New molecular biologist perspective and insight: DNA topoisomerases production by recombinant DNA technology for medical laboratory application and pharmaceutical industry

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Abstract

DNA topoisomerases are essential enzymes that control the topological state of DNA replication during mitosis. These enzymes are classified based on their mechanisms and physical properties. During mitosis, superhelical DNA must be unwound or relaxed by DNA topoisomerases prior to a decoding step by DNA processing enzymes, such as DNA polymerase and RNA polymerase. By blocking the reaction of resealing the breaks in the DNA ultimately can result in cellular death. Compounds that inhibit the catalytic function of these enzymes can serve as potential anticancer agents. DNA topoisomerases are found in nature and used as high quality and well-validated targets for the screening of potential anticancer agents. Our current work focuses on determining potential anticancer agents from natural resources using DNA topoisomerases as the screening targets. Large scale production of these enzymes using recombinant DNA technology in our academic laboratory is utilised to avoid dependence on expensive commercially available enzymes. The in-house produced enzymes can also be used to enhance our research in the field of molecular medicine by providing an enzyme source that can be used to screen potential anticancer agents, and for other newly developed diagnostic and medical research projects in the near future as well as a step in moving our efforts into the industrial sector.

Keywords: DNA topoisomerase; medical laboratory application; pharmaceutical industry; recombinant DNA technology.

INTRODUCTION

DNA topoisomerases are essential enzymes that control the topological state of DNA during cellular processes in both prokaryotic and eukaryotic cells. The cellular processes include DNA replication, transcription, recombination and chromatin segregation, to control the synthesis of proteins and to facilitate DNA replication during mitosis (Champoux, 2001; Durand-Dubief et al. 2011). The types of organisms used in the studies of DNA topoisomerases include *Escherichia coli*, *Staphylococcus aureus*, yeasts, plants from the genus *Arabidopsis*, flies from the genus *Drosophila* and human. Several viruses, such as bacteriophage T4 and animal vaccinia viruses, can also encode DNA topoisomerases. All organisms contain at least two classes of DNA topoisomerases, namely type I and type II DNA topoisomerases in which the enzymes are classified based on their mechanisms and physical properties (Champoux, 2001; Salerno et al. 2010). For example, *E. coli* has two type I DNA topoisomerases (DNA topoisomerase I and DNA topoisomerase III) and two type II DNA topoisomerases (DNA topoisomerase II or gyrase and DNA topoisomerase IV) (Kato et al. 1992; Peng and Marians, 1993). The DNA topoisomerase subtypes (A, B or C) are differentiated by amino acid sequences or structures (Champoux, 2001; Vos et al. 2011). For example, enzymes that cleave only one strand of DNA are defined as type I with a further classification of type I-A subtypes for proteins

linked to a 5'-phosphate and type I-B subtypes for proteins attached to a 3'-phosphate during the relaxation of the cleavable DNA. The enzymes are further divided into subfamilies based on the structural changes induced by gene duplication, such as DNA topoisomerase IIα and IIβ (Wang, 1996; Champoux, 2001).

