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The identification and optimization of a N-hydroxy urea series of flap endonuclease 1 inhibitors

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Abstract—Flap endonuclease-1 (FEN1) is a key enzyme involved in base excision repair (BER), a primary pathway utilized by mammalian cells to repair DNA damage. Sensitization to DNA damaging agents is a potential method for the improvement of the therapeutic window of traditional chemotherapeutics. In this paper, we describe the identification and SAR of a series of low nanomolar FEN1 inhibitors. Over 1000-fold specificity was achieved against a related endonuclease, xeroderma pigmentosum G (XPG). Two compounds from this series significantly potentiate the action of methyl methanesulfonate (MMS) and temozolamide in a bladder cancer cell line (T24). To our knowledge, these are the most potent endonuclease inhibitors reported to date.

Flap endonuclease-1 (FEN1) is a 43 kDa divalent metal-dependent nuclear enzyme that exhibits both DNA structure-specific endonuclease activity and 5′ exonuclease activity. Arguably, the most well-studied aspect of FEN1 function is its role in the cleavage of Okazaki fragments during DNA replication.[1] However, FEN1 also acts to cleave 5′ DNA flaps generated during a variety of other cellular processes including double-strand break repair,[2] homologous recombination,[3] and base excision repair (BER).[4] FEN1 is necessary for normal embryonic development and homozygous mouse knockouts of FEN1 are lethal.[5,6] Interestingly, however, yeast knockouts of FEN1 are viable, albeit at a slower growth rate.[7]

BER is an important cellular mechanism for the repair of DNA damage caused by alkylating agents.[8] The role of FEN1 in BER is clearly exemplified in a recent report that shows nuclease-defective FEN1 results in increased cellular sensitivity to methyl methanesulfonate (MMS), a potent DNA alkylating agent.[9] Sensitization to DNA damaging agents may improve the therapeutic window of classical chemotherapeutics by lowering the minimum effective dose.[10] Recent reports describe several small molecule inhibitors of DNA repair proteins including poly(ADP-ribose) polymerase-1 (PARP)[11] and O^6-alkylguanine-DNA alkyltransferase (ATase or MGMT).[12] These inhibitors are reported to potentiate the activity of various chemotherapeutic agents including temozolamide[13] and topotecan.[11] In light of this recent work, we embarked on a strategy to identify selective small-molecule inhibitors of FEN1 for use as chemopotentiating agents.

FEN1 is highly homologous to a related endonuclease, xeroderma pigmentosum G (XPG).[14] XPG is part of a repair pathway that excises DNA containing pyrimidine dimers, a common form of damage caused by exposure to UV light. Defects in XPG are known to cause hypersensitivity to UV light, resulting in light-induced skin lesions and carcinoma.[15] Therefore, selective inhibition of FEN1 over XPG was a key goal of this program.

A high-throughput screen was performed with our compound library in order to identify compounds with inhibitory activity against FEN1. Selected compounds were subsequently screened against XPG. FEN1 and

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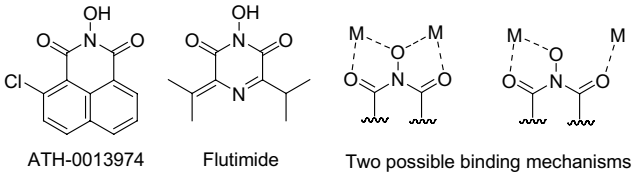
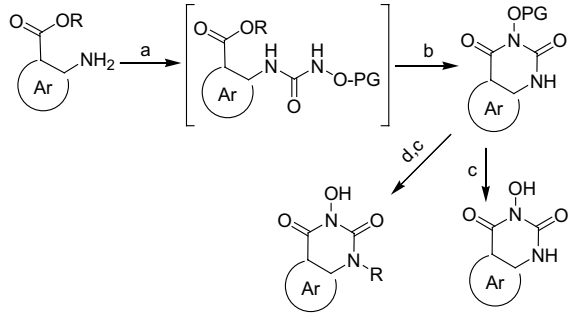


Figure 1. Structures of ATH-0013974 and Flutimide.

XPG inhibition assays were performed using a fluorogenic substrate consisting of a triple-labeled, double-stranded DNA molecule containing an internal 2-nucleotide gap.[16] Several active compounds were identified from the screen, with the most potent one, ATH-0013974, having an IC_{50} of 0.31 μM against FEN1 and 1.98 μM against XPG (Fig. 1).

