PURIFICATION AND CHARACTERIZATION OF TOPOISOMERASE II INHIBITOR FROM *STREPTOMYCES* SP.

CHAPTER 2
2.1 INTRODUCTION

The identification of novel targets and the development of more specific chemotherapeutic agents are two of the most important goals of research in cancer therapy [Cameron and Feuer, 2000]. DNA topoisomerases have been recognized as potential chemotherapeutic targets for antitumor and antiparasitic agents [Chakraborty and Majumder 1988; Burri et al., 1996]. These enzymes modulate DNA superhelicity and act by introducing single (type I) or double (type II) DNA breaks. They are involved in DNA repair, replication, transcription, chromosome segregation during mitosis and catalyze two different natures of reactions [Montaner et al., 2005]. Studies of the subclass type I topoisomerases indicate that they act by a mechanism involving the introduction of temporary breaks in only one strand of the double-stranded DNA whereas, the type II topoisomerases break and religate both strands of the double-stranded DNA [Webb and Ebeler, 2003]. Topoisomerase I changes DNA linking number in one step and does not require a cofactor. Topoisomerase II accomplishes this in two steps and requires ATP which may offer more targets for inhibitors’ action [Hande, 2008].

Topoisomerase II is essential for cell division and cell proliferation since it is required for the completion of mitosis. While differentiated cells express very low levels of topoisomerase II, highly proliferative tumor cells often express 25–300 times higher levels of topoisomerase II than those of quiescent cells [Heck and Earnshaw, 1986]. Mammalian cells express two genetically distinct topoisomerase II isoforms, α and β which are differentially regulated and play different roles in living cells [Champoux, 2001]. Topoisomerase II α is most abundantly expressed in rapidly growing tissues and its expression is cell cycle-regulated, peaking in G2/M, whereas the β-isoform is expressed in virtually all tissues and throughout the cell cycle [Padget et al., 2000; Adachi et al., 2000]. An important feature of topoisomerase II-mediated activity is the formation of a covalent bond between a tyrosyl residue on the enzyme and the phosphate moiety at the 5' terminus of the DNA at the break site, thereby creating an intermediate DNA-enzyme complex referred to as the "cleavable complex" [Akimitsu et al., 2003; Carpenter and Porter , 2004].
Inhibition of topoisomerase enzymes has been considered as an important biomarker in screening chemo preventive and therapeutic properties of potential anticancer agents and the mode of action of many anti-cancer drugs has been based on this inhibitory capacity [Hande, 1998; Cho et al., 2000]. Two general classes of topoisomerase inhibitor mechanisms have been described. A catalytic intermediate stage involving a covalent linkage between the topoisomerase and the 3' end of the broken DNA strand has been shown to be susceptible to interference by certain compounds which essentially prevent the religation of the broken strand and the release of the bound enzyme. Such compounds like camtothecin and etoposide, referred to as topoisomerase poisons, may be considered as a subset of general topoisomerase inhibitors (where inhibition refers to interference with the supercoil relaxation activity of the enzyme) [Falk and Smith, 1992; Kim et al., 2002]. The agents such as alcarubicin and novobiocin acting on any of the other steps in the catalytic cycle are called catalytic inhibitors [Larsena et al., 2003]. Some of these topoisomerase inhibitors are isolated from microorganisms.

The coumarins are natural products of Streptomyces species, which inhibits ATPase activity of DNA gyrase and Topo IV by competing with ATP, Mg\(^{2+}\) for binding with GyrB and ParE subunits of the enzymes, respectively [Trovatti et al., 2008]. Topostins had been reported as novel inhibitors of mammalian DNA topoisomerase I (topo I) isolated from a culture broth of Flexibacter topostinus sp. nov. [Nemoto et al., 1998].

