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Original article

Structure–activity relationships for the inhibition of recombinant human cytochromes P450 by curcumin analogues

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Abstract

Inhibition of cytochrome P450 (CYP) is a major cause of drug–drug interactions. In this work, inhibitory potentials of 33 curcumin analogues, i.e. 2,6-dibenzylidenecyclohexanone (A series), 2,5-dibenzylidenecyclopentanone (B series) and 1,4-pentadiene-3-one (C series) substituted analogues of curcumin towards recombinant human CYP1A2, CYP3A4, CYP2B6, CYP2C9 and CYP2D6, all important for drug metabolism, were studied in vitro. Fluorescence plate reader and high performance liquid chromatography (HPLC) assays were used to evaluate CYP-inhibitory activities. MOE-based Quantitative structure–activity relationship (QSAR) analysis suggested that electrostatic and hydrophobic interactions and lipophilicity are important factors for CYP inhibition. Apart from insights in important molecular properties for CYP inhibition, the present results may also guide further design of curcumin analogues with less susceptibility to drug–drug interactions.

Keywords: Cytochrome P450; Curcumin analogues; Inhibition; Drug–drug interactions; (Q)SAR

1. Introduction

Curcumin is a well-known food additive and constituent of traditional medicine in Southeast Asia and the Indian subcontinent, the latter being an area with low incidence of colorectal cancer [1]. This naturally occurring and synthetic compound is regarded as a promising drug and has received considerable attention due to its antioxidant, anticancer, anti-inflammatory, anti-HIV and antimalarial properties [2–6]. Reports from a phase 1 clinical trial on curcumin have shown that it is non-toxic even at doses as high as 8 g/day [3]. Curcumin is unstable at a pH 7.4, however, this stability is strongly improved by lowering the pH or by adding glutathione (GSH), *N*-acetyl L-cysteine (NAC), ascorbic acid or rat liver microsomes or cytosol [7]. The instability of curcumin at neutral to basic pH conditions has been attributed to the presence of an active methylene group (Fig. 1) [8]. Omitting this methylene group leads to the formation of more stable and potent antioxidative compounds [8]. Removal of the active methylene group and one carbonyl group led to 1,4-pentadiene-3-ones, which still possess antioxidant properties [9]. Modification of groups on the terminal aromatic rings of curcumin has revealed that the electron-donating substituents increase the anti-inflammatory activity [10].

Previously a series of curcumin analogues were synthesized, in which the methylene and one carbonyl group have

Abbreviations: CYP, cytochrome P450; (Q)SAR, (quantitative) structure–activity relationship; HPLC, high performance liquid chromatography; GSH, reduced glutathione; NAC, *N*-acetyl L-cysteine; *n*, number of compounds in the dataset; *s*, standard error; R^2, correlation coefficient; *F*, coefficient of variance.

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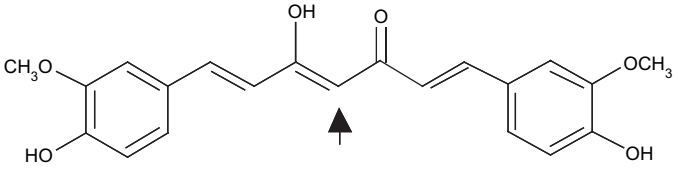


Fig. 1. Chemical structure of curcumin. The arrow is pointing to the active methylene group.

been omitted [9]. These analogues are derivatives of benzylidine having either electron-withdrawing, electron-donating or steric groups. The derivatives include 9 compounds of 2,6-dibenzylidenecyclohexanone (A), 13 of 2,5-dibenzylidenecyclopentanone (B) and 11 of 1,5-diphenyl-1,4-pentadiene-3-one (C) (Schemes 1–3). These analogues exhibit antioxidant, anti-inflammatory, and antibacterial activities that render them potential drug candidates. Interestingly, some of the analogues have shown much stronger antioxidant activities than curcumin [9].

Drug–drug interactions due to inhibition or induction of enzyme activity are among the major causes of attritions in drug development [11]. Inhibition of CYP enzymes is a cause of clinically significant drug–drug interactions [12]. Irrespective of the mechanism, CYP inhibition may result in accumulation of drugs resulting in adverse drug reactions or a decrease in metabolism of drugs and activation of pro-drugs, and hence alter their pharmacokinetic profile. Therefore, in vitro CYP-associated inhibition studies for evaluation of drug candidates during the early stages of drug discovery and development are considered cost-effective for predicting potential clinical drug–drug interactions, since these interactions may result in adverse drug reactions and therapeutic failure [13,14].

Recently the inhibitory effect of curcumin on five major human drug-metabolizing CYPs has been reported [15]. Curcumin showed strong inhibition of CYP2C9 and CYP3A4, with IC_{50} values 4.3 and 16.3 μM, respectively, and moderate inhibition of CYP1A2, CYP2B6 and CYP2D6 (40.0, 24.5, 50.3 μM, respectively). The inhibitory effects of curcumin analogues on human CYPs have not yet been reported. In this study, we investigated the inhibitory potentials of 33 curcumin analogues to the five major recombinant human drug-metabolizing CYPs [16,17] mentioned above. Inhibitor structure–activity relationships (SARs) were subsequently evaluated and quantitative structure–activity relationships (QSARs) were investigated using the program MOE (Molecular Operating Environment). In these studies we tried to identify the molecular features that cause inhibition of the different CYP isoenzymes tested. These studies are important because the resulting information will guide to design the synthesis of new curcumin analogues with less CYP-inhibitory properties and open a way for in silico prediction of xenobiotic inhibition of CYPs.

