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Original article

Chalcones with electron-withdrawing and electron-donating substituents: Anticancer activity against TRAIL resistant cancer cells, structure–activity relationship analysis and regulation of apoptotic proteins

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ABSTRACT

In the present study, a series of 46 chalcones were synthesised and evaluated for antiproliferative activities against the human TRAIL-resistant breast (MCF-7, MDA-MB-231), cervical (HeLa), ovarian (Caov-3), lung (A549), liver (HepG2), colorectal (HT-29), nasopharyngeal (CNE-1), erythromyeloblastoid (K-562) and T-lymphoblastoid (CEM-SS) cancer cells. The chalcone **38** containing an amino (-NH_2) group on ring A was the most potent and selective against cancer cells. The effects of the chalcone **38** on regulation of 43 apoptosis-related markers in HT-29 cells were determined. The results showed that 20 apoptotic markers (Bad, Bax, Bcl-2, Bcl-w, Bid, Bim, CD40, Fas, HSP27, IGF-1, IGFBP-4, IGFBP-5, Livin, p21, Survivin, sTNF-R2, TRAIL-R2, XIAP, caspase-3 and caspase-8) were either up regulated or down regulated.

1. Introduction

Worldwide, cancer is one of the leading causes of death. Patients diagnosed with cancer often experience a poor quality of life due to the adverse events associated with cancer. Chemotherapy is one of the effective approaches in suppressing tumour growth and eradication of tumours. However, many patients undergoing chemotherapy suffer from associated side effects such as nausea, vomiting, cachexia, lethargy and poor oral intake. Although, many research studies have reported potential chemotherapeutic effects of novel compounds, the search for new anti-cancer agents with improved efficacy and reduced side effects continues. [1]

Chalcones have attracted much attention due to their diverse biological activities, such as anti-cancer, anti-oxidant, anti-inflammatory, and/or anti-infective activities. Chalcones consist of two aromatic rings connected by an α,β-unsaturated carbonyl group. It has been shown that the removal of α,β-unsaturated carbonyl system could hinder their biological activities [2]. A number of synthetic modifications, viz., such as oxathiolone fused [3], boron substituted [4], heterocyclic infused [5], biphenyl based [6], imidazolones linked [7], coumarin based chalcones [8] or other substitutions [9–13]; have also been reported to affect the biological activities including anticancer activities of chalcones.

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an attractive target in cancer research because it is capable of selectively inducing apoptosis in cancer cells without affecting normal cells [14]. Five receptors, such as TRAIL-R1 (DR4, death receptor 4), TRAIL-R2 (DR5), DR6, TRAILR-3 (decoy receptor (DcR)1) and TRAIL-R4 (DcR2); have been identified for TRIAL. Two of these receptors DR4 and DR5 have cytoplasmic death domains and trigger TRAIL induced apoptosis [15]. Other receptors, DcR1 and DcR2, are expressed on the cell surface and protect the cancer cells from TRAIL induced apoptosis [16]. The last TRAIL receptor, osteoprotegerin, is a less studied receptor and is known to inhibit the tumouricidal activity of TRAIL [17]. Interaction of TRAIL with DR4 and DR5 results in caspase-8 activation, which induces apoptosis by activating either caspase-3 or the intrinsic mitochondria-mediated apoptotic pathway [18]. However, TRAIL

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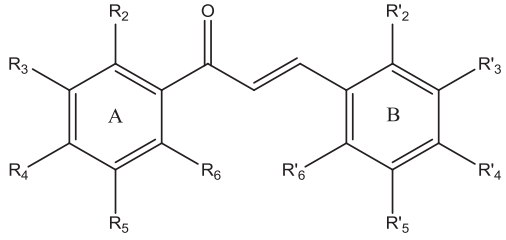
Table 1
Chalcone derivatives and their Lipinski's rule of five parameters.



Chalcone	Ring A					Ring B					Lipinski rule of 5 parameters			
No.	R_2	R_3	R_4	R_5	R_6	R'_2	R'_3	R'_4	R'_5	R'_6	MW	*A* log *P*	HBA	HBD
1	H	H	H	H	H	H	H	H	H	H	208.26	3.70	1	0
2	H	H	H	H	H	OH	H	H	H	H	224.26	3.46	2	1
3	H	H	H	H	H	H	OH	H	H	H	224.26	3.46	2	1
4	H	H	H	H	H	H	H	OH	H	H	224.26	3.46	2	1
5	OH	H	H	H	H	H	H	H	H	H	224.26	3.46	2	1
6	OH	H	H	H	H	OH	H	H	H	H	240.25	3.22	3	2
7	OH	H	H	H	H	H	OH	H	H	H	240.25	3.22	3	2
8	OH	H	H	H	H	H	H	OH	H	H	240.25	3.22	3	2
9	OH	H	H	H	H	H	H	OCH_3	H	H	254.28	3.44	3	1
10	OH	H	H	H	H	H	OCH_3	OCH_3	H	H	284.31	3.43	4	1
11	OH	H	H	H	H	H	OCH_3	OCH_3	OCH_3	H	314.33	3.41	5	1
12	OH	H	H	H	H	H	1,3-dioxolane		H	H	268.26	3.23	4	1
13	OH	H	H	H	H	H	H	CH_3	H	H	238.28	3.95	2	1
14	OH	H	H	H	H	H	H	Cl	H	H	258.70	4.12	2	1
15	OH	H	H	H	H	H	Phenyl		H	H	274.31	4.37	2	1
16	OH	H	OCH_3	H	OCH_3	H	H	$N(CH_3)_2$	H	H	327.37	3.59	5	1
17	OH	H	OCH_3	H	OCH_3	H	H	OCH_3	H	H	314.33	3.41	5	1
18	OH	OCH_3	OCH_3	H	H	H	H	Br	H	H	363.20	4.18	4	1
19	OH	H	OCH_3	H	OCH_3	H	H	H	H	H	284.31	3.43	4	1
20	OH	H	OCH_3	H	OCH_3	NO_2	H	H	H	H	329.30	3.32	6	1
21	OH	H	OCH_3	H	OCH_3	H	H	NO_2	H	H	329.30	3.32	6	1
22	OCH_3	H	OCH_3	H	H	H	H	NO_2	H	H	313.31	3.56	5	0
23	OCH_3	H	OCH_3	H	H	H	H	Cl	H	H	302.75	4.33	3	0
24	OCH_3	H	OCH_3	H	H	H	H	Br	H	H	347.20	4.42	3	0
25	OCH_3	H	OCH_3	H	H	H	1,3-dioxolane		H	H	312.32	3.44	5	0
26	OCH_3	H	H	OCH_3	H	H	1,3-dioxolane		H	H	312.32	3.44	5	0
27	OCH_3	H	H	OCH_3	H	H	OCH_3	OCH_3	OCH_3	H	358.39	3.62	6	0
28	H	OCH_3	OCH_3	H	H	H	H	Cl	H	H	302.75	4.33	3	0
29	H	OCH_3	OCH_3	H	H	H	H	SCH_3	H	H	314.40	4.21	4	0
30	OCH_3	H	H	H	H	H	H	F	H	H	256.27	3.89	2	0
31	H	H	OCH_3	H	H	OCH_3	H	H	H	H	268.31	3.67	3	0
32	H	H	OCH_3	H	H	H	H	Cl	H	H	272.73	4.35	2	0
33	H	H	OCH_3	H	H	H	Phenyl		H	H	288.34	4.59	2	0
34	H	H	CH_3	H	H	H	H	$N(CH_3)_2$	H	H	265.35	4.35	2	0
35	H	H	CH_3	H	H	H	H	F	H	H	240.27	4.39	1	0
36	H	H	CH_3	H	H	H	H	Cl	H	H	256.73	4.85	1	0
37	H	H	CH_3	H	H	H	H	SMe	H	H	268.37	4.73	2	0
38	NH_2	H	H	H	H	H	H	H	H	H	223.27	2.96	2	1
39	NH_2	H	H	H	H	H	H	NO_2	H	H	268.27	2.85	4	1
40	NH_2	H	H	H	H	H	H	Cl	H	H	257.72	3.62	2	1
41	NH_2	H	H	H	H	H	H	OCH_3	H	H	253.30	2.94	3	1
42	NH_2	H	H	H	H	H	Phenyl		H	H	273.33	3.86	2	1
43	H	H	NH_2	H	H	H	H	H	H	H	223.27	2.96	2	1
44	H	H	NH_2	H	H	Cl	H	H	H	H	257.72	3.62	2	1
45	H	H	NH_2	H	H	H	H	Cl	H	H	257.72	3.62	2	1
46	H	H	NH_2	H	H	Cl	H	Cl	H	H	292.16	4.28	2	1

