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## Curcumin and Turmeric Modulate the Tumor-Promoting Effects of Iron In Vitro

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### Abstract

Free or loosely chelated iron has tumor-promoting properties in vitro. Curcumin, a polyphenol derived from the food spice turmeric (*Curcuma longa*), is a potent antioxidant that binds iron. The primary aim of this study was to investigate whether curcuminoids prevent tumor-promoting effects of iron in T51B cells, a non-neoplastic rat liver epithelial cell line. Purified curcuminoids (curcumin) or a standardized turmeric extract similarly reduced oxidative stress and cytotoxicity associated with iron overload (IC<sub>50</sub> values near 10  $\mu$ M,  $P < 0.05$ ). Inhibition of iron-induced tumor promotion (seen upon treatment with 200  $\mu$ M ferric ammonium citrate  $\pm$  curcumin/turmeric for 16 wk in culture; subsequently assayed by soft agar colony formation) was nearly complete at 20  $\mu$ M of total curcuminoids ( $P < 0.05$ ), a concentration predicted to only partially chelate the added iron. Surprisingly, lower curcumin concentrations (10  $\mu$ M) increased tumor promotion ( $P < 0.01$ ). Curcuminoids delivered as a standardized turmeric extract were taken up better by cells, had a longer half-life, and appeared more effective in blocking tumor promotion ( $P < 0.01$ ), suggesting enhanced curcuminoid delivery to cells in culture. The primary finding that curcuminoids can inhibit tumor promotion caused by iron in T51B cells is tempered by evidence for an underlying increase in neoplastic transformation at lower concentrations.

### Introduction

Turmeric is the powdered rhizome (root) of the Asian plant *Curcuma longa* commonly used as a food spice and coloring agent. It also has a long history of medicinal uses generally ascribed to a high content of bioactive compounds including the polyphenolic iron-binding compound curcumin and related curcuminoids (1,2) (Fig. 1). Curcumin has been widely studied in recent years, with a number of documented anti-inflammatory and anticancer properties. Although the molecular effects of curcumin in vitro and in vivo are consistent with cancer prevention activity in the liver, dietary curcumin has not consistently reduced the incidence of primary liver cancer in several rodent models (3,4).

Iron is an essential metal but can be cytotoxic at high levels, where redox cycling of free or loosely chelated iron causes oxidative stress in cells (5). The liver is the main storage organ

for excess body iron, making it particularly vulnerable to toxicities of iron overload. Accumulation of iron in the livers of hemochromatosis patients can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma. The incidence of primary liver cancer is higher in hemochromatosis than in other causes of chronic liver disease and cirrhosis (6–8). Possible carcinogenic effects of iron include direct action as a mutagen (9) as well as tumor-promoting effects (10). We found previously that iron acts as a tumor promoter but is not by itself carcinogenic in the T51B rat liver cell carcinogenesis model (11).

Repeated cycles of hepatotoxicity and regrowth/regeneration are a common theme of agents that act as liver tumor promoters (12). Because of this, any agent that binds iron tightly or otherwise reduces its cytotoxic effects should prevent tumor promotion. In T51B cells, tumor promotion by iron was blocked by the high-affinity iron chelator deferoxamine (11). Curcumin, which can bind iron with micromolar affinity and prevent iron toxicity (13–15), may have similar effects. The aim of this work was to test the *in vitro* anticancer effects of both a defined mixture of pure curcuminoids and a standardized turmeric extract. We evaluated the concentration dependence of these two preparations to inhibit cytotoxicity and tumor promotion associated with iron overload in T51B liver epithelial cells.

## Methods

### Materials

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ferric ammonium citrate (FAC), and 8-hydroxyquinoline (8HQ) were obtained from Sigma-Aldrich (St. Louis, MO). Newborn calf serum was from Atlanta Biologicals (Norcross, GA), and other cell culture reagents were from GIBCO/Invitrogen (Carlsbad, CA). Turmeric and purified curcuminoids (C3 complex®, a defined mixture of curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin) were donated by Sabinsa Corporation (Piscataway, NJ). One lot of purified curcuminoids (C3 complex®) and two lots of turmeric were used for this study. The curcuminoid concentrations of these preparations are listed in Table 1. Low-temperature melting point agarose was obtained from Lonza (Walkersville, MD).

### Test Product Quality

Turmeric and purified curcuminoids (C3 complex®) were evaluated and stored in accordance with the manufacturer's recommendations. These test products met quality standards expected of substances intended for human consumption (Sabinsa Corporation), as required by the Product Integrity Working Group of the National Advisory Council for Complementary and Alternative Medicine, a primary sponsor of this study. Quality standards included specifications of the World Health Organization for *Rhizoma Curcumae Longae* as to identity, product purity and chemical/biological contamination (16). Purified curcuminoids were dissolved in dimethylformamide to a final concentration of 50 mM and stored at –20°C. Turmeric powder (containing not less than 3.0% w/w total curcuminoids) was extracted with 1.6 ml of dimethylformamide/g for 30 min at room temperature, and particulates were removed by 0.45 µm filtration. Extracts were adjusted to a standardized concentration of 50 mM curcuminoids (quantified by absorbance at 425 nm relative to a defined curcuminoid standard) and stored at –20°C. Monitoring of the turmeric extracts and

purified curcuminoid stock solutions showed no change in curcuminoid concentrations over a period of more than 1 yr. Where indicated the curcuminoids or turmeric extract was added along with iron directly to the culture media; control cultures contained an equal amount of dimethylformamide (0.1% by volume) and/or other solvents as appropriate. The curcuminoid composition of the test products was determined by reverse-phase high performance liquid chromatography (HPLC) using an Inertsil ODS-3 column (Agilent, Santa Clara, CA), with an isocratic mobile phase of tetrahydrofuran: 0.1% citric acid (40:60), as detected by absorbance at 425 nm. Integration of peak areas gave the relative amounts of curcuminoids listed in Table 1.

