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Antitumor agents 290. † Design, synthesis, and biological evaluation of new LNCaP and PC-3 cytotoxic curcumin analogs conjugated with anti-androgens

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Abstract

In our continuing study of curcumin analogs as potential anti-prostate cancer drug candidates, 15 new curcumin analogs were designed, synthesized and evaluated for cytotoxicity against two human prostate cancer cell lines, androgen-dependent LNCaP and androgen-independent PC-3. Twelve analogs (**5-12**, **15**, **16**, **19**, and **20**) are conjugates of curcumin (**1**) or methyl curcumin (**2**) with a flutamide- or bicalutamide-like moiety. Two compounds (**22** and **23**) are C4-mono- and difluoro-substituted analogs of dimethyl curcumin (DMC, **21**). Among the newly synthesized conjugates compound **15**, a conjugate of **2** with a partial bicalutamide moiety, was more potent than bicalutamide alone and essentially equipotent with **1** and **2** against both prostate tumor cell lines with IC₅₀ values of 41.8 μM (for LNCaP) and 39.1 μM (for PC-3). A cell morphology study revealed that the cytotoxicity of curcumin analogs or curcumin-antiandrogen conjugates detected from both prostate cancer cell lines might be due to the suppression of pseudopodia formation. A molecular intrinsic fluorescence experiment showed that **1** accumulated mainly in the nuclei, while conjugate **6** was distributed in the cytosol. At the tested conditions, antiandrogens suppressed pseudopodia formation in PC-3 cells, but not in LNCaP cells. The evidence suggests that distinguishable target proteins are involved, resulting in the different outcomes toward pseudopodia suppression.

Keywords

Synthesis; Curcumin analogs; Conjugates; Cytotoxicity; Anti-prostate cancer; Morphology

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1. Introduction

Statistically, prostate cancer is still ranked as the most common cancer in American males (28%) and was the second leading cause of cancer-related death among men in the United States (11%) in 2010.² Constitutive activation of the androgen receptor (AR) by high levels of androgens is presumed to be responsible for the progression of prostate cancer. Most presently available chemotherapeutic agents are antiandrogens, including steroidal antiandrogens and nonsteroidal antiandrogens. The steroidal antiandrogens, such as cyproterone acetate, possess partial agonistic activity to the AR and inter-activity with other hormonal systems, which can induce many complications.³⁻⁵ The nonsteroidal antiandrogens, such as flutamide and bicalutamide, have fewer side effects and were thought to be pure AR antagonists. However, antiandrogen withdrawal syndrome has been discovered in patients treated with nonsteroidal antiandrogens for several months.⁶⁻⁷ One proposed mechanism for this syndrome is a mutation of the AR caused by the long-term use of these nonsteroidal antiandrogens, so that the nonsteroidal antiandrogens exhibit agonistic activity to the mutant AR.⁸ Flutamide and bicalutamide (a racemic mixture) (Figure 1) are two well-known nonsteroidal antiandrogens, widely used for the clinical treatment of prostate cancer.⁹⁻¹⁰ However, induction of antiandrogen-withdrawal syndrome in patients has been a considerable problem. Combination usage with a gonadotropin-releasing hormone agonist, such as goserelin acetate or leuprolide acetate, is currently recommended and used in the clinic to minimize antiandrogen withdrawal syndrome.¹¹ To date, none of the effective clinically available antiandrogens is able to kill prostate cancer cells.

Curcumin (**1**) (Figure 1), a major yellow pigment of *Curcuma longa*, has been reported to have anti-prostate cancer activity *in vitro* and *in vivo*.¹²⁻¹⁴ Although the mechanism of action is still unknown, it has been associated with multiple proteins/signaling pathways, such as NF- κ B,¹⁵ STATs,¹⁶ AP-1,¹⁷ MAPK,¹⁸ and Akt,¹⁹ etc. More recently, Ras has been reported to be a potential target protein related to the anticancer activity.²⁰ However, the metabolic instability of **1** leads to its poor bioavailability, which, in part, prevents **1** from being used as an efficient therapeutic drug.²¹⁻²³ Other reports have identified curcumin analogs that were cytotoxic against androgen-dependent LNCaP cells and androgen-independent PC-3 cells.²⁴⁻²⁷ Some analogs demonstrated potent anti-AR activity in LNCaP cells and PC-3 cells transfected with wild type AR, and were even more potent than hydroxyflutamide, the active metabolite of flutamide,²⁸⁻²⁹ although again, the exact mechanism of action remains unclear.

It is also known that the conjugation of antitumor drugs with other components such as antioxidants or other antitumor agents has provided some advantages in improving the antitumor efficacy and selectivity, while decreasing the systematic toxicity.³⁰⁻³² In our search for new anti-prostate cancer drugs with increased potency and minimized adverse antiandrogen effects, we considered conjugation of curcumin or its analogs with clinically used antiandrogens, such as flutamide and bicalutamide, to be a possible approach to develop new anti-prostate cancer leads, in addition to the conventional structural modification of the curcumin molecule alone. Although **1** has been extensively studied recently in combinations with different cancer therapeutic agents to treat cancers through synergistic activity,³³⁻³⁴ the conjugation of **1** (or its analog) with a cancer therapeutic agent has not been explored. Unlike the principle of drug combination, the biological activity of a conjugate basically results from a single molecule in which two or more active components are tethered through covalent chemical bonds. The mechanism of action of the conjugate, therefore, could be different from that of the combination forms. Ideally, the advancement of the conjugation approach lies in “one molecule double or multiple functions”.³⁵ This field merits exploration, and in this manner, we hope to develop new drug candidates potentially inhibiting proliferation of prostate cancer cells and mitigating the side effects of existing

antiandrogens. Structurally, flutamide and bicalutamide share a *N*-(4-substituted-3-(trifluoromethyl)phenyl)acetamido moiety, which may be an essential pharmacophore for their anti-prostate cancer activity. New conjugates in which curcumin (**1**) or methyl curcumin (**2**) were coupled with a flutamide molecule (or *N*-arylmethacrylamide moiety of bicalutamide) through various linkages were designed and synthesized (Figure 2). The *in vitro* anti-prostate cancer activity of the newly synthesized entities was examined and will be used to guide further optimization of new conjugates. It is also known that an enol-ketone linkage in **1** (and its analogs) is an important structural feature for its biological activities.²⁵ In order to further confirm this interesting structure-activity phenomenon and the effectiveness of both enol-ketone/diketone isomers on prostate cancer, we designed and synthesized compounds **22** and **23**, which contain fluorine as an isostere of hydrogen at the C4 position.³⁶ With the mono-fluoro substitution, we expect that **22** will be stabilized as only the enol-ketone isomer, while the di-fluoro substituted **23** should retain the diketone form without tautomerization to an enol-ketone isomer. We also determined the subcellular distribution of **1** and its conjugate **6** in PC-3 cells by capturing their intrinsic fluorescence, and observed clear dissimilarity. From these preliminary results, we hope to gain information not only on new SAR but also regarding promising new anti-prostate cancer leads and mechanism of action.

2. Results and Discussion

2.1. Chemistry

The starting material **2** was prepared according to a reported synthetic method.³⁷ As illustrated in Scheme 1, the conjugation of **1** or **2** with flutamide-related analogs, utilized 2-bromo-2-methyl propanoic acid, which was reacted with 4-substituted-3-trifluoromethylphenylamines (**3a-e**) in the presence of EDCI and DMAP to give corresponding amides (**4a-e**) in 30-65% yields. Reaction of these amides with **1** or **2** in the presence of anhydrous cesium carbonate (sodium iodide was used as the catalyst for making **5** and **10**) gave the desired compounds **5-12** (2-10% yields) (Scheme 1), along with the recovered starting materials (**1** or **2**) (recovery rates: 39%-92%). Various reaction conditions were tried (such as different bases: Cs₂CO₃ or K₂CO₃; solvents: CH₃CN, DMF, acetone-DMF, CH₂Cl₂ or THF; reaction temperature: rt-80 °C and reaction times: 4 h-overnight) to improve the yields of the target compounds, but were not successful. The low reaction yields are probably due to the low reactivity of **4a-4e**, resulting from the steric hindrance of adjacent dimethyl groups. The synthesis of compounds **15** and **16** (Scheme 2) began with the reaction of methacryloyl chloride with 4-nitro-3-trifluoromethylphenylamine (**3a**) or 4-cyano-3-trifluoromethylphenylamine (**3b**) to afford *N*-(4-nitro-3-trifluoromethylphenyl)-methacrylamide (**13a**) or *N*-(4-cyano-3-trifluoromethylphenyl)-methacrylamide (**13b**). The resulting *N*-arylmethacrylamides were subsequently oxidized to the corresponding racemic epoxides (**14a** and **14b**) by using a combination of hydrogen peroxide and trifluoroacetic anhydride.³⁸ Epoxides **14a** and **14b** were further reacted with **2** in the presence of sodium hydride through a ring-opening procedure to afford the target conjugates **15** in 64% yield and **16** in 41% yield (Scheme 2).

