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Nigericin and grisorixin methyl ester from the Algerian soil-living *Streptomyces youssoufiensis* SF10 strain: a computational study on their epimeric structures and evaluation of glioblastoma stem cells growth inhibition

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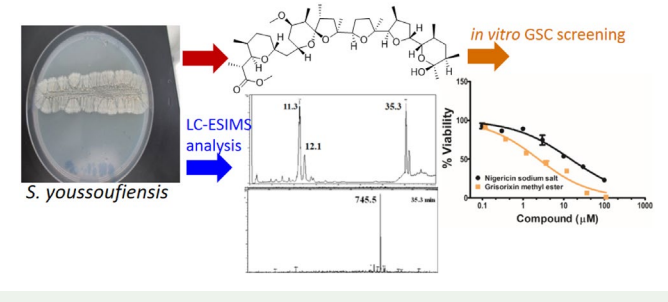
ABSTRACT

The present work describes the metabolites produced by a strain identified as *Streptomyces youssoufiensis*, whose secondary metabolites profile has not been studied so far. The crude ethyl acetate extract was analyzed by high performance liquid chromatography-electrospray ionization mass spectrometry, leading to the detection of the ionophoric polyethers nigericin, epinigericin, abierixin and the newly isolated grisorixin methyl ester. The presence of epimeric forms of nigericin/epinigericin and grisorixin/epigrisorixin has spurred density functional theory computational calculations. This analysis was able to provide the relative stability of the most favored epimers, setting the basis for general structural considerations applicable to several other polyethers. Both nigericin sodium salt and grisorixin methyl ester showed to affect glioblastoma stem cells proliferation in a dose-dependent manner, with a higher activity for the more lipophilic grisorixin methyl ester (GI_{50} values of 3.85 and 3.05 μM for VIPI and COMI human glioblastoma stem cells, respectively).



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1. Introduction

Actinobacteria living in terrestrial habitats have been widely investigated for the production of bioactive metabolites which represent promising candidates for the development of new and selective antibiotic and antitumor agents. About 120 ionophore polyether antibiotics have been isolated from *Streptomyces*, which is the largest genus of Actinobacteria with over 500 species characterized (Dutton et al. 1995; Rutkowski and Brzezinski 2013). The structures of these metabolites display cyclic ethers and branched-chains with terminal carboxylic groups, and are characterized by the presence of several stereogenic centers (Bamdad et al. 1995). Due to their capability of binding monovalent or multivalent cations, they form lipid soluble complex (Sun et al. 2003) able to transport metal cations across cell membranes, therefore causing loss of membrane integrity and subsequent cell death (Smith et al. 2008).

The polyether nigericin is produced by various *Streptomyces* strains (Taechowisan et al. 2013) and displays a broad spectrum of activity, including strong antibacterial properties, whereas the related metabolites grisorixin and abierixin exhibit weak activity against Gram positive bacteria (Rutkowski and Brzezinski 2013). Both nigericin and epinigericin show similar activity against *Toxoplasma gondii*, which is lower for abierixin (Couzinet et al. 1994). Polyethers have also been shown to possess antitumor properties. For instance, nigericin is able to suppress colorectal cancer metastasis (Zhou et al. 2012), to inhibit proliferation of multidrug-resistant lung cancer cells (Yakisich et al. 2017) and to block proliferation of cancer stem cells (Deng et al. 2013; Hegazy et al. 2016). Recently, abierixin has shown a cytotoxic effect in A549, K562 and MCF-7 cells with micromolar IC_{50} values (Wang et al. 2017). Moreover, the more widely studied salinomycin and monesin inhibit cancer stem cells proliferation and resulted to be toxic for chemoresistant cancer cells (Huczynski 2012). Based on these data, polyether ionophores are considered good candidates as anticancer drugs.

Among all the types of primary brain tumors, glioblastoma multiforme (GBM) is the most malignant. Despite the aggressive treatments standardly used, the survival of the patients remains poor (Stupp et al. 2005) therefore highlighting the urgent need for new, more effective molecules. The presence of chemo- and radiotherapy poorly responsive gliobastoma stem cells (GSCs) within the heterogeneous glioblastoma population has been indicated as responsible for the high malignancy of this tumor. GSCs not only promote tumor initiation, growth and angiogenesis but are also resistant to the conventional therapies, therefore contributing to the failure of the treatments used in the clinic. In fact, it has been demonstrated that the limited effect of temozolomide, currently the most widely used drug in GBM treatment, can be largely ascribed to the GSCs population (Zhou et al. 2015). Therefore, GSCs efficient eradication is a critical step to achieve a successful therapy for GBM and new drugs targeting this specific cell population may lead to significant improvement in the treatment of this aggressive brain tumor.