There are some peptides which can also inhibit topoisomerase activity. Synthetic peptides like KWWCRW and KWWWRW have been reported as inhibitor of vaccinia virus topoisomerase type I B enzyme [Klemm et al., 2000]. The ccd toxin, antitoxin system of the F plasmid encodes CcdB, a protein that poisons the essential Escherichia coli DNA gyrase, unique type IIA topoisomerase able to introduce negative supercoils into DNA [Trovatti et al., 2008]. A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia [Willmore et al., 2004].

Chemotherapy regimens using topoisomerase inhibitors have been proved for successful treatment of various cancers. Etoposide and doxorubicin are among the most
commonly used anticancer drugs but nevertheless their use in oncology is limited by secondary effects, such as cardiac toxicity for the anthracyclines or leukemogenesis for the epipodophyllotoxins. There is a strong demand for new, better targeted antitumor agents having fewer unwanted side effects and improved efficacy, in particular for the treatment of chemo- and radio-refractory or metastatic disease. Thus development of novel, potent topoisomerase inhibitors are necessary.

Microbial sources like actinomycetes are well recognized to produce a variety of chemical structures, several of which are most valuable pharmaceuticals, agrochemicals and industrial products like enzymes [Balagurunathan et al., 2010]. For the potential new drug discovery, actinomycetes have attracted scientists from different disciplines, such as pharmacology and biology. The present study revealed that an active actinomycetes strain *Streptomyces* sp. produced quite an unique topoisomerase inhibitor. The inhibitor was further purified and identified. The mode of topoisomerase II inhibition was explored.
2.2 MATERIALS AND METHODS

2.2.1 Preparation of crude nuclear extracts

Crude nuclear extracts were prepared by a modified method [Deffie et al., 1989]. Exponentially growing human erythromyeloblastoid leukemia cell line, K562 cell line (ATCC, Manassas, VA, USA) were collected by centrifugation and washed in ice cold phosphate buffered saline (PBS). The washed cells were resuspended in PBS, mixed well and centrifuged at 1800 rpm for 10 min. The cell pellet were suspended in lysis buffer (Lysis buffer consist of 10mM Tris HCl (pH 7.5), 1mM MgCl₂, 1mM EGTA, 0.5% (v/v) CHAPS, 10%(v/v) glycerol, 5mM β- mercaptoethanol, 1mM PMSF) and incubated in ice for 25 min with intermittent vortexing. Cells were sonicated for 1 min. DNA and nuclear debris was pelleted by centrifugation at 4°C, 1800 rpm for 20 min and the supernatant were collected. Protein concentration in the extract was determined by Folin- Ciocaltaeu method [Lowry et al., 1951] using BSA as standard.

2.2.2 DNA topoisomerase activity assay

Relaxation activities of DNA topoisomerases were determined by detecting the conversion of supercoiled plasmid pBR322 DNA (Fermentas, USA) to its relaxed form [Mizushina et al., 2003]. Topoisomerase II reaction was performed in 20µL reaction mixtures containing 50mM Tris HCl buffer (pH 8.0), 100mM KCl, 10mM MgCl₂, 0.5mM dithiothreitol, 0.5 mM EDTA, 1mM ATP, 30µg/mL BSA, pBR322 plasmid DNA (125 ng), 1-4 µg of topoisomerase II enzyme (or 1U of enzyme), without inhibitor or with inhibitor (10-50µg crude extract / 2-10 µg in the case of purified inhibitor). Etoposide was used as a positive control. According to the earlier reports [Dassonneville et al., 2000] 50µM was used as the positive control which showed 50% inhibition at that particular concentration. The reaction mixtures were incubated at 37°C for 30 min and terminated by adding 3 µL of loading buffer consisting of 40% sucrose, 0.25% bromophenol blue, 0.1mM EDTA. The mixtures were subjected to 1% agarose gel electrophoresis in TBE (Tris- Borate- EDTA) running buffer. The agarose gels were stained with ethidium bromide and DNA was visualized on UV transilluminator. Gel statin, Gel doc LabImage ID L320 (Medicare Scientific, India) was used for densitometric quantitative analysis of the supercoiled DNA.
2.2.3 Extraction of topoisomerase II inhibitor from *Streptomyces* sp.

