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AWARD NUMBER: DAMD17-03-1-0251

TITLE: Rational Inhibitors of DNA Base Excision Repair Enzymes: New Tools for Elucidating the Role of BER in Cancer Chemotherapy

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REPORT DATE: May 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2006	Annual Summary	21 Apr 2003 – 20 Apr 2006

4. TITLE AND SUBTITLE

Rational Inhibitors of DNA Base Excision Repair Enzymes: New Tools for Elucidating the Role of BER in Cancer Chemotherapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0251

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
Baltimore, Maryland 21218-2686

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this funding period we have extended our studies outlined in Tasks 1 and 2 of the approved Statement of Work with the development of a novel and versatile strategy to synthesize focused chemical libraries against DNA repair enzymes. A high-throughput screen of this focused library yielded several potent and specific inhibitors of human uracil DNA glycosylase. Investigations into the effects of inhibiting uracil DNA glycosylase with these small molecules on the cytotoxicity and mechanism of 5-fluorouracil are ongoing. This work has resulted in two publications during this funding period.

15. SUBJECT TERMS
Uracil DNA glycosylase, inhibitors, 5-fluorouracil, breast cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	22	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
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INTRODUCTION

This program seeks to obtain a fundamental understanding of the chemical mechanisms by which enzymes repair damaged DNA, and to use this information to design small molecule inhibitors of these enzymes. The driving force for these efforts is the recognition that the effectiveness of cancer chemotherapy regimes is intimately connected to, and in some cases directly relies on, DNA damage repair pathways. A more sophisticated understanding of the roles of DNA damage repair in the pharmacology of DNA replication inhibitors will allow for the design of better treatments against breast and other cancers.

Studies during this period have extended the earlier work done on Tasks 1 and 2 to the development of potent and selective small molecule inhibitors of human uracil DNA glycosylase (UDG). The discovery of these inhibitors was facilitated by the design of a focused chemical library that exploited the extrahelical damaged base mechanism of UDG. An x-ray co-crystal structure of one of these inhibitors bound to human UDG was solved at 1.3 Å resolution, and revealed that this inhibitor mimics many of the features of extrahelical damaged base recognition by the enzyme. This reporting period has produced two publications in peer-reviewed journals and two more manuscripts are in preparation.

BODY

A major achievement of this period was the discovery of potent and selective small molecule inhibitors of human UDG. These inhibitors, which were published in the Journal of the American Chemical Society and in Bioorganic and Medicinal Chemistry, were discovered through the use of a novel chemical library focused against UDG. The bias of this library was introduced by taking advantage of the specific interactions the enzyme makes with extrahelical uracil in the active site. UDG only binds uracil with modest affinity (K_D ~ 80 μM), but uracil is highly complementary to the active site. Thus, we used uracil as an anchor into the active site of UDG, and then relied on random exploration of adventitious binding sites proximal to the active site by tethering random small molecules to one of three formyluracils (**Figure 1**).

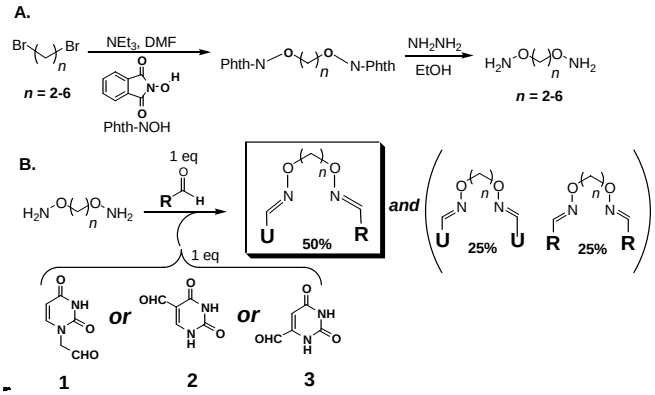

Figure 1. High-throughput synthesis of directed uracil library for human UDG inhibition using oxime chemistry. (A) Synthesis of alkyldioxamine tethers. (B) Mixed oxime formation involves the reaction of an alkyldioxiamine of variable length with an uracil aldehyde and a R-CHO library. The reaction proceeds quantitatively to produce a statistical mixture of oxime derivatives (50% U-R heterosubstituted tethers). The *O*-alkyl oxime libraries are rapidly and efficiently synthesized in 96-well plates without purification.

The oxime chemistry used in the library construction is ideal for use in high-throughput screening (HTS) because the reactions are quantitative, require no purifications, and the resultant mixtures can be used directly in the HTS assay. In addition, the ability to easily vary the linker length between the formyluracil and the variable aldehyde allows for increased chemical diversity in the library and rapid optimization of the spacing between the uracil and the variable binding element

Using these libraries, an initial hit containing a catechol moiety, 3-(3)-13, was identified as an inhibitor of human UDG (**Figure 2**). The affinity of this compound (K_I = 1.6 μM) for human UDG was 50-fold greater than uracil. Several analogs of 3-(3)-13 were made, which led to the discovery of an analog, 3-(3)-27 (**Figure 2**), with a 5-6-fold increase in potency (K_I = 0.3 μM). The chemical instability of these catechol-containing inhibitors toward oxidation limited their usefulness beyond in vitro characterization. However, an analog that replaced the vicinal hydroxyl groups with a carboxylic acid (3-(2)-A8, **Figure 2**) was nearly as potent as the initial catechol hit (K_I = 3.2 μM) and is chemically stable. An x-ray co-crystal structure of 3-(2)-A8 and human UDG was solved at 1.3 Å resolution, and revealed that the inhibitor mimics the shape and interactions of damaged DNA with the enzyme (**Figure 3**). Currently, we are using this co-crystal structure as a guide to design more potent analogs of 3-(2)-A8 that will be useful in cell-based studies (see below).

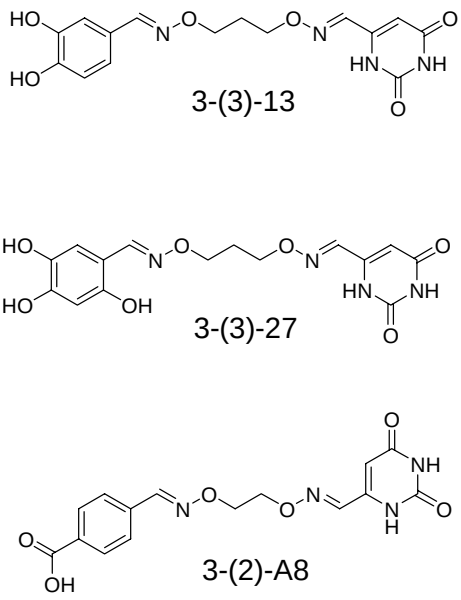

Figure 2. Inhibitor of human UDG isolated from a directed uracil library.

Experiments regarding Task 3 have been initiated during this funding period. The interactions of uracil base excision repair and 5-fluorouracil chemotherapy were elucidated in a yeast model system (Seiple, et al. *Nucleic Acids Res.* **2006**, *34*, 140-51). These studies strongly suggested that UDG potentiates the cytotoxic effects of 5-fluorouracil by initiating futile repair of the high concentrations of deoxyuridine in the DNA introduced as a consequence of thymidylate synthase inhibition. Studies that extend these observations from the

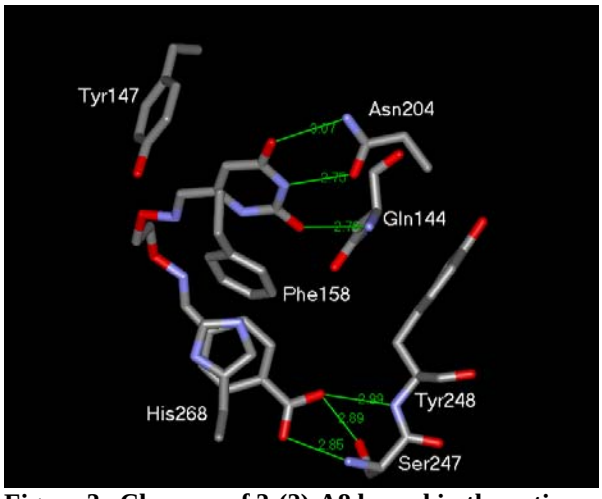

Figure 3. Close-up of 3-(2)-A8 bound in the active site of human UDG from 1.3 Å co-crystal structure.

yeast model system to mammalian cells are ongoing. As a first step, we have demonstrated that 3-(2)-A8 was equipotent against both recombinant human UDG (which contains an 84 amino acid truncation at the N-terminus) and full length human UDG assay in cell lysates derived from MCF-7 breast cancer cells. Future work will involve co-administering 3-(2)-A8 (or a more potent analog) and 5-fluorouracil to MCF-7 cells and measuring changes in the uracil content of DNA and cytotoxicity.

KEY RESEARCH ACCOMPLISHMENTS

- Developed novel chemical libraries based focused against DNA repair enzymes (SOW, Task 1).
- Identified potent and selective inhibitors of human uracil DNA glycosylase (SOW, Task 1 and 2).
- Solved x-ray co-crystal structure of 3-(2)-A8 and human uracil DNA glycosylase at 1.3 Å resolution.

REPORTABLE OUTCOMES

This work has resulted in two published manuscripts:

1. Jiang YL, Krosky DJ, Seiple LM, Stivers JT. Uracil-directed ligand tethering: an efficient strategy for uracil DNA glycosylase (UNG) inhibitor development. *J Am Chem Soc* **2005**, *127*, 17412-20.

2. Jiang YL, Chung S, Krosky DJ, Stivers JT. Synthesis and high-throughput evaluation of triskelion uracil libraries for inhibition of human dUTPase and UNG2. *Bioorg Med Chem* **2006**, in press.

CONCLUSIONS

The long-term goal of this research is to increase the effectiveness of 5-fluorouracil chemotherapy through selective targeting of base excision repair enzymes such as UDG. We have progressed towards this goal by developing methodology to create chemical libraries focused against DNA repair enzymes, and we have successfully used this technology to discover the first non-nucleotide inhibitors of human UDG. At least one of these inhibitors shows evidence of being cell permeable, and will be used to elucidate the mechanism of action of 5-fluorouracil against breast cancer cells.

REFERENCES

None.

APPENDICES

Two manuscripts are included (see above).

J|A|C|S
ARTICLES
Published on Web 11/15/2005

Uracil-Directed Ligand Tethering: An Efficient Strategy for Uracil DNA Glycosylase (UNG) Inhibitor Development

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Abstract: Uracil DNA glycosylase (UNG) is an important DNA repair enzyme that recognizes and excises uracil bases in DNA using an extrahelical recognition mechanism. It is emerging as a desirable target for small-molecule inhibitors given its key role in a wide range of biological processes including the generation of antibody diversity, DNA replication in a number of viruses, and the formation of DNA strand breaks during anticancer drug therapy. To accelerate the discovery of inhibitors of UNG we have developed a uracil-directed ligand tethering strategy. In this efficient approach, a uracil aldehyde ligand is tethered via alkyloxyamine linker chemistry to a diverse array of aldehyde binding elements. Thus, the mechanism of extrahelical recognition of the uracil ligand is exploited to target the UNG active site, and alkyloxyamine linker tethering is used to randomly explore peripheral binding pockets. Since no compound purification is required, this approach rapidly identified the first small-molecule inhibitors of human UNG with micromolar to submicromolar binding affinities. In a surprising result, these uracil-based ligands are found not only to bind to the active site but also to bind to a second uncompetitive site. The weaker uncompetitive site suggests the existence of a transient binding site for uracil during the multistep extrahelical recognition mechanism. This very general inhibitor design strategy can be easily adapted to target other enzymes that recognize nucleobases, including other DNA repair enzymes that recognize other types of extrahelical DNA bases.

