# Inhibitors of the Nonmevalonate Pathway of Isoprenoid Biosynthesis as Antimalarial Drugs

Hassan Jomaa,[1]* Jochen Wiesner,[1] Silke Sanderbrand,[1] Boran Altincicek,[1] Claus Weidemeyer,[1] Martin Hintz,[1] Ivana Türbachova,[2] Matthias Eberl,[1] Johannes Zeidler,[3] Hartmut K. Lichtenthaler,[3] Dominique Soldati,[2] Ewald Beck[1]

A mevalonate-independent pathway of isoprenoid biosynthesis present in *Plasmodium falciparum* was shown to represent an effective target for chemotherapy of malaria. This pathway includes 1-deoxy-D-xylulose 5-phosphate (DOXP) as a key metabolite. The presence of two genes encoding the enzymes DOXP synthase and DOXP reductoisomerase suggests that isoprenoid biosynthesis in *P. falciparum* depends on the DOXP pathway. This pathway is probably located in the apicoplast. The recombinant *P. falciparum* DOXP reductoisomerase was inhibited by fosmidomycin and its derivative, FR-900098. Both drugs suppressed the in vitro growth of multidrug-resistant *P. falciparum* strains. After therapy with these drugs, mice infected with the rodent malaria parasite *P. vinckei* were cured.

Malaria is one of the leading causes of morbidity and mortality in the tropics, with 300 million to 500 million estimated clinical cases and 1.5 million to 2.7 million deaths per year. Nearly all fatal cases are caused by *Plasmodium falciparum*. Because the parasite's resistance to conventional antimalarial drugs such as chloroquine is growing at an alarming rate, new efficient drugs are urgently needed.

In all organisms studied so far, the biosynthesis of isoprenoids such as sterols and ubiquinones depends on the condensation of different numbers of isopentenyl diphosphate units (*1*). In mammals and in fungi, isopentenyl diphosphate is derived from the mevalonate pathway. This pathway depends on the condensation of three molecules of acetyl coenzyme A (acetyl CoA) into 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which is reduced to mevalonate by HMG-CoA reductase. Mevalonate is further converted into isopentenyl diphosphate with mevalonate 5-phosphate as an intermediate. Previous studies revealed very low HMG-CoA reductase activity in *P. falciparum* (*2*), and attempts to establish HMG-CoA reductase inhibitors as antimalarial drugs failed (*3*), suggesting the absence of a mevalonate pathway in *P. falciparum*.

Recently, in some eubacteria, algae, and plants, the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway was described as an alternative nonmevalonate pathway for the early steps in the biosynthesis of isoprenoids. In higher plants, plastidic isoprenoids such as carotenoids are formed through the DOXP pathway (*4*). The sterols, in turn, are formed in the cytosol through the mevalonate pathway. The DOXP pathway is characterized by the condensation of glyceraldehyde 3-phosphate and pyruvate to DOXP and its conversion to 2-*C*-methyl-D-erythritol 4-phosphate by the enzymes DOXP synthase and DOXP reductoisomerase (*5, 6*).

Searching for genes encoding enzymes of the DOXP pathway in *P. falciparum*, we identified similarities between known bacterial and blue algal protein sequences of the enzyme DOXP reductoisomerase and sequences on chromosome 14 of *P. falciparum*, obtained from the *P. falciparum* genome project (*7*). The high degree of similarity suggests the existence of a functional DOXP reductoisomerase in *P. falciparum*. The complete gene encoding the DOXP reductoisomerase of *P. falciparum* was consequently cloned (Fig. 1). In addition, we identified a *P. falciparum* gene that was very similar to the DOXP synthase of different species of bacteria and plants. Expression of these genes in the erythrocytic stages of *P. falciparum* (the stage responsible for the clinical manifesta-