*Streptomyces* sp. ST1 (NCBI accession number GQ423725.1.) was selected for topoisomerase II inhibitor production from our previous work which showed maximum topoisomerase II inhibition activity [Sivasamy, 2010]. *Streptomyces* sp. ST1 was grown in Modified Kuster’s agar medium (g/L) (0.3 casein, 2.0 potassium nitrate, 2.0 sodium chloride, 2.0 dipotassium phosphate, 0.05 magnesium sulphate, 0.02 calcium carbonate, 0.01 ferrous sulphate, 10 starch) [Kuster and Williams, 1964] and incubated at 30ºC in a rotary incubator at 150 rpm for 12 days. After incubation period, culture media was centrifuged at 2500 X g for 20 min. The obtained cell biomass was homogenized with 2 volumes of methanol and extracted at room temperature [Becerra *et al.*, 2001]. The extract was concentrated *in vacuo* and dissolved in 20mM Tris buffer (pH 7.5).

2.2.4 Purification of topoisomerase II inhibitor

The dissolved crude extract was fractionated using a centrifugal filter device (cut off, MW 10000). The resulting solution which showed maximum inhibitory activity against topoisomerase II was loaded onto a sephadex G-75 column (1.5 X 45 cm) pre equilibrated with 20 mM Tris HCl (pH 7.5). Elution of proteins was performed with same buffer at a flow rate of 2mL/min. The fractions containing topoisomerase II inhibitory activity were pooled and concentrated using amicon filters (10 kDa cut off). The concentrated fraction was applied onto a DEAE cellulose column (2.0 X 15 cm), pre equilibrated with 20 mM Tris HCl pH 7.5 and the protein bound to the column was eluted with a linear gradient of 0.1-1.0 M NaCl in the same buffer at a flow rate of 2mL/min. The active fractions were concentrated using amicon filters of 10 kDa cut off for further use. Protein content of crude extract, concentrated fractions and purified protein was determined by Folin – Ciocalteau method using BSA as a standard [Lowry *et al.*, 1951]. The protein content of individual fractions collected from different columns was determined by UV absorbance at 280 nm. Inhibitory activity unit is defined as the amount of protein required for inhibiting 50% of topoisomerase II [Prasad *et al.*, 2010]. Specific activity is defined as the number of inhibitory activity units/ mg protein. Inhibitory percent was calculated by the following equation,
(X-A)
Inhibitory percent = \frac{X-A}{X} \times 100

Where, X - Intensity of supercoiled DNA in control sample (pBR322 lane)
A - Intensity of supercoiled DNA with compound

2.2.5 Electrophoretic analysis

The molecular mass and homogeneity of topoisomerase II inhibitor was determined by SDS-PAGE (12%) [James and Dubery, 2001]. Protein molecular mass standard used was SM041 (Fermentas, USA), which contained β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), REase Bsp981 (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa). Proteins were stained with silver nitrate method [Chevallet et al., 2006].

2.2.6 MALDI TOF Analysis

Identification and determination of molecular masses were performed by MALDI-TOF. Purified topoisomerase inhibitor which showed single band on SDS PAGE was digested overnight with MALDI grade trypsin (Sigma). Gel band was excised, destained and dried before digestion with trypsin. After the sample was mixed with equal volume of matrix solution (50mM) 2, 5 dihydroxybenzoic acid in acetonitrile and 0.1% (w/v) trifluoric acid, dried at room temperature on the sample probe [Kumari et al., 2010]. Sample molecules were ionized, extracted into analyzer region of mass spectrometer and were separated according to their m/z ratio. All mass spectra obtained in reflector mode were calibrated using several matrix ion peaks as internal standards. The mass peak was taken as input in MASCOT using following parameters: (a) fixed modifications – carbamidomethyl (C); (b) variable modifications: oxidation (M); (c) cleavage by trypsin: cuts C-term side of KR unless next residue is P.

