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

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Received July, 26, 2017; Revised October 25, 2017

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 IC50 (such concentration of the compound in *µ*M 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 IC50 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**).

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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-19positions and **2a** or **2b** moiety in the C-11 – C-16 fragment.Thecompounds 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 = COiPr

**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 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:** H2C—CH2 R1 = 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 June2012, near Lovech and Pleven, Bulgaria; *Scutellaria altissima* in June 2011, in Bachkovo, near Assenovgrad, Bulgaria.

*Structural data*: 1H 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. 13C NMR spectra were recorded at 100.61 and 150.903 MHz, respectively, on the corresponding spectrometers. TMS was used as internal standard and CDCl3 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 Me2CO 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. Me2CO (40 % H2O, v/v, 100 mL). This solution was cooled to 4 °C for 24 h and filtered. The filtrate was extracted with CHCl3 and the organic layer was dried (Na2SO4) and evaporated in vacuum to afford a residue (bitter fraction). This residue was subjected to CC (silica gel Merck n. 7734, deactivated with 10% H2O, 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 (1H, 13C including Dept 125) and 2D (HSQC, HMBC, 1H-1H COSY, NOESY) NMR experiments. Absolute configuration of scutalpin A was determined by X-ray diffraction analysis.

Diterpenoids have been kept in the refrigerator at 4oC. 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 *µ*L of DMSO (dimethylsulfoxide). To obtain the tests solutions two or three *µ*L 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 *µ*L of 300, 150, 75, 37.5 and 19 *µ*M. 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%.

МТТ assay: Cells (0.5 × 104 – 1 × 104) were plated in 125 *µ*L 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 МТТ. 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 + МТТ was thrown and the remains, that were bottom purple crystals МТТ (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 IC50.

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 IC50.

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-10*β*). 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-2*β*) with the exception of **14**.

  

R1 R2 R3 14,15-Dihydrojodrellin T(**10**):Otig HOAc Scutecyprol A (**14**): R1, R2 = H, OH Scupolin H (**15**) Neoajugapyrin A (**11**):H OHOtig (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** whichcontain 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 constructedby three diterpenoids, scutalpins A, E and F, as the configuration of the carbon C-13 in

  

R R1 R2 Clerodin (**16**) Scutalpin A (**17**): MeBu Scutalpin E (**19)** Scutegalin D(**20**): Tig H Scutalpin F (**18**): Ac Scutaltisin G (**21**):H CH3

**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*C79.4 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*C79.6 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 | IC50 values | | Compounds | IC50 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*-lactoneas the configurationof the asymmetric center C-13 is S in **17** and **18** and Rin **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 РОДA *SCUTELLARIA*

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Постъпила на 26 юли, 2017 г.; коригирана на 25 октомври, 2017 г.

(Резюме)

Дванадесет природни нео-клероданови дитерпени, изолирани от три вида на родa *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 МТТ (3-/4,5-диметилтиазол-2-ил/-2,5-дифенилтетразолиев бромид) метод. Цитотоксичната активност е изчислена като IC50 стойност (такава концентрация на съединението в *µ*M, при която половината от клетките умират). Три съединения, скуталпини А, Е и F проявяват умерени цитотоксични свойства за двете клетъчни линии. От всички тествани съединения най-висока активност е отчетена за скуталпин А, със стойности на IC50 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**)

|  |  |
| --- | --- |
| h1299 2 data.jpg | h1299 2 reults.jpg |
|  |  |

Scutalpin F (**18**)

|  |  |
| --- | --- |
| huvec 1 data.jpg | huvec 1 results.jpg |
|  |  |

Scutalpin E (**19)**

|  |  |
| --- | --- |
| huvec 3 data.jpg | huvec 3 results.jpg |
|  |  |