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Novel 3,5-bis(arylidiene)-4-piperidone based monocarbonylanalogs of curcumin: Anticancer activity evaluation and mode of action study

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Abstract

A series of eighteen novel 3,5-bis(arylidiene)-4-piperidone based symmetrical monocarbonyl analogs of curcumin were synthesised and representative set screened by National Cancer Institute (NCI), USA for their anticancer activity. Dose response studies and mechanism of action investigation suggest most active compounds are apoptosis inducers.

Introduction

Sequencing of genomes has revealed that the complexity in organisms at the molecular level is derived from many different interactions that proteins undergo. Also, the diversity of biological functions that a protein assumes, depend on the molecular interactions that it makes.Such interactions are critical for practically all cellular, signaling and regulatory pathways. The dysfunction of these pathways is the cause of many diseases including cancer and neurological diseases. Therefore, controlling the damages and alterations due to these protein interactions that causes or accelerate human diseases, is a prime target for drug discovery.[1-5] Most of the efforts in recent decades have been in the discovery and development of therapeutics that modulate individual disease-modifying targets. Though, such approach has led to numerous successful drugs reaching the market, unfortunately few new drugs act at novel molecular targets. This is

also because drugs designed to act against individual molecular targets cannot usually combat multi-genic diseases such as cancer, or diseases that affect multiple tissues. Successful development of first-in-class drugs is challenging, in part because agents directed against individual molecular targets are often found to be less effective at treating disease, and therefore, reach the market later than hoped.[6]Hence, to overcome these challenges, multi-targeting approaches such as combination therapy and design of multi-targeted hybrids could be promising strategies to surpass the existing one chemical for one target for one disease paradigm. However, a trouble in a combination therapy is that the different solubilities of the two or more chemical species necessitate a fine-tuning of the formulation to ensure that their blood levels should be the same. On the other hand multi-targeted hybrids are generally incorporated by linking the framework of two target-selective ligands to provide a therapeutic benefit greater than each ligand.[7,8]Given the importance of this approach and in continuation of our on-going effort to develop multifunctional drugs,[9] we decided covalent hybridization of curcumin with another known pharmacophore. The hope here is to find new hybrid molecules, which could serve as useful 'probes' and help in developing new "molecular leads' for finding new drugs. Considering that this approach is still at its infancy, studies which provide data for that prove inhibition of the growth of cancer cells, are very valuable, give hope to the drug discovery community and should be taken seriously.

Curcuma longa, a perennial herb of the ginger family (Zingiberaceae), has been used extensively as an essential spice and a traditional medicine in India and China since ancient times. Turmeric, a yellow colouring powder derived from the rhizome of this herb has been a source of curcumin (Figure 1) and exhibits various pharmacological activities including anti-inflammatory,[10]   antioxidant,[11]antibacterial,[12]antimalarial,[13]anti-HIV[14]and

anticancer.[15]Toxicological studies conducted in animal models and in humans proved that

curcumin is extremely safe even at a dose level of 12 g/day.[16]However, inspite of its safe

toxicological profiles, curcumin itself is not a good candidate for further clinical development

because of poor solubility, low systemic bioavailability, undesirable absorption and rapid

metabolism when tested *in vivo*.[16]Detailed pharmacological studies confirmed that the central β-

diketone functionality of curcumin is a substrate for liver aldoketo reductases and this may lead

to rapid metabolism of curcumin.[17]
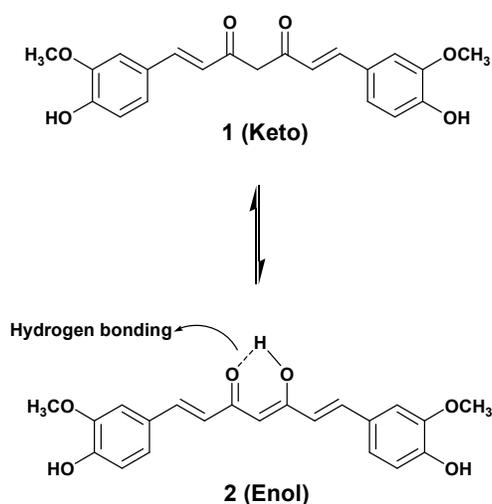
Figure 1: Keto-enoltautomers of curcumin. Six membered cyclic ring in enol form may be the reason for degradation of curcumin.[18]

In order to improve the *in vivo* metabolic stability of curcumin, several types of analogues have

been prepared and among them mono carbonyl analogues of curcumin have shown a variety of

cellular responses with no toxic effects (Figure 2).[19]*In vivo* degradation and pharmacokinetic

studies suggested these compounds have better stability and activity than curcumin.[18]As a result,

much effort has been devoted in developing novel mono-carbonyl analogues of curcumin, in

order to solve the pharmacokinetic problems and at the same time to retain high potency and low

toxicity.
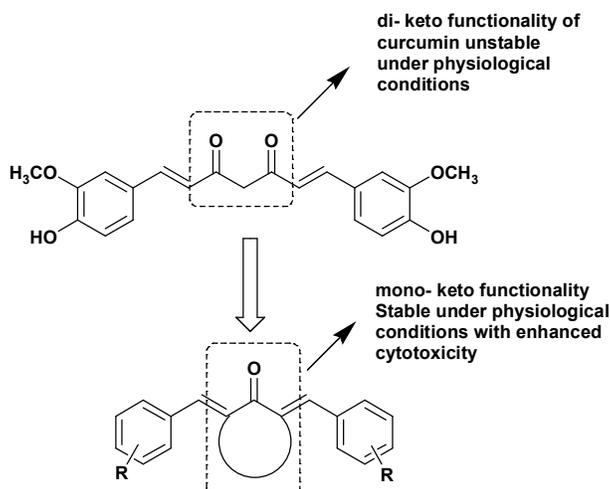
Figure 2: Modification of central β-diketone moiety to mono ketone moiety

Earlier studies on monocarbonyl analogues of curcumin resulted in the exploration of diarylidenyl-piperidone (DAP) based curcumin derivatives as potent anticancer agents.[10] Structurally these compounds can be considered as a Mannich base of dienone and α, β-unsaturated ketones, which shows anticancer properties *via* a mechanism of action comprising interactions with cellular thiols with little or no affinity for hydroxyl and amino groups in nucleic acids.[20] The 1,5-diaryl-3-oxo-1,4-pentadienyl groups reacts at a primary binding site, however, the bioactivity may be influenced by other structural units as well.[21] Structure activity relationship studies confirmed that DAP based compounds are more effective than curcumin in inhibiting the proliferation of a variety of cancer cell lines. For example 3,5-bis[2-(fluoro)benzylidene]-piperidin-4-one commonly known as EF24 (3; Figure 3) with *o*-fluorinated phenyl groups exhibited excellent anticancer activity *in vitro* when tested against breast, colon and ovarian epithelial cancer cell lines.[22,23] Its para fluorinated derivative was also found to be potent against ovarian cancer cells.[23,24] Sulphonamide moiety connected to aromatic/hetrocyclic/aliphatic ring is an important pharmacophore for the generation of

drugs.[25]For example compound E7070 (4; Figure 3) affects cell cycle progression by inhibiting

proliferation of variety of a human tumour cell lines.[26]Therefore, given the importance of this

moiety, we decided covalent hybridization of 3,5-bis(arylidene)-4-piperidone with aryl

sulphonamide moiety and prepared a set of eighteen compounds (8-25) for the present

