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In vitro and *in vivo* studies on the inhibitory effects of myocardial cell culture medium on growth of a human lung adenocarcinoma cell line, A549

Y. Zheng MS,* J. Zhou MS,† S.Z. Fu MD,* J. Fan MS,* and J.B. Wu MS*

ABSTRACT

Background Although the heart is one of the body's vital organs, with an abundant blood supply, metastasis to the heart is considered rare. In a previous study, we found that the myocardial microenvironment might contain a low molecular weight natural tumour suppressor. The present study was designed to investigate the inhibitory effect of cardiac myocyte–conditioned medium (CMCM) on the growth of A549 human lung adenocarcinoma cells *in vitro* and *in vivo*.

Methods An MTT assay was used to detect the inhibition ratio with respect to A549 proliferation. Human lung adenocarcinoma cells (A549 cell strain) were transplanted subcutaneously into nude mice to produce tumours. The xenograft tumour growth in mice was observed after selected drug administration.

Results After treatment with CMCM and cisplatin (Cis), A549 cell viability significantly declined ($p < 0.001$). The cell viability in the CMCM and Cis groups were 53.42% ± 3.45% and 58.45% ± 6.39% respectively. Growth of implanted tumour cells *in vivo* was significantly inhibited in the CMCM group, the group treated with recombinant human adenovirus–p53, and the Cis-treated group compared with a control group. The inhibition rates were 41.44% in the CMCM group, 41.34% in the p53 group, and 64.50% in the Cis group. Lung metastasis capacity was significantly reduced in the presence of CMCM ($p < 0.05$). Lung metastasis inhibition rates in mice were 56.52% in the CMCM group, 47.83% in the p53 group, and 82.61% in the Cis group. With CMCM, the lives of A549-tumour-bearing mice could be significantly prolonged without any effect on weight loss.

Conclusions Use of CMCM has the effect of reducing A549 cell viability, tumour volume, and lung metastasis rate, while prolonging survival duration without severe toxicity.

Key Words Cardiac myocyte–conditioned medium, lung adenocarcinoma, nude mice, tumour inhibition, metastasis

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INTRODUCTION

Although tumours can occur in any part of the body, primary tumours of the heart and great vessels are commonly understood to be rare (approximately 0.001%–0.03% in autopsy studies[1–3]). And of primary heart neoplasms, about 75% are benign[2,4,5].

Metastasis is a basic biologic feature of malignant tumour cells. Metastatic dissemination represents the true cause of the malignant character of cancers. Metastasis involves a number of complex interactions between tumour and stroma, with contributions from adhesion and motility pathways in addition to the proliferation and survival pathways, and is a major feature of cancer and the main cause of patient death[6,7].

Tumour spread or metastasis occurs preferentially to certain organ sites. Evidence from certain experimental tumour systems supports Paget's "seed and soil" hypothesis about the nonrandom distributions of metastases, in which the unique properties of particular tumour cells ("seeds")

Correspondence to: Jing Bo Wu, Department of Oncology, The Affiliated Hospital of Luzhou Medical College, Luzhou 646000 P.R.C.
E-mail: wjb6147@163.com **DOI:** http://dx.doi.org/10.3747/co.23.2844

and the varying characteristics of each organ microenvironment ("soil") together determine the "preferred" organ for metastasis[8]. Lung is the most common site of distant metastasis, followed by liver, brain, bone, and adrenal glands. However, metastasis to the heart—one of the body's vital organs, with an abundant blood supply—is considered to be rare, the incidence being in the 2.3%–18.3% range[5].

In principle, there are two possible explanations for infrequent metastasis to the heart: something in the myocardial microenvironment affects malignant cells either directly or indirectly. To further investigate that hypothesis, we set up an experimental system to test the effect of medium conditioned using newborn rat cardiac muscle cells [cardiac myocyte–conditioned medium (CMCM)] on the proliferation of malignant cells.

