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Original article

Synthesis and biological evaluation of thio-benzodiazepines as novel small molecule inhibitors of the p53–MDM2 protein–protein interaction

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ABSTRACT

A series of thio-benzodiazepine p53–MDM2 inhibitors were designed and synthesized based on the principle of bioisosterism. Most of the thio-benzodiazepines had nanomolar to micromolar affinity toward MDM2. Particularly, compounds **8a** ($K_i = 0.52$ μM) and **8f** ($K_i = 0.32$ μM) showed binding activity comparable to the positive drug nutlin-3a ($K_i = 0.23$ μM). Meanwhile, compound **8j** exhibited excellent antitumor activity against the U-2 OS human osteosarcoma cell line with an IC_{50} value of 1.06 μM, which was about 23 times higher than that of nutlin-3a. The docking model also successfully predicted that this class of compounds mimicked three p53 critical residues binding to MDM2. The thio-benzodiazepines represent a promising class of non-peptide inhibitors of the p53–MDM2 interaction.

1. Introduction

The significance of the p53 tumor suppressor is not only from its pivotal role in DNA-repair and cell-cycle control but also from its overarching regulating role in carcinogenesis [1,2]. In approximately 50% of all human cancers, the function of p53 has been disannulled by deletions or mutations [3]. While in the remaining half of human cancers, p53 retains its wild-type form but its activity is effectively inhibited through direct interaction with the human murine double minute 2 (MDM2) oncoprotein [4].

Using a non-peptide small molecule inhibitor to restore the impaired function of the p53 protein by disrupting the p53–MDM2 interaction offers an innovative avenue for the treatment of a broad spectrum of cancers [4–6]. In recent years, a number of non-peptide small molecule inhibitors of the p53–MDM2 interaction have been reported [6–12]. These inhibitors can mainly be classified into nutlin derivatives, spiro-oxindole derivatives and benzodiazepine derivatives [6,9,10]. The benzodiazepine-based inhibitor was reported by Grasberger *et al.* in 2005 [9]. The scaffold was identified from a library of 300,000 compounds by high-throughput screening (HTS) using the temperature-dependent protein-unfolding assay ThermoFluor [13]. The strongest MDM2-binding compound from this family was further optimized to TDP222669 (Fig. 1), which has a K_i value of 80 nM and was confirmed to be active *in vitro* [14,15].

However, this compound suffered from low bioavailability and rapid *in vivo* clearance. Herein, we reported a series of thio-benzodiazepines as small molecule inhibitors of p53–MDM2 interaction. In view of the sulfur atom's wide application in drug design [16–18], the thio-benzodiazepines were designed based on the principle of bioisosterism. Biological assay showed that the thio-benzodiazepines possessed both p53–MDM2 inhibitory activity and *in vitro* antitumor activity.

2. Chemistry

As depicted in Scheme 1, thio-benzodiazepines were synthesized utilizing the highly efficient and versatile Ugi four-component condensation (Ugi 4CC) reaction, which is a multi-component reaction involving a ketone (or aldehyde), an amine, an isocyanide and a carboxylic acid to form a bis-amide [19]. In this study, we used *p*-chlorobenzaldehyde (**2**) as the aldehyde, methyl amino(4-chlorophenyl)acetate hydrochloride (**1**) as the amine, substituted nitrobenzoic acid (**4a**,**b**) as the carboxylic acid and 1-isocyanocyclohexene (**3**) as the isocyanide to perform the Ugi reaction and the

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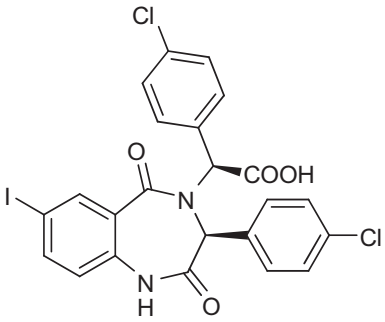


Fig. 1. Structure of TDP222669.

conditions were similar to those in the literature [20]. Followed by treating with iron powder, the key intermediates **6a**,**b** were obtained. Reductive amination reactions were performed between compound **6b** and commercially available aldehydes to give the compounds **7a**–**p**. Finally, treatment of compounds **7a**–**p** with the Lawesson's Reagent afforded the target compounds **8a**–**q** with the yields of 54.9–86.1%.

3. Results and discussion

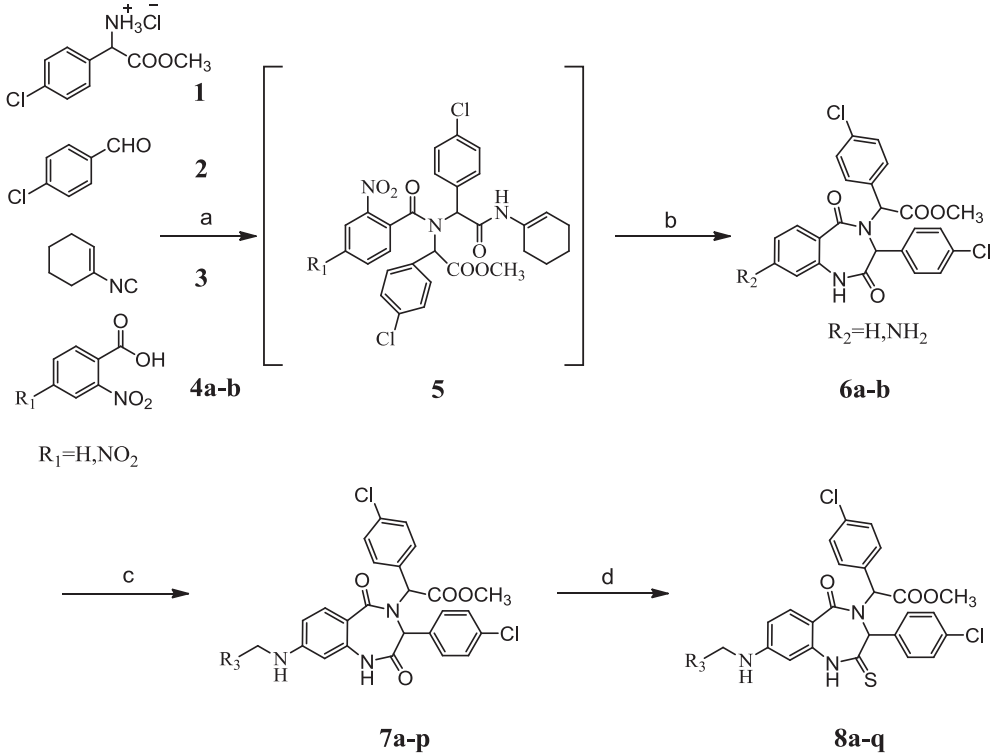
3.1. p53–MDM2 binding assay and structure–activity relationships

