Dispiroindolinone-Glutarimide Conjugates as Potential Hetero-PROTAC Compounds for p53 Reactivation

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Article

Dispiroindolinone-Glutarimide Conjugates as Potential Hetero-PROTAC Compounds for p53 Reactivation

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Abstract

Aiming at p53-reactivating compounds, a convergent scheme for the preparation of conjugates with the dispiro-indolinone-pyrrolidine-thioimidazolone and glutarimide moieties connected via a triazole-containing linker were proposed. Target conjugates were synthesized by azide-alkyne cycloaddition reactions between propargylthio-substituted dispiro-indolinone-pyrrolidine-imidazolones and an azido-glutarimide derivative. The starting compounds were available isothiocyanates, glycine, substituted benzaldehydes, chloroacetamide, and ethyl acrylate. The key azide-alkyne cycloaddition step was carried out using TBTA as a catalyst, achieving >70% product yields. The resulting bifunctional compounds contained a fragment of dispiroindolinone (p53-MDM2 interaction inhibitor) and glutarimide, an ubiquitin ligase ligand. The dispiroindolinone-glutarimide conjugate with 5-bromoisatine and 4-bromophenyl moieties showed a potential for p53 re-activation as determined by preferential cytotoxicity against HCT116 colon carcinoma cells (wild type53) compared to the isogenic HCT116p53$^{-/-}$ subline.

Keywords: spiroindolinones; glutarimide; azide-alkyne cycloaddition; thiohydantoin; imidazolones

1. Introduction

The MDM2 protein, an E3 ligase of the tumor suppressor p53, is overexpressed in many cancer types. As a result, p53 loses its ability to trigger apoptosis [1–3]. In the last 20 years, various compounds of the imidazoline and indolinone classes [4,5] have been proposed to inhibit the p53-MDM2 interaction, thereby activating p53, among them nutlins [6,7], spiroindolinones [8–10], and dispiroindolinones [11] containing an imidazolinone or thioimidazolinone fragments [9,12]. However, most of the known MDM2-p53 inhibitors cause numerous side effects, partly due to high concentrations required to disrupt protein-protein complexes.

A promising strategy to overcome side effects is the PROTAC (PROteolysis Targeting Chimera) approach [13]. This strategy allows for a significant reduction in the amount of the drug used to degrade unwanted proteins with just one molecule of the active compound. The PROTAC molecule consists of two functional fragments connected by a linker. One fragment is capable of binding to a target protein, and the other to the E3 ubiquitin ligase followed by ubiquitinylation and proteasomal cleavage. After the target protein is destroyed, the PROTAC molecule is released and can participate in the next round of protein recognition/ubiquitinylation. In this scenario, a stable cellular effect is achieved along with reduced drug concentrations and side effects [14,15].

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The homo-PROTACs can be used if the functional fragment is a ligand for the target protein as well as for a specific ubiquitin ligase; in this case, it is sufficient to connect two identical functional fragments with linkers to initiate protein degradation [13–17]. In hetero-PROTACs, one functional fragment acts as a ligand for the target protein, and the second one binds ubiquitin ligase [18–21]. Typically, thalidomide [22–26] or similar molecules containing the fragments of glutarimide and phthalimide [27,28], or glutarimide only [29,30], can be used as ubiquitin ligase binders.

Currently, several PROTAC-based drug candidates are in various stages of clinical trials: three are in phase III, 11 compounds in phase II, and more than 30 in phase I (according to [https://www.biochempeg.com/article/434.html] as of May 2025). Ccompounds ARV-110 [31] and ARV-471 [32] for prostate and breast cancers undergo phases II and III, respectively (Figure 1). However, the synthesis of these molecules is a highly complex problem. The original method for synthesis of ARV-741 (Vepdegestrant) includes 16 stages, which leads to a high cost and limited availability of the final compound.

