JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Quantification of *trans*-3,4,5,4′-Tetramethoxystilbene in Rat Plasma by HPLC: Application to Pharmacokinetic Study

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ABSTRACT: A simple HPLC method was established to quantify *trans*-3,4,5,4′-tetramethoxystilbene (MR-4 or DMU-212) in rat plasma. Chromatographic separation was obtained with a reversed-phase HPLC column through an 11 min gradient delivery of a mixture of acetonitrile and water at a flow rate of 1.5 mL/min at 50 °C. The limit of quantification was 15 ng/mL. The intra- and interday precisions in terms of relative standard deviation were <9% at all concentrations. Similarly, the accuracy was good, and the bias rates ranged within ±7%. The pharmacokinetic profiles of MR-4 were subsequently assessed in rats using 2-hydroxypropyl-β-cyclodextrin as a dosing vehicle. Upon intravenous administration, MR-4 displayed moderate clearance (46.5 ± 7.6 mL/min/kg) and terminal elimination half-life (154 ± 80 min). However, the absolute oral bioavailability of MR-4 was low (6.31 ± 3.30%). Future investigation on MR-4 as a chemotherapeutic agent should be focused on colorectal cancers.

KEYWORDS: *trans*-3,4,5,4′-Tetramethoxystilbene, HPLC, pharmacokinetics, absolute oral bioavailability

INTRODUCTION

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) (**1**, Figure 1) is a dietary phytoalexin that has attracted substantial interest in the past 15 years.[1] Its beneficial biological activities such as antiaging, antidiabetic, anti-inflammation, antiobesity, antioxidation, cancer chemoprevention, and cardio- and neuro-protection have been extensively reported.[1] The interest in resveratrol has also been extended to its naturally occurring or synthetic analogues.[2−5]

trans-3,4,5,4′-Tetramethoxystilbene (MR-4, also reported as DMU-212)[2,3] (**2**, Figure 1) is a resveratrol analogue present in the leaves of *Piper caninum*.[6] Its anticancer effects have been noted in a wide variety of tumor cells.[2,3,7−12] The in vitro antineoplastic potency of MR-4 was found to be higher than that of resveratrol.[3,7,10,11] Interestingly, the antiproliferative activity of MR-4 was more specific to transformed cells than to normal cells.[2,10,12] Besides its anticancer effects, MR-4 also displayed anti-inflammatory potential.[13,14] It suppressed tumor necrosis factor-α-induced activation of transcription factor nuclear factor-κB[13] and inhibited both cyclooxygenases-1 and -2.[14] Clearly, MR-4 has emerged as a promising candidate for further therapeutic exploration.

Pharmacokinetics plays an important role in drug discovery and development.[15] Resveratrol is well-known for its unfavorable pharmacokinetic properties such as short half-life, rapid clearance, and limited bioavailability.[1] To date, there is limited information on the pharmacokinetics of MR-4. A previous study has suggested the possible inferior oral pharmacokinetics of MR-4.[3] Given at the same dose, the plasma exposure of MR-4 was about 70% lower than that of resveratrol in mice.[3] However, the kinetic profile was assessed only after oral administration; thus, the absolute oral bioavailability of MR-4 remained unknown.[3] It is therefore of interest to determine the absolute bioavailability of MR-4.

In the present study, a simple and rapid HPLC method was developed and validated to quantify MR-4 in rat plasma. The pharmacokinetic profile of MR-4 was subsequently examined in Sprague−Dawley rats after oral and intravenous administration. The pharmacokinetic parameters of MR-4 were compared with resveratrol and another two tetramethoxystilbenes, namely, *trans*-3,5,2′,4′-tetramethoxystilbene (oxyresveratrol tetramethyl ether, OTE) (**3**, Figure 1) and *trans*-3,5,3′,4′-tetramethoxystilbene (piceatannol tetramethyl ether, PTE) (**4**, Figure 1). To the authors' knowledge, this is the first report on the intravenous pharmacokinetics and the absolute bioavailability of MR-4. Findings from this study would be useful to evaluate the therapeutic potential of MR-4.

