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Anticancer activity of 4-aminoquinoline-triazine based molecular hybrids†

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In this study the potential for anticancer activity of 4-aminoquinoline-triazine based hybrids has been investigated on 60 human cancer cell lines (NCI 60). The representative compounds show activity on a range of cell lines and apoptosis as the mode of growth inhibition.

Introduction

To combat an array of diseases and counter drug resistance problems, the development of newer, cheaper and safer drugs will always remain a current need. In this perspective, the strategy of synthesizing hybrid molecules by covalently connecting two (or more) pharmacophores to generate a new 'dual-drug' species is fast becoming an alternative to other strategies.[1] These dual-drugs are believed to act by inhibiting two biological targets simultaneously. This concept, of hybrids as a dual-sword molecule to create efficient drugs, was first suggested by Meunier *et al.*[2] In the past few years some of these hybrid molecules have shown excellent biological activity.[3]

Chloroquine (CQ, Fig. 1a) and other 4-aminoquinoline based molecules are well known for their antimalarial activity.[4] 4-Aminoquinoline based compounds are also known for their antimicrobial,[5] anti-prion,[6] anti-trypanosomal[7] and anticancer activities.[8] The mechanism of action for their anticancer activity is not clearly understood, however, reports suggest their radiosensitizing effects through lysosome permeabilization as the cause of their activity. The accumulation of CQ in the lysosomes hampers significantly the proteolytic processes and metabolism of neoglycolipids, resulting in an alterations of various cellular signal pathways.[8] Similarly, several other 4-aminoquinoline based derivatives were also reported for their activity against different cancer cell lines.[9] Earlier studies have shown ruthenium(II) complexes of CQ (Fig. 1b) to inhibit the growth of colon cancer cells (HCT-116),[10] and ferrocenyl-aminoquinoline-carboxamide based hybrid bioorganometallics (Fig. 1c) displayed inhibitory activity against colon (Caco-2, HTB-37) and breast (HTB-129) cancer cells.[11]

Previous reports from our lab showed that hybrids of 4-aminoquinoline and triazine moieties when covalently connected *via* different alkyl chain linkers (Fig. 1d), showed improved antimalarial activity against both CQ-sensitive (D6) and CQ-resistant (W2) strains of *P. falciparum*.[12,13] A few of these hybrids were found to be 3–4 times more potent than the reference molecule CQ (IC_{50} = 0.04 μM for D6 clone and 0.4 μM for W2 clone) used in this study. Encouraged by these results, and as a part of our on-going work towards the synthesis of biologically relevant molecules,[14] we were interested to explore the anticancer activity of these hybrids against a panel of different cancer cell lines. Therefore, a series of 4-aminoquinoline-triazine based hybrids (**4–15**) were initially screened against 60 human cancer cell lines (NCI 60) and two representative hybrid molecules (**11** and **14**; Fig. 2) were selected for a dose–response study, followed by investigation of the mechanism-of-action of their anticancer activity. These two hybrids (**11** and **14**) were also tested against VERO, LLC-PK11 and HepG2 mammalian cells and found to be noncytotoxic up to a concentration of 25 μM, proving their safe toxicity profile.[13]

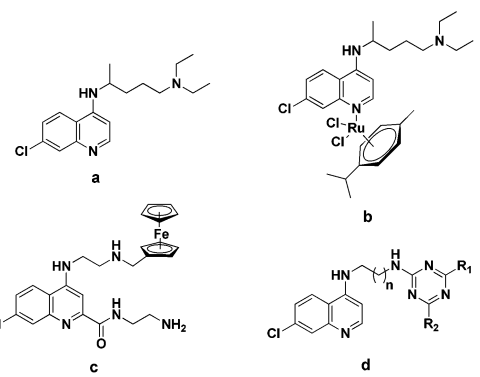

Fig. 1 (a) Chloroquine (CQ); (b) ruthenium(II) complex of CQ; (c) ferrocenyl-aminoquinoline-carboxamide based bio-organometallics; (d) general structure of 4-aminoquinoline-triazine based hybrids.

