P53 upregulation by USP7-engaging molecular glues

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Article

Keywords:

Posted Date: April 5th, 2023
Abstract

Molecular glues are typically small chemical molecules that act on the interface between the target protein and the degradation machinery to trigger ternary complex formation. Identification of molecular glues is challenging, and there has been a lack of target-upregulating molecular glues, which are desired for many targets such as tumor suppressor proteins (TSPs). TSPs are usually degraded by the proteasome through polyubiquitination (poly-ub) by specific E3 ligases, whereas deubiquitinases (DUBs) are capable of removing poly-ub conjugates to counteract these E3 ligases. Thus, small molecular glues that enhance the anchoring of TSPs to DUBs may stabilize them through deubiquitination. Here, through small-molecule microarray-based technology and unbiased screening, we identified three potential molecular glues that may tether P53 to the DUB USP7 and elevate the P53 level. Among them, bromocriptine (BC) is an FDA-approved drug showing the most robust effects. We further demonstrated that BC increased P53 stability via the predicted molecular glue mechanism engaging USP7. To confirm the generality of the screening platform, we identified another USP7-engaging molecular glue that upregulates PTEN, which is another well-known TSP. Taken together, we established a potential screening platform that may facilitate the discovery of novel molecular glues stabilizing TSPs via engaging the DUB USP7. Similar strategies could be applied to the identification of other types of molecular glues that may benefit drug discovery and chemical biology studies.

Introduction

Targeted protein degradation (TPD) technologies such as proteolysis-targeting chimeras (PROTACs) and lysosome-engaging degrader technologies have been booming in the last decade, leading to promising drug discovery strategies and candidate drugs that selectively degrade pathogenic proteins. Meanwhile, many diseases are caused by the loss-of-function mechanism due to the depletion or reduced activities of specific protein targets, and TPD technologies are incapable of tackling them. Instead, technologies that can selectively upregulate or restore the level or function of these targets are desired for these diseases. Gene therapy approaches over-expressing the target protein may achieve this goal, but they typically require delivery of adeno-associated viruses (AAVs), which could raise safety concerns and be prohibitively expensive. In addition, there is a limitation of the gene size that could be packaged into AAVs, precluding the expression of relatively large proteins. Thus, strategies to identify small-molecule compounds that selectively upregulate the target protein are highly desired yet largely unexplored.

A recent study reports the deubiquitinase-targeting chimeras (DUBTACs) technology stabilizes the target protein by heterobifunctional molecules consisting of a covalent DUB recruiter linked to a protein-targeting ligand. DUBs are catalytic enzymes that can specifically disassemble the ubiquitin-protein conjugates by cleaving the isopeptide bonds formed between the C-terminus of ubiquitin and the target proteins, protecting them from degradation and inactivation. DUBTACs engaging the DUB OTUB1 successfully up-regulated ΔF508-CFTR and WEE1 proteins. Meanwhile, DUBTACs are chimeric compounds that are typically large and less druglike according to Lipinski’s Rule of Five. The reported
DUBTACs covalently interact with the DUB and may have certain advantages, such as high efficiency of DUB engagement. Meanwhile, this may lead to possible limitations such as a “permanent” occupancy of the DUB that may influence the DUB's function and prevent recycling of the DUB. In comparison, molecular glues are typically small chemical molecules that act on the interface between the target protein and the degradation machinery to trigger ternary complex formation. They do not have separate chemical moieties interacting with each of the two proteins and the linker connecting the two moieties. Thus, they are usually smaller and more druglike than chimeric compounds. Therefore, molecular glues may exhibit advantages over DUBTACs in certain aspects. The targeted-protein degradation molecular glue lenalidomide has already achieved great clinical success and provided therapeutic benefits for cancer patients. Thus, the identification of small molecular glues upregulating the target protein via engaging specific DUBs (DUBEGs, for DUB-engaging glues) is highly desired to expand the landscape and possibly enhance the drug-likeness of targeted-protein upregulation compounds.

Before the publication of the DUBTAC study, we made an independent attempt to identify DUBEGs that regulate specific targets, especially tumor suppressor proteins (TSPs). TSPs regulate cell proliferation and play an essential role in preventing cancer development. Upregulating TSPs may inhibit cancer cell proliferation and the growth of tumors. TSPs are categorized into five major classes based on their mechanisms of action that are not necessarily mutually exclusive. These classes include proteins controlling the progression of cell cycle stages (e.g., pRB and p16), receptor protein or signal transducers orchestrating signals that inhibit cell proliferation (e.g., transforming growth factor (TGF)-β), checkpoint-control proteins triggering cell cycle arrest in case of DNA damage or chromosomal defects (e.g., p16, p14, and breast cancer type 1 susceptibility protein (BRCA1)), proteins inducing apoptosis (e.g., P53), and proteins involved in the repair of DNA (e.g., DNA mismatch repair protein 2 (MSH2) and P53).

