Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue trans 3,4,5,4’-tetramethoxystilbene

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Resveratrol (trans-3,5,4’-trihydroxystilbene) is a naturally occurring polyphenol with cancer chemopreventive properties in preclinical models of carcinogenesis, including those of colorectal cancer. Recently, a variety of analogues of resveratrol have been synthesised and investigated in vitro assays. One analogue, 3,4,5,4’-tetramethoxystilbene (DMU 212), showed preferential growth-inhibitory and proapoptotic properties in transformed cells, when compared with their untransformed counterparts. As part of a chemoprevention drug development programme, the pharmacokinetic properties of DMU 212 were compared with those of resveratrol in the plasma, liver, kidney, lung, heart, brain and small intestinal and colonic mucosa of mice. DMU 212 or resveratrol (240 mg kg-1) were administered intragastrically, and drug concentrations were measured by HPLC. Metabolites were characterised by cochromatography with authentic reference compounds and were identified by mass spectrometry. The ratios of area of plasma or tissue concentration vs time curves of resveratrol over DMU 212 (AUCres/AUCDMU212) for the plasma, liver, small intestinal and colonic mucosa were 3.5, 5, 0.1 and 0.15, respectively. Thus, resveratrol afforded significantly higher levels than DMU 212 in the plasma and liver, while DMU 212 exhibited superior availability compared to resveratrol in the small intestine and colon. Resveratrol was metabolised to its sulphate or glucuronate conjugates, while DMU 212 underwent metabolic hydroxylation or single and double O-demethylation. DMU 212 and resveratrol inhibited the growth of human-derived colon cancer cells HCA-7 and HT-29 in vitro with IC50 values of between 6 and 26 μM. In the light of the superior levels achieved in the gastrointestinal tract after the administration of DMU 212, when compared to resveratrol, the results provide a good rationale to evaluate DMU 212 as a colorectal cancer chemopreventive agent.

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and Avkiran, 2001; Slater et al, 2003) and decrease in the activity of transcription factors NfκB and AP-1 (Surr et al, 2001; Banerjee et al, 2002). As is the case with many polyphenols with putative cancer chemopreventive properties, the systemic bioavailability of resveratrol is probably poor. This notion is borne out by studies in mice, rats and dogs, which suggest consistently that resveratrol is well absorbed but avidly glucuronidated and sulphated both in the liver and in intestinal epithelial cells (Asensi et al, 2002; Juan et al, 2002; Marier et al, 2002). One study in humans also hints at a poor bioavailability of resveratrol (Goldberg et al, 2003). In the wake of the discovery of the interesting pharmacological properties of resveratrol, the trihydroxystilbene scaffold has become the subject of imaginative synthetic manipulations by medicinal chemists with the aim of generating novel congeners of pharmacological interest and to characterise structural features, which impart activity to the molecule. These structural alterations have been aimed at the optimisation of the cytochrome P450 enzyme-inhibitory and antimutagenic potencies of the molecule (Chun et al, 2001; Kim et al, 2002), its antioxidant activity (Lu et al, 2001), its apoptosis-inducing and growth-inhibitory activity (Lu et al, 2001; Nam et al, 2001; Kim et al, 2002) and its ability to inhibit cell transformation (She et al, 2003). These chemical synthetic attempts have predominantly been concerned with the introduction of additional hydroxy moieties into the trihydroxystilbene framework and with various degrees of methylation of the phenol groups. An especially auspicious finding concerning these analogues is the fact that 3,4,5,4′-tetrahydroxystilbene, resveratrol with an additional hydroxy moiety, and its O-methylated congener, 3,4,5,4′-tetramethoxystilbene (DMU 212, for structure see Figure 1), were capable of preferentially interfering with the proliferation and survival of various transformed human lung-derived cells, with much lower growth-inhibitory and apoptotic properties in their untransformed counterparts (Lu et al, 2001). In contrast, resveratrol did not possess this discriminatory potential. Furthermore, DMU 212 is currently under preclinical evaluation as a potential antitumour prodrug that undergoes metabolic activation by certain cytochrome P450 enzymes (Potter et al, 2002a). In the light of the availability of pharmacologically interesting stilbene analogues, it seems appropriate to compare the cancer chemopreventive potential of resveratrol in preclinical models with those of its congeners, which have been shown to possess increased potency in relevant in vitro assays. Before embarking on such efficacy studies, it is desirable to find out if resveratrol congeners, to be evaluated as potential chemopreventive agents, possess adequate bioavailability in the tissues in which malignancies are to be prevented.

