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Ungeremine effectively targets mammalian as well as bacterial type I and type II topoisomerases

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Abstract

From the methanol extract of the bulbs of *Pancratium illyricum* L., three phenanthridine type alkaloids, ungeremine (**1**), (–)-lycorine (**2**) and (+)-vittatine (**3**) were isolated. For the evaluation of their anticancer and antibacterial potential, compounds **1–3** were tested against human (I, II α) and bacterial (IA, IV) topoisomerases. Our data demonstrated that ungeremine impairs the activity of both, human and bacterial topoisomerases. Remarkably, ungeremine was found to largely increments the DNA cleavage promoted by bacterial topoisomerase IA, a new target in antimicrobial chemotherapy. 2011 Elsevier Ltd. All rights reserved.

Graphical abstract

Keywords

Topoisomerase; alkaloid; anticancer; antibacterial; *Pancratium illyricum* L

Natural product drugs and their semi-synthetic derivatives are widely explored anticancer and antimicrobial therapeutics.¹ An interesting class of cytotoxic compounds that is

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receiving growing interest are phenanthridine type alkaloids obtained from Amaryllidaceae species.² Among them, lycorine, pancratistatin and narciclasine, showed *in vitro* and *in vivo* antitumoral activities.^{3–5} Recent studies have demonstrated that these compounds exhibit selective activity against cancer cells according to a mechanism that is not related to proapoptotic stimuli.^{6, 7} In addition, lycorine has been reported to efficiently act as an antiviral^{8, 9} and antifungal agent.¹⁰ Several SAR studies have been performed to rationalize the anticancer profile of phenanthridine derivatives.^{6, 11–13} Although no clear-cut correlations are available yet, prominent cytotoxic activity has been associated with the presence of a positively charged nitrogen atom in the phenanthridine core.¹¹ This feature characterizes the structure of ungeremine, a lycorine type alkaloid that shows distinct physiological properties.^{5, 11} In particular ungeremine does not induce apoptosis and, additionally, shows antibacterial properties.^{4, 5, 12–15} Interestingly, Barthelmes et al. have shown that ungeremine is able to interfere with the enzymatic activity of human topoisomerases.¹⁶ These enzymes are essential for a fine regulation of the topology of the nucleic acid during the progression of the cell cycle and are present in all living organisms.¹⁷ Topoisomerases are divided into two main types, I and II, according to the number of DNA strands cleaved during the catalytic cycle. They can be further divided into subclasses (A, B and C for type I and A or B for Type II) depending on the enzyme structure and mechanism of action. Small molecules can inhibit these enzymes leading to an altered topological DNA distribution or can poison them causing an accumulation of cleavage sites along the DNA strand. Notable examples are the antitumoral anthracyclines and camptothecin (which are active against human type I and II, respectively) as well as the quinolones, which poison bacterial type II topoisomerase.¹⁸

Among the Amaryllidaceae, the genus *Pancratium*, widely spread in the Eurasian region, is extensively studied for its potential therapeutical use.⁵ In our ongoing search for cytotoxic compounds from sardinian plant species,¹⁹ we focused on *Pancratium illyricum* L. a species endemic to Sardinia, Corsica and the tuscan archipelago²⁰. There are no reports in the literature regarding both, the chemical composition and biological activity of this species. The MeOH extract obtained from the bulbs allowed the isolation of three main phenanthridine alkaloids, namely ungeremine or lycobetaine (**1**), (–)-lycorine (**2**) and (+)-vittatine (**3**) (Fig. 1). Additionally, from the CH₂Cl₂ extract 7,3'-dihydroxy-4'-methoxy-8-methyl flavan, 7,3'-dihydroxy-4'-methoxy flavan, *p*-hydroxyphenethyl *trans*-ferulate and sucrose were isolated.²¹ All their spectral (¹H- and ¹³C-NMR, UV and MS) and physical data (melting point, optical rotation) were in agreement with literature data.^{22–26}

The isolation of three already known alkaloids from *P. illyricum* prompted us to investigate in deeper details their behavior towards topoisomerases, since no such mechanism of action has been explored for lycorine and vittatine up to date. Therefore, the three alkaloids (**1–3**) were assayed against human topoisomerase I (hTopoI) and human topoisomerase II α (hTopoII α) by monitoring the relaxing activity of these enzymes on supercoiled pBR322.²⁷