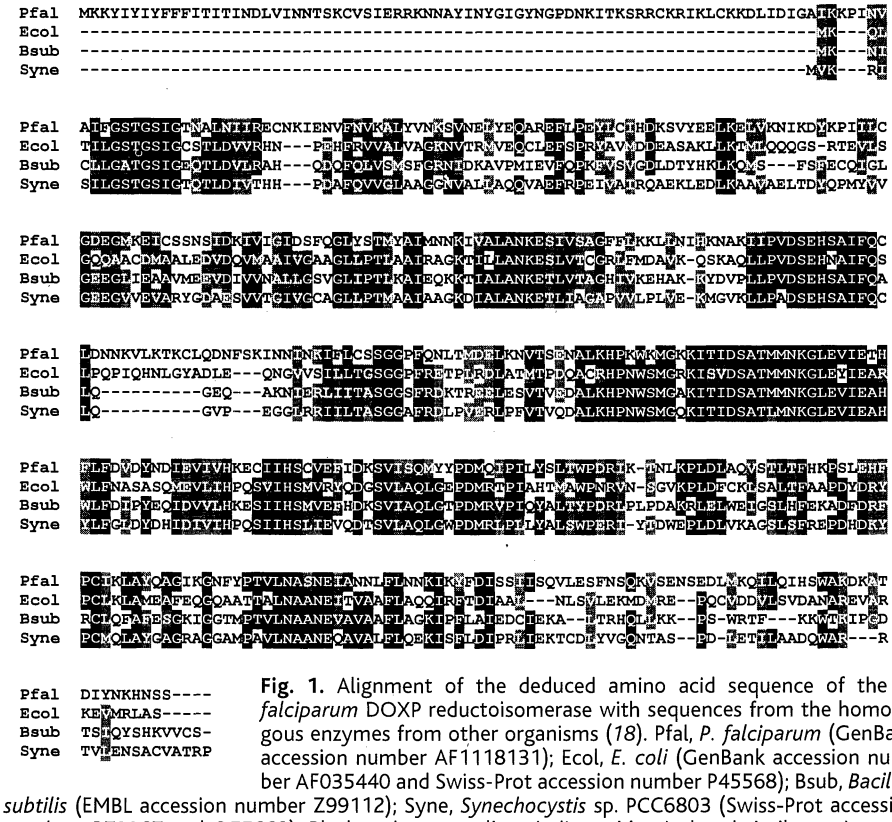


**Fig. 1.** Alignment of the deduced amino acid sequence of the *P. falciparum* DOXP reductoisomerase with sequences from the homologous enzymes from other organisms (*18*). Pfal, *P. falciparum* (GenBank accession number AF1118131); Ecol, *E. coli* (GenBank accession number AF035440 and Swiss-Prot accession number P45568); Bsub, *Bacillus subtilis* (EMBL accession number Z99112); Syne, *Synechocystis* sp. PCC6803 (Swiss-Prot accession numbers P73067 and Q55663). Black and gray outlines indicate identical and similar amino acid residues, respectively.

[1]Institute of Biochemistry, Academic Hospital Centre, Justus-Liebig-University, Friedrichstrasse 24, D-35392 Giessen, Germany. [2]Zentrum für Molekulare Biologie, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany. [3]Botanisches Institut II, University of Karlsruhe, Kaiserstrasse 12, D-76128 Karlsruhe, Germany.

*To whom correspondence should be addressed. E-mail: hassan.jomaa@biochemie.med.uni-giessen.de

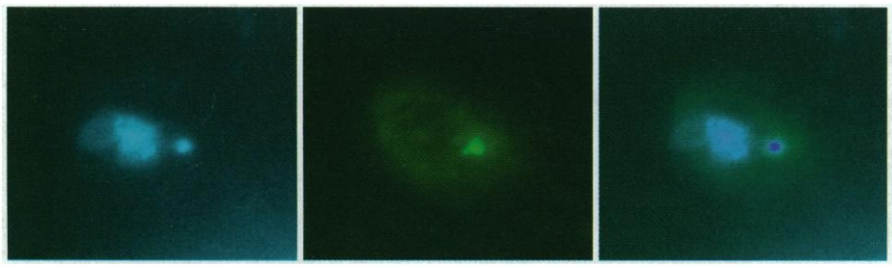

**Fig. 2.** Targeting of GFP to the apicoplast of *T. gondii* by the leader peptide of the *P. falciparum* DOXP reductoisomerase. *Toxoplasma gondii* cells were transiently transfected with a construct encoding the leader peptide fused to the $NH_2$-terminus of GFP. The localization of GFP was analyzed by direct fluorescence (green). Nuclear and apicoplast DNA were counterstained with 4′,6′-diamidino-2-phenylindole (blue). Color images were (left and middle) obtained independently and (right) overlaid.

