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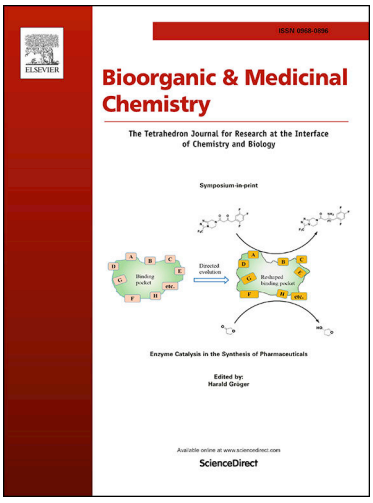

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Design, Synthesis and Biological Evaluation of Novel Thiohydantoin Derivatives as Potent Androgen Receptor Antagonists for the Treatment of Prostate Cancer

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ABSTRACT

Prostate cancer (PC) is the most common malignancy in men worldwide. Here, two series of novel thiohydantoin derivatives of enzalutamide as potent androgen receptor (AR) antagonists were designed and synthesized. Among them, compound **31c** was identified as an AR antagonist which is 2.3−fold more potent than enzalutamide. Molecular docking studies were performed to explain the improved potency of **31c** at AR. In cell proliferation assays, **31c** exhibited similar anti-proliferative activities with enzalutamide against hormone sensitive LNCaP cells and AR-overexpressing LNCaP/AR cells. These data indicate that **31c** can be a good lead compound for further structure optimization for the treatment of prostate cancer.

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1. I

t of CRPC.

Prostate cancer (PC) is the most common malignancy in men worldwide and the second most common cause of cancer-related death worldwide after lung cancer. According to the International Agency for Research on Cancer's Globocan data for 2018, the number of deaths due to prostate cancer was 358,989.[1]

Most PCs are initially androgen-dependent, and the androgen receptor (AR) is highly expressed at all stages of the disease, playing a key role in the development and progression of PC.[2,3] Androgen ablation, by surgical or chemical castration in combination with AR antagonists such as **1** (flutamide) [4], **2** (bicalutamide) [5] and **3** (nilutamide) [6] (**Fig. 1**), has been the standard treatment for advanced PC for many years.[7] This therapy is initially effective in 80−90% of patients, however, more than 50% of the patients will ultimately develop castration resistant prostate cancer (CRPC) because tumor cells will spontaneously synthesize in situ or through the adrenal glands to synthesize low levels of androgens to resume growth.[8] CRPC is attributed to elevated AR gene expression which can be driven by AR gene amplification,[9,10] AR gene mutation,[11,12] or ligand-independent AR activation through other factors such as increased expression of transcriptional coactivators.[13,14]

Since 2012, three AR antagonists (**Figure 1**) have been approved by the FDA, including **4** (enzalutamide) for mCRPC and **5** (apalutamide), **6** (darolutamide) for nonmetastatic CRPC (nmCRPC).[15-17] Despite the promising results achieved in CRPC patients with androgen deprivation therapies (ADT), such as enzalutamide,[15,18] resistance to such therapies has been observed. Recently, an F876L mutation in AR ligand binding domain (AR−LBD) has been identified, which confers an antagonist-to-agonist switch driving the resistance to enzalutamide and apalutamide.[19,20] Therefore, there is an unmet and urgent need to

Fig. 1. Chemical structures of compounds **1**−**6** (representative AR antagonists)

In the setting of CRPC, the first-generation AR antagonists undergo an antagonist-to-agonist switch and stimulate AR activity induced prostate tumor cell growth because of their low affinity for the AR relative to testosterone and dihydrotestosterone (DHT). Therefore, **series 1** compounds (**Fig. 2**) were designed which adopted a ring-closing strategy in order to simulate the structure of endogenous steroid ligands and enhance the binding affinity to AR. In addition, AR agonists and AR antagonists share different modes of action towards the related transcriptional machinery inducing the "closed" and "open" conformations of AR-H12 helix (H12). It is supposed that the ectopic of H12 may play an important role in the antagonist conformation, similar to the estrogen receptor and glucocorticoid receptor antagonist structures.[21-23] So we designed **series 2** compounds (**Fig. 2**) that features the cyclization of the amide group to the right-hand side benzene ring of enzalutamide to increase the steric hindrance between the substituent and H12 to increase the AR antagonistic activity. The design, synthesis and biological evaluation of these series of compounds are reported herein.

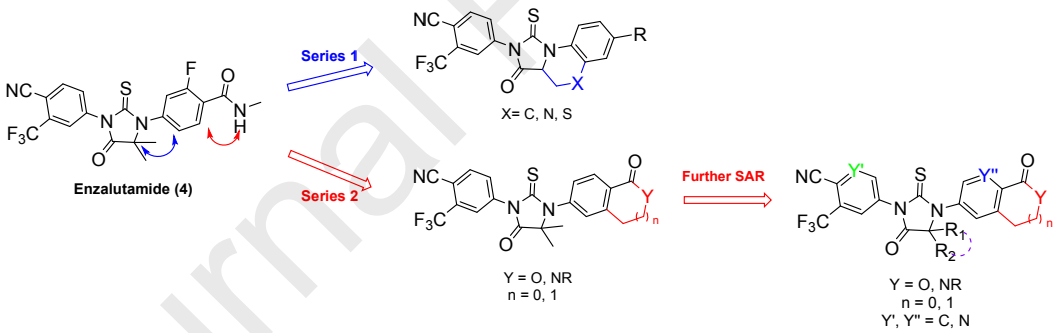
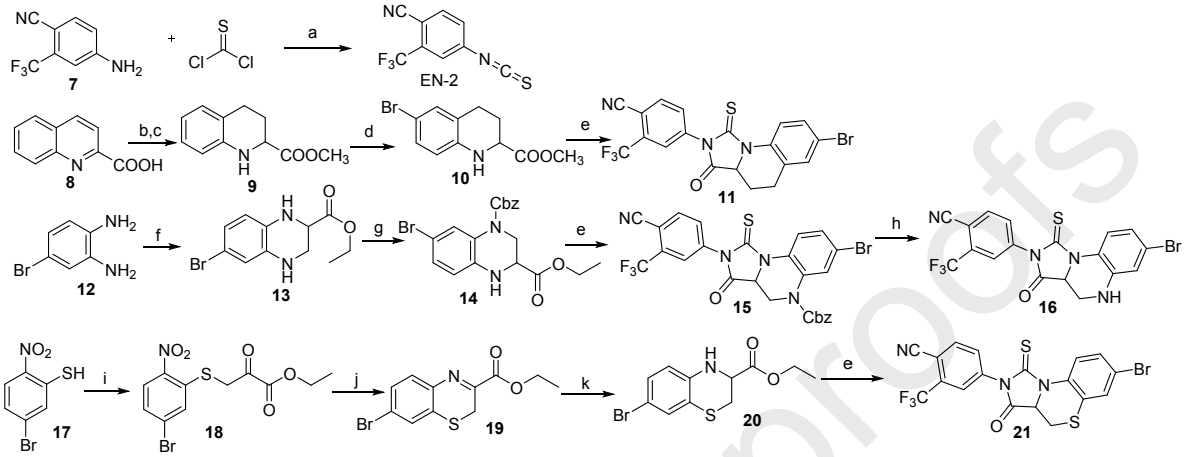


Fig. 2. Design of two novel series of thiohydantoin derivatives

2. Results and discussion

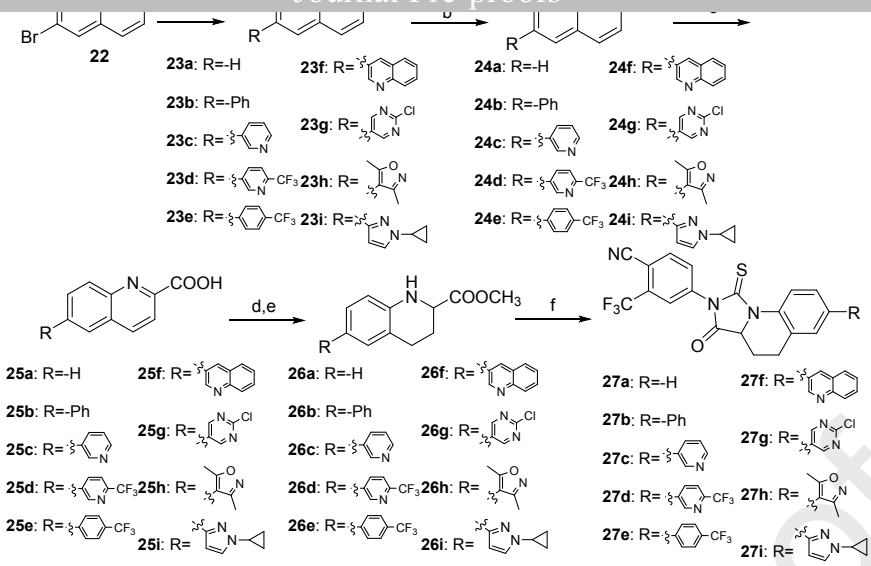
2.1. Chemistry

K[illegible]nate prepared as depicted in **Scheme 1**. Compound 7 was treated with thiophosgene to provide the intermediate **EN-2**. Compound **8** was reduced to tetrahydroquinoline-2-carboxylic acid by hydrogenation in the presence of PtO_2, which was further esterified to give intermediate **9**. Then it was treated with N-Bromosuccinimide (NBS) to afford compound **10**, which was cyclized with **EN-2** to give compound **11**. Compound **12** was treated with ethyl 2,3-dibromopropanoate at 80°C to get (CbzCl) to obtain the mono-protected compound **14**, and **14** was cyclized with **EN-2** to give compound **15**. Removal of the Cbz group yielded compound **16**. Compound **17** and ethyl 2,3-dibromopropanoate underwent a nucleophilic substitution reaction to provide compound **18**, followed by reduction by iron powder to obtain compound **19**. Next, catalytic hydrogenation reduced the imine to obtain compound **20**, which was reacted with **EN-2** to yield compound **21**.



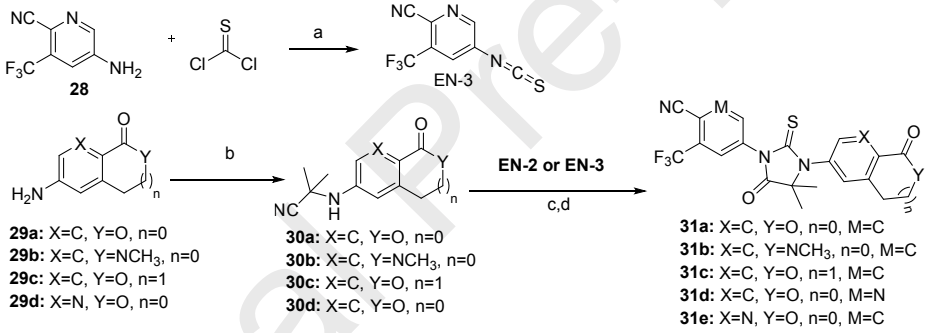
Scheme 1. Synthesis of key intermediate **EN-2** and target compounds (**11, 16, 21**) [a]. [a] Reagents and conditions: (a)H_2O, rt; (b)H_2, PtO_2, MeOH, rt; (c) $SOCl_2$, MeOH, 0°C -rt; (d) NBS, DMF, rt; (e) **EN-2**, DMF, 30°C; (f) ethyl 2,3-dibromopropanoate, Et_3N, DMF, 80°C; (g) CbzCl, K_2CO_3, CH_3CN, rt; (h) 33% HBr.CH_3COOH, rt; (i) ethyl 2,3-bromo-2-oxopropanoate, dithiothreitol, DMF, K_2CO_3, 0°C; (j) Fe, NH_4Cl, EtOH:H_2O=12:1, 70°C; (k) H_2, Pd/C, MeOH, 35°C

As for target compounds **27a**−**27i**, the syntheses are depicted in **Scheme 2**. Suzuki coupling reactions between Compound **22** and various boric acid or borate esters yielded compounds **23a**−**23i**. Then the methyl group was oxidized by selenium dioxide to obtain the corresponding compounds **24a**−**24i**, followed by Pinnick oxidation to obtain the corresponding carboxylic acids **25a**−**25i**. Next, the quinoline core was reduced to tetrahydroquinoline and the carboxylic acids was converted to corresponding esters **26a**−**26i**, under similar conditions as described for **Scheme 1**. Finally, cyclization reactions with **EN-2** yielded compounds **27a**−**27i**.