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 inhibitor, was used as the reference drug. The results were presented in Table 1. Most of the thio-benzodiazepines had nanomolar to micromolar affinity towards MDM2. SAR analysis showed that the replacement of the oxygen atom of the benzodiazepine scaffold (compound **6a**, K_i = 10.6 μM) by the sulfur atom (compound **8a**, K_i = 0.52 μM) led to the increase of the activity toward MDM2. Moreover, the compounds with aliphatic substituents on the benzene ring displayed moderate to high affinity against p53–MDM2. Particularly, compounds **8a** (K_i = 0.52 μM) and **8f** (K_i = 0.32 μM) showed comparable binding activity to nutlin-3a (K_i = 0.23 μM). However, for the compounds with aromatic

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

Compounds	R_1	R_2	R_3	K_i[a](μM)	IC50[b] (μM)	
					Saos-2 (p53 null)	U-2 OS (wt-p53)
6a	H	H	–	10.6	5.58	13.94
8a	H	H	–	0.52	6.55	13.68
8b	$-NO_2$	$-NH_2$	$-CH_2CH_3$	1.02	12.41	91.17
8c	$-NO_2$	$-NH_2$	-Ph	2.26	17.76	>100
8d	$-NO_2$	$-NH_2$	-4-Cl-Ph	>100	13.3	20.9
8e	$-NO_2$	$-NH_2$	$-C(CH_3)_3$	>100	2.19	17.98
8f	$-NO_2$	$-NH_2$	$-CH(CH_3)_2$	0.32	15.74	>100
8g	$-NO_2$	$-NH_2$	$-(CH_2)_3CH_3$	15	20.66	>100
8h	$-NO_2$	$-NH_2$	$-(CH_2)_2CH_3$	49.6	27.85	>100
8i	$-NO_2$	$-NH_2$	-cyclopropyl	3.43	19.04	>100
8j	$-NO_2$	$-NH_2$	-4–CF_3-Ph	5.34	2.87	1.06
8k	$-NO_2$	$-NH_2$	-cyclohexyl	>100	72.23	>100
8l	$-NO_2$	$-NH_2$	-2-naphthyl	>100	91.74	>100
8m	$-NO_2$	$-NH_2$	-4–OCH_3-Ph	>100	88.12	>100
8n	$-NO_2$	$-NH_2$	-3-thienyl	>100	3.37	>100
8o	$-NO_2$	$-NH_2$	-4-Br-Ph	>100	13.8	14.73
8p	$-NO_2$	$-NH_2$	-4-F-Ph	1.71	9.81	30.58
8q	$-NO_2$	$-NH_2$	-2-furyl	43.1	11.28	48.53
Nutlin-3a	___			0.23	54.38	24.61

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



Scheme 1. Synthetic Route. Reagents and conditions: (a) CH_3OH, KOH, r.t., 3 days; (b) Fe powder, CH_3COOH, 65–70 °C, 1 h; (c) R_3CHO, $NaBH_3CN$, r.t., 16 h; (d) Lawesson's Reagent, toluene, 70 °C, 4 h.

substituents, only the fluorine-containing compounds (**8j** and **8p**) revealed moderate affinity. The crystal structure of MDM2 and p53 peptide (residues 15–29) provided insight into p53–MDM2 interaction [21]. The model revealed that p53 formed an amphipathic α-helix interaction on the MDM2 protein surface containing three key amino acid (Phe19, Trp23 and Leu26). Our docking models (Fig. 2) demonstrated that thio-benzodiazepines could interact with MDM2 by filling its Trp23 subpocket with *p*-chlorobenzyl group. The substituted phenyl ring and 1-(4-chlorobenzyl) group are located in the Phe19 and Leu26 pockets, respectively. Based on the previously reported structures of the antagonists binding to MDM2 [8] and our docking model, we proposed a binding model for the thio-benzodiazepine-MDM2 complex (Fig. 3). The binding interaction involves three lyophobic pockets that are filled by the three aromatic rings of the thio-benzodiazepine. This binding model predicts that the ester group is well positioned as hydrogen bond acceptor with Gly16 or Ser17, while the secondary amine functions as a donator to form a hydrogen bond with Gly58 (Fig. 2). This prediction was further validated by the inhibitory activity of compounds **8a** and **8f** (Fig. 2**A** and **C**). Furthermore, with regard to thio-benzodiazepines with aromatic substituents, the affinity with the MDM2 decreased significantly, which may be accounted for the changed binding mode to the hydrophobic pocket Phe19 due to their relatively larger steric volumes (Fig. 2B). In addition, the docking models also illustrated that the sulfur atom with better lipid solubility was easier to be exposed to the solvents, which may explain the fact that the affinity of compound **8a** is 20 times higher than that of **6a**.

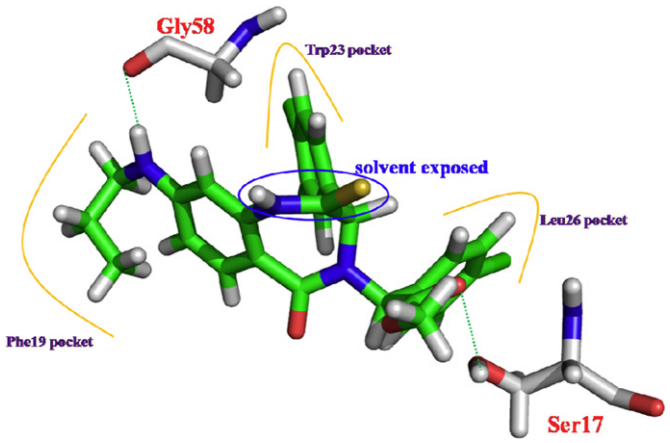


Fig. 3. 3D schematic binding model for the complex of thio-benzodiazepines and MDM2.