Type I and type II enzymes

Type I DNA topoisomerases (or DNA topoisomerase I) are monomeric proteins and ATP-independent enzymes that induce a single strand DNA break *via* a phosphodiester bond between the tyrosine group of the enzyme and the phosphate group of the DNA (Champoux, 2001) (Figure 1a). DNA topoisomerase III is an example of a type I-A enzyme that is more active as a decatenating enzyme than as a DNA-relaxing enzyme (DiGate and Marians, 1988). Type II DNA topoisomerases are tetrameric proteins formed by two different subunits, GyrA2GyrB2 for gyrase and ParC2ParE2 for DNA topoisomerase IV (Kato et al. 1992; Peng and Marians, 1993). Requiring ATP, these enzymes act by making a transient break on the double stranded DNA, passing through an intact duplex DNA *via* the broken strand followed by a resealing of the transient break (Pommier, 1993; Wang, 1996) (Figure 1b). Studies in eukaryotes have shown that type I DNA topoisomerase is associated with actively transcribed genes, whereas type II DNA topoisomerase is required for DNA replication and the successful traverse of mitosis (Champoux, 2001; Wang, 2002). Type I and type IIα DNA topoisomerases are well known targets for chemotherapy of advanced or recurrent human cancers.
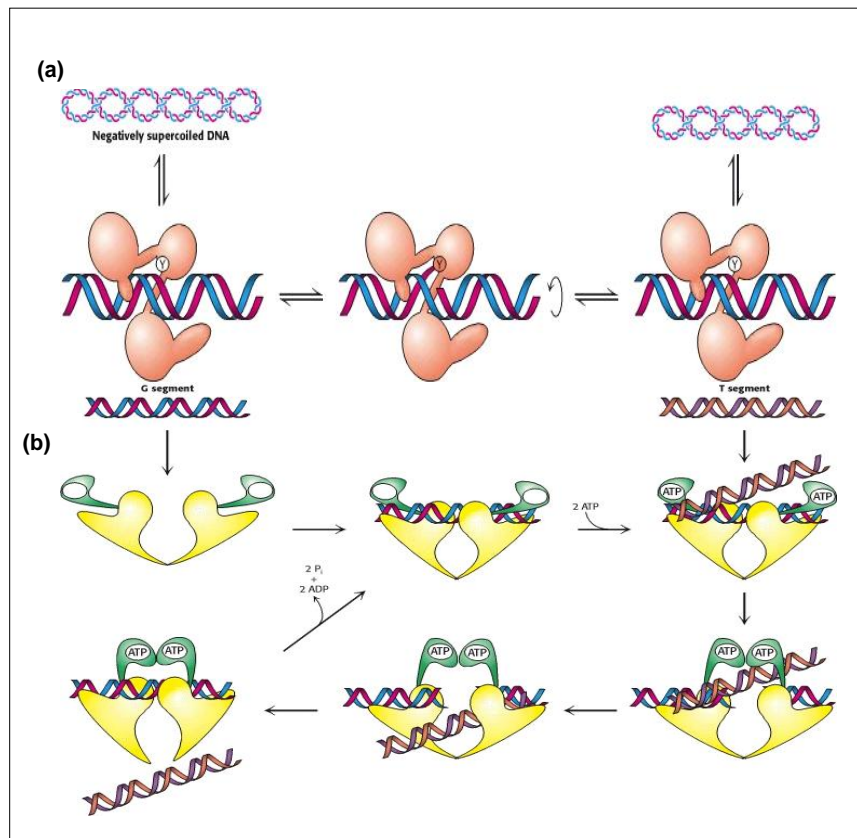

Fig. 1 (a) The mechanism of type I DNA topoisomerase. The enzymes nick a single strand of DNA (pink) and form a phosphodiester bond between the enzyme tyrosine group (Y) and the phosphate group of the DNA. This makes the duplex DNA at the end of the nicking to rotate freely and follow by resealing of the nicking. **(b) Mechanism of type II DNA topoisomerase.** The enzymes cleave both strands of a DNA duplex (G-segment) and pass a second intact duplex DNA (T-segment) through the transient break using 2 ATPs for the strand passage.

In general, DNA is wound around a basic protein called histone in a tightly coiled or supercoiled formation (Durand-Dubief et al. 2010). Sequences of DNA must be unwound or relaxed prior to being read by DNA processing enzymes, such as DNA polymerase and RNA polymerase. Type II DNA topoisomerases (or DNA topoisomerase II) temporarily break the DNA strand, allowing both ends to rotate within the enzyme freely. This mechanism allows the DNA to unwind. The enzyme then reconnects the two ends, leaving a part of the relaxed DNA ready for processing. DNA topoisomerase II inhibitors, such as etoposide, stabilize the enzyme with the DNA strand cut in the enzyme-DNA complex, leaving a permanent break in the double strand of the DNA. This DNA strand break is capped by the remnants of the enzyme and is difficult to repair.

