Accepted Manuscript

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PII: S0223-5234(17)30951-0

DOI: 10.1016/j.ejmech.2017.11.048

Reference: EJMECH 9923

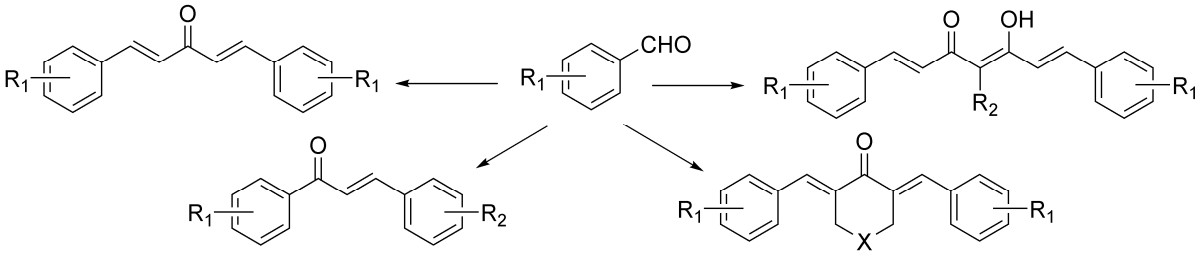
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 22 August 2017

Revised Date: 31 October 2017

Accepted Date: 18 November 2017

Please cite this article as: L.M. Deck, L.A. Hunsaker, T.A. Vander Jagt, L.J. Whalen, R.E. Royer, D.L. Vander Jagt, Activation of anti-oxidant Nrf2 signaling by enone analogues of curcumin, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.11.048.



Activation of Anti-Oxidant Nrf2 Signaling by Enone Analogues of Curcumin

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Abstract

Inflammation and oxidative stress are common in many chronic diseases. Targeting signaling pathways that contribute to these conditions may have therapeutic potential. The transcription factor Nrf2 is a major regulator of phase II detoxification and anti-oxidant genes as well as anti-

inflammatory and neuroprotective genes. Nrf2 is widespread in the CNS and is recognized as an important regulator of brain inflammation. The natural product curcumin exhibits numerous biological activities including ability to induce the expression of Nrf2-dependent phase II and anti-oxidant enzymes. Curcumin has been examined in a number of clinical studies with limited success, mainly owing to limited bioavailability and rapid metabolism. Enone analogues of curcumin were examined with an Nrf2 reporter assay to identify Nrf2 activators. Analogues were separated into groups with a 7-carbon dienone spacer, as found in curcumin; a 5-carbon enone spacer with and without a ring; and a 3-carbon enone spacer. Activators of Nrf2 were found in all three groups, many of which were more active than curcumin. Dose-response studies demonstrated that a range of substituents on the aromatic rings of these enones influenced not only the sensitivity to activation, reflected in EC_{50} values, but also the extent of activation, which suggests that multiple mechanisms are involved in the activation of Nrf2 by these analogues.

Keywords

Nrf2 activation; pyridone; enone; aldol condensation

Abbreviations Used

Nrf2, nuclear factor erythroid 2 related factor 2; CNS, central nervous system; Keap1, Kelch-like ECH associated protein 1; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; Maf, musculoaponeurotic fibrosarcoma oncogene; XRE, xenobiotic response element; ARE, anti-oxidant response element; mTORC1, mammalian target of rapamycin complex 1; GSK-3, glycogen synthase kinase 3; PKB, protein kinase B; ROS, reactive oxygen species

1. Introduction

Transcription factor nuclear factor erythroid 2 related factor 2 (Nrf2), which is a member of the cap'n'collar family of transcription factors, is the master regulator of an inducible cellular system of cytoprotective genes. These genes code for a broad range of proteins, including phase I and II detoxification enzymes, anti-oxidant proteins, as well as anti-inflammatory and neuroprotective factors, growth factors and receptors, and other transcription factors. Interest has emerged in Nrf2 as a therapeutic target, especially for treatment of chronic inflammatory diseases. [1-6] In the absence of stress, Nrf2 forms a cytosolic complex with Kelch-like ECH associated protein 1 (Keap1) and Cul3; Cul3 is an adaptor to link Nrf2 to an E3 ubiquitin ligase complex. Newly synthesized Nrf2 in unstressed cells is degraded by ubiquitination and proteosomal degradation, which limits the cytosolic concentration of Nrf2. In response to oxidative and electrophilic stresses, cysteine residues of Keap1 are modified, which alters the interaction between Nrf2 and Keap1 and unlocks Keap1 from the Nrf2 complex. This allows newly synthesized Nrf2 to accumulate and translocate to the nucleus where Nrf2 interacts with small Maf proteins and then binds to promoters with anti-oxidant response element (ARE) sequences. [7-11]

Numerous Nrf2-activating chemicals have been identified, including natural products such as curcumin (Figure 1, Structure **1**). Curcumin and some other natural product phenols can activate Nrf2 after oxidation to electrophilic quinones, which can modify select cysteine residues in Keap1 by Michael addition. Curcumin may also modify Keap1 by Michael addition involving the enone functionalities of curcumin. Keap1 cysteine residues 273, 288 and 151 appear to be especially important. [12,13] Peptide inhibitors of the Keap1-Nrf2 protein-protein interaction have been developed as well as a variety of small molecules that inhibit the Keap1-Nrf2

interaction. [14-19] These studies have been aided by the availability of several crystal structures of Nrf2. [20-22]

Curcumin, obtained from the rhizomes of *Curcuma longa* (turmeric), exhibits numerous biological activities [23] including ability to induce the expression of Nrf2-dependent phase II and anti-oxidant enzymes such as glutathione S-transferase, aldose reductase and heme oxygenase-1. [24,25] Curcumin appears to utilize more than a single mechanism for activation of Nrf2, including covalent modification of Keap1 [25,26] and activation of upstream kinases. [25,27] Curcumin has been examined in a number of clinical studies with limited success, [28] mainly owing to limited bioavailability and rapid metabolism. Attempts to improve curcumin as a therapeutic agent include development of new formulations that may enhance bioavailability.

There is considerable interest in the development of analogues and derivatives of curcumin with improved therapeutic potential. [29-32] There also is interest in the development of analogues that activate anti-oxidant Nrf2 but simultaneously inhibit pro-inflammatory NF-κB signaling, [33-35] which is consistent with the ability of curcumin to target both of these pathways. [36,37] Here we describe the synthesis and evaluation of the Nrf2-activating potential of enone analogues of curcumin.

2. Results

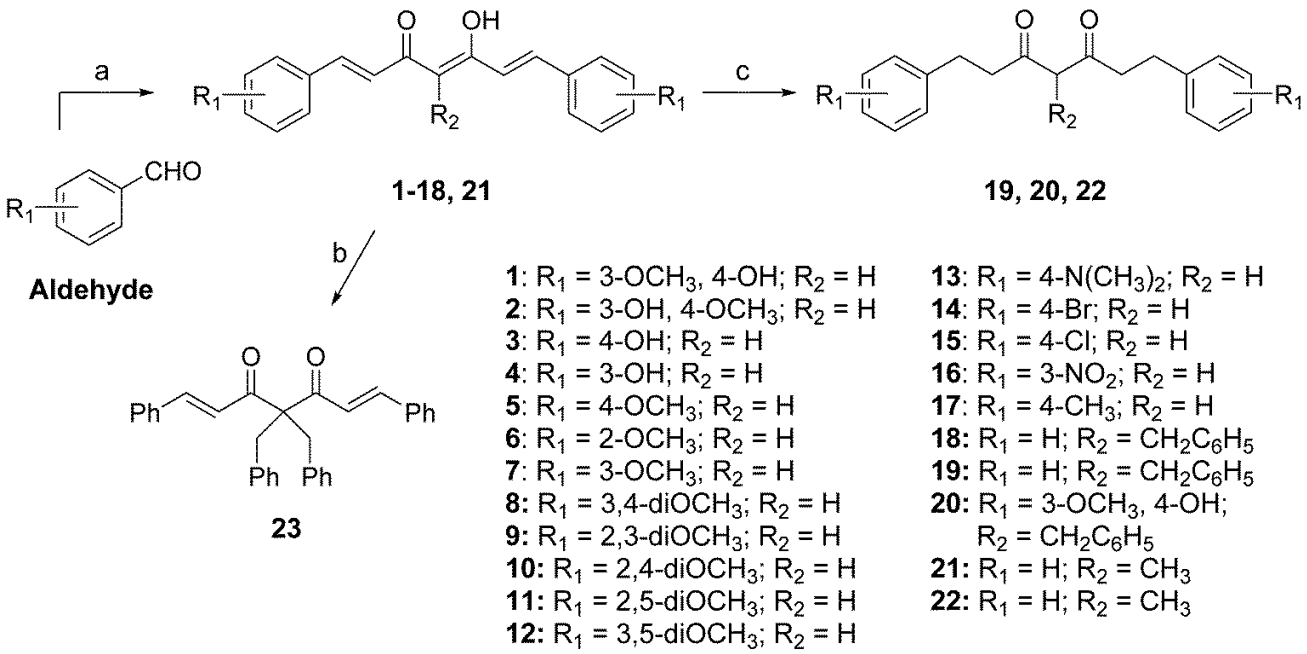
2.1 Synthesis of curcumin analogues

Curcumin (Scheme 1, Structure **1**) is a symmetrical bis-phenolic compound that exists as an equilibrium mixture of the dienone and the keto-enol tautomer; the keto-enol tautomer predominates and is stabilized by intramolecular hydrogen bonding. [38-40] Curcumin has a 7-carbon unsaturated spacer separating two substituted aromatic rings. The current study involves

analogues of curcumin having a 7-carbon spacer, a 5-carbon spacer, a 5-carbon ring-containing spacer and a 3-carbon spacer. We have previously reported the synthesis and characterization of several of these compounds. [41-43]

