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Characterization and inhibition of a p38-like mitogen-activated protein kinase (MAPK) from *Echinococcus multilocularis*: Antiparasitic activities of p38 MAPK inhibitors

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ARTICLE INFO

Article history:
Received 25 June 2008
Accepted 7 August 2008

Keywords:
Echinococcus
Echinococcosis
Cestode
Parasite
p38 MAP kinase
Pyridinyl imidazole

ABSTRACT

Alveolar echinococcosis (AE), caused by the metacestode larval stage of the fox-tapeworm *Echinococcus multilocularis*, is a life-threatening disease with very limited treatment options. In search for novel drug targets, we concentrate on factors of the cellular signaling machinery and report herein the characterization of a novel gene, Em*mpk2*, which is expressed in the parasite's larval stage and which codes for a member of the mitogen-activated protein kinase (MAPK) family. On the amino acid sequence level, the encoded protein, EmMPK2, shares considerable homologies with p38 MAPKs from a wide variety of animal organisms but also displays several distinct differences, particularly in amino acid residues known to be involved in the regulation of enzyme activity. Upon heterologous expression in *Escherichia coli*, purified EmMPK2 showed prominent autophosphorylation activity and strongly elevated basal activity towards a MAPK substrate, when compared to the closest human orthologue, p38-α. EmMPK2 activity could be effectively inhibited in the presence of ML3403 and SB202190, two ATP-competitive pyridinyl imidazole inhibitors of p38 MAPKs, in a concentration-dependent manner. When added to in vitro cultivated metacestode vesicles, SB202190 and particularly ML3403 led to dephosphorylation of EmMPK2 in the parasite and effectively killed parasite vesicles at concentrations that did not affect cultivated mammalian cells. Taken together, these results identify pyridinyl imidazoles as a novel class of anti-*Echinococcus* compounds and EmMPK2 as a promising target for the development of drugs against alveolar echinococcosis.

1. Introduction

The larval stage of the fox-tapeworm *Echinococcus multilocularis* is the causative agent of alveolar echinococcosis (AE) which is considered to be the most lethal helminthic infection in humans [1,2]. AE is characterized by an infiltrative, tumor-like growth of the *E. multilocularis* metacestode, usually affecting the liver of intermediate hosts such as small ruminants or humans. Besides surgery, treatment of patients with anti-helminthic benzimidazole derivatives is the most important form of AE therapy [3,4]. Benzimidazoles have a high affinity for helminth-specific β-tubulin isoforms, thus inhibiting microtubule polymerization which eventually leads to parasite death [1,4,5]. However, in AE these treatments are mostly parasitostatic rather than parasitocidal and, as a consequence, chemotherapy has to be given life-long. Furthermore, long-term benzimidazole therapy of AE patients is often associated with heavy side effects [3–5]. These are due to the interaction of the drugs with β-tubulin of the host [6,7] which, on amino acid sequence level, is to over 90% identical to that of

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doi:10.1016/j.bcp.2008.08.020

Echinococcus [8]. Although there have been several recent reports on the activity of other drugs such as amphothericin B, isoflavones or nitazoxanide on *E. multilocularis* larvae cultivated in vitro [5,9–12], no reliable chemotherapeutic alternative can presently be given to patients who do not tolerate or do not respond to benzimidazoles. Thus, it has become evident that new chemotherapeutic strategies against AE are urgently needed.

As a metazoan organism, *E. multilocularis* strictly depends on cell–cell-communication mechanisms to regulate development and growth [1]. During recent years, several respective components of the parasite, including surface receptor kinases of the insulin-, the epidermal growth factor (EGF)- and the transforming growth factor-β (TGF-β)-receptor families have been characterized by us and displayed considerable structural and functional homologies to the corresponding systems of mammals [1,13–15]. Particularly in the case of receptor tyrosine kinase signaling, eukaryotic cells frequently employ mitogen-activated protein kinase (MAPK) modules as central systems for the transmission of signals from the cell surface to the nucleus [16–20]. Upon activation of surface receptor complexes, the signals are sequentially transmitted through an array of proteins including MAPK kinase kinases (MAPKKK) and MAPK kinases (MAPKK). As final components of the cascades, MAPKs are phosphorylated, and thereby activated, on tyrosine and threonine residues within a conserved T-X-Y motif by specific MAPKKs. Several individual MAPK signal transduction pathways have been identified in mammalian cells of which one, the Erk (extracellular signal-regulated kinase)-pathway, plays an important role in regulating cellular responses to growth factors [1,16,21]. Two further pathways, which are mainly induced in response to different stress stimuli, converge on families of the p38 MAPKs and the c-Jun amino-terminal kinase/stress activated protein kinases (JNK/SAPK) [17,18,20]. In addition to being activated through different stress conditions such as ultraviolet radiation, heat shock, oxidative stress and osmotic stress, p38 MAPKs are also activated in response to several growth factors (e.g. TGF-β) and inflammatory signals [17,18,20,22]. For *E. multilocularis*, only two MAPK cascade components have so far been reported, the MAPKKK EmRaf [23] and the Erk-like MAPK EmMPK1 [21] which form part of an Erk-like MAPK module, as indicated by the activation of EmMPK1 in parasite larvae in response to host-derived EGF [21].

Due to their important role in developmental regulation of all metazoans, including helminths, and reduced homologies to corresponding host factors, when compared to other targets like β-tubulin, the cellular signaling components of helminths have already been suggested as promising drug targets for anthelminthic chemotherapy [1,21,24]. This is further supported by the fact that the biochemistry of these components has been very well worked out, due to cancer research, and that a wealth of small molecules is available to modify their activities [25,26]. In this study, we make use of this knowledge. We present molecular data on a novel MAPK of *E. multilocularis* which belongs to the p38 subfamily and displays significantly elevated basic enzyme activity when compared to human p38 MAPKs. Furthermore, we demonstrate that the activity of the parasite enzyme can be blocked by inhibitors designed against human p38 MAPKs. We also show that these molecules lead to parasite death in vitro at concentrations which do not affect cultured human cells.

2. Materials and methods

2.1. Chemicals, radioactivity and antibodies

p38 MAPK inhibitors SB202190 [27] and ML3403 [28] were purchased from Calbiochem (Gibbstown, NJ, USA), dissolved in dimethyl sulfoxide (DMSO) and stored according to the manufacturers instructions until use. Antisera were used against the V5 epitope (Invitrogen; Karlsruhe, Germany), the *E. multilocularis* protein Elp [29], the phosphorylated (pTGpY) form of p38 MAPK (Promega; Mannheim, Germany; cat. no. V1211), as well as against the non-phosphorylated and the phosphorylated forms of EmMPK1 [21]. Radioactively labeled [γ-^{32}P] ATP was from Hartmann Analytic (Braunschweig, Germany). Oligonucleotides were from Sigma–Aldrich (Taufkirchen, Germany).

2.2. Organisms and culture methods

Experiments were performed with the natural *E. multilocularis* isolate H95 whose properties have been described previously [30]. The isolate was propagated and maintained in Mongolian jirds (*Meriones unguiculatus*) as described [30]. Co-cultivation of metacestode vesicles with host hepatocytes was performed as outlined in detail in ref. [31]. In vitro cultivation of metacestode vesicles under axenic conditions was performed according to a previously established protocol [32] and protoscoleces were isolated from in vivo cultivated material as described [33]. Pepsin activation of protoscoleces was performed essentially as described previously [21]. *E. multilocularis* primary cells were isolated and cultivated as described [31].

2.3. Nucleic acid isolation, cloning and sequencing

Chromosomal DNA and total RNA were isolated from parasite material after cultivation in laboratory hosts as described [34]. For RNA isolation from in vitro cultivated metacestode vesicles, the RNeasy kit (Qiagen; Hilden, Germany) was employed. cDNA synthesis was performed essentially as previously described using oligonucleotide CD3-RT [33]. PCR products were cloned using the TOPO-TA cloning kit (Invitrogen; Karlsruhe, Germany) and sequenced employing a ABI prism 377 DNA sequencer (PerkinElmer; Rodgau-Juegesheim, Germany).

