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Article type : Research Article

Synthesis and evaluation of novel thiazole-based derivatives as selective inhibitors of DNA-binding domain of the androgen receptor

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Abstract

A series of thiazole-based inhibitors selectively targeting DNA-binding domain of the androgen receptor (AR) were synthesized, evaluated and the SAR data were summarized. We identified a novel compound SKLB-C2807 that effectively inhibited the human prostate cancer cell line LNCaP/AR with the IC_{50} value of 0.38 μM without significant antiproliferative effects on other cell lines PC-3

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cbdd.13068

(AR-negative), SW620, MCF-7 (ER-positive) and L-O2 (non-cancerous). This compound also considerably decreased the expression of prostate-specific antigen (PSA). Its binding mode to the AR-DBD was studied. These efforts lay the foundation for developing the next generation of anti-androgens.

Keywords

Antitumor activity; Selective inhibition; Structure-activity relationships (SARs); Androgen receptor; DNA-binding domain

According to the World Health Organization (WHO), prostate cancer is the second most common cancer in men and is becoming a huge public health concern worldwide.[1,2] In United States, it is the most prevalent cancers among males.[3,4] The androgen receptor (AR) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily, which plays a critical role in the occurrence and progression of prostate cancer (PCa).[5-7] Androgen receptor possesses a highly conserved DNA-binding domain (DBD), a moderately conserved ligand-binding domain (LBD) and a little conserved N-terminal domain (NTD).[8,9] All three domains are important for receptor functions.

The biological functions of AR are triggered by binding with testosterone/5α- dihydrotestosterone, which leads to the AR conformational change and its translocation from the cytosol to the nucleus to elicit transcriptional regulation of target genes that can be further modulated by various AR coregulators.[10-12] The AR always play a stimulator role in many different prostate cancer cell types during PCa progression and metastasis.[13] It makes AR the best target for all prostate cancers.

Current chemotherapy for prostate cancer includes inhibition of androgen biosynthesis and blocking the interaction of the hormones with the AR-LBD by anti-androgen drugs.[14-16] Enzalutamide (**1**), an anti-androgen agent that binds to AR-LBD with higher affinity than testosterone, was approved by the Food and Drug Administration (FDA) in 2012.[17, 18] However, after being treated for some time, some

patients developed drug resistance and the disease became castration-resistant prostate cancer (CRPC), which features mutations that express either AR with altered LBD or AR that has no LBD.[19] In both cases, anti-androgens agents become ineffective. Thus, new anti-AR therapeutic avenues are critically needed.

Targeting the AR-DBD represents a novel approach that may overcome anti-androgen resistance. The absence of the LBD would prevent androgens from targeting the androgen-binding site at LBD, thus leaving only the NTD and DBD as viable domains that are targetable by small molecules.[20] The binding of AR to DNA via the AR-DBD is an essential step in the regulation of transcription of genes by both full-length AR and ARVs.[21] Thus, targeting the AR-DBD may have good potential to overcome anti-androgen resistance. A few compounds known to target AR-DBD are showed in **Figure 1**.[22-25] These results encouraged further studies on inhibiting AR activity by targeting AR-DBD.

Reported here are our studies on exploring the AR-DBD as a novel target for small molecule inhibitors to block AR signaling. Although the previous paper by Li et al reported diverse derivatives of 14428, we tried to further investigate the effects of different ring A, ring B and ring C, especially different substituent groups on benzene's *para*- or *meta*- position and heterocyclic-fused group on ring A. We chose 14428 as the start point of this work, then prepared and evaluated analogs of 14428.[24] Firstly, the central thiazole core was replaced by 1,2-substituted five-membered heterocyclic ring, six-membered heterocyclic ring, heterocyclic-fused ring. Then, the benzene on the ring A was explored. Finally, the morpholine moiety on the ring C was investigated. It was demonstrated that SKLB-C2807 (**9**) could effectively inhibit the human prostate cancer cell line LNCaP/AR with the IC_{50} value of 0.38 μM without significant antiproliferative effects on AR-negative human prostate cancer cell line PC-3, human metastatic colon cancer cell line SW620, estrogen receptor (ER)-positive human breast cancer cell line MCF-7 and non-cancerous human liver cell line L-O2, and decreases the expression of prostate-specific antigen (PSA). Its binding mode was studied by docking SKLB-C2807 into the AR-DBD binding site. Conventional anti-androgens are thought to block nuclear localization of the AR, thereby preventing it from initiating transcription.[26,27] In contrast to this mechanism, our compounds do not impede AR nuclear translocation, and they only exert their effect on AR inside the nucleus.

