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Effects of Curcumin on *N-bis*(2-Hydroxypropyl) nitrosamine (DHPN)-induced Lung and Liver Tumorigenesis in BALB/c Mice *In Vivo*

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Abstract. *Curcumin (diferuloylmethane), a phenolic compound from the plant Curcuma longa (Linn.) has been shown to exhibit antitumor activity and apoptosis in many human cancer cell lines including that of lung and liver cancer. In this study, curcumin was evaluated in BALB/c mice for its ability to inhibit pulmonary and liver adenoma formation and growth after they were orally treated with N-bis(2-hydroxypropyl)nitrosamine (DHPN). Animals were treated with DHPN in water for approximately 14 days before multiple doses of curcumin were given intraperitoneally. It was found that 200 μM curcumin reduced lung and liver tumor multiplicity by 37% ($p<0.05$) and 30% ($p<0.05$) respectively. The results indicated that curcumin significantly inhibited pulmonary and liver adenoma formation and growth in BALB/c mice. The precise mechanism by which curcumin inhibits lung and liver tumorigenesis remains to be elucidated. Thus, curcumin appears to be a promising new chemotherapeutic and preventive agent for lung and liver cancer induced by DHPN.*

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Lung and liver cancer are the major causes of cancer-related deaths in Taiwan. The treatment of lung and liver cancer includes radiation, chemotherapy, or combination of radiotherapy with chemotherapy, however, the mortality remains high. Numerous naturally occurring substances are thought to act as antioxidants and cancer preventative agents, or even as cancer therapy drugs (1).

Curcumin (1,7-*bis* (4-hydroxy-3-methoxyphenyl)-1,6.heptadiene-3,5-dione), is a natural product and the active portion of turmeric (*Curcuma longa* L.), widely used as a flavoring agent in food (2). Curcumin inhibits cell proliferation and induces apoptosis in many human cancer cell lines such as leukemia, prostate cancer and non-small cell lung cancer (3-5). Antitumor activity of curcumin has been reported in the colon, skin, stomach, duodenum, soft palate and breasts of rodents (6-8). In our laboratory, we demonstrated that curcumin inhibits *N*-acetyltransferase activity and gene expression (9) and induces apoptosis *via* reactive oxygen species in human colon cancer cells (10). We also found that curcumin induced cell cycle arrest and apoptosis in human acute promyelocytic leukemia HL-60 cells through a mitochondria- and caspase-3-dependent pathway (11).

In colon cancer patients, the phase I clinical trial of oral curcumin showed its high tolerability in humans and proposed the possibility of developing curcumin as an oral cancer preventative or therapeutic agent (12). The antitumor activity of curcumin is attributed to its ability to induce apoptosis *via* caspase-3 activation. Curcumin also induced

Table I. *Experimental designs of curcumin affect lung and liver cancer in BALB/C mice.*

Group	No. of animals	Experimental time (weeks)
		0-----2----------22
1	15	Water
2	15	0.1% DHPN for two weeks then 0 μmoles curcumin in 0.2 ml corn oil
3	15	0.1% DHPN for two weeks then 10 μmoles curcumin in 0.2 ml corn oil
4	15	0.1% DHPN for two weeks then 100 μmoles curcumin in 0.2 ml corn oil
5	15	0.1% DHPN for two weeks then 200 μmoles curcumin in 0.2 ml corn oil

Five groups of BALB/C mice, at the age of 5 weeks, were given drinking water containing 0.1% DHPN [*N-bis*(2-hydroxypropyl) nitrosamine] for 2 weeks, for initiation except group 1 which received water only. From the age of 7 weeks, treatment groups of animals received corn oil alone or containing 0, 10, 100, or 200 μM curcumin in 0.2 ml corn oil. Noncurcumin-treated animals receiving corn oil alone acted as positive control.

apoptosis in scleroderma lung fibroblasts (SLF) without affecting normal lung fibroblasts (NLF) (13).

N-bis(2-hydroxypropyl)nitrosamine (DHPN) has been reported to induce lung and liver cancer in mice and rats (14, 15). There have been extensive investigations to elucidate the mechanism of action of curcumin as an antitumor agent *in vitro*, however, its effects on lung and liver tumorigenesis induced by DHPN *in vivo* is still unclear. Therefore, in the present study, we focused on the effect of curcumin on lung and liver tumorigenesis in an animal model *in vivo*.

