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Synthesis and cytotoxic potential of heterocyclic cyclohexanone analogues of curcumin

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ABSTRACT

A series of 18 heterocyclic cyclohexanone analogues of curcumin have been synthesised and screened for their activity in both adherent and non-adherent cancer cell models. Cytotoxicity towards MBA-MB-231 breast cancer cells, as well as ability to inhibit NF-κB transactivation in non-adherent K562 leukemia cells were investigated. Three of these analogues 3,5-bis(pyridine-4-yl)-1-methylpiperidin-4-one **B1**, 3,5-bis(3,4,5-trimethoxybenzylidene)-1-methylpiperidin-4-one **B10**, and 8-methyl-2,4-bis((pyridine-4-yl)methylene)-8-aza-bicyclo[3.2.1]octan-3-one **C1** showed potent cytotoxicity towards MBA-MB-231, MDA-MB-468, and SkBr3 cell lines with EC_{50} values below 1 μM and inhibition of NF-κB activation below 7.5 μM. The lead drug candidate, **B10**, was also able to cause 43% of MDA-MB-231 cells to undergo apoptosis after 18 h. This level of activity warrants further investigation for the treatment of ER-negative breast cancer and/or chronic myelogenous leukemia as prototypical cellular models for solid and liquid tumors.

1. Introduction

Breast cancer is the most prevalent form of cancer diagnosed in women, and its incidence is steadily rising.[1] Although there are effective treatments for estrogen receptor (ER)-positive breast cancers unfortunately for ER-negative breast cancers, which account for approximately 30% of all breast cancer diagnoses, treatments are limited. Those cancers that overexpress Her-2 can now be treated with trastuzumab (Herceptin) immunotherapy, however, there is still a need for more drug treatments for ER-negative breast cancers that either overexpress or lack Her-2.

In the search for effective drugs for ER-negative breast cancer several natural product lead compounds have been identified. Curcumin (diferuloylmethane), the primary bioactive compound isolated from the rhizome of turmeric (*Curcuma longa* Linn.), is of particular interest.[2] Curcumin has long been known as a chemopreventative and chemotherapeutic agent, and in vivo studies have demonstrated decreased tumorigenesis of many organs, including the mammary gland.[2–8] In vitro studies have also demonstrated that curcumin has potent cytotoxicity towards many cell lines including ER-negative human breast cancer cells.[9–15] Whilst curcumin itself has limited clinical efficacy due to its low bioavailability and stability in physiological media,[3] it has been the subject of many analogue studies.[16–20] From these studies it has been shown that cyclohexanone analogues of curcumin have enhanced activity and stability in biological medium compared to curcumin.[19] The cyclohexanone-containing curcumin derivative 2,6-bis((3-methoxy-4-hydroxyphenyl)methylene)-cyclohexanone (BMHPC) **A13** was found to be cytotoxic towards ER-negative breast cancer cells (EC_{50} of 5.0 μM),[21] although bioavailability and in vivo efficacy were still problematic. More recently several fluorinated cyclohexanone derivatives have been prepared, one of which has shown potent cytotoxicity towards MDA-MB-231 cells (EC_{50} of 0.8 μM).[16,22]

Recently we have reported that the two pyridine analogues, 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone, **A1** and 2,6-bis(pyridin-3-ylmethylene)-cyclohexanone, **A2** are cytotoxic towards MDA-MB-231 cells with EC_{50}s of 1.10 and 1.54 μM, respectively).[23] In order to investigate structure–activity relationships of these potent analogues, and in the search for analogues with even greater activity, we needed to assay a wider range of compounds. We chose to concentrate on nitrogen heterocycles, since these have the advantage of their ability to exist in both a protonated and neutral form, allowing both, solubility in aqueous media, as well as the potential to cross cellular membranes. Our laboratory (Rosengren) uses the aggressive ER-negative MDA-MB-231 human breast cancer cells, amongst others, to identify new potent drugs. Our collaborators (Diederich) use the inhibition of the nuclear transcription factor

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κB (NF-κB) activation. NF-κB is an important target in drug discovery for ER-negative breast cancer as it is over expressed in these tumors.[24] As NF-κB is responsible for increased cell resistance towards possible cytocidal treatments currently applied in clinics, apoptosis induction through NF-κB inhibition and/or extrinsic and intrinsic pathway activation is considered as a main aim in cancer research. NF-κB has largely been proven to be implicated in cell death (apoptosis), cell adhesion, cell proliferation, innate- and adaptive-immune response and cancer development.[25,26]

This work describes the preparation and biological assessment of a series of 18 heterocyclic cyclohexanone curcumin analogues, in both adherent and non-adherent cancer cell models. Curcumin, **A10**, **A12**, **A13** and the two previously studied heterocyclic analogues **A1** and **A2** were included for comparison purposes. All compounds were initially screened for their cytotoxicity towards ER negative breast cancer cells as well as their inhibition of NF-κB in leukemia cells. By including the inhibition of NF-κB we have ensured that only drugs with a dual potency in both adherent and non-adherent cellular models underwent further testing.

