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Relationship between cucurbitacins reversed-phase high-performance liquid chromatography hydrophobicity index and basal cytotoxicity on HepG2 cells

Judit Bartalis, Fathi T. Halaweish*

Department of Chemistry and Biochemistry, South Dakota State University, P.O. Box 2202, Brookings, SD 57007, USA

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Abstract

Drug development of cucurbitacins requires derivatives that have lower cytotoxicity. Therefore, the effect of structural modification on in vitro cytotoxicity has been investigated. Lipophilicity or chromatographic hydrophobicity index (CHI) was chosen as molecular property. CHI was determined by RP-HPLC in both aqueous acetonitrile and aqueous methanol. Compounds CHI range was wide and better defined in acetonitrile (CHI_{ACN} = 46–88 and 38–102) than in methanol (CHI_{MeOH} = 56–78). Higher resolution was achieved in acetonitrile, and higher precision on the shorter C18 column. Cucurbitacins cytotoxicity (IC_{50}) was measured on the hepatocyte-derived HepG2 cells. Strong relationship between CHI and logarithmic IC_{50} was found. As a result, cytotoxicity increased linearly with increasing hydrophobicity ($r \geq 0.90$). Other lipophilicity parameters, such as $\log P$ and $C \log P$ were also estimated. Cytotoxicity correlated well with $\log P$ ($r = 0.95$) and slightly with $C \log P$ ($r = 0.74$). The $\log P$ and $C \log P$ data showed good correlation with CHI ($r > 0.92$). Overall, alkylation of C1 hydroxyl, unsaturation of C1—C2 bond, and acetylation of C25 hydroxyl increased both lipophilicity and cytotoxicity. This assay should prove useful for monitoring cucurbitacin homologues or other drug candidates for their cytotoxicity.
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Keywords: Cucurbitacin; Chromatographic hydrophobicity index; CHI; HepG2; IC50; Cytotoxicity; Structure-activity relationship; SAR; $\log P$; $C \log P$; RP-HPLC

1. Introduction

Plants secondary metabolites represent tremendous resources for scientific and clinical researches as well as for new drug development [1]. Cucurbitacins are particularly known in folk medicine for their strong purgative, anti-inflammatory, and hepatoprotective activities [1,2]. They are positioned on the top of the NCI list as potential antitumor agents in various tumor subpanels [3,5]. However, cucurbitacins strong biological activity was found to be very close to their toxic dose, which renders them unlikely biological agents [6]. On the other side, methylation of the enolic hydroxyl (a.k.a. diosphenol) of cucurbitacin E enhanced the antitumor activity and lowered the toxicity on mice [7–9].

Lipophilicity is one of the major factors that influences the transport, absorption, and distribution of chemicals in biological systems, and it is a predominant descriptor of the pharmacodynamic, pharmacokinetic and toxic aspects of drug activities in quantitative structure-activity relationship (QSAR) studies [10–13]. In the 1960s Hansch's octanol–water partition coefficient P_{oct} ($P_{oct} = C_{oct}/C_{water}$; C: analyte concentration) became the standard parameter to measure lipophilicity for both experimental and theoretical investigations [14]. The octanol–water partition coefficients can be obtained from other solvent systems, with certain restrictions, by applying Collander's [15] equation: $\log P_1 = a \log P_2 + b$. RP-HPLC has been long recognized as a potential method for lipophilicity determination, where

* Corresponding author. Tel.: +1 605 688 4269; fax: +1 605 688 6364.
E-mail address: fathi.halaweish@sdstate.edu (F.T. Halaweish).

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mainly hydrophobic forces dominate the retention process [16–20]. Moreover, the mobile phase/stationary phase interface models better the biological partitioning processes than the solute partitioning in the bulk octanol/water phase [21]. The chromatographic retention data is a linear free-energy related parameter and it is a more reliable descriptor in QSAR than the estimated or calculated hydrophobic, electronic and/or steric parameters [22]. Chromatographic hydrophobicity index, CHI, is deduced from the retention data and reflects not only the lipophilicity of the compound but it approximates the concentration of organic phase required achieving an equal distribution of analyte between the mobile phase and stationary phase. Thus, hydrophobicity index is a useful tool in method development [23].

Drug development would require analogues that retains or enhances the natural cucurbitacins biological activity and reduces toxicity. We choose HepG2 cell line for our in vitro study, because it is one of the best human cell lines to predict basal human cytotoxicity [24–26].

