Introduction

Topoisomerases are enzymes that alter the topology of DNA by breaking and resealing one or both strands in concert, allowing strand passage during the breaking-resealing process [1, 2]. They are crucial for DNA replication, RNA transcription, chromosome condensation, and segregation during mitosis, and attachment of DNA loops to the nuclear matrix and chromosomal scaffold. As a result, induction of DNA damaging by the inhibition of TOP2 becomes an important strategy for clinical anticancer agents [2], such as chemotherapeutic drugs etoposide and doxorubicin. In addition, the development of anti-TOP2 agents is still one of the major goals for oncologists [3]. In the past few years, we have performed screenings of crude extracts and pure compounds with antitumor activities from Chinese herbal medicine. Among them, Lindera megaphylla (Lauraceae) [4] is traditionally used as an antineoplastic and wound healing remedy for relieving pain, swelling, and palsy in Chinese herbal medicine. In a previous study [4], we found antitumor activities of D-dicentrine, a natural isoquinoline alkaloid from the root of L. megaphylla. To generate analogues, series of phenanthrene alkaloids from D-dicentrine were synthesized by degradation with ethyl chloroformate in pyridine, base hydrolysis, and N-alkylation. In this study, we demonstrated that one of the synthesized D-dicentrine analogues (here after designated as analogue 1) exhibited more potent cytotoxic effects than D-dicentrine in colon adenocarcinoma, hepatoma, leukemia, and epidermoid carcinoma cells. We performed cell cycle and apoptotic analysis by flow cytometry, an apoptotic DNA detection ELISA assay, and topoisomerase II activity by the kinetoplast DNA concatenation assay for studying their cytotoxic mechanisms. We found that both D-dicentrine and analogue 1 induced apoptosis and G2/M arrest in HL-60 leukemia cells. The percentage of apoptotic cells induced by analogue 1 was 4.5-fold higher than that induced by D-dicentrine as evident from measuring the amount of histone-bound DNA fragments. Moreover, we found that analogue 1 was 28-fold more potent than D-dicentrine for inhibition of topoisomerase II activity by the kinetoplast DNA concatenation assay. Our findings indicate that D-dicentrine analogue 1 is very promising as a potential antitumor agent for future study.
noline alkaloid isolated from the root of L. megaphylla. Among growth inhibition tests, D-dicentrine shows cytotoxicities to various tumor cell lines, including hepatoma, melanoma, lung carcinoma, colon adenocarcinoma, esophageal carcinoma, lymphoma, and leukemia. IC50 values are between 0.408 and 29.04 µM. Moreover, D-dicentrine possesses antitumor activities as detected by the colony formation assay in human hepatoma and in vivo antitumor assay [4]. In this study, we compared the efficacy between D-dicentrine and its analogues, and analyzed their cytotoxic mechanisms. We found that both D-dicentrine and its analogues induce apoptosis of cancer cells through TOP2 inhibition.

Results

Since D-dicentrine shows effective antitumor activity in vivo and in cell culture systems [4], we tried to modify D-dicentrine to obtain its analogues (with their structures shown in Fig. 1) with a higher efficacy [5]. To test the potential antitumor effects of these analogues, cytotoxic effects were first tested by the MTT assay. As shown in Table 1, analogue 1 is the most effective agent for growth inhibition in various human cancer cell lines.

