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# Synthesis of novel C5-curcuminoid-fatty acid conjugates and mechanistic investigation of their anticancer activity

**David J. Sanabria-Ríos**[a,1], **Yaritza Rivera-Torres**[a], **Joshua Rosario**[a], **Camille Ríos**[a], **Ricardo Gutierrez**[a], **Néstor M. Carballeira**[b], **Christian Vélez**[c], **Beatriz Zayas**[c], **Félix Álvarez-Cólon**[d], **Gabriela Ortiz-Soto**[d], **Victor Serrano**[d], **Joanne Altieri-Rivera**[d], **Eddy Ríos-Olivares**[d], and **José W. Rodríguez**[d]

[a]Faculty of Science and Technology, Inter American University of Puerto Rico, Metropolitan Campus, PO Box 191293, San Juan, Puerto Rico 00919

[b]Department of Chemistry, University of Puerto Rico, Rio Piedras Campus, PO Box 23346, San Juan, Puerto Rico 00931-3346

[c]School of Environmental Affairs, Universidad Metropolitana, San Juan, Puerto Rico, 00928

[d]Department of Microbiology and Immunology, Universidad Central del Caribe School of Medicine, PO Box 60327, Bayamón, Puerto Rico 00960

## Abstract

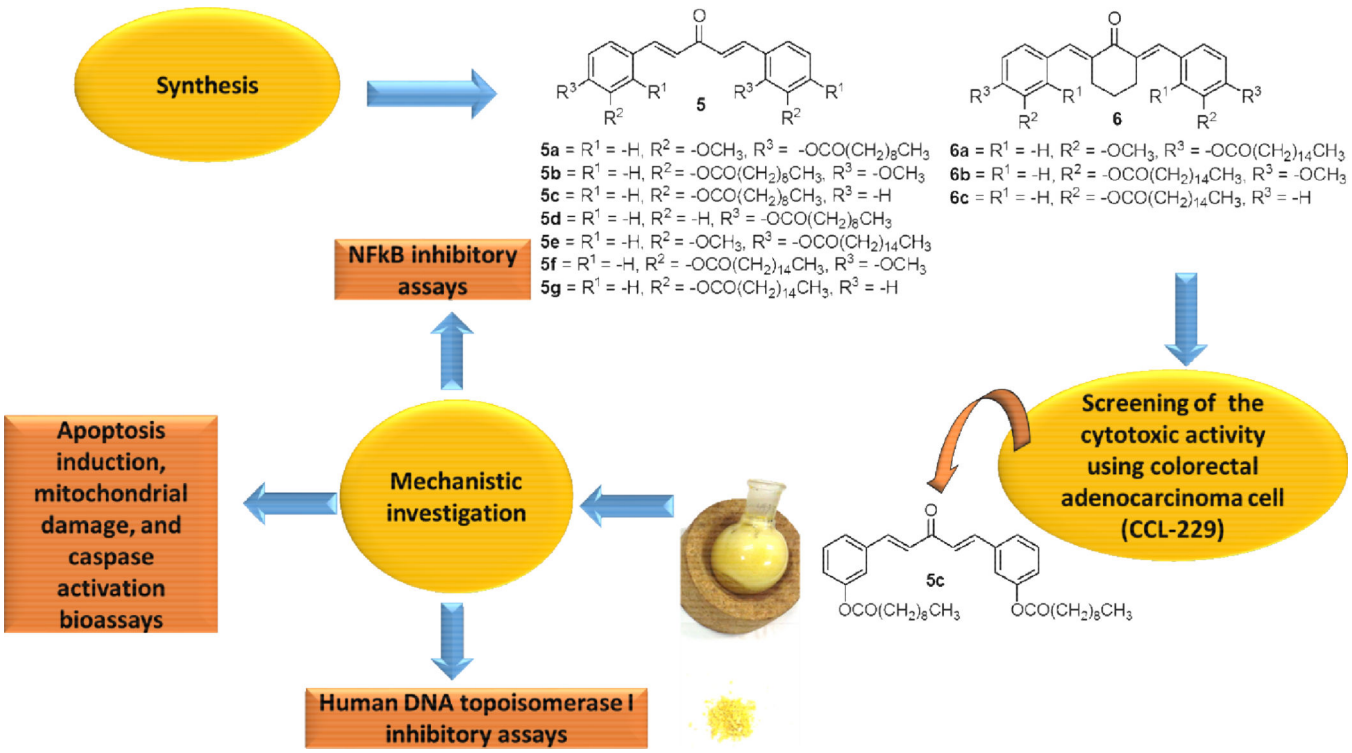
The first synthesis of C5-Curcumin-Fatty Acid (C5-Curc-FA) conjugates was successfully performed. Through a two-step synthetic route, 10 analogs were synthesized for a structure-activity relationship (SAR) study. It was found that C5-Curc-FA conjugates containing either decanoic acid or palmitic acid moieties were cytotoxic against colorectal adenocarcinoma cell (CCL-229) at $IC_{50}$s ranging from 22.5 to 56.1 μg/mL, being **5c** the most active C5-Curc-FA conjugate. Our results strongly suggests that a decanoic acid moiety at the *meta* position in C5-Curc-FA conjugates is important for their anticancer activity effect. Possible mechanisms for the anticancer activity of C5-Curc-FA conjugates were also investigated including apoptosis induction, mitochondrial damage and caspases activation. It was shown that **5c** inhibited the luminescence activity of NFκB, a key signaling molecule involved in cell apoptosis and cell proliferation, at $IC_{50}$ = 18.2 μg/mL. In addition, it was demonstrated that **5c** displayed significant apoptotic effect at $GI_{50}$ = 46.0 μg/mL in colorectal adenocarcinoma cell line (ATCC CCL-222), which can be explained by the significant mitochondrial membrane permeabilization and caspases 3 and 7 activation effect of **5c**. Finally, it was investigated that C5-Curc-FA conjugates can affect the replication process of cancer cells, since compounds **5c, 5e**, and **6c** inhibited the relaxing activity of the human DNA topoisomerase I at minimum inhibitory concentrations (MICs) that range from 50 to 500 μg/mL. Our results strongly support the hypothesis that the inhibition of both



[1]Corresponding author. Tel.: 787-250-1912 ext. 2332; fax: 787-250-8736, dsanabria@intermetro.edu (D. J. Sanabria-Rios).

NFkB and DNA topoisomerase I by C5-Curc-FA conjugates is associated with their anticancer activity.

