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Diamide Linked γ-Cyclodextrin Dimers as Molecular-Scale Delivery Systems for the Medicinal Pigment Curcumin to Prostate Cancer Cells

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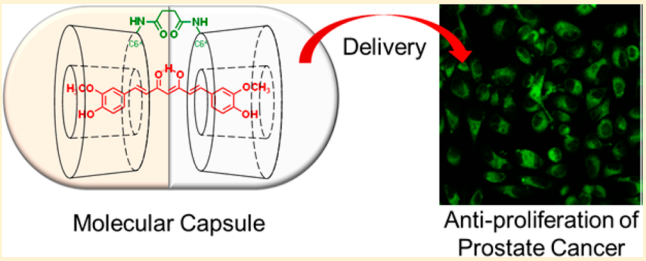
Supporting Information

ABSTRACT: Diamide linked γ-cyclodextrin (γ-CD) dimers are proposed as molecular-scale delivery agents for the anticancer agent curcumin. *N,N′*-Bis(6^A-deoxy-γ-cyclodextrin-6^A-yl)succinamide (66γCD$_2$su) and *N,N′*-bis(6^A-deoxy-γ-cyclodextrin-6^A-yl)urea (66γCD$_2$ur) markedly suppress the degradation of curcumin by forming a strong 1:1 cooperative binding complexes. The results presented in this study describe the potential efficacy of 66γCD$_2$su and 66γCD$_2$ur for intracellular curcumin delivery to cancer cells. Cellular viability assays demonstrated a dose-dependent antiproliferative effect of curcumin in human prostate cancer (PC-3) cells that was preserved by the curcumin-66γCD$_2$su complex. In contrast, delivery of curcumin by 66γCD$_2$ur significantly delayed the antiproliferative effect. We observed similar patterns of gene regulation in PC-3 cells for curcumin complexed with either 66γCD$_2$su or 66γCD$_2$ur in comparison to curcumin alone, although curcumin delivered by either 66γCD$_2$su or 66γCD$_2$ur induces a slightly higher up-regulation of heme oxygenase-1. Highlighting their nontoxic nature, neither 66γCD$_2$su nor 66γCD$_2$ur carriers alone had any measurable effect on cell proliferation or candidate gene expression in PC-3 cells. Finally, confocal fluorescence imaging and uptake studies were used to demonstrate the intracellular delivery of curcumin by 66γCD$_2$su and 66γCD$_2$ur. Overall, these results demonstrate effective intracellular delivery and action of curcumin when complexed with 66γCD$_2$su and 66γCD$_2$ur, providing further evidence of their potential applications to deliver curcumin effectively in cancer and other treatment settings.



KEYWORDS: *anticancer, drug delivery, cyclodextrin, biocompatibility, cell proliferation*

INTRODUCTION

Prostate cancer remains a worldwide health concern, with approximately 913 000 new cases diagnosed each year, or around 14% of all male cancers.[1] The treatment options for men diagnosed with early stage prostate cancer include surgery and/or radiation and are often curative. For men diagnosed with cancer that has already spread beyond the prostate gland, however, treatment options are essentially palliative and often involve removal of the gland and/or surgical or chemical castration. While these treatments are initially effective, many patients eventually relapse, after which the only remaining treatments are chemotherapy or blockade of androgen metabolism, which have limited efficacy and significant side effects.[2−4] It is unsurprising, therefore, that there is considerable interest in developing adjuvant or alternative cancer agents to improve treatment response or prolong progression and/or quality of life or have fewer side effects. One potential agent in this regard is the naturally occurring compound curcumin, found in the Indian spice plant turmeric (*Curcuma longa*), which has previously been shown to possess chemo-preventive properties with low toxicity.[5,6]

Turmeric contains a group of yellow pigments, namely, curcuminoids, which are mainly comprised of curcumin (∼77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%).[7] In the past decade, curcumin has been investigated intensively and is shown to have anticancer,[8−10] anti-inflammatory,[10,11] anti-Alzheimer's,[12] anticystic fibrosis,[13] and

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wound healing activities.[9,10] In a phase I clinical study, Cheng et al. demonstrated that curcumin is nontoxic up to 8 g/day when orally administrated for three months, but at that dose it did not significantly affect a variety of either premalignant or high-risk lesions.[5] Poor bioavailability is likely to be a major contributor to the disparity between *in vitro* and *in vivo* effects of curcumin, which is poorly soluble in water (~11 μg/mL),[14,15] and prone to hydrolysis and fragmentation, resulting in significant degradation within 30 min.[16−19] These challenges must be overcome to increase the practical applicability of this compound. Previous studies have demonstrated effective stabilization of curcumin using a range of potential delivery agents, including micelles,[19−22] liposomes,[23−25] polymers,[26−28] and proteins.[17,29−32] Each of these large-scale supramolecular assemblies may, however, limit intracellular delivery of curcumin, as they typically undergo significant structural perturbation upon contact with cellular membranes.[33] The development of molecular-scale delivery agents for curcumin and other agents is therefore of significant interest, because they have the potential to be more effective at delivering these agents to the intracellular milieu while maintaining their structural integrity.[33] Our previous study has shown that diamide linked γ-cyclodextrin dimers possess many desirable properties for molecular encapsulation and delivery of curcumin, including a high structural stability and the ability to suppress degradation of curcumin under physiological conditions.[16]

