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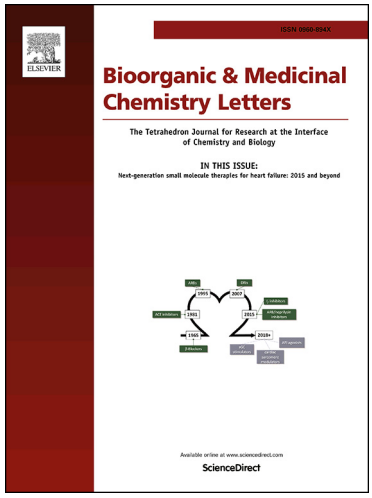


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## Graphical Abstract

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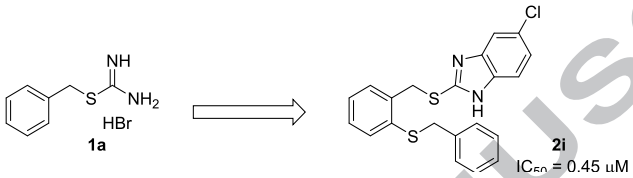

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# Cyclic analogue of *S*-benzylisothiourea that suppresses kynurenine production without inhibiting indoleamine 2,3-dioxygenase activity

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ABSTRACT

Kynurenine is biosynthesised from tryptophan catalysed by indoleamine 2,3-dioxygenase (IDO). The abrogation of kynurenine production is considered a promising therapeutic target for immunological cancer treatment. In the course of our IDO inhibitor programme, formal cyclisation of the isothiourea moiety of the IDO inhibitor **1** afforded the 5-Cl-benzimidazole derivative **2b-6**, which inhibited both recombinant human IDO (rhIDO) activity and cellular kynurenine production. Further derivatisation of **2b-6** provided the potent inhibitor of cellular kynurenine production **2i** ($IC_{50}$ = 0.34 μM), which unexpectedly exerted little effect on the enzymatic activity of rhIDO. Elucidation of the mechanism of action revealed that compound **2i** suppresses IDO expression at the protein level by inhibiting STAT1 expression in IFN-γ-treated A431 cells. The kynurenine-production inhibitor **2i** is expected to be a promising starting point for a novel approach to immunological cancer treatment.



Catabolism of the essential amino acid tryptophan generates several biologically active metabolites, such as the neurotransmitter serotonin, the excitotoxin quinolinic acid, kynurenic acid, and nicotinamide adenine dinucleotide.[1,2] These metabolites are predominantly formed through the kynurenine pathway (>90% of tryptophan in mammals).[3] The initial step in this pathway is the oxidative cleavage of the C-2/C-3 bond of the indole ring of tryptophan to afford *N*′-formylkynurenine. This is the rate-limiting step in the kynurenine pathway and is catalysed by intracellular heme-containing dioxygenases, namely indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase.[4] *N*′-Formylkynurenine is then converted to kynurenine by a formamidase, and the subsequent metabolism of kynurenine provides many biological substances (vide supra).

Kynurenine and its metabolites play a variety of roles in the central nervous system (CNS) and the innate and adaptive immune responses.[5–9] Most metabolites of the kynurenine pathway are neuroactive and play essential roles in regulating the function of the *N*-methyl-D-aspartate (NMDA) receptor. In particular, increased levels of quinolinic acid cause excitotoxicity by stimulating NMDA receptors in the CNS and have been implicated in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases.[10] In addition, kynurenine and its metabolites are responsible for immunological tolerance. It has been reported that kynurenine modulates T cells by suppressing their proliferation and inducing apoptosis, and it also activates regulatory T (Treg) cells.[11] These effects influence the pathology of autoimmune diseases and, in particular, cancer immune tolerance. Furthermore, kynurenine has been identified as an endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor (AhR).[12] Cancer-cell-derived kynurenine promotes tumour-cell survival and motility and suppresses the antitumour immune response through AhR in an autocrine/paracrine manner.[12] With progress in understanding cancer immunology, disruption of the PD-1/PD-L1 immune checkpoint successfully led to the new cancer therapies nivolumab and pembrolizumab.[13,14] Consequently, the abrogation of kynurenine production in cancer cells is considered a promising approach to anticancer therapy.[15]

The importance of the kynurenine pathway in drug discovery has prompted efforts to identify modulators for this pathway. In particular, IDO has received attention as the regulatory enzyme that catalyses the initial and rate-limiting step in the kynurenine pathway. Several IDO inhibitors have been reported,[16] such as the *S*-benzylisothioureas[17] and analogues of the anti-hypertensive agent candesartan[18] that we recently described. Although the *S*-benzylisothiourea analogues inhibited both recombinant human IDO (rhIDO) and cellular kynurenine production in A431 cells, the isothiourea moiety is not a drug-like structure owing to its extremely hydrophilic properties and potential toxicity associated with substitution reactions involving nucleophilic groups of amino acids.

