Diarylidene-N-Methyl-4-Piperidone and Spirobibenzopyran Curcumin Analogues as Antioxidant and Anti-Inflammatory Pharmacophores

R. Siva Kumar,[1,#] Javier A. Mas-Rosario,[2,#] Bishnu P. Joshi, [3] Mayank Joshi,[4] Angshuman Roy Choudhury,[4] Swayamsiddha Kar,[1] Nageswara Rao G,[1,*] and Michelle E. Farkas[2,3,*]

[#] *Denotes equal contributions*
[1]*Department of Chemistry, Sri Sathya Sai Institute of Higher Learning, Puttaparthi 515134(AP), India.*
[2]*Department of Chemistry, University of Massachusetts Amherst, 710 N. Pleasant St., Amherst, MA 01003, USA*
[3]*Molecular & Cellular Biology Graduate Program, University of Massachusetts Amherst, 230 Stockbridge Rd, Amherst, MA 01003, USA*
[4]*Department of Chemical Sciences, Indian Institute of Science Education and Research, Mohali, Sector 81, S. A. S. Nagar, Knowledge City, Manauli P. O. Mohali, Punjab, 140306, India.*
[*]*Authors for correspondence; email addresses: gnageswararao@sssihl.edu.in; farkas@chem.umass.edu*

Abstract

There is a significant need for new small molecule anti-inflammatory compounds. Curcumin, a small molecule natural product from the Turmeric (*Curcuma longa*) plant, has well-known anti-oxidant properties, resulting from its radical scavenging ability and inhibition of inflammation-associated factors. However, its lack of solubility, instability, and poor bioavailability and biodistribution characteristics are an impediment to its use. To circumvent these issues while retaining curcumin's biological activity, we synthesized twenty-one diarylidene-N-methyl-4-piperidones (DANMPs), four diheteroarylidene-N-methyl-4-piperidones (DHANMPs), and five spirobibenzopyran (SBP) derivatives. All were screened in terms of anti-oxidant activity via a cell-free 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and for drug-like properties *in silico*. In the former, some compounds possessed improved radical scavenging behavior versus ascorbic acid, which was used as a benchmark. Conformity to simulated Lipinski's parameters and Absorption, Distribution, Metabolism, and Excretion (ADME) studies indicated the DANMPs, DHANMPs, and SBPs to be potentially useful compounds. A subset of molecules was investigated in terms of their aqueous solubilities, which were significantly improved compared to that of curcumin. *In vitro* assessments of the cellular and anti-inflammatory effects of these compounds were conducted using RAW 264.7 macrophages. RT-PCR and Griess assays were used to evaluate the presence of inflammatory/activated (M1) markers and production of nitric oxide (NO) species,

which are associated with inflammation, respectively. While the compounds did not affect non-stimulated (naïve) macrophages, they did reduce levels of markers and NO to extents similar to or better than curcumin in inflamed cells. Our results indicate that these pharmacophores possess anti-inflammatory properties and can be used as curcumin-substitutes with improved characteristics. Further investigation into their mechanisms of action and potential use in the treatment of inflammatory diseases is merited.

Introduction

Inflammation is the first line of defense in the body. It is the immune system's response to harmful stimuli, such as pathogens, damaged cells, or toxic compounds, and is responsible for mitigating threats and initiating the healing process.[1] When immune cells sense damaged tissues or pathogens, they signal and recruit more immune cells, such as macrophages and T-cells, by releasing various cytokines and chemokines.[2,3] Macrophages are typically activated to a pro-inflammatory (M1) sub-type, which can be replicated *in vitro* by treatment with lipopolysaccharide (LPS) and interferon-gamma (IFNγ). On the other hand, anti-inflammatory (M2) macrophages aid in the resolution of inflammation, generated *in vitro* by treatment with interleukin 4 (IL-4).[4,5] Unfortunately, there are congenital conditions and diseases that force these immune cells to generate too much inflammation (auto-immune diseases) or a lack thereof (immuno-compromised conditions).[6-10]

Prolonged inflammation is a hallmark of chronic diseases including rheumatoid arthritis, atherosclerosis, asthma, cystic fibrosis, and cancer.[1,6,11] Pro-inflammatory stimuli (inflammation-causing stimuli) can be generated externally, including via pathogens, allergens, or stress, or internally, such as via free radical species, interleukin 1β (IL-1β), tumor necrosis factor-alpha (TNF-α), or arachidonic acid (AA).[4,7,12,13] Of particular interest, AA is a polyunsaturated fatty acid covalently bound in esterified form to the cell membranes of most cells in the body. During

inflammation, AA is released and oxygenated by enzymes, leading to the formation of an important group of inflammatory mediators, the eicosanoids, which include prostaglandins and leukotrienes. Prostaglandins and other prostanoids are generated from AA via the cyclooxygenase (COX) enzyme, and have potent inflammatory properties.[13,14] On the other hand, lipoxygenase (LOX) metabolizes arachidonic acid to a group of non-cyclized eicosanoids, the leukotrienes, some of which are also important inflammatory mediators.[13]

When auto-immune diseases like asthma and inflammatory bowel disease occur (leading to the release of AA, COX, LOX, prostaglandins, and leukotrienes), resolution of inflammation, represented by downregulation of TNF-α, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX), is desirable.[15,16] Toward this goal, nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used, with more than 30 million daily consumers.[17-19] Based on data collected from patients in the Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS), concerns associated with NSAIDs include that they are palliative, and can result in serious side-effects including gastrointestinal and renal complications.[20] Thus, there remains a need for efficacious anti-inflammatory lead compounds with fewer side effects. Among natural products having antioxidant and anti-inflammatory activities, phenolics appear to be promising.[21-32]

