Effects of Different Carboxylic Ester Spacers on Chemical Stability, Release Characteristics, and Anticancer Activity of Mono-PEGylated Curcumin Conjugates

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ABSTRACT: We investigated the effects of different carboxylic ester spacers of mono-PEGylated curcumin conjugates on chemical stability, release characteristics, and anticancer activity. Three novel conjugates were synthesized with succinic acid, glutaric acid, and methylcarboxylic acid as the respective spacers between curcumin and monomethoxy polyethylene glycol of molecular weight 2000 ($mPEG_{2000}$): $mPEG_{2000}$–succinyl–curcumin (PSC), $mPEG_{2000}$–glutaryl–curcumin (PGC), and $mPEG_{2000}$–methylcarboxyl–curcumin (PMC), respectively. Hydrolysis of all conjugates in buffer and human plasma followed pseudo first-order kinetics. In phosphate buffer, the overall degradation rate constant and half-life values indicated an order of stability of PGC > PSC > PMC > curcumin. In human plasma, more than 90% of curcumin was released from the esters after incubation for 0.25, 1.5, and 2 h, respectively. All conjugates exhibited cytotoxicity against four human cancer cell lines: Caco-2 (colon), KB (oral cavity), MCF7 (breast), and NCI-H187 (lung) with half maximal inhibitory concentration (IC_{50}) values in the range of 1–6 μM, similar to that observed for curcumin itself. Our results suggest that mono-PEGylation of curcumin produces prodrugs that are stable in buffer at physiological pH, release curcumin readily in human plasma, and show anticancer activity. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:5206–5218, 2011
Keywords: curcumin; biodegradable polymers; conjugation; PEGylation; prodrugs; solubility; stability; polymeric drugs; cytotoxicity

INTRODUCTION

There is considerable public and scientific interest in the use of phytochemicals derived from dietary components to treat human diseases. The dried ground rhizome of turmeric (*Curcuma longa* L., Zingiberaceae) has been widely used in Asian medicine since the second millennium BC and is now on the generally recognized as safe list of the US Food and Drug Administration (FDA).[1,2] Turmeric contains several curcuminoids, including curcumin (**1**, Fig. 1), desmethoxycurcumin, and bisdesmethoxycurcumin, with curcumin being the major constituent.[3] Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a hydrophobic polyphenolic compound with antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer activities.[4–6] Curcumin inhibits proliferation and angiogenesis, induces apoptosis and cell cycle arrest in a variety of tumor cell lines, and promotes tumor regression in a preclinical model.[7,8] A phase I clinical trial of orally administered curcumin for treatment of colorectal cancer has recently been reported.[8,9] In terms of toxicity, curcumin has a long history of safe use in humans.[10] Using absorption and fluorescence spectroscopic methods, Kunwar et al.[11]

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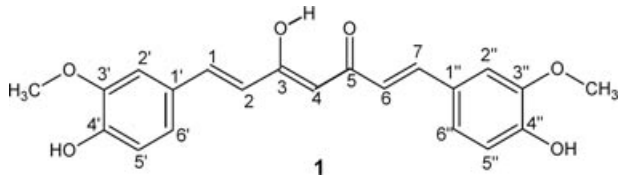


Figure 1. Chemical structure of curcumin.

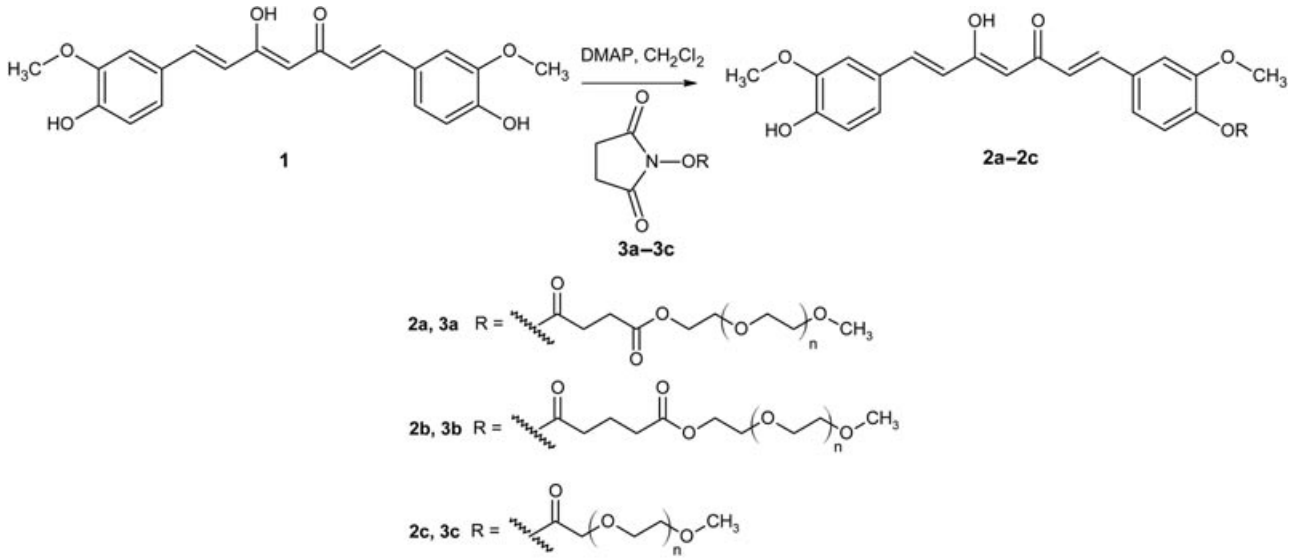
showed that uptake of curcumin was significantly higher in tumor cells compared with normal cells. Therefore, it is possible that preferential uptake of curcumin into tumors cells over normal cells contributes to the safety profile of curcumin.

Although curcumin has promising biological activity and a good pharmacological safety profile, it has poor physicochemical and biopharmaceutical properties that limit its development as a therapeutic agent. Curcumin is unstable at alkaline pH and rapidly degrades to *trans*-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic acid, feruloylmethane, and vanillin.[12] In addition, curcumin has an unfavorable pharmacokinetic profile consisting of poor absorption, rapid metabolism, and elimination, which leads to a short plasma half-life and low concentrations in target tissues.[13–15] Several approaches to enhance the water solubility, chemical stability, and resultant bioavailability of curcumin have been investigated.[16–28] Adding adjuvants such as piperine, which can inhibit glucuronidation metabolic pathways of curcumin, has been used to improve curcumin bioavailability.[16] Development of curcumin formulations as nanoparticles, liposomes, micelles, and phospholipid complexes is an alternative approach to prolong half-life, improve membrane permeability, and increase the metabolic stability of curcumin.[17–21] A chemical modification approach has been used to generate curcumin derivatives with increased biological activity and improved pharmacokinetic properties.[22–28] For example, Qiu et al.[26] synthesized a series of curcumin derivatives by replacing a phenyl ring with furan, and these compounds have higher inhibitory potency than curcumin against growth of several thioredoxin reductase-overexpressing cancer cell lines *in vitro*. Ferrari et al.[27] synthesized glycosylated curcumin analogues with increased water solubility and improved stability, which resulted in higher cytotoxicity against a cisplatin-resistant human ovarian carcinoma cell line.

