Notes

Halogenated Chalcones with High-Affinity Binding to P-Glycoprotein: Potential Modulators of Multidrug Resistance

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Previous studies have shown that flavonoids are modulators of the transmembrane P-glycoprotein (P-gp) which mediates cell multidrug resistance. Some structural elements have been identified which seem to contribute to these compounds' activity. In the present study, a series of halogenated chalcones was prepared to further explore the structural requirements for the P-gp modulation. Four halogenated chalcones have been synthesized and evaluated as potential modulators of P-gp-mediated multidrug resistance of cancer cells by in vitro assays using a purified recombinant domain of the transporter containing the modulator binding site. Halogenated chalcones exhibited high-affinity binding, the 2′,4′,6′-trihydroxy-4-iodochalcone behaving as the most potent compound with a K_D value in the nanomolar range.

Introduction

Development of drug resistance is a major impediment to efficient anticancer chemotherapy. Multidrug resistance (MDR) appears after prolonged exposure of cells to a single drug and is characterized by resistance to a series of structurally unrelated compounds with different subcellular targets.[1] It is now well-established that the major mechanism of MDR in cancer cells involves the overexpression of a 170-kDa transmembrane P-glycoprotein (P-gp) encoded in humans by the *MDR1* gene.[2] P-gp belongs to the ATP-binding cassette superfamily of transporter proteins and is thought to function as an ATP-dependent pump which binds and exports a broad range of drugs out of the cell, decreasing their intracellular concentration below the cytotoxic threshold.[3] P-gp is composed of two homologous halves, each containing a transmembrane domain, involved in drug efflux and a cytosolic nucleotide-binding domain involved in ATP binding and hydrolysis. Therefore, the development of molecules that can reverse multidrug resistance is of high priority.

Since the naturally occurring flavonoid quercetin can restore sensitivity to adriamycin in multidrug-resistant cells[4] by inhibiting the ATP-binding site of P-gp,[5] a screening program aimed at structure−activity relationships was initiated in Lyon, to discover other flavonoids and derivatives with high affinity as potential P-gp antagonists. It was recently shown that P-gp flavonoid-binding site is cytosolic and partly overlaps the ATP-binding site[6] by using a purified recombinant protein corresponding to a cytosolic nucleotide-binding
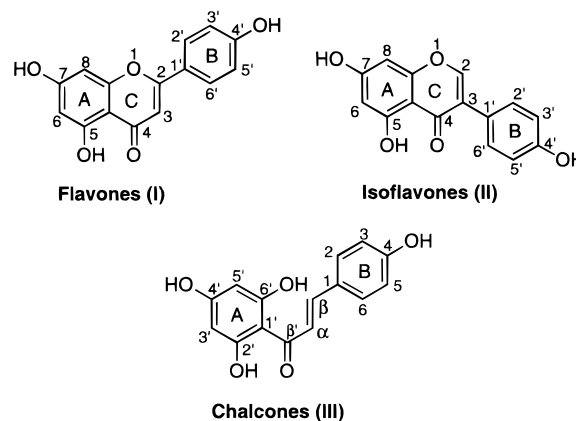

Figure 1. Structures of naturally occurring chalcones, flavones, and isoflavones with affinity binding to P-glycoprotein.

domain;[6−9] the binding affinity appeared highly dependent on both the class of flavonoids and their substituents.