MW: Molecular Weight; *A* log *P*: logarithm of octanol–water partition coefficient; HBA: number of hydrogen bond acceptor; HBD: number of hydrogen bond donor.

resistance in cancer cells have been reported elsewhere in the literature [19–21]. Few recent studies have reported the potential of chalcones in inducing apoptosis in TRAIL-resistant cancer cells [22,23].

In the current study, we have synthesised 46 chalcones consisting wide range of electron-withdrawing and electron-donating substituents that obey Lipinski's rule of five. The apoptosis inducing capability of these chalcones in 10 TRAIL-resistant cancer cells and selective toxicity towards cancer cells compared to normal cells was determined with an aim to derive the important structure activity relationships. Regulation of the various apoptotic proteins in the most sensitive cancer cell, HT-29, by the most potent chalcone **38**, was also determined.

2. Results and discussion

2.1. Chemistry

The chalcones were classified into 12 groups according to their substitution patterns on ring A. Group 1 chalcones (**1**–**4**) have no

Table 2
The anti-proliferative effects of chalcones over cancer cells.

Chalcone No.	MCF-7 IC_{50} (μM)	MDA-MB-231 IC_{50} (μM)	HeLa IC_{50} (μM)	Caov-3 IC_{50} (μM)	A549 IC_{50} (μM)	HepG2 IC_{50} (μM)	HT-29 IC_{50} (μM)	CNE-1 IC_{50} (μM)	K562 IC_{50} (μM)	CEM-SS IC_{50} (μM)
1	>100	>100	>100	>100	69.79 ± 3.15	>100	>100	>100	>100	>100
2	26.96 ± 1.26	25.98 ± 7.79	45.05 ± 0.45	37.21 ± 0.90	65.71 ± 1.70	32.19 ± 1.64	9.95 ± 1.12	78.41 ± 0.70	36.07 ± 0.41	7.94 ± 0.47
3	16.69 ± 1.05	10.01 ± 2.54	61.87 ± 0.20	19.63 ± 0.34	31.43 ± 1.97	21.35 ± 0.63	14.36 ± 2.05	29.84 ± 0.66	34.22 ± 0.96	5.86 ± 0.10
4	>100	32.26 ± 0.34	63.72 ± 0.46	36.75 ± 1.80	77.76 ± 2.63	63.33 ± 2.35	5.81 ± 0.24	46.40 ± 1.37	19.24 ± 0.20	19.33 ± 3.41
5	76.81 ± 3.21	38.41 ± 1.73	77.29 ± 0.38	>100	47.78 ± 1.20	90.74 ± 2.70	70.75 ± 10.08	75.12 ± 1.86	18.00 ± 2.42	76.45 ± 3.22
6	47.16 ± 1.56	16.22 ± 2.98	32.26 ± 0.48	32.30 ± 1.67	24.04 ± 0.53	47.72 ± 1.93	45.25 ± 5.92	37.96 ± 2.02	39.39 ± 1.45	15.97 ± 1.57
7	36.44 ± 2.16	16.32 ± 2.98	19.84 ± 0.29	20.59 ± 0.94	56.36 ± 1.76	42.78 ± 3.15	32.74 ± 4.81	40.20 ± 2.00	32.75 ± 1.30	20.75 ± 2.90
8	>100	43.29 ± 0.06	87.54 ± 1.01	57.96 ± 4.91	67.61 ± 5.88	94.64 ± 2.97	56.25 ± 2.45	71.00 ± 3.05	38.56 ± 1.27	35.20 ± 3.72
9	>100	41.89 ± 0.50	>100	42.65 ± 2.92	61.68 ± 0.36	>100	>100	>100	36.01 ± 1.01	63.81 ± 2.29
10	63.90 ± 1.95	18.33 ± 3.67	>100	75.71 ± 2.20	40.41 ± 0.98	58.11 ± 2.72	32.05 ± 11.00	16.76 ± 4.51	18.91 ± 0.80	25.22 ± 2.16
11	12.00 ± 1.96	20.22 ± 3.08	>100	27.21 ± 5.11	20.97 ± 0.14	18.52 ± 3.91	17.68 ± 5.04	10.06 ± 1.89	23.65 ± 1.12	22.85 ± 1.78
12	16.01 ± 1.27	23.06 ± 0.68	85.81 ± 6.00	68.35 ± 6.86	23.27 ± 0.66	33.24 ± 2.49	22.84 ± 4.92	28.30 ± 1.21	23.28 ± 0.10	35.95 ± 5.01
13	20.96 ± 3.02	62.08 ± 2.00	81.92 ± 4.35	>100	33.50 ± 1.02	69.15 ± 2.12	55.61 ± 3.39	18.40 ± 0.73	20.81 ± 1.26	66.00 ± 3.69
14	27.87 ± 4.83	21.90 ± 1.59	41.50 ± 3.41	>100	34.038 ± 7.67	>100	48.31 ± 0.93	>100	22.36 ± 3.09	55.99 ± 9.48
15	31.86 ± 3.25	>100	>100	>100	15.26 ± 6.03	>100	>100	>100	22.96 ± 2.44	21.66 ± 1.91
16	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
17	>100	66.38 ± 7.24	>100	>100	76.87 ± 4.92	>100	>100	>100	>100	>100
18	14.04 ± 1.50	10.22 ± 0.42	>100	27.81 ± 1.17	16.61 ± 0.68	36.43 ± 1.87	13.67 ± 1.42	82.25 ± 6.54	78.71 ± 1.89	21.26 ± 0.88
19	23.02 ± 6.07	11.43 ± 0.19	>100	11.74 ± 1.76	8.05 ± 1.40	14.37 ± 4.44	6.61 ± 0.72	19.00 ± 1.16	10.04 ± 0.51	9.77 ± 1.34
20	9.58 ± 0.81	66.72 ± 1.82	>100	>100	15.04 ± 5.70	>100	>100	>100	94.90 ± 6.57	88.23 ± 4.20
21	13.45 ± 1.82	16.16 ± 4.02	43.32 ± 2.61	11.72 ± 0.72	11.24 ± 0.03	8.60 ± 0.11	7.66 ± 1.72	8.36 ± 0.80	16.06 ± 2.99	7.72 ± 0.66
22	>100	31.19 ± 3.70	>100	>100	86.12 ± 1.38	>100	>100	26.57 ± 1.18	84.54 ± 6.69	47.00 ± 2.96
