Development of Novel Furanocoumarin Dimers as Potent and Selective Inhibitors of CYP3A4.

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Abstract

Grapefruit juice has been found to cause an increase in the oral bioavailability of many therapeutic agents. Such interactions are believed to result from the mechanism-based inhibition of CYP3A4 activity in the intestine. Furanocoumarin dimers present in the juice have been found to be extremely potent inhibitors of CYP3A4 activity. The aim of this work was to synthesise and test a series of dimers with a view to defining the relationship between structure and inhibitory activity and establish whether they might make suitable probes of CYP3A4 activity. Eleven furanocoumarin dimers were synthesised and evaluated as inhibitors of CYP3A4 using human liver microsomes using testosterone as the marker substrate. Four of the most potent dimers were also investigated for their effects on CYP3A4 activity in the human intestine and on five additional hepatic cytochrome P450 (CYP) isoforms. The dimers showed potent dose-dependent inhibition of CYP3A4 activity in both liver and intestine; IC$_{50}$ values ranged from 0.021 ± 0.002 to 0.146 ± 0.041 µM (mean ± s.d. n = 3). Of the four dimers evaluated further, all showed time-dependent inhibition of CYP3A4 activity. 88Prop showed moderate inhibition of both CYP2C19 and CYP1A2 with IC$_{50}$ values of 4.42 ± 0.01 and 1.98 ± 0.34 µM, 88Octa was found to inhibit CYP2C19 (IC$_{50}$ = 3.16 ± 0.01 µM) and 58Prop to inhibit CYP1A2 (IC$_{50}$ = 2.39 ± 0.77 µM). Minimal inhibition of CYP2D6 and CYP2C9 was observed (IC$_{50}$ < 10µM). In conclusion, all the dimers tested were extremely potent inhibitors of CYP3A4 activity. In particular dimer 55EE was highly selective towards the enzyme, suggesting that this compound is a suitable probe for determining the contribution of CYP3A4 to drug metabolism.
There has been substantial interest in the inhibition of CYP3A4 activity by the constituents of grapefruit juice. This effect was first recognised by Bailey and colleagues from a chance finding in a felodipine-ethanol interaction study where grapefruit juice was used to mask the taste of the alcohol (Bailey et al., 1989). Elevated plasma felodipine concentrations were observed both in the alcohol and control phases of the study, and in a later study, grapefruit juice was found to triple the mean area under the plasma concentration-time curve through inhibition of CYP3A4 (Bailey et al., 2004) Subsequently, a large number of studies have been performed on the effects of grapefruit juice on a range of drugs of therapeutic importance, such as terfenadine (Benton et al., 1996; Rau et al., 1997), felodipine (Bailey et al., 2000; Dresser et al., 2000; Goosen et al., 2004), nifedipine (Mohri et al., 2000), midazolam (Kupferschmidt et al., 1995), diazepam (Ozdemir et al., 1998) and cyclosporine (Yee et al., 1995). The constituents of grapefruit juice that cause inhibition of CYP3A4 have not been conclusively identified. Strong candidates are the furanocoumarins, particularly the bergamottin series (Ohnishi et al., 2000; Kakar et al., 2004) However, there is no clear evidence that any one compound is primarily responsible and it is possible that the interaction arises from the accumulative effect of a number of constituents present in the juice. Of the compounds isolated, three dimers, namely GF-I-1, GF-I-4 and GF-I-6 (Figure 1.), possibly derived from the geranyloxy monomer (6',7'-dihydroxybergamottin), were the most potent inhibitors of CYP3A4 activity (Fukuda et al., 1997; Guo et al., 2000a; Tassaneeyakul et al., 2000). Potencies were found to exceed that of ketoconazole and were shown to be one to two orders of magnitude higher than those observed for the monomers bergamottin, 6',7'-
epoxybergamottin and 6',7'-dihydroxybergamottin, when using testosterone as the marker substrate (Guo et al., 2000b).

Inhibition of CYP3A4 activity by grapefruit juice is believed to be the result of mechanism-based inactivation (SchmiedlinRen et al., 1997; He et al., 1998), indicating that enzyme activity is lost by a time-dependent degradation of the enzyme. It is believed that the interaction of CYP3A4 with furanocoumarins occurs at the unsaturated furan ring. There is initial formation of an epoxide, which then undergoes ring-opening (by hydrolysis or attack from an internal nucleophile) to form a vic-adduct, which is able to covalently bind to the apoprotein (He et al., 1998) of the enzyme, thus irreversibly inactivating it. This mechanism may be similar to the scheme proposed for the inactivation of CYP2B1 and CYP2A6 by furanocoumarins (Koenigs and Trager, 1998).

The naturally occurring dimers in grapefruit juice that inhibit CYP3A4 are those substituted at the 5-position and are derivatives of bergamottin. Position 8-substituted dimers, based on 8-geranyloxypsoralen, which is a much more potent CYP3A4 inhibitor than bergamottin (Guo et al., 2000b), have not been tested. In the present work a series of dimers were synthesised with differing functional groups at either the 5- or the 8- positions on the psoralen (furanocoumarin) ring system, and as well as some 5/8- mixed dimers. A number of dimers were designed incorporating five different interlinking chains including cis or trans-but-2-ene, propyl, octyl and an ethoxy-ethane polyether linkage (Table 1).

The incorporation of cis or trans but-2-ene generates a linkage between the two furanocoumarin molecules similar to that seen for resvatrol. The latter is a component of red wine, which has also been shown to be a potent inhibitor of CYP3A4 (Chan and Delucchi, 2000). Inactivation of CYP3A4 by resvatrol has been
suggested to occur via epoxidation or hydroxylation of the ethylene bridge (Chan and Delucchi, 2000). It is possible that inhibition of CYP3A4 by the cis and trans but-2-ene dimers may also occur in this manner. Alternatively, the inclusion of an alkene substituent may enhance binding by increased Van der Waals interactions. The inclusion of the propyl and octyl derivatives enables the inclusion of more flexible chains into the molecule compared to the but-2-ene derivatives. Incorporation of the polyether linkage enables a direct comparison with the octyl derivative to establish whether enhanced hydrogen bonding along the chain influences CYP3A4 inhibition.