Studies of structure−activity relationships[6] have concluded that (a) flavones **I** are much more efficient than isoflavones **II**, (b) a hydroxyl group at position 5 (position 2′ in chalcones) on ring A is essential for high-affinity binding to P-gp, whereas its substitution at position 7 (position 4′ in chalcones) does not produce any effect, and (c) the affinity is greatly decreased for flavanones when the double bond at position 2,3 on ring C ($α,β$ in chalcones) is reduced (Figure 1). However, chalcones **III** corresponding to disruption of flavone ring C were not investigated. The interaction of flavonoids appears at least partly due to their ability to mimic the adenine moiety of ATP as shown by cocrystallization with either the cyclin-dependent kinase 2[10] or the tyrosine kinase Hck.[11] When the hydroxyl group at position 4′ (position 4 in chalcones) on ring B is replaced

Table 1. Binding Affinity of Chalcones as a Function of the Properties of Substituents at Position 4

chalcone	substituent at position 4	size of the substituent (Å)[19]	hydrophobic substituent constant (π)	K_D (μM)	ΔF_{max} (%)
3a	H	1.20	0.00	4.6 ± 0.3	87.8 ± 1.5
3b	OH	2.37	−0.67	4.8 ± 0.5	86.8 ± 0.5
3c	OMe	2.97	−0.02	2.3 ± 0.2	85.5 ± 1.3
3d	F	1.35	0.14	3.6 ± 0.4	88.1 ± 2.7
3e	Cl	1.80	0.71	1.3 ± 0.1	80.1 ± 1.2
3f	Br	1.95	0.86	0.57 ± 0.08	88.7 ± 2.2
3g	I	2.15	1.12	0.25 ± 0.06	86.6 ± 3.5

Scheme 1[a]

MEM: -CH$_2$OCH$_2$CH$_2$OCH$_3$
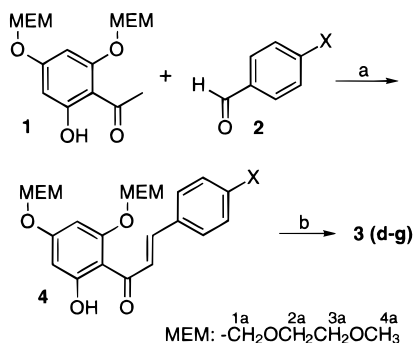
[a] Reagents: (a) KOH, MeOH; (b) HCl, Et$_2$O/MeOH.

by a methoxyl group, as in kaempferol to give kaempferide, the affinity is significantly enhanced which may suggest either that the oxygen atom is participating in hydrogen acceptor bonding with an amino acid located inside the active site or that hydrophobicity of the substituent strengthens the interaction. We raised the question of whether keeping hydroxyl groups at positions 2′, 4′, and 6′ of chalcones and substituting that at position 4 might increase the binding affinity and consequently modulate P-gp activity. Herein, we describe the effects produced by chalcones and derivatives obtained from substitution of the 4-hydroxyl group by a halogen. The latter can function both as hydrogen bond acceptors[12] and as hydrophobic substituents.

Results and Discussion

The naturally occurring chalcones **3a** (R = H), **3b** (R = OH), and **3c** (R = OMe) were prepared according to known methods which are not reported here.[13] Halogenated chalcones **3d**−**g** were prepared from methoxyethoxymethyl (MEM)-protected trihydroxyacetophenone (**1**)[13] and 4-halogenobenzaldehyde (**2**). Since 4-iodobenzaldehyde (**2g**) was not available, it was prepared starting from 4-iodobenzoic acid.[14] The generation of enolate from the acetyl group of MEM-protected acetophenone was ensured by using KOH in MeOH (Scheme 1). Treatment of the enolate with 1.5 equiv of 4-halogenobenzaldehyde afforded the protected chalcones **4d**−**g**. Then, deprotection of di-OMEM by HCl in MeOH/ether gave halogenated chalcones **3** which were purified on LiChroprep DIOL to avoid any cyclization to flavanones.