Cyclodextrins (CDs) are natural cyclic oligosaccharides that are FDA-approved[34−36] and are already utilized in the food and cosmetic industries.[37] γ-Cyclodextrin (γ-CD), which consists of eight glucopyranoside units in a toroidal structure, possesses a hydrophobic interior and hydrophilic exterior (Figure 1A). As such, γ-CD can act as a host to encapsulate and solubilize hydrophobic guest species in water through host−guest complexation.[38,39] Recently, Pham et al. established the synthesis of γ-CD dimers linked with either succinamide or urea substituted onto the $C6^A$ site of a glucopyranose unit in each of the γ-CD, namely, *N*,*N*′-bis(6^A-deoxy-γ-cyclodextrin-6^A-yl)succinamide, $66\gamma CD_2su$, and *N*,*N*′-bis(6^A-deoxy-γ-cyclodextrin-6^A-yl)urea, $66\gamma CD_2ur$ (Figure 1B and C).[40] These diamide linked γ-CD dimers are excellent systems for drug delivery because (i) of their small size relative to other delivery agents mentioned above and (ii) the diamide linker can be hydrolyzed by intracellular enzymes to release the encapsulated species. The close proximity of the two γ-CDs in the dimers results in cooperative binding to the guest molecule. In the case of curcumin, the resulting molecular encapsulation by the diamide linked γ-CD dimers at the 1:1 molar ratio suppresses the rates of curcumin degradation substantially under physiological conditions.[16] Cooperative binding of the diamide linked γ-CD dimers to curcumin results in a high binding constant of 10^6 M^{-1}, which is indicative of an entrapment efficiency of nearly 100% in water. In addition, a high concentration of curcumin of at least 1.3 mg/mL (3.3 mM) in aqueous solution can be achieved with either $66\gamma CD_2su$ or $66\gamma CD_2ur$ at a 1:1 molar ratio, which is more than 100 times higher than the aqueous solubility of curcumin (11 μg/mL).[14,15] Moreover, this high concentration of curcumin is unachievable with single γ-CDs, demonstrating the importance of cooperative binding by $66\gamma CD_2su$ and $66\gamma CD_2ur$. Thus, they possess the potential to be effective and nontoxic delivery systems for curcumin.

Here, we report for the first time the intracellular delivery of curcumin using $66\gamma CD_2su$ and $66\gamma CD_2ur$ and the biological consequences to human prostate cancer (PC-3) cells. Using cell viability assays, we observed a significant, dose-dependent decrease in cellular proliferation in response to encapsulated curcumin without any observable effect from the carrier $66\gamma CD_2su$ or $66\gamma CD_2ur$ alone. The intracellular delivery of curcumin to PC-3 cells by $66\gamma CD_2su$ and $66\gamma CD_2ur$ and the maintenance of biological activity of curcumin delivered by these carriers were verified by a variety of techniques, including confocal fluorescence imaging, uptake studies using fluorescence spectroscopy, and expression of several well-characterized curcumin target genes. Overall, the results indicate the potential of $66\gamma CD_2su$ and $66\gamma CD_2ur$ as effective and nontoxic delivery agents for curcumin in cancer treatment.

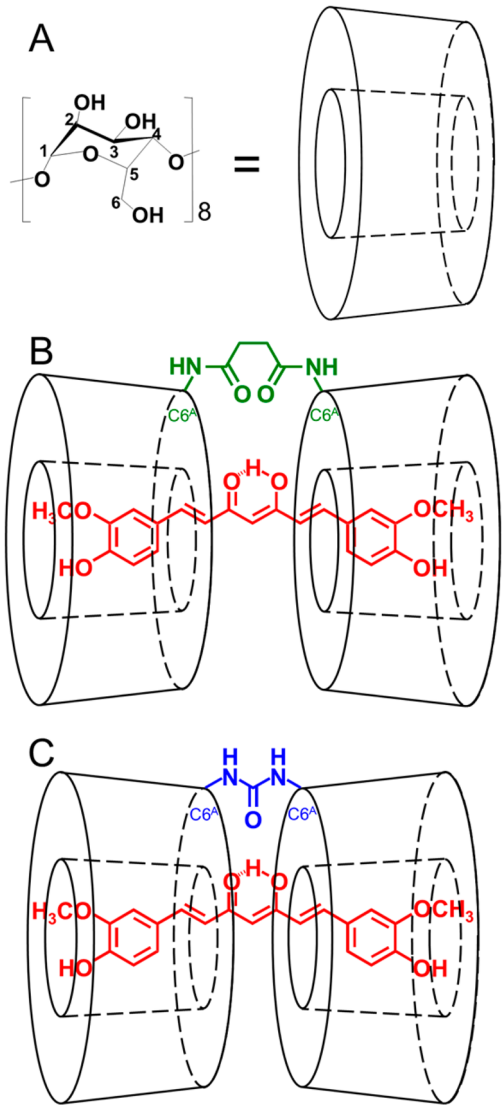

Figure 1. Structures of (A) γ-CD and curcumin complexed in (B) $66\gamma CD_2su$ and (C) $66\gamma CD_2ur$.

EXPERIMENTAL SECTION

Materials. Curcumin was obtained from LKT Laboratories (purity >98%). Methanol (AR grade, 99.5%) from Merck Pty Ltd. was used as received. The phosphate buffer solution (50 mM) used in the stability study was prepared with deionized water from a Millipore Milli-Q NANO pure water system, and the pH was adjusted to 7.4. The human prostate cancer (PC-3)

cell line was obtained from American Type Culture Collection (VA, USA). RPMI 1640 cell culture medium with and without phenol red were purchased from Invitrogen (Mulgrave, VIC, Australia). Dimethyl sulfoxide (DMSO, ≥99.7%, sterile filtered), fetal bovine serum (FBS), dextran-coated charcoal stripped fetal bovine serum (DCC-FBS), bovine serum albumin (BSA), and 0.1% trypan blue diluted with PBS (endotoxin free) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). RNA was extracted using the RNeasy mini kit (Qiagen, VIC, Australia) and cDNA generated using the iScript cDNA synthesis kit (Biorad, NSW, Australia). Cells were maintained in RPMI supplemented with 10% FBS. For cell treatments, phenol red-free (PRF) RPMI was supplemented with 10% DCC-FBS.