This work presents a precise and reliable technique to study the effect of structural modification on cucurbitacins cytotoxicity. The basal cytotoxicity of seventeen cucurbitacin analogues was monitored on HepG2 cells, and their hydrophobicity calculated in different ways. The lipophilic parameters are the CHI, measured by RP-HPLC, and $\log P$ and $C\log P$ estimated with ALOGPS software. In order to have a larger number of compounds, some cucurbitacins were isolated from plants and others generated by alkylation and acetylation of enolic analogues. Cucurbitacins drug development requires derivatives with low cytotoxicity, and correlation of lipophilicity with in vitro toxicity may lead to important conclusions regarding this issue.

2. Experimental

2.1. Extraction, isolation, and identification

Ripe fruits of *Cucurbita texana* (Cucurbitaceae) were received from Dr. D.W. Tallamy (University of Delaware, Newark, Delaware). The fruits were cut and homogenized with methanol (MeOH), filtered, and the solvent removed under reduced pressure. The residue was subjected to flash column chromatography (silica gel G60) with gradient elution (hexane/ethyl acetate and then ethyl acetate/MeOH of increasing polarity) [27] and the fractions were screened using NP-TLC (silica gel, UV_{254}, 250 μm layer). TLC plates were developed with toluene:ethyl acetate 40:60 solvent mixture, and visualized for the $\Delta^{23,24}$ cucurbitacins with vanillin/orthophosphoric acid or for the diosphenols with $FeCl_3$ solution [28]. Fractions were further separated using preparative NP-TLC (silica gel, UV_{254}, 2 mm layer) under similar developing conditions to the analytical TLC and bands were visualized with UV light. Cucurbitacins ^{13}C and ^{1}H NMR spectra (Bruker 400 MHz) were recorded in $CDCl_3$ and compared to published data [29–34]. Additional amount of cucurbitacin glycosides were isolated by preparative HPLC from the concentrate of *Citrullus lanatus* (Cucurbitaceae) (Florida Food Products, Eustis, FL).

2.2. HPLC separation

We used Dynamax liquid chromatograph (Varian Chromatography Systems) with PDA-2 photodiode array UV detector, controlled by the Dynamax PC Chromatography Data System (v. 1.9) software. Dynamax dual pump solvent delivery system, model SD-200. Cucurbitacins final purification and separation was conducted on Econosil C18 (Alltech; 250 mm × 22 mm, 10 μm) preparative column at flow rate of 13.00 ml/min, and at gradient elution in acetonitrile (Pharmco, Brookfield, CT; 20–55% in 50 min), or MeOH (Pharmco; 60–75% in 50 min). Cucurbitacins analytical separation was optimized on Alltima C18 (Alltech; 250 mm × 4.6 mm, 5 μm) HPLC column [4,35,36], at gradient elution in acetonitrile (30–70% ACN in 57 min), and in MeOH (60–75% MeOH in 50 min). Cucurbitacins stock concentration of 10^{-2} M in DMSO:ethanol (1:1) was standardized against pure cucurbitacin I (Indofine Chemical Company, Hillsborough, NJ) by analytical HPLC means. Compounds CHI was measured in both ACN, by using Alltima C18 column, and in MeOH, by using Econosil C18 column (Alltech; 150 mm × 4.6 mm, 5 μm). Analytical separations were conducted at a flow rate of 1 ml/min. The aqueous phase was buffered for the CHI measurement. For this purpose, solid ammonium acetate (Fisher Sci. Co., Fair Lawn, NJ) was dissolved in deionized distilled water at 50 mM final concentration and its pH adjusted to 7.0.

2.3. Chromatographic hydrophobicity index

2.3.1. CHI measurement in ACN

All standard compounds were purchased from Acros (Acros Organics, NJ). The chromatographic lipophilicity or hydrophobicity was determined applying Valkó's technique [37]. A standard mixture of seven compounds was prepared in solution: theophylline (**19**), benzimidazole (**20**), acetophenone (**21**), indole (**22**), propiophenone (**23**), butyrophenone (**24**), and valerophenone (**25**). In the first approach, the mixture of compounds **19**–**25**, dissolved in water:ACN (1:1), was injected at isocratic elution of 40, 45, 50, 55, and 60% ACN. The retention factor, $\log k = \log((t_R - t_0)/t_0)$, was calculated for each analyte from five good injections of 10 μl sample. The dead time (t_0) was measured by injecting $NaNO_3$ together with the sample. Then, the $\log k$ values were plot against isocratic ACN concentrations to establish the linear regression equations for each analyte. From each straight line the isocratic hydrophobicity index was computed, $\varphi_0 = (-\text{intercept/slope})$. Further, the calibration mixture was injected at fast gradient elution, 0–22 min 0–100% ACN, and three additional minutes at 100% ACN. The φ_0 values for the test compounds were plot against gradient retention