## Abstract




### Keywords

C5-curcuminoids synthesis; anticancer agents; NFkB; apoptosis; DNA topoisomerase I

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"*Cancer is characterized by the uncontrolled growth and spread of abnormal cells*". This disease affects approximately 1,665,540 United States citizens, causing 585,720 deaths per year.[1] In Puerto Rico, 51,805 persons have been diagnosed with cancer and the rate of death is 127 per 100,000 cases.[2] One problem that is affecting the nation's health is the high toxicity of the current drugs to treat patients with cancer that destroy blood cells such as T-cells, B-cells, macrophages, NK cells, and neutrophils. This situation is complicated when these treatments break down the tissue of natural infection barriers such as the skin, urogenic, and gastrointestinal tract covering thus allowing cancerous agents to travel through these openings and invade immunocompromised patients. Despite the fact that several compounds are being evaluated as anticancer agents, there is a critical need to develop new cancer chemotherapy treatments with low toxicity.

Among the compounds presently been evaluated as anticancer agents, curcumin has demonstrated to exhibit anticancer properties.[3–7] Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6- heptadiene-3,5-dione, **1**, Figure 1] is a yellowish polyphenolic compound isolated from turmeric, a rhizome of the Indian plant *Curcuma longa*. Although

preclinical and clinical studies have shown that **1** is not toxic towards normal human cells, several pharmacokinetic disadvantages, such as poor bioavailability, fast metabolism and requiring repetitive oral doses were reported, which limits its pharmacological applications.[8–9] One of the reasons for the reduced bioavailabilty of **1** is its high rate of metabolism.[10] Several studies performed in rats and in humans have suggested that orally and intravenously administered **1** is rapidly metabolized in the liver into hexahydrocurcumin and hexahydrocurcuminol, while curcumin glucuronide and curcumin sulfate are generated extrahepatically, probably in the gastrointestinal tract.[11–13] The fast metabolism of **1** can be due to the presence of the β-diketone moiety, which appears to be a specific substrate for a series of aldo-keto reductases.[14–16] Several approaches have been performed in order to improve the pharmacokinetics disadvantages mentioned above including the preparation of liposome-encapsulated **1** and the synthesis of mono-carbonyl analogs of **1** (C5-curcuminoids).[17–20] Recently, the synthesis of (1*E*,4E)-1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (C5-curcumin, **2**, Figure 1) was performed with the objective of evaluating its anticancer properties.[18–19] It was found that **2** was particularly active against both CNE (nasopharyngeal adenocarcinoma) and LS 174T (colorectal adenocarcinoma), thus displaying a good pharmacokinetic profile.[19]

In 2010, Singh and collaborators reported the first total synthesis of curcumin bioconjugates containing fatty acids aimed at studying both its antibacterial and antiviral properties.[21] Results from this study revealed that the conjugation of both decanoic acid and palmitic acid to curcumin improved the antimicrobial activity of **1**. These authors reasoned that the improved biological properties of curcumin-fatty acid (Curc-FA) conjugates is due to the presence of the fatty acid moiety, which decreases the rate of metabolism of **1** needing an additional hydrolysis of ester bonds by carboesterases present in cells; provides structural similarity to the cell wall allowing the uptake of **1** by enhancing its effective concentration; and improves the biological activity of **1** after enzymatic hydrolysis of the ester group in Curc-FA conjugates.[21] However, to the best of our knowledge, the chemical connection of a fatty acid to a monocarbonyl analog of **1** and evaluation of its anticancer activity has not been reported.

In the present study the Singh approach was used to synthesize, for the first time, C5-curcumin-fatty acid (C5-Curc-FA) conjugates in order to determine structure-activity relationship (SAR) using the CCL-229 (colorectal adenocarcinoma) cell line. We chose to use the CCL-229 cell line because colorectal cancer is one of the cancers that mostly affects the adult population in both the United States and Puerto Rico.[2,22] Our approach of connecting FAs to **2** is totally new, since to the best of our knowledge, only one study has been reported addressing the chemical connection of FAs to **1**, which contains a β-diketone moiety in its structure. In this study, we decided to conjugate **2** to FAs because the presence of only one carbonyl moiety in **2** enhances its *in vitro* stability profile, its *in vivo* pharmacokinetic profile, and its cytotoxic activity against several cancer cell lines including CNE and LS 174T adenocarcinomas *in vitro*.[19]

The synthesis of C5-curcumin-fatty acid conjugate **5** started with the conjugation of the substituted phenol **3** with either decanoic acid or palmitic acid through Steglich esterification conditions described in the literature[23], which afforded **4a–g** in 43–80%

yields. Compounds **4a–g** were subsequently reacted with acetone and lithium hydroxide monohydrate in ethanol obtaining the desired C5-Curc-FA conjugates **5a–g** in 47–69% yields. The synthetic strategy displayed in Scheme 1 was also used for preparing C5-Curc-FA conjugates containing cyclohexanone moieties. In this approach, compounds **4e–g** were reacted with cyclohexane and lithium hydroxide monohydrate in ethanol for 24 h. This reaction afforded the desired **6a–c** in 42–62% yields (Scheme 2). Melting points for **5a–g** and **6a–c** were determined with a Melt-Temp apparatus and were uncorrected.

The NMR analyses were performed in a Bruker Avance AV-500 spectrometer. Both $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data are shown in Table 1. In $^{1}$H-NMR two doublets at 7.5 and 6.5 ppm were observed for **5a–5g**. These signals have a coupling constant (*J*) of 16 Hz, which are characteristic of compounds containing a trans alkene moiety.[24] In the case of **6a–6c**, a singlet at 7.6 ppm was also observed in $^{1}$H-NMR, which is in agreement with spectroscopic data from other C5-curcuminoids containing cyclohexanone moieties previously reported in the literature.[18] The purity of **5** and **6** was determined to be > 95% by $^{13}$C-NNR and confirmed by a melting point analysis. For **5** and **6**, two signals at 191 and 170 ppm were observed. These signals are evidence of the presence of the α,β-unsaturated carbonyl and ester carbonyl groups, respectively.[24]

Once **5a–g** and **6a–c** were synthesized, these compounds were tested against colon cancer cells (CCL-229). These tests were performed through the MTT (methyl thiazolyl tetrazolium) assay for determining cell viability. CCL-229 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Kaighn's Modification of Ham's F-12 Medium containing 2 mM L-glutamine and 1500 mg/L sodium bicarbonate (F12K, Mediatech, Inc., Manassas, VA, USA). A 10,000 cells/200 μL/well were seeded into a Corning 96-well microplate (Corning, NY, USA) and compounds **5a–g** and **6a–c** were added to the cell cultures. The final concentrations of C5-Curc-FA conjugates **5a–g** and **6a–c** ranged from 5 to 500 μg/mL. The cells were incubated at 37°C for three days in a humidified 5% $CO_2$ incubator. Results from MTT assays are displayed in Table 2.

