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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

C_5-curcuminoid-4-aminoquinoline based molecular hybrids: Design, synthesis and mechanistic investigation of anticancer activity

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Received (in XXX, XXX) XthXXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Abstract: The privileged scaffolds of curcumin and 4-aminoquinolines are extensively used in the design and synthesis of biodynamic agents having remarkable efficacy against diseases like cancer and malaria. Therefore, we anticipated that covalent hybridization of these two pharmacophore *via* triazole linker may lead to molecules with better anticancer activity. The synthesized hybrid compounds were tested for their anti-cancer activity on 60 human cancer cell lines, which represent diverse histologies. Our study has identified a set of these hybrids that showed excellent growth inhibition at nano-molar concentrations. The mechanistic investigations through a series of assays showed apoptotic induction as a cause for their displayed anticancer activity.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008 and expected to cross the figure of around 13 million deaths by 2030. About 70% cancer related deaths are mainly due to lung, colon, liver, stomach and breast cancers.[1,2] It is well established that there is no single treatment of cancer and patients often receive a combination of therapies and palliative care, such as surgery, radiation, immunotherapy, chemotherapy or gene therapy depending on the type and stage of the cancer, health status, age and personal characteristics.[3] Anticancer drugs such as alkylating agents,[4-8] antimetabolites,[9-11]plant alkaloids,[12-15] topoisomerase inhibitors[16,17] and cytotoxic antibiotics[18] have been used extensively in chemotherapy. Among these, natural products show good promises in the development of anticancer molecules[19,20] and curcumin is one such natural product which has been extensively studied over the past few decades.[21] These studies revealed that curcumin shows anticancer activity against prostate cancer,[22] cervical cancer,[23] colorectal carcinoma,[24] leukemia[25] and human breast cancer cells.[26] The clinical use of curcumin has been hampered due to its poor solubility, absorption, bioavailability and rapid metabolism.[27-30] The pharmacokinetic studies reveals that the β-diketone functionality of curcumin is a substrate for liver aldoketo reductases and this may be one of the reasons for its rapid *in vivo* metabolism.[31] To overcome these limitations, several approaches have been explored and replacement of central diketo functionality with mono carbonyl has resulted many compounds with improved anticancer activity, pharmacokinetic properties and bioavailability.[32-36]

In recent years, concept of molecular hybrids has gained considerable importance due to the problem of drug resistance and it is anticipated that these 'dual-drugs' or 'double-drugs' synthesised by covalent hybridization of two or more pharmacophores may solve the problem of drug resistance.[37-39] In this scenario, the synthesis of molecular hybrids by conjugating C_5-curcuminoid pharmacophore with other biologically active entities, especially those which are known for their anticancer activity may exhibit better anticancer properties in comparison to none conjugate counterparts.[40] Quinoline is one such privileged pharmacophore that possess an array of biological activities including antimalarial, anti-proliferative and antitumor activity. Among the many synthesized molecules containing quinoline scaffold, camptothecin and its synthetic derivatives topotecan and irinotecan are well known for their antitumor activity and these compounds display their anticancer activity by inhibiting DNA enzyme topoisomerase I (Fig. 2).[41] So, based on these observations and in continuation of our efforts of developing structurally diverse molecules of medicinal importance,[42-45] we designed molecular hybrids by clubbing together C_5-curcuminoids and quinoline nucleus by covalently linking it through 1,2,3-triazole ring. Our rational for choosing 1,2,3-triazole linker is based on the fact that triazole shows favourable physiochemical properties in biological system and can be conveniently synthesized by click chemistry.[46,47]

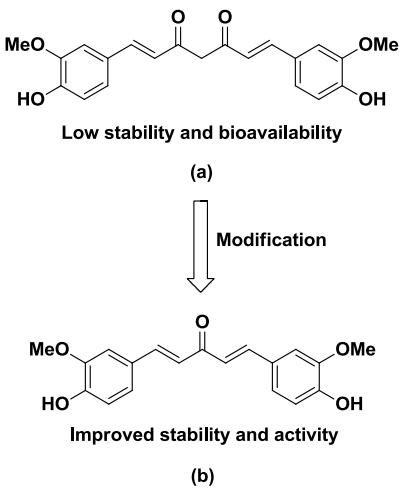


Fig.1: (a) Curcumin; (b) C5-Curcuminoid

2. Results and Discussion

2.1 Chemistry

The targeted C_5-curcuminoid-4-aminoquinoline based hybrids (**7a-g** and **8a-g**) were synthesized as depicted in scheme 1. To start with, firstly commercially available 4,7-dichloroquinoline

(**1**) was reacted with linear chain aminoalcohols under neat conditions to yield 4-aminoquinolines with a free hydroxyl group at the terminal position (**2a-b**) in excellent yield.

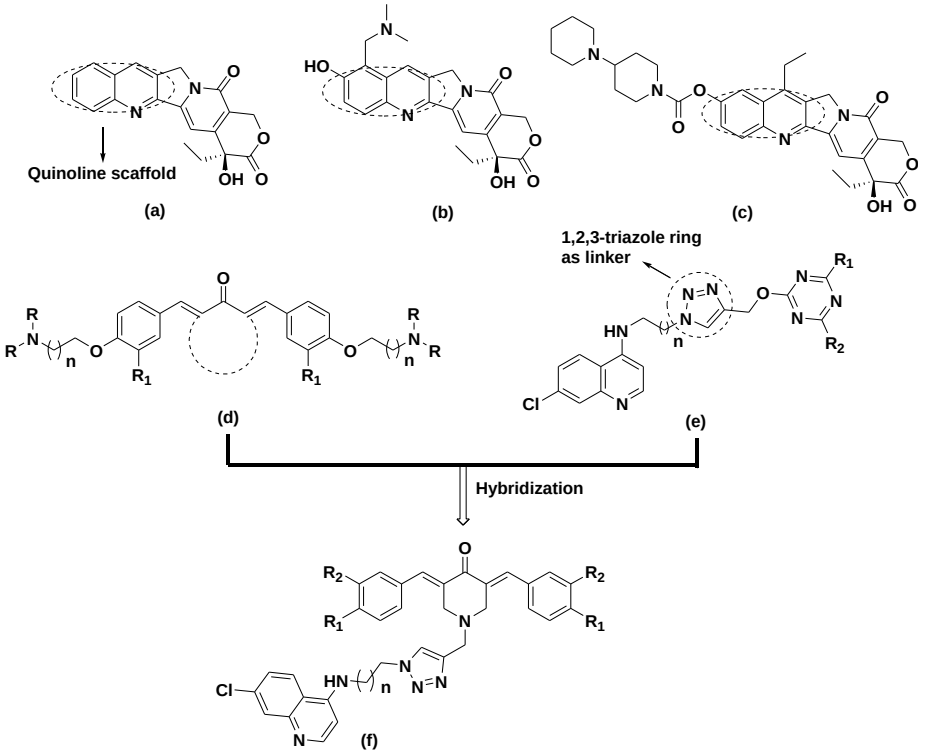
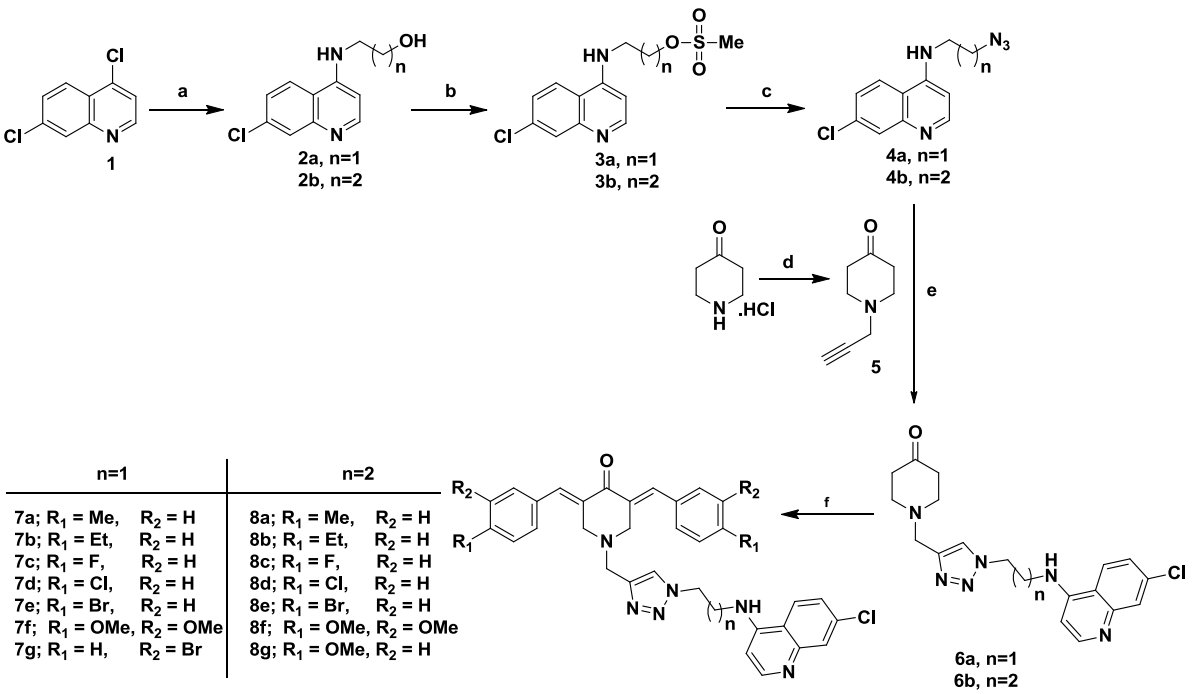