MATERIALS AND METHODS

Special Precautions. All laboratory procedures involving the manipulation of MR-4 and *trans*-stilbene were executed in a dimly lit environment.

Chemicals and Reagents. MR-4, a white powder, was prepared from a previous study (purity > 97% by HPLC).[16] *trans*-Stilbene (purity = 96%) was purchased from Sigma-Aldrich (St. Louis, MO). MR-4 was stored at −20 °C, whereas *trans*-stilbene was kept at room temperature. 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) (degree of substitution ∼ 0.6) was donated by Roquette Freres S.A. (Lestrem, France). HPLC/Spectro grade acetonitrile and methanol were supplied from Tedia (Fairfield, OH). Analytical grade DMSO was obtained from MP Biomedicals (Solon, OH). Purified water (18.2 MΩ·cm at 25 °C) was generated by a Millipore Direct-Q ultrapure water system (Billerica, MA) and used throughout the study. Blank plasma was collected from Sprague−Dawley rats, which did not receive drug treatment.

Liquid Chromatograph. A Shimadzu (Kyoto, Japan) 2010A liquid chromatograph was used for the HPLC analysis. This integrated

Received: November 5, 2010
Accepted: December 16, 2010
Revised: December 15, 2010
Published: January 13, 2011

dx.doi.org/10.1021/jf1043019 | *J. Agric. Food Chem.* 2011, 59, 1072–1077

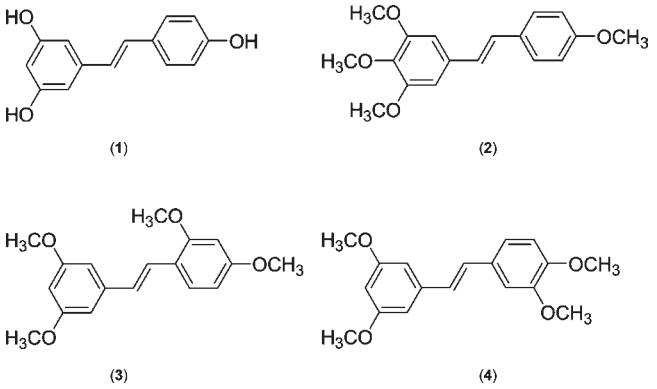

Figure 1. Chemical structures of resveratrol (**1**), *trans*-3,4,5,4′-tetramethoxystilbene (**2**), *trans*-3,5,2′,4′-tetramethoxystilbene (**3**), and *trans*-3,5,3′,4′-tetramethoxystilbene (**4**).

HPLC system consisted of a quaternary gradient low-pressure mixing pump, an online degasser, an autosampler, a column oven, a dual-wavelength UV−vis detector, and a system controller. The HPLC system was controlled by a personal computer through the software of Shimadzu Class-VP version 6.12 SP1. Chromatographic data were analyzed with the same software.

The chromatographic conditions were modified from our recent methods for the quantification of the other methoxylated stilbenes.[17,18] Briefly, a RP-HPLC column (Agilent Zorbax Eclipse Plus C18: 250 × 4.6 mm i.d., 5 μm), which was protected by a guard column (Agilent Zorbax Eclipse Plus C18: 12.5 × 4.6 mm i.d., 5 μm), was applied to quantify MR-4 in rat plasma; chromatographic separation was obtained through an 11 min gradient delivery of a mixture of acetonitrile and Milli-Q water at a flow rate of 1.5 mL/min at 50 °C. The gradient schedule was (a) 0−2 min, acetonitrile, 65%; (b) 2−4 min, acetonitrile, 65→90%; (c) 4−7.5 min, acetonitrile, 90%; (d) 7.5−11 min, acetonitrile, 65%. UV absorbance at 325 and 335 nm was recorded, but only the data acquired at 325 nm were used for the quantification of MR-4.

