Project no. 516017

O2-sensitive targets

Oxygen-sensitive enzymes of the mevalonate-independent isoprenoid biosynthesis pathway as targets for new antimalarial and anti-TB drugs

Instrument: Specific Targeted Research Project

Thematic Priority: Life science, Genomics and Biotechnology for Health

Publishable final activity report

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Draft 1

1. Project execution

Summary

In malaria parasites of the genus *Plasmodium* and in *Mycobacterium tuberculosis*, the causative agent tuberculosis, isoprenoids are synthesised by the mevalonate-independent 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway. Since this pathway is absent in humans the enzymes involved provide valuable new drug targets. The last two steps are mediated by enzymes which contain oxygen-sensitive iron-sulphur clusters and catalyse unique radical-type reactions. A methodology was developed in order to screen for inhibitors of these enzymes under fully oxygen-free conditions. Several natural product extracts were found to contain compounds with promising inhibitory activity.

Contractors

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Introduction

Isoprenoids are essential for all organisms. In humans isoprenoids are synthesised via the well-established mevalonate pathway. However, in most bacteria and some protozoal parasites as well as in the plastids of plants a completely unrelated biosynthetic route, the so-called 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, is used. Work on this pathway represents a quite young field of research. In particular, any attempts to demonstrate the catalytic activity of the enzymes responsible for the last two reaction steps failed until it was shown that these enzymes contain iron-sulphur clusters involved in unique radical-type reactions. These iron-sulphur clusters are highly oxygen-sensitive leading to the immediate destruction of the enzymes after contact with air.

Objectives

The enzymes of the DOXP pathway provide attractive new drug targets for the treatment of tuberculosis and malaria. In several clinical phase II studies it had already been shown that fosmidomycin, which represents an inhibitor of the second enzyme of the DOXP pathway, cures patients with acute uncomplicated *Plasmodium falciparum* malaria. Additional previous experiments had suggested that the oxygen-sensitive enzymes of the DOXP pathway also represent valuable drug targets if the technological challenge of establishing a fully oxygen-free screening technology can be solved. Therefore, the project aimed at setting up a screening technique which combines separation of complex mixtures with biological testing under oxygen-free conditions.

Work performed

Work under oxygen exclusion was achieved by using a transparent tent which was floated with a mixture of nitrogen and hydrogen. Residual oxygen was consumed by reaction with the hydrogen on palladium catalysts. For the establishment and conduct of the assays a comparably high amount of the oxygen-sensitive enzymes was produced in recombinant form. Photometric assays for monitoring the enzymatic activity were first developed in 1 ml cuvettes, later on 96 well plates and finally on 384 well plates. For the rapid identification active compounds in complex mixtures a liquid chromatography-mass spectrometry (LC-MS) based separation technique was coupled to the testing for biological activity. This procedure was used for screening of several synthetic and natural product extract libraries. The libraries comprised a targeted library which was specifically designed based on the structures of the natural enzyme substrates.

Results achieved

A variety of synthetic compounds and complex mixtures with inhibitory activity in the enzyme assays were identified (Fig. 1). After validation, different natural product extracts were found to be most promising. The active ingredients were partially purified and characterised by their chromatographic and spectroscopic properties.

