이학석사학위논문

Single-Molecule FRET based Screening Methodology for Topoisomerase-Targeting Drugs

단일 분자 FRET 기술에 기반한 Topoisomerase 단백질을 표적으로하는 약품 검사의 방법론

2012 년 8 월

서울대학교 대학원
물리천문학부
허 강

Single-Molecule FRET based Screening Methodology for Topoisomerase-Targeting Drugs

지도교수 홍 성 철

이 논문을 이학석사 학위논문으로 제출함

2012 년 6 월

서울대학교 대학원

물리천문학부

허 강

허 강의 이학석사 학위논문을 인준함.

2012 년 8 월

위 원 장 김 기 훈 (인)

부위원장 홍 성 철 (인)

위 원 김 대 식 (인)

Master's thesis

Single-Molecule FRET based Screening Methodology for Topoisomerase-Targeting Drugs

Kang Heo

***Research Advisor*: Professor Sungchul Hohng**

June 2012

School of Physics and Astronomy
Seoul National University

Abstract

Type II topoisomerases are an important target of anti-cancer and antibacterial drug development, but exact mechanisms of many type II topoisomerase-targeting drugs are not understood well. A fast and accurate way to understand how drug candidates interact with type II topoisomerases is required for efficient drug screening and drug development. In this work, we demonstrated that the unique capability of single-molecule FRET technique to monitor all the key reaction intermediates of the cleavage reaction of type II topoisomerases can be utilized for the screening of the three representative types of drugs: etoposide which inhibit the ligation step, ICRF 187 and I93 which trap the enzyme in the N-gate clamped conformation, and PCA which hinders the binding step of the enzyme. Detailed kinetic analysis further revealed that etoposide binding occurs after the cleavage reaction, and N-gate clamping of the enzyme by ICRF187/193 requires the enzyme-DNA interaction. We expect single-molecule FRET methodology will be a useful screening tool for type II topoisomerase-targeting drugs

Keywords: fluorescence resonance energy transfer, etoposide, ICRF 187/193, Protocatechuic acid, anti-cancer drugs, single-molecule manipulation, Type II topoisomerases

Table of Contents

Single-Molecule FRET based Screening Methodology for Topoisomerase-Targeting Drugs

1. Introduction

Type II DNA topoisomerases play important roles in cellular biology enabling cells to escape from topological problems during replication, repair, and transcription by their ability to cleave a DNA duplex, pass a second DNA duplex through the break, and relegate the cleaved DNA (1). Moreover, type II topoisomerases also have been identified as the molecular targets of anticancer drugs. (2) Some anti-topoisomerase drugs have their primary mode of action, inhibition of enzymatic activity. Other drugs targeting the topoisomerases interfere with the enzyme's cleavage and rejoining activities. Depending on the mode of anti-biotic activity, anti-cancer drugs targeting type II topoisomerase typically have been classified in several groups; type II topoisomerase poison, catalytic inhibitor, non-catalytic inhibitor. (3-5) In general, type II topoisomerases poison prevents the re-ligation of DNA, catalytic inhibitors target the N-terminal ATPase domain of type II topoisomerases from turning over and non-catalytic inhibitors prevent topoisomerases from its catalytic cycle.

Although these types of drugs have long been used for either prescribing cancer patient or biological researches, the detailed mechanisms at molecular level were not understood well. At answering this question, single-molecule fluorescence resonance energy transfer (FRET) method assays (6) have many advantages in drug development by following reasons; it enable us (i) to monitor each molecules in real time at the spatial precision of nanometer scale in a high temporal resolution; (ii) to statistically analyze data obtained from them.

Here, we investigated that how these drugs interact with enzyme in detail by introducing single-molecule FRET assays that monitor the individual reaction intermediates between type II topoisomerase IIα and drugs. We could clearly screen different types of drugs depending on its kinetic features. As drug candidates, we tested three types of drug known to be a most successful in clinical use (Figure 1): etoposide (VP-16) as topoII poison (7,8); dexrazoxane (ICRF 187) as a catalytic inhibitor (9-12); protocatechuic acid (PCA) as a non-catalytic inhibitor (13). Apart from efficiently screening each drugs at single-molecule level, results of this study provide several important biological meanings: (i) etoposide interacts with type II topoisomerases in advance before acting on the DNA; (ii) single-stranded DNA break is sufficient for stabilizing DNA-enzyme cleavage complex; (iii) N-gate clamping of the enzyme by ICRF-187/193 requires the enzyme-DNA interaction; (iv) PCA hinders the binding step of enzyme.

2. Materials and Methods

2.1. Protein purification

Wild-type and mutant human topoisomerase IIα (hTopoIIα) proteins were expressed in Saccharomyces cerevisiae JEL1Δtop1 cells and purified as described previously. (14,15)

2.2. Drug preparation

Etoposide, ICRF187 and, PCA were purchased from commercial industries (Sigma). ICRF-193 was gifted from H. Lee at Seoul National University (figure 1).

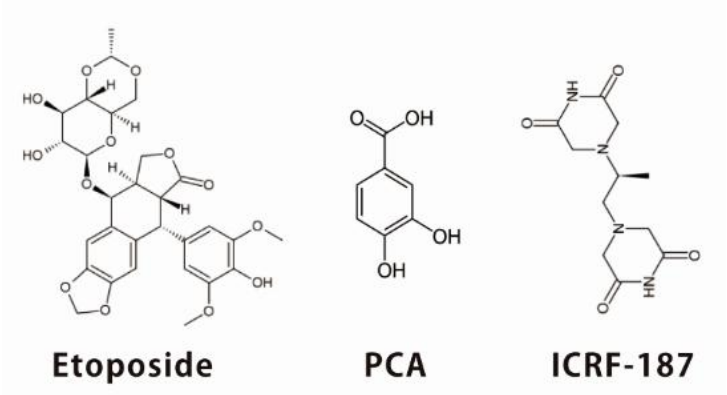
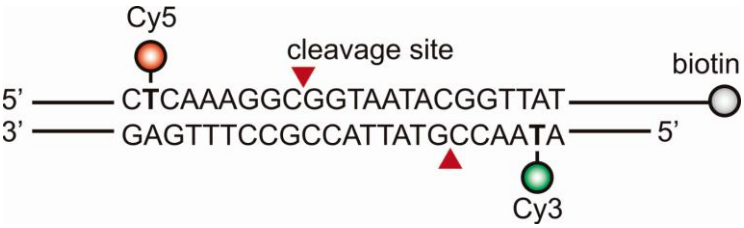


