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Curcumin synergistically potentiates the growth-inhibitory and pro-apoptotic effects of celecoxib in osteoarthritis synovial adherent cells

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Objectives. **Osteoarthritis (OA) is the Western world's leading cause of disability. Cyclo-oxygenase-2 (COX-2) inhibitors are efficient anti-inflammatory agents commonly used in the treatment of osteoarthritis. However, recent studies have shown that their long-term use may be limited due to cardiovascular toxicity. The anti-inflammatory efficacy of the phytochemical curcumin has been demonstrated in several *in vitro* and animal models. This study was undertaken to investigate whether curcumin augments the growth-inhibitory and pro-apoptotic effects of celecoxib in OA synovial adherent cells.**

Methods. **OA synovial adherent cells were prepared from human synovial tissue collected during total knee replacement surgery. The cells were exposed to different concentrations of celecoxib (0–40 μM), curcumin (0–20 μM) and their combination. Flow cytometric analysis was used to measure the percentage of cells with a subdiploid DNA content, the hallmark of apoptosis. COX-2 activity was assessed by measuring production of prostaglandin E_2 by enzyme-linked immunoassay.**

Results. **A synergistic effect was observed in inhibition of cell growth when the cells were exposed to various concentrations of celecoxib combined with curcumin. The inhibitory effect of the combination on cell growth was associated with an increased induction of apoptosis. The synergistic effect was mediated through a mechanism that involves inhibition of COX-2 activity.**

Conclusions. **This effect may enable the use of celecoxib at lower and safer concentrations, and may pave the way for a novel combination treatment in osteoarthritis and other rheumatological disorders.**

KEY WORDS: Celecoxib, COX-2, Curcumin, Osteoarthritis, Synergism.

Osteoarthritis (OA) is a systemic and chronic inflammatory disorder lacking effective treatment [1, 2]. It is the leading cause of disability among the elderly, and it has been estimated that 68% of Americans aged over 55 yr have OA [3].

Non-steroidal anti-inflammatory drugs (NSAIDs) are efficient anti-inflammatory and analgesic agents commonly used in the treatment of OA. NSAIDs inhibit prostaglandin synthesis through inhibition of both cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2) isoenzymes [4]. Generally, the gene for COX-1 is considered to act as a 'house-keeper' gene. It is constitutively expressed in most tissues where it maintains several physiological processes. The inducible COX-2, on the other hand, is not expressed in most normal tissues but is induced by a wide spectrum of pro-inflammatory cytokines and growth factors in specific pathophysiological conditions, such as arthritis [5, 6] and cancer [7].

The effect of COX-2 inhibitors in the treatment of OA is similar to that of aspirin and other non-specific NSAIDs, whereas two large-scale clinical trials have revealed that both celecoxib [8] and rofecoxib [9] cause fewer drug-related gastrointestinal adverse effects than NSAIDs. However, physicians and patients are becoming increasingly concerned regarding the cardiovascular safety of COX-2 inhibitors and non-specific NSAIDs [10].

The molecular mechanism responsible for the anti-inflammatory activity of celecoxib is not completely understood and may involve several pathways. It is generally accepted that celecoxib exerts its effect by inhibiting the COX-2 isoenzyme, which governs the rate-limiting steps in prostaglandin synthesis and is considered to be a key modulator of joint inflammation in arthritis. Recently, Shishodia and Aggarwal [11] showed that celecoxib may inhibit COX-2 via suppression of nuclear factor NF-κB, which regulates the COX-2 protein. Celecoxib may also exert its anti-inflammatory effect via COX-2-independent mechanisms, including inhibition of the activation of Akt [12] and suppression of the ability of the peroxisome proliferator-activated receptor γ (PPARγ) complex to bind to DNA [13].