In a previous study, we found that the myocardial microenvironment might contain a low molecular weight natural tumour suppressor. The cell viability of nasopharyngeal carcinoma was significantly decreased in a CMCM group compared with a control group ($p < 0.01$). However, CMCM had no inhibitory effect on rat mesangial cells ($p > 0.05$)[9]. We also found that CMCM inhibited mouse-transplanted S180 tumours *in vivo*, with no obvious side effects during treatment[10]. However, the latter experiment had defects, such as an extended drug treatment period and interference by immune factors[11].

In the present study, we set out to observe, using an improved experimental method, the antitumour effect of CMCM on A549 lung cancer cells *in vivo* and *in vitro*. We investigated for the first time in lung cancer the effect of CMCM on cell proliferation *in vitro* and the growth of tumour xenografts and lung metastases *in vivo*. Our results might suggest a potential new way to improve cancer treatment.

METHODS

Preparation of CMCM

Cardiac muscle from newborn Wistar rats 24–72 hours old (Teng Xin Biological Technology, Chongqing, P.R.C.) were separated and minced, digested in 0.25% pancreatin and 0.01% type II collagenase, and purified by the differential attachment technique. The cells were then counted and seeded in enriched Dulbecco modified Eagle medium (Gibco, Carlsbad, CA, U.S.A.). At the end of the incubation period, the supernatant was collected, centrifuged, filtered through a 0.22 μm filter, and subjected to ultrafiltration through an Amicon membrane (Merck Millipore, Darmstadt, Germany) with a molecular weight between 5 kDa and 10 kDa. (Our previous work[10] had showed that inhibitory activity is detected at a molecular weight fraction between 5 kDa and 10 kDa.) After the ultrafiltration, the ultrafiltrate was collected and dried by lyophilization in a freeze-drier (EYELA FD-1: Tokyo Rikakikai, Tokyo, Japan) to produce CMCM powder.

MTT Assay: Effect of CMCM on A549 Cells *In Vitro*

Cell viability was evaluated biochemically with MTT and visually with an ethidium bromide/acridine orange fluorescent assay. The MTT assay is based on the capacity of cellular mitochondrial NADPH dehydrogenases to reduce the yellow water-soluble tetrazolium substrate into a dark blue or purple water-insoluble formazan product in viable cells[12].

The A549 cells were seeded into 96-well plates (2000 cells/plate) containing either Dulbecco modified Eagle medium, cisplatin 2.5 μg/mL (Cis), or CMCM. After culturing for 24 hours, the viability of the A549 cells was analyzed by MTT assay. The A549 cells were stained with 100 μL sterile MTT dye 0.5 mg/mL (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 4 hours at 37°C, after which the culture medium was removed, and 150 μL dimethyl sulphoxide (Sigma-Aldrich) was added. Absorbance was measured at 570 nm.

Tumour Growth Inhibition Study

Female BALB/c nude mice (4 weeks of age) weighing between 17 g and 20 g were purchased from the Institute of Laboratory Animal Sciences (Beijing, P.R.C.). Tumour cell injections were carried out using freshly prepared cell suspensions at a concentration of 1×10^7 cells/mL in phosphate-buffered saline. Human lung adenocarcinoma cells from the A549 cell strain were transplanted subcutaneously into the nude mice to produce tumours.

When the tumours reached a diameter of 100 mm^3, the mice were weighed, coded, and randomly divided into 4 groups of 12 mice each that received injections as follows: a control group (saline solution, 25 mL/kg), a CMCM group (freeze-dried CMCM powder, 30 mg/kg), a Cis group (Cis, 2 mg/kg), and a p53 group [recombinant human adenovirus–p53 5×10^{10} viral particles (rhAd-p53)]. The CMCM and saline solution were given intraperitoneally daily for 14 consecutive days. The cisplatin and rhAd-p53 were given intraperitoneally once every other day. At 24 hours after the last treatment, half the animals in each group were euthanized, and tumour tissue was taken. Dynamic changes in tumour volume, body weight, and tumour weight and the lung metastasis rate were determined. Survival duration was analyzed for the remaining mice in each group.