Synthesis of Diamide Linked γ-CD Dimers. The $C6^A$-to-$C6^A$ diamide linked γ-CD dimers, *N,N′*-bis(6^A-deoxy-γ-cyclodextrin-6^A-yl)succinamide, 66γCD_2su, and *N,N′*-bis(6^A-deoxy-γ-cyclodextrin-6^A-yl)urea, 66γCD_2ur, were synthesized using methods established by Pham et al.[40] Briefly, the native γ-CDs were substituted with 4-toluenesulfonylchloride for activation at the $C6^A$ position, which yielded 6^A-*O*-(4-methylbenzenesulfonyl)-γ-cyclodextrin (6γCDTs). For the synthesis of 66γCD_2su, the reaction between 6γCDTs and ammonium bicarbonate produced 6^A-amino-6^A-deoxy-γ-cyclodextrin, 6γ$CDNH_2$, which was then dimerized by the reaction with bis(4-nitrophenyl) succinate as the linker. For the synthesis of 66γCD_2ur, the reaction between 6γCDTs and sodium azide produced 6^A-azido-6^A-deoxy-γ-cyclodextrin, 6γCDN_3, which was then dimerized by the reaction with carbon dioxide as the linker.

Measurement of Cell Viability. A 50 mM solution of curcumin in DMSO and 8 mg/mL solutions of 66γCD_2su and 66γCD_2ur in PBS were used as stock solutions. PC-3 cells (5×10^3 cells/well in 24-well plates) were plated in phenol red free RPMI 1640 (PRF RPMI 1640) media containing 10% dextran-charcoal stripped fetal bovine serum (DCC-FBS) and allowed to attach for 24 h. Cells were washed with PBS once and treated in quadruplicates with curcumin (3.1−50.0 μM), 66γCD_2su (12.5 μM), 66γCD_2ur (12.5 μM), curcumin-66γCD_2su (12.5 μM), or curcumin-66γCD_2ur (12.5 μM) in PRF RPMI 1640 media containing 10% DCC-FBS, and the plates were incubated for 1−5 days. Owing to curcumin's low solubility and stability in PBS, it was delivered to PC-3 cells using a small quantity of DMSO. As a consequence, each solution contained a total of 0.03 vol % DMSO as vehicle control to maintain consistency in each study. The negligible quantity of DMSO was expected to have an insignificant effect on cell viability. The curcumin stock solution was mixed with either the 66γCD_2su or 66γCD_2ur solution to facilitate the diamide linked γ-CD dimer−curcumin complexation before being added to the medium. Viable and dead cells were manually counted using a hemocytometer on the day of treatment (Day 0) and at Days 1−5 post-treatment by trypan blue exclusion as described previously.[41,42]

Curcumin Target Gene Expression in PC-3 Cells. PC-3 cells (2×10^5 cells/well in 6-well plates) were plated in PRF RPMI 1640 containing 5% DCC-FBS and allowed to attach for 24 h. Cells were treated in triplicate for 12 h with curcumin (6.3−25.0 μM), 66γCD_2su (25.0 μM), 66γCD_2ur (25.0 μM), curcumin-66γCD_2su (6.3−25.0 μM), or curcumin-66γCD_2ur (6.3−25.0 μM) in PRF RPMI 1640 containing 5% DCC-FBS. Each solution contained a total of 0.05 vol % DMSO as vehicle control for the reason stated above. For the studies involving a diamide linked γ-CD dimer, the control was either 25.0 μM 66γCD_2su or 66γCD_2ur, in addition to a total of 0.05 vol % DMSO without curcumin. RNA was extracted using the RNeasy mini kit and DNase treated using the RNase free DNase kit according to the manufacturer's instructions (Qiagen, VIC, Australia). RNA was reverse transcribed using the iScript cDNA synthesis kit according to the manufacturer's protocol (Biorad, VIC, Australia). Quantitative real time PCR (QPCR) was performed using iQ SYBR green supermix (Biorad) on a Biorad CFX96 real time PCR machine. Data are presented as the average of three biological replicates in technical duplicates, with gene expression normalized to the reference genes *GAPDH* and *RPL32*.

Qualitative and Quantitative Cellular Uptake of Curcumin. The qualitative cellular uptake studies involved imaging PC-3 cells with curcumin with a laser scanning confocal fluorescence microscope (Leica TCS SP5). The purpose of these studies is to confirm cellular uptake of curcumin. PC-3 cells (1.6×10^3 cells/well in 8-well chamber slides) in PRF RPMI 1640 containing 10% DCC-FBS and allowed to attach for 24 h. Cells were washed once with PBS and treated with 12.5 μM curcumin, 66γCD_2su, 66γCD_2ur, curcumin-66γCD_2su, or curcumin-66γCD_2ur in PRF RPMI 1640 containing 10% DCC-FBS, and incubated for 1−5 days. Each of these solutions contained a total of 0.08 vol % DMSO as vehicle control for the reason stated above. Prior to imaging, cells were washed with PBS twice (0.5 mL/well) so that only intracellular curcumin was detected. The excitation and emission wavelengths used were λ_{ex} = 405 nm and λ_{em} = 470−600 nm, respectively. The excitation source was a PicoQuant PDL 800-B pulse diode laser with a repetition rate of 4 MHz. The average excitation power used was 3 mW. The excitation light was focused onto the sample using a Leica HCX PL APO 63× N.A. 1.20 water-immersion objective with a 220 μm working distance. The emission was collected by the same objective, separated from the excitation source using a dichroic mirror and dispersed using a built-in spectrometer. Each image was acquired using line and frame averaging of 1 and 8, respectively. The images were 700 × 700 μm^2, and each image acquisition time was approximately 3 s.