1. Experimental section

1.1 Chemistry

All commercial reagents and anhydrous solvents were obtained from commercial sources and were used without further purification unless otherwise specified. Reactions were monitored by thin layer chromatography (TLC) on glass plates coated with silica gel containing fluorescence indicator reagents. NMR spectra were recorded on a Bruker AMX 400 spectrometer and were calibrated using TMS or residual deuterated solvent as an internal reference ($CDCl_3$: 7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR or DMSO-d_6: 2.50 ppm for ^1H NMR and 39.5 ppm for ^{13}C NMR). ESI-HRMS spectra were recorded on a commercial apparatus and methanol was used to dissolve the sample.

1.1.1 General procedure for preparation of the intermediates 33a-j

To a solution of 2,4-dibromothiazole (1 eq), base (1.5 eq) in DMF, morpholine (1.5 eq) was added dropwise. The mixture was warmed at 50 °C in a sealed tube. After the reaction had finished, the mixture was cooled to room temperature, diluted with 20 mL of water and extracted with three portions of diethyl ether. The combined organic layer was washed with five portions of water, brine, dried over magnesium sulfate, filtered, and concentrated under vacuum. The residue was chromatographed over SiO_2 using ethyl acetate in hexanes (0-30% gradient) to afford the intermediates.

1.1.2 General procedure for preparation of compounds 6, 8-21, 23, 26-32

A mixture of the obtained intermediate (1 eq), appropriate boronic acid (1.5 eq), [Pd]-catalyst (0.1 eq) and base (2 eq) in dioxane and water (5: 1) was stirred at 80 °C under N_2 overnight. It was extracted and concentrated in vacuum. The residue was purified by column chromatography (silica gel, eluted with EtOAc in hexane) to give the targeted compounds.

1.1.3 General procedure for preparation of compound 7

A mixture of the obtained intermediate (1 eq) and aniline (2 eq) was dissolved in 1-Butanol, followed by slowly addition of TFA (2 eq) under ice-bath. Then the reaction mixture was stirred at 120° C overnight. The progress of the reaction was monitored by TLC. After the reaction had completed, the mixture was diluted with saturated aqueous $NaHCO_3$ until neutrality and extracted with ethyl acetate.

The combined organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated in vacuum. Then the residue is dissolved in 2 ml CH_2Cl_2 and precipitate formed after 3 hours. The resulting solid was collected by filtration and purified by column chromatography (silica gel, eluted with MeOH in CH_2Cl_2) to give the targeted compound.

1.1.4 General procedure for preparation of compounds 22, 24, 25

Dry DMF was added to a mixture of the obtained intermediate (1 eq), K_3PO_4 (2 eq), [Cu]-catalyst (0.2 eq), appropriate ligand (1.5 eq), and appropriate N-heterocyclic (1 eq). Then the reaction mixture was stirred at 120° C for 12 hours under N_2. The progress of the reaction was monitored by TLC. After the reaction had completed, the reaction mixture was diluted with ethyl acetate. The combined organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by column chromatography (silica gel, eluted with EtOAc in hexane) to give the targeted compounds.

1.2 Cell counting kit-8 assay

Cell proliferation assay using cell counting kit-8 (CCK-8; YEASEN) was performed according to the manufacturer's instructions. 14428 was used as the positive control. Briefly, LNCaP/AR cells were seeded into 96-well plates at a density of 2000 cells per well with complete growth medium. After 24-h incubation, the tested compounds at the indicated final concentrations were added into the culture medium and incubated for 144 h. Then, CCK-8 was added to each well at the final concentration of 100 μL/mL and incubated with cells at 37℃ for 2 h. The absorbency at 450 nm was measured with an ELISA reader. All of the compounds were tested three times. The results, expressed as IC_{50} (inhibitory concentration 50%), were the average of three determinations and calculated relative to the vehicle (DMSO) control by the GraphPad Prism (Version 5.00) software.

