European Journal of Pharmaceutical Sciences 45 (2012) 429–435

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Liposomes as carriers for colchicine-derived prodrugs: Vascular disrupting nanomedicines with tailorable drug release kinetics

Bart J. Crielaard [1], Steffen van der Wal [1], Huong Thu Le, Aloïs T.L. Bode, Twan Lammers, Wim E. Hennink, Raymond M. Schiffelers, Marcel H.A.M. Fens, Gert Storm *

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Available online 1 September 2011

Keywords:
Liposomes
Colchicine
Colchicinoids
Cancer
Prodrugs
Vascular disrupting agents

ABSTRACT

Newly formed tumor vasculature has proven to be an effective target for tumor therapy. A strategy to attack this angiogenic tumor vasculature is to initiate local blood vessel congestion and consequently induce massive tumor cell necrosis. Vascular disrupting agents (VDAs) typically bind to tubulin and consequently disrupt microtubule dynamics. Colchicine and its derivatives (colchicinoids) are very potent tubulin binding compounds but have a narrow therapeutic index, which may be improved by employing a liposomal targeting strategy. However, as a result of their physicochemical properties, colchicinoids are problematic to retain in liposomes, as they are released relatively rapidly upon encapsulation. To overcome this limitation, two hydrolyzable PEGylated derivatives of colchicine were developed for encapsulation into the aqueous core of long-circulating liposomes: a moderately rapid hydrolyzing PEGylated colchicinoid containing a glycolic acid linker (prodrug I), and a slower hydrolyzing PEGylated colchicinoid with a lactic acid linker (prodrug II). Hydrolysis studies at 37 °C and pH 7.4 showed that prodrug I possessed relatively rapid conversion characteristics ($t_{1/2}$ = 5.4 h) whereas prodrug II hydrolyzed much slower ($t_{1/2}$ = 217 h). Upon encapsulation into liposomes, colchicine was released rapidly, whereas both PEGylated colchicine derivatives were efficiently retained and appeared to be released only after cleavage of the PEG-linker. This study therefore demonstrates that, in contrast to colchicine, these novel PEGylated colchicine-derived prodrugs are retained within the aqueous interior after encapsulation into liposomes, and that the release of the active parent can be controlled by using different biodegradable linkers.

1. Introduction

Newly formed angiogenic endothelium has become an important target for the design of anticancer agents (Carmeliet and Jain, 2000). As tumor vasculature develops relatively fast during early tumor growth, blood vessels appear immature, disorganized and imperfect, which makes them a vulnerable target for cancer therapy (Baluk et al., 2005; Thorpe, 2004). Vascular disrupting agents (VDAs) initiate local disruption of tumor endothelium by interfering with the immature vasculature, causing site-specific blood vessel congestion. As a consequence, tumor cells are deprived from nutrients and oxygen, leading to rapid and massive tumor cell necrosis (Kanthou and Tozer, 2007; Lippert, 2007; Tozer et al., 2005). Tubulin binding agents (TBAs), such as colchicine and the structurally and pharmacologically similar colchicinoids, display vascular disrupting activity by binding irreversibly to tubulin at its colchicine domain, thereby inhibiting tubulin dynamics and microtubule formation (Bhattacharyya et al., 2008; Ravelli et al., 2004). Because endothelial cells in the tumor vasculature rely more on tubulin than actin to maintain their cell shape, the binding of TBAs to tubulin leads to rapid rounding of these cells, resulting in loss of vascular integrity and, ultimately, to hemostasis (Jordan and Wilson, 2004; Thorpe, 2004). TBAs are considered to be useful in cancer therapy not only because of their ability to disrupt existing angiogenic tumor vasculature, but also because of their capabilities to induce mitotic arrest of tumor cells and inhibition of angiogenesis (Jordan and Wilson, 2004; Lippert, 2007; Pasquier et al., 2006; Pasquier and Kavallaris, 2008).

Colchicine is currently used clinically in low doses for the treatment of acute gout (Keith and Gilliland, 2007; Petersel and Schlesinger, 2007), familial Mediterranean fever (FMF) (Cerquaglia et al., 2005), and dermatologic (Bibas et al., 2005) and autoinflammatory diseases (Terkeltaub, 2009). However, the utility of colchicine for cancer therapy is currently limited, as only doses close to the maximal tolerated dose (MTD) can induce reduction in tumor blood perfusion leading to a high risk for toxicity (Baguley et al., 1991; Nihei et al., 1999).

* Corresponding author. Address: Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands. Tel.: +31 30 2537388; fax: +31 30 2517839.
E-mail address: G.Storm@uu.nl (G. Storm).
[1] These authors contributed equally to this work.

0928-0987/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejps.2011.08.027

A potential strategy for improving the therapeutic index of colchicine, i.e. limiting the side effects and improving the efficacy, may be the employment of a colloidal drug delivery system, such as long-circulating liposomes (Fenske and Cullis, 2008; Lammers et al., 2008). Advantages associated with the use of long-circulating liposomes include their prolonged circulation kinetics, their passive targeting properties, as well as the possibility to encapsulate both hydrophobic and hydrophilic drugs (Torchilin, 2005). However, it remains a challenge to formulate long-circulating liposomes that allow the retention of drugs with moderate lipophilicity, such as colchicinoids, while enabling appropriate release kinetics once accumulated in the target site (Coimbra et al., 2011). Colchicinoids localize mainly in the lipid bilayer and leak out readily, which makes it troublesome to control their retention and release rate (Kulkarni et al., 1997; Mons et al., 2000). A potential way to overcome this challenge is to make use of prodrugs, which are (inactive) bioreversible derivatives of the active drug but often possess different physicochemical properties than the parent compound (Coimbra et al., 2011).

After enzymatic and/or chemical conversion of the prodrug, the parent molecule with its original pharmacological activity is recovered (Rautio et al., 2008). If the prodrug is designed to be very hydrophilic, it may be encapsulated solely in the aqueous core of liposomes, thereby limiting its diffusion over the lipid bilayer. Only upon conversion of the prodrug in the aqueous interior, the parent drug molecule, in this case the colchicinoid, may be released from the liposome by its ability to diffuse over the lipid bilayer. A useful method for developing hydrophilic prodrugs of small molecular weight drugs is the conjugation with poly(ethylene glycol) (PEG). PEG has high aqueous solubility, it is non-toxic, non-immunogenic and non-antigenic (Bell et al., 2007; Parveen and Sahoo, 2006). In addition, by utilizing a PEG-linker that is susceptible to hydrolysis, PEGylated prodrugs that can be cleaved in the liposomal interior are created. The molecular structure of the linker can influence the rate of hydrolysis, and therefore, by employing different linkers, the rate of conversion to the active drug can be controlled (Roberts et al., 2002).