### Cell culture and biological assays

T51B cells are a non-neoplastic rat liver epithelial cell line maintained as described previously (11). These well-characterized cells have been used previously by us and others to document carcinogenesis and tumor promotion activity in vitro (17–19). They display a normal phenotype of density-inhibited and anchorage-dependent proliferation, but can be transformed to grow in soft agar (an accepted measure of neoplastic transformation) by treatment with carcinogens and tumor promoters (20). T51B cells have characteristics of liver oval cells; we believe that their transformation models hepatocellular carcinoma that originates from this hepatocyte precursor (21).

In general, experimental treatments were started while the cells were actively proliferating (one day after plating) except as noted (confluent T51B cells are largely quiescent). Chronic iron overload was induced by adding iron (as FAC) directly to the culture media for the prescribed time. Acute iron overload was induced using the lipophilic iron chelator 8HQ to rapidly deliver iron to intracellular compartments (15). Lipid peroxidation was measured in confluent cells by thiobarbituric reactive substances (TBARS) assay using a kit from Cayman Chemical (Ann Arbor, MI), and the results were expressed relative to control cells as malondialdehyde (MDA) equivalents. To assess cell viability, treated cells were incubated in 0.3  $\mu\text{g}/\text{ml}$  methylthiazolyldiphenyl-tetrazolium bromide (MTT), and the formazan mitochondrial reduction product was dissolved in DMSO and measured by absorbance at 540 nm (15).

Neoplastic transformation was assessed by determining colony formation in soft agar as described (11). The transformation protocol specified a single 24 h treatment with 0.5  $\mu\text{g}/\text{ml}$  MNNG followed by continuous culture with 200  $\mu\text{M}$  FAC with or without added curcuminoids (Supplemental Table 1). Previous work optimized these conditions for iron-related carcinogenesis in the T51B cell model (11). Although this iron concentration is higher than that normally encountered in human iron overload (~10–20  $\mu\text{M}$  serum iron citrate levels (22)), the form of iron is the same. Higher concentrations were justified by the need to observe neoplastic transformation at a reasonable rate within a reasonable time (compared to human liver cancer that generally requires 20–30 yr to appear in the setting of iron overload). In general, the media was renewed every 3–4 days, and the cells were split every 14 days. For splitting, 200,000 cells were replated into a new P60 dish, and treatments resumed 1 day later. At prescribed times (nominally 16 wk), cell aliquots (25,000 cells per P60 soft agar plate) were plated in quadruplicate soft agar dishes, grown without treatment

for 3 wk, stained with 0.5 mg/ml iodinitrotetrazolium violet, and counted. Colonies larger than 0.17 mm in diameter (approximately 100 cells) were scored as positive.

Tumor promotion results are presented from three experiments that differed slightly in curcuminoid treatments. The conditions and number of soft agar dishes evaluated in each experiment are as follows (Supplemental Table 2): Experiment 1 included control ( $n = 8$ ), 0.5  $\mu\text{g/ml}$  MNNG + 200  $\mu\text{M}$  FAC ( $n = 32$ ), MNNG + FAC + 50  $\mu\text{M}$  curcuminoids ( $n = 28$ ), and MNNG + FAC + 30  $\mu\text{M}$  turmeric ( $n = 16$ ). MNNG was given once, while curcuminoids/turmeric and FAC were added the next day and renewed with fresh media approximately twice a week thereafter. Evaluation of 50  $\mu\text{M}$  turmeric was discontinued during the experiment due to poor cell growth. Experiment 2 included control ( $n = 8$ ), 0.5  $\mu\text{g/ml}$  MNNG + 200  $\mu\text{M}$  FAC ( $n = 12$ ), MNNG + FAC + 10  $\mu\text{M}$  ( $n = 8$ ), MNNG + FAC + 20  $\mu\text{M}$  curcuminoids ( $n = 8$ ), MNNG + FAC + 30  $\mu\text{M}$  curcuminoids ( $n = 8$ ), MNNG + FAC + 5  $\mu\text{M}$  turmeric ( $n = 8$ ), MNNG + FAC + 10  $\mu\text{M}$  turmeric ( $n = 8$ ), and MNNG + FAC + 20  $\mu\text{M}$  turmeric ( $n = 8$ ). MNNG was given once, while curcuminoids/turmeric and FAC were renewed every 3–4 days thereafter. Experiment 3 included control ( $n = 8$ ), 0.5  $\mu\text{g/ml}$  MNNG + 200  $\mu\text{M}$  FAC ( $n = 40$ ), MNNG + FAC + 5  $\mu\text{M}$  curcuminoids ( $n = 8$ ), MNNG + FAC + 10  $\mu\text{M}$  curcuminoids ( $n = 8$ ), MNNG + FAC + 20  $\mu\text{M}$  curcuminoids ( $n = 8$ ), MNNG + FAC + 30  $\mu\text{M}$  curcuminoids ( $n = 8$ ), MNNG + FAC + 5  $\mu\text{M}$  turmeric ( $n = 8$ ), MNNG + FAC + 10  $\mu\text{M}$  turmeric ( $n = 8$ ), and MNNG + FAC + 20  $\mu\text{M}$  turmeric ( $n = 8$ ). MNNG was given once, while curcuminoids/turmeric and FAC were renewed every 2–3 days thereafter. Results from these separate experiments were grouped according to treatment conditions as specified.