Fig. 2: (a) Camptothecin; (b) Topotecan; (c) Irinotecan; (d) C_5-curcuminoids and (e) 4-aminoquinoline based hybrid molecules reported previously from our research group; (f) C_5-curcuminoid-4-aminoquinoline based hybrids synthesized in the present investigation.



n=1	n=2
7a; R_1 = Me, R_2 = H	8a; R_1 = Me, R_2 = H
7b; R_1 = Et, R_2 = H	8b; R_1 = Et, R_2 = H
7c; R_1 = F, R_2 = H	8c; R_1 = F, R_2 = H
7d; R_1 = Cl, R_2 = H	8d; R_1 = Cl, R_2 = H
7e; R_1 = Br, R_2 = H	8e; R_1 = Br, R_2 = H
7f; R_1 = OMe, R_2 = OMe	8f; R_1 = OMe, R_2 = OMe
7g; R_1 = H, R_2 = Br	8g; R_1 = OMe, R_2 = H

Scheme 1(Reagents and conditions): (a) Aminoalcohol, neat, 130-150 ^oC, under N_2, 10-12 h; (b) MsCl, Et_3N, THF, 0 ^oC-rt, under N_2, 1h; (c) NaN_3, DMF, 50 ^oC, 4-5 h; (d) Propargyl bromide, K_2CO_3, $CHCl_3$, H_2O, rt, 5-6 h; (e) t-Butanol: H_2O (1:1), $CuSO_4.5H_2O$, sodium ascorbate, 40-60 ^oC, 3h; (f) Substituted benzaldehydes, 20% aq. NaOH (w/v), ethanol, rt, 4-5 h.

The free hydroxyl group was chemoselectively *O*-mesylated by literature method[45] using mesyl chloride and triethylamine as a base to yield mesylated 4-aminoquinolines (**3a-b**). Subsequently, the mesylated products (**3a-b**) were converted into corresponding azides (**4a-b**) using sodium azide as nucleophile and DMF as solvent at 50 °C. These azides (**4a-b**) were reacted with 1-(prop-2-yn-1-yl)piperidin-4-one (**5**) by click chemistry using standard protocols of sodium ascorbate and copper sulfate as catalyst and an equimolar ratio of water and *t*-butanol as solvents to yield 1,2,3-triazole linker contained intermediates (**6a-b**). In the final step, the intermediates (**6a-b**) were subjected to aldol condensation with substituted benzaldehydes in the presence of NaOH as base in ethanol to get the final targeted products (**7a-g** and **8a-g**). It may be noted that, the intermediate compound 1-(prop-2-yn-1-yl)piperidin-4-one (**5**) was synthesized *via* nucleophilic substitution reaction between 4-piperidone hydrochloride monohydrate and propargyl bromide in the presence of anhydrous K_2CO_3 in the biphasic system [$CHCl_3$:H_2O (1:1)] at room temperature in good yield.

2.2 Biological studies

2.2.1 Anticancer activity

In the present study, a series of C_5-curcuminoid-4-aminoquinoline based molecular hybrids (**7a-g** and **8a-g**) were synthesized and in order to explore the therapeutic potential of these hybrids, a representative subset of these compounds was screened for their growth inhibitory activity and cytotoxicity against 60 different human cancer cell lines. The screening was carried out by following a standard procedure described earlier.[48] In the screening panel, 60 human cancer cell lines are organized into nine subpanels representing diverse histology's: leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate, and central nervous system. The cells were grown in supplemented RPM1 1640 medium for 24 h. The test compounds were dissolved in DMSO and incubated with cells at five concentrations with 10-fold dilutions, the highest concentration being 10^{-4} M and the others 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M. The assay was terminated by the addition of cold trichloroacetic acid, and the cells are fixed and stained with sulforhodamine B. The bound stain was solubilized, and the absorbance was 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 screening process was a two-stage process started with the evaluation of compound against the 60 human tumor cell lines with a single dose of 10.0 µM, which was carried out by following the same protocol as for five dose screening. Only the compound which showed more than 60% of growth inhibition in at least 8 tumor cell lines was selected for further testing and the others were assumed inactive. The initial testing at a single dose of 10 µM found compounds **7d** and **7f** causing significant inhibition of several cell lines. Both compounds were further tested for dose response effect at five concentration levels (100, 10, 1.0, 0.1 and 0.01 µM). The mean values for GI_{50} and LC_{50} on all 60 cell lines are given in Table 1. Additional data for both compounds i.e. one dose results, mean graphs, drug response curves, five dose mean graphs and GI_{50} and LC_{50} values are given in the supplementary information. The dose response curves of **7d** and **7f** for all 60 cell lines are illustrated in Fig. 3.

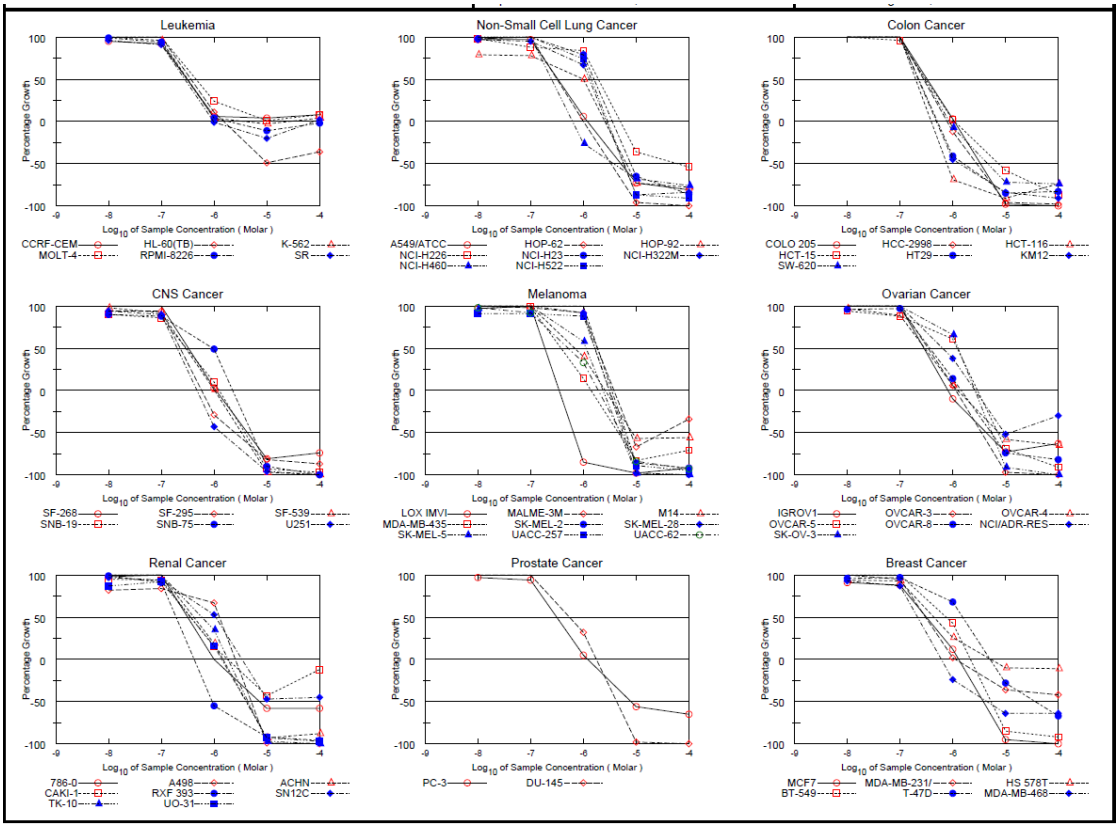
Mean graph patterns reflect the mechanism of action *in vitro*. The graph of a dose-response curve helps in determining effective concentrations of an agent. Fig. 3 & 4 shows the dose-response curves for each of the nine cancer panels. These graphs display the effective variations in sensitivity of cell lines in different cancer panels. As can be seen from these graphs, both compounds have a similar effect on the cell lines suggesting that the class of molecular hybrid apparently attack the same protein target.