Among TSPs, P53 is a promising yet challenging target for cancer treatment. P53 is a potent transcription factor that controls a major pathway protecting cells from malignant transformation, inhibiting cell proliferation, and inducing cell apoptosis mutations. Its malfunctioning mutations may contribute to ~50% of tumorigenesis, and its paramount role in tumor suppression has attracted great interest in drug development. Meanwhile, targeting P53 is extremely difficult due to at least two reasons. First, agonists of the wild-type P53 function are desired, whereas classical drug discoveries typically prefer inhibitors; second, P53 is a transcription factor, which is “undruggable” due to a lack of enzymatic activities and activity-influencing “pockets” for binding of small molecules. The P53 reactivator PC14586 exhibited encouraging effects in clinical trials, but it targets only the P53 Y220C mutant. Upregulation of the wild-type P53 may offer a more universal therapeutic strategy that inhibits cancer growth and tumorigenesis. The previously reported P53 upregulating compounds function via inhibition or lowering of its E3 ligase MDM2. Under normal conditions, the P53 level is usually kept low due to the poly-ub by the E3 ligase MDM2 or MDMX. Thus, MDM2 inhibitors such as Nutlin-3a may increase the P53 level and decrease cancer cell viability in vitro and in vivo in wild-type P53 models. Thus, the Nutlin-
3a derivative RG7388 entered clinical trials. Upregulating P53 could also be achieved by inhibition of the ubiquitin-specific protease 7 (USP7), which is a MDM2-targeting DUB that may deubiquitinate MDM2. USP7 inhibitors have been developed to upregulate P53 and degrade MDM2, without causing a feedback upregulation of MDM2. Meanwhile, E3 ligase inhibition or lowering may influence all its different substrates and increase the risk of side effects. In fact, MDM2 sustains STAT5 stability and contributes to T cell-mediated antitumor immunity, and thus MDM2 deficiency in T cells promotes cancer growth. A very recent study also attempted to upregulate P53 by DUBTACs, but it requires an additional P53-binding DNA oligo in addition to DUBTACs and the mechanism requires quaternary complex formation. Thus, small molecular compounds that upregulate P53 directly are highly desired. Beside MDM2, USP7 has also been reported as a potential P53-targeting DUB that may deubiquitinate endogenous P53. Thus, we hypothesized that DUBEGs enhancing the P53-USP7 interaction without influencing the USP7 enzymatic activity may provide a new way to upregulate P53 and provide proof-of-concept evidence for DUBEGs.

Molecular glues were identified primarily from serendipitous discoveries, and identifying them by screening approaches is emerging but still highly challenging. Notably, no previous studies have been reported to identify upregulating molecular glues. To resolve this problem, we established an unbiased screening platform that may detect potential ternary complex formation in vitro and used it to screen for potential P53 DUBEGs. We further investigated their functions and mechanisms of action. Finally, to validate the system's generality, we used it to screen for potential DUBEGs targeting another TSP, PTEN.

**Results**

**Identification of potential P53-USP7 molecular glues**

To identify compounds that may enhance P53-USP7 interaction via binding to these proteins, we stamped a small molecule microarray (SMM) consisting of 3,375 compounds in duplicates onto isocyanate-functionalized glass slides, on which small molecules were immobilized through covalent bonds. We then flew through the recombinant purified USP7 catalytic domain with the N-terminal maltose binding protein (MBP) tag (MBP-USP7CD) and MBP-P53 (Extended Data Fig. 1a-b) proteins sequentially (Fig. 1a). The binding of the flow-through protein to specific compound spots could then be detected by a label-free oblique-incidence reflectivity difference (OI-RD) microscope. By flowing through the two proteins sequentially, the potential USP7-compound-P53 ternary complex formation could be detected on the SMM (Fig. 1a). Meanwhile, compounds that interact with P53 or USP7 alone were excluded. The flowing through of MBP (Extended Data Fig. 1c) was performed as a negative control for non-specific or the MBP tag-binding compounds.

Based on the screening with two SMMs (4 repeats of each compound), we identified three compounds, bromocriptine (BC) (4L10), liothyronine (8C10), and conivaptan (7E4), that may interact with MBP-USP7 and enhance subsequent MBP-P53 tethering while exhibit no binding to MBP (Fig. 1b). Phenyltoloxamine
(5K18) also exhibited binding signals for MBP-USP7 and MBP-P53, but it is likely a false positive because it may also interact with the control protein MBP (Fig. 1b).

