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Responsible structural features for cytotoxic and other kinds of activity of *neo*-clerodane diterpenes from genus *Scutellaria*

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Twelve natural neo-clerodane diterpenes, isolated from three *Scutellaria* (*Labiatae*) species, were tested for cytotoxicity on two cell lines, from human tumors of the lung designated as H1299 and normal cells from a navel string (HUVEC), using the MTT (3-/4,5-dimethylthiazol-2-yl/-2,5-diphenyltetrazolium bromide) method. The cytotoxic activity was evaluated as rate of IC_{50} (such concentration of the compound in μM by which half of the cells die). Three compounds, scutalpins A, E and F, exhibited moderate cytotoxic properties on both cell lines. Among all tested compounds the highest activity was detected for scutalpin A, with IC_{50} values of 21.35 and 23.9. Some significant aspects of the relationship structure-activity are discussed.

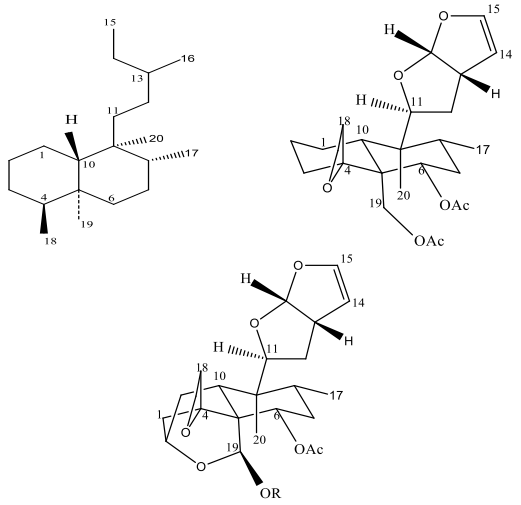
Key words: *Scutellaria*; Diterpenes; Cytotoxic, Antifeedant, Antifungal activities.

INTRODUCTION

The natural *neo*-clerodane diterpenoids, isolated from *Scutellaria* species, draw attention because of their varied biological activities especially as potent insect antifeedants [1-4] and antifungal [5] agents against plant pathogenic fungi.

All diterpenoids isolated from the species of genus *Scutellaria* (Labiatae) are with *neo*-clerodane skeleton (**1**, Fig. 1) which Bruno *et al.* divided in two part substructures bearing different substitutes [1]. The first substructure, including the carbon atoms from C-1 to C-10, is always a *trans*-bound bicyclic system - decalin core. The second one covers the carbon atoms C-11– C-16 and consists of different fragments: tetra- (**2a,** Fig. 2) or hexahydrofurofuran system (**2b)**; α,β-unsaturated-γ-lactone (**3**) junked between C-13 – C-16; γ-lactone 13-spiro, bound up with cyclic ether inclusive C-8 and forming two epimers with C-13S (**4**) and C-13R (**5**) configurations. Finally, in the C-11 – C-16 substructure may be formed a lactol ring that comprises carbons C-11, C-12, C-13, C-16 (**6**) or carbons C-13 – C-16 (**7**).

Clerodanes, obtained from *Scutellaria* plants, displaying the above activities, predominantly possess a decalin ring with C-4-C-18 *spiro*-epoxide and two acetate groups at C-6 and C-19 positions and **2a** or **2b** moiety in the C-11 – C-16 fragment. The compounds clerodin (**8)**, jodrellin A (**9)** and jodrellin B (**10)** exhibit strong antifeedant activity. Particulary **10** was reported to be the most potent antifeedant known to date [4]. These three compounds have been assayed for antifungal activity against the plant pathogenic fungi *Fusarium oxysporum f.* sp. *lycopersici* and *Verticillium tricorpus*. Spore germination of *V. tricorpus* was delayed by clerodin and jodrellin B.



1, 8, 9: R = Ac; **10:** R = CO^{i}Pr

Figure 1. *Neo*-clerodane skeleton (**1)**, clerodin (**8)**, jodrellin A (**9)** and jodrellin B (**10)**

Growth of *F. oxysporum* and *V. tricorpus* was inhibited by the clerodanes in a dose-dependent manner [5].