Figure 1. anti-cancer drugs used in our research

2.3. DNA preparation

HPLC-purified DNA strands (written from 5’ to 3’) were purchased from from Integrated DNA Technologies (Coralville, IA) except the phosphorothiolate modified DNA strand, which was purchased from Purimex (Grebenstein, Germany) DNA oligonucleotides were labelled at the amine group of an internal amino modifier (dTC6) with either Cy3 or Cy5. DNA duplexes were annealed by slowly cooling the mixture of the biotinylated strand and non-biotinylated strand in 2:3 molar ratio at 10 μM concentration in a buffer containing 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl.



2.4. Single-molecule FRET experiment

To prevent nonspecific adsorption of enzyme, quartz microscope slides and coverslips were cleaned, coated with polyethylene glycol (m-PEG-5000; Laysan Bio Inc.), and biotinylated PEG (biotin-PEG-5000; Laysan Bio, Inc.) with 40:1 ratio. DNA duplexes were immobilized on the PEG-coated surface via a streptavidin-biotin interaction (16). Single-molecule fluorescence images were taken in a home-made prism-type total-internal-reflection fluorescence microscope with 50-ms or 1-s time resolution. All measurements were

performed at room temperature with the following buffer composition unless mentioned otherwise: 10mM Tris-HCl (pH 8.0), 135 mM KCl, 5 mM $MgCl_2$, 20 nM enzyme and an oxygen scavenger system (0.4 % (w/v) glucose (Sigma), 1 mM Trolox (Sigma), 1 mg/ml glucose oxidase (Sigma), 0.04 mg/ml catalase (Roche)) to slow photo-bleaching (17). Enzyme was diluted 100–fold for the 20 nM experiments. Therefore, the imaging buffer also contains as much diluted components of the enzyme storage buffer (50 mM Tris (pH 7.7), 5 mM DTT, 1 mM NaEDTA, 750 mM KCl, 40 % (v/v) glycerol). As an excitation source, a green laser (532-nm, Compass215M, Coherent) was used. Fluorescence signals from Cy3 and Cy5 were collected by a water immersion objective (UPlanSApo 60x; Olympus), filtered through 532-nm long-pass filter (LP03-532RU-25; Semrock), separated with a dichroic mirror (635dcxr; Chroma Technology), and imaged on an EM-CCD camera (Ixon DV897, Andor). In buffer exchange experiments, a new buffer was infused in real time into the detection chamber by using a syringe pump (PHD 22/2000; Harvard Apparatus) while single-molecule images were being taken. For calculation of FRET efficiency, which was defined as the ratio of acceptor intensity to the sum of donor and acceptor intensities, background subtraction, and bleed-through correction of the donor signal to the acceptor channel were done, but gamma correction was not performed.

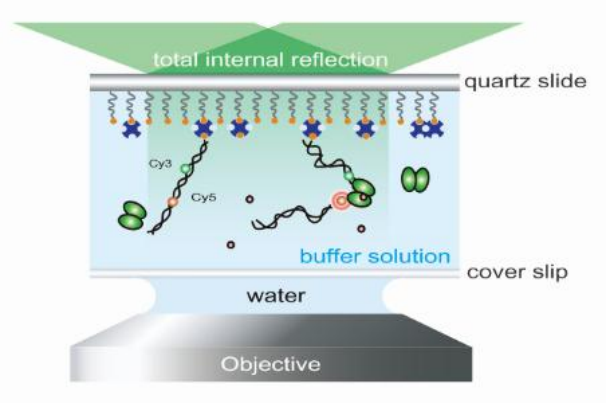


Figure 2. Single-molecule experimental scheme. DNA duplexes were immobilized on the PEG-coated surface via a streptavidin-biotin interaction and single-molecule fluorescence images were taken in a total-internal-reflection microscope.

2.5. Determination of kinetic rates

Association/dissociation and bending/straightening events were determined by the combination of threshold method and eye inspection of fluorescence intensity and FRET time traces, respectively. Dwell time histograms of each state thus obtained were fit by an exponential decay function to obtain corresponding kinetic rates. Since DNA duplexes have two competing reaction pathways of bending and dissociation in the presence of Mg^{2+} ions, the duration time histograms of enzyme-bound state without bending give the sum of bending and dissociation rates, which is multiplied by the relative frequency of each event to obtain individual rates of bending and dissociation rates (18)

3. Results and Discussions

3.1. Basic Mechanism of Type II Human Topoisomerase (hToIIα)

To characterized the actions of several drugs, we developed single-molecule FRET (Fluorescence Resonance Energy Transfer) assays that monitor individual steps in the interaction between human topoisomerase IIα (hTopoIIα) and the DNA segment.

In the first step, we confirmed the kinetics of the hTopIIα which are well-defined from the previous study (19). We used Cy3 and Cy5 as fluorescent dye molecules which are positioned at 8bp apart from the cleavage site of the DNA duplex. The duplex has a biotinylated single-stranded overhang to avoid any possible steric hindrance caused by surface immobilization. Through delivering hTopoIIα into the detection chamber after immobilizing DNA molecules on a polymer coated quartz slide, DNA molecules were imaged by a total-internal reflection fluorescence microscope (TIRFM). By monitoring the FRET signals, we could characterize the each catalytic cycle; binding and bending states. In addition, we could also observe the trap events during the interactions with the etoposide as same as previously reported study (Figure 3).

