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The methylerythritol phosphate pathway for isoprenoid biosynthesis in coccidia: Presence and sensitivity to fosmidomycin

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Abstract

The apicoplast is a recently discovered, plastid-like organelle present in most apicomplexa. The methylerythritol phosphate (MEP) pathway involved in isoprenoid biosynthesis is one of the metabolic pathways associated with the apicoplast, and is a new promising therapeutic target in *Plasmodium falciparum*. Here, we check the presence of isoprenoid genes in four coccidian parasites according to genome database searches. *Cryptosporidium parvum* and *C. hominis*, which have no plastid genome, lack the MEP pathway. In contrast, gene expression studies suggest that this metabolic pathway is present in several development stages of *Eimeria tenella* and in tachyzoites of *Toxoplasma gondii*. We studied the potential of fosmidomycin, an antimalarial drug blocking the MEP pathway, to inhibit *E. tenella* and *T. gondii* growth *in vitro*. The drug was poorly effective even at high concentrations. Thus, both fosmidomycin sensitivity and isoprenoid metabolism differs substantially between apicomplexan species.

Index Description and Abbreviations: Apicomplexa; Coccidia; *Cryptosporidium parvum*; *Cryptosporidium hominis*; *Eimeria tenella*; *Plasmodium falciparum*; *Toxoplasma gondii*; Fosmidomycin; Isoprenoid biosynthesis; MEP, methylerythritol phosphate; MVA, mevalonic acid; RT-PCR, reverse transcription-polymerase chain reaction; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; IPPI, isopentenyl diphosphate isomerase; CMS, 4-diphosphocytidyl methylerythritol synthase; *Etdxs*, *E. tenella* deoxyxylulose 5-phosphate synthase; *Etmecs*, *E. tenella* methylerythritol 2,4-cyclodiphosphate synthase; *Tgdxr*, *T. gondii* deoxyxylulose 5-phosphate reductoisomerase; *Tghdr*, *T. gondii* hydroxymethylbutenyl 4-diphosphate reductase; *Tgmecs*, *T. gondii* methylerythritol 2,4-cyclodiphosphate synthase

1. Introduction

Apicomplexan parasites are some of the most widespread and poorly controlled in the world. It is estimated that 40% of the world's population is at risk of malaria with more than 300 million new cases and 2.7 million deaths annually (Gardner et al., 2002). The largest subgroup of the phylum Apicomplexa is the suborder Eimeriorina which contains organisms collectively referred to as the coccidia (Levine, 1980). Most coccidia are intestinal parasites and infect most phyla of vertebrate classes. The disease they cause, coccidiosis, is still a major health hazard in domestic animal husbandry. There is no effective treatment available for human cryptosporidiosis, a diarrhoeal disease caused by *Cryptosporidium parvum* or *Cryptosporidium hominis*. Current treatments for toxoplasmosis are poorly tolerated and are ineffective against the long-lived encysted bradyzoïte stage. Eimeriosis is the most costly disease affecting the poultry industry; its development is controlled since several years by chemotherapy and more recently for certain productions, by the use of live attenuated

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vaccines. However, the extensive use of chemotherapeutic compounds leads to drug resistance and there is an urgent need for the development of new therapeutic products active against all these diseases. To use the modern approach to drug discovery would involve identification of possible drug targets by exploring the unique metabolism of Apicomplexa. There are many significant biochemical differences between coccidia and their hosts (for review see (Coombs and Muller, 2002)), including polyamine biosynthesis, the shikimate pathway, and the mannitol cycle.

Recently, a unique non-photosynthetic plastid named the apicoplast and which is essential for cell survival was discovered in some apicomplexan parasites including *Plasmodium falciparum* and *Toxoplasma gondii*. This has been followed by interest in the metabolic pathways present in this organelle, particularly type II fatty acid synthesis and isoprenoid biosynthesis (Wilson, 2002).