1.3 Western blot

RIPA buffer (Sigma-Aldrich) was used to lyse cells with Complete Protease Inhibitor Cocktail (Roche). Cell lysates were transferred to 1.5 mL tube and kept at −20°C before use. SDS-PAGE was conducted to separate the cellular proteins. 40 μg of each protein sample was subjected to SDS-PAGE. Proteins were separated by 5% stacking gel and 10% running gel. The molecular weight of candidate

proteins was referred to the Pre-stained SeeBlue rainbow marker (Invitrogen) loaded in parallel. The following antibodies were used: anti-PSA (Abcam), anti-GAPDH (Sigma). Blots were detected using a Kodak film developer (Fujifilm, Japan).

1.4 Transient transfection

The LNCaP cells were starved in RPMI 1640 medium (Hyclone) supplemented with 10% FBS (Gibco) for 72 h and then seeded into a 96-well plate (10000 cells/well). After 24 h, AR-EGFP plasmid was transfected into LNCaP cells using 10.5 μL/well transfection reagents (Lipofectamine®3000, Promega). After the transfection for 24 h, cells were treated with compounds at various concentrations in RPMI 1640 medium supplemented with 5% CSS for 24 h. The AR activation was stimulated with 0.3 μM R1881. After that, cells were treated with Hoechst 33342 at 1μg/mL for 10 min. Images were taken on a Zeiss OBSERVER D1/AX10 cam HRC inverted fluorescence microscope controlled with Zen 2012 software.

1.5 The Construction of a three-dimensional protein structure model of human AR-DBD

Homology modeling aims to build the three-dimensional (3D) protein structure model by using experimentally determined structures of a rat AR-DBD as a template in the SWISS-MODEL because there is no 3D structure of human AR-DBD reported at present. SWISS-MODEL workspace is an integrated system for automated comparative modeling of 3D protein structures for a given target protein.[28, 29] Homology modeling in general consists of four main steps, which can be seen as follow. (i) the amino acid sequence of human AR-DBD, which contains 74 amino acids (residues 556-629), was retrieved from UniProt database (UniProt, entry P10257); (ii) the experimentally solved structures of rat AR (PDB code: 1R4I) was identified in the SWISS-MODEL as the template for modeling the human AR-DBD; (iii) the residues of target sequence was mapped to corresponding residues of template structure by means of sequence alignment methods; (iv) the 3D model of human AR-DBD on the basis of the alignment was built and the quality was evaluated.[30] Then the SWISS-MODEL workspace generated three structure models. The model 1 was selected as an initial model for the succeeding optimization because it possesses the highest values of Global Model Quality Estimation (GMQE) and the lowest values of Qualitative Model Energy Analysis (QMEAN); a model with high values of GMQE and low values of QMEAN indicates a good model in general.

2. Results and discussion

2.1 Chemistry

Compounds were synthesized using a general synthetic route starting from dihalo-compounds as outlined in **Scheme 1**. All compounds were typically obtained by a two-step procedure consisting of a nucleophilic substitution by an amine followed by either a Suzuki coupling with a boronic acid or a displacement reaction with a nitrogen-containing heterocycle.

The synthetic route of the key intermediates **33a-j** is illustrated in the first step of **Scheme 1**. Commercially available dihalo-compounds were reacted with substituted morpholines under the base condition to provide the intermediates. Then the intermediates and appropriate boronic acids were subjected to a Suzuki coupling in the presence of [Pd]-catalyst to afford the target compounds. Also, the intermediates were coupled with appropriate *N*-heterocycles using TFA to give the second part of the target compounds. To prepare the third part of the target compounds, the intermediates were utilized to create a displacement reaction with appropriate *N*-heterocycles under [Cu]-catalyst, appropriate ligand and base reagent K_3PO_4.

