Accepted Manuscript

Synthesis of novel curcuminoids accommodating a central β-enaminone motif and their impact on cell growth and oxidative stress

Rob De Vreese, Charlotte Grootaert, Sander D'hoore, Atiruj Theppawong, Sam Van Damme, Maarten Van Bogaert, John Van Camp, Matthias D'hooghe

PII: S0223-5234(16)30565-7

DOI: 10.1016/j.ejmech.2016.07.017

Reference: EJMECH 8735

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 27 May 2016

Revised Date: 8 July 2016

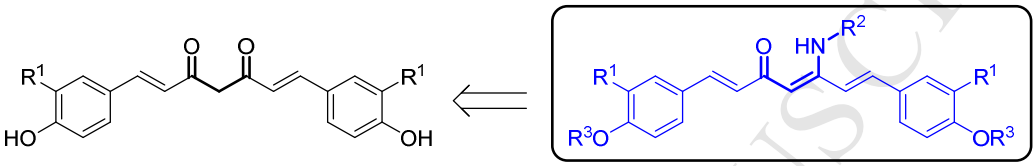
Accepted Date: 9 July 2016

Please cite this article as: R. De Vreese, C. Grootaert, S. D'hoore, A. Theppawong, S. Van Damme, M. Van Bogaert, J. Van Camp, M. D'hooghe, Synthesis of novel curcuminoids accommodating a central β-enaminone motif and their impact on cell growth and oxidative stress, *European Journal of Medicinal Chemistry* (2016), doi: 10.1016/j.ejmech.2016.07.017.

Graphical Abstract

Synthesis and anticancer assessment of novel curcuminoids accommodating a central β-enaminone motif

Rob De Vreese,[a] Charlotte Grootaert,[b] Sander D'hoore,[b] Atiruj Theppawong[a], Sam Van Damme,[a] Maarten Van Bogaert,[a] John Van Camp[b,*] and Matthias D'hooghe[a,*]



R^1 = OMe (curcumin)
R^1 = H (bisdemethoxycurcumin)

(R^1 = H, OMe)
(R^2 = nPr, allyl, nBu, iBu, sBu, cHex)
(R^3 = H, Ac)

***N*-alkyl β-enaminone analogs of (bisdemethoxy)curcumin identified as promising lead structures for oxidative stress-related diseases drug design**

Synthesis of novel curcuminoids accommodating a central β-enaminone motif and their impact on cell growth and oxidative stress

Rob De Vreese,[a] Charlotte Grootaert,[b] Sander D'hoore,[b] Atiruj Theppawong[a], Sam Van Damme,[a] Maarten Van Bogaert,[a] John Van Camp[b,*] and Matthias D'hooghe[a,*]

[a] *SynBioC Research Group, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium, matthias.dhooghe@UGent.be*

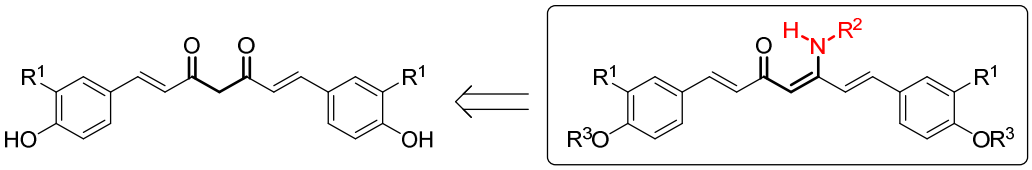
[b] *Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium, John.VanCamp@UGent.be*

Abstract

Curcuminoids are high-potential drugs targeting multiple components of vital signaling pathways without being toxic, and are therefore considered to be valuable lead structures in medicinal chemistry. Unfortunately, most curcuminoids poorly reach their site of action because of low bioavailability issues, (partly) associated with the labile β-diketo structure. In that respect, curcumin derivatives bearing a central β-enaminone fragment may have improved solubility and intestinal stability, and therefore may represent a new class of analogs with higher bioactivity. In that mindset, thirteen *N*-alkyl enaminones were efficiently synthesized via a novel approach, using montmorillonite K10 clay and microwave irradiation. These compounds were then characterized in terms of solubility and chemical anti-oxidant properties, and were applied in screening assays for cell toxicity, growth and oxidative stress using CHO-K1, EA.hy926, HT-29 and Caco-2 cell lines. Compared to native curcumin, many nitrogen derivatives showed a stronger antiproliferative effect, which was highly structure and cell type dependent. In addition, the correlation between cell viability and reactive oxygen species production was limited. Therefore, this set of novel curcumin derivatives may be useful to unravel other mechanisms of oxidative stress-related diseases, and eventually be used as more bioavailable and bioactive alternatives for native curcumin.

Introduction

The natural extracts of *Curcuma Longa*, defined as curcuminoids, have attracted a lot of attention in recent years due to their broad variety of reported biological activities[1] and clean safety profile.[2] However, despite many efforts and realizations in curcuminoid chemistry and biology, their mode of action still remains poorly understood.[3] This could be related to the fact that curcuminoids are regarded as pan-assay interference compounds ("PAINS"), and thus often show unspecific activity across a range of assays.[4] Another drawback associated with the curcuminoid scaffold, besides the mainly unknown mode of action, is its poor bioavailability because of its high intrinsic chemical reactivity (related to the presence of a β-diketone moiety, two phenolic groups and two α,β-unsaturated carbonyl entities), water insolubility, pH dependence and metabolic instability.[5] Nevertheless, curcuminoids target multiple important biological components of vital signaling pathways and thus could serve as key building blocks toward the preparation of more drug-like bioactive compounds.[6] In that respect, the replacement of the β-diketone moiety by a β-enaminone group could possibly improve the bioavailability and/or biological activity of the resulting structures and could therefore represent a new approach in curcuminoid research.[7] β-Enaminones in general have been reported to display anticonvulsant, antibacterial, anti-inflammatory and antitumor activities,[8] but also comprise excellent starting points for the development of bioactive heterocycles in medicinal chemistry.[9] Curcumin-derived structures with a central β-enaminone motif, however, have only been studied sparsely,[10] and a thorough evaluation of this class of compounds is thus lacking. This can be attributed to the fact that no convenient synthetic approach toward these β-enaminone curcumin derivatives is available in the literature. Therefore, the goal of this study was (i) to successfully convert naturally occurring curcuminoids, i.e. curcumin **1a** and bisdemethoxycurcumin **1b** (BDMC), into a set of *N*-alkyl-substituted β-enaminone derivatives **2** (Figure 1) by means of a general and straightforward synthetic methodology, (ii) to characterize these compounds in terms of solubility and antioxidant capacity, and (iii) to test the obtained compounds for their effect on basic cell parameters such as viability, mitochondrial activity, cell growth and oxidative stress as a preliminary investigation of the biological potential of this new class of curcumin derivatives.



1a (R^1 = OMe) - curcumin
1b (R^1 = H) - bisdemethoxycurcumin

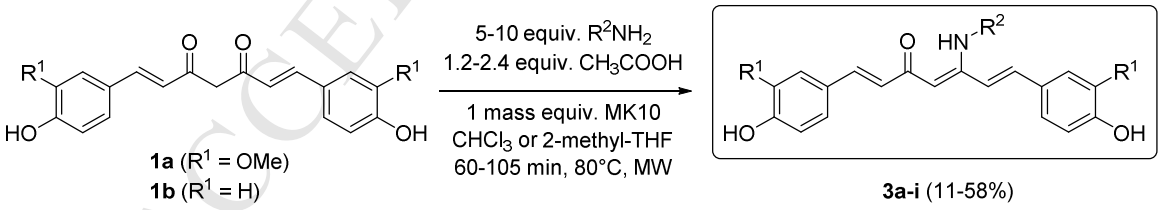
2 (R^1 = H, OMe)
(R^2 = alkyl)
(R^3 = H, Ac)

Figure 1. Article outline – replacing the labile β-diketo structure with a β-enaminone system

Synthesis

Only one synthetic approach toward *N*-alkyl-substituted enaminone derivatives of curcumin has been reported so far.[10a] In that procedure, a Dean-Stark trap with benzene as a solvent and acetic acid as a proton source has been used, providing the desired compounds **2** (R^1 = OMe; R^2 = H, CH_2Ph, $CH(CH_3)_2$, $CH(CH_3)Ph$, CH_2CH_3; R^3 = H, Figure 1) in rather low yields (25-35%). Applying these reaction conditions using other aliphatic amines (*n*-propylamine, *n*-butylamine) in toluene, however, resulted in a low conversion of the starting material to the corresponding enaminones after long reaction times (40 hours), pointing to the limitation of this synthetic strategy. To improve these conversions and to drive the reaction faster to completion, in the present study we enforced microwave irradiation[11] and added montmorillonite K10 clay (MK10 clay) as an additional reagent.[12] Microwave irradiation – considered to be a green approach – often accelerates the reaction speed and gives altered product distributions compared to conventional heating, and MK10 clay is known to act as an efficient catalyst for this type of reactions. So far, the combination of MK10 and microwave irradiation has amply been used in the literature, and almost always resulted in efficient synthesis of the anticipated products.[13] The straightforward synthesis of β-enaminones from methyl acetoacetate, ethyl acetoacetate and acetylacetone, by using both microwave irradiation and MK10, has also been reported recently.[14] Inspired by these promising results, several attempts were made to produce β-enaminone analogs of curcumin, employing a microwave irradiation/MK10 strategy. After thorough evaluation of a variety of reaction condition combinations (by changing the solvent, time, temperature, microwave conditions, amount of acid, amine and MK10 clay used), full conversion of the starting material was eventually achieved in rather short reaction times (60-105 minutes, Scheme 1, Table 1). As shown in Table 1, the optimal reaction conditions to obtain curcumin analogs **3a-e** comprise treatment of curcumin with an excess of amine (5 equiv.), 1.2 equiv. of acetic acid and one mass equiv. of MK10 clay. Stirring of this mixture in chloroform under microwave irradiation at a temperature of 80°C resulted in the formation of compounds **3a-e** in short reaction times (60 minutes) and acceptable yields (40-56%). A double amount of cyclohexylamine (10 equiv.) and acetic acid (2.4 equiv.) was required to fully convert curcumin to enaminone derivative **3f** in a respectable

yield (58%). The enaminone analogs **3a-f** were purified via column chromatography (SiO_2) and finally obtained in high purity (>95%, determined via LC-MS and ^{1}H NMR). As can be noticed from these yields, a substantial amount of product was lost during column chromatography because of the cumbersome separation of the target product from the minor impurities formed during the reaction. Recrystallization of the products was also evaluated as a purification method, albeit without any success. For the synthesis of bisdemethoxycurcumin analogs **3g-i**, slightly longer reaction times (90-105 minutes) and another solvent (2-methyltetrahydrofuran) were required to attain full conversion (Scheme 1, Table 1). The corresponding enaminones **3g-i** were isolated in somewhat lower yields (11-40%), but could be purified more easily through recrystallization from acetone or methanol/water, providing high purity samples (>95%, determined via LCMS and ^{1}H NMR). Again, a substantial amount of product was lost during the purification step because of difficult separation of the product from minor impurities. Although an excess of amine was used in all reactions to effect full conversion, no formation of di-imines was observed, pointing to the reluctance of the obtained enaminones to undergo a second imination step. Spectroscopic NMR analysis of compounds **3a-i** revealed characteristic signals for the β-enaminone group, in accordance with previous literature data of similar structures,[10a] indicating the dominance of the β-enaminone structure over the β-iminoketone form. Both the CH proton and the exchangeable NH proton of enaminones **3a-i** were clearly present at δ-values between 5.5-5.6 and 11.5-11.7 ppm, respectively (^{1}H NMR, $CDCl_3$ and D_6-DMSO), confirming the presence of the β-enaminone entity in these molecules. In summary, this new method was shown to represent a reliable, general approach for the convenient synthesis of *N*-alkyl-substituted β-enaminone curcuminoids in acceptable yields and high purities, demonstrated by the preparation of nine representatives.