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† Electronic supplementary information (ESI) available: One dose mean graphs, drug response curves, five dose mean graphs and GI_{50} and LC_{50} values of compound **11** and **14**. See DOI: 10.1039/c3ra45333b

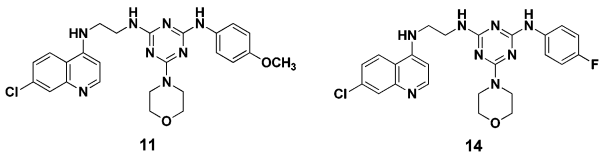


Fig. 2 4-Aminoquinoline-triazine based hybrids selected by the US National Cancer Institute.

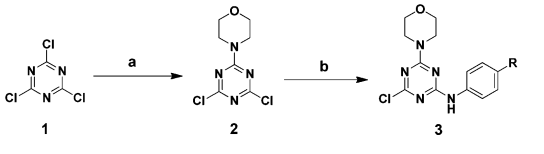
Results and discussion

Chemistry

The synthesis of the compounds has been reported earlier.[12,13] In brief, the synthesis was accomplished by sequential nucleophilic substitution on commercially available cyanuric chloride (**1**) (Scheme 1).[12,13] First, the chlorine of cyanuric chloride (**1**) was substituted with morpholine to yield a monosubstituted triazine (**2**), which upon subsequent reaction with different anilines provided disubstituted triazines (**3**). Thereafter, the remaining chlorine on the triazines (**3**) were substituted by 4-aminoquinoline with different carbon alkyl chain lengths and the targeted 4-aminoquinoline-traizine hybrids (**4–15**) were obtained. All the compounds (**4–15**) were purified by column chromatography and the products were characterized by various spectroscopic techniques and elemental analysis.

Biological studies

(a) Anticancer activity. 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 the lung, colon, kidney, ovary, breast, prostate, and central nervous system. The cells are grown in a supplemented RPM1 1640 medium for 24 h. The test compounds were dissolved in DMSO and incubated with cells at five concentrations with 10-fold dilution, the highest being 10^{-4} M and the others being 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M. The



Comp. No.	n	R
4	1	H
5	2	H
6	3	H
7	1	Et
8	2	Et
9	3	Et
10	2	OMe
11	1	OMe
12	3	OMe
13	2	F
14	1	F
15	3	F

Scheme 1 Reagents and conditions: (a) morpholine, K_2CO_3, THF, 0–5 °C, 3 h, 80%; (b) different anilines, K_2CO_3, THF, rt, 3 h, 70–85%; (c) substituted 4-aminoquinolines, K_2CO_3, DMF, 100–110 °C, 10–12 h, 65–75%.

assay is terminated by addition of cold trichloroacetic acid, and the cells are fixed and stained with sulforhodamine B. The bound stain is solubilized, and the absorbance is read on an automated plate reader. The cytostatic parameter, that is 50% growth inhibition (GI_{50}), was calculated from the absorbance at time zero, in the control growth, and at the five concentration levels. The cytotoxic parameter, that is the inhibitory concentration (LC_{50}), represents the average of two independent experiments. The *in vitro* screening is a two-stage process that started with the evaluation of the compound against the 60 human tumor cell lines with a single dose of 10.0 μM, which is done by following same protocol as for the five-dose screening. Only the compounds which show more than 60% growth inhibition in at least 8 tumor cell lines are selected for further testing, and the others were assumed to be inactive. The compounds (**11**) and (**14**) were initially evaluated at a single dose of 10 μM and found to be active against various cell types. The results of the single-dose screening are given in the ESI† of this manuscript. Subsequently, both compounds were evaluated 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. The additional data for both compounds *i.e.* one-dose mean graphs, drug–response curves, five-dose mean graphs and GI_{50} and LC_{50} values are given the ESI.† As the data show, the 4-aminoquinoline-triazine based hybrids have activity against various cancer panels. Both compounds (**11** and **14**) show inhibitory activity at single digit micromolar concentration against most of the cell lines. The GI_{50} values suggest that these compounds have inhibitory activity against a vast range of cancer cell lines (a low micromolar effect). Also, comparison of the LC_{50} values from this study, show that these compounds are least cytotoxic to most of the cell lines in leukemia, non-small cell lung cancer, colon, ovarian and prostate cancer panels. The high LC_{50} values compared to GI_{50} give a high therapeutic window for both of these compounds (Table 1). The dose–response curves of **1** for all 60 cell lines are illustrated as Fig. 3.

