

Förster Resonance Energy Transfer Based Biosensor for Targeting the hNTH1–YB1 Interface as a Potential Anticancer Drug Target

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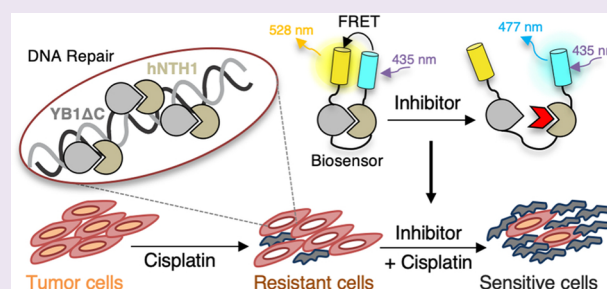


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ABSTRACT: The Y-box binding protein 1 (YB1) is an established metastatic marker: high expression and nuclear localization of YB1 correlate with tumor aggressiveness, drug resistance, and poor patient survival in various tumors. In the nucleus, YB1 interacts with and regulates the activities of several nuclear proteins, including the DNA glycosylase, human endonuclease III (hNTH1). In the present study, we used Förster resonance energy transfer (FRET) and AlphaLISA technologies to further characterize this interaction and define the minimal regions of hNTH1 and YB1 required for complex formation. This work led us to design an original and cost-effective FRET-based biosensor for the rapid *in vitro* high-throughput screening for potential inhibitors of the hNTH1–YB1 complex. Two pilot screens were carried out, allowing the selection of several promising compounds exhibiting IC₅₀ values in the low micromolar range. Interestingly, two of these compounds bind to YB1 and sensitize drug-resistant breast tumor cells to the chemotherapeutic agent, cisplatin. Taken together, these findings demonstrate that the hNTH1–YB1 interface is a druggable target for the development of new therapeutic strategies for the treatment of drug-resistant tumors. Moreover, beyond this study, the simple design of our biosensor defines an innovative and efficient strategy for the screening of inhibitors of therapeutically relevant protein–protein interfaces.



INTRODUCTION

The most common nonsurgical cancer treatments function by generating DNA damage either directly or indirectly. Damage to DNA induces several cellular responses that enable the cell to either eliminate or cope with the damage or activate a programmed cell death process, presumably to eliminate cells with potentially catastrophic mutations.¹ In mammals, several DNA repair pathways are responsible for removal of DNA lesions and thus ensure genomic stability.² Deficiencies in any of these pathways may accelerate the process of carcinogenesis,³ and conversely, enhanced DNA repair pathways provide a resistance mechanism to initially effective cytotoxic therapies.¹ Radiotherapy and several chemotherapeutic drugs induce oxidative stress in exposed tumor cells. The base excision repair (BER) pathway is responsible for the removal of small base lesions, resulting from deamination, oxidation, or methylation;^{4,5} its efficiency can thus reduce the cytotoxic effects of certain anticancer drugs. As a result, the proteins involved in BER are increasingly considered as targets for cancer treatment.^{6–8}

Human NTH1 (endonuclease three) is a bifunctional DNA glycosylase (possessing both DNA glycosylase and apurinic or apyrimidic (AP) lyase activities; Figure 1), responsible for the removal of oxidized pyrimidines.⁹ hNTH1's catalytic activity is tightly regulated.¹⁰ Its N-terminal mammalian-specific domain

(NTD), which is predicted to be largely disordered, plays an autoinhibitory role, which most likely explains its reduced activity compared to that of its *E. coli* homologue.¹¹ Dimerization of hNTH1 occurring via NTD interactions actually disrupts the inhibitory effect of the NTD.¹⁰ hNTH1 also has several interaction partners, which regulate its enzymatic reaction. The nucleotide excision repair (NER) enzyme, XPG, binds directly to hNTH1 and enhances its binding to damaged DNA (Figure 1).^{12,13} Moreover, the AP-endonuclease, APE1, is known to stimulate hNTH1's glycosylase activity against thymine glycol (oxidized form of thymine) paired with adenine.¹⁴ Finally, a direct interaction between hNTH1 and the multifunctional Y-box binding protein 1 (YB1) has been reported and was shown to enhance the AP-lyase activity of hNTH1 (Figure 1).¹⁵

Interestingly, YB1 is also an established metastatic marker, and high expression of YB1 correlates with tumor aggressiveness and poor patient survival in diverse tumor types.^{16–19}

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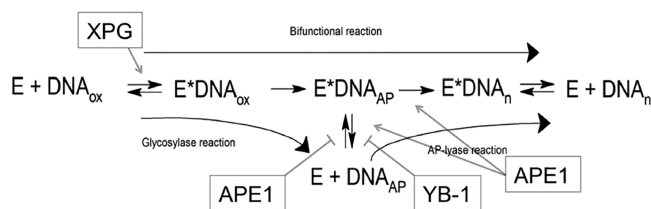


Figure 1. Schematic illustration of the bifunctional reaction mechanism catalyzed by hNTH1 and the effect of XPG, APE1, and YB1 on the regulation of its activity. During the first glycosylase reaction, the enzyme (E) associates with its substrate DNA (DNA_{ox}) to generate an enzyme–substrate complex ($\text{E}^*\text{DNA}_{\text{ox}}$), which leads to the removal of the oxidized base and the formation of an enzyme–abasic site complex ($\text{E}^*\text{DNA}_{\text{AP}}$). In turn, this complex is further processed by the lyase reaction to an enzyme-nicked DNA complex (E^*DNA_n), and finally, the fully processed DNA product after β -elimination is released from the enzyme ($\text{E}+\text{DNA}_n$). In case the two reaction steps are uncoupled, the enzyme dissociates from the abasic site DNA ($\text{E}+\text{DNA}_{\text{AP}}$) after the glycosylase reaction, thereby creating a mixture of DNA substrates containing either abasic sites or oxidized bases. The enzyme can then engage in either one of the two subpathways: (i) a new round of DNA glycosylase activity on DNA_{ox} or (ii) AP-lyase activity on DNA_{AP} . The relative engagement into one or the other pathway is regulated by the abundance of the various species (E, DNA_{ox} , DNA_{AP} , and DNA_n) in the reaction and by various factors that either stimulate or inhibit specific steps of the reaction.

Many reports have also shown a possible link between YB1 and drug resistance both in cell cultures and in clinical human tumor samples. For example, the cellular localization and expression levels of YB1 correlate with cellular sensitivities to the cytotoxic effects of cisplatin and other DNA-damaging agents.²⁰ Several studies have indeed demonstrated that the level of nuclear expression of YB1 is predictive of drug resistance and poor prognosis in breast tumors, ovarian cancers, and synovial sarcomas.^{21–26} The nucleocytoplasmic distribution of YB1 is regulated by a noncanonical nuclear localization signal (NLS) and a cytoplasmic retention site (located in the C-terminal region of YB1), which prevails over the NLS and therefore confers a mainly cytoplasmic localization to YB1.^{17,27} However, in response to environmental stress, including exposure to genotoxic agents, the 20S proteasome cleaves YB1, and a C-terminally truncated form of YB1 (YB1ΔC), corresponding to residues 1–219, is translocated to the nucleus.²⁸

In the nucleus, YB1 becomes involved in transcription regulation, DNA repair and replication, and pre-mRNA splicing.²⁷ YB1 has been shown to interact with and regulate the activities of several nuclear proteins, such as the topoisomerase I,²⁹ and several DNA repair proteins, including hNTH1.¹⁵ In the MCF7 breast adenocarcinoma cell line, Guay and co-workers observed an increase in hNTH1–YB1 complex formation after exposure to UV light or the chemotherapeutic agent, cisplatin, but not to other DNA-damaging agents, such as camptothecin and mitomycin C.³⁰ Both UV light and cisplatin are known to cause oxidative stress and lead to the formation of oxidized bases. Moreover, in this same study the authors also reported that knocking down hNTH1 protein levels sensitized cells to both UV light and cisplatin—a very similar effect to that observed when knocking down YB1.³⁰ Together these findings suggest that YB1 acts in concert with hNTH1 in the response of MCF7 cells to cisplatin and indicate that the hNTH1–YB1 interface may be a valid target for the

development of new anticancer drugs for the treatment of cisplatin-resistant tumors.

In the present study, we have further characterized the interaction between hNTH1 and YB1 *in vitro* and *in cellulo* to define the domains involved in this interaction. This led us to design and optimize a very robust Förster Resonance Energy Transfer (FRET) based biosensor for the *in vitro* screening of chemical libraries in order to identify potential inhibitors of the hNTH1–YB1 complex. Two pilot screens were carried out using this biosensor and allowed the selection of several compounds exhibiting IC₅₀ values in the low micromolar range, which were further validated *in vitro* using an orthogonal assay. Of these, two compounds bind to YB1 and partially restore the sensitivity of MCF7 cells to cisplatin, thereby demonstrating that the hNTH1–YB1 interface is a druggable target for the development of new anticancer agents and that our biosensor represents a highly efficient and low-cost tool for the screening of chemical libraries for the identification of inhibitors of protein–protein interactions (PPIs).

■ RESULTS AND DISCUSSION

YB1 Stimulates the AP-Lyase Activity of hNTH1. Using purified hNTH1 and YB1 proteins, we measured the rates of DNA glycosylase and AP-lyase activities of hNTH1 on a 35mer dsDNA substrate containing thymine glycol paired with adenine in the absence and presence of an excess of wild-type YB1 (Figure 2A). As reported previously, YB1 did not affect the DNA glycosylase activity of hNTH1 but instead was found to specifically stimulate the rate of AP-lyase activity of hNTH1 (Figures 1 and 2A, and Supporting Information, Figure 1) in agreement with earlier data.¹⁵ Indeed, in the absence of YB1, we observed a clear uncoupling of hNTH1's activities on such a substrate with a glycosylase rate significantly faster than the AP-lyase activity, as reported previously.^{14,15,31,32} Upon addition of YB1, however, after the initial lag phase due to the lack of AP-lyase substrate (0–5 min), the steady-state rate of the AP-lyase activity was increased to a level similar to that measured for the DNA glycosylase activity alone (see table in Figure 2A). Through its interaction with hNTH1, YB1 thus appears to favor the coupling of hNTH1's bifunctional activities.

