Journal Pre-proofs

Synthesis of novel 4-Boc-piperidone chalcones and evaluation of their cytotoxic activity against highly-metastatic cancer cells

Carlimar Ocasio-Malavé, Metsiel J. Donate, María M. Sánchez, Jesús M. Sosa-Rivera, Joseph W. Mooney, Tomás A. Pereles-De León, Néstor M. Carballeira, Beatriz Zayas, Christian E. Vélez-Gerena, Magaly Martínez-Ferrer, David J. Sanabria-Ríos

PII: S0960-894X(19)30723-1
DOI: https://doi.org/10.1016/j.bmcl.2019.126760
Reference: BMCL 126760

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 6 August 2019
Revised Date: 25 September 2019
Accepted Date: 15 October 2019

Please cite this article as: Ocasio-Malavé, C., Donate, M.J., Sánchez, M.M., Sosa-Rivera, J.M., Mooney, J.W., Pereles-De León, T.A., Carballeira, N.M., Zayas, B., Vélez-Gerena, C.E., Martínez-Ferrer, M., Sanabria-Ríos, D.J., Synthesis of novel 4-Boc-piperidone chalcones and evaluation of their cytotoxic activity against highly-metastatic cancer cells, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.126760

Synthesis of novel 4-Boc-piperidone chalcones and evaluation of their cytotoxic activity against highly-metastatic cancer cells

Carlimar Ocasio-Malavé[a], Metsiel J. Donate[b], María M. Sánchez[c], Jesús M. Sosa-Rivera[b], Joseph W. Mooney[a], Tomás A. Pereles-De León[a], Néstor M. Carballeira[d], Beatriz Zayas[e], Christian E. Vélez-Gerena[e], Magaly Martínez-Ferrer[b,c], David J. Sanabria-Ríos[a1]

[a]Faculty of Science and Technology, Department of Natural Sciences, Inter American University of Puerto Rico, Metropolitan Campus, PO Box 191293, San Juan, PR 00919, USA
[b] School of Pharmacy, Department of Pharmaceutical Sciences, University of Puerto Rico, Medical Sciences Campus, PO Box 365067, San Juan, PR 00936, USA
[c]University of Puerto Rico Comprehensive Cancer Center, PO Box 363027. San Juan, PR 00936, USA
[d]Department of Chemistry, University of Puerto Rico, Rio Piedras Campus, 17 Ave Universidad STE 1701, San Juan, PR 00925, USA
[e]School of Science, Technology & Environmental, Universidad Metropolitana, PO Box 21150, San Juan, PR 00928, USA

Keywords: Curcumin, curcumin analogs, cytotoxic agents, cancer, apoptosis, cell proliferation

[1]Corresponding author. Tel.: 787-250-1912 ext. 2332; Fax: 787-250-8736
Email address: dsanabria@intermetro.edu

Abstract

In the present study, six curcuminoids containing a *tert*-butoxycarbonyl (Boc) piperidone core were successfully synthesized, five of them for the first time. These compounds were prepared through an aldolic condensation by adding tetrahydropyranyl-protected benzaldehydes or substituted benzaldehyde to a reaction mixture containing 4-Boc-piperidone and lithium hydroxide in an alcoholic solvent. A 44–94% yield was obtained supporting this methodology as a good strategy for the synthesis of 4-Boc-piperidone chalcones. Cytotoxic activity against LoVo and COLO 205 human colorectal cell lines was observed at GI_{50} values that range from 0.84-34.7 μg/mL, while in PC3 and 22RV1 human prostate cancer cell lines, GI_{50} values ranging from 17.1-22.9 μg/mL were obtained. Results from biochemical assays suggest that the cytotoxicity of the 4-Boc-piperidone chalcones can be linked to their ability to induce apoptosis, decrease the activity of NFκB and cellular proliferation. Our findings strongly support the potential of Boc-piperidone chalcones as novel cytotoxic agents against highly-metastatic cancer cells.

Cancer is an enormous health burden reaching every region of the world but affecting mostly underdeveloped countries. Today, it accounts for about one in every seven deaths worldwide; more than HIV/AIDS, tuberculosis, and malaria combined.[1] Since many traditional anticancer therapies are facing numerous problems, such as multidrug resistance, whole body distribution before reaching cancer tissues, and toxic side effects[2], natural compounds like curcumin (Curc), the active component of the Asian plant turmeric (*Curcuma longa*), have been explored for its broad range of pharmacological effects. The properties of Curc are widely used by Oriental Medicine for the treatment of various chronic illnesses. Based on its capability to affect several molecular targets, Curc has the power for the prevention and treatment of allergies, arthritis, obesity, aging, diabetes, psoriasis, atherosclerosis, autoimmune, and neurodegenerative diseases.[3-8] Its efficacy as a potent anti-inflammatory has shown promising results as a novel agent to treat different types of carcinoma.[9-13] The antimetastatic effect of Curc has been revealed in human melanoma, carcinoma, colon, liver, pancreatic, bladder, ovarian and breast cancer.[14,15] This is mediated through regulation of various transcription factors (NFκB, AP-1, STAT), growth factors (VEGF, EGF, FGF), inflammatory cytokines (IL-1, IL-6, TNF) and other enzymes (COX-2, LOX, MMP9, MAPK, mTOR, Akt).[16] Apoptosis related proteins (Bcl-2, DR, Fas, caspases) and the suppression of specific cytochrome P450 enzymes can be inhibited as well by the chemopreventive effects of Curc.[17,18]

Although Curc's low water solubility can lead to poor bioavailability coupled to fast metabolism and minimum chemical stability, the use of nanotechnology can successfully enhance Curc's efficiency for clinical use. Various drug delivery systems have been developed to overcome this problem. Some of them include polysaccharides, micelles, phospholipid complexes, liposomes and nanoparticles.[19-21] However, the chemical structure of Curc plays a vital role in its biological action.[22-24] Given that Curc is a polyphenol with two carbonyl groups (**Figure 1**), reports revealed that its rapid metabolism and deficient absorption are due to the presence of two carbonyl functional groups that severely affect its pharmacokinetics.[25-27]

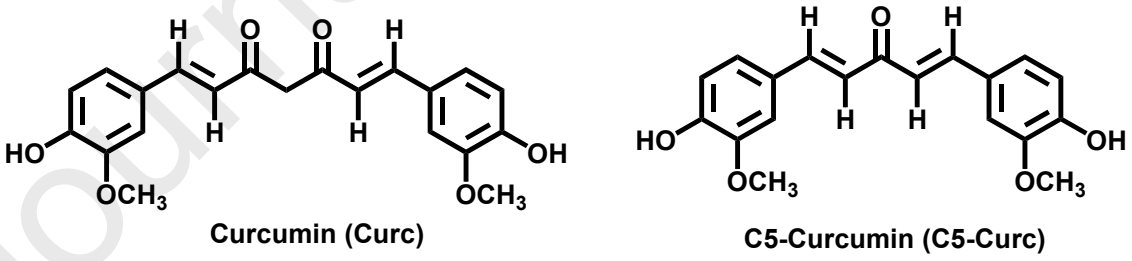


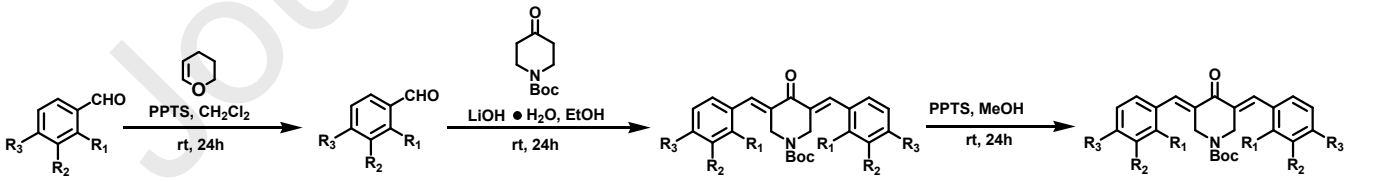
Figure 1. Chemical structures of Curc and C5-Curc.