(b) Mechanism of action studies

Materials and methods. Experimental compound stock solutions: 3 mM stock solutions were prepared in culture grade DMSO under sterile conditions. All experiments were done at the 10 μM 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 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For all experiments, approximately 3×10^6 cells were treated with the test compound and controls (DMSO and camptothecin).

Annexin V: Annexin V staining is a useful tool to determe the type of cell death. It has been used to detect apoptotic cells after exposure to various substances;[15] 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, and propidium iodide (Biotium, Hayward, CA). After staining,

Table 1 Antitumor activity (GI_{50}/μM) and toxicity (LC_{50}/μM) data of compounds selected for 5 dose studies for the NCI 60-cell lines screen[a]

	GI_{50}/μM (**11**)	LC_{50}/μM (**11**)	GI_{50}/μM (**14**)	LC_{50}/μM (**14**)
Leukemia				
CCRF-CEM	2.950	>100	0.457	75.20
HL-60(TB)	1.130	>100	1.800	>100
K-562	2.090	>100	2.910	79.50
MOLT-4	2.010	59.60	4.170	65.30
RPMI-8226	2.750	>100	3.150	74.20
SR	2.010	>100	2.440	>100
Non-small cell lung cancer				
A549/ATCC	4.870	>100	10.50	59.90
EKVX	8.090	60.40	ND	ND
HOP-62	1.160	50.60	13.40	52.40
HOP-92	3.890	51.00	10.30	29.40
NCI-H226	1.370	5.510	14.40	77.00
NCI-H23	13.70	64.40	14.60	62.10
NCI-H322M	10.30	49.60	11.80	51.20
NCI-H460	4.580	79.40	3.440	50.90
NCI-H522	3.850	39.30	4.250	47.00
Colon cancer				
COLO 205	1.530	7.250	1.710	6.230
HCC-2998	2.080	7.240	2.000	7.790
HCT-116	3.010	43.10	4.820	43.90
HCT-15	2.630	71.40	2.460	31.30
HT29	1.070	16.90	2.270	24.00
KM12	5.180	47.40	5.440	48.10
SW-620	3.470	59.90	4.210	51.60
CNS cancer				
SF-268	5.030	48.10	6.850	48.90
SF-295	2.050	7.470	4.190	45.00
SF-539	2.180	31.20	3.790	40.00
SNB-19	10.60	52.90	12.40	54.20
SNB-75	2.090	30.30	3.130	40.50
U251	3.100	42.70	4.72	43.90
Melenoma				
LOX IMVI	3.010	42.40	2.950	42.10
MALME-3M	1.940	6.190	2.760	7.430
M14	1.860	6.450	1.940	6.930
MDA-MB-435	1.820	6.690	2.220	19.20
SK-MEL-2	2.190	8.360	1.830	9.250
SK-MEL-28	1.810	5.940	2.150	5.800
SK-MEL-5	1.590	5.780	2.150	9.940
UACC-257	1.870	6.950	1.900	7.000
UACC-62	1.850	6.990	2.650	26.30
Ovarian cancer				
IGROV1	8.430	58.00	6.300	56.10
OVCAR-3	11.50	51.00	11.90	50.60
OVCAR-4	9.000	62.60	10.70	50.00
OVCAR-5	15.70	62.90	17.50	58.70
OVCAR-8	6.460	91.60	11.60	>100
NCI/ADR-RES	6.380	79.70	9.360	72.10
SK-OV-3	1.170	51.80	11.90	50.90
Renal cancer				
786-0	2.110	38.20	3.350	46.40
A498	0.272	21.90	1.480	31.30
ACHN	4.770	48.60	7.060	46.50
CAK-1	4.170	45.40	5.590	48.80
RXF 393	1.890	35.90	1.890	28.00
SN12C	8.730	57.10	11.60	54.00
TK-10	7.640	46.60	10.30	50.10
UO-31	1.330	39.60	3.520	41.80
Prostate cancer				
PC-3	3.940	60.30	4.870	44.90
DU-145	9.800	48.40	8.680	47.20
Breast cancer				
MCF7	2.860	4.870	3.430	49.60
MDA-MB-231ATCC	2.730	4.280	3.050	39.80
HS 578T	3.910	>100	7.320	97.80
BT-549	9.920	48.90	8.670	51.20
T-47D	11.60	68.00	10.60	69.30
MDA-MB-468	1.620	9.990	1.960	15.90

[a] ND: not determined.

the cells were analyzed using the NucleoCounter 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* Tukey test was also performed.