The search for new anti-inflammatory compounds with minimal toxicity is of particular interest. Curcumin, a diferuloylmethane derived from the plant *Curcuma longa*, is a potent antioxidant that has been used for centuries in several Eastern countries as a dietary factor and as herbal therapy [14]. Similar to NSAIDs, curcumin possesses both anti-inflammatory [15] and antitumour activities [16]. Its anti-inflammatory efficacy has been demonstrated in several *in vitro* and animal models. In a rat model of adjuvant-induced arthritis, curcumin notably lowered paw inflammation in arthritic rats when administered orally (30 mg/kg) for a period of 15 days by gavage [17]. Banerjee *et al.* [18] demonstrated that curcumin significantly inhibited the induction of several inflammatory mediators in a rat chronic inflammation model. Several clinical trials have confirmed that curcumin has anti-inflammatory and analgesic properties [19, 20].

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The anti-inflammatory and anti-neoplastic properties of curcumin have been attributed, at least in part, to its ability to inhibit COX-2, which results in the suppression of prostaglandin synthesis [21]. Goel *et al*. [22] showed that curcumin inhibits the expression of COX-2 at the transcriptional level.

Several studies indicated that the mechanism of action of curcumin is not limited to the inhibition of COX-2. Other pathways are involved, including down-regulation of cellular protein kinases [e.g. JNK, protein kinase C, epidermal growth factor and human epidermal growth factor receptor (EGFR) and ErbB-2], leading to growth inhibition [23–27]. Recent studies demonstrated that curcumin inhibits the activation of NF-κB and blocks the function of c-jJun/AP-1 [28, 29]. In addition, curcumin was shown to suppress several pro-inflammatory cytokines and mediators of their release such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1, IL-8 and nitric oxides synthase (NOS) [30–32].

One of the lessons learned from translational research in recent years is that combinatorial therapy can provide dramatic improvement in safety and efficacy over monotherapy regimens, especially if the drugs differ in their mode of action [33, 34]. The concurrent use of drugs with different mechanisms of action or pharmacokinetics may be more effective and less toxic than each of the monotherapeutic regimens alone. For example, Agarwal *et al*. [35] found that the combination of celecoxib with the HMG-CoA reductase inhibitor lovastatin synergistically induced apoptosis in colon cancer cells.

Recently, we have shown the synergistic growth inhibition effect of this combination in pancreatic and colorectal [36, 37] cancer cells.

The rationale for combining curcumin and celecoxib stems from the fact that both drugs inhibit COX-2 by different mechanisms—curcumin down-regulates COX-2 mRNA and protein levels [21, 22], while celecoxib inhibits COX-2 activity directly by binding to its active site [38]. Here it is demonstrated that curcumin synergistically enhances the growth inhibitory and pro-apoptotic effects of celecoxib in OA synovial adherent cells.

Materials and methods

Reagents and chemicals

Curcumin (purity 97%) was purchased from Merck (White House, NJ, USA). Celecoxib was provided by Pfizer (NY, USA). All other reagents with the highest purity were purchased from Sigma Chemical Co (St Louis, MO, USA).

Culture of osteoarthritis synovial adherent cells

OA synovial adherent cells were prepared from small pieces (2 mm in diameter) of human synovial tissue collected from four patients during total knee replacement surgery. They were grown after trypsinization in monolayers in tissue culture flasks, as previously described [39]. The study was approved by the Institutional Ethics Committee. Synovial tissue was digested for 2 h with 0.2% (weight/volume) bacterial collagenase and then suspended in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Israel) with 10% (volume/volume) fetal calf serum (FCS), 100 units/ml penicillin and 100 mg/ml streptomycin. The cells were incubated at 37°C in 5% CO_2 for 3–5 days, after which the non-adherent cells were removed. Fibroblast-like adherent cells from the first or second passage were used for further studies.

Cell viability assay

OA synovial adherent cells (1.5×10^4/well) were incubated at 37°C in 96-well plastic plates with test drugs in DMEM containing 10% FCS in an atmosphere of 5% CO_2. After 72 h, cell viability was assessed by the ability of metabolically active cells to reduce tetrazolium salt (XTT) to coloured formazan compounds. The absorbance of the samples was measured with a specific enzyme-linked immunosorbentassay (ELISA) reader (wavelength 450 nm, reference wavelength 630 nm). Each measurement was done in triplicate. All experiments were repeated at least three times.

