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Research paper

Molecular and biochemical characterization of methionine aminopeptidase of *Babesia bovis* as a potent drug target

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ABSTRACT

Aminopeptidases are increasingly being investigated as therapeutic targets in various diseases. In this study, we cloned, expressed, and biochemically characterized a member of the methionine aminopeptidase (MAP) family from *Babesia bovis* (*B. bovis*) to develop a potential molecular drug target. Recombinant *B. bovis* MAP (rBvMAP) was expressed in *Escherichia coli* (*E. coli*) as a glutathione S-transferase (GST)-fusion protein, and we found that it was antigenic. An antiserum against the rBvMAP protein was generated in mice, and then a native *B. bovis* MAP was identified in *B. bovis* by Western blot assay. Further, an immunolocalization assay showed that MAP is present in the cytoplasm of the *B. bovis* merozoite. Analysis of the biochemical properties of rBvMAP revealed that it was enzymatically active, with optimum activity at pH 7.5. Enhanced enzymatic activity was observed in the presence of divalent manganese cations and was effectively inhibited by a metal chelator, ethylenediaminetetraacetic acid (EDTA). Moreover, the enzymatic activity of BvMAP was inhibited by amastatin and bestatin as inhibitors of MAP (MAPi) in a dose-dependent manner. Importantly, MAPi was also found to significantly inhibit the growth of *Babesia* parasites both *in vitro* and *in vivo*; additionally, they induced high levels of cytokines and immunoglobulin (IgG) titers in the host. Therefore, our results suggest that BvMAP is a molecular target of amastatin and bestatin, and those inhibitors may be drug candidates for the treatment of babesiosis, though more studies are required to confirm this.

1. Introduction

Babesiosis is a tick-borne protozoan disease caused by the genus *Babesia*. The disability and the poverty associated with this disease constitute large burdens on the health and economic development of low- and middle-income countries in the world. Strategies to control this disease are based on surveillance, early diagnosis, vector control, and treatment. A few drugs are on the market to treat parasitic disease, but they are not universally available in the affected areas. The problems with currently available drugs are inefficiency, toxicity, prolonged treatment schedule, and high cost. Therefore, there is an urgent need for new treatments that are safe, effective, cheap, and easy to administer and for a lead compound with novel mechanisms of action.

In past decades, aminopeptidases have been increasingly investigated as therapeutic and prophylactic targets in various diseases, including many parasitic infections (Molina et al., 2002; Chen et al., 2009; Kang et al., 2012, 2015). Generally, aminopeptidases are classified by the number of amino acids cleaved from the N-terminus of substrates, and classification is performed with respect to the relative efficacy with which residues are removed. Methionine aminopeptidases (MAPs), a family of aminopeptidases, play an important role in N-terminal methionine excision from the polypeptides during protein synthesis, and they have been identified in numerous microorganisms, plants, vertebrates, and invertebrates (Lowther and Matthews, 2000; Addlagatta et al., 2005). The physiological importance of this family of enzymes is apparent in the lethality of its absence in both bacteria and yeast (Chang et al., 1989, 1992; Li and Chang, 1995). Recently, malarial MAPs have been determined to play important roles in parasite biology due to their role in parasite hemoglobin during peptide catabolism (Gavigan et al., 2001; Chen et al., 2006, 2009; Naughton

