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【论 著】

抗癌药物索拉非尼体外抗多房棘球蚴的效果评价

党志胜[1]，奥祐三郎[2]，周晓农[1]，胡薇[1,3]，张颋[1]，陈军虎[1]，徐斌[1]，赵玉敏[4]，肖宁[1*]

【摘要】 目的 对抗癌药物索拉非尼体外抗多房棘球蚴的效果进行评价。 方法 从感染小鼠体内收集多房棘球蚴小囊泡，加入预先接种大鼠肝细胞的 DMEM 培养液（含 10%胎牛血清、100 U/mL 青霉素、100 mg/L 链霉素）中，37 ℃、5% CO_2 培养 2～3 个月后，选取直径 2～4 mm 的完整包囊用于药物效果评价实验。实验分阿苯达唑组（阳性对照），阿苯达唑终浓度分别为 10 μmol/L 和 30 μmol/L；索拉非尼组（实验组），索拉非尼终浓度分别为 10 μmol/L 和 30 μmol/L；DMSO 组（溶剂对照组），加 DMSO（1∶9 溶于 PBS 中）；PBS 组（阴性对照），仅加 PBS。每孔取 25～30 个包囊，每个浓度设 3 个重复。药物处理 48、72、96、120、144 和 168 h 后，光学显微镜和扫描电镜观察包囊形态结构变化，计算包囊抑制率，对其体外抗棘球蚴效果进行评价。 结果 10、30 μmol/L 索拉非尼处理 48、72、96、120、144 和 168 h 后，包囊的抑制率分别为 6.6%、42.4%、68.5%、77.4%、84.0% 、89.5%和 7.1%、45.6%、70.9%、82.6%、84.0%、89.9%。10、30 μmol/L 阿苯达唑在每个时间点的包囊抑制率分别为 2.6%、6.6%、21.4%、47.8%、59.9%、70.6%和 3.8%、12.7%、27.0%、51.4%、54.0%、73.0%。DMSO 和 PBS 对包囊没有明显的抑制效果。索拉非尼的抑制效果均高于阿苯达唑，从处理后 72 h 起，索拉非尼和阿苯达唑的抑制率差异有统计学意义（$P < 0.05$）。光学显微镜观察表明，索拉非尼处理后，包囊向内部陷塌，形成“日全食”状；扫描电镜观察发现，索拉非尼处理后，包囊内部生发层细胞脱落，完全失去活性。 结论 索拉非尼显示出较高的包囊抑制效果，是棘球蚴病治疗的潜在药物。

【关键词】 多房棘球蚴病；生发层；索拉非尼

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In vitro inhibitive effect of the anticancer drug sorafenib on *Echinococcus multilocularis* larvae

DANG Zhi-sheng[1], OKU Yuzaburo[2], ZHOU Xiao-nong[1], HU Wei[1,3], ZHANG Ting[1], CHEN Jun-hu[1], XU Bin[1], ZHAO Yu-min[4], XIAO Ning[1*]

(**1** *National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention; WHO Collaborating Centre for Tropical Diseases; National Center for International Research on Tropical Diseases, Ministry of Science and Technology; Key Laboratory of Parasite and Vector Biology, Ministry of Health, Shanghai* **200025**, *China;* **2** *Parasitology Laboratory, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori,* **680-8553**, *Japan;* **3** *Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai* **200433**, *China;* **4** *Department of Biomedical Engineering, College of Biotechnology, Guilin Medical University, Guilin* **541004**, *China*)

【**Abstract**】 **Objective** To evaluate the effect of an anti-cancer drug sorafenib against alveolar echinococcosis (AE) *in vitro*. **Methods** Metacestode vesicles were obtained from infected mice and cultured with rat hepatocytes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin G and 100 mg/L streptomycin sulphate at 37 ℃ with 5% CO_2. Morphologically intact metacestode vesicles with a minimal diameter of 2–4 mm were manually picked up from the co-culture and washed with PBS. They were then allocated (n = 25–30 cysts/well) into four groups: albendazole-treated group (positive group), sorafenib-treated group (experimental group), DMSO-treated group (solvent group), and PBS group (negative control), and incubated in 24-well