This compound bears an interesting resemblance to Flutimide, a recently reported inhibitor of the influenza endonuclease.[17] We hypothesized that these compounds inhibit the endonucleases by coordination of the two divalent metal ions needed for catalysis, as illustrated in Figure 1. These two metals are essential for catalytic turnover in FEN1[18] and related endonucleases.[19] A crystal structure of *Pyrococcus furiosus* FEN1 shows that the metal ions are ~5 Å apart,[20] only about 0.5 Å more than the distance between the two carbonyl oxygens in ATH-0013974. The hypothesized interaction of the N-hydroxyl with an active site metal is supported by the observation that O-substitution of ATH-0013974 completely abolishes its FEN1 inhibitory activity (data not shown).

One of our first strategies was to eliminate one of the fused aromatic rings in order to avoid any potential problems associated with flat, polyaromatic systems, such as nonselective DNA intercalation and toxicity. As such, we envisioned a related series of N-hydroxy ureas that are readily accessible from *ortho* amino esters, as illustrated in Scheme 1.[21] Generally, the *ortho* amino ester is heated in the presence of carbonyl diimidazole and then treated with an appropriately protected hydroxylamine. Base-promoted ring closure and deprotection affords the desired products.[22] The ring system is highly stable, allowing a variety of N-hydroxy protecting group strategies to be employed including benzyl (removed with HBr), dimethoxy benzyl[23] (removed with TFA), and allyl (removed with $Pd(OAc)_2/PPh_3$).



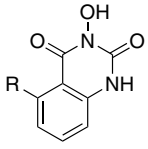
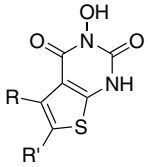
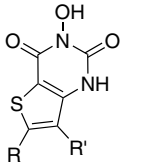
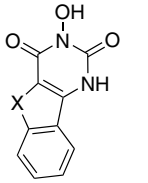
Scheme 1. Synthesis of cyclic ureas (a) CDI/toluene/heat then H_2NO-PG; (b) aq NaOH/heat; (c) TFA or HBr or $Pd(OAc)_2/PPh_3$; (d) DMF/K_2CO_3/alkyl halide/heat.

Compound **1** (R = H) proved to be a 0.08 μM inhibitor of FEN1, significantly more potent than our original lead. Addition of the *ortho*-chlorine, compound **2**, increased potency against FEN1 but not XPG, resulting in a compound approximately 20-fold more potent than the lead, ATH-0013974 (Table 1).

Thiophenes are well-known isosteres of benzene rings. This prompted us to explore heterocyclic N-hydroxy ureas exemplified by compounds **3**–**11**. Compound **3**, while being a very potent inhibitor, was only 4-fold selective for FEN1 over XPG. Interestingly, the addition of a methyl group at the 4-position (compound **4**) resulted in slightly increased potency and significantly improved selectivity (~10-fold). It is likely that the methyl group of **4** mimics the chlorine of compound **2**. Reversal of the thiophene (compounds **6**–**10**) resulted in potent compounds provided that a hydrophobic group was present at the 4 position (compounds **8** and **9**). Hydrophobic substitution at the 5-position generally resulted in decreased potency (compounds **6** and **7**). Interestingly, benzothiophene **10** is approximately 7-fold more potent than the corresponding benzofuran **11**, presumably due to the increased hydrophobicity of sulfur (Table 1).

Alkylation of the urea nitrogen with small alkyl groups such as methyl (**12**) decreased the affinity to both FEN1

Table 1. Activity of compounds **1**–**11**

Compd[a]	Structure		IC_{50} (μM)	
			FEN1	XPG
ATH-13974 (see Fig. 1)			0.310	1.98
1		R = H	0.079	0.160
2		R = Cl	0.014	0.185
3		R,R′ = H	0.025	0.101
4		R = Me, R′ = H	0.014	0.145
5		R,R′ = –$(CH_2)_4$–	0.009	0.157
6		R = *t*Bu, R′ = H	0.134	0.258
7		R = Ph, R′ = H	0.146	0.202
8		R = H, R′ = Ph	0.012	0.028
9		R = H, R′ = SO_2Ph	0.010	0.023
10		X = S	0.043	0.062
11		X = O	0.285	0.257

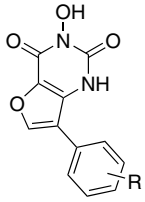
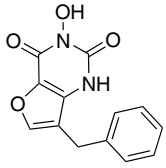
[a] All compounds were purified by preparative HPLC and were evaluated for proper identity and purity by analytical HPLC-MS and by ^1H NMR.

and XPG (Table 2). Unexpectedly, the potency against FEN1 was regained by the addition of a benzyl group (**13**) while the potency against XPG remained unchanged. This dramatically boosted selectivity for the desired target, from ~10-fold (compound **4**) to nearly 150-fold. In many cases, substituted benzyl groups (i.e., **15**–**16**) gave up to 240-fold selectivity.