23	18.17 ± 6.06	24.00 ± 5.13	>100	79.53 ± 3.63	61.53 ± 1.41	82.09 ± 2.28	5.00 ± 0.26	>100	31.37 ± 2.66	30.04 ± 4.48
24	28.26 ± 6.87	10.89 ± 0.18	>100	69.35 ± 1.37	37.06 ± 4.16	29.45 ± 2.18	17.61 ± 3.29	13.59 ± 2.02	11.87 ± 0.04	27.47 ± 1.99
25	>100	>100	>100	>100	74.38 ± 5.56	>100	>100	4.96 ± 0.04	59.11 ± 7.30	78.31 ± 3.96
26	>100	36.28 ± 7.90	>100	39.39 ± 2.31	17.89 ± 1.13	11.31 ± 0.16	5.51 ± 0.71	21.00 ± 0.86	12.06 ± 0.68	8.71 ± 0.74
27	7.53 ± 0.71	36.68 ± 0.66	17.91 ± 0.43	4.92 ± 0.17	5.52 ± 0.11	4.56 ± 0.21	4.54 ± 0.20	>100	4.68 ± 0.29	5.38 ± 0.41
28	13.63 ± 2.04	43.65 ± 0.49	>100	55.40 ± 0.70	93.20 ± 0.96	28.37 ± 5.63	40.25 ± 2.05	>100	21.13 ± 1.05	36.22 ± 2.58
29	>100	>100	>100	>100	95.03 ± 0.63	>100	>100	>100	55.54 ± 5.97	72.97 ± 4.45
30	>100	>100	>100	>100	66.70 ± 2.04	>100	>100	>100	>100	>100
31	>100	36.59 ± 1.61	91.73 ± 10.31	61.15 ± 0.85	45.67 ± 4.81	>100	>100	>100	>100	34.50 ± 0.68
32	14.84 ± 2.17	77.51 ± 1.04	>100	5.07 ± 0.53	92.32 ± 0.48	21.11 ± 1.92	5.70 ± 0.31	>100	61.33 ± 2.80	31.02 ± 0.99
33	22.18 ± 0.58	>100	>100	>100	>100	>100	>100	>100	>100	>100
34	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
35	>100	64.10 ± 5.30	>100	>100	93.01 ± 3.39	>100	>100	>100	>100	15.81 ± 3.36
36	>100	>100	>100	72.83 ± 7.29	>100	>100	>100	>100	>100	31.63 ± 4.56
37	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
38	5.42 ± 0.17	>100	>100	44.35 ± 2.34	5.68 ± 0.12	11.43 ± 0.45	4.39 ± 0.25	63.03 ± 5.83	4.99 ± 0.11	10.29 ± 3.30
39	8.15 ± 1.03	8.27 ± 4.69	45.90 ± 3.17	15.58 ± 0.88	10.97 ± 1.89	13.08 ± 3.22	5.36 ± 0.17	46.15 ± 3.69	8.84 ± 0.38	15.11 ± 3.12
40	14.44 ± 4.65	>100	>100	26.45 ± 6.41	>100	72.11 ± 2.67	5.93 ± 1.56	95.27 ± 3.66	36.29 ± 2.92	36.62 ± 6.86
41	>100	>100	>100	>100	78.13 ± 9.90	>100	>100	>100	69.10 ± 4.01	>100
42	>100	35.58 ± 1.23	>100	>100	15.37 ± 0.13	>100	>100	>100	21.55 ± 0.11	21.06 ± 2.43
43	19.60 ± 3.45	42.84 ± 3.20	>100	>100	69.16 ± 2.64	72.97 ± 3.63	>100	78.10 ± 6.50	42.54 ± 1.58	28.71 ± 7.97
44	36.87 ± 3.87	20.68 ± 0.54	>100	5.32 ± 0.34	12.30 ± 2.25	23.46 ± 1.34	14.93 ± 1.15	49.23 ± 7.97	12.14 ± 0.11	9.38 ± 0.18
45	17.46 ± 4.30	12.50 ± 0.51	23.51 ± 1.29	19.26 ± 0.99	28.52 ± 4.60	18.46 ± 3.88	19.13 ± 4.67	38.56 ± 1.59	10.16 ± 0.36	7.91 ± 0.83
46	>100	>100	>100	93.24 ± 3.03	94.08 ± 3.71	>100	>100	>100	>100	29.82 ± 3.82

ring A substitution. Group 2 chalcones (**5**–**15**) have ring A substituted with hydroxy group at position 2. Groups 3 and 4 are variants of group 2 in which addition of two methoxy groups at positions 4 and 6 resulted in group 3 (**16**–**20**) and at positions 3 and 4 gave rise to group 4 (**21**). Group 5 chalcones (**30**) have ring A substituted with methoxy group at position 2. Groups 6 and 7 are variants of group 5 in which addition of one methoxy at position 4 resulted in group 6 (**22**–**25**) and at position 5 gave group 7 (**26**–**27**). Group 8 chalcones (**31**–**33**) have ring A substituted with methoxy group at position 4. Groups 9 and 10 are variants of group 8 in which an additional methoxy group appeared at position 3 in the case for group 9 (**28**–**29**) and the methyl group is replaced by a methoxy group at position 4 resulted in group 10 (**34**–**37**). Group 11 chalcones (**38**–**42**) had ring A substituted with amino group at position 2. Group 12 chalcones (**43**–**46**) had ring A substituted with amino group at position 4. The major substituents on ring B were the halogens (F, Cl, Br), electron donating groups (OH, OCH_3, SCH_3, 1,3-dioxolane), or electron withdrawing groups (NO_2). In some chalcones (**15**, **33**, **42**) ring B was replaced with naphthalene ring. All the chalcones synthesized in this study obey the Lipinski's rule of five (Table 1) suggesting these chalcones could be potential orally active drug candidates [24].

