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# MDM2-Recruiting PROTAC Offers Superior, Synergistic Antiproliferative Activity via Simultaneous Degradation of BRD4 and Stabilization of p53

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## Abstract

Although the number of proteins effectively targeted for posttranslational degradation by PROTAC has grown steadily, the number of E3 ligases successfully exploited to accomplish this has been limited to the few for which small-molecule ligands have been discovered. Although the E3 ligase MDM2 is bound by the nutlin class of small-molecule ligands, there are few nutlin-based PROTAC. Because a nutlin-based PROTAC should both knockdown its target protein and upregulate the tumor suppressor p53, we examined the ability of such a PROTAC to decrease cancer cell viability. A nutlin-based, BRD4-degrading PROTAC, A1874, was able to degrade its target protein by 98% with nanomolar potency. Given the complementary ability of A1874 to stabilize p53, we discovered that the nutlin-based PROTAC was more effective in inhibiting proliferation of many cancer cell lines with wild-type p53 than was a corresponding VHL-utilizing PROTAC with similar potency and efficacy to degrade BRD4. This is the first report of a PROTAC in which the E3 ligase ligand and targeting warhead combine to exert a synergistic antiproliferative effect. Our study highlights the untapped potential that may be unlocked by expanding the repertoire of E3 ligases that can be recruited by PROTAC.

**Significance:** These findings present the first BRD4-targeting MDM2-based PROTAC that possesses potent, distinct, and synergistic biological activities associated with both ends of this heterobifunctional molecule.

## Introduction

Recent years have seen posttranslational protein degradation harnessed to target and eliminate many cellular proteins of interest to both basic researchers and clinicians. This has been accomplished through development of a variety of heterobifunctional molecules that tether together a ligand that binds the protein of interest with another ligand that engages an E3 ubiquitin ligase (1–3). Upon introduction into living cells, these chimeric molecules facilitate complex formation of the protein of interest with an E3 ligase, resulting in ubiquitination of the former and its subsequent degradation by the 26S proteasome: hence, these heterobifunctional molecules are called "proteolysis-targeting chimeras" or "PROTACs" (4, 5).

From a basic research standpoint, PROTACs serve as an effective tool enabling small-molecule–based target protein knockdown that is more tunable and reversible than some modalities (e.g., RNAi or genetic knockout). Moreover, unlike other small-molecule-based strategies for regulating protein levels posttranslationally (6, 7), PROTACs require no genetic modification of the cells. From a clinical standpoint, PROTACs permit the engagement and elimination of both established drug target proteins as well as the so-called "undruggable" proteome. Because a PROTAC can bind to any available surface feature of its protein target to mediate its dimerization with an E3 ligase and the resultant ubiquitination (8), PROTACs are not restricted to engaging their intended target proteins only at their cognate active sites. This is an advantage for PROTAC therapeutics because many disease-related proteins (scaffolding proteins, transcription factors, etc.) lack a tractable active site through which a conventional small-molecule inhibitor could work—hence the designation of such disease-related proteins as traditionally "undruggable" (9). Furthermore, because transient interaction with the target protein is sufficient for a PROTAC to commit it for degradation, PROTACs can act catalytically and be effective at lower concentrations than those necessary for the sustained, maximum target occupancy that traditional small-molecule inhibitors require to be effective (10, 11). Thus, in both contexts—the research lab and the clinic—PROTACs have received recent attention, leading to the generation of PROTACs that degrade cyclin-dependent kinases (12), lipid kinases (13), peptidases (4), protein isomerases (5), scaffolding proteins (13), transcription factors (3, 11, 14), protein kinases (1, 10, 15–18), and epigenetic regulating proteins (2, 19).

Beyond expanding the repertoire of proteins successfully degraded, research also led to a better understanding of the finer mechanistic details concerning how PROTACs work and expanding the mechanistic paradigm itself. It is now known that the most effective PROTACs facilitate the formation of protein–protein

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interactions between target protein and E3 ligase (20). The importance of these cooperative interactions is underscored by the observation that PROTACs with only modest target affinity can nonetheless induce potent target degradation (18). Moreover, design variations led to the creation of PROTACs that bind irreversibly to either of its recruited proteins via an orthogonal ligand (21, 22), such that target degradation then depends on only a single binding event to permit an even higher degree of targeting efficiency as well as specificity. Other structural refinements led to the development of PROTACs whose target degradation is conditional on the activation of specific signaling pathways in the cell (13), as well as PROTACs that cause the degradation of the harnessed E3 ligase itself (23).

Paradoxically, while the scope of targeted proteins and refinement of PROTAC functionality has been wide-ranging, the selection of E3 ligases through which PROTACs induce target ubiquitination has remained limited. Although 600 E3 ligases are encoded by the human genome (24), only three have been routinely used for PROTAC-mediated target ubiquitination: von Hippel–Lindau, or VHL; cereblon; and the "inhibitor of apoptosis protein," or IAP. Although the first PROTAC (4) worked through recruitment of the E3 ligase, ß-TRCP, it did so by utilization of a phosphopeptide as the E3-binding component and as such was not cell permeable nor projected to be stable *in vivo*. A recent study proposes that, despite the past emphasis on VHL, cereblon, and IAP, many other E3 ligases should be amenable for utilization by PROTACs, including the already mentioned ß-TRCP, as well as parkin and Siah1 (22). The major hurdle for exploiting these E3 ligases is the lack of corresponding small-molecule ligands for them that could be incorporated into the PROTAC structure.

In 2008, our group published the first all-small molecule PROTAC, which degrades the androgen receptor through its recruitment to the E3 ligase, MDM2 (25). Previous PROTACs, including the ones that worked through VHL recruitment, had used peptide sequences to deliver their target proteins to an E3 ligase for their ubiquitination. By coupling the small-molecule MDM2 inhibitor, nutlin 3, with the androgen receptor antagonist hydroxyflutamide, an entirely non-peptidic entity was created that induced the degradation of the androgen receptor, albeit at micromolar concentrations. Further inquiry into the use of MDM2 has lagged, in part due to the generation of VHL-binding small molecules (26), the discovery that phthalmide-based ligands (e.g., thalidomide) bind to cereblon (27), and the availability of bestatin methyl ester and other ligands for IAP (28, 29). These molecules have been used to create all-small molecule PROTACs that induce target degradation at submicromolar and even subnanomolar concentrations. However, in light of the potential wide-ranging amenability of E3 ligases for repurposing into resources for PROTAC-mediated protein degradation, we reasoned that renewed effort concerning nutlin-based PROTACs was worthwhile considering that, among the E3 ligases currently used for PROTACs, MDM2 stands out in that its endogenous substrate, the tumor suppressor p53, plays a crucial tumor suppressor role. Indeed, the ability to manipulate p53 levels using nutlins holds therapeutic promise in the field for cancer treatment (30, 31).

In this study, we present evidence showing not only that MDM2 is indeed amenable to being used for nanomolar-potency PROTAC-mediated target degradation, but moreover a nutlin-based PROTAC retains the p53-stabilizing activity of the parent molecule allowing an MDM2-recruiting PROTAC to be more active against certain cancers than a counterpart VHL-utilizing PROTAC directed against the same target protein.