2.2.7 Kinetoplast DNA decatenation assay

Topoisomerase II catalytic activity was measured by ATP dependent decatenation of kinetoplast DNA (kDNA) [Vanderzee et al., 1993]. The standard decatenation assay mixture (20µL) contained: 50 mM Tris HCl (pH 7.5), 50mM KCl, 10 mM MgCl₂, 1 mM
ATP, 0.5 mM dithiothreitol, and 30 µg/mL bovine serum albumin, 0.2 µg of kDNA (Topogen Inc., USA) and 2 unit of enzyme (1 unit of enzyme activity is defined as the amount of enzyme needed for 50% decatenation of 0.2µg of kDNA networks into minicircles) in the presence or absence of inhibitor. The reaction was carried out at 30°C for 30 min. The reaction was stopped by addition of 1% SDS, and the products were electrophoresed in 1% agarose gel containing 0.5 µg/mL ethidium bromide in TBE buffer.

2.2.8 Topoisomerase II mediated DNA cleavage assay

The ability of an inhibitor to stabilize topo II – DNA complexes was evaluated using the method of Gantchev and Hunting with some modifications [Gantchev and Hunting, 1998]. Reaction mixtures (20 µL) containing 100 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA, 30 µg/mL bovine serum albumin, 125ng of pBR322 DNA, 1U of topoisomerase II enzyme with increasing concentration of inhibitor (2 µg – 10 µg) were incubated at 37 °C for 30 min. The reactions were terminated by adding 1µL of 10% SDS and 1µL of 20 µg/mL proteinase K. After additional 1 h incubation at 37°C, DNA samples were electrophoresed in 1% agarose gel.
2.3 RESULTS AND DISCUSSION

2.3.1 Topoisomerase II relaxation assay

The protein concentration of the topoisomerase II extract from human erythromyeloblastoid leukemia cell line, K562 cell lines was found to be 11.7 µg/µL. The relaxation of the pBR322 by the enzyme was shown in Fig. 2.1. Complete relaxation of 125 ng pBR322 supercoiled DNA was achieved by the enzyme at 4µg protein concentration. One unit of the enzyme was calculated as the amount of enzyme capable of relaxing 0.125 µg DNA in 30 min at 37ºC [Deffie et al., 1989]. The crude intracellular extract of Streptomyces sp. ST1 showed inhibition towards topoisomerase II enzyme (Fig. 2.2). At 40 µg and 50 µg concentration the crude extract showed significant inhibitory activity.

2.3.2 Purification of Streptomyces sp. ST1 topoisomerase II inhibitor (StTI)

The Streptomyces sp. ST1 topoisomerase II inhibitor (StTI) was purified to homogeneity from the crude extract by two step purification procedure as summarized in Table 2.1. Gel filtration using Sephadex G-75 size exclusion chromatography resulted in two protein peaks, one major (peak 19) and other minor peak (peak 37) (Fig. 2.3). Of these, the major peak showed topoisomerase II inhibition activity. In this step, the specific activity was increased to 58.8 Umg⁻¹ protein and the yield was 41% with a purification of two fold.

The major topoisomerase II inhibiting fractions were further purified by DEAE cellulose anion exchange chromatography (Fig. 2.4). DEAE column chromatography enriched the StTI protein by purification fold of 8, yield of 19% and with specific activity of 238 Umg⁻¹. TrmFO, a Gid protein purified from Bacillus subtilis by gel filtration chromatography has showed purification yield of 7% [Hamdane et al., 2010].