In recent years, researchers have devoted their efforts systematically in modifying Curc's structure by the deletion of a methylene and the β-diketone moiety group to synthesize C5-Curcumin (C5-Curc, **Figure 1**) and other related analogs.[28] It was demonstrated that C5-Curcuminoids display an improved pharmacokinetics profile and increase anti-inflammatory activity *in vitro* without noticeable toxicity *in vivo* when compared to Curc.[29,30] To fully understand the mechanism of action of Curc and related analogs, careful structure activity

relationship (SAR) analyses in man-made monocarbonyl Curc analogues (MACs) have shown that (i) strong electron-withdrawing substituents at the 2' position may increase the molecule's bioactivity and the more electronegative is the moiety, the more cytotoxic is the compound; (ii) a weak electron-donating substituent at the 4' position is most favorable for the anti-tumor activity of the compound; and (iii) a strong electron-donating or withdrawing moiety may remove or reduce the bioactivity, respectively.[31] Moreover, compounds that are hexa-substituted can exhibit strong activities, with a 3,4,5-hexasubstitution resulting in the highest potency.[32]

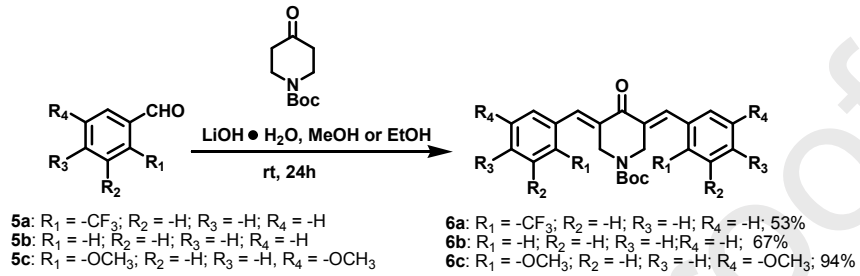
Previously, our group reported on a series of symmetrical MACs with cytotoxicity against colorectal cancer cells.[33] As a continuation of our study, exploration of new analogs were investigated, inspired by the synthetic equivalents made by Gandhy et. al.[34] They reported several 4-Boc-piperidone containing compounds, RL197 being the most active of the series, with very potent effects against colon cancer cells by downregulation of several transcription factors. In addition, they demonstrated that the presence of a *tert*-butoxycarbonyl (Boc) protecting group in the nitrogen atom of the chalcone's six-membered ring core is key for the cytotoxicity. In a recent study performed by Kálai and colleagues, it was demonstrated that the removal of N-acyl protecting group 2-fold decrease the anticancer activity of several N-acyl-3,5-bis (4-fluoro-benzylidene) piperidin-4-ones in three different cancer cell lines[35], which strongly support those Gandhy's results that showed that the presence of the Boc-protecting group is necessary in the design of new MACs containing the piperidone chalcone core. In the present study, six MACs with the Boc piperidone core were synthesized (five of them for the first time) with up to a three-step synthetic route. Their cytotoxicity and some mechanistic aspects were also examined using the highly-metastatic cancer cell lines LoVo, COLO-205, PC3, and 22RV1.

In this work, a recently synthetic methodology reported by Sanabria-Ríos et al.[33] was employed for the preparation of the new 4-Boc-piperidone chalcones (**Schemes 1** and **2)**. In **Scheme 1**, tetrahydropyranyl-protected benzaldehydes were added to a reaction mixture containing 4-Boc-piperidone and lithium hydroxide (LiOH) in ethanol. The resulting tetrahydropyranyl-protected 4-Boc-piperidone chalcones **3a**-**c** were treated with pyridinium p-toluene sulfonate (PPTS) in methanol obtaining the desired products **4a**-**c** in 44-65% yield.



Scheme 1. Total synthesis of 4-Boc-piperidone chalcones **4a**-**c**.

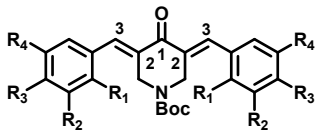
In **Scheme 2**, compounds **6a**-**c** were synthesized by reacting a substituted benzaldehyde and 4-Boc-piperidone with LiOH in either methanol or ethanol. Products **6a** and **6b** were obtained in 53 and 67% yield, respectively. In the case of **6c**, the compound was obtained in 94% yield demonstrating that the use of LiOH as a base was more effective than $NaOCH_3$ in methanol, an alkaline solution that was previously reported useful for the preparation of this product.[34]



Scheme 2. Synthetic approach towards 4-Boc-piperidone chalcones **6a**-**c**.

Both ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Brucker Avance 500 spectrophotometer. ^{1}H-NMR chemical shifts are reported relative to internal tetramethylsilane ($SiMe_4$, 7.26 ppm), while ^{13}C-NMR chemical shifts were reported in parts per million (ppm) relative to $CDCl_3$ (77.00 ppm). FT-IR data were generated on a Thermo Scientific Nicolet iS5 FT-IR spectrophotometer. Melting points for **4** and **6** were determined with a Melt-Temp apparatus and these measurements were uncorrected. **Table 1** summarizes the spectroscopic data obtained from NMR analyses. Detailed chemical characterization of compounds **4** and **6** is described in the Supporting Information (SI) section.

Table 1. ^{1}H-NMR and ^{13}C-NMR characterization of 4-Boc-piperidone chalcones **4** and **6**.[a,b,c]

					
	^{13}C-NMR chemical shift (ppm)			^{1}H-NMR chemical shift (ppm), *multiplicity*	Melting point (°C)
Compound	C1	C2	C3	H3	
4a	192.5	141.4	142.1	7.73, *s*	151-153
4b	190.8	141.4	142.1	7.74, *s*	102-104
4c	187.4	136.9	143.0	7.77, s	183-185
6a	186.8	137.6	143.6	7.72, *s*	90-96
6b	192.2	142.5	142.5	7.83, *m*	146-148
6c	187.7	143.5	143.9	7.97, *s*	164-166

[a] Signals from methyl groups from Boc-protecting groups were observed at 1.26-1.51 ppm in ^{1}H-NMR and 27.9-28.2 ppm in ^{13}C-NMR. Tertiary carbons from *t*-butyl groups were observed at 79.9-80.7 ppm in ^{13}C-NMR. Methylene groups from Boc-piperidone six-membered rings showed signals at 3.97-4.74 ppm in ^{1}H-NMR and 41.1-45.1 ppm in ^{13}C-NMR. Carbonyl groups from Boc-protecting groups showed signals at 154.0-156.5 ppm in ^{13}C-NMR. [b]Signals from methoxy groups in compounds **4b** and **6c** were observed at 3.49-3.83 ppm in ^{1}H-NMR and 55.8-56.0 ppm in ^{13}C-NMR. [c]Signals from aromatic groups were observed at 6.70-7.77 ppm in ^{1}H-NMR and 111.8-137.8 ppm in ^{13}C-NMR.

In ^{1}H-NMR, singlet signals were observed for **4** and **6** in the region of 7.73-7.98 ppm. These signals are characteristic of compounds containing an α, β-unsaturated carbonyl system[36] and demonstrates the success of the synthesis displayed in **Schemes 1** and **2**. In the case of **6b**, a multiplet at 7.83 ppm was also observed in ^{1}H-NMR, which suggests a long-range coupling between H3 and an aromatic hydrogen. In ^{13}C-NMR, signals that were generated by C1, C2, and C3 in the regions of 186.8-192.5 ppm (C1) and also, at 140.5-145.1 ppm (C2-C3) demonstrate the presence of the α, β-unsaturated carbonyl system, which are in accordance with the results from ^{1}H-NMR displayed in **Table 1**.

In 2014, Thakur and collaborators reported the total synthesis of novel 3,5-bis(arylidiene)-4-piperidone based monocarbonylanalogs of curcumin.[37] In the latter publication, the christallographic structure for the (3E,5E)-3,5-Bis-(2,5-difluoro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one was reported demostrating that this compound has an *E* double bond configuration. Because **6c** has a similar structure than the above-mentioned compound, it was compared the spectroscopic data of **6c** with the NMR data reported by Thakur et al.[37] In that study, it was reported a singlet at 7.66 ppm for the vinylic protons in the α, β unsaturated system, while a singlet at 7.97 ppm was observed for **6c**. These results are in agreement with results reported in the literature[34,37] and suggest that there is no long-range coupling between the vinylic protons and the aromatic protons of the aryl substituent as a possible consequence of free rotation of the sigma bond between the sp^2 carbon of the C=C and the sp^2 carbon of the aryl group. Because all vinylic protons showed a singlet in the region

of 7.72-7.97 (see **Table 1**) it is believed that the same phenomena described in the literature is taking place with all the compounds mentioned in **Table 1**. In addition, we were no able to observe the presence of any Z diasteroisomers in ^{13}C-NMR, specifically, in the region of 136-144 ppm, which is in agreement with those findings reported in the literature.[34,37]

Once the syntheses of **4** and **6** were carried out, the cytotoxicity of these compounds against highly-metastatic colorectal cancer cells were also determined by using the CellTiter-Glo® luminescent cell viability approach. In this methodology, the colorectal cancer cell lines LoVo and COLO-205 and the normal colorectal cell line CCD18Co were studied. A detailed description about luminescent cell viability assays is available in the Supplementary Information (SI) section. **Table 2** shows GI_{50} values obtained from CellTiter-Glo® cell viability assays.

Table 2. Cytotoxic activity of 4-Boc-piperidone chalcones **4** and **6** against highly-metastatic colorectal cancer cell lines.[a]

Compounds	**GI_{50} ± SEM, µg/mL[b]**		
	LoVo (SIn[c])	COLO-205 (SIn)	CCD18Co
4a	4.1 ± 1.0 (1.6)	11.7 ± 1.0 (0.5)	6.4 ± 1.0
4b	9.6 ± 1.0 (1.9)	27.4 ± 1.0 (0.7)	18.3 ± 1.0
4c	3.5 ± 1.1 (1.8)	20.9 ± 1.1 (0.3)	6.4 ± 0.3
6a	4.6 ± 1.0 (1.7)	4.5 ± 1.0 (1.7)	7.6 ± 1.0
6b	6.1 ± 1.1 (2.0)	34.7± 1.0 (0.4)	12.4 ± 1.0
6c	2.0 ± 0.2 (1.7)	0.84 ± 0.08 (1.4)	1.2 ± 0.1
C5-Curc[d]	10.0 ± 1.0 (1.5)	19.4 ± 1.9 (0.8)	15.1 ± 1.5
Curc[d]	4.3 ± 1.0 (1.7)	12.7 ± 1.0 (0.6)	7.2 ± 1.0
Cisplatin[d]	1.7 ± 0.1 (0.6)	6.8 ± 0.7 (0.2)	1.1 ± 0.1

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

[b]Results were reported as mean ± SEM from three determinations. GI_{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. 6.01 (GraphPad Software).

