Characterization of DNA Topoisomerase-1 in Spodoptera exigua for Toxicity Evaluation of Camptothecin and Hydroxy-Camptothecin

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Abstract
Camptothecin (CPT), a plant alkaloid originally isolated from the native Chinese tree, Camptotheca acuminate, exerts the toxic effect by targeting eukaryotic DNA topoisomerase 1 (DNA Topo1). Besides as potent anti-cancer agents, CPT and its derivatives are now being explored as potential pesticides for insect control. In this study, we assessed their toxicity to an insect homolog, the Topo1 protein from beet armyworms (Spodoptera exigua Hübner), a worldwide pest of many important crops. The S. exigua Topo1 gene contains an ORF of 2790 base pairs that is predicted to encode a polypeptide of 930 amino acids. The deduced polypeptide exhibits polymorphism at residue sites V420, L530, A653 and T729 (numbered according to human Topo1) among insect species, which are predicted to confer sensitivity to CPT. The DNA relaxation activity of this protein was subsequently examined using a truncated form that contained the residues 337–930 and was expressed in bacteria BL21 cells. The purified protein retained the ability to relax double-stranded DNA and was susceptible to CPT and its derivative hydroxy-camptothecin (HCPT) in a dose-dependent manner. The same inhibitory effect was also found on the native Topo1 extracted from IOZCAS-Spex-II cells, a cell line established from beet armyworms. Additionally, CPT and HCPT treatment reduced the steady accumulation of Topo1 protein despite the increased mRNA expression in response to the treatment. Our studies provide information of the S. exigua Topo1 gene and its amino acid polymorphism in insects and uncover some clues about potential mechanisms of CPT toxicity against insect pests. These results also are useful for development of more effective Topo1-targeted CPT insecticides in the future.

Citation: Zhang L, Ma D, Zhang Y, He W, Yang J, et al. (2013) Characterization of DNA Topoisomerase-1 in Spodoptera exigua for Toxicity Evaluation of Camptothecin and Hydroxy-Camptothecin. PLoS ONE 8(2): e56458. doi:10.1371/journal.pone.0056458

Editor: Subba Reddy Palli, U. Kentucky, United States of America

Received: March 27, 2012; Accepted: January 14, 2013; Published: February 22, 2013

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Funding: The study was financially supported by the National Natural Science Foundation of China (31000851 and 31071707). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction
DNA topoisomerases (DNA Topos), the enzymes that participate in DNA strand breakage and reunion reactions in a series of genetic processes such as DNA replication, transcription and recombination during the cell growth and proliferation, have been a target for cancer therapy [1]. A potent inhibitor of this class of enzymes is camptothecin (CPT), a plant alkaloid isolated originally from the Chinese tree, Camptotheca acuminate Decne. CPT functions by binding to and stabilizing the covalent complex of the nicked DNA-Topo1, which prevents DNA re-ligation and therefore causes irreversible DNA break during ongoing DNA and RNA synthesis [2]. Ever since its discovery in 1988, a plethora of studies have demonstrated that inhibition of eukaryotic Topo1 by CPT and its derivatives hinder cell survival and lead to apoptosis [3–5]. Due to the remarkable anti-cancer activity, CPT has been developed into a classical anticancer agent [3,6]. In addition, two of its classic derivatives, irinotecan and topotecan, have been used for treatment of various cancers throughout the world, and meanwhile some CPT analogues are currently used at various stages of clinical trials [7].

Although CPT was not known till 1966 during a discovery screening of natural products for anti-cancer drugs, the crude extract of C. acuminate that contains the now-known CPT has been traditionally used to control pests in ancient China for centuries. CPT and its derivatives are now being explored as a class of botanical insecticide in agriculture. CPT is a potent chemosterilant against houseflies [8]. It also exhibits significant genotoxicity to fruit flies (Drosophila melanogaster Meigen), which acts through promoting homologous and illegitimate recombination, as well as the whole chromosome loss by clastogenicity, deeply elucidating essential functions of Topo1 in embryogenesis, oogenesis, larva and pupal growth [9–11]. Recently, CPT has been shown to be toxic to several other agricultural pests like brown plant hoppers (Nilaparvata lugens Stål), striped rice borers (Chilo suppressalis Walker), cabbage aphids (Brevicoryne brassicae Linnæus) and small citrus trypetids (Bacterca dorsalis Hendel) [12,13]. To find new CPT-derived insecticides with improved efficacy and to determine the potential structural factors required for the biological activity of...
CPT analogues, a series of novel CPT derivatives have been semi-synthesized, and some of them were found toxic to armyworms (Mythimna separate Walker) and coconut palm beetles (Brontispa longissima Gestro) [14,15]. In our previous studies, we found that 0.1–30 µM CPT treatment to IOZCAS-Spex-II cells (established from beet armyworms, Spodoptera exigua Hubner) induce cell apoptosis [16]. Thus, these previous findings intrigue us to explore CPT as a lead compound of an insect control agent, and to investigate the possibility of exploring a new Topo1-targeted botanical insecticide.