In the current study, two PEGylated colchicinoid prodrugs were synthesized and encapsulated into long-circulating liposomes. The presented strategy allows retention of the colchicinoids – in their prodrug form – in the aqueous core of the liposomes, and at the same time enables release of the cleaved prodrug from the liposome, at a rate that can be tailored by adjustment of the hydrolysis kinetics (Fig. 1). The hydrolysis rate is controlled by using a hydrolysable PEG-linker based on a primary alcohol, which is more prone hydrolysis, or a linker based on a secondary alcohol, which is more resistant to hydrolysis. Since colchicinoids are able to pass the lipid bilayer, the release rate of colchicinoid from the liposome is directly related to the cleavage rate of the colchicinoid prodrug. Obviously, the release kinetics of colchicinoids from the liposomes, once they have accumulated in the tumor, is a critical determinant for therapeutic activity. Therefore, a tailorable drug delivery system for colchicine derivates may prove of great value for improving the therapeutic index of colchicinoids in the therapy of solid tumors.

2. Material and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000}) and dipalmitoylphosphatidylcholine (DPPC) were provided by Lipoid (Germany). All other chemicals were ordered from Sigma–Aldrich (Germany) and used without further purification. Phosphate Buffered Saline (PBS) with pH 7.4 was purchased from B. Braun (The Netherlands). Solvents were obtained from Biosolve (The Netherlands) and used without distillation. Acetonitrile and dichloromethane were stored on molecular sieves prior to use (3 and 4 Å, respectively). All reactions were performed without direct lighting and with flasks covered in aluminum foil to prevent degradation of colchicine. Flash chromatography was performed using silica gel of 0.035–0.070 mm 60 Å mesh (Acros Organics, Belgium). TLC analysis was performed using plastic backed silica 60 F_{254} plates (Merck, Germany) and NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. ESI mass analysis was performed on a Shimadzu LCMS-QP8000 single quadrupole spectrometer in positive ionization mode.

2.2. Synthesis of N-Boc-colchicine (2)

Colchicine (**1**, 4.9 g, 12.3 mmol) and DMAP (1.5 g, 12.5 mmol) were dissolved in acetonitrile (50 mL). Subsequently Boc_2O (17.4 g, 79.7 mmol), dissolved in acetonitrile (50 mL), and dry Et_3N (3 mL, 21.5 mmol) were added. The reaction mixture was refluxed for 1.5 h after which additional Boc_2O was added (1.6 g,

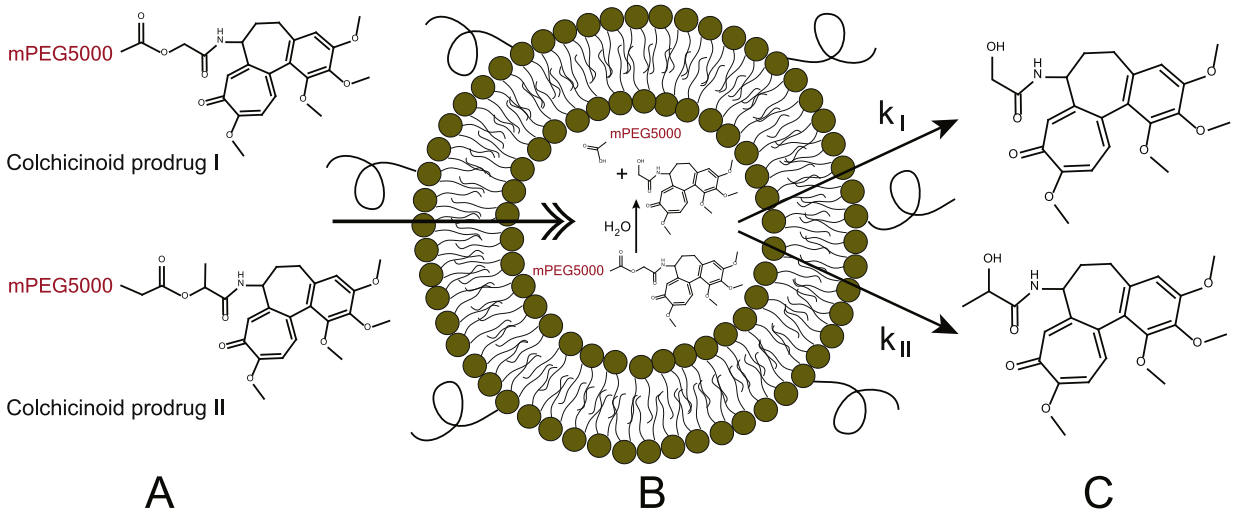

Fig. 1. Schematic outline of the liposomal colchicine-derived prodrug strategy. PEGylated colchicine-derived prodrugs with different hydrolysis kinetics are synthesized by using different biodegradable PEG-linkers (A). These colchicinoid prodrugs are encapsulated into long-circulating liposomes (B). Upon hydrolysis of prodrug I or II in the aqueous core of the liposomes, the pharmacologically active parent is released from the liposomes with rate k_I and k_{II} ($k_I > k_{II}$), respectively (C).

7.3 mmol) and stirred at RT for an additional 2 h. The reaction mixture was concentrated *in vacuo* and re-dissolved in chloroform. The chloroform was extracted three times with saturated aqueous citric acid (pH 2), and the aqueous fractions extracted again with chloroform. The combined organic extracts were washed with brine and dried over $MgSO_4$. After flash chromatography in ethylacetate/acetone 4:1, **2** was obtained as a brownish yellow solid (3.52 g, 57%).

TLC : $R_f = 0.35$ in EtOAc/acetone 4 : 1

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (s, 9H), 1.90–2.16 (m, 2H), 2.28 (s, 3H), 2.44–2.70 (m, 2H), 3.65 (s, 3H), 3.89, 3.93, 3.97 (3 × s, 3 × 3H), 5.14 (dd, 1H), 6.52 (s, 1H), 6.77 (d, 1H, *J* = 10.7 Hz), 7.21 (d, 1H, *J* = 10.7 Hz), 7.60 (s, 1H) ppm.

2.3. Synthesis of N-Boc-deacetylcolchicine (3)

N-Boc-colchicine (**2**, 3.52 g, 7.0 mmol) was dissolved in methanol (40 mL). Sodium methoxide in methanol (9 mL 25% w/v, ~40 mmol) was added at 4 °C and stirred for 45 min. The reaction was stopped by addition of saturated NH_4Cl solution (40 mL). Excess brine was added and the now cloudy suspension was extracted with Et_2O. After drying over $MgSO_4$ and concentration *in vacuo* a light brown solid was obtained.

