Amarogentin, a Naturally Occurring Secoiridoid Glycoside and a Newly Recognized Inhibitor of Topoisomerase I from *Leishmania donovani*

Sutapa Ray, Hemanta K. Majumder, Ajit K. Chakravarty, and Sibabrata Mukhopadhyay*

Indian Institute of Chemical Biology, Calcutta 700032, India

Roberto R. Gil and Geoffrey A. Cordell

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612

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A MeOH extract of *Swertia chirata* found to inhibit the catalytic activity of topoisomerase I of *Leishmania donovani* was subjected to fractionation to yield three secoiridoid glycosides: amarogentin (**1**), amaroswerin (**2**), and sweroside (**3**). Amarogentin is a potent inhibitor of type I DNA topoisomerase from *Leishmania* and exerts its effect by interaction with the enzyme, preventing binary complex formation.

Swertia chirata Buch-Ham (Gentianaceae) has long been used as a bitter tonic, stomachic, febrifuge, and anthelmintic in the Indian system of medicine.[1,2] Previous phytochemical investigations resulted in the isolation of triterpenoids,[3–8] xanthones,[9] lignans,[10] and iridoid glycosides.[11] Herein we report the inhibitory activity of the secoiridoid glycoside, amarogentin (**1**), on DNA topoisomerase I of *Leishmania donovani* strain UR6.

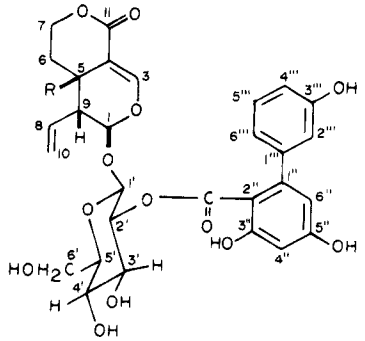
DNA topoisomerases play a pivotal role in modulating the dynamic nature of DNA secondary and higher-order structures, and thus provide essential functions inside cells. They are implicated in the events involving DNA action, for example, replication, transcription, mitosis, and repair.[12–16] The DNA topoisomerases are classified into two types.[17] Most of the type I topoisomerases are single subunit proteins that relax negatively supercoiled plasmid DNA in the absence of ATP. They introduce transient breakage of one strand at a time to permit the passage of another strand through the break. During this process the linking number of DNA changes in steps of one, and the enzyme is found to be covalently linked to the 3′ phosphoryl end of the broken DNA strand via a tyrosyl phosphate bond. Type II enzymes work in a similar manner, but they create breaks on both strands of DNA in the presence of high-energy cofactors, resulting in changes of linking number in steps of two.[18,19] Because DNA topoisomerases play important roles in maintaining cellular functions, they have recently been found to be good targets for many trypanocidal,[20,21] antimicrobial,[22,23] and antitumor[24–28] drugs. Most of these antitumor and trypanocidal drugs are targeted against type II topoisomerases, and there are very few compounds that specifically alter the biological functions of topoisomerase I by inhibiting its catalytic pathway after binding with the enzyme or DNA or enzyme DNA complex. The antitumor drug camptothecin and three of its derivatives, topotecan, 9-amino-camptothecin, and CPT-11, inhibit the catalytic activity of topoisomerase I by stabilizing the cleavable complex between topoisomerase I and DNA.[29,30]

Human leishmaniasis is a globally widespread, parasitic disease caused by members of the genus *Leishmania*.[31,32] Pentavalent antimonials (Sb^V), sodium stibogluconate and *N*-methylglucamine antimonate, are the preferred drugs for treatment of most forms of leishmaniasis.[33] However, various new chemotherapeutic agents are now being administered.[35] The active constituents of some plant extracts have significant activity against various species of *Leishmania*, and iridoid glycosides are one of the types having promising antileishmanial activity.[36]

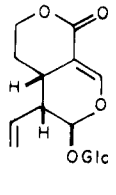
It has been shown in our laboratory that two potent antileishmanial drugs, sodium stibogluconate and urea stibamine, inhibit the relaxation of supercoiled DNA catalyzed by DNA topoisomerase I of *Leishmania donovani*.[34,37] We report here the inhibition of DNA topoisomerase I of *Leishmania donovani* with the secoiridoid glycoside amarogentin.