Despite efforts on the toxicity of CPT to agricultural pests, very limited studies have been focused on insect Topo1 except the fruit fly (D. melanogaster) [11,17–19], and it has remained yet clear of whether CPT cytotoxicity to insect cells is closely associated with Topo1 gene expression and Topo1 enzyme activity. Here, we isolated and characterized the beet armyworm Topo1 gene and evaluated the susceptibility of Topo1 to CPT and its derivative hydroxyl-camptothecin (HCPT). We found that the Topo1 protein, when either purified as a recombinant protein from bacteria or extracted from IOZCAS-Spex-II cells, was susceptible to both CPT and HCPT. Moreover, pretreatment with CPT and HCPT led to reduction in both the enzymatic activity and the steady accumulation of the Topo1 protein in IOZCAS-Spex-II cells despite up-regulation of its mRNA expression in response to the treatment. These results offer important information for understanding CPT-toxicity against insects from the point of adaptive evolution and molecular toxicology and have implications in designing potent Topo1-based pesticides with high-performance and environment safety.

**Results**

**Characterization of S. exigua Topo1 Sequence**

To isolate the *S. exigua* Topo1 gene, three sets of degenerate primers targeting the conserved regions of eukaryotic Topo1 genes were employed to amplify the Topo1 coding region. The 5' and 3' ends were obtained with RACE PCR. The full-length Topo1 mRNA has 3740 nucleotides in length, encoding an ORF of 2790 nt flanked by a 80 nt 5' UTR and a 870 nt 3' UTR. The deduced Topo1 polypeptide has a size of 930 amino acids with a calculated molecular weight of 108 kDa and an estimated pI of 9.47, belonging to the type IB topoisomerases according to the pI. CPT-resistant Topo1s derived from Topo1-based biosynthesis and CPT-resistant Topo1s from the CPT-producing plants [22]. Three amino acid substitutions, N421K, L530I and N722S (numbered according to human Topo1) were found evolutionarily conserved, further explaining a theory of adaptive coevolution between CPT biosynthesis and CPT-resistant Topo1s derived from Topo1-based mutations in the CPT-producing plants [22]. Moreover, the same mutations were also detected in some human cancer cells extracted from patients who were tolerant to CPT treatments. Lots of reports have revealed that mutations in Topo1 that affect CPT binding or catalytic process can confer animal cells effective resistance to various extent [24–26]. Thus, the polymorphism of Topo1 protein provides useful information for prediction of CPT resistance or sensitivity before performing cellular toxicity tests.

To reveal the amino acid polymorphism of insect Topo1s, we performed sequence alignment analysis. The key residues including R408, K532, R590, H632 and Y723 (numbered according to human Topo1) were found evolutionarily conserved, further supporting their essential role in the nicking-closing reaction. Amino acid substitutions were observed at several positions, including V420, L530, A653 and T729, which may affect the sensitivity of insects to CPT and its derivatives. It has been reported that V420I do not alter sensitivity of CPT-producing plants to CPT; in contrast to previous studies that residue substitutions in amino acids 410–429 confer CPT-resistant in human cancer cells [22,27]. Unlike T729 in human and plants, S729 exists in every insect listed in Figure 3 except *Apis mellifera*. Other substitutions at position T729 associated with differential CPT resistance have been documented, but T729S has not been mentioned [28,29].

**Truncated Topo1 Expressed in E. Coli BL21 (DE3) Cells Retained the Same DNA-relaxing Enzyme Activity as Natural Topo1 in S. exigua**

The N-terminal region of Topo1 is highly heterogeneous and has been shown to be not required for the DNA relaxation activity in the case of Human Topo1. As *S. exigua* Topo1 is highly homologous with human Topo1, an N-terminus truncated form containing residues 337–930 of *S. exigua* Topo1 was expressed as a GST fusion protein in *E. coli* BL21 cells to test its enzymatic activity. Single expression of GST served as a negative control. The bacterially expressed truncated Topo1 (named Topo70) was subsequently purified by GSTrap columns and analyzed by SDS-PAGE. As expected, a protein with approximate 97 KDa corresponding to the predicted size of GST-Topo70 was observed on the gel by commassie blue staining (Figure 4). The enzymatic activity...
N-terminal domain

Domain II

Domain I

Domain III

Linkage region

Carboxyl domain
activity of Topo70 from E. coli cell lysates was determined by serial two-fold dilutions. The specific activity of Topo70 from purified fraction and crude lysate was 1,797,600 and 344,000 U mg⁻¹ pro, respectively (Table 1), suggesting that the truncated Topo1 function as well as the natural Topo1 did in IOZCAS-Spex-II cells.