Compounds **19** and **20** were synthesized in three steps beginning from the reaction of vanillin in DMF with ethyl 4-bromobutyrate in the presence of K₂CO₃ (for **17a**) or with 11-bromoundecanoic acid in the presence of NaI and Cs₂CO₃ (for **17b**). The vanillin intermediates **17a** and **17b** were obtained in yields of 99% and 50%, respectively (Scheme 3). Reaction of these intermediates with 6-(3,4-dimethoxyphenyl)-4-hydroxyhexa-3,5-dien-2-one²⁵ to afford the corresponding product **18a** or **18b** was achieved by following a slightly modified literature procedure.³⁹ Hydrolysis in 10% aq. HCl followed by coupling with 4-amino-2-(trifluoromethyl)benzonitrile afforded the desired products **19** and **20**.

Compound **22**, a C4-mono-fluoro analog, was obtained by fluorination of dimethyl curcumin (DMC, **21**) with SelectFluor™ in DMF. The yield of **22** was relatively low (7%) due to the difficulty in controlling mono-substitution. Di-fluoro analog **23** was obtained in the presence of sodium hydride as a major product in 31% yield (Scheme 4).

2.2. Biological Activity and SAR

The newly synthesized analogs were evaluated for cytotoxicity against androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines. The *in vitro* cytotoxicity bioassay was performed according to procedures described in literature.⁴⁰ The test compounds were dissolved in DMSO as stock solutions and stored in the dark at -20 °C. The final concentration of DMSO was < 0.5% (v/v) in all experiments. The human prostate cancer cells were exposed to doses of the compounds for three days. IC₅₀ was defined as the concentration of test compound causing 50% inhibition of cell growth, as compared to the untreated control. IC₅₀ values were calculated by log – linear interpolation of data points. Of the three marketed compounds, **1** was twice as potent as the clinically used anti-androgens flutamide and bicalutamide against both prostate cancer cell lines *in vitro*. The IC₅₀ values for **1** were 36.4 ± 2.4 for LNCaP cell lines and 36.0 ± 1.4 μM for PC-3 cell lines compared with 81.8 ± 2.1 μM and 98.8 ± 3.2 μM, respectively, for flutamide and 74.0 ± 5.9 and 76.4 ± 7.2 μM, respectively, for bicalutamide (Table 1).

Among the 12 conjugates, compound **15**, a conjugate between **2** and *N*-[4-nitro-3-(trifluoromethyl)phenyl]-2-hydroxy-2-methylpropanamide (hydroxyflutamide) exhibited the most potent cytotoxicity against both cell lines with IC₅₀ values of 41.8 ± 3.9 μM against LNCaP and 39.1 ± 3.1 μM against PC-3 proliferation. It was essentially equipotent with its parent compound **2** (IC₅₀ 47.0 ± 3.2 μM against LNCaP and 33.1 ± 2.5 μM against PC-3) as well as **1** and almost twofold more potent than flutamide and bicalutamide (Table 1). Interestingly, **16**, which had only a slight structural variation compared with **15** [the nitro group (NO₂) on the aniline ring of the hydroxyflutamide-related substructure of **15** was replaced with a cyano group (CN) in **16**], was almost twofold less active than **15** against both cancer cell lines (IC₅₀ 73.0 ± 4.2 μM against LNCaP and 100.3 ± 1.9 μM against PC-3). This result suggests that substitutions in the flutamide portion of the conjugate molecule can greatly impact the biological activity.

The curcuminoids **1** and **2** were also linked with several 4-substituted-3-trifluoromethylphenylanilines through an -O-C(CH₃)₂-CO- amide bond generating compounds **5-9** and **10-12**, respectively. Surprisingly, except for **6** and **7**, the anti-prostate cancer activity was significantly reduced relative to the individual parent compounds (**1** or **2** and flutamide). Conjugates **6** and **7**, which contain cyano and fluoro groups, respectively, on the aniline ring in the flutamide substructure, showed significant cytotoxicity against both prostate cancer cell lines, while conjugates with nitro, chloro, and methyl groups did not. The results again suggested that the substituents on the flutamide substructure may be important for interaction of the conjugate molecule with the target protein(s). However, the curcuminoid portion of the molecule is also important, as conjugate **11**, which has the same cyano-flutamide substructure found in **6**, but is conjugated with **2** rather than **1**, showed at least twofold lower cytotoxicity against both LNCaP and PC-3 (IC₅₀ > 100 μM) compared with **6** or **2**, implying that the cytotoxic potency depended on the substituents on both parts of the conjugate. Even very slight structural deviations (e.g., NO₂ vs CN in the flutamide substructure and OCH₃ vs OH in the curcuminoid part) could affect the potency.

The identity of the linker between the curcuminoid phenoxy oxygen and flutamide amido carbonyl also played a role in the cytotoxic activity. As described above, conjugates **15** and **16** [linker = -CH₂C(CH₃)(OH)-] showed significant activity, while the corresponding **10** and

11 [linker = $-\text{C}(\text{CH}_3)_2-$] did not. In addition, conjugates **19** and **20** with unbranched propyl and decyl linkers, respectively, also did not show significant activity.

The antiproliferation activities induced by the conjugates increased in a dose-dependent manner. Figures 3 and 4 show dose-dependent curves of the conjugates **6** and **15** on viability of LNCaP and PC-3 cells compared with bicalutamide and flutamide in RPMI-1640 medium supplemented in 10% FBS, slightly modified from literature reported methods.⁴¹⁻⁴² Both conjugates demonstrate more potent antiproliferation effect than the tested antiandrogens.

Besides the 12 conjugates, two compounds, **22** and **23** were synthesized from dimethyl curcumin **21** to investigate the role of enol-ketone tautomerism in the anti-prostate cancer cytotoxicity. Compound **21** was previously synthesized and reported to show anti-prostate cancer activity.²⁵⁻²⁶ One of the important structural features contributing to the activity is the extended unsaturated system resulting from the enol-ketone bridge between the two phenyl groups. The enol-ketone exists as a stable and predominate tautomer in the equilibrium with its diketone form. Compound **22**, with mono-fluoro substitution at the C4 position, was designed to maximize the enol-ketone form of **21**, while compound **23**, with a di-fluoro replacement at C4, was designed to maintain the structure in the diketone form. Interestingly, compound **22** (IC_{50} $27.3 \pm 3.0 \mu\text{M}$ against LNCaP, $17.7 \pm 2.4 \mu\text{M}$ against PC-3) was almost as potent as the parent compound **21** and twofold more cytotoxic than its di-fluoro analog **23** (IC_{50} $53.8 \pm 4.9 \mu\text{M}$ against LNCaP, $44.3 \pm 4.8 \mu\text{M}$ against PC-3), supporting the postulate that the enol-ketone tautomerism may play a certain role in inhibition of prostate cancer cell growth *in vitro*. The physicochemical property of the fluoro substitution may also affect the potency.