investigation.
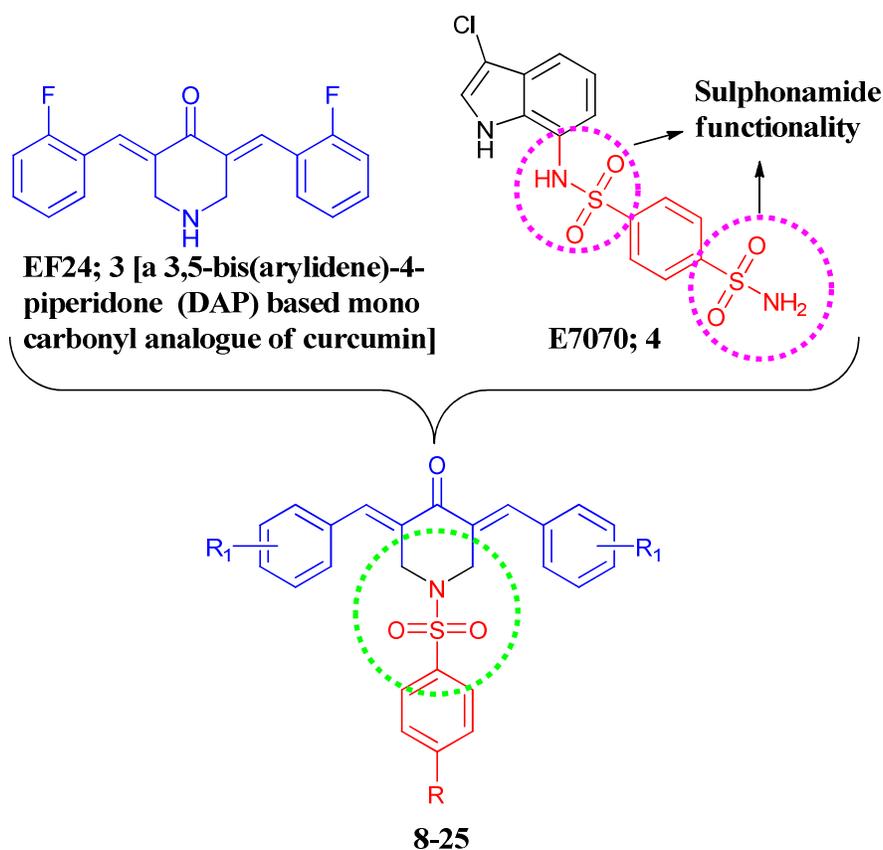
Figure 3: EF24 (**3**); sulphonamide based compound E7070 (**4**); compounds prepared in the present investigation (**8-25**)

Results and discussion

Chemistry

For the synthesis of desired symmetrical mono-carbonyl analogues of curcumin, the -NH group

of 4-piperidone hydrochloride (**5**) was subjected to nucleophillic substitution by

benzenesulpohnyl chloride or *p*-toluenesulphonyl chloride in a bi-phasic medium as reported in

literature to afford intermediates **6** and **7**.[27]The acid catalyzedClaisen-Schmidt condensation of these intermediates (**6** and **7**) with a variety of halo substituted benzaldehydes under reflux condition led to the formation of desired compounds (**8-25**) in good to excellent yield (Scheme 1).
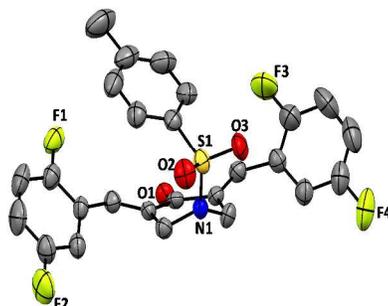
Figure 4: Molecular structure of compound **11** with partial numbering scheme (thermal ellipsoids are drawn at 50% probability level).

Crystal structure of one of the compound (**11**) was also determined (Figure 4) whose structural parameters are given in table **1** and 2.

6; R = H
7; R = CH$_3$

8-25

8; R = CH$_3$, R$_1$ = 3-F	17; R = H, R$_1$ = 3-F
9; R = CH$_3$, R$_1$ = 3,4-di-F	18; R = H, R$_1$ = 3,4-di-F
10; R = CH$_3$, R$_1$ = 3,5-di-F	19; R = H, R$_1$ = 3,5-di-F
11; R = CH$_3$, R$_1$ = 2,5-di-F	20; R = H, R$_1$ = 2,5-di-F
12; R = CH$_3$, R$_1$ = 3-Cl	21; R = H, R$_1$ = 3-Cl
13; R = CH$_3$, R$_1$ = 2,4-di-Cl	22; R = H, R$_1$ = 2,4-di-Cl
14; R = CH$_3$, R$_1$ = 2,6-di-Cl	23; R = H, R$_1$ = 2,6-di-Cl
15; R = CH$_3$, R$_1$ = 3-Br	24; R = H, R$_1$ = 3-Br
16; R = CH$_3$, R$_1$ = 4-CF$_3$	25; R = H, R$_1$ = 4-CF$_3$
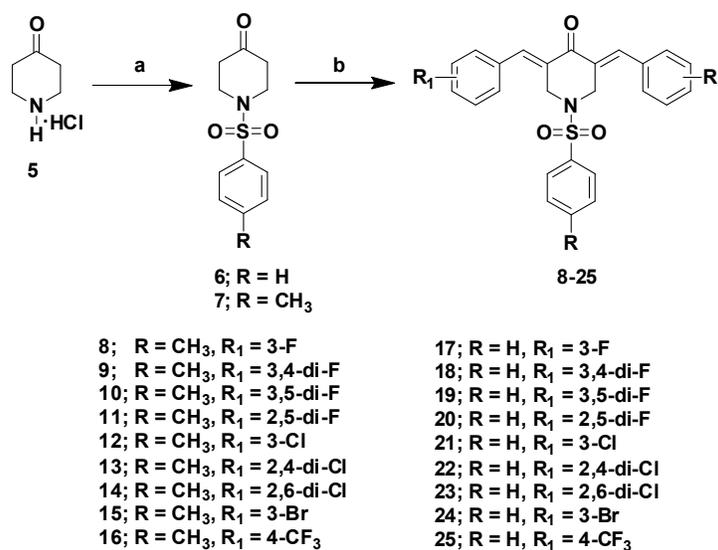
Scheme 1: Reagents and conditions: (a) benzenesulphonyl chloride or p-toluene sulphonyl chloride, K$_2$CO$_3$, CHCl$_3$, H$_2$O, rt, 4 h, 85% for **6** and 90% for **7**; (b) benzaldehydes, conc. HCl,

EtOH, reflux, 6-12 h, 60-80%

Table 1: Bond lengths [Å] for compound **11**

Compound **11**	
C(2)-N(1)	1.634(2)
S(1)-O(2)	1.428(2)
S(1)-O(3)	1.429(2)
C(4)-O(1)	1.224(3)
C(2')-F(1)	1.359(3)
C(5')-F(2)	1.363(4)
C(2")-F(3)	1.351(4)
C(5")-F(4)	1.360(4)

Table 2: Crystallographic data and structural solution parameters for compounds **11**

Compound **11**	
Molecular formula	$C_{26}H_{19}F_4NO_3S$
Fw	501.49
T(K)	293(2)
Crystal system	Triclinic
Space group	P-1
a	8.7795(5)
b	11.4369(8)
c	12.4788(8)
α	102.414(6)
β	100.722(5)
γ	102.556(5)
V ($Å^3$)	1158.28(13)
Z	2
d (g cm^{-3})	1.438
F (000)	516
Goodness of fit (F^2)	1.044
$R_1, wR_1[I > 2 (I)]^a$	$R_1 =$ 0.0611, $wR_1 =$ 0.1028
R_1, wR_2[all data]	$R_2 =$ 0.0999, $wR_2 =$ 0.1156

$^aR = \Sigma||F_o|-|F_c||/ \Sigma|F_o|;$
$wR= \{[\Sigma(|F_o|^2|F_c|^2)^2]\}^{1/2}$