The progress of the reaction was followed by NMR (R_f was also 0.35 in EtOAc: acetone 4:1), which showed complete removal of the acetyl group. The product was deemed pure enough and was used directly without further purification.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37 (s, 9H), 1.63–1.70 (m, 1H), 2.22–2.51 ppm (m, 3H), 3.65 (s, 3H), 3.89, 3.93, 3.99 (3 × s, 9H), 4.36–4.41 (m, 1H), 4.95 (d, 1H, *J* = 7.4 Hz), 6.52 (s, 1H), 6.81 (d, 1H, *J* = 10.7 Hz), 7.26 (d, 1H, *J* = 10.7 Hz), 7.51 (s, 1H) ppm.

2.4. Synthesis of N-deacetylcolchicine (4)

N-Boc-deacetylcolchicine (**3**) was dissolved in CH_2Cl_2 (40 mL) after which TFA (9 mL) was added. The reaction mixture was stirred for 2 h after which a saturated sodium carbonate solution (pH approximately 10) was carefully added to quench the reaction. This was extracted twice with CH_2Cl_2 and once with EtOAc. After pooling the organic fractions, drying over $MgSO_4$ and concentration *in vacuo*, a thick brown oil was obtained. After flash chromatography with CH_2Cl_2/MeOH/Et_3N (90:10:0.1 R_f 0.38) and concentration, **4** was obtained as a slightly yellow solid (1.98 g, 79% in two steps).

ESI − MS$[M + Na]^+ = 380.05$, calculated 380.15

$[M + Na + CH_3CN]^+ = 420.90$, calculated 421.17

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.56–1.62 (m, 1H + broad S, $-NH_2$, disappeared on D_2O addition), 2.26–2.47 (m, 3H), 3.64 (s, 3H), 3.64–3.72 (m, 1H), 3.89 (s, 6H), 3.98 (s, 3H), 6.52 (s, 1H), 6.78 (d, 1H, *J* = 10.7 Hz), 7.17 (d, 1H, *J* = 10.7 Hz), 7.73 (s, 1H) ppm.

2.5. Synthesis of N-(2-hydroxyacetyl)-deacetylcolchicine (5)

N-Deacetylcolchicine (**4**, 250 mg 0.67 mmol), glycolic acid (50 mg, 0.66 mmol), Et_3N (0.3 mL, 2 mmol) and *N*-hydroxysuccinimide (60 mg, 0.5 mmol) were dissolved in CH_2Cl_2 (10 mL). DIC (150 μL, 0.95 mmol) was added and stirred for 16 h. After concentration *in vacuo*, flash column chromatography was performed using CH_2Cl_2/MeOH (90:10). After filtration in water followed by lyophilization, **5** was obtained as a slightly yellow solid (75 mg, 27%).

TLC : $R_f = 0.40$ in $CHCl_3$/MeOH/AcOH 90 : 10 : 0.1

ESI − MS$[M + Na]^+ = 438.20$, calculated 438.15

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.96–2.58 (4 × m, 5H), 3.64 (s, 3H), 3.90, 3.93, 3.98 (3 × s, 9H), 4.04–4.20 (m, 2H), 4.63–4.75 (m, 1H), 6.54 (s, 1H), 6.86 (d, 1H, *J* = 10.7 Hz), 7.33 (d, 1H, *J* = 10.7 Hz), 7.60 (s + broad s, 2H) ppm.

^{13}C NMR (300 MHz, $CDCl_3$) δ 30.1, 37.0, 52.0, 56.3, 56.5, 61.6, 61.7, 62.5, 107.6, 113.0, 125.9, 131.3, 134.4, 135.7, 137.1, 141.9, 151.4, 152.0, 153.8, 164.2, 172.8, 179.9 ppm.

2.6. Synthesis of mPEG-acetyl-N-(2-hydroxyacetyl)-deacetylcolchicine ester (prodrug I, 6)

N-(2-Hydroxyacetyl)-deacetylcolchicine (**5**, 20 mg, 0.048 mmol), DMAP (15 mg, 0.12 mmol) and $mPEG_{5000}$-acetic acid (210 mg, 0.041 mmol) were dissolved in 5 mL CH_2Cl_2. DIC (15 μL, 0.095 mmol) was added and the reaction mixture was stirred for 16 h at room temp. Next, the reaction mixture was concentrated *in vacuo* and dissolved in ammonium acetate buffer (3 mL, pH 5.5, 20 mM). This was filtered and separated on a PD-10 column (GE Healthcare, Belgium) equilibrated with the same buffer. After two gel filtrations, no low molecular weight starting material could be detected on TLC ($CHCl_3$/MeOH/AcOH, 90:10:0.1). The PEG-conjugate was precipitated in diethyl ether, collected by centrifugation, and after drying *in vacuo*, prodrug I (**6**) was obtained as a slightly yellow solid (120 mg, 52%, composed of a mixture of unreacted mPEG-acetic acid and **6**). The amount of colchicine derivate conjugated to PEG was determined with an UPLC system (Waters) equipped with an Acquity BEH C18 1.7 μm column (2.1 × 50 mm) (Waters) and photodiode array detector (Acquity PDA, Waters) set at 350 nm, using a gradient mobile phase from 5% to 95% acetonitrile in water with trifluoroacetic acid (TFA) as modifier.

2.7. Synthesis of N-(2-hydroxypropionyl)-deacetylcolchicine (7)

N-Deacetylcolchicine (**4**, 15 mg, 0.04 mmol), *N*-hydroxysuccinimide (7 mg, 0.06 mmol), DL-lactic acid (5.4 mg, 0.06 mmol) and 10 μL of Et_3N were dissolved in CH_2Cl_2 (3 mL). DCC (12.4 mg, 0.06 mmol) in CH_2Cl_2 (3 mL) was subsequently added and stirred for 16 h. After concentration *in vacuo* the product was purified using flash chromatography in CH_2Cl_2/MeOH 9:1. Further purification with flash chromatography using chloroform/MeOH/Et_3N 91:9:0.2 (R_f = 0.4) yielded **7** as a yellow solid (14 mg, 82%).

