Metabolism of the Antimalarial Endoperoxide Ro 42-1611 (Arteflene) in the Rat: Evidence for Endoperoxide Bioactivation

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ABSTRACT

Ro 42-1611 (arteflene) is a synthetic endoperoxide antimalarial. The antimalarial activity of endoperoxides is attributed to iron(II)-mediated generation of carbon-centered radicals. An α,β-unsaturated ketone (enone; 4-[2,4′ bis(trifluoromethyl)phenyl]-3-buten-2-one), obtained from arteflene by reaction with iron(II), was identified previously as the stable product of a reaction that, by inference, also yields a cyclohexyl radical. The activation of arteflene in vivo has been characterized with particular reference to enone formation. [14C]Arteflene (35 μmol/kg) was given i.v. to anesthetized and cannulated male rats: 42.2 ± 7.0% (mean ± S.D., n = 7) of the radiolabel was recovered in bile over 5 h. In the majority of rats, the principal biliary metabolites were 8-hydroxyarteflene glucuronide (14.2 ± 3.9% dose, 0–3 h) and the cis and trans isomers of the enone (13.5 ± 4.6% dose, 0–3 h). In conscious rats, 15.3 ± 1.6% (mean ± S.D., n = 8) of the radiolabel was recovered in urine over 24 h. The principal urinary metabolite appeared to be a glycine conjugate of a derivative of the enone. Biliary excretion of the glucuronide, but not of the enones, was inhibited by ketoconazole. 8-Hydroxyarteflene was formed extensively by rat and human liver microsomes but no enone was found. Bioactivation is a major pathway of arteflene’s metabolism in the rat. Although the mechanism of in vivo bioactivation is unclear, the reaction is not catalyzed by microsomal cytochrome P-450 enzymes.

Malaria persists as a major cause of morbidity and mortality in tropical regions and, especially, among children in Africa. In recent years, there has been a widespread, if uneven, increase in the resistance of the major malaria parasite Plasmodium falciparum to several standard synthetic antimalarials and, in particular, to the 4-aminoquinoline, chloroquine. Consequently, novel drugs to which chloroquine-resistant parasites are sensitive are required (Vial, 1996).

Endoperoxides represent a new class of antimalarials. Artemisinin (Fig. 1A), a polyoxygenated amorphene endoperoxide obtained from the medicinal plant Artemisia annua, and various of its semisynthetic O-ether and ester derivatives have attracted particular attention (Meshnick et al., 1996). These compounds exhibit potent activity against chloroquine-resistant strains of P. falciparum in vitro, and numerous trials have established clinical efficacy in respect of both uncomplicated and severe malaria (de Vries and Dien, 1996). However, the utility of the first-generation derivatives is compromised to a certain degree by their rapid metabolic clearance, to which has been ascribed the relatively high incidence of recrudescence associated with their use in single-dose monotherapy regimens (de Vries and Dien, 1996). This, in turn, has suggested that chemically or metabolically more stable peroxides might have enhanced activity in vivo (Hofheinz et al., 1994).

A second series of phytochemical endoperoxides, isolated from Artabotrys uncinatus and structurally unrelated to the artemisininoids (Liang et al., 1979), also includes at least one antimalarial compound, yingzhaosu A (Fig. 1B). The chemically stable 2,3-dioxabicyclo [3.3.1] nonane ring of yingzhaosu has been substituted to obtain a number of simplified but highly active antimalarials (Hofheinz et al., 1994), one of which, Ro 42-1611 (arteflene; Fig. 1C), possesses potent and sustained activity in experimental models (Jaquet et al., 1994). A single oral dose of 25 mg/kg (61 μmol/kg) arteflene is a well tolerated and effective treatment for mild P. falciparum malaria in certain (Salako et al., 1994) but not all (Radloff et al., 1996) regions of tropical Africa. The therapeutic effect of arteflene appears to be confined to its parasiticidal action (Jakobsen et al., 1995).

Arteflene has a low bioavailability in humans and experimental animals as a consequence of extensive hepatic C-8 hydroxylation, but 8-hydroxyarteflene, the principal plasma metabolite, retains about a quarter of arteflene’s parasiticidal and antimalarial action (Hofheinz et al., 1994). A single oral dose of 25 mg/kg (61 μmol/kg) arteflene is a well tolerated and effective treatment for mild P. falciparum malaria in certain (Salako et al., 1994) but not all (Radloff et al., 1996) regions of tropical Africa. The therapeutic effect of arteflene appears to be confined to its parasiticidal action (Jakobsen et al., 1995).

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Cidal activity (Girometta et al., 1994; Weidekamm et al., 1994).

The antimalarial activity of endoperoxides generally is ascribed to the drugs’ intracellular nonenzymatic activation to cytotoxic intermediates by *Plasmodium’s* intraerythrocytic stage (Meshnick et al., 1996). Proteolysis of ingested hemoglobin within the parasite’s food vacuole releases heme, which, it is supposed, is not only toxic to the parasite but also effects reductive cleavage of the peroxide function to give sequential oxyl- and carbon-centered radicals. The reductive activation of artemisinin and its derivatives by iron(II) catalysts has been characterized in simple chemical systems (Butler et al., 1998). Although the parasiticidal action(s) of these radical species has not been characterized, it might include the induction of oxidative stress (Postma et al., 1996), the alkylation of specific parasite proteins—which has been demonstrated with arteflene (Asawamahasakda et al., 1994)—and the alkylation of heme to create an adduct that inhibits the polymerization of heme to insoluble hemozoin, and, thereby, detoxification of the monomer (Robert and Meunier, 1998). The reactive intermediates also selectively alkylate the proteins of neuronal cells in vitro, although not as extensively as the *Plasmodium* proteins and, consequently, have been implicated hypothetically in the neurotoxicity of artemisinin and its derivatives (Park et al., 1998).