Bicalutamide and flutamide are well known antiandrogens. They inhibit prostate cancer cell growth through binding to the AR on the prostate and preventing the binding of the natural ligand. Although it has long been known that **1** and its analogs exhibit anti-prostate cancer effects, the mechanism behind the biological activity remains unclear. To identify the target points of cytotoxicity from molecular cell biology level, morphologic changes generated by active compounds were examined. LNCaP and PC-3 prostate tumor cells were first treated with **1**, **2**, and two cytotoxic conjugates, **6** and **15**, as well as the antiandrogen bicalutamide. After incubation with agents for 48 h, the cells were stained with DNA-specific fluorescence dye DAPI, and the morphologic changes in the cells were analyzed. For LNCaP cells, the observed cell morphology indicated that **1**, **2**, and the conjugates strongly inhibited actin-based pseudopodia formation compared with the control. In contrast, the androgen antagonist bicalutamide did not suppress the pseudopodia formation in LNCaP cells, even at a concentration as high as $100 \mu\text{M}$ (Figure. 5). These observations suggested that **1** and its analogs, as well as conjugates might share similar mechanism(s) of action in LNCaP cytotoxicity by inhibiting F-actin formation, not only for the production of pseudopodia, but also the dynamism of cytoskeletal actin filaments. Interestingly, for PC-3 cells, all tested compounds, including antiandrogen bicalutamide significantly suppressed pseudopodia-like extension (Figure 6). In addition, **1** and **2** induced nuclear enlargement, suggesting that nuclear division and cell cleavage were impaired in PC-3 cells, which might induce cell cycle arrest at the G2-phase. In contrast to treatment with **1**, multinucleated cells were observed when cells were treated with conjugate **6** or **15** (Figure. 6), indicating that while **1** itself inhibited cell cycle progression,⁴³ the conjugates induced irregular nuclear division resulting in production of polykaryotic cells that were subject to undergo apoptosis. These findings led to the hypothesis that **1** and its analogs may target the actin cytoskeletal dynamics, but it is still unclear whether the irregular nuclear division induced by the conjugates in PC-3 cells does or does not result from the suppression of F-actin formation. Such morphologic changes were not observed in the cells treated with the antiandrogen, and it is also notable that neither flutamide nor bicalutamide showed significant cytotoxicity

against PC-3 cells within 48 h even at concentration of 100 μ M (data not shown), while suppression of pseudopodia-like formation was observed.

The findings resulting from the current morphology study in LNCaP and PC-3 prostate cancer cells clearly indicated that different cellular molecules are targets of **1**, its analogs, and conjugates, as well as antiandrogens, though it is still unclear whether the cellular defects distinguishable between PC-3 and LNCaP are caused by expression of the AR. Because all test compounds in this study displayed little or no cytotoxic selectivity towards LNCaP or PC-3 tumor cells, we have speculated that the displayed cytotoxicity might not be associated completely or at all with androgen or the AR. Nevertheless, because pseudopodia formation is highly related to cell migration and tumor metastasis,⁴⁴ **1** and its analogs, including conjugates, may effectively inhibit prostate tumor metastasis. The observed morphological change induced by the antiandrogens in PC-3 cells may also lead to their marginal cytotoxicity in a certain degree. Cell invasion assays in the presence of **1**-analogs are currently underway.

The structural uniqueness of **1** and its structural analogs results in detectable molecular intrinsic fluorescence, which enables us to detect the distribution of the drugs in the cells by capturing the intrinsic fluorescence under a fluorescence microscope. Experimentally, PC-3 cells were treated with **1** and conjugate **6**, respectively, for 1 h (this experiment was not performed in LNCaP cells due to extremely slow growth), and as expected, the intrinsic green fluorescence generated by treated compounds was observed in PC-3 cells (Figure. 7). Interestingly, the intrinsic fluorescence captured from **1**-treated cells was accumulated in the cell nuclei, while for **6** treated cells, intrinsic fluorescence was detected only in the cytosol. These findings demonstrated a different subcellular distribution between **1** and the conjugate **6**. Curcumin distributes and accumulates mainly in the nuclear region, while the conjugate accumulates mainly in the cytosol.

3. Conclusions

In this study, we designed and synthesized new **1**-analogs, including conjugates of **1** (or its analogs) and antiandrogens (such as flutamide or a substructure of bicalutamide or modified molecules). The preliminary SAR profile showed that conjugate **15** was equipotent to **1** and **2**, and much more potent than flutamide or bicalutamide. Two additional conjugates, **6** and **7**, also showed more cytotoxic potency than the clinically used antiandrogens flutamide and bicalutamide against both androgen-dependent LNCaP and androgen-independent PC-3 cell lines. These results implied that appropriate accommodation of a large curcuminoid group on the carbon adjacent to the amide carbonyl of flutamide or bicalutamide may enhance the inhibition of prostate cancer cells proliferation through different mechanism of action other than targeting to the AR. Compound **22**, a C4 mono-fluorinated curcuminoid, showed comparable potency to its parent compound **21** and greater potency than its di-fluorinated analog **23**. Morphological and intrinsic fluorescence analysis indicated that **1**, its analogs, and conjugates may target the actin cytoskeletal dynamics in both LNCaP and PC-3 cells. In PC-3 cells, **1** distributed and accumulated mainly in the nuclei of the cells and inhibited cell cycle progression by targeting the functional proteins in the nuclear region, while the conjugates **6** and **15** accumulated mainly in the cytosol and induced irregular nuclear division, which could lead to prokaryotic cells and eventually apoptosis.

4. Experiments

4.1. Chemistry

Melting points were determined on a Fisher-John melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (¹H NMR) and ¹³C NMR spectra were

measured on Varian Gemini 300 or Inova 400 spectrometers with tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in δ (ppm). Mass spectra (MS) were obtained on a Shimadzu LCMS-2010. CombiFlash[®] chromatographic system (Isco Companion) with a Grace silica gel cartridge was used for general separation and purification. Preparative thin layer chromatography (PTLC) on silica gel plates (Kieselgel 60, F254, 1.50 mm) was also used for separation and purification. Precoated silica gel plates (Kieselgel 60, F254, 0.25 mm) were used for thin layer chromatography (TLC) analysis. Shimadzu LC-20AT prominence liquid chromatography was used for HPLC. Alltima 2.1 mm $\times$ 100 mm C18 3 μ m was used as HPLC column. All reagents and solvents were purchased from Aldrich, Fisher, VWR, and other vendors. Some chemicals were used after purification, and others were used as purchased. Compounds **1**, **2**, and **21** were synthesized by following literature methods.²⁵⁻²⁶ All final compounds are >95% pure through HPLC analysis.

4.2. Synthesis of 2-bromo-2-methyl-*N*-(4-substituted-3-(trifluoromethyl)-phenyl)propanamides (**4a-e**)

4.2.1. General procedure—2-Bromo-2-methylpropionic acid (1.5 equiv), EDCI (1.5 or 2 equiv) and DMAP (1.5 or 2 equiv) were added to a solution of 4-substituted-3-(trifluoromethyl)aniline in CH₂Cl₂ (for **3a,b,c,e**) or pyridine (for **3d**), and the mixture was stirred at 35 °C overnight (60 °C, 4 h for pyridine as solvent) under N₂. The mixture was diluted with CH₂Cl₂, washed with sat. NH₄Cl (aq.), sat. NaHCO₃ (aq.), and brine, then dried over anhydrous MgSO₄. After removal of the solvent in vacuo, the crude products were purified by silica gel column chromatography eluting with *n*-hexane-EtOAc.

4.2.2. 2-Bromo-*N*-(4-nitro-3-(trifluoromethyl)phenyl)-2-methylpropanamide (4a**)**—Yield: 12%, white solid powder; ¹H NMR (300 MHz, CDCl₃): δ 2.06 (s, 3H, CH₃), 2.07 (s, 3H, CH₃), 7.98 (s, 1H, aromatic-H), 7.99 (s, 1H, aromatic-H), 8.06 (s, 1H, aromatic-H), ESI MS m/z 355.98 (M+H)⁺.

4.2.3. 2-Bromo-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-methylpropanamide (4b**)**—Yield: 64%; white solid; mp. 168-170 °C; ¹H NMR (400 MHz, CDCl₃): δ 1.90 (3H, s, CH₃), 2.06 (3H, s, CH₃), 7.82 (1H, d, J = 8.6 Hz, Ar-H), 7.95 (1H, dt, J = 8.6, 2.1 Hz, Ar-H), 8.11 (1H, dd, J = 6.6, 1.9 Hz, Ar-H), 8.80 (br, 1H, NH). ESI-MS (negative, m/z): 333.05 [M-H]⁻, 335.00 [M+2-H]⁻.