2.4. Cloning of the Emmpk2 cDNA and genomic locus

To identify cDNAs encoding serine/threonine kinases from the trans-spliced transcriptome of *E. multilocularis* [33,34], a degenerative PCR approach was undertaken. Total RNA was isolated from in vitro cultivated metacestode vesicles and transcribed into cDNA. A first PCR reaction was performed using oligonucleotide ST-C6 (5′-AAG ATG CAN CCN CAN GAC CA-3′), directed against coding regions for the conserved oligopeptide WSVGCIF, and oligonucleotide SL-5PR [33],

directed against the 5′ end of the *E. multilocularis* spliced leader [34], under low stringency annealing conditions (55 °C). The PCR reaction was purified (PCR purification kit; Qiagen; Hilden, Germany) and a 1/100 dilution was then used for nested PCR using oligonucleotides ST-C1 (5′-CAT AAT TTC YGG NGC NCG RTA CCA-3′), directed against regions encoding the conserved peptide WYRAPEIM and oligonucleotide SL-TGT [33], directed against the 3′ region of the *E. multilocularis* spliced leader. The resulting PCR product (~600 bp) was cloned and sequence analysis revealed that it encoded a novel MAPK of *E. multilocularis*. The remaining parts of the cDNA were subsequently isolated by 3′-RACE according to a previously established protocol [33]. Oligonucleotides for 3′-RACE were ROC-p38dw (5′-GAT GGA TGC CGA CCT GGG-3′) and ROC4 (5′-GCT CAG CAG GTG CTC ACT G-3′). The Em*mpk2* genomic locus was PCR-amplified from chromosomal *E. multilocularis* DNA using several oligonucleotides directed against the 5′- and 3′-regions of the cDNA as well as against internal exons. Through sequence analysis of the genomic locus, the correct sequence of the cDNA was verified. The nucleotide sequences of the Em*mpk2* cDNA and chromosomal locus have been deposited in the GenBank data library under the accession numbers AM950261 and AM950262.

2.5. Computer analyses and statistics

Amino acid sequence comparisons were performed using the basic local alignment research tool (BLAST) software on the nr-aa database collection (http://blast.genome.jp). Pileups were constructed employing the software tool BioEdit using the BLOSUM62 matrix. Kinase domain predictions were done as described by Schultz et al. [35] using the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de). For statistical analyses, the GraphPad Prism software (GraphPad Software) version 4 was used. Error bars represent standard deviation. Differences were considered significant for p values below 0.05 (indicated by (*)), very significant for p between 0.001 and 0.01 (**), extremely significant for $p < 0.001$ (***) and non-significant for $p > 0.5$ (ns). Survival curves after drug treatment were compared using the log-rank test. The influences of drugs on metacestode vesicles and mammalian cell lines were analyzed using ANOVA and Tukey's multiple comparison test. For IC_{50} determination, linear regression was performed according to Pearson's correlation. R^2 values indicate the 'goodness of fit'. The confidence interval was calculated as 95% in all analyses.

2.6. RT-PCR

Total RNA was isolated from in vitro cultivated primary cells, metacestode vesicles as well as activated or non-activated protoscoleces using the NucleoSpin kit (Macherey-Nagel; Düren, Germany) according to the manufacturer's instructions. cDNA was produced using the Omniscript RT-PCR kit (Qiagen; Hilden, Germany) and oligonucleotide CD3RT (see above) according to the manufacturer's instructions. Ten-fold serial dilutions of the cDNA were then used as template for PCRs using primers Em10-15 (5′-AAT AAG GTA ATC AGT CGA TC-3′) and Em10-16 (5′-TTG CTG GTA ATC AGT CGA TC-3′) for the constitutively expressed gene *elp* [33,36] as well as Roc6 (5′-GCA AGA AGG ATC TCA AGG ATG-3′) and Roc3 (5′-GGC GGT CAA GGG AAG C-3′) for Em*mpk2*. Cycling conditions were 25 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C. PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

2.7. Heterologous expression in E. coli and purification of recombinant proteins

EmMPK2 and human p38 MAPK-α were expressed in *Escherichia coli* using the pBAD-TOPO system (Qiagen; Hilden, Germany). The entire Em*mpk2* ORF was PCR-amplified from *E. multilocularis* cDNA using primers p38-5EC (5′-GTG CCC GAT GTA AAT GAG CG-3′) and p38-3EC (5′-CGC GTT GAT TGG CGA GTA C-3′) and cloned into the pBAD-TOPO vector, resulting in a C-terminal in frame fusion with the V5 epitope (Invitrogen, Karlsruhe, Germany) and a hexahistidine tag. To clone the ORF encoding human p38-α, total RNA was isolated from HEK-293 cells, reverse transcribed into cDNA and used as a template in a PCR reaction employing primers hsMAPK14-UP (5′-GGA CTC CAT CTC TTC TTG G-3′) and hsMAPK14-dw (5′-TTC TAC CGG CAG GAG CTG-3′). The resulting PCR product was cloned into pBAD-TOPO, resulting in a C-terminal in frame fusion with the V5 epitope and the hexahistidine tag. Production of recombinant proteins after induction with 0.2% L-arabinose was performed essentially according to the pBAD-TOPO user manual (Invitrogen; Karlsruhe, Germany). Fusion proteins were purified from *E. coli* lysates under native conditions by affinity chromatography against the hexahistidine tag. After collecting recombinant *E. coli* (6000 g, 20 min, 4 °C) from 2 l of culture, the pellet was resuspended in 20 ml lysis buffer (300 mM NaCl, 50 mM sodium-phosphate buffer pH 8.0, 10 mM imidazole). Cell debris was removed by centrifugation, the supernatant was transferred to 4 ml slurry nickel beads (ProBond Resin; Invitrogen, Karlsruhe, Germany) and equilibrated with lysis buffer. After overnight rotation at 4 °C, beads were washed three times with 300 ml washing buffer (300 mM NaCl, 50 mM sodium-phosphate buffer pH 8.0, 20 mM imidazole; all from Sigma–Aldrich, Taufkirchen, Germany). Recombinant protein was then extracted with 6 ml elution buffer I (300 mM NaCl, 50 mM sodium-phosphate buffer pH8.0, 250 mM imidazole) and 6 ml elution buffer II (300 mM NaCl, 50 mM sodium-phosphate buffer pH8.0, 500 mM imidazole). Protein containing fractions were pooled and dialyzed overnight against phosphate buffered saline (PBS) at 4 °C.

2.8. MAPK activity assay

EmMPK2 enzymatic activity was measured via integration of radioactively labeled γ-^{32}P into the MAPK substrate myelin basic protein (MBP; Sigma–Aldrich; Taufkirchen, Germany). Purified and normalized protein solutions were incubated in kinase assay buffer (20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 100 μM ATP; all from Sigma–Aldrich, Taufkirchen, Germany), including 0.5 mg/ml MBP, 100 μM leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM PMSF before 4 μCi [γ-^{32}P]-ATP and 1 μl DMSO with or without inhibitor were added. After 60 min at 37 °C, the reaction was stopped (140 mM Tris–HCl pH 6.8, 20% glycerol, 5% SDS, 0.003% bromophenol blue, 7% β-mercaptoethanol; all from

Sigma–Aldrich, Taufkirchen, Germany) and boiled for 10 min. Samples were separated on 15% polyacrylamide gels, blotted onto nitrocellulose membranes (Protran™ nitrocellulose sheets; Schleicher and Schuell Biosciences; Dassel, Germany) and exposed to X-ray films for 48 h.

2.9. Drug treatment of metacestode vesicles and mammalian cell lines

Morphologically intact metacestode vesicles with a minimal diameter of 3 mm were manually picked from co-culture or axenic culture, washed in PBS and incubated in 12-well plates in the presence of culture medium (Dulbecco's minimal essential medium, 10% FCS, antibiotics; GIBCO-BRL, Eggenstein, Germany) and DMSO with or without p38 inhibitors. Viability and integrity of the vesicles were measured microscopically after incubation. Alkaline phosphatase activity in supernatants was measured according to a previously established protocol [37]. Rat Reuber hepatoma cells, human brain microvascular endothelial cells (HBMEC) and HEK-293 human embryonic kidney cells (for references see ref. [32]) were seeded into 24-well plates with 10^4 cells per well and incubated in DMEM, containing 10% fetal calf serum and antibiotics, in the presence of DMSO with or without inhibitors. After 4 days of incubation, crystal violet life staining was performed as described [38] and absorbance at 570 nm was measured. All experiments were performed four times independently.

