

# Screening for inhibitors of topoisomerase I from *Lycoris radiata* by combining ultrafiltration with liquid chromatography/mass spectrometry

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**RATIONALE:** Although crude Amaryllidaceae alkaloids (AAs) extracted from *Lycoris radiata* are reported to exhibit significant anti-cancer activity, both the specific responsible alkaloid(s) and their targets remain elusive. Screening anti-cancer AAs targeted on topoisomerase I from crude AAs could be very helpful in tackling these two challenging questions.

**METHODS:** An ultrafiltration method combined with liquid chromatography/electrospray ionization mass spectrometry (UF-LC/MS) was developed to screen for the inhibitors of topoisomerase I, which has been reported to mediate DNA unwinding during carcinoma proliferation. Enrichment factors (EFs) of different AAs were used to evaluate the binding affinity between AAs and topoisomerase I, and the AAs with higher EFs were further tested to validate the method.

**RESULTS:** Eleven AAs from *Lycoris radiata* (ten of which were identified) were screened using UF-LC/MS, and a glaring discrepancy in EFs was revealed for the first time. One of the AAs, hippeastrine, with the highest EF at 49.3%, was further tested against topoisomerase I, and the IC<sub>50</sub> value of hippeastrine was determined to be 23.0 μmol/L, which is comparable with the well-known anti-cancer drug camptothecin at 19.3 μmol/L.

**CONCLUSIONS:** A simple, rapid and effective screening method using UF-LC/MS was developed and successfully applied to screen candidate inhibitors of topoisomerase I from crude AAs in *Lycoris radiata*, which may pave the way to further understand the potential anti-cancer constituents and mechanisms of *Lycoris radiata*. Copyright © 2016 John Wiley & Sons, Ltd.

Amaryllidaceae alkaloids (AAs) from *Lycoris radiata* have recently attracted increasing interest since crude extracts of AAs exhibited remarkable anti-cancer activities.<sup>[1]</sup> However, neither the specific responsible alkaloid(s) nor their targets are clear to date, or the action mechanisms of AAs. In order to tackle these key questions, we set out to screen for the inhibitors of topoisomerase I (as one of the targets) from crude AAs, since it was reported that inhibitors of topoisomerase I may contribute to the antineoplastic activity by blocking the DNA synthesis and malignant cell proliferation.<sup>[2]</sup> Thus, they have been successfully used as therapy for colorectal, lung, and ovarian cancers.<sup>[3]</sup> Conventional methods for screening topoisomerase I inhibitors mainly included nuclear magnetic resonance (NMR), X-ray crystallography and calorimetric methods, fluorescence monitoring, and surface plasmon resonance (SPR).<sup>[4,5]</sup> These spectroscopic or biophysical-based approaches either required higher amounts of precious samples or provided no or very little information about the structures of the screened inhibitors. Mass spectrometry (MS)-based methods however could overcome both the aforementioned limitations and thus be used to screen

for topoisomerase I inhibitors in this work, which achieved not only high sensitivity, but also provided structural information on the compounds of interest,<sup>[6]</sup> bio-activity screening, and metabolomics research.<sup>[7]</sup> To screen for the anti-cancer candidates that targeted topoisomerase I in this study, ultrafiltration combined with liquid chromatography/electrospray ionization mass spectrometry (UF-LC/MS) was developed. To further verify the selected inhibitors, some targeted AAs were further tested against topoisomerase I *in vitro* based on their determined half maximal inhibitory concentrations (IC<sub>50</sub>). Not only could new inhibitors of topoisomerase I from *Lycoris radiata* be successfully discovered for the first time, but also valuable clues were provided for the study on anti-cancer targets and the action mechanisms of AAs. In addition, the method developed in this study would be very promising for exploring other candidate inhibitors of topoisomerase I from other medicinal plants.

