the micellar dispersions after redispersion (solid bars) and subsequent storage at 2 to 8 °C for 12 h (hatched bars). (*n*=6)

Thus, polymeric films of P2LA(1.1) comprising a Sagopilone content of 5 % have been demonstrated as a novel solid formulation stable for at least 3 months of storage at 6 °C. Adjuvant excipients were not required to maintain the capability to form micellar dispersions in an aqueous medium. This is in contrast to previous publications [31] describing the necessity of the addition of PEG to obtain storable liquid formulations because redispersion had failed without it.

The storage temperature has been identified as a key factor in stability. Although complete film redispersion was still possible after 1 month of storage at 25 °C, the resulting dispersions were not stable, and more than 60 % of the initially solubilized drug precipitated within the following 12 h (Fig. 6, grey bars). This precipitation phenomenon was very likely to be due to an enhanced degradation of PLA resulting in shorter PLA blocks with a subsequent decreased drug loading capacity of the corresponding micelles. After storage for another 2 months, Sagopilone crystallization occurred (Fig. 7D), impeding complete redispersion.

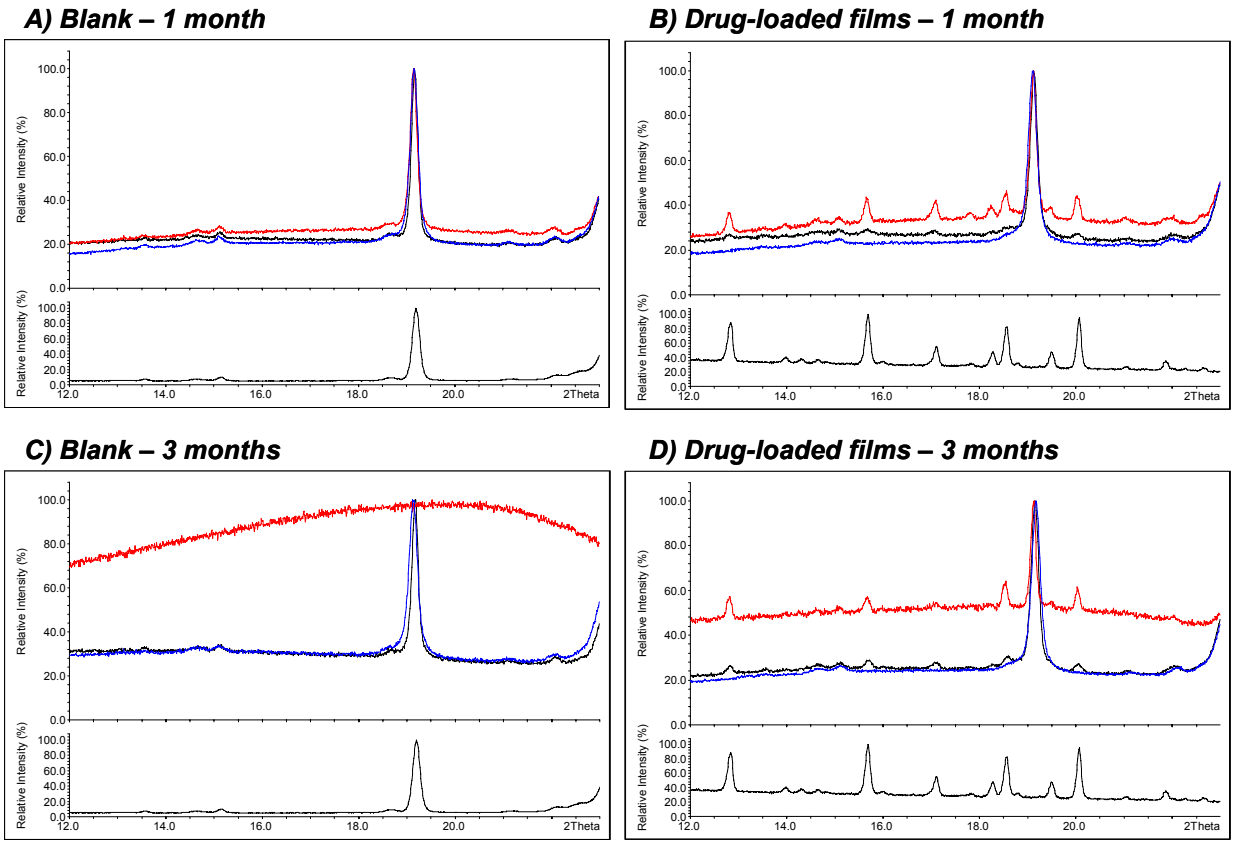

Figure 7: XRPD pattern of polymeric films
XRPD pattern of blank and Sagopilone-loaded P2LA(1.1) films stored at 6 °C (—), 25 °C (—), and 40 °C (—) after 1 and 3 months. As a comparison, the XRPD pattern of PEG (1500 Da) and Sagopilone is displayed below the blank and drug-loaded diffractograms, respectively.

At 40 °C, crystalline Sagopilone patterns were detected even after 1 month (Fig. 7B, red diffractogram) along with incomplete redispersion (Fig. 6, red bars). Crystallization was promoted since the glass transition temperature of PLA (approximately 38 °C) was reached increasing the fluidity and coalescence of drug-loaded phases. Additionally, the blank matrix changed to a liquid state after 3 months with complete disappearance of the PEG peak (Fig. 7C). This is a clear indication of a high degree of degradation of PLA resulting in lactic acid, which is a colourless to slightly yellow syrupy liquid [32] dissolving the residual crystalline PEG. Thus, the storage temperature has to be maintained at 6 °C to prevent matrix degradation and drug crystallization.

3.4 *In vitro* cytotoxicity

The *in vitro* cytotoxic activity of micellar and free Sagopilone was investigated in a proliferation assay using a human cervix carcinoma cell line (HeLa/MaTu). The activity is given as a concentration that inhibits cell proliferation by 50 % (IC_{50}). For comparison, Sagopilone solutions containing HPβCD (drug-excipient-ratio of 1:200) or ethanol (0.1 %) were tested. Paclitaxel (ethanol solution) was used as an internal standard to verify the reliability of the results obtained with the given *in vitro* test system. Sagopilone's high antiproliferative activity ($IC_{50} < 1$ nM) compared to Paclitaxel ($IC_{50} > 1$ nM) [3] was maintained. The IC_{50}-values of the Sagopilone samples tested were in a range of 0.14 to 0.26 nM (Tab. 4), showing no significant differences between the respective formulations and the ethanol solution ($p > 0.05$). Corresponding blanks did not show any cytotoxicity within the effective concentration range of Sagopilone.

Table 4: *In vitro* cytotoxicity of different Sagopilone-loaded polymeric micelles compared to free Sagopilone and Paclitaxel in human HeLa/MaTu cells (*n*=3x8)

Vehicle	Sagopilone	Paclitaxel	IC_{50} (nM)
P2LA(1.1)	x		0.21 ± 0.03
P2CL(1.3)	x		0.17 ± 0.04
P5CL(1.0)	x		0.14 ± 0.05
HPβCD	x		0.19 ± 0.08
ethanol/ medium	x		0.26 ± 0.07
		x	1.17 ± 0.21

Thus, micellar Sagopilone was proven to be still highly active on a cellular level. Encapsulation in polymeric micelles did not prevent drug internalization into the cells, a mandatory premise for Sagopilone's activity. Although *in vitro* experiments do not allow a prediction of the *in vivo* behaviour of nanocarriers, they are considered as a necessary step towards *in vivo* testing.

3.5 *In vivo* toxicity

Following the *in vitro* investigations, the toxicity of the polymeric micelles as well as the maximum tolerated dose (MTD) of the drug-loaded micelles were determined *in vivo*.

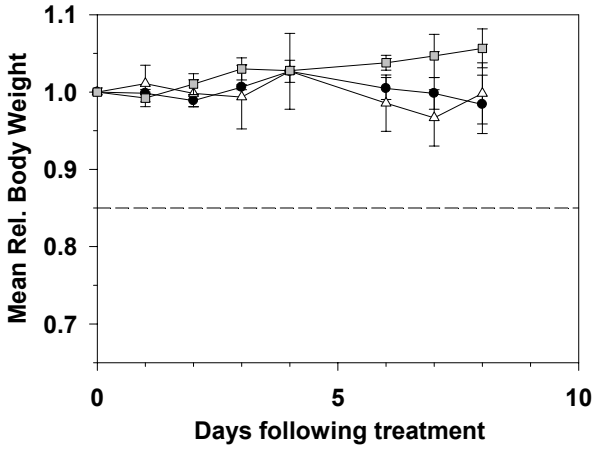

Figure 8: Safety of vehicles
Mean relative body weight after i.v. injection of unloaded polymeric micelles (c = 20 g/L) comprising P2LA(1.1) (●), P2CL(1.3) (△), and P5CL(1.0) (▫) at a dose of 200 mg/kg (Mean ± Min/Max). (*n*=2)

The polymeric micelles (*c*(polymer) = 20 g/L) revealed no acute toxicity or signs of hypersensitivity reactions after i.v. application to non-tumour-bearing nude mice at a polymer dose of 200 mg/kg (Fig. 8). The mice' body weight did not change during 1 week. Thus, the PEG-*b*-PLA as well as the PEG-*b*-PCL vehicles were proven to be safe, which is an important requirement for the subsequent dose-finding study.

For this study, drug-loaded micelles (drug-polymer-ratio 1:20) were administered at increasing doses as shown in Figure 9. The MTD was determined to be 6 mg/kg independent of the polymer used. At the higher dose of 8 mg/kg, the animals died or had to be sacrificed due to a weight loss exceeding 20 % with the exception of the group receiving P2CL(1.3) micelles, in which only 1 of 3 mice died (Fig. 9*). The MTD of micellar Sagopilone was decreased compared to a cyclodextrin-based formulation of Sagopilone (MTD = 10 mg/kg, data not shown).

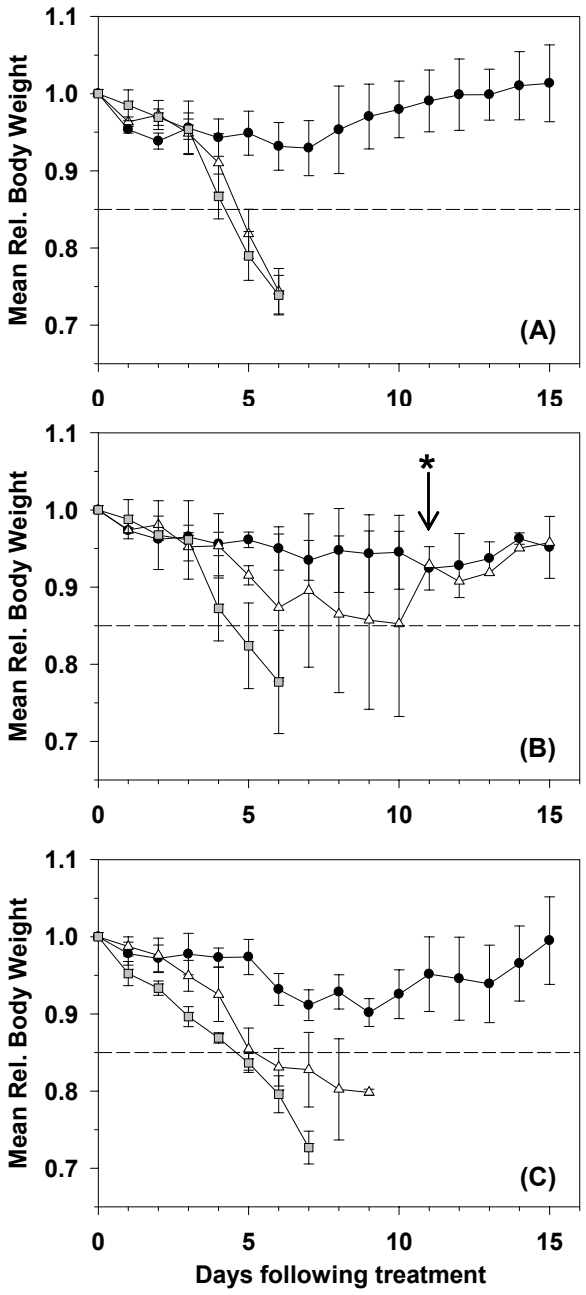

Figure 9: Determination of maximum tolerated dose (MTD)
Mean relative body weight after i.v. application of Sagopilone-loaded polymeric micelles of (A) P2LA(1.1), (B) P2CL(1.3), and (C) P5CL(1.0) at Sagopilone doses of 6 (●), 8 (△), and 10 (□) mg/kg and polymer doses of 120, 160, and 200 mg/kg, respectively.
(*n*=3, except * 8 mg/kg: one animal has died)

This may be due to an enhanced effective dose and biodistribution of Sagopilone accompanied by an enhanced toxicity. Furthermore, the degradation of Sagopilone by serum esterases may be hampered due to its encapsulation within polymeric micelles resulting in higher plasma levels of the effective drug after i.v. administration.