### Potential P53-usp7 Molecular Glues Increased P53 Levels In A Proteasome- And Usp7-Dependent Manner

We then investigated whether these potential P53-USP7 molecular glues may elevate endogenous P53 levels. All three hit compounds (4L10, 8C10, and 7E4) significantly upregulated P53 in a dose-dependent manner in HeLa cells at ~µM concentrations (Fig. 2a-c). We then discovered that these compounds interact with both P53 and USP7 as detected by Bio-Layer Interferometry (BLI) (Extended Data Fig. 2). 5K18 failed to elevate the P53 level (Fig. 2d), consistent with the speculation that it is a false positive as suggested by its possible binding to MBP (Fig. 1b). The hit compounds' 2D structures and information are presented in Fig. 2e.

We further investigated the mechanism of actions of these potential P53-USP7 molecular glues. The P53-expressing mRNA level was not influenced by the treatment of these compounds (Fig. 3a), suggesting that the effects were post-transcriptional. Meanwhile, the half-life of P53 was significantly prolonged (Fig. 3b & Extended Data Fig. 4f, measured by the decay of P53 after the cycloheximide treatment to block protein synthesis), confirming that the upregulation effects were mediated by protein stabilization. Finally, treatment of the proteasome inhibitor epoxomicin significantly blocked P53 upregulation induced by these compounds (Fig. 3c), suggesting that the effects were mediated by the proteasomal degradation of P53. Taken together, the mechanism of action of these potential P53-USP7 molecular glues is consistent with the predicted inhibition of P53’s proteasomal degradation.

We then investigated the predicted USP7-dependence of the compound-induced P53 upregulation. USP7 knockout in HeLa cells largely blocked the compounds’ effects on P53 (Fig. 3d), confirming the involvement of USP7. Expressing USP7 cDNA in the USP7 knockout HeLa cells stored compounds’ capability of upregulating P53 (Fig. 3e-g), further confirming the USP7-dependent mechanism. The baseline level of P53 was increased in the USP7 KO cells (Fig. 3d), consistent with previous reports \(^{27,28}\), although we observed variation of the P53 baseline level in different clones (not shown).

### The Potential P53-usp7 Molecular Glues Decreased Poly-ub Of P53

The P53-USP7 molecular glues are supposed to stabilize P53 by decreasing its poly-ub by tethering it to the DUB USP7. Thus, we tested the poly-ub of P53 by immunoprecipitating it in HeLa cells and detected its poly-ub by Western blots. Since the poly-ub form of P53 is rapidly degraded by the proteasome, we applied the proteasome inhibitor epoxomicin to the cells to enable its detection. As a low-abundance protein, the poly-ub signal of P53 is extremely weak. Thus, we over-expressed His-tagged ubiquitin (His-ub) in the cells to further facilitate its detection. The His-ub transfection was performed in a large plate to ensure the same His-ub transfection efficiency, and the cells were then suspended and plated into
individual wells for the compounds’ treatment. All three potential P53-USP7 molecular glues drastically reduced the poly-ub level (Fig. 3h), consistent with the predicted deubiquitylation mechanism.

**Bromocriptine (Bc) May Tether P53 To Usp7 Via Interacting With Both Proteins**

Among the three hits, BC exhibited the most potent P53 upregulation effects and is an FDA-approved drug for treating type 2 diabetes, pituitary prolactinomas, acromegaly and Parkinson's disease, and thus we focused on it for further characterization and mechanistic studies. The dose dependence of BC exhibited a “hook” effect with an optimal dose at 10 µM (Fig. 4a), suggesting that excessive BC may interact with the two proteins separately, consistent with a bifunctional mechanism. After the BC treatment at 10 µM, P53 elevation appeared at ~ 8 hours and reached a plateau at ~ 24 hours, which is much longer than the endogenous half-life of P53 (Fig. 4b), consistent with the stabilization mechanism. Besides HeLa cells, BC also increased the P53 level in SJSA-1 cells (Fig. 4c), another cancer cell line expressing wild-type P53.

