

Curcumin: a novel Stat3 pathway inhibitor for chemoprevention of lung cancer

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Multiple studies from independent groups find evidence for signal transducer and activator of transcription 3 (Stat3) activation in nearly 50% of lung cancers, suggesting a functional role for this target in subsets of lung cancer. On the basis of the existing evidence, we hypothesized that bioavailable curcuminoid complex may modulate lung carcinogenesis, primarily by inhibiting Stat3 activation. With the safety of this being botanically well established, the objective of these studies was to test our hypothesis *in vitro* and *in vivo* in an effort to inform the design of a phase II chemoprevention trial in former smokers. We treated non-tumor-derived, normal (but immortalized) human bronchial epithelial cells (AALE) (Lundberg *et al.*, 2002; Pillai *et al.*, 2011) and lung adenocarcinoma-derived cells (H441) with bioactive curcumin C3 complex. Asynchronous cells in each case were treated with curcumin for 24 h, followed by immunoblotting for Stat3 and activated Stat3-P, prior signal of which was used for normalization. We also completed a preclinical trial in which 12 mice were randomly divided into three groups and subjected to 3 days or 9 days of curcumin intraperitoneal injections, followed by analysis of lung tissues for Stat3-P changes and growth suppressive effects of the curcumin. The growth suppressive effects were measured using Cyclin D1 and the replicative helicase subunit, Mcm2, as surrogates for the proliferative capacity of the tissues. *In-vitro* studies with curcuminoid complex demonstrated that the activity of Stat3 in both normal bronchoepithelial cells and lung cancer-derived cells is sensitive to curcumin

exposure. In a dose-dependent manner, curcumin treatment resulted in significant suppression of Stat3 phosphorylation and reduction in the proliferative capacity of both cell types. In the preclinical trial with rodent models, curcumin reduced Stat3-P and the proliferative markers CycD1 and Mcm2 in mice lung tissues *in vivo*. These culture and preclinical studies indicate that the activity of the Stat3 pathway can be suppressed by curcumin treatment, concomitant with a reduction in cell proliferation, supporting our hypothesis that inhibition of the Stat3 pathway represents at least one important mechanism by which curcumin elicits its effects on the bronchoepithelium. These data provide a rationale for the use of curcumin as a promising chemopreventive agent in high-risk populations such as former smokers. *European Journal of Cancer Prevention* 00:000–000 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Lung cancer is the leading cause of cancer deaths worldwide and is responsible for 29% of cancer-related deaths in USA (Jemal *et al.*, 2007; Keith *et al.*, 2011). In addition to age and obstructive pulmonary disease, cigarette smoking is the major cause of lung cancer in USA and prevention of tobacco exposure has become critical in reducing lung cancer mortality (Garfinkel and Silverberg, 1991; Irvin and Brandon, 2000; Jemal *et al.*, 2007; Keith *et al.*, 2011). However, recent studies have demonstrated that over 50% of new lung cancers occur in former smokers, who are highly motivated and eagerly seeking strategies to reduce their risk (Tong *et al.*, 1996). Although a number of previous studies to prevent lung cancer in smokers have

failed, our understanding of novel compounds and their molecular targets relevant to pulmonary carcinogenesis, specific to current and former smokers has vastly expanded (Nicholson *et al.*, 2001; O'Shaughnessy *et al.*, 2002; Lynch *et al.*, 2006; Kelly *et al.*, 2009; Keith *et al.*, 2011). Other than smoking cessation as a prevention strategy, there is an urgent need to identify and test effectiveness and safety of promising, novel nutrient-derived substances as chemoprevention agents to modulate lung carcinogenesis.