4.2.4. 2-Bromo-*N*-(4-fluoro-3-(trifluoromethyl)phenyl)-2-methylpropanamide (4c**)**—Yield: 64%; white solid; mp. 111-112 °C; ¹H NMR (400 MHz, CDCl₃): δ 1.89 (3H, s, CH₃), 2.06 (3H, s, CH₃), 7.20 (1H, t, J = 9.3 Hz, Ar-H), 7.73-7.75 (1H, m, Ar-H), 7.84 (1H, td, J = 9.0, 2.7 Hz, Ar-H), 8.52 (br, 1H, NH). ESI-MS (negative, m/z): 325.95 [M-H]⁻, 328.00 [M+2-H]⁻.

4.2.5. 2-Bromo-*N*-(4-chloro-3-(trifluoromethyl)phenyl)-2-methylpropanamide (4d**)**—Yield: 29%; white solid; mp. 134-135 °C; ¹H NMR (400 MHz, CDCl₃): δ 2.05 (6H, s, CH₃), 7.48 (1H, d, J = 8.8 Hz, Ar-H), 7.73 (1H, dd, J = 8.8, 2.5 Hz, Ar-H), 7.91 (1H, d, J = 2.5 Hz, Ar-H), 8.54 (br, 1H, NH). ESI-MS (negative, m/z): 342.05 [M-H]⁻, 344.05 [M+2-H]⁻, 345.95 [M+4-H]⁻.

4.2.6. 2-Bromo-*N*-(4-methyl-3-(trifluoromethyl)phenyl)-2-methylpropanamide (4e**)**—Yield: 49%; white solid; mp. 125-126 °C; ¹H NMR (400 MHz, CDCl₃): δ 2.05 (6H, s, CH₃), 2.46 (3H, s, Ar-CH₃), 7.27 (1H, d, J = 8.2 Hz, Ar-H), 7.64 (1H, dd, J = 8.2, 2.1 Hz, Ar-H), 7.78 (1H, d, J = 2.1 Hz, Ar-H), 8.48 (br, 1H, NH). ESI-MS (negative, m/z): 322.15 [M-H]⁻, 324.00 [M+2-H]⁻.

4.3. Synthesis of *N*-(4-substituted-3-(trifluoromethyl)phenyl)pivalamide-curcumin or methylcurcumin analogs (5-12)

4.3.1 General procedure—Compounds **4a-4e** (1.1, 1.5 or 3.0 equiv) and Cs₂CO₃ (1.5 equiv) were added to a solution of **1** or **2** in CH₃CN, and the mixture was stirred at 60 °C for 3 h, 5 h or overnight under N₂. The mixture was quenched with 0.1N HCl to pH 6 and extracted three times with EtOAc. The combined organic layers were washed with H₂O and brine. After being dried over anhydrous Na₂SO₄, the solvent was removed in vacuo. The crude products were purified by CombiFlash[®] column chromatography or preparative-TLC eluting with *n*-hexane-EtOAc.

4.3.2. *N*-(4-Nitro-3-(trifluoromethyl)phenyl)pivalamide-curcumin (5)—Yield: 10%; amorphous solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.49 (s, 1H), 8.36-8.32 (m 1H), 8.24-8.21 (m, 1H), 7.58-7.53 (m, 2H), 7.42 (br s, 1H), 7.32 (br s, 1H), 7.26-7.14 (m, 2H), 6.96-6.89 (m, 2H), 6.84-6.56 (m, 2H), 6.09 (s, 1H), 3.84 (s, 3H, OCH₃), 3.81 (s, 3H, OCH₃), 1.53 (s, 6H, CH₃); ESI MS *m/z* 643.13 (M+H)⁺.

4.3.3. *N*-(4-Cyano-3-(trifluoromethyl)phenyl)pivalamide-curcumin (6)—Yield: 5% (starting with 0.26 mmol of curcumin), amorphous solid; ¹H NMR (400 MHz, CDCl₃): δ 1.58 (6H, s, CH₃), 3.95 and 3.96 (each 3H, s, OCH₃), 5.83 (1H, s, C=CH), 6.50 and 6.56 (each 1H, d, *J* = 15.8 Hz, CH=CH), 6.94 (1H, d, *J* = 8.2 Hz, Ar-H), 7.06 (1H, d, *J* = 1.7 Hz, Ar-H), 7.08 (1H, d, *J* = 8.0 Hz, Ar-H), 7.136 (1H, dd, *J* = 8.2, 1.7 Hz, Ar-H), 7.141 (1H, d, *J* = 1.9 Hz, Ar-H), 7.17 (1H, dd, *J* = 8.4, 1.9 Hz, Ar-H), 7.61 and 7.62 (each 1H, d, *J* = 15.8 Hz, CH=CH), 7.83 (1H, d, *J* = 8.4 Hz, Ar-H'), 8.03 (1H, dd, *J* = 8.4, 2.1 Hz, Ar-H'), 8.09 (1H, d, *J* = 1.9 Hz, Ar-H'), 10.02 (1H, s). ESI-MS (positive or negative, *m/z*): 623.50 [M+H]⁺, 621.30 [M-H]⁻.

4.3.4. *N*-(4-Fluoro-3-(trifluoromethyl)phenyl)pivalamide-curcumin (7)—Yield: 6% (starting with 0.27 mmol of curcumin), amorphous solid; ¹H NMR 400 MHz, CDCl₃): δ 1.58 (6H, s, CH₃), 3.95 (6H, s, OCH₃), 5.83 (1H, s, C=CH), 6.49 and 6.55 (each 1H, d, *J* = 15.8 Hz, CH=CH), 6.94 (1H, d, *J* = 8.2 Hz, Ar-H), 7.05 (1H, s, Ar-H), 7.07 (1H, d, *J* = 8.6 Hz, Ar-H), 7.13 (1H, s, Ar-H), 7.16 (2H, d, *J* = 8.2 Hz, Ar-H), 7.61 and 7.62 (each 1H, d, *J* = 15.8 Hz, CH=CH), 7.75 (1H, d, *J* = 8.6 Hz, Ar-H'), 7.87 (1H, d, *J* = 8.6 Hz, Ar-H'), 7.90 (1H, s, Ar-H'), 9.68 (1H, s). ESI-MS (positive or negative, *m/z*): 616.25 [M+H]⁺, 614.40 [M-H]⁻.

4.3.5. *N*-(4-Chloro-3-(trifluoromethyl)phenyl)pivalamide-curcumin (8)—Yield: 6% (starting with 0.33 mmol of curcumin), yellow solid; mp. 268-270 °C, ¹H NMR (400 MHz, CDCl₃): δ 1.58 (6H, s, CH₃), 3.95 and 3.96 (each 3H, s, OCH₃), 5.83 (1H, s, C=CH), 6.50 and 6.55 (each 1H, d, *J* = 15.8 Hz, CH=CH), 6.94 (1H, d, *J* = 8.2 Hz, Ar-H), 7.06 (1H, d, *J* = 1.9 Hz, Ar-H), 7.07 (1H, d, *J* = 8.2 Hz, Ar-H), 7.13 (1H, d, *J* = 1.9 Hz, Ar-H), 7.14 (1H, dd, *J* = 8.6, 1.9 Hz, Ar-H), 7.16 (1H, dd, *J* = 8.4 1.9 Hz, Ar-H), 7.49 (1H, d, *J* = 8.7 Hz, Ar-H'), 7.61 and 7.62 (1H, d, *J* = 15.8 Hz, CH=CH), 7.88 (1H, dd, *J* = 8.6, 2.5 Hz, Ar-H'), 7.96 (1H, d, *J* = 2.5 Hz, Ar-H'), 9.74 (1H, s). ESI-MS (positive or negative, *m/z*): 632.10 [M+H]⁺, 634.25 [M+2+H]⁺, 630.35 [M-H]⁻, 632.35 [M+2-H]⁻.