Flow cytometric analysis

OA synovial adherent cells were plated at a density of 5×10^6 per 10 cm dish with the various test drugs at selected concentrations. The adherent and non-adherent cells were collected during exponential growth of the cells and counted. A total of $(1–2) \times 10^6$ cells were washed in phosphate-buffered saline (PBS) and the pellet was fixed in 3 ml ethanol for 1 h at 4°C. The cells were pelleted and resuspended in 1 ml PBS and incubated for 30 min with 0.64 mg/ml ribonuclease (RNAse) at 37°C. The cells were stained with 45 mg/ml propidium iodide (PI) for at least 1 h before analysis by flow cytometry using a standard protocol for cell cycle distribution and cell size [40].

Necrotic cells were counted using trypan blue before fixation. All experiments were done three times. Data acquisition was performed on a FACScan and analysed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). All fluorescence and laser light scatter measurements were made with linear signal processing electronics. Data for at least 10 000 cells were collected for each data file.

Fluorescence microscopy

OA synovial adherent cells were plated at a density of 5×10^6 per 10 cm dish with curcumin, celecoxib and their combination at selected concentrations for 72 h. Apoptotic cells were detected by nuclear morphological changes using PI staining. Cells were washed twice with PBS and fixed for 15 min at room temperature with 4% paraformaldehyde in PBS. The fixative was removed by aspiration, and the monolayer was washed twice in PBS. DNA was incubated with 0.15 mg/ml RNAse for 15 min and stained with 5 μg/ml PI at room temperature. Excess PI stain was removed, and the monolayer was thoroughly washed with PBS. The cover slip was mounted with glycerol The stained nuclei were viewed at ×63 using a Lieca TCS SP2 confocal microscope (Lieca Microsystems, Wetzler, Germany).

Protein extraction and western blotting

Exponentially growing cells were collected and washed three times in ice-cold PBS as described earlier. The cell pellets were resuspended in lysis buffer [20 mM Tris-HCl pH7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), 6 mM 6-mercaptoethanol, 1% NP-40, 0.1% sodium dodecyl sulphate (SDS) and 10 mM NaF, plus the protease inhibitors leupeptin 10 mg/ml, aprotinin 10 mg/ml and 0.1 mM phenylmethylsulphonylfluoride]. The protein concentration of each sample was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). For western blotting, samples containing 50 μg of total cell lysate were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were transferred to 'Hybond-C' membranes (Amersham, Arlington Heights, IL, USA) in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol), using a Trans Blot transfer apparatus at 70 mA for 12–18 h at room temperature. The membranes were blocked with blocking buffer (PBS/0.2% Tween 20/0.5% gelatin) for 1 h at room temperature and subsequently washed three times for 5 min in washing buffer

(PBS/0.05% Tween-20). The membranes were incubated with polyclonal human anti-COX-1, human anti-COX-2 and actin antibodies for 1 h at room temperature. The membranes were washed as described above and incubated with antigoat, secondary antibodies (1:2000) for 1 h at room temperature. Additional washes were carried out as previously described above, and immune detection was performed using the ECL western blotting detection system (Amersham, Arlington Heights, IL, USA).

Measurement of prostaglandin E_2 concentration

The concentration of prostaglandin E_2 (PGE_2) in the medium, as released by the OA synovial adherent cells, was determined by a commercially available PGE_2-specific ELISA (R&D Biosystems, Abingdon, UK) according to the manufacturer's protocol.

Lipid peroxidation assay

The antioxidative properties of celecoxib, curcumin and their combination were evaluated on the basis of their effect on the kinetics of serum lipids, as previously described [41]. Briefly, different concentrations of celecoxib and curcumin were added at time zero to a 50-fold diluted serum in PBS containing sodium citrate (0.72 mM) and $CuCl_2$ (0.1 mM). Copper-induced peroxidation was monitored at 37°C by continuous recording of absorbance at 245 nm and 268 nm using a Kontron (Uvikon 933) double-beam spectrophotometer. The kinetics of copper-induced peroxidation on serum lipids in the presence of selected drugs was analysed as previously described [42].

