**Streptomyces scabrisporus**, an endophyte isolated from *Amphipterygium adstringens* as producer of an anthracycline active against different cancer cell lines

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Abstract

The search for bioactive compounds against cancer cell lines produced by endophytes led to the isolation of an actinomycete identified by 16S rDNA as *Streptomyces scabrisporus*. The organic extracts of this microorganism were evaluated in a bioassay-guided study for cytotoxic activity against breast adenocarcinoma MCF7 and human cervical carcinoma HeLa cell lines. The major and also more active compound was isolated and completely characterized as steffimycin-B (StefB). This compound showed cytotoxic activity against HeLa cells and caused morphological changes in MCF-7 cells with an IC50 value of 2.56 μM. StefB also affected other breast cancer cell lines, including the triple negatives HCC1806 and DU4475 (IC50 values of 18.2 and 4.82 μM, respectively) and exhibited low activity against the non-cancerous line of keratinocytes HaCaT (IC50 156 μM). Besides, StefB decreased expression of the proliferating cell nuclear antigen (PCNA), induced morphological changes and apoptosis on MCF7 cells, which increased with the exposure time, supporting this compound as a promissory drug for breast cancer treatment.

Introduction

Screening and isolation of soil Actinomycetes with potential pharmacological properties has been explored for more than 50 years. Owing to the movement of the pharmaceutical industry away from natural products, in recent years the number of drug approvals has drastically dropped down [1]. However, microbial products with pharmaceutical applications are still big business and the search for new drugs cannot be stopped. Therefore, new screening approaches including new metagenomic and genome-mining techniques and the exploration of non-conventional sources of potentially producer microorganisms, are still being studied [2].

Endophytes are microorganisms that inhabit plants without causing visible disease symptoms [3]. Along the past few years they have become an important source of novel molecules with interesting biological characteristics [4].

Discovery of anticancer compounds in endophytes started with the isolation of taxol from *Taxomyces andreanae*, a molecule widely used in chemotherapy [5]. Towards 2010, 57 fungal and 13 bacterial endophytes producing taxol or some of its precursors were reported [6], reaching over 80 fungi by 2014 [7,8]. A similar story has been observed for other commercially important anticancer compounds. Thus, 11 fungal endophytes able to produce camptothecin and 14 yielding podophyllotoxin, were isolated from different host-plants [9-11].

Diversification of novel compounds produced by endophytes increases every day. Thus, 100 anticancer molecules were isolated between 1990 and 2010, belonging to 19 different chemical classes and showing activity against 45 different cell lines [11]. Additional compounds recently isolated from endophytes exhibited antibiotic [12], antifungal [13], antiparasitic [14] and antiviral activities [15].

Actinomycetes have the potential to produce a huge number of compounds that differ greatly between genera. Some of them contain more than 60 clusters for secondary metabolites, mostly unknown [16]. Screening and isolation of endophytic actinomycetes is important, due to the interaction and evolution developed between host plants and endophytes. The use of medicinal plants as source of actinomycetes has gained interest due to the biotechnological potential in the production of different and novel compounds [17].

The plant selected for this study was *Amphipterygium adstringens*, traditionally known as “Cuachalalate”. This is a medicinal tree widely used in Mexico for a variety of human affections like circulatory problems, stomach cancer, urinary disorders, gastrointestinal, lung and respiratory diseases, among others. It is an endemic Mexican tree, resinous and dioecious, inhabiting in regions with deciduous or sub-deciduous tropical forest [18]. Phytochemical studies of its bark have revealed the presence of a wide variety of compounds like...
masticadienonic acid, 3α-hidroximasticadienonic acid, alkyl phenolic acids, alkyl phenolic aldehydes, tirucallane-type triterpenes, anacardic acids and β-sitosterol, which exhibit different biological activities [19-21].

This report deals with the evaluation of the cytotoxic activity of an endophytic actinomycete isolated from A. adstringens and the chemical characterization of the most active compound to finally discuss its possible mechanism of action.