## EXPERIMENTAL

### Chemicals

Formic acid, ammonium acetate (AA), and acetonitrile (ACN) of HPLC grade were purchased from ROE Scientific Inc, ANROUR Chemicals Supply, and Fisher Scientific, respectively. DNA topoisomerase I was purchased from

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New England Biolabs (Beijing) Ltd (Beijing, China), and centrifugal ultrafiltration filters (YM-30, 30 kDa) were provided by Millipore Co. Ltd (Bedford, MA, USA). Water for HPLC and LC-MS was prepared with EPED (Nanjing Yeap Esselte Technology Development Co., Nanjing, China).

### Sample preparation and analysis

Fresh samples of *Lycoris radiata* (*L. radiata*) were harvested from the Wuhan Botanical Garden, and the crude AAs were prepared as reported in our previous study.<sup>[8]</sup> The ultrafiltration experiments were conducted as follows: 100  $\mu$ L AAs sample solution (2.0  $\mu$ g/ $\mu$ L) was mixed with 10  $\mu$ L topoisomerase I (0.5 U/ $\mu$ L). After incubation and filtration through a YM-30 membrane (30 kDa), the unbound components were washed away. Then, the bound compounds were dissociated, collected, and lyophilized. The resultant sample was dissolved in 50  $\mu$ L of 90% aqueous ACN with 5  $\mu$ g/mL of nuciferin as the internal standard and subjected to the LC-UV/ESI-MS/MS analysis. Following the same procedures above, the negative control sample was prepared from the inactivated topoisomerase I (boiled for 10 min) solution and analyzed.

### Instrumentation

Chromatographic analysis was performed on an Agilent 1100 series high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with an autosampler, a binary pump, an online degasser, and a UV detector. The samples were analyzed under the same LC and LC/MS conditions as reported in our previous study.<sup>[8]</sup>

## RESULTS AND DISCUSSION

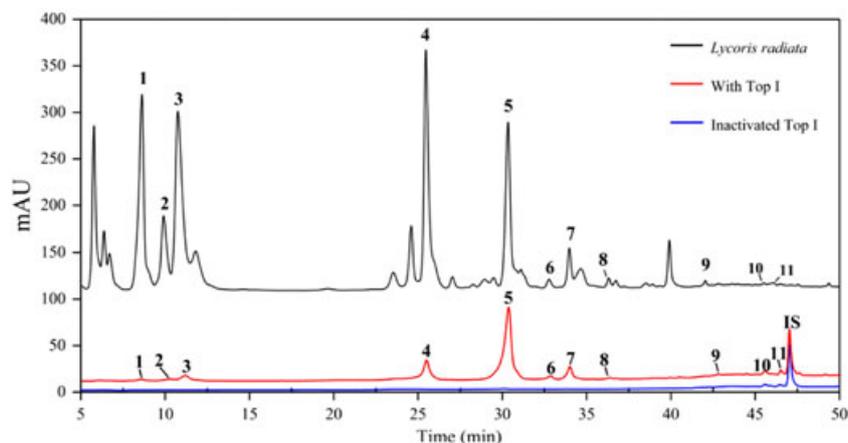
### Ultrafiltration of AAs bound to topoisomerase I

After incubation with topoisomerase I and ultrafiltration, samples were analyzed in parallel as shown in Fig. 1. Eleven AAs were observed and considered as the potent inhibitors, and the LC chromatograms displayed significant discrepancy, which may be attributed to different affinities of the potent

inhibitors to topoisomerase I. To further assess their affinity, the enrichment factor (EF) was introduced and calculated as follows:  $EF = (A_T - A_C)/A_0 \times 100\%$ , where  $A_T$ ,  $A_C$ , and  $A_0$  represent the peak areas of the corresponding AAs incubated with activated, inactivated, and without topoisomerase I, respectively. In this way, EFs for all of the candidate AAs were calculated as shown in Table 1. Peak 5 in the activated group exhibited the highest EF at 49.3%, followed by peaks 7 (24.2%), 4 (12.7%), and 6 (11.1%), which implied these AAs could be the more potent inhibitors of topoisomerase I and may have better anti-cancer activities compared with those with lower EFs, although these potent inhibitors of topoisomerase I should be further verified using other biological assays.