Introduction

DNA repair pathways have been traditionally viewed as the cellular quality control machinery that preserves the coding potential of genomes.[1] However, there is emerging recognition that the repair mechanisms evolved to prevent accumulation of the RNA base uracil in DNA play a much broader role in a number of important areas of biomedicine that are divergent from genome preservation. Remarkable examples include the role of the uracil excision repair machinery in the process of generating genetic diversity during antibody maturation in B cells,[2−4] the importance of uracil incorporation and removal in the life cycles of herpes,[5] cytomegalo,[6] pox,[7,8] and type 1 human immunodeficiency viruses (HIV-1),[9] and the essential role of this pathway in generating pharmacologically active single and double strand DNA breaks during chemotherapy treatment with 5-flurouracil and methotrexate.[10,11] The key enzyme player in all of these remarkably diverse processes is uracil DNA glycosylase (UNG), which cleaves the glycosidic bond between the uracil base and the deoxyribose sugar in DNA by flipping the uracil nucleotide from the DNA duplex into the enzyme active site (Figure 1A).[12] Given that UNG is emerging as a very interesting pharmacologic target, we have sought out methods for the rapid and efficient identification of small-molecule ligands that could inhibit its activity. Although potent nucleic acid-based and proteinaceous inhibitors are available that target UNG,[13−17] there are no small-molecule inhibitors for this enzyme, and strategies for the discovery of such ligands are lacking.

One of the most exciting potential applications of small-molecule human UNG inhibitors are as antiretroviral agents. Recent findings have established that HIV-1 specifically packages human UNG (hUNG) into virus particles via interaction with the virus encoded integrase protein (Int) or perhaps a ternary complex between UNG, Int, and the viral Vpr

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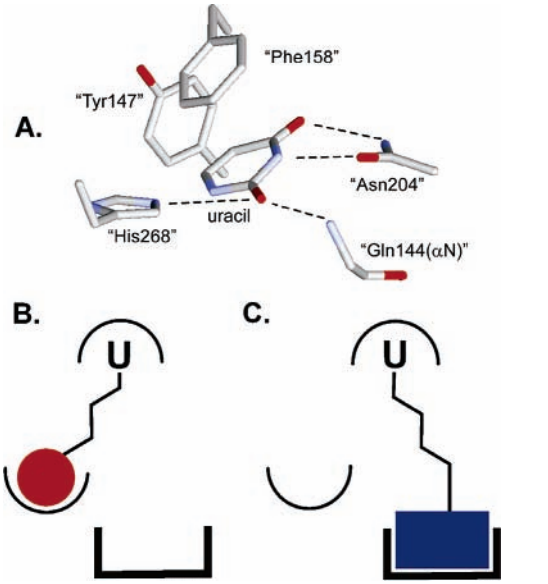

Figure 1. Extrahelical binding of uracil to the UNG active site and the general strategy for uracil-directed ligand tethering. (A) Structure of UNG bound to uracil is shown (pdb code 2eug). The residue numbering is for the human enzyme. (B, C) The uracil ligand (U) that targets the UDG active site is covalently tethered to two different ligands that can interact with distinct binding surfaces near the active site.

protein.[5,18−25] hUNG is required for infection of nondividing cells such as macrophages and resting T cells and helps maintain a viral reservoir in the host that is crucial for virus spread to the lymphoid organs and T-helper lymphocytes and, ultimately, AIDS pathogenesis.[20,26] UNG is apparently recruited to minimize uracil incorporation into the viral genome in these cells, which have naturally high levels of dUTP, a good substrate for the viral reverse transcriptase.[27] In the absence of UNG, the HIV-1 mutation rate is found to increase by 18-fold resulting in extremely inefficient virus replication in nondividing cells,[20] and the virus particles produced from UNG-depleted cells are incapable of infecting new target cells.[9,28] Pharmacologic targeting of a human enzyme required for virus infectivity is extremely attractive because such a target would not be susceptible to the same high mutagenesis rate and resulting drug resistance as viral encoded proteins.[29] Targeting the human enzyme is a viable therapeutic strategy because it is not an essential enzyme. Thus, UNG knock-out mice display no remarkable phenotype, nor do UNG null yeast or human cell lines.[30]

Herein, we report an integrated high-throughput (HTP) platform for discovering small-molecule ligands that inhibit UNG. The strategy takes advantage of the extrahelical uracil recognition mechanism of UNG by using the specificity and binding energy of a uracil ligand to target the UNG active site[14,31,32] and then covalent tethering of random functional groups for exploration of nearby binding pockets (Figure 1B). Library members can be rapidly screened using a robust HTP activity assay, and initial hits are quickly optimized using subsequent structure−activity studies. This tethering approach, which uses efficient oxime chemistry (Figure 2), is related to the "combinatorial target-guided ligand assembly" method of Ellman et al.[33] but differs in that the uracil ligand specifically targets the active site rather than irrelevant regions of the enzyme. Thus, the hit-rate and binding affinities of early hits are higher than the more random approach of Ellman and colleagues. This synthetic and screening strategy should be easily adaptable for the discovery of inhibitors of other enzymes that recognize extrahelical bases in DNA or free nucleosides.

Results and Discussion

Synthesis of Uracil-Tethered Oxime Libraries and General Strategy. We sought an inhibitor development strategy that allowed rapid and economical synthesis of small-molecule ligands that explore binding sites near the UNG active site and which could be used directly in HTP screening applications without purification. One efficient synthesis strategy that meets these criteria is outlined in Figure 2. First, flexible diaminoalkanediol linkers of variable length are synthesized from the corresponding dibromoalkanes (Figure 2A). Then the linkers are used to tether uracil aldehyde binding elements (**1**−**3**) to a library of aldehyde binding elements (RCHO) via the formation of stable oxime linkages (Figure 2B). Each tethering reaction is carried out in one well of a 96-well microtiter plate that contains one equivalent uracil aldehyde, one equivalent RCHO library member, and a mixture of diaminoalkanediol linkers ($n = 2-6$). The reactions typically proceed to 85−99% completion after overnight incubation (DMSO solvent, 37 °C) and produce a 1:2:1 statistical mixture of the homodimeric (U∧U, R∧R) and heterodimeric (U∧R) oximes for each of the five linker lengths present (see Experimental Section and Supporting Imformation Figure S1). Although two geometric configurations are possible, oxime derivatives with bulky substituents are generally found to be ≥95% in the trans configuration.[34] The unpurified oxime mixtures were directly screened for inhibition of UNG at ∼100 μM total oxime concentration to ensure that each component in the mixture is present at a concentration in the range 5−10 μM. If significant inhibition is observed by any mixture, the linker length and RCHO binding element that gave rise to the inhibition can be identified by resynthesis of the individual oximes using a single linker length in each reaction (see below).

An important aspect of this approach is that the uracil homodimers present in some reaction mixtures are inhibitory even in the absence of any active heterodimer. For instance,

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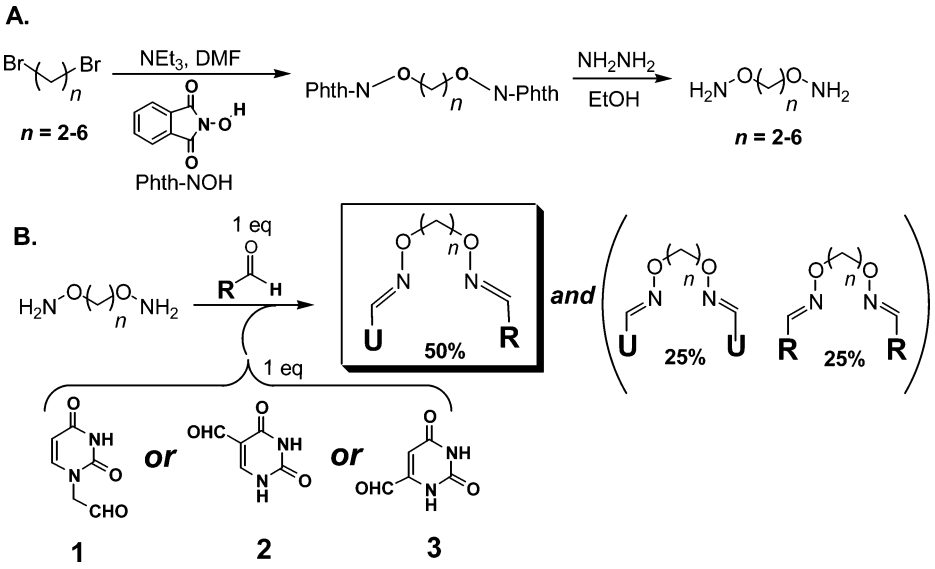

Figure 2. Synthesis of oxime libraries based on uracil and RCHO: (A) synthesis of diaminoalkanediol tethers of variable length; (B) construction of the uracil−oxime library based on the uracil aldehydes (**1**−**3**) and a series of aldehyde compounds (RCHO). The products consist of a 1:2:1 mixture of the heterodimer (U∧R) and the two homodimers (U∧U and R∧R) connected via alkane linkers of lengths 2−6. A 1 equiv amount of total diaminoalkanediol is added to each reaction. Each linker length is present at one-fifth of the total concentration.

the purified homodimers of various lengths that are based on 6-formyluracil (**3**) give rise to about 22% inhibition in all the mixtures based on **3** under the screening conditions (not shown). In contrast, the homodimers of **1** and **2** show no detectable inhibition under the same conditions. Thus, the screening assay must be robust enough to detect any *additional* inhibition resulting from an active heterodimer in the mixture. Spectroscopic results for determining the purity and composition of representative reaction mixtures are available (see Supporting Information).

High-Throughput Screening of Uracil−Oxime Libraries. To test this directed library approach, we tethered the three uracil aldehydes (**1**−**3**) shown in Figure 2 to 14 aldehyde binding elements (RCHO) using the variable-length diaminoalkanediol linkers (see Supporting Information Table S1 for RCHO structures). This library of uracil-linked binding elements was screened for inhibition of hUNG using a high-throughput molecular beacon activity assay **(**Figure 3**)**.[35] In this assay, one DNA strand is labeled with a fluorescent 5′-FAM and the complementary strand is modified with a 3′-dabsyl moiety that serves to efficiently quench the fluorescence of the FAM group through contact quenching. To increase stability, the two DNA strands are linked in a hairpin configuration using an 18 atom poly(ethylene glycol) linker. When the substrate DNA is exposed to UDG, multiple uracils are removed, and eventually the two paired strands of the hairpin spontaneously separate, thus removing the dabsyl quencher from the proximity of the FAM group and resulting in a 6-fold increase in the fluorescence of the system (Figure 3A). Under the assay conditions, the hairpin DNA substrate has a $K_m = 164 \pm 10$ nM and $k_{cat} = 0.33 \pm 0.01$ s^{-1} (Figure 3B). To enhance detection of competitive inhibitors during HTP screening we employed a molecular beacon substrate concentration equivalent to $1/3K_m$ (50 nM). Representative HTP screening results for several inactive and active oxime mixtures are shown in Figure 4 ([total oxime] = 100 μM).

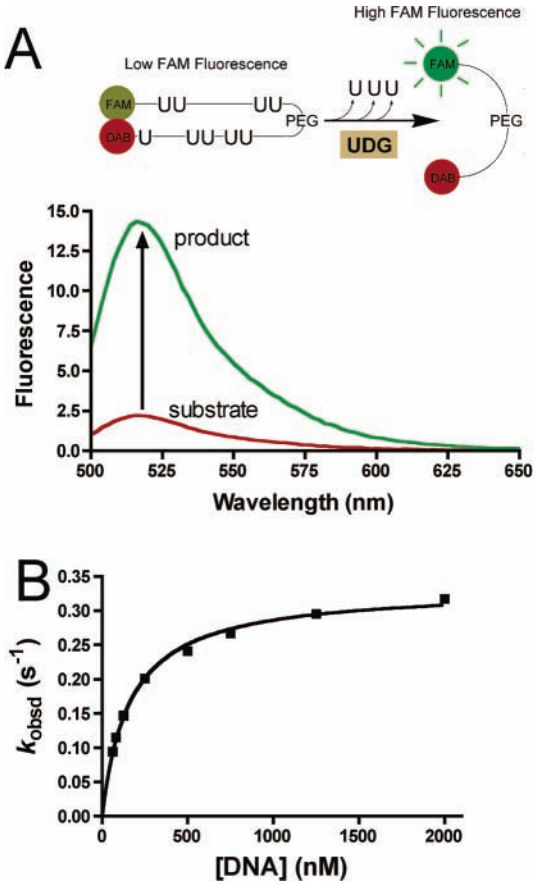

Figure 3. High-throughput (HTP) UDG kinetic assay. (A) The HTP assay relies on molecular beacon technology. Excision of multiple uracil bases by the enzyme destabilizes the hairpin structure thereby releasing the 5′-FAM fluorophore from the quenching effects of the 3′-dabsyl group. (B) Steady-state kinetic analysis is shown of the hUDG reaction using the molecular beacon hairpin substrate.