Based on the above considerations, the aim of this work was to determine the relationship between the chemical structure of furanocoumarin dimers (chain length, chain flexibility, hydrogen bonding capability and lipophilicity) and the inhibition of CYP3A4 activity in liver and intestine. The selectivity of these dimers for CYP3A4 was also investigated by determining their effect on the activities of other human CYPs.

Materials and Methods

Chemicals. Unless otherwise stated all chemicals were of analytical grade or of a higher purity and were purchased from Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK), Fisher Scientific (Loughborough, UK), Roche Diagnostics Ltd (Lewes, UK), VWR International Ltd (Poole, UK), or were donated by SAFC Pharma (formerly Ultrafine, Manchester, UK).

Synthesis and Characterization of Furanocoumarin Dimers. The 5/5- and 8/8-substituted dimers were synthesised under basic conditions in a reaction between the hydroxylated furanocoumarin derivatives and a dihalogenated-interlinking chain (Castellan et al., 1983). Preparation of the 5/8- mixed furanocoumarin dimers was
carried out in two steps. First, the reaction between the 8-hydroxypsoralen and the dibromoalkane gave the intermediate bromopropyl and bromooctyl derivatives. These compounds were then coupled with bergaptol, affording the propyl and octyl mixed furanocoumarin dimers in reasonable yields.

\(^1\)H and \(^{13}\)C NMR were recorded on Bruker AC 250 or Bruker AMX1-400 instruments. Chemical shifts (\(\delta_H, \delta_C\)) are reported in ppm and coupling constants (\(J\)) are in Hertz (Hz). Chemical shifts were referenced to residual non-deuterated solvent present in the deuterated sample, e.g. CHCl\(_3\) in CDCl\(_3\). Infra-red spectra were determined by direct sample analysis using a Bruker Goldengate ATR Vector 22 Spectrometer and are reported by wave numbers (cm\(^{-1}\)). Electron Impact (EI) and Chemical Ionisation (CI) mass spectrometry were carried out on a Micromass Prospec magnetic sector instrument by Sheffield University Mass Spectrometry Department or a Kratos Concept 1S instrument by Manchester University Mass Spectrometry Department. Melting points were determined on a Shardon scientific hot stage microscope apparatus, and are quoted uncorrected in °C. Thin layer chromatography (TLC) was carried out on aluminium backed Merck Kiesgel plates, with detection by UV (254nm) fluorescence. Chromatography was carried out using Merck Silica gel 60 (<63 micron) or Fisher Matrex 35-70 micron.

**General Procedure 1.** The dihalide (0.50 mmol) was added dropwise to a stirred suspension of 8-hydroxypsoralen (200 mg, 0.99 mmol), potassium carbonate (273 mg, 1.98 mmol) and potassium iodide (20 mg, 0.12 mmol) in DMF (10 mL) under argon. The reaction was heated to 80 °C for 3 hours. On completion as judged by TLC aqueous citric acid (10% w/v) was added which, resulted in precipitation of the product. Water (5 mL) was added and the reaction stirred for a further 30 minutes. The product was recovered by filtration and washed with ether (20 mL).
General procedure 2. A solution of 8-hydroxypsoralen (200 mg, 0.99 mmol) in DMF (5 mL) was added dropwise to a stirred suspension of the dibromoalkane (3.96 mmol) and potassium carbonate (273 mg, 1.98 mmol) in DMF (5 mL) under argon. The reaction mixture was heated to 80°C for 45 minutes. On completion as judged by TLC the potassium carbonate was neutralised by the addition of aqueous citric acid (10% w/v). The solution was extracted with ethyl acetate (2 x 20 mL) and the combined organic extracts washed with water, brine and dried (MgSO₄). Removal of the solvent under reduced pressure gave a translucent yellow oil, which was purified by column chromatography affording the bromo compound as colourless crystals. The intermediate bromopropyl or bromooctyl derivative (0.31 mmol) was added to a stirred suspension of bergaptol (63 mg, 0.31 mmol), potassium carbonate (85 mg, 0.62 mmol) and potassium iodide (12 mg, 0.08 mmol) in DMF (10 mL) under argon. The reaction mixture was heated to 80 °C for 6 hours. Aqueous citric acid (10% w/v) was added, resulting in the formation of a pale precipitate, which was recovered by filtration.

**88cBUT** was prepared by general procedure 1, using cis-1,4-dichlorobut-2-ene and xanthotoxol. The desired compound was recrystallised from dichloromethane:hexane (2:1) yielding off-white crystals (186 mg, 0.41 mmol, 83%). Mp 182-183 °C; ν_max cm⁻¹ 1709 (C=O), 1586 (C=C); δ_H (d₆-DMSO; 250MHz) 8.11 (2H, d, J = 9.6 Hz), 8.06 (2H, d, J = 2.1 Hz), 7.65 (2H, s), 7.06 (2H, d, J = 2.1 Hz), 6.40 (2H, d, J = 9.6 Hz), 5.98 (2H, t, J = 4.2 Hz), 5.04 (4H, d, J = 4.2 Hz); δ_C (d₆-DMSO; 63MHz) 159.7, 147.9, 147.5, 145.3, 143.0, 130.3, 129.5, 125.8, 116.5, 114.6, 114.3, 107.2, 68.6; m/z (CI) 457 (15%, [M+H]^+), 203 (75%, [R₂OH + H]^+), Found (EI) 456.0841; C_{26}H_{16}O_8 requires 456.0845.
88tBUT was prepared by general procedure 1, using trans-1,4-dichlorobut-2-ene and xanthotoxol and yielding the desired compound as small cream crystals (212 mg, 0.46 mmol, 94%). Mp 226-227 °C; $\nu_{\text{max}}$ cm$^{-1}$ 1712 (C=O), 1584 (C=C); $\delta_H$ (d$_7$-DMF; 250MHz) 8.27 (2H, d, $J = 9.8$ Hz), 8.20 (2H, d, $J = 2.0$ Hz), 7.79 (2H, s), 7.18 (2H, d, $J = 2.0$ Hz), 6.53 (2H, d, $J = 9.8$ Hz) 6.33 (2H, bs), 5.16 (4H, d, $J = 3.4$ Hz); $\delta_C$ (d$_7$-DMF; 100.6MHz) 165.5, 153.3, 150.9, 148.9, 136.5, 135.2, 131.7, 122.3, 120.0, 119.9, 112.7, 78.3, not found (2 x OC$_{ar}$) possibly obscured by C$_{ar}$OR at 148.9; $m/z$ (CI) 457 (20%, [M+H]$^+$), 203 (100%, [R$_{ar}$OH + H]$^+$), Found (EI) 456.0841; C$_{26}$H$_{16}$O$_8$ requires 456.0845.