Well-known targets of polyphenols and the sources of their anti-inflammatory activities involve arachidonic acid-dependent pathways, including the inhibition of COX, LOX, and phospholipase A_2 (PLA_2).[21,24,26,29,31,33] Curcuminoids and their structurally-related metabolic degradation products, like ferulic acid, share a basic pharmacophore with phenolic acids by possessing a base phenylpropanoid (C6-C3) structure (**Supporting Information Figure S1**).[22,27,29] These metabolites act on the metabolism of arachidonic acid by inhibiting LOX and COX

enzymes.[21,24,26,31] It has also been shown that curcumin has the ability to prevent macrophage polarization towards a pro-inflammatory phenotype through toll-like receptor 4 (TLR4)-mediated signaling pathway inhibition.[34] More specifically, curcumin inhibits nuclear factor-κB (NF-κB), 5-LOX, and COX-2,[35,36] which results in decreased expression of the inflammation marker iNOS and decreased production of pro-inflammatory cytokines such as nitric oxide (NO), TNF-α, IL-1β, prostaglandin E_2 (PGE_2), and interleukin 6 (IL-6) in macrophages stimulated with LPS, a known TLR-4 agonist (**Supporting Information Figure S2**).[37] Curcumin's antioxidant capabilities and its ability to inhibit LOX and COX, and affect pro-inflammatory phenotypes in macrophages, make it an ideal candidate to treat chronic inflammation and auto-immune diseases.

Unfortunately, Curcumin's poor *in vivo* bioavailability and biodistribution in phase I clinical trials has prevented its development as an effective therapeutic.[38,39] Curcumin's hydrophobic nature leads to poor absorption in oral administration, and its instability and rapid metabolism result in low serum bio-availability and poor tissue distribution when administered systemically.[40,41] Attempts to deliver curcumin using drug delivery systems, such as liposomes and nanoparticles, have not been very promising due to issues associated with solubility, bioavailability, and bio-degradability.[42,43]

Curcuminoid metabolites possess a diarylheptanoid base structure consisting of symmetric or asymmetric combination of C_6-C_3 phenolic moieties (e.g., ferulic acid and vanillin), which are responsible for free radical scavenging, anti-oxidant, and anti-inflammatory properties.[29,44] Free radical scavengers, such as antioxidants, protect cells from injury caused by free radicals, which are unstable, reactive entities that can damage biological molecules whose presence can increase the risk of cancer and other diseases.[45,46]

The biological activities of curcumin and related structures are associated with the presence of unsaturated α,β-ketones.[44] However, the di-carbonyl moiety is conformationally unstable due to keto-enol tautomerism mediated by pH, heat, and light.[47,48] To address issues of stability and bio-availability, based on our earlier synthetic work on diarylidenecycloalkanone derivatives as new antiplasmodial pharmacophores,[48,49] we now report the syntheses and investigation of the antioxidant and anti-inflammatory properties of symmetric diarylidene- and diheteroarylidene-N-methyl-4-piperidone derivatives (**Supporting Information Figures S1, S3-S5**).

These symmetric and robust structures facilitate the SAR studies presented here. We have generated molecules bearing electron-donating or –withdrawing substituents to assess antioxidant and anti-inflammatory activities. We hypothesize that by having suitable substituents on the two aryl rings, free radical scavenging will be improved, thereby achieving potent anti-inflammatory activity. In addition, we synthesized and evaluated spirobibenzopyrans derived from mono carbonyl analogues of curcumin (i.e., diarylidenealkanones; (**Supporting Information Figures S1** and **S6**)). These molecules offer structural novelty; to date, there have been no reports investigating anti-inflammatory properties of these compounds, although our group has previously reported spirobibenzopyran molecules with potent anti-cancer properties.[50]

In this study, thirty curcumin derivatives were synthesized of which five were selected for further assessments based on *in silico* evaluations and DPPH assays. The macrophage model cell line RAW264.7 was chosen to test the anti-inflammatory properties of these compounds (Compounds **1**, **3**, **13**, **26**, and **27**). Results from RT-PCR and Griess assay studies indicate that the selected curcumin-derived small molecules were able to decrease the levels of inflammatory

responses in activated macrophages stimulated with LPS and IFNγ. Based on our findings, further studies of these molecules should be undertaken, including in models of disease.

Materials and Methods

Reagents. All chemicals used in syntheses, including aldehydes, N-methyl piperidone, and 3-pentanone were obtained from Merck chemicals, HiMedia, or Sigma-Aldrich. Curcumin was obtained from Sigma-Aldrich. All solvents used were spectral grade or distilled before use. General methods for the four synthetic approaches are provided here. Detailed procedures and complete chemical characterization are included in the supporting information.

General method for the alkali-catalyzed preparation of DANMPs (1-16) and DHANMPs (22-25). To a 1:1 mixture of ethanol-water solution placed in an ice bath, NaOH (0.05 mol) was added and the solution was stirred for 10 minutes. To this, half of a pre-prepared ethanolic mixture of N-methyl piperidone (0.01 mol) and aldehyde (0.02 mol) was added at room temperature and stirred for another 30 minutes. Subsequently the other half of the mixture was added and stirred. The entire process was completed under nitrogen atmosphere. Reaction progress was monitored by TLC and after completion, ice-cold water was added. The product precipitated and the resulting solid was filtered, and washed thoroughly with ice-cold water for removal of alkali. The crude solid was then dried and recrystallized.

General method for the acid-catalyzed preparation of DANMPs (17-21). For the generation of compounds **17**-**21**, 10-40 mL acetic acid (CH_3COOH) was saturated with HCl gas by using Kipp's

apparatus. The acetic acid-hydrochloric acid mixture was cooled to 15 °C and the corresponding aldehyde (0.02 mol) was dissolved in it. To the resulting solution, N-methyl-piperidone (0.01 mol) was added and stirred for 10 minutes, following which the reaction was allowed to warm to room temperature. All reactions were performed under nitrogen atmosphere, and reaction progress was monitored by thin layer chromatography (TLC). After completion of the reaction, 10% aqueous NaOH was added to the reaction mixture to result in neutral pH. The product precipitated and the resulting solid was filtered and washed with ice cold water. The crude solid was purified by recrystallization.

General procedure for the preparation of spirodibenzopyrans (SBPs) (26-30). 3-pentanone (0.01 mol), substituted salicylaldehyde (0.02 mol), and $SiCl_4$ (silicon tetrachloride, 0.03 mol) were added to absolute ethanol (20 mL) at ambient temperature and the mixture was stirred under nitrogen atmosphere. Formation of solid in the reaction vessel indicated completion of the reaction. The contents were poured onto ice and stirred for approximately 15 minutes to quench unreacted $SiCl_4$. The solid obtained was filtered and washed with water, then dried and recrystallized from distilled chloroform.