Sample Preparation. MR-4 was dissolved in DMSO and diluted to 1.00 mg/mL. This stock solution was dispensed into individual vials and stored at room temperature (24 °C). The calibration standards or quality control samples were prepared by diluting the stock solution with blank rat plasma. *trans*-Stilbene (internal standard) was dissolved in acetonitrile and diluted to 200 ng/mL (working solution). During sample preparation, 3 volumes of *trans*-stilbene/acetonitrile working solution was added to 1 volume of rat plasma. After vigorous mixing, the samples were centrifuged at 10000*g* for 10 min at 4 °C. After centrifugation, the supernatant was transferred into a glass insert in a 1.5 mL amber autosampler vial. During HPLC assay, 50 μL of analyte was injected into the system. Only 30 μL plasma was required for a single assay.

Assay Validation. The validation of this HPLC assay was performed by assessing its specificity, sensitivity, linearity (R^2), precision (intra- and interday), accuracy (bias rate), and absolute recovery and the stability profiles of MR-4.

The specificity was examined by comparing the chromatograms of six individual blank rat plasma samples and the corresponding plasma samples spiked with MR-4 and *trans*-stilbene. The specificity of the assay was further confirmed in the actual pharmacokinetic study by chromatographic comparison between predosing and postdosing plasma samples (n = 10).

The sensitivity of this assay was represented by a lower limit of detection (LOD) and a lower limit of quantification (LOQ), which were defined as signal-to-noise ratios equal to 3 and 10, respectively.

The ratio between the peak area of MR-4 and *trans*-stilbene (internal standard) was defined as the analytical response. Linear regression was carried out with GraphPad Prism version 5.03 (La Jolla, CA), where x was the concentration of MR-4, y was the analytical response, and $1/x^2$ was used as a weighting factor. The calibration standards of the following concentrations, 15, 50, 100, 250, 500, 1000, and 1500 ng/mL, were used to assess the linearity. The calibration was carried out on five consecutive days. For intraday assay, five replicate of samples were prepared; for interday assay, duplicate samples were prepared. The intra- and interday relative standard deviations (RSDs) at individual concentration were calculated and used as a precision indicator. Similarly, quality control (QC) samples (40, 400, and 1400 ng/mL) were also prepared and analyzed. The precision, absolute recovery, and accuracy were assessed with such QC samples.

The stability of MR-4 DMSO solution was evaluated after storage at room temperature (24 °C) for 10 days. The stability of MR-4 in rat plasma under different conditions was also profiled with the QC samples. The impact of freeze−thaw on stability was assessed after three freeze (−80 °C)−thaw (24 °C) cycles. Short-term refrigerator storage stability (4 °C, 24 h) and long-term deep freezer storage stability (−80 °C, 21 days) were also examined. The postpreparative stability study at room temperature in autosampler vials was investigated by reanalyzing the samples 24 h later.

Pharmacokinetic Study. This pharmacokinetic study was carried out strictly following the national guidelines on the care and use of animals for scientific purposes.[19] The animal handling protocol had been reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS) (Protocol 107/06). Sprague−Dawley rats (male, 7−8 weeks old, bred by the Center for Animal Resources, NUS) were maintained on a 12 h light/dark cycle with free accesses to food and water. On the day before pharmacokinetic study, a polyethylene tube (i.d. = 0.58 mm, o.d. = 0.965 mm, Becton Dickinson, Sparks, MD) was implanted into the right jugular vein under anesthesia. Intravenous drug administration and blood sampling were carried out through this cannula. HP-β-CD (0.3 M) was used as a dosing vehicle in this pharmacokinetic study. Ten rats were divided into two groups. Group 1 (n = 4) received a single bolus intravenous administration of MR-4 (2 mg/kg); serial blood samples were collected before dosing and at 5, 15, 30, 45, 60, 90, 120, 180, 300, 420, and 600 min post dosing. Group 2 (n = 6) received a single oral administration of MR-4 (5 mg/kg) through oral gavage, and serial blood samples were collected before dosing and at 15, 30, 45, 60, 90, 120, 180, 300, 420, and 600 min post administration. To maintain the patency of the catheter, 0.3 mL of heparin/saline (10 IU/mL) was flushed through catheter after each intravenous injection or blood sampling. The blood samples were centrifuged at 3500*g* at 4 °C for 5 min. The harvested plasma samples were stored at −80 °C until HPLC assay.

