Study on catalytic properties of flap structure : specific endonuclease 1 and establishment of new platforms for screening its inhibitors

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**STUDY ON CATALYTIC PROPERTIES OF FLAP
STRUCTURE - SPECIFIC ENDONUCLEASE 1 AND
ESTABLISHMENT OF NEW PLATFORMS FOR
SCREENING ITS INHIBITORS**

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Abstract

Flap structure-specific endonuclease 1 (FEN1) is known to be essential for reproduction and development of eukaryotic cells. During DNA replication in cell division, a 5' flap structure is generated by polymerase δ . FEN1 subsequently emerges, specifically recognizes the produced flap structure, and further cleaves the phosphodiester bond. However, recent studies reveal few systematic results between catalytic property of FEN1 and different structures of DNA substrates that could emerge during replication. Here, we report the correlations between DNA-cleaving properties of FEN1 and oligonucleotide substrates with various length of 5' flap or double strands. Substrate possessed 5' flap composed of 3 nucleotides and double strands composed of 16 nucleotides demonstrated high activity towards FEN1 catalysis. Since the enzyme FEN1 plays vital role in over-expression of DNA in cancer cells, FEN1 might be a sound target in certain anticancer treatment. Therefore we investigated effect of several small molecules on FEN1 catalysis, including chloroquine, ethidium bromide, netropsin, spermidine and cisplatin as well as inhibitory effect of phosphorothioate modified oligonucleotide substrates. Phosphorothioate bond was designed and modified at or adjacent to FEN1 cleavage site, enzyme kinetics of FEN1 using phosphorothioate-substituted oligonucleotides with modification at different positions was explored. The result showed that

phosphorothioate modification at FEN1 cleavage site inhibited catalysis most efficiently. Based on activity study and inhibitory study, FEN1 oligonucleotide inhibitor was designed and its inhibitory effect was investigated.

In addition, we have established two simple and efficient fluorescence platforms for screening FEN1 inhibitors *in vitro*. The two fluorescence platforms developed were Cy3-(BHQ-2) donor-quencher fluorogenic platform as well as 2-aminopurine (2-AP) platform. The efficiency of FEN1 inhibitors could be measured by decrease of fluorescence emitted by Cy3 or 2-aminopurine. The Cy3-(BHQ-2) was more efficient than 2-AP platform; however 2-AP system was still significant to be utilized as validation by a different signal output for newly-discovered FEN1 inhibitors. Three organic inhibitors including PTPD, NSC-13755, aurintricarboxylic acid and our newly designed oligonucleotide inhibitor had been tested by fluorescence platforms and demonstrated a strong concentration-dependent inhibition. By using Cy3-(BHQ-2) fluorescence platform and 2-aminopurine fluorescence platform, IC₅₀ value of organic inhibitors and oligonucleotide inhibitor was tested. Both platforms have demonstrated great potential to be further developed for screening FEN1 inhibitors.

Besides, FEN1 activity using triplex DNA, RNA modified substrates at 5' flap structure were also explored. Flap-structured plasmid DNA was designed and synthesized for future study on FEN1.

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List of Abbreviations

DNA	Deoxyribonucleic acid
FEN1	Flap structure-specific endonuclease 1

Tris	Tris(hydroxymethyl)aminomethane
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
EB	Ethidium bromide
nts	Nucleotides
dNTP	Deoxynucleotide triphosphate
BSA	Bovine serum albumin
FRET	Förster resonance energy transfer
UV	Ultraviolet
T _m	Melting temperature
PTPD	3-Hydroxy-5-methyl-1-phenylthieno[2,3-d]pyrimidine-2, 4(1H,3H)-dione
ATA	Aurintricarboxylic acid
2-AP	2-Aminopurine
pmol	Picomole
°C	Degree Celsius
min	Minute
h	Hour

Chapter 1 - Introduction

1.1 Function of FEN1 in DNA replication

Flap structure-specific endonuclease 1 (FEN1) is over-expressed in various types of cancer cells (e.g. testis, lung, breast and brain cancer cells), and has been considered in the very recent years as a cancer biomarker and anti-cancer drug target ^[1]. As one of the most ancient proteins, this nuclease is known to be essential for reproduction and development of eukaryotic cells ^[2]. During DNA replication in cell division, for example, syntheses of Okazaki fragments begin discretely on the lagging template strand of DNA through the action of DNA polymerases ^[3]. Unlike prokaryotic cells in which polymerase I alone makes the 5' and 3' ends of two Okazaki fragments juxtaposed towards each other without mutual strand invasion, eukaryotic cells let their polymerase δ elongate one Okazaki fragment continuously until a 5' flap structure is generated on the other (strand displacement, Scheme 1.1) ^[4]. FEN1 subsequently emerges, specifically recognizes the produced flap structure, and further cleaves the phosphodiester bond at which the single-stranded flap starts to allow the further action by DNA ligase I to take place ^[5]. Besides taking part in the abovementioned Okazaki fragment maturation during eukaryotic DNA replication, FEN1 is involved in long-patch base excision repair, DNA recombination, DNA double-strand break repair, stalled replication fork restart as well as preservation of telomere stability and thus a key node in maintaining genomic stability, in which it serves

as either endonuclease or exonucleases ^[6]. Experimental data that have accumulated thus far indicate that FEN1 plays vital roles in the growth of eukaryotic cells.

Scheme1.1 Illustration of function of flap structure-specific endonuclease 1 (FEN1) in replication of DNA in eukaryotic cells.

It has been demonstrated in the past that FEN1 is able to bind and excise a wide variety of DNA conformations such as 5' flap structures, pseudo-Y structures, duplex structures with nicks and gaps, D-loops and bubble structures ^[1]. Kinetic studies demonstrated, however, that FEN1 bind to and cleave 5' flap DNA structures far more effective than any other types of structural entities ^[6]. This observation could imply that removals of 5' flap structure during replications and DNA repairs are the foremost inborn roles of FEN1 in the eukaryotic cells. We therefore focused mainly on the studies of the 5'

flap-structured DNA as FEN1 substrates rather than other types of DNA structures.

Based on crystal structure of FEN1, researchers have found that action of FEN1 involves substantial conformation change and binds 3' and 5' DNA flaps which enforces structure-specific binding. FEN1 binds about 16 base pairs of double-stranded DNA substrates plus nucleotides at 3' and 5' flap. FEN1 recognizes 5' flap of substrate by its ability to form a sharp 100° bend with double-stranded DNA on either side ^[7].

1.2 Study on property of FEN1

1.2.1 Fundamental study of property of FEN1

There are about three billion base pairs in the DNA molecules in the human genomes while half of them are synthesized on the lagging template strands through the abovementioned flap structure-generating pathway (Scheme 1.1). It has been estimated that during each replication, FEN1 molecules carry out their actions around 50 million times for removing flap structures on the produced lagging strands ^[8]. Because the DNA sequence for forming alternative DNA structures (e.g. (i) unordinary Watson-Crick base pairings as exemplified, RNA modified oligonucleotides; and (ii) non-B DNA conformations) are known to be widespread in the three billion base-paired human genomes ^[9], we would speculate that a wide variety of alternative DNA structures could occur at the

single stranded flap segments as well as in the areas adjacent to the flap structures. However, only a very limited number of variety of DNA structures on the DNA-cleaving activity of FEN1 were examined in the past ^[10] while effect of different size or length and types of non-B DNA structures as well as unordinary Watson-Crick base pairings on the catalytic activity of FEN1 have not yet been thoroughly investigated thus far. We systematically examine the response of FEN1 to varieties size or length of substrates. Non-B DNA structures and unordinary Watson-Crick base pairings that likely emerge at and near the flap sites have also been tested. Our objectives in designing these studies are to find out (1) whether alternative DNA structures formed at and near the flap sites could influence the DNA cleaving action of FEN1 and (2) to what extents the influence goes if such effects indeed exist. It is our expectation that such information could be beneficial to our understanding of the principles utilized by eukaryotic cells in their replications.

The projects designed in the current section belong to “basic research” ^[11] in nature, which could have implications in our understanding of the fundamental principles utilized by eukaryotic cells in their replication. More specifically, if some alternative DNA structures examined in our studies could indeed resist the hydrolysis by FEN1, it would be the indication that such flap sites could not properly undergo the currently known replication pathways. As a consequence, replication in a eukaryotic cell will be either held up at the particular flap site. It is our idealistic expectation that the outcomes of our

studies could allow cell biologists and clinical geneticists to rationalize certain replication-related puzzling phenomena and idiopathic genetic disease.

1.2.2 Study on inhibition of FEN1

In addition to the aforementioned fundamental scientific issues related to the correlation between DNA structures at flap sites and FEN1 catalytic activities, various studies on the up-regulation of FEN1 in different types of cancer cells in comparison with matched normal cells have been carried out in the past ten years. Nikolova *et al.* (2009) ^[1], for example, examined FEN1 protein expression in the specimens of several tumour tissues with reference to their corresponding normal tissues from the same patients, and discovered that FEN1 was over-expressed in testis, lung and brain tumours ^[1]. In addition, studies carried out by Lam *et al.* revealed that FEN1 is up-regulated in prostate cancer as compared with matched normal prostate cells and this up-regulation increases with tumour progression ^[12]. Moreover, Wang *et al.* reported last year (2014) that FEN1 was over-expressed in gastric cancer as compared with matched normal tissues, and this over-expression was correlated with tumour size, lymphatic metastasis and degree of differentiation ^[13]. These observations led us to suggest that studies of inhibitory effect of wide variety of small molecules on FEN1 are essential, which could have potential in the long term to serve as an effective inhibitor for treatment of several kinds of cancers. In spite of the fact that FEN1 is over-expressed in various types of cancers, the

effectiveness of inhibition of oligonucleotide inhibitor including phosphorothioate bond has not yet been thoroughly investigated thus far. We accordingly designed oligonucleotides containing phosphorothioate modified linkage at or near FEN1 cleavage site and tested the activity of FEN1 susceptible towards these modified molecules. It is our anticipation that such phosphorothioate modified substrates could be further developed and explored to be used as inhibitor of FEN1 to slow down its over-expression in cancer cells.

Apart from the detection and monitoring of activity of FEN1 as discussed above, on the other hand, various organic compounds have been screened in the past against the activities of FEN1 with the intention of identifying potent FEN1 inhibitors ^[14]. In order to facilitate these screening processes, certain particularly designed methods have also been established very recently, through which organic FEN1 inhibitors can be singled out from large libraries of organic compounds ^[15]. Even though no valid lead compound as FEN1 inhibitor has been identified for further development of anticancer drug candidate thus far, FEN1-specific inhibitors have still been considered to have chemotherapeutic potential against certain types of cancers ^[16]. We designed and prepared new type of FEN1 potential inhibitor, which is different from any of those previously screened FEN1 inhibitors since our designed FEN1 inhibitor is substrate-based molecule (oligonucleotide). It is our expectation that our designed new type of FEN1 inhibitor could serve as useful tool for studying

the responses of cancer cells and normal cells to FEN1's inhibitions in the long term.

In brief summary, we have addressed several fundamental scientific issues related to influence of flap-structured substrates with different size or length on the action of FEN1. Correlations between catalytic activities of FEN1 and alternative DNA structures that could well emerge at the flap sites of FEN1 substrates have been explored. Besides, the resisting effects of several small molecules on catalysis of FEN1 have been measured. In addition, we examined inhibitory effect of phosphorothioate modification of oligonucleotides on action of FEN1 and developed inhibitor that can be used for studying the activities of FEN1. Moreover, two fluorogenic platforms have been established for screening commercially available enzyme inhibitor and our designed FEN1 inhibitor. It is our belief that accomplishment of these research projects (1) provides critical information for us to comprehend in depth eukaryotic replications; and (2) offers the biotech and pharmaceutical companies advantageous experimental data for their evaluation of the viability of the new type of inhibitor as tool for anticancer chemotherapy.

1.3 Experimental approaches

1.3.1 Exploration of property of FEN1

1.3.1.1 Exploration of different lengths of 5' flap of oligonucleotide

substrates

During DNA replication in eukaryotic cells, a 5' flap structure is generated by polymerase δ before action of FEN1. The influence of 5' flap with different length on activity of FEN1 was not yet been thoroughly explored. In order to design the most efficient and short length of flap in substrate, we designed substrates with 5' flap composed of 0, 1, 2, 3, 4, 5, 6, 11, 16, 21, 41 and 61 nucleotides and systematically examined their activity undertaken FEN1 catalysis (Scheme 1.2).

Scheme 1.2 Illustration of oligonucleotides with different lengths of 5' flap composed of various numbers of nucleotides.

When a 5' flap sequence is relatively long, "Tracking Model" and "Threading Model" have often been cited for describing the pathways of recognition and cleavage of flap structures by FEN1 (Scheme 1.3)^[17]. In 2010, Gloor *et al.*^[18] suggested based on their studies that FEN1 would choose to undergo threading mechanism instead of tracking pathway^[19].

In both Tracking Model and Threading Model, the presence of free 5' single stranded terminus is a prerequisite for FEN1's catalytic activity, which presumably evolved for preventing this enzyme from causing scission (1) on its single-stranded parental templates and (2) on adjacent Okazaki fragments. Unlike the tracking pathway in which FEN1 presumably recognize the 5' free end in the first place, on the other hand, threading pathway starts with the binding of FEN1 at the flap site followed by "threading" (Scheme 1.3). One of the distinctive features in the threading pathway is the formation of highly bent conformations by the 5' flapped single stranded sequences, a process that will allow the free 5' end of DNA to "thread" through a needle hole-like structure within FEN1 molecules. Generation of a bent structure at the 5' flap, however, is not required in the tracking pathway.

Scheme 1.3 Illustration of tracking model and threading model of FEN1

cleavage.

1.3.1.2 Exploration of different lengths of double strands in oligonucleotide substrates

We systematically test 5, 6, 8, 10, 12, 16 and 20 base pairs on each side of the flap site in double stranded oligonucleotides (Scheme 1.4). Correlation between lengths of double stranded oligonucleotides and their activities against FEN1 is examined in order to find out the minimum length for our designed oligonucleotide inhibitor that maintains high activity.

Scheme 1.4 Illustration of oligonucleotides with different lengths of double strands composed of various numbers of nucleotides.

1.3.1.3 “Extraordinarily thermo-stable hairpin”

“Extraordinarily thermo-stable mini-hairpins” refers to oligonucleotide hairpin structures that contain only 7 to 9 nucleotides in length such as 5’ CGCGAAGCG 3’^[20]. These particular types of hairpin structures possess their melting points from 76 to 88 °C, which are much higher than those of ordinary

hairpin structures with the same nucleotide length ^[20]. If the sequences for the formation of “extraordinarily thermo-stable mini-hairpins” occur on lagging strands, the discontinuity of complementarity could in theory facilitate these sequences for the formation of hairpin structures. We make use of extraordinarily thermostable mini-hairpin-forming DNA segments among single strand oligonucleotides to form double stranded substrates (Scheme 1.5).

Scheme 1.5 Illustration of well-recognized formation of "Extraordinarily thermostable mini-hairpin" from single-stranded DNA.

1.3.1.4 Examination of correlation between triplex DNA structures and DNA-cleaving activity of FEN1

The duplex sequences of poly (dA/dT) (one strand composed of purely adenosine nucleotides and the complementary strand composed of purely thymidine nucleotides) are known to be plentiful DNA elements in human genomes ^[21]. When such sequences are replicated on the lagging strand, a flap structure that is entirely composed of thymidine nucleotides highly likely occurs (Scheme 1.6). Such thymidine-rich flap will in theory be able to fold back to generate triplex structure with its adjacent duplex poly (dA·dT) sequences (Conformation 2 in Scheme 1.6). Unlike the cytosine-containing

triplex that forms under acidic conditions ($\text{pH} < 6.2$), the thymidine oligonucleotide is able to form triplex at neutral pH with its adjacent duplex thymidine/adenosine oligonucleotides as observed for the formation of poly(dT)·poly(dA)·poly(dT) triplex^[21]. We examined whether the formation of such triplex structure could retard the DNA-cleaving rate of FEN1 through comparing the triplex-forming nucleotides with substrate that do not have the potential to form triplex structure under certain condition.

Scheme 1.6 Illustration of triplex conformation of poly(dA·dT) oligonucleotide.

1.3.1.5 Examination of effect of small molecules interfering with DNA on activity of FEN1

There are various types of small molecules that could intercalate into or

interfere with duplex DNA. We speculated that these DNA intercalators and small molecules would be able to affect the FEN1's DNA-cleaving activity if such small molecules interact with DNA. Flap structured double-stranded DNA was reacted with chloroquine, spermidine, netropsin, cisplatin and ethidium bromide.