A prodrug strategy involving chemical modification often improves the pharmacokinetic and delivery properties of a parent drug molecule by increasing solubility and stability and releasing the drug in a specific target organ. Previous curcumin prodrugs have been synthesized with amino acids and piperic acid as promoieties.[29,30] Recently, polymeric prodrugs with drug molecules conjugated to a polymer backbone such as polyethylene glycol (PEG) and polyvinylpyrrolidone have shown promise in drug delivery.[31–33] The use of PEG as a promoiety to attach to drug molecules known as PEGylation is an accepted strategy for human therapy that takes advantage of the safety profile of PEG.[34,35] In addition to its application as a drug carrier, PEG is used clinically for its chemopreventive properties in colon carcinogenesis.[36,37] PEGylated bovine adenosine deaminase (ADAGEN®), L-asparaginase (ONCASPAR®), and α-interferon (PEG-INTRON®) have been approved by the FDA for treatment of human disease.[32] For oral application, Calceti et al.[38] synthesized insulin–monomethoxy PEG (mPEG) derivatives formulated into poly(acrylic acid)–cysteine tablets. In comparison with regular insulin, this formulation decreased glucose levels for as long as 30 h after oral administration to diabetic mice.

PEGylation has been used previously to overcome the low aqueous solubility and stability of curcumin.[39,40] Safavy et al.[39] synthesized PEGylated curcumin by direct conjugation of curcumin with two different average molecular weight (MW) PEGs of 750 and 3500 Da via either ester or urethane linkages between a hydroxyl group of PEG and a phenolic group of curcumin. Cytotoxicity assays showed that the ester-linked conjugate was inactive, whereas the urethane-linked conjugate had activity against human prostate, colon, and pancreatic carcinoma cell lines, suggesting that the cell-killing activity was linker dependent. In addition, conjugates with various sized PEG had different rates of drug release due to variation in chain-terminal functionalities and molecular size. In contrast, Li et al.[40] showed that PEGylated curcumin with an ester linkage could inhibit pancreatic cancer cell growth with higher potency compared with unconjugated curcumin.

PEGylation of small organic molecules may decrease the therapeutic index. For example, PEGylation of paclitaxel to improve solubility and target delivery gives a prodrug that is less potent as an anticancer agent than the unconjugated drug *in vitro*.[41] However, these problems can be solved by incorporation of specific spacers with suitable linkages between the target drug and the PEG chain.[42] Therefore, our hypothesis tested in this study was that incorporation of spacers between PEG and curcumin would give conjugates with improved stability and anticancer activity, compared with curcumin itself. To this end, we synthesized three novel mono-PEGylated curcumin conjugates (**2a–2c**, Scheme 1) with succinic acid, glutaric acid, and methylcarboxylic acid as the respective spacers between curcumin and mPEG of MW 2000 ($mPEG_{2000}$). Synthesized conjugates consisted of $mPEG_{2000}$–succinyl–curcumin (PSC, **2a**), $mPEG_{2000}$–glutaryl–curcumin (PGC, **2b**), and $mPEG_{2000}$–methylcarboxyl–curcumin (PMC, **2c**).



Scheme 1. Synthesis of $mPEG_{2000}$–curcumin conjugates; $mPEG_{2000}$–succinyl–curcumin (PSC, **2a**); $mPEG_{2000}$–glutaryl–curcumin (PGC, **2b**); $mPEG_{2000}$–methylcarboxyl–curcumin (PMC, **2c**).

The effects of different carboxylic ester spacers of $mPEG_{2000}$–curcumin conjugates on their chemical stability, release characteristics, and anticancer activity were investigated.

MATERIALS AND METHODS

Materials

Three activated $mPEG_{2000}$–*N*-hydroxylsuccinimide (NHS) were purchased from NOF Corporation (Tokyo, Japan): Sunbright® ME-020CS ($mPEG_{2000}$ succinyl succinimidyl ester, $mPEG_{2000}$–succinyl–NHS, **3a**), Sunbright® MEGC-20HS ($mPEG_{2000}$ glutaryl succinimidyl ester, $mPEG_{2000}$–glutaryl–NHS, **3b**) and Sunbright® ME-020AS ($mPEG_{2000}$ methylcarboxyl succinimidyl ester, $mPEG_{2000}$–methylcarboxyl-NHS, **3c**) (Scheme 1). Weight average molecular weights (M_w) determined by matrix-assisted laser desorption–ionization–time-of-flight mass spectrometry (MALDI–TOF MS) were found at 2393.467, 2430.954, and 2417.916 for compounds **3a**, **3b,** and **3c**, respectively. Human plasma was kindly provided by the Thai Red Cross Society and other reagents were obtained from commercial suppliers. Reaction progress was monitored by thin layer chromatography (TLC) on precoated plastic plates (silica gel 60 F_{254}, 0.25mm thickness) purchased from Merck, Thailand. Melting points were determined on a differential scanning calorimeter (DSC823^e, Mettler-Toledo, Columbus, Ohio). Fourier transform infrared (FTIR) spectra were obtained with a PerkinElmer FTIR 1760 X spectrometer (Waltham, Massachusetts). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova Fourier Transform NMR 500 MHz spectrometer (Santa Clara, California). The NMR spectra were obtained in deuterated chloroform ($CDCl_3$). Chemical shifts were reported as δ values in parts per million (ppm) relative to the residual solvent peak and coupling constants were reported as J values in Hertz (Hz). Mass spectra were obtained on either a Reflex IV Bruker TOF-RS-MS (HRMS) or an Autoflex II MALDI–TOF-MS. Purity of synthesized compounds (>95%) and the amount of curcumin and $mPEG_{2000}$–curcumin conjugates were determined using an Agilent 1200 high-performance liquid chromatography (HPLC) system (Agilent, California) equipped with an Alltech Alltima C18 column (150 × 4.6 mm internal diameter, 5 μm; Grace, Illinois).

Methods

Synthesis of Curcumin (1)

Curcumin (**1**) was synthesized using a previously reported method with some modifications.[43,44] Acetyl acetone (1.03 mL, 10 mmol) followed by tributyl borate (10.8 mL, 40 mmol) were added to a solution of boric anhydride (0.35 g, 5.0 mmol) in ethyl acetate (30.0 mL) at 50°C for 15 min. Vanillin (3.04 g, 20 mmol) was added to the resulting boron complex and stirred at 50°C for 5 min. Butylamine (0.4 mL, 4.1 mmol) was then added dropwise over 40 min at 50°C and the reaction mixture was refluxed for 4 h, cooled, combined with 1N HCl (30 mL), and stirred for 30 min. The organic layers were separated, extracted three times with ethyl acetate, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by recrystallization from methanol to give curcumin (**1**, 2.92 g, 81.0%) as a yellow solid; melting point 187°C–188°C [literature (Ref. [45]) 186°C–187°C];

IR (KBr): ν_{max} 3500, 1626, 1601, 1504, 1427, 1261, 1026 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.95 (6H, s), 5.80 (1H, s) 6.48 (2H, d, J = 15.70 Hz), 6.94 (2H, d, J = 8.10 Hz), 7.05 (2H, d, J = 1.80 Hz), 7.13 (2H, dd, J = 8.13 and 1.80 Hz), 7.59 (2H, d, J = 15.70 Hz); ^{13}C NMR (125.76 MHz, $CDCl_3$): δ 55.96, 101.16, 109.62, 114.82, 121.77, 122.86, 127.69, 140.50, 146.77, 147.83, 183.25; HRMS calculated for $C_{21}H_{21}O_6$ [M + H^+]: 369.1338; found 369.1335.