Pharmacokinetic parameters were calculated by WinNonlin standard version 1.0 (Scientific Consulting Inc., Apex, NC). As the plasma pharmacokinetic profile of MR-4 after intravenous administration displayed a typical biexponential decline, the plasma MR-4 concentration−time data were fitted into the classical two-compartment first-order open model. The plasma exposure (area under the plasma MR-4 concentration versus time curve ($AUC_{0\rightarrow last}$)), clearance ($Cl_{0\rightarrow last}$), mean transit time ($MTT_{0\rightarrow last}$), and terminal elimination half-life ($t_{1/2\ \lambda z}$) were calculated using noncompartmental analysis.

RESULTS AND DISCUSSION

The specificity of this HPLC assay was documented. Under our chromatographic conditions, MR-4 and *trans*-stilbene eluted at about 5.1 and 6.4 min, respectively (Figure 2A). No notable interference peak was observed in the chromatograms acquired from either blank plasma samples (n = 6) or predosing plasma samples (n = 10) (a typical chromatogram of a predosing sample is shown in Figure 2B). Moreover, no notable metabolite peak coeluted with either MR-4 or *trans*-stilbene in the chromatograms

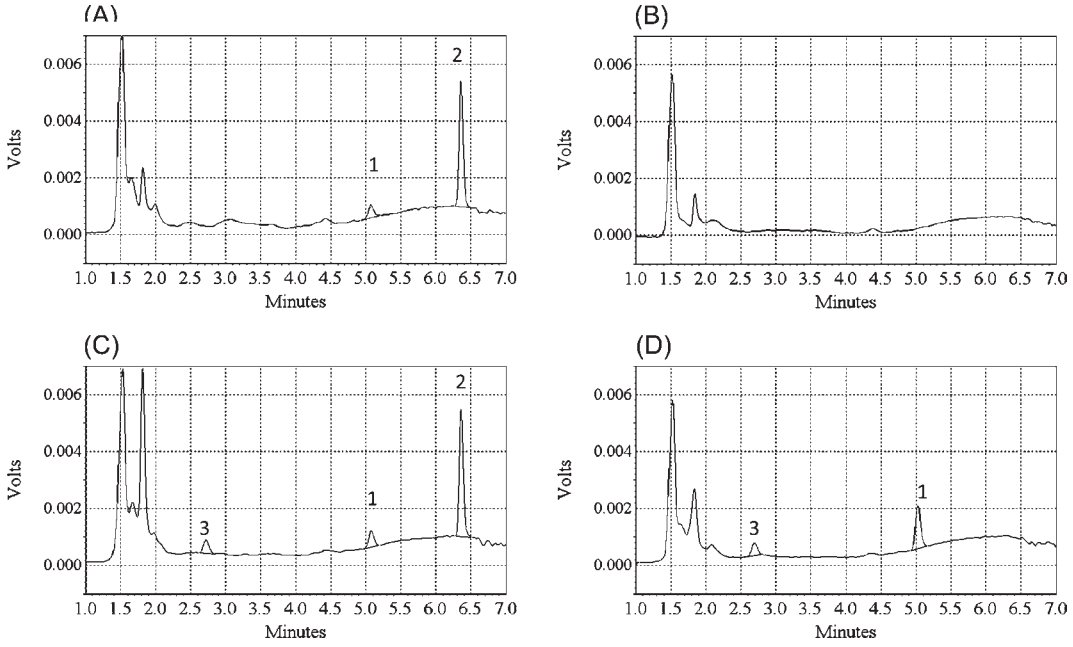

Figure 2. Typical chromatograms (UV absorbance, λ = 325 nm) of (A) a blank plasma sample spiked with MR-4 (40 ng/mL) and *trans*-stilbene (internal standard) (600 ng/mL), (B) a predosing plasma sample, (C) a plasma sample taken from a rat at 15 min after receiving an oral dose of MR-4 (5 mg/kg) (with internal standard), and (D) a plasma sample taken from a rat at 45 min after receiving an intravenous dose of MR-4 (2 mg/kg) (without internal standard). Peaks: 1, MR-4; 2, internal standard; 3, unidentified metabolite.