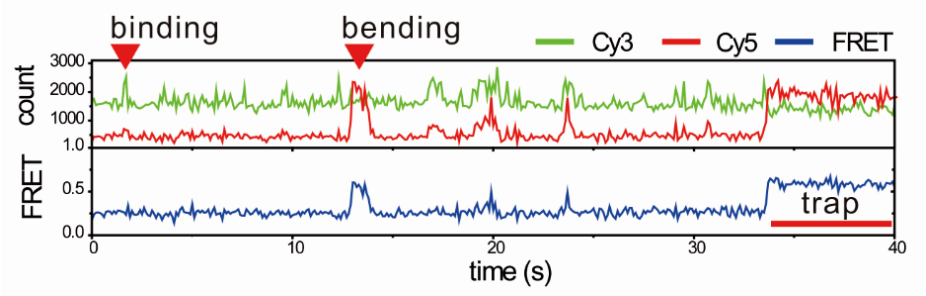


Figure 3. Representative fluorescence signals and FRET efficiency in which binding, bending and trap events are clearly shown.

3.2. When and how does the Etoposide works.

As a topoisomerase II poison, we employed etoposide which is highly successful anticancer drug that has been in clinical use for nearly decades and has been well known to kill cells by increasing levels of topoisomerase II-mediated DNA breaks. Etopoisde stabilizes topoisomerase II-associated double-stranded DNA breaks by inhibiting the ability of the enzyme to ligate cleaved DNA molecules (7,8). The understanding of precise mechanism how it does so, however, was still poor.

With the knowledge that topoisomerase poison stabilizes the cleavage complex, we took time lapse movies after injection of the etoposide in a saturated condition (500uM). By investigating histograms of each movie and fitting with exponential decay function, we could calculate the time constants revealing association time (Figure 4a). In the presence of etoposide, it took

about 8min to associate with the duplex (Figure 4b). Then, after washing flow chamber with buffer without hTopIIα, we analyzed dissociation time in the same manner and it revealed that etoposide requires about 9-fold more time to dissociate. These results explain why etoposide have been used for long time in treating cancer cells because it works very fast and goes a long way until it dissociates.

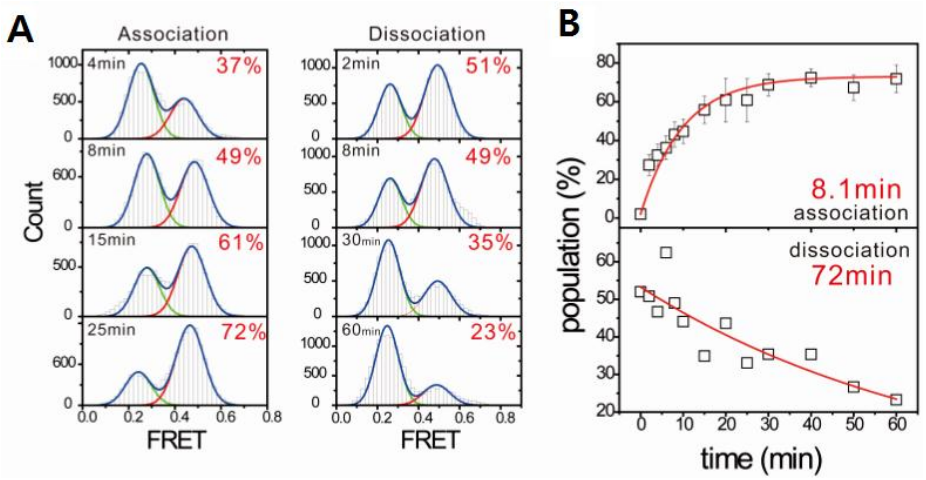


Figure 4. . (a) In addition of etoposide, high FRET population got growing fast indicating that trapped molecules are accumulated as time goes on. After washing free enzymes and etoposide the high FRET population is decreased very slowly. (b) In each reaction, time constants are 8.1min and 73min relatively.

In addition, Comparing kinetics between before and after the etoposide reaction, we could answer the argument with whom the etoposide interact first. Since topoisomerase II-targeting agents interact with both enzyme and DNA,

there are three potential ways to forming the ternary complex. Route 1: drugs may bind specifically to the topoisomerase II-DNA binary complex and have minimal independent interactions with either DNA or the enzyme alone. Route 2: drugs may form the ternary complex primarily through prior interactions with DNA. Route 3: drugs may form the ternary complex primarily through prior interactions with topoisomerase II (figure 5) (20).

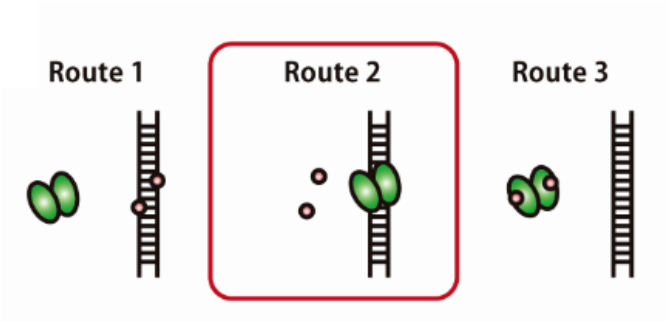


Figure 5. Three possible ways of forming the ternary complex

However, despite the importance of drug-enzyme interactions in the formation of the ternary complex, little has been known about the drug interaction on topoisomerase II. Assuming that etoposide interacts with cleavable site of the duplex before hTopIIα binds to DNA, kinetics measured from the data without etoposide should be significantly different from the kinetics measured in the presence of etoposide. By calculating kinetic parameters which is intimately related with hTopIIα, we couldn't observe any considerable differences (Figure 6). Thus, we conclude that etoposide interacts with enzyme first and then, binds to the duplex.