Several activity trends emerged immediately from the screening results shown in Figure 4. First, none of the mixtures derived from the uracil N1-acetaldehyde binding element (**1**) were inhibitory at the concentration used in the screen. In addition,

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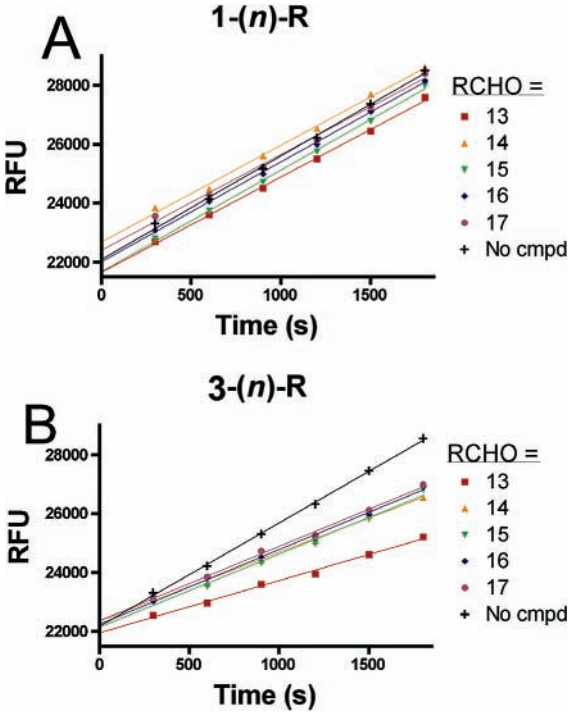


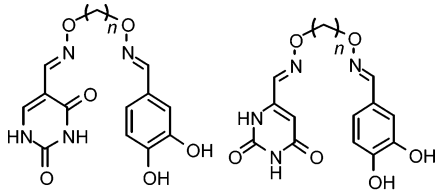
Figure 4. Representative HTP screening results using the molecular beacon substrate. (A) Screen of oxime dimer mixtures derived from uracil aldehyde **1** and aryl aldehydes **13**−**17** is presented. No inhibition was observed for any oxime derived from **1** regardless of linker length (*n*). (B) Screen of oxime dimer mixtures derived from uracil aldehyde **3** and aryl aldehydes **13**−**17** is shown. The mixed oxime derived from **3** and **13** shows significant inhibition, and this derivative was further optimized. For **14**−**17**, the observed inhibition represents that from the **3**−**3** homodimers that are present in the mixtures.

none of the U∧U homodimers derived from **2** were found to be inhibitory, nor were any of the R∧R homodimers regardless of the linker length. (Inhibition by the homodimers is automatically assessed because these are present in multiple reaction mixtures.) In contrast, one oxime mixture derived from uracil aldehydes **2** and **3** and RCHO = 3,4-dihydroxybenzaldehyde (**13**) showed inhibitory activity in the range 15−100%, indicating that active heterodimers were present. The structures of the active heterodimers present in these two oxime mixtures are shown at the top of Table 1.

The two active mixtures were deconvoluted with respect to linker length by individually synthesizing each oxime dimer using a *single* diaminoalkanediol linker/reaction (Table 1). At this stage we did not separate the homodimers from the active heterodimers in the mixtures. For the oxime dimers derived from 5-formyluracil (**2**) and 3,4-dihydroxybenzaldehyde (**13**), a broad dependence on linker length was observed with length $n = 2$ being most favorable for inhibitory activity (i.e. mixed oxime **2**-(*2*)-**13**, Chart 1). In contrast, a very stringent linker length of $n = 3$ was required for maximal inhibitory activity with the oxime mixture derived from 6-formyluracil (**3**) and 3,4-dihydroxybenzaldehyde (**13**) to form mixed-oxime **3**-(*3*)-**13** (Chart 1). To confirm these results, **2**-(*2*)-**13** and **3**-(*3*)-**13** were separated from their respective homodimers using reversed phase HPLC (see Methods), and the concentration dependence of inhibition was determined. The measured IC_{50} values for **2**-(*2*)-**13** and **3**-(*3*)-**13** were 5.8 and 1.1 μM, respectively (Figure 5).

Structure−Activity Relationships. In an effort to find more potent inhibitors based on the **3**-(*3*)-**13** scaffold, 25 commercially

Table 1. Structures of Active Heterodimers and Dependence of Inhibition on Linker Length[a]

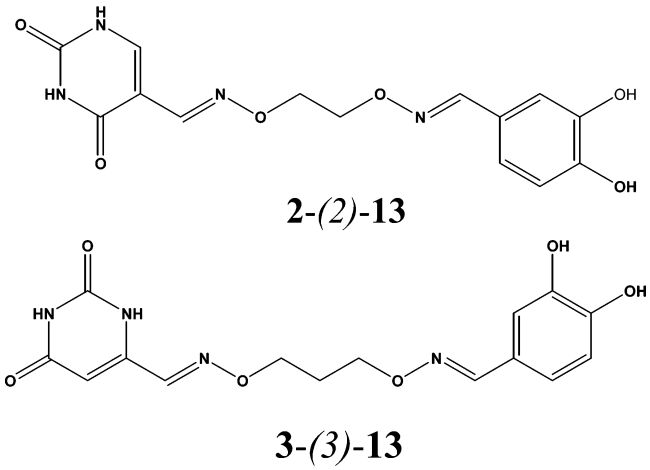


2-(*n*)-**13** **3**-(*n*)-**13**

mixture	linker length (*n*)	% inhibition
2-(*n*)-**13**	2	50
	3	40
	4	20
	5	20
	6	15
3-(*n*)-**13**	2	57
	3	100
	4	51
	5	48
	6	48

[a] Reactions were performed in the presence of 100 μM oxime mixture and 50 nM substrate concentration.

Chart 1. Heterodimer Oximes Identified from Deconvolution of an Active Mixture



2-(*2*)-**13**

3-(*3*)-**13**

available benzaldehyde precursors were purchased (**18**−**42**; cf. Supporting Information Table S2). The HTP screen was then performed on this set of oxime mixtures (**3**-(*3*)-***R***) in an identical fashion as described above. This structure−activity study established that the 3- and 4-hydroxyl groups of **3**-(*3*)-**13** were essential for activity because alkylation or halogen substitution at these positions had a substantial deleterious effect on inhibitory activity (see Supporting Information Table S2). Thus,

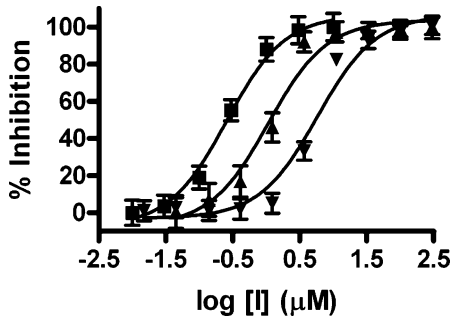
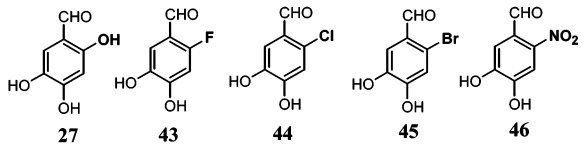


Figure 5. IC_{50} analysis for **2**-(*2*)-**13** (▼), **3**-(*3*)-**13** (▲), and **3**-(*3*)-**27** (■).

Table 2. Inhibitory Activity for Structural Variants of **3**-(*3*)-**27**[a]

3-*(3)*-***R***

RCHO =



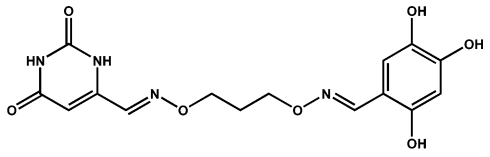
27 **43** **44** **45** **46**

variant	IC_{50} (μm)	2-R
3-(*3*)-**27**	0.26	OH
3-(*3*)-**43**	2.7	F
3-(*3*)-**44**	16	Cl
3-(*3*)-**45**	40	Br
3-(*3*)-**46**	40	NO_2

[a] The concentration dependence of inhibition was determined using 50 nM substrate.

hydrogen bond donating groups at the 3- and 4-positions of the benzyl ring appear to be essential.

One compound in this series with an additional hydroxyl group at the 2-position of the benzyl ring (**3**-(*3*)-**27**) showed a



3-*(3)*-**27**

3-fold greater potency than **3**-(*3*)-**13** (Figure 5, ■) (IC_{50} = 0.3 μM). To further investigate SARs based around the **3**-(*3*)-**27** scaffold**,** we synthesized four more 3,4-dihydroxybenzaldehyde analogues (**43**−**46**, Table 2), where the substituent at the 2-position was varied (R = F, Cl, Br, or NO_2). Within this series there was a strong trend correlating with atomic size for the halogens, with the smaller fluorine substituent binding 16-fold more tightly than bromine. However, no substituent in this series was more effective than the 2-hydroxyl group. In conclusion, the binding pocket for the 2-substituent favors a hydrogen bond donating group with a van der Waals radius smaller than chlorine.

Inhibition Mechanisms of 3-(*3*)-27, 2-(*2*)-13, and Uracil. Although the uracil-directed ligand tethering strategy is expected to produce competitive inhibitors of UNG, we thoroughly investigated whether this assumption was true. The detailed mode of inhibition by **3**-(*3*)-**27** and **2**-(*2*)-**13** was evaluated by varying both substrate and inhibitor concentrations (Figure 6A,B). Standard double reciprocal plots of $1/k_{obsd}$ against 1/[DNA] at increasing concentrations of **3**-(*3*)-**27** showed no significant intercept effects establishing a competitive aspect to the inhibition (Figure 6A). However, a secondary plot of the Lineweaver−Burk slopes against [**3**-(*3*)-**27]** showed a *parabolic* response consistent with the presence of at least two inhibitor binding sites (Figure 6A, inset).[36] Global discrimination fitting of the inhibition data by computer simulation with the program Dynafit using competitive, noncompetitive, uncompetitive, mixed-type, two-site competitive−noncompetitive, and two-site competitive−uncompetitive inhibition mechanisms unambiguously confirmed the presence of two inhibitory binding sites for **3**-(*3*)-**27** (see Supporting Information).[37] Simulations clearly indictated that the first tight site is competitive with respect to substrate. Although the simulations indicated a slight statistical advantage for a partial mixed-type inhibition mode for the second weaker site, it was difficult to eliminate an uncompetitive mode for this site. Using the criterion of Occam's razor, the inhibition parameters for **3**-(*3*)-**27** are reported in Table 3 using the simulation results for the competitive−partial uncompetitive mechanism (Scheme 1).

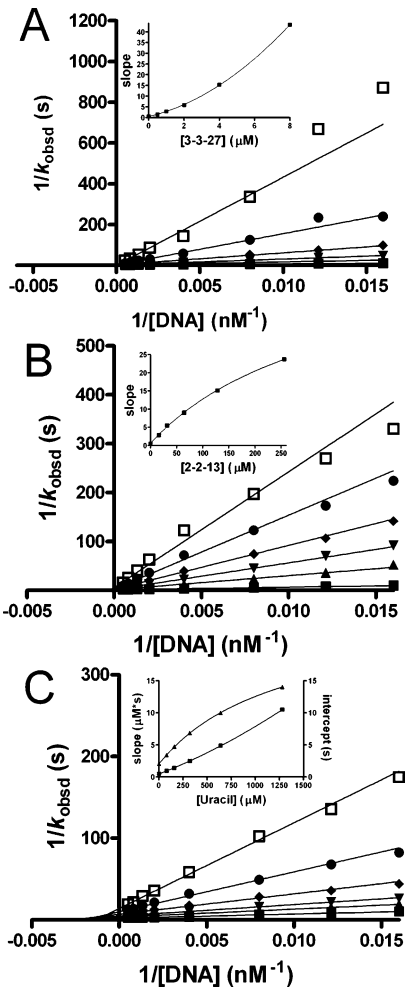


Figure 6. Mode of inhibition analysis, presenting double reciprocal plots and secondary slope and intercept replots for inhibition by increasing concentrations of (A) **3**-(*3*)-**27**, (B) **2**-(*2*)-**13**, and (C) uracil. Slope and intercept effects in the inset to (C) are shown as squares and triangles, respectively.

