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Research Article

Does Curcumin Analogues, Demethoxycurcumin (DMC) and Bisdemethoxycurcumin (BDMC), Enhance the Therapeutic Efficacy of Curcumin in the Treatment of Rheumatoid Arthritis (RA)?

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ABSTRACT

Curcumin has been used as an anti-inflammatory agent, however recent studies have shown that it is a combination of three compounds namely; curcumin, Demethoxycurcumin (DMC), Bisdemethoxycurcumin (BDMC). This research evaluates the most effective curcuminoid for treating rheumatoid arthritis (RA) by monitoring changes in the expression levels of 10 inflammatory response genes namely, COL14A1, CXCL12, CYTL1, HSPA6, IFTIM1, IL-6, IL-7, MMP-1, MMP-13 and TNFSF10.

The expression profiles of the candidate genes were determined by qRT-PCR analysis using untreated and curcumin, DMC, or BDMC treated Human Fibroblast like Synoviocytes (HFLS-RA) cells. Methotrexate (MTX) was used as a positive control. The level of C-reactive protein (CRP) in the cell culture supernatant was determined using ELISA assays.

The untreated HFLS-RA cells exhibited high expression levels of these genes which subsequently was reduced following treatment. Curcumin exhibited increased potency in downregulating the gene expression of COL14A1, CYTL1, IFITM1, IL-7, MMP-1, MMP-13 and TNFSF10 in comparison to the other treatment options. The CRP level decreased on treatment with the curcuminoid mixture, curcumin and DMC, which correlated with the IL-6 transcription profile.

The presence of DMC and BDMC were found to limit curcumin's efficacy as a mixture since curcumin alone was found to be the most effective anti-rheumatic agent.

Keywords: Curcuminoids; Curcumin; Gene expression; C-reactive protein; qRT-PCR; ELISA assay; Rheumatoid arthritis; Anti-rheumatic agents

ABBREVIATIONS

RA: Rheumatoid Arthritis; MTX: Methotrexate; DMC: Demethoxycurcumin; BDMC: Bisdemethoxycurcumin; TNF-α: Tumour Necrosis Factor-α (TNF-α); NFkB: Nuclear Factor Kappa B; HFLS-RA: Human Fibroblast Like Synoviocytes-Rheumatoid Arthritis; SSZ: Sulfasalazine; CBR2: Cannabinoid Receptor 2; ABCC1-3: ATP Binding Cassette Subfamily C Member 1-3; CRP: C-Reactive Protein; IC_{50}: Inhibitory Concentration; mRNA: Messenger RNA; ELISA: Enzyme-Linked Immuno Sorbent Assay; COL14A1: Collagen alpha-1(XIV) chain; CXCL12: C-X-C motif Chemokine 12; CYTL1: Cytokine-Like Protein 1; HSPA6: Heat shock 70 kDa Protein 6; IFTIM1: Interferon-Induced Transmembrane Protein 1; IL-6: Interleukin 6; IL-7: Interleukin 7; IL-7R: Interleukin 7 Receptor; MMP-1: Matrix Metalloproteinase-1; MMP-13: Matrix Metalloproteinase-1; TNFSF10: Tumor Necrosis Factor Ligand Superfamily Member 10; qRT-PCR: Quantitative Real-Time Polymerase Chain

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Reaction; ERK1/2: Extracellular Signal-Regulated Kinases; MAPK: Mitogen-Activated Protein Kinase; JNK: c-Jun N-Terminal Kinases; PKB/AKT: Protein Kinase B; c/EBPβ: CCAAT/Enhancer-Binding Protein Beta; TGF-β: Transforming Growth Factor Beta; HIF-α: Hypoxia-Inducible Factor Alpha; Flt3: Fms Related Tyrosine kinase 3; HSP: Heat Shock Protein; IFNγ: Interferon Gamma; JAK-STAT: Janus Kinase-Signal Transducer and Activator of Transcription; MHC: Major Histocompatibility Complex; PI3K: Phosphoinositide 3-kinase; IL1β: Interleukin 1 beta; AP-1: Activator Protein 1; MIF: Macrophage Migration Inhibitory Factor; CXCR4: C-X-C Chemokine Receptor Type 4; DMSO: Dimethyl Sulfoxide; ECACC: European Collection of Authenticated Cell Cultures.