3. Results

3.1. Isolation and characterization of the Emmpk2 cDNA and genomic locus

Approximately 25% of the *E. multilocularis* metacestode transcriptome is processed via the parasite-specific mechanism of trans-splicing [33,34]. We had previously identified the Erk-like MAPK EmMPK1, the mRNA of which is not trans-spliced [21]. Assuming that mRNAs for MAPKs other than EmMPK1 might be among the trans-spliced fraction, a degenerative PCR approach was undertaken as outlined in detail in Section 2. Using this approach, the full-length cDNA for a novel *E. multilocularis* MAPK, EmMPK2, was successfully cloned and characterized. The respective gene was designated Em*mpk2* (for *E. multilocularis MAPK2*). The full-length cDNA of Em*mpk2* comprised 1371 bp (excluding the polyA tail) and contained the 36 bp *Echinococcus* spliced leader (SL) [34] at the 5′ end. Twenty-three bp downstream of the SL, an ATG start codon was followed by a single ORF of 1104 bp, encoding EmMPK2 (368 amino acids; 42 kDa). On amino acid sequence level, EmMPK2 displayed significant homologies (~50–53%

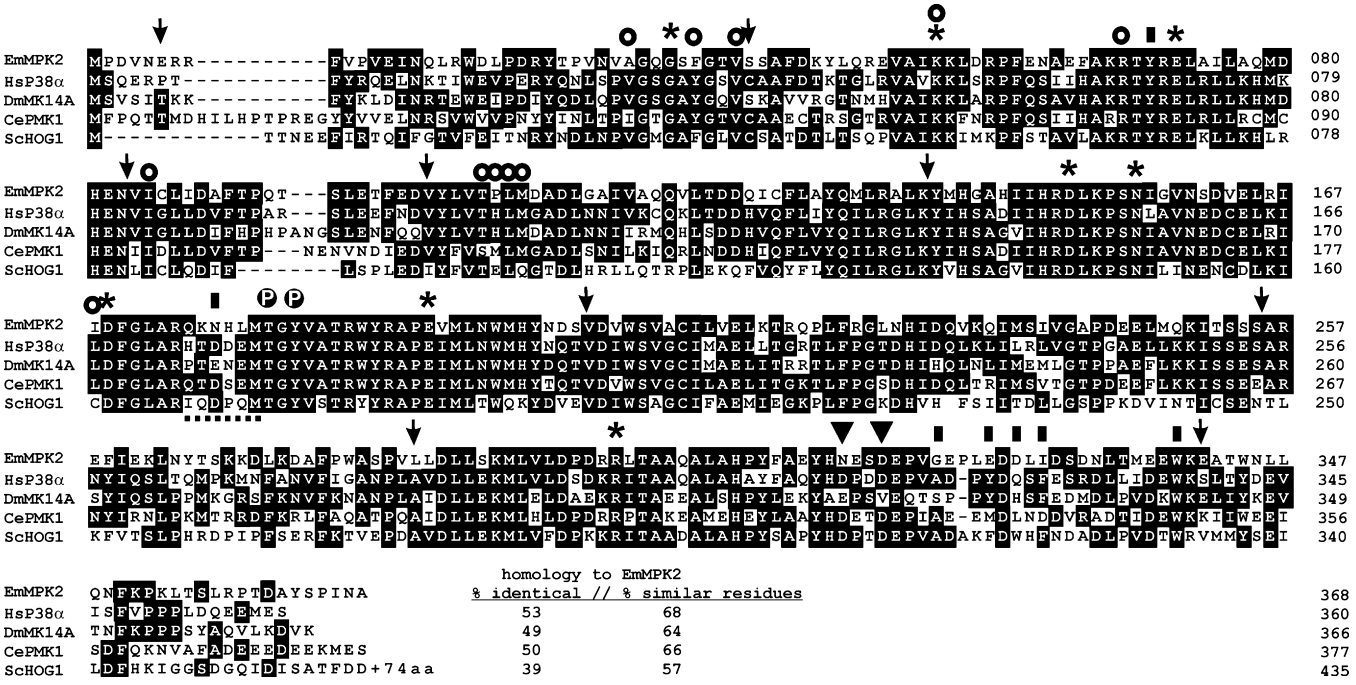

Fig. 1 – Amino acid sequence comparison between EmMPK2 and other p38 MAPKs. Shown are the sequences of EmMPK2 (this work), human p38 MAPK-α (HsP38α; Q16539), *D. melanogaster* p38 MAPK (DmMK14A; O62618), *C. elegans* stress activated protein kinase PMK-1 (CePMK1; Q17446) and yeast HOG-1 (ScHOG1; P32485). Amino acid residues which are conserved in at least two of the sequences are printed in white on black background. Homologies of the aligned proteins to EmMPK2 are indicated at the end of the alignment (%identity, %similarity). Asterisks above the alignment mark residues which are highly conserved in eukaryotic protein kinases [39]. Black triangles indicate conserved Asp residues within the docking domain [44]. Thr and Tyr residues of the conserved T-G-Y motif are marked by 'P'. Arrows indicate the intron positions on the encoding mRNA. Black rectangles above the alignment indicate residues the mutation of which lead to constitutively activated forms of yeast HOG1 [41]. Circles above the alignment indicate residues involved in binding of pyridinyl imidazole inhibitors to human p38 MAPK-α [41]. The activation loop is marked by a dotted line below the alignment.

identical and 65–68% similar residues) to p38 MAPK homologues from a wide variety of phylogenetically distinct animals including mammals, insects and nematodes (Fig. 1). Homologies to human p38 MAPKs were highest for isoform p38-α (53% identity), followed by p38-β (51%), p38-γ (46%) and p38-δ (45%). Significant, albeit lower homologies (~40% identical, 57% similar residues) were also shared with yeast p38 MAPK orthologues (Fig. 1).

Computer analyses using the SMART software package [35] identified a Ser/Thr protein kinase catalytic domain in EmMPK2 between residues Tyr^{25} and Phe^{309}. All amino acid residues previously shown to be invariable in eukaryotic protein kinases [39], were also present in EmMPK2 (Fig. 1). A hallmark of MAP kinases is the presence of a highly conserved T-X-Y motif close to the activation loop of the kinase domain in which both the Thr and the Tyr residues are phosphorylated by upstream MAPKKs. The typical consensus for this motif is T-G-Y in p38 MAPKs while Erk- and JNK/SAPK-like MAPKs harbor T-E-Y and T-P-Y motifs, respectively [40]. EmMPK2 contained the T-G-Y motif which is typical for p38 MAPKs (Fig. 1). Furthermore, the activation loop of EmMPK2 comprised six residues (Fig. 1) which is characteristic of p38 MAPKs while Erk- and JNK/SAPK-MAPKs typically contain activation loops of 12 or 8 residues, respectively [21]. Taken together, these sequence features identified EmMPK2 as a member of the p38 subfamily of MAPKs. Interestingly, EmMPK2 also showed several differences to known p38 MAPKs which included amino acid exchanges at residues previously shown to be involved in the regulation of enzymatic activity of the yeast orthologue HOG1 [41] and human p38 MAPK-α [42,43]. Furthermore, one of the conserved Asp residues in the so-called 'common docking domain' that regulates the interaction of MAPKs with upstream MAPKKs and with phosphatases [44] is replaced by Asn in EmMPK2 (Fig. 1).

Analysis of the chromosomal Em*mpk2* locus revealed that the ORF is contained within 10 exons, separated by 9 introns (Fig. 1) which all displayed canonical GT- and AG-motifs at the splice-donor and -acceptor sites, respectively. Of these 9 introns, 7 shared conserved exon–intron boundaries with the human genes encoding p38 MAPKs while only 2 and 4 conserved boundaries were shared with introns in the human genes encoding Erk-like MAPKs and JNK/SAPKs, respectively (data not shown), indicating a close evolutionary relationship between Em*mpk2* and mammalian p38 MAPK genes.

The *E. multilocularis* genome is currently being sequenced and sequence information representing 4-fold coverage is available under http://www.sanger.ac.uk/Projects/Echinococcus/. When comparing the cDNA sequence of Em*mpk2* with the available data, the chromosomal locus was found on one contig of 21.211 bp and displayed a nucleotide sequence and exon–intron organization identical to that which has been determined by us. As expected, the genomic locus contained a consensus splice acceptor site at the position where the SL was found in the cDNA (data not shown). Extensive BLAST analyses using the sequences of EmMPK2 and human p38 MAPK isoforms as queries did not reveal the presence of p38 MAPK encoding genes other than Em*mpk2* in the *E. multilocularis* genome. Taken together, these data verified our analyses of the Em*mpk2* cDNA and genomic locus and indicated that EmMPK2 is the only p38-like MAPK of the parasite.

It should be noted that a partial sequence from the *E. granulosus* p38-orthologue (Eg*mpk2*) is available under accession number EGC02047 from the LophoDB database (http://www.nematodes.org/NeglectedGenomes/Lopho/LophDB.php). The deposited EST sequence corresponded closely to the Em*mpk2* cDNA sequence determined by us with the exception that, probably due to alternative splicing, exon VIII was directly fused to exon X, thus completely removing the sequence information of exon IX (coding for amino acids 281–341). Due to this splicing event, a stop-codon is introduced directly after the exon VIII sequences, leading to a truncated protein which only encompasses amino acids 1–281. Whether this splicing mode is typical for Eg*mpk2* or whether, by chance, an mRNA containing a splicing error was sequenced, remains to be established. However, within the available parts of the cDNA, the deduced EgMPK2 amino acid sequence only differed in three positions from EmMPK2, making it highly likely that both genes code for proteins that are to over 99% identical.