Curcuminoid uptake in cells was determined under normal adherent monolayer culture conditions at specified times. Cells were placed on ice and then rinsed twice with 0.1% bovine serum albumin in phosphate-buffered solution (PBS), twice with PBS, and scraped in reagent ethanol. The total curcuminoid concentrations in the ethanolic extracts were measured by absorbance at 425 nm and expressed as absorbance units. Relative curcuminoid composition was assessed by HPLC as described above.

## Statistics

To determine the significance of differences between groups (primarily cells treated with curcuminoids/turmeric vs. cells not given these test products), the data were evaluated using a 2-tailed unpaired *t*-test for samples with unequal variance, and significance was noted at  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*). Normalized values (expressed relative to control cells) were used to compile data from the TBARS experiments. A paired *t*-test was used when testing significance relative to the normalizing group itself.

## Results

### Curcuminoids protect against the cytotoxic effects of acute iron overload

Redox cycling of loosely chelated iron causes oxidative stress and lipid peroxidation that contribute to its cytotoxicity (23). The lipophilic iron chelator 8-hydroxyquinoline (8HQ) was used with FAC to induce rapid iron entry (within minutes) and acute iron overload in T51B cells. Fig. 2 illustrates the protective effects of purified curcuminoids and a

standardized turmeric extract on iron-induced lipid peroxidation in T51B cells. By this measure, both preparations were similarly effective, with apparent  $IC_{50}$ s near  $2 \mu M$  total curcuminoids and complete inhibition evident at  $50 \mu M$ .

Table 1 summarizes the curcuminoid composition of the curcumin test products used in this study. Both purified curcuminoids and the turmeric extract contain 3 types of curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin). Compared to the purified curcuminoids, the turmeric extracts contained a slightly higher proportion of bisdemethoxycurcumin and correspondingly less curcumin, as well as additional compounds co-extracted with the curcuminoids. All 3 curcuminoids contain the keto-enol functional group thought to be critical for iron chelation, and all are thought to possess similar antioxidant and biological activities (24). Although the complete chemical profile of the turmeric extract remains to be determined, curcuminoids are the only turmeric components for which ironbinding activity has been reported.

As shown in Fig. 3, purified curcuminoids and the turmeric extract protected T51B cells against the cytotoxic effects of acute iron overload. The potencies of the two preparations were similar in this assay; both displayed minimal effective concentrations near  $20 \mu M$  total curcuminoids. The apparent  $IC_{50}$ s were higher than those required for inhibition of iron-induced lipid peroxidation (Fig. 2), possibly reflecting the degradation of curcuminoids in cell culture over the longer experimental period (2 days for cell viability vs. 2 h for lipid peroxidation).

### **Curcuminoids protect against cytotoxicity and tumor promotion associated with chronic iron overload**

Fig. 4 shows the curcuminoid concentration dependence for preventing growth inhibition caused by  $500\text{-}\mu M$  FAC. This model of chronic iron overload (5 days) depends on physiological iron uptake to overload the cells. Maximal effects of both purified curcuminoids and the standardized turmeric extract were seen at curcuminoid concentrations between  $5$  and  $20 \mu M$ , well below the added concentration of  $500 \mu M$  iron. Turmeric was slightly more effective at the lowest concentration ( $2 \mu M$ ) and more toxic at the highest concentration ( $50 \mu M$ ). These data placed an upper limit on the concentrations that could be tested in long-term cell culture studies. Acceptable growth inhibition of 20% or less was observed at upper curcuminoid concentration limits for purified curcuminoids of  $50 \mu M$  and for the standardized turmeric extract of  $20 \mu M$ .

Iron was shown previously to act as a tumor promoter in T51B cells (11). Neoplastic transformation, as judged by colony formation in soft agar, required an initiating low dose of the carcinogen MNNG followed by prolonged culture in the presence of iron (given as FAC). To investigate effects of curcuminoids on iron-related neoplastic cell transformation, purified curcuminoids or turmeric extracts were given with FAC throughout the 16-wk tumor promotion phase (Supplemental Table 1). Three experiments were conducted using a range of repeated dosing conditions (Supplemental Table 2). As shown in Table 2, curcuminoids had two contrasting dose-dependent effects on iron-treated T51B cells. Low concentrations appeared to increase colony formation in soft agar. For purified curcuminoids given with  $200 \mu M$  iron,  $5\text{--}10 \mu M$  curcuminoids resulted in a 3-fold increase in soft agar growth compared

to iron alone ( $P < 0.01$ ). Similarly, for the turmeric extract, 5  $\mu\text{M}$  curcuminoids appeared to potentiate the tumor-promoting effects of iron (although this trend was not significant).