To the best of our knowledge the *neo*-clerodane diterpenoids from *Scutellaria* species have not been studied for other biological properties. In continuation with our research on this topic we had examined *neo*-clerodane diterpenoids, isolated in our laboratory, for antifeedant activity against *Leptinotarsa decemlineata* (Say) [6,7] and that study confirmed results achieved in previous works. Subsequently, we tested these compounds for antioxidant effects but none of the diterpenes

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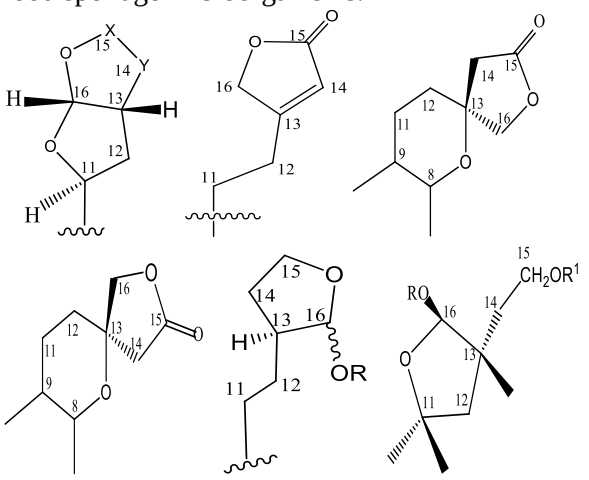
showed such activity. The next step was to evaluate the influence of these clerodanes on pathogenic and food spoilage microorganisms.



X-Y **3** **4**

5 **6** **7**

2a: H**C=CH** R = H; Me R = Alkyl

2b: H_2C—CH_2 R^1 = Acyl

Figure 2. C-11 – C-16 moiety

We concluded that the compounds, containing fragment **4** or **5** in their structures, exhibit antimicrobial activity while the compounds possessing the other kind of C-11 – C-16 moiety are inactive [8].

Herein we report the results from the bioassay of natural *neo*-clerodane diterpenoids, isolated from the acetone extracts of the aerial parts of three *Scutellaria* species, for cytotoxic activity on H1299 and HUVEC cell lines.

EXPERIMENTAL

Plant material: The stems of all plants were collected during their blossoming as follows: *scutellaria alpina* in August 1991, at Pirin Mountains, near Bansko, Bulgaria; *Scutellaria galericulata* in June 2012, near Lovech and Pleven, Bulgaria; *Scutellaria altissima* in June 2011, in Bachkovo, near Assenovgrad, Bulgaria.

Structural data: ^{1}H NMR spectra were recorded on Bruker DRX-250, Varian Mercury-400 or Bruker Avance II+ spectrometers, operating at 250.13 MHz, 400.13 or 600.130 MHz, respectively. ^{13}C NMR spectra were recorded at 100.61 and 150.903 MHz, respectively, on the corresponding spectrometers. TMS was used as internal standard and $CDCl_3$ as solvent. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz. The IR spectra were registered in KBr pellets on a Perkin-Elmer 1750 FT-IR spectrometer from 4000 cm^{-1} to 450 cm^{-1} at resolution 4 cm^{-1} with 9 scans. MS and HRMS spectra were registered on Accela quaternary UHPLC pump with Accela autosampler and HRMS "Q-Exative" detector (Thermo Fisher Scientific, Waltham, MA, USA) with heated electro spray (H-ESI) interphase.