Table 1: Antitumor activity (GI_{50}/µM)[a] and toxicity (LC_{50}/µM)[b] data of compounds **7d** and **7f** selected for five dose studies for theNCI60-cell line screen.

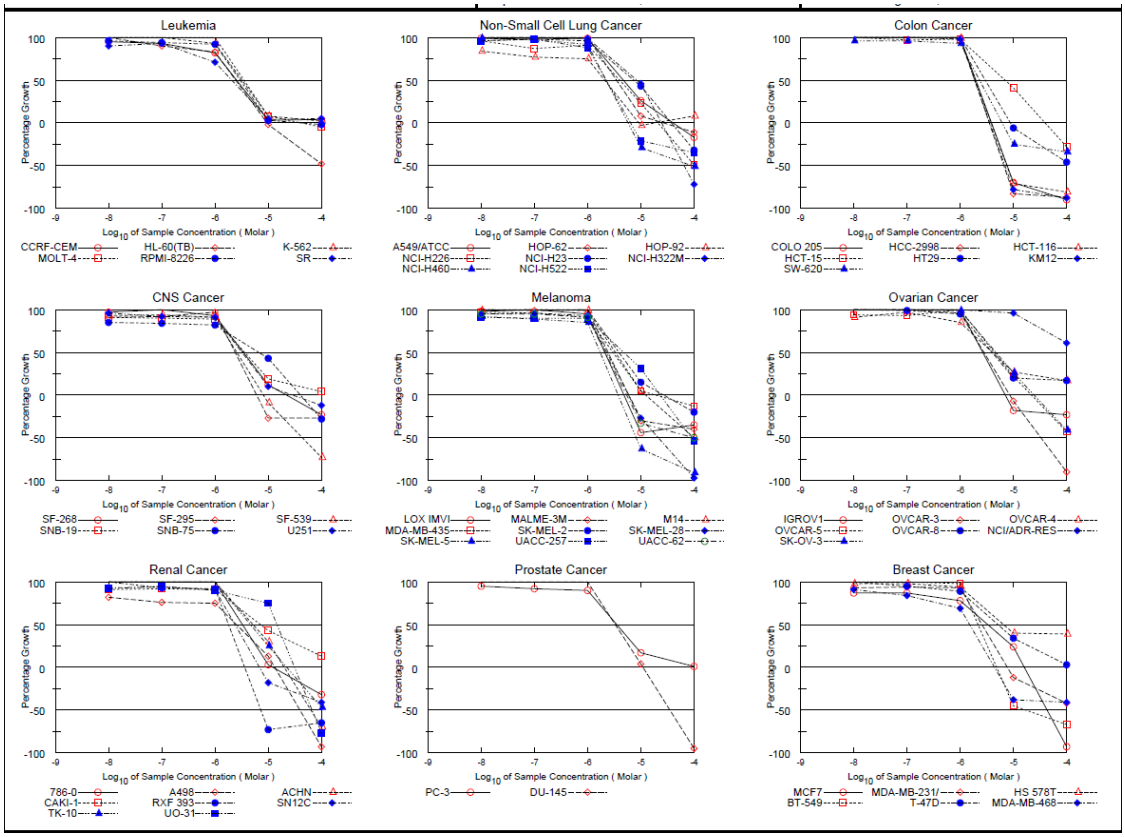
	GI_{50} (µM) **(7d)**	LC_{50} (µM) **(7d)**	GI_{50} (µM) **(7f)**	LC_{50} (µM) **(7f)**
Leukemia				
CCRF-CEM	0.309	>100	2.54	>100
HL-60(TB)	0.373	>100	2.42	>100
K-562	0.317	>100	3.21	>100
MOLT-4	0.499	>100	3.50	>100
RPMI-8226	0.306	>100	3.00	>100
SR	0.280	>100	2.04	>100
Non-small cell Lung Cancer				
A549/ATCC	0.329	5.15	4.73	>100
HOP-62	0.316	3.31	3.90	>100
HOP-92	0.989	6.47	2.09	>100
NCI-H226	1.920	5.96	4.04	>100
NCI-H23	1.580	7.89	7.45	>100
NCI-H322M	1.290	5.77	8.25	64.6
NCI-H460	0.250	3.79	2.22	87.2
NCI-H522	1.410	5.86	2.24	>100
Colon Cancer				
COLO 205	0.354	3.35	1.99	7.65

HCC-2998	0.313	2.83	1.96	6.68
HCT-116	0.205	0.77	1.95	7.57
HCT-15	0.308	7.23	6.87	>100
HT 29	0.241	1.61	3.13	>100
KM 12	0.231	1.36	1.87	6.94
SW-620	0.295	4.55	2.32	>100
CNS Cancer				
SF-268	0.335	4.28	3.36	>100
SF-295	0.228	2.50	2.41	>100
SF-539	0.289	3.29	2.58	43.4
SNB-19	0.300	3.85	3.59	>100
SNB-75	0.936	5.17	6.67	>100
U 251	0.199	1.37	3.20	>100
Melanoma				
LOX IMVI	0.189	0.651	2.10	>100
MALME-3M	1.85	NA	2.78	>100
M 14	0.689	8.50	3.35	>100
MDA-MB-435	0.373	4.56	3.01	>100
SK-MEL-2	2.01	6.55	3.88	>100
SK-MEL-28	1.67	5.59	2.29	21.2
SK-MEL-5	1.12	4.91	1.72	8.15
UACC-257	1.64	6.02	4.80	88.6
UACC-62	0.512	5.14	2.14	>100
Ovarian Cancer				
IGROV1	0.316	4.36	2.68	>100
OVCAR-3	0.383	3.50	2.80	33.0
OVCAR-4	0.297	7.49	4.07	>100
OVCAR-5	1.22	7.12	4.55	>100
OVCAR-8	0.367	5.35	3.94	>100
NCI/ADR-RES	0.673	NA	>100	>100
SK-OV-3	1.26	5.45	4.66	>100
Renal Cancer				
786-0	0.316	7.31	3.35	>100
A498	1.26	5.08	2.53	3.92
ACHN	0.414	4.13	5.25	6.26
CAKI-1	0.362	>100	7.22	>100
RXF 393	0.190	0.920	1.81	7.27
SN 12C	1.08	>100	2.36	>100
TX-10	0.603	4.41	4.96	>100
UO-31	0.352	4.01	14.50	66.20
Prostate Cancer				
PC-3	0.315	8.04	3.57	>100
DU-145	0.580	4.30	3.48	35.40
Breast Cancer				
MCF7	0.318	3.80	3.30	43.00
MDA-MB-231/ATCC	0.303	>100	2.55	>100
HS 578T	0.436	>100	6.49	>100
BT-549	0.792	5.36	2.16	16.80
T-47D	1.53	37.3	5.05	>100
MDA-MB-468	2.17	4.51	1.51	>100

[a]GI_{50}: 50% Growth inhibition; concentration of drug resulting in a 50% reduction in net protein increase compared with control cells.
[b]LC_{50}: Lethal concentration; concentration of drug lethal to 50% of cells.



(a)



(b)

Fig. 3: (a) Dose response curves for individual cancer panel (Compound **7d**); (b) Dose response curves for individual cancer panel (Compound **7f**)

2.2.2 Mechanism of action study: In the initial screening compounds 7d and 7f showed significant effect on the colon cancer panel. Therefore, to understand the mode of their action, subsequent studies were carried out using COLO205 cell lines. These cells provide a system for careful analysis of the immunobiology and antigenicity of human adenocarcinoma of the colon without the complications of histocompatibility differences. The details of our experiments are given below.