All isoprenoids are derived from a basic five-carbon unit, isopentenyl diphosphate (IPP), and its allyl isomer dimethylallyl diphosphate (DMAPP). Both compounds are the substrates for prenyltransferases catalyzing prenyl chain elongation (Liang et al., 2002). There are two known metabolic routes leading to IPP (Kuzuyama, 2002): the classical mevalonic acid (MVA) pathway and the more recently discovered methylerythritol phosphate (MEP) pathway. The detailed steps of these pathways are shown in Fig. 1. The MVA pathway starts with the condensation of acetyl-CoA and acetoacetyl-CoA and terminates with the synthesis of IPP, which is then reversibly converted to DMAPP in a reaction catalyzed by IPP isomerase type 1 (Anderson et al., 1989) or type 2 (Kaneda et al., 2001). The MEP pathway utilizes pyruvate and glyceraldehyde 3-phosphate as the initial precursors and leads to the production of both IPP and DMAPP.

Mammals produce isoprenoids via the MVA pathway, but *P. falciparum*, plastids of higher plants and some bacteria use the MEP pathway. The antibiotic fosmidomycin, originally isolated from *Streptomyces lavendulae*, inhibits the deoxyxylulose 5-phosphate reductoisomerase, the enzyme catalyzing the second step of the MEP pathway (Fig. 1). The group of Jomaa reported that fosmidomycin blocks the development of *P. falciparum in vitro* and cures mice of virulent rodent malaria infection (Jomaa et al., 1999). The combination of fosmidomycin–clindamycin (Borrmann et al., 2004) or fosmidomycin–artenusate (Borrmann et al., 2005) given orally was effective against the development of *P. falciparum* in pediatric outpatients with malaria in Gabon. Fosmidomycin may therefore be a useful lead for the development of novel antimalarial compounds. These results make MEP pathway inhibitors an attractive class of potential broad-spectrum drugs against apicomplexa.

In the present study, we first examined the presence of isoprenoid pathways in four different coccidia: *T. gondii*, *Eimeria tenella, C. parvum* and *C. hominis*. Extensive nucleotide database searches for genes encoding the isoprenoid enzymes identified members of the MEP pathway in both *T. gondii* and *E. tenella* while *Cryptosporidium* species lack the pathways leading to IPP. The operativity of the MEP pathway in *E. tenella* and *T. gondii* was then confirmed by gene expression studies. However, fosmidomycin, even at high concentrations, had only a small effect on the growth of either *T. gondii* or *E. tenella in vitro.*

2. Materials and methods

2.1. Cell culture

Chicken fibroblastoid-like rho0 cell line CS-3 (Gurnett et al., 1995; Morais et al., 1988) was maintained at 41 °C in the presence of 5% CO_2 in Ham's F-12-Glutamax medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml uridine and 1 mM sodium pyruvate.

The CBA/J mouse epithelial cell line, Mode-K (Vidal et al., 1993), was maintained at 37 °C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 5% FCS, 10 mM HEPES, 10 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. Parasites

Eimeria tenella strain PAPt37 was maintained and grown in 3-week-old specific-pathogen-free white Leghorn (PA12). Unsporulated oocysts were purified 7 days after infection from the caecal content. Sporozoites were obtained by excystation of sporulated oocysts (Dulski and Turner, 1988). Second generation merozoites were purified from the caecal content 108 h after inoculation with a high inoculation dose of 10^6 oocysts according to previously published protocol (Binger et al., 1993).

Tachyzoïtes of T. gondii type II 76K and hypervirulent type I RH strains were obtained on immunosupressed Swiss OF1 and CBA/J mice respectively. Parasites were recovered from infected mice by peritoneal wash, passed twice through a 27-gauge needle, filtered on glass wool column in RPMI 10% SVF, then washed twice by 2000*g* centrifugation for 10 min. Cysts of the 76K *T. gondii* strain were obtained from the brains of orally infected CBA/J mice.

After 4 weeks, mature cysts were isolated from the brains of chronically infected mice and purified by isopycnic centrifugation on a Percoll gradient (Cornelissen et al., 1981).

2.3. Drugs

Clomazone was provided by FMC Corporation France. Fosmidomycin was prepared synthetically as previously described (Kamiya et al., 1980). The herbicidal activities of fosmidomycin (Mincheva et al., 2004) and clomazone (data not shown) were confirmed by measuring the inhibition of alkaloid accumulation in *Catharanthus roseus* cell suspension culture. The antimicrobial activity of synthesized