ESI − MS$[M]^+ = 428.10$, calculated 428.46

^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.35 (d, 3H), 1.88–2.59 (4 × m, 5H), 3.62 (s, 3H), 3.89, 3.93, 3.98 (3 × s, 9H), 4.22 (q, 1H), 4.64 (m, 1H), 6.54 (s, 1H), 6.87 (d, 1H, *J* = 11.0), 7.32 (d, 1H, *J* = 10.7), 7.45 (s, 2 × 1H) ppm.

^{13}C NMR (300 MHz, $CDCl_3$) δ 20.8, 30.1, 36.8, 51.8, 56.3, 56.5, 61.4, 61.7, 68.4, 107.6, 113.1, 125.8, 131.2, 134.5, 135.7, 137.0, 141.8, 151.4, 151.9, 153.7, 164.1, 175.6, 179.8 ppm.

2.8. Synthesis of mPEG-propionyl-N-(2-hydroxypropionyl-)-deacetyl colchicine ester (prodrug II, 8)

N-(2-Hydroxypropionyl)-deacetylcolchicine (**7**, 14 mg, 0.033 mmol), DMAP (15 mg, 0.12 mmol) and $mPEG_{5000}$-propionic acid (210 mg, 0.041 mmol) were dissolved in CH_2Cl_2 (5 mL). DIC (15 μL, 0.095 mmol) was added and stirred for 16 h. The reaction mixture was concentrated *in vacuo*, dissolved in ammonium acetate buffer (pH 5.5, 20 mM), filtered and finally separated on a PD-10 column (GE Healthcare, Belgium) equilibrated with the same buffer. No low molecular weight starting material could be detected by TLC ($CHCl_3$/MeOH/AcOH, 90:10:0.1). After lyophilization, prodrug II (**8**) was obtained as a stringy offwhite solid (153 mg, 67%,

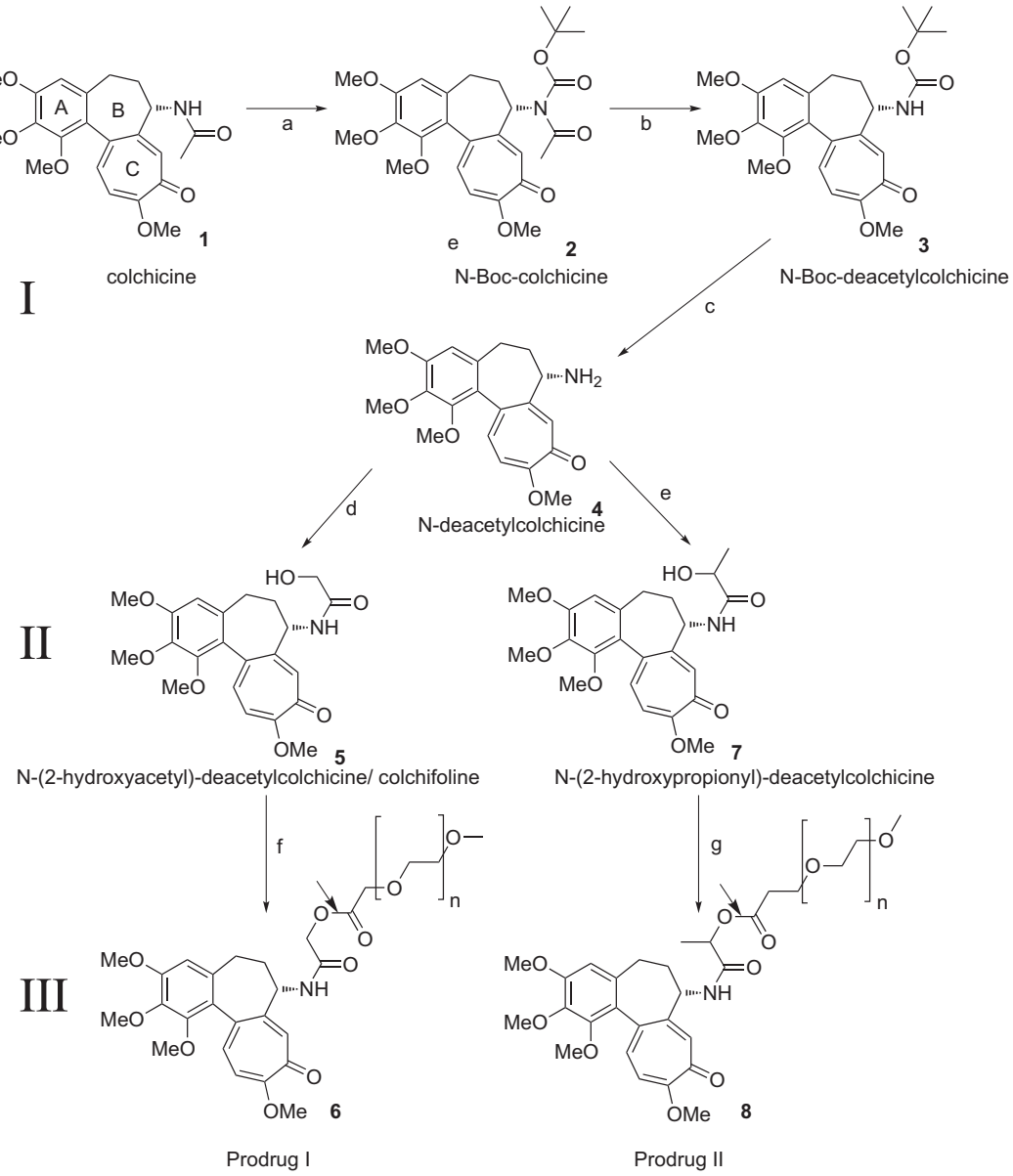

Fig. 2. Synthesis of PEGylated colchicine-derived prodrugs. Prodrug I and II are synthesized in three phases. In phase I, colchicine is deacetyled in three steps. First, the acetamide is Bocylated; second, the acetyl group is removed using methoxide; third, the Boc group is removed using TFA, yielding intermediate **4**. In phase II, the deacetylcolchicine is reacted with glycolic acid or lactic acid by carbodiimide coupling to give the hydroxyl-functionalized colchicine derivatives **5** and **7**, respectively. In phase III, the hydroxyl group is conjugated via a hydrolysable ester bond to PEG bearing a carboxyl moiety (indicated by an arrow).Reagents: (a) Boc_2O, DMAP, Et_3N, Acetonitrile; (b) NaOMe, MeOH; (c) TFA, DCM; (d) glycolic acid, DIC, *N*-hydroxysuccinimide, Et_3N, CH_2Cl_2; (e) lactic acid, DCC, *N*-hydroxysuccinimide, Et_3N, CH_2Cl_2; (f) $mPEG_{5000}$-CH_2COOH, DIC, DMAP, CH_2Cl_2; (g) $mPEG_{5000}$-$(CH_2)_2COOH$, DIC, DMAP, CH_2Cl_2.

composed of a mixture of unreacted mPEG-propionic acid and **8**). The amount of colchicine derivate conjugated to PEG was determined by UPLC, as described in Section 2.6.