Materials and methods

Plant material and collection site

Plant material was collected in Barranca Honda, municipality of Tlaltizapan, Morelos, Mexico (18°48'33.4476"N, 99°07'13.944"W). The first collection was in February 2013 (drought) by sampling stem vascular tissue and seeds from female trees and stem vascular tissue from male trees. There were no leaves on the trees at this season. The second collection was carried out in August 2013 (rainy), taking leaves, stem and seeds from the female trees as well as leaves and stem tissue from male trees. Samples were identified by a specialist at the National Herbarium of Mexico as A. adstringens and incorporated as A. adstringens and asproducer of an anthracycline active against different cancer cell lines [23].

Sample treatment and isolation of endophytes

A stem sample of A. adstringens was exhaustively washed in running water. Then cut out in a small piece and subject to a four-step surface sterilization procedure by sequential immersion (one minute each) in sterile water, 65% ethanol, 3.4% NaClO and sterile water (modified from Sánchez-Fernández et al.). A 500 μl sample of the fourth wash was used to inoculate Nutrient agar to test for sterility of the sample surface. The sample was cut out into 0.3 cm² pieces and placed in a Petri dish containing Nutrient agar Difco® 213000 (Becton Dickinson, San Diego, CA, USA) for at least 60 days at 29°C. Complete isolation of the actinomycete was possible by sub-culturing the colonies in ISP-2 medium [23].

Molecular characterization of endophytic actinomycetes

The Actinomycete DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research Corp, Irvine, CA, USA) commercial kit, following manufacturer's instructions. 16S ribosomal DNA sequence of the actinomycete was amplified using universal primers fwd5’-CCGATTGCTGACAAACAGATTTGATCAGTGCTCA Gandrev 3’-CCCCGGGATCAAAGCTTACGGCTACCTTGTTACGACTTTG TACGACTT, following PCR amplification conditions PCR amplification conditions PCR mp3. The major compound was purified in a Millipore preparative TLC medium [20]. The more active fraction was analyzed by HPLC/UV/ESI-MS in an Agilent 1200 Binary SL pump (Santa Clara, CA, USA) coupled with an ESI-MS Mass Spectrometer Esquire 6000 (Bruker, Billerica, MA, USA) and a Waters 2996 PDA detector (Milford, MA, USA). For High-performance liquid chromatography (HPLC), a Synergi 4u Polar RP 80A 10 X 2.0 mm column (Phenomenex, Torrance, CA, USA) was used with MeOH:H2O (80:20, v/v) to 100% MeOH at 0.2 mL/min flow. The major compound was purified in a Millipore preparative TLC Silica gel 60 F254 Glass plates 20 X 20 cm (Merck-Millipore, Darmstadt, Germany), being the elution system CH2Cl2/MeOH (99.9:0.1 to 0.100) resulting in 14 primary fractions.

The more active fraction was analyzed by HPLC/UV/ESI-MS in an Agilent 1200 Binary SL pump (Santa Clara, CA, USA) coupled with an ESI-MS Mass Spectrometer Esquire 6000 (Bruker, Billerica, MA, USA) and a Waters 2996 PDA detector (Milford, MA, USA). For High-performance liquid chromatography (HPLC), a Synergi 4u Polar RP 80A 10 X 2.0 mm column (Phenomenex, Torrance, CA, USA) was used with MeOH:H2O (80:20, v/v) to 100% MeOH at 0.2 mL/min flow. The major compound was purified in a Millipore preparative TLC Silica gel 60 F254 Glass plates 20 X 20 cm (Merck-Millipore, Darmstadt, Germany), being the elution system CH2Cl2/MeOH (99:1), yielding 20 mg of an orange powder. Nuclear Magnetic Resonance spectrometry (NMR) was done in a Bruker 400 MHz Spectrometer using deuterated chloroform as solvent. A Xevo G2-XS QTOF mass spectrometer (Waters, Milford, MA, USA) was used to compare the fragmentation
pattern of the isolated compound with a commercial standard of Steffimycin B from Enzo Life Sciences ALX-380-220-M001 (Ann Arbor, MI, USA).

