CHAPTER V

DISCUSSION AND CONCLUSION

Topoisomerase I has been recognized as a biological target for anticancer or antimicrobial agents (Holden, 2001; Chen *et al.*, 2007). To determine topoisomerase I activity, the DNA relaxation assay and the DNA cleavage assay using gel electrophoresis were widely used. But these assays were inconvenient in terms of sensitivity, speed and throughput for rapid screening of inhibitors of these enzymes. Because of the substrates and products of topoisomerase I assay are the plasmids. They are identical in UV/visible absorption properties, therefore they could not separate by photometry. The separate technique like gel electrophoresis was useful to detected the products and substrates of the enzyme. The assay was based on the separation of the products from the substrate by agarose gel electrophoresis, and the activity was measured by densitometric scanning.

A separation technique by gel electrophoresis was a time consuming process with a messy quantitative analysis for enzyme activity or the IC_{50} value. After completed incubation, all samples would be diluted with loading dye for putting in agarose gel well. This process took 1 min per sample. Thus, more samples spent more times. In separation step, it took at least 1 hr for finishing task with clear separation. And in visualization step, agarose gel was stained and destained minimally for 1 hr with enough resolution. Therefore, Overall time for gel electrophoresis after incubation was over 2 hr. The maximum numbers of sample which were able to run at the same time was about 80 (referred with our equipment), it quite small when compared with microplate format. Thus, this technique was not suitable to be used for screening compounds from large chemical library. There were several researchers attempted to develop a rapid separation technique such as HPLC (Onishi *et al.*, 1993; Molley *et al.*, 2004) and capillary electrophoresis (Raucci *et al.*, 2000). Although, these methods were faster than gel electrophoresis, they could not support a large number of unknown samples. In this study, microplate format was selected to use in assay development that could manage a lot of samples.

To avoid separation step such in the conventional assay, the enzyme assays were performed in microplate format by many researchers. For example, Hwang *et al.* (2000) developed an ELISA assay and Kwon *et al.* (2004) established a continuous fluorescence assay for topoisomerase I. Nevertheless, both assays needed the modified double-strand DNA substrate instead of plasmid. Another continuous assay was developed by Gabibov *et al.* (2005) monitoring by flow linear dichroism technique. This technique was preferred to determine enzyme kinetics than screen for inhibitors that involved with the limitation of the analytical equipment.

Quantitative fluorescence assays for topoisomerase I based on differences in fluorescence intensity between relaxed and supercoiled DNA have been reported (Foglesong, 1989; Andrea *et al.*, 1991). These assays used a standard enzyme reaction mixture like gel electrophoresis assay. However, these assay methods were not performed in 96-well microplate format and used ethidium bromide, a genotoxic agent in the assay.

To establish the new assay based on 96-well microplate format and using plasmid like in the conventional assay, the fluorescence dyes such as Hoechst (H) 33258, Hoechst (H) 33342, Picogreen and Sybr Green I were taken to study the difference of fluorescence intensity between supercoiled and relaxed DNA, substrate and product of topoisomerase I. There was only H 33342 that had been reported for affecting the difference of the fluorescence intensity between supercoiled and relaxed pBR322 (Sandhu *et al.*, 1985). However, they focused on topological state of DNA but did not emphasize on the topoisomerase activity. In this study, H 33342 and a related compound, H 33258 were examined for their abilities in separating two forms of DNA. The fluorescence intensity of supercoiled DNA was slightly lower than that of relaxed DNA. The result of H 33342 was contrast with the result obtained from Sandhu *et al.* (1985) because of the differences in dye concentration, DNA concentration or a kind of buffer.

Another fluorescence dye such as Picogreen and Sybr Green I using for staining DNA were also included in this experiment. Unlike H 33258 and H 33342, Picogreen and Sybr Green I exhibited intercalative and surface binding properties (Zipper, 2004). There was no any previous report about the fluorescence intensity between supercoiled and relaxed DNA studied by Picogreen or Sybr Green I. In this study, the fluorescence intensity of supercoiled DNA was higher than that of relaxed DNA. In addition, Picogreen displayed the greater difference fluorescence intensity between the two forms of DNA over Sybr Green I.