Results from Table 2 show that **5a–g** and **6a–c** exert direct cytotoxic effect on CCL-229 cells and inhibit their growth in vitro in a dose dependent manner. Among the ten C5-Curc-FA tested, compounds **5c, 5e**, and **6c** were the most active conjugates against CCL-229 displaying $IC_{50}$ values ranging from 22.5–56.1 μg/mL. It can be appreciated from Table 2 that the anticancer activity of C5-Curc-FA increases when decreasing the number of carbon atoms in the fatty acyl chain. This is the case of the pairs of compounds **5f/5b** and **5g/5f** whose $IC_{50}$ values increase 1.1 and 5.0 times, respectively. With the exception of **5g** and **6c**, the cyclohexanone moiety in a C5-Curc-FA conjugates decrease their anticancer activity against CCL-229 cells. For example, when the anticancer activity of **5e** and **6a** are compared, it can be observed that the $IC_{50}$ value of **6a** is 2.5-fold higher than **5e**. A similar pattern was observed when the activity of **5f** and **6b** are compared. It is interesting to observe that **5c** showed similar $IC_{50}$ values than **1**, which demonstrate its efficacy as anticancer agent. Results in Table 2 also suggest that the presence of a methoxy group in a C5-Curc-FA conjugate affects its anticancer activity towards CCL-229. In curcuminoids **5f** and **5g**, which contain 16-carbon chain length FA moieties, the presence of a methoxy group

at the *para* position increases their anticancer activity. On the other hand, the methoxy group at the *para* position in curcuminoid **5b**, decreases its anticancer activity. These results strongly support that the FA moiety plays an important role in the anticancer activity of C5-Curc-FA conjugates towards colorectal cancer cells.

The toxicities of the most active C5-Curc-FA conjugates were determined against peripheral blood mononuclear cells (PBMCs) from healthy volunteers (Table 3). PBMCs were cultured in culture medium supplemented with interleukin-2 (IL-2) and seeded into a 96-well microplate ($4 \times 10^5$–$5 \times 10^4$ cells/ 200 μL/ well). C5-Curc-FA conjugates were added to the cell cultures obtaining final concentrations that ranged from 2 to 100 μg/mL. The cells were incubated at 37°C for 3 days in humidified 5% $CO_2$ incubator. The cytotoxicity of the cells was evaluated by the MTT assay. Results in Table 3 show that **5c, 5e** and **6c** were 2.9–3.3-fold less toxic than **1** suggesting that the presence of a fatty acid moiety and removal of a carbonyl group in **1** decreases the cytotoxic activity of C5-Curc-FA conjugates against PBMC. Singh et al. explained that fatty acids are natural components of cell membranes, thus by conjugating fatty acids to **2** it is expected that this connection enhances the cellular uptake of **2**, increases its lipophilicity, improves its half-life, and reduces its rate of metabolism by carboesterases inside the cell, which ensures its low toxicity and high bioavailability.[21] In fact, it can be noted from Table 3 that the cytotoxicity of C5-Curc-FA conjugates against PBMC decreases when their logP values increase.

One of the mechanisms that has been widely studied for **1**, and its derivatives, is the inhibition of the transcription factor NFκB.[3,25–26] The NfkB is a key signaling molecule in the elaboration of the inflammatory response and apoptosis of cancer cell lines.[27] In order to further investigate the mechanism of action of C5-Curc-FA conjugates, we carried out several NFkB luciferase reporter assays aimed at determining the inhibitory effects of these conjugates on the activity of NFkB. For these assays, we selected **5c** because **5c** displayed the best anticancer activity against the CCL-229 cells. Prior to these assays, NFkB Reporter-HEK293 (BPS bioscience, CA) cells were cultured in MEM (Hyclone, UT) medium with 10% FBS (Life technologies, CA), 1% non-essential amino acid, 1mM Na-pyruvate (Hyclone, UT), 1% Penn-strep (Hyclone, UT), and 100 μg/mL of Hygromycin B (Hyclone, UT). The NFkB luciferase reporter assays were performed by seeding 30,000 cells per well into a white clear-bottom 96-well microplate in 40 μL of assay medium (growth medium without Hygromycin B). Cells were incubated at 37 °C and 5% $CO_2$ overnight to allow them to recover and reattach. After 24 h, compounds **5c** and **1** were diluted in the assay medium and 5 μL of dilution were added to each well. Also, 5 μL of assay medium with DMSO were added to the untreated control wells and cell-free control wells. Cells were treated with the tested compounds for 16 h. Then, the cells were treated with mouse TNFα (5 ng/mL, Sigma-Aldrich, MO) for an additional 6 h. After treatment, cells were lysed and luciferase assays were performed by adding 50 μL of One-Step Luciferase reagent per well and shaking at room temperature for approximately 15 min. Luminescence was measured using a luminometer (BioTek SynergyTM 2 microplate reader).

Results from the NFkB inhibitory assays are displayed in Figure 2. Our results demonstrate that **5c** has the ability of inhibiting the activity of NFkB since its luminescence properties, after treatment with **5c**, exhibited a sigmoidal behavior with the logarithm of the

concentration of the conjugate. A similar dose-response behavior was observed for **1**, since its inhibitory activity against NFkB is known in the literature.[3,25–26,28] Also, it was observed that the luminescence in the presence of **1** is 2.3-fold higher than in the presence of **5c**, which suggests that the lipophilic nature of **5c** is affecting the NFkB inhibitory activity. The fact that **5c** was able to inhibit the NFkB luminescence activity implies that the disruption of the NFkB pathway is taking place. The NFkB pathway is a key mediator of the control of genes that are highly involved in apoptosis and cell proliferation such as c-IAP1, c-IAP2, and IXAP (antiapoptotic genes), the TNF receptor-associated factors (TRAF1 and TRAF2), the Bcl-2 homologue A1/Bfl-1, and IEX-IL.[27] Results from proliferation of CCL-229 cells and NFkB inhibitory assays strongly support the hypothesis that the disruption of the NFkB pathway can be a possible mechanism for **5c**, since its activity as an anticancer agent and an NFkB inhibitor was observed at $IC_{50}$s values of 22.5 and 18.2 μg/mL, respectively.

Due to the fact that **1** and **5c** displayed NFkB inhibitory activity, we hypothesize that these compounds might be able to induce apoptosis in colorectal cancer cells. For this reason, we performed the Annexin V assay in order to determine whether compounds **1** and **5c** induce apoptosis. For this assay the human COLO-205 colorectal adenocarcinoma (ATCC CCL-222) was used and cultures were maintained in RPMI 1640 media (ATCC, Manassas VA) containing 10% fetal bovine serum (ATCC) and incubated at 37°C with humidified atmosphere of 95% air and 5% $CO_2$. Apoptosis endpoint was measured after 24 h of exposure. The Annexin V assay has been used as an indicator of apoptosis. We used Annexin V as the apoptosis indicator because this assay allows for the detection of phosphatidylserine (PS) on the exterior surface of cells, a hallmark in apoptotic cells.[29] For this assay, approximately $2.5 \times 10^6$ cells were treated for 24 h with the $GI_{50}$ dose of each test compound 46.0 μg/mL of **5c** and 7.4 μg/mL of **1**. Controls used in all assays were camptothecin (3.5 μg/mL), and the vehicle (DMSO). After a 24 h exposure, cells were stained with Annexin V conjugate, and propidium iodide (Biotium, Hayward, CA). Samples were analyzed using the Nucleo Counter NC3000 system (Chemometec, Allerod, Denmark). A one way ANOVA with post hoc Tukey test was also performed for determining statistical significance. Results obtained from the Annexin V (Figure 3) assay showed a statistically significant apoptosis induction by **5c** ($P < 0.05$). The experimental compound presented an average of 27.5% apoptotic cells, which contrasts significantly to the negative control that presented an average of 14.3% (Figure 3). Positive controls camptothecin and **1** presented averages of 29.0% and 20.7%, respectively, indicating that all test compounds induced apoptosis when comparing to the negative compound.