In order for an intercalator to fit between base pairs, the DNA must dynamically open a space between its base pairs by unwinding. Ethidium cation (the ionic form of ethidium bromide found in aqueous solution) unwinds DNA by about 26° [22]. This unwinding causes the base pairs to separate, or "rise", creating an opening of about 0.34 nm (3.4 Å). This unwinding induces structural changes to the DNA strands, such as lengthening DNA strands or twisting base pairs. These structural modifications can lead to functional changes, often to the resist of transcription and replication and DNA repair processes, which make intercalators potent mutagens [22]. In nucleic acid chemistry, intercalator inserts into the planar bases of DNA. They slot in between base pairs and interact through pi stacking. Intercalation is utilized as a method to analyze DNA and it is also the basis of certain kinds of poisoning. DNA intercalators are used in chemotherapeutic treatment to resist DNA replication in rapidly growing cancer cells [22].

In order to measure efficiency of resistance of several DNA intercalators and other molecules when DNA substrate undergoes catalysis of FEN1, we

tested IC_{50} value. IC_{50} is half maximal resisting concentration to measure the effectiveness of a substance in resisting a specific biological or biochemical function. This quantitative measurement indicates the amount of a particular substance (inhibitor) is needed to resist a given biological enzymatic process by half in vitro^[23].

1.3.1.6 Design of FEN1 plasmid substrate

Other than oligonucleotide substrates designed for exploration of property of FEN1, a flap-structured plasmid DNA substrate for investigation of FEN1 was designed as shown in Scheme 1.7.

Scheme 1.7 Illustration of preparation and flap removal of phi X 174 plasmid substrate of FEN1.

1.3.2 Exploration of effect of phosphorothioate modification to DNA substrates on FEN1 activity

1.3.2.1 Nuclease-resistance by phosphorothioate bond

Various strategies have been developed so far for substrates to resist nuclease hydrolysis. Among them, incorporations of phosphorothioate bonds is a commonly used method. In order to study the inhibitory effect by phosphorothioate bond on activity of FEN1 hydrolysis and thereafter design phosphorothioate modified potential FEN1 oligonucleotide inhibitor, the activity of FEN1 using DNA substrates incorporated with phosphorothioate bonds was explored.

1.3.2.2 Design of phosphorothioate modified DNA substrates

Phosphorothioate bond were inserted into different positions at or adjacent to the FEN1-cutting site (Scheme 1.8) in order to find out at which position the modified substrate could achieve the highest inhibitory efficiency against FEN1 action. The activity of flap-structured duplex oligonucleotides that contain phosphorothioate bond at different positions was tested, with reference to non-modified FEN1 substrate.

Scheme 1.8 Illustration of oligonucleotides that contain phosphorothioate bonds at different positions as FEN1 substrates.

1.3.2.3 Design, synthesis and characterization of substrate-based FEN1 inhibitor

Being often evaluated as drug candidates, enzyme inhibitors have been commonly used in the past as tools for investigating the mode of action of enzymes in their catalysis in cell-free systems. From the time when the critical roles of FEN1 in various cancer cells were discovered, researchers have started on (1) searching for proper FEN1 inhibitors as well as (2) establishing effective methods for screening lead compounds ^[11, 16]. The FEN1 inhibitors that have been tested and screened thus far, however, are organic in nature whereas description of substrate-based inhibitors against FEN1's activity has not yet been found in literature. We use our designed phosphorothioate-containing oligonucleotide to examine (1) whether FEN1's activity could indeed be reduced and (2) whether the phosphorothioate bond at FEN1 cleaving position

could be effective for high inhibitory efficiency (Scheme 1.9).

Our objectives in designing the assays are (1) to design and synthesize novel oligonucleotide-based potential FEN1 inhibitor, and (2) to determine its inhibitory efficiency against FEN1 in buffer solutions.

A flap-structured duplex oligonucleotide as FEN1 inhibitor was designed and prepared that contains a phosphorothioate bond at the FEN1-cutting site (Scheme 1.9). The inhibitor possessed most efficient length of 5' flap for FEN1 catalysis. The number of base pairs in double strands of inhibitor was designed based on reported FEN1 binding mechanism in order to minimize its size^[7]. We investigated the inhibitory effect of the phosphorothioate-containing FEN1 inhibitor by measuring enzyme kinetics.

Scheme 1.9 Structure of phosphorothioated modified oligonucleotide as FEN1 inhibitor.

IC₅₀ values of our designed oligonucleotide inhibitor has been measured in order to quantify the substrate-based inhibitor against FEN1 nuclease activity.

Substrate-based FEN1 inhibitor was designed and investigated its inhibitory effect. It is our idealistic and optimistic hope that it would be able to used to study (i) the responses of a wide range of normal cells (cell lines) to FEN1 inhibition, (ii) vulnerability of a wide range of normal cells (cell lines) to carcinogens when cellular FEN1 is inhibited by FEN1 inhibitor and (iii) the responses of a wide range of cancer cells (cell lines) to FEN1 inhibition. In addition, our newly designed FEN1 inhibitor would be evaluated and developed for its suitability as anticancer drug candidate in the future when innovative carriers of oligonucleotide drugs will be validated legitimately.

1.3.2.4 Examination of correlation between RNA segment at flap site and activity of FEN1

During DNA replication in eukaryotic cells, along the lagging strand's template, primase builds RNA primers in short bursts. DNA polymerase delta is then able to use the free 3'-OH groups on the RNA primers to synthesize DNA in the 5'→3' direction ^[24]. Murante et al. reported that when RNA primers was left at the flap site and was complementary in part with a DNA template strand during Okazaki fragment maturation, calf RTH-1 Nuclease (a homologue of human FEN-1) excised the RNA at the flap starting position in a relative fast rate in comparison with the cases where DNA is as the substrate ^[25]. In order to examine (1) whether FEN1 could take the oligonucleotides containing RNA flap structures as its substrates, and (2) what the cleaving efficiencies of FEN1

are if FEN1 could indeed excise flapped duplex structures that are composed entirely or partially of ribonucleotides, we prepared flap structures that contain ribonucleotides (RNA) in different lengths and examined the effects of these RNA-containing structures on FEN1's catalytic activity (Scheme 1.10).

Scheme 1.10 Flap-structured substrates containing RNA nucleotides at 5' flap site.

The oligonucleotides were incubated with FEN1 based on reported procedures^[26] followed by polyacrylamide gel electrophoretic analyses.

1.3.3 Establishment of effective methods for screening FEN1 inhibitors

1.3.3.1 Development of fluorophor-quencher-based platform for monitoring FEN1 activity

The BHQ dyes are a class of high-efficiency dark quencher which prevent fluorescence^[27]. These highly specific dyes enable the identification as well as quantification of biomolecules which could be used to measure the degree of biochemical reaction.

On the other hand, the dye molecule Cy3 absorbs light around a wavelength of 500 nm and generates the dye excited state. In the absence of quencher, Cy3 emits fluorescence. When quencher BHQ-2 presents, Förster resonance energy transfer (FRET) occurs. The dye excited state transfers energy to the quencher BHQ-2 ground state, generating quencher BHQ-2 excited state. As a consequence, quencher BHQ-2 returns to the ground state with radiationless decay (Scheme 1.11). In this case, fluorophore Cy3 is not able to emit fluorescence. In our assays, BHQ-2 was present to quench fluorescence of Cy3, after FEN1 catalysis, the quencher BHQ-2 was separated from fluorophore Cy3, therefore Cy3 emitted fluorescence. The degree of completion of FEN1 reactions could be monitored by fluorescent spectrometry.

Step 1: Cy3 absorbs light and generate excited state

Step 2: Quencher BHQ-2 interacts with Cy3

Step 3: After removal of quencher BHQ-2, dye Cy3 emits fluorescence

Scheme 1.11 Illustration of interaction between dye Cy3 and quencher BHQ-2.

1.3.3.2 Design of Cy3-(BHQ-2) fluorescence platform

Fluorescence resonance energy transfer is a highly distance-dependent interaction between fluorophore Cy3 in the excited state and quencher BHQ-2 in its ground state. In order to increase the efficiency of fluorophore quenching, firstly, the fluorescence emission spectrum of the fluorophore must overlap with the absorption spectrum of the quencher to facilitate energy transfer. In this case, fluorophore Cy3 and quencher BHQ-2 pair are selected since their spectra perfectly overlap with each other, with maximum intensity around 570 nm. Secondly, the distance between fluorophore Cy3 and quencher BHQ-2 should be designed closely, which is in 10 to 100 Å range^[28]. This can be achieved by designing and modifying Cy3 and BHQ-2 in the same DNA molecule shown in Scheme 1.12.

Scheme 1.12 Illustration of fluorescence emission after FEN1 catalysis using Cy3-(BHQ-2) platform.

1.3.3.3 Design of 2-aminopurine-based fluorescence platform

The 2-amino purine utilized as fluorescent probe for FEN1 cleaving reaction is sequence dependant. Poly (dA) plays an important role to provide electron transfer and meanwhile provide energy transfer to 2-aminopurine to emit fluorescence at a wavelength of 370 nm, while nucleotide G or poly (dG) could block electron transfer thus to quench the fluorescence. Once the phosphodiester bond at the 5' flap in oligonucleotide (Scheme 1.13) is cleaved by FEN1, 2-aminopurine is separated from nucleotide G, thus poly (dA) could transfer energy to 2-aminopurine without blockage by nucleotide G. The free 5' flap from substrate would increase the intensity of fluorescence of 2-aminopurine. By measurement the change of signal of fluorescence by 2-aminopurine substrate, we could monitor FEN1 cleaving reaction.

Scheme 1.13 Illustration of 2-aminopurine fluorescence platform for monitoring FEN1 reaction.

1.3.3.4 Significance of fluorescence platforms

The development of the fluorescent probes could offer a fast and convenient method for monitoring reaction by FEN1. In addition, the fluorescent signals would be useful for detecting activity of FEN1 in effective and accurate manners, which could provide evidences for cell biologists and molecular oncologists to further study FEN1 and to screen its inhibitors.

1.4 Significance

The specific significance in designing these projects were to establish correlations between catalytic activities of flap structure-specific endonuclease 1 (FEN1) and different kinds of DNA structures that could well emerge at the flap sites of lagging strands during eukaryotic replication. (Significance 1) Besides, we revealed inhibitory effect of a series of small molecules, including DNA intercalators on FEN1 activity. (Significance 2) Moreover, we designed and investigated inhibitory effect of phosphorothioate modified DNA oligonucleotides on catalytic activity of FEN1. (Significance 3) In addition, we investigated effect of RNA primer that could left at flap site on FEN1 cleavage. (Significance 4) We also developed substrate-based FEN1 inhibitor and test its inhibitory effect on FEN1 catalysis. (Significance 5) Last but not least, oligonucleotide fluorescence platforms for monitoring FEN1 activity were developed, which have potency to be used for screening FEN1 inhibitors. (Significance 6)

1.5 Originality

The correlation between flap-structured DNA substrates with different length of 5' flap or double strands and catalytic activity of FEN1 has not yet been systematically investigated thus far. Besides, various alternative structures could emerge at or near 5' flap structures on the lagging strands during eukaryotic replication, effects of which on FEN1's activity have not yet been understood. We systematically examined the activity of oligonucleotides with different length of flap or double strands as FEN1 substrates, as well as the correlations between alternative DNA structures and FEN1's catalytic activity for the first time, knowledge of which could be beneficial to cell biologists and clinical geneticists for rationalizing certain replication-related puzzling phenomena and idiopathic genetic disease. In addition, even though it has been known that FEN1 is over-expressed in various cancer cells, highly specific and potent FEN1 inhibitor has not yet been developed thus far. We designed, prepared and tested substrate-based FEN1 oligonucleotide inhibitor, which has potential to be further developed and optimized for studying responses of cancer cells and normal cells to their FEN1 inhibition. On the other hand, we developed Cy3-(BHQ-2) and 2-aminopurine fluorescence platforms which could serve as effective tools for monitoring the progression of catalytic reaction of FEN1 and efficient screening of FEN1 inhibitors.

Chapter 2 - Study on FEN1 catalytic property

2.1 Examination of correlation between catalytic property of FEN1 and structures of substrates

2.1.1 Correlation between length of 5' flap of DNA substrates and FEN1 activity

During DNA replication in eukaryotic cells, a 5' flap structure is produced before catalysis of nuclease FEN1. In order to explore the correlation between length of 5' flap and catalytic activity of FEN1, flap-structured DNA substrates with different length of flap ranging from 0-61 nucleotides were systematically designed and investigated. The most efficient and short length of flap could be used for designing FEN1 oligonucleotide inhibitor.

2.1.1.1 DNA sequences

Name of DNA	Nucleotides sequence (5' to 3')
Up strand	TCGAGGTCGACGGTATCGATAAGCTTGATAT
Template strand	CCGGGCTGCAGGAATTCGATATCAAGCTTATCGATAC CGTCGACCTCGA
Down strand 0	CGAATTCCTGCAGCCCGG
Down strand 1	TCGAATTCCTGCAGCCCGG
Down strand 2	CTCGAATTCCTGCAGCCCGG

8.3	
Acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
N,N'-methylenebisacrylamide	Sigma-Aldrich (St. Louis, MO, USA)
N,N,N',N'-tetramethylethylene diamine (TEMED)	Bio-rad laboratories (Hercules, CA, USA)
Ammonium persulfate	Bio-rad laboratories (Hercules, CA, USA)
Ethidium bromide solution, 10 mg/ml	Bio-rad laboratories (Hercules, CA, USA)
Electrophoresis power supply	Consort (Turnhout, Belgium)
Nanophotometer	Implen (München, Germany)
Dual accublock digital dry bath	Labnet international (Edison, NJ, USA)
Vortex mixer	Labnet international (Edison, NJ, USA)
G:BOX Chemi gel doc system	Syngene (Cambridge, UK)

Buffer: Ultra pure / biotechnology grade

DNA substrates with different length of 5' flap were annealed by incubating 150 pmol of different length of single-stranded down strand DNA,

100 pmol of up strand and 100 pmol of template strand with 10mM Tris for 5 minutes at 95°C in a whole volume of 20 µl to have a final concentration of substrate of 5 uM. Sample vials were subsequently taken out from heater and chilled in room temperature (25 °C) for 2 hours. This annealing method was compared with other methods, for example added 15 mM sodium chloride or cooled down in the heating bath. The results demonstrated that the above mentioned annealing method is the optimum. (see results in discussion)

All the oligonucleotides with various 5' flap length were treated in identical condition to investigate catalytic activity of human flap structure specific endonuclease 1 (FEN1). Two picomoles of each oligonucleotide was reacted with FEN1 in reaction buffer (250 mM Tris, 50 mM MgCl₂, 5 mM DTT, 500 µg/ml BSA at pH 8.0) for 1 hour at 37 °C in a total reaction volume of 20 µl. After reaction, samples with longer length of flap were analyzed by 15% non-denaturing acrylamide gel electrophoresis at 250 volt for 8 hours, and samples with shorter length of flap were analyzed for 16 hours, respectively. Ethidium bromide was used to stain bands in gel after electrophoresis.

2.1.1.3 Result and discussion

The activity of oligonucleotides as FEN1 substrates possess 5' flap composed of 6, 11, 16, 21, 41 and 61 nucleotides was explored. As shown in Figure 2.1, in each lane, the upper band was unreacted substrate and the lower band was FEN1-cleaving product. The product was A1Dn which had one

nucleotide at 5' flap. The nucleotides in the 5' flap were noncomplementary to template strand except the last nucleotide adjacent to FEN1 cleavage site. This design was based on importance of 3' flap composed of one nucleotide for FEN1 cleavage^[29]. There is an equilibrium of complement of 5' flap and 3' flap to template strand, thus it is difficult for FEN1 to cleave the complementary nucleotide at 5' flap. As a consequence, the product after FEN1 catalysis was A1Dn, rather than nicked double strands.

Comparison between multiple substrates with length of 5' flap ranging from 6 nts to 61 nts, activity was decreased inversely proportional to increasing length of flap. The ratio of bands were analyzed and calculated by GeneTools. Substrate with flap length of 6 nts demonstrated highest activity and generated 54% of product, while substrate with flap length of 61 nts generated 28% of product.

Figure 2.1 Oligonucleotides with different length of 5' flap (6-61 nts) after FEN1 catalysis. Lane 1: A1Dn only; Lane 2: A6Dn + FEN1; Lane 3: A11Dn +

FEN1; Lane 4: A16Dn + FEN1; Lane 5: A21Dn + FEN1; Lane 6: A41Dn + FEN1; Lane 7: A61Dn + FEN1; Lane 8: 100 bp DNA ladder.

