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Affinity ultrafiltration of DNA topoisomerases-targeted compounds determined with HPLC/ESI-MS for drug candidate screening*

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Abstract: A method of screening assay is demonstrated. The approach is based on the affinity of antitumor candidates for topoisomerases. In this method, antitumor candidates are fished out using topoisomerases as targets. Traditional analysis of complex compounds typically encounters signal suppression due to the relatively low concentrations, but enzyme-affinity screening for the active compounds can effectively concentrate the desired analysts into a small volume of high concentration. Active compounds are separated from non-affinity compounds by ultrafiltration. The molecules-enzymes complexes that are retained on the filter are subsequently separated by acidification to obtain the topoisomerases-affinity compounds for analysis on High Performance Liquid Chromatography coupled with electrospray ionization mass spectrometric detection (ESI-MS). This enzyme-affinity based screening assay provides a highly specific and efficient method that can directly screen, identify, and acquire drug candidates thus improving the accuracy and speed of high-throughput screening activities.

Key words: Affinity, High-throughput screening, LC-MS, Topoisomerase, Ultrafiltration
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INTRODUCTION

Variety of scientific advances and economic pressures in recent years called for improved drug discovery screening technology (Burbaum, 1998; Major, 1998). In response to that call, a number of potential therapeutic targets from the field of functional genomics have emerged and large compound libraries derived from parallel and combinatorial chemical synthesis techniques have been developed. These new advances have helped the pharmaceutical industry reduce development costs and enhance commercial competitiveness (Sundberg, 2000).

The demand for screening large compound collections against increasing number of therapeutic targets has stimulated technology development in the areas of assay automation, miniaturization, and detection methodologies. High-throughput screening technology based on 96-well plates and other approaches are widely available, and methods for more efficient, cost-effective screening are maturing rapidly (Gund and Nolan, 1999). The traditional discovery system was developed to handle the physical and chemical properties of purified samples, and assay results for a limited set

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of screens. In that case, for natural products, a separate system usually tracked the crude mixtures and their fractionated components; then a lead structure was purified and characterized, and finally was entered into the traditional discovery system as a conventional and purified compound. When these approaches were used in combinatorial libraries, some of the initial screening results might be for mixtures or uncharacterized samples. If structure-activity analyses of these samples generate hits, further work is then required. Once combinatorial libraries screening is combined with High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS), the structure and activity information could be obtained directly (Brimmel *et al*., 1996). In this paper, a screening method for potential anti-topoisomerase molecules is presented. This method integrates bioaffinity screening and chemical analysis in one process, provides high throughput for bioactive compounds screening.

METHOD

Materials

Centrifugal and micro-plate ultrafiltration were performed with 1000 μl microcon microconcentrators (Millipore, USA). Filters with nominal molecular weight cutoff of 100000 were used without preconditioning. Drug sample was made by our laboratory or obtained as donations from pharmaceutical companies. Supercoiled pBR 322 DNA and other chemical components were purchased from Huadong Chemical Co. (Hangzhou, China).

Enzyme preparation and definition of enzymatic activity

DNA topoisomerases I and II were extracted from rat Ehrlich ascites carcinoma cells from the peritoneal cavity 7 days after tumor inoculation following the procedure of De Isabella *et al*.(1990). The extract was mixed with an identical volume of glycerol and stored at –80 °C. The enzyme containing the activity of DNA topoisomerase I (Topo I) and II (Topo II) activity was nuclease free. Topo I activity was examined by the DNA relaxation reaction. One unit of Topo I activity was defined as the amount of enzyme required to fully relax 0.15 μg of supercoiled DNA under the conditions described in Meng *et al*.(2001). Topo II activity was detected by the kDNA to minicircles under the conditions described by them.