Water solubility studies. 1 mg of each compound was added to a 500 mL conical flask. Water at ambient temperature was then added with continuous stirring until the compounds dissolved to obtain saturated solutions. The volumes of water required for dissolution of each compound are as follows: **1** - 100 mL, **3** - 215 mL, **13** - 180 mL, **26** - 180 mL, **27** - 100 mL, and curcumin - 500 mL. Five standard solutions were prepared by serial dilution (dilution factor of 1.111) from each of these stocks. The optical density of the standard solutions was recorded at 328 nm for **1**, 322 nm for **3**, 301 nm for **13**, 281 nm for **26**, 296 nm for **27**, and 425 nm for curcumin using a Shimadzu

2450 spectrophotometer to generate standard curves. The aforementioned saturated solutions of the compounds were diluted to half of their concentration by taking an equal volume of water, and optical density was recorded. Then the solubility of the compounds in water was calculated from the obtained standard curves.[51]

DPPH assay. Antioxidant activities of the compounds were determined using a stable free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay.[51,52] Briefly, 0.15 mM DPPH was dissolved in 20 mL methanol and separately, 10 μM of each test compound was prepared in 5 mL of DMSO. Then, the DPPH solution was added to the sample in a 2:1 ratio (160 μL DPPH and 80 μL sample). The mixture was kept in the dark for 30 min at ambient temperature. The absorbance of the solutions was then measured at 517 nm via a Varioskan Lux Multimode Microplate Reader (Thermo Scientific). Ascorbic acid (in methanol) was used as a positive control, and prepared similarly. DPPH radical scavenging activity was determined using the following equation: % Inhibition = $(A_c\text{-}A_s) / A_c \times 100$, where A_c = Absorbance of the control and A_s = Absorbance of the test samples.

Cell culture. RAW 264.7 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured at 37 °C under a humidified atmosphere containing 5% CO_2. Standard growth media consisted of high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin). Cells were sub-cultured approximately once every four days and only cells between passages 7 and 15 were used for all experiments.

RT-PCR Assay. RAW 264.7 cells were plated in 24-well plates at a density of 100,000 cells/well in 500 μL of growth media. For experiments involving compound-treated pro-inflammatory cells (IFN-γ/LPS), designated cells were then treated with 50 ng/mL each of IFN-γ and LPS for 24 h. Then, molecules were dissolved in DMSO to give 10 mM stock solutions, which were further diluted in media to give 10 μM with a final DMSO concentration of 0.4% per sample. Media was aspirated from the cells and replaced with media containing designated compounds at 10 μM for 48 hours. Each experiment included three biological replicates per treatment condition. Approximately 1.5 μg RNA was directly harvested from cells using the PureLink RNA Mini Kit (Ambion) following the manufacturer's instructions. SuperScript IV Reverse Transcriptase, RNaseOut, 10mM dNTPs, and 50 μM Random Hexamers were used for the conversion of approximately 150 ng of RNA to cDNA (ThermoFisher), also following the manufacturer's instructions with a sample volume of 20 μL. Briefly, primers were annealed to RNA at 65 °C for 5 min. Then the annealed RNA was combined with the reaction mixture (containing 500 units of reverse transcriptase per sample) and amplified at 53 °C for 10 min and melted at 80 °C for 10 min. cDNA was stored at -20 °C and used for RT-PCR within 1 week. RNA and cDNA were quantified and evaluated using a NanoDrop 2000 spectrophotometer (ThermoFisher). RT-PCR was performed using a CFX Connect real-time system (Biorad) with iTaq Universal SYBR Green Supermix (Biorad). All DNA primers were purchased from Integrated DNA Technologies. The following primer sequences were used: β-actin (forward) 5'-GATCAGCAAGCAGGAGTACGA-3', (reverse) 5'-AAAACGCAGCGCAGTAACAGT-3'; iNOS (forward) 5'-GTTCTCAGCCCA-ACAATACAAGA-3', (reverse) 5'-GTGGACGGGTCGATGT CAC-3'. 200 nM of forward and reverse primers (1 gene per reaction) was mixed with 1 μL (100 ng) of cDNA, 10 μL SYBR Green and H_2O to a final volume of 20 μL. The thermocycler protocol was as follows: the samples were

first activated at 50 °C for 2 min, then 95 °C for 2 min; then denaturing occurred at 95 °C for 30 s followed by annealing at 57 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the Ct value of the gene of interest to that of β-actin control, by the $2^{\Delta\Delta Ct}$ method.[53] Three biological replicates were performed for each treatment condition and three technical replicates were used for each biological replicate. Data was analyzed using CFX Manager 3.1 software. CQ values were generated by using the point at which the sample fluorescence value exceeded the software's default threshold value.

Griess assay. RAW 264.7 cells were plated in phenol red-free DMEM containing 10% FBS and 1% antibiotics in 24-well plates at a density of 1.5×10^5 cells/well 24 h prior to the experiment. Cells were treated as described in the RT-PCR protocol. Then, cell supernatant was collected from the wells and centrifuged at 5000 rpm for 5 min. Griess reagent (Invitrogen) was freshly prepared according to the manufacturer's instructions. 60 μL Griess reagent was combined with 60 μL cell supernatant in a clear 96-well plate. After incubating for 15 min in the dark, absorbance was read using a SpectraMax ID3 plate reader (Molecular Devices) at 548nm. Three biological replicates were performed per treatment condition. Actual $NO2^-$ concentrations were determined by comparing absorbance values to a standard curve generated using $NaNO_2$ solutions.