Table 1. Analytical Accuracy and Precision of MR-4 in Rat Plasma (n = 5)

	intraday			interday		
amount spiked (ng/mL)	amount measured (ng/mL)	precision (RSD, %)	bias range (%)	amount measured (ng/mL)	precision (RSD, %)	bias range (%)
40.0	40.4 ± 1.9	4.8	−6.5 to +5.2	41.0 ± 2.0	4.9	−5.7 to +6.6
400.0	391.2 ± 11.9	3.0	−6.3 to +1.6	393.0 ± 11.9	3.0	−6.3 to +1.8
1400.0	1363.0 ± 48.0	3.5	−7.0 to +0.5	1373.0 ± 21.9	1.6	−4.5 to −0.9

Table 2. Stability Profiles of MR-4[a]

	spiked concentration		
	40 ng/mL	400 ng/mL	1400 ng/mL
stock solution stored at 24 °C for 10 days	96.7 ± 3.5	99.0 ± 1.8	96.3 ± 0.1
plasma samples stored at 4 °C for 24 h	101.2 ± 6.2	98.1 ± 2.5	95.9 ± 2.9
postpreparative samples stored at 24 °C for 24 h	101.0 ± 6.5	100.1 ± 2.8	98.3 ± 1.9
plasma samples after three freeze−thaw cycles	100.6 ± 3.0	97.1 ± 2.2	95.5 ± 3.0
plasma samples stored at −80 °C for 21 days	96.5 ± 2.9	97.3 ± 2.4	95.0 ± 2.5

[a] Results are presented as stability remaining (%, mean ± SD, n = 5).

acquired from the rats after MR-4 administration (Figure 2C,D). The peak eluted at about 2.8 min in the chromatograms of the postdosing samples was presumed to be a metabolite (Figure 2C, D). However, the structure of the presumed metabolite was unidentified, although it appeared to be more polar than MR-4.

The lower LOD and LOQ of MR-4 were found to be 5 and 15 ng/mL, respectively. The calibration curves were all linear with regression correlation coefficients (R^2) of >0.998. The intraday calibration equation was $y = 0.0002949x - 0.008980$, whereas the interday calibration was$y = (0.0002947 \pm 0.000079)x - (0.007744 \pm 0.002629)$. The precision of the assay was demonstrated by the intraday or interday RSDs, which were all <9% (calibration data are not shown; QC data are shown in Table 1). Similarly, the accuracy was also confirmed by the bias rates that were within ±7.0% at all concentrations in the QC samples (Table 1). The absolute recovery rates at the concentrations of 40, 400, and 1400 ng/mL were 102.1 ± 5.6, 101.2 ± 1.5, and 96.7 ± 0.7%, respectively (n = 5). The stability profiles of MR-4 were assessed under different storage conditions. MR-4 was found to be stable under the test conditions (Table 2).

In summary, a simple and rapid HPLC method has been developed and validated to quantify MR-4 in rat plasma. The method was subsequently applied to investigate the pharmacokinetic profiles of MR-4 in Sprague−Dawley rats.

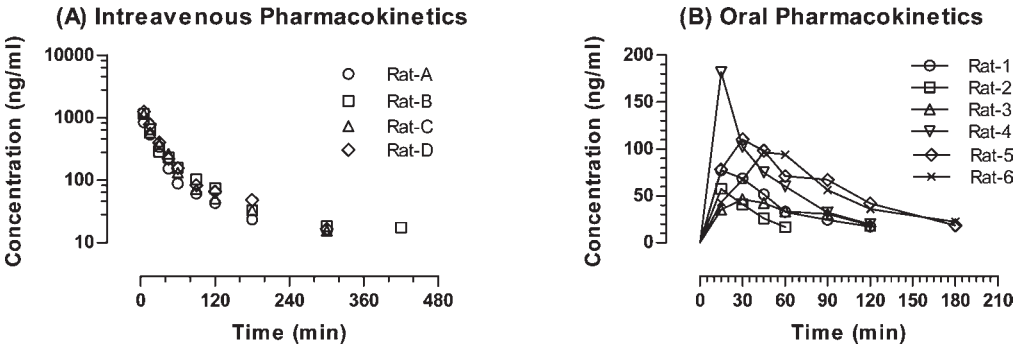

Figure 3. Plasma pharmacokinetic profiles of MR-4 in Sprague−Dawley rats after (A) intravenous administration, 2 mg/kg, and (B) oral administration, 5 mg/kg.

