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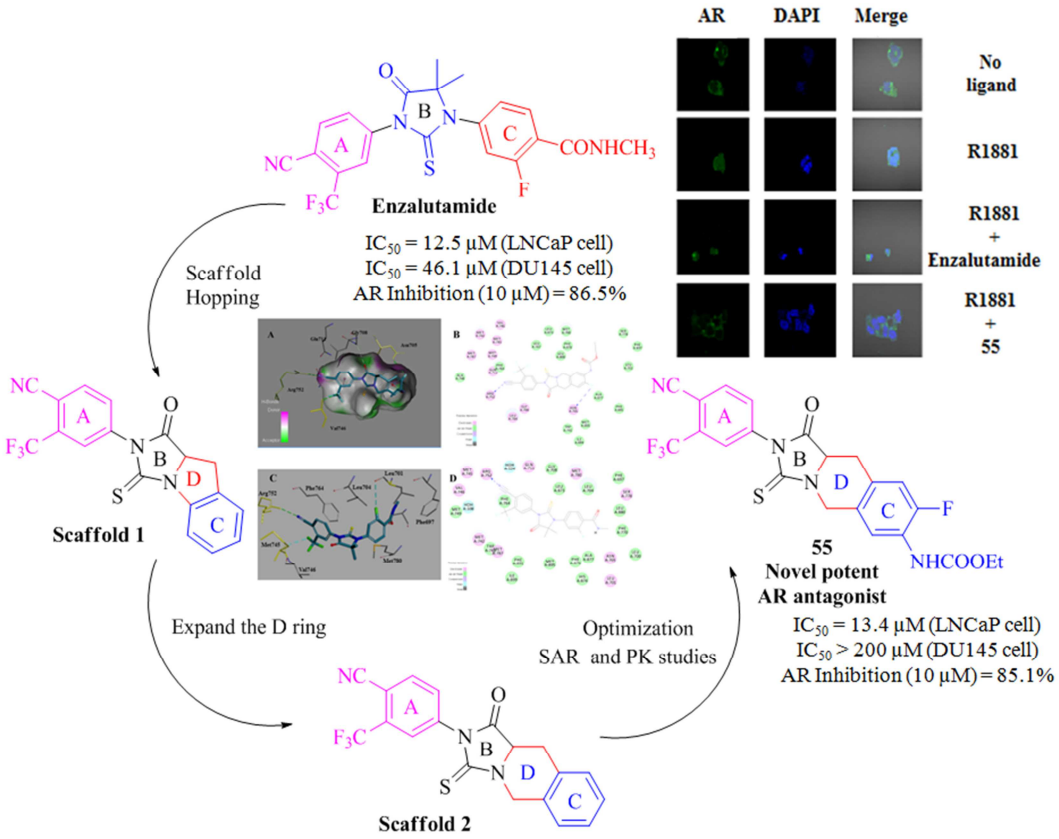

Exploring the tetrahydroisoquinoline thiohydantoin scaffold blockade the androgen receptor as potent anti-prostate cancer agents

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ABSTRACT

Prostate cancer (PC) is a major cause of cancer-related male death in worldwide and the identification of new and improved potent anti-PC molecules is constantly required. A novel scaffold of tetrahydroisoquinoline thiohydantoin was rationally designed based on the enzalutamide structures and our pre-work, leading to the discovery of a series of new antiproliferative compounds. Several new analogues displayed improved androgen receptor (AR) antagonistic activity, while maintaining the higher selective toxicity toward LNCaP cells (AR-rich) versus DU145 cells (AR-deficient) compared to enzalutamide. In fact, compound **55** exhibited promising *in vitro* antitumor activity by impairing AR unclear translocation. More importantly, **55** showed better pharmacokinetic properties compared to the compound **1** reported in our pre-work. These results demonstrate a step towards the development of novel and improved AR antagonists.

Keywords: Prostate cancer, androgen receptor, antagonist, tetrahydroisoquinoline thiohydantoin derivatives

1. Introduction

Prostate cancer (PC) is the second leading cause of cancer-related male death in worldwide and it is expected to account for 19% of all new cancer diagnoses in 2017 [1]. Initially, most patients with advanced prostate cancer are treated with androgen-deprivation therapy (ADT) using either orchiectomy or a luteinising hormone-releasing hormone (LHRH) agonist or antagonist [2, 3]. However, patients invariably develop more aggressive castration-resistant prostate cancer (CRPC) because of several different mechanisms such as: androgen receptor (AR) aberrations, including gene amplifications, point mutations as well as AR splice variants [4-9].

AR is a ligand-dependent transcription factor that belongs to the nuclear receptor superfamily and it plays a critical role in normal prostate development and more over in growth and progression of prostate cancer [10, 11]. AR is composed of an N-terminal domain (NTD), a DNA-binding domain

(DBD), a hinge region and a C-terminal ligand-binding domain (LBD), comprising a Helix-12 (H12). Activation function (AF-1) and activation function (AF-2) are essential for transcriptional activity, which are located in the NTD and LBD, separately. With no androgen binding, inactive AR is in the cytoplasm and binds to heat shock protein 90 (HSP90). After binding, androgen induces a conformational change of AR, leading to dissocation from the chaperones and the relocation of H12. The ligand-bound AR homodimer complex translocates to the nucleus and binds to the androgen-response elements (AREs), then coregulatory proteins, coactivators or corepressors are recruited to the AR complex to regulate AR target gene transcription [12-15].

Several non-steroidal androgen receptor antagonists have been approved for the treatment of PC. Flutamide, hydroxyflutamide and bicalutamide (**Fig. 1**) were first generation non-steroidal AR antagonists and they decreased androgenic effects by competitively inhibiting the binding of androgen to AR and induce a conformational change of H12 by steric clashes. However, these antiandrogens activated AR-LBD point mutants (for example, AR-T877A and AR-W741L, a common mutant triggered by bicalutamide) with the long time of treatment, as a result of switching these antagonists of AR to agonists and leading to the relapse of CRPC [16].

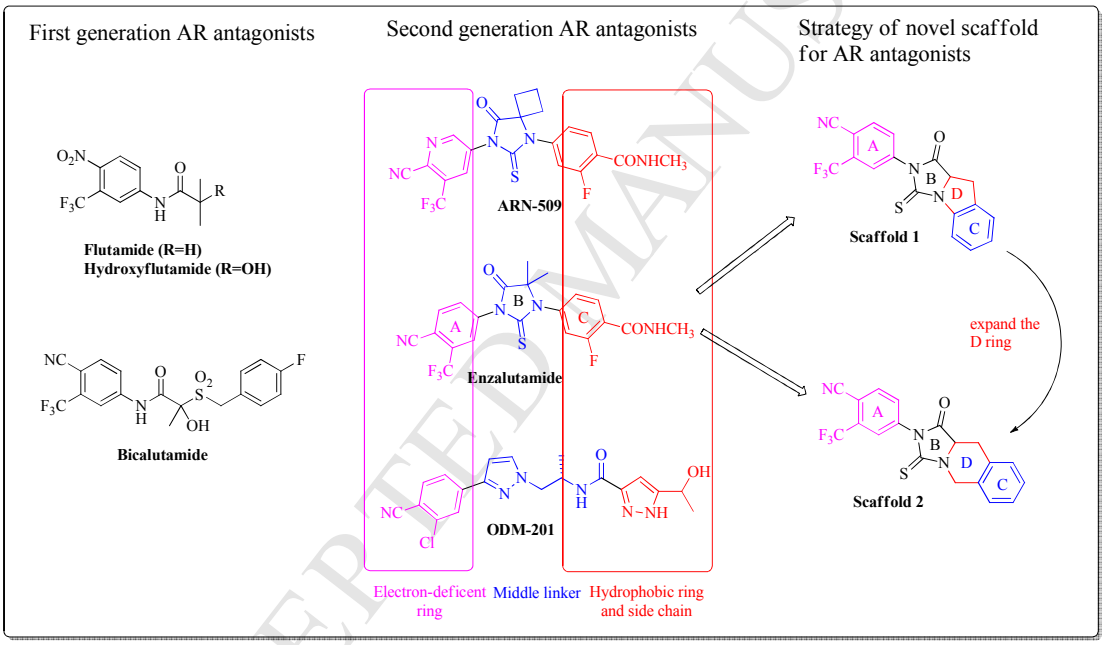


Fig. 1. Structures of non-steroidal AR antagonists and Design of a novel scaffold as AR antagonists.

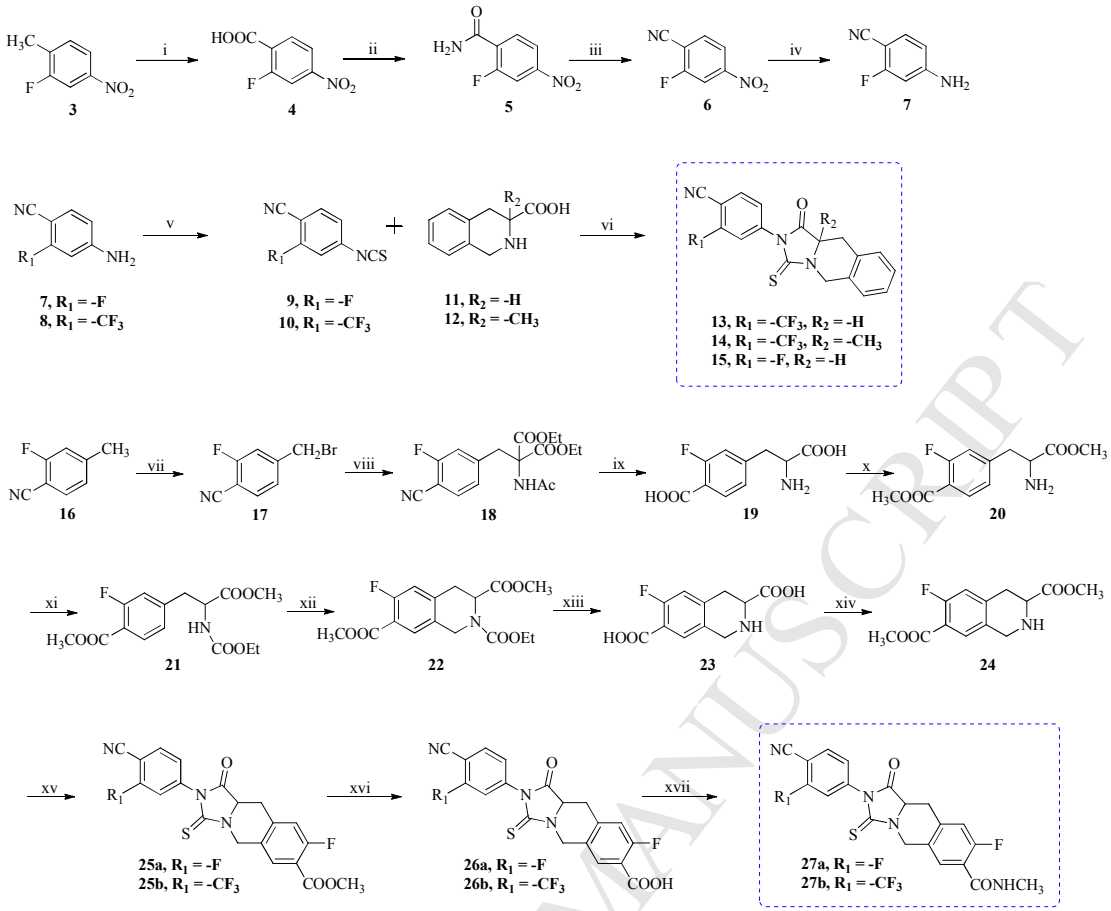
Enzalutamide, ARN-509 and ODM-201 (**Fig. 1**) are second generation non-steroidal antiandrogens with high affinity binding for the AR-LBD. Enzalutamide was approved by FDA in 2012 for the treatment of patients with bicalutamide-resistant CRPC. Enzalutamide, which has higher affinity to AR-LBD than bicalutamide, can block nuclear translocation of AR and recruitment of coactivator. ARN-509 is another competitive AR antagonist with potent activity in both preclinical and clinical trials [17], which shows higher efficacy and is less likely to penetrate the blood-brain barrier than enzalutamide. SPARTAN (NCT01946204), a Phase III trial of ARN-509, is ongoing with patients of CRPC [18]. However, Korpal and coworkers found that enzalutamide and ARN-509 could also be turned into agonists by a mutation of AR-LBD F876L (Phe 876 to Leu). F876L mutant induced the smaller leucine residue leading that the reposition of the benzamide motif of enzalutamide side chain to eliminate H12 dislocation caused by steric clashes [19, 20]. ODM-201, a full antagonist, was

developed for the mutant AR. It shows a similar structure with enzalutamide, but with a longer middle linker for better fitting into the AR-LBD pocket [21, 22]. Based on the second generation non-steroidal AR antagonists, we found that they show a common scaffold composed of electron-deficient ring, middle linker and hydrophobic ring with side chain (**Fig. 1**).

Previously, based on the SAR of enzalutamide, our group introduced a cyclopentane moiety D ring fused with thiohydantoin B ring and benzene C ring to the discovery a novel scaffold (**Fig. 1**) of indoline thiohydantoin **1** [23], which showed potent antiproliferation against LNCaP-hr cell (AR-rich) and little inhibitory activity in DU145 cell (AR-deficient) with the IC_{50} being 15.3 and 143.1 μM. In addition, **1** exhibited considerable antagonistic activity against AR (58.4% inhibition).The three fused rings of indoline thiohydantoin (ring B, C, D, **Fig. 1**) can impose conformational restriction for a greater steric clashes with residues approach to H12 to induce a larger dislocation in H12 and avoid the loss of entropic by the rigid structure [23]. This showed that the introduction of D ring into the enzalutamide is an efficacious and practical method for the finding of novel AR antagonists. Thus, on one hand, to get more effective AR antagonists and furtherly explore the relationship of the fused rings scaffold with AR, this paper designed a novel scaffold of tetrahydroisoquinoline thiohydantoin **2** through changing the D ring from cyclopentane into the cyclohexane and synthesize a novel series of tetrahydroisoquinoline thiohydantoin derivatives (**Fig. 1**). On the other hand, as a result the pharmacokinetic properties of **1** was not satisfactory with low plasma exposure and high clearance, we expected to improve the pharmacokinetic properties of new designed compounds to develop novel and improved AR antagonists.