## Methods and Materials

### Chemical syntheses

See Supplementary Data for synthetic schemes and validations of PROTACs A1874, A1875, and A743.

### Reagents

Antibodies against BRD4 (cat. no. 13440), GAPDH (cat. no. 2118), and $p21^{CIP1/WAF1}$ (cat. no. 2947) were purchased from Cell Signaling Technology. Antibody recognizing c-Myc (cat. no. ab32072) was from Abcam and antibody for p53 (cat. no. OP43) was obtained from EMD Millipore. HRP-linked anti-mouse (cat. no. NA931V) and anti-rabbit (cat. no. NA934V) secondary antibodies were from GE Life Sciences. JQ1 (cat. no. HY-13030) and idasanutlin (cat. no. HY-15676) were obtained from MedChem Express, and all other reagents were procured from Sigma unless otherwise specified.

### Cell lines

HT29 colon cancer cells, NCI-H2030 lung cancer cells, Daudi cells, and SJSA1 osteosarcoma cells were purchased from ATCC. A375 cells were a gift from Neal Rosen (Memorial Sloan Kettering Cancer Center, New York, NY) and HCT116 cell lines were donated by Gary Kupfer (Yale University, New Haven, CT). MOLM-13 cells were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. NCI-H2030, SJSA1, MOLM-13, and Daudi cells were cultured in RPMI medium 1640 from ATCC, HT29 cells were grown in McCoy's 5A medium, and A375 and HCT116 cells were grown in DMEM from Gibco. All media were supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries) and 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco). Multiple early-passage frozen stocks of each cell line in this study were stored under liquid nitrogen: all cell lines were used in experimentation for no longer than 10 passages from thaw. *Mycoplasma* testing of all cultured cell lines was performed every 3 weeks using the MycoAlert detection kit from Lonza. Cultures that yielded a test ratio score of <1 were considered negative for *Mycoplasma* exposure; a test ratio score of 1 to 2 indicated an early-stage *Mycoplasma* exposure and use of the culture was discontinued; cultures that yielded a test ratio score of > 2 were designated as testing positive for significant *Mycoplasma* contamination, and affected experiments were repeated using *Mycoplasma*-negative cultures.

### Immunoblotting

Cultured cells were incubated in the presence of the PROTACs or component ligands for 24 hours, after which, they were rinsed once with ice-cold PBS and harvested in buffer containing 25 mmol/L Tris HCl pH 7.4, 1% Nonidet P-40, 0.25% deoxycholic acid and supplemented with 10 μg/mL pepstatin A, 10 μg/mL leupeptin, 30 μg/mL bestatin, and 0.3 TIU/mL aprotinin. Lysis proceeded on ice for 15 minutes with occasional vortexing, followed by centrifugation at 16,000 × *g* for 15 minutes. Supernatant was removed and subjected to protein SDS polyacrylamide gel electrophoresis (Tris-glycine buffer), followed by electrophoretic transfer to nitrocellulose membrane.

Membranes were probed for the proteins indicated and visualized using a ChemiDoc MP Imaging System. Protein band intensity on immunoblots was quantitated using Image Lab software v5.2.1 (Bio-Rad Labs) and graphed using Prism v7.0 (GraphPad Software).

**Cell viability assay**

Cells were seeded into 96-well plates and incubated with the indicated concentrations of the PROTACs or component ligands for 48 hours, at which time MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; Promega Corp.] and PMS (phenazine methosulfate; Sigma] were added to a final concentration of 330 μg/mL and 25 μmol/L, respectively. Viable cells converted the MTS to its colored formazan derivative, which was quantitated by measuring absorbance at 490 nm using a Victor$^2$ plate reader (PerkinElmer). Data were graphed and analyzed using Prism v7.0 software (GraphPad Software). The combination index (CI) value to determine whether the observed activity of A1874 ($E_A$) at each treatment concentration was synergistic compared with the calculated additive effect of JQ1 ($E_J$) and idasanutlin ($E_N$) was determined using the Bliss independence model equation: $CI = (E_J + E_N - E_J E_N)/E_A$, as previously described in ref. 32.

## Results

We chose the bromodomain-containing protein BRD4 for our degradation target in this study for practical reasons: due to its role in driving hematologic and solid tumors (33, 34), BRD4 has already been successfully targeted for degradation by PROTAC recruitment of either cereblon (2, 19) or VHL (20, 35), providing a solid foundation from which to begin our investigation. In order to create a PROTAC capable of degrading BRD4 by recruiting it to MDM2, we elected to tether the BET ("bromodomain and extra-terminal") inhibitor JQ1 (Fig. 1A; ref. 36) from its diazepine ring to the methoxyphenyl group of the MDM2 inhibitor, idasanutlin (Fig. 1B; ref. 37), by way of a 13-atom long PEG-based linker. The full synthesis protocol and scheme for the resultant PROTAC, A1874 (Fig. 1C), are included in the Supplementary Information.

Once synthesized, the ability of the new PROTAC to induce BRD4 degradation in cells was evaluated by immunoblotting (Fig. 2A). The colon cancer cell line HCT116 was selected for the initial evaluation of A1874 given the reported importance of BRD4/c-Myc signaling in colon cancer (33, 38) and the wild-type status of p53 of this cell line. Treatment of HCT116 cells for 24 hours with increasing concentrations of A1874 induced a dose-dependent knockdown of BRD4 levels, with near-maximum knockdown by 100 nmol/L and a maximum degradation ($D_{max}$) of BRD4 of 98% of the levels in control (0.1% DMSO-treated) cells. By contrast, treatment with equivalent concentrations of unmodified inhibitor JQ1 did not cause BRD4 loss—indeed, at higher concentrations of JQ1, there appears to be compensatory BRD4 upregulation (Fig. 2B). There was no detectable rebounding of BRD4 expression levels at higher PROTAC concentrations (up to 10 μmol/L), which contrasts with other PROTACs in which we and others have reported such a "hook effect" (1, 21, 39). It is worth highlighting that the extent of target protein knockdown and potency ($DC_{50}$ = 32 nmol/L) are substantially improved compared with our previous report on nutlin-based PROTACs (25), in which 10 μmol/L was needed to produce ∼50% target knockdown. Thus, A1874 is a much improved nutlin-based protein degrader, approaching the activity of our other reported PROTACs that work through recruitment of the more frequently-used E3 ligases, cereblon and VHL.