2. Results and discussion

2.1. Chemistry

A series of heterocyclic analogues of curcumin containing a cyclic ketone as a core, with aromatic groups linked to the core via two methylidene groups (Fig. 1) were prepared. Series A contain a cyclohexanone core; series B, an *N*-methylpiperidone core; series C a tropinone core and series D a cyclopentanone core unit. The aromatic groups include fluorine substituted pyridines, since there are examples of fluorine substitution of aromatics giving enhanced activity.[16] We also included the electron rich five-membered aromatic units of pyrrole, imidazole and indole, as well as electron rich trimethoxyphenyl and two dimethoxyphenyl groups because there has been some evidence that electron rich aromatics can have enhanced activity.[27] Structures are shown in Figure 2. The synthesis of compounds was carried out via condensation of two equivalents of the appropriate aldehyde with the core cyclic ketone following the procedures of Liang et al.[28] and Vatsadze et al.[29] ^{1}H NMR spectroscopy showed that the compounds were all isomerically pure. It is known[30] that the *Z* isomers of =CH are characterized by chemical shifts at $\delta\sim$6.8 ppm, whereas the signals for the *E* isomers should appear at higher field than 7.2 ppm. For all these compounds =CH signals in the 7.5–8.5 ppm region indicated the *E* configuration. Additionally X-ray crystallography of 8-methyl-2,4-bis(4-methoxybenzylidene)-8-azabicyclo[3.2.1]octan-3-one,[31] 3,5-bis(benzylidene)-1-methyl-4-piperidones, and 3,5-bis(benzylidene)-cyclohexanones[29] have all shown that the olefinic double bonds adopt the *E* configuration. Hence, the assumption was made that all of the compounds were *E,E* isomers. The synthetic yields, melting points, elemental analyses, ^{1}H NMR, and ESI-MS of unreported compounds are described in Section 4. ^{13}C NMR data are presented in Table 1.

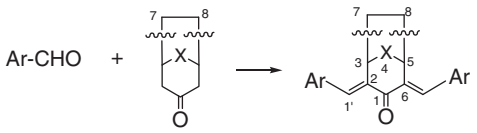

Figure 1. Generic structure of curcumin analogues.

2.2. Cytotoxicity of curcumin analogues towards MDA-MB-231 cells and inhibition of NF-κB in K562 cells

Cytotoxicity of the analogues towards MDA-MB-231 cells as well as inhibition of NF-κB activity in K562 cells was determined and the results presented in Table 2. The results demonstrated that there were 12 analogues with a greater or similar NF-κB inhibition compared to curcumin and 12 analogues that were more cytotoxic towards MDA-MB-231 cells than curcumin. Of these, five analogues **(A1, B1, B10, B12**, and **C1)** showed potent cytotoxicity (EC_{50} values of $\sim$1 μM) and potent NF-κB inhibition (IC_{50} values of <7.5 μM).

2.3. Structure–activity relationship analysis

We have previously demonstrated that the two pyridine analogues, 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone, **A1** and 2,6-bis(pyridin-3-ylmethylene)-cyclohexanone, **A2** were cytotoxic towards MDA-MB-231 cells.[23] In an extension of this work we wanted to explore the structural requirements for activity, in particular in relation to other heterocyclic analogues with differing electronic effects. Previous studies have reported that the six-membered cyclohexanone ring system is in general superior to the five-membered cyclopentanone system for inhibitory activity in a group of seven cancer cell lines.[19] Evidence that this was also the case for MBA-MB-231 cells was found when the five-membered cyclopentanone derivative **D2** was compared with cyclohexanone **A2** and showed a fivefold decrease in activity. We therefore decided to restrict our investigation to six-membered ring ketones. In general it was found that the inhibition of NF-κB activation paralleled cytotoxicity against MBA-MB-231 cells, so we have examined structure–activity relationships mainly in terms of the cytotoxicity against MBA-MB-231 cells. Concentrating initially on the cyclohexanone core **A** series we then examined fluorine substituted pyridine rings. Two examples **A3** and **A4** were readily prepared from commercially available starting materials. These derivatives were found to be 2–3 times less active than the parent pyridine compounds **A1** and **A2**. We next turned our attention to replacement of the pyridine ring with different heterocyclic rings. Thiophene **A5**, *N*-methylpyrrole **A6**, *N*-methylindole **A7** and 4-substituted *N*-methylimidazole **A9** exhibited no activity (EC_{50} >30 μM). 2-Substituted *N*-methylimidazole **A8** showed good cytotoxicity towards MBA-MB-231 cells, but no inhibition of NF-κB activation. We also replaced the pyridyl groups with electron rich trimethoxyphenyl and dimethoxyphenyl to give analogues **A10** and **A12** but neither analogue showed any activity in either assay.

For the *N*-methylpiperidone core series **B** analogues, the heteroaromatic analogues generally had similar or slightly increased activity over cyclohexanone core, series **A** analogues. Thus 4-pyridyl **B1** showed some increased activity over **A1**, the 3-pyridyl analogue **B2** slightly less activity than the corresponding **A2**, and thiophene **B5** retained the lack of activity of **A5**. Surprisingly *N*-methylimidaz-2-yl analogue **B8**, showed no activity compared to **A8** which showed good activity. In contrast the tri- and dimethoxyphenyl **B** series derivatives showed good activity compared to the **A** series where little activity was observed. In fact the trimethoxyphenyl analogue **B10** exhibited a level of activity about three times greater than any other compound examined. The dimethoxyphenyl analogues were also active, with **B11** being less active than **B12**, further indicating that the fluorine group conferred no improvement in activity in these compounds.

