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ORIGINAL RESEARCH

Curcuminoid Analogs Differentially Modulate Nuclear Factor Kappa-Light-Chain-Enhancer, P65 Serine276, Mitogen- and Stress-activated Protein Kinase 1 And MicroRNA 148a Status

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ABSTRACT

Background: Curcumin has been used successfully to treat inflammatory conditions; however, reliability and repeatability of clinical and bench research results have been a challenge. Curcumin is comprised of 3 curcuminoid analogs that can vary in proportion from one extract to another, even from batch to batch from the same commercial supplier. A better understanding of how each curcuminoid analog comprising the curcumin extract can partake in the overall curcumin pharmacology might give us better insight on the polypharmacology involved.

Methods/Results: Applied as pretreatment drugs, all 3 curcuminoids, curcumin I (diferuloylmethane), curcumin II (demethoxycurcumin), and curcumin III (bisdemthoxycurcumin), are shown here to modulate 3 key subcellular drug targets differentially. Nucleotranslocation by the curcuminoids is not apparent in the lipopolysaccharide-induced BV2 (immortalised murine microglial cell line) cells. At a nuclear level, multiple compounding findings related to curcumin pharmacology, regulate transactivation of the Nuclear Factor kappa-light-chain-enhancer (NF-kB) heterodimer once it has translocated. Each of the 3 curcuminoid analogs seems to inhibit phosphorylation of p65 (REL-associated protein involved in NF-κB protein formation) at residue serine^{276} of the transcription factor's transactivation domain with curcumin I showing a significant hindrance. On the other hand, curcumin III and not curcumins I or II is found to significantly downregulate mitogen- and stress-activated protein kinase 1 status in both the cytosol and the nucleus of these cells. The upstream mechanism repressing mitogen- and stress-activated protein kinase 1 status is also shown to be the upregulation of MIR 148a by curcumin III. Curcumin II shows downregulatory activity of microRNA (MIR) 148a in opposition to curcumin III's upregulatory activity; whereas curcumin I remains neutral with regards to this target.

Conclusions: These results demonstrate that although there are some common targets and biochemical activity by the curcuminoid analogs, a differential activity by each can also be observed on other targets. These new findings show us that the common curcumin extract can be utilized with greater selectivity against specific drug targets and the associated disease pathologies. It also demonstrates the importance of establishing a standardization process that takes into account these curcuminoid proportions with the objective of improving reliability of pharmacology and repeatability of research outcomes.

Key words: anti-inflammatory, curcumin, microRNA 148a, MSK1, NF-kB

Introduction

Curcuma longa (turmeric) comprised of many active constituents, but the curcumin fraction is the most-studied active component of the herb. The curcumin extract comprised of 3 principal curcuminoid analogs: curcumin I (diferuloylmethane), curcumin II (demethoxycurcumin), and curcumin III (bisdemthoxycurcumin). The proportion of these curcuminoid analogs in the extract is not consistent in research standards or consumer products. Many subcellular targets of the curcumin extract have been found and many therapeutic effects of the extract have been reported including a significant amount of anecdotal and clinical evidence.[1–6] Our understanding of the mechanisms by which curcumin alters cellular activity, however, continues to evolve due, in part, to the lack of standardization in chemical composition of curcumin products.

Nuclear Factor kappa-light-chain-enhancer (NF-kB) is an important transcription factor regulating the expression of as many as 150 genes involved in the inflammatory and immune responses.[7–10] The NF-kB signaling pathway plays a central role in the regulation of cel-

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lular response and survival and is itself regulated by multiple kinases and inhibitory proteins through a complex network of positive and negative feedback signals.[11] Although the curcumin extract is known to modulate inflammation through NF-kB regulation, the mechanisms involved are not clear cut.[12] The NF-kB signaling pathway cross-talks with other central signaling pathways such as the MAPK signaling pathway,[13] which ultimately shapes NF-kB's transcriptional activity. These 2 pathways are highlighted in Fig 7A, B—a figure designed to help navigate the Discussion of this document.

Our experiments are designed to determine how daily prophylactic use of a curcuminoid compound could help mitigate exacerbated inflammatory activity by a trigger or other predisposing activity. Curcumin has been shown for decades to inhibit NF-kB[12,14,15] and its inflammatory and immune escalating transcription products.[16–18] The curcumin extract's inhibitory activity on NF-kB is primarily known to be through upstream inhibition of I-kappa-B kinase (IKK)[19,20] as Fig 7 demonstrates. IKK activation results in separation by the NF-kB protein from its inhibitory protein in the cytosol allowing the transcription factor to nucleotranslocate to engage in transactivation and subsequent cytokine transcription. NF-kB activation can be experimentally induced by way of lipopolysaccharide (LPS) stimulation.[21] Curcumin can inhibit this activation.[12] However, knowing that the curcumin extract is made up of multiple curcuminoid analogs begs the question: How do each of these naturally inherent curcuminoid analogs factor into the total polypharmacology? Our research is designed to help identify this compartmentalized pharmacology.

The term "curcumin" is used to refer to the principal curcuminoid, curcumin I. However, the term curcumin is confusingly also used in the literature and commercial applications to describe the typical curcumin extract that contains all 3 curcuminoid analogs (I, II, and III). The proportion of curcuminoids I, II, and III in the curcumin extract can vary from sample to sample contributing to a lack of standardization when comparing research executed with curcumin. Although these 3 curcuminoid analogs are homologous, they also display differential structural features, differences that are expected to exhibit differential chemical and pharmacologic properties.

The research outcomes reported here demonstrate, for the first time, critical inhibitive activity by the curcuminoid analogs on 3 different drug targets: p65 (REL-associated protein involved in NF-κB protein formation) serine^{276}, microRNA (MIR) 148a, and mitogen- and stress-activated protein kinase 1 (MSK1). The cell model chosen is the immune cell, BV2 macrophage. These research outcomes justify the need to know the proportions of the constituent curcuminoids in the curcumin extract sample to better predict pharmacology. A standardization model that sets the stage for consistent results by properly quantifying the label claim of the curcumin-based therapeutic agent is the starting point of a much-needed corrective process for more reliable curcumin extract standardization. Each curcuminoid must be quantified on the label claim of a curcumin extract-containing product.

Our work evolved in natural stages as we investigated the upstream mechanisms that led to an unexpected finding. Although investigating how each curcuminoid analog in isolation differentially inhibits p65 serine^{276} phosphorylation—a key activation site in the transcription factor's transactivation domain—differential inhibitory activity for the curcuminoid analogs was apparent. Investigation of upstream kinases associated with the phosphorylation of this activation site led us to investigate MSK1 activity. MSK1 is known to activate p65 by way of serine^{276} phosphorylation.[22] The first unexpected finding was the irrefutable change in status for nuclear and cytosolic concentrations of MSK1—changes induced by curcuminoid treatment, but not all the curcuminoids. It was quickly established that curcumin III and not curcumin II or I repressed MSK1 status in both the cytosolic and nuclear compartments of the cell model. In search of upstream mechanisms for this MSK1 target, MIR 148a was found to be a logical candidate. Prior independent work by Fujita et al[23] showed that MSK1 expression was intimately associated with MIR 148a activity.