3.2. *Expression of Em*mpk2* in E. multilocularis larval stages*

The expression of Em*mpk2* in larval stages was analyzed by semi-quantitative RT-PCR. As shown in Fig. 2A, Em*mpk2* transcripts were easily detectable in metacestode vesicles as well as in protoscoleces before or after treatment with pepsin at low pH, mimicking the transition of the larvae to the digestive system of the definitive host. Hence, at least in these larval stages, Em*mpk2* is constitutively expressed.

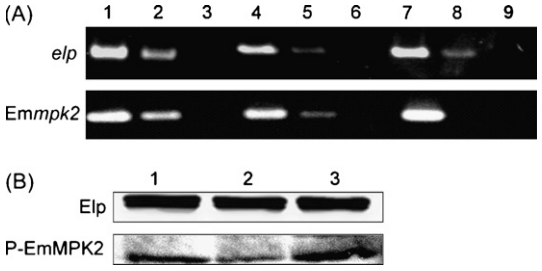

Fig. 2 – Expression of Em*mpk2* in *E. multilocularis* larval stages. (A) Semi-quantitative RT-PCR analysis of Em*mpk2* expression in different *E. multilocularis* larval stages. Serial, 10-fold dilutions of cDNA preparations from in vitro cultivated metacestode vesicles (lanes 1–3) and protoscoleces prior to (lanes 4–6) and after (lanes 7–9) activation with low pH/pepsin were used as templates for PCR reactions specific for Em*mpk2* and the constitutively expressed control gene *elp* [33,36]. (B) Detection of phosphorylated EmMPK2 in larval stages. Cell lysates from in vitro cultivated metacestode vesicles (lane 1), non-activated protoscoleces (lane 2) and activated protoscoleces (lane 3) were separated on a 12% acrylamide gel and transferred to nitrocellulose membranes. Western blot detection was performed using an anti-phospho p38 MAPK antibody. As a control for equal protein concentrations, the ezrin-radixin-moesin like protein Elp was detected using antibody mAb2810 [29].

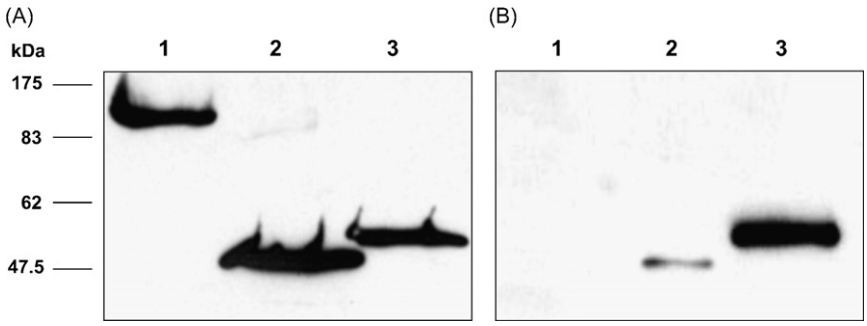

Fig. 3 – Heterologous expression and autophosphorylation of EmMPK2 in *E. coli*. EmMPK2 (lanes 3), human p38 MAPK-α (lanes 2) and β-galactosidase (lanes 1; control) were expressed in *E. coli*, purified via a hexahistidine tag and separated on a 12% acrylamide gel. Western blot detection was performed with antibodies against the V5-epitope (A) and an antiserum against phosphorylated human p38 MAPK (B). Marker sizes are indicated to the left.

The amino acid sequence context around the T-G-Y motif that is phosphorylated through MAPKKs or through p38 MAPK autoactivity is well conserved between EmMPK2 and human p38 MAPK-α (Fig. 1). We therefore tested whether a commercially available antibody against the phosphorylated form of the human enzyme, α-P-p38, can also be used to detect activated EmMPK2. As further outlined below (Section 3.3), the α-P-p38 antibody intensely cross-reacted with heterologously expressed, autophosphorlyated EmMPK2 and should therefore be suitable to detect the phosphorylated form of the enzyme also in parasite cells. When the α-P-p38 antibody was used in Western blots against lysates of the parasite, a pattern of 5–6 interacting protein bands was detected of which only one had the expected size of 42 kDa. This band was present at equal amounts in the metacestode and in the activated protoscolex (Fig. 2B). Furthermore, this band was also clearly detectable in the resting protoscolex, albeit to a somewhat lower extent than in the metacestode or the activated protoscolex (Fig. 2B). Altogether, these data indicate that EmMPK2 is expressed and present in a phosphorylated state in metacestode vesicles and protoscoleces.

3.3. *Enzymatic activity and inhibition of EmMPK2*

Heterologous expression in *E. coli* has previously been used to study p38 MAPK kinase biochemistry [41–43,45]. These studies revealed that constitutively active, mutant forms of p38 MAPKs from yeast and mammals are capable of autophosphorylation and of phosphorylating MBP as a downstream target [42,43,45]. We employed a similar approach to study the activity of EmMPK2. Both human p38 MAPK-α and EmMPK2 were expressed in *E. coli* as C-terminal fusions to the V5 antibody epitope and a hexahistidine tag. After purification of the recombinantly expressed proteins under native conditions, autophosphorylation activity was measured using the α-P-p38 antibody. As shown in Fig. 3, EmMPK2 displayed a significantly higher capability of autophosphorylation than the human enzyme.

We then tested the purified enzymes in MBP-based MAPK activity assays in the presence or absence of the known p38 MAPK inhibitors SB202190 [27] and ML3403 [28]. Under these conditions, EmMPK2 displayed both a marked autophosphorylation activity and a pronounced activity to phosphorylate MBP while the assay was not sensitive enough to detect activities of wild-type p38 MAPK-α (Fig. 4). EmMPK2 activity

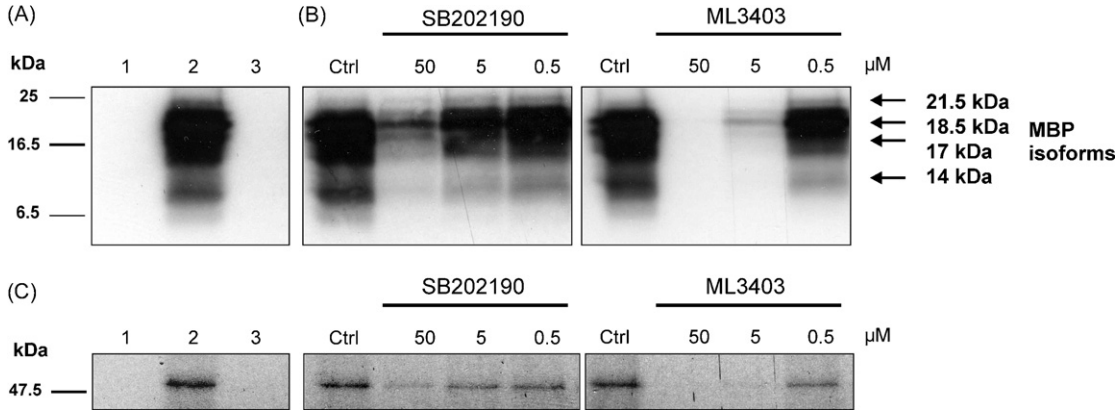

Fig. 4 – In vitro activity of EmMPK2. (A) Heterologously expressed and purified β-galactosidase (lane 1), EmMPK2 (lane 2) and human p38 MAPK-α (lane 3) were used in MAPK activity assays using myelin basic protein (MBP; four isoforms of 21.5, 18.5, 17 and 14 kDa, as indicated to the right) as the substrate. (B) Activity assays were performed as in (A) but in the presence of indicated concentrations of the p38 MAPK inhibitors SB202190 and ML3403. Ctrl. indicates the DMSO control. The incorporation of radioactively labeled phosphate into MBP (A and B) and into EmMPK2 (C; through autophosphorylation) was detected.

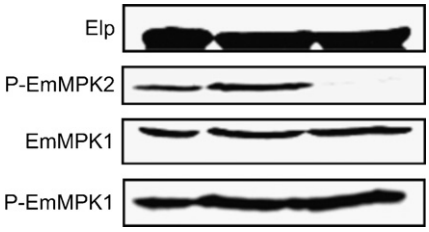

Fig. 5 – EmMPK2 activity in inhibitor-treated metacestode vesicles. Metacestode vesicles were incubated in the presence of inhibitor ML3403 (10 μM) for 3.5 h. Lysates were produced, separated on a 12% acrylamide gel and transferred onto nylon membranes. Western blot detection was performed using antibodies against phosphorylated p38 MAPK (P-EmMPK2) as well as against non-phosphorlyated (EmMPK1) and phosphorylated (P-EmMPK1) Erk1/2 kinase. As a control for equal protein concentrations, antibody mAB2810 [29] against the constitutively expressed protein Elp was used. Lane 1: untreated vesicles. Lane 2: DMSO-treated vesicles. Lane 3: ML3403-treated vesicles.

was clearly inhibited in the presence of SB202190 and even more pronounced in the presence of ML3403 in a concentration-dependent manner (Fig. 4).