Biological Studies

a) Anticancer activity

Four representative molecules (**10, 14, 22** and **23**) were selected by US National Cancer Institute's 60 human cancer cell line (NCI 60) for screening of their growth inhibition activity and cytotoxicity against 60 different human cancer cell lines covering wide histologies. Details of the methodology for NCI 60 cell line screening are described at http://dtp.nci.nih.gov/branches/btb/ivclsp.html. Briefly, the panel is organized into nine subpanels representing diverse histologies: leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate, and central nervous system. The *in vitro* screening is a two-stage process which starts with the evaluation of a compound against 60 human tumor cell lines with a single dose of 10.0 μM. Only the compounds which show more than 60% of growth inhibition in at least 8 tumor cell lines are selected for further testing for dose response effect at five concentration levels (100, 10, 1.0, 0.1 and 0.01 μM) and the others are assumed inactive. The cells are grown in supplemented RPM1 1640 medium for 24 h. The test compounds are dissolved in DMSO and incubated with cells at five concentrations with 10-fold dilutions, the highest being 10^{-4} M and the others being 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M. The assay is terminated by addition of cold trichloroacetic acid, and the cells are fixed and stained with sulforhodamine B. Bound stain is solubilized, and the absorbance is read on an automated plate reader. The cytostatic parameter i.e. 50% growth inhibition (GI_{50}) was calculated from time zero, control growth, and the five concentration level absorbance. The cytotoxic parameter i.e. inhibitory concentrations (LC_{50}) represent the average of two independent experiments. The compounds **10, 14, 22** and **23**were initially evaluated at a single dose of 10 μM and found to be active against various cell types. The results of single dose screening are given as supplementary information in this manuscript. Among these compounds **10&22** showed promising results, and therefore, both

were further selected for 5 dose studies. The mean values for GI_{50}, TGI and LC_{50} on all 60 cell lines are given in Table 3. The additional data for both compounds i.e. one dose mean graphs, drug response curves, five dose mean graphs, GI_{50} and LC_{50} values are given in supplementary information. The dose response curves of **10** for all 60 cell lines are illustrated in Figure 5. Detailed data on dose response investigation of **10&22** is available in the supplementary information to this paper.

Table 3. Antitumor activity (GI_{50}/µM), total growth inhibition (TGI/ µM) and toxicity (LC_{50}/µM) data of compounds selected for 5 dose studies for the NCI60-cell lines screen.

Cancer cell lines	Compound 10			Compound 22		
	GI_{50}(µM)	TGI (µM)	LC_{50} (µM)	GI_{50}(µM)	TGI(µM)	LC_{50}(µM)
Leukemia						
CCRF-CEM	0.0748	0.951	>100	0.286	0.994	>100
HL-60(TB)	0.289	1.74	>100	0.562	3.97	>100
K-562	0.0772	1.37	>100	0.346	>100	>100
MOLT-4	0.299	2.11	>100	0.594	9.05	>100
RPMI-8226	0.0372	0.331	93.8	0.225	0.605	>100
SR	0.0348	0.252	>100	0.185	0.865	>100
Non-small cell Lung Cancer						
A549/ATCC	2.86	14.6	>100	0.277	0.887	>100
HOP-62	2.04	4064	11.9	0.385	>100	>100
HOP-92	1.11	3.30	9.78	1.70	5.55	>100

NCI-H226	8.62	39.6	>100	1.04	2.87	7.92
NCI-H23	1.75	4.99	26.8	0.423	2.71	>100
NCI-H322M	4.7	22.8	>100	0.680	3.56	>100
NCI-H460	0.828	2.87	9.22	0.208	0.447	0.959
NCI-H522	0.316	0.962	3.85	0.854	2.99	9.73
Colon Cancer						
COLO 205	0.612	2.11	6.14	0.424	2.01	>100
HCC-2998	0.602	1.91	4.85	0.383	1.36	4.54
HCT-116	0.209	0.543	1.94	0.198	0.424	0.908
HCT-15	0.250	1.30	30.7	0.247	0.759	>100
HT 29	0.229	0.638	3.19	0.293	0.843	>100
KM 12	0.327	1.00	4.04	0.256	0.602	25.7
SW-620	0.0721	0.305	1.45	0.317	2.79	>100
CNS Cancer						
SF-268	0.431	2.15	14.4	0.447	>100	>100
SF-295	0.466	2.50	15.7	0.345	6.67	>100
SF-539	1.21	2.87	6.81	0.424	2.07	>100
SNB-19	0.220	0.752	2.82	0.313	2.88	>100
SNB-75	1.99	5.90	26.2	0.432	7.29	>100
U 251	0.188	0.530	2.02	0.173	0.373	0.805
Melanoma						
LOX IMVI	0.175	0.339	0.656	0.203	0.508	ND
MALME-3M	0.274	0.742	3.81	3.21	ND	>100
M 14	0.359	1.40	5.91	3.66	>100	>100

MDA-MB-435	0.241	0.685	3.63	0.98	3.21	>100
SK-MEL-2	1.97	4.66	14.8	6.01	>100	>100
SK-MEL-28	0.473	1.79	4.82	4.44	>100	>100
SK-MEL-5	0.286	1.08	3.60	ND	>100	>100
UACC-257	0.939	2.90	8.60	4.08	>100	>100
UACC-62	0.212	0.514	1.62	0.804	2.31	5.81
Ovarian Cancer						
IGROV1	0.436	1.55	5.17	0.317	0.649	>100
OVCAR-3	0.441	1.44	4.56	0.310	0.818	5.01
OVCAR-4	0.441	5.23	36.3	0.279	>100	>100
OVCAR-5	1.02	2.75	7.40	2.31	7.43	>100
OVCAR-8	0.301	1.77	16.3	0.286	0.958	>100
NCI/ADR-RES	0.439	10.1	7.67	1.37	>100	>100
SK-OV-3	1.84	5.62	37.2	7.95	>100	>100
Renal Cancer						
786-0	0.587	1.91	4.95	0.241	0.544	1.71
A498	1.88	4.05	8.76	0.727	0.439	>100
ACHN	0.246	0.622	2.39	0.874	4.56	>100
CAKI-1	0.365	0.243	61.9	1.84	5.75	>100
RXF 393	0.723	2.14	5.13	1.06	2.42	5.54
SN 12C	0.342	1.31	4.26	0.245	0.718	>100
TX-10	1.03	2.96	8.46	ND	ND	ND
UO-31	1.09	2.37	5.17	0.236	0.639	4.75

Prostate Cancer						
PC-3	0.248	0.803	9.28	0.291	1.03	>100
DU-145	1.24	3.08	7.62	0.446	18.8	>100
Breast Cancer						
MCF7	0.175	1.45	6.88	0.305	>100	>100
MDA-MB-231/ATCC	0.270	0.784	8.48	0.466	2.16	ND
HS 578T	0.399	18.6	>100	ND	ND	ND
BT-549	0.340	0.931	3.52	0.987	4.21	>100
T-47D	1.79	6.09	54.3	1.276	>100	>100
MDA-MB-468	0.349	1.71	5.77	1.13	3.89	>100

