

© 2002, Acta Pharmacologica Sinica
ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Acetazolamide suppresses tumor metastasis and related protein expression in mice bearing Lewis lung carcinoma¹

XIANG Yang, MA Bing, LI Tao, YU He-Ming², LI Xue-Jun³

Department of Pharmacology, School of Basic Medical Sciences, Peking University, Beijing 100083;

²National Research Institute for Family Planning, Beijing 100081, China

KEY WORDS acetazolamide; neoplasm metastasis; aquaporins; actin

ABSTRACT

AIM: To study the suppressing effect of acetazolamide on tumor metastasis *in vivo* and observe the protein alteration of lung in mice bearing Lewis lung carcinoma. **METHODS:** The functional role of aquaporin-1 (AQP₁) was investigated in tumor tissues by SDS-PAGE and Western blot. The effect of acetazolamide on tumor metastasis was analyzed by Lewis-lung-carcinoma model. Differential protein was identified by SDS-PAGE, isoelectrofocusing (IEF) methods, and peptide mass fingerprinting (PMF). **RESULTS:** Acetazolamide (40 mg·kg⁻¹·d⁻¹ *po* for 21 d) dramatically reduced the numbers of lung metastasis after sc inoculating Lewis lung carcinoma. The inhibition rate of lung metastases was 83.9%. Simultaneously, the AQP₁ protein level and actin-cytoplasmic in lungs containing metastatic tumor deposits were found to be higher than that in the normal tissue. After treated with acetazolamide for 21 d, the expression of AQP₁ was obviously inhibited. **CONCLUSION:** Acetazolamide can suppress tumor metastasis, at least in part, by inhibiting the expression of AQP₁. AQP₁ and actin-cytoplasmic may be new prognostic molecules as well as new therapeutic targets for the prevention and treatment of metastatic tumor.

INTRODUCTION

Water transport occurs across plasma membranes in all cells for housekeeping functions such as volume regulation. The recent discovery of “aquaporins”, a large family of membrane proteins that function as highly selective water channels, has drawn attention to their role in physiology and several human

diseases involving rapid water transport and have identified them as potential targets for therapeutic intervention.

Aquaporin-1 (AQP₁), the first characterized water channel protein^[1], was identified in erythrocyte membranes, renal proximal tubule, choroid plexus, eye, lung, vascular endothelium, hepatobiliary epithelium, and some tumor cells themselves^[2]. Most tumors have been shown to exhibit high vascular permeability and high interstitial fluid pressure, but the transport pathways for water within tumors remain unknown.

Acetazolamide is a kind of sulfanilamide served as carbonic anhydrase inhibitor. To be interesting, the

¹ Project supported by the National Natural Science Foundation of China, No. 39770286 and 30171090.

³ Correspondence to LI Xue-Jun. Phn 86-10-6209-1421.

Fax 86-10-6217-9119. E-mail lixj@mail.bjmu.edu.cn

Received 2001-10-29

Accepted 2002-05-30

tissue distribution and even the subcellular localization of AQP₁ are similar to that of carbonic anhydrase, suggesting that there are some relation between the two kinds of proteins in their structure and function. Parkkilas *et al*^[3] have shown that acetazolamide alone can inhibit the invasive potential of cancer cells *in vitro*, but the mechanism remains unclear. We have established a AQP₁ expression system by using *Xenopus* oocytes to study the effect of acetazolamide on water transport function of AQP₁, and confirmed that acetazolamide was a direct inhibitor of AQP₁^[4-5]. We hypothesized that the reductive action of acetazolamide on AQP₁ gene expression and water transportation might be contributed to their effects on cancer invasions.

Therefore, our goal in this study was to characterize and evaluate the expression of AQP₁ in tumor tissues and the effect of acetazolamide on tumor metastasis *in vivo*. The well-characterized Lewis-lung-carcinoma model was chosen for these studies because the tumor is relatively resistant to many cancer therapies and spontaneously metastasizes to the lungs.