Taken together, these data indicate that EmMPK2 is an enzymatically active MAPK with significantly higher basal activity (towards MBP and towards itself as substrates) than human p38 MAPK-α and that its activity can be effectively inhibited by p38 MAPK inhibitors designed to bind to the mammalian enzyme.

3.4. *Effects of p38 MAPK inhibitors on in vitro cultivated metacestode vesicles*

To test the effects of p38 MAPK inhibitors on *E. multilocularis* larvae, we first isolated intact metacestode vesicles that had been cultivated in vitro in the presence of rat Reuber hepatoma feeder cells [31,32]. Even when cultivated in the absence of host cells in serum-containing medium, such vesicles remain fully viable for at least 14 days [33]. Using these vesicles, we tested the effect of ML3403 and SB202190 on the phosphorylation state of EmMPK2 since our above experiments already indicated that the parasite enzyme is capable of pronounced autophosphorylation. While phospho-EmMPK2 was clearly detectable in control settings with or without DMSO, the reactive 42 kDa band fully disappeared after incubation in the presence of 10 μM ML3403 for 3.5 h (Fig. 5), indicating that the p38 MAPK inhibitor effectively blocked EmMPK2 autophosphorylation activity in the parasite. A similar, albeit less pronounced effect was observed when SB202190 was used instead of ML3403 (data not shown). On the other hand, no effects were observed on double phosphorylation of the previously described [21] Erk-like MAPK EmMPK1 (Fig. 5).

For *E. multilocularis* metacestode vesicles, the release of alkaline phosphatase (AP) into the culture supernatant has previously been used as an indicator to demonstrate loss of viability upon drug treatment [37]. We therefore performed AP activity assays with supernatant of ML3403-treated metacestode vesicles. Interestingly, although inhibitor concentrations up to 0.5 mM led to a slight increase in AP activity, clear effects were only observed using concentrations as high as 1 mM (data not shown).

Next we measured the effect of p38 MAPK inhibitors on vesicle integrity. As shown in Fig. 6, starting from day 2 of incubation in the presence of inhibitors, vesicle integrity started to decline depending on the concentration of the inhibitors. After 4 days of incubation in the presence of 50 μM of ML3403, no intact vesicle remained and only 20% intact vesicles were present after 4 days of treatment with 5 μM of this inhibitor (Fig. 6F and G). Although SB202190 also showed significant effects on vesicle integrity, these were never as pronounced as with ML3403 (Fig. 6E and G). The IC_{50} values we measured for both inhibitors in this assay were 3.8 μM for SB202190 and 55 nM for ML3403 (Fig. 6H).

Although treatment with 50 μM ML3403 for 4 days showed drastic effects on vesicle integrity, it was not clear whether all cells of the germinal layer were effectively inactivated. We have very recently demonstrated that isolated *E. multilocularis* germinal cells are capable of fully regenerating infective metacestode vesicles even when they are no longer part of an intact germinal layer [31]. Thus, to test the regeneration capacity of p38 inhibitor-treated *Echinococcus* cells, primary cell cultures from inhibitor-treated vesicles and from DMSO controls were set up and maintained for 4 weeks. While primary cells from DMSO-treated vesicles (Fig. 6A) were capable of regeneration (i.e. formed small vesicles and cell aggregates that give rise to vesicles [31]; Fig. 6C), no vesicle regeneration or formation of cell aggregates was observed for primary cells that derived from ML3403-treated vesicles (Fig. 6B and D). Similarly, no cyst-forming capacity was observed for primary cells that derived from vesicles after 4 days treatment with 50 μM SB202190 (data not shown).

Taken together, the above experiments demonstrated that ML3403 and, to a lesser extent, SB202190 effectively inactivated in vitro cultivated metacestode vesicles and that this treatment was associated with strongly reduced phosphorylation of EmMPK2.

3.5. *Effects of p38 MAPK inhibitors on cultivated parasite cells*

After having demonstrated that p38 MAPK inhibitors inactivated cultivated metacestode vesicles and that no regeneration from treated vesicles occurred, we also tested the situation in primary *E. multilocularis* cells. Primary cell cultures were set up as previously described [31] and, after 1 week of cultivation, Em*mpk2* expression was investigated using RT-PCR. As shown in Fig. 7A, the Em*mpk2* mRNA was clearly detectable in DMSO- and in ML3403-treated cultures. Furthermore, the 42 kDa protein band which reacted with the α-P-p38 antibody was present in primary cultures (Fig. 7B). Upon treatment with 5 μM ML3403 for 1 week, phospho-EmMPK2 was no longer detectable (Fig. 7B). As previously described [31], during 4 weeks of cultivation, DMSO-treated primary cells proliferated and formed massive cell aggregates with central cavities, indicative of vesicle formation (Fig. 7C). On the other hand, in SB202190-treated cultures and even more