Topoisomerase affinity screening

A mixture of the selected components was prepared in methanol. DNA topoisomerases II and I were prepared in 100 μl 1/100 mmol/L PBS buffer at pH 7.9. The binding was accomplished by incubation of the mixture at room temperature for 30 min. The solutions were then transferred onto Microcon 100 centrifugal filters and centrifuged at 10000 g for 8 min, or until the filter appeared dry. Two washing steps were required to reduce the retention of non-binding analysts to a minimum. PBS buffer at pH 7.9 was used to wash the filter with subsequent ultrafiltration until the filter appeared dry. The eluate collected from the wash steps and containing the non-binding components of the mixture was discarded. Finally, disruption of the affinity complexes collected on the filter was performed by the addition of 30 μl of 1% trifluoroacetic acid in water. The released components were eluted by ultrafiltration into fresh collection vessels, and the topoisomerases II and I remained trapped on the filter. The eluate from this final extraction step was then analyzed by LC/MS.

High-performance liquid chromatography

High-performance liquid chromatography was carried out on a Hewlett-Packard system, 1100A pump and diode array detector, using a Lichrosher C18 analytical Column (5 μm, 250×4.6 mm) with 70% methanol and 30% water (content 0.02% trifluoroacetic acid), flow rate 0.8 ml/min, detection wavelength of 232 nm, 254 nm or 445 nm.

ESI-Mass spectrometry

Mass spectrometry was performed on a Bruker Esquire 3000+ system with electrospray interface. A range of 112.00–2000.00 m/z scanned with a sampling dwell time of 12616 μs was typically used.

RESULTS AND DISCUSSION

DNA topoisomerases

DNA topoisomerases are ubiquitous enzymes that act to change the topological state of the DNA double helix. Topoisomerases of two highly conserved classes, namely topoisomerase I and topoisomerase II, have been identified so far. These two classes differ in their reaction mechanisms and physical properties. Topoisomerase I alters DNA topology by making a transient single-stranded break in the DNA backbone, thereby, allowing the passage of another DNA strand. In contrast, topoisomerase II introduces transient double-stranded DNA that breaks and transfers an intact DNA duplex through the break before resealing it (Wiehodt *et al*., 1997; Froelich and Osheroff, 1995; Wang, 1996; Wang *et al*., 2000; Bjergbaek *et al*., 2000). Both activities are especially crucial during DNA transcription and replication, when the DNA helix must be unwound to allow proper functioning of large enzymatic machinery.

Topoisomerase is normally localized in the nucleolus (Chang *et al*., 1992) of most cells but in very low levels or are even absent from mature granulocytes. Resting lymphocytes express topoisomerase and increase the level of expression upon stimulation (Kaufmann *et al*., 1995). Topoisomerase levels have been reported to be elevated in some tumors (Husain *et al*., 1994). Topoisomerases are the targets of anti-tumor drugs that are usually inhibitors of topoisomerases. The functions and structures of the two topoisomerase types, and the inhibitors against them are different. Recent research revealed that almost all of the known inhibitors of DNA topoisomerases displayed potential anticancer activities. If high-throughput screening is applied to affinity ultrafiltration of DNA topoisomerases to study natural products, chances to identify new inhibitors of DNA topoisomerases would be increased, and more potent anticancer drugs would be discovered (Husain *et al*., 1994; Liu, 1992).

Enzyme-affinity screening

In this approach, a method of enzyme-affinity screening was established by using DNA topoisomerase I or II as a target to capture active compounds. Table 1 shows the library compound for the screening model. All the substances, individual or mixed, were co-cultured with topoisomerase to form active compounds-topoisomerase complexes. These complexes are separated from the mixture by ultrafiltration. Subsequently, the link between active compounds and topoisomerase is broken, and the active compounds are released. The active compounds, drug candidates, were analyzed and identified by HPLC/EIS-MS. Fig.1 is the flow chart of the enzyme-affinity screening.

Step I, culture: Topoisomerases were mixed with selected substances to form topoisomerase-bound complexes, with the non-binding substances remaining free in solutions.

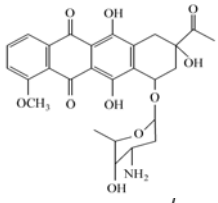
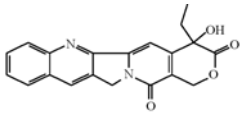
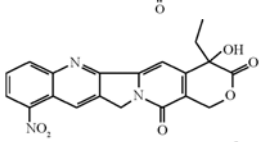
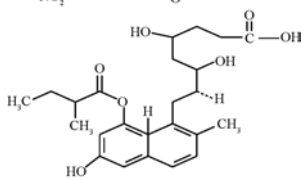
Step II, the first ultrafiltration: The overloaded drugs and non-affinitive components were discarded by ultrafiltration and topoisomerase-bound complexes were obtained.