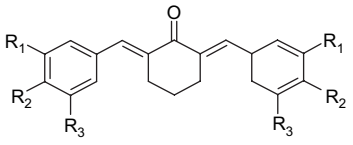
2. Results

2.1. Stability of curcumin analogues in buffer

The stability of curcumin analogues (25 μM) in 0.1 M phosphate buffer was measured spectrophotometrically at 200–600 nm. At pH 7.4 the curcumin analogues decomposed to various extents after 30 min of incubation. Table 1 shows the percent decomposition of the analogues. Generally, compounds of group B (i.e. 2,5-dibenzylidenecyclopentanones) were more stable at pH 7.4 than those in groups A and C (2,6-dibenzylidenecyclohexanones and 1,5-diphenyl-1,4-pentadiene-3-ones). Group A, demonstrated the greatest instability, with A2 having the highest (97.5%) and B14 the lowest (3.0%) percent decomposition. The degradation of the compounds in buffer pH 7.4 was significantly blocked in the presence of CYP enzyme and GSH resulting in 8.2% and 19% decomposition, respectively. These effects are similar to those reported on the stability of curcumin in buffer, by Oetari et al. [7].

2.2. CYP inhibition

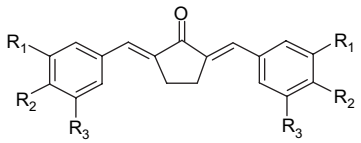
All the 33 curcumin analogues (each at 100 μM concentration) were screened for inhibitory potentials towards human CYP1A2, CYP3A4, CYP2B6, CYP2C9 and CYP2D6.



Cyclohexanones (A)

	R_1	R_2	R_3		R_1	R_2	R_3
A0	H	OH	H	**A8**	H	$N(CH_3)_2$	H
A2	H	H	H	**A10**	Cl	H	H
A4	H	OCH_3	H	**A11**	CH_3	OH	CH_3
A5	H	CH_3	H	**A14**	t-C_4H_9	OH	t-C_4H_9
A7	H	CF_3	H				

Scheme 1.



Cyclopentanone (B)

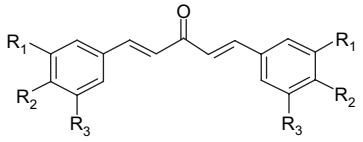
	R_1	R_2	R_3
B0	H	OH	H
B1	OCH_3	OH	H
B2	H	H	H
B3	H	Cl	H
B4	H	OCH_3	H
B7	H	CF_3	H
B8	H	$N(CH_3)_2$	H
B9	Cl	Cl	H
B10	Cl	H	H
B11	CH_3	OH	CH_3
B12	C_2H_5	OH	C_2H_5
B13	i-C_3H_7	OH	i-C_3H_7
B14	t-C_4H_9	OH	t-C_4H_9
B15	OCH_3	OH	OCH_3

Scheme 2.

Preceding the inhibitor screening experiments, tests were conducted with and without GSH [7] to determine whether another stabilization factor was necessary to mitigate decomposition. The results indicated that inhibition by curcumin analogues in the presence of GSH, did not influence the inhibitory effect of the compounds on CYP activity (data not shown). Initial screening results indicated that the curcumin analogues demonstrated a wide range of inhibitory activities towards CYP-mediated metabolism of probe substrates (data not shown). Results on 29 selected compounds (with % inhibition >20%) revealed 75.8% (22), 27.5% (8), 13.7% (4), 62.0% (18) and 41.3% (12) of the compounds inhibiting CYP1A2, CYP3A4, CYP2B6, CYP2C9 and CYP2D6, respectively. In general the compounds showed a comparatively stronger inhibitory potency towards CYP1A2 and CYP2C9 than towards CYP3A4, CYP2B6 and CYP2D6 activities. The least inhibited enzyme was CYP2B6, with only four compounds, i.e. B13, B15, C11 and C15 exhibiting >20% inhibition at 100 μM concentration of compounds, and IC_{50} values being 74.3, 70.0, 44.8 and 24.8 μM, respectively. Compounds of group A showed very weak or no inhibitory activities towards CYP3A4, CYP2B6 and CYP2D6.

2.3. Similarity studies

Concerning the structures of curcumin analogues, similarity studies were performed using the procedure as described by Labute et al. [18]. Accordingly, calculations were carried out using the flexible alignment module of MOE, employing the MMFF94 force field. The best scoring fit both in terms of similarity and objective function are shown in Table 2 and Fig. 2. The respective superimpositions indicated no significant difference between the A, B and C series of compounds, since they appear to be perfectly superposed. However, differences



1,4-Pentadiene-3-ones (C)

	R_1	R_2	R_3
C0	H	OH	H
C1	OCH_3	OH	H
C2	H	H	H
C3	H	Cl	H
C5	H	CH_3	H
C6	H	t-C_9H_4	H
C7	H	CF_3	H
C9	Cl	Cl	H
C10	Cl	H	H
C11	CH_3	OH	CH_3
C15	OCH_3	OH	OCH_3

Scheme 3.

Table 1
Percent decomposition of curcumin analogues in buffer (pH 7.4)

Cmpd	Decomp (%)	Cmpd	Decomp (%)	Cmpd	Decomp (%)
A0	17.5	B3	12.0	C1	nd
A2	97.5	B4	30.5	C2	35.0
A4	54.5	B7	25.5	C3	46.0
A5	65.5	B8	17.0	C6	73.0
A7	74.0	B9	18.5	C7	82.5
A8	35.5	B10	40.5	C9	25.0
A10	63.0	B11	7.0	C10	46.0
A11	13.0	B12	25.5	C11	7.5
A14	57.5	B13	41.5	C15	6.5
B0	13.5	B14	3.0	Curcumin	74.0
B1	7.4	B15	nd		
B2	13.0	C0	53.0		

Cmpd, compound; Decomp, decomposition; nd, not determined. Concentration of each curcumin analogues used was 25 μM. The experiment performed is described in the Section 5.

may also arise due to steric bulk. In the conformation A0–B0, the potential energies of A0 and B0 were 5.7 and 3.9 kcal/mol, above the respective global minima. For the conformation A0–C0, the potential energy of A0 was 3.7 kcal above, and that of C0 was exactly at the global minimum. The conformation B0–C0 resulted in potential energies of B0 and C0, 0.6 and 0.4 kcal/mol, respectively, above the global minima.