The first set of quantitative cellular uptake studies was performed using a FLUOstar OPTIMA microplate reader (λ_{ex} = 400 nm, λ_{em} = 520 nm). PC-3 cells (5×10^4 cells/well in 24-well plates) were plated in the same culture media and allowed to attach for 24 h. Cells were washed with PBS once and treated in sextuplicates with 12.5 μM curcumin, 66γCD_2su, 66γCD_2ur, curcumin-66γCD_2su, or curcumin-66γCD_2ur in PBS and incubated for the time periods. Cells were subsequently washed twice with PBS. At each incubation time point, 200 μL of chilled 100% methanol was used to lyse the cells in the six replicate wells. Lysates were combined and transferred to a well of a 96-well plate on ice in order to reduce methanol evaporation. Curcumin fluorescence intensity relative to untreated cells was measured. Data were normalized to the saturation intensity of curcumin (Supporting Information, Table S1) and was obtained by fitting with first-order binding kinetics.

The second set of quantitative studies was aimed at investigating the role of BSA in cellular uptake of curcumin. A 4.0 mg/mL BSA solution was prepared in PBS, which corresponds to the concentration of BSA in PRF RPMI 1640 containing 10% DCC-FBS used in our cell viability and confocal fluorescence imaging assays.[43−45] Cells were incubated with either 0.0 or 4.0 mg/mL BSA solution supplemented with

12.5 μM curcumin, curcumin-66γCD$_2$su, or curcumin-66γCD$_2$ur for 10 or 90 min, where fluorescence intensity was found to be within the error range of the half or full saturation value described above. The curcumin stock solution was mixed with either 66γCD$_2$su or 66γCD$_2$ur solution prior to dilution with the BSA solution. The curcumin fluorescence intensity of 200 μL of chilled 100% methanol lysates was measured using the microplate reader described above with identical wavelength settings. Fluorescence intensities were normalized to the half or full saturation intensity of each sample in PBS without BSA after 10 min or 90 min incubation, respectively.

Statistical Analyses. All data, except for cellular viability assays, are presented as mean ± standard deviation for three independently performed experiments unless described. The data from cellular viability assays are presented as mean ± standard error of the mean (SEM) of viable or dead cells per well in quadruplicates. Statistical analyses were performed by two-way ANOVA for paired comparisons of means. Values of $p > 0.05$ were indicative of insignificant differences, whereas those of $p < 0.001$ were indicative of very significant differences.

RESULTS

PC-3 Viability in the Presence of Curcumin and Encapsulated by Diamide Linked γ-CD Dimers. The antiproliferative effects of curcumin on PC-3 cells were evaluated using trypan blue exclusion, as described previously.[41,42] We observed a dose-dependent decrease in cell proliferation between 3.1 μM and 50.0 μM curcumin, with a 50% maximal inhibitory response (IC50) observed at 12.5 μM over a 5 day period (Figure 2A). The duration of our studies is consistent with that of previous studies.[46−51] Curcumin treatment did not appear to lead to an increased incidence of dead cells determined by trypan blue exclusion over the course of the experiments (Supporting Information; Figure S2). An IC50 concentration of 12.5 μM was determined and used for further investigation using 66γCD$_2$su and 66γCD$_2$ur as delivery agents.

The effect of curcumin encapsulated in either 66γCD$_2$su or 66γCD$_2$ur, namely curcumin-66γCD$_2$su and curcumin-66γCD$_2$ur, on PC-3 cells was investigated next over a 5 day period. Figure 2B shows the antiproliferative effect of curcumin in the absence and presence of 66γCD$_2$su or 66γCD$_2$ur. Compared to vehicle, curcumin alone and curcumin-66γCD$_2$su were equally effective at inhibiting cell proliferation at all time points ($p > 0.05$). While curcumin-66γCD$_2$ur also inhibited cell proliferation effectively compared to vehicle at all time points ($p < 0.001$), it was only 73% as effective as curcumin or curcumin-66γCD$_2$su after Day 3 ($p < 0.001$; Figure 2B), indicating a delayed response. Importantly, neither 66γCD$_2$su nor 66γCD$_2$ur alone affected PC-3 cell proliferation compared to vehicle control ($p > 0.05$; Figure 2C).

Curcumin-Induced Gene Expression in the PC-3 Cell Line. Heme oxygenase 1 gene (*HMOX1*) is an inducible stress response gene forming part of the nuclear factor like 2 (NRF2) pathway, a primary cellular defense against cytotoxic effects of oxidative stress. Curcumin-induced expression of *HMOX1* has been well-characterized in several cellular systems.[52−54] Here we used *HMOX1* regulation to infer and quantitate the intracellular delivery and biological activity of curcumin, curcumin-66γCD$_2$su, and curcumin-66γCD$_2$ur in PC-3 cells. At 12.5 μM, a clear up-regulation of *HMOX1* expression with curcumin alone was observed relative to vehicle control (58-fold, $p < 0.001$; Figure 3A). Similar upregulation (53- or 70-fold) was observed with curcumin encapsulated in 66γCD$_2$su or 66γCD$_2$ur, respectively ($p < 0.001$). Furthermore, at 25.0 μM, both curcumin-66γCD$_2$su and curcumin-66γCD$_2$ur had a significantly greater effect on *HMOX1* upregulation (140 fold) than curcumin alone (112-fold, $p < 0.001$). *HMOX1* expression did not increase above baseline levels in PC-3 cells treated with either 66γCD$_2$su or 66γCD$_2$ur alone (i.e., 0 μM curcumin; Figure 3A and inset), consistent with the results in Figure 2. To support this finding further, we investigated four other curcumin target genes,[55−62] one of which was upregulated (*GADD45A*) and three down regulated (*TNFRSF10B, BRCA2*, and *NFκBIA*) in response to curcumin treatment. The diamide linked γ-CD dimer encapsulated forms of curcumin were equally as effective as curcumin alone in increasing or decreasing expression of these candidate genes (Figure 3B−E), and again 66γCD$_2$su or 66γCD$_2$ur alone did not affect gene expression (Figure 3B−E). Together, these results demonstrate that encapsulation with 66γCD$_2$su or 66γCD$_2$ur permits intracellular delivery and biological activity of curcumin.