Scheme 1 describes the synthesis of symmetrical analogues, which maintain a 7-carbon keto-enol spacer between aromatic rings as in curcumin. The aryl rings contain different substituents in various positions on the ring. These analogues were designed to test the importance of the type of functional group and its location on the aromatic ring. Compounds **1**-**17** were synthesized from aromatic aldehydes by condensation with 2,4-pentanedione in an aldol type reaction as described by Pabon. [44] This involves base-catalyzed condensation in the presence of a trialkylborate to complex with the carbonyl groups of 2,4-pentanedione to protect C-3 from a Knoevenagel condensation, allowing the reaction to occur only on the terminal methyl groups. Compounds **1**-**17** exist predominately in the keto-enol form (99-100%) as exhibited by the spectral data. [39,40] The proton NMR spectra have a tall peak at 5.47 ppm corresponding to the enol proton and a short peak at 3.48 ppm for the two central methylene protons of the diketo form when it is present. There are also doublets in the aromatic region with *J* values of 15.5-16.5 Hz for alkene protons present in the spacer, evidence that supports the E configuration. [39,40] The carbon spectra have a large signal at approximately 100 ppm for the enol carbon and a very small signal at approximately 50 ppm for the central methylene carbon in the diketo form.

Scheme 1



Scheme 1. Reagents for 7-carbon spacer compounds: (a) $CH_3C(O)CH_2C(O)CH_3$ (for compounds **1**-**17**) or $CH_3C(O)CH(CH_3)C(O)CH_3$ (for compound **21**) or $CH_3C(O)CH(CH_2C_6H_5)C(O)CH_3$ (for compound **18**) (0.5 equiv), B_2O_3, $B(OC_4H_9)_3$, EtOAc, $nBuNH_2$, HCl/H_2O, 20-85%; (b) $C_6H_5CH_2Br$ (1.0 equiv.), NH_4Cl, NaOH, CH_2Cl_2, 61%; (c) H_2, Pd/C, EtOAc, 80-90%.

Aldol condensation of benzaldehyde with 3-methyl-2,4-pentanedione or 3-benzyl-2,4-pentanedione in the presence of a trialkylborate afforded compounds **18** and **21** respectively, [44] which have a methyl and a benzyl group on the central methylene carbon spacer. These compounds were designed to test the importance of the central methylene hydrogens. Use of monosubstituted pentanediones avoided disubstitution of the methylene carbon on the spacer which was observed when methyl iodide or benzyl bromide and base were used in an S_N2 reaction on 1,7-diphenyl-1,6-heptadiene-3,5-dione. Pedersen reported monosubstitution when reacting an alkyl halide with curcumin in the presence of a base but in our hands we observed disubstitution. [40] When a single substituent such as a methyl or benzyl group is placed on the

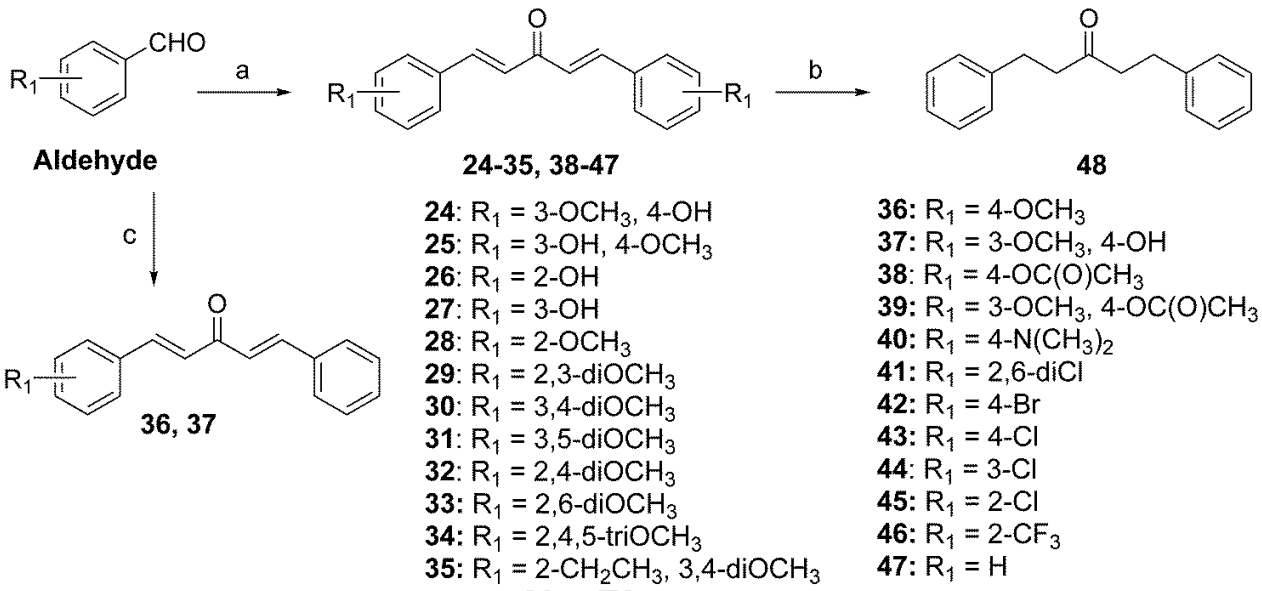
central methylene carbon, a pattern in the proton and carbon NMR spectra is observed that is similar to analogues without the central group. Reaction of **18** with benzyl bromide in the presence of base gave the dibenzylated compound **23,** which exhibits no tautomerization because the molecule is trapped in the diketo form. [40]

The saturated compounds, **19, 20**, and **22** were synthesized by hydrogenation of unsaturated compounds, **18, 1** and **21** using hydrogen in the presence of palladium on charcoal as reported by Venkateswarlu et al. [45] In the proton and carbon NMR spectra of **22** a mixture of the diketo (10-15%) and enol (85-90%) forms was observed due to the lack of conjugation. This is not surprising since the observation of both forms has also been reported in 1,5-diaryl-2,4-pentanediones. [46]

The analogues shown in Scheme 2 contain a 5-carbon spacer and two identical aryl rings, except for **36** and **37**. These compounds were designed to test the importance of the length of the spacer between two aryl rings. Analogues **24-35** and **40-47** were prepared as described by Masuda et al [47] by reaction of the appropriate benzaldehyde with acetone in a base-catalyzed aldol reaction. In the case of phenolic benzaldehydes, **26** and **27**, the phenol was protected with a methoxymethyl group prior to the aldol reaction and deprotected later to give the free phenol. [47] Compounds **38** and **39** were prepared from 1,5-bis(4-hydroxyphenyl)-1,4-pentadien-3-one and **24** respectively by reaction with acetic anhydride in the presence of pyridine in an esterification reaction as described by Suarez. [48] Compounds **36** and **37,** which are not symmetrical, were prepared using two consecutive base-catalyzed aldol reactions. [47] The substituted benzaldehyde needed to prepare compound **35**, which is a new compound, was synthesized by reacting 2,3-dimethoxyethylbenzene and N-methylformanilide with phosphorus oxychloride. [49] The proton NMR of the compounds exhibited a pair of doublets in the aromatic

region with *J* values between 15.5 to 16.5 Hz for the alkene protons present in the spacer, again providing evidence of (E)-stereochemistry. The carbon NMR has two signals in the aromatic region for the alkene carbons and a downfield signal for the carbonyl carbon.

Scheme 2



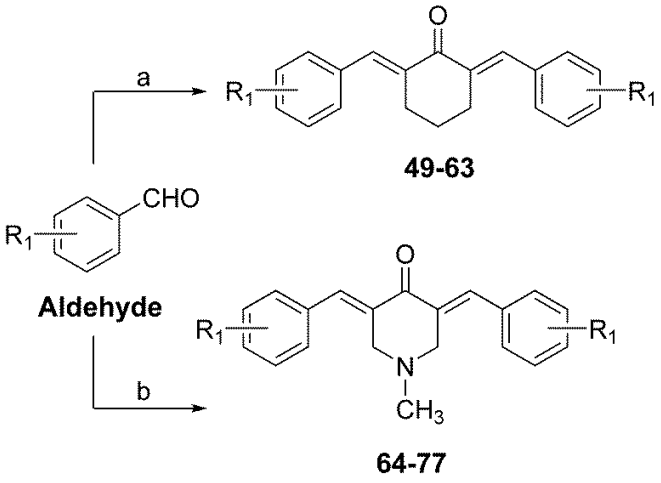
Scheme 2. Reagents for 5-carbon spacer compounds: (a) $CH_3C(O)CH_3$ (0.5 equiv.), NaOH, CH_3CH_2OH, H_2O, 67-96%; (b) H_2, Pd/C, EtOAc, 80%; (c) $CH_3C(O)CH_3$ (1.0 equiv.), NaOH, CH_3CH_2OH, H_2O, PhCHO, 94-99%.

