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NEW ALKALOID ANTIBIOTICS THAT TARGET THE DNA TOPOISOMERASE I OF *STREPTOCOCCUS PNEUMONIAE**

María Teresa García[1], María Amparo Blázquez[2], María José Ferrándiz[1], María Jesús Sanz[2], Noella Silva-Martín[3], Juan A. Hermoso[3] and Adela G. de la Campa[1*]

From Unidad de Genética Bacteriana, Centro Nacional de Microbiología and CIBER Enfermedades Respiratorias, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid[1], Departamento de Farmacología. Facultat de Farmàcia, Universitat de València, Vicent Andrés Estellés s/n. 46100 Burjasot, Valencia[2], and Grupo de Cristalografía Macromolecular y Biología Estructural, Instituto de Química-Física "Rocasolano", CSIC, Serrano 119, 28006 Madrid[3], Spain.

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Address correspondence to: Adela G. de la Campa, PhD, Carretera a Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain. Fax: 34-91-509-7919; E-mail: agcampa@isciii.es.

***Streptococcus pneumoniae* has two type II DNA-topoisomerases (DNA-gyrase and DNA topoisomerase IV) and a single type I enzyme (DNA-topoisomerase I, TopA), as demonstrated here. While fluoroquinolones target type II enzymes, antibiotics efficiently targeting TopA have not yet been reported. Eighteen alkaloids (7 aporphine and 11 phenanthrenes) were semisynthesized from boldine and used to test both inhibition of TopA activity and of cell growth. Two phenanthrenes (seconeolitsine and N-methylseconeolitsine) effectively inhibited both TopA activity and cell growth at equivalent concentrations (~17 μM). Evidence for *in vivo* TopA targeting by seconeolitsine was provided by the protection of growth inhibition in a *S. pneumoniae* culture in which the enzyme was overproduced. Additionally, hypernegative supercoiling was observed in an internal plasmid after drug treatment. Furthermore, a model of pneumococcal TopA was made based on the crystal structure of *E. coli* TopA. Docking calculations indicated strong interactions of the alkaloids with the nucleotide-binding site in the closed protein conformation, which correlated with their inhibitory effect. Finally, although seconeolitsine and N-methly-seconeolitsine inhibited TopA and bacterial growth, they did not affect human cell viability. Therefore, these new alkaloids can be envisaged as new therapeutic candidates for the treatment of *S. pneumoniae* infections resistant to other antibiotics.**

Antibiotic resistance in bacterial pathogens is a serious clinical problem. This problem affects to *Streptococcus pneumoniae* (the pneumococcus) that in addition to be one of the principal human pathogens, is the main ethyological agent of community-acquired pneumonia. Annually, about 1 million children aged <5 years die of pneumococcal pneumonia, meningitis, and/or sepsis worldwide (1). Resistance to currently used antimicrobial drugs for the treatment of pneumococcal infections, including beta-lactams and macrolides, has spread worldwide in the last two decades (2). The new fluoroquinolones, such as levofloxacin and moxifloxacin, which act on type II DNA topoisomerases, are nowadays therapeutic alternatives for treatment of adult patients with community-acquired pneumonia, (3). However, although resistance to fluoroquinolones in *S. pneumoniae* is still lower than 3% (4), an increase in resistance is likely to occur.

DNA topoisomerases participate in almost all cellular functions involving DNA transactions (5). They solve the topological problems associated with DNA replication, transcription, and recombination. In addition, these enzymes fine-tune the steady-state level of DNA supercoiling both facilitating protein interactions with the DNA and preventing excessive supercoiling that is deleterious. In bacteria, the homeostasis of DNA supercoiling is maintained by the opposing activities of topoisomerases that relax DNA, and gyrase that introduces negative supercoils. The transcriptional response to DNA relaxation involves genes coding for all the DNA

topoisomerases from *S. pneumoniae*: topoisomerase I, topoisomerase IV, and DNA gyrase. Relaxation of DNA triggers the transcriptional up-regulation of gyrase and the down-regulation of topoisomerase I and topoisomerase IV (6).

The enzymatic activity of topoisomerases involves DNA cleavage and the formation of a transient phosphodiester bond between a catalytic tyrosine residue in the protein and one of the ends of the broken strand. DNA topology is modified during the lifetime of the covalent intermediate, and the enzyme is released as the DNA is religated. Topoisomerases are classified into two types based on their DNA cleavage pattern: type I enzymes that only cleave one DNA strand, and type II enzymes that cleave both strands. The type II topoisomerases are tetrameric proteins formed by two different subunits: $GyrA_2GyrB_2$ for gyrase and $ParC_2ParE_2$ for topoisomerase IV. Fluoroquinolones inhibit type II enzymes and mainly affect chromosome replication. They also stabilize a reaction intermediate in which the enzymes are covalently linked to the DNA originating double-stranded breaks that lead to cell death (7). Genetic and biochemical studies have shown that in Gram-positive bacteria, including *S. pneumoniae*, topoisomerase IV is the primary target for most fluoroquinolones and gyrase is a secondary target (8, 9). The type I enzymes are classified as either subfamily type IA if the protein links to the 5' phosphate or subfamily type IB when the protein attaches to the 3' phosphate. There is extensive sequence similarity among members of the same subfamily (10) but almost no sequence or structural similarity between the two subfamilies (11,12). All bacterial type I topoisomerases are type IA enzymes. The overall structure of *Escherichia coli* topoisomerase I presents four domains with the active site located at the intersection of domains I and III where the catalytic Tyr319 residue is placed (13). The proposed mechanism of action involves the opening of the enzyme through a large conformational change that separates domains II and III from the rest of the protein (14). A nucleotide-binding site has been identified in *E. coli* at the interface of domains I, III and IV. This nucleotide-binding site, the only one formed by residues from the three domains in the closed conformation, has been proposed to bind the region of the 3'-OH end of the cleaved DNA strand (14).