Extraction and isolation: Dried and finely powdered aerial parts of the plants were extracted with Me_2CO threefold at room temperature for a week. After filtration, the solvent was evaporated to dryness under reduced pressure and low temperature (<40 °C) yielding a gum, which was dissolved in aq. Me_2CO (40 % H_2O, v/v, 100 mL). This solution was cooled to 4 °C for 24 h and filtered. The filtrate was extracted with $CHCl_3$ and the organic layer was dried (Na_2SO_4) and evaporated in vacuum to afford a residue (bitter fraction). This residue was subjected to CC (silica gel Merck n. 7734, deactivated with 10% H_2O, w/w). Pure petroleum ether, followed by a gradient of petroleum ether - EtOAc mixtures (10:1 to 4:1) and dichloromethane were used first as eluting solvents. The diterpene fractions (TLC monitoring) were eluted with 1% to 3% methanol in DCM. Rechromatography or repeated prep TLC of these fractions (2% methanol in DCM or EtOAc as eluent) afforded crude compounds. Crystallization and recrystallization from acetone yielded pure diterpenes.

Test compounds: All tested neo-clerodane diterpenoids (Fig. 3) were available from previous investigations. 14,15-dihydrojodrellin T (**10**), neoajugapyrin A (**11**), scutegalerin A (**12**), scuecolumnin C (**13**) and scutegalin D (**20**) were retrieved from *Scutellaria galericulata L.* as described above [10, 11]. Scutecyprol A (**14**), scupolin H (**15**), clerodin (**16**) and scutaltisin G (**21**) were isolated from *Scutellaria altissima L.* [12, 13]. Diterpenoids scutalpin A (**17**), scutalpin F (**18**) and scutalpin E (**19**) were obtained from *Scutellaria alpina L.* [14, 15]. The molecular structures of the compounds were established by spectroscopic means: IR spectroscopy, MS, 1D (^{1}H, ^{13}C including Dept 125) and 2D (HSQC, HMBC, ^{1}H-^{1}H COSY, NOESY) NMR experiments. Absolute configuration of scutalpin A was determined by X-ray diffraction analysis.

Diterpenoids have been kept in the refrigerator at 4°C. Before doing the cytotoxicity bioassays we measured and compared with the literature the melting points and the IR spectra of the tested compounds. The purity of the compounds was checked by TLC with different solvents (diethyl ether, ethyl acetate, from 1% to 3% methanol in DCM).

Cytotoxicity bioassays. Summary. Cell lines: For the *in-vitro* cytotoxic activity screening, two cell lines were selected: from human tumors of the lung, designated as H1299, and normal cell lines HUVEC (cells from a navel string). The cell lines

were obtained from BPS Bioscience, Recombinant Cell Lines & Assay Kits. The bought cells were sustained in the artificial medium (DMEM – HUVEC; RPMI – H1299) and often resewed. Stock solutions: The working probes were prepared by dissolution of 1 mg of diterpenoides in 50 μL of DMSO (dimethylsulfoxide). To obtain the tests solutions two or three μL from the DMSO solutions of the compounds were diluted in 1 mL culture medium as the concentration of DMSO in the tests solutions became 0.2 – 0.3 %.. For bioassays we took so much quantity of the tests solutions that after subsequent diluting in the culture medium, to ensure a concentration in 125 μL of 300, 150, 75, 37.5 and 19 μM. In such conditions the diterpenoids remained well soluble and did not react with the solvent DMSO and the components of the culture medium, based on the TLC monitoring. Pure water was used for the control probe. Four repetitions of assays were made for each concentration. The influence of different concentrations of DMSO on the cytotoxic activity on the cell lines was studied. This step is important because the terpenoids have only poor water solubility and usage of compatible organic solvents such as DMSO is required. Cytotoxic effect for the cells was observed in the mixture of DMSO/water with w/w of DMSO of 1%. Concentration of DMSO in the working probes ranged between 0.1 and 0.007%.