INTRODUCTION

Rheumatoid Arthritis (RA) is a debilitating, chronic autoimmune disease leading to progressive joint destruction due to a cycle of perpetual inflammation [1]. The current first line 'gold-standard' treatment for RA is Methotrexate (MTX), however undesirable side effects such as hepatotoxicity results in early treatment termination and poor patient compliance (Figure 1) [2,3]. Therefore, natural therapeutic agents with high safety profiles are required.

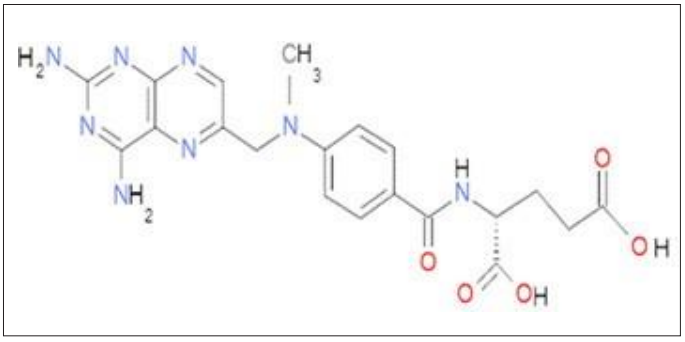

Figure 1: Chemical structure of methotrexate, the current first line treatment for RA.

Turmeric is an oriental spice frequently used as a food-flavoring ingredient and is obtained from the rhizome of Curcuma Longa grown abundantly in South Asian countries. Turmeric contains an active compound called curcumin and its two analogues, Demethoxycurcumin (DMC) and Bisdemethoxycurcumin (BDMC) (Figure 2) [4]. *In vitro* and *in vivo* reports have shown different therapeutic activities exerted by these curcuminoids. Curcumin is the most potent curcuminoid, followed by DMC and then BDMC. This is attributed to the varying number of methoxy groups present in the structure, which contributes towards their differing efficacies. Reports have suggested that curcumin and DMC exhibit the most potent anti-inflammatory and antioxidant properties whereas BDMC is more effective in the treatment of malignancies [5].

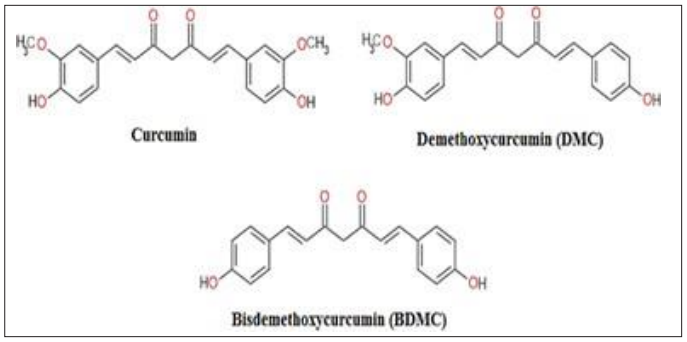

Figure 2: Chemical structure of the active ingredient present in turmeric, curcumin, alongside its analogues DMC and BDMC.

Commercially available supplements of curcumin contain varying proportions of the curcuminoids, which is not specifically labelled on the product packaging and thereby misleads consumers into purchasing curcumin supplements claiming a high degree of purity [6]. Research has shown that curcumin lowers the expression of tumour necrosis factor (TNF-α), downregulates nuclear factor kappa B (NFκB) an dprevents the release of pro-inflammatory cytokines (IL-1, IL-2, IL-6, IL-8 and IL-12). Furthermore, clinical trials have reported a high safety profile of curcumin in RA patients with a significant reduction in joint swelling and stiffness [7].

Further work is required to verify the efficacy of curcumin, individually and in a mixture in order to establish the best therapeutic reagent for RA treatment. Recent microarray studies carried out within our laboratory identified 10 candidate genes, COL14A1, CXCL12, CYTL1, HSPA6, IFTIM1, IL-6, IL-7, MMP-1, MMP-13 and TNFSF10 that were differentially expressed in untreated Human Fibroblast-like Synoviocytes-Rheumatoid Arthritis (HFLS-RA) cells and MTX treated cells [8]. Herein, we report the outcome of the curcuminoid mixture, curcumin, DMC and BDMC treatments on the expression levels of these 10 candidate genes using qRT-PCR analysis. MTX was selected as a positive control and the untreated HFLS-RA cells were used as the negative control in this study.