Nd: Not Determined

As the data shows, our covalent hybrids of 3,5-bis(arylidene)-4-piperidone with aryl sulphonamide are active against various cancer panels. Both compounds (**10** and **22**) show cytostatic potential at sub-micromolar concentrations against several cell lines especially against leukemia (upto 34 nM) and colon cancer as seen from the GI_{50} values. In summary low growth inhibitory concentration of these compounds coupled with high lethal concentrations indicate broad therapeutics index of these hybrid molecule. Therefore, motivating further investigation for their mode of action. Figure **5**: shows drug response curve from five dose study against compound **10.**
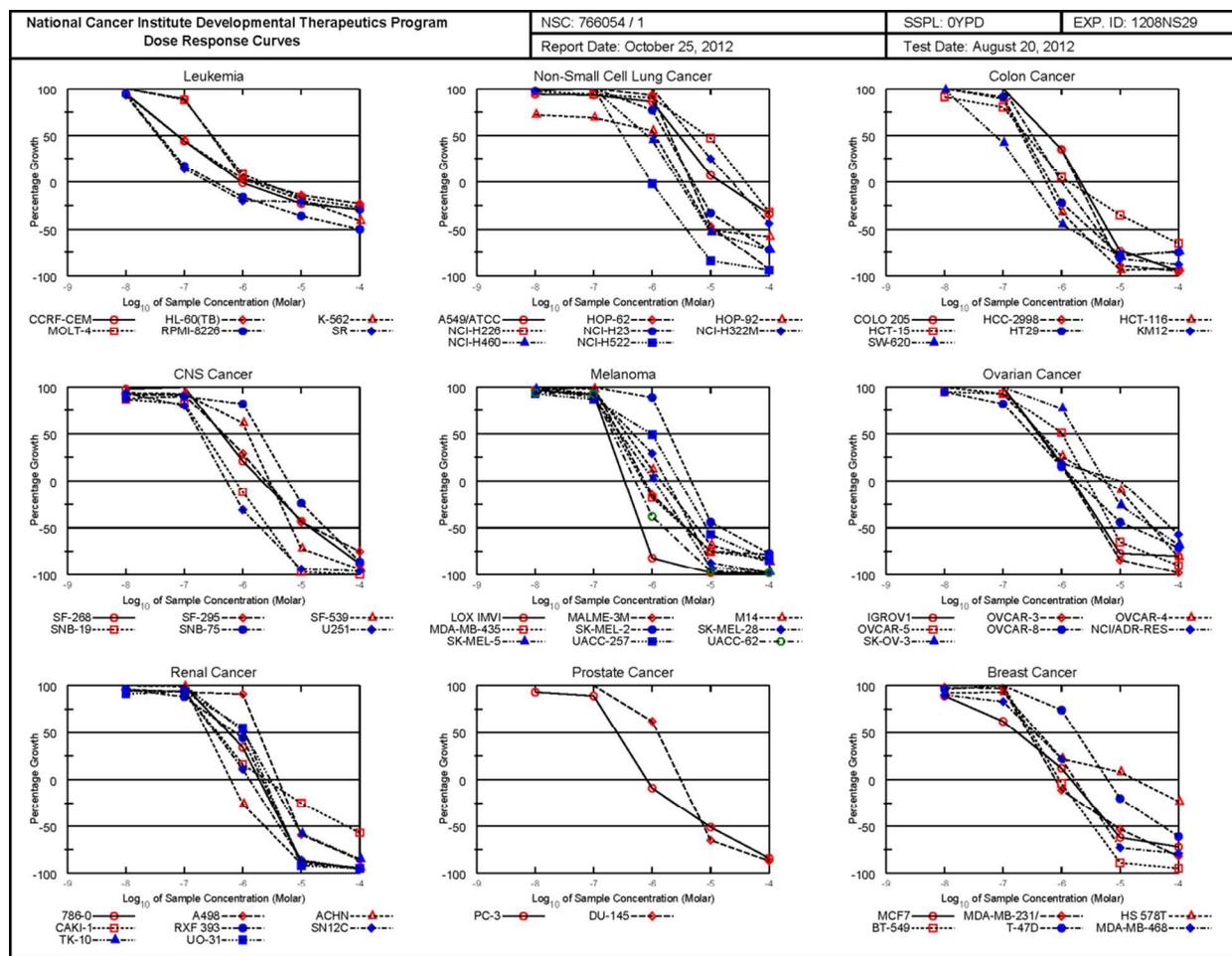
Figure 5: Dose response curve of compound **10**

(b) Mechanism of action studies

(I) Annexin Staining

Annexin staining was performed as a screening for apoptosis. Cells exposed to the compounds presented significant apoptotic activity. Compound **10** presented 70% of apoptotic cells while **22** presented 66% (Figure 6a). Compound **10** presented activation similar to the camptothecin positive control (53%). Apoptotic cells exposed to **10** were distributed between early and late apoptotic (figure 6b) whereas **22** did not present cells at late apoptotic stage.
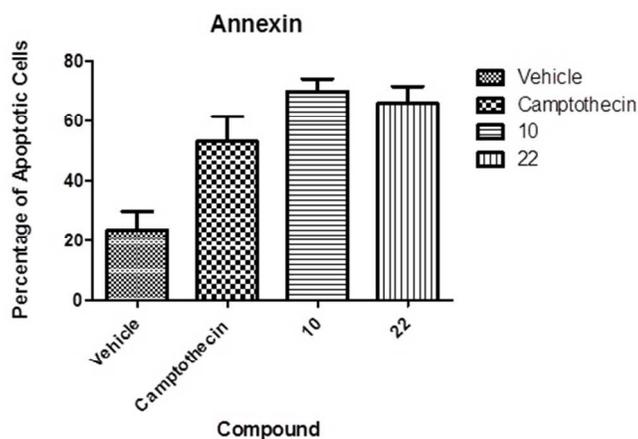
Figure 6a: Annexin Staining. Both compounds presented significant (P<0.05) apoptotic activity. Compound **10** presented 70% apoptotic cells while compound **22** presented 66%.
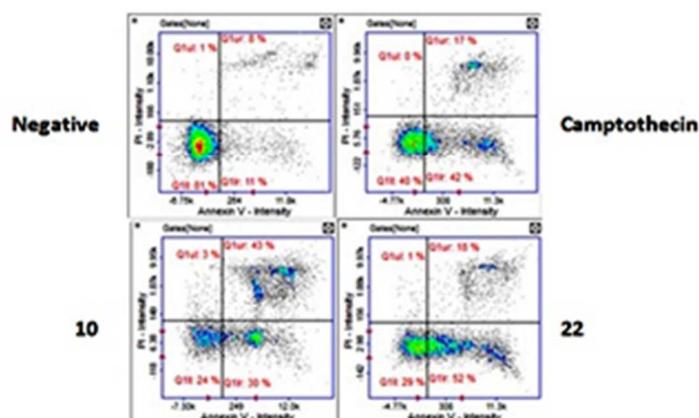
Figure 6b: Apoptotic cell distribution. Compound **10** presented a majority of cells in the apoptotic stage with a high number at the late apoptotic stage. Compound **22** presented cells mostly at the early apoptotic stage

(II) Caspase activation

(i) Caspase 3 and 7

Activation of effector caspases was measured to determine apoptotic cell death process. Our compounds caused moderate activation of these effector caspases. Compound **10** presented 41.5% of caspase activation while **22** presented 35.5%of activation (Figure 7). Both experimental compounds were significantly below the positive camptothecin control (62%). These moderate results contrast with the high annexin positive results which demonstrated a high apoptosis activity.
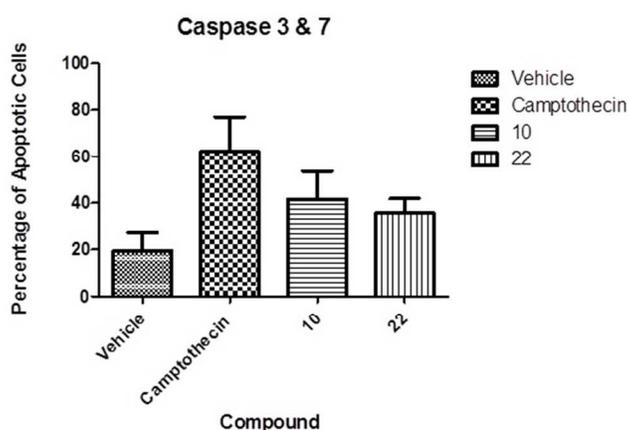
Figure 7: Caspase 3 and 7 activation. Compound **10** presented 41.5% of caspase activation while **22** presented 35.5%of activation. Both experimental compounds were significantly lower than the positive camptothecin control (62%).