3.2. *In vitro antitumor activity*

All the compounds were further evaluated in MTT assays for ascertaining whether their *in vitro* antitumor activity was consistent with the inhibitory activity of p53–MDM2 binding. According to the results (Table 1), compounds **6a**, **8a**, **8d**, **8e**, **8j** and **8o** showed better biological activity against the U-2 OS cell line with wild-type p53 than nutlin-3a. Especially, compound **8j** showed the best antitumor activity (IC_{50} = 1.06 μM). However, compounds **8k**, **8l**, **8m** and **8n** displayed weak *in vitro* antitumor activity, which were consistent with the results of the p53–MDM2 binding assays. Interestingly, compound **8j** was more active against the U-2 OS cell line with wild-type p53 than the Saos-2 cell line with p53 deficient. Therefore, compound **8j** can be used as a lead compound and further structural modification on solvent exposed position is necessary for enhancing its antitumor activity. Further pharmacological and toxicological evaluation of this promising compound is in progress.

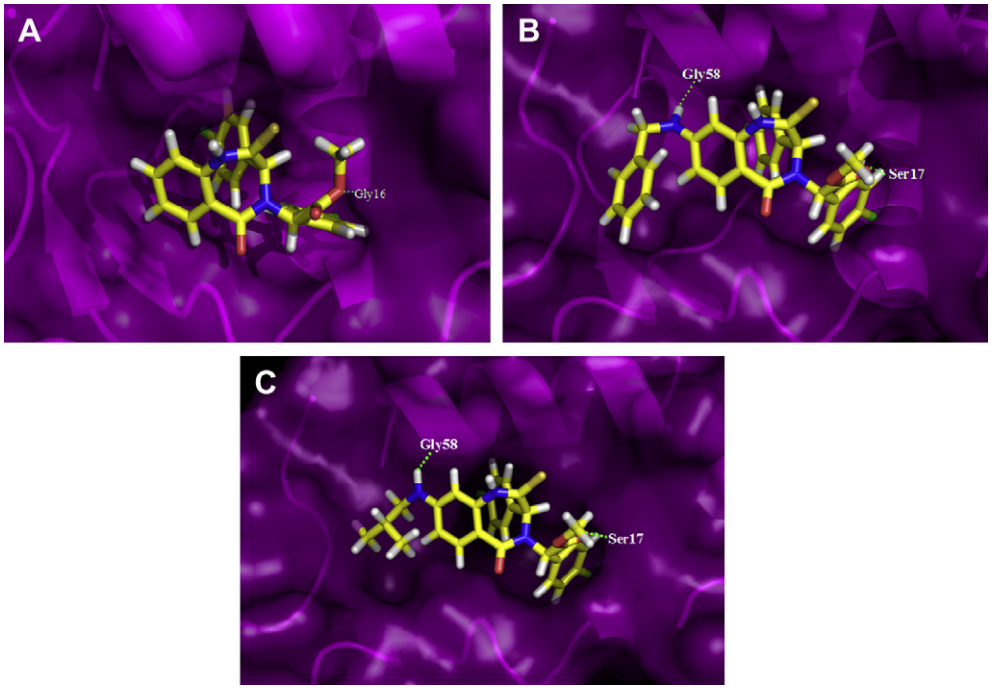


Fig. 2. The docking models of thio-benzodiazepines inhibitors with MDM2. The inhibitors are shown in stick models with carbon atoms colored yellow, nitrogen blue, oxygen red and sulfur deep yellow. Hydrogen bonds are depicted as green dashed lines. The amino acid residues which were formatted H-bonds are labeled. (A) Compound **8a**; (B) Compound **8b**; (C) Compound **8f**. The figures were prepared from PDB entry: 1T4E, Using PyMol (http://pymol.souceforge.net/). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

A series of thio-benzodiazepines were designed and synthesized via Ugi reaction with the purpose to find more potent p53–MDM2 inhibitors. In the p53–MDM2 binding assay, the affinity of thio-benzodiazepines with MDM2 had been largely increased compared with the benzodiazepine derivative. Moreover, compounds **8a** and **8f** showed good binding activity in nanomolar range, which were comparable to the positive drug nutlin-3a. In addition, all the compounds were evaluated for *in vitro* antitumor activity against two human osteosarcoma cell lines. For the U-2 OS cell line, compounds **8a**, **8d**, **8e**, **8j** and **8o** showed higher *in vitro* inhibitory activity than nutlin-3a. Furthermore, compound **8j** showed potent antitumor activity with an IC_{50} value of 1.06 μM. Several highly potent compounds are being further evaluated utilizing *in vivo* models. In conclusion, this class of thio-benzodiazepines can be used as promising lead structures 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 500 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. Elemental analyses were performed with a MOD-1106 instrument and were consistent with theoretical values within ±0.4%. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China). Flash column chromatography was carried out on silica gel 300–400 mesh. Anhydrous solvent and reagents were all analytical pure and dried through routine protocols.

5.2. Synthesis of target compounds

5.2.1. General procedure A: synthesis of benzodiazepines via Ugi 4CC

Powdered 2-nitrobenzoic acid or 2, 4-dinitrobenzoic acid (**4a**,**b**) (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 (**1**) (10 mmol), a solution of 1-isocyanocyclohexene (**3**) (11 mmol) in CH_3OH (2 mL) and a solution of *p*-chlorobenzaldehyde (**2**) (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 3 days. 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 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 45 °C and treated under vigorous stirring with iron powder (50 mmol) in one portion. When the exothermic reaction had subsided, the reaction mixture was heated at 65–70 °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 **6a**,**b**, yields: 37.5–41.3%.

5.2.2. General procedure B: reductive amination

Aldehyde (10 mmol) and **6b** (10 mmol) were mixed in CH_3OH (10 mL), adjusted to pH 6–7 with acetic acid. After stirring at room temperature for 30 min, $NaBH_3CN$ (15–20 mmol) was then added to the solvent, stirred for another 16 h. Removal of the solvent under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2–CH_3OH, 100:1) to give **7a**–**q**, yields: 25.0–74.3%.

5.2.3. General procedure C: synthesis of thio-benzodiazepines

A dry toluene solution of **7a**–**q** (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 **8a**–**q**, yields: 54.9–86.1%.