2.9. In vitro degradation kinetics

The hydrolysis kinetics of the prodrugs were studied at 4 °C and 37 °C in phosphate buffer (20 mM, pH 7.4) for both prodrug I (**6**) and prodrug II (**8**), and at 37 °C in sodium hydrogen carbonate buffer (20 mM, pH 9.0) for prodrug II (**8**). Samples were taken at several time points during 72 h, and stored at −20 °C before analysis. The concentration of both PEGylated and hydrolyzed colchicine derivatives was determined by UPLC, as described in Section 2.6.

2.10. Encapsulation in liposomes

DPPC, DPSE-PEG_{2000} and cholesterol were dissolved in ethanol in a molar ratio of 1.85:0.15:1. Lipid films were formed by rotary evaporation, which were dried further under a nitrogen flow for 30 min. The lipid films were hydrated with solutions of colchicine (40 mg/mL), prodrug I (90 mg/mL) or prodrug II (60 mg/mL) in PBS to form liposomes. The size and polydispersity of the liposomes were decreased by repeated extrusions using an extruder (LIPEX, Northern Lipids Inc, Canada) equipped with polycarbonate filters with pore sizes of 200 nm and 100 nm (Whatman, USA). Colchicine and prodrugs not encapsulated into the liposomes was removed by ultracentrifugation (Beckman Coulter, The Netherlands) for 45 min at 250,000 g. After resuspending the liposome pellet in PBS, the particle size and size distribution were measured by dynamic light

scattering using a Malvern ALV CGS-3 (Malvern Instruments, UK). The concentration of colchicine and colchicine-derived prodrugs in the liposome dispersions was determined by UPLC, as described in Section 2.6.

2.11. In vitro release kinetics

The *in vitro* release kinetics of colchicine and colchicine-derived prodrugs from long-circulating liposomes were determined by dialysis of the liposome formulations against PBS at 4 °C and 37 °C. 1 mL of liposome dispersion was added to a Slide-A-Lyzer Cassette (Thermo Scientific, USA) with a molecular weight cut-off of 10 kDa, which was dialyzed against 200 mL of PBS. Samples were taken from the dialysate at several time points during 72 h, and stored at −20 °C before measurement by UPLC, as described in Section 2.6.

2.12. Data analysis

The hydrolysis and liposomal release data were analysed using Graphpad Prism v5.03 (Graphpad Software Inc, USA). LogP calculations were performed with MarvinSketch 5.3.2 (ChemAxon Ltd, Hungary).

3. Results and discussion

3.1. Synthesis of colchicine-derived prodrugs

Two novel types of PEGylated colchicine-derived prodrugs were synthesized following the scheme depicted in Fig. 2. Since colchicine lacks functional groups suitable for conjugating PEG, derivatives were synthesized and modified at the acetamide moiety at the B-ring of colchicine, which is not part of the pharmacophore and not essential for its tubulin binding activity (Bhattacharyya et al., 2008; Nguyen et al., 2005). Earlier studies have shown that modification at this site indeed does not result in a loss of activity, provided that the modification does not cause too much steric hindrance (Bagnato et al., 2004; Bombuwala et al., 2006; Lagnoux et al., 2005). The *N*-acetyl moiety of colchicine was substituted with an *N*-2-hydroxyacetyl- or *N*-2-hydroxypropionyl moiety, which are both suitable for esterification (Fig. 2). These colchicinoids (**5** and **7**) were synthesized in two steps, monitored by ESI-MS and NMR. First, the acetamide moiety of colchicine (**1**) was protected (**2**), deacetylated (**3**) and deprotected (**4**), with a final yield of 40%, using a strategy based on earlier reported methodology (Fig. 2, phase I) (Bagnato et al., 2004; Lagnoux et al., 2005). Then, the obtained deacetylcolchicine was re-acylated with glycolic acid (**5**, yield 27%) and lactic acid (**7**, yield 82%), respectively, allowing for conjugation with an acid-functionalized methoxy PEG (Fig. 2, phase II). The glycolic acid derivative, also known as colchifoline, has similar tubulin binding properties and toxicity as its parent compound colchicine (Brossi et al., 1983). Although the synthesis of colchifoline has been described in the literature (Iorio et al., 1981), a more straightforward method for the synthesis of both colchifoline and the related compound **7** is presented here. By using of *N*,*N*′-dicyclohexylcarbodiimide (DCC) instead of acid chlorides in the modification of colchicine, a higher yield of colchicine derivatives could be achieved (Sharma et al., 1984).

Finally, the conjugation of PEG_{5000} by esterification was performed using methoxy PEG-acetic acid to obtain prodrug I (**6**) and methoxy PEG-propionic acid to obtain prodrug II (**8**) (Fig. 2, phase III). After conjugation, the product consisted of a mixture of unreacted PEG and colchicine-derived prodrug. The amount of colchicinoid prodrug present in each synthesized product was quantified by UPLC, which was 34% (w/w) for prodrug I and 74% (w/w) for prodrug II.