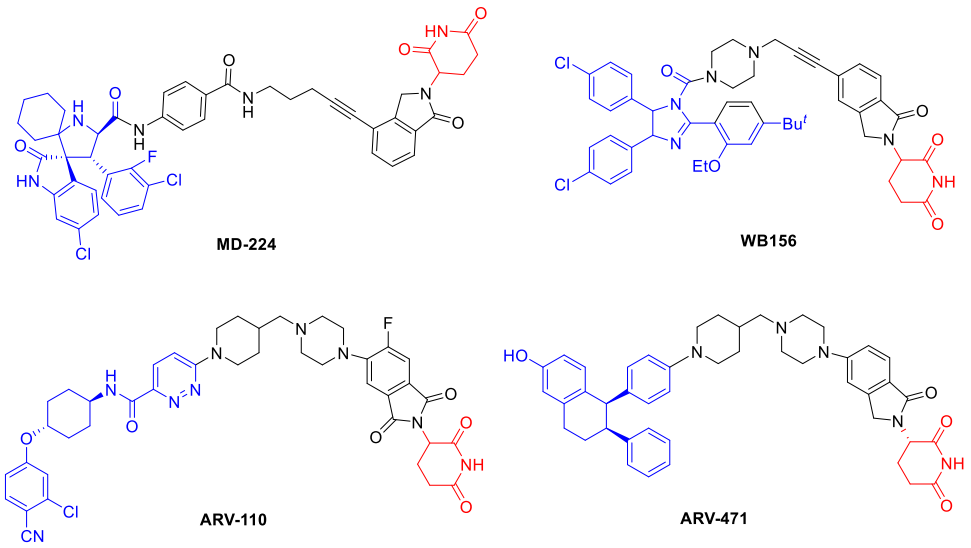

Figure 1. Structures of glutarimide-containing hetero-PROTAC compounds in development and in clinical trials [18,20,31–33]. The fragment that binds to the target protein is shown in blue, and the fragment that binds to ubiquitin ligase is shown in red.

Previously, we have synthesized a series of dispiroindolinone type inhibitors of p53-MDM2 interaction with significant cytotoxicity against HepG2 liver, LNCaP prostate and MCF-7 breast cancer cell lines [9,34–37] (Figure 2, compounds **A**). Since MDM2 is a specific E3 ubiquitin ligase for p53, the dispiroindolinone moiety may be potentially utilized to generate homo- and hetero-PROTAC molecules. In our dispiroindolinone-based homo-PROTACs **B** and **C** ([38,39], Figure 2)) two identical dispiro-indolinone-pyrrolidine-thioimidazolone moieties were connected via a polymethylene linker. However, these compounds did not demonstrate a significant increase of cytotoxicity compared to dispiroindolinones **A**, indicating the need to change the overall design of the target molecules.

In the present study we report the design and synthesis of novel small molecular weight conjugates containing fragments of the MDM2 ligand, dispiro-indolinone-pyrrolidine-thioimidazolone, and the E3 ligase ligand, glutarimide, connected via a triazo-containing linker (Figure 2). The dispiroindolinone-glutarimide conjugate with 5-bromoisatine and 4-bromophenyl moieties showed preferential cytotoxicity for human colon carcinoma cells carrying wild type p53 compared to the isogenic p53-null subline. These data suggested a possibility to target MDM2 with these chemotypes, thereby re-activating p53 for induction of cell death.