88Prop was prepared by general procedure 1, using the 1,3-dibromopropane and xanthotoxol, and yielding the desired compound as pale brown crystals (76 mg, 0.17 mmol, 34%). Mp 183-185 °C; $\nu_{\text{max}}$ cm$^{-1}$ 1727 (C=O), 1588 (C=C); $\delta_H$ (CDCl$_3$; 250MHz) 7.75 (2H, d, $J = 9.6$ Hz), 7.67 (2H, d, $J = 2.2$ Hz), 7.33 (2H, s), 6.80 (2H, d, $J = 2.2$ Hz), 6.34 (2H, d, $J = 9.6$ Hz), 4.89 (4H, t, $J = 6.0$ Hz), 2.44 (2H, quin, $J = 6.0$ Hz); $\delta_C$ (d$_6$-DMSO; 63MHz) 159.6, 147.8, 147.3, 145.2, 142.8, 131.0, 125.8, 116.5, 114.2, 107.1, 70.2, 30.6; $m/z$ (EI) 444 (45%, M$^+$), 202 (90%, [R$_{ar}$OH + H]$^+$), Found (EI) 444.0845; C$_{25}$H$_{16}$O$_8$ requires 444.0845.

88Octa was prepared by general procedure 1, using 1,8-dibromoctane and xanthotoxol, and yielding the desired compound as light yellow crystals (89 mg, 0.17 mmol, 70%). Mp 143-144 °C, Lit.(Castellan et al., 1983) 145-146 °C; $\nu_{\text{max}}$ cm$^{-1}$ 1714 (C=O), 1581 (C=C); $\delta_H$ (CDCl$_3$; 250MHz) 7.78 (2H, d, $J = 9.6$ Hz), 7.72 (2H, d, $J = 2.2$ Hz), 7.37 (2H, s), 6.83 (2H, d, $J = 2.2$ Hz), 6.39 (2H, d, $J = 9.6$ Hz), 4.51 (4H, t, $J = 6.7$ Hz), 1.89 (4H, quin, $J = 6.7$ Hz), 1.62-1.31 (8H, m); $\delta_C$ (CDCl$_3$; 63MHz) 160.6, 148.2, 146.6, 144.4, 143.4, 132.1, 126.0, 116.5, 114.7, 112.9, 106.7, 70.1, 29.7, 29.2,
25.6; m/z (EI) 514 (10%, M⁺), 202 (37%, R₆OH), Found (EI) 514.1635; C₃₀H₂₆O₈ requires 514.1628.

**88EE** was prepared by general procedure 1, using 1,2-bis-(2-iodoethoxy)ethane and xanthotoxol. The product was recrystallised from ethyl acetate:hexane (1:1) furnishing light yellow crystals of the desired compound (193 mg, 0.37 mmol, 75%). Mp 120-121 °C, Lit.(Castellan et al., 1983) 124-125 °C; υ max cm⁻¹ 1703 (C=O), 1580 (C=C); δΗ (CDCl₃; 250MHz) 7.77 (2H, d, J = 9.6 Hz), 7.71 (2H, d, J = 2.2 Hz), 7.37 (2H, s), 6.82 (2H, d, J = 2.2 Hz), 6.37 (2H, d, J = 9.6 Hz), 4.64 (4H, t, J = 4.8 Hz), 3.92 (4H, m), 3.74 (4H, s); δC (CDCl₃; 63MHz) 160.4, 148.1, 146.7, 144.3, 143.3, 131.9, 125.9, 116.4, 114.6, 113.2, 106.7, 73.1, 70.9, 70.4; m/z (EI) 518 (10%, M⁺), 202 (77%, R₆OH), Found (EI) 518.1209; C₂₈H₂₂O₁₀ requires 518.1213.

**55cBUT** was prepared by general procedure 1, using *cis*-1,4-dichlorobut-2-ene and bergaptol. The product recrystallised from dichloromethane:hexane (2:1) yielding an off-white solid (181 mg, 0.40 mmol, 80%). Mp 244-246 °C; υ max cm⁻¹ 1714 (C=O), 1626 (C=C); δΗ (d₇-DMF; 250MHz) 8.33 (2H, d, J = 9.5 Hz), 8.14 (2H, s), 7.51 (2H, s), 7.43 (2H, s), 6.42 (2H, d, J = 9.5 Hz), 6.28 (2H, s), 5.42 (4H, s); δC (d₇-DMF; 100MHz) 165.8, 163.5, 158.3, 154.1, 151.7, 145.1, 134.7, 119.5, 118.2, 112.8, 111.2, 99.3, 74.4; m/z (EI) 456 (10%, M⁺) 202 (100%, R₆OH), Found (EI) 456.0861; C₂₆H₁₆O₈ requires 456.0845.