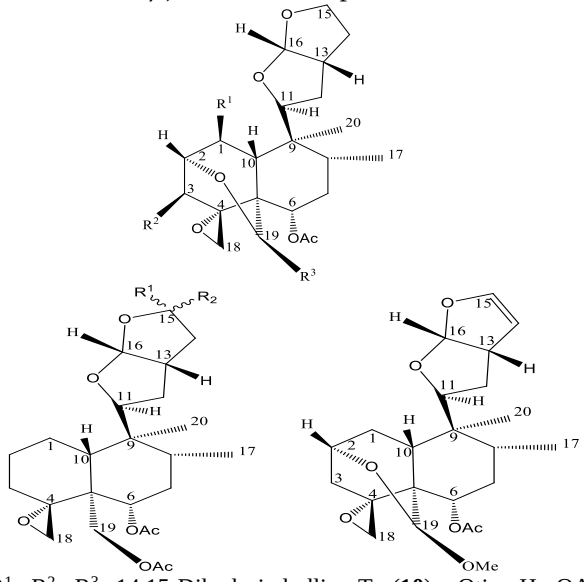
MTT assay: Cells ($0.5 \times 10^4 - 1 \times 10^4$) were plated in 125 μL of medium/well in a 96–well plate. After seventy-two hours we threw the old nutritive medium together with the compound and added a new medium with the dissolved MTT. The cell mitochondrial respiratory chain should include MTT and if the cell is alive and breaths its color changes from yellow to purple. After four hours the medium + MTT was thrown and the remains, that were bottom purple crystals MTT (phurmazanic), were dissolved with 2% solutions of formic acid in *iso*-propanol. The intensity of the purple tinge was measured with the device, ELISA reader. The data were processed with the program GraphPadPrism. Activity was evaluated as rate of IC_{50}.

RESULTS AND DISCUSSIONS

Twelve natural neo-clerodane diterpenes (Fig. 3), isolated from *Scutellaria galericulata* L., *Scutellaria alpina* L. and *Scutellaria altissima* L., growing in Bulgaria, were tested for cytotoxicity on two cell lines, from human tumors of the lung designated as H1299 and normal cells from a navel string (HUVEC), using the MTT method. The cytotoxic activity was evaluated as rate of IC_{50}.

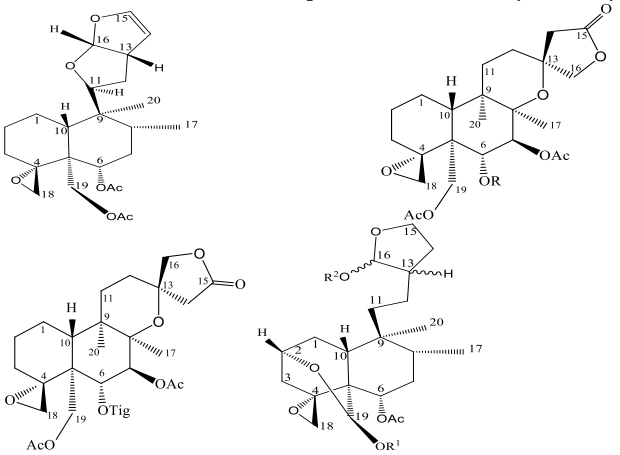
Characteristic signals for the neo-clerodane skeleton in all tested diterpenoids were easily determined at δ_H in the region 1.10-1.20 s (Me-20), 0.78-0.95 d (Me-17) and 1.75-2.25 d or dd (H-10β). The decalin moiety contains some constant functional features: the decalin junction is always trans; methyl groups C-17 and C-20 are always α-orientated; α-hydroxy or α-acyloxy groups are always present on carbons C-6 and C-19; while carbon C-10 is never functionalized; bearing C-4–C-18 *spiro*-epoxide. The two doublets from the AB quartet corresponding to the C-18 two hydrogens appeared at the region of δ_H 2.40-2.50 for H-18A and 2.95-3.05 for the H-18B, respectively. In compounds having substitute at third position the signal for 18A is replaced at δ_H 2.85-2.92.

In accordance with the aim of this investigation the compounds were separated into four groups **I-IV** on the basis of the presented C-11 – C-16 substructures (Fig. 3). Members of the **I** (diterpenoids from **10** to **14**) have hexahydrofurofuran moiety (**2b**) (1H, δ_H 2.80-2.95 br tt, H-13; 1H, δ_H 5.60-5.70 d, H-16) and display a 2α,19-hemiacetal or acetal function (1H, δ_H 3.98-4.10 ddd, H-2β) with the exception of **14**.