5.2.4. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-2, 5-dioxo-2, 3-dihydro -1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (*6a*)

Following General Procedure A, 2-nitrobenzoic acid (**4a**) (10 mmol), compound **1** (10 mmol), *p*-chlorobenzaldehyde (**2**) (10 mmol) and 1-isocyanocyclohexene (**3**) (11 mmol) got compound **6a** as a white solid (1.755 g, yield: 37.5%). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.78 (s, 1H), 6.84–7.53 (m, 12H, Ar-H), 6.29 (s, 1H), 5.32 (s, 1H), 3.77 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 169.97, 169.65, 167.15, 135.77, 134.12, 133.57, 133.48, 132.84, 132.52, 131.96, 130.60, 129.21, 128.78, 126.78, 126.70, 124.36, 120.56, 65.35, 64.73, 52.92. ESI-MS (m/z): 467.57 $[M-1]^-$. Anal. calcd. for $C_{24}H_{18}Cl_2N_2O_4$: C, 61.42; H, 3.87; N, 5.97. Found: C, 61.53; H, 3.86; N, 5.95.

5.2.5. Methyl 2-(8-amino-3-(4-chlorophenyl)-2, 5-dioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4(5H)-yl)-2-(4-chlorophenyl) acetate (*6b*)

Following General Procedure A, 2, 4-dinitrobenzoic acid (**4b**) (10 mmol), compound **1** (10 mmol), *p*-chlorobenzaldehyde (**2**) (10 mmol) and 1-isocyanocyclohexene (**3**) (11 mmol) gave compound **6b** as a light-yellow solid (2.0 g, yield: 41.3%). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 6.18–7.48 (m, 11H, Ar-H), 5.90 (s, 1H), 5.72 (s, 2H), 5.20 (s, 1H), 3.72 (s, 3H). ESI-MS (m/z): 485.29 $[M+1]^+$. Anal. calcd. for $C_{24}H_{19}Cl_2N_3O_4$: C, 59.52; H, 3.95; N, 8.68. Found: C, 59.40; H, 3.96; N, 8.70.

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

Following General Procedure B, propaldehyde (28 mg) and **6b** (240 mg) gave **7a** (200 mg) as a white solid, yield: 76.5%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 6.19–7.48 (m, 11H, Ar-H), 6.19 (s, 1H), 5.90 (s, 1H), 5.21 (s, 1H), 3.72 (s, 3H), 2.84 (t, 2H, J = 5.5 Hz), 1.46 (m, 2H), 0.85 (t, 3H, J = 7.3 Hz). ESI-MS (m/z): 526.80 $[M+1]^+$, 524.43 $[M-1]^-$. Anal. calcd. for $C_{27}H_{25}Cl_2N_3O_4$: C, 61.60; H, 4.79; N, 7.98; Found: C, 61.45; H, 4.80; N, 7.99.

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

Following by General Procedure B, benzaldehyde (53.6 mg) and **6b** (240 mg) gave **7b** (170 mg) as a white solid, yield: 58.1%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.45 (s, 1H), 6.21–7.45 (m, 16H, Ar-H), 6.20 (s, 1H), 5.96 (s, 1H), 5.20 (s, 1H), 4.17 (d, 2H, J = 5.9 Hz), 3.72 (s, 3H). ESI-MS (m/z): 572.59 $[M-1]^-$. Anal. calcd. for $C_{31}H_{25}Cl_2N_3O_4$: C, 64.81; H, 4.39; N, 7.31; Found: C, 64.69; H, 4.40; N, 7.33.

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

Following General Procedure B, 4-chlorobenzaldehyde (30 mg) and **6b** (100 mg) gave **7c** (31.3 mg) as a white solid, yield: 25.0%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.45 (s, 1H), 6.20–7.46 (m, 15H, Ar-H), 6.17 (s, 1H), 5.94 (s, 1H), 5.20 (s, 1H), 4.17 (d, 2H, J = 6.0 Hz), 3.71 (s, 3H). ESI-MS (m/z): 606.62 $[M - 1]^-$. Anal. calcd. for $C_{31}H_{24}Cl_3N_3O_4$: C, 61.15; H, 3.97; N, 6.90; Found: C, 61.05; H, 3.98; N, 6.92.

*5.2.9. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-(neopentylamino) –2, 5-dioxo-2, 3-dihydro-1H-benzo[e] [1,4] diazepin-4 (5H)-yl) acetate (**7d**)*

Following General Procedure B, pivalaldehyde (18 mg) and **6b** (100 mg) gave **7d** (51.4 mg) as a white solid, yield: 45.0%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.40 (s, 1H), 6.22–7.47 (m, 11H, Ar-H), 6.19 (s, 1H), 5.98 (s, 1H), 5.21 (s, 1H), 3.72 (s, 3H), 2.75 (m, 2H), 0.85 (s, 9H). ESI-MS (m/z): 554.82 $[M - 1]^-$. Anal. calcd. for $C_{29}H_{29}Cl_2N_3O_4$: C, 62.82; H, 5.27; N, 7.58; Found: C, 62.72; H, 5.28; N, 7.60.

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

Following General Procedure B, isobutyraldehyde (13 mg) and **6b** (87 mg) gave **7e** (41.6 mg) as a white solid, yield: 41.8%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.42 (s, 1H), 6.20–7.47 (m, 11H, Ar-H), 6.19 (s, 1H), 5.92 (s, 1H), 5.22 (s, 1H), 3.72 (s, 3H), 2.72 (m, 2H), 1.74 (m, 1H), 0.85 (d, 6H, J = 6.6 Hz). ESI-MS (m/z): 1103.59 $[2M + Na]^+$. Anal. calcd. for $C_{28}H_{27}Cl_2N_3O_4$: C, 62.23; H, 5.04; N, 7.78; Found: C, 62.10; H, 5.05; N, 7.80.

*5.2.11. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-2, 5-dioxo-8-(pentyl amino)-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7f**)*

Following General Procedure B, pentanal (21.5 mg) and **6b** (120 mg) gave **7f** (90 mg) as a white solid, yield: 65.6%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 6.18–7.47 (m, 11H, Ar-H), 6.17 (s, 1H), 5.90 (s, 1H), 5.21 (s, 1H), 3.72 (s, 3H), 2.87 (t, 2H, J = 5.8 Hz), 1.44 (m, 2H), 1.25 (m, 4H), 0.85 (t, 3H, J = 6.4 Hz). ESI-MS (m/z): 554.95 $[M + 1]^+$. Anal. calcd. for $C_{29}H_{29}Cl_2N_3O_4$: C, 62.82; H, 5.27; N, 7.58; Found: C, 62.69; H, 5.28; N, 7.60.