In contrast, higher concentrations of curcuminoids blocked neoplastic transformation. At 20  $\mu\text{M}$  curcuminoids or higher, either purified curcuminoids or the standardized turmeric extract inhibited the tumor-promoting effect of 200  $\mu\text{M}$  iron (Table 2). At 10  $\mu\text{M}$ , the turmeric extract also appeared to inhibit tumor promotion by iron (although statistical significance was not achieved). Fig. 5 illustrates the compiled results for curcuminoid concentrations 20  $\mu\text{M}$  for either purified curcuminoids (20–50  $\mu\text{M}$ ) or the standardized turmeric extract (20–30  $\mu\text{M}$ ). Both preparations decreased tumor promotion by iron to near background levels at the 16-wk time point ( $P < 0.01$ ). Analysis of the duration of this effect in a single experiment suggested that the prevention by 20  $\mu\text{M}$  could be maintained through at least 20 wk of iron exposure (Fig. 6).

These data indicate increased potency of the turmeric extracts compared to the purified curcuminoids, presumably due to additional noncurcuminoid compounds in turmeric. These compounds may independently inhibit tumor promotion, or they may alter curcuminoid uptake and/or degradation. As shown in Fig. 7, cells treated with 20  $\mu\text{M}$  turmeric extract contained nearly twice the curcuminoid levels 4 h later as cells treated with 20  $\mu\text{M}$  purified curcuminoids. Curcuminoids recovered from the cells after 1 h had essentially the same ratios of curcumin: demethoxycurcumin: bisdemethoxycurcumin as the starting preparations. For purified curcuminoids recovered from cells, the ratio was 75:22:3 (vs. 75:22:3 in the starting material, Table 1) and for turmeric, it was 53:28:19 (vs. 58:24:18 in the starting material, Table 1). Evaluation of the kinetic data shown in Fig. 7 using a one-phase decay model gave an apparent half-life in T51B cell culture for the turmeric extract curcuminoids of 43 h compared to 15 h for purified curcuminoids.

## Discussion

### Curcuminoids as cancer prevention agents in iron overload

The incidence of primary liver cancer in humans with severe iron overload is nearly 10% (8,9), higher than any other known risk factor. Identifying non-invasive preventive therapies is hampered by the long latency period (up to 30 yr in humans) and paucity of cell and animal models. T51B rat liver epithelial cells are a non-neoplastic cell line used previously to document the tumor-promoting properties of iron (11). A single subcarcinogenic initiating dose of MNNG followed by an extended culture in 200  $\mu\text{M}$  FAC resulted in neoplastic transformation of the cells. FAC was chosen as the form of iron because iron citrate is found in the serum of hereditary hemochromatosis patients (22). Thus, this in vitro model depends on a physiologically relevant form of iron to progress to a physiologically relevant end point (growth in soft agar), making it uniquely useful for investigating preventive therapies. The present paper shows that 20  $\mu\text{M}$  curcuminoids can block tumor-promoting effects of iron in T51B liver cells (Fig. 5). The specific concentration is significant in that it is insufficient to completely chelate the iron used in the experiment, given a binding stoichiometry of 1 or more curcumin molecules per iron atom (13,14). Toxic effects of iron were similarly inhibited by substoichiometric concentrations of curcuminoids [Figs. 2–4 and (15)]. Curcumin acts differently than the iron-specific chelator deferoxamine, which was required



in stoichiometric amounts to prevent toxicity (15) and in vitro tumor promotion by iron (11). Thus, block of tumor promotion is not linked to chelation, but instead appears dependent on curcumin entry into cells and subsequent inhibition of one or more events downstream of iron.

By definition, tumor promoters favor the selective growth of preneoplastic cells. This may occur if iron directly stimulated proliferation of those cells, as might be expected, if low iron levels were limiting growth. This is likely not relevant in vitro for FAC, since transferrin-bound iron from the serum/media is readily available, and nontransferrin-bound iron does not stimulate cell proliferation (11,25,26). Another possibility is that free or loosely chelated iron is toxic to normal cells but not the transformed cells. The idea that neoplastic cells become resistant to effects of a growth inhibitor was proposed by Farber and colleagues as the “resistant hepatocyte model” of tumor promotion (27). FAC can be toxic, but we have not been able to detect an iron-resistant phenotype in T51B daughter cells isolated from soft agar, and for that reason, we do not believe that the transformed cells have acquired resistance. A third possibility is the related concept of compensatory proliferation, thought to be particularly relevant in the liver (12,28). According to this theory, cytotoxicity speeds the carcinogenesis process because otherwise quiescent preneoplastic cells are triggered to proliferate, thereby accumulating the multiple mutations required for carcinogenesis. Compensatory proliferation in the context of cytotoxic free iron seems most consistent with effects of iron in the T51B cell carcinogenesis model (11). We propose that curcuminoids reduce iron toxicity and/or compensatory proliferation in vitro, accounting for the observed block of the tumor-promoting effects of iron. This occurred at curcuminoid concentrations that are high enough to reduce iron toxicity but below the threshold where the curcuminoids themselves become toxic through some other mechanism.