DNA fragmentation: DNA fragmentation as an apoptosis marker is a widely used assay in apoptosis studies.[16] We utilized the NC3000 system for this experiment. DNA fragmentation can be quantified using DNA content and measuring cells containing less than 1 DNA 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.[17] Caspase activation was measured using the Fluorescent Labeled Inhibitors of Caspases (FLICA). These probes 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.) then analyzed using the NucleoCounter NC3000 instrument.

Results

Annexin V. Our results indicate that only SM334 (**14**) presented apoptotic activity in a statistically significant ($P < 0.05$) manner. Compound (**14**) presented a barely significant average of 39.0% of apoptotic cells (Fig. 4) whereas SM332 (**11**) did not present statistically significant activation with 34.5% of apoptotic cells. Both compounds where significantly lower than the positive camptothecin control (53%).

Caspase activation. Caspase 8: Caspase 8 activation is measured as a marker for an extrinsic apoptotic mechanism. Our data (Fig. 5) shows that compound (**11**) presented the highest average activation at 50.5% (Fig. 5). Compound (**14**) presented an average activation of 26%. Only compound (**11**)

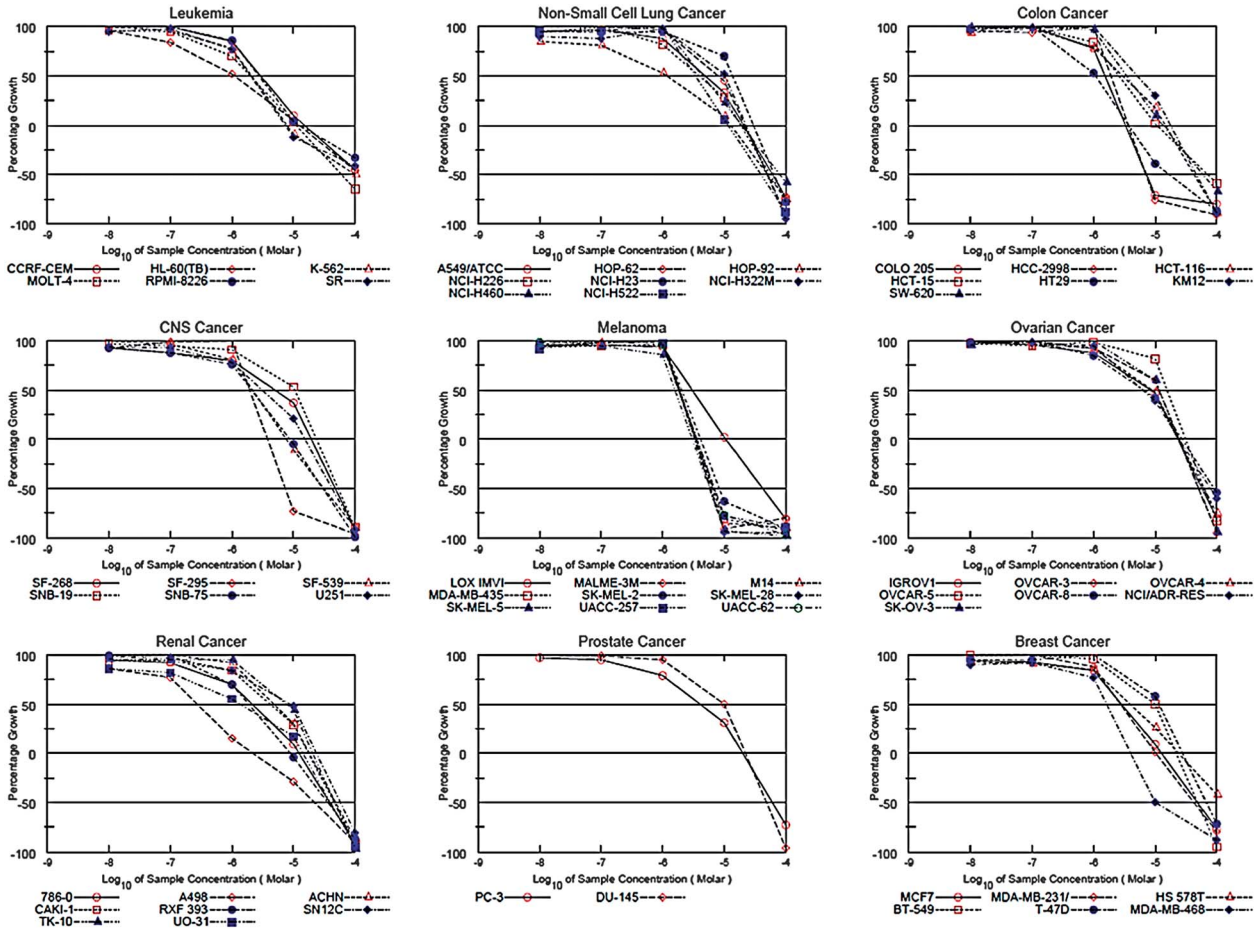