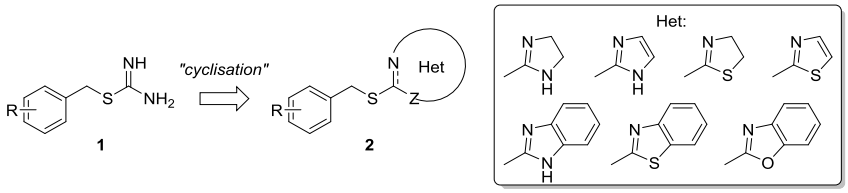
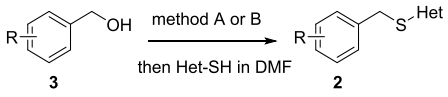



**Fig. 1**. Synthetic concept: conversion of the isothiourea to drug-like heterocycles.

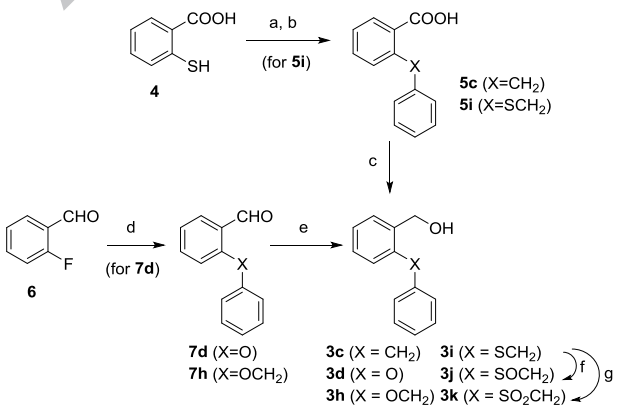
Therefore, conversion of the isothiourea moiety to a drug-like structure is essential for exploiting these promising compounds as drugs for cancer immunotherapy. Herein, we describe the design and synthesis of cyclic derivatives **2** of isothiourea **1** (Fig. 1), as well as the installation of substituents on the parent phenyl ring. The biological activities of these compounds with respect to rhIDO inhibition and cellular kynurenine production in A431 cells are also reported. In particular, one derivative exhibited unexpectedly potent inhibition of cellular kynurenine production with very low rhIDO inhibition, and the underlying mechanism of action was elucidated.

All of the target molecules were synthesised by the condensation of a mercaptoheterocycle (Het-SH) and benzyl alcohol **3** by activation of the hydroxyl group using MsCl (method A) or $PBr_3$ (method B), except for the commercially available compound **2a** (Scheme 1).