Members of the signal transducer and activator of transcription (Stat) family of transcription factors are potential targets in lung cancer and other cancers (Yu and Jove, 2004). Janus kinase/Stat signaling can be a common pathway activated by diverse upstream signaling proteins, including growth factor receptors, cytokines, and non-receptor tyrosine kinases such as Src and Abl. Stat proteins

Publications/Presentations: To date, the results of this trial in part or as a whole have not been reported or presented elsewhere.

are latent transcription factors that are activated by upstream tyrosine kinase signaling and control genes that regulate cancer hallmarks. Indirect or direct inhibition of Stat3 has been shown to affect tumor formation through inhibition of cell growth, induction of apoptosis, or inhibition of tumor angiogenesis (Yu and Jove, 2004). Stat proteins, in particular Stat3, are oncogenic in part by activating a gene transcription program that affects multiple cancer hallmarks. This includes cell proliferation (cyclin D, Myc), antiapoptotic signaling (Mcl-1, Bcl-xL), angiogenesis (vascular endothelial growth factor), and immune evasion (Yu and Jove, 2004). Although nontumor cells have robust systems that allow only transient activation of this pathway, tumor cells acquire persistent pathway activation through various mechanisms. Targeting strategies such as small molecule inhibitors, natural products such as curcumin, RNA interference, and tyrosine kinase inhibitors are potential strategies to target Stat3 signaling in cancer (Haura *et al.*, 2005a). Multiple studies from independent groups find evidence for Stat3 activation in nearly 50% of lung cancers, suggesting a functional role for this target in subsets of lung cancer (Haura *et al.*, 2005b; Cortas *et al.*, 2007; Gao *et al.*, 2007). IL-6 and Janus kinase signaling regulate Stat3 activity in lung cancer cells through an autocrine mechanism (Gao *et al.*, 2007). Our previous study found IL-6 to be a strong activator of Stat3 in lung cancer cells and along with its expression in lung cancer tumors suggests that this pathway could be responsible for constitutive Stat3 levels in lung cancer tumor cells (Song *et al.*, 2003; Haura *et al.*, 2006; Yeh *et al.*, 2006). There is evidence in mouse models that tobacco smoke exposure leads to activation of the IL-6/Stat3 pathway (Halappanavar *et al.*, 2009). Finally, overexpression of Stat3 in alveolar type II epithelial cells in mice leads to severe inflammation (associated with increased production of cytokines and chemokines) and spontaneous generation of adenocarcinomas (Li *et al.*, 2007). For these reasons, targeting Stat3 activation could be an important approach toward the prevention of lung cancers.

Curcumin (diferuloylmethane) is a natural compound derived from the rhizome of *Curcuma longa*, an East Indian plant, commonly called turmeric. Historically, curcumin has been used to treat inflammatory disorders, including various respiratory conditions, with no toxicity observed with use (Aggarwal *et al.*, 2006; Lao *et al.*, 2006; Anand *et al.*, 2008). The major curcuminoids present in turmeric are demethoxycurcumin, bisdemethoxycurcumin and cyclocurcumin, together termed the curcuminoid complex (Kiuchi *et al.*, 1993; Bansal *et al.*, 2011). Extensive research over the past two decades suggests that curcumin has multiple molecular targets and influences several biochemical and molecular cascades involved in cell cycle regulation, apoptosis, proliferation, survival, invasion, angiogenesis, metastasis and inflammation, providing support for the chemoprevention potential of curcumin (Choudhuri *et al.*, 2005; Cho *et al.*, 2007; Goel *et al.*, 2008; Kunnumakkara *et al.*,

2008; Sameermahmood *et al.*, 2008; Aggarwal and Harikumar, 2009; Lin *et al.*, 2009; Bill *et al.*, 2010; Chen *et al.*, 2010a, 2010b; Wu *et al.*, 2010). In further support of this, studies with curcumin in lung carcinogenesis have demonstrated that it can induce apoptosis in human lung adenocarcinoma A549 and nonsmall cell lung cancer NCI-H460 cells, in a dose-dependant manner through mitochondria-dependent pathways (Chen *et al.*, 2010b; Wu *et al.*, 2010). Curcumin has also been shown to inhibit the migration and invasion of A549 lung cancer cells through the inhibition of matrix metalloproteinase-2, matrix metalloproteinase-9, and vascular endothelial growth factor, demonstrating its antimetastatic potential (Lin *et al.*, 2009).