BC interacts with both P53 and USP7 at ~ µM affinity as validated by BLI (Extended Data Fig. 2) and Isothermal Titration Calorimetry (ITC) (Extended Data Fig. 3a) using recombinant purified proteins (Extended Data Fig. 1b & Extended Data Fig. 1d). Thus, we further investigated their potential binding sites for BC. We performed hydrogen-deuterium exchange (HDX) analyses and revealed BC’s potential binding site at the FLQKTDPKDPAN peptide region in the USP7’s catalytic domain (Extended Data Fig. 3b-c). Such studies were technically challenging for P53, which contains flexible regions. Thus, we expressed different P53 protein fragments (Extended Data Fig. 1e) and tested their potential binding with BC, guided by computational docking analyses. We revealed that BC likely interacts with the TAD I- TAD II domain (LP47) in the N-terminus of P53 (Extended Data Fig. 3d-e). The binding sites revealed by these experiments are also consistent with the ones predicted by docking (Extended Data Fig. 3f). To further validate the potential molecular glue mechanism, we performed the pull-down experiments using recombinant purified proteins (Extended Data Fig. 1b-d) and confirmed that BC significantly enhanced the P53-USP7 interaction (Fig. 4d-e).

**Bc Did Not Influence Usp7 Enzymatic Activities**

The binding of BC to USP7 may enable BC's molecular glue's function by engaging USP7. Meanwhile, this may also lead to possible changes in the USP7’s enzymatic activity. Thus, we tested the other substrates of UPS7, DAXX and PTEN. We observed no changes in their levels (Extended Data Fig. 4a), suggesting that the USP7 activity was unchanged. We further confirmed this by the in vitro enzymatic assay measuring the enzymatic activity of USP7 and observed no changes (Extended Data Fig. 4b). The MDM2 level and activity may also influence the level of P53 and its poly-ub. We thus investigated its level and observed a significant increase rather than a decrease in the HeLa cells (Extended Data Fig. 4c). The increase of MDM2 cannot be the contributor to the increased P53 level because the direction of its
change is predicted to lower P53. The observed MDM2 elevation is likely due to the compensatory transcriptional feedback response to the P53 elevation, which has been reported previously.

**The D2-like Dopamine Receptors Are Not Involved**

BC is known as a selective D2-like dopamine receptor agonist. Meanwhile, BC is unlikely to regulate P53 through the D2-like dopamine receptors, because the observed hook effects and effective concentration range (several µM) are inconsistent with the D2-like receptor activation mechanism (EC50 ~ nM)\(^43\). To further exclude this possibility, we tested another potent D2-like dopamine receptor agonist quinpirole hydrochloride and observed no significant changes in the P53 level (Extended Data Fig. 4d), validating the D2-like dopamine receptor-independent mechanism. We also blocked D2-like dopamine receptors with trifluoperazine, and then treated the cells with BC versus the DMSO control. The P53 upregulation by BC was unaffected (Extended Data Fig. 4e), further confirming that D2-like dopamine receptors were not involved.

**The P53 Upregulation Effects Could Be Competitively Inhibited**

In contrast to quinpirole, the D2-like dopamine receptor agonist that shares similar activities with BC on the known target but not P53, a BC's structural analog BC-AN1 was capable of elevating P53 with similar efficacy to the one of BC (Fig. 5a). In comparison, we also identified another structural analog BC-AN2, which was incapable of elevating the P53 level (Fig. 5b). These structural analogs (Fig. 5c) provide chemical biology tools to further elucidate the mechanism of action of BC as a potential P53-USP7 molecular glue.

To elucidate why BC and BC-AN1 could elevate P53 whereas BC-AN2 could not, we tested their binding with P53 or USP7 by the Cell Thermal Shift Assay (CETSA) for intracellular compound-protein interactions. All three compounds obviously shifted the thermal stability of P53 but not TUBB (Extended Data Fig. 5a-c), suggesting that all compounds may interact with P53 in the cells. Meanwhile, only BC and BC-AN1 but not BC-AN2 shifted the thermal stability of USP7 (Extended Data Fig. 5a-c), suggesting that BC-AN2 may not interact with USP7 in the cells. We further confirmed these observations and measured the compound-protein affinities by real time OI-RD (Extended Data Fig. 6a-c)\(^44\). Taken together, that BC and BC-AN1 can interact with both P53 and USP7, whereas BC-AN2 only interacts with P53 but not USP7CD. These results possibly explain why only BC and BC-AN1 but not BC-AN2 could elevate the P53 level, suggesting that interacting with both proteins might be required.

Taking advantage of BC-AN2's binding to only P53 but not USP7, we performed a competition experiment to test if the inactive structural analog BC-AN2 may compete with the active ones for the binding of P53 to inhibit their P53 upregulation effects. We pre-incubated the cells with BC-AN2 at 50 µM for two hours, and then treated the cells with BC or BC-AN1 at 10 µM. In this case, BC and BC-AN1 could no longer upregulate p53 (Fig. 5d-e). The observed competition effects confirmed that BC and BC-AN1 upregulated
P53 via the molecular glue mechanism and the intracellular binding to both P53 and USP7 was required for their regulation of P53.

