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Mutasynthesis of a Potent Anticancer Sibiromycin Analog

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Abstract

Pursuit of the actinomycete pyrrolobenzodiazepine natural product sibiromycin as a chemotherapeutic agent has been limited by its cardiotoxicity. Among pyrrolobenzodiazepines, cardiotoxicity is associated with hydroxylation at position 9. Deletion of the methyltransferase gene *sibL* abolishes the production of sibiromycin. Supplementation of growth media with 4-methylantranilic acid can substitute for its native 3-hydroxy congener that is natively produced. Cultures grown in this fashion yielded 9-deoxysibiromycin. In this study, we characterize the structure and biological activity of sibiromycin and 9-deoxysibiromycin methyl carbinolamines. Preliminary in vitro evidence suggests that 9-deoxysibiromycin exhibits reduced cardiotoxicity while gaining antitumor activity. These results strongly support further exploration of the production and evaluation of monomeric and dimeric glycosylated pyrrolobenzodiazepine analogs of sibiromycin.

The pyrrolobenzodiazepines (PBDs) represent a diverse family of natural products produced by diverse actinomycetes including *Streptomyces*, *Micromonospora*, and *Streptosporangia*(1). The PBDs consist of a tricyclic 6-7-5 system and exhibit antibacterial and anticancer activity by alkylating DNA. In this reaction the imine of the diazepine ring undergoes nucleophilic attack by an exocyclic guanine nitrogen upon binding in the minor groove of DNA. In solution, the imine form of the molecule is in dynamic equilibrium with the preferred carbinolamine form, but evidence(1) suggests that the imine form is the reactive species. The chirogenic 5-membered ring imparts curvature to the molecule in such a way that the molecule nearly perfectly fits inside the minor groove(2). DNA thus modified is believed to evade error-correction mechanisms, which typically sense errors by changes in DNA curvature(3).

Although sibiromycin is one of the most potent anticancer PBDs, a major obstacle to animal testing is its cardiotoxicity attributable to the C-9 hydroxyl group (Figure 1)(1, 4, 5). Supplementation of coenzyme Q in cardiomyocyte cultures appears to mitigate this cardiotoxicity; possibly because C-9 hydroxyl containing PBDs disrupt the mitochondrial electron transport system(5). Sibiromycin is one of only two known glycosylated PBDs (Figure 1), the other being sibanomicin(6), and its remarkable potency is dependent on the presence of the cationic sugar sibirosamine(7). Unfortunately, while some non-glycosylated PBDs are synthetically accessible(1, 8, 9), yielding molecules such as SJG-136 (Figure 1) currently in clinical trials(9), sibiromycin is synthetically intractable(1). The producer strain

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for sibanomicin, which also features the sibirosamine group but not the C-9 hydroxyl group, is not available.

The PBD scaffold is assembled via a two-component nonribosomal peptide synthetase (NRPS) that merges an anthranilic acid derived from chorismate (as for tomaymycin(10)) or tryptophan (as for anthramycin and sibiromycin(11, 12)) with a 3-substituted proline derived from tyrosine. Sibirosamine is derived from glucose-1-phosphate and added after completion of the PBD scaffold(11). The convergent nature of PBD biosynthesis strongly suggests the use of mutasynthesis to first validate if the elimination of the C-9 hydroxyl group in sibiromycin mitigates cardiotoxicity while retaining its anticancer properties, and later to derivatize this molecule. This strategy became possible when we identified the gene cluster responsible for sibiromycin biosynthesis(11). Here we report the first preparation of a glycosylated PBD analog, 9-deoxysibiromycin (Figure 1), and characterization of its DNA binding affinity, cardiotoxicity and anticancer properties.

The 3-hydroxy-4-methylanthranilate unit of sibiromycin derives from kynurenine obtained by tryptophan degradation(11). Recent biochemical studies have shown that 3-hydroxykynurenine is methylated by the SAM-dependent methyltransferase SibL, converted to 3-hydroxy-4-methylanthranilic acid by the PLP-dependent kynureninase SibQ and loaded on the NRPS SibE for formation of the PBD scaffold(13). We have obtained a *Streptosporangium sibiricum* Δ *sibL* strain that, grown in unsupplemented media, does not produce sibiromycin (Figure 2). The inability of this strain to produce sibiromycin despite SibQ and SibE's recognition of 3-hydroxykynurenine and 3-hydroxyanthranilic acid as substrates(13), respectively, is likely due to competitive depletion of 3-hydroxykynurenine by shunt pathways(11, 14) combined with a decreased catalytic efficiency of SibQ for this substrate (Figure 1). Supplementation with 4-methylanthranilic acid at 1 mM results in a peak that by reversed-phase high performance liquid chromatography exhibits a retention delay compared to sibiromycin and possesses a mass corresponding to 9-deoxysibiromycin (Figure 2). The production of the sibiromycin analog in this mutant strain upon addition of 4-methylanthranilic acid shows that the broad substrate specificity of SibE(13) extends to this compound.