Statistical analysis

The results were assessed as mean ± S.D. The difference between treatments with each of the drugs and with their combination was evaluated by the one-way analysis of variance test using the SPSS software package (SPSS Inc., Chicago, IL, USA). Statistical significance ($P < 0.05$) was established by *post hoc* Tukey's pairwise comparison.

To determine if the combination was additive or synergistic we used the analysis of Loewe [43, 44], which is used where the effects of two drugs are mutually exclusive (i.e. the drugs possess similar modes of action, such as COX-2 inhibition). In brief, in an isobologram the *x*- and *y*-axes represent doses of drugs 1 and 2. A straight line is then drawn for any F_a (fraction of cells effected by the treatment) value of interest representing doses of drug 1 + drug 2 that would be required to achieve the given F_a value if the two drugs were additive. The observed experimental concentrations at which combined treatment generated the given F_a value are plotted in the isobologram: synergism is indicated if these points lie to the lower left of the curve/straight line, defining additivity at that F_a value; additivity is indicated if the experimental points lie on the curve/straight line; antagonism is indicated if they lie to the upper right of the curve/straight line.

Results

Celecoxib and curcumin synergistically inhibited cell growth

The effect of celecoxib and curcumin on cell growth of OA synovial adherent cells was assessed alone and in combination using the XTT assay. Treatment with celecoxib ($IC_{50} = 40\ \mu M$) and curcumin ($IC_{50} = 35\ \mu M$) inhibited cell growth in a dose-dependent manner (Fig. 1). The addition of curcumin (10–20 μM) synergistically potentiated the effect of celecoxib on cell growth (Fig. 2A). In the presence of 10 μM curcumin, the IC_{50} of celecoxib was 10 μM, a 4-fold lower concentration than the IC_{50} (40 μM) of celecoxib alone. The Loewe isobologram method, as described in Materials and methods, was used to confirm synergism at F_a values of 0.5 and 0.8 (Fig. 2B).

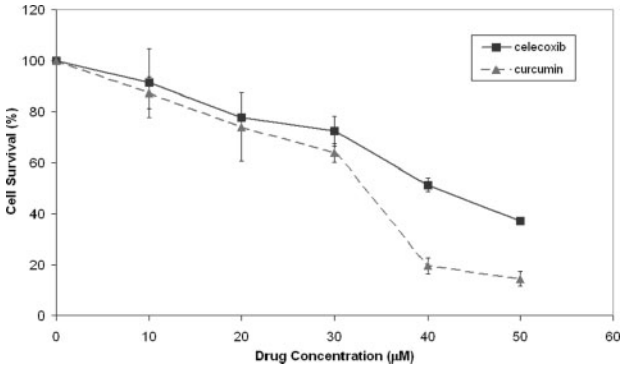

FIG. 1. Curcumin and celecoxib inhibit growth of OA synovial adherent cells in a dose-dependent manner. The OA synovial adherent cells were exposed for 72 h to different concentrations of celecoxib and curcumin as indicated. Cell viability was determined by the XTT method as described in Materials and methods. The data are mean ± S.D. values from three individual experiments.