### Identification of the screened AAs

After EFs for all of the candidate AAs were obtained, the next task was to identify these AAs. As shown in Table 1, among ten peaks detected and screened, peaks 1, 2, 3, 4, 5, 6, 7, and 8 were identified based on our previous study.<sup>[8]</sup> For the identification of peak 5 ( $[M + H]^+$  at  $m/z$  316), the MS/MS fragments at  $m/z$  298, 280, 239, 191, 126, and 96 were observed as shown in Fig. 2. The characteristic fragments at  $m/z$  191 and 126 were produced by retro-Diels\_Alder (RDA) rearrangements. The further neutral loss of  $CH_2O$  generated the fragment ion at  $m/z$  96. The fragments at  $m/z$  298, 280, and 239 were formed due to the neutral loss of  $H_2O$ ,  $2H_2O$ , and  $C_3H_{11}NO$ . The MS/MS spectra and proposed fragment pathway are shown in Fig. 2. For other peaks like peak 9, with the  $[M + H]^+$  ion at  $m/z$  332, the fragments at  $m/z$  300, 282, and 264 were 2 Da higher than those of peak 5 and could thus be tentatively identified as 5-hydroxyhomolycorine as reported.<sup>[9]</sup> As for peak 10, by comparing the MS/MS data with those of haemanthamine,<sup>[10]</sup> similar abundant fragments at  $m/z$  211, 181, and 168 were observed, indicating the structural similarity of the two alkaloids. The fragment at  $m/z$  288 was produced by the loss of  $C_2H_4NO$  due to the RDA cleavage. Other fragments at  $m/z$  241, 239, 193, and 183 were also deduced by the corresponding neutral losses, and peak 10 was identified