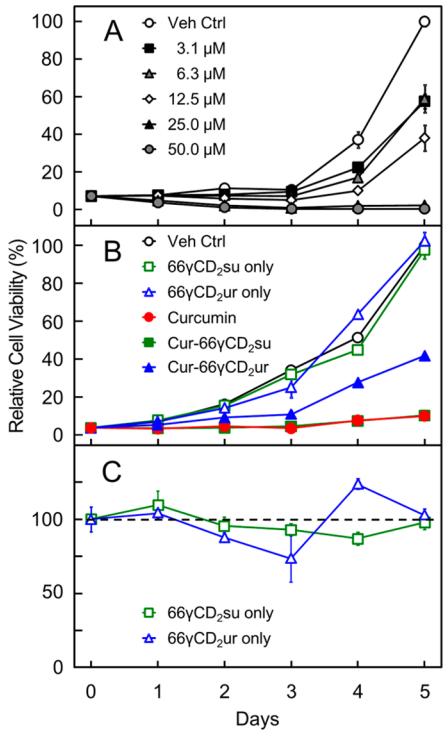

Figure 2. Cell viability assays performed using human prostate cancer PC-3 cells. (A) Viable PC-3 cells were treated with indicated concentrations of curcumin over 5 days, and viable cells were counted in the presence of trypan blue, demonstrating that curcumin exhibits dose-dependent antiproliferative activity. (B) Viable PC-3 cells were counted using trypan blue assays after treatment with 12.5 μM curcumin, curcumin-66γCD$_2$su, or curcumin-66γCD$_2$ur over 5 days. (C) Viable PC-3 cells were counted using trypan blue assays and treated with 66γCD$_2$su or 66γCD$_2$ur with respect to vehicle control (shown as dashed line), indicating their nontoxic nature ($p > 0.05$).

Qualitative and Quantitative Cellular Uptake Studies of Curcumin. Next, we assessed the amount of curcumin delivered to PC-3 cells qualitatively using confocal fluorescence microscopy. Figure 4 shows fluorescence images of PC-3 cells treated with 12.5 μM curcumin, curcumin-66γCD$_2$su or curcumin-66γCD$_2$ur for 1 and 4 days. The fluorescence intensity at 1 day of each treatment was substantially greater

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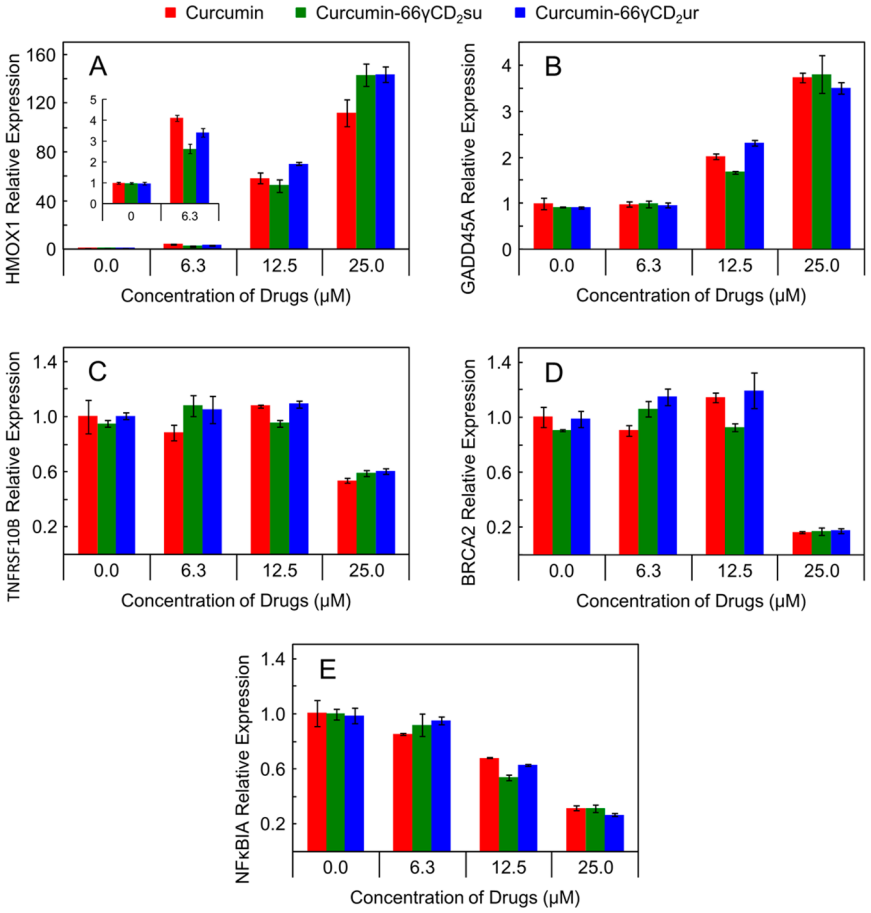

Figure 3. Relative expression of (A) *HMOX1*, (B) *GADD45A*, (C) *TNFRSF10B*, (D) *BRCA2*, and (E) *NFκBIA* in PC-3 cells treated with different concentrations of curcumin (red), curcumin-66γCD$_2$su (green), or curcumin-66γCD$_2$ur (blue). The gene expression was normalized to those of *GAPDH* and *RPL32*. The 0.0 μM represents vehicle control with 0.05 vol % DMSO (red), 25.0 μM 66γCD$_2$su (green), and 25.0 μM 66γCD$_2$ur (blue).

than the detection limit (Figure 4, upper panels), while the intrinsic fluorescence of untreated cells was negligible (Supporting Information; Figures S3−5, panel A). Irrespective of 66γCD$_2$su or 66γCD$_2$ur encapsulation, curcumin fluorescence was still detectable within cells after single dose treatment at Day 4, with an intensity roughly half of that observed at Day 1, with slightly lower intensities for both treatments with encapsulated curcumin (Figure 4, lower panels).