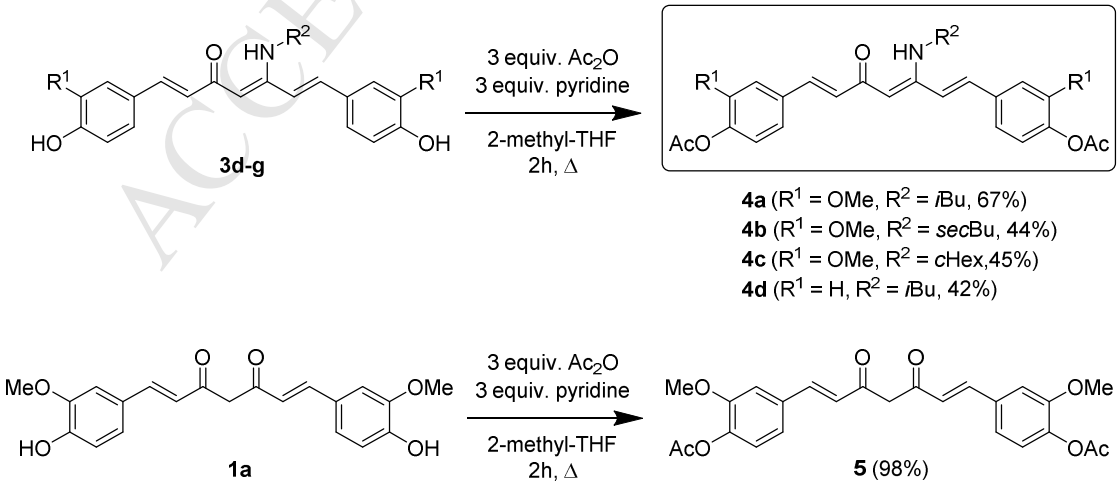


Scheme 1. Microwave-assisted synthesis of β-enaminones **3a-i**.

Table 1. Reaction conditions and yields for the synthesis of β-enaminones **3a-i**.

Compound	R^1	R^2	Time	Solvent	Equiv.	Equiv.	Yield (%)
3a	OMe	*n*Pr	60	$CHCl_3$	1.2	5	40
3b	OMe	allyl	60	$CHCl_3$	1.2	5	48
3c	OMe	*n*Bu	60	$CHCl_3$	1.2	5	47
3d	OMe	*i*Bu	60	$CHCl_3$	1.2	5	56
3e	OMe	*sec*Bu	60	$CHCl_3$	1.2	5	48
3f	OMe	*cyclo*Hex	60	$CHCl_3$	2.4	10	58
3g	H	*i*Bu	90	2-methyl-THF	2.4	5	30
3h	H	*sec*Bu	90	2-methyl-THF	2.4	5	40
3i	H	*cyclo*Hex	105	2-methyl-THF	2.4	7	11

In a following part, four of the obtained β-enaminones (**3d-g**) were *O*-acetylated to assess the effect of free *versus* protected phenolic groups on the biological activity of these compounds. To achieve this, a previously reported procedure for bis-acetylated curcumin synthesis was slightly modified in terms of the solvent used (the more environmentally friendly solvent 2-methyltetrahydrofuran was used instead of dichloromethane, Scheme 2).[15] Otherwise, no adjustments were made to the reported procedure and compounds **4a-d** were obtained in 42–67% yield upon treatment of enamines **3d-g** with three equiv. of acetic anhydride and three equiv. of pyridine. Bis-acetylated curcumin **5** was also prepared as a reference compound, to more easily compare the enamine analogs with their respective oxygenated counterparts. Hence, the previously mentioned modified procedure was used to synthesize the earlier documented bis-acetylated curcumin **5** in an excellent yield as well (98%, Scheme 2).[15]



Scheme 2. Synthesis of acetylated β-enaminones **4a-d** and bis-acetylated curcumin **5**.

In conclusion, thirteen β-enaminones (**3a-i** and **4a-d**) were efficiently synthesized, including nine compounds with free phenolic groups and four *O*-acetyl-protected structures. Nine of these molecules were derived from curcumin and four from bisdemethoxycurcumin. This small set of compounds allows for a preliminary SAR study concerning the influence of free or *O*-acetylated phenols, dimethoxy or bisdemethoxy analogs, and the nature of the *N*-alkyl group on the biological activity of this class of β-enaminone curcuminoids.

Characterization of solubility and anti-oxidant activity

In a next step, the solubility of the curcumin derivatives in cell culture medium was evaluated to have an idea of the bioaccessibility of the compounds to the cells, which may then reflect also differences in bioactivity. To this end, a concentration range of 0, 0.5, 1, 5, 10, 25 and 50 µM was prepared in serum-free DMEM, transferred to a 96-well plate and incubated for 1 day at 37°C and 10% CO_2 conditions. Crystal formation was checked microscopically. Table 2 presents the concentration at which crystals were detected.

Table 2. Chemical characterization of the curcumin derivatives in terms of solubility in cell culture medium and antioxidant capacity using the DPPH and FRAP test.

Compound	R^1	R^2	R^3	Solubility (µM)	DPPH (% inhibition per µM)	FRAP (Trolox equivalent per µM)
1a	OMe	n.a.	H	0.5	0.15	1.00
1b	H	n.a.	H	25	0.02	0.28
3a	OMe	*n*Pr	H	50	0.10	0.87
3b	OMe	allyl	H	25	0.10	1.14
3c	OMe	*n*Bu	H	5	0.08	1.29
3d	OMe	*i*Bu	H	25	0.09	0.89
3e	OMe	*sec*Bu	H	50	0.13	1.08
3f	OMe	*cyclo*Hex	H	25	0.13	0.83
3g	H	*i*Bu	H	25	0.02	0.19
3h	H	*sec*Bu	H	25	0.03	0.18
3i	H	*cyclo*Hex	H	5	0.03	0.56
4a	OMe	*i*Bu	Ac	5	0.00	0.04
4b	OMe	*sec*Bu	Ac	5	0.00	0.00
4c	OMe	*cyclo*Hex	Ac	5	0.01	0.04
4d	H	*i*Bu	Ac	25	0.00	0.01
5	OMe	n.a.	Ac	0.5	0.00	0.03

It was seen that β-diketone **5** and curcumine **1a** were highly insoluble (crystal formation at 0.5 μM), followed by compounds with an acetyl group on the R^3 position (crystal formation at 5 μM) with exception of compound **4d**, and compound **3c** and **3i**. Two compounds **3a** and **3e** showed a better solubility then the other compounds and only started to form crystals at a concentration of 50 μM.

To assess the antioxidant properties of the newly synthetized structures, two commonly used tests were used including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the ferric reducing ability of plasma (FRAP) assay. The DPPH assay is based on the neutralization of the stable DPPH radical and measures the percentage inhibition of the radical activity, whereas the FRAP assay is based on the reduction of a ferric-tripyridyltriazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) and is measured in trolox equivalents. The DPPH results in Table 2 are expressed as percentage inhibition per μM, based on the slope of the linear curve in the (%) inhibition in function of concentration graphs. Similarly, the values of the FRAP assay are expressed as trolox equivalents, i.e., the anti-oxidant potential of the analog compared to that of trolox as a reference molecule, per μM based on the slope of the linear curve of trolox equivalents in function of concentration of the analog. The higher the values of the DPPH and FRAP assay, the higher the anti-oxidant capacity of the analog.[16] From Table 2 it can be seen that high antioxidant activities were observed by both the FRAP and DPPH assay for compounds **3a-f** (0.08-0.13 % inhibition/μM and 0.83-1.29 trolox equiv./μM, respectively). Reference compound curcumin **1a** showed activity in the same range on both the FRAP and DPPH assay (0.15 % inhibition/μM and 1 trolox equiv./μM respectively). All compounds exerting good antioxidant activity (curcumin **1a** and enaminones **3a-f**) carry a methoxy group in *ortho*-position with respect to the phenol group on the aromatic rings. The favorable impact of the *ortho*-methoxy group on the antioxidative activity has previously been established for curcumin analogs and explained by the formation of an intramolecular hydrogen bond between the hydrogen atom of the phenol and the *ortho*-methoxy group, leading toward a facilitated removal of the hydrogen atom and thus an improved antioxidant activity.[17] For β-enaminone analogs **3g-i** and reference molecule bisdemethoxycurcumin **1b** (not containing an *ortho*-methoxy group), only small antioxidant effects were observed in both assays (0.2-0.3% inhibition/μM and 0.18-0.56 trolox equiv./μM), again pointing to the importance of the *ortho*-methoxy group for the antioxidant activity of curcumin analogs. Protection of both phenolic groups through acetylation (in compounds **4a-d** and bis-acetylated curcumin **5**) resulted in complete abolition of the antioxidant activity in both assays, further supporting the established importance of the free phenolic groups for the antioxidant activity. It should also be noted that the obtained *N*-alkyl enaminone derivatives of (bisdemethoxy)curcumin in general display a similar aqueous solubility as compared to the original substrates.

Evaluation of cell growth and oxidative stress

It is known that curcuminoids have a beneficial effect on, amongst others, inflammation[18] and chronic diseases such as cancer,[19] cardiovascular diseases,[20] diabetes,[21] metabolic syndrome,[22] aging.[23] Cell growth, viability and oxidative stress are common cell markers involved in these health aspects. [24] Therefore, in this study, we have applied the newly synthesized nitrogen analogs **3a-i** and **4a-d**, as well as the non-nitrogen-containing reference molecules **1a**,**b** and **5** on cell lines and have performed high throughput assays for protein content as marker for cell growth (SRB), mitochondrial respiration (MTT) and intracellular ROS production (DCFH). Cell lines included human-derived intestinal cells such as the HT-29 cell line and the Caco-2 cell line under undifferentiated and differentiated conditions, to simulate the gut which is considered to be the first barrier exposed to curcuminoids. In addition, the EA.hy962 cell line, which shows characteristics of endothelial cells, was used as well because of the influence of angiogenesis in many of the chronic diseases mentioned above. Finally, the analogs were also applied on the Chinese hamster ovarian cell line CHO-K1, because this fast-growing and relatively stable cell line is, in contrast to the other cell lines, not from a cancerous origin. Concentrations of the curcumin analogs applied onto the cells were 0, 1, 5, 10 and 25 µM in cell culture medium.