MATERIALS AND METHODS

Tissue culture conditions

Human Fibroblast-like Synoviocytes (HFLS-RA) were purchased at passage 2 from the European Collection of Cell Cultures (ECCAC, UK) and propagated in Synoviocytes Growth Medium (ECACC, UK) containing basal medium and growth supplements. The cells were limited to between 5-7 population doublings and were cultured to 70%-80% confluence in a humidified incubator at 37°C, 5% CO_2 and filtered air.

Drug preparation

The pure powder forms of the compounds were purchased and reconstituted in DMSO at stock concentrations of 13.5 mM turmeric (curcumin from Curcuma Longa (Sigma Aldrich, UK)), 13.5 mM curcumin (Tocris, UK), 14.7 mM demethoxycurcumin (Sigma Aldrich, UK), 16.2 mM bisdemethoxycurcumin (Sigma Aldrich, UK) and positive control: 38.9 mM MTX (Tocris, UK). The DMSO content present in the final concentrations prepared and used in tissue culture treatment did not exceed 0.02% (non-toxic threshold for the cells).

Cell viability assay

The HFLS-RA cells were seeded in triplicate in 96-well plates at a density of 2×10^3 cells/well and incubated for 24 h at 37°C before the addition of various drug concentrations (turmeric, curcumin, DMC, BDMC and MTX). The control for each drug condition was the untreated cells and the plates were incubated for 48 h. The change in cell viability was monitored using CellTiter-Glo luminescent cell viability assay (Promega, UK) and Tecan GENios Pros (Tecan, Austria). The inhibitory concentration (IC_{50}) values (mean ± standard deviation) was established by analyzing the dose dependent inhibitory effect of the compounds.

Drug treatment for qRT-PCR analysis

HFLS-RA cells were grown in 75 cm^2 culture flasks and treated with IC_{50} concentrations of the five compounds turmeric, curcumin, DMC, BDMC and MTX. Following 48 h incubation at 37°C, the untreated cells (control) and drug treated cells were harvested and stored at -20°C for qRT-PCR analysis.

mRNA isolation and cDNA synthesis

Messenger RNA (mRNA) was isolated using mRNA isolation kit (Roche Diagnostics, Germany) per the manufacturer's instructions. The concentration and purity of the isolated mRNA samples were determined using Nano Drop (ND-1000) Spectrophotometer (ThermoFisher Scientific, USA). The mRNA samples were reverse transcribed into cDNA using First-Strand cDNA Synthesis Kit (Sigma Aldrich, UK). For cDNA synthesis, 100 ng of mRNA was aliquoted into 11.8 µl of the reaction mixture containing 2 µl 10X reaction buffer, 4 µl 25 mM $MgCl_2$ stock solution, 2 µl Primer Oligo-$p(dT)_{15}$, 2 µl Deoxynucleotide Mix, 1 µl RNase Inhibitor and 0.8 µl Reverse Transcriptase AMV. The final volume of 20 µl was attained by the addition of PCR grade water. The cDNA reaction mixtures were incubated at 25°C for 10 min, followed by 42°C for 1 h and then at 99°C for 5 min and then stored at -37°C [9].

qRT-PCR analysis

The expression levels of COL14A1, CXCL12, CYTL1, HSPA6, IFITM1, IL-6, IL-7, MMP-1, MMP-13 and TNFSF10 before and after treatment were monitored using Applied Biosystems 7000/Real-Time PCR system (ThermoFisher Scientific, USA) with the primers (Table 2) (TIB MOLBIOL, Germany) and LightCycler® FastStart DNA Master PLUS SYBR Green I kit (Roche Diagnostics, Germany). Each primer was reconstituted in PCR grade water as per manufacturer's instructions and stored at -20°C [10-14]. LightCycler® FastStart DNA Master PLUS SYBR Green master mix was prepared by the addition of LightCycler® FastStart enzyme to the reaction mix and stored away from light at 40°C. The 20 µl PCR mix comprised of 2 µl cDNA template, 12 µl PCR grade water with 1 µl of sense and 1 µl antisense primer and 4 µl of SYBR. qRT-PCR run was performed using the following cycle parameters; pre-incubation stage at 50°C for 2 min, 95°C for 10 min, amplification stage for 35 cycle at 95°C for 10 min, variable primer temperature for 10 s, 72°C for 45 s and dissociation stage at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s.