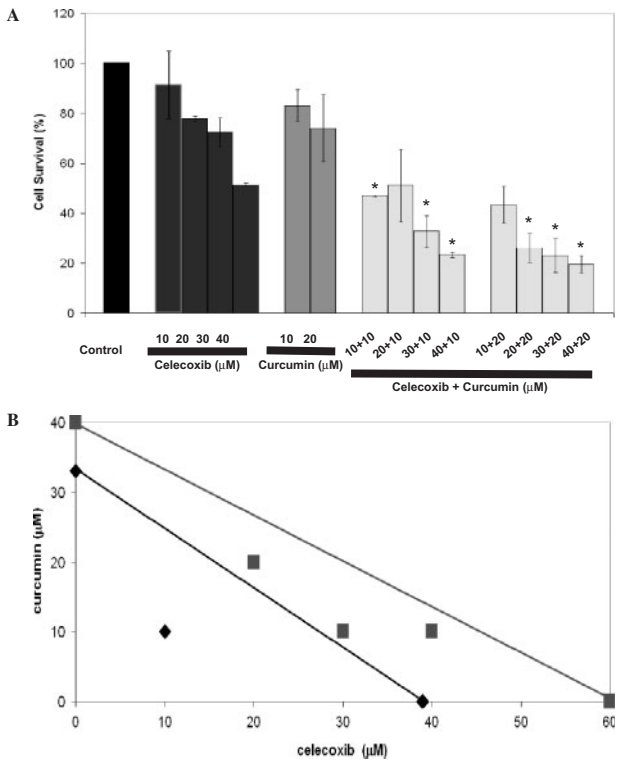

FIG. 2. Combining celecoxib and curcumin has a synergistic effect on growth of OA synovial adherent cells. The OA synovial adherent cells were exposed for 72 h to a combination of celecoxib and curcumin as indicated. Cell viability was determined by the XTT method as described in Materials and methods. (A) Effect of combining curcumin and celecoxib on the growth of OA synovial adherent cells. Differences in cell growth after exposure to curcumin and celecoxib separately and to their combination was determined using one-way ANOVA test. *Significant differences, $P < 0.05$. (B) The Loewe isobologram method, as described in Materials and methods, was used to confirm synergism at F_a values of 0.5 and 0.8. The data are mean ± S.D. values from three individual experiments.

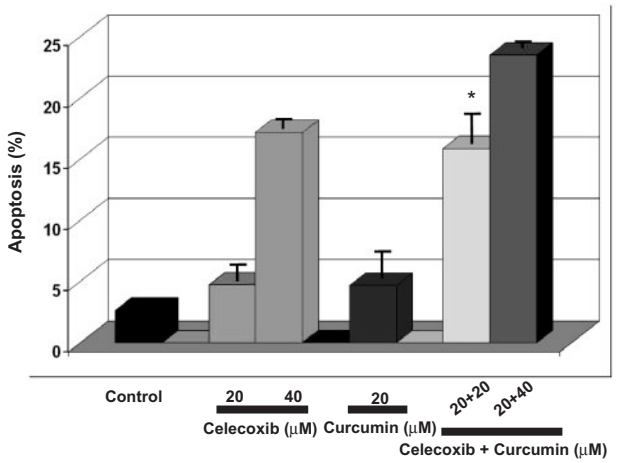

FIG. 3. Celecoxib and curcumin synergistically induce apoptosis. The OA synovial adherent cells were treated and incubated with curcumin and celecoxib and harvested for quantification of apoptosis by flow cytometry as described in Materials and methods. The extent of apoptosis was assessed by the size of the sub-G_1 population seen during DNA flow cytometry and is expressed as the *fold increase* relative to the untreated control. The values are means ± S.D. of triplicate assays. Differences in induction of apoptosis after exposure to curcumin and celecoxib separately and to their combination was determined using a one-way ANOVA test. *Significant differences, $P < 0.05$.

Curcumin augmented the effect of celecoxib on the induction of apoptosis

To investigate whether the effect of the combination on cell growth could result from its effect on apoptosis we examined the effect of the different treatments on the percentage of cells with subdiploid DNA content, the hallmark of apoptosis, using flow cytometric analysis.

Celecoxib at concentrations of 20–40 μM demonstrated a moderate effect (4.8–19.2%) on cell apoptosis (Fig. 3). Curcumin at a concentration of 20 μM caused a minor effect (4.7%), but it demonstrated a significantly enhanced effect on cell apoptosis when it was applied together with celecoxib (20–40 μM).