R^1 R^2 R^3 14,15-Dihydrojodrellin T (**10**): Otig H OAc Scutecyprol A (**14**): R^1, R^2 = H, OH Scupolin H (**15**) Neoajugapyrin A (**11**): H OH Otig (R and S form) Scutegalerin A (**12**): OH H Otig Scutecolumnin C (**13**):H OH

Separate compounds differ from one another by the substitutes at C-1, C-3 or C-19 position. In the second group (**II**) are included two clerodanes **15** and **16** which contain the fragment **2a** (1620 cm^{-2} vinyl ether; 1H, δ_H 4.81 t, H-14 / δ_C 102.0 d; 1H, δ_H 6.45 t, H-15 / δC 146.7 d). The series **III** was constructed by three diterpenoids, scutalpins A, E and F, as the configuration of the carbon C-13 in



R R^1 R^2 Clerodin (**16**) Scutalpin A (**17**): MeBu Scutalpin E (**19**) Scutegalin D (**20**): Tig H Scutalpin F (**18**): Ac Scutaltisin G (**21**): H CH_3

Figure 3. Molecular structures of the tested *neo*-clerodane diterpenoids

scutalpin E (1H, 2.48 d, H-14*α*; 1H, 3.12 d, H-14*β* / δ_C 44.3 t, C-14; 1H, 4.30 d, H-16*α*; 1H, 4.10 d, H-16*β* / δ_C 79.4 t, C-16 and δ_C 173.1 s, C-15)0 is opposed to that in the scutalpins A and F (1H, 2.57 d, H-14*α*; 1H, 2.73 d, H-14*β* / δ_C 42.6 t, C-14; 1H, 4.13 d, H-16*α*; 1H, 4.35 d, H-16*β* / δ_C 79.6 t, C-16 and δ_C 174.6 s, C-15). The representatives **20** and **21**, possessing the lactol ring **6** (δ_H 3.86 td, 1H, H-15A; 3.93 td, 1H, H-15B; 4.64 d, 1H, H-16), form the last group **IV**.

Scutalpins A, E and F, exhibited moderate cytotoxic properties on both cell lines (Table 1), but the rest of the compounds were inactive within the studied concentration range. Based on these results, it could be concluded that the tetra- or hexahydrofurofuran substructures are not the responsible moiety for cytotoxic action, which is in discrepancy with the affirmation from Kojima and Kato, that these structural features of the molecules are accountable conditions for the significant antifeedant activity [15].

Table 1. Cytotoxic activity of neo-clerodane diterpenes on H1299 and HUVEC cell lines

Compounds	IC_{50} values		Compounds	IC_{50} values	
	H1299	HUVEC		H1299	HUVEC
14,15-Dihydrojodrellin T (**10**)	242,21	244,43	Clerodin (**16**)	236,35	236,84
Neoajugapyrin A (**11**)	288,65	287,59	Scutalpin A (**17**)	21,35	23,89
Scutegalerin A (**12**)	452,01	458,00	Scutalpin F (**18**)	26,62	31,28
Scutecolumnin C (**13**)	667,44	665,79	Scutalpin E (**19**)	34,24	32,48
Scutecyprol A (**14**)	335,76	365,32	Scutegalin D (**20**)	888,35	883,47
Scupolin H (**15**)	578,71	574,55	Scutaltisin G (**21**)	892,02	892,22

Presumably, there is no correlation between the two kinds of biological activities, cytotoxic and antifeedant. All three compounds with cytotoxic effect, have 13-spiro connected *γ*-lactone as the configuration of the asymmetric center C-13 is S in **17** and **18** and R in **19,** respectively. Diterpenoids differ from one another by the substitute at carbon C-6. The most active among them scutalpin A contains 2-methylbutyrate, while the less active scutalpin F and scutalpin E are presented with acetyl or (E)-2-methyl-2-butenoyl ester. It is uncertain that only changing of the 2-methylbutyrate function with the tiglate one causes the bigger decrease in the action of **19**, because the C-11 – C-16 substructure is not with 13S configuration like in scutalpin A, but it is with the other possible orientation - 13R.