CPT and HCPT Exhibited a Dose-dependent Inhibition on the Enzyme Activity of Topo1 Extracted in IOZCAS-Spex-II Cells

To investigate whether the natural S. exigua Topo1 is sensitive to CPT and HCPT treatment as expected, the Topo1 crude extracted from IOZCAS-Spex-II cells was used. 1 U Topo1 enzyme was incubated with the reaction buffer which pre-mixed with different concentrations of CPT or HCPT. The natural Topo1 completely relaxed supercoiled DNA (Figure 5A and 5B), line Topo1), and was susceptible to the treatment of both CPT and HCPT in a similar dose-dependent manner. The two compounds had respective EC50 values of 42.5 μM (95% fiducial limits, 20.8–86.9 μM) and 48.9 μM (95% fiducial limits, 11.5–207 μM). As for the recombinant Topo1, a similar dose-dependent inhibitory effect was also observed for both CPT and HCPT (Figure 6A and 6B). HCPT reached the maximum inhibition (Figure 6B, the percent of supercoiled DNA, 73.4%) at 100 μM and CPT at 50 μM (Figure 6A, the percent of supercoiled DNA, 93.0%). The respective EC50 values were 4.42 μM (95% fiducial limits, 2.22–8.32 μM) and 15.2 μM (95% fiducial limits, 8.05–28.7 μM), an inhibitory effect that was much higher than that from cell extracts, suggesting some unknown factors may interfere with the activity of CPT and HCPT.

CPT and HCPT Treatment of IOZCAS-Spex-II Cells

Reduced Topo1 Specific Activities

To test whether CPT and HCPT treatment can reduce the enzymatic activity of Topo1 in vivo, IOZCAS-Spex-II cells were pretreated with different doses of these chemicals for various times. As shown in Figure 7A, Topo1 specific activity dropped significantly in cells when treated with increased concentrations of CPT and HCPT for 24 h with the exception of Topo1 in cells treated with 0.5 μM HCPT (Figure 7B). In contrast, there was no significant change in Topo1 specific activity in the DMSO treated cells for all the test time points (Figure 8A and 8B). In the presence of CPT and HCPT, Topo1 specific activity (the relative specific activity, from 0.03 to 0.52 for CPT; from 0.22 to 0.57 for HCPT) decreased significantly with the increase of incubation time (p<0.05). There was no appreciable difference in Topo1 specific activity between CPT and HCPT pretreated cells. Thus, the above observations indicated that CPT/HCPT pretreatment induced a time- and dose-dependent loss of Topo1 enzyme activity in IOZCAS-Spex-II cells.

Upregulation of Topo1 Gene Expression in IOZCAS-Spex-II Cells Pretreated with CPT and HCPT

To test whether or not the decreased enzymatic activity of Topo1 upon CPT and HCPT pretreatment is due to the reduced Topo1 gene expression, the mRNA expression of Topo1 was measured by Real-time PCR with beta-actin as an internal control. Surprisingly, the mRNA expression of Topo1 was significantly up-regulated in CPT and HCPT treated IOZCAS-Spex-II cells. The Topo1 gene expression was increased in cells treated with all concentrations of CPT (the relative expression, from 4.90 to 8.37) and HCPT (the relative expression, from 1.48 to 3.88) for 24 h compared to the mock treated cells (Figure 9). HCPT-treated cells showed less Topo1 expression than CPT-treated cells with all corresponding concentrations. At all the tested time points, there was no significant change in Topo1 expression in the control group (Figure 10, Blank). However, Topo1 gene expression was up-regulated at 12, 24 and 48 h in cells treated with 10 μM CPT (1.58–5.19) and HCPT (1.96–2.38).

Figure 1. Multiple sequence alignment of Topo1. The amino acid sequences of Topo1 proteins from six representative species were aligned using ClustalX 1.83 with standard parameters and then rendered with ESPript 2.2 for clear illustration. Identical amino acids were highlighted in filled black columns. N-terminus region, aa 1–380; Domain I, aa 486–599; Domain II, aa 381–485; Domain III, aa 600–800; Carboxyl domain, aa 861–931; Linkage region, aa 801–859 (numbered according to human Topo1). The asterisk represents the key residue involved in the catalytic process.