The common curcumin extract is well known to modulate a myriad of microRNAs and has been studied in this context for some time.[24,25] However, curcumin has never been shown to have a modulatory influence on MIR 148a and rationale for believing, hypothetically, we might be looking at something novel was based on a very simple premise: the common curcumin extract historically studied in the context of microRNAs does not have curcumin III levels high enough to participate in the overall curcumin extract pharmacology. In our current work, we are studying the curcuminoid analogs: curcumin I, curcumin II, and curcumin III, in isolation and full concentration. The modulatory influence on MSK1 by curcumin III and not the other 2 curcuminoids could very well be a function of MIR 148a influence by curcumin III where regular curcumin extract will not have had a curcumin III level high enough inherently to deliver an efficacious pharmacology on the microRNA.

Eventually, we show that the different curcuminoid analogs differently modulate cellular MIR 148a status and, based on the dialogue in the coming pages, this too could have significant implications on how curcuminoid proportions in a curcumin extract ultimately influence cell behavior and disease symptoms. MIR 148a is aberrantly expressed in various cancers[26] and can play a role in cancer drug resistance.[23] Last, the fact that the curcumin III analog significantly modulates MSK1 status could lead to an understanding of how incremental curcumin III levels in a common curcumin extract might play a significant role in the heightened regulation of NF-kB transactivation[27] by way of MAPK signaling cross-talk on the NF-kB signaling pathway.

These 3 targets are common drug targets for good reasons. Constitutively nucleotranslocated NF-kB p65–p50 can be a function of an I-kappa-B (or more accurately NFKB Inhibitor Alpha [*NFKBIA*] gene) mutation.[28] Inhibition of MSK1 translation could theoretically mitigate ultimate NF-kB transactivation despite nucleotranslocation of NF-kB p65–p50. Fig 7 shows these pathways and kinases interacting at multiple points cytosolically and within the nucleus. In cases of immune system dysregulation by triggering-receptor-expressed-on-myeloid-cells-2 (TREM2) mutation such as that seen in variants of Alzheimer's disease,[29] this innate immune cell mutation might also theoretically be mediated by inhibition or downregulation of MSK1 to inhibit p65 serine^{276} phosphorylation and other targets crucial to NF-kB transactivation.

Our research on these targets has led us to what can be significant findings about curcuminoid pharmacology. Due to historical research showing curcumin inhibits PKC, we also had to take precautions to eliminate the possibility that PKC inhibition could be inhibiting the MAPK signaling pathway upstream of MSK1 as Fig 7 also demonstrates. PKC inhibitors were used in the experimental model strategically to clarify our findings in this context. These discoveries on these new targets by the isolated curcuminoids and the continued compartmentalized research of the curcuminoid analogs might change how the natural drug will be used as a medicinal agent and how it might be designed to be more effective, more selective, and more reliable in the future.

Methods

Cell Culture

BV2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Oakville, Ontario, Canada) complete me-

dium (DMEM + 1% ampicillin + 10% fetal bovine serum). Approximately 2×10^7 BV2 cells per well were seeded in each well of a 6-well plate with 2.0 ml complete medium and cultured overnight at 37°C and 5% carbon dioxide. The BV2 cell line is a good model to use in the evaluation of the inflammatory model because this microglial cell line reproduces many of the reactions and interactions of the primary microglial cell in an in vivo setting, including a functional sensitivity to LPS induction and subsequent NF-kB activation.[30] Based on our historical work and an initiative to stay consistent in our expanded experiment series, we chose to use an LPS concentration of 100 µg/ml.

Chemicals/Drug Preparation

The drugs were procured as follows: Bay 11–7082 NF-kB Inhibitor (Santa Cruz, Calif.); Dexamethasone (Sigma-Aldrich); Ibuprofen (Sigma-Aldrich); Commercial Curcuminoid Preparation from Biologic Nutrigenomic Health Research Corp (Surrey, BC, Canada); Curcumin I research standard [ChromaDex, Irvine, Calif. (CoA 97.7% Purity water excluded)]; Synthetic Curcumin I (ChromaDex); Curcumin II research standard [ChromaDex (CoA 97.3% Purity water excluded)]; Curcumin III research standard [Sigma-Aldrich (CoA 97.7% Purity)]; Curcuminoids/Curcumin Extract (curcumin I 77.7%, curcumin II 16.9%, curcumin III 0.9%) research standard [ChromaDex (CoA 95.3% Purity water excluded)]; and LPS from *Escherichia coli* (Sigma-Aldrich). Curcumin/curcuminoids and other drugs were all prepared to various concentrations in DMEM containing 0.2% dimethyl sulfoxide (DMSO).

MTT Assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich) was performed on BV2 to study the cytotoxicity of curcuminoid analogs. For MTT assay, 3×10^4 cells were seeded on each well of a 96-well plate. The drugs used were the same ones described above: commercial curcumin preparation, curcumin I, curcumin II, curcumin III, and synthetic curcumin I. Each curcumin preparation was tested at the following concentrations on each cell line to generate the corresponding graph: 5.0, 10.0, 20.0, 40.0, and 80.0 µg/ml. Absorbance was measured at 570 nm using a Perkin Elmer Envision 2103 Multilabel Reader. Results were expressed as percent of the absorbance found in control cells (n = 4).

Drug Treatment and Diagnostic

Curcumin drug pretreatment of the wells is executed as described in the following order: Bay 11 (Santa Cruz, CA, USA; 30 µM); no treatment/cells only; DMSO only (0.2%)—control, Curcumin Extract 95% (research standard) (22.0 µg/ml); curcumin extract 95% (research standard) (22.0 µg/ml); commercial curcumin extract [off-the-shelf (22.0 µg/ml)]; curcumin I (research standard) (22.0 µg/ml), curcumin II (research standard) (22.0 µg/ml); and curcumin III (research standard) (22.0 µg/ml). BV2 cells were stimulated with 100 µg/ml (final concentration per well) of LPS and incubated after LPS induction for 30 minutes. After stimulation, drug pretreated cells were washed with 100–150 µl phosphate-buffered saline and treated with Lysis Buffer (Sigma-Aldrich). Protein concentration of the samples was determined in order to finalize gel-loading volume of the sample lysates. These lysates were harvested and processed for Luciferase Assay (Sigma-Aldrich) according to the manufacturer's instruction using a Perkin Elmer Envision 2103 Multilabel Reader (Shelton, CT, USA; n = 3).