Like its 6-substituted analogue, initial inspection of the Lineweaver−Burk analysis of **2**-(*2*)-**13** indicates mixed-type inhibition with a strong preference for binding to the free enzyme (i.e. slope effects, Figure 6B). However, in contrast to **3**-(*3*)-**27**, the secondary plot of the Lineweaver−Burk slopes versus **2**-(*2*)-**13** concentration is *hyperbolic*, indicating that binding of **2**-(*2*)-**13** results in partial inhibition (Figure 6B, inset).[36] Because binding to the active site would result in complete inhibition, **2**-(*2*)-**13** most likely binds to the noncompetitive site observed for **3**-(*3*)-**27.** Global discrimination fitting of the inhibition data by computer simulation confirmed this inhibition mechanism (Scheme 1) and provided the inhibition

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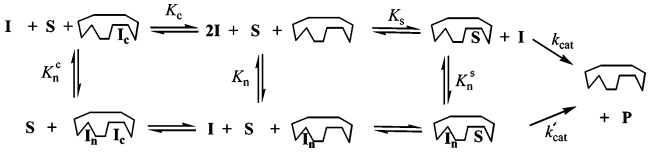
Table 3. Inhibition Constants for Uracil and Its Derivatives[a]

param	**3**-(*3*)-**27**	**2**-(*2*)-**13**	uracil	**51**
K_s (mM)	0.19 ± 0.02	0.23 ± 0.03	0.23 ± 0.02	0.16 ± 0.01
k_{cat} (s^{-1})	0.41 ± 0.01	0.50 ± 0.02	0.47 ± 0.01	0.33 ± 0.01
k_{cat} (s^{-1})	0.16 ± 0.04	0.012 ± 0.02	0.06 ± 0.01	
K_c (mM)	0.32 ± 0.02		80 ± 7	45 ± 2
K_n (mM)		2.8 ± 0.1		
K_n^c (mM)	1.2 ± 0.2		300 ± 55	
K_n^s (μM)	1 ± 0.3	125 ± 46	104 ± 7	
mode of inhibitn	two sites, competitive, partial uncompetitive	one site, partial mixed-type	two sites, competitive, partial uncompetitive	one site, competitive

[a] Parameters correspond to the mechanisms shown in Scheme 1. K_c and K_n represent dissociation constants for inhibitor binding sites that are competitive and noncompetitive with substrate, respectively. K_n^c and K_n^s represent the dissociation constants for inhibitor binding to the noncompetitive site when the active site is occupied by the competitively bound inhibitor or substrate, respectively. In these simulations the Michaelis−Menten parameters for the substrate were fixed using values from nonlinear regression fits (Figure 6). Other parameters were obtained from simulations to the data using the program Dynafit (cf. Supporting Information).

Scheme 1. Inhibition Mechanisms for **3**-(*3*)-**27** and **2**-(*2*)-**13** and Uracil[a]



[a] Only **3**-(*3*)-**27**, **2**-(*2*)-**13**, and uracil have mechanisms that include the k_{cat}' step. The mechanisms for **3**-(*3*)-**27** and uracil do not include the equilibrium constant K_n, and the mechanism for **2**-(*2*)-**13** does not include the equilibria K_c or K_n^c.

constants reported in Table 3. These observations strongly indicate that **2**-(*2*)-**13** binds to a site distinct from the active site, although DNA binding is strongly antagonistic to inhibitor binding (Table 3). In summary, the inhibition mechanisms of **3**-(*3*)-**27** and **2**-(*2*)-**13** indicate that two inhibition modes exist for these uracil derivatives: one mode competitively targets the active site, and the second weaker mode is noncompetitive or uncompetitive with respect to substrate binding. These data, quite surprisingly, suggested the presence of two uracil binding sites on human UNG.

To further investigate the interesting possibility of two uracil binding sites on UNG, we performed a mode of inhibition analysis for uracil itself (Figure 6C). In confirmation of this initial expectation, inhibition by uracil involves two sites. The first site is competitive, and the second is partially uncompetitive. Accordingly, the Lineweaver−Burk slope replot was slightly parabolic indicating that inhibition involved binding of more than one molecule of uracil, and the intercept replot was hyperbolic indicating a partial uncompetitive mode. These characteristics of the inhibition by uracil combine the features observed for **3**-(*3*)-**27** and **2**-(*2*)-**13** and establish that the two site binding of **3**-(*3*)-**27** is not attributable to the trihydroxybenzaldoxime moiety but, instead, arises from the uracil functionality itself.

Implications for Two Uracil Binding Sites. Why would UNG have a second uracil binding site? Although the answer to this question cannot be firmly established by inhibition data alone, an intriguing role for this site during the mechanism of uracil base flipping is supported by several different experimental findings. First, kinetic experiments following the pathway of uracil flipping from duplex DNA have detected a weakly bound intermediate state of uracil that precedes its attainment of the final extrahelical state seen in the crystal structure (Figure 1A).[15,38−40] Solution- and solid-state NMR studies of uracil flipping support the existence of a weak uracil binding site because UNG is found to transiently stabilize thymine and other uracil congeners in an extrahelical conformation, without these bases gaining full access to the uracil active site pocket.[41,42] Relevant to these observations, the crystal structure of herpesvirus UDG bound to pTTTp shows that the 5′ T is bound in the mouth of the active site pocket in a manner that is consistent with a transient state on the pathway for base flipping of uracil.[43] Finally, the crystal structure of another base-flipping DNA repair enzyme, human 8-oxoguanine DNA glycosylase, suggests that this related enzyme can flip the normal base guanine into a discrimination pocket that was distinct from the active site pocket that only accommodates 8-oxoguanine.[44] These combined data provide a compelling case for a generalized pathway for base flipping involving transient enzyme stabilization of at least one extrahelical intermediate state before the base is docked into the active site. On the basis of the observation that **2**-(*2*)-**13** is excluded from the active site but that **3**-(*3*)-**27** and uracil can occupy both sites**,** we surmise that the relative binding affinities for each site might depend on the bulkiness of the substituent at the 5-position of uracil. In other words, uracil congeners with small substituents at the five position (such as hydrogen in the case of **3**-(*3*)-**27**) would favor binding to the active site and uracil derivatives with bulkier substituents (such as the dihydroxybenzaldoxime of **2**-(*2*)-**13**) would be sterically excluded from the active site but could gain access to the weaker less selective site. Indeed, it is well-known that the active site of UNG uses the bulky side chain of a tyrosine to exclude thymidine (5-methyluracil),[14,45−47] yet 6-substituted uracil derivatives such as **3**-(*3*)-**27** have been generally observed to bind to the active site.[14] Thus, the uracil-based inhibitors found here have revealed a possible pyrimidine discrimination site that may be employed during the multistep extrahelical uracil recognition mechanism. It should be noted that the noncompetitive inhibition

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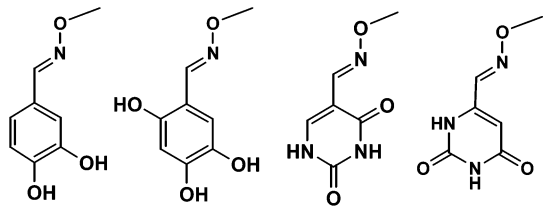
Chart 2. *O*-Methyl Oxime Derivatives of the Aldehyde Binding Elements of **2**-(*2*)-**13** and **3**-(*3*)-**27**



47 **48** **50** **51**

mode for **2**-(*2*)-**13** requires that the final extrahelical state can be attained, albeit inefficiently, even when the transient uracil binding site is occupied by the inhibitor. In contrast, the partial uncompetitive mechanism for binding of **3**-(*3*)-**27** to its second site does not present the same apparent discrepancy, because, for uncompetitive inhibition, the compound binds after the substrate is fully inside the active site pocket (see above).

Inhibition by the Untethered Parts. It is of interest to ask how well uracil-directed ligand tethering has performed. To dissect the energetic contributions of the formyluracil and hydroxybenzaldoxime binding elements of **3**-(*3*)-**27** and **2**-(*2*)-**13,** we synthesized the methyl oxime derivatives of aldehydes **2**, **3**, **27**, and **13** as shown in Chart 2. These methyl oxime derivatives are reasonable mimics of the two individual binding elements and in principle could provide an energetic analysis of the binding affinities of the two separate elements. If the sum of the binding energies of each element equals the entire binding free energy of the whole tethered molecule, then it may be concluded that (i) the tether is energetically inert with respect to binding and (ii) the binding of one element does not affect the other by induced strain or forcing a tighter fit. If the whole tethered molecule binds much more weakly or tightly than expected from the summation of the binding free energies of the two individual binding elements, then nonadditive energetic effects are present. Such effects would indicate either an energetic penalty for tethering (antagonistic binding of the parts) or, alternatively, a nonadditive energetic benefit (synergistic binding of the parts).[48,49]

Comparison of the binding affinity of **3**-(*3*)-**27** to its competitive site ($K_c^{\mathbf{3}-(3)-\mathbf{27}} = 0.32\ \mu$M) with that of the 6-formyluracil *O*-methyl oxime binding element alone (**51)** allows estimation of the free energy benefit of tethering the trihydroxybenzaldoxime binding element to the 6-formyluracil oxime part. Conversely, comparison of the binding affinity of **3**-(*3*)-**27** with that of the trihydroxybenzaldoxime *O*-methyl ether (**48)** allows estimation of the free energy benefit of tethering the 6-formyluracil oxime binding element to the trihydroxybenzaldoxime part. The 6-formyluracil *O*-methyl oxime **51** shows a cleanly competitive mode of inhibition with $K_c^{\mathbf{51}} = 45 \pm 2\ \mu$M (Table 4, data not shown). Thus, the enhancement in the free energy of binding upon addition of the trihydroxybenzaldoxime (THB) part to the 6-formyluracil oxime element is $\Delta\Delta G^{\mathrm{THB}} = -RT \ln(K_i^{\mathbf{3}-(3)-\mathbf{27}}/K_i^{51}) = -3$ kcal/mol. We were unable to perform a similar energetic analysis with the trihydroxybenzaldoxime *O*-methyl ether (**48**) due to its extremely weak binding (9% inhibition at 1 mM concentration, data not shown). Similarly, an energetic analysis of the binding elements comprising **2**-(*2*)-**13** was not possible because of the extremely weak inhibition by the 5-formyluracil *O*-methyl oxime (**50**) and the dihydroxybenzaldoxime *O*-methyl ether (**47**). Nevertheless, the 140-fold greater binding affinity of **3**-(*3*)-**27** as compared to the 6-formyluracil *O*-methyl oxime binding element (**51**) alone indicates that a large benefit can be derived from tethering.[50]

Experimental Section

Reagents and General Methods. All chemicals were purchased from commercial sources without further purification unless otherwise stated. The ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded on a 400 MHz Varian Innova instrument. The spectra were recorded in deuteriochloroform ($CDCl_3$) or in hexadeuteriodimethyl sulfoxide (DMSO-d_6). The chemical shifts of protons are given in ppm with TMS as internal standard. The chemical shifts of carbons are obtained in ppm with solvents as internal standards. That of fluorine is given in ppm with 1% trifluoroacetic acid in DMSO-d_6 as an external standard. Most of oximes were purified by HPLC using aqueous triethylammonium acetate (TEAA) as a running buffer. Therefore, TEAA was not completely removed and it appeared in the NMR spectra. Accordingly, proton and carbon chemical shifts of TEAA were not listed during the characterizations of the oximes. During the purification of the oxime **3**-(*3*)-**27**, 2-mercaptoethanol was used as an antioxidant. Therefore, small amounts of this compound and its oxidation product are also present in the oxime **3**-(*3*)-**27**. Flash chromatographies were performed with silica (70−230 mesh from Sorbent Technologies) and monitored by thin-layer chromatography (TLC) with silica plates (Merck, Kieselgel 60 F254).