In this research, as lung tissue proteins were analyzed by SDS-PAGE, we accidentally discovered that a M_r 42 000 protein increased clearly in tumor lung tissues compared with normal. We evaluated this protein with the technology of peptide mass fingerprinting (PMF).

MATERIALS AND METHODS

Lewis-lung-carcinoma *in vivo* model Female C57BL/6 mice weighing 18–20 g, were used and purchased from the Experimental Animal Center of Peking University (Certificate No. 11-00-0004). Lewis lung carcinoma provided by Chinese Medical Science Institute was maintained in C57BL/6 mice by sc injection in the axillary region of 0.2 mL of homogenized tumor tissue [tumor tissue (g):0.9 % sodium chloride (mL)=1:3] prepared from donors similarly inoculated for experimental tumor transplantation.

Drug preparation and treatment Acetazolamide was purchased from Sigma and given at a volume of 0.1 mL/mice, corresponding to 40 mg·kg⁻¹·d⁻¹ *po*. This dose has been confirmed to reduce AQP₁ protein

level to 59.95 % after 14 d treatment by our laboratory (unpublished results). NaHCO₃ 30 mg/kg *po* was applied once every 3 d to correct the metabolic acidosis as our previous studies. Control mice received the same volume of vehicle (*po*). As a positive control, cyclophosphamide 100 mg/kg *ip* were given once a week.

The animals were divided into four groups: 1) control, without tumor implantation; 2) model, with tumor implantation; 3) acetazolamide treatment; 4) cyclophosphamide treatment. On d 21, 8 mice per group were killed to calculate the ratio of tumor weight to body weight (g/g) and the lung weight. The numbers of lung metastases were counted under microscopy as well. Primary tumor and lungs were then surgically resected and tissue specimens were snap-frozen in liquid nitrogen for analysis.

SDS-PAGE and Western blot analysis Homogenized lung tissues in lysis buffer were solubilized in sample buffer^[6] and heated to 60 °C for 15 min. Total protein concentration was measured by Lowry's method, using bovine serum albumin as standard. These samples containing 50 µg of protein were loaded on a 12 % polyacrylamide gel and electroblotted onto nitrocellulose membranes, SDS-PAGE gels were stained with Coomassie brilliant blue to confirm equivalence of samples. The nitrocellulose membranes were blocked in blotting buffer containing 5 % nonfat dry milk and 0.05 % Tween 20 in TBS (TBST), followed by incubation with anti-AQP₁ antibody (rabbit anti-human IgG, a gift from Prof YU He-Ming, National Research Institute for Family Planning, Beijing, China) diluted 1:300 in blotting buffer at 4 °C overnight. The membranes were washed three times for 5 min with TBST buffer and incubated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:5000 in TBS buffer. After washing three times for 5 min with TBST buffer, the M_r 28 000 protein were stained with BCIP/NBT. Stained bands were scanned and the pixel intensity was quantified using Gel Doc 2000 Image system (Bio-Rad Ltd, USA). All of the reagents were from Bio-Rad Ltd or Sigma Chemical Co. The experimental procedures were carried out at room temperature.

Isoelectrofocusing (IEF) electrophoresis The

reagents and electrophoresis apparatus were from Bio-Rad Ltd and operated as enclosed instruction. The monomer-ampholyte solution for two 45 mm×125 mm×0.8 mm gels: H₂O 5.5 mL, monomer concentrate 2.0 mL, 25 % (w/v) glycerol 2.0 mL, ampholyte 0.5 mL, 10 % (w/v) ammonium persulfate 15 μL, 0.1 % (w/v) FMN (riboflavin-5'-phosphate) 50 μL, TEMED (N,N'-tetramethylene-ethylenediamine) 3 μL. Irradiate the solution for approximately 1 h, then load the samples 8–12 μL.