Western Blot Analysis of Cytoplasmic and Nuclear Fractions

Using the ThermoFisher Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit, cells were lysed and the cytoplasmic and nuclear protein fractions were separated from BV2 cells pretreated with drugs and LPS. Total protein concentration for each sample extracted was determined by Bio Rad Protein Assay according to the manufacturer's instruction. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis, the samples were blotted to a nitrocellulose membrane (ThermoFisher Scientific, Surrey, BC, Canada), blocked with skim milk solution (5% skim milk in tris-buffered saline with TWEEN) and prepared for primary antibody treatment for each target. Primary antibody (1:3,000) incubation term was overnight (approximately 13 hours) at 4°C. The antibodies used for the targets were rabbit polyclonal pan anti-p65, anti-p65 serine^{276}, anti-MSK1, and anti-MSK1 serine^{376} phosphorylation (Sigma-Aldrich). Incubation with the secondary antibody conjugated to horseradish peroxidase (Goat Anti-rabbit IgG; ThermoFisher Scientific) was prepared in skim milk powder solution diluted to 1:10,000 and applied to each for 1 hour at room temperature. Membrane scans were executed with a Bio Rad ChemDoc MP Imaging System (Mississauga, ON, Canada); bands were quantified using Image J software (https://imagej.nih.gov/i). The internal control used was total protein, determined also by Image J software quantification of total protein chemiluminescence and divided into each measurement of the quantified target protein measurement. This is defined reliable in previous research[31] to serve as a control (n = 3).

qRT-PCR of mRNA

Reverse transcription polymerase chain reaction (RT-PCR) was utilized to determine mRNA status representing MSK1 expression. In this case, drug treatment included the above-described curcumin extract and the curcuminoid analog preparations along with a protein kinase C inhibitor (PKCI; Sigma-Aldrich) cohort. The treatments were executed as such: LPS only; DMSO only; curcumin extract only; curcumin extract + PKCI + LPS; curcumin extract + LPS; PKCI + LPS; curcumin I + LPS; curcumin I + PKCI + LPS; curcumin III + PKCI + LPS; and curcumin III + LPS.

The experimental supplies also include Kapa SYBR FAST One-Step quantitative reverse transcription polymerase chain reaction (qRT-PCR) Universal kit (D-Mark Biosciences, Toronto, Ontario, Canada); PCR primers were custom prepared and procured (Invitrogen/Life Technologies, Burlington, Ontario, Canada): MSK1 (1) forward 5′-TGC TGA AGG TCC TAG GAA CT-′3; reverse 5′-GCATACAGCTTTCCAGCATC-′3; MSK1 and (2) forward 5′-AGCCACATGCACGATGTAGGA-3′; reverse, 5′-AGGCGTGCAAACCCAAAGT-3′. DNAse 1 (product code: AMPD1-1Kt) was procured (Sigma-Aldrich, Oakville, Ontario, Canada).

BV2 cells were treated and harvested as described for the Western blot experimentation above. Ribonucleic acid (RNA) was extracted using QuickExtract RNA Extraction Kit (Mandel Scientific Company, Guelph, Ontario, Canada). PCR was executed using a 1-step qRT-PCR Kit (Sigma-Aldrich). Bar graphs are generated from the 3 iterations to present the data for MSK1 expression changes.

qRT-PCR of MicroRNA

The status of MIR 148a was tested by qRT-PCR in BV2 cells treated with the 3 different curcuminoids and the curcumin extract to determine if expression patterns of this MSK1-regulating microRNA correlate with the MSK1 status changes found in the Western blot experimentation. A microRNA extraction kit (MicroRNeasy Mini Kit;

Qiagen, Valencia, Calif.); miScript II RT kit (cDNA synthesis kit; Qiagen); and Hs miR-148a primer (Qiagen) were used in this process.

Statistical Analysis

Image J software was used to quantify the data. The *t* test was used to evaluate each test condition against the control, where a *P* value of <0.05 was considered as significant.

Results

Cytotoxicity of Curcumin

The MTT assay results show that the relative toxicity of the different curcumin- and curcuminoid-based preparations tested in the BV2 microglia cell line. The results shown at Fig 1 demonstrate that curcumin extract and curcuminoid concentrations used for the treatment (22.0 µg/ml) in the experimental series are not cytotoxic.

There is a clear concentration-dependent cytotoxicity by all curcumin compounds. Cell survival dropped to approximately 50% of the total cell count in the concentration range between 40 and 60 µg/ml (Fig 1). Each compound induced a consistently steep drop in cell survival after 40 µg/ml, and 100% cell killing was seen at 80 µg/ml. The IC50 (concentration of an inhibitor to half the activity) for the curcumin-based drugs is approximately 60 µg/ml in the BV2 cell line (Fig 1).

Curcumin Does Not Block LPS-induced P65 Nuclear Translocation

The immunoblot results in this experimental series in the BV2 cell line demonstrate that the curcumin extract does not reduce LPS-induced nuclear NF-kB translocation at our selected curcumin concentration (22.0 µg/ml). This series uses the BV2 microglial cells stimulated with 100.0 µg/ml LPS. Results are presented at Fig 2 with quadrants A and B displaying total p65 status in the cytosol and nucleus, respectively. Our data also show at Fig 2 that none of the curcuminoid analogs at the test concentration significantly inhibit NF-kB nucleotranslocation in the LPS-stimulated cell line as presented in quadrants A and B. There is no significance to report. Fig 2 also shows that the curcumin extract applied at the test concentration, 22.0 µg/ml, did not influence nuclear p65 status in nonstimulated cells either. Total protein was used as the control to process these Western blot data; scanned directly from the BioRad Stain-Free Gel before protein transfer and represented with samples at Fig 3.

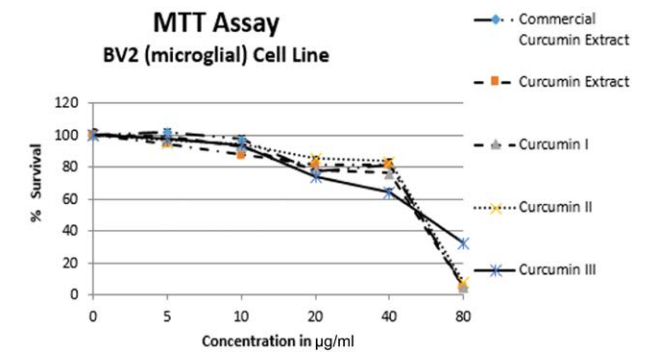

Fig 1. Graphed MTT assay results showing the relative toxicity of the different curcuminoid-based preparations tested. Results demonstrate 75%–80% survival with treatment concentrations as high as 80 µg/ml. Curcumin III treatment is an outlier in the graphed results potentially demonstrating lower toxicity, or a possible increased resilience to toxicity by these cell lines to curcumin III. N of 3.

Curcumin Significantly Inhibits p65 Serine^{276} Phosphorylation

Where a profound inhibitive pharmacology by the curcumin drug is significant in the context of this cell line and LPS treatment dose is at the nuclear p65 serine^{276} phosphorylation site. Quadrants C and D of Fig 2 display phosphorylation status of this key site within the cytosol and nucleus, respectively. The results demonstrate in quadrants C and D that, in nonstimulated BV2 cells, the curcumin extract inhibited nuclear and cytosolic p65 serine^{276} phosphorylation by 70% ($P = 0.0003$) and 40% ($P = 0.0008$), respectively. This is a novel finding that may help further define new curcuminoid applications.