2.2 *In vitro* antiproliferative activities and structure-activity relationships (SAR)

All the synthesized compounds were assayed for *in vitro* antiproliferative activities against human prostate cancer cell line LNCaP/AR using standard CCK-8 assay. Compound 14428 was used as the standard reference compound, and the results were summarized in **Table 1-4**.

Our initial modification focused on central core ring B. As showed in **Table 1**. Among all heterocycles we explored, thiazole group remains the best. 3-Chloropyrimidine group was tolerated, however, was about eight-fold less potent than 14228. The bulkier heterocycles (**7**) reduced potency even further. Altering the 1,3-substitution pattern to 1,2-substitution pattern by using 1,2,5-thiadiazole completely diminished the activity.

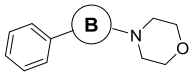
For ring A modification, we first explored various substitutions to the benzene. The phenyl moiety appeared to be rather sensitive to structural changes. Some *meta*-substituent and *para*-substituents were

introduced, and the results were summarized in **Table 2**. The activity was retained when acetyl or methyl ester was at benzene's *meta*-position, whereas when acetyl or methyl ester was at *para*-position of benzene, the activity was completely lost. Reducing the methylketone **9** to alcohol **15** significantly reduced the activity. When benzene's meta-position was replaced with amino, *N*-methylacetamide or sulfonyl groups, the activity was remarkably decreased.

Further modification to ring A by exploring heterocycles was showed in **Table 3**. Thiophene is the heterocycle that is close to aromatic moiety. Indeed, when ring A was replaced with thiophene, the activity was retained. 2-Thiophene (**19**) appears to be slightly less active than 3-thiophene (**18**). We then tried cyclohexene, in which the double bond was applied to help maintain planarity. As showed in **Table 3**, compound **20** showed an IC_{50} of 0.99 μM, which is similar to that of compound **18**. Unfortunately, the potency diminished when the ring A was switched to 3-pyridine (**21**). All fused heterocycles resulted in significantly reduced potencies.

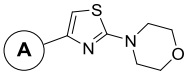
In the next set of experiment, we focused on modification of ring C (**Table 4**). Replacing the oxygen in morpholine with sulfur (**28)** gave the best activity (IC_{50} = 0.40 μM) in this series. All other attempts such as replacing the morpholine oxygen with carbon (**29**), introducing methyl to morpholine (**30**), replacing the morpholine oxygen with an extended hydroxyl group (**31**) or a carbonyl group (**32**) resulted in reduced activity.

Table 1 Ring B modification.



Compound	Ring B	LNCaP/AR IC_{50}±SEM[a] (μM)
14228 (**4**)		0.31±0.05
6		3.00±0.72
7		5.27±1.72
8		>30

Table 2 Synthesized analogues by modifying the benzene's *para*- or *meta*-position.



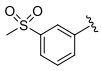
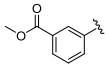
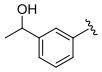
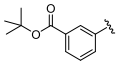
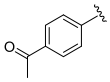
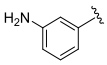
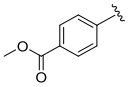
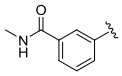
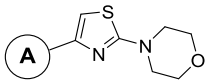
Compound	Ring A	LNCaP/AR $IC_{50}\pm SEM^a$ (μM)	Compound	Ring A	LNCaP/AR $IC_{50}\pm SEM^a$ (μM)
9 (SKLB-C2807)		0.38±0.01	**14**		12.66±1.32
10		1.03±0.29	**15**		13.86±3.00
11		3.88±0.70	**16**		>30
12		5.98±1.62	**17**		>30
13		11.06±0.54			

Table 3 Analogues with ring A modifications.



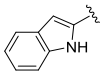
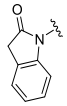
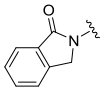
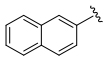
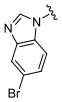
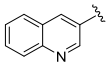
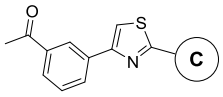
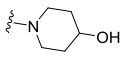
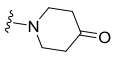
Compound	Ring A	LNCaP/AR $IC_{50}\pm SEM^a$ (μM)	Compound	Ring A	LNCaP/AR $IC_{50}\pm SEM^a$ (μM)
18		1.10±0.80	**23**		6.66±1.66
19		1.58±1.45	**24**		12.84±1.75
20		0.99±0.17	**25**		>30
21		>30	**26**		4.88±0.50
22		20.99±2.17	**27**		4.85±0.63

Table 4 Synthesized analogues by modifying the ring C.