Statistical Analysis

Data are expressed as mean ± standard deviation. Statistical analyses used analysis of variance. Kaplan–Meier curves for the survival duration of mice in each group were established and compared using the log-rank test. Values of $p < 0.05$ were considered significant. All statistical tests were carried out using the SPSS software application (version 13.0: SPSS, Chicago, IL, U.S.A.).

RESULTS

Inhibition of A549 Cell Proliferation by CMCM

Viability of the cells treated with CMCM and Cis for 24 hours was 53.42% ± 3.45% and 58.45% ± 6.39% respectively. Compared with the viability of cells from the control group, those results were significantly different ($p < 0.001$), indicating that CMCM has an inhibitory effect on tumour growth *in vitro* (Table I).

Antitumour Effects of CMCM on Transplanted Tumours in Nude Mice

Growth of A549 Cell Xenografts in Nude Mice

Tumour-bearing mice were divided into 4 groups to examine the anticancer efficacy of CMCM *in vivo* and to compare it with the anticancer efficacy of Cis and rhAd-p53. The

tumour volume in the mice was measured at 3, 6, 12, and 15 days after treatment began. Growth of the implanted tumours was significantly inhibited in the groups treated with CMCM, Cis, and rhAd-p53 ($p < 0.05$) compared with the control group. The difference was most obvious at day 15. Tumour volume in the Cis group (96.82 ± 21.53 mm^3) was less than that in the CMCM (132.62 ± 33.28 mm^3) and rhAd-p53 groups (134.83 ± 14.97 mm^3, $p < 0.05$). Tumour growth in the group treated with CMCM was similar to that in the rhAd-p53 group ($p > 0.05$). Those results suggest that CMCM can significantly inhibit tumour growth in A549 tumour–bearing mice. The inhibition effect in the CMCM and rhAd-p53 groups was similar (Figure 1).

Tumour Weight and Rate of Inhibition of A549 Cell Xenografts in Nude Mice

Tumour tissue taken from the mice euthanized at 24 hours after the last treatment was photographed and weighed, and the average tumour weight in each study group was calculated. The weight of the tumours was significantly lower in the treatment groups than in the control group: 0.60 ± 0.21 g in the control group, 0.35 ± 0.02 g in the CMCM group, 0.35 ± 0.02 g in the rhAd-p53 group, and 0.21 ± 0.03 g in the Cis group. The inhibition rate was 41.44% in the CMCM group, 41.34% in the rhAd-p53 group, and 64.50% in the Cis group. Compared with the control group, the differences in the inhibition rate were statistically significant ($p < 0.001$); however, the difference between the CMCM and rhAd-p53 groups was nonsignificant [$p > 0.05$, Table II, Figure 2(A,B)].

TABLE I Effect of cardiac myocyte–conditioned medium (CMCM) on A549 lung cancer cells *in vitro*

Group	Cell viability (%)
Control	100
CMCM	53.42±3.45[a]
Cisplatin	58.45±6.39[a]

[a] $p < 0.05$ compared with control group.

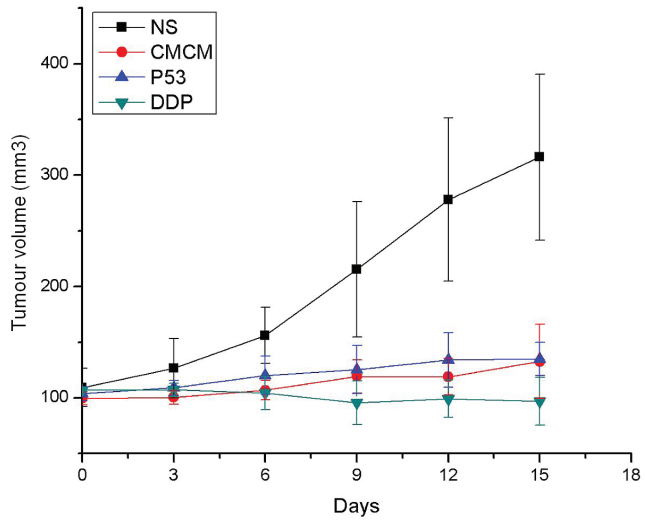

FIGURE 1 Growth curves for lung cancer tumour xenografts implanted in nude mice. NS = control group (normal saline); CMCM = treated with cardiac myocyte–conditioned medium; P53 = treated with recombinant human adenovirus–p53; DDP = treated with cisplatin.