In preclinical trials with rodent models, studies have demonstrated curcumin's chemopreventive potential in lung cancer. In a K-ras-induced mouse model, Moghaddam *et al.* (2009) administered curcumin, 1% in diet, before and during weekly nontypeable *Haemophilus influenzae* (NTHi) exposure. This significantly reduced the number of visible lung tumors in the absence of NTHi exposure by 85% and in the presence of NTHi exposure by 53%. Mechanistically, curcumin markedly suppressed NTHi-induced increased levels of the neutrophil chemoattractant keratinocyte-derived chemokine by 80% and neutrophils by 87% in bronchoalveolar lavage fluid. In-vitro studies of murine K-ras-induced lung adenocarcinoma cell lines (LKR-10 and LKR-13) indicated direct antitumoral effects of curcumin by reducing cell viability, colony formation, and inducing apoptosis. The researchers concluded that curcumin suppresses the progression of K-ras-induced lung cancer in mice by inhibiting intrinsic and extrinsic inflammation and by direct antitumoral effects. These findings suggest that curcumin could be used to protract the premalignant phase and inhibit lung cancer progression in high-risk chronic obstructive pulmonary disease patients (Moghaddam *et al.*, 2009). It has also been shown that curcumin increases antioxidant defenses in lung, ameliorates radiation-induced pulmonary fibrosis, and improves survival in mice (Lee *et al.*, 2010), although not impairing tumor-cell killing by radiation.

On the basis of the existing evidence, we hypothesized that bioavailable curcuminoid complex may modulate lung carcinogenesis, primarily by inhibiting Stat3 activation. With the safety of curcumin well established because of the long use as a component of food in India, the objective of our studies is to test our hypothesis *in vitro* and *in vivo*, in an effort to inform the design of a phase II chemoprevention trial of curcumin in former smokers.

Materials and methods

Curcuminoid compound

We selected Sabinsa's curcumin C3 complex, which is composed of three main chemical compounds – curcumin, demethoxycurcumin, and bisdemethoxycurcumin – collectively known as curcuminoids. C3 curcuminoid

capsules, provided in a single batch by the Sabinsa Corporation (Piscataway, New Jersey, USA) were used in these studies. Sabinsa Corporation received generally recognized as safe status for this branded and patented ingredient curcumin C3 complex – *C. longa* (turmeric), after a comprehensive review of safety and toxicology data by an independent panel of scientists with international repute assembled by Soni & Associates Inc. (Vero Beach, Florida, USA). This formulation was selected on account of its reproducibility and bioavailability of curcuminoid content as demonstrated in previous trials in humans.

Cell culture

AALÉ normal human bronchoepithelial cells were provided by Melissa Hector (Dana–Farber Cancer Institute, Boston, Massachusetts, USA), and H441 human lung adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, Virginia, USA). AALÉ were cultured in bronchial epithelial basal medium with Bronchial Epithelial Growth Medium supplements (Lonza, Walkersville, Maryland, USA), and H441 were cultured in Roswell Park Memorial Institute medium with 10% fetal bovine serum.

Antibodies and immunoblotting

The antiCycD1 and antiphosphotyrosine-Stat3 (P-705) rabbit polyclonal antibodies were obtained from Cell Signaling (Danvers, Massachusetts, USA) and the total Stat3 rabbit polyclonal antibody was obtained from Santa Cruz Biotech (Santa Cruz, California, USA). Rabbit polyclonal antiMcm2 was generated by the Alexandrow lab (Moffitt Cancer Center, Tampa, Florida, USA) and has been reported previously (Mukherjee *et al.*, 2009, 2010). Mouse monoclonal anti-Actin was obtained from Sigma-Aldrich (St Louis, Missouri, USA) and used at a dilution of 1:10 000 for immunoblotting. All other primary antibodies were used at a dilution of 1:1000 for immunoblots. Immunoblotting experiments were performed using standard techniques and enhanced chemiluminescence.