Such pharmacokinetic exploration should be an essential part of the chemopreventive drug discovery process. Mindful of these considerations, we chose DMU 212, one of the most interesting resveratrol analogues described thus far (Lu et al, 2001; Potter et al, 2002a), and compared its levels in murine tissues after oral administration with those of resveratrol. Thus, the hypothesis was tested that a replacement of the phenol functionalities in resveratrol by methoxy moieties and an addition of a further methoxy group impinge on the pharmacokinetic properties of the parent molecule. Additionally, DMU 212 was compared with resveratrol in terms of their metabolism in the mouse in vivo and in liver homogenate preparations in vitro. The mouse is the animal species frequently used in cancer chemoprevention efficacy studies. Finally, we compared the ability of DMU 212 and resveratrol to interfere with the growth of human-derived colon cancer cells. Overall, the study was designed to help rationalise the choice of resveratrol analogues for further testing for potential usefulness as cancer chemopreventive agents.

**MATERIALS AND METHODS**

**Reagents, animals and cells**

Resveratrol, DMU 212 and its congeners referred to in this work are exclusively the trans-isomers. Resveratrol was purchased from Changchun Kingherb International Co., Ltd (Changchun, China) and its purity established as 99% by HPLC analysis. Authentic resveratrol-3-sulphate was a gift from Dr Tristan Booth (Mount Royal Pharma, Montreal, Canada), and its identity was corroborated by mass spectrometry. DMU 212 (3,4,5,4′-tetramethoxystilbene) was synthesised by Wittig olefination involving the reaction of 4-methoxybenzyl-tri-phosphonium chloride with 3,4,5-trimethoxybenzaldehyde (Potter et al, 1999). This reaction yielded the cis and trans geometric isomers, which were separated by preparative column chromatography. The trans isomer was purified by recrystallisation from ethanol. The DMU 212 analogues to be used for metabolite identification of 4,4′-di-dihydroxy-3,5′-dimethoxystilbene (4,4′-di-desmethyl-DMU 212, DMU 295), 4′- hydroxy-3,4,5′-trimethoxystilbene (4′-desmethyl-DMU 212, DMU 281), 4-hydroxy-3,5,4′-trimethoxystilbene (4-desmethyl-DMU 212, DMU 291) and 3-hydroxy-4,5,4′-trimethoxystilbene (3-desmethyl-DMU 212, DMU 807) (for structures, see Figure 2) were synthesised in a similar fashion using the tert-butylmethylsilyl-protected Wittig precursors, and final deprotection with tetra-butylammonium fluoride (Potter et al, 1999). 3′-Hydroxy-3,4,5,4′-tetramethoxystilbene (3′-hydroxy-DMU 212, DMU 214, trans isomer of combretastatin A4) was synthesised according to the method of Pettit et al (1995). The identity of newly synthesised compounds was confirmed by mass spectrometry, nuclear magnetic resonance spectrometry and infrared and ultraviolet spectroscopy. Purity was established as at least 99% by HPLC analysis. The stability of resveratrol in solution is affected by light. Therefore, care was taken to protect the solutions of compounds from light. Laboratory chemicals were purchased from Sigma Chem. Comp. (Poole, UK). Male C57BL/6J mice were obtained from Charles River Laboratories (Margate, UK). Mice were chosen for this study, as they frequently are the experimental model of carcinogenesis used in preclinical chemoprevention studies. Human-derived malignant colorectal carcinoma cell lines HT-29 and HCA-7 were obtained from Prof C Paraskeva (Bristol University, Bristol, UK) and Dr S Kirkland (Hammersmith Hospital, Imperial

Figure 1  Chemical structures of (A) resveratrol and (B) DMU 212.
was suspended in Tris buffer (50 mM, pH 7.4), recentrifuged at High Wycombe, Buckinghamshire, UK). The microsomal pellet in a Beckman L-8-60 ultracentrifuge (Beckman Coulter UK Ltd, resveratrol or DMU 212 (1 mM) for 20 min (final volume: 0.2 ml). Two to four mice were pooled. Microsomes (0.5 mg protein ml$^{-1}$C0$^2$) were snap-frozen (liquid nitrogen) and stored at $-80^\circ$C until analysis.