(ii) Caspase 8

Caspase 8 activation was assessed with high activation by both experimental compounds. **10** presented the highest activation at a 61% of cells with active caspase 8 (Figure 8). Compound **22** presented 23% similar to the positive camptothecin control (23.5%). Both compounds presented statistically significant ($P < 0.05$) when compared to the negative control.
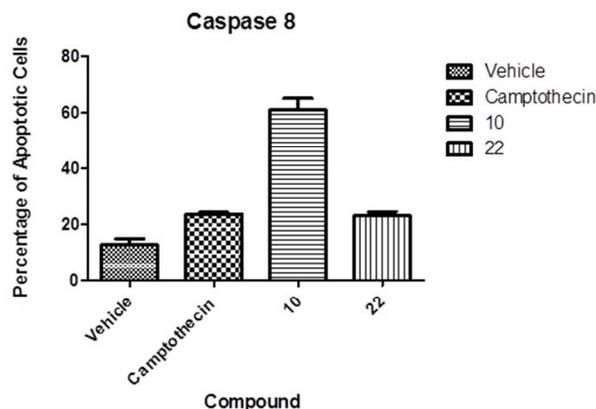
Figure 8: Caspase 8 activation.10 presented the highest activation at a 61% of cells with active caspase 8. Compound **22**presents 23% similar to the camptothecin positive control (23.5%).

(iii) Caspase 9

Caspase 9 is a key marker for the intrinsic apoptotic mechanism. Our compounds presented very different activation of this caspase. Compound **10** once again caused activation of caspase 9with the highest mean value of 40.5% (Figure 9). Compound **22** presented 22% of activation which was comparable to the positive camptothecin control (19.5%). Upon further analysis of these values we can see that camptothecin did not activate caspase 9 in a significant manner (P>0.05) and neither did **22** since this compound caused activation similar to camptothecin. We thus can see that only **10** caused activation in a statistically significant manner (P<0.05).
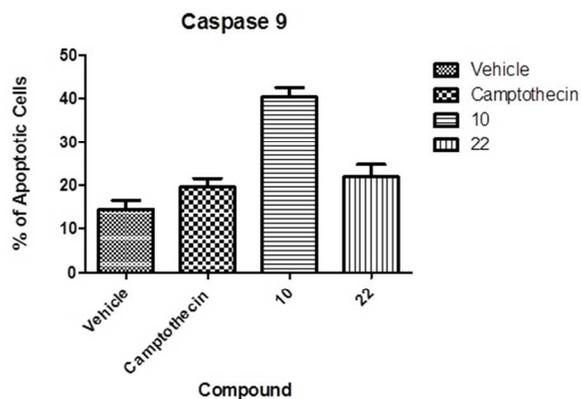
Figure 9: Caspase 9 activation.10 caused activation of caspase 9 with a mean value of 40.5%. Compound **22** presented 22% of activation comparable to the positive camptothecin control (19.5%).

(III) DNA Fragmentation

DNA fragmentation as a confirmatory test for apoptosis was performed after exposure to the compounds. Both compounds presented a high DNA fragmentation activity. Compound **10** presented 69.5% of cells with fragmented DNA. Compound **22** presented a 45% of cells with fragmented DNA. Both compounds presented high activation although less than the positive camptothecin control which presented a mean of 80.5% (Figure 10).
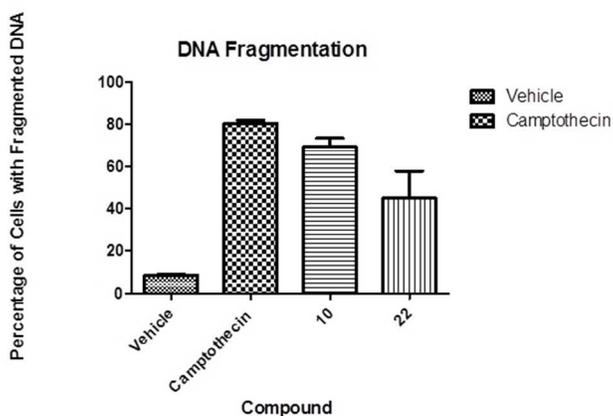
Figure 10: DNA Fragmentation. Compound **10** presented 69.5% of cells with fragmented DNA, while compound **22** presented a 45% of cells with fragmented DNA.

Discussion

The occurrence of apoptosis, a natural death process in cells, most commonly is associated with arrested mitotic activity, decreased DNA replication, fragmentation of DNA, and activation of caspase type enzymes. Accordingly, we examined COLO 205 cells treated with compounds **10&22** to determine whether these indications of apoptosis appeared. A commonly held view is that uncontrolled cell proliferation in malignant tissues derives from a combination of two circumstances: increased cell multiplication unresponsive to normal control processes and decreased occurrence of the normal process of cell death, that is, apoptosis. Our present state of knowledge, however, offers little insight regarding the intrinsic or extrinsic signals that might govern the opposing processes of cell multiplication and cell death to maintain normal cell populations. Our studies showed that both compounds (**10&22**) caused varied biological activities. Compound **10** was the more biologically active of the two compounds. This compound presented apoptotic cells evidenced by the presence of phosphatidylserine on the cell membrane as evidenced by the annexin V staining. This compound also presented desirable traits for potential anti-cancer compounds. Among these is the activation of caspases. Compound **10** appears to activate both caspase 8, 9, and effector caspase 3 (this one moderately) which combined with the DNA fragmentation event would suggest an apoptotic mechanism. Additionally this compound presented cells with fragmented DNA with moderate effector caspase activation which corroborates the apoptotic cell death mechanism. Activation of both apoptotic pathways has been observed on a number of substances including

etoposide,[28]pyrazole[29]and quinolone [30]derivatives. While, compound **22** presented a mechanistically different apoptotic cell death. This compound presented annexin positive results, DNA fragmentation and activation of effector caspase typical of apoptotic cells. However, it did activate caspase 8 but not caspase 9, which suggests an extrinsic mechanism. In conclusion both of these compounds are apoptosis inducers.

Experimental Section

Instrumentation and Chemicals

All of the chemicals used in the synthesis were purchased from Sigma-Aldrich and were used as such. Thin layer chromatography was used to monitor the progress of the reactions and checked by precoated TLC plates (E. Merck Kieselgel 60 F_{254}) with spots being visualized by iodine vapors. Compounds were purified by precipitation or recrystallization technique with suitable solvents. Solvents were distilled before using for purification purposes. Meting points were recorded on an ERS automated melting point apparatus and are uncorrected. IR spectra were recorded using Perkin-Elmer spectrophotometer and .the values are expressed as λ_{max} cm^{-1}. Mass spectral data were recorded on a Jeol-AccuTOF JMS-T100LC and micromass LCT Mass Spectrometer/Data system. Elemental analyses were performed on Carlo Erba Model EA-1108 elemental analyzer and data of C, H and N is within ± 0.4 of calculated values. ^{1}H NMR and ^{13}C NMR spectra were recorded on JeolSpectrospin spectrometer at 400 MHz and 100 MHz respectively using TMS as an internal standard. The chemical shift values are recorded on δ scale and the coupling constants (J) are in Hz.

(a) Synthesis and characterization of compounds

Procedure for the synthesis of 1-(Phenylsulfonyl)piperidin-4-one (6)[27]and related

compound (7)[19]**:** The title compound was prepared by literature method.[27]In brief, 4-piperidone hydrochloride monohydrate (**5**) (5 g, 32.5 mmol) was dissolved in 20 mL of biphasic system of $CHCl_3:H_2O$ (1:1). To this reaction mixture, K_2CO_3 (13.47 g, 97.6 mmol) was added followed by the addition of benzenesulphonyl chloride (7.78 g, 32.5 mmol). The reaction was stirred at room temperature for 4 h and progress of the reaction was monitored by thin layer chromatography. After completion, reaction mixture was extracted with chloroform. Organic layer was dried over Na_2SO_4 and excess of solvent was removed under vacuum and crude product was purified by silica gel column using EtOAc/Hexane as an eluent to afford the desired compound **6** as white solid. Yield: 85%; mp 118-119 $^{\circ}$C (Litt. 117.5-118.5 °C).[31]

1-Tosylpiperidin-4-one (7): Yield: 90%; mp: 132-134 $^{\circ}$C (Litt. 130-132 $^{\circ}$C)[32]; IR (cm^{-1}, Film): 2966, 2927, 2879, 1713, 1369, 1348, 1225, 1171, 965, 691; ^{1}H NMR (CDCl$_3$): 2.44 (s, 3H), 2.53 (t, 4H, J = 5.8 Hz,), 3.38 (t, 4H, J = 5.8 Hz), 7.33 (d, 2H, J = 8.0 Hz), 7.67 (d, 2H, J = 8.0 Hz); ESI-MS (m/z): 254.08 (M+H)$^{+}$, Anal. Calcd for C$_{12}$H$_{15}$NO$_3$S: C, 56.90; H, 5.97; N, 5.53; Found: C, 56.98; H, 6.13; N, 5.55.