Step III, the second ultrafiltration: Wash off the non-specific binding components.

Step IV, de-binding to free drug: Release the active compounds from topoisomerase binding by denaturing the protein enzyme, topoisomerase.

Step V, the third ultrafiltration: Ultrafiltrated again to allow the topoisomerase to remain on the

Table 1 Names, structures and exact mass of selected components

Name	Chemical structure	Mass
Daunorubicin		527.18
Camptothecin		348.36
9-NO_2-camptothecin		393.36
Pravastatin		446.52

filter but the desired active compounds to pass through; collect the filtrate for analysis.

Step VI, analysis and identification: Screened drugs were analyzed and identified by HPLC/EIS-MS.

HPLC and ESI-MS

High-performance liquid chromatography was performed to decrease mobile phase flow rate in 0.2 ml/min to the range suitable for direct introduction into the ion source without splitting to waste. For separation of more complex compositions, the mobile phase proportion was adjusted; or the step was performed with grading-program elution to increase resolving capability. In the above screening process, DNA topoisomerases II and I were targets, respectively. The LC-MS chromatogram for the library compounds is shown in Fig.2. In the library contained four compounds: daunorubicin, camptothecin, 9-NO_2 camptothecin and pravastain.

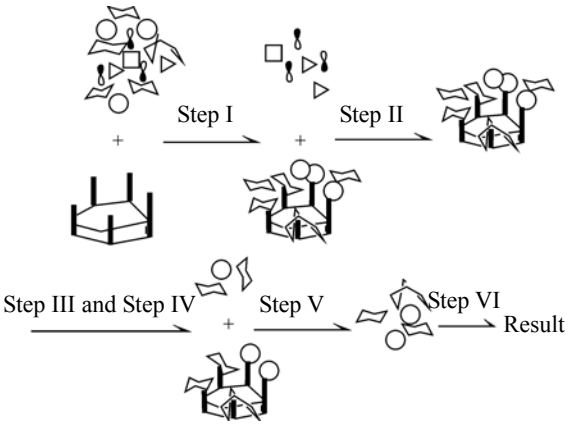
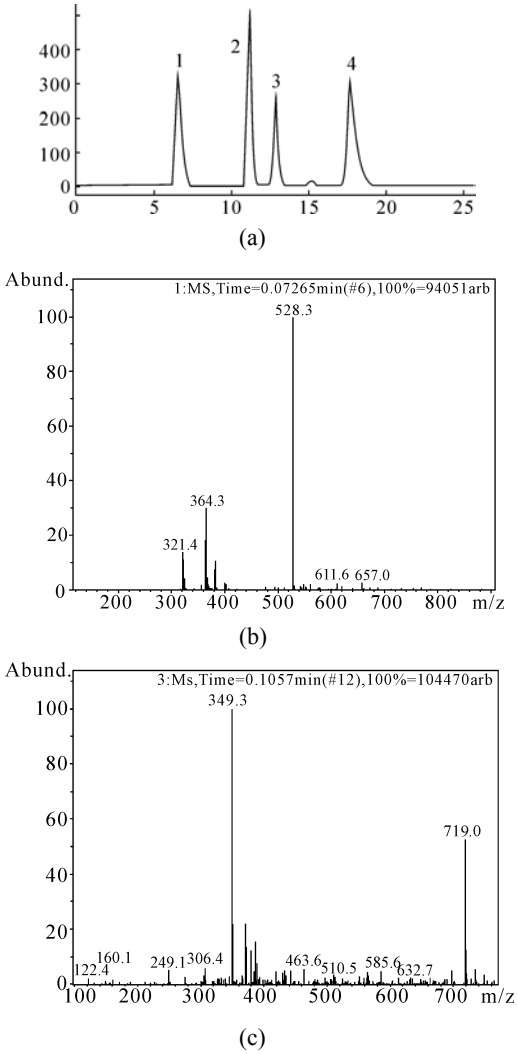