time and the linear equation determined from the following equation:

$$\varphi_0 = \text{CHI} = At_R + B \quad (1)$$

A mixture of 18 cucurbitacin analogues was injected under similar gradient elution and from the peaks retention time their CHI values were deduced applying Eq. (1). In the second approach, Eq. (1) was generated from the correlation between the published CHI values [37] and the fast gradient elution of compounds **19–25**, colchicine (**26**), and phenyltheophylline (**27**). The gradient elution conditions were similar to the one from the first approach.

2.4. CHI measurement in MeOH

A standard mixture of 10 compounds including **19–21**, **23–27**, aniline (**28**) and bromobenzene (**29**), dissolved in MeOH, was injected at five isochratic elution, at 40, 45, 50, 55, and 60% MeOH. Then the mixture was injected at fast gradient elution to establish the correlation from Eq. (1). The fast linear gradient elution was optimized for 30–100% MeOH in aqueous buffer with 10 min runtime.

2.5. Structural modification

2.5.1. Alkylation

The C2 hydroxyl of enolic analogues, such as cucurbitacin E cucurbitacin I, was alkylated by the Williamson ether synthesis [38]. Pure cucurbitacin (2 mg) and freshly dried anhydrous K_2CO_3 (3 g) were mixed and refluxed in acetone under N_2 with continuous stirring for 3 days. During this period, two portions of alkyl iodide, or RI (R: Me-, Et-, *i*Pr-, or *n*Pr-; 50 ml) were added at 24 h intervals. The solution was filtered and the salt washed twice with acetone. The combined filtrate and washings was evaporated under air and the residue further purified by preparative RP-HPLC.

2.5.2. Acetylation

Cucurbitacin E-Me ether (2 mg) was acetylated at C16 position overnight at room temperature in dry pyridine (5 ml) and acetic anhydride (5 ml) [38]. The mixture was decomposed with cold water and the product extracted in methylene chloride, then evaporated and further purified by preparative RP-HPLC.

2.6. Enzymatic hydrolysis

Additional amount of aglycons were generated by the enzymatic hydrolysis of saponins cucurbitacin E β-glucoside and I β-glucoside, using β-glucosidase enzyme (Worthington, Lakewood, NJ). A ratio of 1:4 saponin to enzyme was suspended in acetate buffer at pH 5 and stirred continuously under N_2 for 3 days in a water bath, at 37 °C [39]. Half portion of enzyme was added to the mixture after 2 days of stirring.

2.7. Cell culture and induction of toxicity

HepG2 (human hepatocellular carcinoma, ATCC) cells were grown in EMEM (Gibco, Grand Island, NY) supplemented with 10% FBS, and 1% penicillin/fungizone mixture (Gibco). Thabrew's [40] optimized procedure was followed. Cells were batch cultured for 10 days, then seeded at concentration of 30,000 cells/well in fresh media in 96-well microtiter plastic plates at 37 °C for a day. Then cells were exposed to different concentrations of cucurbitacins at final volume of 100 μl/well. Five-fold serial dilution of compounds was carried out in the plate for five consecutive wells. After 24 h of incubation with chemicals, live cells were visualized by the MTT assay (Promega, Madison, WI). The absorbance was measured at 570 nm. Negative (without cells) and positive (without test chemicals) controls were also incubated with each plate. The endpoint was determined from the exponential curve of viability versus concentration as IC_{50}, which represents the concentration of compound that kills 50% of the cells. At least three reproducible experiments were performed per compound with three replicate wells per concentration.