Cell lines culture

Breast adenocarcinoma (MCF-7 ATCC HTB-22), human cervical carcinoma (HeLa ATCC CCL-2) and immortal keratinocytes (HaCaT) were donated by Centro Médico Nacional Siglo XXI, México, and authenticated by Instituto Nacional de Medicina Genómica, México. MCF-7 and HaCaT cell lines were cultured in RPMI-1640 medium supplemented with L-glutamine Caisson RPL03 (Caisson Laboratories, East Smithfield, UT, USA), 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. HeLa cultures were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with high glucose, L-glutamine, and sodium pyruvate Caisson DM1J0, 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin. Cells were incubated at 37°C with 98% humidity and a 5% CO₂ gas environment.

For the pure compound evaluation, different breast cancer cell lines including triple negative were also tested, with HCC1806 ATCC crl-2335, HCC1937 ATCC CRL-2336, DU4475 ATCC HTB-123 and MBD5 (kindly donated from Instituto Nacional de Ciencias Médicas y Nutrición), all of them cultured in RPMI-1640 medium as described above.

In Vitro cytotoxicity assay

Cell survival and proliferation were determined by a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) in a 96-well plate containing 5000 cells per well and incubated for 24hr. Crude extract, fractions and the pure compound were added from DMSO-diluted stocks to achieve different concentrations of the extract per well (0.1 to 100 μg/mL). In all cytotoxicity assays, DMSO was tested as the diluent control (vehicle); sterile media was used as a negative control and doxorubicin in concentrations at 100 μg/mL), the activity for DMSO was subtracted from the result, being 20% mortality for MCF7 and 22% for HeLa cells, in concentrations at 100 μg/mL. Cell survival and proliferation were determined by a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Plates were observed using the Attune cytometric software (Thermo Fisher Scientific, Waltham, MA, USA) and images were obtained in a Carestream Kodak BIOMAX Light Film CAT 178 8207 (Sigma-Aldrich, St. Louis, MO, USA).

Evaluation of senescence and morphological changes

24-well plates containing 10,000 MCF7 cells per well were treated as flow cytometry assay. To detect senescence cells, a β-Galactosidase Staining Kit (Cell Signaling Technology, INC., Danvers, MA, USA) was used following manufacturer’s instructions. Plates were observed in an Olympus IX-71 microscope (Olympus Corporation, Tokio, Japón) adapted with an Olympus LUCPlanFL N 20x (N.A. 0.45) objective. Images were acquired with an Evolution VF camera (Media Cybernetics, Rockville, MD, USA).

Results

Isolation and identification of the NF3 strain

The NF3 strain was isolated from a small piece of a female stem of A. adstringens incubated in nutrient agar medium. The strain presented small mycelium at X1000 magnification and irregular shape colonies at X250, a morphology characteristic of actinomycetes. The 16S ribosomal DNA from NF3 strain with 1272 bp (base pairs) showed 99% identity with different strains of Streptomyces scabrisporus. The phylogenetic tree of the NF3 (Figure 1), exhibited this strain in a clade where all the S. scabrisporus reported up today are included, with a high value of bootstrap, supporting its identity with this species. Its identification was corroborated as S. scabrisporus via 16S rRNA, gyrB, rpoB and recG genes [26].

Figure 2 shows the S. scabrisporus NF3 strain growing as soft pink colonies in rough aggregates, changing to darker colonies with white spores that appeared at the time of incubation, as well as producing a red pigmentation in agar. Gram-positive mycelium was observed under optic microscopy while in SEM micrographs linear spore chains presenting some areas with free spores were found.

Identification of the major bioactive compound of S. scabrisporus NF3.