[c]SIn = Selectivity Index. SIn values were calculated as described by Sanabria-Ríos et al.[38]

[d]C5-Curc was synthesized as described by Liang et al.[28] C5-Curc, Curc, and cisplatin were used as reference compounds.

It can be noted that compounds **4** and **6** were more cytotoxic against LoVo cells than C5-Curc suggesting that the presence of the 4-Boc-piperidone core is needed for the cytotoxicity (**Table 2**). In the case of **4a**, **4c**, **6a**, and **6c**, these compounds displayed similar GI_{50} values as Curc or cisplatin, which makes them attractive for further studies in cancer research. In fact, these results suggest that the presence of an electron donating group, such as -OH, at either the *meta* or *para* position in **4a** or **4c**, or the presence of an electron withdrawing group, such as -CF_3, at the *ortho* position in **6a**, or the presence of methoxy groups at both *ortho* and *para* positions in **6c**, are needed for their cytotoxicity against LoVo cells. These findings are in agreement with SAR studies reported in the literature.[31]

Table 2 also revealed that compounds **4** and **6** were almost 2-fold more selective towards LoVo cells than towards the normal colorectal cell line CCD18Co. However, the opposite was

observed for COLO-205. Except for compounds **6a** and **6c**, almost all tested compounds, including the reference compounds, were more cytotoxic towards CCD18Co than towards COLO-205 suggesting that COLO-205 is more resistant towards anticancer drugs than LoVo. It can be appreciated from **Table 2** that the presence of a -CF_3 group at the *ortho* position in **6a** or methoxy groups at both *ortho* and *para* positions in **6c**, are pivotal for their cytotoxicity towards COLO-205 colorectal cancer cell lines. In general, the cytotoxicity of 4-Boc-piperidone chalcones against highly-metastatic colorectal cancer cells followed the order: **6c** > **6a** > **4a** > **4c**.

To further understand the mechanism of 4-Boc-piperidone chalcones, we employed Annexin V apoptosis analysis to determine whether **6a** or **6c** can induce apoptotic effect in COLO-205 cancer cells. Detailed description of Annexin V assays can be found in the SI section. **Figure 2** shows the apoptotic induction of **6a** and **6c** on human COLO-205 colorectal adenocarcinoma cells.

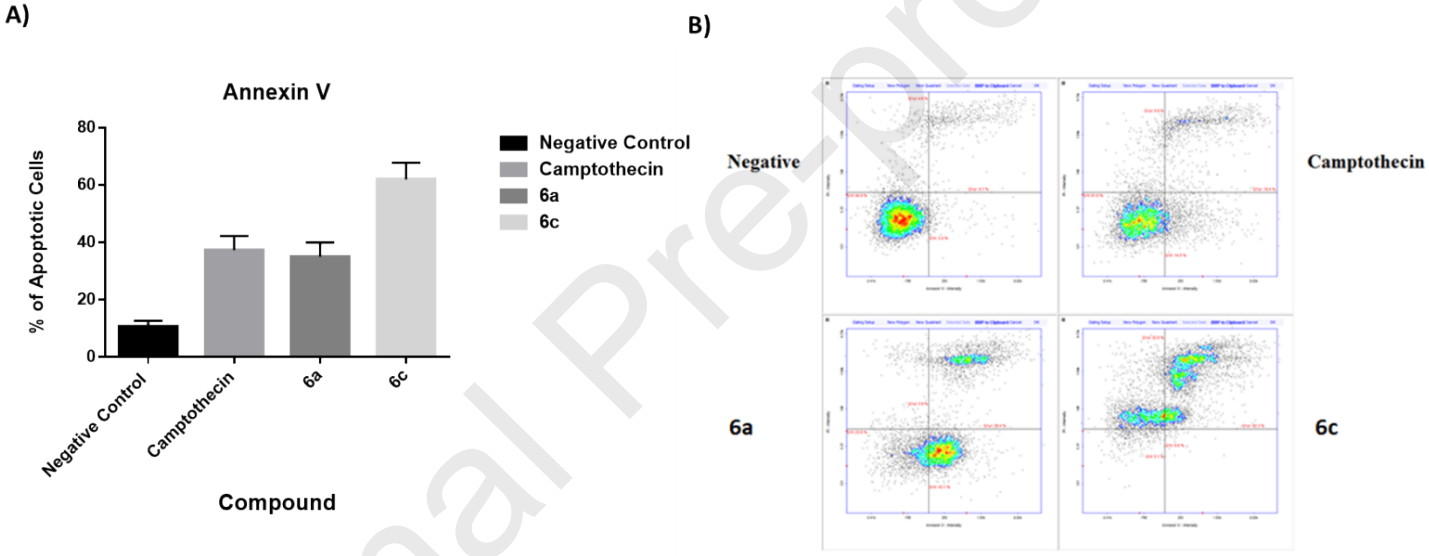


Figure 2. (**A**) Apoptotic induction comparison of **6a** and **6c** on human COLO-205 colorectal adenocarcinoma cells. Annexin V experiments for both **6a** and **6c** were performed at their corresponding GI_{50} displayed in **Table 2**. (**B**) The Annexin V Assay FACS showing the distribution of apoptotic COLO-205 cells treated with either **6a** or 6**c**.

Significant apoptotic induction of **6a** and **6c** was observed compared to the positive control Camptothecin. The negative control showed an average of 10.7% apoptotic cells, while the positive Camptothecin control presented an average of 37.3% apoptotic cells. Compound **6a** displayed an average of 35% of apoptotic cells, while **6c** presented the highest average with 62% apoptotic cells, considerably higher than previously reported with Curc (27.5%).[33]

The activation of caspases such as caspase 3 and 7 (known as effector caspases), are important components of the apoptotic process.[39] These enzymes can be activated by signaling caspases such casp 8 and 9 and participate in the activation of enzymatic degradation of cell structures and content.[39] Therefore, it is possible that compounds **6a** and **6c** can induce

apoptosis through the inhibition of effector caspases. For this reason, it was carried out *in vitro* assays using the fluorescent inhibitor probe FAM-DEVD-FMK to label active caspases 3 and 7 enzymes in COLO-205. Results from these assays are displayed in **Figure 3**.

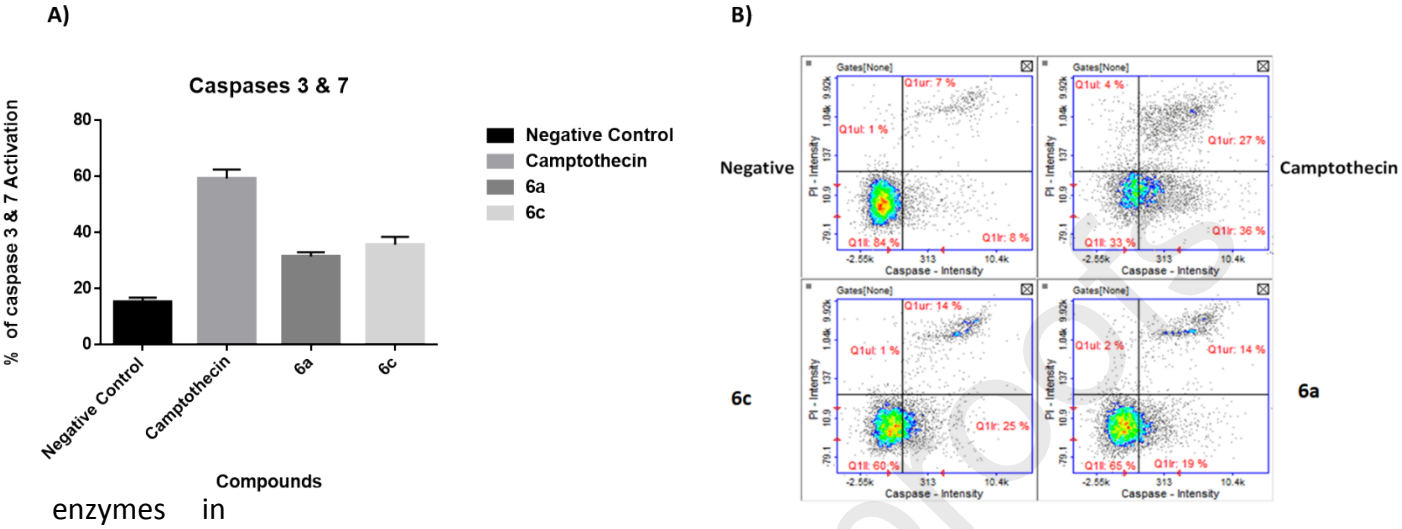


Figure 3. (**A**) Histograms showing the activation effect of compounds **6a** and **6c** on caspases 3 & 7. Annexin V experiments for both **6a** and **6c** were performed at their corresponding GI_{50} displayed in **Table 2.** Experiments were carried out in triplicate (N = 3). (**B**) FACS plot showing distribution of apoptotic COLO-205 cells treated with either **6a** and **6c**.