Synthesis of mPEG$_{2000}$–Curcumin Conjugates (2a–2c)

The general procedure for synthesis of $mPEG_{2000}$–curcumin conjugates (**2a–2c**) is shown in Scheme 1. An activated $mPEG_{2000}$ succinimidyl ester ($mPEG_{2000}$–NHS, **3a–3c**, 50 mg, 0.022 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and the solution was added to a mixture of curcumin (**1**, 79 mg, 0.22 mmol) and 4-(*N,N*-dimethylamino)pyridine (DMAP; 13 mg, 0.11 mmol) in CH_2Cl_2 (10 mL) in a round bottomed flask. The reaction was monitored by TLC. The mixture was stirred for 24 h at room temperature until no $mPEG_{2000}$–NHS was detected by TLC. After the reaction was complete, glacial acetic acid (5 μL) was added to neutralize DMAP. Subsequently, the reaction mixture was dried on a rotary evaporator to remove the excess of glacial acetic acid and CH_2Cl_2. The residue was dissolved in methanol (2 mL), loaded onto a Sephadex LH-20 column (60 × 2 cm), and eluted with methanol. The clear methanolic solution was evaporated to afford a bright yellow, water-soluble solid product of $mPEG_{2000}$–curcumin conjugate.

$mPEG_{2000}$–succinyl–curcumin (PSC, **2a**): $mPEG_{2000}$–succinyl–NHS (**3a**) was used as an activated PEG succinimidyl ester. The yield was 90.8%; IR (KBr): ν_{max} 2883, 1771, 1736, 1627, 1588, 1511, 1466, 1359, 1341, 1279, 1240, 1204, 1144, 1100, 1060, 960, 841, 528 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 2.76 (2H, t, J = 6.80Hz,), 2.90 (2H, t, J = 6.80Hz,), 3.35 (3H, s), 3.46–3.76 (repeat ethylene units, s + m), 3.68 (2H, t, J = 4.92 Hz,), 3.84 (3H, s), 3.92 (3H, s), 4.25 (2H, t, J = 4.92 Hz,), 5.81 (1H, s), 6.47 (1H, d, J = 15.72 Hz), 6.53 (1H, d, J = 15.80 Hz), 6.91 (1H, d, J = 8.31 Hz), 7.03 [1H, s(*br*)], 7.05 (1H, d, J = 8.33 Hz), 7.09 (1H, d, J = 1.80 Hz,), 7.11 (1H, dd, J = 8.31 and 1.80 Hz), 7.12 (1H, dd, J = 8.32 and 1.80 Hz), 7.57 (1H, d, J = 15.76 Hz), 7.58 (1H, d, J = 15.80 Hz,); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 28.91, 29.06, 55.94, 58.99, 63.93, 69.02, 70.51 (repeat ethylene units), 71.89, 101.47, 109.70, 111.43, 114.91, 120.92, 121.73, 123.01, 123.24, 124.26, 127.49, 134.08, 139.34, 141.11, 146.87, 148.06, 151.33, 170.22, 171.97, 181.75, 184.47; Calculated MW = 2646.574, MALDI–TOF MS = 2646.858.

$mPEG_{2000}$–glutaryl–curcumin (PGC, **2b**): $mPEG_{2000}$–glutaryl–NHS (**3b**) was used as an activated PEG succinimidyl ester. The yield was 94.3%; IR (KBr): ν_{max} 2883, 1771, 1736, 1627, 1588, 1511, 1466, 1359, 1341, 1279, 1240, 1145, 1101, 1060, 959, 841, 528 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.06 (2H, m), 2.50 (2H, t, J = 7.18 Hz), 2.64 (2H, t, J = 7.18 Hz), 3.35 (s, 3H), 3.45–3.70 (s + m, repeat ethylene units), 3.75 (2H, t, J = 4.90 Hz), 3.84 (s, 3H), 3.92 (s, 3H), 4.23 (2H, t, J = 4.90 Hz), 5.81 (s, 1H), 6.47 (1H, d, J = 15.76 Hz), 6.53 (1H, d, J = 15.80 Hz), 6.90 (1H, d, J = 8.30 Hz,), 7.01 (1H, d, J = 8.30 Hz), 7.03 [1H, s(*br*)], 7.09 (1H, d, J = 1.84 Hz), 7.11 (1H, dd, J = 8.30 and 1.80 Hz), 7.12 (1H, dd, J = 8.30 and 1.80 Hz), 7.57 (1H, d, J = 15.78 Hz,), 7.58 (1H, d, J = 15.82 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 20.14, 29.95, 32.94, 55.86, 55.93, 58.98, 63.56, 69.09, 70.52 (repeat ethylene units), 71.89, 101.46, 109.71, 111.35, 114.92, 120.95, 121.72, 123.00, 123.18, 124.24, 127.47, 134.05, 139.35, 141.10, 146.88, 148.08, 151.31, 170.77, 172.83, 181.76, 184.48; Calculated MW = 2684.061, MALDI–TOF MS = 2684.146.

$mPEG_{2000}$–methylcarboxyl–curcumin (PMC, **2c**): $mPEG_{2000}$–methylcarboxyl–NHS (**3c**) was used as an activated PEG succinimidyl ester. The yield was 93.8% yield; IR (KBr): ν_{max} 2883, 1779, 1628, 1589, 1511, 1466, 1359, 1341, 1279, 1240, 1145, 1100, 1060, 960, 841, 528 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 3.35 (3H, s), 3.45–3.75 (s + m, repeat ethylene units), 3.84 (3H, s), 3.92 (3H, s), 4.42 (2H, s), 5.81 (1H, s), 6.46 (1H, d, J = 15.91 Hz), 6.53 (1H, d, J = 15.92 Hz), 6.90 (1H, d, J = 8.30 Hz), 7.03 [1H, s (*br*)], 7.05 (1H, d, J = 8.30 Hz), 7.09 (1H, d, J = 1.80 Hz), 7.10 (1H, d, J = 8.30 and 1.80 Hz), 7.13 (1H, d, J = 8.30 and 1.80 Hz), 7.57 (1H, d, J = 15.90 Hz), 7.58 (1H, d, J = 15.86 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 55.68, 55.85, 58.94, 68.28, 70.47 (repeat ethylene units), 71.83, 101.46, 109.68, 111.34, 114.93, 120.86, 121.60, 122.98, 123.24, 124.32, 127.35, 134.21, 140.53, 146.90, 148.11, 151.14, 139.14, 141.13, 168.34, 181.57, 184.53; Calculated MW = 2671.023, MALDI–TOF MS = 2670.875.

Chemical Stability of mPEG$_{2000}$–Curcumin Conjugates in a Buffer Solution

Stock solutions of curcumin in methanol and $mPEG_{2000}$–curcumin conjugates in water were prepared at 40 μM. The stock solutions were diluted with 0.1 M potassium phosphate buffer (pH 7.4) to give a final concentration of 4 μM. The solution was left to stand at 37°C for 24 h. The amount of curcumin or conjugate was determined at appropriate time intervals using a previously reported HPLC method with some modifications.[46] Chromatography was performed using a gradient system with an autosampler temperature of 15°C, a column temperature of 33°C, a flow rate of 2.0 mL/min, and a detection wavelength of 400 nm. Gradient elution consisted of eluents A (2%, v/v aqueous acetic acid) and B (acetonitrile). The elution program was optimized and conducted as follows:

initial 0–4 min, isocratic elution A–B (70:30, v/v); 4–5 min, linear change to A–B (50:50, v/v); 5–8 min, isocratic elution A–B (50:50, v/v); 8–9 min, linear change to A–B (45:55, v/v); 9–10 min, isocratic elution A–B (45:55, v/v); 10–11 min, linear change to A–B (30:70, v/v); 11–12 min, isocratic elution A–B (30:70, v/v); 12–13 min, linear change to A–B (70:30, v/v); 13–15 min, isocratic elution A–B (70:30, v/v). The injection volume was 20 μL. Experiments were performed in triplicate. Kinetic parameters were determined by a logarithmic plot of concentration versus time and calculated using a linear least-squares regression analysis.