Procedure for the synthesis of (*3E,5E*)-3,5-Bis-(3-fluoro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one (8) and related compounds (9-25): Compound **6** or **7** (0.2 g, 0.79 mmol) and 3-fluorobenzaldehyde (0.19 g, 1.50 mmol) were dissolved in 10 mL of EtOH at 0 $^{\circ}$C. To this reaction mixture 1 mL of 35-38% HCl was added. After 15-20 min of stirring at room temperature, the reaction mixture was refluxed for 10-12 h. The yellow precipitate was filtered and washed with EtOH. Crude product was crystallized in EtOH to get compound **8** in pure form. Yield: 75%; mp 193-195 $^{\circ}$C; IR (cm^{-1}, Film): 3024, 1611, 1583, 1443, 1348, 1213, 1164, 956; ^{1}H NMR (400 MHz, CDCl$_3$): 2.43 (s, 3H), 4.56 (s, 4H), 7.01 (d, 2H, J = 9.5 Hz), 7.11-7.16 (m, 4H), 7.25 (d, 2H, J = 7.3 Hz), 7.41-7.47 (m, 2H), 7.51 (d, 2H, J = 8.7 Hz), 7.66 (s, 2H); ^{13}C

NMR (100 MHz, CDCl$_3$): 21.57, 47.09, 116.62 (d, J = 5.75 Hz), 116.84 (d, J = 5.75 Hz), 125.96, 127.57, 129.79, 130.50 (d, J = 7.67 Hz), 130.84, 134.34, 136.27 (d, J = 7.67 Hz), 137.28, 144.32, 162.69 (d, J = 247.28 Hz), 184.46; ESI-MS (m/z): 466.18 (M+H)$^+$; Anal. Calcd for C$_{26}$H$_{21}$F$_2$NO$_3$S: C, 67.08; H, 4.55; N, 3.01; Found: C, 67.21; H, 4.59; N, 3.21.

(*3E,5E*)-3,5-Bis-(3,4-difluoro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one (9): Yield: 75%; mp 184-186 ^{o}C; IR (cm^{-1}, Film): 2920, 1593, 1511, 1238, 1162,997, 946, 742; ^{1}H NMR (400 MHz, CDCl$_3$): 2.44 (s, 3H), 4.52 (s, 4H), 7.08-7.17 (m, 4H), 7.23-7.30 (m, 4H), 7.52 (d, 2H, J = 8.0 Hz), 7.60 (s, 2H); ESI-MS (m/z): 502.16 (M+H)$^+$; Anal. Calcd for C$_{26}$H$_{19}$F$_4$NO$_3$S: C, 62.27; H, 3.82; N, 2.79; Found: C, 62.43; H, 3.71; N, 2.93.

(*3E,5E*)-3,5-Bis-(3,5-difluoro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one (10): Yield: 65%; mp 183-185 ^{o}C; IR (cm^{-1}, Film): 2919, 1591, 1430, 1347, 1238, 1220, 1163, 1121, 997; ^{1}H NMR (400 MHz, CDCl$_3$): 2.45 (s, 3H), 4.52 (s, 4H), 6.82-6.94 (m, 6H), 7.28 (d, 2H, J = 8.0 Hz), 7.53 (d, 2H, J = 8.0 Hz), 7.60 (s, 2H); ESI-MS (m/z): 502.16 (M+H)$^+$; Anal. Calcd for C$_{26}$H$_{19}$F$_4$NO$_3$S: C, 62.27; H, 3.82; N, 2.79; Found: C, 62.40; H, 3.80; N, 2.85.

(*3E,5E*)-3,5-Bis-(2,5-difluoro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one (11): Yield: 72%; mp 179-181 ^{o}C; IR (cm^{-1}, Film): 3078, 2925, 1679, 1618, 1598, 1488, 1348, 1226, 1163, 963; ^{1}H NMR (400 MHz, CDCl$_3$): 2.44 (s, 3H), 4.47 (s, 4H), 6.89-6.96 (m, 2H), 7.10-7.19 (m, 4H), 7.22-7.28 (m, 2H), 7.46 (d, 2H, J = 8.0 Hz), 7.66 (s, 2H); ESI-MS (m/z): 502.18 (M+H)$^+$; Anal. Calcd for C$_{26}$H$_{19}$F$_4$NO$_3$S: C, 62.27; H, 3.82; N, 2.79; Found: C, 62.35; H, 3.73; N, 2.71.

(*3E,5E*)-3,5-Bis-(3-chloro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one (12): Yield: 70%; mp 170-172 ^{o}C; IR (cm^{-1}, Film): 3024, 2924, 1678, 1615, 1593, 1474, 1351, 1240, 1190, 1162, 1089, 963; ^{1}H NMR (400 MHz, CDCl$_3$): 2.44 (s, 3H), 4.55 (s, 4H), 7.21-7.31 (m, 6H),

7.41 (d, 4H, *J* = 5.1 Hz,), 7.51 (d, 2H, *J* = 8.0 Hz), 7.63 (s, 2H); ESI-MS (m/z): 498.09 (M+H)$^+$,

499.07 (M+2)$^+$; Anal. Calcd for C$_{26}$H$_{21}$Cl$_2$NO$_3$S: C, 62.65; H, 4.25; N, 2.81; Found: C, 62.77; H,

4.36; N, 2.73.

(*3E*,*5E*)-3,5-Bis-(2,4-dichloro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one   (13):

Yield: 80%; mp 200-202 ^{o}C; IR (cm^{-1}, Film): 2915, 1585, 1465, 1347, 1237, 1163, 956; ^{1}H NMR

(400 MHz, CDCl$_3$): 2.42 (s, 3H), 4.47 (s, 4H), 7.13 (d, 2H, *J* = 8.0 Hz), 7.21 (d, 2H, *J* = 8.0 Hz),

7.34 (d, 2H, *J* = 9.52 Hz), 7.43 (d, 2H, *J* = 8.0 Hz), 7.53 (d, 2H, *J* = 1.4 Hz), 7.79 (s, 2H); ^{13}C

NMR (100 MHz, CDCl$_3$): 21.61, 46.88, 127.29, 127.49, 129.93, 130.17, 130.84, 131.05, 131.59,

134.59, 134.87, 135.82, 136.15, 144.35, 183.73; ESI-MS (m/z): 565.97 (M+H)$^+$, 566.96 (M+2)$^+$;

Anal. Calcd for C$_{26}$H$_{19}$Cl$_4$NO$_3$S: C, 55.05; H, 3.38; N, 2.47; Found: C, 55.19; H, 3.54; N, 2.54.

(*3E*,*5E*)-3,5-Bis-(2,6-dichloro-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one   (14):

Yield: 66%; mp 185-187 ^{o}C; IR (cm^{-1}, Film): 2923, 2853, 1719, 1606, 1429, 1349, 1163, 1090,

958, 778; ^{1}H NMR (400 MHz, CDCl$_3$): 2.41 (s, 3H), 4.23 (s, 4H), 7.23 (d, 2H, *J* = 8.0 Hz), 7.30

(d, 2H, *J* = 8.7 Hz), 7.40 (d, 4H, *J* = 8.0 Hz), 7.52 (d, 2H, *J* = 8.0 Hz), 7.65 (s, 2H); ESI-MS

(m/z): 565.99 (M+H)$^+$, 566.97 (M+2)$^+$; Anal. Calcd for C$_{26}$H$_{19}$Cl$_4$NO$_3$S: C, 55.05; H, 3.38; N,

2.47; Found: C, 55.21; H, 3.35; N, 2.62.