**Table 1.** Sensitivity of different *P. falciparum* strains to fosmidomycin, FR-900098, chloroquine, and pyrimethamine. Concentrations causing half-maximal inhibition ($IC_{50}$) were determined in vitro with the indicated *P. falciparum* strains (*15*). Mean values and standard deviations from three to four independent experiments are shown.

| *Plasmodium falciparum* strain | $IC_{50}$ (nM) | | | |
|---|---|---|---|---|
| | Fosmidomycin | FR-900098 | Chloroquine | Pyrimethamine |
| HB3 | 350 ± 170 | 170 ± 100 | 20 ± 5 | 60 ± 42 |
| A2 | 370 ± 45 | 170 ± 45 | 37 ± 7 | 4 ± 2 |
| Dd2 | 290 ± 130 | 90 ± 20 | 200 ± 30 | 2500 ± 1000 |

tion of the disease) was confirmed by reverse transcriptase–polymerase chain reaction (*8*).

In contrast to the protein sequences of the DOXP reductoisomerases from bacteria and blue algae, a unique $NH_2$-terminal extension of the corresponding homologous enzyme of *P. falciparum* was identified (Fig. 1). The first 30 amino acids of the $NH_2$-terminal extension of the *P. falciparum* DOXP reductoisomerase resemble an endoplasmic reticulum signal peptide, whereas the following 44 amino acids exhibit the characteristics of plastidial targeting sequences (*9*). Because *Toxoplasma gondii*, in contrast to *P. falciparum*, can be transfected efficiently by means of established vectors, we transfected *T. gondii* with a construct containing the $NH_2$-terminal sequence of the *P. falciparum* DOXP reductoisomerase fused to green fluorescent protein (GFP) (*10*). GFP accumulated in a restricted region of the cell that colocalizes with extranuclear apicoplast DNA (Fig. 2). The apicoplast is a plastidlike organelle acquired by members of the phylum Apicomplexa by secondary endosymbiosis of an alga. The metabolic function of the apicoplast is not known, but it is essential for the survival of the parasites (*11*).

Recombinant *P. falciparum* DOXP reductoisomerase was produced in *Escherichia coli* (Fig. 3A) (*12*). The conversion of DOXP to 2-*C*-methyl-D-erythritol 4-phosphate by the recombinant enzyme was determined in an assay based on the NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) dependency of the reaction. This conversion was inhibited by fosmidomycin and its derivative, FR-900098, in a dose-dependent manner (Fig. 3B) (*13*). Fosmidomycin has recently been shown to inhibit the DOXP reductoisomerase from bacteria and plants (*14*).

To investigate whether the DOXP pathway is essential for the survival of the parasites, we treated *P. falciparum* cultures with fosmidomycin and FR-900098. The viability of *P. falciparum* was determined by [$^3$H]-hypoxanthine incorporation in the presence of different concentrations of the drugs (*15*). Both drugs inhibited the growth of *P. falciparum* in submicromolar concentrations (Fig. 4). Three *P. falciparum* strains with different sensitivity patterns to chloroquine and pyrimethamine were efficiently inhibited (Table 1).

Because the DOXP pathway is absent in mammals and fosmidomycin and FR-900098 are known to have low toxicity (lethal doses of >8000 and >5000 mg/kg after oral and subcutaneous administration, respectively, in rats), their antimalarial activity was tested in an animal model (*13*). Mice infected with the rodent malaria parasite *P. vinckei,* which is fatal if untreated, were treated with the drugs from day 1 after infection to 4 days later (*16*). The drugs were well tolerated, even at the highest dosage of 300 mg/kg. Untreated control animals developed parasitemias of >60% on day 5 and died on day 7 after infection. Animals treated intraperitoneally with dosages of >10 mg/kg of fosmidomycin or 5 mg/kg of FR-900098 were apparently free of parasites (Table 2). After treatment with 2 mg/kg of FR-900098 or 5 mg/kg of fosmidomycin, parasitemias were <1%. Animals treated orally with 50 or 100 mg/kg of either fosmidomycin or FR-900098 were apparently free of parasites, and parasitemias were <1% after treatment with 20 mg/kg of either drug. Recrudescence was observed when the treat-