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

Following General Procedure B, n-butanal (17.9 mg) and **6b** (120 mg) gave **7g** (70 mg) as a white solid, yield: 52.5%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 6.19–7.48 (m, 11H, Ar-H), 6.18 (s, 1H), 5.90 (s, 1H), 5.22 (s, 1H), 3.72 (s, 3H), 2.88 (t, 2H, J = 6.1 Hz), 1.44 (m, 2H), 1.25 (m, 2H), 0.86 (t, 3H, J = 7.3 Hz). ESI-MS (m/z): 540.90 $[M + 1]^+$, 538.68 $[M - 1]^-$. Anal. calcd. for $C_{28}H_{27}Cl_2N_3O_4$: C, 62.23; H, 5.04; N, 7.78; Found: C, 62.40; H, 5.03; N, 7.77.

*5.2.13. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-(cyclopropyl methylamino)-2, 5-dioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7h**)*

Following General Procedure B, cyclopropanecarbaldehyde (14.5 mg) and **6b** (100 mg) gave **7h** (65 mg) as a white solid, yield: 58.6%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 6.22–7.48 (m, 11H, Ar-H), 6.19 (s, 1H), 5.93 (s, 1H), 5.22 (s, 1H), 3.72 (s, 3H), 2.80 (t, 2H, J = 6.1 Hz), 0.94 (m, 1H), 0.40 (t, 2H, J = 4.0 Hz), 0.15 (t, 2H, J = 4.5 Hz). ESI-MS (m/z): 536.70 $[M - 1]^-$. Anal. calcd. for $C_{28}H_{25}Cl_2N_3O_4$: C, 62.46; H, 4.68; N, 7.80; Found: C, 62.35; H, 4.69; N, 7.82.

*5.2.14. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-2, 5-dioxo-8-(4 -(trifluoromethyl) benzylamino)-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7i**)*

Following General Procedure B, 4-(trifluoromethyl)benzaldehyde (37 mg) and **6b** (100 mg) gave **7i** (82 mg) as a white solid, yield: 61.8%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 6.19–7.66 (m, 15H, Ar-H), 6.17 (s, 1H), 5.94 (s, 1H), 5.20 (s, 1H), 4.29 (d, 2H, J = 5.8 Hz), 3.71 (s, 3H). ESI-MS (m/z): 642.92$[M + 1]^+$. Anal. calcd. for $C_{32}H_{24}Cl_2F_3N_3O_4$: C, 59.82; H, 3.77; N, 6.54; Found: C, 59.72; H, 3.78; N, 6.55.

*5.2.15. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-(cyclohexyl methylamino)-2, 5-dioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7j**)*

Following General Procedure B, cyclohexanecarbaldehyde (23.2 mg) and **6b** (100 mg) gave **7j** (72 mg) as a white solid, yield: 60.2%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.41 (s, 1H), 6.19–7.47 (m, 11H, Ar-H), 6.18 (s, 1H), 5.90 (s, 1H), 5.21 (s, 1H), 3.72 (s, 3H), 2.74 (m, 2H), 1.98 (m, 1H), 1.65–0.85 (m, 10H). ESI-MS (m/z): 1184.27 $[2M + Na]^+$. Anal. calcd. for $C_{31}H_{31}Cl_2N_3O_4$: C, 64.14; H, 5.38; N, 7.24; Found: C, 64.24; H, 5.37; N, 7.21.

*5.2.16. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-(naphthalen-2-yl methylamino)-2, 5-dioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7k**)*

Following General Procedure B, 2-naphthaldehyde (32.3 mg) and **6b** (100 mg) gave **7 k** (93 mg) as a white solid, yield: 72.2%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 7.86 (m, 2H), 7.84 (m,1H), 7.79 (s, 1H), 7.37–7.67 (m, 7H), 7.14 (m, 1H), 7.03 (m, 2H), 7.00 (m, 3H), 6.25 (d, 1H, Ar-H, J = 8.6 Hz), 6.18 (s, 1H), 6.01 (s, 1H), 5.19 (s, 1H), 4.34 (d, 2H, J = 5.9 Hz), 3.71 (s, 3H). ESI-MS (m/z): 624.41 $[M + 1]^+$. Anal. calcd. for $C_{35}H_{27}Cl_2N_3O_4$: C, 67.31; H, 4.36; N, 6.73; Found: C, 67.42; H, 4.35; N, 6.71.

*5.2.17. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-(4-methoxy benzylamino)-2, 5-dioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7l**)*

Following General Procedure B, 4-methoxybenzaldehyde (28.1 mg) and **6b** (100 mg) gave **7l** (78 mg) as a white solid, yield: 62.6%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.44 (s, 1H), 6.21–7.46 (m, 15H, Ar-H), 6.18 (s, 1H), 5.97(s, 1H), 5.20 (s, 1H), 4.09 (d, 2H, J = 5.8 Hz), 3.72 (s, 3H), 3.71 (s, 3H). ESI-MS (m/z): 1231.41 $[2M + Na]^+$. Anal. calcd. for $C_{32}H_{27}Cl_2N_3O_5$: C, 63.58; H, 4.50; N, 6.95; Found: C, 63.68; H, 4.49; N, 6.94.

*5.2.18. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-2, 5-dioxo-8- (thiophen-3-ylmethylamino)-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7m**)*

Following General Procedure B, thiophene-3-carbaldehyde (23.2 mg) and **6b** (100 mg) gave **7m** (86 mg) as a white solid, yield: 71.9%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.46 (s, 1H), 6.23–7.47 (m, 14H, Ar-H), 6.19 (s, 1H), 5.99 (s, 1H), 5.22 (s, 1H), 4.13 (d, 2H, J = 5.7 Hz), 3.72 (s, 3H). ESI-MS (m/z): 580.74 $[M + 1]^+$. Anal. calcd. for $C_{29}H_{23}Cl_2N_3O_4S$: C, 60.00; H, 3.99; N, 7.24; Found: C, 60.11; H, 3.98; N, 7.22.

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

Following General Procedure B, 4-bromobenzaldehyde (38.2 mg) and **6b** (100 mg) gave **7n** (78 mg) as a white solid, yield: 58.0%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.45 (s, 1H), 6.20–7.49 (m, 15H, Ar-H), 6.18 (s, 1H), 5.93 (s, 1H), 5.20 (s, 1H), 4.15 (d, 2H, J = 5.9 Hz), 3.71(s, 3H). ESI-MS (m/z): 654.47 $[M + 1]^+$. Anal. calcd. for $C_{31}H_{24}BrCl_2N_3O_4$: C, 56.99; H, 3.70; N, 6.43; Found: C, 56.87; H, 3.71; N, 6.41.