Results and Discussion

The concentrated MeOH extract of *S. chirata* was fractionated successively with $CHCl_3$ and *n*-BuOH. Chromatographic resolution of the butanol-soluble fraction gave three bitter iridoid glycosides, amarogentin (**1**), amaroswerin (**2**), and sweroside (**3**), along with three polyoxygenated xanthones and magniferin.



1 R = H
2 R = OH



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The ^1H- and ^{13}C-NMR spectral data of amarogentin (**1**) were identical to those reported earlier.[11,38] The results were further corroborated by 2D NMR studies

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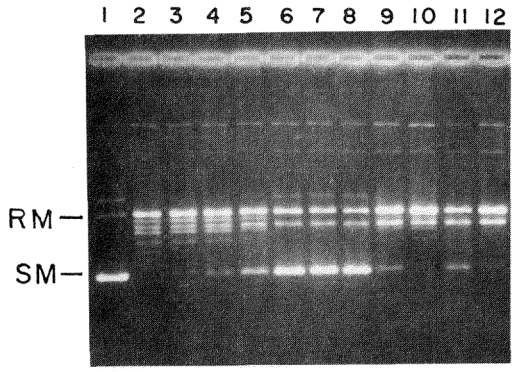

Figure 1. Inhibition of DNA relaxation activity by amarogentin and its reversal by increasing enzyme concentration. Supercoiled plasmid DNA was incubated alone (lane 1), in presence of 2 units of *Leishmania donovani* topoisomerase I (lane 2) or in presence of 2 units of enzyme plus 30, 60, and 120 μM amarogentin, respectively (lanes 3–5). Lanes 6–8: same as lanes 3–5, but preincubation of the enzyme with the compound. Lanes 9 and 10: same as lane 7, but 4 and 8 units of enzyme were added later. Lanes 11 and 12: same as lane 8, but 4 and 8 units of enzyme were added later. SM: supercoiled monomer; RM: Relaxed monomer.

(e.g., DEPT, COSY, HETCOR, and selective INEPT experiments). The structures of two other iridoid glycosides, amaroswerin (**2**) and sweroside (**3**), were established by comparison of their spectral data with those of amarogentin (**1**) as well as with the published data on amaroswerin[39] and sweroside.[40]

While studying the *in vitro* effect of amarogentin on the relaxation of supercoiled pGEM4Z catalyzed by topoisomerase I from *Leishmania donovani* strain UR6, we have found that the relaxation activity was inhibited in the presence of increasing concentrations of amarogentin (Figure 1, lanes 3–5). Inhibition of enzyme activity by amarogentin is more predominant when the enzyme is preincubated with the compound for 10 min at 37 °C in the relaxation assay mixture before addition of the DNA substrate (lanes 6–8). The compound-mediated inhibition can be overcome by increasing the enzyme concentrations. Lanes 7 and 8 show inhibition of two units of enzyme by 60 μM and 120 μM concentration of amarogentin, respectively. Inhibitions by 60 μM and 120 μM of amarogentin are relieved with increasing concentrations of enzyme (i.e., 4 and 8 units) (lanes 9–12). Moreover, the above inhibition of relaxation by the compound cannot be relieved with increasing concentrations of pGEM4Z DNA (data not shown).

This inhibition of relaxation activity by amarogentin occurs in a highly dose-dependent manner. Densitometric analysis of the agarose gel shows that when the enzyme, DNA (pGEM4Z), and amarogentin are added simultaneously in the standard relaxation assay mixture, 28% inhibition was found at the 30-μM concentration. Simultaneously, at this concentration, 80% inhibition was found when the enzyme was preincubated with amarogentin before the addition of a supercoiled DNA substrate (Figure 2).