Comparatively, the MTD of micellar Paclitaxel (Genexol®-PM, 60 mg/kg) was threefold higher than that of conventional Paclitaxel (Taxol®, 20 mg/kg) using a polymer similar to P2LA(1.1) in a nude mouse model [17]. Taxol® uses Cremophor®EL, which is known to cause severe side effects limiting the dose of Paclitaxel. Since the polymeric micelles (Genexol®-PM) did not exhibit any hypersensitivity reactions dosing of Paclitaxel could be increased, resulting in a higher MTD. As shown in the present study, the comparison with a cyclodextrin-based formulation revealed a decreased MTD suggesting an improved stability after i.v. administration and an enhanced lysosomal internalization of micellar Sagopilone into cells. The recommended dose was identified to be 6 mg/kg. The described micelles are believed to show an increased *in vivo* antitumour efficacy due to a decreased degradation of the drug and an enhanced permeation and retention of micellar Sagopilone in solid tumours.

4. Conclusion

Polymeric micellar dispersions of P2LA(1.1), P2CL(1.3), and P5CL(1.0) were successfully used to solubilize Sagopilone at a clinically relevant concentration of 1 g/L, requiring a drug-polymer-ratio as low as 1:20 (w/w). The resulting micellar dispersions exhibited sufficient stability independent of the polymer type and composition. Precipitation phenomena due to a 'supersaturation' following particular preparation methods such as the film formation must not be mistaken with instability of the micelles and could be circumvented by simply adjusting the drug loading to values not exceeding the loading capacity as seen for P2LA(1.1). The demonstrated lyophilization of these dispersions, shown for the first time with PEG-*b*-PCL, promotes the further development of this kind of block copolymers as solubilizing agents. A novel solid formulation concept, namely drug-loaded polymeric films for redispersion prior to parenteral administration, has been demonstrated to be feasible for PEG-*b*-PLA even without additional excipients, in contrast to previous studies. This approach could be of considerable commercial interest due to the prevention of complex and costly lyophilization and the absence of water during the production process. Altogether, amphiphilic block copolymers should lend themselves well to become standard solubilizing excipients. The PEG-*b*-PCL micelles revealed a distinctly higher kinetic stability both in the presence of isotonic additives and upon dilution. For this reason, they may demonstrate superior stability after i.v. application and passive tumour targeting. The *in vivo* evaluations revealed no carrier-related side effects and decreased MTDs of Sagopilone-loaded polymeric micelles independent of the polymer used and despite their different kinetic stability. The polymeric micelles are believed to be superior in terms of delivering higher amounts of drugs to tumour tissue despite lower dosing due to an increased stability of the encapsulated drug against blood esterases as well as an enhanced permeation and retention of the delivery system in solid tumours. To provide evidence of this effect, tumour efficacy studies are needed, preferably using tumour models that represent the *in vivo* situation of leaky vessels.

References

1. G.S. Kwon. Polymeric micelles for delivery of poorly water-soluble compounds. *Crit. Rev. Ther. Drug Carr. Syst.* 20 (2003) 357-403.

2. A.J. ten Tije, J. Verweij, W.J. Loos, A. Sparreboom. Pharmacological effects of formulation vehicles: implications for cancer chemotherapy. *Clin. Pharmacokinet.* 42 (2003) 665-685.

3. U. Klar, B. Buchmann, W. Schwede, W. Skuballa, J. Hoffmann, R.B. Lichtner. Total synthesis and antitumor activity of ZK-EPO: the first fully synthetic epothilones in clinical development. *Angew. Chem. Int. Edit.* 45 (2006) 7942-7948.

4. J. Hoffmann, I. Vitale, B. Buchmann, L. Galluzzi, W. Schwede, L. Senovilla, W. Skuballa, S. Vivet, R.B. Lichtner, J.M. Vicencio, T. Panaretakis, G. Siemeister, H. Lage, L. Nanty, S. Hammer, K. Mittelstaedt, S. Winsel, J. Eschenbrenner, M. Castedo, C. Demarche, U. Klar, G. Kroemer. Improved cellular pharmacokinetics and pharmacodynamics underlie the wide anticancer activity of Sagopilone. *Cancer Res.* 68 (2008) 5301-5308.

5. H. Reichenbach, G. Höfle. Discovery and development of the epothilones: A novel class of antineoplastic drugs. *Drugs R. D.* 9 (2008) 1-10.

6. U.S. National Institutes of Health. Clinical Trials of Sagopilone. http://www.clinicaltrial.gov/ct2/results?term=Sagopilone (accessed 01/02/2010).

7. European Medicines Agency. Doc.Ref. EMEA/602569/2008 - Questions and answers on recommendation for the refusal of the marketing authorisation for Ixempra. http://www.emea.europa.eu/pdfs/human/opinion/IxempraQ&A_60256908en.pdf (accessed 10/09/2009).

8. Y. Matsumura, H. Maeda. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* 46 (1986) 6387-6392.

9. H. Maeda, G.Y. Bharate, J. Daruwalla. Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. *Eur. J. Pharm. Biopharm.* 71 (2009) 409-419.

10. Y. Bae, K. Kataoka. Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. *Adv. Drug Deliv. Rev.* 61 (2009) 768-784.

11. V.P. Torchilin. Micellar nanocarriers: pharmaceutical perspectives. *Pharm. Res.* 24 (2007) 1-16.

12. N. Nishiyama, K. Kataoka. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. *Pharmacol. Ther.* 112 (2006) 630-648.

13. H.M. Aliabadi, A. Lavasanifar. Polymeric micelles for drug delivery *Expert Opin. Drug Deliv.* 3 (2006) 139-162.

14. R.T. Liggins, H.M. Burt. Polyether-polyester diblock copolymers for the preparation of paclitaxel loaded polymeric micelle formulations. *Adv. Drug Deliv. Rev.* 54 (2002) 191-202.

15. H.M. Aliabadi, A. Mahmud, A.D. Sharifabadi, A. Lavasanifar. Micelles of methoxy poly(ethylene oxide)-b-poly(ε-caprolactone) as vehicles for the solubilization and controlled delivery of cyclosporine A. *J. Control. Release* 104 (2005) 301-311.

16. O. Molavi, Z. Ma, A. Mahmud, A. Alshamsan, J. Samuel, R. Lai, G.S. Kwon, A. Lavasanifar. Polymeric micelles for the solubilization and delivery of STAT3 inhibitor cucurbitacins in solid tumors. *Int. J. Pharm.* 347 (2008) 118-127.

17. S.C. Kim, D.W. Kim, Y.H. Shim, J.S. Bang, H.S. Oh, S.W. Kim, M.H. Seo. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *J. Control. Release* 72 (2001) 191-202.

18. T. Kawaguchi, T. Honda, M. Nishihara, T. Yamamoto, M. Yokoyama. Histological study on side effects and tumor targeting of a block copolymer micelle on rats. *J. Control. Release* 136 (2009) 240-246.

19. H.M. Aliabadi, M. Shahin, D.R. Brocks, A. Lavasanifar. Disposition of drugs in block copolymer micelle delivery systems: from discovery to recovery. *Clin. Pharmacokinet.* 47 (2008) 619-634.

20. A.S. Mikhail, C. Allen. Block copolymer micelles for delivery of cancer therapy: Transport at the whole body, tissue and cellular levels. *J. Control. Release* 138 (2009) 214-223.

21. A. Richter, C. Olbrich, M. Krause, T. Kissel. Solubilization of Sagopilone, a poorly water-soluble anticancer drug, using polymeric micelles for parenteral delivery. *Int. J. Pharm.* 389 (2010) 244-253.

22. Y.H. Bae, H. Yin. Stability issues of polymeric micelles. *J. Control. Release* 131 (2008) 2-4.

23. J. Liu, F. Zeng, C. Allen. In vivo fate of unimers and micelles of a poly(ethylene glycol)-block-poly(caprolactone) copolymer in mice following intravenous administration. *Eur. J. Pharm. Biopharm.* 65 (2007) 309-319.

24. X. Shuai, T. Merdan, A.K. Schaper, F. Xi, T. Kissel. Core-cross-linked polymeric micelles as paclitaxel carriers. *Bioconjugate Chem.* 15 (2004) 441-448.

25. M.G. Carstens, P.H.J.L.F. de Jong, C.F. van Nostrum, J. Kemmink, R. Verrijk, L.G.J. de Leede, D.J.A. Crommelin, W.E. Hennink. The effect of core composition in biodegradable oligomeric micelles as taxane formulations. *Eur. J. Pharm. Biopharm.* 68 (2008) 596-606.

26. M.H. Seo, Y.W. Yi, J.W. Yu. Stable polymeric micelle-type drug composition and method for the preparation thereof. European Patent EP1282447B1 (2009).

27. W. Kueng, E. Silber, U. Eppenberger. Quantification of cells cultured on 96-well plates. *Anal. Biochem.* 182 (1989) 16-19.

28. R. Liu, N. Sadrzadeh, P.P. Constantinides. Micellization and Drug Solubility Enhancement. In: R.Liu (Ed.). Water-Insoluble Drug Formulation. Interpharm Press, Englewood (2000) 213-354.

29. S. Cai, K. Vijayan, D. Cheng, E.M. Lima, D.E. Discher. Micelles of different morphologies – advantages of worm-like filomicelles of PEO-PCL in paclitaxel delivery. *Pharm. Res.* 24 (2007) 2099-2109.

30. F. De Jaeghere, E. Allémann, J. Feijen, T. Kissel, E. Doelker, R. Gurny. Freeze-drying and lyopreservation of diblock and triblock poly(lactic acid)-poly(ethylene oxide) (PLA-PEO) copolymer nanoparticles. *Pharm. Dev. Technol.* 5 (2000) 473-483.

31. M.H. Seo, I.J. Choi. Polymeric composition for solubilizing poorly water soluble drugs and process for the preparation thereof. U.S. Patent US6616941B1 (2003).

32. Sigma-Aldrich. Safety Data Sheet: DL-Lactic Acid. http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do (accessed 04/09/2009).

CHAPTER 4

NON-IONIC DENDRITIC GLYCEROL-BASED AMPHIPHILES: NOVEL EXCIPIENTS FOR THE SOLUBILIZATION OF POORLY WATER-SOLUBLE ANTICANCER DRUG SAGOPILONE

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Abstract

The purpose of this study was to investigate dendritic glycerol-based amphiphiles as novel solubilizers using the poorly water-soluble anticancer drug Sagopilone. The effect of different core structures on the solubilization, formulation stability, and cytotoxicity using human umbilical vein endothelial cells (HUVECs) were investigated and compared to standard excipients. Structurally, all amphiphiles were composed of 2nd generation polyglycerol (PG[G2]) as the hydrophilic part and a single C_{18}-chain (PG[G2]-C_{18}), a C_{18}-chain coupled by a diaromatic spacer (PG[G2]-DiAr-C_{18}), a C_{18}-chain with a naphthyl or bisphenyl end group (PG[G2]-C_{18}-Naph/ -BiP), or two C_{18}-chains (PG[G2]-$(C_{18})_2$) as the hydrophobic part. They formed small (7-10 nm), monodisperse (PDI 0.04-0.20) micelles with the exception of PG[G2]-$(C_{18})_2$. The amphiphiles revealed a 2-3-fold higher solubilization of Sagopilone than Cremophor® ELP and polysorbate 80 independent of the core structure. PG[G2]-DiAr-C_{18} exhibited the highest solubilization capacity (56.7±1.3 mg/g) compared to Cremphor® ELP (18.5±0.1 mg/g). The micellar dispersions were stable in drug content over 3 days (≥ 97 %). In contrast to polysorbate 80, dilutions did not show any precipitation after 3 days at 37 °C (remaining drug content: > 95 %). They did not induce significant cytotoxicity at a concentration of 0.01 g/L after 24 h, and PG[G2]-C_{18}-Naph was the least cytotoxic structure after 72 h with values comparable to Cremophor® ELP and polysorbate 80. Overall, these amphiphiles possess superior solubilization properties compared to standard excipients used in parenteral formulations with an excellent formulation stability profile and comparable cytotoxicity.

