Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism

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Curcumin, a component of turmeric (Curcuma longa), has been shown to exhibit chemopreventive activity. Whether analogs of curcumin (Cur), such as demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC), tetrahydrocurcumin (THC) and turmerones, modulate inflammatory signaling and cell proliferation signaling to same extent as curcumin was investigated. The results indicate that the relative potency for suppression of tumor necrosis factor (TNF)-induced nuclear factor-κB (NF-κB) activation was Cur > DMC > BDMC; thus suggesting the critical role of methoxy groups on the phenyl ring. THC, which lacks the conjugated bonds in the central seven-carbon chain, was completely inactive for suppression of the transcription factor. Turmerones also failed to inhibit TNF-induced NF-κB activation. The suppression of NF-κB activity correlated with inhibition of NF-κB reporter activity and with down-regulation of cyclooxygenase-2, cyclin D1 and vascular endothelial growth factor, all regulated by NF-κB. In contrast to NF-κB activity, the suppression of proliferation of various tumor cell lines by Cur, DMC and BDMC was found to be comparable; indicating the methoxy groups play minimum role in the growth-modulatory effects of curcumin. THC and turmerones were also found to be active in suppression of cell growth but to a much lesser extent than curcumin, DMC and BDMC. Whether suppression of NF-κB or cell proliferation, no relationship of any of the curcuminoid was found with reactive oxygen species (ROS) production. Overall, our results demonstrated that different analogs of curcumin present in turmeric exhibit variable anti-inflammatory and anti-proliferative activities, which do not correlate with their ability to modulate the ROS status.

Introduction

Traditional medicine is inexpensive and believed to be beneficial but generally neither active principles nor their molecular targets are well defined. Therefore, the understanding of the active component and its mechanism of action can make such medicine more acceptable. With >2000 citations, turmeric, which is derived from the rhizome of the plant Curcuma longa, is one such remedy that has been used for centuries on the Southeast Asian continent. Extensive research in the past half a century has indicated that curcumin (also called diferuloyl methane), a yellow coloring agent present in turmeric, is an antioxidant more potent than even vitamin E (1). Curcumin has been linked with the suppression of mutagenesis; has been used as a chemopreventive agent for a wide variety of cancers, including colon, breast, prostate, esophagus, lung and oral; inhibition of atherosclerosis and inhibition of viral and bacterial growth (2–7). Mechanistically, curcumin has been shown to mediate anti-inflammatory effects through the suppression of nuclear factor-κB (NF-κB) activation (8), anti-proliferative effects through suppression of cyclin D1 and anti-apoptotic gene products (9–11), induce cytochrome C release, activate caspases (12,13) and p53 (14) and have anti-angiogenic effects through the down-regulation of vascular endothelial growth factor (VEGF) (9,15). On the basis of the results from these studies, curcumin is currently in clinical trials for treatment of various cancers (16,17) and for Alzheimer’s disease (18).

Commercially available curcumin used for research and for clinical trials (curcumin mix) contains ~77% pure curcumin (Cur), 17% demethoxycurcumin (DMC) and 3% bisdemethoxycurcumin (BDMC). Additionally when cells are treated with curcumin, one of the major metabolites is tetrahydrocurcumin (THC) (19). These studies also showed that curcumin and DMC were less stable than BDMC, whereas reductive metabolite THC is most stable. Besides curcumin, turmeric also contains turmeric oil that has been linked with anti-fungal (20), antibacterial (21), insecticidal (22), mosquitocidal (23), antioxidant and anti-mutagenic (24) and anticancer (25) activities. Turmeric oil consists of aromatic turmerone (α-turmerone), α-turmerone and β-turmerone.

How various forms of curcumin and turmerones differ with respect to anti-inflammatory and anti-proliferative effects is not understood; and thus was investigated in the current study. Anti-inflammatory effects were examined by investigating their effect on tumor necrosis factor (TNF)-induced NF-κB activation and on NF-κB-regulated gene products, whereas anti-proliferative effects were examined by investigating their effect on the proliferation of wide variety of tumor cell lines. Whether these effects were mediated through the modulation of redox signaling was also addressed. We found that both anti-proliferative and anti-inflammatory effects of various curcuminoids may be linked to glutathione (GSH) status but not to the reactive oxygen species (ROS) status of the cell.