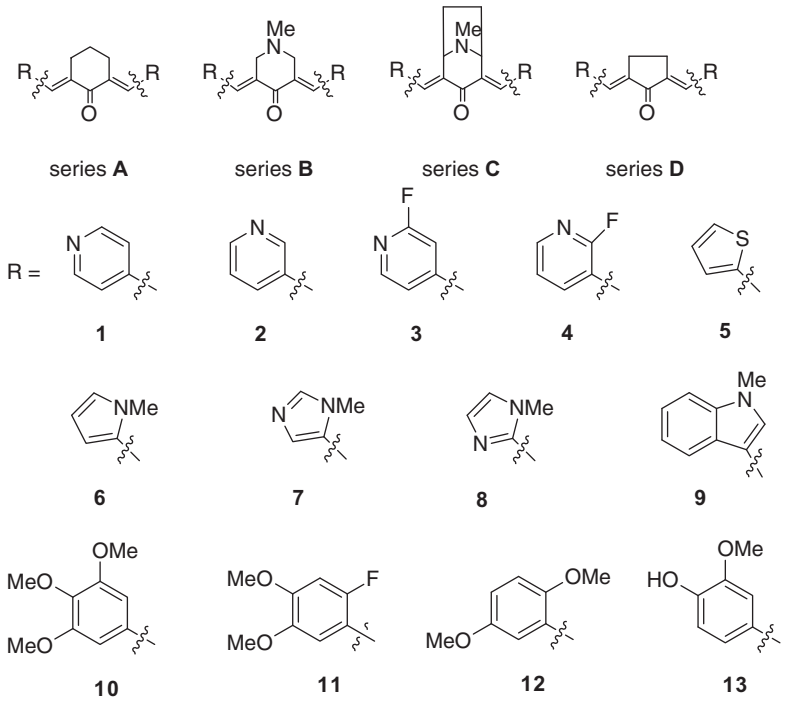

Figure 2. Structures of the heterocyclic curcumin analogues.

Table 1
^{13}C NMR data for heterocyclic curcumin analogues[a]

Compound	A3[b]	A4[b]	A7	A8	A9	B8	B10	B11[b]	B12	C1	C2	C10
Position												
1	188.35	188.55	188.09	190.23	188.71	187.39	186.61	186.01	186.83	187.14	187.05	187.28
2 and **6**	140.79	139.4	134.18	138.36	131.53	135.28	132.40	133.17	133.4	142.33	140.23	138.98
3 and **5**	28.38	28.34	28.62	28.45	29.39	57.22	56.96	57.06	57.06	60.88	61.00	61.01
4	22.31	22.58	21.65	21.63	22.48	—	—	—	—	—	—	—
7 and **8**										29.99	30.11	30.38
1′	127.46	128.32	133.69	130.37	130.35	130.76	136.71	129.68	132.34	133.44	132.69	136.97
4-NMe						46.02	43.61	45.47	45.58	36.12	36.09	35.69
Ar-1	131.03	118.67	129.25	144.41	128.95	143.83	130.73	114.08	125.31	141.62	130.87	130.52
2	138.81	161.08	—	—	127.59	—	107.93	155.76	152.89	123.97	150.81	107.72
3	156.94	—	140.16	122.79	—	123.16	153.12	100.19	111.8	150.16	—	153.10
4	—	147.62	—	119.00	136.56	117.79	139.18	151.07	114.76	—	149.63	137.51
5	145.41	121.11	121.26	—	112.59	—	153.12	145.01	152.89	150.16	123.45	153.10
6	124.06	140.85			122.83		107.93	112.62	116.34	123.97	137.06	107.72
7					120.64							
8					119.52							
9					109.35							
NMe			31.95	33.25	33.29	33.30						
OMe							56.26	56.56	56.11			56.12
OMe							56.26	56.21	55.83			56.12
OMe							60.97					60.97

[a] Non-systematic numbering has been used to facilitate comparisons between compounds. Numbering is based on numbering shown in Figure 1. Assignments have been determined by HSQC and HMBC 2D NMR experiments.
[b] ^{13}C–^{19}F coupling not included.

For the tropinone core, series **C** analogues which have a more rigid structure, as well as being more sterically hindered, we found either no change in activity, as in the case of the 4-pyridyl compound **C1**, or else reduced activity, as seen for compounds **C2** or **C10**.

In summary, we have found that pyridine heteroaromatic substituted bismethylene cyclohexanones are the most active of the heteroaromatic analogues tested to date, and that the five-membered heteroaromatic in general have no activity in this cell line. Replacement of the cyclohexanone core with *N*-methylpiperidone can give some improvement in activity for the heteroaromatic analogues, although this varied. In contrast the polymethoxyphenylmethylene *N*-methylpiperidone derivatives had good activity compared to the cyclohexanone core derivatives which had no activity. The use of the more sterically hindered tropinone led to equal to or reduced activity in all cases. Additionally, fluorine substituents on the aromatic groups do not increase the activity of these analogues. These results suggest that activity in this cell line is not determined by the electronic effects of the aromatic groups. It also appears that a nitrogen heteroatom in either the aromatic group or in the core cyclic ketone provides analogues with good activity.

