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Original article

Structure–activity relationship and antitumor activity of thio-benzodiazepines as p53–MDM2 protein–protein interaction inhibitors

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ABSTRACT

In order to discuss the structure–activity relationship (SAR) of the thio-benzodiazepine compounds which showed excellent activity against p53–MDM2 protein–protein interaction, we designed and synthesized twenty compounds with electrophilic and nucleophilic groups on the benzene ring. Among them, compounds **8i** (K_i = 91 nM) and **8n** (K_i = 89 nM) showed better binding activity than that of the reference drug Nutlin-3a (K_i = 121 nM). In addition, *in vitro* antitumor activity against Saos-2, U-2 OS, A549 and NCI-H1299 cell-lines were assayed by the MTT method. Especially, compounds **8i** and **8n** possessed excellent biological activity and good selectivity comparable to Nutlin-3a, which were promising candidates for further evaluation.

1. Introduction

The tumor suppressor p53 is one of the most important proteins in human cancers [1,2]. Its main functions are cell-cycle arrest, DNA repair, and apoptosis [3]. The p53 mutations are very common in human tumors, however it remains wild-type in approximately 50% of human cancers [4]. Although 50% of all human tumors express wild-type p53, many are thought to have inadequate p53 function due to abnormalities in p53 regulation or defective signaling in the p53 pathway [2]. Murine double minute 2 (MDM2) is a protein which can inhibit p53's ability to bind to DNA, activate transcription and promote rapid degradation of p53. In turn, p53 activates the expression of the MDM2 protein in an autoregulatory negative feedback loop [5].

Due to the crucial role of p53 in tumor suppression, reactivation of the p53 function by disruption of the p53–MDM2 interaction using non-peptide small-molecule inhibitors is now recognized as a new and promising strategy for anticancer drug design [6]. So far, many series of small-molecule inhibitors were described, including benzodiazepinediones, nutlins, spiro-oxindoles, quinolinols, isoindolinones, chlorofusin, norbornanes, sulfonamides, chalcones, terphenyls, and piperazine-4-phenyl derivatives [3,7–15], many of which showed relatively weak bioactivity, only the nutlins, the spiro-oxindoles and the benzodiazepinediones are particularly valuable [3,7]. Nultins were the first potent and selective p53–MDM2 interaction inhibitors developed by Roche, and promising candidates RG7112 [16] and RO5503781 [17] entered phase I clinical trials, but their detailed structures were not reported [18]. The spiro-oxindoles developed by Wang's group possessed good pharmacokinetic properties as well as a high binding affinity to MDM2. Among them, one representative candidate MI-219 with a K_i value of 5 nM was a potent and orally active small-molecule inhibitor [19].

In 2005, Grasberger and his co-workers firstly reported 1,4-benzodiazepine-2,5-dione compounds as the small molecule inhibitors of the p53–MDM2 protein–protein interaction [7]. The benzodiazepine was thought to be a "privileged structure" in medicinal chemistry [20]. In our previous work, we first reported and synthesized a series of thio-benzodiazepines with the principle of bioisosterism and found that many compounds had nanomolar affinity toward MDM2 and exhibited potent antitumor activities against the U-2 OS human osteosarcoma cell line (Fig. 1) [21], which is worthy of further structural optimization. Herein, we designed and synthesized a series of thio-benzodiazepines with electrophilic

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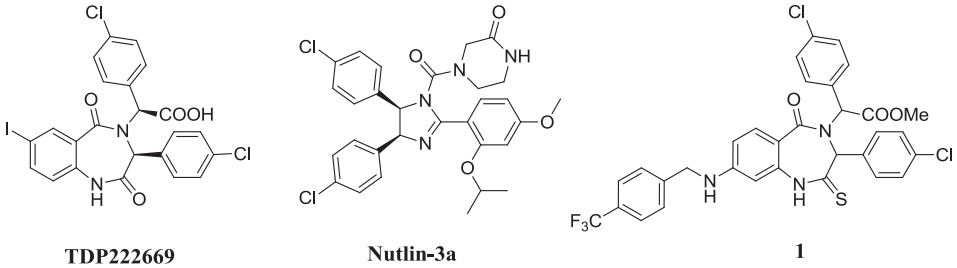


Fig. 1. The structures of TDP222669, Nutlin-3a and **1**.

and nucleophilic groups on the benzene ring to extend the SAR and find promising lead compounds with excellent biological properties.

2. Chemistry

A general pathway for the synthesis of target compounds **8a**–**t** (Table 2) was outlined in Scheme 1. The skeleton of thio-benzodiazepines was synthesized utilizing the highly efficient and versatile Ugi four-component condensation (Ugi 4CC reaction) [22]. We used substituted benzaldehydes (**5**) as the aldehyde, methyl amino(4-chlorophenyl)acetate hydrochloride or methyl 2-amino-2-(4-fluorophenyl) acetate hydrochloride (**3**) as the amine, substituted nitrobenzoic acids (**2**) as the carboxylic acid and 1-isocyanocyclohexene (**4**) as the isocyanide to perform the Ugi reaction. In this synthetic process, the purity of the starting material **4** had significant influence on the yield of Ugi reaction. The obtained key intermediate **7** was treated with the Lawesson's reagent to afford the compounds **8a**–**r** with the yield of 50–87%. In order to increase the structural diversity, two compounds **8s** and **8t** with substituents on sulfur atom were synthesized by the nucleophilic substitution reaction in presence of 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) at room temperature. Owing to the stronger nucleophilic activity of sulfur atom, the substitution reaction took place on sulfur instead of nitrogen atom.

Table 1
Binding constants (K_i) of the MDM2 ligands and IC_{50} values of *in vitro* antitumor activity.