We have investigated the bioactivation of arteflene in vivo and in vitro by taking the enone as a marker of peroxide activation and now report that the enone is a major metabolite of arteflene in nonparasitized rats.

**Materials and Methods**

**Materials.** Ro 42-1611 [arteflene; (1R,4S,5S,8R)-4-[(Z)-2,4-bis(trifluoromethyl)styryl]-4,8-dimethyl-2,3-dioxabicyclo[3.3.1]nonan-7-one] and [14C]arteflene (58.9 mCi/mmol; Fig. 1C) were synthesized and provided by Hoffmann-La Roche Ltd. (Basel, Switzerland). Ketocanazole was a gift from Jansen Pharmaceuticals (Beerse, Belgium). H-2 β-glucuronidase-sulfohydrolase preparation (ca. 100 × 10^3 U/μl), glucurase (beef liver β-glucuronidase; ca. 5000 U/ml), BSA, and reduced glutathione were obtained from Sigma.

![Fig. 1. Endoperoxide antimalarials.](image1)

![Fig. 2. Iron-mediated degradation of arteflene in vitro (Jefford et al., 1996; O’Neill et al., 1997).](image2)
Chemical Co. (Poole, U.K.). Chemical reagents were obtained from Aldrich Chemical Co. (Gillingham, U.K.). HPLC-grade solvents were products of Fisher Scientific Ltd. (Loughborough, U.K.).

**Chemical Synthesis of Arteflene Enone.** The cis and trans isomers of the α,β-unsaturated ketone fragment of arteflene (4-[2,4-bis(trifluoromethyl)phenyl]-3-buten-2-one), a bis trifluoromethyl derivative of methyl styryl ketone (MSK), were prepared by iron(II)-mediated degradation of arteflene (Fig. 2) according to the method used by O’Neill et al. (1997) to obtain the cis isomer alone. The former was prepared and characterized as before. The trans form, separated from its isomer by silica column chromatography, gave electrospray (ES) 

\[
\text{m/z } 300 \left(\text{[M + NH}_4]^+\right); \quad \text{H}-NMR (CDCl}_3, 300 \text{ MHz): } \\
\delta 7.93 (1 \text{ H, s, Ar-H}), 7.77 (1 \text{ H, d, } J = 8.10 \text{ Hz, Ar-H}), 7.51 (1 \text{ H, d, } J = 7.69 \text{ Hz, Ar-H}), 7.11 (1 \text{ H, d, } J = 12.36 \text{ Hz, vinyl-H}), 6.45 (1 \text{ H, d, } J = 12.36 \text{ Hz, vinyl-H}), 2.11 (3 \text{ H, s, COCH}_3).
\]

Both purified forms yielded a mixture of the isomers, resolvable by HPLC [Ultracarb 5-μm C8 column; methanol (45–85%, 30 min) – 0.1 M ammonium acetate, 0.9 ml/min; Rc, cis = 28.0 min, trans = 29.5 min], when methanol solutions were left at room temperature for several days.

**Animal Experiments.** Male Wistar rats (220–270 g) obtained from a breeding colony maintained by the University of Liverpool were administered \(^{14}C\)Arteflene (35 μmol/kg; 2.5 μCi) in DMSO (35 μmol/ml) i.p. and placed in metabolism cages with access to food and water. Urine was collected for 24 h.

Male Wistar rats (220–250 g) were anesthetized with urethane (1.4 g/ml isotonic saline; 1.0 ml/kg, i.p.), and cannulas were inserted into the jugular vein and common bile duct. The penis was ligated. \(^{14}C\)Arteflene (35 μmol/kg; 2.5 μCi) in DMSO (35 μmol/ml) was administered i.v. Some of the rats received either ketonazole (94 μmol/kg) in DMSO (94 μmol/ml; solution prepared immediately before administration) or an equal volume of the vehicle by slow i.v. injection (10 min) 1 h before administration of \(^{14}C\)Arteflene. Bile was collected hourly for 5 h. After 5 h, urine was aspirated from the bladder. The rats were sacrificed by cervical dislocation, and tissues were removed immediately and frozen in liquid nitrogen. The tissues were stored at −80°C.

Radioactivity in bile, urine, and tissues was determined by liquid scintillation counting as described previously (Maggs et al., 1995). The bile and urine were either analyzed immediately by radiometric HPLC and ESP mass spectrometry (MS) or stored at −80°C.

**Derivatization of Urinary Metabolite.** Aliquots (10 ml) of urine from 0- to 24-h collections (15 ml; 180 × 10^3 dpm) were concentrated on C18 Sep-Pak Plus cartridges (Waters Corp., Milford, MA) preconditioned with washing with methanol (10 ml) and water (15 ml). The metabolite was eluted with methanol (5 ml). The effluent was evaporated to dryness under nitrogen and reconstituted in methanol (5 ml). The methanolic solution was mixed with 8 ml of freshly prepared diazomethane (de Boer and Backer, 1963) and left at room temperature for 10 min. Excess diazomethane was destroyed with four drops of glacial acetic acid. The solution was concentrated to low volume under nitrogen and resuspended in 500 μl of methanol before radiochromatographic analysis.