Among the tested alkaloids, only ungeremine was found to cause a reduction of the relaxation activity of hTopoII α and hTopoI with IC₅₀ values of 25.8 μ M and 6.1 μ M, respectively (Table 1). Several agents known to impair topoisomerase functions are efficient DNA binders and interfacial inhibition has been proposed as a common mechanism for the

efficient trapping of topoisomerase cleavage complexes.²⁸ Ungeremine is known to bind DNA by an intercalation process²⁹ likely promoted by its planar nature and the positively charged nitrogen, which can reinforce the interaction by forming an ionic pair with phosphate groups. The alkaloid-DNA complex formation can easily be followed by monitoring the change of the absorption spectrum of the ligand upon nucleic acid addition (Fig. 2A). In the same experimental condition, DNA does not affect the spectrum of 2 and 3 (Fig. 2B) suggesting that their structural features fully prevent an efficient interaction with the nucleic acid. This behavior can explain why they 2 and 3 do not act as topoisomerase inhibitors and supports the relevance of a DNA-drug complex for the activity against topoisomerase.

We therefore focused on ungeremine and, since some eukaryotic and prokaryotic topoisomerases have common mechanistic and structural features, we decided to assess if bacterial topoisomerases can equally be targeted by this alkaloid. As a first target we chose topoisomerase IV from *Escherichia coli* (EcTopoIV), a type II topoisomerase that shares a relevant mechanistic similarity with the human enzyme. We found that ungeremine potently inhibits the EcTopoIV relaxation activity with an IC₅₀ of 7.3 μ M, thereby showing a lower IC₅₀ than against the human homologue (Table 1).

Each topoisomerase can recognize DNA of different topologies and process them with variable efficiency. In addition to removal of negative supercoils, both hTopoII and EcTopoIV efficiently decatenate DNA, too.^{30–31} Intriguingly, when we included ungeremine in the decatenation reaction of kinetoplast DNA (kDNA) mediated by these type II topoisomerases, we observed differential effects of the alkaloid towards the two enzymes. In particular, ungeremine was able to prevent the formation of monomeric circles from kDNA, only with hTopoII α and not with EcTopoIV (Fig. 3). The decatenation reaction results from the crossing of two DNA molecules whereas the supercoiled relaxation involves only one DNA molecule³¹. We can infer that differences in the protein-DNA complex geometry can cause a differential stabilization of the ternary complex by ungeremine. Nevertheless, both reactions are the results of specific sequential enzymatic steps, which are differently regulated by the tested topoisomerases.³² As previously reported for other inhibitors of topoisomerase type II α ,³³ we cannot exclude a preferential impairment of one single reaction at different catalytic steps. This would equally results into a different distribution of reaction products.

In our case, however, the selective inhibition of the EcTopoIV mediated relaxation process caused by ungeremine is relevant. Indeed, this result, merged with the previously reported selective poisoning of hTopoII β (and not of hTopoII α),¹⁵ clearly underlines that ungeremine has the ability to specifically impair selected enzymatic activities of different, although related, type II topoisomerases.

In order to assess whether this selective behavior involves only type II enzymes we extended our analysis to *E. coli* topoisomerase I (EcTopoI). In this case the bacterial enzyme belongs to the IA family and thus processes DNA by a different mechanism in comparison to hTopoI (a member of the IB family). Indeed, camptothecin, a well-known hTopoI poison, did not affect the relaxation activity of EcTopoI. On the opposite, ungeremine showed a good

inhibition of the relaxation activity of EcTopoI (IC_{50} 15.0 μ M). For a deeper investigation of the molecular mechanism of this activity, we monitored drug promotion of enzyme-mediated cleavage sites, using a ^{32}P 5'-labelled sequence (32-residues) as substrate (Fig. 4). In line with the mechanism reported to occur in the presence of hTopoII β ,¹⁵ our results confirmed that ungeremine stabilizes the EcTopoI DNA cleavage complex. The cleavage induction is characterized by a bell-shaped dependence that might reflect the drug binding to the nucleic acid, which ultimately prevents DNA accessibility to the enzyme. Remarkably, the alkaloid increments the enzyme-mediated DNA cleavage up to about 250 folds (Fig. 4). This result is noteworthy since few inhibitors for this bacterial topoisomerase have been reported up-to-date. Among them some compounds related to fluorene or phenanthrene (stephananthrene) were confirmed to inhibit bacterial type IA topoisomerases with IC_{50} values in the 5–10 μ M range but they were less efficient in promoting DNA cleavage (maximal DNA cleavage enhancement 80–150%).³⁴ Additionally, two semi-synthetic antibiotics deriving from boldine, which also contain the phenanthrene nucleus were found to selectively inhibit the TopoIA from *S. pneumoniae* (IC_{50} 17 μ M) although in this case poisoning of the enzyme was not assessed.³⁵ Interestingly, all these compounds bind DNA, which reinforces the hypothesis of a common mechanism of action against bacterial Topo IA strictly related to the formation of a stable ternary DNA-drug-enzyme complex.