Mouse experiments

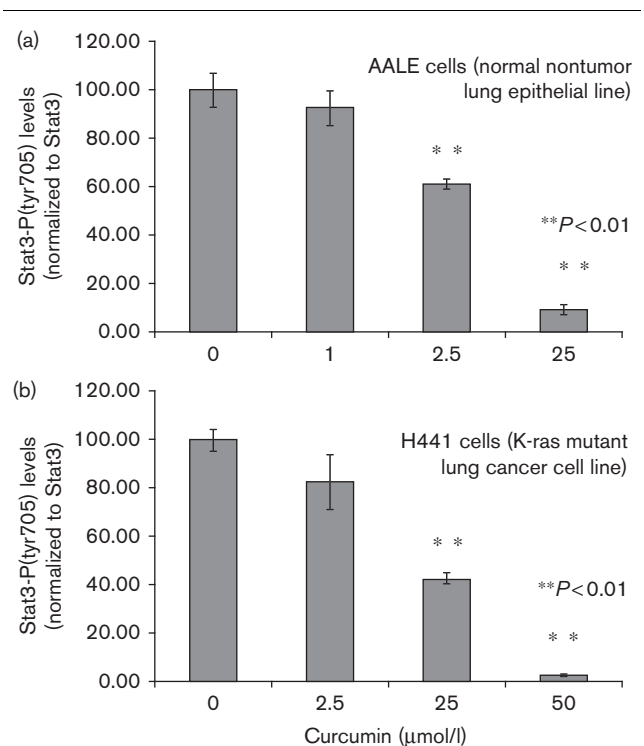
CD-1 female nude mice were obtained from Charles River (Wilmington, Massachusetts, USA). The mice were treated with curcumin after 6–7 weeks, and the weight of each mouse was ~22 g. Curcumin dissolution with dimethyl sulfoxide (DMSO) was given to mice at a dose of 50 mg/2.5 ml/kg by daily intraperitoneal injection. Equal amounts of DMSO diluted in water were administered to the control group. Twelve mice were randomly divided into three groups of four. Two groups of mice were given curcumin for 3 days or 9 days. The control group was given DMSO for 9 days. The mice were sacrificed and whole lung tissue was removed for generating the protein extracts. The total protein extract of 80 µg was used for immunoblotting with indicated antibodies.

Results

Curcumin suppresses Stat3 activation and proliferation of lung cells in culture

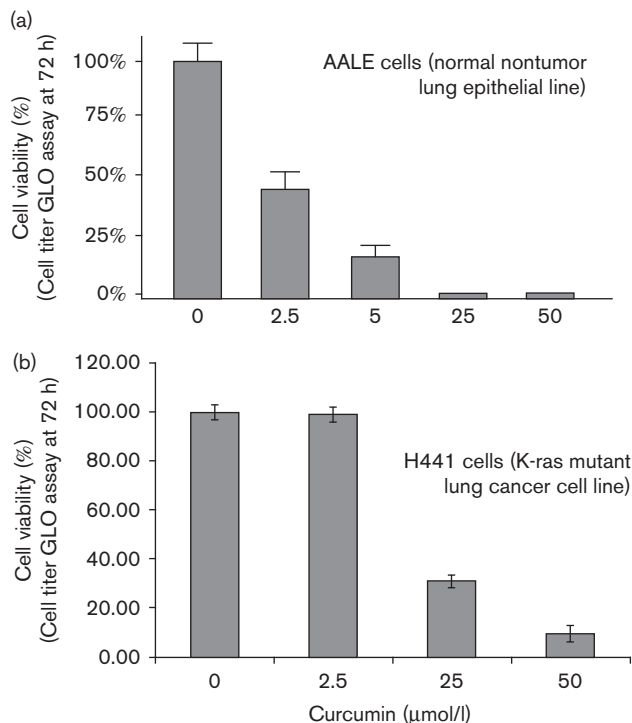
We treated non-tumor-derived, normal (but immortalized) human bronchial epithelial cells (Fig. 1a, AALÉ cells) and human lung adenocarcinoma-derived cells (Fig. 1b, H441 cells) with bioactive curcumin C3 complex. Asynchronous cells in each case were treated with curcumin at the doses indicated for 24 h, followed by Luminex assays for Stat3-P and unphosphorylated Stat3 in total protein extracts. The Stat3 signal was used for normalization to determine changes in Stat3-P levels. In both AALÉ and H441 cells, treatment with curcumin resulted in a dose-dependent reduction in the levels of activated Stat3, as measured by the levels of Stat3 phosphorylated on Tyr-705 (Stat3-P). In addition, curcumin treatment resulted in reduced cell proliferation in a dose-dependent manner for both the AALÉ and H441 cells (Fig. 2a and b). We conclude from these in-vitro culture studies that the activity of the Stat3 pathway in normal and lung carcinoma cells can be suppressed by curcumin treatment, concomitant with a reduction in cell proliferation. Although we do not have