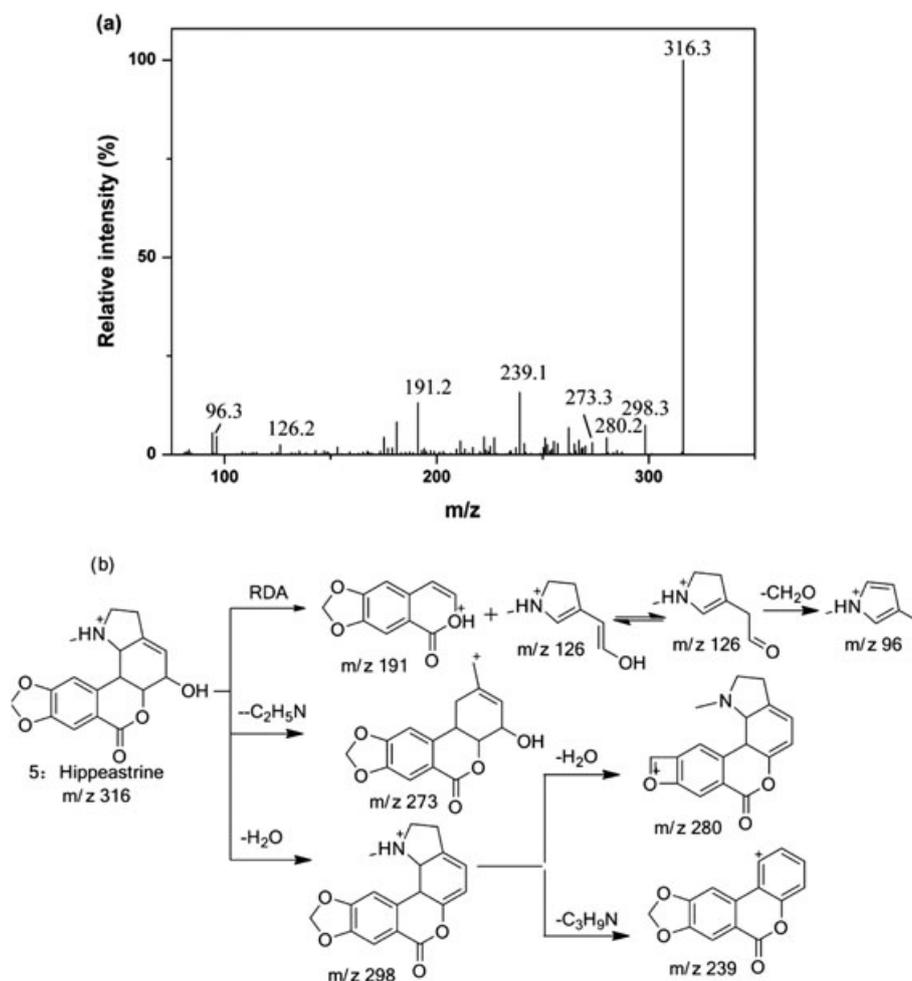


**Figure 1.** HPLC chromatograms of crude AAs in *Lycoris radiata* (black line), activated (red line) and inactivated (blue line) topoisomerase I (Top I), respectively.

**Table 1.** Identification, relative amounts, enrichment factors (EFs), and MS/MS data of AAs screened from *Lycoris radiata* using UF-LC-MS/MS

No.	RT (min)	[M + H] <sup>+</sup>	Relative amount (μg/mL)		EFs (%)	MS/MS data	Identification and references
			AAs -T	AAs -C			
1	8.5	288	0.15	0	0.4	270,252,222,177,147, 119	Lycorine <sup>[10]</sup>
2	10.2	290	0.14	0	1.3	272,233,215,189	Lycoramine <sup>[10]</sup>
3	11.1	288	0.78	0	2.3	270,231,225,213,198	Galanthamine <sup>[9]</sup>
4	25.4	332	4.96	0	12.7	300,282,264,234,225,213,199,169	Ambelline <sup>[10]</sup>
5	30.3	316	15.93	0	49.3	298,280,273,239, 191,126,96	Hippeastrine <sup>[14]</sup>
6	32.8	334	0.28	0	11.1	316,298,270,267,255, 238,173	2α-hydroxy-6-O -methyldoline <sup>[14]</sup>
7	33.9	316	1.62	0	24.2	298,280,267,239,237,207,191,176	(+)-8,9-methylenedioxyhomolycorine-N-oxide <sup>[11]</sup>
8	36.3	332	0.08	0	4.1	300,282,271,257,243,191	2-methoxyoduline <sup>[14]</sup>
9	42.7	332	0.05	0	2.6	314,300,282,271,257,191,181,175	5-hydroxyhomolycorine <sup>[9]</sup>
10	45.5	346	0.28	0.14	8.3	288,271,241,239,211,193,183,181,168	(+)-3α-hydroxy-6β-acetylbulbispermine <sup>[11]</sup>
11	46.4	556	0.22	0.10	6.1	282,267,266,251,220	Unidentified

AAs-T: Ultrafiltration with activated topoisomerase I.  
AAs-C: Ultrafiltration with inactivated topoisomerase I.

**Figure 2.** The MS/MS spectrum (a) and proposed fragment pathways (b) of peak 5.

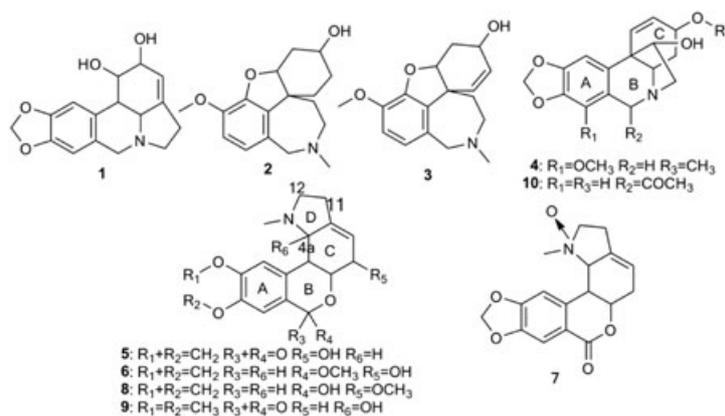


Figure 3. Structures of AAs identified from *Lycoris radiata*.