Incubation with liver microsomes

Microsomes were prepared by differential centrifugation of mouse liver homogenate first at $9 \times 10^7$ g (20 min, 4 $^\circ$C), then at $10^7$ g (1 h) in a Beckman L-8-60 ultracentrifuge (Beckman Coulter UK Ltd, High Wycombe, Buckinghamshire, UK). The microsomal pellet was suspended in Tris buffer (50 mM, pH 7.4), recentrifuged at $10^7$ g (1 h) and resuspended in Tris buffer. Liver microsomes from two to four mice were pooled. Microsomes (0.5 mg protein ml$^{-1}$) were incubated at 37$^\circ$C with NADPH (1 mM), MgCl$_2$ (1 mM) and resveratrol or DMU 212 (1 mM) for 20 min (final volume: 0.2 ml). The addition of one volume of ice-cold methanol terminated the reaction. The mixture was vortexed (30 s), centrifuged (3 min, 13,400 g) and the supernatant was collected and analysed by HPLC. For the biosynthesis of resveratrol glucuronide for use as a reference compound, microsomes were incubated with resveratrol (1 mM) as described above, except that NADPH was replaced by uridine-diphosphoglucuronic acid (1 mM).

Extraction of agents from plasma and tissues

Tissues were homogenised (1:1 volume to tissue mass ratio) in 50 mM Hepes buffer (Sigma, Poole, UK) using a hand-held glass homogeniser. An aliquot (250 ml) of homogenate, to which an internal standard had been added was vortexed, followed by the addition of acetonitrile (1 ml). After vigorous shaking, mixtures were kept on ice (5–10 min) and centrifuged (2800 g, 10 min, 4$^\circ$C). The supernatants were dried under a stream of nitrogen and reconstituted in mobile phase (100 ml).

HPLC analysis

HPLC analysis was performed using a Varian Prostar HPLC system (Varian, UK) with a Pro-Star 230 solvent delivery system, a Pro-Star 310 UV–Vis detector, a Pro-Star 363 Fluorescence detector, a 410 Varian autosampler and an Ultracarb C$_{18}$ column (4.6 mm × 250 mm, 5 $\mu$m, Phenomenex, UK). The mobile phase consisted of the three components: aqueous ammonium acetate (pH 6.5, 50 mM), propan-2-ol and acetonitrile. The gradient system that determined the composition of the eluent concerning these three components was as follows: for resveratrol 80:10:10 at the start, 75:10:15 at 10 min, 70:10:20 at 15 min, 60:10:30 at 17 min, 50:10:40 at 20 min and 20:10:70 at 25 min; for DMU 212 45:10:45 at the start for up to 10 min, 20:10:70 at 15 min and 10:10:80 at 20 min. The flow rate for both methods was 1 ml min$^{-1}$.

Internal standards were carbamazepine and 4′-methoxy-4-methyl-trans-stilbene for resveratrol and DMU 212, respectively. Resveratrol and its metabolites was analysed using UV detection (325 nm), and DMU 212 and its metabolites were determined with fluorescence detection (335 nm excitation, 395 nm emission). The injection volume of samples reconstituted in the mobile phase was 50 $\mu$l. The preliminary characterisation of metabolites was achieved by cochromatography using either synthetic standards or biosynthetically generated resveratrol glucuronide.

Figure 2  Structures of five putative metabolites of DMU 212.
Quantification of resveratrol and DMU 212 was performed using standard curves constructed with relevant drug concentrations. The curves were characterised by regression coefficients of $R^2 = 0.99$ or above. The extraction efficiencies (in percent) for resveratrol and DMU 212 were as follows: from plasma 102 ± 17 and 67 ± 2, respectively, from tissues 86 ± 7 and 78 ± 15, respectively (mean ± s.d., $n = 5–7$). The recovery of the resveratrol conjugates was approximately 50% that of the DMU 212 metabolites 60%. Therefore, metabolites were not quantitated.