TABLE II Effect of cardiac myocyte–conditioned medium (CMCM) on the tumour weight of lung cancer xenografts in nude mice

Group	Mice (*n*)	Mean tumour weight (g)	Average inhibitory rate (%)
Control	6	0.60±0.21	—
CMCM	6	0.35±0.02[a]	41.44
rhAd-p53	6	0.35±0.02[a]	41.34
Cisplatin	6	0.21±0.03[a]	64.50

[a] $p < 0.001$ compared with control group.
rhAd-p53 = recombinant human adenovirus–p53.

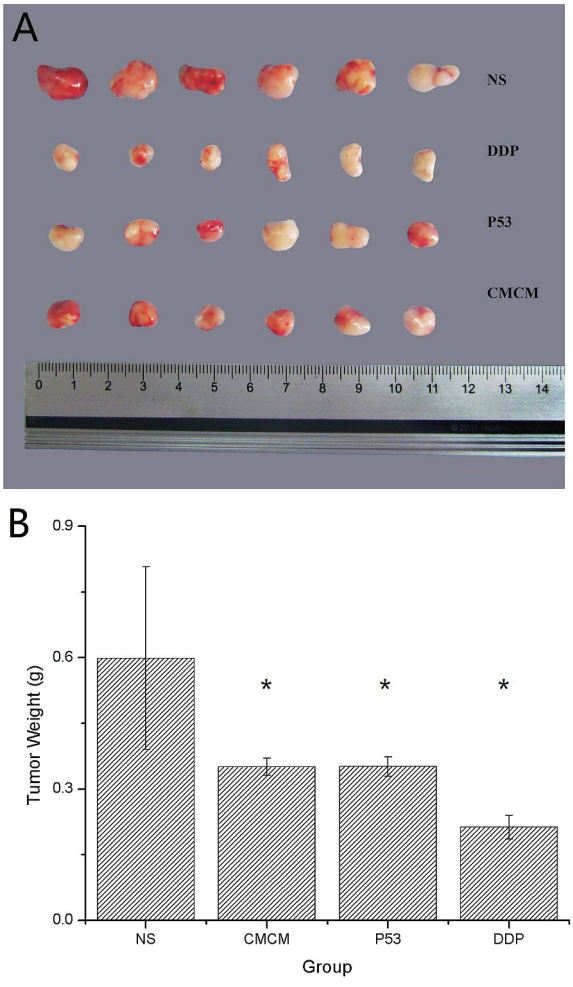

FIGURE 2 Antitumour efficacy of cardiac myocyte–conditioned medium (CMCM) *in vivo*. (A,B) An inhibitory effect of CMCM on the weight of lung cancer tumour xenografts implanted in nude mice is observed. NS = control group (normal saline); DDP = treated with cisplatin; P53 = treated with recombinant human adenovirus–p53; CMCM = treated with CMCM. * $p < 0.001$ compared with the control group.

Survival Duration of Mice

The median survival duration of mice bearing A549 tumours and treated with normal saline or rhAd-p53 was 17 ± 2.45 days and 17 ± 2.44 days respectively. Survival was significantly prolonged in the Cis (26 ± 6.12 days) and CMCM groups (29 ± 10.41 days). The CMCM was more effective than Cis or rhAd-p53 at prolonging survival in tumour-bearing mice [log-rank $p < 0.05$, Figure 3(A)].