Materials and methods

Reagents

Commercial curcumin (>95% pure) was purchased from LKT Laboratories (St Paul, MN). A 25 mM solution was prepared in dimethyl sulfoxide, stored as small aliquots at −20°C and diluted as needed in cell culture medium. THC was purified and supplied by Sabinsa Corporation (Piscataway, NJ). Turmerones were purified by Dr A.M. (Kyoto University, Japan). Stock solutions (100 mM) of Cur, DMC, BDMC, THC, α-turmerone, δ-turmerone and β-turmerone were prepared in dimethyl sulfoxide and stored as small aliquots at −20°C. Bacteria-derived human recombinant TNF, purified to homogeneity with a specific activity of 5 × 107 U/mg, was kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, Iscove’s modified Dulbecco’s medium, Dulbecco’s modified Eagle’s medium, RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY). Antibody against β-actin was obtained from Sigma–Aldrich (St Louis, MO). Antibody against VEGF was purchased from NeoMarkers (Fremont, CA). Dichlorodihydrofluorescein diacetate (DCF-DA) and monobromobimane were purchased from Molecular Probes (Eugene, OR). Antibody against cyclin D1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against cyclooxygenase-2 (COX-2) was obtained from BD Biosciences (San Diego, CA).

Abbreviations: α-turmerone, aromatic turmerone; BDMC, bisdemethoxycurcumin; COX-2, cyclooxygenase-2; Cur, pure curcumin; DCF-DA, dichlorodihydrofluorescein diacetate; DMC, demethoxycurcumin; FBS, fetal bovine serum; GSH, glutathione; HPLC, high-performance liquid chromatography; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; THC, tetrahydrocurcumin; THF, tetrahydrofuran; TLC, thin-layer chromatography; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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Cell lines

U937 (histiocytic myeloid lymphoma), KBM-5 (chronic myeloid leukemia), Jurkat (T-cell leukemia), H1299 (lung adenocarcinoma), Calu-6 (non-small cell lung carcinoma), A549 (lung adenocarcinoma), SCC-4 (squamous cell carcinoma), Panc-1 (pancreatic duct cell carcinoma), MCF-7 (breast adenocarcinoma), DU145 (prostate carcinoma) and A293 (embryonic kidney carcinoma) were obtained from American Type Culture Collection (Manassas, VA). U937, Jurkat, H1299, Calu-6, A549, Panc-1, MCF-7 and DU145 cells were cultured in RPMI 1640 medium with 10% FBS. K562 cells were cultured in Iscove’s modified Dulbecco’s medium with 15% FBS. A293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS. SCC-4 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, 100 μM non-essential amino acids, 1 mM pyruvate, 6 mM L-glutamine and 1 μM vitamins. Culture media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Extraction and fractionation of curcuminoids

Turmeric rhizomes purchased from a local market in Chiang Mai, Thailand, were dried and blended to a powder form. The powder was extracted with 95% ethanol for 24 h. The ethanolic extract was filtered through Whatman filter paper no. 2 and ethanol was removed by using a rotary evaporator. One kilogram of laboratory-made crude curcuminoids were dried and blended to a powder form. The powder was extracted with 95% methanol with increasing polarity to yield pure fractions of Tur, DMC and BDMC (Figure 1B). The fractions were collected and spotted on thin-layer chromatography (TLC) aluminum sheets coated with silica gel 60 F254. Fractions that showed the same pattern on TLC were pooled and the organic solvent was removed to obtain the powder form (26). The identity and purity of each curcuminoids were verified using TLC, high-performance liquid chromatography (HPLC), Infrared (IR), mass spectrometry and Nuclear Magnetic resonance (NMR) analyses (27). The purity of curcumin, DMC and BDMC by HPLC analysis was in the range of 95–99%.