Table 3 shows the IC_{50} of the mitochondrial activity (MTT) and protein content of the cells (SRB), two parameters that indicate cell growth inhibition when they both decrease in parallel. A first observation is that inhibition of mitochondrial activity and protein content was strongly dependent on the cell type. Interestingly, none of the enaminones caused cytotoxic effects on differentiated Caco-2 cells. As differentiated Caco-2 cells show high similarities with *in vivo* enterocytes lining the intestinal tract, we may conclude that none of these analogs disturb the intestinal barrier by cell damage, which is desirable for therapeutic use. In addition, chemical compounds specifically targeting and killing undifferentiated cells of tumor origin without damaging differentiated cells are interesting candidates for anti-cancer drugs.[25] Secondly, when examining the nitrogen-lacking reference compounds **1a,b** and **5**, curcumin decreased the mitochondrial activity of the CHO ovarian cell line to a large extent, and none of the reference compounds had an effect on the undifferentiated cell lines from cancerous origin. In contrast, β-enaminone analogs of bisdemethoxycurcumin **3g-i** were cytotoxic for all undifferentiated cell lines at the concentrations tested. Especially compound **3i**, bearing a cyclohexyl-substituted enamine structure, was more cytotoxic compared to curcumin (**3i**: IC_{50} CHO = 9.2 µM, IC_{50} Caco-2 undiff. = 7.8 µM, IC_{50} HT-29 = 4.0 µM).

Table 3. Cell growth inhibition (IC_{50}-values in µM) measured after 72 h of curcumin derivative treatment using assays for mitochondrial activity (MTT) and protein content (SRB).

	IC_{50} CHO (µM)		**IC_{50} Caco-2 undiff (µM)**		**IC_{50} Caco-2 diff (µM)**		**IC_{50} HT-29 (µM)**		**IC_{50} EA.hy926 (µM)**	
	MTT	SRB	MTT	SRB	MTT	SRB	MTT	SRB	MTT	SRB
1a	7.5	>25	>25	>25	>25	>25	>25	>25	>25	>25
1b	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25
3a	>25	>25	>25	22.0	>25	>25	22.7	>25	>25	>25
3b	7.4	>25	>25	>25	>25	>25	>25	24.6	>25	>25
3c	7.0	20.4	>25	23.8	>25	>25	19.7	21.2	>25	>25
3d	>25	>25	>25	>25	>25	>25	24.5	24.0	>25	>25
3e	>25	>25	>25	>25	>25	>25	>25	22.3	>25	>25
3f	24.7	>25	>25	5.1	>25	>25	>25	>25	>25	>25
3g	17.9	23.7	23.0	18.5	>25	>25	20.1	22.5	>25	>25
3h	13.1	15.1	9.4	5.8	>25	>25	13.0	15.6	22.0	>25
3i	9.2	11.6	7.8	6.9	>25	>25	4.0	5.9	3.3	4.2
4a	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25
4b	>25	>25	>25	24.0	>25	>25	>25	>25	>25	>25
4c	10.1	>25	9.7	4.4	>25	>25	>25	>25	>25	>25
4d	>25	>25	>25	>25	>25	>25	13.7	22.6	>25	>25
5	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25

Despite the high potency of these compounds to target undifferentiated cells, their potential use as anticancer drugs requires further optimization studies, as it is not desirable to destroy other dividing cells of non-cancerous origin. Therefore, other nitrogen derivatives may be more interesting in this context. Some of the curcumin nitrogen derivatives **3** and bis-acetylated analogs **4** were more selective in their cytotoxic activity. Especially compounds **3a, 3d, 3e, 4b** and **4d** were effective against cancer-related cell lines without cytotoxicity to the CHO-K1 cell line. In addition, these specific compounds did not affect the viability of the endothelial-like cell line EA.hy926, which may indicate that their circulation in the blood stream may not damage the endothelial cells. Interestingly, a methoxy group on the R^1 position in combination with a cyclohexyl group on the R^2 position specifically targeted undifferentiated Caco-2 cells (compounds **3f**, and **4c**), whereas the viability of the HT-29 cells was unaffected. A reason why both intestinal cell lines react differently is currently unclear, although differences in both cell lines in terms of morphology, differentiation behavior and gene expression have been reported previously.[26] Overall, the selectivity of the different curcumin derivatives towards certain cell types is probably caused by their different uptake mechanisms by the

cells and/or their influence on different pathways. In a next set of experiments, intracellular ROS was evaluated in the different cell lines because of its possible role in cytotoxicity-related pathways.

In most of the chronic diseases, such as metabolic syndrome, inflammatory diseases etc., higher ROS levels are considered to be hazardous and may be counteracted by anti-oxidants. Yet, in cancer development, high levels of intracellular ROS may have a dual effect. On the one hand, high ROS levels may cause DNA damage,[27] and therefore increase the risk on mutated cells in the first stage of cancer development. On the other hand, higher ROS levels may also cause cell apoptosis through the mitochondrial intrinsic pathway, which is the permeabilization of the mitochondrial outer membrane and the consequent release of pro-apoptotic proteins.[28] In general, cancer cells have higher intrinsic ROS levels compared to normal cells and therefore, pro-oxidant compounds may specifically target and destroy cancer cells. In general, many polyphenols have both anti-oxidant and pro-oxidant activities, dependent on structure and intracellular concentration. To investigate whether the observed cytotoxic effects may be correlated with pathways affected by ROS, we have measured intracellular ROS using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) method.[29] We have applied a mild (1 µM) and high (10 µM) concentration of the curcuminoids, which is in the range of plasma concentrations reported by Jäger *et al*.[30]

A first observation (Table 4) is that the ROS modulating effect of the curcuminoids is strongly dependent on the cell type. For undifferentiated cells of intestinal cancer origin (Caco-2 and HT-29), curcuminoids significantly decreased intracellular ROS, especially at a concentration of 10 µM. HT-29 cells were more sensitive than Caco-2 cells, as also at 1 µM, decreases were observed. For CHO-cells, a mild increase in ROS was observed for some curcuminoids at a concentration of 1 µM. In contrast, EA.hy926 cells were strongly responsive to 10 µM of most of the curcuminoids, leading towards ROS increases up to more than 4-fold for **3c** and **3h**. Interesting exceptions to this effect was compound **4d**, which caused a significant decrease of ROS, and **3a** and **4b**, which showed variable effects.

The comparison of our results with existing literature is not always easy because of differences in experimental setup including the type of cell lines, the type of analogues and the concentrations and incubation times with the test compounds. ROS measurements after curcumin incubation has been described for fibroblast L929,[31] HGF[32] and CCD-18Co cells,[33] human colon carcinoma RKO, SW480,[33-34] HT29,[35] pancreatic PC-3 and DU145 cells,[36] monocyte U937 cells[37] and nasopharyngeal CNE1 and CNE2[38] cell lines. In all cases, a ROS increase of 2- to even 30-fold was reported depending on cell line, concentration and incubation time. Our results however showed a limited ROS effect of curcumin for the tested cell lines. As far as we know, no literature is available on the impact of curcuminoids on the intracellular ROS production in the Caco-2, CHO-K1 or EA.hy926 cell line. For the HT-29 cell line, the study of Chen et al. (2012) showed a significant 1.5- to 2.5-fold increase of cellular

ROS levels after 6 and 12 hours of incubation with 40 μM curcumin. In contrast, our results demonstrate a ROS-decreasing effect at similar incubation conditions, but at the lower concentrations of 1 and 10 μM. In addition, we have applied the more frequently used DCFA method instead of the dihydrorhodamine method, but although small differences exist in the outcome of these assays,[39] we do not expect that this has contributed to the differences in ROS trend. Furthermore, this decrease could not be correlated to decreased viability. Therefore, we conclude that the anti-oxidant or pro-oxidant activity of curcumin in the HT-29 cell line is concentration dependent, and that physiologically relevant concentrations of curcumin result in an anti-oxidant effect.

So far, the effect of similar nitrogen-containing analogues on ROS production has not been reported, but the effect of other types of analogues has been published. Gandhy et al. (2012)[33] showed that the concentration of the piperidone analogue (3*E*,5*E*)-3,5-bis(2,5-dimethoxybenzylidene)-1-butoxycarbonylpiperidin-4-one needed for the same ROS stimulating effect on RKO cells was 15 to 30 times lower than curcumin. In a study of Atsumi et al. (2007),[32] a one hour incubation with curcumin resulted in a 4-fold increase of ROS for 10 μM, and a 12-fold increase for 30 μM, whereas no ROS increase was detected with tetrahydrocurcumin in HSG cells at concentrations of 30 and 100 μM. This illustrated the importance of the unsaturated bonds in the middle chain of the curcumin molecule in its ROS-scavenging capacity. Zhang et al. (2015) have demonstrated that a nitrogen-containing monocarbonyl analogue WZ35 [1-(4-hydroxy-3-methoxyphenyl)-5-(2-nitrophenyl)penta-1,4-dien-3-one] was able to induce a 2-fold increase in ROS in pancreatic cell lines after 24 hours of incubation with 10 μM of the compound. These conditions and results are in the range of those obtained with our experimental setup for the CHO and especially the EA.hy926 cell lines. This study, and the one from Zou et al. (2015),[40] has demonstrated that the anticancer effect of this analogue WZ35 was mediated through ROS and its subsequent activation of JNK mitochondrial and endoplasmatic reticulum (ER) stress apoptotic pathways. In our studies, only two compounds, i.e. **3h** and **3i**, showed a correlation between ROS induction and decreased cell viability in especially the EA.hy926 cell line. Therefore, in future research it may be interesting to investigate whether apoptosis through JNK mitochondrial and ER stress is at the basis of this effect. For the other cell lines and analogues, other ROS mediated pathways should be explored. For instance, ROS modulation may also impact NF-κB activity, thereby regulating downstream factors involved in apoptosis, proliferation, tumor promotion, metastasis, angiogenesis, inflammation and immortality.[41] An interesting study in this context is the one from Sandur et al. (2007),[42] in which curcumin was shown to produce ROS while TNF-mediated NF-κB activation was inhibited. The authors suggested a concentration effect of ROS or the possibility that the apoptotic effects of curcumin are mediated through ROS while NF-κB activation was supressed in other ways. In future research, pathway

analysis using molecular techniques may further elucidate the mechanisms by which the nitrogen-containing curcuminoids may impact cell-related processes in health and disease conditions.

We conclude that in our results, no correlation could be found between chemical anti-oxidant capacity and anti-oxidant effects in the cells. In addition, no correlation could be found between pro-oxidant effects and decreased viability, and therefore, none of the curcuminoids seemed to exert anti-cancer effects through ROS induction. Instead, other mechanisms should be explored in the future.

Table 4. Intracellular reactive oxygen species (ROS), expressed as percentage compared to the untreated cells (mean ± standard deviation). *(p<0.05)/**(p<0.01) and §(p<0.05)/§§(p<0.01) indicate significant decreases and increases, respectively, compared to the untreated cells according to a two-tailed Student t-test with unequal variances (n≥6).