Cheng *et al* (15) have recently described one phenanthrene alkaloid able to inhibit the relaxation activity of *E. coli* topoisomerase I. However, no significant inhibition in cell growth was observed. The aim of the present study was to investigate the therapeutic potential of targeting pneumococcal topoisomerase I. For this purpose new alkaloid inhibitors of its enzymatic activity have been developed and tested for their effects on DNA supercoiling. Furthermore, their effect on cell growth was also evaluated.

Experimental Procedures

Bacterial strains, growth and transformation of bacteria- S. pneumoniae was grown in a casein hydrolysate-based medium with 0.3% sucrose (AGCH) as energy source and transformed with chromosomal or plasmid DNA as described previously (16). Minimal inhibitory concentrations (MICs) were determined in the same medium by the microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) (17). The MIC was defined as the lowest concentration of drug without visible growth.

Structures of N-metilseconeolitsine and seconeolitsine- The structural elucidation of compounds 16 (N-metilseconeolitsine,) and 17 (seconeolitsine) were determined by ^{1}H and ^{13}C-NMR using one-dimensional and two-dimensional (homonuclear COSY, heteronuclear multiple quantum coherence HMQC, and heteronuclear multiple bond connectivity HMBC) experiments in conjunction with mass spectral analysis and UV spectrum. Parameters were the following:

-Seconeolitsine: $C_{19}H_{17}NO_4$; mp 111-113ºC; UV (MeOH): λ_{max} (log ε) = 262 (0.40), 289 (0.12), 325 (0.07) nm; ^{1}H-RMN (DMSO-d_6) δ (ppm): 2.32 (3H, *s*, N-CH_3), 2.76 (2H, *m*, $CH_{2\alpha}$), 3.14 (2H, *m*, $CH_{2\beta}$), 6.16 (2H, *s*, OCH_2O-6,7), 6.24 (2H, *s*, OCH_2O-3,4), 7.22 (1H, *s*, H-2), 7.40 (1H, *s*, H-8), 7.56 (1H, *d*, *J*= 9.3 Hz, H-9), 7.81 (1H, *d*, *J*= 9.3 Hz, H-10), 8.39 (1H, *s*, H-5); ^{13}C-RMN (DMSO-d_6) δ (ppm): 32.88 ($CH_{2\beta}$), 35.75 (NCH_3), 52.73 ($CH_{2\alpha}$), 100.93 (OCH_2O, C-3,4),

101.48 (OCH_2O, C-6,7), 104.37 (C-5), 105.19 (C-8), 110.29 (C-2), 116.28 (C-4a), 121.31 (C-10), 123.44 (C-5a), 124.32 (C-9), 125.01 (C-10a), 128.15 (C-8a), 131.33 (C-1), 140.64 (C-4), 144.03 (C-3), 147.03* (C-7) 147.10* (C-6); EI-MS *m/z* (int. rel.)= 323 (10), 280 (100), 279 (32), 221 (2), 163 (14).

-*N*-metilseconeolitsine: C_{20} H_{19} N O_4; mp 132-134ºC; UV (MeOH): λ_{max} (log ε) = 262 (0.40), 289 (0.12), 325 (0.07) nm; ^{1}H-RMN (DMSO-d_6) δ (ppm): 2.22 (6H, *s*, N-$(CH_3)_2$), 2.49 (2H, *m*, $CH_{2\alpha}$), 3.12 (2H, *m*, $CH_{2\beta}$), 6.17 (2H, *s*, OCH_2O-6,7), 6.25 (2H, *s*, OCH_2O-3,4), 7.25 (1H, *s*, H-2), 7.41 (1H, *s*, H-8), 7.57 (1H, *d*, *J*= 9.3 Hz, H-9), 7.75 (1H, *d*, *J*= 9.3 Hz, H-10), 8.39 (1H, *s*, H-5); ^{13}C-RMN (DMSO-d_6) δ (ppm): 30.79 ($CH_{2\beta}$), 45.10 ($N(CH_3)_2$), 60.51 ($CH_{2\alpha}$), 100.94 (OCH_2O, C-3,4), 101.48 (OCH_2O, C-6,7), 104.39 (C-5), 105.19 (C-8), 110.29 (C-2), 116.28 (C-4a), 121.15 (C-10), 123.47 (C-5a), 124.40 (C-9), 124.90 (C-10a), 128.12 (C-8a), 131.50 (C-1), 140.60 (C-4), 144.05 (C-3), 146.80 (C-6) 146.80 (C-7); EI-MS m*/z* (int. rel.)= 337 (100), 279 (63), 221 (5), 191 (5), 163 (58).

Cloning and expression of topA in E. coli- The *topA* gene was amplified by PCR with 0.1 µg of chromosomal DNA from *S. pneumoniae* R6 and 1 µM (each) synthetic oligonucleotide primers. Oligonucleotides used were topAUP2 (5'-GTGGCTACGGCAACAAAAAAGAA-3') and topADOWN (5'-cgcgcgcatgcTTATTTAATCTTTTCTTCCTC-3'). The 5' end of topADOWN contained a sequence including a *PaeI* restriction site (lower case letters), topAUP2 included the GTG initiation codon and topADOWN the sequence complementary to the TAA stop codon (underlined). Amplification was achieved with an initial cycle of 2 min denaturation at 94 ºC, 1 min annealing at 55ºC, and 2.5 min polymerase extension with Pfu high-fidelity polymerase (Fermentas) at 72 ºC. Then 30 cycles of 2 min 94 ºC, 1 min at 55 ºC and 2.5 min at 72 ºC with slow cooling at 10 ºC. Oligonucleotides were removed (QIAquick PCR Purification Kit, QIAGEN), cut with PaeI, cloned into plasmid pQE1 digested with PaeI + PvuII, and established in *E. coli* M15 (pREP4) (QIAGEN). Sequencing with oligonucleotides pQE-seq3 (5'-AGCTAGCTTGGATTCTCACC-3') and pQE-seq5 (5'-GAGGCCCTTTCGTCTTCA-3') was performed to confirm the cloning. The pQE1 vector/M15(pREP4) host cloning system permits the hyperproduction of 6× His-tagged recombinant proteins encoded by genes placed under the control of a phage T5 promoter and two *lac* operator sequences. The host strain contains the low-copy plasmid pREP4 which constitutively expresses the LacI repressor. Expression of recombinant proteins encoded by pQE vectors was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the LacI protein and inactivates it. This inactivation allows the host cell's RNA polymerase to transcribe the sequences downstream from the T5 promoter. A culture of *E. coli* M15 (pREP4)/ pQE-SPNtopA was grown at 37 ^{o}C in LB medium containing 250 µg/ml of ampicilin (to select pQE1) and 25 µg/ml kanamicin (to select pREP4) to DO_{620} = 0.6. IPTG (1mM) was added and incubation continued for 30 min. Cells were collected by centrifugation, lysed at 4°C for 30 min in buffer A (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10 mM imidazol) and lisozyme (1mg/ml) and sonicated for 5 min (10 times for 30 s, with 1 min cooling between each sonication) in a Sonifier B-12 (Branson Co, Connetticut). Debris was removed by centrifugation at 10.000 × g for 20 min and the resulting supernatant dialyzed overnight against buffer A. The TopA protein was purified by affinity chromatography in Ni-NTA (QIAGEN) column following manufacturer's instructions. Briefly, column (1 ml) was washed with 10 ml of buffer A and the bound proteins eluted with 10 ml of buffer containing imidazol at 60, 100, and 200 mM. Fractions of 2 ml were collected and analyzed by SDS-10% polyacrylamide gel and stained with comassie blue. Fractions containing a protein of the expected size were dialyzed against buffer B (20 mM TrisHCl pH 8, 50 mM KCl, 1mM DTT, 50% glicerol). The purified protein was conserved at -20 ^{o}C. In these conditions, TopA remains active for at least 12 months.