**Bc And Bc-an1 Activated P21 Expression And Inhibited Cancer Cell Proliferation**

p21 (WAF1/CIP1) is a major downstream factor of P53 and a potent tumor suppressor that prevents uncontrolled cell proliferation by inducing cell cycle arrest.\(^{45}\) It is an inhibitor of Cyclin-dependent kinases (CDK) and is transcriptionally activated by P53.\(^{46}\) Consistent with the observed P53 upregulation, treatment of BC significantly increased the level of p21-expressing mRNA (Fig. 6a), illustrating the potential functional impacts of these compounds via P53 upregulation.

We further investigated the compounds’ effects on the proliferation of cancer cells. BC and BC-AN1 significantly suppressed the proliferation of HeLa cells (Fig. 6b). Such effects were obviously reduced in USP7-KO HeLa cells or HeLa cells with P53 knocked down (Fig. 6c-d). The data demonstrate that the effects at the cellular level depend on the USP7-mediated P53 upregulation, confirming the functional impact of the molecular glue mechanism. The suppression of cell proliferation by BC or BC-AN1 was also observed in another cancer cell line SJSA-1, with a significant elevation of apoptosis signals as well (Fig. 6e-f).

**Discovery Of A Potential Pten-usp7 Molecular Glue Using The Same Screening Platform**

To validate the generality of our screening platform for upregulating molecular glues, we performed a similar screen aiming at identifying DUBEGs for another TSP, PTEN. Through highly similar screening procedures except replacing MBP-P53 with MBP-PTEN, we identified one possible PTEN-USP7 molecular glue, WZ8040. WZ8040 significantly upregulated PTEN in the cancer cell line DU145 at \(~\)µM concentrations (Fig. 7a) and it interacted with both PTEN and USP7 (Extended Data Fig. 7a-b). We further investigated the mechanism of action of this potential PTEN-USP7 molecular glue. Treatment of the proteasome inhibitor epoxomicin or MG132 largely blocked the PTEN upregulation induced by WZ8040. In contrast, the treatment of the autophagy inhibitor chloroquine (CQ) did not (Fig. 7b & Extended Data Fig. 7c), confirming the proteasome-dependence of the effect. Note that the caspase inhibitor Z-VAD-FMK (FMK) was added to prevent drastic cell death of DU145 cells caused by epoxomicin (Fig. 7b). USP7 knockdown in DU145 cells largely blocked the WZ8040’s effects on PTEN (Fig. 7c), confirming the USP7-dependence.

Since the WZ8040 is an inhibitor of a mutated form of EGFR, T790M, we investigated whether EGFR is involved. Knock-down of EGFR did not block the PTEN upregulation effect of WZ8040 (Extended Data Fig. 7e), suggesting that WZ8040 regulates PTEN in an EGFR-independent manner.
Treatment of WZ8040 significantly suppressed the proliferation of several cancer cell lines, including DU145, C33A, and HeC-1-a, in a dose-dependent manner (Fig. 7d-f). It also significantly elevated the apoptosis signals of DU145 cells (Extended Data Fig. 7d). The 2D structure and information of WZ8040 is presented in Fig. 7g.

Taken together, WZ8040 may function as a PTEN-USP7 molecular glue that upregulates PTEN and suppresses relevant cancer cell growth.

Discussion

Our study identified potential P53 DUBEGs through an in vitro screening platform. As the famous “guardian of the genome”, P53 is a promising drug target for cancer and other related diseases. While certain P53 upregulating compounds have been discovered and entered clinical trials, our study provides the proof-of-concept of upregulating P53 via a novel molecular glue mechanism, which may open a direction for P53-targeted drug discovery. Besides cancer, P53 also contributes to the etiology of other non-neoplastic diseases and rare hereditary diseases such as the Li–Fraumeni Syndrome, which may all benefit from new P53-targeting strategies.