*5.2.20. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-(4-fluorobenzyl amino)-2, 5-dioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7o**)*

Following General Procedure B, 4-fluorobenzaldehyde (25.6 mg) and **6b** (100 mg) gave **7o** (90 mg) as a white solid, yield: 73.7%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.45 (s, 1H), 6.21–7.47 (m, 15H, Ar-H), 6.18 (s, 1H), 5.93 (s, 1H), 5.20 (s, 1H), 4.15 (d, 2H, J = 5.9 Hz), 3.71 (s, 3H). ESI-MS (m/z): 1207.96 [2M + Na]$^+$. Anal. calcd. for $C_{31}H_{24}FCl_2N_3O_4$: C, 62.85; H, 4.08; N, 7.09; Found: C, 62.74; H, 4.09; N, 7.11.

*5.2.21. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-(furan-2-yl methylamino)-2, 5-dioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**7p**)*

Following General Procedure B, furfural (19.9 mg) and **6b** (100 mg) gave **7o** (89 mg) as a white solid, yield: 74.3%. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.48 (s, 1H), 6.18–7.54 (m, 14H, Ar-H), 6.17 (s, 1H), 6.01 (s, 1H), 5.21 (s, 1H), 4.14 (d, 2H, J = 5.9 Hz), 3.72 (s, 3H). ESI-MS (m/z): 1151.93 [2M + Na]$^+$. Anal. calcd. for $C_{29}H_{23}Cl_2N_3O_5$: C, 61.71; H, 4.11; N, 7.44; Found: C, 61.82; H, 4.10; N, 7.42.

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

Following General Procedure C, **6a** (100 mg) and Lawesson's Reagent (47.5 mg) gave **8a** (80.9 mg) as a yellow solid, yield: 78.3%, mp: 249–252 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.77 (s, 1H), 7.08–7.50 (m, 12H, Ar-H), 6.26 (s, 1H), 5.31 (s, 1H), 3.77 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.63, 169.48, 166.33, 136.54, 134.12, 133.79, 133.17, 132.80, 132.52, 132.42, 129.30, 128.96, 128.80, 127.91, 127.27, 126.03, 120.82, 70.86, 66.11, 52.83. ESI-MS (m/z): 483.56 [M − 1]$^-$. Anal. calcd. for $C_{24}H_{18}Cl_2N_2O_3S$: C, 59.39; H, 3.74; N, 5.77; Found: C, 59.29; H, 3.75; N, 5.78.

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

Yield: 75.4%, a light-yellow solid, mp: 210–211 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.43 (s, 1H), 6.26–7.53 (m, 11H, Ar-H), 6.11 (s, 1H), 6.09 (s, 1H), 5.75 (s, 1H), 3.70 (s, 3H), 2.86 (t, 2H, J = 5.6 Hz), 1.48 (m, 2H), 0.86 (t, 3H, J = 7.3 Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.01, 169.76, 166.41, 152.22, 138.26, 134.41, 133.80, 133.65, 132.26, 132.08, 128.79, 128.59, 127.15, 114.59, 110.61, 100.95, 70.94, 65.75, 52.57, 44.49, 21.92, 11.85. ESI-MS (m/z): 542.24 [M + 1]$^+$. Anal. calcd. for $C_{27}H_{25}Cl_2N_3O_3S$: C, 59.78; H, 4.65; N, 7.75; Found: C, 59.68; H, 4.66; N, 7.77.

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

Yield: 72.1%, a light-yellow solid, mp: 150–151 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.45 (s, 1H), 6.12–7.52 (m, 16H, Ar-H), 6.10 (s, 1H), 6.06 (s, 1H), 5.75 (s, 1H), 4.18 (d, 2H, J = 6.0 Hz), 3.73 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.10, 169.67, 166.34, 151.88, 139.36, 138.17, 134.32, 133.77, 133.60, 132.23, 132.17, 132.01, 128.75, 128.66, 128.52, 127.44, 127.18, 127.09, 115.23, 110.79, 101.58, 70.92, 65.73, 52.55, 46.06. ESI-MS (m/z): 590.15 [M + 1]$^+$, 588.42 [M − 1]$^-$. Anal. calcd. for $C_{31}H_{25}Cl_2N_3O_3S$: C, 63.05; H, 4.27; N, 7.12; Found: C, 63.15; H, 4.26; N, 7.11.

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

Yield: 62.9%, a yellow solid, mp: 150–151 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.45 (s, 1H), 6.10–7.52 (m, 15H, Ar-H), 6.10 (s, 1H), 6.08 (s, 1H), 5.74 (s, 1H), 4.18 (d, 2H, J = 6.0 Hz), 3.70 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.13, 169.73, 166.33, 151.67, 138.52, 138.18, 134.35, 133.80, 133.63, 132.26, 132.20, 132.08, 131.74, 129.30, 128.80, 128.64, 128.57, 127.13, 115.43, 110.89, 101.68, 70.97, 65.79, 52.61, 45.36. ESI-MS (m/z): 622.61 [M − 1]$^-$. Anal. calcd. for $C_{31}H_{24}Cl_3N_3O_3S$: C, 59.58; H, 3.87; N, 6.72; Found: C, 59.66; H, 3.86; N, 6.70.

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

Yield: 86.0%, a light-yellow solid, mp: 150–151 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.40 (s, 1H), 6.33–7.53 (m, 11H, Ar-H), 6.15 (s, 1H), 6.10 (s, 1H), 5.75 (s, 1H), 3.71 (s, 3H), 2.74 (m, 2H), 0.85 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.01, 169.70, 166.36, 152.30, 138.22, 134.38, 133.70, 133.65, 132.23, 132.18, 131.98, 128.73, 128.52, 127.12, 114.40, 110.70, 100.95, 70.94, 65.73, 52.53, 48.59, 33.12, 27.19. ESI-MS (m/z): 570.70 [M − 1]$^-$. Anal. calcd. for $C_{29}H_{29}Cl_2N_3O_3S$: C, 61.05; H, 5.12; N, 7.37; Found: C, 61.20; H, 5.11; N, 7.35.