Materials and Methods

Materials and reagents. Curcumin, DHPN and olive oil were obtained from Sigma (MO, USA). RPMI-1640, fetal bovine serum, penicillin-streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

BALB/c mice. Male BALB/c mice (approximately 22-28 g) were obtained at the age of 5 weeks from the Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan). Animals were quarantined for 1 week and housed with woodchip bedding in environmentally controlled cages, with a 12-hour light–dark cycle and 50% relative humidity. Drinking water and diet were supplied *ad libitum*. The animals were maintained at the Animal Center of the China Medical University for 2 weeks under animal guidelines before the grouping and experiments were performed.

Tumor induction and curcumin treatment. Seventy-five BALB/c mice were split into 5 groups. Four groups of BALB/C mice, at the age of 6 weeks, were given drinking water containing 0.1% DHPN for 2 weeks, while group 1 received water only. From the age of 7 weeks, animals received water alone (group 1), 0.2 ml corn oil alone (group 2), or 0.2 ml corn oil supplemented with 10 (group 3), 100 (group 4), or 200 (group 5) μmoles curcumin intraperitoneally twice per week. The noncurcumin-treated animals acted as control. Positive control animals were treated with corn oil throughout the study. DHPN continued to be administered throughout the entire experiment (22 weeks). Overall, the experimental treatments are summarized in Table I.

Throughout the study, the health of the mice was monitored every day and body weights were measured every week. Mice were sacrificed by CO_2 asphyxiation 22 weeks after DHPN treatment. For the phenotyping of lung and liver tumors in all bioassays described above, lung and liver tissues were dissected, individually fixed with buffered 10% formalin and routinely processed to paraffin-embedded and hematoxylin and eosin (H&E)-stained sections for histological examination. Lung and liver tumor development was estimated based on the histopathological examinations performed and the number of tumors were recorded.

Statistical analysis. Student's *t*-test was used to analyze the differences between the curcumin-treated and control groups in the experiment.

Results

In this study, BALB/c mice were used as test animals, curcumin as an antitumor agent and DHPN as an inducer of lung and liver tumorigenesis *in vivo*. This protocol is designed to determine any suppressing effects on the stages of lung and liver tumorigenesis (promotion and progression).

The effects of curcumin on final liver and lung weight of mice treated with DHPN. The animals in the control and corn oil-treated positive control groups showed no signs of gross toxicity or loss of body weight during the experiment (data not shown). However, the control and DHPN-treated groups showed significant differences ($p<0.05$) (Tables II and III), but the 10 μM curcumin treated mice were not significantly different from these of the positive control (Tables II and III). The effects of 100 and 200 μM curcumin treatment were significantly different from those of the positive control (Tables II and III) ($p<0.05$; $p<0.01$).

The effects of curcumin on DHPN-induced liver and lung tumorigenesis in BALB/c mice. The *i.p.* administration of curcumin reduced tumor numbers. The treatment of curcumin (10, 100 and 200 μM) led to a decrease of lung tumor number by approximately 15% , 36% and 37% , and of liver tumor number by approximately 5% , 23% and 32% , respectively when compared to the positive controls (Figure 1A and B).

Table II. *Final lung weight of mice treated with DHPN and curcumin.*

Treatment	No. of mice	Body weight (g)	Lung weight (g)	
H_2O	15	24.8±3.90	0.25±0.04	*
0.1% DHPN	15	23.91±4.64	0.28±0.08	
0.1% DHPN + 10 μM curcumin	15	25.22±4.80	0.26±0.06	*
0.1% DHPN + 100 μM curcumin	15	24.60±2.74	0.23±0.04	**
0.1% DHPN + 200 μM curcumin	15	25.18±4.62	0.20±0.04	

*$p<0.05$, **$p<0.01$.

Table II. *Final liver weight of mice treated with DHPN and curcumin.*

Treatment	No. of mice	Body weight (g)	Liver weight (g)	
H_2O	15	24.82±3.90	1.36±0.68	
0.1% DHPN	15	23.94±4.66	1.48±0.46	*
0.1% DHPN + 10 μM curcumin	15	25.22±4.83	1.38±0.74	
0.1% DHPN + 100 μM curcumin	15	24.63±2.74	1.30±0.64	*
0.1% DHPN + 200 μM curcumin	15	25.12±4.36	1.24±0.49	

*$p<0.05$, **$p<0.01$.