Table 2
Cytotoxicity and inhibition of NF-κB activation

Compound	MDA-MB-231 EC_{50} (μM)	NF-κB activation IC_{50} (μM)
Curcumin	7.6	15 ± 5
A1	1.1	2.5 ± 0.2
A2	1.5	2.5 ± 0.5
A3	3.3	2.2 ± 0.3
A4	3.2	3.8 ± 0.5
A5	>30	>200
A6	>30	>200
A7	>30	140 ± 20
A8	1.9	48 ± 7
A9	>10	>200
A10	>30	Not tested
A12	>10	>200
A13 (BMHPC)	2.6	10 ± 3
B1	0.8	1.0 ± 0.3
B2	2.1	0.8 ± 0.2
B5	>30	>200
B8	>30	47 ± 3
B10	0.3	0.9 ± 0.1
B11	3.8	4.0 ± 0.5
B12	1.1	6.3 ± 0.3
C1	1.1	1.5 ± 0.2
C2	12.9	4.0 ± 0.5
C10	>30	>200
D2	8.6	34 ± 5

2.4. Cytotoxicity of curcumin analogues in MDA-MB-468 and SKBr3 cells

The analogues which showed potent cytotoxicity (EC_{50} values of ~1 μM) and potent NF-κB inhibition (IC_{50} values of <7.5 μM) were further examined for their ability to elicit cytotoxicity in other ER-negative cell lines. The results are presented in Table 3. **A1, B1, B10, B12**, and **C1** were examined in cell lines that both contained (SkBr3) and lacked (MDA-MB-468) the Her-2, isoform of the epidermal growth factor receptor. All five compounds were found to be more potent than curcumin towards these two breast cancer cell lines, as all elicited EC_{50} values that were 2- to 30-fold lower than that for curcumin. In the SkBr3 cell line **A1** showed the greatest cytotoxicity (EC_{50} 0.2 μM), followed by **B10** (0.4 μM) and **B1** (0.6 μM) whereas in the MDA-MB-468 cell line compound **B10** (EC_{50} 0.3 μM) showed the greatest cytotoxicity, followed by **B1** (0.5 μM) and **C1** (0.6 μM). Within the three cell lines, curcumin and **A1** showed a variation in activity between the different cell lines, with the greatest activity in the SkBr3 cell line, whilst **B1**, **C1**, **B10**, and **B12** showing similar activity in all three cell lines. Looking at comparisons between NF-κB inhibitory activity and cytotoxicity for these five compounds, in general these do seem to be related, with the ratio of the NF-κB inhibitory activity IC_{50}/cytotoxicity EC_{50} being similar for four of the five compounds with both MDA-MB-231 and MDA-MB-468 cell lines. This implies that reduction in the level of the cell signalling protein, NF-κB, is at least in part related to the cytotoxicity of these analogues. There are however differences in the ratio of NF-κB inhibitory activity IC_{50}/cytotoxicity EC_{50} between both curcumin and **A1** cytotoxicity in the SkBr3 cell line and NF-κB inhibitory activity and also between cytotoxicity of **B12** in all cell lines and NF-κB inhibition. This indicates that other factors are involved in the cytotoxicity.

Table 3
EC_{50} (μM) values of curcumin analogues in other ER-negative human breast cancer cells

Compound	MDA-MB-468 EC_{50} (μM)	SKBr3 EC_{50} (μM)
Curcumin	9.7	2.4
A1	1.9	0.2
B1	0.5	0.6
B10	0.3	0.4
B12	1.1	1.1
C1	0.6	0.7

2.5. Induction of apoptosis

To determine mechanisms for the cytotoxicity elicited, the two analogues with the overall greatest potency (**B1** and **B10**) were examined for their ability to induce apoptosis. The results (Fig. 3) showed that the two analogues had differing abilities to induce apoptosis in MDA-MB-231 cells. **B10** (1 μM) caused a significant increase in the proportion of apoptotic cells versus control. Treatment of MDA-MB-231 cells with **B1** (2 μM) induced significant but much lower apoptosis versus control. Furthermore, **B10** had a higher apoptotic potency compared to **A1** and **A2**, as we have previously shown that these analogues at concentration of 2–3 μM caused less than 20% of MDA-MB-231 cells to undergo apoptosis following 18 h of treatment.[23] However, **A1** is a stronger inducer of apoptosis than **B1**, as **A1** exhibited a peak apoptotic induction of ~40% of cells after 36 h,[23] compared to both **A2** and **B1** which showed less than 20% of cells undergoing apoptosis at this time-point. Therefore, **B10** displays the greatest apoptotic induction potential of all the compounds examined.