Abbreviations: SE, Spodoptera exigua (GenBank ID: JN258956); BM, Bombyx mori (KAIKOGA029083); DM, Drosophila melanogaster (GenBank ID: NM078606); HM, Homo sapiens (GenBank ID: J03250); CE, Caenorhabditis elegans (GenBank ID: NM060936); CA, Camptotheca acuminata (GenBank ID: AB372511).

doi:10.1371/journal.pone.0056458.g001

Figure 2. Phylogenetic analysis of Topo1. The amino acid sequences of Topo1 from different spices, including beet armyworm (S. exigua), fruit fly (D. melanogaster), silkworm (B. mori), nematode (C. elegans), CPT-producing plant (C. acuminata) and human (H. sapiens), were aligned with MEGA5.0 program. A phylogenetic tree was constructed by the neighbor-joining method with 1,000 replicates. The genetic distance was drawn to scale and the bootstrap value illustrated above the line was marked in numbers.

doi:10.1371/journal.pone.0056458.g002
Pretreatment of IOZCAS-Spex-II cells with CPT and HCPT resulted in reduction in steady accumulation of Topo1 protein.

It has been demonstrated that prolonged treatment of human cancer cells with CPT led to down-regulation of Topo1 in a dose- and time-dependent manner due to Topo1 protein degradation and re-localization [30]. Also, the Topo1 mRNA level in the CPT-resistant cell lines has been found to be reduced, which accordingly reduced cellular Topo1 protein level as measured by western blotting in nuclear extracts [5,31,32]. However, it is not always unusual that Topo1 proteins and CPT-stabilized Topo1 cleavable complexes are not altered accordingly in some CPT-resistant cancer cells, suggesting that the CPT cytotoxicity may depend on cell types for other unknown mechanisms [33].

To determine whether CPT and HCPT treatment altered the expression of the Topo1 protein in IOZCAS-Spex-II cells, a polyclonal antibody (anti-Topo70) against beet armyworm Topo1 was produced by immunizing rabbits with purified recombinant Topo1 (Topo70) by Immunosoft Ltd. (Zhoushan, China). This polyclonal antibody strongly reacted with the recombinant GST-Topo70 fusion protein. Detection of native beet armyworm Topo1 from cell crude extracts revealed a band of 130 kD, which was different from the predicted size of 108 kD, suggesting further posttranslational modifications. The specificity was further supported by a competition assay. Pre-incubation of the antibody to purified GST-Topo70 blocked the recognition of the 130-kD polypeptide, suggesting that the 130-kD polypeptide is the beet armyworm Topo1.

![Figure 3. Amino acid polymorphism in Topo1s related to catalytic functions and direct/indirect CPT binding capacity.](image)

The five residues essential for catalytic activity are colored white, while the residues involved in binding to CPT are marked gray. The amino acid substitutions were highlighted in light gray. The residues are numbered according to the relative position in human Topo1. Abbreviations: SE, *Spodoptera exigua* (GenBank ID: JN258956); BM, *Bombyx mori* (KAIKOGA002908); DM, *Drosophila melanogaster* (GenBank ID: NM078606); AM, *Apis mellifera* (GenBank ID: XM_396203); AP, *Acythosiphon pisum* (GenBank ID: XM_001942991); NV, *Nasonia vitripennis* (GenBank ID: XM_001605054); CQ, *Culex quinquefasciatus* (GenBank ID: XM_001845544); AA, *Aedes aegypti* (GenBank ID: XM_001655563); TC, *Triatoma castaneum* (GenBank ID: XM_966102); HM, *Homo sapiens* (GenBank ID: J03250); CA, *Camptotheca acuminate* (GenBank ID: AB372511); CE, *Caenorhabditis elegans* (GenBank ID: NM060936).

doi:10.1371/journal.pone.0056458.g003

![Figure 4. Analysis of purified Topo1 preparations by SDS-PAGE.](image)

A truncated form of *S. exigua* Topo1 containing residues 337–930 was expressed as a GST fusion protein in *E. coli* BL21 cells, and purified using the trap column as described in Materials and Methods. Lane 1, cell lysates of bacteria BL21 expressing GST alone; Lane 2, cell lysates of bacteria expressing GST-Topo70; Lane 3–9, serial fractions of eluted Topo1 (#1–#7).

doi:10.1371/journal.pone.0056458.g004
Figure 5. CPT and HCPT inhibited the DNA relaxation activity of *S. exigua* Topo1 extracted from IOZCAS-Spex-II cells. To evaluate the toxicity of CPT or HCPT to *S. exigua* Topo1, an in vitro double-stranded DNA relaxation assay was performed by incubating Topo1 crude extracts from IOZCAS-Spex-II cells with 0.5 μg pBR322 DNA and various concentrations of CPT or HCPT at 26°C for 30 min. The DNA was subsequently assessed by agarose gel electrophoresis, and the DNA bands were densitometrically quantified with Quantity One (Gel Doc XR, Bio-Rad, USA). The experiments were repeated three times. The inhibition rate of the Topo1 enzymatic activity by CPT or HCPT was calculated as the percentage of supercoiled DNA over total pBR322 DNA. Each bar represents the mean ± SD. A: CPT; B: HCPT; Sc: supercoiled DNA; R: relaxed DNA; N: nicked DNA; pBR322: pBR322+0.50% DMSO; Topo1: Topo1+pBR322+0.50% DMSO. doi:10.1371/journal.pone.0056458.g005