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作者单位：1 中国疾病预防控制中心寄生虫病预防控制所，世界卫生组织热带病合作中心，科技部国家级热带病国际联合研究中心，卫生部寄生虫病原与媒介生物学重点实验室，上海 200025；2 日本鸟取大学农学部兽医学院寄生虫学教研室，鸟取 680-8553，日本；3 复旦大学生命科学院微生物学与微生物工程系，上海 200433；4 桂林医学院生物技术学院生物医学工程系，桂林 541004

* 通讯作者，E-mail：ningxiao116@126.com

plates in the presence of DMEM without phenol red. Albendazole and sorafenib were dissolved in DMSO (1∶9 dissolved in PBS) and used at 10 μmol/L and 30 μmol/L respectively. The experiment was performed in triplicates per sample. The integrity of the cysts was assessed with light microscopy and scanning electron microscopy (SEM). The inhibitory effects of albendazole and sorafenib on cysts were evaluated at 48, 72, 96, 120, 144 and 168 h post-treatment. **Results** The cyst inhibitory rate of sorafenib was 6.6%, 42.4%, 68.5%, 77.4%, 84.0% and 89.5% at 10 μmol/L, and 7.1%, 45.6%, 70.9%, 82.6%, 84.0% and 89.9% at 30 μmol/L, respectively, at 48, 72, 96, 120, 144 and 168 h post-treatment. While the cyst inhibitory rate of albendazole was 2.6%, 6.6%, 21.4%, 47.8%, 59.9% and 70.6% at 10 μmol/L, and 3.8%, 12.7%, 27.0%, 51.4%, 54.0% and 73.0% at 30 μmol/L, respectively. DMSO and PBS showed less toxic to the cysts. Sorafenib exerted a stronger inhibitory effect than albendazole at the corresponding time points, and the difference began to be statistically significant from 72 h post-treatment. Light microscopy and SEM showed observable collapse of the germinal layer and overall damage of germinal cells in the experiment groups. **Conclusion** Sorafenib shows a significant inhibitory effect on *Echinococcus* cysts, suggesting a potential drug candidate for echinococcosis treatment.

【Key words】 Alveolar echinococcosis; Germinal layer; Sorafenib

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* Corresponding author, E-mail: ningxiao116@126.com

Alveolar echinococcosis (AE), caused by the metacestode of the fox-tapeworm *Echinococcus multilocularis*, is a life-threatening disease with very limited treatment options. Echinococcosis is an endemic disease world-wide[1-2]. In China, echinococcosis is endemic in Qinghai-Tibet Plateau[3-5].

AE exhibits malignant features, such as tumor-like growth of the vesicle-like metacestodes and occasionally metastatic spread into other organs. Chemotherapeutic anti-AE treatment is currently relying on benzimidazoles (BZs) that target parasite β-tubulin. However, due to certain affinities of the drugs to host β-tubulin, BZ treatment of AE is often associated with severe side effects. Moreover, it exerts only a parasitostatic action rather than parasitocidal. BZs therapy often is life-long. Against this background, there is an urgent need for identification of novel drug targets and inhibitory drugs against AE.

Components of cellular signalling systems have been suggested as promising targets in anthelminthic chemotherapy owing to their crucial participation in regulating development and proliferation of metazoans[6-7]. Furthermore, owing to their important role in carcinogenesis, the biochemistry of respective signaling molecules such as kinases and phosphatases is well studied, and a broad range of inhibitory compounds is available. The ATP-competitive kinase inhibitor Imatinib (Glivec®; STI-571) is currently used in cancer therapy against chronic myelogenous leukaemia (CML), caused by the expression of a constitutively active BCR-ABL kinase fusion protein, as well as against gastrointestinal stromal tumors (GISTs) that are mostly caused by constitutive activation of the receptor tyrosine kinase c-Kit[8]. Imatinib belongs to the 2-phenylaminopyrimidine class of compounds and specifically targets ABL kinases as well as a limited number of related enzymes from the type **III** group of tyrosine kinases, such as c-Kit or the platelet-derived growth factor receptor (PDGFR), which are all involved in the regulation of cell proliferation, survival, cell adhesion and migration.