Fig. 1



Curcumin inhibits the Stat3 pathway in normal and lung cancer-derived human bronchoepithelial cells. Asynchronous AALÉ (a) or H441 (b) cells were treated with curcumin at the doses indicated for 24 h, following which Luminex assays were performed on total protein extracts for P-Tyr705-Stat3 and total Stat3. Readings were used to normalize P-Stat3:Stat3, and resulting data are shown \pm 1SD. Untreated samples were arbitrarily set at 100% Stat3-P level (first column in each set). Statistically significant differences are indicated with *P* values.

Fig. 2



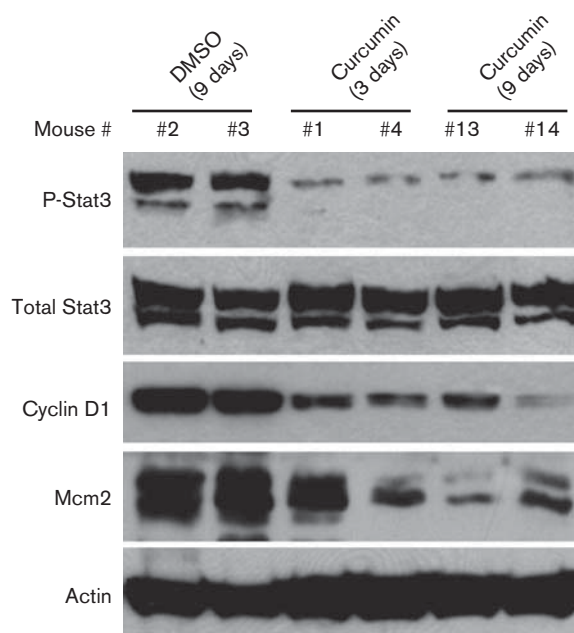
Curcumin inhibits the proliferation of human normal and lung cancer cells. Asynchronous AALE (a) or H441 (b) cells were treated with curcumin at the doses indicated for 72 h, following which cell viability (Proteosome-Glo assay; Promega Corporation, Madison, Wisconsin, USA) assays were performed to determine growth suppressive effects of curcumin on each cell type. Triplicate samples were tested, and average results are shown \pm 1SD. Samples were normalized to untreated samples shown in first column in each graph, arbitrarily set to 100% viability.

access to true dysplastic cell lines in culture, we further conclude from these results, using immortalized normal (and tumor-derived) lung cells as a surrogate, that curcumin likely has the propensity to suppress nonneoplastic cells that will be present in dysplastic lesions. Importantly, such findings provide a rationale for performing a phase II chemopreventive curcumin trial in former smokers.

