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Polymeric micelles for the solubilization and delivery of STAT3 inhibitor cucurbitacins in solid tumors

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Abstract

Poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) and newly developed poly(ethylene oxide)-*block*-poly(α-benzyl carboxylate ε-caprolactone) (PEO-*b*-PBCL) micelles were evaluated for the solubilization and delivery of cucurbitacin I and B, poorly water soluble inhibitors of signal transducer and activator of transcription 3 (STAT3). Encapsulation of cucurbitacins in PEO-*b*-PCL and PEO-*b*-PBCL by co-solvent evaporation technique resulted in polymeric micelles <90 nm in diameter. The aqueous solubility of both derivatives increased from less than 0.05 mg/mL in the absence of the copolymer to around 0.30–0.44 and 0.65–0.68 mg/mL in the presence of 5000–5000 and 5000–24,000 PEO-*b*-PCL micelles, respectively. Maximum cucurbitacin solubilization was achieved with PEO-*b*-PBCL micelles for both derivatives. PEO-*b*-PCL micelles having longer PCL block were found to be more efficient in sustaining the rate of release for cucurbitacins. The anti-cancer and STAT3 inhibitory activity of polymeric micellar cucurbitacins were comparable with free drugs in B16.F10 melanoma cell line *in vitro*. Intratumoral injection of 1 mg/kg/day cucurbitacin I resulted in the regression of established B16.F10 mouse melanoma tumors *in vivo*. In comparison to free cucurbitacin I, PEO-*b*-PBCL micellar cucurbitacin I was found to provide comparable anti-cancer effects against B16.F10 tumors and limit drug levels in animal serum while maintaining high drug levels in tumor following intratumoral administration. The results indicate the potential of polymeric micelles as suitable vehicles for the delivery of cucurbitacin- I and B.

Keywords: Polymeric micelles; Cucurbitacin; Solubilization; Drug delivery; STAT3

1. Introduction

Cucurbitacin- I and B (Fig. 1) are potent anti-cancer agents with selective inhibitory effect on signal transducer and activator of transcription 3 (STAT3) pathway (Blaskovich et al., 2003; Jayaprakasam et al., 2003; Sun et al., 2005). Cucurbitacin I and B belong to a group of natural products called cucurbitacins, tetracyclic triterpinoid substances which have been isolated from various plant families such as Cucurbitaceae. Cucurbitacins, possess a broad range of potent biological activity derived largely from their cytotoxic properties (Chen et al., 2005). A number of compounds in this group have been investigated for their hepatoprotective, anti-inflammatory, anti-microbial and most importantly anti-cancer properties (Miro, 1995; Duncan and Duncan, 1997; Jayaprakasam et al., 2003; Chen et al., 2005). The molecular mechanism of the various biological activities of cucurbitacins has not been fully investigated. Various functions of cucurbitacins have been related to their polarity and chemical structure (Dinan et al., 1997; Sun et al., 2005). For instance, it has been demonstrated that cytotoxic effects of cucurbitacins increase linearly with their hydrophobicity (Bartalis and Halaweish, 2005). Greige-Gerges et al. have also shown that the ability of cucurbtiacins to modify the binding of bilirubin to albumin is related to their structure. In this study, cucurbitacin I was found not to affect the binding of bilirubin to albumin and considered to be less active in affecting the interaction of albumin with small molecules in comparison to cucurbitacin E and D (Greige-Gerges et al., 2007). The anti-cancer activity of

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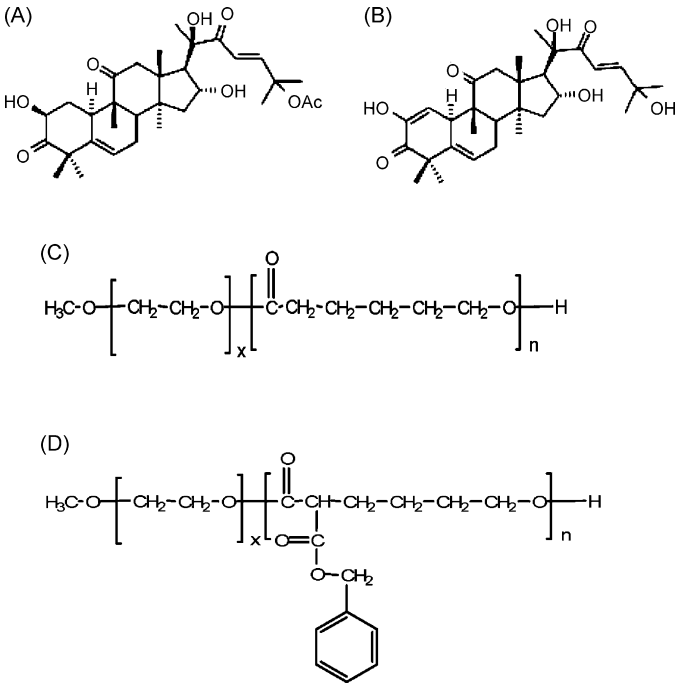

Fig. 1. Chemical structure of (A) cucurbitacin B; (B) cucurbitacin I; (C) PEO-*b*-PCL; and (D) PEO-*b*-PBCL.

cucurbitacin I and B have been shown in several cancer cell lines *in vitro* and there are some reports on their anti-tumor activity *in vivo* (Blaskovich et al., 2003; Jayaprakasam et al., 2003; Shi et al., 2006; Tannin-Spitz et al., 2007). Furthermore, cucurbitacin I and B have been shown to have selective signal transducer and activator of transcription 3 (STAT3) inhibitory activity in several cancer cell lines *in vitro* (Blaskovich et al., 2003; Sun et al., 2005; Shi et al., 2006).