Synthesis of Alkyl Hydroxyamines. *O*,*O*′-Diaminoalkanediol linkers of variable length (ethyl, propyl, butyl, pentyl, hexyl) were prepared from the corresponding dibromoalkanes in two steps according to literature procedures (Figure 2A).[33,51,52]

General Synthesis of Tethered Oxime Dimers. A set of 14 aryl aldehydes (**4**−**17**; cf. Supporting Information Table S1) was selected for library synthesis for coupling to the three uracil containing aldehydes (**1**−**3**, Figure 2) using the *O*,*O*′-diaminoalkanediol linkers as follows. To each 0.5-mL well of a Matrix microtiter plate was added a DMSO stock solution of AcOH (20 μL, 150 mM, 3 μmol), uracil aldehyde **1**−**3** (20 μL, 150 mM, 3 μmol), and a single aryl aldehyde (20 μL, 150 mM, 3 μmol). The plate was carefully agitated to make the solutions homogeneous. To each of the uracil−aryl aldehyde mixture was added a DMSO solution of the *O*,*O*′-diaminoalkanediol linkers containing each of the five linker lengths in equal proportion (22 μL, 150 mM, 3.3 μmol total amine equivalents). The plate was sealed, further agitated, and incubated in an oven for 12 h at 37 °C.

The most potent inhibitors from this first screen **2**-(*2*)-**13** and **3**-(*3*)-**13**) were synthesized in larger scale and thoroughly characterized after HPLC purification of the heterodimers as follows.

2-(*2*)-13: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 (s, 1 H), 7.91 (s, 1 H), 7.78 (s, 1 H), 7.04 (s, J = 2.4 Hz, 1 H), 6.86 (m, 1 H), 6.74 (d, J = 8.0 Hz, 1 H), 4.26 (s, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.40, 151.04, 149.25, 147.92, 145.75, 142.74, 140.66, 123.05, 119.88, 115.74, 113.10, 104.31, 71.82, 71.54; UV/vis λ_{max} 275 nm; HRMS (*m*/*z*) [M + Na]$^+$ calcd for $C_{14}H_{14}N_4O_6Na$ 357.08, found 357.08.

3-(*3*)-13: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (bs, H), 8.01 (s, 1 H), 7.94 (s, 1 H), 7.04 (d, J = 1.6 Hz, 1 H), 6.82 (d, J = 7.6 Hz, 1 H), 6.74 (d, J = 7.6 Hz, 1 H), 5.78 (s, 1 H), 4.26 (t, J = 6.8 Hz, 2 H), 4.12 (t, J = 6.0 Hz, 2 H), 2.06 (t, J = 6.8 Hz, 2 H); ^{13}C NMR (125 MHz,

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(50) The uracil N1-acetaldehyde *O*-methyl oxime (**49**) showed undetectable inhibition (K_c > 10 mM).
(51) Kung, P. P.; Bharadwaj, R.; Fraser, A. S.; Cook, D. R.; Kawasaki, A. M.; Cook, P. D. *J. Org. Chem.* **1998**, *63*, 1846−1852.
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DMSO-d_6) δ 163.95, 151.15, 148.96, 148.04, 145.89, 144.73, 142.23, 123.12, 119.83, 115.81, 113.15, 101.60, 71.94, 69.76, 28.46; UV/vis λ_{max} 273 nm; HRMS (*m*/*z*) $[M + H]^+$ calcd for $C_{15}H_{17}N_4O_6$ 349.11, found 349.11.

The second set of oxime dimers based on the **3**-(*3*)-**13** hit discovered in the first screening round were synthesized in an identical fashion as described above using uracil aldehyde **3** and hydroxybenzaldehydes **18**−**42** and the *O*,*O*′-diaminopropanediol linker (cf. Supporting Information Table S2). The most potent inhibitor identified from this second round of screening (**3**-(*3*)-**27**) was synthesized in larger scale and thoroughly characterized. **3**-(*3*)-**27**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (bs, H), 8.21 (s, 1 H), 7.94 (s, 1 H), 6.88 (s, 1 H), 6.31 (s, 1 H), 5.78 (s, 1 H), 4.28 (t, J = 6.0 Hz, 2 H), 4.10 (t, J = 6.0 Hz, 2 H), 2.06 (m, 2 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.91, 151.04, 150.25, 149.17, 146.90, 144.63, 142.20, 138.73, 112.76, 107.78, 103.56, 101.69, 71.90, 69.77, 28.38; UV/vis λ_{max} 286 nm; ESI (*m*/*z*) for $[M + H]^+$ calcd for $C_{15}H_{18}N_4O_7$ 366, found 366; ESI (*m*/*z*) for $[M + Na]^+$ calcd for $C_{15}H_{17}N_4O_7Na$ 388, found 388; ESI (*m*/*z*) for $[M - H]^-$ calcd for $C_{15}H_{16}N_4O_7$ 364, found 364.

Isolation and Purification of Oxime Dimers using HPLC. All of the most active oxime heterodimers were purified by HPLC using a Phenomenex Aqua reversed phase C-18 HPLC column (250 mm, 10 mm, 5 μm). Most of the oximes were purified using gradient elution from 0 to 30% CH_3CN in 0.1 M aqueous TEAA over the course of 2 h using UV detection at 254 nm. An exception was oxime **3**-(*3*)-**27**, which is prone to air oxidation. In this case, 25 mM 2-mercaptoethanol was added to both of the running buffers. The oximes all eluted with baseline resolution in the order U−U homodimer, U−R heterodimer, followed by the R−R homodimer. This HPLC method was also used to confirm the expected 1:2:1 stoichiometries of homodimer and heterodimer oxime formation, using 10 representative uracil and aryl aldehydes from the library (see Supporting Information Figure S1). Additional NMR evidence supporting the expected stoichiometries is detailed in the Supporting Information Figures S2 and S3.

Synthesis of 2-R-Substituted 3,4-Dihydroxybenzaldehydes and the Corresponding Mixed Oximes with 3. Aldehyde **43** was synthesized by removing the methyl groups of the commercially available 3,4-dimethoxy-6-fluorobenzaldehyde using BBr_3 in CH_2Cl_2.[53] The aldehydes **44** and **45** were synthesized by removing the methylene group of the corresponding 2-halogenated piperonal using $AlCl_3$ and 6N HCl.[54] Aldehyde **46** was commercially available. These four aldehydes (**43**−**46**) were reacted with 6-formyluracil **3** and the *O*,*O*′-diaminopropanediol linker using the procedure described above, and the mixed oxime dimer was obtained after HPLC purification.

3-(*3*)-**43:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.11 (s, 1 H), 7.94 (s, 1 H), 7.05 (d, J = 7.2 Hz, 1 H), 6.58 (d, J = 6.8 Hz, 1 H), 5.77 (s, 1 H), 5.10 (bs, H), 4.28 (t, J = 6.8 Hz, 2 H), 4.16 (t, J = 6.0 Hz, 2 H), 2.07 (m, 2 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.91, 155.50, 153.09, 151.08, 149.87, 149.76, 144.65, 142.72, 142.30, 142.21, 110.91, 110.87, 108.62, 108.49, 103.21, 102.96, 101.67, 71.85, 70.06, 28.38; ^{19}F NMR (DMSO-d_6) δ −54.33, −54.35, −54.36, −54.38; UV/vis λ_{max} 268 nm; HRMS (*m*/*z*) $[M + Na]^+$ calcd for $C_{15}H_{15}FN_4O_6Na$ 389.09, found 389.09.

3-(*3*)-**44:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (s, 1 H), 7.94 (s, 1 H), 7.18 (s, 1 H), 6.77 (s, 1 H), 5.78 (s, 1 H), 4.27 (t, J = 5.6 Hz, 2 H), 4.16 (t, J = 6.0 Hz, 2 H), 2.08 (m, 2 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.90, 151.08, 149.71, 145.48, 145.16, 144.64, 142.21, 123.00, 119.09, 116.08, 112.33, 101.67, 71.85, 70.22, 28.37; UV/vis λ_{max} 275 nm; HRMS (*m*/*z*) $[M + Na]^+$ calcd for $C_{15}H_{15}ClN_4O_6Na$ 405.06, found 405.06.

3-(*3*)-**45:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (s, 1 H), 7.94 (s, 1 H), 7.19 (s, 1 H), 6.93 (s, 1 H), 5.78 (s, 1 H), 4.27 (t, J = 6.4 Hz, 2 H), 4.16 (t, J = 6.4 Hz, 2 H), 2.08 (m, 2 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.91, 151.10, 150.07, 147.41, 146.02, 144.66, 142.22, 120.58, 119.15, 112.89, 112.29, 101.69, 71.86, 70.24, 28.38; UV/vis λ_{max} 278 nm; HRMS (*m*/*z*) $[M + Na]^+$ calcd for $C_{15}H_{15}BrN_4O_6Na$ 449.01, found 449.01.

3-(*3*)-**46:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 (d, J = 1.2 Hz, 1 H), 7.95 (s, 1 H), 7.37 (s, 1 H), 6.74 (s, 1 H), 6.26 (bs, H), 5.78 (d, J = 1.2 Hz, 1 H), 4.28 (t, J = 6.0 Hz, 2 H), 4.16 (t, J = 6.0 Hz, 2 H), 2.09 (m, 2 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.88, 160.27, 151.05, 148.62, 148.00, 144.62, 142.22, 133.36, 122.38, 113.80, 109.23, 101.73, 71.85, 70.07, 28.39; UV/vis λ_{max} 269 nm; HRMS (*m*/*z*) $[M + H]^+$ calcd for $C_{15}H_{16}N_5O_8$ 394.10, found 394.10.

Synthesis of Methyl Oxime Derivatives of 1−3, 13, and 27. The *O*-methyl oxime of 3,4-dihydroxybenzaldehyde (**47**) is known and was synthesized using **13** and *O*-methylhydroxylamine hydrochloride.[55] *O*-Methyloximes **48**−**51** were made using a similar method.

48: To a solution of **27** (308 mg, 2.0 mmol) in 4.0 mL of EtOH−H_2O−THF (0.45/0.3/0.25) were added sodium acetate (264 mg) and *O*-methylhydroxylamine hydrochloride (183 mg), and the solution was stirred at room temperature for overnight. The solvents were removed in vacuo, and the residue was extracted with chloroform three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexanes) to give a product amount of 347 mg in 95% yield: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.38 (s, 1 H), 9.21 (s, 1 H), 8.52 (s, 1 H), 8.18 (s, 1 H), 6.88 (s, 1 H), 6.30 (s, 1 H), 3.80 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 150.17, 148.88, 146.52, 138.61, 112.51, 107.77, 103.46, 61.28; UV/vis λ_{max} 239, 274 nm; HRMS (*m*/*z*) $[M + H]^+$ calcd for $C_8H_{10}NO_4$ 184.06, found 184.06.

49: To a solution of **1** (10.8 mg, 0.063 mmol) in hot DMF (0.5 mL) were added sodium acetate (5.2 mg, 0.063 mmol) solution in water (0.1 mL) and *O*-methylhydroxylamine hydrochloride (5.3 mg, 0.063 mmol), and the solution was stirred at room temperature for overnight. The solvents were removed in vacuo, and the residue was purified by column chromatography using 10−15% (v/v) methanol in CH_2Cl_2, resulting in 90% yield (10.3 mg 50/50 mixture of trans and cis geometric isomers): ^{1}H NMR (400 MHz, chloroform-*d*) δ 9.56 (s, 1 H), 7.43 (t, J = 5.2 Hz,0.5 H), 7.20 (m, 1 H), 6.80 (t, J = 4.4 Hz,0.5 H), 5.78 (m, 1 H), 4.55 (d, J = 4.4 Hz,1 H), 4.48 (d, J = 5.6 Hz,1 H), 3.94 (s, 1.5 H), 3.87 (s, 1.5 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.92, 163.87, 151.06, 150.99, 144.72, 144.54, 143.93, 143.53, 103.14, 102.97, 62.68, 62.37, 46.52, 43.76; UV/vis λ_{max} 263 nm; HRMS (*m*/*z*) $[M + H]^+$ calcd for $C_7H_{10}N_3O_3$ 184.07, found 184.07.