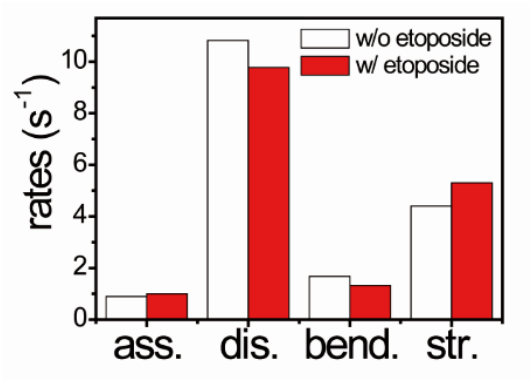


Figure 6. Comparative kinetics analysis of type II human topoisomerase with (red) and without 500 uM of etoposide (white).

Moreover, we addressed whether cleavage at one strand is sufficient for etoposide to stabilize cleavage complex or not. To prove this, we first prepared a DNA duplex whose 3'-bridging oxygen at one of the scissile bonds was replaced with a sulfur atom (Figure 7). The sulfur atom at the cleavage site stabilizes the cleavage complex by blocking the ligation process. Thus, we expected that irreversable trapping events were also observed even in the absence of hTopIIα. Figure 7 shows comparative trapping rates in different samples. In the presence of etoposide with hTopIIα in clv, trapping rate was $0.13s^{-1}$. And in the absence of etoposide with hTopIIα in clv-S, trapping rate was significantly decreased to about half $0.07\,s^{-1}$. These results look plausible because the former, re-ligation on both of each strand might be disturbed by etoposide but not in the latter, only the sulfur modified strand was inhibited to re-ligate. Next, we performed same experiment in the

presence of etoposide in clv-S. In this case, trapping rate return to the value $0.13s^{-1}$ same with the clv experiment. In the case of clv-S without enzyme, the trapping rate indicates the time required for cleavage at one strand. So, if two drug molecules are required to stabilize the cleavage complex, trapping rate obtained from the clv-S in the presence of etoposide should be observed below to the $0.07s^{-1}$ supporting that cleavage at one strand is sufficient for etoposide to stabilize cleavage complex.

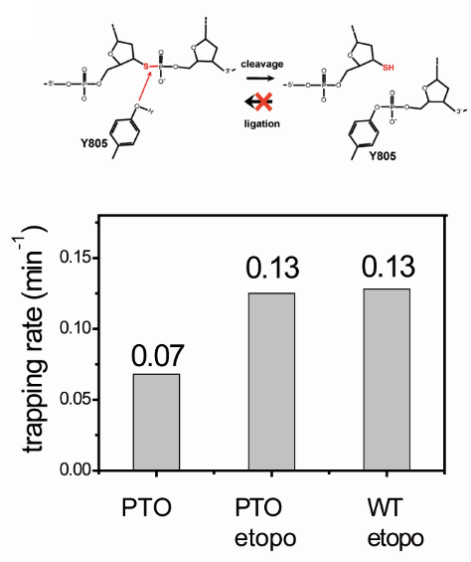


Figure 7. In the absence of etoposide, sulfur-modified sample (PTO) shows lower trapping rates than the others. However, in addition of etoposide trapping rates are recoved at the level of unmodified sample.

In addition to the kinetic analysis during the catalytic cycle, by flowing denaturing buffer into the reaction chamber, we could investigate the ratio between double stranded breaks and single stranded breaks. To this end,

etoposide was pre-incubated in the reaction chamber for 40min enough to saturate the level of hTopIIα-mediated DNA breaks. 50 second after getting start to take movie, we flew denaturing buffer into the chamber (Figure 8). Right after the injection, we could observe that fluorescent signals were abruptly changed indicating that denatured enzyme was released from bent DNA duplex. Some of the duplex were cleaved in double stranded, and the others were single stranded. And in former case, only biotinylated part of duplex lebeled with Cy3 at one strand only remain immobilized and the other duplex lebeled with Cy5 diffuse away in the chamber. So, the existence of Cy5 signal in each trace could be an evidence for determining whether it's double or single stranded break. Throughout the procedure, we could show that single and double stranded break were about 56%, 44% relatively (Figure 2E). It is similar result reported before. However, we could quantify the exact number of molecules in each state at the single molecule level and provide more accurate way to characterizing molecular behavior than biochemical researches.

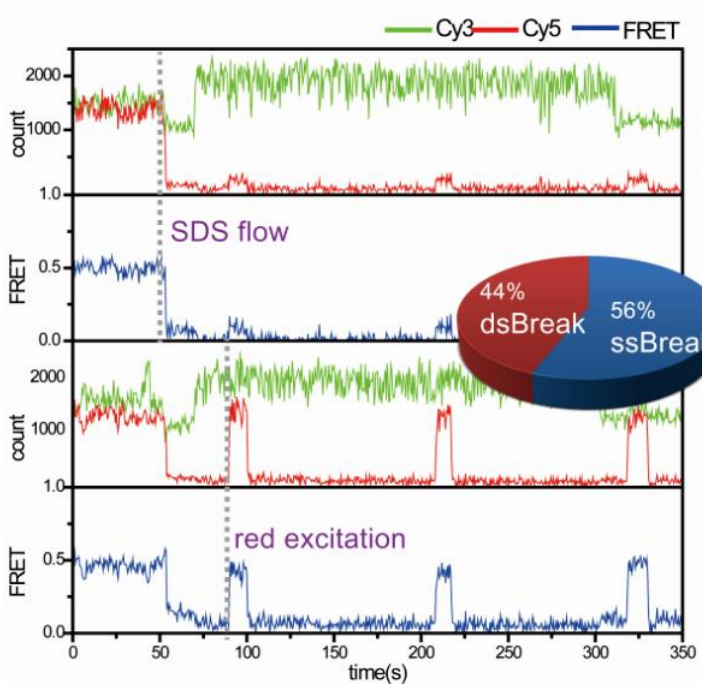


Figure 8. Flowing SDS(sodium dodecyl sulfate) to characterizing of the cleavage products. Both single strand and double strand cleavage products are observed.