The dose-dependent inhibition and its reversal by increasing enzyme concentration suggests that the compound exerts its inhibitory effect by binding with the enzyme and prevents binary complex formation between topoisomerase I and DNA. We have compared the inhibition of *Leishmania donovani* type I topoisomerase by amarogentin and camptothecin. These compounds have similar inhibitory effects that are more

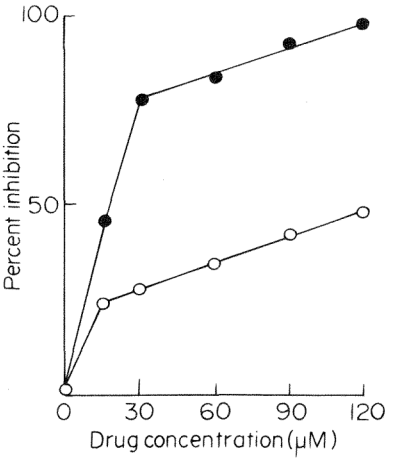

Figure 2. Preincubation of enzyme with amarogentin enhanced the inhibitory potency. (○): simultaneous addition of enzyme, compound and DNA in the reaction mixture. (●): preincubation of enzyme and the compound.

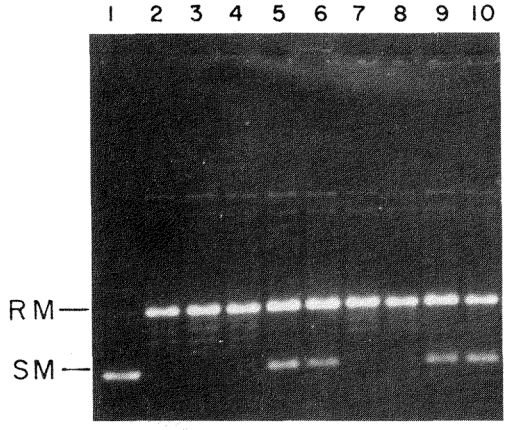

Figure 3. Comparative inhibition of DNA relaxation activity by amarogentin and camptothecin. Supercoiled plasmid DNA was incubated alone (lane 1), in presence of 2 units of *Leishmania donovani* topoisomerase I (lane 2), or in presence of 2 units of enzyme plus 5 and 10 μM amarogentin (lanes 3 and 4). Lanes 5 and 6: same as lanes 3 and 4, but enzyme was preincubated with amarogentin. Lanes 7 and 8: same as lane 2, but in presence of 5 and 10 μM camptothecin, added simultaneously. Lanes 9 and 10: same as lanes 7 and 8, but enzyme was preincubated with camptothecin.

pronounced when the enzymes and compounds are preincubated prior to the addition of DNA in the relaxation reaction (Figure 3).

These observations suggest that amarogentin, a secoiridoid glycoside isolated from *S. chirata* inhibits type I DNA topoisomerase. This potent inhibitory effect on the topoisomerization of DNA catalyzed by type I topoisomerase from *Leishmania* may present a "lead" for the design of more effective drugs against leishmaniasis.

Experimental Section

General Experimental Procedures. ^{1}H-NMR, ^{13}C-NMR (broad band decoupled and DEPT), and COSY spectra were recorded in CD_3OD, with TMS as an internal standard, employing a Nicolet NMC-360 spectrometer, which was also used for the selective INEPT experiments. The HETCOR experiment was carried out using a Varian XL-300 instrument. For the selective INEPT experiments, data sets of 16K covering a spectral width of 10 KHz were acquired. Proton pulse widths were calibrated using a sample of acetic acid in 10%

C_6D_6 (^{IR}J = 6.7 Hz) in a 5-mm NMR tube. The radio frequency field strength for the soft pulse was on the order of 25 Hz for these experiments. For aromatic and vinylic protons 7 Hz was used as $^3J_{C-H}$ and for aliphatic and hydroxyl protons, 5 Hz.

Plant Materials. The aerial part of *S. chirata* was collected from the eastern Himalayan region up to an altitude of 2500 m during January–February 1992. A voucher specimen is kept in Indian Institute of Chemical Biology, Calcutta, India.