	CHO (%)		Caco-2 undiff (%)		HT-29 (%)		EA.hy926 (%)	
	1 µM	**10 µM**	**1 µM**	**10 µM**	**1 µM**	**10 µM**	**1 µM**	**10 µM**
1a	112 (±19.5)	97 (±8.7)	84 (±12.2)**	77 (±17.6)**	67 (±9.0)**	65 (±10.7)**	91 (±8.0)	88 (±2.4)
1b	101 (±2.1)	105 (±4.1)	98 (±19.7)	83 (±13.2)**	75 (±5.3)**	69 (±2.7)**	92 (±6.7)	103 (±16.3)
3a	116 (±12.8)§	118 (±11.8)	97 (±21.1)	88 (±11.9)*	88 (±9.2)*	92 (±10.8)	78 (±6.4)**	141 (±3.8)§§
3b	115 (±8.0)§	104 (±7.8)	89 (±13.3)	76 (±15.6)**	99 (±5.4)	84 (±9.3)**	124 (±33.9)	267 (±23.4)§§
3c	129 (±20.8)§	110 (±7.2)	91 (±8.4)*	83 (±18.5)*	99 (±9.3)	98 (±6.8)	110 (±29.9)	439 (±65.0)§§
3d	104 (±5.1)	118 (±19.0)	91 (±11.5)	74 (±15.4)**	91 (±6.7)**	92 (±8.6)*	83 (±22.8)	100 (±16.4)
3e	107 (±13.9)	112 (±4.1)§	91 (±11.7)	66 (±9.5)**	99 (±6.5)	90 (±7.0)**	85 (±18.6)	184 (±55.5)§
3f	137 (±36.0)§	122 (±17.4)	88 (±15.8)	63 (±17.9)**	80 (±8.8)**	74 (±10.6)**	91 (±8.4)	191 (±12.1)§§
3g	109 (±4.9)	97 (±4.2)	100 (±19.0)	82 (±20.4)*	77 (±3.1)**	75 (±8.7)**	100 (±11.6)	119 (±12.6)§
3h	104 (±12.7)	111 (±9.0)§	102 (±23.2)	102 (±13.8)	76 (±6.4)**	76 (±4.5)**	99 (±4.8)	414 (±46.1)§§
3i	111 (±11.2)	150 (±15.7)§§	97 (±16.1)	82 (±20.4)**	77 (±12.1)**	80 (±9.6)**	129 (±6.0)§	299 (±11.4)§§
4a	111 (±20.5)	111 (±7.6)	96 (±22.6)	77 (±20.5)**	95 (±4.6)**	96 (±7.2)	104 (±10.2)	240 (±55.3)§§
4b	88 (±8.2)	92 (± 7.1)	95 (±12.6)	86 (±18.5)	92 (±6.2)**	89 (±7.5)**	68 (±8.5)**	105 (± 10.1)
4c	89 (±16.8)	122 (±33.5)	89 (±14.3)	62 (±7.5)**	96 (±2.4)*	94 (±6.4)*	101 (±8.3)	113 (±4.6)
4d	152 (±30.4)§	142 (±28.5)§	102 (±10.3)	85 (±15.9)*	92 (±6.3)**	99 (±10.2)	60 (±3.2)**	53 (±3.5)**
5	128 (±19.0)§	112 (±11.7)	93 (±17.9)	90 (±17.7)	79 (±10.0)**	76 (±16.8)**	114 (±18.9)	189 (±57.7)§

Conclusion

The efficient synthesis of thirteen β-enaminone analogs of curcumin was established through a new approach, based on the use of microwave irradiation and montmorillonite K10 clay, thus replacing the labile β-diketo structure with a β-enaminone fragment. These thirteen structures were subjected to a preliminary bioactivity screening using different cell lines, and their reactive oxygen species (ROS)-generating potential and antioxidative activity was evaluated. We conclude that (i) *N*-analogs of curcumin strongly increase the cellular responses compared to the non-nitrogen analogs (**1a**, **1b**,

5), (ii) all compounds have no toxic effect on differentiated intestinal cells, which is desirable, (iii) no correlation between chemical anti-oxidant capacity and intracellular ROS could be found, which also shows the limitations of the chemical anti-oxidant assays to predict biological effect, and (iv) viability and ROS results are strongly cell type dependent, thereby indicating the involvement of other targets than ROS-dependent pathways. Because of their strong cytotoxic effects, compounds **3c**, **3g**, **3h** and **3i** are considered to be suitable candidates for further elaboration in terms of lead scaffold optimization studies. In addition, we also recommend to investigate other oxidative stress-related diseases than cancer. Table 5 gives an overview of the properties of the tested substrates.

Table 5. Overview of the properties of the tested substrates. For chemical characteristics, - and + indicate ranking according to the characteristic. For cell effects, 0 = no effect, - = decrease and + = increase compared to untreated cells.

						Intestinal (cancer)		**Ovarian (non-cancer)**		**Endothelial (cancer)**	
	R^1	**R^2**	**R^3**	**Solub.**	**Anti-ox.**	**Viability**	**ROS**	**Viability**	**ROS**	**Viability**	**ROS**
1a	OMe	n.a.	H	-	++	0, 0	--,--	0	0	0	0
1b	H	n.a.	H	++	-	0, 0	--,--	0	+	0	0
3a	OMe	*n*Pr	H	+++	+	-, -	-,-	0	+	0	--, +
3b	OMe	allyl	H	++	++	0, -	-,-	0	+	0	++
3c	OMe	*n*Bu	H	+	++	-, -	-, 0	-	+	0	+++
3d	OMe	*i*Bu	H	++	+	0, -	-, -	0	+	0	0
3e	OMe	*sec*Bu	H	+++	++	0, -	--, 0	0	+	0	+
3f	OMe	*cyclo*Hex	H	++	+	---, 0	--,--	0	++	0	+
3g	H	*i*Bu	H	++	-	-,-	--,--	-	0	0	+
3h	H	*sec*Bu	H	++	-	--,--	0, --	--	+	0	+++
3i	H	*cyclo*Hex	H	-	+	--,---	--,--	--	+	---	++
4a	OMe	*i*Bu	Ac	+	--	0, 0	--, -	0	+	0	++
4b	OMe	*sec*Bu	Ac	+	--	-, 0	0, -	0	0	0	-, 0
4c	OMe	*cyclo*Hex	Ac	+	--	---, 0	-, 0	0	+	0	0
4d	H	*i*Bu	Ac	++	--	0, --	-, 0	0	+	0	--
5	OMe	n.a.	Ac	-	--	0, 0	0, -	0	+	0	+

In summary, this study serves as a valuable starting point for the design of new curcuminoids bearing a β-enaminone motif and their deployment as promising drugs for oxidative stress-related diseases. Future research should focus on elaborate bioactivity and bioavailability optimization through chemical modification of the lead structures identified in this work.

Experimental section

Chemistry

General methods

^{1}H NMR spectra were recorded at 300 MHz (Jeol Eclipse+) or 400 MHz (Bruker Advance III Nanobay) with $CDCl_3$ or D_6-DMSO as solvent. ^{13}C NMR spectra were recorded at 75 MHz (Jeol Eclipse+) or 100.6 MHz (Bruker Anvance III Nanobay) with $CDCl_3$ or D_6-DMSO as solvent. Mass spectra were obtained with a mass spectrometer Agilent 1100, 70 eV. IR spectra were measured with a Spectrum One FT-IR spectrophotometer. High resolution electron spray (ES) mass spectra were obtained with an Agilent Technologies 6210 series time-of-flight instrument. Melting points of crystalline compounds were measured with a Kofler Bench, type WME Heizbank of Wagner & Munz. Microwave reactions were carried out with a CEM Discover microwave at fixed temperature. The purity of all tested compounds was assessed by ^{1}H NMR analysis and/or HPLC analysis, confirming a purity of ≥95%.

Representative procedure for the synthesis of β-enaminones 3a-i

Curcumin **1a** (5 mmol, 1.84 g) was dissolved in chloroform (15 mL) and mixed with five equiv. of isobutylamine (25 mmol, 2.48 mL) and 1.2 equiv. of glacial acetic acid (6 mmol, 0.34 mL). Montmorillonite K10 clay (MK10, 1.83 g) was added and the resulting mixture was stirred for 60 minutes at 80°C under microwave irradiation. After reaction, the mixture was filtered over celite and the filter cake was thoroughly rinsed with chloroform (200 mL). The filtrate was extracted with sodium bicarbonate (50 mL) and brine (50 mL), and dried over magnesium sulfate. Evaporation of chloroform and purification by means of column chromatography on silica gel (EtOAc/PE 3/2) resulted in 1.19 g (1*E*,4*Z*,6*E*)-1,7-bis-(4-hydroxy-3-methoxyphenyl)-5-isobutylamino-1,4,6-heptatrien-3-one **3d** (2.81 mmol, 56%).

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-hydroxy-3-methoxyphenyl)-5-propylamino-1,4,6-heptatrien-3-one 3a

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.04 (3H, t, *J* = 7.2 Hz), 1.71 (2H, sextet, *J* = 7.2 Hz), 3.37-3.39 (2H, m), 3.92 and 3.95 (2 × 3H, 2 × s), 5.52 (1H, s), 6.63 and 6.67 (2 × 1H, 2 × d, *J* = 15.4 Hz), 6.91-7.09 (6H, m), 7.21 and 7.46 (2 × 1H, 2 × d, *J* = 15.4 Hz), 11.54 (1H, bs). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 11.6, 23.6, 45.5, 56.0, 56.1, 93.4, 109.4, 114.7, 115.0, 118.1, 121.8, 122.3, 126.9, 128.2, 128.8, 137.1, 137.6, 146.8, 146.9, 147.0, 147.4, 162.9, 185.5. **IR** (ATR, cm^{-1}): $\nu_{NH/OH}$ = 3000; $\nu_{C=O}$ = 1567. **MS** (70 eV): m/z (%) 410 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{24}H_{28}NO_5$ $[M+H]^+$ calcd 410.1962, found 410.1916. Orange crystals. Column chromatography R_f (SiO_2) = 0.18, EtOAc/PE (3/2), yield 40%. Mp = 160˚C.

(1*E*,4*Z*,6*E*)-5-Allylamino-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatrien-3-one 3b

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.93 and 3.95 (2 × 3H, 2 × s), 4.04 (2H, t, *J* = 5.3 Hz), 5.22-5.37 (2H, m), 5.57 (1H, s), 5.80 and 5.85 (2 × 1H, 2 × bs), 5.90-5.99 (1H, m), 6.65 (2H, d, *J* =15.9 Hz), 6.89-6.97 (3H, m), 7.04-7.11 (3H, m), 7.20 and 7.47 (2 × 1H, 2 × d, *J* = 15.9 Hz), 11.48 (1H, bs). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 45.9, 56.0, 56.1, 93.8, 109.3, 109.4, 114.7, 114.9, 116.7, 118.1, 121.8, 122.4, 126.8, 128.2,

128.7, 134.3, 137.5, 137.7, 146.8, 146.9, 147.0, 147.4, 163.0, 186.0. **IR** (ATR, cm^{-1}): $\nu_{NH/OH}$ = 3006; $\nu_{C=O}$ = 1562. **MS** (70 eV): m/z (%) 408 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{24}H_{26}NO_5$ $[M+H]^+$ calcd 408.1806, found 408.1816. Orange crystals. Column chromatography R_f (SiO_2) = 0.15, EtOAc/PE (7/6), yield 48%. Mp = 160˚C.