After determining apoptosis activity with the annexin assay, several hallmarks of this process were analyzed. Among these endpoints is mitochondrial membrane permeabilization (MMP) and effector caspase activation. Mitochondrial membrane permeabilization (MMP) is one of the hallmarks of the apoptotic process.[30] A total of $1 \times 10^6$ cells were exposed to compounds and the controls camptothecin and **1** at the previously described doses, the samples were labeled with 200 μg/ml of 5, 5', 6, 6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanineiodide (JC-1). An additional counter stain with 1 μg/ml DAPI in PBS was also applied, then analyzed using the Nucleo Counter NC3000 system. A one way ANOVA with post hoc Tukey test was also performed. Our results present a

statistically significant permeabilization of the experimental compound comparable to the Camptothecin control. All compounds except the negative control (14.0%) presented average statistically significant ($P<0.05$) depolarization. The test compounds show varied activation rates; Camptothecin presented 48.5% and **1** 23.0% average depolarization (Figure 4a and 4b). The experimental compound **5c** presented and average of 68.0% depolarized cells.

The activation of effector caspases 3 and 7 is a key event in the apoptosis process.[31] These enzymes activate the nucleases tasked with degradation of nuclear material. Cell cultures were exposed to the test compounds as described previously in the annexin assay. Cells were harvested then stained with the Fluorescent Labeled Inhibitors of Caspases (FLICA). These markers bind to the active caspase enzymes, the green FAM FLICA kit (Immunochemistry Technologies, Bloomington Min.) was utilized as per manufacturer's specification then analyzed using the Nucleocounter instrument. Data obtained presents statistically significant ($P<0.05$) effector caspase activation in the experimental **5c** compound and positive controls (Figure 5). The background negative samples presented an average of 8% cell with active caspase 3 whereas the positive camptothecin presented 30% activation. Compound **5c** and its analog **1** presented 19.5% and 23.3% respectively demonstrating statistically significant caspase activation.

Other studies have demonstrated that curcumin and other curcuminoids displayed inhibitory activity against DNA topoisomerase I and II.[32–34] DNA topoisomerases are enzymes involved in generating the necessary topological and conformational changes in DNA, which are essential for several cellular processes such as replication, recombination, and transcription.[35] In addition to their normal cellular functions, DNA topoisomerases are known to be important molecular targets for some anticancer drugs.[36] In an effort to further understand the mechanism of action of **5** and **6**, we studied the inhibitory activity of **5c, 5e** and **6c** against the human topoisomerase I (Figure 6). The inhibitory activity of these curcuminoids towards topoisomerase I was studied because they were the most active anticancer conjugates based on their $IC_{50}$ values (Table 2).

The enzyme activity of topoisomerase I was assessed by using human DNA topoioserase I kit (TopoGen, Port Orange, FL, USA, 10 unit of enzyme relaxes 0.06 μg of pHOT1 supercoiled DNA in 30 min at 37 °C). Compounds **5c, 5e**, and **6c** were dissolved in 50% DMSO and were tested at different concentrations that range from 10 to 1,500 μg/mL. Reactions (final volume 20 μL) were carried out for 30 min at 37 °C. Reactions were stopped by adding 2 μL of 10% sodium dodecyl sulfate (SDS). Bound proteins were digested by incubation with proteinase K (0.05 mg/mL) for 15 min at 37 °C. After an incubation period, 2 μL of electrophoresis universal loading buffer were added. Samples were loaded followed by electrophoresis in a 1% agarose gel (72V) until the dye front of bromophenol blue is about 70% down gel. To visualize the reaction products, the gel was stained with ethidium bromide for 1 h and distained for 30 min in distilled water. DNA bands were detected and quantified in a Min BIS bioimaging system (model 241016P1, Israel).

Results from Figure 6 show that **5c** inhibits the relaxing activity of the human DNA topoisomerase I at 50 μg/mL, while **5e** and **6c** completely inhibit the activity of the enzyme at minimum inhibitory concentrations (MICs) of 100 and 250 μg/mL, respectively. These results are comparable with those findings described by Roth et al., where they reported that curcumin inhibits the relaxing activity of DNA topoisomerase I at 50 μg/mL.[32] It is very interesting to observe that the inhibitory activity of **5c** is 5–10-fold higher than **5e** and **6c** implying that the decanoic acid moiety at the *meta* position is important for the topoisomerase inhibitory activity of C5-Curc-FA conjugates. Also, it was observed that the methoxy group in **5e** and the cyclohexanone moiety in **6c** decrease their inhibitory activity against the DNA topoisomerase I. These topoisomerase inhibitory results are in agreement with the anticancer activity displayed by these compounds (Table 2) and they suggest that the inhibition of the relaxing activity of DNA topoisomerase I can be another mechanism of action for these C5-Curc-FA conjugates.

Our results clearly demonstrated that **5c, 5e** and **6c** warrant further investigation for the development of valuable anticancer agents. In the case of **5c**, this compound displayed a similar anticancer activity as **1** towards CCL-229 cells, but resulted to be less toxic towards PBMC. Therefore, **5c** displayed the best therapeutic index. We are in the process of synthesizing other C5-Curc-FA conjugates in order to further explore their anticancer properties as well as their mechanism of action.