Table 1 Analogue 1 is the most effective agent for growth inhibition in various human cancer cell lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell line</th>
<th>COLO 201</th>
<th>MS-G2</th>
<th>KB</th>
<th>HeLa</th>
<th>HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dicentrine</td>
<td>17.4 ± 4.0</td>
<td>16.0 ± 1.7</td>
<td>21.8 ± 3.4</td>
<td>35.0 ± 2.1</td>
<td>55.0 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>analogue 1</td>
<td>3.9 ± 0.4*</td>
<td>3.9 ± 0.5**</td>
<td>3.5 ± 0.6**</td>
<td>2.6 ± 0.3***</td>
<td>11.0 ± 0.8***</td>
<td></td>
</tr>
<tr>
<td>analogue 2</td>
<td>5.8 ± 1.2*</td>
<td>5.1 ± 1.8**</td>
<td>6.4 ± 1.5*</td>
<td>14.1 ± 1.9**</td>
<td>14.0 ± 0.8***</td>
<td></td>
</tr>
<tr>
<td>analogue 3</td>
<td>25.0 ± 4.1</td>
<td>23.1 ± 3.7</td>
<td>24.6 ± 6.1</td>
<td>35.1 ± 4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>analogue 4</td>
<td>31.7 ± 2.7</td>
<td>29.6 ± 2.6</td>
<td>26.4 ± 6.1</td>
<td>28.2 ± 3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>analogue 5</td>
<td>4.0 ± 1.1*</td>
<td>4.4 ± 0.8**</td>
<td>3.3 ± 0.1**</td>
<td>3.6 ± 0.5***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>analogue 6</td>
<td>4.8 ± 1.1*</td>
<td>4.0 ± 1.2**</td>
<td>3.5 ± 0.1**</td>
<td>9.9 ± 0.9***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>analogue 7</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>4.9 ± 1.1**</td>
<td>3.1 ± 0.9***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>analogue 8</td>
<td>5.4 ± 1.1*</td>
<td>14.7 ± 2.1</td>
<td>7.0 ± 0.9*</td>
<td>8.5 ± 0.9***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camptothecin</td>
<td></td>
<td></td>
<td></td>
<td>0.9 ± 0.1**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells as indicated were treated with 0.25% DMSO (solvent control), D-dicentrine, its analogues, or camptothecin (positive control) for 72 h, and IC50 values (µM) of D-dicentrine and its analogues on the viability of various human tumor cell lines were determined by the MTT assay; Each value represents the mean ± SEM obtained from three repeats; *P < 0.05; **p < 0.01; ***p < 0.001 (t-test) between treated and DMSO control cells.

Fig. 1 Structures of D-dicentrine and its analogues (analogues 1–8).
hepatoma MS-G2 and epidermoid carcinoma KB cells. In general, most analogues show a better inhibition effect than D-dicentrine (Table 1). Further study of D-dicentrine and analogue 1 led to the conclusion that the cytotoxic potency of analogue 1 (IC50 = 11.0 µM) is higher than D-dicentrine (IC50 = 55.0 µM) in human HL-60 leukemia cells (Table 1). To test whether non-tumorigenic cells are more resistant to these compounds, we compared drug sensitivity of both wild-type mouse embryonic fibroblasts (MEFs, normal diploid cells) and its tumorigenic counterpart, E1A/Ras transformed MEFs [6, 7]. Both cell lines share the same genetic background except for overexpression of E1A and Ras oncogenes in the latter cells. As shown in Fig. 2, non-tumorigenic MEFs were more resistant to D-dicentrine, analogue 1, and camptothecin compared to the dramatically lower viability of E1A/Ras transformed MEFs treated with these compounds. These data suggest that we can obtain the potent D-dicentrine analogues through modification, and analogue 1 is the most effective agent for growth inhibition in various human cancer cell lines. In this paper, we will focus on D-dicentrine and analogue 1 for further studies of cytotoxic mechanisms.

To further study the growth inhibition mechanisms of D-dicentrine and analogue 1, apoptosis induction was first estimated by double staining with annexin V plus PI using flow cytometry in HL-60 and analogue 1, apoptosis induction was first estimated by double staining with annexin V plus PI using flow cytometry in HL-60 cells (Fig. 3). The percentage of early apoptotic cells represented by annexin V-FITC positive and PI fluorescence negative cells increased in a dose-dependent manner after cells were treated with D-dicentrine or analogue 1. These results reveal that both D-dicentrine and analogue 1 caused apoptosis in HL-60 cells, and analogue 1 is more potent than D-dicentrine.