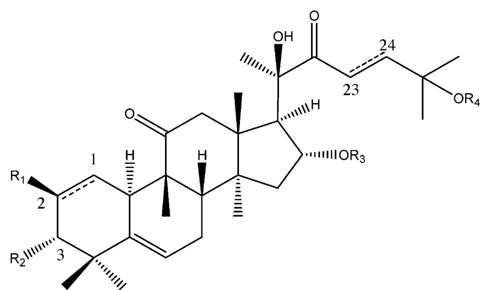
2.8. Calculations

The estimated $\log P$ and $C\log P$ octanol/water partition coefficients for cucurbitacins were obtained by means of the on-line software ALOGPS v. 2.1 (Virtual Computational Chemistry Laboratory, www.vcclab.org). The $\log P$ calculation is based on the neural network ensemble analysis, where the molecular structure was represented by the electrotopological state indices and the number of hydrogen and non-hydrogen atoms [41]. The $C\log P$ partition coefficient is based on the fragmentation principle developed by Leo et al. [42]. The CLOGP program version 4.0 uses improved $C\log P$ calculation theory [43] and it is running under evaluation license of BioByte Corporation [44].

The data analysis was carried out using the Microsoft™ Excel 2000 software package. The correlation coefficient "r", F-test, and t-test were the basis for testing the significance of fitting quality. In addition, the S/O was introduced as a specific fitting error. It represents the ration of standard error and range of observation. The statistical residual variance RV was considered in assessment of the prediction error. RV is the ratio of prediction sum of squares (PRESS) and the total number of data n, and PRESS is:

$$\text{PRESS} = \sum_{j=1}^{n} (\text{Obs}_j - \text{Pred}_j)^2 \quad (2)$$

where Obs_j and Pred_j are the collected and predicted values. High quality models should give S/O and RV values close to zero.



No.	Cucurbitacin	R_1	R_2	R_3	R_4	Other
1	I Gluc[a, b]	Glu	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
2	E Gluc[a, b]	Glu	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
3	D[b]	OH	=O	H	H	$\Delta^{23,24}$
4	iso-D[b]	=O	OH	H	H	$\Delta^{23,24}$
5	I [b]	OH	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
6	I-Me[c]	O-Me	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
7	L-Me[c]	O-Me	=O	H	H	$\Delta^{1,2}$
8	I-Et[c]	O-Et	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
9	B[b]	OH	=O	H	Ac	$\Delta^{23,24}$
10	iso-B[b]	=O	OH	H	Ac	$\Delta^{23,24}$
11	I-iPr[c]	O-iPr	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
12	I-nPr[c]	O-nPr	=O	H	H	$\Delta^{1,2}$, $\Delta^{23,24}$
13	E[b]	OH	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
14	E-Me[c]	O-Me	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
15	E-Et[c]	O-Et	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
16	E-iPr[c]	O-iPr	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
17	E-Me-Ac[c]	O-Me	=O	Ac	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$
18	E-nPr[c]	O-nPr	=O	H	Ac	$\Delta^{1,2}$, $\Delta^{23,24}$

[a] β-D-glucopyranose; [b] isolated from plants; [c] generated by semi-synthesis

Scheme 1. Cucurbitacins used for the assay.

3. Results and discussion

Cucurbitacin analogues were isolated from *C. texana* and *C. lanatus*, and diosphenols **5** and **13** were further modified by alkylation and esterification (Scheme 1). We generated alkyloxy derivatives to follow up earlier studies [7,8] that demonstrated five times lower toxicity for cucurbitacin E-Me ether in Swiss mice than for the parent compound cucurbitacin E. The alkylation of compounds **5** and **13** and acetylation of **14** yielded 100% the product. On the other hand, methylation of a mixture containing non-separable cucurbitacins I and L generated only L-Me ether. The enzymatic hydrolysis of **1** and **2** yielded 35% of cucurbitacin I and 100% of cucurbitacin E, respectively; the transformation was not complete for **1** even though both **1** and **2** have β-glucosidic bond. Several attempts have been made to methylate the C2 hydroxyl of cucurbitacin B. Unfortunately, alkylation in the presence of a strong base (NaH, THF, RI, 50 °C) [45] or reaction with diazomethane (freshly prepared CH_2N_2, HBF_4, CH_2Cl_2, 0 °C) [46], destroyed the functional groups.