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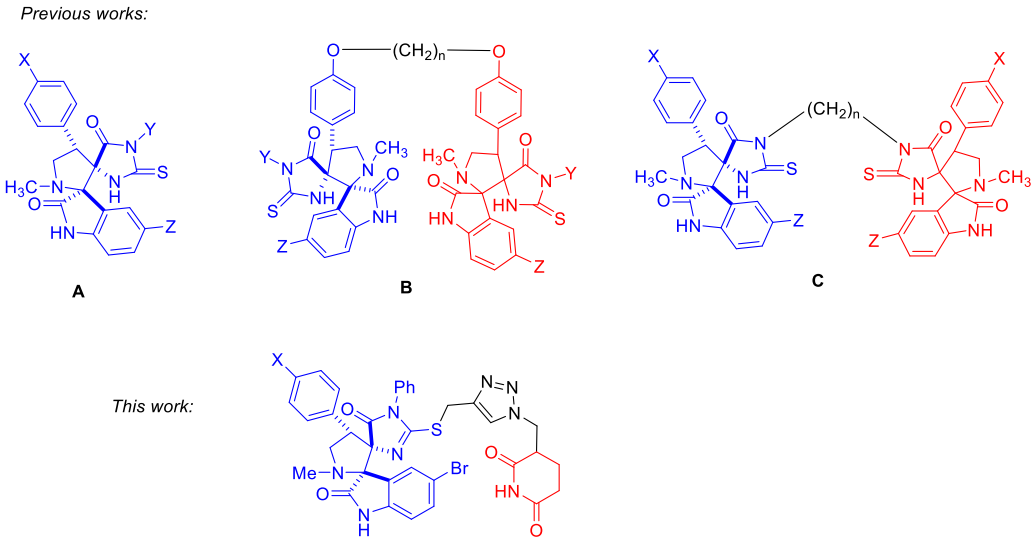

Figure 2. Structures of dispiro-indolinone-pyrrolidine-thioimidazolones **A** [9,34–37], bis-dispiro-indolinone-pyrrolidine-thioimidazolones **B**, **C** [38,39] and newly synthesized compounds.

2. Results and Discussion

2.1. Synthesis

We proposed a simple and convenient method for the preparation of dispiroindolinone-glutarimide conjugates **6a-c** by a convergent scheme starting from available 5-arylidene-2-thiohydantoins **1a-c** and 3-methylidene glutarimide **4** (Scheme 1). Thiohydantoins **1a-c** were synthesized from phenyl isothiocyanate, the corresponding benzaldehydes, and glycine [40]. S-Propargylation of thiohydantoins **1** to obtain 2-propargylthioimidazolones **2a-c** was carried out similarly to the procedure described for 5-pyridylmethylene-2-thioimidazolones, with minor modifications [41]. At the final step of the assembly of the dispiro-indolinone functional fragment, the dipolarophiles **2** were introduced into regio- and diastereoselective (3+2)-cycloaddition reactions with azomethine ylide **D**, generated in situ from isatin and sarcosine, similarly to the synthesis of compounds **A** (Figure 2; no propargyl fragment in the starting dipolarophile). The resulting products **3** were isolated by column chromatography. The selectivity of the reaction was confirmed by a single set of signals in ^{1}H and ^{13}C NMR spectra, containing characteristic three-spins system signals–of protons of the central pyrrolidine ring in the range of 3.4-4.1 ppm (Supplementary Information). For compound **3b**, the complete assignment of signals in the NMR spectra was carried out using two-dimensional correlation methods HMBC, HSQC (see Supplementary Information). Relative *2′S*,4R*,4′R** configuration of compounds **3** is consistent with the one determined for the products of azomethine ylide addition to 5-arylidene-2-thioimidazolones [9,34–37].

The starting 3-methylenepiperidine-2,6-dione **4** was synthesized according to [42,43] from chloro acetamide and ethyl acrylate. 3-(Azidomethyl)piperidine-2,6-dione **5** was prepared by Michael addition of trimethylsilyl azide to compound **4** in a mixture of acetic acid and triethylamine [29].