Analogue **48** was prepared by hydrogenation of **47** in the presence of palladium on charcoal. [45] Compound **48** was verified by the appearance of triplets at 2.76 ppm and 2.97 ppm for the alkane protons in the proton NMR along with the loss of doublets in the aromatic region for the alkene protons.

Analogues in Scheme 3 contain two identical aryl rings separated by a five carbon unsaturated spacer having both a carbonyl and a saturated ring. They were designed to test the importance of a ring in the spacer. Compounds **49-63** contain a cyclohexanone ring whereas compounds **64-77**

contain an N-methylpiperidone ring. The compounds were synthesized using a procedure described by Masuda et al [47] by reaction of a substituted benzaldehyde with cyclohexanone or N-methylpiperidone in a base-catalyzed aldol reaction. The phenolic benzaldehydes needed to prepare **49** and **60** were protected before the aldol reaction and deprotected after condensation as described earlier. The formation of products was verified by proton NMR with the appearance of a singlet at 7.7 ppm for the alkene protons on the spacer. The carbon NMR spectra have two new signals in the aromatic region for the alkene carbons in the spacer.

Scheme 3



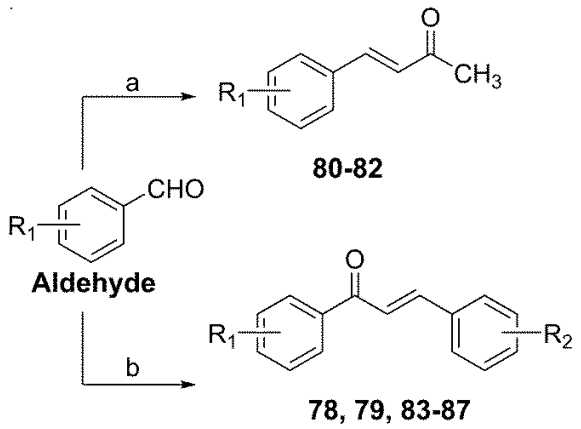
49: R_1 = 3-OCH_3, 4-OH
50: R_1 = 3,4-diOCH_3
51: R_1 = 3,5-diOCH_3
52: R_1 = 2,5-diOCH_3
53: R_1 = 4-OCH_3
54: R_1 = 3,4,5-triOCH_3
55: R_1 = 3-OCH_3
56: R_1 = 2,4-diOCH_3
57: R_1 = 2,3-diOCH_3
58: R_1 = 2,3,4-triOCH_3
59: R_1 = 2-OCH_3
60: R_1 = 3-OH
61: R_1 = 3-F
62: R_1 = 2,6-diCl
63: R_1 = 4-Br
64: R_1 = 3-OCH_3
65: R_1 = 2,3-diOCH_3
66: R_1 = 4-OCH_3
67: R_1 = 2,4-diOCH_3
68: R_1 = 3,4,5-triOCH_3
69: R_1 = 2,3,4-triOCH_3
70: R_1 = 3,5-diOCH_3
71: R_1 = 3-Br
72: R_1 = 4-Br
73: R_1 = 3-CF_3
74: R_1 = 4-$CH(CH_3)_2$
75: R_1 = 4-SCH_3
76: R_1 = 4-OCH_2CH_3
77: R_1 = 3-CH_3

Scheme 3. Reagents for 5-carbon ring containing spacer compounds: (a) Cyclohexanone (0.5 equiv), NaOH, CH_3CH_2OH, H_2O, 75-95%; (b) 1-Methyl-4-piperidone (0.5 equiv), NaOH, CH_3CH_2OH, H_2O, 77-99%.

Analogues **80-82,** shown in Scheme 4, contain a single aryl ring with an unsaturated three-carbon chain and a single carbonyl. Also shown in the scheme are compounds **78, 79** and **83-87**, which contain two aryl rings with the three carbon chain. All of the compounds were designed to test the importance of a three-carbon chain and the importance of one aryl ring versus two aryl

rings. Analogues **80-82** were prepared from excess acetone and a substituted benzaldehyde in a base-catalyzed aldol reaction. [47] The proton NMR spectra of these compounds show a pair of doublets in the aromatic region having *J* values of approximately 16 Hz for the alkene protons and a signal at 2.3 ppm for the methyl protons. The carbon NMR spectra have two signals in the aromatic region for the alkene carbons and a carbonyl carbon signal. Compounds **78, 79** and **83-87** were prepared in a similar manner using a substituted acetophenone and a substituted benzaldehyde in a base-catalyzed aldol condensation. [47,50,51] Analogues **78** and **79** were protected with a methoxymethyl group before reaction and deprotected after condensation. Compounds **83** and **85**, which contain a carboxyl group, were synthesized following a procedure described by Cleeland. [51] The proton and carbon NMR data is similar to that reported for compounds **80-82** except for the absence of the methyl signal.

Scheme 4



78: $R_1 = R_2 = 3\text{-}OCH_3, 4\text{-}OH$
79: $R_1 = 3\text{-}OCH_3, 4\text{-}OH$; $R_2 = H$
80: $R_1 = 3\text{-}OCH_3, 4\text{-}OH$
81: $R_1 = 4\text{-}OCH_3$
82: $R_1 = 2\text{-}OH$
83: $R_1 = 4\text{-}COOH$; $R_2 = H$
84: $R_1 = 4\text{-}CN$; $R_2 = H$
85: $R_1 = H$; $R_2 = 4\text{-}COOH$
86: $R_1 = 2,4\text{-}diCH_3$; $R_2 = H$
87: $R_1 = R_2 = H$

Scheme 4. Reagents for 3-carbon spacer compounds: (a) $CH_3C(O)CH_3$ (1.0 equiv), NaOH, CH_3CH_2OH, H_2O, 30-75%; (b) $PhC(O)CH_3$, NaOH, CH_3CH_2OH, H_2O, 55-91%.

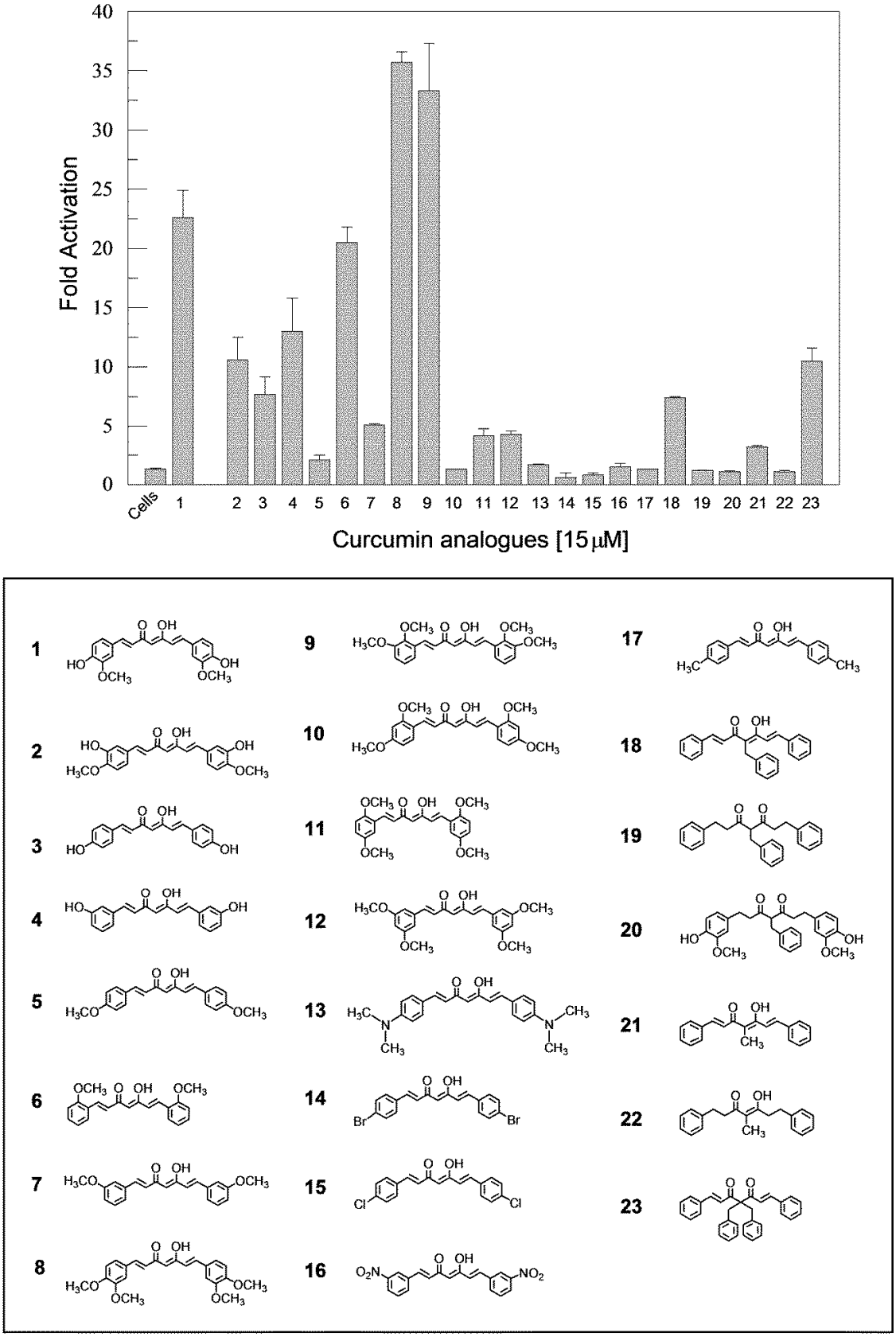
An additional three-carbon spacer analogue, compound **88**, was prepared to test the importance of two carbonyls in the spacer. Analogue **88,** whose structure is shown in Figure 4, was prepared by reacting acetophenone with methylbenzoate and sodium methoxide in a condensation reaction. [52] The proton NMR has a signal for the enol proton in the spacer at 6.8 ppm and the carbon NMR has a signal at 93 ppm for the enol carbon and a signal at 185 ppm for the carbonyl carbon, indicating **88** exists 100% in the keto-enol form.