^{1}H and ^{13}C NMR data of the isolated and modified cucurbitacins matched the published data [29–33]. The new carbon

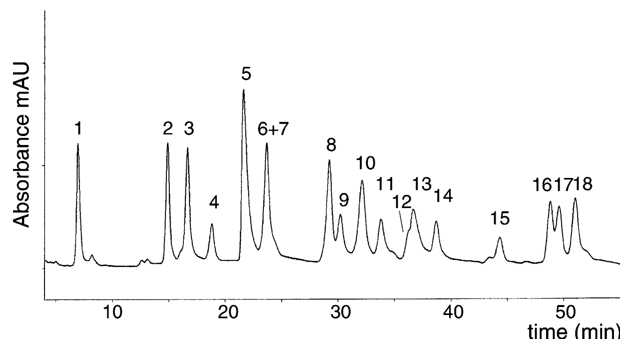


Fig. 1. Cucurbitacins HPLC separation on Alltima C18 (250 mm × 4.6 mm, 5 μm). Conditions: 30–70% ACN in water in 57 min, flow rate 1 ml/min.

shifts for the semi-synthesized compounds were identified, for the R_1 side chain: 55.0 ppm (CH_3–O) for compounds **6–8** and **17**; 14.4 ppm (CH_3) and 63.4 ppm (CH_2–O) for **8** and **14**; 21.5 ppm (CH_3) and 70.3 ppm (CH–O) for **11** and **16**; 10.4 ppm (CH_3), 22.1 ppm (CH_2), and 69.4 ppm (CH_2–O) for **12** and **18**. The R_3 group ^{13}C–NMR shift of **17** was found at 19.9 ppm (CH_3) and 169.8 ppm (C=O).

The RP-HPLC separation of cucurbitacin analogues was conducted in both aqueous ACN and MeOH. Chromatograms are illustrated in Figs. 1 and 2, where peaks are numbered following the order in Scheme 1. Higher resolution was achieved in ACN than in MeOH organic phase. Interestingly enough, Alltima C18 HPLC column showed different selectivity toward the C25–OH derivatives **8**, **11**, and **12** in the two organic phase. Methanol is a good proton acceptor and tends to interact with hydroxylated molecules [47]. This would suggest that compounds **8**, **11**, and **12**, with an extra hydroxyl group relative to other derivatives, would elute faster in MeOH relative to ACN, contrary to what was actually happening. We can explain it with the fact that there are some complex interactions taking place between the solute and stationary phase. Abraham quantified these interactions [48], and Valkó tailored Abraham's equation for various organic phases finding that both solute dipolarity and hydrogen-bond acidity had weaker influence over solute elution in methanol than in acetonitrile [49].

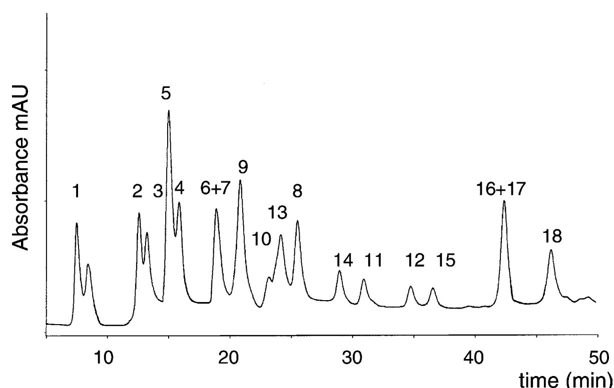


Fig. 2. Cucurbitacins HPLC separation on Alltima C18 (250 mm × 4.6 mm, 5 μm). Conditions: 60–75% MeOH in water in 50 min, flow rate 1 ml/min.

Table 1
Linear equations and statistical data for the standard compounds and cucurbitacins[a]

Number	Compounds[b]	Equation	r	S/O	RV
CHI vs. t_R					
2	$n=7$ (**19**–**25**)	$CHI_{ACN1}=\varphi_{0\,ACN}=3.983t_R-5.473$	0.962	0.13	25.79
3	$n=9$ (**19**–**27**)	$CHI_{ACN2}=6.172t_R-42.993$	0.998	0.03	2.68
4	$n=10$ (**19**–**21**, **23**–**29**)	$CHI_{MeOH}=\varphi_{0\,MeOH}=6.951t_R+2.046$	0.996	0.03	1.84
$C\log P$ vs. CHI and biological data					
5	$n=18$ (**1**–**18**)	$CHI_{ACN2}=21.495C\log P+6.290$	0.927	0.11	44.11
6	$n=18$ (**1**–**18**)	$CHI_{MeOH}=7.252C\log P+45.304$	0.959	0.08	2.69
7	$n=17$ (**1**–**9**, **11**–**18**)	$\log IC_{50}=-0.553C\log P+3.052$	0.742	0.21	0.15
$\log P$ vs. CHI and biological data					
9	$n=18$ (**1**–**18**)	$CHI_{ACN2}=20.769\log P-3.985$	0.920	0.11	47.99
10	$n=18$ (**1**–**18**)	$CHI_{MeOH}=6.896\log P+42.261$	0.937	0.10	4.08
11	$n=17$ (**1**–**9**, **11**–**18**)	$\log IC_{50}=-0.688\log P+3.905$	0.948	0.10	0.03
$\log IC_{50}$ vs. CHI					
12	$n=17$ (**1**–**9**, **11**–**18**)	$\log IC_{50}=-0.026CHI_{ACN2}+3.264$	0.824	0.17	0.11
13	$n=17$ (**1**–**9**, **11**–**18**)	$\log IC_{50}=-0.083CHI_{MeOH}+6.996$	0.847	0.16	0.10