Cloning of topA under the control of Pmal in S. pneumoniae- The *topA* gene was amplified by PCR from strain R6 as described above except that primer topAUP4 (5'-CGCGCtctagaAGGTGTGATACTATGGCT -3') was used instead topAUP2. The 5' end of topAUP4 contained a sequence including an

XbaI restriction site (lower case letters) and included an ATG initiation codon (underlined). The amplified fragment was cut with PaeI + XbaI, cloned into plasmid pLS1RGFP (18) digested with the same enzymes and established in strain R6. Transformed bacteria were selected in AGCH containing erythromycin (1 µg/ml). Recombinant plasmids were identified by digestion with HindIII + EcoRI and confirmed by sequencing with oligonucleotides pLS1rF (5'- GAGTATACTTATAAGTAACGCAAAC-3') and pLS1rR (5'-TAGGTTGAGGCCGTTGAGCACC -3').

Relaxation of pBR322 by TopA and human TOPO1- Reactions were carried out in 15 or 100 µl containing 0.5 µg of supercoiled plasmid pBR322 in 20 mM Tris HCl pH 8, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50 µg BSA/ml. After 1 h incubation at 37°C in the presence on TopA, the reaction was terminated by 2 min incubation at 37ºC with 50 mM EDTA and 1 h at 37°C with 1% SDS, 100 µg/ml proteinase K. Treatment with human TOPO1 (Inspiralis, Norwich, UK) was performed with the buffer and conditions recommended by the supplier. When required, samples were ethanol precipitated and resuspended in H_2O. Reaction products were analyzed by electrophoresis in 1.2% agarose gels run at 18 V for 20 h. Treatment with the alkaloids was performed by preincubation of TopA or TOPO1 during 10 min at 4 °C in a final volume of 15 µl. DNA quantification of agarose gels was done by scanning densitometry after electrophoresis and ethidium bromide (EtBr) staining. Quantification of TopA activity was calculated by gel densitometry using the Quantity One program (Bio-Rad laboratories). To calculate activity, the amount of the CCC form was determined and divided by the total amount of DNA in each well. IC_{50} (mean of at least three independent determinations) was defined as the concentration of drug required for a 50% reduction of enzymatic activity.

Analysis of the topology of covalently closed circles- Circular DNA molecules were analyzed in neutral/neutral two-dimensional agarose gels. The first dimension was run at 1.5 V/cm in a 0.4% agarose (Seakem; FMC Bioproducts) gel in 1 × Tris-borate-EDTA (TBE) buffer for 17−19 h at room temperature. The second dimension was run at 7.5 V/cm in 1% agarose gel in 1 × TBE buffer for 7−9 h at 4ºC. Chloroquine (Sigma) was added to the TBE buffer in both, the agarose and the running buffer. After electrophoresis gels were subjected to Southern hybridization. Two probes were used on two-dimensional agarose gels transferred to nylon membrane (Inmobylon NY^+, Millipore). For the analysis of the supercoiling level in pLS1 *in vivo*, a 240-bp PCR fragment obtained from pLS1 as described (6) was used. For the analysis of the enzymatic activity of TopA, pBR322 plasmid was digested with EcoRI + EcoRV and the ends filled with byotinilated dNTPs by the Klenow polymerase Fragment (Fermentas) and used as a probe. Chemiluminiscent detection of DNA was performed with the Phototope® -Star kit (New England Biolabs). Images were captured in a VersaDoc MP400 system and analyzed with the Quantity One program (BioRad).

Cytofluorometric analysis of neutrophil apoptosis and survival. Human peripheral blood neutrophils were obtained from buffy coats of healthy donors by Ficoll Hypaque density gradient centrifugation, as previously described (19). Freshly isolated neutrophils were resuspended in supplemented RPMI at 2 x 10^6 cells/ml. Twenty-five µl were cultured in a 96-well plate containing 200 µl supplemented RPMI during 24 h in the absence or presence of compounds 16 and 17. Assessment of apoptosis was performed by flow cytometry using annexin V-FITC and propidium iodide (PI). The protocol indicated by the manufacturer (Annexin-V-Fluos; Roche Applied Science) was used as previously outlined (20). Cells (1 x 10^4) were analyzed in a Beckman Coulter Epics XL (Fullerton, CA) and differentiated as early or viable apoptotic (annexin V^+, PI^-), late apoptotic and/or necrotic (annexin V^+ and PI^+), and viable nonapoptotic (annexin V^- and PI^-) cells.