Table 1. Topo1 protein purification and the enzyme activity.

| Usage       | Gene  | Forward primers (5’–3’)                           | Reverse primers (5’–3’)                           | Size(bp) |
|-------------|-------|--------------------------------------------------|--------------------------------------------------|----------|
| RT-PCR      | Topo1 | GAACCNCCNGNYTNTTCCMGNG                            | ATNGCNACGNGCGRRTNG                                | 770      |
|             |       | AAAAGGATGATGACCAGGCGGAC                           | CNGGNCCNGCATTATCGCCTATGCCC                      | 805      |
|             |       | CAYAARGNCGCHTNNTYGCWECGH                          | CGAGGAGGTGTAGTTAGTA                               | 530      |
| 5′ RACE     | Topo1 | CGCGGATCCACAGCTACTGATGATGATGATGATG               | GCATGAAATCCGGCTACTTCCCTACG                      | 1419     |
| 3′ RACE     | Topo1 | GCTGTCCCAAGGTCCATTCA                               | CGCGGATCTCCACTAGTATTGCCTACTATAGG                | 1294     |
| Full-length cDNA | Topo1 | ATGAGCTCGAATCCGCTACTGAC                           | TTAGAAATATTCGCCGCCGCC                           | 2793     |
| Truncated cDNA | Topo70| GCTGCGATCCAAAGCAAGTCAAAACACTCAAGT               | CATGTCCGAAGATATATTCGCCGCCGCCCCGCC               | 1782     |
| Real-time PCR | Topo1 | AGGTTGTGAAAGTTGGAAGTTGAGA                        | CATGATCATTACCTGACGAC                             | 198      |
|             |       | TCCAGCCTCTCTTCTGTATT                            | GGGAGCGATGATCTGTGAT                              | 213      |
|             | Beta- | AGGTTGGAATGTTGGAAGTTGAGA                        | CATGATCATTACCTGACGAC                             | 198      |

doi:10.1371/journal.pone.0056458.t001
site residues (R488, K532, R590 and H632) and the catalytic Y723. The amino acid polymorphism of Topo1s at the sites directly/indirectly related to CPT binding illustrates the important footprint of coevolution between CPT-producing plants and animals [22,34,35]. As shown in Figure 3, the amino acid polymorphism of Topo1s is detectable and widespread in different unrelated species, offering themselves a chance of CPT resistance or sensitivity [25]. Sikikantarmas et al. reported the survival strategy of CPT-producing plants against the CPT self-toxicity through amino acid substitutions in Topo1 compared to H. sapiens [35]. Three amino acid substitutions, N421K, L530I and N722S existing naturally in Topo1 of CPT-producing plants, are related to CPT-resistance. In particular, the substitution N722S is identical to that observed in CPT-resistant cancer cell lines. Additionally, more than ten mutations have been reported to be associated with resistance to CPT and its derivatives in human cancer cell lines established from screening Topo1 mutants stepwisely with CPT [24,26,35]. These findings reveal a very exciting example to understand the coevolution not only between the CPT biosynthetic pathway and self-resistance mechanism in CPT-producing plants, but also between plants and their natural enemies including insects and human. In nine reported insect Topo1s, four amino acid substitutions were also found at the sites involved in DNA binding or structural interaction with CPT. The effect of these four amino acid polymorphisms on CPT-sensitivity/resistance in insects is worthy of further studies, which may be useful for designing better CPT analogues.

CPT and HCPT are poisonous to a broad spectrum of Topo1s from different eukaryotic species with no exception to S. exigua Topo1. Generally, CPT derivatives are expected to have improved solubility, stability and long lifetime of the lactone form. HCPT is formed by hydroxy substitution at position 10 of CPT, which also is a stronger Topo1 inhibitor than CPT in many studies [19]. Modifications at the specified positions help
alter the equilibrium of the carboxylate/lactone form of the E ring and are directly associated with drug toxicity [36,37]. CPT and its derivatives with a closed E ring are more effective than those with an open E ring [38]. Our results showed that both CPT and HCPT exhibited a dose- and time-dependent effect on Topo1 enzyme activity. Because the lactone form of the E ring in HCPT is preferentially pH-dependent, HCPT showed lower inhibitory effects in vitro than CPT in a reaction buffer of pH 7.5 (Figures 5 and 6). In addition, the Topo1 gene expression in the HCPT-treated cells was lower than that in the CPT-treated sample for 24 h treatment (Figure 9, Figure 10). These results suggest that CPT is more toxic to S. exiguus Topo1 than HCPT. The difference of CPT and HCPT toxicity on the S. exiguus Topo1 requires further investigation in the future.