1. Introduction

Solubilization currently represents one of the major challenges in drug development because approximately 40 % of the new compounds are poorly water-soluble [1]. This is of particular concern in the field of parenteral delivery, but also impacts oral delivery and drug development in general [2]. The range of approved excipients used as solubilizers in parenteral formulations is limited to a select few such as Cremophor® EL, polysorbate 80, poloxamers, or cyclodextrins [3, 4]. Apart from these limitations regarding formulation development, the problem of poor aqueous solubility also affects the early development process [5]. For this purpose formulations comprising multiple doses of drug exposure are required for toxicity testing and existing solubilization/ dissolution strategies frequently fail.

Furthermore, solubilizers currently used have been implicated in clinically important adverse effects such as peripheral neuropathy and hypersensitivity reactions (HSR) [6, 7]. One prominent example is Taxol®, a formulation of Paclitaxel in Cremophor® EL and ethanol, exhibiting potentially life-threatening HSRs in clinical practice [8]. Szebeni et al. suggested complement activation and subsequent histamine release by Cremophor® EL as an important contribution to these reactions [9]. Block copolymers like poloxamer 188 seem to trigger complement activation as well, and adverse responses after administration of poloxamer-based formulations are very likely to be secondary to this effect [10]. Even polyethylene glycol (PEG), which is generally regarded as safe and biocompatible, was found to trigger complement activation in a concentration- and molecular-weight-dependent manner [11]. These findings may provide a plausible explanation for the unexplained anaphylaxis in species that have received medicines containing high levels of PEG as a solubilizer or carrier [11]. In addition to the potentially harmful intrinsic effects, solubilizers may modulate the disposition profiles of various drugs after parenteral administration, especially known from cancer therapy [6]. For example, the formulation of

Paclitaxel and Docetaxel in Cremophor® EL- and polysorbate 80-micelles, respectively, is accompanied by an increased systemic drug exposure, a simultaneously decreased clearance, and a reduced cellular drug uptake [6].

Besides causing these undesirable effects in humans, Cremophor® EL and polysorbate 80 are known to be very effective histamine releasers in certain species like dogs [12]. This fact poses a major problem in preclinical studies using this sensitive animal model.

Thus, there is a need for the development of novel solubilizers taking into account both preclinical and clinical implications.

With regard to the structural aspects, systematic studies of histamine release in dogs showed that unsaturated oxethylated oleic acid was the effective principle in Cremophor® EL [12] and saturated 12-hydroxystearic acid was the least harmful [13]. Moghimi et al. discovered a similar effect, namely the termination of complement activation after the removal of double bonds in diblock copolymers present in poloxamer 188 by catalytic hydrogenation [10]. Furthermore, complement activation was shown to be an intrinsic property of the macromolecular components instead of the small molecular weight constituents [10].

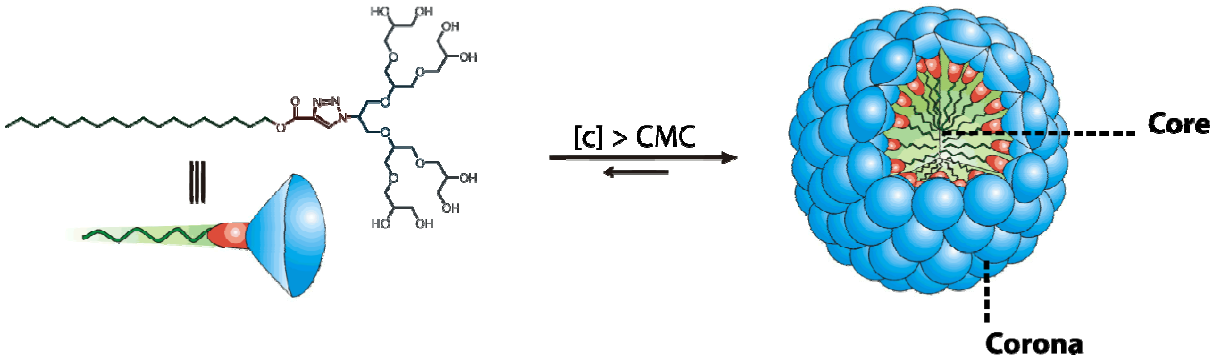

Figure 1: Micelles composed of dendritic amphiphiles for drug solubilization

On the basis of these observations, a novel class of amphiphiles based on glycerol dendrons coupled to saturated alkyl chains may provide favourable properties in terms of solubilization and safety. Their synthesis was performed by the group of R. Haag and will be reported

elsewhere. As a result, they found that structures comprising a glycerol dendron of 2nd and 3rd generation are optimal for the formation of small, spherical micelles (Fig. 1) showing a typical core-corona structure.

The modular approach in their synthesis and the use of simple click-chemistry as the final coupling reaction lead to well-defined amphiphiles instead of mixtures of variable composition like Cremophor® EL. This is a good prerequisite for structure-response relationship investigations with regard to (a) solubilization, (b) micelle stability, and (c) first orientating toxicology testing *in vitro*.

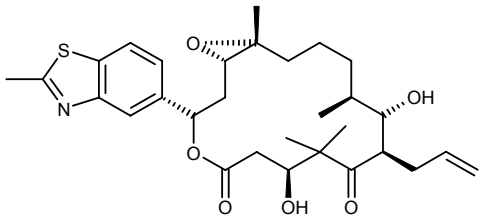

Figure 2: Structural formula of Sagopilone

The aim of the present study was to investigate five non-ionic dendritic glycerol-based amphiphiles comprised of different hydrophobic modifications with respect to solubilization and micelle stability using the novel anticancer drug Sagopilone (Fig. 2). It is a poorly soluble drug with a water solubility of 12 mg/L. Its application in chemotherapy requires a final formulation concentration of 1 g/L, and for this reason, solubilization is mandatory.

First, the micelle formation and Sagopilone solubilization by the dendritic amphiphiles, as shown in Figure 3, were studied and compared to Cremophor® ELP, polysorbate 80, and Pluronic® F68. Structurally, all dendritic amphiphiles were composed of a 2nd generation polyglycerol dendron (PG[G2]) as the hydrophilic part and a single C_{18}-chain coupled by a triazol ring as the hydrophobic part (PG[G2]-C_{18}) (Fig. 3). The effect of different core structures was investigated by the introduction of a second C_{18}-chain (PG[G2]-$(C_{18})_2$) or

an additional aromatic group. The latter was incorporated through an additional phenyl group on the hydrophobic-hydrophilic interface of the amphiphile (PG[G2]-DiAr-C_{18}) or a naphthyl or bisphenyl end group ((PG[G2]-C_{18}-Naph/ -BiP). On the basis of the solubility investigations final formulations representing clinically relevant concentrations of Sagopilone (1 g/L) were prepared, and their stability was assessed both undiluted and after dilution. Second, an MTT assay was performed to assess the *in vitro* cytotoxicity using primary endothelial cells.

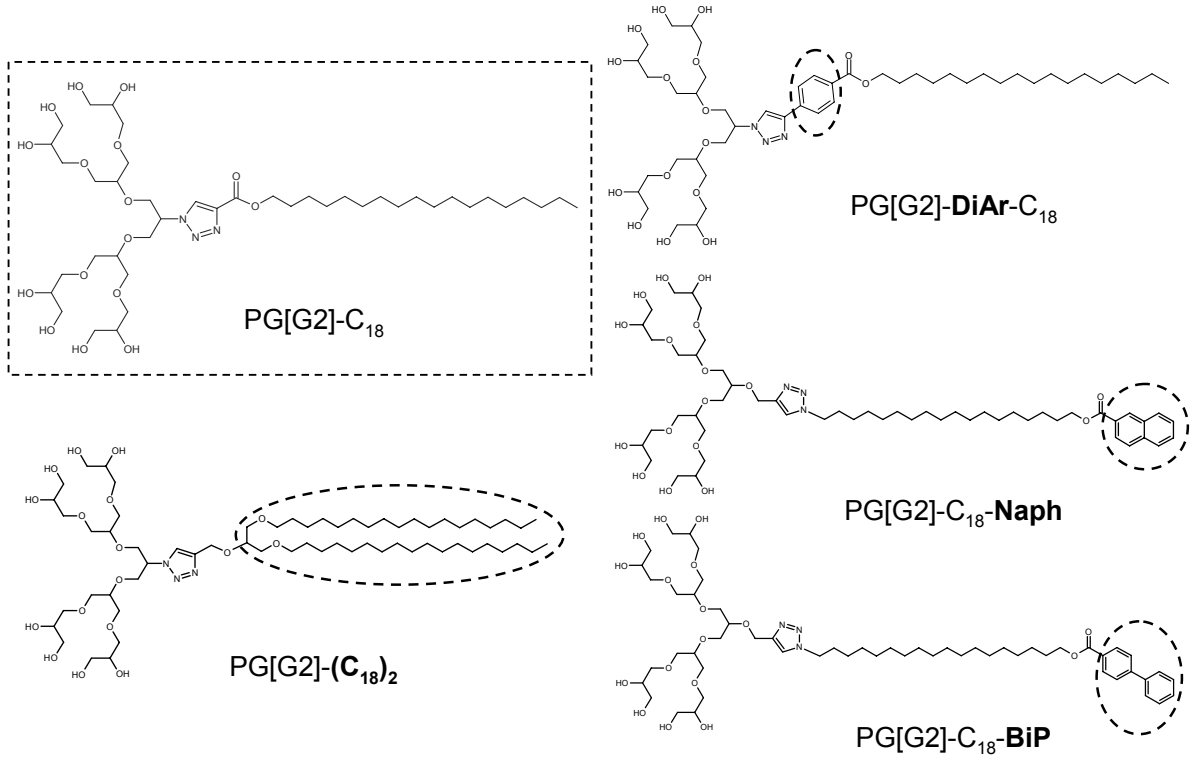

Figure 3: Structural formulas of dendritic glycerol-based amphiphiles

2. Materials and methods

2.1 Materials

The dendritic amphiphiles with the different hydrophobic structures were provided by the group of Prof. R. Haag (FU Berlin), and were used directly without further modification. Sagopilone was obtained from Bayer Schering Pharma AG (Berlin, Germany). The standard excipients Cremophor® ELP (BASF, Ludwigshafen, Germany) and polysorbate 80 (Merck KGaA, Darmstadt, Germany) were used in European Pharmacopoeia grade. Pluronic® F68 was purchased from Sigma Aldrich (St. Louis, USA). All other ingredients were obtained in analytical quality.

2.2 Molecular modelling

Molecular mechanics calculations were performed for conformational searching of PG[G2]-C_{18} using MMFF94 of Spartan'08 software (Wavefunction, Inc., Irvine, USA). In brief, MMFF94 is a computational tool for automatic conformational analysis in the lowest dielectric. All possible conformations are calculated automatically and an optimized gas-phase structure is indicated afterwards. The latter was used to estimate the linear extension of the molecule.

2.3 Preparation and characterization of unloaded and drug-loaded micellar dispersions

Micellar dispersions were prepared by direct dissolution of the particular dendritic amphiphiles in phosphate buffer (0.05 M, pH 7.4) with additional heating to 50 °C for 5 min, if necessary. The loading capacity of the micelles was determined by an excess method. In a typical experiment, 2.0 mg Sagopilone was weighed in screw-top glass vials and 2.0 ml of the micellar dispersion (1 or 10 g/L in phosphate buffer (0.05M, pH 7.4)) was added. The suspensions were stirred at ambient temperature for 24 h using a magnetic

stirrer (IKA® GmbH, Staufen, Germany). Afterwards, the remaining drug substance was removed by filtration through a syringe filter (Millex®-GV 0.22 µm, Millipore, USA). These experiments were performed in triplicate.