2.4. Quantitative structure–activity relationships (QSARs)

Summaries of the relevant datasets employed for generating the QSARs relating the various molecular descriptors to the CYP-inhibitory potencies of curcumin analogues used in this work are shown in Tables 3–6. Table 3 shows the data for 20 curcumin analogues that exhibited inhibitory activities towards CYP1A2, with five relatively important descriptors, i.e. standard dimension 3 − the standard deviation along a principal component axis (std_dim3), polar surface area (TPSA), number of oxygen atoms (a_nO), number of hydrogen bond atoms (a_acc) and potential energy with bonded terms disabled (E_nb). A weak correlation ($R^2 = 0.682$) was found between experimental and predicted IC_{50} data (Fig. 3A) based on these molecular descriptors. However, exclusion of four outliers (B1, B13, C3 and C6) resulted in a good correlation ($R^2 = 0.907$), with the descriptors being an electrostatic parameter (PEOE_VSA_FPNEG), a lipophilicity feature (S log P_VSA0), molecular weight (weight), molecular refractivity (SMR_VSA5) and potential energy with bonded terms disabled (E_nb).

The relevant dataset on seven curcumin analogues inhibiting CYP3A4, employed for generating QSARs, is found in Table 4. A fairly good correlation ($R^2 = 0.804$) was found between experimentally derived and predicted activities based on the descriptor, VdistEq, a distance matrix parameter (Fig. 3B). However, these results are biased due to one outlier (i.e. B0) that appears to influence the outcome significantly. Exclusion of the outlier resulted in no correlation between experimental and predicted inhibitory activities. The dataset on 12 compounds inhibiting CYP2C9 activity is presented in Table 5. Three relatively important descriptors that appear in the QSAR equation are, fractional polar negative van der Waals surface area (PEOE_VSA_FPNEG), fractional hydrophobic van der Waals surface area (PEOE_VSA_FHYD), and log P of accessible van der Waals surface areas for each atom (S log P_VSA4). Experimental inhibitory activities of the analogues towards CYP2C9 weakly correlated ($R^2 = 0.738$) with predicted activities based on these descriptors (Fig. 3C). Elimination of the outlier, B0 resulted in a fairly good correlation ($R^2 = 0.836$) with the same descriptors. Table 6 contains dataset for six curcumin analogues with inhibitory activity towards CYP2D6. The descriptor present in the QSAR equation and as apparently related to the observed inhibitory activity is PEOE_VSA_FPNEG. A weak correlation ($R^2 = 0.522$) was found between experimental and predicted data based on the descriptor (Fig. 3D). A good correlation ($R^2 = 0.903$) was obtained upon removal of outlier C15.

Table 2
The best scoring fits using the flexible alignment module of MOE

	F	*S*	d*U*
A0-B0	119.6379	177.1833	1.7428
A0-C0	119.5509	171.6317	0.0000
B0-C0	119.0929	168.9706	0.0000

F, similarity; *S*, objective function; d*U*, potential energy difference between the lowest minimal energy conformation and the global minimal.

3. Discussion

Inhibition of CYPs can lead to drug–drug interactions and therefore it is considered important to evaluate potential drug candidates for CYP-inhibitory activities. Inhibitory potentials of curcumin towards recombinant human CYP1A2, CYP3A4, CYP2B6, CYP2C9 and CYP2D6 recently have been evaluated in vitro [15]. The inhibitory potencies (IC_{50} values) towards CYP3A4 and CY2C9 suggested a potential for relevant drug–drug interaction in the intestine upon oral co-administration with other drugs metabolized by CYP3A4, considering the required high dose for therapeutic effects. Less potent activities were observed with CYP1A2, CYP2B6 and CYP2D6. Three groups of curcumin analogues [9], i.e. 9 of 2,6-dibenzylidenecyclohexanone (group A), 13 of 2,5-dibenzylidenecyclopentanoe (group B) and 11 of 1,5-diphenyl-1,4-pentadiene-3-one (group C) were analogously tested experimentally for inhibition towards five important human drug-metabolizing CYPs mentioned above [15]. QSAR analysis employing molecular descriptors that have >18% correlations with activity that were used as inputs for artificial neural networks (ANNs) [http://www.natural-selection.com/library/2006/NN_antihiv_ligand_gbf.pdf] and were generated by MOE.

Most of the curcumin analogues exhibited low inhibitory activities towards the CYPs tested. Six of them, A2, A8, A10, C2, C7 and C10, showed potent inhibitory activities with IC_{50} values in the range 0.9–4 μM, towards CYP1A2. These compounds showed about 10- to 40-fold greater potency towards inhibition of CYP1A2 than curcumin itself. However, these six compounds have not been reported to

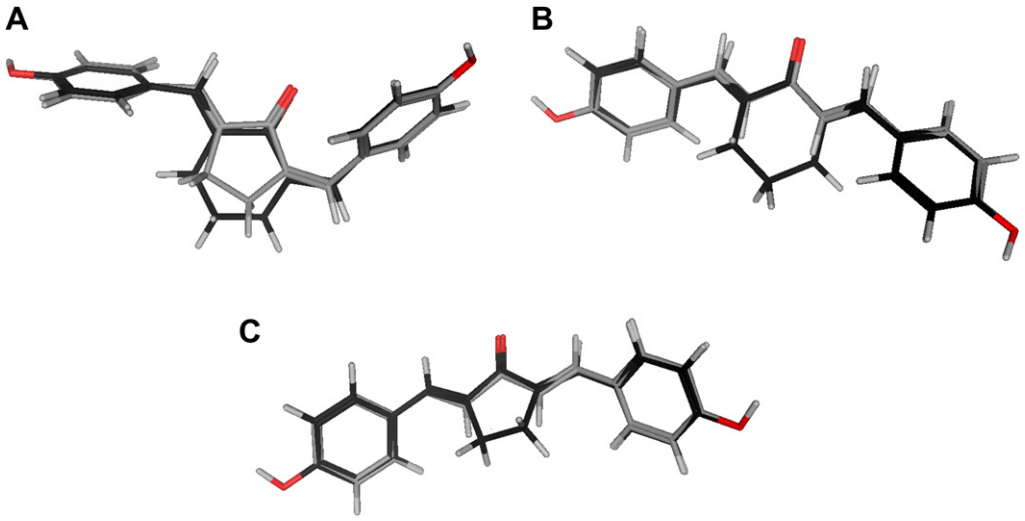


Fig. 2. Flexible alignment of curcumin analogues (A): A0 (black), B0 (grey); (B): A0 (black), C0 (grey); and (C): B0 (black), C0 (grey).