Since the mitogen-activated protein kinase (MAPK) signaling pathway is involved in both physiological and pathological cell proliferation, recent studies have focused on some MAPK transducers as therapeutic targets. Some of inhibitors of the p38, such as SB203580, SB202190 and ML3403 are evaluated in their effects on inhibiting parasite replication or development, including *Plasmodium*, *Leishmania*, *Toxoplasma* and *Echinococcus*[9-13]. Akt/PKB (protein kinase B) was found to be an essential player in regulating cell/organ growth at the adult stage in the hard tick[14]. Study showed that cell growth by 8-bromo-7-methoxychrysin (BrMC) was inhibited

through inactivation of Akt in human epidermal growth factor receptor 2 (HER-2)/neu-overexpressing breast cancer cells[15].

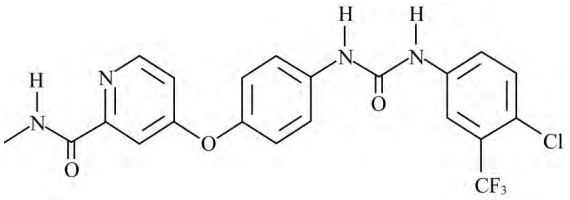
Sorafenib (known as Nexavar®), which targeting B-rapidly accelerated fibrosarcoma (B-Raf), rapidly accelerated fibrosarcoma 1(Raf-1), vascular endothelial growth factor receptor 2 (VEGFR2), platelet-derived growth factor beta-receptor (PDGFR-b), Fms-like tyrosine kinase 3 (Flt-3), c-Kit and fibroblast growth factor receptor 1 (FGFR-1), was used to treat a type of kidney cancer called renal cell carcinoma (RCC) and a type of liver cancer called hepatocellular carcinoma (HCC). It might be used to treat other types of cancer as part of a research trial (Phase Ⅱ–Ⅲ clinical trial). Meanwhile, it also has inhibitory activity for which the receptors are responsible for blood vessel renascence.

The most common adverse reactions (more than 20%) related to sorafenib in patients with HCC or RCC are fatigue, weight loss, rash/desquamation, hand-foot skin reaction, alopecia, diarrhea, anorexia, nausea and abdominal pain.

In the present study, we evaluated the inhibitory effect of a MAPK inhibitor sorafenib (in comparison with albendazole) on *E. multilocularis*. The results will be useful for further therapeutic application of this kind of drug in treating AE.

1 Materials and Methods

1.1 Materials *E. m ultilocularis* vesicles were obtained from an experimentally infected KM mouse, and cultured as described by Hemer *et al* [8]. Reuber rat hepatocytes were kindly provided by the Parasitology Laboratory, Tottori University. Sorafenib (systematic name: 4-[4-[[4-chloro-3-(trifluoromethyl) phenyl]carbamoylamino] phenoxy]-N-methyl-2-pyridinecarboxamide. Formula: $C_{21}H_{16}ClF_3N_4O_3$. Molecular mass: 464.8) was purchased from Cayman, USA. The chemical structure of sorafenib was shown in Fig. 1.



Systematic name: 4-[4-[[4-chloro-3-(trifluoromethyl) phenyl] carbamoylamino] phenoxy] -N-methyl-2-pyridinecarboxamide, formula: $C_{21}H_{16}ClF_3N_4O_3$, molecular mass: 464.8.

Fig. 1 Chemical structure of sorafenib

1.2 Reuber rat hepatocyte cultivation Reuber rat hepatocytes (stored in liquid nitrogen) were thawed at 37 ℃ and cultured with RPMI-1640 (with 10% FBS, 100 U penicillin G and 100 mg/L streptomycin sulphate) at 37 ℃, 5% CO_2 one week before use.