LPS treatment does not alter the phosphorylation status of p65 serine^{276}. The fact that the NF-kB transcription factor nucleotranslocates may result in p65 serine^{276} phosphorylation in anticipation of transactivation. The curcumin extract treatment lowers basal p65 serine^{276} phosphorylation ($P = 0.0003$). In LPS-stimulated cells, each curcumin extract inhibited nuclear p65 serine^{276} phosphorylation as well. Phosphorylation of p65 serine^{276} is robust in both LPS-induced and noninduced (controls) BV2 cells.

This stimulation-independent phosphorylation indicates that phosphorylation of this site might be mandated by a mechanism designed to maximize transactivation potential of all nucleotranslocated NF-kB. Although the averaged results seem to support that each curcuminoid (I, II, and III) in isolation inhibited nuclear p65 serine^{276} phosphorylation by between 50% and 60% across the range of the different drugs, the *P* values only support the curcumin-based treatments that boast a curcumin I-dominant constitution (see quadrants C and D of Fig 2). These include the curcumin extracts (both the curcumin research standard and the commercial extract in the nuclear fraction) with a range between 75% and 80% curcumin I ($P = 0.064$ and $P = 0.036$, respectively) of the 22.0 µg/ml treatment dose and the isolated curcumin I ($P = 0.036$) analog with an isolated purity of approximately 97% of the 22.0 µg/ml treatment dose.

Phosphorylation of p65 serine^{276} in the transcription factor's transactivation domain 2 is essential for effective transactivation.[32,33] The inhibition of nuclear p65 serine^{276} phosphorylation plays a significant role in transactivation inhibition despite p65 nucleotranslocation as per the citations. Curcumin I inhibits p65 serine^{276} phosphorylation effectively in the nuclear space (Fig 2, quadrant D), but it does so with insignificant activity in the cytosolic space (Fig 2, quadrant C).

Curcumin III, But Not Curcumins I or II, Downregulates MSK1 Status and MSK1 Serine^{376} Phosphorylation in BV2 Cells

These data are presented at Fig 4 as a percent of control—"DMSO Only" group where quadrants A and B represent total MSK1 status in the cytosol and nucleus, respectively. Quadrants C and D of Fig 4 represent MSK1 phosphorylation status in the cytosol and nucleus, respectively. Bay 11 is a common anti-inflammatory research drug also used as a reference/control. An off-the-shelf commercial curcumin extract (95%) was tested in the group of test drugs, and unlike the curcumin extract (95%) research standard, it did not have a significant influence on MSK1 status. The typical curcumin extract (95%) is also represented in the test group by a research standard. This curcumin extract (95%), comprising a very low inherent curcumin III content (<2%), does seem to inhibit or downregulate MSK1 status mildly but without statistical significance in the nuclear space.

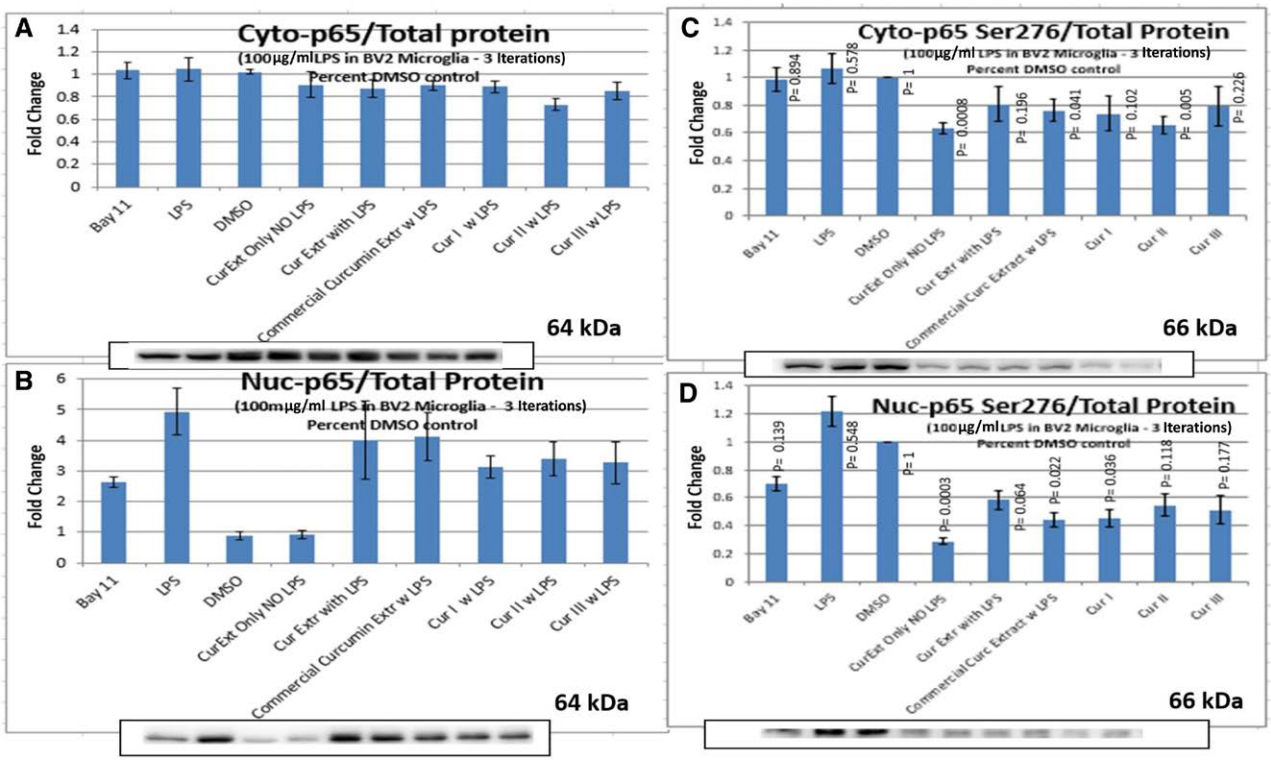

Fig 2. Results from immunoblot experimentation showing p65 total protein status in the cytosol (quadrant A) and in the nucleus (quadrant B), with representation of p65 serine^{276} phosphorylation status in the cytosol (quadrant C) and the nucleus (quadrant D) also being presented in this figure. The BV2 microglia cell line has been "stimulated by 100 µg/mg LPS" with results presenting the appearance of inhibition of p65 serine^{276} phosphorylation by each isolated curcuminoid (I, II, and III) and variable curcumin extract preparations. However, *P* values strongly support inhibition of basal (and LPS stimulated) p65 serine^{276} phosphorylation by the curcumin extract in both the cytosolic and nuclear compartments. *P* values also support the curcumin extracts' inhibition of nuclear p65 serine^{276} phosphorylation in LPS-stimulated cells. Statistical significance also supports curcumin *I*'s inhibitive activity on nuclear p65 serine^{276} in stimulated and nonstimulated cells but not that of curcumins II or III. LPS does not seem to induce phosphorylation of the p65 serine^{276} residue over that which is found basally in the nucleus. Expressed as a "fold change" of DMSO control. Three iterations compiled and averaged. N of 3. *The wells displayed in the Western blot images are displayed in the same order as the bar graphs. Commercial Curcumin Extr W LPS indicates commercial curcumin extract with LPS; CurExt Only NO LPS, curcumin extract only no LPS; CurExt with LPS, curcumin extract with LPS; Cur I, curcumin I; Cur II, curcumin II; Cur III, curcumin III; Cur I W LPS, curcumin I with LPS; Cur II W LPS, curcumin II with LPS; Cur III W LPS, curcumin III with LPS. Cyto-p65 = Cytoplasmic-p65 protein status; Nuc-p65= Nuclear-p65 protein status; Ser276= p65Serine^{276} phosphorylation site.