Among A6Dn to A61Dn, since the substrate with shorter flap demonstrated the higher efficiency, the activity of oligonucleotides with flap composed of 0 to 6 nucleotides was further examined (Figure 2.2). Substrate with flap composed of 3 nucleotides (A3Dn) demonstrated highest activity and generated 77% of product, followed by A4Dn generated 75% of product, A5Dn generated 70% of product, A2Dn generated 68% product and A6Dn generated 67% product. A1Dn was not excised because of equilibrium of free 3' flap and 5' flap. And nicked double-stranded DNA was compared as reference to confirm product's length of flap.

Figure 2.2 Oligonucleotides with different length of 5' flap (0-6 nts) after FEN1 catalysis. Lane 1: A0Dn only; Lane 2: A1Dn + FEN1; Lane 3: A2Dn + FEN1; Lane 4: A3Dn + FEN1; Lane 5: A4Dn + FEN1; Lane 6: A5Dn + FEN1; Lane 7: A6Dn + FEN1.

To summarize, the activity of FEN1 using substrates with different length

of 5' flap has been explored. Substrate A3Dn which possessed 3 nucleotides at 5' flap demonstrated highest activity. When flap was longer, the activity of substrate was declined accordingly. The substrate with flap that only has one nucleotide could not be cleaved by FEN1 under moderate condition. The result indicated that 3-nucleotide-containing 5' overhang structure would be the form that most frequently generated by polymerase delta during DNA replication. Combined with following study of modification of oligonucleotide in the next chapter, this result could be used for designing FEN1 oligonucleotide inhibitor which has efficient and tiny structure to prevent FEN1 from involvement in over-expression of DNA in cancer cells.

Before investigating activity of FEN1 using different substrates, the annealing method for flap-structured substrates was explored. Single-stranded up stream, down stream 21 and template strand were reacted with 10 mM Tris at pH 8.0 in a total reaction volume of 20 μ l. Sample was incubated at 95 °C for 5 minutes, followed by different chilling methods. Subsequently, samples were analyzed by 20% non-denaturing acrylamide gel electrophoresis for 12 hours at 200 volt. As shown in Figure 2.3, the sample which chilled in heating bath from 95 °C to 25 °C demonstrated a series of bands of impurities. On the other hand, the sample which was chilled at room temperature (25 °C) demonstrated one clear band. Therefore, in order to produce flap-structured substrate with high purity, samples were chilled at room temperature (25 °C).

Figure 2.3 Comparison of annealing methods for flap-structured substrates.

Lane 1: Substrate was chilled in incubator from 95 °C to 25 °C; Lane 2: Substrate was chilled at room temperature (25 °C).

On the other hand, sodium chloride is widely used when annealing single-stranded DNA. We compared substrate annealing method including sodium chloride and without sodium chloride. A21Dn annealed with Tris and 15 mM NaCl in a total reaction volume of 20 µl was heated for 5 minutes at 95 °C, followed by chilled in room temperature (25 °C). The sample was analyzed by 20% non-denaturing gel electrophoresis for 15 hours at 300 volt. The result as shown in Figure 2.4 indicated sodium chloride was not appropriate to be added for annealing flap-structured DNA substrate, as the sample contained many impurities.

Figure 2.4 Substrate annealed with NaCl. Lane 1: A21Dn annealed with 15 mM NaCl; Lane 2: A21Dn + 0.3 ug of FEN1, reacted for 1 hour at 37 °C; Lane 3: A21Dn + 0.2 ug of FEN1, reacted for 1 hour at 37 °C; Lane 4: A21Dn annealed without NaCl.

2.1.2 Correlation between length of double strands of DNA substrates and FEN1 activity

Activity of flap-structured oligonucleotides with different lengths of double strands reacted with enzyme FEN1 was explored for designing small and efficient enzyme oligonucleotide inhibitor. Double strands designed were composed of 10, 12, 16, 20, 24, 32 and 40 nucleotides, respectively and flap structure was in the middle of double strands.

2.1.2.1 DNA sequences

Name of DNA	Nucleotides sequence (5' to 3')
A10	ATCGCTCCGCGAAGCGGATACGCGAAGCGTAT
A12	ATCGCTCGCGCGAAGCGCGATATCGCGAAGCGATAT
A16	ATCGCTCGAACGCGAAGCGTTCGATATCACGCGAAG

	CGTGATAT
A20	ATCGCTCGAATTCGCGAAGCGAATTCGATATCAAGC GCGAAGCGCTTGATAT
A24	ATCGCTCGAATTCCCGCGAAGCGGGAATTCGATATC AAGCTCGCGAAGCGAGCTTGATAT
A32	ATCGCTCGAATTCCTGCACGCGAAGCGTGCAGGAAT TCGATATCAAGCTTATCCGCGAAGCGGATAAGCTTGA TAT
A40	ATCGCTCGAATTCCTGCAGCCCCGCGAAGCGGGGCT GCAGGAATTCGATATCAAGCTTATCGATACGCGAAG CGTATCGATAAGCTTGATAT

2.1.2.2 Method

Different length of flap-structured substrates at a concentration of 10 μ M was annealed with 10 mM Tris at pH 8.0 and heated at 95 °C for 5 minutes in a total reaction volume of 20 μ l, followed by cooling at 25 °C for 2 hours. After annealing, 4 pmol of substrates was incubated with FEN1 and 250 mM Tris, 50 mM $MgCl_2$, 5 mM DTT and 500 μ g/ml BSA at pH 8.0 in a total reaction volume of 20 μ l. Samples were reacted with FEN1 at 37 °C for 10, 20, 40 and 60 minutes to monitor progress of reaction, respectively. After reaction, samples were added with loading buffer and kept at -20 °C to freeze and quench reaction. Samples were analyzed by 20% non-denaturing acrylamide gel electrophoresis

at 300 volt for 15 hours.

2.1.2.3 Result and discussion

Activity of substrates A24, A32, A40 had been examined (Figure 2.5). After incubation at 37 °C for 10 minutes, about 70% of the substrates with different length had been cleaved to products. Within reaction time of 20 minutes, shorter substrate A24 was totally reacted, while substrates A32 and A40 were incompletely reacted. After 40 minutes, the catalysis had been completed for A32 and A40. The result indicated shorter double-stranded substrate possessed higher activity towards FEN1 catalysis.

Figure 2.5 Activity of substrates with different length of double strands (24-40 nts). Lane 1: A40 only; Lane 2: A32 only; Lane 3: A24 only; Lane 4: A40 + FEN1, reacted for 10 minutes; Lane 5: A32 + FEN1, reacted for 10 minutes; Lane 6: A24 + FEN1, reacted for 10 minutes; Lane 7: A40 + FEN1, reacted for 20 minutes; Lane 8: A32+ FEN1, reacted for 20 minutes; Lane 9: A24+ FEN1,

reacted for 20 minutes; Lane 10: A40 + FEN1, reacted for 40 minutes; Lane 11: A32+ FEN1, reacted for 40 minutes; Lane 12: A24+ FEN1, reacted for 40 minutes; Lane 13: A40 + FEN1, reacted for 1 hour; Lane 14: A32+ FEN1, reacted for 1 hour; Lane 15: A24 + FEN1, reacted for 1 hour.

Activity of flap-structured substrates with shorter length of double strands was thereafter explored. Oligonucleotides A10, A12, A16 and A20 were incubated with FEN1 and reaction buffer at 37 °C for 10 minutes, 20 minutes, 40 minutes and 1 hour, respectively. Samples were afterwards analyzed by gel electrophoresis. Gel was stained by ethidium bromide as shown in Figure 2.6 and 2.7. A12, A16 and A20 were gradually cleaved by enzyme FEN1 to generate products, while shortest substrate A10 remained unreacted under this condition. Within one hour incubation, A16 and A20 were almost completely reacted. Substrate A12 produced about 65% of product after 1 hour reaction. The result indicated that length of double strands of substrate affects activity of FEN1. A16, A20 and A24 possessed high activity, whereas longer substrates A32 and A40 or short substrates A10 and A12 would reduce activity.

Figure 2.6 Activity of substrates with different length of double strands (16-20 nts). Lane 1: A20 only. Lane 2: A16 only; Lane 3: A12 only; Lane 4: A10 only; Lane 5: A20 + FEN1, reacted for 10 minutes; Lane 6: A20 + FEN1, reacted for 20 minutes; Lane 7: A20 + FEN1, reacted for 40 minutes; Lane 8: A20 + FEN1, reacted for 1 hour; Lane 9: A16 + FEN1, reacted for 10 minutes; Lane 10: A16 + FEN1, reacted for 20 minutes.

Figure 2.7 Activity of substrates with different length of double strands (10-16 nts). Lane 1: A16 + FEN1, reacted for 40 minutes. Lane 2: A16 + FEN1, reacted for 1 hour; Lane 3: A12 + FEN1, reacted for 10 minutes; Lane 4: A12 + FEN1, reacted for 20 minutes; Lane 5: 1 µl A12 + FEN1, reacted for 40 minutes; Lane 6: A12 + FEN1, reacted for 1 hour; Lane 7: A10 + FEN1, reacted

for 10 minutes; Lane 8: A10 + FEN1, reacted for 20 minutes; Lane 9: A10 + FEN1, reacted for 40 minutes; Lane 10: A10 + FEN1, reacted for 1 hour.

2.2 Activity of triplex DNA as FEN1 substrate

The flap-structured triplex DNA was designed and explored its activity as FEN1 substrate. We synthesized flap-structured duplex oligonucleotides that contained poly (dT) segment as part of 5' flap sequence. Unlike conventional DNA triplex that usually forms under acidic condition ($\text{pH} < 6.2$) for its formation, this triplex entity can be assembled under neutral condition ^[30]. It will be able to form triplex structure with its neighbouring complementary duplex.

2.2.1 DNA sequences

Name of DNA	Nucleotides sequence (5' to 3')
Triplex T	TTTTTTTTTTTTTTTTTTTTTACTCAAAAAAAAAAAAAA AAAAAACGCGAAGCGTTTTTTTTTTTTTTTTTTTC GCGATGAACTAGCGCGAAGCGCTAGTTCATCGCGA
Duplex CT	CTCTTCCCTTCTCCTTTCCTACTCAGGAAAGGAGAA GGGAAGAGCGCGAAGCGCTCTTCCCTTCTCCTTCC TCGCGATGAACTAGCGCGAAGCGCTAGTTCATCGCG A

We inserted nucleotides ACTC to make an ordinary stable hairpin structure

within 5' flap to assist to form triplex structure. Other than hairpin structure, the flap of triplex T was merely composed of thymine; while the flap of duplex CT was composed of cytosine and thymine. From the result shown in section 2.1.1 on study of length of 5' flap, 5' flap as long as 21-mer was still active towards FEN1 catalysis. The flap of triplex T and duplex CT were designed to have 24 nts, rather than short ones in order to maintain stable triplex structure.

In order to observe whether triplex T had formed triplex structure, we tested melting temperature for nucleic acid thermal stability analysis (Figure 2.8). Melting curve analysis is an assessment of dissociation characteristics of double-stranded DNA during heating. Along with rising temperature, double strand begins to dissociate leading to a rise in UV absorbance (Figure 2.9)^[31]. G-C base pairing has 3 hydrogen bonds between them while A-T base pairing has 2 hydrogen bonds. DNA with a higher G-C content will have a higher melting temperature than DNA with a higher A-T content^[32].

Figure 2.8 Typical UV-melting profiles of duplexes and triplexes. (A) UV-melting profiles of duplexes reacted under different conditions; (B) UV melting profiles of triplexes reacted under different conditions^[33].

Figure 2.9 Illustration of change of UV absorbance when duplex DNA decomposed into single strands.

2.2.2 UV-melting test

In UV-Tm analysis, Temperature was increased from 23 °C to 95 °C at a rate of 0.2 °C/min, and oligonucleotides were held at 95 °C for 5 minutes. Afterwards, temperature was declined at same rate to 23 °C. Readings of UV absorbance at wavelength of 260 nm were recorded at every 0.5 °C.

The result of UV-Tm analysis showed that the UV absorbance of triplex T was higher than duplex CT (Figure 2.10). More importantly, triplex T demonstrated two slopes which was due to decomposition of triplex structure. However duplex CT had only one slope in the curve of UV absorbance against temperature because of decomposition of double strands.

Figure 2.10 Correlation between UV absorbance and temperature for (a) triplex T and (b) duplex CT.

2.2.3 Test by gel electrophoresis

The UV-T_m analysis proved the formation of triplex structure. In order to compare activity of triplex T and duplex CT towards FEN1 catalysis to investigate effect of triplex formation on catalysis, substrates were reacted with FEN1. Substrate A3Dn which had the most efficient length of flap for catalysis was reacted as reference. Adequate amount of FEN1 (0.16 ug) for substrate A3Dn and inadequate amount of FEN1 (0.064 ug) were added into samples, respectively. Concentration of triplex T and duplex CT was tested using UV-photometer for exactly taking 2 picomoles substrates into mixtures. After 1 hour reaction incubated at 37 °C, loading buffer was added into each sample and samples were separated by 15% non-denaturing acrylamide gel electrophoresis at 250 volt for 2 hours. Gel was stained by ethidium bromide and visualized by UV illuminator.

As shown in the Figure 2.11, A3Dn could completely reacted with 0.16 ug

of enzyme and generate product, on the other hand, triplex CT and duplex CT were not completely reacted. Duplex CT generated around 60% of product, meanwhile triplex T generated around 40% of product. A3Dn reacted with 0.064 ug of FEN1 generated about 30% of product, while triplex T and duplex CT generated a little product. The result demonstrated higher activity of flap-structured duplex substrate, compared with triplex substrate, which suggested triplex structure could slow down FEN1 catalysis. Compared to substrate A3Dn that had short 5' flap composed of 3 nucleotides, the reactivity of both triplex T and duplex CT is slower due to long flap.

Figure 2.11 Comparison of FEN1 catalysis using triplex T, duplex CT and A3Dn as substrates. Lane 1: 100 bp DNA ladder. Lane 2: Triplex T only. Lane 3: Triplex T + 0.064 ug of FEN1. Lane 4: Triplex T + 0.16 ug of FEN1. Lane 5: Duplex CT only. Lane 6: Duplex CT + 0.064 ug of FEN1. Lane 7: Duplex CT + 0.16 ug of FEN1. Lane 8: A3Dn only. Lane 9: A3Dn + 0.064 ug of FEN1. Lane 10: A3Dn + 0.16 ug of FEN1.

2.3 Effect of several small molecules on FEN1 substrates.

Several molecules that could interact with DNA duplex were investigated

their effect on FEN1 catalytic reactions. Among which, intercalators are molecules that could insert into DNA duplex. Intercalators interact between cationic, planar, polycyclic aromatic systems of the correct size (on the order of a base pair)^[2-4]. One proposed mechanism of intercalation states that in aqueous isotonic solution, the cationic intercalator is attracted electrostatically to the surface of the polyanionic DNA. The ligand displaces a sodium and/or magnesium cation present in the "condensation cloud" of such cations that surrounds DNA (to partially balance the sum of the negative charges carried by each phosphate oxygen), thus forming a weak electrostatic association with the outer surface of DNA. From this position, the ligand diffuses along the surface of the DNA and may slide into the hydrophobic environment found between two base pairs that may transiently "open" to form an intercalation site, allowing intercalators to move away from the hydrophilic (aqueous) environment surrounding the DNA and into the intercalation site. The base pairs transiently form such openings due to energy absorbed during collisions with solvent molecules.

2.3.1 Effect of chloroquine on DNA substrate against FEN1 cleavage

The effect of chloroquine binding to DNA substrate under FEN1 catalysis was assessed^[34]. The effect of intercalator chloroquine on FEN1 substrate was quantified by IC₅₀ value. According to previous result, the most efficient substrate A3Dn was used for FEN1 catalysis to test effect by chloroquine. Two

picomoles of oligonucleotide A3Dn was reacted with FEN1 (0.16 μ g) for 50 minutes at 37 °C. The reaction buffer was 250 mM Tris, 50 mM MgCl_2 , 5 mM DTT and 500 μ g/ml BSA at pH 8.0. An increasing concentration from 0.25 mM to 20 mM chloroquine was added into samples. After reaction, gel electrophoresis was carried out at 250 volt by 15% non-denaturing acrylamide gel for 8 hours.

As shown in the Figure 2.12, chloroquine demonstrated resisting effect on FEN1 activity. The upper bands were substrate A3Dn and the lower bands were product A1Dn. Along with increasing concentration of chloroquine, the band of product became lighter, whereas the band of substrate became darker. When concentration of chloroquine changing from 0.25 mM to 20 mM, we observed progressive resistance of production. The sample with 12 mM chloroquine after FEN1 catalysis was almost completely unreacted, which indicated complete resistance by chloroquine.