Table 3
The IC_{50} values of chalcones over HEK-293 and HT-29 cells with corresponding selectivity ratios.

Chalcone no.	HEK-293 IC_{50} (μM)	HT-29 IC_{50} (μM)	Selectivity ratio
1	86.93 ± 6.97	>100	<1
2	67.98 ± 4.59	9.95 ± 1.12	6.83
3	22.23 ± 0.49	14.36 ± 2.05	1.55
4	33.77 ± 5.21	5.81 ± 0.24	5.81
5	48.68 ± 0.55	70.75 ± 10.08	0.69
6	48.20 ± 9.80	45.25 ± 5.92	1.07
7	53.79 ± 3.49	32.74 ± 4.81	1.64
8	>100	56.25 ± 2.45	>1.78
9	22.93 ± 0.278	>100	<1
10	71.47 ± 8.92	32.05 ± 11.00	2.23
11	37.88 ± 1.80	17.68 ± 5.04	2.14
12	45.66 ± 3.83	22.84 ± 4.92	2.00
13	46.30 ± 1.77	55.61 ± 3.39	0.83
14	42.75 ± 2.70	48.31 ± 0.93	0.88
15	73.40 ± 4.70	>100	<1
16	>100	>100	n.d.
17	>100	>100	n.d.
18	>100	13.67 ± 1.42	>7.31
19	21.71 ± 0.14	6.61 ± 0.72	3.28
20	66.85 ± 1.4	>100	<1
21	81.42 ± 0.93	7.66 ± 1.72	10.63
22	>100	>100	n.d.
23	>100	5.00 ± 0.26	>20
24	>100	17.61 ± 3.29	>5.68
25	>100	>100	n.d.
26	22.95 ± 0.13	5.51 ± 0.71	4.17
27	60.44 ± 8.71	4.54 ± 0.20	6.94
28	88.83 ± 2.07	40.25 ± 2.05	2.21
29	>100	>100	n.d.
30	>100	>100	n.d.
31	83.85 ± 10.58	>100	<1
32	>100	5.70 ± 0.31	>17.54
33	>100	>100	n.d.
34	>100	>100	n.d.
35	>100	>100	n.d.
36	>100	>100	n.d.
37	>100	>100	n.d.
38	>100	4.39 ± 0.25	>22.78
39	36.31 ± 3.02	5.36 ± 0.17	6.77
40	>100	5.93 ± 1.56	>16.86
41	>100	>100	n.d.
42	44.42 ± 1.17	>100	<1
43	95.34 ± 2.48	>100	<1
44	72.39 ± 9.65	14.93 ± 1.15	4.84
45	86.18 ± 0.35	19.13 ± 4.67	4.50
46	83.43 ± 5.07	>100	<1

n.d. = cannot be quantified.

2.2. Biological activity

2.2.1. Anti-proliferative effects of chalcones on cancer cells and its structure–activity relationships

Chalcones synthesised in this study were tested for their anti-proliferative effects for 72 h at 37 °C using the methyl thiazolyl tetrazolium (MTT) assay on 10 different cancer cell lines, viz., human oestrogen receptor positive breast cancer cells (MCF7), human oestrogen receptor negative breast cancer cells (MDA-MB-231), human cervical cancer cells (HeLa), human ovarian cancer cells (Caov-3), human lung cancer cells (A549), human liver cancer cells (HepG2), human colorectal cancer cells (HT-29), human nasopharyngeal cancer cells (CNE-1), human T-lymphoblastoid leukaemia cells (CEM-SS), human erythromyeloblastoid leukaemia cells (K562) and normal human embryonic kidney (HEK-293) cells respectively.

Table 2 lists the IC_{50} values of the chalcones against human cancer cell lines (MCF-7, MDA-MB-231, HeLa, Caov-3, A549, Hep G2, HT-29, CNE-1, K562, CEM-SS) and Table 3 lists the IC_{50} values of chalcones against normal human embryonic kidney (HEK-293) cells. Therefore selectivity ratios (IC_{50} HEK-293/IC_{50} HT-29) of the chalcones were calculated using the following formula:

$$\text{Selectivity ratio} = \frac{IC_{50} \text{ of chalcones against HEK} - 293 \text{ cells}}{IC_{50} \text{ of chalcones against HT} - 29 \text{ cells}}$$

The selectivity ratios of chalcones are shown in Table 3. Arbitrary thresholds of IC_{50} against HT-29 $\leq$ 10 μM and selectivity ratio $\geq$ 5 (Table 3) were used to identify promising compounds. Eight (chalcone **2**, **4**, **21**, **23**, **27**, **32**, **38** and **39**) out of forty-six chalcones fulfilled the threshold limits for IC_{50} and selectivity ratio. The contribution of either the electron-withdrawing or electron-donating substituents to anti-proliferative effect was assessed by making the following comparisons.

Firstly, we compared the activities (based on IC_{50} HT-29 values) of the unsubstituted chalcone **1** with mono-hydroxy substituted chalcones **2**–**5**. The chalcones with –OH group at either *ortho*- or *para*- positions on aromatic ring B were shown potent and selective antiproliferative effect on HT-29 cells. The –OH groups on both rings A and B of chalcones **6**–**8** did not improve the anticancer activity compared to that of mono hydroxy substituted chalcones in which –OH group is present on either A or B ring. Hydroxyl group substitutions in chalcones have been shown to play a key role in anti-cancer activities of chalcones [25]. These results suggest that the presence of –OH group and its position on ring B are important in determining the potency and selectivity.

Secondly, comparisons were made to determine if having another substituent (–OCH_3, –1,3-dioxolane, –CH_3, –Cl, –C_6H_5, –$N(CH_3)_2$, –Br) in addition to –OH group (Chalcones **9**–**21**) made a difference to activity. There was no specific trend observed in antiproliferative activity. Chalcone **21** containing –NO_2 group at *para*-position on ring B was the most potent and selective.

Thirdly, comparisons were made to determine if having more than one electron withdrawing/donating groups (-OCH_3, –CH_3 – NO_2, –F, –Cl, –Br, –1,3-dioxolane, –SCH_3, –$N(CH_3)_2$, –C_6H_5) on either ring (chalcones **22**–**37**) made a difference in activity. There was no specific trend and all the chalcones were neither potent nor selective. Chalcones **23** and **32** with a substituent –Cl on ring B and chalcone **27** with three –OCH_3 groups were potent and selective indicating that the substituents –Cl and –OCH_3 on ring B contribute to the antiproliferative activity of chalcones.