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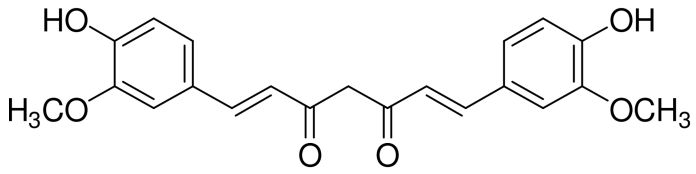
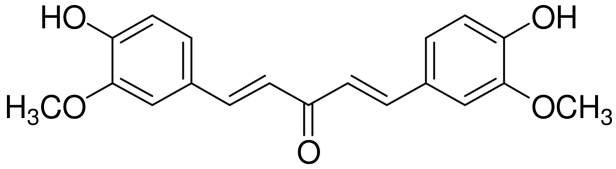



Curcumin (1)




C5-Curcumin (2)

**Figure 1.**
Chemical structures of Curcumin (**1**) and C5-Curcumin (**2**).

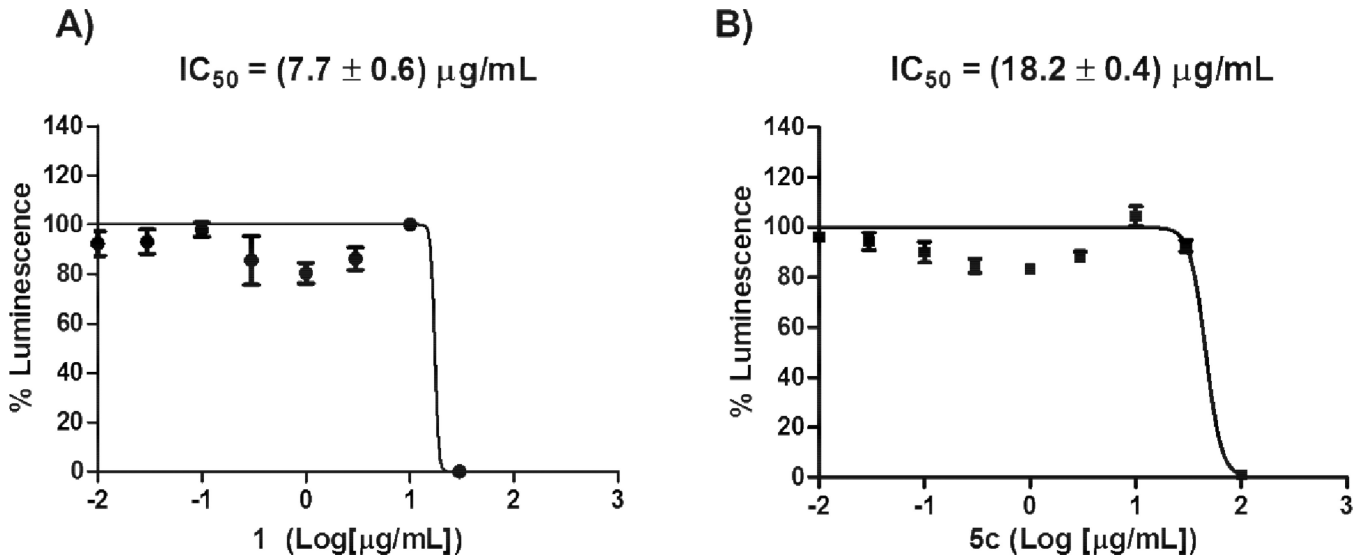



**Figure 2.**

Dose-response curves for the NFkB inhibitory activity for both **1** (A) and **5c** (B).a,b

[a]Experiments were performed in triplicate (N =3). Results were reported as mean ± SEM from three determinations.

[b] Non-linear regression analysis was performed by using GraphPad Prism v. 5.01 (GraphPad Software). The $IC_{50}$ values were determined by the concentration causing a half-maximal percent activity.

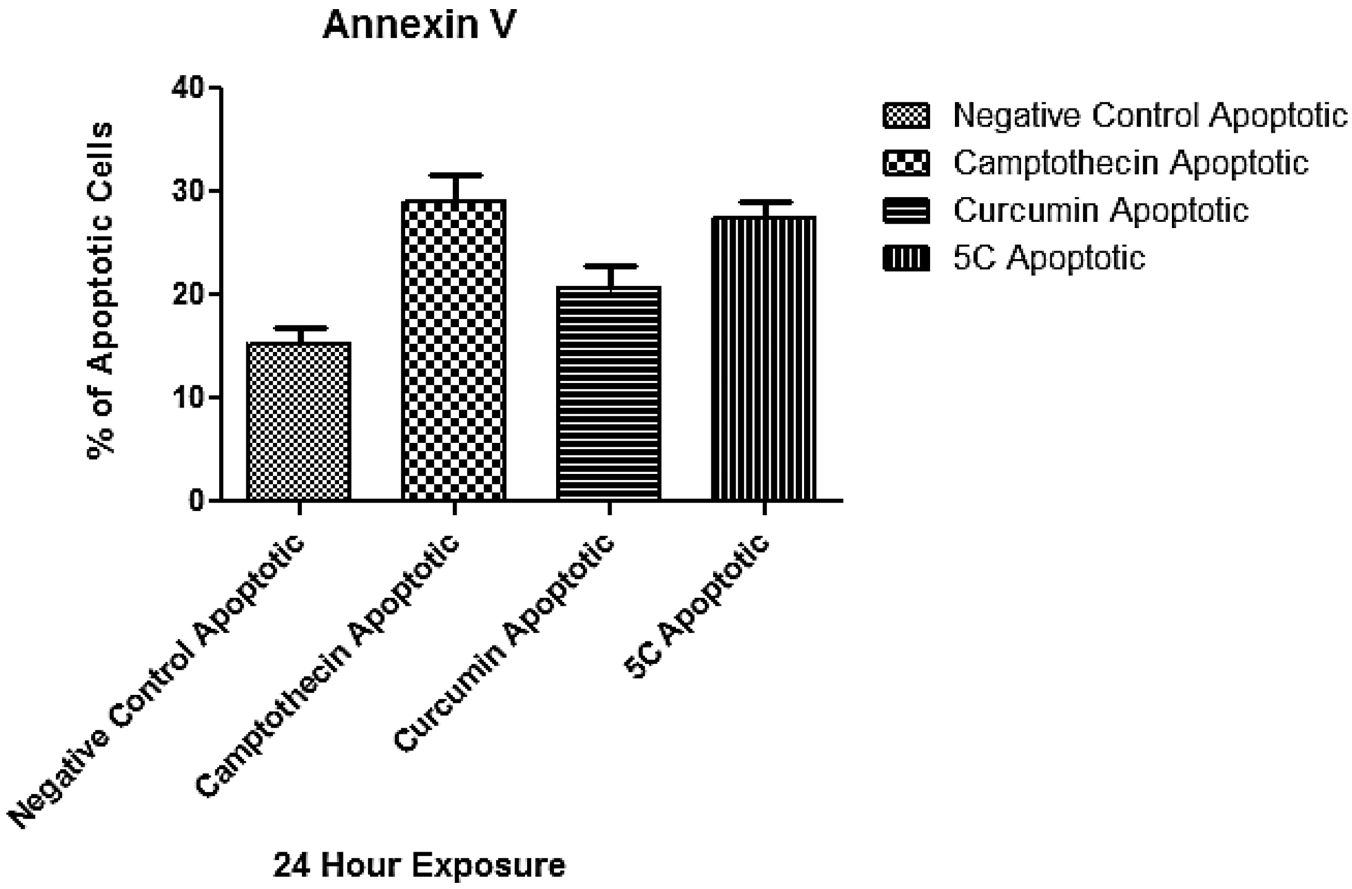



**Figure 3.**

(**A**) Annexin V assay results showing the apoptotic effect of compounds **5c** and curcumin (**1**). Statistical distribution showing the percentage of apoptotic cells per compound tested.a

[a]Experiments were performed in triplicate (N =3). Negative control presented an average of 14.3% apoptotic cells, positive controls camptothecin and **1** (curc) presented averages of 29.0 and 27.5% respectively. Compound **5c** presented an average of 27.5% which is statistically significant ($P<0.05$) when comparing to the negative control.