**55tBUT** was prepared by general procedure 1, using *trans*-1,4-dichlorobut-2-ene and bergaptol. The desired compound was recovered as small cream crystals by filtration and washing with ether. The insolubility of this compound in most organic solvents led to difficulties with purification. Numerous attempts were made, but a purity of only 77.4% was obtained. Thus, the compound was not evaluated as an inhibitor of CYP3A4. (454 mg, 0.99 mmol, 80%). Mp 230-232 °C; υ max cm⁻¹ 1716 (C=O), 1624
(C=C); δ_H (d_6-DMSO; 250MHz) 8.17 (2H, d, J = 9.8 Hz), 8.03 (2H, d, J = 2.1 Hz), 7.37 (2H, s), 7.30 (2H, d, J = 2.1 Hz), 6.33 (2H, d, J = 9.8 Hz), 6.21 (2H, s), 5.09 (4H, s), impurities not included; δ_C (d_7-DMF; 63MHz) 159.8, 157.7, 152.2, 148.2, 145.7, 139.0, 128.6, 113.4, 112.2, 106.5, 105.0, 93.3, 71.8; m/z (LCMS, ES) 457 (100%, [M+H]^+).

55Prop was prepared by general procedure 1, using 1,3-dibromopropane and bergaptol, and yielding off-white crystals (185 mg, 0.42 mmol, 84%). Mp decomposed at 285 °C; ν_max cm⁻¹ 1703 (C=O), 1626 (C=C); δ_H (d_6-DMSO; 250MHz) 8.16 (2H, d, J = 9.8 Hz), 8.03 (2H, d, J = 2.1 Hz), 7.34 (2H, s), 7.31 (2H, s), 6.21 (2H, d, J = 9.8 Hz), 4.76 (4H, t, J = 6.0 Hz), 2.39 (2H, quin, J = 6.0 Hz); δ_C (d_7-DMF; 100.6MHz) 161.0, 158.9, 153.5, 149.9, 146.6, 140.1, 113.9, 112.9, 106.3, 93.9, 70.2; not found (2 x C_ARCOR), expected to lie around 107.0 ppm, The signal for CH² around 30 ppm was possibly obscured by the DMF peak; m/z (EI) 444 (47%, M^+), 203 (5%, [R_AROH + H]^+), Found (EI) 444.0851; C₂₅H₁₆O₈ requires 444.0845.

55Octa was prepared by general procedure 1, using 1,8-dibromoctane and bergaptol, and yielding light brown crystals (140 mg, 0.27 mmol, 55%). Mp 174-176 °C; ν_max cm⁻¹ 1717 (C=O), 1621 (C=C); δ_H (CDCl₃; 250MHz) 8.17 (2H, d, J = 9.7 Hz), 7.61 (2H, s), 7.16 (2H, s), 6.96 (2H, s), 6.29 (2H, d, J = 9.7 Hz), 4.47 (4H, t, J = 6.2 Hz), 1.93-1.88 (4H, m), 1.59-1.33 (8H, m); δ_C (d_7-DMF; 100.6MHz) 165.9, 163.9, 158.4, 155.0, 151.5, 145.2, 118.9, 118.0, 112.0, 111.4, 98.8, 86.6, 40.8, 39.6, 31.3; m/z (EI) 514 (5%, M^+), 202 (100%, R_AROH), Found (EI) 514.1630; C₃₀H₂₆O₈ requires 514.1628.

55EE was prepared by general procedure 1, using 1,2-bis-(2-iodoethoxy)ethane and bergaptol. The product was recrystallised from dichloromethane, affording pale mauve crystals (170 mg, 0.33 mmol, 66%). Mp 207-209 °C; ν_max cm⁻¹ 1715 (C=O),
1626 (C=C); $\delta_H$ (CDCl$_3$; 250MHz) 8.12 (2H, d, $J = 9.7$ Hz), 7.59 (2H, d, $J = 2.3$ Hz), 7.11 (2H, s), 6.96 (2H, d, $J = 2.3$ Hz), 6.26 (2H, d, $J = 9.7$ Hz), 4.55 (4H, t, $J = 4.5$ Hz), 3.89 (4H, t, $J = 4.5$ Hz), 3.77 (4H, s); $\delta_C$ (d$_7$-DMF; 100.6MHz) 160.9, 158.7, 153.2, 149.7, 146.9, 140.3, 114.9, 113.1, 107.8, 105.9, 94.4, 73.6, 71.1, 70.4; m/z (EI) 518 (23%, M$^+$), 203 (100%, [R$_{ar}$OH + H]$^+$), Found (EI) 518.1214; C$_{28}$H$_{22}$O$_{10}$ requires 518.1213.

58Prop was prepared by general procedure 2, using 1,3-dibromopropane and xanthotoxol. The desired compound was recrystallised from dichloromethane:hexane (2:1), yielding the desired compound as off-white crystals (82 mg, 0.18 mmol, 59%). Mp 222-224 °C; $\nu_{\text{max}}$ cm$^{-1}$ 1716 (C=O), 1623 (C=C); $\delta_H$ (CDCl$_3$; 250MHz) 8.09 (1H, d, $J = 9.8$ Hz), 7.75 (1H, d, $J = 9.6$ Hz), 7.63 (1H, d, $J = 2.4$ Hz), 7.59 (1H, d, $J = 2.2$ Hz), 7.34 (1H, s), 7.17 (1H, d, $J = 2.4$ Hz), 7.11 (1H, s), 6.81 (1H, d, $J = 2.2$ Hz), 6.36 (1H, d, $J = 9.6$ Hz), 6.18 (1H, d, $J = 9.8$ Hz), 4.88 (2H, t, $J = 6.0$ Hz), 4.79 (2H, t, $J = 5.7$ Hz), 2.45 (2H, quin, $J = 5.9$ Hz); $\delta_C$ (d$_7$-DMF; 100.6MHz) 165.9, 165.5, 163.9, 158.3, 154.7, 153.4, 153.4, 151.6, 150.8 1, 149.0, 145.0 1, 137.0, 131.8, 122.4, 119.9, 118.9, 117.9, 112.8, 112.0, 111.3 1, 98.0, 76.0, 75.0, 36.1; m/z (EI) 444 (100%, M$^+$), 202 (60%, R$_{ar}$OH), Found (EI) 444.0854; C$_{25}$H$_{16}$O$_{8}$ requires 444.0845.