have potent antioxidant activities [9]. Compounds A2 and C2, both lack substituents, in all investigated substitutions. Similarly, compounds A10 and C10 both have chloride at R_1, with the R_2 and R_3 positions unsubstituted (Schemes 1 and 3). The only difference between series A and C compounds is the absence of a central six-membered ring in the latter. Therefore it appears that these substitutions together with the presence or absence of a central six-membered ring favour increased CYP1A2 inhibitory potency of compounds of A and C series. However, compound B2, having the same substituents as A2 and C2 but a central five-membered ring, showed lower inhibitory potency. Generally, compounds of the B series showed rather weak inhibitory activities towards CYP1A2. Apparently the central five-membered ring renders these compounds less active towards CYP1A2. The present QSAR analysis suggest that five descriptors influence inhibition of CYP1A2, being as standard dimension 3(std_dim3) – the standard deviation along a principal component axis, potential energy with bonded terms disabled (E_nb), number of oxygen atoms (a_nO), number of hydrogen bond acceptor atoms (a_acc) and total polar surface area (TPSA). A weak correlation ($R^2 = 0.682$) was obtained between experimental and predicted inhibitory activities. However, exclusion of four outliers resulted in a good correlation ($R^2 = 0.907$), with electrostatic and lipophilicity descriptors, as well as molecular refractivity, molecular weight and potential energy of bonded terms disabled. The reason for the outliers is not clear, and subject to ongoing research, that also includes the molecular

Table 3
Dataset for QSARs in CYP1A2 inhibitors

Cmpd	IC_{50} (mM)	log $1/IC_{50}E$[a]	log $1/IC_{50}P$[b]	std_dim3	TPSA	a_nO	a_acc	E_nb
A2	0.0009	3.046	2.334	0.432	17.07	1	1	310.29
A4	0.0110	1.959	1.842	0.822	35.53	3	1	706.87
A5	0.0210	1.678	1.839	0.669	17.07	1	1	746.37
A7	0.0104	1.983	2.273	0.538	17.07	1	1	344.92
A8	0.0026	2.585	2.554	0.491	23.55	1	1	413.26
A10	0.0034	2.469	2.492	0.460	17.07	1	1	303.64
B0	0.0456	1.341	1.744	0.334	57.53	3	3	263.25
B1	0.0389	1.410	1.173	0.961	75.99	3	5	677.95
B2	0.0366	1.437	1.520	0.713	17.07	1	1	1054.22
B12	0.0367	1.435	1.591	0.875	57.53	3	3	280.39
B13	0.0284	1.547	1.101	1.181	57.53	3	3	692.98
B14	0.0428	1.369	1.382	1.018	57.53	3	3	453.52
C1	0.0415	1.382	1.342	1.104	75.99	3	5	473.23
C2	0.0037	2.432	2.168	0.556	17.07	1	1	445.43
C3	0.0266	1.575	2.073	0.900	17.07	1	1	453.90
C6	0.0280	1.553	1.915	1.193	17.07	1	1	538.34
C7	0.0040	2.398	2.010	1.016	17.07	1	1	487.56
C10	0.0034	2.469	2.285	0.077	17.07	1	1	448.75
C11	0.0304	1.517	1.227	1.668	57.53	3	3	444.70
C15	0.0752	1.124	1.181	1.651	94.45	3	7	498.76

QSAR expressions: $n = 20$, $s = 0.356$, $R^2 = 0.682$, $F = 19.51$. log $1/IC_{50} = 3.249 - 0.252$std_dim3 $+ 0.052$TPSA $- 0.479$a_nO $- 0.904$a_acc $- 0.001$E_nb.

[a] Experimental IC_{50} value.

[b] Predicted IC_{50} value.

Table 4
Dataset for QSARs in CYP3A4 inhibitors

Cmpd	IC_{50} (mM)	log $1/IC_{50}E$[a]	log $1/IC_{50}P$[b]	VDistEq
B0	0.0051	2.292	2.125	3.492
B1	0.0649	1.188	1.473	3.628
B12	0.0735	1.134	1.157	3.694
B13	0.0776	1.110	1.166	3.693
B15	0.0383	1.417	1.157	3.694
C11	0.0403	1.395	1.540	3.614
C15	0.0132	1.119	1.027	3.722

QSAR expressions: $n = 7$, $s = 0.205$, $R^2 = 0.804$, $F = 20.39$. log $1/IC_{50} = 18.867 - 4.793$VDistEq.
[a] Experimental IC_{50} value.
[b] Predicted IC_{50} value.

features of the target site. Earlier QSAR studies have indicated that CYP activities are related to substrate lipophilicity [19–21], and these results lend some support to that suggestion. Substrates and inhibitors of CYP1A2 are usually planar small-volume molecules that are neutral or weakly basic. However, a proposal has been made that the binding pocket of CYP1A2 enzyme is composed of mostly hydrophobic and aromatic amino acids with polar amino acids for hydrogen bonding being present near the heme centre [22]. Thus it is possible that hydrogen bonding and hydrophobic interactions contribute to the observed inhibitory activities. The presence of an *ortho*- or *para*-hydroxyl group has been earlier shown to be relevant for the antioxidant activity of these and other curcumin analogues [9,23,24]. However, in contrast to these activities the most potent inhibitors of CYP1A2 lack the *para*-hydroxyl moiety. It is worth noting that CYP1A2 is known to be involved in the activation of procarcinogens [25] and consequently, that inhibition of CYP1A2 could be pharmacologically beneficial.