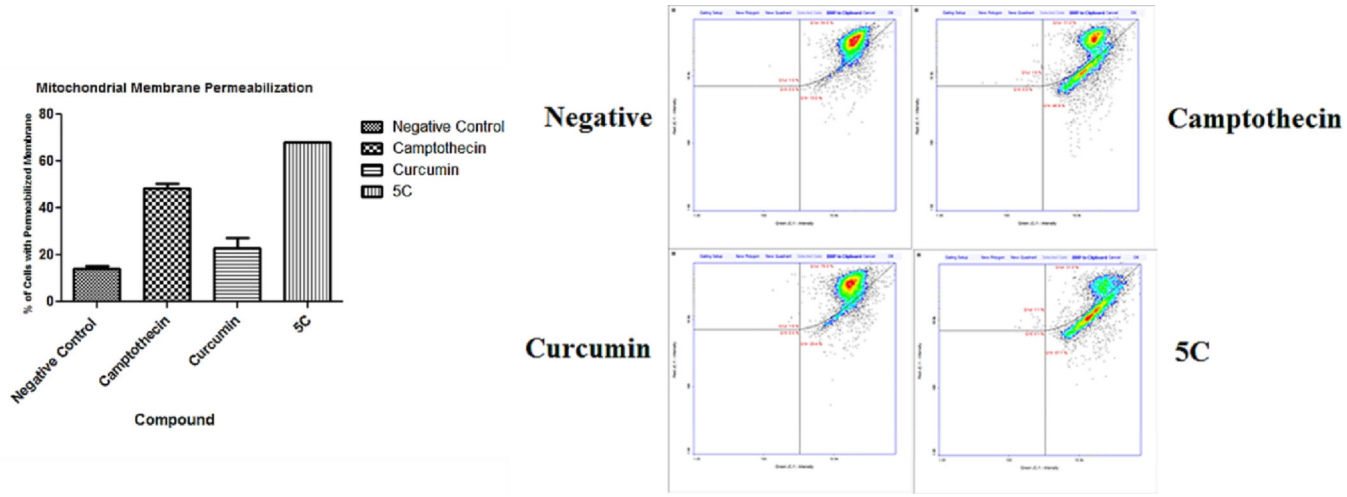



**Figure 4.**

(**A**) Mitochondrial membrane permeabilization effects of **5c**.a (**B**) Diagram showing the distribution cells with permeabilized mitochondrial membrane among all tested compounds [a] Experiments were performed in triplicate (N =3).Control sample presented an average of 14% permeabilized cells whereas the Camptothecin positive control 48.5% and Curcumin analog 23.0%. Compound **5c** presented 68% of cells with permeabilized mitochondrial membrane which is statistically significant ($P<0.05$).

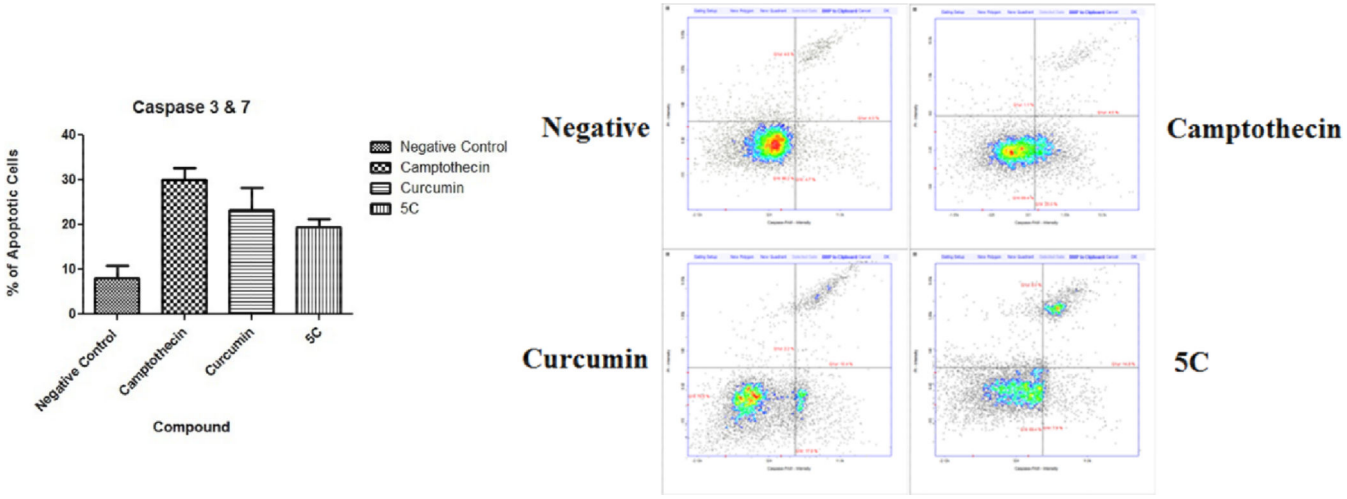



**Figure 5.**

Caspase 3 and 7 activation effect of **5c**.a

[a] Experiments were performed in triplicate (N =3). Results present a statistically significant ($P<0.05$) activation in the experimental compound **5c** and positive controls. Negative control presented a mean of 8% of cells with active caspase 3. Camptothecin, curcumin and the experimental **5c** compound presented averages of 30%, 23.3% and 19.5% respectively.