Preparation of THC

THC was prepared by dissolving curcumin mix in mixture of acetone:water (95:5) and hydrogenated in presence of hydrogen gas under pressure using a metal catalyst (palladium on carbon).

Purification of turmerones

Turmerones were purified as described earlier (28). Briefly, C. longa L. was extracted with chloroform and the chloroform layer was subjected to silica gel column chromatography. This was eluted with increasing amounts of ethyl acetate in n-hexane. The 2.5% ethyl acetate fraction was further separated on silica gel (0–5% ethyl acetate in n-hexane), which had been pre-treated with 5% AgNO3 in water and dried. Each fraction was subjected to HPLC (column, YMC ODS AQ-302, Yamamura Chemical Laboratories, Kyoto, Japan; mobile phase, 85% acetonitrile in H2O; flow rate, 1.0 ml/min; detection, 254 nm) to purify sesquiterpenes (retention time, min): α-turmerone (4.0), β-turmerone (5.2) and γ-turmerone (5.3). The chemical identity of isolated sesquiterpenes was confirmed by comparing their spectral data (NMR and mass spectrometry). The purity of each compound was >95%.

Separation of curcuminoids from curcumin mix by HPLC

Fifty milligram of curcuminoids fractionated from curcumin mix on silica gel 60 column chromatography was dissolved in 10 ml tetrahydrofuran (THF). The column was coupled with a 4.6 × 30 mm, C8 guard column.

One milliliter aliquot of THF solution was diluted to the final concentration of 5 mg/ml with 40% THF/60% deionized distilled water. Curcuminoids were separated and quantified by HPLC using detection at a wavelength of 240 nm, 30°C. An aliquot (10 ml, 50 μg) of the diluted sample was injected onto a reversed-phase C18 column, ODS (hypersil) 4.6 × 50 mm, 5 μm and eluted with a mobile phase of 40% THF and 60% deionized water containing 1% citric acid, pH 3.0. The system was run isocratically at a flow rate of 0.7 ml/min. The quantitation of curcuminoids is by peak area ratio.

Electrophoretic mobility shift assay

To examine NF-κB activation, we performed electrophoretic mobility shift assay as described previously (29). Briefly, nuclear extracts prepared from treated KBM-5 cells were incubated with 32P-end-labeled, 45mer double-stranded NF-κB oligonucleotides (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat (5′-TGTTA-CAAGGGACCTTCCCGCTGGGACCTTCAGGAGGCGTG-3′; boldface indicates NF-κB-binding sites) for 30 min at 37°C. The DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized and radioactive bands were quantified with a PhosphorImager (Amersham Biosciences, Piscataway, NJ) using ImageQuant software.

NF-κB-dependent reporter gene expression assay

NF-κB-dependent reporter gene expression assay was as described previously (30). To examine TNF-induced reporter gene expression, we transfected A293 cells with 0.5 μg of the secretory alkaline phosphatase expression plasmid and 2 μg of the control plasmid pCMV-FLAG1 for 24 h. We then treated the cells with curcumin mix or curcuminoids. TNF (1 nM) was added after

Fig. 1. (A) Chemical structure of curcuminoids: curcumin (Cur), DMC and BDMC; the curcumin metabolite THC and turmeric oil components α-turmerone (ar), γ-turmerone and β-turmerone (αβ). (B) HPLC separation of the curcuminoids present in curcumin mix and validation of each peak were done by comparing the retention times of the purified curcuminoids.
4 h and the cell culture medium was harvested after 24 h of TNF treatment. The culture medium was then analyzed for secretory alkaline phosphatase activity, essentially as described by the manufacturer’s instructions (Clontech, Palo Alto, CA), using a Victor 3 microplate reader (Perkin Elmer Life and Analytical Sciences, Boston, MA) with excitation at 360 nm and emission at 460 nm.

Western blot analysis
To determine the levels of protein expression, we prepared whole-cell extracts from treated KBM-5 cells (31) and fractionated them by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the appropriate antibodies and detected by enhanced chemiluminescence (Amersham Biosciences). The bands obtained were quantified using NIH imaging software (Bethesda, MD).