A. Lung

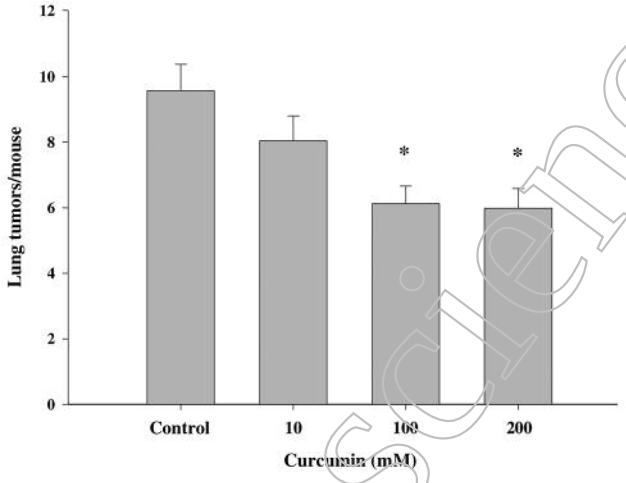
Group	No. of tumors	SE	*p*-value	Inhibition+
Control (groups)	9.56	0.82		
Curcumin (μM)				
10	8.04	0.76	>0.05	15%
100	6.12	0.54	<0.05	35.98%
200	5.98	0.60	<0.05	37.44%

+Reduction in tumor number compared to the control group.

B. Liver

Group	No. of tumors	SE	*p*-value	Inhibition+
Control (groups)	8.46	0.54		
Curcumin (μM)				
10	8.06	0.62	>0.05	5%
100	6.49	0.48	<0.05	23.28%
200	5.86	0.51	<0.05	30.73%

+Reduction in tumor number compared to the control group.



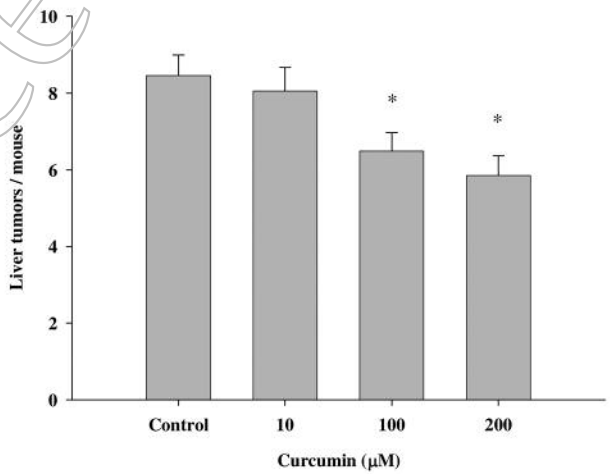


Figure 1. *Effects of curcumin on DHPN-induced lung and liver tumorigenesis in BALB/c mice. After 2 weeks of administration of 0.1% DHPN mice were treated with different doses of curcumin in corn oil. The total treatment duration was 22 weeks. A: Effect of curcumin on lung tumor multiplicity. B: Effect of curcumin on liver tumor load. *p<0.05 compared with the positive control group.*

Discussion

It is well known that antitumor agents can block tumor initiation by blocking carcinogen activation, scavenging reactive carcinogens, enhancing DNA damage repair, inducing apoptosis of tumor initiative cells, or by suppressing the progression of initiated cells. Aiming to identify novel chemotherapy agents for lung and liver cancer, we have determined the efficacy of curcumin in blocking lung and liver tumorigenesis induced by DHPN in mice. We found that curcumin was an effective chemopreventive agent in our mouse model of lung cancer at doses (10-200 μM) that caused no significant toxicity. Such mice have been used widely in experimental research. In 1978, it was reported that oral treatment with DHPN led to the development of lung and liver cancer (14). DHPN is a very potent mutagen and a wide-

spectrum carcinogen in rodents. DHPN causes carcinomas of the lung, thyroid and kidney in F344 and Wistar rats (16-19). It was also reported that DHPN induced lung and liver cancer in ddY mice (20). DHPN acting as a tumor initiator is a useful tool for screening chemopreventive as well as tumorigenic activities of chemicals (21). In the present study, we also confirmed that DHPN induced lung and liver cancer in BALB/c mice. Curcumin is a naturally occurring compound present in turmeric which possesses both anti-inflammatory and anti-oxidant properties, and has been tested for its chemopreventive properties in colon carcinogenesis *in vivo* (22), as well as in skin and forestomach (23, 24). Curcumin has been demonstrated to prevent chemically induced cancer in several different animal tumor bioassay systems, such as the inhibition of benzo[*a*]pyrene (BaP*)-induced forestomach tumorigenesis in A/J mice, *N*-ethyl-N9-nitro-*N*-nitrosoguanidine- duodenal tumorigenesis in C57BL/6 mice and azoxymethane-induced colon carcinogenesis in CF-1 mice (25-27). Curcumin co-inhibited COX-2 and EGFR expression and decreased Erk1/2 activity. This inhibition was associated with decreased survival and enhanced induction of apoptosis in lung and pancreatic adenocarcinoma cells (28).