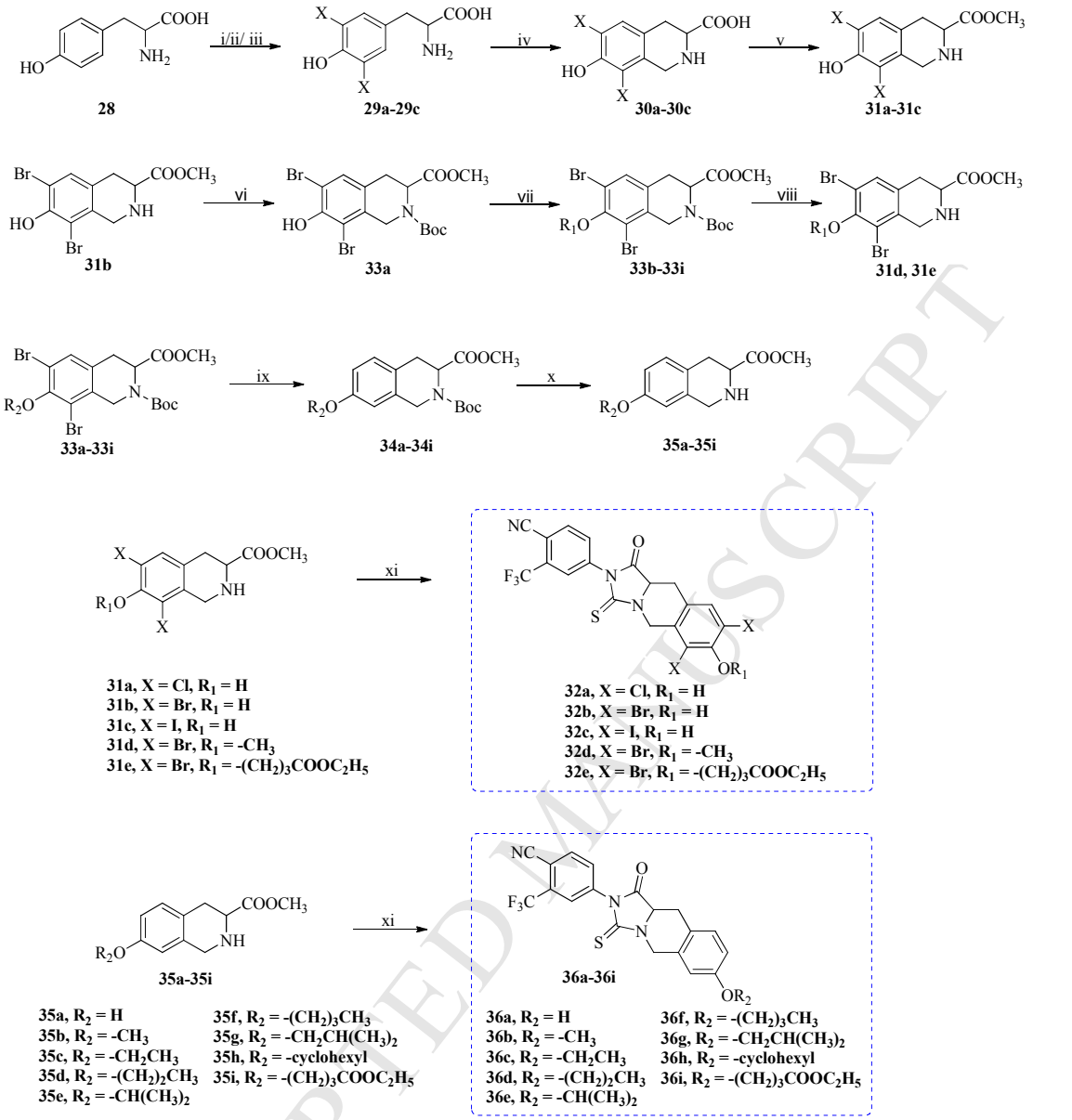
2. Chemistry

Target compounds **13-15** were synthesized as **Scheme 1**. 2-Fluoro-4-nitrotoluene (**3**) was oxidized with potassium permanganate to obtain 2-fluoro-4-nitrobenzoic acid (**4**). We got 2-fluoro-4-nitrobenzamide (**5**) through **4** reacting with aqueous ammonia in the presence of thionyl chloride. **5** was converted to 4-amino-2-fluorobenzonitrile (**7**) by the treatment with phosphorus oxychloride and the reduction with hydrogen and palladium on carbon. Two important intermediates 4-isothiocyanato-2-fluorobenzonitrile (**9**) and 4-isothiocyanato-2-trifluoromethylbenzonitrile (**10**) were readily obtained from **7** and **8** by reacting with thiophosgen [24]. 1,2,3,4-Tetrahydroisoquinoline-3-formic acid (**11**) and 3-methyl-1,2,3,4-tetrahydroisoquinoline-3-formic acid (**12**) were cyclized upon the treatment with **9** and **10** into the target compounds **13-15** [25]. Next, 2-fluoro-4-methylbenzonitrile (**16**) was converted to 4-(2-amino-2-methoxycarbonyl-ethyl)-2-fluoro-benzoic acid methyl ester (**20**) through the treatment with *N*-bromosuccinimide, diethyl acetamidomalonate, hydrolysis and methylation. After protection the $-NH_2$ group of **20** with ethyl chloroformate, we got 4-(2-ethoxycarbonylamino-2-methoxycarbonyl-ethyl)-2-fluoro-benzoic acid methyl ester (**21**), which was cyclized to obtain 6-fluoro-3,4-dihydro-1H-isoquinoline-2,3,7-tricarboxylic acid 2-ethyl ester 3,7-dimethyl ester (**22**). Based on the mechanism of Pictet-Spengler reaction [26, 27], which was used for the synthesis of isoquinoline **22,** we utilized ethyl chloroformate to decrease the electron cloud density of the $-NH_2$ group so as to increase the reaction activity of **20**. Through the hydrolysis and methylation of **22**, we synthesized significant intermediate 6-fluoro-1,2,3,4-tetrahydro-isoquinoline-3,7-dicarboxylic acid dimethyl ester (**24**). Compound **24** was further cyclized with **9** and **10** to yield **25a** and **25b**, respectively. Target compounds **27a** and **27b** were obtained by the hydrolysis with sodium hydroxide and amidation of **25a** and **25b**.



Scheme 1. Reagents and conditions: (i) $KMnO_4$, H_2O, tert-butanol, reflux, 8 h; (ii) $SOCl_2$, toluene, 40 ^oC, 12 h, $NH_3 \cdot H_2O$, DCM, 5 ^oC, 30 min; (iii) $POCl_3$, DMF, H_2O, 25 ^oC, 12 h; (iv) H_2/Pd-C, MeOH, rt, 12 h; (v) $CSCl_2$, $CHCl_3$, H_2O, rt, 15 h; (vi) TEA, DMF, 30 ^oC, 1-2 h; (vii) NBS, BPO, $CHCl_3$, reflux, 12 h; (viii) EtONa, EtOH, diethyl acetamidomalonate, reflux, 18 h; (ix) HCl, reflux, 15 h; (x) $SOCl_2$, MeOH, reflux, 12 h; (xi) $ClCOOC_2H_5$, pyridine, DCM, rt, 4.5 h; (xii) HCHO, CH_3COOH, H_2SO_4, rt, 5 h; (xiii) HCl, reflux, 18 h; (xiv) $SOCl_2$, MeOH, reflux, 12 h; (xv) **9** or **10**, TEA, DMF, rt, 2 h; (xvi) NaOH, THF, H_2O, rt, 1 h; (xvii) NH_2CH_3, DMT-MM, DCM, rt, 3 h.

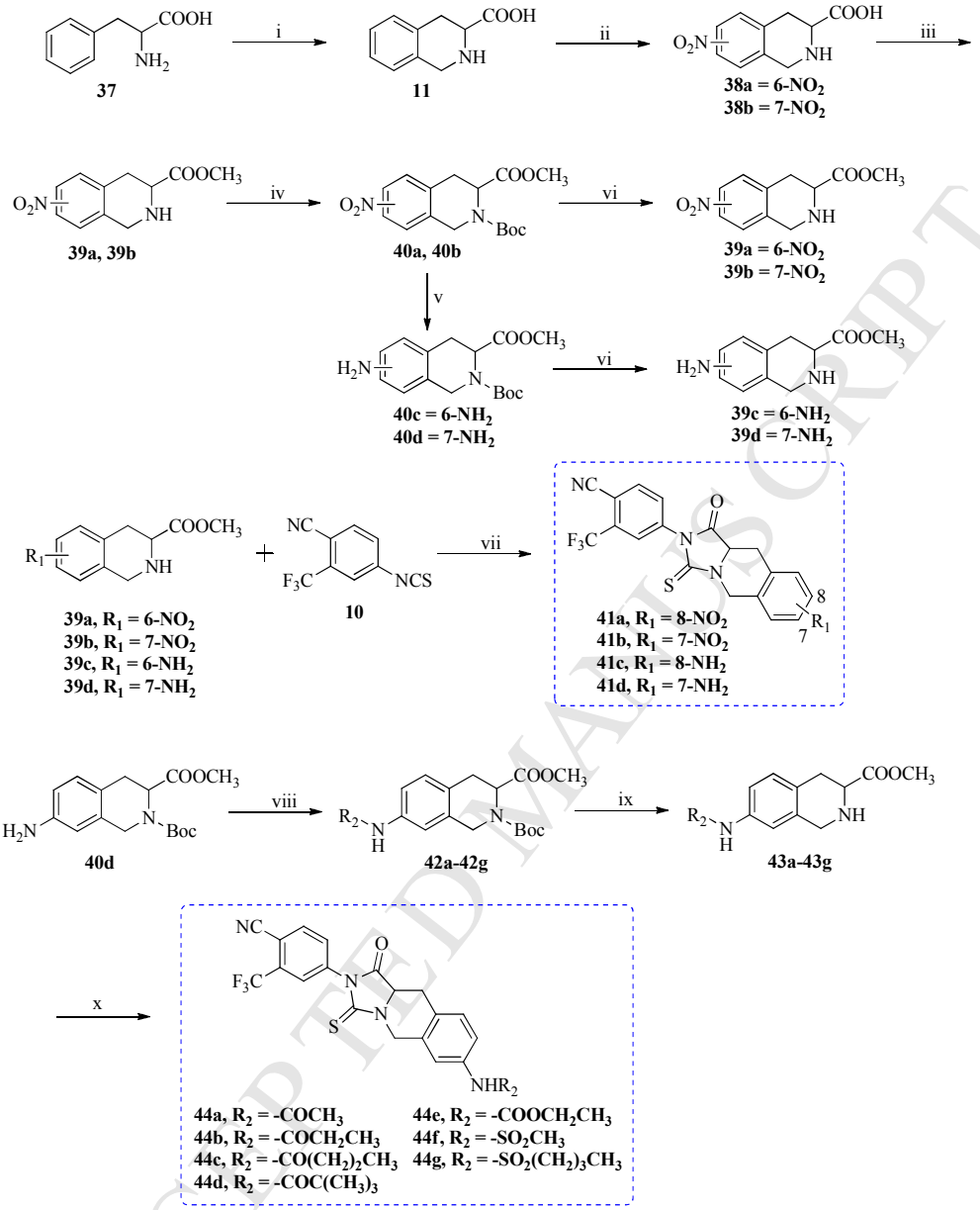
Scheme 2 outlines the synthesis of compounds **32a-32e** and **36a-36i**. Starting from commercially available tyrosine (**28**), substituents Cl, Br and I were introduced into the ortho of -OH of **28** to obtain **29a-29c**. Then **29a-29c** were cyclized by reacting with formaldehyde into the tetrahydroisoquinoline derivatives **30a-30c**. The methylation of **30a-30c** afforded intermediates **31a-31c.** After protecting the -NH of 6,8-dibromo-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid methyl ester (**31b**) with di-tert-butyl dicarbonate $(Boc)_2O$, **33a** was treated with different halogenated hydrocarbons to provide the corresponding aromatic ethers **33b-33i** in high yields. **33b** and **33i** were deprotected by trifluoroacetic acid to yield **31d** and **31e,** respectively. **31a-31e** were converted to the final compounds **32a-32e** upon the treatment with **10** under basic conditions. **35a-35i**, which were synthesized through the cleavage of the 5,7-dibromo and the *N*-Boc of **33a-33i**, were cyclized with **10** into the final products **36a-36i**.



Scheme 2. Reagents and conditions: (i) Cl_2, MeOH, rt, 12 h; (ii) Br_2, CH_3COOH, rt, 4 h; (iii) I_2, 30%H_2O_2, CH_3COOH, HCl, 60 ^oC, 1 h; (iv) HCHO, 33%HBr, CF_3COOH, 55 ^oC, 72 h; (v) $SOCl_2$, MeOH, reflux, 12 h; (vi) $(Boc)_2O$, TEA, 1,4-dioxane, H_2O, rt, 24 h; (vii) R_1X, K_2CO_3, DMF, rt, 2 h; (viii) CF_3COOH, DCM, rt, 30 min; (ix) TEA, H_2/Pd-C, MeOH, 55 ^oC, 12 h; (x) CF_3COOH, DCM, rt, 30 min; (xi) **10**, TEA, DMF, rt, 1-2 h.

The synthesis of compounds **41a-41d** and **44a-44g** were shown in **Scheme 3**. We used phenylalanine (**37**) as raw material, which was converted to **38a** and **38b** by the Pictet-Spengler reaction and nitrification. However, it was difficult to separate **38a** and **38b** purely due to the similar polarity of the two molecules. After trying many methods, we found that decreasing the polarity of the mixture of **38a** and **38b** by treating with methylation and $(Boc)_2O$ could provide **40a** and **40b**, respectively. Then **40a** and **40b** were reduced by hydrogen and palladium on carbon to **40c** and **40d**. With **40a-40d** in hand, it was easy to get tetrahydroisoquinoline derivatives **39a-39d** by the treatment of trifluoroacetic acid. **39a-39d** were cyclized with **10** into the final products **41a-41d.** Through the condensation reaction of **40d** with different acid chloride or sulfonyl chloride and the deprotection of

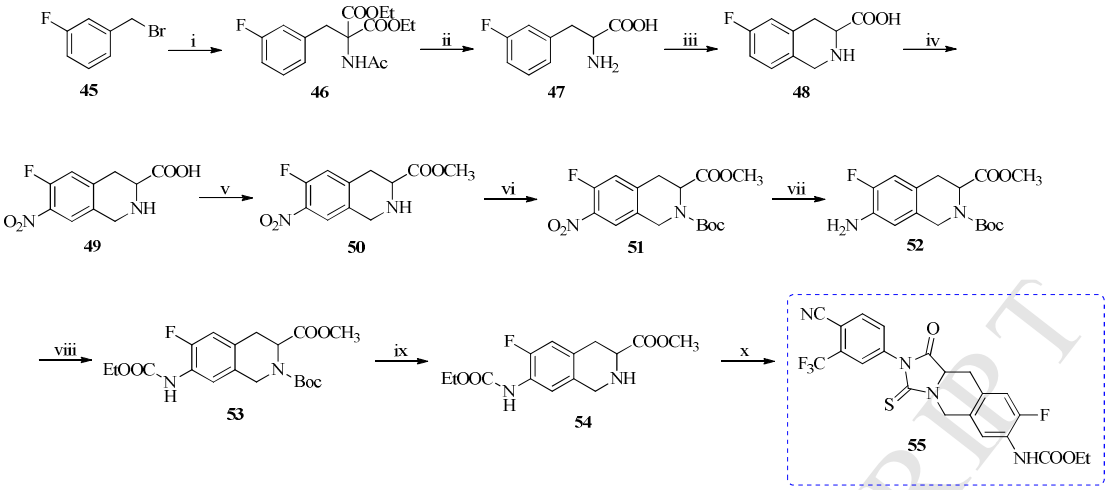
N-Boc, we obtained a series of amide **43a-43g**. Finally, target compounds **44a-44g**, bearing different long side chains were synthesized through the reacting of **43a-43g** with **10**, respectively [28-30].



Scheme 3. Reagents and conditions: (i) HCHO, HCl, reflux, 6 h; (ii) KNO_3, H_2SO_4, rt, 1 h; (iii) $SOCl_2$, MeOH, reflux, 12 h; (iv) $(Boc)_2O$, TEA, 1,4-dioxane, H_2O, rt, 24 h; (v) H_2/Pd-C, MeOH, rt, 12 h; (vi) CF_3COOH, DCM, rt, 30 min; (vii) TEA, DMF, rt, 1 h; (viii) RCl, pyridine, DCM, rt, 3 h; (ix) CF_3COOH, DCM, rt, 12 h; (x) **10,** TEA, DMF, rt, 1-2 h.

To expand our SAR study, target compound **55**, introducing a fluorine atom into the **44e**, was prepared as depicted in **Scheme 4**. 1-(Bromomethyl)-3-fluorobenzene (**45**) was used as raw material. Due to the reactions of **Scheme 4** were similar to **Scheme 1** and **3**, we did not describe them in this repeatedly.

The structures of all target compounds were verified using NMR spectroscopy, ESI mass spectrometry and Infrared spectroscopy. Detailed information about synthesis and characterization of all compounds is described in the experimental and supporting information sections of this manuscript.



Scheme 4. Reagents and conditions: (i) EtONa, EtOH, diethyl acetamidomalonate, reflux, 18 h; (ii) HCl, reflux, 15 h; (iii) HCHO, HCl, reflux, 6 h; (iv) KNO_3, H_2SO_4, rt, 1 h; (v) $SOCl_2$, MeOH, reflux, 12 h; (vi) $(Boc)_2O$, TEA, 1,4-dioxane, H_2O, rt, 24 h; (vii) H_2/Pd-C, MeOH, rt, 12 h; (viii) ClCOOEt, pyridine, DCM, rt, 3 h; (ix) CF_3COOH, DCM, rt, 12 h; (x) **10,** TEA, DMF, rt, 1 h.

3. Results and discussion

3.1. Antiproliferative and androgen receptor antagonist assay

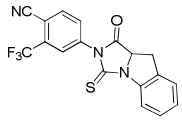
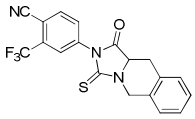
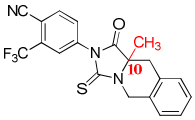
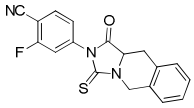
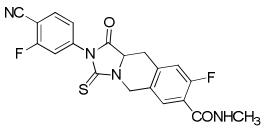
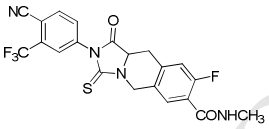
Antiproliferative activity of the newly synthesized compounds was initially evaluated in LNCaP-hr cell line using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Additionally, in order to verify the probability of synthesized compounds as novel AR antagonists, the potential cytotoxic effects were also investigated in DU145 cell. LNCaP shows androgen-dependent cell proliferation whereas DU145 is hormone-independent. Moreover, to clarify whether the antiproliferative activity is related to any interference with the AR function, the AR antagonist effect was evaluated using the luciferase reporter gene assay.

The results demonstrated that the novel compound **13** showed a little less potent inhibitory activity in LNCaP cell than **1** with the IC_{50} being 31.1 μM vs 15.3 μM (Table 1). However, to our delight, the antagonistic activity against AR of **13** (89.0% inhibition) is much higher than compound **1** (58.4% inhibition). This is an encouraging fact that the size of bone skeleton of molecule has a very important influence on the inhibition activity of AR. Next, we decided to fully and thoroughly study the SAR of novel scaffold **13** with AR.

Firstly, to simulate the dimethyl moiety of enzalutamide, **14** was synthesized by introducing a methyl into the position of 10 in compound **13**. However, compound **14** did not show a satisfying result with weak antiproliferative activity in LNCaP (IC_{50} = 65.3 μM) and antagonistic activity against AR (inhibition rate 26.2%, @ 10 μM). This demonstrated that the 10-CH_3 was not necessary for the activity of novel scaffold. Previously, we have found that the electron-withdrawing group -CN of aromatic ring A is required for binding to AR with the respect to direct interaction with Arg752 [23]. Next, we want to know whether the trifluoromethyl moiety is necessary for maintaining the activity. Replacement of the trifluoromethyl with the fluorine atom is associated with decreased activity (i.e. **13** vs **15**; **27b** vs **27a**). Especially, compared with **13**, the antagonistic activity against AR of **15** was abolished completely. Modifications of -CF_3 on aromatic ring A do appear to decrease antiproliferative and antagonistic activity.

Table 1

Antiproliferative and androgen receptor antagonist activity of compounds **1**, **13-15, 27a** and **27b.** All data are mean values from triplicate experiments.

