In Vitro Metabolism Study of Combretastatin A-4 in Rat and Human Liver Microsomes

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ABSTRACT:

The phase I biotransformation of combretastatin A-4 (CA-4) 1, a potent tubulin polymerization inhibitor with antivascular and antitumoral properties, was studied using rat and human liver subcellular fractions. The metabolites were separated by high-performance liquid chromatography and detected with simultaneous UV and electrospray ionization (ESI) mass spectrometry. The assignment of metabolite structures was based on ESI-tandem mass spectrometry experiments, and it was confirmed by comparison with reference samples obtained by synthesis. O-Demethylation and aromatic hydroxylation are the two major phase I biotransformation pathways, the latter being regioselective for phenyl ring B of 1. Indeed, incubation with rat and human microsomal fractions led to the formation of a number of metabolites, eight of which were identified. The regioselectivity of microsomal oxidation was also demonstrated by the lack of metabolites arising from stilbene double bond epoxidation. Alongside the oxidative metabolism, Z-E isomerization during in vitro study was also observed, contributing to the complexity of the metabolite pattern. Moreover, when 1 was incubated with a cytosolic fraction, metabolites were not observed. Aromatic hydroxylation at the C-6' of phenyl ring B and isomerization led to the formation of M1 and M2 metabolites, which were further oxidized to the corresponding para-quinone (M7 and M8) species whose role in pharmacodynamic activity is unknown. Metabolites M4 and M5, arising from O-demethylation of phenyl ring B, did not form the ortho-quinones. O-Demethylation of phenyl ring A formed the metabolite M3 with a complete isomerization of the stilbene double bond.

The combretastatins are a large family of natural products extracted from Combretum caffrum (Pettit et al., 1987, 1989). In particular, combretastatin A-4 [CA-4; (Z)-3'-hydroxy-3,4,4',5-tetramethoxy-stilbene] 1; Fig. 1] is the most potent of these compounds. In the original articles, 1 was described as a strong cell growth and tubulin inhibitor (Lin et al., 1988; Pettit et al., 1989). These features ultimately induced the disruption of microtubular function (McGown and Fox, 1989), causing the selective and irreversible damage to the neovascularature of tumors. Because of its poor solubility in water, a more soluble bifunctional molecule of the family to have entered clinical trials. AVE8062 2, has been developed as the selected lead for in vivo and human studies (Mealy et al., 2006). 1 is not the only molecule of the family to have entered clinical trials. AVE8062 3, a synthetic analog bearing a different substitution on ring B, has recently done so as well (Lippert, 2007). Indeed, combretastatin A-1 phosphate (Oxi4503) 4, which retains the same biological and structural signature, has been shown to possess features that should make it suitable for therapeutic intervention (Lippert, 2007). Treatment with 2, which is rapidly converted by nonspecific endogenous phosphatas, present in plasma and in endothelial cells, to the active compound 1 (Pettit et al., 1995), disrupts selectively the tumoral vascularuature causing reduction of the blood flow within the tumor and subsequent massive hemorrhagic necrosis (Grosios et al., 1999; Tozer et al., 1999). These results have been demonstrated at doses less than 1/10 of the maximum tolerated (Dark et al., 1997), in contrast with other structurally related tubulin-binding agents (e.g., vincristine, vinblastine podophyllotoxin, and colchicine). The pharmacokinetics of 2 has also been investigated during preclinical and clinical studies (Rustin et al., 2003; Stevenson et al., 2003; Kirwan et al., 2004), whereas no data are available for the metabolic fate of 1. Stratford and Dennis (1999) postulated the presence of combretastatin A-4 metabolites without determining their structures. Similarly, Kirwan et al. (2004) stated that the metabolic profile of the drug is complex, but, also in this case, no effort was made to identify the metabolites. Finally, Rustin et al. (2003), in their phase I clinical study, confirmed the rapid transformation of 2 into the active 1, and they reported the formation of the CA-4 glucuronide.

These considerations, linked to our interest in the medicinal chemistry of CA-4 1 (Tron et al., 2006), led us to study the metabolic fate of 1 in rat and human microsomal preparations. In the present article,
the major in vitro phase I biotransformation pathways of 1 with the formation of quinonic species is described.

Materials and Methods

Reagents and Chemicals. Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Milan, Italy). Water (HPLC grade) was obtained from Milli-Q Reverse Osmosis system (Millipore Corporation, Billerica, MA). Titrilplex V, Extrelut 3, and Extrelut 1 columns were purchased from Sigma-Aldrich (Milan, Italy). Water (HPLC grade) was obtained from Milli-Q Reverse Osmosis system (Millipore Corporation). Tetrahydrofuran (THF) was dried over lithium aluminum hydride to remove peroxides, and it was stored on activated molecular sieves (4 Å). DMF was used without further purification, and they were purchased from Sigma-Aldrich, lithium aluminum hydride, pyridinium dichromate, sodium hydride, dimethyl sulfate, Fremy’s salt (potassium nitrosodisulfonate), imidazole, iodomethane, butyllithium (1.6 M in tetrahydrofuran), diisopropylethylamine, and trifluoroacetic acid were used without further purification, and they were purchased from Sigma-Aldrich. Aldrich. Titriplex V, Extrelut 3, and Extrelut 1 columns were acquired and processed using Xcalibur software (Thermo Electron Corporation).