2.2 Nrf2 activation by curcumin analogues with a 7-carbon spacer

Curcumin (Scheme 1, Structure **1**) was evaluated with an Nrf2 reporter assay. Dose-response studies demonstrated an EC_{50} = 21 μM and maximum activation 23-fold above untreated cells (Table 1). Curcumin was used as the reference compound for studies of curcumin analogues. A series of analogues that contained 7-carbon spacers between the aromatic rings was screened initially at 15 μM, as shown in Figure 1. Several analogues (**6**,**8** and **9**) were comparable to curcumin as activators of Nrf2. Dose-response studies (Table 1) demonstrated that analogues **8** and **9** activated Nrf2 36-fold and 33-fold, respectively, which is considerably higher than the activation of Nrf2 by curcumin, suggesting that more than a single mechanism is likely involved. This is consistent with reports that curcumin can activate Nrf2 by multiple mechanisms. [25-27] Other analogues that gave moderate activation (**2**,**3**,**4**,**6**,**8**,**9**) shared with curcumin the presence of methoxy and/or hydroxy functional groups. However, the presence of these functional groups

does not consistently predict activation of Nrf2. Analogues **5** and **7**, for example, exhibit very little activity compared with analogue **6**, which is an isomer of **5** and **7**. Likewise, analogues **10**, **11** and **12** exhibit very little activity compared with their isomers **8** and **9**. Analogues of curcumin with other ring substituents (**13**-**17**) exhibited no significant activity. Analogues **18**, **21** and **23**, which contained alkyl or aralkyl groups attached to the central carbon but contained no ring substituents, demonstrated modest activity, whereas compounds **19** and **22**, which are the products from reduction of the double bonds in **18** and **21**, showed no activity. This suggests that those analogues that exhibit activity as activators of Nrf2 function, at least in part, as Michael acceptors, consistent with one of the mechanisms of activation of Nrf2 by curcumin. [25,26]

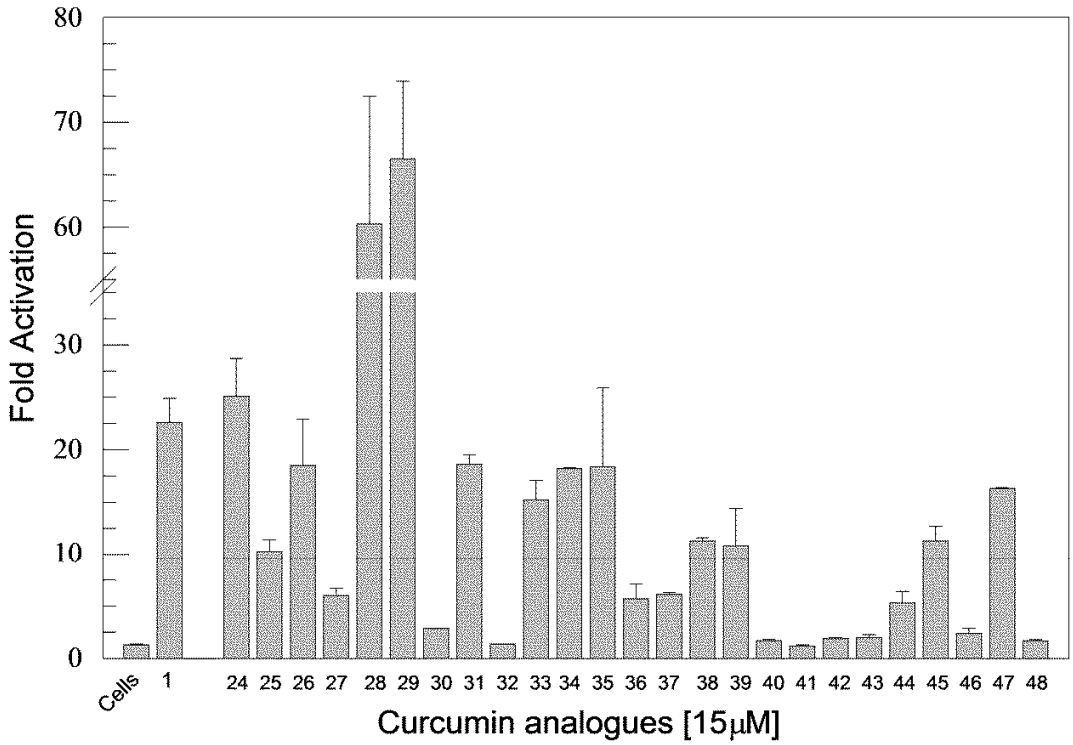
Figure 1. Nrf2 activation by curcumin analogues with a 7-carbon spacer

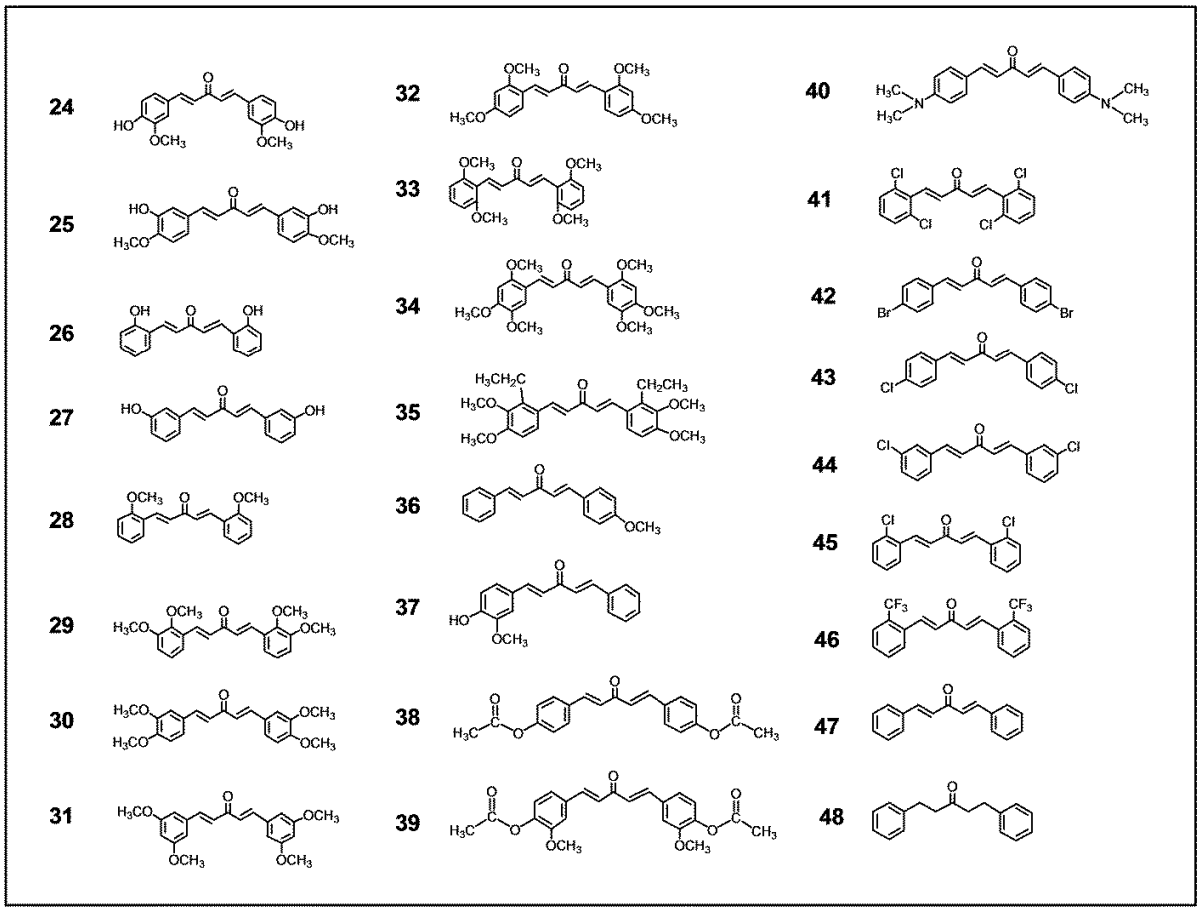


2.3 Nrf2 activation by curcumin analogues with a 5-carbon spacer

A series of 12 analogues of curcumin with 5-carbon spacers between the aromatic rings was screened at 15 μM concentration of analogue and compared with curcumin (Figure 2). A number of analogues activated Nrf2 comparable to curcumin (analogues **24**, **26**, **28**, **29**, **34**, **35**). Dose-response analyses demonstrated that most of these analogues were better than curcumin, both in terms of higher fold activations and lower EC_{50} values (Table 1). Analogues **28** and **29**, for example, gave 60.3 and 66.5 fold activations. Analogues **28** and **34** are the most active when expressed as the ratio of fold activation divided by EC_{50}. These active analogues with 5-carbon spacers shared with active analogues with 7-carbon spacers the presence of hydroxy and/or methoxy functional groups. Analogues with other functional groups (analogues **40**-**44**, **46**) showed little or no activity, except for analogue **45**, which contains *ortho* chloro functional groups. Interestingly, analogue **47**, which contains no functional groups, showed significant activity, whereas its reduction product **48** showed no activity, consistent with the data in Figure 1 and with the conclusion that an enone functionality is essential for activity.