Although fluorescence imaging revealed the intracellular presence of curcumin, the influence of serum proteins on curcumin delivery, specifically the effect of the most abundant protein in FBS, BSA, was not revealed. Therefore, the uptake of curcumin by PC-3 cells 10 and 90 min after treatment was measured using a fluorescent plate reader. The treatments involved using solutions of 12.5 μM curcumin, curcumin-66γCD$_2$su, or curcumin-66γCD$_2$ur in PBS alone or PBS spiked with 4.0 mg/mL BSA, which is the approximate concentration found in 10% DCC-FBS/PRF RPMI 1640 and 5 times higher than the 12.5 μM diamide linked γ-CD dimers used in our experiments.[43−45] The relative fluorescence intensity of each curcumin solution in the presence of BSA at 10 or 90 min was normalized to that in the absence of BSA (Figure 5). Importantly, the relative fluorescence intensity in cells treated with unencapsulated curcumin was 72% lower when BSA was present in the solution ($p < 0.001$). However, BSA had a minor effect on the relative fluorescence intensity in cells treated with either curcumin-66γCD$_2$su or curcumin-66γCD$_2$ur (*n.s.*, $p > 0.05$), as shown in Figure 5. In addition, the fluorescence intensity of cells treated with curcumin alone in the presence of BSA resulted in a similar level to that of curcumin delivered by either 66γCD$_2$su or 66γCD$_2$ur (Supporting Information, Figure S6). Furthermore, the treatments were also applied to PC-3 cells in serum-free PBS to determine the amount of curcumin in cells within the time scale that cellular proliferation was negligible. Consistent with first-order binding kinetics, the fluorescent intensity following treatment with curcumin alone reached 50% of the maximum value within the first 10 min and saturated around 90 min, increasing at a rate constant of 0.050 ± 0.012 min^{-1} (Supporting Information, Figure S7). The maximum fluorescence remained constant over an extended period of time (60 − 180 min; Supporting Information, Figure S8). In the presence of either 66γCD$_2$su or 66γCD$_2$ur (Supporting Information, Figure S7), the curcumin fluorescence intensity increased at a rate constant of 0.076 ± 0.031 min^{-1} and 0.029 ± 0.018 min^{-1}, respectively. The maximum fluorescence intensity of curcumin delivered by either 66γCD$_2$su or 66γCD$_2$ur is 5−9 times lower than that of curcumin alone.

DISCUSSION

Curcumin has been shown to have significant medicinal effects including anticancer and anti-inflammatory activities.[8−11]

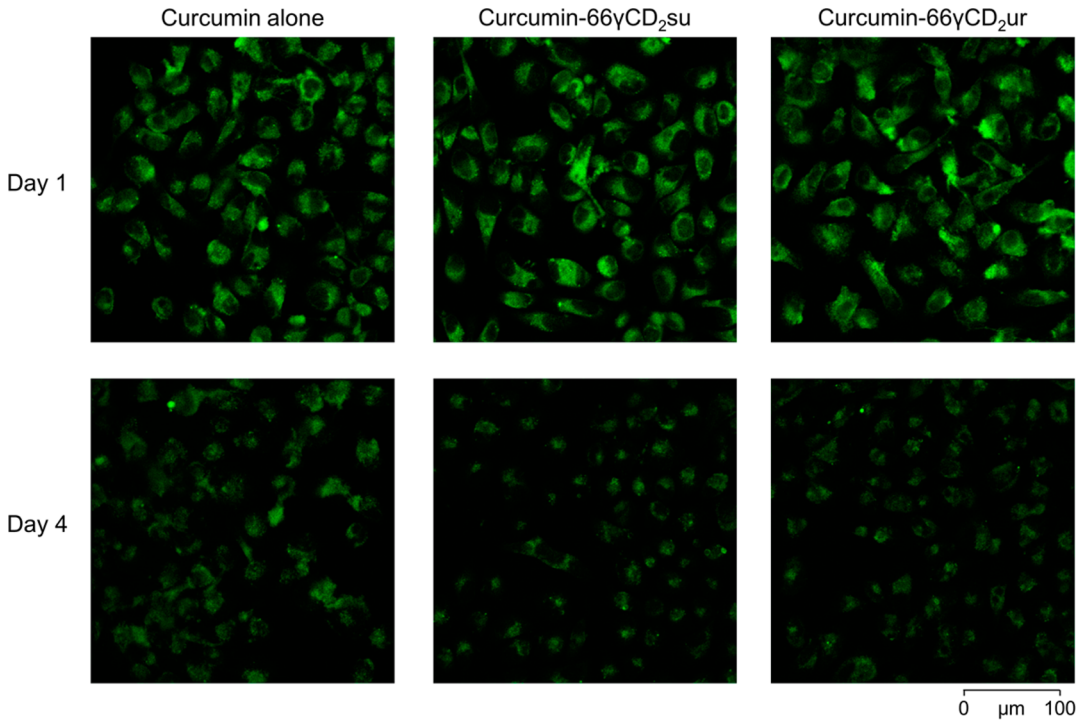

Figure 4. Confocal fluorescence images of PC-3 cells treated with 12.5 μM curcumin, curcumin-66γCD$_2$su, and curcumin-66γCD$_2$ur 1 day (top) and 4 days (bottom) after single dose treatment.