STAT3, a common oncogenic signaling pathway, is constitutively activated in many types of cancers (Yu and Jove, 2004), including 82% of prostate cancers (Mora et al., 2002), 70% of breast cancers (Dolled-Filhart et al., 2003), more than 82% of the carcinomas of the head and neck (Nagpal et al., 2002), and 71% of nasopharyngeal carcinoma (Hsiao et al., 2003). Constitutive activation of STAT3 has been shown to play a major role in tumor cell growth, resistance to apoptosis and immune evasion by cancer. Accumulating evidence shows that blocking aberrant activation of STAT3 in tumor results in the inhibition of cancer cell growth, induction of apoptosis and enhancement of anti-cancer immune responses (Yu and Jove, 2004; Burdelya et al., 2005; Darnell, 2005; Kortylewski et al., 2005; Yu et al., 2007). Cucurbitacin I has been shown to modulate tumor-induced immunosuppression and enhance anti-tumor activity of cancer immunotherapy *in vivo* (Nefedova et al., 2005). STAT3 inhibitory and potent anti-proliferative activity of cucurbitacin I and B make them excellent and novel drug candidates in cancer therapy. However, problems of poor water solubility and non-specific toxicity have restricted their clinical benefit. Application of nanoscopic carriers such as polymeric micelles for the delivery of cucurbitacins is expected to overcome both limitations and enhance the therapeutic benefit of this important and emerging category of anti-cancer drugs.

Polymeric micelles are nanoscopic carriers (20–100 nm in size) with a hydrophilic shell/hydrophobic core structure that have shown great promise in the solubilization and controlled delivery of hydrophobic drugs (Aliabadi and Lavasanifar, 2006). Polyethylene oxide block used as hydrophilic shell of micelles, masks the hydrophobic core from biological milieu leading to their prolonged circulation following intravenous (i.v.) administration. Longevity in blood circulation is followed by improved tumor accumulation through enhanced permeation and retention (EPR) effect leading to enhanced drug delivery with reduced toxicity (Nishiyama et al., 2003; Hamaguchi et al., 2005). To date, only a limited number of polymeric micellar systems have shown positive results in tumor targeted delivery of poorly soluble drugs after systemic administration (Aliabadi and Lavasanifar, 2006; Kwon and Forrest, 2006). The key to success is to find the right drug–block copolymer combination that can withstand the destabilizing effect of biological environment and provide a proper pattern of drug release in the biological system.

Poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) is a biocompatible copolymer which have been successfully used for the solubilization and controlled delivery of a number of hydrophobic drugs (Allen et al., 1998, 2000; Kim et al., 1998; Aliabadi et al., 2005a). PEO-*b*-PCL micelles were also shown to cause a favorable shift in the pharmacokinetics and biodistribution of cyclosporine A and hydroxylcampthotecin after i.v. administration (Aliabadi et al., 2005b; Shi et al., 2005). In this study, we have compared the potential of micelle-forming PEO-*b*-PCLs of different PCL molecular weights and the newly developed block copolymer, poly(ethylene oxide)-*block*-poly(α-benzyl carboxylate ε-caprolactone) (PEO-*b*-PBCL), as nanoscale drug delivery systems for the solubilization and controlled delivery of cucurbitacin I and B. The anti-cancer activity of polymeric micellar formulations as well as of cucurbitacins in a mouse melanoma tumor model has also been evaluated and compared to the activity of free drug *in vitro* as we *in vivo* after intratumoral administration.

2. Materials and methods

2.1. Materials

Cucurbitacin I (white powder with molecular weight of 514.7, soluble in acetone, DMSO, ethanol and methanol) was purchased from Calbiochem (San Diego, CA 92121, USA). Cucurbitacin B (white powder with molecular weight of 558, soluble in acetone and methanol) was obtained from PhytoMyco Research Corporation (Greenville, North Carolina, USA). Methoxy PEO (average molecular weight of 5000 g mol^{-1}), diisopropyl amine (99%), benzyl chloroformate (tech. 95%), sodium (in kerosin), butyl lithium (Bu-Li) in hexane (2.5 M solution), palladium coated charcoal and thiazolyl blue tetrazolium bromide were purchased from Sigma (St. Louis, MO, USA). Caprolactone was purchased from Lancaster Synthesis, UK. Stannous octoate was purchased from MP Biomedicals Inc., Germany. All other chemicals were reagent grade.

2.2. Preparation and characterization of micellar formulations of cucurbitacin B and I

PEO-*b*-PCL block copolymers having an identical PEO molecular weight of 5000 g mol^{-1} and PCL molecular weights of 5000 or 24,000 g mol^{-1} were synthesized and characterized as reported previously (Aliabadi et al., 2005a). PEO-*b*-PBCL block copolymer was also synthesized and characterized as described recently (Mahmud et al., 2006). A nomenclature, e.g., 5000–4700, 5000–5000 and 5000–24,000, in which the left number corresponds to the theoretical molecular weight of the shell forming block and the right number corresponds to the molecular weight of the core forming block, is used throughout the manuscript to distinguish between prepared block copolymers. Encapsulation of cucurbitacins in PEO-*b*-PCL (5000–5000 and 5000–24,000) and PEO-*b*-PBCL (5000–4700) micelles was achieved by a co-solvent evaporation method (Aliabadi et al., 2005a). Briefly 20 mg of copolymer and 2 mg of either cucurbitacins B or I were dissolved in 0.5 mL acetone. This solution was added to 2 mL doubly distilled water (ddH_2O) in a drop-wise manner. After 4 h stirring at room temperature, the remaining acetone was removed by vacuum. The aqueous solution of the micellar formulation was then centrifuged at 3000 × *g* for 5 min to remove free cucurbitacin precipitates. Polymeric micellar cucurbitacin formulations were used freshly in all *in vitro* and *in vivo* studies. Mean diameter and polydispersity of micelles were defined by light scattering (3000 HS_A Zetasizer Malvern Zeta-Plus^{TM} zeta potential analyzer, Malvern Instrument Ltd., UK).