The binding of chalcones to the purified C-terminal cytosolic domain of P-gp was directly measured by the quenching of protein intrinsic fluorescence, as previously described for other classes of flavonoids.[6] The dissociation constant (K_D) and the maximal fluorescence quenching (ΔF_{max}) were determined by using the Grafit program as detailed.[8] For drug design perspectives,

chalcones appear attractive since they can be regarded as potential nonpeptidic synthons with a wide range of pharmacological actions.[15] Flavonoids are particularly promising as efficient modulators since, on one hand, they are assumed not to be transported by P-gp, as opposed to most commonly used compounds such as verapamil or cyclosporin A,[16] and, on the other hand, they are able to inhibit the ATP-dependent drug efflux catalyzed by the liposome-reconstituted purified transporter.[5] The interaction of the different chalcones was monitored by high quenching (>80%) of the intrinsic fluorescence of P-gp C-terminal cytosolic domain. The lead, natural, product chalcone **3a** exhibited a K_D of 4.6 ± 0.3 μM (Table 1), which is in the same concentration range as the modularory effects reported for quercetin in both restoring the sensitivity to adriamycin of multidrug-resistant cells[4] and inhibiting the ATP-dependent drug efflux by reconstituted P-gp.[5] The affinity was nearly 2-fold higher than that of the corresponding flavone chrysin (K_D = 8.9 ± 0.3 μM), indicating that disruption of the flavone ring C could allow a better adjustment of the flavonoid inside the binding site and strengthen the interaction. Substitution of the chalcone hydrogen at position 4 with a hydroxyl group (**3b**) did not increase the binding affinity (K_D = 4.8 ± 0.5 μM) in contrast to a methoxyl group (**3c**) which doubled it (K_D = 2.3 ± 0.2 μM). Substitution by different halogen atoms gradually enhanced the binding affinity, with a strong dependence on the nature of the halogen: I > Br > Cl > F. These results indicate that iodine in derivative **3g**, which produced a nearly 20-fold increase in affinity (K_D = 0.25 ± 0.06 μM) by comparison to the natural chalcone **3a**, appears to optimally fit the active site of the protein and to play a critical role at position 4. The halogen effect would clearly not be due to the strength of the hydrogen bond acceptor, as estimated according to Raevsky et al.[17] (OMe > OH > F > Cl > Br > I), but appears to be correlated to the lipophilicity substituent constant π (I > Br > Cl > F > OMe > OH) as illustrated in Table 1.[18] The size of the halogen could also contribute to the effect since the values of the van der Waals radius follow the same sequence (I > Br > Cl > F), although the correlation was not observed for hydroxyl and methoxyl groups.[19]

Chalcones having highly hydrophobic substituents at position 4 are being made to investigate further the role of hydrophobicity at the B ring. Also, further efforts to examine the effects produced on binding to P-glycoprotein by differently substituted chalcones on ring A and to understand the action mechanism of these compounds are underway and will be reported in due course.

Experimental Section

General Chemistry Methods. ¹H and ¹³C NMR spectra were recorded on a Bruker AC-200 instrument (200 MHz for

^{1}H, 50 MHz for ^{13}C). Chemical shifts are reported as δ values (ppm) relative to Me$_4$Si as an internal standard. EI mass spectra were obtained at 70 eV using a Fisons Trio 1000 instrument. The ionization current and the chamber temperature were 150 mA and 200 °C, respectively. Elemental analyses were performed by the Analytical Department of CNRS, Vernaison, France. Thin-layer chromatography (TLC) was carried out using E.Merck silica gel F-254 plates (thickness 0.25 mm). Flash chromatography was carried out using Merck silica gel 60, 200−400 mesh, or LiChroprep DIOL (40−63 μm). All solvents were distilled prior use. Diethyl ether was purchased as anhydrous and used as received.

4-Halogeno-2′-hydroxy-4′,6′-di-*O*-MEM-chalcones 4. A solution of 2′-hydroxy-4′,6′-di-*O*-MEM-acetophenone (1.45 mmol), 4′-halogenobenzaldehyde (1.5 equiv), and aqueous solution of KOH (50%, 1.2 mL) in methanol (10 mL) was heated at 70 °C. The reaction was monitored by TLC until completion (1.5−2 h). Water (20 mL) was added and methanol evaporated followed by an extraction with CH$_2$Cl$_2$. The organic layer was dried and evaporated to dryness, then purified by column chromatography (silica gel − AcOEt:Hexane 1:1), to afford a yellow powder of chalcones **4**.