**Hydrolysis of Biliary Metabolites.** To obtain deconjugated metabolites, aliquots of bile (120 μl) each were made up to 1 ml with sodium acetate buffer (0.1 M, pH 5.0). H-2 enzyme preparation (30 μl) was added, and the incubation was left for 15 h in a capped glass test tube at 37°C. The incubations were extracted thrice with tert-butyl methyl ether (TBME) (10 ml) for 10 min. The combined extracts were evaporated to dryness under nitrogen and reconstituted in methanol (150 μl) for chromatographic analysis.

Bile (250 μl) also was incubated with glucurase (30 μl) at 37°C for 15 h, and the whole incubation was used for chromatographic analysis.

**Microsomal Incubations.** Microsomes were prepared from the freshly removed livers of male Wistar rats and portions (10 g) of two human livers stored at −80°C. The histologically normal human livers, obtained from renal transplant donors (female, 10 years, asphyxia; male, 41 years, cerebral hemorrhage), were removed, portioned, and frozen in liquid nitrogen within 30 min of death; approval was granted by the relevant ethical committees, and prior consent was obtained from the donor’s relative. Liver was minced in two volumes of ice-cold potassium phosphate buffer (67 mM; pH 7.4) containing 0.15 M KCl and homogenized using a motor-driven homogenizer. Homogenates were centrifuged at 10,000g for 20 min, and the resulting supernatants were centrifuged at 105,000g for 60 min. The microsomal pellets were resuspended in the phosphate buffer and sedimented at 105,000g for 60 min. The washed pellets were resuspended in chloride-free phosphate buffer. Protein was assayed by the method of Lowry et al. (1951) using BSA as standard.

**Incubations of \(^{14}C\)Arteflene were based on the method of Girometta et al. (1994). The incubation mixture contained 1.25 μCi of \(^{14}C\)Arteflene (final concentration, 1.5, or 10 μM) in acetone (1/v/v in the complete 1.0-ml incubation mixture), microsomal suspension (final protein concentration, 0.3 mg/ml) in sodium phosphate buffer (67 mM; pH 7.4), MgCl2·6H2O (final concentration, 20 μM) in water (5 μl), and NADPH (final concentration, 1.0 μM) in water (100 μl). The incubations were performed in silanized glass flasks in a shaking-water bath at 37°C for 1 h; the reactions were started by adding the NADPH solution after the rest of the incubation mixture had been warmed at 37°C for 10 min. The mixtures were extracted with TBME (10 ml × 3). The combined extracts were evaporated to dryness and reconstituted in methanol (150 μl) for radiochromatographic analysis. The recovery of incubated radioactivity was ca. 80%.

A mixture of the isomeric \(^{14}C\)enone metabolites was extracted from pooled rat bile (3.5 ml) with TBME (10 ml × 3), reconstituted in methanol, and isolated by HPLC [Ultracarb 5-μm C8 column; methanol (45–85%, 30 min) – 0.1 M ammonium acetate, 0.9 ml/min]. An aliquot (1 μCi; 4.7 μCi dissolved in acetone (10 μl) was incubated with rat liver microsomes in the same manner as \(^{14}C\)Arteflene. The material recovered after ether extraction was analyzed by radioimmunoassay (HPLC).

**Incubations of \(^{14}C\)Arteflene with Blood and Bile.** \(^{14}C\)Arteflene (8.57 μmol; 2.5 μCi) in DMSO (35 μmol/ml) was added to freshly drawn rat blood (17 ml) stirred in a heparinized plastic tube at 37°C. Aliquots (1 ml) were removed hourly for 5 h and centrifuged at 2200 rpm for 10 min. Plasma samples (50 μl; 10 × 10^3 dpm) were taken for radiochromatographic analysis. \(^{14}C\)Arteflene (0.17 μmol; 0.2 μCi) in DMSO (10 μl) was added to freshly collected rat bile (300 μl) stirred in a silanized glass tube at 37°C. Aliquots (40 μl) were removed hourly for 5 h and analyzed by radioimmunoassay HPLC.

**Chromatographic Analysis.** The HPLC system consisted of two Jasco PU980 pumps (Jasco Corporation, Tokyo, Japan), an HG-980–30 mixing module, a Jasco UV-975 absorbance detector (254 nm), and a Canberra-Packard Radiomatic Flo-One/particle activity detector in series. The entire eluate was directed to the radioactivity detector, where it was mixed with Flow-Scint A scintillation fluid (Packard Instrument, Groningen, Netherlands) delivered at 1 ml/min. Bile (60–100 μl; 60–100 × 10^3 dpm), urine (100–200 μl; 25 × 10^3 dpm), and the reconstituted extracts of biliary hydrolysates (50–70 μl; 60–100 × 10^3 dpm) and microsomal incubations (30–50 μl; 50–70 × 10^3 dpm) were eluted from an Ultracarb 5-μm C8 column (25 × 0.32 cm; Phenomenex, Macclesfield, U.K.) with gradients of methanol in 0.1 M ammonium acetate (pH 6.9): for bile and urine, 45–85% over 30 min; for the microsomal and biliary extracts, 60–85% over 30 min. The flow rate was 0.9 ml/min.