2.2.2.1 Annexin V

Annexin V screening was performed to assess apoptotic cells. The values obtained from the percentage of apoptotic cells were 97% for compound **7f** and 63.5% for compound **7d**. Results obtained revealed statistically significant ($P < 0.05$) activity when compared to the negative control suggesting apoptosis activity (Fig. 4). Compound **7d** presented 30% of cells at the late apoptotic stage (Fig. 5) whereas compound **7f** presented a majority of cells (79%) at an early apoptotic stage. These results indicated that both compounds are apoptosis inducers although with varying degrees since there are differences between both experimental compounds indicated by the early/late apoptotic stages.

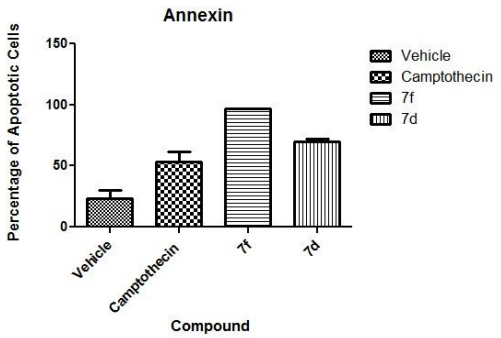


Fig.4: Annexin V

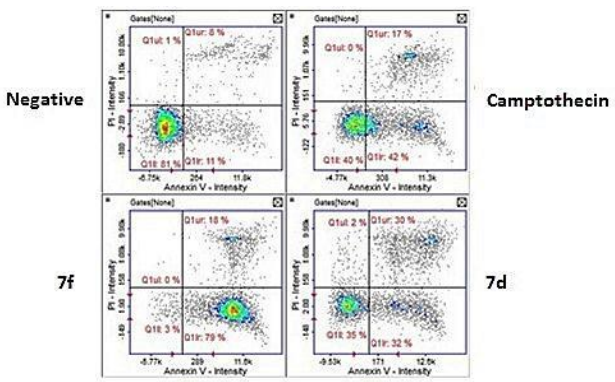


Fig. 5: Distribution of apoptotic cells

2.2.2.2 DNA Fragmentation

Cells with fragmented DNA are characteristic of an apoptotic death mechanism. This event is measured as a confirmatory event in the apoptotic pathway. Our experimental data present a statistically significant ($P < 0.05$) activity of both experimental compounds. Compound **7f** presented 47% of cells with fragmented DNA while it was 54% in the case of compound **7d**. These results are significant when compared to the negative control which presented 8.5% of cells with fragmented DNA (Fig. 6).

2.2.2.3 Caspase activation

(a) Caspase 3 and 7: The activation of effector caspases is one of the key late events in apoptosis. Our test compounds demonstrated high activation of these caspases. Compound **7f** gave the highest activation at 98% of cells while compound **7d** presented 59.5% of cells with activated caspases (Fig. 7). Although both compounds caused significant activation, results of compound **7f** were comparable ($P < 0.05$) to the camptothecin positive control. A key difference between these two compounds is the apoptotic stage after the 24 hour exposure. Compound **7f** exposed cells were present at the early apoptotic stage while the majority of compound **7d** exposed cells are at the late apoptotic stage (Fig. 8).

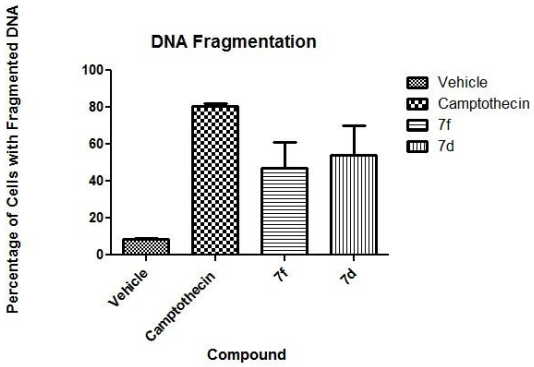


Fig.6: DNA Fragmentation on COLO 205 cells

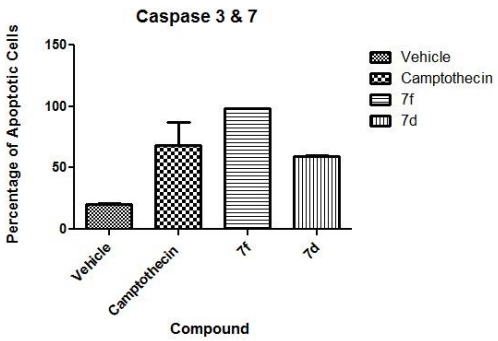


Fig.7: Caspase 3 and 7 activation.

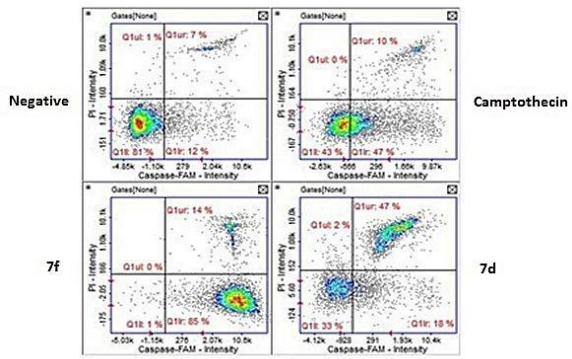


Fig. 8: Distribution of effector caspases

(b) Caspase 8: Caspase 8 activation is one of the key effects in extrinsic mechanism apoptosis.[49] Our experiments presented varied activity among the test compounds. Compound **7f** presented the highest activation at 86.5% of cells with activated caspase 8 (Fig. 9). The majority of these cells were at the early apoptotic stage (Fig. 10). Compound **7d** presented moderate induction of caspase 8 at 41% with the majority of apoptotic cells at the late apoptotic stage. Both compounds caused higher induction than the camptothecin positive control.

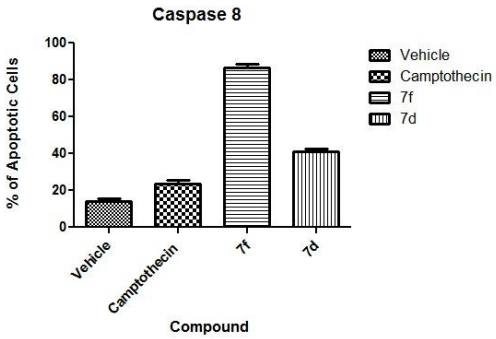


Fig. 9: Caspase 8 activation.

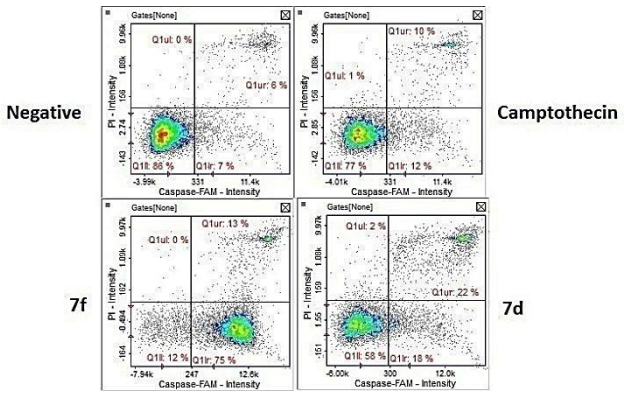


Fig. 10: Caspase 8 apoptotic cell distribution

(c) Caspase 9: Caspase 9 activation is characteristic of the intrinsic apoptotic pathway.[50] Our compounds caused distinct activation of caspase 9. Compound **7f** caused the highest activation of caspase 9 with an average at 91.5% of cells (Fig. 11) with apoptotic cells at the early apoptotic stage (Fig. 12). Compound **7d** did not activate caspase 9 in a significant manner suggesting an extrinsic mechanism.

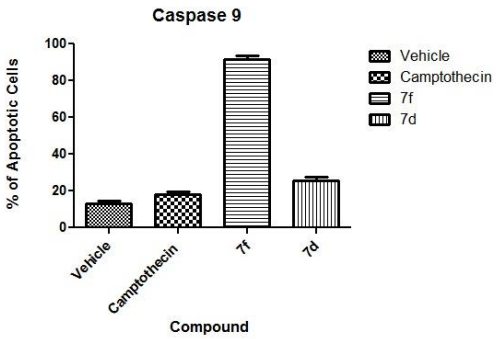


Fig. 11: Caspase 9 activation.