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

Yield: 76.6%, a yellow solid, mp: 206–208 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.43 (s, 1H), 6.30–7.53 (m, 11H, Ar-H), 6.07 (s, 1H), 6.07 (s, 1H), 5.75 (s, 1H), 3.71 (s, 3H), 2.72 (t, 2H, J = 6.3 Hz), 1.74 (m, 1H), 0.85 (d, 6H, J = 6.6 Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 198.99, 169.71, 166.35, 152.34, 138.23, 134.38, 133.75, 133.65, 132.22, 132.17, 131.98, 128.74, 128.54, 127.12, 114.49, 110.72, 100.86, 70.93, 65.72, 52.53, 50.44, 27.57, 20.60, 20.56. ESI-MS (m/z): 1103.59 [2M + Na]$^+$. Anal. calcd. for $C_{28}H_{27}Cl_2N_3O_3S$: C, 60.43; H, 4.89; N, 7.55; Found: C, 60.33; H, 4.90; N, 7.57.

*5.2.28. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-5-oxo-8-(pentyl amino)-2-thioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**8g**)*

Yield: 78.1%, a light-yellow solid, mp: 150–151 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.44 (s, 1H), 6.25–7.52 (m, 11H, Ar-H), 6.10 (s, 1H), 6.05 (s, 1H), 5.75 (s, 1H), 3.71 (s, 3H), 2.89 (t, 2H, J = 4.5 Hz), 1.44 (m, 2H), 1.25 (m, 4H), 0.85 (t, 3H, J = 6.8 Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.02, 169.83, 166.41, 152.19, 138.27, 134.48, 133.81, 133.75, 132.29, 132.17, 132.11, 128.83, 128.67, 127.19, 114.57, 110.19, 100.92, 71.05, 65.84, 52.66, 42.65, 29.17, 28.34, 22.38, 14.38. ESI-MS (m/z): 572.56 [M + 1]$^+$. Anal. calcd. for $C_{29}H_{29}Cl_2N_3O_3S$: C, 61.05; H, 5.12; N, 7.37; Found: C, 61.16; H, 5.11; N, 7.35.

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

Yield: 80.8%, a light-yellow solid, mp: 205–206 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.44 (s, 1H), 6.25–7.53 (m, 11H, Ar-H), 6.10 (s, 1H), 6.06 (s, 1H), 5.75 (s, 1H), 3.71 (s, 3H), 2.88 (t, 2H, J = 4.6 Hz), 1.44 (m, 2H), 1.25 (m, 2H), 0.86 (t, 3H, J = 6.7 Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.02, 169.82, 166.41, 152.21, 138.26, 134.47, 133.80, 133.74, 132.28, 132.18, 132.10, 128.82, 128.66, 127.19, 114.57, 110.60, 100.95, 71.04, 65.83, 52.65, 42.38, 30.80, 20.13, 14.18. ESI-MS (m/z): 554.81 [M − 1]$^-$. Anal. calcd. for $C_{28}H_{27}Cl_2N_3O_3S$: C, 60.43; H, 4.89; N, 7.55; Found: C, 60.31; H, 4.90; N, 7.57.

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

Yield: 77.0%, a light-yellow solid, mp: 210–211 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.44 (s, 1H), 6.28–7.53 (m, 11H, Ar-H), 6.10

(s, 1H), 6.09 (s, 1H), 5.75 (s, 1H), 3.71 (s, 3H), 2.80 (t, 2H, $J = 6.1$ Hz), 0.94 (m, 1H), 0.41 (m, 2H), 0.15 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 198.99, 169.72, 166.37, 152.18, 138.20, 134.75, 133.82, 133.68, 132.23, 132.20, 132.06, 128.76, 128.58, 127.13, 114.65, 110.59, 101.11, 70.94, 65.73, 52.55, 47.00, 10.59, 3.75. ESI-MS (m/z): 554.87 $[M + 1]^+$. Anal. calcd. for $C_{28}H_{25}Cl_2N_3O_3S$: C, 60.65; H, 4.54; N, 7.58; Found: C, 60.75; H, 4.53; N, 7.57.

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

Yield: 82.0%, a yellow solid, mp: 151–152 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.44 (s, 1H), 6.30–7.67 (m, 15H, Ar-H), 6.10 (s, 1H), 6.09 (s, 1H), 5.74 (s, 1H), 4.30 (d, 2H, $J = 6.1$ Hz), 3.70 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.15, 169.67, 166.30, 151.58, 144.54, 138.19, 134.31, 133.79, 133.58, 132.24, 132.16, 132.11, 128.76, 128.50, 128.03, 127.11, 125.53, 125.50, 115.59, 110.85, 101.68, 70.92, 65.75, 52.55, 45.58. ESI-MS (m/z): 656.60 $[M - 1]^-$. Anal. calcd. for $C_{32}H_{24}Cl_2F_3N_3O_3S$: C, 58.36; H, 3.67; N, 6.38; Found: C, 58.50; H, 3.66; N, 6.36.

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

Yield: 86.1%, a yellow solid, mp: 151–152 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.42 (s, 1H), 6.25–7.53 (m, 11H, Ar-H), 6.10 (s, 1H), 6.05 (s, 1H), 5.74 (s, 1H), 3.71 (s, 3H), 2.74 (m, 2H), 1.98 (m, 1H), 1.65–0.85 (m, 10H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 198.99, 169.72, 166.36, 152.37, 138.24, 134.66, 133.78, 133.68, 132.22, 132.17, 131.96, 128.74, 128.52, 127.12, 114.40, 110.76, 100.76, 70.92, 65.71, 52.52, 49.08, 37.07, 30.92, 26.45, 25.84. ESI-MS (m/z): 594.66 $[M - 1]^-$. Anal. calcd. for $C_{31}H_{31}Cl_2N_3O_3S$: C, 62.41; H, 5.24; N, 7.04; Found: C, 62.52; H, 5.23; N, 7.02.

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

Yield: 54.9%, a yellow solid, mp: 259–260 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.44 (s, 1H), 7.87 (m, 2H), 7.68 (m, 1H), 7.51 (s, 1H), 7.43–7.50 (m, 7H), 7.24 (d, 1H, $J = 8.8$ Hz), 7.14 (m, 5H), 6.35 (d, 1H, $J = 8.6$ Hz), 6.17 (s, 1H), 6.10 (s, 1H), 5.72 (s, 1H), 4.36 (d, 2H, $J = 5.7$ Hz), 3.69 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.04, 169.68, 166.32, 151.89, 138.17, 136.98, 134.31, 133.76, 133.59, 133.28, 132.57, 132.22, 132.06, 128.75, 128.51, 128.31, 127.93, 127.89, 127.08, 126.55, 125.99, 125.56, 115.26, 110.85, 101.90, 70.91, 65.73, 52.55, 46.30. ESI-MS (m/z): 642.75 $[M + 1]^+$. Anal. calcd. for $C_{35}H_{27}Cl_2N_3O_3S$: C, 65.62; H, 4.25; N, 6.36; Found: C, 65.75; H, 4.24; N, 6.34.