To further compare the apoptotic induction effect between D-dicentrine and analogue 1, the apoptotic DNA degradation was quantified with an ELISA method, which measures the amount of histone-bound DNA fragments in the cytosol. In this experiment, the drug exposure time was constant (24 h), while the D-dicentrine concentration varied from 0 to 50 µM. As shown in Fig. 4, the apoptosis induction by D-dicentrine and analogue 1 were both dose dependent. Compared to the OD value of 0.25% in DMSO-treated cells, the OD values of 50 µM D-dicentrine- and 10 µM analogue 1-treated cells increased from 0.13 to 0.303 and 1.39, respectively (Fig. 4A, B). Using this technique, the OD values of 10 µM analogue 1-treated cells was about 7-fold higher than the OD values of 10 µM D-dicentrine-treated cells, which is consistent with the higher percentage of apoptosis in the analogue 1-treated cells in Fig. 3. This suggests that analogue 1 induces more apoptotic cells than D-dicentrine. To gain insights into the mechanism, cell cycle profiles from both drug-treated cells were analyzed by DNA content analysis. As shown in Figs. 5 and 6, both D-dicentrine and analogue 1 caused G2/M cell cycle arrest in HL-60 cells. Compared with the DMSO solvent-treated control, the populations of G2/M cells increased from 23% to 33% or 42%, respectively, for treatment with 50 µM D-dicentrine or 25 µM analogue 1 (Figs. 5 and 6). As shown in Fig. 6, the G2/M phase populations also dramatically decreased and the S phase populations also dramatically increased for cells treated with 25 µM analogue 1. We confirmed both D-dicentrine and analogue 1 induce apoptosis and cell cycle arrest, as shown in Fig. 4 to 6. In addition, we also found a reduction of caspase-3 (apoptosis marker) and an elevation of phospho Histone H3 (M-phase arrested marker) by these compounds (Fig. 4C). These data suggest that D-dicentrine and analogue 1 cause G2/M cell cycle arrest in a dose-dependent manner.

In this paper, the inhibition effects of TOP2 by both D-dicentrine and analogue 1 were also studied. A well-known anticancer drug camptothecin (lane 10, Fig. 7A, C), belonging to topoisomerase I inhibitors, was used as the negative control, while the clinical anticancer drug novantrone, a TOP 2 inhibitor, was used as the positive control (lane 11, Fig. 7A, C). As shown in Fig. 7, D-dicentrine and analogue 1 inhibited DNA concatenation catalyzed by TOP2, with IC50 values of 78.4 and 2.8 µM, as estimated by the kDNA concatenation assay using HeLa cell nuclear extract. In conclusion, analogue 1 is 28-fold more potent than D-dicentrine in TOP2 inhibition. On the other hand, D-dicentrine and analogue 1 show no effect on topoisomerase I-induced DNA relaxation (data not shown). These results indicate D-dicentrine and analogue 1 induce apoptosis through TOP2 inhibition.

Discussion

The main purpose of our study was to discover novel topoisomerase inhibitors, by isolating active constituents from medicinal plants, to serve as potential antitumor drugs. In this study, a series of phenanthrene alkaloids were synthesized from D-dicentrine to evaluate the cytotoxic mechanisms of D-dicentrine and its analogues [5]. We found most synthetic analogues of D-dicentrine are more potent than D-dicentrine on cytotoxicity. There is a major structural difference as analogues are tetracyclic while parent compound D-dicentrine is pentacyclic, suggesting possi-
ble binding mode difference. In addition, analogues 1, 2, 3, and 4 carry alkyl groups on the nitrogen atom; however, elongation of alkyl groups for N-hexyl and N-octyl analogues 3 and 4 hampers their cytotoxic effects. Analogues 5, 6, and 8 also exhibit potent activity, but their structural features are different from those of analogues 1–4 with alkyl groups. Analogue 5 with nitrile is thought to be potent on cytotoxicity. According to our results (Table 1), analogues 5 and 6 are as potent as analogue 1 on growth inhibition in human COLO 201 colon adenocarcinoma, MS-G2 hepatoma, and KB epidermoid carcinoma cells. However, the potential immediate first-pass N-dealkylation in vivo, together with the fact that the N-dealkylated analogue 8 was not active,
has to be considered further on. As a result, we will continue to study cytotoxic mechanisms of other analogues in these tumor cells.

Specifically, in HL-60 leukemia cells, apoptosis induction by analogue 1 is more evident than D-dicentrine by using annexin V/PI double staining and flow cytometry (Fig. 3), and an apoptotic DNA detection ELISA assay (Fig. 4). Although both D-dicentrine and analogue 1 induce G2/M arrest in HL-60 leukemia cells by cell cycle analysis, the latter is more effective (Figs. 5 and 6). In addition, we found the cytotoxic mechanisms of D-dicentrine and analogue 1 through TOP2 inhibition by the kDNA concatenation assay (Fig. 7), and analogue 1 is 28 times more potent than D-dicentrine.

