Investigation of human flap structure-specific endonuclease 1 (FEN1) activity on primer-template models and exploration of a substrate-based FEN1 inhibitor

Wu, Haixia; Ye, Ruijuan; Huang, Dejian; Li, Tianhu; Ba, Sai; Zhang, Hao; Lee, Jasmine Yiqin

2016

Ba, S., Zhang, H., Lee, J. Y., Wu, H., Ye, R., Huang, D., et al. (2016). Investigation of human flap structure-specific endonuclease 1 (FEN1) activity on primer-template models and exploration of a substrate-based FEN1 inhibitor. *Bioorganic & Medicinal Chemistry*, 24(9), 1988-1992.

<https://hdl.handle.net/10356/85086>

<https://doi.org/10.1016/j.bmc.2016.03.025>

© 2016 Elsevier. This is the author created version of a work that has been peer reviewed and accepted for publication by *Bioorganic & Medicinal Chemistry*, Elsevier. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<http://dx.doi.org/10.1016/j.bmc.2016.03.025>].

Downloaded on 26 Apr 2023 21:52:48 SGT

Graphical abstract

Investigation of human flap structure-specific endonuclease 1 (FEN1) activity on primer-template models and exploration of a substrate-based FEN1 inhibitor

pp xxx-xxx

Sai Ba, Hao Zhang, Jasmine Yiqin Lee, Haixia Wu, Ruijuan Ye, Dejian Huang, Tianhu Li *

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Investigation of human flap structure-specific endonuclease 1 (FEN1) activity on primer-template models and exploration of a substrate-based FEN1 inhibitor

Sai Ba^a, Hao Zhang^a, Jasmine Yiqin Lee^a, Haixia Wu^a, Ruijuan Ye^a, Dejian Huang^b, Tianhu Li^{a,*}^a Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, Singapore 637371, Singapore^b Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

ARTICLE INFO

Article history:

Received 29 January 2016

Revised 9 March 2016

Accepted 14 March 2016

Available online xxxx

Keywords:

FEN1

Phosphorothioate

Inhibitor

Primer

Electrophoresis

ABSTRACT

Flap structure-specific endonuclease 1 (FEN1) is one of the enzymes that involve in Eukaryotic DNA replication and repair. Recent studies have proved that FEN1 is highly over-expressed in various types of cancer cells and is a drug target. However, a limited number of FEN1 inhibitors has been identified and approved. Herein, we investigate the catalytic activity of FEN1, and propose a substrate-based inhibitor. As a consequence, one of the phosphorothioate-modified substrates is proved to exhibit the most efficient inhibitory effect in our in vitro examinations. A novel-designed substrate-based FEN1 inhibitor was accordingly constructed and determined a remarkable IC₅₀ value.

© 2016 Published by Elsevier Ltd.

1. Introduction

Human flap structure-specific endonuclease 1 (FEN1) is known to play critical roles in DNA replication and DNA repair process in Eukaryotic cells. The action of FEN1 in vivo is proved to cleave the 5' overhanging flap (primer) on lagging strand, generating nicked DNA duplex with a phosphate group attached to 5'-end.¹ DNA ligase I takes further action to seal the nicked duplex.² A number of studies on the up-regulation of (FEN1) in cancer cells have been conducted in the past few decades.^{1–4} Nikolova et al. (2009) examined FEN1 expression in the specimens of several tumor tissues, and proved that FEN1 is highly over-expressed in testis, lung and brain tumors in Homosapiens and FEN1 is a drug target.^{1,2} Additional studies carried out by Lam et al. revealed that FEN1 is up-regulated in prostate cancer cells in human and Wang et al. reported in 2014 that over-expression of FEN1 in gastric cancer is correlated with tumor size, lymphatic metastasis and degree of differentiation.^{3,4} From the time when the roles of FEN1 in various cancer cells were discovered, researchers have started on searching for proper FEN1 inhibitors. Even though no valid lead compound as FEN1 inhibitor has been identified for further development of anti-cancer drug candidate thus far, FEN1-specific inhibitors have still been considered to have chemotherapeutic potential against cer-

