CHAPTER II

LITERATURE REVIEW

Topoisomerases

DNA topoisomerases are important nuclear enzymes involved in many aspects of DNA metabolism, such as DNA replication, RNA transcription, chromosome condensation, and segregation. DNA topoisomerases can be divided into two classes that depend on their mechanism of action. Type I DNA topoisomerases trigger transient single-strand breaks in DNA and passes the other strand through the cleavage site before resealing the break, whereas Type II DNA topoisomerases introduce transient double-strand breaks (Wang, 2002).

Eukaryotic topoisomerase I is type IB topoisomerase that cleaves the DNA on 3′ DNA terminus by hydroxyl group of tyrosine residue of the enzyme and relaxes positively and negatively supercoiled plasmid. They bind only to double-stranded DNA. The topoisomerase I catalytic mechanism can be divided into four steps. Firstly, they bind non-covalently to the DNA. Secondly, strand scission occurred through a transesterification in which a tyrosine hydroxyl group of topoisomerase I attacked to the 3′ phosphate of a phosphodiester bond, liberating the 5′ hydroxyl to generate a trand break leaded to a topoisomerase I cleavage complex. Thirdly, single-strand passage occurred by rotation of the cleaved strand. Fourthly, ligation is a second transesterification reaction in which the 5′ hydroxyl group of the the cleaved DNA strand now serves as a nucleophile, attacking the phosphytyrosin linkage, and rejoining the cleavage DNA (Pommier *et al.*, 1998; Wang, 2002).

There were know to be at least two topoisomerase II in mammalian, α and ß isoform. Topoisomerase II acted by inducing double-strand breaks that involved a covalent attachment at the 5' phosphate end. Strand passage required the translocation of DNA through both strands of the bound DNA were cleaved to yield nicked DNA. A second DNA strand was then passed through this gate in the DNA, and the nicked DNA was religated. The hydrolysis of ATP was required to drive allosteric changes in enzyme structures for enzyme activity and magnesium ion (Mg^{2+}) was necessary in the enzyme reaction (Cortes *et al.*, 2003).

Topoisomerase inhibitors

Topoisomerase I inhibitors can be divided into two classes, topoisomerase poisons and catalytic inhibitors due to different mechanism of action. Topoisomerase poisons were characterized by the stabilization of cleavable complexes, a topoisomerase intermediate and catalytic inhibitors were characterized by competitive inhibition of the enzyme (Pommier *et al.*, 1998). Unlike topoisomerase poisoning, the

catalytic inhibition was required preincubation in *in vitro* assay to obtain a maximum inhibition. Each classes of inhibitor can be subclassified by DNA binding properties. The DNA binder was categorized into two types, intercalator and minor groove binder. Some topoisomerase inihibitors was summarized in Table 1.

Camptothecin, a cytotoxic alkaloid isolated from *Camptotheca acuminata*, a tree found in China, selectively inhibited eukaryotic topoisomerase I. The anticancer activity of CPT was tested in clinical trials against leukaemia and lung cancer in the early 1970s. It caused dose-limiting haemorrhagic cystitis and myelosuppression. Structure-activity studies to improve the anticancer activity and reduced the toxicity of CPT indicated that the six-membered lactone ring was essential for anticancer activity. A number of derivatives of CPT have been synthesized by modifying the A-ring of the parent compound included irinotecan and topotecan, for example.

Flavonoids, ubiquitously occurring and widely consumed secondary metabolites of plants, were among the active components in vegetables and fruits that prevented or inhibited cancer development (Lopez-Lazaro *et al.*, 2002). Flavonoids were a diverse group of naturally occurring polyphenolic compounds with profound pharmacological properties. They were reported to have antiviral, anticancer and antiparasitic activities. Among naturally occurring flavonoids, quercetin, the lead compound, was most widely studied *in vitro* and *in vivo*. It was known to interfere with several pathways of intermediary metabolism and various components of signal transduction cascades. Quercetin and related flavonoids, genistein, hesperetin, naringinin and epigenin were known to inhibit the growth of tumor cells. Moreover, quercetin and some related compounds had been shown to induce topoisomerase II-mediated DNA cleavage in mammalian cells. Thus, topoisomerase-mediated DNA damage seemed to be a candidate mechanism, by which some flavonoids may exert their cytotoxic potential. However, some flavonoids including EMD 21 388 and EMD 50 689 inhibited topoisomerase via direct binding to enzyme. Unlike quercetin, these compounds did not stabilize enzyme covalent complex (Boege *et al.*, 1996).

Naphthoquinone derivatives such as ß-lapachone, menadione and diospirin were reported their topoisomerase inhibition. Likewise flavonoids, they exerted catalytic inhibition or enzyme poisons. Moreover, some derivatives exhibited antiparasitic activity, which included bis-naphthoquinone, diospirin (Bailly, 2000).

There was interesting evidence that some polysaccharides could inhibit topoisomerase. Heparin were firstly reported as topoisomerase I inhibitor (Ishii *et al.*, 1987). It could induce the cleavage complex like camptothecin. Umemura *et al.* (2003) had introduced a marine microalgal polysaccharide, GA3P as topoisomerase I and II inhibitor. Unlike heparin, it could not stabilize cleavage complex. Those polysaccharide was previously reported their cytotoxic properties on cell lines. There were evidences of heparin uptake in cancer cells (Berry *et al.*, 2004).