Compound	Ring C	LNCaP/AR $IC_{50}\pm SEM^{a}$ (μM)
28		0.40±0.83
29		1.86±1.10
30		2.21±0.21
31		6.86±2.41
32		25.55±9.81

[a]IC_{50} = compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean ± SEM from the dose-response curves of at least three independent experiments.

2.3 Binding mode analysis

To elucidate the binding mode, molecular docking of the most potent compound SKLB-C2807 was performed in the binding site cavity of AR-DBD. Molecular docking studies have been executed using the GOLD (version 5.0) program integrated into the Discovery Studio 3.1 (Accelrys, Inc., USA) software package. The three-dimensional (3D) protein structure model of human androgen receptor

DNA-Binding Domain (AR-DBD) was established by SWISS-MODEL. The structure model was prepared in the Discovery Studio 3.1, which includes adding hydrogen and assigning the CHARMm force field. Then the binding site was defined as a sphere containing the active residues of residues SER579, VAL582, PHE583, ARG586, GLN592, TYR594, and LYS610 in human AR-DBD. The parameters in the docking processes were set as 'GOLD Default'.

Figure 2 presents a possible binding mode of SKLB-C2807 in the AR-DBD. SKLB-C2807 locates at the surface of AR-DBD, and three hydrogen bonds are formed between SKLB-C2807 and AR-DBD. The first one corresponds to that formed between the oxygen (namely O18 in **Figure 2C**) of morpholine ring and the TYR-594 residue, the remaining two hydrogen bonds are between carbonyloxy (namely O9 in **Figure 2C**) of the benzene ring and ARG-609 residue in the AR-DBD. Additionally, the benzene group in SKLB-C2807 forms a hydrophobic interaction with residues Lys610.

2.4 Anti-proliferative activities in PC-3, SW620, MCF-7 and L-O2 cell lines

SKLB-C2807 was further assessed for its selectivity in AR-negative cell line PC-3 (human prostate cancer), estrogen receptor (ER)-positive cell line MCF-7 (human breast cancer), SW620 cells (human metastatic colon cancer) and non-cancerous human liver cell line L-O2 using standard CCK-8 assay. Both enzalutamide and 14428 were used as the control compounds. As expected, SKLB-C2807 showed no potency at 30 μM to PC-3, MCF-7 and SW620. This experiment results also indicated that SKLB-C2807 doesn't bind to AR-LBD. Although SKLB-C2807 showed an $IC_{50}{}^{a}$ value of 28.38 ± 6.57 μM to L-O2, it was significantly lower than the value of enzalutamide ($IC_{50}{}^{a}$ = 23.59 ± 2.28 μM) and 14428 ($IC_{50}{}^{a}$ = 7.17 ± 1.99 μM).

($^{a}IC_{50}$ = compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean ± SEM from the dose−response curves of at least three independent experiments.)

2.5 The PSA suppression by compounds

SKLB-C2807 was further assessed for its capability to down regulate the expression of PSA, which is a glycoprotein enzyme encoded in humans by the KLK3 gene in LNCaP/AR cell line using Western Blot assay. Our data showed that the PSA expression level remained at the dose of 1 μM compared with the control group, so the higher concentrations (5 μM and 10 μM) have been chosen to this experiment. As illustrated in **Figure 3**, SKLB-C2807 may effectively decreased the expression of PSA in comparison with the control group and might be better than 14428 at 10μM.