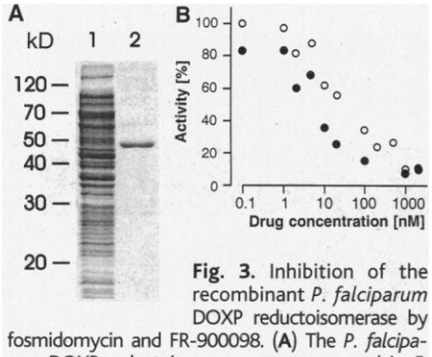


**Fig. 3.** Inhibition of the recombinant *P. falciparum* DOXP reductoisomerase by fosmidomycin and FR-900098. (**A**) The *P. falciparum* DOXP reductoisomerase was expressed in *E. coli* with an $NH_2$-terminal His tag. Crude bacterial lysates (lane 1) and the purified enzyme (lane 2) were analyzed by SDS-PAGE. (**B**) The enzymatic activity of the recombinant enzyme was monitored by NADPH oxidation in the presence of different concentrations of fosmidomycin (open circles) and FR-900098 (solid circles).

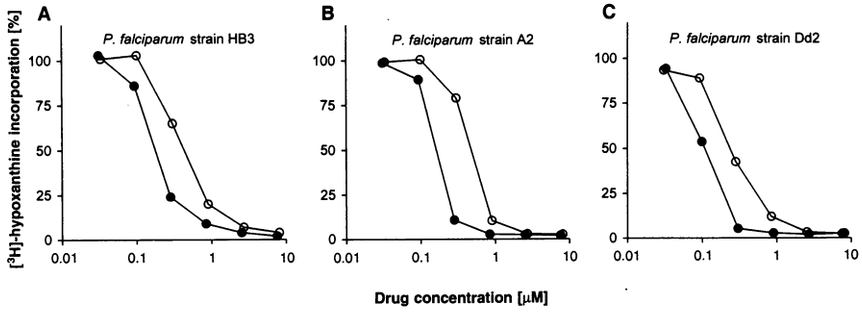


**Fig. 4.** In vitro antimalarial activity of fosmidomycin and FR-900098. *Plasmodium falciparum*–infected erythrocytes were cultured in the presence of different amounts of fosmidomycin (open circles) or FR-900098 (solid circles). The viability of the parasites was determined by [$^3$H]-hypoxanthine incorporation. The antimalarial activity was measured with the *P. falciparum* strains (**A**) HB3, (**B**) A2, and (**C**) Dd2. Mean values from three to four independent experiments are shown.

**Table 2.** In vivo antimalarial activity of fosmidomycin and FR-900098. The in vivo antimalarial activity was determined by a 4-day suppressive test with *P. vinckei*–infected mice (*16*). The indicated dosages of fosmidomycin and FR-900098 in PBS were administrated intraperitoneally or orally three times per day over a period of 4 days. Parasitemias were determined on day 5 by Giemsa-stained blood smears. Mean values and standard deviations from three to four independent experiments, each containing four to six mice per dosage, are shown.

| Dosage (mg/kg) | Parasitemia (%) | |
|---|---|---|
| | Fosmidomycin | FR-900098 |
| | *Intraperitoneal administration* | |
| 300 | 0.0 | 0.0 |
| 30 | 0.0 | 0.0 |
| 10 | 0.0 | 0.0 |
| 5 | 0.1 ± 0.2 | 0.0 |
| 2 | 12 ± 17 | 0.9 ± 0.4 |
| | *Oral administration* | |
| 100 | 0.0 | 0.0 |
| 50 | 0.0 | 0.0 |
| 20 | 0.2 ± 0.3 | 0.1 ± 0.2 |
| Control | 68 ± 21 | 68 ± 21 |

ment was terminated after 4 days. Mice treated with 30 mg/kg of fosmidomycin or FR-900098 over a period of 8 days were totally cured. The efficiency of these drugs at low dosages and their activity after oral administration demonstrate that fosmidomycin and FR-900098 are potential antimalarial drugs.

In summary, we provide evidence for the presence of the DOXP pathway in the apicoplast of *P. falciparum* and for the inhibition of one of the key enzymes of this pathway, DOXP reductoisomerase, with fosmidomycin and FR-900089. In addition, we have demonstrated the antimalarial activity of these drugs in vitro and in vivo. In former studies with volunteers and patients suffering from bacterial infections, fosmidomycin was well tolerated (*17*). The efficacy of these drugs against multidrug-resistant parasites and their low manufacturing costs and high stability make them very attractive as a potential new class of antimalarial drugs.