While there are many P53 mutations related to tumorigenesis, we utilized the wild-type P53 protein for the screening based on several considerations. First, there are many P53 mutations associated with cancer risks and many cancer cell types carry more than one P53 mutation. Screenings using all the different P53 mutants may be inefficient. Second, while somatic TP53 mutations occur in almost every kind of cancer at a rate from 5–50%, most of the cancer cell types have at least one copy of wild-type P53. Thus, elevating the wild-type P53 level is feasible in most cases. Third, while several P53 mutations may function through a gain-of-function (GOF) mechanism, most P53 mutants still function via the loss-of-function or the dominant negative mechanisms, justifying the rationale of increasing wild-type P53. Nonetheless, it is desirable to test the compounds’ binding to different P53 mutants or to screen for mutant-specific DUBEGs in future studies. Based on our mapping of the compound’s binding site in P53, we may predict that mutations outside the LP47 region may not abolish the compound’s effects, although direct experiments are needed to confirm the results.

We chose USP7 as the potential DUB for DUBEGs mainly because it is relatively easy to purify with its catalytic domain’s crystal structure reported and it is a potential DUB for P53 deubiquitylation. USP7 inhibitors can also upregulate P53, likely via lowering MDM2. Meanwhile, the activity of USP7 may also influence many other substrates and USP7-engaging molecular glues may provide an additional strategy to upregulate P53.

The compounds that we identified likely upregulate P53 through the molecular glue mechanism. They upregulated P53 in a proteasome and USP7-dependent manner (Fig. 3c-e) and decreased the poly-ubiquitination of P53 (Fig. 3h). The top hit compound BC also enhanced the USP7-P53 interaction (Fig. 4d-e). Its P53-regulating effect is competitively blocked by an inactive structural analog that only
interacts with P53 but not USP7 (Fig. 5d), further confirming the molecular glue mechanism in cells. Consistent with this, BC seems to interact with both P53 at its TAD I- TAD II domain and USP7 at its FLQKTDPKDPAN peptide region in the catalytic domain (Extended Data Fig. 3), exhibiting a hook effect of P53 upregulation (Fig. 4a). This differs from immunomodulatory imide drugs, which are molecular glues that mainly interact with the E3 ligases but not the target proteins. The critical next step in developing this concept would be resolving the compound–P53 and compound-USP7 interaction interfaces, which may further confirm the mechanism and enable the development of potential platform technologies for targeted protein upregulation. The activity of BC and its active/inactive analogs provide some preliminary clues, but extensive medicinal chemistry and structural biology work are necessary to elucidate the answers in the future.

BC is indicated in the symptomatic treatment of idiopathic or postencephalitic Parkinson's disease, likely via its dopamine receptor agonist activity. BC may also benefit prolactin-secreting adenomas and reduce the tumor size as observed decades ago while the mechanisms were unclear. The P53 upregulating activity of BC may also contribute to tumor size reduction, although this has not been studied. The potency and efficacy of BC or BC-AN1 are still low compared to the MDM2 inhibitor that entered clinical trials (Nutlin-3a and its analogs). Extensive medicinal chemistry efforts are needed to further improve the potency and efficacy of the compounds.

While both our DUBEGs and the reported DUBTACs function through DUBs, their mechanisms are distinct. Besides having distinct chemical structures and functioning through different DUBs, the compound-protein interaction mechanisms are also different. DUBTACs interact with the DUB OTUB1 covalently, triggering persistent occupation and allosteric changes of OTUB1. While this leads to the high-affinity binding that may contribute to the substantial effect size observed, it may also lead to long-term alterations of the substrates and prevent the possible recycling of degraders inside the cells. Meanwhile, the DUBEGs we identified have weaker but more reversible interactions with the DUB, and thus likely have less influence on the DUB's substrate and allow the compounds to function in a catalytic manner. Finally, the DUBTACs are designed by assembly of different chemical moieties and are typically large, and DUBEGs could be smaller than DUBTACs.

We validated the generality of the screening platform by identifying DUBEGs of another target PTEN. Taking advantage of SMM and OI-RD, the screening system can detect potential ternary complex formation in vitro (Fig. 1a), which is critical to identify molecular glue that induce or enhance the ternary complex formation. We realize the potential caveat that the system may capture false positives due to the in vitro setting, but this could be addressed by cellular experiments including the CETSA assay that we performed to validate the binding (Extended Data Fig. 5). In addition, a strong in vitro baseline binding of the target and DUB without compounds’ treatment may also lead to false positives. In this case, almost all the positive compounds showing signals when flowing through the first protein will also exhibit signals when flowing through second protein, which may raise alerts of the screening results and more detailed measurement of compound-induced affinity changes could be performed to address this.
In summary, we have identified DUBEGs capable of stabilizing P53 or PTEN through engaging USP7 and established a screening platform that could be utilized to screen for DUBEGs or potentially other types of molecular glues for disease-relevant proteins.