tain types of cancers.⁵ Most of the previous reported FEN1 inhibitors are small organic molecules which are known to have serious side effects to other metabolic processes.⁶ Moreover, organic inhibitors may cause drug resistance due to the genetic mutation occurred in vivo.^{7,8} A new category of effective and specific FEN1 inhibitors is therefore required to overcome these obstacles in anti-cancer drug design. Here for the first time, we designed and prepared a novel FEN1 potential inhibitor, the structure of which possesses a phosphorothioate functional core positioned within a dumbbell-shape DNA duplex structure. It is our anticipation that this pseudo-substrate molecule could be of great use to slow down FEN1-catalyzed DNA hydrolysis reaction, and could act as an alternative inhibitor against FEN1.

As illustrated in Figure 1A, FEN1 is demonstrated to cleave the phosphodiester bonds between the nucleotide subunits of nucleic acids.⁹ Various strategies have been developed so far for substrates to resist FEN1 hydrolysis, among which incorporation of phosphorothioate bonds is effective.^{10,11} As reported by Agrawal in 1988, phosphorothioate bond is incorporated to replace phosphodiester bond.¹² This modification is considered to render the internucleotide linkage resistant against nuclease degradation.¹¹

Here in our studies, phosphorothioate modification was introduced into different positions in the primer-template FEN1 substrates, which consist of an upstream DNA strand, a primer strand and a template strand (Fig. 1). Furthermore, we examined inhibitory effects of these phosphorothioate-modified substrates

* Corresponding author. Tel.: +65 6513 7364; fax: +65 6791 1961.

E-mail address: thli@ntu.edu.sg (T. Li).

Figure 1. Schematic illustration of (A) natural FEN1 substrate hybridized from upstream, primer and template strands, and (B) our newly-designed phosphorothioate-modified FEN1 substrates.

on the action of FEN1 hydrolysis. Substrates containing phosphorothioate linkage located at exactly FEN1 cleavage site of DNA substrate or adjacent positions were therefore designed and examined in order to determine the most efficient modified-substrate to design FEN1 inhibitor.

2. Material and methods

2.1. Preparation of primer-template models

Three oligonucleotide segments (upstream strand, primer strand and template strand) were mixed in 10 mM Tris buffer and incubated at 95 °C for 5 min, and gradually cooled down to room temperature during a period of 2 h, followed by storage at –20 °C, as shown in Supporting information.

2.2. FEN1-catalyzed DNA hydrolysis reaction

Modified or unmodified DNA substrates (2 pmol) were incubated with FEN1 (0.2 µg) in the presence of 50 mM Tris at pH 8.0, 10 mM MgCl₂, 0.5 mg/ml BSA and 5 mM DTT in a 20 µl reaction volume at 37 °C for 25 min (except indicating). FEN1-catalyzed DNA cleavage was terminated by adding 4 µl of reaction stopping buffer (15% Ficoll-400, 66 mM EDTA, 19.8 mM Tris–HCl, 0.1% SDS and 0.09% bromophenol blue) followed by storage at –20 °C. Reaction mixtures were analyzed by 15% non-denaturing acrylamide gel at 10 V/cm at room temperature. Gel was stained by ethidium bromide before visualization by G:BOX Chemi gel documentation systems (Syngene, Cambridge, UK). Band intensity was quantified by GeneTools software (Syngene, Cambridge, UK).

2.3. Determination of efficiency of substrate-based FEN1 inhibitor

Substrate **1** (2 pmol) was reacted with FEN1 (0.23 µg) at 37 °C for 30 min in a total volume of 20 µl, in the absence or the presence of 0.4–2.8 µM of substrate-based inhibitor. Reaction buffer consists of 50 mM Tris at pH 8.0, 10 mM MgCl₂, 0.5 mg/ml BSA and 5 mM DTT. Their inhibitory effects were analyzed by 15% non-denaturing acrylamide gel electrophoresis at 10 V/cm at room temperature. After electrophoresis, gel was stained by ethidium bromide and visualized under UV irradiation.

