Identification of Functional MKK3/6 and MEK1/2 Homologs from *Echinococcus granulosus* and Investigation of Protoscolecidal Activity of Mitogen-Activated Protein Kinase Signaling Pathway Inhibitors *In Vitro* and *In Vivo*

Chuanshan Zhang,^{a,b} Jing Li,^c Tuerganaili Aji,^d Liang Li,^a Xiaojuan Bi,^a Ning Yang,^a Zhide Li,^b Hui Wang,^a Rui Mao,^e Guodong Lü,^a Yingmei Shao,^d Dominique A. Vuitton,^f Hao Wen,^d Renyong Lin^{a,b}

^aState Key Laboratory of Pathogenesis, Prevention, and Treatment of Central Asian High Incidence Diseases, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China

^bXinjiang Key Laboratory of Echinococcosis, WHO-Collaborating Centre, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China

^cBasic Medical College, Xinjiang Medical University, Urumqi, Xinjiang, China

^dDepartment of Hepatic Echinococcosis and Hepatobiliary Surgery, Digestive and Vascular Surgery Centre, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China

^eDepartment of Radiation Oncology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China

^fWHO-Collaborating Centre for the Prevention and Treatment of Human Echinococcosis, Department of Parasitology, University Bourgogne Franche-Comté (EA 3181) and University Hospital, Besançon, France

ABSTRACT Cystic echinococcosis is a zoonosis caused by the larval stage of *Echinococcus granulosus sensu lato*. There is an urgent need to develop new drugs for the treatment of this disease. In this study, we identified two new members of mitogen-activated protein kinase (MAPK) cascades, MKK3/6 and MEK1/2 homologs (termed EgMKK1 and EgMKK2, respectively), from *E. granulosus sensu stricto*. Both EgMKK1 and EgMKK2 were expressed at the larval stages. As shown by yeast two-hybrid and coimmunoprecipitation analyses, EgMKK1 interacted with the previously identified Egp38 protein but not with EgERK. EgMKK2, on the other hand, interacted with EgERK. In addition, EgMKK1 and EgMKK2 displayed kinase activity toward the substrate myelin basic protein. When sorafenib tosylate, PD184352, or U0126-ethanol (EtOH) was added to the medium for *in vitro* culture of *E. granulosus* protoscoleces (PSCs) or cysts, an inhibitory and cytolytic effect was observed via suppressed phosphorylation of EgMKKs and EgERK. Nonviability of PSCs treated with sorafenib tosylate or U0126-EtOH, and not with PD184352, was confirmed through bioassays, i.e., inoculation of treated and untreated protoscoleces into mice. *In vivo* treatment of *E. granulosus sensu stricto*-infected mice with sorafenib tosylate or U0126-EtOH for 4 weeks demonstrated a reduction in parasite weight, but the results did not show a significant difference. In conclusion, the MAPK cascades were identified as new targets for drug development, and *E. granulosus* was efficiently inhibited by their inhibitors *in vitro*. The translation of these findings into *in vivo* efficacy requires further adjustment of treatment regimens using sorafenib tosylate or, possibly, other kinase inhibitors.

KEYWORDS *Echinococcus granulosus*, MAPK kinases, chemotherapy, cystic echinococcosis, inhibitor

Cystic echinococcosis (CE), caused by the larval stage of the cestode parasite *Echinococcus granulosus sensu lato*, affects both humans and a wide range of mammalian intermediate hosts and remains an increasing public health and socio-economic concern in many areas of the world (1). Its life cycle involves dogs and other

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Address correspondence to Hao Wen, Dr.wenhao@163.com, or Renyong Lin, renyong_lin@sina.com.

C.Z., J.L., and T.A. contributed equally to this article.

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canids as definitive hosts for the intestinal tapeworm and domestic and wild ungulates as intermediate hosts for the tissue-invading metacestode, which is the larval stage of the tapeworm (2). Currently, treatment of CE involves curative surgical removal of the entire cyst, partial removal of the cyst combined with protoscoleces, nonsurgical interventional methods such as PAIR (puncture, aspiration, injection, reaspiration), and anti-infective treatment based on the use of benzimidazole (BZ) carbamate derivatives, albendazole (ABZ), and mebendazole (3, 4), alone or in combination with interventions. However, except for complete cyst resection, none of the current treatments is fully satisfactory, especially because dissemination of the protoscoleces (PSCs) present in the cyst may generate new active cysts, and novel therapeutic strategies centered on new target identification are urgently needed.

As parasitic helminths, the various species of *E. granulosus sensu lato* depend on host molecular signals for their growth and development (5–7). The microenvironment for *Echinococcus* spp. involves some host-derived cytokines and hormones, which are thought to bind to parasite receptors and in turn influence the parasite's survival through their impacts on the relevant evolutionarily conserved signaling pathways within the parasite (8). For example, host epidermal growth factor (EGF), insulin, and transforming growth factor β (TGF- β) bind to the surface receptors of the metacestode and then activate several downstream components of the metacestode mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and TGF- β /BMP signaling pathways (9–12). This suggests the presence of signaling pathways by which *E. granulosus sensu lato* can detect host signals and respond to them in a way that presumably enhances parasite development and, ultimately, survival within its host. Investigating signaling pathways in *E. granulosus sensu lato* may therefore allow us to better understand how these pathways are affected by existing drugs and/or could serve as targets for new therapeutic agents against CE.

The MAPK pathway is an evolutionarily conserved signal transduction pathway that utilizes a series of protein kinases to transmit extracellular signals such as cytokines, hormones, and growth factors. The activation of a MAPK cascade occurs in a module of three consecutive phosphorylation events; i.e., after a previous stimulus, each MAPK is phosphorylated by upstream MAPKs. A MAPK module comprises a mitogen-activated protein kinase kinase kinase (MAP3K) that phosphorylates and activates a mitogen-activated protein kinase kinase (MAP2K), which in turn phosphorylates and activates a MAPK. Activated MAPKs phosphorylate and regulate their substrates, most of which are transcription factors, membrane and cytoplasmic proteins, as well as other protein kinases, triggering downstream stress-related responses. Several individual MAPK modules have been identified, two of which, the p38 MAPK and the c-Jun NH₂-terminal kinase (JNK1/2) pathways, are mainly activated in response to diverse stress stimuli, and another of which, the Erk1/2/5-like MAPK pathway, plays an important role in regulating cellular responses to growth factors (13–15). The Erk-like pathway comprises G-protein-coupled receptors (GPCRs); growth factor receptor-bound protein 2 (Grb2); small GTPases of the Ras family; three Raf-like MAPK kinase kinases (MAPKKKs) (Raf-1, A-Raf, and B-Raf); the three MAPK kinases (MAPKKs) MEK1, MEK2, and MEK5 (MAP/Erk kinase 1/2/5); as well as the related MAPKs Erk1, Erk2, and Erk5. MEK1/2/5 specifically recognizes Erk-like MAPKs, harboring a T-E-Y motif in the activation loop, while MKK3/6—as well as MKK4/7-like MAPKKs are specific for p38 (T-G-Y) and JNK-like (T-P-Y) MAPKs, respectively (15, 16). Recent evidence indicates that therapies targeted toward MAPK pathway components stand out as potential alternative drugs in many tumor types (17–19).