The crucial role of Topo1 for cell survival and differentiation requests a strict regulation of its expression. Upon CPT treatment, Topo1 is the first protein to be regulated both in redistribution and in Topo1 protein level in a single clone H1299-cherry [30]. Topo1 intensity in the nucleoli dropped in less than 2 min and it accumulated in the cytoplasm up to 5 hours in a CPT dose-dependent manner. Topo1 protein was finally degraded into detectable fragments of ~40 kD in the nucleus and then Topo1 fragments exited the nucleus into cytoplasm [30]. In this paper, the Topo1 enzyme activities and protein levels in drug-treated cells decreased despite the improved Topo1 mRNA levels (Figures 7, 8, 9, 10 and 11). Although the degraded fragments of Topo1 proteins were not detected both in cytosolic and nuclear fractions, the down-regulation of Topo1 protein explained well the decreased enzymatic activity. Because CPT/HCPT-treated cells need a large quantity of Topo1 proteins to resolve DNA topological problems in DNA repair or other apoptosis-associated DNA gene expression [39,40], the Topo1 mRNA level was accordingly increased to ensure that enough Topo1 protein is synthesized for the efficient

Figure 7. Pre-treatment of IOZCAS-Spex-II cells with CPT and HCPT decreased Topo1 specific activity in a dose-dependent manner. (A) The IOZCAS-Spex-II cells were pretreated with various concentrations of CPT or HCPT for 24 h prior subjected to Topo1 extraction. The CPT and HCPT toxicity was assessed by incubating serial of 2-fold diluted Topo1 extracts with 0.5 ug DNA at 26°C for 30 min. The DNA relaxation ability was then analyzed by agarose-gel electrophoresis. The picture shown was from a single representative experiment out of three repeats. Sc: supercoiled DNA; R: relaxed DNA; N: nicked DNA; 2^0, 2^1, 2^2, 2^3, 2^4, 2^5: the serial two-fold dilutions; Blank: pretreated with only 0.50% DMSO for 24 h. (B) The relative specific activity of Topo1 was expressed as the ratio of the specific activity of Topo1 from CPT or HCPT treated cells over that from Blank. Each bar represents the mean ± SD. Means with the same letter are not significantly different (Student’s t-test, p=0.05). Small letters represent the comparison within different dosage scales. Capital letters represent the comparison between CPT (gray) and HCPT (dark).

doi:10.1371/journal.pone.0056458.g007
DNA repair or other gene expression in response to Topo1 down-regulation induced by CPT and HCPT in IOZCAS-Spex-II cells. In summary, our studies suggest that *S. exigua* Topo1 can be inactivated in the presence of CPT and HCPT with time- and dose-effect, which provides a suitable experimental model for further studies focused on exploring derivates of CPT as insecticides.

**Materials and Methods**

**Culture of Insect Cells and CPT and HCPT Preparation**

IOZCAS-Spex-II cells, a cell line derived from *S. exigua* mid-gut fat bodies, were provided by the Institute of Zoology of Chinese Academy of Science (Beijing, China), and maintained at 27°C in Grace’s insect medium supplemented with 10% fetal bovine serum (FBS, Heat-inactivated, Invitrogen, CA, USA) in T25 cm² tissue culture flasks (Corning, USA). The cultures were sub-cultured every 6 days.

CPT (99.17%) and HCPT (99.44%) were purchased from Sichuan Nanbu Chenxin Technology Co., Sichuan, China and dissolved in 100% dimethyl sulfoxide (DMSO).

**Isolation of *S. exigua* Topo1 Gene**

Total RNA was isolated from IOZCAS-Spex-II cells with RNeasy® Mini kit (Qiagen GmbH, Germany). cDNA was synthesized with 1 μL total RNA using the Omniscript® RT Kit (QIAGEN GmbH, Germany) following the manufacturer’s instructions. The degenerate and specific primers listed in Table 2 were designed to target the conserved regions obtained from the three Topo1 sequences.
from the multiple alignment analysis of Topo1s from different species with DNAMAN (Lynnon Biosof, USA).

The PCR amplification was conducted in a 25 μL reaction volume containing 200 μM dNTPs, 1×PCR buffer and 1U Taq DNA polymerase (Takara, Dalian, China), with an initial denaturation step of 94°C for 5 min and followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. A final step for 10 min at 72°C was used to fully extend the amplicons. The target PCR product was purified using QIAquick Gel Extraction Kit (QIAGEN) and ligated into pGEM-T easy vector (Promega,
USA) for sequencing at Sangon Bio-engineering (Shanghai, China). The 5' and 3' ends were amplified with gene-specific primers listed in Table 1 and the adaptor primers were provided by the kits of 5'-Full RACE Core Set Ver.2.0 and 3'-Full RACE Core Set Ver.2.0 (Takara, Dalian, China). The full-length cDNA was assembled by overlapping all the amplified fragments, and deposited in National Center for Biotechnology Information (NCBI) with GenBank ID: JN258956.