The expressions of DNA topoisomerases in cancer tissues and cells

DNA topoisomerases are over-expressed at different levels in both normal and cancerous tissues. The expression of DNA topoisomerase I in colorectal cancer, as detected by immunohistochemical methods, was a biological marker for the chemosensitivity of tumour against DNA topoisomerase I inhibitors (Ataka et al. 2007). Using the investigation with reverse transcription PCR, differential expression studies in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) demonstrated that the DNA topoisomerase IIα gene in tumours was expressed at significantly higher level than in normal lung tissues, and the relative expression in SCLC was significantly higher than in NSCLC (Syahruddin et al. 1998). Meanwhile, immunostaining for topoisomerase IIIβ was found in 33.9% of breast carcinoma and the immunopositivity was correlated with distant metastasis and death (Oliveira-Costa et al. 2010). Decreased expression of topoisomerase IIIβ was correlated with low expression of Ki67, positivity to BRCA1, but negativity for HER2 and p53 expressions in the study. Our research using cancer cell lines had also indicated that DNA topoisomerases are expressed at different levels in human cancer cell lines (Figure 2).
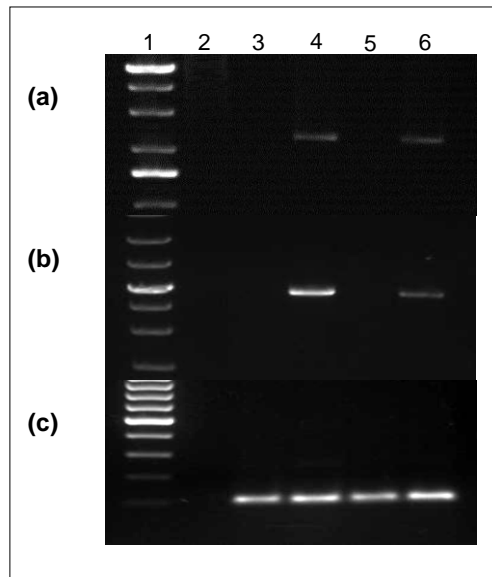

Fig. 2 PCR of (a) DNA topoisomerase I cDNA (2,300 bp), (b) DNA topoisomerase II cDNA (4,600 bp), and (c) beta-actin (100 bp) in cancerous cells. The cDNAs were isolated from cancer cells. Lane 1: 1 kb plus DNA ladder marker, 2: negative control, 3: amplification of DNA topoisomerase I cDNA from HeLa, 4: amplification of DNA topoisomerase I cDNA from HT-29, 5: amplification of DNA topoisomerase I cDNA from MCF-7, and 6: amplification of DNA topoisomerase I cDNA from MDA-MB-231. The PCR products were separated by 0.7% agarose gel electrophoresis at 80 V for 90 min.

The highly invasive human cancer cell lines HT-29 and MDA-MB-231 expressed higher levels of DNA topoisomerases than the levels measured in HeLa and MCF-7. Both DNA topoisomerases I and II were not detected in HeLa and MCF-7 by conventional PCR. These results indicated that both HT-29 and MDA-MB-231 may be sensitive to DNA topoisomerase inhibitors whereas HeLa and MCF-7 may not be sensitive to these compounds. Expression levels of DNA topoisomerase II were observed to be higher than DNA topoisomerase I in both HT-29 and MDA-MB-231, indicating that DNA topoisomerase II plays a more important role than DNA topoisomerase I in these highly invasive human cancer cells. In addition, these cells might be more sensitive to DNA topoisomerase II inhibitors than DNA topoisomerase inhibitor I. Decreased expression of DNA topoisomerase I and II had been demonstrated to indicate a resistance against certain anticancer agents (Sugimoto et al. 1990a; Sugimoto et al. 1990b), suggesting that these enzymes are promising targets for anticancer agents.

Application of DNA topoisomerases for preliminary anticancer agent screening

Chemicals or compounds that inhibit the catalytic function of DNA topoisomerases, by blocking the reaction that reseals the breaks in the DNA, can lead to permanent DNA strand breaks and ultimately cell death. Anti-DNA topoisomerase agents, which are capable of stabilizing the covalent intermediate formed by the DNA topoisomerases, can serve as potential anticancer drugs for human cancer treatments (Cheng et al. 2007; Tsai et al. 2010). DNA topoisomerases have been widely used as molecular targets for the *in vitro* screening of potential anticancer agents from various sources (Liu, 1989; Wang, 1996; Topcu, 2001; Hurley, 2002).

Anticancer agents that act against the activities of DNA topoisomerases can be categorized into class I and class II based on their effects on these enzymes. Class I drugs are known as DNA topoisomerase poisons as these drugs transform the enzyme into a potent cellular toxin. Class II drugs are DNA topoisomerase inhibitors that act by interfering with conventional cellular functions, such as DNA transcription and replication as well as the prevention of DNA supercoiling (Topcu, 2001). The DNA topoisomerase class I drugs that act on the covalent enzyme-DNA complexes are intermediate products formed during the catalytic function of the enzyme. To date, multiple drugs in this category, including acridines (Nelson et al. 1984; Langer et al. 1999), anthracyclines (Chow et al. 1988; Wassermann et al. 1990; Foglesong et al. 1992), actinomycins (Wassermann et al. 1990), ellipticines (Arguello et al. 1998), alkaloids (Hsiang and Liu, 1988; Aflalo et al. 1994; Bendetz-Nezer et al. 2004), epipodophyllotoxins (Ross et al. 1984; Chow et al. 1988), isoflavodins and quinolones (Shen and Pernet, 1985; Langer et al. 1999) have been identified. DNA topoisomerase class II drugs that have been identified, including the coumarine antibiotics (Maxwell, 1997) and the fostriecin analogues (Boritzki, 1988). These types of drugs react on the catalytic function of the enzyme without stabilizing the intermediate covalent complex. Table 1 shows the summary of topoisomerase I and II inhibitors.