(1*E*,4*Z*,6*E*)-5-Butylamino-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatrien-3-one 3c

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.96 (3H, t, *J* = 7.2 Hz), 1.47 (2H, sextet, *J* = 7.2 Hz), 1.67 (2H, quin, *J* = 7.2 Hz), 3.41 (2H, q, *J* = 6.6 Hz), 3.92 and 3.95 (2 × 3H, 2 × s), 5.52 (1H, s), 6.01 (2H, bs), 6.63 and 6.68 (2 × 1H, 2 × d, *J* = 15.4 Hz), 6.89-7.09 (6H, m), 7.21 and 7.46 (2 × 1H, 2 × d, *J* = 15.4 Hz), 11.54 (1H, bs). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 13.9, 20.1, 32.3, 43.4, 56.0, 56.1, 93.3, 109.4, 114.7, 115.0, 118.1, 121.8, 122.3, 126.9, 128.2, 128.8, 137.1, 137.6, 146.8, 146.9, 147.0, 147.4, 162.9, 185.4. **IR** (ATR, cm^{-1}): $\nu_{NH/OH}$ = 3004; $\nu_{C=O}$ = 1567. **MS** (70 eV): m/z (%) 424 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{25}H_{30}NO_5$ $[M+H]^+$ calcd 424.2119, found 424.2129. Orange crystals. Column chromatography R_f (SiO_2) = 0.14, EtOAc/PE (3/2), yield 47%. Mp = 169˚C.

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-hydroxy-3-methoxyphenyl)-5-isobutylamino-1,4,6-heptatrien-3-one 3d

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.04 (6H, d, *J* = 6.6 Hz), 1.94 (1H, nonet, *J* = 6.6 Hz), 3.24 (2H, t, *J* = 6.6 Hz), 3.92 and 3.96 (2 × 3H, 2 × s), 5.52 (1H, s), 6.64 (1H, d, *J* = 15.7 Hz), 6.67 (1H, d, *J* = 15.9 Hz), 6.90 (1H, d, *J* = 8.2 Hz), 6.95 (1H, d, *J* = 8.2 Hz), 6.98-6.99 (1H, m), 7.05-7.10 (3H, m), 7.21 (1H, d, *J* = 15.9 Hz), 7.47 (1H, d, *J* = 15.7 Hz), 11.64-11.67 (1H, m). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 20.4, 29.4, 51.5, 56.0, 56.1, 93.5, 109.40, 109.42, 114.7, 115.0, 118.4, 121.8, 122.3, 126.9, 128.3, 128.8, 137.1, 137.6, 146.8, 146.9, 147.0, 147.4, 163.1, 185.5. **IR** (cm^{-1}): $\nu_{C=O}$ = 1567. **MS** (70 eV): m/z (%) 424 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{25}H_{30}NO_5$ $[M+H]^+$ calcd 424.2119, found 424.2119. Orange crystals. Column chromatography R_f (SiO_2) = 0.18, EtOAc/PE (3/2), yield 56%. Mp = 84°C.

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-hydroxy-3-methoxyphenyl)-5-*sec*-butylamino-1,4,6-heptatrien-3-one 3e

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.00 (3H, t, *J* = 7.4 Hz), 1.29 (3H, d, *J* = 6.5 Hz), 1.61-1.69 (2H, m), 3.67-3.74 (1H, m), 3.93 and 3.96 (2 × 1H, 2 × s), 5.48 (1H, s), 5.77 and 5.84 (2 × 1H, 2 × bs), 6.63 (1H, d, *J* = 15.7 Hz), 6.69 (1H, d, *J* = 15.9 Hz), 6.90 (1H, d, *J* = 8.1 Hz), 6.95 (1H, d, *J* = 8.1 Hz), 6.97-6.98 (1H, m), 7.05-7.10 (3H, m), 7.21 (1H, d, *J* = 15.9 Hz), 7.47 (1H, d, *J* = 15.7 Hz), 11.56 (1H, d, *J* = 8.8 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 10.6, 21.8, 30.8, 50.9, 56.0, 56.1, 93.4, 109.3, 109.4, 114.7, 115.0, 118.5, 121.7, 122.4, 127.0, 128.4, 128.9, 137.1, 137.4, 146.8, 146.89, 146.92, 147.3, 162.4, 185.4. **IR** (cm^{-1}): $\nu_{C=O}$ = 1564. **MS** (70 eV): m/z (%) 424 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{25}H_{30}NO_5$ $[M+H]^+$ calcd 424.2119, found 424.2123. Orange crystals. Column chromatography R_f (SiO_2) = 0.16, EtOAc/PE (3/2), yield 48%. Mp = 88°C.

(1*E*,4*Z*,6*E*)-5-Cyclohexylamino-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatrien-3-one 3f

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27-1.52 (5H, m), 1.57-1.62 (1H, m), 1.78-1.83 (2H, m), 1.93-1.96 (2H, m), 3.55-3.64 (1H, m), 3.92 and 3.96 (2 × 3H, 2 × s), 5.48 (1H, s), 6.63 (1H, d, *J* = 15.7 Hz), 6.69 (1H, d, *J*

= 15.9 Hz), 6.90 (1H, d, *J* = 8.1 Hz), 6.95 (1H, d, *J* = 8.1 Hz), 6.97-6.98 (1H, m), 7.05-7.09 (3H, m), 7.21 (1H, d, *J* = 15.9 Hz), 7.47 (1H, d, *J* = 15.7 Hz), 11.67 (1H, d, *J* = 8.3 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 24.4, 25.5, 34.0, 52.0, 56.0, 56.2, 93.5, 109.4, 109.6, 114.7, 115.0, 118.4, 121.6, 122.3, 127.0, 128.4, 128.9, 137.0, 137.4, 146.8, 146.9, 147.0, 147.3, 161.8, 185.3. **IR** (cm^{-1}): $\nu_{NH/OH}$ = 3008; $\nu_{C=O}$ = 1565. **MS** (70 eV): m/z (%) 450 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{27}H_{32}NO_5$ $[M+H]^+$ calcd 450.2275, found 450.2275. Orange-yellow crystals. Column chromatography R_f (SiO_2) = 0.16, EtOAc/PE (4/3), yield 58%. Mp = 99°C.

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-hydroxyphenyl)-5-isobutylamino-1,4,6-heptatrien-3-one 3g

^{1}H NMR (400 MHz, D_6-DMSO): δ 0.96 (6H, d, *J* = 6.5 Hz), 1.82 (1H, nonet, *J* = 6.5 Hz), 3.28 (2H, t, *J* = 6.5 Hz), 5.61 (1H, s), 6.67 (1H, d, *J* = 15.7 Hz), 6.77 and 6.80 (2 × 2H, 2 × d, *J* = 8.6 Hz), 6.88 (1H, d, *J* = 16.0 Hz), 7.27 (1H, d, *J* = 15.7 Hz), 7.32 (1H, d, *J* = 16.0 Hz), 7.44 and 7.55 (2 × 2H, 2 × d, *J* = 8.6 Hz), 9.79 (2H, bs), 11.67-11.70 (1H, m). **^{13}C NMR** (100 MHz, D_6-DMSO): δ 19.8, 28.8, 49.9, 92.1, 115.7, 116.7, 126.4, 126.6, 126.7, 129.2, 129.4, 135.9, 137.2, 158.5, 158.9, 162.4, 184.1. **IR** (cm^{-1}): $\nu_{C=O}$ = 1565. **MS** (70 eV): m/z (%) 364 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{23}H_{26}NO_3$ $[M+H]^+$ calcd 364.1907, found 364.1922. Orange-yellow crystals. Recrystallization from methanol/water, yield 30%. Mp = 140°C.

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-hydroxyphenyl)-5-*sec*-butylamino-1,4,6-heptatrien-3-one 3h

^{1}H NMR (400 MHz, D_6-DMSO): δ 0.91 (3H, t, *J* = 7.4 Hz), 1.18 (3H, d, *J* = 6.4 Hz), 1.46-1.59 (2H, m), 3.85-3.95 (1H, m), 5.57 (1H, s), 6.66 (1H, d, *J* = 15.7 Hz), 6.77 and 6.80 (2 × 2H, 2 × d, *J* = 8.5 Hz), 6.90 (1H, d, *J* = 16.0 Hz), 7.25 (1H, d, *J* = 15.7 Hz), 7.32 (1H, d, *J* = 16.0 Hz), 7.43 and 7.55 (2 × 2H, 2 × d, *J* = 8.5 Hz), 9.78 (2H, bs), 11.57 (1H, d, *J* = 8.9 Hz). **^{13}C NMR** (100 MHz, D_6-DMSO): δ 9.9, 21.5, 30.1, 49.2, 92.0, 115.7, 116.9, 126.5, 126.6, 126.7, 129.2, 129.5, 135.8, 137.1, 158.5, 159.0, 161.7, 184.1. **IR** (cm^{-1}): $\nu_{NH/OH}$ = 3021; $\nu_{C=O}$ = 1561. **MS** (70 eV): m/z (%) 364 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{23}H_{26}NO_3$ $[M+H]^+$ calcd 364.1907, found 364.1903. Orange-yellow crystals. Recrystallization from aceton, yield 40%. Mp = 133°C.

(1*E*,4*Z*,6*E*)-5-Cyclohexylamino-1,7-bis-(4-hydroxyphenyl)-1,4,6-heptatrien-3-one 3i

^{1}H NMR (400 MHz, D_6-DMSO): δ 1.29-1.42 (5H, m), 1.52-1.55 (1H, m), 1.66-1.70 (2H, m), 1.84-1.87 (2H, m), 3.76-3.84 (1H, m), 5.57 (1H, s), 6.66 (1H, d, *J* = 15.7 Hz), 6.77 and 6.81 (2 × 2H, 2 × d, *J* = 8.6 Hz), 6.90 (1H, d, *J* = 15.9 Hz), 7.26 (1H, d, *J* = 15.7 Hz), 7.32 (1H, d, *J* = 15.9 Hz), 7.43 and 7.55 (2 × 2H, 2 × d, *J* = 8.6 Hz), 9.80 (2H, bs), 11.70 (1H, d, *J* = 8.6 Hz). **^{13}C NMR** (100 MHz, D_6-DMSO): δ 23.6, 25.0, 33.5, 50.2, 92.1, 115.7, 116.7, 126.5, 126.6, 126.8, 129.2, 129.6, 135.9, 137.2, 158.5, 159.0, 161.2, 184.1. **IR** (cm^{-1}): $\nu_{NH/OH}$ = 3244; $\nu_{C=O}$ = 1567. **MS** (70 eV): m/z (%) 390 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{25}H_{28}NO_3$ $[M+H]^+$ calcd 390.2064, found 390.2066. Orange-yellow crystals. Recrystallization from aceton, yield 11%. Mp = 254°C.