**MS.** Positive-ion ESP mass spectra of analytes resolved by HPLC were obtained using a Quattro II tandem quadrupole instrument fitted with the standard liquid chromatography-MS (LCMS) interface (Micromass Ltd., Manchester, U.K.). The configuration of the system for parallel mass spectrometric and radiometric analysis has been described elsewhere (Maggs et al., 1995). Elute flow to the LCMS interface was ca. 40 μl/min. Nitrogen was used as the nebulizing and drying gas. The interface temperature was 60°C, the capillary voltage was 3.9 × 10^6 V, the standard cone voltage was 30
V, the high voltage and radio frequency lens voltage was 0.6 × 10^3 V and 0.1 V, respectively, and the photomultiplier voltage was 650 V. Spectra were acquired between m/z 100 and 1050 over a scan duration of 4.91 s. Selective ion monitoring of analytes resolved by HPLC was performed with a dwell time of 200 ms and an interchannel delay of 20 ms. Data were processed via MassLynx 2.1 software (Micromass Ltd.).

For direct-probe chemical ionization MS (CIMS), the source temperature was 150°C, the electron energy was 25 eV, and the reagent gas (ammonia) pressure was 1 × 10^-3 mBar.

Chemical Derivatizations of α,β-Unsaturated Ketones. Two methods of chemical derivatization of the enone were employed to confirm the structure of the metabolite. Conditions for oxime formation and carbonyl reduction were established by use of the putative parent compound of the metabolite, MSK (4-phenyl-3-buten-2-one). MSK (0.68 mmol) was dissolved in ethanol (10 ml). Methoxylamine (0.82 mmol) was added, and the solution was stirred at room temperature for 48 h. The O-methyl oxime derivative was extracted into dichloromethane (10 ml × 2), washed with distilled water, and dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure to yield 0.55 mmol of product: LCMS [methanol (45–85%, 30 min) – 0.1 M ammonium acetate] Rf = 28 min, m/z 176 (M + 1)^+; 1H-NMR (300 MHz), δ 7.26–7.47 (7 H, m, Ar-H and vinyl-H), 3.95 (3 H, s, OCH₃), 2.07 (3 H, s, CH₃).

MSK (0.68 mmol) was reduced with sodium borohydride (0.68 mmol) in ethanol (10 ml) stirred at room temperature for 35 min. The methyl styryl alcohol was extracted with dichloromethane, washed with distilled water, and dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure to yield 0.55 mmol of product: LCMS [acetonitrile (40–80%, 20 min) – 0.1 M ammonium acetate] Rf = 11 min, m/z 131 [M + OH]⁺; 1H-NMR (300 MHz), δ 7.13–7.40 (5 H, m, Ar-H), 6.49 (1 H, d, J = 15.95 Hz, vinyl-H), 6.20 (1 H, dd, J = 15.95 Hz and 1.65 Hz, vinyl-H), 4.41 (1 H, m, CHOH), 1.31 (3 H, d, J = 6.95 Hz, CH₃).

Arteflene enone (3.3 μmol; mixture of cis and trans isomers) prepared by iron(II)-mediated degradation of arteflene (ONeill et al., 1997) was dissolved in ethanol (10 ml) and reacted as for MSK with either methoxylamine (4 μmol) or sodium borohydride (3.96 μmol). Samples of the reaction mixtures were analyzed by either LCMS or HPLC [methanol (45–85%, 30 min) – 0.1 M ammonium acetate] and CIMS. For the oxime (four isomeric products resolved): Rf = 33.2, 33.9 (major), 34.8, and 35.6 (major) min; LCMS, m/z 329 (M + 1)^+.

For the enol: Rf = 28.7 min and 29.2 min; CIMS, m/z 339 ([M + NH₄]⁺). The solvent was removed under reduced pressure to yield 0.55 mmol of product: LCMS [methanol (45–85%, 30 min) – 0.1 M ammonium acetate] Rf = 11 min, m/z 131 [M + OH]⁺; 1H-NMR (300 MHz), δ 7.13–7.40 (5 H, m, Ar-H), 6.49 (1 H, d, J = 15.95 Hz, vinyl-H), 6.20 (1 H, dd, J = 15.95 Hz and 1.65 Hz, vinyl-H), 4.41 (1 H, m, CHOH), 1.31 (3 H, d, J = 6.95 Hz, CH₃).

Arteflene enone (60,000 dpm) extracted from rat bile with TBME (8 volumes gas (ammonia) pressure was 1 atm, temperature was 150°C, the electron energy was 25 eV, and the reagent gas (ammonia) pressure was 1 × 10^-3 mBar. Data were processed via MassLynx 2.1 software (Micromass Ltd.).

The first hourly bile fraction contained substantial amounts of a polar metabolite (Rf = 21 min), which always was present in much smaller proportions in later fractions (Fig. 3, B and C). It corresponded to a minor peak in the ion-current chromatogram for m/z 618 (Fig. 4B) and, therefore, might be a positional isomer of 8-hydroxyarteflene glucuronide.

The second and third prominent radiolabeled metabolites (II and III) were coincident with peaks of absorbance at 254 nm (Fig. 4A) and with peaks in the ion-current chromatogram for m/z 300, which corresponds to that of the ammonium adduct of arteflene enone (Fig. 4C); the latter were absent from “drug blank” bile collected immediately before injection of the [14C]arteflene. They cochromatographed with synthetic standards of the cis and trans isomers of the enone, respectively. All of the rats examined excreted both metabolites although in somewhat variable ratios (Table 1). It was noticed that the isomers differed considerably in respect of their ESP relative response factors (Chi et al., 1991), with the cis form giving the much more intense signal at m/z 300. The signal intensity also was critically dependent on the organic component of the eluent: no peak of m/z 300 was obtained from either isomer when the methanol was replaced with acetonitrile. Arteflene enone was the sole metabolite extracted from bile into TBME.