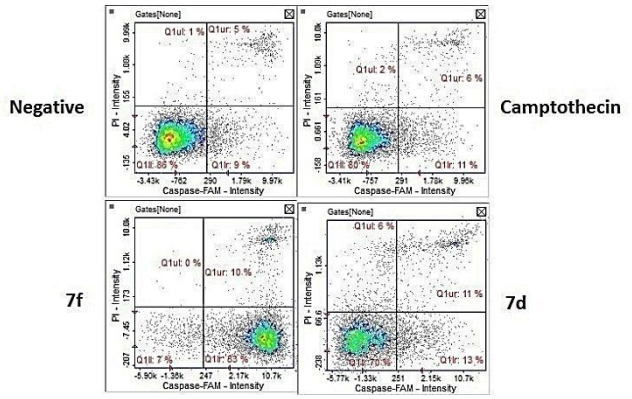


Fig. 12: Apoptotic cell distribution

3. Conclusion

We have synthesized a new class of C_5-curcuminoid-4-aminoquinoline molecular hybrid and a detailed screening of representative compounds has demonstrated their potential as anticancer agents**.** Mechanistic studies of test compounds **7d** and **7f** presented positive annexin V staining, which is characteristic of apoptotic cell death after chemical insult.[51]After initial screening, we decided to explore further markers for apoptosis to determine any additional mechanistic insights, and tested for DNA fragmentation which is another hallmark of apoptosis. This nuclease mediated event occurs after activation of DNase by effector caspases.[52] Our results confirmed that indeed both compounds (**7d** and **7f**) cause apoptotic cell death since the cells exposed to them presented fragmentation of DNA and activation of effector caspases in a significant manner. Interestingly, both compounds caused activation of caspase 8 while compound **7f** also caused activation of caspase 9. This simultaneous activation of both extrinsic and intrinsic apoptosis has been observed in heterocyclic compounds such as quinoline derivatives,[53] where an increase in the pro apoptotic protein Bax causes the release of cytochrome C activation of caspase 9 and 8 followed by the effector caspase 3. These results have established the anticancer activity of our newly synthesized molecular hybrids which merits for their development as potential drug agents.

4. Experimental Section

4.1 Chemistry

4.1.1. Instrumentation and Chemicals

All the starting materials used in the synthesis were purchased from Sigma-Aldrich (Bangalore, Karnataka, India) and were used as such. Thin layer chromatography (E. Merck Kieselgel 60 F_{254}) was used to monitor the progress of the reactions. The synthesized compounds were purified using the silica gel column. Distilled solvents were used for purification purposes. Melting points were measured in open capillary tubes on an ERS automated melting point apparatus. IR spectra were recorded using a Perkin-Elmer FT-IR spectrophotometer and the values are expressed as λ_{max} cm^{-1}. The ^{1}H and ^{13}C NMR spectra were recorded on Jeol Spectrospin 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. Mass spectral data were recorded on a Jeol-AccuTOF JMST100LC and micromass LCT Mass Spectrometer/Data system. Elemental analyses were performed on a Carlo Erba Model EA-1108 elemental analyzer and data of C, H and N are within ±0.4% of calculated values.

Procedure for synthesis of 1-((1-(2-((7-chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl) piperidin-4-one (6a) and similar compound (6b):

To a stirred solution of compound 5 (2 g, 14.59 mmol) and compound 4a (3.60 g, 14.59 mmol) in *t*-butanol (40 mL) at room temperature, a solution of $CuSO_4.5H_2O$ (0.7279 g, 2.918 mmol) and sodium ascorbate (1.1566 g, 5.836 mmol) in water (40 mL) was added. The temperature was increased to 40-60 ^oC and the reaction mixture was stirred for 3-4 h. After completion of reaction as evident by TLC, $CHCl_3$ (100 mL) was added and organic layer was washed with water (3 × 500 mL) and finally with brine (200 mL). The organic layer was then dried over Na_2SO_4 and excess of solvent was evaporated to dryness to get the pure product. Yield: 72%; mp: 161-162 ^oC; IR (cm^{-1}, Film): 3233, 3065, 2950, 1715, 1609, 1579, 1544, 1354; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.40 (t, 4H, *J* = 5.95 Hz), 2.76 (t, 4H, *J* = 5.95 Hz), 3.75 (s, 2H), 3.87-3.91 (m, 2H), 4.72 (t, 2H, *J* = 5.50 Hz), 5.96 (br s, 1H), 6.39 (d, 1H, *J* = 5.50 Hz), 7.35 (dd, 1H, J_{12} = 2.29 Hz, J_{13} = 8.70 Hz), 7.52 (s, 1H), 7.70 (d, 1H, *J* = 8.70 Hz), 7.94 (d, 1H, *J* = 1.83 Hz), 8.52 (d, 1H, *J* = 5.50 Hz); ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 41.0, 42.7, 48.6, 52.2, 52.7, 98.7, 117.1, 121.1, 123.5, 125.8, 128.6, 135.3, 144.8, 148.9, 149.0, 151.7, 208.3; ESI-MS (*m/z*): 385.15 $(M+H)^+$, 386.15 $(M+2)^+$; Anal. Calcd for $C_{19}H_{21}ClN_6O$: C, 59.29; H, 5.50; N, 21.84; Found: C, 59.30; H, 5.51; N, 21.83%.

1-((1-(3-((7-Chloroquinolin-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperidin-4-one (6b): Yield: 71%; mp: 120-121 ^oC; IR (cm^{-1}, Film): 3338, 3054, 2923, 1717, 1610, 1581, 1050, 730; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.36 (p, 2H, *J* = 6.41 Hz), 2.42 (t, 4H, *J* = 5.95 Hz), 2.77 (t, 4H, *J* = 5.95 Hz), 3.41-3.47 (m, 2H), 3.75 (s, 2H), 4.54 (t, 2H, *J* = 5.95 Hz), 5.84 (br s, 1H), 6.36 (d, 1H, *J* = 5.50 Hz), 7.35 (dd, 1H, J_{12} = 2.29 Hz, J_{13} = 8.70 Hz), 7.55 (s, 1H), 7.75 (d, 1H, *J* = 8.70 Hz), 7.92 (d, 1H, *J* = 1.83 Hz), 8.47 (d, 1H, *J* = 5.50 Hz); ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 28.3, 40.1, 40.9, 47.9, 52.2, 52.7, 98.7, 117.1, 121.4, 122.9, 125.5, 128.2, 135.1, 144.9, 148.6, 149.6, 151.4, 208.4; ESI-MS (*m/z*): 399.16 $(M+H)^+$, 400.17 $(M+2)^+$; Anal. Calcd for $C_{20}H_{23}ClN_6O$: C, 60.22; H, 5.81; N, 21.07; Found: C, 60.23; H, 5.80; N, 21.09%.

Procedure for the synthesis of (3*E*,5*E*)-1-((1-(2-((7-chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl) methyl)-3,5-bis(4-methylbenzylidene)piperidin-4-one (7a) and similar compounds (7b-g and 8a-g):

To a well stirred solution of compound 6a (0.2 g, 0.51 mmol) and 4-methylbenzaldehyde (0.131 g, 1.09 mmol) in ethanol (10 mL), aqueous NaOH solution (2 mL, 20% w/v) was added drop-wise and kept for stirring at rt for 4-5 h. After completion of reaction as evident by TLC, a saturated solution of NH_4Cl was added to quench the reaction followed by extraction of reaction mixture with ethylacetate (2 × 20 mL). The organic layer was dried with anhydrous Na_2SO_4 and the crude product obtained was further purified by column chromatography. Yield: 70%; mp: 230-232 ^oC; IR (cm^{-1}, Film): 3351, 3067, 2922, 1609, 1583, 1440, 1266, 1179, 1058, 877, 512; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.34 (s, 6H), 3.71-3.75 (m, 2H), 3.86 (s, 6H), 4.59 (t, 2H, *J* = 5.86 Hz), 5.84 (br s, 1H), 6.34 (d, 1H, *J* = 5.13 Hz), 7.18 (d, 4H, *J* = 8.05 Hz), 7.25 (d, 4H, *J* = 8.05 Hz), 7.31 (dd, 1H, J_{12} = 1.46 Hz, J_{13} = 8.79 Hz), 7.37 (s, 1H), 7.62 (d, 1H, *J* = 8.79 Hz), 7.75 (s, 2H), 7.92 (d, 1H, *J* = 2.2 Hz), 8.48 (d, 1H, *J* = 5.13 Hz); ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 21.3, 42.7, 48.5, 52.3, 54.6, 98.7, 117.0, 121.2, 123.6, 125.9, 128.2, 129.3, 130.5, 132.1, 132.2, 135.4, 136.5, 139.5, 144.9, 148.5, 149.1, 151.3, 187.2; ESI-MS (*m/z*): 589.25 $(M+H)^+$, 590.24 $(M+2)^+$; Anal. Calcd for $C_{35}H_{33}ClN_6O$: C, 71.36; H, 5.65; N, 14.27; Found: C, 71.38; H, 5.66; N, 14.26%.