Similar to our findings, D-dicentrine shows an antiproliferative effect [8]. Recently, S-(+)-dicentrine demonstrated an antinociceptive effect in pain models induced by inflammation [9]. In addition, it has been shown that dicentrine inhibits α1-adrenoceptor [10] and the platelet aggregation induced by arachidonic acid (AA), thrombin, collagen, and platelet-activating factor (PAF) [11, 12].

DNA TOP2 is known to be involved in a number of vital cellular processes, and it is an important nuclear enzyme controlling DNA topology through catalysis of a transient breakage of double-stranded DNA in an ATP-dependent fashion, allowing for the passage of double-stranded DNA followed by a resealing of the DNA [1–3]. Consistent with our study, recently, D-dicentrine isolated from the root of *L. megaphylla* was reported to be an inhibitor of mammalian DNA TOP2 *in vitro* [13]. However, due to concerns regarding TOP2A overexpression in HCC with a high incidence of chemoresistance [14], the importance of finding new antitumor agents against TOP2 cannot be overemphasized.

In conclusion, by evaluation of synthetic analogues of D-dicentrine, we have identified analogue 1 as a potent inhibitor for the catalytic activities of TOP2. By comparing the inhibition activities of TOP2 with several clinically important anticancer drugs, including etoposide (IC50 value of 100 µM), epipodophyllotoxin GL-331 (IC50 value of 15 µM), genistein (IC50 value of > 100 µM), and F11782, which is a novel epipodophylloid (IC50 value of 1.3 µM) [15], we found that the efficacy of analogue 1 is comparable to that of etoposide, epipodophyllotoxin, and genistein [3,16]. In short, our findings strongly suggest that analogue 1 is very promising as a potential cytotoxic drug, and deserves further study and development.

**Materials and Methods**

**Cell lines and cell culture**

Some cell lines used in this study were kindly provided by the Cell Bank of General Veterans Hospital, Taipei, Taiwan. Five human tumor cell lines were used in our study: COLO201 human colon adenocarcinoma, KB human epidermoid carcinoma, MS-G2 human hepatoma [17], HeLa human cervical carcinoma, and HL-60 human leukemia cell lines. Human and mouse embryonic fibroblasts (MEFs) [6,7] cell lines were maintained in either DMEM (Gibco Laboratories) or RPMI medium 1640 (for HL-60,
Gibco Laboratories) supplemented with 10% (v/v) fetal calf serum (Gibco Laboratories), 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 1% nonessential amino acids (Gibco Laboratories). Exponentially growing cultures with more than 95% plating efficiency were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

**Analalogues**

To create more powerful antitumor agents, analogues were obtained by modification of D-dicentrine by the following methods (Fig. S1, Supporting Information) [5]: the benzylic C–N bond of D-dicentrine was cleaved by ethyl chloroformate in pyridine to form analogue 7 ([Ethyl-N-[2-(9,10-dimethoxyphenanthro-[3,4-d][1,3]-dioxol-5-yl)-ethyl]-N-methylcarbamate], followed by base hydrolysis with KOH in refluxing aqueous ethanol to form analogue 8 ([2-(9,10-dimethoxyphenanthro-[3,4-d][1,3]-dioxol-5-yl)-ethyl]-methylamine). The N-alkylation of analogue 8 with various alkyl iodides in DMF led to the formation of analogue 4 (Octyl-[2-(9,10-dimethoxyphenanthro-[3,4-d][1,3]-dioxol-5-yl)-ethyl]-methylamine). analogue 3 (Hexyl-[2-(9,10-dimethoxyphenanthro-[3,4-d][1,3]-dioxol-5-yl)-ethyl]-methylamine), analogue 4 (Octyl-[2-(9,10-dimethoxyphenanthro-[3,4-d][1,3]-dioxol-5-yl)-ethyl]-methylamine), analogue 5 (2-[2-(9,10-dimethoxyphenanthro-[3,4-d][1,3]-dioxol-5-yl)-ethyl]-methylaminol) ethanenitrite), and analogue 6 (2-[2-(9,10-dimethoxyphenanthro-[3,4-d][1,3]-dioxol-5-yl)-ethyl]-methylaminol]-ethanol). The purities of D-dicentrine and the analogues were ≥ 95% and ≥ 99%, respectively.