Compounds	K_i[a](μM)	IC_{50}[b] (μM)			
		Saos-2 (p53 null)	U-2 OS (wt-p53)	A549 (wt-p53)	NCI-H1299 (p53 null)
8a	1.37	11.1	12.1	37.2	16.5
8b	>100	61.8	88.5	>100	>100
8c	0.721	8.91	3.50	18.7	24.7
8d	0.707	6.59	12.0	8.44	16.5
8e	>100	3.84	2.61	11.3	5.73
8f	1.72	12.7	44.3	25.6	>100
8g	0.315	6.48	7.23	19.7	29.7
8h	8.18	>100	>100	>100	>100
8i	0.0910	6.78	5.69	26.9	11.1
8j	3.02	13.7	11.8	9.44	14.0
8k	>100	20.0	35.3	>100	>100
8l	>100	19.1	27.8	>100	73.3
8m	37.7	25.5	32.7	>100	73.6
8n	0.0890	11.9	7.53	12.9	14.5
8o	1.05	32.1	28.0	19.4	25.0
8p	3.98	11.0	35.7	>100	35.4
8q	0.518	9.23	27.4	23.7	39.5
8r	11.9	>100	33.9	>100	>100
8s	>100	>100	67.6	>100	>100
8t	21.1	>100	>100	>100	>100
Nutlin-3a	0.121	12.1	19.6	15.0	20.4
1	6.70	25.3	15.9	25.7	42.6

[a] Values were determined by fluorescence polarization assay.
[b] Values were measured with MTT method.

3. Results and discussion

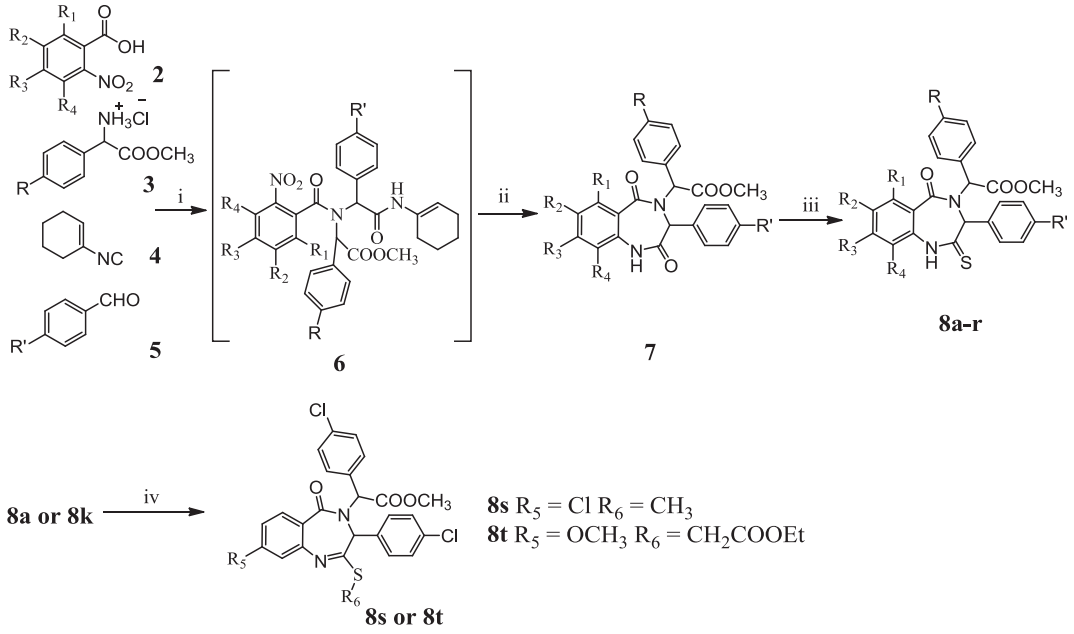
3.1. p53–MDM2 binding assays

The binding K_i constants of small molecule ligands were measured by fluorescence polarization (FP) binding assay. Nutlin-3a, one of the most active small molecule p53–MDM2 inhibitors, was used as the reference drug. The results were listed in Table 1. Most of the targeted thio-benzodiazepines had nanomolar to micromolar affinity toward MDM2. The detailed SAR analysis demonstrated that the proper substituents on the position 7 and 8 were beneficial to the binding activity. In particular, most fluorine-containing compounds on the position 7 or 8 exhibited moderate to excellent affinity. For example, compounds **8i** (K_i = 91 nM) and **8n** (K_i = 89 nM) with trifluoromethyl group and fluorine atom on position 8 showed excellent binding activity superior to the reference compound (K_i = 121 nM). In addition, compounds **8d**, **8f**, **8g**, **8j**, **8o** and **8q** also displayed good binding affinity comparable to Nutlin-3a. However, the difluorinated compound **8e** had relatively weak activity. Interestingly, compound **8c** with three chlorine atoms also showed good binding affinity with a K_i value of 721 nM.

The results also revealed that the substituents on the position 9 generated negative influence on the activity. For instance, compounds **8b**, **8h** and **8l** exhibited weak binding activity. Furthermore, we found that the substituents on sulfur atom led to significant decrease of activity, such as compounds **8s** and **8t**. According to the previously reported structures of the inhibitors binding to MDM2 and our docking model, we presented

Table 2
The structures of thio-benzodiazepines compounds **8a**–**8r**.

compounds	R_1	R_2	R_3	R_4	R	R′
8a	H	H	Cl	H	Cl	Cl
8b	H	H	H	Cl	Cl	Cl
8c	H	Cl	H	H	Cl	Cl
8d	H	H	F	H	Cl	Cl
8e	H	F	F	H	Cl	Cl
8f	H	H	Cl	H	F	F
8g	H	Cl	H	H	F	Cl
8h	H	H	H	OCH_3	Cl	Cl
8i	H	H	CF_3	H	Cl	Cl
8j	H	H	CF_3	H	F	Cl
8k	H	H	OCH_3	H	Cl	Cl
8l	H	H	H	CH_3	Cl	Cl
8m	H	CH_3	H	H	Cl	Cl
8n	H	F	H	H	Cl	Cl
8o	H	H	F	H	F	Cl
8p	H	H	Br	H	Cl	Cl
8q	H	H	Cl	H	Cl	CF_3
8r	H	H	Cl	H	Cl	OCH_3



Scheme 1. Reagents and conditions: (i) MeOH, KOH, r.t., 24 h; (ii) Fe powder, AcOH, 70 °C, 1 h; (iii) Lawesson's reagent, toluene, 70 °C, 4 h (iv) MeI or $BrCH_2COOEt$, DBU, DCM, r.t., 0.5–1 h.