[a] All equations show $\alpha<0.01$ for the F- and t-test.
[b] Compounds identification number is indicated in parentheses.

Cucurbitacins lipophilicity was measured by RP-HPLC. The selectivity differences in the two organic phase prompted us to measure CHI in both ACN and MeOH organic phase. Due to its high viscosity, aqueous MeOH required a shorter column than the one applied for ACN. First, the C18 columns were calibrated against a standard mixture, and the relationships established between the fast gradient t_R and φ_0 or published CHI (see Eqs. (2)–(4) from Table 1). The CHI of the standard compounds is listed in Table 2. Second, cucurbitacins were injected at fast gradient elution under similar conditions, and their CHI calculated (Table 3) from Eqs. (2)–(4). Eqs. (2) and (4) involve the isochratic hydrophobicity index, φ_0, while Eq. (3) employs the earlier established gradient CHI in buffered ACN [37]. Faster gradient elution did not improve statistically Eqs. (2)–(4). The fitting quality and predictive power of Eq. (3) (CHI_{ACN2}) and Eq. (4) (CHI_{MeOH}) are relatively high, while the predictive power of Eq. (2) (CHI_{ACN1}) is lower, therefore the latest equation was not included in the QSAR studies. The CHI_{ACN2} and CHI_{MeOH} data correlated well with one another ($n=18$, $r=0.979$). Furthermore, the $\log P$ and $C\log P$ of cucurbitacins were calculated using ALOGPS program (Table 3).

Table 2
Standard mixtures chromatographic hydrophobicity indexes in buffered acetonitrile and methanol using three different approaches[a]

Standard compound	CHI_{ACN}[b]	CHI_{ACN}[c]	CHI_{MeOH}[b]
Theophylline (**19**)	32.63 ± 0.07	15.76	25.76 ± 0.05
Aniline (**28**)	–	–	29.94 ± 0.05
Benzimidazole (**20**)	43.18 ± 0.12	30.71	41.07 ± 0.04
Acetophenone (**21**)	61.93 ± 0.05	64.90	52.47 ± 0.05
Colchicine (**26**)	–	41.37	57.56 ± 0.04
Indole (**22**)	67.73 ± 0.13	69.15	–
Propiophenone (**23**)	71.72 ± 0.15	78.41	60.41 ± 0.04
Ph-theophylline (**27**)	–	52.04	61.61 ± 0.04
Butyrophenone (**24**)	79.32 ± 0.14	88.49	66.79 ± 0.05
Bromobenzene (**29**)	–	–	69.43 ± 0.07
Valerophenone (**25**)	86.66 ± 0.08	97.67	73.05 ± 0.12

[a] All data has less than ±1% error.
[b] Isochratic and gradient elution of standard mixture.
[c] Gradient elution of standard mixture and correlation with published data [37].

It has been reported that CHI values depend on the type of stationary phase, the type of organic phase and, for acidic or basic compounds, the pH [37]. The pH affected only the elution of benzimidazole, one of the compounds from the standard mixture; therefore, we employed buffered mobile phase to measure correctly the hydrophobicity. We recommend the selected test mixture, compounds **19**–**21**, and **23**–**29**, for the calibration of any 150 mm long RP-HPLC C18 column to measure CHI_{MeOH}. This standard mixture covers a range of CHI between 25 and 73. However, shorter columns are more convenient for less polar or larger compounds [48]. For the CHI_{ACN} measurement of the standard mixture, Valkó et al. [37] applied ODS-2 Interstil column of 150 mm. We chose Alltima C18 column of 250 mm and so we generated different values for these compounds (Table 2). This indicates that the column parameters have influence over the data. Nevertheless, any column can be calibrated by applying known CHI values for the standard compounds at fast gradient elution. Thus, CHI_{ACN1} translates the standard mixture and cucurbitacins lipophilicity on our column, while CHI_{ACN2} gives the calibrated values against published data for inter-laboratory purposes.

The cytotoxicity of 17 cucurbitacin analogues on HepG2 cells is listed in Table 3. This is the first in vitro assay of cucurbitacins on HepG2 cells to study the effect of structure alteration on cellular toxicity. Cells were challenged with cucurbitacins at various concentrations for a day and then live cells quantified with MTT dye. This period of time measures exclusively compounds cytotoxicity, while longer incubation time may lead to interference from metabolites [25]. We did not have enough amount from iso-cucurbitacin B to include it into the biological assay.

Table 3
Cucurbitacins cytotoxicity on HepG2 cells, chromatographic hydrophobicity indexes in buffered acetonitrile and methanol using three different approaches, and the software estimated $C\log P$ values[a]

Compound	IC_{50} (μM)	CHI_{ACN1}	CHI_{ACN2}	CHI_{MeOH}	$C\log P$	$\log P$
I Gluc	390.0 ± 10.0	46.48	37.50	56.41	1.84	2.09
E Gluc	226.7 ± 15.3	53.98	49.13	62.27	2.75	2.28
D	77.3 ± 8.7	58.31	55.83	60.40	2.05	3.12
iso-D	80.3 ± 3.5	60.59	59.37	62.27	2.22	3.07
I	15.8 ± 6.7	63.27	63.53	63.86	2.44	3.33
I-Me	15.0 ± 5.6	64.84	65.95	66.58	2.69	3.81
L-Me	19.0 ± 1.0	64.84	65.95	66.58	3.55	3.79
I-Et	5.5 ± 0.5	69.99	73.94	69.61	3.08	4.15
B	27.7 ± 9.0	70.93	75.40	67.33	2.96	3.69
iso-B	–	72.86	78.38	68.50	3.12	3.68
I-*i*Pr	7.0 ± 1.0	73.87	79.94	71.55	3.38	4.54
I-*n*Pr	5.0 ± 0.5	75.92	83.13	72.88	3.6	4.52
E	15.3 ± 4.2	75.92	83.13	69.61	3.35	3.72
E-Me	12.0 ± 3.0	77.84	86.09	71.55	3.59	4.15
E-Et	5.1 ± 0.9	82.76	93.73	74.25	3.98	4.68
E-*i*Pr	4.3 ± 0.5	86.84	100.04	76.30	4.29	4.78
E-Me-Ac	26.0 ± 1.0	86.84	100.04	76.30	4.30	4.29
E-*n*Pr	3.7 ± 0.1	88.34	102.37	77.66	4.51	4.93

[a] All CHI values has less than ±1% error.

Correlations between CHI_{ACN2} or CHI_{MeOH} and logarithmic IC_{50}, as a measure of cytotoxicity, have been investigated (Fig. 3), and found statistically significant correlations (Table 1). These equations suggest that compounds lipophilicity increases in vitro cytotoxicity, with the exception of cucurbitacin E-Me-Ac (**17**). This compound lipophilicity is increasing while its toxicity is decreasing relative to cucurbitacin E and E-Me ether analogues. Acetylation of C-16 hydroxyl diminishes toxicity in accordance with published data [7,50]. Equations on Fig. 3 present the improved QSAR when **17** was not considered. Glycosides **1** and **2** showed much lower toxicity (Table 3) than their aglycon counterparts, cucurbitacins I and E. It should be associated with the glucose molecule, which increases greatly both the polarity and the volume of the structure. Contrary to the in vivo data mentioned above, we noticed an increase in cytotoxicity for the alkylated derivatives on HepG2 cells. Additionally, cytotoxicity increased proportionally with increasing alkyl chain at C2 hydroxyl (compounds **6**, **8**, **11**, **12**, **14–16**, and **18**).