4-Fluoro-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (4d): 22% yield; ^{1}H NMR (CDCl$_3$) δ 3.36 (s, 3H), 3.39 (s, 3H), 3,56 (m, 4H), 3.83 (m, 4H), 5.27 (s, 2H), 5.36 (s, 2H), 6.24 (d, 1H, $J =$ 2.3 Hz), 6.34 (d, 1H, $J =$ 2.3 Hz), 7.10 (m, 2H), 7.60 (m, 2H), 7.73 (d, 1H, $J =$ 15.6 Hz), 7.86 (d, 1H, $J =$ 15.6 Hz), 12.86 (s, 1H); ^{13}C NMR (CDCl$_3$) δ 192.63 (Cβ′), 167.26 (C2′), 163.46 (C4′), 161.12 (d, $J =$ 236 Hz, C4), 159.70 (C6′), 141.13 (Cβ), 132.50 (C1), 130.16 (d, $J =$ 8.6 Hz, C2 + C6), 128 (Cα), 115.90 (d, $J =$ 22 Hz, C5 + C3), 107.41 (C1′), 97.53 (C3′), 94.81 (C5′), 94.21 (C1a), 93.00 (C1a), 71.42 (2C3a), 68.61 (C2a), 68.14 (C2a), 59.94 (2C4a); MS (EI) *m/e* 450 [M]$^+$; HRMS (C$_{23}$H$_{27}$FO$_8$) calcd 450.2299, found 450.2320. Anal. (C$_{23}$H$_{27}$FO$_8$·0.35H$_2$O) C, H; F: calcd, 4.16; found, 4.98.

4-Chloro-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (4e): 12% yield; ^{1}H NMR (CDCl$_3$) δ 3.36 (s, 3H), 3.40 (s, 3H), 3,53 (m, 4H), 3.83 (m, 4H), 5.26 (s, 2H), 5.37 (s, 2H), 6.26 (d, 1H, $J =$ 2.2 Hz), 6.33 (d, 1H, $J =$ 2.2 Hz), 7.37 (d, 2H, $J =$ 8.5 Hz), 7.54 (d, 2H, $J =$ 8.5 Hz), 7.70 (d, 1H, $J =$ 15.7 Hz), 8.05 (d, 1H, $J =$ 15.7 Hz), 13.73 (s, 1H); ^{13}C NMR (CDCl$_3$) δ 193.32 (Cβ′), 168.08 (C2′), 164.33 (C4′), 160.52 (C6′), 141.64 (Cβ), 134.15 (C4), 139.90 (C1), 130.14 (C2 + C6), 129.85 (C3 + C5), 128.65 (Cα), 104.10 (C-1′), 98.32 (C3′), 95.61 (C5′), 94.96 (C1a), 93.76 (C1a), 72.21 (2C3a), 69.44 (C2a), 68.92 (C2a), 59.74 (2C4a); MS (EI) *m/e* 466 [M]$^+$. Anal. (C$_{23}$H$_{27}$ClO$_8$) C, H, Cl.

4-Bromo-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (4f): 28% yield; ^{1}H NMR (CDCl$_3$) δ 3.35 (s, 3H), 3.39 (s, 3H), 3.55 (m, 4H), 3.82 (m, 4H), 5.28 (s, 2H), 5.37 (s, 2H), 6.26 (d, 1H, $J =$ 2.3 Hz), 6.33 (d, 1H, $J =$ 2.3 Hz), 7.46 (d, 2H, $J =$ 8.7 Hz), 7.54 (d, 2H, $J =$ 8.7 Hz), 7.69 (d, 1H, $J =$ 15.6 Hz), 7.90 (d, 1H, $J =$ 15.6 Hz), 13.72 (s, 1H); ^{13}C NMR (CDCl$_3$) δ 193.33 (Cβ′), 168.01 (C2′), 164.32 (C4′), 161.00 (C6′), 141.63 (Cβ), 135.11 (C1), 132.86 (C3 + C5), 130.37 (C2 + C6), 128.75 (Cα), 124.99 (C4), 108.20 (C1′), 98.32 (C3′), 95.60 (C5′), 94.96 (C1a), 93.76 (C1a), 72.21 (2C3a), 69.44 (C2a), 68.92 (C2a), 59.74 (2C4a); MS (EI) *m/e* 512 [M]$^+$. Anal. (C$_{23}$H$_{27}$BrO$_8$·0.25AcOEt) C, H, Br.