In recent years, several members of the MAPK pathway have been identified in *Echinococcus* spp. (20, 21). One member of the novel receptor tyrosine kinases, EmER of the human parasite *Echinococcus multilocularis* (isolate H-95), was found to be functionally responsive to EGF stimulation from the host (11). Apart from the two MAPKs EmMPK1 (Erk-like) and EmMPK2 (p38-like), only one member of the MAPKKK family, EmRaf (Raf-like kinase); two members of the MAPKK family, EmMKK1 (MKK3/6-like kinase) and EmMKK2 (MEK1/2-like kinase); and EmRas (Ras-like kinase) were identified

from *E. multilocularis* (isolate H-95, K188, or JAVA) (22–25). A recent study demonstrated that human EGF-mediated EGF receptor (EGFR)/extracellular signal-regulated kinase (ERK) signaling in the parasite promotes germinal cell proliferation in *E. multilocularis* that results in stimulated growth and development of metacystode larvae (26). In addition, inhibition of signaling using either the EGFR inhibitors CI-1033 and BIBW2992, the MEK/ERK inhibitor U0126, or the MKK/p38 inhibitors ML3403 and SB202190 impairs germinal cell proliferation and larval growth. The aim of our study was to identify a protoscolecidal effect of inhibitors of the MAPK pathway on the larval stage of *E. granulosus sensu stricto*, the species used for our experiments. In this work, we describe the first characterization of EgMKK1 and EgMKK2, which adds two new members to the *E. granulosus sensu stricto* MAPKK family. We defined their expression levels and localization at the larval stages, protein interaction properties, and functional kinase activities. Furthermore, we investigated the inhibitory and cytolytic efficacy of the MAPK pathway inhibitors *in vitro* and in mice infected with *E. granulosus sensu stricto*.

RESULTS

Cloning and characterization of *egmkk1* and *egmkk2* cDNAs. To experimentally identify an *E. granulosus sensu stricto* MEK1/2 homolog, specific primers were designed and used to amplify sequences by PCR from *E. granulosus sensu stricto* cDNA. Sequence analysis of amplified cDNA revealed two MAPKK homologs, *egmkk1* and *egmkk2*.

The full-length EgMKK1 reading frame (GenBank accession no. [JN573355.1](#)) comprised 1,017 bp and coded for a protein of 338 amino acids, EgMKK1, with a calculated molecular mass of 37,959 Da. The protein contained a typical serine/threonine protein kinase domain (data not shown). In addition to the conserved motif of protein kinases (substrate binding pocket), this region included an HRDL/VKxxN motif (catalytic loop). Based on sequence homology analysis, EgMKK1 shared significant sequence identity with EmMKK1 (identity, 99%). All these data confirmed that *E. granulosus sensu stricto* expresses the same MKK3/6 subgroup as *E. multilocularis* (25).

The full-length EgMKK2 reading frame (GenBank accession no. [HQ677766.1](#)) comprised 1,563 bp and coded for a protein of 520 amino acids, EgMKK2, with a calculated molecular mass of 57,016 Da. The protein contained the typical features of MEK1/2, including a typical serine/threonine protein kinase domain (data not shown). In addition to the conserved motif of protein kinases (substrate binding pocket), this region included an HRDL/VKxxN motif (catalytic loop). By aligning homologous sequences, EgMKK2 shared significant sequence identity with the MEK1/2 ortholog EmMKK2 from *E. multilocularis* (identity, 98%), and only 9 of the 520 residues were different (25).

Expression patterns of EgMKK1 and EgMKK2. Quantitative real-time PCR (qRT-PCR) experiments were performed to determine the relative abundances of EgMKK1 and EgMKK2 transcripts in larval developmental stages, which included nonactivated PSCs, pepsin-activated PSCs (mimicking the natural process of PSC activation and the stage of invasion into the definitive host in the parasite life cycle), and cysts after *in vitro* cultivation. As shown in Fig. 1A, the EgMKK1 and EgMKK2 transcripts were approximately 10-fold more abundant in cysts than in nonactivated and activated PSCs. In addition, EgMKK1 exhibited lower levels in nonactivated and activated PSCs. These data indicated that EgMKK1 and EgMKK2 were clearly detectable but presented different expression levels at all larval stages.

Identification and immunolocalization of EgMKK1 and EgMKK2. Expression at the protein level was verified at larval stages by immunoblot analysis using serum obtained by immunization of rabbits with the purified EgMKK1 and EgMKK2 recombinant proteins. As shown in Fig. 1C, anti-EgMKK1 antibodies recognized recombinant His-tagged EgMKK1; immunoblotting detected a single protein in extracts from non-activated PSCs, pepsin-activated PSCs, and *in vitro*-cultivated cysts. Similarly, the anti-EgMKK2 antibody recognized recombinant His-tagged EgMKK2 and revealed a protein in nonactivated PSCs, pepsin-activated PSCs, and cyst stages. The sizes of native EgMKK1 (37,959 Da) and EgMKK2 (57,016 Da) were in agreement with the deduced amino acid sequences from the open reading frames (ORFs).

FIG 1 mRNA and protein levels of EgMCK1 and EgMCK2 expression in the larval stages. (A) Quantitative PCR analysis of EgMCK1 and EgMCK2 mRNA levels. Quantitative real-time PCR analysis was performed on total RNA isolated from nonactivated PSCs, activated PSCs, and hydatid cysts. Shown is a bar graph comparing the relative expression levels (means $\pm$ SD) of EgMCK1 and EgMCK2 normalized to the levels of Egelp. The relative expression value was averaged from triple samples. (B and C) Immunoblot detection of the EgMCK1 (B) and EgMCK2 (C) proteins. Cell lysates of nonactivated PSCs (lane 1), activated PSCs (lane 2), *in vitro*-cultivated cysts (lane 3), and *Escherichia coli* BL21 expressing a His-EgMCK1 fusion protein or a His-EgMCK2 fusion protein (lane 4) were separated on a 12% SDS-polyacrylamide gel. Protein marker sizes are indicated to the left (M).

In situ localizations of EgMCK1 and EgMCK2 were performed on PSCs and cysts by immunofluorescence analysis. In PSCs, EgMCK1 was predominantly expressed in the parenchymal region, tegument tissue, and hooks (Fig. 2E to H). In addition, EgMCK2 was seen mostly in the suckers and tegument of the PSCs. However, EgMCK2 expression was stronger than EgMCK1 expression in the tegument and suckers (Fig. 2M to P). Localizations of EgMCK1 and EgMCK2 were also observed in the germinal layer of hydatid cysts (Fig. 2I to L and Q to T). In both cases, EgMCK1 and EgMCK2 were seen in the cytoplasm of PSCs and cysts. There was nonspecific staining of the laminated layer by EgMCK2 antisera. No fluorescence signals were detected in the negative controls (Fig. 2A to D).