Release Study of $mPEG_{2000}$–Curcumin Conjugates in Human Plasma

The plasma (pH 7.4) was spiked with a stock aqueous solution of each $mPEG_{2000}$–curcumin conjugate to give a final concentration of 4 μM. The spiked plasma was left to stand at 37°C for 3 h. The released curcumin and the remaining $mPEG_{2000}$–curcumin conjugate were determined at appropriate time intervals by extraction with acetonitrile and analysis using the HPLC conditions described above. Experiments were performed in triplicate. Kinetic parameters were determined by a semilogarithmic plot of concentration versus time and calculated using a linear least-squares regression analysis.

The control experiment was performed by measuring plasma aspirin esterase activity. The experiment followed the method previously reported by Williams et al.[47] with a modification. Briefly, 1.0 mL freshly prepared aspirin solution (10 mM) was incubated for 40 min at 37°C with 0.6 mL plasma, 3.4 mL buffer solution pH 7.4 (containing 600 mM Tris–HCl and 400 mM $CaCl_2$), and 5.0 mL water. The final concentration of aspirin solution was 1 mM. The amount of salicylate produced by hydrolysis was measured against the calibration curve of aqueous salicylate solution (0.022–0.22 mM) using the first derivative continuous spectrophometric measurement at 310 nm. Plasma aspirin esterase enzyme activity was expressed as nmol salicylate formed mL^{-1} plasma min^{-1} (nmol mL^{-1} min^{-1}). The experiment was performed in triplicate. The human plasma used in this study has the aspirin esterase activity of 64.2 ± 0.5 nmol mL^{-1} min^{-1}.

Cytotoxicity Evaluation of $mPEG_{2000}$–Curcumin Conjugates

Cytotoxicity of curcumin and $mPEG_{2000}$–curcumin conjugates was evaluated in four human cancer cell lines: Caco-2 (human colon adenocarcinoma, American Type Culture Collection (ATCC) Catalogue Number HTB-37), KB (human epidermoid carcinoma of oral cavity, ATCC CCL-17), MCF7 (human breast adenocarcinoma, ATCC HTB-22), and NCI-H187 (human small cell lung carcinoma, ATCC CRL-5804). Cytotoxicity against Caco-2 cells was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) microplate assay.[48] Cytotoxicity against KB, MCF7, and NCI-H187 cells was evaluated using a resazurin microplate assay (REMA).[49]

Stock solutions of curcumin and $mPEG_{2000}$–curcumin conjugates were prepared in dimethylsulfoxide (DMSO) at 1000 μM. The solutions were then serially diluted with the culture medium of cells to obtain sample solutions at appropriate concentrations. The cells were exposed to the sample at a concentration range of 10 to 0.078 μM. The incubation time was 24 h for Caco-2, 72 h for KB and MCF7, and 5 days for NCI-H187 cells. After incubation, the samples were removed from the cell cultures prior to either MTT[48] or REMA[49] assays. Ellipticine and doxorubicin were used as positive controls and 1% DMSO was used as the negative control. Results were expressed as the concentration of the sample required to kill 50% of the cells [half maximal inhibitory concentration (IC_{50})] compared with control. Experiments were performed in triplicate.

Cells were observed for altered morphology following incubation with the tested compounds at 4 μM concentration. The incubation time was 24 h for Caco-2, 72 h for KB and MCF7, and 5 days for NCI-H187 cells. After incubation, the cells were washed four times with phosphate-buffered saline (PBS) and reconstituted with fresh culture medium. The cells were then fixed with 3.7% aqueous formaldehyde for 15 min and washed three times with PBS. Negative control experiments were performed under the same conditions by replacing the test solution with an equal volume of 0.5% DMSO. Cell morphological images were scanned under the light microscope (10×).

RESULTS AND DISCUSSION

Synthesis of $mPEG_{2000}$–Curcumin Conjugates

Curcumin (**1**) contains two phenolic hydroxyl groups and ionization of these groups destabilizes curcumin toward oxidation and hydrolysis of the 1,3-diketone group.[12] PEG conjugation of these hydroxyl groups could protect against degradation and increase water solubility. However, PEG has two alcoholic hydroxyl groups and PEGylation of the phenolic hydroxyl groups of curcumin requires an appropriately protected PEG. Several suitable PEG derivatives are available, including mPEG, in which one end of the PEG chain is capped with a methoxy group; PEG carboxylic acid (PEG-COOH), in which a terminal hydroxyl group is oxidized to a carboxylic acid; and activated PEG succinimidyl ester (PEG-NHS), in which the carboxyl group of PEG-COOH is esterified with NHS.

Low MW PEG is commonly used to enhance the water solubility and stability of hydrophobic and/or labile drugs. PEGylation with low MW PEG may also allow easier enzymatic cleavage of the conjugated bond, compared with that of high MW PEG. Previously, direct conjugation of curcumin with low MW PEG (750 and 3500 Da) via either ester or urethane linkages has shown to improve the water solubility and the stability of curcumin.[39] In this work, we designed three $mPEG_{2000}$–curcumin conjugates with succinic acid, glutaric acid, and methylcarboxylic acid as spacers: PSC (**2a**), PGC (**2b**), and PMC (**2c**), respectively (Scheme 1). The modified PEG with a MW of 2000 Da and a methyl ether as a terminal functional group was used to conjugate with curcumin for investigating the effects of spacers. These conjugates were synthesized using three activated $mPEG_{2000}$ succinimidyl esters ($mPEG_{2000}$–NHS) containing succinic acid, glutaric acid, and methyl carboxylic acid as spacers: $mPEG_{2000}$–succinyl–NHS, **3a**; $mPEG_{2000}$–glutaryl–NHS, **3b**); and $mPEG_{2000}$–methylcarboxyl–NHS, **3c** (Scheme 1).

The general synthesis of the $mPEG_{2000}$–curcumin conjugates (**2a–2c**) (Scheme 1) involved esterification of curcumin with the corresponding activated $mPEG_{2000}$–NHS derivatives in the presence of DMAP as a catalyst. The reaction was initiated by mixing curcumin and each activated $mPEG_{2000}$–NHS derivative in the ratio of 10:1 to give products in good yield. The use of a large excess of curcumin ensured that (1) the reaction had gone to completion, (2) there was no remaining unreacted mPEG derivatives, and (3) a monosubstitued $mPEG_{2000}$–curcumin conjugate was predominantly formed with a minimal amount of the disubstituted impurity. Gel chromatography was used to purify each monosubstituted $mPEG_{2000}$–curcumin conjugate based on the substantial difference in MW between the conjugate and free curcumin.