2.6 Compounds do not impede AR nuclear translocation

Conventional anti-androgen inhibitors, such as enzalutamide, bind to AR-LBD are thought to block nuclear localization of the AR, thereby preventing it from initiating transcription. Different from this mechanism, our compounds were designed to exert their effect on nuclear AR. To confirm this, we transfected LNCaP cells with plasmid encoding EGFP-tagged full-length AR. The previous experiment demonstrated that the EGFP tag did not affect AR transcriptional activity or compound inhibition. Upon treatment with R1881 and enzalutamide, fluorescence microscope images revealed considerable levels of AR-EGFP in the cytosol compared with control experiments. SKLB-C2807 and bicalutamide (a first-generation anti-androgen) did not prevent R1881-stimulated nuclear localization of AR-EGFP with no fluorescence signal observed in the cytosol, while enzalutamide successfully stopped. Control experiments showed that SKLB-C2807 and enzalutamide could not stimulate any nuclear localization in the absence of R1881. AR-T877A mutant existing in LNCaP cells makes bicalutamide from an antagonist to an agonist, and it leads that bicalutamide still stimulates nuclear localization in the absence of R1881. (**Figure 4**) Together, these results suggest that our compounds influence the activity of the AR inside the cell nucleus, consistent with directly affecting DBD functions.

3. Conclusions

We prepared and tested a series of small molecules aiming to target the DNA-binding domain of the androgen receptor. We identified novel compound SKLB-C2807 as a candidate for further studies. It showed decent potency against LNCaP/AR cell line. Docking studies suggested the compounds bind

to the DNA binding domain. Western Blot assay showed that SKLB-C2807 may effectively inhibit the expression of PSA. Finally, our compounds just exert their effect on nuclear AR, and they don't block nuclear localization of the AR. Further pharmacological profiling of these AR-DBD targeted inhibitors is on-going.

Conflict of interest

The authors declare no conflict of interest.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure legends

Figure 1 Structure of enzalutamide and inhibitors that bind to AR-DBD.

Figure 2 A. Chemical structure of SKLB-C2807; B. SKLB-C2807 is docked into the human AR-DBD, showing interactions between SKLB-C2807 and AR-DBD; C. A 2-dimensional interaction map of SKLB-C2807 and AR-DBD.

Figure 3 The PSA suppression by compounds.

Figure 4 Effect of compounds on AR nuclear localization. LNCaP cells were transfected with EGFP-AR plasmid and treated with 0.3 μM R1881 and 10 μM of indicated compounds. Cells were fixed, mounted and stained with Hoechst 33342. Fluorescence microscope reveals the location the EGFP-AR (488 nm) and cell nucleus (Hoechst 33342, 350 nm) in the first two rows of images. The third row depicts the merged images of EGFP-AR and Hoechst 33342 visualizations.

Scheme 1 The synthetic routes of compounds. Reagents and conditions: (Ⅰ) appropriate boronic acid, [Pd]-catalyst, base, dioxane and water (5:1), 80 °C; (Ⅱ) appropriate *N*-heterocycles, TFA, 1-Butanol, 120 °C; (Ⅲ) appropriate *N*-heterocycles, appropriate ligand, [Cu]-catalyst, K_3PO_4, DMF, 120 °C.

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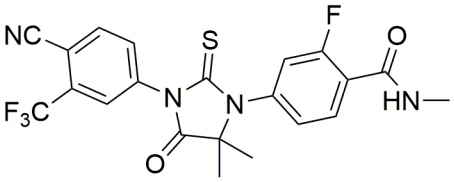

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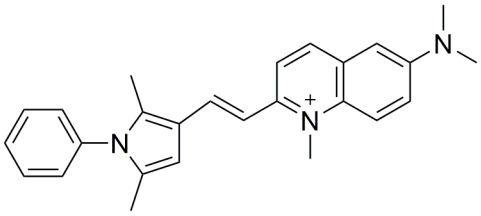

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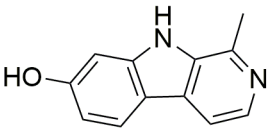

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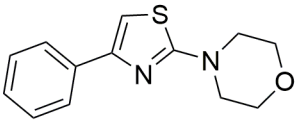

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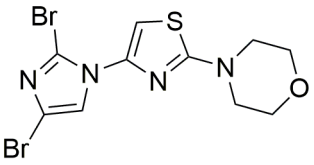

14449 **(5)**