Sibiromycin and 9-deoxysibiromycin were isolated by extraction and subjected to silica gel and size exclusion chromatography. Per volume yields of ~ 2.5 mg L⁻¹ of sibiromycin and ~ 1.3 mg L⁻¹ of 9-deoxysibiromycin were obtained from wild-type and Δ *sibL* culture, respectively. Full confirmation of the identity of the PBD produced by the Δ *sibL* supplemented with 4-methylanthranilic acid as the 9-deoxysibiromycin was obtained by 1D and 2D NMR characterizations of the methyl carbinolamine adduct as present in CD₃OD solvent (NMR spectra provided in the Supporting Information). Compared to the prior assignment of sibiromycin conducted in D₆-DMSO(15), which left some resonances unresolved, our assignment completely accounts for resonances of all expected protons and carbon nuclei (Supporting Table 2). We also report resonances for both diastereomers for 9-deoxy-sibiromycin and sibiromycin (Supporting Tables 1 and 2; Supporting Figures 2–12) resulting from methanol attack on opposite faces of the diazepine ring. NMR analysis also indicated 9-deoxysibiromycin modestly prefers solvent attack in *cis* with respect to the pyrrole ring, whereas sibiromycin prefers *trans* attack.

Biological assays were performed to assess the relative activities of both sibiromycin and 9-deoxysibiromycin relative to a reference PBD, anthramycin. 9-Deoxysibiromycin was tested in the calf thymus DNA melting assay routinely used to evaluate the DNA binding affinity of a PBD compared to well characterize PBDs(1). 9-Deoxysibiromycin shows far stronger stabilization ($\Delta T_m = 22$ °C) of the DNA against melting than either anthramycin ($\Delta T_m = 13$ °C) or sibiromycin ($\Delta T_m = 16$ °C). Thus, removal of the 9-hydroxy group has not adversely

affected the DNA affinity but rather it has increased it. In an initial evaluation on the cardiotoxicity properties we tested sibiromycin and 9-deoxysibiromycin on the human cardiac cell line AC16(16, 17). This assay suggests that 9-deoxysibiromycin is approximately five-fold less cardiotoxic than its parent compound (Supporting Figure 1). Unfortunately, we were unable to obtain SJG-136 to establish a baseline acceptable cardiotoxicity for anticancer PBDs.

Sibiromycin and 9-deoxysibiromycin were submitted to the NCI cancer screen (http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html). *In vitro* anti-tumor activities against selected cell lines are detailed in Table 1 (for the full list of lines screened and activities see Tables S3 and S4). The average concentration for 50 % growth inhibition (GI₅₀) is 5 fold less for 9-deoxysibiromycin (24 nM) than for sibiromycin (117 nM). GI₅₀ values for 9-deoxysibiromycin vary from 3 nM to 239 nM. While concentrations of sibiromycin higher than 1 μ M are necessary to obtain 50 % lethality in all cell lines (LC₅₀), concentrations as low as 58 nM and 25 nM of 9-deoxysibiromycin were sufficient for LC₅₀ and total growth inhibition (TGI) particularly in some melanoma and renal cancer cell lines. Overall, sibiromycin and 9-deoxysibiromycin display different cytotoxicity profiles based on the mean graph patterns. 9-Deoxysibiromycin is generally more potent than sibiromycin.

A COMPARE analysis(18) provided a comparison of the cytotoxicity profiles with other anticancer compounds tested in the NCI cancer screen. This indicated that sibiromycin and 9-deoxysibiromycin exhibit patterns of relative activity similar to DNA alkylating compounds as a class. However, sibiromycin and 9-deoxysibiromycin were each most similar to different compounds in the class and also distinct from SJG-136(19). Although all three compounds are PBDs, the DNA sequence adjacent to the reactive guanine base interacts differently with the different substituents on the PBD scaffold. The PBD substitution also creates differences in the twist of the PBD scaffold and different modes of binding in the minor groove(1). Combined, these differences in binding to DNA are likely the major factor contributing to the different cytotoxicity profiles that are observed in these three compounds. Other less studied properties such as cellular transport may also contribute to the different activities of these PBDs.