(*3E*,*5E*)-3,5-Bis-(3-bromo-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one (15): Yield:

76%; mp 168-170 ^{o}C; IR (cm^{-1}, Film): 3062, 2927, 1611, 1584, 1351, 1188, 1164, 997; ^{1}H NMR

(400 MHz, CDCl$_3$): 2.45 (s, 3H), 4.53 (s, 4H), 7.25-7.31 (m, 4H,), 7.33-7.37 (m, 2H), 7.45 (s,

2H), 7.52 (d, 2H, *J* = 8.0 Hz), 7.56 (d, 2H, *J* = 8.0 Hz,), 7.63 (s, 2H); ESI-MS (m/z): 585.99

(M+H)$^+$ 586.97 (M+2)$^+$; Anal. Calcd for C$_{26}$H$_{21}$Br$_2$NO$_3$S: C, 53.17; H, 3.60; N, 2.38; Found: C,

53.32; H, 3.75; N, 2.31.

(*3E*,*5E*)-3,5-Bis-(4-trifluoromethyl-benzylidene)-1-(toluene-4-sulfonyl)-piperidin-4-one

(16): Yield: 68%; mp 189-191 ^{o}C; IR (cm^{-1}, Film): 2921, 2852, 1613, 1325, 1164, 1121, 1070, 986, 840; ^{1}H NMR (400 MHz, CDCl$_3$): 2.44 (s, 3H), 4.55 (s, 4H), 7.24-7.28 (m, 2H), 7.45 (d, 4H, J = 8.0 Hz,), 7.51 (d, 2H, J = 8.0 Hz), 7.72-7.74 (m, 6H); ESI-MS (m/z): 566.17 (M+H)$^+$; Anal. Calcd for C$_{28}$H$_{21}$F$_6$NO$_3$S: C, 59.47; H, 3.74; N, 2.48; Found: C, 59.58; H, 3.65; N, 2.59.

(3E,5E)-3,5-Bis(3-fluorobenzylidene)-1-(phenylsulfonyl) piperidin-4-one (17): Yield: 80%; mp 183-185 ^{o}C; IR (cm^{-1}, Film): 2923, 1612, 1582, 1352, 1214, 1166, 1089, 996, 964; ^{1}H NMR (400 MHz, CDCl$_3$): 4.61 (s, 4H), 7.02 (d, 2H, J = 9.5 Hz), 7.11-7.18 (m, 4H), 7.42-7.49 (m, 4H), 7.59-7.65 (m, 5H); ESI-MS (m/z): 452.15 (M+H)$^+$; Anal. Calcd for C$_{25}$H$_{19}$F$_2$NO$_3$S: C, 66.51; H, 4.24; N, 3.10; Found: C, 66.55; H, 4.38; N, 3.12.

(3E,5E)-3,5-Bis(3,4-difluorobenzylidene)-1-(phenylsulfonyl) piperidin-4-one(18): Yield: 80%; mp 210-212 ^{o}C; IR (cm^{-1}, Film): 2922, 2852, 1602, 1514, 1350, 1233, 1167, 974; ^{1}H NMR (400 MHz, CDCl$_3$): 4.56 (s, 4H), 7.07-7.19 (m, 4H), 7.24-7.31 (m, 2H), 7.44-7.50 (m, 2H), 7.58-7.65 (m, 5H); ^{13}C NMR (100 MHz, CDCl$_3$): 46.94, 118.07 (d, J = 18.21 Hz), 119.02 (d, J = 18.21 Hz), 126.90, 127.46, 129.24, 130.39, 131.17, 133.45, 136.51, 137.49, 149.51 (d, J = 64.22 Hz), 152.00 (d, J = 69.01 Hz), 184.11; ESI-MS (m/z): 488.14 (M+H)$^+$; Anal. Calcd for C$_{25}$H$_{17}$F$_4$NO$_3$S: C, 61.60; H, 3.52; N, 2.87; Found: C, 61.75; H, 3.68; N, 2.93.

(3E,5E)-3,5-Bis(3,5-difluorobenzylidene)-1-(phenylsulfonyl) piperidin-4-one (19): Yield: 62%; mp 211-213 ^{o}C; IR (cm^{-1}, Film): 3083, 1591, 1431, 1347, 1216, 1165, 1120, 997; ^{1}H NMR (400 MHz, CDCl$_3$): 4.57 (s, 4H), 6.81-6.95 (m, 6H), 7.47-7.51 (m, 2H), 7.57-7.66 (m, 5H); ^{13}C NMR (100 MHz, CDCl$_3$): 47.00, 105.29, 112.78, 127.50, 129.31, 131.55, 133.54, 136.44, 136.97, 137.37, 164.36 (d, J = 287.54 Hz), 183.86; ESI-MS (m/z): 488.16 (M+H)$^+$; Anal. Calcd for C$_{25}$H$_{17}$F$_4$NO$_3$S: C, 61.60; H, 3.52; N, 2.87; Found: C, 61.72; H, 3.67; N, 2.71.

(3E,5E)-3,5-Bis(2,5-difluorobenzylidene)-1-(phenylsulfonyl) piperidin-4-one (20): Yield:

72%; mp 174-176 °C; IR (cm^{-1}, Film): 2924, 2853, 1624, 1490, 1353, 1223, 1167, 1090, 982; ^{1}H NMR (400 MHz, CDCl$_3$): 4.50 (s, 4H), 6.90-6.95 (m, 2H), 7.10-7.19 (m, 4H), 7.43-7.49 (m, 2H), 7.56-7.66 (m, 5H); ESI-MS (m/z): 488.15 (M+H)$^+$; Anal. Calcd for C$_{25}$H$_{17}$F$_4$NO$_3$S: C, 61.60; H, 3.52; N, 2.87; Found: C, 61.76; H, 3.65; N, 2.85.

(*3E,5E*)-3,5-Bis(3-chlorobenzylidene)-1-(phenylsulfonyl) piperidin-4-one (21): Yield: 70%; mp 167-169 °C; IR (cm^{-1}, Film): 3066, 2925, 2853, 1677, 1614, 1352, 1166, 1090, 992; ^{1}H NMR (400 MHz, CDCl$_3$): 4.59 (s, 4H), 7.21-7.24 (m, 2H), 7.28-7.31 (m, 2H), 7.39-7.49 (m, 6H), 7.58-7.64 (m, 5H); ^{13}C NMR (100 MHz, CDCl$_3$): 47.10, 127.48, 128.14, 129.17, 129.72, 129.97, 130.16, 130.78, 133.35, 134.83, 135.86, 137.19, 137.46, 184.14; ESI-MS (m/z): 484.06 (M+H)$^+$ 485.07 (M+2)$^+$; Anal. Calcd for C$_{25}$H$_{19}$Cl$_2$NO$_3$S: C, 61.99; H, 3.95; N, 2.89; Found: C, 62.11; H, 4.02; N, 2.89.

(*3E,5E*)-3,5-Bis(2,4-dichlorobenzylidene)-1-(phenylsulfonyl)

piperidin-4-one(22): Yield: 75%; mp 222-224 °C; IR (cm^{-1}, Film): 2922, 2852, 1609, 1466, 1350, 1165, 960, 772; ^{1}H NMR (400 MHz, CDCl$_3$): 4.50 (s, 4H), 7.12 (d, 2H, *J* = 8.0 Hz), 7.34 (d, 2H, *J* = 8.0 Hz), 7.41-7.47 (m, 2H), 7.52-7.61 (m, 5H), 7.78 (s, 2H); ^{13}C NMR (100 MHz, CDCl$_3$): 46.98, 127.33, 127.48, 129.35, 130.22, 130.85, 131.00, 131.40, 133.42, 135.03, 135.81, 136.23, 137.75, 183.51; ESI-MS (m/z): 551.98 (M+H)$^+$, 552.95 (M+2)$^+$; Anal. Calcd for C$_{25}$H$_{17}$Cl$_4$NO$_3$S: C, 54.27; H, 3.10; N, 2.53; Found: C, 54.31; H, 3.18; N, 2.50.