Characterization of $mPEG_{2000}$–Curcumin Conjugates

The formation of monosubstitued $mPEG_{2000}$–curcumin conjugates between $mPEG_{2000}$ and curcumin at the phenolic hydroxyl group was confirmed by several spectroscopic evidences. These included IR, ^{1}H NMR, and MALDI–TOF MS.

Infrared spectra of the three $mPEG_{2000}$–curcumin conjugates showed common frequencies at 2883, 1627–1628, and 1100 cm^{-1}, corresponding to C–H stretching (methylene) in the PEG chain, carbonyl stretching of the enolizable 1,3-diketone of curcumin, and C–O stretching of the ethylene ether in the PEG chain, respectively. For PSC and PGC, the C=O stretching of the aliphatic and phenolic esters appeared at 1736 and 1771 cm^{-1}, respectively. For PMC, the C=O stretching of the phenolic ester appeared at 1779 cm^{-1}.

^{1}H NMR spectra of the $mPEG_{2000}$–curcumin conjugates showed signals for the curcumin, spacer, and PEG moieties with an integration ratio of 1:1 between curcumin and spacer, indicating the monosubstitution of the $mPEG_{2000}$–curcumin conjugates (Fig. 2). All conjugates showed a singlet signal with one proton at 5.8 ppm, representing a typical spectral pattern of a keto-enolic moiety, as also found in the curcumin structure. The molecular structure

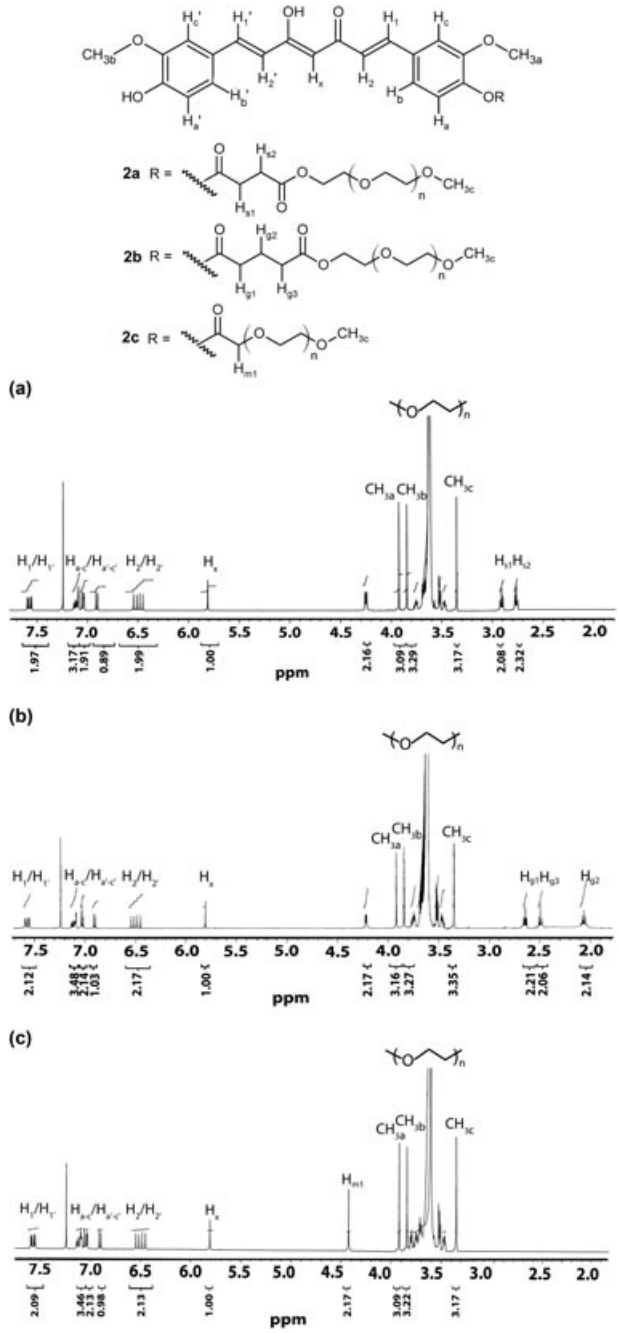


Figure 2. ^{1}H NMR spectra of $mPEG_{2000}$–curcumin conjugates; (a) $mPEG_{2000}$–succinyl–curcumin (PSC, **2a**); (b) $mPEG_{2000}$–glutaryl–curcumin (PGC, **2b**); (c) $mPEG_{2000}$–methylcarboxyl–curcumin (PMC, **2c**).

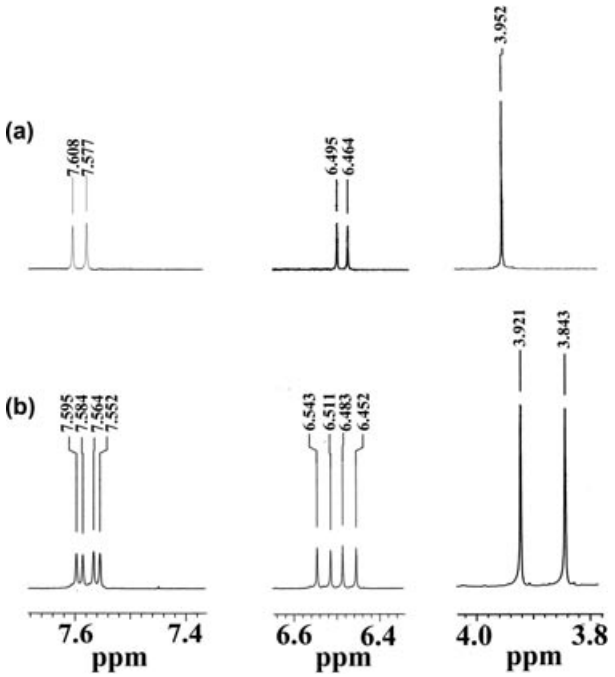


Figure 3. (a) Resonance symmetrical effect of curcumin on ^{1}H NMR spectrum leading to two doublets of *trans*-vinylic protons ($H_1/H_{1'}$ and $H_2/H_{2'}$) and one singlet of the phenolic methoxy groups. (b) Nonequivalent coupling signals of *trans*-vinylic protons and phenolic methoxy groups after PEGylation.

of curcumin contains a 1,3-diketone group, which can undergo keto-enol tautomerism. In nonpolar ($CDCl_3$) and aprotic (DMSO-d_6) solvents, curcumin exists in an enolic form due to intramolecular hydrogen bonding. The formation of the enolic structure of curcumin results in a labile enolate proton, which is exchangeable with the deuterium ion of $CDCl_3$. Therefore, this proton is not detected in NMR spectra. This observation is consistent with several previous reports.[50–53] Two singlet signals at about 3.84 and 3.92 ppm indicated nonequivalence of the two methoxy groups of the curcumin moiety of the conjugates (Fig. 2 and 3b), whereas these methoxy groups are equivalent and appear as a singlet signal at 3.95 ppm in unconjugated curcumin (Fig. 3a). The splitting pattern (two doublets) of *trans*-coupling signals at 6.4–6.5 and 7.5–7.6 ppm reflects the nonequivalence of the vinylic protons at position $H_2/H_{2'}$ and $H_1/H_{1'}$, respectively (Fig. 2 and 3b), whereas these vinylic protons appear as one doublet in unconjugated curcumin (Fig. 3a). The nonequivalence of the methoxy and vinylic protons of the curcumin moiety in the conjugates provides strong evidence for the monosubstitution of the $mPEG_{2000}$–curcumin conjugates.