We report the first production of a glycosylated PBD, 9-deoxysibiromycin, and the biological characterization of this compound and its parent compound, sibiromycin. 9-Deoxysibiromycin showed diminished cardiotoxicity but increased potency against cancer cell lines relative to sibiromycin. Variations in relative potency of these compounds towards the cell lines in the panel are likely to be due to different DNA selectivity. The improved overall activity can be rationalized by the stronger binding towards DNA as reflected by the increase in ΔT_m . This rationale is consistent with the results observed for SJG-136(20). This is mechanistically distinct from the electron transport disruption caused by the 9-hydroxyl group present in sibiromycin parent compound. We speculate that an evolutionary preference for two moderate antibiotic activities may be preferable to a single potent activity in the host's environmental context to provide broad-spectrum activity or to hinder the development of resistance to the compound. To the best of our knowledge this is the first reported instance of a targeted mutasynthesis performed specifically to remove an undesirable side effect from a natural product compound.

METHODS

Bacterial strains and plasmids

Escherichia coli ET12567(21), and plasmids pIJ790 and pIJ773 were a generous gift from Dr. B. Gust (University of Tübingen). Growth and maintenance of *S. sibiricum* (DSM 44039) was performed as previously described.(11)

***sibL* Inactivation by Gene Replacement**

Single gene inactivation experiments were carried out in *E. coli* using the protocol based in REDIRECT technology(22) and as previously described(11). Insertional inactivation of the target gene was confirmed as previously described(11).

Batch production and purification of sibiromycin and 9-deoxysibiromycin

Twenty 500 mL Erlenmeyer flasks with metal springs and filled with 100 mL of sibiromycin media (30 g L⁻¹ of corn starch (Spectrum), 15 g L⁻¹ of Bacto soytone (BD), 50 mM CaCO₃ (Fisher) and 70 mM of NaCl (Fisher) were inoculated with 1 mL culture plug of wild-type or Δ *sibL* *S. sibiricum*. Cultures of Δ *sibL* *S. sibiricum* were supplemented upon inoculation with 15 mg of 2-amino 4-methylbenzoic acid (Maybridge) dissolved in 0.1 mL DMSO (Fisher) for a final concentration of 1 mM. Cultures were grown for ~36 h at 30 °C and harvested by centrifugation. The mycelial pellet was removed and the media was filtered. The filtered media (~1.5 L) was washed 5 times in 200 mL of 5 % isopropanol: 95 % pentanes. The aqueous layer was partitioned into 250 mL portions and extracted 3 times in 250 mL of dichloromethane. The organic and emulsion layers were pooled and frozen overnight to further separate the aqueous components. Frozen extract was cold-filtered at 4 °C and pooled; the dichloromethane was then removed by rotary evaporation at 10 °C. The remaining, dried crude extract was resuspended in 10 mL of methanol and stored at -20 °C. PBDs exist in aqueous solution in equilibrium between the imine and carbinolamine forms(1). Because neither form is ideal for long-term storage, PBDs are usually stored in methanol as the carbinolamine methyl ether.

Silica gel (Fisher) chromatography was performed by dry-packing the column (4.5 cm diameter, ~20 cm vertical length; liquid column volume ~200 mL) under positive pressure into 100 % chloroform, followed by equilibration by gravity drip (1 cv) and under positive pressure (1.5 cv) of 5 % methanol, 95 % chloroform. The stored crude extract in methanol was dried under rotary evaporation at 10 °C, reconstituted in a minimal volume of 5 % methanol, 95 % chloroform, and loaded on the silica gel column. The column was washed with 300 mL of 5 % methanol, 95 % chloroform. Sibiromycin or 9-deoxysibiromycin was eluted with 20 % methanol, 80 % chloroform. Collected fractions were analyzed by spot TLC of 10 μ L. UV-active fractions were analyzed by HPLC; fractions assessed to be of high yield and purity were pooled and dried under rotary evaporation at 10 °C, resuspended in 10 mL of methanol and stored at -20 °C.