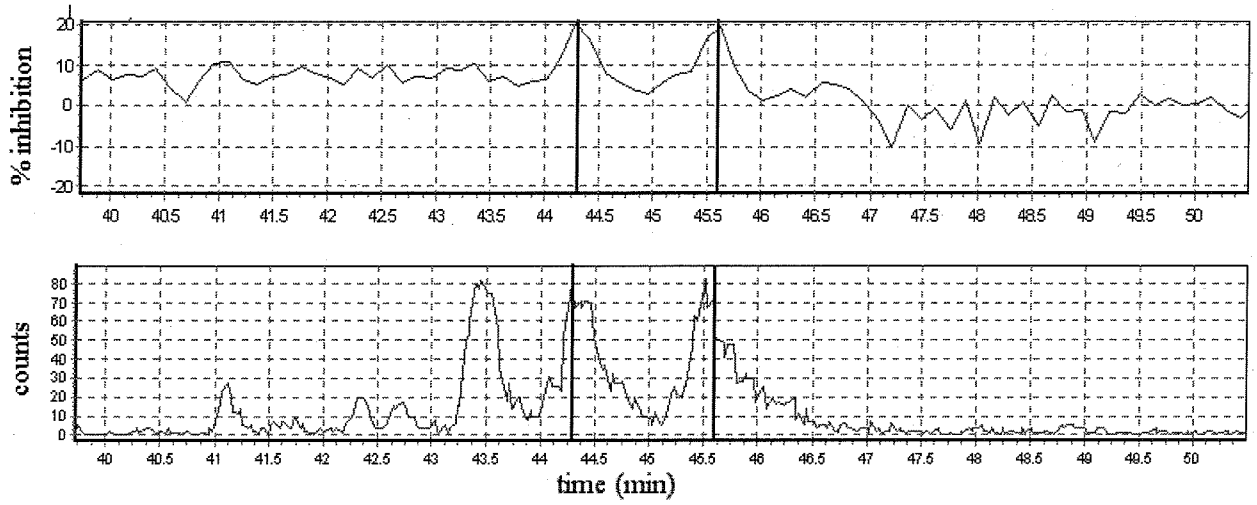

Figure 1. Assignment of bioactivity to individual compounds contained in a complex natural product extract. The correlation of the inhibitory activity in the enzyme assay (upper panel) to the reconstructed ion current of a compound with a defined m/z ratio (bottom panel) is shown. The observed two bioactive peaks with the same m/z ratio may be caused by the presence of two isomers that show distinct elution behaviours in liquid chromatography but are both biologically active.

Intentions for use and impact

The project will be continued with the short-term goal to elucidate the chemical structures of the active compounds present in the identified natural product extracts. These compounds will be developed through additional medicinal chemistry work into drug candidates. In the longer term, the development of a synergistic drug combination inhibiting two enzymes of the DOXP pathway simultaneously is envisaged. The drugs finally expected to emerge from the project will be of particular value for the treatment of infections with pathogens resistant to conventional drugs and provide the potential to become a new mainstay in anti-TB and anti-malarial therapy.

2. Dissemination and use

Section 1 – Exploitable knowledge and its use

Exploitable Knowledge (description)	**Exploitable product(s) or measure(s)**	**Sector(s)** of application	Timetable for commercial use	Patents or other IPR protection	**Owner** & Other Partner(s) involved
1. Production of recombinant enzymes of the DOXP pathway	Enzyme inhibition assays	Research	Not predictable	Not planned	**Kiadis** Uni Giessen
2. Oxygen-free screening technology	New screening procedure	Research	Not predictable	Not planned	**Kiadis** Uni Giessen
3. Enzyme inhibitors	Potential lead compounds for new drugs	Research	Not predictable	Patents planned depending on further results	**Kiadis** Uni Giessen
4. Synthesis of substrate analogues	Potential lead compounds for new drugs	Research	Not predictable	Patents planned depending on further results	**Kiadis** Uni Ghent

Ad 1. Procedures for the recombinant production of high amounts of the target enzymes under oxygen-free conditions were established. This provided the base for the development of specific inhibitors as new antimicrobial drugs throughout the project and beyond.

Ad 2. The technology for the conduct of high throughput screening (HTS) under complete exclusion of oxygen was established. Oxygen-free HTS apparently was never performed before. The technology may be applied to other oxygen-sensitive target enzymes beyond the scope of the current project.

Ad 3. The inhibitors identified so far provide the potential to be developed into new antimalarial and anti-TB drugs. However, additional medicinal chemistry work is required in order to identify a drug candidate which finally may undergo preclinical and clinical development.

Ad 4. Homologues of the natural substrates of the target enzymes were synthesised and demonstrated to inhibit those enzymes. For the identification of therapeutically useful compounds the synthesis of further derivatives with improved activity is required.