2.3. Determination of the cucurbitacin loaded levels by liquid chromatography–mass spectrometry (LC–MS)

To determine the level of encapsulated cucurbitacin in PEO-*b*-PCL and PEO-*b*-PBCL micelles, aqueous solution of polymeric micelles were placed in centrifugal filter tubes (M. wt. cut-off = 100,000 g mol^{-1}) and centrifuged at 3000 × *g* for 5 min to separate free and micelle-incorporated drug. Then 50 μL aliquot of the micellar solution (the top layer) was diluted in 0.95 mL methanol to disrupt the micellar structure and release the incorporated drug. Diluted solution (0.1 mL) was added to 0.1 mL of 4-hydroxybenzophenone solution (0.01 mg/mL methanol), which was used as internal standard (I.S.). This solution (10 μL) was injected to Waters Micromass ZQ 4000 LC–MS spectrometer. Quantitative analysis of cucurbitacin I by LC–MS was performed as described previously (Molavi et al., 2006). For the quantification of cucurbitacin B by LC–MS, mass spectrometer was operated in negative ionization mode with selected ion recorder acquisition. Then the analytes were quantified with single ion recording (SIR) at *m/z* 557 corresponding to [$M-H$] and *m/z* 539 corresponding to [$M-H_2O$–H] for cucurbitacin B and at *m/z* 196.8 for I.S. For chromatographic separation a mobile phase consisting of a mixture of acetonitrile water containing 0.2% ammonium hydroxide (40:60) was employed for 3 min. This was followed by a non-linear gradient to a final ratio of 60:40 (v/v) over 8 min at a constant flow rate of 0.2 mL/min. Calibration curves were constructed over the quantification range of 5–10,000 ng/mL for both cucurbitacin I and B. The ratios of cucurbitacin to I.S. peak areas were calculated and plotted versus cucurbitacin concentration. Cucurbitacin loading and encapsulation efficiency were calculated by the following equations:

$$\text{cucurbitacin loading (w/w)} = \frac{\text{amount of loaded cucurbitacin in mg}}{\text{amount of polymer in mg}}$$

$$\text{encapsulation effeciency (\%)} = \frac{\text{amount of cucurbitacin loaded in mg}}{\text{amount of cucurbitacin added in mg}} \times 100$$

2.4. In vitro release study

Release study was preformed using dialysis method as reported previously (Forrest et al., 2006). As a control, free cucurbitacin I and B were dissolved in water at a concentration of 1 mg/mL with the aid of methanol (2% v/v). The solubility of cucurbitacin I and B in water containing 2% methanol was verified using LC–MS. Aqueous solution of polymeric micellar cucurbitacin I and B were also prepared at a similar concentration. One milliliter of each sample was placed in a Spectra/Pore dialysis bag (M.wt. cut-off = 12,000–14,000 g mol^{-1}). The dialysis bag was located in 100 mL ddH_2O and whole system was placed in a rotating water bath where temperature was kept at 37 °C during the experiment. In order to maintain "perfect sink" conditions, the bath was overflowed with fresh ddH_2O so that the bath volume was refreshed every 1 h. Aliquots were taken at each time point from inside cassettes and drug concentrations were measured by LC–MS. Three parallel measurements were performed in each time point. Remaining drug in cassettes was determined and measurements were corrected for drug removed in the previous samples.

2.5. Cell viability assay

Anti-proliferative activity of free and PEO-*b*-PCL micellar cucurbitacin was assessed in B16.F10, a melanoma of C57/black origin (American Type Culture Collection, ATCC). B16.F10 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 IU/mL penicillin/streptomycin in 5% CO_2 atmosphere. Cell viability was monitored using - 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). To evaluate cytotoxicity, B16.F10 cells were obtained from exponentially growing 90–95% confluent cultures and seeded at a density of 2000 cells/well in 96-well plates. After two days incubation the cells were washed twice in serum-free medium and treated with control vehicles (2% methanol and empty PEO-*b*-PCL micelles) or four different concentrations of either cucurbitacins I or B, dissolved in methanol or encapsulated in 5000–24,000 PEO-*b*-PCL micelles (micellar cucurbitacin were diluted in cell culture media by serial dilution). After 24 or 48 h incubation at 37 °C the medium was removed and cells were washed three times with

the media followed by addition of 20 μL of 2 mg/mL solution of MTT dye. The plates were returned to the incubator for a period of 4 h. The residual MTT solutions were removed from wells then 0.2 mL of DMSO was added to each well and the plates were read at 570 nm using plate reader (PowerWave 340, Bio-Tek Instruments Inc.). Concentrations required for 50% inhibition in cell growth (IC_{50}) were determined from log-linear dose–response curves for each agent.

2.6. Western blot analysis

To evaluate the effect of cucurbitacin I and B formulations on STAT3 phosphorylation, B16.F10 cells were plated into six-well tissue culture plates at 2×10^6 cells/well. Cells were then incubated with cucurbitacin I at a concentration of 10 μM or cucurbitacin B at concentration of 40 μM in either soluble form in 2% methanol or encapsulated in 5000–24,000 PEO-*b*-PCL micelles for 24 h at 37 °C. Cells were then collected and washed twice with ice-cold PBS and then lysed in a buffer containing 30 mM Hepes (pH 7.5), 2 mM Na_3VO_4, 25 mM NaF, 2 mM EGTA, 2% Nonidet P-40, 1:100 protease inhibitor mixture (Sigma–Aldrich), 0.5 mM DTT and 6.4 mg/mL Sigma 104 Phosphatase Substrate (Sigma–Aldrich). Cell lysates were centrifuged for 20 s at $16{,}000 \times g$. Total protein extract was determined by Micro BCA Protein Assay. Equal amounts of protein (20 μg) were loaded in 8% SDS-PAGE gel. SK-MEL-28 + IFN-γ cell lysate (Santa Cruz Biotech) was used as positive control. Proteins were transferred into PVDF membrane and were probed with anti-p-STAT3 antibody (Santa Cruz Biotech). To confirm equal loading, membranes were stripped and re-probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotech). Membranes were developed using ECL plus detection kit (Amersham).

2.7. In vivo anti-tumor activity

The animal studies were carried out in accordance with procedures approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. A group of 16 C57Bl/6 mice were injected subcutaneously with 10^6 B16.F10 melanoma cells at their left flank. Tumors were allowed to grow until they became palpable. Animals were then randomly assigned to four groups (4 mice per group). Control groups were kept untreated or received daily intratumoral injection of empty PEO-*b*-PBCL micelles. Test groups were treated with 1 mg/kg cucurbitacin I in either soluble form (in 20% ethanolic solution) or encapsulated in PEO-*b*-PBCL micelles daily through intratumoral injection. Tumor size was measured during treatment three times a week by vernier caliper. Tumor volume (mm^3) was calculated using the following equation:

$$\text{tumor volume} = \frac{(\text{longer diameter}) \times (\text{shorter diameter})^2}{2}$$

Tumor-bearing animals were sacrificed 8–10 days after treatment and blood and tumor samples were collected. Cucurbitacin I was extracted from mice serum and tumor samples and quantified by LC–MS as described previously (Molavi et al., 2006).