EtBr displacement assay-A total of 6 µg (300 µl) of calf thymus DNA was diluted to 3 ml with buffer (20 mM NaCl, 2 mM HEPES, 10 µM EDTA, pH 7.4). Prior to analysis, 3 µl of EtBr (0.5 mg/ml) were added and allowed to equilibrate 1 min. Aliquots of alkaloids were then added and the fluorescence measured on an Cary Eclipse Varian fluoresce spectrophotometer after 1 min equilibration, using excitation and emission wavelengths of 546 and 660 nm, respectively.

Molecular modelling of topoisomerase I from S. pneumoniae- The model was built on the basis of the crystal structure of *E. coli* topoisomerase I (PDB code: 1CY1). Amino acid changes along the entire sequence were performed using the O program (21), running in a Silicon Graphics workstation. Side-chain rotamers were chosen from a database of more common conformers. The overall fold of the model was energy minimized using the minimiser algorithm implemented in the CNS package (22). The stereo chemical quality of the model was checked with the PROCHECK program (23).

Three-dimensional structures of alkaloid ligands and molecular docking- Boldine, secoboldine, seconeolitsine, and N-methyl seconeolitsine were used in the docking calculations. The three-dimensional structures of boldine and secoboldine were obtained from the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/) with reference codes HIHXUR and OLIDES respectively. The three-dimensional structures of seconeolitsine and N-methyl seconeolitsine were modelled on the basis of the crystallographic structures of boldine and secoboldine by using the Dundee PRODRG2 Server (24). Stereochemistry of ligands was checked with Mercury program (25). Molecular docking was carried out using GOLD (Genetic optimization for Ligand Docking) software (26), that uses the Genetic Algorithm (GA). This method allows a partial flexibility of protein and full flexibility of the ligand. The cavity was defined from to 10 Å around Ser487, giving freedom of movement to Asp101, Arg102, Arg156, Arg300, Arg485, Asp543 and Glu546 in side chains rotamers. For each of the 25 independent GA runs, a maximum number of 100000 GA operations were performed on a set of five groups with a population size of 100 individuals. Default cut-off values of 2.5 Å (dH-X) for hydrogen bonds and 4.0 Å for van der Waals distance were employed. When the top three solutions attained RMSD values within 1.5 Å, GA docking was terminated. The RMSD values for the docking calculations are based on the RMSD matrix of the ranked solutions. We observed that the best-ranked solutions were always among the first 10 GA runs, and the conformation of molecules based on the best fitness score was further analyzed.

RESULTS

Characterization of the DNA topoisomerase I of S. pneumoniae. We performed a BLAST search for DNA topoisomerases coding sequences in the *S. pneumoniae* R6 genome (27) (http://www.streppneumoniae.com/) and found that, besides the genes coding the two type II enzymes (gyrase and topoisomerase IV), gene *topA* (spr1141) encodes an homologue of bacterial topoisomerase I. The topoisomerase I enzymes from *E. coli* and *S. pneumoniae* show 46.9% similarity on the whole sequence and 62% in the cleavage/ strand passage module (Fig. 1A). The *topA* gene was amplified by PCR from R6 chromosomal DNA with specific oligonucleotides and cloned into the *E. coli* plasmid pQE-1 rendering pQE-SPNtopA, which carries the Met-$(His)_6$-Gln-TopA fusion protein under the control of the T5 promoter. This plasmid was established into *E. coli* M15 (pREP4) and its T5 promoter was induced by IPTG. The recombinant plasmid overproduced a protein with apparent Mr 84 kDa, and an apparent purity of 98% (Fig. 1B), which is in agreement with the expected size of the fusion protein (79.37 kDa for TopA + 5.23 kDa for the N-terminal fusion). Total yield of purification was 0.8 mg of purified TopA per liter of culture, with a concentration of 80 µg/ml and a specific relaxation activity (see below) of 2.4 x 10^4 units/ mg.

To test DNA relaxation activity of TopA, covalently-closed negatively supercoiled (CCC) pBR322 plasmid (1.77 nM) was incubated in the presence of different amounts of the purified enzyme and the reaction products analyzed in mono-dimensional agarose as described (28, 29). TopA activity converted the CCC plasmid in topoisomers with different degrees of supercoiling that were observed as discrete bands when the gel was run in the absence of EtBr (Fig. 2A). However, all relaxed topoisomers (RC) migrated as a single band when the gel was run in the presence of saturating concentrations of EtBr (Fig. 2B). A unit of TopA enzymatic activity was defined as the amount of enzyme being able to relax 50% of the CCC substrate in 1h at 37ºC, and corresponded to 42 ng (5 nM) of the purified enzyme. As observed in other bacterial

topoisomerase I enzymes, the pneumococcal TopA did not relax positively-supercoiled DNA (Fig. 2C). This was shown by the absence of activity on CCC pBR322 treated with EtBr (Fig. 2C, lane +EtBr), which, at the high concentration used, introduces positive supercoiling. The activity was partially recovered when the intercalating agent was removed with isoamyl alcohol (Fig. 2C, lane ± EtBr). An additional method to examine pBR322 topoisomers distribution after TopA treatment was two-dimensional agarose gel electrophoresis in the presence of chloroquine (30). This technique, which differs from the classical two-dimensional chloroquine gels, makes it possible to separate DNA molecules by mass and shape. In the two-dimensional gel, topoisomers appeared distributed in the autoradiograms in a bubble-shaped arc, negative supercoiled molecules were located on the right-side and positive supercoiled ones on the left (Figs 2D and 2E). In the absence of enzyme, 100% of the topoisomers had negative supercoiling (Figs 2D). In the presence of 1 unit (5 mM) of enzyme 56.8% and 43.2% were negative and positive supercoiled topoisomers, respectively. In the presence of 3 units (15 mM) of TopA, these figures were of 16.8% and 83.2% for negative and positive supercoiled topoisomers, respectively (Fig. 2D).