The S. scabrisporus NF3 crude extract obtained from a 10-liter bioreactor was evaluated against MCF7, HeLa and HaCaT cell lines.
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**Figure 1.** Phylogenetic tree of the NF3 strain using the maximum likelihood method based on the Jukes-Cantor model. Bootstrap values were calculated from 1,000 re-samples with values over 50% shown at the respective node.
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Figure 2. Micrographs of S. scabrisporus at: a) stereoscopic microscope (X250); b) Optical microscope (X1,000); c) SEM micrograph (X10,000) of linear mycelia; d) SEM micrograph (X15,000) of spore formation

Because of its high activity against the cancer cell lines and low activity against keratinocytes, fraction 4 was chosen for subsequent purification. Besides, 197.6 mg were obtained from this fraction. The HPLC/UV/ESI-MS analysis showed 10 peaks in the chromatogram, being the peak 8 the major compound (Figure 3a) and the other compounds in that fraction seemed to be closely related because of its yellow color and proximity of mass profiles (data not shown). The mass and the UV spectra of this compound can be seen in Figures 3b and 3c, respectively. These data and the 1H and 13C NMR spectra supported StefB as the active molecule (Figure 3d). The ESI-MS results are as follow: m/z 587.5 [M-H]; UV in methanol: λmax at 235.5 nm. 1H NMR (CDCl3, 400 MHz): 12.91 (s, 1H), 12.0 (s, 1H), 8.29 (s, 1H), 7.35 (d, J = 2.8 Hz, 1H), 6.64 (d, J = 2.4 Hz, 1H), 5.54 (s, 1H), 5.12 (d, J = 2.8 Hz, 1H), 3.89 (s, 3H), 3.72 (m, 1H), 3.69 (d, J = 2.5 Hz, 1H), 3.61 (m, 1H), 3.56 (s, 1H), 3.52 (s, 3H), 3.51 (s, 3H), 3.49 (d, J = 3.6 Hz, 1H), 3.00 (t, 9.4 Hz, 1H), 2.34 (d, J = 8.8 Hz, 1H), 1.45 (s, 3H), 1.33 (s, 3H); 13C NMR (CDCl3, 100MHz): δ 198.94, 190.64, 180.41, 167.5, 165.84, 162.15, 135.53, 135.07, 133.55, 133.52, 118.77, 117.59, 110.18, 109.27, 106.75, 100.6, 85.67, 83.3, 80.46, 76.68, 71.95, 71.27, 69.00, 60.94, 59.98, 58.85, 56.29, 22.9, 17.95. Fragmentation patterns of the compound isolated in this study, named 4D along the purification steps (Figure 3e), and a commercial standard of StefB (Figure 3f) were acquired, obtaining similar results.

Cytotoxic activity of StefB and its effect on MCF7 cells

Cytotoxic activity of the pure compound was tested against different cell lines, including three triple negative breast cancer cell lines (HCC1806, HCC1937 and DU4475). As shown in Table 3, StefB affected MCF7, DU4475, HCC1806 and MBCD5 cell lines, with MCF7 as the most sensitive one. As seen in the same table, although the IC50 values were higher than doxorubicin, this commercial compound also affected normal keratinocytes.

To elucidate the possible effects of StefB, treated MCF7 cells were analyzed by flow cytometry and Western blot. Figure 4a shows the presence of apoptotic cells, after treating the cell line with 2.56 µM StefB, with 48 h exposure as the time to get the maximum value. Furthermore,

Table 1. Cytotoxic effect of NF3 crude extract grown in ISP-2 medium and the positive control Doxorubicin, against different cell lines

<table>
<thead>
<tr>
<th>EXTRACTS</th>
<th>IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>HeLa</td>
</tr>
<tr>
<td>NF3 EtOAc</td>
<td>0.96</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 2. Cytotoxic effect of the fractions obtained from an open chromatography column from the S. scabrisporus NF3 strain