Compound	Structure	IC_{50}/μM[a] (LNCaP)	IC_{50}/μM[b] (DU145)	AR antagonistic activity % (10 μM)[c]
1		15.3 ± 0.3	143.1 ± 5.8	58.4 ± 0.6
13		31.1 ± 1.7	> 200	89.0 ± 3.1
14		65.3 ± 1.9	> 200	26.2 ± 1.2
15		70.3 ± 0.8	37.1 ± 3.2	N.E
27a		116.5 ± 2.3	85.4 ± 2.7	26.7 ± 0.2
27b		28.1 ± 1.1	> 200	47.1 ± 2.3

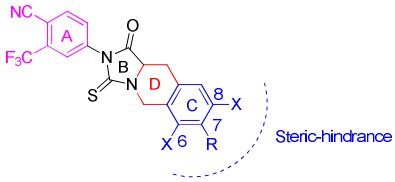
[a]The cell proliferation inhibition of LNCaP cell line; See the Experimental Section for details; [b]The cell proliferation inhibition of DU145 cell line; See the Experimental Section for details; [c]Inhibition rate was shown as a ratio to the R1881 control; See the Experimental Section for details; N.E, no antagonistic effect.

Based on our previous work, structure modifications in the C ring profoundly influence the activity and drug-like properties of compounds. For the purpose of exploring the SAR of substituent group in aromatic ring C, we firstly studied the steric hindrance of substituents by designing compounds **32a-32e** and **36a** (**Table 2**). The results indicated that **32a-32e** showed a comparable activity against LNCaP cell line as enzalutamide. However, the five compounds also exhibited potent inhibitory activity in DU145. Worse more, none of these compounds expressed apparent antagonistic behavior, especially **32e** which had no antagonistic activity. These findings suggested that **32a-32e** might not AR antagonists but cytotoxic agents to exhibit the inhibitory activity in either LNCaP and DU145. We speculated that the molecular size of compounds **32a-32e** might be too large to fit the receptor cavity. Based on the ideas, we obtained compound **36a** by removing the halogen atom of 6 and 8 position to "thin" the molecules **32a-32e**. Removing the halogen atoms might make compound **36a** interacting with AR binding pocket more better. To our delighted, **36a** showed potent antagonistic activity against AR (85.7% inhibition) and no inhibitory activity in DU145 (IC_{50} > 200 μM) which were consistent with our design. So, compound **36a** by removal of halogen atoms showed potent inhibitory activity toward proliferation of LNCaP through AR antagonistic activity but not cytotoxic

effect in DU145.

Table 2

Antiproliferative and androgen receptor antagonist activity of compounds **32a-32e** and **36a** designed from **13.** All data are mean values from triplicate experiments.



Compound	X	R	IC_{50}/μM[a] (LNCaP)	IC_{50}/μM[b] (DU145)	AR antagonistic activity % (10 μM)[c]
32a	-Cl	-OH	14.0 ± 0.6	17.1 ± 1.5	36.0 ± 3.9
32b	-Br	-OH	11.1 ± 0.8	11.4 ± 0.4	6.1 ± 0.9
32c	-I	-OH	16.3 ± 2.1	22.1 ± 0.7	27.3 ± 1.8
32d	-Br	$-OCH_3$	19.1 ± 1.6	33.8 ± 2.1	4.6 ± 0.3
32e	-Br	$-O(CH_2)_3COOEt$	6.3 ± 2.0	21.9 ± 1.3	N.E
36a	H	-OH	50.7 ± 0.4	> 200	85.7 ± 2.3

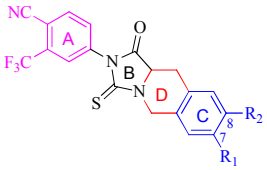
[a]The cell proliferation inhibition of LNCaP cell line; See the Experimental Section for details; [b]The cell proliferation inhibition of DU145 cell line; See the Experimental Section for details; [c]Inhibition rate was shown as a ratio to the R1881 control; See the Experimental Section for details; N.E, no antagonistic effect.

Next, to explore the influence of the position of substituents in the ring C on activity, we designed four compounds **41a-41d** with substituents 7- or 8- position (**Table 3**). Compound **41a** and **41c**, the 8-substituted derivatives, did not show significant AR antagonist activity with the inhibition value being N.E and 14.2%. And their antiproliferative activity against LNCaP cell line were also not high with the IC_{50} being 56.4 and 73.0 μM. However, **41b** and **41d** with the same substituents shifting to 7-position showed an increasement activity (IC_{50} values: 43.0 and 18.9 μM). The antagonistic activity suggested the similar result with the inhibition rate being 59.8% and 37.8%. The results indicated that compounds with substituents at 7-position were more appropriate than at 8-position for maintaining the activity.

Previously, we have studied the steric hindrance and the position of substituent group in C ring. Among the synthesized compounds, **36a** exhibited similar AR antagonistic activity compared to enzalutamide (inhibition: 85.7% vs 86.5%). However, antiproliferative activity against LNCaP of **36a** was not satisfied with the IC_{50} value being 50.7 μM. Thus, to increase the antiproliferative activity, we synthesized target compounds **36b-36i** through extending the length of side chain (**Table 3**). Most of the newly compounds showed increased inhibition activity in LNCaP with IC_{50} being almost 20 μM. Compound **36f**, bearing four carbon atoms, exhibited a little higher activity than enzalutamide (IC_{50}: 10.2 vs 12.5 μM) at the same time maintaining considerable AR antagonistic activity (inhibition: 65.3%). According to the result, we concluded that on the one hand side chain extension indeed increased the anti-proliferation activity in LNCaP cell, however on the other hand the extension exhibited a little reduce in AR antagonistic activity. Three or four atom length was best appropriate for increasing inhibition activity in LNCaP and maintaining AR antagonistic activity.

Table 3

Antiproliferative and androgen receptor antagonist activity of compounds **41a-41d**、**36b-36i**、**44a-44g** and **55**. All data are mean values from triplicate experiments.



Compound	R_1	R_2	IC_{50}/μM[a] (LNCaP)	IC_{50}/μM[b] (DU145)	AR antagonistic activity % (10 μM)[c]
41a	H	$-NO_2$	56.4 ± 2.1	> 200	N.E
41b	$-NO_2$	H	43.0 ± 0.5	> 200	59.8 ± 1.2
41c	H	$-NH_2$	73.0 ± 1.6	> 200	14.2 ± 0.5
41d	$-NH_2$	H	18.9 ± 3.7	89.7 ± 1.1	37.8 ± 0.2
36b	$-OCH_3$	H	100.1 ± 3.1	60.4 ± 4.7	N.E
36c	$-OCH_2CH_3$	H	22.6 ± 1.4	40.9 ± 3.7	47.1 ± 3.1
36d	$-OCH_2CH_2CH_3$	H	11.6 ± 0.6	62.3 ± 0.5	54.0 ± 2.9
36e	$-OCH(CH_3)_2$	H	8.6 ± 1.2	91.6 ± 3.5	45.4 ± 4.2
36f	$-O(CH_2)_3CH_3$	H	10.2 ± 2.5	54.2 ± 2.1	65.3 ± 2.5
36g	$-OCH_2CH(CH_3)_2$	H	10.6 ± 1.9	52.6 ± 0.8	61.5 ± 2.2
36h	-Ocyclohexyl	H	8.5 ± 0.7	98.1 ± 1.6	61.6 ± 0.4
36i	$-O(CH_2)_3COOEt$	H	18.8 ± 4.1	15.4 ± 2.3	43.9 ± 4.1
44a	$-NHCOCH_3$	H	83.8 ± 1.9	> 200	25.0 ± 1.9
44b	$-NHCOCH_2CH_3$	H	19.5 ± 0.8	> 200	4.5 ± 3.0
44c	$-NHCOCH_2CH_2CH_3$	H	10.8 ± 1.6	> 200	45.5 ± 4.1
44d	$-NHCOC(CH_3)_3$	H	21.2 ± 4.5	83.2 ± 4.4	N.E
44e	$-NHCOOCH_2CH_3$	H	6.3 ± 0.4	90.1 ± 3.5	45.4 ± 0.7
44f	$-NHSO_2CH_3$	H	31.1 ± 5.9	> 200	13.6 ± 1.9
44g	$-NHSO_2(CH_2)_3CH_3$	H	3.12 ± 0.3	> 200	29.5 ± 2.5
55	$-NHCOOCH_2CH_3$	F	13.4 ± 1.1	> 200	85.1 ± 3.6
Enzalutamide			12.5 ± 0.3	46.1 ± 0.2	86.5 ± 1.1

[a]The cell proliferation inhibition of LNCaP cell line; See the Experimental Section for details; [b]The cell proliferation inhibition of DU145 cell line; See the Experimental Section for details; [c]Inhibition rate was shown as a ratio to the R1881 control; See the Experimental Section for details; N.E, no antagonistic effect.

AR-LBD is more hydrophilic in antagonistic conformation compared with agonistic conformation. Based on compound **41d**, we obtained **44a-44g** (**Table 3**) through introducing hydrophilic moiety to favor the antagonistic conformation "H12-open." Most of the compounds had no inhibition in DU145 cell with the IC_{50} value being more 200 μM, indicating that these compounds had no cytotoxicity. **44f** and **44g**, introducing the sulfonamide group, had no effect in AR antagonistic activity with the inhibition rate decreasing to 13.6% and 29.5%. Amide derivatives **44a-44e** exhibited improvement activity, especially for compound **44e** (IC_{50} = 6.33 μM; inhibition rate = 45.4%), showed the improved anti-proliferation activity in LNCaP and the AR antagonistic activity compared to **41d** (IC_{50} = 18.9 μM; inhibition rate = 37.8%).

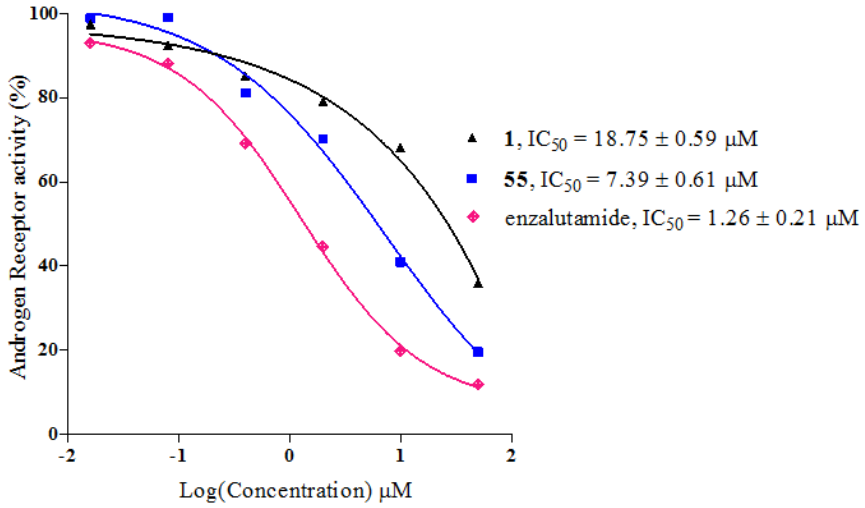


Fig. 2. Dose-dependent androgen receptor antagonist assay for compound **1**, **55** and enzalutamide. Compounds tested at 6 different concentrations. All data are mean values from triplicate experiments.

Finally, to further increase the antagonistic activity and improve pharmacokinetic properties, introducing a polar fluorine atom in the ortho position of the side chain got target compound **55**. The result indicated that compound **55** showed profoundly AR antagonistic activity compared to **44e** (inhibition rate: 85.1% vs 58.6%). Moreover, compound **55** showed comparable effect in inhibiting the LNCaP cell growth and AR antagonistic activity with enzalutamide (the IC_{50} being 13.4 μM vs 12.5 μM and the inhibition rate being 85.1% vs 86.5%). In addition, **55** exhibited no inhibition activity in DU145 cell compared to enzalutamide (IC_{50}: > 200 μM vs 46.1 μM). A 6-concentrations dose-dependent AR antagonism assay (**Fig. 2**) showed that **55** possess an antagonistic IC_{50} in the same range of enzalutamide and the AR antagonistic activity of **55** was two-fold higher than compound **1**. On the basis of these findings, we summarized the SAR studies for compound **55** in **Fig. 3**.

No correlation between the inhibitory activity toward LNCaP proliferation and AR antagonism can be identified just as previously report[2], implying that other modes of action or other parameters such as: physicochemical property, cell membrane permeability or target activity could have an significant role in the antiproliferative activity of our novel compounds.

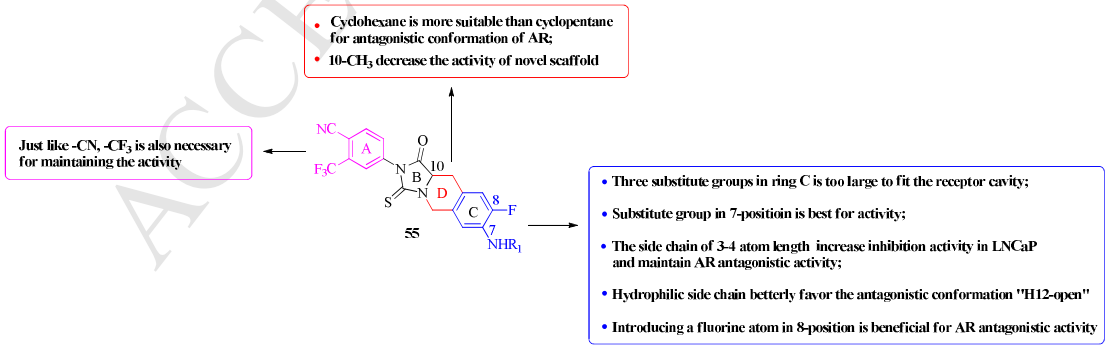


Fig. 3. SAR derived from tetrahydroisoquinoline thiohydantoin derivatives.

3.2 Immunofluorescence assay of compound 55

The resultant strong antiandrogenic effect of compound **55** can be explained by molecular basis for its antagonism. To explore molecular basis of **55**, the dynamic of AR subcellular distribution was

analyzed by immunofluorescence assay with a confocal microscope (LSM700, Zeiss) [31]. When an agonist R1881 binds to AR, the R1881-AR complex translocated from the cytoplasm into the nucleus, causing the localization of the liganded AR proteins in the nucleus (**Fig. 4**). However, treatment of enzalutamide and **55** in the presence of R1881 resulted in a distinct distribution pattern in which the liganded AR proteins were instead dispersed throughout the cytoplasm, indicating that they effectively interfere with nuclear translocation of the AR proteins. Moreover, the green nuclear AR staining of compound **55** was much weaker than that of enzalutamide, indicating that **55** can significantly impair AR nuclear translocation and reduce more levels of nuclear AR than enzalutamide.

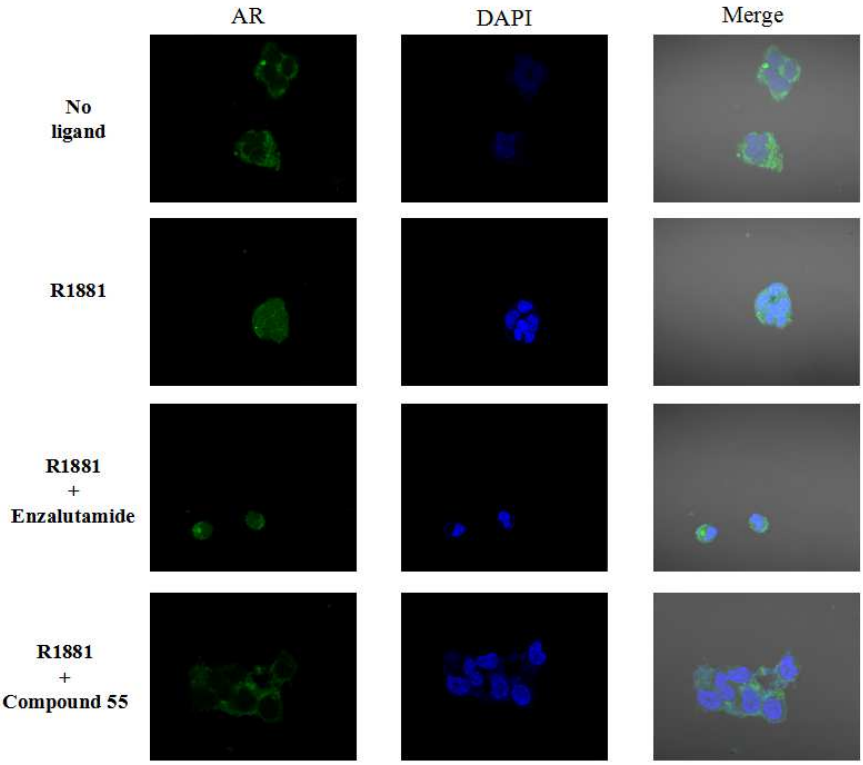


Fig. 4. Molecular basis of antiandrogenic effect of compound **55**: green staining represents AR, blue staining represents nuclei and merge represents both. AR and nuclei was labeled with Alexa Fluor® 488 (green) goat anti-rabbit IgG (H + L) and DAPI, respectively. LNCaP were treated with R1881 (10 nM) alone or in combination with compound **55** (10 μM) or enzalutamide (10 μM), and compared to the no ligand group without treating with R1881.

*3.3 Pharmacokinetic Properties of compound **1, 13, 44e,** and **55***

Having identified several compounds with excellent in vitro antiproliferative and androgen receptor antagonist activity, pharmacokinetic evaluations were conducted in rats following gavage administration at a dose of 5 mg/kg [32], and the results were summarized in **Table 4**.

Table 4

Pharmacokinetic properties of selected compound **1**, **13**, **44e**, and **55.**

compound	AUC_{0-t}(ng·h/mL)	$T_{1/2}$(h)	T_{max}(h)	C_{max} (ng/mL)	CL(mL/h/kg)
1	2863	10.36	2	452	1588
13	3865	5.13	2	387	1230
44e	7034	34.38	2	641	570
55	29995	24.68	4	2290	146

Abbreviations: AUC_{0-t}, area under the concentration-time curve up to last sampling time; $T_{1/2}$, elimination half-life; CL, clearance. Rats dosed at 5 mg/kg gavage administration, n = 3. Data are the mean values.