The micelle characteristics were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK). In brief, the principle is based on the measurement of the backscattered light fluctuations at an angle of 173° and the calculation of an autocorrelation function. The samples were measured undiluted at 25 °C, adjusted to the temperature for 1 min prior to the measurement. The autocorrelation functions were analysed using the DTS v5.1 software provided by Malvern, and the hydrodynamic diameter of the micelles (d_H, equal to Z-Ave) and their size distribution (PDI: polydispersity index) were calculated. Measurements were done in triplicate with 15 - 20 runs per single measurement and the calculated mean values were used.

The critical micelle concentration (CMC) was determined by measuring the surface tension of the amphiphiles in deionized water (Millipore system Milli-Q plus) by the pendant drop method. The contact angle tensiometer OCA20 (DataPhysics Instruments GmbH, Filderstadt, Germany) was used for these measurements and the temperature was set at 25 ± 0.5 °C. Calculation of the surface tension was done by using the Young-Laplace-Equation. The accuracy of measurements, checked by one replicate experiment and by control of γ for pure water, was ± 0.4 mN/m. The behaviour of the amphiphiles in water was studied over the concentration range of $4 \cdot 10^{-7}$ to $1 \cdot 10^{-3}$ M. Aqueous solutions were prepared 24 h before measurement. The surface tension γ was determined two times per minute and the measurement was stopped when the value γ did not change by more than 0.1 mN/m over three minutes. Equilibration time was generally between 50 - 80 min below the CMC and 25 - 50 min at higher concentrations.

The Sagopilone content was determined by High Performance Liquid Chromatography in combination with UV-detection (HPLC-UV) using an Agilent 1100 Series chromatography system (Agilent Technologies, Santa Clara, USA) consisting of a quaternary pump, an auto-injector, a column heater at 25 °C, and a UV-detector. Two Chromolith® Performance RP-18e columns (100 x 4.6 mm, Merck, Germany) were used. A gradient was run from acetonitrile (ACN)/ water (25/75, v/v) to ACN/ water (45/55, v/v) in 10 min followed by isocratic elution for 15 min at a flow rate of 1 mL/min. Afterwards, the solvent was set to ACN/ water (70/30, v/v) to elute the dendritic amphiphiles with subsequent equilibration of the system using ACN/ water (25/75, v/v) for 8 min. Samples were diluted in a ratio of 1:5 to 1:10 with ACN/ water (50/50, v/v) prior to analysis. The injection volume of the samples was 10 µL, and Sagopilone was detected at a wavelength of 220 nm. The data were analysed using Empower™ 2 software (Waters Corporation, Milford, USA), and the amount of Sagopilone was determined by an external standard calibration. To validate the method, the recovery of Sagopilone was determined in the presence of the amphiphiles. Therefore, Sagopilone solution (0.2 g/L in acetonitrile) and the particular micellar dispersion (1 g/L in water) were mixed in a ratio of 1:1 (v/v) and the drug content was measured. It was calculated according to Equation (1).

$$\text{Recovery (\%)} = \frac{c_{Sagopilone}\ (\text{g/L})}{0.1\ \text{g/L}} \times 100 \qquad (1)$$

2.4 Stability investigations

Final formulations were prepared containing Sagopilone at a concentration of 1 g/L. To achieve this target concentration, the selected amphiphiles and the standard excipients were dissolved in phosphate buffer (0.05 M, pH 7.4) at a concentration of 30 and 60 g/L, respectively. Sagopilone (2.0 mg) was weighed in screw-top glass vials, 2.0 mL of the particular dispersion was added, and the suspensions were heated to 60 °C for 10 min

while stirring. After cooling to ambient temperature the volume was adjusted to 2.0 mL and the dispersions were filtered through a syringe filter (Millex®-GV 0.22 µm, Millipore, USA). Subsequent dilutions were prepared in a ratio of 1:10 with phosphate buffer (0.05 M, pH 7.4). The physicochemical stability for the formulations and the particular dilutions were investigated at storage temperatures of 21 °C (ambient temperature) and 37 °C, respectively. Samples were taken at designated time points for determining the physical stability and the remaining drug content. The latter was done after filtration to remove drug substance that precipitated during storage.

2.5 *In vitro* cytotoxicity

The colorimetric MTT assay was performed to study the *in vitro* cytotoxicity for human umbilical vein endothelial cells (HUVECs). Amphiphile and excipient dispersions with concentrations ranging from 0.01 to 1 g/L were prepared in endothelial cell growth medium (Clonetics® EGM, Lonza, Basel, Switzerland) and sterilized by filtration (Millex®-GV 0.22 µm, Millipore, USA). The compounds were soluble in the cell culture medium at all concentrations used. HUVECs (Clonetics®, Lonza, Basel, Switzerland) were seeded onto 96-well plates (TPP) with a density of 25 000 cells per well. After a 24 h recovery at 37 °C in a humidified atmosphere with 5 % CO_2, the growth medium was replaced by 100 µL serial dilutions of the amphiphile and excipient dispersions. The cells were incubated for 24 h and 72 h. Twenty microlitres of sterile filtered MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Aldrich, St. Louis, USA) stock solution (5 g/L in phosphate buffer saline) were added to each well 2 h prior to the final incubation time. After the completion of incubation, the medium was replaced by 100 µL of lysis medium (65.5 % SDS (30 %), 33 % DMF, 1.5 % glacial acetic acid). After 5 min at 37 °C and shaking for an additional 10 min at ambient temperature to ensure complete dissolution of the dark

blue formazan crystals, the absorption at 580 nm (test) and 675 nm (reference) was measured using an ELISA reader (Fluostar Optima, BMG Labtech GmbH, Offenburg, Germany).

The cell viability (%) relative to the control wells containing cell growth medium without any excipients was calculated according to Equation (2). All experiments were run 8 times.

$$\text{Cell viability}\,(\%) = \frac{\text{A(test)}}{\text{A(control)}} \times 100 \qquad (2)$$

2.6 Statistics

Data were recorded as mean ± standard deviation. All experiments were done at least in triplicate as specified in the results section. Means were analyzed for statistical significance using the unpaired Student's *t*-test. Differences were considered statistically significant at *p*-values < 0.05, if not mentioned specifically.

3. Results and discussion

3.1 CMC, micelle characteristics, and molecular modelling

All amphiphiles tested were water-soluble at a concentration of at least 10 g/L. The critical micelle concentration of the new amphiphiles was in a range of $3 \cdot 10^{-6}$ to $7 \cdot 10^{-6}$ M (Tab. 1). Thus, the minimum concentration of amphiphiles for the formation of micelles is remarkably lower compared to the standard excipients Cremophor® EL ($3.6 \cdot 10^{-5}$ M) [14], polysorbate 80 ($1.1 \cdot 10^{-5}$ M) [15], and poloxamer 188 ($1.1 \cdot 10^{-4}$ M) [16]. This may be beneficial with regard to formulation stability especially after dilution prior to or after intravenous application.

Table 1: CMC and particle characteristics of micellar dispersions

PG[G2]-	CMC[a]		c = 1 g/L[b]		c = 10 g/L[b]	
	(g/L)	(M)	d_H (nm)	PDI	d_H (nm)	PDI
-C_{18}	$3.2 \cdot 10^{-3}$	$3.7 \cdot 10^{-6}$	7.7	0.16	7.5	0.08
-$(C_{18})_2$	n.d.	n.d.	11.8	0.33	10.8	0.24
-DiAr-C_{18}	$6.7 \cdot 10^{-3}$	$6.9 \cdot 10^{-6}$	9.2	0.04	9.2	0.04
-C_{18}-BiP	$5.5 \cdot 10^{-3}$	$5.2 \cdot 10^{-6}$	9.2	0.20	9.0	0.11
-C_{18}-Naph	$5.7 \cdot 10^{-3}$	$5.5 \cdot 10^{-6}$	8.6	0.12	8.3	0.04

[a] Standard deviation ± 5 % (n=3)
[b] Concentration of amphiphile in micellar dispersion

As shown in Table 1, the amphiphiles formed small (7-10 nm), monodisperse (PDI: 0.04-0.11) micelles with the exception of PG[G2]-$(C_{18})_2$. Due to the low polydispersity indices they are very likely to be spheres, whereas the higher polydispersity of the latter may be due to the formation of non-spherical micelles such as worm-like micelles.

The correlation between the molecular length of the amphiphiles and the measured sizes of the corresponding micelles was exemplified for PG[G2]-C_{18} (Fig. 4). The optimized gas-phase conformation was calculated using conformational analysis by "MMFF94", and its length dimension was estimated to be 3.3 nm (Fig. 4A). This is very likely to be the maximum length of this molecule. Assuming spherical micelles, their theoretical size is twice this length

in diameter, which is approximately 6.6 nm. The theoretical value (6.6 nm) correlated very well with the hydrodynamic diameter measured using DLS (7.5 nm) since the latter displays the size of the micelle including its hydrodynamic water shell, which is slightly larger than the pure micelle size by definition.

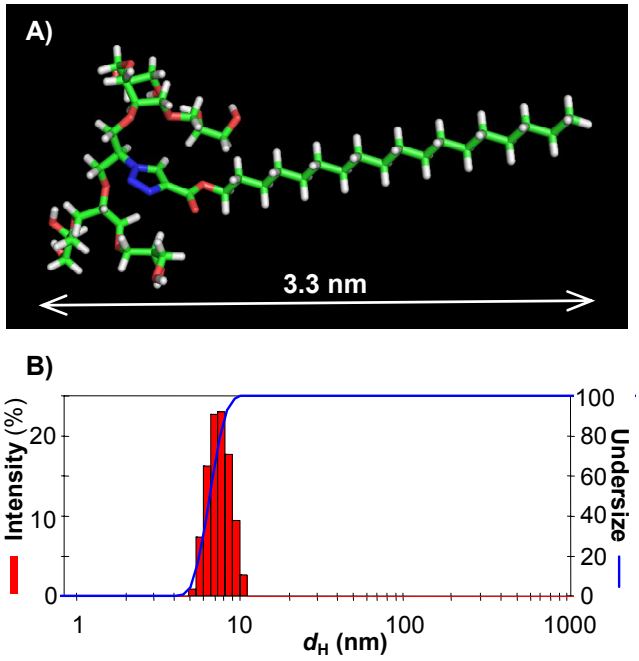

Figure 4: PG[G2]-C_{18} molecular and micellar sizes
(A) Conformation of PG[G2]-C_{18} and (B) size distribution by intensity of the corresponding micelles in phosphate buffer (*c*(amphiphile) = 10 g/L) measured by DLS. (*n*=3)

3.2 Solubilization capacity compared to standard excipients

The solubilization capacity for Sagopilone of the micelles described was determined using HPLC-UV. Prior to these investigations, the drug recovery was tested in the presence of the amphiphiles. Sagopilone as well as the dendritic amphiphiles were detectable at a wavelength of 220 nm, as shown in Figure 5, with the exception of PG[G2]-$(C_{18})_2$. Sagopilone (t_R = 20-21 min) was clearly separated from the detected excipients (t_R = 37-38 min) by the HPLC method used. The absence of an excipient peak of PG[G2]-$(C_{18})_2$ may be due to a different absorption maximum or an increased hydrophobic interaction with the C_{18}-modified column material and a remarkably increased retention time.

However, subsequent measurements were not affected, and the drug recovery was in a range of 98 - 105 % for all amphiphiles tested. Thus, the method suitability was proven.