Spontaneous Lung Metastasis in Mice

Lungs of mice in the normal saline group contained multiple metastasized tumours of various sizes on their surface; lung metastases were significantly fewer in the CMCM and rhAd-p53 groups ($p < 0.05$). Metastatic nodules in lung numbered 3.83 ± 1.83 in the control group, 1.67 ± 1.21 in the CMCM group, 2.00 ± 0.89 in the rhAd-p53 group, and 0.67 ± 0.82 in the Cis group. The lung metastasis inhibition rates were 56.52% in the CMCM group, 47.83% in the rhAd-p53 group, and 82.61% in the Cis group. The CMCM was able to effectively reduce the lung-metastasizing capacity of A549 tumours [Table III, Figure 4(A-E)].

Body Weight Change in Tumour-Bearing Mice

All animals were randomly assigned to their groups, and there was no statistically significant difference in in body weight between the groups before treatment ($p > 0.05$). At the end of 15 days, mice in all groups except the CMCM group had lost weight.

Compared with the other groups, mice in the Cis group experienced a statistically significant reduction in body weight starting at 3 days after first administration until the end of the study ($p < 0.05$), indicating that the tumour burden and Cis toxicity resulted in loss of body weight. In contrast, body weight of mice in the CMCM group had increased significantly at the end of the experiment. The results indicate that CMCM is not associated with weight loss [Figure 3(B)].

DISCUSSION

Tumour spread by metastasis is well-known to have organ specificity. Many clinical studies show that various tumour cells have a certain affinity for a specific organ or tissue during the early phase of metastasis. For instance, the common metastatic sites for lung cancer are bone, adrenal gland, and brain; bone is the most likely metastasis site for the primary malignancies of prostate cancer, renal cell carcinoma, and thyroid carcinoma. However, metastasis is rare in some organs, even if they have an abundant blood supply. Organs such as kidney, striated muscle, and thyroid take up almost a quarter of an individual's entire blood supply, and yet metastases are rare in those organs and tissues.

Metastasis is associated with the tumour microenvironment. The tumour microenvironment includes cells such as fibroblasts, immune cells, endothelial cells, extracellular matrix, proteases, and cytokines. Together, those components participate in complex crosstalk with neoplastic tumour cells, affecting growth, angiogenesis, and metastasis[13]. Recent research has shown that some organs and tissues express and secrete factors closely