Although all 4 dyes had the difference intensity dependent on the form of DNA, it did not mean that they could be applied to analyse the topoisomerase I activity. To investigate the detection ability of each dyes, Z′ value was taken to demonstrate the assay performance. Z′ value was usually utilized to validate in assay development especially high-throughput assay (Zhang *et al.*, 1999). Maximum signal and minimum signal was defined by fluorescent characteristic of each dyes. To compare ability of each dye to be used for enzyme assay, Z′ value was applied. The Z′ value $\geq$ 0.4 was accepted (Eli Lilly and Company and NIH Chemical Genomics Center, 2008). H 33258, H 33342 and Syrbr Green I had negative value implied that those dyes were inappropriate to be used for assay. Picogreen was the only one which passed the accepted criterion. Howerver, if it based on Zhang's criteria, this assay system did not show an excellent result.

Interday assay was performed only for Sybr Green I and Picogreen to comfirm reproducibility following the assay guidance of Eli Lilly and Company and NIH Chemical Genomics Center. Picogreen demonstrated no significant change of Z′ value and Sybr Greeen I presented negative result correspond to intraday assay experiments.

The assay ability of Picogreen at various DNA concentrations were examined. The Z′ value at 10 ng/ml of DNA concentration was lower than 0.4 that implied fluorescence intensity of Picogreen might be insufficient to separate two form of DNA at this concentration. The appropriate DNA concentrations to be used in this assay system are between 50 to 1,000 ng/ml. However, Z′ value depend on CV which would be changed in each occasion of experiment.

Time-course study of enzyme activity was performed by proposed assay compared with conventional assay using various DNA concentrations. The results demonstrated that fluorescence microplate assay was more sensitive than gel-based assay since the fluorescence intensity had a greater change over band intensity at the same range of time of incubation. Nevertheless, this experiment was not examined from the same sample.

To illustrate proposed assay could be used to screen for inhibitor, enzyme inhibition was studied using known topoisomerase I inhibitors, enzyme poisons (camptothecin, heparin, quercetin) and catalytic inhibitor (menadione) and topoisomerase II inhibitors (etoposide and ellipticine). Conventional assay and proposed assay were run in parallel to confirm each other.

The IC_{50} values of camptothecin obtained from gel-based assay and fluorescence-based assay were 3.46 ± 1.20 and 11.6 ± 9.2 μM, respectively. It was similar to the IC_{50} values that were reported as 2.9 μM from gel-based assay and 9.6 μM from fluorescence assay that was developed by Maxwell *et al.* (2006). Although there were differences in the kind of substrates, enzyme reactions, testing solutions or assay procedures between the assays. The estimated IC_{50} value of camptothecin from the ethidium fluorescence assay performed by Andrea *et al.* (1991) was about 50 μM. It was higher than the proposed fluorescence assay. This result suggested that this proposed assay has higher sensitivity over Andrea's method.

In previous study, heparin was reported as a topoisomerase I inhibitor with the IC_{50} value of 1.8 and 5.0 μg/ml (Ishii *et al.*, 1987). In this study showed the IC_{50} values obtained from gel-based assay and fluorescence-based assay were 2.45±1.02 and 4.43±1.24 μg/ml, respectively.

The fluorescence intensity of both supercoiled and relaxed DNA were interfered by quercetin. Quercetin was reported as intercalator (Webb and Ebeler,

2004). Since DNA was bound with quercetin, Picogreen could not bind to the DNA effectively, therefore the fluorescence intensity was decreased. The assay was inappropriate to examine the DNA binder. From this data, false negative result prone to be appeared in fluorescence assay. Thus, unknown compounds should be always examined for the interference of the signal before or within the assay.

Inactivation of topoisomerase I by Menadione was reported by Frydman *et al.* (1997) without reporting the IC_{50} value. It is the only one catalytic inhibitor using in this experiment. The results given the IC_{50} values as 51.0±28.7 and 58.1±6.3 μM that performed by gel-based assay and fluorescence-based assay, respectively. The fact that preincubation requirement for this study to gain a desirable inhibition, concentration error had been occured easily in both assays.

Although the IC_{50} values of those inhibitors gained from the fluorescence assay were slightly higher than the gel-based assay, correlation analysis between two methods were significant with $p < 0.01$. This finding showed that the ability of the proposed method to screen for inhibitors was comparable to that of gel-based assay.

Etoposide and ellipticine, topoisomerase II inhibitors were used as negative control to validate the proposed assay. Etoposide at the concentration of 1 mM had no effect on topoisomerase I when tested in both assays. This data was consistent with previous paper (Minocha and Long, 1984). Nevertheless, ellipticine exhibited complete inhibitory effect on topoisomerase I at 20 μM as shown in gel electrophoresis. This result disagreed with several previous studies that ellipticine was a specific topoisomerase II inhibitor (Teywey *et al.*, 1984). It was possible that it has never been examined with topoisomerase I in the first time of discovery. In addition, the fluorescence intensity was also disturbed by ellipticine due to DNA intercalating properties.