### References and Notes


1. E. D. Beytia and J. W. Porter, *Annu. Rev. Biochem.* **45**, 113 (1976).
2. H. J. Vial, J. R. Philippot, D. F. Wallach, *Mol. Biochem. Parasitol.* **13**, 53 (1984).
3. P. Grellier, A. Valentin, V. Millerioux, J. Schrevel, D. Rigomier, *Antimicrob. Agents Chemother.* **38**, 1144 (1994).
4. D. Arigoni *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10600 (1997); A. Disch, J. Schwender, C. Müller, H. K. Lichtenthaler, M. Rohmer, *Biochem. J.* **333**, 381 (1998); H. K. Lichtenthaler, J. Schwender, A. Disch, M. Rohmer, *FEBS Lett.* **400**, 271 (1997); M. Rohmer, M. Knani, P. Simonin, B. Sutter, H. Sahm, *Biochem. J.* **295**, 517 (1993); H. K. Lichtenthaler, M. Rohmer, J. Schwender, *Physiol. Plant.* **101**, 643 (1997); J. Schwender *et al.*, *FEBS Lett.* **414**, 129 (1997).
5. G. A. Sprenger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12857 (1997).
6. B. M. Lange, M. R. Wildung, D. McCaskill, R. Croteau, *ibid.* **95**, 2100 (1998); L. M. Lois *et al.*, *ibid.*, p. 2105; M. A. Mandel, K. A. Feldmann, L. Herrera-Estrella, M. Rocha-Sosa, P. Leon, *Plant J.* **9**, 649 (1996); S. Takahashi, T. Kuzuyama, H. Wanatabe, H. Seto, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9879 (1998); H. K. Lichtenthaler, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 47 (1999).
7. Sequences encoding the $NH_2$-terminal and COOH-terminal part of the *P. falciparum* DOXP reductoisomerase were identified by a TBLASTN screen with the *E. coli* DOXP reductoisomerase protein sequence against different databases [the following were from the *P. falciparum* database at the Institute for Genomic Research (TIGR) (available at www.tigr.org/tdb/edb/pfdb/pfdb.html): PNADW48TF, PNAAR51TR, PNABS84TF, and PNACV29TR]. The complete coding sequence was amplified from genomic DNA (*P. falciparum* strain HB3) by polymerase chain reaction (PCR) with the primers PfREDfor (5′-CTGAATTTCATATTACAAAATTAATAGATG-3′) and PfREDrev (5′-GTACTATGAAGAATTATGTTTGTTGTATAT-3′). Similarly, sequences encoding $NH_2$-terminal and COOH-terminal parts of the *P. falciparum* DOXP synthase were identified [the following were from the TIGR database: PNAEL30TR, PNAEP81TF, and PNAEP81TR (contiguous sequence 749); the following was from the database at the Sanger Centre (available at www.sanger.ac.uk/Projects/P_falciparum/ftp.shtml): M13Q12a4.plt (contiguous sequence 09086)]. A central part of the DOXP synthase gene missing in the databases was amplified with the primers PfSYNfor (5′-AATAAATTAAAAAGAGCTACTGTTCTTC-3′) and PfSYNrev (5′-TAAGATAAATCATATATACCTTGATGTG-3′) (GenBank accession number AF1118141). For sequencing, the PCR products were cloned with the TA-cloning kit (Invitrogen).
8. *Plasmodium falciparum*–infected erythrocytes were cultured as described [W. Trager and J. B. Jensen, *Science* **193**, 673 (1976)]. mRNA was isolated from 20 ml of packed infected erythrocytes at a parasitemia of 3 to 4% after saponin lysis with the Fast Track 2.0 kit (Invitrogen). mRNA (0.5 μg) was treated with 2U RQ1 RNase-free DNase (Promega) for 15 min. The mRNA sample was extracted with phenol:chloroform and reverse transcribed with random hexanucleotide primers and SuperScript II reverse transcriptase (Gibco-BRL). A portion of the resulting cDNA was used as a template for PCR amplification with primers defining expressed *P. falciparum* genes as follows: DOXP reductoisomerase with PfREDfor and PfREDint (5′-AGAATATAATCCTTGAAAAGAATCAATACC-3′) and DOXP synthase with PfSYNfor and PfSYNrev (H. Jomaa *et al.*, data not shown).
9. S. D. Schwartzbach, T. Osafune, W. Loffelhardt, *Plant Mol. Biol.* **38**, 247 (1998).
10. The sequence encoding the first 79 amino acids of the DOXP reductoisomerase was remodeled with the average codon usage of *T. gondii* and amplified by PCR as described [W. Pan *et al.*, *Nucleic Acids Res.* **27**, 1094 (1999)]. The sequence was ligated to GFP and inserted into a modified version of the *T. gondii* expression vector pTUBGFPhxgprt under the control by the Tg*TUB1* promoter [R. F. Waller *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12352 (1998); D. Soldati and J. C. Boothroyd, *Mol. Cell. Biol.* **15**, 87 (1995)]. *Toxoplasma gondii* tachyzoites (RH strain *hxgprt*$^-$) were transfected with this construct by electroporation [D. Soldati and J. C. Boothroyd, *Science* **260**, 349 (1993)]. Intracellular parasites grown for 24 hours in human foreskin fibroblasts on glass slides were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde for 20 min. After fixation, slides were briefly rinsed in phosphate-buffered saline (PBS) and 0.1 M glycine and mounted in Vectashield. Micrographs were obtained with a Axiophot microscope (Zeiss) equipped with a camera (type CH-250, Photometrics, Tucson, AZ). Adobe Photoshop (Adobe Systems, Mountain View, CA) was used for image processing. The expression of GFP coupled to the leader sequence of DOXP reductoisomerase appears to be toxic for *T. gondii* because all positive transfectants failed to divide at 24 and 48 hours after transfection.