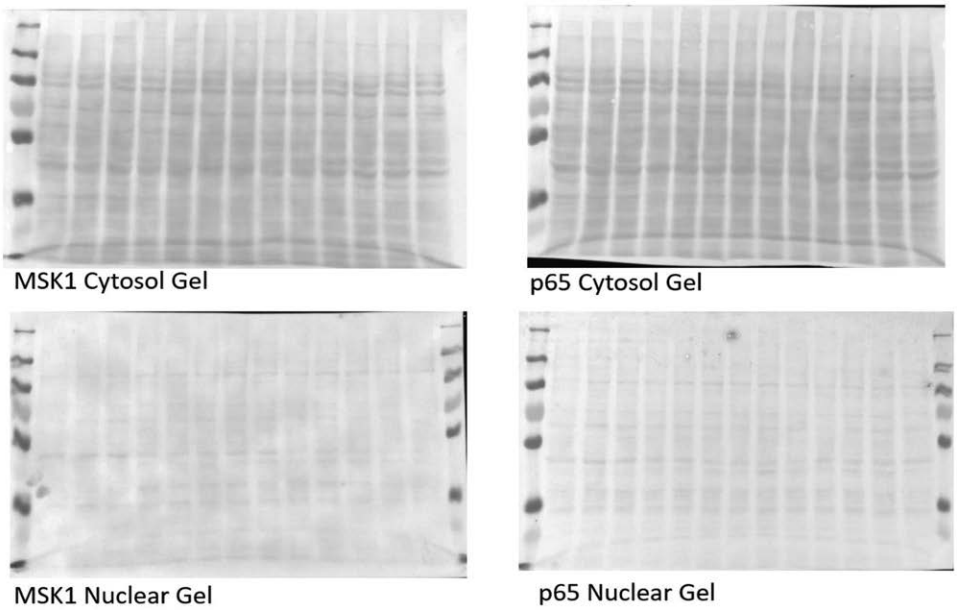

Fig 3. Four samples of the scanned BioRad Stain-Free Gel before protein transfer to establish quantification of total protein concentration per well are presented. Each gel presents 13 lanes (1–13 from left to right). Lanes 10–13 are redundant lanes that were not reported on in the final data output. The lanes represent the same order seen in the previous Figure 2 and the following Figure 4. Lanes from left to right (1–9) are as follows: Bay 11, LPS only, DMSO only, curcumin extract only, curcumin standard with LPS, commercial curcumin with LPS, curcumin I with LPS, curcumin II with LPS, and curcumin III with LPS.

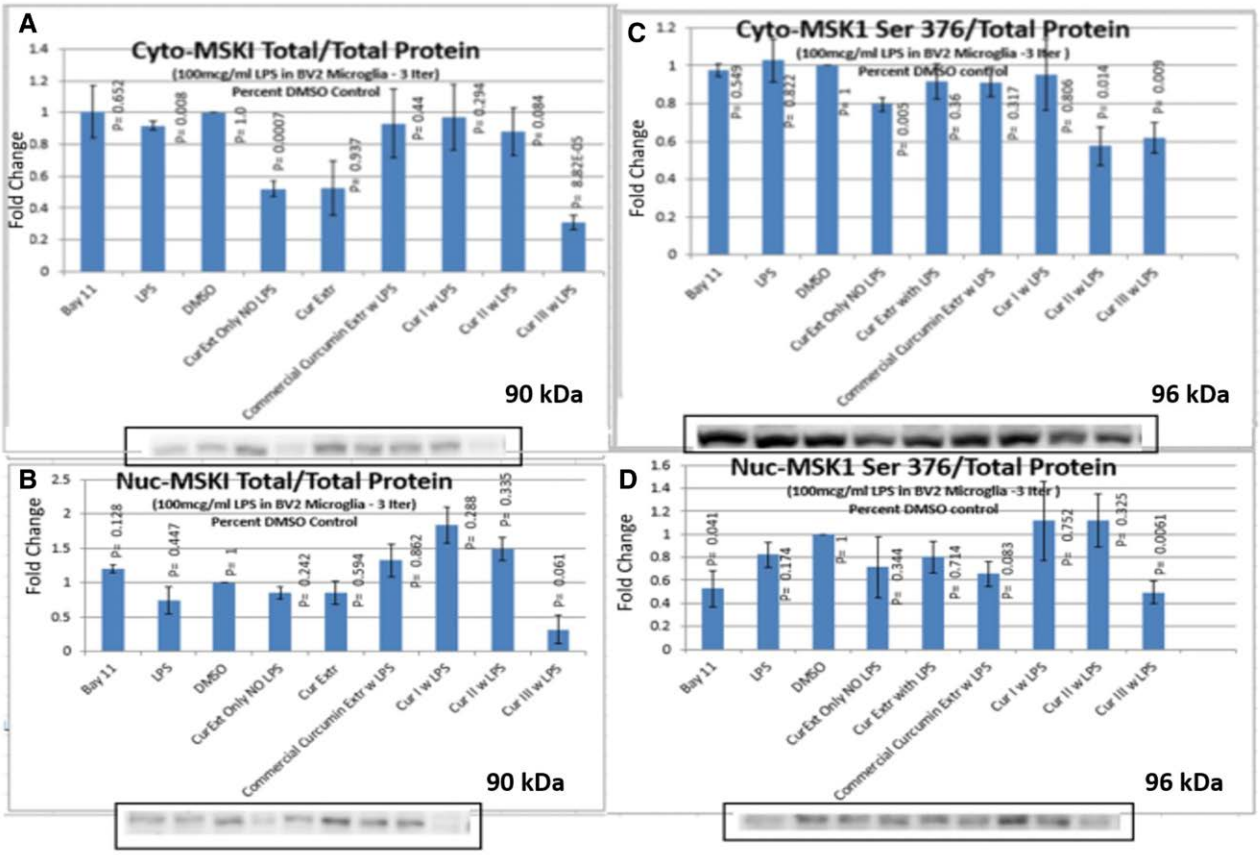

Fig 4. Graphed results from Western blot work probing for MSK1 status in the nuclear fraction vs. cytosolic fraction. Quadrants A and B represent total protein status in the cytosol and nucleus, respectively. Quadrants C and D represent MSK1 phosphorylation status on this key activation site in the cytosol and nucleus, respectively. Pretreatment with each curcumin-based drug of BV2 cells is followed by LPS stimulation to evaluate the curcumin-based drug's influence on the target. Pan MSK1 status is significantly reduced in both the cytoplasmic and nuclear compartments by curcumin III and not by the other curcuminoid analogs nor the curcumin extract. Interestingly, it is also shown that curcumin II escalates MSK1 status with statistical significance in the cytoplasmic compartment. N of 3. *The wells displayed in the Western blot images are displayed in the same order as the bar graphs. Commercial Curcumin Extr W LPS indicates commercial curcumin extract with LPS; CurExt Only NO LPS, curcumin extract only no LPS; CurExt with LPS, curcumin extract with LPS; Cur I W LPS, curcumin I with LPS; Cur II W LPS, curcumin II with LPS; Cur III W LPS, curcumin III with LPS. Cyto = Cytoplasmic; mcg/ml = μg/ml; Nuc = Nuclear; Ser376 = serine^{376} phosphorylation site.