As MR-4 possesses poor aqueous solubility, HP-β-CD was used to form a water-soluble formulation. This HP-β-CD-based formulation was used for both intravenous and oral administrations. The intravenous pharmacokinetic profile of MR-4 is shown in Figure 3A. Similarly to several other methoxylated stilbenes,[17,18,20−23] upon intravenous injection, MR-4 was cleared from plasma through a biexponential process, that is, a distribution phase followed by a terminal elimination phase. Therefore, the plasma MR-4 versus time data were fitted into the classical two-compartment first-order open model. The correlation of the pharmacokinetic modeling was good (R^2 = 0.995, 0.954, 0.978, and 0.999, respectively), indicating that an appropriate model was chosen. The pharmacokinetic parameters are listed in Table 3. MR-4 had a moderate apparent volume of distribution of the central compartment (V_c = 1.68 ± 0.38 L/kg), clearance (Cl = 46.5 ± 7.6 mL/min/kg), and terminal elimination half-life ($t_{1/2\lambda z}$ = 154 ± 80 min). The plasma MR-4 concentration dropped to unquantifiable levels (<15 ng/mL) 5 or 7 h after intravenous administration.

The oral pharmacokinetic profile of MR-4 is shown in Figure 3B. When given in a water-soluble formulation, MR-4 was absorbed rapidly after oral gavage, and the plasma concentration peaked within 45 min. The mean transit times (MTT) after intravenous and oral administration were similar, also indicating rapid absorption. However, the maximal plasma concentration (C_{max}) was low (C_{max} = 94.8 ± 48.7 ng/mL). After achieving C_{max}, the plasma MR-4 level declined very quickly and became unquantifiable 1−3 h after oral administration. Subsequently, the absolute oral bioavailability was low (F = 6.31 ± 3.30%). To the authors' knowledge, this is the first report of the absolute oral bioavailability of MR-4.

The oral bioavailability of a given compound is determined by its aqueous solubility, membrane permeability, and metabolic stability.[24,25] Although MR-4 is insoluble in water, when formulated in HP-β-CD solution, the solubility might not be a problem. As the HP-β-CD-based formulation of MR-4 was rapidly absorbed with a t_{max} achieved within 45 min, the membrane permeability of MR-4 was unlikely to cause problems in absorption. Therefore, the poor bioavailability of MR-4 should be attributed to its metabolic instability and extensive first-pass effect. A previous mouse pharmacokinetic study did support such a hypothesis as various polar metabolites were identified in mouse livers extract or microsomes.[3] In addition, the former study indicated that the MR-4 exposure in intestinal mucosa was about 850-fold higher than that in liver, whereas the hepatic MR-4 exposure was about 70% higher than in plasma.[3] The great difference of MR-4 exposure among intestinal mucosa, liver, and plasma could be explained by extensive first-pass metabolism. Moreover, the intestinal mucosa rather than the liver appeared to be the major organ for MR-4 metabolism.[3]

Table 3. Pharmacokinetic Parameters of MR-4[a]

parameter	intravenous (n = 4)	oral (n = 6)
dose (mg kg^{-1})	2	5
A (μg mL^{-1})	1.14 ± 0.26	
B (ng mL^{-1})	95.4 ± 44.1	
α (10^{-2} min^{-1})	4.70 ± 0.96	
β (10^{-3} min^{-1})	5.46 ± 1.61	
V_c (L kg^{-1})	1.68 ± 0.38	
$AUC_{0\rightarrow last}$ (10^4 min ng^{-1} mL^{-1})	4.01 ± 0.63	0.63 ± 0.33
Cl (mL min^{-1} kg^{-1})	46.5 ± 7.6	
$t_{1/2\ \lambda z}$ (min)	154 ± 80	
$MTT_{0\rightarrow last}$ (min)	55.7 ± 9.1	53.9 ± 18.0
C_{max} (ng mL^{-1})		94.8 ± 48.7
t_{max} (min)		15 - 45
F (%)		6.31 ± 3.30

[a] Results are presented as the mean ± SD.