Results and Discussion

Synthesis and Characterization of Curcumin Analogues. Curcumin, the principal component of Turmeric (*Curcuma longa*), has been extensively studied due to its anti-inflammatory, antioxidant, and radical scavenging abilities, which can assist in the prevention and treatment of some inflammatory diseases.[34] Unfortunately, curcumin possesses characteristics that result in its

poor bioavailability and biodistribution *in vivo*,[40,41] which is an obstacle for drug delivery. To harness curcumin's therapeutic qualities while circumventing its negative aspects, we synthesized three series of curcumin derivatives for further study. A total of thirty compounds, twenty-one diarylidene-N-methyl-4-piperidones (DANMPs; **1-21**), four diheteroarylidene-N-methyl-4-piperidones (DHANMPs; **22-25**), and five spirobibenzopyrans (SBPs; **26-30**) were synthesized (**Figure 1**, **Supporting Information Figures S3-S6**). The compounds were purified by re-crystallization and characterized using UV-Vis, FT-IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry; purity was assessed via HPLC. Complete details of syntheses and characterizations are provided in **Supporting Information**. As a representative model, one of the compounds (**22**) was further characterized by single crystal XRD (**Supporting Information Figure S7**, **Table S1**).

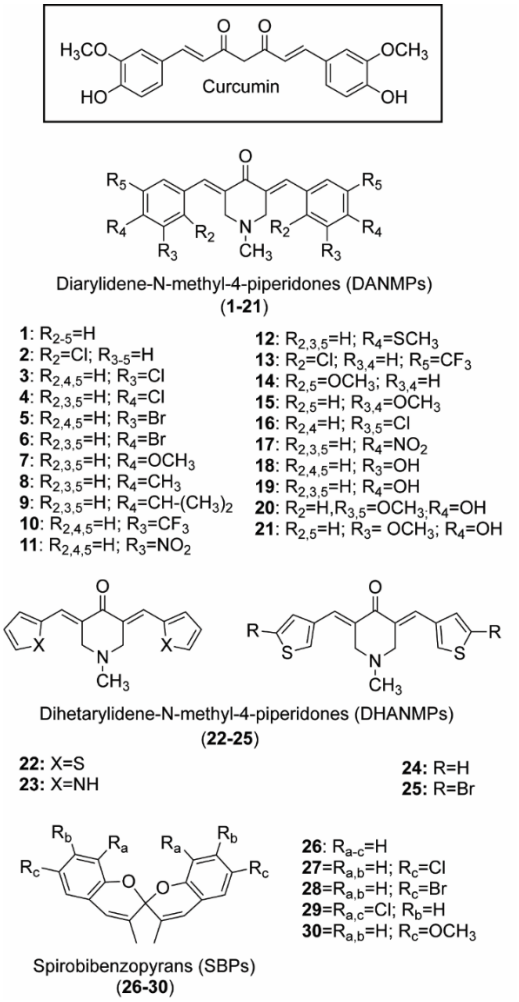

Figure 1: Molecular structure of curcumin, and three classes of derivatives generated: diarylidene-N-methyl-4-piperidones (DANMPs; **1-21**), diheteroarylidene-N-methyl-4-piperidones (DHANMPs; **22-25**), and spirobibenzopyrans (SBPs; **26-30**).

***In Silico* Evaluations of Compounds for Drug-Like Properties.** To assess the drug-like characteristics and potential pharmacokinetic properties of the DANMP, DHANMP, and SBP compounds, computational analyses were performed (methods described in **Supporting Information**). We found that the majority of the structures are in accordance with Lipinski's rule of five (**Supporting Information Table S2**). Specifically, the molecular weights (MW) were under 500 Daltons (the range was 267 to 494), there were fewer than five hydrogen bond donors (HBD; the range was zero to two), there were fewer than ten hydrogen bond acceptors (HBA; the

range was two to eight), and molar refractivity (MR) was within the desired range of 40-130 (with our compounds having MR from 77.14 to 120.87. The partition coefficients (Log P) of the compounds were in the range 2.32 to 6.19. The only compounds that were outside of the desired range of -0.4 to +5.6 were **9**, **10**, and **28**, whose LogP values slightly exceeded this, with 5.91, 5.78, and 6.19, respectively. It is important to note that curcumin itself has a LogP value of -0.053; the lowest value in our series was analyzed to be 2.32 (for **23**). The results of *in silico* ADME predictions showed that most of the compounds should have reasonable pharmacokinetic characteristics (**Supporting Information Table S3**). Most of the compounds have very good bioavailability scores, moderate to good water solubilities (Log S), and high gastrointestinal (GI) absorptivities and Blood Brain Barrier (BBB) permeabilities. Furthermore, skin permeability (Log Kp) values were also within acceptable limits. Overall, the computationally determined properties of the compounds supported their potential as useful anti-inflammatory agents.

DPPH assay. To evaluate the radical scavenging activity of the curcumin-related compounds, a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was performed (**Figure 2**, **Supporting Information Table S4**). This method facilitates the evaluation of the antioxidant potential of a compound by exposing it to DPPH, which is a violet, stable free radical solution that loses its color upon gain of a hydrogen atom.[54] In this assay, ascorbic acid (a.a.) was used as a positive control and benchmark for the new molecules. The majority of the compounds were found to have activity similar to that of a.a, with only one (**13**, which contained R = 2-Cl-5-CF_3) being significantly better ($p<0.05$). Among the DANMPs, only **12** (R = 4-SCH_3) and **20** (R = 3,5-di-OCH_3, 4-OH) were significantly worse ($p>0.05$) than a.a. Overall, most of the best-performing DANMPs contained electron-withdrawing substituents, likely due to improved stabilization of the

added electron. Among the DHANMPs and the SBPs, all showed significantly reduced radical scavenging activity versus a.a. All DHANMPs had similar effects to one another with no statistical difference among them, while among the five spiro derivatives, **26** (R = H) and **27** (R = 5-Cl) showed better scavenging activities than the others. Statistical comparison of the DHANMPs showed that there was no significant difference between compounds **26** and **27**, or among compounds **26**, **28**, **29**, and **30**. Compound **27** was, however, statistically different ($p<0.001$) when compared to compounds **28**, **29**, and **30**; there was no statistical difference found among **28**, **29**, and **30**.