***In vitro* interaction of EgMCK1 and EgMCK2 with other MAPK pathway members.** The yeast two-hybrid system was used to study interactions of EgMCKs with the downstream partners of the MAPK cascade. As shown in Fig. 3A and Table 1, EgMCK1 interacted strongly with Egpp38 but not with EgERK under high-stringency conditions, and EgMCK2 interacted weakly with EgERK but not with Egpp38 under low-stringency conditions. Furthermore, double transformants of EgMCK1 fused to the Gal4 activation domain (Gal4-AD) and the Gal4 DNA binding domain (Gal4-BD) or of EgMCK2 grew

FIG 2 Immunolocalization of the EgMKK1 and EgMKK2 proteins on parasite sections. Sections were labeled with purified EgMKK1-immunized rabbit sera (E to L), EgMKK2-immunized rabbit sera (M to T), and F(ab')₂ goat anti-rabbit antibody conjugated to Alexa 488 fluorochrome. Column I, phase-contrast images; column II, nuclear staining images (4',6-diamidino-2-phenylindole dihydrochloride [DAPI]); column III, anti-EgMKK1 or anti-EgMKK2 fluorescence images; column IV, nuclear staining (blue) image merged with anti-EgMKK1 or anti-EgMKK2 fluorescence (green). Negative controls (panels A to D) were incubated with rabbit preimmune sera. Panels E to H and M to P represent invaginated PSCs. Panels I to L and Q to T represent *in vitro*-cultivated hydatid cysts. Tg, tegument; H, hooks; su, suckers; GL, germinal layer; LL, laminated layer.

under low- or high-stringency conditions, respectively, indicating the ability of the *E. granulosus sensu stricto* MAPKK to form homodimers. Finally, EgMKK2 also interacted strongly with Eg14-3-3.2, which was not the case for EgMKK1 and Eg14-3-3.2.

For further verification of the interaction between EgMKK1, EgMKK2, EgERK, Egp38, and Eg14-3-3.2, coimmunoprecipitation (co-IP) experiments were performed. As shown in Fig. 3B and C, V5-tagged EgMKK1, V5-tagged EgMKK2, Myc-tagged EgERK, Myc-tagged Egp38, and Myc-tagged Eg14-3-3.2 could be stably and highly expressed in 293T cells, which was critical for the following coimmunoprecipitation experiment. The Myc-tagged Egp38 protein was detected in the precipitates from cell lysates cotransfected with both the EgMKK1 and Egp38 proteins. In addition, the Myc-tagged EgERK or Eg14-3-3.2 protein was detected in the precipitates from cell lysates cotransfected with both EgMKK2 and EgERK or the Eg14-3-3.2 protein (Fig. 3D and E). In summary, these results confirmed the interactions between EgMKK1 and Egp38 and between EgMKK2 and EgERK, as well as Eg14-3-3.2, in mammalian cells.

Kinase activity of EgMKK1 and EgMKK2. To demonstrate the kinase activity of EgMKK1 and EgMKK2, we expressed V5-tagged EgMKK1 or EgMKK2 in 293T cells and measured their activity *in vitro*. Immunoprecipitated EgMKK1 and EgMKK2 were assayed for their ability to phosphorylate the substrate protein myelin basic protein

FIG 3 Interaction of EgMKK1 and EgMKK2 proteins with downstream signaling partners. (A) Yeast two-hybrid analyses. Translational fusions were produced between the Gal4 activation domain (pGADT7 vector) or the Gal4 DNA binding domain (pGBKT7 vector) and EgMKK1, EgMKK2, EgERK, Egp38, or Eg14-3-3.2. Double transformants of yeast strain Y2HGold were assessed for colony growth after 3 to 5 days of incubation. Growth of a representative number of double transformants on selection plates is shown. As positive (pGBKT7-p53 $\times$ pGADT7-T) and negative (pGBKT7-Lam $\times$ pGADT7-T) controls, the recommended plasmids of the Matchmaker kit (Clontech, Japan) were used. (B to E) Co-IP experiments. (B and C) 293T cells were cotransfected with expression vectors for V5-tagged EgMKK1 and Myc-tagged Egp38 (lane 3) as well as V5-tagged EgMKK2 and Myc-tagged EgERK (lane 2) or Eg14-3-3.2 (lane 6), and whole-cell lysates were directly analyzed (input) (B) or precipitated with V5 antibody (co-IP) (C) and subjected to immunoblot analysis with the indicated antibody. Extracts from V5-EgMKK1 or EgMKK2 and pCMV-Myc-N coexpression in 293T cells were used as negative controls. (D and E) The immunoblot was probed with anti-Myc antibody to detect interacting proteins. (B, D, and E) Lanes 1 and 5, pCMV-Myc-N; lane 2, V5-tagged EgMKK2 plus Myc-tagged Egp38; lane 3, V5-tagged EgMKK1 plus Myc-tagged Egp38; lanes 4 and 7, 293T cells; lane 6, V5-tagged EgMKK2 plus Myc-tagged Eg14-3-3.2. (C) Lane 1, V5-tagged EgMKK2 plus pCMV-Myc-N; lane 2, V5-tagged EgMKK2 plus Myc-tagged Egp38; lane 3, V5-tagged EgMKK1 plus Myc-tagged Egp38; lane 4, V5-tagged EgMKK1 plus pCMV-Myc-N; lane 5, V5-tagged EgMKK2 plus Myc-tagged Eg14-3-3.2; lane 6, 293T cells. Protein marker sizes are indicated to the left (M).

TABLE 1 Summary of EgMKK1 and EgMKK2 interaction with downstream signaling partners analyzed by yeast two-hybrid assay

Vector	Growth with interaction partner ^a	
	pGBKT7-p53	pGBKT7-lamC
pGADT7-T	++	—
pGADT7-EgMKK1	++	—
pGADT7-EgMKK2	\	+
pGADT7-EgERK	—	+
pGADT7-Egp38	++	—
pGADT7-Eg14-3-3.2	—	++

^aNo growth (—) or growth under low-stringency (+) or high-stringency (++) conditions after being cotransformed into yeast strain Y2HGold and incubated for 3 to 5 days. \, not determined.

(MBP). Both EgMKK1 and EgMKK2 were expressed and immunoprecipitated, as judged by immunoblot analysis (Fig. 4A). The results indicated that EgMKK1 and EgMKK2 displayed the enzymatic activity and could phosphorylate the MBP substrate (Fig. 4B).