Representative procedure for the synthesis of *O*-acetylated compounds 4a-d

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-hydroxy-3-methoxyphenyl)-5-isobutylamino-1,4,6-heptatrien-3-one **3d** (3 mmol, 1.27 g), acetic anhydride (9 mmol, 0.85 mL) and pyridine (9 mmol, 0.73 mL) were dissolved in 2-methyltetrahydrofuran and refluxed for two hours. When the reaction was finished, the liquids were evaporated and the resulting solid was recrystallized from methanol, which lead to the formation of 1.02 g (1*E*,4*Z*,6*E*)-1,7-bis-(4-acetoxy-3-methoxyphenyl)-5-isobutylamino-1,4,6-heptatrien-3-one **4a** (2.01 mmol, 67%).

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-acetoxy-3-methoxyphenyl)-5-isobutylamino-1,4,6-heptatrien-3-one 4a

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.04 (6H, d, *J* = 6.6 Hz), 1.94 (1H, nonet, *J* = 6.6 Hz), 2.32 and 2.34 (2 × 3H, 2 × s), 3.23 (2H, t, *J* = 6.6 Hz), 3.87 and 3.89 (2 × 3H, 2 × s), 5.52 (1H, s), 6.71 (1H, d, *J* = 15.7 Hz), 6.77 (1H, d, *J* = 15.9 Hz), 7.02 (1H, d, *J* = 8.6 Hz), 7.05-7.06 (1H, m), 7.07 (1H, d, *J* = 8.6 Hz), 7.11-7.15 (3H, m), 7.23 (1H, d, *J* = 15.9 Hz), 7.50 (1H, d, *J* = 15.7 Hz), 11.62-11.65 (1H, m). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 20.3, 20.8, 29.5, 51.6, 56.0, 56.1, 94.1, 111.1. 111.2. 120.1, 120.9, 121.2, 123.1, 123.4, 129.2, 134.7, 135.2, 136.5, 137.0, 140.4, 140.8, 151.3, 151.5, 162.9, 169.1, 185.3. **IR** (cm^{-1}): $\nu_{NH/OH}$ = 3358; $\nu_{C=O}$ = 1758, 1579. **MS** (70 eV): m/z (%) 508 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{29}H_{34}NO_7$ $[M+H]^+$ calcd 508.2330, found: 508.2326. Orange-yellow crystals. Recrystallization from methanol, yield 67%. Mp = 90°C.

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-acetoxy-3-methoxyphenyl)-5-*sec*-butylamino-1,4,6-heptatrien-3-one 4b

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.99 (3H, t, *J* = 7.2 Hz), 1.29 (3H, d, *J* = 6.5 Hz), 1.65 (2H, pentet, *J* = 7.2 Hz), 2.32 and 2.34 (2 × 3H, 2 × s), 3.64-3.75 (1H, m), 3.87 and 3.89 (2 × 3H, 2 × s), 5.48 (1H, s), 6.70 (1H, d, *J* = 15.7 Hz), 6.80 (1H, d, *J* = 16.0 Hz), 7.02 (1H, d, *J* = 8.6 Hz), 7.05-7.06 (1H, m), 7.07 (1H, d, *J* = 8.6 Hz), 7.11-7.14 (3H, m), 7.24 (1H, d, *J* = 16.0 Hz), 7.49 (1H, d, *J* = 15.7 Hz), 11.55 (1H, d, *J* = 8.9 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 10.5, 20.8, 21.8, 30.8, 51.2, 56.0, 56.1, 93.9, 111.1, 111.3, 120.1, 120.9, 121.3, 123.1, 123.4, 129.2, 134.7, 135.3, 136.5, 136.9, 140.4, 140.8, 151.3, 151.5, 162.2, 169.1, 169.2, 185.2. **IR** (cm^{-1}): ν_{NH} = 3017; $\nu_{C=O}$ = 1760, 1573. **MS** (70 eV): m/z (%) 508 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{29}H_{34}NO_7$ $[M+H]^+$ calcd 508.2330, found 508.2325. Orange-yellow crystals. Recrystallization from methanol, yield 44%. Mp = 84°C.

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-acetoxy-3-methoxyphenyl)-5-cyclohexylamino-1,4,6-heptatrien-3-one 4c

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30-1.51 (5H, m), 1.57-1.61 (1H, m), 1.79-1.82 (2H, m), 1.93-1.96 (2H, m), 2.32 and 2.34 (2 × 3H, 2 × s), 3.54-3.62 (1H, m), 3.87 and 3.89 (2 × 3H, 2 × s), 5.48 (1H, s), 6.70 (1H, d, *J* = 15.7 Hz), 6.79 (1H, d, *J* = 16.0 Hz), 7.02 (1H, d, *J* = 8.4 Hz), 7.05-7.06 (1H, m), 7.07 (1H, d, *J* = 8.4 Hz), 7.11-7.14 (3H, m), 7.24 (1H, d, *J* = 16.0 Hz), 7.49 (1H, d, *J* = 15.7 Hz), 11.67 (1H, d, *J* = 8.3 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 20.8, 24.4, 25.5, 34.0, 52.2, 56.0, 56.1, 94.0, 111.2, 111.4, 120.1, 120.9, 121.3, 123.1, 123.5, 129.3, 134.8, 135.3, 136.5, 136.8, 140.5, 140.9, 151.3, 151.6, 161.6, 169.1, 185.2. **IR** (cm^{-1}): $\nu_{C=O}$ = 1761, 1572. **MS** (70 eV): m/z (%) 534 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{31}H_{36}NO_7$

$[M+H]^+$ calcd 534.2486, found 534.2489. Orange crystals. Recrystallization from methanol, yield 45%. Mp = 88°C.

(1*E*,4*Z*,6*E*)-1,7-Bis-(4-acetoxyphenyl)-5-isobutylamino-1,4,6-heptatrien-3-one 4d

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.03 (6H, d, *J* = 6.6 Hz), 1.94 (1H, nonet, *J* = 6.6 Hz), 2.31 and 2.32 (2 × 3H, 2 × s), 3.23 (2H, t, *J* = 6.6 Hz), 5.51 (1H, s), 6.72 (1H, d, *J* = 15.4 Hz), 6.79 (1H, d, *J* = 16.1 Hz), 7.09 and 7.14 (2 × 2H, 2 × d, *J* = 8.6 Hz), 7.24 (1H, d, *J* = 15.4 Hz), 7.52 (1H, d, *J* = 16.1 Hz), 7.52 and 7.55 (2 × 2H, 2 × d, *J* = 8.6 Hz), 11.63-11.66 (1H, m). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 20.4, 21.3, 29.5, 51.6, 94.0, 121.1, 122.0, 122.3, 128.6, 128.9, 129.2, 133.5, 134.0, 136.1, 136.5, 151.2, 151.6, 162.9, 169.4, 169.5, 185.4. **IR** (cm^{-1}): ν_{NH} = 3044; $\nu_{C=O}$ = 1752, 1571. **MS** (70 eV): m/z (%) 448 ($[M+1]^+$, 100). **HRMS** (ESI): m/z for $C_{27}H_{30}NO_5$ $[M+H]^+$ calcd 448.2119, found 448.2117. Orange crystals. Recrystallization from methanol, yield 42%. Mp = 149°C.

Biology

Reagents and cell lines

Dimethylsulfoxide, triethylamine, trichloroacetic acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), trolox, sulforhodamine B (SRB), MEM non-essential amino acid solution (NEAA), tris(hydroxymethyl)aminomethane, 2,2-diphenyl-1-picrylhydrazyl (DPPH), penicillin /streptomycin (P/S), formic acid and 2,7-dichlorofluorescein diacetate were purchased from Sigma-Aldrich. Both thiazolyl blue tetrazolium bromide and trypan blue were obtained from Amresco. Dulbecco's phosphate-buffered saline (PBS^{D-}, no calcium and no magnesium), Dulbecco's modified eagle medium (DMEM, high glucose + glutamax and RPMI 1640) and trypsin/EDTA solution were obtained from Life Technologies (Merelbeke, Belgium) and fetal bovine serum (FBS) from Greiner Bio-one (Vilvoorde, Belgium).

The cell lines, Caco-2 (intestinal), HT-29 (intestinal), CHO (ovarian), and EA.hy926 (endothelial) cells were originated from ATCC. These cells were cultivated in the same growth medium containing DMEM + glutamax, 1% NEAA, 1% P/S and 10% FBS in order to avoid interferences of specific medium compounds with the results. Upon treatment, no FBS was added to the medium. Curcumin and its compounds were dissolved in DMSO to a concentration of 100 mM. When the cells were exposed, solutions of the compounds were prepared from this stock solution in medium immediately before treatment. The final concentration of DMSO in this growth medium was less than 0.1% to avoid cytotoxic effects caused by DMSO.

MTT assay

An aliquot of cells were counted in a Bürker counting chamber after trypan blue staining. They were seeded at a concentration of 20000 cells in a 96 well plate and after one day the cells were treated without (control group) and with curcumin or its analogs **3a-i, 4a-d** and **5** at a concentration of 1, 5,

10 and 25 µM. After three days of treatment, 100 µL of culture medium was removed and 20 µL of MTT solution (5 mg MTT /ml PBS) was added. After two hours of incubation in the dark at 37°C and 10% CO_2, the medium was removed and the 96 well plate was dried. Solubilisation of the formazan crystals was achieved by adding 200 µL DMSO to each well. Finally, the absorbance was recorded at 570 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer. Each condition was performed in triplicate.

SRB assay[43]

Cells were seeded at a concentration of 20000 per well and treated with or without (control) compound at 1, 5, 10 and 25 µM after one day. Three days after this treatment the cells were fixed by addition of 50 µL of 50% trichloroacetic acid in milliQ-water for one hour at 4°C. The plate was carefully rinsed for 5 times with tap water and dried, after which the cells were stained with an SRB solution (0.4% sulforhodamine B in 1% glacial acetic acid). After 30 minutes the plate was rinsed for five times with 1% glacial acetic acid and dried. Tris buffer in a concentration of 10 mM was used to dissolve the stain. Finally the absorbance was measured using the Bio-Rad Benchmark Plus microplate spectrophotometer at a wavelength of 490 nm. Each condition was performed in triplicate.

Reactive oxygen species (ROS) assay

Cells were seeded in a black 96 well plate with transparent bottom at a concentration of 20000 cells per well. At confluency, the cells were treated overnight with or without (control) the compounds. After one day, the cells were washed and incubated at 37°C and 10% CO_2 with 2',7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA, 20 µM) for 30 minutes. After one hour, the cells were washed again and culture medium without phenol red was added. The plate was incubated for one hour, and again the medium was replaced by serum-free medium. Fluorescence of the wells was immediately measured with a Gemini XPS Microplate Reader with an excitation of 485 nm and an emission of 535 nm.