To determine whether BRD4 knockdown by A1874 has a functional impact on downstream signaling, c-Myc expression was measured by immunoblotting (Fig. 2A). c-Myc, a transcription factor that drives cell proliferation, is itself dependent on functional BRD4 for its own expression (40, 41); thus, BRD4 knockdown is predicted to reduce c-Myc expression. In response to treatment with A1874, c-Myc expression is reduced by 85% relative to control HCT116 cells, which is greater than the 70%

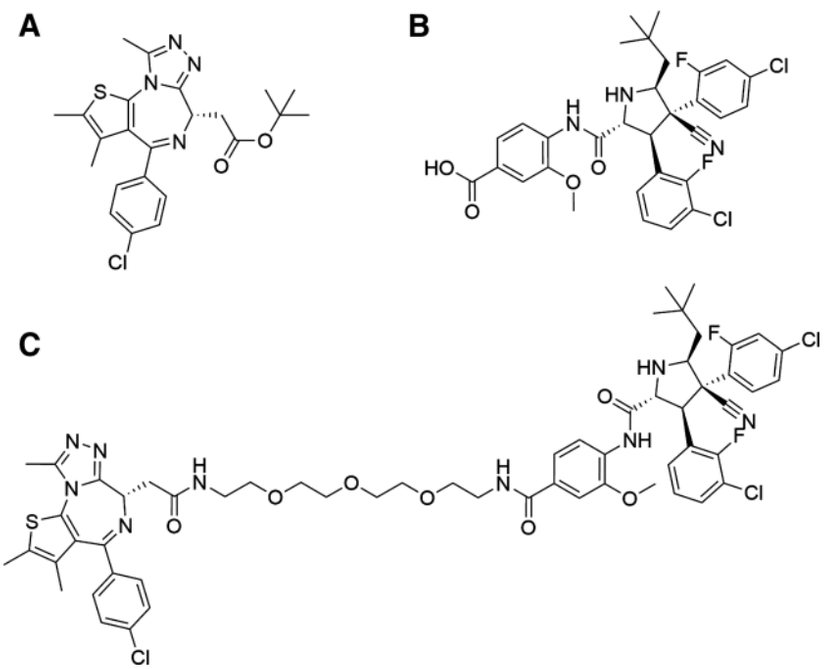


**Figure 1.**
Schematic of nutlin-based PROTAC and its component ligands. **A,** Structure of the BRD4/BET inhibitor, JQ1. **B,** structure of idasanutlin (RG7388), an MDM2 antagonist. **C,** structure of A1874, an MDM2-recruiting, BRD4-degrading PROTAC.

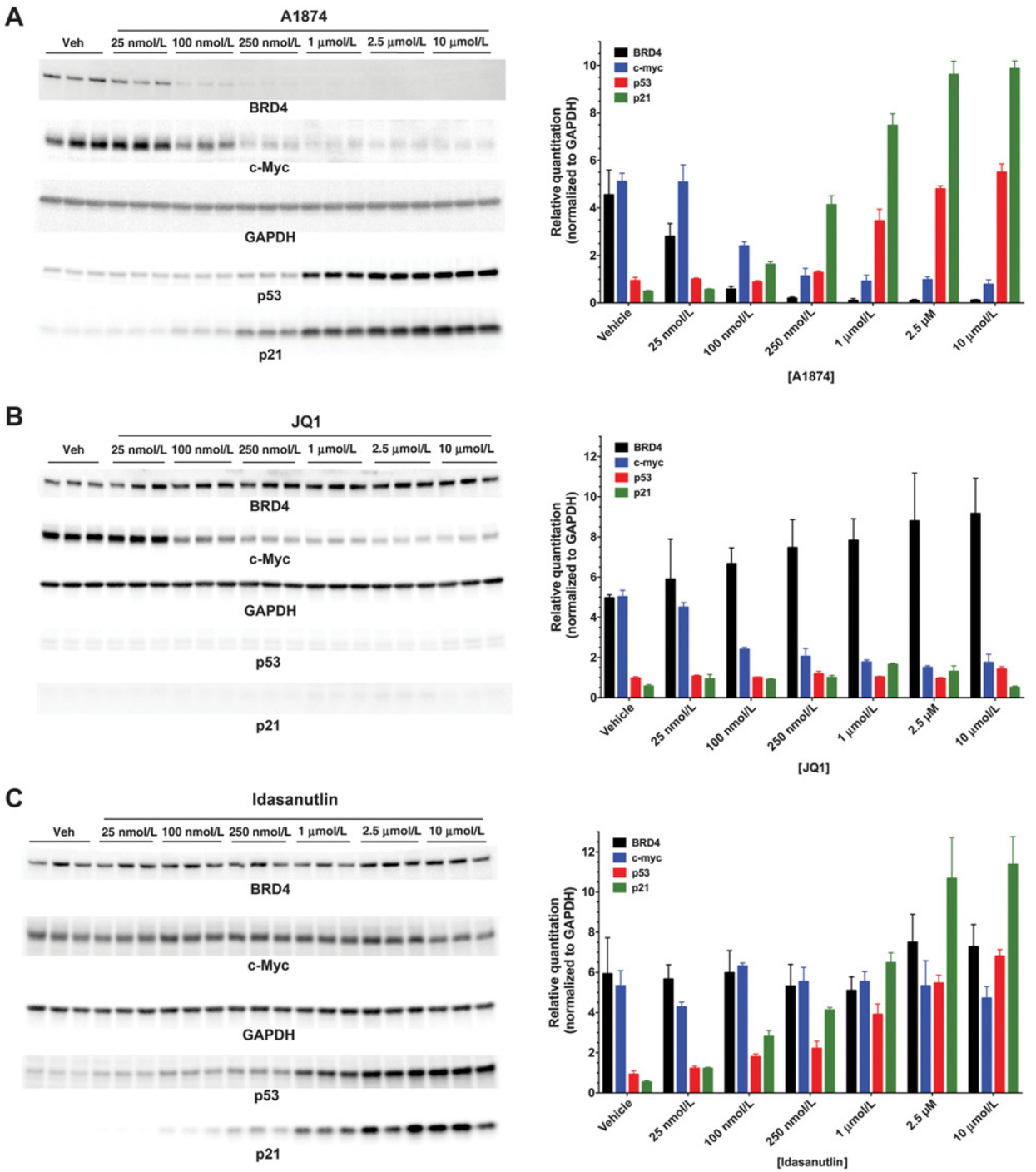


**Figure 2.**

A1874 combines the biochemical activities of both JQ1 and idasanutlin into a single molecule. **A,** Representative immunoblots from HCT116 cells treated with increasing concentrations of A1874 (left) and quantified results (right). **B,** Representative immunoblots from HCT116 cells treated with increasing concentrations of JQ1 (left) and quantified results (right). **C,** Representative immunoblots from HCT116 cells treated with increasing concentrations of idasanutlin (left) and quantified results (right).

suppression observed in response to JQ1 itself (Fig. 2B) as well as consistent with observations with other BRD4-targeting PROTACs (2, 19, 35). Thus, despite the limited degradation demonstrated by the first all-small molecule PROTAC (25), we show here that using MDM2 to ubiquitinate target proteins for proteasomal degradation can be an effective PROTAC strategy.