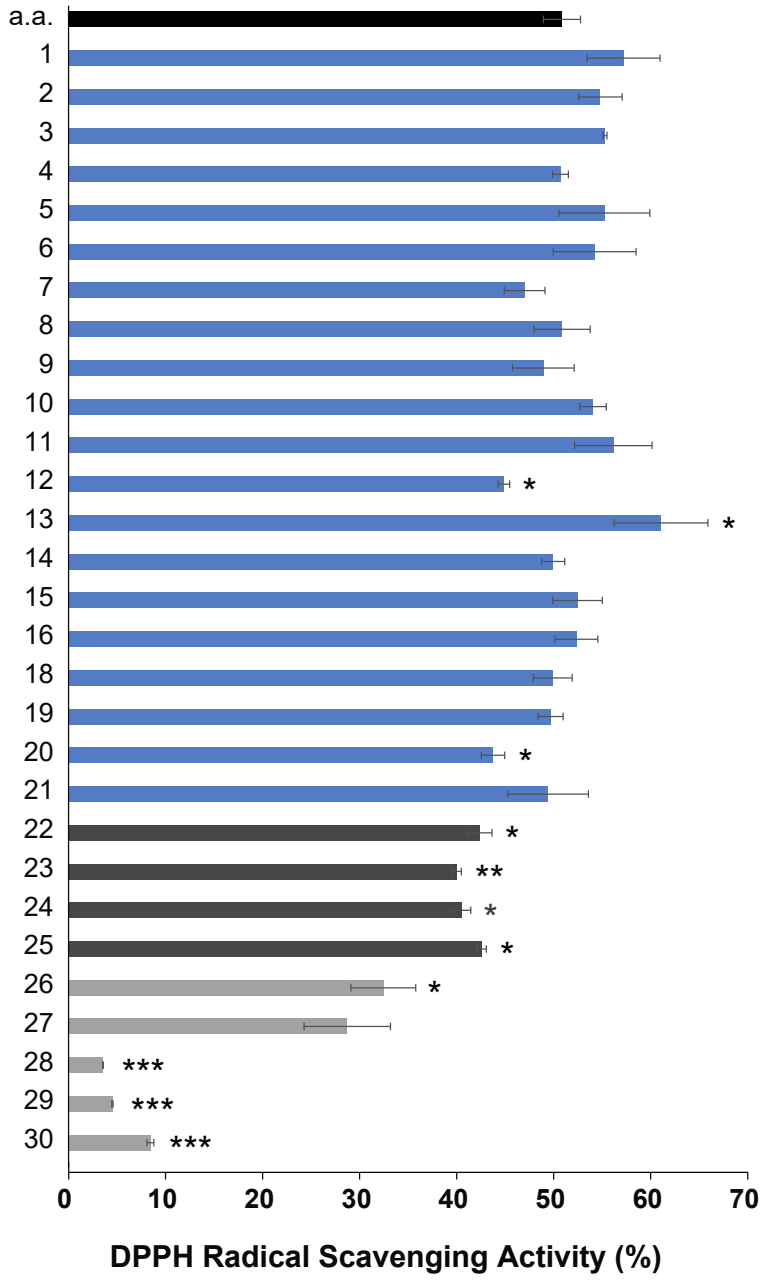

Figure 2: DPPH radical scavenging activity of curcumin derivatives. Thirty synthesized molecules were evaluated and benchmarked against ascorbic acid (a.a.), which was used as a positive control. All compounds were dosed at the same concentration, 10 μM. Error bars represent standard error. Student T-test was performed to compare each treatment to ascorbic acid. ($p > 0.05$ = not significant (not marked), $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = ***).

Solubility Studies. From the 30 synthesized derivatives, we selected five compounds for further evaluation based on a combination of results from computational assessments (**Supporting Information Tables S2** and **S3**), DPPH radical scavenging activity (**Figure 2** and **Supporting Information Table S4**), and structural diversity. Compounds **1**, **3**, **13**, **26**, and **27** were used in additional studies. As described above, a major challenge preventing the use of curcumin is its lack of bioavailability. In many instances low bioavailability and biodistribution may attributed to poor aqueous solubility resulting in low permeability.[55] To address whether the newly generated compounds had improved solubilities relative to curcumin, we employed UV absorption spectroscopy. As compared to the water solubility of curcumin (cited in the literature as 0.0006 mg/mL),[56] experimentally determined by us as 0.00056 mg/mL, all of the tested compounds are substantially improved in this regard (**Table 1**). Strikingly, compounds **1** and **27** showed the greatest solubility (both 0.01 mg/mL), while **3**, **13**, and **26** were all similar (0.00477, 0.00652, and 0.00693 mg/mL, respectively).

Table 1: Water solubilities of curcumin versus derivatives.

Compound	Water solubility (mg/mL)
Curcumin	0.00060 (lit); 0.00056 (det)
1	0.01
3	0.00477
13	0.00652
26	0.00693
27	0.01

Lit = literature cited; det = experimentally determined

Cell-Based Evaluations of Anti-Inflammatory Activity. For cell-based *in vitro* studies of anti-inflammatory activity of the compounds, the commonly employed RAW 264.7 macrophage cell line was used.[57,58] Prior to additional *in vitro* studies, we determined whether there were any effects of the five selected curcumin derivatives on RAW264.7 cell viability (**Supporting Information**

Figure S8). None of the compounds resulted in dramatic changes. Curcumin has the ability to inhibit COX, LOX, and NF-κB, which in turn reduces the expression of inducible nitric oxide synthase (*iNos*) and the resulting secretion of nitric oxide (NO).[59,60] At the same time, enhanced levels of *iNos* and NO are also markers of inflammation. Therefore, we elected to track *iNos* and NO to assess the activities of the curcumin derivatives. To monitor the effects of the selected compounds on *iNos* expression, Real Time Polymerase Chain Reaction (RT-PCR) was used.