Figure 2. Nrf2 activation by curcumin analogues with a 5-carbon spacer.

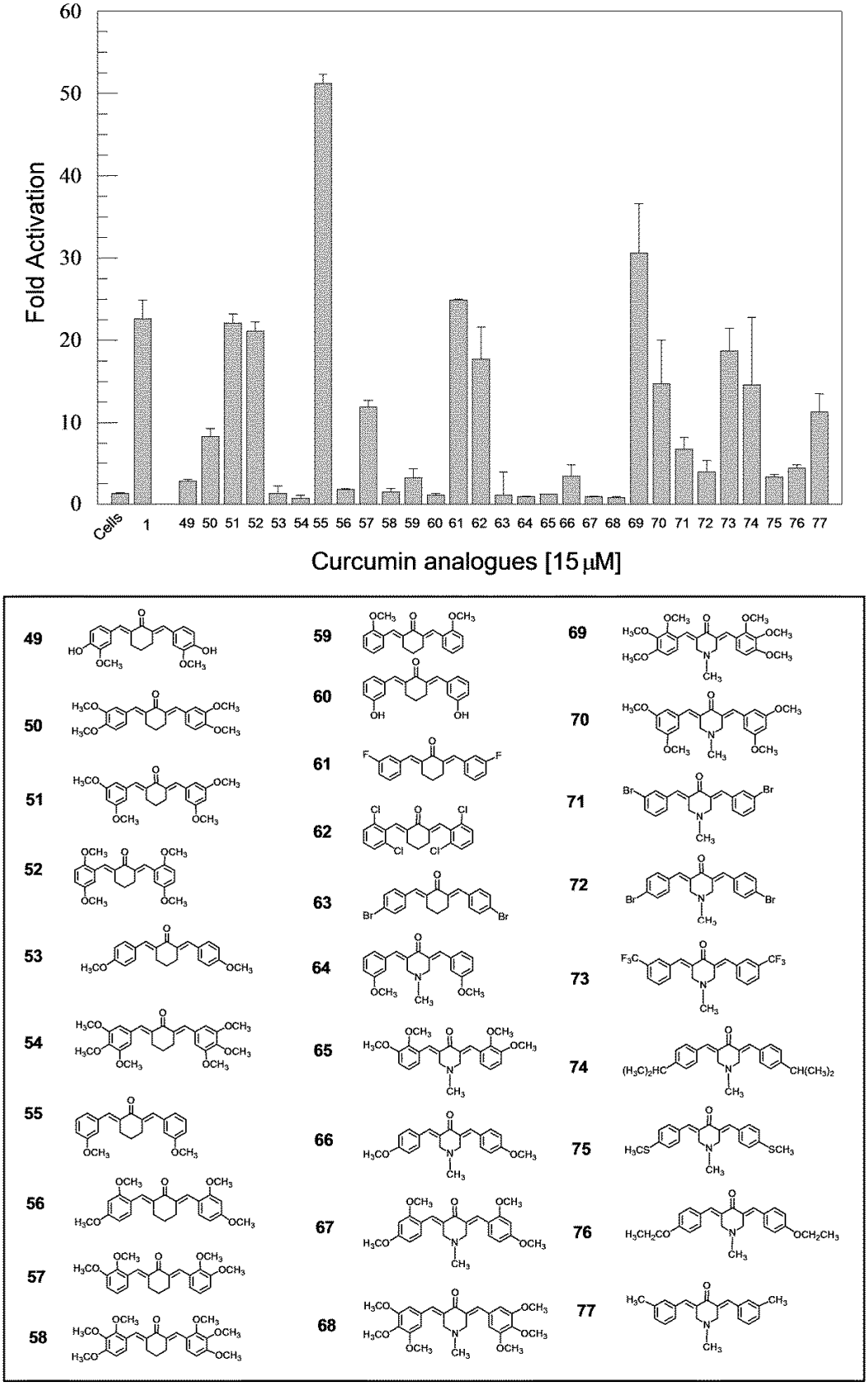




2.4 Activation of Nrf2 by curcumin analogues with a 5-carbon, ring-containing spacer

A series of 29 analogues of curcumin with 5-carbon spacers, in which the spacer contained a 6-member ring, was screened at 15 μM analogue and compared with curcumin (Figure 3). A number of analogues (**51**,**52**,**55**,**61**,**62**,**69**,**70**,**73**,**74**) showed significant activity with **51**,**52**,**61** and **69** showing activity comparable to curcumin. Unlike the results in Figures 1 and 2 where active analogues contained hydroxyl or methoxy substituents, the analogues with a 5-carbon, ring-containing spacer included numerous examples with substituents other than hydroxyl or methoxy (analogues **61**,**62**,**71**,**73**,**74**,**77**). Analogue **61**, for example, contains *meta* fluoro substituents and is comparable to curcumin in activity. Similar to the results in Figures 1 and 2, there are numerous analogues that are isomers yet show markedly different activities. Analogue **55** is active whereas its isomers **53** and **59** show little or no activity. Analogues **51**, **52** and **69** are active whereas isomers **56** and **68** are inactive.

Figure 3. Nrf2 activation by curcumin analogues with a 5-carbon, ring-containing spacer.



2.5 Activation of Nrf2 by curcumin analogues with a 3-carbon spacer

A series of 11 analogues of curcumin with 3-carbon spacers was screened at 15 μM analogue and compared with curcumin (Figure 4). Analogues **78**,**79** and **85**-**88**, all of which contained two aromatic rings, showed significant activity, whereas none of the analogues with a single ring showed activity. Analogue **88**, which contained no ring substituents, was considerably more active than curcumin.

Figure 4. Nrf2 activation by curcumin analogues with a 3-carbon spacer.

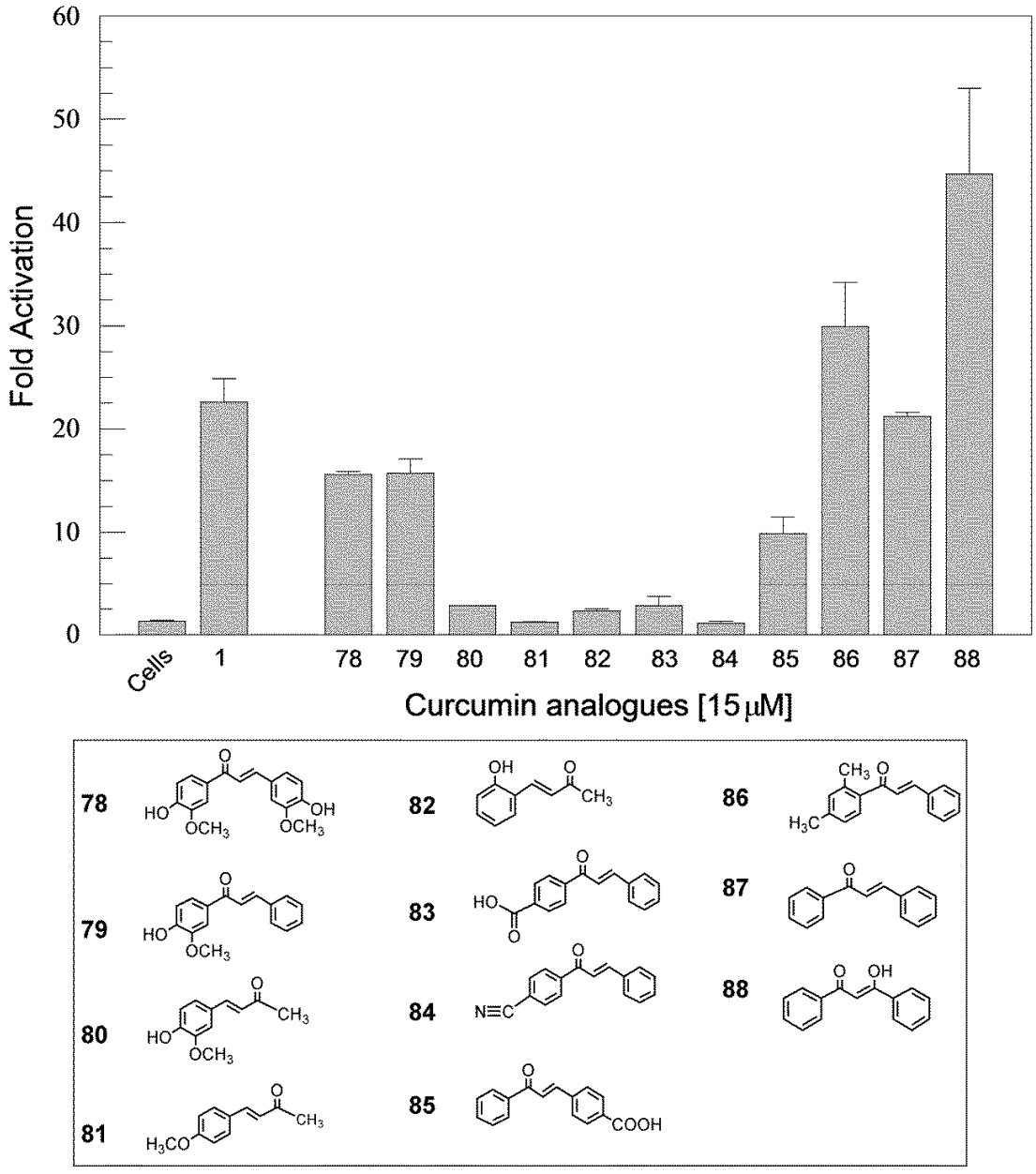
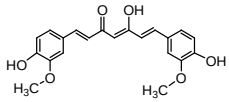
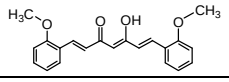
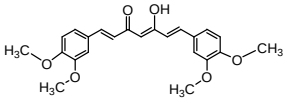
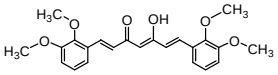
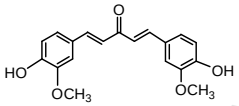
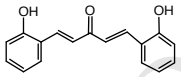
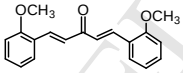
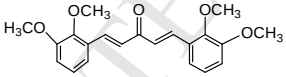
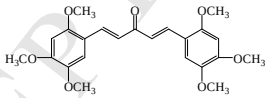
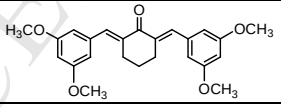
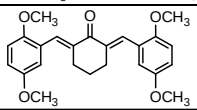
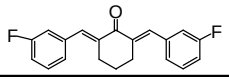
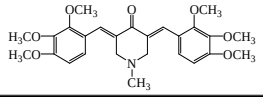
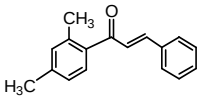
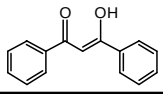