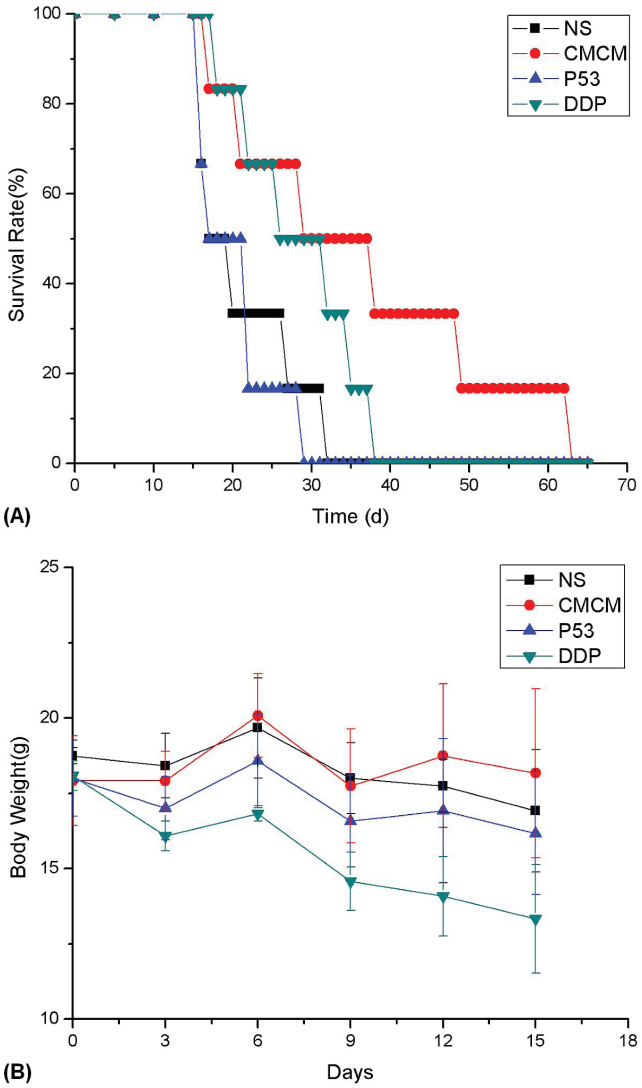

FIGURE 3 Effects of cardiac myocyte–conditioned medium (CMCM) on survival rate and side effects in tumour-bearing mice. (A) Treatment with CMCM was more effective than treatment with cisplatin (DDP) or recombinant human adenovirus–p53 (P53) for increasing the survival of tumour-bearing mice. (B) Treatment with CMCM had no effect on weight loss in tumour-bearing mice. NS = control group (normal saline).

TABLE III Inhibition of tumour metastasis caused by A549 lung carcinoma xenografts in nude mice

Group	Mice (*n*)	Mean metastases (*n*)	Average inhibitory rate (%)
Control	6	3.83±1.83	—
CMCM	6	1.67±1.21[a]	56.52
rhAd-p53	6	2.00±0.89[a]	47.83
Cisplatin	6	0.67±0.82[a]	82.61

[a] $p < 0.05$ compared with control group.
CMCM = cardiac myocyte–conditioned medium; rhAd-p53 = recombinant human adenovirus–p53.

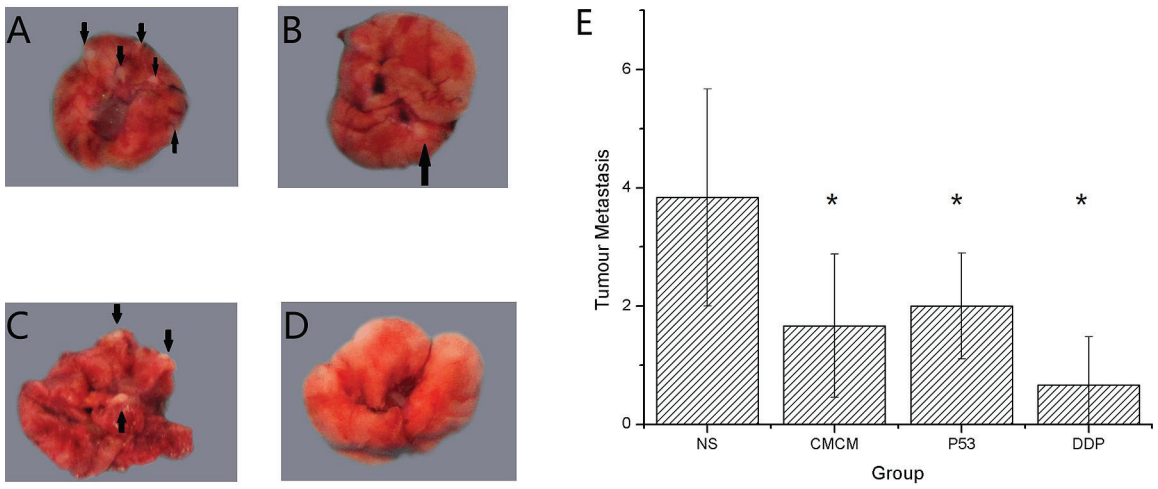

FIGURE 4 Inhibition of tumour metastasis (A549 lung carcinoma cells) in mice. (A) Control group [NS (normal saline)], (B) group treated with cardiac myocyte–conditioned medium (CMCM), (C) group treated with recombinant human adenovirus–p53 (P53), (D) group treated with cisplatin (DDP), and (E) overall occurrence of metastases in each group. * $p < 0.05$ compared with control group.

associated with invasion and metastasis in a variety of tumours. Proteins such as cytokines, chemokines, and growth factors have been found to play a key role in the regulation of organ selectivity. Overexpression of chemokine receptors by specific organs is clearly associated with organ-specific metastasis[14]. Other organs might secrete factors that inhibit the growth of tumour cells.