Table 1. Different classes of DNA topoisomerase I and II directed drugs (Van Gijn et al. 2000; Topcu, 2001).

Class	Subclass	Group	Example drug	Target on DNA topoisomerase
Class I (Poisons)	Intercalative	Acridines Anthracyclines Actinomycins Ellipticines	M-AMSA Doxorubicin Actinomycin D 2-methyl-9-OH-epllipticinium acetate	II II I & II II
Class I (Poisons)	Non-intercalative	Alkaloids Epipodophyllotoxins Quinolones	Camptothecin Etoposide Ciprofloxacin	I II
Class II (Catalytic inhibitors)	Intercalative	Coumarins	Novobiocin Aclarubicin	II I
Class II (Catalytic inhibitors)	Non-intercalative	Fostriecin analogues	Fostriecin	II

DNA topoisomerase inhibitors can be further characterized into type I and type II, based on the inhibitor targets. Type I DNA topoisomerase inhibitors are mainly extracted from the bark and wood of Chinese ornamental trees, such as *Camptotheca acuminata* (Wall and Wani, 1995). These inhibitors work by forming a complex with the DNA topoisomerase I, suppressing the conventional biological function of the enzyme. Type II DNA topoisomerase inhibitors have been extracted from the alkaloids that are found in the rhizomes and roots of *Podophyllum emodi* wall and the leaf blades of the North American mayapple (*P. peltatum* L.) (Meijer, 1974; Imbert, 1998; Meresse et al. 2004). These inhibitors work in the late phases S and G2 of cell cycle (Kelly and Hartwell, 1954; Heck et al. 1988; Woessner et al. 1991; Kimura et al. 1994; Meresse et al. 2004). DNA topoisomerase inhibitors are often used in combination with other drugs, such as 5-fluorouracil, in cancer chemotherapy (Ewesuedo and Ratain, 1997). Significant efforts have been made to determine the best combinations of DNA topoisomerase inhibitors with other chemotherapy agents for cancer treatment in order to minimize the severe sequential or cumulative adverse effects, such as life-threatening neutropenia and anaemia. Current chemotherapeutic agents can be expensive, reducing patient affordability. Therefore, isolating anticancer agents from more affordable resources, such as traditional medicinal plants, is warranted. Our country, Malaysia, has abundant pharmacologically beneficial flora and natural resources that may be advantageous in this search for new affordable agents for human cancer treatments.

DNA relaxation assays using DNA topoisomerases are utilised in our current research for preliminary anticancer agent screening instead of cell-based assays, as the later are very expensive, requiring specific equipment, expertise and specialized facilities. The enzymes used are major contributors to the total cost in the DNA relaxation assay development, and hence, limiting the wide-spread use of this approach in many developing countries that are actively involved in the research of potential anticancer agents from traditional medicinal plants. Besides, improvements in the sensitivity of the commercially available assays using DNA topoisomerases have been limited as these assays are not as popular as the cell-based MTT assay. Therefore, the production of in-house enzymes and the development of in-house assays may be beneficial for many researchers who would prefer to not depend on costly commercial enzymes and kits. This type of assays may facilitate also research at those institutes that do not have cell culture facilities to perform preliminary screening using cell culture approach for potential anticancer agents from plant extracts and synthetic compounds. With this approach, only those agents demonstrating positive effects in the DNA relaxation approach would then be subjected to secondary screening approaches using more costly cell-based assays. This framework has the potential to reduce research costs, making the screening procedures more practical and versatile.