Operating conditions on the ion trap mass spectrometer in positive ion mode were as follows: spray voltage, 3.50 kV; source current, 80 μA; capillary temperature, 350°C; capillary voltage, 11.60 V; tube lens offset, 5 V; multipole 1 offset, −7.00 V; multipole 2 offset, −8.50 V; and sheath gas flow (N2), 60 arbitrary units. Data were acquired in MS/MS product ion scan mode using mass scan range m/z 90 to 400, and the collision energy was optimized at 32%.

Reagents and Chemicals.

LC-DAD-UV. A Thermo Finnigan LCQ Deca XP Plus system equipped with a quadrupole pump, a Surveyor AS autosampler, and a vacuum degasser was used for LC-MS analysis (Thermo Electron Corporation, Waltham, MA). The chromatographic separation was performed on a Phenomenex Luna 5μ C18(2) (50 × 4.6 mm) as the stationary phase protected by a Security Guard C18. The eluate was injected into the electrospray ion source (ESI), with a splitting of 20%, and MS/MS spectra were acquired and purchased from Sigma-Aldrich.

Instrumentation and Chromatographic Conditions. LC-DAD-UV. An HPLC system (Shimadzu, Kyoto, Japan) with two LC-10ADVp module pumps, an SLC-10ADVp system controller, and a DGU-14-A online degasser was used for the analysis. The chromatographic separations were performed on a Phenomenex Luna 5μ C18(2) (250 × 4.6 mm) as the stationary phase protected by a C18-Security Guard (Phenomenex, Torrance, CA). A model 7725i Rheodyne valve was used for the injection of samples (20 μl). The SPD-M10Awp photodiode array detector was used to detect the analytes at 330 nm. ClassVp 5.03 software was used to process the chromatograms. The isocratic mobile phase (flow rate 1.0 ml/min) consisted in water/acetonitrile [60:40 (v/v)] (0.5% formic acid) mixture. The eluants were filtered through a 0.45-μm pore size polyvinylidene difluoride membrane filter before use. All of the analyses were carried out at room temperature.

A standard incubation mixture, in 10-ml polyethylene tubes, contained 1.3 mM MgCl2·6H2O, 0.4 mM NADPNa2, 3.6 mM glucose 6-phosphate, and 0.4 U/ml glucose 6-phosphate dehydrogenase in a 0.1 M phosphate buffer, pH 7.4, containing 1.5 mM Tiritplex V (an ethylenediamine tetraacetic acid analog), 0.84 mg/ml of surfactant Tween 80, and 1 mM CA-4. The mixture was brought to a final volume of 3 ml. After pre-equilibration of the mixture, an appropriate volume of microsomal suspension or cytosol was added to give a final protein concentration of 1 or 1.5 mg/ml for microsomal suspensions or cytosol, respectively. The mixture was shaken for 60 min at 37°C. Control incubations were done without the NADPH-regenerating system or with boiled microsomes. The incubation mixtures were then extracted on Extrelut3 using 15 ml of chloroform/ethyl acetate/2-propanol (45:45:10) as eluant. The organic phase was evaporated under reduced pressure, and the crude extract was reconstituted in acetonitrile (0.5 ml) and analyzed by LC-DAD-UV and LC-ESI-MS.

Human liver fractions incubation. Cryopreserved CYP3A4 human liver microsomes (pooled mixed sex; 10 individuals donors; protein concentration,
mg; 61% yield) was obtained using the conditions used for 36% yield). Starting from compound (1% of incubation total volume), and 38 or 100 
ether/ethyl acetate (99:1) as eluant to give (3-
iene). The residue was purified by column chromatography using petroleum 
ether/ethyl acetate (7:3 and then 6:4) as eluants to give compound 
440 m/z. 

**Synthesis of Putative Metabolites.** Synthesis of (E)-3',4-dihydroxy-3,4,5-
trimethoxy-stilbene 5. (2 g; 5.5 mmol) was dissolved in dichloromethane (2.5 ml) and cooled to −78°C. Boron tribromide (1 M solution in dichlo-
oromethane; 0.8 ml) was added dropwise under magnetic stirring. After com-
pletion of the reaction, ethyl acetate was added, and the mixture was neutral-
ized with saturated NaHCO₃ solution. The organic layer was washed with 
brine, dried over anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (7.5:3 and then 6:4) as eluants to give compound 5 (46; 19% yield).