Compound **55** exhibited favorable plasma exposure (AUC_{0-t} = 29995 ng·h/mL) and relatively low clearance (CL = 146 mL/h/kg). Compound **1** displayed poor pharmacokinetic properties with low plasma exposure (AUC_{0-t} = 2863 ng·h/mL) and relatively high clearance (CL = 1588 mL/h/kg), whereas the hit compound **13** showed a litter higher plasma exposure and a litter decrease of clearance when compared with that of **1**. The results indicated that the pharmacokinetic properties of novel scaffold of tetrahydroisoquinoline thiohydantoin was better than that of indoline thiohydantoin. Interestingly, the compound **44e** exhibited the longest elimination half-life ($T_{1/2}$ = 34.38 h) among these analogues, while its plasma exposure was low (AUC_{0-t} = 7034 ng·h/mL). Based on the pharmacokinetic properties, therefore, we chose compound **55** for further *in vivo* studies in our next work.

3.4 Molecular Modeling

In order to better elucidate the mechanism of compound **55** antagonism at the molecular level, we performed molecular modeling calculations and compared them to those of enzalutamide. The compound **55** and enzalutamide were docked into the active site of AR (PDB code 2OZ7) using Accelrys Discovery Studio (DS) 3.0 (Accelrys Inc., San Diego, CA, USA). As shown in **Fig. 5**, compound **55** could fit into the binding site by the formation of the key hydrogen bonding interaction with Arg752 similar to that of enzalutamide, while the side chain on ring C was located to different orientation, formed as a result of the conformational rearrangement of the active site. Enzalutamdie formed additional hydrogen bond interactions through its fluorine atom on ring C with the side chain of Leu701. Similarly, **55** kept the interaction between the fluorine atom and the side chain of Asn705, and formed an additional hydrogen bond between the side chain of Gly708 and the oxygen atom on ring B.

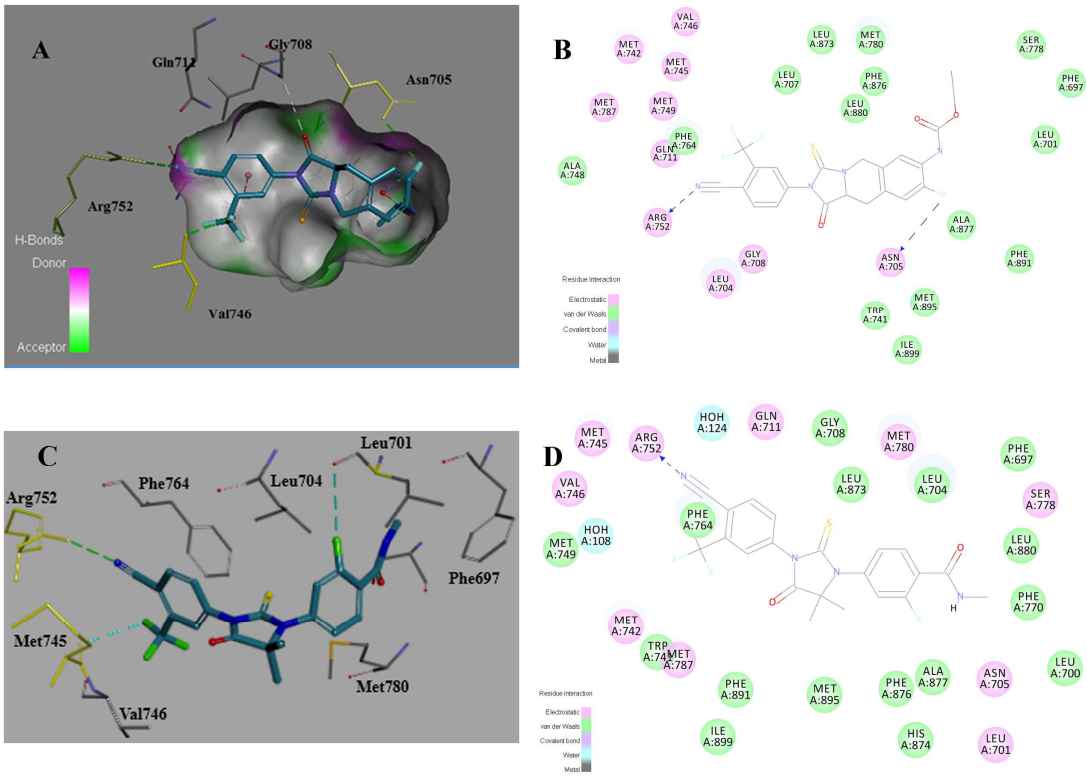


Fig. 5. (**A**) The predicted binding mode of compound **55** in complex with AR; (**B**) The 2D diagram of binding mode of compound **55** in complex with AR; (**C**) The predicted binding mode of enzalutamide in complex with AR; (**D**) The 2D diagram of binding mode of enzalutamide in complex with AR.

4. Conclusion

Herein we report the synthesis and biological *in vitro and vivo* evaluation of a novel class of tetrahydroisoquinoline thiohydantoin derivatives as potent androgen receptor antagonists. Firstly, compound **13** exhibited much increasement in AR antagonistic activity compared to compound **1** (inhibition rate: 89.1% vs 58.4%) and on the same time **13** maintained potent antiproliferative activity in LNCaP cell. Next, we roundly investigated the SAR of novel scaffold tetrahydroisoquinoline thiohydantoin to obtain compound **55**, a potent androgen receptor antagonist. **55** showed comparable effect in inhibiting the LNCaP cell growth (IC_{50} = 13.4 μM) and AR antagonistic activity (inhibition rate = 85.1%) with enzalutamide (IC_{50} = 12.5 μM, inhibition rate = 86.5%). In addition, **55** exhibited no inhibition activity in DU145 cell (IC_{50} > 200 μM) compared to enzalutamide (IC_{50} = 46.1 μM). More importantly, **55** showed favorable pharmacokinetic properties with high plasma exposure and relatively low clearance compared to **1**. Finally, the most promising compound **55** has been selected for *in vivo* preclinical studies in mouse models, which will be reported in later paper. We believe that it may be a novel candidate in the treatment of advanced human prostate cancer.

5. Experimental procedures

5.1 Chemistry

All reagents and solvents were from commercial sources and were used without further purification. With tetramethylsilane (TMS) as internal standard, the ^{1}H and ^{13}C NMR spectra were

recorded on Bruker AV-300 (300 and 75 MHz) apparatus at 25 ^{o}C. Samples were prepared as solutions in deuterated solvent. EI-MS was collected on shimadzu GCMS-2010 instruments. HR-MS spectral data was obtained on Agilent technologies 6520 Accurate-Mass Q-TOF LC/MS instruments. All target compounds were purified via silica gel (60 Å, 70-230 mesh) column chromatography. Melting points were measured by XT-4 melting point apparatus. All target compounds were found to have > 95% purity.

*5.1.1 2-Fluoro-4-nitro-benzoic acid (**4**)*

To the solution of 2-fluoro-4-nitrotoluene **3** (15.0 g, 96.69 mmol) in tert-butanol/H_2O (500 mL, *v:v* = 2:3), tetrabutylammonium chloride (1.5 g, 5.40 mmol) and potassium permanganate (76.4 g, 0.48 mol) were added. After refluxed for 8 h, the reaction mixture was filtered. The filtrate was then acidified to pH 1 with 1 M HCl and extracted with ethyl acetate (EA). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product. It was obtained as a white solid in 89% yield. **4** was ready for the next step without the further purification. HRMS (ESI): m/z, calculated for $C_7H_4FNO_4$ 184.0079 $(M - H)^-$, found 184.0059.

*5.1.2 2-Fluoro-4-nitro-benzamide (**5**)*

To a solution of **4** (10 g, 54.05 mmol) in toluene (100 mL) was added thionyl chloride (12.86 g, 0.1 mol) dropwisely, and the reaction mixture was stirred at 40 ^{o}C for 12 h. The solvent was removed under reduced pressure to afford the acid chloride as a viscous oil. This oil was dissolved in CH_2Cl_2 (150 mL) and cooled to 0 ^{o}C. Aqueous ammonia (28%, 200 mL) was slowly added and the reaction was stirred at 4 ^{o}C for 30 min. The mixture was extracted with EA, and the organic layer was dried with Na_2SO_4, filtered, and concentrated under reduced pressure to afford a yellow solid in 89.33% yield. HRMS (ESI): m/z, calculated for $C_7H_5FN_2O_3$ 185.0365 $(M + H)^+$, found 185.0388.

*5.1.3 2-Fluoro-4-nitro-benzonitrile (**6**)*

To a solution of 5 (10 g, 54.35 mmol) in DMF (100 mL) was added phosphorus oxychloride (35 mL) dropwisely. And the mixture was stirred at 25 ^{o}C for 12 h. This mixture was treated with ice water (300 mL) for 30 min. Then the mixture was extracted with EA, and the organic layer was washed with sat, dried with Na_2SO_4, filtered, and concentrated under reduced pressure to afford product **6** (7.82 g, 86.73%) as a yellow solid. HRMS (ESI): m/z, calculated for $C_7H_3FN_2O_2$ 167.0261 $(M + H)^+$, found 167.0283.

*5.1.4 4-Amino-2-fluoro-benzonitrile (**7**)*

A solution of **6** (10.0 g, 60.24 mmol) in methanol (100 mL) was hydrogenated with 10% Pd/C (1.0 g) under hydrogen atmosphere at room temperature for 12 h. After filtration, the filtrate was evaporated to give the corresponding product. It was obtained as a gray solid in 88% yield. **7** was ready for the next step without the further purification. HRMS (ESI): m/z, calculated for $C_7H_5FN_2$ 137.0487 $(M + H)^+$, found 137.0452.

*5.1.5 2-Fluoro-4-isothiocyanato-benzonitrile (**9**)*

Thiophosgen (9.3 g, 80.62 mmol) was added to a suspension of intermediate **7** (10.0 g, 73.53 mmol) in $H_2O/CHCl_3$ (450 mL, *v:v* = 8:1). The mixture was stirred at room temperature for 15 h. After the reaction was completed, the reaction mixture was extracted with $CHCl_3$ (3 × 50 mL). The combined

organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (petroleum ether (PE)/ethyl acetate (EA) 6:1) to give the corresponding product. It was obtained as a pale yellow oil in 57% yield. HRMS (ESI): m/z, calculated for $C_8H_3FN_2S$ 179.0098 $(M + H)^+$, found 179.0028.

*5.1.6 4-Isothiocyanato-2-trifluoromethyl-benzonitrile (**10**)*

The synthesis of intermediate **10** was similar to **9**, just replacing the material **7** into 4-amino-2-trifluoromethyl-benzonitrile **8**. m.p. 41-42 ^oC; ^1H-NMR (300 MHz, $CDCl_3$): δ 7.84 (d, J = 8.25 Hz, 1H, Ar-H), 7.59 (s, 1H, Ar-H), 7.48 (d, J = 8.25 Hz, 1H, Ar-H) ppm; MS-EI (m/z): 69, 186, 228 $[M]^+$.

*5.1.7 4-(1-Oxo-3-thioxo-4,9-dihydro-10,10a-dihydroimidazol[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)-2-(trifluoromethyl)benzonitrile (**13**)*

To the solution of **10** (0.23 g, 1 mmol) in DMF (10 mL), 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid **11** (0.21 g, 1 mmol) and triethylamine (0.15 mol) were added. The mixture was stirred at 30 ^oC for 1 h. Then the solution was added into ice water (50 mL), extracted with CH_2Cl_2. The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (PE/EA 6:1) to give the corresponding product. It was obtained as a yellow solid in 39% yield. m.p. 215-218 ^oC; ^1H-NMR (300 MHz, $CDCl_3$): δ 7.88 (d, 2H, Ar-H), 7.48 (d, J = 5.94 Hz, 1H, Ar-H), 7.39-7.31 (m, 4H, Ar-H), 5.79 (d, 1H, -CH-), 4.74 (d, J = 6.87 Hz, 1H, -C<u>H</u>H-), 4.51 (q, J = 6.87 Hz, 1H, -CH<u>H</u>-), 3.45 (d, 1H, -C<u>H</u>H-), 3.08 (q, 1H, -CH<u>H</u>-) ppm; ^{13}C-NMR (75 MHz， DMSO-d_6): δ 178.3, 172.0, 138.1, 136.3, 133.6, 131.2, 130.8, 129.2, 127.1 (q, J = 267.9 Hz, -CF_3), 124.0, 115.0, 108.3, 57.6, 45.6, 29.2 ppm; IR (KBr): 3397, 1742, 1311, 1283, 1134, 809 cm^{-1}; MS-EI (m/z): 104, 130, 387 $[M^+]$; HRMS (ESI): m/z, calculated for $C_{19}H_{12}F_3N_3OS$ 388.0726 $[M + H]^+$, found 388.0724.

*5.1.8 4-(10a-Methyl-1-oxo-3-thioxo-4,9-dihydro-10,10a-dihydroimidazol[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)-2-(trifluoromethyl)benzonitrile (**14**)*

The synthesis of **14** was similar to **13**. The product **14** was yellow solid in 35% yield. m.p. 193-195 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 8.38 (d, J = 8.34 Hz, 1H, Ar-H), 8.32 (s, 1H, Ar-H), 8.08 (d, J = 8.34 Hz, 1H, Ar-H), 7.37 (m, 4H, Ar-H), 5.58 (d, J = 18.09 Hz, 1H, -C<u>H</u>H-), 4.73 (d, J = 18.09 Hz, 1H, -CH<u>H</u>-), 3.55 (d, J = 16.14 Hz, 1H, -C<u>H</u>H-), 3.08 (d, J = 16.14 Hz, 1H, -CH<u>H</u>-), 1.50 (s, 3H, -CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 177.3, 175.0, 138.0, 136.1, 134.0, 131.2, 130.8, 129.9, 128.1 (q, J = 268.2 Hz, -CF_3), 124.0, 115.0, 108.5, 61.8, 43.6, 19.3 ppm; IR (KBr): 3127.38, 1763.72, 1399.48, 1311.14, 1136.40 cm^{-1}; MS-EI (m/z): 104, 130, 144, 401 $[M^+]$; HRMS (ESI): m/z, calculated for $C_{20}H_{14}F_3N_3OS$ 402.0882 $[M + H]^+$, found 402.0888.

*5.1.9 2-Fluoro-4-(1-oxo-3-thioxo-10,10a-ihydroimidazo[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)benzonitrile (**15**)*

The synthesis of **15** was similar to **13**. The product **15** was yellow solid in 56% yield. m.p. 210-214 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 8.38 (s, 1H, Ar-H), 8.15 (d, J = 8.04 Hz, 1H, Ar-H), 8.12 (d, J = 3.6 Hz, 1H, Ar-H), 7.74 (m, 1H, Ar-H), 7.65 (d, J = 8.46 Hz, 1H, Ar-H), 7.55 (m, 1H, Ar-H), 5.49 (t, J = 18.66 Hz, 1H, -CH_2-), 4.83 (dd, J = 5.43 Hz, 1H, -CH_2-), 3.47 (t, J = 2.97 Hz, 1H, -CH-) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 172.7, 171.4, 160.8, 141.2, 134.0, 130.4, 128.6, 127.5, 126.9, 126.3, 125.7, 121.3, 110.8, 108.1, 108.0, 72.5, 53.8, 28.1 ppm; IR (KBr): 3125, 2231, 1752,

1617, 1525, 1400, 1347, 1069, 860, 515 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{18}H_{12}FN_3OS$ 360.0577 $(M + Na)^+$, found 360.0585.

*5.1.10 4-Bromomethyl-2-fluoro-benzonitrile (**17**)*

To the solution of 2-fluoro-4-methylbenzonitrile **16** (10 g, 74.07 mmol) in $CHCl_3$ (200 mL), dibenzoyl peroxide (1 g, 4.13 mmol) and *N*-Bromosuccinimide (19.77 g, 11.11 mmol) were added. The reaction was stirred at refluxing for 12 h. The mixture was treated with saturated sodium bicarbonate solution, washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product **17**. It was obtained as a yellow oil in 88% yield. HRMS (ESI): m/z, calculated for C_8H_5BrFN 213.9678 $(M + H)^+$, found 213.9667.

*5.1.11 2-Acetylamino-2-(4-cyano-3-fluoro-benzyl)-malonic acid diethyl ester (**18**)*

To the solution of Na (0.76 g, 33.16 mmol) in EtOH (100 mL), diethyl acetamidomalonate (6.45 g, 30.15 mmol) was added. The mixture was stirred for 30 min. Then **17** (6.42 g, 30.15 mmol) was added into the solution. After the reaction mixture was stirred at refluxing for 18 h, the mixture was treated with ice water (400 mL) and filtered to give the corresponding **18**. It was obtained as a white solid in 71% yield. HRMS (ESI): m/z, calculated for $C_{17}H_{19}N_2O_5$ 350.1321 $(M + H)^+$, found 350.1423.

*5.1.12 4-(2-Amino-2-carboxy-ethyl)-2-fluoro-benzoic acid (**19**)*

The intermediate 18 (7.50 g, 21.43 mmol) was added into HCl (80 mL). The reaction mixture was stirred at refluxing for 15 h and TLC analysis indicated that the reaction was completed. The mixture was filtered to give the white solid **19** in 86% yield. HRMS (ESI): m/z, calculated for $C_{10}H_{10}NO_4$ 228.0603 $(M + H)^+$, found 228.0606.