Other applications of DNA topoisomerases

DNA topoisomerases can also be used for other molecular biology laboratory applications. The DNA topoisomerase I from the *vaccinia* virus has been widely used to produce the DNA topoisomerase-activated adapter for various modifications and cloning of PCR products by inserting a DNA fragment into a plasmid without DNA ligase. This cloning method rapidly incorporates the T7 promoter into a plasmid for a PCR product with 3' deoxyadenosine (A) overhangs, using a plasmid that has been engineered to have a linearized strand with 3' deoxythymidine (T) overhangs. The DNA topoisomerase I from *vaccinia* virus covalently binds to the linearized plasmid at a specific site, cleaving the phosphodiester backbone after the 5'-CCCTT in a single strand of the plasmid DNA (Figure 3). The phosphotyrosyl bond between the DNA and enzyme can be subsequently replaced by the 5'-hydroxyl of the original strand, releasing the enzyme after a religation with the external DNA, such as PCR product or DNA fragment (Shuman, 1994). The PCR product, complement of the 3'-T overhang of the plasmid, is then allowed to quickly ligate with the DNA topoisomerase I in salt and without requiring a DNA ligase. This application has been developed by different manufacturers, including Invitrogen, to produce a number of plasmids for the effective cloning of a PCR product, providing a powerful and convenient tool for the rapid modification of a PCR product.

DNA topoisomerases are also used as biomarkers in cancer diagnostic and chemotherapeutic efficacy evaluations (Pfister et al. 2009). Over-expressions of DNA topoisomerases are associated with tumour growth, differentiation and poor survival prognosis in colorectal cancer (Braun et al. 2008), ovarian carcinoma (Faggad et al. 2009), breast cancer (Oakman et al. 2009), lung cancer (Dingemans et al. 1999) and oral squamous carcinoma (Oliveira-Costa et al. 2012). The expression levels of these enzymes have been determined using reverse transcription PCR technique, microarray and qualitative analyses of immuno-stained tissues. Multivariate analysis showed that the topoisomerase IIIβ expression was a significant and independent predictor of survival and the expression of

topoisomerase IIIβ can be a useful marker in assessing the prognosis of patients with breast cancer (Oliveira-Costa et al. 2010). The expression patterns of these enzymes would need to be evaluated in large scale clinical trials, to determine the specific roles of the enzymes prior for the use in diagnostic applications and predictions of the responses to chemotherapy in various types of human cancers.
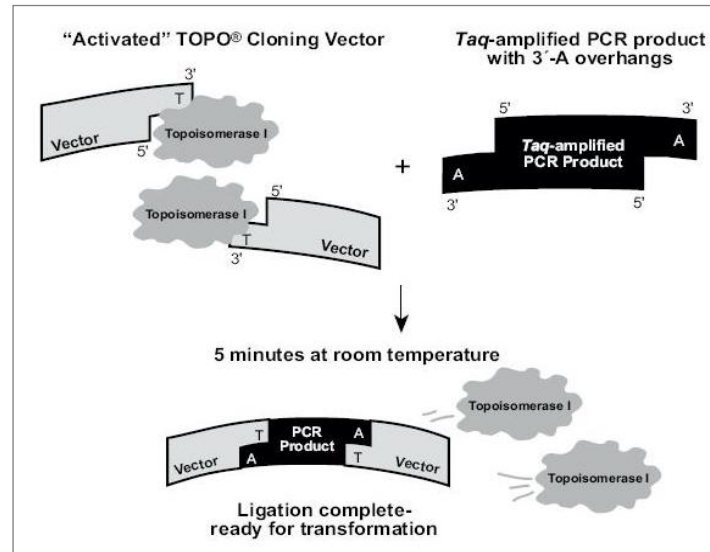

Fig. 3 Taq-amplified PCR product with 3'-A overhangs is ligated to activated Topo cloning vector. This figure was adapted from http://2007.igem.org/wiki/index.php/Boston_University/TOPO_Cloning (Shuman, 1994).

In addition to being targeted for anticancer agents, DNA topoisomerases can also be used as targets for antibacterial agents and toxins. Antibiotic resistance in microbial pathogens is a serious clinical problem. Investigations on the therapeutic potential of natural products on microbial DNA topoisomerases would be beneficial. Novel alkaloid inhibitors of enzymatic activities can be developed and tested for their effects on DNA supercoiling and cell growth. In bacteria, the homeostasis of DNA supercoiling is maintained by the opposing activities of DNA topoisomerases that relax the DNA and gyrase to introduce negative supercoils. Gyrase is a unique enzyme capable of introducing negative supercoils in bacteria at the expense of an ATP hydrolysis (Gellert et al. 1976). This feature makes bacterial DNA gyrases valuable as antibiotic targets. Modulation of bacterial chromosomal supercoiling is a function of DNA topoisomerase-catalysed strand breakage and rejoining. Antibiotic and DNA topoisomerase inhibitors can trap the DNA topoisomerase molecule at the DNA cleavage stage. Double stranded DNA breaks are introduced and the replication machinery is arrested at blocked replication forks, resulting in bacteriostasis and cell death (Dwyer et al. 2007). Certain bacterial toxins have similar properties to those of the quinolones; the second largest group of medically important antibacterial drugs that primarily target the bacterial gyrases. The bacterial toxins, CcdB (coupled cell division or control of cell death) and microcin B17 both act on DNA gyrase, killing the cells by stabilizing the complex between the gyrase and the DNA (Maxwell, 1997; Kampranis et al. 1999). CcdB acts at a different stage of the catalytic cycle of the DNA-gyrase than the quinolone drugs. There is no cross-resistance between the quinolones and CcdB, indicating that these compounds do not interact at the same site on the DNA gyrase. The interactions in the gyrase:bacterial toxin complex provide new perspectives for the design of novel classes of antibiotics (Dao-Thi et al. 2005).