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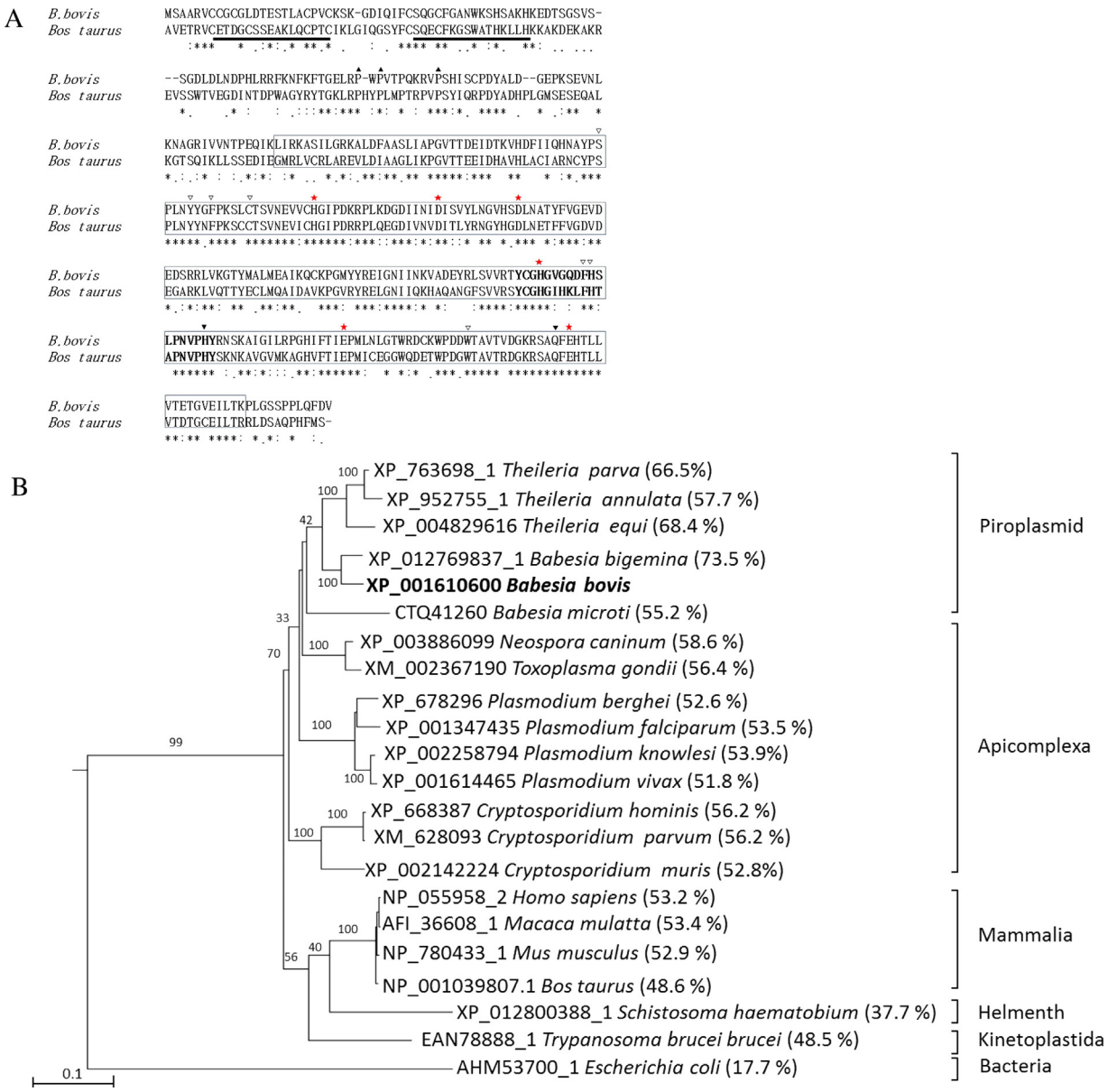

Fig. 1. Bioinformatic analysis of translated BvMAP polypeptide. (A) MAP amino acid sequences for *B. bovis* and *Bos taurus* were aligned using the CLUSTAL X program. Gaps were introduced to maximize the alignment. The putative zinc finger motifs are underlined in the sequence. Prolines conserved in the connector region are shown in closed triangles. The signature of the MAP1 subfamily is in boldface type, and the metallopeptidase family M24 domain is boxed in blue in the sequence. The conserved metal-binding residues are marked with red stars. (B) Phylogenetic tree of the deduced amino acid sequence of the BvMAP with the MAP protein identified from apicomplexan parasites and other organisms using the neighbor-joining method with the NJplot program. Gene bank accession numbers of the amino acid sequences used to construct the phylogenetic tree: *Babesia bovis* (XP_001610600), *Babesia bigemina* (XP_012769837), *Babesia microti* (XP_0126492711), *Theileria parva* (XP_763698), *Theileria annulata* (XP_952755), *Theileria equi* (XP_004829616), *Toxoplasma gondii* (XM_002367190), *Plasmodium falciparum* (XP_001347435), *Plasmodium vivax* (XM_001614465), *Plasmodium berghei* (XP_678296), *Plasmodium knowlesi* (XP_002258794), *Neospora caninum* (XP_003886099), *Cryptosporidium hominis* (XP_668387), *Cryptosporidium parvum* (XM_628093), *Cryptosporidium muris* (XP_002142224), *Trypanosoma brucei brucei* (EAN78888), *Schistosoma haematobium* (XP_012800388), *Bos taurus* (NP_001095507), *Mus musculus* (NP_7804331), *Macaca mulatta* (AF1366081), *Homo sapiens* (NP_05595582), and *Escherichia coli* (AHM53700). Numbers shown at branch nodes indicate bootstrap values. Percent identities between amino acid sequences were calculated using EMBOSS Needle, an online program (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

et al., 2010). Furthermore, several previous studies documented that inhibitors of MAP have been shown to have a therapeutic effect on *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Cryptosporidium parvum* (*C. parvum*), *Enterocytozoon bieneusi* (*E. bieneusi*), and *Encephalitozoon hellem* (*E. hellem*) both *in vitro* and *in vivo* (Molina et al., 2002; Chen et al., 2009; Kang et al., 2012, 2015). Among the inhibitors of MAP, bestatin (ubenimex) and amastatin are well known to cause antimalarial activity in cultures and in mouse models (Nankya-Kitaka et al., 1998; Naughton et al., 2010). Importantly, bestatin induces immunomodulatory effects on certain immune effector cells but has very low toxicity in experimental animals and humans (Abe et al., 1989; Inoi et al., 1995). In a case of babesial MAP, although a MAP gene of *B. bovis* (BvMAP) is available in the genome of *B. bovis* (BBOV_IV006760), it is not clear whether the gene encodes a functional enzyme crucial for the survival of the parasite. Therefore, in the present study, we characterized a member of the MAP family from highly pathogenic *B. bovis* as a potential molecular drug target for babesiosis.