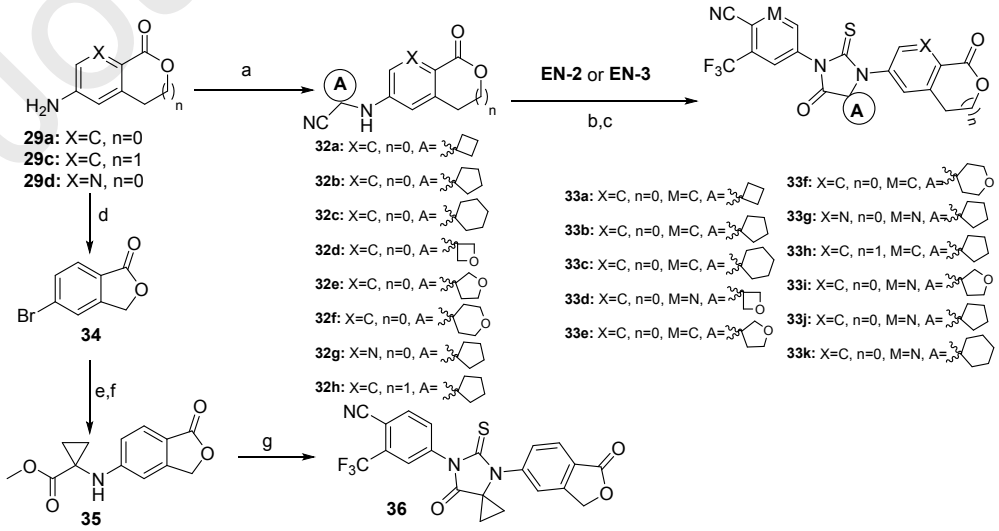
Scheme 2. Synthesis of **series 1** compounds (**27a−27i**) [a]. [a] Reagents and conditions: (a) various boric acid or borate ester reagents, K_2CO_3, Pd $(PPh_3)_4$, dioxane: H_2O = 4:1, 80°C; (b) SeO_2, dioxane, 100°C; (c) $NaClO_2$, $NaH_2PO_4.2H_2O$, 2-methyl-2-butene, *t*-BuOH, rt; (d) H_2, PtO_2, MeOH, rt; (e) $SOCl_2$, MeOH, 65°C; (f) **EN-2**, DMF, 30°C

The preparation of compounds **31a−31e** is depicted in **Scheme 3.** Compound **28** was treated with thiophosgene to get the intermediate **EN-3**. Intermediates **29a−29d** (prepared as described in **Supplementary material)** were treated under Strecker reaction conditions with acetone and trimethylsilyl cyanide to generate the cyanoamines **30a−30d.** Then cyclization reaction with **EN-2** or **EN-3** using lithium bistrimethylsilylamine at the temperature of -78°C yielded the imine compounds, followed by acidic hydrolysis to provide target compounds **31a−31e**.



Scheme 3. Synthesis of **series 2** compounds (**31a−31e**) [a]. [a] Reagents and conditions: (a) H_2O, rt; (b) TMSCN, $ZnCl_2$, Acetone, 40°C; (c) LiHMDS, THF, -78°C; (d) 2N HCl, MeOH, 80°C

Compounds **33a−33k** and **36** were prepared as depicted in **Scheme 4.** The synthetic route of **33a−33k** is basically the same as that of **31a−31e**. Through diazotization of 5-aminoisobenzofuran-1(3H)-one (**29a**), compound **34** was obtained. Next, compound **35** was obtained from **34** through a Copper-catalyzed Ullman coupling reaction and a methylation reaction with by methyl iodide. Finally it was cyclized with **EN-2** to yield compound **36**.



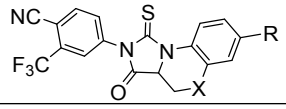
Sch 40° C; (b) LiHMDS, THF, -78°C; (c) 2N HCl, MeOH, 80°C; (d) $NaNO_2$, 47% HBr, CuBr, 0°C -rt; (e) N,N-Dimethylglycine hydrochloride, CuI, DMSO, K_2CO_3, 120°C; (f) K_2CO_3, CH_3I, DMF, rt; (g) **EN-2**, LiHMDS, THF, -78°C

2.2. Androgen receptor (AR) antagonist assay

All the synthesized compounds were evaluated for their AR antagonistic activity using the GeneBLAzer® Betalactamase reporter technology for nuclear receptors (NRs) (see **Experimental Section**). Enzalutamide (**4**) was used as the reference, and the data are shown in **Table 1** and **Table 2**.

As shown in **Table 1**, the activity of the tested compounds varies in a wide range. Compounds **11**, **16** and **21** were first tested for their AR antagonistic activity, and it was found that their activities were all weaker than enzalutamide (**Table 1**). However, among them, compound **11** showed relatively better activity than that of **16** and **21** for which the X is a carbon. Therefore, we set the X of the tricyclic series compounds as a carbon, and explored the substituent R at the 3-position of the right-side benzene ring and synthesized compounds **27a−27i**, but the activity is still far weaker than enzalutamide. So **series 1** compounds were discontinued for further structural modifications.

Table 1. AR Antagonist Effect of series 1 compounds



Compd.	X	R	IC_{50}/nM
11	C	Br	4377
16	N	Br	9862
21	S	Br	18140
27a	C	H	2837
27b	C	Ph	3438
27c	C		3906
27d	C		2183
27e	C		>50000
27f	C		8953
27g	C		8075
27h	C		5978
27i	C		4527
enzalutamide			646.9

In the discovery process of enzalutamide, it was reported that compounds with an ester substituent on the right benzene ring are more active than compounds with an amide substituent.[24] Since the ester group is easier to metabolize than an amide, ring-closing strategy was adopted by closing the ester group to the benzene ring, forming lactones, in order to improve metabolic properties and increase the steric hindrance to push H12 away at the same time. Based on this, compounds **31a** and **31b** were first synthesized and evaluated. It was surprised to find that the activity of **31a** is better than **31b** and enzalutamide (**Table 2**). So the right side was chosen as a lactone instead of a lactam. In order to further explore the ring size of the benzolide compounds, compounds **31c** and **33h** were then synthesized. It was found that **31c** exhibit weaker activity than that of **31a,** and **33h** is also weaker than **33b.** These results indicate that the five-membered ring lactone is preferred.

At the same time, in order to prevent the right benzene ring from metabolism via oxidation of the electron-rich aromatic ring,[24] we also replaced the right benzene ring with a pyridine ring and synthesized **31e**. It was found that the activity was greatly diminished, which means that the right benzene ring cannot be replaced.

In addition, compounds **33a−33c**, **33e, 33f** and **36** were synthesized. Because part A plays an important role in AR antagonist activity based on the structure-activity relationship study of enzalutamide. It was found that their activities are better than enzalutamide except for **36**, and the substituent with cyclopentane shows the best activity.

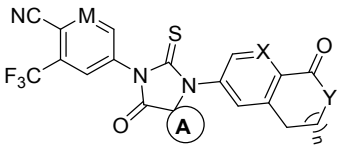
Since the benzene ring on the left side of the enzalutamide was replaced with a pyridine ring in apalutamide (**5**), which showed improved activity, we next synthesized compounds **31d, 33d,** and **33i−33k**. Among them, the activity of **33j** and **33k** are much better than enzalutamide, but **31d**, **33d** and **33i** are weaker than enzalutamide.

Finally, we also synthesized **33g**, where the left and right benzene rings were both replaced with pyridine rings, and found that the activity was reduced by nearly 5-fold compared to enzalutamide, this is further confirmed that the right benzene ring cannot be replaced.

2.3. Antiproliferative assay

Compounds with potent AR antagonist activity were selected for further studies in antiproliferative assays toward normal LNCaP (hormone sensitive) cells and LNCaP/AR cells, which were engineered (using viral infection with a cDNA encoding for the AR) to express 3- to 5-fold higher levels of the AR to mimic the clinical setting of CRPC. The data were listed in **Table 3**. Enzalutamide (**4**) was used as the reference drug. For LNCaP cells, the antiproliferative activity of **33h** is better than that of the control drug enzalutamide, while **31a** and **31c** are equivalent as enzalutamide, and the other compounds (**33a−33c, 33e, 33f, 33j,** and **33k**) are weaker. For LNCaP/AR cells, the anti-proliferative activity of **33e** is the most potent, which is slightly better than compound enzalutamide. Compound **31c** showed comparable activity to that of enzalutamide, while the activities of other compounds (**31a, 33b, 33c, 33f−33k**) are weaker. Taken together, the anti-proliferative activity of compound **31c** is comparable to that of compound enzalutamide in both types of cells, and it has great development potential.

Table 2. AR Antagonist Effect of series 2 compounds



Co						
31a	C		C	O	0	148.6
31b	C		C	NCH_3	0	590.2
31c	C		C	O	1	195.5
31d	N		C	O	0	941.1
31e	C		N	O	0	2292
33a	C		C	O	0	216.7
33b	C		C	O	0	80.7
33c	C		C	O	0	95.2
33d	N		C	O	0	26735
33e	C		C	O	0	238.8
33f	C		C	O	0	140.9
33g	N		N	O	0	2219
33h	C		C	O	1	134.6
33i	N		C	O	0	516.2
33j	N		C	O	0	63.8
33k	N		C	O	0	93.3
36	C		C	O	0	669.6
Enzalutamide						454.6

Table 3. Antiproliferative activity for novel thiohydantoin Derivatives

Compd.	IC_{50}/μM(LNCaP)	IC_{50}/μM(LNCaP/AR)
31a	1.982	1.472
31c	1.936	0.730
33b	3.687	1.086
33c	2.192	17.00
33e	2.663	0.569
33h	1.287	5.588
33k	4.77	2.036
enzalutamide	1.900	0.730

2.4. Molecular modelling studies

Since the AR antagonistic activity of **31c** is 2.3−fold more potent than enzalutamide (**4**) and its anti-cell proliferation activity for LNCaP cells and LNCaP/AR cells is equivalent to that of enzalutamide, we investigated the possible binding modes of **31c** to AR (PDB ID: 2OZ7) which was obtained from Protein Database online(www.rcsb.org). The possible binding modes of compound **31c** with the key residues are shown in **Fig. 3A**. Overall, the binding pose of compound **31c** is very similar to that of enzalutamide. The cyano group of **31c** formed two important hydrogen bonds with Arg752 and Gln711, and there is a π-π interaction between the benzene ring and Phe764, which is similar to enzalutamide (**Fig. 3B**). In addition, the carbonyl group and the ester oxygen of **31c** formed two hydrogen bonds with Arg779, while only the amide NH group of enzalutamide formed a hydrogen bond with Ser778. One more hydrogen bond in binding patterns may be the reason for the high potency **31c** toward AR.

