Design and Development of Water-Soluble Curcumin Conjugates as Potential Anticancer Agents£

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Conjugates of curcumin to two differently sized poly(ethylene glycol) molecules were synthesized in an attempt to overcome the low aqueous solubility of this natural product with cytotoxic activity against some human cancer cell lines. The soluble conjugates exhibited enhanced cytotoxicity as compared to that of the parent drug. Synthesis, analyses of the rate of drug release, and cytotoxicity studies are herein reported. The water-soluble conjugates may provide information useful for the development of injectable curcumin conjugates.

Introduction

Phytochemicals have proved to be valuable sources of clinically useful drugs including antitumor agents. Paclitaxel (5β,20-epoxy-1,2a,4,7β,10β,13a-hexahydroxytax-11-en-9-one 4,-10-diacetate 2-benzoate 13-ester with (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine), an anticancer drug originally extracted from the pacific yew tree (*Taxus brevifolia*), has impressive activities against several human solid tumors. It has been in clinical use since the last decade and is still the subject of extensive research in oncology. Camptothecin (4-ethyl-4-hydroxy-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14-(4*H*,12*H*)-dione), extracted from *Camptotheca acuminata*, is the active ingredient of the anticancer drugs irinotecan and topotecan used in the treatment of lung and metastatic colorectal cancers.

Curcumin (CCMN,[a] diferuloylmethane, Figure 1) is the yellow pigment isolated from rhizomes of the perennial herb *Curcuma longa*, which has been in traditional medicinal use since the second millennium BC. In addition to stimulative and taste-enhancing effects utilized in dietary applications, CCMN has strong antioxidant properties and has been also used as a topical treatment for wound healing. In recent times, much attention has been devoted to this natural product due to its putative cancer-preventing and even anticancer properties.[1,2] The apoptosis-inducing effects of CCMN and its inhibitory activities against protein kinase C and epidermal growth factor receptor tyrosine kinase have been clearly demonstrated, and cytotoxic activity against some cancer cell lines of human origin has been reported for this compound. It has been demonstrated that CCMN acts on or inhibits several important cellular targets such as NF-κB.[3] This interaction, in turn, induces apoptosis and blocks the function of protein kinase C, epidermal growth factor receptor tyrosine kinase, and HER-2.[4,5] Significant cytotoxicity was observed in CCMN-treated MCF-7 human breast carcinoma cells, which was demonstrated to be through the mitotic spindle disruption and G2/M phase arrest.[6] In HT29 human colon carcinoma, application of 9 μM CCMN resulted in cell shrinkage and DNA degradation and an overall reduction in cell volume.[7] Furthermore, CCMN has shown cytotoxicity against the cell lines of other malignancies such as prostate,[8,9] kidney,[10] hepatocellular,[11] lymphoid and myeloid,[12] and melanoma.[13] In addition to being a direct-acting drug and on the basis of its ability to induce G2/M phase arrest, CCMN may be useful also as a radiosensitizer for tumor radiotherapy.[14]

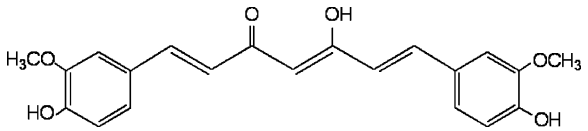

Figure 1. Structure of curcumin.

At the same time, and as evident from its long-time dietary use, CCMN has no toxicity to human body. In a phase I clinical trial, Cheng et al. have demonstrated the nontoxic nature of CCMN at a daily oral dose of 8 g.[15] On the basis of the results of another phase I study, Sharma et al. have proposed a daily oral dose of 3.6 g for a phase II trial as no dose-limiting toxicity could be reached in the study.[16] On the basis of these findings, CCMN may be considered as a potential compound for the development of anticancer drugs.