**1H NMR (300 MHz, CDCl₃): δ 7.1 (d, J = 2.2 Hz, H-2'), 6.9 (dd, J = 8.2/2.2 Hz, H-6'), 6.8 (s, 2H), 6.82 (d, J = 8.2 Hz, H-5'), 6.7 (s, 2H), 5.6 (s, OH), 5.5 (s, OH), 3.94 (s, 2OHCH₃), 3.90 (s, OH).**

**13C NMR (300 MHz, CDCl₃): δ 147.2 (2C ring A), 146.3 (C), 145.8 (C), 134.5 (C), 131.2 (C), 129.2 (C), 127.3 (CH), 126.5 (CH), 119.1 (CH), 111.6 (CH), 110.7 (CH), 103.1 (2CH ring A), 56.4 (2OCH₃), 56.3 (OCH₃).**

**UV (CH₂CN): λmax 245, 300 (broad) nm.**

**3,5-di[(t-butyldimethylsilyloxy)-4-methoxy-benzaldehyde 14.** Compound 13 (2.9 g; 7.3 mmol) was dissolved in diethyl ether (32 ml), and the solution was cooled to 0°C. Lithium aluminum hydride (0.6 g; 16 mmol) was added in small portions under magnetic stirring. After an hour, the mixture was treated with silica gel (4.8 g) and saturated NH₄Cl solution. The mixture was filtered through a pad of Celite and washed with diethyl ether. The filtrate was brought to a final volume of 2 ml. After completion of the reaction, ethyl acetate was added, and the mixture was neutralized with 2 N HCl to give compound 14 as a viscous colorless oil (2.9 g; 98% yield), which was used directly without further purification.

**1H NMR (300 MHz, CDCl₃): δ 6.5 (s, 2H), 4.5 (s, CD₂CH₃), 3.7 (s, OCH₃), 1.0 [s, 2Si(CH₃)₂], 0.2 [s, 2SiC(CH₃)₃].**

**UV (CH₂CN): λmax 245, 300 nm.**

**Synthesis of 3,5-di[(t-butyldimethylsilyloxy)-4-methoxy-benzaldehyde**

To a solution of compound 14 (2.9 g; 7.3 mmol) in dichloromethane (30 ml), pyridinium dichromate (3.8 g; 10 mmol) was added and the mixture stirred for 12 h. The reaction was worked up by dilution with dichloromethane. The organic layer was washed with 2 N HCl and brine, and then it was dried over Na₂SO₄ and filtered. Evaporation of the solvent gave a crude product that was purified by column chromatography using petroleum ether/ethyl acetate (98:2) to give compound 15 as a white solid (1.8 g; 70% yield).

**1H NMR (300 MHz, CDCl₃): δ 9.8 (s, CHO), 7.0 (s, 2H), 3.8 (s, OCH₃), 1.0 [s, 2Si(CH₃)₂], 0.2 [s, 2SiC(CH₃)₃].**

**Synthesis of (Z)-3',5,5',5'-tetramethoxy-stilbene 16. Under a N₂ atmosphere, the phosphonium salt 6 (2.9 g; 6.5 mmol) was dissolved in dry THF (15 ml), and the suspension was cooled to −15°C. Sodium hydride (60%; 545 mg; 13.6 mmol) was then added. The reaction mixture was stirred for 1 h until the solution became red. Subsequently, compound 7 (2 g; 5.5 mmol), dissolved in dry THF (10 ml), was added dropwise and the reaction was stirred overnight. The mixture was diluted with ethyl acetate and neutralized with 2 N HCl. The organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (95:5 and then 9:1) as eluant to give 3,4,4-di[butyl(dimethyl)silyloxy]benzaldehyde 7 (4.73 g; 93% yield).

**1H NMR (300 MHz, CDCl₃): δ 6.4 (m, broad 4H), 6.3 (2H), 3.8 (s, OCH₃), 3.7 (s, 2OCH₃), 3.6 (OCH₃), 0.9 [s, 2Si(CH₃)₂], 0.1 [s, 2SiCH₂CH₃].**

**Synthesis of (Z)-3',5'-di[(t-butyldimethylsilyloxy)-4,4',5'-tetramethoxy-
stilbene 17. Compound 16 (80 mg; 0.14 mmol) was dissolved at 0°C in dry THF (1 ml). TBAF was added (1 M in tetrahydrofuran; 440 µl). After completion of the reaction, the mixture was diluted with dichloromethane and washed with water. The organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (8:2 and then 6:4) as eluants to give compound 10 (24 mg; 36% yield). Starting from compound 9 (167 mg; 0.3 mmol), compound 11 (56 mg; 61% yield) was obtained using the conditions used for 10.**

**Compound 10.** 1H NMR (300 MHz, CDCl₃): δ 6.8 (s, H), 6.74 (s, H), 6.73 (s, H), 6.5 (s, 2H), 6.4 (d, J = 12.1 Hz, H-olefinic), 6.3 (d, J = 12.1 Hz, H-olefinic), 4.8 (broad peak 2OH), 3.8 (s, OCH₃), 3.6 (s, 2OCH₃).

**MS-ESI: m/z 303 [M + H⁺]⁺.**

**UV (CH₂CN): λmax 245, 300 (broad) nm.**

**Synthesis of 3,5-di[(t-butyldimethylsilyloxy)-4-methoxy-benzaldehyde 14**

To a solution of compound 14 (2.9 g; 7.3 mmol) in dichloromethane (30 ml), pyridinium dichromate (3.8 g; 10 mmol) was added and the mixture stirred for 12 h.