Cytotoxicity assay
The cytotoxic effects of curcumin mix, curcuminoids, THC and turmerones on the various cell lines were determined by the MTT (3-(4,5-Dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide) uptake method as described previously (32).

Measurement of ROS
To detect intracellular ROS, KBM-5 cells were pre-incubated with 20 µM DCF-DA for 15 min at 37°C before being treated with 1 µm curcumin mix, curcuminoids, THC or turmerones. After 30 min of incubation, the increase in fluorescence resulting from oxidation of DCF-DA to DCF was measured by flow cytometry (33). The mean fluorescence intensity at 530 nm was calculated. Data were collected from at least 10,000 cells at a flow rate of 250–300 cells/s.

Intracellular GSH measurement
To measure intracellular GSH, KBM-5 cells were incubated with 1 µm curcumin mix, curcuminoids, THC or turmerones for 24 h. Monobromobimane (final concentration, 40 µM) was loaded into cells (34). Fluorescence emission from cellular sulfhydryl-reacted monobromobimane was recorded using a flow cytometer. Data were collected from at least 10,000 cells at a flow rate of 250–300 cells/s.

Results
The objective of the present study was to determine whether different isoforms of curcumin differ in their ability to suppress inflammation and cellular proliferation and whether redox signaling mediates these effects. Inflammatory effects were measured by measuring TNF-induced NF-κB activation and NF-κB-regulated gene products. The structure of various curcuminoids examined is shown in Figure 1A. While curcumin (Cur) contains two methoxy groups at its ortho position, DMC contains only one and BDMC contains none. Several methods have been used to separate these curcuminoids (35,36). In comparison, THC contains both the methoxy groups but lacks conjugated bonds in the central seven-carbon chain.

Curcuminoids have different HPLC retention times
Whether curcumin mix consists of different chemical entities was examined by HPLC. We found that curcumin mix consisted of three components viz Cur, DMC and BDMC, in the order Cur > DMC > BDMC. Curcumin was eluted first with retention volume at 4.96 ml followed by DMC and BDMC at 5.69 and 6.50 ml, respectively. The Cur, DMC and BDMC preparations were found to be homogeneous and their retention times were 7.08, 8.13 and 9.28 min, respectively (Figure 1B).

Curcuminoids differentially suppress TNF-induced NF-κB activation
Both TNF and NF-κB are major mediators of inflammation; TNF mediates its inflammatory effects through the activation of NF-κB. Whether different curcuminoids modulate TNF-induced NF-κB activation to a similar extent was investigated. Results in Figure 2 indicate that treatment of KBM-5 cells with curcumin mix (panel A), Cur (panel B), DMC (panel C) or BDMC (panel D) inhibited the TNF-induced NF-κB activation in a dose-dependent manner, but the potency varied. At 25 µM, curcumin mix was the most potent, followed by Cur, DMC and BDMC (Figure 2E). The reconstitution from pure preparations also indicated that curcumin mix was more potent than any individual component (Figure 2F). That the maximum NF-κB inhibitory activity was associated with Cur and less so with DMC and least of all with BDMC suggests the critical role of both phenyl methoxy groups in curcumin in suppressing NF-κB activation.

THC is less active than curcumin mix to suppress TNF-induced NF-κB activation
THC contains both methoxy groups but lacks conjugated bonds in the central seven-carbon chain. Whether THC can modulate TNF-induced NF-κB activation to a similar extent as curcumin mix was investigated. The results showed that treatment of KBM-5 cells with THC inhibited TNF-induced NF-κB activation but only at a concentration 10 times higher than that required for the curcumin mix (Figure 3A). Thus, these results suggest that THC is not a very potent anti-inflammatory agent and that the conjugated bonds in the central seven-carbon chain are needed for its activity.