*5.1.13 4-(2-Amino-2-methoxycarbonyl-ethyl)-2-fluoro-benzoic acid methyl ester (**20**)*

To the solution of **19** (10 g, 44.05 mmol) in methanol (100 mL), thionyl chloride (10.48 g, 88.10 mmol) was added dropwisely at 0 ^oC. After adding, the mixture was stirred for 30 min maintaining the temperature no more 5 ^oC. Then the solution was stirred at refluxing for 8 h. The reaction mixture was cooled to room temperature and filtered to give the white solid **20** in 75% yield. HRMS (ESI): m/z, calculated for $C_{12}H_{14}NO_4$ 256.0917 $(M + H)^+$, found 256.0914.

*5.1.14 4-(2-Ethoxycarbonylamino-2-methoxycarbonyl-ethyl)-2-fluoro-benzoic acid methyl ester (**21**)*

To the solution of **20** (4 g, 15.69 mmol) in CH_2Cl_2 (40 mL), pyridine (4.96 g, 62.74 mmol) was added. Then carbonochloridicacid ethylester (1.70 g, 15.69 mmol) was dropped into the solution solwly. The reaction was stirred at room temperature for 4.5 h. The solvent was removed under reduced pressure. The the crude was treated with water (100 mL), extracted with EA and washed with brine. The organic layer was dried over Na_2SO_4, filtered and evaporated to give the corresponding product **21**. It was obtained as a white solid in 60% yield. HRMS (ESI): m/z, calculated for $C_{15}H_{18}NO_6$ 328.1119 $(M + H)^+$, found 328.1123.

*5.1.15 6-Fluoro-3,4-dihydro-1H-isoquinoline-2,3,7-tricarboxylic acid 2-ethyl ester 3,7-dimethyl ester (**22**)*

To the solution of **21** (3.83 g, 11.71 mmol) in CH_3COOH (12 mL) and H_2SO_4 (4 mL), paraformaldehyde (4.20 g, 58.56 mmol) was added. The reaction mixture was stirred at room

temperature for 5 h. Then the solution was treated with water, extracted with EA and washed with brine. The organic layer was dried over Na_2SO_4, filtered and evaporated to give the corresponding product **22**. It was obtained as a white solid in 92% yield. HRMS (ESI): m/z, calculated for $C_{16}H_{18}NO_6$ 340.1118 $(M + H)^+$, found 340.1113.

*5.1.16 6-Fluoro-1,2,3,4-tetrahydro-isoquinoline-3,7-dicarboxylic acid (**23**)*

The intermediate **22** (5.00 g, 14.74 mmol) was added into HCl (50 mL). The reaction mixture was stirred at refluxing for 18 h and TLC analysis indicated that the reaction was completed. The mixture was cooled and filtered to give the white solid **23** in 75% yield. HRMS (ESI): m/z, calculated for $C_{11}H_{10}NO_4$ 240.0709 $(M + H)^+$, found 240.0706.

*5.1.17 6-Fluoro-1,2,3,4-tetrahydro-isoquinoline-3,7-dicarboxylic acid dimethyl ester (**24**)*

To the solution of **23** (4.22 g, 17.65 mmol) in methanol (50 mL), thionyl chloride (4.20 g, 35.30 mmol) was added dropwisely at 0 ^oC. After adding, the mixture was stirred for 30 min maintaining the temperature no more 5 ^oC. Then the solution was stirred at refluxing for 8 h. The reaction mixture was cooled to room temperature and filtered to give the white solid **24** in 78% yield. HRMS (ESI): m/z, calculated for $C_{13}H_{14}NO_4$ 268.0917 $(M + H)^+$, found 268.0918.

*5.1.18 2-(4-Cyano-3-fluoro-phenyl)-8-fluoro-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydro-imidazo[1,5-b] isoquinoline-7-carboxylic acid methyl ester (**25a**)*

To the solution of **9** (0.18 g, 1 mmol) in DMF (10 mL), **24** (0.28 g, 1 mmol) and triethylamine (0.5 g, 0.80 mmol) were added. The mixture was stirred at 30 ^oC for 1 h. Then the solution was added into ice water (50 mL), extracted with EA and washed with brine. The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (PE/EA 6:1) to give the corresponding product **25a**. It was obtained as a yellow powder in 70% yield. m.p. 228-230 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.14 (t, J = 5.4 Hz, 1H, Ar-H), 7.95 (d, J = 5.4 Hz, 1H, Ar-H), 7.71 (d, J = 7.8 Hz, 1H, Ar-H), 7.56 (d, J = 8.1 Hz, 1H, Ar-H), 7.41 (d, J = 8.1 Hz, 1H, Ar-H), 5.41 (m, 1H, -CH-), 4.78 (m, 2H, -CH_2-), 3.87 (s, 3H, -CH_3), 3.37 (q, J = 6.0 Hz, 2H, -CH_2-) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 172.7, 171.4, 165.9, 160.8, 158.5, 142.0, 141.2, 134.0, 130.5, 129.6, 121.3, 115.6, 114.4, 110.8, 108.1, 108.0, 72.5, 53.8, 51.5, 28.1 ppm; IR (KBr): 3482, 2236, 1662, 1444, 1367, 1200, 1140, 890, 558 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{20}H_{13}F_2N_3O_3S$ 414.0747 $(M + H)^+$, found 414.0713.

*5.1.19 2-(4-Cyano-3-trifluoromethyl-phenyl)-8-fluoro-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydro-imid-azo[1,5-b]isoquinoline-7-carboxylic acid methyl ester (**25b**)*

The synthesis of compound **25b** was similar to **25a**, just replacing the material **9** into **10**. It was obtained as a yellow powder in 69% yield. m.p. 220-225 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.38 (d, J = 8.4 Hz, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 8.01 (d, J = 8.4 Hz, 1H, Ar-H), 7.96 (d, J = 8.4 Hz, 1H, Ar-H), 7.42 (d, J = 7.5 Hz, 1H, Ar-H), 5.43 (s, 1H, -CH-), 4.78 (m, 2H, -CH_2-), 3.88 (s, 3H, -CH_3), 3.40 (d, J = 4.8 Hz, 1H, -CH_2-), 3.34 (s, 1H, -CH_2-) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 172.7, 171.4, 165.9, 158.5, 142.0, 139.9, 134.7, 132.7, 130.5, 129.6 (q, J = 267.3 Hz, -CF_3), 119.6, 118.1, 115.8, 115.7, 115.6, 114.4, 105.0, 72.5, 53.8, 51.5, 28.1 ppm; IR (KBr): 3409, 2957, 1777, 1628, 1444, 1310, 1268, 1147, 854, 558 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{21}H_{13}F_4N_3O_3S$ 464.0616 $(M + H)^+$, found 464.0671.

*5.1.20 2-(4-Cyano-3-fluoro-phenyl)-8-fluoro-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydro-imidazo[1,5-b] isoquinoline-7-carboxylic acid (**26a**)*

The compound **25a** (0.20 g, 0.48 mmol) was dissolved in THF (5 mL) and 1 N aqueous NaOH (5 mL). The mixture was stirred at room temperature for 50 min. The organic solvent was removed under reduced pressure. The the solution was treated with 1 N HCl to adjust pH being 6 and filtered to give the yellow powder **26a** in 77% yield. m.p. 246-248 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 13.26 (s, 1H, -COOH), 8.13 (t, J = 8.1 Hz, 1H, Ar-H), 7.91 (m, 1H, Ar-H), 7.71 (d, J = 8.1 Hz, 1H, Ar-H), 7.56 (d, J = 7.8 Hz, 1H, Ar-H), 7.35 (d, J = 8.1 Hz, 1H, Ar-H), 5.37 (d, J = 17.4 Hz, 1H, -CH-), 4.84-4.75 (m, 2H, -CH_2-), 3.38-3.30 (m, 2H, -CH_2-) ppm; IR (KBr): 3531, 2234, 1760, 1628, 1449, 1376, 1251, 1139, 896, 558 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{19}H_{11}F_2N_3O_3S$ 398.0416 (M - H)$^-$, found 398.0436.

*5.1.21 2-(4-Cyano-3-trifluoromethyl-phenyl)-8-fluoro-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydro-imid-azo [1,5-b]isoquinoline-7-carboxylic acid (**26b**)*

The synthesis of compound **26b** was similar to **26a**, just replacing the material **25a** into **25b**. It was obtained as a yellow powder in 76% yield. m.p. 160-162 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 13.33 (s, 1H, -COOH), 8.40 (t, J = 8.1 Hz, 1H, Ar-H), 8.06 (m, 1H, Ar-H), 7.96 (d, J = 8.1 Hz, 1H, Ar-H), 7.56 (d, J = 7.8 Hz, 1H, Ar-H), 7.35 (d, J = 8.1 Hz, 1H, Ar-H), 5.39 (d, J = 17.4 Hz, 1H, -CH-), 4.84-4.75 (m, 2H, -CH_2-), 3.41-3.36 (m, 2H, -CH_2-) ppm; ^{13}C-NMR (75MHz, DMSO-d_6): δ 172.7, 171.1, 165.1, 158.9, 142.9, 139.9, 134.7, 132.7, 130.9, 129.6 (q, J = 265.1 Hz, -CF_3), 119.6, 118.1, 115.8, 115.7, 115.7, 114.4, 105.0, 72.5, 53.8, 28.1 ppm; IR (KBr): 3413, 2235, 1718, 1627, 1443, 1313, 1253, 1140, 804, 558 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{20}H_{11}F_4N_3O_3S$ 448.0384 (M - H)$^-$, found 448.0384.

*5.1.22 2-(4-Cyano-3-fluoro-phenyl)-8-fluoro-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydro-imidazol[1,5-b] isoquinoline-7-carboxylic acid methylamide (**27a**)*

To the solution of **26a** (0.1 g, 0.25 mmol) in CH_2Cl_2 (15 mL), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmpholinium chlide, DMTMM (0.04 g, 0.37 mmol) was added at 0 ^{o}C, followed by stirring for 0.5 h. Triethylamine (0.13 g, 1.25 mmol) and methylamine (0.05 g, 0.49 mmol) (33% in ethanol) were then added into the solution and the mixture was reacted at room temperature for 3 h. After the reaction was completed, the solvent was removed under reduced pressure. The resulting slurry was taken up in 1 N HCl and then extracted with EA. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product **27a**. It was obtained as a yellow powder in 78% yield. m.p. 306-309 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.25 (s, 1H, -NH-), 8.13 (t, J = 8.4 Hz, 1H, Ar-H), 7.69 (m, 2H, Ar-H), 7.55 (d, J = 8.4 Hz, 1H, Ar-H), 7.32 (d, J = 8.4 Hz, 1H, Ar-H), 5.33 (d, 1H, -CH-), 4.75 (m, 2H, -CH_2-), 3.33 (s, 2H, -CH_2-), 2.77 (d, J = 8.4 Hz, 3H, -CH_3) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 172.7, 171.4, 167.8, 160.8, 156.1, 141.2, 141.1, 134.0, 129.8, 128.1, 122.1, 121.3, 114.6, 110.8, 108.1, 108.0, 72.5, 53.4, 28.1, 26.7 ppm; IR (KBr): 3424, 2244, 1748, 1620, 1450, 1380, 1247, 1168, 819, 512 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{20}H_{14}F_2N_4O_2S$ 413.0811 (M + H)$^+$, found 413.0884.

5*.1.23 2-(4-Cyano-3-trifluoromethyl-phenyl)-8-fluoro-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydro-imid-azo[1,5-b]isoquinoline-7-carboxylic acid methylamide (**27b**)*

The synthesis of compound **27b** was similar to **27a**, just replacing the material **26a** into **26b**. It

was obtained as a yellow powder in 80% yield. m.p. 329-333 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.38 (d, J = 8.4 Hz, 1H, Ar-H), 8.32 (s, 1H, -NH-), 8.22 (s, 1H, Ar-H), 8.02 (d, J = 8.4 Hz, 1H, Ar-H), 7.65 (d, J = 8.4 Hz, 1H, Ar-H), 7.33 (d, J = 7.5 Hz, 1H, Ar-H), 5.39 (d, J = 7.5 Hz, 1H, -CH-), 4.76 (m, J = 7.5 Hz, 2H, -CH_2-), 3.28 (t, J = 7.5 Hz, 2H, -CH_2-), 1.12 (t, J = 6.9 Hz, 3H, -CH_3) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 172.7, 171.4, 167.8, 156.1, 141.1, 139.9, 134.7, 132.7, 129.8, 128.1 (q, J = 272.0 Hz, -CF_3), 122.1, 119.6, 118.1, 115.8, 115.7, 114.6, 105.0, 72.5, 53.4, 28.1, 26.7 ppm; IR (KBr): 3446, 2963, 1748, 1649, 1487, 1310, 1254, 1140, 856, 561 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{21}H_{14}F_4N_4O_2S$ 463.0854 $(M + H)^+$, found 463.0759.

*5.1.24 2-Amino-3-(3,5-dichloro-4-hydroxyphenyl)propanoic acid (**29a**)*

To the solution of tyrosine **28** (5.00 g, 27.6 mmol) in methanol (100 mL), chlorine gas was added at 0 ^{o}C. The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The remainder was treated with water and filtered. The filtrate was adjusted pH being 5 with ammonia water to generate precipitate. Then the precipitate was filtered and washed with water to give brown solid **29a** in 45% yield. m.p. > 250 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-D_2O): δ 7.17 (s, 2H, Ar-H), 4.30 (m, 1H, -CH-), 3.10 (m, 2H, -CH_2-).

*5.1.25 2-Amino-3-(3,5-dibromo-4-hydroxyphenyl)propanoic acid (**29b**)*

To the solution of tyrosine **28** (10.00 g, 55.2 mmol) in ice acetic acid (100 mL), the solution of bromine (17.3 g, 0.11 mol) in ice acetic acid (30 mL) was dropped at 0 ^{o}C. The reaction mixture was stirred at room temperature for 4 h. The solvent was removed under reduced pressure. The crude was recrystallized with acetone:PE = 1:1 to give white solid **29b** in 67% yield. m.p. > 250 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-D_2O): δ 9.92 (s, 1H, -COOH), 7.44 (s, 2H, Ar-H), 4.22 (m, 1H, -CH-), 3.00 (m, 2H, -CH_2-).

*5.1.26 2-Amino-3-(3,5-diiodo-4-hydroxyphenyl)propanoic acid (**29c**)*

To the solution of tyrosine **28** (2.00 g, 11.0 mmol) in ice acetic acid (14 mL) and HCl (8 mL), iodo (2.8 g, 11.0 mmol) was added. Then 30% H_2O_2 was dropped into the solution at 65 ^{o}C. The mixture was stirred for 30 min maintaining the temperature. The solution was cooled to 15 ^{o}C and treated with water (100 mL). The mixture was adjusted pH being 5 with ammonia water to generate precipitate. Then the precipitate was filtered and washed with water to give brown solid **29c** in 80% yield. m.p. > 250 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-D_2O): δ 7.46 (s, 2H, Ar-H), 4.10 (t, 1H, -CH-), 2.93 (m, 2H, -CH_2-).

*5.1.27 Typical procedure for the preparation of (**30a-30c**)*

To the solution of **29a-29c** (3.60 mmol) in CF_3COOH (20 mL), 33% HBr in CH_3COOH was dropped. Then paraformaldehyde (0.22 g, 7.34 mmol) was added into the solution. The reaction was stirred at 55 ^{o}C for 72 h and the TLC indicated that the reaction was completed. The mixture was cooled to 0 ^{o}C to generate precipitate. Then the precipitate was filtered and washed with EA. The crude was recrystallized with methanol to give corresponding product **30a**-**30c**. And **30a**-**30c** were ready for the next step without the further purification.

*5.1.28 Typical procedure for the preparation of (**31a-31c**)*

To the solution of **30a**-**30c** (16.99 mmol) in methanol (150 mL), thionyl chloride (50.97 mmol)

was added dropwisely at 0 °C. After adding, the mixture was stirred for 30 min maintaining the temperature no more 5 °C. Then the solution was stirred at refluxing for 14 h and the TLC indicated that the reaction was completed. The reaction mixture was cooled to room temperature and filtered to give corresponding product **31a**-**31c**. And **31a**-**31c** were ready for the next step without the further purification.

*5.1.29 Typical procedure for the preparation of (**32a**-**32c**)*

To the solution of compound **10** (1.00 mmol) and TEA (1.00 mmol) in DMF (10 mL), **31a**-**31c** (1.00 mmol) was added. The reaction mixture was stirred at room temperature for 1 h and the TLC indicated that the reaction was completed. The solution was added into ice water and extracted with EA (3 × 20 mL). The combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography (PE/EA 3:1) to give the corresponding product.

*5.1.30 4-(6,8-Dichloro-7-hydroxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)-2-(trifluoromethyl)benzonitrile (**32a**)*

It was obtained as a yellow solid in 44.1% yield. m.p. 260-262 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.27 (s, 1H, -OH), 8.39 (d, J = 8.28 Hz, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, J = 8.28 Hz, 1H, Ar-H), 7.48 (s, 1H, Ar-H), 5.35 (s, 1H, -CH-), 4.76 (d, J = 18.18 Hz, 1H, -C<u>H</u>H-), 4.55 (d, J = 18.18 Hz, 1H, -CH<u>H</u>-), 3.32 (s, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.6, 171.7, 147.9, 137.8, 136.1, 133.7, 131.3, 130.8, 128.6 (q, J = 270.2 Hz, -CF_3), 127.8, 124.3, 120.8, 120.2, 114.9, 108.4, 57.0, 44.2, 28.4 ppm; IR (KBr): 3414, 3127, 1762, 1503, 1400, 1314 cm^{-1}; MS-EI (m/z): 214, 471 [M$^+$]; HRMS (ESI): calculated for $C_{19}H_{10}Cl_2F_3N_3O_2S$ 471.9896 (M + H)$^+$, found 471.9898.

