

Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells

Frédéric Colland,¹ Etienne Formstecher,¹ Xavier Jacq,¹ Céline Reverdy,¹ Cécile Planquette,¹ Susan Conrath,¹ Virginie Trouplin,¹ Julie Bianchi,¹ Vasily N. Aushev,² Jacques Camonis,² Alessandra Calabrese,¹ Catherine Borg-Capra,¹ Wolfgang Sippl,³ Vincent Collura,¹ Guillaume Boissy,¹ Jean-Christophe Rain,¹ Philippe Guedat,¹ Rémi Delansorne,¹ and Laurent Daviet¹

¹Hybrigenics Pharma; ²Institut Curie, INSERM, Paris, France and ³Department of Pharmaceutical Chemistry, Martin-Luther-University Halle-Wittenberg, Halle, Germany

Abstract

Deregulation of the ubiquitin/proteasome system has been implicated in the pathogenesis of many human diseases, including cancer. Ubiquitin-specific proteases (USP) are cysteine proteases involved in the deubiquitination of protein substrates. Functional connections between USP7 and essential viral proteins and oncogenic pathways, such as the p53/Mdm2 and phosphatidylinositol 3-kinase/protein kinase B networks, strongly suggest that the targeting of USP7 with small-molecule inhibitors may be useful for the treatment of cancers and viral diseases. Using high-throughput screening, we have discovered HBX 41,108, a small-molecule compound that inhibits USP7 deubiquitinating activity with an IC₅₀ in the submicromolar range. Kinetics data indicate an uncompetitive reversible inhibition mechanism. HBX 41,108 was shown to affect USP7-mediated p53 deubiquitination *in vitro* and in cells. As RNA interference-mediated USP7 silencing in cancer cells, HBX 41,108 treatment stabilized p53, activated the transcription of a p53 target gene without inducing genotoxic stress, and inhibited cancer cell growth. Finally, HBX 41,108 induced p53-dependent

apoptosis as shown in p53 wild-type and null isogenic cancer cell lines. We thus report the identification of the first lead-like inhibitor against USP7, providing a structural basis for the development of new anticancer drugs. [Mol Cancer Ther 2009;8(8):2286-95]

Introduction

Deregulation of the ubiquitin-proteasome system has been implicated in the pathogenesis of many human diseases, including cancer (1), neurodegenerative disorders (2), and viral diseases (3). The recent approval of the proteasome inhibitor Velcade (bortezomib) for the treatment of multiple myeloma and mantle cell lymphoma has established this system as a valid target for cancer treatment (4, 5). A promising alternative to targeting the proteasome itself would be to interfere with the upstream ubiquitin conjugation/deconjugation machinery to generate more specific, less toxic anticancer agents.

Ubiquitin-specific proteases (USP) constitute the largest subfamily of deubiquitinating enzymes, with >60 human members (6). They remove ubiquitin from specific protein substrates, thus preventing their targeting to the proteasome or regulating their subcellular localization and activation (7). USPs are emerging as potential targets for pharmacologic interference with the ubiquitin regulation machinery based on their protease activity and involvement in several human diseases.

USP7 has been shown to interact with viral proteins, such as ICP0 (Vmw 110), a herpes simplex virus immediate-early gene stimulating initiation of the viral lytic cycle (8), and Epstein-Barr nuclear antigen-1 (9). Human proteins, such as p53 and the major E3 ligase of p53, Mdm2, have also been identified as partners and substrates of USP7 (10–13). It is becoming clear that Mdm2, rather than p53, is the preferred substrate of USP7 under normal unstressed conditions. This results in Mdm2 stabilization through antagonization of its autoubiquitination and consequently in induction of p53 degradation. Structural and biochemical data have shown that Mdm2 and p53 associate with USP7 in a mutually exclusive manner but that USP7 has a higher binding affinity for Mdm2 (14). More generally, USP7 can deubiquitinate different targets, including Mdm2 and p53, and the net deubiquitination of these latter targets ultimately determines functional p53 levels. Recently, RNA interference was used in cancer-relevant cellular assays to systematically screen all USPs predicted in the human genome. In this screen, USP7 silencing was shown to selectively exert antiproliferative effect through induction of cell cycle arrest (15). Interestingly, this phenotype was observed only in cancer cells retaining wild-type p53. Consistent with recent reports, USP7 silencing has also been shown to increase steady-state p53 levels by promoting Mdm2 degradation. More recently, both up-regulation and

Received 2/4/09; revised 5/15/09; accepted 5/15/09; published OnlineFirst 8/11/09.

Grant support: Association de Recherche sur le Cancer (grant 3131) and Association Christelle Bouillot (J. Camonis) and Ministère Français des Affaires Etrangères fellowship (V.N. Aushev).

Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Frédéric Colland, Hybrigenics Pharma, 3-5 Impasse Reille, 75014 Paris, France. Phone: 33-1-58103823; Fax: 33-1-58103849. E-mail: fcolland@hybrigenics.com

Copyright © 2009 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-09-0097

down-regulation of USP7 have been shown to inhibit colon cancer cell proliferation *in vitro* and tumor growth *in vivo*, by resulting in constitutively high p53 levels (16). USP7 has also been shown to deubiquitinate FOXO4, provoking its nuclear export and hence its inactivation; consequently, the oncogenic phosphatidylinositol 3-kinase/protein kinase B signaling pathway was activated (17). The tumor suppressor PTEN is also deubiquitinated by USP7, resulting in its nuclear export and inactivation (18). In the same study, USP7 overexpression in prostate cancer was directly associated with tumor aggressiveness (18). Finally, USP7 plays an important role in p53-mediated cellular responses to various stress, such as DNA damage and oxidative stress (17, 19, 20).

The phenotypes associated with USP7 silencing and the known connections between USP7 and essential viral proteins and oncogenic pathways, such as the p53/Mdm2 and phosphatidylinositol 3-kinase/protein kinase B pathways, strongly suggest that targeting USP7 with small-molecule inhibitors may be beneficial in the treatment of cancers and viral diseases. We describe here the identification of HBX 41,108, an optimized cyano-indenopyrazine derivative that inhibits USP7 catalytic activity and stabilizes and activates p53 in a nongenotoxic manner, inhibiting cell growth and leading to p53-dependent apoptosis.

Materials and Methods

Protein Production and Purification

Full-length wild-type human USP7 and its catalytic mutant (cysteine 223 replaced by alanine, C223A) were produced as NH₂-terminally His-tagged fusions in *Spodoptera frugiperda* cells (Sf9; Invitrogen) using the Bac-to-Bac Baculovirus System (Invitrogen) according to the manufacturer's instructions. Fusion proteins were then allowed to bind to TALON beads (BD Biosciences) for 30 min at 4°C with gentle rocking. Beads were extensively washed and bound proteins were eluted with 250 mmol/L imidazole (Sigma). Eluted fractions were resolved on 4% to 12% NuPAGE gels (Novex; Invitrogen). Fractions containing high concentrations of purified proteins (purity >95%) were dialyzed, aliquoted, and snap-frozen in liquid nitrogen before storage at -80°C.