Fig.1 Flow chart of enzyme-affinity ultrafirtation



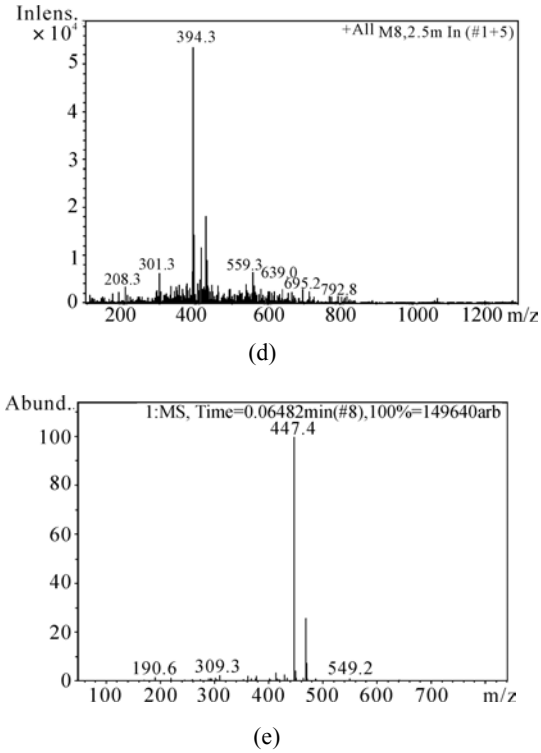


Fig.2 HPLC/ESI-MS graphs of pre-screening compounds

(a) Chromatogram of pre-screening compounds. 1: daunorubicin; 2: camptothecin; 3: 9-NO_2 camptothecin; 4: Pravastain; (b) Mass spectrum of daunorubicin; (c) Mass spectrum of camptothecin; (d) Mass spectrum of 9-NO_2 camptothecin; (e) Mass spectrum of pravastain

Fig.3 is the LC-MS chromatogram for post-screening sample. $[M+H]^+$=528 for daunorubicin, $[M+H]^+$=349 for camptothecin and $[M+H]^+$=394 for 9-NO_2 camptothecin. Comparing Fig.3 with Fig.2, camptothecin and 9-NO_2 camptothecin as well as daunorubicin showed affinity to topoisomerases. These results are consistent with the anticancer properties of camptothecin and 9-NO_2 camptothecin since they are strong inhibitors of topoisomerase I (Li *et al*., 1996). Daunorubicin was a strong inhibitor of topoisomerase II (Li *et al*., 1996). Pravastain was not recovered during the affinity assaying process, and so was not detected in the final filtrate as shown in Fig.3. Pravastain was obviously not affinitive to topoisomerase II or I. Therefore, it is conceivable that pravastain does not affect the activity of topoisomerases.

High-throughput screening

The method employing affinity ultrafiltration of DNA topoisomerase to recover and discover anti-topoisomerase compounds is useful and feasible for high-throughput screening. Ultrafiltration process achieved by 96-well micro-plate ultrafiltrator and analyzed by HPLC/ESI-MS with automatic injector of 96-well micro-plate would allow the screening of thousands of components in oneshort period of time. Using this method, the potential anticancer compounds from the callus cultures (Hengel, 1992) of Camptotheca acuminata were screened. The result showed that the method described above was applicable and easy to repeat. Twenty-one samples out of ninety cell cultures were identified as containing camptothecin and its derivative. Two samples were especially noteworthy. These two samples were different from the others in that camptothecin and its derivative were not detected by thin layer chromatography (TLC) techniques, despite the fact that these cell cultures caused the death of cancer cells, strongly suggesting that they contained anticancer compounds. The affinity ultrafiltration screening showed that the two samples indeed contained camptothecin, not detectable with TLC, but detectable after the affinity