Despite these advantages, CCMN suffers from a low, if at all, aqueous solubility. In the natural form, only one minor component of turmeric, namely turmerin, is water-soluble. Turmerin has no medicinal significance, however, and constitutes only about 7.4% of the natural mixture.[17] The observed poor bioavailability and less-than-favorable biodistribution of CCMN may be, in fact, the results of its low solubility. In patients with hepatic metastatic colorectal cancer receiving a dose of 0.45−3.6 g/day, only low-nanomolar quantities of CCMN and its metabolites were present in the serum. No CCMN was detected in the liver, although trace amounts of its metabolites were observed in this organ.[18] Accordingly, the investigators concluded that therapeutically significant doses of CCMN to the liver were not achievable in humans. In another

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[a] Abbreviations: BNPC, bis(*4*-nitrophenyl)carbonate; CCMN, curcumin; DAPI, *4′,6*-diamidino-*2*-phenylindole; DIEA, *N,N*-diisopropylethylamine; DPBS, Dulbecco's PBS; EET, effective exposure time; H, high; L, low; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MW, molecular weight; HPLC, high-performance liquid chromatography; PEG, poly(ethylene glycol); PBS, phosphate-buffered saline; RP, reversed-phase.

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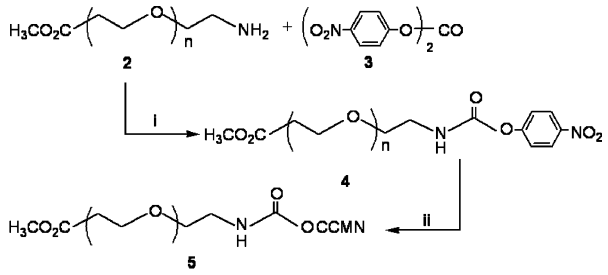
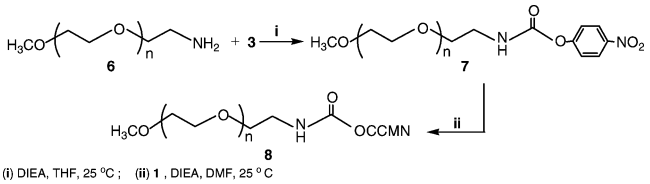
Scheme 1



Scheme 2



clinical pilot study, a low oral bioavailability was observed for CCMN in patients with advanced colorectal cancer refractory to standard treatments.[19] Other studies have shown the absence of carcinogenesis inhibition of CCMN against lung and breast tumors, which has been ascribed to its low bioavailability.[20] In general, the aqueous insolubility of CCMN may hinder the development of clinically applicable formulations of this otherwise promising natural product.

In an attempt to overcome this shortcoming, we have prepared water-soluble CCMN−PEG conjugates and herein report the synthesis and some biological properties of these conjugates.

Results and Discussion

Conjugates were synthesized using the hydrophilic and biocompatible polymer PEG, with high (H, 3500 Da) and low (L, 750 Da) molecular weights. In the first synthesis, a direct carboxylic ester linkage to connect the CCMN nucleus to the HMW polymer was used. Thus, the *N*-acetylamino PEG−carboxylic acid was directly conjugated to CCMN by a standard carbodiimide-mediated esterification. Purification by RP-HPLC and MALDI MS analysis of this product indicated a one-to-one addition of CCMN to the polymer. Cytotoxicity assays against PC-3 human prostate carcinoma cells, however, revealed no activity for this conjugate at a concentration range of 5−20 μM. Under the same conditions, unconjugated CCMN showed 65 and 90% cell-killing activity at 10 and 20 μM, respectively, whereas at a lower dose (5 μM), it had negligible toxicity against this cell line (data not shown). Accordingly, the ester-linked conjugate was considered inactive and was not further studied.

In a second approach (Scheme 1), the HMW methylamino−PEG carboxylate **2** was converted to the activated urethane **4** through condensation with bis(4-nitrophenyl)carbonate (BNPC, **3**). Compound **4** was subsequently conjugated to CCMN through a direct coupling reaction under basic conditions, to afford conjugate **5**. This compound was a solid, which displayed an intense yellow color, had a solubility in water of over 1.5 g/mL, and produced a viscous solution at high concentrations. Similar to the ester-linked compound, this conjugate was a 1:1 adduct with an average CCMN content of 9.4%. It was used only as a model for testing the aqueous solubility as well as the effect of conjugation on the cytotoxic activity of CCMN (see below).