1.3 Co-cultivation of metacestode vesicles with host hepatocytes Metacestode vesicles were obtained from previously infected mice and 1 ml of metacestodes were cultured in 50 ml DMEM medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS), 100 U/ml penicillin G, 100 mg/L streptomycin sulphate) with 5×10^6 rat hepatocytes. These co-cultures were incubated in culture flasks at 37 ℃ and 5% CO_2, with medium changes once a week. Splitting of cultures was carried out when a total metacestode vesicles volume of 15 ml was exceeded. Cysts were used for experimental procedures when they reached diameters of 2–4 mm.

1.4 Drug treatment of metacestode cysts Morphologically intact metacestode vesicles with a minimal diameter of 2–4 mm were manually picked from co-culture and washed with PBS. Cysts were allocated into four groups: albendazole-treated group (positive group), sorafenib-treated group (experimental group), DMSO-treated group (solvent group) and PBS group (negative control) (25–30 cysts/well), and were incubated in 24-well plates in the presence of culture medium (DMEM, 10% FCS, antibiotics). Albendazole and sorafenib were dissolved in DMSO (1∶9 dissolved in PBS) and used at concentrations 10 μmol/L and 30 μmol/L respectively. Experiment was performed triplicate per sample. The effect of drugs to cysts was evaluated by inhibition rate as following formula:

Inhibition rate = number of cysts that germinal layer damaged / total number of cysts used in experiment × 100%

1.5 Observation of cysts morphological change Cyst samples in sorafenib-treated group (Experimental group) and negative group (PBS group) were prepared and their morphology was observed under light microscopy. For inspecting detailed morphological changes, scanning electron microscopy (SEM) analysis

was conducted. The samples were washed several times with deionized water and fixed with 3% glutaraldehyde for 24 h at 4 ℃. Wash samples 3 times with deionized water and fixed with 1% osmium tetroxide. The samples were washed 3 times with deionized water and dehydrated gradually by sequential incubations in 70%, 80%, 90% and 100% ethanol, and finally immersed in 2-methyl-2-propanol and stored in a freezer until frozen. They were then sputter-coated with gold and observed on a scanning electron microscope.

1.6 Statistical analyses The data were analyzed using one-way ANOVA followed by a multiple comparison Tukey's test. A value of $P < 0.05$ was considered significant.

2 Results

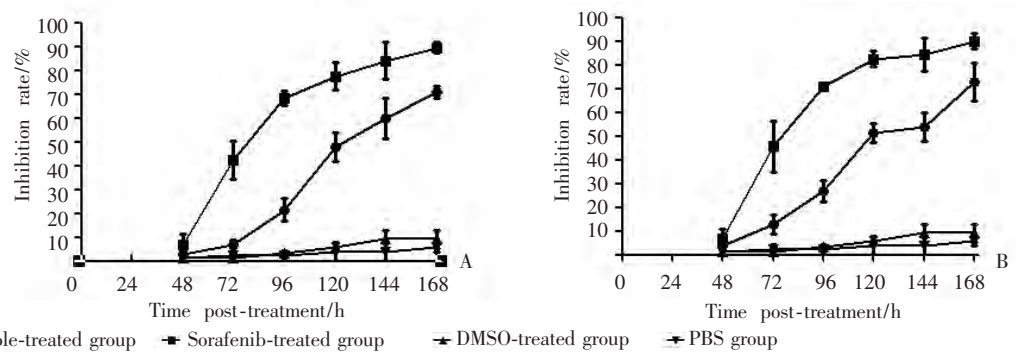
2.1 Inhibition of the cyst As shown in table 1, the inhibitive effects of sorafenib on cysts were observed at 48, 72, 96, 120, 144 and 168 h post-treatment and the inactivation rate were 6.6%, 42.4%, 68.5%, 77.4%, 84.0% and 89.5% at a concentration of 10 μmol/L, and were 7.1%, 45.6%, 70.9%, 82.6%, 84.0% and 89.9% at a concentration of 30 μmol/L, respectively, while in the same observed time point, the inhibitive effects by albendazole were 2.6%, 6.6%, 21.4%, 47.8%, 59.9% and 70.6% at a concentration of 10 μmol/L, and were 3.8%, 12.7%, 27.0%, 51.4%, 54.0% and 73.0% at a concentration of 30 μmol/L, respectively. Beginning from 72 h post-treatment, there were significant differences between sorafenib and other groups including albendazole (Table 1). In both of positive group (albendazole-treated group) and experimental group (sorafenib-treated group), the inhibitive effect was most likely to be time-dependent (Fig. 2A and B). DMSO and PBS showed less toxic to the cysts.