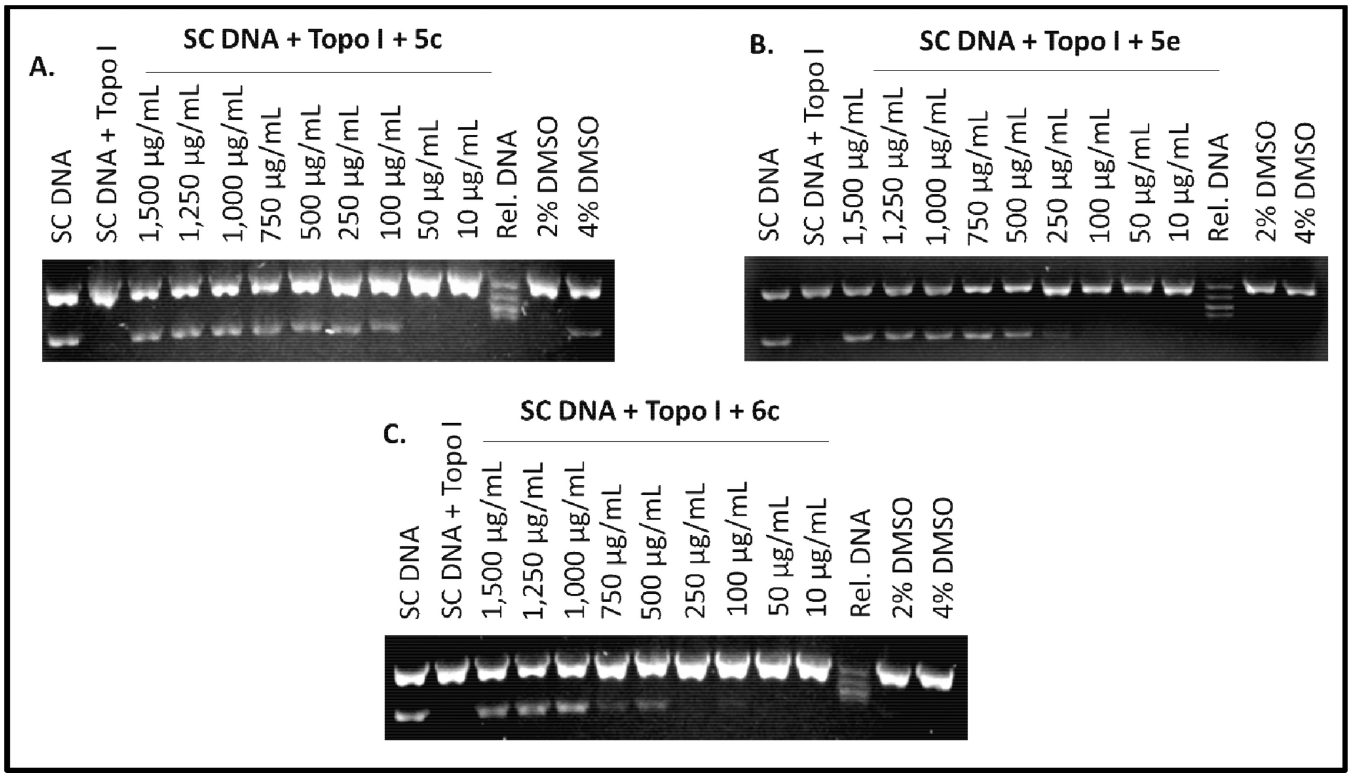
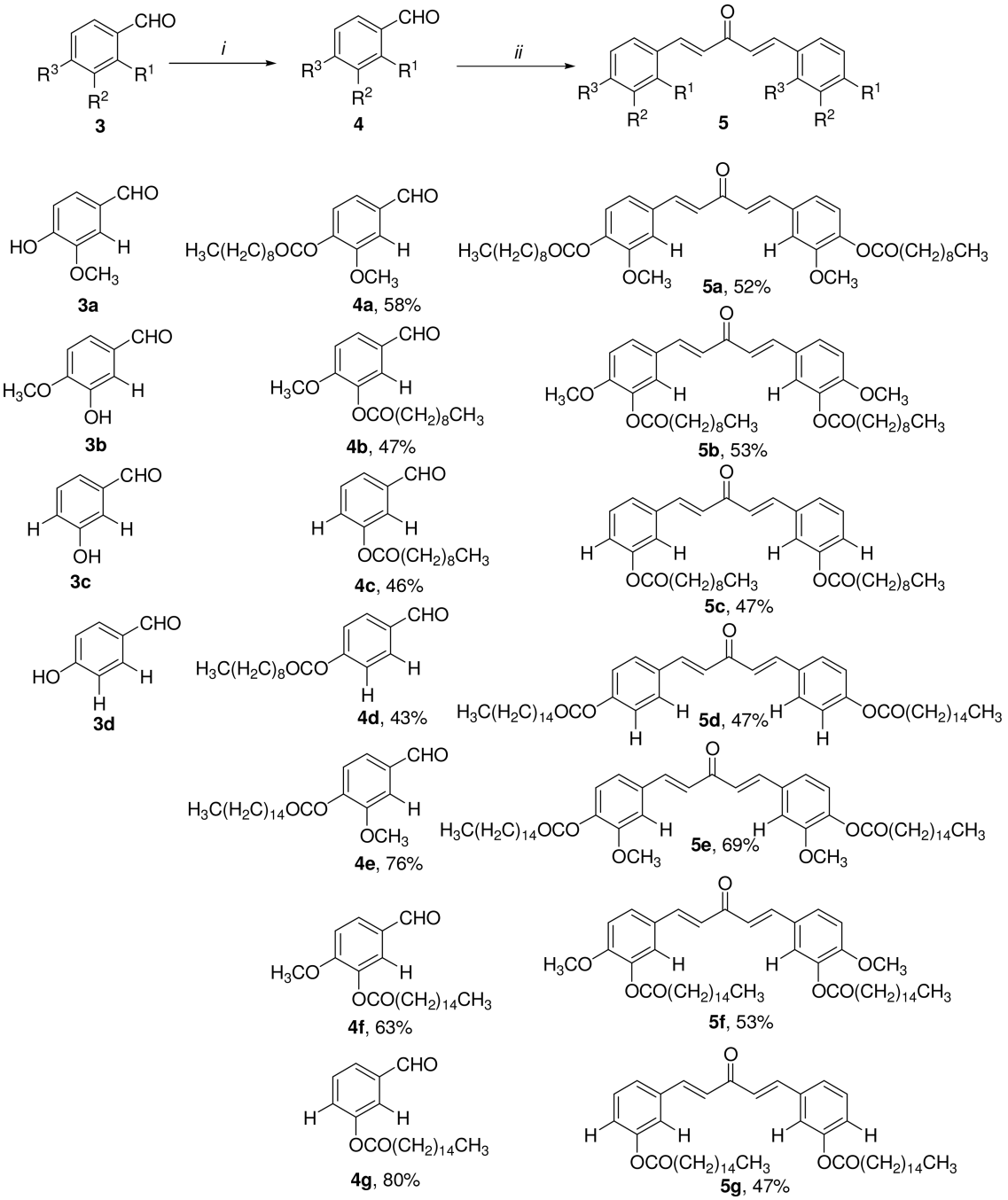



**Figure 6.**

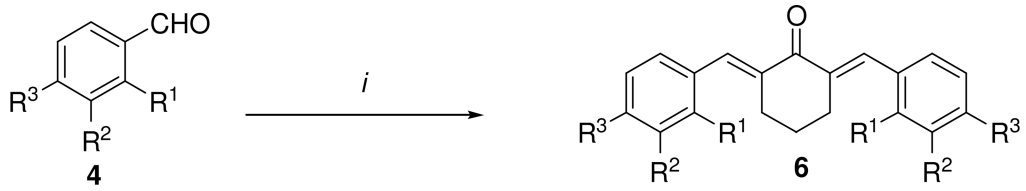
Representative ethidium bromide stained gels showing the inhibitory activity of C5-Curc-FA conjugates **5c, 5e**, and **6c**.a

[a] Experiments were performed in triplicate (N =3).