The reaction was worked up by dilution with dichloromethane. The organic layer was washed with 2 N HCl and brine, and then it was dried over Na₂SO₄ and filtered. Evaporation of the solvent gave a crude product that was purified by column chromatography using petroleum ether/ethyl acetate (98:2) to give compound 15 as a white solid (1.8 g; 70% yield).

**1H NMR (300 MHz, CDCl₃): δ 9.8 (s, CHO), 7.0 (s, 2H), 3.8 (s, OCH₃), 1.0 [s, 2Si(CH₃)₂], 0.2 [s, 2SiC(CH₃)₃].**

**Synthesis of (Z)-3',5,5',5'-tetramethoxy-stilbene 16. Under a N₂ atmosphere, the phosphonium salt 6 (1.0 g; 2.3 mmol) was dissolved in dry THF (10 ml). The solution was cooled to −15°C, and butyl lithium (1.6 M solution in tetrahydrofuran; 1.8 ml) was added. The reaction mixture was stirred until the solution became red. Subsequently, compound 15 (0.9 g; 2.3 mmol) dissolved in dry THF (7 ml) was added dropwise and stirred at room temperature for 1 h. The mixture was diluted with ethyl acetate and neutralized with saturated NH₄Cl solution. The organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (95:5) as eluant to give in order compound 16 (Z) pure (0.5 g; 37% yield) and then 0.4 g of mixture (Z + E) compounds both as yellow oils.

**1H NMR (300 MHz, CDCl₃): δ 6.4 (m, broad 4H), 6.3 (2H), 3.8 (s, OCH₃), 3.7 (s, 2OCH₃), 3.6 (OCH₃), 0.9 [s, 2Si(CH₃)₂], 0.1 [s, 2SiCH₂CH₃].**

**Synthesis of (Z)-3',5'-di[(t-butyldimethylsilyloxy)-4,4',5'-tetramethoxy-stilbene 17. Compound 16 (80 mg; 0.14 mmol) was dissolved at 0°C in dry THF (1 ml). TBAF was added (1 M in tetrahydrofuran; 300 µl). After 1 h, the reaction mixture, which rapidly developed an intense dark red color, was neutralized with cold 2 N HCl and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (6:4) as eluant to give compound 10 (46 mg; 98% yield) as a white solid.
**Synthesis of (E)-3',5'-di-[([butyldimethylsilyl]oxy]-4-methoxy-benzaldehyde 25.** Compound 24 (243 mg; 1.4 mmol) and imidazole (490 mg; 7.2 mmol) were dissolved in dry DMF (10 ml), and the reaction mixture was cooled to 0°C. TBDMSI (650 mg; 4.3 mmol) was added, and the reaction was stirred overnight at room temperature. The mixture was diluted with ethyl acetate and washed with 1 N HCl, and dried over anhydrous Na2SO4. Filtration and evaporation of the solvent gave a residue that was purified by column chromatography using petroleum ether and then petroleum ether/ethyl acetate (9:1) as eluants to give compound 25 (490 mg; 85% yield) as a white solid.

**MS-ESI:** m/z 169 [M + H]+.

**Synthesis of 2,5-di-[([butyldimethylsilyl]oxy]-4-methoxy-benzaldehyde 25.** Compound 24 (243 mg; 1.4 mmol) and imidazole (490 mg; 7.2 mmol) were dissolved in dry DMF (10 ml), and the reaction mixture was cooled to 0°C. TBDMSI (650 mg; 4.3 mmol) was added, and the reaction was stirred overnight at room temperature. The mixture was diluted with ethyl acetate and washed with 1 N HCl, and dried over anhydrous Na2SO4. Filtration and evaporation of the solvent gave a residue that was purified by column chromatography using petroleum ether/ethyl acetate (9:1) as eluant to give compound 25 (490 mg; 85% yield) as a white solid.

**MS-ESI:** m/z 169 [M + H]+.

**Synthesis of 2,5-di-[([butyldimethylsilyl]oxy]-4-methoxy-benzaldehyde 25.** Compound 24 (243 mg; 1.4 mmol) and imidazole (490 mg; 7.2 mmol) were dissolved in dry DMF (10 ml), and the reaction mixture was cooled to 0°C. TBDMSI (650 mg; 4.3 mmol) was added, and the reaction was stirred overnight at room temperature. The mixture was diluted with ethyl acetate and washed with 1 N HCl, and dried over anhydrous Na2SO4. Filtration and evaporation of the solvent gave a residue that was purified by column chromatography using petroleum ether/ethyl acetate (9:1) as eluant to give compound 25 (490 mg; 85% yield) as a white solid.

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**MS-ESI:** m/z 169 [M + H]+.
with ethyl acetate and neutralized with saturated Na2CO3 solution. The organic and methanol (20 ml), cooled to 0°C, 20 ml of trifluoroacetic acid (67% (s, OCH3), 3.84 (s, OCH3). 