*5.1.31 4-(6,8-Dibromo-7-hydroxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)-2-(trifluoromethyl)benzonitrile (**32b**)*

It was obtained as a yellow solid in 39.2% yield. m.p. 242-245 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.03 (s, 1H, -OH), 8.39 (d, J = 8.22 Hz, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.03 (d, J = 8.22 Hz, 1H, Ar-H), 7.64 (s, 1H, Ar-H), 5.28 (d, J = 18.18 Hz, 1H, -CH-), 4.76 (d, J = 17.01 Hz, 1H, -C<u>H</u>H-), 4.48 (d, J = 17.01 Hz, 1H, -CH<u>H</u>-), 3.28 (s, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.5, 171.7, 149.9, 137.8, 136.2, 133.6, 130.7, 127.8 (q, J = 264.8 Hz, -CF_3), 126.1, 124.0, 120.5, 115.1, 112.1, 108.5, 57.1, 47.2, 28.3, 14.0 ppm; IR (KBr): 3408, 3107, 1760, 1502, 1313 cm^{-1}; MS-EI (m/z): 57, 89, 116, 170, 304, 558, 560 [M$^+$]; HRMS (ESI): m/z, calculated for $C_{19}H_{10}Br_2F_3N_3O_2S$ 559.8885 (M + H)$^+$, found 559.8882.

*5.1.32 4-(6,8-Diiodo-7-hydroxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)-2-(trifluoromethyl)benzonitrile (**32c**)*

It was obtained as a yellow solid in 36.1% yield. m.p. 239-242 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.66 (s, 1H, -OH), 8.39 (d, J = 8.28 Hz, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 8.03 (d, J = 8.28 Hz, 1H, Ar-H), 7.80 (s, 1H, Ar-H), 5.22 (d, 1H, -CH-), 4.74 (d, J = 17.10 Hz, 1H, -C<u>H</u>H-), 4.40 (d, J = 17.10 Hz, 1H, -CH<u>H</u>-), 3.23 (d, J = 8.31 Hz, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.6, 171.7, 154.5, 139.0, 137.8, 136.2, 134.2, 133.8, 127.1 (q, J = 263.4 Hz, -CF_3), 114.8, 91.2, 85.2, 57.2, 52.6, 27.8 ppm; IR (KBr): 3127, 1735, 1477, 1400, 1316 cm^{-1}; MS-EI (m/z): 89, 116, 144, 170, 272, 398, 529, 655 [M$^+$]; HRMS (ESI): calculated for $C_{19}H_{10}F_3I_2N_3O_2S$ 655.8608 (M + H)$^+$, found

655.8603.

*5.1.33 2-Tert-butyl-3-methyl-6,8-dibromo-7-hydroxy-3,4-dihydroisoquinoline-2,3(1H)-dicaroxylate (**33a**)*

To the solution of intermediate **31b** (3.60 g, 10.0 mmol) and TEA (1.52 g, 15.0 mmol) in 1,4-dioxane/ H_2O (180 mL, *v:v* = 8:1), the di-*tert*-butyl dicarbonate (2.4 g, 11.0 mmol) was added dropwise slowly at 0 ^oC. The mixture was stirred at room temperature for 24 h and TLC analysis indicated that the reaction was completed. The solvent was removed under reduced pressure. The crude residue was treated with water and adjusted pH being 1 with 5 N HCl. Then the solution was extracted wtih EA and washed with water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product **33a**. **33a** was ready for the next step without the further purification.

*5.1.34 Typical procedure for the preparation of (**33b**-**33i**)*

To the solution of **33a** (2.00 g, 4.43 mmol) and K_2CO_3 (2.52 g, 17.74 mmol) in DMF (25 mL), different alkylation reagents RX (17.74 mmol) was added. The reaction mixture was stirred at room temperature for 1 h and TLC analysis indicated that the reaction was completed. The solution was treated with water and extracted with EA. The crude material was purified by silica gel column chromatography (PE/EA 3:1) to give the corresponding product. **33b**-**33i** was ready for the next step without the further purification.

5.1.35 *Typical procedure for the preparation of (**31d** and **31e**)*

To the solution of **33b** or **33i** (3.26 mmol) in CH_2Cl_2 (20 mL), CF_3COOH (48.3 mmol) was added. The reaction mixture was stirred at room temperature for 30 min and TLC analysis indicated that the reaction was completed. The solution was adjusted pH being 7 with saturated aqueous sodium bicarbonate. The organic layer was washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product **31d** and **31e**. **31d** and **31e** were ready for the next step without the further purification.

*5.1.36 4-(6,8-Dibromo-7-methoxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)-2-(trifluoromethyl)benzonitrile (**32d**)*

The synthesis of **32d** was similar to **32a**. It was obtained as yellow powder in 30% yield. m.p. 162-165 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.39 (d, J = 8.22 Hz, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, J = 8.22 Hz, 1H, Ar-H), 7.79 (s, 1H, Ar-H), 5.30 (d, J = 18.09 Hz, 1H, -CH-), 4.76 (m, J = 17.91 Hz, 1H, -C<u>H</u>H-), 4.48 (d, J = 17.91 Hz, 1H, -CH<u>H</u>-), 3.82 (s, 3H, $-OCH_3$), 3.35 (d, J = 5.64 Hz, 2H, $-CH_2-$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.55, 171.60, 152.33, 137.80, 136.19, 133.72, 131.51, 127.68 (q, J = 264.0 Hz, $-CF_3$), 117.69, 115.47, 108.46, 60.40, 56.74, 46.91, 28.50; IR (KBr): 3422.75, 3127.97, 1763.71, 1638.01, 1400.37 cm^{-1}; MS-EI (m/z): 170, 318, 575 [M^+]; HRMS (ESI): calculated for $C_{20}H_{12}Br_2F_3N_3O_2S$ 573.9042 $(M + H)^+$, found 573.9028.

*5.1.37 4-(6,8-Dibromo-7-(4-ethoxy-4-oxobutoxy)-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H, 3H, 5H)-yl)-2-(trifluoromethyl)benzonitrile (**32e**)*

The synthesis of 32e was similar to 32a.It was obtained as yellow powder in 52% yield. m.p. 158-160 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.41 (d, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.04 (d, 1H,

Ar-H), 7.80 (s, 1H, Ar-H), 5.32 (d, 2H, -CH_2-), 4.50 (d, 2H, -CH_2-), 4.10 (q, 4H, -O-C$\underline{H_2}$-, -COOC$\underline{H_2}$-), 3.98 (t, 1H, -CH-), 2.08 (m, 4H, -C$\underline{H_2}$C$\underline{H_2}$-), 1.20 (t, 3H, -CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) : δ 178.3, 172.5, 157.3, 134.0, 136.1, 133.7, 131.9, 130.1, 127.7 (q, J = 272.5 Hz, -CF_3), 122.9, 114.0, 111.9, 66.6, 59.8, 57.9, 30.1, 28.5, 24.1, 14.1 ppm; IR (KBr): 3127.66, 2360.55, 2342.23, 1400.41 cm^{-1}; HRMS (ESI): calculated for $C_{25}H_{20}Br_2F_3N_3O_4S$ 673.9566 $(M + H)^+$, found 673.9554.

*5.1.38 Typical procedure for the preparation of (**34a-34i**)*

A solution of **33a-33i** (10 mmol) and TEA (10 mmol) in methanol (100 mL) was hydrogenated in the presence of 10% Pd/C and stirred at 55 ^{o}C for 12 h. The reaction mixture was filtered to remove Pd/C and the solvent was removed under reduced pressure. The crude residue was treated with CH_2Cl_2 and washed with water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product. **34a-34i** were ready for the next step without the further purification.

*5.1.39 Typical procedure for the preparation of (**35a-35i**)*

To the solution of **34a-34i** (3.26 mmol) in CH_2Cl_2 (20 mL), CF_3COOH (48.3 mmol) was added. The reaction mixture was stirred at room temperature for 30 min and TLC analysis indicated that the reaction was completed. The solution was adjusted pH being 7 with saturated aqueous sodium bicarbonate. The organic layer was washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product. **35a-35i** were ready for the next step without the further purification.

*5.1.40 Typical procedure for the preparation of (**36a-36i**)*

To the solution of compound **10** (1.00 mmol) and TEA (1.00 mmol) in DMF (10 mL), **35a-35i** (1.00 mmol) was added. The reaction mixture was stirred at room temperature for 1 h and the TLC indicated that the reaction was completed. The solution was added into ice water and extracted with EA (3 x 20 mL). The combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography (PE/EA 3:1) to give the corresponding product.

*5.1.41 4-(7-Hydroxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36a**)*

It was obtained as yellow powder in 39% yield. m.p. 234-237 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.48 (s, 1H, -OH), 8.39 (d, 1H, Ar-H), 8.36 (s, 1H, Ar-H), 8.22 (d, 1H, Ar-H), 7.13 (d, 1H, Ar-H), 6.73-6.68 (m, 2H, Ar-H), 5.28 (d, 1H, -CH-), 4.74-4.58 (m, 2H, -CH_2-), 3.17 (d, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 175.7, 169.6, 153.7, 135.5, 133.6, 131.2, 129.1, 128.3 (q, J = 265.7 Hz, -CF_3), 125.2, 118.5, 112.3, 110.2, 105.9, 55.6, 43.2, 26.1; IR (KBr): 3397, 3127, 1742, 1503, 1400 cm^{-1}; MS-EI (m/z): 66, 73, 84, 120, 146, 403 [M^+]; HRMS (ESI): m/z, calculated for $C_{19}H_{13}F_3N_3O_2S$ 404.0675 $(M + H)^+$, found 404.0676.

*5.1.42 4-(7-Methoxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36b**)*

It was obtained as yellow powder in 66% yield. m.p. 234-236 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.38 (d, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.25 (d, 1H, Ar-H), 6.99 (s, 1H, Ar-H), 6.88 (d, 1H, Ar-H), 5.38 (d, 2H, -CH_2-), 4.71 (m, 2H, -CH_2-), 3.4-3.2 (m, 4H, -C$\underline{H_3}$, -C$\underline{H}$-); ^{13}C-NMR

(75 MHz, DMSO-d_6): δ 178.4, 172.2, 158.1, 138.2, 136.2, 133.8, 130.2, 122.8 (q, J = 265.3 Hz, -CF_3), 114.9, 113.5, 111.5, 57.9, 55.5, 46.8, 28.5 ppm; IR (KBr): 3126.93, 1745.66, 1502.63, 1400.34, 1135.62 cm^{-1}; MS-EI (m/z): 84, 134, 160, 417 [M^+]; HRMS (ESI): m/z, calculated for $C_{20}H_{14}F_3N_3O_2S$ 418.0832 $(M + H)^+$, found 418.0827.

*5.1.43 4-(7-Ethoxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36c**)*

It was obtained as yellow powder in 43% yield. m.p. 230-233 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.38 (d, 1H, Ar-H), 8.24 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.24 (d, 1H, Ar-H), 6.96 (s, 1H, Ar-H), 6.86 (d, 1H, Ar-H), 5.38 (d, 1H, -CH-), 4.77 (m, 2H, -CH_2-), 4.06 (q, 2H, -CH_2-), 3.23 (q, 2H, -CH_2-), 1.35 (t, 3H, -CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.3, 172.1, 157.4, 138.0, 136.1, 133.7, 131.8, 130.1, 127.7 (q, J = 268.1 Hz, -CF_3), 122.6, 115.0, 113.9, 111.8, 63.1, 57.9, 45.7, 28.5, 14.6 ppm; IR (KBr): 3133.37, 1763.04, 1507.09, 1400.14, 1127.84 cm^{-1}; MS-EI (m/z): 91, 120, 146, 174, 431 [M^+]; HRMS (ESI): m/z, calculated for $C_{21}H_{16}F_3N_3O_2S$ 432.0988 $(M + H)^+$; found 432.0992.

*5.1.44 4-(7-Propoxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36d**)*

It was obtained as yellow powder in 34% yield. m.p. 202-205 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.39 (d, 1H, Ar-H), 8.24 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.23 (d, 1H, Ar-H), 6.96 (s, 1H, Ar-H), 6.84 (d, 1H, Ar-H), 5.34 (d, 2H, -CH_2-), 4.65 (d, 2H, -CH_2-), 3.95 (m, 3H, -C<u>H</u>-, C<u>H$_2$</u>-), 1.72 (q, 2H, -CH_2-), 0.98 (t, 3H, -CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.4, 172.2, 157.8, 138.1, 136.2, 133.8, 130.2, 127.8 (q, J = 272.6 Hz, -CF_3), 122.7, 115.1, 113.9, 111.8, 69.1, 57.8, 45.8, 28.5, 21.8, 10.4 ppm; IR (KBr): 3127.72, 1766.88, 1507.56, 1400.34 cm^{-1}; MS-EI (m/z): 91, 120, 146, 188, 445 [M^+]; HRMS (ESI): m/z, calculated for $C_{22}H_{18}F_3N_3O_2S$ 446.1145 $(M + H)^+$, found 446.1152.

*5.1.45 4-(7-Isopropoxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36e**)*

It was obtained as yellow powder in 38% yield. m.p. 220-223 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.36 (d, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.24 (d, 1H, Ar-H), 6.96 (s, 1H, Ar-H), 6.83 (d, 1H, Ar-H), 5.37 (d, 1H, -CH-), 4.80-4.58 (m, 3H, -OC<u>H</u>-, -C<u>H$_2$</u>-), 3.22 (d, 2H, -CH_2-), 1.28 (d, 6H, -CH(C<u>H$_3$</u>)<u>$_2$</u>); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.1, 156.3, 138.0, 136.1, 133.7, 131.9, 130.8, 127.6 (q, J = 268.9 Hz, -CF_3), 124.0, 115.0, 113.2, 108.3, 69.2, 57.9, 45.7, 28.5, 27.8 ppm; IR (KBr): 3127.75, 1744.00, 1502.36, 1400.32, 1312.74 cm^{-1}; MS-EI (m/z): 91, 120, 146, 445 [M^+]; HRMS (ESI): m/z, calculated for $C_{22}H_{18}F_3N_3O_2S$ 446.1145 $(M + H)^+$, found 446.115.

*5.1.46 4-(7-Butoxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36f**)*

It was obtained as yellow powder in 44% yield. m.p. 203-205 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.39 (d, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.24 (d, 1H, Ar-H), 6.97 (s, 1H, Ar-H), 6.85 (d, 1H, Ar-H), 5.37 (d,1H, -CH-), 4.73-4.68 (m, 2H, -CH_2-), 3.99 (t, 2H, -OCH_2-), 3.22 (d, 2H, -CH_2-)1.72 (m, 2H, -CH_2-), 1.47 (m, 2H, -CH_2-), 0.95 (t, 3H, -CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.0, 157.6, 138.0, 136.1, 133.7, 131.8, 130.0, 127.7, 124.0, 122.6, 120.4, 115.2, 114.0, 111.91, 108.4, 67.7, 58.0, 45.8, 30.6, 28.6, 18.7, 13.8 ppm; IR (KBr): 3127.04, 1744.74, 1400.25, 1311.92, 1173.64 cm^{-1}; MS-EI (m/z): 91, 120, 146, 202, 403, 459 [M^+]; HRMS (ESI): m/z, calculated for

$C_{23}H_{20}F_3N_3O_2S$ 460.1301 $(M + H)^+$, found 460.1308.

*5.1.47 4-(7-Isobutoxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36g**)*

It was obtained as yellow powder in 38% yield. m.p. 208-210 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 8.38 (d, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.03 (d, 1H, Ar-H), 7.24 (d, 1H, Ar-H), 6.99 (s, 1H, Ar-H), 6.89 (d, 1H, Ar-H), 5.37 (d, 1H, -CH-), 4.73-4.62 (m, 2H, -CH_2-), 3.75 (d, 2H, -OCH_2-), 3.22 (d, 2H, -CH_2-), 2.02 (m, 1H, -CH-), 0.98 (d, 6H, -CH($C\underline{H}_3$)$_2$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.1, 172.3, 157.8, 138.1, 136.5, 133.6, 131.8, 130.1, 126.5 (q, J = 264.0 Hz, -CF_3), 122.5, 114.8, 113.8, 111.9, 73.8, 57.9, 45.6, 28.6, 27.7, 19.1 ppm; IR (KBr): 3127.80, 1750.31, 1508.03, 1400.09, 1312.76 cm^{-1}; MS-EI (m/z): 57, 91, 120, 146, 403, 459 [M^+]; HRMS (ESI): m/z, calculated for $C_{23}H_{20}F_3N_3O_2S$ 460.1301 $(M + H)^+$, found 460.1304.

*5.1.48 4-(7-Cyclohexyloxy-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**36h**)*

It was obtained as yellow powder in 53% yield. m.p. 218-220 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 8.38 (d, 1H, Ar-H), 8.24 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.23 (d, 1H, Ar-H), 6.99 (s, 1H, Ar-H), 6.86 (d, 1H, Ar-H), 5.37 (d, 1H, -CH-), 4.77 (m, 2H, -CH_2-), 4.33 (s, 1H, -CH-), 3.21 (d, 2H, -CH_2-), 2.01-1.15 (m, 10H, -($C\underline{H}_2$-)$_5$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.2, 156.4, 137.9, 135.9, 133.6, 131.8, 130.1, 127.8 (q, J = 271.4 Hz, -CF_3), 122.8, 115.0, 113.4, 74.5, 57.9, 46.9, 31.3, 28.6, 24.8, 23.1 ppm; IR (KBr): 3127.55, 2939.85, 1753.82, 1503.09, 1400.35, 1312.91 cm^{-1}; MS-EI (m/z): 85, 91, 146, 341, 356, 403, 554 [M^+]; HRMS (ESI): m/z, calculated for $C_{25}H_{22}F_3N_3O_2S$ 486.1458 $(M + H)^+$, found 486.1463.