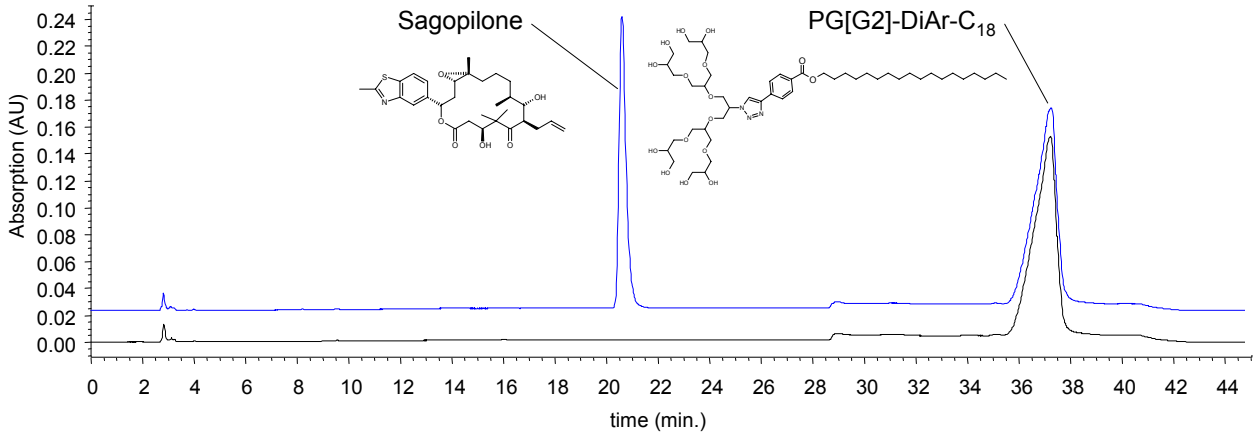

Figure 5: Analysis of the Sagopilone content by HPLC-UV
Chromatograms by HPLC-UV (λ = 220 nm) of unloaded (black) and drug-loaded (blue) micellar dispersions of PG[G2]-DiAr-C_{18}.

The solubilization effect for Sagopilone was determined after 24 h stirring at ambient temperature at a uniform excipient concentration of 1 and 10 g/L. As presented in Figure 6A, the dendritic amphiphiles showed a remarkably higher solubilization of Sagopilone (3- to 4.7-fold increase in water solubility) compared to Cremophor® ELP (CELP) and polysorbate 80 (PS80) (1.5- to 1.6-fold increase) as well as Pluronic® F68 (F68) (no solubilization enhancement at all). These results were achieved at a constant excipient concentration as low as 1 g/L, and the trend was equivalent at the higher concentration of 10 g/L (Fig. 6B). However, the increase in the total factor of solubilization (5- to 8-fold) was non-linear with respect to the increase in the excipient concentration (10-fold). This effect was very likely to be due to the solubilization method itself, which is a slow distribution process. Nevertheless, a high solubilization of the poorly water-soluble drug was achieved with this method in the absence of any organic solvents or heat. The micelle characteristics did not change after drug loading (data not shown). Comparing the different hydrophobic structures of the dendritic amphiphiles, the best solubilization was achieved in the presence of a diaromatic spacer (Fig. 6, red bar). The introduction of aromatic end groups did result in

a similar solubilizing effect compared to the single C_{18}-chain amphiphile, whereas a second C_{18}-chain led to a slightly decreased solubilization.

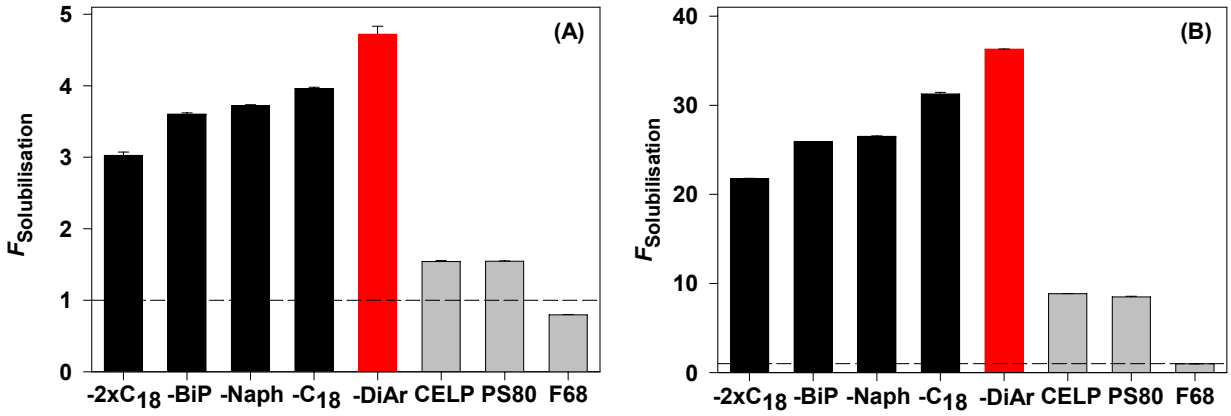

Figure 6: Solubilization of Sagopilone
Factor of Sagopilone solubilization (F: x-fold increase in solubility compared to water solubility) of dendritic amphiphiles (black, red bars) and standard excipients (grey bars) at an excipient concentration of (A) 1 g/L and (B) 10 g/L. (*n*=3)

3.3 Stability investigations

Three amphiphiles were selected for formulation stability investigations in comparison to Cremophor® ELP and polysorbate 80. For this purpose final formulations were prepared comprising a clinically relevant Sagopilone concentration of 1 g/L equal to the formulation already used in clinical trials. The amounts needed of the particular excipients were estimated according to the solubilization capacity experiments to be 30 and 60 g/L for the novel and standard excipients, respectively (Tab. 2).

The primary purpose to solubilize Sagopilone at a concentration of 1 g/L was successfully obtained with the novel as well as the standard excipients (Tab. 2). However, the amount needed of the latter was twice that of the dendritic amphiphiles. No drug substance was lost during the preparation process and subsequent sterile filtration. This fact displays a solubilization efficiency of 100 %, and meets primary requirements for parenteral formulation development. In addition, the method itself was convenient and feasible for scale-up.

Table 2: Solubilization of clinically relevant concentrations of Sagopilone (*n*=3)

Excipient	Drug-excipient-ratio[a]	Final Formulations for Stability Testing			
		c(excip.) (g/L)	*c*(drug) (g/L)	d_H (nm)	PDI
PG[G2]-C_{18}	1 : 21	30	1.04 ± 0.04	7.4 ± 0.04	0.06
PG[G2]-C_{18}-Naph	1 : 22	30	1.03 ± 0.01	8.1 ± 0.01	0.01
PG[G2]-DiAr-C_{18}	1 : 18	30	1.07 ± 0.02	8.4 ± 0.02	0.02
Cremophor® ELP	1 : 54	60	0.98 ± 0.02	11.8 ± 0.02	0.02
Polysorbate 80	1 : 54	60	0.97 ± 0.03	10.4 ± 0.03	0.08

[a] Calculated from solubilization experiments with excipient concentration of 1 g/L (w/w)

Second, the stability was investigated with regard to clinical application both prior to and after dilution. All formulations tested were stable for at least three days at ambient temperature as shown in Figure 7. Precipitation of the drug substance was not observed, and at least 98 % of Sagopilone still remained solubilized after three days of storage. In addition, the micellar characteristics also did not change (data not shown).

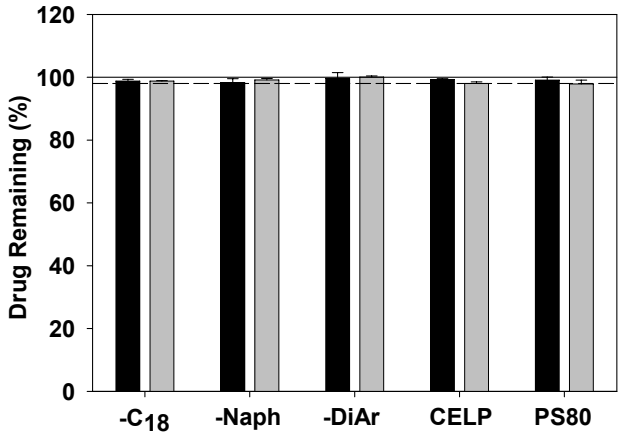

Figure 7: Formulation stability at ambient temperature
Drug remaining (-- 98 %) after 24 h (▬) and 3 days (▭) of storage at ambient temperature. (*n*=3)

There was no difference in the solubilization stability between the different core structures of the dendritic amphiphiles comprised of an unmodified C_{18}-chain, a C_{18}-chain coupled by a diaromatic spacer or a C_{18}-chain modified by a naphthyl end group. In a previous publication, Carstens et al. reported an increased stability of mPEG-*b*-oligo(ε-caprolactone) micelles in the presence of an aromatic end group such as a benzoyl or naphthyl group [17]. A similar effect

has also been described for hydrophobically modified polymeric micelles [18, 19]. In contrast to these effects, dendritic amphiphiles exhibited stable Sagopilone solubilization both in the presence and absence of hydrophobic modifications of the micellar core. This may be due to a high kinetic stability with a slow exchange rate between amphiphilic unimers and micelles. Another explanation is the formation of mixed micelles composed of Sagopilone and the dendritic amphiphiles. This micelle type, e.g. mixed surfactant micelles, often performs better than a single surfactant with respect to solubilization and/ or stability [20].

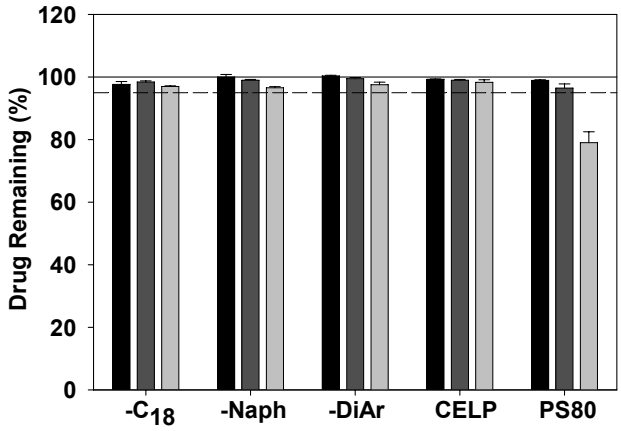

Figure 8: Stability of dilutions at elevated temperature
Dilution of final formulations (1:10 with phosphate buffer pH 7.4) and drug remaining (-- 95 %) after 6 h (▬), 24 h (▬), and 3 days (▭) of storage at 37 °C. (*n*=3)

With regard to their clinical application, aliquots of the formulations were diluted at a ratio of 1:10 with phosphate buffer (0.05 M, pH 7.4) and stored at an elevated temperature of 37 °C. The resulting dilutions were stable for drug content with a remaining drug content in solution of at least 95 % after three days of storage with the exception of the polysorbate 80-based formulation (Fig. 8). The latter exhibited Sagopilone precipitation if stored for more than 24 h. The others did not show any change in the micelle characteristics (data not shown). Again, no difference was observed between the dendritic amphiphiles with and without a hydrophobic modification in terms of stability upon dilution.

Overall, the amphiphilic dispersions exhibited an excellent formulation stability profile, and additional co-solvents such as ethanol were not needed.

3.4 *In vitro* cytotoxicity

The cytotoxicity was determined by measuring the metabolic activity after incubation of human umbilical vein endothelial cells (HUVECs). The choice of this cell line was governed by their potential exposure with the amphiphilic carriers after i.v. application [21]. Thus, this test may provide an indication of the local tolerance at the injection site. Figure 9 shows the cell viability relative to the viability of control cells upon exposure to different excipient concentrations for (a) 24 h and (b) 72 h. The concentrations tested were in a range from 0.01 to 1 g/L for both the novel and the standard excipients, allowing direct comparisons.