2.8. Statistical analysis

The results are expressed as mean ± one S.D. for each group. The significance of differences among groups was analyzed either by one-way or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test for multiple comparisons. Before executing the ANOVA, data were tested for normality and equal variance. If any of those tests failed, data were compared using a Kruskal–Wallis one-way ANOVA on ranks. A P value of ≤ 0.05 was set for the significance of difference among groups. The statistical analysis was performed with SigmaStat software (Jandel Scientific, San Rafael, CA).

3. Results

3.1. Encapsulation of cucurbitacin B and I in polymeric micelles

Table 1 summarizes the characteristics of 5000–5000 and 5000–24,000 PEO-*b*-PCL and 5000–4700 PEO-*b*-PBCL micelles loaded with cucurbitacin B and I. Polymeric micelles were able to increase the water solubility of cucurbitacins. The aqueous solubility of both derivatives increased from less than 0.05 mg/mL in the absence of the copolymer to around 0.30–0.44 and 0.65–0.68 mg/mL in the presence of 5000–5000 and 5000–24,000 PEO-*b*-PCL micelles, respectively. Water solubility of cucurbitacin I in the presence of 5000–5000 PEO-*b*-PCL was found to be higher than that of B derivative (0.44 mg/mL versus 0.30 mg/mL) ($P < 0.05$, ANOVA). Between the two PEO-*b*-PCL micelles, those having longer PCL blocks were found to be more efficient in encapsulating both cucurbitacin derivatives. Maximum cucurbitacin solubilization was achieved with

Table 1
Characteristics of cucurbitacin-loaded PEO-*b*-PCL and PEO-*b*-PBCL micelles ($n = 3$)

Compound	Block copolymer	Cucurbitacin loading ± S.D. (mg/mg)	Encapsulation efficiency ± S.D. (%)	Micellar size (nm)	Polydispersity index
Cucurbitacin B	PEO-*b*-PCL 5000–5000	0.030 ± 0.003	30.2 ± 3.9	73.3 ± 1.15	0.27 ± 0.02
	PEO-*b*-PCL 5000–24000	0.065 ± 0.003	65.1 ± 3.8	78.2 ± 1.11	0.26 ± 0.01
	PEO-*b*-PBCL 5000–4700	0.092 ± 0.005	92.9 ± 4.6	76.3 ± 7.12	0.26 ± 0.02
Cucurbitacin I	PEO-*b*-PCL 5000–5000	0.044 ± 0.010	44.1 ± 10	72.2 ± 2.01	0.27 ± 0.03
	PEO-*b*-PCL 5000–24000	0.068 ± 0.006	68.4 ± 6.6	77.2 ± 2.01	0.17 ± 0.03
	PEO-*b*-PBCL 5000–4700	0.074 ± 0.007	74.1 ± 7.5	74.1 ± 7.50	0.24 ± 0.03

PEO-*b*-PBCL micelles for both derivatives (Table 1) despite a lower molecular weight of the PBCL block (4700 g mol^{-1}). The difference in the solubilization of cucurbitacins by 5000–4700 PEO-*b*-PBCL in comparison to 5000–24,000 PEO-*b*-PCL was more evident for the more hydrophobic derivative; i.e, cucurbitacin B. The aqueous solubility of cucurbitacins B and I was found to be 0.92 and 0.74 mg/mL in the presence of PEO-*b*-PBCL micelles corresponding to an encapsulation efficiency of 92.9 and 74.1%, respectively.

3.2. In vitro release of cucurbitacin B and I from polymeric micellar formulations

The release profile of cucurbitacin B and I from different vehicles evaluated by dialysis method are shown in Fig. 2A and B, respectively. The transfer of solubilized cucurbitacins from methanolic solution through the dialysis bag was found to be relatively rapid (>60 and 90% transfer in 1 and 4 h, respectively,

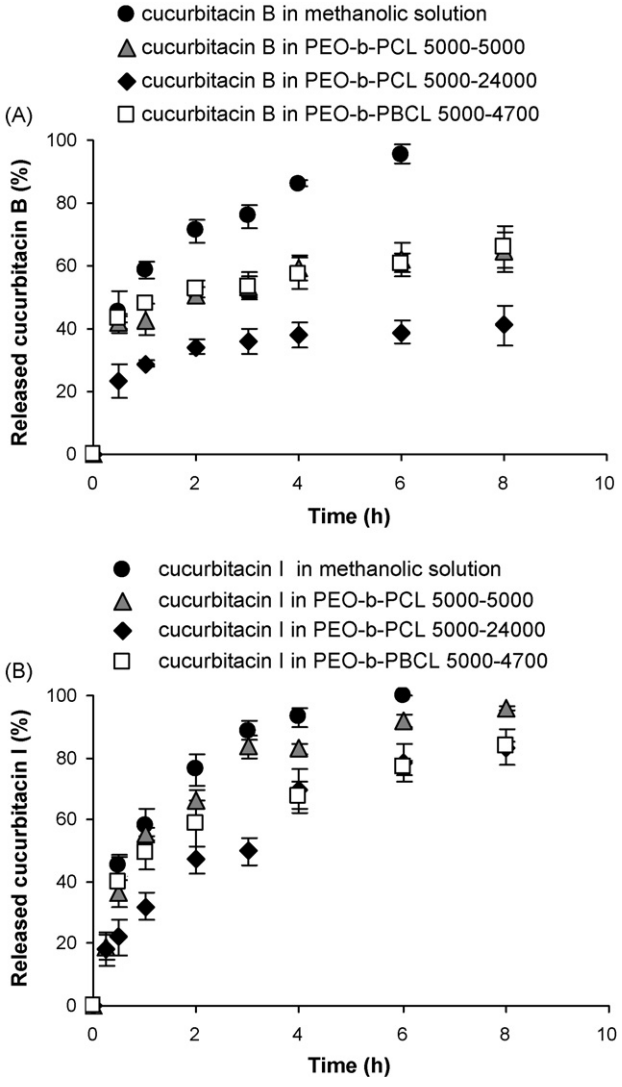

Fig. 2. The effect of solubilizing vehicle on the *in vitro* rate of (A) cucurbitacin B and (B) cucurbitacin I release. Data shown are representative of three independent experiments and the values for each time point are mean of triplicates ± S.D.