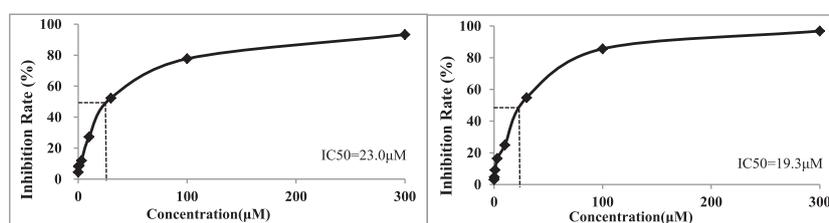


Figure 4. Inhibition curve of hippeastrine (left) and camptothecin (right) against topoisomerase I.

as (+)-3 $\alpha$ -hydroxy-6 $\beta$ -acetylbulbispermine as reported.<sup>[11]</sup> Thus, the structures of these ten AAs were successfully identified and are shown in Table 1 and Fig. 3.

#### Determination of IC<sub>50</sub> of selected candidate AAs

To further validate the screening method, peak 5 identified as hippeastrine with the highest EF value was selected for further *in vitro* test against topoisomerase I. The IC<sub>50</sub> of hippeastrine was evaluated in a concentration range of 0.1–300  $\mu\text{mol/L}$  using camptothecin as the positive control, which was one of the tested inhibitors of topoisomerase I, and was developed as a series of very promising anti-cancer drugs for advanced digestive carcinoma.<sup>[12]</sup> In this work, it was found for the first time that hippeastrine and camptothecin exhibited similar dose-dependent inhibition against topoisomerase I with IC<sub>50</sub> values of 23.0 and 19.3  $\mu\text{mol/L}$  as shown in Fig. 4, respectively. This also implied that hippeastrine could be a very promising candidate inhibitor of topoisomerase I and could be another anti-cancer drug candidate, which is consistent with the ultrafiltration binding assay and further validates the use of this approach to rank the order of ligands according to their affinity for a specific receptor. Since topoisomerase I relaxes supercoils by reversibly nicking duplex DNA to control DNA replication,<sup>[13]</sup> the hypothesis is that hippeastrine could reversibly block topoisomerase I mediated cleavage of the DNA complex and ultimately lead to DNA strand breaks and subsequent activation of apoptosis.<sup>[4]</sup> These results confirmed that the screening method developed in this work was very successful and effective and could be applied for screening new anti-cancer candidates and further new drug discovery and development from other medicinal plants.

## CONCLUSIONS

A fast, simple and effective screening method by UF-LC/MS was developed to screen candidate inhibitors of topoisomerase I from crude alkaloids in *Lycoris radiata*. Eleven alkaloids exhibited potential inhibitory activity, ten of which were identified with four candidate inhibitors showing EFs above 10%. Hippeastrine with the highest EF value was selected for further *in vitro* testing against topoisomerase I and found to be comparable with that of camptothecin in terms of IC<sub>50</sub>, which verified our screening method, and implied that hippeastrine could target topoisomerase I for its anti-cancer activity and could be another potent anti-tumor candidate. To the best of our knowledge, the current method for the first time succeeded in screening candidate inhibitors of topoisomerase I from *Lycoris radiata* and implied that topoisomerase I could be one of the anti-cancer targets of AAs. It is expected that, with some modifications, this new method would be very useful to explore more important applications in new anti-cancer drug discovery and development from other medicinal plants.