Turmerones do not suppress TNF-induced NF-κB activation
Although numerous activities have been associated with turmerones, their structure is significantly different from that of curcumin (Figure 1A). Whether turmerones can modulate TNF-induced NF-κB activation to a similar extent as curcumin mix was investigated. Results in Figure 3B show that treatment of KBM-5 cells with α,-turmerone and γ-/β-turmerone had no effect on TNF-induced NF-κB activation, suggesting that turmerones lack the anti-inflammatory activity commonly known to be associated with curcumin.

Suppression of TNF-induced NF-κB activation correlates with NF-κB reporter activity
Although we have shown by electrophoretic mobility shift assay the relative ability of various components of turmeric to block NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (37). To determine the effect of curcumin mix and curcuminoids on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected A293 cells with the NF-κB-regulated secretory alkaline phosphatase reporter construct and incubated the cells with an equimolar concentration of curcumin mix, curcuminoids or THC and then stimulated the cells with TNF. We found that TNF induced an almost 8-fold increase in NF-κB-regulated reporter gene expression and that curcumin mix suppressed the activity by 85% (Figure 3C). Again curcumin mix was most potent compound, followed by Cur, DMC and BDMC. Both DMC and THC showed marginal suppression of reporter activity. These data are in agreement with the ability of these compounds to suppress NF-κB.

NF-κB suppression correlates with NF-κB-regulated gene products
NF-κB activation has been linked with the regulation of the expression of COX-2, cyclin D1 and VEGF, all of which regulate cell proliferation and inflammation. Whether suppression of TNF-induced NF-κB activation correlates with the expression of these gene products was investigated. We found that TNF induced the expression of COX-2, cyclin D1 and VEGF in KBM-5 cells and that 25 µM curcumin mix suppressed the expression of these gene products (Figure 3D). The same concentration of Cur was almost as effective as curcumin mix, but DMC was less effective, and BDMC and THC were almost completely ineffective in suppressing the gene expression. These results correlate well with the data on suppression of NF-κB activation.

Curcuminoids, THC and turmerones differ in their ability to inhibit cell proliferation
NF-κB-regulated gene products are known to affect cellular proliferation and apoptosis. For instance, cyclin D1 is required for G1 to S transition. Besides NF-κB-suppressive activity, we also investigated the ability of curcumin mix, curcuminoids, THC and turmerones to inhibit the proliferation of tumor cells. Curcumin mix inhibited the
proliferation of U937, KBM-5, Jurkat, H1299, Calu-6, A549, SCC-4, Panc-1, MCF-7 and DU145 cells in a dose-dependent manner (Figure 4A). Although Cur, DMC, BDMC, THC and turmerones also suppressed the proliferation of these cell lines, their potency varied. When examined at a given concentration (25 μM), the results show that curcumin mix, Cur, DMC and BDMC suppressed the proliferation of various tumor cell lines with similar potency, but THC and turmerones were much less effective (Figure 4B). These results suggest that the suppression of NF-κB and the inhibition of cell proliferation by the various curcuminoids are not strictly interrelated.

Curcuminoids, THC and turmerones differ in their ability to induce ROS

That exposure of cells to curcumin can generate ROS has been demonstrated (38). Whether curcuminoids, THC and turmerones differ in their ability to generate ROS was examined by treating KBM-5 cells and using DCF-DA as a probe to measure the increase in ROS levels inside cells. Figure 5A shows overlapped histograms of flow cytometric fluorescence intensity patterns of ROS produced by cells on treatment with each compound. Although curcumin mix, Cur, DMC and BDMC induced significant levels of ROS, BDMC showed highest amount (7.7-fold) as compared with control cells (Figure 5B). Cur and DMC showed 2-fold increase in ROS levels. These results suggest that phenyl methoxy groups in curcumin negatively regulate ROS production. THC and turmerones had no effect on ROS production from the cells.