50: To a solution of **2** (70 mg, 0.5 mmol) in hot DMF (1 mL) were added sodium acetate (41 mg, 0.5 mmol) solution in water (0.5 mL) and *O*-methylhydroxylamine hydrochloride (42 mg, 0.5 mmol), and the solution was stirred at room temperature for 4 h. The solvents were removed in vacuo, and the residue was collected by filtration and washed with cold water 2 × 1 mL, resulting in 76% yield (70 mg 87/13 mixture of trans and cis geometric isomers): ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.40 (bs, 2 H), 8.52 (s, 0.13 H), 7.87 (s, 0.87 H), 7.74 (s, 0.87 H), 7.29 (s, 0.13 H), 3.89 (s, 0.39 H), 3.80 (s, 2.61 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.98, 162.36, 150.80, 150.27, 146.08, 142.26, 140.09, 137.31, 104.41, 103.43, 62.32, 61.44; UV/vis λ_{max} 288 nm; HRMS (*m*/*z*) $[M + H]^+$ calcd for $C_6H_8N_3O_3$ 170.06, found 170.06.

51: To a solution of **3** (79 mg, 0.5 mmol) in hot DMF (2.0 mL) were added sodium acetate (46 mg, 0.5 mmol) solution in water (0.5 mL) and *O*-methylhydroxylamine hydrochloride (46 mg, 0.5 mmol), and the solution was stirred at 50 °C for 4 h. The solvents were removed in vacuo, and the residue was washed by cold water. After the filtration, product was obtained in 62% yield (53 mg): ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.18 (s, 1 H), 10.77 (s, 1 H), 7.91 (s, 1 H), 5.77 (s, 1 H), 3.96 (s, 3 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.87, 151.02,

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144.51, 142.17, 101.41, 62.84; UV/vis λ_{max} 292 nm; HRMS (*m*/*z*) [M + Na]$^+$ calcd for $C_6H_7N_3O_3Na$ 192.04, found 192.04.

High-Throughput Inhibitor Screening. The substrate in this HTS assay was synthesized using standard phosphoramidite DNA solid-phase chemistry using reagents purchased from Glen Research. The DNA was purified using anion exchange chromatography followed by desalting using reversed phase methods. The sequence and size was confirmed using analytical denaturing polyacrylamide gel electrophoresis and MALDI-MS. The substrate is a double-stranded 14-mer DNA containing nine U·A base pairs (5′-FAM-GCA CUU AAG AAU UG: 3′-DABSYL-CA AUU CUU AAG UGC). The UNG HTS assay is performed as follows. To a 96-well microtiter plate was added 5 μL (2 mM total) of compound in DMSO, followed by 75 μL (33.3 pM) of human UNG in reaction buffer (10 mM Tris·HCl, pH 8.0, 20 mM NaCl, 7.5 mM $MgCl_2$, 0.002% brij-35). The reactions were initiated by the addition of 20 μL (250 nM) of molecular beacon substrate in reaction buffer. The plates are incubated at ambient temperature in a fluorescence plate reader for 30 min, and the progress of the reaction was monitored every 5 min (ex 485 nm/em 520 nm). The final concentrations of the reagents in the assay are 10 mM Tris·HCl, pH 8.0, 20 mM NaCl, 7.5 mM $MgCl_2$, 0.002% Brij-35, 25 pM human UNG, 50 nM molecular beacon substrate, 100 μM total compound, and 5% DMSO. The $MgCl_2$ is essential to increase the stability of the double-stranded DNA substrate and, thus, decrease the initial fluorescence of the molecular beacon and increase the maximum signal of the assay. Addition of Brij-35, a nonionic detergent, is essential to stabilize human UNG at the low concentration used in this assay. A similar assay has been described by Maksimenko et al. that utilizes a 39-mer hairpin DNA.[56] However, the synthesis and purification of this more complex substrate proceeds with low efficiency and requires higher temperature to induce strand separation (Krosky and Stivers, unpublished data). In contrast, the 14-mer double-stranded molecular beacon is routine and allows screening to be performed conveniently at room temperature.

Mechanism of Inhibition. The substrate used in mechanism of inhibition studies was a modified DNA hairpin where the two strands described above are connected by a hexakis[poly(ethylene glycol)} linker (PEG-U9). This substrate was easier to synthesize and purify than an all-DNA hairpin and, unlike the double stranded DNA substrate, does not require $MgCl_2$ to achieve minimum fluorescence. To a 96-well plate was added 5 μL of compound in DMSO, followed by 75 μL of PEG-U9 hairpin in reaction buffer (20 mM Tris·HCl, pH 8.0, 50 mM KCl, 0.2 mM $MgCl_2$, 0.002% Brij-35, 1 mM DTT). Eight different DNA concentrations were used in the range 62.5−2000 nM. Reactions were initiated by the addition of 20 μL of 0.5 nM human UNG in reaction buffer. The final concentrations of reagents in the assay are 20 mM Tris·HCl, pH 8.0, 50 mM KCl, 0.2 mM $MgCl_2$, 0.002% Brij-35, 1 mM DTT, 5% DMSO, 0.1 nM human UNG, 62.5−2000 nM PEG-U9 hairpin DNA, and variable amounts of inhibitor. The plates were incubated at ambient temperature in a fluorescence plate reader for 60 min, and the progress of each reaction was monitored every 5 min (ex 485 nm/em 520 nm). Afterward, *Escherichia coli* UNG was added to each well to drive the reactions to completion, and the overall change in fluorescence values were measured. These values were used to convert initial velocities from units of fluorescence units/s to [product]/s. Mechanisms of inhibition and their corresponding inhibitor dissociation constants were determined by Lineweaver−Burk slope and intercept replot analysis and by computational simulations of the initial velocity against inhibitor concentration data using Dynafit v.3.28 (see Supporting Information)

Conclusions

We have developed an efficient strategy to develop small-molecule inhibitors of UNG that have the potential for activity in cell culture or in vivo. The method is quite general and could be adapted to target other enzymes that bind extrahelical bases or free nucleosides. Two future targets of the current uracil mixed oxime library would be the essential bacterial enzyme deoxyuridine nucleotidylhydrolase, which converts dUTP to dUMP,[23,57−60] and human thymidine phosphorylase, an enzyme implicated in vascularization of tumors.[61] Such inhibitors could serve as useful tools to study the life cycle of pathogenic human viruses, the biology of uracil base excision repair in normal cell lines and tissues, and mechanisms of tumor vascularization.

Acknowledgment. This work was supported by NIH Grant GM56834-10 to J.T.S. D.J.K was supported by the DOD Breast Cancer Research Program (Grant DAMD17-03-1-1251).

Supporting Information Available: Tables of aryl aldehydes and hydroxybenzaldehydes used to construct oxime libraries, HPLC traces and NMR spectra to ascertain stoichiometries of oxime mixtures, ^{1}H and ^{13}C NMR spectra of **3**-(*3*)-**13** and **3**-(*3*)-**27**, computer simulations of inhibition data, and analytical equations for each inhibition mechanism. This material is available free of charge via the Internet at http://pubs.acs.org.

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Synthesis and high-throughput evaluation of triskelion uracil libraries for inhibition of human dUTPase and UNG2

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Received 22 February 2006; revised 10 April 2006; accepted 13 April 2006

Abstract—Human nuclear uracil DNA glycosylase (UNG2) and deoxyuridine triphosphate nucleotidohydrolase (dUTPase) are the primary enzymes that prevent the incorporation and accumulation of deoxyuridine in genomic DNA. These enzymes are desirable targets for small molecule inhibitors given their roles in a wide range of biological processes ranging from chromosomal rearrangements that lead to cancer, viral DNA replication, and the formation of toxic DNA strand breaks during anticancer drug therapy. To accelerate the discovery of such inhibitors, we have developed a high-throughput approach for directed library synthesis and screening. In this efficient technology, a uracil-aldehyde ligand is covalently tethered to one position of a trivalent alkyloxyamine linker via an oxime linkage, and then the vacant linker positions are derivatized with a library of aldehydes. The resulting triskelion oximes were directly screened for inhibitory activity and the most potent of these showed micromolar binding affinities to UNG2 and dUTPase.

1. Introduction

From the classic view of DNA repair and mutagenesis, the uracil base has no place in genomic DNA.[1] Accordingly, elaborate DNA repair mechanisms have evolved to exclude dUTP from the nucleotide pool used for DNA replication,[5,6] and to remove uracil from DNA when it arises from spontaneous deamination of cytosine bases.[7] However, the uracil base has recently been found to play a much more diverse role in human biology, disease, and anticancer therapy (Fig. 1). Surprisingly, the uracil excision repair machinery has been found to participate in the process of generating somatic mutations during antibody maturation in B cells,[8–10] and uracil incorporation and/or removal is critical in the life cycles of herpes,[11] cytomegalo,[12] pox,[13,14] and type 1 human immunodeficiency viruses (HIV-1).[15] Furthermore, this pathway also generates the pharmacologically active single and double strand DNA breaks that are the essential tumor killing lesions produced by the widely used anticancer drugs 5-fluorouracil and methotrexate,[16,17] and generates the characteristic chromosomal translocations found in some B cell lymphomas. Thus, pharmacologic agents that inhibit these processes are desirable for both investigational and therapeutic purposes.

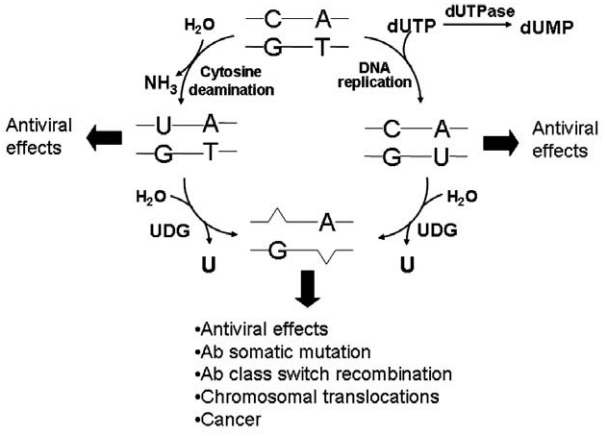

Figure 1. Biological effects of uracil in DNA.[2–4]

Human nuclear uracil DNA glycosylase (UNG2) and deoxyuridine triphosphate nucleotidohydrolase

Keywords: Uracil DNA glycosylase; dUTPase; Enzyme inhibition; Directed chemical libraries; High-throughput screening.
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doi:10.1016/j.bmc.2006.04.022

(dUTPase) are the primary enzymes that prevent the incorporation and accumulation of deoxyuridine in genomic DNA.[17,18] Given that these enzymes are emerging as interesting pharmacologic targets, we have sought out methods for the rapid and efficient identification of small molecule ligands that can inhibit their activity. One of the most exciting potential applications of UNG2 and dUTPase inhibitors would be as antiretroviral agents. Recent findings have established that HIV-1 specifically packages UNG2 into virus particles via interaction with the virus encoded integrase protein (Int), or perhaps a ternary complex between UNG, Int and the viral Vpr protein.[11,19–26] UNG2 is required for infection of nondividing cells such as macrophages and resting T cells, and virus particles produced from UNG depleted cells are incapable of infecting new target cells.[15,27] Infection of macrophages helps maintain a viral reservoir in the host that is crucial for virus spread to the lymphoid organs and T-helper lymphocytes, and ultimately, AIDS pathogenesis.[21,28] UNG is apparently recruited to minimize uracil incorporation into the viral genome in these cells, which have naturally high levels of dUTP, a good substrate for the viral reverse transcriptase.[29] Inhibition of dUTPase would be expected to further increase dUTP levels in macrophages, resulting in even more uracil misincorporation into the viral genome (Fig. 1). Pharmacologic targeting of a UNG2 and dUTPase is extremely attractive because these targets would not be susceptible to the same high mutagenesis rate and resulting drug resistance as viral encoded proteins.[30] Targeting UNG2 is a viable therapeutic strategy because it is not an essential enzyme. Thus, UNG knock-out mice display no remarkable phenotype, nor do UNG null yeast or human cell lines.[31] Although dUTPase is an essential enzyme in all organisms, it would be expected that rapidly replicating viruses such as HIV-1 would show higher sensitivity than the host, providing a potential therapeutic window.