Many DNA binders exhibited topoisomerase inhibition. Intercalator such as intoplicine was dual topoisomeras I/II inhibitor (Nabiev *et al.*, 1994). Minor groove binders such as Hoechst 33342 and distamycin were topoisomerase I inhibitors (Cheng *et al.*, 1993). Unlike Hoechst 33342, distamicin exerted catalytic inhibition while Hoechst 33342 exhibited topoisomerase poisoning.

Like topoisomerase I inhibitors, topoisomerase II inhibitors had divided into two categories: catalytic inhibitors and enzyme poisons and subclassified by DNA binding properties. Merbarone and fostriecin were catalytic inhibitors without DNA binding properties. Etoposide and teniposide were non-DNA binder that exhibited enzyme poisoning. Duanomycin and ellipticine were intercalators that induced cleavage complex, the specific byproduct of enzyme poisons. Netropsin was a minor groove binder which exhibited only catalytic inhibition (Topcu, 2001).

Table 1 Summary of topoisomerase inhibiotors.

		Topoisomerase I inhibitors		Topoisomerase II inhibitors	
		Catalytic inhibitors	**Enzyme poisons**	**Catalytic inhibitors**	**Enzyme poisons**
Non-DNA binders		Napthaquinone: menadione, lapachone, diospirin Flavonoids: EMD21 388, EMD 50 689	Camptothecins: camptothecin, irinotecan, topotecan Polysaccharide: heparin, GA3P	Merbarone, fostriecin	Etoposide, teniposide
DNA binders	**Intercalators**	-	Intoplicine Flavonoids: quercetin, genistein	-	Duanomycin, ellipticine
	Minor groove binders	Distamycin	H 33342	Netropsin	-

Topoisomerase agarose-gel electorphoresis assays

Many experimental approaches had been employed to identify topoisomerase inhibitors and to characterize their mechanism of action. Agarose-gel electrophoresis was a common *in vitro* cell-free assay using topoisomerase I and plasmid DNA substrate had been manipulated to present relaxation activity of enzyme or inhibitory effect of inhibitors. There were two assays used for classifying inhibitors. First assay was called DNA relaxation assay which could not indicate whether inhibitors were catalytic inhibitor or enzyme poisons. The latter was called DNA cleavage assay which confirmed poisoning effect of inhibitors.

DNA relaxation assay

A plasmid relaxation assay for topoisomerase I activity by method of agarose-gel electrophoresis had been described by Keller in 1975. With this technique, the compact nature of supercoiled topoisomers enables them to migrate through the porous gel matrix with less resistance than relax topisomers. Agarose-gel electrophoresis is now the method of choice for visualizing the products of topoisomerase relaxation assays. After enzyme incubation, the enzyme reaction mixture was aliquoted and diluted with loading buffer. And then the diluted sample was loaded in gel well placing in buffer-contained chamber. The current was applied in rang of 2-10 V/cm depend on DNA molecular weight. Time of electrophoresis was at least 1 hr or overnight as desirable band resolution. For DNA visualization, gel was soaked with ethidium bromide solution and destained by distilled water to decrease the fluorescence background after that gel was observed under UV illuminator or gel documentation system (Stewart and Champoux, 2001). Sybr Green I or Sybr Gold could be used as alternative choice for DNA visualization on gel in the meaning of safety.

DNA cleavage assay

Topoisomerase I catalyzed the reversible single strand nicked DNA. Specific studies often required the addition of denaturant such as SDS result in trapping the cleavage complex. In some case, the addition of proteinase K to hydrolyze bond between the enzyme and DNA was required for nicked DNA band resolution in agarose-gel electrophoresis. Unlike DNA relaxation assay, molar ratio of enzyme to DNA of 10-100 are sufficiency to observe cleavage complex which was induced in absence or presence of topoisomerase I poisons (Champoux, 2001). Since relaxed DNA and nicked DNA closely migrated at the overlapped position on gel, an intercalator such as ethidium bromide or chloroquine was used to separate relax DNA from nicked DNA. Closely relaxed DNA would be change to compact structure when it was bound by intercators, while the nicked DNA was not change. In the experiment, an intercalator was included in electrophoretic buffer (Bailly, 2001).

Topoisomerase assay development

Because of the cumbersome of gel electrophoresis techniques of the enzyme for studying mechanism or for screening topoisomerase inhibitors, many researchers put an effort to develop new assays (Table 2). Some methods were described below.

Foglesong (1989) established fluorometric methods employing low concentration of ethidium bromide for DNA topoisomerase activity detection. The volume of enzyme reactions was 250 µl which much higher than normal reaction using in gel electrophoresis. This assay was prone to waste enzyme, substrate or inhibitors.

In 1991, Andrea and his coworkers developed fluoremetric method that resembled to that of Foglesong (Andrea *et al.*, 1991). The difference between two assays was the volume of enzyme reactions. Andrea prepared enzyme reaction in the same condition as using in gel electrophoresis method. However, the assay was not performed in microplate format.