The area under the plasma or tissue concentration vs time curve (AUC) values were calculated using the trapezoidal rule (WinNonlin v.1.1, Scientific Consultants, USA).

Mass spectrometry

Mass spectrometry was performed using a Quattro Bio-Q tandem quadrupole mass spectrometer upgraded to Quattro MK II specifications (Micromass, Manchester, UK) with a pneumatically assisted electrospray interface. Samples were analysed in positive ion mode. The temperature was maintained at 120°C, and the operating voltage of the electrospray capillary was 3.88 kV and the cone voltage was 32 V. HPLC conditions used for the on-line HPLC-mass spectrometric analyses were as described for DMU 212 above.

Effect of agents on growth of HT-29 and HCA-7 cells

Cells were seeded (10^4 per well) in 24-well plates and cultured in DMEM containing Glutamax I (Life Technologies, Paisley, UK) and glucose (4.5 g l^{-1}) and 10% foetal calf serum (Gibco, Paisley, UK). Resveratrol or DMU 212 (1–100 μM) dissolved in DMSO was added to cellular incubates 24 h postplating. Cells were counted 72, 96, 120, 144 and 168 h postaddition of agents using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, High Wycombe, UK). Control cultures contained the vehicle only. The amount of DMSO added to the incubate did not exceed 0.1%, which on its own failed to affect the cell growth. The IC_{50} values inserted in Figure 5 were calculated from the linear portion of the cell number vs agent concentration curves at the 168 h time point.

RESULTS

Plasma and tissue levels of resveratrol and DMU 212

Mice received intragastric resveratrol or DMU 212 (1 or 0.8 mmol kg^{-1}), and drug levels were measured in the plasma and liver, kidney, lung, brain, small intestinal mucosa and colonic mucosa. These are all tissues in which resveratrol might prevent malignancy, or delay its onset. For comparison, levels in the heart, small intestinal and colonic mucosae, respectively. DMU 212 levels peaked after 10 min, the first of the time points chosen, and peak concentrations were 32 μmol in plasma and 51, 16, 50, 1.2, 75, 960 and 30 nmols g^{-1} tissue in the liver, kidney, lung, brain, heart, small intestinal and colonic mucosae, respectively. DMU 212 levels reached a peak after 10 min, except in the case of the colon, in which the peak occurred after 30 min. The peak levels for DMU 212 were 5 μM in the plasma and 8, 11, 11, 5, 10, 7600 and 330 nmols g^{-1} tissue in the liver, kidney, lung, brain, heart, small intestinal and colonic mucosae, respectively. We performed a preliminary experiment in order to explore whether the difference in gastrointestinal levels would also be observed when the agents are administered with the diet, the route often used in preclinical cancer chemoprevention studies. Agents were admixed with the diet at 0.2% and fed for 2 weeks. Colonic levels of DMU 212 after DMU 212 administration exceeded those of resveratrol after resveratrol administration by two-fold (result not shown).

Table 1 Area under the plasma or tissue concentration time curve (AUC) for resveratrol and DMU 212 in mice that received these agents (240mg/kg) i.g.

| Tissue                  | AUC (nmol ml^{-1} or g^{-1} min) |
|------------------------|----------------------------------|
|                        | Resveratrol | DMU 212 | AUC_{plasm}/AUC_{DMU} |
| Plasma                 | 863         | 246     | 3.5                   |
| Liver                  | 2150        | 432     | 5                     |
| Kidney                 | 785         | 566     | 1.5                   |
| Lung                   | 1123        | 778     | 1.5                   |
| Heart                  | 2072        | 750     | 3                     |
| Brain                  | 103         | 193     | 0.5                   |
| Intestinal mucosa      | 36690       | 369315  | 0.1                   |
| Colonic mucosa         | 2869        | 19256   | 0.15                  |

AUC values were calculated from the curves shown in Figure 2 using the mean plasma or tissue concentration values between 0 and 120 min postadministration.
Metabolism of resveratrol and DMU 212