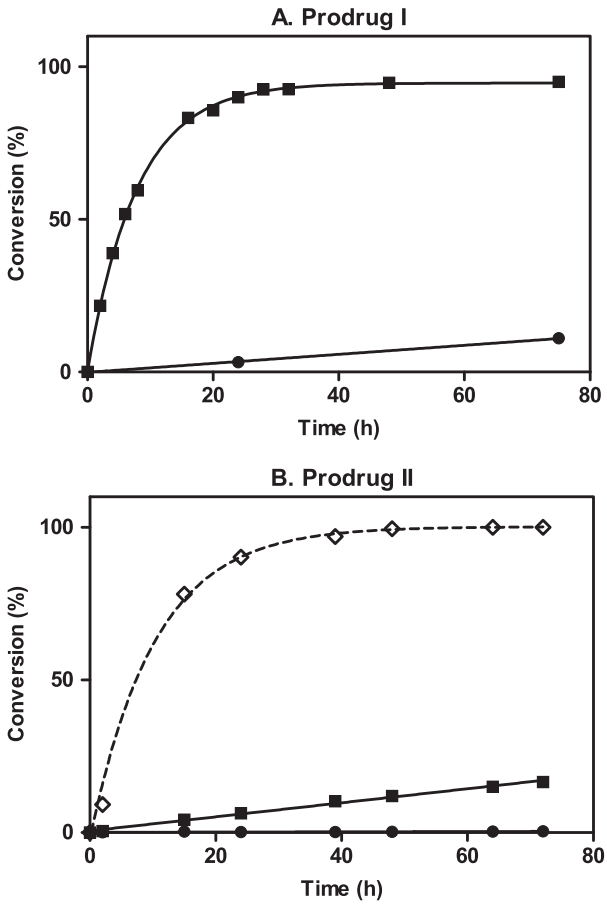

Fig. 3. Hydrolysis of PEGylated colchicine-derived prodrugs. The hydrolysis in phosphate buffer of pH 7.4 at 4 °C (●) and 37 °C (■) was measured for both prodrug I (A) and prodrug II (B). For prodrug I, the calculated hydrolysis half-life ($t_{1/2}$) at 4 °C was 14 d (zero-order kinetics, r^2 0.64) and at 37 °C 5.4 h (first-order kinetics, r^2 1.00). For prodrug II in phosphate buffer of pH 7.4 at 4 °C (●), the calculated hydrolysis half-life ($t_{1/2}$) was 500 d (zero-order kinetics, r^2 0.69) and at pH7.4 at 37 °C (■) it was 9 d (zero-order kinetics, r^2 0.87). In order to demonstrate the liability of prodrug II to hydrolysis, the hydrolysis was also determined in carbonate buffer of pH 9 at 37 °C (◇), resulting in a calculated hydrolysis half-life of 7.1 h (first-order kinetics, r^2 = 0.99).

3.2. In vitro hydrolysis kinetics

It was anticipated that the synthesized PEG-colchicinoids are hydrolyzed at different rates under physiological conditions. The main difference between prodrug I and prodrug II is that prodrug I is an ester of a primary alcohol, which is more prone to hydrolysis than esters of secondary alcohols, such as prodrug II. Additionally, the closer proximity of the ether moiety to the carboxylic end group of the mPEG in prodrug I further influences the degradation rate due to electronic destabilization of the ester bond (Roberts et al., 2002). Incubation of prodrug I in 20 mM phosphate buffer with pH 7.4 at 37 °C resulted in first-order hydrolysis kinetics with a calculated half-life ($t_{1/2}$) of 5.4 h and degradation of approximately 90% of the total amount after 24 h incubation (Fig. 3A). Furthermore, the hydrolysis in PBS at 4 °C appeared to be slow (zero-order kinetics, calculated hydrolysis $t_{1/2}$ = 14 d). Prodrug II was found to be more resistant to hydrolysis at pH 7.4, with a calculated half-life of approximately 9 d at 37 °C and a virtually absent degradation at 4 °C (0.4% after 72 h) (Fig. 3B). When the pH was increased to pH 9, to assess the susceptibility of prodrug II to hydrolysis, the calculated half-life at 37 °C dropped to 7.1 h.