58Octa was prepared by general procedure 2, using 1,8-dibromoocanate and xanthotoxol. The desired compound was purified by column chromatography eluting with ethyl acetate:hexane (2:3); removal of solvent under reduced pressure furnished off-white crystals (82 mg, 0.16 mmol, 64%). Mp 136-137 °C; $\nu_{\text{max}}$ cm$^{-1}$ 1708 (C=O), 1620 (C=C); $\delta_H$ (CDCl$_3$; 250MHz) 8.18 (1H, d, $J = 9.8$ Hz), 7.77 (1H, d, $J = 9.6$ Hz), 7.70 (1H, d, $J = 2.2$ Hz), 7.59 (1H, d, $J = 2.4$ Hz), 7.36 (1H, s), 7.13 (1H, s), 6.98 (1H, d, $J = 2.4$ Hz), 6.83 (1H, d, $J = 2.2$ Hz), 6.37 (1H, d, $J = 9.6$ Hz), 6.27 (1H, d, $J = 9.8$ Hz), 4.49 (4H, ot, $J = 6.5$, 6.5 Hz), 1.90 (4H, quin, $J = 6.7$ Hz), 1.65-1.39 (8H, m); $\delta_C$
Source and Preparation of Tissue Samples. Samples of liver tissue were obtained with written consent from patients undergoing surgery for the removal of a hepatocellular tumour secondary to colon cancer. Macroscopically normal tissue close to the resection line was used. Samples of upper intestine (duodenum & jejunum) were obtained with written consent from patients undergoing total gastrectomy. These studies were approved by the appropriate Hospital Ethics Committee.

Human liver microsomes and intestinal supernatant S9 fraction were prepared as described previously. Intestinal S9 samples were pooled from five patients due to limited tissue availability.

Incubation Conditions for the Characterisation of CYP3A Inhibition. The $6\beta$-hydroxylation of testosterone was used as the index of CYP3A4 activity. Human liver microsomes and intestinal S9 (0.2 mg/mL) were incubated with testosterone (37 µM) and the test compounds (concentration range 0.5-100 µM) at 37°C in the presence of KCl (1.15%), phosphate buffer (0.2M, pH7.4) and a NADPH generating system, for 10 min in a total volume of 1 mL (Crewe, 1997) Incubations were performed in triplicate. The reaction was terminated by the addition of ethyl acetate (2 mL). 16-$\alpha$-Hydroxytestosterone (1.5 µg) or 11-$\beta$-hydroxytestosterone (1 µg) (depending on the retention time of the test compound) was added as the internal standard. Samples were gently mixed for 15 minutes, before centrifugation at 1500g for 15 minutes. The
upper organic layer was evaporated to dryness under reduced pressure, and the residue was stored at −20 °C until analysis.

**Characterisation of the Inhibition of the Activities of Other Human CYP’s**

The O-demethylation of dextromethorphan (10 µM) was used as the index of CYP2D6 activity (Gorski et al., 1994), the 7-hydroxylation of (S)-warfarin (4 µM) the index of CYP2C9 activity, the O-deethylation of 7-ethoxyresorufin (1 µM) the index of CYP1A2 activity (Hanioka et al., 2000), the 4-hydroxylation of (S)-mephenytoin (200 µM) the index of CYP2C19 activity (Wrighton et al., 1993), and the 6-hydroxylation of chlorzoxazone (20 µM) the index of CYP2E1 activity (Leclercq et al., 1998). The concentrations in brackets are the mean $K_m$ values for the reactions and were those used in the present work.

**Mechanism-Based Inhibition of CYP3A4.** In order to minimise competitive inhibition by the furanocoumarin dimers, incubations were performed at a testosterone substrate concentration of 250 µM. Human liver microsomes (2 mg/mL) were preincubated with furanocoumarin dimers or DMSO in the presence of an NADPH-generating system for various times, ranging from 0 to 180 seconds at 37 °C. An aliquot (100 µL) was then transferred to another incubation vial containing the testosterone and NADPH-generating system (final volume 1 mL) and assayed for remaining CYP3A4 activity. This resulted in a 1 in 10 dilution of protein (final concentration 0.2 mg/mL) and in final inhibitor concentrations of 12.5, 31, 62.5, 125 and 250 nM. The reaction was terminated after 3 minutes by the addition of ethyl acetate (2 mL). Samples were then treated as described above. Incubations were performed in triplicate.

**Analysis of 6β-Hydroxytestosterone.** Sample residues were reconstituted with mobile phase (150 µL) and an aliquot was injected onto the HPLC. A Hypersil C8
BDS column (150 mm x 4.6 mm; 5 µm particle size) was used. The mobile phase was methanol:water (55:45 v/v) delivered at a flow rate of 1 mL/min. Eluents were detected by UV at 254 nm. The lower limit of determination of the assay was 30 pmol mL\(^{-1}\), and the coefficient of variation at 328 pmol mL\(^{-1}\) was less than 5%.

**Data Analysis.** Values of IC\(_{50}\) were obtained by non-linear regression using Grafit version 5.0 (Erithacus Software Ltd, Staines, UK). The inactivation parameters \(k_{\text{inact}}\) and \(K_I\) were calculated according to the method of Silverman (Silverman, 1988). The initial rate constant (\(k\) min\(^{-1}\)) for the inactivation at each inhibitor concentration was estimated from the slope of the log of the remaining CYP3A4 activity vs the preincubation time. Values for the inactivation parameters were determined from the double reciprocal plot of the initial inactivation rate constants vs the respective inhibitor concentrations (\(1/k\) vs \(1/[I]\)). These values were used as initial estimates in solving the following equation with GraFit:

\[
    k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{K_I + [I]}
\]

where \([I]\) is the initial inhibitor concentration, \(k_{\text{inact}}\) is the maximum rate constant for inactivation when \([I]\) approximates infinity, \(K_I\) is the inhibitor concentration that produces half the maximal rate of inactivation.