Fig. 3 Drug response curve from the five-dose study of compound **11**.

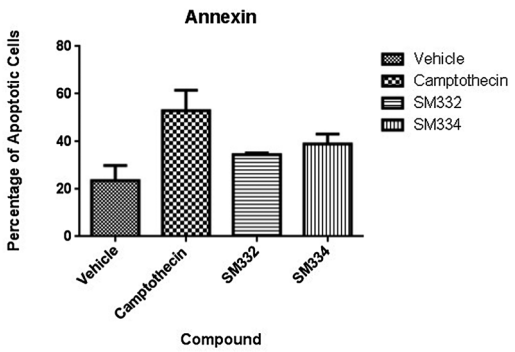


Fig. 4 Annexin V staining. Only compound (**14**) presented significant apoptotic activity which was comparable to the positive control.

presented significant activation when compared to the negative control ($P < 0.05$) and higher than the camptothecin control.

Caspase 9: Caspase 9 activation is an important hallmark of the intrinsic apoptotic pathway. Our results show both compounds present high activation of caspase 9 (Fig. 6). Compound (**11**) presented a 45.5% of cells with active caspase 9 while compound (**14**) presented 43%. Both of these compounds presented higher activation than the camptothecin control (21%).

Caspase 3 and 7: Effector caspase activation was measured yielding negative results. None of the compounds caused statistically significant ($P < 0.05$) activation. Compound (**11**) presented an average of 39% of cells with activated caspase 3 (Fig. 7) whereas compound (**14**) presented 33.5% activation. In order to verify these results DNA fragmentation was performed.

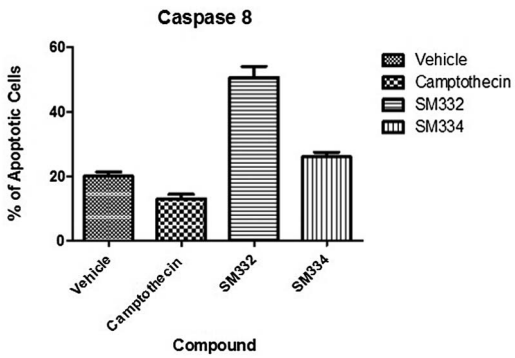


Fig. 5 Caspase 8 activation. Compound (**11**) caused significant ($P < 0.05$) activation of caspase 8. Compound (**11**) caused 50.5% activation whereas (**14**) caused 26%.