for both derivatives). In general polymeric micelles were able to reduce the rate of drug transfer from the dialysis membrane to outside medium in the in vitro release experiment. For cucurbitacin B, the 5000–5000 PEO-*b*-PCL micelles showed a burst release (42% release) within 1 h followed by an accumulative 64% of encapsulated drug release within 8 h. An increase in the molecular weight of PCL block from 5000 to 24,000 g mol^{-1} significantly reduced the burst release of cucurbitacin B in the first hour of experiment from 42 to 23% and resulted in a significant decrease in accumulative drug release ($P < 0.0001$, ANOVA). This system released 40% of its drug content within 8 h (Fig. 2A). The release profile of cucurbitacin B from PEO-*b*-PBCL micelles was similar to that for 5000–5000 PEO-*b*-PCL micelles, i.e., a burst release (around 45%) within 1 h followed by an accumulative 64% of encapsulated drug release within 8 h. In the case of cucurbitacin I, 5000–5000 PEO-*b*-PCL micelles, the pattern of drug transfer showed minimal differences with the transfer of free drug. In this case, a burst release of 55% in the first hour followed by 90% drug release within 8 h was observed (Fig. 2B). PEO-*b*-PCL micelles having 24,000 g mol^{-1} of PCL significantly reduced the burst release of cucurbitacin I as well as accumulative drug release percent in 8 h ($P < 0.0001$, ANOVA). This system showed a burst release of 30% within 1 h followed by an accumulative release of 80% for incorporated cucurbitacin I in 8 h. Micelles of PEO-*b*-PBCL showed a similar release profile for cucurbitacin I to that of 5000–5000 PEO-*b*-PCL micelles in initial time points (50% drug release at 1 h), but a decreased rate of cucurbitacin I release at later times (80% drug release at 8 h) (Fig. 2B).

3.3. Anti-proliferative activity of free and polymeric micellar cucurbitacin B and I against B16.F10 cell line in vitro

Fig. 3A and B depicts the anti-proliferative activity of methanolic solution or polymeric micellar formulation of cucurbitacin I and B against B16.F10 cells after 24 and 48 h incubation. Treatment of the murine B16.F10 melanoma cells with increasing concentrations of cucurbitacin I or B as free or encapsulated in 5000–24,000 PEO-*b*-PCL micelles resulted in a significant loss of cell viability assessed by MTT assay. Cucurbitacin I exerted more potent anti-proliferative activity than cucurbitacin B against the B16.F10 melanoma cells. Treatment of B16.F10 cells with cucurbitacin I in methanolic solution for 24 h at concentrations of 1, 5, 10 and 20 μM reduced the viability of treated cells to 81, 51, 43 and 22% of untreated cells, respectively (Fig. 3B), whereas cucurbitacin B incubation with B16.F10 melanoma cells for the same incubation period at concentrations of 1 and 5 μM did not result in any significant loss of cell viability (data not shown) and the presence of B derivative at concentrations of 10 and 20 μM left 64 and 47% of B16.F10 cells viable, respectively (Fig. 3A). As it has been shown in Table 2 the IC_{50} values for cucurbitacin I against the B16.F10 melanoma cells after 24 and 48 h incubation were significantly lower than those for cucurbitacin B at the same incubation time ($P < 0.0001$, ANOVA). The IC_{50} values of free cucurbitacins B and I after 24 h incubation were 18.9 and 4.7 μM. After 48 h

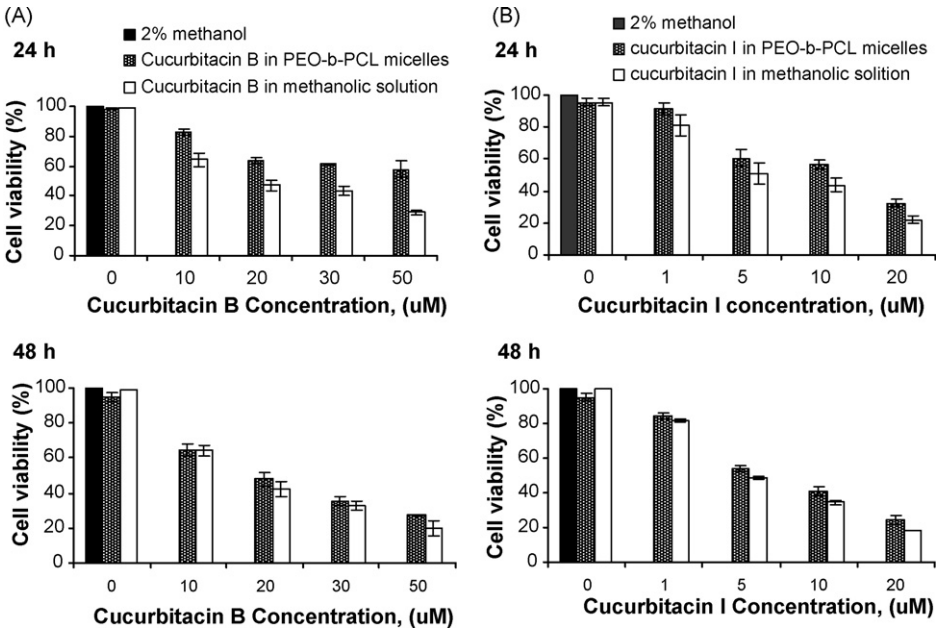

Fig. 3. Anti-cancer activity of free and polymeric micellar (A) cucurbitacin B; (B) cucurbitacin I formulations against B16 melanoma cell line after 24 and 48 h incubation, *in vitro*. B16 cells were treated with five different concentrations of either cucurbitacin I or B in free or polymeric micellar form. After 24 or 48 h incubation, cell viability was estimated by MTT assay and expressed as percentage of untreated controls. The data represent the mean ± S.D. of three independent experiments.

incubation, the IC_{50} was calculated at 16.1 and 4.6 μM for the B and I derivatives, respectively.