Effects of MAPK pathway inhibitors on *E. granulosus sensu stricto* PSC viability *in vitro*. As previous and present studies have fully confirmed that *E. granulosus sensu stricto* expressed EgER, EgMKK1, EgMKK2, EgERK, and Egp38, five members of the MAPK pathway, in the larval stages, we performed specific experiments to test the role of the MAPK pathway in the growth and development of *E. granulosus sensu stricto* and potential effects of inhibitors (sorafenib tosylate, PD184352, and U0126-ethanol [EtOH]) using an *in vitro* cultivation system. As shown in Fig. 5, the sensitivity of PSCs to damage by the three inhibitors was dose dependent. PSC viabilities were $84.8\% \pm 1.3\%$, $49.3\% \pm 1.8\%$, $38.7\% \pm 5.3\%$, $16.8\% \pm 2.2\%$, and $17.0\% \pm 4.6\%$ after incubation with 25, 50, 100, 200, and 400 μM sorafenib tosylate for 2 days, respectively. After 3 days, all PSCs were dead in medium containing more than 50 μM sorafenib tosylate. For the

FIG 4 Kinase activities of EgMKK1 and EgMKK2 proteins. (A) pcDNA3.1/V5-EgMKK1 (lane 2) and -EgMKK2 (lane 3) vectors were transfected into 293T cells and expressed as V5 fusion proteins. Whole-cell lysates were precipitated with V5 antibody (co-IP) and subjected to immunoblot analysis with anti-V5 antibody. Extracts from 293T cells transfected with the pcDNA3.1/V5 vector were used as negative controls (lane 1). (B) V5-tagged EgMKK1 (lane 3) or EgMKK2 (lane 2) was determined from extracts by immunoprecipitation with a V5 antibody followed by an *in vitro* kinase assay with MBP as a substrate. Extracts from NIH 3T3 cells were used as positive controls (lane 4). Protein marker sizes are indicated to the left (M).

FIG 5 Effects of MAPK pathway inhibitors on *E. granulosus sensu stricto* PSC viability *in vitro*. (A) Loss of viability of *E. granulosus sensu stricto* PSCs at 1, 25, 50, 100, 200, and 400 μ M for sorafenib tosylate (a potent and selective inhibitor of Raf) over 4 days; (B) loss of viability of *E. granulosus sensu stricto* PSCs at 1, 25, 50, 100, 200, and 400 μ M for PD184352 (a potent and selective inhibitor of MEK) over 4 days; (C) loss of viability of *E. granulosus sensu stricto* PSCs at 1, 25, 50, 100, 200, and 400 μ M for U0126-EtOH (a specific inhibitor of MEK) over 8 days. Albendazole at 1 and 400 μ M was used as a reference anti-infective agent. PSCs incubated in the absence of an inhibitor (supplemented with 0.2% DMSO) were used as negative controls. Each point represents the mean percentage of viable PSCs from three different experiments. Asterisks indicate a statistically significant difference ($P < 0.05$) from the corresponding control.

PD184352 inhibitor at concentrations of 200 and 400 μ M, the viabilities of PSCs were $28.0\% \pm 6.01\%$ and $2.31\% \pm 0.5\%$ after 4 days of incubation, respectively. The percentages of viable PSCs decreased to $92.0\% \pm 2.3\%$ and $11.6\% \pm 2.2\%$ after 4 days of incubation with 200 and 400 μ M U0126-EtOH, respectively. After 8 days, PSC viability was $18.9\% \pm 7.5\%$ for 200 μ M U0126-EtOH. The 50% inhibitory concentration (IC_{50}) values for sorafenib tosylate, PD184352, and U0126-EtOH were 26.6 μ M, 165.9 μ M, and 221.9 μ M, respectively. In contrast, control PSCs incubated in the absence of an

inhibitor but with a similar concentration of dimethyl sulfoxide (DMSO) (final concentration, 0.2%) were not altered and remained viable ($99.2\% \pm 0.2\%$ and 93.8% after 8 and 30 days of incubation, respectively) (Fig. 5; see also Fig. S3 in the supplemental material). The viabilities of PSCs treated with ABZ remained high for all durations of the short-term *in vitro* experiments; they were $96.1\% \pm 2.7\%$ and $93.3\% \pm 2.6\%$ for incubation with 1 and 400 μM ABZ after 8 days, respectively. At 30 days of culture, the viability of PSCs incubated with ABZ was still $27.9\% \pm 2.5\%$, while the viability of those incubated with all 3 inhibitors was less than 2.2% (Fig. S3). These results indicated that sorafenib tosylate emerged as the most promising compound, showing far faster and higher killing efficacy than the reference anti-infective agent in CE, ABZ, against *E. granulosus sensu stricto* PSCs at a concentration of 25 μM .

Effects of MAPK pathway inhibitors on morphology and ultrastructures of *E. granulosus sensu stricto* in vitro. To analyze the effects of specific inhibitors of the MAPK pathway on *E. granulosus sensu stricto* full metacestodes (Fig. 6A), we first incubated PSCs or cysts for 30 days in the presence of 25 μM sorafenib tosylate, 100 μM PD184352, and 100 μM U0126-EtOH, and we then tested the phosphorylation levels of EgMKKs and EgERK. As shown in Fig. 6B and D, the basal levels of EgMKK and EgERK phosphorylation in both larval stages were downregulated under these conditions, while the overall expression level of the parasite's β -actin was not affected.

We then observed the effects of *in vitro* inhibitor treatment by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). As shown in Fig. 6C and E, PSCs treated with sorafenib tosylate or U0126-EtOH presented a disintegration or absence of tegument with their microtriches, a large vacuolization of the cytoplasm in many cells, a high degree of chromatin condensation of some nuclei, and the presence of some residual lamellar bodies. PD184352 treatment resulted in the truncation and loss of microtriches, the formation of residual lamellar bodies, and chromatin condensation, while in control cultures, no damage could be seen in parasite tissue during the whole incubation period. Furthermore, *E. granulosus sensu stricto* cysts treated with sorafenib tosylate presented a complete loss of cells in the germinal layer of cysts. In addition, PD184352 or U0126-EtOH treatment led to partial wrinkling and separation of the germinal layer from the inner surface of the laminated layer. These results suggested that the inhibitors of the MAPK pathway could impair the activation of the parasite's MKK and ERK kinase, leading to significant morphological and structural alterations in larval stages.

Viability assessment of *in vitro* inhibitor-treated *E. granulosus sensu stricto* PSCs by a bioassay in mice. In order to determine whether the MAPK pathway inhibitors had a truly killing effect on the protoscoleces, *E. granulosus sensu stricto* PSCs treated for 30 days *in vitro* were tested by a mouse bioassay (Table 2; see also Fig. S3 in the supplemental material). PSC viabilities were $1.54\% \pm 0.39\%$, $1.03\% \pm 0.14\%$, and $1.70\% \pm 0.45\%$ in the presence of 25 μM sorafenib tosylate, 100 μM PD184352, and 100 μM U0126-EtOH, respectively, before intraperitoneal injection, whereas $93.8\% \pm 4.28\%$ of the protoscoleces were still viable on day 30 after treatment with DMSO alone as a control (final concentration, 0.2%). Six months after inoculation of parasite tissue, the average weight of the cysts that developed from the injected protoscoleces was 7.4 ± 6.0 g for the control group (DMSO). In the group of mice infected with PD184352-treated PSCs, the average weight was 1.1 ± 1.0 g. However, mice inoculated with PSCs treated with sorafenib tosylate (25 μM) or U0126-EtOH (100 μM) for 30 days had no cyst tissue, indicating that these treatments achieved a complete protoscolecidal effect.