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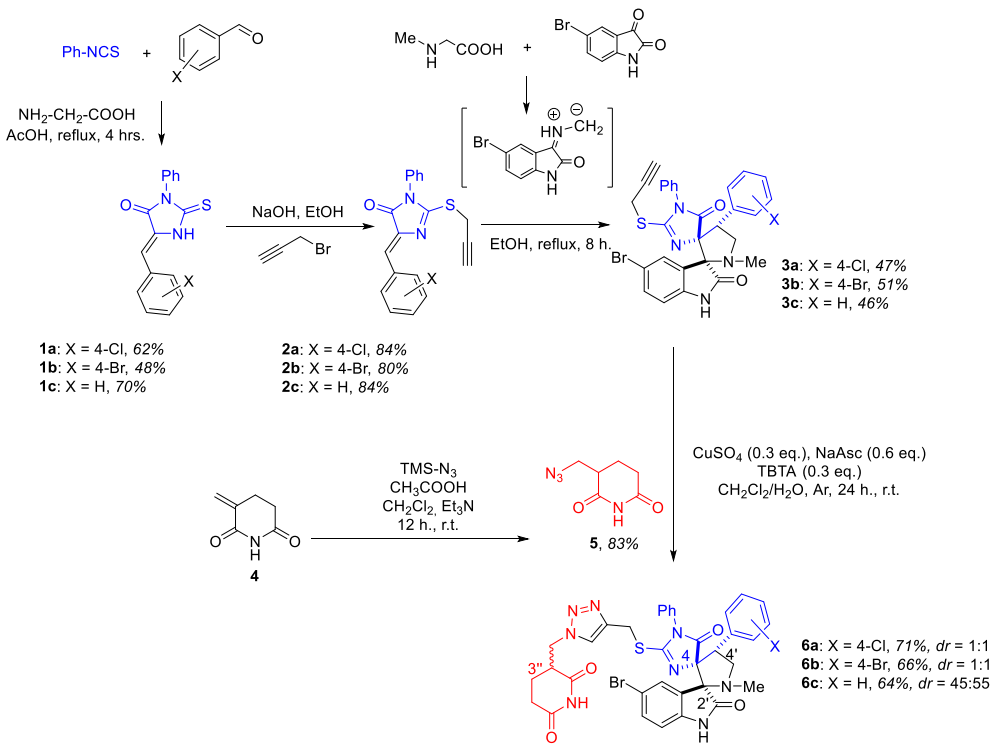

Scheme 1. General scheme of synthesis of dispiroindolinone-glutarimide conjugates **6a-e**.

To obtain target conjugates **6** via a click reaction, we initially employed a standard azide-alkyne cycloaddition procedure between alkyne **3a** and azide **5** using a $CuSO_4$/NaAsc catalytic system in a CH_2Cl_2/H_2O mixture. However, this reaction yielded no conversion of the starting spiro compound **3** (TLC monitoring). Therefore, we modified the conditions by adding (tris(benzyltriazolylmethyl)amine (TBTA) and conducting the reaction under argon atmosphere to prevent Cu(I) oxidation. By varying TBTA quantities we found that its optimal amount was 0.3 equivalents relative to the starting spiro derivative **3**; with less TBTA the yield of the product decreased, and further TBTA increase did not improve the yield of the target product. Under these conditions **6a** was obtained within 24 h at room temperature.

Once the conditions were optimized, we synthesized a series of conjugates **6a-c** in reasonable yields (64-72%) and isolated them using silica gel column chromatography. Structures were confirmed by ^1H and ^{13}C NMR spectroscopy and high resolution mass spectrometry. Since **3a-c** contain three chiral carbon centers (C2', C4, C4' atoms; Scheme 1) and azidomethyl glutarimide **5** also contains a chiral center at the C3 atom, all conjugates **6a-c** formed by combination of these structural fragments were isolated as the mixtures of diastereomers with *2'S*,4R*,4'R*,3''R** and *2'S*,4R*,4'R*,3''S** relative configurations (*ca* 1:1). Due to the presence of diastereomers, two sets of signals were observed in NMR spectra of **6**. A complete assignment of signals in the ^1H and ^{13}C NMR spectra using two-dimensional HMBC, HSQC, and ^1H-^1H COSY techniques unambiguously confirming the structure of the product for compound **6c** (Supplementary Information).

The nature and position of halogen substituents in **6a-c** did not significantly affect the yield of target compounds. The halogen-substituted arylidentiohydanthins **1** and 5-bromoisatin were chosen to obtain adducts **6**, since spiro derivatives with similar substituents demonstrated the highest cytotoxicity among dispiro derivatives of the structural type **A** (Figure 2) [9,34–37].