Inhibition of TopA activity by aporphine and phenanthrene alkaloids. A total of 18 compounds (6 aporphine and 12 phenanthrene alkaloids, supplemental Fig. S1) semisynthesized from the natural alkaloid boldine (31, and unpublished results) were selected to test inhibition of growth and inhibition of *S. pneumoniae* TopA activity. As shown in Table 1, previously characterized clinical isolates (4), either susceptible or resistant to various antibiotics (including fluoroquinolone resistant) were tested. The alkaloids showed equivalent activities against all isolates, independently of their antibiotic resistance, suggesting the absence of cross-resistance. Two phenanthrene alkaloids, with numbered compounds 16 and 17 (Fig. 3), showed the greatest inhibition of growth. The structural elucidation of these compounds was carried out as described in material and methods.

The *in vitro* inhibition of TopA relaxation activity was tested with the 18 compounds using 1 unit of enzyme, i.e. the amount of enzyme yielding 50% of activity. Under these conditions, TopA relaxation activity was inhibited in a concentration-dependent manner, with IC_{50} values (average ± SD) of 559±72.0 (n=3) for secoboldine (compound 2), 58.2± 3.0 (n=3) for neolitsine (compound 14), 17±0.4 (n=5) for N-methyl-seconeolitsine (compound 16) and 17±0.4 μM (n=4) for seconeolitsine (compound 17) (Figs 4A and 4B). A good correlation (r^2= 0.96) between the inhibition of TopA activity and the inhibition of cell growth was observed for these compounds. These results imply that topoisomerase I is indeed the *in vivo* target of the compounds assayed. The inhibition by the alkaloids was enhanced by preincubation of the enzyme with the drug. Lower inhibition was observed when preincubation was avoided (Fig 4C). These results suggest that the alkaloid would interact with the enzyme at the DNA binding site (see below).

Some inhibition of the relaxation activity over pBR322 was observed for compounds 2 and 5 at concentrations ≥100 μM (Fig. 4A). However, no inhibition for boldine (Fig. 4A) and the rest of the compounds (data not shown) was observed at concentrations ≤ 500 μM. The inhibition of TopA was tested with EtBr, a typical DNA intercalant compound. A pattern compatible with the intercalation of EtBr into the CCC pBR322 form was observed (Fig. 4C). In addition, preincubation did not change the observed pattern. These results suggest that TopA was not inhibited by EtBr.

Human topoisomerase I (TOP1), a prototype IB topoisomerase, was tested in relaxation assays with compounds 16 (N-metilseconeolitsine) and 17 (seconeolitsine) (Fig. 5A). Activation was observed at low concentrations and partial inhibition at 50 μM, a concentration at which the pneumococcal TopA showed full inhibition (data not shown). These effects on TOP1 could be attributed to the intercalation of compounds 16 and 17 in the DNA. As showed in Fig. 5B, both compounds displace EtBr from calf thymus DNA. At 50% displacement, the ratio alkaloid/ EtBr was 2.5 (compound 16) and 2.8 (compound 17).

Targeting of TopA in vivo by seconeolitsine. To asses that TopA is the *in vivo*

target of the alkaloids, different attempts were made to obtain resistant mutants to these compounds. About 1.2 × 10^{10} cells were plated on agar plates containing 1 to 8 × MIC of seconeolitsine and no resistant bacteria were obtained. Therefore, to establish that the primary cellular target for the phenanthrene alkaloids was indeed topoisomerase I, the *topA* gene was cloned under the control of the P*mal* promoter into plasmid pLS1R, yielding plasmid pLS1R-topA. The activity of this promoter was induced by maltose (18). The effect of the drug was tested in the wild type strain R6 (seconeolitsine MIC = 16 μg/ml) carrying either pLS1R or pLS1R-topA plasmids, at drug concentrations ranging from 0.25× to 1× MIC. These results shown that cell growth (Fig. 6A) and division (Fig. 6B) were affected in a drug concentration-dependent manner. The growth of R6 carrying either of these plasmids showed similar kinetics in the absence or presence of seconeolitsine at 1 × MIC the drug (Fig. 6). In contrast, differences both in growth inhibition and viability were observed at 0.25 × MIC and 0.5 × MIC, where inhibition by seconeolitsine was attenuated by the induction of TopA in plasmid pLS1R-topA (Fig. 6).

Further evidence of the *in vivo* inhibition of TopA by seconeolitsine was obtained from the analysis of topoisomers distribution of the internal plasmid pLS1 by two-dimensional agarose gel electrophoresis, a suitable approach for studying supercoiling levels (6). This technique allows to separate DNA molecules by mass and shape. First, pLS1 linking number (ΔLk) was quantified using two-dimensional agarose gel electrophoresis with 15 μg/ml chloroquine in the second dimension. Under these conditions, the induced ΔLk of monomers was −31. Treatment with compound 16 at 0.5 × MIC and 1 × MIC resulted in a time-dependent increase of plasmid supercoiling (Fig. 7). At 0.5 × MIC, supercoiling densities (σ) were −0.059, −0.086, and −0.093 at 0, 5 and 30 min after drug addition, respectively, indicating that plasmids became hypernegatively- supercoiled. Similar results were obtained after treatment at 1 × MIC, where supercoiling densities were −0.093 and −0.100 at 5 and 30 min respectively. Although direct extrapolation from observations made in small plasmids is not completely equivalent to the bacterial chromosome, our results indicate that supercoiling significantly increases in the presence of seconeolitsine, and support that TopA is its *in vivo* target.