**Results**

All the synthetic furanocoumarin dimers were found to inhibit CYP3A4 in a dose dependent manner (Table 2) with potencies comparable to and in most cases exceeding those obtained for their naturally occurring counterparts (Guo et al., 2000b). Within this series 88Prop, 88tBUT, 88Octa and 55EE are found to be the most potent analogues, with IC\(_{50}\) values of less than 0.030 µM and causing maximum inhibition of more than 90% over the concentration range studied. The least potent analogue was the 55Octa derivative with an IC\(_{50}\) value of 0.140 µM. 88Prop showed
approximately a two-fold increase in potency over the 5/5- and 5/8- derivatives. 88Octa was found to be the most potent inhibitor of the octyl derivatives, showing a 4.8 and 7-fold higher potency compared to the 5/8- and 5/5-octyl dimers, and 58Octa was 1.4-fold more inhibitory than its 5/5-substituted counterpart. Within the 8/8-substituted series the potency of inhibition appeared to increase with lipophilicity (Table 2). However, for the 5/5- and 5/8-substituted dimers a decrease in potency was observed as the lipophilicity of the compounds increased.

Four of the most potent dimers (88Prop, 58Prop, 88Octa and 55EE) were selected for further study (Figure 2 & 3). Inhibition of CYP3A4 by these compounds was assessed in human microsomes from additional livers, again using testosterone as the marker substrate (Table 3). Overall there was about a three-fold variation in inhibitory potency between the livers. Furanocoumarin dimers were found to inhibit CYP3A4 activity in intestinal supernatant S9 fractions to a similar extent as that seen with liver microsomes (Table 4). The effects of these four furanocoumarin dimers on the activity of five different CYP isoforms (Figure 4) were investigated to demonstrate whether the compounds were selective for CYP3A4. All were found to be weak inhibitors of CYP2D6 and CYP2C9 activity, giving IC$_{50}$ values of more than 10 µM (Table 5). No data were obtained for the inhibition of CYP2E1 activity by the dimers, since DMSO and DMF (both 0.2% v/v), used to dissolve the analogues, were found to be potent inhibitors of chlorzoxazone-6-hydroxylase activity in vitro (producing a more than a 90% loss), data which were consistent with previous findings (Hickman et al., 1998). Moderate inhibition of CYP2C19 activity was seen for the two 8/8-substituted derivatives, giving IC$_{50}$ values approximately two orders of magnitude higher than those observed for CYP3A4. 88Prop, 58Prop and 88Octa produced moderate inhibition of CYP1A2 activity. Inclusion of the propyloxy
interlinking chain led to a 2.5-fold higher potency when compared to the longer chain octyl derivative. The 5/5-ethoxyethane compound (55EE) showed minimal inhibition of the five CYP isoforms.

These four furanocoumarin dimers were found to inhibit testosterone CYP3A4 activity in a time- as well as a concentration-dependent manner. Pseudo first order kinetics were observed for the inactivation of enzyme activity. The mean (± s.d. from triplicate experiments) $K_I$ values obtained for 88Prop, 58Prop, 88Octa and 55EE were $0.098 ± 0.015$, $0.123 ± 0.041$, $0.040 ± 0.013$, $0.123 ± 0.007$ µM, respectively, and their inactivation rate constant $k_{\text{inact}}$ were $0.088 ± 0.006$, $0.058 ± 0.009$, $0.036 ± 0.004$ and $0.045 ± 0.001$ min$^{-1}$, respectively, in microsomes from liver HL7.

**Discussion**

The dimers synthesised showed a dose-dependent inhibitory effect on CYP3A4 activity in human liver microsomes. The potency of these compounds, which were active at low nanomolar concentrations, was found to exceed ketoconazole and other known inhibitors of CYP3A4 (Guo et al., 2000b). The dimers exhibited comparable potencies with little or no difference arising from the length of the interlinking chain. This lack of variability may be attributed to the lipophilic nature of these chains, which possibly causes them to "fold up" on themselves within the aqueous medium of the active site of CYP3A4. The highly lipophilic nature of the compounds as a whole may give rise to strong interactions within hydrophobic region(s) of the active site. The IC$_{50}$ values for the inhibition of CYP3A4 activity were of the same order of magnitude for each dimer in microsomes from five different livers.

Dose-dependent inhibition of the formation of 6β-hydroxytestosterone in human intestinal (S9) supernatant was also observed. Complete abolition of enzyme activity
was found at a concentration of 1 µM for all four dimers, indicating a similar degree of inhibition as that observed in liver. This suggests that the susceptibility of enteric and hepatic CYP3A4 to inhibition is similar, a result that is consistent with published data (Lown et al., 1998; Obach et al., 2001). The furanocoumarin dimers synthesised in the present work were shown (a) to inhibit CYP3A4 activity in human liver microsomes in a time- and concentration-dependent manner, and (b) along with the naturally occurring dimers GF-I-1 and GF-I-4 (Tassaneeyakul et al., 2000), to be extremely potent mechanism based inhibitors. Their potencies (range of $K_I$ values = 0.04-0.32 µM) are one to two orders of magnitude greater than many other known mechanism-based inhibitors (e.g. verapamil = 2.3 µM) (Yeo and Yeo, 2001), gestodene = 46 µM (Guengerich, 1990), mibedrafil = 2.3 µM (Yeo and Yeo, 2001) and L-754 394 = 7.5 µM (Lightning et al., 2000)).