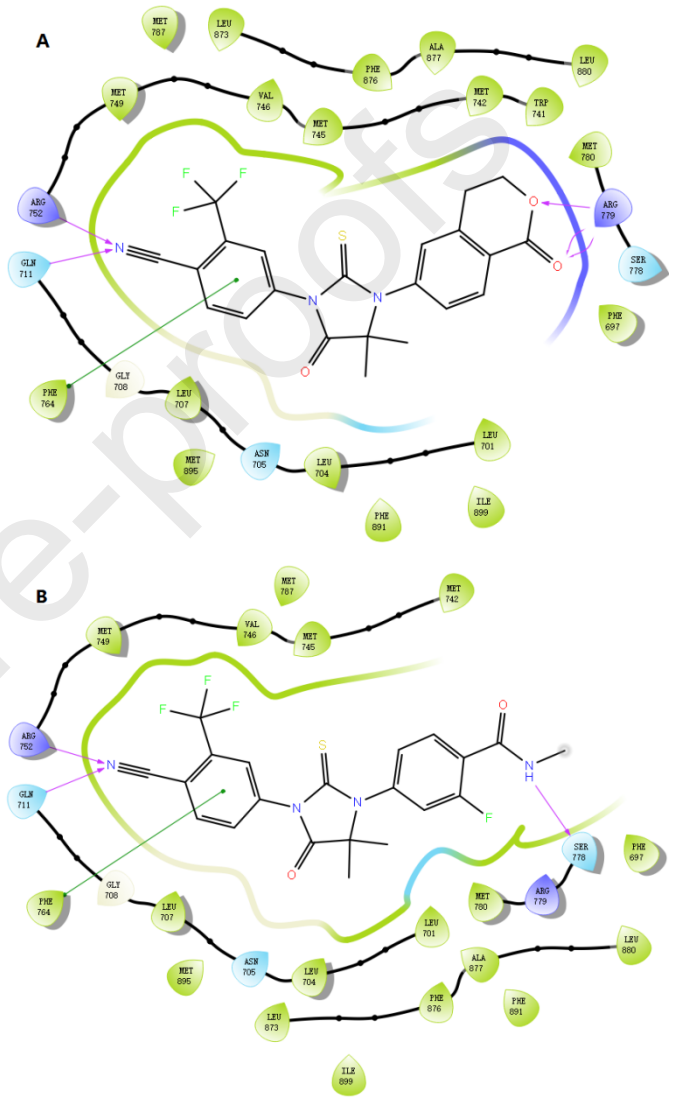


Fig. 3. The predicted binding modes and 2D diagram of (**A**) compound **31c** and (**B**) enzalutamide in complex with AR (PDB ID: 2OZ7)

3. Conclusion

In conclusion, two series of novel thiohydantoin derivatives were designed, synthesized and evaluated for AR antagonist effect and antiproliferative activities. **series 2** compounds showed excellent AR antagonistic activity, ten compounds (**31a, 31c, 33a−33c, 33e, 33f, 33h,33j, 33k**) are 2−7 fold more potent than the control drug enzalutamide. Among them, the compound **31c** exhibited comparable anti-proliferative activities to that of enzalutamide in both LNCaP and LNCaP/AR cell proliferation assays. In addition, Molecular docking studies were performed to show that one more hydrogen bond in binding patterns may be the reason for the high potency **31c** toward AR. These data indicate

that structural optimization for the treatment of prostate cancer.

4. Experimental Section

4.1. General Chemistry Methods

All solvents and chemicals were used as purchased without further purification. Room temperature refers to 20−25°C. Intermediates not described below were purchased from commercial vendors and were used as supplied unless stated otherwise. All reactions were monitored using thin-layer chromatography (TLC) on silica gel F-254 TLC plates. Column chromatography was carried out using silica gel (200−300 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400, a Bruker 500, or a Bruker 600 NMR spectrometer using solvent residual as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) are reported in hertz (Hz). Electron ionization-mass spectra were obtained on a Finnigan MAT95 spectrometer, and electrospray ionization (ESI) mass spectra were obtained on a Krats MS 80 mass spectrometer. All final compounds were purified to >95% purity as determined by analytical high-performance liquid chromatography (PLATISIL ODS 250 mm × 4.6 mm, particle size 5 μm) with acetonitrile/water as the mobile phase.

4.1.1. Synthesis of 4-isothiocyanato-2-(trifluoromethyl) benzonitrile (**EN-2**)

Thiophosgene (0.45 mL, 5.91 mmol, 1.1 equiv) was dissolved in 20 mL water stirred at room temperature, **7** (1 g, 5.37 mmol, 1 equiv) was added to the solution in batches. The reaction mixture was stirred at room temperature for 4 h and TLC analysis indicated the reaction was completed. Then the solution was extracted with CH_2Cl_2, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (0−10% EtOAc/hexanes) to give the desired product. It was obtained as a yellow oil 898 mg in 73.25% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.87 (d, J = 8.3 Hz, 1H), 7.61 (d, J = 2.1 Hz, 1H), 7.51 (dd, J = 8.3, 2.1 Hz, 1H).

4.1.2. Synthesis of Methyl 1,2,3,4-tetrahydroquinoline-2-carboxylate (**9**)

8 (10 g, 57.75 mmol, 1 equiv) and PtO_2 (0.656 g, 2.88 mmol, 0.05 equiv) were dissolved in methanol stirred at room temperature under the protection of Hydrogen. The reaction mixture was stirred at room temperature for 4 h and TLC analysis indicated the reaction was completed. Then the solution was filtered through celite, the filtrate was collected and concentrated, the reaction mixture was cooled to 0°C, and 6.28 mL $SOCl_2$ was added dropwise over 10 min. Then the reaction mixture was allowed to warm slowly to rt. The reaction mixture was stirred at room temperature for 6.5 h and TLC analysis indicated the reaction was completed. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (5% EtOAc/hexanes) to give the desired product. It was obtained as a yellow oil 3.58 g in 32.42% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.07 – 7.01 (m, 1H), 7.00 (dt, J = 7.6, 1.2 Hz, 1H), 6.69 (td, J = 7.4, 1.2 Hz, 1H), 6.62 (dd, J = 7.9, 1.1 Hz, 1H), 4.41 (s, 1H), 4.08 (dd, J = 8.7, 3.8 Hz, 1H), 3.81 (s, 3H), 2.83 (dddt, J = 21.9, 16.4, 11.2, 5.5 Hz, 3H), 2.32 (dtd, J = 12.9, 5.7, 3.8 Hz, 1H), 2.09 – 1.99 (m, 1H).

4.1.3. Synthesis of Methyl 6-bromo-1,2,3,4-tetrahydroquinoline-2-carboxylate (**10**)

Methyl 1,2,3,4-tetrahydroquinoline-2-carboxylate (**9**) (0.2 g, 1.05 mmol, 1 eqviu) was put into a three-necked flask, dissolved in anhydrous DMF stirred at room temperature under the uiv) which was dissolved in anhydrous DMF was slowly added. The reaction was stirred at room temperature for 1.5 h, then the solution was added some ice water, extracted with EtOAc. The organic phase was washed with saturated brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a light yellow solid 252 mg in 89.2% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.12 – 7.08 (m, 2H), 6.50 – 6.47 (m, 1H), 4.41 (s, 1H), 4.05 (dd, J = 8.6, 3.8 Hz, 1H), 3.80 (s, 2H), 2.77 (dddt, J = 22.2, 16.5, 11.4, 5.5 Hz, 2H), 2.33 – 2.25 (m, 1H), 2.00 (dtd, J = 13.0, 8.8, 5.2 Hz, 1H).

4.1.4. Synthesis of 4-(7-bromo-3-oxo-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**11**)

Methyl 6-bromo-1,2,3,4-tetrahydroquinoline-2-carboxylate (**10**) (0.25 g, 925.49 umol, 1 equiv) was reacted with **EN-2** (0.211 g, 925.49 umol, 1 eqviu) in 20 mL anhydrous DMF at room temperature for 1 h. After work up, the crude product was poured into ice water. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (20%-50% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 0.288 g in 66.74% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.76 – 8.71 (m, 1H), 8.02 (d, J = 8.2 Hz, 1H), 7.91 (d, J = 2.0 Hz, 1H), 7.81 – 7.77 (m, 1H), 7.47 – 7.42 (m, 2H), 4.47 (dd, J = 12.4, 3.2 Hz, 1H), 3.17 (dd, J = 9.2, 4.2 Hz, 2H), 2.75 – 2.68 (m, 1H), 2.25 – 2.16 (m, 1H). ^{13}C NMR (126 MHz, DMSO) δ 177.30, 170.87, 138.09, 136.31, 134.43, 134.26, 131.94, 131.43, 128.60, 128.30, 123.26, 117.86, 115.03, 108.81, 60.50, 25.39, 23.57. HRMS (ESI) m/z calcd for $C_{19}H_{10}BrF_3N_3OS$ 463.969, found: 463.9686.

4.1.5. Synthesis of 4-(7-bromo-3-oxo-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinoxalin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**16**)

Ethyl 6-bromo-1,2,3,4-tetrahydroquinoxaline-2-carboxylate (**13**)

12 (3 g, 16.04 mmol, 1 equiv) was dissolved in DMF, Et_3N (4.46 ml, 32 mmol, 2 equiv) and ethyl 2,3-dibromopropanoate (2.33 mL, 16.04 mmol, 1 equiv) were added to the solution, then the reaction mixture was slowly warmed to 80℃. The reaction mixture was stirred at this temperature for 24h and TLC analysis indicated the reaction was completed. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (10%-15% EtOAc/hexanes) to give the mixture brown oil 1.63 g.

1-benzyl 3-ethyl 7-bromo-3,4-dihydroquinoxaline-1,3(2H)-dicarboxylate (**14**)

The mixture was dissolved in CH_3CN stirred at room temperature, then K_2CO_3 (1.18 g, 8.61 mmol, 1.5 equiv) and 0.8 mL CbzCl were added to the solution, the reaction mixture was stirred at room temperature for 4 h and TLC analysis indicated the reaction was completed. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (10%-30% EtOAc/hexanes) to give the desired product. It was obtained as a yellow oil 908 mg.

Benzyl 7-bromo-2-(4-cyano-3-(trifluoromethyl)phenyl)-3-oxo-1-thioxo-2,3,3a,4-tetrahydroimidazo[1,5-a]quinoxaline-5(1H)-carboxylate (**15**)

The mixture (2.478 g, 5.91 mmol, 1 equiv) was reacted with **EN-2** (1.6 g, 7.01 mmol, 1.19 eqviu) in 12 mL anhydrous DMF

for 5 g in Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (10%-20% EtOAc/hexanes) to give the desired product. It was obtained as a yellow oil 2.226 g in 62.74% yield for the next step.

Then the previous step compound was added to 33% $HBr.CH_3COOH$, stirred at room temperature for 0.5 h and TLC analysis indicated the reaction was completed. Then slowly add 10% NaOH solution to adjust the pH of the solution to alkaline, then the solution was extracted with EtOAc. The organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (50%−100% EtOAc/hexanes) to give the desired products. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.71 (d, J = 2.3 Hz, 1H), 8.41 (d, J = 8.2 Hz, 1H), 8.24 (d, J = 1.8 Hz, 1H), 8.03 (dd, J = 8.3, 1.9 Hz, 1H), 7.18 (dd, J = 8.8, 2.3 Hz, 1H), 6.91 (d, J = 4.4 Hz, 1H), 6.75 (d, J = 8.7 Hz, 1H), 4.72 (dd, J = 10.9, 4.4 Hz, 1H), 3.81 (dt, J = 11.6, 4.5 Hz, 1H), 3.64 (t, J = 11.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 177.07, 169.52, 138.29, 137.36, 136.83, 134.83, 129.53, 128.68, 124.27, 121.56, 116.59, 115.47, 109.33, 105.27, 57.00. HRMS (ESI) m/z calcd for $C_{18}H_{11}BrF_3N_4OS$ 466.9779, found: 466.9784.