Results in **Figure 3** reveal that the activation of caspases 3 & 7 is statistically significant ($P < 0.05$) when compared to the negative vehicle control. The negative control (no treatment) presented an average of 15.3% of cells undergoing apoptosis, while the positive control (Camptothecin) presented an average of 59.3%. Compounds **6a** and **6c** presented an average of 31.5% and 35.7%, respectively (**Figure 3**). The results above are in accordance with results in **Figure 2**, which revealed significant amounts of cells undergoing apoptosis as caspase 3 & 7 activation is closely associated with apoptotic cell death.

Previously, Sanabria-Ríos and colleagues synthesized C5-curcumin-fatty acid (C5-Curc-FA) conjugates to establish the structure-activity relationship (SAR) of their anticancer activity.[33] Interestingly, they found that one of the conjugates, C5-Curc-decanoic acid (DA), inhibits the activity of NFκB at an GI_{50} of 18.2 µg/mL.[33] Moreover, they showed that C5-Curc-DA causes an apoptotic effect at a GI_{50} of 46.0 µg/mL in the colorectal adenocarcinoma cell line COLO-205, which is likely due to the observed significant mitochondrial membrane permeabilization, and caspase 3 and 7 activation, that occurs following C5-Curc-DA treatment. Based on these findings, it is hypothesized that the 4-Boc-piperidone chalcones will also inhibit NFκB activity. To test this hypothesis, we carried out NFκB luciferase reporter assays aimed at determining the inhibitory effects of **6a** on the activity of NFκB. Further description of NFκB luciferace reporter assays is available in the SI.

Results displayed in **Figure 4** show the inhibitory effect of **6a** on the reporter activity of NFκB. It can be observed that the luminescence properties, after treatment with **6a**, showed a sigmoidal response associated with the logarithm of the concentration of the chalcone. This behavior was also observed for Curc, a well-known inhibitor of NFκB.[40-43] The fact that Curc is 3.5-fold higher than **6a** suggests that the presence of a monocarbonyl moiety and the presence of -CF_3 groups are influencing the inhibition of the reporter activity of NFκB. The results above are important because the NFκB pathway controls the genes that are linked to 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.[44] Apoptosis results displayed in **Figure 2**, in combination with the ones of **Figure 4**, do not discard the hypothesis that the inhibition of NFκB pathway can be also involved in the apoptosis effect of 4-Boc-piperidone chalcones in COLO-205.

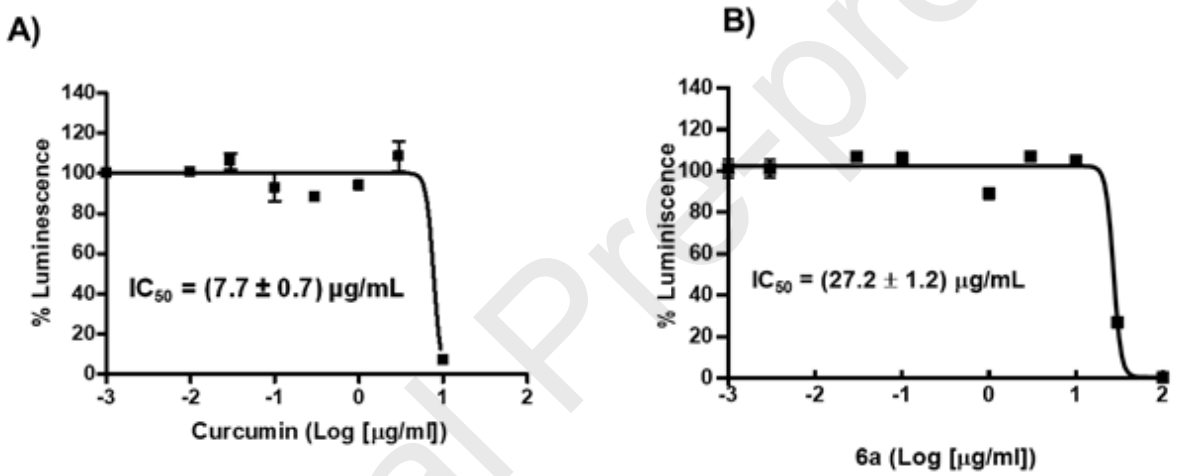


Figure 4. Drug-response curves showing the inhibitory effect of both Curc and **6a** on the NFκB Reporter-HEK293 cells. Experiments were performed in triplicate (N=3). Results were reported as mean ± SEM from three determinations. Non-linear regression analysis was performed with GraphPad Prism v. 6.01 (GraphPad Software). The IC_{50} values were determined by the concentration causing a half-maximal percent activity.

Intended to further explore the potential of 4-Boc-piperidone chalcones as cytotoxic agents against highly-metastatic cancer cell lines, the MTS approach was employed for determining the cytotoxicity of these compounds on PC3 and 22RV1 cells. Compound **4c** was tried first because it displayed significant cytotoxicity against LoVo cells (**Table 2**).

Figure 5 shows the cytotoxic effect of **4c** and related analogs on the cell viability of PC3 and 22RV1. It was observed that C5-Curc significantly decreased cell viability in PC3 and 22RV1 cells, when compared to the control (DMSO). At similar ranges of concentrations than C5-Curc, **4c** showed a significant decrease ($P < 0.05$) in the cell viability when compared with the control (DMSO) in 22RV1 cells, with GI_{50} lower than PC3 cells, suggesting that 22RV1 is more susceptible towards **4c**. In the case of Curc, this compound significantly decreased the cell

viability in both PC3 cells and 22RV1 cells when compared to the control (DMSO) at similar concentration range of **4c**. Significant cytotoxicity was observed as well when both PC3 and 22RV1 were treated with Dxtl ($P < 0.05$). In both prostate cancer cell lines, the GI_{50} values were remarkably lower than **4c** and other analogs.

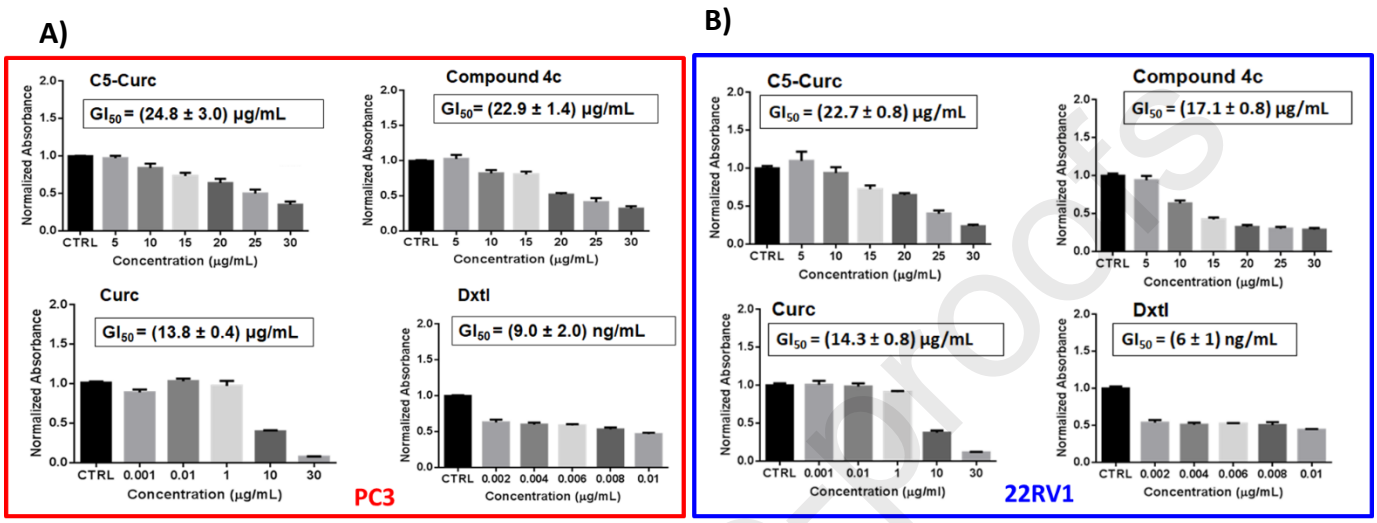


Figure 5. Inhibitory effect of **4c** and related analogs on the cell viability of both PC3 (**A**) and 22RV1 (**B**). PC3 and 22RV1 cells were treated with **4c** at concentrations ranging from 5 to 30 µg/mL. C5-Curc, Curcumin, and Docetaxel (Dxtl) were used as reference compounds. PC3 and 22RV1 were treated with C5-Curc at concentrations ranging from 5 to 30 µg/mL, Curcumin at concentrations ranging from 0.001 to 30 µg/mL and Dxtl at concentrations that range from 0.002 to 0.01 µg/mL. Cell viability was determined after 48h of incubation with the MTS reagent (see SI section). Experiments were performed in triplicates (N=3). Statistical analysis was performed using One-way ANOVA, followed by Dunnett's test. Mean ± SEM with $P<0.05$ was statistically significant. Graphs and statistical analyses were performed with GraphPad Prism v. 6.01 (GraphPad Software).