11. G. I. McFadden, M. E. Reith, J. Munholland, N. Lang-Unnasch, *Nature* **381**, 482 (1996); S. Köhler *et al.*, *Science* **275**, 1485 (1997); M. E. Fichera and D. S. Roos, *Nature* **390**, 407 (1997).
12. The amino acid sequence of DOXP reductoisomerase minus the leader peptide (amino acids 1 to 72) was back-translated in the codon usage of *E. coli* because the original AT-rich *P. falciparum* sequence was not expressed in *E. coli*. The complete synthetic gene was obtained by custom synthesis from Oligos Etc. (Bethel, ME) and cloned into a modified pQE31 vector (Qiagen, Valencia, CA). The vector-derived sequences resulted in the following extensions of the original sequence: MHHHHHHGSACELGTM (*18*) at the $NH_2$-terminus and GSRSA (*18*) at the COOH-terminus. Bacteria (Top10F′, Invitrogen) harboring this construct were induced for 4 hours with 1 mM isopropyl-β-D-thiogalactopyranoside and disintegrated by sonication in buffer A [100 mM NaCl, 10 mM tris-HCl, and 2 mM 2-mercaptoethanol (pH 8.0)]. The enzyme was purified from the soluble fraction by affinity chromatography on Talon Superflow resin (Clontech, Heidelberg, Germany) with a high-performance liquid chromatography device (Gynkotek, Germering, Germany). The column was washed successively with 4 and 50 mM imidazole in buffer A. The protein was eluted with 100 mM imidazole in buffer A, resulting in a purity of ~80% as determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The enzymatic activity of the recombinant DOXP reductoisomerase was determined in 100 mM tris-HCl (pH 7.5), 1 mM $MnCl_2$, 0.3 mM NADPH, and 1 mM DOXP while the oxidation of NADPH in a spectrophotometer at 365 nm was monitored. The specific activity of *P. falciparum* DOXP reductoisomerase was 6 U/mg as compared to 41 U/mg of DOXP reductoisomerase from *E. coli*. *Escherichia coli* DOXP reductoisomerase was overexpressed with a pQE9 vector (Qiagen). DOXP as substrate for the enzyme assay was enzymatically synthesized as described in (*5*) with the recombinant DOXP synthase from *E. coli*. The identity of DOXP was confirmed by $^{13}C$ nuclear magnetic resonance spectroscopy.
13. Fosmidomycin [3-(*N*-formyl-*N*-hydroxyamino)propylphosphonate] and FR-900098 [3-(*N*-acetyl-*N*-hydroxyamino)propylphosphonate] were synthesized by a combination of the protocols from work by Kamiya *et al.* and Öhler and Kanzler [T. Kamiya, M. Hashimoto, K. Hemmi, H. Takeno (Fujisawa Pharmaceutical Company), U.S. Patent 4, 206, 156 (1980); E. Öhler and S. Kanzler, *Synthesis* **1995**, 539 (1995)]. Detailed protocols are available from the authors on request.
14. J. Zeidler *et al.*, *Z. Naturforsch.* **53C**, 980 (1998); T. Kuzuyama, T. Shizimu, S. Takahashi, H. Seto, *Tetrahedron Lett.* **39**, 7913 (1998).
15. Infected erythrocytes (200 μl per well, with 2% hematocrit and 0.4% parasitemia) were incubated with a serial dilution of the drugs for 48 hours. After the addition of 0.8-μCi [$^3H$]-hypoxanthine in 30 μl of medium per well, the plates were further incubated for 24 hours. Incorporated radioactivity was counted after filtration on glass-fiber filters [M. L. Ancelin *et al.*, *Blood* **91**, 1 (1998)]. Three *P. falciparum* strains were used for the sensitivity test (HB3, Honduras; A2, The Gambia; and Dd2, Indochina).
16. The in vivo antimalarial activity was determined in *P. vinckei*–infected mice with a modified 4-day suppressive test as described in work by Peters [W. Peters, *Malaria*, J. P. Kreier, Ed. (Academic Press, New York, 1980), vol. 1, pp. 160–161]. Mice were intraperitoneally inoculated with $10^7$ infected erythrocytes on day 0. On day 1, successful infection was confirmed, and the treatment was started. The drugs were administrated every 8 hours, three times per day, with respect to their short plasma half-life time as previously published [T. Murakawa, H. Sakamoto, S. Fukada, T. Konishi, M. Nishida, *Antimicrob. Agents Chemother.* **21**, 224 (1982)].
17. H. P. Kuemmerle, T. Murakawa, F. De Santis, *Chemioterapia* **6**, 113 (1987).
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.