Modelling of S. pneumoniae TopA and docking with alkaloids. We have observed a 46.9% sequence similarity between topoisomerase I enzymes from *E. coli* and *S. pneumoniae* (Fig. 1A). Therefore, a structural model of the neumococal TopA was performed based on the structure of the *E. coli* enzyme. The model shows that residues forming the active site are mainly conserved between *E. coli* and *S. pneumoniae*. The catalytic Tyr319 of *E. coli* corresponds to Tyr314 of *S. pneumoniae* (Fig. 8A). The nucleotide-binding site (binding of the 3'-OH end of the cleaved DNA strand) has been identified in *E. coli* at the interface of domains I, III and IV (14). The equivalent site in *S. pneumoniae* TopA presents a strong salt bridge interaction network at both sides of the cavity. This network is formed by residues R485-D543-R102-E546 on one side and E103-R316-D101 on the other side (Fig. 7C). All these residues are also found in *E. coli* and *S. aureus* except for D546 that is an Ala residue in *E. coli* and *S. aureus.*

Furthermore, drug recognition by the neumococal TopA enzyme was modelled by docking using GOLD program with boldine, secoboldine, seconeolitsine, and N-methyl seconeolitsine (Fig. 3 and S2). All these ligands are placed in a similar orientation, stacked between P486 and R102 in the nucleotide binding site (supplemental Fig. S2), as observed for the *E. coli* topoisomerase I in complex with nucleotide (13). Among all the tested ligands, N-methyl-seconeolitsine displays more interactions with the protein than the remaining alkaloids (Fig. 8C, supplemental Fig. S2). The larger number of interactions for phenanthrene alkaloids (secoboldine, seconeolitsine, N-methyl seconeolitsine) compared with those with aporphine skeleton (boldine), can be explained by the presence of a third benzylic ring. This extra ring may further stabilize the structures through both cation-π interaction with R102 and a stacking interaction with P486. Interestingly, R102, which is critical for the cation-π interaction with the ligand, is also stabilized by a double salt-bridge interaction with D543 and E546 (the last one unique to *S. pneumoniae).* Moreover, oxygen atoms from methylenedioxy

groups are establishing polar contacts with D103, R156 and D543. Furthermore, additional polar interaction can be predicted between the secondary amine group of N-methyl-seconeolitsine and E546. Taking into account all these data, the large salt bridge network in *S. pneumoniae* TopA results in a rigid and narrow cavity in which N-methyl-seconeolitsine is strongly stabilized through both the interactions with different protein residues and by a perfect fitting in the cavity (Fig. 8B).

Effect of seconeolitsine and N-methly-seconeolitsine in human cell viability. At the two concentrations assayed (30 and 100 µM), neither N-methyl-seconeolitsine nor seconeolitsine affected neutrophil apoptosis (Fig. 9). In contrast, seconeolitsine, at the highest concentration tested (100 µM), caused a small but significant decrease in neutrophil survival (Fig. 9). Nevertheless this concentration was nearly 5 fold higher than that necessary to exert antibiotic activity.

DISCUSSION

Two main approaches can be used for the discovery of new antibiotics. The first approach is based on the screening of natural products for bacteria growth inhibition followed by the subsequent identification of the cellular target. The second uses the knowledge of the structure of essential enzyme targets to design different compounds. In this study both approaches have been successfully used to identify new alkaloid antibiotics. We have characterized for the first time the topoisomerase I enzyme of *S. pneumoniae* and completed the characterization of the DNA topoisomerase complement of this pathogen. Topoisomerase I is the unique DNA topoisomerase of type I in this bacterium and has enzymatic features similar to those found in Gram-negative bacteria, being able to relax negatively-supercoiled DNA, but not positively-supercoiled (Fig. 2).

A previous study carried out by Tse-Dinh's group evaluated the capability of different compounds to inhibit topoisomerase I enzymes of Gram-negative bacteria (*E. coli* and *Yersinia pestis*) (15). Among them, stephenanthrine (20-100 µM), was found to inhibit the relaxation activity of *E. coli* topoisomerase I via stabilization of the cleavage complex. However, this alkaloid did not affect bacterial growth at these concentrations, being the MIC of 60 and 100 µM for *Bacillus subtilis* (Gram-positive organism) and *E. coli*, respectively. These findings led us to test the activity of various boldine-derived compounds presenting a chemical structure similar to stephenanthrine. Among them, N-methyl-seconeolitsine and seconeolitsine displayed the most potent inhibitory activity of bacterial growth at concentrations within the range required for inhibition of the relaxation activity of *S. pneumoniae* topoisomerase I (Fig. 4, Table 1). Moreover, these compounds also inhibited the growth of *S. pneumoniae* clinical isolates resistant to other chemically unrelated antibiotics used to treat pneumococcal infections (Table 1); including fluoroquinolones that target type II topoisomerases.

Attempts to obtain mutants resistant to the alkaloids were unsuccessful. These results suggest that the alteration of the single topoisomerase of type IA in *S. pneumoniae* could be lethal, especially if the alteration would be at the DNA-binding site. In agreement, *E.coli* cells lacking their two type IA topoisomerases (TopA and TopB) are found to be nonviable (32).

Evidence for *in vivo* targeting of TopA by seconeolitsine was provided by the protection against growth inhibition when TopA was overproduced (Fig. 6). In fact, the increase in supercoiling of an internal plasmid in the presence of the alkaloid suggest an inhibition of TopA. This increase in supercoiling could be also caused by activation of gyrase or by inhibition of topoisomerase IV. However, these effects were not observed in the *in vitro* activities of purified pneumococcal gyrase and topoisomerase IV in the presence of N-methyl-seconeolitsine (data not shown). These results suggest that TopA is the intracellular target of compounds N-methyl-seconeolitsine and seconeolitsine and that these alkaloids might be useful in the treatment of infectious diseases caused by multidrug-resistant isolates. Although pharmacokinetic studies are required, our toxicological studies suggest that N-methyl-seconeolitsine and seconeolitsine (30 µM) did not affected human neutrophil viability (Fig. 8).

It has been proposed that the reaction cycle of topoisomerase I involves the opening and closing of the enzyme. In order for DNA to bind near the active site, the enzyme must adopt an open (or partially open) conformation (13). After the cleavage reaction takes place, the protein adopts an open conformation in which the protein forms a bridge between the two ends of the broken DNA strand. The opening of the enzyme would allow the entrance of another strand into the central hole. For the religation step to take place, the enzyme must bring the two ends of the broken strand together by bringing domains I and III together while keeping the passing strand inside the central hole of the enzyme. Clearly, different openings of the enzyme are required to display full activity. In this way, docking calculations with alkaloids indicate that they can be accommodated at the nucleotide-binding site of the closed conformation of topoisomerase I. In this site alkaloids are in close contact with domains I, III and IV (Fig. 7, supplemental Fig. S2) and establish strong interactions (cation-π, hydrophobic and polar interactions) with residues of the nucleotide-binding site. Boldine presents a more flexible structure without the extended π cloud of the seconeolitsine or N-methyl-seconeolitsine, therefore preventing a strong stabilization through cation-π interaction, as predicted for seconeolitsine or N-methyl-seconeolitsine. These strong interactions together with the fine fitting observed between the nucleotide-binding site and the alkaloid, should block the opening mechanism required for topoisomerase I to be fully active. Therefore, avoidance of DNA binding may explain the strong inhibitory effect observed for these ligands. In agreement with that hypothesis, the inhibitory effect exerted by these alkaloids was enhanced prior to DNA binding. Thus, alkaloid-topoisomerase interactions seem to occur at the initial steps (i.e. in the closed conformation) and very likely in simultaneous interaction with the different domains involved in the opening mechanism of the enzyme.