Figure 2.12 Resisting effect of chloroquine on FEN1 catalysis. Lane 1: A3Dn only; Lane 2: A3Dn + FEN1; Lane 3: A3Dn + FEN1 + 0.25 mM Chloroquine; Lane 4: A3Dn + FEN1 + 1 mM Chloroquine; Lane 5: A3Dn + FEN1 + 2 mM

Chloroquine; Lane 6: A3Dn + FEN1 + 3 mM Chloroquine; Lane 7: A3Dn + FEN1 + 4 mM Chloroquine; Lane 8: A3Dn + FEN1 + 5 mM Chloroquine; Lane 9: A3Dn + FEN1 + 8 mM Chloroquine; Lane 10: A3Dn + FEN1 + 12 mM Chloroquine; Lane 11: A3Dn + FEN1 + 20 mM Chloroquine.

Substrate A3Dn that incubated for 50 minutes at 37 °C without adding enzyme was the reference which contained trace amount of product due to degradation. The ratio of product in reference was deducted. In order to quantify resisting effect by chloroquine, the correlation between resisting effect of intercalation and concentration of chloroquine was plotted as shown in Figure 2.13 and IC_{50} value was tested to measure the ability of resistance. Resisting effect was proportional to increasing concentration of intercalator in millimolar scale. After 8 mM chloroquine was added into FEN1 reaction mixture, the reaction activity was 97% resisted. IC_{50} value of chloroquine to FEN1 catalysis was measured to be 3.55 mM.

Figure 2.13 Effect of resistance by different concentrations of chloroquine on

FEN1 catalysis.

2.3.2 Effect of ethidium bromide on DNA substrate against FEN1 cleavage

Ethidium bromide is an intensively studied intercalator that often used for staining double strands of nucleic acid. It has an appropriate size and chemical nature to fit itself in between base pairs of DNA.

Two picomoles of A3Dn was incubated with various concentrations of ethidium bromide, and reacted with 0.16 μ g of FEN1, 250 mM Tris, 50 mM MgCl_2 , 5 mM DTT and 500 μ g/ml BSA at pH 8.0. In order to explore effect of resistency by ethidium bromide, different concentrations of ethidium bromide were added into reaction mixture, ranging from 1 μ M to 80 μ M. Samples were incubated for 50 minutes at 37 °C in a total reaction volume of 20 μ l. Gel electrophoresis was carried out at 250 volt by 15% non-denaturing acrylamide gel for 8 hours. The result of electrophoresis (Figure 2.14) showed that resisting effect of EB on FEN1 catalysis was proportional to concentration of EB. 10 μ M ethidium bromide demonstrated a little effect of resistance and 80 μ M ethidium bromide completely resisted the action of FEN1.

Figure 2.14 Resisting effect of ethidium bromide on FEN1 catalysis. Lane 1: A3Dn only; Lane 2: A3Dn + FEN1; Lane 3: A3Dn + FEN1 + 1 μ M EB; Lane 4: A3Dn + FEN1 + 10 μ M EB; Lane 5: A3Dn + FEN1 + 20 μ M EB; Lane 6: A3Dn + FEN1 + 30 μ M EB; Lane 7: A3Dn + FEN1 + 40 μ M EB; Lane 8: A3Dn + FEN1 + 50 μ M EB; Lane 9: A3Dn + FEN1 + 60 μ M EB; Lane 10: A3Dn + FEN1 + 80 μ M EB.

Effect of intercalator ethidium bromide on enzymatic reaction was plotted as shown in Figure 2.15. The intercalating effect of EB on reaction was significant since the concentration of EB was in micromolar scale. The resisting effect reached 100% adding 60 μ M ethidium bromide. IC_{50} value of ethidium bromide was thereafter calculated to be 27.56 μ M.

Figure 2.15 Resisting effect by different concentrations of ethidium bromide on FEN1 catalysis.

2.3.3 Effect of netropsin on DNA substrate against FEN1 cleavage

Netropsin binds to the minor groove of AT-rich sequences of double stranded DNA [35]. The sequence of flap-structured oligonucleotide for FEN1 cleavage was designed to contain AATT. Effect of netropsin on FEN1 cleavage was explored and its IC_{50} value was tested. FEN1 (0.16 ug) was added into 2 pmol of substrate A3Dn and reacted for 50 minutes at 37 °C with 50 mM Tris, 50 mM $MgCl_2$, 5 mM DTT and 500 $\mu g/ml$ BSA at pH 8.0, in a total reaction volume of 20 μl . Concentrations of netropsin dihydrochloride ranging from 0.1 mM to 10 mM were tested. After reaction, samples were analyzed by 15% non-denaturing acrylamide gel at 250 volt for 8 hours, followed by staining with EB.

Result of gel electrophoresis (Figure 2.16) revealed the influence of binding of netropsin and DNA substrate on catalytic reaction. The band of unreacted substrate became darker and product became less along with

increasing concentration of netropsin. 0.2 mM netropsin demonstrated a little effect on enzymatic catalysis, while after 10 mM netropsin binded to DNA susbstrate, the reaction was mostly resisted.

Figure 2.16 Resisting effect of netropsin on FEN1 catalysis. Lane 1: A3Dn only; Lane 2: A3Dn + FEN1; Lane 3: A3Dn + FEN1 + 0.1 mM netropsin; Lane 4: A3Dn + FEN1 + 0.2 mM netropsin; Lane 5: A3Dn + FEN1 + 0.5 mM netropsin; Lane 6: A3Dn + FEN1 + 1.0 mM netropsin; Lane 7: A3Dn + FEN1 + 1.5 mM netropsin; Lane 8: A3Dn + FEN1 + 2.0 mM netropsin; Lane 9: A3Dn + FEN1 + 5 mM netropsin; Lane 10: A3Dn + FEN1 + 10 mM netropsin.

Based on the data of percent yield, the resisting effect of various concentrations of netropsin was plotted as shown in Figure 2.17. As the concentration of netropsin increased from 0.1 mM to 10 mM, the resisting effect was proportionally increased. The resistance reached 95% until 10 mM netropsin binded with DNA substrate. IC_{50} value of netropsin for FEN1 catalysis was 1.69 mM.

Figure 2.17 Correlation between effect of binding and concentration of netropsin.

2.3.4 Effect of spermidine on DNA substrate against FEN1 cleavage

Spermidine binds DNA oligonucleotides ^[36]. The influence of binding between spermidine and DNA oligonucleotide on FEN1 cleavage was investigated. Two picomoles of A3Dn was reacted with 0.16 μ g of FEN1, 250 mM Tris, 50 mM $MgCl_2$, 5 mM DTT and 500 μ g/ml BSA at pH 8.0 for 50 minutes at 37 °C. Concentration of spermidine ranging from 5 mM to 15 mM was added into each sample. Effect of spermidine on FEN1 catalysis was analyzed by 15% non-denaturing acrylamide gel at 250 volt for 8 hours.

As shown in Figure 2.18, the effect of binding of spermidine and DNA substrate was significant. Along with increasing concentration of spermidine, the percent yield by FEN1 catalysis declined. The bands of unreacted DNA substrate became darker while bands of excised compound became lighter. After 6 mM spermidine was mixed with substrate, the gel demonstrated a little effect on reaction, whereas after 14 mM spermidine was incubated with mixture,

there was only a little product shown on gel.

Figure 2.18 Resisting effect of spermidine on FEN1 catalysis. Lane 1: A3Dn only; Lane 2: A3Dn + FEN1; Lane 3: A3Dn + FEN1 + 5 mM spermidine; Lane 4: A3Dn + FEN1 + 6 mM spermidine; Lane 5: A3Dn + FEN1 + 7 mM spermidine; Lane 6: A3Dn + FEN1 + 8 mM spermidine; Lane 7: A3Dn + FEN1 + 9 mM spermidine; Lane 8: A3Dn + FEN1 + 10 mM spermidine; Lane 9: A3Dn + FEN1 + 11 mM spermidine; Lane 10: A3Dn + FEN1 + 12 mM spermidine; Lane 11: A3Dn + FEN1 + 13 mM spermidine; Lane 12: A3Dn + FEN1 + 14 mM spermidine; Lane 13: A3Dn + FEN1 + 15 mM spermidine.

In order to quantify resisting effect of spermidine on DNA substrate to affect FEN1 catalysis, the correlation between effect of binding and concentration of spermidine was plotted as shown in Figure 2.19. The effect of binding on reaction was proportional to concentration of spermidine. Along with increasing concentration of spermidine, the binding of spermidine and DNA substrate became stronger which affected the reaction activity. While concentration was increased from 5 mM to 15 mM, resisting effect was increased from 25% to 95%; meanwhile resisting effect on sum of intermediate and final products was increased to 80%. The IC_{50} value of spermidine was

measured to be 7.86 mM.

Figure 2.19 Correlation between effect of binding and concentration of spermidine.

2.3.5 Effect of cisplatin on DNA substrate against FEN1 cleavage

Cisplatin is a platinum-containing compound known to treat various types of cancers. Cisplatin is a small molecule that can both actively transport and passively diffuse into cell membranes. It forms adduct with two consecutive guanine bases in DNA. The molecule loses its chlorine atoms and binds to nitrogen atoms in guanine bases ^[37]. Our designed substrates contained consecutive guanine bases.

Two picomoles of A3Dn was fully catalyzed by 0.16 μ g of FEN1 with 250 mM Tris pH 8.0, 50 mM MgCl_2 , 5 mM DTT and 500 μ g/ml BSA. Samples were prepared in a reaction volume of 20 μ l and reacted for 50 minutes at 37 $^{\circ}\text{C}$. A concentrations series of cisplatin ranging from 0.075 mM to 2.5 mM were added accordingly into each sample. Result was analyzed by gel electrophoresis at 250 volt for 8 hours using 15% non-denaturing acrylamide gel. Likewise, gel

was stained by ethidium bromide, followed by visualization using UV illuminator.

As shown in Figure 2.20, the effect of cisplatin on FEN1 catalysis is different from the effect of small molecule intercalators. The bands on gel were dispersed upwards along with increasing concentration of cisplatin, which indicated more formation of adduct by cisplatin and two consecutive guanine bases in DNA oligonucleotides, therefore the molecular weight increased and the adducts could not run as fast as product on gel.

Figure 2.20. Effect of cisplatin on FEN1 catalysis. Lane 1: A3Dn only; Lane 2: A3Dn + FEN1; Lane 3: A3Dn + FEN1 + 0.075 mM cisplatin; Lane 4: A3Dn + FEN1 + 0.25 mM cisplatin; Lane 5: A3Dn + FEN1 + 0.5 mM cisplatin; Lane 6: A3Dn + FEN1 + 0.75 mM cisplatin; Lane 7: A3Dn + FEN1 + 1 mM cisplatin; Lane 8: A3Dn + FEN1 + 1.25 mM cisplatin; Lane 9: A3Dn + FEN1 + 1.5 mM cisplatin; Lane 10: A3Dn + FEN1 + 1.75 mM cisplatin; Lane 11: A3Dn + FEN1 + 2 mM cisplatin; Lane 12: A3Dn + FEN1 + 2.25 mM cisplatin; Lane 13: A3Dn + FEN1 + 2.5 mM cisplatin.

2.4 Design and synthesis of plasmid DNA as FEN1 substrate

Other than small oligonucleotide substrates, plasmid DNA substrate was designed and synthesized to study action of FEN1. Phi X 174 virion single-stranded circular DNA was used as template. A 5' flap primer was annealed with template and subsequently added T4 DNA polymerase and dNTP to do polymerization to form a double-stranded plasmid DNA with flap structure. T4 polymerase polymerizes to the 3' end and does not involve strand displacement to lengthen 5' flap. FEN1 was thereafter involved to excise the flap to generate a nicked circular plasmid. Since flap is composed of 5 nucleotides to ensure activity of plasmid substrate towards catalysis, the cleaved product cannot be separated from substrate by electrophoresis due to similar molecular weight. In order to confirm the structure of the sample, ligation by T4 ligase was done to seal the nick. Through agarose gel electrophoresis, the position of band of closed circular plasmid differed from position of nicked or flapped circular plasmid. In case the FEN1 catalysis was not completed, plasmid with flap could not undergo ligation.

2.4.1 DNA sequence

Name of DNA	Nucleotides sequence (5' to 3')
Phi X 174-Dn	TTTTTCAATAGTCACACAGTCCTTG

2.4.2 Materials and Methods

DNA, Enzymes, Chemicals and equipments

Product(s)	Manufacturer
Phi X 174 virion	New England Biolabs (Ipswich, MA, USA)
Deoxynucleotide (dNTP) solution mix	New England Biolabs (Ipswich, MA, USA)
Human flap endonuclease 1 (FEN1)	US Biological (Salem, MA, USA)
T4 DNA polymerase	New England Biolabs (Ipswich, MA, MA, USA)
T4 DNA ligase	New England Biolabs (Ipswich, MA, MA, USA)
Phi X 174 RF II DNA	New England Biolabs (Ipswich, MA, MA, USA)
BAL 31 exonuclease	New England Biolabs (Ipswich, MA, USA)
1 kb DNA ladder	New England Biolabs (Ipswich, MA, MA, USA)
6X DNA loading dye	Fermentas (Waltham, MA, USA)
10X Tris-acetate-EDTA (TAE) buffer, pH 8.0	1st Base (Singapore)
Agarose	1st Base (Singapore)

Ethidium bromide solution, 10 mg/ml	Bio-rad laboratories (Hercules, CA, USA)
Electrophoresis power supply	Consort (Turnhout, Belgium)
Nanophotometer	Implen (München, Germany)
Dual accublock digital dry bath	Labnet international (Edison, NJ, USA)
Vortex mixer	Labnet international (Edison, NJ, USA)
Concentrator	Eppendorf (Hauppauge, NY, USA)
Centrifuge	Eppendorf (Hauppauge, NY, USA)
G:BOX Chemi gel doc system	Syngene (Cambridge, UK)

Buffer: Ultra pure / biotechnology grade

Single-stranded plasmid phi X 174 was annealed with primer followed by polymerization to form double-stranded plasmid which could be excised by FEN1. To prepared plasmid substrate, 0.294 pmol of phi X 174 virion single strand DNA (0.5 ug) and excess phi X 174-Dn (5' primer) were annealed with 20 mM NaCl and 50 mM Tris-HCl for 1.5 hours at 25 °C. Polymerization was subsequently carried out by adding excess dNTP, 3 units of T4 DNA polymerase and 2 ug of BSA and incubated for 3 hours at 37 °C. After polymerization, pH was adjusted to 8.0 in order to optimize condition for FEN1 catalysis. Subsequently, 0.33 ug of phi X 174 flap substrate and 0.35 ug of FEN1 were incubated with 50mM Tris-HCl, 10mM MgCl₂, 1mM ATP, 10mM

DTT for 1 hour at 37 °C. After FEN1 reaction, pH was adjusted to 7.5, sample was added with excess T4 DNA ligase and T4 ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP and 10 mM DTT at pH 7.5) and reacted for 1.5 hours at 25 °C. Electrophoresis was done by 1.2% agarose gel for 10 hours at 80 volt.

One unit of T4 polymerase is defined as the amount of enzyme that incorporates 10 nmol of dNTP into acid insoluble material in 30 minutes at 37 °C [38]. And one unit of T4 DNA ligase is defined as the amount of enzyme required to give 50% ligation of HindIII fragments of λ DNA (5' DNA termini concentration of 0.12 μM, 300 μg/ml) in a total reaction volume of 20 μl in 30 minutes at 16 °C.

2.4.3 Result and discussion

Single-stranded circular phi X 174 plasmid was annealed and polymerized. The polymerized product was compared with phi X 174 RF II DNA, which was double-stranded nicked circular form of phi X 174 DNA (relaxed form) which was 5,386 base pairs in length. Compared to phi X 174 DNA, the polymerized product contains a flap composed of 5 nucleotides. Molecular weight of polymerized product and phi X 174 RF II DNA didn't much differ from each other. By 1.5% agarose gel electrophoresis at 80 volt for 6 hours, the band of product after polymerization was at same level with the band of phi X174 RF II DNA which indicated that the polymerization was completed (Figure 2.21).

Figure 2.21 Result of electrophoresis of polymerized plasmid substrate. Lane 1: Phi X 174 single-stranded DNA; Lane 2: Phi X 174 RF II DNA; Lane 3: Phi X 174 single-stranded DNA after polymerization.

The result of synthesis of plasmid substrate and cleavage by FEN1 was revealed by gel electrophoresis as shown in Figure 2.26. Samples were tested in common agarose gel as well as in gel containing 0.5 ug/ml ethidium bromide. After FEN1 catalysis, the band of product was present at same level before catalysis. In order to identify structure of product, ligation was carried out. The product of ligation was supercoiled double-stranded plasmid. The supercoiled plasmids had same size but in different shapes which could be resolved by agarose gel. Several bands in the last lane as shown in Figure 2.22 (a) were all supercoiled double-stranded circular plasmid DNA.