Similar difference between the activity of encapsulated cucurbitacin I and B was observed. Treatment of B16.F10 cell with polymeric micellar cucurbitacin B at concentrations of 10, 20, 30 and 50 μM for 24 h reduced the cell viability to 83, 63, 61 and 58%, which were significantly higher than the viability of the cells treated with free drug at identical concentrations ($P < 0.0001$, ANOVA). However, when the incubation time was increased to 48 h, the viability of B16.F10 cells treated with polymeric micellar cucurbitacin B was not significantly different from that of free drug (Fig. 3A) ($P > 0.05$, ANOVA). When B16.F10 cells were treated with polymeric micelles of cucurbitacin I, at concentrations of 1, 5, 10 and 20 μM, the viability of cells was 91, 60, 56 and 32% of untreated cells after 24 h incubation, respectively. As Fig. 3B indicates, the viability of B16.F10 cells treated with free cucuribitacin I at the same concentrations for the same period of time were significantly lower than what was observed with polymeric micellar cucurbitacin I ($P < 0.0001$, ANOVA). Treatment of B16.F10 cell with polymeric micellar cucurbitacin I at the same concentrations for 48 h left 84, 53, 40 and 24% of the cells viable, which were not significantly different from what was observed with free cucurbitacin I ($P > 0.05$, ANOVA). As illustrated in Table 2, IC_{50} values for free form of both derivatives are significantly different from those for their polymeric micellar formulation after 24 h incubation ($P < 0.05$, ANOVA). However, there is no significant differences in IC_{50} values between free and encapsulated forms of both derivatives after 48 h incubation ($P > 0.05$, ANOVA). PEO-*b*-PCL and PEO-*b*-PBCL block copolymers did not show any cytoxicity against B16.F10 cells when incubated at concentrations as high as 100 μg/mL or below (data not shown).

Table 2
The IC_{50} values of free and polymeric micellar cucurbitacins against B16.F10 cells ($n = 3$)

Drug	Vehicle	IC_{50} ± S.D. (μM)	
		24 h	48 h
Cucurbitacin B	2% methanol	18.9 ± 2.1	16.1 ± 2.1
	5000–24,000 PEO-*b*-PCL micelles	52.9 ± 2.8[a]	17.1 ± 0.3
Cucurbitacin I	2% methanol	4.7 ± 1.6[b]	4.6 ± 0.1[b]
	5000–24,000 PEO-*b*-PCL micelles	9.7 ± 1.1[a,b]	5.9 ± 0.5[a,b]

[a] Significantly different from free drug ($P < 0.05$).
[b] Significantly different from cucurbitacin B ($P < 0.05$).

3.4. STAT3 inhibitory activity of free and polymeric micellar formulations of cucurbitacin B and I in B16.F10 cell line in vitro

Cucurbitacin I and B are selective inhibitors of Junes kinase (JAK)/STAT3 pathway and block STAT3 activation through reduction in the phosphorylated STAT3 (p-STAT3) level in tumor cells. Measuring the level of p-STAT3 in B16.F10 cells using Western blot analysis provided means to evaluate the anit-STAT3 activity of cucurbitacin formulations in this study. As it is shown in Fig. 4A, treatment of B16.F10 cells with free or in 5000–24,000 PEO-*b*-PCL micellar cucurbitacin B at concentration of 40 μM for 24 h resulted in the suppression of p-STAT3 level. Free and 5000–24,000 PEO-*b*-PCL micellar cucurbitacin I were found to reduce the level of p-STAT3 at a concentration of 10 μM (Fig. 4B).

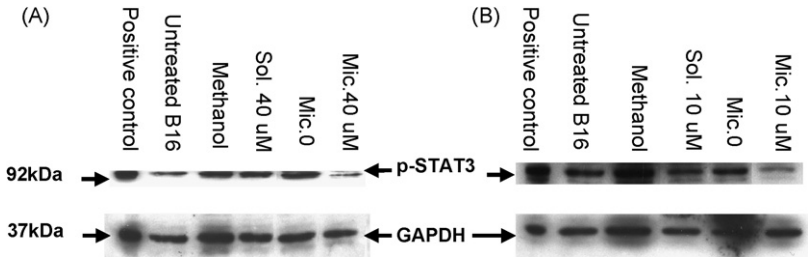

Fig. 4. Inhibition of p-STAT3 in B16.F10 melanoma cells by soluble (Sol) and polymeric micellar (Mic) (A) cucurbitacin B at a concentration of 40 μM and (B) cucurbitacin I at a concentration of 10 μM. Methanol-treated group (Methanol) or empty-micelle treated group (Mic.0) were controls. SK-MEL-28 + IFN-γ cell lysate was used as positive control. p-STAT3 was detected by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were also detected and served as a loading control.

3.5. In vivo anti-tumor activity of cucurbitacin I and its micellar formulation in B16.F10 melanoma mouse model after intratumoral injection

Based on the result of *in vitro* anti-cancer and STAT3 inhibitory activity, between two cucurbitacins, derivative I was more potent against cell growth as well as reduction of p-STAT3 level in B16.F10 cell line. Therefore, this derivative was chosen for *in vivo* efficacy study in a B16 melanoma tumor model. Among all the micellar formulations evaluated for the encapsulation of cucurbitacin I, PEO-*b*-PBCL micelles was the most efficient one in the solubilization of this compound and the level of drug release from this polymeric micellar system was also relatively slow and, except for the initial burst effect, was comparable to that of 5000–24,000 PEO-*b*-PCL micelles. Thus, this block copolymer was selected to form the micellar structure of choice for cucurbitacin I delivery during *in vivo* studies. As it has been shown in Fig. 5, B16 tumors from the mice in control groups, either untreated or treated with empty micelles, grew to about 3200 mm^3 within 14 days after tumor implantation, whereas the tumor growth was efficiently inhibited in both test groups that received 1 mg/kg/day of intratumoral cucurbitacin I (∼25 μg/mouse) as free or encapsulated in PEO-*b*-PBCL micelles. In fact, at the end of treatment period, in