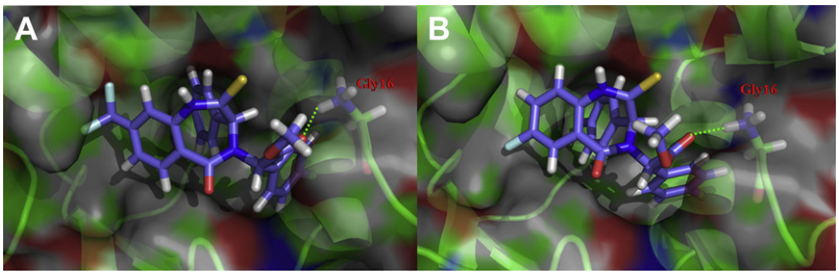


Fig. 2. The docking models of thio-benzodiazepines inhibitors with MDM2. The inhibitors are shown in stick models with carbon atoms light purple, nitrogen blue, oxygen red, fluorine light green and sulfur yellow. Hydrogen bonds are depicted as green dashed lines. The amino acid residues which were formatted H-bonds are labeled. (A) Compound **8i**; (B) Compound **8n**. The figures were prepared from PDB ID: 1T4E, using PyMol (http://pymol.souceforge.net/).

a hypothetical binding model for the thio-benzodiazepine-MDM2 complex (Fig. 2). The binding interactions involved three hydrophobic pockets that were filled by three aromatic rings of the thio-benzodiazepine. This binding model also predicted that the ester group was well positioned as hydrogen bond acceptor with Gly16, which may account for the enhancement of binding activity.

3.2. *In vitro* antitumor activity

All the compounds were further evaluated in MTT assays for ascertaining whether their *in vitro* antitumor activity showed good relevance with the inhibitory activity of p53–MDM2 binding. In order to demonstrate the rationality of this class of thio-benzodiazepines, they were compared with the most potent thio-compound **1** reported by our group [21]. The results (Table 1) revealed that most compounds showed moderate to excellent *in vitro* antitumor activity. With regard to the human osteosarcoma U-2 OS cell line (wild-type p53), many compounds, such as **8a**, **8c**, **8d**, **8e**, **8g**, **8i**, **8j** and **8n**, showed better biological activity than Nutlin-3a and **1**. For the lung cancer A549 cell line (wild-type p53), compounds **8d**, **8e**, **8j** and **8n** displayed higher *in vitro* anti-proliferative activity than Nutlin-3a. At the same time, eight compounds, namely **8a**, **8c**, **8e**, **8g**, **8i**, **8n**, **8p** and **8q**, also exhibited better antitumor activity than the most potent compound **1**. Meaningfully, most compounds revealed good selectivity against the U-2 OS cell line with wild type p53 and the Saos-2 cell line with p53 deficiency. Several compounds also had good inhibitory selectivity against the A549 cell line (wild type p53) and the NCI-H1299 cell line (p53 null). Importantly, based on the data in Table 1, we found that most compounds showed good *in vitro* antitumor activity, which were consistent with the results in the p53–MDM2 binding assays. Two promising compounds, **8i** and **8n** showed excellent p53–MDM2 binding affinity and excellent *in vitro* antitumor activity, which were promising candidates for further evaluation.

4. Conclusion

In conclusion, we designed and synthesized a series of novel thio-benzodiazepines by Ugi reaction with the purpose to explore its SAR. In the p53–MDM2 binding assay, most of targeted compounds have good binding affinity comparable to the reference drug. Moreover, many compounds displayed good activity in *in vitro* antitumor activity assay compared with Nutlin-3a. Interestingly, several compounds exhibited good correlation between the binding activity and *in vitro* antitumor activity. In particular,

two representative compounds, namely **8i** and **8n**, showed excellent biological activity in both p53–MDM2 binding affinity and *in vitro* antitumor activity. A better understanding of this SAR could provide meaningful insights for further optimization.

5. Experimental protocols

5.1. General methods

All reagents and solvents were purchased from commercial suppliers and used as received unless otherwise stated. Melting points were measured on an uncorrected X-5 digital melting point apparatus (Gongyi City Yuhua Instrument Co., Ltd.; China).^{1}H NMR and ^{13}C NMR spectra were recorded on a BRUKER AVANCE 600 spectrometer (Bruker Company, Germany), using TMS as an internal standard and $CDCl_3$ or DMSO-d_6 as solvents. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China). Flash column chromatography (Biotage, LCD 2073 A) was carried out on silica gel 300–400 mesh. Anhydrous solvent and reagents were all analytical pure and dried through routine protocols.