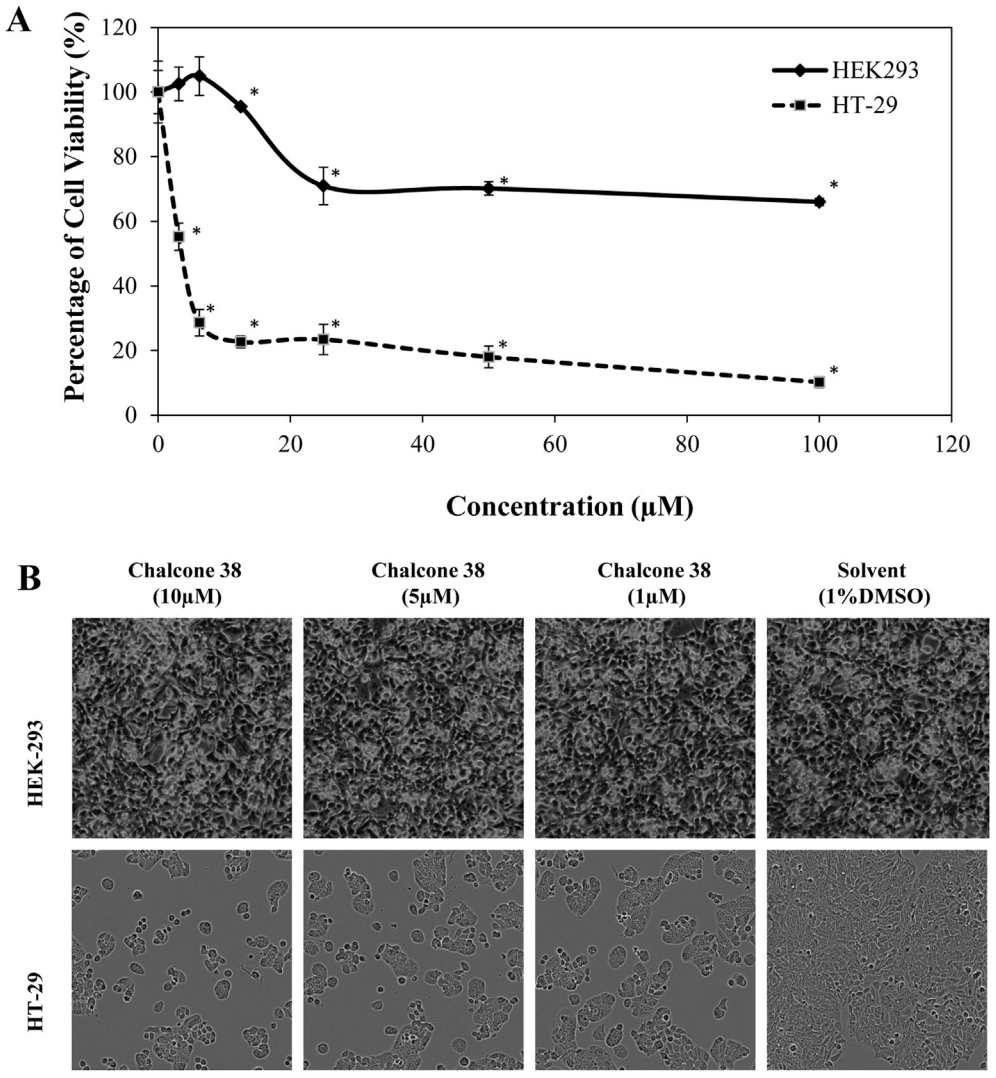


Fig. 1. (A) The dose response curve of the chalcone **38** against HEK-293 and HT-29 cells, (B) morphological changes in HEK-293 and HT-29 cells upon treatment with chalcone **38**.

Fourthly, comparisons were made to determine the effect of $-NH_2$ group on ring A and other substituent ($-NO_2$, $-Cl$, $-OCH_3$, $-C_6H_5$) on ring B (chalcones **38**–**46**) made a difference in activity. Chalcones **38** and **42** showed the most potent anti-proliferative effect against HT-29 cells indicating that the $-NH_2$ group on ring A plays an important role.

Among all those tested, chalcone **38** exhibits the most potent anti-proliferative effects with lowest IC_{50} (4.39 ± 0.25 μM) demonstrated against HT-29 cells. The dose response curves (Fig. 1A) showed that the chalcone **38** exhibited lower percentage of cell viability in HT-29 cells at all concentrations when compared to HEK-293 cells. The anti-proliferative effects exhibited by the chalcone **38** were also dose dependent. Observation under the microscope (Fig. 1B) showed no significant morphological change in HEK-293 cells treated 1–10 μM of chalcone **38,** as compared to HEK293 cells treated with negative control (1% dimethylsulfoxide, DMSO). However we observed that the number of viable HT-29 cells was reduced when HT-29 cells were exposed to 1–10 μM of chalcone **38.** When compared to the negative control, treatment of chalcone **38** caused HT-29 cells to be shrunken and/or rounded in shape. Some cells began to detach thereby loosing their cell–cell and cell–plate interactions. The above observations were similar to the

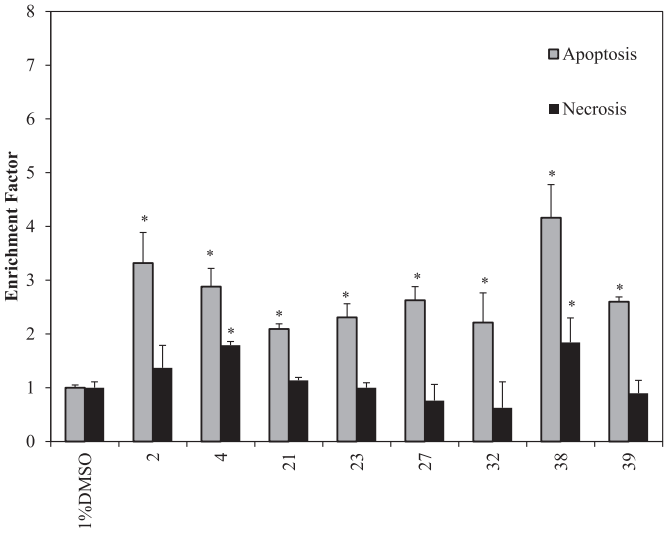


Fig. 2. The mode of cell death induced by the selected chalcones in HT-29 cells.

morphology of the apoptotic bodies, the preferred programme cell death [26–28].

2.2.2. Chalcones induce cell death through apoptosis rather than necrosis in cancer cells

Cancer cell death induced by chemotherapy could be activated through apoptosis or necrosis of which apoptosis is the preferred mode of cell death [29,30]. Chemoresistance to conventional chemotherapy could also be due to the dysregulated apoptotic pathways [31–33]. Reversal of chemo-resistance and thus enhancement of apoptosis by drug or drug-like molecule could potentiate apoptotic cell death in cancer [34–37].