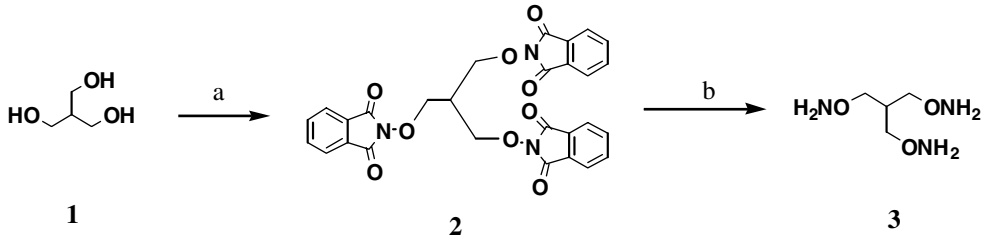
Herein, we report an integrated high-throughput (HTP) platform for the synthesis and evaluation of uracil-directed small molecule libraries based upon triskelion oxyamine scaffolds. The strategy is to attach a uracil-aldehyde ligand to one or two arms of the triskelion scaffold and then derivatize the vacant position(s) with a random library of aldehydes (RCHO). The uracil moiety is expected to weakly target the fully functionalized compound to the active site rather than irrelevant regions of the enzyme, and the random functional groups can then explore nearby binding pockets resulting in increased affinity over that of the uracil alone. Library compounds are rapidly screened using robust HTP activity assays, from which several inhibitors of UNG2 and dUTPase have been identified.

2. Results and discussion

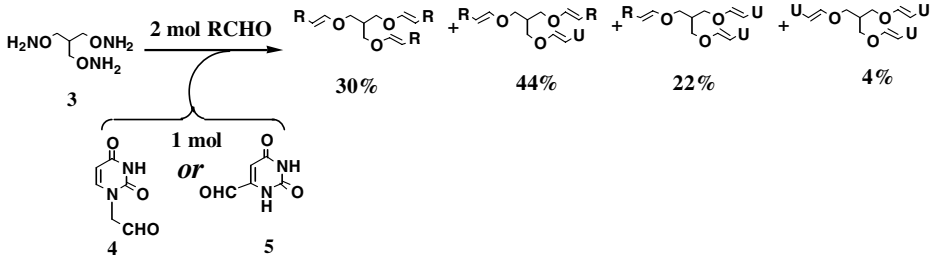
2.1. Synthesis of uracil triskelion oxime libraries

We sought an inhibitor development strategy that allowed rapid and economical synthesis of small molecule ligands that explore binding sites near the UNG2 and dUTPase active sites, and which could be used directly in HTP screening applications without purification. One efficient synthesis strategy that meets these criteria is outlined in Schemes 1 and 2. First, a triskelion oxyamine scaffold is synthesized in two steps from tris(hydroxymethyl)methane (Scheme 1). Then the three oxyamine groups are derivatized with a uracil-aldehyde and a library of 215 aldehyde binding elements (RCHO, Table 1) via the formation of stable oxime linkages (Scheme 2).

Each linking reaction is carried out in one well of a 96-well microtiter plate that contains one molar equivalent

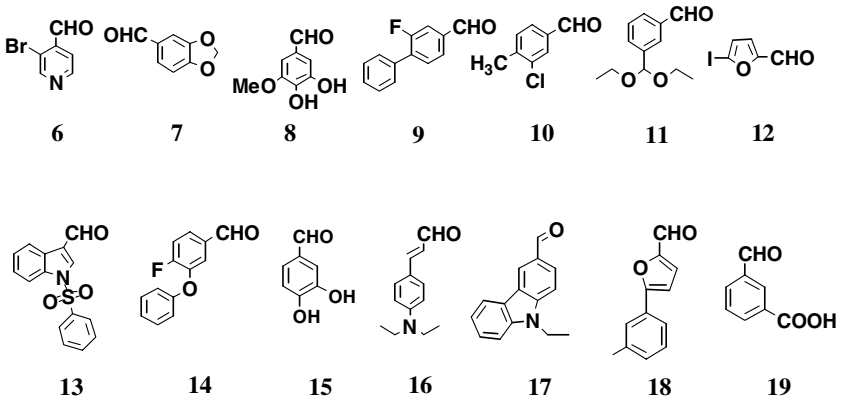


Scheme 1. Reagents and conditions: (a) triphenylphosphine, diisopropyl azodicarboxylate, *N*-hydroxyphthalimide, anhydrous THF, overnight, 0 °C, 53% yield; (b) anhydrous NH_2NH_2, 95% ethanol, room temperature, 2 h, 67%. See Ref. 32.



Scheme 2. Reagents and conditions: AcOH (20% v/v), DMSO, 12 h, 37 °C.

Table 1. Representative aryl aldehydes (RCHO) used in library synthesis[a]



[a] The entire library consisted of 215 aryl aldehydes.

uracil aldehyde, two molar equivalents RCHO library member, and one molar equivalent oxyamine triskelion scaffold (Scheme 2). The reactions typically proceed to 85–99% completion after overnight incubation (DMSO, 37 °C), and produce a statistical mixture of the homotrimeric (UUU, RRR) and heterotrimeric (UUR, RRU) oximes in the approximate amounts indicated in Scheme 2 (see supplemental materials). No compound purification is required before screening the library for active compounds.

2.2. High-throughput screening of triskelion libraries against UDG

We have previously developed a high-throughput fluorescence assay for UNG2 that allows screening of chemical libraries.[33] This assay was used to screen the 215 oxime mixtures obtained from reaction of the triskelion oxyamine scaffold with uracil **5** and library aldehydes **6** through **34**. Mixtures that showed inhibitory activity were subjected to fractionation using reversed-phase HPLC, and then the individual purified components were reevaluated to determine which species was responsible for the inhibition. After identifying the inhibitory molecules, they were resynthesized in larger scale, purified, and complete IC_{50} curves were determined as previously described.[33]

Two RCHO groups were found to be inhibitory when attached to uracil in the triskelion scaffold (Table 2). The IC_{50} values were found to fall in the range ~0.9 to 11 μM. When RCHO = 3,4 dihydroxybenzaldehyde, both the RRU (**45**) and UUR (**46**) variants showed nearly equal activity, suggesting that the enzyme recognizes the UR element, but not the third substituent on the scaffold (U or R). Consistent with this interpretation, the corresponding bifunctional oxime (UR, **47**, Table 2)[33] showed a similar IC_{50} value as **45** and **46**. In contrast, when RCHO = 3-carboxybenzaldehyde, the trivalent forms **48** and **49** were found to be 6- to 12-fold more potent than the bifunctional oxime **50**. Thus, in this latter case the third substituent has a significant effect on binding affinity.

2.3. High-throughput screening of triskelion libraries against dUTPase

A similar strategy was used to screen the 215 oxime mixtures obtained from reaction of the triskelion scaffold with uracil aldehyde **4** and library aldehydes **6** through **34** against human dUTPase. In this case, only one inhibitory RCHO group was found (Table 3), and both the RRU (**51**) and UUR (**52**) variants provided comparable IC_{50} values in the range 3–5 μM. To the best of our knowledge, **51** and **52** are the most potent nonnucleotide inhibitors of human dUTPase yet reported.

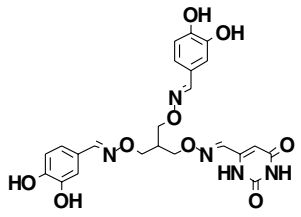
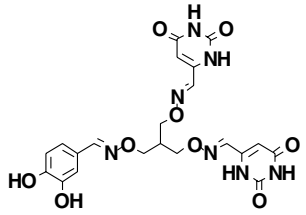
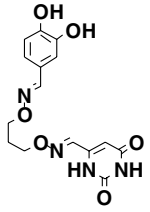
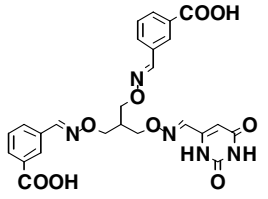
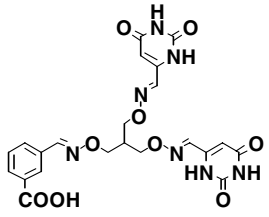
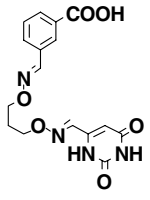
2.4. Inhibition by the untethered parts

The key question in determining the effectiveness of this tethering strategy is the inhibitory capacities of the untethered uracil and aldehyde components. For UNG2, the methyl oxime of uracil **5** has an IC_{50} value of 75 μM, and no inhibition by the methyl oxime of 3,4 dihydroxybenzaldehyde (**15**) could be detected even at concentrations as high as 1 mM. Thus, tethering **15**–**5** produced an increase in binding affinity of 75-fold relative to uracil alone, and tethering **5**–**15** brought about at least a 1000-fold increase in binding affinity relative to **15** alone. For dUTPase, the methyl oximes of **4** and **8** were not inhibitory even at concentrations as high as 1 mM. Thus in this case, tethering of the two parts has brought about increases in binding affinity of at least 700-fold as compared to the separate components. A trivial but potentially useful modification of the tethering approach would be to incorporate two different R groups into the triskelion scaffold. This is easily accomplished by first synthesizing and isolating the monoderivatized uracil compound and then reacting the remaining two oxyamine positions with a mixture of two aldehydes (unpublished).

3. Conclusion

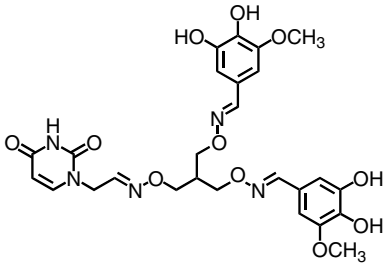
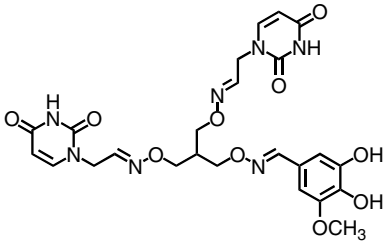
We have established that triskelion libraries of uracil derivatives can efficiently yield inhibitors with micromolar affinities for two different enzymes that recognize the

Table 2. Structures and inhibitory constants for UNG2 inhibitors[a]

Triskelion compound	IC_{50} (μM)
 **45**	1.6 ± 0.1
 **46**	0.9 ± 0.1
 **47**	1.3 ± 0.1
 **48**	1.7 ± 0.5
 **49**	0.9 ± 0.1
 **50**	10.9 ± 0.5

[a] See Ref. 33 for representative HTS screening data and IC_{50} curves.

Table 3. Structures and inhibitory constants for dUTPase inhibitors

Triskelion compound	IC_{50} (μM)
 **51**	3.3 ± 1.1
 **52**	5.5 ± 1.0

uracil base. Unlike previous nucleic acid-based inhibitors of UNG.[34,35] and nucleotide-based inhibitors of dUTPase,[36] these library compounds are expected to be cell permeable. A useful extension of this approach is currently being developed where the length of the linker arms is varied. This modified approach is expected to generate more diverse libraries that allow more comprehensive probing of potential binding sites near the uracil pocket.

With respect to the in vivo utility of such oxime libraries, there are a number of currently used drugs with oxime functional groups: the selective serotonin reuptake inhibitor, fluvoxamine,[37,38] the monobactam antibiotic, aztreonam,[38] and several preclinical antimicrobial drugs.[39,40] The activity of these drugs indicates that oxime linkages are stable and useful in real clinical applications. Nevertheless, oximes are susceptible to reduction in metabolic reactions involving cytochrome P450-mediated transformations.[40,41] Depending on the pharmacokinetic and pharmacodynamic properties of the individual oximes, this may or may not pose a problem. For instance fluvoxamine, although extensively processed in first-pass metabolism, has a reasonable serum half-life of 12 h.[37,38] We anticipate that triskelion libraries based on substrate fragments will be useful for rapid inhibitor development against a variety of enzymes.

4. Experimental

4.1. Reagents and general methods

All chemicals were purchased from commercial sources without further purification unless otherwise stated. The ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Varian Innova instrument. The spectra were recorded in deuteriochloroform ($CDCl_3$) or in hexadeu-

teriodimethylsulfoxide (DMSO-d_6). The chemical shifts of protons are given in ppm with TMS as internal standard. The chemical shifts of carbons are obtained in ppm with solvents as internal standards. Oximes were purified by HPLC using aqueous triethylammonium acetate (TEAA) as a running buffer. Therefore, TEAA was not completely removed and it appeared in the NMR spectra. Accordingly, proton and carbon chemical shifts of TEAA were not listed during the characterizations of the oximes. During purification of the oximes **45** and **46**, 2-mercaptoethanol was used as an anti-oxidant. Therefore, small amounts of this compound and its oxidation product are also present in these oximes. Flash chromatographies were performed with silica (70–230 mesh from Sorbent Technologies) and monitored by thin-layer chromatography (TLC) with silica plates (Merck, Kieselgel 60 F254).