Protein identification by MALDI-TOF The difference protein band was cut out from SDS-PAGE gel and the Coomassie stain was removed by 50 % acetonitrile NH₄HCO₃ 2.5 mmol/L. Additional gels in the tubes were digested with trypsin (modified, sequencing grade from Boehringer Corp) at 37 °C for 20 h and dried under vacuum. A sample of 120 μL of 5 % trifluoroacetic acid (TFA) was added to the tube and incubated at 40 °C for 1 h in a water bath. After collecting the supernatant another 120 μL of TFA solution [2.5 % TFA, 50 % ACN (acetonitrile)] were added to the tube and incubated at 30 °C for 1 h in water bath. The supernatant was combined and dried to a powder under vacuum. The sample of generated peptides were dissolved in 5 μL of 0.5 % TFA and measured by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS)^[7].

The experiments were performed using a Bruker Inc Biflex III MALDI-TOF mass spectrometer equipped with 337 nm nitrogen laser. Both matrices 4-hydroxy-α-cyanocinnamic acid and sample were dissolved in 1:1 (v/v) acetonitrile:water with 1 % trifluoroacetic acid. And 0.5 μL of this mixture solution was placed on a

metal sample plate and air-dried at ambient temperature. Mass spectra were acquired in positive linear mode and using an acceleration voltage of 19 kV. External mass calibration was performed using a standard peptide mixture. Spectra were obtained by setting the laser power close to the threshold of ionization and generally 100 pulses were acquired and averaged. The data obtained from PMF were applied in searching the protein database to determine the identity of the protein.

Statistical analysis Data were expressed as mean±SD and comparisons were done by *t* test or Mann-Whitney test depending on whether the data were normally distributed. All statistical analysis were done in SPSS version 10.0, *P*<0.05 was considered to be statistically significant.

RESULTS

Effect of acetazolamide on primary tumor growth and the formation of spontaneous lung metastasis in mice bearing Lewis lung carcinoma The murine Lewis lung carcinoma is known to produce spontaneous lung metastasis. To determine whether treatment with acetazolamide could reduced the number of lung metastasis, groups of 8-mice were implanted with Lewis lung carcinoma and killed on d 21. The data reported in Tab 1. Tumor weight index means the ratio of tumor weight to body weight (g/g)×100. Inhibition rate of lung metastases %=($W_{\text{model}}-W_{\text{treatment}}$)/($W_{\text{model}}-W_{\text{control}}$)×100 %, here *W* is lung wet weight. Acetazolamide could not inhibit the primary tumor growth (20±5) compared with vehicle-treated mice (24±4, *P*>0.05). Both acetazolamide and cyclophosphamide could significantly reduce the number of lung metastasis. The

Tab 1. Effect of acetazolamide on experimental tumor metastasis in mice. Mean±SD. **P*<0.01 vs model.

| Group | Number of survival | Tumor weight index | Number of metastasis | Number of metastasis larger than 2 mm | Inhibition rate of lung metastases % |
|---|--------------------|----------------------|----------------------|---------------------------------------|--------------------------------------|
| Model | 7/8 | 24±4 | 24±6 | 6.4±2.8 | |
| Acetazolamide 40 mg·kg ⁻¹ ·d ⁻¹ , <i>po</i> | 7/8 | 20±5 | 11±3 ^c | 1.6±2.2 ^c | 83.9 |
| Cyclophosphamide 100 mg·kg ⁻¹ , <i>ip</i> | 8/8 | 1.7±1.3 ^c | 0.9±1.6 ^c | 0.4±1.1 ^c | 97.3 |

inhibition rate of lung metastasis in acetazolamide treated group was 83.9 %.

AQP1 protein expression Western blot analysis showed (Tab 2) that the protein levels of AQP1 in lungs with metastatic tumor deposits were significant higher compared to normal lung tissues. Treated with acetazolamide or cyclophosphamide clearly inhibited the expression of AQP1 compared to the tumor transplanted model group (Fig 1).