Preliminary assessment of inhibitor treatment in mice experimentally infected with *E. granulosus sensu stricto*. All infected animals involved in this study developed echinococcosis cysts in their abdominal cavities. Cysts weights were determined (Fig. 7A). After oral administration of 30 mg/kg of body weight sorafenib tosylate and 30 mg/kg U0126-EtOH to mice, the cyst weights (2.5 ± 0.5 g and 3.0 ± 0.5 g, respectively) were lower than those obtained in the control group (3.5 ± 0.6 g), but the difference was not statistically significant. Treatment with 60 mg/kg PD184352 did not have a dramatic effect on the parasite weight ($P > 0.05$).

FIG 6 *In vitro* effects of inhibitor treatments on *E. granulosus sensu stricto* PSCs and cysts by targeting MAPK pathway members for 30 days. (A) Model of the *E. granulosus* ERK/p38-like MAPK pathway. (B and D) Representative immunoblots of EgMKKs and EgERK revealed with heterologous antibodies against the phosphorylated forms of human MEK1/2 and ERK1/2. Shown are total protein extracts from PSCs (B) or hydatid cysts (D) cultured in the presence of medium containing DMSO (control) (lane 1), sorafenib tosylate (25 μ M) (lane 2), PD184352 (100 μ M) (lane 3), or U0126-EtOH (100 μ M) (lane 4). Actin was used as a loading control. (C) Ultrastructural changes of PSCs detected by TEM after incubation with either DMSO (control) or inhibitors. (E) Morphology changes of germinal layers from hydatid cysts detected by SEM after incubation with either DMSO (control) or inhibitors. (a to c) Control PSCs or cysts; (d to f) 25 μ M sorafenib tosylate treatment; (g to i) 100 μ M PD184352 treatment; (k to m) 100 μ M U0126-EtOH treatment. mt, microtriches; nu, nucleus; mu, muscle cells; cc, chromatin condensation; b, residual lamellar bodies; GL, germinal layer; LL, laminated layer. The white arrows point to local tissue magnification.

Some cysts were processed for SEM to assess the efficacy of the treatment. As shown in Fig. 7B, all cysts in the control group had no observable collapse of the germinal layer or changes in ultrastructure. The cysts that developed in mice treated with sorafenib tosylate exhibited a breakdown of the structural integrity of the germinal layer. However, only a wrinkling of the germinal layer was observed in mice treated with PD184352 or U0126-EtOH, and there was no dramatic effect on the morphology of the germinal layer. Thus, among the *in vivo* treatments targeting the parasite's MAPK pathway, only the use of the inhibitor sorafenib tosylate had an effect observable histologically with clear killing efficacy and, at the dosage used in the study, partially prevented cyst growth.

TABLE 2 Weights of parasites recovered from mice after a bioassay, following inoculation of *E. granulosus sensu stricto* PSCs treated *in vitro* with sorafenib tosylate, PD184352, or U0126-EtOH^a

Treatment	No. of infected mice/ total no. of mice	Mean cyst wt (g) $\pm$ SD
DMSO	5/5	7.4 $\pm$ 6.0
Sorafenib tosylate (25 μ M)	0/5	0
PD184352 (100 μ M)	5/5	1.1 $\pm$ 1.0
U0126-EtOH (100 μ M)	0/5	0

^aA control group was inoculated with *E. granulosus sensu stricto* PSCs treated with the solvent DMSO alone at the same concentration as that used in the experimental groups (0.2%). Data are shown as means $\pm$ standard deviations (SD) (5 mice per group).

DISCUSSION

MAPK kinases (MAPKKs), the intermediate downstream kinases in signal transduction cascades, are highly conserved serine/threonine-specific protein kinases present in all eukaryotes. MAPKKs have been studied extensively in a variety of organisms, including mammals, protozoa, bacteria, and helminths (27–30). They relay external signals and can ultimately lead to changes in the gene expression profiles. Moreover, MAPK pathway members can be used as drug targets in the treatment of cancer and bacterial and parasitic infections. However, the MAPKK gene had not yet been described in *E. granulosus*. This study characterized *E. granulosus sensu stricto* MAPKKs, including EgMKK1 and EgMKK2, and it confirmed that various compounds that are able to block this pathway could represent a potential therapeutic approach for CE treatment. Recently, several related members of the MAPK pathway have been found and characterized in *E. multilocularis*, including EmER, EmRas, EmRal, EmRaf, EmMPK1, EmMPK2, EmMKK1, and EmMKK2 (21).

In the present work, we describe the characterization of EgMKK1 and EgMKK2 from *E. granulosus sensu stricto*. Sequence alignment (blastp search) indicated that EgMKK1

FIG 7 *In vivo* treatment of secondary *E. granulosus sensu stricto*-infected BALB/c mice with MAPK pathway inhibitors. (A) Box plots indicating the distribution of cyst weights (grams) in the different treatment groups. Reductions in the weights of recovered parasites in relation to those in the control groups were achieved by treatment with 30 mg/kg sorafenib tosylate and 30 mg/kg U0126-EtOH, but they were not statistically significant (ns) ($P = 0.1994$ and $P = 0.5360$, respectively). (B) Representative images obtained by SEM of hydatid cysts from inhibitor-treated mice. (a and b) control cysts; (c and d) mice treated with 30 mg/kg sorafenib tosylate; (e and f) mice treated with 60 mg/kg PD184352; (g and h) mice treated with 30 mg/kg U0126-EtOH. GL, germinal layer; LL, laminated layer. The white arrows point to local tissue magnification. Each treatment group comprised 10 animals.

exhibits high homology with the MKK3/6 subgroup family from different species. The MKK3/6 orthologs from *Schistosoma mansoni*, CeSEK1, and the closely related MAPKK from *E. multilocularis*, EmMKK1, attained the highest alignment scores with EgMKK1. The second MAPKK factor identified in this study, EgMKK2, constitutes a typical member of its respective protein MEK1/2 subfamily. Our data indicate that EgMKK2 is a close homolog of *E. multilocularis* EmMKK2. In addition, EgMKK1 and EgMKK2 contain a typical serine/threonine kinase domain in the activation loop, which is typically present in this subclass of MAPKKs. Moreover, EgMKK1 and EgMKK2 were constitutively expressed concomitantly, at least in the larval stages (including larval vesicles, nonactivated PSCs, and activated PSCs). This is consistent with the important function of MAPKKs in cellular signaling events and reflects the situation in other animals where MAPKKs are also expressed in a wide variety of tissues and developmental stages (31).