### **Other compounds in turmeric improve curcuminoid delivery in cell culture experiments**

The standardized turmeric extract appeared more effective at blocking tumor promotion by iron than the purified curcuminoids (Fig. 5 and Table 2). This is likely not the result of differences in curcuminoid composition between the turmeric extract and the purified preparation (i.e., a 5–6 fold higher content of bisdemethoxycurcumin in turmeric) (Table 1). There are no clear differences in biological activities between the three curcuminoids (for example, all contain the keto-enol structure important for iron binding), and we did not detect differences in uptake between them. More likely, other constituents in the turmeric extract account for the observed increase in potency. Such constituents could act independently of the curcuminoids, or they could increase the intracellular concentration of curcuminoids by facilitating/extending uptake or preventing degradation. The kinetic data in Fig. 7 argue for improved delivery of curcuminoids to cells in culture. The roughly twofold increase in potency was similar to the enhanced cellular uptake and prolonged half-life of curcuminoids given as the turmeric extract. How this occurs is unknown; further work is required to identify the molecules and characterize their interactions with curcuminoids in vitro and in vivo.



## Chelation by curcuminoids may increase the tumor-promoting effects of iron

Concentrations of curcuminoids below that required to block tumor promotion by iron had the surprising effect of increasing neoplastic transformation in the presence of iron (Table 2). It seems likely that this occurs only in the setting of iron overload, since many studies done in the absence of added iron have failed to observe carcinogenic and/or tumor-promoting effects of curcuminoids. There are reports that the carcinogenic effect of free iron is increased by lipophilic iron chelators like nitrilotriacetate (NTA) (29–32). Curcumin may act similarly to NTA as a membrane-permeating agent, thereby increasing the mutagenic (9) or tumor-promoting activities of iron. However, purified curcuminoids and turmeric extracts inhibited iron toxicity in short-term experiments that depended on 8HQ for rapid iron entry to the cells (Figs. 2 and 3). Furthermore, a significant increase in iron levels in curcumin-treated cells was not detected (15), and curcumin does not alter liver iron levels in mice, although important regulators of iron homeostasis including ferritin are dysregulated (33,34). These points argue that transmembrane iron permeating activity of curcuminoids, if it exists, is significantly less than seen with 8HQ or NTA. However, even if curcuminoids do not transport iron across membranes, their demonstrated ability to bind iron with micromolar affinity is predicted to increase the solubility of ferric iron, by analogy to citrate and other physiological iron chelators. An increase in redox-active free iron may result, especially under cell culture or disease conditions in which options for iron sequestration in transferrin or ferritin are limited. Thus, although iron chelation does not explain inhibition of tumor promotion by curcuminoids, it may contribute to the increased tumor promotion activity of iron observed here.