2.3.3 Electrophoretic and matrix assisted desorption ionization time of flight (MALDI-TOF) analysis of StTI

Electrophoretic separation of StTI showed a single clear band in SDS – PAGE. The relative molecular mass of the purified StTI was estimated to be 42 kDa (Fig. 2.5), by both SDS PAGE and gel filtration chromatography. The band was excised, digested in situ with trypsin and the digestion mixture was analysed directly by MALDI TOF
peptide mass spectrum. The m/z ratio value obtained from MALDI TOF spectra corresponding to peptide of StTI were matched with the MASCOT database (Table 2.2). The output displayed match with tRNA uridine 5-carboxymethyl amino methyl modification enzyme MnmG (GidA – Glucose inhibited division protein A) of Natranaerobius thermophilus with accession MNMG_NATTJ (http://www.matrixscience.com).

Twelve peptides in the spectrum (Table 2.2 and Fig. 2.6) correspond to tryptic peptides of GidA protein of Natranaerobius thermophilus were significantly identified by MASCOT database search by MOWSE score of 72 and E value of 0.036. α-amylase from soybean was identified by MOWSE score of 67 and E value of 0.012 [Kumari et al., 2010]. The MOWSE score obtained in the present study was more than 70 which implied that the result was highly significant.

The molecular mass of Streptomyces sp. ST1 GidA protein was estimated to be 42 kDa from the SDS PAGE which shared high identity with a large family of Gid proteins. In this, these findings corroborate with previous findings that GidA range in size from 48 kDa in Bacillus subtilis to 53 kDa in Deinococcus radiodurans. [White et al., 2001]. GidA range in size from 59 kDa in Lactococcus lactis to 70 kDa in E.coli [Meyer et al., 2008]. This indicated considerable diversity in GidA molecular mass and structure from different microbes. The calculated isoelectric point of GidA protein from the MASCOT search was 5.36 which coincided with the theoretical isoelectric point of untagged TrmFO (a group of Gid protein) at 5.83 [Hamdane et al., 2010]. These findings strongly suggest the identified StTI inhibitor as GidA protein.

### 2.3.4 GidA inhibited the catalytic activity of DNA topoisomerase II

The effect of GidA on the catalytic activity of DNA topoisomerase II was measured by relaxation of supercoiled plasmid DNA as shown in Fig. 2.7. Lane 1 showed pBR322 supercoiled form. The supercoiled form (SC) was compact and hence moved faster when compared to open or closed circular and relaxed form [Vance and Bastow, 1999]. The pBR322 and topoisomerase II (1U) added in lane 2 showed relaxed form (RX) which migrated slower than the supercoiled form. The relaxation was inhibited by GidA starting at 2µg (lane 4) and at 10 µg (lane 8) the reaction was totally inhibited. When the GidA 2-10 µg was added in lane 4-8 there was gradual increase in the supercoiled DNA formation.
These findings showed the inhibition by the GidA occurred in a highly dose dependent manner. Etoposide a well known topo poison [Benchokroun et al., 1995; Dassonneville et al., 2000] (50 µM) added with topoisomerase II and DNA showed higher inhibition of topoisomerase by reappearance of the supercoiled form, in lane 3. Dicentrine, an aphporine alkaloid from Stephania sp. inhibited topoisomerase II catalyzed relaxation of supercoiled DNA with IC₅₀ value of 27 µM [Woo et al., 1999]. In the present study complete inhibition was achieved at 10 µg of GidA protein. This result suggested GidA protein had completely inhibited topoisomerase II activity at lesser concentration when compared to etoposide and other topoisomerase II inhibitors.

GidA by itself did not unwind DNA. To confirm this, supercoiled pBR322 was incubated with GidA at concentration up to 50 µg without addition of topoisomerase II. No relaxation and no apparent DNA conformational changes took place because of the unwinding of DNA. This suggested GidA protein can act as topoisomerase II enzyme inhibitor. Since the crude nuclear extract of K562 cell lines were used for topoisomerase activity assay, the presence of topoisomerase II enzyme activity conformation was investigated by decatenation assay [Deffie et al., 1989].