Our results demonstrated that EgMKK2 significantly interacted with EgERK and Eg14-3-3.2. These data agree with previous studies that have reported interactions between EmMKK2 and EmMPK1 or Em14-3-3.2. In addition, we found a strong interaction between EgMKK1 and Egp38 that was inconsistent with previous data for *E. multilocularis* indicating no interaction between EmMKK1 and EmMPK2 (p38-like) (25). This phenomenon may be due to several amino acid changes that affect the binding characteristics of EgMKK1 and EmMKK1, respectively. Furthermore, a V5 fusion protein of EgMKK1 and EgMKK2 was able to phosphorylate MBP *in vitro*, thereby demonstrating that EgMKK1 or EgMKK2 is a functional protein kinase. Previous studies have also reported that EmMPK2 displays significantly higher basal activity (toward MBP and toward itself as a substrate) than p38 MAPK orthologs from humans (22). These data suggest that there are two separate subpathways in the larval stage of *E. granulosus sensu stricto*, including the Egp38 (EgMKK1) pathway and the EgERK (EgMKK2) pathway, which are known to be components of signaling cascades controlling cell proliferation, differentiation, and survival (32).

One important aspect of this study is the use of chemical probes to study parasitic helminth signaling pathways. Synthetic inhibitors provide an alternative chemical and biological approach to study gene function in these organisms (33, 34). MAPK pathway inhibitors have been developed as drugs for the treatment of patients, such as sorafenib tosylate, which is an oral multikinase inhibitor by a dual mechanism, through inhibition of Raf and Kit signaling, and/or of vascular endothelial growth factor (VEGF) receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) signaling, but not MEK- or Erk-like kinases (35–37). The other two inhibitors, PD184352 and U0126, are potent and selective inhibitors with specific activity against MEK1/2-like MAPKKs, and they do not exert effects on related MKK3/6- or MKK4/7-like MAPKKs (38). In parasites such as *Plasmodium falciparum*, *Trypanosoma brucei*, and *S. mansoni*, MAPK pathway members have long been investigated as potential drug targets (30, 39–42). In addition, the antiparasitic activities of sorafenib, PD184352, and U0126 were assessed previously (43–45). The Raf kinase inhibitor sorafenib has been shown to prevent trypanosome proliferation and the maturation of *P. falciparum* parasites from the ring to the schizont stage. Moreover, the MEK inhibitor U0126, which was shown to attenuate *S. mansoni* ERK activation, decreased egg output and affected worm motion. This is the first report suggesting that low-molecular-weight inhibitors of the MAPK pathway have the potential for further development as useful anthelmintics for the treatment of CE (Fig. 6A). Our study demonstrated that sorafenib tosylate (25 μ M), PD184352 (100 μ M), and U0126-EtOH (100 μ M) induced the dephosphorylation (i.e., inactivation) of EgMKKs and EgERK while decreasing PSC vitality. Moreover, the biological activity of sorafenib tosylate may be due, in addition, to its actions against other targets (e.g., VEGFR, PDGFR, and Flt3) in this situation. However, according to recent studies, there are no known orthologs of VEGFR, PDGFR, or Flt3 expressed in *Echinococcus* spp., although the literature mentions the presence of a VEGF signaling pathway in *E. multilocularis* and *E. granulosus* (7, 46). Therefore, at present, the parasitocidal effect of sorafenib tosylate could be only partially attributed to its inhibition of the MAPK pathway. In addition, PSCs treated with sorafenib tosylate were killed more effectively than those treated

with PD184352 and U0126. PSCs in *E. granulosus sensu lato* infections are important for both infection of the definitive host and metacestode dissemination in the intermediate host. By spreading numerous infectious PSCs, the rupture of the cyst, either spontaneously or during surgical operations, leads to multifocal formation of new cysts, exophytic growth, and peritoneal seeding (47). PSCs are thus crucial for the pathological potential of *E. granulosus sensu lato* in humans and represent a target for drug intervention by either systemic or local administration (at surgery or PAIR); however, the protoscolecidal agents currently available for local administration are only chemicals with a certain degree of inefficacy and of side effects (48, 49). ABZ, which is the reference systemic anti-infective drug for the treatment of CE, has only a delayed and incomplete effect on protoscoleces (50), as confirmed in our short- and long-term *in vitro* experiments (Fig. 5), and flubendazole, which exhibits faster activity on protoscoleces, has poorer efficacy on the *E. granulosus sensu lato* germinal layer *in vivo* and is not recommended for the treatment of CE in humans (48). As assessed by murine bioassays performed with inhibitor-treated PSCs, *ex vivo*, sorafenib tosylate and U0126-EtOH exhibited the highest parasiticidal activity against *E. granulosus sensu stricto* PSCs. In addition to its protoscolecidal effect, we demonstrated that inhibition of EgMKKs and EgERK caused morphological and ultrastructural alterations, including cell loss in the germinal layer or the partial wrinkling and separation of the germinal layer from the laminated layer in hydatid cysts. Similarly, parasite development and growth were significantly affected *in vitro* when metacestodes of *E. multilocularis* were treated with Raf, MEK, and p38 kinase inhibitors (BAY 43-9006, PD184352, MK3403, or SB202190) (22, 23). Similar changes in the germinal layer have also been reported after administration of “reference” drugs, such as albendazole and flubendazole (51, 52).

In an attempt to translate these *in vitro/ex vivo* findings into *in vivo* efficacy, experimentally infected mice were treated with sorafenib tosylate, PD184352, or U0126-EtOH. Treatment of mice with sorafenib tosylate or U0126-EtOH showed a reduction in the parasite weight compared to the control group, but there was no significant difference, although the morphological assessments reflected the breakdown of the structural integrity of the germinal layer of cysts from mice treated with sorafenib tosylate. Several factors may have contributed to this reduced effectiveness compared to *in vitro* and *ex vivo* results. First, the present treatment regimen was administered on well-established cysts (6 months), which normally show a reduced response to treatment regimens compared to those therapeutic regimens that aim at treating the cysts just after the intraperitoneal inoculation of the PSCs. Fully developed *E. granulosus sensu stricto* cysts include the laminated layer as well as the fibrous adventitia, and to a certain extent, this barrier, which encysts the germinal layer and the fluid containing the protoscoleces, contributes to the reduced action of antiparasitic drugs (53). It has been reported that benzimidazole compounds are more effective on young metacestodes than on metacestodes that have developed their laminated layer and fibrous shell (54). Intracystic use of the inhibitors could be an alternative route, using them as protoscolecidal agents, in order to prevent protoscolex dissemination and, thus, disease recurrence. Second, the inhibitors were perhaps not delivered to the parasite target tissue in adequate quantities *in vivo*. Actually, for our preliminary experiments on a parasitic agent, we voluntarily administered inhibitor doses that were lower than those used to treat malignant tumors. In further experiments, based on the convincing morphological changes that we observed, we intend to increase the dosage of the applied inhibitors and/or to prolong the treatment duration. In reported studies, to show significant therapeutic effects, animals treated with either sorafenib or PD184352 as individual agents received noticeably higher drug dosages than those administered here. For example, Wilhelm et al. treated animals carrying breast, lung, and colon tumor xenografts for 25 to 27 days with 60 mg/kg sorafenib to achieve tumor regression (55). Similarly, Sebolt-Leopold et al. and McDaid et al. used PD184352 concentrations in the range of 48 to 300 mg/kg (with drug administration over each of 14 to 20 days) to achieve antitumor effects (56, 57). Third, the lack of efficacy of sorafenib or PD184352 treatment against murine CE *in vivo* stresses the problem of the