3.3. Protocatechuic acid(PCA) inhibit the binding step during the catalytic cycle

PCA is a simple phenolic compound widely distributed in nature. It has known to have mixed effects on normal and cancer cells in *in vitro* and *in vivo* studies and chemopreventive activity as well (13,21,22). Recent years, PCA also widely used as an anti-oxidant agent in biochemical studies (23). During the catalytic cycle, however, the detailed step in which PCA was implicated has not been reported.

So, we characterized the effect of PCA on the hTopIIα catalytic cycle in different range of concentration. In each experiment, we increased the concentration of drug from 3mM to 7mM. Then, we could observe the significant decrease in the binding events in proportion to the concentration indicating that hTopIIα was inhibited to bind to the DNA by PCA (Figure 9a). This result clearly shows on which catalytic cycle PCA hinders the hTopIIα.

Furthermore, to demonstrate whether PCA only affect the binding step rather than the bending step, we also analyzed kinetic parameters before and after the reaction of PCA in different concentrations. In the presence of 7mM PCA, association rate were decreased more about 2 fold over the absence of PCA. In contrast to the association rates, the other parameters related to the bending reaction didn't show any significant changes (Figure 9b). From this, we could confirm that PCA only affects the biding step during the hTopIIα catalytic cycle. Similar results were observed from the hTopIIβ, which differ from hTopIIα in their subcellular localization, biochemical properties and susceptibility to inhibition by anti-cancer drugs (24,25), indicating that the binding inhibition is general process in which PCA affects Topoisomerases.

PCA have long been used for treating cancer cells, but the details on reaction cycle had not been well known. In this work, by investigating kinetics of PCA on hTopIIα, we could first determine that PCA only hinder the binding step during the type II topoisomerase reaction cycle.

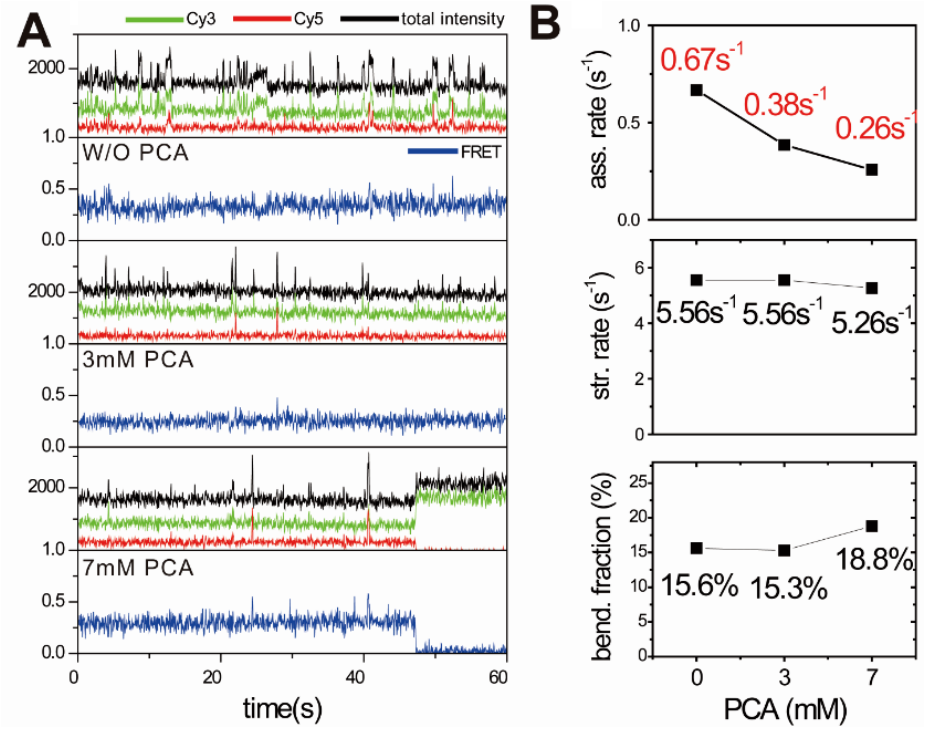


Figure 9. (A) Representative traces at different concentration of protocatechuic acid (PCA). As the concentration get higher, the association events were decreased significantly. (B) In contrast to the association rate, once they are bound to DNA straightening/bending rates weren't affected by the PCA.

3.4. N-gate clamping of the enzyme by ICRF-187/193 requires the enzyme-DNA interaction

As discussed previously, chemotherapeutic drugs such as etoposide exert part of their action by inhibiting hTopII-mediated resealing of DNA strand breaks;

other drugs, such as protocatechuic acid(PCA), inhibit the binding of hTopII to its DNA substrate. In contrast, the inhibitory effect of bis-dioxopiperazines on the catalytic activity of type II topoisomerase is due to inappropriate binding and stabilization of the protein/DNA complex (11). Bis-dioxopiperazines are anticancer agents that associate with ATP-bound topoisomerase and convert the enzyme into an inactive, salt-stable clamp around DNA by stabilizing closed N-gate region of type II topoisomerase (26) and also can be considered as a prodrug analog of EDTA that is activated upon hydrolysis to its one-ring open intermediates, and then to its fully rings-opened form ADR-925 (10,27). Although there're plenty of studies about biochemical effects and clinical uses of bis-dioxopiperazines and its analogues, the understanding of detailed kinetic analysis is still poor in molecular level.