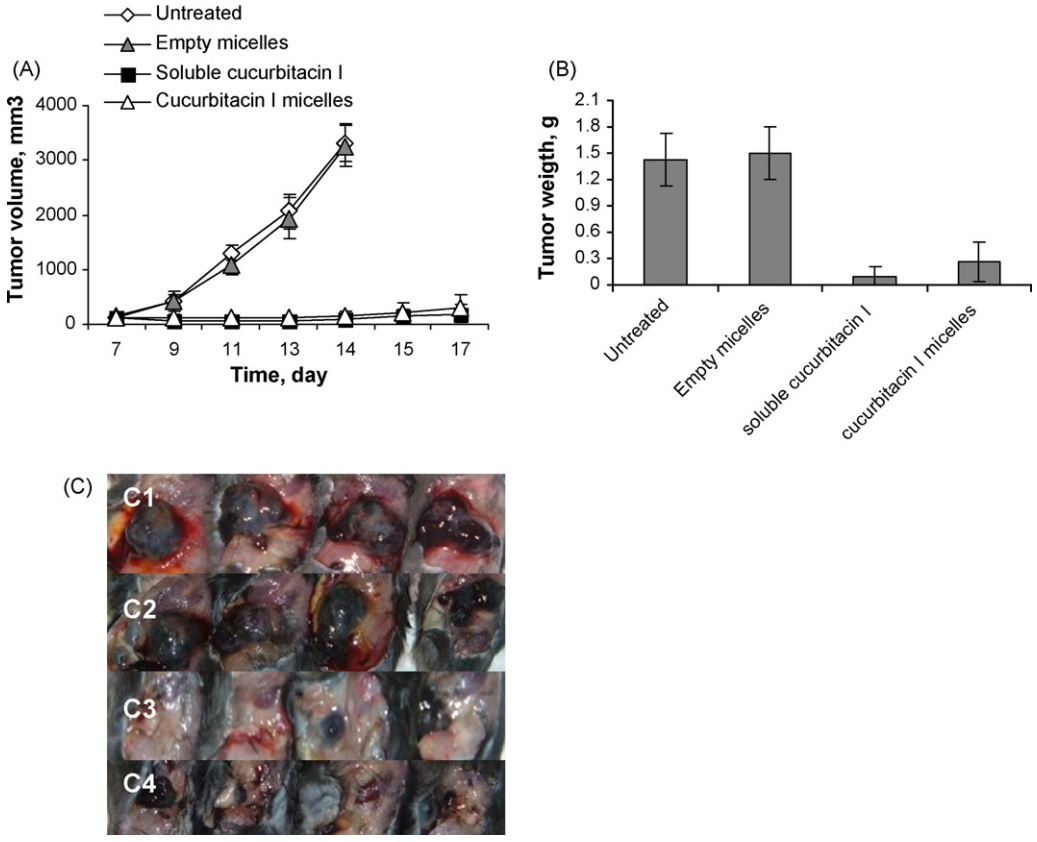

Fig. 5. Anti-tumor activity of cucurbitacin I and its PEO-*b*-PBCL micellar formulation in B16 melanoma tumor-bearing mice. Mice bearing B16 melanoma tumor at their left flank received intratumoral injection of 1 mg/kg/day cucurbitacin I, solubilized in 20% ethanolic solution or encapsulated in polymeric micelles for 10 days. (A) average tumor volume in animals during treatment; (B) average tumor weight in each group at the end point of the experiment (day 15 and 17 after tumor implantation in control and test groups, respectively); and (C) photographs of tumor-bearing animals at the end of experiment: (C1) untreated, (C2) treated with empty micelles, (C3) soluble cucurbitacin I, and (C4) polymeric micellar cucurbitacin I.

groups that received free or polymeric micellar cucurbitacin, 2 and 1 out of 4 mice were tumor free, respectively (Fig. 5C). Average tumor size in mice treated with free and encapsulated cucurbitacin I was below 150 mm^3 at day 17 after tumor cell injection. The average weight of tumors in control animals that were untreated or received empty PEO-*b*-PBCL micelles was five times higher than that in test groups treated with free or polymeric micellar cucurbitacin I (Fig. 5B and C). Quantification of cucurbitacin I in the serum samples obtained from mice ($n = 4$) in test groups 24 h after the last treatment revealed detectable level of cucurbitacin I in the serum of mice treated with free drug (58 ± 24 ng/mL), but cucurbitacin I was below detectable level in the serum of animals receiving the micellar formulation of this drug (<10 ng/mL). Tumor samples from mice in the test groups (animals that received free and encapsulated drug and had visible tumor at the end of treatment period) was harvested and analyzed for drug concentration 24 h after the last treatment. Cucurbitacin I was present at a concentration of 0.9 μg/g tumor tissue isolated from animals receiving free drug. The level of drug in tumor sample isolated from animals treated with PEO-*b*-PBCL micellar cucurbitacin I was 2.0 μg/g tumors.

4. Discussion

Cucurbitacins I and B are of great interest due to their selective STAT3 inhibitory activity and strong anti-proliferative function against a number of human carcinoma cell lines (Blaskovich et al., 2003; Jayaprakasam et al., 2003; Sun et al., 2005). The IC_{50} values of cucurbitacins against several cancer cells are comparable with doxorubicin, a widely used anti-cancer drug (Jayaprakasam et al., 2003). Moreover, selective STAT3 inhibitory activity of cucurbitacins makes them excellent drug candidates for delivery to tumor microenvironment to overcome tumor-induced immunosuppression, which may eventually lead to potent anti-tumor immune responses through inhibition of STAT3 (Yu et al., 2007). The main limitations to the clinical application of cucurbitacins are their poor water solubility and non-specific toxicity. Development of a polymeric micellar carrier that can solubilize cucurbitacins effectively and control their rate of release was purposed to overcome both limitations. For this purpose micelle-forming PEO-*b*-poly(ester)s having different poly(ester) blocks (PCL molecular weights of 5000 or 24,000 g mol^{-1} and PBCL molecular weight of 4700) were utilized (Fig. 1).

Between two PEO-*b*-PCLs, those with longer hydrophobic block showed better efficiency for the solubilization of both cucurbitacins (Table 1). This is predictable since longer PCL block provide more hydrophobic core leading to better interaction with hydrophobic molecules in the micellar core. Besides, owing to a lower critical micellar concentration (CMC), block copolymers having more hydrophobic core structures may form of higher number of micelles at a certain concentration (assuming similar aggregation numbers) and load higher drug content in total as a result.