![Figure 11. CPT and HCPT treatment induced Topo1 protein down-regulation.](image)

Table 2. Primers used in this study.

| Enzyme     | Total Protein (µg) | Total Activity (U) | Specific Activity (U mg⁻¹ pro) | Yield (%) |
|------------|--------------------|--------------------|-------------------------------|-----------|
| Crude Extracts | 372                | 128,000            | 344,000                       | 100       |
| Fraction #2 | 32,000             | 128,000            | 344,000                       | 100       |

Y = C or T; R = A or G; H = A, C or T; M = A or C; W = A or T; D = A, G or T; N = A, T, C or G.

*Y bases underlined are the restriction site for BanHI.
*Bases underlined are the restriction site for SalI.

doi:10.1371/journal.pone.0056458.t002
Phylogenetic Analysis of Topo1s

The protein sequences of Topo1s retrieved from National Center for Biotechnology Information (NCBI) and KAIObase were aligned with ClustalX 1.83 by using standard parameters and then rendered with ESPript 2.2 (http://esprit.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). A phylogenetic tree was constructed with MEGA version 5.0 program using the neighbor-joining method with 1,000 replicates [41,42].

Recombinant Protein Expression and Purification

The Topo1 encoding the residues 337–939 (Topo70) was amplified by PCR with the specific primers and cloned into the vector pGEX-4T-1 at the restriction sites of BamHI I and Sal I (Table 2) to generate plasmid pGST-Topo70. E.coil BL21 (DE3) cells were transformed with pGST-Topo70 or pGEX-4T-1 and grown at 37°C. Exponentially growing bacteria in LB medium including 100 μg/mL ampicillin (OD600 = 0.6) were treated with 0.6 mM isoproyl-1-thio-β-D-galactopyranoside (IPTG) to induce target protein expression for 4 hours at 30°C.

The cultures were harvested by centrifugation at 8,000 rpm for 5 min, washed with the PBS buffer (pH 7.4, 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.5 mM KH2PO4) twice, and resuspended in lysis buffer (50 mM Tris-HCl, pH7.5, 250 mM KCl, 0.5% Triton X-100, 1 mM DTT, 2 mM EDTA and 1 mM PMSF) with a final concentration of 250 μg/mL lysozyme on ice for 15 min. The samples were then spun at 15,000 × g for 30 min to remove cell debris and insoluble materials. The Topo1 proteins in the collected supernatants were purified with the GSTrap 4B columns (GE Healthcare, UK) following the manufacturer’s instructions. The recombinant Topo1 was eluted to seven fractions with an equal volume of 500 μL. The concentrations of the protein were determined by Bradford methods [43] and the purified Topo1 was confirmed by SDS-PAGE. The recombinant Topo1 was kept in 50% glycerol with aliquots at −80°C.

The Activity Unit and Specific Activity of Topo1 Expressed in E.coil

The DNA topoisomerase-1 relaxation assay was performed according to the procedures as previously reported with some modifications [44]. One unit (U) of Topo1 activity was defined as the amount of enzyme activity that will relax 0.5 μg pBR322 DNA fully at 26°C in 30 min. The specific activity was expressed in units of enzyme per milligram protein (U mg⁻¹ pro).

In the absence of Mg²⁺ for avoidance from interference of prokaryotic Topo1, protein samples were diluted by two-folds and each dilution was incubated with the reaction buffer (150 mM KCl, 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 0.1 mg/mL BSA) and 25 ng/mL DNA in 20 μL volumes under 26°C for 30 min to ensure the maximum relaxation of the DNA. The reaction was terminated by adding proteinase K (250 μg/mL) and 0.5% SDS (final concentration), and then incubated at 50°C for additional 30 min to remove extra proteins from DNA. Subsequently, three different forms of DNA (the supercoiled, relaxed and nicked form) were separated by electrophoresis in 1% agarose gel.

Inhibition of CPT and HCPT on the Activity of Recombinant and Natural Topo1s

Both recombinant and natural Topo1s were used for the toxicity analysis. The natural Topo1 was extracted from IOZCAS-Spex-II cells according to the modified method [45]. After removal of the insect medium, insect cells were harvested by centrifugation at 400 × g for 5 min and washed with PBS three times. The cell pellet was resuspended in 180 μL lysis buffer (pH 7.5, 50 mM KCl, 10 mM Tris-HCl, 13 mM DTT, 2 mM MgCl2, 1% Triton X-100, 1 mM PMSE) by vigorously shaking for 5 min on ice. The nuclei was collected by sedimentation of 600 x g for 10 min and then stirred in 120 μL resuspension buffer (pH 7.5, 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl2) containing 15 mM DTT, 1 mM PMSE. The pelleted nuclei were spun at 600 x g for 10 min. The collected nuclei were then pelleted in 50 μL resuspension buffer containing 25 mM DTT, 1 mM PMSE and 10 mM EDTA. 50 μL of 2X nuclear extraction buffer (2M NaCl, 50 mM Tris-HCl (pH 7.5), 20% glycerol, 2mM EDTA) was added to lyse the nuclei for 15 min. Crude extracts were collected by discarding the precipitated nucleic acids in PEG buffer (1M NaCl, 18% PEG, 10% glycerol) by centrifugating at 10,000 x g for 30 min.