Table 1 summarizes the most active of all of the analogues where both fold activation and EC_{50} values are considered.

Table 1. Fold activation of Nrf2 by curcumin analogues and EC_{50} values.

Number	Structure	Fold Activation	EC_{50} (μM)
Curcumin **1**		22.6 ± 2.3	**21 ± 0.02**
6		20.5 ± 1.3	**14.4 ± 4**
8		35.7 ± 0.9	**16.6 ± 0.2**
9		33.3 ± 4.0	**7.3 ± 1.5**
24		25.1 ± 3.6	**9.9 ± 0.6**
26		18.5 ± 4.4	**3.3 ± 0.5**
28		60.3 ± 12	**3.3 ± 0.1**
29		66.5 ± 7.4	**10.3 ± 0.4**
34		18.2 ± 0.08	**1.32 ± 0.2**
51		22.1 ± 1.1	**5.3 ± 0.3**
52		21.1 ± 1.7	**18.1 ± 0.9**
61		24.9 ± 0.4	**7.6 ± 0.3**
69		30.6 ± 6.8	**8.0 ± 0.2**
86		29.9 ± 4.3	**21.1 ± 0.5**
88		44.7 ± 8.3	**7.2 ± 1.2**

3. Discussion and Conclusions

The importance of inflammation and oxidative stress in many chronic diseases supports the concept that activation of anti-oxidant Nrf2 signaling may have therapeutic potential. A number of Nrf2 activators have entered into clinical trials. Bardoxolone methyl, an oleanolic acid-derived synthetic triterpene, is a potent Nrf2 activator that was evaluated for its ability to slow progression to end-stage renal disease in patients with type 2 diabetes and stage 4 chronic kidney disease. Bardoxolone methyl is able to modify Keap1 as a Michael acceptor. This clinical trial was terminated in phase III owing to an increase in heart failure. [53] However, bardoxolone methyl is currently in phase III studies for pulmonary hypertension (ClinicalTrials.gov). Dimethyl fumarate, a simple derivative of the metabolic intermediate fumaric acid, has recently been FDA-approved for the treatment of relapsing-remitting multiple sclerosis. [54] Dimethyl fumarate is active as the monomethyl derivative formed by the action of non-specific esterases. Monomethyl fumarate modifies Keap1 through electrophilic addition. Among the natural product activators of Nrf2, sulforaphane has been extensively studied, including in a number of clinical trials. Sulforaphane has shown promise in treatment of recurrent prostate cancer, [55,56] schizophrenia, [57] and autism spectrum disorder. [55] Sulforaphane also modifies Keap1 through electrophilic addition. [58]

Although numerous Nrf2-activating chemicals are electrophiles that modify Keap1 by Michael addition, there are other mechanisms for activation of Nrf2. Epigenetic modifications of CpG methylation status of Nrf2 by the anti-cancer drug 3,3'-diindolylmethane resulted in enhanced expression of Nrf2 and of Nrf2-target genes in cell-based studies. [55] This was suggested as an explanation for the chemopreventive activity of this drug. The promoter of the gene encoding Nrf2 contains XRE sequences, which can recruit the aromatic hydrocarbon receptor AhR; this

allows the Nrf2 gene to be activated by polycyclic aromatic hydrocarbons. [56] The promoter of the Nrf2 gene also contains ARE-like sequences, which allows Nrf2 to regulate its own expression. [57] The autophagy cargo receptor and signaling adaptor protein p62 contains a binding site for Keap1. The attraction of Keap1 to this site is further regulated by phosphorylation by mTORC1, which leads to autophagosome–mediated destruction of Keap1 and activation of Nrf2. [58-60] This is one example of proteins other than Nrf2 that interact specifically with Keap1, which indicates a broad role for Keap1 and a complex set of mechanisms that impact Nrf2 levels. [61] There are also mechanisms for activation of Nrf2 that do not appear to involve Keap1. Nrf2 activity is inhibited by the serine/threonine kinase GSK-3. GSK-3 constitutive activity is inhibited by PKB/Akt phosphorylation. It is conceivable that analogues of curcumin might activate Nrf2 independently of Keap1 through inhibition of GSK-3 or activation of kinases such as Akt. [61]

In the present study, we demonstrated that a wide range of curcumin analogues can activate Nrf2. This includes dienones with a 7-carbon spacer, enones with a 5-carbon spacer, and enones with a 3-carbon spacer (Table 1). It is likely that these analogues function to modify Keap1 through Michael addition, similar to curcumin. [25,26] However, they may utilize other mechanisms, again similar to curcumin. [25,27] Some of these curcumin analogues have an extensive literature. Analog **26**, for example, was originally identified as a Michael acceptor with high reactivity for sulfhydryl groups and ability to induce the expression of phase II enzymes that protect against carcinogenesis. [62] Keap1 was subsequently identified as the source of the critical intracellular sulfhydryl groups. [63] Analog **26**, which we initially developed as an inhibitor of the activation of pro-inflammatory NF-κB signaling [41,42] and AP-1 signaling, [43] was subsequently shown to down-regulate expression of the androgen receptor in human prostate

cancer cells. [64] This involved an oxidative stress-mediated mechanism supported by development of reactive oxygen species (ROS), activation of Nrf2, and induction of Nrf2-dependent genes for NAD(P)H quinone oxidoreductase-1 and aldo-keto reductase 1C1. Analog **26** has been shown to exhibit complex concentration-dependent effects on growth of leukemia cells. [65] Recently, it was reported that analog **26** activates Nrf2 through a Keap1-Cys151-dependent process. [32]

The results with analog **26** raise several issues with respect to development of Nrf2 activators as therapeutic agents. First, as mentioned above, there is the issue of selectivity for electrophilic activators that target sulfhydryl groups in Keap1. Such agents likely would also modify other sulfhydryl groups in numerous proteins. Second, there is the issue of activators that produce ROS, which then leads to activation of Nrf2 through oxidative modification of Nrf2. Since the goal is to develop therapeutic activators of Nrf2 to combat the oxidative stress associated with many chronic inflammatory diseases, it seems counterproductive to focus on activators that produce oxidative stress. It should be noted, however, that Nrf2 activators such as bardoxolone methyl, dimethyl fumarate and sulforaphane also covalently modify Keap1, yet are promising therapeutic agents. Moreover, curcumin itself has been studied extensively in clinical trials and is considered to have low toxicity. In this study numerous analogues of the natural product cucumin containing 7-carbon dienone spacers as in curcumin, 5-carbon enone spacers including ring-containing spacers, and 3-carbon enone spacers were found to be activators of the anti-oxidant transcription factor Nrf2. The presence of an enone functionality appears to be essential.

4. Experimental Section

4.1 Assay Validation

The evaluation of the ability of enone analogues of curcumin to activate Nrf2 was determined with a luciferase assay under the following conditions: 1) each analogue was evaluated in triplicate assays with untreated cells as the negative control and curcumin-treated cells as the positive control; 2) cells were washed extensively to remove drug before the luciferase activity was measured; 3) before measurement of luciferase activity, the cells were lysed with buffer, which further reduced the concentration of any remaining drug; 4) only a portion of the lysate was used in the final assay, which diluted any remaining drug even further; 5) the measurement of final luciferase activity was verified as a valid measurement by adding back low concentrations of drug to determine whether this treatment either inhibited or enhanced the luciferase activity. It was observed that this treatment with low concentrations of drug did not alter the luciferase activity. We conclude, therefore, that the luciferase measurement conducted under these conditions is a valid determination of the level of luciferase gene expression.