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Immunology and Transfusion Medicine for supplying blood components. Sequence data for *P. falciparum* chromosome 13 were obtained from the Sanger Centre (available at www.sanger.ac.uk/Projects/P_falciparum/). Sequencing of *P. falciparum* chromosome 13 was accomplished as part of the Malaria Genome Project, with support by the Wellcome Trust. Preliminary sequence data for *P. falciparum* chromosome 14 were obtained from TIGR (available at www.tigr.org). Sequencing of chromosome 14 was part of the international Malaria Genome Sequencing Project and was supported by awards from the Burroughs Wellcome Fund and the U.S. Department of Defense.

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# Functional Interaction of BRCA1-Associated BARD1 with Polyadenylation Factor CstF-50

**Frida E. Kleiman and James L. Manley***

**Polyadenylation of messenger RNA precursors requires a complex protein machinery that is closely integrated with the even more complex transcriptional apparatus. Here a polyadenylation factor, CstF-50 (cleavage stimulation factor), is shown to interact in vitro and in intact cells with a nuclear protein of previously unknown function, BRCA1-associated RING domain protein (BARD1). The BARD1-CstF-50 interaction inhibits polyadenylation in vitro. BARD1, like CstF-50, also interacts with RNA polymerase II. These results indicate that BARD1-mediated inhibition of polyadenylation may prevent inappropriate RNA processing during transcription, perhaps at sites of DNA repair, and they reveal an unanticipated integration of diverse nuclear events.**

Almost all eukaryotic mRNAs have a polyadenylated [poly(A)] tail at the 3′ end. Formation of this structure involves endonucleolytic cleavage of the mRNA precursor coupled with poly(A) synthesis, a reaction that requires a complex set of protein factors (*1*). Although polyadenylation can be reconstituted in vitro with purified components and a synthetic mRNA precursor, considerable evidence now exists that, in the cell nucleus, 3′ end formation is normally tightly coupled to transcription by RNA polymerase II (RNAP II). Polyadenylation factors associate with RNAP II at the promoter (*2*) and appear to remain with it during elongation (*3*). When RNAP II reaches the site of polyadenylation, it participates directly in the processing reaction together with the other polyadenylation factors (*4*); it also receives a signal needed for subsequent transcription termination (*5*). How these complex interactions are orchestrated is not known. However, polyadenylation can be regulated, for example, during the cell cycle (*6*) and cellular differentiation (*7*).

Cleavage stimulation factor (CstF) is a polyadenylation factor that helps specify the site of processing (*8, 9*). It is a heterotrimeric protein with subunits of 77, 64, and 50 kD (CstF-77, -64, and -50) that recognizes the G+U–rich element, a sequence located downstream of the cleavage site. RNA binding is mediated by CstF-64 (*10*). CstF-77, or Suppressor-of-forked in *Drosophila*, bridges CstF-64 and -50 (*11*) and interacts with another multisubunit factor, the AAUAAA-binding cleavage-polyadenylation specificity factor (CPSF), to define the poly(A) site (*12*). CstF-50 contains seven WD-40 repeats (*13*), is required for CstF activity in vitro (*11*), and interacts with the COOH-terminal domain of the RNAP II largest subunit (CTD) (*3*).