As comparison, same samples were loaded into gel containing EB. Circular DNA is more strongly affected by ethidium bromide than linear DNA if ethidium bromide is present in the gel during electrophoresis. Ethidium bromide which intercalates into circular DNA can change the charge, length, as well as the superhelicity of the DNA molecules, therefore presence of EB

during electrophoresis can affect its movement in gel ^[39]. DNA in EB-containing gel migrated faster than common agarose gel, especially the ligated circular double-stranded plasmid DNA as shown in Figure 2.22 (b).

Figure 2.22 Result of gel electrophoresis of phi X 174 flap-structured substrate preparation. (a) Agarose gel. Lane 1: 1 kb DNA ladder; Lane 2: Annealed primer and Phi X 174-Dn; Lane 3: Polymerized plasmid; Lane 4: FEN1 catalyzed plasmid; Lane 5: Ligased plasmid. (b) Agarose gel containing ethidium bromide. All the lanes contained the same samples as (a).

The cleaved and ligated susbtrate had been carried out nuclease BAL-31 reaction. Nuclease BAL-31 exonuclease is a highly specific single-stranded endonuclease which cleaves at nicks, gaps and single-stranded regions of duplex DNA. In order to examine whether nicks in plasmid substrate had been fully ligated, excess nuclease BAL-31 was added.

The reaction was carried out with ligated 0.667 ug of phi X 174 plasmid,

0.3 units of nuclease BAL-31 and reaction buffer (20 mM Tris-HCl, 12 mM MgCl₂, 600 mM NaCl, 12 mM CaCl₂, 1 mM EDTA at pH 8.0) for 1 hour at 30 °C in a total volume of 10 µl. Subsequently, mixture was analyzed by 1.2% agarose gel electrophoresis at 80 volt for 10 hours. Gel was stained by ethidium bromide for 30 minutes, followed by visualization using G:BOX Chemi. One unit of nuclease BAL-31 is defined as the amount of enzyme required to remove 200 base pairs from each end of linearized double-stranded phi X 174 DNA (40 µg/ml) in a total reaction volume of 50 µl for 10 minutes at 30 °C.

As shown in Figure 2.23, bands of sample and reference were similar and there was no typical progressive shortening of duplex DNA demonstrated on gel. Thus we inferred that there was few single stranded region (flap or nick site) unreacted after preparation. The result indicated that plasmid substrate had been completely excised by FEN1 and subsequently ligated.

Figure 2.23 Result of BAL-31 exonuclease reacted with ligated plasmid substrate.

Before polymerization, annealing method was explored. Primer and

single-stranded circular plasmid was annealed at 25 °C which was different from annealing short linear single-stranded DNA due to heat sensitive of single-stranded circular plasmid. 0.5 ug of phi X 174 single strand was diluted to 20 µl and incubated at 95 °C for 5 minutes before cooled down to room temperature for 2 hours. Result of stability of circular DNA with reference to non-heated one was analyzed by 1.5% agarose gel electrophoresis at 80 volt for 6 hours. As shown in Figure 2.24, the upper band in the non-heated sample was circular single-stranded phi X 174 plasmid DNA, and the lower band was linear single strand which was impurity from the plasmid we purchased. After heated at 95 °C, single-stranded circular plasmid DNA was mostly decomposed. Therefore, in order to maintain stable circular structure, sample was annealed at 25 °C rather than heated at high temperature.

Figure 2.24 Plasmid DNA single strand heated at high temperature. Lane 1: 1kb DNA ladder; Lane 2: Single-stranded circular plasmid DNA; Lane 3: Single-stranded plasmid DNA after heated at 95 °C.

2.4.4 Summary

A new flap-structured plasmid DNA substrate for FEN1-related study was designed and prepared. Single-stranded circular phi X 174 plasmid was firstly annealed with primer to form a 5' flap structure, followed by reaction with T4 polymerase to produce double-stranded DNA. This substrate could be catalyzed by enzyme FEN1 to generate a nicked double-stranded circular plasmid and the nick could be ligated by T4 ligase to form supercoiled double-stranded circular DNA.

2.5 Conclusion

Property of FEN1 using different DNA oligonucleotide substrates was systematically explored. Flap-structured substrate which possessed 3 nucleotides at 5' flap demonstrated high activity towards FEN1 catalysis. Oligonucleotides with double strands composed of 16, 20 and 24 nucleotides revealed high activity. Additionally, flap-structured duplex substrate demonstrated higher activity than triplex substrate, which suggested that triplex structure could slow down FEN1 catalysis. Besides, resistance of several small molecules, including chloroquine, ethidium bromide, spermidine, netropsin and cisplatin, on action of FEN1 were investigated and their corresponding IC_{50} value had been measured by gel electrophoresis. In addition, a flap-structured plasmid DNA substrate for FEN1 study was designed and synthesized.

Chapter 3 - Investigation of inhibition by phosphorothioate modification and design of oligonucleotide inhibitor

Nuclease FEN1 is capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids. FEN1 is structure specific nuclease, rather than sequence specific nuclease ^[40]. Phosphorothioate bond is widely used for replacement of phosphodiester bond between nucleotides in molecular biochemistry studies. The phosphorothioate linkage is supposed to be resistant to degradation by nucleases. Phosphorothioates are tetrahedral anions, with phosphorus atom at the center of the tetrahedron (Figure 3.1). Among which, mono-phosphorothioate is an analogue of phosphate of substitution interference because of its stability, solubility and relative simplicity in synthesis ^[41, 42]. This modification renders the internucleotide linkage resistant to nuclease degradation. The base composition of phosphorothioate oligonucleotides does not affect the inhibitory effect. Furthermore, the binding of phosphorothioate modified oligonucleotides to enzymes is independent on phosphorus chirality at the internucleotide linkage site ^[43].

Figure 3.1 Phosphorothioate modification between nucleotides.

3.1 Inhibition of phosphorothioate modified substrates on FEN1 activity

3.1.1 Design of phosphorothioate modified substrates

Phosphorothioate bonds at different positions in DNA substrates were explored. Phosphorothioate linkage was substituted at FEN1 cleavage site, adjacent to FEN1 cleavage site in downstream strand and in template strand as well as at the end of 3' upstream strand, respectively. The activity of FEN1 using substrates containing phosphorothioate linkage at different positions was compared with non-modified substrate A3Dn, as illustrated in Figure 3.2.

Figure 3.2 Phosphorothioate modification at different positions in flap-structured DNA substrates.

3.1.2 DNA sequences

Phosphorothioate modified and non-modified substrates for FEN1 cleavage contained same nucleotides sequence.

Name of DNA	Nucleotides sequence (5' to 3')
ASDn	GCT ^{thiol} CGAATTCCTGCAGCCCGG
AS4Dn	GCTC ^{thiol} GAATTCCTGCAGCCCGG
AS2Dn	GC ^{thiol} TCGAATTCCTGCAGCCCGG
ASUp	TCGAGGTCGACGGTATCGATAAGCTTGATA ^{thiol} T
AST	CCGGGCTGCAGGAATTCG ^{thiol} ATATCAAGCTTATCGATA CCGTCGACCTCGA
Down strand 3	GCTCGAATTCCTGCAGCCCGG

Up strand	TCGAGGTCGACGGTATCGATAAGCTTGATAT
Template strand	CCGGGCTGCAGGAATTCGATATCAAGCTTATCGATAC CGTCGACCTCGA
A3S12_6	TTT ^{thiol} CGCCTAGACGCGAAGCGTCTAGGCGACGCCGC GAAGCGGCGT
A4S12_6	TTTT ^{thiol} CGCCTAGACGCGAAGCGTCTAGGCGACGCCG CGAAGCGGCGT
A6S12_6	TTTTTT ^{thiol} CGCCTAGACGCGAAGCGTCTAGGCGACGC CGCGAAGCGGCGT
Down strand 6	ATCGCTCGAATTCCTGCAGCCCGG

3.1.3 Materials and Methods

DNA, Enzyme, Chemicals and equipments

Product(s)	Manufacturer
Human flap endonuclease 1 (FEN1)	US Biological (Salem, MA, USA)
100 bp DNA ladder	Promega (Madison, WI, USA)
6X DNA loading dye	Fermentas (Waltham, MA, USA)
10X Tris-borate-EDTA (TBE) buffer, pH 8.3	1st Base (Singapore)
Acrylamide	Sigma-Aldrich (St. Louis, MO, USA)

N,N'-methylenebisacrylamide	Sigma-Aldrich (St. Louis, MO, USA)
N,N,N',N'-tetramethylethylene diamine (TEMED)	Bio-rad laboratories (Hercules, CA, USA)
Ammonium persulfate	Bio-rad laboratories (Hercules, CA, USA)
Ethidium bromide solution, 10 mg/ml	Bio-rad laboratories (Hercules, CA, USA)
Electrophoresis power supply	Consort (Turnhout, Belgium)
Nanophotometer	Implen (München, Germany)
Dual accublock digital dry bath	Labnet international (Edison, NJ, USA)
Vortex mixer	Labnet international (Edison, NJ, USA)
G:BOX Chemi gel doc system	Syngene (Cambridge, UK)

Buffer: Ultra pure / biotechnology grade

Annealing method for phosphorothioate modified DNA substrates was the same as non-modified DNA substrate. 150 pmol of downstream single strand DNA, 130 pmol of upstream single strand DNA and 100 pmol of template single strand DNA were incubated in 10 mM Tris buffer in a total reaction volume of 20 µl for 5 min at 95°C. Samples were kept in room temperature (25

°C) to cool down for 2 hours to form 5 uM substrates.

Modified and non-modified oligonucleotides (2 pmol) were incubated with 0.18 ug of FEN1, 50 mM Tris, 10 mM MgCl₂, 500 µg/ml BSA and 5 mM DTT at pH 8.0 for 25 minutes at 37 °C. Result was analyzed by 15% non-denaturing acrylamide gel at 250 volt for 8 hours. Ethidium bromide was used to stain gel, followed by visualization using G:BOX Chemi gel doc system.

3.1.4 Result and discussion

Gel analysis revealed the inhibitory effect of phosphorothioate bond on FEN1 catalysis and phosphorothioate modification was position dependent, as shown in Figure 3.3. Modified oligonucleotides generated fewer products than non-modified DNA substrate after FEN1 cleavage. Modification designed at FEN1 cleavage site demonstrated the most efficient inhibition. ASDn didn't generate final product and only generated intermediate product, with the most amount of substrate unreacted. The second and third efficient inhibitions were presented by substrates with phosphorothioate modifications adjacent to FEN1 cleavage site in downstream strand. Modification in template strand near FEN1 cleavage site showed less efficiency, followed by modification at the end of 3' upstream strand near FEN1 cleavage site. Substrate AST generated more than half of final product, meanwhile ASUp directly generated final product and presented little intermediate product.

Figure 3.3 Reactivity comparison of phosphorothioate modified substrates after FEN1 cleavage.

Based on result of gel electrophoresis, comparison of reactivity using phosphorothioate substituted oligonucleotides was created. The percent yield of final product as well as both final and intermediate products was shown in the column graph (Figure 3.4). The ratio of products was measured by GeneTools. The order of reactivity of phosphorothioate modified substrates carried out FEN1 catalysis was ASUp > AST > AS2Dn > AS4Dn > ASDn. Therefore the order of inhibitory effect of phosphorothioate bond at different positions on FEN1 catalysis was in reverse, which was ASDn > AS4Dn > AS2Dn > AST > ASUp.

Figure 3.4 Comparison of percent yield using different phosphorothioate modified substrates after FEN1 catalysis.

3.2 Kinematic study of FEN1 using phosphorothioate modified DNA substrates

In order to further analyze and understand the process of FEN1 catalysis and inhibitory effect of phosphorothioate bond modified at different positions in the substrates, enzymatic kinetics were tested using different substrates.

3.2.1 Method

Substrates were reacted with FEN1 for 5, 10, 20, 40, 60 and 120 minutes at 37 °C respectively. Phosphorothioate substituted oligonucleotides were incubated with 0.23 ug of FEN1 in reaction buffer containing 250 mM Tris, 50 mM MgCl₂, 5 mM DTT and 500 µg/ml BSA at pH 8.0. After reaction, loading buffer was immediately added and samples were stored in -20 °C to quench reaction before analysis by 15% non-denaturing acrylamide gel electrophoresis at 250 volt for 8 hours.

3.2.2 Result and discussion

Non-modified oligonucleotide A3Dn was tested as reference. Result of FEN1 enzyme kinetics was shown in Figure 3.5. After reacted for 10 minutes, removal of flap structure was mostly finished and after reacted for 20 minutes, the sample had little unreacted substrate. Intermediate product which contained 2 nucleotides at 5' flap could be observed when the reaction was incomplete. After incubation for 2 hours, the intermediate product was almost completely further excised to product A1Dn, which had 1 nucleotide at 5' flap.

Figure 3.5 FEN1 enzyme kinetics study using substrate A3Dn.

According to the result from gel electrophoresis, the enzyme kinetics of FEN1 using non-modified flap-structured oligonucleotide as substrate was drawn (Figure 3.6). FEN1 efficiently cleaved substrate under this condition. Along with increasing reaction time, products of A3Dn accumulated. The ratio of products was calculated by GeneTools. Within reaction time of 5 minutes, final product had been generated more than 30%. After 20 minutes, substrate had been completely reacted and final product was accumulated to 80%.

Figure 3.6 Correlation between percentage of product and reaction time using substrate A3Dn for FEN1 catalysis.

Among phosphorothioate substituted DNA substrates, the oligonucleotide with modification at FEN1 cleavage site demonstrated the most efficient inhibition effect. Compared to A3Dn, the activity of FEN1 catalysis using substrate ASDn was significantly decreased (Figure 3.7). After reaction time of 1 hour, ASDn still presented unreacted substrate, and after 2 hours, the substrate was almost reacted. On the other hand, within 2 hours, ASDn only generated intermediate product which contained 2 nucleotides at 5' flap, therefore FEN1 catalysis was incompleted. If excess amount of FEN1 is reacted with ASDn, it could be cleaved to generate final product (data not shown).

Figure 3.7 FEN1 enzyme kinetics study using substrate ASDn.

Phosphorothioate bond designed at FEN1 cleavage site demonstrated the

most efficient inhibition that only produced intermediate compound. The correlation between ratio of intermediate substance and reaction time was plotted (Figure 3.8). After the sample had been reacted for 40 minutes, ASDn generated less than 40% intermediate product and within 2 hours' incubation, percentage of intermediate compound was consistently increased until 90%.

Figure 3.8 Correlation between percentage of product and reaction time using substrate ASDn for FEN1 catalysis.

The reactivity of substrate AS4Dn with phosphorothioate bond adjacent to FEN1 cleavage site, in the duxplex DNA was tested. Result of enzyme kinetics was shown in Figure 3.9. After catalysis by FEN1 for 20 minutes, a little substrate was reacted. After 40 minutes, the reaction was almost finished yielding 80 percent final product and the sum of intermediate and final compounds reached more than 90%.

Figure 3.9 FEN1 enzyme kinetics study using substrate AS4Dn.

Based on gel analysis by GeneTools, the ratio of final and intermediate products of AS4Dn was plotted as shown in Figure 3.10. Within 40 minutes, the accumulation of final product increased rapidly. The reaction rate between 20 minutes and 40 minutes was rapid. 60% of final product had been generated in this period of time. After 40 minutes, there was little intermediate compound further cleaved into final product.

Figure 3.10 Correlation between percentage of product and reaction time using substrate AS4Dn for FEN1 catalysis.

Enzyme kinetics using substrate AS2Dn with phosphorothioate bond in the 5' flap adjacent to FEN1 cleavage site was studied (Figure 3.11). According to result of gel electrophoresis, the percent yield kept increasing until 40 minutes. At 20 minutes, the final product was more than 30% and within 40 minutes,

there was no unreacted substrate and most of the product was final product. Between reaction time of 1 hour and 2 hours, intermediate product was further catalyzed by FEN1. After reacted for 2 hours, AS2Dn had been completely transformed into final product.

Figure 3.11 FEN1 enzyme kinetics study using substrate AS2Dn.

Analysis of enzyme kinetics was plotted in Figure 3.12. Within 40 minutes, the reaction was rapid shown by the steep gradient. Within reaction time of 10 minutes, substrate AS2Dn generated 20% final product and in 20 minutes, AS2Dn produced 40% final product, respectively. After reacted for 40 minutes, the products were composed of 70% final product and 30% intermediate product. Subsequently, intermediate product gradually took further reaction to yield final product.

Figure 3.12 Correlation between percentage of product and reaction time using substrate AS2Dn for FEN1 catalysis.