To assess the abilities of the compounds to resolve inflammation, RAW 264.7 cells were first treated with interferon gamma (IFN-γ) and lipopolysaccharide (LPS) to achieve a pro-inflammatory (M1) phenotype. Macrophages were treated with curcumin and molecules **1**, **3**, **13**, **26**, and **27** to assess and compare their abilities to reduce *iNos* expression relative to a non-treated (N.T.), M1-polarized control group (**Figure 3**). Both curcumin and all five derivatives resulted in dramatically decreased levels of *iNos* expression, within a ten-fold range of levels present in naïve (non-polarized) macrophages. Among the five compounds, compound **1** was the only one that showed a significant decrease in expression ($p<0.01$) relative to the curcumin-treated group, while compound **13** was not statistically different from the curcumin-treated group. Compounds **3**, **26**, and **27**, however, showed slight statistically significant increases ($p<0.05$). Treatment of naïve (non-polarized) RAW264.7 macrophages with curcumin and the derivative molecules **1**, **3**, **13**, **26**, and **27** did not reduce *iNos* below basal levels, but rather slightly increased it (**Supporting Information Figure S9**). In this instance, statistical analyses comparing curcumin's effect on the RAW 264.7 cells to the other compounds show no significant change between curcumin and compounds **1**, **3**, and **13**, but increases ($p<0.001$) for compounds **26** and **27**.

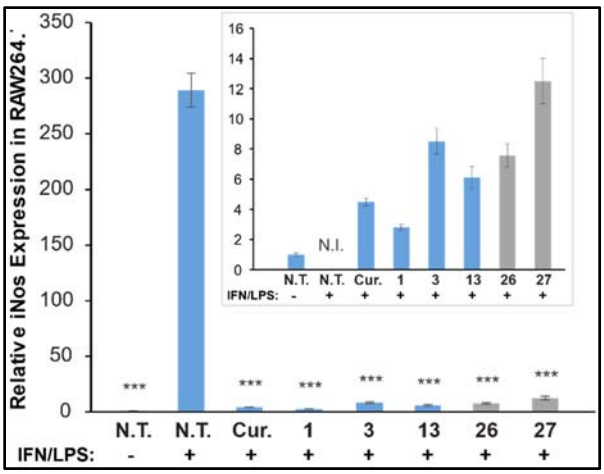

Figure 3: Relative expression of inducible nitric oxide (*iNos*) following treatment of M1-activated macrophages with anti-inflammatory compounds. M1 phenotype was produced via IFN-γ/LPS-mediated polarization in indicated samples. N.T = non-treated; cur. = Curcumin. Error bars represent standard error. Student T-test was performed to compare each treatment to the M1-polarized, non-compound-treated control (N.T.; $p < 0.001$ = ***). Inset shows same assay results but without the IFN-γ/LPS N.T. sample; N.I. = not included.

To support these results and correlate *iNos* mRNA transcript levels with NO production, Griess assays were performed to determine the amounts of NO produced following treatments with curcumin and derivatives versus non-treated (M1-polarized and naïve) controls. Treatment with each of the compounds resulted in dramatically decreased levels of NO relative to the pro-inflammatory control group (N.T. treated with IFN-γ/LPS; **Figure 4**). Statistical analyses indicate that there is no difference when comparing curcumin with compounds **1, 13, 26** or **27**, but there is statistically significant decrease for compound **3** ($p<0.05$). Altogether, these results demonstrate the ability of the described curcumin analogues to reduce NO secretion in pro-inflammatory, M1 macrophages. It also suggests that compound **3** reduces NO secretion when compared to other compounds, including curcumin. Assessment of NO levels following compound treatment of naïve macrophages showed no substantial changes (**Supporting Information Figure S10**). Compound **13** slightly reduced NO levels beyond baseline, while curcumin and molecules **1**, **3**, **26**, and **27** did not yield any significant effects.

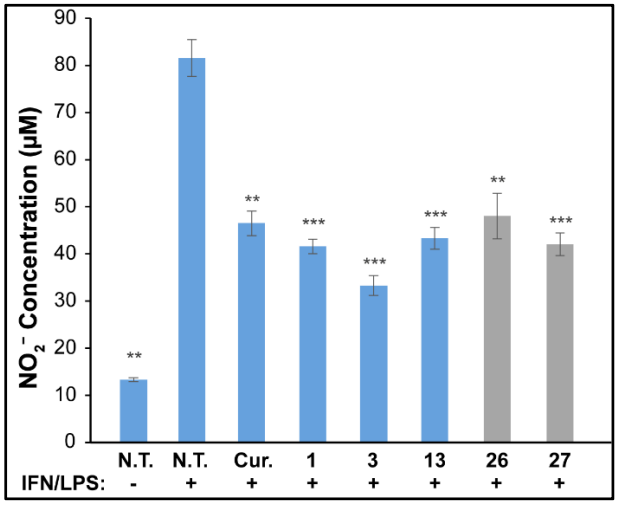

Figure 4: Effects of anti-inflammatory compounds on cellular nitrite production via Griess assay. M1 phenotype macrophages were generated via IFN-γ/LPS-mediated polarization in indicated samples, which were then treated with molecules as designated. N.T = non-treated; cur. = Curcumin. Error bars represent standard error. Student T-test was performed to compare each treatment to the M1 polarized, non-compound-treated control ($p < 0.01$ = **, and $p < 0.001$ = ***).

Conclusion

There is a need for more drug-like molecules that can resolve inflammation and ameliorate associated symptoms in the contexts of chronic inflammation and autoimmune diseases. The use of curcumin has attracted the attention of the immunology field due to its anti-inflammatory properties. While curcumin has shown promising results and has anti-inflammatory properties, its poor biodistribution and bioavailability have limited its use. Therefore, in this study, we generated curcumin-derivatives aiming to overcome these issues, while maintaining the induction of anti-inflammatory responses in immune cells.

In summary, twenty-one DANMPs (**1-21**), four DHANMPs (**22-25**) and five SBPs (**26-30**) were synthesized and characterized. Using both *in silico* and cell-free experiments, we determined

the drug-like properties of the molecules and quantified their radical scavenging activities, respectively. Our results displayed improved Lipinksi and ADME parameters (**Supporting Information Tables S2 and S3**) and solubilities (**Table 1**) compared with those of curcumin itself. DPPH radical scavenging assay data indicated that all generated compounds largely retained this capability (**Figure 2**).