**Cytotoxicity assay**

Cytotoxicity in vitro was done according to the method described by Carmichael et al. [18]. In general, 2 × 10⁴ cells/well were incubated in a 96-well plate in the presence or absence of one of the five doses (i.e., 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ M) of compounds for 72 h. Then, 1 µg/mL MTT (Sigma) was added, and the plates were incubated at 37°C for 4 h. After the culture medium was removed, 100 µL DMSO (Merck) were added to the wells and mixed thoroughly to dissolve the dark blue crystals. To ensure that all of the crystals were dissolved, the OD values of the plates were then measured by a Power Wavex Microelisa reader (Bio-tek) using a test wavelength of 540 nm. The experiment was done in triplicate and the percent of inhibition was calculated as follows: % inhibition = [1-OD (540 nm) of sample well/OD (540 nm) of control well] × 100. IC₅₀ was given as the concentration in µM required for 50% inhibition of cell growth. Results of the positive controls, camptothecin (Sigma, purity ≥ 95%) or novantrone (Sigma, purity ≥ 90%), were compared with the tested compounds for this assay and the following assays.

**Flow cytometric assessment of apoptosis using Annexin V assay**

Both 12-h drug-treated and untreated HL-60 cells were harvested and washed twice with PBS buffer, and the measurement of PS exposure was carried out using the annexin V/PI assay as described by the manufacturer (R&D). Briefly, 1 × 10⁶ cells were collected and resuspended in 1 mL annexin V buffer containing 1.5 µL annexin V, and incubated for 8 min. PI (2.5 µg/mL) was then added and analyzed by flow cytometry (FACSCalibur, Becton Dickenson).

**Apoptotic DNA detection enzyme-linked immunosorbsent assay**

Cells from the control and drug treatment groups were processed and analyzed for cytosolic histone-bound DNA fragments using the Cell Death Detection ELISA PLUS Kit (Roche Biochemicals) as described by the manufacturer. Briefly, HL-60 cells were plated in 96-well culture plates at an initial seeding density of 2 × 10⁴ cells/well. In dose-response experiments, cell cultures were treated for 24 h with 0, 1, 10, or 50 µM of the assay drug. Following treatment for the required period, the culture plate was centrifuged (20 min at 750g) to collect non-adherent cells, and the spent medium was removed carefully by aspiration. Two hundred µL of lysis buffer was then added to each well, and the plate

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was incubated with shaking for 30 min at room temperature. 

Lysates were subsequently transferred to Eppendorf microcentrifuge tubes and were centrifuged at 16000 × g for 5 min. Triplicate dilutions of cell lysates were placed into wells of a microtiter plate that was coated with streptavidin. Subsequently, a mixture of anti-histone-biotin (that binds to the histone component of the nucleosome) and anti-DNA conjugate to horseradish peroxidase (that reacts with the DNA component of the nucleosome) was added. The plate was then incubated at room temperature for 2 h. Afterwards, the wells were washed three times to remove unbound antibodies, and nucleosomes were quantified by determining the amount of peroxidase retained within the immunocomplex. Peroxidase activity was detected colorimetrically after the addition of 2,2'-Azino-di-[3-ethylbenzthiozolin-sulfonate] as a substrate. The absorbance of the product was measured at 405 nm using an ELISA reader (Bio-tek). The absorbance of the blank, which contained all reagents but no sample, was subtracted from the test results. ELISA results were normalized for the cell number.

**Western blot analysis**

After compound treatment for 24 h, cells were harvested and lysed with lysis buffer (Sigma) containing protease inhibitors (Roche Biochemicals). Cell lysates were subjected into 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis, and immunoblotted with antibodies against caspase-3 (Active Motif), caspase-8 (BD Biosciences), phopho histone H3 (Santa Cruz), and actin (Sigma).