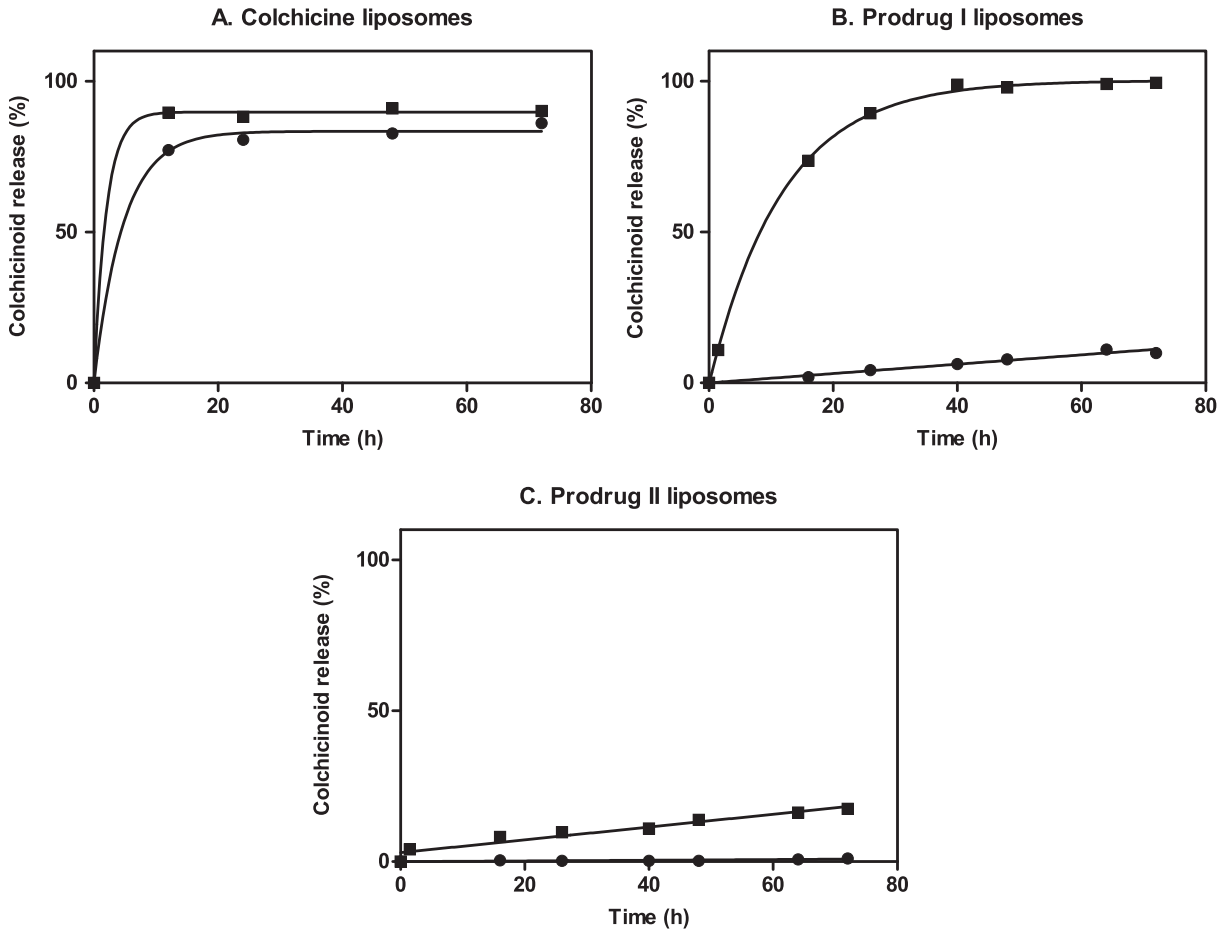

Fig. 4. Release of colchicine and colchicine derivatives from long-circulating liposomes. Colchicine and colchicine-derived prodrugs were encapsulated into long-circulating liposomes. Upon dialysis against PBS at 4 °C (●) and 37 °C (■), unmodified colchicine leaked out very rapidly (A). The release kinetics of colchicinoid from liposomes encapsulating prodrug I (B) was more controlled, with slow release at 4 °C (zero-order kinetics, r^2 0.96, release $t_{1/2}$ = 13 d) and faster release at 37 °C (first-order kinetics, r^2 1.00, release $t_{1/2}$ = 8.2 h). The colchicinoid release from liposomes encapsulating prodrug II (C) was much slower: at 4 °C the release was negligible (zero-order kinetics, r^2 0.73, release $t_{1/2}$ = 181 d), while at 37 °C the release was higher (zero-order kinetics, r^2 0.94, release $t_{1/2}$ = 10 d), but still slow as compared to prodrug I.

3.3. Liposomal encapsulation and release

3.3.1. Liposomes containing colchicine

Colchicine was encapsulated in long-circulating liposomes with a mean particle size of around 100 nm and low polydispersity, using the well-known lipid film hydration method. As expected, due to its physicochemical properties, colchicine was released readily (Fig. 4A). This has been observed before (Kulkarni et al., 1997). Colchicine has moderate water solubility with a partition coefficient (logP) of around 1.0 (Quinn et al., 1981; Zamora et al., 1988), and localizes in the lipid bilayer (Mons et al., 2000). The rapid release, i.e. 100% within 24 h, indicates that colchicine will also leak out of the liposomes when they are circulating in the bloodstream before they reach the target site, making this formulation unsuitable for therapeutic applications.

3.3.2. Liposomes containing colchicine-derived prodrugs

By conjugating colchicine to PEG_{5000} using a biodegradable linker, prodrugs with large molecular weight and high aqueous solubility (calculated logP: −4.44 (prodrug I) and −3.64 (prodrug II)) were obtained. The improved solubility allows encapsulation into the aqueous core of long-circulating liposomes. Both prodrug I and II were entrapped into long-circulating liposomes with a mean diameter of around 100 nm and low polydispersity. At pH 7.4, the two liposomal prodrug formulations showed a release profile of colchicinoid that strongly resembled the hydrolysis kinetics of each prodrug. At 37 °C, long-circulating liposomes loaded with prodrug I showed a rapid release of colchicinoid (first-order kinetics, release $t_{1/2}$ = 8.2 h) (Fig. 4B), whereas liposomes containing prodrug II released the colchicinoid much slower (zero-order kinetics, release $t_{1/2}$ = 10 d) (Fig. 4C). At 4 °C, the release of colchicinoid from liposomes loaded with prodrug I was slow (zero-order kinetics, release $t_{1/2}$ = 13 d h), whereas almost no release was observed from liposomes loaded with prodrug II (zero-order kinetics, release $t_{1/2}$ = 181 d). These data demonstrate that the release of colchicinoid is mainly determined by the hydrolysis rate of the prodrug, as both prodrugs have similar physicochemical characteristics as such. The partitioning coefficients of the colchicinoids used for the synthesis of prodrug I and II are similar to that of colchicine (calculated logP: 0.6 and 1.2 respectively), which indicates that these colchicinoids, like colchicine, will be rapidly released from the long-circulating liposomes. Both prodrugs, however, are retained within the aqueous core of the liposomes, and only after cleavage of the PEG chain by hydrolysis of the ester bond, the formed colchicinoid can diffuse over the lipid bilayer. These results demonstrate that by adjusting the PEG-linker, and thus modifying the hydrolysis rate of the colchicine-derived prodrugs, the release rate of colchicinoid from liposomes can be tailored. After i.v. administration, targeted nanomedicines such as liposomes need time to localize passively in the target tissue, and during this period the drug should not be released. On the other hand, there should be efficient drug release after localization in the target area

to induce a therapeutic effect. Although the optimal release profile at the target site needs to be established experimentally, the presented prodrug strategy shows that premature leakage in the circulation is prevented.

4. Conclusions

This work demonstrates that the release kinetics of colchicinoids from long-circulating liposomes can be tailored by encapsulating rationally designed PEGylated colchicinoid prodrugs that hydrolyze in the aqueous interior. Two PEGylated colchicine-derived prodrugs were synthesized by substituting the acyl-moiety with a hydroxyl containing moiety to allow for esterification with an acid-functionalized PEG. By employing glycolic or lactic acid to functionalize colchicine, and by using different PEG acids, the hydrolysis rate of the prodrug could be controlled. Both designed prodrugs were retained in liposomes, whereas colchicine leaked out instantaneously. Indeed, the release kinetics of colchicinoids from the liposomal prodrug formulations appeared determined by the hydrolysis rate of the prodrug.

Consequently, by using different biodegradable linkers, the release of colchicinoids from long-circulating liposomes can be tailored.

Acknowledgements

This work was supported by MediTrans, an Integrated Project funded by the European Commission under the ''nanotechnologies and nano-sciences, knowledge-based multifunctional materials and new production processes and devices'' (NMP), thematic priority of the Sixth Framework Program.

References

Bagnato, J.D., Eilers, A.L., Horton, R.A., Grissom, C.B., 2004. Synthesis and characterization of a cobalamin-colchicine conjugate as a novel tumor-targeted cytotoxin. J. Org. Chem. 69, 8987–8996.

Baguley, B.C., Holdaway, K.M., Thomsen, L.L., Zhuang, L., Zwi, L.J., 1991. Inhibition of growth of colon 38 adenocarcinoma by vinblastine and colchicine: evidence for a vascular mechanism. Eur. J. Cancer 27, 482–487.

Baluk, P., Hashizume, H., McDonald, D.M., 2005. Cellular abnormalities of blood vessels as targets in cancer. Curr. Opin. Genet. Dev. 15, 102–111.

Bell, S.J., Fam, C.M., Chlipala, E.A., Carlson, S.J., Lee, J.I., Rosendahl, M.S., Doherty, D.H., Cox, G.N., 2007. Enhanced circulating half-life and antitumor activity of a site-specific pegylated interferon-α protein therapeutic. Bioconjug. Chem. 19, 299–305.

Bhattacharyya, B., Panda, D., Gupta, S., Banerjee, M., 2008. Anti-mitotic activity of colchicine and the structural basis for its interaction with tubulin. Med. Res. Rev. 28, 155–183.