For the biological testing, we synthesized dispiroindolinones **7a**,**b** (Scheme 2, [44]) that are similar to **6a** and **6b** but lack a glutarimide fragment. Compounds **7** were used as the reference to judge whether the glutarimide moiety affects cytotoxicity.

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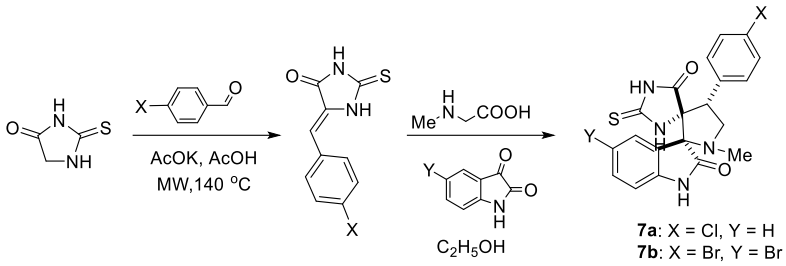

Scheme 2. Synthesis of compounds **7a**,**b**.

2.2. Biological Testing

Initially, we performed MTT assays for **6a-c** and **7a**,**b** on the HCT116 cell line (wild type p53). Table 1 shows that **6a**, **6b** and **7b** caused an antiproliferative effect (IC_{50} 10-15 μM after 72 h of cell exposure). To clarify the role of p53 in cell response, these compounds were tested against the isogenic HCT116p53$^{-/-}$ subline with deletion of both alleles of the *p53* gene (see Materials and Methods). As shown in Figure 3, two out of three derivatives were less cytotoxic in HCT116p53$^{-/-}$ cells than in the wild type counterparts.

Table 1. Cytotoxicity of compounds **6, 7** in HCT116 cell line (wild type p53).

Compound	Mean IC_{50}, μM
6a	11.2±2.3
6b	12.5±3.4
6c	22.5±3.3
7a	44.3±3.2
7b	13.0±2.3

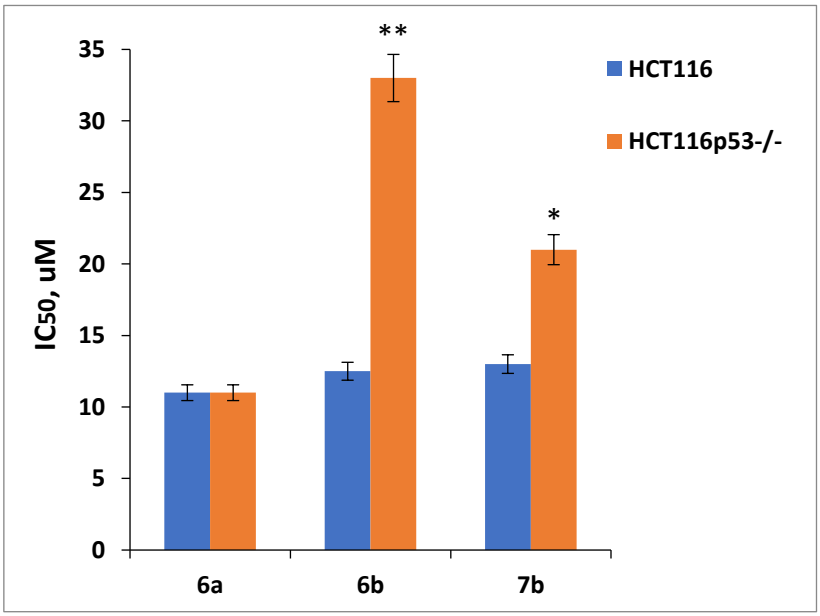

Figure 3. Cytotoxicity of selected new compounds for HCT116 cells (wild type p53) and HCT116p53$^{-/-}$ subline. Here, in Table 1 and in Figures S1, S2: mean±S.D. (MTT tests) after 72 h of cell exposure. Experiments were performed three times, each concentration tested in duplicate. *p<0.05, **p<0.001 compared to the respective HCT116 group.