In the present work we described (i) the LC-MS analysis of the metabolite profile of a *Streptomyces* strain obtained from a semi-arid Algerian soil, (ii) the isolation and structural characterization of polycyclic polyethers including nigericin and the newly isolated grisorixin methyl ester (iii) the computational study on the structural epimerization of nigericin and grisorixin and (iv) the evaluation of the cytotoxic effect of the most abundant polyether metabolites on glioblastoma cancer stem cells *in vitro*.

2. Results and discussion

2.1. Taxonomic identification of the strain

Due to the temperature, radiation and salt concentration conditions, arid and semi-arid areas are peculiar ecosystems able to affect the metabolite profile of the extremophilic Actinobacteria, which have been slightly studied so far (Mohammadipanah and Wink 2016). The Actinobacteria colonies investigated in the present study were collected from an Algerian semi-arid soil and the SF10 strain was selected due to its ability of inhibiting pathogenic bacteria. Based on morphological, physiological and chemical evidences, the strain was characterized as belonging to the *Streptomyces* genus. Scanning electron microscopy (SEM) showed that it formed a pale yellow aerial mycelium with rectiflexible spore chains bearing smooth-surfaced spores (Figure S1). The SF10 strain physiological and biochemical features are reported in Table S1. The SF10 strain sequence was deposited in the GenBank database under accession number KU 373054. BLAST analyses revealed a similarity of 99.8% between the partial 16S rRNA gene sequence of this strain and that of *Streptomyces youssoufiensis* strain (FT421338) that has been previously isolated from a Moroccan phosphate mine in Youssoufia (Hamdali et al. 2011) and deposited in the German Collection of Microorganisms and Cell Cultures (DSM 41920 code). *S. youssoufiensis* resulted very close to *S. zagrosensis* recently isolated from an Iranian soil (Mohammadipanah et al. 2014).

2.2. Metabolite profile by LC-ESI-MS/MS analysis of the crude extract

An online HPLC-MS/MS analysis (RP18, MeOH/H_2O + 0.1% trifluoroacetic acid, TFA) was performed on crude ethyl acetate extract obtained from the strain grown in solid state fermentation as single culture in Bennett's medium supplemented with glucose as carbon source at pH 7. The peaks at 11.3 and 12.1 min corresponded to the same $[M + Na]^+$ ion at *m/z* 747.5 (Figure S2(a)), indicative of isomeric compounds. Tandem fragmentation experiments showed a fragment at *m/z* 729.4 attributable to $[M\text{-}H_2O + Na]^+$, whereas the loss of carbon dioxide from the $[M\text{-}H]^-$ ion at *m/z* 723 indicated the presence of a carboxylic group. The peak at 11.3 min was assigned to nigericin (**2**, Figure 1) by comparison with a commercial sample of nigericin sodium salt (Figure S2(b)). The peak at 12.1 min was attributed to epinigericin (**3**, Figure 1), showing inverted configuration at C-28 compared to nigericin (Berrada et al. 1987), as confirmed by next injection of the purified metabolite. The signal at 35.3 min associated to *m/z* 745.5 (Figure S2(c)) was identified as grisorixin methyl ester (**4**, Figure 1) after preparative HPLC purification and structural characterization.

2.3. Purification and structural identification of the metabolites

The crude EtOAc extract (0.80 g) gave pure metabolites after a sequence of chromatographic separations (Scheme S1). Abierixin (**1**, Figure 1) was isolated in very small amount and identified by comparison with reported data (David et al. 1985, Supplementary). Nigericin (**2**) and the minor epinigericin (**3**) were identified by comparing the MS and NMR data to those previously reported (Taechowisan et al. 2013, Supplementary). The relative amounts of the isolated metabolites (Scheme S1) were in line with the 75:25 ratio, for nigericin and epinigericin respectively, observed in the total ion chromatogram in the LC-MS analysis.