5.2. General procedure for the synthesis of benzodiazepines (**7**) [23]

Powdered substituted 2-nitrobenzoic acid (10 mmol) was added to a well stirred solution of KOH (10 mmol) in CH_3OH (10 mL). The resulting suspension was stirred at room temperature for 10 min and then cooled to 0 °C and treated with methyl 2-amino-2-(4-chlorophenyl)acetate hydrochloride (10 mmol) or 2-amino-2-(4-fluorophenyl)acetate hydrochloride (10 mmol) (**3**), a solution of 1-isocyanocyclohexene (**4**) (11 mmol) in CH_3OH (2 mL) and a solution of *p*-chlorobenzaldehyde or 4-fluorobenzaldehyde (**5**) (10 mmol) in CH_3OH (2 mL), in the order given. The cooling bath was removed and the reaction mixture was stirred at room temperature for 1 day. Removal of the solvent under reduced pressure left a residue which was stirred with H_2O (10 mL) and CH_2Cl_2 (80 mL). The organic layer was dried (Na_2SO_4) and evaporated to dryness. The residue was stirred with boiling hexanes (50 mL) for 5 min. The supernatant liquid was discarded while still warm. An analogous treatment was performed with boiling H_2O (50 mL × 2). The resulting solid product was stirred with AcOH (60 mL). The resulting solution was heated at 50 °C and treated under vigorous stirring with iron powder (40 mmol) in one portion. When the exothermic reaction had subsided, the reaction mixture was heated at 70 °C for 1 h and then allowed to cool and stirred with CH_2Cl_2 (50 mL) and H_2O (50 mL). The resulting suspension was filtered to remove the unreacted iron and the filtrate transferred to a separating funnel. The organic layer was washed with H_2O (50 mL), $NaHCO_3$ (aq. 2%, 50 mL), H_2O (50 mL) and then separated, dried (Na_2SO_4), and evaporated to dryness. The residue was purified by column chromatography (*n*-hexane–ethyl acetate, 5:1) to give **7a**–**r**, yield: 45–52%.

The ^{1}H NMR data of the partly representative benzodiazepines.

5.2.1. Methyl 2-(8-chloro-3-(4-chlorophenyl)-2,5-dioxo-2,3-dihydro-1H-benzo [e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (**7a**)

^{1}H NMR (DMSO-d_6, 600 MHz): δ 10.89 (s, 1H, N–H), 7.52–7.47 (m, 5H, C–H), 7.23–7.06 (m, 5H, Ar–H), 6.92 (s, 1H, Ar–H), 6.29 (s, 1H, C–H), 5.33 (s, 1H, C–H), 3.76 (s, 3H, C–H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 171.35, 170.02, 166.94, 138.40, 135.76, 135.69, 133.87, 132.46, 131.38, 131.24, 129.66, 128.68, 125.77, 125.15, 124.90, 119.82, 62.62, 61.77, 52.91; HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{24}H_{17}Cl_3N_2O_4$: 503.0332; found: 503.0327.

5.2.2. Methyl 2-(9-chloro-3-(4-chlorophenyl)-2,5-dioxo-2,3-dihydro-1H-benzo[e] [1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (**7b**)

^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.39 (s, 1H, N–H), 7.55–7.53 (d, 2H, J = 8.55 Hz, Ar–H), 7.48–7.46 (d, 2H, J = 8.55 Hz, Ar–H), 7.43–7.38 (m, 2H, Ar–H), 7.20–7.04 (m, 5H, Ar–H), 6.19 (s, 1H, C–H), 5.38 (s, 1H, C–H), 3.77 (s, 3H, C–H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 169.69, 169.46, 166.67, 135.78, 133.80, 132.45, 131.45, 131.32, 131.19, 131.17, 129.65, 129.61, 128.61, 128.49, 125.49, 125.23, 123.59, 62.80, 61.69, 52.91; HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{24}H_{17}Cl_3N_2O_4$: 503.0332; found: 503.0333.

5.2.3. Methyl 2-(7-chloro-3-(4-chlorophenyl)-2,5-dioxo-2,3-dihydro-1H-benzo [e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (**7c**)

^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.91 (s, 1H, N–H), 7.51–7.35 (m, 6H, Ar–H), 7.24–7.22 (d, 2H, J = 9.9 Hz, Ar–H), 7.10–7.09 (d, 2H, J = 8.05 Hz, Ar–H), 6.88–6.86 (d, 2H, J = 8.6 Hz, Ar–H), 6.28 (s, 1H, C–H), 5.33(s, 1H, C–H), 3.76 (s, 3H, C–H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 170.92, 169.87, 166.38, 135.76, 133.93, 133.16, 132.77, 131.30, 131.21, 130.59, 130.26, 129.62, 128.70, 127.62, 125.71, 121.41, 62.66, 61.94, 52.88; HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{24}H_{17}Cl_3N_2O_4$: 503.0332; found: 503.0336.

5.3. General procedure for the synthesis of thio-benzodiazepines (**8a**–**r**)

A dry toluene solution of **7a**–**r** (10 mmol) under an atmosphere of nitrogen was treated with Lawesson's reagent (2,4-bis(4-methoxyphenyl)-2,4-disulfide, 5.5 mmol) under 70 °C for 4 h, cooled to room temperature, concentrated under vacuum and purified by column chromatography(*n*-hexane–ethyl acetate, 10:1) to give compounds **8a**–**r**, yield: 50–80%.

5.3.1. Methyl 2-(8-chloro-3-(4-chlorophenyl)-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (**8a**)

According to the general procedure, compound **7a** (5 mmol), Lawesson's reagent (3 mmol) gave compound **8a** as a light-yellow solid (1.619 g, yield: 62%), m.p. 156–158 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.79 (s, 1H, N–H), 7.56–7.46 (m, 5H, C–H), 7.26–7.22 (m, 4H, Ar–H), 7.16 (dd, 1H, J = 10.1, 1.7 Hz, Ar–H), 7.07 (d, 1H, J = 1.4 Hz, Ar–H), 6.21 (s, 1H, C–H), 5.85 (s, 1H, C–H), 3.74 (s, 3H, C–H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 200.03, 169.35, 165.45, 137.49, 136.75, 134.17, 133.52, 132.90, 132.70, 132.61, 132.37, 128.98, 128.95, 127.26, 126.64, 125.96, 120.10, 70.63, 66.03, 52.86; ESI-MS (m/z): 517.25 (M − H).