Drug-treated OA synovial adherent cells were examined for morphological characteristics evidence of apoptosis using fluorescence microscopy. Typical apoptotic features of chromatin condensation and nuclear fragmentation confirmed the FACS analysis results (data not shown).

Curcumin potentiates the effect of celecoxib on synthesis of PGE_2

In order to evaluate whether the inhibition of cell growth by the combination of drugs was correlated with COX-2 inhibition, we assessed the effect of various treatments on COX-2 activity: 0.5 μM of celecoxib inhibited PGE_2 production by 80% (Fig. 4A) and the addition of curcumin (5 μM) to celecoxib almost totally diminished (>95%) PGE_2 synthesis (Fig. 4A).

Combination therapy did not alter levels of COX-1 or COX-2

Western blot analysis demonstrated that the levels of COX-1 and COX-2 (data not shown) proteins were not altered by treatment with celecoxib, curcumin or their combination (celecoxib 0.5 μM and curcumin 5 μM) (Fig. 4B). This is in agreement with previous data showing that neither celecoxib nor curcumin inhibits COX-1 proteins [22, 45].

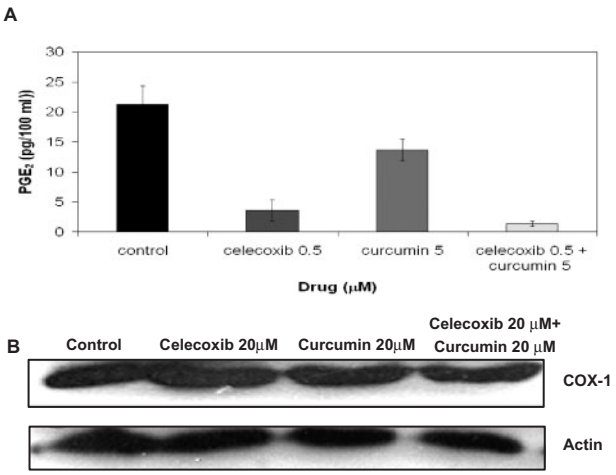

FIG. 4. (A) Curcumin potentiates the effect of celecoxib on PGE_2 synthesis. The OA synovial adherent cells were treated and incubated with celecoxib curcumin and their combination for 72 h at selected doses. PGE_2 levels in the culture medium were measured by ELISA as described in Materials and methods. The data are mean values ± S.D. from three independent experiments. (B) Lack of effect of the combination therapy on COX-1 levels. The OA synovial adherent cells were incubated with curcumin and celecoxib for 72 h and then collected for western blot analysis as described in Materials and methods. The combination treatment did not alter the expression of COX-1 protein. Lane 1, untreated OA synovial adherent cells (control); lane 2, OA synovial adherent cells treated with 20 μM celecoxib; lane 3 OA synovial adherent cells treated with 20 μM curcumin; lane 4, OA synovial adherent cells treated with 20 μM celecoxib + 20 μM curcumin. Lower panel: actin expression.

Celecoxib does not augment curcumin's antioxidative properties

The antioxidative properties of celecoxib and curcumin were assessed using the *ex vivo* procedure as described in Materials and methods. Curcumin was found to be a very potent antioxidant even at low (5 mM) concentrations, whereas celecoxib (20 mM) had no antioxidant activity whatsoever (Fig. 5). Furthermore, the addition of celecoxib did not alter the antioxidative properties of curcumin (Fig. 5).

Discussion

In the present study we have shown that the phytochemical curcumin synergistically augments the effect of celecoxib on inhibition of cell growth and induction of apoptosis in OA synovial adherent cells.

Combination therapy in arthritis is based on the multifactorial nature of chronic inflammation. The concurrent use of drugs with different mechanisms of action or pharmacokinetics may be more effective and less toxic than each of the monotherapeutic regimens alone. Furthermore, toxicity can be reduced, particularly when the treatment comprises a commonly used dietary factor such as curcumin. Curcumin is commonly consumed (as turmeric spice) in large quantities (up to 100 mg/day) in certain countries. Moreover, a recent clinical trial [46] showed that curcumin is not toxic even at significantly higher doses (up to 8 g/day).