Nevertheless, this same curcumin extract (95%) research standard does induce a significant (P = 0.0007) reduction of the MSK1 protein in the cytosolic compartment by as much as 48% (1.0–0.51847) where the inhibition cannot significantly impact transactivation changes directly. Moreover, it is shown that the curcumin III research standards and not the curcumins I or II research standards, in isolated form, at equimolar concentration (22 μg/ml) downregulate MSK1 protein status in both the cytosolic and nuclear fractions. For the first time, it is shown that MSK1 total protein status in these BV2 cells is reduced with significant implications in both the cytoplasmic and nuclear compartments by curcumin III (bisdemethoxycurcumin) treatment when compared with the DMSO only control, whereas the other 2 curcuminoids do not change MSK1 status. Curcumin III downregulates MSK1 status in the cytosolic compartment by 76% (1.0–0.2409) (P = 0.0001), whereas it induces a significant reduction of nuclear MSK1 status by 54% (1.0–0.46227) (P = 0.0611). Total protein was used as the control to process the Western blot data as previously described with scan samples displayed at Fig 3.

qRT-PCR

The RT-PCR results show us the curcumin extract inhibits MSK1 transcription reducing it by as much as 40% of basal activity. It does not do so, however, any more than LPS does. The inhibition by LPS was unexpected but may be a function of feedback controls designed to downregulate an activated transcriptional signaling pathway responding to generate the inflammatory response. Nevertheless, each of the curcuminioid analogs tested, II and III, performs similarly across the board as presented in Fig 5. Here, 3 iterations as one biological experiment with 2 internal repeats are averaged.

The short experiment is designed to help give us some insight on the next steps to be taken. The curcumin extract is comprised primarily of curcumins I and II. P values support the significance of the results. Comparing the results of the RT-PCR experiment for the 2 curcuminoid analogs tested does not offer a rationale or reasonable mechanism for the MSK1 protein status shown to be significantly downregulated by curcumin III in the Western blot data.

Addition of the PKC inhibitor to the curcumin extract or each of the curcuminoid analogs tested does not change the performance of the test drug. This tells us that the upstream inhibition by curcumin known to us from the literature is not factoring into curcumin III's pharmacology in relation to the MSK1 changes. In addition, it must be considered that the typical curcumin extract studied and shown in historical research to inhibit PKC, upstream of the MAPK pathway, would have a low bisdemethoxycurcumin (curcumin III) content that would not likely participate in the overall pharmacology. From here it must be concluded that another

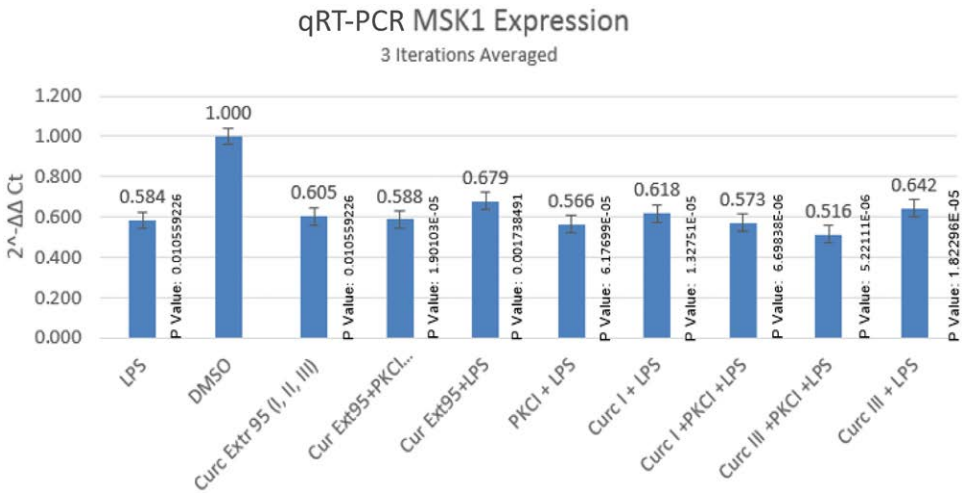

Fig 5. Results from qRT-PCR showing little change of MSK1 mRNA by curcumin or the curcuminoids over changes induced by LPS. *P* Values are included. Nevertheless, the curcumin extract does have a downregulatory influence on MSK1 transcription as well. Curc Extr 95, Curcumin Extract 95%; Curc I, curcumin I; Curc II, curcumin II; Curc III, curcumin III.

mechanism is involved in the downregulation of the MSK1 protein in both the cytoplasmic and nuclear compartments as seen in the graphed Western blot data of Fig 4.

qRT-PCR of MicroRNA

It is well established that the curcumin extract induces a significant influence on a myriad of microRNAs.[34–37] Some are upregulated and others downregulated. Nevertheless, the typical curcumin extract does not contain significant levels of bisdemethoxycurcumin (curcumin III), and the "curcumin" studied and reported on historically is the typical extract. It is therefore reasonable to assume that the full microRNA spectrum influenced by curcumin could be void of the microRNA(s) that may be modulated by a functional curcumin III concentration. Historical results evaluating curcumin's activity on microRNAs do not include modulation of MIR 148a. It is well established that MSK1 status correlates inversely with MIR 148a status, and it has been concluded historically that MIR 148a inhibits MSK1 translation.[23,38,39]

This rationale led to our next target, MIR 148a, as a possible candidate to be involved in the mechanism for MSK1 modulation by curcumin III. Fig 6 presents our findings showing curcumin I has little or no influence on MIR 148a status. Nevertheless, as speculated, curcumin III has a profound upregulatory influence on MIR 148a. The curcumin III test shows an upregulation to increase MIR 148a status by 11.85%. The curcumin III + LPS test results in an upregulation of the microRNA by an incremental 29.25%. These data are generated from 2 biological repeats with a compilation of internal repeats to produce 6 iterations in total that are averaged. *P* values are also presented to support the data.

Curcumin II surprisingly exhibited an opposing pharmacology to that of curcumin III to downregulate MIR 148a by 17.25% for the curcumin II only treatment group. The MIR 148a status was reduced by as much 37.16% for the curcumin II plus LPS group.