5.3.2. Methyl 2-(9-chloro-3-(4-chlorophenyl)-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (**8b**)

A light-yellow solid. Yield: 70%. M.p. 151–153 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.17 (s, 1H, N–H), 7.59 (d, 2H, J = 8.55 Hz, Ar–H), 7.47–7.42 (m, 4H, Ar–H), 7.23–7.12 (m, 5H, Ar–H), 6.16 (s, 1H, C–H), 5.92 (s, 1H, C–H), 3.77 (s, 3H, C–H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 204.20, 173.19, 169.29, 138.06, 137.55, 137.34, 137.19, 136.97, 136.80, 136.50, 135.67, 132.94, 132.82, 132.77, 131.86, 130.66, 129.48, 75.48, 70.41, 56.85; ESI-MS (m/z): 517.26 (M − H).

5.3.3. Methyl 2-(7-chloro-3-(4-chlorophenyl)-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (**8c**)

A yellow solid. Yield: 59%. M.p. 260–262 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.84 (s, 1H, N–H), 7.55 (d, 2H, J = 8.55 Hz, Ar–H), 7.48–7.45 (m, 3H, Ar–H), 7.42 (dd, 1H, J = 8.7, 2.6 Hz, Ar–H),

7.26–7.22 (m, 4H, Ar–H), 7.02 (d, 1H, J = 8.7 Hz, Ar–H), 6.22 (s, 1H, C–H), 5.87 (s, 1H, C–H), 3.76 (s, 3H, C–H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 199.93, 169.21, 165.67, 135.92, 133.92, 133.82, 132.68, 131.84, 131.59, 130.91, 130.44, 129.50, 128.88, 128.72, 126.21, 120.59, 67.13, 64.27, 52.77; ESI-MS (m/z): 517.36 (M − H).

*5.3.4. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-fluoro-5-oxo-2- thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl) acetate (**8d**)*

A light-yellow solid. Yield: 58%. M.p. 140–142 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.79 (s, 1H, NH), 7.57–7.55 (m, 3H, Ar–H), 7.46 (d, 2H, J = 8.52 Hz, Ar–H), 7.23 (s, 4H, Ar–H), 6.98–6.95 (m, 1H, Ar–H), 6.82 (dd, 1H, J = 9.9, 2.4 Hz, Ar–H), 6.21 (s, 1H, CH), 5.85 (s, 1H, CH), 3.74 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 200.48, 169.51, 166.40, 165.04, 163.35, 137.20, 137.13, 135.93, 133.89, 133.82, 133.78, 131.99, 131.88, 130.40, 129.53, 128.64, 126.23, 124.14, 124.12, 113.75, 113.60, 106.43, 106.26, 67.15, 64.30, 52.79; ESI-MS (m/z): 501.35 (M − H).

*5.3.5. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-7,8-difluoro-5-oxo-2- thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl) acetate (**8e**)*

A yellow solid. Yield: 69%. M.p. 94–95 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.77 (s, 1H, NH), 7.52 (d, 2H, J = 8.52 Hz, Ar–H), 7.48 (dd, 1H, J = 8.58, 10.62 Hz, Ar–H), 7.44 (d, 2H, J = 8.64 Hz, Ar–H), 7.25 (d, 2H, J = 8.82 Hz, Ar–H), 7.21 (d, 2H, J = 7.98 Hz, Ar–H), 7.04 (dd, 1H, J = 11.2, 7.00 Hz, Ar–H), 6.20 (s, 1H, CH), 5.86 (s, 1H, CH), 3.73 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 200.05, 169.42, 165.29, 164.28, 164.26, 164.14, 136.06, 134.53, 134.42, 134.07, 134.02, 133.96, 131.87, 131.80, 130.14, 129.58, 128.84, 126.13, 67.08, 64.43, 52.89; ESI-MS (m/z): 519.53 (M − H).

*5.3.6. Methyl 2-(8-chloro-3-(4-fluorophenyl)-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-fluorophenyl) acetate (**8f**)*

A yellow solid. Yield: 70%. M.p. 143–144 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.75 (s, 1H, NH), 7.59 (d, 1H, J = 5.52 Hz, Ar–H), 7.58 (d, 1H, J = 5.46 Hz, Ar–H), 7.47 (d, 1H, J = 8.58 Hz, Ar–H), 7.24–7.19 (m, 4H, Ar–H), 7.13 (dd, 1H, J = 2.10, 8.52 Hz, Ar–H), 7.06 (d, 1H, J = 1.98 Hz, Ar–H), 6.98 (t, 2H, J = 8.82 Hz, Ar–H), 6.23 (s, 1H, CH), 5.78 (s, 1H, CH), 3.29 (s, 3H, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 200.43, 169.64, 165.49, (163.71, 162.60), (161.75, 160.66), 137.52, 136.63, 132.91, 132.84, 130.64, 130.03, 127.50, 126.77, 125.83, 120.02, 116.01, 115.89, 70.01, 65.72, 52.80; ESI-MS (m/z): 487.43 (M + H).