2.4. Nucleotide sequences of DNA fragments

The nucleotide sequences of single-stranded DNA fragments used to construct FEN1 substrates are listed in the following Table 1.

3. Results and discussion

The position of phosphorothioate bonds on the DNA substrate was explored in the first part. Phosphorothioate linkage was incorporated at FEN1 cleavage site (**2**), adjacent to FEN1 cleavage site in downstream strand (**3**, **4**) and at the end of 3' upstream strand (**5**) as well as in template strand (**6**), respectively (Fig. 1B). The activity of FEN1 using substrates containing phosphorothioate linkage at different positions was compared with unmodified substrate (**1** in Fig. 1).

As shown in Figure 2, our electrophoretic result revealed that the inhibitory effect of phosphorothioate bond on FEN1 catalysis and the inhibition was position-dependent. The band that migrates slowly on the gel in Figure 2B is the unreacted DNA substrate. After FEN1 cleavage, compared to lane 1 in Figure 2B, the remaining part is believed to migrate faster, which appears lower on the gel. The phosphorothioate-modified FEN1 substrates generate less product than unmodified DNA substrate (**1**) after FEN1 cleavage. As shown in lane 3 in Figure 2B, substrate **2** with modification designed at FEN1 cleavage site demonstrates the highest inhibition. Substrate **2** generates intermediate compound rather than product under this reaction condition, and demonstrates more unreacted substrate (lane 3 in Fig. 2B). Meanwhile, substrates with phosphorothioate modification adjacent to FEN1 cleavage site in primer strands (**3** and **4**) exhibit moderate inhibitory effect (lanes 4 and 5 in Fig. 2B). Substrate with phosphorothioate-modification in template strand near FEN1 cleavage site (**6**) shows lower inhibitory effect. However, substrate with modification at the end of 3' upstream strand near FEN1 cleavage site (**5**) demonstrates the lowest inhibitory efficiency.

Moreover, comparison of FEN1 activity on different phosphorothioate-modified substrates was shown in the column graph (Fig. 2C). The order of FEN1 activity on these substrates is as fol-

Table 1
Name and nucleotide sequences of DNA fragments

Name	Oligonucleotide sequences (5' to 3')
Primer 1	GCTCGAATTCCTGCAGCCCGG
Primer 2	GCT [*] CGAATTCCTGCAGCCCGG
Primer 3	GCTC [*] GAAATTCCTGCAGCCCGG
Primer 4	GC [*] TGCAATTCCTGCAGCCCGG
Upstream 5	TCGAGGTCGACGGTATCGATAAGCTTGATA [*] T
Template 6	CCGGGCTCGAGGAATTCG [*] ATATCAAGCTTATCGATACCGTCGACCTCGA
Upstream	TCGAGGTCGACGGTATCGATAAGCTTGATAT
Template	CCGGGCTCGAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGA
Inhibitor	TTT [*] CGCCTAGACGCGAAGCGTCTAGCGACGCCGCGAAGCGGCGT

^{*} Phosphorothioate bond.

Figure 2. (A) Illustration of FEN1 catalyzed flap-structured DNA cleavage process. (B) Examination of FEN1 activities on different phosphorothioate-modified or unmodified substrates. (C) Comparison of product yield after FEN1 catalysis on different DNA substrates.

Figure 3. Dynamic studies of FEN1 activity on (A) substrate 1, (B) substrate 2, (C) substrate 3, (D) substrate 4, (E) substrate 5 or (F) substrate 6. Reaction mixtures were analyzed by acrylamide gel electrophoresis at 10 V/cm at room temperature.

lows: $1 > 5 > 6 > 4 > 3 > 2$. Therefore, the order of inhibitory effect of phosphorothioate bond at different positions on DNA substrates after FEN1 catalysis is $2 > 3 > 4 > 6 > 5$.

In order to further analyze the process of FEN1 catalysis and inhibitory effect of phosphorothioate bond modified at different positions, each substrate was reacted with FEN1 individually. Unmodified substrate 1 has demonstrated highest reactivity towards FEN1 cleavage, as shown in Figure 3A. The activity of FEN1 on substrate 2 is significantly decreased, compared with the unmodified substrate 1 (shown in Fig. 3B). After reacted for 1 hour, substrate 2 still presents unreacted substrate and only intermediate compound has been generated under this reaction condition. On the other hand, the activities of FEN1 on substrates 3–6 are higher than the activity of FEN1 on 2.