bioavailability of the drug when given orally. The clinical use of sorafenib is effectively limited by its low oral bioavailability (~8.43%), owing to its poor aqueous solubility (58). In recent years, some studies have focused on increasing the antitumor efficacy of sorafenib, PD184352, or U0126-ETOH by improving its solubility and developing injectable intravenous (i.v.) formulations (59, 60). One advantage of sorafenib over novel drugs for the treatment of CE is that this drug is commercially available for hepatocellular carcinoma (HCC) therapy and has been extensively characterized in terms of bioavailability, pharmacokinetics, and toxicity (35, 36). Thus, further adjustment of treatment regimens is easy to explore, and pilot studies in humans would be ethically acceptable in selected cases of CE. In addition, new MAPK cascade inhibitors provide novel starting points for the development of new chemotherapeutic agents.

MATERIALS AND METHODS

Ethics statement. All protocols involving animals were approved by the Animal Welfare Committee of the First Affiliated Hospital of Xinjiang Medical University (IACUC-20140716014). Surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize suffering.

Molecular cloning of EgMKK1 and EgMKK2. *E. granulosus sensu stricto* PSCs were aspirated from liver hydatid cysts and then cultured *in vitro* according to a previously established protocol (61). Pepsin activation of PSCs was performed as previously described (61). Different RNA extractions were carried out as previously described (6). Total RNA was reverse transcribed using a reverse transcription system (Promega, USA), according to the manufacturer's instructions.

By aligning the conserved regions of MEK1/2-like proteins (MKK1 [GenBank accession no. [FN434110](#)] of *E. multilocularis*) and MKK3/6-like proteins (MKK2 [GenBank accession no. [FN434111](#)] of *E. multilocularis*) and the sequence available in a supercontig database of *E. granulosus sensu lato* (https://www.sanger.ac.uk/cgi-bin/blast/submitblast/e_granulosus), we found two homologous sequences producing high-scoring segment pairs (pathogen_EgG_scaffold_0009 and pathogen_EgG_scaffold_0004) from *E. granulosus sensu lato* and designed specific primers for EgMKK1 (F1/R1) (see Table S3 in the supplemental material) and EgMKK2 (F2/R2) (Table S3) amplification. PCR products were cloned into the pMD19-T vector (TaKaRa, China) and sequenced (Sangon Biotech Inc., China).

Gene structure of *egmkk1* and *egmkk2*. In order to determine the EgMKK1 and EgMKK2 gene structures, the partial sequences available in the supercontig database of *E. granulosus sensu lato* were subjected to BLASTN analysis with the EgMKK1 and EgMKK2 cDNA sequences. The exon-intron junctions were located, and specific primers were then designed for intron amplification of *egmkk1* (F3/R3-F11/R11) (see Table S3 in the supplemental material) and *egmkk2* (F12/R12-F16/R16) (Table S3). PCR products were cloned into the pMD19-T vector and sequenced (Sangon Biotech Inc., China).

Phylogenetic studies of EgMKK1 and EgMKK2. Sequences were aligned using Lasergene (DNASTAR Inc.) (62). Phylogenetic analysis was performed on the predicted amino acid sequences of EgMKK1 or EgMKK2 and the other MAPKs using the Clustal function of the MegAlign program (DNASTAR Inc.). The phylogenetic tree was built by MEGA5.2 (63), using the neighbor-joining method with previously described parameters (64).

Expression analysis of EgMKK1 and EgMKK2 in larval developmental stages. Total RNA extracted from *E. granulosus sensu stricto* PSCs or cysts was reverse transcribed (as described above). cDNAs were used as the templates for PCR amplification using SYBR green PCR master mix. qRT-PCR experiments were performed as previously described (6). Three PCR primer pairs, F17/R17, F18/R18, and F19/R19 (Table S3), were used to amplify the total transcripts of EgMKK1, EgMKK2, and Egelp. The data were analyzed against the reference gene Egelp by using the $2^{-\Delta\Delta CT}$ method (65).

Immunoblot and immunohistochemical analyses. Antibodies were produced against the open reading frames (ORFs) of EgMKK1 and EgMKK2, which were amplified using primer pairs F20/R20 and F21/R21 (see Table S1 in the supplemental material), subcloned into the pET-28α(+) expression vector, and expressed in *Escherichia coli*. Antisera were produced in rabbit after immunization against the recombinant protein under conditions previously described (6).

Total protein extracts were obtained by homogenizing *E. granulosus sensu stricto* PSCs or cysts in lysis buffer (Life Technologies, USA). Analyses of the parasite and recombinant proteins were performed as previously described (6). The following primary antibodies were used to detect total EgMKK1 and EgMKK2: anti-EgMKK1 (1:2,000), anti-EgMKK2 (1:2,000), or preimmune rabbit serum (1:2,000), overnight at 4°C. Alkaline phosphatase-conjugated goat secondary antibody was used as the secondary antibody (1:1,000) (Cell Signaling Technology, USA). Immunoreacting bands were visualized using the alkaline phosphatase substrate kit (Thermo Fisher Scientific, USA).

To investigate the immunolocalization of EgMKK1 and EgMKK2, fresh PSCs and cysts were generated as previously described (6). The sections were fixed for 15 min and then blocked in blocking buffer and incubated with rabbit polyclonal antibody (EgMKK1 at 1:1,000 and EgMKK2 at 1:2,000) overnight at 4°C. At the end of the incubation, samples were washed with phosphate-buffered saline (PBS) and reacted with Alexa 488 fluorochrome-conjugated F(ab')₂ antibody (Cell Signaling Technology, USA) for 2 h at room temperature in darkness. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI; Cell Signaling Technology, USA) for 5 min. The sections were imaged with a confocal laser scanning fluorescence microscope (Leica, Wetzlar, Germany). Negative controls were prepared with preimmune rabbit serum.

Interaction of EgMKK1 and EgMKK2 with other MAPK members. Both methods, i.e., yeast two-hybrid assays and coimmunoprecipitation, were used to detect the interaction of EgMKK1 and EgMKK2 with other signaling partners. Yeast two-hybrid assays were performed according to the manual for the yeast transformation system kit (Clontech, Japan). The sequences of EgMKK1 and EgMKK2 ORFs were amplified by PCR with primer pairs F22/R22 and F21/R23, respectively, and then inserted into pGADT7 vectors to generate the fused Gal4 activation domain (Gal4-AD) (see Table S3 in the supplemental material). In addition, the sequences of EgMKK1, EgMKK2, EgERK, Egp38, and Eg14-3-3.2 ORFs were amplified using primer pairs F22/R22, F21/R21, F24/R24, F25/R25, and F26/R26, respectively, and cloned into pGBKT7 vectors to generate the Gal4 DNA binding domain (Gal4-BD) (Table S3) (66, 67). Interaction trap analyses of cotransformants into yeast strain Y2HGold were performed as previously described (6).