*5.1.49 Ethyl 4-((2-(4-cyano-3-(trifluoromethyl)phenyl)-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydroimidazo[1,5-b]isoquinolin-7-yl)oxy)butanoate (**36i**)*

It was obtained as yellow powder in 52% yield. m.p. 158-160 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 8.39 (d, 1H, Ar-H), 8.19 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.23 (d, 1H, Ar-H), 6.99 (s, 1H, Ar-H), 6.86 (d, 1H, Ar-H), 5.32 (d, 2H, -CH_2-), 4.50 (d, 2H, -CH_2-), 4.10 (q, 4H, -O-$C\underline{H}_2$-, -$COOC\underline{H}_2$-), 3.98 (t, 1H, -CH-), 2.08 (m, 4H, -$C\underline{H}_2C\underline{H}_2$-), 1.20 (t, 3H, -$CH_3$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.3, 172.5, 157.3, 134.0, 136.1, 133.7, 131.9, 130.1, 127.7 (q, J = 263.1 Hz, -CF_3), 122.9, 114.0, 111.9, 66.6, 59.8, 57.9, 30.1, 28.5, 24.1, 14.1 ppm; IR (KBr): 3127.66, 2360.55, 2342.23, 1400.41 cm^{-1}; MS-EI (m/z): 115, 146, 472, 517 [M^+]; HRMS (ESI): m/z, calculated for $C_{25}H_{22}F_3N_3O_4S$ 518.1356 $(M + H)^+$, found 518.1358.

*5.1.50 1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (**11**)*

To the solution of phenylalanine **37** (16.5 g, 0.1 mol) in HCl (150 ml), 33% formaldehyde solution (70 ml, 1 mol) was added. The reaction mixture was stirred at refluxing for 6 h and TLC analysis indicated that the reaction was completed. The mixture was filtered to give white solid **11** in 33% yield. m.p. 245-247 ^oC; ^1H-NMR (300 MHz, DMOS-d_6): δ 10.07 (s, 1H, -COOH), 7.27 (s, 4H, Ar-H), 4.43-4.40 (m, 2H, -CH_2-), 3.81 (s, 1H, -CH-), 3. 45-3.10 (m, 2H, -CH_2-).

*5.1.51 Typical procedure for the preparation of (**38a** and **38b**)*

The compound **11** (2.5 g, 14.1 mmol) was dissolved in H_2SO_4 (12.5 mL) at -10 ^oC. The solution of

KNO_3 (1.48 g, 14.7 mmol) in H_2SO_4 (5.00 mL) was added dropwise slowly into the mixture. The reaction mixture was stirred at room temperature for 1 h and TLC analysis indicated that the reaction was completed. The solution was treated with ice water (100 mL) and adjusted pH being 7 with ammonia water to generate precipitation. Then the mixture was filtered to give yellow solid. **38a** and **38b** were ready for next step without the further purification.

*5.1.52 Typical procedure for the preparation of (**39a** and **39b**)*

To the solution of **38a** and **38b** (10.00 mmol) in methanol (50 mL), thionyl chloride (20.00 mmol) was added dropwisely at 0 ^oC. After adding, the mixture was stirred for 30 min maintaining the temperature no more 5 ^oC. Then the solution was stirred at refluxing for 14 h. The reaction mixture was cooled to room temperature and filtered to give the responding compound. **39a** and **39b** were ready for next step without the further purification.

*5.1.53 Typical procedure for the preparation of (**40a** and **40b**)*

To the solution of intermediate **39a** and **39b** (10.0 mmol) and TEA (1.52 g, 15.0 mmol) in 1,4-dioxane/H_2O (180 mL, v:v = 8:1), the di-*tert*-butyl dicarbonate (2.4 g, 11.0 mmol) was added dropwise slowly at 0 ^oC. The mixture was stirred at room temperature for 24 h and TLC analysis indicated that the reaction was completed. The solvent was removed under reduced pressure. The crude residue was treated with water and adjusted pH being 1 with 5 N HCl. Then the solution was extracted wtih EA and washed with water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product. **40a** and **40b** were ready for the next step without the further purification.

*5.1.54 Typical procedure for the preparation of (**40c** and **40d**)*

A solution of **40a** and **40b** (10 mmol) in methanol (100 mL) was hydrogenated in the presence of 10% Pd/C and stirred at room temperature for 12 h. The reaction mixture was filtered to remove Pd/C and the solvent was removed under reduced pressure to give the corresponding product. **40c** and **40d** were ready for the next step without the further purification.

*5.1.55 Typical procedure for the preparation of (**39c** and **39d**)*

To the solution of **40c** and **40d** (3.26 mmol) in CH_2Cl_2 (20 mL), CF_3COOH (48.3 mmol) was added. The reaction mixture was stirred at room temperature for 30 min and TLC analysis indicated that the reaction was completed. The solution was adjusted pH being 7 with saturated aqueous sodium bicarbonate. The organic layer was washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product. **39c** and **39d** were ready for the next step without the further purification.

*5.1.56 Typical procedure for the preparation of (**41a-41d**)*

To the solution of compound **10** (1.00 mmol) and TEA (1.00 mmol) in DMF (10 mL), **39a-39d** (1.00 mmol) was added. The reaction mixture was stirred at room temperature for 1 h and the TLC indicated that the reaction was completed. The solution was added into ice water and extracted with EA (3 × 20 mL). The combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography (PE/EA 3:1) to give the corresponding product.

*5.1.57 4-(8-Nitro-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**41a**)*

It was obtained as a yellow powder in 34% yield. m.p. 171-173 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.40 (d, 1H, Ar-H), 8.32 (s, 1H, Ar-H), 8.25 (s, 1H, Ar-H), 8.15 (d, 1H, Ar-H), 8.04 (d, 1H, Ar-H), 7.69 (d, 1H, Ar-H), 5.55 (d, 1H, -CH-), 4.81 (d, 2H, -CH_2-), 3.45 (m, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.0, 137.6, 136.0, 133.5, 132.5, 129.1, 128.2 (q, J = 266.3 Hz, -CF_3), 127.5, 123.8, 121.9, 114.9, 108.4, 57.3, 45.2, 29.1 ppm; IR (KBr): 3448, 3133, 2371, 1637, 1400, 1313 cm^{-1}; MS-EI (m/z): 129, 175, 432 [M^+]; HRMS (ESI): calculated for $C_{19}H_{11}F_3N_4O_3S$ 433.0577 (M + H)$^+$, found 433.0583.

*5.1.58 4-(7-Nitro-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**41b**)*

It was obtained as a yellow powder in 60% yield. m.p. 219-221 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.39 (d, 2H, Ar-H), 8.23 (s, 1H, Ar-H), 8.12 (d, 1H, Ar-H), 8.01 (d, 1H, Ar-H), 7.65 (d, 1H, Ar-H), 5.57 (d, 1H, -CH-), 4.86 (q, 2H, -CH_2-), 3.48-3.41 (m, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.6, 171.8, 146.4, 139.4, 137.9, 136.2, 133.7, 132.9, 130.6, 127.6 (q, J = 267.1 Hz, -CF_3), 122.0, 56.9, 45.4, 29.4 ppm; IR (KBr): 3439, 2348, 1614, 1312, 1048, 889; MS-EI (m/z): 129, 175, 432 [M^+]; HRMS (ESI): calculated for $C_{19}H_{11}F_3N_4O_3S$ 433.0577 (M + H)$^+$, found 433.0583.

*5.1.59 4-(8-Amino-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**41c**)*

It was obtained as a yellow powder in 31% yield. m.p. 225-227 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.39 (d, 1H, Ar-H), 8.24 (s, 1H, Ar-H), 8.02 (d, 1H, Ar-H), 7.38 (d, 1H, Ar-H), 7.10 (s, 2H, Ar-H), 5.38 (d, 1H, -CH-), 4.72 (m, 2H, -CH_2-), 3.76 (s, 2H, -NH_2-), 3.32 (d, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.0, 137.6, 136.0, 133.5, 132.5, 129.1, 128.2 (q, J = 272.8 Hz, -CF_3), 127.5, 123.8, 121.9, 114.9, 108.4, 57.3, 45.2, 29.1 ppm; IR (KBr): 3133, 2371, 1400, 1313 cm^{-1}; MS-EI (m/z): 119, 145, 402 [M^+]; HRMS (ESI): calculated for $C_{19}H_{13}F_3N_4OS$ 403.0835 (M + H)$^+$, found 403.0573.

*5.1.60 4-(7-Amino-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**41d**)*

It was obtained as a yellow powder in 31% yield. m.p. 158-160 ^{o}C; ^{1}H-NMR (300 MHz, D_2O): δ 8.40 (s, 1H, Ar-H), 8.28 (s, 1H, Ar-H), 8.08 (s, 2H, Ar-H), 7.44 (d, 2H, Ar-H), 5.42 (s, 1H, -CH-), 4.80 (t, 2H, -CH_2-), 3.36 (t, 2H, -CH_2-); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 179.2, 178.1, 172.1, 144.2, 137.6, 137.1, 135.9, 133.5, 131.2, 129.2 (q, J = 269.2 Hz, -CF_3), 127.8, 122.1, 120.1, 115.3, 114.2, 108.4, 101.8, 57.6, 45.4, 28.8 ppm; IR (KBr): 3127, 1763, 1526, 1400, 1346, 1312, 1140, 1057 cm^{-1}; MS-EI (m/z): 91, 119, 145, 402 [M^+]; HRMS (ESI): m/z, calculated for $C_{19}H_{13}F_3N_4OS$ 403.0835 (M + H)$^+$, found 403.0839.

*5.1.61 Typical procedure for the preparation of (**42a-42g**)*

To the solutino of **40d** (2.00 mmol) and TEA (4.00 mmol) in CH_2Cl_2, different acylation and sulfonylation reagents (4.00 mmol) were added dropwise slowly. The reaction mixture was stirred at room temperature for 3 h and TLC analysis indicated that the reaction was completed. The mixture was treated with water and extracted with CH_2Cl_2. The organic layer was washed with saturated sodium

bicarbonate solution, washed with brine and drived over Na_2SO_4. The solvent was evaporated to give corresponding product.

*5.1.62 Typical procedure for the preparation of (**43a-43g**)*

To the solution of **42a-42g** (2 mmol) in CH_2Cl_2 (20 mL), CF_3COOH (2 mL) was added. The reaction mixture was stirred at room temperature overnight and TLC analysis indicated that the reaction was completed. The solution was adjusted pH being 7 with saturated aqueous sodium bicarbonate. The organic layer was washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product.

*5.1.63 Typical procedure for the preparation of (**44a-44g**)*

To the solution of compound **10** (1.00 mmol) and TEA (1.00 mmol) in DMF (10 mL), **43a**-**43g** (1.00 mmol) was added. The reaction mixture was stirred at room temperature for 1 h and the TLC indicated that the reaction was completed. The solution was added into ice water and extracted with EA (3 × 20 mL). The combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography (PE/EA 3:1) to give the corresponding product.

*5.1.64 N-(2-(4-cyano-3-(trifluoromethyl)phenyl)-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydroimidazo[1,5-b]isoquinolin-7-yl)acetamide (**44a**)*

It was obtained as yellow powder in 21% yield. m.p. 288-290 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 10.00 (s, 1H, -NH-), 8.37 (d, J = 8.1 Hz, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, J = 8.1 Hz, 1H, Ar-H),7.60 (s, 1H, Ar-H), 7.44 (d, J = 8.1 Hz, 1H, Ar-H), 7.26 (d, J = 8.4 Hz, 1H, Ar-H), 5.34 (d, J = 17.7 Hz, 1H, -CH-), 4.79-4.71 (m, 2H, -CH_2-), 3.24 (d, J = 6.9 Hz, 2H, -CH_2-), 2.06 (s, 3H, -CH_3) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.0, 168.3, 137.9, 136.1, 133.7, 131.2, 127.6 (q, J = 267.3 Hz, -CF_3), 125.4, 123.9, 120.3, 118.1, 116.6, 108.3, 57.7, 45.6, 28.7, 23.9 ppm; IR (KBr): 3363, 3128, 2238, 1753, 1600, 1401, 1348, 1256, 1172, 1052, 888, 851 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{21}H_{15}F_3N_4O_2S$ 467.0760 $(M + Na)^+$, found 467.0769.

*5.1.65 N-(2-(4-cyano-3-(trifluoromethyl)phenyl)-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydroimidazo[1,5-b]isoquinolin-7-yl)propionamide (**44b**)*

It was obtained as yellow powder in 27% yield. m.p. 243-245 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 9.92 (s, 1H, -NH-), 8.37 (d, J = 8.1 Hz, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 8.01 (d, J = 8.1 Hz, 1H, Ar-H), 7.62 (s, 1H, Ar-H), 7.44 (d, J = 7.5 Hz, 1H, Ar-H), 7.26 (d, J = 8.1 Hz, 1H, Ar-H), 5.34 (d, J = 17.7 Hz, 1H, -CH-), 4.79-4.65 (m, 2H, -CH_2-), 3.32-3.20 (m, 2H, -CH_2-), 2.32 (q, J = 7.5 Hz, 2H, -CH_2-), 1.09 (t, J = 7.5 Hz, 3H, -CH_3) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.0, 138.1, 137.9, 136.1, 133.7, 131.2, 130.8, 129.3, 127.6 (q, J = 266.0 Hz, -CF_3), 125.3, 123.9, 120.3, 118.1, 116.6, 115.0, 108.3, 57.7, 45.6, 29.4, 9.5 ppm; IR (KBr): 3125, 2238, 1687, 1400, 1312, 1279, 1137, 1067, 851, 830, 560 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{22}H_{17}F_3N_4O_2S$ 459.1097 $(M + H)^+$, found 459.1119.

*5.1.66 N-(2-(4-cyano-3-(trifluoromethyl)phenyl)-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydroimidazo[1,5-b]isoquinolin-7-yl)butyramide (**44c**)*

It was obtained as yellow powder in 46% yield. m.p. 141-143 ^oC; ^1H-NMR (300 MHz, DMSO-d_6): δ 9.93 (s, 1H, -NH-), 8.37 (d, J = 8.1 Hz, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, J = 8.1 Hz, 1H, Ar-H),

7.64 (s, 1H, Ar-H), 7.45 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.26 (d, *J* = 8.4 Hz, 1H, Ar-H), 5.34 (d, *J* = 17.4 Hz, 1H, -CH-), 4.70 (t, *J* = 17.4 Hz, 2H, -CH_2-), 3.24 (d, *J* = 6.9 Hz, 2H, -CH_2-), 2.30 (t, *J* = 7.5 Hz, 2H, -CH_2-), 1.62 (q, *J* = 7.5 Hz, 2H, -CH_2-), 0.92 (t, *J* = 7.5 Hz, 3H, -CH_3) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.0, 171.1, 138.1, 136.1, 133.7, 130.8, 129.3 (q, *J* = 264.2 Hz, -CF_3), 125.3, 57.7, 45.6, 28.1, 18.5, 13.5 ppm; IR (KBr): 3129, 1761, 1682, 1400, 1284, 1258, 1174, 1132, 1073, 802 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{23}H_{19}F_3N_4O_2S$ 473.1254 $(M + H)^+$, found 473.1261.

*5.1.67 N-(2-(4-cyano-3-(trifluoromethyl)phenyl)-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydroimidazo[1,5-b]isoquinolin-7-yl)pivalamide (**44d**)*

It was obtained as yellow powder in 25% yield. m.p. 229-231 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.27 (s, 1H, -NH-), 8.38 (d, *J* = 7.8 Hz, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, *J* = 7.8 Hz, 1H, Ar-H), 7.67 (s, 1H, Ar-H), 7.51 (d, *J* = 7.5 Hz, 1H, Ar-H), 7.26 (d, *J* = 8.7 Hz, 1H, Ar-H), 5.34 (d, *J* = 17.4 Hz, 1H, -CH-), 4.76-4.64 (m, 2H, -CH_2-), 3.25 (d, *J* = 6.3 Hz, 2H, -CH_2-), 1.23 (s, 9H, -$(CH_3)_3$) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.8, 176.9, 172.5, 138.6, 136.6, 134.2, 132.4, 131.1, 129.6 (q, *J* = 265.8 Hz, -CF_3), 126.1, 118.4, 58.2, 46.1, 29.2, 27.6 ppm; IR (KBr): 3129, 1658, 1615, 1478, 1400, 1351, 1279, 1132, 1077, 833, 559 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{24}H_{21}F_3N_4O_2S$ 487.1410 $(M + H)^+$, found 487.1417.

*5.1.68 Ethyl (2-(4-cyano-3-(trifluoromethyl)phenyl)-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydroimidazo[1,5-b]isoquinolin-7-yl)carbamate (**44e**)*

It was obtained as yellow powder in 24% yield. m.p. 236-238 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.68 (s, 1H, -NH-), 8.37 (d, *J* = 8.4 Hz, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.02 (d, *J* = 8.4 Hz, 1H, Ar-H),7.45 (s, 1H, Ar-H), 7.35 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.24 (d, *J* = 8.4 Hz, 1H, Ar-H), 5.32 (d, *J* = 17.7 Hz, 1H, -CH-), 4.79-4.69 (m, 2H, -CH_2-), 4.17-4.10 (m, 2H, -CH_2-), 3.25-3.22 (m, 2H, -CH_2-), 1.28-1.20 (m, 3H, -CH_3-) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.7, 172.5, 154.0, 138.4, 136.6, 134.2, 131.3, 129.9, 128.1 (q, *J* = 264.6 Hz, -CF_3), 125.2, 120.8, 117.8, 116.2, 115.5, 108.8, 60.6, 58.2, 46.1, 29.1, 14.9 ppm; IR (KBr): 3477, 3364, 3124, 2238, 1618, 1400, 1314, 1284, 1136, 888, 851 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{22}H_{17}F_3N_4O_3S$ 475.1046 $(M + H)^+$, found 475.1082.