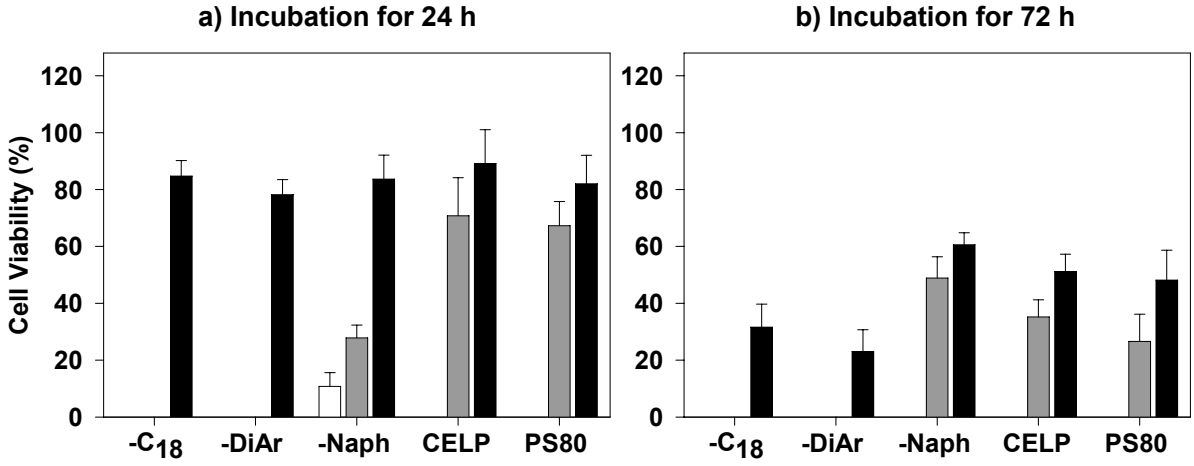

Figure 9: In vitro cytotoxicity determined by MTT assay
HUVEC viability after incubation with decreasing excipient concentrations (1 (▭), 0.1 (▬), and 0.01 (■) g/L) for (a) 24 h and (b) 72 h in comparison to cells treated with medium. (n=8)

Generally, the viability after 72 h differed from the short-term effects after 24 h. As shown in Figure 9, cells treated with the compounds at the lowest concentration of 0.01 g/L (black bars) exhibited a high viability (~ 85 %) after 24 h independent of the compounds' structure ($p > 0.01$). An incubation time of 72 h using identical excipient concentrations resulted in an overall decreased viability (20-65 %). Structurally, cells treated with PG[G2]-C_{18}-Naph showed a significantly higher viability ($p = 0.01$) compared to the standard excipients. And in turn, the latter were significantly better tolerated compared to PG[G2]-C_{18} and PG[G2]-DiAr-C_{18} ($p < 0.01$). At the next higher concentration of 0.1 g/L (grey bars),

no viability was observed for the dendritic structures PG[G2]-C_{18} and PG[G2]-DiAr-C_{18}, whereas cells treated with PG[G2]-C_{18}-Naph showed an interesting behaviour. The long-term viability after 72 h (49 %) was significantly higher than the viability after an incubation for 24 h (28 %). This is in contrast to the viability trend observed with the standard excipients comprising 67-71 % and 27-35 % viability after 24 h and 72 h, respectively. Thus, PG[G2]-C_{18}-Naph exhibited a significantly lower long-term cytotoxicity ($p < 0.01$) compared to the standard excipients. Nevertheless, the actual viability values were in a comparable range. At the highest concentration of 1 g/L (white bars), viability was no longer observed for any of the excipients with the exception of PG[G2]-C_{18}-Naph, which showed a low percentage of viable cells (11 %) after the short-term incubation of 24 h.

The results obtained with polysorbate 80 were in good agreement with previously accomplished *in vitro* cytotoxicity testing using human fibroblasts [22], and this fact signifies the reliability of these results. The cytotoxic effects observed are very likely caused by an interaction with the cellular membranes, a well-known characteristic of surfactants [23]. As an example, the *in vitro* cytotoxicity of Cremophor® EL was postulated to be due to peroxidation of polyunsaturated fatty acids and subsequent formation of free radicals and/or a direct pertubing effect in the cell membrane, which causes fluidity and leakage [7]. Generally, Oros et al. have shown that the strength of the biological effects of non-ionic surfactants mainly depends on their hydrophobicity [24]. With regard to the chemical structure of the dendritic amphiphiles, the introduction of a hydrophobic naphthyl end group has had a significant impact on lowering cytotoxicity. This may be due to higher micelle stability and subsequent decreased surfactant-membrane interactions. Despite the membrane-interacting effect, there are other parameters that may influence the results like a simple detachment of viable cells from the flasks during the experiment. However, the occurrence of the latter is very unlikely because detachment mainly results from cell death [23].

Overall, an excipient concentration of 0.01 g/L was well tolerated by HUVECs after 24 h incubation, but higher concentrations showed less viability compared to the standard excipients. Therefore, the novel dendritic amphiphiles may be considered biocompatible in terms of short-term cytotoxicity at concentrations of 0.01 g/L and below. Furthermore, these data indicate an acute effect on a cellular basis towards HUVECs at higher concentrations. A measure to address this could be to use highly diluted dendritic amphiphiles at a low infusion speed to avoid acute site reactions during injection. Long-term exposition revealed an overall superior viability of the dendritic amphiphile comprising a naphthyl end group and its actual cytotoxic effect was comparable to the standard excipients Cremophor® ELP and polysorbate 80.

Kojima et al. have shown that *in vitro* cytotoxicity testing of surfactants required a treatment period longer than 24 h to acquire a good correlation with the *in vivo* Draize eye score [25]. Thus, the incubation for 72 h may reflect the *in vivo* situation better than the results obtained after 24 h.

In general, cytotoxicity testing is an important step, but results largely depend on the cell type and test conditions used. The use of primary endothelial cells like HUVEC give an indication of local tolerability effects at the injection/ infusion site, but may be of limited predictive value due to short contact times in typical infusion protocols. Non-toxic behaviour on a cellular basis is often described as evidence of biocompatibility and a prerequisite for safety *in vivo*. However, *in vitro – in vivo* correlation studies, such as for *in vivo* ocular irritancy [23], are missing for the prediction of the toxicity after intravenous application. Thus, biocompatibility, especially a structure-response relationship of the histamine release and complement activation potential *in vitro* as well as *in vivo*, will be studied further and correlated to the findings of cytotoxicity.

4. Conclusion

The novel dendritic glycerol-based amphiphiles showed superior solubilization behaviour for the poorly water-soluble anticancer drug Sagopilone compared to standard excipients used in parenteral formulations. The structure comprising a diaromatic spacer (PG[G2]-DiAr-C_{18}) provided the best solubilization effect for Sagopilone out of the different dendritic amphiphiles tested. This indicates preferential drug localization on the interface between the hydrophobic core and the hydrophilic corona of the micelle. In contrast to the standard excipients, the dendritic amphiphiles are compounds of definite composition allowing easy analysis by HPCL-UV without any pre-treatment. In combination with their excellent formulation stability profile, both undiluted and after dilution, they fulfil important requirements to be considered as alternative solubilizing agents. Hence, they are worthy of being studied further as drug carriers. Cytotoxicity studies of the different structures showed a clear structure-response relationship with the structure comprising a naphthyl end group being the least cytotoxic. Its actual cytotoxicity values were comparable to the standard excipients Cremophor® ELP and polysorbate 80. Further *in vitro* and, particularly important, *in vivo* studies have to be performed to assess their biocompatibility, especially in terms of histamine release as well as complement activation.

Overall, the PG[G2]-C_{18} derivatives and, in particular, the simple synthesis and variation of their chemical structure as well as their definite composition offer possibilities for optimizing a lead that may be developed as a solubilizing compound for preclinical and clinical use considering additional aspects such as histamine release potential.

References

1. G.S. Kwon. Polymeric micelles for delivery of poorly water-soluble compounds. *Crit. Rev. Ther. Drug Carr. Syst.* 20 (2003) 357-403.

2. S. Sweetana, M.J. Akers. Solubility principles and practices for parenteral drug dosage form development. *PDA J. Pharm. Sci. Technol.* 50 (1996) 330-342.

3. M.F. Powell, T. Ngueyen, L. Baloian. Compendium of Excipients for Parenteral Formulations. *PDA J. Pharm. Sci. Technol.* 52 (1998) 238-311.

4. R.G. Strickley. Solubilizing excipients in oral and injectable formulations. *Pharm. Res.* 21 (2004) 201-230.

5. P. Li, L. Zhao. Developing early formulations: Practice and perspective. *Int. J. Pharm.* 341 (2007) 1-19.

6. A.J. ten Tije, J. Verweij, W.J. Loos, A. Sparreboom. Pharmacological effects of formulation vehicles: implications for cancer chemotherapy. *Clin. Pharmacokinet.* 42 (2003) 665-685.

7. H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. *Eur. J. Cancer* 37 (2001) 1590-1598.

8. R.B. Weiss, R.C. Donehower, P.H. Wiernik, T. Ohnuma, R.J. Gralla, D.L. Trump, J.R. Baker, Jr., D.A. Van Echo, D.D. Von Hoff, B. Leyland-Jones. Hypersensitivity reactions from taxol. *J. Clin. Oncol.* 8 (1990) 1263-1268.

9. J. Szebeni, F.M. Muggia, C.R. Alving. Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. *J. Natl. Cancer Inst.* 90 (1998) 300-306.

10. S.M. Moghimi, A.C. Hunter, C.M. Dadswell, S. Savay, C.R. Alving, J. Szebeni. Causative factors behind poloxamer 188 (Pluronic F68, Flocor^{TM})-induced complement activation in human sera: A protective role against poloxamer-mediated complement activation by elevated serum lipoprotein levels. *Biochim. Biophys. Acta Mol. Basis Dis.* 1689 (2004) 103-113.

11. I. Hamad, A.C. Hunter, J. Szebeni, S.M. Moghimi. Poly(ethylene glycol)s generate complement activation products in human serum through increased alternative pathway turnover and a MASP-2-dependent process. *Mol. Immunol.* 46 (2008) 225-232.

12. W. Lorenz, H.-J. Reimann, A. Schmal, P. Dormann, B. Schwarz, E. Neugebauer, A. Doenicke. Histamine release in dogs by Cremophor El and its derivatives: Oxethylated oleic acid is the most effective constituent. *Inflamm. Res.* 7 (1977) 63-67.

13. W. Lorenz, A. Schmal, H. Schult, S. Lang, C. Ohmann, D. Weber, B. Kapp, L. Lüben, A. Doenicke. Histamine release and hypotensive reactions in dogs by solubilizing agents and fatty acids: Analysis of various components in cremophor El and development of a compound with reduced toxicity. *Inflamm. Res.* 12 (1982) 64-80.

14. D. Kessel. Properties of Cremophor EL micelles probed by fluorescence. *Photochem. Photobiol.* 56 (1992) 447-451.

15. L.S.C. Wan, P.F.S. Lee. CMC of polysorbates. *J. Pharm. Sci.* 63 (1974) 136-137.

16. I.R. Schmolka. A review of block polymer surfactants. *J. Am. Oil Chem. Soc.* 54 (1977) 110-116.

17. M.G. Carstens, P.H.J.L.F. de Jong, C.F. van Nostrum, J. Kemmink, R. Verrijk, L.G.J. de Leede, D.J.A. Crommelin, W.E. Hennink. The effect of core composition in biodegradable oligomeric micelles as taxane formulations. *Eur. J. Pharm. Biopharm.* 68 (2008) 596-606.

18. P. Opanasopit, M. Yokoyama, M. Watanabe, K. Kawano, Y. Maitani, T. Okano. Block copolymer design for camptothecin incorporation into polymeric micelles for passive tumor targeting. *Pharm. Res.* 21 (2004) 2001-2008.

19. O. Molavi, Z. Ma, A. Mahmud, A. Alshamsan, J. Samuel, R. Lai, G.S. Kwon, A. Lavasanifar. Polymeric micelles for the solubilization and delivery of STAT3 inhibitor cucurbitacins in solid tumors. *Int. J. Pharm.* 347 (2008) 118-127.

20. R. Liu, N. Sadrzadeh, P.P. Constantinides. Micellization and Drug Solubility Enhancement. In: R.Liu (Ed.). Water-Insoluble Drug Formulation. Interpharm Press, Englewood (2000) 213-354.

21. J. Djordjevic, M. Barch, K.E. Uhrich. Polymeric micelles based on amphiphilic scorpion-like macromolecules: Novel carriers for water-insoluble drugs. *Pharm. Res.* 22 (2005) 24-32.