Bibas, R., Gaspar, N.K., Ramos-e-Silva, M., 2005. Colchicine for dermatologic diseases. J. Drugs Dermatol. 4, 196–204.

Bombuwala, K., Kinstle, T., Popik, V., Uppal, S.O., Olesen, J.B., Vina, J., Heckman, C.A., 2006. Colchitaxel, a coupled compound made from microtubule inhibitors colchicine and paclitaxel. Beilstein J. Org. Chem. 2, 13.

Brossi, A., Sharma, P.N., Atwell, L., Jacobson, A.E., Iorio, M.A., Molinari, M., Chignell, C.F., 1983. Biological effects of modified colchicines. 2. Evaluation of catecholic colchicines, colchifolines, colchicide, and novel *N*-acyl- and *N*-aroyldeacetylcolchicines. J. Med. Chem. 26, 1365–1369.

Carmeliet, P., Jain, R.K., 2000. Angiogenesis in cancer and other diseases. Nature 407, 249–257.

Cerquaglia, C., Diaco, M., Nucera, G., La Regina, M., Montalto, M., Manna, R., 2005. Pharmacological and clinical basis of treatment of Familial Mediterranean Fever (FMF) with colchicine or analogues: an update. Curr. Drug Targets Inflamm. Allergy 4, 117–124.

Coimbra, M., Isacchi, B., van Bloois, L., Torano, J.S., Ket, A., Wu, X., Broere, F., Metselaar, J.M., Rijcken, C.J.F., Storm, G., Bilia, R., Schiffelers, R.M., 2011. Improving solubility and chemical stability of natural compounds for medicinal use by incorporation into liposomes. Int. J. Pharm. 416, 433–442.

Fenske, D.B., Cullis, P.R., 2008. Liposomal nanomedicines. Expert. Opin. Drug. Deliv. 5, 25–44.

Iorio, M.A., Molinari, M., Brossi, A., 1981. Synthesis of colchifoline from deacetylcolchiceine. Can. J. Chem. 59, 283–284.

Jordan, M.A., Wilson, L., 2004. Microtubules as a target for anticancer drugs. Nat. Rev. Cancer 4, 253–265.

Kanthou, C., Tozer, G.M., 2007. Tumour targeting by microtubule-depolymerizing vascular disrupting agents. Expert Opin Ther Targets 11, 1443–1457.

Keith, M.P., Gilliland, W.R., 2007. Updates in the management of gout. Am. J. Med. 120, 221–224.

Kulkarni, S.B., Singh, M., Betageri, G.V., 1997. Encapsulation, stability and in-vitro release characteristics of liposomal formulations of colchicine. J. Pharm. Pharmacol. 49, 491–495.

Lagnoux, D., Darbre, T., Schmitz, M.L., Reymond, J.L., 2005. Inhibition of mitosis by glycopeptide dendrimer conjugates of colchicine. Chem. Eur. J. 11, 3941–3950.

Lammers, T., Hennink, W.E., Storm, G., 2008. Tumour-targeted nanomedicines: principles and practice. Br. J. Cancer 99, 392–397.

Lippert, J.W., 3rd, 2007. Vascular disrupting agents. Biorg. Med. Chem. 15, 605-615.

Mons, S., Veretout, F., Carlier, M.F., Erk, I., Lepault, J., Trudel, E., Salesse, C., Ducray, P., Mioskowski, C., Lebeau, L., 2000. The interaction between lipid derivatives of colchicine and tubulin: Consequences of the interaction of the alkaloid with lipid membranes. BBA-Biomembranes 1468, 381–395.

Nguyen, T.L., McGrath, C., Hermone, A.R., Burnett, J.C., Zaharevitz, D.W., Day, B.W., Wipf, P., Hamel, E., Gussio, R., 2005. A common pharmacophore for a diverse set of colchicine site inhibitors using a structure-based approach. J. Med. Chem. 48, 6107–6116.

Nihei, Y., Suzuki, M., Okano, A., Tsuji, T., Akiyama, Y., Tsuruo, T., Saito, S., Hori, K., Sato, Y., 1999. Evaluation of antivascular and antimitotic effects of tubulin binding agents in solid tumor therapy. Jpn. J. Cancer Res. 90, 1387–1395.

Parveen, S., Sahoo, S.K., 2006. Nanomedicine: clinical applications of polyethylene glycol conjugated proteins and drugs. Clin. Pharmacokinet. 45, 965–988.

Pasquier, E., Honore, S., Braguer, D., 2006. Microtubule-targeting agents in angiogenesis: where do we stand? Drug Resist. Update 9, 74–86.

Pasquier, E., Kavallaris, M., 2008. Microtubules: a dynamic target in cancer therapy. IUBMB Life 60, 165–170.

Petersel, D., Schlesinger, N., 2007. Treatment of acute gout in hospitalized patients. J. Rheumatol. 34, 1566–1568.

Quinn, F.R., Neiman, Z., Beisler, J.A., 1981. Toxicity and quantitative structure-activity relationships of colchicines. J. Med. Chem. 24, 636–639.

Rautio, J., Kumpulainen, H., Heimbach, T., Oliyai, R., Oh, D., Jarvinen, T., Savolainen, J., 2008. Prodrugs: design and clinical applications. Nat. Rev. Drug Discov. 7, 255–270.

Ravelli, R.B.G., Gigant, B., Curmi, P.A., Jourdain, I., Lachkar, S., Sobel, A., Knossow, M., 2004. Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain. Nature 428, 198–202.

Roberts, M.J., Bentley, M.D., Harris, J.M., 2002. Chemistry for peptide and protein PEGylation. Adv. Drug Deliv. Rev. 54, 459–476.

Sharma, P.N., Brossi, A., Silverton, J.V., Chignell, C.F., 1984. Synthesis and binding to tubulin of colchicine spin probes. J. Med. Chem. 27, 1729–1733.

Terkeltaub, R.A., 2009. Colchicine update: 2008. Semin. Arthritis Rheum. 38, 411–419.

Thorpe, P.E., 2004. Vascular targeting agents as cancer therapeutics. Clin. Cancer Res. 10, 415–427.

Torchilin, V.P., 2005. Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. 4, 145–160.

Tozer, G.M., Kanthou, C., Baguley, B.C., 2005. Disrupting tumour blood vessels. Nat. Rev. Cancer 5, 423–435.

Zamora, J.M., Pearce, H.L., Beck, W.T., 1988. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. Mol. Pharmacol. 33, 454–462.