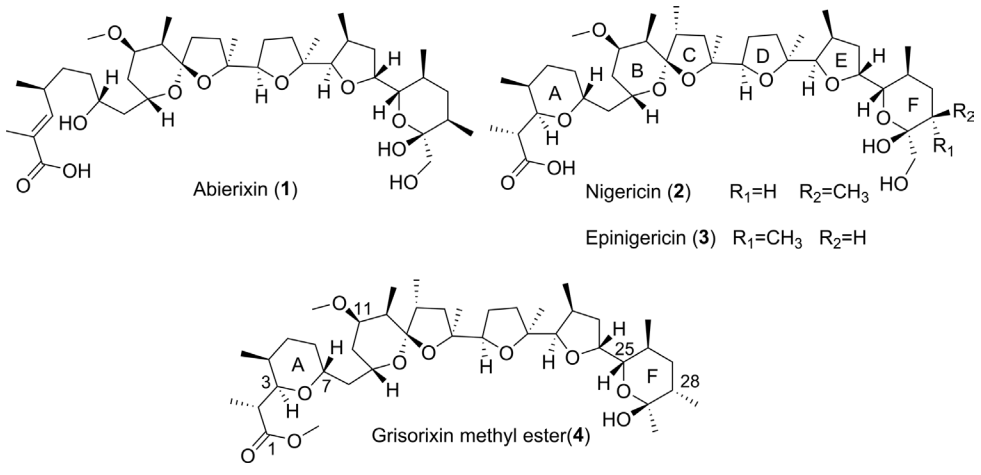

Figure 1. Molecular structures of polyethers isolated from the *Streptomyces youssoufiensis* SF10 strain.

For grisorixin methyl ester (**4**, Figure 1), HR-ESIMS experiments revealed a composition of $C_{41}H_{70}O_{10}$ by the values *m/z* 745.48526 (Calcd 745.486120) for $[M + Na]^+$ ion and *m/z* 721.48169 (Calcd 721.489622) for $[M–H]^-$ ion. The molecular structure was defined upon comparison with NMR data reported for grisorixin (Gachon et al. 1970; Cuer et al. 1983; Oikawa et al. 1992) and epigrisorixin (Mouslim et al.1993). ^{13}CNMR spectra allowed to distinguish between grisorixin and epigrisorixin, which present chemical shift changes for the carbons belonging to the F-ring unit, especially for the C26-C28 signals undergoing a marked upfield shift (C26 signal goes from 32.9 to 25.3 ppm) (Mouslim et al. 1993). We found ^{13}CNMR signals attributable to the F-ring unit that are superimposable to the data reported for grisorixin in $CDCl_3$ (Oikawa et al. 1992). The replacement of the carboxylic unit with a methyl ester was confirmed by HMBC experiment, where the singlet at 3.72 ppm, assigned to the COOMe group, correlates with the only detected signal in the C=O region at 176.4 ppm. The intense IR band at 1725 cm^{-1} assigned to the carboxylic C=O present in the grisorixin structure (Cuer et al. 1983) was replaced by a strong band at 1739 cm^{-1} attributed to the ester group. Grisorixin methyl ester has not been previously isolated as a metabolite, but it has been reported as a grisorixin derivative in an oxidation reaction of the natural product (Gachon and Kergomard 1975). LC-ESIMS profile of the crude extract (Figure S2) dispelled the doubt that the presence of a methyl ester in the structure of metabolite **4** could be an artifact due to the isolation procedures. In fact, **4** was detected as methyl derivative whereas nigericin and epinigericin as free acids, under the same chromatographic conditions. As a further support, 1-methyl ester–nigericin, showing a similar COOMe unit as compound **4**, has been reported as a metabolite isolated by elution from a silica gel column using hexane/ethyl acetate (Taechowisan et al. 2013).