The perspectives of our current research

Current cancer drugs and treatment strategies have been reported to cause many adverse side effects to cancer patients. The side effects include hair loss, weakened immune system, vomiting, sickness etc. Improvements in effective cancer treatments have been limited despite the advances made in cancer research in the medical laboratory. Moreover, drugs used for chemotherapy can be excessively expensive (more than RM 1,000 per dose), reducing affordability for some patients. This restriction in patient access makes the search for effective and affordable anticancer agents from natural products

more urgent and critical as these resources may be more affordable to cancer patients with low living wages. Natural products, such as garlic, ginseng, grape, shallot, green tea and onion, contain a high level of flavonoids that have been proven *in vitro* to have anticancer properties, including inhibitory effect on cancer cell growth *via* induction of apoptosis and autophagy (Singletary and Milner, 2008). For example, chrysin, which has been found to be the most effective of all the flavonoids tested, has equal potency to the drugs used for chemotherapy, such as tamoxifen. Indeed, natural products may be ideal resources for anticancer agents. Indeed, many natural products have yet to be scientifically proven to have anticancer effects using the above mentioned mechanisms. Therefore, large amounts of DNA topoisomerases are required to perform preliminary screening of potential anticancer agents from natural products. Large quantity of in-house produced DNA topoisomerases are also needed to develop standard, robust, rapid and sensitive preliminary screening assays for cancer diagnoses and predictions of the responses to chemotherapy, as well as for other molecular biology applications in our laboratory.

A process to produce a reliable high-yield of the purified recombinant enzymes is critically needed to enable the development of the abovementioned applications. However, one factor that limits our establishment of an anticancer agent screening project and other projects related to molecular research using these enzymes is the applicability of current *E. coli* system to express large amounts of eukaryotic enzymes that are functionally compatible. DNA topoisomerases can also be extracted from plants. The plant DNA topoisomerase I have been purified and characterized from wheat germ, *Triticum aestivum* (Dynan et al. 1981), cultured tobacco cells, *Nicotiana tabacum* (Heath-Pagliuso et al. 1990), broccoli, *Brassica* oleracea (Kieber et al. 1992a), pea and *Pisum sativum* (Chiatante et al. 1993; Tuteja et al. 2003). Several reports have established the procedures for the isolation of DNA topoisomerase I genes from plants, such as plants from the genus *Arabidopsis* (Kieber et al. 1992b), carrots, *Daucus carota* (Balestrazzi et al. 1996; Balestrazzi et al. 2000) and peas (Reddy et al. 1998). Isolations and studies of the DNA topoisomese II gene from plants have also been performed in plants from the genus *Arabidopsis* (Makarevitch and Somers, 2005), peas (Reddy et al. 1999) and tobacco (Singh et al. 2004). However, the reported strategies to extract the enzymes from plants are not sustainable and are contrary to the green practices in our country. Therefore, producing in-house DNA topoisomerases using recombinant DNA technology may represent "a good practice" and be preferable to a dependence on expensive commercial enzymes. Perhaps, potent dual DNA topoisomerase inhibitors that target both DNA topoisomerase I and II may be found in local natural resources. To the best of our knowledge, this has not been done by any research group. Indeed, dual DNA topoisomerase inhibitors may have improved anticancer effects as compared with single target DNA topoisomerase inhibitors. Therefore, large amounts of both DNA topoisomerase I and II are required for above mentioned screening purposes, and it may be cost effective to produce in-house DNA topoisomerases using a selected expression system as the first step to facilitate the project to screen DNA topoisomerase inhibitors from natural resources.

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