Matrix-assisted laser desorption–ionization–time-of-flight mass spectrometry was used to determine the MW of the $mPEG_{2000}$–curcumin conjugates. The spectra are shown in Fig. 4. The M_w, M_n, and

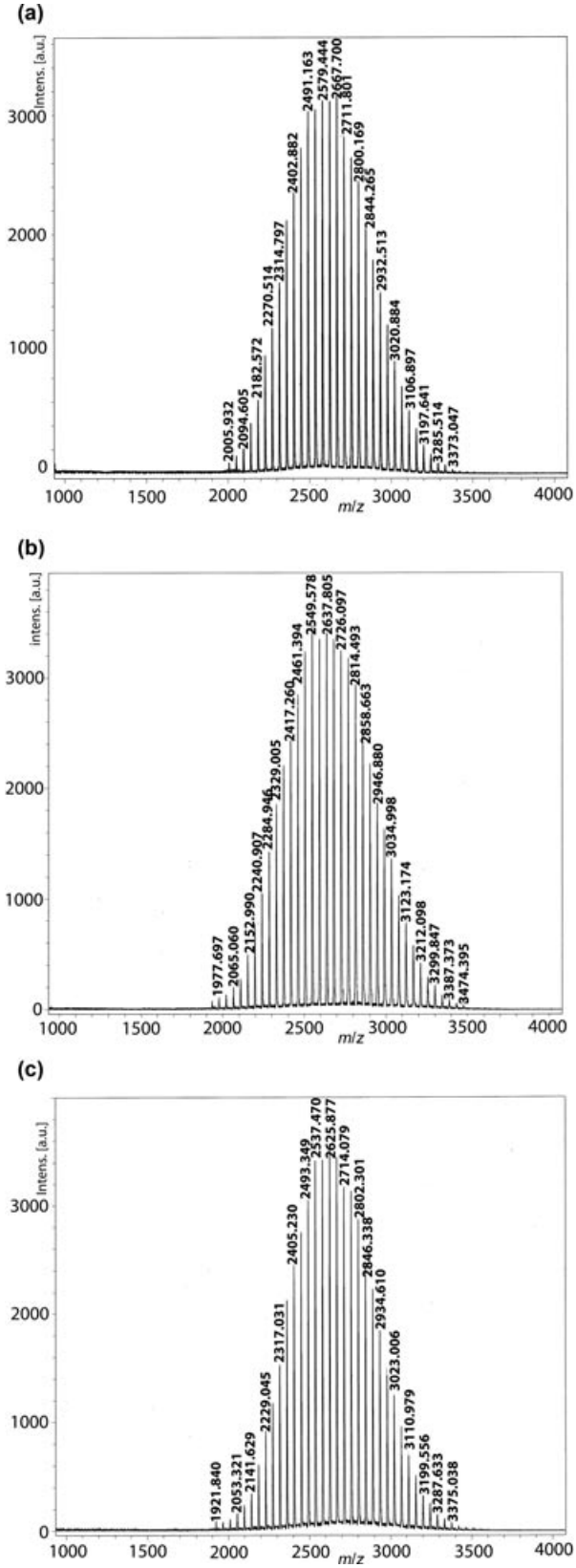


Figure 4. MALDI–TOF mass spectra of $mPEG_{2000}$–curcumin conjugates; (a) $mPEG_{2000}$–succinyl–curcumin (PSC, **2a**); (b) $mPEG_{2000}$–glutaryl–curcumin (PGC, **2b**); (c) $mPEG_{2000}$–methylcarboxyl–curcumin (PMC, **2c**).

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Table 1. Weight Average Molecular weight (M_w), Number Average Molecular Weight (M_n), and Polydispersity Index (PDI) of $mPEG_{2000}$–Curcumin Conjugates

Conjugates	Weight Average Molecular Weight (M_w)[a]	Number Average Molecular Weight (M_n)[b]	Polydispersity Index (PDI)[c]	Repeat Units (n)
PSC (**2a**)	2646.858	2624.514	1.009	35–66
PGC (**2b**)	2684.146	2659.030	1.009	34–68
PMC (**2c**)	2670.875	2647.912	1.009	33–66

[a] $M_w = (\sum N_i M_i^2) / (\sum N_i M_i)$
[b] $M_n = (\sum N_i M_i) / (\sum N_i)$
[c] $PDI = M_w / M_n$
where M_i is the weight of different kinds of polymer chains, N_i is the number of polymer chains of different masses, as determined from MALDI mass spectrometry data.

polydispersity index (PDI) were calculated for each conjugate (Table 1). All conjugates had M_w and M_n values in the range of 2625 –2684 Da, which is consistent with the calculated sum of the MWs of $mPEG_{2000}$, spacer, and curcumin, indicating mono-substituted conjugates with 33–68 methyleneoxy $(-CH_2O-)_n$ repeats. The PDI values were about 1.009, suggesting that the synthetic $mPEG_{2000}$–curcumin conjugates had a relatively narrow mass distribution (PDI < 1.1).[54–56]

Chemical Stability of $mPEG_{2000}$–Curcumin Conjugates in a Buffer Solution

The chemical stability of curcumin and $mPEG_{2000}$–curcumin conjugates in a 0.1 M phosphate buffer (pH 7.4) at 37°C was determined using reversed-phase HPLC with detection at 400 nm. Semilogarithmic plots of the concentration of curcumin and conjugates in the buffer versus time were linear for all of the compounds tested (Fig. 5), indicating that the degradation followed pseudo first-order kinetics. The overall degradation rate constants (k_{obs}) and half-lives ($t_{1/2}$) of curcumin and $mPEG_{2000}$–curcumin conjugates in the buffer are shown in Table 2. The k_{obs} and $t_{1/2}$ values indicated an order of stability of PGC > PSC > PMC > curcumin. These results show that PEGylation significantly enhanced the chemical stability of curcumin against hydrolytic degradation at the 1,3-diketone group. We also attempted to measure the amount of released curcumin as a hydrolytic product from each $mPEG_{2000}$–curcumin conjugate, and found that curcumin was undetectable or detected in a very small amount. This is explained by the slow release of curcumin from the conjugate and subsequent rapid decomposition of the free curcumin, as indicated by k_{obs} and $t_{1/2}$ values (Table 2). The differing stability among the three $mPEG_{2000}$–curcumin conjugates indicated that the spacer linking $mPEG_{2000}$ and curcumin plays a key role in the chemical stability of the conjugates. The spacers in PGC and PSC contain both phenolic and aliphatic esters, whereas PMC contains only a monocarboxyl phenolic ester spacer. The phenolic ester is more susceptible to hydrolysis than the aliphatic ester because the phenoxy group is a better leaving group than the alkoxy group. Therefore, the chemical stability of the three $mPEG_{2000}$–curcumin conjugates depends on hydrolytic cleavage at the phenolic ester. PMC has the fastest degradation rate because the oxygen atom adjacent to the alpha carbon of the methylcarboxyl linkage has a strong inductive effect on the ester carbonyl, resulting in a significant increase of the rate of hydrolysis in comparison with PGC and PSC hydrolysis. Therefore, these results suggested that the spacer played a key role in the stability profile of conjugates.