Synthesis of (Z)-2-methoxy-5-[2-(3,4,5-trimethoxy-phenyl)-vinyl]-[1,4]benzoazone 29. To a mixture of Aliquat 336 (91 μl) and NaH2PO4.H2O (162 mg; 1.17 mmol) in water (50 ml), a solution of 1 (50 mg; 0.16 mmol) dissolved in dichloromethane (3.5 ml) was added. Then, Fremy’s salt (potassium nitrosodisulfonate) (106 mg; 0.40 mmol) was added, and the mixture was stirred for 30 min (the color changes from mauve to red). The reaction was worked up by dilution with dichloromethane and washed with water. The aqueous layer was further washed with dichloromethane, and the combined organic extracts were washed with brine. After drying over Na2SO4, filtration and evaporation of the solvent, the crude product was purified by column chromatography using petroleum ether/ethyl acetate (65:35) as eluant to give compound 29 (17 mg; 31% yield). 

1H NMR (300 MHz, CDCl3): δ 6.9 (d, J = 12.2 Hz, H-olefinic), 6.7 (s, H-quinoine, meta to OCH3), 6.5 (s, 2H), 6.4 (dd, J = 12.5/1.2 Hz, H-olefinic next to quinone ring), 5.9 (s, H-quinoine, ortho to OCH3), 3.85 (s, OCH3), 3.82 (s, OCH3), 3.7 (s, 2OCH3). 

UV (CH3CN): λmax: 235, 295 (broad) nm. 

Synthesis of (Z)-2’-5’-dihydroxy-3,4,4’-5-tetramethoxy stilbene 30. To a solution of compounds 26 and 27 (mixture) (265 mg; 0.47 mmol) in THF (10 ml) and methanol (20 ml), cooled to 0°C, 20 ml of trifluoroacetic acid (67% aqueous solution) was added. The reaction was stirred for 5 h and then diluted with ethyl acetate and neutralized with saturated Na2CO3 solution. The organic layer was washed with brine, dried over anhydrous Na2SO4, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (6:4) as eluant to give compound to give compound 30 (7 mg; 4.5% yield). 

1H NMR (300 MHz, CDCl3): δ 6.7 (s, H), 6.6 (d, J = 11.9 Hz, H-olefinic), 6.50 (s, 2H), 6.45 (s, overlapped with 6.44, H), 6.44 (d, J = 11.9 Hz, H-olefinic), 5.2 (s, broad OH), 4.7 (s, broad OH), 3.83 (s, OCH3), 3.81 (s, OCH3), 3.6 (s, 2OCH3). 

13C NMR (300 MHz, CDCl3): δ 153.0 (2C), 146.9 (C), 146.0 (C), 139.4 (C), 137.8 (C), 132.1 (CH), 131.4 (C), 123.8 (CH), 115.5 (C), 114.4 (C), 105.7 (2CH), 99.6 (CH), 60.9 (OCH3), 56.1 (OCH3), 55.9 (2OCH3). 

ESI-MS: m/e: 333 [M + H]+. 

UV (CH3CN): λmax: 235, 275 (broad) nm. 

Synthesis of (Z)-2’-5’-dihydroxy-3,4,4’-5-tetramethoxy stilbene 31. To a solution of compound 28 (39 mg; 0.12 mmol) in diethyl ether (2 ml), an excess of an aqueous solution of Na2S2O4 was added under vigorous magnetic stirring. After 30 min, when the color changed from dark red to yellow, the aqueous layer was removed and the organic layer was evaporated under reduced pressure to give compound 31 (32 mg; 80% yield). 

1H NMR (300 MHz, CDCl3): δ 7.1 (d, J = 16.2 Hz, H-olefinic), 7.0 (s, H), 6.8(d, J = 16.2 Hz, H-olefinic), 6.7 (s, 2H), 6.4 (s, H), 3.88 (s, 2OCH3), 3.85 (s, OCH3), 3.84 (s, OCH3). 

13C NMR (300 MHz, CDCl3): δ 153.3 (2C), 146.8 (C), 141.5 (C), 139.8 (C), 137.5 (C), 133.6 (C), 128.2 (CH), 122.0 (CH), 117.0 (C), 111.6 (CH), 103.3 (2CH), 100.2 (CH), 61.1 (OCH3), 56.1 (OCH3), 56.0 (2OCH3). 

ESI-MS: m/e: 333 [M + H]+. 

UV (CH3CN): λmax: 245, 295, 350 nm. 

Results 

Synthesis of Putative Metabolites. The Wittig reaction was used for the preparation of the putative metabolites 10, 11, 17, 19, 23, 28, 29, 30, and 31. Indeed, appropriate protected aldehydes and phosphonium salt intermediates were prepared and reacted to obtain the Z/E mixture of combretastatin analogs. 