The poisoning activity of ungeremine towards eukaryotic and prokaryotic topoisomerases correlates with its reported cytotoxic and antibacterial profile. In line with previously reported results using a viability assay³⁶, we confirmed a prominent activity of the alkaloid against the highly proliferative and non-adherent leukemia cells (IC_{50} = 1.4 μ M for HL-60) in comparison with a human breast adenocarcinoma cell line (IC_{50} >40 μ M for MCF-7).^{6, 13} Additionally, we evaluated the antibacterial activity against selected gram-positive and negative bacteria strains (MIC values of 31.25 and 125 μ g/mL for *S. epidermidis* and *S. tify*, respectively) with the agar dilution method.³⁷

Our findings show that ungeremine inhibits both, human and bacterial (*E. coli*) topoisomerases with comparable efficiency *in vitro*. More importantly, we identified ungeremine as a new scaffold for the effective poisoning of bacterial TopoIA, a new target in antimicrobial chemotherapy. Such a result is useful in the search for new antibacterial strategies, which has become imperative since many pathological bacterial strains developed multi-resistant mechanisms against most of the available antibacterial therapeutics. Diverging the eukaryotic and prokaryotic topoisomerase inhibitory activity represents, however, a major challenge. A critical analysis of literature data evidenced that the positively charged nitrogen in ungeremine derivatives is essential for the cytotoxic activity in eukaryotic cells.¹³ Distinctly, the bacterial TopoIA inhibitors reported up to date are structurally related to ungeremine but do not contain permanently charged nitrogens.^{34, 35} Therefore, we suggest that the synthesis of ungeremine derivatives containing uncharged nitrogen(s) could represent a possible first step for a rational SAR study. This would help in transforming ungeremine into an efficient antibacterial agent characterized by a novel mechanism of action.

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affording four subfractions (F4.1-F4.4). F4.2 (0.32 g) was further chromatographed over alumina using CH₂Cl₂-MeOH (9.75:0.25) as an eluent resulting in (+)- vittatine (**3**, 16.2 mg). F4.4 (0.15 g) was chromatographed over an open silica gel-column and eluted with MeOH yielding ungeremine (**1**, 4 mg). Using a similar fractionation procedure, from the CH₂Cl₂ extract the known compounds 7,3'-dihydroxy- 4'-methoxy-8-methyl flavan, 7,3'-dihydroxy-4'-methoxy flavan, *p*-hydroxyphenethyl *trans*-ferulate and sucrose were isolated.

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Figure 1.
Chemical structures of isolated alkaloids

Figure 2.

UV absorption spectra of ungeremine (PANEL A) and vittatine (PANEL B) upon addition of increasing concentrations (X – Y μ M) of calf thymus DNA in 10 mM Tris, 50 mM KCl, pH 8.0. The contribution of the nucleic acid has been properly subtracted. The arrow indicates the spectral change upon nucleic acid addition.

Figure 3.

Inhibition of decatenation activity of hTopoIIα (in 50 mM Tris, 120 mM KCl, 10 mM MgCl 0.5 mM ATP, 0.5 mM DTT, pH 8.0) or EcTopoIV (in 40 mM HEPES-KOH, 100 mM potassium glutamate, 10 mM magnesium acetate, 10 mM DTT, 1 mM ATP, 50 μg/ml albumin, pH 7.6) by ungeremine (**1**) on kDNA (0.15 μg) after 1h incubation at 37°C. a, kDNA; b, resolved minicircles; b1, monomeric relaxed minicircles, b2, monomeric supercoiled minicircles.

Figure 4. Stabilization of DNA cleavage sites induced by EcTopoI in the presence of ungeremine or camptothecin on a 32 bases single stranded DNA ^{32}P -TAACCCTGAAAGATTATGCAATGCGCTTTGGG) after 20 min incubation at 37°C in 20 mM Tris, 0.5 mM EDTA, 100 mg/ml BSA, 12 % glycerol, pH 8.0. B, untreated DNA; 0, DNA in the presence of enzyme.

Table 1

Ungeremine concentrations required to inhibit by 50% (IC₅₀) the relaxation of supercoiled pBR322 by selected topoisomerases

Topoisomerase	IC ₅₀ (μM)
hTopoIIα	25.8 ± 2.3
hTopoI	6.1 ± 0.4
EcTopoIV	7.3 ± 1.1
EcTopoI	15.0 ± 1.2