(3*E*,5*E*)-1-((1-(2-((7-Chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(4-ethylbenzylidene)piperidin-4-one (7b): Yield: 66%; mp: 181-183 ^oC; IR (cm^{-1}, Film): 3357, 3056, 2963, 1604, 1578, 1447, 1196, 1140, 1074, 1056, 553; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 1.22 (t, 6H, *J* = 8.05 Hz), 2.64 (q, 4H, *J* = 8.05 Hz), 3.72-3.78 (m, 2H), 3.87 (s, 6H), 4.61 (t, 2H, *J* = 5.49 Hz), 6.02 (br s, 1H), 6.32 (d, 1H, *J* = 5.86 Hz), 7.21 (d, 4H, *J* = 8.05 Hz), 7.27 (d, 4H, *J* = 8.05 Hz), 7.30 (s, 1H), 7.41 (s, 1H), 7.66 (d, 1H, *J* = 8.79 Hz), 7.75 (s, 2H), 7.90 (d, 1H, *J* = 1.46 Hz), 8.49 (d, 1H, *J* = 5.13 Hz), ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 15.2, 28.7, 42.7, 48.4, 52.3, 54.6, 98.6, 116.9, 121.5, 123.7, 126.0, 128.2, 127.7, 130.6, 132.1, 132.4, 135.6, 136.5, 144.8, 145.8, 147.9, 149.5, 150.7, 187.2; ESI-MS (*m/z*): 617.29 $(M+H)^+$, 618.28 $(M+2)^+$; Anal. Calcd for $C_{37}H_{37}ClN_6O$: C, 72.00; H, 6.04; N, 13.62; Found: C, 72.02; H, 6.05; N, 13.62%.

(3*E*,5*E*)-1-((1-(2-((7-Chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(4-fluorobenzylidene) piperidin-4-one (7c): Yield: 54%; mp: 217-219 ^oC; IR (cm^{-1}, Film): 3246, 3065, 2928, 1610, 1581, 1266, 1187, 1104, 1079; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 3.75-3.78 (m, 2H), 3.83 (s, 4H), 3.86 (s, 2H), 4.64 (t, 2H, *J* = 5.49 Hz), 5.93 (br s, 1H), 6.34 (d, 1H, *J* = 5.86 Hz), 7.06 (d, 4H, *J* = 8.05 Hz), 7.29-7.34 (m, 5H), 7.43 (s, 1H), 7.65 (d, 1H, *J* = 8.79 Hz), 7.72 (s, 2H), 7.90 (d, 1H, *J* = 2.20 Hz), 8.46 (d, 1H, *J* = 5.13 Hz), ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 42.3, 48.0, 51.2, 53.2, 98.5, 115.6, 115.8, 116.9, 121.2, 123.8, 126.0, 128.12, 131.1, 132.3, 132.4, 135.6, 135.4, 144.6, 148.3, 151.1, 161.6, 186.3; ESI-MS (*m/z*): 597.20 $(M+H)^+$, 598.20 $(M+2)^+$; Anal. Calcd for $C_{33}H_{27}ClF_2N_6O$: C, 66.38; H, 4.56; N, 14.08; Found: C, 66.40; H, 4.54; N, 14.09%.

(3*E*,5*E*)-3,5-Bis(4-chlorobenzylidene)-1-((1-(2-((7-chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl) piperidin-4-one (7d): Yield: 74%; mp: 229-231 ^oC; IR (cm^{-1}, Film): 3368, 3065, 2924, 1611, 1585, 1404, 1095, 525; ^1H NMR (400 MHz; DMSO-d_6; Me_4Si): 3.63 (s, 4H), 3.72 (q, 2H, *J* = 5.95 Hz), 3.78 (s, 2H), 4.58 (t, 2H, *J* = 5.95 Hz), 6.41 (d, 1H, *J* = 5.50 Hz), 7.29 (dd, 1H, J_{12} = 2.29, J_{13} = 8.70 Hz), 7.39-7.41 (m, 5H), 7.47(d, 4H, *J* = 8.70 Hz), 7.52 (s, 2H), 7.65 (d, 1H, *J* = 2.29 Hz), 8.00 (s, 1H), 8.07 (d, 1H, *J* = 9.16 Hz), 8.29 (d, 1H, *J* = 5.04 Hz); ^{13}C NMR (100 MHz; DMSO-d_6; Me_4Si): 42.3, 48.0, 51.1, 53.1, 98.6, 117.2, 123.6, 124.3, 124.7, 127.4, 128.7, 132.1, 133.3, 133.5, 133.8, 134.1, 141.9, 148.8, 149.7, 151.7, 186.3; ESI-MS (*m/z*): 629.14 $(M+H)^+$, 630.13 $(M+2)^+$; Anal. Calcd for $C_{33}H_{27}Cl_3N_6O$: C, 62.92; H, 4.32; N, 13.34; Found: C, 62.93; H, 4.34; N, 13.35%.

(3*E*,5*E*)-3,5-Bis(4-bromobenzylidene)-1-((1-(2-((7-chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl) piperidin-4-one (7e): Yield: 71%; mp: 225-227 ^oC; IR (cm^{-1}, Film): 3423, 3056, 2926, 1604, 1581, 1458, 1138, 1073, 522; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 3.76-3.80 (m, 2H), 3.82 (s, 4H), 3.85 (s, 2H), 4.63 (t, 2H, *J* = 6.59 Hz), 5.90 (br s, 1H), 6.35 (d, 1H, *J* = 5.86 Hz), 7.19 (d, 4H, *J* = 8.05 Hz), 7.32 (dd, 1H, J_{12} = 2.2 Hz, J_{13} = 8.79 Hz), 7.39 (s, 1H), 7.51 (d, 4H, *J* = 8.05 Hz), 7.64 (d, 1H, *J* = 8.79 Hz), 7.68 (s, 2H), 7.91 (d, 1H, *J* = 2.20 Hz), 8.48 (d, 1H, *J* = 5.27 Hz) ; ESI-MS (*m/z*): 717.03 $(M+H)^+$, 718.03 $(M+2)^+$, 720.04 $(M+4)^+$; Anal. Calcd for $C_{33}H_{27}Br_2ClN_6O$: C, 55.14; H, 3.79; N, 11.69; Found: C, 55.15; H, 3.77; N, 11.70%.

(3*E*,5*E*)-1-((1-(2-((7-Chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(3,4-dimethoxybenzylidene) piperidin-4-one (7f): Yield: 76%; mp: 153-154 ^oC; IR (cm^{-1}, Film): 3370, 3054, 2929, 1583, 1510, 1458, 1021,541; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 3.71-3.76 (m, 2H), 3.86-3.89 (m, 18 H), 4.58 (t, 2H, *J* = 5.50 Hz), 5.92 (br s, 1H), 6.32 (d, 1H, *J* = 5.50 Hz), 6.84-6.95 (m, 6H), 7.25-7.28 (m, 1H), 7.41 (s, 1H), 7.62 (d, 1H, *J* = 8.70 Hz), 7.71 (s, 2H), 7.88 (d, 1H, *J* = 1.83 Hz), 8.46 (d, 1H, *J* = 5.5 Hz); ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si):

42.7, 48.5, 52.3, 54.6, 55.8, 55.9, 98.6, 110.9, 113.6, 117.0, 121.2, 123.8, 124.0, 125.8, 127.9, 128.2, 131.1, 135.3, 136.4, 144.5, 148.5, 148.7, 149.1, 150.0, 151.3, 186.8; ESI-MS (*m/z*): 681.25 $(M+H)^+$, 682.26 $(M+2)^+$; Anal. Calcd for $C_{37}H_{37}ClN_6O_5$: C, 65.24; H, 5.47; N, 12.34; Found: C, 65.25; H, 5.45; N, 12.32%.