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Compounds **6c** and **7a** turned out to be less potent (IC_{50} > 22 μM). This result is in the agreement with [9,35,36] confirming that the halogen atom in the aryl substituent and bromine in the isatin fragment of dispiroindolinone increase the cytotoxicity.

Altogether, one may hypothesize that combinations of **6b/7b** with treatments contingent on p53 responses, such as DNA damaging chemotherapeutic drugs or γ-irradiation, can potentiate the therapeutic efficacy in tumors carrying wild type p53.

3. Materials and Methods

3.1. General

All solvents used were purified and dehydrated using the standard techniques. All starting reagents were purchased from commercial sources (Sigma-Aldrich, ABCR, AKSci, Burlington, VT). Unless otherwise stated, all reactions were monitored by TLC analysis using silica plates with a fluorescent indicator (254 nm) and visualized with a UV lamp.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance-400 (Bruker Biospin, Karlsruhe, Germany) and Agilent 400-MR (Agilent Technologies, Santa Clara, California, USA) spectrometers (400 MHz for ^{1}H, 101 MHz for ^{13}C) or on Q.One Quantum I - 600 spectrometer (Q.One Instruments Ltd./ Wuhan Zhongke-Niujin Magnetic Resonance Technology Co., Ltd, China; 600 MHz for ^{1}H, 151 MHz for ^{13}C). All chemical shifts (δ) are reported in parts per million (ppm) with ^{1}H and ^{13}C NMR referenced to solvent signals [^{1}H NMR: $CDCl_3$ (7.27), DMSO-d_6 (2.50); ^{13}C NMR: $CDCl_3$ (77.16), DMSO-d_6 (39.52)]. Coupling constants (J) are reported in Hertz (Hz).

High resolution mass spectra were obtained using electrospray ionization (ESI) in positive ion mode on a TripleTOF 5600+ quadrupole time-of-flight mass spectrometer (ABSciex, Concord, Canada) equipped with DuoSpray ion source. The following MS parameters were applied: capillary voltage 5.5 kV; nebulizing and curtain gas pressure were 15 psi and 25 psi, respectively; ambient ion source temperature; declustering potential 20 V; m/z range 100–1200. Elemental compositions of the detected ions were determined based on accurate masses and isotope distributions using Formula Finder software (ABSciex). Maximum allowed deviation of the experimental vs calculated molecular mass was 5 ppm. Experimental details and characteristic data for all synthesized compounds are given in the Supplementary Information.

3.2. Synthesis

3-Methylenepiperidine-2,6-dione **4** was synthesized according to [42,43] from chloroacetamide and ethyl acrylate. 3-(Azidomethyl)piperidine-2,6-dione **5** was synthesized according to [29]. The TBTA catalyst was synthesized as in [45,46]. The starting thiohydantoins were prepared using methods described in [47–51].

General procedure for the synthesis of 2-thiohydantoins 1a-e. The substituted benzaldehyde (1.1 eq.), glycine (1.1 eq.) and phenyl isothiocyanate (1 eq.) were dissolved in glacial acetic acid. The mixture was refluxed for 3-4 h, the reaction was monitored by TLC (eluent, *n*-hexane/ethyl acetate 3:1). After completion of the reaction the mixture was poured in an excess of cold water, the resulting precipitate was filtered, washed with cold water and dried on air to obtain a yellow solid followed by recrystallization from ethanol or glacial acetic acid or purification by column chromatography on silica gel (eluent, *n*-hexane/ethyl acetate 3:1, Rf ~ 0.35-0.4).

General procedure for the synthesis of S-propargyl-2-thiohydantoins 2a-e. 2-Thiohydantoin (1 eq.) was dissolved in 95% ethanol (10 ml/1 mmol), after which potassium hydroxide (1.2 eq.) was added. After the solution changed the color, propargyl bromide (1.2 eq.) was added dropwise, and the mixture was stirred at room temperature overnight. An excess of cold water was added. The precipitate was filtered and washed with 10% aqueous sodium hydroxide, water, and cold petroleum ether. The resulting residue was purified by column chromatography (eluent, petroleum ether/ethyl acetate gradient from 4:1 to 1:1) to yield a bright yellow solid.