*5.1.69 4-(7-(Methylsulfonyl)-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**44f**)*

It was obtained as yellow powder in 28% yield. m.p. 121-123 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.72 (s, 1H, -NH-), 8.38 (d, *J* = 8.5 Hz, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 8.01-8.09 (m, 1H, Ar-H), 7.31 (d, *J* = 7.8 Hz, 1H, Ar-H), 7.17 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.03 (d, *J* = 6.3 Hz, 1H, Ar-H), 5.34 (d, *J* = 17.7 Hz, 1H, -CH-), 4.50 (dd, *J* = 16.02, 16.08 Hz, 2H, -CH_2-), 3.60 (s, 3H, -CH_3), 3.17 (s, 2H, -CH_2-) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 206.4, 178.2, 172.0, 137.1, 133.7, 132.7, 131.7, 126.8 (q, *J* = 270.5 Hz, -CF_3), 118.7, 117.1, 57.5, 53.6, 52.3, 45.5, 43.8, 30.4, 28.6 ppm; IR (KBr): 3416, 3253, 2960, 2851, 2361, 1742, 1655, 1400, 1318, 1152, 970, 804, 510 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{20}H_{15}F_3N_4O_3S_2$ 481.0610 $(M + H)^+$, found 481.0610.

*5.1.70 4-(7-(Butylsulfonyl)-1-oxo-3-thioxo-10,10a-dihydroimidazo[1,5-b]isoquinolin-2(1H,3H,5H)-yl)-2-(trifluoromethyl)benzonitrile (**44g**)*

It was obtained as yellow powder in 29% yield. m.p. 112-114 ^{o}C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.76 (s, 1H, -NH-), 8.37 (d, *J* = 8.5 Hz, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 8.09-8.01 (m, 1H, Ar-H), 7.28

(d, *J* = 7.8 Hz, 1H, Ar-H), 7.17-7.12 (m, 1H, Ar-H), 7.02 (d, *J* = 7.8 Hz, 1H, Ar-H), 5.34 (d, *J* = 17.7 Hz, 1H, -CH-), 4.87-4.84 (m, 2H, $-CH_2-$), 4.50 (dd, *J* = 16.02, 16.08 Hz, 2H, $-CH_2-$), 3.29-3.18 (m, 2H, $-CH_2-$), 1.62 (t, *J* = 6.9 Hz, 2H, $-CH_2-$), 1.39-1.33 (m, 2H, $-CH_2-$), 0.88-0.80 (m, 3H, $-CH_3$) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6): δ 178.2, 172.0, 171.0, 137.9, 136.7, 133.7, 132.9, 131.7, 130.0 (q, *J* = 268.1 Hz, $-CF_3$), 126.1, 118.3, 117.0, 116.7, 115.0, 108.3, 57.5, 53.5, 52.3, 50.3, 45.5, 43.9, 28.6, 25.0, 20.6, 13.7 ppm; IR (KBr): 3448, 3252, 2962, 2342, 1739, 1617, 1400, 1140, 1014, 959, 801, 645 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{23}H_{21}F_3N_4O_3S_2$ 521.0934 $(M - H)^-$, found 521.0902.

*5.1.71 2-Acetylamino-2-(3-fluoro-benzyl)-malonic acid diethyl ester (**46**)*

To the solution of Na (0.76 g, 33.16 mmol) in EtOH (100 mL), diethyl acetamidomalonate (6.45 g, 30.15 mmol) was added. The mixture was stirred for 30 min. Then **45** (6.42 g, 30.15 mmol) was added into the solution. After the reaction mixture was stirred at refluxing for 18 h, the mixture was treated with ice water (400 mL) and filtered to give the corresponding **46**. It was obtained as a white solid in 71% yield. HRMS (ESI): m/z, calculated for $C_{16}H_{20}NO_5$ 326.3321 $(M + H)^+$, found 326.1429.

*5.1.72 2-Amino-3-(3-fluorophenyl)propionic acid (**47**)*

The intermediate **46** (7.50 g, 21.43 mmol) was added into HCl (80 mL). The reaction mixture was stirred at refluxing for 15 h and TLC analysis indicated that the reaction was completed. The mixture was filtered to give the white solid **47** in 86% yield. HRMS (ESI): m/z, calculated for $C_9H_{10}NO_2$ 184.0715 $(M + H)^+$, found 184.0767.

*5.1.73 6-Fluoro-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**48**)*

To the solution of **47** (16.50 g, 0.3 mol) in HCl (150 mL), 40% formaldehyde solution (70 mL, 1 mol) was added. The reaction mixture was stirred at refluxing for 6 h and TLC analysis indicated that the reaction was completed. The mixture was cooled to room temperature and filtered to give white solid **48** in 48% yield. HRMS (ESI): m/z, calculated for $C_{10}H_{10}FNO_2$ 196.0702 $(M + H)^+$, found 196.0783

*5.1.74 6-Fluoro-7-nitro-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**49**)*

The compound **48** (2.5 g, 14.11 mmol) was dissolved in H_2SO_4 (12.5 mL) at -10 ^oC. The solution of KNO_3 (1.48 g, 14.74 mmol) in H_2SO_4 (5.00 mL) was added dropwise slowly into the mixture. The reaction mixture was stirred at room temperature for 1 h and TLC analysis indicated that the reaction was completed. The solution was treated with ice water (100 mL) and adjusted pH being 7 with ammonia water to generate precipitation. Then the mixture was filtered to give yellow solid **49** in 45% yield. HRMS (ESI): m/z, calculated for $C_{10}H_9FN_2O_4$ 241.0587 $(M + H)^+$, found 241.0621.

*5.1.75 6-Fluoro-7-nitro-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid methyl ester (**50**)*

To the solution of **49** (1.00 g, 4.48 mmol) in methanol (30 mL), thionyl chloride (1.07 g, 8.96 mmol) was added dropwisely at 0 ^oC. After adding, the mixture was stirred for 30 min maintaining the temperature no more 5 ^oC. Then the solution was stirred at refluxing for 14 h. The reaction mixture was cooled to room temperature and filtered to give yellow solid **50** in 48% yield. HRMS (ESI): m/z, calculated for 255.0719 $(M + H)^+$, found 255.0788.

*5.1.76 2-Tert-butyl 3-methyl 6-fluoro-7-nitro-3,4-dihydroisoquinoline-2,3(1H)-dicarboxylate (**51**)*

To the solution of intermediate **50** (1.50 g, 5.91 mmol) and DMAP (0.16 g, 1.53 mmol) in CH_2Cl_2 (20 mL), the di-*tert*-butyl dicarbonate (2.50 g, 15.32 mmol) was added dropwise slowly at 0 ^oC. The mixture was stirred at room temperature for 30 min and TLC analysis indicated that the reaction was completed. The solvent was removed under reduced pressure. The crude residue was treated with water and adjusted pH being 1 with 5 N HCl. Then the solution was extracted with EA and washed with water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give yellow oil **51** in 83% yield. HRMS (ESI): m/z, calculated for $C_{16}H_{19}FN_2O_6$ 377.1105 $(M + Na)^+$, found 377.1122.

*5.1.77 2-Tert-butyl 3-methyl 6-fluoro-7-amino-3,4-dihydroisoquinoline-2,3(1H)-dicarboxylate (**52**)*

A solution of **51** (1.00 g, 4.48 mmol) in methanol (30 mL) was hydrogenated in the presence of 10% Pd/C and stirred at 40 ^oC for 12 h. The reaction mixture was filtered to remove Pd/C and the solvent was removed under reduced pressure to give white solid **52** in 89% yield. HRMS (ESI): m/z, calculated for $C_{16}H_{21}FN_2O_4$ 325.1515 $(M + H)^+$, found 325.1572.

*5.1.78 2-Tert-butyl 3-methyl 7-((ethoxycarbonyl)amino)-6-fluoro-3,4-dihydroisoquinoline-2,3(1H)-dicarboxylate (**53**)*

To the solution of **52** (0.20 g, 0.22 mmol) and pyridine (0.09 g, 0.33 mmol) in CH_2Cl_2 (15 mL), carbonochloridic acid ethyl ester (0.20 g, 0.44 mmol) was added. The reaction was stirred at room temperature for 3 h and TLC analysis indicated that the reaction mixture was completed. The mixture was treated with water and extracted with EA. The organic layer was washed with saturated sodium bicarbonate solution, washed with brine and dried over Na_2SO_4. The crude was purified by silica gel column chromatography (PE/EA 6:1) to give the corresponding product. It was obtained as yellow powder in 75% yield. HRMS (ESI): m/z, calculated for $C_{19}H_{25}FN_2O_6$ 397.1765 $(M + H)^+$, found 397.1752.

*5.1.79 Methyl 7-((ethoxycarbonyl)amino)-6-fluoro-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (**54**)*

To the solution of **54** (0.79 g, 2 mmol) in CH_2Cl_2 (20 mL), CF_3COOH (2 mL) was added. The reaction mixture was stirred at room temperature overnight and TLC analysis indicated that the reaction was completed. The solution was adjusted pH being 7 with saturated aqueous sodium bicarbonate. The organic layer was washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to give the corresponding product. It was obtained as yellow powder in 85% yield. HRMS (ESI): m/z, calculated for $C_{14}H_{17}FN_2O_4$ 297.1265 $(M + H)^+$, found 297.1254.

*5.1.80 Ethyl (2-(4-cyano-3-(trifluoromethyl)phenyl)-8-fluoro-1-oxo-3-thioxo-1,2,3,5,10,10a-hexahydr-oimidazo[1,5-b]isoquinolin-7-yl)carbamate (**55**)*

To the solution of compound **10** (1.00 mmol) and TEA (1.00 mmol) in DMF (10 mL), **54** (1.00 mmol) was added. The reaction mixture was stirred at room temperature for 1 h and the TLC indicated that the reaction was completed. The solution was added into ice water and extracted with EA (3 x 20 mL). The combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography (PE/EA 3:1) to give the corresponding product. It was obtained as yellow powder in 45% yield. m.p. 236-240 ^oC; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.37 (s, 1H, -NH-), 8.38 (d, J = 8.5 Hz, 1H, Ar-H), 8.23 (d, J = 8.5 Hz, 1H, Ar-H), 8.01 (d, J = 8.5 Hz, 1H, Ar-H), 7.63 (d, 1H, Ar-H), 7.25 (d, J = 7.8 Hz, 1H, Ar-H), 5.34 (d, J = 17.7 Hz, 1H, -CH-), 4.77 (t, J = 18.02 Hz, 1H, -C<u>H</u>H-), 4.65 (d, J = 16.02 Hz, 1H, -CH<u>H</u>-), 4.13 (q, J = 16.02 Hz, 2H, -CH_2-), 3.28 (d, J = 8.5 Hz, 2H, -CH_2-), 1.24 (t, J = 8.5 Hz, 3H, -CH_3) ppm; ^{13}C-NMR

(75 MHz, DMSO-d_6): δ 172.7, 171.4, 161.0, 153.8, 139.9, 134.7, 133.3, 132.7, 129.9 (q, J = 268.1 Hz, -CF_3), 121.7, 119.6, 118.1, 116.1, 115.8, 115.7, 114.7, 105.0, 72.5, 61.7, 53.8, 28.1, 13.8 ppm. IR (KBr): 3414, 2360, 1761, 1603, 1454, 1341, 1314, 1183, 1136 cm^{-1}; HRMS (ESI): m/z, calculated for $C_{22}H_{16}F_4N_4O_3S$ 491.0806 $(M - H)^-$, found 491.0786.

5.2 Biology

5.2.1. LNCaP cell proliferation assay

LNCaP cells were cultured in RPMI-1640 supplemented with 10% Fetal Bovine Serum(FBS) at 37 ^oC in a 5% CO_2 humidified incubator. All experiments were performed in triplicate or more. Cells were trypsinized and diluted to 100,000 cells/mL with RPMI-1640 supplemented with 10% FBS. This cell suspension was seeded in 96-well plates at a volume of 100 μL(10,000 cells/well) and incubated at 24 h. After removal of 100 μL of medium from each well, 200 μL of the drug solution, which was supplemented with serial dilutions of the test compounds or DMSO, was added. Then the plates were incubated at 37 ^oC under 5% CO_2 for 3 d. A 20 μL aliquot of MTT (5mg/mL) was added to each well of microcultures, and the cells were incubated for 4 h. Carefully remove the supernatant, then add 150 μL of DMSO so as to melt the crystal. The absorbance at 492 nm was measured. This parameter relates to the number of living cells in the culture.

5.2.2. DU145 cell growth inhibition assay

DU145 cells were cultured in RPMI-1640 supplemented with 10% FBS at 37 ^oC in a 5% CO_2 humidified incubator. All experiments were performed in triplicate or more. Cells were trypsinized and diluted to 100,000 cells/mL with RPMI-1640 supplemented with 10% FBS. This cell suspension was seeded in 96-well plates at a volume of 100 μL(7,000 cells/well) and incubated at 24 h. After removal of 100 μL of medium from each well, 200 μL of the drug solution, which was supplemented with serial dilutions of the test compounds or DMSO, was added. Then the plates were incubated at 37 ^oC under 5% CO_2 for 3 d. A 20 μL aliquot of MTT (5 mg/mL) was added to each well of microcultures, and the cells were incubated for 4 h. Carefully remove the supernatant, then add 150 μL of DMSO so as to melt the crystal. The absorbance at 492 nm was measured. This parameter relates to the number of living cells in the culture.

5.2.3. AR reporter gene assay

Assay of androgenic activity was performed by means of ARE-luciferase reporter assay using Cos-7 cells. Cos-7 (5 x 10^6 cells) were sown in a 150 cm^2 flask (Corning), and cultured in culture medium (DMEM medium containing 10% Dextran Charcoal (DCC)-Fetal Bovine Serum (FBS), 2 mM glutamine) for 24 h. pcDNA3.1-AR, pRL-SV40 and pMMTV-Luc vector containing luciferase gene bound at the downstream of an AR promoter derived from Mouse Mammary Tumor Virus (MMTV) were co-transfected by using Lipofectamine 2000. After culturing at 37 ^oC in a 5% CO_2 atmosphere for 4 h, these cells were harvested and plated in a 96 well plate (10,000 cells/well) and cultured for 2 h. Twenty-four hours after addition of the sample (final concentration, 10 μM) and 1 nM R1881, cells

were harvested with 20 μL of cell passive lysis buffer (Promega), and the firefly and Renilla luciferase activities were determined with a Dual Luciferase Asaay Kit (Promega) by measuring luminescence with a Wallac Micro-Beta scintillation counter (Perkin-Elmer Life Sciences). The data were obtained in triplicate and expressed as inhibition rate over the R1881 control. Inhibition% = 1 - (RLU_{test} - RLU_{blank})/(RLU_{R1881} - RLU_{blank})*100%. RLU = relative light unit

5.2.4. Immunofluorescence assay

LNCaP cells were cultured in fresh RPMI-1640 containing 10% DCC FBS and treated with 10 nM R1881, or 10 nM R1881 in combination with each of 10 μM **55** and enzalutamide for 12 h. Cells were fixed with 4% paraformaldehyde for 15 min and stored either on ice. Immediately before the assay, samples were permeabilized with a solution of Triton-X 0.3% in PBS. Afterwards the samples were stained sequentially with a 1st and 2nd antibody. The first antibody was anti-androgen receptor antibody-ChIP Grade (ab74272). The secondary antibody was Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate. Cells were mounted onto microscope slides for fluorescent microscopy using the fluorescent microscope (LSM700, Zeiss). The nucleus was stained with 10 μg/mL 4'6-diamidino-2-phenylindole (DAPI, Sigma).

5.2.5. Rat Pharmacokinetic Studies

Pharmacokinetic parameters of compounds **1**, **13**, **44e**, and **55** were measured in rats weighing between 180 and 220 g, with three animals in each group. The tested compounds were dissolved in water and administered by gavage at a dose of 5.0 mg/kg. Serial specimens were collected via the retrobulbar vein 0.25, 0.75, 1.5, 2, 4, 6, 8, 12, 24, 48 and 72 h after administration and quantified by HPLC-MS/MS. Pharmacokinetic parameters were calculated from the mean plasma concentration by noncompartmental analysis. The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals.

5.3. Molecular modeling

The binding modes for compound **55** and enzalutamide to androgen receptor were generated by CDOCKER in Discovery Studio 3.0 (Accelrys Software Inc.). In CDOCKER, random ligand conformations were generated through molecular dynamics, and a variable number of translations/rotations were applied to each conformation to generate low-energy orientations of the ligand within the active site of rigid receptor. Final ligand conformations were sorted by CHARMm energy (interaction energy plus ligand strain). The crystal structures of AR (PDB entry code: 2OZ7) were extracted from the Protein Database. All ligands were docked in all possible stereoisomeric forms in an active site located sphere with 12 Å radius for AR, which was generated with the Create-Sphere function around the subsequently removed crystal structure ligand. A total of 30 dockings for each ligand were performed, and the conformers with the lowest CHARMm energy were chosen for interpreting the docking results.

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Highlights:

- A series of tetrahydroisoquinoline thiohydantoin derivatives were synthesized and evaluated for its binding affinity to AR, its inhibition of cell growth in bicalutamide-resistant cells as well as AR nuclear translocation.
- The most potent compound **55** in our paper shows comparable ability with enzalutamide in proliferation inhibition of LNCaP cells and AR antagonistic activity.
- Compound **55** has less cytotoxic to AR-negative cells compared with Enzalutamide.
- More importantly, **55** displays good pharmacokinetic properties compared to our pre-work.
- The bicalutamide-resistant mechanism was clarified and overcome by compound **55**.