*5.3.7. Methyl 2-(7-chloro-3-(4-chlorophenyl)-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-fluorophenyl) acetate (**8g**)*

A light-yellow solid. Yield: 73%. M.p. 120–123 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.70 (s, 1H, NH), 7.53 (d, 1H, J = 5.52 Hz, Ar–H), 7.52 (d, 1H, J = 5.28 Hz, Ar–H), 7.49 (d, 1H, J = 2.52 Hz, Ar–H), 7.38 (dd, 1H, J = 2.58, 8.70 Hz, Ar–H), 7.25–7.17 (m, 6H, Ar–H), 6.97 (d, 1H, J = 8.7 Hz, Ar–H), 6.16 (s, 1H, CH), 5.76 (s, 1H, CH), 3.71 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 199.70, 169.59, 165.92, 164.13, 162.47, 139.19, 134.17, 133.84, 132.61, 132.55, 132.50, 131.97, 131.45, 130.73, 129.84, 128.80, 128.69, 126.23, 124.45, 123.95, 120.98, 67.10, 64.33, 52.78; ESI-MS (m/z): 503.64 (M + H).

*5.3.8. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-9-methoxy-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)acetate (**8h**)*

A light-yellow solid. Yield: 62%. M.p. 123–125 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 11.70 (s, 1H, NH), 7.59 (d, 2H, J = 5.52 Hz, Ar–H), 7.42 (d, 2H, J = 8.58 Hz, Ar–H), 7.17 (s, 4H, Ar–H), 7.06–7.01 (m, 2H, Ar–H), 6.98 (dd, 1H, J = 1.50, 7.92 Hz, Ar–H), 6.12 (s, 1H, CH), 5.86 (s, 1H, CH), 3.73 (s, 6H, CH_3). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 198.88, 169.33, 165.91, 149.60, 133.96, 133.86, 133.37, 132.45, 132.39, 129.63, 128.79, 128.66, 127.26, 126.81, 126.27, 121.33, 114.93, 71.42, 66.33, 57.09, 52.78; ESI-MS (m/z): 515.52 (M + H).

*5.3.9. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-5-oxo-2-thioxo-8-(trifluoromethyl)-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)acetate (**8i**)*

A light-yellow solid. Yield: 53%. M.p. 151–152 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.89 (s, 1H, N–H), 7.68 (d, 1H, J = 8.15 Hz, Ar–H), 7.56 (d, 2H, J = 8.5 Hz, Ar–H), 7.48–7.43 (m, 3H, Ar–H), 7.36 (s, 1H, Ar–H), 7.26–7.21 (m, 4H, Ar–H), 6.24 (s, 1H, C–H), 5.90 (s, 1H, C–H), 3.76 (s, 3H, C–H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 200.19, 169.26, 165.23, 136.84, 134.21, 133.31, 132.76, 132.73, 132.26, 132.20, 131.08, 130.54, 129.70, 129.00, 128.92, 127.28, 126.61, 124.25, 122.44, 122.14, 117.60, 70.58, 66.08, 52.92; ESI-MS (m/z): 553.24 (M + H).

*5.3.10. Methyl 2-(3-(4-chlorophenyl)-5-oxo-2-thioxo-8-(trifluoromethyl)-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-fluorophenyl)acetate (**8j**)*

A light-yellow solid. Yield: 59%. M.p. 132–135 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.89 (s, 1H, N–H), 7.68 (d, 1H, J = 8.25 Hz, Ar–H), 7.62–7.60 (m, 2H, Ar–H), 7.44 (d, 1H, J = 7.45 Hz, Ar–H), 7.36 (s, 1H, Ar–H), 7.26–7.21 (m, 6H, Ar–H), 6.27 (s, 1H, C–H), 5.84 (s, 1H, C–H), 3.75 (s, 3H, C–H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 200.50, 169.69, 166.14, 164.20, 162.54, 135.76, 134.23, 134.00, 133.95, 132.56, 132.51, 132.25, 131.91, 130.28, 128.73, 127.46, 126.22, 122.16, 116.56, 116.42, 66.91, 64.40, 52.82; ESI-MS (m/z): 537.13 (M + H).

*5.3.11. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-methoxy-5-oxo-2-thioxo- 2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)acetate (**8k**)*

A light-yellow solid. Yield: 56%. M.p. 135–136 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.64 (s, 1H, N–H), 7.54 (d, 2H, J = 8.40 Hz, Ar–H), 7.54–7.45 (m, 3H, Ar–H), 7.23 (s, 4H, Ar–H), 6.69 (dd, 1H, J = 2.00, 6.65 Hz, Ar–H), 6.54 (d, 1H, J = 1.6 Hz, Ar–H), 6.18 (s, 1H, C–H), 5.82 (s, 1H, C–H), 3.73 (s, 3H, C–H), 3.69 (s, 3H, C–H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 199.57, 169.57, 166.00, 162.11, 138.05, 134.03, 133.93, 133.24, 132.75, 132.47, 132.33, 128.91, 128.78, 127.20, 120.48, 112.23, 105.25, 70.75, 65.92, 55.94, 52.74; ESI-MS (m/z): 513 (M − H).

*5.3.12. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-9-methyl-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)acetate (**8l**)*

A light-yellow solid. Yield: 50%. M.p. 240–241 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 11.97 (s, 1H, C–H), 7.58 (d, 2H, J = 8.5 Hz, Ar–H), 7.44 (d, 2H, J = 8.55 Hz, Ar–H), 7.30 (d, 1H, J = 7.65 Hz, Ar–H), 7.21–7.12 (m, 5H, Ar–H), 7.01 (t, 1H, J = 7.7 Hz, Ar–H), 6.12 (s, 1H, C–H), 5.88 (s, 1H, C–H), 3.76 (s, 3H, C–H), 2.18 (s, 3H, C–H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 199.92, 169.38, 166.52, 135.09, 133.93, 133.83, 133.47, 132.50, 132.28, 130.11, 128.77, 128.65, 127.86, 126.71, 126.64, 71.51, 66.42, 52.81, 17.94; ESI-MS (m/z): 499.28 (M + H).