The reaction progress using oligonucleotide AST with phosphorothioate bond designed in template strand near FEN1 cleavage site was also explored. As shown in Figure 3.13, the reaction rate was rapid between 10 minutes and 20 minutes since most of the final product was generated during this period of time. After reaction time of 20 minutes, there was only a little amount of unreacted AST. During reaction time from 40 minutes to 2 hours, intermediate product was gradually further cleaved by FEN1 into final product.

Figure 3.13 FEN1 enzyme kinetics study using substrate AST.

The reaction process was plotted in Figure 3.14. The gradient of curve before 20 minutes was very high, compared with gradient after 20 minutes. After reaction time of 20 minutes, intermediate product was further reacted with

FEN1 to generate final product.

Figure 3.14 Correlation between percentage of product and reaction time using substrate AST for FEN1 catalysis.

The substrate ASUp containing phosphorothioate bond at the end of 3' upstream strand presented the fastest reactivity as shown in Figure 3.15. Within reaction time of 5 minutes, percentage of final product was highest compared to other modified oligonucleotides. Within 10 minutes, above 50% of the final product had been produced. Moreover, after 20 minutes, only trace amount of substrate ASUp remained unreacted. The gel analysis demonstrated that ASUp was the least effective substrate to inhibit FEN1 reaction.

Figure 3.15 FEN1 enzyme kinetics study using substrate ASUp.

Based on result of gel electrophoresis, percent yield of substrate ASUp had been plotted (Figure 3.16). The production rate of final product before 20

minutes was quite high because of the steep gradient of the curve and above 70% of final product had been produced. The intermediate product was also accumulated to about 20% with little remaining substrate. During the subsequent reaction with FEN1, the intermediate product had been fully reacted with FEN1 and further cleaved to final product.

Figure 3.16 Correlation between percentage of product and reaction time using substrate ASUp for FEN1 catalysis.

3.2.3 Summary

We placed phosphorothioate modifications near or at the FEN1-cutting site. The inhibitory effects of flap-structured oligonucleotides containing phosphorothioate linkages have been examined to identify the most potent position for modification in order to design oligonucleotide inhibitor. Result of enzyme kinetics revealed that phosphorothioate bond substituted at FEN1 cleavage site demonstrated the most effective inhibition to FEN1 catalysis. Phosphorothioate bond modified beside FEN1 cleavage site also demonstrated significant inhibition. Phosphorothioate bond in template strand near FEN1

cleavage site also showed inhibitory effect. The least effective inhibition was placed at the end of 3' in upstream strand.

3.3 Design and study of FEN1 oligonucleotide inhibitor

Based on the results shown by gel electrophoresis, the inhibitory effect of phosphorothioate modified oligonucleotides on FEN1 catalysis was significant. The inhibitory effect of phosphorothioate modification at different positions in oligonucleotides differed from each other, among which the most efficient modification was substituted at FEN1 cleavage site. In order to design the most efficient oligonucleotide inhibitor to resist action of FEN1, the phosphorothioate bond was modified at FEN1 cleavage site (Scheme 3.1). The length of flap was designed to be composed of 3 nucleotides and length of double strands was designed based on mechanism of FEN1 recognition and binding to substrate for minimizing the size of oligonucleotide inhibitor ^[7].

Scheme 3.1 Illustration of interaction between oligonucleotide inhibitor and FEN1.

The annealing condition for oligonucleotide inhibitor A3S12-6 was the

same as for non-modified FEN1 substrate A3Dn. Single strand of phosphorothioate substituted inhibitor was annealed in 10 mM Tris for 5 minutes at 95°C in a total reaction volume of 20 µl. Sample was subsequently chilled at 25 °C for 2 hours.

Oligonucleotide A3Dn was the substrate for testing oligonucleotide inhibition. It had been investigated to be the most efficient substrate for FEN1 catalysis. A3Dn (2 pmol) was reacted with 0.23 ug of FEN1 for 30 minutes at 37°C in a reaction volume of 20 µl. Reaction buffer was composed of 250 mM Tris, 50 mM MgCl₂, 5 mM DTT and 500 µg/ml BSA at pH 8.0. Various concentrations of oligonucleotide inhibitor A3S12-6 was added into each sample. Concentration was ranged from 0.37 uM to 2.81 uM. The inhibitory effect of our newly designed oligonucleotide inhibitor A3S12-6 was analyzed by 15% non-denaturing acrylamide gel electrophoresis at 250 volt for 8 hours, followed by ethidium bromide staining.

The bands on the gel were A3Dn and its according intermediate and final product after catalysis by FEN1 (Figure 3.17). Both oligonucleotide inhibitor and cleaved oligonucleotide inhibitor were not presented on gel since the molecular weight of them were much smaller than A3Dn, therefore these compounds ran much faster than A3Dn. The inhibitory effect of oligonucleotide inhibitor was efficient. The amount of unreacted substrate A3Dn kept increasing proportionally to concentration of inhibitor A3S12_6. Meanwhile the amount of

intermediate product was declined inversely proportional to increasing concentration of oligonucleotide inhibitor.

Figure 3.17 Result of inhibition by different concentrations of oligonucleotide inhibitor.

The substrate that was not added with FEN1 as reference presented trace amount of products due to degradation. The percentage of products in the reference had been deducted when calculating percent yield. In order to calculate the half maximal inhibitory concentration, the correlation between activity and concentration of substrate-based oligonucleotide inhibitor was plotted, as shown in Figure 3.18. The activity was reduced from 90% to 20% when the concentration of inhibitor progressively increased to 2.81 uM. IC_{50} value of oligonucleotide inhibitor was 1.43 uM.

Figure 3.18 Correlation between activity and $\log_{10}$ concentration of oligonucleotide inhibitor after FEN1 catalysis.

Phosphorothioate inhibitor could be excised if excess amount of FEN1 was added (Figure 3.19). Six picomoles of A4S12_6 and A6S12_6 were totally reacted to generate products after reacted for 1 hour at 37 °C with 50 mM Tris, 50 mM MgCl_2 , 5 mM DTT and 500 $\mu\text{g/ml}$ BSA at pH 8.0, in a reaction volume of 20 μl . Result was analyzed by 20% non-denaturing acrylamide gel for 4 hours at 300 volt.

Figure 3.19 Phosphorothioate oligonucleotide inhibitors were fully reacted with excess FEN1. Lane 1: A4S12_6 only; Lane 2: A4S12_6 + excess FEN1; Lane 3: A6S12_6 only; Lane 4: A6S12_6 + excess FEN1.

The inhibition of oligonucleotide inhibitors were further studied when

reacted in a much shorter reaction time and added with excess FEN1 (Figure 3.20). The substrate was A6Dn. Two picomoles of A6Dn was reacted with 1.10 ug of FEN1 for 3 minutes at 37 °C with 250 mM Tris, 50 mM MgCl₂, 5 mM DTT and 500 µg/ml BSA at pH 8.0 in a total reaction volume of 20 µl. The gel analysis demonstrated that 54.1% of A6Dn was excised to product. When 1 uM oligonucleotide inhibitor A3S12_6 was added, the percent yield was decreased to 33.0%. A6Dn was also reacted for 5 minutes with 0.96 ug of FEN1, and generated 73.3% of product. After 1 uM inhibitor A3S12_6 was added, the percent yield was reduced to 54.1%. When inhibitor A3S12_6 was incubated with FEN1 for 10 minutes before added into sample, the percent yield was decreased to 35.7%.

Figure 3.20 The inhibitory effect of oligonucleotide inhibitors within short reaction time on FEN1 catalysis. Lane 1: A6Dn only; Lane 2: A6Dn + 1.10 ug of FEN1, reacted for 3 min; Lane 3: A6Dn + 1.10 ug of FEN1 + 1 uM A3S12_6, reacted for 3 min; Lane 4: A6Dn + 0.96 ug of FEN1, reacted for 5 min; Lane 5: A6Dn + 1 uM A3S12_6 + 0.96 ug of FEN1, reacted for 5 min; Lane 6: 1 uM A3S12_6 incubated with 0.96 ug of FEN1 for 10 min + A6Dn, reacted for 5 min.

Reaction rate was significantly reduced by phosphorothioate modified oligonucleotide inhibitor conducting FEN1 catalysis compared with non-modified substrate. FEN1 was inhibited, rather than blocked. The data were consistent with the study indicated that phosphorothioate bond slowed down reactivity of enzyme but not actually blocked it ^[44].

3.4 Effect of RNA modified flaps in DNA oligonucleotides on catalytic activity of FEN1

During the replication process of DNA in eukaryotic cell, before action of FEN1, RNA primers were essentially involved for polymerization. In order to investigate activity of DNA substrates with remaining RNA primers at 5' flap structure, several DNA substrates were designed to contain different numbers of RNA nucleotides.

3.4.1 DNA sequences

Name of DNA	Nucleotides sequence (5' to 3')
2 Rna Dn	GC[dT][dC][dG][dA][dA][dT][dT][dC][dC][dT][dG][dC][dA][dG][dC][dC][dG][dG]
3 Rna Dn	GCU[dC][dG][dA][dA][dT][dT][dC][dC][dT][dG][dC][dA][dG][dC][dC][dG][dG]
4 Rna Dn	GCUC[dG][dA][dA][dT][dT][dC][dC][dT][dG][dC][dA][dG][dC][dC][dG][dG]

5 Rna Dn	GCUCG[dA][dA][dT][dT][dC][dC][dT][dG][dC][dA][dG][dC][dC][dC][dG][dG]
Up strand	TCGAGGTCGACGGTATCGATAAGCTTGATAT
Template strand	CCGGGCTGCAGGAATTCGATATCAAGCTTATCGATAC CGTCGACCTCGA

DNA substrates with RNA nucleotides could not be annealed using same condition for annealing DNA oligonucleotides, since RNA nucleotides especially in single strands could not endure high temperature. If these substrates were annealed at 95 °C followed by visualized by gel electrophoresis, there were no clear or obvious bands due to decomposition (data not shown). Thus we annealed two single strands (template strand and up strand) which only composed of DNA nucleotides at a ratio of 1:1 with 10 mM Tris, 50 mM sodium chloride at pH 8.0 for 5 minutes at 95 °C, and subsequently cooled at 25 °C for 1.5 hours. Twice the amount of single strand DNA containing RNA nucleotides was added and incubated at 60 °C for 5 minutes. Substrates were cooled down in heating bath to 25 °C for 2 hours. After annealing, oligonucleotides were tested by acrylamide gel. Although the numbers of nucleotides and base sequences were exactly the same for oligonucleotides containing different numbers of RNA nucleotides, a little difference in height on vertical acrylamide gel was observed which should be caused by RNA nucleotides at flap sites as shown in Figure 3.21.

Figure 3.21 Result of DNA oligonucleotides containing different numbers of RNA nucleotides.

DNA substrates with different lengths of RNA at flap to mimic RNA primer in DNA replication were tested. The reactivity of FEN1 using these substrates was explored. DNA substrate A3Dn was utilized as reference. Modified substrates were composed of 2, 3, 4 and 5 RNA nucleotides in flap-structured DNA substrates, respectively (Scheme 3.2). The 5' flap of substrate A2RNA contained 2 RNA nucleotides and 1 DNA nucleotide. Substrate A3RNA was composed of 3 RNA nucleotides at 5' flap structure. On the other hand, oligonucleotide A4RNA contained 3 RNA nucleotides at 5' flap and 1 adjacent RNA nucleotide in duplex. Similarly, oligonucleotide A5RNA had 3 RNA nucleotides at flap and 2 adjacent RNA nucleotides in duplex.

Scheme 3.2 Illustration of RNA modified flap-structured DNA substrates for FEN1 catalysis.

The activity of substrates containing RNA nucleotides at 5' flap around FEN1 cleavage site was compared with DNA substrate A3Dn. Two picomoles of oligonucleotides of A3Dn, A2RNA, A3RNA, A4RNA and A5RNA were reacted with inadequate amount of FEN1 (0.17 ug) for 20 minutes at 37°C in total reaction volume of 20 µl. Reaction buffer was composed of 250 mM Tris, 50 mM MgCl₂, 5 mM DTT and 500 µg/ml BSA at pH 8.0. After reaction, samples were analyzed by 15% non-denaturing acrylamide gel electrophoresis at 250 volt for 8 hours.

The result of gel electrophoresis was shown in Figure 3.22. All the samples were not completely reacted with enzyme. The activity of A2RNA was the only substrate that was higher than reference A3Dn, since more reactant had been cleaved. Activity of A3RNA was similar to reference A3Dn. Meanwhile substrate A4RNA was less active than A3Dn; and A5RNA was the least active

substrate as it left most amount of substrate unreacted.

Figure 3.22 Comparison of activity of DNA substrates with different numbers of RNA nucleotides for FEN1 catalysis.

3.4.2 Enzyme kinetics of FEN1 using RNA modified DNA substrates

In order to further investigate reactivity of DNA substrates with RNA nucleotides at flap, the enzyme kinetics was measured. Reaction was carried out at 37 °C with 2 pmol of RNA modified DNA substrates, 0.23 ug of enzyme FEN1 and reaction buffer in a total reaction volume of 20 µl. Reaction buffer was prepared with 250 mM Tris, 50 mM MgCl₂, 5 mM DTT and 500 µg/ml BSA at pH 8.0. Reaction time was 5 minutes, 10 minutes, 20 minutes, 40 minutes, 1 hour and 2 hours, respectively. After reaction, samples were immediately treated with loading buffer and stored in -20 °C to freeze to quench reaction. In order to analyze activity, 15% non-denaturing acrylamide gel electrophoresis was carried out at 250 volt for 8 hours.

3.4.2.1 Exploration of activity of FEN1 using substrate A2RNA

The activity of flap-structured DNA substrate with two RNA nucleotides at

5' flap was tested (Figure 3.23). A2RNA was quite active since after reacted for 10 minutes, more than 50% of product had been produced. And after reacted for 20 minutes, the reactant had completely turned into product. There was little amount of intermediate product shown on gel which indicated that A2RNA was favored by FEN1, compared with non-modified A3Dn which generated a little intermediate product during reaction and reaction rate was a little slower.

Figure 3.23 Enzyme kinetics study of FEN1 using substrate A2RNA.

The enzyme kinetics using A2RNA as substrate for FEN1 cleavage was plotted as shown in Figure 3.24. Within 5 minutes, A2RNA generated more than 50% of final product and after reacted for 10 minutes, 80% of reactant had been excised to final product. After a reaction period of 20 minutes, the catalysis had been completed. Compared with non-modified DNA substrate A3Dn, within 5 minutes, A3Dn generated less than 40% of final product and within 10 minutes, it produced less than 70% of final product. Besides, between reaction time of 20 minutes and 2 hours, 20% of intermediate product wasn't further excised to final product. Therefore, enzyme kinetics study demonstrated that A2RNA was more active than A3Dn for FEN1 reaction. Result indicated that DNA replication process was very efficient with RNA primers when FEN1

catalysis took place.

Figure 3.24 Correlation between percentage of product and reaction time of FEN1 catalysis using substrate A2RNA.

3.4.2.2 Exploration of activity of FEN1 using substrate A3RNA

Flap-structured DNA substrate with 3 RNA nucleotides at 5' flap was explored (Figure 3.25). The activity was less efficient than A2RNA when conducted catalysis. The reaction was incompleted after 20 minutes. After reaction time of 40 minutes, the modified substrate had been completely excised by enzyme.

Figure 3.25 Enzyme kinetics study of FEN1 using substrate A3RNA.

Enzyme kinetics using A3RNA as susbtrate was plotted in Figure 3.26. Within 10 minutes' reaction time, 35% of final product as well as 40% of

intermediate product had been produced. After reacted for 20 minutes, there was 60% of final product and 30% of intermediate product generated.

Figure 3.26 Correlation between percentage of product and reaction time of FEN1 catalysis using substrate A3RNA.

3.4.2.3 Exploration of activity of FEN1 using substrate A4RNA

Activity of 4 RNA nucleotides modified flap-structured substrate was explored (Figure 3.27). The activity was a little slower than A3RNA. After reacted for 20 minutes, more than 50% of final product had been generated. The reaction was almost completed after 1 hour.

Figure 3.27 Enzyme kinetics study of FEN1 using substrate A4RNA.

Correlation of percentage of product and reaction time of oligonucleotide A4RNA was plotted as shown in Figure 3.28. The generation of final product

kept increasing along with extension of reaction time. Substrate had been cleaved 90% within 20 minutes, among which 60% was final product. After reaction period of 1 hour, 95% of final product had been produced. After 2 hours' catalysis, A4RNA had been completely excised to final product.

Figure 3.28 Correlation between percentage of product and reaction time of FEN1 catalysis using substrate A4RNA.