4.1.6. Synthesis of Ethyl 3-((5-bromo-2-nitrophenyl)thio)-2-oxopropanoate (**18**)

17 (2.82 g, 12.05 mmol, 1 equiv) and dithiothreitol (1.85 g, 12 mmol, 1 equiv) were put into a three-necked flask, dissolved in anhydrous DMF stirred at 0℃ under the protection of argon, then K_2CO_3 (1.99 g, 18.25 mmol, 1.5 equiv) and ethyl 2,3-dibromopropanoate (1.8 mL, 14.46 mmol, 1.2 equiv) were added to the solution. The reaction mixture was stirred at 0℃ for 2 h and TLC analysis indicated the reaction was completed. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (10%-20% EtOAc/hexanes) to give the desired product. It was obtained as a yellow solid 1.65g in 39.34% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.11 (d, J = 8.8 Hz, 1H), 7.65 (d, J = 2.0 Hz, 1H), 7.48 (dd, J = 8.8, 2.0 Hz, 1H), 6.46 (s, 1H), 6.43 (d, J = 1.3 Hz, 1H), 4.43 (q, J = 7.1 Hz, 2H), 1.43 (t, J = 7.1 Hz, 3H).

*4.1.7. Synthesis of Ethyl 7-bromo-2H-benzo[b][1,4]thiazine-3-carboxylate (**19**)*

Ethyl 3-((5-bromo-2-nitrophenyl)thio)-2-oxopropanoate (**18**) (550 mg, 1.58 mmol, 1 equiv) was dissolved in 12 mL EtOH and 1 mL water stirred for a few minutes, Fe (352 mg, 6.32 mmol, 4 equiv) and NH_4Cl (422 mg, 7.88 mmol, 5 equiv) were added to the solution, then the reaction mixture was slowly warm to 70℃. The reaction mixture was stirred at 70℃ for 2.5 h and TLC analysis indicated the reaction was completed. The solution was filtered with celite to get filtrate, extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (5% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 200 mg in 42.18% yield for the next step.

4.1.8. Synthesis of Ethyl 7-bromo-3,4-dihydro-2H-benzo[b][1,4]thiazine-3-carboxylate (**20**)

Ethyl 7-bromo-2H-benzo[b][1,4]thiazine-3-carboxylate (**19**) (466 mg, 1.55 mmol, 1 equiv) was dissolved in methanol stirred at room temperature, and then Pd/C (170 mg, 1.55 mmol, 1 equiv) was added to the solution. The reaction mixture was stirred at room temperature for 8 h under the protection of hydrogen and TLC analysis indicated the reaction was completed. After completed, filter off Pd/C with celite to get filtrate, concentrated and purified by silica gel chromatography (5% EtOAc/hexanes) to give the 12.79% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.16 – 7.13 (m, 0H), 7.04 (ddd, J = 8.6, 2.4, 1.0 Hz, 1H), 6.49 (dd, J = 8.6, 1.0 Hz, 1H), 4.63 (s, 0H), 4.34 (d, J = 2.4 Hz, 0H), 4.32 – 4.26 (m, 1H), 3.29 (ddd, J = 12.6, 3.1, 0.9 Hz, 1H), 3.07 (ddd, J = 12.6, 7.9, 1.0 Hz, 1H), 1.33 (td, J = 7.1, 1.0 Hz, 2H).

4.1.9. Synthesis of 4-(7-bromo-3-oxo-1-thioxo-3a,4-dihydro-1H-benzo[b]imidazo[1,5-d][1,4]thiazin-2(3H)-yl)-2-(trifluoromethyl)benzonitrile (21)

Ethyl 7-bromo-3,4-dihydro-2H-benzo[b][1,4]thiazine-3-carboxylate (**20**) (56 mg, 0.185 mmol, 1 equiv) was reacted with **EN-2** (50 mg, 0.219 mmol, 1.19 eqviu) in 10 mL anhydrous DMF for 5.5h. After work up, the crude product was poured into ice water. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (10%-20% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 55 mg in 61.28% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.60 (d, J = 8.9 Hz, 1H), 8.42 (d, J = 8.2 Hz, 1H), 8.26 (d, J = 1.8 Hz, 1H), 8.04 (dd, J = 8.3, 1.9 Hz, 1H), 7.64 (d, J = 2.2 Hz, 1H), 7.40 (dd, J = 8.9, 2.2 Hz, 1H), 4.89 (dd, J = 11.6, 3.5 Hz, 1H), 3.87 (t, J = 12.0 Hz, 1H), 3.61 (dd, J = 12.4, 3.5 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 178.00, 169.14, 138.56, 136.94, 134.81, 131.74, 128.86, 127.18, 125.63, 119.02, 115.67, 109.33, 57.98, 26.77. HRMS (EI) m/z calcd for $C_{18}H_9ON_3BrF_3S_2$ 482.9317, found: 482.9306.

4.1.10. Synthesis of 4-2-methyl-6-phenylquinoline (**23b**)

22 (0.3 g, 1.35 mmol, 1 equiv) was coupled to Phenylboronic acid (0.198 g, 1.62 mmol, 1.2 equiv) using K_2CO_3 (0.559 g, 4.86 mmol, 3 equiv) and $Pd(PPh_3)_4$ (0.156 g, 0.162 mmol, 0.1 equiv) in 12 mL dioxane and 3 mL water for 16 h at the temperature of 80℃. After work up, some of water was poured into the solution, extracted with EtOAc. The organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (10% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 0.221g in 74.61% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.13 – 8.09 (m, 2H), 8.00 – 7.96 (m, 2H), 7.76 – 7.71 (m, 2H), 7.55 – 7.49 (m, 2H), 7.44 – 7.39 (m, 1H), 7.33 (d, J = 8.4 Hz, 1H), 2.79 (s, 3H).

4.1.11. Synthesis of 6-phenylquinoline-2-carbaldehyde (**24b**)

2-methyl-6-phenylquinoline (**23b**) (221 mg, 1.01 mmol,1 equiv) and SeO_2 (450 mg, 4.06 mmol, 4 equiv) were dissolved in dioxane, the reaction was slowly warmed to 100℃. The reaction mixture was stirred at 100℃ for 4 h and TLC analysis indicated the reaction was completed. Then the solution was cooled to room temperature and extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a yellow solid 230 mg in 97.83% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 10.14 (d, J = 0.8 Hz, 1H), 8.64 (d, J = 8.4 Hz, 1H), 8.43 (d, J = 2.0 Hz, 1H), 8.32 – 8.22 (m, 2H), 8.02 (d, J = 8.4 Hz, 1H), 7.91 – 7.86 (m, 2H), 7.56 (dd, J = 8.3, 6.8 Hz, 3H), 7.50 – 7.45 (m, 1H).

4.1.12. Synthesis of 6-phenylquinoline-2-carboxylic acid (**25b**)

The compounds $NaClO_2$ (0.87 g, 9.62 mmol, 9 equiv) and $NaH_2PO_4.2H_2O$ (1 g, 6.90 mmol, 7 equiv) were dissolved in 8 mL of water, and then slowly added to the tert-butanol solution of 6-phenylquinoline-2-carbaldehyde (**24b**) (230 mg, 0.986 mmol, 1 equiv), after the dropwise addition, 5 mL of 2-methyl-2-butene was added and reacted at room temperature. The reaction mixture was stirred at room temperature for 12 h and TLC analysis indicated the reaction was completed. The solution was

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collect the filter cake. Finally put the filter cake into a vacuum drying oven to dry. It was obtained as a white solid 218 mg in 88.70% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.58 (d, J = 8.5 Hz, 1H), 8.39 (d, J = 2.0 Hz, 1H), 8.27 (d, J = 8.8 Hz, 1H), 8.23 – 8.19 (m, 1H), 8.16 (d, J = 8.5 Hz, 1H), 7.91 – 7.86 (m, 2H), 7.56 (t, J = 7.7 Hz, 2H), 7.49 – 7.43 (m, 1H).

4.1.13. Synthesis of Methyl 6-phenyl-1,2,3,4-tetrahydroquinoline-2-carboxylate (**26b**)

6-phenylquinoline-2-carboxylic acid (**25b**) (218 mg, 0.87 mmol, 1 equiv) and PtO_2 (10 mg, 0.04 mmol, 0.05 equiv) were added to the methanol solution, and then hydrogen gas was bubbled to react at room temperature for 8 h. Then the reaction mixture was cooled to 0°C, and 0.1 mL $SOCl_2$ was added dropwise, next the reaction mixture was slowly warmed to 65℃. The reaction mixture was stirred at this temperature for 4h and TLC analysis indicated the reaction was completed. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (8% EtOAc/hexanes) to give the desired product. It was obtained as a yellow solid 55 mg in 23.53% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.58 – 7.52 (m, 3H), 7.45 – 7.38 (m, 3H), 7.33 – 7.23 (m, 4H), 6.70 (d, J = 8.3 Hz, 1H), 4.49 (s, 1H), 4.12 (dd, J = 8.9, 3.7 Hz, 1H), 3.82 (s, 4H), 2.98 – 2.79 (m, 3H), 2.35 (dtd, J = 12.7, 5.5, 3.7 Hz, 1H), 2.08 (dtd, J = 12.6, 8.9, 5.2 Hz, 1H).

4.1.14. Synthesis of 4-(3-oxo-7-phenyl-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27b**)

Methyl 6-phenyl-1,2,3,4-tetrahydroquinoline-2-carboxylate (**26b**) (49 mg, 0.18 mmol, 1 equiv) was reacted with **EN-2** (50 mg, 0.22 mmol, 1.2 eqviu) in 5 mL anhydrous DMF for 4 h. After work up, the crude product was poured into ice water. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (20%-50% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 62 mg in 72.98% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.97 (d, J = 8.6 Hz, 1H), 8.41 (d, J = 8.2 Hz, 1H), 8.28 (d, J = 1.9 Hz, 1H), 8.06 (dd, J = 8.2, 1.9 Hz, 1H), 7.75 – 7.68 (m, 2H), 7.68 – 7.60 (m, 2H), 7.48 (t, J = 7.7 Hz, 2H), 7.41 – 7.35 (m, 1H), 4.87 (dd, J = 12.0, 3.6 Hz, 1H), 3.20 (pd, J = 15.5, 13.4, 5.5 Hz, 2H), 2.47 – 2.27 (m, 3H). ^{13}C NMR (126 MHz, DMSO) δ 176.65, 171.02, 139.12, 138.18, 137.19, 136.28, 134.45, 134.32, 129.12, 128.97, 128.38, 127.58, 126.52, 123.90, 121.86, 115.06, 108.74, 60.73, 25.78, 23.94. HRMS (ESI) m/z calcd for $C_{25}H_{15}F_3N_3OS$ 462.0896, found: 462.0893.

4.1.15. General procedure for the preparation of compounds (**27a, 27c-27i**)

The experimental procedure for synthesizing compounds (**27a, 27c-27i**) is basically the same as **27b**.

4-(3-oxo-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27a**): It was obtained as a white solid. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.80 (dd, J = 8.3, 1.1 Hz, 1H), 8.01 (d, J = 8.2 Hz, 1H), 7.92 (d, J = 2.0 Hz, 1H), 7.81 (dd, J = 8.2, 2.0 Hz, 1H), 7.37 – 7.23 (m, 4H), 4.49 (dd, J = 12.3, 3.3 Hz, 1H), 3.23 – 3.14 (m, 2H), 2.75 – 2.67 (m, 1H), 2.28 – 2.17 (m, 1H). ^{13}C NMR (126 MHz, DMSO) δ 176.79, 171.10, 138.20, 136.27, 134.91, 134.45, 129.54, 128.62, 128.36, 125.67, 121.54, 115.06, 108.72, 60.61, 25.54, 24.01. HRMS (ESI) m/z calcd for $C_{19}H_{11}F_3N_3OS$ 386.0573, found: 386.058.