3. Conclusions

In this study, a series of 18 heterocyclic cyclohexanone analogues of curcumin together with two previously studied analogues **A1** and **A2** and three non-heterocyclic analogues of curcumin were synthesised and evaluated for inhibitory activity against NF-κB transactivation in non-adherent K562 leukemia cells as well as cytotoxicity by using ER-negative breast cancer cell models. A structure–activity relationship study showed that the more electron poor pyridine heteroaromatics were the most active of those tested to date, and that the electron rich pyrrole based structures in general, had no activity in this cell line. The *N*-methylpiperidone analogues did show some improvement in activity over cyclohexanone analogues, although this was not consistent. The more sterically hindered tropinone analogues showed reduced activity in all cases, indicating that bulky groups in this area of the molecule led to decreased activity. Additionally, the incorporation of a fluorine substituent into the aromatic rings did not increase the activity of these compounds. Three of these analogs 3,5-bis(pyridine-4-yl)-1-methylpiperidin-4-one **B1**, 3,5-bis(3,4,5-trimethoxybenzylidene)-1-methylpiperidin-4-one **B10** and 8-methyl-2,4-bis((pyridine-4-yl)methylene)-8-aza-bicyclo[3.2.1]octan-3-one **C1** showed potent cytotoxicity towards MBA-MB-231, MDA-MB-468, and SkBr3 cell lines with EC_{50} values

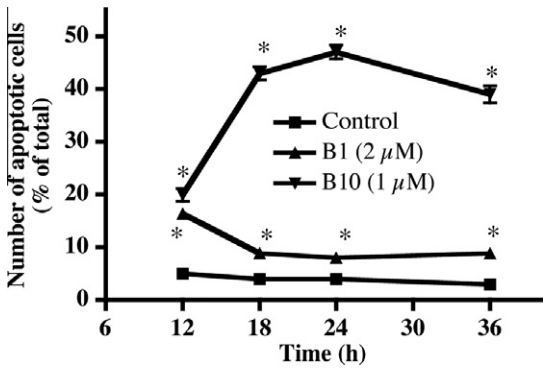

Figure 3. Apoptosis following treatment of MDA-MB-231 cells with **B1** and **B10**. *Significantly different from control p <0.001.

below 1 μM and inhibition of NF-κB activation below 7.5 μM. The lead drug candidate, **B10** was also able to cause 43% of MDA-MB-231 cells to undergo apoptosis after 18 h.

However, only **B10** was more potent at eliciting apoptosis than curcumin, BMHPC (**A13**), and our previously studied cyclohexanone curcumin derivatives **A1** and **A2**.[32] This level of activity warrants further investigation for the treatment of ER-negative breast cancer and/or chronic myelogenous leukemia as prototypical cellular models for solid and liquid tumors.

4. Experimental section

4.1. Chemical synthesis

Melting points were determined on a Mettler Toledo FP62 melting block and were uncorrected. High resolution mass spectrometry was recorded using a VG70-250S double focusing magnetic sector mass spectrometer. NMR spectra, at 25 °C, were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on Varian INOVA-500 spectrometer. Chemical shifts are given in ppm on the δ scale referenced to the solvent peaks C**H**Cl$_3$ at 7.26 and **C**DCl$_3$ at 77.00. Cyclohexanone, *N*-methylpiperidone, tropinone, pyridine-3-carbox aldehyde, pyridine-4-carboxaldehyde, 2-fluoropyridine-3-carboxaldehyde, 2-fluoropyridine-4-carboxaldehyde, thiophen-2-carbox aldehyde, 1-methylpyrrole-2-carboxaldehyde, 1-methylindole-3-carboxaldehyde, 1-methylimidazole-2-carboxaldehyde, 1-methylimidazole-5-carboxaldehyde, 3,4,5-trimethoxybenzaldehyde, 2-fluoro-4,5-dimethoxybenzaldehyde, and 2,5-dimethoxybenzaldehyde were all purchased from the Aldrich Chemical Company. 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone (**A1**), 2,6-bis(pyridin -3-ylmethylene)-cyclohexanone (**A2**), 2,6-bis(thiophen-2-ylmethylene)-cyclohexanone (**A5**) and 2,6-bis((3,4,5-trimethoxyphenyl) methylene)-cyclohexanone (**A10**), were prepared following the method of Bhagat et al.[33] 2,6-Bis((1-methyl-1H-pyrrol-2-yl)methylene)-cyclohexanone (**A6**) was prepared as described by Liang et al.[19] 2,6-Bis((3-methoxy-4-hydroxyphenyl)methylene)-cyclohexanone (**A13**) was prepared as described previously.[34] 1-Methyl-3,5-bis[(*E*)-(4-pyridyl)methylidene]-4-piperidone (**B1**), 1-methyl -3,5-bis[(*E*)-(3-pyridyl)methylidene]-4-piperidone (**B2**), 1-methyl-3,5-bis[(*E*)-(2-thienyl)methylidene]-4-piperidone (**B5**) and 2,5-bis(3-pyridylmethylene)-cyclopentanone (**D2**) were prepared according to the method of Vatsadze et al.[29]

4.1.1. General procedure A[29]

To a mixture of the appropriate ketone (0.75 mmol) and aldehyde (1.50 mmol) in ethanol (5 ml) was added sodium hydroxide (1 M in water, 1 ml) and the mixture was stirred for 18 h at room temperature. The resulting precipitate was removed by filtration, washed with cold ethanol, and purified by recrystallisation from ethanol.

4.1.2. General procedure B[28]

To a mixture of the appropriate ketone (0.75 mmol) and aldehyde (1.50 mmol) in methanol (10 ml) was added sodium methoxide (5 M in methanol, 0.15 ml) and the mixture was stirred for 18 h at room temperature. The resulting precipitate was removed by filtration then washed with cold methanol and purified by recrystallisation from ethanol.