4-Iodo-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (4g): 32% yield; ^{1}H NMR (CDCl$_3$) δ 3.36 (s, 3H), 3.38 (s, 3H), 3,55 (m, 4H), 3.82 (m, 4H), 5.28 (s, 2H), 5.37 (s, 2H), 6.26 (d, 1H, $J =$ 2.3 Hz), 6.33 (d, 1H, $J =$ 2.3 Hz), 7.37 (d, 2H, $J =$ 8.5 Hz), 7.60 (d, 2H, $J =$ 8.5 Hz), 7.74 (d, 1H, $J =$ 15.6 Hz), 7.91 (d, 1H, $J =$ 15.6 Hz), 12.91 (s, 1H); ^{13}C NMR (CDCl$_3$) δ 193.17 (Cβ′), 167.96 (C2′), 164.21 (C4′), 162.10 (C6′), 141.66 (Cβ), 136.71 (C3 + C5), 134.50 (C1), 130.31 (C2 + C6), 128.72 (Cα), 104.60 (C1′), 98.17 (C3′), 96.61 (C5′), 95.45 (C5′), 94.65 (C1a), 93.66 (C1a), 72.11 (2C3a), 69.29 (C2a), 68.63 (C2a), 59.62 (2C4a); MS (EI) *m/e* 558 [M]$^+$. Anal. (C$_{23}$H$_{27}$IO$_8$·0.5AcOEt) C, H, I.

2′,4′,6′-Trihydroxy-4-halogenochalcones 3. To a stirred solution of MEM-protected chalcone **4** (1 mmol) in MeOH (30 mL) was added 10 mL of HCl (1% in ether). The solution was heated at 60 °C and monitored by TLC; after completion (2−3

h), the reaction was cooled to room temperature and 30 mL of water was added. The solution was diluted with CH$_2$Cl$_2$ (100 mL), and the organic layer was separated, washed twice with water, dried, and concentrated. The solid obtained was washed three times with hexane, and the solid was collected by filtration to afford a crude material which was purified by column chromatography (LiChroprep DIOL, CHCl$_3$).

4-Fluoro-2′,4′,6′-trihydroxychalcone (3d): 59% yield; ^{1}H NMR (DMSO-d_6) δ 12.42 (s, 2H), 10.43 (s, 1H), 8.04 (d, 1H, $J =$ 15.6 Hz), 7.66 (d, 1H, $J =$ 15.6 Hz), 7.72 (dd, 2H, $J_1 =$ 5.6 Hz, $J_2 =$ 8.8 Hz), 7.27 (dd, 2H, $J_1 = J_2 =$ 8.8 Hz), 5.84 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 191.58 (Cβ′), 165.31 (C4′), 165.00 (d, $J =$ 240 Hz, C4), 164.53 (C2′ + C6′), 140.01 (Cβ), 130.55 (d, $J =$ 8.6 Hz, C2 + C6), 128.90 (d, $J =$ 5 Hz, C1), 127.58 (Cα), 116.15 (d, $J =$ 21.7 Hz, C5 + C3), 104.41 (C1′), 95.02 (C-3′ + C-5′); SM (EI) *m/e* 274 [M]$^+$. Anal. (C$_{15}$H$_{11}$FO$_4$·0.2H$_2$O) C, H; F: calcd, 6.83; found, 6.15.