Tab 2. Pixel intensity of AQP₁ expression by Western blot in each group. Mean±SD. °P<0.01 vs control. °P<0.05, °P<0.01 vs model.

| Group | n | Pixel intensity |
|--|---|----------------------|
| Control | 8 | 2.1±0.6 |
| Model | 7 | 4.0±0.7 [°] |
| Acetazolamide 40 mg·kg ⁻¹ ·d ⁻¹ , po | 7 | 2.5±1.1 [°] |
| Cyclophosphamide 100 mg·kg ⁻¹ , ip | 8 | 2.2±0.8 [°] |

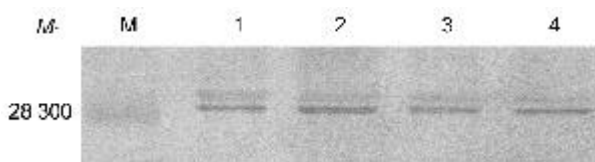


Fig 1. The protein levels of aquaporin-1 from lung specimens in normal lungs (lane 1), lungs containing metastatic tumor deposits (lane 2), treated with acetazolamide (lane 3), and cyclophosphamide (lane 4).

Characterization and quantification of the differential protein

When analyzed the proteins obtained from lung specimens, we accidentally discovered a significant increase of a *M_r* 42 000 component from lungs containing metastatic tumor deposits compared with normal lungs (Fig 2). The isoelectrofocusing electrophoresis method determined that the pI of *M_r* 42 000 protein was 5.3 (Fig 3).

MALDI-TOF-MS studying and protein database searching showed that the *M_r* 42 000 protein was actin-cytoplasmic analogue. The matching masses represented more than 43 % of the fragment masses obtained, with a difference between measured and calculated

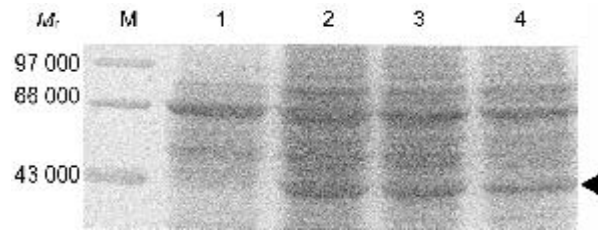


Fig 2. Total lung proteins (50 mg) were electrophoresed into 12% SDS-PAGE gels and stained with Coomassie blue. Lane 1: normal lungs; lane 2: lungs containing metastatic tumor deposits; lane 3: treated with acetazolamide; lane 4: treated with cyclophosphamide. Arrow points to *M_r* 42 000 protein.

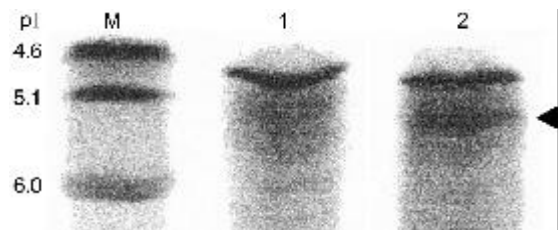


Fig 3. Isoelectrofocusing electrophoresis analysis. Lane 1: normal lungs; lane 2: lungs containing metastatic tumor deposits. Arrow points to the differential protein pI was 5.3.

masses below 0.2 (Fig 4).

DISCUSSION

The present results indicated AQP₁ protein expression in the tumor was higher than that in the normal tissue. Treated with acetazolamide or cyclophosphamide dramatically inhibited the expression of dramatically AQP₁. This study demonstrated the possibility that the tumors growing might have a higher water permeability since a general function of AQP₁ is to increase membrane water permeability. We proposed that appropriate management of fluid in the vascular, interstitial, and cells would be in parallel to tumor growth. Furthermore, the expression of AQP₁ in tumor may reflect a role of this water channel in pathological processes including the development of effusions or edema due to changes in hydrostatic or tumor oncotic pressures. This finding is consistent with the view that transcript levels of AQP₁ may serve as a new molecular prognostic marker in patients with renal cell carcinoma following nephrectomy^[8].