3.4.2.4 Exploration of activity of FEN1 using substrate A5RNA

FEN1 substrate A5RNA demonstrated least activity with reference to activity of other RNA substituted substrates as well as non-modified substrate A3Dn. Result of gel electrophoresis of kinematic study using A5RNA to conduct FEN1 catalysis was shown in Figure 3.29. There was about one third of final product generated after cleavage by FEN1 for 10 minutes. Within 40 minutes, there was still a little amount of unreacted substrate demonstrated on gel. After 40 minutes, remaining substrate as well as intermediate product were gradually excised.

Figure 3.29 Enzyme kinetics study of FEN1 using substrate A5RNA.

Based on enzyme kinetics study using A5RNA as substrate, the correlation between ratio of products and reaction time was plotted as shown in Figure 3.30. The production of final product kept increasing along with extension of reaction time. 30% of substrate had been transformed into final product at 10 minutes. Within 20 minutes, half of final product had been generated. The ratio of final product was increased to 70% after 40 minutes and increased to 84% after reaction time of 1 hour.

Figure 3.30 Correlation between percentage of product and reaction time of FEN1 catalysis using substrate A5RNA.

3.5 Conclusion

Reactivity of substrates with phosphorothioate bond at different positions at or near FEN1 cleavage site was explored in order to investigate inhibitory effect

of phosphorothioate bond on FEN1 catalysis. Based on result of enzyme kinetics study of FEN1, substrate which had phosphorothioate linkage at FEN1 cleavage site demonstrated highest inhibitory efficiency to FEN1.

Oligonucleotide inhibitor with phosphorothioate bond at FEN1 cleavage site was designed for nuclease-resistance purpose. Inhibiting property was measured and IC_{50} value was tested to be 1.20 μ M.

On the other hand, flap-structured DNA substrates were modified with RNA nucleotides at 5' flap or at both 5' flap and double strand. Activity of these substrates had been explored, compared to non-modified flap-structured FEN1 substrate. The substrate that contained two RNA oligonucleotides at 5' flap demonstrated the highest activity towards FEN1 catalysis, which indicated the efficient process of DNA replication which involved RNA primers in vivo. With more RNA nucleotides possessed by substrates, the lower activity was demonstrated. This phenomenon may due to specific recognition process by enzyme FEN1 thus to ensure accurate cutting.

Chapter 4 - Development of Cy3-(BHQ-2)-based and aminopurine-based fluorescence platforms for screening FEN1 inhibitors

We would like to develop new methods aiming at monitoring FEN1 reaction and screening its inhibitors. Fluorogenetic platforms are efficient than gel electrophoresis when used to analyze progress of catalytic reaction.

4.1 Development of Cy3-(BHQ-2) fluorescence platform

Cy3 is one of the most popular cyanine fluorescent dyes which are used recently in labeling and analysis in biomedical imaging. Cyanine fluorescence dyes advantageously replace conventional dyes such as Fluorescein, yielding brighter and more stable fluorescence at 570 nm. The chemical structure of Cy3 was shown in Scheme 4.1, two nitrogen atoms are joined by a polymethine chain, and both nitrogen atoms are independently part of a heteroaromatic moiety (indole) ^[45]. The Cy3 nomenclature simply indicates its structure, with Cy stands for cyanine and 3 is the number of methines between heteroaromatic structures. The R groups used are short aliphatic chains ^[45]. Cyanine dye Cy3 can be applied to label nucleic acid. In our study, Cy3 was modified internally in single-stranded oligonucleotide at a distance of around 14 nucleotides in space from quencher. Fluorophor Cy3 was detected and quantified by fluorometer. The fluorescence intensity is proportional to the concentration of Cy3 when no quencher presents.

Scheme 4.1 Chemical structures of fluorophore Cy3 and its specific quencher BHQ-2.

The BHQ-2 dark quencher has strong absorption from 599 nm to 670 nm, which provides excellent quenching effect on fluorophore Cy3 with maximum emission at 570 nm. In our study, BHQ-2 was modified at the 5' termini of the oligonucleotide labeled with Cy3. BHQ-2 dye quenches fluorescence of Cy3 by FRET mechanism. The process of FRET describes energy transfer between two light-sensitive molecules ^[46]. A donor chromophore (Cy3), while in its electronic excited state, can transfer energy to an acceptor chromophore (quencher BHQ-2) through nonradiative process ^[47]. The efficiency of this energy transfer process is inversely proportional to the sixth power of the distance between donor chromophore and acceptor chromophore ^[48]. This phenomenon makes FRET extremely sensitive to distance. BHQ-2 quenches fluorescence of Cy3 at a quite short distance between 10 Å to 100 Å ^[27]. In our preliminary study, Cy3 and BHQ-2 were labeled on different molecules and we could hardly observe obvious quenching effect done by BHQ-2. (data not

shown) Therefore, we labeled both Cy3 and BHQ-2 in the same oligonucleotide for quenching effect.

Based on quenching distance, Cy3 was designed at double strand of oligonucleotide, while BHQ-2 was synthesized at 5' flap site. BHQ-2 quenches fluorescence of Cy3 significantly. After FEN1 cleaves 5' flap of oligonucleotide, the flap containing BHQ-2 separates from Cy3. In aqueous solution, distance between Cy3 and BHQ-2 is relatively quite far to achieve fluorescence quenching. Thus Cy3's fluorescent signal increases significantly after action of FEN1. By monitoring fluorescent intensity, it is simple to measure the degree of FEN1 reaction. This Cy3-(BHQ-2) fluorescent system could be utilized to screen FEN1 inhibitors.

4.1.1 DNA sequence

Name of DNA	Nucleotides sequence (5' to 3')
ACB	^{BHQ2} CTATCGAATTCCTGCAGCGCGAAGCGCTGCAGG AATTCGAGCCCGC ^{Cy3} GAAGCGGGCT

4.1.2 Materials and Methods

Enzyme, Chemicals and equipments

Product(s)	Manufacturer
Human flap endonuclease 1 (FEN1)	US Biological (Salem, MA, USA)
3-hydroxy-5-methyl-1-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione (PTPD)	Glix Laboratories (Southborough, MA, USA)
NSC-13755	Aobious Inc (Gloucester, MA, USA)
Aurintricarboxylic acid	Sigma-Aldrich (St. Louis, MO, USA)
Nanophotometer	Implen (München, Germany)
Dual accublock digital dry bath	Labnet international (Edison, NJ, USA)
Vortex mixer	Labnet international (Edison, NJ, USA)
Fluorescence Spectrophotometer	Varian (Santa Clara, CA, USA)

Buffer: Ultra pure / biotechnology grade

Single-stranded oligonucleotide containing internal modified fluorophore Cy3 and quencher BHQ-2 was annealed with 10mM Tris at pH 8.0 for 5 minutes at 88°C. Sample was cooled at 25 °C for 2 hours. The concentration of annealed double-stranded oligonucleotide ACB was 5 uM.

Substrate ACB (4 pmol) containing both fluorophore and quencher was reacted with adequate amount of FEN1 at 37 °C for 30 minutes, in FEN1

reaction buffer (250 mM Tris, 50 mM MgCl₂, 5 mM DTT and 500 µg/ml BSA at pH 8.0). Reaction was taken place in darkness in order to prevent degradation of fluorophore Cy3. The total reaction volume was 20 µl. After reaction, 50 µl of distilled water was added into sample in order to increase volume for measuring fluorescence.

Fluorescence excitation was set at 500 nm to excite fluorophore Cy3, and fluorescence emission was read from 450 to 650 nm to cover signal of Cy3. Both excitation and emission slits were set at 10 nm. Scan rate was 120 nm/min. The fluorescence of substrate in FEN1 reaction buffer was tested as reference.

4.1.3 Result and discussion

After catalysis by FEN1, Cy3 was separated from quencher BHQ-2 since BHQ-2 was modified at 5' flap site which was excised by FEN1. By fluorescence spectroscopy, the spectrum revealed a significant increase of fluorescence emitted by Cy3, compared to reference (Figure 4.1). Result indicated that quencher BHQ-2 was effective and efficient in this fluorescence platform. When Cy3 was quenched by BHQ-2 before catalysis, the spectrum presented a low fluorescence peak for reference sample.

Figure 4.1 Fluorescence emission by Cy3-(BHQ-2) platform at different wavelength after FEN1 catalysis.

4.2 Screening FEN1 inhibitors by Cy3-(BHQ-2) fluorescence platform

Recent research has revealed that FEN1 is over-expressed and plays an essential role in cancer progression. For discovery and development of FEN1 inhibitors, we established a simple and effective method for screening inhibitors. Cy3-(BHQ-2) fluorescence platform was effective and efficient to monitor FEN1 reaction. Therefore we utilized this platform to test effectiveness of several small molecular FEN1 inhibitors.

There are limited numbers of reported FEN1 organic inhibitors in the literature and most of these inhibitors are not commercially available. PTPD (3-hydroxy- 5- methyl- 1- phenylthieno [2, 3-d] pyrimidine- 2, 4 (1H, 3H)- dione) is one of N-dydroxy urea compounds serves as FEN1 inhibitor ^[14] (Scheme 4.2).

Besides, aurintricarboxylic acid (ATA) is a general inhibitor of nucleases. ATA inhibits enzymes that bind nucleic acids, presumably as a competitive inhibitor at the polynucleotide binding sites ^[49].

Reported effective FEN1 inhibitors also included NSC-13755 which had submicromolar inhibitory potency. NSC-13755 possesses arylstibonic core, which has recently been reported by Seiple et al to have inhibition for enzyme FEN1 ^[50]. There is structure-activity relationship demonstrated between structure of NSC-13755 and its inhibitory effect on endonuclease. According to comparison of inhibition between NSC-13755 and its analogues, the pentavalent stibonic acid moiety attached to a benzene ring as well as the carboxylate and nitro group substituted meta or para on the benzene ring are essential to inhibition, indicating polar or negatively charged substituents are of great importance. Besides, arylstibonate is similar to phosphonate and is hypothesized to mimic phosphate backbone in DNA. The lower electronegative Sb (V) with reference to P (V) lengthens the Sb-O bond, compared with length of P-O bond. Meanwhile negative charge density on the oxygen atom of NSC-13755 presented is larger than the phosphonate analogue ^[50].

Scheme 4.2 Structures of organic inhibitors for FEN1. (a) PTPD. (b) ATA. (c) NSC-13755.

Organic inhibitors for FEN1 were tested by Cy3-(BHQ-2) system, including PTPD, NSC-13755 and ATA in order to investigate function of fluorescence platform. Firstly, the amount of FEN1 required for Cy3-(BHQ-2) platform was tested in order to achieve effective fluorescence increase and to avoid adding excess FEN1 when measuring IC₅₀ value of inhibitors. Different concentrations of organic inhibitors were added into Cy3-(BHQ-2) sample and monitored inhibition on FEN1 catalysis, respectively. The effect of inhibition could be measured by decrease of fluorescence intensity of Cy3. 4 picomoles of flap-structured Cy3-(BHQ-2) DNA substrate was reacted with 0.5 ug of FEN1 and incubated at 37 °C for 15 minutes. Various concentrations of organic inhibitors was added into each sample. After catalysis by FEN1, all the samples were tested their fluorescence intensity immediately and organic inhibitors were calculated their IC₅₀ value.

4.2.1 Study of inhibition by PTPD tested by Cy3-(BHQ-2) platform

Aqueous stock solution of PTPD in 1.82 μM concentration was prepared. Reaction buffer was composed of 250 mM Tris, 50 mM MgCl_2 , 5 mM DTT and 500 $\mu\text{g/ml}$ BSA at pH 8.0. The concentration of organic inhibitor PTPD added into each sample was ranging from 0.52 nM to 260.71 nM. The decrease of fluorescence intensity of Cy3 was plotted against wavelength as shown in Figure 4.2. Maximum emission wavelength was at around 565 nm. Intensity of fluorescence was decreased along with increasing concentration of organic inhibitor PTPD.

Figure 4.2 Inhibitory effect of different concentrations of PTPD tested by Cy3-(BHQ-2) platform.

In order to calculate the half maximal inhibitory concentration of inhibitor PTPD, the correlation between activity and concentration of PTPD was plotted as shown in Figure 4.3. The activity was measured at maximum Cy3 emission wavelength 565 nm. The activity declined inversely proportional to increasing

$\log_{10}$ concentration of organic inhibitor PTPD. By dose response function, the IC_{50} value of PTPD tested by Cy3-(BHQ-2) fluorophore-quencher platform was 2.68 nM.

Figure 4.3 Correlation between activity and $\log_{10}$ concentration of PTPD.

4.2.2 Study of inhibition by NSC-13755 tested by Cy3-(BHQ-2) platform

Organic inhibitor NSC-13755 (an aryl stibonic inhibitor) was a recently reported FEN1 inhibitor ^[50]. The compound was initially prepared as 59 μ M aqueous stock solution. 4 picomoles of flap-structured Cy3-(BHQ-2) DNA substrate was reacted with 0.5 μ g of FEN1 at 37 °C for 15 minutes. The reaction buffer for FEN1 cleavage was the same as in previous sections, which was composed of 250 mM Tris, 50 mM $MgCl_2$, 5 mM DTT and 500 μ g/ml BSA at pH 8.0. The concentration of organic inhibitor NSC-13755 added into each sample had a range from 0.19 nM to 0.79 μ M. Fluorescence intensity of Cy3 was measured and IC_{50} value of NSC-13755 was calculated (Figure 4.4).

Figure 4.4 Inhibitory effect of different concentrations of NSC-13755 tested by Cy3-(BHQ-2) platform.

At Cy3 maximum emission wavelength of 565 nm, the activity of FEN1 reduced along with increase of $\log_{10}$ concentration of organic inhibitor NSC-13755, as shown in Figure 4.5. The half maximal inhibitory concentration IC_{50} value of NSC-13755 tested by Cy3-(BHQ-2) fluorophore-quencher platform was 2.97 nM.

Figure 4.5 Correlation between activity and $\log_{10}$ concentration of NSC-13755.

4.2.3 Study of inhibition by ATA tested by Cy3-(BHQ-2) platform

We subsequently measured inhibitory effect of aurintricarboxylic acid (ATA), a compound previously revealed to be a broad-specificity inhibitor for multiple classes of DNA-processing enzymes, such as nucleases and polymerases^[51]. In aqueous solution, aurintricarboxylic acid forms stable free radical that inhibits protein-nucleic acid interactions. ATA prevents the binding of nucleic acid to the enzymes^[52]. NMR studies indicate that the mechanism of action involves competition between the nucleic acid and the polymeric ATA for binding to the active site of the enzymes^[53].

Organic inhibitor aurintricarboxylic acid (ATA) aqueous stock solution in 23.7 μ M concentration was prepared. 4 picomoles of flap-structured Cy3-(BHQ-2) DNA substrate was reacted with 0.5 μ g of FEN1 at 37 °C for 15 minutes. The reaction buffer of FEN1 was identical as in previous sections. The concentration of organic inhibitor ATA added into each sample was ranging from 0.15 nM to 79.23 nM. Reaction volume was 20 μ l. Fluorescence intensity of Cy3 was measured and IC₅₀ value of aurintricarboxylic acid (ATA) was calculated (Figure 4.6).

Figure 4.6 Inhibitory effect of different concentrations of ATA tested by Cy3-(BHQ-2) platform.

Similarly, the activity of FEN1 measured at maximum emission wavelength of Cy3 was decreased along with the increase of $\log_{10}$ concentration of aurintricarboxylic acid as shown in Figure 4.7. Based on the data, the IC_{50} value of aurintricarboxylic acid tested by Cy3-(BHQ-2) fluorophore-quencher platform was 4.90 nM.

Figure 4.7 Correlation between activity and $\log_{10}$ concentration of ATA.

4.2.4 Study of inhibition by oligonucleotide inhibitor tested by Cy3-(BHQ-2) platform

Our newly designed oligonucleotide inhibitor A3S12_6 was explored using Cy3-(BHQ-2) fluorescence platform. Flap-structured Cy3-(BHQ-2) DNA substrate (4 pmol) was reacted with 0.5 ug of FEN1 at 37 °C for 15 minutes. FEN1 reaction buffer was 250 mM Tris, 50 mM MgCl₂, 5 mM DTT and 500 µg/ml BSA at pH 8.0. The concentration of oligonucleotide inhibitor A3S12_6 added into each sample was ranging from 0.014 uM to 7.39 uM (Figure 4.8).

Figure 4.8 Inhibitory effect of different concentrations of oligonucleotide inhibitor tested by Cy3-(BHQ-2) platform.

At maximum fluorescence emission wavelength of fluorophore Cy3, the correlation between activity and log₁₀ concentration of our newly designed phosphorothiate-modified oligonucleotide inhibitor was plotted (Figure 4.9). The activity declined along with the increasing log₁₀ concentration of

oligonucleotide inhibitor. The half maximal inhibitory concentration IC_{50} value of oligonucleotide inhibitor A3S12_6 tested by Cy3-(BHQ-2) fluorophore-quencher platform was 0.37 μ M.