Reversal of the thiophene also provided exceptionally potent and selective compounds (**17**–**21**). *meta*-Substituted biaryl groups (**19**–**20**) provided the best potency in this series. Interestingly, the addition of a methyl group at the 4-position (**21**) lowers the activity by over 300-fold. We believe that the presence of the methyl group forces the conformation of the benzyl group to be shifted toward the urea carbonyl. This conformation is evidently not tolerated in the binding site.

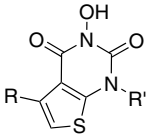
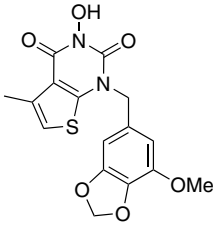
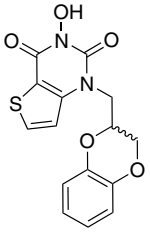
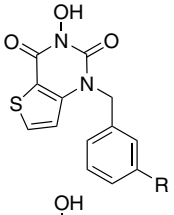
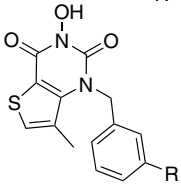
A related series of furans was also investigated (Table 3). These compounds were synthesized by the route illus-

Table 3. Activity of furan derivatives **22**–**25**

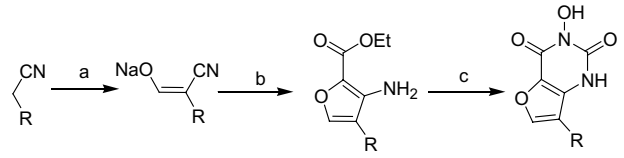
Compd[a]	Structure		IC_{50} (μM)	
			FEN1	XPG
22		R = H	0.143	0.276
23a		R = 4-Me	0.082	0.128
23b		R = 4-OMe	0.034	0.230
23c		R = 4-Br	0.076	0.327
23d		R = 4-Ph	0.023	0.136
24		R = 3-OMe	0.031	0.215
25			0.038	0.061

[a] All compounds were purified by preparative HPLC and were evaluated for proper identity and purity by analytical HPLC-MS and by ^{1}H NMR.

Table 2. Activity of N-alkylated compounds **12**–**21**

Compd[a]	Structure		IC_{50}, (μM)	
			FEN1	XPG
12		R = Me, R′ = Me	0.338	4.62
13		R = Me, R′ = Bn	0.028	3.99
14		R = H, R′ = Bn	0.230	2.00
15		R = Me, R′ = Bn-4-OMe	0.035	8.45
16			0.018	3.04
17			0.011	0.292
18		R = Br	0.072	0.550
19		R = 3-(NHAc)-Ph	0.004	0.586
20		R = 4-($CONH_2$)-Ph	0.003	0.226
21		R = 4–($CONH_2$)–Ph	1.08	30.2

[a] All compounds were purified by preparative HPLC and were evaluated for proper identity and purity by analytical HPLC-MS and by ^{1}H NMR.



Scheme 2. Synthesis of furan-based cyclic ureas (a) ethyl formate, NaOMe, MeOH, heat; (b) Diethyl chloromalonate/DMF then DBN/EtOH/heat; (c) (1) CDI/toluene/heat; (2) H_2NO-allyl; (3) aq NaOH/heat; (4) $Pd(OAc)_2/PPh_3$.

trated in Scheme 2.[24] Substituted acetonitriles were treated with ethyl formate then alkylated with diethyl chloromalonate. Subsequent treatment with base promoted decarboxylation and cyclization (Scheme 2). The amino ester was converted to the N-hydroxy urea by the aforementioned pathway.[25] Consistent with the previous observation of benzofuran **11** being less potent than benzolthiophene **10**, furan **22** was significantly less active than thiophene **8**. Interestingly, however, *para*-substituents (**23a–d**) seem to significantly increase the activity. We believe that the *para*-biaryl motif in **23d** mimics the *meta*-biaryl motif in compounds **19–20**. Unlike the thiophene series, substitution on nitrogen generally had a detrimental effect on activity (data not shown).

The high selectivity of compounds **15–20** against XPG, also a metalo-endonuclease, makes it implausible that the inhibitors act strictly via metal chelation. Moreover, it is highly unlikely that metal chelation alone would result in sub-10 nM inhibitors such compounds as **19** and **20**. With these potent and selective inhibitors now in hand, the compounds described in Tables 1–3 were screened for their ability to sensitize cells to DNA damaging agents. Previous studies have shown that FEN1 null-mutations in a T24 cell line (bladder carcinoma) resulted in increased sensitivity to MMS.[9] Accordingly, the compounds above were initially screened for their ability to sensitize T24 cells to MMS. The most potent sensitizers were studied further for their ability to potentiate the effects of other chemotherapeutic agents.