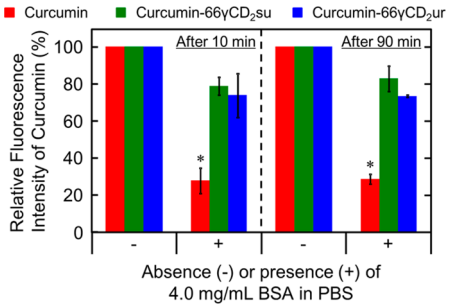

Figure 5. Effects of BSA on cellular uptake of curcumin in the absence (−) and presence (+) of either 66γCD$_2$su or 66γCD$_2$ur. PC-3 cells were treated with 12.5 μM of each solution prepared with either PBS alone or PBS spiked with 4.0 mg/mL BSA, for 10 (left) and 90 min (right). The asterisks in the figure represent a statistically significant decrease in fluorescence intensity of curcumin due to the presence of BSA ($p < 0.001$).

However, the poor solubility and stability of curcumin that limit the *in vivo* availability are major problems for the development of curcumin as a therapeutic agent. The association of curcumin with a delivery agent is an approach to address these issues. Our previous study showed effective and significant aqueous stabilization of curcumin using diamide linked γ-CD dimers, 66γCD$_2$su and 66γCD$_2$ur.[16] Here, we propose the use of 66γCD$_2$su and 66γCD$_2$ur as novel delivery systems for curcumin without perturbing its medicinal efficacy.

We demonstrated that curcumin inhibits the proliferation of PC-3 cells in a dose-dependent manner, which is consistent with previous studies.[63,64] Importantly, our results reveal that the use of the delivery agents 66γCD$_2$su and 66γCD$_2$ur does not prevent the antiproliferative effect of curcumin, indicating effective intracellular delivery and biological activity of curcumin. In addition, we demonstrated that 66γCD$_2$su and 66γCD$_2$ur alone do not affect cellular proliferation or death, supporting a general nontoxic nature of these delivery agents. This observation is consistent with single γ-CDs being nontoxic[34] and the diamide linker of 66γCD$_2$su and 66γCD$_2$ur being hydrolyzed enzymatically in the cellular environment.[65−68] It is also important to stress that delivery of curcumin using 66γCD$_2$su and 66γCD$_2$ur here was achieved at a 1:1 molar ratio, whereas delivery systems with a much higher cyclodextrin-to-curcumin molar ratios have been reported in previous studies.[69−71] Curcumin delivery using 66γCD$_2$su and 66γCD$_2$ur is therefore more effective and efficient than other cyclodextrin-based delivery systems, and a higher concentration of encapsulated curcumin can be achieved.

Intracellular delivery of curcumin and the negligible effect of 66γCD$_2$su and 66γCD$_2$ur were observed on the expression of curcumin target genes. The increase or decrease in gene regulation was evident from curcumin treatment and exhibited a strong dependence on the dose of curcumin, which has been shown previously.[54] Our results showed that the gene expression of PC-3 cells treated with 66γCD$_2$su-curcumin or 66γCD$_2$ur-curcumin is also dose-dependent and comparable to curcumin alone, indicating that these delivery agents do not prevent the intracellular efficacy or uptake of curcumin. In addition, we demonstrated that neither 66γCD$_2$su nor 66γCD$_2$ur alone has any effect on these curcumin responsive target genes, which further supports their nontoxic nature up to 25.0 μM, consistent with our viability assay results.

Fluorescence imaging and spectroscopic studies provide further, direct evidence of curcumin−cell interaction owing to the sensitivity of the fluorescence properties of curcumin to the polarity of the surrounding environment.[22,72,73] For instance, the fluorescence quantum yields of curcumin in BSA and sodium dodecyl sulfate (SDS) micelles, which have hydrophobic characteristics similar to cell membranes, are approx-

imately 2−5%, while that in a pH 7.4 phosphate buffer solution is negligible.[16,22,30] In addition, our previous study showed that curcumin is essentially nonfluorescent in the presence of either 66γCD$_2$su or 66γCD$_2$ur in buffer solution.[16] Our observation of intracellular fluorescence here therefore indicates that curcumin is present in hydrophobic regions, for example, membranes, within the intracellular space. The results can be used to directly assess the delivery of curcumin to PC-3 cells.

Confocal fluorescence images demonstrate a minor difference between the fluorescence intensity of curcumin alone and curcumin delivered by either 66γCD$_2$su or 66γCD$_2$ur, similar to our cellular viability and gene expression results. Together, all of these data indicate efficient intracellular delivery of curcumin by 66γCD$_2$su and 66γCD$_2$ur. Another interesting observation in the confocal fluorescence images is a lower fluorescence level in the nucleus, implying that curcumin is present at a higher concentration in the cytoplasm than the nucleus. This result was also observed in a previous study.[74] Moreover, the slightly lower fluorescence intensity of curcumin delivered by 66γCD$_2$su and 66γCD$_2$ur than curcumin alone at Day 4 is unlikely to arise from lower curcumin levels. This is because nearly identical antiproliferative effects of curcumin alone and curcumin delivered by 66γCD$_2$su were observed, as shown in Figure 2b. It is likely that similar levels of curcumin were present in all cases.

We also considered the role of serum proteins in the cellular uptake of curcumin. It is possible that a significant proportion of curcumin in either 66γCD$_2$su or 66γCD$_2$ur are bound to serum proteins as the concentration of BSA was substantially higher than that of either 66γCD$_2$su or 66γCD$_2$ur in the medium. Previous studies showed that curcumin (in the absence of 66γCD$_2$su and 66γCD$_2$ur) is captured and stabilized by serum proteins and model membranes.[16,17,22,29,30,75] The stabilization of curcumin by these systems may result in a similar level of antiproliferative effect and fluorescence intensity of curcumin in the absence of 66γCD$_2$su and 66γCD$_2$ur. To further understand curcumin delivery to PC-3 cells, we performed quantitative curcumin uptake studies.