Biliary Metabolites of Arteflene in Cannulated Rats. [14C]Arteflene given i.v. to anesthetized male rats was excreted in bile to the extent of 17.2 ± 3.7% (mean ± S.D., n = 7), 8.5 ± 4.1%, 7.9 ± 3.3%, and 42.2 ± 7.0% of the administered radiolabel over 0 to 1 h, 1 to 2 h, 2 to 3 h, and 0 to 5 h, respectively. After 5 h, only 0.1 ± 0.05% was recovered in urine taken from the bladder.

The hepatic residues of radioactivity represented 3.4 ± 1.1% of the dose.

The first and subsequent hourly bile fractions usually contained the three prominent radiolabeled components I through III (Fig. 3); they were quantified by radiometric HPLC (Table 1). LCMS revealed the most polar of these (I; Rf = 25.5 min) to be coincident with a peak in the ion-current chromatogram for m/z 618, which corresponds to that of the ammonium adduct ([M + NH₄]⁺) of hydroxyarteflene glucuronide (Fig. 4B). However, two of the nine male rats examined after dosing with [14C]arteflene alone excreted no more than trace amounts of this metabolite in bile over 5 h (Fig. 3A). These animals have not been included in the data shown. When the six rats preloaded with vehicle (DMSO, as a control for the ketoconazole-preloaded animals) also were taken into account, this figure became 3 of 15. The recovery of administered radioactivity in the bile of the two groups was 40.3 ± 6.9 (n = 3) and 40.0 ± 6.7 (n = 12), respectively, over 5 h. From the work of Girometta et al. (1994) it was known that 8-hydroxyarteflene is the major metabolite of arteflene formed by hepatic microsomes of male rats. When the microsomal incubation of [14C]arteflene was repeated, the major metabolite was found by LCMS to be hydroxyarteflene (Fig. 5). Rat bile that had been incubated with a β-glucuronidase preparation no longer contained the hydroxyarteflene glucuronide at 25.5 min and, instead, contained a radiolabeled metabolite (Rf = 26.5 min) that yielded an ion at m/z 442 corresponding to [M + NH₄]⁺ for hydroxyarteflene. Radiolabeled 8-hydroxyarteflene recovered from microsomal incubations by TBME extraction and the hydroxyarteflene metabolite extracted from enzymic hydrolysates of bile chromatographed when the mixture was analyzed by radiometric HPLC and ESP MS.

The first hourly bile fraction contained substantial amounts of a polar metabolite (Rf = 21 min), which always was present in much smaller proportions in later fractions (Fig. 3, B and C). It corresponded to a minor peak in the ion-current chromatogram for m/z 618 (Fig. 4B) and, therefore, might be a positional isomer of 8-hydroxyarteflene glucuronide.

The second and third prominent radiolabeled metabolites (II and III) were coincident with peaks of absorbance at 254 nm (Fig. 4A) and with peaks in the ion-current chromatogram for m/z 300, which corresponds to that of the ammonium adduct of arteflene enone (Fig. 4C); the latter were absent from “drug blank” bile collected immediately before injection of the [14C]arteflene. They cochromatographed with synthetic standards of the cis and trans isomers of the enone, respectively. All of the rats examined excreted both metabolites although in somewhat variable ratios (Table 1). It was noticed that the isomers differed considerably in respect of their ESP relative response factors (Chi et al., 1991), with the cis form giving the much more intense signal at m/z 300. The signal intensity also was critically dependent on the organic component of the eluent: no peak of m/z 300 was obtained from either isomer when the methanol was replaced with acetonitrile. Arteflene enone was the sole metabolite extracted from bile into TBME.

Derivatizations of [14C]Arteflene Enone. Samples of the derivatives of the biliary [14C]arteflene enones were an-
analyzed by radiometric HPLC and ESP MS [methanol (45–85%, 30 min) 0.1 M ammonium acetate]. For the oximes, two isomeric products (Rt = 33.1 and 35.6 min) were resolved: LCMS, m/z 282 ([M + NH₄⁺ - CH₂O]⁺). They corresponded to the two major oxime derivatives of the synthetic cis and trans arteflene enones. For the enols, two isomeric products (Rt = 28.7 and 29.2 min) were resolved: LCMS, m/z 302 ([M + NH₄⁺]; they corresponded to the derivatives of the synthetic compound.