Right after adding of 100 uM ICRF-187, kinetic rates was not so much different from that of without the ICRF-187 (Figure 10).

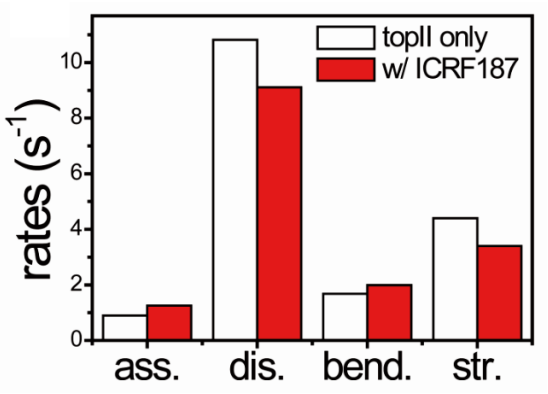


Figure 10. Comparative kinetics analysis of type II human topoisomerase with (red) and without 100 uM of ICRF-187 (white).

However after some time later after the addition of ICRF-187, we observed that hTopIIα stayed longer time in the bended state and the level of the high FRET states are accumulated (Figure 11).

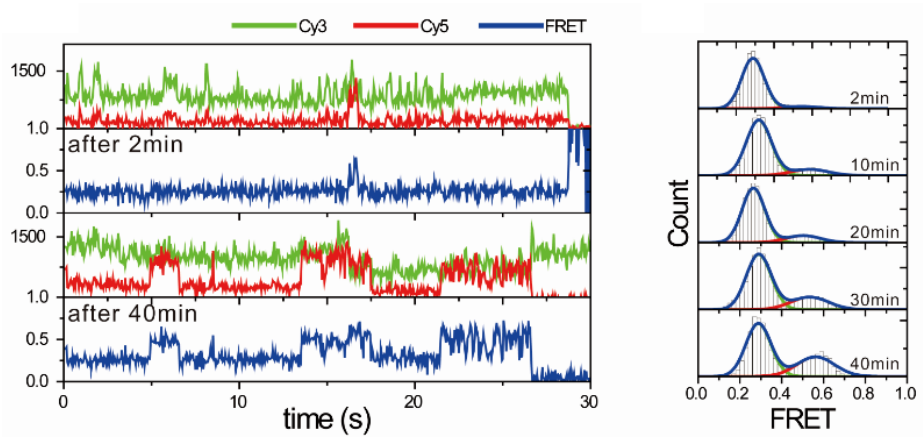


Figure 11. Representative time traces in addition of 100 uM of ICRF-187 and (B) corresponding FRET histogram

The time constant calculated from time-dependent histogram (Figure 4B) is about 48min more slow than etoposide. This is very interesting in the way that although we have known that ICRFs stabilize the protein/DNA complex, the fact that bending state is crucial for its effect had been unknown. Additionally, ICRF-193 which have been known to be the most potent of the analogs (28) showed similar but much fast time constant to reach to the

equilibrium as expected (Figure 12).

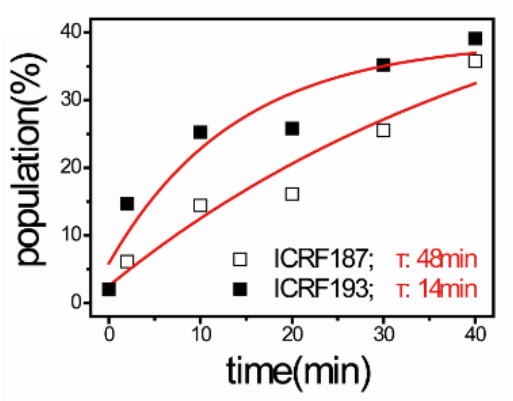


Figure 12. Time constants of ICRF-187/193 calcultated from FRET histogram.

Then, does the time constants obtained from ICRFs mean that they requires that time to stabilize the dimer interface between two ATPase protomers? To address this question, we assumed that loner bending states should be observed right after the injection of ICRF, if it were incubated with hTopIIα for sufficient time in the presence of ATP. Thus, we pre-incubated 100 uM ICRF-187 for 40min in the imaging buffer containing 1 mM ATP and then, take time lapse movie as same procedure as before. Interestingly, we couldn't any significant difference in the time dependent behavior (data not shown). This is the indirect evidence that at least DNA substrates should be required for stabilizing clamping N-gate region and in other words, ICRF-187 requires the enzyme-DNA interaction.

Next, we characterized dissociation properties of ICRF-mediated enzyme/DNA complex by exchanging buffer. Figure 13 shows representative images and corresponding histograms of a buffer exchange experiment. 40min after the addition of 100 uM ICRF, we exchage buffer in the chamber for the new buffer without ICRF. In contrast to the case of etoposide, hTopIIα dissociated immediately revealing that the stability of enzyme/DNA complex mediated by ICRF is not so tight. It indicate that ICRFs doesn't interact with DNA substrates directly as expected from considering X-ray crystal structure (26).

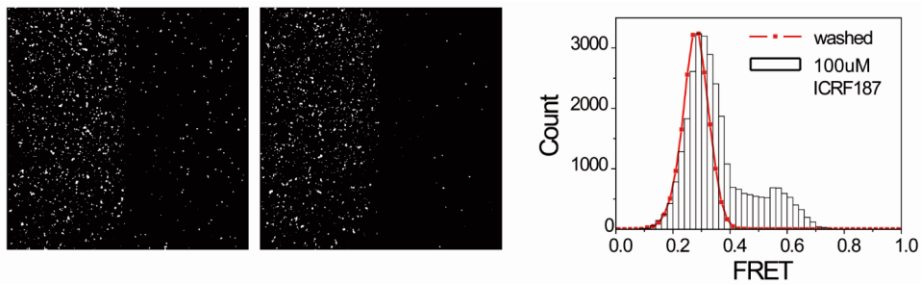


Figure 13. Dissociation of 100 uM ICRF-187 (A) representative images before (left) and after (right) washing drug, and (B) corresponding normalized FRET histograms indicates that the stability of enzyme/DNA complex mediated by ICRF is not so tight.