Plasma and tissues of mice, which had received resveratrol or DMU 212, were investigated using HPLC cochromatography and mass spectrometry in order to detect and characterise metabolites. HPLC analysis of liver samples from animals on resveratrol displayed two peaks (Figure 4A) in addition to the parent compound. These two species were also observed in the liver, lung and kidney, but not in the plasma (result not shown). Solvent eluting from the column with the two peaks was collected, and peak constituents were isolated and subjected to mass spectrometric analysis. Mass spectrometric analysis of peak 1 afforded the molecular ion $m/z = 403$, consistent with a resveratrol glucuronide. Biosynthesis of resveratrol glucuronide using UDP glucuronyl transferase and liver microsomes generated material, which on cochromatography eluted with peak 1 (Figure 4A). Mass spectral analysis did not allow unequivocal confirmation of its structural assignment as either the 3 or 4' glucuronide. Peak 2 eluted with the same retention time as authentic resveratrol-3-sulphate and yielded $m/z = 307$, consistent with resveratrol sulphate.

Extracts of plasma and liver samples from animals that had received DMU 212 exhibited several peaks in addition to that of the parent drug (Figure 4B). Extracts were subjected to cochromatography with several authentic putative metabolic products of mono-hydroxylation or O-demethylation of DMU 212. The results suggest that five of the peaks were 4,4'-di-dihydroxy-3,5-dimethoxystilbene (DMU 295, 4,4'-di-desmethyl-DMU 212, for structures see Figure 2), 4'-hydroxy-3,4,5-trimethoxystilbene (DMU 281, 4'-desmethyl-DMU 212), 3'-hydroxy-3,4,5,4'-tetramethoxystilbene (DMU 214, 3'-hydroxy-DMU 212), 4-hydroxy-3,5,4'-trimethoxystilbene (DMU 291, 4-desmethyl-DMU 212), and 3-hydroxy-4,5,4'-trimethoxystilbene (DMU 807, 3-desmethyl-DMU 212). Collection of solvent eluting individual peaks and subsequent tandem mass spectrometric analysis of solvent extracts confirmed the putative structural assignment for the four metabolites 4,4'-di-desmethyl-DMU 212 (m/z = 273, Table 2), 4'-desmethyl-DMU 212 (m/z = 287), 3'-hydroxy-DMU 212 (m/z = 317) and 3-desmethyl-DMU 212 (m/z = 287). In extracts of lung and kidney tissues, these metabolites were also detected, albeit at lower concentrations (result not shown).

For confirmatory purposes, the metabolism of resveratrol and DMU 212 was also studied in vitro in suspensions of liver microsomes fortified with cofactors of cytochrome P450 enzymes. While HPLC analysis of extracts of the incubation mixture with resveratrol did not yield any peak in addition to that of the parent substrate, analysis of incubates with DMU 212 afforded peaks in

![Figure 4](image-url)
addition to that of parent DMU 212 (Figure 4C). In analogy to the analysis of liver tissue from mice that had received DMU 212, the incubation mixture was extracted and the extract was subjected to cochromatographic analysis. The results (Figure 4C) suggest that 4,4'-di-desmethyl-DMU 212, 4'-desmethyl-DMU 212, 3'-hydroxy-DMU 212, 4-desmethyl-DMU 212 and 3-desmethyl-DMU 212 were products of the microsomal biotransformation of DMU 212.

**Effect of resveratrol and DMU 212 on the growth of colon cancer cells**

The growth-modulating ability of resveratrol and DMU 212 were investigated in a preliminary manner in HT-29 and HCA-7 colon cancer cells *in vitro*. The IC$_{50}$ values computed form the growth curves (Figure 5) range from 6 to 26 μM, and DMU 212 appears to be a slightly more potent growth inhibitor than resveratrol.