Table 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>8-Hydroxyarteflene glucuronide (% of Radiolabeled Dose)</th>
<th>Cis enone (% of Radiolabeled Dose)</th>
<th>Trans enone (% of Radiolabeled Dose)</th>
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<td>0–1</td>
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<td>0.9 ± 0.3</td>
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<td>1–2</td>
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<td>2.8 ± 0.8</td>
<td>1.4 ± 0.6</td>
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<td>2–3</td>
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<td>4.1 ± 1.6</td>
<td>1.1 ± 0.3</td>
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<tr>
<td>0–3</td>
<td>14.2 ± 3.9</td>
<td>10.1 ± 3.4</td>
<td>3.4 ± 1.2</td>
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Inhibition of Arteflene Metabolism in Rats. The rats predosed with ketoconazole excreted approximately the same fraction of administered radioactivity in bile over 5 h (34.5 ± 4.1%, n = 5) as the rats that received DMSO alone (37.8 ± 6.9, n = 5; Fig. 6) but they displayed a distinctive change in biliary metabolite profile (Table 2). The excretion of 8-hydroxyarteflene glucuronide but not of the enones was inhibited partially by ketoconazole; the recovery of radioactivity as the glucuronide over the first hour was 6.0 ± 2.4% and 0.4 ± 0.1% (mean ± S.D.; n = 5) of the dose in the control and test group, respectively. After 5 h, 8-hydroxyarteflene glucuronide excretion in all but one of the test animals had become equal to that in the control animals. The data for each group do not include one rat that excreted only trace amounts of 8-hydroxyarteflene glucuronide over the entire period of bile collection.

Urinary Metabolites of Arteflene in Rats. Metabolites of [¹⁴C]arteflene given i.p. to male Wistar rats were excreted in urine to the extent of 15.3 ± 1.6% (mean ± S.D.; n = 8) of the dose over 24 h (Fig. 7).

The urine contained one prominent radiolabeled metabolite (Fig. 7A). This was coincident with a peak in the ioncurrent for m/z 361 (Fig. 7B), although a slightly earlier
component, yielding \( m/z \) 496, was separated from it by only 0.3 min. The ion \( m/z \) 361 was taken to be the ammonium adduct of the analyte. It was not subject to cone-voltage fragmentation. Derivatization of the metabolite with diazomethane resulted in a mass increase of 14 amu, corresponding to the addition of a methyl group, presumably to a carboxyl function, and an increase in retention time on the reversed-phase column of 6.6 min. The derivatization also increased the mass of the \( m/z \) 496 ion by 14 amu, but this produced a smaller increase in retention time (5.8 min) and a greater resolution from the radiolabeled metabolite.

From the molecular weight of the metabolite, the presence of a carboxyl group, the retention of the \( ^{14} \)C label, and the presumed presence of an intact \( \text{bis} \) trifluoromethylphenyl moiety, it is postulated that the compound is derived from arteflene enone via \( \alpha \)-oxidation (forming a carboxyl group), reduction of the double bond, and conjugation with glycine. MSK is metabolized to glycine conjugates in the rat, but this involves oxidative side-chain cleavages to phenylacetic acid and benzoic acid (Sauer et al., 1997), not, as in the present case, the more complicated pathway leading to phenylpropanolic acid.

The proposed major pathways of arteflene metabolism are schematized in Fig. 8.

Metabolism of Arteflene by Hepatic Microsomes. After incubation of arteflene (1, 5, or 10 \( \mu \)M) with microsomes from rat and human livers (final protein concentration, 0.3 mg/ml), the unchanged compound was detected in the presence of one major radiolabeled metabolite (Fig. 5A). This was coincident with a peak in the ion-current chromatogram for \( m/z \) 442 (Fig. 6B), which was taken from the work of Girometta et al. (1994), who identified 8-hydroxyarteflene as the sole major metabolite of arteflene in rat liver microsomes, as the ammonium adduct of 8-hydroxyarteflene. No enone was recovered from the microsomal incubations.

An isomeric mixture of \( [^{14} \text{C}] \)arteflene enone extracted from pooled rat bile was incubated with microsomes from rat and human livers in the same manner as \( [^{14} \text{C}] \)arteflene. Recovery of radiolabel after ether extraction was ca. 75%. Radio-HPLC analysis of the reconstituted extracts demonstrated that no metabolism of the enones had occurred.

Reaction of \( \alpha, \beta \)-Unsaturated Ketones with Glutathione. The addition of glutathione (500 \( \mu \)M) to MSK (400 \( \mu \)M) yielded appreciable amounts of two isomeric glutathione conjugates (\( m/z \) 454 ([M + 1]+), \( R_t \) = 15.8 and 16.5 min) within 30 s and almost complete reaction within 5 min.

The reaction of synthetic arteflene enone (400 \( \mu \)M) with glutathione (500 \( \mu \)M) was much slower, but almost complete.
turnover to two glutathione conjugates (m/z 590 ([M + NH₄]⁺, m/z 442). The minor peak in B did not correspond to a radiolabeled compound and would appear to be of endogenous origin.

**Discussion**

The currently favored mechanism of action of endoperoxide antimalarials, derived primarily from studies on iron-perox-ide chemistry in vitro, involves intracellular reduction of the peroxide group and the subsequent formation of a carbon-centered radical species (Butler et al., 1998). These reactive species may give rise to specific parasite toxicity by engendering oxidative stress, alkylating parasite proteins, and inhibiting the detoxification of heme. If such activation occurs within mammalian (host) cells other than the parasitized erythrocyte, it might have toxicological implications for the malaria patient: the artemisinin-type antimalarial endoperoxides are generally well tolerated by volunteers and patients (de Vries and Dien, 1996) but, under certain circumstances, their administration to experimental animals has been associated with neurotoxicity and embryotoxicity (Hofheinz et al., 1994; Park et al., 1998). The investigations reported here were undertaken primarily to determine the magnitude, nature, and site of bioactivation of a synthetic endoperoxide in the nonparasitized rat.