We observed under very specific cell culture conditions that 20  $\mu\text{M}$  curcuminoids was the threshold concentration for inhibition of tumor promotion in T51B cells. If a comparable effect occurs in vivo, a similar concentration should be required. However, this concentration is greater than that has been observed in the blood of animals or humans given curcuminoids in the diet (35). Unless bioavailability can be improved, it seems unlikely that curcuminoids given orally would confer this benefit to humans. Minimally, the curcumin would have to be injected, a daunting requirement for long-term prevention therapy. Importantly, lower concentrations of curcuminoids (that we found to increase in vitro tumor promotion by iron) approach serum levels of curcuminoids and iron-binding curcuminoid metabolites achievable from dietary curcumin (36). This previously unrecognized and potentially harmful outcome may negate any potential health benefits of curcumin or turmeric when iron levels are high. Further investigation of the risk/benefit of curcumin and turmeric in the context of elevated body iron, as exists in humans with hemochromatosis and other iron overload disorders, is needed.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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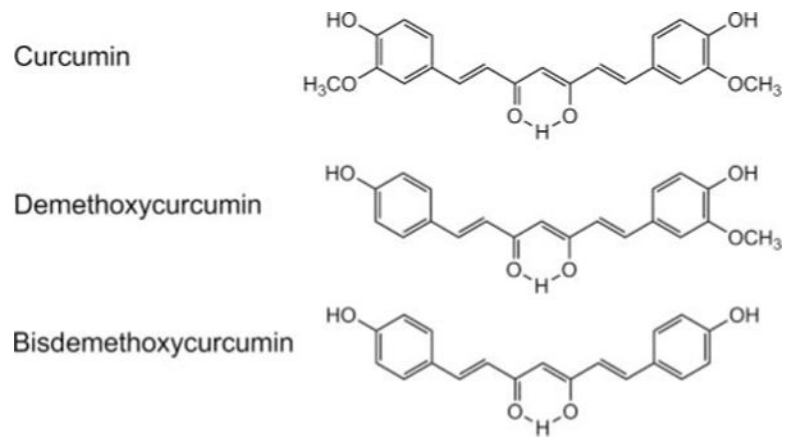
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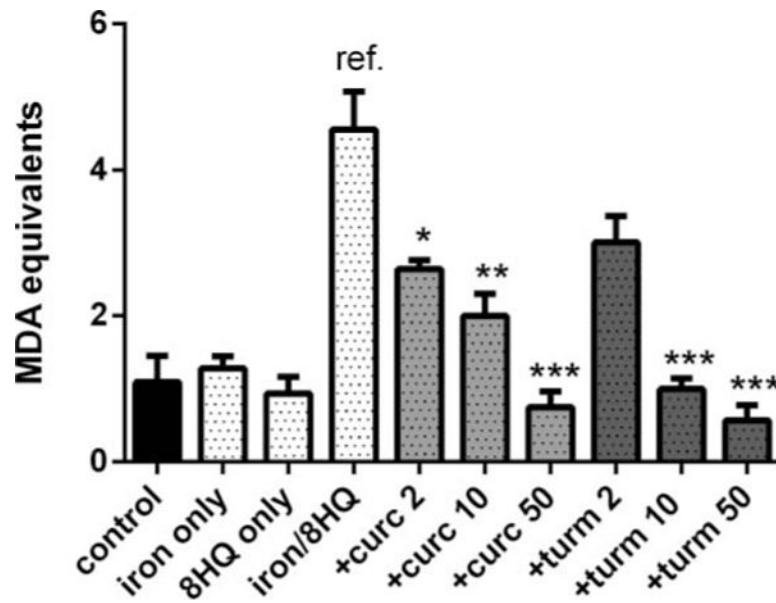
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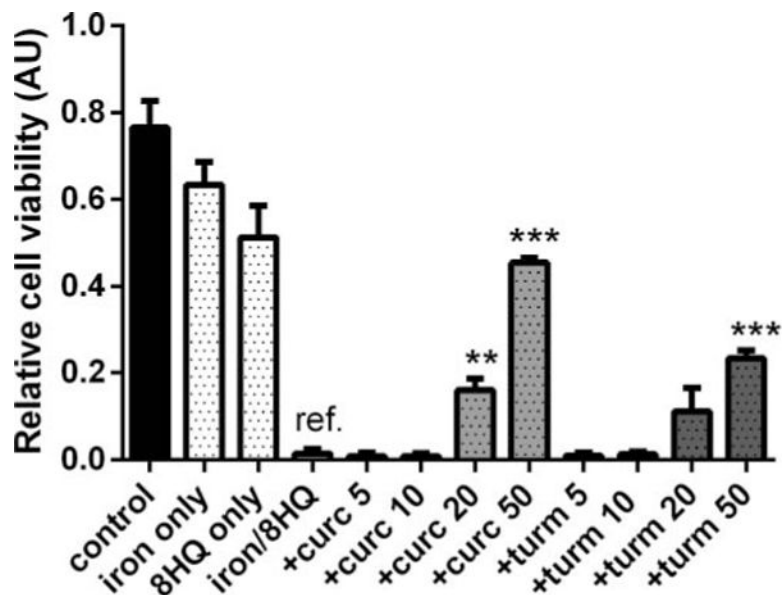


**Figure 1.**  
Structures of curcumin and related curcuminoids.

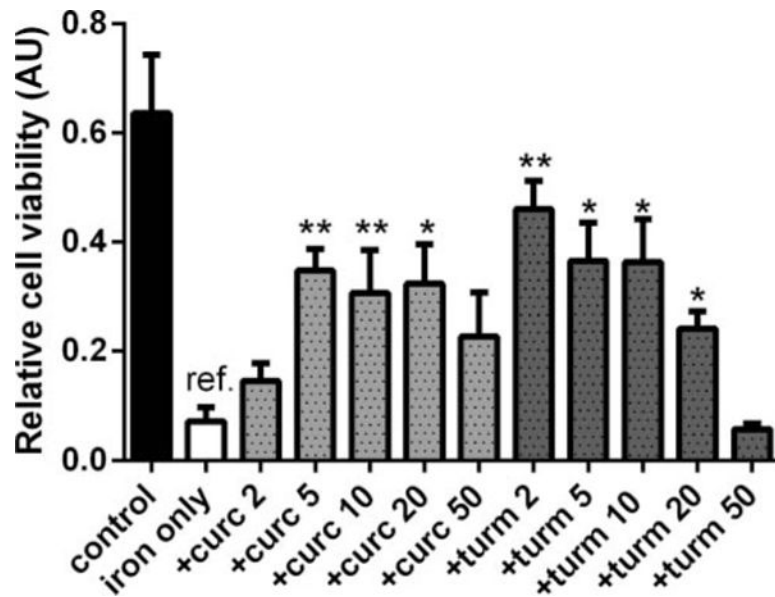


**Figure 2.**

Curcuminoids prevent iron-induced lipid peroxidation. Confluent T51B cells were treated for 2 h  $\pm$  200  $\mu$ M iron and  $\pm$ 10  $\mu$ M 8-hydroxyquinoline (8HQ) as indicated. Curcuminoids were added with iron/8HQ as either purified curcuminoids (curc at 2, 10, or 50  $\mu$ M) or a standardized turmeric extract (turm at 2, 10, or 50  $\mu$ M). Cell lysates were analyzed by TBARS assay and results expressed in MDA equivalents. Means  $\pm$  SE from 4 replicate experiments are presented. (\*\*\*)  $P < 0.001$  vs. iron + 8HQ reference (ref.); \*\*  $P < 0.01$ ; \*  $P < 0.05$ .)