Low concentrations of seconeolitsine and N-methyl-seconeolitsine seemed to activate the *in vitro* relaxation activity of human topoisomerase I (TOPO1), a prototype IB topoisomerase. However, the *in vitro* intercalation of these compounds in the DNA (Fig. 5B) would suggest that the alterations of TOPO1 activity could be attributed to this intercalation. In agreement, a similar effect was observed when the relaxing activity of TopA was tested in the presence of EtBr (Fig. 5B).

Higher alkaloid concentrations seemed to inhibit the *in vitro* activity of human TOPO1 without inducing apoptosis in human cells. In contrast, camptothecin, a selective inhibitor of TOPO1 used as antitumor drug, induce apoptosis. It binds simultaneously both to the DNA and to TOP1 and estabilize the cleavage complex formed by TOP1-DNA and the drug. Then, the collision of these cleavage complexes with replication forks induce double-strand breaks that led to apoptosis (33). Therefore, it is likely that either alkaloid interaction with TOPO1 occurs before the single-stranded DNA cleavage is produced, since apoptosis was not detected, or that did not interact with the enzyme.

In conclusion, in the present study we have isolated and purified a new antibiotic target from *S. pneumoniae,* topoisomerase I. Through the semi-synthesis of two new antibiotics, seconeolitsine and N-methly-seconeolitsine, we have proved that topoisomerase I inhibition results in the blockade of bacteria growth without affecting the viability of human cells. These compounds did not cause any cytotoxic effect on human neutrophils at the concentrations required to exert antibiotic activity. Thus, seconeolitsine and N-methyl-seconeolitsine can be envisaged as two new therapeutic candidates for the treatment of *S. pneumoniae* infections resistant to other antibiotics and open a new scenario for designing more specific antibiotics targeting this pivotal enzyme.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1**. Identification and purification of *S. pneumoniae* DNA topoisomerase I**. *A*, alignment of *E. coli* and *S. pneumoniae* TopA proteins. Colour codes correspond to the four structural domains showed in Fig. 7. The catalytic Tyr residue (Tyr319 for *E. coli* and Tyr314 for *S. pneumoniae*) is coloured in magenta, shadowed in yellow and labelled with a magenta asterisk. Residues in boldface labelled with a black asterisk and shadowed in yellow form part of the nucleotide binding site and are involved in the salt-bridge interaction network. *B,* expression of TopA and steps of purification. Cultures of M15 (pREP4) containing pQE-SPNtopA were grown in LB medium and induced with IPTG as described in Materials and Methods. Samples of 10 µl were electrophoresed in a SDS-10% polyacrylamide gel. Polypeptides were revealed by Coomassie blue staining. Lanes: CE (12 µg) after 0 and 30 min induction; Ni-NTA column, fraction that did not bind to the column and eluted in the loading (9 µg), fraction eluted with 10 mM (2 µg), with 60 mM (0.5 µg) and 100 mM (1 µg) imidazol. Sizes of protein markers (Mw) are indicated to the left.

FIGURE 2**. TopA has nicking and closing activities.** Plasmid pBR322 (1.77 nM) was incubated with purified topoisomerase I at the indicated concentrations for 1h at 37ºC. Samples were processed and analysed as described under "Material and Methods". OC, relaxed open circles; CCC, covalently closed circles; RC, relaxed circular plasmids forms. Mw, molecular weight standard. *A*, gel run in the absence of EtBr. *B,* the same samples run in A were run in the presence of 0.5 µg/ml of EtBr. *C*, TopA activity on positively-supercoilied pBR322. In wells labeled as (+) for EtBr, the pBR322 was incubated in the presence of 2 µg/ml of EtBr for 15 min at 4ºC before the incubation with TopA. In the well labeled as (+,-), pBR322 previously treated with 2 µg/ml of EtBr was treated with isoamylalcohol to remove EtBr and further incubated with TopA. *D*, topoisomer distribution of pBR322 after TopA treatment. Plasmid DNA was subjected to two-dimensional agarose electrophoresis. *E*, illustration of pBR322 topoisomer distribution after treatment with 15 mM TopA in two-dimensional electrophoresis in agarose gels run in

the presence of 1 and 2 µg/ml chloroquine in the first and second dimensions, respectively. Negative supercoiled topoisomers are in white and positive supercoiled in black. A black arrowhead indicates the topoisomer that migrated with $\Delta Lk = 0$ in the second dimension.

FIGURE 3**. Chemical structures of the most relevant aporfine and phenantrene alkaloids used in this study.**

FIGURE 4**. TopA is inhibited by phenanthrene alkaloids**. *A*, inhibition of TopA by boldine, compound 2 (secoboldine) and compound 5 (N-methyl-secoboldine). Supercoiled pBR322 (0.5 µg) was treated with 1 unit of purified TopA in 15 µl reactions containing the alkaloids at the concentrations (µM) indicated. Mw, molecular weight standard, S, covalently-closed supercoiled pBR322 used as substrate. *B*, inhibition of TopA by compounds 16 (N-metilseconeolitsine), 17 (seconeolitsine) and 14 (neolitsine). Reactions were carried out as in A. *C*, the inhibition of TopA by seconeolitsine is enhanced by preincubation of the enzyme with the alkaloid. pBR322 was treated with 1 unit of purified TopA in the absence of the inhibitor (0), or in the presence of 30 µM of compound 17 with (+) or without (–) preincubation during 10 min at 4ºC. *D*, effect of EtBr on TopA activity. Reactions were carried out as in A containing the compound at the indicated concentrations (µM).