High-Throughput Screening

Deubiquitinating enzyme activity was monitored in a fluorometric assay, using ubiquitin-7-amido-4-methylcoumarin (Ub-AMC; Boston Biochem; U-550) as the substrate, as described previously (21). USP7 inhibitors were identified by screening our compound library for inhibition of the enzyme-mediated cleavage of the AMC fluorophore from Ub-AMC. High-throughput screening (HTS) was done with a Perkin-Elmer Minitrak automat in a 384-well format (Greiner black plates). Both enzyme and substrate were freshly prepared in USP7 reaction buffer [50 mmol/L Tris-HCl (pH 7.6), 0.5 mmol/L EDTA, 5 mmol/L DTT, 0.01% Triton X-100, and 0.05 mg/mL serum albumin] for each run. Each well (except substrate control wells) in a typical assay contained 0.5 nmol/L USP7, 20 μmol/L of compound, or 5% DMSO. The wells were incubated for 30 min to attain equilibrium and the enzymatic reaction was then initiated by adding

the substrate (400 nmol/L). The reaction mixture was incubated at room temperature for 2 h and reaction was stopped by adding 250 mmol/L acetic acid. Fluorescence emission intensity was measured on a PHERAstar (BMG Labtech). The Z' factor, a standard statistical parameter for HTS assays, was 0.77, indicative of a robust HTS assay (22). The intrinsic fluorescence of the compounds was evaluated by measuring their fluorescence emission intensity alone. False positives due to the quenching of AMC amine fluorescence were eliminated by a series of control experiments in which each compound was incubated at a concentration of 10 μmol/L with a 200 nmol/L AMC amine solution. The assay was optimized and a proprietary compound library of 65,092 chemically diverse compounds was screened for USP7 activity (23).

Compound Characterization

We characterized the inhibitory mechanism of HBX 41,108 by adding various concentrations of HBX 41,108 to USP7 (500 pmol/L final concentration) in USP7 reaction buffer and incubating the mixture at room temperature for 30 min. Deubiquitinating activity was initiated by adding 10 μL of various concentrations of Ub-AMC substrate. Reactions were monitored with a PHERAstar (BMG Labtech) and initial velocity was determined with GraphPad Prism software.

We also evaluated the effect of HBX 41,108 on other proteases. (a) Recombinant SENP1 was purified from *Escherichia coli* and its substrate (SUMO-AMC) purchased from Boston Biochem. (b) Ubiquitin COOH-terminal hydrolase-L1 (UCH-L1) was purchased from Boston Biochem. The other proteases tested were purified and evaluated with their specific fluorescent substrates as recommended by MDS Pharma Services.

For reversibility studies, we incubated USP7 (260 pmol/L) for 30 min in the presence of HBX 41,108 (10 μmol/L) or iodoacetamide (50 mmol/L), an irreversible thiol alkylating agent (24). We passed a fraction of each sample through a G50 gel filtration system (Sephadex, G50 superfine; Sigma-Aldrich). The deubiquitinating activity of the G50 eluates was assessed with Ub-AMC, as described above, and compared with that of samples not subjected to G50 filtration. To characterize the effect of HBX 41,108 on USP7 inactivation by iodoacetamide, various amounts of HBX 41,108 were incubated with USP7 (500 pmol/L) for 5 min before the initiation of the Ub-AMC reaction followed by addition of iodoacetamide (50 mmol/L). The reactions were monitored using a PHERAstar (BMG Labtech).

Molecular Modeling and Docking

All calculations were done on a Pentium IV 2.2 GHz-based Linux cluster. The molecular structures of the inhibitors were generated using the Sybyl6.8 modeling software (Tripos). The structures were energy minimized using the Tripos force field, Gasteiger-Hueckel partial charges, and the conjugate gradient method until the default derivative convergence criterion of 0.01 kcal/(mol Å) was met. The X-ray coordinates of the USP7 in free form (1NB8.pdb) and ubiquitin aldehyde complexed form (1NBF.pdb; ref. 25) were retrieved from the Protein Databank (26) and

prepared using the Sybyl software. Hydrogen atoms were added and the protein structures were minimized using the Kollmann all-atom force field. The protein structures were subsequently used in combination with our in-house docking program ParaDock to dock the inhibitors into the different USP7 structures. Ten different docking poses were calculated for each inhibitor and ranked using the X-Score scoring function (27). The top-ranked docking solutions were visually analyzed using the Sybyl software.

Cell Lines

Human prostatic adenocarcinoma PC3 cells (American Type Culture Collection) were maintained in F-12K medium supplemented with 7% fetal bovine serum. Human embryo kidney HEK293 cells (European Cell Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 1% nonessential amino acids. Human colon carcinoma HCT116 cells and isogenic HCT116 cells (kindly provided by B. Vogelstein) were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and 3 mmol/L glutamine. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Antibodies

Antibodies used were p53 (DO-1; Santa Cruz Biotechnology), phospho-S15-p53 (Cell Signaling; 9286S), p21 (BD Pharmingen; 556430), poly(ADP-ribose) polymerase (Cell Signaling; 9542), Mdm2 (SMP14; Santa Cruz Biotechnology), USP7 (Bethyl Lab; A300-034A), caspase-3 (Cell Signaling; 9661), and actin (Sigma; A2066).

DNA Intercalating Assay

DNA intercalating assays were carried out as described previously (28). Various concentrations of the tested compounds were incubated with supercoiled pLAZ plasmid DNA (130 ng) before the addition of 4 units of human topoisomerase I and incubation at 37°C for 45 min in relaxation buffer [50 mmol/L Tris (pH 7.8), 50 mmol/L KCl, 10 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L EDTA, and ATP]. Reactions were stopped by adding SDS (0.25%) and proteinase K (250 µg/mL) and by incubating the samples at 50°C for 30 min. DNA samples were electrophoresed through a 1% agarose gel and then stained with ethidium bromide.

Ubiquitination Assay

HEK293 cells were transfected with the indicated constructs. Forty hours after transfection, cells were treated for 24 h with various concentrations of HBX 41,108 and lysed and ubiquitinated proteins were precipitated from using Ni-NTA agarose beads (Qiagen), essentially as described elsewhere (17), and analyzed by Western blotting for the presence of p53.

For the *in vitro* deubiquitination of p53 by USP7, the purified enzyme (3 nmol/L final concentration) was incubated for 30 min at room temperature with HBX 41,108 at the indicated concentration before the addition of purified p53 multi-monoubiquitinated by Mdm2 (kindly provided by O. Coux). Reactions were incubated at 37°C for 90 min and stopped by adding denaturing 1× NuPAGE LDS Sample Buffer (Invitrogen) and 0.1 mol/L DTT (final concen-

tration). Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-p53 antibody.

Caspase-3 Assay

Caspase-3 activity, following HBX 41,108 treatment at the indicated concentrations for 24 h, was quantified using the fluorometric CaspACE Assay System (Promega). For sample preparation, cells were treated with trypsin and floating and free cells were then collected by centrifugation. Cell pellets were resuspended in native conditions in NP40 buffer (10 mmol/L HEPES, 142.5 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L EDTA, 1% NP40, and 1× protease inhibitor cocktail; Sigma). Samples were subjected to five freeze/thaw cycles in liquid nitrogen. The supernatants were collected after centrifugation. A fixed amount of protein (20 µg) was then used for caspase-3 assays according to the manufacturer's instructions.