2.4. Computational analysis on the structural epimerization

The concomitant isolation of nigericin/epinigericin and grisorixin/epigrisorixin could be explained by a biosynthetic process involving an enzymatic pathway in a detoxification process as occurring in *S. hygroscopicus* (Mouslim et al. 1993). In order to identify the most stable form we have therefore evaluated the energy values associated to the different

isomers. Density functional theory (DFT) calculations are able to provide energy-minimized structures and their energy values. More specifically, a study of the energy values associated to each isomer enables to identify the thermodynamically favored epimeric molecule. An effective example of the use of DFT method to study epimers has been recently reported for gluco-and galactoside derivatives, differing in their stereochemical arrangement of a hydroxyl group (Ahmadi et al. 2017). To facilitate the calculations, we considered simplified molecules as model for the F-ring of nigericin and grisorixin structures. Specifically, 2-(hydroxymethyl)-3,5,6-trimethyltetrahydro-2H-pyran-2-ol was selected for the nigericin-like series and 2,3,5,6-tetramethyltetrahydro-2H-pyran-2-ol for the grisorixin-like series. The energy values of the models obtained from the computational analysis at a B3LYP/6-31G(d,p) level of theory for nigericin or grisorixin were compared with epinigericin or epigrisorixin respectively and those of their corresponding C-29 isomers. The data were obtained *in vacuo* and in water conditions, the latter one considered more representative of the biological environment. In the nigericin-like series, epinigericin unit has been shown to be slightly more stable than nigericin (ΔE = 0.4129 kJ/mol *in vacuo* and ΔE = 2.5992 kJ/mol in water), whereas the structures showing inverted configuration at the C-29 emiacetalic centre resulted significantly less stable if compared to nigericin (ΔE = 5.4877 kJ/mol *in vacuo* and ΔE = 6.2003 kJ/mol in water). In the grisorixin-like series, the grisorixin configuration at F-ring has resulted the most stable if compared with the C-28 epimer epigrisorixin (ΔE = 9.9357KJ/mol *in vacuo* and ΔE = 6.1825KJ/mol in water), and especially with the C-29 epimeric forms of both grisorixin and epigrisorixin (Table S2). These data could provide a thermodynamic explanation of the epimerization process occurring in these metabolites. The C-28 epimers, epinigericin and epigrisorixin, are both reported in literature as isolated metabolites, whereas the corresponding isomers coming from the emiacetalic epimerization at C-29 have not been isolated so far, in line with their higher energy values obtained by calculation. These considerations are of general interest and can be applied to the wide class of naturally occurring polycyclic polyethers. In fact, the F-ring structural moiety considered for nigericin is also present in endusamycin, CP-120509, monensin, laidlomycin, octacyclomycin, moyukamycin X-14931A, and the one considered for grisorixin is shown in mutalomycin, senduramycin, emduramicin, CP-91243, CP-91244 and W341C (Rutkowski and Brzezinski 2013).

2.5. In vitro *cytotoxicity*

It has been recently shown that nigericin is able to disrupt glioma cells' energy balance and to suppress malignant phenotypes of human patient-derived GBM cells both *in vitro* and *in vivo* (Hegazy et al. 2016). Based on the therapeutic potential of nigericin for human GBM treatment, it was worth to evaluate the cytotoxic activity of the newly isolated grisorixin methyl ester (**4**) in comparison with nigericin (**2**). In particular, the commercially available nigericin sodium salt has been used rather than the free acid form because the conjugated base is effectively present at pH 7.4 used in the assay, as supported by ChemSketch calculation (99.93% at pH 7.40). It is to note that the presence of TFA during the HPLC purification affected the isolation of the free acid form, but both nigericin and its carboxylate salt are reported as isolated metabolites (Wang et al. 2017).

Two glioblastoma stem cell lines, namely COMI and VIPI lines, were treated with increasing concentration of nigericin sodium salt or grisorixin methyl ester for 48 h. The cells were subsequently stained with Hoechst 33342 and propidium iodide (PI) and analyzed using

Operetta High Content Imaging System (Perkin Elmer). The number of living cells upon each treatment was estimated by counting the total number of cells stained using Hoechst and subtracting PI positive cells. Dose-response curves were plotted and growth inhibition 50 (GI_{50}) values were calculated as a parameter to evaluate cytotoxicity. Figure 2 shows that both the compounds affected cell viability in a dose-dependent manner. The calculated GI_{50} values resulted in 14.40 ± 3.24 and 12.60 ± 2.30 µM for nigericin sodium salt, and in 3.05 ± 0.92 and 3.85 ± 0.78 µM for grisorixin methyl ester, on COMI and VIPI respectively. The data indicate that grisorixin methyl ester is at least three time more potent in inhibiting glioblastoma stem cells proliferation as compared to nigericin sodium salt, besides being at least ten times more efficient than temozolomide included in the assay as a positive control (data not shown).