Compound 5 was easily prepared by selective demethylation using boron tribromide (see Scheme 1 in Supplemental Data); it is worth mentioning that the reaction occurred with complete Z-E isomerization of the double bond. The syntheses of 10 and 11 were performed by reacting compounds 7 and 6 (see Scheme 2 in Supplemental Data). The E and Z isomers were separated by column chromatography and subsequently deprotected in the presence of TBAF. The Wittig reaction with 15 and the phosphonium salt 6 provided the Z isomer 16 and a Z/E mixture of isomers, which could not be separated by column chromatography to give the pure E isomer. Compound 17 was easily obtained by deprotection of 16 (see Scheme 3 in Supplemental Data). The E isomer 19 was prepared starting from 16 by using I2-induced isomerization (Gaukroger et al., 2001) and deprotection with TBAF. The synthesis of the putative metabolites 23 required the preparation of aldehyde intermediate 21, which was subsequently reacted with phosphonium salt 6 to give 22 as a Z/E mixture, which could not be separated by column chromatography (see Scheme 4 in Supplemental Data). For this reason, after deprotection, compound 23 was obtained as a 1:1 mixture. Chromatographic separation of two geometric isomers Z/E of compound 23 was achieved from elution into LC-DAD-UV system. Compounds 30 and 31 were obtained starting from 2,4,5-trimethoxy benzaldehyde, which was selectively demethylated to 24 by BBrr treatment. To establish the correct structure, NOE was performed by selective irradiation of the methoxy group, resulting in NOE enhancement (3.3%) of the singlet signal at 6.5 ppm (see Scheme 5 in Supplemental Data). The protected aldehyde intermediate 25 was then reacted with phosphonium salt 6 to give 26 and 27. TBAF deprotection of the isomeric mixture only resulted in the formation of the (E)-quinonic compound 28. The reduced form 31 was obtained by reacting 28 in the presence of sodium dithionite. The Z isomer 30 was obtained starting from the isomeric mixture of 26 and 27, which was deprotected by using trifluoroacetic acid to give both compounds 30 and 31 separated by column chromatography. 

Compounds 28, 29, 30, and 31 were also prepared in a more convenient and straightforward way by using Fremy’s salt as a bio-mimetic oxidant. Indeed, the reaction of 1 with Fremy’s salt in a heterogeneous system (water/dichloromethane) and in the presence of Aliquat 336, as a phase transfer catalyst, afforded the quinone 29, which was easily prepared with sodium dithionite to 30. The same procedure was also used to obtain compounds 28 and 31 starting from E-CA-4. 

In Vitro CA-4 Metabolism in Rat and Human Liver Microsomes. 1 was incubated in rat and human liver microsomal fractions in the presence of an NADPH-regenerating system. Because of the lipophilic character of the drug, Tween 80 or acetonitrile [<1% (v/v)] were used to increase the solubility in the incubation medium: no difference was observed in the metabolite pattern. To enhance the formation of metabolites, 1 mM of 1 was used through rat study. Indeed, when a lower concentration (100 μM) was used, no significant differences in the metabolite pattern were observed. Metabolites were recovered from incubation media by a liquid-liquid extraction on Extrelut columns. 

LC-DAD-UV Analysis of Rat and Human Microsomal Incubations. 1 and its metabolites were separated by HPLC using a C18 reverse-phase column and a mixture of H2O/CH3CN acidified with 0.5% formic acid as eluant. The LC-DAD-UV analysis of rat liver microsome incubations (Fig. 2A) showed the presence of at least eight metabolites (M1–M8), which were not observed in incubations performed in the absence of the NADPH-regenerating system (Fig. 2C). Moreover, any metabolic transformation occurred when 1 was incubated with boiled microsomes or with the cytosolic fraction. The analysis of human liver microsome incubations, as reported in Fig. 2B, afforded a very similar metabolic pattern to that observed with rat liver preparations. The DAD-UV analysis of the chromatographic peaks of rat liver incubations allowed us to assign the geometric
isomerism to metabolites M1–M8. Indeed, it has been reported that UV data are of relevance to characterize the geometric isomerism of stilbene derivatives (Yu et al., 2002). In particular, 1 showed two absorbance maxima at 245 and 300 nm, whereas a batochromic shift to 330 nm was observed for the E isomer (Fig. 3). Consequently, Z and E configurations were attributed to the M5-M6 and M3-M4 metabolites, respectively (Supplemental Data Fig. 1).

The UV spectra of M1, M2, M7, and M8 showed a slightly different absorbance pattern, suggesting a more marked modification of the stilbenic scaffold. In particular M1 and M2 spectra were characterized by three absorbance maxima; however, batochromic shifts (275–295 nm for M8 and 330–350 nm for M2) were again observed, allowing us to assign the E configuration to M2 and M8 and Z configuration to M1 and M7.