The enzymatic activity of Topo1s was determined according to methods mentioned above. Both 1U of recombinant and natural Topo1s were incubated with various concentrations (from 0.01 to 100 μM) of CPT and HCPT in a total of 20 μL reaction buffer under the same reaction condition. DNA bands were visualized with a digital UV transilluminator and quantified with Quantity one (Gel Doc XR, Bio-Rad, USA). The inhibitory rate (%) of the enzymatic activity by CPT and HCPT was calculated as the percentage of supercoiled over total pBR322 DNA. The EC50 value was calculated by using Probit analysis with DPS (v 9.5, Zhejiang University, China) [46].

Effects of CPT and HCPT Pre-treatment on the Specific Activity of Topo1 in S. exigua

Two series of experiments were conducted to analyze the effects of CPT and HCPT treatment on the catalytic activity of S. exigua Topo1. The first series examined changes of Topo1 activity caused by 10 μM CPT and HCPT pre-treatment for different times (0, 2, 4, 6, 12, 24 and 48 h). The second series of experiments examined the effects on the Topo1 specific activity with different concentrations (0.10, 0.50, 1.00, 5.00, 10.0, 50.0, 100 μM) of CPT and HCPT treatment for 24 h.

In all experiments, IOZCAS-Spex-II cells were pretreated with CPT and HCPT according to the procedure described in our previous studies [16]. The Topo1 was extracted from IOZCAS-Spex-II cells, and the specific activity of the nuclear extract in each sample was determined using the method described above.

Real-time PCR Analysis

The Topo1 mRNA level in CPT and HCPT treated cells was measured by Real-time PCR. The reactions were carried out in strip tubes with caps (0.2 mL, AXYGEN, USA) in a total volume of 20 μL including 10 μL SYBR Premix Ex Taq™ (Takara, Dalian, China), 0.4 μL forward primer and reverse primer (10 μM), 0.4 μL ROX reference dye II, 2.0 μL reverse transcribed cDNA and 6.8 μL H2O. The procedure was carried out with two steps as follows: 30 sec at 95°C; 40 cycles of 5 sec at 95°C and 30 sec at 60°C. Gene expression was normalized to beta-actin with 2⁻ΔΔCt method [47]. Data was analyzed with 7500 software V 2.0.3 (Applied Biosystems, CA, USA).

Western Blot Analysis

IOZCAS-Spex-II cells were pretreated with 10 μM CPT and HCPT for 24 h prior to fractionation with the method as described by Fu et al. [48]. Total protein concentration was determined by Bradford methods [43]. The equal amount of samples were separated by 8% SDS-PAGE, transferred to nitrocellulose membranes and blocked in blocking buffer contain-
ing 5% non-fat milk (25.0 mM Tris, 150 mM NaCl, 0.1% Tween20, pH 7.5) for 1 h at the room temperature. The blots were then probed with rabbit polyclonal antibodies to topoisomerase I produced with the recombinant TopoI (Topo70) by Immunol (15050), Zhuhuahan, China, followed by mouse anti-rabbit IgG-HRP antibody (1:10,000; R&D Sysyms, Inc., MN, USA). For loading control, beta-actin was probed with mouse anti-actin monoclonal antibody (Abcam Ltd., Hongkong) and secondary primary, anti-mouse IgG-HRP antibody (R&D Systems, Inc., MN, USA). The signals were detected with a DAB detection kit (Boster, Wuhan, China).

**Statistical Analysis**

All experiments were repeated at least three times and results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out by one-way ANOVA followed by Student’s t-test with a statistically significant value of p<0.05.

**Acknowledgments**

We thank Dr. Xiu Gao for technical assistance. We are grateful to Dr. Qilian Qin and Dr. Huan Zhang for kindly providing us insect cell lines. We also thank Dr. Guoliang Wang for critical reading of the manuscript.

**Author Contributions**

Conceived and designed the experiments: HJ LZ CL. Performed the experiments: DM LZ JY YZ WH. Analyzed the data: DM LZ. Contributed reagents/materials/analysis tools: LZ HJ. Wrote the paper: LZ DM.
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