*5.2.34. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8–((4-methoxy benzyl) amino)-5-oxo-2-thioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**8m**)*

Yield: 78.6%, a yellow solid, mp: 222–223 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.45 (s, 1H), 6.29–7.53 (m, 15H, Ar-H), 6.11 (s, 1H), 6.10 (s, 1H), 5.74 (s, 1H), 4.10 (d, 2H, $J = 5.7$ Hz), 3.72 (s, 3H), 3.70 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.07, 169.70, 166.34, 158.65, 151.88, 138.15, 134.34, 133.77, 133.62, 132.23, 132.16, 131.99, 131.15, 128.75, 128.53, 127.11, 115.12, 114.12, 110.81, 101.56, 70.94, 65.73, 55.46, 52.53, 45.53. ESI-MS (m/z): 618.64 $[M - 1]^-$. Anal. calcd. for $C_{32}H_{27}Cl_2N_3O_4S$: C, 61.94; H, 4.39; N, 6.77; Found: C, 61.81; H, 4.40; N, 6.79.

*5.2.35. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-5-oxo-8-((thiophen -3-ylmethyl) amino)-2-thioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**8n**)*

Yield: 75.3%, a light-yellow solid, mp: 165–166 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.46 (s, 1H), 6.20–7.53 (m, 14H, Ar-H), 6.15 (s, 1H), 6.11 (s, 1H), 5.75 (s, 1H), 4.16 (d, 2H, $J = 5.9$ Hz), 3.71 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.11, 169.70, 166.36, 151.78, 140.30, 138.17, 134.34, 133.77, 133.63, 132.25, 132.20, 132.00, 128.77, 128.56, 127.75, 127.12, 126.60, 115.28, 110.90, 101.54, 70.95, 65.75, 52.57, 41.78. ESI-MS (m/z): 594.64 $[M - 1]^-$. Anal. calcd. for $C_{29}H_{23}Cl_2N_3O_3S_2$: C, 58.39; H, 3.89; N, 7.04; Found: C, 58.49; H, 3.88; N, 7.02.

*5.2.36. Methyl 2-(8–((4-bromobenzyl) amino)-3-(4-chlorophenyl)-5-oxo-2-thioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl)-2-(4-chlorophenyl) acetate (**8o**)*

Yield: 70.8%, a yellow solid, mp: 236–237 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.44 (s, 1H), 6.27–7.95 (m, 15H, Ar-H), 6.10 (s, 1H), 6.08 (s, 1H), 5.73 (s, 1H), 4.17 (d, 2H, $J = 6.1$ Hz), 3.71(s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.11, 169.67, 166.30, 151.62, 138.93, 138.16, 134.31, 133.77, 132.23, 132.17, 132.04, 131.51, 129.62, 128.75, 128.51, 127.09, 120.15, 115.44, 110.87, 101.67, 70.92, 65.74, 52.55, 45.40. ESI-MS (m/z): 668.79 $[M + 1]^+$. Anal. calcd. for $C_{31}H_{24}BrCl_2N_3O_3S$: C, 55.62; H, 3.61; N, 6.28; Found: C, 55.51; H, 3.62; N, 6.30.

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

Yield: 75.2%, a yellow solid, mp: 147–148 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.45 (s, 1H), 6.30–7.52 (m, 15H, Ar-H), 6.10 (s, 1H), 6.10 (s, 1H), 5.74 (s, 1H), 4.17 (d, 2H, $J = 5.9$ Hz), 3.70 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.12, 169.73, 166.34, 151.74, 138.18, 135.52, 134.36, 133.80, 133.64, 132.26, 132.20, 132.08, 129.47, 129.40, 128.79, 128.58, 127.13, 115.50, 115.33, 110.88, 101.64, 70.98, 65.78, 52.61, 45.36. ESI-MS (m/z): 1239.59 $[2M + Na]^+$. Anal. calcd. for $C_{31}H_{24}FCl_2N_3O_3S$: C, 61.19; H, 3.98; N, 6.91; Found: C, 61.29; H, 3.97; N, 6.89.

*5.2.38. Methyl 2-(4-chlorophenyl)-2-(3-(4-chlorophenyl)-8-((furan-2-yl methyl) amino)-5-oxo-2-thioxo-2, 3-dihydro-1H-benzo [e] [1,4] diazepin-4 (5H)-yl) acetate (**8q**)*

Yield: 81.2%, a yellow solid, mp: 180–181 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.43 (s, 1H), 6.30–8.51 (m, 14H, Ar-H), 6.09 (s, 1H), 6.09 (s, 1H), 5.74 (s, 1H), 4.28 (d, 2H, $J = 5.9$ Hz), 3.70 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 199.12, 169.69, 166.33, 152.44, 151.51, 142.50, 138.07, 134.95, 133.87, 133.69, 132.24, 131.99, 128.77, 128.57, 127.11, 115.51, 110.79, 110.73, 107.58, 101.79, 70.95, 65.75, 52.56, 40.43. ESI-MS (m/z): 1181.31 $[2M + Na]^+$. Anal. calcd. for $C_{29}H_{23}Cl_2N_3O_4S$: C, 60.00; H, 3.99; N, 7.24; Found: C, 60.11; H, 3.98; N, 7.23.

5.3. Computational protocol

Molecular docking was used to predict the binding mode of the synthesized thio-benzodiazepine derivatives. The crystal structure [9] of MDM2 (PDB code: 1T4E) was prepared by removing the benzodiazepine and adding hydrogen atoms in GOLD 4.1.2. 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 GOLD were similar to the literatures [15,22]. We also used MVD 4.3.0 for docking to confirm the robustness of the docking pose. The active site was defined to encompass all MDM2 atoms within a 10 Å radius sphere from the center of the Trp23 of the p53 peptide ligand. Other parameters were set by default. As a result, these two docking programs obtained approximate binding poses.

5.4. 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 were 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 H4 with a 480 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.

5.5. 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. Thiobenzodiazepines were dissolved with 100 μL of DMSO. The absorbance (OD) was quantitated with microplate using Biotek Synergy H4 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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