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General procedure for the synthesis of dispiroindolinones 3a-e. To a boiling solution of 2-(prop-2-yn-1-ylthio)hydantoins **2a–2e** (1 eq.) and sarcosine (3 eq.) in 95% ethanol, 5-bromoisatin (2 eq.) was added in two portions 15 min apart; the resulting mixture was boiled for 6-8 h (TLC monitoring). After completion of the reaction and cooling the solution, a 3-fold volume of cold water was added, and the obtained precipitate was filtered out. The residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate 3:1) to yield a white solid.

General procedure for the synthesis of 6a-e. To a solution of the terminal alkyne **3a–3e** (1 equiv.) and TBTA (0.3 equiv.) in CH_2Cl_2 (5 ml/0.1 mmol of alkyne) under argon, a solution of $CuSO_4$*$5H_2O$ (0.3 equiv.) in 200 μL of dH_2O was added, followed by a solution of sodium ascorbate (0.6 equiv.) in 200 μL of dH_2O. The reaction mixture was stirred for 30 min, then a solution of the N_3-glutarimide (1 equiv.) in CH_2Cl_2 (2.5 ml/0.1 mmol of azide) was added dropwise to the resulting dark red solution. The mixture was stirred overnight at room temperature under argon. Then the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (eluent — CH_2Cl_2/CH_3OH 99:1 → 30:1) to obtain a white solid.

3.3. Cell Culture and Treatment

3.3.1. Cell Culture and Cytotoxicity Assays

The human HCT116 colon carcinoma cell line (American Type Culture Collection; Manassas, VA) and its isogenic HCT116p53$^{-/-}$ subline with deletion of both alleles of the *p53* gene (generated in Vogelstein laboratory at Johns Hopkins University, Baltimore, MD; provided by B.P.Kopnin) were cultured in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone; Logan, UT), 100 μg/ml penicillin and 100 U/ml streptomycin at 37 °C, 5% CO_2 in a humidified atmosphere. Newly synthesized compounds were dissolved in DMSO as 10 mM stock solutions followed by serial dilution in the culture medium immediately before the experiments. Cell viability was determined in MTT tests [52]. Experiments were performed three times, each concentration tested in duplicate.

3.3.2. Statistics

One-way or two-way analyses of variance (ANOVA) followed by Sidak's post hoc test for multiple comparisons were used (GraphPad Prism 9; GraphPad Software, San Diego, CA). The p value <0.05 was taken as evidence of statistical significance.

4. Conclusions

Aiming at the design of agents that re-activate p53 via inhibition of its antagonist MDM2, we developed a convenient method for the preparation of potential hetero-PROTAC conjugates containing dispiro-indolinone-pyrrolidine-thioimidazolone and glutarimide moieties connected via a triazole-containing linker, starting from available precursors: aryl isothiocyanates, glycine, substituted benzaldehydes, chloroacetamide, and ethyl acrylate. The proposed strategy represents a convergent scheme of no more than four sequential steps for each starting compound. Two newly synthesized compounds, 5''-bromo-4'-(4-bromophenyl)-2-(((1-((2,6-dioxopiperidin-3-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-1'-methyl-1-phenyldispiro[imidazole-4,3'-pyrrolidine-2',3''-indoline]-2'',5(1H)-dione **6b** and 5''-bromo-4'-(4-bromophenyl)-1'-methyl-2-thioxodispiro[imidazolidine-4,3'-pyrrolidine-2',3''-indoline]-2'',5-dione **7b** showed preferential cytotoxicity against HCT116 cells (wild type p53) compared to the isogenic p53-null subline, suggesting a potential for stabilization of the pro-apoptotic p53.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org (experimental details, NMR spectra and cells survival curves).

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Data Availability Statement: The data are available from the authors upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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