Studies with other irreversible CYP3A4 inhibitors such as 8-methoxypsoralen, furafylline and coumarin compounds suggest that the loss of catalytic activity may be attributed to modification of the haem, denaturation of the apoprotein or covalent binding of a metabolite of the inhibitor to the enzyme active site. The furanocoumarin monomers bergamottin and 6',7'-DHB are also mechanism based inhibitors of CYP3A4 with $k_{inact}$ and $K_I$ values of 40, 5.6 µM and 0.08, 0.06 min$^{-1}$, respectively (Tassaneeyakul et al., 2000). The $k_{inact}$ values for the naturally occurring furanocoumarin dimers are very similar to those obtained for the synthetic dimers, suggesting the possibility that the mechanism of inactivation is similar. Thus, the dimers may interact with the enzyme at the unsaturated furan moiety in a similar manner as that described for the inactivation of CYP3A4, CYP2B1 and CYP2A6 by furanocoumarin monomers (He et al., 1998; Koenigs and Trager, 1998). Initially an epoxide is formed on the unsaturated furan ring. This epoxide then undergoes ring-
opening (by hydrolysis or attack from an internal nucleophile) to form a vic-adduct, which is able to covalently bind to the apoprotein of the enzyme, thus irreversibly inactivating it. Further studies are required to confirm this hypothesis with respect to the synthetic dimers.

Our finding suggests that the 8/8-octyl and propyl derivatives are slightly more selective towards CYP3A4 than their 5/5- and 5/8- substituted counterparts. Weak inhibition of CYP2D6 and CYP2C9 was observed for the four dimers chosen for further study, with IC₅₀ values exceeding 10 µM. 88Octa and 88Prop were found to be moderate inhibitors of CYP2C19 activity but with IC₅₀ values approximately two orders of magnitude greater than those observed for inhibition of CYP3A4 activity. Little difference in potency towards CYP2C19 was observed with increasing chain length, suggesting that the binding sites on the enzyme mainly consist of hydrophobic regions. 88Prop, 88Octa and 88Prop showed moderate inhibition of CYP1A2 activity but with IC₅₀ values exceeding those observed for CYP3A4 by two orders of magnitude. An increase in potency was observed with a decrease in chain length. CYP1A2, like CYP3A4, catalyses the metabolism of neutral/basic, lipophilic or planar molecules with at least one hydrogen-bonding site, making both the furanocoumarin monomers and dimers candidates for an interaction with this enzyme. In addition to the compounds tested, flavonoids (Zhai et al., 1998; Murray et al., 2001), methylxanthines (Murray et al., 2001), grapefruit juice, 6',7'-dihydroxybergamottin, bergamottin and the naturally occurring dimers GF-I-1 and GF-I-4 (Tassaneeyakul et al., 2000) have been shown to be potent to moderate inhibitors of CYP1A2 activity, using phenacetin as the marker substrate. The increased selectivity of the 55EE analogue suggests different orientations for the 5- and 8-substituted ring systems within the binding domains for CYP1A2 and
CYP2C19. For CYP1A2 it appears that the 8-substituted furanocoumarins have a greater affinity for the binding site than the 5/5-substituted dimers. As inhibitory activity was observed with the mixed 5/8-propyl dimer, it is proposed that the dimer is binding at its 8- rather than its 5-substituted end.

Unfortunately no data were obtained for CYP2E1, as the DMSO (0.2% v/v) used to dissolve the dimers was found to be a potent inhibitor of chlorzoxazone 6-hydroxylase activity (>90%), the probe reaction used. However, it is unlikely that any interaction would occur between these dimers and CYP2E1, since the enzyme is principally involved with the metabolism of small molecules with a molecular weight of <200. Furthermore, as neither naturally occurring furanocoumarin monomers nor dimers are reported to be potent inhibitors of CYP2E1 activity (Tassaneeyakul et al., 2000), no significant inhibitory effects would be envisaged with the synthetic furanocoumarin dimers tested in the present study. The selectivity of our synthetic compounds is similar to that observed by Tassaneeyakul et al. (2000), where furanocoumarin dimers isolated from grapefruit juice significantly inhibited CYP1A2, CYP2C9, CYP2C19 and CYP2D6 activities, but with IC$_{50}$ values being at least one order of magnitude higher compared to those for CYP3A4 inhibition.

In addition to CYP3A4, P-glycoprotein has been reported to play a role in the grapefruit juice interaction (Soldner et al., 1999; Spahn-Langguth and Langguth, 2001; Wang et al., 2001). In this context, a small pilot study was performed on four of the furanocoumarin dimers synthesised. Using P-glycoprotein over expressed human leukaemia CEM$_{VBL100}$ cell line, inhibition of its transport function by all four dimers was observed (data not shown). These preliminary findings are consistent with other published data, showing that the furanocoumarins GF-I-1, 6’,7’-dihydroxybergamottin, bergamottin, bergapten and bergaptol (10 µM) inhibit the efflux of [$^{3}$H]-VBL in
Caco-2 cells, increasing uptake by 601, 357, 138, 244 and 270 %, respectively (Ohnishi et al., 2000).

In conclusion, the synthetic furanocoumarin dimers 88prop, 58Prop, 88Octa and 55EE have been shown to inhibit the activity of CYP3A4 in a highly selective and potent fashion, suggesting that these compounds are suitable probes for determining the contribution of the enzyme to drug metabolism.
References


Footnotes

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Figure 1. Furanocoumarin dimers isolated from grapefruit juice (Fukuda et al., 2000; Guo et al., 2000a)

Figure 2. Inhibition of CYP3A4 activity microsomes from liver HL7 by four of the most potent dimers, which were chosen for further study. Where ■ = 88Prop, ▲ = 58Prop, ▼ = 55EE and ♦ = 88Octa. Mean (± s.d. from triplicate experiments)

Figure 3. The four furanocoumarin dimers chosen for further study had propyl octyl or ethoxy-ethane linkages substituted at either the 5- or the 8-position of the ring system.