(3*E*,5*E*)-3,5-Bis(3-bromobenzylidene)-1-((1-(2-((7-chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl) piperidin-4-one (7g): Yield: 68%; mp: 218-220 ^oC; IR (cm^{-1}, Film): 3233, 3058, 2965, 1608, 1587, 1448, 1320, 1178, 855, 568; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 3.74-3.79 (m, 2H), 3.81 (s, 4H), 3.86 (s, 2H), 4.6 (t, 2H, *J* = 5.55 Hz), 5.87 (br s, 1H), 6.34 (d, 1H, *J* = 5.13 Hz), 7.20-7.23 (m, 4H), 7.31 (dd, 1H, J_{12} = 2.2 Hz, J_{13} = 8.79 Hz), 7.41-7.44 (m, 5H), 7.63 (d, 1H, *J* = 8.79 Hz), 7.67 (s, 2H), 7.92 (d, 1H, *J* = 2.20 Hz), 8.49 (d, 1H, *J* = 5.13 Hz); ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 42.7, 48.6, 52.0, 54.1, 98.7, 117.0, 121.1, 122.6, 123.6, 126.0, 128.6, 130.1, 131.9, 132.9, 133.7, 135.2, 135.4, 136.9, 139.1, 142.4, 144.4, 151.4, 186.5; ESI-MS (*m/z*): 717.03 $(M+H)^+$, 718.04 $(M+2)^+$, 720.05 $(M+4)^+$; Anal. Calcd for $C_{33}H_{27}Br_2ClN_6O$: C, 55.14; H, 3.79; N, 11.69; Found: C, 55.16; H, 3.78; N, 11.68%.

(3*E*,5*E*)-1-((1-(3-((7-Chloroquinolin-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(4-methylbenzylidene) piperidin-4-one (8a): Yield: 73%; mp: 159-161 ^oC; IR (cm^{-1}, Film): 3346, 3068, 2953, 1610, 1583, 1440, 1079, 537; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.17 (p, 2H, *J* = 5.86 Hz), 2.32 (s, 6H), 3.14 (q, 2H, *J* = 5.86 Hz), 3.86 (s, 2H), 3.88 (s, 4H), 4.37 (t, 2H, *J* = 6.59 Hz), 5.56 (br s, 1H), 6.29 (d, 1H, *J* = 4.39 Hz), 7.18 (d, 4H, *J* = 8.05 Hz), 7.26 (d, 4H, *J* = 7.32 Hz), 7.36 (m, 2H), 7.70 (d, 1H, *J* = 8.79 Hz), 7.77 (s, 2H), 7.94 (d, 1H, *J* = 2.20 Hz), 8.48 (d, 1H, *J* = 5.37 Hz); ^{13}C NMR (100 MHz; DMSO-d_6; Me_4Si): 21.3, 28.1, 30.9, 39.4, 47.5, 52.3, 54.6, 98.6, 117.1, 121.2, 122.9, 125.5, 128.6, 129.3, 130.5, 132.1, 135.0, 136.6, 139.5, 145.1, 149.1, 149.2, 151.8, 187.0; ESI-MS (*m/z*): 603.25 $(M+H)^+$, 604.24 $(M+2)^+$; Anal. Calcd for $C_{36}H_{35}ClN_6O$: C, 71.69; H, 5.85; N, 13.93; Found: C, 71.70; H, 5.86; N, 13.92%.

(3*E*,5*E*)-1-((1-(3-((7-Chloroquinolin-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(4-ethylbenzylidene)piperidin-4-one (8b): Yield: 71%; mp: 148-150 ^oC; IR (cm^{-1}, Film): 3336, 3041, 2963, 1602, 1580, 1431, 1176, 1137, 1002, 805, 505; ^1H NMR (400 MHz; DMSO-d_6; Me_4Si): 1.10 (t, 6H, *J* = 7.33 Hz), 2.08 (p, 2H, *J* = 6.87 Hz), 2.54 (q, 4H, *J* = 7.33 Hz), 3.11 (q, 2H, *J* = 5.95 Hz), 3.78 (s, 6H), 4.38 (t, 2H, *J* = 6.87 Hz), 6.28 (d, 1H, *J* = 5.50 Hz), 7.22 (d, 4H, *J* = 8.24 Hz), 7.28 (br s, 1H), 7.32 (d, 4H, *J* = 7.79 Hz), 7.40 (dd, 1H, J_{12} = 1.83 Hz, J_{13} = 9.16 Hz), 7.52 (s, 2H), 7.73 (d, 1H, *J* = 2.75 Hz), 7.99 (s, 1H), 8.18 (d, 1H, *J* = 8.70 Hz), 8.28 (d, 1H, *J* = 5.48 Hz); ^{13}C NMR (100 MHz; DMSO-d_6; Me_4Si): 15.2, 28.0, 28.4, 40.0, 47.2, 51.5, 53.8,98.6, 117.4, 124.1, 124.2, 124.3, 127.3, 128.2, 130.6, 132.0, 132.9, 133.5, 134.8, 143.0, 145.4, 148.8, 150.0, 151.7, 186.6; ESI-MS (*m/z*): 631.28 $(M+H)^+$, 632.30 $(M+2)^+$; Anal. Calcd for $C_{38}H_{39}ClN_6O$: C, 72.31; H, 6.23; N, 13.31; Found: C, 72.30; H, 6.25; N, 13.33%.

(3*E*,5*E*)-1-((1-(3-((7-Chloroquinolin-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(4-fluorobenzylidene) piperidin-4-one (8c): Yield: 58%; mp: 166-168 ^oC; IR (cm^{-1}, Film): 3248, 3065, 2929, 1611, 1582, 1276, 1188, 1104, 1077, 850; ^1H NMR (400 MHz; DMSO-d_6; Me_4Si): 2.08 (p, 2H, *J* = 6.4 Hz), 3.10 (m, 2H), 3.73 (s, 2H), 3.77 (s, 4H), 4.40 (t, 2H, *J* = 6.41 Hz), 6.27 (d, 1H, *J* = 5.50 Hz), 6.95-6.97 (m, 4H), 7.20-7.25 (m, 2H), 7.37-7.41 (m, 4H), 7.51 (s, 2H), 7.73 (d, 1H, *J* = 2.29 Hz), 8.01 (s, 1H), 8.18 (d, 1H, *J* = 9.6 Hz), 8.29 (d, 1H, *J* = 5.50 Hz); ESI-MS (*m/z*): 611.20 $(M+H)^+$, 612.22 $(M+2)^+$; Anal. Calcd for $C_{34}H_{29}ClF_2N_6O$: C, 66.83; H, 4.78; N, 13.75; Found: C, 66.84; H, 4.79; N, 13.75%.

(3*E*,5*E*)-3,5-Bis(4-chlorobenzylidene)-1-((1-(3-((7-chloroquinolin-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl) piperidin-4-one (8d): Yield: 74%; mp: 212-213 ^oC; IR (cm^{-1}, Film): 3245, 3065, 2924, 1611, 1579, 1458, 1094, 525; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.18 (p, 2H, J=6.22 Hz), 3.23 (q, 2H, J=5.49 Hz), 3.84 (s, 4H), 3.85 (s, 2H), 4.41 (t, 2H, *J* = 6.22 Hz), 5.44 (br s, 1H), 6.31 (d, 1H, *J* = 5.86 Hz), 7.28 (d, 4H, *J* = 8.05 Hz), 7.35-7.39 (m, 6H), 7.67 (d, 1H, *J* = 8.79 Hz), 7.73 (s, 2H), 7.94 (d, 1H, *J* = 1.10 Hz), 8.49 (d, 1H, *J* = 5.13 Hz); ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 28.3, 39.7, 47.7, 52.2, 54.3, 98.8, 117.1, 121.0, 122.8, 125.6, 128.8, 128.9, 131.5, 133.1, 133.3, 135.1, 135.2, 135.4, 144.8, 149.1, 149.2, 151.8, 186.7; ESI-MS (*m/z*): 643.15 $(M+H)^+$, 644.15 $(M+2)^+$; Anal. Calcd for $C_{34}H_{29}Cl_3N_6O$: C, 63.41; H, 4.54; N, 13.05; Found: C, 63.40; H, 4.55; N, 13.07%.