We observed that the most prominent anti-proliferative effects were exhibited by chalcones on HT-29, the most sensitive of all cancer cells, with the mode of cell death yet to be identified. Microscopic observation suggests apoptotic cell death. Therefore, eight (chalcone **2**, **4**, **21**, **23**, **27**, **32**, **38** and **39**) out of forty-six chalcones that fulfilled the requirement of IC_{50} against HT-29 cells $\leq$ 10 μM and selectivity ratio $\geq$ 5 (Table 3) were selected for a cell death study by using the Cell Death Detection ELISA^{PLUS} Kit (Roche, Germany). The results showed that all chalcones induced a higher enrichment factor in apoptosis as compared to necrosis (Fig. 2). All enrichment factors of apoptosis induced by chalcones were significantly different ($p < 0.05$) as compared with cells treated with 1% DMSO. These selected chalcones induced up to 4.16 fold (chalcone **38**) of apoptosis on HT-29 as compared to cells treated with 1% DMSO. The results support the above microscopic observation in which chalcone **38** induced apoptosis. These results were also in agreement with the findings from literature which suggests that chalcones can induce apoptosis in cancer cells. [38–47]

*2.2.3. Chalcone **38** induces early apoptosis in cancer cells*

To further understand the stage of apoptosis induced by the chalcone **38** on HT-29, we subjected the cancer cells treated with the chalcone **38** to double staining of acridine orange (AO)-propidium iodide (PI) fluorescence microscopy. In viable cells and cells undergone early apoptosis, most of the cells will have intact cell membrane which excludes PI and only allows AO to cross the cell membrane. In the late apoptosis and necrotic cells, the cell membrane will be disrupted and allows both AO and PI to cross the membranes and stain the cells with a predominance of PI fluorescence. Therefore, the double staining approach would able to differentiate the viable cells (green intact nucleus), early apoptosis cells (dense green areas of chromatin condensation in the nucleus), late apoptosis cells (red-orange areas of chromatin condensation in the nucleus) and necrosis cells (red-orange intact nucleus or fragmented cells).

For HT-29 cells treated with 1% DMSO and IC_{50} of the chalcone **38**, fluorescence microscopic observation showed that majority of the HT-29 cells were stained with green fluorescence with minimum or negligible red fluorescence (Fig. 3). HT-29 cells treated with the chalcone **38** exhibited a higher number of cells with a dense green core, as compared to the HT-29 cells treated with 1% DMSO. These observations suggest treatment with chalcone induced early apoptosis. As a comparison, we treated HT-29 cells with 5 μM of 5-fluorouracil, the conventional chemotherapy agent for treating colorectal cancer patients. We observed that a large

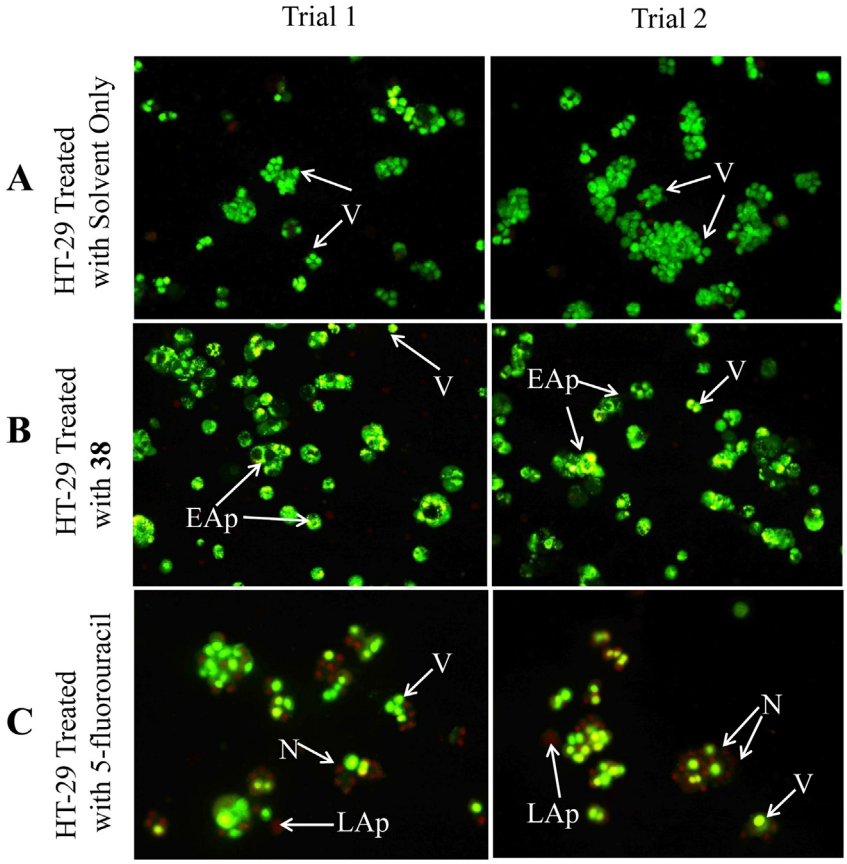


Fig. 3. Fluorescence microscopic examination of HT-29 cells treated with the (A) solvent, (B) chalcone **38** (4.5 μM) and (C) 5-fluorouracil (5 μM). Viable cells (V) were stained green with intact nucleus; early apoptotic (EAp) cells were stained green with chromatin condensation; apoptotic cells (LAp) were stained red with chromatin condensation; necrotic cells (N) were stained red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

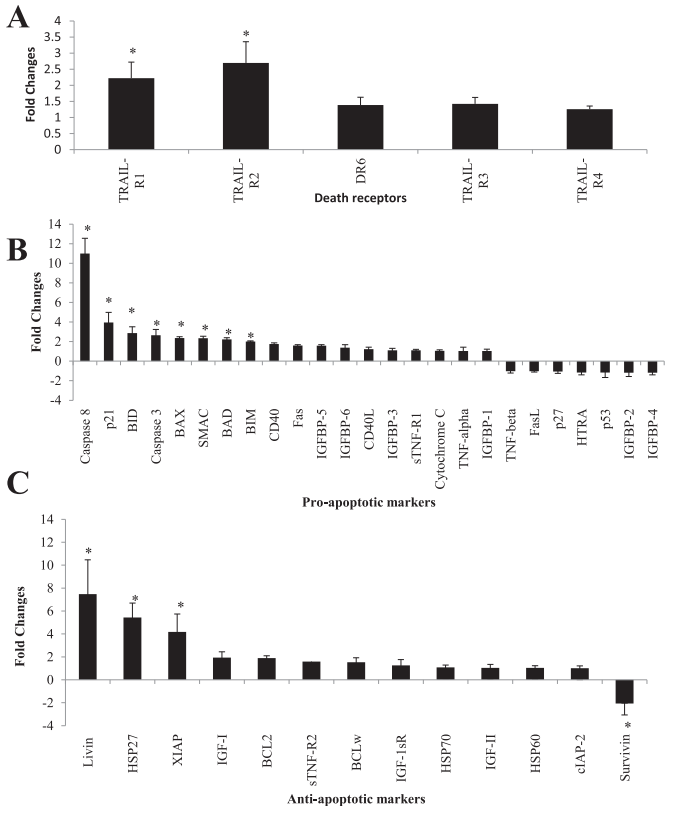


Fig. 4. The effect of the chalcone **38** on the expression of (A) death receptors, (B) pro-apoptotic markers, (C) anti-apoptotic markers in HT-29 cells. Fold changes more than 2 is indicated with symbol (*).