4.3.6. *N*-(4-Methyl-3-(trifluoromethyl)phenyl)pivalamide-curcumin (9)—Yield: 2% (starting with 0.27 mmol of **1**), amorphous solid; ¹H NMR (400 MHz, CDCl₃): δ 1.58 (6H, s, CH₃), 2.46 (3H, s, Ar-CH₃), 3.80 and 3.95 (each 3H, s, OCH₃), 5.85 (1H, s, C=CH), 6.56 and 6.57 (each 1H, d, *J* = 15.8 Hz, CH=CH), 6.77 (1H, dd, *J* = 8.4, 2.5 Hz, Ar-H), 6.88 (1H, d, *J* = 8.0 Hz, Ar-H), 6.97 (1H, d, *J* = 2.3 Hz, Ar-H), 7.07 (1H, d, *J* = 8.2 Hz, Ar-H), 7.08 (1H, s, Ar-H), 7.15 (1H, d, *J* = 8.0 Hz, Ar-H), 7.28 (1H, d, *J* = 8.7 Hz, Ar-H'), 7.61 and

7.63 (each 1H, d, $J = 15.8$ Hz, CH=CH), 7.80 (1H, d, $J = 8.4$ Hz, Ar-H'), 7.83 (1H, s, Ar-H'), 9.61 (1H, s). ESI-MS (positive or negative, m/z): 612.25 [M+H]⁺, 610.15 [M-H]⁻.

4.3.7. *N*-(4-Nitro-3-(trifluoromethyl)phenyl)pivalamide-methylcurcumin (10)—

Yield: 3%; amorphous solid; ¹H NMR (300 MHz, acetone-*d*₆): δ 8.55 (s, 1H), 8.32 (s, br, 1H), 8.18-8.20 (m, 1H), 7.66-7.61 (m, 2H), 7.46 (br s, 1H), 7.39-7.14 (m, 4H), 7.00-7.03 (m, 1H), 6.86-6.73 (m, 2H), 6.03 (s, 1H), 3.98 (s, 3H, OCH₃), 3.88 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 1.57 (s, 6H, CH₃); ESI MS m/z 657.3 (M+H)⁺.

4.3.8 *N*-(4-Cyano-3-(trifluoromethyl)phenyl)pivalamide-methylcurcumin (11)—

Yield: 5% (starting with 0.13 mmol of methylcurcumin), amorphous solid; ¹H NMR (400 MHz, CDCl₃): δ 1.79 (6H, s, CH₃), 3.80, 3.93 and 3.94 (each 3H, s, OCH₃), 4.96 (1H, s), 5.84 (1H, s, C=CH), 6.51 and 6.55 (each 1H, d, $J = 16.0$ Hz, CH=CH), 6.79 (1H, dd, $J = 8.4$, 2.3 Hz, Ar-H'), 6.89 (1H, d, $J = 8.4$ Hz, Ar-H), 6.90 (1H, d, $J = 8.0$ Hz, Ar-H), 6.96 (1H, d, $J = 2.3$ Hz, Ar-H'), 7.08 (1H, d, $J = 1.7$ Hz, Ar-H), 7.10 (1H, s, Ar-H), 7.14 (1H, dd, $J = 8.0$, 1.7 Hz, Ar-H), 7.15 (1H, dd, $J = 8.2$, 1.7 Hz, Ar-H), 7.59 and 7.63 (each 1H, d, $J = 15.8$ Hz, CH=CH), 7.61 (1H, d, $J = 8.6$ Hz, Ar-H'). ESI-MS (positive, m/z): 637.30 [M+H]⁺.

4.3.9. *N*-(4-Chloro-3-(trifluoromethyl)phenyl)pivalamide-methylcurcumin (12)—

Yield: 3% (starting with 0.26 mmol of methylcurcumin), amorphous solid; ¹H NMR (400 MHz, CDCl₃): δ 1.72 (6H, s, CH₃), 3.78, 3.91 and 3.92 (each 3H, s, OCH₃), 4.36 (1H, s), 5.82 (1H, s, C=CH), 6.49 and 6.53 (each 1H, d, $J = 15.6$ Hz, CH=CH), 6.75 (1H, dd, $J = 8.7$, 2.7 Hz, Ar-H'), 6.87 (2H, d, $J = 8.2$ Hz, Ar-H), 6.98 (1H, d, $J = 2.7$ Hz, Ar-H'), 7.067 (1H, s, Ar-H), 7.074 (1H, s, Ar-H'), 7.11 (1H, dd, $J = 8.0$, 1.7 Hz, Ar-H), 7.13 (1H, dd, $J = 8.0$, 1.7 Hz, Ar-H'), 7.27 (1H, d, $J = 8.7$ Hz, Ar-H'), 7.57 and 7.61 (each 1H, d, $J = 15.6$ Hz, CH=CH). ESI-MS (positive, m/z): 646.15 [M+H]⁺.

4.4. Synthesis of *N*-(4-substituted-3-(trifluoromethyl)phenyl) methacrylamide (14a,b): *N*-(4-substituted-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide-methylcurcumin (15-16)

4.4.1. General procedure—Methacryloyl chloride (1.2-3.0 eq.) was added dropwise to a solution of 4-nitro-3-trifluoromethylphenylamine (**3a**) or 4-cyano-3-trifluoromethylphenylamine (**3b**) in CH₂Cl₂ containing triethylamine (1.2-3.0 eq.) at 0 °C. After stirring at room temperature for 4-12 h with TLC monitoring, the reaction mixture was diluted with CH₂Cl₂ and washed with H₂O. The CH₂Cl₂ extract was then dried over Na₂SO₄, concentrated, and purified by flash column chromatography to afford the desired compounds.

4.4.2. *N*-(4-Nitro-3-trifluoromethylphenyl)methacrylamide (13a)—Yield: 41%; amorphous solid; ¹H NMR (300 MHz, CDCl₃): δ 1.98 (s, 3H, CH₃), 5.70 (s, 1H, ethylene-H), 5.79 (s, 1H, ethylene-H), 8.38-8.17 (m, 3H, aromatic-H); ESI MS m/z 274.2 (M+H)⁺.

4.4.3. *N*-(4-Cyano-3-trifluoromethylphenyl)methacrylamide (13b)—Yield: 20%; amorphous solid; ¹H NMR (300 MHz, CDCl₃): δ 2.08 (s, 3H, CH₃), 5.60 (s, 1H, ethylene-H), 5.86 (s, 1H, ethylene-H), 8.05-7.78 (m, 3H, aromatic-H); ESI MS m/z 254.2 [M+H]⁺.

4.5. Synthesis of *N*-(4-substituted-3-(trifluoromethyl)phenyl)-2-methyloxirane-2-carboxamide (14a,b)

4.5.1. General procedure—To a solution of *N*-arylmethacrylamides (**13a** or **13b**, 1.7 mmol) in CH₂Cl₂ (25 mL) was added 0.31 mL of hydrogen peroxide (30%, w/w) followed by 1.25 mL of trifluoroacetic anhydride. After stirring at rt for 2 h, the reaction was quenched by addition of sodium bisulfide and extracted with CH₂Cl₂. The organic layer was

washed with sat. NaHCO_3 then brine, and dried over MgSO_4 . After removal of the solvent by evaporation, the crude was purified through a CombiFlash[®] chromatograph system to afford the desired products as racemic epoxides.

4.5.2. *N*-(4-Nitro-3-(trifluoromethyl)phenyl)-2-methyloxirane-2-carboxamide

(14a)—Yield: 81%; pale-yellow powder; ^1H NMR (300 MHz, CDCl_3) δ 1.70 (s, 3H, CH_3), 3.04 (s, 2H, epoxide-H), 7.23 (s, 1H, NH), 7.82 (d, 1H, J = 8.7 Hz, aromatic-H), 8.17 (d, 1H, J = 8.7 Hz, aromatic-H), 8.23 (s, 1H, aromatic-H); ESI MS m/z 291.7 $[\text{M}+\text{H}]^+$.

4.5.3. *N*-(4-Cyano-3-(trifluoromethyl)phenyl)-2-methyloxirane-2-carboxamide

(14b)—Yield: 70%; white powder; ^1H NMR (300 MHz, CDCl_3) δ 1.68 (s, 3H, CH_3), 3.01 (s, 2H, epoxide-H), 7.78 (d, 1H, J = 8.7 Hz, aromatic-H), 7.91 (dd, 1H, J = 2.4, 8.7 Hz, aromatic-H), 8.03 (d, J = 2.1 Hz, 1H, aromatic-H), 8.42 (s, 1H, NH); ESI MS m/z 292.7 $[\text{M}+\text{Na}]^+$.