2.2 Morphological change of cysts under light microscope By light microscopy, the morphological changes of the cysts in experiment group (sorafenib-treated group) and negative group (PBS group) were observed. No observable collapse of the germinal layer was seen in negative group (Fig. 3A), while in sorafenib-treated group, germinal layer fell off from laminated layer and germinal cells gathered to the center of cyst (Fig. 3B) formed a'total solar eclipse'like shape.

Table 1 Inhibition rate of cysts in different time-points treated by drugs at concentrations 10 and 30 μmol/L respectively

Group	48 h	72 h	96 h	120 h	144 h	168 h
Albendazole-treated group						
10 μmol/L	2.6%	6.6%	21.4%	47.8%	59.9%	70.6%
30 μmol/L	3.8%	12.7%	27.0%	51.4%	54.0%	73.0%
Sorafenib-treated group[a]						
10 μmol/L	6.6%	42.4%	68.5%	77.4%	84.0%	89.5%
30 μmol/L	7.1%	45.6%	70.9%	82.6%	84.0%	89.9%
DMSO-treated group	1.2%	1.2%	3.5%	5.8%	9.1%	9.1%
PBS group	1.2%	2.4%	2.4%	3.6%	3.6%	6.0%

Note: a, Beginning from 72 h post-treatment, the inhibition rates of sorafenib-treated group were significantly different from that of other groups ($P < 0.05$)

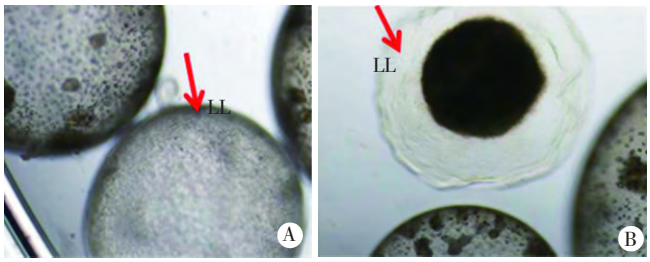


The inhibition rate indicates the mean value of 3 replicates within a group and the short bar is the standard deviation within a group.

Fig. 2 Effects of drugs at 10 μmol/L (A) and 30 μmol/L (B) at different times post-treatment

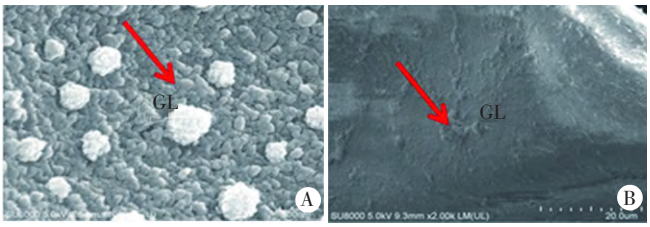
2.3 Ultrastructural changes of cysts under SEM

Ultrastructural changes of the germinal and laminated layers in cysts were observed by SEM (Fig. 4). Cysts treated by DMSO showed intact structure in germinal layer (without observable collapse of the germinal layer mainly comprised by germinal cells) (Fig. 4A). Meanwhile, ultrastructural observation in sorafenib-treated cysts, which showed morphological changes by light microscopy, showed significant morphological alteration-overall damage of germinal cells and thus the collapse of germinal layer (Fig. 4B).



A: In DMSO-treated group, no observable collapse of the germinal layer from laminated layer (LL) was observed; B: In sorafenib-treated group, germinal layer (GL) fell off from laminated layer and germinal cells gathered to the center of systs formed a 'total solar eclipse' like shape.