4-(3-oxo-7-(pyridin-3-yl)-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-... solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.01 (d, J = 8.7 Hz, 1H), 8.95 (d, J = 2.4 Hz, 1H), 8.59 (dd, J = 4.8, 1.6 Hz, 1H), 8.41 (d, J = 8.2 Hz, 1H), 8.29 (d, J = 1.9 Hz, 1H), 8.12 (dt, J = 8.1, 2.0 Hz, 1H), 8.08 – 8.04 (m, 1H), 7.75 (d, J = 2.2 Hz, 1H), 7.69 (dd, J = 8.6, 2.3 Hz, 1H), 7.50 (dd, J = 8.0, 4.7 Hz, 1H), 4.88 (dd, J = 12.0, 3.6 Hz, 1H), 3.21 (pd, J = 15.1, 13.2, 5.3 Hz, 2H), 2.40 (dtt, J = 31.2, 12.4, 6.6 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 176.81, 170.98, 148.58, 147.53, 138.15, 136.30, 134.90, 134.55, 134.45, 134.04, 133.89, 129.38, 128.37, 127.81, 124.11, 123.88, 121.99, 115.05, 108.77, 60.69, 25.77, 23.85. HRMS (ESI) m/z calcd for $C_{24}H_{16}F_3N_4OS$ 465.0983, found: 465.0991.

4-(3-oxo-1-thioxo-7-(6-(trifluoromethyl)pyridin-3-yl)-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27d**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.16 (d, J = 2.2 Hz, 1H), 9.06 (d, J = 8.7 Hz, 1H), 8.42 (dd, J = 8.2, 3.7 Hz, 2H), 8.29 (d, J = 1.8 Hz, 1H), 8.07 (dd, J = 8.2, 1.9 Hz, 1H), 8.01 (d, J = 8.3 Hz, 1H), 7.86 (d, J = 2.2 Hz, 1H), 7.80 (dd, J = 8.7, 2.3 Hz, 1H), 4.90 (dd, J = 12.0, 3.6 Hz, 1H), 3.31 – 3.17 (m, 2H), 2.50 – 2.32 (m, 1H). ^{13}C NMR (126 MHz, DMSO) δ 177.45, 171.38, 148.55, 138.60, 138.31, 136.78, 136.24, 136.15, 134.91, 132.86, 130.03, 128.84, 125.09, 122.50, 121.35, 115.50, 109.26, 61.11, 26.29, 24.21. HRMS (ESI) m/z calcd for $C_{25}H_{13}F_6N_4OS$ 531.0716, found: 531.072.

4-(3-oxo-1-thioxo-7-(4-(trifluoromethyl)phenyl)-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27e**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.02 (d, J = 8.6 Hz, 1H), 8.41 (d, J = 8.2 Hz, 1H), 8.28 (s, 1H), 8.07 (d, J = 8.3 Hz, 1H), 7.95 (d, J = 8.1 Hz, 2H), 7.83 (d, J = 8.1 Hz, 2H), 7.76 (s, 1H), 7.71 (d, J = 8.9 Hz, 1H), 4.91 – 4.83 (m, 1H), 3.22 (dq, J = 17.6, 11.2, 8.6 Hz, 2H), 2.49 – 2.30 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 177.31, 171.42, 143.55, 138.61, 136.76, 135.90, 135.63, 134.91, 129.82, 128.83, 128.49, 127.73, 126.26, 124.78, 123.77, 122.41, 115.50, 109.24, 61.15, 26.23, 24.29. HRMS (ESI) m/z calcd for $C_{26}H_{14}F_6N_3OS$ 530.0764, found: 530.0767.

4-(3-oxo-7-(quinolin-3-yl)-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27f**): It was obtained as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.32 (d, J = 2.3 Hz, 1H), 9.06 (d, J = 8.7 Hz, 1H), 8.71 (d, J = 2.4 Hz, 1H), 8.42 (d, J = 8.2 Hz, 1H), 8.30 (d, J = 1.8 Hz, 1H), 8.07 (dd, J = 8.2, 1.2 Hz, 3H), 7.92 (d, J = 2.2 Hz, 1H), 7.86 (dd, J = 8.7, 2.3 Hz, 1H), 7.79 (ddd, J = 8.3, 6.8, 1.5 Hz, 1H), 7.67 (ddd, J = 8.2, 6.9, 1.2 Hz, 1H), 4.91 (dd, J = 12.0, 3.6 Hz, 1H), 3.25 (ddd, J = 17.0, 11.8, 7.9 Hz, 1H), 2.43 (ddd, J = 24.3, 12.4, 6.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 177.29, 171.44, 149.71, 147.27, 138.63, 136.78, 135.41, 134.92, 134.47, 133.14, 132.24, 130.10, 129.95, 129.12, 128.86, 128.53, 128.12, 127.59, 124.84, 122.52, 115.51, 109.23, 61.18, 26.27, 24.33. HRMS (ESI) m/z calcd for $C_{28}H_{17}ON_4F_3S$ 514.1070, found: 514.1069.

4-(7-(2-chloropyrimidin-5-yl)-3-oxo-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27g**): It was obtained as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.18 (s, 2H), 9.04 (d, J = 8.7 Hz, 1H), 8.42 (d, J = 8.2 Hz, 1H), 8.28 (d, J = 1.8 Hz, 1H), 8.06 (dd, J = 8.2, 1.9 Hz, 1H), 7.87 (d, J = 2.2 Hz, 1H), 7.79 (dd, J = 8.7, 2.2 Hz, 1H), 4.89 (dd, J = 12.0, 3.5 Hz, 1H), 3.21 (dtd, J = 23.1, 17.5, 17.0, 6.1 Hz, 2H), 2.40 (ddp, J = 25.0, 12.6, 6.7, 6.2 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 177.43, 171.32, 159.32, 158.24, 138.54, 136.75, 136.17, 134.87, 131.77, 130.01, 129.75, 128.79, 128.41, 124.67, 122.43, 115.46, 109.21, 61.05, 26.16,

499.0479.

4-(7-(3,5-dimethylisoxazol-4-yl)-3-oxo-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27h**): It was obtained as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.96 (d, J = 8.5 Hz, 1H), 8.41 (d, J = 8.2 Hz, 1H), 8.28 (d, J = 1.9 Hz, 1H), 8.06 (dd, J = 8.2, 1.9 Hz, 1H), 7.38 – 7.30 (m, 2H), 4.88 (dd, J = 11.9, 3.6 Hz, 1H), 3.17 (ddt, J = 24.2, 17.7, 8.5 Hz, 2H), 2.41 (s, 0H), 2.35 (dq, J = 12.4, 6.0 Hz, 1H), 2.25 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 177.28, 171.47, 165.72, 158.58, 138.59, 136.75, 134.91, 134.66, 130.23, 129.72, 128.73, 127.62, 126.65, 122.22, 115.69, 115.50, 109.21, 61.08, 26.09, 24.35, 11.86, 10.98. HRMS (EI) m/z calcd for $C_{24}H_{17}O_2N_4F_3S$ 482.1019, found: 482.1023.

4-(7-(1-cyclopropyl-1H-pyrazol-4-yl)-3-oxo-1-thioxo-3,3a,4,5-tetrahydroimidazo[1,5-a]quinolin-2(1H)-yl)-2-(trifluoromethyl)benzonitrile (**27i)**: It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.84 (d, J = 8.6 Hz, 1H), 8.40 (d, J = 8.2 Hz, 1H), 8.27 (d, J = 1.1 Hz, 2H), 8.04 (dd, J = 8.2, 1.9 Hz, 1H), 7.89 (d, J = 0.8 Hz, 1H), 7.56 (d, J = 2.1 Hz, 1H), 7.50 (dd, J = 8.6, 2.1 Hz, 1H), 4.83 (dd, J = 11.9, 3.7 Hz, 1H), 3.75 (tt, J = 7.4, 3.9 Hz, 1H), 3.23 – 2.99 (m, 2H), 2.38 (ddq, J = 31.0, 12.4, 6.8 Hz, 2H), 1.13 – 1.05 (m, 2H), 1.02 – 0.96 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 176.28, 171.02, 138.19, 136.27, 136.12, 134.43, 132.84, 129.93, 128.99, 128.39, 127.01, 125.61, 122.22, 121.78, 120.83, 115.06, 108.68, 60.72, 32.82, 25.66, 23.98, 6.29. HRMS (EI) m/z calcd for $C_{25}H_{18}ON_5F_3S$ 493.1179, found: 493.1185.

4.1.16. Synthesis of 5-isothiocyanato-3-(trifluoromethyl)picolinonitrile (**EN-3**)

Thiophosgene (0.489 mL, 6.4 mmol, 1.2 equiv) was dissolved in 20 mL water stirred at room temperature, **28** (1 g, 5.37 mmol, 1 equiv) was added to the solution in batches. The reaction mixture was stirred at room temperature for 4 h and TLC analysis indicated the reaction was completed. Then the solution was extracted with CH_2Cl_2, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (0-10% EtOAc/hexanes) to give the desired products. It was obtained as a light yellow oil 540 mg in 44.09% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.76 (d, J = 2.3 Hz, 1H), 7.88 (d, J = 2.3 Hz, 1H).

4.1.17. Synthesis of 5-aminoisobenzofuran-1(3H)-one (**29a**)

$CuSO_4$ (35 mg, 0.22 mmol, 0.007 equiv) was dissolved in 1.5 mL water, then Zn (5.5 g, 84.11 mmol, 2.8 equiv) was added to the solution followed by 20% NaOH solution 12.75 g, the reaction was stirred at ice bath, next 5-aminoisoindoline-1,3-dione (5.00 g, 30.84 mmol, 1 equiv) was added after a few minutes. The reaction mixture was stirred at 60℃ for 4 h and TLC analysis indicated the reaction was completed. The reaction was cooled to room temperature for suction filtration, and then the filter cake was washed with a small amount of water to obtain a filtrate, and then the pH was adjusted to 3-4 with concentrated hydrochloric acid, at which time a large amount of solid precipitated out, and suction filtration was performed to obtain a yellow solid. It was dissolved with 10 mL water and 10 mL concentrated hydrochloric acid, slowly raise the temperature to 100°C for 1 h, and then adjust the pH to 8-9 with 20% NaOH solution. At this time, a large amount of solid is precipitated, and suction filtration is performed to obtain a pink solid, which is placed in a vacuum drying cabinet at 50 °C, 3.2 g of solid was finally obtained with a yield of 69.58%.^{1}H NMR (400 MHz, DMSO-d6) δ 7.45 (d, J = 8.4 Hz, 1H), 6.67 (dd, J = 8.4, 2.0 Hz, 1H), 6.59 (d, J = 1.8 Hz, 1H), 6.26 (s, 2H), 5.16 (s, 2H).

4.1.18. Synthesis of 3-aminofuro[3,4-b]pyridin-5(7H)-one (**29b**)

Methyl 2-methyl-4-nitrobenzoate (5g, 25.62mmol, 1equiv) and NBS (6.8 g, 38.2 mmol, 1.5 equiv) were dissolved in CCl_4 at room temperature under the protection of Argon, then AIBN (420 mg, 2.56 mmol, 0.1 equiv) was added to the solution. The reaction was slowly warmed to 80 ℃ and stirred for 11.5 h. After completed, the mixture was concentrated and purified by silica gel chromatography (5%−20% EtOAc/hexanes) to give the desired product (**EN-4**). It was obtained as a white solid 6.3 g in 89.73% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.35 (d, J = 2.3 Hz, 1H), 8.21 (dd, J = 8.6, 2.2 Hz, 1H), 8.13 (d, J = 8.5 Hz, 1H), 4.99 (s, 2H), 4.02 (d, J = 0.9 Hz, 3H).

EN-4 (6 g, 21.89 mmol, 1 equiv) was dissolved in MeOH stirred at room temperature, and then 10 mL 2M $MeNH_2$/MeOH was added to the solution. The reaction mixture was stirred at room temperature for 12 h and TLC analysis indicated the reaction was completed. Then the solution was concentrated and extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (30%−50% EtOAc/hexanes) to give the desired product (**EN-5**). It was obtained as a light yellow solid 1.6 g in 38.03% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.39 – 8.33 (m, 2H), 8.00 (d, J = 8.2 Hz, 1H), 4.53 (s, 2H), 3.27 (s, 2H).