Based on the gel electrophoretic results shown in Figure 3, the correlation between product yield of different phosphorothioate-modified substrates and reaction time was plotted in Figure 4. The reaction rate of FEN1 is very fast within 20 min on substrate 1. The reaction rate of substrate 2 was the most moderate and after FEN1 catalysis, substrate 2 only generated intermediate compound thus only one curve is presented in Figure 4B. Based on these

graphs, the reaction rate of all the phosphorothioate-modified substrates was lower than that of unmodified substrate 1.

Based on the above results, the slow-down effects of phosphorothioate modified substrates on FEN1 catalysis are significant. In addition, the slow-down effect of phosphorothioate modification at different positions in substrates shows significant difference, among which 2 with modification at FEN1 cleavage site shows the highest slow-down effect. In order to design a substrate-based inhibitor to efficient and specific resist the action of FEN1, the phosphorothioate bond was incorporated at FEN1 cleavage site, as illustrated in Figure 5. The length of double-stranded region of this substrate-based inhibitor was optimized based on reported FEN1 binding mechanism for the purpose of in vivo import of liposome.^{13,14} Hairpin structures were introduced at the end of duplex, which were utilized to stabilize the inhibitor and to protect it from in vivo exonuclease hydrolysis, as shown in Supporting information. Half maximal inhibitory concentration (IC_{50} value) of this substrate-based FEN1 inhibitor has been measured in order to quantify its ability against FEN1.

In order to examine the inhibitory efficiency of substrate-based FEN1 inhibitor 1, substrate 1 was utilized as platform for testing

Figure 4. Correlation between product yield and reaction time of FEN1 catalysis on (A) substrate **1**, (B) substrate **2**, (C) substrate **3**, (D) substrate **4**, (E) substrate **5** or (F) substrate **6**.

efficiency of FEN1 inhibitor. An increasing concentration of inhibitor **1** was added into each reaction mixture, respectively. Inhibitor **1** slowed down the flap removal by FEN1 reaction. The upper bands on the gel are unreacted substrate **1** and the bands migrate faster on the gel are intermediate compound and product, respectively, as indicated in Figure 6. The molecular weight of inhibitor **1** is much lower than substrate **1** and moves out of the gel shown in Figure 6. The amount of unreacted substrate **1** kept increasing with the increasing concentration of inhibitor, while the amount of intermediate compound declines.

In order to further quantitatively characterize the efficiency of our newly-developed substrate-based FEN1 inhibitor, half maximal inhibitory concentration (IC_{50} value) was determined. The cor-

relation between FEN1 activity and logarithm of concentration of FEN1 inhibitor **1** was plotted and shown in Figure 7. Inhibition of 50% FEN1 activity (IC_{50}) was observed when concentration of inhibitor **1** comes to around 1.4 μM .

As shown in Figure 7, the reaction rate of FEN1 was significantly reduced by substrate-based FEN1 inhibitor, whose IC_{50} value reaches micromolar scale. Competition of inhibitory effect of inhibitor **1** and competitive substrate has been carried out, as shown in Supporting information. On the other hand, assays of competition of substrate and inhibitor **1** towards FEN1 catalysis have been conducted (Supporting information). Therefore substrate-based inhibitor **1** shows great potential to be an effective and specific

Figure 5. Illustration of the structure of substrate-based FEN1 inhibitor that contains a phosphorothioate modification.

Figure 6. Examination of inhibitory effect of substrate-based FEN1 inhibitor (inhibitor 1).

Figure 7. Determination of half maximal inhibitory concentration (IC₅₀) of our newly-developed inhibitor (inhibitor 1).

inhibitor for FEN1, and benefits our knowledge on drug design and development against malignant tumor.