DPPH radical scavenging assay

A solution of 40 µg/ml DPPH in methanol was prepared. This solution was mixed in a 9:1 ratio with the solution of the compound in DMSO having a concentration ranging between 1 and 500 µM. This mixture was incubated at 30 °C in the dark for 30 minutes. Subsequently, this mixture was added to a 96 well plate and measured in the microplate spectrophotometer at a wavelength of 517 nm. Blanks without derivative and blanks without DPPH were run simultaneously. The last blank was necessary to check for absorption of the derivatives themselves. Percentage inhibition was calculated using the formula:

$$I(\%) = \frac{Absorption_{blank} - Absorption_{compound}}{Absorption_{blank}} * 100$$

Ferric reducing ability of plasma (FRAP) assay

Acetate buffer of 300 mM was prepared by adding 3.1 g of sodium acetate trihydrate to 16 mL of acetic acid, which was diluted to 1000 mL with milliQ-water. The TPTZ (2,4,6-tripyridyl-s-triazine) solution of 10 mM was prepared by adding 0.156 g of TPTZ to 50 mL of ethanol. Lastly, a 20 mM solution of iron(III) chloride hexahydrate was prepared by mixing 0.54 g of $FeCl_3.6H_2O$ with 2 mL 37% HCl and 98 mL milliQ-water. The TPTZ and iron solutions were freshly prepared on the day of the assay. These three mixtures were added in a 10:1:1 ratio to obtain the FRAP reagent. Finally, 100 µL of the samples were mixed with 900 µL of the FRAP reagent and after four minutes the absorbance was measured at 593 nm in a Cary 50 bio UV-visible spectrophotometer. Trolox (a water soluble analog of vitamin E) was used as a standard and the FRAP value was calculated as trolox equiv. (µmol/L) via a linear regression of the trolox standard curve. This method is similar to the protocol used by previous workers.[16b, 44]

References

1. (a) Anand, P.; Thomas, S. G.; Kunnumakkara, A. B.; Sundaram, C.; Harikumar, K. B.; Sung, B.; Tharakan, S. T.; Misra, K.; Priyadarsini, I. K.; Rajasekharan, K. N.; Aggarwal, B. B., Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. *Biochem. Pharmacol.* **2008,** *76* (11), 1590-611; (b) Bairwa, K.; Grover, J.; Kania, M.; Jachak, S. M., Recent developments in chemistry and biology of curcumin analogues. *RSC Adv.* **2014,** *4* (27), 13946; (c) Chen, Q. H., Curcumin-Based Anti-Prostate Cancer Agents. *Anti-Cancer Agent Me.* **2015,** *15*, 138-156.
2. (a) Shankar, T. N.; Shantha, N. V.; Ramesh, H. P.; Murthy, I. A.; Murthy, V. S., Toxicity studies on turmeric (Curcuma longa): acute toxicity studies in rats, guineapigs & monkeys. *Indian J. Exp. Biol.* **1980,** *18*, 73-75; (b) Lao, C. D.; Ruffin, M. T. t.; Normolle, D.; Heath, D. D.; Murray, S. I.; Bailey, J. M.; Boggs, M. E.; Crowell, J.; Rock, C. L.; Brenner, D. E., Dose escalation of a curcuminoid formulation. *BMC Complem. Altern. M.* **2006,** *6*, 10; (c) Teiten, M. H.; Gaascht, F.; Eifes, S.; Dicato, M.; Diederich, M., Chemopreventive potential of curcumin in prostate cancer. *Genes Nutr.* **2010,** *5* (1), 61-74; (d) Gupta, S. C.; Patchva, S.; Aggarwal, B. B., Therapeutic roles of curcumin: lessons learned from clinical trials. *AAPS J.* **2013,** *15* (1), 195-218.
3. (a) Shehzad, A.; Lee, Y. S., Molecular mechanisms of curcumin action: signal transduction. *BioFactors* **2013,** *39* (1), 27-36; (b) Ingolfsson, H. I.; Thakur, P.; Herold, K. F.; Hobart, E. A.; Ramsey, N. B.; Periole, X.; de Jong, D. H.; Zwama, M.; Yilmaz, D.; Hall, K.; Maretzky, T.; Hemmings, H. C., Jr.; Blobel, C.; Marrink, S. J.; Kocer, A.; Sack, J. T.; Andersen, O. S., Phytochemicals perturb membranes and promiscuously alter protein function. *ACS Chem. Biol.* **2014,** *9* (8), 1788-98.
4. (a) Baell, J. B.; Holloway, G. A., New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays. *J. Med. Chem.* **2010,** *53*, 2719-2740; (b) Baell, J.; Walters, M. A., Chemical con artists foil drug discovery. *Nature* **2014,** *513*, 481-483.
5. (a) Wang, J. Y.; Pan, M. H.; Cheng, A. L.; Lin, L. I.; Ho, Y. S.; Hsieh, C. Y.; Lin, J. K., Stability of curcumin in buffer solutions and characterization of its degradation products. *J. Pharmaceut. Biomed.* **1997,** *15*, 1867-1876; (b) Anand, P.; Kunnumakkara, A. B.; Newman, R. A.; Aggarwal, B. B., Bioavailability of Curcumin: Problems and Promises. *Mol. Pharm.* **2007,** *4* (6), 807-818.
6. (a) Marchiani, A.; Rozzo, C.; Fadda, A.; Delogu, G.; Ruzza, P., Curcumin and Curcumin-like Molecules: From Spice to Drugs. *Curr. Med. Chem.* **2013,** *21* (2), 204-222; (b) Heger, M.; van Golen, R. F.; Broekgaarden, M.; Michel, M. C., The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. *Pharmacol. Rev.* **2014,** *66* (1), 222-307.
7. Eddington, N. D.; Dox, D. S.; Roberts, R. R.; Stables, J. P.; Powell, C. B.; Scott, K. R., Enaminones-versatile therapeutic pharmacophores. Further advances. *Curr. Med. Chem.* **2000,** *7* (4), 417-436.

8. Foster, J. E.; Nicholson, J. M.; Butcher, R.; Stables, J. P.; Edafiogho, I. O.; Goodwin, A. M.; Henson, M. C.; Smith, C. A.; Scott, K. R., Synthesis, Characterization and Anticonvulsant Activity of Enaminones. Part 6: Synthesis of Substituted Vinylic Benzamides as Potential Anticonvulsants. *Bioorgan. Med. Chem.* **1999,** *7*, 2415-2425.

9. (a) Negri, G.; Kascheres, C.; Kascheres, A. J., Recent Development in Preparation Reactivity and Biological Activity of Enaminoketones and Enaminothiones and Their Utilization to Prepare Heterocyclic Compounds. *J. Heterocyclic Chem.* **2004,** *41*, 461-491; (b) Elassar, A.-Z. A.; El-Khair, A. A., Recent developments in the chemistry of enaminones. *Tetrahedron* **2003,** *59* (43), 8463-8480; (c) Cacchi, S.; Fabrizi, G.; Filisti, E., *N*-Propargylic b-enaminones: Common Intermediates for the Synthesis of Polysubstituted Pyrroles an Pyridines. *Org. Lett.* **2008,** *10* (13), 2629-2632; (d) Saito, A.; Konishi, T.; Hanzawa, Y., Synthesis of Pyrroles by Gold(I)-Catalyzed Amino-Claisen Rearrangement of *N*-Propargyl Enaminone Derivatives. *Org. Lett.* **2010,** *12* (2), 372-374; (e) Goutham, K.; Ashok Kumar, D.; Suresh, S.; Sridhar, B.; Narender, R.; Karunakar, G. V., Gold-Catalyzed Intramolecular Cyclization of N-Propargylic beta-Enaminones for the Synthesis of 1,4-Oxazepine Derivatives. *J. Org. Chem.* **2015,** *80* (21), 11162-8.

10. (a) Simoni, D.; Rizzi, M.; Rondanin, R.; Baruchello, R.; Marchetti, P.; Invidiata, F. P.; Labbozzetta, M.; Poma, P.; Carina, V.; Notarbartolo, M.; Alaimo, A.; D'Alessandro, N., Antitumor effects of curcumin and structurally beta-diketone modified analogs on multidrug resistant cancer cells. *Bioorg. Med. Chem. Lett.* **2008,** *18* (2), 845-9; (b) Lal, J.; Gupta, S. K.; Thavaselvam, D.; Agarwal, D. D., Biological activity, design, synthesis and structure activity relationship of some novel derivatives of curcumin containing sulfonamides. *Eur. J. Med. Chem.* **2013,** *64*, 579-88; (c) Chandrasekar, T.; Pravin, N.; Raman, N., Biosensitive metal chelates from curcumin analogues: DNA unwinding and anti-microbial evaluation. *Inorg. Chem. Commun.* **2014,** *43*, 45-50.

11. Kappe, C. O., Controlled microwave heating in modern organic synthesis. *Angew. Chem. Int. Edit.* **2004,** *43* (46), 6250-84.

12. Kaur, N.; Kishore, D., Montmorillonite: An efficient, heterogeneous and green catalyst for organic synthesis. *J. Chem. Pharm. Res.* **2012,** *4* (2), 991-1015.

13. (a) Varma, R. S.; Saini, R. K.; Kumar, D., An Expeditious Synthesis of Flavones on Montmorillonite K 10 Clay with Microwaves. *J. Chem. Res. (S)* **1998**, 348-349; (b) Varma, R. S.; Kumar, D.; Liesen, P. J., Solid state synthesis of 2-aroylbenzo[*b*]furans, 1,3-thiazoles and 3-aryl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazoles from α-tosyloxyketones using microwave irradiation. *J. Chem. Soc. Perk. T. 1* **1998**, 4093-4096; (c) Gajare, A. S.; Shaikh, N. S.; Bonde, B. K.; Deshpande, V. H., Microwave accelerated selective and facile deprotection of allyl esters catalyzed by Montmorillonite K-10. *J. Chem. Soc. Perk. T. 1* **2000,** (5), 639-640; (d) Marvi, O.; Fekri, L. Z.; Takhti, M., Montmorillonite K10 and KSF clays as acidic and green catalysts for effective esterification of phenols and alcohols under MWI. *Russ. J. Gen. Chem.* **2014,** *84* (9), 1837-1840; (e) Jaiswal, S.; Siddiqui, I. R., Montmorillonite K-10 Supported Solvent-Free Synthesis of Novel 2-thioxoimidazole-4-one N-nucleosides. *Int. J. Pharm. Sci. Rev. Res.* **2014,** *26* (1), 50-54; (f) Suresh, D.; Kanagaraj, K.; Pitchumani, K., Microwave promoted one-pot synthesis of 2-aryl substituted 1,3,4-oxadiazoles and 1,2,4-oxadiazole derivatives using Al3+-K10 clay as a heterogeneous catalyst. *Tetrahedron Lett.* **2014,** *55* (27), 3678-3682; (g) Sakhuja, R.; Shakoor, S. M. A.; Kumari, S.; Kumar, A., Microwave-Assisted Expedite Synthesis of 2-Phenylimidazo[1,2-a]pyridylquinoxalin-2(1H)-ones. *J. Heterocyclic Chem.* **2015,** *52* (3), 773-779; (h) Pagadala, R.; Chidurala, P.; Jetti, V.; Meshram, J. S.; Maddila, S.; Jonnalagadda, S. B., Synthesis of New 1, 10-Phenanthroline Analogs as Potent Antimicrobial Agents Using Montmorillonite K-10 as Catalyst. *J. Heterocyclic Chem.* **2015,** *52* (2), 397-402.