Declarations

Acknowledgements: We’d like to thank Jingwen Li and Xiaoxu Tian (the staff members of the Large-scale Protein Preparation System at the National Facility for Protein Science in Shanghai (NFPS), Shanghai Advanced Research Institute, Chinese Academy of Sciences, China) for providing technical support and assistance in data collection and analysis.

Funding: We thank the following for funding support: National Natural Science Foundation of China (82050008, 92049301, 81925012, 32200797, 32271510, 32200602), the Science and Technology Commission of Shanghai Municipality (Grant 20JC1410900), the Innovation Program of Shanghai Municipal Education Commission (2021-01-07-00-07-E00074), the Shanghai Municipal Science and Technology Major Project (Grant No. 2018SHZDZX01) and ZJLab, and the China Postdoctoral Science Foundation (BX20200093 and 2021M690038).

Author contributions: B.L. perceived the idea and supervised the project; Y.D. and Y.F. co-supervised the project; Z.L. designed, performed, and analyzed most of the cellular and biochemical experiments on P53 studies; Z.W. designed, performed, and analyzed most of the protein purification and compound-protein interaction measurement experiments; P.A. designed, performed, and analyzed most of PTEN-related experiments; H.Z. and Y.F. performed the primary screens; C.Z., R.L., Z.M., J.L., C.P. and D.X. performed other essential experiments.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Identification of potential P53-USP7 molecular glues by in vitro screenings. a, The schematic illustration of the screening design. The SMM was stamped in duplicates onto isocyanate-functionalized glass slides. OI-RD screenings were performed by flowing through the indicated recombinant purified proteins, including MBP, MBP-USP7 catalytic domain (MBP-USP7CD), and MBP-P53. Flowing through of the BSA-containing buffer was applied before and after flowing through of each tested protein to wash out non-specific binding. The catalytic domain of USP7 was used to facilitate the protein purification and enable direct engagement of the domain by potential molecular glues. The compounds binding to each protein
could be identified in the difference images obtained by subtracting the image before flowing through from one after flowing through, as illustrated by the bright circles. Compounds that exhibit positive signals in both difference images of MBP-USP7CD and MBP-P53 but not MBP were selected as hits. b, The representative screening results (from two replicated SMM chips) showing the three positive hits (in solid red rectangles) and one false positive hit that also binds with MBP (in the dashed red rectangle). The compounds were annotated by their positions in the microplates used to print SMM.

![Figure 2](image)

**Figure 2**

The potential P53 DUBEGs upregulated P53 in HeLa cells. a-d, Representative Western blots and quantification of P53 levels in HeLa cells treated with the indicated compounds. e, the 2D compound structures of the three hit compounds. For all panels, n indicates the number of independently plated wells from different batches; data are mean ± s.e.m., analyzed by one-way ANOVA and Dunnett’s post hoc tests; ****p<0.0001. Statistical parameters are shown in the figure.
**Figure 3**

Upr egulation of P53 by potential DUBEGs is dependent on USP7 and the proteasome.

a, qPCR measurements of the P53mRNA levels in the HeLa cells treated with DMSO or the indicated compounds. The two-tailed unpaired t test. b, Representative Western blots and quantifications of P53 degradation in HeLa cells upon treatment of the indicated compounds or the DMSO control. 10 μg/mL
cycloheximide (CHX) was applied to block protein synthesis for the measurement of protein degradation. Two-Way ANOVA. c, Representative Western blots and quantifications of P53 levels in compound-treated HeLa cells co-treated with or without the proteasome inhibitor epoxomicin. One-Way ANOVA with Dunnett’s post hoc tests. d, Representative Western blots and quantifications of compound-treated USP7 knockout (KO) HeLa cells versus the parental HeLa cells. One-Way ANOVA with Dunnett’s post hoc tests. e, Representative IP-Western blots (from 3) showing that the potential P53 DUBs (BC\8C10\7E4) decreased the poly-ubiquitination (poly-ub) level of P53. Knock-down of MDM2 (si:\_MDM2) was used as a control confirming the specificity of the poly-ub signals. f, Representative Western blots and quantifications of P53 levels in USP7 KO HeLa cells transfected with the indicated cDNA plasmids. USP7 KO abolished BC’s effects on P53 whereas the expression of USP7 restored the effects. One-Way ANOVA with Bonferroni’s post hoc tests. g, Similar to f, but using compound 8C10. h, Similar to f, but using compound 7E4. For all panels, n indicates the number of independently plated wells from different batches; data are mean ± s.e.m. Statistical parameters are shown in the figure.