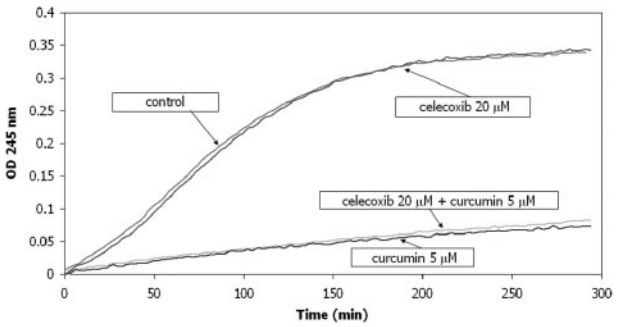

FIG. 5. Celecoxib does not augment the antioxidative properties of curcumin. The antioxidative effect of curcumin, celecoxib and their combination was measured during copper-induced oxidation of 50-fold diluted serum as described in Materials and methods. Peroxidation was monitored by recording absorbance at 245 nm. The temperature was maintained at 37°C.

Several combinations in the treatment of arthritis were previously demonstrated to be more efficacious than monotherapies [33, 34]. Although COX-2 inhibitors are considered safer than NSAIDs, recent studies indicated that long-term use of COX-2 inhibitors may cause serious cardiovascular toxicity [47]. Therefore, the development of a regimen consisting of a phytochemical with a very low profile of side-effects in combination with a selective COX-2 inhibitor that does not suppress COX-1 activity would be highly desirable for long-term treatment of osteoarthritis.

Synovial fibroblasts secrete mediators of inflammation and joint destruction [48] and are recognized as important players in the pathogenesis of arthritis. Therefore, induction of apoptosis of these cells to induce long-term remission is an attractive therapeutic goal [49]. Kusunoki *et al*. [50] showed that celecoxib reduced the viability of rheumatoid synovial fibroblasts by the induction of apoptosis, in a concentration-dependent manner. We have now revealed that curcumin potentiates the effect of celecoxib to induce enhanced apoptosis in OA synovial adherent cells, a finding that may enable the use of celecoxib at lower and safer concentrations.

Our data suggest that the combined therapy is COX-2 dependent. The addition of curcumin (5 μM) to celecoxib (0.5 μM) augmented its effect by almost totally abolishing PGE_2 production (>95%), indicating that curcumin augments the inhibitory effect of celecoxib on COX-2 activity.

Previous studies have shown that curcumin is an efficient scavenger of the free radicals generated by macrophages [51]. In view of the pivotal role of oxygen free radicals generated by macrophages in arthritis, we were interested in evaluating whether celecoxib potentiates the antioxidant properties of curcumin. The lack of such an effect (Fig. 5) clearly shows that the observed synergism cannot be attributed to the potentiation by celecoxib of the antioxidant effects of curcumin.

A series of new molecular targets and signalling pathways are involved in the growth inhibition and induction of apoptosis exerted by curcumin. These targets include epidermal growth factor receptor (EGFR) [26], HER-2 [27], NF-κB [52], cyclin D1 [53], TNF [54], NOS [55], matrix metalloproteinase 9 (MMP9) [56] and Akt [57]. Interestingly, several of these molecular targets, such as NF-κB [58] cyclin D1 [59], Akt [60] and NOS [61] have been found to be involved in the growth inhibitory and pro-apoptotic effects of celecoxib. Therefore, it is possible that the effect of combining the drugs is also mediated via one of these targets. Additional experiments will be required to identify other possible non-COX-2 mechanisms.

In conclusion, this study reveals that curcumin synergistically augments the inhibition of growth of OA synovial adherent cells and enhances the induction of apoptosis by celecoxib. The synergistic effect was mediated through a mechanism that involves inhibition of COX-2 activity. This may enable the use of celecoxib at lower and safer concentrations and may pave the way for a novel combinatorial treatment of arthritis.

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The authors have declared no conflicts of interest.

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