This study demonstrated for the first time that curcumin reduced the tumor number in mice *in vivo*. Our data indicated that DHPN induced liver and lung cancer, in agreement with other investigators findings (29). Although curcumin appears to be safe in both large and small animal models, the systematic studies of the pharmacology and toxicology of curcumin in humans are few (30-32). Minimal toxicity of doses up to 8,000 mg have been reported in humans (29, 30), however, the maximum tolerated dose has not yet been defined. The peak plasma concentration has been identified 1 to 2 hours after single dose oral administration of 4,000 mg and higher (30). Recently, it was reported that the tolerance of curcumin (*C*3 Complex™, Sabinsa Corporation, Piscataway, NJ, USA) in single oral doses up to 12,000 mg appears to be excellent and warrants further investigation for its utility as a long-term chemopreventive intervention (31). Therefore, the dose of curcumin selected in this study was reasonable. In conclusion, curcumin may be a chemotherapeutic or/and preventive agent against lung and liver cancer induced by DHPN.

References

1 Hassan MA, Braam SR and Kruyt FA: Paclitaxel and vincristine potentiate adenoviral oncolysis that is associated with cell cycle and apoptosis modulation, whereas they differentially affect the viral life cycle in non-small-cell lung cancer cells. Cancer Gene Ther *13*: 1105-1114, 2006.

2 Huang MT, Lou YR, Xie JG, Ma W, Lu YP, Yen P, Zhu BT, Newmark H and Ho CT: Effect of dietary curcumin and dibenzoylmethane on formation of 7,12-dimethylbenz[a] anthracene-induced mammary tumors and lymphomas/leukemias in Sencar mice. Carcinogenesis *19*: 1697-1700, 1998.

3 Guatam SC, Xu YX, Pindolia KR, Janakiraman N and Chapman RA: Nonselective inhibition of proliferation of transformed and transformed cells by anticancer agent curcumin (diferuloyl-methane). Biochem Pharmacol *55*: 1333-1337, 1998.

4 Dorai T, Cao YC, Dorai B, Buttyan R and Katz AE: Therapeutic potential of curcumin in human prostate cancer. III. Curcumin inhibits proliferation, induces apoptosis, and inhibits angiogenesis of LNCaP prostate cancer cells *in vivo*. Prostate *47*: 293-303, 2001.

5 Pillai GS, Srivastava AS, Hassanein TL, Chauhan DP and Carrier E: Induction of apoptosis in human lung cancer cells by curcumin. Cancer Lett *208*: 163-170, 2004.

6 Rao CV, Rivenson A, Simi B and Reddy BS: Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res *55*: 259-266, 1995.

7 Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV and Reddy BS: Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res *59*: 597-601, 1999.

8 Verma SP, Salamone E and Goldin B: Curcumin and genistein plant natural products, show synergistic inhibitory effects on the growth of human breast cancer MCF-7 cells induced by estrogenic pesticides. Biochem Biophys Res Commun *233*: 692-696, 1997.

9 Chen JC, Hwang JM, Chen GW, Tsou MF, Hsia TC and Chung JG: Curcumin decreases the DNA adduct formation, arylamines *N*-acetyltransferase activity and gene expression in human colon tumor cells (colon 205). In Vivo *17(3)*: 301-309, 2003.

10 Su CC, Lin JG, Li TM, Chung JG, Yang JS, Ip SW, Lin WC and Chen GW: Curcumin-induced apoptosis of human colon cancer colon 205 cells through the production of ROS, Ca^{2+} and the activation of caspase-3. Anticancer Res *26(6B)*: 4379-4389, 2006.

11 Tan TW, Tsai HR, Lu HF, Lin HL, Tsou MF, Lin YT, Tsai HY, Chen YF and Chung JG: Curcumin-induced cell cycle arrest and apoptosis in human acute promyelocytic leukemia HL-60 cells *via* MMP changes and caspase-3 activation. Anticancer Res *26(6B)*: 4361-4371, 2006.

12 Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M and Gescher AJ: Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res *10*: 6847-6854, 2005.

13 Khar A, Ali AM, Pardhasaradhi BV, Begum Z and Anjum R: Antitumor activity of curcumin is mediated through the induction of apoptosis in AK-5 tumor cells. FEBS Lett *445*: 165-168, 1999.

14 Konishi Y, Kondo H, Inui S, Denda A, Ikeda T and Kojima K: Organotropic effect of *N-bis*(2-hydroxypropyl)nitrosamine: production of lung and liver tumors by its oral administration in mice. Gann *69(1)*: 77-84, 1978.

15 Cho YM, Imai T, Hasumura M, Onose JI, Ueda M and Hirose M: Lack of prepubertal administration of ethinyl estradiol on susceptibility to multiple organ carcinogenesis in rats exposed to 7,12-dimethylbenz[a]anthracene and *N-bis*(2-hydroxypropyl)-nitrosamine during adolescence. Cancer Lett *223*: 37-46, 2005.

16 Mohr U, Reznik G and Pour P: Carcinogenic effects of diisopropanolnitrostine in Sprague-Dawley rats. J Natl Cancer Inst *58*: 361-X16, 1977.

17 Konishi Y, Kondo H, Ikeda T, Kawabata A, Shoji Y and Denda A: Effect of dose on the carcinogenic activity of orally administered *N-bis*(2-hydroxypropyl)nitrosamine in rats. Gann *69*: 573-577, 1978.
18 Hiasa Y, Kitahori Y, Konishi N, Shimoyama T and Lin JC: Sex differential and dose dependence of phenobarbital-promoting activity in *N-bis*(2-hydroxypropyI)- nitrosamine-initiated thyroid tumorigenesis in rats. Cancer Res *45*: 4087-4090, 1985.
19 Hiasa Y, Kitahori Y, Katoh Y, Ohshima M, Konishi N, Shimoyama T, Sakaguchi Y, Hashimoto H, Minami S and Murata Y: Effects of castration before and after treatment with *N-bis*(2-hydroxypropyl)-nitrosamine (DHPN) on the development of thyroid tumors in rats treated with DHPN followed by phenobarbital. Jpn J Cancer Res *78(10)*: 1063-1067, 1987.
20 Yamamoto H: Tumorigenesis and cystic lesion of the liver by *N-bis*(2-hydroxypropyl)nitrosamine in ddY mice. Gann *69(1)*: 77-84, 1978.
21 Ito N, Hasegawa R, Imaida K, Hirose M and Shirai T: Medium-term liver and multi-organ carcinogenesis bioassay for carcinogens and chemopreventive agents. Exp Toxicol Pathol *48*: 113-119, 1996.
22 Rao CV, Rivenson A, Simi B and Reddy BS: Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res *55(2)*: 259-266, 1995.
23 Wright TI, Spencer JM and Flowers FP: Chemoprevention of nonmelanoma skin cancer. J Am Acad Dermatol *54(6)*: 933-946, 2006.
24 Singh SV, Hu X, Srivastava SK, Singh M, Xia H, Orchard JL and Zaren HA: Mechanism of inhibition of benzo[a]pyrene-induced forestomach cancer in mice by dietary curcumin. Carcinogenesis *19(8)*: 1357-1360, 1998.
25 Huang MT, Smart RC, Wong CQ and Conney AH: Inhibitory effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumor promotion in mouse skin by 12-*O*-tetradecanoyl-phorbol-13-acetate. Cancer Res *48*: 5941-5946, 1988.
26 Huang MT, Lou YR, Ma W, Newmark HL, Reuhl KR and Conney AH: Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. Cancer Res *54*: 5841-5847, 1994.
27 Huang MT, Wang ZY, Georgiadis CA, Laskin JD and Conney AH: Inhibitory effects of curcumin on tumor initiation by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene. Carcinogenesis *13*: 2183-2186, 1992.
28 Lev-Ari S, Starr A, Vexler A, Karaush V, Loew V, Greif J, Fenig E, Aderka D and Ben-Yosef R: Inhibition of pancreatic and lung adenocarcinoma cell survival by curcumin is associated with increased apoptosis, down-regulation of COX-2 and EGFR and inhibition of Erk1/2 activity. Anticancer Res *26(6B)*: 423-430, 2006.
29 Konishi Y, Kondo H, Inui S, Denda A, Ikeda T and Kojima K: Organotropic effect of *N-bis*(2-hydroxypropyl)nitrosamine: production of lung and liver tumors by its oral administration in mice. Gann *69(1)*: 77-84, 1978.
30 Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC and Hsieh CY: Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res *21(4B)*: 2895-2900, 2001.
31 Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ and Steward WP: Pharmacodynamic and pharmacokinetic study of oral Curcuma extract in patients with colorectal cancer. Clin Cancer Res *7(7)*: 1894-1900, 2001.
32 Shoba G, Joy D, Joseph T, Majeed M, Rajendran R and Srinivas PS: Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med *64(4)*: 353-356, 1998.

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