portion of HT-29 cells had undergone late apoptosis (dense red core) and/or necrosis (red fragmented cells or cells debris) after treatment with 5-fluorouracil. These findings further supported the results from the microscopic observation and the Cell Death Detection ELISA^{PLUS} kit, in which the chalcone **38** induces apoptosis rather than necrosis in HT-29 cells.

*2.2.4. Multiple apoptosis markers induced by chalcone **38** in cancer cells*

In order to further investigate the apoptotic mechanisms induced by chalcone **38** in cancer cells, HT-29 cells treated with the chalcone **38** and subjected to Raybio® Human Apoptosis Antibody Array (Norcoss, GA, USA). We studied the effect of the chalcone **38** on the receptors for the TNF related apoptosis inducing ligand (TRAILR). TRAIL-R1 (also known as death receptor 4) and TRAIL-R2 (also known as death receptor 5) were reported to be up-regulated to sensitise cancer cells to TRAIL induced apoptosis [48]. The TRAIL-R3 and TRAIL-R4 were decoy receptors lacking intracellular death domain and therefore dysfunctional. Over-expression of TRAIL-R3 and TRAIL-R4 could possibly prevent TRAIL to induce apoptosis. TRAILR-3 prevents the formation of TRAIL-R2 associated death inducing signalling complex (DISC). On the other hand, TRAIL-R4 binds to TRAIL-R2 DISC to obstruct the recruitment of caspases within DISC as well as activates NF-κB to inhibit TRAIL induced apoptosis [49–52]. Over-expression of the tumour necrosis factor receptor superfamily member 21 (DR6 or death receptor 6) also induces apoptosis [53,54]. Our results showed that HT-29 cells treated with the chalcone **38,** showed 2.221 and 2.696 fold increases of TRAIL-R1 and TRAIL-R2 respectively as compared to cells treated with 1% DMSO (Fig. 4A). The chalcone **38** did not affect the expression of death receptor 6 (DR6), TRAIL-R3 and TRAIL-R4.

The chalcone **38** upregulated various pro-apoptotic markers in HT-29 cells as compared to HT-29 cells treated with 1% DMSO (Fig. 4B). Caspase 8, an initiator cysteine–aspartic acid protease (caspase), was the highest upregulated pro-apoptotic marker in HT-29 cells treated with the chalcone **38** (11.000 fold). We also observed up regulation of caspase 3 by 2.651folds in HT-29 cells, which implies that caspase 8 dependent activation of caspase 3 results in amplification of apoptosis [55,56]. Also, we observed a 2.343 fold increase in the second mitochondrial derived activator (SMAC) of caspase in HT-29 cells treated with the chalcone **38** as compared to control. SMAC induces apoptosis as a result of the activation of caspases [57]. Treatment with the chalcone **38** has induced a high level of pro-apoptic bcl-2 markers such as bcl-2 homology 3-interacting domain death agonist (BID, 2.867 fold increase) [58], bcl-2 like protein 4 (BAX, 2.363 fold increase) [59,60], bcl-2 antagonist of cell death (BAD, 2.225 fold increase) [61], and bcl-2 like protein 11 (BIM, 2.011 fold increase) [62] in HT-29 cells treated with the chalcone **38** as compared to control. Up-regulation of cyclin dependent kinase inhibitor 1 (p21) is associated with enhancement of apoptosis, probably through BAX [63]. The treatment of compound **38** increased p21 (3.961fold) when compared to the control. The other pro-apoptotic markers which had less than twice the increase included tumour necrosis factor receptor superfamily member 5 (CD40), necrosis factor receptor CD40 ligand (CD40L), superfamily member 6 (Fas), Fas ligand (FasL), insulin-like growth factor binding protein (IGFBP)-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, tumour necrosis factor receptor superfamily 1A (sTNF-R1), cytochrome C, TNF-alpha, TNF-beta, p27, p53, and high temperature required protein (HTRA) (Fig. 4B).

We also observed that a great reduction in baculoviral inhibitor of apoptosis proteins repeat containing protein 5 (survivin), one of the inhibitor of apoptosis proteins (IAP). Survivin exerts its anti-apoptotic effect via binding with baculoviral IAP repeat containing protein 4 (XIAP) and thus further increase XIAP's inhibitory effect against caspase 9 [64]. One study suggests that survivin delays SMAC release during an apoptotic stimulus [65]. Treatment of the chalcone **38** reduced the survivin's level (2.06 fold, Fig. 4C) in HT-29 cells as compared to the control. The unbounded XIAP could thus results in a 4.176 ± 1.56 fold increase of XIAP in cells treated with chalcone **38**. Just like other IAPs, baculoviral IAP repeat containing protein 7 (livin) is one of the anti-apoptotic markers. Cancer cells with high expression of livin are commonly associated with poorer prognosis [66–69]. However the role of livin as solely an anti-apoptotic protein is remain questionable as livin could have both pro-apoptotic and anti-apoptotic effect, as per highlighted by Abd-Elrahman and coworkers recently. Under a strong apoptotic stimulus, livin was cleaved by caspases to result in a truncated protein with paradoxical pro-apoptotic and tumour suppressive effect [70]. Our results showed an increase in livin level after treatment with the chalcone **38**. Therefore the chalcone **38** induces apoptosis in HT-29 cells by inhibiting survivin and inducing livin's pro-apoptotic effect. The up-regulation of heat shock proteins (HSP) were associated with poorer clinical outcomes and anti-apoptotic effect [71–73]. However, the treatment implication of HSP is still under investigation because the elevated HSP could acts as biological adjuvants to break tolerance to tumour antigens. We observed an induction of 5.440 ± 1.26 fold increase in HSP27 and less than 2 fold increase in HSP60 and HSP70 for HT29 cells treated with the chalcone **38**. The other anti-apoptotic markers which had less than 2 folds, were BCL-2, bcl-2 like protein 2 (BCLw), tumour necrosis factor receptor superfamily 1B (sTNF-1B), insulin-like growth factor 1 receptor (IGF-1sR), insulin-like growth factor 1 (IGF-I), insulin-like growth factor-2 (IGF-II) and baculoviral IAP repeat containing protein 3 (cIAP) (Fig. 4C).