Curcumin suppresses Stat3 activation and proliferation in lung tissue in vivo

We performed a preclinical trial in which 12 mice were randomly divided into three groups and subjected to 3 days or 9 days of curcumin intraperitoneal injections (or DMSO control), followed by analysis of lung tissues for Stat3-P changes and growth suppressive effects of the curcumin (Fig. 3). The growth suppressive effects were measured using Cyclin D1 and Mcm2 as surrogates for the proliferative capacity of the tissues. Cyclin D1 is a well-known cell cycle regulatory protein expressed in proliferating cells, and Mcm2 is a member of the DNA replicative helicase hexameric complex active and expressed in

Fig. 3



Curcumin suppresses the proliferative capacity of normal lung tissue *in vivo* in mice. Curcumin in dimethyl sulfoxide (DMSO) (or DMSO as a control) was given to mice at 50 mg/2.5 ml/kg daily by intraperitoneal injection, for 3 or 9 days. Twelve mice were randomly divided into three groups (9 days DMSO, 3 days curcumin, 9 days curcumin), and results from two mice for each condition are shown. Immunoblots were performed on total protein extracts using antibodies obtained from mice that were sacrificed to obtain whole lung tissue for protein samples. Similar results were obtained in the remaining mice for each condition.

proliferating cells in culture and in animal tissues (Bell and Dutta, 2002; Mukherjee *et al.*, 2009, 2010). Intriguingly, curcumin exposure dramatically suppressed Stat3-P (but not Stat3 total levels), and further suppressed Cyclin D1 and Mcm2 markers; the latter two are indicative of a reduced proliferative capacity of the lung tissues in the presence of curcumin for 3 or 9 days. We conclude from this *in-vivo* preclinical study that curcumin treatment indeed has the ability to suppress the proliferative capacity of lung tissues in animals, which is accompanied by a significant reduction in Stat3-P activation. This further supports our rationale for a chemopreventive curcumin trial for former smokers, and our hypothesis that Stat3-P suppression is an important mechanism by which curcumin reduces cell growth.

Discussion

These results show that the activity of the Stat3 pathway in both normal human bronchoepithelial cells and lung cancer-derived cells is sensitive to curcumin exposure. In a dose-dependent manner, curcumin treatment results in significant suppression of Stat3 phosphorylation, indicative of Stat3 pathway suppression, and concomitantly reduces the proliferative capacity of both cell types. In agreement with this, preclinical trials using rodent

models (Moghaddam *et al.*, 2009; Lee *et al.*, 2010), and similar results herein, demonstrate that curcumin reduces Stat3-P and proliferation of murine lung tissue *in vivo*. Altogether, these findings support our hypothesis that inhibition of the Stat3 pathway represents at least one important mechanism by which curcumin elicits its growth suppressive effects on the bronchoepithelium.

Future directions

There is a pressing need to identify novel agents for lung cancer chemoprevention beyond smoking cessation. Several novel nutrient-derived substances such as black and green tea polyphenols, resveratrol, isoflavones, indole-3-carbinol, and anthocyanins (Pastorino, 1994; Riboli, 1996; Yu *et al.*, 1997; Bianchini and Vainio, 2003; Banerjee *et al.*, 2005) have shown promise in preclinical and laboratory studies for lung cancer chemoprevention. Among these naturally derived compounds, as demonstrated in our preclinical and in-vitro studies, curcumin appears most promising in modulating lung carcinogenesis. Several recently completed phase I–II clinical trials targeting gastrointestinal tract cancers have also reported that curcuminoid formulations, especially with piperine to be bioavailable, show no dose-limiting toxicity at doses from 3.6 g up to 12 g/day (Shoba *et al.*, 1998; Cheng *et al.*, 2001; Sharma *et al.*, 2004; Janakiram *et al.*, 2010; Suresh and Srinivasan, 2010; Bansal *et al.*, 2011; Carroll *et al.*, 2011). These data provide a scientific rationale and support further evaluation of the safety and effectiveness of curcumin compounds in modulating lung carcinogenesis in well powered, phase II randomized chemoprevention trials, targeting populations at high risk for lung cancer such as former smokers and thus advancing our knowledge of the chemopreventive effects of curcumin. If the safety and efficacy of curcumin on valid intermediate endpoint biomarkers of lung cancer can be demonstrated in these clinical trials, this coupled with our provocative mechanistic rationale and consistent animal studies, can identify this agent as a potential chemopreventive agent that can be used in the form of oral supplements as well as in the daily diet in preventing lung cancer in healthy and high-risk populations such as former smokers.

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Conflicts of interest

There are no conflicts of interest.

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