HPLC assay for topoisomerase activity was introduced by Onishi group (Onishi *et al.* 1993). The method was sensitive to detect DNA concentration at nanogram level. Nevertheless, a large number of samples could not be applied by this technique. Specific column such as anion-exchange column and purified samples were required.

Capillary electrophoresis was used to apply for topoisomerase activity (Raucci *et al.* 2000). Although this method was rapid, limitations were plasmid purification and expensive equipment.

The first 96-well microplate assay of topoisomerase was presented by Hwang group (Hwang *et al.* (2000). The application based on ELISA using modified DNA substrate. Because this technique was end-point assay, it was inappropriate to use for studying enzyme kinetics.

Another topoisomerase assay using modified substrate was developed by Kwon laboratory (Kwon *et al.*, 2004). Their study demonstrated an advantage in kinetic study and showed ribonuclease activity of topoisomerase I. The enzyme activity or enzyme inhibition was observed in a real time manner. However, the method required special designed substrate which was expensive.

The flow linear dichroism technique was applied to study topoisomerase activity by Gibibov and coworkers (Gabibov *et al.*, 2005). This technique was continuous assay that provided enzyme kinetic information and can be use to screening inhibitors. The limitations of this study were inseparable types of inhibitors and limited number of samples was performed in assay.

Recently, Maxwell group had developed two assay applied to topoisomerases (Maxwell *et al.*, 2006). The assay used some special probes, fluorescent-labelled triplexing oligo or radio-labelled oligo to detect topoisomer state alteration of plasmid by topoiosomerases. However, this assay procedure was performed like ELSA technique, multi-washing steps were required.

Other assay for screening topoisomerase inhibitors was demonstrated by Zhang group (Zhang *et al.*, 2004). This assay based on the affinity of antitumor candidates for topoisomerases. The enzyme activity did not involve in the assay.

Previous studies had been demonstrated that their developed assays still need to include a conventional substrate such as plasmid together with fluorescence dyes and used in microplate format.

Table 2 Summary of topoisomerase assay.

Principle	substrate	Separation technique	detection	Sample capacity	Remark	Reference
HPLC	Plasmid	Yes	UV detector	96		Onishi *et al.*, 1993
Capillary electrophoresis	Plasmid	Yes	UV detector	96		Raucci *et al.*, 2000
Fluorometry	Plasmid	No	Spectrofluorometer	10	Large volume of enzyme reaction	Foglesong, 1989
Fluorometry	Plasmid	No	Spectrofluorometer	10		Andrea *et al.*, 1991
Flow linear dichroism technique	Plasmid	No	UV detector	No data	Large volume of enzyme reaction	Gabibov *et al.*, 2000
ELISA	Oligonucleotide	No	UV-visible spectrometer	96	Microplate format	Hwang *et al.*, 2000
Fluorometry	Oligonucleotide	No	Spectrofluorometer	≥ 96	Microplate format	Kwon *et al.*, 2004
Fluorometry	Plasmid	No	Spectrofluorometer	96	Microplate format	Maxwell *et al.*, 2006
HPLC/ESI-MS	No	Yes	UV detector/ Mass detector	96	Bioaffinity ultrafiltration	Zhang *et al.*, 2004

Fluorescence dyes and topological state of DNA

To observe topological state of DNA, agarose-gel electrophoresis was used to separate DNA molecules on the basis of size and shape. The most commonly used technique for measuring DNA concentration involved in absorbance at 260 nm. This technique was inability to observe topological change of DNA because of similar UV absorption properties of DNA topoisomer. In addition, UV/visible spectrophotometry has disadvantages compared with fluorescence spectroscopy in DNA determination, including lower sensitivity and specificity (Haque *et al.*, 2003).

There were a lot of fluorescence dyes used widely as DNA probes. Those fluorescence dyes included ethidium bromide (intercalator), Bisbenzimide including Hoecht (H) 33258 and Hoecht (H) 33342 as minor groove binder and cyanine dyes, Picogreen and Sybr Green I. Ethidium bromide, H 33258 and H 33342 were auto-fluorescent molecules because of its conjugated system, condesnsed system of fused rings, with one or more heteratoms or electron-donating groups where as Picogreen and Sybr Green I were non-intrinsic fluorescence. Thus, Picgreen and Sybr Green I was sensitive more than ethidium bromide and bisbenzimide dyes because of low background (Rengarajan *et al.*, 2002). There were a small number of studies in DNA topology with fluorescence dyes. The fluorescence intensity change of ethidium bromide with alteration of DNA topology was observed in previous study (Lee and Morgan, 1978). Sandhu laboratory (Sandhu *et al.*, 1985) studied fluorescence effect of Hoecht 33342 on supercoiled and relaxed pBR322. This basis was applied to observe DNA condensation depending on cell-cycle or apoptosis in cell line (Prosperi *et al.*, 1994). The cyanine dyes such as Picogreen and Sybr Green I were employed only for DNA quantitation but fluorescence intensity of cyanine dyes on DNA topology had not been reported yet.