In addition to enabling engagement of MDM2 for the purpose of target ubiquitination, we hypothesized that idasanutlin incorporation into the PROTAC could also have an additional, potentially complementary biological activity: the stabilization of the tumor suppressor p53. The natural protein target of MDM2 is p53, a transcription factor that causes cell-cycle arrest and/or activates apoptotic signaling in cells that have damaged DNA. Although p53 is constitutively synthesized, it is normally present only at low levels in normal cells because it is also constitutively degraded via the action of MDM2 and the ubiquitin-proteasome system. However, DNA damage, hypoxia, and activation of oncogenes, which could result in transformation into a cancerous phenotype, cause disruption of the interaction between p53 and MDM2. This disruption prevents degradation of the former and permits its level to rise, triggering the aforementioned tumor suppressing actions. Immunoblotting for p53 levels in the HCT116 cells as treatment concentration with A1874 increased showed dose-dependent p53 stabilization (Fig. 2A), up to 5.9-fold over steady-state levels. The p53 increase was slightly less than the 7.2-fold increase seen upon treatment with unmodified idasanutlin (Fig. 2C). On the other hand, this p53 upregulation is not detected in HCT116 cells treated with JQ1 (Fig. 2B), just as BRD4 knockdown and c-Myc suppression are not observed in cells treated with idasanutlin (Fig. 2C). Only the PROTAC itself combines these activities into a single agent. Stabilization of p53 by A1874 was sufficient to upregulate the levels of its well-characterized effector protein $p21^{CIP1/WAF1}$ (Fig. 2A). It is noteworthy that significant amounts of $p21^{CIP1/WAF1}$ are induced at 250 nmol/L of A1874, a concentration causing only small increases in p53 levels, reflecting the amplification often associated as a signal is transduced downstream. Thus, from the perspective of downstream effectors, the effective dose ranges of the two antimitogenic mechanisms of A1874 (c-Myc suppression and p21 induction) begin to overlap, allowing for the possibility of a combined biological effect even in the nanomolar concentration range.

Suppression of c-Myc or induction of $p21^{CIP1/WAF1}$—either one alone—will inhibit cell proliferation. HCT116 cells incubated with JQ1 or idasanutlin for 48 hours showed clear dose-dependent loss of viability (Fig. 3A). Treatment with JQ1 resulted in 25% loss of MTS signal compared with control cells; and idasanutlin treatment caused a 62% loss. However, treatment with A1874, which combines the activities of the two inhibitors, ultimately resulted in a 97% loss in HCT116 cell viability. That the effect of A1874 was greater than either idasanutlin or JQ1 alone, and even slightly more effective than a combined treatment (Supplementary Fig. S1) demonstrates that combining both activities into a single PROTAC does not result in one activity diminishing the other. In fact, the evidence demonstrates the opposite—that their combined effect in A1874 can be "synergistic": were the two activities in A1874 merely additive, their predicted combined effect would be an 87% reduction (the sum of the individual effects of JQ1 and idasanutlin). That the actual loss of HCT116 viability is greater than that suggests a synergistic antiproliferative effect of the PROTAC; and indeed, subjecting the viability losses to Bliss independence analysis of drug effects interactions (32) determined that at concentrations of 100 nmol/L or greater, the antiproliferative effects of the two activities of A1874 are synergistic (Bliss independence CI values: <1; Table 1). This reflects the observation that 100 nmol/L is the lowest concentration at which A1874 begins to affect both c-Myc and $p21^{CIP1/WAF1}$. To confirm this finding, A1874 was applied to a different cell line: A375 melanoma cells (Fig. 3B). Similar to the HCT116 cells, the 98% loss of cell viability when A375 cells are treated with A1874 is greater than the sum of the effects of JQ1 alone and idasanutlin alone (15% loss and 64% loss, respectively). Analysis showed that in A375 cells, the activities of A1874 synergize at concentrations of 250 nmol/L or greater (Supplementary Table S1). Thus, the superior anticancer activity of A1874 is not unique to a single-cell line.

Although A375 and HCT116 cells have different oncogenic driving mutations, they both express wild-type p53—given the targeted protein level changes caused by A1874 treatment, this would appear to be a key element for its maximum effectiveness. To investigate whether the maximum effect of A1874 requires wild-type p53, the treatments were performed in two other cell lines: NCI-H2030 lung cancer cells and HT-29 colon cancer cells. These cell lines were chosen because (i) they both possess p53 mutations that render it inactive and (ii) their driving oncogenic mutations match those of the previously tested A1874-sensitive cell lines. More specifically, HT-29 cells possess the same B-Raf–activating V600E mutation as A375 cells, whereas NCI-H2030 cells and HCT116 cells both possess constitutively active K-Ras. However, when treated with up to 1 μmol/L A1874, the NCI-H2030 and HT-29 cells exhibited nominal loss of viability (Fig. 3C and D, respectively). Only at 10 μmol/L A1874, a concentration that is far above that necessary to maximally decrease viability in the wild-type p53 cells, was there limited reduction (22.4%) in viability, and then in only the NCI-H2030 cells. In fact, the mutant B-Raf–expressing cells seemed to be affected less potently than the mutated K-Ras–expressing cells regardless of p53 status. To confirm that the p53-null status underlies the loss of effectiveness of A1874, we tested the PROTAC's activity against an isogenic, p53-deficient version of the HCT116 cells and observed diminished loss of viability (Fig. 3E). The effectiveness of A1874 in this cell line was similar to that of unmodified JQ1, consistent with the predicted absence of p53 signaling in these cells (corroborated by their insensitivity to idasanutlin). In this cellular context, the PROTAC has been reduced to having a single biological activity—targeting BRD4 to suppress c-Myc levels. These data clearly indicate that the maximum activity of A1874 depends on the cells ability to effectively mobilize p53 in response to the idasanutlin moiety.

Further support that MDM2 recruitment is crucial for the PROTAC's pronounced biological activity was provided by testing A1875, an inactive version of the PROTAC with identical physicochemical properties but discrete stereochemical alterations in the idasanutlin moiety that diminish its MDM2 binding (Fig. 4A and Supplementary Information). This reduces its activity both to degrade BRD4 and to stabilize p53 (Fig. 4B and C). Another approach to the evaluation of nutlins as E3 ligase-targeting ligands would be comparing the activity of A1874 against a PROTAC that degrades BRD4 with similar $DC_{50}$ and suppresses c-Myc with a similar efficacy, but that works through a different E3 ligase. A743 is an analogous BRD4 degrader, but it incorporates a VHL-binding ligand (Fig. 4D and Supplementary Information) rather than idasanutlin. As constructed, A743 degrades BRD4 with similar potency ($DC_{50} = 23.1$ nmol/L) and efficacy ($D_{max} = 89\%$) as A1874 (Fig. 4E and F). Like some PROTACs, A743 demonstrates a "hook effect" at concentrations $\geq 2.5$ μmol/L; however, the hook effect on BRD4 degradation at higher concentrations does not influence the downstream effect of A743 because it suppresses c-Myc levels by 84%—nearly equal to A1874.