*5.3.13. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-7-methyl-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)acetate (**8m**)*

A light-yellow solid. Yield: 71%. M.p. 220–222 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.67 (s, 1H, N–H), 7.54 (d, 2H, J = 8.5 Hz, Ar–H), 7.45 (d, 2H, J = 8.55 Hz, Ar–H), 7.32 (d, 1H, J = 1.25 Hz, Ar–H), 7.24–7.20 (m, 4H, Ar–H), 7.14 (dd, 1H, J = 1.55, 8.35 Hz, Ar–H), 6.88 (d, 1H, J = 8.3 Hz, Ar–H), 6.17 (s, 1H, C–H), 5.87 (s, 1H, C–H), 3.74 (s, 3H, C–H), 2.16 (s, 3H, C–H); ^{13}C NMR (150 MHz,

DMSO-d_6) δ: 198.84, 169.41, 166.23, 135.38, 134.39, 133.96, 133.81, 133.57, 133.31, 132.46, 132.31, 130.44, 128.85, 128.78, 127.48, 127.21, 120.81, 71.10, 66.20, 52.78, 20.59; ESI-MS (m/z): 499.54 (M + H).

5.3.14. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-7-fluoro-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl) acetate(**8n**)

A light-yellow solid. Yield: 66%. M.p. 146–148 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.76 (s, 1H, N–H), 7.55 (d, 2H, J = 8.55 Hz, Ar–H), 7.45 (d, 2H, J = 8.55 Hz, Ar–H), 7.24–7.20 (m, 6H, Ar–H), 7.06–7.02 (m, 1H, Ar–H), 6.20 (s, 1H, C–H), 5.85 (s, 1H, C–H), 3.74 (s, 3H, C–H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 199.19, 169.31, 165.07, (159.83, 158.21), 134.18, 133.54, 133.35, 132.89, 132.66, 132.40, (129.65, 129.60), 128.98, 128.93, 127.24, (123.52, 123.46), (120.40, 120.25), (116.28, 116.11), 70.76, 66.12, 52.90; ESI-MS (m/z): 503.28 (M + H).

5.3.15. Methyl 2-(3-(4-chlorophenyl)-8-fluoro-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-fluorophenyl) acetate (**8o**)

A light-yellow solid. Yield: 80%. M.p. 106–107 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.79 (s, 1H, N–H), 7.56 (d, 2H, J = 8.5 Hz, Ar–H), 7.46 (d, 2H, J = 8.5 Hz, Ar–H), 7.26–7.23 (m, 6H, Ar–H), 7.06–7.04 (m, 1H, Ar–H), 6.22 (s, 1H, C–H), 5.87 (s, 1H, C–H), 3.75 (s, 3H, C–H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 199.18, 169.28, 165.04, (159.98, 158.04), 134.15, 133.52, 133.33, 132.89, 132.63, 132.38, (129.65, 129.59), 128.96, 128.90, 127.22, (123.51, 123.45), (120.39, 120.20), (116.27, 116.08), 70.77, 66.11, 52.87; ESI-MS (m/z): 487.69 (M + H).

5.3.16. Methyl 2-(8-bromo-3-(4-chlorophenyl)-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (**8p**)

A yellow solid. Yield: 73%. M.p. 139–141 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.76 (s, 1H, N–H), 7.55 (d, 2H, J = 8.58 Hz, Ar–H), 7.46 (d, 2H, J = 8.58 Hz, Ar–H), 7.42 (d, 1H, J = 8.46 Hz, Ar–H), 7.29 (dd, 1H, J = 1.86, 8.46 Hz, Ar–H), 7.26–7.24 (m, 4H, Ar–H), 7.21 (d, 1H, J = 1.86 Hz, Ar–H), 6.21 (s, 1H, C–H), 5.75 (s, 1H, C–H), 3.74 (s, 3H, C–H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 200.00, 169.35, 165.57, 137.47, 134.17, 133.51, 132.89, 132.71, 132.60, 132.36, 128.97, 128.83, 127.26, 126.93, 125.47, 122.98, 70.63, 66.03, 52.86; ESI-MS (m/z): 560.89 (M − H).

5.3.17. Methyl 2-(8-chloro-5-oxo-2-thioxo-3-(4-(trifluoromethyl) phenyl)-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl)acetate (**8q**)

A light-yellow solid. Yield: 68%. M.p. 160–162 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.83 (s, 1H, N–H), 7.56 (d, 4H, J = 8.52 Hz, Ar–H), 7.50 (d, 1H, J = 8.58 Hz, Ar–H), 7.46 (d, 4H, J = 8.52 Hz, Ar–H), 7.14 (dd, 1H, J = 2.04, 7.38 Hz, Ar–H), 7.06 (d, 1H, J = 2.04 Hz, Ar–H), 6.23 (s, 1H, C–H), 5.97 (s, 1H, C–H), 3.76 (s, 3H, C–H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 199.84, 169.54, 166.46, 138.47, 137.58, 136.38, 136.09, 132.65, 131.91, 130.26, 130.14, 130.04, 129.63, 126.33, 125.91, 125.51, 125.48, 125.29, 124.37, 122.57, 119.37, 67.25, 64.45, 52.89; ESI-MS (m/z): 551.37 (M − H).

5.3.18. Methyl 2-(8-chloro-3-(4-methoxyphenyl)-5-oxo-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl)acetate (**8r**)

A yellow solid. Yield: 75%. M.p. 137–138 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 12.72 (s, 1H, N–H), 7.57–7.47 (m, 5H, Ar–H), 7.15–7.08 (m, 4H, Ar–H), 6.70 (d, 2H, J = 8.8 Hz, Ar–H), 6.23 (s, 1H, C–H), 5.78 (s, 1H, C–H), 3.74 (s, 3H, C–H), 3.61 (s, 3H, C–H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 201.03, 169.45, 165.60, 158.85, 137.54, 136.52, 134.12, 132.97, 132.56, 132.41, 128.95, 126.74, 126.61, 125.99, 125.69, 119.93, 114.26, 70.54, 65.87, 55.42, 52.77; ESI-MS (m/z): 515.83 (M + H).