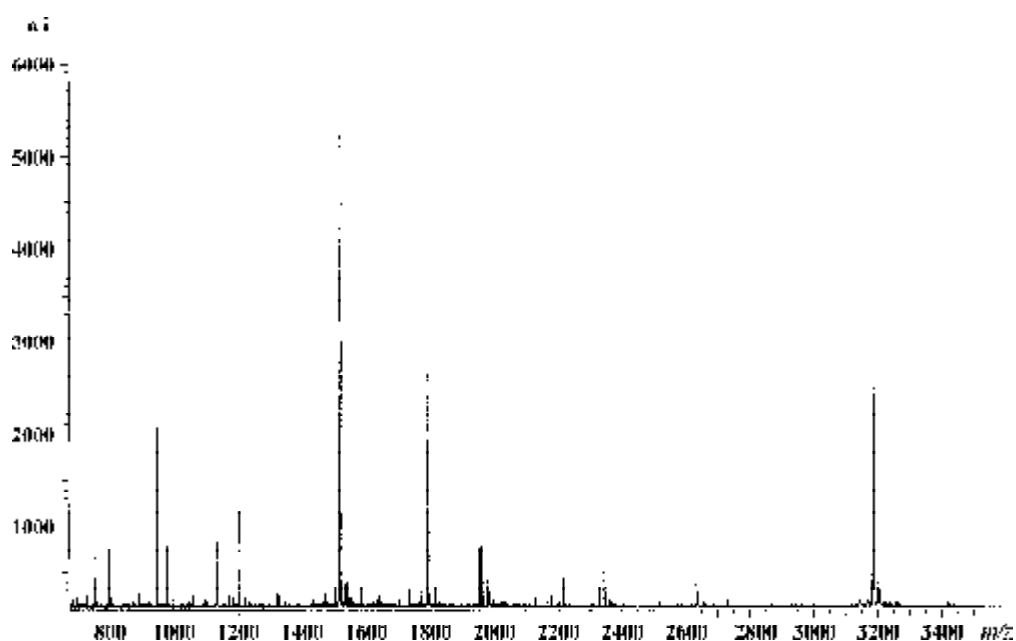


Fig 4. MALDI-TOF peptide-mass fingerprint spectrum of the tryptic digest of differential protein.

Treatment with acetazolamide or cyclophosphamide could significantly reduced the number of lung metastasis. The effects of acetazolamide *in vivo* were surprising. Its inhibition rate of lung metastases was 83.9 %. This non-cytotoxic drug produced a robust response to the tumor metastases. As we know, when acetazolamide was introduced as a chemotherapeutic agent, a notable side effect was metabolic acidosis. Therefore, to correct acidification of the extracellular milieu. The animals were treated with acetazolamide and combined with NaHCO_3 (once every 3 d). The present results raise two interesting questions. First, how does acetazolamide reduce the metastasis rate? Second, what is the target for acetazolamide in the analyzed spontaneous metastasis of Lewis lung carcinoma in mice? Cyclophosphamide might inhibit cell proliferation [tumor weight index (1.7 ± 1.3) vs model group (24 ± 4), $P < 0.01$] and consequently reduce the tumor metastasis and the expression of AQP_1 , while acetazolamide [tumor weight index 20 ± 5 vs model group, $P > 0.05$] is not attributable to reduced proliferation of the cancer cells. Our previous study has demonstrated that the acetazolamide could significantly decrease AQP_1 both on mRNA and protein level expression, which was not influenced by acid-base condition^[5]. Long-term

treatment (14 d) with acetazolamide could increase the expression of carbonic anhydrase (CA) II and IV, and recover the repressed CA activity gradually, this indicates acetazolamide can directly affect AQP_1 (unpublished results). Therefore, the most straightforward explanation for our finding is that acetazolamide suppresses tumor metastases, at least in part, by inhibiting the expression of AQP_1 . The present data only represent an initial step toward an understanding of the complex pathways of acetazolamide in inhibiting tumor metastases, and the possible mechanisms remain to be explored in detail.