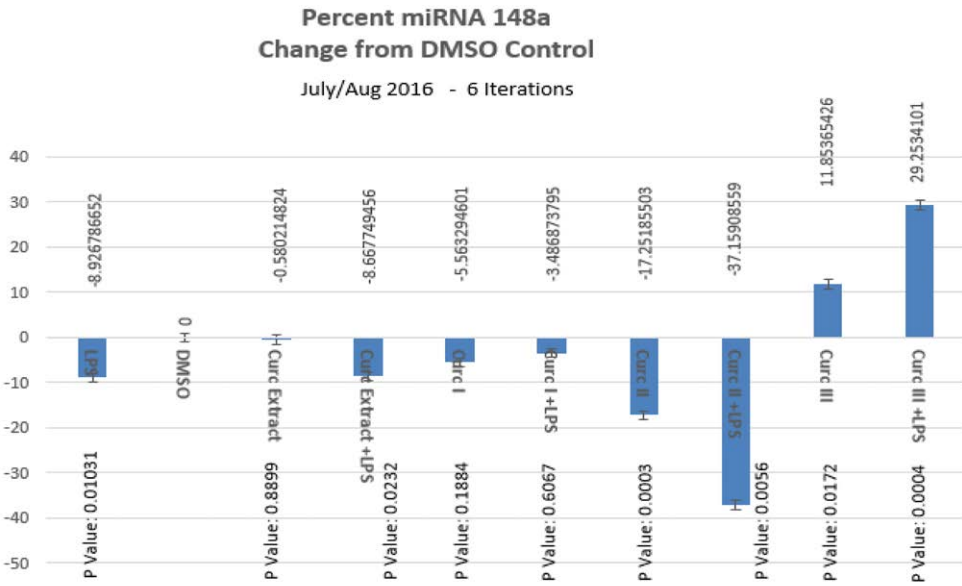

Fig 6. Curc III upregulates MIR 148a by an incremental 11.85%. The Curc III + LPS test results in an incremental 29.25% MIR 148a status. Curc II downregulates MIR 148a by 17.25% for the Curc II only treatment group, and reduced MIR 148a status by 37.16% for the Curc II + LPS group. *P* values are also presented to support the data. Curc I indicates curcumin I; Curc II, curcumin II; Curc III, curcumin III.

This finding sheds some light on how the typical curcumin extract can be utilized with greater precision if the inherent curcuminoid analog proportions are altered. It also sheds light on how the regular curcumin extract (95%) with a typical curcumin II concentration above 15% and a relatively low counter force by a low curcumin III concentration below 2% could theoretically downregulate an already suppressed MIR 148a status to subsequently escalate MSK1 where it might adversely influence some disease pathologies.

Discussion

The research results reported here may help define curcumin's pharmacology on key nuclear targets that participate in shaping the activity of the NF-kB transcription factor transactivation and at the point of translation where cytokine transcription might be better regulated. The work is designed to identify the ultimate influence by the extract's principal components individually in their isolated forms. This is proposed to help produce a better picture of the mechanism(s) by which the curcumin polypharmacology is exhibited. Not only does it allow us to map cumulatively the regulation of inflammatory events by the extract's components, this work will also help define guidelines that support the engineering of unique curcuminoid compositions with more target or indication specificity.

It is well known that curcumin inhibits NF-kB activation by way of IKK inhibition and even inhibits transactivation of the transcription factor.[14,40,41] However, the method previously understood—inhibition of IKK, and ultimately inhibition of I-kappa-B phosphorylation and p65–p50 translocation[40,42,43]—may not be the whole story. Translocation inhibition may be a very small factor (and not a factor at all according to our findings with this macrophage model) in the overall pharmacology of curcumin under some conditions as our research shows.

It appears that the BV2 experimental data do not support an incremental change in p65 serine^{276} phosphorylation status by LPS induction. Phosphorylation of this site in nuclear p65 is relatively high for both LPS-induced BV2 cells and those not induced by LPS. The presence of p65 in the nucleus may coincide with other factors that promote activation or more specifically, p65 serine^{276} phosphorylation, with an objective to maximize transactivation. Nevertheless, the curcuminoid analogs do have an inhibitive influence on phosphorylation at this residue whether LPS stimulation is applied or not. The inhibitive pharmacology reported here by each of the curcuminoids on p65 serine^{276} phosphorylation helps define a more selective pharmacology for this drug and a better understanding for why and how it might be used to treat disease. In this role, the curcuminoid treatment might serve to counter dysregulated NF-kB circumventing transcriptional activity of constitutively activated and nucleotranslocated NF-kB p65–p50. I-kappa-B mutations, for example, can result in constitutive p65–p50 translocation, disease pathology characteristics associated with autoinflammatory and autoimmune disease pathologies.[7,44]

The search in this process for the upstream mechanism that might be responsible for p65 serine^{276} phosphorylation inhibition led to the MAPK pathway. The MAPK pathway interacts and shapes NF-kB signaling activity at multiple points including MSK1's pivotal role in the nucleus as a regulator of NF-kB (p65–p50) transactivation.[45] MSK1 takes part by orchestrating multiple requisite transactivation elements including key transcription factors and cofactors at the promoter region of NF-kB's cognate genes.[46,47] In addition to the conventional therapeutic potential of curcumin, individuals with predispositions to NF-kB (p65–p50) dysregulation might be served well by the administration of a properly designed curcumin drug as a reasonable low risk and, now, even more substantiated prophylactic application. MSK1 is shown to play a role in p65 serine^{276} phosphorylation[27,48]; therefore, our findings that this upstream kinase is modulated by the curcuminoids is not a surprise. More work is needed to demonstrate the therapeutic potential in clinical applications.

The quest to search for upstream mechanisms responsible for this MSK1 modulation by the curcuminoids led to MIR 148a. It is shown in the literature that ectopic expression of MSK1 is associated with disease and the common upstream factor contributing to, or more simply associated with, the MSK1 dysregulation is MIR 148a downregulation.[23] MicroRNAs have increasingly been reported to be important role players in the regulation of a wide range of biologic processes.[49] More and more microRNAs are implicated in disease pathology including immune-mediated diseases and tumorigenesis[50]; their downregulation is shown to be a function of epigenetic influences that silence the upstream gene by hypermethylation.[51] Interestingly, MIR 148a expression is shown to subsequently contribute to hypomethylation of disease implicated genes such as the case for lupus cluster of differentiation 4 (CD4+) T cell DNA.[52]

Upon studying the effects of isolated curcumin III in its pure form in our experimental models to unveil the upstream mechanism for MSK1 downregulation, upregulation of MIR 148A was, in fact, seen to be a significant discovery and a likely mechanism for the MSK1 changes. This posttranscriptional influence by MIR 148a unveils the likely mechanism by which MSK1 expression is downregulated by curcumin III. It also showed us more about the discovery in the context of health and disease management, including diseases that may be set in motion by epigenetic influences. Furthermore, it was soon established by our work that curcumin II displayed an opposing pharmacology to downregulate MIR 148a with significance as shown in Fig 6.