4.6. Synthesis of *N*-(4-substituted-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide-methylcurcumin (15, 16)

4.6.1 General procedure—Methylcurcumin (**2**) (0.26 mmol in 3 mL of THF) was cooled to 0 °C. Sodium hydride (60%) (0.37 mmol) was added. After stirring at rt for 5 min, **14a** or **14b** (0.27 mmol) was added. The resulting mixture was heated to reflux for 24 h. The reaction mixture was diluted with CH_2Cl_2 and washed with water twice and extracted with CH_2Cl_2 . After purification through a Combiflash[®] system, the desired product was obtained.

4.6.2. *N*-(4-Nitro-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamido-methylcurcumin (15)

—Yield: 64%; amorphous solid; ^1H NMR (400 MHz, CDCl_3): δ 1.57 (s, 3H, CH_3), 3.92-3.91 (m, 9H, OCH_3), 3.96 (d, J = 9.6 Hz, 1H), 4.46 (d, J = 9.6 Hz, 1H), 5.81 (s, 1H), 6.49 (dd, J = 15.6, 1.2 Hz, 2H), 6.87 (d, J = 8.4 Hz, 1H), 7.06 (s, 2H), 7.13 (dd, J = 8.4, 1.6 Hz, 2H), 7.60 (d, J = 16.0 Hz, 1H), 7.62-7.54 (m, 1H), 7.95 (d, J = 8.4 Hz, 1H), 8.00-7.97 (m, 2H), 8.07 (dd, J = 8.8, 2.0 Hz, 1H), 9.30 (s, 1H), 8.52 (s, 1H); ^{13}C NMR (400 MHz, CDCl_3): δ 20.6 (CH_3); 56.2 ($\text{OCH}_3 \times 3$), 75.1 (CH_2), 84.9 [$-\text{C}(\text{OH})(\text{CH}_3)\text{CO}$], 101.6 ($\text{COCH}=\text{COH}$), 122.4 (CF_3), 110.0, 111.0, 111.4, 116.2, 118.2, 122.1, 122.4, 123.4, 127.3 (aromatic CH), 120.5 ($\text{CHOHCH}=\text{CH}$), 139.7 ($-\text{CH}=\text{CH}-\text{Ph}$), 122.9 ($\text{COCH}=\text{CH}-\text{Ph}$), 141.0 ($\text{COCH}=\text{CH}-\text{Ph}$), 128.1, 128.2, 130.6, 141.9, 143.4 (aromatic C), 149.0, 149.5, 150.0, 151.4 (aromatic $\text{C}-\text{OCH}_3$); 173.5 ($-\text{CONH}-$), 182.6 (enol COCHCOH), 184.2 (carbonyl $\text{COCH}=\text{COH}$); ESI MS m/z 656.2 $[\text{M}+\text{H}]^+$.

4.6.3. *N*-(4-Cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide-methylcurcumin (16)

—Yield: 41%; amorphous solid; ^1H NMR (300 MHz, acetone- d_6): δ 1.56 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.16 (d, J = 9.6 Hz, 1H), 4.42 (d, J = 9.6 Hz, 1H), 6.00 (s, 1H), 6.74 (d, J = 15.6 Hz, 2H), 7.02 (d, J = 15.9 Hz, 1H), 7.05 (d, J = 15.9 Hz, 1H), 7.23 (t, J = 13.8, 7.2 Hz, 2H), 7.31 (d, J = 4.8 Hz, 2H), 7.59 (d, J = 15.9 Hz, 1H), 7.62 (d, J = 15.9 Hz, 1H), 8.06 (d, J = 8.7 Hz, 1H), 8.31 (d, J = 8.7 Hz, 1H), 8.52 (s, 1H), 10.03 (s, 1H); ESI MS m/z 653.4 $[\text{M}+\text{H}]^+$.

4.7. Ethyl 4-(4-formyl-2-methoxyphenoxy)butanoate (17a)

To a solution of vanillin (1.0 g, 6.6 mmol) in DMF (20 mL), ethyl 4-bromobutyrate (2.8 mL, 19.6 mmol) and K_2CO_3 (360 mg, 9.8 mmol) were added and the mixture was stirred at 80 °C for 2 days under N_2 . The mixture was quenched with 0.1N HCl to pH 6 and extracted three times with EtOAc. The combined organic layer was washed with H_2O and brine, and then dried over anhydrous MgSO_4 , and the solvent was removed in vacuo. The crude was purified by CombiFlash[®] column chromatography eluting with *n*-hexane-EtOAc gradient to give the desired compound (1.7 g, 99%), white solid; mp. 75-76°C; ^1H NMR (400 MHz,

CDCl₃): δ 1.26 (3H, t, J = 7.2 Hz, OCH₂CH₃), 2.20 (2H, quintet, J = 6.8 Hz, OCH₂CH₂CH₂CO), 2.55 (2H, t, J = 7.2 Hz, OCH₂CH₂CH₂CO), 3.92 (3H, s, OCH₃), 4.15 (2H, q, J = 7.2 Hz, OCH₂CH₃), 4.17 (2H, t, J = 6.4 Hz, OCH₂CH₂CH₂CO), 6.99 (1H, d, J = 8.2 Hz, Ar-H), 7.41 (1H, d, J = 1.7 Hz, Ar-H), 7.44 (1H, dd, J = 8.2, 1.7 Hz, Ar-H), 9.85 (1H, s, CHO). ESI-MS (positive, m/z): 267.05 [M+H]⁺, 289.00 [M+Na]⁺.

4.7.1. 4-(4-Formyl-2-methoxyphenoxy)undecanoic acid (17b)—Compound **17b** was synthesized using a similar procedure to that of making **17a** with slight modification. To a solution of vanillin (502 mg, 3.3 mmol) in DMF-dioxane (3: 1, 20 mL), 11-bromoundecanoic acid (2.6 g, 9.9 mmol), NaI (2.5 g, 16.4 mmol) and Cs₂CO₃ (2.1 g, 6.6 mmol) were added and the mixture was stirred at 100 °C for 4 days under N₂. The workup and purification steps were as described above from **17a**, to give **17b** (552 mg, 50%) as an amorphous solid; ¹H NMR (400 MHz, CDCl₃): δ 1.55-1.59 (2H, m, CH₂), 1.58-1.63 (12H, m, CH₂), 1.88 (2H, quintet, J = 7.6 Hz, CH₂CH₂CH₂), 2.29 (2H, t, J = 7.6 Hz, CH₂CH₂CO), 3.93 (3H, s, OCH₃), 4.06 (2H, t, J = 6.6 Hz, OCH₂CH₂), 6.97 (1H, d, J = 8.0 Hz, Ar-H), 7.41 (1H, s, Ar-H), 7.44 (1H, d, J = 8.4 Hz, Ar-H), 9.85 (1H, s, CHO). ESI-MS (negative, m/z): 335.10 [M-H]⁻.

4.8. O-Butanoic acid substituted methylcurcumin (18a)

To a solution of 6-(3,4-dimethoxyphenyl)-4-hydroxyhexa-3,5-dien-2-one²⁵ (837 mg, 3.1 mmol) in EtOAc (8 mL) was added boric anhydride (171 mg, 2.5 mmol). The solution was stirred at 70 °C for 1 h. To this solution were added **17a** (1247 mg, 4.7 mmol) and tributyl borate (1.3 mL, 4.8 mmol). The mixture was stirred for 0.5 h and a solution of *n*-butylamine (0.46 mL, 4.7 mmol) in EtOAc (4 mL) was added dropwise over 15 min at 85 °C. The stirring was continued for 3 h at 100 °C. The mixture was then hydrolyzed by adding 0.1 N HCl and stirred at 50 °C for 0.5 h. The organic layer was separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed H₂O and brine and dried over anhydrous Na₂SO₄. After removal of solvent in vacuo, the crude products were purified by CombiFlash[®] column chromatography eluting with *n*-hexane-EtOAc gradient to give the ethyl ester precursor of **18a** (393 mg, 25%), amorphous solid, ¹H NMR (400 MHz, CDCl₃): δ 1.26 (3H, t, J = 7.2 Hz, OCH₂CH₃), 2.17 (2H, quintet, J = 6.8 Hz, OCH₂-CH₂-CH₂-CO), 2.54 (2H, t, J = 7.0 Hz, OCH₂-CH₂-CH₂-CO), 3.91, 3.92 and 3.93 (each 3H, s, OCH₃), 4.11 (2H, t, J = 6.2 Hz, OCH₂-CH₂-CH₂-CO), 4.15 (2H, q, J = 7.0 Hz, OCH₂CH₃), 5.82 (1H, s, C=CH), 6.49 and 6.50 (each 1H, d, J = 15.6 Hz, CH=CH), 6.878 (1H, d, J = 8.2 Hz, Ar-H), 6.883 (1H, d, J = 8.4 Hz, Ar-H), 7.07 (2H, s, Ar-H), 7.13 (2H, t, J = 8.4 Hz, Ar-H), 7.59 and 7.60 (each 1H, d, J = 15.6 Hz, CH=CH). ESI-MS (positive or negative, m/z): 497.20 [M+H]⁺, 495.10 [M-H]⁻.