2.3.5 Decatenation assay unique for topoisomerase II

Decatenation assay is a highly specific assay for type II topoisomerases. Topoisomerases II catalyzed strand passage of double stranded kinetoplast DNA yielding two types of decatenated kinetoplast DNA: the covalently closed circular relaxed form and the nicked open circular form [Lis et al., 1993]. Fig. 2.8 lane 2, showed the decatenation of kDNA (lane 1) by 2U of topoisomerase II. The catenated kDNA in lane 1 moved slower and the band appeared near the well whereas; in lane 2 the decatenated kDNA moved faster than catenated DNA and appeared away from the well [Yang et al., 2009]. Lane 3-7 referred to the decatenation of kDNA in presence of 2, 4, 6,8,10 µg of GidA protein. There was increase in the intensity of catenated DNA which was observed with increased concentration of GidA. Lane 8 referred to the decatenation of kDNA in presence of 60 µM of etoposide.

Etoposide also inhibited decatenating activity of topoisomerase II and does so with greater potency. GidA protein inhibited the topoisomerase II catalyzed decatenation
Purification and characterization of topoisomerase II inhibitor from Streptomyces sp.

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of kDNA in a dose dependent manner and with greater efficiency than etoposide. The concentration required for complete inhibition of topoisomerase II activity for GidA protein is 10µg, whereas NK 314, a novel topoisomerase II inhibitor completely inhibited decatenation activity at 10 µmol/L concentration [Onda et al., 2008]. The result suggested topoisomerase II in the crude nuclear extract was inhibited by the GidA protein. Topoisomerase II can be inhibited by binding of GidA protein to DNA itself (catalytic inhibitor) or to the DNA enzyme cleavable complex (topo poison).

2.3.6 GidA induced topoisomerase II mediated DNA cleavage

Topoisomerase II introduces double strand nick in the phosphodiester bond of the DNA, allows an intact strand to pass through the nick, and rejoins the nicked strand of the DNA. A covalent bond is formed between the 3’OH group of the DNA backbone and the tyrosine group at the active site of topoisomerase II [Tse et al., 1980]. The covalent enzyme DNA complex is the putative reaction intermediate termed the ‘cleavable complex’ which can be detected when the reaction is terminated with a strong detergent, SDS and proteinase K. [Champoux, 1981]. Etoposide is an established antitumor drug and well characterized inhibitor of topoisomerase II. Etoposide has been shown to stabilize the cleavable complex, which is probably the mechanism of its enzymatic inhibition [Meyer et al., 2008].

To understand the mechanism of GidA inhibition, the effect of GidA on the cleavable complex formation between the topoisomerase II and pBR322 DNA was investigated (Fig. 2.9). The supercoiled pBR322 DNA (125ng) was relaxed by the enzyme (1U) in the absence of the inhibitor (lane 2). In the presence of etoposide (50 µM), (lane 8) a band corresponding to linear DNA could be clearly seen, attesting to the fact that etoposide inhibited the religation of DNA once the double helix had been cleaved by the enzyme [Dassonneville et al., 2000]. With increasing concentrations of GidA (2-10 µg), the same band could be detected. The gradual increase in the linear band (lane 3-7) indicated the GidA stabilized the “cleavable complex”. According to the earlier reports, it has been proved that there was interaction between GidA and tRNA [Meyer et al., 2008].
tRNA binding ability was demonstrated by gel retardation analysis, strongly suggested GidA was responsible for the tRNA binding during tRNA modification process through the formation of ternary complex [Osawa et al., 2009].

Earlier evidences suggested there are some proteins capable of binding to both DNA and RNA. TIA-1 related protein (TIAR) is a high-affinity RNA-binding protein that promotes apoptosis. It possesses three RNA recognition motifs (RRMs) and shares 80% amino acid homology with TIA-1. Truncation of TIAR indicated that the high affinity DNA-binding site overlaps with the RNA-binding site involving RNA recognition motif 2 (RRM2). These results provide a potential mechanism by which TIAR can shuttle between RNA and DNA ligands [Morales et al., 1998]. Another germ cell Y-box protein, MSY2, constitutes 0.7% of total protein in male germ cells, binds to a consensus promoter element. These Y-box proteins, consisting of variable N and C termini and a highly conserved cold shock domain, are a family of evolutionarily conserved DNA- and RNA-binding proteins that function in both transcription and translation [Suswam et al., 2005]. Testis–brain RNA-binding protein (TB-RBP) is a single stranded DNA- and RNA-binding protein prominent in the nuclei and cytoplasm of specific stages of differentiating male germ cells [Yang et al., 2005].