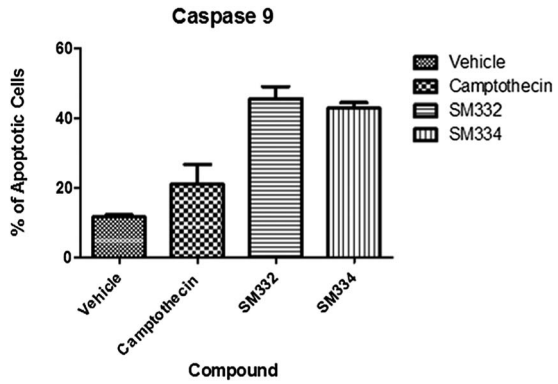


Fig. 6 Caspase 9 activation. Both experimental compounds presented significant activation of caspase 9. Compound (**11**) presented 45.5% of cells with active caspase while compound SM334 (**14**) presented 43%.

DNA fragmentation: As Fig. 8 shows, none of the hybrid compounds presented statistically significant DNA fragmentation. Compound (**11**) presented an average of 8.5% and compound (**14**) presented an average of 9%. These denotes a clear absence of cells with fragmented DNA, especially when compared to the negative control which had an average of 8.5% and positive camptothecin control with 80.5%. These results confirm the absence of effector caspase activation.

Discussion

The results obtained varied between our test compounds SM332 (**11**) and SM334 (**14**). Compound SM332 (**11**) presented activation of caspase 8 and 9, but not the typical hallmarks of this event such as the migration of phosphatidylserine, DNA fragmentation and effector caspase activation. It is known that caspase 8 mediates apoptotic cell death in various ways, such as direct activation of effector caspase 3 or through the Bid mitochondrial mediated pathway.[18] Activation of messenger caspases but not effectors is a phenomenon that has been described in other apoptotic cell death settings, however it has been suggested by other researchers that other caspases might be involved.[19] Compound SM334 (**14**) presented significant migration of phosphatidylserine, indicating apoptotic cells.

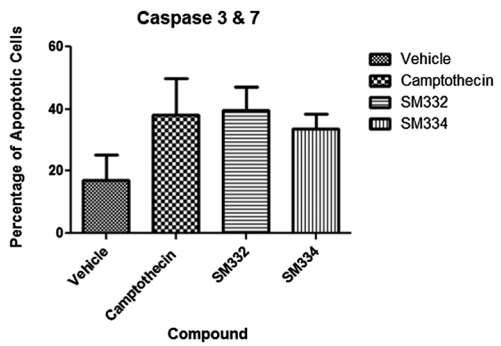


Fig. 7 Effector caspase activation. None of the compounds caused statistically significant activation of effector caspases.

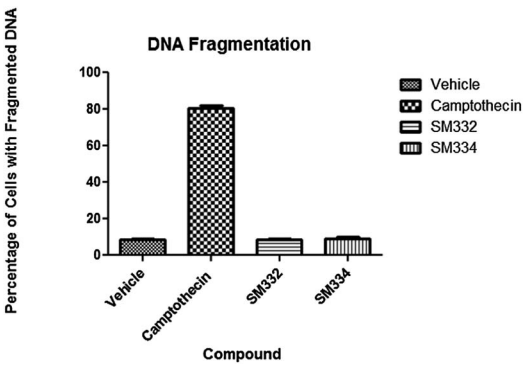


Fig. 8 DNA fragmentation: none of the experimental compounds caused significant DNA fragmentation.

Additionally this compound activated caspase 9, but not caspase 8, in a significant manner. This compound presented no activation of effector caspases or DNA fragmentation. Although activation of caspase 9 and phosphatidylserine migration suggest an intrinsic apoptotic pathway, the lack of effector caspase activation and DNA fragmentation remains unexplained. Further experiments should include protein expression pathways, especially the apoptosis related proteins.

Conclusion

The potential for the anticancer activity of 4-aminoquinoline-triazine based molecular hybrids has been explored. Our study of two representative compounds (**11**) and (**14**) showed significant inhibition against NCI 60 human tumor cell lines. Compound (**11**) presented activation of caspase 8 and 9 but not the typical hallmarks of this event, such as migration of phosphatidylserine, DNA fragmentation and effector caspase activation. Compound (**14**) presented significant migration of phosphatidylserine, indicating apoptotic cells. It also activated caspase 9 but not caspase 8. Also, no activation of effector caspases or DNA fragmentation was seen. Activation of caspase 9 and phosphatidylserine migration suggest an intrinsic apoptotic pathway as the mode of action for these compounds.

Acknowledgements

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