By comparing the $t_{1/2}$, the chemical stability of the $mPEG_{2000}$–curcumin conjugates was about 6 to 25 times greater than that of curcumin under the physiological pH at 37°C. Our results suggest that the significant increase in the stability of the curcumin conjugates would be useful for development of injectable formulation of curcumin.

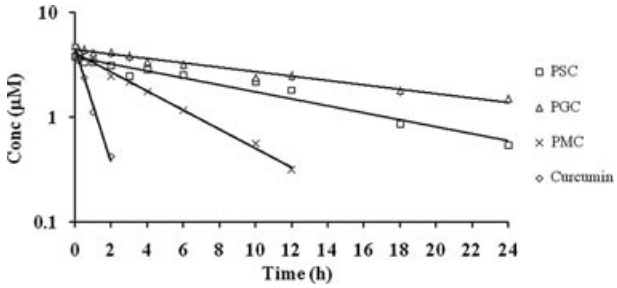

Figure 5. Pseudo first-order plots for the degradation of curcumin and $mPEG_{2000}$–curcumin conjugates in 0.1 M phosphate buffer (pH 7.4) at 37°C.

Table 2. Stability Kinetic Parameters of Curcumin and $mPEG_{2000}$–Curcumin Conjugates in 0.1 M Phosphate buffer (pH 7.4) at 37°C

Compounds	Kinetic Parameters	
	k_{obs} (h^{-1})	$t_{1/2}$ (h)
Curcumin	1.239	0.56
PSC (**2a**)	0.077	9.05
PGC (**2b**)	0.052	13.25
PMC (**2c**)	0.209	3.32

Kinetic Release Study of mPEG$_{2000}$–Curcumin Conjugates in Human Plasma

Release of curcumin from the mPEG$_{2000}$–curcumin conjugates in human plasma was examined by measuring the decrease in the conjugate concentration and the increase of released curcumin using reversed-phase HPLC with detection at 400 nm. The amount of curcumin formed was consistent with the decreased amount of each conjugate shown in Fig. 6.

The kinetics for hydrolysis of the mPEG$_{2000}$–curcumin conjugates in human plasma was evaluated by plotting the logarithmic concentration of each conjugate versus time (Fig. 7). These plots were linear for the three conjugates, indicating that the degradation in human plasma followed pseudo first-order kinetics. The k_{obs} and $t_{1/2}$ of the mPEG$_{2000}$–curcumin conjugates in human plasma are shown in Table 3. The k_{obs} and $t_{1/2}$ values indicated an order of stability of PSC > PGC > PMC. The stability of all three conjugates in human plasma was much lower than that in buffer, suggesting the participation of an enzyme catalyzed process. These results also indicated that the different spacers have an impact on the release rate of curcumin from conjugates. Other PEGylated drugs, such as PEG-ibuprofen,[57] have also been shown to release drug as a result of catalysis by plasma esterases.

Esterase enzymes such as carboxylesterase, butyrylcholinesterase, paraoxonase, albumin esterase, phenylacetate esterase, and aspirin esterase[58,59] catalyze hydrolysis of a variety of ester-containing substrates with different activity and specificity, depending upon the structure of substrate. Butyrylcholinesterase, paraoxonase, and albumin esterase are present in human plasma at high enough concentrations to contribute significantly to ester hydrolysis.[58] In contrast, Li et al.[58] were unable to detect carboxylesterase in human plasma, and therefore this enzyme may be less important for hydrolysis of the conjugates. Although further study will be needed to identify the specific enzyme or enzymes involved in the accelerated hydrolysis of mPEG$_{2000}$–curcumin conjugates, it is clear that release of curcumin in human plasma fractions was under enzyme control. The observed drug release from the conjugates in human plasma suggested that esterase enzymes can access the ester bond and catalyze the hydrolytic reaction. Therefore, the groove of the binding site of the enzyme(s) is large enough to accommodate the cleavage site of the conjugate. However, the molecular mechanism of catalytic hydrolysis of the mPEG$_{2000}$–curcumin conjugates with esterase enzymes requires further investigation in order to understand the relationship between the spacers and the rate of hydrolysis.

Table 3. Release Kinetic Parameters of mPEG$_{2000}$–Curcumin Conjugates in Human Plasma at 37°C

Compounds	Kinetic Parameters		
	k_{obs} (h^{-1})	$t_{1/2}$ (h)	t_{max}(h)
PSC (**2a**)	0.933	0.75	4.00
PGC (**2b**)	2.312	0.30	1.50
PMC (**2c**)	33.87	0.02	1.00

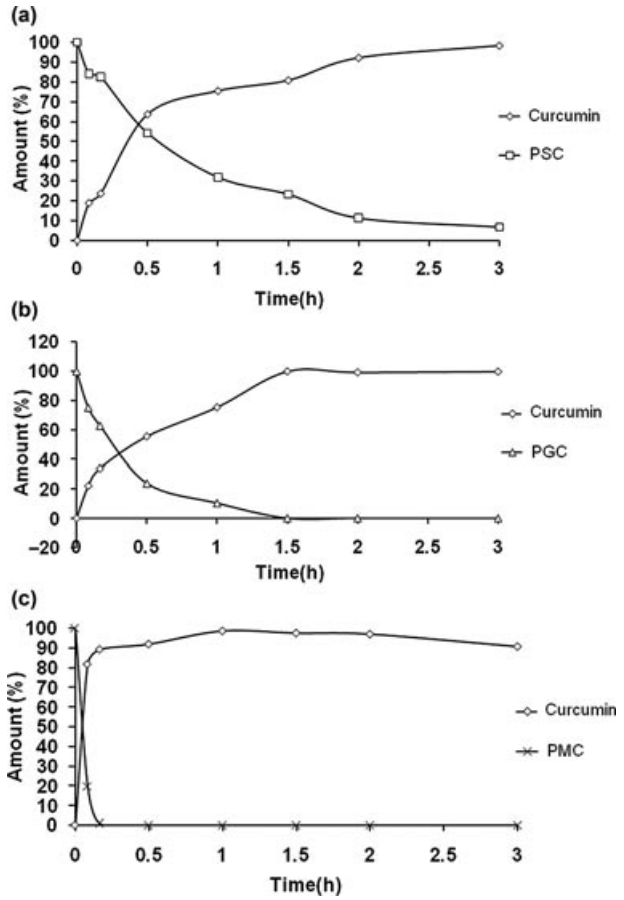

Figure 6. Release profiles of curcumin in human plasma at 37°C from (a) mPEG$_{2000}$–succinyl–curcumin (PSC, **2a**), (b) mPEG$_{2000}$–glutaryl–curcumin (PGC, **2b**), (c) mPEG$_{2000}$–methylcarboxyl–curcumin (PMC, **2c**).

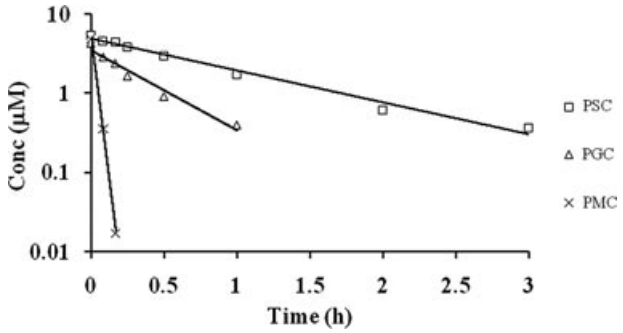

Figure 7. Pseudo first-order plots for the degradation of mPEG$_{2000}$–curcumin conjugates in human plasma at 37°C.

Table 4. *In Vitro* Cytotoxicity of Curcumin and $mPEG_{2000}$–Curcumin Conjugates Against Four Human Cancer Cell Lines

	IC_{50} (μM)			
Compounds	Caco-2	KB	MCF7	NCI-H187
Curcumin	3.31 ± 0.16	3.53 ± 0.09	1.63 ± 0.23	1.23 ± 0.14
PSC (**2a**)	5.97 ± 0.31	1.56 ± 0.21	3.07 ± 0.25	1.10 ± 0.15
PGC (**2b**)	3.74 ± 0.26	2.97 ± 0.13	2.99 ± 0.13	1.29 ± 0.08
PMC (**2c**)	2.94 ± 0.28	3.39 ± 0.15	2.50 ± 0.17	1.40 ± 0.12

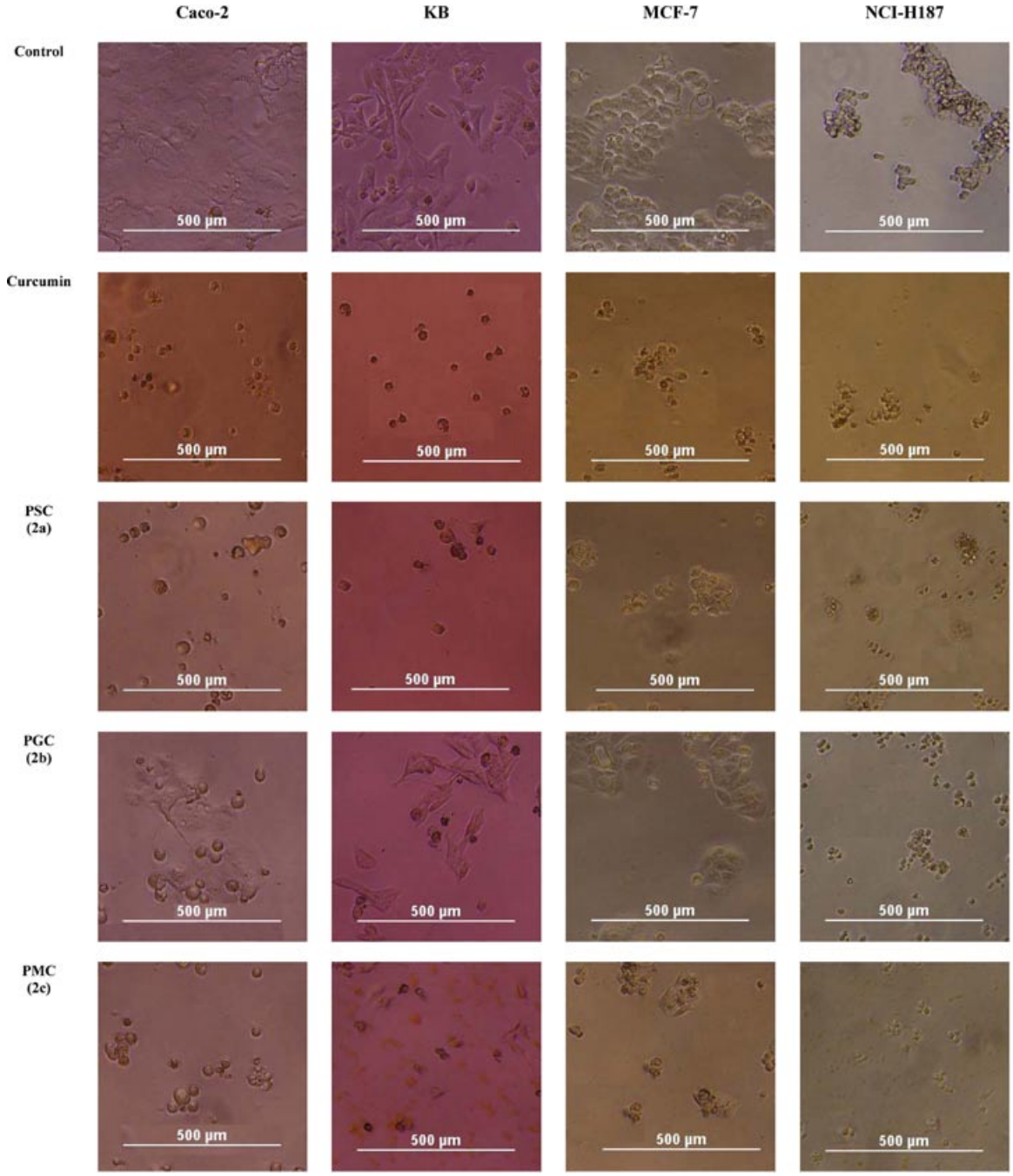

Figure 8. Four human cancer cell lines treated with 4 μM of curcumin, $mPEG_{2000}$–succinyl–curcumin (PSC, **2a**), $mPEG_{2000}$–glutaryl–curcumin (PGC, **2b**), and $mPEG_{2000}$–methylcarboxyl–curcumin (PMC, **2c**) compared with control.

Cytotoxicity Evaluation of $mPEG_{2000}$–Curcumin Conjugates

The $mPEG_{2000}$–curcumin conjugates were active against four human cancer cell lines: Caco-2 (colon), KB (oral cavity), MCF7 (breast), and NCI-H187 (lung) with IC_{50} values in the range of 1–6 μM (Table 4). For Caco-2 cell, IC_{50} values were in the range of 3–6 μM. For KB, MCF7, and NCI-H187 cells, IC_{50} values were in the range of 1–3 μM. The IC_{50} values in these ranges are considered to have a very similar potency and we therefore conclude that the conjugates exhibited cell growth inhibition with potency comparable to that of curcumin. These results suggest that the curcumin conjugates are effective prodrugs that could release curcumin in effective concentrations for anticancer activity. The morphology of the control/untreated cell lines and the curcumin and $mPEG_{2000}$–curcumin conjugates treated cell lines are shown in Fig. 8. The treated cells were much smaller in size in comparison with untreated cells and had lost intercellular adhesion. Because curcumin and $mPEG_{2000}$–curcumin conjugates are yellow, the yellow coloring observed inside the treated cells suggests that curcumin and $mPEG_{2000}$–curcumin conjugates penetrated into the cells. Our results are consistent with previous reports showing internalization of PEG and PEGylated conjugates into cells.[60,61]

CONCLUSION

Three $mPEG_{2000}$–curcumin conjugates were successfully synthesized with high purity and good yield. The solubility and hydrolytic stability profiles of the conjugates were dramatically improved in comparison with the profile of curcumin. In addition, all the conjugates were able to release free curcumin and inhibit cell growth. The properties of the $mPEG_{2000}$–curcumin conjugates determined in this study provide the basis for optimization of the pharmacokinetics and biological activity of water-soluble curcumin conjugates for clinical applications. The variable effects of the different carboxylic ester spacers on the chemical stability and release profile indicate that the choice of spacer is important for pharmaceutical formulation and drug delivery. Use of different carboxylic acid spacers in PEG conjugates may permit control of the pharmacokinetic profiles of parent compounds in prodrugs. In a broader sense, curcumin can be considered as a model compound of other small-molecule drug candidates that are hindered by undesirable physical and chemical properties. The ultimate goal is to apply selective PEGylation strategies to the development of drug candidates that would otherwise be eliminated from consideration because of poor bioavailability.

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