3. Conclusions

A series of chalcones containing electron-withdrawing and electron-donating substituents was synthesised as a continuation of our ongoing anticancer development research project. The target compounds were evaluated for antiproliferative activities against ten human TRAIL-resistant cancer cells and their selectivity indices. A structure activity relationship (SAR) study has been made to correlate between the chalcones structures and antitumour activities. Chalcones **2**, **4**, **21**, **23**, **27**, **32**, **38** and **39** possessing substituents $-NH_2$ on ring A and $-OH$ and $-NO_2$ on ring B showed potent and selective antitumour activity.

In addition, chalcone **38**, the most potent, induces apoptosis in TRAIL-resistant human colon cancer (HT-29) cells by increasing the death receptors expression (TRAIL-R1 and TRAIL-R2) and pro-apoptotic markers (p21, BAD, BIM, BID, BAX, SMAC, caspase 3 and caspase 8) as well as reducing the anti-apoptotic markers (livin, XIAP and HSP27).

The high potency of chalcone **38** over the ten tested TRAIL-resistant tumours, *in silico* studies which predicted oral bioavailability, and regulation of 43 apoptosis related proteins would pave a way for future development of anticancer agents.

4. Experimental

4.1. Chemistry

A total of 46 chalcone derivatives (**1-46**, Table 1) were synthesised by following a Claisen–Schmidt condensation reaction [74,75]. The Lipinski's rule of five [76,77] parameters for all the chalcones were predicted using Discovery Studio 2.5, Accelrys (San Diego, CA, USA). The details of synthesis and spectroscopic data are presented in the supplementary information.

4.2. Biological activity

4.2.1. Cell lines

The human oestrogen receptor positive breast cancer cells (MCF7; HTB-22™), human oestrogen receptor negative breast cancer cells (MDA-MB-231; HTB-26™), human cervical cancer cells (HeLa; CCL-2™), human ovarian cancer cells (Caov-3; HTB-75™), human lung cancer cells (A549; CCL-185™), human liver cancer cells (Hep G2; HB-8065™), human colorectal cancer cells (HT-29; HTB-38™), human erythromyeloblastoid leukaemia cells (K-562; CCL-243™), and non-cancerous human embryonic kidney cells (HEK-293; CRL-1573™) were obtained from the American Tissue Culture Collection (Rockville, MD, USA); while human T-lymphoblastoid leukaemia cells (CEM-SS) was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CEM-SS (Cat# 776) from Dr. Peter L. Nara. Human nasopharyngeal cancer cells (CNE-1) were a kind gift from Professor Sam C. K. (Institute of Biological Sciences, University of Malaya, Malaysia).

4.2.2. Cell culture

All cancerous and non-cancerous cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum, 100IU/mL of penicillin, 100 μg/mL of streptomycin, with L-glutamine, 4.5 g/L glucose and sodium pyruvate (Sigma–Aldrich, St Louis, MO, USA). All cells were maintained at 37 °C under 5% CO_2 in a humidified incubator.

4.2.3. Cell proliferation assay

Inhibition of cell proliferation by chalcones was determined using the methyl thiazolyl tetrazolium (MTT) cell viability assay, as described previously [78]. All the chalcones were tested in the concentration range of 1–100 μM.

4.2.4. Detection of mode of cancer cells deaths by quantitative sandwich enzyme immunoassay (ELISA)

The degree of mode of cancer cell deaths induced by chalcones were quantified using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Indianapolis, USA) as per the manufacturer's instruction and in our previous studies [79,80].

4.2.5. Acridine orange (AO)-propidium iodide(PI) double staining fluorescence microscopy

In order to further understand the degree of morphological changes, HT-29 cells were challenged with chalcone **38** for 72 h and stained with a mixture of acridine orange (AO)-propidium iodide (PI) as described in the literature [81–83]. The stained cells were observed under a Nikon Eclipse 80i fluorescence microscope (Tokyo, Japan). Viable cells are presented as a green intact nucleus; early apoptosis cells are presented as dense green areas of chromatin condensation in the nucleus; late apoptosis cells exhibit dense red-orange areas of chromatin condensation in the nucleus while necrosis cells have a red-orange intact nucleus or fragmented cells.

4.2.6. Cellular apoptotic markers analysis using antibody array

Apoptosis or programmed cell death is a complex mechanism with multiple possible pathways involved. Raybio® Human Apoptosis Antibody Array (Norcoss, GA, USA) was thus used to simultaneously determine the effect exerted by chalcone **38** on HT-29 cells. All reagents and chemicals were supplied by the manufacturer unless specified. The array was prepared in accord with the manufacturer's instructions. In brief, HT-29 cells were seeded for 24 h and treated with chalcone **38** at IC_{50} or 1% DMSO (negative control) for 3 days. Cells were rinsed with cold phosphate buffer saline (PBS) (Sigma–Aldrich, St Louis, MO, USA) to remove any dead cells or debris. Cells were lysed using the 1 × Lysis Buffer containing Protease Inhibitor Cocktail as supplied by the manufacturer. Cells were re-suspended through gentle pipetting and subsequently the lysates were rocked gently for 30 min at 4 °C. The mixture was centrifuged at 14,000× g for 10 min. The protein lysates (supernatant) were collected and the protein concentration was estimated using a Bio-Rad Protein Assay Kit (Hercules, CA, USA). All the lysates were diluted with the 1× Blocking Buffer to 200 μg/mL. The diluted lysates were added to the antibody array membranes and incubated for 2 h at room temperature. The membranes were washed using 1× Wash Buffer I and 1× Wash Buffer II as specified by the manufacturer. A cocktail of biotin-conjugated and anti-apoptotic protein antibodies were incubated with the membranes for 2 h at room temperature. Remaining unbound antibodies were washed away by rinsing buffers before the membranes were incubated in the dark with horseradish peroxidase (HRP)-streptavidin for 2 h at room temperature. The arrays were scanned using the Axon Gene Pix 4000B (Molecular Devices, Sunnyvale, CA, USA) and the values were extracted using the Axon Gene Pix Pro 6.1 (Molecular Devices, Sunnyvale, CA, USA) software. The data was then analysed using the RayBio® Analysis Tool (RayBiotech, Norcross, VA, USA).

4.3. Statistical analysis

All data were reported as mean ± standard deviation from a minimum three independent experiments. Statistical significant difference was analysed using one-way analysis of variance (ANOVA) or independent sample *T*-test through SPSS (version 18.0)

for Windows. A p-value of less than 0.05 ($p < 0.05$) was considered significantly different.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.002.

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