Cytotoxic activity of organic compounds can be explained by several mechanisms, one of them being cellular proliferation. Cellular proliferation is key in cancer development and progression, particularly in prostatic diseases.[45,46] This mechanism can be analyzed by using Ki67 antigen, a marker that works against nuclear antigens in specific phases in the cell cycle.[47,48] In the present study, immunofluorescence assays were performed to determine whether **4c** is able to decrease cell proliferation in both PC3 and 22RV1 after 48h of exposition.

Results in **Figure 6** show that **4c** decreased cell proliferation in both PC3 and 22RV1. In PC3, **4c** was the only compound that significantly inhibited cell proliferation when compared to control (DMSO), Curc, C5-Curc, and Dxtl. In 22RV1, all compounds tested (i.e. **4c**, Curc, C5-Curc, and Dxtl) inhibited the Ki67 protein expression. However, Dxtl was the compound that better inhibited the Ki67 protein expression (**Figure 6B**). These results clearly demonstrate the potential of 4-Boc-piperidone chalcones as cytotoxic agents against highly metastatic prostate

cancer cells, and are similar to previous studies addressing the synthesis of Curc analogs to inhibit the proliferation of prostate cancer cells.[49]

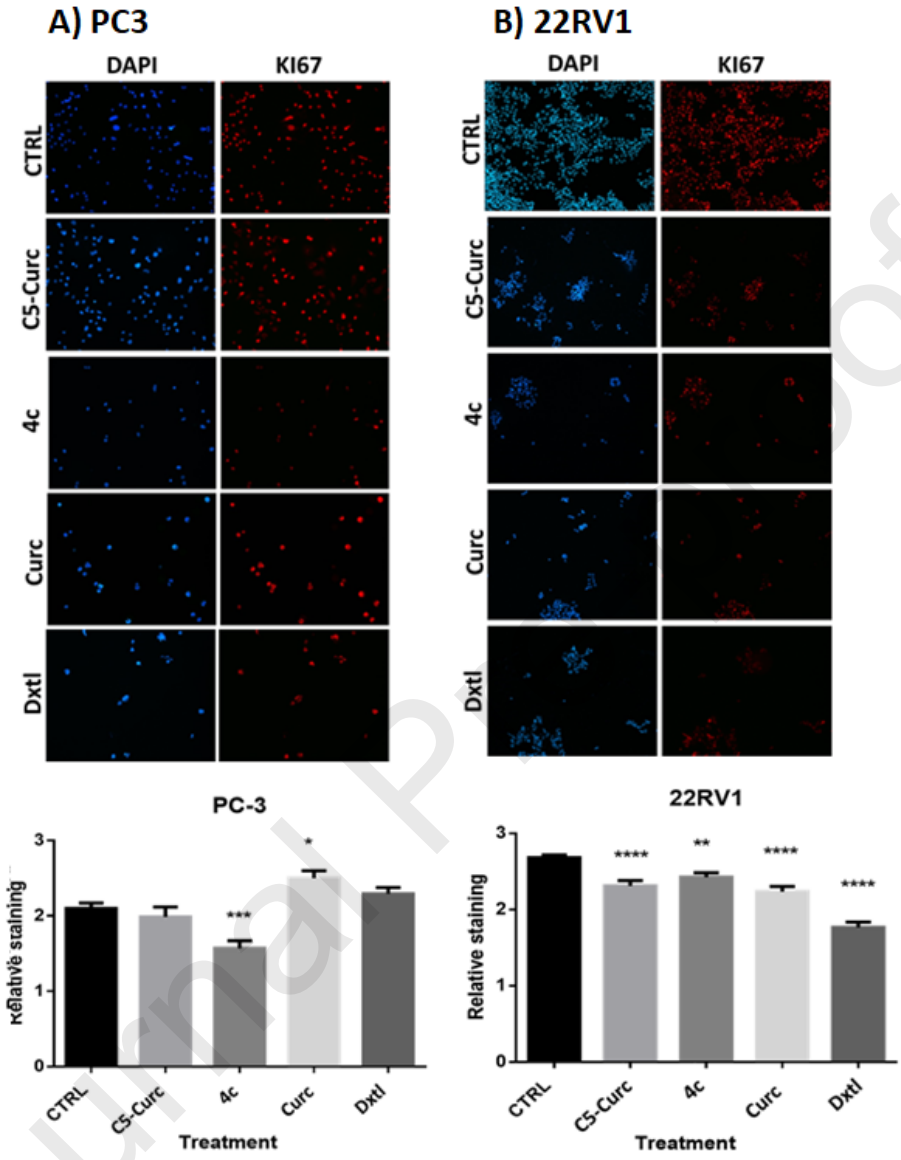


Figure 6. Inhibitory effect on the proliferation activity (Ki67 antibody) in treated PC3 (A) or 22RV1 (B) cells with **4c** after 48h of incubation. Immunofluorescence assay was performed to determine Ki67 activity. PC3 cells were treated with **4c** (22.9 µg/mL), C5-curc (24.8 µg/mL), Curc (13.8 µg/mL) and Dxtl (9 ng/mL); while 22RV1 cells were treated with **4c** (17.1 µg/mL), C5-Curc (22.7 µg/mL), Curc (14.3 µg/mL), and Dxtl (6 ng/mL). Representative images of Ki67 protein staining PC3 or 22RV1 cells treated with **4c**, Curc, C5-Curc, and Dxtl. Nuclei are stained with blue DAPI (left panels in **Figures 5A** and **5B**) and Ki67 protein is stained in red (right panels in **Figures 6A** and **6B**). Statistical analysis shows that Ki67 activity was decreased with **4c** when compared to control (DMSO), Curc, and C5-Curc. Dxtl decreased Ki67 protein expression more efficiently when compared to control (DMSO), **4c**, C5-Curc, and Curc. Statistical analysis was performed

using One-way ANOVA, followed by Dunnett's test. Mean ± SEM (*P<0.05). Experiments were performed in triplicates (N=3).

Since, the ability of **6a**, a synthetic analog of **4c**, in inhibiting the reporter activity of NFκB in HEK293 cells (**Figure 4**) was demonstrated, we hypothesized that the cytotoxic activity of **4c** against PC3 and 22RV1 can be linked to the inhibition of NFκB. To test this hypothesis, immunofluorescence assays were carried out to determine whether **4c** inhibits the translocation of NFκB in the nucleus of both PC3 and 22RV1. For these assays, primary antibodies against NFκB were used, as well as secondary anti-mouse, and DAPI for staining the nucleus of both PC3 and 22RV1 prostate cancer cell lines (see SI section for further details).

Our results show that **4c** significantly decrease the activity of NFκB in PC3 cells and 22RV1 cells (**Figure 7**) when compared to control (DMSO), Curc, and C5-Curc (compounds that most significantly inhibited the activity of NFκB). Curc only showed significant difference when compared to control (DMSO) in PC3 cells (**Figure 7A**). In the case of Dxtl, this compound significantly inhibited the activity of NFκB in both prostate cancer cells when compared to control (DMSO). Results in **Figure 7** agree with findings from NFκB reporter activity assays displayed in **Figure 4**. In addition, these results strongly support the hypothesis that the inhibition of NFκB can be involved in the cytotoxicity activity of **4c** in either PC3 or 22RV1 prostate cancer cells.