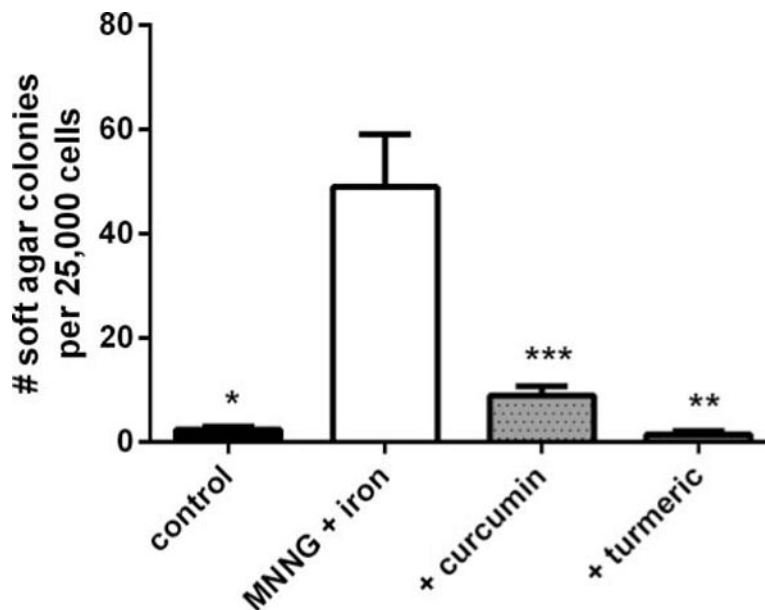
**Figure 3.** Curcuminoids reduce acute iron cytotoxicity. Subconfluent, proliferating T51B cells were treated for 2 days ± 200 μM iron and ± 10 μM 8-hydroxyquinoline (8HQ) as indicated. Curcuminoids were added with iron/8HQ as either purified curcuminoids (curc at 5, 10, 20, or 50 μM) or a standardized turmeric extract (turm at 5, 10, 20, or 50 μM). Cell number and viability were determined by MTT assay. Means ± SE from 3 replicate experiments are presented. (\*\**P* < 0.001 vs. iron + 8HQ reference (ref.); \*\**P* < 0.01; \**P* < 0.05.)



**Figure 4.**

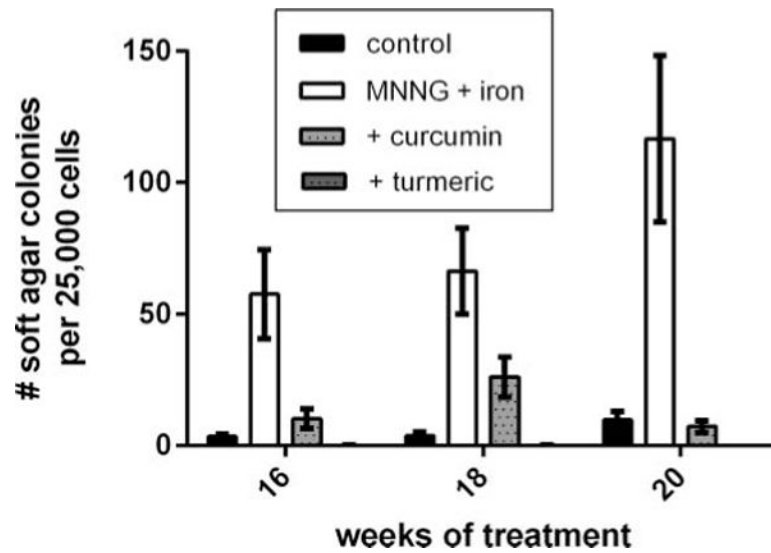
Curcuminoids reduce the cytotoxicity of chronic iron loading. Proliferating T51B cells were treated for 5 days  $\pm$  500  $\mu$ M iron. Curcuminoids were added with iron as either purified curcuminoids (curc at 2, 5, 10, 20, or 50  $\mu$ M) or a standardized turmeric extract (turm at 2, 5, 10, 20, or 50  $\mu$ M). Cell number and viability were determined by MTT assay. Means  $\pm$  SE from 3 replicates are presented. (\*\* $P$  < 0.01 vs. 500  $\mu$ M iron only reference (ref.); \* $P$  < 0.05.)