An effective treatment of GBM requires targeting of the cancer stem cell compartment by molecules able to cross the blood-brain barrier (BBB). Polar surface area (PSA) is one of the most important parameters used to characterize the transport properties of drugs, giving a very good correlation with BBB penetration (Dréan et al. 2016). *In silico* data prediction performed by MarvinSketch software provided the values of 145.20 Å^2 for nigericin sodium salt, 142.37 Å^2 for its free acid, 131.37 Å^2 for its methyl ester and 111.14 Å^2 for grisorixin methyl ester. The value obtained for grisorixin methyl ester is closer to the value of 105.94 Å^2 obtained for temozolomide, which is known to cross the BBB. The preliminary results reported here confirm the potential use of polyethers as anticancer drugs (Huczynski 2012), suggesting that even small structural modifications could improve their potency and the BBB penetration capacity.

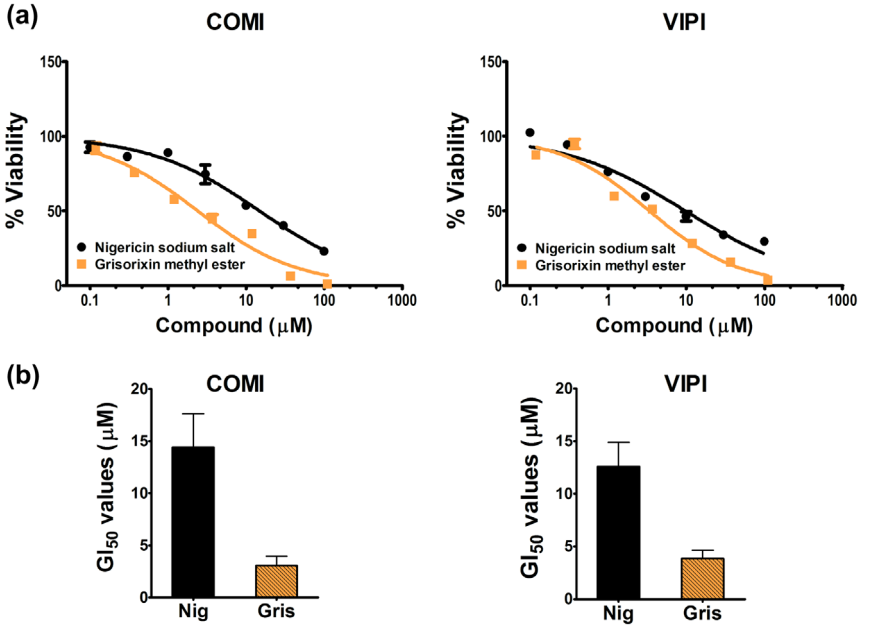

Figure 2. (a) Dose-response curves of human glioblastoma stem cells (COMI and VIPI) treated with nigericin sodium salt or grisorixin methyl ester. The metabolites affect cell proliferation in a dose-dependent manner. (b) Calculated GI_{50} values for nigericin sodium salt and grisorixin methyl ester. The GI_{50} value is defined as the compound concentration causing the 50% inhibition of the cell growth.

3. Conclusion

The polycyclic polyethers nigericin (**2**) and grisorixin methyl ester (**4)** have been isolated, purified and characterized as the major metabolites, together with abierixin (**1**) and epinigericin (**3**), from the strain SF10 collected in an Algerian semi-arid soil and identified as *S. youssoufiensis*. Moreover, the grisorixin methyl ester (**4**) has never been previously described as a natural product. Density functional theory (DFT) calculations allowed us to compare the relative stability of nigericin and grisorixin epimeric forms and set the basis for general structural considerations applicable to several other polyethers. The clear antiproliferative activity of grisorixin methyl ester on glioblastoma stem cells represents a promising starting point for further structure activity relationship (SAR) investigation aimed to the development of new drug candidates in the treatment of glioblastoma multiforme.

Supplementary material

Experimental details, together with Figures S1, S2, Tables S1, S2 and Scheme S1 related to this paper are available online.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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