Resveratrol is well-known to undergo extensive first-pass metabolism, and it is subjected to phase II conjugation such as glucuronidation and sulfation.[1] As a fully methoxylated stilbene, phase II metabolism is not applicable to MR-4. Phase I metabolism such as hydroxylation and demethylation appeared to be the major metabolic pathways of MR-4.[3] In view of their structural differences, resveratrol and MR-4 are likely to be subjected to different biotransformation pathways. When compared with resveratrol,[26] the intravenous pharmacokinetic profile of MR-4 was more favorable as it was found to have more plasma exposure, longer terminal elimination half-life, and lower clearance. However, in contrast, MR-4 possessed poorer oral pharmacokinetics than resveratrol as it displayed lower oral bioavailability and dose normalized C_{max}.

Fortunately, the anticancer mechanism of MR-4 appeared to be different from that of resveratrol.[11] Resveratrol is a well-known inhibitor of cyclooxygenase,[1] which is considered as a molecular target for cancer prevention and treatment.[27] Although MR-4 did not inhibit cyclooxygenase, it displayed cancer-preventive efficacy that was comparable to that of resveratrol in a mouse model of intestinal carcinogenesis.[5] Moreover, MR-4 is a pro-drug, and it is activated by aromatic hydroxylation, a process that is catalyzed by cytochrome p450 1A1 (CYP 1A1) and also by O-demethylation, which is mediated by CYP 1B1.[28] Expression of CYP 1A1

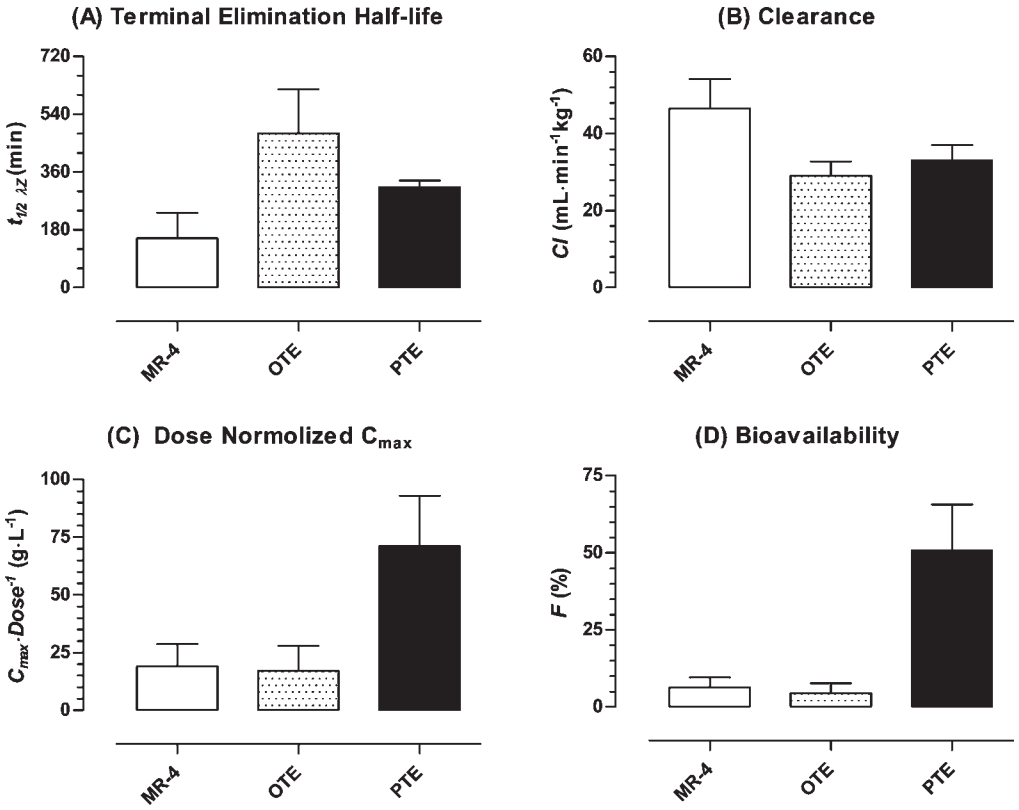

Figure 4. Pharmacokinetic comparison among MR-4, OTE, and PTE. Symbols represent mean values, and error bars represent SD. MR-4: intravenous, $n = 4$; oral, $n = 6$. OTE: intravenous, $n = 3$; oral, $n = 5$. PTE: intravenous, $n = 3$; oral, $n = 5$.