The copy number calculation of qRT-PCR data analysis was carried out using a standard curve of known genomic DNA concentrations and mRNA copy numbers were extrapolated from the Ct values of the untreated and treated samples [9].

C-reactive protein ELISA assay

HFLS-RA cell culture supernatant was used to determine the levels of C-reactive proteins (CRP) present in the samples (untreated and treated) using the CRP enzyme-linked immunosorbent assay (ELISA) kit (Abcam, USA) per manufacturer's instructions. FLUOstar OPTIMA (BMG Labtech, Germany) plate reader measured the optical density at 450 nm and samples CRP levels were extrapolated using a standard curve of known protein concentrations.

Statistical analysis

The inhibitory concentration (IC_{50}) for cell viability and qRT-PCR data were analyzed using Microsoft Excel. The mean and standard deviation were obtained from three independent experiments. The copy numbers of untreated and treated HFLS-RA cells were matched by pair wise comparison in order to derive the fold change difference. The gene expression data was analyzed using IBM SPSS Statistics 23 software by one-way ANOVA analysis [25-30]. This was followed by post-hoc analysis in which Tukey HSD (honest significant difference) test was carried out. The threshold level for significance was set at *≤ 0.05 and ** ≤
0.001 and the p values below this threshold were considered as statistically significant.

RESULTS AND DISCUSSION

IC_{50} of the compounds on HFLS-RA cells

The IC_{50} concentration determination for each compound was carried out following 48 h incubation at 37°C. The treatment of HFLS-RA cells with turmeric, curcumin, DMC, BDMC and MTX induced apoptosis of 50% cell population at concentrations of 18.1 (± 1.1) µM, 24.1 (± 0.6) µM, 24.2 (± 3.2) µM, 38.8 (± µM and 278.7 (± 2.0) µM, respectively. The concentrations determined for curcuminoids present in turmeric in the ratio of 80:16:4-curcumin: 14.48 µM, DMC: 2.89 µM and BDMC: 0.72 µM.

Gene expression analysis

The mRNA transcription levels of the 10 candidate genes COL14A1, CXCL12, CYTL1, HSPA6, IFTIM1, IL-6, IL-7, MMP-1, MMP-13 and TNFSF10 were measured using qRT-PCR in untreated and treated HFLS-RA cells with turmeric, curcumin, DMC, BDMC and MTX. In this study, the negative control was the untreated HFLS-RA cells and the positive control was the MTX treated HFLS-RA cells. Treated HFLS-RA cells showed varying expression of the 10 candidate genes compared to the untreated condition (Table 1). Fold change difference was determined for the 10 candidate genes by comparing each treated condition against untreated (Table 2).

CRP ELISA analysis

C-reactive protein Human SimpleStep ELISA kit was used to determine the concentration of CRP present in the tissue culture supernatant of HFLS-RA cells for untreated and treated conditions. The unknown samples were quantified using a standard curve of known CRP concentrations (0-1000 pg/ml). The average CRP values for untreated cells, HFLS-RA cells treated with turmeric, curcumin, DMC, BDMC and MTX were 14.98 (± 1.06) pg/ml, 3.15 (± 0.99) pg/ml, 2.77 (± 0.33) pg/ml, 4.51 (± 1.95) pg/ml, 6.29 (± 0.57) pg/ml and 1.31 (± 0.69) pg/ml, respectively (Figure 3).

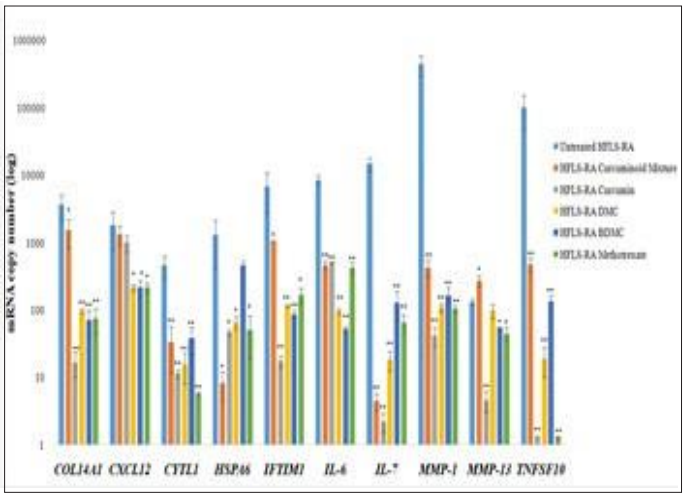

Figure 3: qRT-PCR results for the 10 candidate genes using HFLS-RA cells.