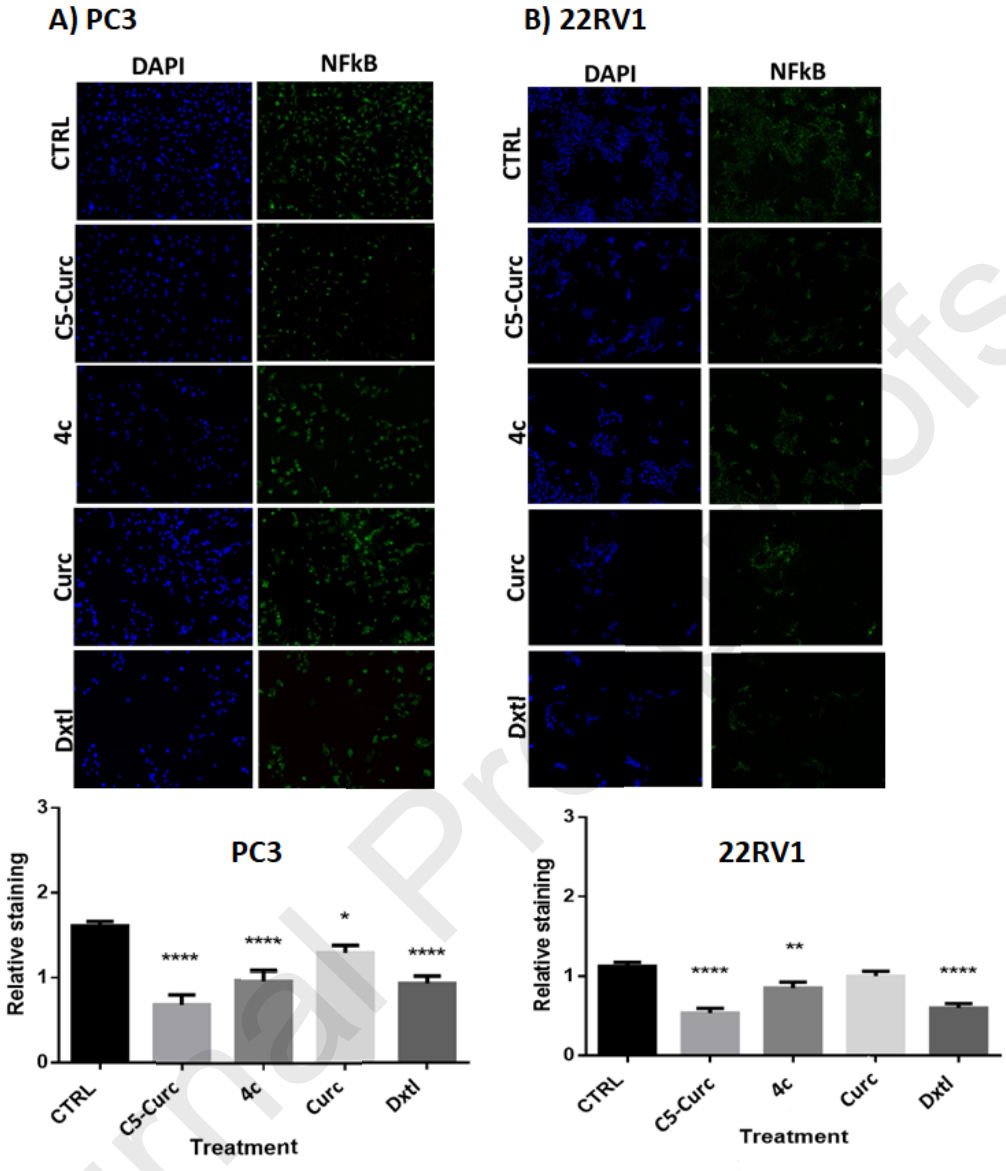


Figure 7. Inhibitory effect on the activity of NFκB in both PC3 (A) and 22RV1(B) cells after treatment with **4c**, C5-Curc, Curc, and Dxtl at 48h of incubation**.** Immunofluorescence assay was performed to determine NFκB inhibitory activity. PC3 cells were treated with **4c** (22.9 µg/mL), C5-curc (24.8 µg/mL), Curc (13.8 µg/mL) and Dxtl (9 ng/mL); while 22RV1 cells were treated with **4c** (17.1 µg/mL), C5-Curc (22.7 µg/mL), Curc (14.3 µg/mL), and Dxtl (6 ng/mL). Representative images of NFκB staining PC3 and 22RV1 treated with **4c**, C5-Curc, Curc and Dxtl. Nuclei were stained with blue DAPI (left panels in **Figures 7A** and **7B**) and NFκB is stained in green (right panels in **Figures 7A** and **7B**). Statistical analysis showed that NFκB activity was decreased with **4c** treatment when compared to control (DMSO) and Curc and C5-Curc. Statistical analysis was performed using One-way ANOVA, followed by Dunnett's test. Mean ± SEM (*P<0.05). Experiments were performed in triplicates (N=3).

The NFkB immune-fluorescence activity results in **Figure 7** were confirmed by using the PathScan® Total NFkB p65 Sandwich ELISA kit (Cell Signaling Technology, Danvers, MA). This methodology consists of a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that allows the detection and quantification of endogenous levels of total NFkB p65 protein in either PC3 or 22RV1. **Figure 8** shows the inhibitory activity of **4c**, C5-Curc, Curc, and Dxtl on the expression of NFkB in both PC3 and 22RV1. It can be observed in **Figure 8A**, that both **4c** and Curc (positive control) displayed a significant inhibitory effect on the transcriptional activity of NFkB when compared to the control (P <0.0001). Also, it is appreciated that Dxtl displayed decreased expression of NFkB than Dxtl in PC3. Moreover, we observed that **4c**, C5-Curc, and Dxtl displayed inhibitory activity on the expression of NFkB in 22RV1 (**Figure 8B**), which are comparable with those findings displayed in **Figure 7**.

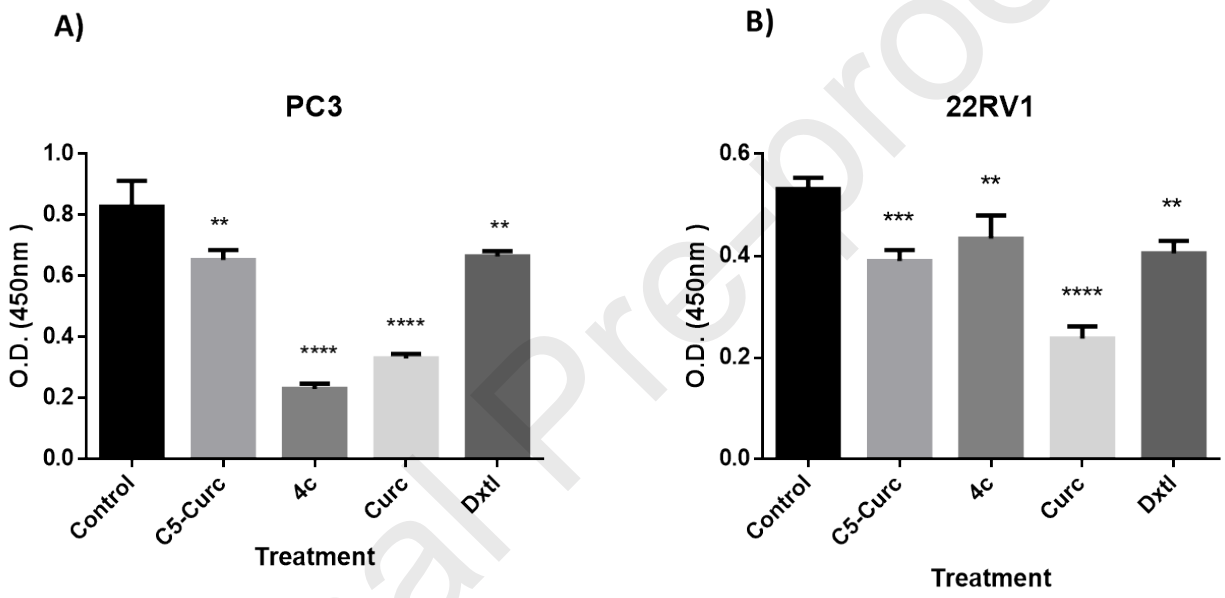


Figure 8. Histograms showing inhibitory effect of **4c**, C5-Curc, Curc, and Dxtl on the transcriptional activity of NFkB p65 in both PC3 (**A**) and 22RV1 (**B**). Both prostate cancer cell lines were exposed to the above-mentioned treatments at their corresponding GI_{50}'s reported in **Figure 5**. Experiments were performed in triplicate (N=3) using protocols that were provided by the manufacturer. Statistical analysis was performed using One-way ANOVA followed by the Dunnett's multiple comparisons test. Results were reported as mean ± SEM and experimental means were compared with the control. (**) = P<0.01, (***) = P < 0.001, (****) = P < 0.0001.

In summary, our results clearly demonstrated that **4** and **6** are promising for further investigations addressing the development of valuable cytotoxic agents against highly-metastatic cancer cells. In the case of **6a** and **6c**, these compounds displayed potent cytotoxicity as cisplatin towards LoVo and COLO-205 cells but less toxicity towards the normal colorectal CCD18Co cells. In addition, the cytotoxicity of **6a** and **6c** can be linked to their ability to induce apoptosis or inhibit the activity of NFκB. Moreover, we gathered preliminary results that suggest that **4c** displayed cytotoxicity towards highly-metastatic PC3 and 22RV1 prostate cancer cell lines by decreasing cell proliferation and the activity of NFκB. To better understand the

cytotoxicity of **4** and **6** in both colorectal and prostate cancer cells, several biological studies are already being pursued.

Acknowledgements

This project was supported by the National Center for Research Resources and the National Institute of General Medical Sciences of the National Institutes of Health through Grant Number P20GM103475-16. Dr. D.J. Sanabria-Ríos would like to thank the IAUPR-MC Seed Money for sponsoring part of this project. Special thanks to the Materials Characterization Center (MCC) for the use of NMR and to Dr. Christian Morales, Ms. Denisse Alequin-Torres, and Ms. Ashley Rivera for assisting in the chemical characterization in this study. Mr. Joseph Mooney and Mr. Tomás Pereles-De León acknowledge the support of the PR-INBRE program for the undergraduate research fellowship provided. Dr. M. Martínez-Ferrer thanks to the financial support provided by the University of Puerto Rico Comprehensive Cancer Center to perform part of this work involving highly-metastatic prostate cancer cells (PC3 and 22RV1). Finally, we want to acknowledge Dr. Denniss Phillips and the Proteomics and Mass Spectrometry Core Facility at University of Georgia for carrying out ESI-HRMS experiments. Also, we acknowledge Crown Bioscience, Inc., and BPS Bioscience, Inc. for their assistance in performing cytotoxicity assays (involving LoVo, COLO-205, and CCD18Co cells), and conducting NFκB activity studies, respectively.

References

(1) American Cancer Society. Cancer Facts & Figures 2018. Atlanta: American Cancer Society; 2018.