**DISCUSSION**

The results presented above suggest that the introduction of four methoxy groups into the stilbene framework, three of which replaced the hydroxy moieties in resveratrol, fails to increase the systemic availability of the molecule in comparison to resveratrol. This conclusion is borne out by the comparison between resveratrol and DMU 212 in terms of the concentrations of these agents in the plasma, liver and heart after oral administration, in which the availability of DMU 212 was inferior to that of resveratrol. In contrast, in comparison to resveratrol, DMU 212 was found to be more available in intestinal and colonic mucosae and in the brain. *A priori*, it is difficult to predict on theoretical grounds in what manner such a structural modification might alter the pharmacokinetic profile of the stilbene molecule. One of the physicochemical corollaries of this alteration, which undoubtedly impacts on the pharmacokinetics of the molecule, is the increase in systemic availability of the molecule.
lipophilicity conferred on the stilbene species in DMU 212 by four methoxy functionalities vis-à-vis resveratrol, which has three hydroxy groups instead. The difference in lipophilicity between DMU 212 and resveratrol is reflected by the fact that reversed phase HPLC analysis of a mixture of both agents using the gradient system described under Materials and Methods for DMU 212 afforded a retention time of 17 min for DMU 212, while resveratrol eluted with the solvent front.

Another factor, which undoubtedly determines the differential pharmacokinetic properties of resveratrol and DMU 212, is the divergence in their metabolic profile. Resveratrol is known to undergo metabolic phase II reactions involving conjugation with sulphate and glucuronic acid (Yu et al., 2002). Consistent with this report, we recently demonstrated the occurrence of glucuronide in the liver and other tissues of mice that had received resveratrol. In contrast to resveratrol, DMU 212 was subjected to hepatic metabolic oxidation, especially single or double O-demethylation reactions in the 3, 4 or 4' positions of the molecule. In addition, we could identify a hydroxylated metabolic species (3'-hydroxy-DMU 212, DMU 214). The spectrum of O-demethylated and hydroxylated metabolites found in vivo accurately reflected by the metabolic profile obtained on incubation of DMU 212 with NADPH-fortified liver microsomes. These findings are consistent with the results of recent in vitro experiments using cytochrome P450 isoenzyme preparations, in which DMU 212 was found to undergo both aromatic hydroxylation and O-demethylation reactions primarily catalysed by isoenzymes of the CYP1 family (Wilsher et al., unpublished). In analogy, resveratrol was recently found to undergo metabolic oxidation in vitro to piceatannol (3,5,2'-tetra-hydroxystilbene), when incubated with a source of CYP1B1 (Potter et al., 2002b). In the study described here, piceatannol was not identified as a metabolite of resveratrol in mice in vivo or in mouse liver microsomes in vitro. It is conceivable that piceatannol was present at very low levels, which might have confounded detection by the procedures used here. The ability to convert resveratrol to piceatannol has been surmised to be a relatively specific property of CYP1B1 (Potter et al., 2002b). The lack of the presence of piceatannol at detectable concentrations in our study in the mouse is consistent with the notion that CYP1B1 is not expressed at appreciable levels in mouse liver (Shimada et al., 2003). Overall, the results described here suggest that hydroxylation to piceatannol is probably not a major metabolic route for resveratrol in the mouse.

DMU 212 is devoid of any toxicity in rats when administered at single doses of up to 400 mg kg$^{-1}$ per day) by factors of 32 and 760, respectively. Furthermore, the results of our preliminary assessment of the colon cancer cell growth-inhibitory properties of resveratrol and DMU 212 suggest that the concentrations of either agent required to inhibit growth, with IC50 values between 6 and 26 µM, are comfortably within the range achieved in the colorectal tract of mice that received these agents at the oral doses used in the study described here, 240 mg kg$^{-1}$.

In conclusion, the work described here provides an initial pharmacokinetic groundwork, which can contribute to rational decision making as to the choice of resveratrol analogues that should be selected for comparative testing for cancer chemopreventive potency in preclinical models. DMU 212 showed more favourable pharmacokinetic properties than resveratrol, in that it yielded higher levels of drug in the small intestinal, colonic mucosa and brain. Buxtressed by our recent finding that DMU 212 is devoid of any toxicity in rats when administered at single doses of up to 40 mg kg$^{-1}$ via the i.v. route or up to 400 mg kg$^{-1}$ when administered p.o. (Verschoyle et al., unpublished), the results presented here render the exploration of DMU 212 side by side with resveratrol for chemopreventive efficacy in rodent models of colorectal carcinogenesis propitious.
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