22. B. Arechabala, C. Coiffard, P. Rivalland, L.J.M. Coiffard, Y. De Roeck-Holtzhauer. Comparison of cytotoxicity of various surfactants tested on normal human fibroblast cultures using the neutral red test, MTT assay and LDH release. *J. Appl. Toxicol.* 19 (1999) 163-165.

23. M. Marinovich, E. Tragni, A. Corsini, C.L. Galli. Quantification of *in vitro* cytotoxicity of surfactants: correlation with their eye irritation potential. *Cutan. Ocul. Toxicol.* 9 (1990) 169-178.

24. G. Oros, T. Cserháti, E. Forgás. Use of spectral mapping and stepwise regression analysis for the assessment of the relationship between chemical structure and biological activity of surfactants. *Chemometr. Intell. Lab. Syst.* 39 (1997) 95-101.

25. H. Kojima, A. Hanamura, A. Sato, H. Konishi. Comparison of cytotoxicity of surfactants on a range of mammalian cells cultured under various conditions. *AATEX* 3 (1995) 111-120.

CHAPTER 5

SUMMARY AND PERSPECTIVES
ZUSAMMENFASSUNG UND AUSBLICK

Summary

The present thesis describes the study of polymeric micelles and novel dendritic amphiphiles for the solubilization and parenteral administration of Sagopilone, a novel anticancer drug. Suitable colloidal carriers were identified and characterized in terms of solubilization, stability, formulation feasibility, and toxicity.

Chapter 2 describes the systematic study of polymeric micelles using PEG-*b*-PLA (poly(ethylene glycol)-*b*-poly(lactide)) and PEG-*b*-PCL (poly(ethylene glycol)-*b*-poly(ε-caprolactone)) block copolymers as drug delivery systems for Sagopilone. It was aimed to identify suitable copolymers and to assess the predictive power of solubility parameters. Besides the copolymer type, the hydrophobic/hydrophilic-ratio (w/w) of the block copolymers and the preparation method were hypothesized to play a decisive role with regard to solubilization, stability, and micelle morphology. PEG_{2000}-*b*-$PDLLA_{2200}$, PEG_{2000}-*b*-PCL_{2600}, and PEG_{5000}-*b*-PCL_{5000}, abbreviated as P2LA(1.1), P2CL(1.3), and P5CL(1.0), were identified as being most suitable in terms of efficient solubilization (≥ 70 %) and stability. The number in parentheses depicts the hydrophobic/hydrophilic-ratio indicating its optimum at approximately 1. The corresponding micelles were shown to be small (< 100 nm), monodisperse, spherical micelles. Sonication was applied to P2CL(1.3) and P5CL(1.0), whereas P2LA(1.1) micelles require preparation by film formation. As a result (93 ± 0.4) %, (96 ± 6) %, and (80 ± 12) % of the drug still remained solubilized after 24 h, respectively. Contrary to previous reports, calculated solubility parameters were not predictive since they showed a reversed order of preference relative to experimental data, and the substantial difference in the solubilization capacity of the two stereoisomers of PLA was not covered. ‘Supersaturation’ is a preparation-specific phenomenon following film formation. Its cause as well as the solubilization of Sagopilone within the block copolymer films were elucidated by the evidence of glass solutions that exceeded the solubilization

capacity of the corresponding micelles in terms of Sagopilone content. The apparent solid-state saturation solubility of Sagopilone in the block copolymer was determined using the Couchman-Karasz approach and showed a good correlation with the loading capacity of the respective micelles.

Chapter 3 describes the stability investigations of the resulting polymeric micelles, novel formulation approaches, and the toxicity testing *in vitro* as well as *in vivo*. A drug-polymer-ratio as low as 1:20 (w/w) was sufficient to effectively solubilize Sagopilone and to obtain stable dispersions that did not show any supersaturation (24 h: drug content $\geq$ 95 %). Although the micelles exhibited a similar thermodynamic stability (CMC: 10^{-7} – 10^{-6} M), PEG-*b*-PCL micelles were kinetically more stable than P2LA(1.1) prior to and after further dilution as well as at elevated temperatures. Lyophilization of PEG-*b*-PCL micelles was shown to be feasible in the presence of additional excipients that prevent PCL crystallization. Sagopilone-loaded polymeric films of P2LA(1.1) have been shown to be stable and easily redispersible for at least 3 months (3 months, 6 °C: drug content of (95.6 ± 1.4) %). This was demonstrated for the first time and the storage temperature was identified as the key parameter. *In vitro*, Sagopilone-loaded polymeric micelles were equipotent to Sagopilone cyclodextrin-based reference solutions using a cervix carcinoma cell line. *In vivo*, no carrier-associated side effects were observed, and the maximum tolerated dose (MTD) of micellar Sagopilone was determined to be 6 mg/kg using nude mice. However, the latter was decreased compared to a cyclodextrin-based formulation of Sagopilone (MTD = 10 mg/kg) possibly due to a hampered drug degradation by serum esterases resulting in an enhanced effective dose. Overall, these results highlight that polymeric micelles, especially PEG-*b*-PCL micelles, fulfil key requirements for their use in parenteral formulations. In particular, they offer an excellent potential for further preclinical and clinical cancer studies using Sagopilone.

Chapter 4 describes structure-response relationship investigations of novel dendritic glycerol-based amphiphiles in terms of Sagopilone solubilization, micelle stability, and cytotoxicity. These amphiphiles are composed of a hydrophilic headgroup composed of dendritic polyglycerol (2^{nd} generation, PG[G2]) coupled to an alkyl chain (C_{18}) and various hydrophobic modifications. They showed superior solubilization capacities of Sagopilone compared to standard excipients used in parenteral formulations. Looking at their different core structures, the best solubilization was achieved with a diaromatic spacer group (PG[G2]-DiAr-C_{18}). This indicates that the hydrophobic-hydrophilic interface of the micelles is the main locus of drug solubilization. The definite chemical composition as well as the convenient analysis of these amphiphiles using HPLC-UV without any pre-treatment marks a further advantage of these solubilizers. They showed an excellent formulation stability profile both undiluted and after dilution independent of their core structure, which is another important requirement for novel solubilizers. Cytotoxicity testing in primary endothelial cells revealed the least toxicity in the presence of a naphthyl end group with values comparable to Cremophor® EL and polysorbate 80. Overall, these results highlight the potential offered by these novel dendritic amphiphiles.

Perspectives

Besides their excellent solubilization behaviour and safety, as shown in this work, polymeric micelles offer the potential for an improved drug therapy, especially in the treatment of cancer. Concerning stability issues the preparation of "mixed polymeric micelles" may provide a straightforward method for stability enhancement. Previous investigations revealed ambiguous results regarding the *in vivo* behaviour of PEG-*b*-Polyester, in particular the absence or presence of an EPR-effect. Thus, appropriate studies are needed with special emphasis on a comparison of different drugs, polymers, and tumour models. The toxicity evaluation presented here gives valuable information, especially compared to the

cyclodextrin-based formulation. Since previous studies primarily addressed the replacement of toxic solubilizers such as Cremophor® EL, the comparison with other solubilizers such as cyclodextrins in terms of the *in vivo* performance would be the next development step. This would in particular be necessary to further exploit the whole potential of these solubilizers, to assess their rank among currently used excipients, and to promote their entrance into standard formulation development. Hopefully, it will lead to an increased number of solubilizers available for parenteral formulation development with better tolerability properties such as Cremophor® EL, and that development in the area of polymeric micelles will make a significant contribution towards safer drug therapy.

In contrast to the block copolymers, the dendritic amphiphiles studied are farther from clinical application. Nevertheless, the results obtained point to their utility as solubilizing agents fulfilling the physicochemical aspects for developing stable parenteral formulations. Furthermore, these results are particularly valuable for an optimization of a lead structure concerning additional physiological aspects such as histamine release.

Generally, a high demand for alternative formulation vehicles exists both in the preclinical and clinical situation, and the polymeric micelles as well as the dendritic amphiphiles described herein present promising approaches towards its satisfaction.

Zusammenfassung

Die vorliegende Dissertation beschreibt die Entwicklung neuer parenteraler Formulierungen für Sagopilon, einem neuen Wirkstoff für die Krebstherapie, basierend auf Polymermizellen sowie neuartigen dendritischen Amphiphilen. Geeignete kolloidale Trägersysteme wurden identifiziert und hinsichtlich ihres Solubilisationsvermögens, der Machbarkeit verschiedener Formulierungsansätze und ihrer Verträglichkeit bzw. Toxizität charakterisiert.

Kapitel 2 beschreibt die systematische Untersuchung von Polymermizellen bestehend aus PEG-*b*-PLA (Polyethylenglykol-*b*-Polylaktid) und PEG-*b*-PCL (Polyethylenglykol-*b*-Polycaprolakton) Block-Copolymeren als Wirkstoffträgersysteme für Sagopilon. Ziel dieser Studie war es, geeignete Copolymere zu identifizieren und die Vorhersagekraft von Löslichkeitsparametern zu bewerten. Es wurde vermutet, dass neben der Natur der einzelnen Polymerblöcke auch der Quotient „hydrophob/hydrophil“ des Block-Copolymers und die Herstellmethode entscheidenden Einfluss auf die Löslichkeitsverbesserung, die Stabilität der Formulierungen und die Mizellmorphologie ausüben. PEG_{2000}-*b*-$PDLLA_{2200}$, PEG_{2000}-*b*-PCL_{2600} und PEG_{5000}-*b*-PCL_{5000}, abgekürzt als P2LA(1.1), P2CL(1.3) und P5CL(1.0), wurden als bestgeeignete Polymere hinsichtlich einer effizienten Wirkstoffsolubilisation (≥ 70 %) und Formulierungsstabilität identifiziert. Der in Klammern angegebene Wert beschreibt den Quotient „hydrophob/hydrophil“ des jeweiligen Block-Copolymers und zeigt, dass dessen Optimum um den Wert 1 liegt. Die resultierenden kolloidalen Strukturen waren nachweislich kleine (< 100 nm) Kugelmizellen mit monomodaler Größenverteilung. Zur Mizellbildung konnte für die Blockcopolymere P2CL(1.3) und P5CL(1.0) eine Dispergierung mittels Ultraschall angewendet werden, während P2LA(1.1) eine Herstellung mittels Filmbildungsmethode erforderte. Dementsprechend lagen nach 24-stündiger Lagerung der Dispersionen noch (93 ± 0.4) %, (96 ± 6) % und (80 ± 12) % des Wirkstoffs in solubilisierter Form vor. Entgegen vorheriger

Berichte waren die berechneten Löslichkeitsparamter nicht prädiktiv, da sie eine gegensätzliche Präferenz zu den experimentell gewonnenen Daten aufwiesen. Außerdem wurde der beobachtete substanzielle Unterschied im Solubilisationsvermögen der beiden Stereoisomere des Polylaktids nicht abgedeckt. „Übersättigung" der Mizellen ist ein herstellspezifisches Phänomen, welches nach Anwendung der Filmmethode beobachtet wurde. Die Ursache sowie die Löslichkeit von Sagopilon innerhalb der gebildeten Filme konnte durch den Nachweis der Bildung von Glaslösungen aufgeklärt werden, welche einen die Beladungskapazität der entsprechenden Mizellen überschreitenden Sagopilongehalt aufwiesen. Die Sättigungslöslichkeit des Sagopilons im Polymerfilm wurde unter Verwendung der Formel nach Couchman-Karasz bestimmt und wies eine gute Korrelation mit der korrespondierenden Mizellbeladungskapazität auf.