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## REFERENCES

- [1] G. V. Goietsenoven, V. Mathieu, F. Lefranc, A. Kornienko, A. Evidente, R. Kiss. Narciclasine as well as other *Amaryllidaceae isocarbostryls* are promising GTP-ase targeting agents against brain cancers. *Med. Res. Rev.* **2013**, *33*, 439.
- [2] A. R. Chaudhuri, Y. Hashimoto, R. Herrador, K. J. Neelsen, D. Fachinetti, R. Bermejo, A. Cocito, V. Costanzo, M. Lopes. Topoisomerase I poisoning results in PARP-mediated replication fork reversal. *Nat. Struct. Mol. Biol.* **2012**, *19*, 417.
- [3] J. L. Holleran, R. A. Parise, A. E. Yellow-Duke, M. J. Egorin, J. L. Eiseman, J. M. Covey, J. H. Beumer. Liquid chromatography–tandem mass spectrometric assay for the quantitation in human plasma of the novel indenoisoquinoline topoisomerase I inhibitors, NSC 743400 and NSC 725776. *J. Pharm. Biomed. Anal.* **2010**, *52*, 714.
- [4] C. X. Yao, N. Na, L. Y. Huang, D. C. He, J. Ouyang. High-throughput detection of drugs binding to proteins using desorption electrospray ionization mass spectrometry. *Anal. Chim. Acta* **2013**, *794*, 60.
- [5] T. Kamei, T. Uchimura, K. Nishimiya, T. Kawanishi. Method development and validation of the simultaneous determination of a novel topoisomerase I inhibitor, the prodrug, and the active metabolite in human plasma using column-switching LC-MS/MS, and its application in a clinical trial. *J. Chromatogr. B* **2011**, *879*, 3415.
- [6] V. Mulabagal, A. I. Calderon. Development of an ultrafiltration-liquid chromatography/mass spectrometry (UF-LC/MS) based ligand-binding assay and an LC/MS based functional assay for *Mycobacterium tuberculosis* shikimate kinase. *Anal. Chem.* **2010**, *82*, 3616.
- [7] X. Li, Q. Wang, C. Xu, F. Song, Z. Liu. The method and application on mass spectrometry analysis of Chinese medicine research. *Sci. Chin. Chem.* **2014**, *44*, 701.
- [8] Y. Q. Tian, C. Y. Zhang, M. Q. Guo. comparative analysis of amaryllidaceae alkaloids from three lycoris species. *Molecules* **2015**, *20*, 21854.
- [9] A. Jin, X. Li, Y. Y. Zhu, H. Y. Yu, H. F. Pi, P. Zhang, H. L. Ruan. Four new compounds from the bulbs of *Lycoris aurea* with neuroprotective effects against CoCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>-induced SH-SY5Y cell injuries. *Arch. Pharm. Res.* **2014**, *37*, 315.
- [10] D. Katoch, S. Kumar, N. Kumar, B. Singh. Simultaneous quantification of Amaryllidaceae alkaloids from *Zephyranthes grandiflora* by UPLC-DAD/ESI-MS/MS. *J. Pharm. Biomed. Anal.* **2012**, *71*, 187.
- [11] B. Hao, S. F. Shen, Q. J. Zhao. Cytotoxic and antimalarial amaryllidaceae alkaloids from the bulbs of *Lycoris radiata*. *Molecules* **2013**, *18*, 2458.
- [12] C. Zhang, S. S. Li, L. Y. Ji, S. Liu, Z. J. Li, S. C. Li, X. B. Meng. Design, synthesis and antitumor activity of non-camptothecin topoisomerase I inhibitors. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 4693.
- [13] N. Kim, S-y. N. Huang, J. S. Williams, Y. C. Li, A. B. Clark, J-E. Cho, T. A. Kunkel, Y. Pommier, S. Jinks-Robertson. Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. *Science* **2011**, *332*, 1561.
- [14] N. Liao, M. Z. Ao, P. Zhang, L. J. Yu. Extracts of *Lycoris aurea* induce apoptosis in murine sarcoma S180 cells. *Molecules* **2012**, *17*, 3723.