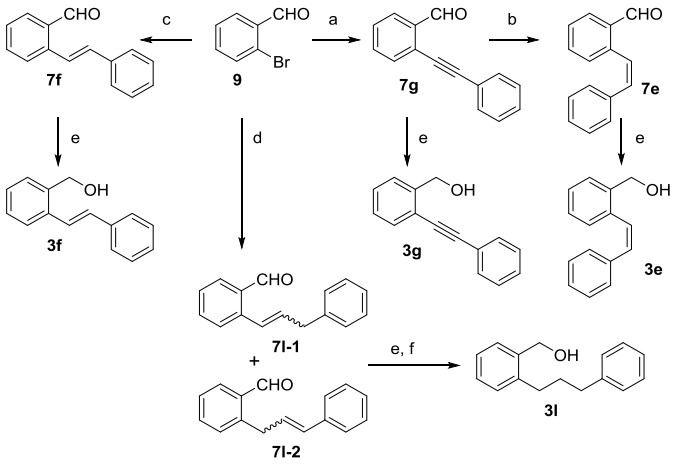

**Scheme 1.** (method A) MsCl, $Et_3N$, 4-DMAP, $CH_2Cl_2$, 0 °C to RT; (method B) $PBr_3$, THF, 0 °C.

Commercially available 2-phenethylbenzyl alcohol (**3b**) was used to prepare the compound **2b** series (Table 1). The preparation of the requisite benzyl alcohols (**3c**, **3d**, **3h**–**3k**) for the target molecules **2c**, **2d**, and **2h**–**2k** is illustrated in Scheme 2. Reduction of carboxylic acids **5c** and **5i** with $BH_3$–THF provided the corresponding alcohols **3c** (X = $CH_2$) and **3i** (X = $SCH_2$) in good yield. The starting material 2-benzylbenzoic acid (**5c**) was commercially available, whereas 2-(benzylthio)benzoic acid (**5i**) was obtained by benzylation of thiosalicylic acid **4** followed by hydrolysis with aqueous NaOH. Compounds **3j** (X = $SOCH_2$) and **3k** (X = $SO_2CH_2$) were synthesised by the oxidation of **3i** with different stoichiometric amounts of mCPBA. Furthermore, reduction of aldehydes **7d** and **7h** with $NaBH_4$ provided the corresponding alcohols **3d** (X = O) and **3h** (X = $OCH_2$) in good yield. The starting material 2-phenoxybenzaldehyde (**7d**) was synthesised by $S_NAr$ reaction of 2-fluorobenzaldehyde (**6**) with phenol, whereas 2-benzyloxybenzaldehyde (**7h**) was purchased from a commercial supplier (Scheme 2).




**Scheme 2.** (a) BnBr, $K_2CO_3$, DMF, 100 °C, 95%; (b) 1 mol/L NaOH (aq.), EtOH, 50 °C, 76%; (c) $BH_3$–THF, THF, 0 °C to RT, 86%; (d) phenol, $K_2CO_3$, DMF, 120 °C, 77%; (e) $NaBH_4$, MeOH, 0 °C, 87% for **3d**, 90% for **3h**; (f) mCPBA (0.77 eq.), $CH_2Cl_2$, 58%; (g) mCPBA (2.3 eq.), $CH_2Cl_2$, 89%.

Unsaturation (**2e**–**2g**) and extension (**2l**) of the ethylene chain in the 2-phenethyl moiety of compound **2b-6** (Table 1) were achieved via palladium chemistry (Scheme 3). 2-Bromobenzaldehyde (**9**) served as the starting material for benzyl alcohols **3e**–**3g** and **3l** via the corresponding aldehydes. Sonogashira–Hagihara cross-coupling with phenylacetylene afforded 2-(phenylethynyl)benzaldehyde (**7g**) in excellent yield. (*Z*)-2-Styrylbenzaldehyde (**7e**) was synthesised by partial hydrogenation of **7g** using Lindlar catalyst in MeOH. Following unsuccessful attempts to directly convert the (*Z*)-olefin **7e** to the (*E*)-olefin **7f** using $I_2$ or light, the Mizoroki–Heck reaction of **9** with styrene selectively provided the (*E*)-styryl analogue **7f**. Reduction of the aldehydes **7g**, **7e**, and **7f** with $NaBH_4$ afforded the corresponding benzyl alcohols **3g** (-C≡C-), **3e** ((*Z*)-CH=CH-), and **3f** ((*E*)-CH=CH-), respectively. The use of allylbenzene instead of styrene in the Mizoroki–Heck reaction of **9** furnished a mixture of alkene regioisomers (**7l-1**, **7l-2**), which was reduced using $NaBH_4$ and subsequently hydrogenated to afford the sole product 2-(3-phenylpropyl)benzyl alcohol (**3l**).

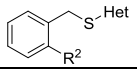


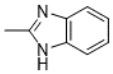
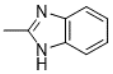
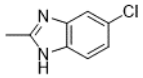
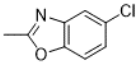
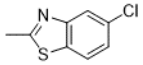

**Scheme 3.** (a) PhC≡CH, $Pd(PPh_3)_4$, dppf, CuI, Et*i*$Pr_2N$, THF, reflux, 92%; (b) $H_2$, Lindlar catalyst, MeOH, RT, 60%; (c) $PhCH{=}CH_2$, $Pd(OAc)_2$, BINAP, Et*i*$Pr_2N$, dioxane, reflux, 51%; (d) $PhCH_2CH{=}CH_2$, $Pd(OAc)_2$, BINAP, Et*i*$Pr_2N$, dioxane, reflux, quant.; (e) $NaBH_4$, MeOH, 0 °C, 81% for **3e**, quant. for **3f**, 100% for **3g**, 88% for **3l**; (f) $H_2$, 10% Pd/C, MeOH, 2.5 atm, RT, 54%.