Size exclusion chromatography was performed by gravity-packing 40 g of LH-20 resin (GE Healthcare) in 200 mL of methanol into a 1.5 m column, inner diameter 1.5 cm, followed by elution and pressure equilibration at a flow rate of 1 mL min⁻¹. Silica purified material was subjected to rotary evaporation at 10 °C (~50 % yield) and resuspended in a total of 1 mL of methanol and loaded onto the column, three times, to minimize volume and maximize yield. Collected fractions were analyzed and pooled as previously described. The purified sibiromycin or 9-deoxysibiromycin was resuspended in 10 mL of methanol, and stored at -20 °C. Production of sibiromycin and 9-deoxysibiromycin were performed in 2 L batches as described, netting ~30 mg of sibiromycin from a total 12 L of wild-type culture and ~40 mg of 9-deoxysibiromycin from a total 16 L of Δ *sibL* culture.

HPLC-ESI

Production of sibiromycin or sibiromycin analog was tested by HPLC-ESI on a Zorbax Eclipse XDB-C8 column (4.6 $\times$ 150 mm, Agilent) pre-equilibrated in 90 % solvent A (0.1 % (v/v) TFA in H₂O) and 10 % solvent B (methanol) at a flow rate of 1 mL min⁻¹. A linear gradient method (hold for 2 min solvent A at 90 %, 10–70 % solvent B for 18 min) was applied to the column. The various compounds were detected at 230 and 310 nm using an

Agilent 1100 HPLC system (Agilent), and by ESI-MS in the positive ion mode using a JEOL AccuTOF-CS mass spectrometer. Depending on the conditions of the analytical technique used, one or more forms were detected. Under the ESI-MS conditions three forms are detected for sibiromycin $[M + H]^+$ at $m/z = 458.2$ for the imine form, $m/z = 476.3$ for the carbinolamine form and $m/z = 490.3$ for the carbinolamine methyl ether form consistent with the molecular formula $C_{24}H_{31}N_3O_6$ (calculated 457.2), $C_{24}H_{33}N_3O_7$ (calculated 475.2) and $C_{25}H_{35}N_3O_7$ (calculated 489.3), respectively(11). Similarly, for 9-deoxysibiromycin three forms are detected $[M + H]^+$ at $m/z = 442.1$ for the imine form, $m/z = 460.2$ for the carbinolamine form and $m/z = 474.2$ for the carbinolamine methyl ether form consistent with the molecular formula $C_{24}H_{31}N_3O_5$ (calculated 441.2), $C_{24}H_{33}N_3O_6$ (calculated 459.2) and $C_{25}H_{35}N_3O_6$ (calculated 473.2), respectively. Fragmentation at the anomeric bond is observed with the appearance of peaks at 287.1 and 301.1 for the aglycone fragment in addition to 174.1 m/z assigned to the sibirosamine fragment. Similar fragmentation has been observed for sibiromycin(11).

NMR Spectroscopy

Samples of sibiromycin and 9-deoxysibiromycin were prepared in 1 mL of CD_3OD to a final concentration of approximately 10 mM and 5 mM, respectively. 1H and ^{13}C NMR spectra (Supporting Information) were acquired on a Bruker DRX-400 at 300 K and 2D NMR spectra (1H - 1H COSY, ^{13}C - 1H HSQC and ^{13}C - 1H HMBC spectra; Supporting Information) were acquired on a Bruker AVIII-600 at 300 K. Standard pulse sequences were used and spectra chemical shifts were referenced against solvent CHD_2OD or $^{13}CD_3OD$ peaks. Simulations of certain peak sets for sibiromycin were performed by fitting the frequency-domain data to a sum of Gaussians separated by fixed intervals whose integration correspond to the appropriate binomial coefficients corresponding to the splitting conditions. This simulation was performed using Mathematica's Nonlinearregress function with the Levenburg-Marquadt algorithm and used to extract chemical shift and J-coupling values for the following resonances: 1β (both diastereomers) $5'$ (both diastereomers), 11a (minor diastereomer), and 13 (both diastereomers) (Figure S3).