Gene transcriptional levels of the 10 candidate genes, the untreated HFLS-RA cells were compared to the treated condition (curcuminoid mixture, curcumin, DMC, BDMC and MTX). Data presented (n=3, ± SD).

Name of gene	Untreated	Curcuminoid Mixture	Curcumin	DMC	BDMC	MTX
COL14A1	3676 ± 1498	1526 ± 689	17 ± 7	98 ± 7	71 ± 29	75 ± 30
CXCL12	1820 ± 1011	1351 ± 436	1005 ± 277	216 ± 23	217 ± 56	216 ± 34
CYTL1	464 ± 172	34 ± 22	12 ± 2	16 ± 10	39 ± 17	6 ± 0
HSPA6	1287 ± 883	8 ± 4	47 ± 4	66 ± 14	471 ± 66	51 ± 31
IFITM1	6841 ± 4047	1071 ± 6	18 ± 4	117 ± 2	88 ± 11	169 ± 45
IL-6	8504 ± 1403	462 ± 64	510 ± 4	97 ± 8	53 ± 5	428 ± 87
IL-7	14840 ± 3214	5 ± 1	2 ± 1	19 ± 6	130 ± 60	65 ± 19
MMP-1	444768 ± 146191	425 ± 123	42 ± 14	110 ± 15	167 ± 50	105 ± 14
MMP-13	134 ± 13	270 ± 48	5 ± 2	99 ± 23	54 ± 1	45 ± 10
TNFSF10	101003 ± 53172	480 ± 112	1 ± 0	19 ± 9	135 ± 29	1 ± 0

Table 1: mRNA copy numbers for the candidate genes using the HFLS-RA cells. mRNA copy numbers observed for 10 candidate genes for each condition (untreated and treated HFLS-RA cells). Data presented (n=3, ± SD).

Name of gene	Curcuminoid Mixture	Curcumin	DMC	BDMC	MTX
COL14A1	-2	-216	-38	-52	-49
CXCL12	-1	-2	-8	-8	-8
CTL1	-14	-39	-29	-12	-77
HSPA6	-161	-27	-20	-3	-25
IFITM1	-6	-380	-58	-78	-40
IL-6	-18	-17	-88	-160	-20
IL-7	-2968	-7420	-781	-114	-228
MMP-1	-1047	-10590	-4043	-2663	-4236
MMP-13	2	-27	-1	-2	-3
TNFSF10	-210	-101003	-5316	-748	-101003

Table 2: Fold change difference determined using qRT-PCR analysis.

Fold change difference calculated for 10 candidate genes of the treated HFLS-RA cells against the untreated condition (Table 3).

Name of gene	Stimulus	Activates	Activity trend of compounds highest to lowest	Literature Findings	Ref
COL14A1	Cartilage oligomeric matrix protein and fibromodulin	Fibrillogenesis	CUR>BDMC>DMC>MIX	Curcumin inhibits fibril formation and destabilizes preformed fibrils	[10-12]
CXCL12	Hypoxia, toxins, irradiation, IL-1, IL-6	ERK1/2, MAPK, JNK and AKT effectors	DMC=BDMC>CUR>MIX	Curcuminoids are reported to target and inhibit c/EBPβ and TGF-β/ HIF-α pathways	[13-16]
CTL1	Fms Related Tyrosine Kinase 3 (Flt3), Thrombopoietin, stem cell factor	Recruitment of inf lammat or y cells to synovium	CUR>DMC>MIX>BDMC	Studies have shown curcuminoids decreasing Flt3 expression in leukemic cell lines	[17-19]
HSPA	Pro-inf lammatory cytokines and chemokines, cellular stress	MAPK, NFkB, HSP70 pathway induction	MIX>CUR>DMC>BDMC	Turmeric is a well-documented inhibitor of MAPK, NFkB signal transduction pathways	[20,21]
IFITM1	Interferon-gamma (IFNγ)	JAK-STAT pathway	CUR>BDMC>DMC>MIX	Curcumin is reported to be an inhibitor of IFNγ signalling by regulating Stat1 phosphorylation and transcription of IFNγ inducible MHC-II genes	[22-24]