In this context, we found a dramatically increase in M_r 42 000 protein level in SDS-PAGE and the protein was identified as actin-cytoplasmic analogue by MALDI-TOF-MS, which suggests that it may be useful as a prognostic marker in cancer metastasis. Actin, the principle component of the cortical cytoplasm, is a M_r 42 000 globular protein possessing the unique ability to self associate into long polymers under intracellular conditions. Cells are supported by a network of polymerized actin filaments configured into a variety of structures by actin structural proteins. For cell locomotion to occur, this network must be reversibly disassembled to allow protrusion, then re-assembled to stabilize the

resulting extension^[9,10]. Thus, proteins to promote both solation and gelation of actin are important for efficient cell locomotion. Several investigators have found malignant transformation prominently down-regulated actin structural protein-gelsolin^[11,12]. Tumor cell motility is an important step in the progression of metastasis and cancer metastasis is dependent on increased cell motility. Tumor cells migrate from the primary tumor, intravasate into tumor blood vessels, extravasate into the secondary tumor site, and migrate through tissue to establish a secondary metastatic site. Currently there is no prognostic molecule of distinguishing these patients from those in which the tumor will progress to metastasis. This is an important clinical problem. On the basis of our data *in vivo*, knowledge of the actin-cytoplasmic in tumor cells may lead to a new prognostic molecule as well as a new therapeutic targets for the prevention and treatment of metastatic tumor. This study also confirms that proteomic analysis is a powerful tool for the discovery of such molecular markers.

In conclusion, the results presently suggested that acetazolamide, as a non-cytotoxic drug, can significantly suppress the tumor metastases *in vivo*. And we provided the first evidence that the mechanism of action of acetazolamide on tumor metastasis could be involved in reducing AQP1 protein expression. The increases of AQP1 and actin level in tumor tissue may provide further impetus for exploring useful prognostic marker and therapeutic targets as well.

ACKNOWLEDGEMENT We thank Prof XIONG Shao-Xiang (National Center of Mass Spectrometry, Beijing 100080) for his assistance in the PMF analyses.

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乙酰唑胺抑制肿瘤转移及荷瘤小鼠肺相关蛋白质的表达¹

项阳, 马兵, 李涛, 于和鸣², 李学军³
(北京大学医学部药理学系, 北京 100083; ² 国家计划生育委员会科学技术研究所, 北京 100081, 中国)

关键词 乙酰唑胺; 肿瘤转移; 水通道蛋白; 肌动蛋白类

目的: 研究乙酰唑胺对肿瘤转移的抑制及相关蛋白质的表达. **方法:** 以 Lewis 肺癌为肿瘤转移模型, 观察乙酰唑胺对肿瘤转移的影响; 用 SDS/PAGE、

Western blot、IEF 和肽质量指纹谱的方法观察相关蛋白质的变化。结果：乙酰唑胺可以显著抑制肿瘤转移，肺转移抑制率为 83.9 %。荷瘤小鼠肺组织中水通道蛋白 - 1 和肌动蛋白表达明显增高，用乙酰唑胺治疗后水通道蛋白 - 1 表达降低。结论：乙

酰唑胺具有抗肿瘤转移作用，其机制可能与抑制水通道蛋白 - 1 的表达有关；水通道蛋白 - 1 和肌动蛋白可望成为肿瘤肺转移诊疗的新靶标。

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Info: P Sikiric MD, PhD

Organization Committee Chairman

Department of Pharmacology

Medical Faculty University of Zagreb

Salata 11, POB 916

10 000 Zagreb, Croatia

Phn +385 1-456-6833 +385 1-456-6834

Fax +385 1-492-0050

E-mail sikiric@mef.hr