EN-5 (1.1 g, 5.72 mmol, 1 equiv) was dissolved in methanol stirred at room temperature, and then Pd/C (150 mg, 0.014 mmol, 0.05 equiv) was added to the solution. The reaction mixture was stirred at room temperature for 8 h under the protection of hydrogen and TLC analysis indicated the reaction was completed. After completed, filter off Pd/C with celite to get filtrate, concentrated to give the desired product (**29b**). It was obtained as a gray solid 0.9 g in 96.94% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.28 (d, J = 8.1 Hz, 1H), 6.62 – 6.56 (m, 2H), 5.72 (s, 2H), 4.24 (s, 2H), 2.97 (s, 3H).

4.1.19. Synthesis of 3-aminofuro[3,4-b]pyridin-7(5H)-one (**29d**)

Methyl 3-methylpicolinate (5g, 33.08mmol, 1equiv) was dissolved in CH_2Cl_2 stirred at 0℃ under the protection of nitrogen, then Bu_4NNO_3 (11 g, 36.13 mmol, 1.1 equiv) which was dissolved in CH_2Cl_2 and trifluoroacetic anhydride (4.6 mL, 33 mmol, 1 equiv) were added to the solution stirred for 2 h. Then the reaction mixture was stirred at room temperature for 10 h, the solution was concentrated, extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (10%-15% EtOAc/hexanes) to give the desired product (**EN-6**). It was obtained as a white solid 3.79 g in 58.41% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 9.33 – 9.30 (m, 1H), 8.44 (dq, J = 2.2, 0.7 Hz, 1H), 4.05 (s, 3H), 2.73 (t, J = 0.7 Hz, 3H).

EN-6 (2.96 g, 15.09 mmol, 1 equiv) and NBS (4.03 g, 22.63 mmol, 1.5 equiv) were dissolved in CCl_4 at room temperature under the protection of argon, then AIBN (246mg, 1.5mmol, 0.1equiv) was added to the solution. The reaction was slowly warmed to 80℃ and stirred for 11.5 h. After completed, the mixture was concentrated and purified by silica gel chromatography (10%−20% EtOAc/hexanes) to give the desired product (**EN-7**). It was obtained as a white solid 1.84 g in 44.33% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 9.41 (d, J = 2.4 Hz, 1H), 8.71 (d, J = 2.4 Hz, 1H), 4.97 (s, 2H), 4.09 (s, 3H).

EN-7 (3.27 g, 11.89 mmol, 1 equiv) was dissolved in 20 mL dioxane and 7 mL water stirred at 100℃, the reaction mixture was stirred at this temperature for 12 h, then the solution was cooled to room temperature, extracted with EtOAc, the organic phase was

was... concentrated and purified by silica gel chromatography (20%−50% EtOAc/hexanes) to give the desired product (**EN-8**). It was obtained as a light yellow solid 880 mg in 41.10% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.58 (dd, J = 2.3, 0.9 Hz, 1H), 9.03 (dd, J = 2.3, 0.8 Hz, 1H), 5.56 (t, J = 0.8 Hz, 2H).

EN-8 (0.88 g, 4.89 mmol, 1 equiv) was dissolved in methanol stirred at room temperature, and then Pd/C (26 mg, 0.244 mmol, 0.05 equiv) was added to the solution. The reaction mixture was stirred at room temperature for 8 h under the protection of hydrogen and TLC analysis indicated the reaction was completed. After completed, filtered off Pd/C with celite to get filtrate, concentrated and purified by silica gel chromatography (5% MeOH/CH_2Cl_2) to give the desired product (**29d**). It was obtained as a light yellow solid 343 mg in 46.76% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.10 (d, J = 2.4 Hz, 1H), 6.95 (d, J = 2.4 Hz, 1H), 6.52 (s, 2H), 5.23 (s, 2H).

4.1.20. Synthesis of 6-aminoisochroman-1-one (**29c**)

Methyl 2-bromo-4-nitrobenzoate (10 g, 38.46 mmol, 1 equiv) was dissolved in 80 mL ethanol and 20 mL water stirred for a few minutes, Fe (6.44 g, 115.3 mmol, 3 equiv) and NH_4Cl (8.23 g, 153.8 mmol, 4 equiv) were added to the solution, then the reaction mixture was slowly warm to 70℃. The reaction mixture was stirred at 70℃ for 5.5 h and TLC analysis indicated the reaction was completed. The solution was filtered with celite to get filtrate, extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered and concentrated to give the desired product (**EN-9**). It was obtained as a white solid 8.05 g in 90.99% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.64 (d, J = 8.6 Hz, 1H), 6.87 (d, J = 2.2 Hz, 1H), 6.55 (dd, J = 8.6, 2.2 Hz, 1H), 6.17 (s, 2H), 3.73 (s, 3H).

EN-9 (844 mg, 3.67 mmol, 1 equiv), DMAP (45 mg, 0.367 mmol, 0.1 equiv) and Et_3N (1.53 mL, 11.01 mmol, 3 equiv) were dissolved in anhydrous THF stirred at room temperature, then Boc_2O (1.68 mL, 7.34 mmol, 2 equiv) was added to the solution drop by drop until completed. The reaction mixture was purified by silica gel chromatography (10%−15% EtOAc/hexanes) to give the desired products 523 mg for the next step.

The above compounds were dissolved in 5 mL DMF, tributyl(vinyl)stannane (0.5 mL, 1.72 mmol, 1.5 equiv) and $Pd(PPh_3)_4$ (0.13 g, 0.11 mmol, 0.1 equiv) were added to the solution stirred at 110℃ in microwave at a rate of 100 W for 2.5 h. then the solution was cooled to room temperature, extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (0-10% EtOAc/hexanes) to give the desired product (**EN-11**). It was obtained as a white solid 570 mg. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.75 (s, 0H), 7.79 (d, J = 6.4 Hz, 0H), 7.78 (s, 0H), 7.51 (dd, J = 8.7, 2.2 Hz, 0H), 7.44 (dd, J = 17.5, 10.9 Hz, 0H), 5.59 (dd, J = 17.4, 1.4 Hz, 0H), 5.34 (dd, J = 10.9, 1.4 Hz, 0H), 3.79 (s, 0H), 1.49 (s, 1H).

EN-11 (570 mg, 2.06 mmol, 1 equiv) was dissolved in anhydrous THF stirred at ice bath, then 0.5M 9-BBN in THF (20 mL, 10.3 mmol, 5 equiv) was added to the solution drop by drop until completed. Then the mixture was slowly warmed to room temperature stirred for 12 h, next 1N NaOH (1 mL) and 30% H_2O_2 (1.8 mL) were added to the solution. The reaction mixture was stirred at room temperature for 2 h and TLC analysis indicated the reaction was completed. Then the solution was concentrated, extracted with EtOAc. The organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (20%−50% EtOAc/hexanes) to give the desired product (**EN-12**). It was obtained as a colorless ...6) δ 9.68 (s, 1H), 7.75 (d, J = 8.7 Hz, 1H), 7.44 (d, J = 2.2 Hz, 1H), 7.40 (dd, J = 8.6, 2.2 Hz, 1H), 3.78 (s, 3H), 3.54 (td, J = 7.2, 5.4 Hz, 3H), 3.08 – 3.00 (m, 2H), 1.48 (s, 9H).

EN-12 (276 mg, 0.934 mmol, 1 equiv) was dissolved in 9 mL dioxane and 3 mL water, 0.5 mL 2N HCl was added to the solution, the mixture was slowly warmed to 100℃ stirred for 2 h. After the reaction was completed, the mixture was cooled to room temperature and adjusted to neutral by $NaHCO_3$, then extracted with EtOAc. The organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (30%−60% EtOAc/hexanes) to give the desired product (**29c**). It was obtained as a white solid 70 mg in 45.90% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.58 (d, J = 8.5 Hz, 1H), 6.51 (dd, J = 8.5, 2.3 Hz, 1H), 6.37 (d, J = 2.2 Hz, 1H), 6.14 (s, 2H), 4.34 (t, J = 6.0 Hz, 2H), 2.84 (t, J = 6.0 Hz, 2H).

4.1.21. Synthesis of 2-methyl-2-((1-oxo-1,3-dihydroisobenzofuran-5-yl)amino)propanenitrile (**30a**)

29a (0.5 g, 3.35 mmol, 1 equiv) was reacted with TMSCN (1.26 mL, 10.06 mmol, 3 eqviu) using $ZnCl_2$ (46 mg, 0.33 mmol, 0.1 equiv) in 2mL Acetone at the temperature of 40℃ for 2 h. After work up, the solution was concentrated. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (30%-60% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 576 mg in 78.22% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.63 (d, J = 9.0 Hz, 0H), 7.18 (s, 0H), 6.97 (d, J = 2.1 Hz, 0H), 6.96 – 6.94 (m, 0H), 5.29 (s, 0H), 1.72 (s, 1H).

4.1.22. Synthesis of 4-(4,4-dimethyl-5-oxo-3-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxoimidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile (**31a**)

2-methyl-2-((1-oxo-1,3-dihydroisobenzofuran-5-yl)amino)propanenitrile (**30a**) (300 mg, 1.39 mmol, 1 equiv) was dissolved in anhydrous THF stirred at -78℃, then 1M LiHMDS in THF (1.39 mL, 1.39 mmol, 1 equiv) was added to the solution drop by drop until completed. The mixture was stirred at -78℃ for 0.5 h, then EN-2 (380 mg, 1.66 mmol, 1.2 equiv) which was dissolved in THF was added to the solution. Then the reaction mixture was stirred at room temperature for 1h and TLC analysis indicated the reaction was completed. Then the solution was purified by silica gel chromatography (30%-60% EtOAc/hexanes) to give the compound. Then 8 mLMeOH and 5 mL 2N HCl were added stirred at 80℃ for 2 h. The solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (30%-100% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 36 mg in 4.85% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.42 (d, J = 8.3 Hz, 1H), 8.33 (d, J = 1.9 Hz, 1H), 8.12 (dd, J = 8.2, 1.9 Hz, 1H), 8.07 (d, J = 8.1 Hz, 1H), 7.75 (dd, J = 1.8, 0.8 Hz, 1H), 7.63 (dd, J = 8.1, 1.7 Hz, 1H), 5.52 (s, 2H), 1.56 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 181.90, 176.61, 171.61, 150.56, 142.51, 139.82, 138.12, 135.87, 132.96, 129.89, 127.97, 127.46, 126.45, 116.89, 110.59, 71.71, 68.62, 24.90. HRMS (EI) m/z calcd for $C_{21}H_{14}O_3N_3F_3S$ 445.0702, found: 445.0693.

4.1.23. General procedure for the preparation of compounds (**31b-31e, 33a-33k**)

The experimental procedure for synthesizing compounds (**31b-31e, 33a-33k**) is basically the same as **25a**.

4-(4,4-dimethyl-5-oxo-3-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxoimidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile (**31b**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.42 (d, J = 8.3 Hz, 1H), 8.32 (d, J = 1.9 Hz, 1H), 8.11 (dd, J = 8.2, 1.9 Hz, 1H), 7.86 (d, J = 8.0 Hz, 1H), 7.64 (d, J = 1.7 Hz, 1H), 7.50 (dd, J = 8.1, 1.7 Hz, 1H), 4.57 (s, 2H), 3.11 (s, 2H), 1.55 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 180.00, 174.86, 166.36, 142.92, 138.03, 137.90, 136.21, 134.00, 133.11, 129.77, 128.07, 124.90, 123.64, 115.03, 108.66, 66.56, 51.29, 29.09, 23.01. HRMS (EI) m/z calcd for $C_{22}H_{17}O_2N_4F_3S$ 458.1019, found: 458.1016.