We then performed interaction studies using AlphaLISA technology to identify the regions of hNTH1 and YB1 involved in this specific interaction. For this, His-tagged hNTH1 was bound to Anti-His coated acceptor beads, and biotinylated YB1 was bound to streptavidin-coated donor beads. We performed various titration experiments using different combinations of hNTH1 and YB1 constructs, and the results presented in [Figure 2B](#) indicate that the NTD of hNTH1, which is known to play a key role in regulating hNTH1's catalytic activity,¹¹ is critical and sufficient for interaction with YB1. In contrast, the C-terminal region of YB1, which is missing in the nuclear form of YB1 (i.e., YB1ΔC), is dispensable for this interaction. To estimate the binding affinity of the hNTH1–YB1ΔC complex, we additionally performed a competition assay in which increasing amounts of untagged hNTH1 were titrated into an AlphaLISA reaction containing biotinylated YB1ΔC and His-tagged hNTH1 ([Figure 2C](#)). The IC₅₀ derived from this data provides an estimate of the binding affinity of the hNTH1–YB1ΔC complex and was found to be $0.4 \pm 0.2 \mu\text{M}$.

hNTH1–YB1 Interaction *in Vitro* and *in Cellulo*. To validate these findings, we decided to evaluate the interaction

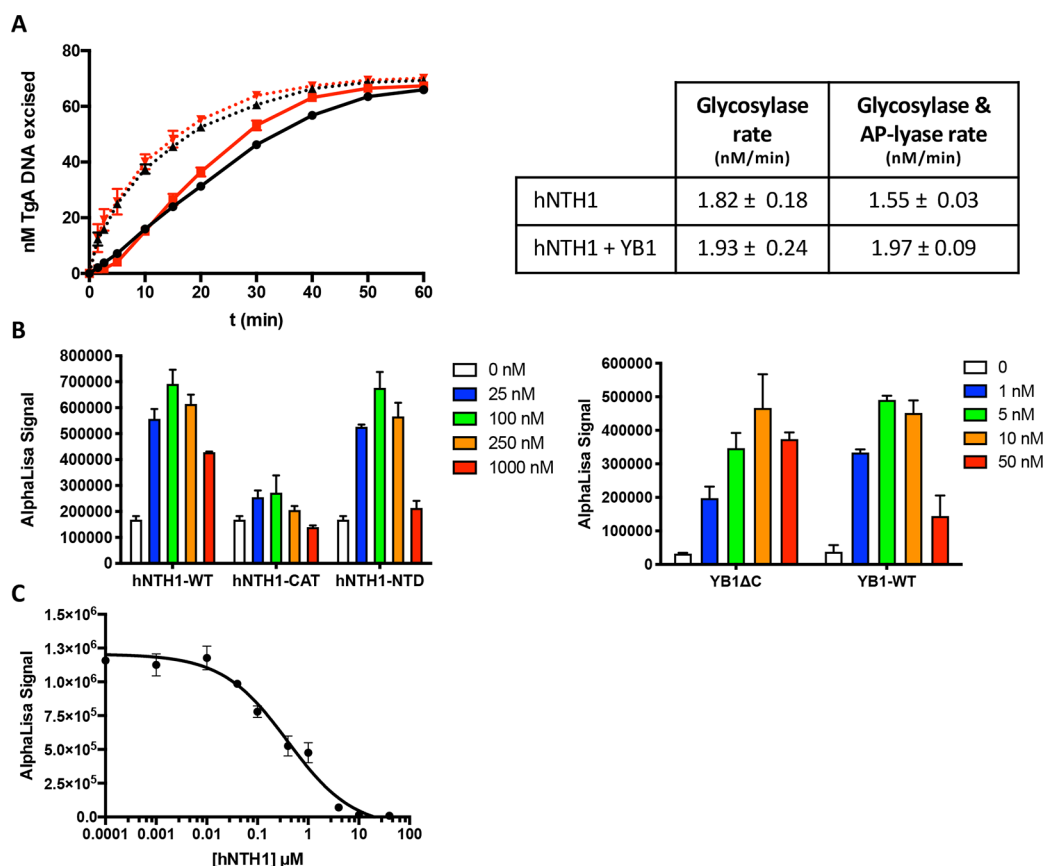


Figure 2. hNTH1–YB1 interaction. (A) Left: Time course experiments of the DNA glycosylase (dotted line) and lyase activities (full line) of 3 nM hNTH1 on 75 nM 35 mer dsDNA containing thymine glycol (Tg) in position 14 and a fluorescein-labeled DNA substrate in the absence (black) and presence (red) of 30 nM YB1. The graphs represent the mean of three replicates, and error bars are the standard deviation. Right: Table presenting the glycosylase and bifunctional (glycosylase/AP-lyase) rates (nM/min) derived from the linear region of the curves (5 to 20 min) using GraphPad Prism 6. (B) Left: AlphaLISA titration of increasing amounts (0, 25, 100, 250, and 1000 nM) of hNTH1 (WT, wild-type; hNTH1–CAT, residues 90–326; hNTH1–NTD, residues 1–89) into reactions containing 10 nM biotinylated YB1ΔC. Right: AlphaLISA titration of increasing amounts (0, 1, 5, 10, and 50 nM) of wild-type (WT) and nuclear YB1 (YB1ΔC, residues 1–219) into reactions containing 100 nM His-tagged hNTH1. The histograms represent the mean of three replicates, and error bars are the standard deviation. The binding profiles are typical of specific binding. The reduced signal obtained at the highest concentration of proteins is due to a saturation of the binding sites on the streptavidin-coated donor beads. (C) AlphaLISA competition assay. Increasing concentrations of hNTH1 (without His-tag) were added to a reaction containing 10 nM biotinylated YB1ΔC and 100 nM His-hNTH1. The data points correspond to the mean of three replicates, and error bars are the standard deviation. The untagged hNTH1 replaces the His-tagged hNTH1 in the complex leading to a decrease in the AlphaLISA signal. The data were fitted to a standard sigmoidal inhibition model in GraphPad Prism 6, and the derived IC₅₀ was found to be 0.4 μM, providing an estimate of the apparent binding affinity of the YB1ΔC–hNTH1 complex.

between hNTH1 and YB1ΔC using FRET measurements, which can be applied both *in vitro* and *in cellulo*. For the *in vitro* studies, we expressed and purified different forms of hNTH1 and YB1ΔC coupled to mTurquoise2 (mTQ2) and super yellow fluorescent protein 2 (SYFP2), respectively. These two fluorescent proteins are two of the brightest fluorescent proteins suitable for FRET experiments.^{33,34} We mixed SYFP2–YB1ΔC or YB1ΔC–SYFP2 with either mTQ2 alone, mTQ2–hNTH1, or hNTH1–mTQ2 and measured the FRET efficiencies of these mixes (Figure 3A). As expected, the mixtures with mTQ2 alone did not show any FRET signal (<0.5%). In contrast, both mTQ2–hNTH1 and hNTH1–mTQ2 containing mixes produced significant FRET levels, thereby confirming that YB1ΔC efficiently interacts with hNTH1. We noticed, however, that the FRET efficiency was significantly influenced by the position of the fluorescent proteins: the highest FRET efficiency was obtained when the fluorescent proteins were fused to the C-termini of both hNTH1 and YB1ΔC constructs. Indeed, in the presence of

YB1ΔC–SYFP2, the hNTH1–mTQ2 construct produced a FRET signal three times higher than that obtained with mTQ2–hNTH1.

To investigate the interaction between hNTH1 and YB1 in cells, we prepared a stably transfected MCF7 cell line expressing hNTH1–mTQ2 and transiently transfected this cell line with either SYFP2–NLS (nuclear localized form of SYFP2 used as a negative control), YB1ΔC–SYFP2, or SYFP2–YB1ΔC containing plasmids (Figure 3B–E). *In cellulo* FRET measurements using the acceptor photobleaching method were carried out on MCF7 cells displaying coexpression of hNTH1–mTQ2 and SYFP2 constructs in the nucleus. Because the transfection efficiency was low, such cells were rare (<1%). The expression levels of YB1ΔC constructs were also variable from cell to cell, with most of the cells showing intense SYFP2 signals. The gradual acceptor photobleaching experiments were performed on such cells, which clearly also exhibited the highest FRET levels. The mean FRET efficiencies measured on MCF7 cells stably transfected

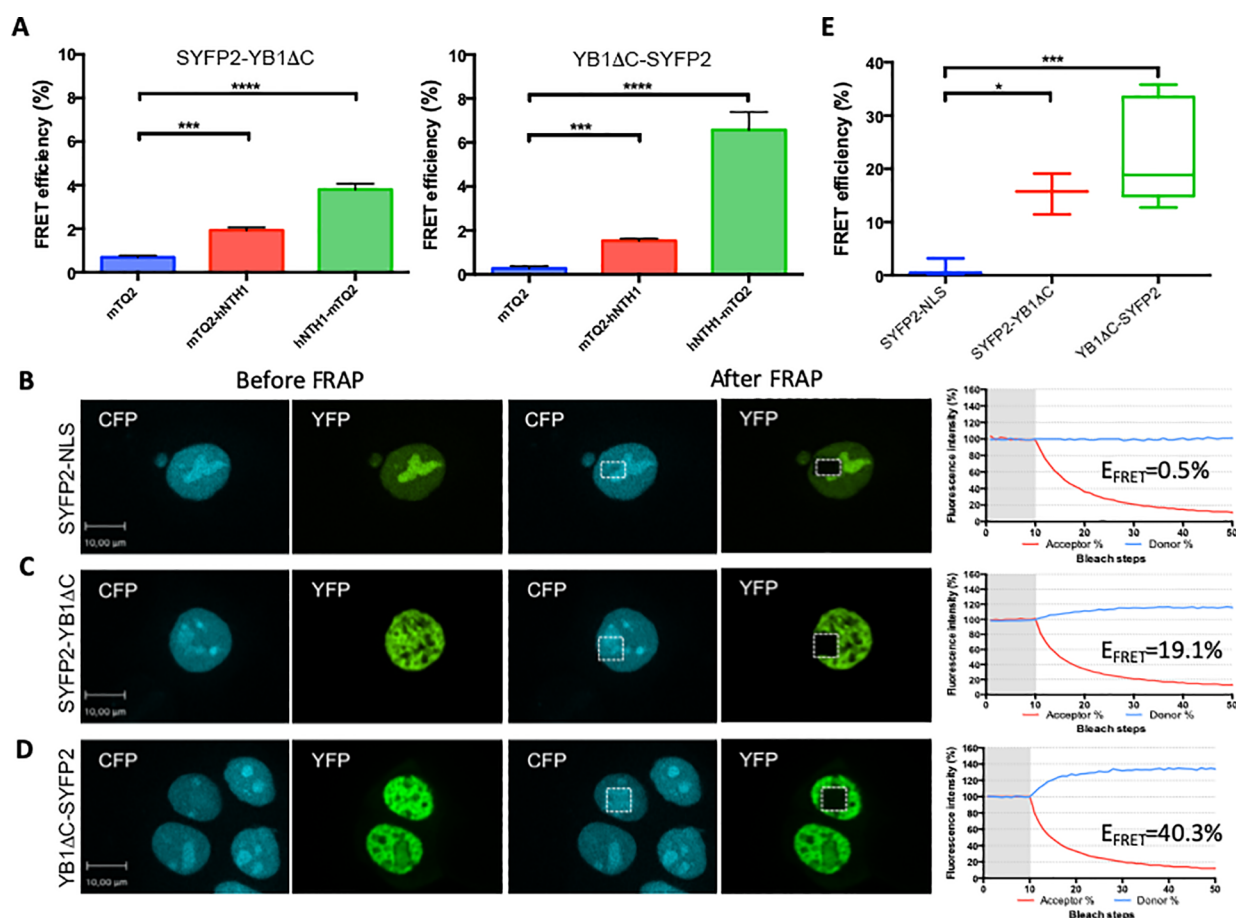


Figure 3. *In vitro* and *in cellulo* FRET analysis of the hNTH1–YB1ΔC interaction. (A) *In vitro* FRET efficiencies of protein mixtures containing mTQ2 alone, mTQ2–hNTH1, or hNTH1–mTQ2 mixed with SYFP2–YB1ΔC or YB1ΔC–SYFP2 at 5 μ M final concentration in FRET buffer. The highest FRET signal was obtained with the fluorescent proteins fused to the C-termini of hNTH1 and YB1ΔC. The histograms represent the mean of three replicates, and error bars correspond to the standard deviation. (B)–(D) hNTH1–YB1ΔC FRET measurements *in cellulo*. Stably transfected MCF7 cells expressing hNTH1–mTQ2 were transfected with either SYFP2–NLS alone (B), SYFP2–YB1ΔC (C), or YB1ΔC–SYFP2 (D). mTQ2 is seen in the CFP channel (blue). SYFP2 is seen in the YFP channel (green). *In cellulo* FRET measurements were performed using the acceptor photobleaching method. The white rectangle indicates the area of the nucleus that was photobleached by the FRAPPA device in the images. Scale bar: 10 μ m. Right panel: Normalized fluorescence intensities of the acceptor (SYFP2) and donor (mTQ2) fluorophores recorded during the FRET experiment. Photobleaching of the acceptor was initiated after the acquisition of 10 images. The calculated FRET efficiency (E_{FRET}) for each of these experiments is provided. (E) Mean FRET efficiencies in the MCF7 stable cell line expressing hNTH1–mTQ2 and transfected with either SYFP2–NLS ($N = 3$), SYFP2–YB1ΔC ($N = 3$), or YB1ΔC–SYFP2 ($N = 8$). The presented data are the mean of at least three replicates, and error bars indicate standard deviation. Data in the graphs A and E were analyzed by unpaired *t* test with GraphPad Prism 6 (* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $0.0001 < P < 0.001$; **** $P < 0.0001$).

with hNTH1–mTQ2 and transiently expressing SYFP2–NLS, YB1ΔC–SYFP2, or SYFP2–YB1ΔC were of 1.3, 15.4, and 22.0%, respectively (Figure 3E). The strongest FRET signal was obtained from stably transfected MCF7 cells transiently expressing YB1ΔC–SYFP2 (Figure 3D). Thus, as with our *in vitro* measurements, a configuration in which both mTQ2 and SYFP2 are fused to the C-termini of hNTH1 and YB1ΔC resulted in the strongest FRET levels. In this configuration, the FRET efficiency was significantly higher than that of the negative control, clearly indicating that hNTH1 and YB1ΔC interact in the nuclei of MCF7 cells.

The levels of nuclear YB1 have been shown to be largely increased in cells exposed to genotoxic stress, and high levels of YB1 are predictive of drug resistance and poor prognosis.^{21–26} Interestingly, we observe strong FRET signal in the absence of exposure to anticancer agents, but only in cells overexpressing high levels of YB1ΔC. Taken together, these findings suggest that the increased abundance of nuclear YB1, resulting from

cleavage and translocation of YB1 to the nucleus in response to genotoxic stress, favors its interaction with hNTH1 and the stimulation of its DNA repair activity. In turn, this enhanced DNA repair activity may contribute to the development of drug resistance. Interfering with the hNTH1–YB1 interaction could thus provide clinicians with a new therapeutic strategy for the treatment of drug-resistant solid tumors.

Design of a FRET-Based Biosensor. Targeting PPIs for drug development is attracting increasing attention,³⁵ but discovering small molecules that disrupt PPIs is very challenging. Several databases and chemical libraries have been developed in order to facilitate the search for potential inhibitors,^{36,37} and various experimental approaches are used to identify initial lead compounds even in the absence of high-resolution structural information. The most common assays rely on either AlphaScreen (and its related AlphaLISA assay), time-resolved FRET (TR-FRET), or time-resolved fluorescence (TRF) assays, all of which have both advantages and

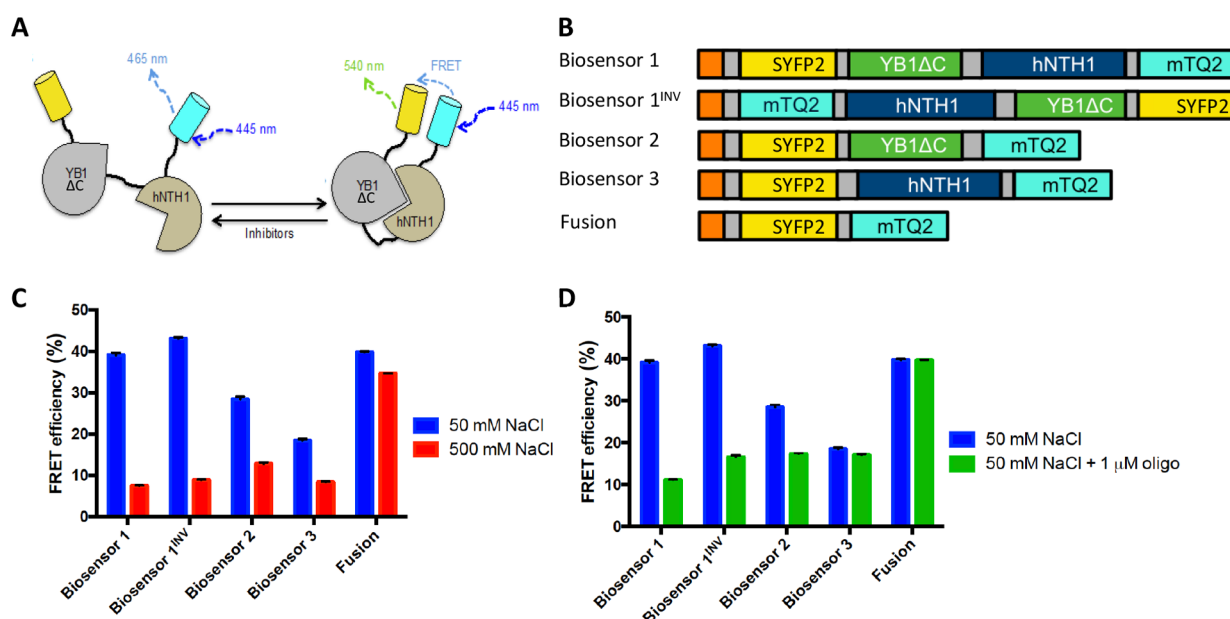


Figure 4. Design and optimization of a FRET-based biosensor. (A) Illustration of the biosensor design. The yellow and blue cylinders represent SYFP2 and mTQ2 proteins, respectively. Upon interaction of hNTH1 with YB1ΔC, mTQ2 and SYFP2 come close together, and energy transfer between the FRET donor and FRET acceptor occurs and can be measured. (B) Illustration of the different biosensor and fusion constructs used in this study. Orange: N-terminal, cleavable His-tag. Gray: short linker sequences. (C) Comparison of the FRET efficiencies of 1 μM biosensor and fusion constructs in FRET buffers containing either 50 mM (blue) or 500 mM (red) NaCl concentrations. (D) Comparison of the FRET efficiencies of 1 μM biosensor and fusion constructs in the absence (blue) and presence (green) of 1 μM THF-oligo in FRET buffer containing 50 mM NaCl. (C),(D) Plotted histograms are the mean of three replicates, and error bars represent the standard deviation.

disadvantages.³⁸ Here we used the highly sensitive AlphaLISA assay to study the *in vitro* interaction of hNTH1 with YB1 and determine the apparent affinity of the complex. However, the major limitations of this assay for high-throughput screening (HTS) reside in its cost and the sensitivity of the beads to ambient light, which causes high variability in the measurements and reduces storage time.

The success of our FRET measurements prompted us to design a cost-effective FRET-based biosensor adapted to HTS technology for the selection of potential inhibitors of the hNTH1–YB1 complex. FRET-based HTS assays relying on the use of genetically encoded fluorescent proteins are mostly performed by mixing donor- and acceptor-labeled protein partners.^{39–41} In contrast, the basic design of this biosensor consisted of a single fusion polypeptide chain encoding both the fluorescent tags and the target proteins with 5–10 amino acid linkers in between each protein (Figure 4A), similar in design to the previously reported constructs of FRET-based biosensors.⁴² Based on our *in vitro* and *in cellulo* FRET data, SYFP2 was placed at the amino terminus and mTQ2 at the C-terminus of the construct, while the YB1ΔC and hNTH1 proteins were inserted in between forming a fusion protein composed of SYFP2–YB1ΔC–hNTH1–mTQ2, which from hereon we will refer to as Biosensor 1. This large (116 kDa) protein chain was overexpressed in *E. coli* and purified for *in vitro* FRET measurements. Protein purity was critical for our assays since DNA or protein (and particularly fluorescent protein) contamination would interfere with subsequent fluorescence measurements and calculations of FRET efficiencies in which we assume to have a 1:1 ratio of mTQ2 and SYFP2 in our samples. In the case of Biosensor 1, the protein yield was high (2 mg L^{−1} culture), and less than 10 mg of protein was sufficient to perform the screens described below.

Having both protein partners within a single polypeptide chain greatly simplifies the protein–protein interaction assay by reducing the number of samples to purify and ensuring a strict 1:1 molar ratio of the two components. As controls, several alternative biosensor constructs were prepared (Figure 4B), missing either one of the two target proteins (SYFP2–YB1ΔC–mTQ2, corresponding to Biosensor 2, and SYFP2–hNTH1–mTQ2, corresponding to Biosensor 3) or inverted (mTQ2–hNTH1–YB1ΔC–SYFP2 corresponding to Biosensor 1^{INV}). A positive FRET control corresponding to SYFP2 fused directly to mTQ2 was also prepared, which we refer to as the fusion construct.

We first optimized the buffer conditions for measuring FRET and found that the hNTH1–YB1ΔC interaction was salt-sensitive and was disrupted above 0.2 M NaCl (Figure 4C and Supporting Information Figure 2). Indeed, the FRET efficiencies of both Biosensor 1 and Biosensor 1^{INV} were comparable to that of the fusion positive control at 50 mM NaCl (~40%), while increasing the NaCl concentration above 200 mM led to a dramatic decrease (~30%) of the FRET efficiencies of these constructs. In contrast, high salt concentrations only mildly affected the FRET of the fusion construct (~5%). Biosensors 2 and 3, missing one of the two partners, respectively, YB1ΔC or hNTH1, displayed strongly reduced FRET efficiencies in low salt conditions (50 mM NaCl) compared to Biosensor 1 and in the case of Biosensor 2 also increased basal FRET levels at high salt (above 200 mM) concentrations. Therefore, the change in FRET efficiency (ΔFRET) between 50 and 500 mM NaCl observed for Biosensor 1 and 1^{INV} constructs reflects a specific interaction between the two partners, hNTH1 and YB1ΔC.

To further demonstrate that the FRET signals observed with Biosensor 1 constructs are the result of a specific interaction between hNTH1 and YB1ΔC, we performed FRET measure-

ments in the presence of a highly specific DNA substrate of hNTH1 (called hereafter “THF-oligo”), a 12mer duplex containing a stable abasic site analogue tetrahydrofuran (THF) in the central position paired with guanine. Such a substrate binds to hNTH1 with nanomolar affinity.³² Addition of the DNA substrate reduced the FRET efficiencies of Biosensor 1 and 1^{INV} constructs by nearly 30% but, as expected, had no effect on the FRET levels of the fusion control and only a limited effect on Biosensors 2 and 3 (Figure 4D). These experiments thus confirm that the observed FRET levels of Biosensors 1 and 1^{INV} are the result of a specific interaction between hNTH1 and YB1ΔC and indicate that this interaction is most likely disrupted by the binding of hNTH1 to its DNA substrate. The observation that the FRET efficiency of Biosensor 3, containing solely hNTH1, did not significantly change upon addition of the DNA substrate suggests that the decrease in FRET efficiency seen with Biosensors 1 and 1^{INV} does not result from a conformational change in hNTH1 induced by substrate binding, which could have positioned the fluorescent proteins in an unfavorable configuration for FRET.

The characterization of the different biosensor constructs thus clearly indicated that Biosensors 1 and 1^{INV} are suitable for probing the hNTH1–YB1ΔC interaction. For subsequent experiments, we focused on the Biosensor 1 construct because the purification yield for this construct and its stability were higher compared to Biosensor 1^{INV}.

Biosensor 1 as a Tool for HTS. We next evaluated the suitability of Biosensor 1 for HTS to search for chemical inhibitors of the hNTH1–YB1ΔC complex. First, we determined the minimal amount of Biosensor 1 needed to obtain reliable FRET measurements in view of assay miniaturization in 384-well microplates (Supporting Information Figure 3A). We observed that the FRET efficiency of Biosensor 1 rapidly dropped when lowering the concentration below 0.2 μM. This was not the case with the Fusion protein, which exhibited a constant FRET efficiency irrespective of its concentration, as expected for intramolecular FRET. We thus set the Biosensor 1 concentration to 0.2 μM in our subsequent screening experiments, corresponding to a FRET efficiency of ~30%.

Because compounds are typically dissolved in DMSO, we evaluated the effect of DMSO on the FRET levels of Biosensor 1 (Supporting Information Figure 3B). We observed only a slight decrease in the FRET efficiency as a function of DMSO concentration, indicating that high concentrations of DMSO (up to 5% DMSO) were compatible with our assay.

Next, we performed a dose response curve with the THF-oligo, which we planned to use as a bioactive control (inducing strong reduction in the FRET level of Biosensor 1) in our subsequent screening experiments. The THF-oligo was found to inhibit the hNTH1–YB1ΔC interaction with an IC₅₀ value of 0.1 μM (Supporting Information Figure 3C). We therefore chose to use it at a final concentration of 0.5 μM to induce maximal inhibition of the FRET signal. Finally, we examined the stability of the Biosensor 1 FRET efficiency over a 2 h period in the presence and absence of the DNA substrate and 0.5 or 1.3% DMSO (Supporting Information Figure 3D). The FRET signal of Biosensor 1 was relatively stable over time with only a slight decrease in the signal after 2 h of incubation, and the difference in FRET levels observed in the absence and presence of the THF-oligo remained around 20%. One of the major limitations of the previously reported FRET-based

biosensors is the small to medium changes in FRET signal.⁴² With the Biosensor 1, in contrast, the large change in FRET signal (~20%) makes this assay ideally suited for miniaturization and HTS.

Finally, to test the statistical robustness of our Biosensor 1-based assay, we performed a Z' factor test⁴³ on 30 technical replicates of 0.2 μM Biosensor 1 in the absence and presence of 0.5 μM THF-oligo. A Z' factor between 0.5 and 1.0 indicates that the assay quality is high and suitable for HTS. We obtained a Z' factor of 0.9, indicating that our Biosensor 1 is particularly well-suited for HTS. It is fast, robust, simple, and low cost.

Chemical Library Screening with Biosensor 1. As a pilot experiment, we screened two relatively small libraries (Life Chemicals PPI library and Prestwick Chemical library) composed of, respectively, 800 and 1280 compounds that are well adapted to test the suitability of Biosensor 1 as a screening assay and are sufficiently diverse to provide us with some initial, promising hits, to define the hNTH1–YB1 interface as a druggable target in cancer therapy. Moreover, the advantage of the Prestwick Chemical library is that it contains 95% approved drugs (FDA, EMA, and other agencies), which can facilitate and accelerate subsequent drug development processes. In both cases, a primary screen was performed on a robotic setup in triplicate using a single compound concentration (50 μM), and a confirmation of primary hits was then carried out at three compound concentrations (50, 10, and 1 μM) each in triplicate on the 30 most potent molecules (respectively, 3.75 and 2.35% of the screened compounds), which showed at least 25% inhibition. The overall results of these screens are presented in the Supporting Information Figures 4 and 5.

No primary hits from the Life Chemicals library were validated in the confirmation screen. Indeed, three compounds from this library (Supporting Information Figure 4) were found to significantly decrease the FRET levels of Biosensor 1, but additional measurements in which the autofluorescence of each of these three compounds was monitored revealed that the apparent decrease in FRET efficiencies was largely due to their intrinsic fluorescence. Although our FRET measurements, like other FRET-based assays,^{39–41,44,45} were performed as ratiometric assays to reduce the effects of autofluorescence and spectral interference of media and test compounds, false positives can nonetheless be selected when the autofluorescence of compounds strongly overlaps with donor emission signal. These three compounds were also found to cause a significant reduction in the FRET efficiency of the fusion construct, clearly indicating that they correspond to false positive hits (Supporting Information Figure 4D).

In contrast, screening of the Prestwick Chemical library allowed us to select eight hits (corresponding to 0.60% of screened compounds) as potential inhibitors of the hNTH1–YB1ΔC complex (indicated with a star in Supporting Information Figure 5). The intrinsic fluorescence of these selected compounds was verified before proceeding with further experiments. This strategy allowed us to rapidly eliminate compounds that either strongly enhanced or quenched the fluorescence signal of the fluorescent proteins used in this assay (Supporting Information Figure 5). Eight compounds were selected that induced a clear dose-dependent inhibition of the FRET efficiency of Biosensor 1 with inhibition levels over 40% at 50 μM (Figure 5A). The names, properties, and molecular structures of these eight

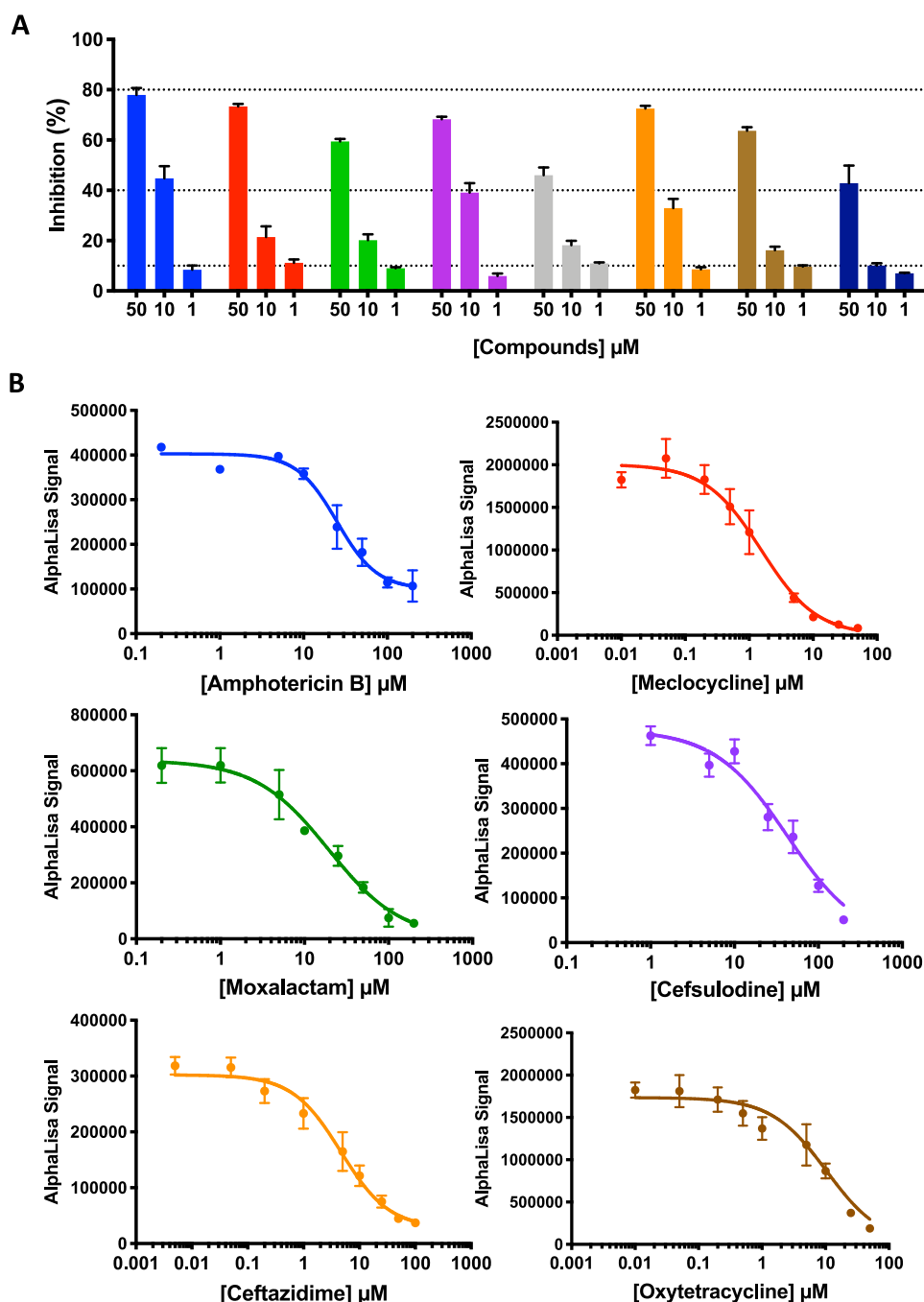


Figure 5. Identification and characterization of selected hits. (A) Extent of Biosensor 1 FRET inhibition by 8 selected compounds (blue: amphotericin B, red: meclocycline, green: moxalactam, purple: cefsulodine, gray: mitoxantrone, orange: ceftazidime, brown: oxytetracycline, dark blue: cefotaxime) at 50, 10, and 1 μM compound concentrations, respectively. Plotted histograms are the mean of three replicates, and error bars represent the standard deviation. (B) Dose-dependent inhibition of the hNTH1–YB1 Δ C interaction by amphotericin B (blue), meclocycline (red), moxalactam (green), cefsulodine (purple), ceftazidime (orange), and oxytetracycline (brown), probed using the AlphaLisa assay. The data points corresponding to the means of triplicate measurements were fitted to a three-parameter sigmoidal inhibition model in GraphPad Prism 8, except for the amphotericin B data for which a four-parameter sigmoidal inhibition model was used with a *Hill Slope* value of -1.81 .

compounds are provided in [Supporting Information Table 1](#). Amphotericin B is a polyene antifungal antibiotic produced by *Streptomyces nodosus*, with antifungal activity. Ceftazidime pentahydrate, cefsulodine sodium, moxalactam disodium, and cefotaxime sodium are β -lactams and cephalosporin antibiotics with bactericidal activity. Meclocycline sulfosalicylate and oxytetracycline are tetracycline derivative antibiotics. Mitoxantrone is an anthracenedione antibiotic with antineoplastic activity and a topoisomerase II inhibitor. Cefotaxime sodium

was left out of the following characterization procedure because it was the least effective of the four β -lactam antibiotics (Figure 5A).

Validation of Selected Hits. In order to further validate the seven selected hits, we prepared in-house stock solutions starting from commercially available powders of amphotericin B, meclocycline sulfosalicylate, moxalactam disodium, cefsulodine sodium, mitoxantrone dihydrochloride, ceftazidime pentahydrate, and oxytetracycline dihydrate. Manual FRET

Table 1. Characterization of the Inhibitory Potential and Molecular Targets of Selected Prestwick Hits^a

compound	IC ₅₀ (μM) Biosensor 1 assay	IC ₅₀ (μM) AlphaLISA assay	T _m hNTH1 (°C)	T _m YB1ΔC (°C)
DMSO	ND	ND	55.00 ± 0.00	37.20 ± 0.76
amphotericin B	9.55 ± 0.47	25.00 ± 4.01	58.83 ± 0.29	37.42 ± 0.74
cefsulodine	8.94 ± 0.39	42.92 ± 11.76	59.50 ± 0.00	36.70 ± 0.45
ceftazidime	15.36 ± 4.70	4.65 ± 0.80	59.17 ± 0.29	36.70 ± 1.04
moxalactam	75.29 ± 177.30	19.36 ± 3.61	55.50 ± 0.00	37.00 ± 0.71
meclocycline	19.48 ± 2.64	1.54 ± 0.26	56.17 ± 1.04	39.50 ± 1.08/42.75 ± 1.32
oxytetracycline	17.94 ± 5.07	10.35 ± 2.89	57.67 ± 0.29	37.50 ± 0.41/42.63 ± 0.75
mitoxantrone	31.88 ± 1.62	ND	ND	ND

^aND: not determined.

measurements were first performed on the fusion construct (Supporting Information Figure 5C). None of these compounds affected the FRET signal produced by the fusion control, indicating that they do not interfere with the FRET process itself.

Next, we evaluated the inhibitory effects of the freshly prepared compounds on Biosensor 1 (Supporting Information Figures 5D and 6). The inhibitory effects of these seven hits at 50 μM were globally similar (or slightly higher) to those seen in our confirmation screen (Supporting Information Figure 5D). The inhibitory effects of amphotericin B, meclocycline, and oxytetracycline were the highest (>100% inhibition), surpassing the inhibitory effect of the THF-oligo. Ceftazidime, cefsulodine, and mitoxantrone were found to be potent inhibitors irrespective of their source with inhibitions of ~90, ~65, and ~45%, respectively. In the case of moxalactam, the fresh solution showed similar inhibitory effects as seen in our confirmation HTS screen (~60%), while the compound provided by the CMBA platform was found to be less potent in this assay, causing only a 20% inhibition. Long-term storage of this compound may have reduced its efficiency. Overall, these experiments confirmed that these seven compounds strongly inhibit the Biosensor 1 FRET signal as seen in the primary and confirmation screens.

Dose–response curves (Supporting Information Figure 6) were then performed with these seven confirmed hits to determine their IC₅₀ values (Table 1). All seven compounds exhibited clear dose-dependent inhibition of Biosensor 1 FRET efficiency. Amphotericin B, meclocycline, ceftazidime, oxytetracycline, and cefsulodine were found to be the more potent inhibitors of the hNTH1–YB1ΔC interaction with IC₅₀ values between 8 and 20 μM (Table 1). Mitoxantrone and moxalactam exhibited higher IC₅₀ values (>30 μM) and thus appear to be weaker inhibitors.

Finally, the inhibitory potentials of these compounds were further assessed using an orthogonal protein–protein interaction assay—the AlphaLISA assay. For six of these compounds (amphotericin B, meclocycline, moxalactam, cefsulodine, ceftazidime, and oxytetracycline), dose-dependent inhibition of the hNTH1–YB1ΔC interaction was also detected using the AlphaLISA assay (Table 1 and Figure 5B). The dark color of mitoxantrone precluded its use in this luminescence-based assay (due to quenching of the signal). No IC₅₀ value could therefore be determined for this compound using this alternative technique. For the other compounds, IC₅₀ values were obtained from the AlphaLISA assay (Table 1). These values were in a similar range to those determined using the FRET-based Biosensor 1, although slightly lower (except for amphotericin B and cefsulodine). Six compounds thus exhibited clear dose-dependent inhibition of the

hNTH1–YB1ΔC interaction using two distinct PPI assays. Of these, three compounds stand out with IC₅₀ values below 20 μM for both assays: meclocycline, ceftazidime, and oxytetracycline, two of which are tetracycline derivatives, indicating that the shared tetracycline structure may be directly implicated in the inhibition process.

Molecular Target of Selected Hits. To determine the molecular targets of each of these six compounds, we performed thermal shift assays on the individual hNTH1 and YB1ΔC proteins in the absence and presence of a molar excess of the selected hits. hNTH1 alone or in the presence of DMSO exhibits a melting temperature (T_m) of 55 °C, which in the presence of several of these compounds was significantly increased (Figure 6A–C and Table 1). In particular, amphotericin B, cefsulodine, ceftazidime, and, to a lesser extent, oxytetracycline clearly enhanced the thermal stability of hNTH1—their effects were very similar to those measured upon addition of the hNTH1 DNA substrate, the THF-oligo (Figure 6A). These four compounds thus clearly target hNTH1. In contrast, addition of moxalactam had no detectable effect on hNTH1, and meclocycline only slightly increased the T_m of hNTH1, an effect that may be caused by its perturbation of the hNTH1 melting curve, which was much noisier than for the other measurements (Figure 6B).

YB1ΔC, unlike hNTH1, contains several disordered domains, and its melting curve was more complex but nonetheless exhibited a distinctive shape that allowed us to derive the T_m of YB1ΔC, which was found to be close to 37.0 °C (Figure 6D–F). Upon addition of the six selected compounds, only two of these, the two tetracycline antibiotics, meclocycline and oxytetracycline, induced a noticeable change in the curve profile and the derived T_m (Figure 6D and Table 1), indicating that they interact with YB1ΔC. Meclocycline caused the main peak of the primary derivative of the melting curve to be significantly shifted toward higher temperatures (39.5 °C) and the appearance of a clear shoulder centered around 42.5 °C, while oxytetracycline only caused the latter, with the appearance of a marked shoulder around 42.5 °C. These observations suggest that YB1ΔC may offer multiple binding sites for these two compounds and that these sites are not fully occupied under the experimental conditions used in the present study.

Our experiments thus indicate that amphotericin B, cefsulodine, and ceftazidime clearly target hNTH1, while meclocycline binds to YB1ΔC and oxytetracycline interacts with both proteins. Moxalactam, however, showed no detectable interaction with hNTH1 and YB1ΔC in these assays, possibly as a result of a low affinity for its molecular target, in agreement with its lower potency as an inhibitor (highest IC₅₀ values of the six selected compounds).

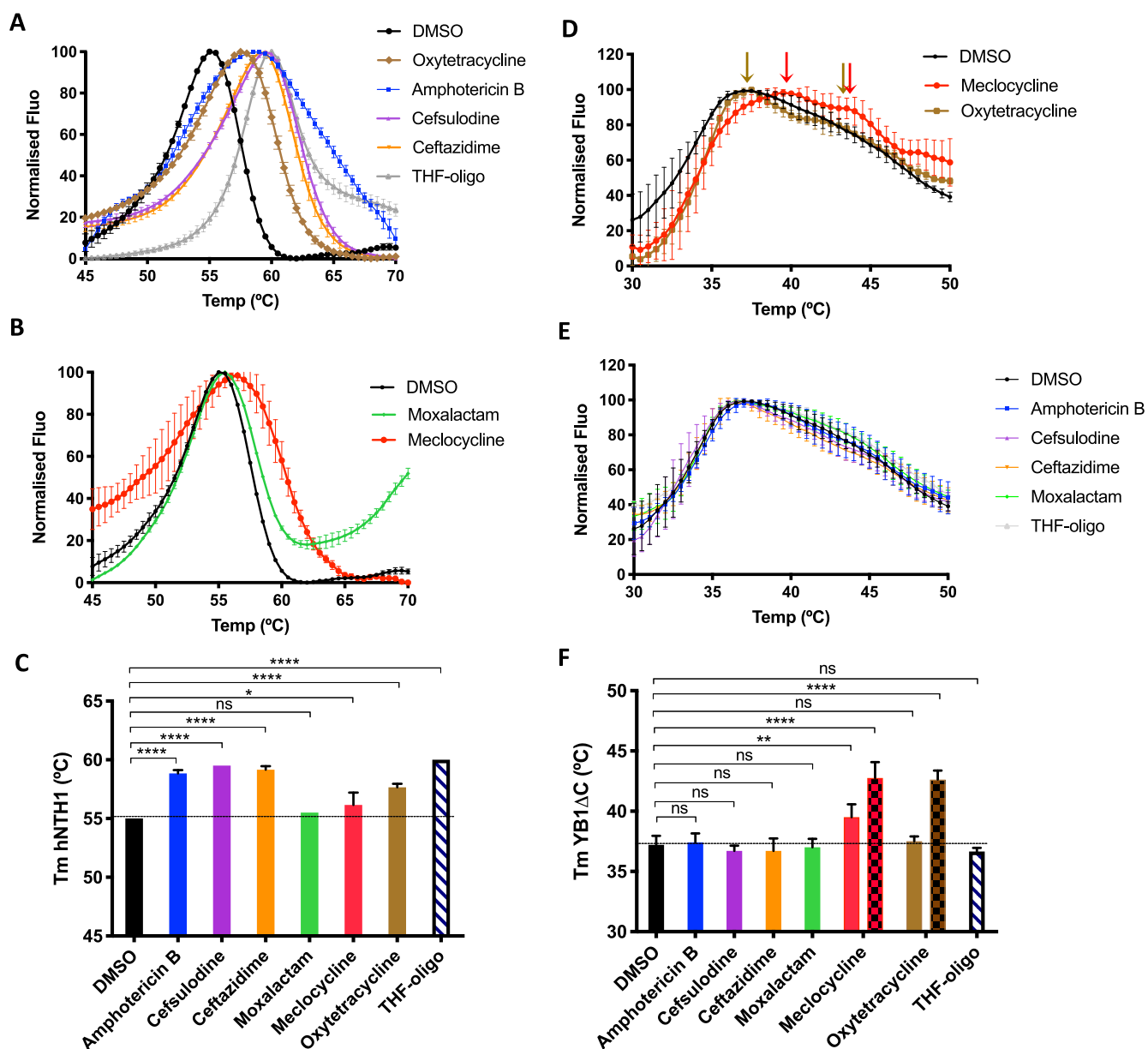


Figure 6. Characterization of the molecular targets of selected hits. (A)–(C) Effects of the selected compounds (100 μ M), DMSO only (1%), or the THF-oligo (20 μ M) on the melting temperature of 5 μ M hNTH1. (A),(B) Normalized primary derivatives of the melting curves of hNTH1 in the presence of compounds that either significantly shift the T_m values of hNTH1 (A) or for which no obvious change in the T_m was observed (B). The mean T_m values of hNTH1 derived from these graphs are shown in (C). The data points correspond to the mean of at least three replicates, and the error bars correspond to the standard deviation. (D)–(F) Effects of the selected compounds (150 μ M), DMSO only (1.5%), or the THF-oligo (30 μ M) on the melting temperature of 15 μ M YB1ΔC. (D)–(E) Normalized primary derivatives of the melting curves of YB1ΔC in the presence of compounds that either significantly shift the T_m values of YB1ΔC (D) or for which no obvious change in the T_m was observed (E). The mean T_m values of YB1ΔC derived from these graphs are shown in (F). The data points correspond to the mean of at least three replicates, and the error bars correspond to the standard deviation. In the presence of meclocycline and oxytetracycline, the primary derivative of the melting curve of YB1ΔC exhibited two peaks (arrows in panel D), hence the two histograms for these compounds in (F). The histogram corresponding to the second peak/shoulder is illustrated with a checkered pattern. (C) and (F) Statistical analysis was performed using a one-way ANOVA test (Dunnett's test) in GraphPad Prism 8 (ns: nonsignificant, * 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** 0.0001 < P < 0.001; **** P < 0.0001).

Sensitization of MCF7 Cells to Cisplatin. As mentioned in the Introduction, the hNTH1–YB1 complex formation increases after exposure to cisplatin, and knocking down hNTH1 or YB1 protein levels sensitizes cells to this chemotherapeutic agent.³⁰ We therefore evaluated the ability of these six compounds to restore the sensitivity of MCF7 cells to cisplatin. MCF7 cells were grown for 24 or 48 h in the presence of increasing amounts of cisplatin. In agreement with previous observations,³⁰ we observed that below 5 μ M

cisplatin, the viability of MCF7 cells after 48 h was not significantly affected. We thus chose to perform our experiments with either 5 μ M cisplatin for 48 h or 20 μ M cisplatin for 24 h (Figure 7 and Supporting Information Figure 7), two concentrations that only moderately reduce the viability of MCF7 cells by 5–15% (Supporting Information Figure 7). First, MCF7 cells were treated with the six selected compounds alone at various concentrations to determine their effects on MCF7 cell viability after 24 and 48 h. We

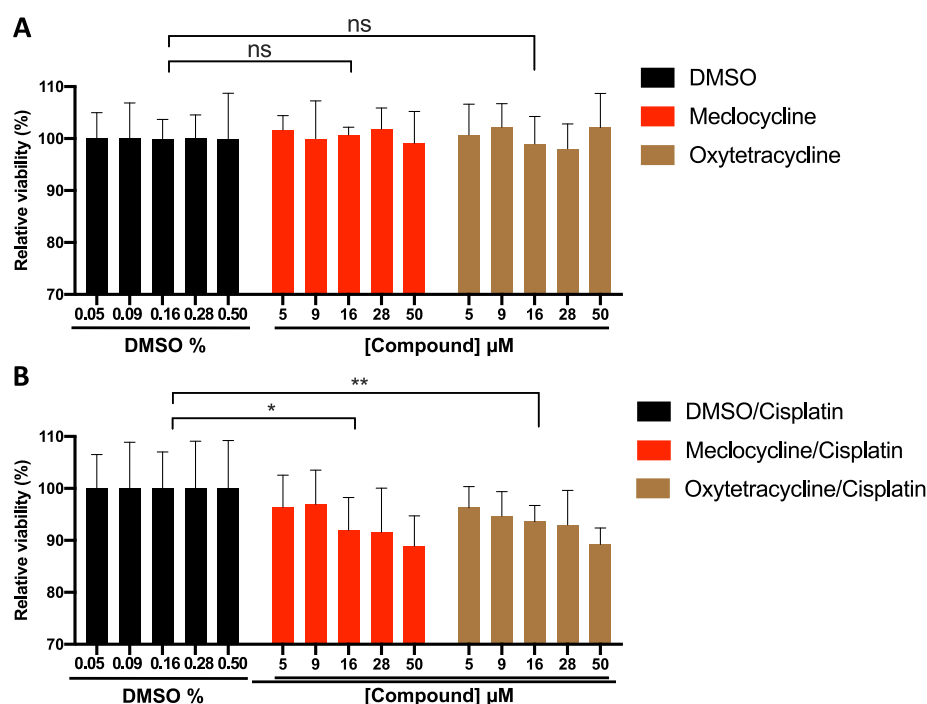


Figure 7. Effects of selected hits on the sensitivity of MCF7 cells to cisplatin. (A) Relative viability of MCF7 cells treated with either 0.05–0.5% DMSO (black) vehicle alone or 5–50 μM meclocycline (red) or oxytetracycline (brown) for 24 h. (B) Relative viability of MCF7 cells treated with 5 μM cisplatin supplemented with either 0.05–0.5% DMSO (black) vehicle alone or 5–50 μM meclocycline (red) or oxytetracycline (brown) for 24 h. Plotted histograms are the mean of four replicates, and error bars represent the standard deviation. The viability of DMSO-treated cells at each concentration was set to 100% and was used as a reference for calculating the relative viability of compound-treated cells. Statistical analysis performed using a one-way ANOVA test (Dunnett's test) in GraphPad Prism 8 revealed that the concentration-dependent decrease in cell viability induced by meclocycline and oxytetracycline in the presence of cisplatin was significant (ns: nonsignificant, * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$).

observed that amphotericin B and the three beta-lactam antibiotics significantly stimulated the growth of MCF7 cells in a concentration-dependent manner at both 24 and 48 h (Supporting Information Figure 7). In contrast, the two tetracycline derivatives, meclocycline and oxytetracycline, had no effect on the viability of MCF7 cells (Figure 7A and Supporting Information Figure 7). Next, we treated MCF7 cells with both cisplatin and the six selected compounds and measured cell viability after 48 h for 5 μM cisplatin and after 24 h for 20 μM cisplatin (Figure 7B and Supporting Information Figure 7). In these conditions, the stimulatory effect of amphotericin B and the three beta-lactam antibiotics was significantly reduced, resulting in cell viabilities close to that of control cells treated with DMSO only. Meclocycline and oxytetracycline, however, reproducibly induced a small, but significant, concentration-dependent decrease in the viability of MCF7 cells treated with 5 μM cisplatin (Figure 7B), indicating that these compounds moderately sensitize MCF7 cells to cisplatin. A similar effect was observed with cells treated for 24 h with 20 μM cisplatin (Supporting Information Figure 7). The finding that compounds disrupting the hNTH1–YB1 interaction can sensitize MCF7 cells to cisplatin strongly argues in favor that the complex, rather than the independent proteins is responsible for cisplatin resistance of breast cancer cells. Interestingly, these two potent compounds both target YB1 ΔC , which suggests that YB1 ΔC -targeting molecules may constitute promising leads for future drug development. Taken together, these results indicate that targeting the hNTH1–YB1 complex through binding to YB1 may represent a valid strategy to fight cisplatin chemoresistance of breast cancer cells.

CONCLUSION

Taken together, this study clearly demonstrates that the hNTH1–YB1 interface is a druggable target for the development of new therapeutic strategies to fight chemoresistance of breast cancer cells, and our results indicate that targeting the hNTH1–YB1 complex through binding to YB1 may represent a valid strategy. Moreover, our work identifies the Biosensor 1-based assay as a remarkable tool for robotic screening of chemical libraries in the search for inhibitors of the hNTH1–YB1 interaction. Beyond this system, the innovative design of the Biosensor 1 construct offers a new strategy to study PPIs and to screen for potential inhibitors of other therapeutic PPI targets in a rapid, cost-effective manner.

MATERIALS AND METHODS

Cloning. The pmTQ2–N1, pSYFP2–N1, and pSYFP2–C1 plasmids were prepared by inserting the mTurquoise2 (*mTQ2*) and super yellow fluorescent protein (*SYFP2*) genes kindly provided by T.W.J. Gadella and A. Royant in place of the GFP in pEGFP–N1 and pEGFP–C1 vectors. The SYFP2–mTQ2 Fusion construct was obtained by cloning *mTQ2* into the pSYFP2–C1 vector and then transferring SYFP2–mTQ2 into the bacterial expression plasmid pProexHtB. pSYFP2–C1–NLS was prepared by inserting the *SYFP2* gene in place of the *GFP* gene in the pAcGFP–Nuc (Clontech) vector that adds three copies of the NLS of the simian virus 40 large T-antigen to the C-terminus of the fluorescent protein. Human *nth1* and *yb1* cDNAs were gene synthesized (Shinegene), and full-length *nth1* and a truncated form of *yb1* (YB1 ΔC) were cloned into pmTQ2–N1 for *hnth1* and both pSYFP2–N1 and pSYFP2–C1 for *yb1* for expression in MCF7 cells. Full-length *nth1* and constructs corresponding to either the catalytic domain only (residues 90–326; hNTH1–CAT) or the N-terminal region (residues 1–89; hNTH1–

NTD) were cloned into pET21a and pProexHtB, respectively, for bacterial expression either as single constructs or as fusion proteins with mTQ2 (hNTH1–mTQ2 and mTQ2–hNTH1). Full-length *yb1* was cloned into pProexHtB for bacterial expression, and *yb1*ΔC was cloned into pProexHtB for expression as a fusion protein with SYFP2 (SYFP2–YB1ΔC and YB1ΔC–SYFP2). To create the Biosensor 1 (SYFP2–YB1ΔC–hNTH1–mTQ2) construct, SYFP2–YB1ΔC and hNTH1–mTQ2 constructs were amplified by PCR and cloned into pProexHtB with a 10 amino acid linker (SGGGASGGGT) between the two constructs containing a *NheI* restriction site to facilitate the cloning. A similar procedure was used for the other biosensor constructs.

Protein Expression and Purification. All protein constructs expressed in *E. coli* BL21 (DE3) cells possessed a TEV cleavable N-terminal hexahistidine tag. The cultures were made in LB medium with 100 μg mL^{−1} of ampicillin and were induced with 1 mM IPTG at 20 °C overnight. Cell pellets were resuspended in 50 mM Tris pH 8.0, 2 M NaCl, 2 mM MgCl₂, 0.5 mM EDTA, 0.02% (v/v) Triton X-100, 1 mM β-mercaptoethanol (βME), and 10% (w/v) sucrose, supplemented with EDTA-free complete protease inhibitor tablets, 1 mM PMSF, lysozyme, and DNaseI (Sigma). In addition, for constructs containing YB1, S7 nuclease (Sigma) was added to the cell lysate. Cells were lysed by sonication at 4 °C, and the cell lysates were loaded onto 5 mL HisTrap FF columns (GE Healthcare) equilibrated in Ni Buffer A (50 mM Tris pH 8.0, 0.5 M NaCl, 2 mM MgCl₂, 0.5 mM EDTA, 0.02% Triton X-100, 10% (v/v) glycerol). His-tagged proteins were eluted with an imidazole gradient after extensive washes with Ni buffer A supplemented with 10 mM and then 50 mM imidazole. To eliminate DNA contamination, the samples were loaded onto a 5 mL HiTrap Heparin affinity column (GE Healthcare) and eluted with a salt gradient. After removal of the His-tag by TEV protease cleavage overnight at 4 °C, the samples were loaded onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in a buffer containing 20 mM Tris pH 8.0, 500 mM NaCl, 0.2 mM EDTA, 1 mM TCEP, and 10% glycerol. For the AlphaLISA assays, His-tagged hNTH1 was used—for this purpose, uncleaved protein was gel filtered. The purity of proteins was confirmed by TGX Stain-Free gel electrophoresis (Bio-Rad) at each step of the purifications, and only the highly pure fractions were retained. Purified proteins were stored at −80 °C.

hNTH1 Activity Assay. hNTH1 activity measurements were performed using 5'-FAM (fluorescein amidite) labeled 35mer duplex DNA with a thymine glycol (Tg) in position 14 described previously.³² The oligonucleotide strand containing Tg was FAM-labeled and synthesized by Midland Certified Reagent Co., and its complementary strand was purchased from MWG Eurofins. 75 nM DNA and 3 nM hNTH1 were incubated at 37 °C in a master mix with 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM βME, 0.01% Triton X-100, and 0.1 mg mL^{−1} of BSA, in the presence and absence of 30 nM YB1. At each time point, 10 μL samples were removed from the master mix and transferred to new tubes containing 10 μL of 2X denaturing loading buffer (2X TBE, 8 M urea, 0.025% (w/v) bromophenol blue, 0.025% (w/v) SDS) and were stored on ice. At the end of the experiment, 10 μL of each sample was transferred to a new tube with 1 μL of 1 M NaOH and heat treated for 2 min in a heating block at 95 °C in order to analyze the DNA glycosylase activity. The other half remained and was used to analyze AP-lyase activity. All samples were separated on 20% 8 M urea-(1X) TBE denaturing acrylamide gels prerun for 30 min at 5 W per gel. Gels were run for 30 min at 5 W per gel in 1X TBE, and DNA bands were visualized using a ChemiDoc MP imager (Biorad) and analyzed using ImageLab (Biorad). Lanes and bands were fitted manually with the lane profile tool to quantify the bands. To determine the rates of glycosylase and AP-lyase activities, time points between 5 and 20 min were fitted to a linear regression in GraphPad Prism 6; the derived slopes correspond to the steady-state rates of excision.

FRET Measurements in Vitro. A 5 μM hNTH1 and YB1ΔC fused to mTQ2 and SYFP2, respectively, were mixed in FRET buffer composed of 20 mM Tris pH 8.0, 50 mM NaCl, 0.2 mM EDTA, and 10% glycerol in a reaction volume of 50 μL. FRET measurements on

the fusion and biosensor constructs were performed at a final concentration of 0.2 or 1 μM in FRET buffer. The samples were transferred into 384-well low flange black flat bottom polystyrene microplates (Corning), and the fluorescence emission was measured with a microplate reader (CLARIOstar, BMG Labtech). Two excitation wavelengths were used: 435/15 nm to excite mTQ2 and 485/15 nm to excite SYFP2. After excitation at 435/15 nm, mTQ2 has an emission peak at 477/20 nm (*I*_{DD}). With FRET, another emission peak at 528/20 nm (*I*_{DA}) can be observed which results from the energy transferred from mTQ2 to SYFP2. When the sample is excited at 485/15 nm, an emission peak at 528/20 nm (*I*_{AA}) can be observed which is from the direct excitation of the acceptor, SYFP2. The FRET efficiency, *E*, was calculated after correction of overlapped signals by the intensity-based ratiometric method^{46,47} using the following equations:

$$E = \frac{A}{1 + A} \text{ with}$$

$$A = \frac{1}{\alpha} \times \frac{I_{DA} - (I_{DD} \times S_1) - (I_{AA} \times S_2) + (I_{DD} \times S_2 \times S_3)}{I_{DD}}$$

The α factor was determined to be 1.37 for the mTQ2/SYFP2 FRET pair, and *S*₁ is the contribution of the donor alone to the FRET signal (*I*_{DA}/*I*_{DD}) and was calculated to be 0.42 for mTQ2. *S*₂ is the contribution of the acceptor alone to the FRET signal (*I*_{DA}/*I*_{AA}) and was calculated to be 0.04 for SYFP2. *S*₃ is the contribution of the donor alone to the acceptor signal (*I*_{AA}/*I*_{DD}) and was calculated to be 0.04 for mTQ2.

Cell Culture and Transfection. MCF7 cells (ATCC HTB-22) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX (Gibco), supplemented with 10% FBS and 1% penicillin/streptomycin, nonessential amino acids (0.1 mM), insulin (10 μg mL^{−1}), and sodium pyruvate (1 mM). To generate the stable cell line, expressing hNTH1–mTQ2 protein, MCF7 cells were transfected with pmTQ2–N1–hNTH1 using FuGENE HD (Promega) in 35 mm dishes and subsequently diluted and selected with 1 mg mL^{−1} of Geneticin G418 (Gibco). For FRET experiments, this stably transfected MCF7 cell line expressing hNTH1–mTQ2 was transiently transfected with pSYFP2–N1–YB1ΔC, pSYFP2–C1–YB1ΔC, or pSYFP2–C1–NLS using FuGENE HD (Promega) in 35 mm dishes. To assess the effects of cisplatin and selected hits on the viability of MCF7 cells, cells were seeded in 96-well clear-bottom black microplates (Greiner) with 7000 cells per well in 80 μL of complete DMEM medium as described above. After 24 h incubation, 10 μL of compounds or DMSO alone diluted in complete medium was added, to obtain a range of final concentrations from 50 μM to 5 μM; then 10 μL of cisplatin diluted in sterile 0.9% (w/v) NaCl solution was added in order to obtain the desired final concentration (5 μM or 20 μM). After a 24 or 48 h incubation, 10 μL of PrestoBlue cell viability reagent (Thermo Fisher) was added, and a fluorescence signal (excitation at 560 nm/emission at 590 nm) was measured after a 30 min incubation to quantify the cell viability in response to treatment. Presented data were extracted from two independent experiments each of which was performed in triplicate.

Gradual Acceptor Photobleaching. Gradual acceptor photobleaching experiments to detect FRET signals were performed on fixed (4% (w/v) paraformaldehyde) MCF7 cells 24 h post-transfection. 50 sequential images were acquired using a spinning disk confocal system (Yokogawa CSUX1 head, Olympus IX81 microscope, and Andor iXon Ultra EMCCD camera) at 100 ms exposure time, using AOTF-controlled lasers at 445 nm (40 mW) for mTQ2 with 80% power and at 515 nm (50 mW) for SYFP2 with 5% power. After 10 acquired images, the acceptor was gradually photobleached (100 ms exposure time, laser 515 nm, and 1–3% power) in a selected area of the nucleus using a FRAPPA device (fluorescence recovery after photobleaching (FRAP) and photoactivation (PA); Andor). Images were analyzed to calculate FRET efficiencies using the acceptor photobleaching (donor dequenching) method described previously,⁴⁸ which is independent of the donor-to-acceptor ratio. Briefly, we used Volocity (Quorum Technologies) to

measure the background-corrected mean intensities of the donor (I_D) and the acceptor (I_A) channels in the photobleached region—ROI1—and a control nonbleached region—ROI2—expressed after normalization to the intensity of each channel before photobleaching. We then calculated I'_D and I'_A as the corrected values of I_D and I_A in ROI1 to account for the fluorescence intensity decay linked to imaging, using the intensities of both channels in the control area (ROI2). FRET efficiency E is then calculated as $E = I'_0 - 1$, where I'_0 is the coefficient of the linear regression ($I'_D = \alpha I'_A + I'_0$, σ^2) of the normalized intensity variations of the donor in ROI1 as a function of the acceptor intensities. The slope, α , is the apparent intensity ratio between the donor and the acceptor in the target cell. The correlation coefficient σ^2 was used to estimate the validity of each calculated FRET efficiency value.

Robotic High-Throughput Screening. Screen reliability and robustness was evaluated by estimating the Z' factor.⁴³ For this, the FRET efficiencies of 30 technical replicates of the Biosensor 1 (0.2 μ M) alone as positive control and in the presence of 0.5 μ M THF-oligo as negative “bioactive” control (12mer dsDNA oligonucleotide with a tetrahydrofuran in position 7 paired with a guanine: 5'-TGTCCAXGTCTC-3' with X = THF and complementary strand: 5'-GAGACGTGGACA-3') in FRET buffer were measured. The Z' factor was calculated using the following formula:

$$Z' = 1 - \frac{(3SD \text{ of positive controls} + 3SD \text{ of negative controls})}{\text{mean of positive controls} - \text{mean of negative controls}}$$

For HTS, Biosensor 1 protein at a final concentration of 0.2 μ M in FRET buffer was transferred into 384-well low-flange black flat-bottom polystyrene microplate (Corning) using the robotic setup of the CMBA platform. Each compound from the Life Chemicals PPI library and the Prestwick Chemical library was then added at a final concentration of 50 μ M in the FRET buffer supplemented with either 1.3 or 0.5% (v/v) DMSO, respectively. Each molecule was tested in triplicate. Positive control wells (9/384 per assay plate) contained Biosensor 1 in 1.3% (v/v) DMSO for Life Chemicals and 0.5% DMSO for Prestwick Library and negative control wells (3/384 per assay plate) contained Biosensor 1 with 0.5 μ M THF-oligo. Fluorescence in each well was measured with a TECAN Infinite M1000 microplate reader at different time points after preparing the reactions: 15, 30, and 60 min. Only the 60 min measurements are presented in this study. Confirmation screens were performed with 30 selected compounds from the primary screening at three different compound concentrations (50, 10, and 1 μ M) in triplicate.

Validation Experiments. Selected hits from our secondary HTS were ordered from Sigma, and 10 mM stock solutions were prepared in 100% DMSO. When stock solutions were used immediately after preparation (or after thawing aliquots stored at -20°C), their inhibitory effects were significantly reduced. Storage of the compounds at 4°C for at least 7 days was necessary to reproduce the results of the HTS experiments. UV/vis absorbance spectra of the compounds were performed on a Nanodrop spectrophotometer immediately after preparation and after storage at 4°C and were compared to that of compounds provided by the CMBA screening platform and used for the HTS (Supporting Information Figure S8). For several compounds, substantial changes in these spectra were observed upon storage of the compound at 4°C . This may be caused by low solubility or poor stability of these compounds. The active compounds in these stored solutions may indeed correspond to modified forms of the expected chemicals, which would explain their enhanced inhibitory potential after storage. Such compound loss or instability during storage has previously been reported.⁴⁹ For the validation experiments (dose–response experiments using Biosensor 1 or AlphaLISA assay), only the active compounds stored at 4°C were used. Compounds were serially diluted in reaction buffer supplemented with 1% DMSO. Dose–response curves were established by measuring the FRET (as described above) on 50 μ L reactions containing 0.2 μ M Biosensor 1 in FRET buffer in the presence of 0–100 μ M compound or DMSO alone. The data points corresponding to the means of triplicate measurements were fitted to

a four-parameter sigmoidal inhibition model $\left(Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / \left[1 + \left(\frac{\text{IC}_{50}}{X} \right)^{\text{Hill Slope}} \right] \right)$ in GraphPad Prism 8. Reliable IC_{50} values could be determined for all compounds except moxalactam for which only partial inhibition of the FRET signal was achieved at the highest concentration.

AlphaLISA Measurements. Donor beads coated with streptavidin and AlphaLISA acceptor beads coated with anti-His antibodies were used to bind biotinylated YB1 Δ C and His-tagged hNTH1, respectively. To biotinylate YB1 Δ C, 0.5 mg of YB1 Δ C was transferred into a phosphate buffer (20 mM Na phosphate pH 8.0, 0.5 M NaCl, 1 mM MgCl_2 , 5% glycerol, 0.005% Triton X-100, and 1 mM β ME) using a 5 mL HiTrap Desalting column (GE Healthcare). YB1 Δ C was then incubated with 0.25 mM NHS-Biotin (Sigma) at 25°C for 2 h before the reaction was stopped by the addition of 50 mM Tris pH 8.0. The excess of NHS-Biotin was then removed by a second run on the desalting column equilibrated in 20 mM Tris pH 8.0, 500 mM NaCl, 0.2 mM EDTA, 1 mM TCEP, and 10% glycerol. Fractions containing biotinylated YB1 Δ C were pooled, aliquoted, and stored at -80°C . AlphaLISA assays were performed in white, flat-bottom, small-volume 384-well plates (PerkinElmer). The final reaction volume was 20 μ L including 8 μ L of AlphaLISA buffer (composed of 20 mM Tris pH 7.5, 150 mM NaCl, 0.01% Triton X-100, 0.01% (v/v) Tween 20, 0.5 mM EDTA, 1 mM β ME, and 0.1 mg mL^{-1} of BSA), 2 μ L of biotinylated YB1 Δ C (100 nM), 2 μ L of His-tagged hNTH1 (1 μ M), 4 μ L of acceptor beads (0.1 mg mL^{-1}), and 4 μ L of donor beads (0.1 mg mL^{-1}). All reagents were diluted in AlphaLISA buffer. The plate was read on the CLARIOstar plate reader (BMG Labtech) fitted with an aperture spoon (to avoid excitation of neighboring wells) and the appropriate filters (excitation filter at 680/40 nm and emission filter at 570/100 nm). Reactions were prepared in triplicate. To estimate the apparent binding affinity of the hNTH1–YB1 Δ C complex, increasing amounts of untagged hNTH1 diluted in AlphaLISA buffer were titrated into the AlphaLISA reactions in place of the 8 μ L of buffer. For dose–response experiments, with selected hits, the latter were diluted in AlphaLISA buffer supplemented with 1% DMSO and were also titrated into the reactions in place of the 8 μ L of buffer. The data points corresponding to the means of triplicate measurements were fitted to a three-parameter sigmoidal inhibition model $\left(Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / \left[1 + \left(\frac{X}{\text{IC}_{50}} \right) \right] \right)$ in GraphPad Prism 8, except for the amphotericin B data for which a four-parameter sigmoidal inhibition model was used $\left(Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / \left[1 + \left(\frac{\text{IC}_{50}}{X} \right)^{\text{Hill Slope}} \right] \right)$ with a Hill Slope value of -1.81 .

Thermal Shift Assay. Thermal shift assays were carried out as described previously.⁵⁰ hNTH1 at a final concentration of 5 μ M and YB1 Δ C at a final concentration of 15 μ M were incubated with, respectively, 100 μ M or 150 μ M compounds (or DMSO alone) in 20 mM Tris pH 8.0, 50 mM NaCl, and 5% glycerol for 1 h at RT before addition of Sypro Orange 8X. Reactions of 25 μ L were then distributed in 0.2 mL of PCR strips that were placed in a real-time qPCR machine (Bio-Rad) and heated from 20 to 90°C with temperature increments of $0.5^\circ\text{C}/\text{min}$. The fluorescence signal of the Sypro Orange was recorded at each step, and the T_m values were determined using the primary derivatives of the fluorescence signal. Presented data were extracted from two independent experiments each of which was performed in triplicate.

Statistical Analysis. Statistical tests were performed with GraphPad Prism 6 and 8. P values below 0.05 were considered as significant and are indicated in figures with asterisks as follows: * 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** 0.0001 < P < 0.001; **** P < 0.0001.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.9b01023>.

Supplemental Table S1 and supplemental Figures S1–S8 (PDF)

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Notes

The authors declare no competing financial interest.

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