IL-6	T cell, macrophages and fibroblasts	B and T cell activation and proliferation, inflammation, NFkB, MAPK, STAT	BDMC>DMC>MIX>CUR	Anti-inflammatory effects of curcuminoids have been reported on IL-6 expression by inhibiting NFkB, MAPK and STAT signalling pathways	[25,26]
IL-7	TNF-α/β, IL-1, IL-17, stromal cells, epithelial, fibroblasts, smooth muscle and dendritic cells	Stimulates macrophages to produce TNF-α (maintains positive feedback), JAK/STAT, PI3K, AKT pathways	CUR>MIX>DMC>BDMC	Curcumin exerts its anti-inflammatory effect by blocking the signal transduction pathways, JAK/STAT, phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) which are activated upon binding of IL-7 to its receptor, IL-7R	[27,28]
MMP-1	TNF-α, IL-1β, IL-6, growthfactors matrix molecules, MAPK p38δ	Type III collagen degradation	CUR>DMC>BDMC>MIX	Curcumin inhibits transcription factors, AP-1, MAPK/ERK1/2, IL-6, TNF- α	[29-31]
MMP-13	IL-1β, TNF-α	Type II collagen degradation, T cell infiltration	CUR>BDMC>DMC>MIX	Curcumin decreases the levels of IL-1 and TNF-α by regulating the transcription of NFκB, AP-1, MIF and MAPK	[32-34]
TNFSF10	MMPs and pro- inflammatory cytokines	Caspases, PI3 kinase/MAPK signalling pathway	CUR>DMC>BDMC>MIX	Curcumin inhibits p38 and ERK1/2 MAPK pathways and thereby reducing TNFSF10 proliferative activity on synovial fibroblasts by suppressing downstream activation of pro-inflammatory cytokines	[33-35]

Table 3: The activity trend observed for the 10 genes for curcuminoids treated HFLS-RA cells.

Name of gene	Sequence of Primers	Amplicon size (bp)	Annealing Temp (℃)
COL14A1	Sense: 5' AGACGAGGTGGTGGTAGATG 3'	106	56
	Antisense: 5' AGCAGTGTGGGCATAGATTG 3'		
CXCL12	Sense: 5' GACAAGTGTGCATTGACCCG 3'	173	58
	Antisense: 5' CTCATGGTTAAGGCCCCCTC 3'		
CYTL1	Sense: 5' AGATCACCCGCGACTTCAAC 3'	77	58
	Antisense: 5' GTACAGCCTGGGCAGGTATC 3'		
HSPA6	Sense: 5' AATCTGTCGCCCCATCTTCTC 3'	174	59
	Antisense: 5' GCCCATAGCATAGCCCTGAC 3'		
IFITM1	Sense: 5' CGCCAAGTGCCTGAACATC 3'	87	57
	Antisense: 5'GTCACAGAGCCGAATACCAGT 3'		

IL-6	Sense: 5'-GGTACATCCTCGACGGCATCT-3'	81	59
	Antisense: 5'-GTGCCTCTTTGCTGCTTTCAC-3'		
IL-7	Sense: 5' GTGACTATGGGCGGTGAGAG 3'	141	59
	Antisense: 5'GCTACTGGCAACAGAACAAGG 3'		
MMP-1	Sense: 5'-AGTGACTGGGAAACCAGATGCT-GA-3'	249	62
	Antisense: 5'-GCTCTTGGCAAATCTGGCCTG-TAA-3'		
MMP-13	Sense: 5'-CGCGTCATGCCAGCAAATTC-CATT-3'	206	64
	Antisense: 5'-TCCATGTGTCCCATTTGTGGT-GTG-3'		
TNFSF10	Sense: 5' TGGGCATTCATTCCTGAGCA 3'	525	63
	Antisense: 5' GGTTGTGGCTGCTCTACTCA 3'		

Table 4: Primer sequences used in qRT-PCR analysis.