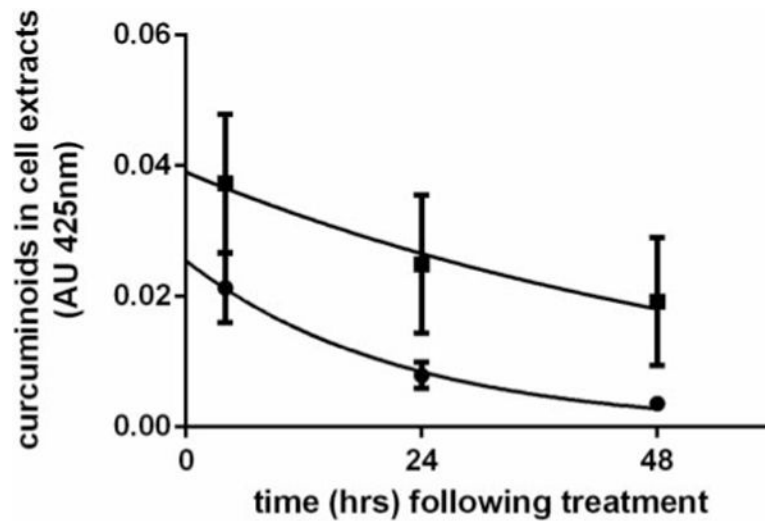
**Figure 5.**

Curcuminoids inhibit tumor promotion by iron. Proliferating T51B cells were treated with 0.5  $\mu\text{g}/\text{ml}$  MNNG for 24 h (initiated) and cultured for 16 wk with 200  $\mu\text{M}$  iron and  $\pm$ curcuminoids or turmeric (20  $\mu\text{M}$ ) as described under “Methods” section. Control cells (no MNNG or iron) were run in parallel. Colony formation in soft agar was measured and results compiled from three independent experiments. The total number of soft agar dishes and treatment conditions were as follows: Control ( $n = 24$  total); MNNG + iron ( $n = 75$  total); purified curcuminoids (curcumin;  $n = 60$  total: 16 at 20  $\mu\text{M}$ , 16 at 30  $\mu\text{M}$ , and 28 at 50  $\mu\text{M}$ ); and turmeric ( $n = 32$  total: 16 at 20  $\mu\text{M}$  and 16 at 30  $\mu\text{M}$ ). (\*\*\*)  $P < 0.001$  vs. MNNG + iron; \*\*  $P < 0.01$ ; \*  $P < 0.05$ .)



**Figure 6.**

Inhibition of tumor promotion by curcuminoids through 20 wk. Proliferating T51B cells were initiated with MNNG and cultured with 200  $\mu\text{M}$  iron and  $\pm 20 \mu\text{M}$  curcuminoids or turmeric with media renewal every 2.6 days on average. Colony formation in soft agar was assessed at 16, 18, and 20 wk. The number of soft agar dishes in this experiment was: Control ( $n = 8$  each at weeks 16–20); MNNG + iron ( $n = 40$  each at weeks 16–20); 20  $\mu\text{M}$  purified curcuminoids (curcumin;  $n = 8$  each at weeks 16–20); 20  $\mu\text{M}$  turmeric ( $n = 8$  each at weeks 16–20).



**Figure 7.**

The *in vitro* pharmacokinetic properties of purified curcuminoids differ from those of the turmeric extract. Confluent T51B cells were treated at time zero with 200  $\mu\text{M}$  iron and 20  $\mu\text{M}$  curcuminoids, given either as purified curcuminoids (circles) or standardized turmeric extract (squares). Curcuminoid levels in the cells at the indicated times were determined by absorbance at 425 nm as described under “Methods” section. The data represent means and SE from  $n = 6$  independent experiments, each set with a best fit line drawn using a one-phase decay model.

**Table 1**

Relative curcuminoid composition of study test products (% of total).

	<b>Purified curcuminoids (%)</b>	<b>Turmeric 1 (%)</b>	<b>Turmeric 2 (%)</b>
Curcumin	75.0	62.4	58.4
Demethoxycurcumin	21.7	21.3	23.6
Bisdemethoxycurcumin	3.35	16.3	18.0

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**Table 2**

Inhibition of tumor promotion by iron in vitro (16 weeks): dependence on curcuminoid concentrations (average number of soft agar colonies/25,000 cells).

Treatment	# Colonies $\pm$ SE ( <i>n</i> )	<i>P</i> -value
Control	2.5 $\pm$ 0.5 (24)	<0.001
MNNG + 200- $\mu$ M iron <sup>#</sup>	44.3 $\pm$ 9.3 (84)	Reference
+5- $\mu$ M curcuminoids	27.5 $\pm$ 5.6 (8)	ns
+10- $\mu$ M curcuminoids	247 $\pm$ 63 (16)	<0.01
+20- $\mu$ M curcuminoids	17.4 $\pm$ 5.2 (16)	<0.05
+30- $\mu$ M curcuminoids	1.7 $\pm$ 0.2 (16)	<0.001
+50- $\mu$ M curcuminoids	8.1 $\pm$ 2.1 (28)	<0.001
+5- $\mu$ M turmeric	162 $\pm$ 70 (16)	ns
+10- $\mu$ M turmeric	29.1 $\pm$ 8.8 (16)	ns
+20- $\mu$ M turmeric	0.2 $\pm$ 0.1 (16)	<0.001
+30- $\mu$ M turmeric	2.8 $\pm$ 1.3 (16)	<0.001

<sup>#</sup>MNNG (N-methyl-N'-nitro-N-nitrosoguanidine; FAC (ferric ammonium citrate).

*n* = total number of soft agar assay dishes; SE = standard error; ns = not significant.