Coimmunoprecipitation analysis was used to verify the results of yeast two-hybrid assays. Plasmids pcDNA3.1/V5-EgMKK1 and pCMV-Myc-Egp38 and plasmids pcDNA3.1/V5-EgMKK2 and pCMV-Myc-EgERK or pCMV-Myc-Eg14-3-3.2 were cotransfected into 293T cells (see Table S3 in the supplemental material). Plasmid pcDNA3.1/V5-EgMKK1 or pcDNA3.1/V5-EgMKK2 was cotransfected with pCMV-Myc-N into 293T cells and served as a negative control. After 48 h posttransfection, the cells were resuspended in immunoblot and immunoprecipitation lysis buffer, and the lysate was used for co-IP assays utilizing the anti-V5 antibody and protein A/G-agarose beads, according to the manufacturer's protocol (Santa Cruz Biotechnology, USA). The beads were washed using lysis buffer four times. Immunoprecipitates and total cell lysates were boiled in SDS loading buffer for 10 min and then subjected to immunoblot analysis using anti-V5 antibody (Thermo Fisher Scientific, USA) and anti-Myc antibody (Clontech, Japan).

In vitro EgMKK1 and EgMKK2 kinase assays. Plasmid pcDNA3.1/V5-EgMKK1 or pcDNA3.1/V5-EgMKK2 was transfected into 293T cells. After 48 h posttransfection, the cell extracts were pretreated with protein A/G-agarose beads (Santa Cruz Biotechnology, USA), followed by immunoprecipitation using anti-V5 antibody and protein A/G-agarose beads, according to the manufacturer's protocol. The beads were washed using lysis buffer four times. After keeping a small amount of the beads for kinase assays, the rest of the beads were suspended in SDS loading buffer and boiled for immunoblot analysis.

Kinase reactions of the immunoprecipitated proteins were performed in 40 μ l of kinase buffer containing 10 μ l assay dilution buffer (100 mM morpholinepropanesulfonic acid [MOPS] [pH 7.2], 125 mM β -glycerol phosphate, 25 mM EGTA, 5 mM sodium orthovanadate, and 5 mM dithiothreitol), 10 μ l inhibitor cocktail (20 mM protein kinase C [PKC] inhibitor peptide, 2 mM protein kinase A [PKA] inhibitor peptide, and 20 mM compound R24571), 10 μ l substrate cocktail II (2 mg/ml MBP), and 10 μ l Mg^{2+} -ATP cocktail (75 mM magnesium chloride and 500 mM ATP), according to the manufacturer's protocol (Sigma-Aldrich, USA). The protein kinase reactions were performed at 30°C for 20 min. The reactions were stopped by the addition of SDS loading buffer. The phosphorylation of MBP was analyzed by using anti-phospho-MBP antibody.

In vitro effects of inhibitors on *E. granulosus sensu stricto* viability. *E. granulosus sensu stricto* PSCs and cysts were obtained as described above. The cysts were used for experimental studies for *in vitro* cultivation when they were 2 to 4 mm in diameter. Viable PSCs and cysts were placed in 96-well flat-bottom plates and 6-well plates, respectively, in complete RPMI 1640 with 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

For short-term inhibitor experiments, *E. granulosus sensu stricto* PSCs were treated *in vitro* with 1, 25, 50, 100, 200, and 400 μ M sorafenib tosylate (a potent and selective inhibitor of MAPKKK, Raf-1, and Selleck); 1, 25, 50, 100, 200, and 400 μ M PD184352 (a potent and selective inhibitor of MAPKK, MEK1/2, and Selleck); and 1, 25, 50, 100, 200, and 400 μ M the inhibitor U0126-EtOH (a specific inhibitor of MAPKK, MEK/2, and Selleck). As controls, medium with an identical amount of DMSO (without an inhibitor; final concentration, 0.2%) or ABZ (1 and 400 μ M) was used. *In vitro* incubations were performed at 37°C, and the medium was changed every 4 days. The initial viability of PSCs was determined by microscopy to confirm that more than 95% of the PSCs were alive with motile behavior and the ability to exclude eosin staining (68). The IC_{50} of each inhibitor was calculated by nonlinear regression using GraphPad Prism.

For long-term treatment, PSCs and cysts were cultivated for 30 days with sorafenib tosylate (25 μ M), PD184352 (100 μ M), or U0126-EtOH (100 μ M). Randomly chosen PSCs and cysts were analyzed by scanning electron microscopy (SEM) (JSM-6390; Japan) and transmission electron microscopy (TEM) (JEM-1230; Japan) for ultrastructure studies, which were performed as previously described (50). Additionally, lysates of PSCs and cysts were prepared as described above and analyzed by immunoblotting using antibodies against anti-phospho-MEK1/2, anti-phospho-ERK1/2, and anti- β -actin, as previously described (22).

Assessment of *E. granulosus sensu stricto* PSC viability by mouse bioassays. To investigate the viability of inhibitor-treated PSCs, female BALB/c mice (five animals per group, 6 weeks old) were infected by intraperitoneal injection of a suspension of 1,000 inhibitor-treated PSCs. Before inoculation, the PSCs had been treated *in vitro* with either 25 μ M sorafenib tosylate, 100 μ M PD184352, or 100 μ M U0126-EtOH for 30 days (see above), and the viability of PSCs was confirmed by eosin staining. A control group was infected with PSCs treated with DMSO alone (final concentration, 0.2%). At 6 months postinoculation, the mice were euthanized, and the parasite tissue weight was determined.

In vivo efficacy of inhibitor treatment in mice experimentally infected with *E. granulosus sensu stricto*. Six-week-old female BALB/c mice were infected intraperitoneally with 50 echinococcosis cysts, as previously described (61). For *in vivo* experiments, the inhibitors were dissolved in Cremophor EL-ethanol-water (12.5:12.5:75 mixture of Cremophor EL, 95% ethyl alcohol, and water; Sigma) (55, 69). At 6 months postinfection, all mice were randomly distributed into 4 groups of 10 mice each. Subsequently, these animals received the following treatments for a period of 4 weeks: group 1 received solvent

(control group), group 2 received sorafenib tosylate orally (30 mg/kg three times a week) (70), group 3 received PD184352 orally (60 mg/kg three times a week) (60), and group 4 received U0126-EtOH orally (30 mg/kg three times a week) (71). At the end of the treatment period, mice were euthanized, and necropsy was carried out immediately thereafter. The peritoneal cavity was opened, and the cysts were carefully removed. The parasite weight was determined. Several samples of the recovered cysts were processed for SEM.

Statistical analysis. Results are expressed as means $\pm$ standard deviations (SD) of the means and were analyzed using the GraphPad Prism software package. Statistical analyses were performed by Student's *t* test and a Kruskal-Wallis test for comparison between groups. *P* values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01043-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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We have reviewed the manuscript and declare that we have no competing interests.

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