A notable discontinuous variation in the biliary excretion of 8-hydroxyarteflene glucuronide was observed. Although the precise metabolic origin of this phenomenon was not determined, discontinuous variations in glucuronyltransferase activity toward 3α-hydroxy steroids (Tephly et al., 1988) and the biliary excretion of hydroxydesoxyfluoroamidequine glucuronide (Jewell et al., 1995) in the Wistar rat have been reported previously. However, in the present study, total biliary excretion of the radiolabel was not diminished appreciably in the rats that excreted only trace amounts of 8-hydroxyarteflene glucuronide, and there was no detectable appearance of hydroxyarteflene in the bile. Incubation of [14C]arteflene with microsomes prepared from the livers of rats that had excreted only trace amounts of 8-hydroxyarteflene glucuronide did not show any turnover to hydroxyarteflene (data not shown), suggesting that the metabolic variation observed in vivo may be a result of a deficiency of hydroxylation, although an additional lack of glucuronyltransferase activity cannot be discounted.

Little is known about the extent or the mechanism(s) of endoperoxide bioactivation in mammalian systems. Nonenzymatic (bioinorganic) reactions remain a possibility, although, as a matter of biological necessity, the intracellular and extracellular concentrations of iron as low-molecular-weight complexes are extremely low. The observed stability of arteflene in blood ex vivo would appear to conform with the highly restricted availability of chemically reactive iron (Ponka et al. 1998), although artemether undergoes reduc-tive rearrangements in rat blood under similar conditions (Blum et al., 1998). Bilirubin, a physiological reductant known to generate free radicals from phenazine methosulfate in vitro (Lott and Slater, 1973), might conceivably reduce endoperoxides in biological media, but the present and earlier findings (Maggs et al., 1998) have shown that arteflene and artemisinin endoperoxides are stable in rat bile. The metabolic fates of only a few antimalarial endoperoxides have been described, and all the available information relates to the simple ether and ester prodrugs of DHA. The principal pathway of metabolism of DHA in the rat is O-glucuronidation (Maggs et al., 1997), which yields a pharmacologically inactive conjugate. Nevertheless, the activation of artemisinin compounds in vivo can be inferred from the presence in plasma and bile of isomeric forms of the drugs and deoxygenated derivatives (Chi et al., 1991; Maggs et al., 1997), generated in vitro by reaction with iron(II), whose

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**Fig. 5.** A, radiochromatogram of the metabolites of [14C]arteflene formed by rat liver microsomes. B, LCMS ion-current chromatogram for hydroxyarteflene ([M + NH₄]⁺; m/z 442). The minor peak in B did not correspond to a radiolabeled compound and would appear to be of endogenous origin.

**Fig. 6.** Excretion of radioactivity in bile after the administration of [14C]arteflene (35 μmol/kg, i.v.) with (n = 5) or without (n = 5) prior administration of ketoconazole (94 μmol/kg, i.v.) to anesthetized and cannulated male rats. Points represent mean ± S.D.
TABLE 2
Inhibition of [14C]arteflene metabolism in vivo

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>8-Hydroxyarteflene glucuronide</th>
<th>Cis enone</th>
<th>Trans enone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>KC</td>
<td>DMSO</td>
</tr>
<tr>
<td>0-1</td>
<td>6.0 ± 2.4</td>
<td>0.4 ± 0.1</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>1-2</td>
<td>5.2 ± 2.1</td>
<td>1.8 ± 0.8</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>2-3</td>
<td>2.1 ± 0.7</td>
<td>2.3 ± 0.8</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>0-3</td>
<td>13.3 ± 5.2</td>
<td>3.8 ± 1.7</td>
<td>7.1 ± 2.2</td>
</tr>
</tbody>
</table>

Fig. 7. A, radiochromatogram of the urinary metabolites (0- to 24-h urine collection) of [14C]arteflene in a male rat. B, LCMS ion-current chromatogram for m/z 361.

formation is ascribable to the rearrangement of radical intermediates (Jefford et al., 1996; Butler et al., 1998). Although these minor metabolites serve as chemically stable markers of bioactivation, as yet they provide no indications as to the site and biochemical route (enzymatic versus non-enzymatic) of activation. In addition, the presence of isomers of artemisinin endoperoxides in urine has to be interpreted with caution because it would appear that the iron concentration of normal human urine, perhaps by virtue of the low concentration of iron-binding urinary protein, is sufficient to effect the rapid isomerization of DHA glucuronide ex vivo (Maggs et al., 1998).

The mechanistic principles underlying the iron-induced rearrangement of artemisinin (Jefford et al., 1996; Butler et al., 1998) have been applied successfully to predicting the formation of free radicals (P.M.O., et al., unpublished observations) and the α,β-unsaturated ketone from arteflene in vitro (O’Neill et al., 1997). The present studies, by identifying enone in rat bile, have confirmed the general biomimetic character of reactions between inorganic iron and endoperoxides, although the apparent absence from the metabolite profile of arteflene diol (notably, metabolic reduction of a peroxide function to a diol has not been reported for antimalarial endoperoxides) reveals that a model chemical system (Fig. 2) may be less predictive of the metabolic fate of the peroxide function in simple bicyclic endoperoxides. Furthermore, the extensive bioactivation of arteflene in rats contrasts with the metabolism of DHA (Maggs et al., 1997), arteether (Bell et al., 1998), and arteether (Chi et al., 1991) in the same species, which consists of glucuronidation and P-450-catalyzed hydroxylations with only minimal disturbance of the peroxide group. These observations imply that attempts to abridge the complex carbon-oxygen framework of artemisinin, which is not essential for antimalarial activity, to open routes to more accessible and wholly synthetic endoperoxide drugs (Jefford, 1996) might be counterbalanced by an unforeseen and potentially damaging enhancement of bioactivation within the nonparasitized tissues.