4.1.2.1. (2*E*,6*E*)-2,6-Bis((2-fluoropyridine-4-yl)methylene)cyclohexanone (A3). From cyclohexanone and 3-fluoro-4-pyridine-carboxaldehyde following general procedure A, yellow solid, 12% yield; mp 152–153 °C. Anal calcd for $C_{18}H_{14}F_2N_2O\frac{1}{4}H_2O$: C, 68.24; H, 4.61; N, 8.84. Found: C, 68.28; H, 4.59; N, 8.72. ^{1}H NMR ($CDCl_3$) δ: 1.82 (quin, J = 6 Hz, 2H), 2.80 (t, J = 6 Hz, 4H), 7.27 (t, J = 6 Hz, 2H), 7.70 (s, 2H), 8.45 (d, J = 5 Hz, 2H), 8.53 (d, J = 1.5 Hz, 2H); HRMS (+ve ESI) calcd for $C_{18}H_{14}F_2N_2NaO$ 335.0966 *m*/*z* [MNa$^+$], found 335.0957 *m*/*z*.

4.1.2.2. (2*E*,6*E*)-2,6-Bis((2-fluoropyridine-3-yl)methylene)cyclohexanone (A4). From cyclohexanone and 2-fluoro-3-pyridine-carboxaldehyde following general procedure A, yellow solid, 58% yield; mp 145–146 °C. Anal calcd for $C_{18}H_{14}F_2N_2O\frac{2}{3}H_2O$: C, 66.66; H, 4.77; N, 8.64. Found: C, 66.64; H, 4.64; N, 8.70. ^{1}H NMR ($CDCl_3$) δ: 1.82 (quin, J = 6 Hz, 2H), 2.80 (t, J = 6 Hz, 4H), 7.24 (m, 2H), 7.73 (s, 2H), 7.79 (m, 2H), 8.21 (d, J = 5 Hz, 2H); HRMS (+ve ESI) calcd for $C_{18}H_{14}F_2N_2NaO$ 335.0966 *m*/*z* [MNa$^+$], found 335.0973 *m*/*z*.

4.1.2.3. (2*E*,6*E*)-2,6-Bis((1-methyl-1*H*-imidazol-5-yl)methylene) cyclohexanone (A7). From cyclohexanone and 1-methyl-5-imidazole-carboxaldehyde following general procedure B, yellow solid, 68% yield; mp >220 °C. Anal calcd for $C_{16}H_{18}N_4O$: C, 68.06; H, 6.43; N, 19.84. Found: C, 67.95; H, 6.53; N, 20.15. ^{1}H NMR ($CDCl_3$) δ: 1.92 (quin, J = 6 Hz, 2H), 2.83 (t, J = 6 Hz, 4H), 3.73 (s, 6H, NMe), 7.38 (s, 2H), 7.57 (s, 2H), 7.63 (s, 2H); HRMS (+ve ESI) calcd for $C_{16}H_{19}N_4O$: 283.1553 *m*/*z* [MH$^+$], found: 283.1560 *m*/*z*.

4.1.2.4. (2*E*,6*E*)-2,6-Bis((1-methyl-1*H*-imidazol-2-yl)methylene) cyclohexanone (A8). From cyclohexanone and 1-methyl-2-imidazole-carboxaldehyde following general procedure B, yellow solid, 68% yield; mp 217–219 °C. Anal calcd for $C_{16}H_{18}N_4O$: C, 68.06; H, 6.43; N, 19.84. Found: C, 67.81; H, 6.52; N, 20.04. ^{1}H NMR ($CDCl_3$) δ: 1.86 (quin, J = 6 Hz, 2H), 3.35 (t, J = 6 Hz, 4H), 3.78 (s, 6H, NMe), 6.97 (s, 2H), 7.26 (s, 2H), 7.54 (s, 2H); HRMS (+ve ESI) calcd for $C_{16}H_{19}N_4O$: 283.1553 *m*/*z* [MH$^+$], found: 283.1560 *m*/*z*

4.1.2.5. (2*E*,6*E*)-2,6-Bis((1-methyl-1*H*-indol-3-yl)methylene) cyclohexanone (A9). From cyclohexanone and indole-3-carboxaldehyde following general procedure B, orange yellow solid, 23% yield; mp >220 °C. Anal calcd for $C_{26}H_{24}N_2O.H_2O$: C, 78.24; H, 6.58; N, 7.03. Found: C, 78.24; H, 6.32; N, 6.95. ^{1}H NMR ($CDCl_3$) δ: 1.96 (quin, J = 6 Hz, 2H), 2.87 (br t, J = 6 Hz, 4H), 3.85 (s, 6H, NMe), 7.25 (m, 2H), 7.31 (m, 2H), 7.34 (m, 2H), 7.35 (s, 2H) 7.94 (d, J = 7.5 Hz, 2H), 8.24 (s, 2H);HRMS (+ve ESI) calcd for $C_{26}H_{25}N_2O$ 381.1961 *m*/*z* [MH$^+$], found 381.1978 *m*/*z*.