Figure 4. Inhibitory effects of furanocoumarin dimers on five different CYP450 isoforms in liver microsomes from liver HL7. ■ = 88Prop, ▲ = 58Prop, ▼ = 55EE and ♦ = 88Octa. Results are expressed as a mean of triplicate incubations ± s.d
Table 1. Structures of the furanocoumarin dimers synthesised, where R denotes either the 5 or the 8-furanocoumarin ring system

<table>
<thead>
<tr>
<th>Ring System</th>
<th>Dimer Linkage (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/5 Series</td>
<td></td>
</tr>
<tr>
<td>8/8 Series</td>
<td></td>
</tr>
<tr>
<td>5/8 Series</td>
<td></td>
</tr>
</tbody>
</table>

- **55cBUT 88cBUT** -
- **55cBUT 88tBUT** -
- **55Prop 88Prop** 58Prop
- **55Octa 88Octa** 58Octa
- **55EE 88EE** -
Table 2. IC₅₀ values for inhibition of CYP3A4 activity in human microsomes from a single liver (HL7) by furanocoumarin dimers (Mean (± s.d) data from triplicate experiments.). Log P values were calculated using Accord for Excel v.5.0.

<table>
<thead>
<tr>
<th>Dimer</th>
<th>IC₅₀ (µM)</th>
<th>Maximum inhibition (%)</th>
<th>Calculated Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>55cBUT</td>
<td>0.030 ±0.001</td>
<td>90</td>
<td>2.17</td>
</tr>
<tr>
<td>55Prop</td>
<td>0.047 ± 0.008</td>
<td>91</td>
<td>2.04</td>
</tr>
<tr>
<td>55Octa</td>
<td>0.146 ± 0.041</td>
<td>86</td>
<td>3.03</td>
</tr>
<tr>
<td>55EE</td>
<td>0.029 ± 0.018</td>
<td>95</td>
<td>1.17</td>
</tr>
<tr>
<td>88cBUT</td>
<td>0.037 ± 0.001</td>
<td>99</td>
<td>2.17</td>
</tr>
<tr>
<td>88tBUT</td>
<td>0.023 ± 0.001</td>
<td>92</td>
<td>2.17</td>
</tr>
<tr>
<td>88Prop</td>
<td>0.026 ± 0.008</td>
<td>97</td>
<td>2.04</td>
</tr>
<tr>
<td>88Octa</td>
<td>0.021 ± 0.002</td>
<td>95</td>
<td>3.03</td>
</tr>
<tr>
<td>88EE</td>
<td>0.043 ± 0.006</td>
<td>97</td>
<td>1.17</td>
</tr>
<tr>
<td>58Prop</td>
<td>0.053 ± 0.007</td>
<td>95</td>
<td>2.04</td>
</tr>
<tr>
<td>58Octa</td>
<td>0.100 ± 0.004</td>
<td>95</td>
<td>3.03</td>
</tr>
</tbody>
</table>
Table 3. Variation between human livers in the inhibition of microsomal CYP3A4 activity by four furanocoumarin dimers. Mean (± s.d. from triplicate experiments) IC$_{50}$ values.

<table>
<thead>
<tr>
<th>Liver</th>
<th>88Prop (µM)</th>
<th>58Prop (µM)</th>
<th>88Octa (µM)</th>
<th>55EE (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL7</td>
<td>0.026 ± 0.008</td>
<td>0.053 ± 0.007</td>
<td>0.021 ± 0.002</td>
<td>0.029 ± 0.008</td>
</tr>
<tr>
<td>HL12</td>
<td>0.012 ± 0.004</td>
<td>0.021 ± 0.005</td>
<td>0.021 ± 0.005</td>
<td>0.047 ± 0.007</td>
</tr>
<tr>
<td>HL21</td>
<td>0.017 ± 0.001</td>
<td>0.029 ± 0.005</td>
<td>0.029 ± 0.001</td>
<td>0.039 ± 0.012</td>
</tr>
<tr>
<td>HL25</td>
<td>0.035 ± 0.009</td>
<td>0.054 ± 0.002</td>
<td>0.076 ± 0.001</td>
<td>0.084 ± 0.002</td>
</tr>
<tr>
<td>HL31</td>
<td>0.055 ± 0.017</td>
<td>0.019 ± 0.005</td>
<td>0.037 ± 0.009</td>
<td>0.051 ± 0.004</td>
</tr>
<tr>
<td>Mean</td>
<td>0.029 ± 0.017</td>
<td>0.035 ± 0.017</td>
<td>0.037 ± 0.023</td>
<td>0.050 ± 0.021</td>
</tr>
</tbody>
</table>
Table 4. Comparison of the inhibition of CYP3A4 activity in human intestine (S9) (pooled from five patients, mean (± s.d) IC_{50} data from triplicate experiments.) and human liver microsomes (HLM, mean (± s.d) IC_{50} data from five livers (Table 3)) by furanocoumarin dimers.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Intestine (µM)</th>
<th>HLM (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>88Prop</td>
<td>0.012 ± 0.002</td>
<td>0.029 ± 0.017</td>
</tr>
<tr>
<td>58Prop</td>
<td>0.022 ± 0.001</td>
<td>0.035 ± 0.017</td>
</tr>
<tr>
<td>88Octa</td>
<td>0.011 ± 0.003</td>
<td>0.037 ± 0.023</td>
</tr>
<tr>
<td>55EE</td>
<td>0.060 ± 0.005</td>
<td>0.050 ± 0.021</td>
</tr>
</tbody>
</table>
Table 5. IC₅₀ values (mean ± s.d. from triplicate experiments) obtained for the inhibition of the activities of five human CYP’s by four furanocoumarin dimers in microsomes from liver HL7.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CYP2D6</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2E1</th>
<th>CYP1A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>88Prop</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>3.53 ± 0.35</td>
<td>NR</td>
<td>1.82 ± 0.46</td>
</tr>
<tr>
<td>58Prop</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>NR</td>
<td>1.84 ± 0.92</td>
</tr>
<tr>
<td>88Octa</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>2.86 ± 0.07</td>
<td>NR</td>
<td>4.65 ± 0.93</td>
</tr>
<tr>
<td>55EE</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>NR</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>
Figure 4

Graphs showing the % Remaining Enzyme Activity in CYP2C19, CYP2D6, CYP2C9, and CYP1A2 at different inhibitor concentrations (μM).