Seven curcumin analogues exhibited IC_{50} values towards CYP3A4 within the concentration range used in the experiments. Weak inhibitory activities were obtained, except in the cases of B0 and C12 where potencies of 5.1 ± 4.0 and 13.2 ± 3.0 μM were found. These activities are comparable with that determined for curcumin [15]. Five of these compounds belong to the cyclopentanone (B) group and the remaining two to the 1,4-pentadiene-3-one (C) group. No inhibition of CYP3A4 was observed for compounds from series A, which suggests that the presence of the central six-membered ring renders them less active towards CYP3A4. The QSAR equation found in the present study contained a distance matrix feature, VdisEq (Vertex distance equation), which suggests that the observed inhibition of CYP3A4 by the analogues was related to distance matrices of the compounds. The correlation found ($R^2 = 0.804$) was biased due to an outlier, resulting in an over-estimation of the outcome. Exclusion of the outlier resulted in no correlation between experimental and predicted inhibitory activities. Hydrophobicity of compounds has been reported to play an important role in oxidation of CYPs and binding to liver microsomes [26,27]. Thus, considering the hydrophobicity of curcumin analogues they would be expected to bind significantly to the hydrophobic pockets of the CYP3A4 active site. However, this was not observed in most cases perhaps due to other more significant factors, as shown in these results.

The present curcumin analogues generally exhibited weak inhibitory activities towards CYP2B6. IC_{50} values were obtained for only 4 compounds and were similar or weaker than that of curcumin, which is a weak inhibitor of CYP2B6 [15]. Compounds from the A series did not show any inhibitory activity towards CYP2B6. The QSAR analysis was considered unreliable, due to the small number of significant inhibitors. Evaluation of inhibitory potentials of curcumin analogues towards CYP2C9 resulted in 12 compounds most of which had lower inhibitory potencies than that reported for curcumin [15]. Among these compounds A0, B1 and C0 exhibited strong inhibitory activities (range 1.0–2.8 μM). Since all three series of compounds (A, B and C) are represented in this list, it appears that the absence or presence of the central five- or six-membered ring in the structure of the compounds is not influencing their inhibitory effect towards CYP2C9, but rather the aromatic ring substituents. B0 and C1, with similar substituents (OCH_3 and/or OH) as the strong inhibitors above (Schemes 1–3), are moderately strong inhibitors of CYP2C9 having IC_{50} values of 9.9 ± 0.79 and 7.5 ± 0.30 μM,

Table 5
Dataset for QSARs in CYP2C9 inhibitors

Cmpd	IC_{50} (mM)	log $1/IC_{50}E$[a]	log $1/IC_{50}P$[b]	P_V_FH	P_V_FP	S log P_VSA4
A0	0.0010	3.000	2.599	0.835	0.105	6.371
B0	0.0099	1.341	2.533	0.825	0.103	6.371
B1	0.0028	2.553	2.115	0.843	0.098	6.371
B11	0.0283	1.548	1.216	0.859	0.083	19.113
B12	0.0377	1.424	1.385	0.882	0.069	19.113
B14	0.0550	1.260	1.565	0.918	0.048	19.113
C0	0.0018	2.745	2.544	0.821	0.105	6.371
C1	0.0075	2.125	2.077	0.840	0.100	6.371
C2	0.0673	1.172	1.188	0.947	0.053	6.371
C10	0.0213	1.672	1.493	0.954	0.046	6.371
C11	0.0628	1.202	1.221	0.857	0.084	19.113
C15	0.0590	1.229	1.811	0.853	0.096	6.371

QSAR expressions: $n = 12$, $s = 0.329$, $R^2 = 0.730$, $F = 11.85$. P_V_FP, PEOE_VSA_FPNEG; P_V_FH, PEOE_VSA_ FHYD. log $1/IC_{50} = 53.151 - 92.429$P_V_FP $- 48.904$P_V_FH $- 0.118$S log P_VSA4.
[a] Experimental IC_{50} value.
[b] Predicted IC_{50} value.

Table 6
Dataset for QSARs in CYP2D6 inhibitors

Cmpd	IC_{50} (mM)	log $1/IC_{50}E$[a]	log $1/IC_{50}P$[b]	P_V_FP
B14	0.1187	0.926	0.911	0.048
C0	0.0020	2.699	2.497	0.105
C1	0.0006	3.222	2.358	0.100
C2	0.0685	1.164	1.050	0.053
C11	0.0132	1.879	1.913	0.084
C15	0.0827	1.082	2.247	0.096

QSAR expressions: $n = 6$, $s = 0.352$, $R^2 = 0.522$, $F = 27.88$. log $1/IC_{50} = -0.782 + 35.382$P_V_FP.
[a] Experimental IC_{50} value.
[b] Predicted IC_{50} value.

respectively. The present QSAR analysis suggested that the observed inhibitory activities are related to the descriptors, PEOE_VSA_FHYD, PEOE_VSA_FPNEG and S log P_VSA4. A weak correlation ($R^2 = 0.73$) was obtained between experimental and predicted data based on these descriptors. However, exclusion of the outlier B0 resulted in a better correlation with the same descriptors ($R^2 = 0.836$). The reason for this compound being an outlier is not clear. However electrostatic and hydrophobic interactions as well as lipophilicity are likely implicated in the observed inhibitory activities towards CYP2C9. Compound lipophilicity has been suggested to play a role in overall substrate binding affinity of CYPs and molecular modeling studies also indicated possible electrostatic interactions at the CYP2C9 active site due to the presence of basic amino acid residues [21,28]. Among others the relevance of hydrophobic interactions in the inhibition of CYP2C9 was also observed. Hydrophobic interactions are primary driving forces and contributors to binding affinity and specificity of a ligand to an active site [Ma et al., 2006 http://www.natural-selection.com/library/2006/NN_antihiv_ligand_gbf.pdf]. This factor is therefore a potential candidate for systematic modulation when developing SARs leading to more potent and specific inhibitors of CYP2C9. Furthermore, the presence of a hydroxyl substituent at the *para*-position appears to be prevalent in the strong inhibitors of CYP2C9 and that has been suggested to have similar implication for other biological activities in curcumin analogues [9].