**Scheme 1.**

Synthetic route towards the C5-Curcumin-Fatty acid conjugates **5a–g**. *i*) Decanoic acid or palmitic acid, DIC, DMAP, $CH_2Cl_2$/THF (1:1 v/v), 0°C (for 1 hr), 24 h and *ii*) acetone, LiOH-$H_2O$, EtOH, rt, 24 h.




**4e** = $R^1$ = -H, $R^2$ = $-OCH_3$, $R^3$ = $-OCO(CH_2)_{14}CH_3$
**4f** = $R^1$ = -H, $R^2$ = $-OCO(CH_2)_{14}CH_3$, $R^3$ = $-OCH_3$
**4g** = $R^1$ = -H, $R^2$ = $-OCO(CH_2)_{14}CH_3$, $R^3$ = -H

**6a** = $R^1$ = -H, $R^2$ = $-OCH_3$, $R^3$ = $-OCO(CH_2)_{14}CH_3$ (42%)
**6b** = $R^1$ = -H, $R^2$ = $-OCO(CH_2)_{14}CH_3$, $R^3$ = $-OCH_3$ (50%)
**6c** = $R^1$ = -H, $R^2$ = $-OCO(CH_2)_{14}CH_3$, $R^3$ = -H (62%)

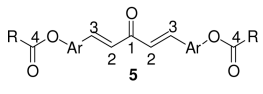
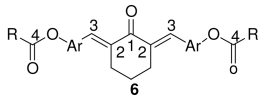
**Scheme 2.**

Synthesis of C5-Curc-FA conjugates containing the cyclohexanone moiety. *i*) Cyclohexanone, LiOH-$H_2O$, EtOH, rt, 24 h.

*Bioorg Med Chem Lett*. Author manuscript; available in PMC 2016 May 15.

**Table 1**

$^{1}$H-NMR and $^{13}$C-NMR characterization of C5-Curc-FA conjugates.[a,b,c]

| Compound |  5 | | | |  6 | | |
|---|---|---|---|---|---|---|---|
| | **$^{13}$C-NMR chemical shift (ppm)** | | | | **$^{1}$H-NMR chemical shift (ppm), *multiplicity*** | | **$H_2$–$H_3$ coupling constant (Hz)** |
| | **C1** | **C2** | **C3** | **C4** | **H2** | **H3** | |
| **5a** | 190.0 | 121.8 | 148.3 | 168.6 | 6.45, *d* | 7.60, *d* | 15.8 |
| **5b** | 191.9 | 122.2 | 147.6 | 170.8 | 6.56, *d* | 7.53, *d* | 16.1 |
| **5c** | 190.8 | 122.0 | 147.2 | 167.0 | 6.67, *d* | 7.56, *d* | 16.2 |
| **5d** | 190.3 | 122.6 | 148.0 | 173.0 | 6.45, *d* | 7.60, *d* | 15.8 |
| **5e** | 190.0 | 122.3 | 147.9 | 168.9 | 6.44, *d* | 7.60, *d* | 15.8 |
| **5f** | 192.1 | 120.9 | 141.1 | 172.9 | 6.52, *d* | 7.48, *d* | 15.8 |
| **5g** | 188.9 | 121.1 | 147.1 | 171.6 | 6.48, *d* | 7.37, *d* | 16.3 |
| **6a** | 190.0 | 137.4 | 139.7 | 168.9 | None | 7.53, *s* | None |
| **6b** | 192.6 | 136.4 | 139.8 | 174.4 | None | 7.62, *s* | None |
| **6c** | 190.7 | 136.9 | 139.5 | 173.4 | None | 7.52, *s* | None |

[a] Signals from FA moieties were observed at 0.85–2.44 ppm in $^{1}$H-NMR and 13.1–34.0 ppm in $^{13}$C-NMR. Signals from cyclohexanone moieties were observed at 1.62–2.28 ppm and 2.95–3.33 ppm in $^{1}$H-NMR and 22.1–23.8 ppm and 25.6–29.4 ppm in $^{13}$C-NMR.

[b] Signals from methoxy groups in compounds **5a, 5b, 5e, 5f, 6a**, and **6b** were observed at 3.78–3.83 ppm in $^{1}$H-NMR and 54.1–56.0 ppm in $^{13}$C-NMR.

[c] Signals from aromatic groups were observed at 6.59–7.80 ppm in $^{1}$H-NMR and 107.2–157.3 ppm in $^{13}$C-NMR.

## Table 2

Cytotoxic activity C5-Curc-FA conjugates **5a–g** and **6a–c** against colorectal cancer cells (CCL-229).[a]

| Compound | $IC_{50}$ ± SEM, μg/mL[b] |
|---|---|
| **5a** | 143.1 ± 14.6 |
| **5b** | 66.0 ± 6.5 |
| **5c** | 22.5 ± 3.0 |
| **5d** | 179.7 ± 31.1 |
| **5e** | 56.1 ± 1.5 |
| **5f** | 70.5 ± 16.9 |
| **5g** | 112.9 ± 7.1 |
| **6a** | 139.0 ± 23.3 |
| **6b** | 129.9 ± 24.1 |
| **6c** | 29.3 ± 0.7 |
| **1**[c] | 26.8 ± 1.6 |

[a] Experiments were performed in triplicate (N =3).

[b] Results were reported as mean ± SEM from three determinations. $IC_{50}$ represents the sample concentration that is required to achieve 50% cell viability and this value was determined from dose-response curves that were generated by using Graph-Pad Prism v. 5.01.

[c] Curcumin was used as positive control.

## Table 3

Toxicity of C5-Curc-FA conjugates against PBMC from healthy volunteers. [a]

| Compound | $IC_{50}$ ± SEM, μg/mL | logP[b] |
|---|---|---|
| **5c** | 35.9 ± 0.4 | 11.66 |
| **5e** | 40.6 ± 0.9 | 16.68 |
| **6c** | 36.3 ± 0.4 | 17.77 |
| **1**[c] | 12.3 ± 0.4 | 4.12 |

[a] Experiments were performed in triplicate (N =3). Results were reported as mean ± SEM from three determinations. $IC_{50}$ represents the sample concentration that is required to achieve 50% cell viability and this value was determined from dose-response curves that were generated by using Graph-Pad Prism v. 5.01 (GraphPad Software).

[b] LogP values were predicted by using MarvinSketch v. 6.2 (ChemAxon).

[c] Curcumin was used as positive control.