Kapitel 3 beschreibt Stabilitätsuntersuchungen und verschiedene Formulierungsansätze für die aus der vorangegangenen Studie resultierenden Polymermizellen sowie deren Verträglichkeitstestung *in vitro* als auch *in vivo*. Ein Wirkstoff-Polymer-Verhältnis von 1:20 war ausreichend, um stabile Formulierungen zu erhalten, welche Sagopilon effektiv solubilisieren und keine Übersättigung aufweisen (Wirkstoffgehalt nach 24 h: $\geq$ 95 %). Im Gegensatz zu der vergleichbaren thermodynamischen Mizellstabilität (CMC: 10^{-7} – 10^{-6} M) unterschieden sich die untersuchten Mizellen deutlich in ihrer kinetischen Stabilität. Dabei waren die PEG-*b*-PCL Mizellen sowohl unverdünnt als auch nach Verdünnung und bei erhöhten Temperaturen deutlich stabiler. Die Herstellung redispergierbarer Lyophilisate von PEG-*b*-PCL Mizellen war in Anwesenheit zusätzlicher, kristallisationsverhindernder Hilfsstoffe machbar. Ein neuer Formulierungsansatz für PEG-*b*-PLA Mizellen bestehend aus Sagopilon-beladenen Polymerfilmen zur späteren Dispergierung wies eine Mindesthaltbarkeit von 3 Monaten hinsichtlich Stabilität und Redispergierbarkeit auf (Wirkstoffgehalt nach 3 Monaten bei 6 °C: (95.6 ± 1.4) %). Die Machbarkeit einer solchen Formulierung sowie der Nachweis der Temperatur als wichtigster stabilititätsbestimmender Faktor konnten zum ersten

Mal gezeigt werden. Unabhängig des verwendeten Polymers waren die Sagopilon-haltigen Polymermizellen gleichermaßen wirksam gegenüber Zevixkarzinomzellen wie die Cyclodextrin- und Ethanol-haltige Referenzlösung. *In vivo* wurde eine sehr gute Verträglichkeit der unbeladenen Trägersysteme beobachtet und eine maximal tolerierbare Sagopilon-Dosis von 6 mg/kg bestimmt. Dieser Wert ist erniedrigt gegenüber einer Cyclodextrin-haltigen Sagopilonformulierung (10 mg/kg) und möglicherweise auf einen verlangsamten Wirkstoffabbau im Serum und somit einer höheren effektiven Dosis zurückzuführen. In ihrer Gesamtheit machen diese Ergebnisse deutlich, dass Polymermizellen, insbesondere auf Basis von PEG-b-PCL, wichtige Bedingungen zur Herstellung und Anwendung parenteraler Formulierungen erfüllen. Außerdem weisen sie ein exzellentes Potenzial für die weitergehende präklinische als auch klinische Testung von Sagopilon in verschiedenen Tumormodellen auf.

Kapitel 4 beschreibt Untersuchungen zur Struktur-Wirkungs-Beziehung neuartiger Glycerol-basierter Amphiphile hinsichtlich des Solubilisationsvermögens für Sagopilon, der Stabilität entsprechender Formulierungen und deren Zytotoxizität. Die untersuchten Amphiphile bestehen aus einer hydrophilen Kopfgruppe aus dendritischem Polyglycerol (2. Generation, PG[G2]) gekoppelt an einen C_{18}-Alkylrest und verschiedenen hydrophoben Modifikationen. Sie wiesen deutlich höhere Solubilisationskapazitäten verglichen mit parenteralen Standardhilfsstoffen auf. Die größte Löslichkeitsverbesserung wurde in Anwesenheit einer diaromatischen Struktur an der Schnittstelle zwischen hydrophober Schwanz- und hydrophiler Kopfgruppe erzielt und macht die bevorzugte Wirkstofflokalisation an der Grenzfläche zwischen Mizellkern und –hülle deutlich. Die definierte Zusammensetzung sowie die einfache und bequeme HPLC-UV Analytik ohne jegliche Probenvorbehandlung markieren weitere Vorteile dieser neuen Hilfsstoffe. Unabhängig von der vorliegenden Kernstruktur wiesen alle Formulierungen ein ausgezeichnetes Stabilitätsprofil auf und erfüllen somit weitere wichtige Voraussetzungen für

neue Hilfsstoffe. Die geringste Toxizität gegenüber primären Endothelzellen zeigte die Struktur, welche eine Naphthylendgruppe enthielt, mit vergleichbaren Werten wie Cremophor® EL und Polysorbate 80. Somit weisen diese neuartigen Amphiphile ein großes Potenzial hinsichtlich der Solubilisation und Formulierung schwerlöslicher Wirkstoffe auf.

Ausblick

Neben ihres ausgezeichneten Solubilisationsvermögens und ihrer guten Verträglichkeit, wie in der vorliegenden Arbeit gezeigt, besitzen Polymermizellen das Potenzial zur Verbesserung der Arzneitherapie, insbesonders in der Krebstherapie. Bezüglich der notwendigen Mizellstabilität könnte die Herstellung von „Mischpolymermizellen“ einen unkomplizierten Ansatz zur Stabilitätsverbesserung bieten. Frühere Studien, welche das *in vivo* Verhalten von PEG-*b*-Polyestern untersuchen, kommen nicht zu einem eindeutigen Resultat, insbesondere im Hinblick auf die Ab- oder Anwesenheit eines EPR-Effekts. Demzufolge bedarf es hier geeigneter Studien, welche ihren Schwerpunkt auf den Vergleich verschiedener Wirkstoffe, Polymere und Tumormodelle legen. Die vorliegenden Ergebnisse der Toxizitätstestung enthalten wertvolle Informationen, besonders im Vergleich zu der Cyclodextrin-haltigen Formulierung. Während vorangegangene Studien primär auf den Ersatz potentiell toxischer Solubilisatoren wie Cremophor® EL ausgelegt waren, stellt der Vergleich hinsichtlich des *in vivo* Verhaltens mit anderen parenteralen Hilfsstoffen, beispielsweise den Cyclodextrinen, den nächsten Entwicklungsschritt dar. Dieser Schritt ist wichtig, um das gesamte Potenzial dieser Block-Copolymere auszuschöpfen, ihre Stellung innerhalb der derzeit zugelassenen Hilfsstoffe zu bewerten und somit ihren Eintritt in die Standardformulierungsentwicklung zu fördern. Im Ergebnis wird dies hoffentlich dazu führen, dass in Zukunft eine größere Auswahl parenteral anwendbarer Solubilisatoren zur Verfügung steht, welche eine besseres Verträglichkeitsprofil aufweisen als beispielsweise Cremophor® EL. Außerdem ist zu

erwarten, dass die Weiterentwicklung auf dem Gebiet der Polymermizellen einen bedeutenden Beitrag zu einer sichereren Arzneitherapie leisten wird.

Im Gegensatz zu den getesteten Block-Copolymeren sind die untersuchten dendritischen Amphiphile weiter von einer klinischen Anwendung entfernt. Die erhaltenen Ergebnisse zeigen deutlich deren Nutzen als Solubilisatoren, welche die physikochemischen Aspekte für die Entwicklung stabiler parenteraler Formulierungen erfüllen. Außerdem enthalten Sie nützliche Informationen für die weitergehende Optimierung einer Leitstruktur, welche zusätzliche physiologische Aspekte wie Histaminausschüttung einbeziehen sollte.

Kurz zusammengefasst: Sowohl die hier beschriebenen Polymermizellen als auch die dendritischen Amphiphile stellen viel versprechende Ansätze dar alternative Formulierungsvehikel für die präklinische und klinische Forschung zur Verfügung zu stellen.

APPENDICES

ABBREVIATIONS

^{1}H NMR	^{1}H nuclear magnetic resonance spectroscopy
abbr.	Abbreviated
API	Active pharmaceutical ingredient
AUC	Area under plasma concentration-time curve
CARPA	Complement activation related pseudo-allergy
CD	Cyclodextrin
CELP	Cremophor® ELP
CMC	Critical micelle concentration
conc.	Concentration
Corp.	Corporation
cryoTEM	Cryogenic transmission electron microscopy
DF_{Core}	Degree of core functionalization
d_H	Hydrodynamic diameter
DLS	Dynamic light scattering
DMAc	N,N-dimethylacetamide
DSC	Differential scanning calorimetry
EMEA	European Medicines Agency
EPR-effect	Enhanced permeation and retention effect
F68	Pluronic® F68
FDA	Food and Drug Administration
Fig.	Figure
GRAS	Generally regarded as safe
HPLC	High performance liquid chromatography
UV	Ultraviolett
HPβCD	Hydroxypropyl-*β*-cyclodextrin
HSR	Hypersensitivity reactions
HTS	High throughput screening
HUVEC	Human umbilical vein endothelial cells
i.v.	Intravenous
IC_{50}	Inhibitory concentration
Inc.	Incorporation
KOH	Kalium hydroxide

MDR	multi-drug resistance
MPS	mononuclear phagocytic system
MTD	maximum tolerated dose
OECD	Organization for Economic Co-operation and Development
PAMAM	Poly(amido amine)
PDI	Polydispersity index
PDLLA	Poly(D,L-lactide)
PEG	Poly(ethylene glycol)
PEG-*b*-PCL	Poly(ethylene glycol)-*b*-poly(ε-caprolactone)
PEG-*b*-PGA	Poly(ethylene glycol)-*b*-poly(glycolide)
PEG-*b*-PLA	Poly(ethylene glycol)-*b*-poly(lactide)
PEI	Poly(ethylene imine)
PEO	Poly(ethylene oxide)
PG	Polyglycerol
Ph. Eur.	European Pharmacopoeia
PK	Pharmacokinetic
PLA	Poly(lactide)
PLLA	Poly(L-lactide)
PPO	Poly(propylene oxide)
PS80	Polysorbate 80
PVP	Poly(N-vinyl-pyrrolidone)
RP-HPLC	Reversed-phase HPLC
SBEβCD	Sulfobutylether-β-cyclodextrin
SE	Solubilization efficiency
SEC	Size exclusion chromatography
syn.	Synonym
$t_{1/2}$	Plasma half life
Tab.	Table

LIST OF PUBLICATIONS

Research Articles

A. Richter, C. Olbrich, M. Krause, T. Kissel. Solubilization of Sagopilone, a poorly water-soluble anticancer drug, using polymeric micelles for parenteral delivery.
Int. J. Pharm. 389 (2010) 244-253.
DOI: 10.1016/j.ijpharm.2010.01.032

A. Richter, C. Olbrich, M. Krause, J. Hoffmann, T. Kissel. Polymeric micelles for parenteral delivery of Sagopilone: Physicochemical characterization, novel formulation approaches and their toxicity assessment *in vitro* as well as *in vivo*.
Accepted for publication in *Eur. J. Pharm. Biopharm.* (2010).
DOI: 10.1016/j.ejpb.2010.02.010

A. Richter, A. Wiedekind, M. Krause, T. Kissel, R. Haag, C. Olbrich. Non-ionic dendritic glycerol-based amphiphiles: Novel excipients for the solubilization of poorly water-soluble anticancer drug Sagopilone.
Accepted for publication in *Eur. J. Pharm. Sci.* (2010).
DOI: 10.1016/j.ejps.2010.02.008

Conference Proceedings

A. Richter, C. Olbrich, M. Krause, T. Kissel. Polymeric Micellar Formulation Development of Sagopilone: The use of Differential Scanning Calorimetry as a prediction tool for stable formulations (Poster). Bayer Schering Pharma AG – Young Scientist Poster Session 2008, Berlin, Germany (10/29/2008).

A. Richter, C. Olbrich, M. Krause, T. Kissel. Polymeric Micelles for Sagopilone: The use of Differential Scanning Calorimetry as a prediction tool for stable drug solubilization (Poster). 36th Annual Meeting & Exposition of the Controlled Release Society, Copenhagen, Denmark (07/2009).

A. Richter, A. Wiedekind, M. Krause, T. Kissel, R. Haag, C. Olbrich. Dendritic glycerol-based amphiphiles: A novel class of solubilizing agents for poorly water-soluble drugs (Poster). Bayer Schering Pharma AG – Young Scientist Poster Session 2009, Berlin, Germany (10/27/2009).

Patent Applications

A. Richter, C. Olbrich, M. Krause, T. Kissel. Stable polymeric composition comprising an epothilone and an amphiphilic block copolymer. EP 09151405.9, submitted on Jan. 27, 2009.

Reports

A. Richter. Conference report: ISPT – International Symposium on Polymer Therapeutics. *Bayer HealthCare: GCD-TO newsletter* 14 (2007) 14-15.

CURRICULUM VITAE

Die Seite 143 (Curriculum Vitae) enthät persönliche Daten. Sie ist deshalb nicht Bestandteil der Online-Veröffentlichung.

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