Fig. 3 Representative images of *E. multilocularis* cysts treated/untreated with sorafenib by light microscopy



A: Cysts treated by DMSO showed intact structure in germinal layer (GL) (without observable collapse of the germinal layer); B: Ultrastructural alterations in cysts treated by sorafenib showed significant morphological alteration-overall damage of germinal cells and thus the collapse of germinal layer.

Fig. 4 Representative images of *E. multilocularis* cysts treated/untreated with sorafenib by scanning electronic microscopy

3 Discussion

Sorafenib, a small-molecule anti-cancer compound[16], is a novel multi-kinase inhibitory drug for the treatment of primary kidney cancer (approved by FDA and SFDA) and advanced primary liver cancer (approved by FDA). Sorafenib mainly targets Raf (Raf-1 and B-Raf) kinase and a vascular endothelial growth factor receptor (VEGFR) and thus inhibits tumor cell proliferation and tumor angiogenesis[17].

E. multilocularis metacestode, which is called as cancer of parasites, performs a cancer-like proliferation in the liver of intermediate host. The metacestodes share some features to malignant tumors, such as the capacity for continuous cell proliferation, the induction of angiogenesis and the capacity of metastasis formation. These similarities suggested that anti-proliferative compounds could also affect *E. multilocularis* metacestodes[18]. Studies have been carried out to evaluate the effect of anti-cancer drugs on *E. multilocularis* [8, 19-21]. This opens the door to tap the potential of anticancer drugs on anti-echinococcosis.

In the present study, both albendazole and sorafenib exhibited high inhibitive effect on cultured vesicles, even at a low concentration (10 μmol/L) (Table 1, Fig. 2 A and B). However, compared with albendazole, the anti-cancer inhibitor sorafenib showed higher effect in inhibiting *Echinococcus* cysts all the time during testing. One week post-treatment, sorafenib showed 89.5% and 89.9% inhibition rate on cysts at 10 μmol/L and 30 μmol/L, respectively. Albendazole is a suspension form in DMSO solvent, while sorafenib absolutely dissolved in DMSO. It appears that sorafenib is easier to be absorbed into cysts and acts early and fast (Table 1, Fig. 2 A and B).

Although the action mechanism of albendazole on *Echinococcus* is unknown, one of its inhibiting functions on the parasite has been revealed. Albendazole can cause degenerative alterations in the intestinal cells of nematode by binding to the colchicine-sensitive site of tubulin, thus inhibiting its polymerization or assembly into microtubules. The loss of the cytoplasmic microtubules leads to impaired uptake of glucose at the larval and adult stages of the susceptible parasites, and depletes their glycogen stores.

Sorafenib targets multiple kinase (Raf-1 and B-Raf) and VEGFR, which plays a very important role in signal transduction associated with cell proliferation and blood vessels regeneration. From the morphological study, falling-off of the germinal layer from laminated layer (Fig. 3), overall damage of germinal cells and the collapse of germinal layer (Fig. 4) were observed. Combined with the higher cysts inhibition rate of

sorafenib, it might be speculated that sorafenib blocked more than one factors/transducers responsible for cell proliferation and parasite development, and thus contributed to a higher effect of sorafenib on *Echinococcus* in this study. Another important fact to consider regarding sorafenib activity *in vivo* is that it can stop the cancer cells developing new blood vessels. In fact, when *E. multilocularis* metacestodes are developing in host tissues (usually in liver and abdominal cavity), new blood vessels normally generate around the focus. Sorafenib is possibly able to block function of VEGFR during *Echinococcus* infection, which reduces supply of oxygen and nutrients to them, thereby causing the shrinking or stopping growth of cysts.

Since sorafenib has been approved for the treatment of primary kidney cancer and primary liver cancer clinically, and has performed well in Phase **III** trials, the progress of its application in human echinococcosis should be accelerated once the *in vivo* (mouse) efficacy is confirmed. Recently, our research group planned to evaluate *in vivo* efficacy of sorafenib on *Echinococcus*, including evaluation of its effect on regeneration of new blood vessels surrounding *Echinococcus* metacestodes.

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