Curcuminoids, THC and turmerones differ in their ability to induce GSH synthesis

Besides ROS, curcumin has been shown to induce GSH synthesis in cells. Whether curcuminoids, THC and turmerones differ in their ability to generate intracellular GSH levels in KBM-5 cells were measured using monobromobimane by flow cytometry. Figure 5C shows increase in levels of GSH 24 h after treatment with curcumin mix and curcuminoids. At equimolar concentrations, curcumin mix, Cur, DMC and BDMC increased GSH levels over control, but the
induction by BDMC was noticeably less. THC, ar-turmerone and α/β-turmerones were completely inactive in inducing GSH levels, thus suggesting a role for conjugated bonds in the central seven-carbon chain (Figure 5C). THC, in fact, decreased the GSH levels below the control levels.

Discussion

Turmeric, commonly referred to in the Western World as ‘Curry powder’, contains curcumin (diferuloylmethane), DMC, BDMC and turmeric oil (ar-turmerones and α/β-turmerones). THC is one of the major metabolites of curcumin. Although the ability of curcumin to inhibit the inflammatory response and suppress the proliferation of tumor cells is well established, how different components of turmeric differ in their ability to modulate these responses is not known. Our results indicate that curcumin mix is most active in suppressing TNF-induced NF-κB activation; and the activity of others is in the order Cur > DMC > BDMC. THC found to be much less active and turmerones failed to inhibit TNF-induced NF-κB activation. The suppression of DNA-binding activity of NF-κB correlated with inhibition of the NF-κB reporter activity and with down-regulation of NF-κB-regulated COX-2, cyclin D1 and VEGF. In contrast, the inhibition of proliferation of various tumor cell lines by curcumin, DMC and BDMC, was found to be comparable. THC and turmerones were much less active as anti-proliferative agents. When examined for ROS production, BDMC was maximally active in inducing ROS; curcumin and DMC had minimal activity. No ROS was produced in response to THC or turmerones. The production of GSH, however, was comparable for curcumin, DMC and BDMC but none in response to THC and turmerones.

We found that curcuminoids vary in their ability to suppress NF-κB activation. Curcumin contains two phenyl methoxy groups, DMC contains one and BDMC contains none. Since curcumin is maximally active, DMC is intermediate and BDMC is least active, this suggests that the phenyl methoxy groups do contribute to the suppression of NF-κB activation. How phenyl methoxy groups mediate NF-κB suppression, however, is unclear. Earlier theoretical studies (39) showed that hydrogen bonding between ortho-methoxy oxygen and phenolic hydrogen influences the planarity, conformation and ability to undergo oxidation. This may probably influence the interaction with the proteins such as NF-κB. Recently, Chen et al. (40) demonstrated that the antioxidant activity of curcumin is due not only to number of phenolic group but also the ortho-methoxy phenolic functionality. The ortho-methoxy group can form an intramolecular hydrogen bond with the phenolic hydrogen, making the H-atom abstraction from the ortho-methoxy phenols surprisingly easy. Therefore, in the present study differential activities of curcumin, DMC and BDMC can arise both from the ortho-methoxy phenol and from a central methylenic hydrogen in the central seven-carbon chain and β-diketone moiety. Furthermore, studies of Somparn et al. (41) showed that...
methoxy group of curcuminoids are also related with the antioxidant activity, therefore, differential NF-κB suppression potential of curcumin, DMC and BDMC may be connected with their antioxidant potential. A similar difference in the potency of curcuminoids has also been shown for the modulation of the function of the multidrug resistance-linked ATP-binding cassette transporter ABCG2 protein (27).

Our data also indicate that the commercially available curcumin mix (mixture of Cur, DMC and BDMC) is more active than Cur. Reconstitution experiments also indicated that the mixture is more
active in suppressing NF-κB activation than the individual components. These results are in agreement with the findings of Tonnesan et al. (42) who showed that the anti-inflammatory activities of curcumin are synergistically enhanced by DMC and BDMC. The nematocidal activity of curcumin has also been found to be synergistically enhanced by the mixture as compared with either one of the curcuminoid alone (43). The mechanism of synergism between Cur, DMC and BDMC is unknown. Also, our results differ from the observations of Huang et al. (44) who showed that Cur, DMC and BDMC had about the same inhibitory potency on 12-O-tetradecanoylphorbol-13-acetate-induced inflammation of mouse ears. The differences could be due to differences in the system or inducer applied.