Section 2 – Dissemiantion of knowledge

Planned / actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
April 2005	Conference	Research	Mainly Germany	70	Uni Giessen
May 2005	Conference	Research	Mainly Europe	150	Uni Giessen
Sept 2005	Conference	Research	Mainly Europe	200	Uni Giessen
Oct 2005	Conference	Research	Mainly Germany	70	Uni Giessen
Nov 2005	Scientific publication	Research	All		Uni Giessen
May 2006	Conference	Research	Mainly Europe	150	Uni Giessen
April 2006	Conference	Research	Mainly Europe	300	Uni Giessen
June 2006	Conference	Research	Mainly Europe	60	Uni Giessen
Jan 2007	Review article	Research / higher education	All		Uni Giessen
Jan 2007	Scientific publication	Research	All		Uni Giessen

The results on the recombinant production and enzymatic characterisation of an oxygen-sensitive *P. falciparum* enzyme were published in a scientific journal. In addition to providing a base for antimalarial drug development these data are of general interest with respect to their relevance for the understanding of the metabolic functions and the phylogenetic origin of the plastid-like organelle present in parasites of the phylum Apicomplexa.

> Röhrich RC, Englert N, Troschke K, Reichenberg A, Hintz M, Seeber F, Balconi E, Aliverti A, Zanetti G, Kohler U, Pfeiffer M, Beck E, Jomaa H, Wiesner J. Reconstitution of an apicoplast-localised electron transfer pathway involved in the isoprenoid biosynthesis of *Plasmodium falciparum*. FEBS Lett. 2005 Nov 21;579(28):6433-8.

A review on the isoprenoid biosynthesis pathway of apicomplexan parasite, in particular *P. falciparum*, was published.

> Wiesner J, Jomaa H. Isoprenoid biosynthesis of the apicoplast as drug target. Curr Drug Targets. 2007 Jan;8(1):3-13.

In an informal cooperation with the group of Evert C Duin, Auburn University, USA, experiments on the molecular mechanism of the reaction catalysed by an oxygen-free enzyme of the DOXP pathway were published. Detailed understanding of this reaction may facilitate the rational design and optimisation of specific inhibitors.

> Adedeji D, Hernandez H, Wiesner J, Kohler U, Jomaa H, Duin EC. Possible direct involvement of the active-site [4Fe-4S] cluster of the GcpE enzyme from *Thermus thermophilus* in the conversion of MEcPP. FEBS Lett. 2007 Jan 23;581(2):279-83.

Different aspects of the isoprenoid biosynthesis pathway as new drug target with emphasise on the oxygen-sensitive enzymes were presented at several conferences.

28 – 29 April 2005. 6th Drug Development Seminar 2005 (Antiparasitic Chemotherapy), Hamburg, Germany; Oxygen-sensitive enzymes of the DOXP pathway as targets for new antimalarial drugs.

08 – 11 May 2005. 2nd annual workshop of the COST 857 Action, Apicomplexan biology in the post-genomic era, Beatenberg, Switzerland; The 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway and its significance as a novel antimalarial drug target.

29 September – 1 October 2005. 2nd COST B22 Congress on "Drug Discovery & Development for Parasitic Disease" Siena, Italy; Identification of an apicoplast-localised electron transfer pathway involved in the isoprenoid biosynthesis of *P. falciparum.*

28 – 29 October 2005. 2. Malaria-Treffen der Paul-Ehrlich-Gesellschaft für Chemotherapie, Hamburg, Germany; Oxygen-sensitive enzymes of the DOXP pathway as targets for new antimalarial drugs.

17 – 20 May 2006. 3rd annual workshop of the COST 857 Action, Apicomplexan biology in the post-genomic era, Dresden, Germany; Iron-sulphur proteins involved in the isoprenoid biosynthesis of *P. falciparum* as novel antimalarial drug targets.

05 – 07 April 2006. Second Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite; Reconstitution of an apicoplast-localised electron transfer pathway involved in the isoprenoid biosynthesis.

05 – 07 June 2006. COST B-22 Expert Meeting – Cell Organelles of Protozoan Parasites; Biosynthesis of isoprenoid precursors by the apicoplast of *P. falciparum.*