4. Conclusion and Outlook

Single-molecule FRET techniques have several key benefits for drug screening and also would provide very useful information on drug development over conventional in vitro methods as they have the unique ability to monitoring conformational changes in each molecule in high temporal and special resolution. Despite its powerful capabilities on drug discoveries, most of researches in which single molecule FRET technique used tend to focus on dynamics of proteins with DNA/RNA.

Here, we investigated several anti-cancer drug candidates targeting hTopIIα such as etoposide, ICRF's and PCA possessing unique capabilities on inhibiting catalytic cycles of hTopIIα each other. By using single molecule FRET technique, it's possible to provide an overall mechanism of such drugs acting on hTopIIα; not only a strong evidence for terminating argument about the model of etoposide action but also general mechanisms on which each drugs are acting during the catalytic cycle. Furthermore, for decades, the combination effects of such drugs have been receiving remarkable interest for prescribing patient (29). So, understanding how these drugs are acting on enzyme each other would enable researchers to study the mixed effects for chemotherapy more deeply. For instance, when combined with ICRF-187, we observed that etoposide show stronger effects on cleavage reaction rather than

working alone (data not shown).

Recently, there have been a few attempts to apply single molecule techniques including smFRET, optical tweezers to a drug screening in vivo and in vitro (30-32). The pharmaceutical trends are growing around the development of anti-cancer drugs, however, researches adopting single molecule technique for characterizing its effects are not enough. In this point of view, we expect that single molecule FRET technique, which is specialized for monitoring the behavior of individual molecule, will be used for drug discovery and thus, expand our knowledge of chemotherapy rather than before.

REFERENCES

1. Wang, J.C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol*, **3**, 430-440.
2. Kathiravan, M.K., Khilare, M.M., Nikoomanesh, K., Chothe, A.S. and Jain, K.S. Topoisomerase as target for antibacterial and anticancer drug discovery. *J Enzyme Inhib Med Chem*.
3. Martincic, D. and Hande, K.R. (2005) Topoisomerase II inhibitors. *Cancer Chemother Biol Response Modif*, **22**, 101-121.
4. Nitiss, J.L. (2009) Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer*, **9**, 338-350.
5. Fortune, J.M. and Osheroff, N. (2000) Topoisomerase II as a target for anticancer drugs: when enzymes stop being nice. *Prog Nucleic Acid Res Mol Biol*, **64**, 221-253.
6. Ha, T., Enderle, T., Ogletree, D.F., Chemla, D.S., Selvin, P.R. and Weiss, S. (1996) Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. *Proc Natl Acad Sci U S A*, **93**, 6264-6268.
7. Yang, J., Bogni, A., Schuetz, E.G., Ratain, M., Dolan, M.E., McLeod, H., Gong, L., Thorn, C., Relling, M.V., Klein, T.E. *et al.* (2009) Etoposide pathway. *Pharmacogenet Genomics*, **19**,

552-553.

8. Wu, C.C., Li, T.K., Farh, L., Lin, L.Y., Lin, T.S., Yu, Y.J., Yen, T.J., Chiang, C.W. and Chan, N.L. Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. *Science*, **333**, 459-462.

9. Hasinoff, B.B. (1989) The interaction of the cardioprotective agent ICRF-187 [+)-1,2-bis(3,5-dioxopiperazinyl-1-yL)propane); its hydrolysis product (ICRF-198); and other chelating agents with the Fe(III) and Cu(II) complexes of adriamycin. *Agents Actions*, **26**, 378-385.

10. Schroeder, P.E., Davidson, J.N. and Hasinoff, B.B. (2002) Dihydroorotase catalyzes the ring opening of the hydrolysis intermediates of the cardioprotective drug dexrazoxane (ICRF-187). *Drug Metab Dispos*, **30**, 1431-1435.

11. Weiss, G., Loyevsky, M. and Gordeuk, V.R. (1999) Dexrazoxane (ICRF-187). *Gen Pharmacol*, **32**, 155-158.

12. Hasinoff, B.B., Abram, M.E., Barnabe, N., Khelifa, T., Allan, W.P. and Yalowich, J.C. (2001) The catalytic DNA topoisomerase II inhibitor dexrazoxane (ICRF-187) induces differentiation and apoptosis in human leukemia K562 cells. *Mol Pharmacol*, **59**, 453-461.

13. Tanaka, T., Tanaka, T. and Tanaka, M. Potential Cancer

Chemopreventive Activity of Protocatechuic Acid. *Journal of Experimental & Clinical Medicine*, **3**, 27-33.

14. Kingma, P.S., Greider, C.A. and Osheroff, N. (1997) Spontaneous DNA lesions poison human topoisomerase IIalpha and stimulate cleavage proximal to leukemic 11q23 chromosomal breakpoints. *Biochemistry*, **36**, 5934-5939.

15. Worland, S.T. and Wang, J.C. (1989) Inducible overexpression, purification, and active site mapping of DNA topoisomerase II from the yeast Saccharomyces cerevisiae. *J Biol Chem*, **264**, 4412-4416.

16. Roy, R., Hohng, S. and Ha, T. (2008) A practical guide to single-molecule FRET. *Nat Methods*, **5**, 507-516.

17. Rasnik, I., McKinney, S.A. and Ha, T. (2006) Nonblinking and long-lasting single-molecule fluorescence imaging. *Nat Methods*, **3**, 891-893.