The extensive biliary elimination of enone by rats implicates the liver as a major site of bioactivation in this species, although the apparent inability of rat liver microsomes to form the metabolite while catalyzing appreciable C-8 hydroxylation of arteflene implies that activation does not occur in the hepatic endoplasmic reticulum. The same result was obtained with two samples of human hepatic microsomes, but it is not known whether they were representative of the general population. It is interesting to note that the heme moiety of the P-450(s) catalyzing hydroxylation of arteflene at C-8 (the reaction was inhibited by ketoconazole in vivo) reacts with the carbon adjacent to the peroxide bridge but not with the oxygens themselves. In marked contrast, arteether incubated with rat liver microsomes undergoes attack on the peroxide moiety to give an isomerized (hydroxydesoxy) product, whereas DHA derived from arteether yields both desoxy-DHA and isomerized metabolites (Baker et al., 1989). However, the metabolism of antimalarial endoperoxides by hepatic microsomes can be qualitatively unrepresentative of metabolism in vivo: the hydroxylation of DHA by rat liver microsomes is not reproduced in the whole animal (Chi et al., 1991; Maggs et al., 1997). The microsomal enzymes catalyzing the rearrangements of the peroxide moiety have yet to be identified. P-450 isozymes appear the most likely candi-
dates because the heme thiolate enzymes prostacyclin synthase and thromboxane synthase, although not closely related to microsomal P-450, actively catalyze the isomerization of prostaglandin H₂ endoperoxide, which is also a substrate for phenobarbitone-induced rat hepatic P-450 (Ullrich and Brugger, 1994). The catalytic mechanism of the synthases is analogous in detail to that of the biomimetic iron(II)-mediated isomerization of artemisinin (Jefford et al., 1996; Butler et al., 1998). The localization of the synthases in the endoplasmic reticulum of endothelial cells and platelets, respectively, raises the possibility of diffuse extrahepatic biotransformation of endoperoxide drugs.

If the bioactivation of arteflene in rats is not to be assigned to hepatic microsomal enzymes, two alternative possibilities might be considered. First, the endoperoxide reductase activity of hepatic cytosol does catalyze the NAD/NADH-dependent conversion of arteether to desoxy-DHA, but the rate of arteether turnover in microsomes is an order of magnitude greater (Leskovac and Theoharides, 1991). Second, and at present, hypothetically, cleavage of the endoperoxide bridge is effected by nonheme iron, i.e., by the poorly characterized intracellular labile iron pool, in which the metal may exist as low-molecular-weight complexes (Ponka et al., 1998).

The ratio of cis- to trans-enone in bile displayed considerable interindividual variation. By analogy with the formation of cis and trans isomers during iron(II)-mediated arteflene degradation, and allowing for the slow rate of isomerization of the purified compounds in vitro, it is concluded that the presence of both forms in vivo derives ultimately from the stereochemistry of their metabolic formation. Thereby, if the variation in isomer ratio is principally of metabolic origin, it might hypothetically be a consequence of arteflene’s degradation by independently variable pathways, for example, cleavage of the peroxide group by cytosolic reductases and the prostacyclin and thromboxane synthases.

α,β-Unsaturated carbonyl compounds such as the enone metabolite of arteflene are potentially reactive and toxic species in biological systems by virtue of their ability to undergo electrophilic addition to the sulphhydril and other nucleophilic centers of macromolecules (Cooper et al., 1992). However, MSK, at least, is reported to lack overt toxicity in rodents (Sauer et al., 1997), although it can be metabolically activated to a mutagen in vitro (National Cancer Institute, 1994). Because the proposed mechanism of enone formation requires proportional creation of a carbon-centered cyclohexyl radical (O’Neill et al., 1997), it follows that there also will be a substantial challenge to the radical defense mechanisms at the site(s) of bioactivation. An assessment of the potential impact on host cells of arteflene’s bioactivation must also consider that the simplified cyclohexyl species, in comparison with artemisinin radicals, with their several possibilities for intramolecular rearrangement and deactivation (Butler et al., 1998), is more likely to be deactivated by reacting with other molecules. The fate of the cyclohexyl radical in both chemical and biological systems remains unknown, but the enone, in rats and, contrary to expectations, was eliminated extensively by biliary excretion rather than by the metabolic routes usually associated with α,β-unsaturated carbonyl compounds and, specifically, with MSK in rodents (Sauer et al., 1997). Most notably, and notwithstanding MSK is a substrate for glutathione S-transferase in vitro (Habig et al. 1974), the enone was not combined with glutathione and eliminated in bile as thioether conjugates, a common pathway for the disposal of electrophilic species (Jewell et al., 1995; Maggs et al., 1995). Studies on the rate of chemical reaction of α,β-unsaturated carbonyl analogues with glutathione reveal that the reactivity of ketones is second only to that of aldehydes (Chien et al., 1994). The enone’s intrinsic reactivity with glutathione was established in vitro but it was clearly much lower than that of MSK, which itself...
is not metabolized extensively via glutathione conjugation in rats (Sauer et al., 1997).

The extensive formation of such a chemically and metabolically stable metabolite as the enone suggests that arteflene might serve as a convenient “probe compound” for assessing endoperoxide bioactivation in both mammalian cells and the malaria parasite.

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