Recent research has shown that a new low molecular weight factor released by muscle cells inhibits proliferation of multiple tumour cells *in vitro* and *in vivo*, is highly specific with respect to tumour cells, and has no observable effect on the proliferation of normal cells[15,16]. Skeletal muscle–conditioned medium exerts a cytostatic effect on tumour cell growth and arrests the cells in the G0/G1 phase of the cell cycle. Partial purification of skeletal muscle–conditioned medium revealed that the active component is a non-proteinaceous compound with a molecular weight of about 500 Da[17]. Using high-performance liquid chromatography separation, mass spectra, and nuclear magnetic resonance analyses, adenosine was identified as one of the active components in the skeletal muscle–conditioned medium. By similar methods, additional inhibitory components in the 600–800 Da molecular mass range were detected in the skeletal muscle–conditioned medium[18,19].

Cardiac muscle and skeletal muscle are both striated muscle. A low molecular weight inhibitory factor might be released by cardiac muscle cells. Proliferation of the S180 carcinoma cell line was significantly inhibited when cultured with CMCM, while murine benign renal cells remained unaffected. The unknown tumour suppressor retained its activity after treatment with pancreatin, but was thermolabile[9,20]. We further found that the low molecular weight tumour suppressor from cardiac muscle inhibited S180 tumour transplanted into mice *in vivo*, with no obvious side effects during treatment[10].

The use of CMCM has not been examined in the context of lung cancer models, and nothing is known about its potential effects on metastasis. Here, we assessed the activity of CMCM in lung cancer, including its anti-proliferation and anti-metastasis activity *in vitro* and *in vivo*, using the highly metastatic A549 cell line.

We used an MTT assay to corroborate the antitumour effects of CMCM on A549 cells *in vitro*. The MTT assay indicated that tumour cell proliferation declined significantly after CMCM treatment. Previous studies have suggested that CMCM might cause functional and structural changes in tumour cells, such as blebbing of the membrane and major ionic changes[21].

We also found that CMCM could inhibit the proliferation of A549 cells *in vivo*, finding CMCM-mediated effects on tumour growth, volume, weight, and survival duration in treated mice. In addition, lung metastases were analyzed for the first time. Our results indicated that CMCM significantly prolongs the life of A549 tumour-bearing mice and inhibits tumour growth and metastasis. It had the effect of inhibiting tumour growth, and its antitumour activity was found to be similar to that of rhAd-p53. The trend of inhibition of tumour weight was consistent with the inhibition of tumour volume. Those data accord with the *in vitro* findings.

No side effects of using CMCM were detectable in our study, and treatment was relatively safe to administer. However, our research on safety of CMCM was somewhat limited. A rigorous safety assessment must be performed.

The results of our study confirm that CMCM inhibits tumour growth, prolongs survival duration, and reduces the lung-metastasizing capacity of A549 tumours, with no severe toxicity. The unique features of the inhibitory factors described in the present study include low molecular weight, inhibition of proliferation and the invasive characteristics of several tumour cell lines, and continued activity after treatment with pancreatin. Those unique characteristics could be important to the potential clinical use of these compounds in the development of novel anticancer therapies. Further studies into the purification of these factors and the biochemical characteristics of CMCM and its antitumour activity should be carried out.

CONCLUSIONS

In vitro and *in vivo*, CMCM has an inhibitory effect on tumour growth and also reduces cell viability, tumour volume, and the lung metastasis rate, thus prolonging survival duration, without severe toxicity.

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CONFLICT OF INTEREST DISCLOSURES

We have read and understood *Current Oncology*'s policy on disclosing conflicts of interest, and we declare that we have none.

AUTHOR AFFILIATIONS

*Department of Oncology, The Affiliated Hospital of Luzhou Medical College, Luzhou, P.R.C.; †Third Military Medical University, Chongqin, P.R.C.

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