(2) Ulla, M. F. Cancer multidrug resistance (MDR): A major impediment to effective chemotherapy. *Asian Pac. J. Cancer Prev.* **2008**, *9* (1), 1.

(3) Hussain, H. E. Hypoglycemic, hypolidemic and antioxidant properties of combination of curcumin from Curcuma longa, Linn. in streptozotocin induced diabetes. *Ind. J. Clin. Biochem.* **2002**, *17* (2), 33.

(4) Xu, P.; Long, Y.; Dai, F.; Liu, Z. The relaxant effect of curcumin on porcine coronary arterial ring segments. *Vascular Pharmacology* **2007**, *47* (1), 25.

(5) Mukhopadhyay, A.; Basu, N.; Ghatak, N.; Gujral, P. K. Anti-inflammatory and irritant activities of curcumin analogues in rats. *Agents and Actions* **1982**, *12* (4), 508.

(6) Moghadamtousi, S. Z.; Kadir, H. A.; Hassandarvish, P.; Tajik, H.; Abubakar, S.; Zandi, K. A Review on antibacterial, antiviral, and antifungal activity of curcumin. *BioMed Res. Int.* **2014**, *2014*, 12.

(7) Gupta, S. C.; Patchva, S.; Aggarwal, B. B. Therapeutic roles of curcumin: Lessons learned from clinical trials. *AAPS J.* **2013**, *15* (1), 195.

(8) Song, K.; Peng, S.; Sun, Z.; Li, H.; Yang, R. Curcumin suppresses TGF-β signaling by inhibition of TGIF degradation in scleroderma fibroblasts. *Biochem. Biophys. Res. Commun.* **2011**, *411* (4), 821.

(9) Ammon, H. P. T.; Safayhi, H.; Mack, T.; Sabieraj, J. Mechanism of antiinflammatory actions of curcumine and boswellic acids. *Journal of Ethnopharmacology* **1993**, *38* (2), 105.

(10) Reddy, A. C. P.; Lokesh, B. R. Studies on anti-Inflammatory activity of spice principles and dietary n-3 polyunsaturated fatty acids on carrageenan-induced inflammation in rats. *Annals of Nutrition and Metabolism* **1994**, *38* (6), 349.

(11) Xu, Y. X.; Pindolia, K. R.; Janakiraman, N.; Noth, C. J.; Chapman, R. A.; Gautam, S. C. Curcumin, a compound with anti-inflammatory and anti-oxidant properties, down-regulates chemokine expression in bone marrow stromal cells. *Exp. Hematol.* **1997**, *25* (5), 413.

(12) Banerjee, M.; Tripathi, L. M.; Srivastava, V. M.; Puri, A.; Shukla, R. Modulation of inflammatory mediators by ibuprofen and curcumin treatment during chronic inflammation in rat. . *Immunopharmacol. Immunotoxicol.* **2003**, *25* (2), 213.

(13) Lantz, R. C.; Chen, G. J.; Solyom, A. M.; Jolad, S. D.; Timmermann, B. N. The effect of turmeric extracts on inflammatory mediator production. *Phytomedicine : international journal of phytotherapy and phytopharmacology* **2005**, *12* (6), 445.

(14) Chadalapaka, G.; Jutooru, I.; Chintharlapalli, S.; Papineni, S.; Smith, R.; Li, X.; Safe, S. Curcumin decreases specificity protein expression in bladder cancer cells. *Cancer Res.* **2008**, *68* (13), 5345.

(15) Shanmugam, M.; Rane, G.; Kanchi, M.; Arfuso, F.; Chinnathambi, A.; Zayed, M.; Alharbi, S.; Tan, B.; Kumar, A.; Sethi, G. The multifaceted role of curcumin in cancer prevention and treatment. *Molecules* **2015**, *20* (2), 2728.

(16) Shishodia, S. Molecular mechanisms of curcumin action: Gene expression. *BioFactors* **2013**, *39* (1), 37.
(17) Aggarwal, S.; Takada, Y.; Singh, S.; Myers, J. N.; Aggarwal, B. B. Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor-κB signaling. *Int. J. Cancer* **2004**, *111* (5), 679.
(18) Choi, H.; Chun, Y.; Shin, Y.; Ye, S.; Kim, M.; Park, J. Curcumin attenuates cytochrome P450 induction in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin by ROS-dependently degrading AhR and ARNT. *Cancer Sci.* **2008**, *99* (12), 2518.
(19) Chang, P. Y.; Peng, S. F.; Lee, C. Y.; Lu, C. C.; Tsai, S. C.; Shieh, T. M.; Wu, T. S.; Tu, M. G.; Chen, M. Y.; Yang, J. S. Curcumin-loaded nanoparticles induce apoptotic cell death through regulation of the function of MDR1 and reactive oxygen species in cisplatin-resistant CAR human oral cancer cells. *Int. J. Oncol.* **2013**, *43* (4), 1141.
(20) Dende, C.; Meena, J.; Nagarajan, P.; Nagaraj, V. A.; Panda, A. K.; Padmanaban, G. Nanocurcumin is superior to native curcumin in preventing degenerative changes in experimental cerebral Malaria. *Sci. Rep.* **2017**, *7*, 10062.
(21) Shome, S.; Talukdar, A. D.; Choudhury, M. D.; Bhattacharya, M. K.; Upadhyaya, H. Curcumin as potential therapeutic natural product: a nanobiotechnological perspective. *J. Pharm. Pharmacol.* **2016**, *68* (12), 1481.
(22) Straganz, G. D.; Glieder, A.; Brecker, L.; Ribbons, D. W.; Steiner, W. Acetylacetone-cleaving enzyme Dke1: A novel C-C-bond-cleaving enzyme from Acinetobacter johnsonii. *The Biochemical journal* **2003**, *369*, 573.
(23) Rosemond, M. J.; St John-Williams, L.; Yamaguchi, T.; Fujishita, T.; Walsh, J. S. Enzymology of a carbonyl reduction clearance pathway for the HIV integrase inhibitor, S-1360: role of human liver cytosolic aldo-keto reductases. *Chemico-biological interactions* **2004**, *147* (2), 129.
(24) Grogan, G. Emergent mechanistic diversity of enzyme-catalysed beta-diketone cleavage. *The Biochemical journal* **2005**, *388*, 721.
(25) Yang, C.; Su, X.; Liu, A.; Zhang, L.; Yu, A.; Xi, Y.; Zhai, G. Advances in clinical study of curcumin. *Curr.Pharm. Des.* **2013**, *19* (11), 1966.
(26) Aggarwal, B. B.; Sung, B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. *Trends Pharmacol. Sci.* **2009**, *30* (2), 85.
(27) Anand, P.; Kunnumakkara, A. B.; Newman, R. A.; Aggarwal, B. B. Bioavailability of curcumin: Problems and promises. *Mol. Pharm.* **2007**, *4* (6), 807.
(28) Liang, G.; Yang, S.; Jiang, L.; Zhao, Y.; Shao, L.; Xiao, J.; Ye, F.; Li, Y.; Li, X. Synthesis and anti-bacterial properties of mono-carbonyl analogues of curcumin. *Chem. Pharm. Bull.* **2008**, *56* (2), 162.
(29) Vyas, A.; Dandawate, P.; Padhye, S.; Ahmad, A.; Sarkar, F. Perspectives on new synthetic curcumin analogs and their potential anticancer properties. *Curr.Pharm. Des.* **2013**, *19* (11), 2047.
(30) Yin, S.; Zheng, X.; Yao, X.; Wang, Y.; Liao, D. Synthesis and anticancer activity of mono-carbonyl analogues of curcumin. *Journal of Cancer Therapy* **2013**, *4* (1), 113.
(31) Liang, G.; Shao, L.; Wang, Y.; Zhao, C.; Chu, Y.; Xiao, J.; Zhao, Y.; Li, X.; Yang, S. Exploration and synthesis of curcumin analogues with improved structural stability both in vitro and in vivo as cytotoxic agents. *Bioorg. Med. Chem.* **2009**, *17* (6), 2623.

(32) Yamakoshi, H.; Ohori, H.; Kudo, C.; Sato, A.; Kanoh, N.; Ishioka, C.; Shibata, H.; Iwabuchi, Y. Structure-activity relationship of C5-curcuminoids and synthesis of their molecular probes thereof. *Bioorg. Med. Chem.* **2010**, *18* (3), 1083.

(33) Sanabria-Rios, D. J.; Rivera-Torres, Y.; Rosario, J.; Rios, C.; Gutierrez, R.; Carballeira, N. M.; Velez, C.; Zayas, B.; Alvarez-Colon, F.; Ortiz-Soto, G.; Serrano, V.; Altieri-Rivera, J.; Rios-Olivares, E.; Rodriguez, J. W. Synthesis of novel C5-curcuminoid-fatty acid conjugates and mechanistic investigation of their anticancer activity. *Bioorg Med Chem Lett.* **2015**, *25* (10), 2174.

(34) Gandhy, S. U.; Kim, K.; Larsen, L.; Rosengren, R. J.; Safe, S. Curcumin and synthetic analogs induce reactive oxygen species and decreases specificity protein (Sp) transcription factors by targeting microRNAs. *BMC cancer* **2012**, *12*, 564.