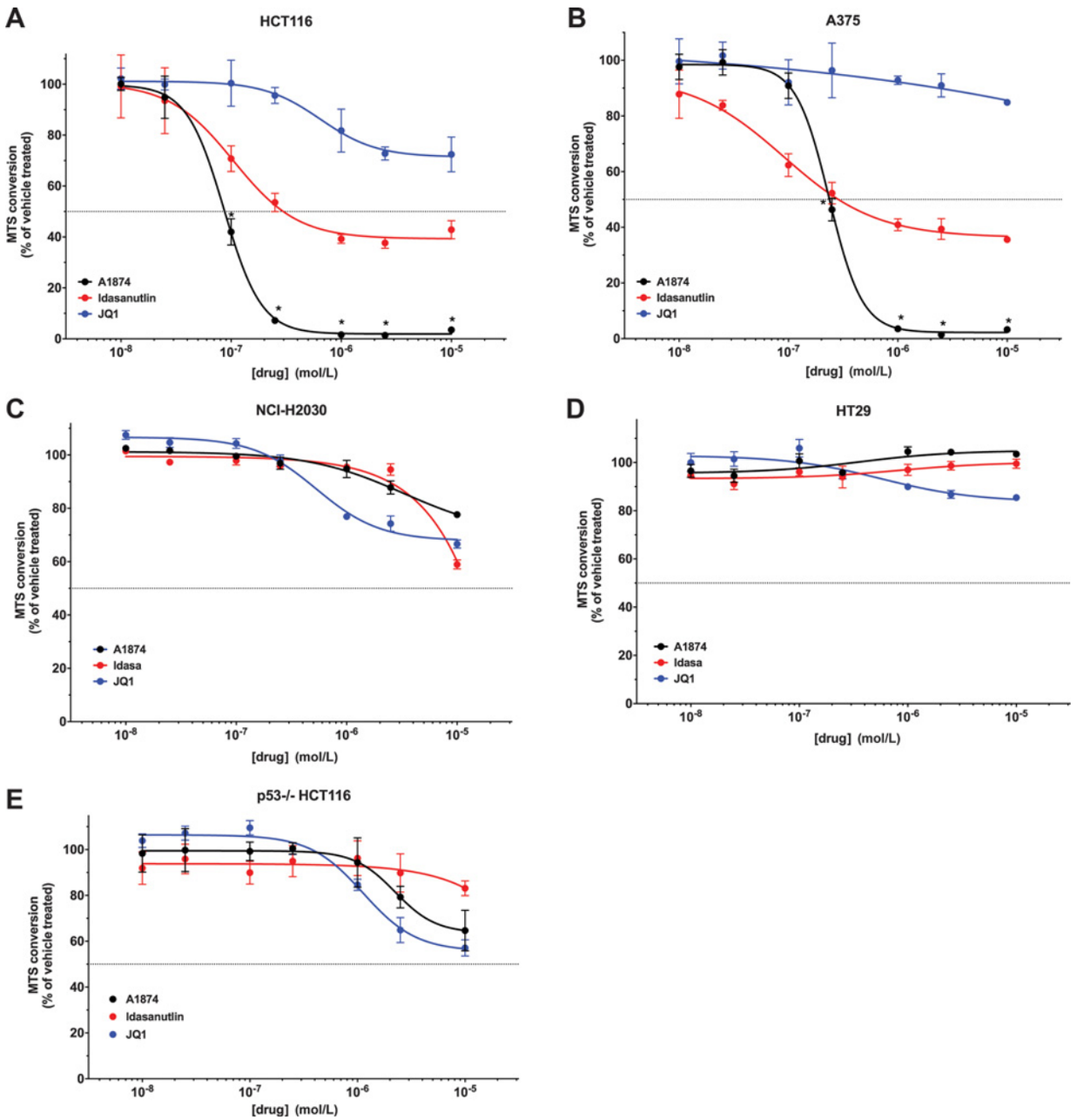


**Figure 3.**

Cell viability assay of cell lines treated with A1874 compared with its component ligands. **A,** HCT116 colorectal cancer cells. **B,** A375 melanoma cells. **C,** NCI-H2030 lung cancer cells. **D,** HT29 colorectal cancer cells. **E,** $p53^{-/-}$ HCT116 colorectal cancer cells (isogenic with **A**). A1874 data points shown in asterisk exhibit synergistic activity compared with the sum of the component warhead activities at the same concentration according to the Bliss independence model (32).

Importantly, A743 does not affect p53 levels because it induces BRD4 ubiquitination by VHL recruitment and not MDM2 recruitment.

In each of the cell lines tested (Fig. 5A–E), the epimeric A1875 had substantially diminished effectiveness in MTS assay relative to A1874, reinforcing that the activity of the PROTAC is dependent on efficient engagement of MDM2. The results from comparisons of A743 against A1874 across the five cell lines showed a dose-dependent loss of viability (Fig. 5). However, in each case, the more effective PROTAC was dependent on the p53 status of the cell line. In the wild-type p53 cell lines (Fig. 5A and B), A1874 was more effective than A743: in HCT116 cells, A1874 caused a 97% decrease in viability compared with the 69% loss resulting from A743 treatment; and in A375 cells, A1874 caused a 98% loss of viability, which is greater than the 76% loss caused by A743. However, in the mutant or null p53 cell lines (Fig. 5C–E), A743

**Table 1.** Bliss independence analysis of A1874 activities in HCT116 cells

| Compound concentration (μmol/L) | % Inhibition by JQ1 | $E_J$ | % Inhibition by idasanutlin | $E_N$ | % Inhibition by A1874 | $E_A$ | CI |
|---|---|---|---|---|---|---|---|
| 0.01 | 0.0% | 0.000 | 0.9% | 0.009 | 0.0% | 0.000 | () |
| 0.025 | 0.1% | 0.001 | 6.5% | 0.065 | 5.1% | 0.051 | 1.29 |
| 0.10 | 0.0% | 0.000 | 29.2% | 0.292 | 58.0% | 0.580 | 0.50 |
| 0.25 | 4.4% | 0.044 | 46.4% | 0.464 | 92.8% | 0.928 | 0.53 |
| 1.00 | 18.2% | 0.182 | 60.8% | 0.608 | 98.4% | 0.984 | 0.69 |
| 2.50 | 27.2% | 0.272 | 62.3% | 0.623 | 98.6% | 0.986 | 0.74 |
| 10.0 | 27.6% | 0.276 | 57.2% | 0.572 | 96.5% | 0.965 | 0.71 |

NOTE: The percentage cell inhibition (compared with vehicle-treated control cells) for each compound at each concentration from Fig. 3A was converted to their decimal equivalents ($E_J$ for JQ1; $E_N$ for idasanutlin; $E_A$ for A1874) and the combination index (CI) value was derived as explained in Materials and Methods and ref. 32. CI values <1 indicate synergy.

was the more effective PROTAC at decreasing viability: in NCI-H2030 cells, HT29 cells, and the $p53^{-/-}$ HCT116 line, A743 decreased viability by 68%, 25% and 69%, respectively. Conversely, A1874 decreased viability by only 22%, 0%, and 36%, respectively, making it the less effective PROTAC in these cell lines.

To determine whether different cellular contexts beyond p53 status impact the relative effectiveness of A1874, we tested it against another, smaller panel of cells that are known to have a more pronounced sensitivity to either of its component ligands (Fig. 6). Daudi cells and MOLM-13 are hematologic cancer cells that are reported to possess an accentuated sensitivity to BRD4 inhibition (34, 42). On the other hand, SJSA1 osteosarcoma cells (wild-type p53) express unusually high levels of MDM2, which renders them especially susceptible to MDM2 inhibition. Against both hematologic cancer lines (Fig. 6A and B), JQ1 reduced viability similarly by 58%. It is, perhaps, a product of this enhanced dependency on BRD4 that even A1875, whose ability to bind to MDM2 is greatly reduced such that it can only inhibit BRD4, was able to cause some loss of viability at the highest concentration tested—31% and 32% in Daudi and MOLM-13 cells, respectively. Although these two lines reacted similarly to the JQ1 inhibitors, their sensitivities to idasanutlin were quite distinct from each other: MOLM-13 cells, which are wild-type p53, were inhibited by idasanutlin to an equal extent as they were by JQ1 (58% reduction), whereas the mutant p53-expressing Daudi cells were minimally affected (9% reduction) by idasanutlin up to 10 μmol/L. Mirroring this pattern, MOLM-13 cells were also more susceptible to inhibition by A1874 than were the Daudi cells, although in both cases the PROTAC was more active than either of the component ligands: A1874 reduced viability of Daudi cells by 70% and of MOLM-13 cells by 95%. Although there was no synergism observed, A1874 was clearly the most active against these cancer lines. Interestingly, in Daudi cells the VHL-recruiting A743 outperformed the MDM2-recruiting A1874, although this appears to be more a result of a relatively diminished activity of the latter than a substantial increase in activity of A743 in this cell line.