(3*E*,5*E*)-3,5-Bis(4-bromobenzylidene)-1-((1-(3-((7-chloroquinolin-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl) piperidin-4-one (8e): Yield: 72%; mp: 213-215 ^oC; IR (cm^{-1}, Film): 3400, 3065, 2929, 1610, 1579, 1485, 1072, 523; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.17 (p, 2H, *J* = 6.35 Hz), 3.23 (q, 2H, *J* = 5.86 Hz), 3.83 (s, 4H), 3.85 (s, 2H), 4.41 (t, 2H, *J* = 6.59 Hz), 5.42 (br s, 1H), 6.32 (d, 1H, *J* = 4.39 Hz), 7.21 (d, 4H, *J* = 8.05 Hz), 7.34 (s, 1H), 7.39 (dd, 1H, J_{12} = 2.93 Hz, J_{13} = 9.52 Hz), 7.52 (d, 4H, *J* = 8.05 Hz), 7.68 (d, 1H, *J* = 9.52 Hz), 7.71 (s, 2H), 7.95 (d, 1H, *J* = 2.20 Hz), 8.50 (d, 1H, *J* = 5.13 Hz); ^{13}C NMR (100 MHz; DMSO-d_6; Me_4Si): 28.3, 40.0, 47.2, 51.3, 53.5, 98.6, 117.4, 122.8, 124.1, 127.4, 131.6, 131.7, 132.3, 13.4, 133.7, 133.8, 134.2, 142.9, 148.8, 149.9, 151.7, 186.4; ESI-MS (*m/z*): 731.06 $(M+H)^+$, 732.05 $(M+2)^+$, 734.05 $(M+4)^+$; Anal. Calcd for $C_{34}H_{29}Br_2ClN_6O$: C, 55.72; H, 3.99; N, 11.47; Found: C, 55.70; H, 3.97; N, 11.45%.

(3*E*,5*E*)-1-((1-(3-((7-Chloroquinolin-4-yl)amino)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(3,4-dimethoxybenzylidene) piperidin-4-one (8f): Yield: 76%; mp: 185-186 ^oC; IR (cm^{-1}, Film): 3312, 3065, 2958, 1611,1597, 1458, 1084, 804, 618, 548; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.14(p, 2H, *J* = 5.86 Hz), 3.17 (q, 2H, *J* = 5.86 Hz), 3.85 (s, 4H), 3.87 (s, 2H), 3.90 (s, 12H), 4.39 (t, 2H, *J* = 6.59 Hz), 5.51 (br s, 1H), 6.29 (d, 1H, *J* = 5.13 Hz), 6.86 (s, 1H), 6.90 (m, 3H), 6.96-6.99 (m, 2H), 7.36-7.39 (m, 2H), 7.72-7.75 (m, 3H), 7.94 (d, 1H, *J* = 2.20 Hz), 8.49 (d, 1H, *J* = 5.13 Hz) ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 28.2, 39.5, 47.5, 52.4, 54.6, 55.3, 98.7, 114.1, 117.1, 121.1, 122.9, 125.5, 127.7, 128.7, 131.0, 132.3, 135.0, 136.2, 145.3, 149.1, 149.2, 151.9, 160.3, 187.0; ESI-MS (*m/z*): 695.25 $(M+H)^+$, 696.24 $(M+2)^+$; Anal. Calcd for $C_{38}H_{39}ClN_6O_5$: C, 65.65; H, 5.65; N, 12.09; Found: C, 65.66; H, 5.66; N, 12.10%.

(3*E*,5*E*)-1-((1-(2-((7-Chloroquinolin-4-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl)-3,5-bis(4-methoxybenzylidene) piperidin-4-one (8g): Yield: 70%; mp: 190-192 ^oC; IR (cm^{-1}, Film): 3356, 3052, 2928, 1600, 1583, 1327, 1081, 1060, 831, 530; ^1H NMR (400 MHz; $CDCl_3$; Me_4Si): 2.14 (p, 2H, *J* = 6.22 Hz), 3.15 (q, 2H, *J* = 6.59 Hz), 3.78 (s, 6H), 3.82-3.88 (m, 6H), 4.39 (t, 2H, *J* = 6.59 Hz), 5.46 (br s, 1H), 6.30 (d, 1H, *J* = 5.86 Hz), 6.90 (d, 4H, *J* = 8.79 Hz), 7.33 (d, 4H, *J* = 8.79 Hz), 7.36-7.37 (m, 2H), 7.70 (d, 1H, *J* = 9.52 Hz), 7.75 (s, 2H), 7.94 (d, 1H, *J* = 2.20 Hz), 8.49 (d, 1H, *J* = 5.13 Hz) ^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si): 28.2, 39.5, 47.5, 52.4, 54.6, 55.2, 98.7, 114.0, 117.1, 121.1, 122.9, 125.5, 127.7, 128.7, 131.0, 132.3, 135.0, 136.2, 145.2, 149.1, 151.8, 160.2, 187.0; ESI-MS (*m/z*): 635.24 $(M+H)^+$, 636.25 $(M+2)^+$; Anal. Calcd for $C_{36}H_{35}ClN_6O_3$: C, 68.08; H, 5.55; N, 13.23; Found: C, 68.10; H, 5.56; N, 13.22%.

4.2 Anticancer screening

The anticancer screening was carried out following the procedure described earlier.[48]

4.3 Mechanism of action studies

Materials and methods

Compound Stock Solutions

Compounds were prepared at a 3mM stock concentration in DMSO culture grade under sterile conditions. All experiments were done at the 10µM dose as determined by the NCI 60 cell line screening protocol previously performed for these compounds.

Cell culture

The cell line used in this study was COLO-205 human colorectal adenocarcinoma
(ATCC CCL-222). These cells were maintained in RPMI 1640 (ATCC, Manassas VA) supplemented with 10% fetal bovine serum (ATCC). Cultures were maintained at 37ºC with humidified atmosphere of 95% air/ 5% CO_2.

Annexin V

The annexin V assay has been used as an imaging tool for the detection and indication of phosphatidylserine (PS) on the surface of cells; a key event in apoptotic cells.[54]Approximately $3x10^6$ cells were treated with the IC_{50} dose of each compound and controls (vehicle DMSO and camptothecin). After exposure, cells were stained with annexin V conjugate, andpropidium iodide (Biotium, Hayward, CA). Samples where then analysed using the Nucleo Counter NC3000 (Chemometec, Allerød, Denmark). 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 commonly used assay in drug-cell interaction studies.[55,56]This nuclease mediated event can be quantified using DNA content and measuring cells containing less than 1DNA equivalent known as Sub-G_1. The NC3000 system was used for this experiment. The NC3000 assay is based on removal of small DNA fragments and retention of 4',6-diamidino-2-phenylindole (DAPI) stained higher weight fragments. After treatment with the tested compounds at the dose and conditions described above, cells were fixed with ethanol 70%, stained with 1µg/ml DAPI, and analysed by differential image analysis using the NC3000 instrument measuring DAPI intensity. A one way ANOVA was performed. If significant results were found in the ANOVA, a Post Hoc Test Tukey was also performed.

Caspase activation

Activation of effector caspases is a key event in the progression of apoptotic cell death.[57] This activation was measured using the Fluorescent Labeled Inhibitors of Caspases (FLICA) probes that bind covalently with active caspase effector enzymes. After treatment as described above, cells were harvested and stained using the green FAM FLICA kit (Immunochemistry Technologies, Bloomington Min.). Samples were then analysed using the Nucleocounter NC3000 instrument. A one way ANOVA was performed. If significant results were found in the one way ANOVA, a Post Hoc Test Tukey was also performed.

Acknowledgments

DSR acknowledges University Grant Commision [41-202/2012 (SR)], New Delhi, India and DU-DST PURSE, University of Delhi, Delhi, India for financial support. S. K. K. and S. M. are thankful to CSIR for the award of junior and senior research fellowship. S.V.M. would like to acknowledge the support from National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. Authors are also thankful to CIF-USIC, University of Delhi, Delhi for NMR spectral data and RSIC, CDRI, Lucknow for mass data. We thank Alfredo Blakeley, Julie Early, Stephanie Florio, and Juliane Ollinger for echnical assistance.

Notes and references

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*†Electronic Supplementary Information (ESI) available: [Single dose data of compounds **7d** and **7f,** One dose mean graph (compound 7**d**),Five dose results (compound **7d**), One dose mean graph (compound **7f**), Five dose results (compound **7f**),*[1]*H and* [13]*C NMR of representative compounds (**6a-b**, **7d** and **7f**)]. See DOI: 10.1039/b000000x/*

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