Six curcumin analogues inhibited CYP2D6 with a wide range of IC_{50} values (0.6–111.7 μM), with C0 and C1 being the most potent. No inhibition of CYP2D6 was observed with compounds from the A series. Therefore, it appears that the presence of central six-membered ring in the A series contributes to the observed effect. The QSAR analysis of the CYP2D6 inhibitors revealed that the electrostatic descriptor, PEOE_VSA_FPNEG, relates best with CYP2D6 inhibition

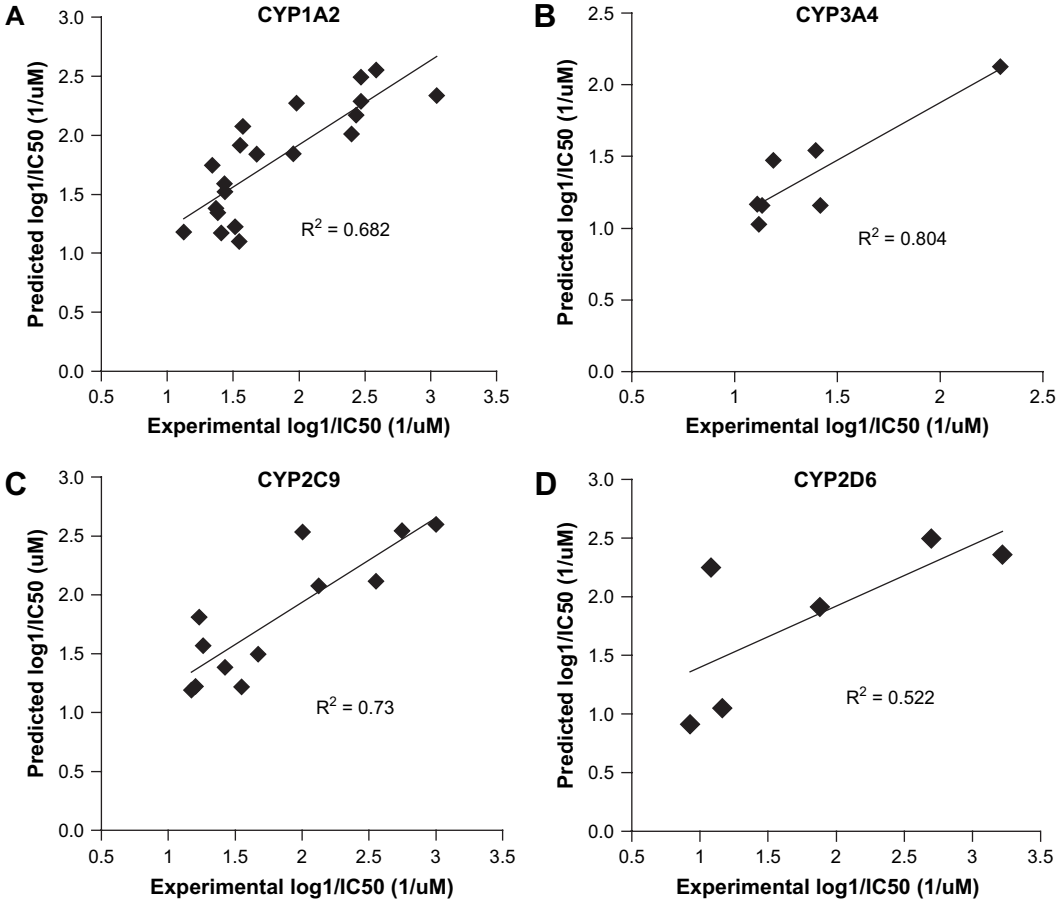


Fig. 3. Plots of experimental and predicted CYP-inhibitory activities (log $1/IC_{50}$). Equations for these plots are shown in Tables 3–6 (CYP1A2, CYP3A4, CYP2C9 and CYP2D6, respectively) and the compounds involved are listed in the tables.

resulting in a weak correlation ($R^2 = 0.522$) between experimental and predicted activities. However removal of the outlier, C15 resulted in a good correlation with the same descriptor. The reappearance of the electrostatic parameter indicates that the inhibitory potencies of curcumin analogues towards CYPs is possibly related to the fraction of polar negative van der Waals surface area present in the compounds. A limitation to our QSAR analysis however, is the small number of compounds especially in case of CYP2D6. Previous SAR studies on analogue series of stereoisomers of quinidine and quinine suggested that hydrogen bonding by the hydroxyl group and not the basic nitrogen interaction with an active site residue would control the inhibitory potency of quinidine [29]. It is likely that the presence of the hydroxyl substituent at the *para*-position will contribute to the observed inhibitory activity towards CYP2D6, since it is present in five of the six CYP2D6 inhibitors. It is interesting to note that compound B15, which has *meta*-methoxy and *para*-hydroxyl substituents quite similar to curcumin and has been shown to also have over 10 times greater antioxidant activity than the latter [9] did not exhibit any significant inhibitory activities towards the five CYPs tested.

Similarity studies indicated no significant differences between the ring structures of the three series of compounds (Fig. 2), although differences may still lie in steric bulkiness of substituents. Compound C0 clearly showed more flexibility than A0 and B0, and possessed the lowest minimal potential energy in each conformation as determined by a stochastic conformational search. This is to be attributed to the absence of the central aliphatic ring occurring in the other compounds. Clearly the results of flexible alignments are unable to explain the observed activities of the compounds, but we consider the similarity studies as a useful approach in comparing curcumin analogues and other structurally related compounds.