Hypermethylation of the gene promoter and the consequential downregulation of MIR 148a are common characteristics in human cancers, including breast and prostate cancers.[53] MIR 148a expression is shown to suppress tumor cell invasion and metastasis in gastric cancer; whereas its suppression is found to be >4 times higher in cancer cells over normal healthy cells.[54,55] MIR 148a is shown to suppress epithelial–mesenchymal transition and metastasis in hepatic cancer.[56]

Hepatitis B virus is shown to repress MIR 148a and promote tumorigenesis.[57] This microRNA is otherwise shown to repress mTOR signaling inhibitive of tumorigenesis.[57] Silencing of MIR 148a is also recently shown to be an early event in pancreatic cancer.[51] Glioblastoma is characterized by changes in microRNA status including downregulation of MIR 148a.[58] This implicates MIR 148a as a central target for cancer regulation in line with the recent trend toward microRNA investigations in the context of cancer and immune-mediated diseases.

However, before drug design guidelines can be defined and the proper role of curcumin in such therapies determined, much work is needed to redefine a standardization protocol for the extract and its subfraction curcuminoids, including the acquisition of more knowledge about the pharmacology of each curcuminoid analog in isolation. Identification, quantification, and labeling of all 3 curcuminoid analogs on supplement or drug labels containing the typical curcumin extract are inconsistent in industry today. This lack of consistency persists due to lack of knowledge about the biologic activity of each of the curcuminoid analogs comprising the curcumin extract and an insistence that curcumin I (diferuloylmethane) is the principal effector.

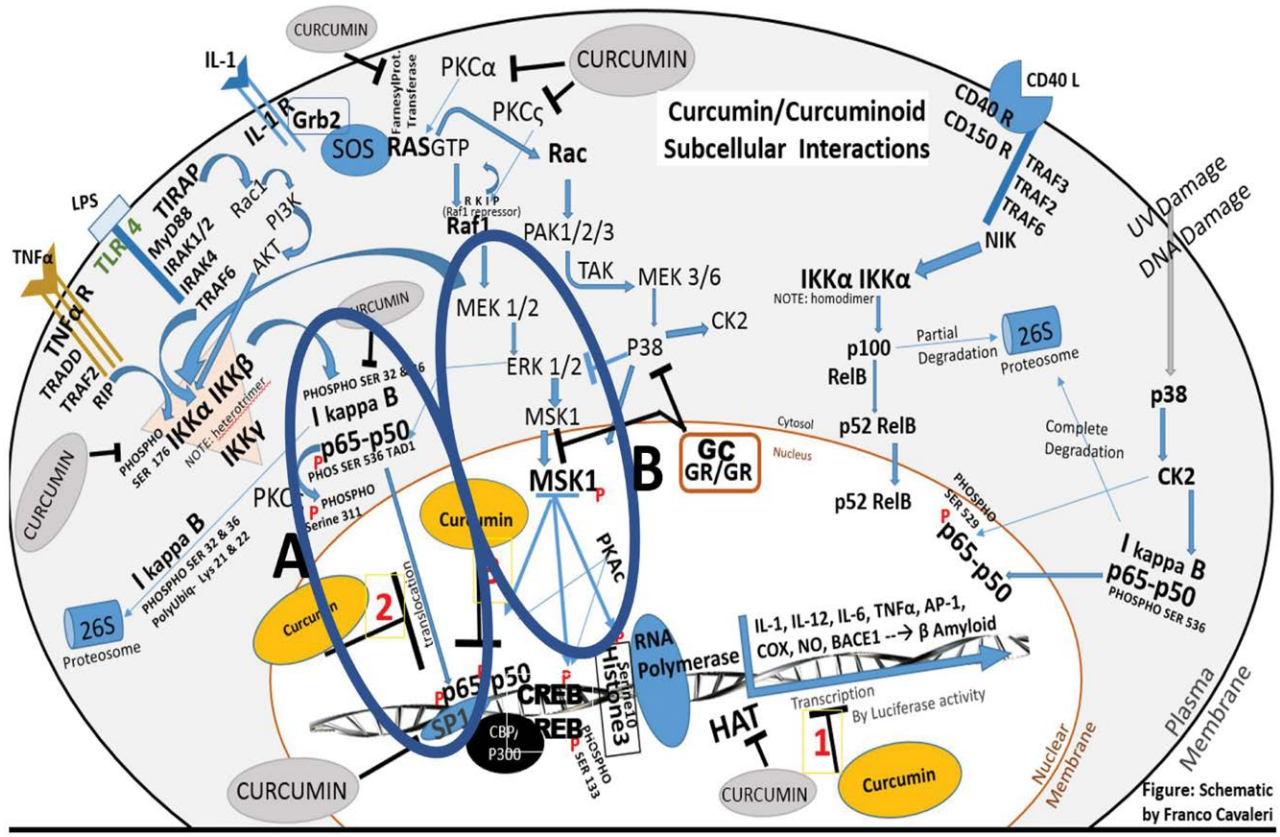

Fig 7. Subcellular pathways related to NF-kB p65–p50 translocation and transactivation (NF-kB signalling pathway segment being studied, labeled "A"). This image includes the portion of the MAPK signalling pathway and the MSK1 cross-talk with p65 being studied, labeled "B."

Complex natural medicines must be studied one constituent at a time to better understand the pharmacology of the whole. Curcumin is demonstrating to be a formidable model by which we can study other natural medicines to better understand, hone, and predict therapeutic potential. As portrayed by the schematic of Fig 7, it does seem that curcumin's influence on multiple subcellular targets contributes to shaping of NF-kB (p65–p50) transactivation. These activities are shown to ultimately converge on the transcription factor to modulate multiple points in the signaling pathway. The 2 pathways being studied here—NF-kB signaling and MAPK signaling—are highlighted and labeled A and B on this Fig 7 schematic showing the MSK1 cross-talk with p65 in the nucleus.

Our findings also reveal for the first time that curcumin III does not typically participate in curcumin pharmacology. Nevertheless, if the curcumin composition allows for a higher curcumin III portion, it may play a role in the pharmacology on key drug targets associated with disease pathology; namely, MSK1 and MIR 148a. The curcuminoid analog pharmacology interacts with the complex NF-kB activity in a comprehensive manner. When we consider the evolutionary development of this polypharmacologic agent or others like it, it should not come as a complete surprise to find what is being discovered here and how it might be considered a controlled polypharmacology with a strategic outcome. This may be especially so in the context of highly conserved signaling pathways that have themselves coevolved for thousands of years to regulate transcriptional events.[59]

These findings help justify curcumin pharmacology as a complementary or alternative drug treatment where anti-inflammatory applications such as glucocorticoid drugs may be required to treat inflammatory and immune-mediated disease. Research shows that, for example, a mechanism by which glucocorticoids exhibit pharmacology is through altered subcellular distribution of MSK1 contributing to NF-kB inhibition in a similar way that is shown here for curcumin III.[60] The newly discovered curcuminoid pharmacology reveals a mechanism by which curcuminoids may inhibit cancer cell survival even in the context of oncogene expression.[61,62]

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Disclosure

The author is the owner of curcumin-based intellectual properties. The author is the owner, CEO, and a primary investigator of a research corporation (Biologic Pharmamedical Research) that funds and executes research on nutraceutical and pharmaceutical pharmacology including research of curcuminoids and development of curcumin-based therapeutic agents. The Article Processing Charge was paid for by the author.

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