Using the model macrophage cell line RAW 264.7, we assessed the biological effects of five selected compounds (**1**, **3**, **13**, **26**, and **27**). None of these molecules affected cell viability and largely did not influence the presence of basal levels of the inflammation-associated marker *iNos* or reactive NO in naïve cells (**Supporting Information Figures S8-S10**). However, in macrophages activated to pro-inflammatory/M1 phenotypes with LPS and IFN-γ, the assessed compounds markedly decreased the expression of *iNos* and levels of NO with activities largely similar to those of curcumin (**Figures 3** and **4**). Altogether, these data demonstrate the potential of curcumin-derived small molecules to reduce inflammation and their usefulness in the treatment of inflammation-associated diseases. Having shown promise here, these molecules should be evaluated in terms of target validation and specificity, and can be tested in other inflammatory models, including disease-specific ones. Structure-activity relationship studies will also be pursued to assist in further optimization to improve upon the compounds' anti-inflammatory properties and other characteristics.

References

1. Medzhitov R. Inflammation 2010: new adventures of an old flame. *Cell.* **2010**; 140:771–776.
2. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol.* **2005** 6(12): 1182-1190.

3. Sadik CD, Luster AD. Lipid-cytokine-chemokine cascades orchestrate leukocyte recruitment in inflammation. *J Leukoc Biol.* **2012**; 91(2):207-15.
4. Italiani P, Boraschi D. From Monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *Front Immunol.* **2014**; 5:514.
5. Huang X, Li Y, Fu M, et al. Polarizing macrophages in vitro. *Methods Mol Biol*. **2018**; 1784:119-126.
6. Coussens LM, Werb Z. Inflammation and cancer. *Nature.* **2002**; 420(6917):860-867.
7. Giardino G, Gallo V, Prencipe R, et al. Unbalanced immune system: immunodeficiencies and autoimmunity. *Front Pediatr.* **2016**; 4:107.
8. Bagatini MD, Cardoso AM, Reschke CR, et al. Immune system and chronic diseases 2018. *J Immunol Res.* **2018**; 2018:8653572
9. Krishnamoorthy S, Honn KV. Inflammation and disease progression. *Cancer Metastasis Rev*. **2006**; 25(3):481-491.
10. Newcombe EA, Camats-Perna J, Silva ML, et al. Inflammation: the link between comorbidities, genetics, and Alzheimer's disease. *J Neuroinflammation.* **2018**; 15(1):276.
11. Furman D, Campisi J, Verdin E, et al. Chronic inflammation in the etiology of disease across the life span. *Nat Med* **2019**; 25, 1822–1832.
12. Opal SM. Endotoxins and other sepsis triggers. *Contrib Nephrol.* **2010**; 167:14-24.
13. Wang B, Wu L, Chen J, et al. Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. *Signal Transduct Target Ther.* **2021**;6(1):94.
14. Higgins AJ, Lees P. The acute inflammatory process, arachidonic acid metabolism and the mode of action of anti-inflammatory drugs. *Equine Vet J.* **1984**;16(3):163-75.

15. Insuela DBR, Ferrero MR, Coutinho DS, et al. Could arachidonic acid-derived pro-resolving mediators be a new therapeutic strategy for asthma therapy? *Front Immunol* **2020**;11:580598.
16. Boroumand N, Samarghandian S, Hashemy SI. Immunomodulatory, anti-inflammatory, and antioxidant effects of curcumin. *J Herbmed Pharmacol.* **2018**; 7(4):211-219.
17. Davis JS, Lee HY, Kim J, et al. Use of non-steroidal anti-inflammatory drugs in US adults: changes over time and by demographic. *Open Heart* **2017**;4:e000550.
18. Conaghan PG. A Turbulent decade for Nsaids: update on current concepts of classification, epidemiology, comparative efficacy, and toxicity. *Rheumatol Int.* **2012**; 32(6):1491-502.
19. Rao CV, Reddy BS. NSAIDs and chemoprevention. *Curr Cancer Drug Targets*. **2004**; 4(1):29-42.
20. Singh G. Gastrointestinal complications of prescription and over-the-counter nonsteroidal anti-inflammatory drugs: a view from the ARAMIS database. Arthritis, Rheumatism, and Aging Medical Information System. *Am J Ther*. **2000**; 7(2):115-121.
21. Huang MT, Lysz T, Ferraro T, et al. Inhibitory effects of curcumin on in vitro lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Res.* **1991**; 51(3):813-819.
22. Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev.* **1998**; 56(11):317-333.
23. Seo WG, Pae HO, Oh GS, et al. Inhibitory effect of ethyl acetate fraction from Cudrania tricuspidata on the expression of nitric oxide synthase gene in RAW 264.7 macrophages stimulated with interferon-gamma and lipopolysaccharide. *Gen Pharmacol.* **2000**; 35(1):21-28.

24. Skrzypczak-Jankun E, McCabe NP, Selman SH, et al. Curcumin inhibits lipoxygenase by binding to its central cavity: theoretical and X-ray evidence. *Int J Mol Med.* **2000**; 6(5):521-526.
25. Kanski J, Aksenova M, Stoyanova A, et al. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure-activity studies. *J Nutr Biochem.* **2002**; 13(5):273-281.
26. Yoon JH, Baek SJ. Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei Med J.* **2005**; 46(5):585-596.
27. Sandur SK, Ichikawa H, Pandey MK, et al. Role of pro-oxidants and antioxidants in the anti-inflammatory and apoptotic effects of curcumin (diferuloylmethane). *Free Radic Biol Med.* **2007**; 43(4):568-580.
28. Chang YC, Lee FW, Chen CS, et al. Structure-activity relationship of C6-C3 phenylpropanoids on xanthine oxidase-inhibiting and free radical-scavenging activities. *Free Radic Biol Med.* **2007**; 43(11):1541-1551.
29. Basnet P, Skalko-Basnet N. Curcumin: an anti-inflammatory molecule from a curry spice on the path to cancer treatment. *Molecules.* **2011**; 16(6):4567-4598.
30. Yang G, Lee K, Lee M, et al. Inhibition of lipopolysaccharide-induced nitric oxide and prostaglandin E2 production by chloroform fraction of Cudrania tricuspidata in RAW 264.7 macrophages. *BMC Complement Altern Med.* **2012**; 12:250.
31. Jantan I, Bukhari SN, Adekoya OA, et al. Studies of synthetic chalcone derivatives as potential inhibitors of secretory phospholipase A2, cyclooxygenases, lipoxygenase and pro-inflammatory cytokines. *Drug Des Devel Ther.* **2014**; 8:1405-1418.