Calf-thymus DNA Melting Point Assay

Calf-thymus DNA (CT-DNA) type I, highly polymerized (Sigma-Aldrich D1501) was reconstituted in 10 mM sodium phosphate, 1 mM EDTA, 0.02 % (w/v) NaN_3 , pH 7.0, at room temperature for 2 h and aliquoted to 100 μM ($OD_{260} = 0.6$). Samples were prepared to a final concentration ratio of DNA:PBD = 1:20. DNA was subjected to thermal denaturation from 25 $^{\circ}C$ to 94 $^{\circ}C$ at a melting rate of 1 $^{\circ}C$ per minute. Two different melting curves were collected at time zero and after 18 h incubation at 37 $^{\circ}C$. Experiments were performed in triplicate. The melting curve for CT-DNA treated with 9-deoxysibiromycin did not show a wide post-transition region necessary for thermodynamic modeling, so the melting temperature T_m for all samples was estimated by calculating the discrete derivative of the melting curve using $\Delta OD_{260}/\Delta T$ for each pair of successive data points on the melting curve. T_m was assigned to the maximum of this derivative. Data processing for this step was performed in Mathematica.

Cardiomyocyte-based Assay

The human cardiac cell line AC16(17) was maintained in DMEM:F12 (1:1) supplemented with 10 % FBS, 1 mM HEPES buffer, 2 mM L-glutamine, 100 units mL^{-1} penicillin and 100 $\mu g mL^{-1}$ streptomycin. Two- to four-day-old cultures (~70 % confluent) were used for the experiments. The human cardiac AC16 cells were seeded in black wall, clear bottom, tissue culture treated 96-well plates (Costar) at a density of 250 cells per well, in Opti-MEM supplemented with 5 % FBS, 1 mM HEPES buffer, 2 mM L-glutamine, 100 units mL^{-1} penicillin and 100 $\mu g mL^{-1}$ streptomycin, 0.05 $mg mL^{-1}$ $CaCl_2$ and incubated overnight at

37 °C. Serial dilutions of sibiromycin and 9-deoxysibiromycin (stocks 20 mM in EtOH) were prepared in Hank's Balanced Salt Solution (HBSS) at concentrations ranging from 20 μ M to 0.2 nM and then diluted 1:1 with Opti-MEM supplemented with 0.8 mg mL⁻¹ BSA, 2 mM HEPES buffer, 4 mM L-glutamine, 200 units mL⁻¹ penicillin, 200 μ g mL⁻¹ streptomycin and 0.1 mg mL⁻¹ CaCl₂. The medium from the cells was removed and replaced immediately by the diluted test compounds or similarly diluted HBSS (vehicle control) and incubated 24 h and 48 h at 37 °C after which cell viability was evaluated by resazurin reduction assay(17). Briefly, 10 μ L per well of resazurin (500 μ M, PBS) was added to each well and incubated for 2–3 h at 37 °C. Viable cells reduce resazurin to the highly fluorescent resorufin dye, which is quantitated in a multiwell plate fluororeader (Ex/Em 530/590 nm, Tecan Safire2). Cell viability was calculated as percentage of fluorescence relative to cells treated with vehicle only (100 % viability) after subtraction of blank fluorescence (wells without cells). All experimental conditions were performed in triplicate. Averages and SEM corresponding to 2 independently performed experiments were calculated. To obtain experimental EC₅₀ values the data were analyzed by nonlinear regression using a sigmoidal dose response curve model with variable slope (GraphPrism). The model R² values were >0.98 in all cases (Supporting Figure 1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

PBDs	pyrrolobenzodiazepines
NRPS	nonribosomal peptide synthetase

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Figure 1.

A) Structures of natural (anthramycin and sibanomycin) and synthetic (SJG-136) PBDs discussed. B) Biosynthesis of sibiromycin. C) Mutasynthesis of 9-deoxysibiromycin, a sibiromycin analog. The analog was obtained by feeding 4-methylantranilic acid to an unproductive mutant strain of *S. sibiricum*.

Figure 2.

HPLC analysis of the secondary metabolites produced by wild-type and the $\Delta sibL$ mutant *S. sibiricum*. ESI-MS analysis of the 17.31 min shows mass peaks corresponding to the imine, carbinolamine and carbinolamine methylether forms(11) of 9-deoxysibiromycin (442.1, 460.2 and 474.2 m/z, respectively) and of the sibirosamine and aglycone fragments (174.1, 287.1 and 301.1, respectively).

Table 1Selected GI₅₀ (nM) NCI 60 Cell Line Screen Results

Cell line/type	Sibiromycin	9-Deoxysibiromycin
SR/leukemia	25.1	3.2
NCI-H522/lung	120.2	7.6
OVCAR-8/ovarian	195.0	17.8
MCF7/breast	131.8	8.5
SK-MEL-5/melanoma	154.9	14.1