4-Chloro-2′,4′,6′-trihydroxychalcone (3e): 83% yield; ^{1}H NMR (DMSO-d_6) δ 12.41 (s, 2H), 10.46 (s, 1H), 8.09 (d, 1H, $J =$ 15.6 Hz), 7.70 (m, 2H), 7.64 (d, 1H, $J =$ 15.5 Hz), 7.50 (m, 2H), 5.84 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 191.58 (Cβ′), 165.31 (C4′), 164.53 (C2′ + C6′), 139.87 (Cβ), 134.67 (C4), 129.93 (C2 + C6), 129.16 (C3 + C5), 128.60 (C1), 128.40 (Cα), 103.04 (C1′), 95.14 (C3′ + C5′); SM (EI) *m/e* 290 [M]$^+$. Anal. (C$_{15}$H$_{11}$ClO$_4$·1.1AcOEt) C, H, Cl.

4-Bromo-2′,4′,6′-trihydroxychalcone (3f): 74% yield; ^{1}H NMR (DMSO-d_6) δ 12.40 (s, 2H), 10.46 (s, 1H), 8.10 (d, 1H, $J =$ 15.6 Hz), 7.72−7.57 (m, 5H), 5.84 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 191.65 (Cβ′), 165.50 (C4′), 164.59 (C2′ + C6′), 140.01 (Cβ), 134.53 (C1), 132.13 (C3 + C5), 130.19 (C6 + C2), 128.49 (Cα), 125 (C4), 104.90 (C1′), 95.05 (C3′ + C5′); SM (EI) *m/e* 370 [M· 2H$_2$O]. Anal. (C$_{15}$H$_{11}$BrO$_4$·1.1H$_2$O) C, H, Br.

4-Iodo-2′,4′,6′-trihydroxychalcone (3g): 90% yield; ^{1}H NMR (DMSO-d_6) δ 8.11 (d, 1H, $J =$ 15.8 Hz), 7.81 (d, 2H, $J =$ 8.4 Hz), 7.59 (d, 1H, $J =$ 15.8 Hz), 7.44 (d, 2H, $J =$ 8.4 Hz), 5.83 (s, 2H); ^{13}C NMR (CDCl$_3$) δ 192.83 (Cβ′), 165.16 (C4′), 163.33 (C2′ + C6′), 142.10 (Cβ), 137.12 (C3 + C5), 133.20 (C1), 131.06 (C2 + C6), 128.14 (Cα), 105.11 (C1′), 97.58 (C4), 95.92 (C3′ + C5′); SM (EI) *m/e* 382 [M]$^+$. Anal. (C$_{15}$H$_{11}$IO$_4$·0.3H$_2$O) C, H, I.

Biological Assays. The recombinant C-terminal cytosolic domain of mouse P-glycoprotein was overexpressed in bacteria and purified by affinity chromatography as described earlier.[7] Fluorescence experiments were performed at 25 ± 0.1 °C, using a SLM-Aminco 8000C spectrofluorometer with spectral bandwidths of 2 and 4 nm, respectively, for excitation and emission. The tryptophan-specific intrinsic fluorescence of 1 μM recombinant protein in 1.2 mL of 20 mM potassium phosphate buffer at pH 6.8, containing 0.5 M NaCl, 20% glycerol, and 0.01% methyl 6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside, was scanned from 310 to 380 nm upon excitation at 295 nm. Contribution for Raman effect of buffer was subtracted, and the fluorescence spectra were integrated. The interaction with chalcones was monitored by the increasing quenching of emission fluorescence produced by successive additions of compound, up to 20 μM. The measurements were corrected for innerfilter effect of chalcones, as determined in parallel experiments with *N*-acetyltryptophanamide. Curve fitting of ligand binding related to fluorescence decrease was performed with Grafit (Erithacus software) as previously described.[6−9]

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