18. Nahas, M.K., Wilson, T.J., Hohng, S., Jarvie, K., Lilley, D.M. and Ha, T. (2004) Observation of internal cleavage and ligation reactions of a ribozyme. *Nat Struct Mol Biol*, **11**, 1107-1113.

19. Lee, S., Jung, S.R., Heo, K., Byl, J.A., Deweese, J.E., Osheroff, N. and Hohng, S. DNA cleavage and opening reactions of human topoisomerase IIalpha are regulated via

Mg2+-mediated dynamic bending of gate-DNA. *Proc Natl Acad Sci U S A*, **109**, 2925-2930.

20. Lock, R.B. and Ross, W.E. (1987) DNA topoisomerases in cancer therapy. *Anticancer Drug Des*, **2**, 151-164.

21. Lin, H.H., Chen, J.H., Huang, C.C. and Wang, C.J. (2007) Apoptotic effect of 3,4-dihydroxybenzoic acid on human gastric carcinoma cells involving JNK/p38 MAPK signaling activation. *Int J Cancer*, **120**, 2306-2316.

22. Stagos, D., Kazantzoglou, G., Magiatis, P., Mitaku, S., Anagnostopoulos, K. and Kouretas, D. (2005) Effects of plant phenolics and grape extracts from Greek varieties of Vitis vinifera on Mitomycin C and topoisomerase I-induced nicking of DNA. *Int J Mol Med*, **15**, 1013-1022.

23. Aitken, C.E., Marshall, R.A. and Puglisi, J.D. (2008) An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments. *Biophys J*, **94**, 1826-1835.

24. Drake, F.H., Hofmann, G.A., Bartus, H.F., Mattern, M.R., Crooke, S.T. and Mirabelli, C.K. (1989) Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry*, **28**, 8154-8160.

25. Sandri, M.I., Hochhauser, D., Ayton, P., Camplejohn, R.C.,

Whitehouse, R., Turley, H., Gatter, K., Hickson, I.D. and Harris, A.L. (1996) Differential expression of the topoisomerase II alpha and beta genes in human breast cancers. *Br J Cancer*, **73**, 1518-1524.

26. Classen, S., Olland, S. and Berger, J.M. (2003) Structure of the topoisomerase II ATPase region and its mechanism of inhibition by the chemotherapeutic agent ICRF-187. *Proc Natl Acad Sci U S A*, **100**, 10629-10634.

27. Schroeder, P.E. and Hasinoff, B.B. (2005) Metabolism of the one-ring open metabolites of the cardioprotective drug dexrazoxane to its active metal-chelating form in the rat. *Drug Metab Dispos*, **33**, 1367-1372.

28. Huang, K.C., Gao, H., Yamasaki, E.F., Grabowski, D.R., Liu, S., Shen, L.L., Chan, K.K., Ganapathi, R. and Snapka, R.M. (2001) Topoisomerase II poisoning by ICRF-193. *J Biol Chem*, **276**, 44488-44494.

29. Holm, B., Jensen, P.B. and Sehested, M. (1996) ICRF-187 rescue in etoposide treatment in vivo. A model targeting high-dose topoisomerase II poisons to CNS tumors. *Cancer Chemother Pharmacol*, **38**, 203-209.

30. Tian, H., Ip, L., Luo, H., Chang, D.C. and Luo, K.Q. (2007) A high throughput drug screen based on fluorescence resonance

energy transfer (FRET) for anticancer activity of compounds from herbal medicine. *Br J Pharmacol*, **150**, 321-334.

31. Hintersteiner, M. and Auer, M. (2008) Single-bead, single-molecule, single-cell fluorescence: technologies for drug screening and target validation. *Ann N Y Acad Sci*, **1130**, 1-11.

32. Skinner, G.M. and Visscher, K. (2004) Single-molecule techniques for drug discovery. *Assay Drug Dev Technol*, **2**, 397-405.

국문 초록

Type II DNA 회전효소는 항암 및 항균 약품 개발에 있어서 중요한 표적이 된다. 하지만 Type II DNA 회전효소를 표적으로 하는 약품에 대한 정확한 기작은 아직 이해가 부족한 실정이다. 효과적으로 약품을 검사하고 새로운 약품을 개발에 있어서 Type II DNA 회전효소가 어떻게 이러한 약품들과 상호작용하는지 이해하기 위해서는 빠르고 정확한 방법론이 요구되고 있다. 본 연구를 통하여, 우리는 Type II DNA 회전효소와 관련한 절단 작용의 핵심적 과정을 추적하는 단일 분자 FRET 기술의 고유한 특성이 Type II DNA 회전효소를 표적으로하는 대표적인 세 가지의 항암제의 특성을 검사하는데 효과적으로 이용될 수 있음을 검증하였다. 그 중 첫 번째 항암제는 DNA의 재접합 과정을 방해하는 etoposide이며, 둘 째는 Type II DNA 회전효소의 N-gate를 닫힌 상태로 고정 시킴으로써 작용하는 ICRF-187 및 193, 마지막으로 효소가 DNA로부터 결속되는 것을 방해하는 PCA가 있다. 구체적인 동력학적 분석을 통하여 우리는 etoposide가 DNA의 절단 이후에 바로 작용할 수 있다는 점과 ICRF-187 및 193에 의해 Type II DNA 회전효소의 N-gate가 닫힘 고정되는 현상이 DNA와 효소의 우선적 작용을 전제한다는 점을 밝혀 내었다. 이러한 연구를 통하여 우리는 단일 분자 FRET 기술이 Type II DNA 회전 효소 및 다른 여러 가지 효소를 표적으로 삼는 항암제들을 연구하는데 유용한 기술로 자리잡기를 기대한다.

핵심단어: 형광공명 에너지 전달, etoposide, ICRF 187/193, protocatechuic acid, 항암제, 단일 분자 제어, Type II DNA 회전 효소