Figure 4.9 Correlation between activity and $\log_{10}$ concentration of oligonucleotide inhibitor.

4.3 Development of 2-aminopurine fluorescence platform

Studies demonstrate that there is energy transfer from normal DNA bases to 2-aminopurine base in DNA oligonucleotide. The energy transfer can be identified directly by fluorescence spectroscopy. Stacks of adenine (dA) adjacent to 2-aminopurine (2-AP) act as an antenna for 2-AP excitation to emit fluorescence, which are the most efficient energy donor ^[54]. We designed poly (dA) composed of 3 adenines. More adenines would play insignificant role in energy transfer. On the other hand, a guanine (dG), cytosine (dC) or thymine (dT) base adjacent to adenine and 2-aminopurine effectively blocks energy transfer from stacks of adenine to 2-aminopurine. Moreover, energy transfer from adenine to 2-aminopurine is both efficient from 5' and 3' sides ^[16]. Based

on the theory of sequence-dependent energy transfer to 2-aminopurine, two sequences of oligonucleotides were designed and tested to identify which was more effective for establishment of our fluorescence platform in order to screen FEN1 inhibitors, as illustrated in Scheme 4.3. In addition, in DNA double strands, thymine (dT) and 2-aminopurine (2-AP) can form base pairs, therefore dT is designed opposite to the position of 2-AP.

Scheme 4.3 Oligonucleotide sequences designed for 2-aminopurine modified fluorescence platform.

The originality of our design was that we modified 2-aminopurine between poly (dA) and guanine for blockage of energy transfer, instead of putting guanine in between. The reason was that the oligonucleotide was designed to be cleaved by FEN1. If we put guanine between energy donor poly (dA) and 2-AP, no matter FEN1 excised at which side of guanine, the energy donor would be separated from 2-AP. However, if 2-AP was designed in between, after FEN1 cleavage, the energy donor would be separated from energy blocker guanine. Before we used 2-AP platform to screen FEN1 inhibitors, it was essential to

explore whether guanine could block energy transfer and resisted fluorescence emission by 2-AP. Moreover, after FEN1 catalysis, the fluorescence intensity should be increased without presence of energy transfer blocker guanine.

4.3.1 DNA sequences

Name of DNA	Nucleotides sequence (5' to 3')
A2AP-A	AAA ^{2AP} GGAATCTCTGCAGCGCGAAGCGCTGCAGAG ATTCCTACCACGCGAAGCGTGGTA
A2AP-G	TGG ^{2AP} AAAGTCCCTGCAGCGCGAAGCGCTGCAGGG ACTTTTAATCCGCGAAGCGGATTA

4.3.2 Method

Single-stranded oligonucleotides containing internal 2-aminopurine modification were annealed in 10mM Tris at pH 8.0 for 5minutes at 88°C, based on melting points of single strand DNA. Substrates were chilled at 25 °C for 2 hours. The concentration of annealed double-stranded DNA (A2AP-A and A2AP-G) was 5 uM.

The fluorescence efficiency of 2-aminopurine is comparatively lower than fluorophore Cy3. In order to observe notable fluorescence peaks, more substrates and FEN1 were required in each sample. Fifty picomoles of flap-structured DNA containing 2-aminopurine was reacted with FEN1 for 40 minutes at 37 °C in FEN1 reaction buffer (250 mM Tris, 50 mM MgCl₂, 5 mM

DTT, 500 $\mu\text{g/ml}$ BSA at pH 8.0). Reaction was taken place in darkness to protect fluorescence of 2-aminopurine. The reaction volume was 20 μl , but after reaction, 50 μl of distilled water was added into sample to increase volume for test by fluorescence spectrometry. As reference, the fluorescence intensity of DNA substrate with presence of FEN1 reaction buffer was also measured.

Fluorescence excitation was set at 260 nm for 2-aminopurine and fluorescence emission was read from 240 to 480 nm to cover maximum fluorescence emission by 2-AP around 370 nm. Both excitation and emission slits were set at 10 nm. Scan rate was 120 nm/min.

4.3.3 Result and discussion

As shown in Figure 4.10, although d (GG) nucleotides sequence blocked energy transfer from poly (dA) to 2-aminopurine, the substrate demonstrated an obvious fluorescence peak. After catalytic reaction by FEN1, the blockage of energy transfer to 2-aminopurine was removed as d (GG) nucleotides separated from poly (dA) by FEN1 cleavage. By fluorescence spectroscopy, the spectrum revealed an increase of fluorescence emitted by 2-aminopurine in A2AP-A with poly (dA) at 5' flap. However, fluorescence of A2AP-G with d (GG) at flap site did not demonstrate significant increase after identical amount of excess FEN1 added (Figure 4.11). On the other hand, the curve presenting the correlation between fluorescence intensity and wavelength was relatively flat. Based on this result, A2AP-A oligonucleotide possessed potential to be developed as

platform for monitoring FEN1 catalysis, however A2AP-G would not be suitable to be further explored as platform.

Figure 4.10 Effect of FEN1 catalysis on fluorescence intensity of 2-aminopurine modified oligonucleotide A2AP-A.

Figure 4.11 Effect of FEN1 catalysis on fluorescence intensity of 2-aminopurine modified oligonucleotide A2AP-G.

4.4 Screening FEN1 inhibitors by 2-aminopurine fluorescence platform

4.4.1 Study of inhibition by PTPD tested by 2-aminopurine platform

Single-stranded substrate A2AP-A with internal 2-aminopurine modification was annealed in 10mM Tris (pH 8.0) at 88°C for 5minutes, followed by chilled at 25 °C for 2 hours. The concentration of annealed double stranded DNA was prepared to be 25 uM, which was more concentrated from previous study, due to limited reaction volume when organic inhibitors added into samples.

Organic inhibitors for resistance of FEN1 were explored by 2-aminopurine fluorescence platform. Inhibitory effect by PTPD, NSC-13755, ATA as well as our newly designed phosphorothioate modified oligonucleotide inhibitor was measured by decrease of fluorescence intensity of 2-AP. Since the fluorescence efficiency of 2-aminopurine was lower than Cy3, a larger reaction scale was applied to each reaction. Adequate amount of FEN1 and according reaction time required were explored. 62.5 picomoles of flap-structured 2-aminopurine DNA substrate was reacted with 3.8 ug of FEN1 and incubated at 37 °C for 30 minutes. A concentration series of organic inhibitors were added into each sample. Subsequently, fluorescence intensity was tested immediately and IC₅₀ values of enzyme inhibitors were calculated.

PTPD aqueous stock solution in 1.82 uM concentration was prepared. The concentration of organic inhibitor PTPD added into each sample was ranging from 0.52 nM to 260.71 nM, which was the same as concentrations explored in

Cy3-(BHQ-2) fluorescence platform. The fluorescence intensity of 2-aminopurine at different wavelengths was plotted in Figure 4.12.

Figure 4.12 Inhibitory effect of PTPD on fluorescence intensity of 2-aminopurine platform.

The 2-aminopurine fluorescence platform was different from Cy3 fluorescence platform. Since nucleotide G could not completely quench the fluorescence of 2-aminopurine and the substrate presented an inevitable fluorescence peak, we conducted the experiments to monitor the effect of different concentrations of inhibitors on the fluorescence of 2-aminopurine. Different concentrations of PTPD, NSC-13755, ATA and our oligonucleotide inhibitor were added into mixture of 2-aminopurine substrate and reaction buffer. Samples were reacted under the same condition (at 37 °C for 30 minutes) as the FEN1 catalytic reaction. At low concentration, inhibitors had little influence on the 2-aminopurine substrate; however at high concentration,

inhibitors decreased the fluorescence of 2-AP platform (data not shown). Since these influential concentrations were much higher than the IC_{50} value and concentration required to totally inhibit fluorescence, the factor of concentration didn't affect fluorescence of 2-aminopurine substrate significantly when testing IC_{50} value of inhibitors. Besides, in order to minimize the intrinsic influence of 2-aminopurine fluorescence platform for screening of FEN1 inhibitors, the effect of inhibitors themselves on the platform was deducted when plotting the graphs of correlation between fluorescence intensity and wavelength, as well as calculating IC_{50} value.

After corrected the influence of different concentrations of PTPD on the fluorescence emission by 2-aminopurine substrate, the correlation between activity and $\log_{10}$ concentration of inhibitor PTPD was plotted. When $\log_{10}$ concentration of PTPD became higher, the fluorescence of 2-aminopurine substrate declined, as shown in Figure 4.13. In order to quantify the ability of inhibition, the IC_{50} value of PTPD tested by 2-aminopurine fluorescence platform was 2.07 nM.

Figure 4.13 Correlation between activity and $\log_{10}$ concentration of PTPD.

4.4.2 Study of inhibition by NSC-13755 tested by 2-aminopurine platform

The inhibitory effect of organic inhibitor NSC-13755 on 2-aminopurine modified DNA oligonucleotide (A2AP-A) and IC_{50} value was tested. The arylstibonic acid derivative NSC-13755 was initially prepared as 59 μ M aqueous stock solution. 62.5 picomoles of flap-structured 2-aminopurine modified DNA substrate was reacted with 3.8 μ g of FEN1, incubated at 37 °C for 30 minutes. The reaction buffer of FEN1 catalysis was 250 mM Tris, 50 mM $MgCl_2$, 5 mM DTT and 500 μ g/ml BSA at pH 8.0. The concentration of organic inhibitor NSC-13755 added into each sample had a wide range from 0.78 nM to 197.23 nM. Fluorescence intensity of 2-aminopurine was measured and IC_{50} value of NSC-13755 was calculated (Figure 4.14). The maximum emission wavelength of 2-aminopurine was affected by NSC-13755 to be 340 nm.

Figure 4.14 Inhibitory effect of NSC-13755 on fluorescence intensity of 2-aminopurine platform.

Furthermore, the correlation between FEN1 activity measured at maximum emission wavelength of 2-aminopurine oligonucleotide and $\log_{10}$ concentration of organic inhibitor NSC-13755 had been drawn in Figure 4.15. The fluorescence of 2-aminopurine was sensitively inhibited by NSC-13755. From the graph, IC_{50} value of NSC-13755 was 0.47 nM which demonstrated great inhibitory effect on FEN1 catalysis.

Figure 4.15 Correlation between activity and $\log_{10}$ concentration of NSC-13755.

4.4.3 Study of inhibition by ATA tested by 2-aminopurine platform

Aurintricarboxylic acid (ATA) as organic inhibitor for FEN1 was also explored using 2-aminopurine fluorescence platform. Aqueous stock solution of ATA in 23.7 μM concentration was prepared. Same amount of 62.5 picomoles of flap-structured 2-AP DNA substrate was incubated with 3.8 μg of FEN1 at 37 $^{\circ}\text{C}$ for 30 minutes. The reaction buffer for FEN1 cleavage was 250 mM Tris, 50 mM MgCl_2 , 5 mM DTT and 500 $\mu\text{g/ml}$ BSA at pH 8.0. The concentrations of organic inhibitor ATA added into each sample were ranging from 0.15 nM to 9.89 nM. Reaction volume was 20 μl , subsequently each sample was added with 50 μl of distilled H_2O to test fluorescence. Fluorescence intensity of 2-AP was measured and IC_{50} value of aurintricarboxylic acid (ATA) was calculated.

As shown in Figure 4.16, other than signals of 2-AP at 340 nm, there were fluorescence signals at 430 nm, with increasing intensity along with concentration of aurintricarboxylic acid. We could infer that there peaks were

generated by aurintricarboxylic acid itself. The maximum emission wavelength of 2-aminopurine was affected by ATA which shifted to around 340 nm.

Figure 4.16 Inhibitory effect of ATA on fluorescence intensity of 2-aminopurine platform.

According to the data of fluorescence intensity of 2-aminopurine reacted with FEN1 which was inhibited by ATA, correlation between FEN1 activity and $\log_{10}$ concentration of aurintricarboxylic acid was plotted as shown in Figure 4.17. Activity of FEN1 declined with increasing $\log_{10}$ concentration of ATA. The IC_{50} value of FEN1 inhibitor aurintricarboxylic acid was 0.186 nM tested by 2-aminopurine fluorescence platform.

Figure 4.17 Correlation between activity and $\log_{10}$ concentration of ATA.

4.4.4 Study of inhibition by oligonucleotide inhibitor tested by 2-aminopurine platform

Our newly designed oligonucleotide inhibitor A3S12_6 was explored using 2-aminopurine fluorescence platform. Inhibitor, 2-AP substrate and FEN1 were reacted at 37 °C for 30 minutes. FEN1 reaction buffer was composed of 250 mM Tris, 50 mM MgCl_2 , 5 mM DTT and 500 $\mu\text{g/ml}$ BSA at pH 8.0. The concentration of oligonucleotide inhibitor A3S12_6 added into each sample was ranging from 0.014 μM to 7.39 μM .

The maximum emission wavelength of 2-aminopurine was at 370 nm. Within a certain range of concentrations of our newly designed phosphorothioate-modified oligonucleotide inhibitor, the fluorescence emission of 2-AP substrate was reduced as shown in Figure 4.18.

Figure 4.18 Inhibitory effect of oligonucleotide inhibitor on fluorescence intensity of 2-aminopurine platform.

The correlation between FEN1 activity and $\log_{10}$ concentration of newly designed oligonucleotide inhibitor A3S12_6 was drawn, as shown in Figure 4.19. The activity was measured at maximum 2-AP emission wavelength of 370 nm. The activity declined along with the increase of $\log_{10}$ concentration of our designed oligonucleotide inhibitor. In order to quantify the inhibitory ability of oligonucleotide inhibitor, IC_{50} value was tested to be 6.07 μ M.

Figure 4.19 Correlation between activity and $\log_{10}$ concentration of oligonucleotide inhibitor.

In the assays of testing fluorescence emission by Cy3-(BHQ-2) and 2-aminopurine platforms, all the organic inhibitors and our newly designed oligonucleotide inhibitor demonstrated a strong concentration-dependent inhibition towards FEN1 cleavage.

By using Cy3-(BHQ-2) fluorescence platform and 2-aminopurine fluorescence platforms, we tested the IC_{50} value of organic inhibitors, which was close to originally reported data, thus validating establishment of our fluorescence platforms ^[14, 56]. Besides, the IC_{50} values derived from the two fluorescence platforms presented revealed very little assay-to-assay variation. We believe that both platforms have potential to be further developed for screening FEN1 inhibitors.

4.5 Conclusion

To conclude, two effective fluorescence platforms have been established

for screening FEN1 inhibitors. The Cy3-(BHQ-2) platform is relatively more efficient than 2-aminopurine platform since it requires less amount of substrate to monitor the change of fluorescence. Besides, high concentration of inhibitors may affect the fluorescence of 2-aminopurine platform, therefore correction is required. On the other hand, the Cy3-(BHQ-2) fluorescence platform is stable despite the increasing concentrations of inhibitors, thus it is more convenient for screening of FEN1 inhibitors. Furthermore, quenching effect of BHQ-2 on Cy3 is more efficient than sequence-dependent blocking effect on 2-aminopurine.

A concentration-dependent inhibition was demonstrated for chemical inhibitors (PTPD, NSC-13755, ATA) and our designed phosphorothioate-modified oligonucleotide inhibitor A3S12_6 for FEN1 catalysis. The IC_{50} value of organic inhibitors tested was in nanomolar scale; while oligonucleotide inhibitor was in micromolar scale, which demonstrated that the organic inhibitors were more efficient to resist FEN1 catalysis. The IC_{50} value calculated by Cy3-(BHQ-2) platform and 2-aminopurine platform were consistent.

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Appendix

Table A1. DNA sequence of plasmid phi X 174 virion DNA.

Nucleotides sequence (5' to 3')
GAGTTTTATCGCTTCCATGACGCAGAAGTTAACACTTTCGGATATTTTC
TGATGAGTCGAAAAATTATCTTGATAAAGCAGGAATTACTACTGCTTG
TTTACGAATTAAATCGAAGTGGACTGCTGGCGGAAAATGAGAAAATT
CGACCTATCCTTGCGCAGCTCGAGAAGCTCTTACTTTGCGACCTTTTCG
CCATCAACTAACGATTCTGTCAAAAACCTGACGCGTTGGATGAGGAGA
AGTGGCTTAATATGCTTGGCACGTTTCGTCAAGGACTGGTTTAGATATG
AGTCACATTTTGTTCATGGTAGAGATTCTCTTGTTGACATTTTAAAAG
AGCGTGGATTACTATCTGAGTCCGATGCTGTTCAACCACTAATAGGTA
AGAAATCATGAGTCAAGTTACTGAACAATCCGTACGTTTCCAGACCG
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