5.4. General procedure for the synthesis of **8s** and **8t**

To a dry CH_2Cl_2 solution of **8a** or **8k** (5 mmol) was added MeI (5 mmol) or ethyl bromoacetate (5 mmol), then DBU (5 mmol) was added to the resulting solution and stirred at room temperature for 30–60 min. After that, the reaction mixture was concentrated under vacuum and purified by column chromatography (n-hexane–ethyl acetate, 10:1) to give **8s** or **8t**, yield: 90–95%.

5.4.1. Methyl 2-(8-chloro-3-(4-chlorophenyl)-2-(methylthio)-5-oxo-3H-benzo[e] [1,4]diazepin-4(5H)-yl)-2-(4-chlorophenyl)acetate (**8s**)

A light-yellow solid. Yield: 95%. M.p. 185–187 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 7.53 (d, 1H, J = 8.46 Hz, Ar–H), 7.49 (d, 2H, J = 8.58 Hz, Ar–H), 7.46 (d, 2H, J = 8.76 Hz, Ar–H), 7.21 (d, 2H, J = 8.58 Hz, Ar–H), 7.17 (d, 2H, J = 8.46 Hz, Ar–H), 7.11 (dd, 1H, J = 2.16, 8.58 Hz, Ar–H), 7.03 (d, 1H, J = 2.10 Hz, Ar–H), 6.02 (s, 1H, CH), 5.44 (s, 1H, CH), 3.75 (s, 3H, CH_3), 2.52 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 170.10, 169.58, 167.38, 145.98, 137.63, 135.83, 133.48, 132.97, 131.57, 131.50, 130.74, 129.52, 128.34, 126.25, 125.71, 125.33, 125.29, 64.31, 60.60, 52.76, 14.38; ESI-MS (m/z): 533.52 (M + H).

5.4.2. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-2-((2-ethoxy-2-oxoethyl) thio)-8-methoxy-5-oxo-3H-benzo[e][1,4] diazepin-4(5H)-yl)acetate (**8t**)

A light-yellow solid. Yield: 90%. M.p. 130–131 °C. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 7.49–7.44 (m, 5H, Ar–H), 7.25–7.19 (m, 4H, Ar–H), 6.66 (dd, 1H, J = 2.45, 8.85 Hz, Ar–H), 6.28 (d, 1H, J = 2.4 Hz, Ar–H), 5.98 (s, 1H, C–H), 5.49 (s, 1H, C–H), 4.20–4.16 (m, 2H, C–H), 4.01 (s, 2H, C–H), 3.75 (s, 3H, C–H), 3.69 (s, 3H, C–H), 1.21 (t, 3H, J = 7.1 Hz, C–H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 170.36, 167.96, 167.82, 167.29, 162.16, 145.86, 135.72, 133.19, 133.07, 132.15, 131.92, 131.16, 129.54, 127.93, 127.21, 119.95, 112.53, 109.40, 62.09, 61.71, 60.01, 55.23, 52.54, 33.37, 14.21; ESI-MS (m/z): 601.59 (M + H).

5.5. Computational protocol

Molecular docking was used to predict the binding mode of the synthesized thio-benzodiazepine derivatives. The crystal structure [7] of MDM2 (PDB code: 1T4E) was prepared by removing the benzodiazepine and adding hydrogen atoms in MVD 4.3.0. We used two known potent inhibitors of the p53–MDM2 interaction with different chemical structures (TDP222669 and nutlin-3a) as positive controls. The docking parameters of MVD were similar to the literatures [24]. The active site was defined to encompass all MDM2 atoms within a 12 Å radius sphere from the center of the Trp23 of the p53 peptide ligand. Other parameters were set by default.

5.6. p53–MDM2 binding assay

The dose-dependent binding experiments were carried out with serial dilution in DMSO of compounds. A 5 μL sample of the tested sample and preincubated (for 30 min) MDM2 binding domain (1–118) (10 nM) and PMDM-F peptide (Anaspec) (10 nM) in the assay buffer (100 mM potassium phosphate, pH 7.5; 100 μg/mL bovine gamma globulin; 0.02% sodium azide was added into black 96-well microplates with F-bottom and chimney wells (Corning) to produce a final volume of 115 μl. For each assay, the controls included the MDM2 binding domain and PMDM-F. The polarization values were measured after 1 h of incubation at room temperature using Biotek Synergy H2 with a 485 nm excitation filter, a 528 nm static and

polarized filter. The K_i values were determined from a plot using nonlinear least-squares analysis. And curve fitting was performed using GraphPad Prism software. Nutlin-3a (Sigma–Aldrich), the first potent and specific non-peptide small-molecule MDM2 inhibitor was used as reference compound for validating the assay in each plate.

5.7. In vitro antitumor activity

The cellular growth inhibitory activity was determined using two human osteosarcoma cell lines [U-2 OS (wild type p53), Saos-2 (p53 null)]. $5–6 \times 10^4$ cells per well were plated in 96-well plates. After culturing for 24 h, test compounds were added onto triplicate wells with different concentrations and 0.1% DMSO for control. After 72 h of incubation, 20 μL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-di phenyltetrazoliumbromide) solution (5 mg/mL) was added to each well, and after the samples were shaken for 1 min the plate was incubated further for 4 h at 37 °C. Thio-benzodiazepines were dissolved with 100 μL of DMSO. The absorbance (OD) was quantitated with microplate using Biotek Synergy H2 at 570 nm. Wells containing no drugs were used as blanks. Concentration of the compounds that inhibited cell growth by 50% (IC_{50}) was calculated.

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