Our results in Figure 5 show that a significant fraction of curcumin binds to BSA in the absence of either 66γCD$_2$su or 66γCD$_2$ur. Previous studies determined that the binding constants of curcumin to serum proteins are approximately 10^5 M^{-1}.[17,29,30] Therefore, the binding of curcumin to the hydrophobic pockets of BSA may be sufficiently strong to prevent curcumin from making contact with cell membranes and thereby inhibit transfer of curcumin by diffusion. In contrast, the intracellular delivery of 66γCD$_2$su- or 66γCD$_2$ur-complexed curcumin appears to be BSA-independent as there is only an insignificant decrease in curcumin uptake between the results with the absence and presence of BSA. This phenomenon is attributable to high binding constants of curcumin to either 66γCD$_2$su or 66γCD$_2$ur, of which are on the order of 10^6 M^{-1}.[16] Here, we propose that curcumin is directly delivered into PC-3 cells by either 66γCD$_2$su or 66γCD$_2$ur as follows. Curcumin may exist in the annuli of either 66γCD$_2$su or 66γCD$_2$ur or be transferred to the hydrophobic pocket of BSA, and/or bind to cell membranes, by diffusion. Our uptake results with and without BSA indicate that a high portion of curcumin remains in the annuli of 66γCD$_2$su and 66γCD$_2$ur rather than being transferred to BSA, in spite of a roughly five times higher BSA concentration. In addition, previous studies suggest a lack of interaction between either β-CD or diamide linked γ-CD dimers and the cell membrane based on SDS model membrane experiments.[16,75] Hence, the results here strongly suggest that 66γCD$_2$su and 66γCD$_2$ur deliver curcumin directly to the cell membrane, independent of the presence of BSA. Moreover, the fluorescence signal of curcumin alone in the presence of BSA is significantly weaker than that in its presence (Supporting Information, Figure S6), indicating that BSA reduces curcumin availability. However, the presence of BSA has a negligible effect on the fluorescence signals of curcumin in either 66γCD$_2$su or 66γCD$_2$ur (Supporting Information, Figure S6), supporting the high entrapment efficiency of the γ-CD dimers.

Our cellular uptake results in the serum-free environment further shows that curcumin and curcumin delivered by 66γCD$_2$su and 66γCD$_2$ur possess similar rate constants for uptake by PC-3 cells, indicating that similar cell membrane diffusion processes are involved for free curcumin and curcumin delivered using either 66γCD$_2$su or 66γCD$_2$ur. In the absence of the diamide linked γ-CD dimers, curcumin is able to partition into the cell membrane by diffusion,[16] which results in the maximum amount of curcumin in the cell. In contrast, in the presence of either 66γCD$_2$su or 66γCD$_2$ur, curcumin partitions largely into the diamide linked γ-CD dimers,[16] leading to an overall lower level of fluorescence because curcumin in the latter environment is essentially nonfluorescent.[16] However, observable fluorescence signal in addition to similar uptake rate constants illustrates that a moderate level of curcumin is released from 66γCD$_2$su or 66γCD$_2$ur by diffusion.

The advantage of the BSA-independent delivery of curcumin by 66γCD$_2$su and 66γCD$_2$ur is the control of the effective dose of curcumin in cancer treatment. The direct delivery of curcumin to cells raises the possibility of protecting the highly labile curcumin against serum proteins and potentially lipoproteins in the circulatory system for an extended period of time by encapsulation. Furthermore, curcumin forms a more stable complex with 66γCD$_2$su and 66γCD$_2$ur than with BSA,[17] and our uptake results suggest a possible gradual increase in intracellular curcumin due to sustained delivery from either 66γCD$_2$su or 66γCD$_2$ur even in the presence of BSA. This phenomenon is consistent with the delayed and reduced effects observed in our cell viability and fluorescence images. Therefore, our delivery agents, 66γCD$_2$su and 66γCD$_2$ur, potentially offer a prolonged delivery of curcumin in cancer therapy, and may therefore protect against rapid hepatic or renal clearance. Finally, while this study is concerned with the delivery of curcumin by 66γCD$_2$su and 66γCD$_2$ur *in vitro*, these agents may also be efficacious to deliver other therapeutic agents in other experimental systems.

A combination of either 66γCD$_2$su or 66γCD$_2$ur with curcumin will be investigated in our future *in vivo* studies to investigate their toxicity and pharmacological profiles, with an ultimate goal of developing curcumin-66γCD$_2$su and curcumin-66γCD$_2$ur as naturally derived chemotherapeutic drugs or their supplements.

CONCLUSIONS

We report here for the first time the direct intracellular delivery of curcumin using diamide linked γ-CD dimers, 66γCD$_2$su and 66γCD$_2$ur. These delivery agents offer molecular-scale encapsulation of curcumin at concentrations ranging from micromolar to millimolar and a high structural integrity under physiological conditions. While encapsulation of curcumin using either 66γCD$_2$su or 66γCD$_2$ur strongly suppresses its

degradation, cellular viability and uptake assays combined with gene expression and fluorescence microscopy reveal that 66γCD_2su and 66γCD_2ur alone do not produce measurable toxicity on viability or gene expression. The 66γCD_2su and 66γCD_2ur are both effective means to deliver curcumin into cells, resulting in inhibition of cellular proliferation, while initiating changes in gene expression similar to that exhibited by curcumin alone. Furthermore, the formation of 66γCD_2su and 66γCD_2ur with curcumin appears to protect curcumin from binding to BSA, which may result in a more efficient intracellular delivery via cell membranes. Together, our results demonstrate the promise of these novel nontoxic molecular-scale agents to deliver curcumin and other highly labile compounds to mammalian cells effectively and therefore may present a more effective means of delivering these agents *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

Release of curcumin from 66γCD_2su and 66γCD_2ur, relative cell death determined by trypan blue exclusion assay, confocal fluorescence images of cells treated with nascent and encapsulated curcumin from Day 1 to Day 5, and curcumin uptake results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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