4.2. 2,2′,2″-[(2-Methyloxy-1,3-propanedioxy)tris]-1*H*-isoindole-1, 3(2*H*)-dione (2)

To a suspension of 2-hydroxymethyl-1,3-propanediol **1** (0.848 g, 8.0 mmol), triphenylphosphine (7.32 g, 28 mmol), and *N*-hydroxyphthalimide (6.52 g, 40 mmol) in anhydrous THF (60 ml) was added diisopropyl azodicarboxylate (5.64 ml, 28.0 mmol) dropwise at 0 °C.[42–44] The mixture was stirred overnight, and the precipitate was filtered and washed with cold THF. After removal of the THF in vacuo, product **2** was obtained (2.3 g) in 53% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (m, 12H), 4.66 (d, J = 6.4 Hz, 6H), 2.78 (septet, J = 6.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.43, 134.39, 128.97, 123.47, 75.52, 37.68. HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{28}H_{20}N_3O_9$, 542.12; found, 542.12.

4.3. 2-[(Aminooxy)-methyl]-1,3-bis(aminooxy)-propane (3)

To a suspension of **2** (2.43 g, 4.5 mmol) in 95% ethanol (9.5 ml), was added anhydrous hydrazine (0.67 ml, 20.2 mmol) dropwise within 10 min at room temperature. The mixture was stirred for 2 h, filtered, and washed with 95% ethanol. The filtrate was concentrated in vacuo to give a residue. To the residue was added methylene chloride (10 ml). The resulting mixture was kept overnight at room temperature, filtered the following morning, and the filtrate was concentrated in vacuo, giving product **3** (0.46 g) in 67% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.71 (d, J = 6.0 Hz, 6H), 2.41 (septet, J = 6.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 74.66, 37.05; HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_4H_{14}N_3O_3$, 152.10; found, 152.10.

4.4. General synthesis of tethered triskelion oximes

A set of 39 commercially available aldehydes were linked to the two uracil-containing aldehydes **4** and **5** using the triskelion alkyloxyamine linker **3** (see Scheme 2 and Table 1, and supplementary materials). To each well of a 0.5-ml Matrix microtiter plate were added DMSO stock solutions of AcOH (20 μL, 150 mM, 3 μmol), uracil-containing aldehyde **4** or **5** (20 μL, 150 mM, 3 μmol), and one library aldehyde (40 μL, 150 mM, 6 μmol). The synthesis and characterization of **4** and **5** has been previously reported. The plate was carefully agitated to make the solutions homogeneous, and 22 μL of a DMSO solution of the triskelion oxyamine was then added (150 mM, 3.3 μmol). The plate was sealed and further agitated and incubated in an oven for 12 h at 37 °C. The expected statistical ratios of the oxime products were confirmed by ^{1}H NMR analysis (see supplementary materials).

4.5. Isolation and purification of inhibitory triskelion oximes

The most potent inhibitors were resynthesized in larger scale and thoroughly characterized after HPLC purification of the mixed oximes using a Phenomenex Aqua reversed-phase C-18 HPLC column (250 mm, 10 mm, 5 μm). Gradient elution from 0% to 65% CH_3CN in 0.1 M aqueous TEAA over the course of 2 h with UV detection at 254 nm was used. An exception was oxime **47**, which is prone to air oxidation. In this case, 25 mM 2-mercaptoethanol was added to both of the running buffers. The oximes all eluted with baseline resolution in the order (1) the homotrimer oxime derived from **4** or **5**, (2) the heterotrimer oxime derived from **4** or **5** and either **8**, **15** or **19**, and (3) the homotrimer oxime derived from **8**, **15** or **19**.

4.6. 2-[*O*-(6-Uracilcarboxaldoximyl)-methyl]-1,3-bis[*O*-(3,4-dihydroxybenzaldoximyl)]-propane (45)

^{1}H NMR (400 MHz, DMSO-d_6): δ 11.15 (br s, 1H), 9.30 (br s, 1H), 8.09 (s, 2H), 7.98 (s, 1H), 7.05 (d, J = 1.6 Hz, 1H), 6.84 (dd, J = 8.0, 2.0 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 5.78 (s, 1H), 4.32 (d, J = 6.0 Hz, 2H), 4.17 (d, J = 5.6 Hz, 4H), 2.66 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.85, 151.04, 149.12, 147.70, 145.57, 144.51, 142.43, 123.09, 119.95, 115.60, 112.91, 101.78, 73.25, 71.13, 38.67; HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{23}H_{24}N_5O_9$, 514.16; found, 514.16.

4.7. 2-[*O*-(3,4-Dihydroxybenzaldoximyl)-methyl]-1,3-bis[*O*-(6- uracilcarboxaldoximyl)]-propane (46)

^{1}H NMR (400 MHz, DMSO-d_6): δ 11.15 (br s, 1H), 8.05 (s, 1H), 7.97 (s, 2H), 7.04 (d, J = 2.0 Hz, 1H), 6.84 (dd, J = 8.0, 2.0 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 5.78 (s, 2H), 4.32 (d, J = 6.4 Hz, 4H), 4.16 (d, J = 6.0 Hz, 2H), 2.66 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.50, 151.72, 149.84, 148.39, 146.24, 145.16, 143.13, 123.68, 120.61, 116.25, 113.61, 102.40, 73.73, 71.60, 38.98; HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{22}N_7O_9$, 516.15; found, 516.15.

4.8. 1-[*O*-(6-Uracilcarboxaldoximyl)]-3-[*O*-(3,4-dihydroxybenzaldoximyl)]-propane (47)

The synthesis and characterization of this oxime heterodimer has been previously described[33].

4.9. 2-[*O*-(6-Uracilcarboxaldoximyl)-methyl]-1,3-bis[*O*-(3-carboxybenzaldoximyl)]-propane (48)

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.34 (s, 2H), 8.15 (s, 2H), 8.0 (s, 1H), 7.91 (d, J = 6.8 Hz, 2H), 7.65 (d,

J = 8.0 Hz, 2H), 7.38 (t, J = 8.4 Hz, 2H), 5.77 (s, 1H), 4.36 (d, J = 6.4 Hz, 2H), 4.28 (d, J = 5.6 Hz, 4H), 2.82 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.54, 163.88, 151.07, 149.16, 144.57, 142.60, 137.36, 131.43, 130.62, 128.75, 128.27, 127.55, 101.86, 73.12, 71.50, 38.67; HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{25}H_{24}N_5O_9$, 538.16; found, 538.16.

4.10. 2-[*O*-(3-Carboxybenzaldoximyl)-methyl]-1,3-bis[*O*-(6-uracilcarboxaldoximyl)]-propane (49)

^{1}H NMR (400 MHz, DMSO-d_6): δ 11.17 (br s, 1H), 8.32 (s, 1H), 8.16 (s, 1H), 8.0 (s, 2H), 7.92 (dd, J = 6.8, 1.6 Hz, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.39 (t, J = 7.2 Hz, 1H), 5.79 (s, 2H), 4.33 (d, J = 5.6 Hz, 4H), 4.25 (d, J = 5.6 Hz, 2H), 2.82 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.29, 163.85, 151.10, 149.15, 144.54, 142.46, 131.33, 130.67, 128.22, 127.58, 101.70, 72.99, 71.39, 38.56; HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{22}H_{22}N_7O_9$, 528.15; found, 528.15.

4.11. 1-[*O*-(6-Uracilcarboxaldoximyl)]-3-[*O*-(3-carboxybenzaldoximyl)]-propane (50)

The synthesis and characterization of this oxime heterodimer has been previously described[33].

4.12. 2-{*O*-[2-(N1-uracil)-acetaldoximyl]-methyl}-1,3-bis[*O*-(3,4-dihydroxy-5-methoxybenzaldoximyl)]-propane (51)

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.32 (d, 2H), 9.14 (s, 1H), 8.72 (s, 1H), 8.03 (q, 1H), 7.65 (dd, 1H), 7.56 (d, 1H), 7.52 (dd, 1H), 6.92 (dd, 1H), 6.69 (s, 1H), 5.55 (d, 2H), 4.50 (dd, 2H), 4.43 (t, 2H), 4.17–4.00 (m, 6H), 3.70 (s, 3H), 1.28 (m, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ (164.5, 164.4), 153.2, (151.8, 151.5), (150.8, 150.0), (149.1, 147.2), (146.5, 146.3), 143.5, 137.1, 122.7, 108.6, 103.2, (101.9, 101.8), 72.4, 72.0, 59.9, (56.9, 56.5), (46.5, 45.2); HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{24}H_{28}N_7O_{10}$ 574.1892, found 574.1885.

4.13. 2-[*O*-(3,4-Dihydroxy-5-methoxybenzaldoximyl)-methyl]-1,3-bis{*O*-[2-(N1-uracil)-acetaldoximyl]}-propane (52)

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (d, 1H), 9.14 (s, 2H), 8.72 (s, 2H), 8.03 (d, 2H), 7.65 (d, 0.5H), 7.56 (d, 0.5H), 7.53 (t, 0.5H), 6.93 (t, 0.5H), 6.69 (s, 2H), 5.55 (d, 1H), 4.50 (d, 1H), 4.43 (d, 1H), 4.42-4.00 (m, 6H), 3.70 (s, 6H), 1.24 (m, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) δ (164.5, 164.4), 153.2, (151.8, 151.5), (150.8, 150.0), (149.1, 147.2), (146.5, 146.3), 143.5, 137.1, 122.7, 108.7, 103.1, (101.9, 101.8), 72.4, 71.9, 59.9, (56.9, 56.4), (46.5, 45.2); HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{26}H_{30}N_5O_{11}$ 588.1936, found 588.1920.

5. In vitro inhibition studies

5.1. High-throughput inhibitor screening of UDG

The molecular beacon-based HTS assay for UDG has been previously described.[33] Briefly, to a 96-well microtiter plate was added 5 μL of 2 mM total compound in DMSO, followed by 75 μL 33.3 pM human UNG in reaction buffer (10 mM Tris–HCl, pH 8.0, 20 mM NaCl, 7.5 mM $MgCl_2$, 0.002% brij-35). The reactions were initiated by the addition of 20 μL of 250 nM molecular beacon substrate in reaction buffer. The plates are incubated at ambient temperature in a fluorescence plate reader for 30 min, and the progress of the reaction was monitored every 5 min (Ex. 485 nm/Em. 520 nm). The final concentrations of the reagents in the assay are 10 mM Tris–HCl, pH 8.0, 20 mM NaCl, 7.5 mM $MgCl_2$, 0.002% Brij-35, 25 or 100 pM human UNG, 50 nM molecular beacon substrate, 100 μM total compound, and 5% DMSO. IC_{50} analysis was performed using the same conditions except that the concentration of compound was varied in the range 0.01–100 μM.

5.2. High-throughput inhibitor screening of dUTPase

To a 96-well microtiter plate were added 20 μL of reaction buffer (50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 0.05% Tween 20) and 5 μL of compounds (2 mM) in DMSO, followed by 50 μL of dUTPase (1 nM) and pyrophosphatase (10 U/mL) in reaction buffer. The reactions were initiated by the addition of 25 μL dUTP (40 μM). The plates are incubated at ambient temperature for 50 min and the reactions were quenched by 25 μL of malachite green color reagent.[45] The mixtures were then allowed to stand for 10 min and the absorbances were measured using a microtiter plate reader with a 620 nm bandpass filter. IC_{50} analysis was performed using the same conditions except that the concentration of compound was varied in the range 0.1–75 μM.

Acknowledgments

This work was supported by NIH Grant GM56834-10 to J.T.S. D.J.K. was supported by the DOD Breast Cancer Research Program (DAMD17-03-1-1251).

Supplementary data

Tables of aryl aldehydes and hydroxybenzaldehydes used to construct oxime libraries, NMR confirmation of product ratios for representative oximes, and IC_{50} curves for active compounds. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.04.022.

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