4. Conclusion

Reactivity of substrates with phosphorothioate bond located exactly at or adjacent to FEN1 cleavage site of flap-structured DNA substrate has been explored in our studies in order to investigate slow-down effect of phosphorothioate modification on FEN1 catalysis. Our findings revealed that substrate containing phospho-

rothioate linkage at FEN1 cleavage site in DNA substrate demonstrated highest inhibitory efficiency against FEN1. Highly specific substrate-based inhibitor was designed and examined. The inhibitory effect of our newly-designed inhibitor against FEN1 was remarkable high with a micromolar level of IC₅₀ value. It is our belief that this inhibitor could be of great potential to be anti-cancer drug candidate which could in theory slow down DNA replication in certain cancer cells and help improve cancer therapy.

Acknowledgements

This work was supported by Nanyang Technological University through research grants, MOE Tier 2 grant (M4020244.110), MOE Tier 1 grant (M4011444.110), iFood research grant award (M4081461.110), MOE Tier 1 grant (M4011125.110) and MOE Tier 1 grant (M4011062.110) to Tianhu Li.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2016.03.025>.

References and notes

1. Nikolova, T.; Christmann, M.; Kaina, B. *Anticancer Res.* **2009**, 29, 2453.
2. Zheng, L.; Jia, J.; Finger, L. D.; Guo, Z.; Zer, C.; Shen, B. *Nucleic Acids Res.* **2011**, 39, 781.
3. Lam, J. S.; Seligson, D. B.; Yu, H.; Li, A.; Eeva, M.; Pantuck, A. J.; Zeng, G.; Horvath, S.; Beldegrun, A. S. *BJU Int.* **2006**, 98, 445.
4. Wang, K.; Xie, C.; Chen, D. *Int. J. Mol. Med.* **2014**, 33, 1268.
5. Dorjsuren, D.; Kim, D.; Maloney, D. J.; Wilson, D. M., 3rd; Simeonov, A. *Nucleic Acids Res.* **2011**, 39, e11.
6. Tan, G.; Gyllenhaal, C.; Soejarto, D. D. *Curr. Drug Targets* **2006**, 7, 265.
7. Tumey, L. N.; Bom, D.; Huck, B.; Gleason, E.; Wang, J.; Silver, D.; Brunden, K.; Boozer, S.; Rundlett, S.; Sherf, B.; Murphy, S.; Dent, T.; Leventhal, C.; Bailey, A.; Harrington, J.; Bennani, Y. L. *Bioorg. Med. Chem. Lett.* **2005**, 15, 277.
8. Tumey, L. N.; Huck, B.; Gleason, E.; Wang, J.; Silver, D.; Brunden, K.; Boozer, S.; Rundlett, S.; Sherf, B.; Murphy, S.; Bailey, A.; Dent, T.; Leventhal, C.; Harrington, J.; Bennani, Y. L. *Bioorg. Med. Chem. Lett.* **2004**, 14, 4915.
9. Pickering, T. J.; Garforth, S. J.; Thorpe, S. J.; Sayers, J. R.; Grasby, J. A. *Nucleic Acids Res.* **1999**, 27, 730.
10. Fisher, T. L.; Terhorst, T.; Cao, X. R.; Wagner, W. *Nucleic Acids Res.* **1993**, 21, 3857.
11. Vosberg, H. P.; Eckstein, F. *J. Biol. Chem.* **1982**, 257, 6595.
12. Agrawal, S.; Goodchild, J.; Civeira, M. P.; Thornton, A. H.; Sarin, P. S.; Zamecnik, P. C. *Proc. Natl. Acad. Sci.* **1988**, 85, 7079.
13. Tsutakawa, S. E.; Classen, S.; Chapados, B. R.; Arvai, A. S.; Finger, L. D.; Guenther, G.; Tomlinson, C. G.; Thompson, P.; Sarker, A. H.; Shen, B.; Cooper, P. K.; Grasby, J. A.; Tainer, J. A. *Cell* **2011**, 145, 198.
14. Geertsma, E. R.; Mahmood, N. A. B. N.; Schuurman-Wolters, G. K.; Poolman, B. *Nat. Protoc.* **2008**, 3, 256.