32. Xin LT, Yue SJ, Fan YC, et al. Cudrania tricuspidata: an updated review on ethnomedicine, phytochemistry and pharmacology. *RSC Adv.* **2017**; 7(51):31807-31832.
33. Rao CV. Regulation of COX and LOX by curcumin. *Adv Exp Med Biol.* **2007**; 595:213-226.
34. Zhou Y, Zhang T, Wang X, et al. Curcumin modulates macrophage polarization through the inhibition of the toll-like receptor 4 expression and its signaling pathways. *Cell Physiol Biochem.* **2015**; 36(2):631-641.
35. Camacho-Barquero L, Villegas I, Sánchez-Calvo JM, et al. Curcumin, a curcuma longa constituent, acts on MAPK p38 pathway modulating COX-2 and iNos expression in chronic experimental colitis. *Int Immunopharmacol*. **2007**;7(3):333-342.
36. Zhou H, Beevers CS, Huang S. The targets of curcumin. *Curr Drug Targets.* **2011**; 12(3):332-47.
37. Phumsuay R, Muangnoi C, Dasuni Wasana PW, et al. Molecular insight into the anti-inflammatory effects of the curcumin ester prodrug curcumin diglutaric acid in vitro and in vivo. *Int J Mol Sci*. **2020**;21(16):5700.
38. Cheng AL, Hsu CH, Lin JK, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.* **2001**; 21(4B):2895-2900.
39. Jurenka JS. Anti-inflammatory properties of curcumin, a major constituent of curcuma longa: a review of preclinical and clinical research. *Altern Med Rev*. **2009**; 14(2):141-153.
40. Anand P, Kunnumakkara AB, Newman RA, et al. Bioavailability of curcumin: problems and promises. *Mol Pharm.* **2007**; 4(6):807-818.
41. Jiang F, Yu T, Liu X, et al. Biodistribution of curcumin and its derivatives new aspects for curcumin administration. *In 2011 International Conference on Remote Sensing, Environment and Transportation Engineering.* **2011**; 3878-3881.

42. Cui J, Yu B, Zhao Y, et al. Enhancement of oral absorption of curcumin by self-microemulsifying drug delivery systems. *Int J Pharm.* **2009**; 371(1-2):148-155.
43. Moballegh Nasery M, Abadi B, Poormoghadam D, et al. Curcumin delivery mediated by bio-based nanoparticles: a review. *Molecules*, **2020**; *25*(3), 689.
44. Shen L, Ji HF. The pharmacology of curcumin: is it the degradation products? *Trends Mol Med.* **2012**; 18(3):138-144.
45. Diplock AT, Charleux JL, Crozier-Willi G, et al. Functional food science and defense against reactive oxygen species. *Br J Nutr.* **1998**; 80(1):S77-S112.
46. Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* **2007**; 39(1):44-84.
47. Weber WM, Hunsaker LA, Abcouwer SF, et al. Anti-oxidant activities of curcumin and related enones. *Bioorg Med Chem.* **2005**; 13(11):3811-3820.
48. Joshi BP, Mohanakrishnan D, Mittal G, et al. Synthesis, mechanistic and synergy studies of diarylidenecyclohexanone derivatives as new antiplasmodial pharmacophores. *Med Chem Res*. **2018**; 27, 2312–2324.
49. Rokkam SK, Yadav M, Joshi M, et al. Synthesis, *in vitro* anti-plasmodial potency, *in-silico*-cum-SPR binding with inhibition of *Pf*Pyridoxal synthase and rapid parasiticidal action by 3,5-bis{(E) arylidene}-N-methyl-4-piperidones, *New J Chem.* **2021**;45:22150–22165.
50. Kar S, Shivalingegowda N, Krishnappagowda LN, et al. SiCl 4 mediated one-pot synthesis of novel spirobibenzopyrans as potent anticancer agents. *New J Chem.* **2019**; 43(12), 4669-4673.
51. Sivakumar PM, Prabhakar PK, Doble M. Synthesis, antioxidant evaluation, and quantitative structure – activity relationship studies of chalcones. *Med Chem Res.* **2011**;20:482–492.

52. Fang X, Fang L, Gou S, et al. Design and synthesis of dimethylaminomethyl-substituted curcumin derivatives/analogues: potent antitumor and antioxidant activity, improved stability and aqueous solubility compared with curcumin. *Bioorg Med Chem Lett.* **2013**;23:1297–1301.
53. Schmittgen TD, Livak KJ, Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* **2008**;3;1101–1108.
54. Kedare SB, Singh RP. Genesis and development of DPPH method of antioxidant assay. *J Food Sci Technol.* **2011**;48:412-22
55. Savjani KT, Gajjar AK, Savjani JK. Drug solubility: importance and enhancement techniques. *ISRN Pharm*. **2012**;195727.
56. Jain M, Marfatia A, Imam N, et al. Ionic liquid-based catanionic vesicles: A de novo system to judiciously improve the solubility, stability and antimicrobial activity of curcumin. *J Mol Liq.* **2021**;341:117396.
57. Rutledge HR, Jiang W, Yang J, et al. Gene expression profiles of RAW264.7 macrophages stimulated with preparations of LPS differing in isolation and purity. *Innate Immun.* **2012**;18:80-8.
58. Bao Z, Huang Y, Chen J, et al. Validation of reference genes for gene expression normalization in raw264.7 cells under different conditions. *Biomed Res Int.* **2019**;2019:6131879.
59. Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life Sci.* **2004**;75:639-53.
60. Robinson MA, Baumgardner JE, Otto CM. Oxygen-dependent regulation of nitric oxide production by inducible nitric oxide synthase. *Free Radic Biol Med.* **2011**;51:1952-65.