Predictably, idasanutlin was effective at reducing viability (down by 82%) of the MDM2-overexpressing SJSA-1 cells (Fig. 6C); unexpectedly, JQ1 was also more effective against SJSA1 cells (46% reduction in viability) than it had been against the cells in Figs. 3 and 5, and approached the sensitivity exhibited by the Daudi and MOLM-13 cells. Nevertheless, the MDM2-recruiting A1874 demonstrated the highest effectiveness to decrease SJSA-1 cell viability (97.5% viability loss), also outperforming the VHL-recruiting A743 (36% viability loss). The increased levels of MDM2 clearly narrowed the effectiveness differential between A1874 and idasanutlin, such that the latter was nearly as effective as the former, limiting how far the PROTAC could outperform the MDM2 inhibitor. The increased MDM2 levels in the SJSA1 cells only modestly increased the potency of A1874 itself: down to an $IC_{50}$ of 46.5 nmol/L in SJSA1 cells versus 86.3 nmol/L and 236 nmol/L in HCT116 cells and A375 cells, respectively. In the end, A1874 was the most effective PROTAC at reducing viability of p53 wild-type cells here, consistent with the earlier results in Fig. 5.

## Discussion

PROTAC-mediated protein degradation combines the lasting effects of nucleic acid-based methods of protein knockdown with the more readily adjustable attributes of application of small molecules, i.e., delivery, dosing, timing, etc. Given their modular design, PROTACs are also adjustable. Studies have shown that changes in all three PROTAC components—target protein ligand, linker, and E3 ligand—can have a profound impact on their target specificity as well as their potency and efficacy to cause degradation. Changes in linker length and hydrophilicity can mean the difference between highly effective versus nominal degradation (21, 39, 43). Linkers that are too short will prevent formation of the trimer complex (target protein:PROTAC:E3 ligase) to favor only dimers (target protein:PROTAC or PROTAC:E3 ligase), while linkers that are too long provide excess steric freedom and fail to facilitate a sufficiently tight interaction of its binding partners to establish stabilizing protein:protein interactions. Likewise, we have documented that structurally dissimilar ligands with high affinity for a common protein will not necessarily each give rise to an effective target degrader when incorporated into PROTACs (1). Nevertheless, effective PROTACs have been synthesized and reported to degrade an ever-increasing number of target proteins, including but not limited to FKBP12 (5), the androgen receptor (11, 44), HER1/HER2 (16), BRD4 and other BET proteins (2, 19, 39), RIPK2 (10), the estrogen receptor (3), $p38^{MAPK}$ (18), the retinoic acid binding protein (45), MetAP2 (4), c-Met (18), CDK9 (12), BCR-Abl (1), FRS2α (13), and BTK (15, 17).

Compared with the variety of proteins successfully degraded, the paucity of options for recruiting an E3 ligase is notable. With few exceptions, PROTACs to date have been constructed to recruit one of the following E3 ligases: VHL, cereblon, or IAP. This is striking because the human genome encodes for hundreds of different E3 ligases (46); while some may not be amenable for use with PROTACs, a recent study suggests that many of them can mediate target protein degradation if successfully harnessed (22). However, the limiting factor has been the availability of small-molecule ligands to harness them. In this report, we have revisited the strategy of constructing PROTACs using the small molecule,

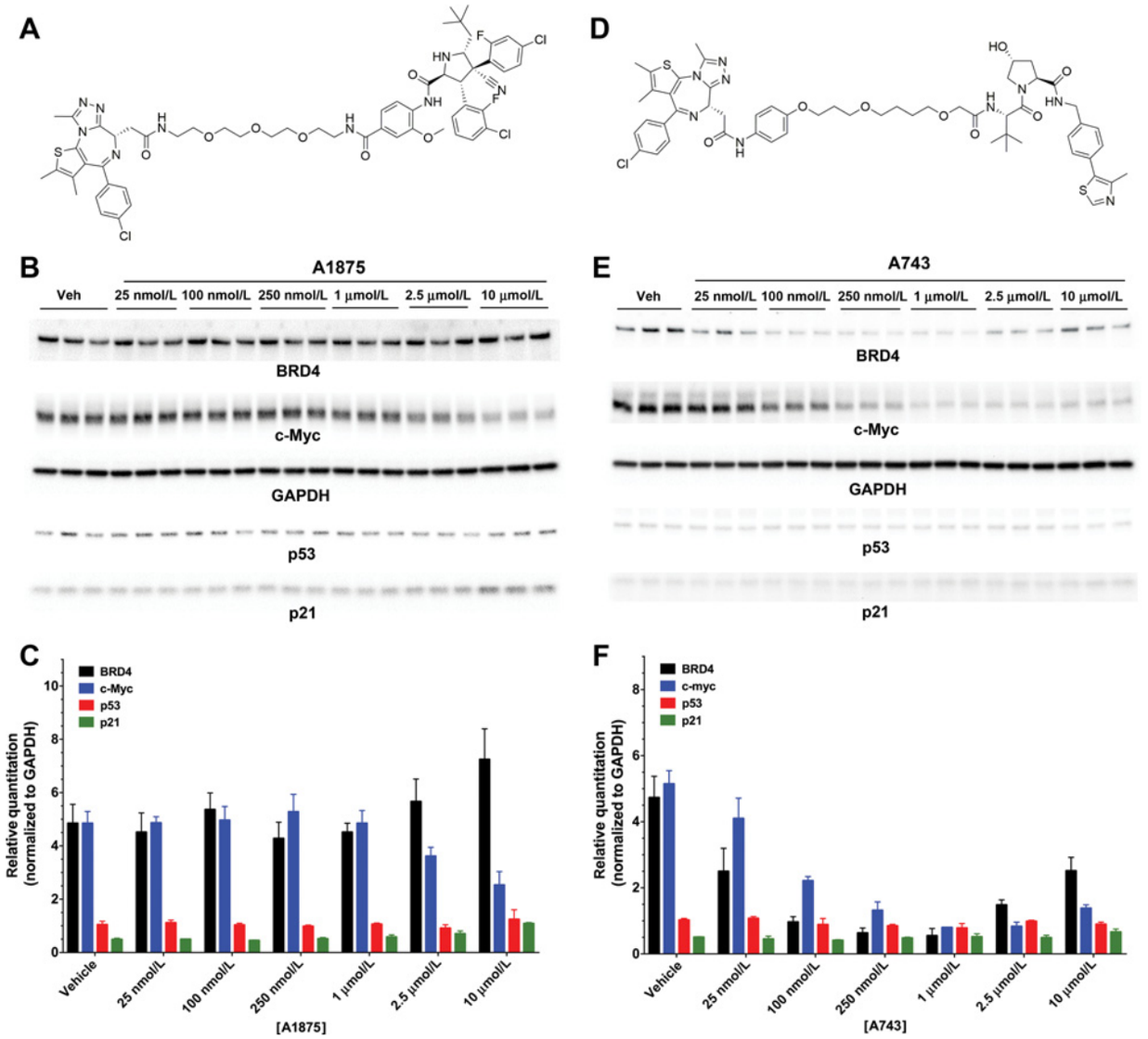


**Figure 4.**

Structures and activities of PROTAC variants of A1874. **A,** Structure of A1875, a diastereomeric analogue of A1874 with greatly reduced affinity for MDM2. **B,** Representative immunoblots from HCT116 cells treated with increasing concentrations of A1875. **C,** Quantified results of immunoblots in **B**. **D,** Structure of A743, a VHL-recruiting BRD4-degrading PROTAC. **E,** Representative immunoblots from HCT116 cells treated with increasing concentrations of A743. **F,** Quantified results of immunoblots in **E**.

idasanutlin—a ligand with high affinity for the E3 ligase MDM2 (37). Nutlins were initially developed to act as a pharmacologic means to elevate expression of the tumor suppressor p53 and there have been numerous reports (47–49) concerning the potential of nutlins as anticancer drugs/adjuvants. Our original study (25) indicated that nutlin-based PROTACs were limited in their ability to facilitate degradation: a PROTAC that recruited MDM2 to ubiquitinate the androgen receptor caused only limited target degradation at micromolar concentrations. However, that study was limited in its scope, and given what has been learned about the structural and mechanistic considerations of PROTAC functioning (50, 51), we have revisited nutlin-based PROTACs here. This study has shown that not only can nutlin-based PROTACs mediate degradation far more potently and effectively than previously realized, but that by virtue of the p53-stabilizing activity particular to nutlins, the PROTACs derived from them can have biological activity surpassing that of equipotent degraders that harness other E3 ligases.

We synthesized a PROTAC by joining the BET protein inhibitor JQ1 to the MDM2 antagonist idasanutlin. JQ1 inhibits BRD4, resulting in c-Myc suppression and subsequent reduced proliferation of a variety of cancers (52–54). Idasanutlin disrupts the interaction between MDM2 and the tumor suppressor protein, p53, leading to accumulation of the latter and activation of tumor suppressing mechanisms that also inhibit cell proliferation and reduce viability. We reasoned that if these complementary

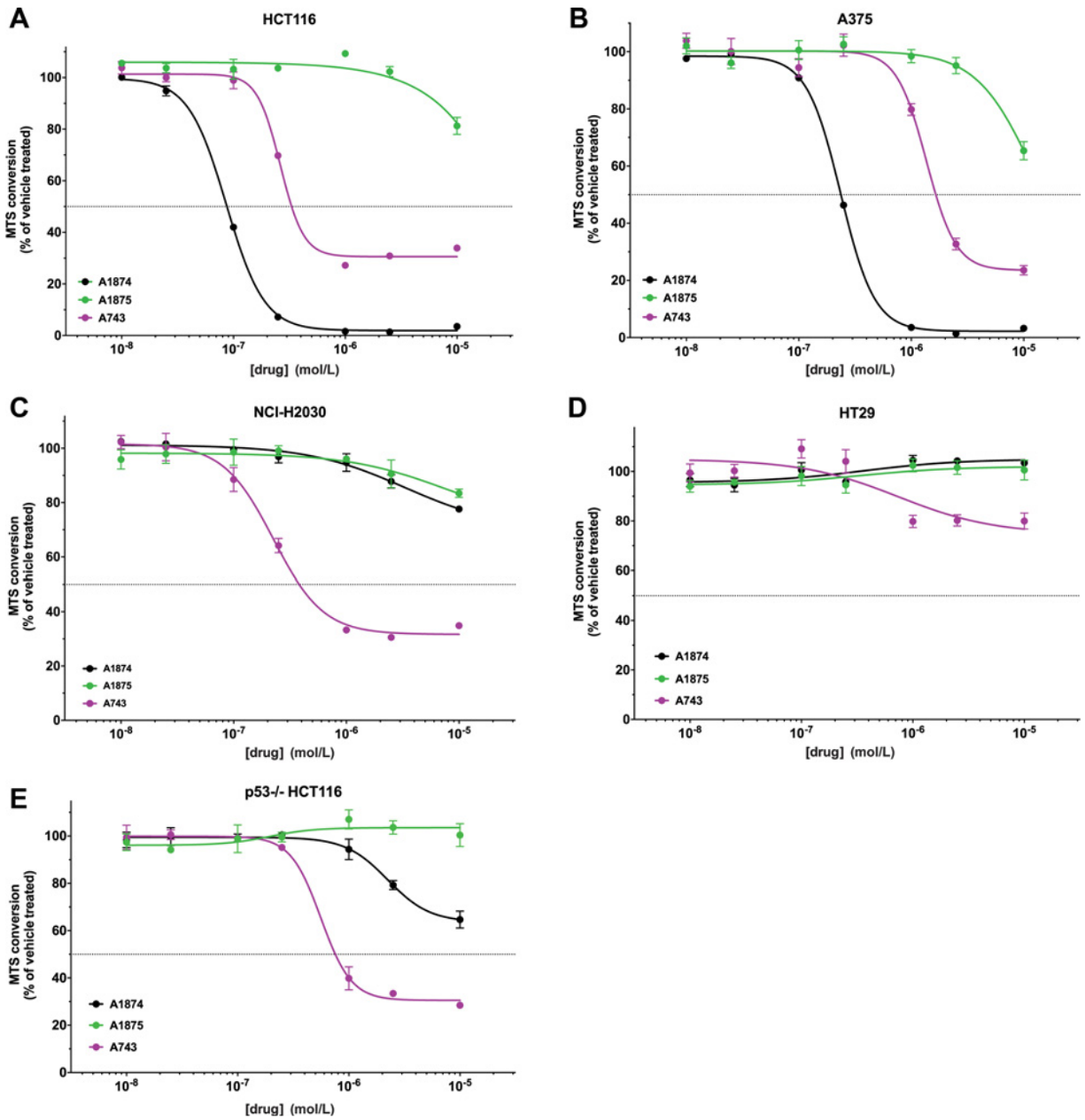


**Figure 5.**

Engagement of MDM2 by PROTAC enhances anticancer activity beyond that of BRD4 degradation in cells with wild-type p53. **A,** HCT116 colorectal cancer cells. **B,** A375 melanoma cells. **C,** NCI-H2030 lung cancer cells. **D,** HT29 colorectal cancer cells. **E,** $p53^{-/-}$ HCT116 colorectal cancer cells (isogenic with **A**).

activities would be incorporated in a PROTAC synthesized from both molecules, a degrader capable of dual-mode anticancer activity would be created. Indeed, the resultant PROTAC, A1874, mediates both BRD4 degradation/c-Myc suppression and p53 stabilization when incubated on HCT116 cells. The degree of c-Myc suppression mediated by A1874 was greater than that by JQ1 itself in the same cells, perhaps owing to degradation having a more profound effect than inhibition of the same target protein (16, 19). Moreover, the level of p53 upregulation in response to A1874 approaches that of unmodified idasanutlin. Unsurprisingly, when HCT116 cells were measured for viability following A1874 incubation, the reduction in viability was striking compared with that due to either of the individual ligand components; and even synergistic compared with the sum of their effects. Enhanced effects have been demonstrated between nutlins combined with other anticancer drugs (30, 31, 55); however, such studies to date have relied on protein inhibition rather than on PROTAC-mediated protein degradation.