(35) Kálai, T.; Kuppusamy, M. L.; Balog, M.; Selvendiran, K.; Rivera, B. K.; Kuppusamy, P.; Hideg, K. Synthesis of N-substituted 3,5-bis(arylidene)-4-piperidones with high antitumor and antioxidant activity. *J. Med. Chem.* **2011**, *54* (15), 5414.

(36) Lambert, J. B.; Shurvell, H. F.; Lightner, D. A.; Graham Cooks, R. *Organic Structural Spectroscopy*; 1st ed.; Prentice Hall: New Jersey, 1998.

(37) Thakur, A.; Manohar, S.; Vélez Gerena, C. E.; Zayas, B.; Kumar, V.; Malhotra, S. V.; Rawat, D. S. Novel 3,5-bis(arylidiene)-4-piperidone based monocarbonyl analogs of curcumin: anticancer activity evaluation and mode of action study. *Med. Chem. Commun.* **2014**, *5*, 576.

(38) Sanabria-Rios, D. J.; Rivera-Torres, Y.; Maldonado-Dominguez, G.; Dominguez, I.; Rios, C.; Diaz, D.; Rodriguez, J. W.; Altieri-Rivera, J. S.; Rios-Olivares, E.; Cintron, G.; Montano, N.; Carballeira, N. M. Antibacterial activity of 2-alkynoic fatty acids against multidrug-resistant bacteria. *Chem. Phys. Lipids* **2014**, *178*, 84.

(39) Pfeffer, C. M.; Singh, A. T. K. Apoptosis: A target for anticancer therapy. *Int. J. Mol. Sci.* **2018**, *19* (2), 448.

(40) Aggarwal, B. B.; Kumar, A.; Bharti, A. C. Anticancer potential of curcumin: Preclinical and clinical studies. *Anticancer research* **2003**, *23* (1A), 363.

(41) Aggarwal, S.; Ichikawa, H.; Takada, Y.; Sandur, S. K.; Shishodia, S.; Aggarwal, B. B. Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IkappaBalpha kinase and Akt activation. *Molecular pharmacology* **2006**, *69* (1), 195.

(42) Tharakan, S. T.; Inamoto, T.; Sung, B.; Aggarwal, B. B.; Kamat, A. M. Curcumin potentiates the antitumor effects of gemcitabine in an orthotopic model of human bladder cancer through suppression of proliferative and angiogenic biomarkers. *Biochem. Pharmacol.* **2010**, *79* (2), 218.

(43) Prakobwong, S.; Khoontawad, J.; Yongvanit, P.; Pairojkul, C.; Hiraku, Y.; Sithithaworn, P.; Pinlaor, P.; Aggarwal, B. B.; Pinlaor, S. Curcumin decreases cholangiocarcinogenesis in hamsters by suppressing inflammation-mediated molecular events related to multistep carcinogenesis. *Int. J. Cancer* **2011**, *129* (1), 88.

(44) Yamamoto, Y.; Gaynor, R. B. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *The Journal of clinical investigation* **2001**, *107* (2), 135.

(45) Feitelson, M. A.; Arzumanyan, A.; Kulathinal, R. J.; Blain, S. W.; Holcombe, R. F.; Mahajna, J.; Marino, M.; Martinez-Chantar, M. L.; Nawroth, R.; Sanchez-Garcia, I.; Sharma, D.; Saxena, N. K.; Singh, N.; Vlachostergios, P. J.; Guo, S.; Honoki, K.; Fujii, H.; Georgakilas, A. G.; Bilsland, A.; Amedei, A.; Niccolai, E.; Amin, A.; Ashraf, S. S.; Boosani, C. S.; Guha, G.; Ciriolo, M. R.; Aquilano, K.; Chen, S.; Mohammed, S. I.; Azmi, A. S.; Bhakta, D.; Halicka, D.; Keith, W. N.; Nowsheen, S. Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. *Seminars in Cancer Biology* **2015**, *35*, S25.

(46) Housman, G.; Byler, S.; Heerboth, S.; Lapinska, K.; Longacre, M.; Snyder, N.; Sarkar, S. Drug resistance in cancer: An overview. *Cancers* **2014**, *6* (3), 1769.

(47) Feneley, M. R.; Young, M. P.; Chinyama, C.; Kirby, R. S.; Parkinson, M. C. Ki-67 expression in early prostate cancer and associated pathological lesions. *Journal of Clinical Pathology* **1996**, *49* (9), 741.

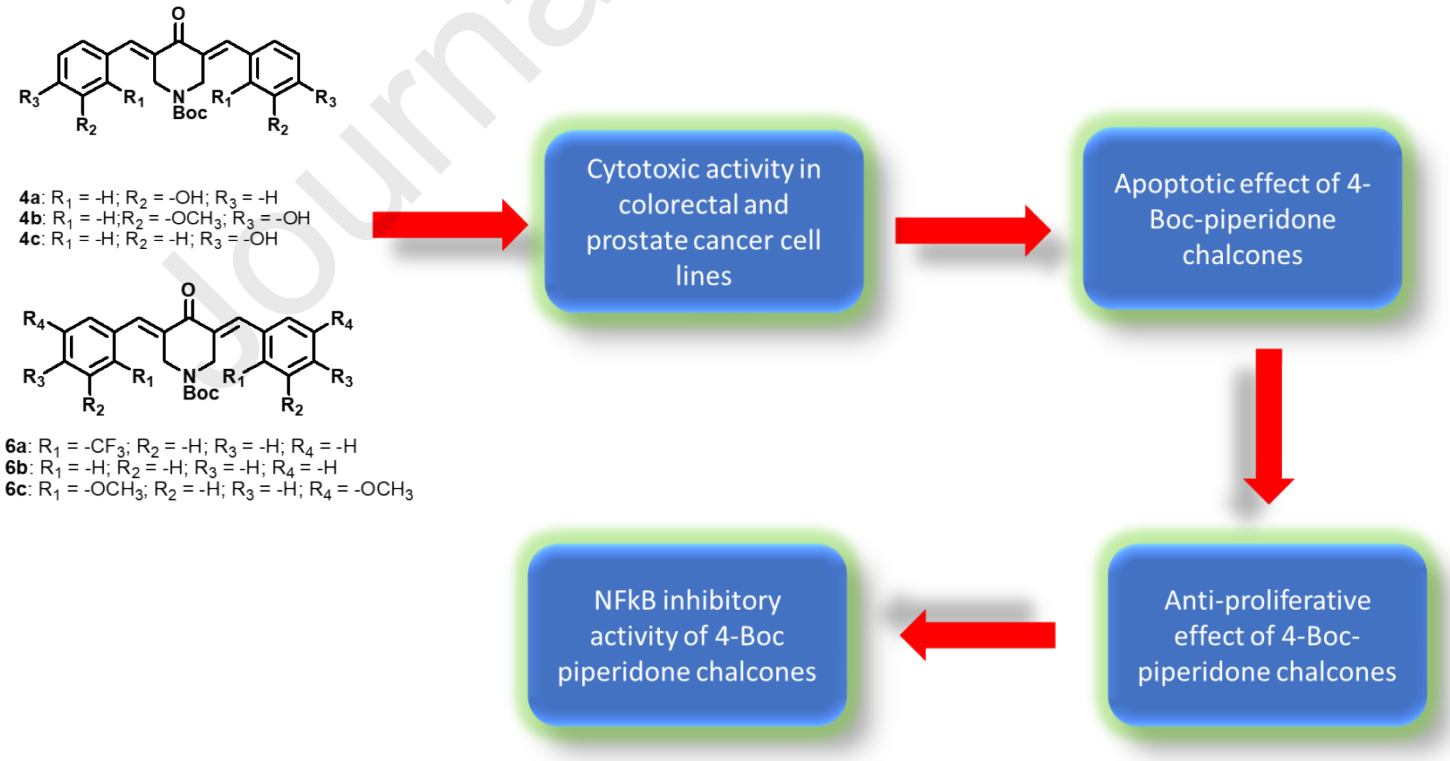
(48) Sulik, M.; Maruszak, K.; Puchalska, J.; Misiunkiewicz-Poc, M. Expression of Ki-67 as a proliferation marker in prostate cancer. *Pol. Ann. Med.* **2011**, *18* (1), 12.

(49) Zhou, D.; Zhao, S.; Du, Z.; Zheng, X.; Zhang, K. Pyridine analogues of curcumin exhibit high activity for inhibiting CWR-22Rv1 human prostate cancer cell growth and androgen receptor activation. *Oncol. Lett.* **2016**, *11* (6), 4160.

Conflict of Interests Declaration

The authors declare no conflict of interests.

Graphical abstract



Highlights

- 4-Boc piperidone chalcones are prepared, for the first time, to investigate their cytotoxic activity against colorectal and prostate cancer cells.
- 4-Boc piperidone chalcones are characterized by ^{1}H-NMR, ^{13}C-NMR, and melting point.
- Significant cytotoxic activity is observed when LoVo, COLO-205, PC3, and 22RV1 are treated with 4-Boc piperidone chalcones.
- 4-Boc-piperidone chalcones induce apoptosis in COLO-205 possibly through the inhibition of NFKB.
- 4-Boc-piperidone chalcones decrease cellular proliferation and nuclear NFkB activity in both PC3 and 22RV1 cells.