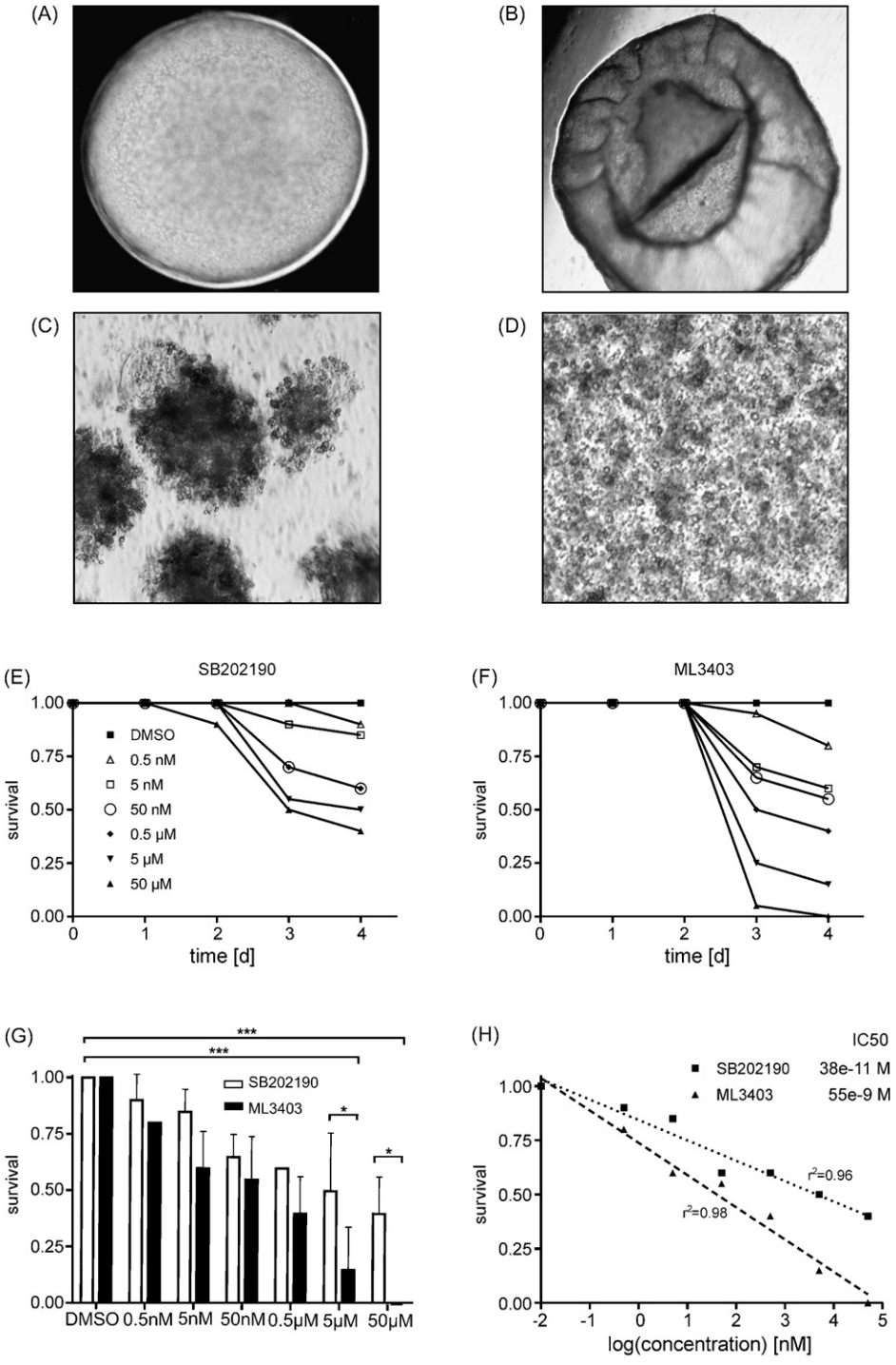

Fig. 6 – Effects of p38 MAPK inhibitors on the viability of cultivated larvae. (A) Intact, viable metacestode vesicle from in vitro culture. (B) Metacestode vesicle after treatment with ML3403 for 3 days. (C) Regeneration of metacestode vesicles from primary cells isolated from DMSO-treated vesicles after 4 weeks of in vitro culture. (D) Primary cell culture (identical to C) from ML3403-treated vesicles after 4 weeks. Note that no cell aggregates or vesicles are formed. (E) Effect of different concentrations of SB202190 on vesicle integrity and survival. (F) Same as in (E) with ML3403. (G) Survival of vesicles after 4 days treatment with different concentrations of SB202190 and ML3403. (H) Determination of IC_{50} values for the activities of SB202190 and ML3403 on metacestode vesicle survival.

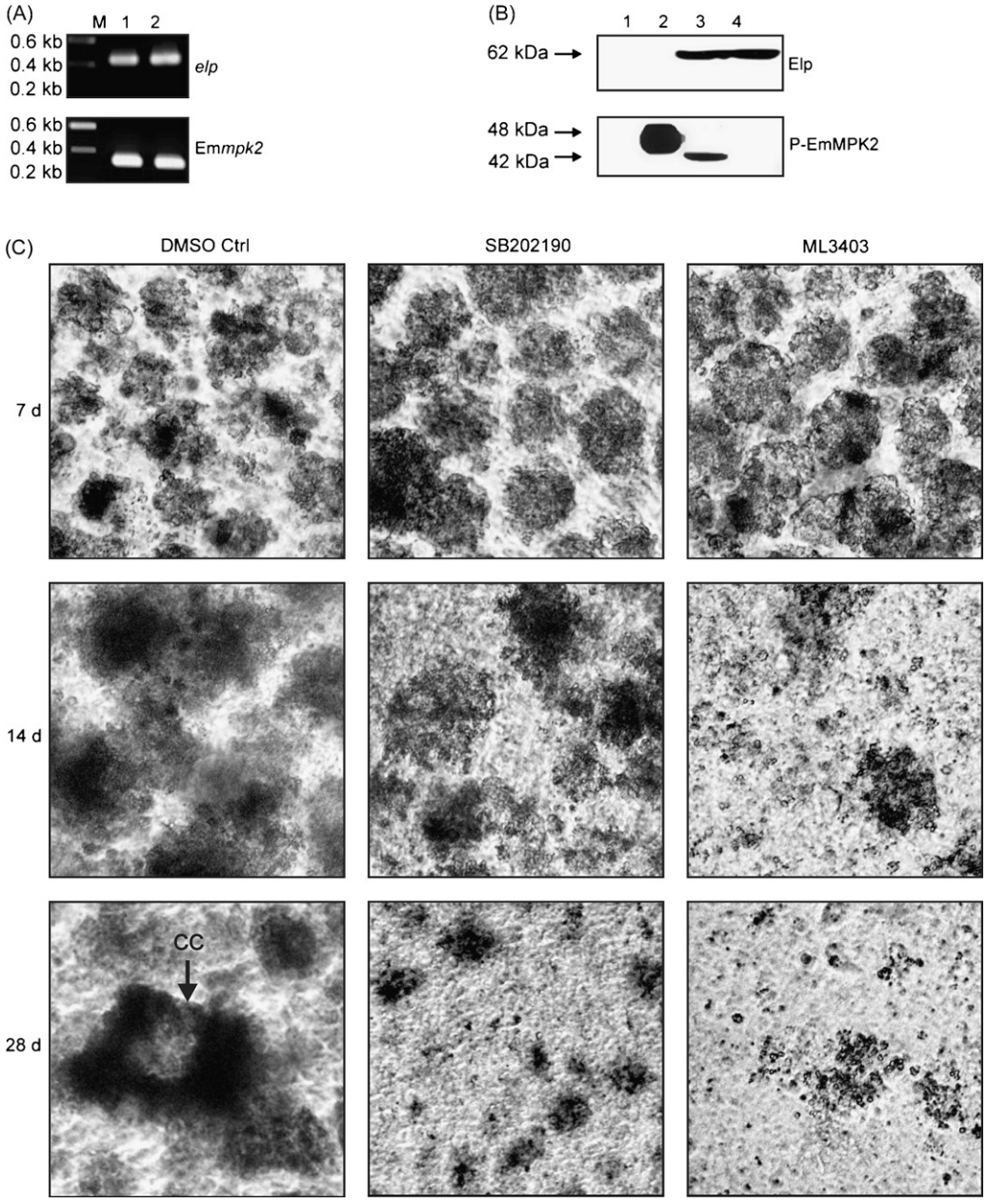

Fig. 7 – Em*mpk2* expression in *E. multilocularis* primary cells and effects of p38 MAPK inhibitors. (A) Semi-quantitative RT-PCR analysis of Em*mpk2* expression in *E. multilocularis* primary cells. Primary cell cultures were established from metacestode vesicles [31] and kept for 1 week in the presence of DMSO (lane 1; control) or 5 μM ML3403 (lane 2). cDNA preparations were then PCR analyzed using primers specific for Em*mpk2* or the constitutively expressed control gene *elp* [33,36]. (B) Detection of phosphorylated EmMPK2 in inhibitor-treated primary cells. Primary parasite cell cultures [31] were kept for 7 days in the presence of DMSO (lane 3) or 5 μM ML3403 (lane 4). Cell lysates were then separated on a 12.5% acrylamide gel, transferred to nitrocellulose membranes and Western blot detection was carried out using the anti-phospho p38 MAPK antibody (below; P-EmMPK2) and, as a control for equal protein concentrations, antibody mAb2810 [29] against the ezrin-radixin-moesin-like protein Elp (above). As controls, lysates were produced from *E. coli* recombinantly expressing EmMPK2 fused to the V5 antigen epitope and the hexahistidine tag (lane 2) or a control vector expression lacZ (lane 1). (C) Effect of p38 MAPK inhibitors on primary cells. *E. multilocularis* primary cell cultures were established as previously described [31] and cultivated for up to 4 weeks in the presence of DMSO (control) as well as 5 μM SB202190 or 5 μM ML3403 as indicated. After 7, 14, and 28 days, light microscopy was performed and the formation of cell aggregates was analyzed. A central cavity (CC), indicative of metacestode vesicle formation [31], is marked by an arrow.

pronounced in ML3403-treated cultures, the parasite cell aggregates degenerated and never formed central cavities. Particularly in the case of ML3403-treated cultures, full degeneration of cell aggregates was observed after 4 weeks of treatment, leaving only single cells and cell debris (Fig. 7C).

Taken together, these data indicated that, like in metacestode vesicles, EmMPK2 was present in its activated state in primary cells and its activity could be blocked by p38 MAPK inhibitors. The complete degeneration of primary cell aggregates and the occurrence of cell debris, particularly in the presence of ML3403, indicated effective killing of parasite cells upon treatment.