Experiments on the stability of curcumin analogues in buffer (pH 7.4) resulted in varying degrees of degradation of the compounds. Generally compounds of group A were more susceptible to degradation than those of groups B and C. The instability of group A compounds may be attributed to the central six-membered ring since that is the basic difference between the three series of compounds. Compounds in series B appeared to be more stable in the buffer. The analogues were more stable in buffer than curcumin, except A2, A7, C6 and C7 which were equally or less stable than curcumin with A2 being the most unstable and B14 the most stable. Instability of group A compounds at neutral to basic pH, may be due to aromatization of the central six-membered ring in the presence of slightly basic conditions and subsequent dissociation of resulting single bonds. Previous studies however, clearly demonstrated that the degradation of curcumin in buffer at neutral to basic pH could be blocked by the incorporation of enzyme, GSH, NAC or ascorbic acid in the incubations [7]. The present results obtained indicate that like curcumin, degradation of its analogues in buffer at pH 7.4 is also significantly blocked (90%) in the presence of enzymes.

4. Conclusion

Thirty-three curcumin analogues were investigated for inhibition of human recombinant CYP1A2, CYP3A4, CYP2B6, CYP2C9 and CYP2D6. Most of the curcumin analogues showed low or negligible activities towards the CYPs tested. Six of the compounds (A2, A8, A10, C2, C7 and C10) showed strong inhibitory activities towards CYP1A2, while one compound (B0) strongly inhibited CYP3A4. CYP2C9 and CYP2D6 were strongly inhibited by three (A0, B1, C0) and two (C0 and C1) compounds, respectively. The MOE-based QSAR analyses suggest that electrostatic and/or hydrophobic descriptors notably PEOE_VSA_FPNEG and PEOE_VSA_FHYD, are important factors of the compounds relating to inhibition of CYP1A2, CYP2C9 and CYP2D6. Consideration of these (Q)SAR results might be relevant in the optimization of curcumin analogues with less potential to cause CYP-mediated drug–drug interactions.

5. Experimental protocols

5.1. Materials

Methoxyresorufin (MRes) and benzyloxyresorufin (BRes) were synthesized by the method of Burke et al. [30], and the purity was determined by HPLC, mass spectrometry and ^{1}H NMR. The plasmid, pSP19T7LT_2D6, containing human CYP2D6 bicistronically co-expressed with human cytochrome P450 NADPH reductase was kindly provided by Prof. M. Ingelman-Sundberg (Stockholm, Sweden). The plasmids, BMX100/h1A2 and pCWh3A4 with human cytochrome P450 NADPH reductase were kindly donated by Dr. M. Kranendonk (Lisbon, Portugal). Expression plasmids, pCWh2B6hNPR and pCWh2C9hNPR with human cytochrome P450 NADPH reductase were kindly provided by Prof. F.P. Guengerich (Nashville, Texas, USA). Curcumin analogues were kindly donated by Dr. S. Sardjiman (Jakarta, Indonesia). All other chemicals were of analytical grade and obtained from standard suppliers.

5.2. CYP expression and membrane isolation

The plasmids containing cDNA of five human CYPs were transformed into Escherichia coli strain JM109. Expression of the CYPs was carried out in 3-litre flasks containing 300 ml terrific broth (TB) medium, with 1 mM δ-aminolevulinic acid, 0.5 mM thiamine, 400 μl/l trace elements, 100 μg/ml ampicillin, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.5 mM $FeCl_3$ (for CYP2D6 and CYP3A4 only), 1 mg/l chloramphenicol (for CYP2B6 only) and 30 μg/ml kanamycin (for CYP3A4 only). The culture media were inoculated with 3 ml overnight cultures of bacteria containing plasmids for the various CYPs. The cell cultures were incubated for about 40 h at 28 °C and 125 rpm, and CYP contents were determined using the carbon monoxide (CO) difference spectra as described by Omura and Sato [31]. Cells were pelleted by centrifugation

(4000*g*, 4 °C, 15 min) and resuspended in 30 ml Tris–Sucrose–EDTA (TSE) buffer (50 mM Tris–acetate buffer pH 7.6, 250 mM sucrose, 0.25 mM EDTA). Cells were treated with 0.5 mg/ml lysozyme prior to disruption by French press (1000 psi, 3 repeats). The membranes containing the human CYPs were isolated by ultracentrifugation in a Beckmann 50.2Ti rotor (60 min, 40,000 rpm, 4 °C), resuspended in TSE buffer and stored at −80 °C until use.

5.3. Stability of curcumin analogues in buffer

Decomposition of curcumin analogues was investigated as described [7], in 0.1 M potassium phosphate buffer of pH 7.4. Solutions of 25 μM curcumin analogues in buffer (in 0.5% DMSO) were scanned every 5 min between 200 and 600 nm for 30 min using an Ultrospec 2000 Pharmacia Biotech UV/visible spectrophotometer. The above experiment was also performed in the presence of 1 mM GSH and 13.2 nM enzyme (CYP) as described [7], to determine the effects of these factors on the stability of the analogues in buffer.

5.4. CYP inhibition assays

5.4.1. 7-Methoxy-, 7-benzyloxyresorufin and O-dealkylation

Inhibition of the activities of human CYP isoforms 1A2, 3A4 and 2B6, by curcumin analogues was determined by microplate reader assays using fluorescent substrates. Incubation conditions (eg. enzyme concentration, substrates, incubation time) and wavelengths for detection for each of the inhibition assays are shown in Table 7. In general, the incubations were carried out in a total volume of 200 μl, and in the presence of 100 μM NADPH (freshly prepared) in a black coaster 96-well plate. Membranes were preincubated for 5 min at 37 °C with 0.1 M potassium phosphate buffer (pH 7.4), substrates, and inhibitors (curcumin analogues) with minimal use of DMSO, i.e. always 1.0% (v/v) or less. Experiments were performed in the absence and presence of GSH, to determine the influence of a second curcumin stabilizing factor on the experiments. Subsequently, the analogues (100 μM each) were all screened for CYP-inhibitory activity. The screening procedure for all CYPs involved pooling compounds into groups of three or four and testing for CYP inhibition of the mixture. Groups showing >20% inhibition were selected for further screening, for identification of potential CYP inhibitors.