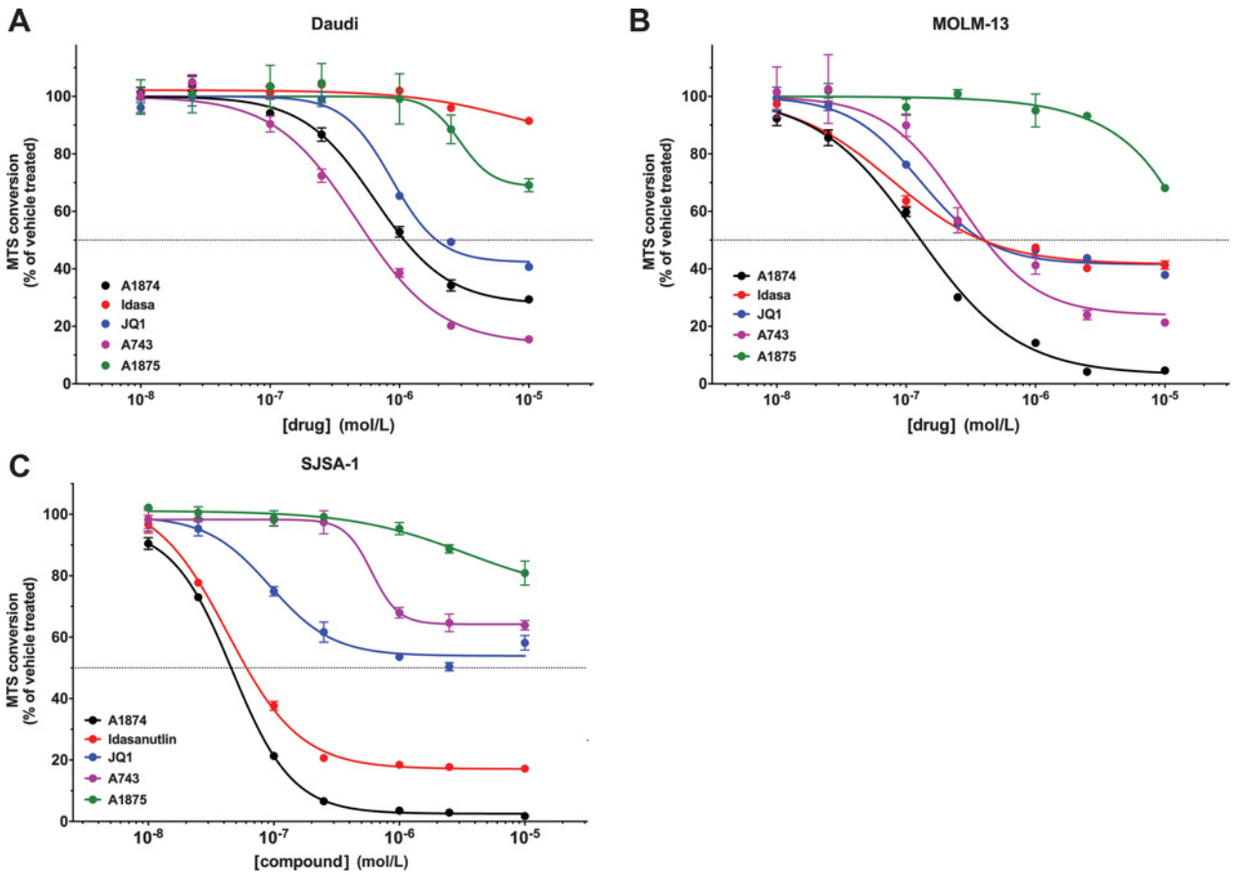


**Figure 6.**

Comparative effectiveness of MDM2-recruiting PROTAC versus component ligands and VHL-recruiting PROTAC in cells with accentuated JQ1 sensitivity and/or elevated MDM2 levels. **A,** Daudi lymphoma cells. **B,** MOLM-13 myeloid leukemia cells. **C,** SJSA-1 osteosarcoma cells.

A similar result as with the HCT-116 cells was obtained in testing the PROTAC on A375 melanoma cells. Because both of these cell lines have wild-type p53, the greater effect of the combined reagent seems intuitive. Even in instances where the effect of either component ligand alone was substantial in terms of reducing cancer cell viability, the PROTAC A1874 was nonetheless the most effective treatment in cells with wild-type p53 (e.g., MOLM-13 and SJSA-1 cells), indicating a combined effect. In order to confirm the involvement of MDM2 and the p53 response in mediating the profound anticancer response of A1874, the PROTAC was both (i) tested in comparable cell line contexts that were p53 mutant or null and (ii) compared with a similarly potent BRD4-degrading PROTAC, A743, that works through recruitment of VHL as its E3 ligase. In cell lines that lacked functional p53, A1874 was much less capable to reduce cell viability than it had been in wild-type p53 cell lines; similarly, in cell lines with wild-type p53, A1874 was more active at reducing cell viability than the cognate VHL-recruiting PROTAC, while the reverse was true in cell lines with mutant p53. Hence, in utilizing idasanutlin, there has developed a selectivity of cell susceptibility to the PROTAC that stands apart from that typically determined from the expression pattern of the target protein itself; in this instance, selectivity is also reliant on signaling events downstream from the E3 ligase. This has recently been shown for some IAP-recruiting degraders (29), although for those molecules, the contribution from the E3 ligase side of the molecule toward the overall observed activity is minor.

To summarize, this study shows that the E3 ligase MDM2 can serve as a valuable addition to the small number of E3 ligases that when harnessed can produce nanomolar PROTAC-mediated target protein degradation. Moreover, with further refinement, nutlin-based PROTACs could become extremely effective candidate anticancer therapeutics due to their dual-mode mechanism of action—elimination of a proto-oncogene/oncogene and activation of a tumor suppressor—where one anticipates that development of resistance would be more difficult because a single mutation in either partner binding protein may not abrogate all activities of the PROTAC.

## Disclosure of Potential Conflicts of Interest

H. Dong has ownership interest (including stock, patents, etc.) in Arvinas stock. Y. Qian has ownership interest (including stock, patents, etc.) in Arvinas stock. C.M. Crews reports receiving a commercial research grant from Arvinas, Inc., has ownership interest (including stock, patents, etc.) in Arvinas, Inc., is a consultant/advisory board member for Arvinas, Inc, and has provided an expert testimony for Amgen. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** J. Hines, H. Dong, Y. Qian, C.M. Crews
**Development of methodology:** S. Lartigue, Y. Qian, C.M. Crews
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J. Hines, S. Lartigue
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J. Hines, S. Lartigue, Y. Qian
**Writing, review, and/or revision of the manuscript:** J. Hines, S. Lartigue, Y. Qian, C.M. Crews
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S. Lartigue
**Study supervision:** J. Hines, C.M. Crews
**Other (designed and supervised the synthesis of PROTAC compounds):** H. Dong

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