Twelve unknown compounds, their structures related to compounds previously reported for their cytotoxic activity or their DNA binding property were tested by gel-based assay. The twelve compounds were flavonoids included genistin, hesperidine, naringin and vitexin, isoquinoline alkaloids included chelidonine, chelerythrine, sanguinarine, tetrahydropalmatine, and ancistrotectorine, and other compounds included oxostephanine (oxoaporphine), clausenidin (coumarins), and γ-fagarine (furoquinoline). All compounds were tested at 50 μM and 100 μM concentrations and compared with 50 μM of camptothecin as positive control.

The topoiosomerase I inhibition was not observed by all those glycosidic flavonoids. However, aglycone related compounds including genistin, hesperetin, naringenin and apigenin were previously reported for their topoisomerase I inhibition (Constantinou *et al.*, 1995; Boege *et al.*, 1996; Webb and Ebeler, 2004). Even if those flavonoid glycosides had no topoisomerase I inhibitory effect, some other flavonoid

glycosides such as orientin, mercetin-3galactoside and phloridzin, for example, were reported to inhibit topoisomerase I (Lopez-Lazaro *et al.*, 2002; Webb and Ebeler, 2004).

From previous study, the benzophenanthridine alkaloids (isoquinoline alkaloids), chelidonine, chelerythrine, and sanguinarine were accounted as cytotoxic agents, which induced DNA damage (Kaminskyy *et al.*, 2008). In this study, there were only chelerythrine and sanguinarine that inhibited topoisomerase I in a concentration-dependent manner. This finding comfirmed a cytotoxic mechanism of both compounds. In addition, sanguinarine was a topoisomerase II inhibitor which reported by Holy *et al.* (2006). Other isoquinoline alkaloid, tetrahydropalmatine and ancistrotectorine, a naphthalene-isoquinoline alkaloid were ineffective on topoisomerase I inhibition.

Oxostephanine and γ-fagarine showed strong inhibitory effect on topoisomerase I. The inhibition was not concentration-dependent. This result implied that they might prefer interacting with DNA more than with the enzyme or might destroy enzyme structure that affecting the enzyme activity. Oxostephanine and γ-fagarine were reported to have anticancer activity by Wirasathien *et al.* (2006) and Jansen *et al.* (2006), respectively. However, Kawaii *et al.* (2001) demonstrated that clausenidin had antiproliferative activity, its inhibitory effect on topoisomerase I was negative in this study. Therefore, clausenidin exhibited cytotoxic effect through other target than topoisomerase I.

In addition, four new positive topoiosomerase I inhibitors, chelerythrine, sanguinarine, oxostephanine and γ-fagarine should be further studied for IC_{50} value determination and specific cytotoxic effect via topoisomerase I in cell lines to confirm their inhibition behavior.

Four compounds from the gel-based assay including vitexin, a putative flavonoid, chelidonine, chelerythrine and sanguinarine were selected to test in the fluorescence microplate assay. There were negative compounds (vitexin and chelidonine) and positive compounds (chelerythrine and sanguinarine) in this study. Unfortunately, the fluorescence intensity was interfered by chelerythrine and sanguinarine due to intercalative properties of such compounds similar to that of quercetin. Moreover, chelidonine, a related analogue did not interfere the fluorescence intensity. It was previously demonstrated as a non-intercalator (Kaminskyy *et al.*, 2008). The interference of fluorescence intensity of Picogreen by intercalators agreed with the study of Koba *et al.* (2007).

Assay procedure time of the proposed assay was obviously faster than gel-based assay. The proposed assay was performed completely in 1 hr after while the gel-based assay was running in electrophoresis step. Moreover, the proposed assay

could manage a lot of samples (96 samples) in one run while the gel-based assay took only 24 samples per time or maximum at 80 samples if the large elcetrophoresis chamber was used.

When compared with Andrea's system (Andrea *et al.*, 1991), Picogreen was safer than ethidium bromide to be used and 96-well microplate format of proposed assay could manipulate a large number of samples more than Andrea. In addition, plasmid was used as substrate which was generally available compared with modified substrate of Hwang et al. and Kwon *et al.* (2004). Their substrates were uncommon with expensive value. The washing step was not required in the proposed method, unlike ELISA technique (Hwang *et al.*, 2000) or fluorescence assay established by Maxwell *et al.* (2006). This step took times because of 3 times washing and was prone to error if it performed manually or vigorously. In contrast, the proposed assay had only two steps after incubation including dilution step and reagent addition.