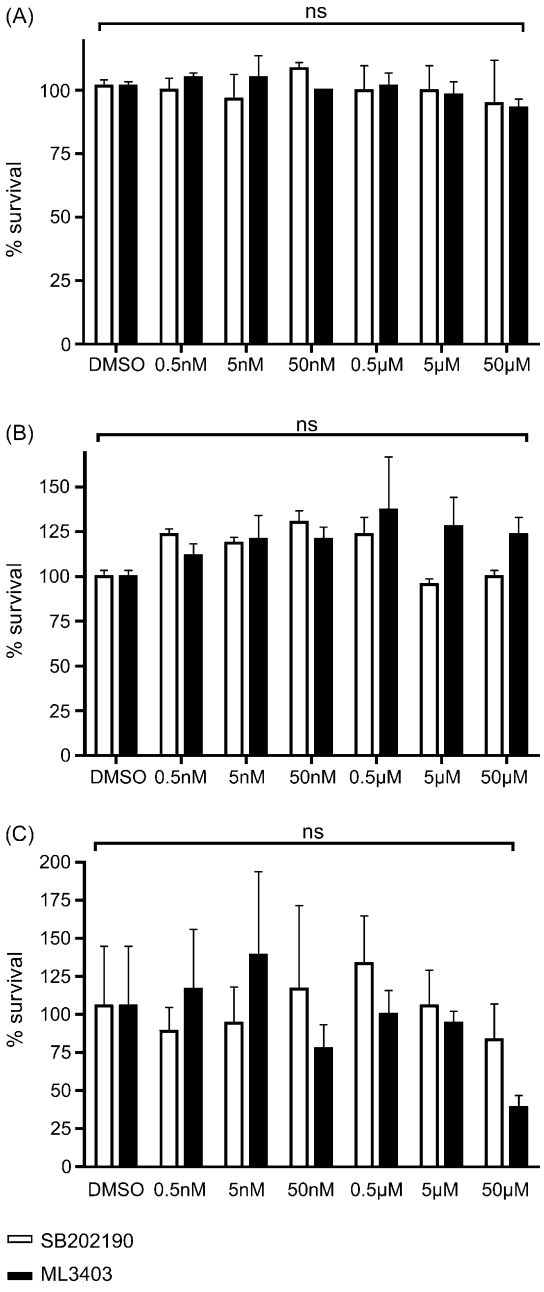

Fig. 8 – Effects of SB202190 and ML3403 on the viability of mammalian cell lines. (A) Survival of human brain microvascular endothelial (HBMEC) cells after 4 days of treatment. (B) Same as in (A) with human embryonic kidney (HEK-293) cells. (C) Same as in (A) with rat Reuber hepatoma (Rh^-) cells.

3.6. *Effects of p38 MAPK inhibitors on mammalian cells*

We finally tested whether concentrations of ML3403 and SB202190 which effectively inactivated in vitro cultivated metacestode vesicles had an affect on the viability of cultivated mammalian (host) cells. Cells of the three cell lines Rh^- (rat Reuber hepatoma), HEK-293 (human embryonic kidney) and HBMEC (human brain microvascular endothelial) were chosen which all express p38 MAPK-α as investigated by us through RT-PCR (data not shown). After cultivation for 4 days in the presence of 0.5 nM to 50 μM of both inhibitors, cell viability was assessed using a crystal violet staining assay. As shown in Fig. 8, no significant effects on viability were observed for either of these cell lines up to inhibitor concentrations of 5 μM. The only deleterious effect was observed for ML3403 at 50 μM on rat hepatoma cells. Hence, p38 MAPK inhibitor concentrations of 0.5 and 5 μM which effectively inactivated more than 50% of metacestode vesicles after 4 days of treatment in vitro had no measurable effect on the viability of Rh^-, HEK-293 and HBMEC cells cultivated under comparable conditions.

4. Discussion

Cell–cell-communication systems involving soluble cytokines, surface receptor kinases and intracellular signal transducers of the MAPK cascades have evolved very early in metazoan evolution and are, therefore, still present in all animals where they regulate a variety of important processes such as proliferation, differentiation and apoptosis [1]. In humans, these systems play pivotal roles in the formation of cancer and have been extremely well studied on the molecular and biochemical levels. Furthermore, many well-studied chemical compounds are available to modify the activities of signaling factors, particularly of tyrosine and serine/threonine kinases [24–26]. To also utilize these compounds for chemotherapeutic treatment of parasitic diseases is sensible, but requires that parasite molecules can be identified which display structural and/or functional differences to their human counterparts.

In the present study, we have identified such a molecule from the human parasitic cestode *E. multilocularis*. Several lines of evidence clearly indicate that EmMPK2 is a functionally active member of the p38 MAPK subfamily. First, EmMPK2 displays strongest homologies to p38 MAPKs from phylogenetically different organisms and contains a T-G-Y motif in the activation loop which is typically present in this subclass of MAPKs. Second, the exon–intron structure of the encoding gene, Em*mpk2*, is highly similar to that of mammalian p38 MAPK genes and differs from genes encoding Erk- or JNK/SAPK-like proteins. Third, EmMPK2 displays autophosphorylation activity which involves phosphorylation at the T-G-Y motif that is recognized by an antibody originally raised against human p38 MAPK. Fourth, recombinantly expressed, purified EmMPK2 phosphorylated MBP, a typical substrate of MAPKs. Fifth, the activity of EmMPK2 could be blocked by inhibitors specifically designed against mammalian p38

MAPKs. Genomic analyses further indicate that EmMPK2 is the only member of the p38 MAPK family in *E. multilocularis*. Due to the important role of p38 MAPKs in cellular signaling of animal cells, it is therefore not surprising that we could measure constitutive expression of Em*mpk2*, at least in the larval stages that are involved in the infection of the intermediate host (including primary cells which give rise to metacestode vesicles). Compared to metacestode vesicles and activated protoscoleces, a somewhat lower level of phosphorylated EmMPK2 was detected in resting protoscoleces. As will be discussed below, one of the functions of EmMPK2 could be, like in the case of other p38 MAPKs, the induction of stress responses. The lower levels of phospho-EmMPK2 in resting protoscoleces might therefore be due to the fact that this stage is located within metacestode vesicles and thus relatively well protected from the host's immune response (having the metacestode's germinal and laminated layers as first lines of defense). On the molecular level, it still remains to be established whether the lower phospho-EmMPK2 levels in resting protoscoleces are due to diminished translation of the protein, due to differences in the activity of upstream regulators, or due to lower autophosphorylation activity. However, based on the robust autophosphorylation activity of EmMPK2 after expression in *E. coli* and the fact that we could not yet identify a corresponding upstream MAPK kinase (see below), we rather favor translational effects. As an alternative, corresponding dual-specific phosphatases which are present in the parasite's genome (our own unpublished observations), could have elevated activities in the resting protoscolex.

One of the most important findings of this study is that EmMPK2 displays a significantly higher basal activity (towards MBP and towards itself as a substrate) than p38 MAPK orthologues from humans. Although we have only tested the physiologically most important human isoform p38-α, no other isoform (β, γ, δ) has been reported to display a basal activity higher than that of p38-α [18,42]. The molecular basis of the high basal activity of EmMPK2 is unknown at present. Overall, the protein displays significant homologies to human p38 MAPKs but also contains many amino acid exchanges which could affect folding and thus basal activity, the interaction with upstream regulators and the responsiveness to extracellular signals. Interestingly, when screening the yeast p38 MAPK orthologue HOG1 for activating mutations, Bell et al. [41] and Diskin et al. [43] identified several hyperactive forms that carried exchanges within domains that are important in forming the interface for p38 MAPK dimerization [46]. When introduced into human p38 MAPKs, exchanges in three of these residues ($Asp^{176}Ala$, $Tyr^{323}Ser$, $Tyr^{323}Leu$, $Phe^{327}Leu$, $Phe^{327}Ser$ in p38-α) also led to constitutively hyperactive forms [42]. In EmMPK2, the residues at the corresponding positions are Asn^{177} (instead of Asp), Glu^{325} (instead of Tyr) and Ile^{329} (instead of Phe) (Fig. 1). Although none of the residues in EmMPK2 exactly matches those which had been exchanged in the constitutively active p38 mutants, an involvement of these amino acids in the elevated basal activity of EmMPK2 is likely (particularly for Ile^{329} which should be very similar to Leu at that position). Hence, it appears that mutations which have to be introduced into yeast and mammalian p38 MAPKs to yield constitutively hyperactive forms, are already present in the wild-type form of EmMPK2. A plausible explanation for the expression of a constitutively active stress kinase with high basal activity by the metacestode could lie in the fact that this larval stage is under constant attack by the host immune system throughout development, so that specific mechanisms for stress-induced kinase upregulation are simply not required.