and/or CYP 1B1 in tumor cells led to 1000-fold enhancement in MR-4 cytotoxicity.[28] As CYP 1B1 is usually overexpressed in human colorectal tumors and MR-4 had abundant exposure in intestinal mucosa,[3,29−31] MR-4 appears to be an ideal chemotherapeutic agent for colorectal tumors.

The pharmacokinetic profiles of three *trans*-tetramethoxystilbenes, namely, *trans*-3,4,5,4′-tetramethoxystilbene (**2**, Figure 1, MR-4), 3,5,2′,4′-*trans*-tetramethoxystilbene (**3**, Figure 1, oxyresveratrol tetramethyl ether, OTE),[17] and 3,5,3′,4′-*trans*-tetramethoxystilbene (**4**, Figure 1, piceatannol tetramethyl ether, PTE),[22] have been assessed in the same animal model using HP-β-CD as delivery excipient, allowing an accurate comparison on their pharmacokinetic profiles. Upon intravenous administration, both compounds **3** and **4** had fairly long terminal elimination half-lives, whereas MR-4 possessed a moderate half-life, suggesting that MR-4 is metabolically less stable (Figure 4A). Similarly, the clearance of compounds **3** and **4** was slightly lower than that of MR-4 (Figure 4B). Clearly, the intravenous pharmacokinetic properties of these tetramethoxystilbenes could not be a major factor that would defer their medical application if administered intravenously. However, the poor oral pharmacokinetic profiles of MR-4 and compound **3** could hamper their oral application. Even when given in a solution form, both MR-4 and compound **3** did not have much plasma exposure, and their absolute oral bioavailability was low (Figure 4C,D). Clearly, compound **4** possessed the best pharmacokinetic characteristics among these three isomeric compounds. From the pharmacokinetic comparison, it is concluded that the location of methoxyl groups on the aryl ring has an important impact on the pharmacokinetics of stilbene.

In summary, a simple and reliable HPLC method has been developed and validated to quantify MR-4 in rat plasma. This HPLC method was successfully applied to investigate the pharmacokinetic profiles of MR-4 in Sprague−Dawley rats. Although MR-4 has a good intravenous pharmacokinetic profile, its oral bioavailability is poor. Future investigation on MR-4 as a chemotherapeutic agent should be focused on colorectal cancers.

■ ABBREVIATIONS USED

Cl, clearance; C_{max}, maximal plasma concentration; CYP, cytochrome p450; DMU-212, *trans*-3,4,5,4′-tetramethoxystilbene; *F*, absolute oral bioavailability; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MR-4, *trans*-3,4,5,4′-tetramethoxystilbene; OTE, *trans*-3,5,2′,4′-tetramethoxystilbene; PTE, *trans*-3,5,3′,4′-tetramethoxystilbene; QC, quality control; RP-HPLC, reversed phase HPLC; t_{max}, time to the maximal concentration; $t_{1/2\lambda z}$, terminal elimination half-life; RSD, relative standard deviation; UV, ultraviolet; V_c, apparent volume of distribution of the central compartment.

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Funding Sources

The work of H.-S.L. and P.C.H. was supported a research grant from the Agency for Science, Technology and Research (BMRC 06/1/21/19/441), Republic of Singapore. The work of W.Z. and M.L.G. was supported by a research grant from NUS (R-148000084112). The work of C.S. and C.T. was supported by a grant of the Università degli Studi di Catania (Progetti di

Ricerca di Ateneo, Catania, Italy) and by MIUR, Ministero dell'Università e della Ricerca (PRIN 2007, Rome, Italy).

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