Good correlations were found between $\log P$ or $C\log P$ and CHI, and between $\log P$ or $C\log P$ and $\log IC_{50}$ (Table 1). While the RP-HPLC hydrophobicity data is experimental, it confirms the good quality of the estimated octanol/water partition data. Overall, $C\log P$ shows better correlation with both CHI_{ACN2} and CHI_{MeOH}, and the $\log P$ correlates better with $\log IC_{50}$. As mentioned in Section 2, different mathematical approaches were used to calculate $l\log P$ or $C\log P$. In addition, the $\log P$ values were reported to be more accurate than $C\log P$ [41]. While $\log P$ correlates better than CHI with $\log IC_{50}$, estimated lipophilicity is usually not as reliable as measured values. More research is necessary to validate the $\log P$ values calculated with the ALOGPS program. The scale of hydrophobicity defined as CHI_{ACN}, CHI_{MeOH}, $\log P$ or $C\log P$ (Table 3) indicates that CHI_{ACN} has the largest range, and therefore it should provide a highly sen-

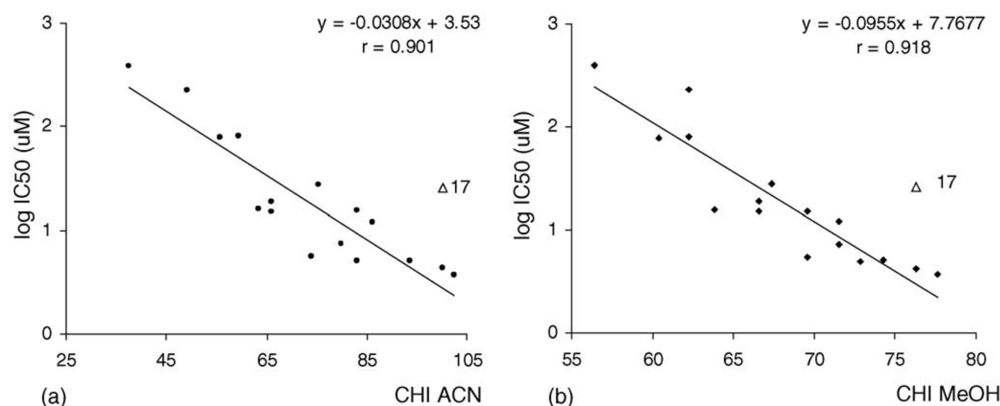


Fig. 3. Relationship between cucurbitacins toxicity on HepG2 cells and CHI measured in acetonitrile (a) or in methanol (b).

sitive measure, allowing more discrimination among similar compounds. Yet CHI_{ACN} is not correlating the best with the cytotoxicity.

The steroid-like cucurbitacins diffuse through the biological membrane by nonmediated transport [51,52]. Only the presence of C19 methyl group at position 9 instead of the usual position 10 for steroids differentiates the cucurbitacin skeleton from steroids [1]. Consequently, the more lipophilic compounds can cross the lipid bilayer easier than their polar homologues, leading to differentiation in their partitioning between the media and cells. Lipophilicity also plays a dominant role in ligand-receptor interactions, e.g. in binding drug to the target molecule inside the cell [53]. We may speculate that cytotoxicity of cucurbitacins involves hydrophobic interaction with the target molecule within the cell, and analogues with higher lipophilicity may have stronger interaction. Furthermore, it has been reported that cucurbitacins are activated within several hours in the cytoplasm and only their metabolites are implicated in the mechanism of action [51,52,54,55]. If the metabolites are involved in the interaction, their hydrophobicity may proportionally change with the hydrophobicity of the original compound, demonstrated by the strong relationship between lipophilicity and cytotoxicity.

4. Conclusion

RP-HPLC is a fast, high-throughput and highly precise technique to determine compounds hydrophobicity, which is an important descriptor in drug design. Cucurbitacins CHI indicates a wide range of lipophilicity. The ACN mobile phase leads to a better resolution and wider range of CHI data than MeOH. On the other hand, a shorter HPLC column generates more accurate data than a longer column. High correlations have been found between the software-estimated $\log P$ or $C\log P$ and CHI, which validates the estimated lipophilicity data. Overall, lipophilicity increases the basal toxicity of cucurbitacins on HepG2 cells. The presence of $\Delta^{1,2}$ generally increases toxicity. The extension of R_1 alkyloxy chain or acetylation of C25–OH increases lipophilicity as well as toxicity. The alkylation of diosphenol increases toxicity on HepG2 cells, in opposite to the lower toxicity demonstrated by others in animals. While the trend is true for most analogues, acetylation of C16–OH group leads to relatively higher lipophilicity but lower toxicity. Although we did not generate other C16 ester derivatives, the result may well demonstrate that SAR studies require very closely related compounds, for which just one functional group is allowed to alter. The significant correlation between cytotoxicity and lipophilicity does not guarantee that no other physicochemical properties are involved in cellular toxicity [14], and it helps us rank the analogues in their order of toxicity and guides us to the next step in our search for less cytotoxic analogues.

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