Since the eventual goal of this project was to develop soluble CCMN derivatives for therapeutic applications, we proposed

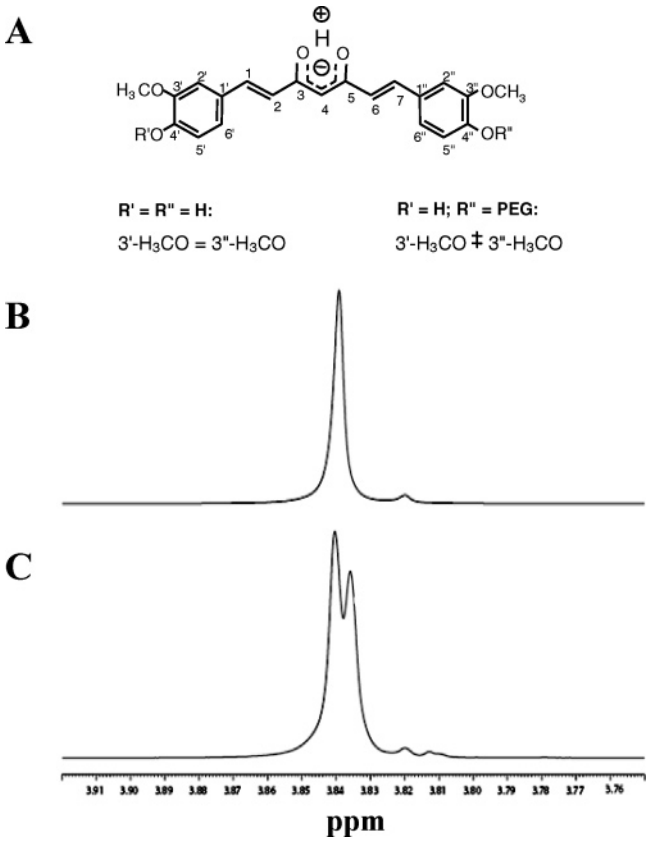


Figure 2. Resonance-symmetric structure of curcumin (A) resulting in one singlet for the ^{1}H NMR spectrum of the ring methoxy groups (B). The signal splits after conjugation of one of the neighboring hydroxy oxygens to PEG (C).

the synthesis of PEG conjugates with smaller MWs, and thus higher drug-to-polymer (D/P) ratios. Practically, a high D/P ratio would have the advantage of reducing the amount of the conjugate required in a given clinical formulations. Considering that CCMN has an IC_{50} in the micromolar range, a high concentration of its HMW conjugates may indeed be required to produce a therapeutically significant dose. To this end, a urethane-linked conjugate, compound **8**, was prepared by the procedure shown in Scheme 2. It should be noted that in this preliminary phase, an important aspect of the drug delivery practice, the relationship between the molecular size and pharmacokinetics, was not taken into account. Only water solubility and retention of the drug activity were of interest at this stage.

In this synthesis, a 750-Da methoxy-truncated amino−PEG (**6**, Scheme 2) was coupled to BNPC to afford the intermediate activated urethane **7**. The latter compound was condensed with CCMN to yield the final conjugate **8**.

Conjugate MWs were identified by MALDI mass spectrometry, which, in all cases, showed a CCMN/PEG ratio of unity. Also, combination of MS and ^{1}H NMR data showed one of the CCMN phenolic oxygens to be the site of conjugation to the polymeric linkers. The conjugation of one phenolic oxygen was evident from formation of a split in the chemical shifts of the neighboring methoxy protons of the CCMN moiety. These chemically equivalent protons (Figure 2A) appeared as a singlet with a chemical shift of 3.84 ppm in the unconjugated CCMN (Figure 2B). After conjugation, this signal was split into a pair with one peak showing a slight upfield shift of 0.01 ppm, apparently due to the attachment of the neighboring oxygen to the linker. These signals are shown in Figure 2C. At the same