Table 1 lists the rhIDO inhibitory activities of the synthesised compounds in which the isothiourea moiety had been formally replaced with a heterocycle. Contrary to our expectations, the simple benzimidazole derivative **2a**, an analogue of compound **1a** containing a cyclised isothiourea moiety, was completely inactive with respect to rhIDO inhibition. However, the introduction of a 2-phenethyl group into the benzene ring (**2b-1**) led to obvious rhIDO inhibition (43% at 10 μM). Therefore, we examined a variety of heterocyclic rings attached to the 2-phenethylbenzylthio moiety in an effort to enhance the rhIDO inhibition. Replacement of the benzimidazole ring with imidazole

(**2b-2**), imidazoline (**2b-3**), thiazole (**2b-4**), and thiazoline (**2b-5**) rings reduced the activity. In contrast, the introduction of 5-Cl into the benzimidazole ring (**2b-6**) retained the rhIDO inhibition. Analogues with other Cl-containing heterocycles (**2b-7**, **2b-8**) were weak rhIDO inhibitors.

**Table 1.** Structures and synthetic methods for compounds **2a** and **2b**, and their inhibitory activities towards rhIDO and cellular kynurenine production.



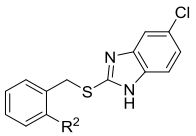

| Compound | $R^2$ | Het | Synthetic method[a] | rhIDO % inhibition @10 μM | KYN production[b] % inhibition @1 μM |
|---|---|---|---|---|---|
| **1a** | H |  | | ($IC_{50}$ = 61 μM) | (44% @10 μM) |
| **2a** | H |  | | −42% | 12% |
| **2b-1** | $CH_2CH_2Ph$ |  | A | 43% | 43% |
| **2b-2** | $CH_2CH_2Ph$ |  | B | 19% | 1% |
| **2b-3** | $CH_2CH_2Ph$ |  | B | 17% | 7% |
| **2b-4** | $CH_2CH_2Ph$ |  | B | 31% | 7% |
| **2b-5** | $CH_2CH_2Ph$ |  | A | 0% | 0% |
| **2b-6** | $CH_2CH_2Ph$ |  | A | 47% | 48% |
| **2b-7** | $CH_2CH_2Ph$ |  | B | 21% | −18% |
| **2b-8** | $CH_2CH_2Ph$ |  | B | 9% | −8% |

[a] Synthetic method for final condensation with Het-SH.
[b] Kynurenine production in A431 cells.

In addition, we examined the cellular inhibition of kynurenine production, which is mediated by IDO, by the synthesised compounds, using interferon-γ (IFN-γ)-treated human epithelial carcinoma A431 cells (Table 1). Among the compounds that displayed obvious rhIDO inhibition, compounds **2b-1** and **2b-6** also exhibited cellular inhibition of kynurenine production without cytotoxicity (data not shown).

We next examined the influence of the ethylene linker of the 2-phenethyl moiety of the 5-Cl-benzimidazole analogue **2b-6**. Table 2 lists the inhibitory activities of the resulting compounds **2c**–**2l**. Replacement of the 2-phenethyl moiety with -$CH_2Ph$ (**2c**), (*Z*)-CH=CHPh (**2e**), and -C ≡ CPh (**2g**) groups resulted in comparable rhIDO inhibition as the parent compound **2b-6**, whereas the -OPh (**2d**), (*E*)-$CH_2$=CHPh (**2f**), -$OCH_2Ph$ (**2h**), -$SCH_2Ph$ (**2i**), -$SOCH_2Ph$ (**2j**), -$SO_2CH_2Ph$ (**2k**), and -$(CH_2)_3Ph$ (**2l**) analogues displayed reduced activity. These results indicate that the distance and orientation of the phenyl ring from the benzimidazole moiety are important and that heteroatoms exert a detrimental effect on the interaction with rhIDO. Furthermore, we evaluated the cellular inhibition of kynurenine production in IFN-γ-treated A431 cells by the synthesised compounds (Table 2). Among the compounds that exhibited obvious rhIDO inhibition, compound **2e** demonstrated potent inhibition ($IC_{50}$ = 0.34 μM) of kynurenine production without causing cytotoxicity even at the higher concentration of 100 μM (data not shown), whereas compounds **2c** and **2g** showed no obvious cellular activity. Surprisingly, compound **2i** exhibited potent inhibition of kynurenine production in A431 cells ($IC_{50}$ = 0.45 μM) without substantial rhIDO inhibition, which occurred without cytotoxicity even at 100 μM (data not shown). We therefore considered that the potent inhibition of cellular kynurenine production could possibly arise from a distinct mechanism of action.