**Flow cytometry analysis of DNA content**

Both 24-h drug-treated and untreated HL-60 cells were harvested and washed twice with PBS buffer. The cell pellets were resuspended (5 × 10^5/mL) in 200 µL citrate buffer (250 mM sucrose, and 20 mM trisodium citrate, 5% DMSO, pH 7.6) with 20 µg/µL of PI and 200 µg/µL of RNase for 60 min at 4°C [19–21]. The intensity of PI staining was analyzed on cell populations to determine the DNA content by flow cytometry (FACScan, Becton–Dickinson) and, hence, the cell cycle profiles of these cells using ModFIT cell cycle analysis software (Becton Dickinson).

**Kinetoplast DNA concatenation activity of topoisomerase II**

For preparation of the nuclear extracts, 5 × 10^5 HeLa cells were harvested after treatment with DMSO, D-dicentrine, analogue 1, camptothecin, or novantrone (Lederle). Cells were washed in ice-cold PBS and harvested in 750 µL sucrose buffer I (0.32 M sucrose, 3 mM CaCl2, 2 mM MgOAc, 0.1 mM EDTA, 1 mM DTT, 2 mM PMSF, 0.5% Nonidet P-40) and then were centrifuged at 500×g for 10 min at 4°C. The pellet was washed with 750 µL sucrose buffer II (0.32 M sucrose, 3 mM CaCl2, 2 mM MgOAc, 0.1 EDTA, 1 mM DTT, 2 mM PMSF) and centrifuged at 500×g for 10 min at 4°C. The pellet was resuspended in 50 µL of low salt buffer (20 mM HEPES-NaOH, pH 7.9, 25% glycercol, 1.5 mM MgCl2, 20 mM KCl, 1 mM DTT, 2 mM PMSF), then 50 µL of high salt buffer (20 mM HEPES-NaOH, pH 7.9, 1.5 mM MgCl2, 0.8 M KCl, 0.2 mM EDTA, 1 mM DTT, 2 mM PMSF, 1% Nonidet P-40) was added, and incubated on ice for 20 min with occasional gentle shaking. Then, after adding 100 µL 2.5× diluent buffer (25 mM HEPES-NaOH, pH 7.9, 25% glycerol, 0.1 mM EDTA, 1 mM DTT, 2 mM PMSF), samples were centrifuged at 10000 × g for 10 min at 4°C and the concentration of nuclear extract contained in the supernatant was determined by a protein assay (Bio–Red). The kDNA concatenate assay [22] as described by Larsen et al. was used with minor modifications.

Briefly, 18 µL of buffer A (50 mM Tris pH 8.0, 120 mM KCl, 0.5 mM DTT, 0.5 mM ATP, and 10 mM MgCl2) containing 200 ng of kDNA (TOPogen) and 1 µL (2 µg) nuclear extract from HeLa cells (the amount of enzyme which resulted in the complete concatenation of 200 ng of kDNA) after a 30-min incubation were added to 1 µL of either the solvent (DMSO) alone or a solution of the test drug. After 10 min of incubation at 37°C, the reaction mixture was analyzed on a 1% agarose gel and run at 35 mA for 2 h in TBE buffer. Gels were stained with EtBr and scanned under UV illumination using an Amersharm Pharmacia Biotech ImageMaster VDS imager. Assays to determine the inhibition of concatenation by quantification of the amount of concatenated DNA were carried out on at least three separate occasions, and the results are expressed as IC50 values.

**Statistical analysis**

The results are expressed as the mean ± SEM. The significance of the difference between the DMSO control and the test compound groups was analyzed by Student’s t-test.

**Supporting information**

A flow chart for the synthetic procedures of the analogues is available as Supporting Information.

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**Conflict of Interest**

The authors of this paper declare we have no conflicts of interest.

**References**

9 Montrucchio DP, Cordova MM, Santos AR. Plant derived aporphinic alkaloid S- (+)-dicentrine induces antinecrotic effect in both acute and chronic inflammatory pain models: evidence for a role of TRPA1 channels. PLoS One 2013; 8: e67730
22 Larsen AK, Grondard I, Couprie J, Desoize B, Comile J, Jardillier JC, Riou JF. The antileukemic alkaloid fagaronine is an inhibitor of DNA topoisom- erases I and II. Biochem Pharmacol 1993; 46: 1403–1412