Questions concerning the cellular function of EmMPK2 and its participation in signaling pathways in *E. multilocularis* cannot be conclusively answered at present. At least on the basis of our inhibitor studies (see below), an important role of the kinase in parasite viability is apparent. When investigating the influence of different stress conditions (e.g. hydrogen peroxide, paraquat or DMSO as producers of reactive oxygen intermediates) on EmMPK2 activity, we only observed a slight increase in phosphorylation upon physical disruption of metacestode vesicles (data not shown). This could indicate a role in osmotic stress regulation but surely requires further investigation. Likewise it is unclear whether EmMPK2 is regulated in a similar fashion as p38 MAPKs in other organisms. Up to now, three different ways of p38 MAPK activation have been reported of which two are unlikely to occur in *E. multilocularis*. These are the activation through phosphorylation at a non-canonical activating residue, Tyr^{323} in p38-α, which occurs in mammalian T cells upon activation of the T cell antigen receptor (TCR) and the TGF-β-dependent activation involving direct interaction between p38-α and TAB1 (Transforming growth factor-β-activated protein 1 (TAK1)-binding protein) [17]. According to our analyses of genomic *E. multilocularis* sequences, the necessary compounds (such as TCR- or TAB1- orthologues) are absent in the parasite. On the other hand, since EmMPK2 carries a possibly phosphomimetic Glu^{325} at a position equivalent to Tyr^{323}, this could be one mechanism which contributes to the high basal activity of the parasite enzyme. The best studied pathway for p38 MAPK activation is dual-phosphorylation at the T-G-Y motif by upstream MAPKKs [17]. For these interactions, however, the conserved Asp residues of the common docking domain are highly important [44] and at least one of these residues is replaced by Asn in EmMPK2 (Fig. 1). A similar exchange in the *Drosophila* MAPK, Rolled, renders the insect enzyme constitutively active and severely weakens the interaction with regulators such as MAPKKs or phosphatases [46]. Since we could already identify two different members of the MAPKK family in *E. multilocularis* of which none interacted with EmMPK2 in yeast two-hybrid and in activity assays (our own unpublished results), it is indeed possible that EmMPK2 is only under very limited control by upstream regulatory pathways. This is further supported by analyses in which we never observed effects of cytokines such as EGF, insulin, TGF-β or bone morphogenetic protein on the EmMPK2 phosphorylation state when added exogenously to metacestode vesicles (data not shown), although, under similar conditions, the Erk-like MAPK EmMPK1 is clearly activated, at least by EGF-treatment [21].

Hence, taken together all structural and functional data we suggest that the accumulation of activating mutations, particularly the possibly phospho-mimetic Glu at position 325 (which was invariably present in all genomic clones) and the alteration of the common docking domain, rendered EmMPK2 a deregulated, constitutively active MAPK. As a

consequence, EmMPK2 phosphorylation at the conserved T-G-Y motif should solely depend on EmMPK2 autophosphorylation activity which is supported by our data in which the treatment of metacestode vesicles or primary cells with p38 MAPK inhibitors led to the complete dephosphorylation of EmMPK2. In this context, it still remains to be established whether EmMPK2 autoactivity leads to dual-phosphorylation of both Thr^{181} and Tyr^{183}, as has been reported for TCR-activated p38-MAPKα [47] or only to the phosphorylation of Thr^{181}, as observed for hyperactive L16-loop mutants of HOG-1 and human p38 MAPKs [43]. In principle, our data support both scenarios since antibodies against the dual-phosphorylated pT-G-pY motif of p38 MAPKs very frequently also recognize mono-phosphorylated pT-G-Y [43] and a similar situation cannot be excluded for our experiments with EmMPK2. For our model of EmMPK2 being a constitutively hyperactive p38 MAPK, on the other hand, this distinction seems not to be critical since it has already been firmly established that threonine-, but not tyrosine-phosphorylation within the T-G-Y motif is necessary for obtaining hyperactive forms of the enzyme [43].

Our biochemical analyses showed that EmMPK2 can be effectively inhibited by the p38 MAPK inhibitors SB202190 and, in particular, by ML3403. Both substances are cell-permeable pyridinyl imidazole compounds that act on p38 MAPKs in an ATP-competitive manner [28,48]. While SB202190 belongs to the first generation of pyridinyl imidazole inhibitors which exhibit severe liver toxicity through interference with hepatic cytochrome P450 enzymes, the toxic side effects have been minimized in second generation inhibitors such as ML3403 [48]. In studies on the widely used model compound SB203580, it has previously been shown that two residues in the ATP-binding pocket of target kinases, Met^{109} and Lys^{53} of p38-α, are in direct contact with pyridinyl imidazoles and contribute decisively to inhibition [18,45]. In EmMPK2, these two residues are invariably present (Met^{110} and Lys^{54}). Furthermore, it is known that target specificity is mainly conferred through one single residue, Thr^{106} in p38-α [18]. Mutation of this residue in p38-α renders the enzyme insensitive to pyridinyl imidazole inhibitors while introduction of the residue into Erk- or JNK/SAPK-like MAPKs leads to sensitivity towards this class of compounds [18,49]. In EmMPK2, this residue is present at the corresponding position (Thr^{107}) which, together with the presence of Met^{110} and Lys^{54}, explains the sensitivity of EmMPK2 towards pyridinyl imidazoles. In EmMPK1, on the other hand, Thr^{107} is replaced by Asp^{102} [21], which is in concordance with our data showing that the Erk-like MAPK of the parasite is not affected by p38 inhibitor treatment. Apart from the three residues mentioned above, structural studies by Gum et al. [45] revealed 10 additional residues that are involved in p38-α inhibition through SB203580, the exchanges of which either lead to higher or lower affinity between enzyme and drug. Four of these are different between EmMPK2 and human p38-α (Fig. 1). Taken into consideration that different pyridinyl imidazole compounds display a wide variety of activities towards the four human p38 MAPK isoforms (SB203580 strongly inhibits isoforms p38-α and β, but not γ or δ), depending on amino acid residue exchanges in critical positions [18], it should be possible to obtain, by chemical modification of ML3403, compounds that display high activity against EmMPK2 while being only weakly active on human p38 MAPKs. On the basis of the EmMPK2 activity assay established in this work, we are currently setting up screening assays in which different pyridinyl imidazoles are tested for high affinity towards EmMPK2 while exerting diminished inhibitory activity towards human p38 isoforms.

Both p38 MAPK inhibitors tested in this study, but particularly ML3403, proved to be effective in inactivating in vitro cultured parasite vesicles. After 4 days of ML3403 treatment at concentrations of 0.5–5 μM, which showed no effects on cultured mammalian cells that express p38-α, more than 50% of metacestode vesicles lost structural integrity and displayed no further capacity to regenerate novel vesicles. It was unexpected that much higher doses of the drug had to be given to see effects in the alkaline phosphatase (AP) assay since, in former studies using benzimidazoles or nitazoxanide as drugs, this method proved to be very sensitive [9,12,37]. However, at least in mammalian cells it is well established that AP expression is controlled through the p38 MAPK pathway [50,51] and a similar scenario cannot be ruled out in *E. multilocularis*. As a consequence, ML3403 treatment and the inhibition of EmMPK2 could have led to decreased overall levels of the enzyme in metacestode vesicles so that significant activities in the supernatant were only detectable after killing of many parasite cells in the presence of 1 mM of the drug.

As yet it is not clear whether the elevated sensitivity of *E. multilocularis* towards ML3403, when compared to cultured mammalian cells, is due to better binding of the drug to EmMPK2 than to mammalian p38 MAPKs. This will be revealed once comparable activity assays are available. We rather suppose, however, that it is the difference in the basal activity of EmMPK2 and mammalian p38 MAPKs which renders the parasite more sensitive. While the mammalian isoforms are only activated under certain stress conditions or in response to specific cytokines, the parasite's cell physiology seems to be highly adapted to constitutive p38 MAPK activity which could result in high sensitivity if parts of this activity are knocked down by specific drugs. If EmMPK2 is also constitutively activated during in vivo infections, which must be assumed, ML3403 could already be an effective drug for AE treatment. At least in *Toxoplasma gondii* infected mice, curative, long-term treatment with second generation p38 MAPK inhibitors did not show detrimental effects on host viability or immune responses [52]. Furthermore, orally administered doses of ML3403 that led to plasma concentrations of the drug in the range between 0.5 and 5 μM were well tolerated by the animals [28]. In parallel to screening pyridinyl imidazole libraries for compounds with high affinity for EmMPK2, we are therefore also investigating the effect of ML3403 on *E. multilocularis* larvae in infected intermediate hosts.

In conclusion, in the present study we demonstrated that ATP-competitive p38 MAPK inhibitors, a class of compounds which has already been successfully used to treat parasitic infection of laboratory animals [52] and which is subject of clinical trials to treat p38 MAPK-associated human diseases [53], display a pronounced activity against *E. multilocularis* larvae cultivated in vitro. In addition, we identified and characterized the cellular target which most likely mediates these inhibitory effects and set up biochemical assays by

which pyridinyl imidazole compound libraries can be screened for substances with high antiparasitic specificity and diminished activity against host p38 MAPKs. In future investigations, this can lead to a novel class of chemotherapeutic drugs for effective AE treatment as an alternative or a supplement to benzimidazoles which are used today.

Acknowledgements

This work was supported by grants SFB 479 and the international graduate college 1141 (both to K.B.) from the Deutsche Forschungsgemeinschaft. We wish to thank Dirk Radloff and Monika Bergmann for excellent technical assistance. The authors address special thanks to Dr. Peter D. Olson (Natural History Museum, London) for critically reading the manuscript. Sequence data to screen the *E. multilocularis* genome for p38 MAPK orthologues have been produced by the Pathogens Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/Echinococcus.

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