Proliferation and Viability Assays

Following HBX 41,108 treatment at the indicated concentrations for 24 h, HCT116 cells were incubated for 30 min in culture medium containing 10 µmol/L 5-bromo-2-deoxyuridine. Cells were then harvested by trypsin treatment. Cells were collected by centrifugation and the pellet was resuspended and incubated in 70% ethanol for 30 min at 4°C. After centrifugation and supernatant elimination, DNA was denaturated by incubation for 30 min with 2 N HCl at room temperature. The percentage of 5-bromo-2-deoxyuridine-positive, proliferating cells was then determined by flow cytometry (Epics XL; Beckman Coulter).

Cell viability was evaluated after treatment with HBX 41,108 (0.37 µmol/L) and/or 5-fluorouracil (5-FU; 1.1 µmol/L) for 24 h using the MTS-based assay (Promega). This assay was carried out according to the manufacturer's instructions.

Results

HTS for USP7 Inhibitors

We produced a full-length His-tagged USP7 protein as a functional enzyme in baculovirus-infected insect cells and purified it using the His-tag. A catalytically inactive form of USP7 was generated by mutating the cysteine 223 into an alanine residue (C223A). This form was purified in the same conditions than wild-type USP7 and used as a control to exclude the copurification of contaminating deubiquitinating activities. Using the previously described substrate Ub-AMC (21), we developed a HTS assay for USP7. This assay is based on the hydrolysis of Ub-AMC by USP7, releasing AMC, which can be monitored by fluorescence spectroscopy. Both wild-type and mutant enzymes were tested in parallel in dose-dependent reactions using this Ub-AMC assay (Supplementary Fig. S1). The wild-type USP7 protein hydrolyzed Ub-AMC 1,000 times more efficiently than the mutant protein, indicating that no interference with a potential copurified contaminating deubiquitinating activity would be detected in our assay. A library of 65,092 compounds was screened, yielding 133 primary hit compounds that inhibited USP7 activity by

>50% at a concentration of 20 $\mu\text{mol/L}$. We confirmed 117 of these hits (tested in duplicate, with structural analysis by liquid chromatography/mass spectrometry) and 45 compounds displayed an IC_{50} below 10 $\mu\text{mol/L}$. Value for the Z' factor, standard statistical parameter for HTS assays, was 0.77, indicating that the HTS assay was robust (22).

Identification and Biochemical Characterization of HBX 41,108

By optimizing active hits using traditional medicinal chemistry, we identified a cyano-indenopyrazine derivative, HBX 41,108, which was characterized in greater detail (Fig. 1A). Submicromolar inhibition of Ub-AMC hydrolysis by USP7 was obtained with HBX 41,108 (median inhibitory concentration $\text{IC}_{50} = 424 \text{ nmol/L}$; Fig. 1B). By contrast, no effect was observed with HBX 91,490, a related compound in which the chloride group was replaced by a hydroxyl group (Fig. 1A and B). We confirmed this finding using a more physiologic USP7 substrate, p53 polyubiquitinated *in vitro* by the ubiquitin ligase Mdm2 (see Materials and Methods). The deubiquitination of polyubiquitinated p53 by USP7 was observed (Fig. 1C), but no such reaction was seen with the catalytically inactive C223A USP7 mutant (data not shown). We then as-

sessed the effect of HBX 41,108 on USP7-mediated p53 deubiquitination and showed that this molecule had a dose-dependent effect on USP7 activity (estimated $\text{IC}_{50} = 0.8 \mu\text{mol/L}$). This showed the USP7-inhibiting properties of HBX 41,108 in a physiologic isopeptide bond cleavage assay.

We characterized the inhibitory mechanism of HBX 41,108 by adding various amounts of Ub-AMC to USP7 in the presence of different concentrations of HBX 41,108. Eadie-Hofstee analysis indicated that HBX 41,108 was an uncompetitive inhibitor of USP7 activity (Fig. 1D). Thus, HBX 41,108 preferentially inhibited USP7 after formation of the enzyme-substrate complex rather than preventing the interaction of the substrate with USP7. We assessed the reversibility of HBX 41,108-mediated inhibition by carrying out gel filtration experiments. In the absence of filtration, USP7 activity was strongly inhibited by HBX 41,108 and the irreversible thiol alkylating agent, iodoacetamide (Supplementary Fig. S2A, prefiltration). Using a sample containing HBX 41,108 alone, we confirmed that HBX 41,108, filtered through a G50 column, was entirely retained on the column (data not shown). The filtration of samples containing USP7 and HBX

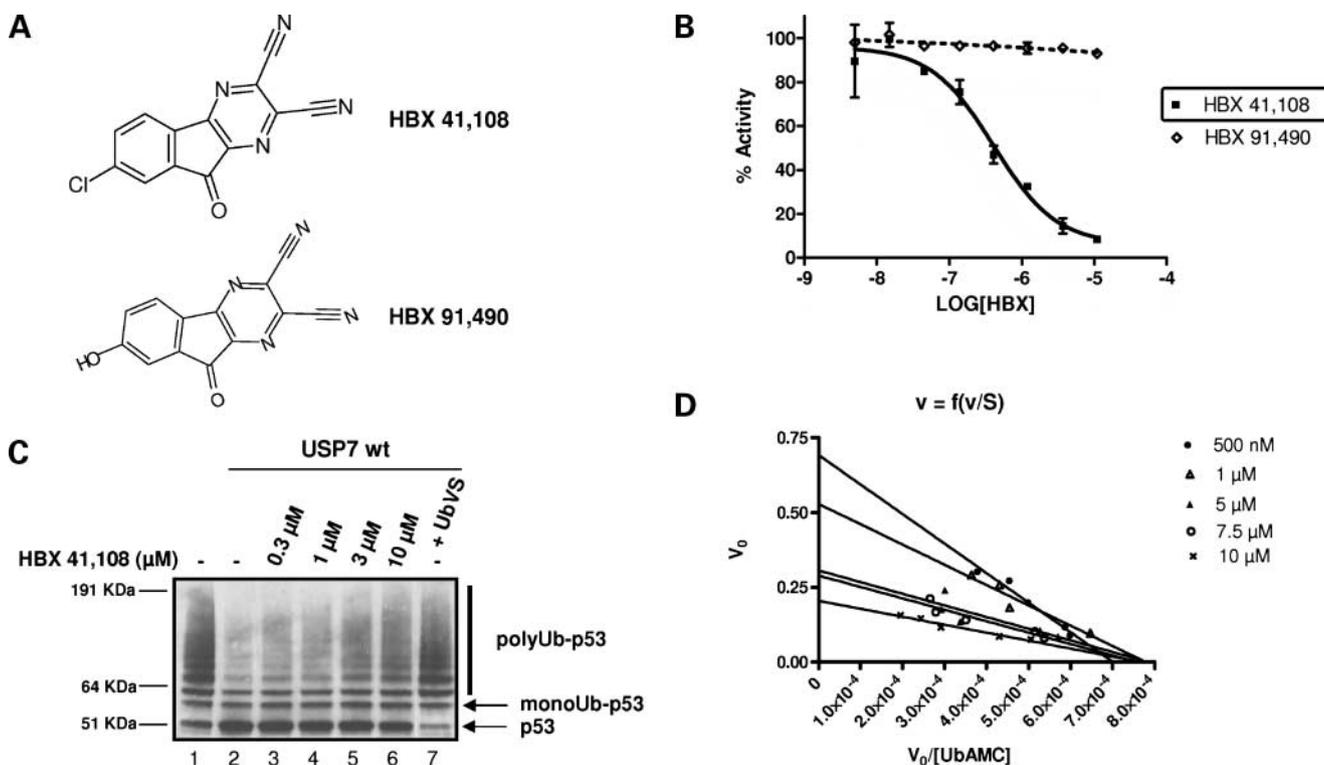


Figure 1. Identification of HBX 41,108 as a USP7 inhibitor. **A**, structures of the cyano-indenopyrazine derivatives, HBX 41,108 and HBX 91,490. **B**, dose-dependent inhibition of USP7 activity by HBX 41,108. Mean \pm SD of triplicate values. Representative of four independent experiments. **C**, HBX 41,108 inhibits USP7 deubiquitination of polyubiquitinated p53. The purified wild-type USP7 enzyme (3 nmol/L final concentration) was incubated for 30 min at room temperature with different doses of HBX 41,108 (lanes 3-6) or ubiquitin vinyl sulfone (UbVS; 0.1 $\mu\text{mol/L}$; lane 7) or without any inhibitors (lane 2). Purified polyubiquitinated p53 was next added to these different samples (lanes 2-7) or used alone (lane 1) and reactions were incubated at 37°C for 90 min. **D**, HBX 41,108 acts through a uncompetitive inhibition mechanism. Various concentrations of Ub-AMC (0.15, 0.2, 0.4, 0.6, and 0.8 $\mu\text{mol/L}$) were added to USP7 (500 pmol/L) in the presence of various concentrations of HBX 41,108 (0.5, 1, 5, 7.5, and 10 $\mu\text{mol/L}$) for initial rate measurements. Data sets from two independent experiments.

Table 1. HBX 41,108 specificity on a panel of proteases

Family	Name	IC ₅₀ (μ mol/L)
Aspartic proteases	Renin	>10
	Cathepsin D	>10
	Cathepsin E	>10
	HIV-1 protease	>10
Serine proteases	Trypsin	>10
	Factor Xa	>10
	Thrombin	>10
	Plasmin	>10
Metallo proteases	ACE	>10
Cysteine proteases	Calpain-1	>10
	Cathepsin B	>1
	Cathepsin L	>1
	Cathepsin S	>1
	UCH-L1	>1
	SEN1	>10
	USP7	0.42

41,108 through a G50 column led to the total restoration of USP7 activity, whereas USP7 inhibition by iodoacetamide could not be reversed by filtration (Supplementary Fig. S2A). These findings show that HBX 41,108 is a reversible USP7 inhibitor. To determine if the cyano-indenopyrazine derivative binds at the active site of USP7, iodoacetamide was added to various concentrations of HBX 41,108. In the presence of iodoacetamide alone, USP7 enzymatic activity is decreased presumably by derivatization and inactivation of the active site cysteine (Supplementary Fig. S2B). Interestingly, combined treatment of USP7 with increasing amounts of HBX 41,108 and iodoacetamide further decreased USP7 activity, which may indicate the absence of any competition between both compounds. These results thus suggest that binding of HBX 41,108 does not protect the USP7 active site cysteine, a finding that may be consistent with the uncompetitive inhibition properties of HBX 41,108.

To further understand the structural basis of HBX 41,108 binding to USP7, we carried out molecular modeling and docking studies. USP7 has been crystallized in apo-form (unproductive conformation) and together with covalently bound ubiquitin aldehyde (productive conformation; ref. 25). In the ubiquitin-bound conformation of USP7, the residues of the catalytic triad are in close proximity, whereas in the uncomplexed form the two catalytic site residues Cys²²³ and His⁴⁶⁴ are separated by >9 Å. The analysis of the two USP7 structures revealed a small hydrophobic pocket in the USP7-ubiquitin complex, which is close to the ubiquitin binding site (Supplementary Fig. S3A). The docking results showed that HBX 41,108 preferentially interacts with this pocket formed by Val²⁵⁶, Phe²⁸³, Trp²⁸⁵, His²⁹⁴, Leu²⁹⁹, and Val³⁰² (Supplementary Fig. S3B). The chloro-substituent is interacting with a highly hydrophobic subsite (Val²⁵⁶, Phe²⁸³, and Val³⁰²). The binding of HBX 41,108 nearby the

covalently bound ubiquitin suggests that the binding of HBX 41,108 modulates allosterically the catalytic reaction. However, further experimental data such as crystal structures are necessary to confirm this hypothesis.

We evaluated the specificity of HBX 41,108 for USP7 as opposed to other proteases by assessing the inhibitory effects of this compound *in vitro* against a representative set of proteases from the major peptidase families. HBX 41,108 was only weakly active (IC₅₀ > 10 μ mol/L) against the serine, aspartic, and metalloproteases tested (Table 1). As USP7 is a thiol protease, we investigated the effects of HBX 41,108 on other proteases of this family. HBX 41,108 had only a marginal activity against the prototypical thiol protease, calpain-1 (IC₅₀ > 10 μ mol/L), but displayed some activity (IC₅₀ > 1 μ mol/L) against cathepsins B, L, and S (Table 1). We then assessed the specificity of HBX 41,108 for USP7 by comparison with the related deubiquitinating enzyme, ubiquitin COOH-terminal hydrolase-L1, and SENP1, a SUMO protease. HBX 41,108 was less active against these ubiquitin and ubiquitin-like proteases than against USP7 (Table 1). Overall, these results suggest that there is some selectivity for HBX 41,108 in the inhibition of USP7 activity but that an effect on unrelated thiol proteases and additional deubiquitinating enzymes could also be measured.

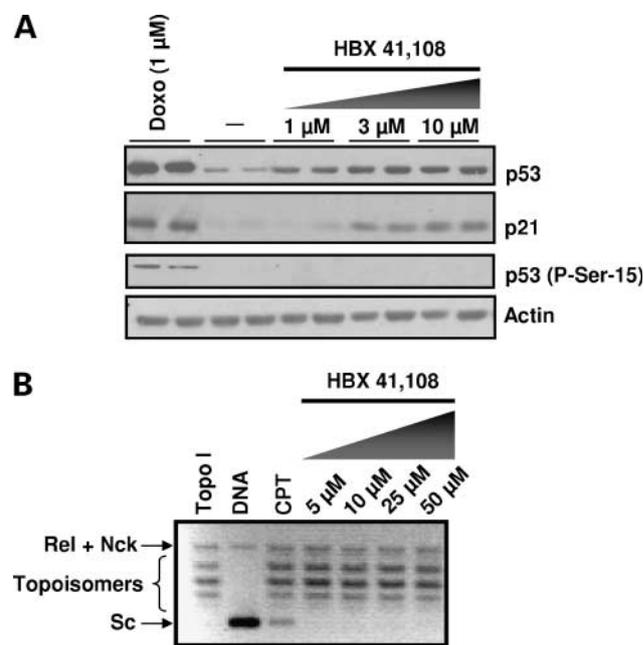


Figure 2. HBX 41,108 increases p53 levels in a nongenotoxic manner. **A**, Western blot analysis of p53, phospho-p53, and p21 protein levels in HCT116 colon cancer cells treated with various doses of HBX 41,108 (1, 3, and 10 μ mol/L) for 24 h. **B**, effect of HBX 41,108 on the relaxation of plasmid DNA by human topoisomerase I in the absence of ethidium bromide during the electrophoresis. Native supercoiled pLAZ (0.13 μ g; lane DNA) was incubated with topoisomerase I (4 units) in the absence (lane Topo I) or presence of camptothecin (CPT) and HBX 41,108 at the indicated concentration (μ mol/L). Reactions were stopped by adding SDS and proteinase K. DNA samples were separated by electrophoresis in 1% agarose gels. Nck, nicked; Rel, relaxed; Sc, supercoiled. Duplicate experiments.

Induction of Functional p53 by HBX 41,108

As USP7 silencing results in the stabilization and activation of p53, we evaluated the effect of HBX 41,108 on endogenous levels of p53 in cells. The incubation of exponentially growing HCT116 cells with HBX 41,108 for 24 h led to a dose-dependent increase in the levels of p53 and of the products of its target genes, such as the cyclin-dependent kinase inhibitor p21^{cip1/waf} (Fig. 2A). Thus, HBX 41,108 induces the functional activation of p53 in mammalian cells. This phenotype was not observed with HBX 91,490 (Supplementary Fig. S4A), a compound that had no effect on USP7 *in vitro* (Fig. 1B). We checked that HBX 41,108 did not activate p53 through genotoxic stress by monitoring the phosphorylation status of serine 15 of p53 (29). Western blot analysis revealed that HBX 41,108 induced p53 accumulation without serine 15 phosphorylation in contrast to the genotoxic drug, doxorubicin (Fig. 2A). Absorption and fluorescence spectra, melting temperature, and circular dichroism studies showed that HBX 41,108 did not bind to DNA (data not shown). Finally, plasmid DNA relaxation experiments with human topoisomerase I showed that HBX 41,108 had no effect on the distribution of the topoisomerase population (Fig. 2B), unlike a diamino derivative of rebeccamycin shown previously to act as a DNA intercalating agent (28). These findings show that HBX 41,108 has no DNA intercalating properties.

Further experiments were done to exclude the possibility that HBX 41,108 stabilized p53 via proteasome inhibition. We therefore investigated whether HBX 41,108 affected

the three major protease activities of the proteasome *in vitro*. Bortezomib has been shown to inhibit all three protease activities, albeit with different potencies (5). By contrast, HBX 41,108 did not inhibit any of these activities (IC₅₀ > 100 μmol/L; Supplementary Fig. S5). Thus, the stabilization of p53 induced by HBX 41,108 does not involve any genotoxicity or proteasome inhibition.

HBX 41,108 Inhibits USP7 Activity in Cells

We investigated whether HBX 41,108 was active against USP7 in cells by developing a USP7-dependent deubiquitination assay in HEK293 cells. To this end, His-tagged ubiquitin and USP7 were expressed together in HEK293 cells. Subsequently, ubiquitinated proteins were pulled down and the polyubiquitinated forms of p53 were detected by immunoblotting with an anti-p53 antibody. In this context, far smaller amounts of polyubiquitinated forms of p53 were observed in the presence of wild-type USP7 than in the presence of the catalytically inactive form of USP7 (Fig. 3A, compare lanes 3 and 4) as reported previously (13, 20). This assay was used to investigate the effect of HBX 41,108 on USP7-mediated p53 deubiquitination. The incubation of USP7-overproducing HEK293 cells with various amounts of HBX 41,108 for 24 h resulted in levels of the polyubiquitinated forms of p53 significantly higher than those seen for the DMSO control (Fig. 3B, compare lanes 3 and 5). Thus, HBX 41,108 partly reverses the USP7-mediated p53 deubiquitination process in HEK293 cells, suggesting that HBX 41,108 is active against USP7 in cells. We also monitored the levels of the other well-characterized USP7 substrate,

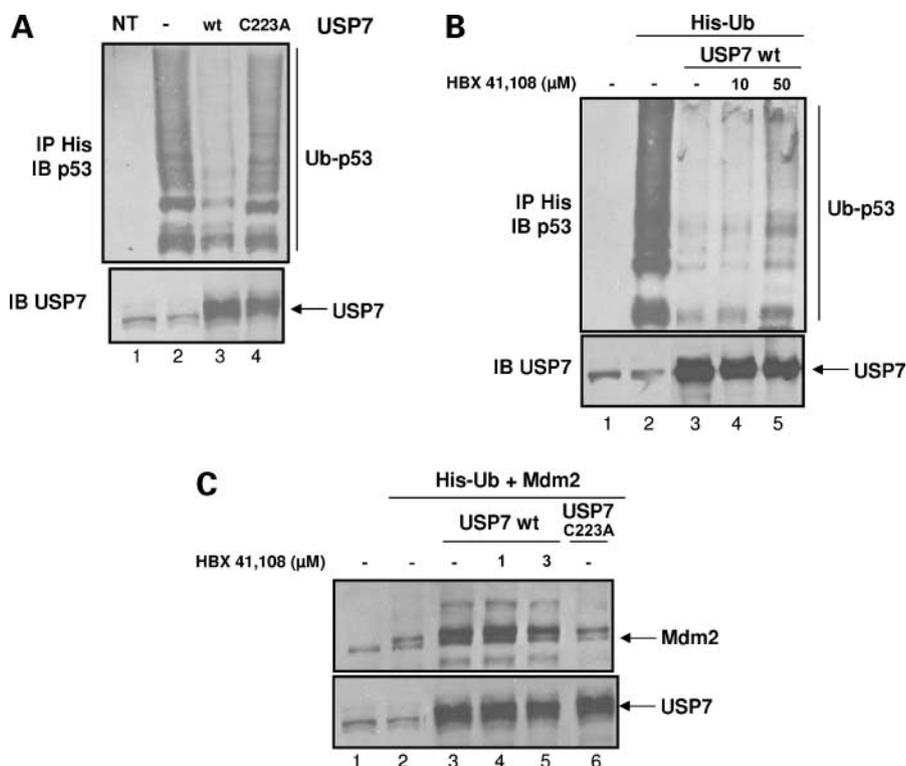


Figure 3. HBX 41,108 inhibits USP7 activity in cells. **A**, ubiquitinated proteins were precipitated, using Ni-NTA agarose, from HEK293 cells transfected (lanes 2-4) or not (lane 1) with His-tagged ubiquitin and Mdm2 ± wild-type (lane 3) or mutant USP7 (lane 4). Western blots were analyzed for the presence of p53 and USP7. **B**, experiment was done as in **A** using HBX 41,108 (10-50 μmol/L). **C**, HEK293 cells were transfected with wild-type Mdm2, His-tagged ubiquitin, and wild-type or mutant USP7 constructs and treated with HBX 41,108 (1-3 μmol/L). Western blots were analyzed for the presence of Mdm2 and USP7.

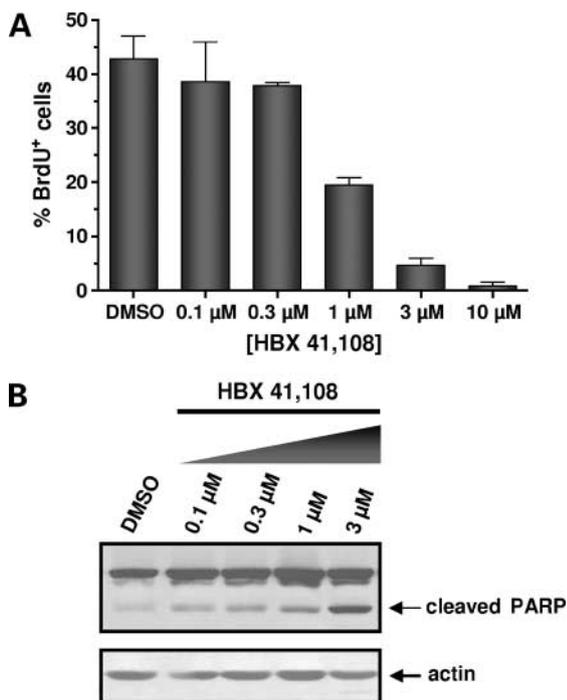


Figure 4. HBX 41,108 inhibits HCT116 cancer cell growth and induces apoptotic cell death. **A**, treatment for 24 h with HBX 41,108 decreases HCT116 cell proliferation in a dose-dependent manner (5-bromo-2-deoxyuridine incorporation). **B**, poly(ADP-ribose) polymerase cleavage following the treatment of HCT116 cells with HBX 41,108 (0.1, 0.3, 1, and 3 μmol/L) was detected by Western blotting.

Mdm2. Mdm2 was stabilized in the presence of wild-type USP7, but not in the presence of the catalytically inactive form of USP7 (Fig. 3C, compare lanes 3 and 6), as shown previously (13, 20). The addition of HBX 41,108 significantly decreased Mdm2 levels, indicating that this compound partly reversed the USP7-mediated Mdm2 stabilization in HEK293 cells (Fig. 3C, compare lanes 3 and 5).

We next addressed whether HBX 41,108 may have USP7-independent effects. To this end, we monitored the induction level of p53 following HBX 41,108 treatment in the presence or absence of endogenous USP7 using a HCT116 cell line stably transfected by an inducible USP7 short hairpin RNA construct. We observed a significant induction of p53 level following USP7 silencing, as expected from literature (refs. 10, 12, 20; Supplementary Fig. S4B). In addition, p53 was also induced following treatment with increasing doses of HBX 41,108, as already shown in Fig. 2A. Interestingly, no such p53 induction was observed when cells deleted for USP7 were treated with the same increasing doses of HBX 41,108 (Supplementary Fig. S4B). To ensure that p53 is inducible further even after USP7 silencing, we evaluated the effect of Nutlin-3, an inhibitor of the p53-Mdm2 interaction. Interestingly, we observed a dose-dependent increase of p53 whatever the endogenous level of USP7, thus indicating that the absence of p53 induction by HBX 41,108 in USP7 silenced cells is not due to a saturation of the system (Supplementary

Fig. S4C). We thus show that p53 induction mediated by HBX 41,108 is likely USP7-dependent.

Inhibition of Cell Proliferation and Induction of p53-Dependent Apoptosis by HBX 41,108

Activation of p53 in cells leads to inhibition of cell proliferation and induction of apoptotic cell death. We investigated whether HBX 41,108 affected cell proliferation by treating exponentially growing HCT116 colon cancer cells with a range of concentrations of HBX 41,108 for 24 h and assessing proliferation in 5-bromo-2-deoxyuridine incorporation assays. HBX 41,108 inhibited HCT116 proliferation in a dose-dependent manner, with an IC_{50} of ~ 1 μmol/L (Fig. 4A), whereas the control HBX 91,490 compound had no such effect (data not shown).

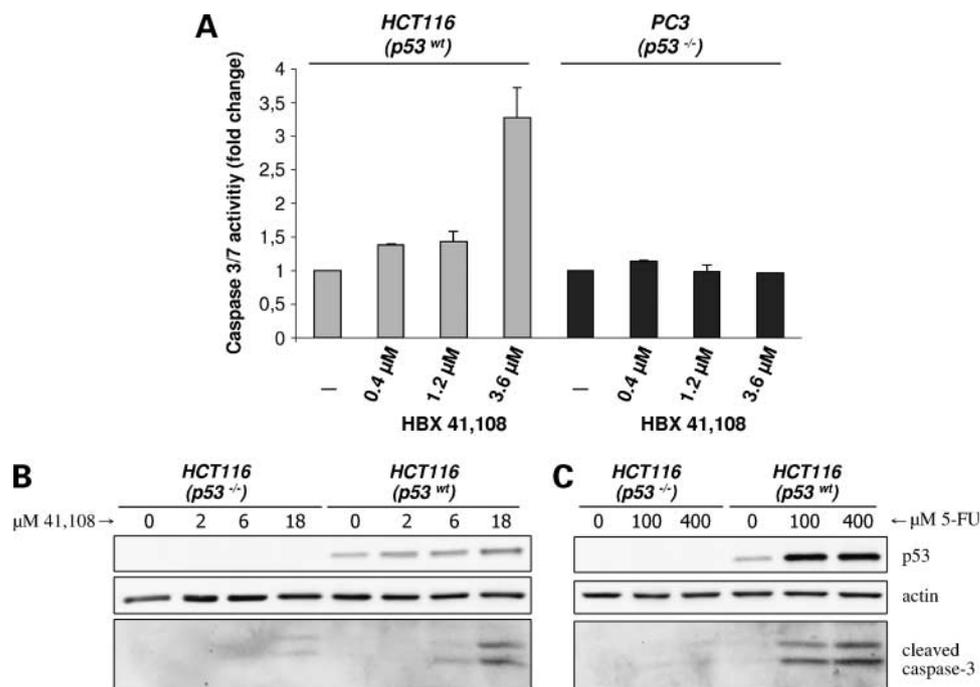
We then investigated the effect of the USP7 inhibitor on cell apoptosis. HCT116 cells were treated with various concentrations of HBX 41,108, and the induction of apoptosis was assessed by monitoring poly(ADP-ribose) polymerase cleavage. HBX 41,108 induced the apoptosis of HCT116 cells in a dose-dependent manner (Fig. 4B). We investigated whether the observed effect was dependent on a functional p53 pathway by treating wild-type (HCT116) and mutant p53 (PC3) cancer cell lines with HBX 41,108 and assessing caspase-3/7 activity. The proapoptotic activity of HBX 41,108 differed between the two cell lines (Fig. 5A). We then investigated the proapoptotic effect of HBX 41,108 on HCT116 cell lines in which the p53 pathway was specifically disrupted by targeted homologous recombination (29). Consistent with observations described above, a marked difference in caspase-3 activation was observed between cell lines treated with HBX 41,108 depending on their p53 status (Fig. 5B). As a control, the same couple of isogenic cell lines was treated with the genotoxic drug 5-FU; this resulted in the specific stabilization of p53 and the induction of apoptosis in the parental HCT116 cell line as reported previously (ref. 29; Fig. 5C). These observations were confirmed in a second set of experiments in which the induction of apoptosis was monitored by poly(ADP-ribose) polymerase cleavage (Supplementary Fig. S6).

Nutlins, nongenotoxic small-molecule activators of p53, have recently been developed as small-molecule inhibitors of the p53-Mdm2 interaction (30). By stabilizing p53, these compounds increase the cytotoxicity of genotoxic drugs (31). Because the USP7 inhibitor also promotes the nongenotoxic activation of p53, we therefore investigated whether it would trigger a similar phenotype. HCT116 cells were incubated for 72 h with suboptimal concentrations of HBX 41,108 (IC_{20}), 5-FU, or a combination of the two compounds. A combination of HBX 41,108 and 5-FU markedly decreased cell viability (Fig. 6), suggesting that treatment with USP7 inhibitor and genotoxic drugs may provide a new therapeutic approach in combinatorial drug therapy.

Discussion

The observation that p53 function is lost in about half of human cancers makes it an attractive cancer target. Because p53 can induce tumor cell death, the identification of small

Figure 5. HBX 41,108 induces apoptosis in a p53-dependent manner. **A**, caspase activity was assessed in p53^{+/+} (HCT116) and p53^{-/-} (PC3) cell lines following HBX 41,108 treatment (0.4, 1.2, and 3.7 μmol/L) using the caspase assay described in Materials and Methods. **B**, isogenic HCT116 cancer cells (p53^{+/+}, p53^{-/-}) were analyzed for the effects of HBX 41,108 (2, 6, and 18 μmol/L) on caspase-3 cleavage as an indicator of apoptosis induction. **C**, experiment was done as in **B** using the genotoxic drug 5-FU.



molecules that will allow the reactivation of p53 is currently one of the most actively explored fields in cancer research. For cancers that retain wild-type p53, compounds have been described that target Mdm2. These include small molecules such as Nutlin-3, which block the interaction of p53 with Mdm2, or HLI98, which targets the ubiquitin-ligase activity of Mdm2 (30, 32).

One of the possible caveats of this approach is that these p53 reactivating drugs are likely to activate p53 in both normal and tumor cells. However, the recent observations that cancers are more sensitive to p53-dependent apoptotic stimuli or senescence than normal cells raise the possibility that these compounds will be sufficiently selective in their killing to be useful cancer therapeutics (33, 34). In line with these observations, Nutlin-3 and HLI98 were reported to have a differential killing effect on tumor versus normal cells (30, 32). Altogether, these experimental results support efforts to treat human cancers by way of pharmacologic reactivation of p53.

Here, we adopted an alternative approach to activate p53 based on the identification of small-molecule inhibitors of USP7, a USP required for Mdm2-dependent p53 destabilization. We reasoned that proteases should be a priori more tractable drug targets than the Mdm2/p53 interaction or the nonclassic "enzymatic activity" of E3 ligases. Our chemical approach to USP7 inhibition led to the identification of HBX 41,108, a cyano-indenopyrazine derivative that inhibits USP7 in a reversible manner. Kinetic analysis showed that HBX 41,108 inhibited USP7 in an uncompetitive manner, indicating that it preferentially interacts with the enzyme-substrate complex rather than directly competing with substrate binding. As the USP7 catalytic core switches from an inactive to a catalytically competent

conformation on ubiquitin binding, it is tempting to speculate that HBX 41,108 specifically interacts with the active, ubiquitin-bound conformation of the core (25). In addition, the absence of any competition between iodoacetamide, a thiol alkylating agent, and HBX 41,108 indicates that binding of HBX 41,108 does not protect the USP7 active site cysteine, a finding that is consistent with the uncompetitive inhibition properties of HBX 41,108. Molecular modeling and docking hypotheses based on the structure of the ubiquitin-bound USP7 core are consistent with such a mechanism.

In cells, HBX 41,108 treatment stabilized p53 in a nongenotoxic manner and increased levels of the p53-induced cell cycle inhibitor p21. Furthermore, using isogenic cell lines

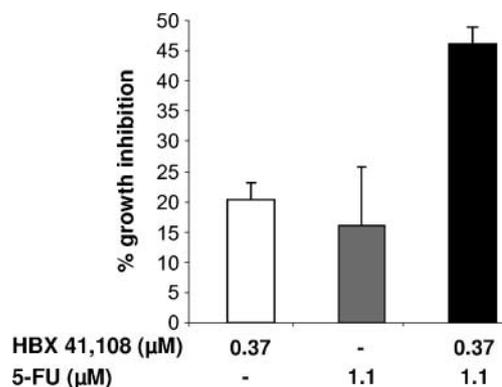


Figure 6. Cooperative effect of HBX 41,108 and 5-FU in inhibiting the growth of HCT116 cells. The IC₂₀ (corresponding to the concentration of HBX 41,108 at which 20% growth inhibition was observed) was used and the growth inhibition obtained at 72 h of drug treatment was determined using the MTS assay. Mean of triplicate experiments.

wild-type and null for p53, we showed that HBX 41,108 targeted p53-positive cells selectively for apoptosis. Thus, our results strongly suggest that the presence of a functional p53 is a dominant determinant of susceptibility to cell death induced by HBX 41,108. However, because we observed a slight poly(ADP-ribose) polymerase and caspase-3 cleavage even in the absence of p53, we cannot exclude that USP7 regulates the function of a known (PTEN) or not yet known substrate involved in p53-independent apoptosis or that HBX 41,108 has additional off-target, p53-independent proapoptotic effects.

Of importance, we observed using a cell viability assay that HCT116 colon tumor cells are more sensitive to HBX 41,108 ($IC_{50} = 0.27 \mu\text{mol/L}$) than normal diploid NIH-3T3 fibroblasts (p53 wild-type) with a 7-fold differential effect ($IC_{50} = 1.77 \mu\text{mol/L}$). In addition, HBX 41,108 did not affect the viability of normal human hepatocytes at $3 \mu\text{mol/L}$ in a MTT assay (viability: $95 \pm 3\%$ of nontreated control). Altogether, these data are in agreement with a differential effect of HBX 41,108 on tumor versus normal p53 wild-type cells and suggest that this compound would have a favorable therapeutic index.

USP7 has been shown to target other key regulatory proteins in addition to Mdm2 and p53, including histone 2B, PTEN, and FOXO4, and to be involved in epigenetic silencing and oxidative stress response pathways (17, 18, 35). There are probably other USP7 substrates not identified yet. The involvement of USP7, and probably USPs in general, in many key regulatory processes is not entirely consistent with current focus on developing highly targeted treatments. However, drugs with general effects on protein activity/fate or gene regulation may have clinically useful therapeutic indexes. This is, for example, the case for geldanamycin, which inhibits Hsp90, and proteasome inhibitors such as bortezomib or vorinostat, which targets HDAC. USP inhibitors are particularly interesting as they provide an intermediate level of "selectivity," targeting a narrower range of substrates than proteasome inhibitors, for example.

This study provides proof-of-principle that lead-like inhibitors of the USP class of proteases can be identified. Further studies are required to assess the true therapeutic potential of this approach, but the identification of HBX 41,108 represents an important first step toward the preclinical evaluation of USP inhibitors for the treatment of cancer.

Disclosure of Potential Conflicts of Interest

F. Colland, E. Formstecher, X. Jacq, C. Reverdy, C. Planquette, S. Conrath, V. Trouplin, J. Bianchi, A. Calabrese, C. Borg-Capra, V. Collura, G. Boissy, J.-C. Rain, P. Guedat, R. Delansorne, and L. Daviet: current or former employees, Hybrigenics. J. Camonis: founder, Hybrigenics. No other potential conflicts of interest were disclosed.

Acknowledgments

We thank all the staff of Hybrigenics for contributing to this study, Olivier Coux for providing the ubiquitinated p53 substrate, Bert Vogelstein for providing the HCT116 isogenic cell lines, Sandra Aresta for contribution to assay development, Matteo Colombo and Stefania Vallese (Nikem Research) for synthesizing HBX 41,108 and HBX 91,490, and François Bellamy and Donny Strosberg for many stimulating discussions.

References

- Hoeller D, Hecker CM, Dikic I. Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. *Nat Rev Cancer* 2006;6:776–88.
- Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* 2006;443:780–6.
- Gao G, Luo H. The ubiquitin-proteasome pathway in viral infections. *Can J Physiol Pharmacol* 2006;84:5–14.
- Adams J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* 2004;4:349–60.
- Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* 1999;59:2615–22.
- Nijman SM, Luna-Vargas MP, Velds A, et al. A genomic and functional inventory of deubiquitinating enzymes. *Cell* 2005;123:773–86.
- Daviet L, Colland F. Targeting ubiquitin specific proteases for drug discovery. *Biochimie* 2008;90:270–83.
- Everett RD, Meredith M, Orr A, Cross A, Kathoria M, Parkinson J. A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J* 1997;16:1519–30.
- Holowaty MN, Zeghouf M, Wu H, et al. Protein profiling with Epstein-Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7. *J Biol Chem* 2003;278:29987–94.
- Cummins JM, Rago C, Kohli M, Kinzler KW, Lengauer C, Vogelstein B. Tumour suppression: disruption of HAUSP gene stabilizes p53. *Nature* 2004;428. 1 p following 486.
- Cummins JM, Vogelstein B. HAUSP is required for p53 destabilization. *Cell Cycle* 2004;3:689–92.
- Li M, Brooks CL, Kon N, Gu W. A dynamic role of HAUSP in the p53-2 pathway. *Mol Cell* 2004;13:879–86.
- Li M, Chen D, Shiloh A, et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* 2002;416:648–53.
- Hu M, Gu L, Li M, Jeffrey PD, Gu W, Shi Y. Structural basis of competitive recognition of p53 and MDM2 by HAUSP/USP7: implications for the regulation of the p53-MDM2 pathway. *PLoS Biol* 2006;4:e27.
- Colland F. Ubiquitin and cancer: from molecular targets and mechanisms to the clinic—AACR Special Conference. *IDrugs* 2006;9:179–81.
- Becker K, Marchenko ND, Palacios G, Moll UM. A role of HAUSP in tumor suppression in a human colon carcinoma xenograft model. *Cell Cycle* 2008;7:1205–13.
- van der Horst A, de Vries-Smits AM, Brenkman AB, et al. FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Nat Cell Biol* 2006;8:1064–73.
- Song MS, Salmena L, Carracedo A, et al. The deubiquitylation and localization of PTEN are regulated by a HAUSP-PML network. *Nature* 2008;455:813–7.
- Marchenko ND, Wolff S, Erster S, Becker K, Moll UM. Monoubiquitylation promotes mitochondrial p53 translocation. *EMBO J* 2007;26:923–34.
- Meulmeester E, Maurice MM, Boutell C, et al. Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol Cell* 2005;18:565–76.
- Dang LC, Melandri FD, Stein RL. Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes. *Biochemistry* 1998;37:1868–79.
- Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67–73.
- Monge A, Arrault A, Marot C, Morin-Allory L. Managing, profiling and analyzing a library of 2.6 million compounds gathered from 32 chemical providers. *Mol Divers* 2006;10:389–403.
- Liu Y, Lashuel HA, Choi S, et al. Discovery of inhibitors that elucidate the role of UCH-L1 activity in the H1299 lung cancer cell line. *Chem Biol* 2003;10:837–46.
- Hu M, Li P, Li M, et al. Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde. *Cell* 2002;111:1041–54.

26. Berman HM, Westbrook J, Feng Z. The Protein Data Bank. *Nucleic Acids Res* 2000;28:235–42.
27. Wang R, Lu Y, Wang S. Comparative evaluation of 11 scoring functions for molecular docking. *J Med Chem* 2003;46:2287–303.
28. Moreau P, Gaillard N, Marminon C, et al. Semi-synthesis, topoisomerase I and kinases inhibitory properties, and antiproliferative activities of new rebeccamycin derivatives. *Bioorg Med Chem* 2003;11:4871–9.
29. Bunz F, Hwang PM, Torrance C, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 1999;104:263–9.
30. Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004;303:844–8.
31. Coll-Mulet L, Iglesias-Serret D, Santidrian AF, et al. MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. *Blood* 2006;107:4109–14.
32. Yang Y, Ludwig RL, Jensen JP, et al. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. *Cancer Cell* 2005;7:547–59.
33. Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell* 2006;127:1323–34.
34. Ringshausen I, O'Shea CC, Finch AJ, Swigart LB, Evan GI. Mdm2 is critically and continuously required to suppress lethal p53 activity *in vivo*. *Cancer Cell* 2006;10:501–14.
35. van der Knaap JA, Kumar BR, Moshkin YM, et al. GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7. *Mol Cell* 2005;17:695–707.

Molecular Cancer Therapeutics

Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells

Frédéric Colland, Etienne Formstecher, Xavier Jacq, et al.

Mol Cancer Ther 2009;8:2286-2295. Published OnlineFirst August 11, 2009.

Updated version Access the most recent version of this article at:
doi:[10.1158/1535-7163.MCT-09-0097](https://doi.org/10.1158/1535-7163.MCT-09-0097)

Supplementary Material Access the most recent supplemental material at:
<http://mct.aacrjournals.org/content/suppl/2009/08/19/1535-7163.MCT-09-0097.DC1>

Cited articles This article cites 34 articles, 5 of which you can access for free at:
<http://mct.aacrjournals.org/content/8/8/2286.full#ref-list-1>

Citing articles This article has been cited by 30 HighWire-hosted articles. Access the articles at:
<http://mct.aacrjournals.org/content/8/8/2286.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://mct.aacrjournals.org/content/8/8/2286>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.