Commercially available turmeric exists as a mixture of curcumin and its analogues, DMC and BDMC in a ratio of 80:16:4. In this study, HFLS-RA cells isolated from the joints of RA patients were used as an in vitro model to assess the anti-rheumatic efficacy of drug treatments (Table 4). Fibroblast-like synoviocytes are major contributors in the secretion of pro-inflammatory mediators, cytokines and proteolytic enzymes for extracellular matrix degradation, which provides a suitable model for measuring gene expression in RA [36]. Therefore, a comprehensive understanding of RA pathogenesis could be achieved by targeting HFLS-RA cells, thereby improving the clinical outcome of the disease and lowering adverse effects associated with the use of conventional therapies such as MTX and sulfasalazine (SSZ).

HFLS-RA cells were treated with the curcuminoids, individually and as a mixture. The curcuminoid mixture was found to be the most potent and reduced cell viability by 50% after 48 h at a concentration of 18 μM. This was followed by curcumin (24 μM), DMC (24 μM) and BDMC (39 μM). The methoxy groups present in curcumin and DMC contribute towards apoptosis and the analogues in combination exert a synergistic effect [37]. A plausible explanation for increased potency of the curcuminoid mixture includes, the activation of intrinsic and extrinsic pathways by curcumin and DMC, whereas BDMC induces apoptosis via cannabinoid receptor 2(CBR2) [38]. These results collectively indicate that curcuminoids in a mixture have a higher efficacy in initiating cell death, compared to the individual compounds.

Here we also report the ability of curcuminoids to lower mRNA transcription levels of the inflammatory response genes before and after treatment by qRT-PCR analysis (Figure 3). We have previously demonstrated that these genes are significantly upregulated in HFLS-RA cells when compared to normal healthy HFLS cells [8]. As an important mediator of fibril development, the increased expression of COL14A1 contributes towards fibrosis which often occurs after inflammation [39]. The curcuminoid mixture failed to exhibit increased potency in downregulating this target gene, whereas the analogues on their own exerted a highly significant response ($p<0.001$). Curcumin displayed the most significant downregulatory response which correlates with its role in suppressing fibrillogenesis.This is attributed to the increased affinity of curcumin to bind to β-amyloid peptides [10]. The destabilization of pre-formed fibrils counteracts the effects of bone destruction, thereby maintaining the integrity of fibrous structures in the synovium.

Tissue damage as a result of inflammation leads to elevated cellular levels of CXCL12 which codes for the ligand of the CXCR4 receptor. Induction of CXCL12 gene transcription is caused by IL-1 and IL-6 through the CAAT/-enhancer binding protein β(C/EBPβ) pathway and by hypoxia through the Transforming Growth Factor-β/Hypoxia-inducible factor 1-α(TGF-β/HIF-1α) axis [11]. Interestingly, the curcuminoid mixture together with curcumin, failed to effectively decrease mRNA transcription levels, whereas DMC and BDMC displayed a significant downregulation of CXCL12 ($p<0.05$). Curcuminoids are reported to inhibit C/EBPβ and TGF-β/HIF-1α pathways, suggesting a possible mechanism by which CXCL12 expression is decreased. Hypoxia exposure may lead to curcumin resistance in synovial fibroblasts through ATP Binding Cassette Subfamily C Member 1-3(ABCC1-3) which could be a possible reason for the increased potency of DMC and BDMC [40,41].

CYTL1 plays a role in signaling via the CCR2 chemokine receptor in order to recruit inflammatory cells to the synovium [13]. Our results once again highlighted the potent therapeutic efficacy of curcumin in comparison to the curcuminoid mixture. Blocking the activity of CCR2 is an attractive target for the treatment of RA since *in vivo* studies have characterized CCR2 activation as being pro-inflammatory. Stem cell maintaining factors including Flt3

enhances the expression of CYTL1. Curcuminoids have been reported to inhibit Flt3 expression, subsequently downregulating CYLT1 [42].

HSPA6 expression in the inflamed synovium is expected to increase in response to a cocktail of pro-inflammatory chemokines and cytokines which correlate with the protective role of HSPA6 in prolonging the survival of synovial fibroblasts through the activation of MAPK, NFκB and HSP70 pathways [17]. In this investigation, exposure of HFLS-RA cells to the treatment groups showed the curcuminoid mixture to be the most effective in downregulating HSPA6 expression, followed by curcumin, DMC and BDMC. Turmeric is a well-documented inhibitor of MAPK and NFκB signal transduction pathways, and eventually helps in alleviating cellular stress [20]. However, repair mechanisms could be compromised by the downregulation of HSPA6 to basal levels due to its critical involvement in the refolding of denatured proteins. Therefore, low levels of HSPA6 could negatively impact RA cell survival and curcumin could possibly be a more favorable natural agent for RA treatment.