**Table 2.** Structures and synthetic methods for compounds **2c**-**2l**, and their inhibitory activities towards rhIDO and cellular kynurenine production.[a]




| Compound | $R^2$ | rhIDO % inhibition @10 μM | KYN production[b] % inhibition @1 μM or $IC_{50}$ |
|---|---|---|---|
| **2c** | $CH_2Ph$ | 46% | 13% |
| **2d** | OPh | 30% | 39% |
| **2e** | CH=CHPh (*Z*) | 49% | $IC_{50}$ = 0.34 μM |
| **2f** | CH=CHPh (*E*) | −6% | 15% |
| **2g** | C≡CPh | 52% | 6% |
| **2h** | $OCH_2Ph$ | 3% | 30% |
| **2i** | $SCH_2Ph$ | 19% | $IC_{50}$ = 0.45 μM |
| **2j** | $SOCH_2Ph$ | 11% | 30% |
| **2k** | $SO_2CH_2Ph$ | 24% | 1% |
| **2l** | $CH_2CH_2CH_2Ph$ | 19% | 44% |

[a] All compounds were synthesised by method A.
[b] Kynurenine production in A431 cells.

To investigate the mechanism of action of compound **2i**, namely its potent inhibition of kynurenine production in A431 cells without rhIDO inhibition, the IDO expression level of the cells was measured by Western blotting. IFN-γ induced upregulation of the IDO protein level, whereas compound **2i** suppressed IDO expression in a concentration-dependent manner (Fig. 2). This phenomenon was only observed for the cyclic analogue **2i**, that is, we have previously reported that the parent *S*-benzylisothiourea **1** does not affect the IDO expression level in IFN-γ-treated A431 cells.[17] Therefore, we next explored the effect on Janus kinase/signal transducer and activator of transcription 1 (JAK/STAT1) signalling, because IFN-γ induces IDO expression through this pathway.[19] As shown in Fig. 2, compound **2i** reduced STAT1 expression, which was upregulated by IFN-γ treatment, in a concentration-dependent manner. Taken together, these results indicate that compound **2i** suppressed IDO expression by inhibiting STAT1 expression in IFN-γ-treated A431 cells.

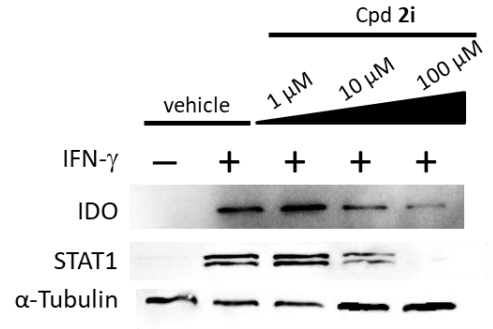



**Fig. 2.** Western blotting analysis of the effects of compound **2i** on IDO and STAT1 expression in IFN-γ-treated A431 cells.

In summary, we synthesised cyclic analogues of our IDO inhibitor *S*-benzylisothiourea in an effort to circumvent its possible toxicity. The 5-Cl-benzimidazole derivative **2b-6** was a moderate rhIDO inhibitor with cellular inhibition of kynurenine production. Compound **2i** was found to exhibit potent inhibition of cellular kynurenine production despite its unexpectedly minor effect on the enzymatic activity of rhIDO. Analysis of the mechanism of action revealed that compound **2i** suppresses IDO expression at the protein level by inhibiting STAT1 expression in IFN-γ-treated A431 cells. With its distinct mechanism of action, the kynurenine-production inhibitor **2i** is expected to be a novel lead compound for immunological cancer treatment.

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**Conflict of Interest (COI)**

The authors declare no conflict of interest.

**Supplementary Material**

The online version of this article contains supplementary material.

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- Modifying *S*-benzylisothiourea provided IDO inhibitors with kynurenine production.
- Cpd **2i** demonstrated potent inhibition for kynurenine production.
- Cpd **2i** had barely effect on the enzymatic activity of IDO unexpectedly.
- Cpd **2i** suppressed the expression of IDO by inhibiting STAT1 expression in cells.