The results for compounds **16** and **17** illustrate the chemopotentiation ability of this class of molecules (Table 4). These two compounds show little or no effect on the growth of T24 cells at the tested concentrations, indicating that the compounds have surprisingly little effect on DNA replication. However, they enhance the

Table 4. Survival assay (colony forming) in T24 bladder carcinoma cells[a]

Compd	+DMSO	+110 μM MMS	+150 μM MMS	+200 μM MMS
DMSO	100	44	22	5
16 (1 μM)	100	24	12	2
16 (3 μM)	86	11	6	0
17 (3 μM)	96	18	6	4
17 (6 μM)	87	11	5	5

[a] Represents the average of two independent experiments.

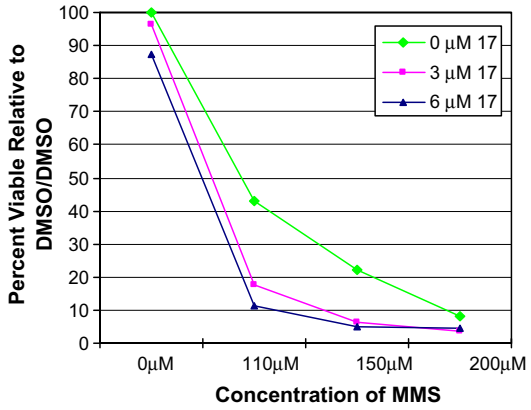


Chart 1. T24 Colony forming assay of MMS +/− compound **17**.

toxicity of MMS by approximately 4-fold at 3 and 6 μM, respectively (Table 4). The data for compound **17** is plotted graphically in Chart 1. As can be seen, the data is suggestive of a dose-dependent enhancement. Compounds **16** and **17** also significantly enhanced the effects of temozolomide, a recently approved chemotherapy agent that acts by methylating DNA (data not shown). Interestingly, none of the FEN1 inhibitors potentiated the activity of bleomycin. Unlike MMS and temozolomide, bleomycin does not induce damage that is repaired by the BER pathway. Therefore, no enhancement of its activity would be expected with a FEN1 inhibitor. Taken together, these results suggest that these FEN1 inhibitors are sensitizing cells to damage that is normally repaired by the BER pathway. Moreover, these results confirm the FEN1 knockout phenotype reported by Shibata and Nakamura.[9]

In summary, we have identified a novel and potent series of FEN1 inhibitors. These compounds were shown to sensitize bladder carcinoma cells to DNA damage that is normally repaired by the BER pathway. Further studies have been undertaken to evaluate the clinical usefulness of this approach as a cancer therapy. To the best of our knowledge, these compounds are approximately 100-fold more potent than any endonuclease inhibitor reported to date. Moreover, this series of compounds may illustrate a useful chemotype for the inhibition of other metallo-nucleases.

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22. General protocol for the synthesis of compounds **12**–**21**: The *ortho* amino ester was refluxed in toluene (0.1 M) along with CDI (1.25 equiv) for 2 h. The protected hydroxylamine (1.5 equiv) was added and refluxing was continued for 1 h. The solution was concentrated, suspended in EtOH and treated with 2 M NaOH. The solution was heated for 1 h and then cooled. The resultant O-protected imide generally precipitated upon cooling and acidification with HOAc. The cyclic imide was dissolved in DMF (0.1 M) and treated with K_2CO_3 followed by an alkyl halide. After heating to 80 °C for 2–12 h, the reaction mixture was cooled and partitioned between water and EtOAc. The organic layer was evaporated to give the crude product, which was generally used without purification. The O-protecting group was removed by standard procedures.
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25. General protocol for the synthesis of the furan amino esters: The substituted acetonitrile was added to a solution of ethyl formate (1.1 equiv) and sodium methoxide (1.1 equiv) in MeOH (0.1 M). After refluxing for 2 h, the resulting precipitate was filtered and washed with ether to give the crude hydroxyacrylonitrile. Diethylchloromalonate (1.0 equiv) was added to a stirred solution of the crude hydroxyacrylonitrile in DMF (0.1 M). After stirring 5 h at rt, the solvent was removed and the resulting oil was dissolved in ethanol. DBN (1.0 equiv) was added and the reaction mixture was refluxed overnight. After concentration, the crude product was dissolved in EtOAc, washed with water, and purified by silica gel chromoatography (EtOAc/Hex) to afford the desired furan amino ester.