Determination of IC_{50} values for curcumin analogues showing over 20% inhibition of CYP activities was performed. The concentration range of the curcumin analogues used was from 0.195 to 100 μM. Incubations were started by the addition of 100 μM NADPH, and maintained at 37 °C for the periods defined (Table 7). Reactions were terminated with 75 μl of 80% acetonitrile and 20% 0.5 M Tris solution. Product formation was linear for all incubation times. Concentrations of the probe substrates in all reaction mixtures were chosen near the Michaelis–Menten's constant (K_m) value for each of the CYPs tested. The K_m values obtained using the alkoxyresorufins and all other substrates were within the range of reported literature values [32]. All measurements were performed in triplicate. Metabolite formation was measured spectrophotometrically on a Victor^2 1420 multilabel counter.

Table 7
Experimental conditions for fluorescence CYP assays

CYP	Enzyme amount (nM)	Incubation time (min)	Substrate	Substrate concn (μM)	Excitation wavelength (nm)	Emission wavelength (nm)
1A2	13.2	10	MRes	5.0	530	586
3A4	14.3	30	BRes	5.0	530	586
2B6	15.3	30	BRes	20.0	530	586

MRes, methoxyresorufin; BRes, benzyloxyresorufin.

5.4.2. Diclofenac hydroxylation

For the CYP2C9 inhibition assay, reaction mixtures in 500 μl total volume consisted of 49 nM enzyme, 100 μM NADPH, 0.1 M potassium phosphate buffer (pH 7.4), 6 μM diclofenac and inhibitor. Each of the analogues (100 μM) was screened for CYP2C9 inhibitory activity. For IC_{50} determinations on curcumin analogues with >20% inhibition, concentrations of analogues used were of the range 0.39–200 μM. After preincubation for 5 min at 37 °C, reactions were initiated by adding NADPH and terminated after 10 min upon the addition of 200 μl methanol. The reaction mixtures were centrifuged at 14,000 rpm for 3 min. Product formed was measured using an isocratic HPLC method [33]. A C18 column (150 mm × 3.2 mm, 5 μm particle size, Phenomenex) was used and the carrier flow rate was 0.6 ml/min. The mobile phase consisted of 60% (v/v) 20 mM potassium phosphate buffer (pH 7.4), 22.5% (v/v) methanol and 17.5% (v/v) acetonitrile. Peaks were monitored at the wavelength of 280 nm. Retention times for 4-hydroxydiclofenac and diclofenac were 5.0 and 24.1 min, respectively.

5.4.3. Dextromethorphan O-demethylation

Inhibition of CYP2D6 activity by curcumin and its decomposition products was evaluated by a method described [34]. The reaction mixture had a total volume of 500 μl and consisted of 18.2 nM enzyme, 4.5 μM dextromethorphan, 90.9 μM NADPH, 0.1 M potassium phosphate buffer and curcumin analogues. Each of the analogues (100 μM) was screened for CYP2D6 inhibitory activity, and IC_{50} was determined for analogues showing >20% inhibition (concentration range 0.39–200). Reactions were initiated by the addition of NADPH and allowed to proceed for 45 min before termination with the addition of 60 mM zinc sulphate solution. Product formed was measured using an isocratic HPLC fluorescence detection method and a C18 column (100 mm × 3 mm, 5 μm particle size, Chromspher). The mobile phase consisted of 24% (v/v) acetonitrile and 0.1% (v/v) triethylamine adjusted to pH 3 with perchloric acid. The carrier flow rate was 0.6 ml/min. Peaks were monitored at 280 nm (excitation) and 310 nm (emission). The retention times of dextrorphan and dextromethorphan were 3.4 and 24.5 min, respectively.

Table 8
List of all molecular descriptors used in this study, obtained from QuaSAR-Descriptor MOE 2005.06 version

No.	Descriptor	Description
1	a_acc	Number of hydrogen bonding atoms
2	a_nO	Number of oxygen atoms
3	E_nb	Value of potential energy with all bonded terms disabled
4	PEOE_VSA_FHYD	Fractional hydrophobic van der Waals surface area
5	PEOE_VSA_FPNEG	Fractional negative polar van der Waals surface area
6	PEOE_VSA_FPPOS	Fractional positive van der Waals surface area
7	S log P_VSA0	VSA with sum of surface area vi such that contribution of log of octanol/water partition coefficient calculated from the given structure Li is −0.4
8	S log P_VSA4	(See 7) Sum of vi such that Li is in 0.1, 0.15
9	SMR_VSA5	Molecular refractivity/VSA, sum of vi such that molecular refractivity from atom I, Ri is in 0.44, 0.485
10	Std_dim2	Square root of second largest eigenvalue of covariance matrix of the atomic coordinates
11	Std_dim3	Square root of the third largest eigenvalue of covariance matrix of the atomic coordinate
12	TPSA	Total polar surface area
13	VdistEq	Sum of $\log_2 m - p_i/m$ where m and p_i are sum and number of distance matrix entries, respectively
14	Weight	Molecular weight

5.4.4. SAR and QSAR analysis

Percent inhibition of CYP activities by the curcumin, analogues was calculated from the ratios of the activities of inhibited to control samples. The IC_{50} values were calculated using GraphPad Prism 4.0 version (GraphPad Prism software Inc. San Diego CA). Subsequent Quantitative structure–activity relationship (QSAR) analysis of the respective CYP-inhibitory activities of the curcumin analogues were performed using the MOE software (Version 2005.06, Chemical Computing Group Inc, Montreal). Structural descriptors were obtained from QuaSAR-Descriptor in MOE 2005.06 version. One hundred and thirty seven descriptors, including both 2D and 3D molecular descriptors were calculated using the MOE program. Structure–activity models were generated by multiple stepwise regression analysis (MRA) of the biological and structural variables using the statistical analysis software SPSS for Windows version 13. Fischer coefficients (F values) were also calculated using the latter. Table 8 provides information on MOE-selected descriptors used in this study.

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