14. Marvi, O.; Giahi, M.; Ayub, P.; Nikpasand, M., K-10 clay as a reusable catalyst for the solvent-free MW-induced synthesis of enaminones. *J. Serb. Chem. Soc.* **2014,** *79* (8), 921-926.

15. (a) Pedersen, U.; Rasmussen, P. B.; Lawesson, S.-O., Synthesis of Naturally Occurring Curcuminoids and Related Compounds. *Liebigs. Ann. Chem.* **1985,** *1985* (8), 1557-1569; (b) Liu, B.; Xia, M.; Ji, X.; Xu, L.; Dong, J., Synthesis and Antiproliferative Effect of Novel Curcumin Analogues. *Chem. Pharm. Bull.* **2013,** *61* (7), 757-763.

16. (a) Xie, J.; Schaich, K. M., Re-evaluation of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity. *J. Agric. Food Chem.* **2014,** *62* (19), 4251-60; (b) Benzie, I. F. F.; Strain, J. J., The Ferric Reducing Ability of Plasma (FRAP) as a Measure of ''Antioxidant Power'': The FRAP Assay. *Anal. Biochem.* **1996,** *239*, 70-76.

17. (a) Barclay, L. R. C.; Vinqvist, M. R.; Mukai, K.; Goto, H.; Hashimoto, Y.; Tokunaga, A.; Uno, H., On the Antioxidant Mechanism of Curcumin: Classical Methods Are Needed To Determine Antioxidant Mechanism and Activity. *Org. Lett.* **2000,** *2* (18), 2841-2843; (b) Chen, W. F.; Deng, S. L.; Zhou, B.; Yang, L.; Liu, Z. L., Curcumin and its analogues as potent inhibitors of low density lipoprotein oxidation: H-atom abstraction from the phenolic groups and possible involvement of the 4-hydroxy-3-methoxyphenyl groups. *Free Radic. Biol. Med.* **2006,** *40* (3), 526-35; (c) Somparn, P.; Phisalaphong, C.; Nakornchai, S.; Unchern, S.; Morales, N. P.,

Comparative Antioxidant Activities of Curcumin and Its Demethoxy and Hydrogenated derivatives. *Biol. Pharm. Bull.* **2007,** *30* (1), 74-78.

18. He, Y.; Yue, Y.; Zheng, X.; Zhang, K.; Chen, S.; Du, Z., Curcumin, inflammation, and chronic diseases: how are they linked? *Molecules* **2015,** *20* (5), 9183-213.

19. (a) Wilken, R.; Veena, M. S.; Wang, M. B.; Srivatsan, E. S., Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. *Mol. Cancer* **2011,** *10* (12), 1-19; (b) Das, L.; Vinayak, M., Long term effect of curcumin in regulation of glycolytic pathway and angiogenesis via modulation of stress activated genes in prevention of cancer. *PloS one* **2014,** *9* (6), 1-12.

20. Han, J.; Pan, X. Y.; Xu, Y.; Xiao, Y.; An, Y.; Tie, L.; Pan, Y.; Li, X. J., Curcumin induces autophagy to protect vascular endothelial cell survival from oxidative stress damage. *Autophagy* **2012,** *8* (5), 812-25.

21. Zhang, D. W.; Fu, M.; Gao, S. H.; Liu, J. L., Curcumin and diabetes: a systematic review. *Evid-Based Compl. Alt.* **2013,** *2013*, 636053.

22. Yang, Y. S.; Su, Y. F.; Yang, H. W.; Lee, Y. H.; Chou, J. I.; Ueng, K. C., Lipid-lowering effects of curcumin in patients with metabolic syndrome: a randomized, double-blind, placebo-controlled trial. *Phytother. Res.* **2014,** *28* (12), 1770-7.

23. Shen, L. R.; Parnell, L. D.; Ordovas, J. M.; Lai, C. Q., Curcumin and aging. *BioFactors* **2013,** *39* (1), 133-40.

24. (a) Anand, P.; Sundaram, C.; Jhurani, S.; Kunnumakkara, A. B.; Aggarwal, B. B., Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett.* **2008,** *267* (1), 133-64; (b) Sa, G.; Das, T., Anti cancer effects of curcumin: cycle of life and death. *Cell Div.* **2008,** *3*, 14.

25. Jumarie, C.; Malo, C., Caco-2 Cells Cultured in Serum-Free Medium as a Model for the Study of Enterocytic Differentiation In Vitro. *J. Cell Physiol.* **1991,** *149* (1), 24-33.

26. Ahmed, D.; Eide, P. W.; Eilertsen, I. A.; Danielsen, S. A.; Eknaes, M.; Hektoen, M.; Lind, G. E.; Lothe, R. A., Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* **2013,** *2*, 1-8.

27. Waris, G.; Ahsan, H., Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* **2006,** *5*, 14.

28. Circu, M. L.; Aw, T. Y., Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.* **2010,** *48* (6), 749-62.

29. Kalyanaraman, B.; Darley-Usmar, V.; Davies, K. J.; Dennery, P. A.; Forman, H. J.; Grisham, M. B.; Mann, G. E.; Moore, K.; Roberts, L. J., 2nd; Ischiropoulos, H., Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic. Biol. Med.* **2012,** *52* (1), 1-6.

30. Jäger, R.; Lowery, R. P.; Calvanese, A. V.; Joy, J. M.; Purpura, M.; Wilson, J. M., Comparative absorption of curcumin formulations. *Nutr. J.* **2014,** *13* (11), 1-8.

31. Thayyullathil, F.; Chathoth, S.; Hago, A.; Patel, M.; Galadari, S., Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and -independent apoptosis in L929 cells. *Free Radical Biology and Medicine* **2008,** *45* (10), 1403-1412.

32. Atsumi, T.; Tonosaki, K.; Fujisawa, S., Comparative cytotoxicity and ROS generation by curcumin and tetrahydrocurcumin following visible-light irradiation or treatment with horseradish peroxidase. *Anticancer Res* **2007,** *27* (1A), 363-71.

33. Gandhy, S. U.; Kim, K.; Larsen, L.; Rosengren, R. J.; Safe, S., Curcumin and synthetic analogs induce reactive oxygen species and decreases specificity protein (Sp) transcription factors by targeting microRNAs. *BMC Cancer* **2012,** *12* (564), 1-12.

34. Noratto, G. D.; Jutooru, I.; Safe, S.; Angel-Morales, G.; Mertens-Talcott, S. U., The drug resistance suppression induced by curcuminoids in colon cancer SW-480 cells is mediated by reactive oxygen species-induced disruption of the microRNA-27a-ZBTB10-Sp axis. *Molecular nutrition & food research* **2013,** *57* (9), 1638-48.

35. Chen, M. B.; Wu, X. Y.; Tao, G. Q.; Liu, C. Y.; Chen, J.; Wang, L. Q.; Lu, P. H., Perifosine sensitizes curcumin-induced anti-colorectal cancer effects by targeting multiple signaling pathways both in vivo and in vitro. *Int J Cancer* **2012,** *131* (11), 2487-98.

36. Zhang, X.; Chen, M.; Zou, P.; Kanchana, K.; Weng, Q.; Chen, W.; Zhong, P.; Ji, J.; Zhou, H.; He, L.; Liang, G., Curcumin analog WZ35 induced cell death via ROS-dependent ER stress and G2/M cell cycle arrest in human prostate cancer cells. *BMC Cancer* **2015,** *15* (1), 1-11.

37. Sánchez, Y.; Simón, G. P.; Calviño, E.; de Blas, E.; Aller, P., Curcumin Stimulates Reactive Oxygen Species Production and Potentiates Apoptosis Induction by the Antitumor Drugs Arsenic Trioxide and Lonidamine in Human Myeloid Leukemia Cell Lines. *Journal of Pharmacology and Experimental Therapeutics* **2010,** *335* (1), 114-123.

38. Wang, D.; Hu, J.; Lv, L.; Xia, X.; Liu, J.; Li, X., Enhanced inhibitory effect of curcumin via reactive oxygen species generation in human nasopharyngeal carcinoma cells following purple-light irradiation. *Oncology letters* **2013,** *6* (1), 81-85.

39. (a) Boulton, S.; Anderson, A.; Swalwell, H.; Henderson, J. R.; Manning, P.; Birch-Machin, M. A., Implications of using the fluorescent probes, dihydrorhodamine 123 and 2 ',7 '-dichlorodihydrofluorescein diacetate, for the detection of UVA-induced reactive oxygen species. *Free Radical Res* **2011,** *45* (2), 115-122; (b) Gomes, A.; Fernandes, E.; Lima, J. L. F. C., Fluorescence probes used for detection of reactive oxygen species. *J Biochem Bioph Meth* **2005,** *65* (2-3), 45-80.

40. Zou, P.; Zhang, J.; Xia, Y.; Kanchana, K.; Guo, G.; Chen, W.; Huang, Y.; Wang, Z.; Yang, S.; Liang, G., ROS generation mediates the anti-cancer effects of WZ35 via activating JNK and ER stress apoptotic pathways in gastric cancer. *Oncotarget* **2015,** *6* (8), 5860-76.

41. Aggarwal, B. B., Nuclear factor-kappa-B: The enemy within. *Cancer Cell* **2004,** *6* (3), 203-208.

42. Sandur, S. K.; Ichikawa, H.; Pandey, M. K.; Kunnumakkara, A. B.; Sung, B.; Sethi, G.; Aggarwal, B. B., Role of pro-oxidants and antioxidants in the anti-inflammatory and apoptotic effects of curcumin (diferuloylmethane). *Free Radic Biol Med* **2007,** *43* (4), 568-80.

43. Voigt, W., Sulforhodamine B assay and chemosensitivity. *Methods in molecular medicine* **2005,** *110*, 39-48.

44. (a) Liao, H.; Dong, W.; Shi, X.; Liu, H.; Yuan, K., Analysis and comparison of the active components and antioxidant activities of extracts from Abelmoschus esculentus L. *Pharmacogn. Mag.* **2012,** *8* (30), 156-161; (b) Thaipong, K.; Boonprakob, U.; Crosby, K.; Cisneros-Zevallos, L.; Hawkins Byrne, D., Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J. Food Compos. Anal.* **2006,** *19* (6-7), 669-675.

Highlights

- A new and convenient synthetic access to *N*-alkyl β-enaminone curcuminoids
- Strong increase of the cellular responses compared to the native molecules
- No correlation between chemical anti-oxidant capacity and intracellular ROS
- Viability and ROS results are strongly cell type dependent
- Valuable new building blocks for oxidative stress-related diseases research