CONCLUSION

The achieved results by the series of bioassays on antifeedant activity confirm the previously reported, by Blaney *et al*. [16]; Houghgoldstein & Whalen [17], dependence of activity on the presence in the molecule of tetra- or hexahydrofurofuran ring. Neo-clerodane diterpenoids do not display antioxidant activity. Compounds which exhibit cytotoxic and antimicrobial activity possess 13-*spiro*-α,β-unsaturated-γ-lactone. Thus, it be concluded that the higher activity depends on the characteristic features of the whole molecular structure. Responsible structural features for biological activity vary for the separate kinds of properties.

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НЯКОИ СТРУКТУРНИ ОСОБЕНОСТИ, ОПРЕДЕЛЯЩИ ЦИТОТОКСИЧНОСТТА И ДРУГИ ВИДОВЕ АКТИВНОСТИ НА НЕО-КЛЕРОДАНОВИ ДИТЕРПЕНИ ОТ РОДА *SCUTELLARIA*

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(Резюме)

Дванадесет природни нео-клероданови дитерпени, изолирани от три вида на рода *Scutellaria* (Labiatae), са тествани за цитотоксичност на две клетъчни линии, от тумори на човешки бял дроб, означени с H1299 и нормални клетки от пъпна връв (HUVEC) с помощта на MTT (3-/4,5-диметилтиазол-2-ил/-2,5-дифенилтетразолиев бромид) метод. Цитотоксичната активност е изчислена като IC_{50} стойност (такава концентрация на съединението в μM, при която половината от клетките умират). Три съединения, скуталпини A, E и F проявяват умерени цитотоксични свойства за двете клетъчни линии. От всички тествани съединения най-висока активност е отчетена за скуталпин A, със стойности на IC_{50} съответно 21,35 и 23,9.

Обсъдени са някои значими аспекти на взаимовръзката структура - активност.

SUPPLEMENTARY DATA

In this section the GraphPadPrism experimental results for the cytotoxic active compounds are shown.

Scutalpin A (**17**)

Table format: XY	X	A			
	concentration	h1299 2			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1	Title	0.01	0.316	0.295	0.290	0.276
2	Title	19.00	0.113	0.160	0.144	0.149
3	Title	37.50	0.107	0.133	0.139	0.117
4	Title	75.00	0.030	0.077	0.077	0.063
5	Title	150.00	0.006	0.007	0.006	0.005
6	Title	300.00	0.008	0.009	0.008	0.011

Nonlin fit	A
	h1299 2
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.329
4 HILLSLOPE	-1.144
5 IC50	21.35
6 Std. Error	
7 LOGIC50	0.04359
8 HILLSLOPE	0.1406
9 95% Confidence Intervals	
10 LOGIC50	1.239 to 1.420
11 HILLSLOPE	-1.436 to -0.8527
12 IC50	17.33 to 26.29
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9604
16 Absolute Sum of Squares	1089
17 Sy.x	7.035

Table format: XY	X	A			
	concentration	huvec 2			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1	Title	0.01	0.243	0.232	0.229	0.241
2	Title	19.00	0.109	0.117	0.104	0.153
3	Title	37.50	0.085	0.116	0.120	0.120
4	Title	75.00	0.018	0.033	0.042	0.055
5	Title	150.00	0.006	0.006	0.007	0.006
6	Title	300.00	0.009	0.010	0.010	0.008

Nonlin fit	A
	huvec 2
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.378
4 HILLSLOPE	-1.275
5 IC50	23.89
6 Std. Error	
7 LOGIC50	0.04098
8 HILLSLOPE	0.1669
9 95% Confidence Intervals	
10 LOGIC50	1.293 to 1.463
11 HILLSLOPE	-1.621 to -0.9285
12 IC50	19.64 to 29.06
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9520
16 Absolute Sum of Squares	1372
17 Sy.x	7.897