In view of the earlier reports, GidA which has the ability of tRNA binding, may also stabilize cleavable complex by binding with topo II- DNA ternary complex in the present study. Therefore GidA can be suggested as a novel “topoisomerase II poison” which inhibited topoisomerase II by binding to topoisomerase II – DNA cleavable complex.

Recently developed topoisomerase inhibitors are either natural products or are derivatives of natural products; several of these compounds are effective antitumor and antibacterial agents [Drlica and Franco, 1988]. Consequently, there is a lot of interest in discovering novel topoisomerase poisons from microbial sources as potential lead compounds for drug development. These data suggests that GidA of Streptomyces sp. ST1 is a novel inhibitor of topoisomerase type II enzyme. If the efficacy of GidA protein is improved by binding with metal complex this inhibitor might turn out to be one of the promising series of antitumor drugs of microbial origin. For binding, the selection of ruthenium metal complex possessing topoisomerase II inhibiting activity was dealt in next chapter.
2.4 REFERENCES


Champoux, J.J., 1981. DNA is linked to the rat liver DNA nicking closing enzyme by a phosphodiester bond to tyrosine. J. Biol. Chem., 256:4805-4809.


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Sivasamy, R., 2010. Ph.D thesis entitled “Anticancer potential of nucleoside diphosphate kinase from native isolate Streptomyces sp. by topoisomerase II inhibition” Cancer Therapeutics Laboratory, Department of Microbial Biotechnology, Bharathiar University, Coimbatore.


Table 2.1 Purification of *Streptomyces* ST1 topoisomerase II inhibitor (StTI)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total inhibitory activity (U)(^a)</th>
<th>Specific activity (U/mg)(^b)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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<tbody>
<tr>
<td>Crude extract</td>
<td>168</td>
<td>4941</td>
<td>29.4</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Sephadex G 75 chromatography</td>
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<td>2058</td>
<td>58.8</td>
<td>41</td>
<td>2</td>
</tr>
<tr>
<td>DEAE cellulose chromatography</td>
<td>4</td>
<td>952</td>
<td>238</td>
<td>19</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\) One StTI inhibitory activity unit is defined as the amount StTI protein required to inhibit 50% of topoisomerase II.

\(^b\) Specific activity is defined as the number of StTI units/ mg protein.
Table 2.2 Sorted peptides of GidA protein according to their residue number and masses along with the peptide match obtained from the MALDI TOF MS analysis

<table>
<thead>
<tr>
<th>No</th>
<th>Start – end</th>
<th>Observed mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Peptide sequence identified</th>
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<tbody>
<tr>
<td>1</td>
<td>84 – 88</td>
<td>650.51</td>
<td>649.32</td>
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<td>2</td>
<td>98 – 102</td>
<td>532.25</td>
<td>531.26</td>
<td>AQADK</td>
</tr>
<tr>
<td>3</td>
<td>109 – 122</td>
<td>1766.53</td>
<td>1764.93</td>
<td>MKYILENEENLLLK</td>
</tr>
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<td>4</td>
<td>123 – 137</td>
<td>1803.50</td>
<td>1801.88</td>
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<td>5</td>
<td>138 – 142</td>
<td>503.17</td>
<td>502.31</td>
<td>GVVTK</td>
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<tr>
<td>6</td>
<td>143 – 161</td>
<td>2274.26</td>
<td>2273.05</td>
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<td>190 – 199</td>
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<td>1231.64</td>
<td>QHGISLMRFK</td>
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<td>8</td>
<td>210 – 227</td>
<td>2252.27</td>
<td>2250.96</td>
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<tr>
<td>9</td>
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<td>1399.68</td>
<td>FSFYHSSERL</td>
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<td>254 – 261</td>
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<td>IINDSLSR</td>
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DNA topoisomerase II activity assay was performed by incubating pBR322 DNA (125 ng) with topoisomerase II enzyme extracted from K562 cell lines. Lane 1, pBR322 DNA as control; Lane 2-5, pBR322 DNA with 1-4 µg of enzyme extract. OC, Open circular DNA; RX, Relaxed; SC, Supercoiled.
Fig. 2.2 Effect of crude extract of *Streptomyces* sp. ST1 on relaxation of plasmid DNA by topoisomerase II