<table>
<thead>
<tr>
<th>Fractions*</th>
<th>% Mortality (at 100 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF7</td>
</tr>
<tr>
<td>F1</td>
<td>0</td>
</tr>
<tr>
<td>F2</td>
<td>0</td>
</tr>
<tr>
<td>F3</td>
<td>7.1±2.0</td>
</tr>
<tr>
<td>F4</td>
<td>69.4±0.2</td>
</tr>
<tr>
<td>F5</td>
<td>80.8±0.4</td>
</tr>
<tr>
<td>F6</td>
<td>77.5±1.3</td>
</tr>
<tr>
<td>F7</td>
<td>79.1±0.6</td>
</tr>
<tr>
<td>F8</td>
<td>79.8±0.8</td>
</tr>
<tr>
<td>F9</td>
<td>80.0±0.2</td>
</tr>
<tr>
<td>F10</td>
<td>0.04±9.9</td>
</tr>
<tr>
<td>F11</td>
<td>0.1±1.3</td>
</tr>
<tr>
<td>F12</td>
<td>97.6±0.3</td>
</tr>
<tr>
<td>F13</td>
<td>97.0±0.4</td>
</tr>
<tr>
<td>F14</td>
<td>89.4±1.1</td>
</tr>
</tbody>
</table>

*Fractions 1 to 8 were diluted in DMSO, and DMSO activity was subtracted to the final result. Fractions 9 to 14 were diluted in water.
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Table 3. IC₅₀ values of the cytotoxic activity of StefB in different cell lines

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>StefB</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>71.90</td>
<td>4.34</td>
</tr>
<tr>
<td>MCF7</td>
<td>2.56</td>
<td>1.32</td>
</tr>
<tr>
<td>HaCaT</td>
<td>156.00</td>
<td>4.67</td>
</tr>
<tr>
<td>HCC1806</td>
<td>18.20</td>
<td>0.07</td>
</tr>
<tr>
<td>HCC1937</td>
<td>456.00</td>
<td>0.45</td>
</tr>
<tr>
<td>MBCD5</td>
<td>25.70</td>
<td>1.59</td>
</tr>
<tr>
<td>DU4475</td>
<td>4.81</td>
<td>&lt;0.09</td>
</tr>
</tbody>
</table>
the presence of StefB, negatively affected cell proliferation, as judged by a Figure 4 western blot analysis of the PCNA protein (Figure 4b). Finally, strong morphological changes were microscopically detected after StefB treatment with the presence of senescence cells (stained in blue), with a maximum effect at 48 h treatment (Figure 4c).

**Discussion**

In this study, an actinomycete (NF3 strain) isolated from a female stem from the medicinal tree *A. adstringens* was selected by their ability to produce secondary metabolites, property frequently found
in endophytic actinomycetes [27-29]. Its molecular identification highlighted NF3 strain as \textit{S. scabrisporus}, a species never reported as a plant endophyte.

The first report on \textit{S. scabrisporus} KM-4927\textsuperscript{T} (=JCM 11712\textsuperscript{T} = NRRL B-24202\textsuperscript{T}) described this microbe as a gray aerial cell mass producer with spiral spore chains and a rough spore surface [30] unable to produce soluble pigments on ISP-2 medium and other solid media. A recent study also reported another strain of \textit{S. scabrisporus} with spiral aerial mycelium and unable to produce soluble pigments when grown in ISP-2 medium [30,31]. Contrary to the \textit{S. scabrisporus} NF3 strain, those strains were isolated from soil. Interestingly, the NF3 strain produced pigments in different media, including ISP-2. Regarding its morphology, SEM micrographs showed lineal spore chains formation instead of the multiple-spiral spore chains reported for other \textit{S. scabrisporus} strains [30], further supporting differences between these microbes. These differences could be related to the activation of pathways used in the symbiotic relationship between NF3 strain, and \textit{A. adstringens}.

The genome sequence of various \textit{S. scabrisporus} strains, have been already released. Their analysis suggested variations between KM-4927\textsuperscript{T} and DSM 41855 strains with changes in some of their polyketide biosynthetic genes [32]. Furthermore, a recent comparison made by our research group between the strains DSM 41855 and NF3, suggested important differences in their putative gene clusters encoding for secondary metabolites production [33]. Finally, the presence of 2 clades in the \textit{S. scabrisporus} phylogenetic tree, with more than 60% bootstrap value, may support significant dissimilarities within this species.