4.1.2.6. (3*E*,5*E*)-1-Methyl-3,5-bis((1-methyl-1*H*-imidazol-2-yl) methylene)piperidin-4-one (B8). From 1-methylpiperidone and 1-methyl-2-imidazole-carboxaldehyde following general procedure B, orange yellow solid, 63% yield; mp 196–198 °C. Anal calcd for $C_{16}H_{19}N_5O$: C, 64.63; H, 6.44; N, 23.55. Found: C, 64.58; H, 6.50; N, 23.69. ^{1}H NMR ($CDCl_3$) δ: 2.58 (s, 3H, NMe), 3.80 (s, 6H, 2 × NMe), 4.15 (s, 4H), 6.99 (s, 2H), 7.28 (s, 2H), 7.51 (s, 2H);. HRMS (+ve ESI) calcd for $C_{16}H_{20}N_5O$: 298.1662 *m*/*z* [MH$^+$], found 298.1662 *m*/*z*.

4.1.2.7. (3*E*,5*E*)-3,5-Bis(3,4,5-trimethoxybenzylidene)-1-methylpiperidin-4-one (B10)[35]. From 1-methylpiperidone and 3,4,5-trimethoxybenzaldehyde following general procedure A, orange yellow solid 35% yield; mp 151–152 °C. Anal calcd for $C_{26}H_{31}NO_7\frac{1}{3}H_2O$: C, 65.67; H, 6.71; N, 2.95. Found: C, 65.70; H, 6.61; N, 3.00. ^{1}H NMR ($CDCl_3$) δ: 2.48 (s, 3H, NMe), 3.80 (s, 4H), 3.89 (s, 12H, 4 × OMe), 3.90 (s, 6H, 2 × OMe), 6.63 (s, 4H), 7.75 (s, 2H); HRMS (+ve ESI) calcd for $C_{26}H_{32}NO_7$ 470.2173 *m*/*z* [MH$^+$], found 470.2161 *m*/*z*.

4.1.2.8. (3*E*,5*E*)-3,5-Bis(2-fluoro-4,5-dimethoxybenzylidene)-1-methylpiperidin-4-one (B11). From 1-methylpiperidone and 2-fluoro-4,5-dimethoxybenzaldehyde following general procedure A, orange yellow solid, 41% yield; mp 189–191 °C. Anal calcd for $C_{24}H_{25}F_2NO_5$: C, 64.71; H, 5.66; N, 3.14. Found: C, 64.77; H, 5.85;

N, 2.97. ^{1}H NMR ($CDCl_3$) δ: 2.42 (s, 3H, NMe), 3.65 (s, 4H), 3.87 (s, 6H, 2 × OMe), 3.90 (s, 6H, 2 × OMe), 6.68 (d, J = 12 Hz, 2H), 6.76 (d, J = 7 Hz, 2H), 7.82 (s, 2H); HRMS (+ve ESI) calcd for $C_{24}H_{26}F_2NO_5$ 446.1774 m/z [MH^+], found 446.1782 m/z.

4.1.2.9. (3*E*,5*E*)-3,5-Bis(2,5-dimethoxybenzylidene)-1-methylpiperidin-4-one (B12). From 1-methylpiperidone and 2,5-dimethoxybenzaldehyde following general procedure A, orange yellow solid, 66% yield; mp 133–134 °C. Anal calcd for $C_{24}H_{27}NO_5$: C, 70.40; H, 6.65; N, 3.42. Found: C, 70.38; H, 6.81; N, 3.29. ^{1}H NMR ($CDCl_3$) δ: 2.38 (s, 3H, NMe), 3.66 (s, 4H), 3.78 (s, 6H, 2 × OMe), 3.81 (s, 6H, 2 × OMe), 6.76 (d, J = 3 Hz, 2H), 6.84 (d, J = 8 Hz, 2H), 6.88 (dd, J = 3, 8 Hz, 2H), 8.00 (s, 2H); HRMS (+ve ESI) calcd for $C_{24}H_{28}NO_5$ 410.1962 m/z [MH^+], found 410.1954 m/z.

4.1.2.10. (2*E*,4*E*)-8-Methyl-2,4-bis((pyridine-4-yl)methylene)-8-aza-bicyclo[3.2.1]octan-3-one (C1). From tropinone and pyridine-4-carboxaldehyde following general procedure A, yellow solid, 92% yield; mp 175–176 °C. Anal calcd for $C_{20}H_{19}N_3O$: C, 75.69; H, 6.03; N, 13.24. Found: C, 75.44; H, 6.10; N, 13.54. ^{1}H NMR ($CDCl_3$) δ: 2.00 (d, J = 7 Hz, 2H), 2.31 (s, 3H, NMe), 2.61 (t, J = 7 Hz, 2H), 4.31 (d, J = 7 Hz, 2H), 7.23 (d, J = 6 Hz, 4H), 7.68 (s, 2H), 8.68 (d, J = 6 Hz, 4H); HRMS (+ve ESI) calcd for $C_{20}H_{20}N_3O$ 318.1601 m/z [MH^+], found 318.1602 m/z.