**Figure 4**
BC may tether P53 to USP7 to stabilize it.

a, Representative Western blot and quantifications of BC-treated HeLa cells showing the dose-dependent “hook” effects. One-Way ANOVA with Dunnett’s post hoc tests. b, Representative Western blots and quantifications of P53 levels in HeLa cells treated with 10 μM BC for the indicated length of time. The effect appeared at 8 hours and peaked at 24 hours. One-Way ANOVA with Dunnett’s post hoc tests; ****p<0.0001. c, similar to a, but in SJSA-1 cells. d, Representative co-immunoprecipitation experiments (from 3) showing that 10 μM BC obviously enhanced P53-USP7 interactions in HeLa cells. e, Representative in vitro pull-down experiments (from 3) showing that 10 μM BC obviously enhanced P53-USP7 interactions in vitro. For all panels, n indicates the number of independently plated wells from different batches; data are mean ± s.e.m.
Figure 5

BC's analogs could competitively block BC's effects on P53.

a-b, Representative Western blots and quantifications of P53 level in HeLa cells treated with the indicated compounds. One-Way ANOVA with Dunnett's post hoc tests; ****p<0.0001. BC-AN1 was also able to upregulate P53 whereas BC-AN2 was not. Both BC-AN1 and BC-AN2 are BC analogs.

c. 2D structures of BC-AN1 and BC-AN2.
BC-AN1 and BC-AN2. d-e, Representative Western blots and quantifications of P53 levels in HeLa cells treated with the indicated compounds for the competitive experiment design. Pretreatment of the inactive analog BC-AN2 that only interacts with P53 but not USP7 in the cells (Extended Data Fig. 5) blocked the P53 upregulation effects of P53 by BC or BC-AN1. One Way ANOVA with Dunnett’s post; ****p<0.0001. For all panels, n indicates the number of independently plated wells from different batches; data are mean ± s.e.m.
Figure 6

BC treatment suppressed cancer cell proliferation.

a, qPCR measurements of the *P21* mRNA levels in the HeLa cells treated with DMSO or 10 μM BC. The *P21* mRNA levels were first corrected by the housekeeping gene *ACTB* and then normalized to the average of the DMSO control group. b, Representative live cell phase-contrast images and quantifications of the confluence of HeLa cells showing that 10 μM BC or BC-AN1 treatment drastically inhibited cell proliferation. Images and analyses were performed by InCucyte. Two-way ANOVA with Dunnett’s post hoc tests; ****p<0.0001. c-d, similar to b, but in USP7 KO HeLa cells (c) or HeLa cells with P53 knocked down (d, with representative Western blots validating the knock-down). Ten μM BC still suppressed cell proliferation, but the effect sizes were obviously smaller. e-f, similar to b, but in SJSA-1 cells and with the fluorescent dyes detecting apoptotic cells. The green dye detects caspase-3 activity (e) and propidium iodide (3 μM) is only permeant to dead cells and detects their double-stranded DNA (f). Two-way ANOVA, ****p<0.0001. For all panels, n indicates the number of independently plated wells from different batches; data are mean ± s.e.m.
Figure 7

WZ8040 is a potential PTEN DUBEG identified from a similar screening platform.

a, Representative Western blots and quantifications of DU145 cells treated with WZ8040 at the indicated concentrations. One-way ANOVA. ****p<0.0001. b, similar to a, but with co-treatment of the caspase inhibitor Z-VD-FMK (FMK, 20 μM) or FMK plus the proteasome inhibitor epoxomicin (100 nM). FMK was
applied to prevent the severe cell death caused by epoxomicin that may influence PTEN measurements. c, similar to a, but in DU145 cells transfected with USP7 siRNA versus the non-targeting control siRNA (Neg siRNA). Two-tailed unpaired t-tests. d, live cell phase-contrast images and quantifications of the confluence of DU145 cells showing that WZ8040 inhibited cancer cell proliferation. Two-way ANOVA with Dunnett’s post hoc tests; ****p<0.0001. e-f, similar to d, but in Hec-1-a cells or C33A cells. g, the 2D compound structures of WZ8040. For all panels, n indicates the number of independently plated wells from different batches; data are mean ± s.e.m.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Figure2data.xlsx
- Figure3data.xlsx
- Figure4data.xlsx
- Figure5data.xlsx
- Figure6data.xlsx
- Figure7data.xlsx
- ExtendedDataFigure2data.xls
- ExtendedDataFigure3data.xls
- ExtendedDataFigure4data.xlsx
- ExtendedDataFigure5data.xlsx
- ExtendedDataFigure6data.xlsx
- ExtendedDataFigure7data.xlsx
- SupplementaryMaterials.docx