Chromatography and HPLC/UV/ESI-MS analysis of the \textit{S. scabrisporus} NF3 organic extracts identified the major fraction with cytotoxic activity as StefB, a bioactive anthracycline initially reported in 1974 in \textit{Streptomyces elgreteus} [34]. Similar fragmentation patterns of the isolated compound with those of a commercial standard of StefB, supported further the authenticity of the cytotoxic compound.

Other \textit{S. scabrisporus} strains have been reported to produce bioactive compound like the macrocyclic hitachimycin [32], the polyketide okilactomycin [35] and the polyether ionophore alborixin [36], all of them colorless compounds with antibiotic and cytotoxic activities. In addition to StefB, \textit{S. scabrisporus} NF3 was found to produce two uncharacterized bioactive pigments (red and yellow), not previously described for this species.

The activity of StefB has been reported against Gram-positive bacteria with low inhibitory effect on L.1210 mouse leukemia cells [34]. Antimalarial and antinflammatory activities have also been reported for this molecule, as well as against both malignant (KB, NCi-H187) and non-malignant (Vero) cells [37,38]. On the contrary, antifungal activity against fourteen human pathogenic fungi was not detected [34]. Studies to improve the activity and solubility of this molecule either via chemical, microbiological or molecular biology have also been performed by different groups [39-41]. Furthermore, isolation, characterization and heterologous expression of the StefB gene cluster, was also explored [42].

When StefB was tested over different cell lines not previously evaluated, this compound affected from major to minor scale the breast adenocarcinoma MCF7, the triple breast cancer cell lines DU4475, HCC1806, MBCD5 and Hela, showing a high IC50 value against the non-tumor cell line HaCaT. Among them, the triple negative HCC1806 and DU4475 have gained importance in the last years mainly due to its resistance to targeted treatments. When compared to doxorubicin, StefB resulted less aggressive or toxic than the above anticanceur compound, making it an attractive drug for further studies. Doxorubicin was selected as positive control in this study since also belongs to the anthracycline family of bioactive compounds [43].

Regarding to its mechanism of action, StefB binds to double stranded DNA as showed by difference spectroscopy and an increase of the DNA thermal stability in the presence of the anthracycline [44]. Furthermore, the molecular and crystal structure of StefB has been described and used for modeling the intercalative binding with DNA, finding that StefB has a higher CpG sequence specificity over the TpA step, similar to that of daunorubicin and nogalamycin [45].

The behavior of the tumor cells to the presence of StefB has not been characterized. In our study we evaluated this response in the MCF7 cell line, as this was the most sensitive to the StefB effect. In this line, a decrease of cell proliferation via PCNA expression was observed with the presence of apoptotic cells and strong morphological changes with the presence of senescence cells that increased with the exposure time to StefB. Although apoptosis seems to be the cell death pathway as occurs with doxorubicin, the extremely morphological changes caused by StefB makes necessary to perform additional studies to know why these structural modifications occur in the cells and their subsequent implications.

Conclusion

In conclusion, an actinomycete with potential to produce bioactive compounds was isolated from \textit{Amphipterygium adstringens}. This strain identified as \textit{S. scabrisporus} was selected by a bioassay-guided study showing a strong cytotoxic potential against various cancer cell lines (breast and cervical carcinoma). A major compound purified from crude extracts of this strain and responsible of the biological activity was identified as StefB. This compound, formerly known for its different biological activities, strongly affected different breast cancer cell lines, including triple negative ones, with low toxicity against keratinocytes, making it a promissory drug for breast cancer treatment.

Acknowledgments

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Competing interest

The authors declare that they have no conflict of interest

Ethical statement

This article does not contain any studies with human participants performed by any of the authors

Authorship and contributor ship

KRP, SS, MLMR, LRZ and RRS contributed equally to conception and design of the project, data analysis and interpretation. KRP and...
MTU contributed to acquisition data. All authors revised the manuscript and gave the final approval of it and agree to act as guarantors of the work.

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