FIGURE 5. **Human TOPO1 is not inhibited by phenantrene alkaloids.** *A*, effect of compounds 16 (N-metylseconeolitsine) and 17 (seconeolitsine) on the enzymatic activity. Supercoiled pBR322 was treated with 1 unit of enzyme in the absence (0) or in the presence of various concentrations of the alkaloids with or without (–) preincubation 30 µM of compound 17. *B*, EtBr displacement assay of compounds 16 and 17. Fluorescence is expressed as the percentage of the maximum fluorencence signal when EtBr was bound to the DNA in the absence of the alkaloids and was corrected for background fluorencence of EtBr in solution.

FIGURE 6**. The overexpression of TopA allows cell survival in the presence of seconeolitsine.** Exponentially-growing cultures of R6 carrying pLS1R (open symbols) or pLS1R-topA (full symbols) were grown in AGCH containing 0.8% sucrose to $DO_{620} = 0.6$. At this moment, cultures were diluted 20-fold in medium containing 0.4% sucrose and 0.4% maltose. *A*, growth and *B*, viability in the absence or the presence of compound 17 (seconeolitsine) at different concentrations were determined. Initial OD_{620} values were 0.17 for pLS1R and 0.15 for pLS1R-topA. Initial viable values were 6.6×10^7 cfu/ml for pLS1R and 3.2×10^7 cfu/ml for pLS1R-topA.

FIGURE 7**. Treatment with seconeolitsine causes hypernegative supercoiling in plasmid DNA.** An exponentially growing culture of R6 in AGCH carrying plasmid pLS1 was treated at $OD_{620}=0.4$ with seconeolitsine (compound 17) at 0.5 × MIC and 1 × MIC. Purified plasmid DNA from samples collected at 5 and 30 min after treatment was subjected to two-dimensional agarose gel electrophoresis in the presence of 5 µg/ml chloroquine in the first dimension and 15 µg/ml in the second dimension. It was previously determined that 15 µg/ml of chloroquine introduces 31 positive supercoils. A black arrowhead indicates the topoisomer that migrated with $\Delta Lk = 0$ in the second dimension. An empty arrowhead indicates the most abundant topoisomer. The corresponding supercoiling density (σ) is indicated below each autoradiogram.

FIGURE 8**. Structural modelling of the interaction of *S. pneumoniae* topoisomerase I with alkaloid antibiotics.** *A***,** overall structure of the 67 kDa fragment of *S. pneumoniae* TopA showing the four structural domains and the catalytic Tyr314 residue (magenta sticks). *B*, N-methyl-seconeolitsine

(represented as blue spheres) bound to the nucleotide-binding site of topoisomerase I. *C*, stereo view representation describing details of ligand recognition by topoisomerase I as obtained by docking calculations. The view shows the interactions between the nucleotide-binding site and N-methyl-seconeolitsine. The residues forming the binding site are drawn as capped sticks. Carbon atoms of the ligand are in yellow. Hydrogen bonds and salt-bridge interactions are represented as dashed lines.

FIGURE 9. **Effect of compounds 16 and 17 on cell viability**. *A*, *B*, effects of compounds 16 and 17 on early apoptosic (annexin V^+, PI^-), late apoptotic and/or necrotic (annexin V^+ and PI^+), and viable nonapoptotic (annexin V^- and PI^-) cells at 24 h of culture of human neutrophils. Columns are mean ± SEM. of 7-9 independent replicates; *$P<0.05$ compared to vehicle. A representative flow cytometry panels showing the effect of compounds 16 and 17 on human neutrophil apoptosis have been included.

TABLE 1.

Susceptibilities of *S. pneumoniae* strains to boldine derivatives

Strains	Resistance Pattern[a]	MIC (μM)[a] of												
		2	**3**	**4**	**5**	**6**	**7**	**11**	**12**	**13**	**14**	**16**	**17**	**23**
R6	S	500	500	>250	500	250	250	125	125	500	125	16	16	62
ATCC 6303	S	1000	1000	>250	500	>250	250	125	125	500	125	16	16	125
CipS8	S	500	500	>250	500	>250	125	125	125	500	125	16	16	62
CipS9	S	1000	1000	>250	1000	>250	250	125	125	500	125	16	16	125
CipR20	PTCEClSxTCip	1000	500	>250	250	>250	250	125	125	500	62	8	16	125
CipR16	PSxTCip	1000	1000	>250	500	>250	500	125	125	500	125	16	16	62
CipR8	PSxTCip	500	500	>250	500	>250	250	62	125	500	62	8	8	125
CipR42	PTEClCip	1000	1000	>250	500	>250	250	125	125	500	62	8	8	125
CipR45	TEClCip	500	500	>250	1000	>250	250	125	125	500	62	8	16	125
CipR68	Cip	500	500	>250	500	>250	250	125	125	500	62	8	8	125
CipR5	EClCip	500	500	>250	500	>250	250	125	125	500	62	16	16	62
CipR15	SxTCip	1000	1000	>250	500	>250	250	125	125	500	62	16	8	62

[a] S, susceptible to all antibiotics tested; P, resistant to penicillin (MICs of 0.12 to 4 μg/ml); T, resistant to tetracycline (MICs ≥4 μg/ml); C, resistant to chloramphenicol (MICs ≥8μg/ml); E, resistant to erythromycin (MICs ≥0.5 μg/ml); Cl, resistant to clindamycine (MICs ≥1 μg/ml) Cp, resistant to ciprofloxacin (MICs ≥4 μg/ml), SxT, resistant to cotrimoxazole (MIC ≥4 μg/ml for trimethoprim and ≥76 μg/ml for sulfamethoxazole).

[b]MICs for compounds B (boldine), 10, 19, 20, 21 and 22 were 1000 μM in all cases. The chemical structures of the compounds used are showed in supplemental Fig. S1.