4.1.2.11. (2*E*,4*E*)-8-Methyl-2,4-bis((pyridine-3-yl)methylene)-8-aza-bicyclo[3.2.1]octan-3-one (C2). From tropinone and pyridine-3-carboxaldehyde following general procedure A, pale yellow solid, 85% yield; mp 181–182 °C. Anal calcd for $C_{20}H_{19}N_3O$: C, 75.69; H, 6.03; N, 13.24. Found: C, 75.63; H, 6.18; N, 13.45. ^{1}H NMR ($CDCl_3$) δ: 2.02 (d, J = 7 Hz, 2H), 2.31 (s, 3H, NMe), 2.63 (t, J = 7 Hz, 2H), 4.33 (d, J = 7 Hz, 2H), 7.38 (dd, J = 5, 7 Hz, 2H), 7.69 (dm, J = 8 Hz 2H), 7.77 (s, 2H), 8.61 (dd, J = 1.5, 5 Hz, 2H), 8.67 (d, J = 1.5 Hz, 2H); HRMS (+ve ESI) calcd for $C_{20}H_{20}N_3O$ m/z 318.11601 m/z [MH^+], found 318.1605 m/z.

4.1.2.12. (2*E*,4*E*)-2,4-Bis(3,4,5-trimethoxybenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (C10). From tropinone and 3,4,5-trimethoxybenzaldehyde following general procedure A, pale yellow solid, 55% yield; mp 185–186 °C. Anal calcd for $C_{28}H_{33}NO_7$: C, 67.86; H, 6.71; N, 2.83. Found: C, 67.75; H, 6.91; N, 2.72. ^{1}H NMR ($CDCl_3$) δ: 2.07 (d, J = 7 Hz, 2H), 2.35 (s, 3H), 2.62 (m, 2H), 3.90 (s, 6H), 3.90 (s, 12H), 4.47 (d, J = 7 Hz, 2H), 6.65 (s, 4H), 7.78 (s, 2H); HRMS (+ve ESI) calcd for $C_{28}H_{34}NO_7$ 496.2330 m/z [MH^+], found 496.2291 m/z.

4.2. Cell line and reagents

MDA-MB-231, SkBr3 and MDA-MB-468 cells were purchased from ATCC (Manassas, VA). Curcumin, 99% purity, was purchased from Cayman Chemical (Ann Arbor, MI). Fetal bovine serum and trypsin were purchased from Life Technologies (Auckland, New Zealand). Tumor necrosis factor α (TNFα) was purchased from Sigma. K562 (human chronic myeloid leukemia) cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

4.2.1. Cell maintenance

Breast cancer cells were maintained in Modified Eagle's Medium-α modification media (MEM) (pH 7.4) supplemented with 10% FBS, 1% antibiotic/antimycotic solution and 0.2% $NaHCO_3$. Leukemia cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured in 5% CO_2/95% humidified air at 37 °C.

4.2.2. Cytotoxicity assays

Human breast cancer cells were seeded in 12-well plates (7×10^4 cells/well) in 1 ml DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l $NaHCO_3$, and incubated for 24 h at 37 °C. For dose–response assays, cells were treated with a range of concentrations of curcumin, or analogues for 5 days. Vehicle control cells were treated with DMSO (0.1%). Cell number in each well was determined using the sulforhodamine B (SRB) assay.[36] The concentration of each compound required to decrease the cell number by 50% of vehicle control (EC_{50}) was determined by non-linear regression using Prism software.

4.2.3. Apoptosis analysis by flow cytometry

Cells were seeded in six-well culture plates (2×10^5 cells per well) in 2 ml of DMEM/HamF12 supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l $NaHCO_3$. Cells were treated as above for 12–36 h. Apoptosis was assessed using Annexin-V-FLUOS/propidium iodide staining, as described.[8] Samples were analyzed using a FACScalibur flow cytometer (Becton Dickinson) and the proportion of apoptotic cells was determined using CellQuest Pro software. MDA-MB-231 cells were treated with **B1** (2 μM) or **B10** (1 μM) for 12, 18, 24 or 36 h. Vehicle control cells were treated with 0.1% DMSO. Values are expressed as the number of apoptotic cells as a % of the total number of cells ± SEM from three independent experiments conducted in triplicate. Data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test.

4.2.4. Measurement of NF-κB-inhibitory activity

The inhibitory activity against NF-κB was determined using a Dual-Glo™ Luciferase assay system from Promega. Electroporation of K562 cells was realised as described previously.[37] Equal amounts (each 5 μg per pulse (2.5×10^6 cells)) of a NF-κB plasmid (Stratagene, p5 × NF-κB containing five repeats of a consensus NF-κB site) and of a *Renilla* plasmid (phRG-TK from Promega) were added to each pulse. After transient transfection cells were transferred to culture medium. After 24 h, transfected K562 cells were re-solubilized at a concentration of 1×10^6 cells/ml in RPMI 1640-medium containing 0.1% FCS and 1% antibiotic–antimycotic. After 30 min, the cells were incubated with different concentrations of curcumin analogues for 2 h. TNF-α (20 ng/ml) was then added for 6 h. Expression of NF-κB was assessed according to the manufacturer's protocol by luminescence measurement using a Berthold Orion Luminometer (integration time 10 s).

4.3. Statistical analysis

All data presented were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test, where $p < 0.05$ denotes a statistically significant difference.

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Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra for all previously unreported compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.063.

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