4-(4,4-dimethyl-5-oxo-3-(1-oxoisochroman-6-yl)-2-thioxoimidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile (**31c**): It was obtained as a white solid. ^{1}H NMR (600 MHz, DMSO-d6) δ 8.41 (d, J = 8.2 Hz, 0H), 8.32 (d, J = 2.0 Hz, 0H), 8.14 – 8.09 (m, 0H), 7.48 (d, J = 6.9 Hz, 0H), 4.58 (t, J = 6.0 Hz, 0H), 3.17 (t, J = 6.0 Hz, 0H), 1.55 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 180.30, 175.23, 164.20, 142.22, 140.43, 138.43, 136.70, 134.48, 131.34, 129.49, 129.32, 128.58, 126.13, 115.48, 109.19, 67.71, 67.12, 27.39, 23.51. HRMS (EI) m/z calcd for $C_{22}H_{16}O_3N_3F_3S$ 459.0859, found: 459.0860.

5-(4,4-dimethyl-5-oxo-3-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxoimidazolidin-1-yl)-3-(trifluoromethyl)picolinonitrile (**31d**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.28 (d, J = 2.0 Hz, 1H), 8.85 (d, J = 2.0 Hz, 1H), 8.08 (d, J = 8.1 Hz, 1H), 7.76 (d, J = 1.7 Hz, 1H), 7.62 (dd, J = 8.1, 1.7 Hz, 1H), 5.52 (s, 2H), 1.58 (s, 7H). ^{13}C NMR (126 MHz, DMSO) δ 180.03, 174.93, 170.16, 154.17, 149.21, 140.84, 136.35, 133.64, 131.43, 126.63, 126.17, 124.98, 114.70, 70.31, 67.45, 23.47. HRMS (EI) m/z calcd for $C_{20}H_{13}O_3N_4F_3S$ 446.0655, found: 446.0649.

4-(4,4-dimethyl-5-oxo-3-(7-oxo-5,7-dihydrofuro[3,4-b]pyridin-3-yl)-2-thioxoimidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile (**31e**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.90 (d, J = 2.0 Hz, 1H), 8.44 (d, J = 8.2 Hz, 1H), 8.32 (dd, J = 15.0, 2.0 Hz, 2H), 8.13 (d, J = 8.0 Hz, 1H), 5.56 (s, 2H), 1.60 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 180.77, 174.61, 167.66, 153.36, 143.54, 142.88, 137.79, 136.34, 135.52, 133.95, 133.40, 127.96, 114.99, 108.86, 68.04, 66.93, 22.92. HRMS (EI) m/z calcd for $C_{20}H_{13}O_3N_4F_3S$ 446.0655, found: 446.0655.

4-(8-oxo-5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-6-thioxo-5,7-diazaspiro[3.4]octan-7-yl)-2-(trifluoromethyl)benzonitrile (**33a**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.41 (d, J = 8.3 Hz, 1H), 8.28 (s, 1H), 8.10 (dd, J = 8.5, 5.8 Hz, 2H), 7.79 (s, 1H), 7.65 (d, J = 8.1 Hz, 1H), 5.55 (s, 2H), 2.66 (t, J = 10.1 Hz, 3H), 2.50 – 2.41 (m, 2H), 2.04 – 1.91 (m, 1H), 1.55 (dd, J = 11.0, 5.8 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 180.59, 175.06, 170.22, 149.32, 141.28, 138.45, 136.64, 134.28, 131.84, 126.72, 126.16, 125.44, 115.51, 108.93, 70.34, 68.03, 31.62, 13.90. HRMS (EI) m/z calcd for $C_{22}H_{14}O_3N_3F_3S$ 457.0702, found: 457.0701.

4-(4-oxo-1-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxo-1,3-diazaspiro[4.4]nonan-3-yl)-2-(trifluoromethyl)benzonitrile (**33b**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.41 (d, J = 8.3 Hz, 1H), 8.32 (d, J = 1.9 Hz, 1H), 8.11 (dd, J = 8.3, 1.9 Hz, 1H), 8.06 (d, J = 8.1 Hz, 1H), 7.82 (s, 1H), 7.68 (dd, J = 8.1, 1.7 Hz, 1H), 5.52 (s, 2H), 2.33 (dt, J = 14.0, 6.8 Hz, 2H), 2.19 (dt, J = 13.8, 6.0 Hz, 2H), 1.78 – 1.65 (m, 2H), 1.43 – 1.30 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 182.09, 177.54, 171.60, 150.60, 143.17, 139.92, 138.04, 135.78, 133.27, 129.83, 127.99, 127.49, 126.81, 116.91, 110.42, 77.30, 71.69, 37.48, 26.42. HRMS (EI) m/z calcd for $C_{23}H_{16}O_3N_3F_3S$ 471.0859, found: 471.0858.

4-(4-oxo-1-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxo-1,3-diazaspiro[4.5]decan-3-yl)-2-(trifluoromethyl)benzonitrile (**33c**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.42 (d, J = 8.3 Hz, 1H), 8.31 (d, J = 1.8 Hz, 1H), 8.13 – 8.09 (m, 1H), 8.07 (d, J = 8.1 Hz, 1H), 7.69 (s, 1H), 7.55 (d, J = 8.1 Hz, 1H), 5.52 (s, 2H), 2.43 (d, J = 12.8 Hz, 2H), 2.00 – 1.84 (m, 2H), 1.67 – 1.45 (m, 5H), 0.98 (d, J = 13.5 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 180.02, 173.52, 169.74, 148.66, 140.25, 137.92, 136.18, 134.15, 131.89, 128.12, 126.04, 125.72, 125.51, 115.05, 108.61, 69.83, 67.52, 31.56, 23.62, 20.38. HRMS (EI) m/z calcd for $C_{24}H_{18}O_3N_3F_3S$ 485.1015, found: 485.1015.

5-(8-oxo-5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-6-thioxo-2-oxa-5,7-diazaspiro[3.4]octan-7-yl)-3-(trifluoromethyl)picolinonitrile (**33d**): It was obtained as a white solid. ^{1}H NMR (600 MHz, DMSO-d6) δ 9.18 (d, J = 2.1 Hz, 1H), 8.58 (d, J = 2.1 Hz, 1H), 8.05 (d, J = 8.2 Hz, 1H), 8.02 (d, J = 1.7 Hz, 1H), 7.90 (dd, J = 8.1, 1.8 Hz, 1H), 5.50 (s, 2H), 3.74 (d, J = 11.4 Hz, 2H), 3.66 (d, J = 11.4 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 171.17, 170.28, 152.05, 149.33, 139.56, 132.31, 132.10, 128.76, 126.68, 125.04, 122.47, 121.11, 114.82, 70.30, 67.51, 33.13. HRMS (EI) m/z calcd for $C_{20}H_{11}O_4N_4F_3S$ 460.0448, found: 460.0444.

4-(4-oxo-1-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxo-7-oxa-1,3-diazaspiro[4.4]nonan-3-yl)-2-(trifluoromethyl)benzonitrile (**33e**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.42 (d, J = 8.3 Hz, 1H), 8.29 (d, J = 1.8 Hz, 1H), 8.10 (dd, J = 8.3, 1.9 Hz, 1H), 8.05 (d, J = 8.1 Hz, 1H), 7.86 (d, J = 1.6 Hz, 1H), 7.72 (dd, J = 8.2, 1.6 Hz, 1H), 5.52 (s, 2H), 4.41 (d, J = 10.6 Hz, 1H), 3.98 (d, J = 10.6 Hz, 1H), 3.75 (q, J = 7.8 Hz, 1H), 3.49 (q, J = 7.5 Hz, 1H), 2.59 (p, J = 6.8 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 181.04, 173.38, 170.18, 149.07, 141.81, 138.47, 136.70, 134.33, 131.57, 128.29, 128.26, 126.52, 126.07, 125.11, 115.48, 109.10, 75.44, 74.79, 70.26, 68.17, 36.97. HRMS (EI) m/z calcd for $C_{22}H_{14}O_4N_3F_3S$ 473.0652, found: 473.0653.

4-(4-oxo-1-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxo-8-oxa-1,3-diazaspiro[4.5]decan-3-yl)-2-(trifluoromethyl)benzonitrile (**33f**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.43 (d, J = 8.3 Hz, 1H), 8.32 (d, J = 1.9 Hz, 1H), 8.13 (dd, J = 8.2, 1.9 Hz, 1H), 8.09 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 1.6 Hz, 1H), 7.56 (dd, J = 8.0, 1.7 Hz, 1H), 5.53 (s, 2H), 4.00 – 3.87 (m, 2H), 3.80 (dd, J = 11.6, 4.9 Hz, 2H), 2.43 (d, J = 13.2 Hz, 2H), 1.80 (td, J = 12.9, 5.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 180.75, 174.02, 170.18, 149.30, 140.35, 138.21, 136.67, 134.58, 132.37, 128.49, 126.63, 126.33, 126.00, 115.48, 109.18, 70.31, 65.26, 62.33, 31.72. HRMS (EI) m/z calcd for $C_{23}H_{16}O_4N_3F_3S$ 487.0808, found: 487.0805.

5-(4-oxo-1-(7-oxo-5,7-dihydrofuro[3,4-b]pyridin-3-yl)-2-thioxo-1,3-diazaspiro[4.4]nonan-3-yl)-3-(trifluoromethyl)picolinonitrile (**33g**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.31 – 9.22 (m, 1H), 8.97 – 8.87 (m, 1H), 8.86 – 8.80 (m, 1H), 8.41 – 8.33 (m, 1H), 5.57 (s, 2H), 2.39 (dt, J = 13.8, 6.6 Hz, 2H), 2.21 (dt, J = 14.0, 6.5 Hz, 3H), 1.73 (dt, J = 16.5, 8.4 Hz, 2H), 1.49 – 1.35 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 181.02, 175.75, 168.06, 153.89, 144.26, 143.40, 136.37, 136.15, 134.13, 133.63, 129.52, 129.44, 129.17, 123.12, 114.71, 76.13, 68.50, 36.28, 25.04. HRMS (EI) m/z calcd for $C_{21}H_{14}O_3N_5F_3S$ 473.0764, found: 473.0764.

4-(4-oxo-1-(1-oxoisochroman-6-yl)-2-thioxo-1,3-diazaspiro[4.4]nonan-3-yl)-2-(trifluoromethyl)benzonitrile (**33h**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.40 (d, J = 8.3 Hz, 0H), 8.30 (s, 0H), 8.10 (dd, J = 8.0, 4.5 Hz, 1H), 7.53 (d, J = 10.4 Hz, 1H), 4.57 (t, J = 6.0 Hz, 1H), 3.16 (t, J

= 6. d in 1.72 (s, 1H), 1.37 (d, J = 27.7 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 180.49, 176.16, 164.19, 142.26, 141.05, 138.53, 136.62, 134.38, 131.36, 129.80, 129.69, 128.44, 128.41, 126.08, 115.50, 109.01, 75.77, 67.73, 36.04, 27.35, 25.04. HRMS (EI) m/z calcd for $C_{24}H_{18}O_3N_3F_3S$ 485.1015, found: 485.1016.