To a solution of resulting compound (393 mg, 0.79 mmol) in THF (10 mL) was added 10% HCl (6 mL) and then the solution was stirred at 70 °C overnight. To the mixture was added H₂O (20 mL) and extracted with EtOAc. The organic layer was washed with H₂O and brine and dried over anhydrous Na₂SO₄. After removal of the solvent in vacuo, the crude was purified by CombiFlash[®] column chromatography eluting with *n*-hexane-EtOAc gradient to afford **18a** (263 mg, 71%), amorphous solid, ¹H NMR (400 MHz, CDCl₃): δ 2.19 (2H, quintet, J = 6.8 Hz, OCH₂-CH₂-CH₂-CO), 2.63 (2H, t, J = 7.0 Hz, OCH₂-CH₂-CH₂-CO), 3.91, 3.93 and 3.94 (each 3H, s, OCH₃), 4.13 (2H, t, J = 6.2 Hz, OCH₂-CH₂-CH₂-CO), 5.82 (1H, s, C=CH), 6.50 (2H, d, J = 15.8 Hz, CH=CH), 6.89 (2H, d, J = 8.4 Hz, Ar-H), 7.08 (2H, s, Ar-H), 7.14 (2H, t, J = 8.2 Hz, Ar-H), 7.60 and 7.61 (each 1H, d, J = 15.6 Hz, CH=CH). ESI-MS (positive or negative, m/z): 469.10 [M+H]⁺, 467.15 [M-H]⁻.

A similar procedure was used to prepare **18b**.

4.8.1. O-Undecanoic acid substituted methylcurcumin (18b)—Yield: 25%; amorphous solid, $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 1.45-146 (2H, m, CH_2), 1.61-163 (12H, m, CH_2), 1.86 (2H, quintet, $J = 7.6$ Hz, CH_2), 2.29 (2H, t, $J = 7.6$ Hz, CH_2), 3.92, 3.93 and 3.94 (each 3H, s, OCH_3), 4.05 (2H, t, $J = 6.6$ Hz, OCH_2), 5.82 (1H, s, $\text{C}=\text{CH}$), 6.49 (1H, d, $J = 15.8$ Hz, $\text{CH}=\text{CH}$), 6.50 (1H, d, $J = 15.6$ Hz, $\text{CH}=\text{CH}$), 6.87 and 6.89 (each 1H, d, $J = 8.4$ Hz, Ar-H), 7.08 (2H, s, Ar-H), 7.13 (1H, d, $J = 8.2$ Hz, Ar-H), 7.15 (1H, d, $J = 8.4$ Hz, Ar-H), 7.61 (2H, d, $J = 15.8$ Hz, $\text{CH}=\text{CH}$). ESI-MS (negative, m/z): 565.35 $[\text{M-H}]^-$.

4.9. *N*-(4-Cyano-3-(trifluoromethyl)phenyl)butyramide-methylcurcumin (19)

4-Amino-2-(trifluoromethyl)benzonitrile (97 mg, 0.52 mmol), EDCI (100 mg, 0.52 mmol) and DMAP (63 mg, 0.52 mmol) were added to a solution of **18a** (120 mg, 0.26 mmol) in CH_2Cl_2 (8 mL), and the mixture was stirred at 35°C overnight under N_2 . The mixture was diluted with CH_2Cl_2 and then washed with sat. NH_4Cl (aq.), sat. NaHCO_3 (aq.), and brine, then dried over anhydrous Na_2SO_4 . After removal of the solvent in vacuo, the product was purified by CombiFlash[®] column chromatography eluting with *n*-hexane-EtOAc gradient to give the desired **19** (60 mg, 37%), amorphous solid, $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 2.27 (2H, quintet, $J = 6.8$ Hz, $\text{OCH}_2\text{-CH}_2\text{-CH}_2\text{-CO}$), 2.68 (2H, t, $J = 7.0$ Hz, $\text{OCH}_2\text{-CH}_2\text{-CH}_2\text{-CO}$), 3.84, 3.92 and 3.93 (each 3H, s, OCH_3), 4.17 (2H, t, $J = 5.8$ Hz, $\text{OCH}_2\text{-CH}_2\text{-CH}_2\text{-CO}$), 5.82 (1H, s, $\text{C}=\text{CH}$), 6.49 and 6.50 (each 1H, d, $J = 15.8$ Hz, $\text{CH}=\text{CH}$), 6.88 and 6.90 (each 1H, d, $J = 8.4$ Hz, Ar-H), 7.075 and 7.080 (each 1H, d, $J = 2.1$ Hz, Ar-H), 7.12 and 7.14 (each 1H, dd, $J = 8.4, 1.7$ Hz, Ar-H), 7.58 and 7.61 (each 1H, d, $J = 15.8$ Hz, $\text{CH}=\text{CH}$), 7.75 (1H, d, $J = 8.4$ Hz, Ar-H'), 7.95 (1H, s, Ar-H'), 7.97 (1H, dd, $J = 8.4, 1.9$ Hz, Ar-H'), 8.62 (1H, s, NH). ESI-MS (positive, m/z): 637.40 $[\text{M+H}]^+$.

4.9.1. *N*-(4-Cyano-3-(trifluoromethyl)phenyl)undecanamide - methylcurcumin (20)—A similar procedure to that described above for **19** gave the desired product **20** (46 mg, 15%), amorphous solid, $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 1.43-1.45 (2H, m, CH_2), 1.61-1.68 (10H, m, CH_2), 1.72-1.76 (2H, m, CH_2), 1.83-1.86 (2H, m, CH_2), 2.29 (2H, t, $J = 7.2$ Hz, CH_2CO), 3.91, 3.93 and 3.94 (each 3H, s, OCH_3), 4.05 (2H, t, $J = 6.4$ Hz, OCH_2), 5.83 (1H, s, $\text{C}=\text{CH}$), 6.49 and 6.50 (each 1H, d, $J = 15.8$ Hz, $\text{CH}=\text{CH}$), 6.88 (1H, d, $J = 8.2$ Hz, Ar-H), 6.89 (1H, d, $J = 8.4$ Hz, Ar-H), 7.08 (2H, s, Ar-H), 7.12 (1H, d, $J = 8.9$ Hz, Ar-H), 7.14 (1H, d, $J = 8.4$ Hz, Ar-H), 7.61 (2H, d, $J = 15.8$ Hz, $\text{CH}=\text{CH}$), 7.76 (1H, d, $J = 8.4$ Hz, Ar-H'), 7.96 (1H, d, $J = 8.4$ Hz, Ar-H'), 8.00 (1H, s, Ar-H'). ESI-MS (positive, m/z): 735.50 $[\text{M+H}]^+$.

4.10. 4-Fluoro substituted dimethylcurcumin (22)

Dimethylcurcumin (**21**) (198 mg, 0.5 mmol) in DMF (5 mL) was added to SelectFluorTM (0.51 mmol). The mixture was stirred at rt overnight under N_2 . The solvent was removed by distillation and the remaining solid was dissolved in CH_2Cl_2 and washed with water. After being dried over Na_2SO_4 the crude product was purified by flash column eluting by *n*-hexane: EtOAc = 6: 1 to afford the desired product **22** as an orange powder in 7% yield. $^1\text{H NMR}$ (300 MHz, CDCl_3): δ 3.94-3.92 (m, 12H, OCH_3), 6.88 (d, $J = 8.4$ Hz, 2H), 6.98 (dd, $J = 15.9, 3.0$ Hz, 2H), 7.12-7.11 (m, 2H), 7.21 (dd, $J = 8.4, 1.8$ Hz, 2H), 7.65 (d, $J = 15.9$ Hz, 2H); ESI MS m/z 413.2 $[\text{M-H}]^-$, 437.2 $[\text{M+Na}]^+$.

4.11. 4,4-Difluoro substituted dimethylcurcumin (23)

A solution of dimethylcurcumin (**21**) (80 mg, 0.2 mmol) in THF (5 mL) was added to a suspension of 10 mg of NaH (80% in mineral oil) at 0°C . The mixture was stirred at 0°C for 0.5 h, then rt for 1h. A solution of SelectFluorTM (80 mg, 0.22 mmol) in DMF (2 mL) was then added and the mixture was stirred for additional 8 h. After additional 30 mg of SelectFluorTM in DMF (1 mL) was added, the reaction mixture was continually stirred for

0.5 h. The solvent was removed by distillation and the remained solid was purified by flash column chromatography (eluting by *n*-hexane: EtOAc = 6: 1) and PTLC (*n*-hexane: EtOAc = 1: 2) to give the desired product **23** (27 mg, 31% yield) as yellow solid powder. ¹H NMR (300 MHz, CDCl₃): δ 3.92 (s, 12H, OCH₃), 6.87 (d, *J* = 8.4 Hz, 2H), 6.96 (d, *J* = 15.6 Hz, 2H), 7.11 (d, *J* = 2.1 Hz, 2H), 7.21 (dd, *J* = 8.4, 2.1 Hz, 2H), 7.48 (d, *J* = 15.6 Hz, 2H); ESI MS *m/z* 433.2 [M+H]⁺, 455.1 [M+Na]⁺.

4.12. Cell culture

PC-3 (ATCC CRL 1435) and LNCaP clone FGC (ATCC CRL 1740) cell lines were obtained from Lineberger Comprehensive Cancer Center (UNC-CH). PC-3 cells were cultured in RPMI-1640 medium containing 25 mM HEPES and 2 mM L-glutamine (Mediatech), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 IU penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Mediatech). In addition, 1 mM sodium pyruvate (Mediatech) was added to the medium for LNCaP cells. Cells were incubated in 5 % CO₂ and 95% air at 37 °C.

4.13. Antiproliferative activity assay

Antiproliferative activity was determined by the sulforhodamine B (SRB) colorimetric assay as previously described.⁴⁵ In brief, the cells (3-5 × 10³ cells/well) were seeded in 96-well plates filled with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) containing various concentrations of samples, and incubated for 72 h. At the end of the exposure period, the attached cells were fixed with cold 50% trichloroacetic acid for 30 min followed by staining with 0.04% SRB (Sigma Chemical Co.) for 30 min. The bound SRB was solubilized in 10 mM Tris-base and the absorbance was measured at 515 nm on a Microplate Reader ELx800 (Bio-Tek Instruments, Winooski, VT) with a Gen5 software. All results were representative of three or more experiments.

4.14. Morphological analysis and intrinsic fluorescence of compounds in cells

PC-3 and LNCaP tumor cells were maintained in RPMI-1640 medium supplemented with 10% FBS in 8-well chamber slides (Lab-Tech) for 12 h prior to treatment with DMSO, **1** (30 μM), **2** (40 μM), **6** (50 μM), **15** (40 μM), and flutamide (100 μM), or bicalutamide (100 μM) for 48 h at 37 °C. For intrinsic fluorescence analysis, PC-3 cells were treated with 40 μM compound for 1 h at 37 °C. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, Sigma), and cells were mounted with VECTASHIELD (vector labs). Fluorescence labeled cells were observed using a Nikon ECLIPSE E400 fluorescence microscope and images were captured by a SPOT cooled digital camera controlled by the SPOT Software (Diagnostic Instruments Inc.). Final images were prepared using Adobe Photoshop ver 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of flutamide, hydroxyflutamide, bicalutamide, and curcumin

Figure 2.
Design of curcumin analogs conjugated with hydroxyflutamide or bicalutamide moiety.

Figure 3.

Dose-response curves of conjugates and antiandrogens in LNCaP cells. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS in the presence of compound for 72 h. All data were calculated as the mean $\pm$ SE of triplicate samples and are representative of at least three individual experiments.

Figure 4.

Dose-response curves of conjugates and antiandrogens in PC-3 cells. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS in the presence of compound for 72 h. All data were calculated as the mean $\pm$ SE of triplicate samples and are representative of at least three individual experiments.

Figure 5.

Suppression of pseudopodia formation in LNCaP cells by curcumin analogs. Androgen-sensitive LNCaP cells were cultured for 48 h in RPMI-1640 medium supplemented with 10% FBS in the presence of compound. Cells were fixed and stained with DAPI for DNA. Vigorous pseudopodia formation was seen in untreated (DMSO) or 100 μ M bicalutamide-treated cells (arrows), while pseudopodia formation was limited in cells treated with **6** or **15**, and completely impaired in cells treated with **1** or **2**. These observations implied that curcumin and its analogs strongly inhibited pseudopodia formation in LNCaP cells. Bar, 50 μ m.

Figure 6.

Induction of nuclear fragmentation in PC-3 cells by curcumin analogs. Androgen-independent PC-3 cells were treated with compound as indicated for 48 h followed by staining with DAPI for DNA. PC-3 cells treated with **1** or **2** often showed nuclear enlargement (black arrows), suggesting that PC-3 cells induced cell cycle arrest at G2/M. Interestingly, when PC-3 cells were treated with **6** or **15**, polykaryocytes (multinucleated cells) (arrow heads) were frequently observed. In addition, pseudopodia-like extension (white arrow) was not seen in agent-treated cells. Bar, 50 μm .

Figure 7. Subcellular distribution of curcumin (1) and conjugate 6 in PC-3 cells

PC-3 cells were briefly treated with **1** or **6**. The intrinsic fluorescence of compound was observed under the fluorescence microscope (left panels, green). DAPI was used for staining nuclei (center panels, red), and merged with intrinsic fluorescence (right panels). The intrinsic fluorescence of **1** or **6** was detected and showed subcellular distribution of these compounds in PC-3 cells. Bar, 50 μ m.

Scheme 1.
Synthesis of conjugates **5-12**

Scheme 2.
Synthesis of conjugates **15** and **16**

19: i) Ethyl 4-bromobutyrate, K_2CO_3 , DMF, ii) 1) B_2O_3 , 2) **17a** ($R = CH_2CH_3$, $n = 1$), $(nBuO)_3B$, 3) $nBuNH_2$, 4) 1% HCl, iii) 10% HCl, THF, iv) EDCI, DMAP, CH_2Cl_2
20: i) 4-Bromoundecanoic acid, Cs_2CO_3 , NaI, DMF-dioxane, ii) 1) B_2O_3 , 2) **17b** ($R = H$, $n = 8$), $(nBuO)_3B$, 3) $nBuNH_2$, 4) 1% HCl, iv) EDCI, DMAP, CH_2Cl_2

Scheme 3.
Synthesis of conjugates **19** and **20**

Scheme 4.
Synthesis of conjugates **22** and **23**

Table 1

Cytotoxicity data for curcumin and curcumin analogs against LNCaP and PC-3 human prostate cancer cell lines.^a

Compound	IC ₅₀ ± SE (μM)	
	LNCaP	PC-3
Curcumin (1)	36.4 ± 2.4	36.0 ± 1.4
Bicalutamide	74.0 ± 5.9	76.4 ± 7.2
Flutamide	81.8 ± 2.1	98.8 ± 3.2
2	47.0 ± 3.2	33.1 ± 2.5
5	> 100	> 100
6	55.0 ± 4.1	48.1 ± 4.6
7	54.8 ± 2.5	52.1 ± 4.8
8	> 100	> 100
9	> 100	> 100
10	> 100	> 100
11	> 100	> 100
12	> 100	> 100
15	41.8 ± 3.9	39.1 ± 3.1
16	73.0 ± 4.2	100.3 ± 1.9
19	96.3 ± 8.3	> 100
20	> 100	> 100
21	15.3 ± 2.8	23.7 ± 1.4
22	27.3 ± 3.0	17.7 ± 2.4
23	53.8 ± 4.9	44.3 ± 4.8

^aCell proliferation was determined by SRB assay as described in "Antiproliferative Activity Assay". Data are expressed as the means ± SE of at least three or more independent experiments