Scutalpin F (**18**)

Table format: XY	X	A			
	concentration	huvec 1			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.243	0.232	0.229	0.241
2 Title	19.00	0.112	0.151	0.142	0.141
3 Title	37.50	0.064	0.136	0.124	0.120
4 Title	75.00	0.073	0.101	0.099	0.110
5 Title	150.00	0.004	0.005	0.008	0.008
6 Title	300.00	0.004	0.006	0.006	0.006

Nonlin fit	A
	huvec 1
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.495
4 HILLSLOPE	-1.127
5 IC50	31.28
6 Std. Error	
7 LOGIC50	0.05258
8 HILLSLOPE	0.1742
9 95% Confidence Intervals	
10 LOGIC50	1.386 to 1.604
11 HILLSLOPE	-1.488 to -0.7654
12 IC50	24.34 to 40.21
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9138
16 Absolute Sum of Squares	2413
17 Sy.x	10.47

Table format: XY	X	A			
	concentration	h1299 1			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.316	0.295	0.290	0.276
2 Title	19.00	0.160	0.158	0.154	0.137
3 Title	37.50	0.129	0.133	0.134	0.157
4 Title	75.00	0.064	0.124	0.117	0.110
5 Title	150.00	0.007	0.016	0.018	0.016
6 Title	300.00	0.009	0.014	0.008	0.013

Nonlin fit	A
	h1299 1
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.425
4 HILLSLOPE	-1.015
5 IC50	26.62
6 Std. Error	
7 LOGIC50	0.05046
8 HILLSLOPE	0.1364
9 95% Confidence Intervals	
10 LOGIC50	1.320 to 1.530
11 HILLSLOPE	-1.297 to -0.7319
12 IC50	20.92 to 33.87
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9393
16 Absolute Sum of Squares	1575
17 Sy.x	8.461
18 Number of points	

Scutalpin E (**19)**

Table format: XY	X	A			
	concentration	huvec 3			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.2430	0.232	0.229	0.241
2 Title	19.00	0.1460	0.132	0.151	0.125
3 Title	37.50	0.1240	0.117	0.121	0.094
4 Title	75.00	0.1070	0.092	0.099	0.087
5 Title	150.00	0.0090	0.010	0.009	0.007
6 Title	300.00	0.0070	0.006	0.005	0.007

Nonlin fit	A
	huvec 3
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.512
4 HILLSLOPE	-1.130
5 IC50	32.48
6 Std. Error	
7 LOGIC50	0.04314
8 HILLSLOPE	0.1438
9 95% Confidence Intervals	
10 LOGIC50	1.422 to 1.601
11 HILLSLOPE	-1.428 to -0.8313
12 IC50	26.43 to 39.91
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9380
16 Absolute Sum of Squares	1680
17 Sy.x	8.738

Table format: XY	X	A			
	concentration	h1299 3			
	X	A:Y1	A:Y2	A:Y3	A:Y4
1 Title	0.01	0.316	0.295	0.290	0.276
2 Title	19.00	0.185	0.182	0.183	0.183
3 Title	37.50	0.145	0.168	0.189	0.139
4 Title	75.00	0.092	0.095	0.088	0.045
5 Title	150.00	0.020	0.020	0.021	0.011
6 Title	300.00	0.010	0.010	0.011	0.008

Nonlin fit	A
	h1299 3
	Y
1 log(inhibitor) vs. normalized response -- Va	
2 Best-fit values	
3 LOGIC50	1.535
4 HILLSLOPE	-1.336
5 IC50	34.24
6 Std. Error	
7 LOGIC50	0.02905
8 HILLSLOPE	0.1294
9 95% Confidence Intervals	
10 LOGIC50	1.474 to 1.595
11 HILLSLOPE	-1.604 to -1.067
12 IC50	29.80 to 39.33
13 Goodness of Fit	
14 Degrees of Freedom	22
15 R?	0.9633
16 Absolute Sum of Squares	1037
17 Sy.x	6.864