4.2 Reporter Assays

Nrf2-ARE reporter-HepG2 stable cell line (BPS Bioscience, San Diego, CA) was grown in a humidified atmosphere at 37°C in 5% CO_2/95% air. The cells were maintained in MEM medium with Earles balanced salts and L-glutamine supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% non-essential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin, and 400 μg/mL Geneticin. One day prior to treatment, the Nrf2-ARE cells were plated into 24-well cell culture plates at approximately 30% confluency in the above media without Geneticin. The following day, fresh media with or without curcumin analogues was applied to the cells. DMSO concentrations were kept at 0.1%. The cells were again placed in a

humidified atmosphere at 37°C in 5% CO_2/95% air for 5 hours. Plate wells were gently washed with phosphate buffered saline (PBS) pH 7.4 and lysed with 1x passive lysis buffer.

4.3 General Chemical Procedures

Reagents were purchased from commercial sources (Aldrich, Acros). The reactions were monitored by thin layer chromatography (TLC), which was carried out on silica gel $60F_{254}$ plates and visualized using UV light. If necessary products were purified by column chromatography using EM type 60 silica gel (230-400 mesh). Melting points were taken on a Thomas-Hoover Uni-Melt capillary melting point apparatus and reported uncorrected. Infrared spectra were obtained using a Bruker ALPHA spectrometer. Unless otherwise noted, ^{1}H spectra were recorded by using $CDCl_3$ solutions at 250, 300 or 500 MHz; ^{13}C NMR spectra were recorded in $CDCl_3$ at 63, 75 or 125 MHz. The data were collected on a Bruker Avance III HD 500 MHz, a Bruker Avance III 300 MHz or a Bruker AC 250 MHz spectrometer. Chemical shifts are reported in ppm relative to TMS at 0.0 ppm for both ^{1}H NMR and ^{13}C NMR. Proton NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, sept = septet, dd = doublet of doublets, td = triplet of doublets, ddd = doublet of doublets of doublets, m = multiplet), coupling constant (*J* in Hz) and integration. Peak assignments were made with the aid of DEPT and HMBC spectra. The samples for accurate mass analysis (HRMS) were run by electrospray ionization in positive mode on a Waters LCT Premier time of flight mass spectrometer after they were dissolved in ethyl acetate with subsequent dilution into methanol and water (037 with formic acid). High resolution mass spectra (HRMS) were obtained at the UNM Mass Spectrometry Facility, Albuquerque, New Mexico. Compounds **1-3, 5, 6, 8, 9, 13, 18-24, 26-33, 36-40, 43-49, 78-88** were prepared and characterized according to procedures

described by Weber et al. [41-43] Compounds **35, 64, 67, 69, 70, 73-77** are new analogues of curcumin that were not included in our previous studies and were prepared and characterized according to procedures described below. The purity of these compounds was found to be greater than 95% by analysis with high performance liquid chromatography (HPLC). HPLC analysis was performed with an Agilent 1290 Infinity instrument using a Phenomenex Luna 5u C18(2) column, 250 x 4.60 mm, 100 Å, detecting at either 345 nm or 360 nm on a diode-array detector. The solvent gradient employed was 0.1% aqueous H_3PO_4 to 100% acetonitrile over 15 minutes, followed by 5 minutes elution with acetonitrile, at a flow rate of 1 mL/min. Samples were dissolved in acetonitrile as the diluent. Detailed procedures and characterization data are described in the supporting information.

4.4 General Procedure for Synthesis of Compounds 24-47 and 49-77 [47]

To a solution of the aromatic aldehyde (10 mmol, 2 equiv.) dissolved in 10 ml of ethanol was added the appropriate ketone (5 mmol, 1 equiv) such as acetone (compounds **24-47**), cyclohexanone (compounds **49-63**), or N-methylpiperidone (compounds **64-77**) and stirred for 15 minutes. Sodium hydroxide (0.4 g) dissolved in water (10 mL) was added and the resulting mixture was stirred at room temperature overnight. The separated solid was filtered, washed with cold ethanol and recrystallized from ethanol or ethanol/water.

4.4.1. 1,5-Bis((E)-2-ethyl-3,4-dimethoxyphenyl)-pentadien-3-one (**35**): 85% yield, yellow crystals: mp 166-167 °C; IR (ATR): ν_{max} 1613, 1575 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.00 (d, *J* = 15.6 Hz, 2H, Ar-CH=C x 2), 7.46 (d, *J* = 8.7 Hz, 2H, Ar-H^6 x 2), 6.91 (d, *J* = 15.6 Hz, 2H, =CHC(O) x 2), 6.83 (d, *J* = 8.7 Hz, 2H, Ar-H^5 x 2), 3.91 (s, 6H, OCH_3 x 2), 3.84 (s, 6H, OCH_3 x 2), 2.87 (q, *J* = 7.5 Hz, 4H, Ar-CH_2 x 2), 1.19 (t, *J* = 7.5 Hz, 6H, CH_3 x 2); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 188.7 (C=O), 154.4 (ArC^3 x 2), 147.0 (ArC^4 x 2), 140.4 (Ar-CH= x 2), 139.0 (ArC^2 x 2), 126.8 (ArC^1 x 2), 125.5 (=CH x 2), 123.0 (ArC^6H x 2), 110.0 (ArC^5H x 2), 60.9 (OCH_3 x 2), 55.7 (OCH_3 x 2), 19.5 (CH_2 x 2), 15.9 (CH_3 x 2). HRMS (EI) calcd for $C_{25}H_{30}O_5$ $[M+H]^+$: 411.2171; found, 411.2174. HPLC t_R (min): 17.201. P_{HPLC}/%: 95.78.

4.4.2. 3,5-Bis((E)-3-methoxybenzylidene)-1-methylpiperidin-4-one (**64)**: 89% yield, yellow crystals; mp 98°C; IR (ATR): ν_{max} 1603, 1574 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.78 (s, 2H, Ar-CH=C x 2), 7.34 (t, *J* = 7.9 Hz, 2H, Ar-H^5 x 2), 6.99 (d, *J* = 7.8 Hz, 2H, Ar-H^6 x 2), 6.93 (s, 2H, Ar-H^2 x 2), 6.91 (d, *J* = 8.9 Hz, 2H, Ar-H^4 x 2), 3.84 (s, 6H, OCH_3 x 2), 3.76 (s, 4H, CH_2NCH_2), 2.45 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.8 (C=O), 159.5 (ArC^3 x 2), 136.5 (ArC^1 x 2), 136.2 (CH= x 2), 133.4 (=C< x 2), 129.5 (ArC^5H x 2), 122.7 (ArC^6H x 2), 115.9 (ArC^4H x 2), 114.5 (ArC^2H x 2), 57.1 (CH_2NCH_2), 55.3 (OCH_3 x 2), 45.8 (NCH_3). HRMS (EI) calcd for $C_{22}H_{23}NO_3$ $[M+H]^+$: 350.1756; found, 350.1754. HPLC t_R (min): 10.213. P_{HPLC}/%: 96.73.

4.4.3. 3,5-Bis((E)-2,4-dimethoxybenzylidene)-1-methylpiperidin-4-one (**67)**: 77% yield, yellow crystals; mp 131-132 °C; IR (ATR): ν_{max} 1588, 1564 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.04 (s, 2H, Ar-CH=C x 2), 7.14 (d, *J* = 8.4 Hz, 2H, Ar-H^6 x 2), 6.50 (dd, *J* = 8.5, 2.3 Hz, 2H, Ar-H^5 x 2), 6.46 (d, *J* = 2.3 Hz, 2H, Ar-H^3 x 2), 3.83 (s, 6H, OCH_3 x 2), 3.82 (s, 6H, OCH_3 x 2), 3.66 (s, 4H, CH_2NCH_2), 2.40 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.7 (C=O), 161.9 (ArC^4 x 2), 160.0 (ArC^2 x 2), 131.6 (=C< x 2), 131.4 (CH= x 2), 131.2 (ArC^6H x 2), 117.4 (ArC^1 x 2), 104.3 (ArC^5H x 2), 98.2 (ArC^3H x 2), 57.3 (CH_2NCH_2), 55.4 (OCH_3 x 2), 55.3 (OCH_3 x 2), 45.7 (NCH_3). HRMS (EI) calcd for $C_{24}H_{27}NO_5$ $[M+H]^+$: 410.1967; found, 410.1966. HPLC t_R (min): 9.945. P_{HPLC}/%: 99.87.

4.4.4. 3,5-Bis((E)-2,3,4-trimethoxybenzylidene)-1-methylpiperidin-4-one (**69)**: 95% yield, yellow crystals; mp 192 °C; IR (ATR): ν_{max} 1593, 1577 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.00 (s, 2H, Ar-CH=C x 2), 6.95 (d, *J* = 8.7 Hz, 2H, Ar-H^6 x 2), 6.70 (d, *J* = 8.7 Hz, 2H, Ar-H^5 x 2), 3.90 (s, 6H, OCH_3 x 2), 3.89 (s, 6H, OCH_3 x 2), 3.88 (s, 6H, OCH_3 x 2), 3.68 (s, 4H, CH_2NCH_2), 2.42 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.5 (C=O), 154.8 (ArC^4 x 2), 153.7 (ArC^2 x 2), 142.4 (ArC^3 x 2), 132.3 (=C< x 2), 131.8 (CH= x 2), 125.2 (ArC^6H x 2), 122.5 (ArC^1 x 2), 106.9 (ArC^5H x 2), 61.7 (OCH_3 x 2), 61.0 (OCH_3 x 2), 57.4 (CH_2NCH_2), 56.0 (OCH_3 x 2), 45.7 (NCH_3). HRMS (EI) calcd for $C_{26}H_{31}NO_7$ $[M+H]^+$: 470.2179; found, 470.2181. HPLC t_R (min): 9.642. P_{HPLC}/%: 99.87.