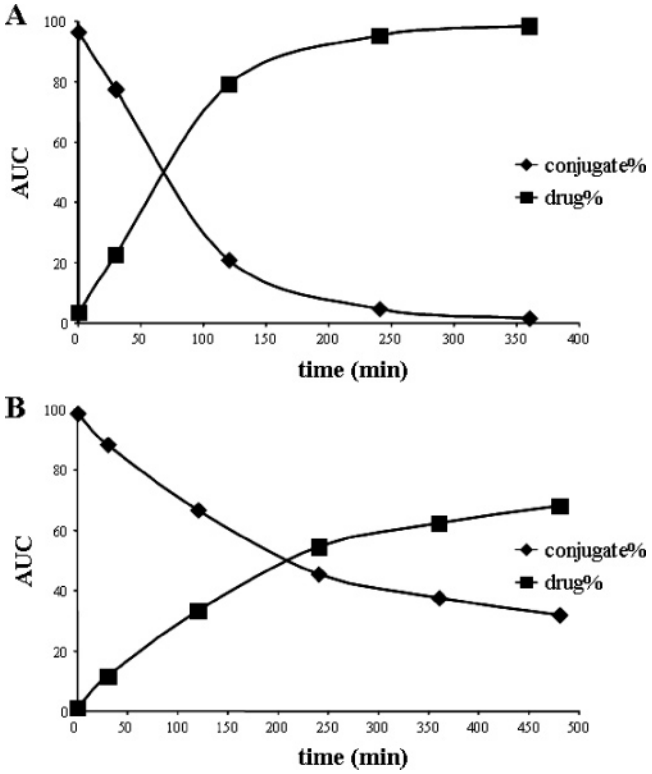


Figure 3. Patterns of drug release from the HMW (A) and LMW (B) CCMNPEG conjugates at pH 7.4 and 37 °C. Reactions were monitored by RP-HPLC at 280 nm.

time, conjugation of the enolic oxygen of CCMN was ruled out based on both the spectroscopy data and the existence of the resonating structures shown in Figure 2A.

To investigate the drug−polymer bond stability and to determine the rate of drug release, conjugate solutions in phosphate-buffered saline (PBS, pH 7.4) were incubated at 37 °C. Disappearance of the conjugate and formation of free CCMN were monitored by RP-HPLC at 280 nm. The CCMN signal was identified by referencing to that of an authentic sample. As shown in Table 1, the differently sized conjugates released the drug moiety at different rates, that is, about 60 and about 200 min for compounds **5** and **8**, respectively. The conjugate decomposition patterns for **5** and **8** are shown in Figure 3. Although no experiments were performed to identify the reasons for this half-life ($t_{1/2}$) difference, it may be related to that between the terminal functionalities in the PEG moiety, that is, a methyl ester in **5** and a methyl ether in **8**. This would be in addition to the difference in the PEG chain length, which may or may not be effective as a rate-determining factor in this process.

Table 1. Half-Life ($t_{1/2}$) of CCMN Release from the High- and Low-MW PEG Conjugates[a]

conjugate	$t_{1/2}$ (min)
CCMNPEG^{3500}, **5**	60
CCMNPEG^{750}, **8**	200

[a] In PBS at pH 7.4 and 37 °C.

Table 2. Cytotoxicity[a] of CCMN and CCMNPEG Conjugates against Four Human Carcinoma Cell Lines

treatment	PC-3	LS-174T	MIA PaCa-2	BxPC-3
CCMN, **1**	12.0 ± 1.35	6.5 ± 1.4	9.0 ± 2.5	2.9 ± 0.3
CCMNPEG^{3500}, **5**	5.0 ± 0.1	ND[b]	ND[b]	ND[b]
CCMNPEG^{750}, **8**	5.6 ± 0.2	4.0 ± 0.7	2.6 ± 0	2.1 ± 0.3

[a] Shown as IC_{50} (μM) ± standard error of the mean. [b] Not determined.

The cell-killing activities of the polymer-bound CCMN were evaluated through a series of controlled cytotoxicity assays and against a number of cancer cell lines of human origin. (Carcinoma cell types: PC-3, prostate; LS-174T, colon; MIA PaCa-2 and BxPC-3, pancreatic. All of human origin.) The cells were incubated with either CCMN as a positive control or the test conjugates for a 24 h exposure time and were counted after 96 h of treatment. The water-insoluble CCMN was applied as a DMSO solution, which was added to the cell-containing media. The viable cell numbers were normalized against untreated controls and were taken as a measure of treatment cytotoxicity. While the ester-linked HMW conjugate was inactive against BxPC-3 cells, the urethane-linked conjugates **5** and **8** were more cytotoxic than the unconjugated CCMN in a dose-dependent fashion. A representative example of the cytotoxicity assays is shown in Figure 4 for conjugate **8** and against BxPC-3 cells. The results for PC-3 and all other cell lines are shown also in

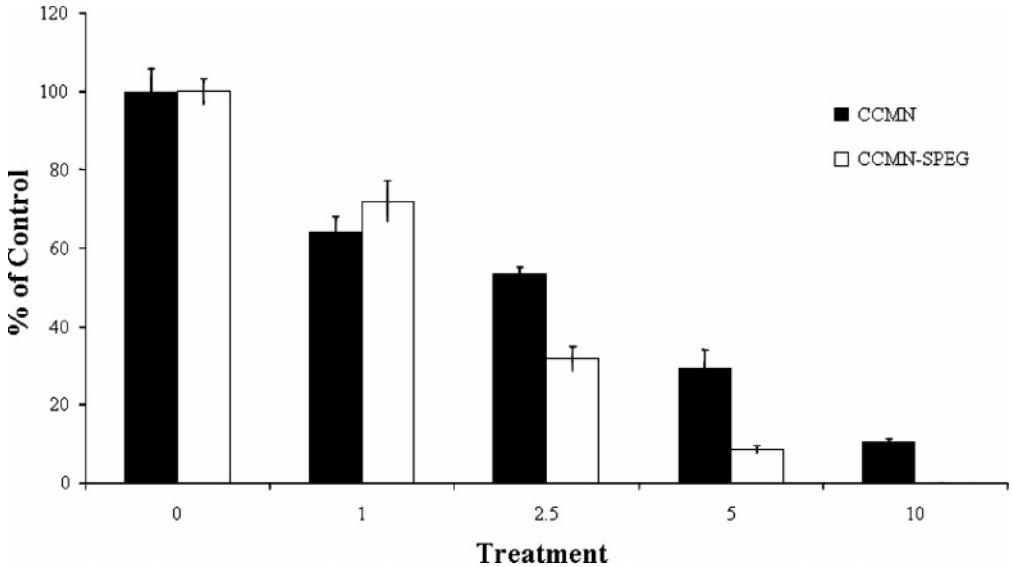


Figure 4. Cell-killing activity of CCMN, **1**, and the CCMNPEG^{750} conjugate, **8**. PC-3 human pancreatic carcinoma cells were treated with the two agents at different concentrations, and the surviving cell numbers were normalized against untreated controls. The number of the surviving cells in the 10-μM conjugate groups is too small to be visible.

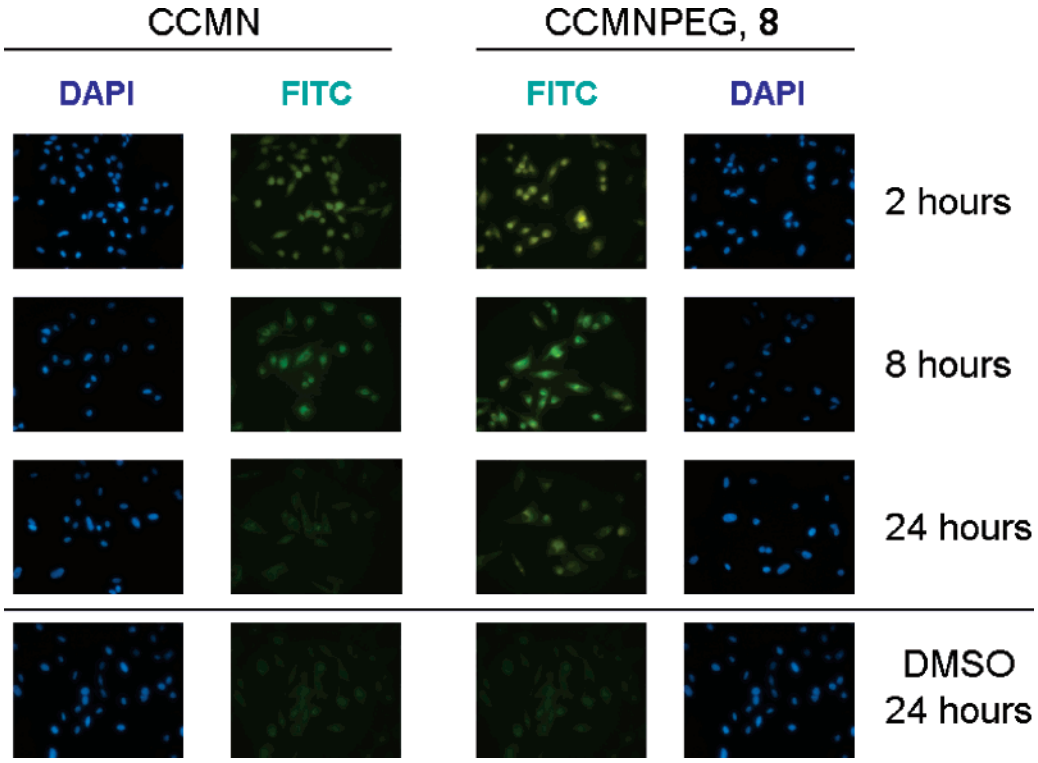


Figure 5. Internalization of CCMN and conjugate **8** in PC-3 human prostate carcinoma cells at different time points and as illustrated by fluorescent microscopy (FITC columns). The 24 h frame of the conjugate demonstrates the nuclear retention at this time point as compared to that of CCMN, which shows the same fluorescent intensity as that of the blank DMSO (bottom row). Locations of the cells' nuclei are confirmed by DAPI staining (DAPI columns).

Table 2 in the form of IC_{50} concentrations (equivalent concentration of CCMN to induce 50% cell death). In these cytotoxicity experiments, a DMSO solution of CCMN was used due to the lack of aqueous solubility. Attempts to dissolve a workable concentration of the drug in ultrapure water were not successful, and no detectable quantity of CCMN could be traced by spectrophotometry in this solvent (see Supporting Information). It is important to note, however, that the amount of the DMSO cosolvent (10 μL, 1% of the total volume in each well) was so small that its overall effect on the cells might be considered negligible. Still, to ensure elimination of such effects, a blank DMSO control was included in each assay, against which the CCMN cell counts were normalized.

It is expected that the improvement in the conjugates' cytotoxicity was due to their water solubility and cell internalization ability. Complete solubilization might have provided the cells with a longer "effective exposure time (EET)" to the drug. In contrast, the cells treated with unconjugated CCMN might have experienced a short EET due to a premature drug precipitation. Also, a facile passage of the pegylated CCMN through the cell membrane, leading to efficient drug internalization, might have further enhanced the activities.[21−23] A facilitated internalization would be favorable to the cytotoxicity of CCMN as one of the mechanisms of action of this drug is inhibition of the nuclear factor κB.[24] That PEG conjugation of CCMN did not affect its cell internalization was demonstrated by fluorescent microscopy experiments and in PC-3 as a model cell line. Thus, the cells were incubated with either DMSO, CCMN, or conjugate **8** in four-chamber microscope slides, were fixed at certain incubation times, and were viewed by fluorescent microscopy (Figure 5). At all time points, the fluorescent emission intensities of the conjugate-treated cells were higher than those of CCMN. In particular, at the 24 h time point, the CCMN emission was the same as that of the background, while that of the conjugate was visibly higher, indicating the presence of **8** in the nuclei. This experiment demonstrated that not only could both CCMN and the conjugate undergo nuclear internalization, but the conjugate had a prolonged internalization time possibly due to a resistance to cellular efflux. This resistance might explain the observed higher cytotoxicity of the conjugates.

Conclusions

Synthesis of novel water-soluble PEG conjugates of CCMN were described in this report. Two different molecular sizes of PEG were covalently attached to the drug through a urethane linkage. Spectroscopic analyses showed formation of conjugates with 1:1 CCMN/PEG molar ratios in which only one of the phenolic oxygens of the drug was attached to the polymer. At pH 7.4 and 37 °C, differently sized conjugates had different rates of drug release, which might have been due to the variation in the nature of chain-terminal functionalities and the molecular size. Furthermore, it was demonstrated that the conjugated drug had a higher cytotoxicity as compared to that of the free CCMN and that the conjugates cell-killing activity was linker-dependent. At this time, it is expected that the relative enhanced cytotoxicities are the result of aqueous solubility and polymer-mediated prolongation of drug internalization. The latter effect was demonstrated by fluorescent microscopy and the observation that the conjugate remained internalized for a longer period as compared to the free drug.

These results warrant further investigation of these conjugates toward the design and development of water-soluble and injectable curcumins, suitable for clinical applications in cancer treatment.

Experimental Section

Methyl-*N*-(4-nitrophenyloxycarbonyl)amino−PEG^{3500} Carboxylate, 4. Methylamino−PEG carboxylate (**2**, Scheme 1, MW ~ 3500) (104 mg, 0.03 mmol) in 10 mL of dry THF was added

within 40 min to a solution of bis(4-nitrophenyl)carbonate (**3**, 37.2 mg, 122.4 mmol) and DIEA (13.3 μL, 0.076 mmol) in 2 mL of dry THF. The mixture was stirred at room temperature (RT) under an argon atmosphere for 15 h. Additional portions of **3** (10.5 mg, 0.035 mmol) and DIEA (21 μL, 0.12 mmol) were added, and stirring was continued for another 2.5 h.

The solvent was distilled in vacuum, and the crude mixture was purified in a 2.5 × 25 cm silica gel column, using a 0−20% methanol (MTL) gradient in chloroform (CHL) containing 0.2% HOAc to afford 81 mg (78%) of the pure product **4** as a highly viscous oil. Calculated MW, 3665; MALDI MS, 3686; RP-HPLC t_R, 21.4 min.

CCMNPEG^{3500}−CO$_2$CH$_3$, 5. Compound **4** (75 mg, 0.022 mmol) was dissolved in 4 mL of dry DMF containing 17.4 μL (0.1 mmol) of DIEA. A solution of CCMN (36.8 mg, 0.1 mmol) in 2 mL of the same solvent was added, and the mixture was stirred under argon and at RT for 3 days. The solvent was distilled in vacuum, and the residue was redissolved in ethyl acetate (ETA) and was loaded into a 2.5 × 8.5 cm silica gel column. The column was eluted with 20% hexanes in ETA, ETA, and then 5−10% MTL/CHL containing 1% HOAc. Distillation of solvents afforded a solid. This was redissolved in 2 mL of distilled water, and the slight quantity of fine particles was separated by filtration through a syringe-tip, 0.2 μm cellulose membrane. The clear solution was lyophilized to afford a bright-yellow, water-soluble solid product in 67 mg (82%) yield. Calculated MW, 3894; MALDI MS, 3928; RP-HPLC t_R, 24.1 min.

Methoxy-*N*-(4-nitrophenyloxycarbonyl)amino−PEG^{750}, 7. The same reaction as that for the preparation of **4**, above, was employed using the methoxyamino−PEG^{750} (**6**, 980 mg, 1.3 mmol) as the starting compound. The crude product mixture was purified in a 2.5 × 10 cm silica gel column, eluted with 0−0.5% MTL/CHL to afford 1 g (83%) of a light-yellow oil. Calculated MW, 916; MALDI MS, 906; RP-HPLC t_R, 19.5 min; ^{1}H NMR, see Supporting Information Table 1, NMR1, and NMR2.

CCMNPEG^{750}−OCH$_3$, 8. Compound **7** (500 mg, 0.55 mmol) was reacted with CCMN according to the procedure for the preparation of **5**, above. Column chromatography on silica gel afforded 252 mg (40%) of the pure product as a deep-red and highly viscous oil. Calculated MW, 1145; MALDI MS, 1145; RP-HPLC t_R, 24.25 min; ^{1}H NMR, see Supporting Information NMR3−NMR6 and Tables 2 and 3.

Supporting Information Available: Experimental details on drug release kinetics, water-solubility, cytotoxicity, fluorescent microscopy, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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