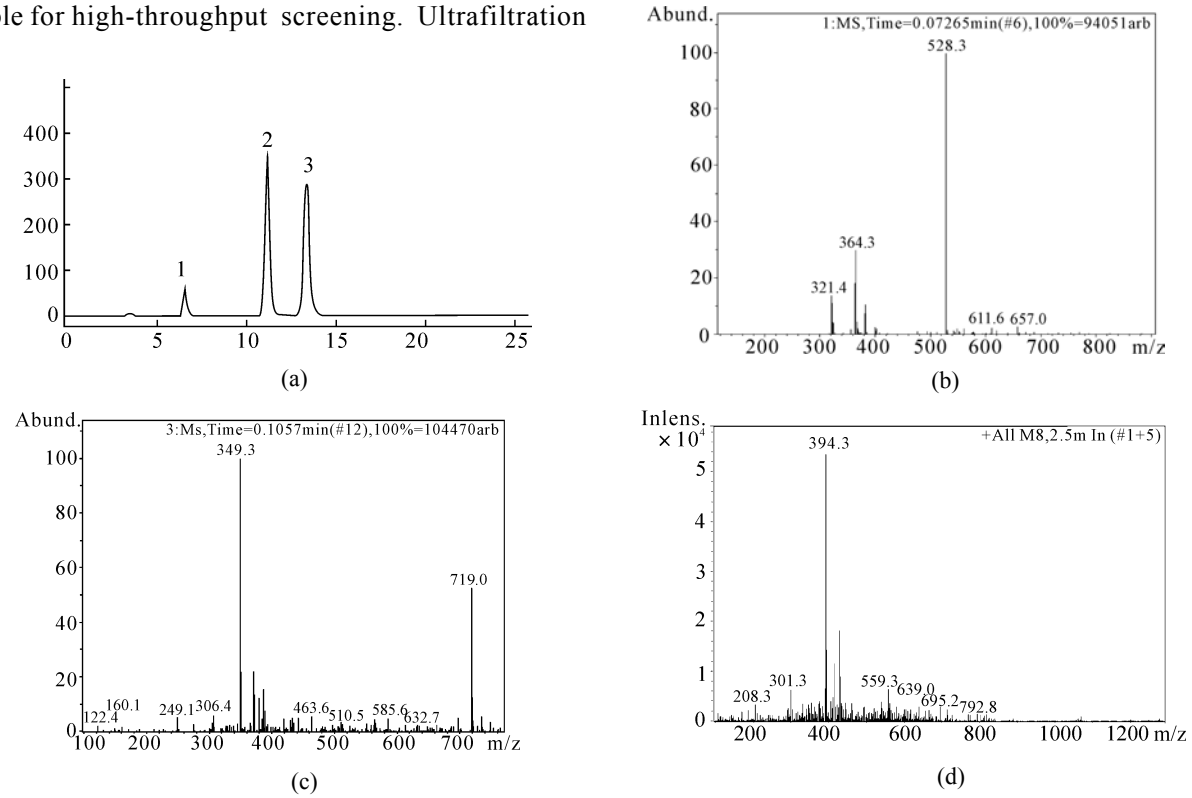


Fig.3 HPLC/ESI-MS graphs of post-screening compounds (the filtrate from Step V)

(a) Chromatogram of pre-screening compounds. 1: daunorubicin; 2: camptothecin; 3: 9-NO_2 camptothecin; (b) Mass spectrum of daunorubicin; (c) Mass spectrum of camptothecin; (d) Mass spectrum of 9-NO_2 camptothecin

assay. This illustrates the power and accuracy of the affinity-screening assay in detecting low concentration plant samples.

CONCLUSION

DNA topoisomerase affinity screening assay is a powerful and useful tool for screening out compounds with potential anticancer activities.

This assay method can separate the active compounds from topoisomerases by a series of ultrafiltrations, and then confirm the success of this separation by HPLC/ESI-MS. By applying this method for high-throughput screening, information on the structure and activity of candidate drugs could be obtained in a simultaneous run. This method integrates bioaffinity screening and chemical analysis in one process; and is highly specific, fast, and cost-effective. It can also be used to screen compounds from combinatorial and other natural products libraries, especially when these compounds are limited in quantity thus offering a new way to improve the yield of high-throughput screening.

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