The cleavage complex was an intermediate product of topoisomerase I that induced by topoisomerase I poison, one of two classes of topoisomerase inhibitor. The cleavage complex was usually analysis by DNA cleavage assay using gel electrophoresis. The enzyme reaction of DNA cleavage assay resembled to DNA relaxation assay but there were differences in the amounts of DNA substrate and enzyme. Enzyme amounts were typically used at high molar ratio to DNA (10-100 folds). In electrophoresis step, it needed an intercalator in electrophoresis buffer to resolve the nicked DNA (the cleavage complex) from relaxed DNA (Champoux, 2001). In this study, enzyme reaction of DNA cleavage assay was prepared in the same way of DNA relaxation assay in order to examine nicked products from the same reaction.

To detect the nicked DNA product in the enzyme reaction, the proposed fluorescence assay was modified by method of Rock *et al.* (2003). The assay based on the denaturation properties of double stranded DNA in alkaline solutions and used Picogreen to monitor denaturation. Owing to stop reaction process of the conventional assay using 10% SDS and 1mg/ml proteinase K that might disturb the fluorescence intensity of Picogreen, the other processes were designed in the experiment. The enzyme reaction was incubated in PCR device as incubator, therefore, stop processes were done by heating at 95° C for 1 min or cooling at 4° C.

The results showed that dectectable nicked DNA was found in substrate control without topoisomerase I and no detectable nicked DNA was found in topoisomerase I reaction control. This finding suggested that topoisomerase I resealed the nicked DNA more than induced nicked DNA. In the heating process, relative fluorescence dropped lower than cooling process and the process that added SDS with proteinase K. The nicked DNA was partially denatured by heating. Thus, the heating process was inappropriate to be used in the assay. When stop process was

compared between the cooling process and SDS/proteinase K addition, the cooling process was superior to SDS/proteinase K addition in the variation of relative fluorescence.

Camptothecin was included in the experiment to compare nicked DNA product. The nicked DNA could be detected in the presence of camptothecin but DNA amount was lower than substrate control. This result implied that camptothecin might induce nicked DNA or camptothecin might prevent nicked DNA residue from topoisomerase I resealing.

In order to test the hypothesis, two inhibitors, camtothecin and menadione were enrolled in concentration-response experiment. The results demonstrated that the % inhibition direct proportion with the increasing inhibitor concentrations. The calculated IC_{50} values of camptothecin and menadione were 7.31±4.76 μM and 61.7±16.9 μM, respectively. Their IC_{50} values were slightly higher than those of DNA relaxation assay. Because of menadione was a topoisomerase I catalytic inhibitor which did not induce the cleavage complex, the fluorescence ratio assay could not detect the nicked DNA was induced by topoisomerase I in absence or presence of topoisomerase I poison. But it could examine resealing activity of the enzyme.

Time-course study of topoisomerase I was performed by the fluorescence ratio assay. The % conversion was increased with increasing time of incubation. This conversion was expected to reseal nicked DNA by topoisomerase I. This suggested that the assay might be used for kinetic study in resealing mechanism.

The advantages of the fluorescence microplate assay were rapid, minimal step, not require separation step and can handle with a large number of samples. However, this proposed assay was not perfomed completely in 96-well microplate format due to a high concentration of DNA in the enzyme reaction was out of rang of Picogreen DNA determination that indicated by Singer *et al.* (1997). To modify incubation step for reducing time of assay, enzyme reactions were incubated in PCR device which could be adapted to 96-well PCR plate and transferred samples to 96-well fluorescence black microplate by multichannel pipette to manage a large number of samples. In further study, enzyme reaction might be incubated in microplate format by using v-shaped 96-well microplate or microplate that supporting volume of enzyme reaction. However, ratio between dye and DNA concentration should be studied again to obtain optimized value. Even if the proposed assay was discontinuous assay that was inappropriate for enzyme kinetic study, it might be developed to continuous assay by incorporating Picogreen in enzyme reaction mixture. However, the enzyme inhibition of the dye should be tested before.

In conclusion, the fluorescence microplate assay that had been developed was appropriate to use for screening topoisomerase I inhibitors. It might be adapted for

other topoisomerases. However, unknown compounds should be screened for DNA binding properties before use in this method. If those compounds have DNA binding properties, another assay including gel-based assay should be used instead of the fluorescence microplate assay.