Our results also suggest that curcumin metabolite, THC had no NF-κB-suppressive activity; thus pointing to the critical role of double bonds in NF-κB suppressive activity of curcumin. Because of lack of double bonds in THC, the extended conjugation is not possible resulting in loss of interconnectivity between the phenolic ring and the diketo moiety which otherwise stabilizes not only the parent molecule but also the resultant radicals produced by interaction with ROS. As a result of this, THC is not expected to possess the similar properties as that of curcuminoids.

Our results also suggest that turmerones had no NF-κB-suppressive activity. Since ortho-methoxy phenolic group are essential for the activity of the curcumin and as turmerones lack these groups, they show no activity toward inhibition of TNF-induced NF-κB.

The suppression of DNA-binding activity of NF-κB correlated with the inhibition of the NF-κB reporter activity and the down-regulation of NF-κB-regulated gene products COX-2, cyclin D1 and VEGF. Cur was again the most effective at suppressing the gene products, DMC moderately effective and BDMC least effective. Similar differences in curcuminoid potency have been reported for heme oxygenase-1 induction in endothelial cells (45). While cyclin D1 is linked with proliferation of cells, COX-2 has been linked with inflammation and VEGF with angiogenesis. The suppression of cyclin D1 suggests that curcumin will also suppress the proliferation of tumor cells.

We found that at equimolar concentration, Cur, DMC and BDMC had similar anti-proliferative activity against all various cell lines. These results are in agreement with those from previously published reports (46,47). It is unclear why the different cell lines exhibited different sensitivity in terms of suppression of proliferation to each of the curcuminoids. This could be due to varying endogenous levels of GSH in these cell lines. Fang et al. (48) showed that curcumin irreversibly modifies thioredoxin reductase directly, which may contribute to its cancer preventive activity. Curcumin mix, Cur and DMC have also been found to exhibit similar activity in suppressing TPA-induced tumor promotion in mouse skin, whereas BDMC and THC

Fig. 5. Effect of curcumin mix, Cur, DMC, BDMC and THC on production of cellular ROS and GSH. (A) KBM-5 cells were labeled with DCF-DA, treated with 1 μM of each compound for 1 h and examined for ROS production by flow cytometer. (B) Quantitation of ROS production is shown as histogram. Data represent the mean of three measurements ± SD *** P<0.001; ** P<0.05, significant with respect to control. (C) KBM-5 cells were incubated with 1 μM curcumin mix, Cur, DMC, BDMC, THC and turmerones for 24 h; stained with monobromobimane (40 μM) and examined by flow cytometer. Data represent the mean of three measurements ± SD ** P<0.001, significant with respect to control.
were less active (44,49). Our results indicate a lack of a direct relationship between suppression of cyclin D1 and cell proliferation, implying that there are other factors involved in cell proliferation. THC and turmerones, however, were minimally effective in suppression of cell proliferation in our study, which correlated with their inability to inhibit NF-kB activation.

The production of ROS has been linked to the anti-proliferative effects of most agents. In our study, curcumin mix, Cur produced ROS, but the highest level of ROS was induced by BDMC. Thus, the level of ROS production by various curcuminoids correlated with their effect on ROS generation, which may account in part for their lack of effect on NF-kB activation and cell proliferation.

An increase in intracellular GSH levels has been linked with the suppression of NF-kB activation by most agents (50,51). We observed that Cur, DMC and BDMC induced GSH to a similar extent at equimolar concentrations. These results are similar to those from previous studies that showed that all three curcuminoids are equipotent in suppressing iron-induced lipid peroxidation (1) or for nitric oxide scavenging activity (52) and differ from those that showed that Cur is more potent antioxidant than DMC or BDMC (53–55). Interestingly, neither turmerones nor THC induced GSH production. Thus, the production of GSH appears to correlate well with the suppression of NF-kB activation and cell proliferation induced by various components of turmeric. Thus, both anti-proliferative and anti-inflammatory effects of various curcuminoids may be linked to GSH status but not to the ROS status of the cell.

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