5-(4-oxo-1-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxo-7-oxa-1,3-diazaspiro[4.4]nonan-3-yl)-3-(trifluoromethyl)picolinonitrile (**33i**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.24 (d, J = 2.0 Hz, 0H), 8.78 (d, J = 2.1 Hz, 0H), 8.06 (d, J = 8.1 Hz, 0H), 7.87 (d, J = 1.6 Hz, 0H), 7.72 (dd, J = 8.1, 1.7 Hz, 0H), 4.42 (d, J = 10.6 Hz, 0H), 4.01 (d, J = 10.5 Hz, 1H), 3.81 – 3.71 (m, 0H), 3.51 (q, J = 7.5 Hz, 0H), 2.70 – 2.53 (m, 1H). ^{13}C NMR (126 MHz, DMSO) δ 180.55, 173.20, 170.15, 153.96, 149.14, 141.53, 136.04, 133.70, 131.47, 126.61, 126.21, 125.06, 114.70, 75.61, 74.81, 70.28, 68.20, 36.99. HRMS (EI) m/z calcd for $C_{21}H_{13}O_4N_4F_3S$ 474.0604, found: 474.0604.

5-(4-oxo-1-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxo-1,3-diazaspiro[4.4]nonan-3-yl)-3-(trifluoromethyl)picolinonitrile (**33j**): It was obtained as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.27 (d, J = 2.1 Hz, 0H), 8.83 (d, J = 2.1 Hz, 0H), 8.07 (d, J = 8.1 Hz, 0H), 7.82 (dd, J = 1.7, 0.8 Hz, 1H), 7.67 (dd, J = 8.1, 1.7 Hz, 1H), 5.53 (s, 1H), 2.36 (dt, J = 13.6, 6.6 Hz, 1H), 2.25 – 2.14 (m, 1H), 1.73 (q, J = 6.4 Hz, 1H), 1.38 (dt, J = 18.3, 9.7 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 180.22, 175.89, 170.15, 154.11, 149.26, 141.48, 136.21, 133.75, 131.75, 126.67, 126.21, 125.34, 114.72, 76.07, 70.30, 36.10, 25.01. HRMS (EI) m/z calcd for $C_{22}H_{15}O_3N_4F_3S$ 472.0811, found: 472.0807.

5-(4-oxo-1-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-2-thioxo-1,3-diazaspiro[4.5]decan-3-yl)-3-(trifluoromethyl)picolinonitrile (**33k**): It was obtained as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.27 (d, J = 2.0 Hz, 1H), 8.84 (d, J = 2.1 Hz, 1H), 8.07 (d, J = 8.1 Hz, 1H), 7.69 (d, J = 1.6 Hz, 1H), 7.54 (dd, J = 8.1, 1.7 Hz, 1H), 5.52 (s, 2H), 2.43 (d, J = 12.5 Hz, 2H), 1.99 – 1.84 (m, 3H), 1.57 (ddd, J = 29.8, 13.9, 4.5 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 180.03, 173.67, 170.16, 154.34, 149.19, 140.46, 136.43, 133.61, 132.23, 129.39, 126.59, 126.32, 125.91, 114.73, 70.31, 68.29, 32.03, 24.03, 20.86. HRMS (EI) m/z calcd for $C_{23}H_{17}O_3N_4F_3S$ 486.0968, found: 486.0966.

4.1.24. Synthesis of 5-bromoisobenzofuran-1(3H)-one (**34**)

29a (2 g, 13.41 mmol, 1 equiv) was dissolved in 3 mL 47%HBr solution, stirred for a few minutes at ice bath, then $NaNO_2$ (1.02 g, 14.74 mmol, 1.1 equiv) dissolved in some water was added drop by drop. 1h later, CuBr (2.11 g, 14.74 mmol, 1.1 equiv) which was dissolved in 3 mL 47%HBr solution was added to the solution drop by drop, then reaction mixture was stirred at room temperature for 3 h and TLC analysis indicated the reaction was completed. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (0-20% EtOAc/hexanes) to give the desired product. It was obtained as a pink solid 1.38 g in 48.31% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.81 (d, J = 8.5 Hz, 1H), 7.70 (dtd, J = 4.8, 1.7, 0.8 Hz, 2H), 5.33 (s, 2H).

4.1.25. Synthesis of Methyl 1-((1-oxo-1,3-dihydroisobenzofuran-5-yl)amino)cyclopropanecarboxylate (**35**)

5-bromoisobenzofuran-1(3H)-one (**34**) (400 mg, 1.88 mmol, 1 equiv), 1-aminocyclopropane -1-carboxylic acid (380 mg, 3.76 mmol, 2 equiv), K_2CO_3 (777 mg, 5.64 mmol, 3 equiv), CuI (215 mg, 1.12 mmol, 0.6 equiv) and N,N-Dimethylglycine DMSO under the protection of Argon, then the reaction was slowly warmed to 120℃. The reaction mixture was stirred for 15 h and TLC analysis indicated the reaction was completed. Then some water was added to the solution, extracted with EtOAc. The aqueous phase was collected, then 2N HCl was added to the solution drop by drop to adjust PH to about 6. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated for the next step.

The collected compound was dissolved in anhydrous DMF, then K_2CO_3 (777 mg, 5.64 mmol, 3 equiv) and CH_3I (0.24 mL, 3.76 mmol, 2 equiv) were added to the solution stirred at 30℃ for 4 h. Then the solution was extracted with EtOAc, the organic phase was washed with saturated brine, dried over Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (30%-50% EtOAc/hexanes) to give the desired product. It was obtained as a light yellow solid 135 mg in 29.08% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.60 (s, 1H), 7.54 (d, J = 8.4 Hz, 1H), 6.74 (dd, J = 8.5, 2.0 Hz, 1H), 6.68 (d, J = 1.9 Hz, 1H), 5.22 (s, 2H), 3.59 (d, J = 1.1 Hz, 2H), 1.55 (d, J = 4.5 Hz, 2H), 1.14 (d, J = 4.3 Hz, 2H).

4.1.26. Synthesis of 4-(7-oxo-4-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-5-thioxo-4,6-diazaspiro[2.4]heptan-6-yl)-2-(trifluoromethyl)benzonitrile (**36**)

Methyl 1-((1-oxo-1,3-dihydroisobenzofuran-5-yl)amino)cyclopropanecarboxylate (**35**) (100 mg, 0.4 mmol, 1 equiv) was dissolved in anhydrous THF stirred at -78℃ ,then 1M LiHMDS in THF (0.4 mL, 0.4 mmol, 1 equiv) was added to the solution drop by drop until completed. The mixture was stirred at -78℃ for 0.5h, then **EN-2** (111 mg, 0.486 mmol, 1.2 equiv) which was dissolved in THF was added to the solution. Then the reaction mixture was stirred at room temperature for 1 h and TLC analysis indicated the reaction was completed. Then the solution was purified by silica gel chromatography (30−60% EtOAc/hexanes) to give the desired product. It was obtained as a white solid 57 mg in 31.78% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.42 (d, J = 8.3 Hz, 1H), 8.31 (d, J = 1.9 Hz, 1H), 8.11 (dd, J = 8.2, 1.9 Hz, 1H), 8.04 (d, J = 8.1 Hz, 1H), 7.73 (d, J = 1.6 Hz, 1H), 7.64 (dd, J = 8.1, 1.7 Hz, 1H), 5.50 (s, 2H), 1.65 – 1.58 (m, 2H), 1.47 – 1.41 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 179.83, 171.86, 169.69, 148.81, 139.27, 138.16, 136.34, 133.79, 130.73, 127.62, 126.28, 125.75, 124.41, 114.74, 108.36, 70.01, 49.67, 14.12. HRMS (EI) m/z calcd for $C_{21}H_{12}O_3N_3F_3S$ 443.0546, found: 443.0552.

4.2. Molecular Docking

All docking procedures were completed with programs as implemented in Schrodinger Suite.

The crystal structure of androgen receptor (PDB: 2OZ7) was prepared in Protein Preparation Wizard with all water molecules deleted and bond orders assigned. The 2D structures of **31c** and Enzalutamide subjected to LigPrep and possible tautomeric states at pH 7.0 ± 2.0 were generated using Epik. Induced-fit docking was performed using the program Induced Fit and briefly consisted of several steps: first, the initial docking using Glide, and then sampling and minimization of sidechain residues within 5 Å of the docked ligand using Prime, followed by re-docking using Glide. In the initial docking, the receptor van der Waals radii scaling was set at 0.50 and the ligand van der Waals radii was scaled at 0.5 to soften the potentials. Finally, the binding energy for each output pose were estimated as IFDScore.

4.3. Cell culture and antiproliferative assays

with 10% Fetal Bovine Serum (FBS) and 1% Pen-Step-Solution (PSS) at 37℃ in a 5% CO_2 humidified Incubator. Cultivated culture were plated into 96-well plates at 3000 cells per well in 100ul and incubated for 24 h. After 24 h, an equal volume of medium containing various concentrations of compounds was added to each well (9-point and 3 replicate wells per concentration). Cells were treated for 6 days and then 100 ul CellTiter-Glo was added to each well. The reagents were mixed on a decolorizing shaker for 2 minutes to induce cell lysis. The culture plate was incubated at room temperature for 10 minutes to stabilize the fluorescence signal value. Finally, the chemiluminescence signal value was measured in an automatic microplate reader. The proliferation inhibition rate was calculated. Inhibition rate (%) = (Lum negative control-Lum test product)/ (Lum negative control-Lum blank control) *100 GraphPad Prism 6 software was used for drawing, and SPSS software calculated IC50 (half the inhibitory concentration).

LNCaP cells were cultured in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) at 37℃ in a 5% CO_2 humidified incubator. Cells were trypsinized and diluted to 37,500 cells/mL with medium. This cell suspension was seeded in 384-well plates at a volume of 40 uL (1,500 cells/well), then the plate was incubated at 37℃ under 5% CO_2 for 24 h. A 10uL aliquot of drug solution, which supplemented with 4-fold serial dilutions of compounds or DMSO, was added to each well, and incubated at 37℃ in a CO_2 humidified incubator for 6 days. A 25 uL aliquot of CellTiter-Glo was added into each well of the plate, shaked the plate at room temperature for 10 minutes, and read the plateon Envision. IC_{50} is defined as the drug concentration causing 50% cell growth inhibition, determined by dose-response curves.

4.4. Cell culture and AR antagonistic activity assay

HEK293 cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) at 37℃ in a 5% CO_2 humidified incubator. Cells were trypsinized and counted. Then 10uL/well transfection solution was prepared with opti-MEM containing 5 ng androgen receptor clone, 100 ng pGL4.36 vector and 315 nL fugene. Cell suspension was diluted with DMEM without phenol red supplemented with 10% Dialyzed FBS and 1% GlutaMax, and mixed with transfection solution to reach 444,444 cells/mL. This diluted cell suspension was seeded in 96-well plates at a volume of 90 uL (40,000 cells/well), and then the plate was incubated for 24 h. A 10 uL of drug solution, which supplemented with serial dilutions of compounds or DMSO, was added. Then the plate was incubated for 30 mins. 10 nL of DHT (1.5 nM final) was added to each well of the plate, then the plate was incubated at 37℃ under 5% CO_2. After 24 h of incubation, 100 uL of Steady-Glo was added into each well of the plate, shaked the plate at room temperature for 20 minutes, and read the plate on Envision. IC_{50} is defined as the drug concentration causing 50% luciferase expression inhibition, determined by dose-response curves.

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Supplementary Material

Synthesis of intermediate **29b-29d**. ^{1}H NMR, ^{13}C NMR data of all target compounds

Graphical Abstract

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Design, Synthesis and Biological Evaluation of Novel Thiohydantoin Derivatives as Potent Androgen Receptor Antagonists for the Treatment of Prostate Cancer

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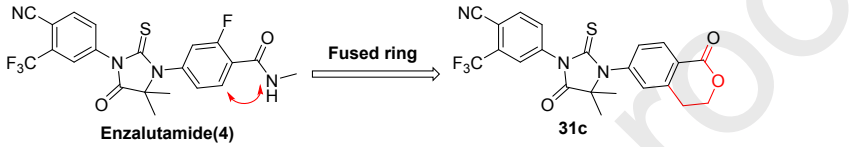

AR antagonist effect: IC_{50}:454.6nM → IC_{50}:195.5nM

2.3-fold improvement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: