Captopril as a Potential Inhibitor of Lung Tumor Growth and Metastasis

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Lung cancer is the most common form of cancer in the world, and 90% of patients die from their disease. The angiotensin converting enzyme (ACE) inhibitors are used widely as antihypertensive agents, and it has been suggested that they decrease the risk of some cancers, although available data are conflicting. Accordingly, we investigated the anticancer activity of the ACE inhibitor, captopril, in athymic mice injected with highly tumorigenic LNM35 human lung cells as xenografts. Using this model, we demonstrated that daily IP administration of captopril (2.8 mg/mouse) for 3 weeks resulted in a remarkable reduction of tumor growth (58%, $P < 0.01$) and lymph node metastasis (50%, $P = 0.088$). There were no undesirable effects of captopril treatment on animal behavior and body weight. In order to determine the mechanism by which captopril inhibited tumor growth, we investigated the impact of this drug on cell proliferation, apoptosis, and angiogenesis. Immunohistochemical analysis demonstrated that captopril treatment significantly reduced the number of proliferating cells (*Ki*-67) in the tumor samples but was not associated with inhibition of tumor angiogenesis (CD31). Using cell viability and fluorescent activated cell sorting analysis tests, we demonstrated that captopril inhibited the viability of LNM35 cells by inducing apoptosis, providing insight about the mechanisms underlying its antitumorigenic activities. In view of these experimental findings, we conclude that captopril could be a promising option for the treatment of lung cancer.

Key words: **captopril; lung tumor; metastasis**

Introduction

The angiotensin converting enzyme (ACE), or kininase II, is a dipeptidyl carboxy metalolpeptidase that plays a major role in blood pressure, volume, and electrolyte regulation. ACE is present as a membrane-bound enzyme in endothelial and epithelial cells in the lung, kidney, intestine, testes, and brain and as a soluble form in blood.[2] Endothelial and circulating ACE converts inactive angiotensin I into the vasoactive octapeptide, angiotensin II (Ang II), and also inactivates the vasodilator peptide bradykinin.

Ang II via its type 1 receptor promotes angiogenesis through activation of vascular endothelial growth factor (VEGF) and VEGF receptor type 2 (KDR) expression in endothelial cells.[3–6] The Ang II type 2 receptor is a 7-transmembrane G-protein coupled receptor that regulates signal transducer and activator of transcription-3 (STAT3).[7] We recently established heterotrimeric G-proteins and STAT3 as new therapeutic targets in lung and colon cancer.[8,9] Ang II acts as an anti-apoptotic agent

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and as a growth factor acting through the epidermal growth factor receptor transactivation-ERK signaling pathway.[10,11,12]

Lever and colleagues (1998) have suggested that long-term use of ACE inhibitors protects against some cancers.[1] In this regard, we demonstrated that some of the long-term beneficial effects of ACE inhibitors could be mediated by a peroxisome proliferator activator receptor (PPAR).[13] Activators of PPAR alpha and gamma inhibit colorectal tumor progression, possibly via inhibition of proliferation and, remarkably, inhibit the growth of human lung cancer cells through induction of apoptosis.[14,15]

In this context, ACE inhibition with captopril, an ACE inhibitor widely used in the management of hypertension, has been shown experimentally to inhibit tumor angiogenesis and to induce apoptosis.[16,17] In contrast, ACE inhibition with quinaprilat or perindopril reportedly promotes angiogenesis in a rabbit model of hindlimb ischemia.[18,19] In agreement with these results, Ebrahimian and co-workers demonstrated that ACE inhibition promoted neovascularization through activation of bradykinin B2 receptor signaling independently of VEGF expression, whereas it reduced blood vessel growth through inhibition of the Ang II pathway.[19,20] Thus, ACE inhibitors with high tissue affinity appear to have proangiogenic effects. In another report, perindopril inhibited angiogenesis and metastasis of hepatocellular carcinoma.[21] In conclusion, the effect of ACE inhibitors on angiogenesis and tumor growth remains controversial.

In the present study, we investigated the impact of the ACE inhibitor, captopril, on human lung tumor growth, angiogenesis, and metastasis *in vivo* and on cell viability and apoptosis *in vitro*.

Methods

Cell Culture and Reagents

Human non-small cell lung cancer cells LNM35 were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (FBS) (Roche Molecular Biochemicals, Meylan, France) and antibiotics. The pharmacological inhibitor of ACE (captopril) was purchased from Sigma (Saint-Quentin Fallavier, France). Primary antibodies were purchased from the following manufacturers: monoclonal rat antimouse CD31 antibody (BD PharMingen, San Jose, CA), monoclonal mouse antihuman *Ki*-67 antibody (Dako, Copenhagen, Denmark). The secondary antibodies were goat antirat antibody (Sigma, St. Louis, MO) and rabbit antimouse antibody (Dako, Copenhagen, Denmark).

Cell Viability

LNM35 cells were seeded at a density of 5000 cells/well onto 96-well plates in RPMI plus 10% FBS. After 24 h, cells were treated for 24 h with increasing concentrations of captopril (0.01–10 mM), in triplicate assays. The effect of captopril on cell viability was determined using a CellTiter-Glo Luminescent Cell Viability assay (Promega Corporation, Madison, WI), which is based on quantification of the ATP level, which signals the presence of metabolically active cells. Luminescent signal was measured in the GLOMAX Luminometer system (Promega, Corp.). The data are presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which was assumed to be 100%.

Cell Apoptosis

LNM35 cells (1×10^6) were plated and cultured at 37°C on 100-mm Petri dishes in RPMI supplemented with 10% FBS. After 24 h, cells were treated or not (controls) with increasing concentrations of captopril (0.001–10 mM) for 24–48 h in RPMI 0.5% FBS. For flow cytometric analysis, adherent and floating cells were combined, washed once with phosphate-buffered saline (PBS), and fixed overnight at 4°C in 70% ethanol. Subsequently cells were washed with PBS, incubated for 30 min at 37°C with 1 μg/mL RNase

A, and stained with propidium iodide. The stained cells were analyzed on a FACScan flow cytometer for relative DNA content (FACSCalibur; Becton Dickinson, Le Pont de Claix, France). About 10,000 cells were recorded per assay.

Tumor Growth Assays

Six-week-old athymic NMRI female nude mice (nu/nu; Elevage Janvier, Le Genest Saint Isle, France) were housed in filtered-air laminar flow cabinets and handled under aseptic conditions. Procedures involving animals and their care were conducted in conformity with Institutional guidelines that are in compliance with Faculty of Medicine and Health Sciences, UAE University, national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; and NIH Guide for Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985). Human Pulmonary LNM35 cells (1×10^6 cells) were injected subcutaneously into the lateral flank of the nude mice. One week after inoculation, when tumors had reached the volume of approximately 100 mm^3, animals (six in each group) were treated with captopril (2.8 mg/mouse) or control (carrier solution alone) during three cycles of 6 days and one rest day for a total of 21 days. Tumor dimensions were measured with calipers every 3 days. Tumor volume (V) was calculated using the formula: $V = 0.4 \times a \times b^2$, with a being the length and b the width of the tumor. The animals were sacrificed 21 days after treatment initiation, and the tumors excised, weighed, and fixed for immunohistochemical analysis.

Immunohistochemical Determination of Proliferating Cell Nuclear Antigen *Ki*-67 and CD31/Platelet-endothelial Cell Adhesion Molecule 1 for Microvessel Density

Five micrometer paraffin-embedded tissue sections were deparaffinized, microwaved for 5 min for antigen retrieval, and then incubated with a monoclonal mouse antihuman antibody against *Ki*-67 (DAKO, clone MIB-1, 1:50) for 1 h at room temperature. The samples were then washed and incubated with secondary antibody for 1 h at room temperature, followed by incubation with the streptavidin-peroxidase complex. Ten high-power fields (0.159 mm^2) per section of four to five tumors per treatment group were examined microscopically, and the average number of cells that stained positive for *Ki*-67 per treatment group was evaluated.

The effect of captopril on angiogenesis was evaluated by CD31 immunostaining. The tumor tissues were quickly frozen in isopentane at $-130°C$ and stored at $-70°C$ until further processing. Frozen sections (8 μm) were fixed in acetone and incubated overnight with a CD31 antibody (1:400). Slides were then washed three times in PBS and incubated with secondary antibody (goat antirat 1:200) for 1 h at room temperature. The sections were then stained with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. Vessel density was determined by counting the number of microvessels. The area occupied by CD31-positive microvessels and total tissue area per section were quantified and compared between treated and control mice. For individual tumors, the microvessel count was scored by averaging the counts from all fields. All analyses were performed in a blind fashion.

Statistical Analysis

Results are expressed as the mean ± SEM of the number of experiments indicated. The difference between experimental and control values were assessed by the Independent Samples Test and ANOVA and $P < 0.05$ was considered as a statistically significant difference.

Results

Impact of Captopril on Tumor Growth and Metastasis

The anticancer activity of captopril was investigated in athymic mice inoculated with

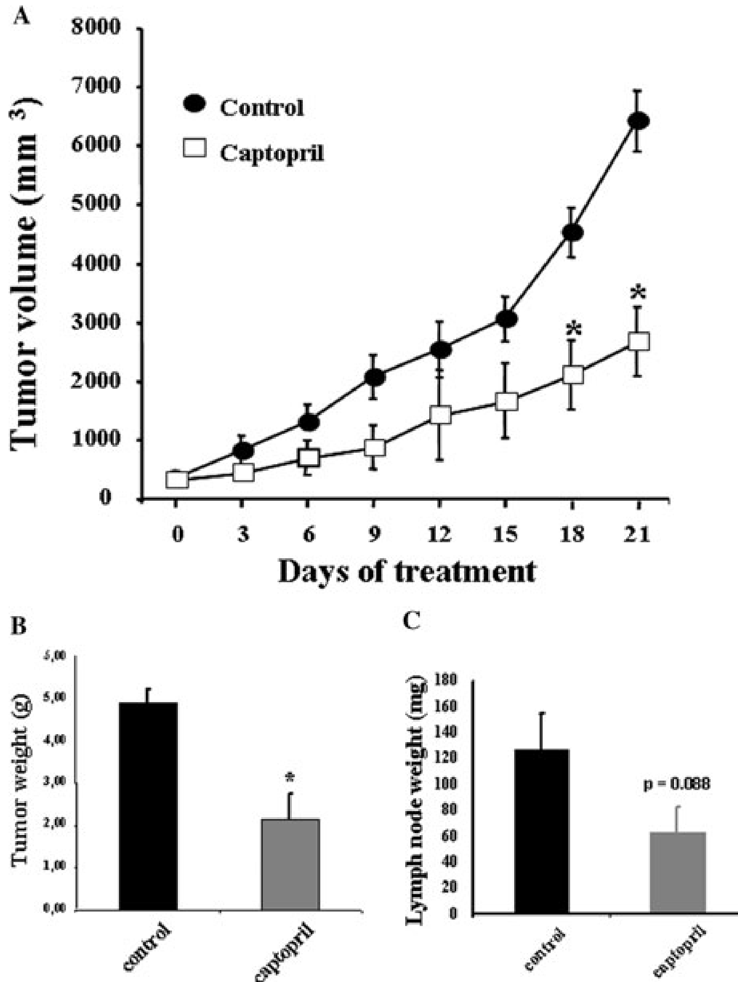

Figure 1. Impact of the ACE inhibitor captopril on the tumor volume **(A)**, weight **(B)**, and lymph node metastasis weight **(C)** of established human lung cancer xenografts. Nude mice were xenografted s.c. with human lung LNM35 cancer cells (10^6 cells per animal) and treated with captopril (2.8 mg/mouse during three cycles of 6 days and one rest day for a total of 21 days) or with saline solution alone. Data points represent the mean ± SEM of four to five mice per group. Statistically significant differences are indicated in **A** for tumor volume (*, $P < 0.01$ versus control) and in **B** for tumor weight (*, $P < 0.01$ versus control).

highly tumorigenic LNM35 human lung cells as xenografts. The IP administration of captopril (2.8 mg/mouse) significantly reduced the growth of LNM35 human tumor xenografts by 52% at day 18 and 58% at day 21 (*, $P < 0.01$ versus control) (Fig. 1A). A similar difference between captopril-treated and control animals was also found in tumor weight (4.92 ± 0.33 g versus 2.13 ± 0.63 g) (Fig. 1B). There was no loss of body weight or other sign of toxicity from captopril administration.

The metastatic dissemination of human cancer cells constitutes the most aggressive aspect of the neoplastic progression leading to a fatal outcome. In order to determine whether inhibition of ACE by captopril affects the dissemination of human cancer cells, we assessed the metastatic behavior of the human pulmonary cell line LNM35 by examining axillary lymph nodes. In the captopril-treated group, the mean lymph node weight was 63.5 mg compared with 127 mg in the vehicle-treated

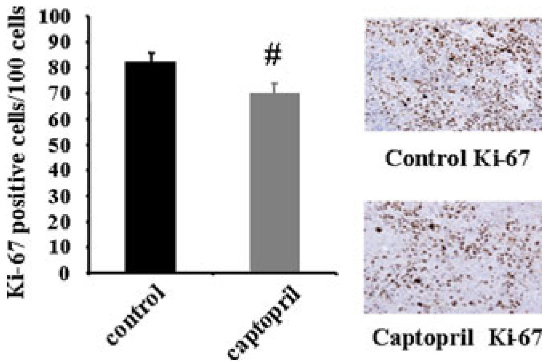

Figure 2. Immunohistochemical staining for *Ki-67* in LNM35 human lung cancer growing in nude mice treated with saline (control) or captopril. $^{\#}P < 0.05$ versus control.

control group. This difference, though sizable, was in a small group of animals and failed to reach conventional levels of statistical significance ($P = 0.088$) (Fig. 1C).

Effect of Captopril on Tumor Angiogenesis and Cell Proliferation *In Vivo*

The effect of captopril on tumor angiogenesis *in vivo* was assessed by CD31-immunostaining of LNM35 tumor tissue xenografts. CD31 is specifically expressed on the surface of endothelial cells and is weakly expressed on lymphoid cells and platelets. Immunohistochemical analysis indicated that when the CD31-positive areas or number of microvessels were normalized to tumor area, captopril versus control caused nonsignificant vascular regression in the tumors (data not shown).

Next, we analyzed the effect of captopril on tumor-cell proliferation *in vivo* by assessing the levels of the nuclear antigen *Ki*-67, which is present in all phases of the cell cycle except G0. There was a significant decrease in the mean number of *Ki*-67-positive cells in the captopril-treated group in comparison with the control group (Fig. 2, $P < 0.05$). Thus, the highly significant inhibition of tumor growth by captopril appears to be due, at least in part, to a direct effect on the proliferation of tumor cells.

Effect of Captopril on Cell Viability and Apoptosis *In Vitro*

To determine the mechanism by which captopril reduced tumor growth, we investigated the effect of captopril on cellular viability. Proliferating LNM35 cells were incubated in the absence or presence of various concentrations (0.01, 0.1, 1, and 10 mM) of captopril for 24 h. At all these concentrations, captopril had an inhibitory impact on the LNM35 cell viability at 24 h ($^{\#}$, $P < 0.05$; *, $P < 0.01$ versus control) (Fig. 3A).

Fluorescent activated cell sorting (FACS) analysis assays were performed to determine whether the antitumor effect of captopril was due to cell death. We demonstrated that treatment of LNM35 cells for 48 h with high concentrations of captopril (1 and 10 mM) was associated with induction of apoptosis from 2.5% for control to 9% and 15.5%, respectively, for captopril-treated cells (Fig. 3B). These results suggest that upon treatment with captopril, LNM35 cells underwent apoptosis, and that there is a good correspondence between apoptosis rate and inhibition of cell viability.

Discussion and Conclusion

Lung cancer is the most common form of cancer in the world, with the highest mortality rate (90% of lung cancer patients die from their disease). A retrospective study of 5207 patients showed that long-term use of ACE inhibitors may protect against cancer.[1] This observation, however, is contrary to a somewhat similar study in elderly people who participated in a Swedish trial of antihypertensive drugs in which ACE inhibitors reportedly had no effect on the risk of developing cancer.[22] ACE inhibitors are widely used as antihypertensive agents and, in general, are not associated with serious side effects. The present study using the non-small cell lung carcinoma LNM35 preclinical model revealed that the ACE inhibitor, captopril, significantly inhibited tumor growth. Our findings

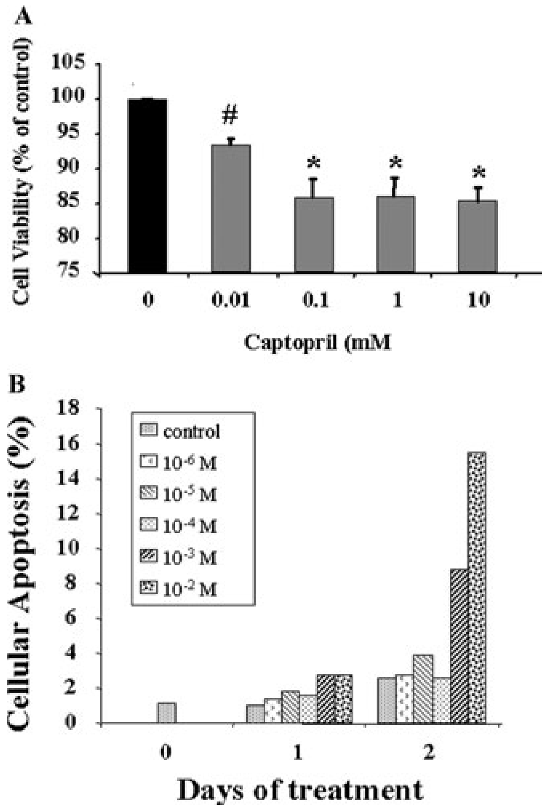

Figure 3. Effect of captopril on the cell viability and apoptosis of LNM35 cancer cells. **(A)** The data are presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which was assumed to be 100%. Statistical significance was evaluated with Independent Samples Test and ANOVA ($^{\#}$, $P < 0.05$ versus control; *, $P < 0.01$ versus control). **(B)** Flow cytometry analysis of captopril-induced apoptosis. LNM35 cells were cultured for 24–48 h in the presence or absence of graded concentrations of captopril and analyzed at the sub-G1 fraction.

are in agreement with other studies showing that captopril and other ACE inhibitors inhibit tumor growth of human renal cell carcinoma and head and neck squamous carcinoma.[23,24] In this context, Chisi and colleagues reported that captopril (100 mg/kg or 2.8 mg/mouse) may be of use in protecting primitive hematopoietic cells against the toxicity of anticancer therapeutic regimens.[25] The acquisition of metastatic ability leads to clinically incurable disease. Our data demonstrate that captopril also inhibited the LNM35 lymph node metastasis in nude mice, although this inhibition, while apparently substantial, failed to reach conventional levels of statistical significance. These results are in agreement with evidence that captopril inhibits human glioma cell migration and invasion related to a reduction in matrix metalloproteinase activity.[26]

Tumor invasion and metastasis are critical events in cancer progression, and angiogenic factors are now considered to play a major role in metastasis. In this context, Ang II reportedly promoted the invasive potency of cervical carcinoma cells by increasing the secretion of VEGF.[27] These results are in agreement with our recent work showing that VEGF induces colon cancer cell invasion.[28] In this regard, we demonstrate here that captopril treatment resulted in a weak and non significant decrease of angiogenesis in the LNM35 tumors. In contrast, the Sukhatme group demonstrated that treatment with captopril induced anti-angiogenic activity *in vitro* and *in vivo*.[29] In conclusion, the effect of ACE inhibitors on angiogenesis remains controversial.

To explain the antitumor effect of captopril, we considered two theories: first, that captopril directly inhibits the proliferation of LNM35 tumor cells. To address this theory, we performed immunohistochemical analysis of proliferating cells (*Ki*-67) in the tumor samples treated with captopril. Quantification of the *Ki*-67-positive cells showed a decreased number of dividing tumor cells after treatment with captopril. However, the *in vitro* proliferation assay for LNM35 cells revealed no antiproliferative effect as a result of ACE inhibition. These results are in agreement with the earlier observation that captopril was ineffective in preventing cell proliferation in several neoplastic cell lines (K562, HeLa, and MDA-MB-361).[30]

The effect of captopril on tumor growth appears to be due, at least in part, to direct inhibition of the survival of tumor cells. There was a significant difference in the percentage of viable cells after incubation with captopril (0.01–10 mM) versus control. It was confirmed by FACS assay that the inhibition of

cell viability observed after treatment with captopril was due to therapy-induced cell death. Then, exposure of LNM35 cells to increasing concentrations of captopril resulted in an inhibition of cell viability accompanied by induction of apoptosis.

In conclusion, we report an antitumor effect of captopril in a highly aggressive human lung cancer xenograft in nude mice. In view of the available experimental findings, we contend that captopril may have clinical potential as an anticancer agent. The first line chemotherapeutic protocol in non-small cell lung cancer is cisplatin in combination with paclitaxel, docetaxel, vinorelbine, gemcitabine, or irinotecan. It is possible that captopril will turn out to be a therapeutic option in the management of lung cancer, perhaps in combination with standard anticancer drugs, such as cisplatin.

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Conflicts of Interest

The authors declare no conflicts of interest.

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