Extraction and Isolation of Amarogentin (1). The defatted, dried aerial parts (20 kg) of *S. chirata* were extracted with 95% EtOH for 30 h using a Soxhlet extraction apparatus. The EtOH extract was concentrated under reduced pressure and kept overnight at room temperature to give a residue (50 g). The filtrate was washed with $CHCl_3$ (500 mL × 3), and the aqueous layer was further extracted with *n*-BuOH (500 mL × 3) to give *n*-BuOH residue (40 g). The *n*-BuOH extract on chromatography over silica gel yielded two fractions on elution with $CHCl_3$–MeOH mixture (19:1 and 9:1). These two fractions, on repeated chromatography over the same adsorbent, afforded amarogentin (1) (2.58 g), amorphous powder, mp 230–232 °C [lit.[11] mp 229–230 °C]; $[\alpha]^{25}D$ −109.2° (*c* 0.5, MeOH); [lit.[11] $[\alpha]^{25}D$ −116.6° (MeOH)]; selective INEPT experiment: proton irradiated at δ(carbon enhanced): H-1 at 5.39 (C-3, C-8, C-1′, C-5), H-3 at 7.43 (C-11, C-4, C-1), H-5 at 2.74 (C-11, C-8, C-4, C-2′, C-7, C-6), H-6 at 1.57 (C-4, C-7, C-5), H-6 at 1.69 (C-5), H-7 at 4.36 (C-11), H-7 at 4.25 (C-11, C-5), H-8 at 5.43 (C-1, C-1′), H-9 at 2.58 (C-11, C-8, C-4, C-1), H-10a at 5.25 (C-8, C-10, C-1, C-9), H-10b at 5.22 (C-8, C-10, C-1), H-1′ at 4.29 (C-11, C-1, C-5′), H-2′ at 4.74 (C-7″, C-1′, C-3′), H-3′ at 2.82 (C-4, C-2′, C-4′), H-4′ at 3.24 (C-5′, C-3′, C-6′), H-5′ at 3.09 (C-1′, C-3′, C-6′), H-6′a at 3.84 (C-4′), H-6′b at 3.61 (C-5′, C-6′), H-4″ at 6.30 (C-3″, C-5″, C-2″), H-6″ at 6.16 (C-3″, C-2″, C-4″), H-2‴ and H-6‴ at 6.72 (C-3‴, C-1″, C-6‴, C-2‴), H-4‴ at 6.78 (C-3‴, C-5‴, C-6‴, C-2‴), H-5‴ at 7.17 (C-3‴, C-1‴); fabms *m/z* 587 $(M + H)^+$, 391, 229.

Parasite Culture and Growth Condition. *Leishmania donovani* strain UR6 (MHOM/IN/1978/UR6) promastigotes were grown in Ray's modified media and subcultured at 72-h intervals.

Purification of Enzyme. Type I DNA topoisomerase was purified from nuclei of *Leishmania donovani* strain UR6 promastigotes as described previously.[34]

Enzyme Assay. The enzyme was assayed by decreased mobility in an agarose gel of supercoiled DNA (pGEM4Z) after treatment with topoisomerase. The standard type I topoisomerase assay mixture (25 μL) contained: 25 mM Tris–HCl (pH 7.5), 5% glycerol, 50 mM KCl, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg/mL BSA, 0.5 μg of pGEM4Z DNA, and 1 unit of enzyme (one unit of topo-I activity is the amount of enzyme needed for 50% relaxation of supercoiled pGEM4Z DNA under the conditions of assay). The reaction was carried out at 37 °C for 30 min. Reactions were stopped by adding 1% SDS, 10 mM EDTA, 0.25 μg/mL bromophenol blue, and 15% glycerol. Samples were applied to a horizontal 1% agarose gel and subjected to electrophoresis in TAE buffer at 1.5 v/cm for 14–16 h at room temperature. The gels were stained with ethidium bromide (5 μg/mL), destained in water, and photographed under UV illumination.

Percent relaxation was measured by microdensitometry of negative photographs of supercoiled monomer DNA band fluorescence after ethididum bromide staining with a microdensitometer (LKB BROMMA 2202 ultroscan) and the area under the peak calculated.

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