PEO-*b*-PBCL micelles were found to be the most efficient vehicle for the solubilization of both cucurbitacins despite higher CMC of 5000–4700 PEO-*b*-PBCL compared to 5000–24,000 PEO-*b*-PCL (Mahmud et al., 2006; Aliabadi et al., 2005a). The presence of aromatic ring on PCL block increases the hydrophobicity and compatibility of core forming block with the hydrophobic structure of cucurbitacin derivatives. More effective solubilization of cucurbitacin B (the more hydrophobic derivative) in PEO-*b*-PBCL micelles provided further evidence for this explanation.

Consistent with findings on the solubilization of cucurbitacin derivatives by polymeric micelles, PEO-*b*-PCL based polymeric micelles were found to be more efficient in controlling the rate of release for the B derivative. In general, PEO-*b*-PCL micelles having longer PCL blocks were found to be more efficient in sustaining the rate of release for both drugs, *in vitro* (Fig. 2). The rate of cucurbitacin B release from PEO-*b*-PBCL micelles was comparable to that of 5000–5000 PEO-*b*-PCL. For cucurbitacin I, the rate of drug release from PEO-*b*-PBCL was similar to 5000–5000 PEO-*b*-PCL micelles at the initial time point (<1 h) but it became slow and similar to that of 5000–24,000 PEO-*b*-PCL micelles afterwards.

In the next step, we assessed the *in vitro* anti-cancer and STAT3 inhibitory activity of free and 5000–24,000 PEO-*b*-PCL micellar cucurbitacins I and B in B16.F10 melanoma cells in which STAT3 has been shown to be constitutively activated (Niu et al., 1999). Our results indicated a more potent anti-proliferative activity characterized by a significantly lower IC_{50} value for cucurbitacin I in comparison to cucurbitacin B (Table 2). Treatment of B16.F10 cells with polymeric micellar cucurbitacin I resulted in a comparable loss of cell viability to that of free drug at equal concentrations when cells were treated for 48 h. However, in shorter incubation time (24 h), free cucurbitacin I was found to have better anti-proliferative activity than encapsulated drug (Table 2). Identical findings were observed for cucurbitacin B (Table 2). The higher IC_{50} values of polymeric micellar cucurbitacins in shorter incubation times (24 h) may reflect slow release of incorporated drug from the micellar formulation (Fig. 2). Investigation of anit-STAT3 activity of cucurbitacin I and B formulations in B16.F10 cell line, revealed the activity of encapsulated cucurbitacins in reducing the level of p-STAT3 (Fig. 4). Taken together, the results of anti-cancer and STAT3 inhibitory activity show the ability of micellar formulations in the delivery of functional cucurbitacins to tumor cells.

The *in vivo* anti-cancer activity of more potent derivate, cucurbitacin I, and its PEO-*b*-PBCL micellar formulation was also assessed in a mouse melanoma tumor model following intratumoral injection of free and encapsulated drug. The selection of PEO-*b*-PBCL formulation for *in vivo* studies was made based on the higher efficiency of PEO-*b*-PBCL micelles in the solubilization of cucurbitacin I. Our results indicated that intratumoral injection of 1 mg/kg/day cucurbitacin I, as free or encapsulated in PEO-*b*-PBCL micelles, efficiently inhibits the growth of B16.F10 tumor, *in vivo* (Fig. 5). This is consistent with a pervious study that show intraperitoneal injection of 1 mg/kg/day of cucurbitacin I inhibits tumor growth in a mouse melanoma tumor model (Blaskovich et al., 2003). The tumor growth inhibitory effect of STAT-3 inhibitor cucurbitacin

may be a result of direct anti-cancer activity and/or induction of anti-tumor immune response by the drug. Further studies are required to define the possible extent of contribution from both mechanisms.

Quantitative analysis of cucurbitacin I in the serum samples of the animals treated with cucurbitacin I revealed detectable drug level in the serum of the mice treated with free drug, but drug level was below detectable concentration in the serum of mice treated with cucurbitacin I micelles. On the other hand, the level of cucurbitacin I in tumors isolated from the mice treated with cucurbitacin I micelles was found to be higher than animals that received free drug. This points to the retention of micellar cucurbitacin I within the tumor tissue and a lower dissemination of drug encapsulated in micelles to the organs following its intratumoral administration which may lead to a lower systemic toxicity for the polymeric micellar formulation of cucurbitacin I. Limited *in vivo* studies from our laboratory implied a potential for PEO-*b*-PCL micelles in causing a favorable change in the pharmacokinetic profile of cucurbitacin I and a reduction in its *in vivo* toxicity after systemic administration (Molavi et al., 2006). These findings are consistent with previous reports on the *in vitro* release and pharmacokinetics of PEO-*b*-PCL micellar formulation of cyclosporine A and hydroxycompthotecin (Aliabadi et al., 2005b; Shi et al., 2005). It is; however, not clear whether this degree of change in drug release and normal pharmacokinetics of cucurbitacin I by its polymeric micellar formulation will be sufficient to increase drug accumulation in solid tumor and reduce cucurbitacin toxicity after systemic administration. Further studies are required to assess the potential of polymeric micellar formulations of cucurbitacin I in enhancing its therapeutic index after intravenous administration.

5. Conclusion

Our results reveal a potential for PEO-*b*-PCL based micelles, especially PEO-*b*-PBCL and PEO-*b*-PCL having longer PCL blocks, as suitable vehicles for the solubilization and controlled delivery of cucurbitacin I and B. PEO-*b*-PCL micelles were shown superiority in controlling the rate of drug release for the more hydrophobic derivative, i.e., cucurbitacin B, but the I derivative was found to be more potent in suppression of p-STAT3 level and inhibition of cell proliferation in a STAT3 over expressing murine cancer cell line. In comparison to free drug, PEO-*b*-PBCL micellar cucurbitacin I was found to provide comparable anti-cancer effects against B16.F10 tumors in mice, limit drug levels in animal serum while retaining enhanced drug levels in tumor tissue after intratumoral administration.

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