LC-ESI-MS/MS Analysis of Rat Microsomal Incubation. To obtain further information on the M1–M8 structures, positive LC-ESI-MS/MS analyses of the incubations with rat microsomes were performed. The structural features of 1 suggest that the metabolites could arise from three putative metabolic pathways: O-demethylation, aromatic hydroxylation, and epoxidation. Indeed, the LC-ESI-MS analysis performed in ion positive MS/MS mode (m/z 303) allowed the detection of O-demethylated metabolites (Fig. 4). In particular, the peaks at 14.13, 15.25, 18.36, and 20.40 min correspond to M3, M4, M5, and M6 peaks in the UV traces. The MS/MS experiments demonstrated the presence of a similar fragmentation pattern for these metabolites (Table 1). Nonetheless, these data are not sufficient to correctly assign their structures. Hence, the CA-4 O-demethylated analogs: (E)-3',4'-dihydroxy-3,4,5-trimethoxy-stilbene 5, (Z)-3',4'-dihydroxy-3,4,5-trimethoxy-stilbene 10, and (E)-3',4'-dihydroxy-3,4,5-trimethoxy-stilbene 11 were synthesized. The chromatographic properties, the mass spectrometry and UV data for 5, 11, and 10 completely matched with those of M3, M4, and M5, respectively. Possibly, M6 could also arise from O-demethylation of one of the two meta-methoxy groups at the positions C-3 and C-5 on phenyl ring A of 1. To detect the metabolites arising from aromatic hydroxylation, LC-ESI-MS analysis was performed in ion positive MS/MS mode (m/z 333) (Fig. 4). The peaks detected at 10.71 and 11.87 min corresponded to metabolites M1 and M2; MS/MS experiments revealed a very similar fragmentation pattern (Table 1). This feature, together with UV data, suggests that the metabolites were isomers. All of the positional isomers, (Z)- and (E)-3',5'-dihydroxy-3,4,4',5-tetramethoxy-stilbene 17, 19, respectively; (Z/E)-2',3'-dihydroxy-3,4,4',5-tetramethoxy-stilbene 23; and (Z)- and (E)-2',5'-dihydroxy-3,4,4',5-tetramethoxy-stilbene 30, 31 were synthesized. In particular,
FIG. 3. UV spectra of CA-4.

FIG. 4. Positive ion mode LC-ESI-MS/MS chromatogram of rat liver microsomal incubations. Unlabeled peaks are matrix-related.

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mol. wt.</th>
<th>Correlated Peak</th>
<th>MS [M + H]^+</th>
<th>MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>332</td>
<td>M1</td>
<td>333</td>
<td>318–301(100%)-273–269–169–165</td>
</tr>
<tr>
<td>31</td>
<td>332</td>
<td>M2</td>
<td>333</td>
<td>318–301(100%)-273–269–169–165</td>
</tr>
<tr>
<td>5</td>
<td>302</td>
<td>M3</td>
<td>303</td>
<td>288–271(100%)-243</td>
</tr>
<tr>
<td>10</td>
<td>302</td>
<td>M4</td>
<td>303</td>
<td>288(100%)-271–243–167</td>
</tr>
<tr>
<td>11</td>
<td>302</td>
<td>M5</td>
<td>303</td>
<td>288–271(100%)-243–167</td>
</tr>
<tr>
<td>29</td>
<td>330</td>
<td>M7</td>
<td>331</td>
<td>316–303(100%)-300–299</td>
</tr>
<tr>
<td>28</td>
<td>330</td>
<td>M8</td>
<td>331</td>
<td>316–303(100%)-300–299</td>
</tr>
</tbody>
</table>
compounds 30 and 31 showed the same chromatographic, mass spectral, and UV properties as metabolites M1 and M2. The presence of catechol and para-hydroquinone moieties in the M4, M5, and the M1, M2 structures, respectively, suggest the possible formation of the corresponding ortho- and para-quinone species in incubation medium. Indeed, LC-ESI-MS analysis performed in ion positive MS/MS mode (m/z 331) revealed two peaks at 22.58 and 31.15 (Fig. 4), which correspond to metabolites M7 and M8 in the UV traces. The pseudomolecular ion [M + H]^+ at m/z 331, the fragmentation patterns, and the UV properties allowed us to assign a quinone structure to M7 and M8. The synthesis of isomers 29 and 28 confirmed the assigned structures. On the contrary, metabolites arising from the oxidation of the catechol function (M4, M5) were not found.

LC-ESI-MS/MS Analysis of Human Microsomal Incubation.

LC-ESI-MS/MS analysis of the human microsomal incubation extracts, reported in Fig. 5, confirmed the presence of metabolites M1–M8 and the complexity of metabolic fate of 1. Hence, as previously shown by UV traces, the metabolite pattern was similar to that obtained from rat microsomal incubations, except that the unknown M6 metabolite was apparently the more abundant metabolite arising from the O-demethylation pathway; indeed, M3, M4, and M5 were only formed in low amounts. Even if a reference standard corresponding to M6 was not synthesized, however, the structure of (Z)-3,3′,4,4′,5-trimethoxy-stilbene could be proposed for this metabolite. Indeed the O-demethylation of 1 at the C-4 and C-4′ methoxy functions resulted in the formation of M3 and M4, M5, respectively. Hence, the only other O-demethylation pathway should occur at one of the two meta-methoxy groups on ring A. Interestingly M6 shows the typical UV spectrum of a Z-stilbene derivative (Supplemental Data Fig. 1) being different with that of M3. Possibly the metabolic O-demethylation pathway occurred with or without olefin bond isomerization depending on the position of the methoxy group. The formation of M1 and M2 as well as the related quinone metabolites M7 and M8 was also observed (Fig. 5; MS/MS, m/z 331). Finally two other peaks at 20.51 and 24.55 min were observed in the LC-MS trace (Fig. 5; MS/MS, m/z 331), which having the same MS/MS data obtained from M7 and M8 should be attributed to another couple of quinone metabolites. In theory, the quinone metabolites could arise from the related catechols obtained by aromatic hydroxylation at C-2 and C-6. In particular the ortho-quinone of ring B could be formed from the corresponding catechol with the same structure of CA-1. However chromatographic, MS/MS, and UV data of both geometric isomers of CA-1, synthesized as reference standard 23, were not detected in human LC-MS trace, possibly indicating their ready oxidation to the quinone species.