(*3E,5E*)-3,5-Bis(2,6-dichlorobenzylidene)-1-(phenylsulfonyl) piperidin-4-one (23): Yield: 72%; mp 172-174 °C; IR (cm^{-1}, Film): 2922, 2852, 1686, 1627, 1429, 1352, 1167, 1091, 959, 780; ^{1}H NMR (400 MHz, CDCl$_3$): 4.27 (s, 4H), 7.28-7.33 (m, 2H), 7.38-7.48 (m, 6H), 7.56-7.60 (m, 1H), 7.63-7.68 (m, 4H); ^{13}C NMR (100 MHz, CDCl$_3$): 47.03, 127.53, 128.35, 129.28, 130.48, 131.95, 133.10, 133.47, 133.78, 134.25, 137.63, 182.64; ESI-MS (m/z): 551.99 (M+H)$^+$,

552.96 (M+2)$^+$; Anal. Calcd for $C_{25}H_{17}Cl_4NO_3S$: C, 54.27; H, 3.10; N, 2.53; Found: C, 54.30; H, 3.21; N, 2.62.

(*3E,5E*)-3,5-Bis(3-bromobenzylidene)-1-(phenylsulfonyl) piperidin-4-one (24): Yield: 68%; mp 188-190 ^{o}C; IR (cm^{-1}, Film): 2923, 2852, 1612, 1589, 1352, 1188, 1165, 1089, 961; ^{1}H NMR (400 MHz, CDCl$_3$): 4.58 (s, 4H), 7.26-7.29 (m, 2H), 7.33-7.38 (m, 2H), 7.45-7.50 (m, 4H), 7.55-7.64 (m, 7H); ^{13}C NMR (100 MHz, CDCl$_3$): 47.11, 122.93, 127.50, 128.55, 129.19, 130.39, 130.80, 132.61, 132.89, 133.36, 136.15, 137.09, 137.36, 184.11; ESI-MS (m/z): 571.96 (M+H)$^+$, 572.94(M+2)$^+$; Anal. Calcd for $C_{25}H_{19}Br_2NO_3S$: C, 52.38; H, 3.34; N, 2.44; Found: C, 52.40; H, 3.55; N, 2.46.

(*3E,5E*)-3,5-Bis(4-trifluoromethyl-benzylidene)-1-(phenylsulfonyl)piperidin-4-one (25): Yield: 65%; mp 216-218 ^{o}C; IR (cm^{-1}, Film): 2921, 1612, 1326, 1167, 1121, 1070, 956; ^{1}H NMR (400 MHz, CDCl$_3$): 4.60 (s, 4H), 7.43-7.50 (m, 6H), 7.60-7.64 (m, 3H), 7.72-7.76 (m, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): 47.00, 125.87, 125.91, 127.49, 129.45, 130.24, 131.21, 131.51, 133.46, 137.17, 137.43, 137.55, 184.28; ESI-MS (m/z): 552.16 (M+H)$^+$; Anal. Calcd for $C_{27}H_{19}F_6NO_3S$: C, 58.80; H, 3.47; N, 2.54; Found: C, 58.92; H, 3.61; N, 2.36.

Crystallography Data

Single crystals suitable for the X-ray diffraction studies for compound **11** were grown by the slow diffusion of diethyl ether to a DMF solution of compound at room temperature. The diffraction data were collected on an Oxford CCD diffractometer having Xcalibur, sapphire diffraction measurement device at 293 K, using graphite-monochromated Mo-Ka radiation (λ = 0.71073 Å).[33]The multi-scan absorption correction was applied using CrysalisPRO. The crystal structures were solved by the direct methods using SIR-92 and refined by full-matrix least-squares refinement techniques on F^2 using SHELXL97. Hydrogen atoms were placed into the

calculated positions and included in the last cycles of the refinement. All calculations were done using WinGX software.[34]The crystallographic data collection and structure refinement parameters forall complexes are summarized in table 2.CCDC 971312 contains the supplementary crystallographic data for compound 11. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

(b) Anti-cancer screening:

   The anti-cancer screening was carried to following the procedure described earlier.
 http://dtp.nci.nih.gov/branches/btb/ivclsp.html.

(c) Mechanism of action studies

Materials and methods

Experimental Compound Stock Solutions

   Three mMstock solutions were prepared in culture grade DMSO under sterile conditions. All experiments were done at the 10µM dose as used in the NCI 60 cell line screening protocol which was previously performed for these compounds.

Cell culture

   The cell line used in this study was the COLO 205 human colorectal adenocarcinoma (ATCC CCL-222). These cultures were maintained in RPMI 1640 media (ATCC, Manassas VA) supplemented with 10% fetal clone serum (ATCC). Cultures were maintained at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. For all experiments approximately $3x10^6$ cells were treated with the test compound and controls (DMSO and camptothecin).

Annexin V

Annexin V staining is a useful tool in determining the type of cell death. It has been used to detect apoptotic cells after exposure to various substances,[35] the expression of phosphatidylserine (PS) on the surface of cells can be used to determine if cells are undergoing apoptosis. Cells were exposed to the test compounds for 24 hours then stained with annexin V conjugate, andpropidium iodide (Biotium,Hayward, CA). After staining cells were analyzed using the Nucleo Counter NC3000 instrument (Chemometec, Allerød, Denmark) this instrument utilizes differential microscopy to detect fluorophores. A one way ANOVA was performed. If significant results were found in the ANOVA, a Post Hoc Test Tukey was also performed.

DNA Fragmentation

DNA Fragmentation as an apoptosis marker is a widely used assay in apoptosis studies.[36] We utilized the NC3000 system for this experiment. DNA fragmentation can be quantified using DNA content and measuring cells containing less than 1DNA equivalent (known as Sub - G_1). The NC3000 fragmentation assay is based on removal of small DNA fragments and retention of 4',6-diamidino-2-phenylindole (DAPI) stained higher weight fragments.

Caspase activity

Activation of effector caspases is key event in apoptosis. These enzymes are responsible for many of the typical hallmarks of apoptotic cell death.[37] Caspase activation was measured using the Fluorescent Labeled Inhibitors of Caspases (FLICA). These probes bind covalently with active caspase effect or enzymes. After treatment as described above, cells were harvested and

stained using the green FAM FLICA kit (Immunochemistry Technologies, Bloomington Min.) then analyzed using the Nucleocounter NC3000 instrument.

Conclusions

A series of novel 3,5-bis(arylidiene)-4-piperidone based monocarbonyl analogues of curcumin were synthesized and screened for their potential anticancer activity. Two compounds (**10**) and (**22**) showed significant inhibition against various human tumor cell lines. Mechanism studies with COLO205 cell suggests compound **10** activates both caspase 8, 9, and effector caspase 3 (this one moderately), which combined with the DNA fragmentation event suggest an apoptotic mechanism. While, compound **22** gives the characteristic Annexin positive result, DNA fragmentation, and caspase 3 activation. The activation of caspase 8 but not caspase 9 however, suggests an apoptosis extrinsic mechanism.

Notes and references

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†Electronic Supplementary Information (ESI) available: [one dose mean graphs, drug response curves, five dose mean graphs, GI_{50} and LC_{50} values of compound **10, 14, 22** and **23.** ^{1}H and ^{13}C NMR spectra of compound **8-25**]. See DOI: 10.1039/b000000x .

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Graphical abstract

Novel 3,5-bis(arylidiene)-4-piperidone based monocarbonyl analogues of curcumin: Anticancer activity evaluation and mode of action study

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Piperidone-sulphonamide and curcumin based molecular hybrids were synthesised, which showed anti-cancer activity on 60 human tumor cell lines panel and their inhibitory effect due to apoptosis.