The influence of crude extract on relaxation of plasmid DNA by topoisomerase II. Lane 1, pBR322 DNA as control; Lane 2, pBR322 DNA with 1U of topoisomerase; Lane 3-7, 10-50 µg of crude extract of inhibitor. OC, open circular DNA; RX, Relaxed; SC, Supercoiled.
Fig. 2.3 Gel filtration chromatography

Purification of StTI from Streptomyces sp. ST1 on Sephadex G-75 column

- Absorbance at 280 nm
- % Inhibition of topoisomerase II
Fig. 2.4 DEAE cellulose anion exchange chromatography

Purification of StTI from Streptomyces sp. ST1 on DEAE cellulose anion exchange column

- ♦ Absorbance at 280 nm
- ○○ % Inhibition
Fig. 2.5 SDS PAGE profile of *Streptomyces* sp. ST1 topoisomerase II inhibitor fractions

SDS – PAGE (12%) of the fractions showing StTI activity obtained during purification and stained with silver nitrate. Lane 1- Molecular mass standards (Fermentas, SM041), Lane 2 active fraction pool from DEAE column, Lane 3- active fraction pool from G75 column, Lane 4 – crude extract
Peptide mass fingerprint spectra of the tryptic digest of single band from SDS-PAGE after purification of GidA protein. Identified mass peaks were labeled. 12 peptide masses correspond to tryptic peptides of tRNA uridine 5'-carboxymethyl amino methyl modification enzyme MnmG (GidA – Glucose inhibited division protein A) from *Natrananaerobius thermophilus* (accession MNMG_NATTJ) significantly identified by MASCOT database search by MOWSE score of 72.
Fig. 2.7 Topoisomerase II activity inhibition assay of GidA protein from *Streptomyces* sp.

Effect of increasing concentrations of GidA on the relaxation of plasmid DNA by topoisomerase II. Native supercoiled pBR322 DNA (125 ng) (lane 1) was incubated with 2 units of topoisomerase II in the absence (lane 2) or presence of GidA protein at the increasing concentration of 2µg – 10 µg (lane 4-8). Etoposide (lane 3) was used at 50 µM instead of GidA protein. OC, open circular DNA; RX, Relaxed; SC, Supercoiled.
Inhibitory effect of GidA protein on decatenation of kinetoplast DNA by topoisomerase II. The kinetoplast catenated DNA 0.2µg (lane 1) was incubated with 2 U of topoisomerase II (lane 2) in the presence or absence of GidA protein at 2-10 µg concentration (lane 3-7) and in presence of etoposide (60 µM) in lane 8.
Effect of GidA on DNA cleavage mediated by topoisomerase II. Topoisomerase II was incubated with supercoiled pBR322 plasmid DNA in the presence of GidA protein or etoposide. Lane 1 supercoiled pBR322 plasmid DNA (125 ng); lane 2, pBR322 DNA with topoisomerase II (1U); lane 3-7, same as lane 2 with GidA protein (2-10 µg concentration), lane 8, same as lane 2 with etoposide (50 µM). NI, Nicked DNA; LI, Linear DNA; SC, Supercoiled DNA.