Discussion

Up to now, the oxidative biotransformation of combretastatin A-4 has not been studied; hence, an in vitro study of metabolic stability in the presence of rat and human liver subcellular preparations was undertaken. To maximize the metabolite formation from 1, rat liver incubations were performed at 1 mM substrate concentration. It is worthwhile to note that the incubations in the presence of a lower concentration (100 μM) did not show significant qualitative differ-
ences. Alternatively, for the study with human liver microsomes, a 38 μM concentration of 1 was used. Indeed, in the clinical studies, the administered dose of CA-4 phosphate was in the range 52 to 68 mg/m², affording a plasma concentration of CA-4 phosphate and 1 of 30.3-46.3 and 1.9-2.3 μM, respectively (Dowlati et al., 2002; Rustin et al., 2003). Rationally, a 2 μM substrate concentration would have been ideal for our study. However, the obtained results with rat liver incubations suggested that this concentration was too low to detect the metabolites, preventing also the determination of geometric isomerism by DAD-UV on-column analysis. These considerations led us to choose an about 20-fold greater concentration similar to that achieved in plasma by the CA-4 phosphate prodrug. The in vitro human and rat hepatic microsomal metabolism of 1 involves two main metabolic pathways (Fig. 6): O-demethylation and aromatic hydroxylation. In particular, aromatic hydroxylation was observed only on phenyl ring B. The steric hindrance of the trimethoxy substituents possibly prevented the hydroxylation on phenyl ring A and, in this case, only an O-demethylation pathway was observed. O-demethylation on ring B affords two isomeric catechol metabolites M4 and M5. Interestingly, the metabolic O-demethylation of 1 to M3 and M4 occurred with isomerization of the olefin bond. On the contrary, in human microsomes, the Z-E conversion was not observed during the formation of M6. Initially, we thought that the formation of metabolites with E configuration could be attributed to the transformation of E-CA-4 present in small amounts (<2%) in our 1 sample. However, this hypothesis was ruled out because when pure E-CA-4 was incubated with rat liver microsomes, only two metabolites were formed (data not shown); moreover, the percentage of the E isomer in metabolites was greater than that of E-CA-4 present in our Z-CA-4. Finally, a stability study of 1 in phosphate buffer, using the same conditions applied in the incubations showed only a small isomerization. These data suggest the Z-E isomerization of the olefin bond occurred mainly during metabolic O-demethylation and aromatic hydroxylation of 1. In particular the isomerization seems to occur when a para-methoxy group was demethylated both on ring A and B, whereas it did not when the methoxy group was in meta-position. Actually, no study has been performed on this topic; hence, from the available data a mechanism of isomerization could not be assumed. Metabolites M4 and M5 were characterized by a catechol function whose oxidation to ortho-quinone is a well known metabolic pathway for various classes of

![Fig. 6. Proposed scheme for CA-4 in vitro rat and human liver microsomal metabolism. ‡, metabolite lacking of reference standard.](image-url)
where

Hence, we propose the following in vitro metabolic scheme (Fig. 6), the presence of other putative quinone metabolites was revealed. Differences in incubation with rat and human microsomes, except that established.

Moreover, the relevance of the quinone species of CA-1 may be due to the formation of a reactive ortho-quinone metabolite. Nonetheless, no conclusive evidence was obtained about the glucuronidation and the reduction by DT diaphorase. Taken to-

E

combretastatins A-4 and A-1 and the relevance of their quinone metabolites in the pharmacokinetic and pharmacodynamic phases. Generally, quinones represent a class of toxic intermediates that can create a variety of harmful effects through different mechanisms (Bolton et al., 2000). However, in a study on CA-1, a hydroxylated analog of CA-4, Kirwan et al. (2004) postulated that the marked antitumor activity of CA-1 may be due to the formation of a reactive ortho-quinone metabolite. Nonetheless, no conclusive evidence was obtained about the metabolic formation of the CA-1 related quinone metabolite. Moreover, the relevance of the quinone species of CA-1 in vivo metabolism could also depend on other metabolic pathways, such as the glucuronidation and the reduction by DT diaphorase. Taken together, these considerations suggest that the role of quinone metabolites in the pharmacodynamics of combretastatins remains to be established.

Overall, the metabolic profile of CA-4 did not show significant differences in incubation with rat and human microsomes, except that the metabolite M6 was more abundant in human preparations and that the presence of other putative quinone metabolites was revealed. Hence, we propose the following in vitro metabolic scheme (Fig. 6), where I undergoes oxidative biotransformation in rat and human microsomes leading to an array of metabolites characterized by both E and Z configurations. The formation of para-quinone metabolites was also unequivocally demonstrated. Further work will be necessary to completely assess the structure-metabolism relationship of the combretastatins A-4 and A-1 and the relevance of their quinone metabolites in the pharmacokinetic and pharmacodynamic phases.

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