IFNγ has been implicated in the pathophysiology of RA, mainly due to its production by T helper cells and regulatory B cells. IFITM1 expression is induced in response to IFNγ which stimulates the JAK-STAT signaling pathway. This contributes towards cell death resistance in fibroblast-like synovial cells through the suppression of TRAIL-mediated apoptosis [21]. The curcuminoid mixture did not display a more potent downregulatory effect in comparison to the individual curcuminoid treatment groups. Curcumin is reported to be an inhibitor of IFNγ signalling by regulating STAT1 phosphorylation and transcription of IFNγ inducible MHC-II genes, which have been previously associated with a susceptibility to RA. Furthermore, the processes of ubiquitination are activated by curcumin, which leads to lysosomal degradation of the IFN-α/β receptor [22].

Exposure of HFLS-RA cells to the treatment of curcumin and its analogues, individually and as a mixture, lowered the expression of the pro-inflammatory cytokine, IL-6. Interestingly, BDMC exhibited greater potency in downregulating IL-6 compared to the mixture, curcumin and DMC. Curcuminoids exert an anti-inflammatory effect by inhibiting NFκB, MAPK and STAT signalling pathways that are responsible for IL-6 expression [23]. In vitro and in vivo experiments have shown IL-6 to also be the primary inducer of the acute phase protein, C-reactive protein (CRP) [25]. The serum CRP levels have been used to assess systemic inflammation in RA. However, the reliability of CRP measurement as a diagnostic tool is questionable since it is a non-specific indicator of systemic inflammation. Normal levels of CRP are found in up to 40% of RA patients which makes accurate diagnosis challenging. The results revealed that CRP levels decreased after treatment with the curcuminoid mixture, curcumin and DMC, which correlates with IL-6 mRNA transcription levels. However, the CRP analysis failed to reflect the mRNA transcriptional downregulation exhibited with the BDMC treatment. This suggests that BDMC used alone may not be an appropriate agent for RA treatment as it was unsuccessful in suppressing eventual protein expression. IL-1 and IL-17 are also involved in the production of CRP and therefore, the change in expression of these interleukins need to be monitored after BDMC treatment [43].

Curcumin significantly downregulated IL-7 expression which is often found to be elevated in RA serum and correlates with disease activity ($p<0.001$). Curcumin is able to suppress the activity of the JAK/STAT and P13K/Akt signalling pathways that are responsible for IL-7 activity where a state of perpetual inflammation is maintained through a positive feedback mechanism [44]. This is a beneficial approach in RA as it may eventually lead to a decrease in bone resorption.

The level of two important collagen and cartilage degradation enzymes, MMP-1 and MMP-13, were remarkably reduced by curcumin ($p<0.001$). Curcumin inhibits TNF-α and IL-1β, both of which elevate the expression of MMP-1 and MMP-13. Furthermore, the inhibition of MAPK/ERK1/2 signalling pathways by curcumin, blocks the transcription of both MMPs, thereby reducing the levels in the serum [27,29]. The activity of MMPs can also induce the expression of TNFSF10, which has a dual role in apoptosis and proliferation of synovial fibroblasts [32]. The levels of TNFSF10 was significantly improved after curcumin treatment ($p<0.001$). The inhibitory activity of curcumin on the downstream regulators of TNFSF10 (p38, MAPK/ERK1/2) has a positive outcome by lowering the proliferative activity of RA cells [35,45].

This study is based on an in vitro model of RA which uses HFLS-RA cells alongside qRT-PCR analysis for gene expression. Further investigations including in vivo experiments and clinical studies in humans are required to fully understand the therapeutic efficacy of curcumin and its analogues for the potential treatment of RA.

CONCLUSION

In conclusion, our data revealed that curcumin had greater anti-inflammatory action compared with its two analogues and the curcuminoid mixture. The response indicated that DMC and BDMC could hinder the therapeutic efficacy of curcumin in turmeric. However, further in vivo studies should be explored in order to gain further insight into the effect of the curcuminoids by monitoring the levels of the 10 candidate genes, indicative of rheumatic activity. In addition, curcumin could possibly be used alongside current therapies to help minimise side effects and improve the quality of life of those suffering from RA.

DISCLOSURE

The authors have no conflicts of interest to disclose.

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