To identify additional CstF-50–interacting proteins, we performed a yeast two-hybrid assay (*14*); for bait we used CstF-50 fused to the LexA DNA binding domain (*15*). Among the strongest interacters recovered was BARD1 (*16*), a protein known to associate with the breast cancer–associated tumor suppressor BRCA1 in vivo. Both BARD1 and BRCA1 possess $NH_2$-terminal RING motifs and COOH-terminal BRCT domains, with the former responsible for the BARD1-BRCA1 interaction (*17*). Although the function of BARD1 is unknown, inhibiting its expression in cultured cells results in changes that suggest a premalignant phenotype (*18*). BARD1 likely plays a role in BRCA1-mediated tumor suppression and may itself be a target for tumorigenic mutations in some cancers (*19*). BARD1, in association with BRCA1, can colocalize with the DNA replication and repair factors PCNA and Rad51, perhaps participating in the cellular response to DNA damage (*20*).

The BARD1 cDNAs obtained from the two-hybrid screen encoded amino acids 457 to 777 (Fig. 1A), which includes two of three ankyrin repeats and the BRCT domain. We used essentially full-length BARD1 (amino acid residues 13 to 777), which interacted as efficiently as the smaller fragment, to confirm the interaction in yeast. CstF-50 (amino acid residues 92 to 431), which lacks the $NH_2$-terminal region of CstF-50, also interacted strongly with BARD1. As observed with BRCA1 (*21*), yeast strains expressing BARD1 displayed a slow-growth phenotype (*22*).

We next characterized the BARD1–CstF-50 interaction in vitro. The two proteins were translated separately in vitro, mixed, and subjected to immunoprecipitation with antibodies to BARD1 (anti-BARD1 antibodies) (*23*) (Fig. 2A). CstF-50 was precipitated in the presence (lane 3), but not in the absence (lane 2), of BARD1, which strongly suggests a specific interaction between the two proteins. In another approach, a glutathione S-transferase (GST)–BARD1 fusion protein (BARD1 residues 13 to 777) was purified from *Escherichia coli* and used in protein interaction assays, again with in vitro translated CstF-50 (Fig. 2B). A significant fraction of the input CstF-50 bound to GST-BARD1 (lane 3) but not to GST alone (lane 2). COOH-terminal truncations of CstF-50 (see Fig. 1B) eliminated binding (lanes 4 to 7), consistent with the requirement of at least the seventh WD-40 repeat for interaction. To elucidate the region of BARD1 required, we used additional GST-BARD1 derivatives (Fig. 1B) in binding assays with CstF-50 (Fig. 2C). A COOH-terminal truncation removing most of

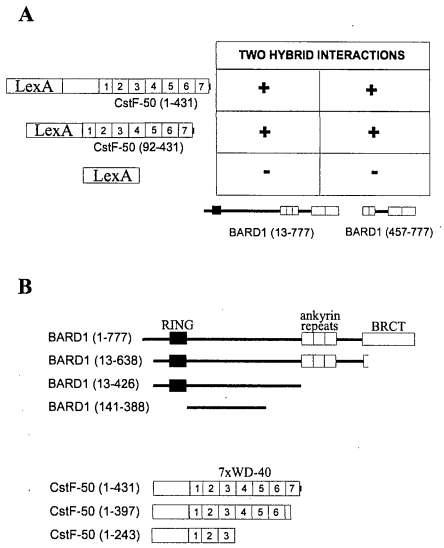


**Fig. 1.** Interaction of BARD1 and CstF-50 in yeast. (**A**) Two-hybrid screening identifies a CstF50–BARD1 interaction. LexA–CstF50 fusion proteins are indicated on the left and BARD1 derivatives are shown below. Features of proteins are indicated and numbers represent amino acid residue. Interactions were detected by LEU2 and LacZ expression. (**B**) Diagram of BARD1 and CstF-50 derivatives used in in vitro experiments. Proteins were produced by in vitro translation or as $NH_2$-terminal GST fusions in *E. coli*.

Department of Biological Sciences, Columbia University, New York, NY 10027, USA.

*To whom correspondence should be addressed. E-mail: jlm2@columbia.edu