4.4.5. 3,5-Bis((E)-3,5-dimethoxybenzylidene)-1-methylpiperidin-4-one (**70)**: 86% yield, yellow crystals; mp 143-144 °C; IR (ATR): ν_{max} 1619, 1588 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.72 (s, 2H, Ar-CH=C x 2), 6.53 (d, *J* = 2.2 Hz, 4H, Ar-H^2 x 2, Ar-H^6 x 2), 6.48 (d, *J* = 2.2 Hz, 2H, Ar-H^4 x 2), 3.82 (s, 12H, OCH_3 x 4), 3.75 (s, 4H, CH_2NCH_2), 2.44 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.8 (C=O), 160.7 (ArC^3 x 2, ArC^5 x 2), 137.0 (ArC^1 x 2), 136.4 (CH= x 2), 133.6 (=C< x 2), 108.4 (ArC^2H x 2, ArC^6H x 2), 101.0 (ArC^4H x 2), 57.0 (CH_2NCH_2), 55.4 (OCH_3 x 4), 45.8 (NCH_3). HRMS (EI) calcd for $C_{24}H_{27}NO_5$ $[M+H]^+$: 410.1967; found, 410.1972. HPLC t_R (min): 10.483. P_{HPLC}/%: 99.91.

4.4.6. 3,5-Bis((E)-3-trifluoromethylbenzylidene)-1-methylpiperidin-4-one (**73)**: 80% yield, yellow crystals; mp 142-143 °C; IR (ATR): ν_{max} 1617, 1592 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.82 (s, 2H, Ar-CH=C x 2), 7.63 (m, 4H, Ar-H^6 x 2, Ar-H^2 x 2), 7.56 (m, 4H, Ar-H^5 x 2, Ar-H^4 x 2), 3.75 (d, *J* = 1.4 Hz, 4H, CH_2NCH_2), 2.47 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.3 (C=O), 135.9 (ArC^1 x 2), 134.9 (CH= x 2), 134.4 (=C< x 2), 133.3 (ArC^6H x 2), 131.2 (q, *J* = 32.5 Hz, ArC^3 x 2), 129.2 (ArC^5H x 2), 126.9 (d, *J* = 3.6 Hz, ArC^2H x 2), 125.6 (d, *J* = 3.3 Hz, ArC^4H x 2), 120.3 (d, *J* = 272.4 Hz, CF_3 x 2), 56.8 (CH_2NCH_2), 45.8 (NCH_3). HRMS (EI)

calcd for $C_{22}H_{17}F_6NO$ $[M+H]^+$: 426.1293; found, 426.1295. HPLC t_R (min): 11.245. P_{HPLC}/%: 100.

4.4.7. 3,5-Bis((E)-4-isopropylbenzylidene)-1-methylpiperidin-4-one (**74)**: 93% yield, yellow crystals; mp 98-100 °C; IR (ATR): ν_{max} 1599, 1581, 1557 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.80 (s, 2H, Ar-CH=C x 2), 7.35 (d, *J* = 8.2 Hz, 4H, Ar-H^2 x 2, Ar-H^6 x 2), 7.28 (d, *J* = 8.2 Hz, 4H, Ar-H^3 x 2, Ar-H^5 x 2), 3.78 (s, 4H, CH_2NCH_2), 2.94 (sept, *J* = 6.9 Hz, 2H, CH x 2), 2.46 (s, 3H, NCH_3), 1.27 (d, *J* = 6.9 Hz, 12H, CH_3 x 4); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.9 (C=O), 150.1 (ArC^4 x 2), 136.3 (CH= x 2), 132.9 (=C< x 2), 132.5 (ArC^1 x 2), 130.6 (ArC^2H x 2, ArC^6H x 2), 126.7 (ArC^3H x 2, ArC^5H x 2), 57.2 (CH_2NCH_2), 45.8 (NCH_3), 34.0 (CH x 2), 23.8 (CH_3 x 4). HRMS (EI) calcd for $C_{26}H_{31}NO$ $[M+H]^+$: 374.2484; found, 374.2481. HPLC t_R (min): 11.522. P_{HPLC}/%: 97.04.

4.4.8. 3,5-Bis((E)-4-methylthiobenzylidene)-1-methylpiperidin-4-one (**75)**: 99% yield, yellow crystals; mp 122-123 °C; IR (ATR): ν_{max} 1573 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.75 (s, 2H, Ar-CH=C x 2), 7.33 (d, *J* = 8.5 Hz, 4H, Ar-H^3 x 2, Ar-H^5 x 2), 7.26 (d, *J* = 8.5 Hz, 4H, Ar-H^2 x 2, Ar-H^6 x 2), 3.75 (s, 4H, CH_2NCH_2), 2.51 (s, 6H, SCH_3 x 2), 2.47 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.6 (C=O), 140.6 (ArC^4 x 2), 135.7 (CH= x 2), 132.5 (=C< x 2), 131.7 (ArC^1 x 2), 130.9 (ArC^2H x 2, ArC^6H x 2), 125.7 (ArC^3H x 2, ArC^5H x 2), 57.1 (CH_2NCH_2), 45.9 (NCH_3), 15.1 (SCH_3 x 2). HRMS (EI) calcd for $C_{22}H_{23}NOS_2$ $[M+H]^+$: 382.1299; found, 382.1292. HPLC t_R (min): 11.008. P_{HPLC}/%: 99.01.

4.4.9. 3,5-Bis((E)-4-ethoxybenzylidene)-1-methylpiperidin-4-one (**76)**: 84% yield, yellow crystals; mp 162-163 °C; IR (ATR): ν_{max} 1601, 1581 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.76 (s, 2H, Ar-CH=C x 2), 7.35 (d, *J* = 8.7 Hz, 4H, Ar-H^2 x 2, Ar-H^6 x 2), 6.92 (d, *J* = 8.7 Hz, 4H, Ar-H^3 x 2, Ar-H^5 x 2), 4.07 (q, *J* = 6.9 Hz, 4H, OCH_2 x 2), 3.76 (s, 4H, CH_2NCH_2), 2.48 (s, 3H, NCH_3), 1.43 (t, *J* = 7.1 Hz, 6H, CH_3 x 2); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 186.7 (C=O), 159.6 (ArC^4 x 2), 135.9 (CH= x 2), 132.3 (ArC^2H x 2, ArC^6H x 2), 131.3 (=C< x 2), 127.8 (ArC^1 x 2), 114.5 (ArC^3H x 2, ArC^5H x 2)), 63.5 (OCH_2 x 2), 57.2 (CH_2NCH_2), 45.9 (NCH_3), 14.7 (CH_3 x 2). HRMS (EI) calcd for $C_{24}H_{27}NO_5$ $[M+H]^+$: 378.2069; found, 378.2069. HPLC t_R (min): 10.704. P_{HPLC}/%: 100.

4.4.10. 3,5-Bis((E)-3-methylbenzylidene)-1-methylpiperidin-4-one (**77)**: 88% yield, yellow crystals; mp 79-80 °C; IR (ATR): ν_{max} 1601, 1582 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.79 (s, 2H, Ar-CH=C x 2), 7.31 (t, *J* = 7.6 Hz, 2H, Ar-H^5 x 2), 7.21 (s, 2H, Ar-H^2 x 2), 7.20 (d, J = 8.7 Hz, 2H, Ar-H^6 x 2), 7.18 (d, J = 8.3 Hz, 2H, Ar-H^4 x 2), 3.76 (s, 4H, CH_2NCH_2), 2.46 (s, 3H, NCH_3), 2.39 (s, 6H, CH_3 x 2); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 186.8 (C=O), 138.1 (ArC^3 x 2), 136.5 (CH= x 2), 135.2 (ArC^1 x 2), 133.0 (=C< x 2), 131.2 (ArC^5H x 2), 129.8 (ArC^4H x 2), 128.4 (ArC^2H x 2), 127.3 (ArC^6H x 2), 57.0 (CH_2NCH_2), 45.7 (NCH_3), 21.4 (CH_3 x 2). HRMS (EI) calcd for $C_{22}H_{23}NO$ $[M+H]^+$: 318.1858; found, 318.1861. HPLC t_R (min): 10.563. P_{HPLC}/%: 99.05.

Acknowledgments

This work was supported in part by grant BC043125 from the U.S. Army/DOD Breast Cancer Program. HRMS data were obtained by Ken Sherrell at the UNM Mass Spectrometry Facility, Albuquerque, New Mexico**.**

Appendix A. Supporting Information

General synthetic procedures for compounds **1-23, 48, 78-88** and characterization data for compounds **4, 7, 10-12, 14-17, 25, 34, 41, 42, 50-63, 65, 66, 68, 71, 72** can be found at

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Highlights for “Activation of Anti-Oxidant Nrf2 Signaling by Enone Analogues of Curcumin,” EJMECH-S-17-02421-2

- Transcription factor Nrf2 regulates anti-oxidant genes and is a potential drug target.
- The natural product curcumin is an inducer of Nrf2-dependent anti-oxidant enzymes.
- Enone analogues of curcumin were examined with an Nrf2 reporter assay.
- Numerous enone activators of Nrf2 were found that are more active than curcumin.
- Both the sensitivity of Nrf2 to activation by enones and the maximum activation vary.