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S-Benzylisothiourea derivatives as small-molecule inhibitors of indoleamine-2,3-dioxygenase

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ABSTRACT

S-Benzylisothiourea **3a** was discovered by its ability to inhibit indoleamine-2,3-dioxygenase (IDO) in our screening program. Subsequent optimization of the initial hit **3a** lead to the identification of sub-μM inhibitors **3r** and **10h**, both of which suppressed kynurenine production in A431 cells. Synthesis and structure–activity relationship of *S*-benzylisothiourea analogues as small-molecule inhibitors of IDO are described.

Indoleamine 2,3-dioxygenase (IDO) is an extrahepatic heme-containing dioxygenase that catalyzes the addition of oxygen across the C-2/C-3 bond of the indole ring of tryptophan (Trp).[1,2] This is the initial and rate-limiting step in the catabolism of the essential amino acid Trp to *N*-formylkynurenine along the kynurenine pathway, which leads to biologically active metabolites such as the neurotransmitter serotonin, excitoxin quinolinic acid, *N*-methyl-D-aspartate (NMDA) receptor antagonist kynurenic acid, and nicotinamide adenine dinucleotide (NAD).[3–5]

IDO is expressed ubiquitously but predominately in cells within the immune system where it is specifically induced in dendritic cells and macrophages at the sites of inflammation by cytokines.[1] It is known that IDO is overexpressed in a variety of diseases, including cancer,[6] neurodegenerative disorders (e.g., Alzheimer's disease),[7] age-related cataract,[8] and HIV encephalitis.[9] Among these, IDO has been shown to play an important role in the process of immune escape by tumors.[1] In environments where the Trp concentration has been depleted by IDO, killer T cells cannot be activated by antigens, and they undergo G1 cell cycle arrest leading to apoptosis and immunosuppression.[10] Consequently, tumor cells escape from the immune response and survive. This is consistent with the observation that increased expression of IDO in tumor cells is correlated with poor prognosis for survival in patients with serious ovarian and colorectal cancers.[11,12]

Several IDO inhibitors have been reported to date (Chart 1). 1-Methyltryptophan (1-MT) is the most frequently used inhibitor with a weak K_i of 34 μM and is in clinical development at the National Cancer Institute.[13–15] The natural product brassinin, which possesses a dithiocarbamate group, has been reported to be a weak IDO inhibitor (K_i >10 μM).[16] In 2008–2009, naphthoquinones (e.g., menadione),[17] exiguamine A,[18] phenylimidazoles **1**,[19] and 4-amino-1,2,5-oxadiazole-3-carboximidamide **2**[20] were newly reported as IDO inhibitors, some of which exhibited in vivo efficacy to reveal preclinical proof of concept (POC) of IDO inhibition in

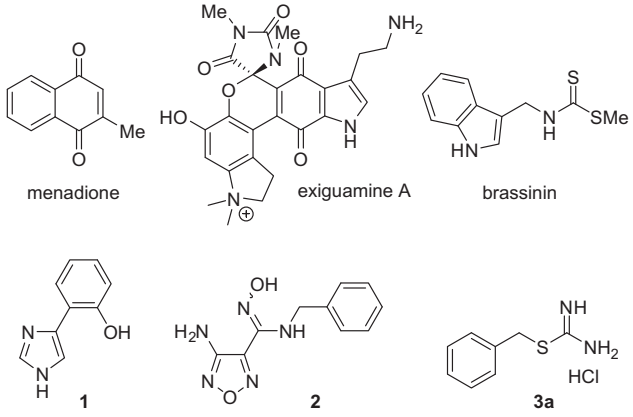

Chart 1. IDO inhibitors.

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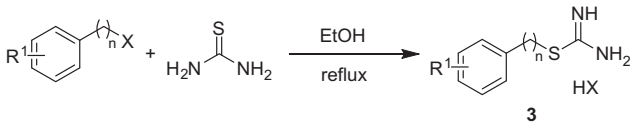


Scheme 1. Preparation of isothioureas **3**.

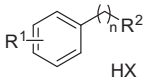
cancer treatment. The combination of menadione (also known as vitamin K_3) with the cytotoxic agent paclitaxel led to regression of tumors in mouse models (e.g., MMTV-Neu transgenic and B16-F10 xenograft) that showed no response to the cytotoxin administered alone.[17] A similarly positive in vivo response has been obtained by using siRNA to silence the IDO gene in B16-F10 tumor-bearing mice.[21] By screening campaign for our chemical library, we have also found that the *S*-benzylisothiourea derivative **3a** is an IDO inhibitor. Herein we report the structure–activity relationship (SAR) for IDO inhibition including cellular activity; specifically, we investigated substituents on the benzene ring, and modification of the linker to and replacement of the isothiourea moiety including isosters. In addition, we carried out kinetic analysis of *S*-benzylisothiourea analogues.

The tested compounds were purchased from commercial sources or synthesized. The isothioureas **3** were obtained by alkylation of thiourea with the corresponding phenylalkyl halide (Scheme 1). Treatment of iso(thio)cyanate with ammonia provided the (thio)ureas (**6** and **7**).

The inhibitory activity of the isothioureas **3** on human IDO is listed in Table 1.[22] For the *S*-benzylisothiourea series (**3a**–**3s**), the substituent on the benzene ring was found to be important for IDO inhibition; incorporation of 2-Cl (**3b**) enhanced inhibitory activity, and the 3-Cl (**3c**) and 4-Cl (**3d**) analogues were more potent inhibitors. Among the 4-substituted-*S*-benzylisothioureas, the 4-Br (**3f**) and 4-CF_3 (**3k**) analogues retained potent activity, while replacement with 4-F (**3e**), 4-Me (**3g**), 4-Et (**3h**), 4-OMe (**3l**), 4-CN (**3m**), and 4-NO_2 (**3n**) reduced activity. Moreover, analogues with 4-*i*Pr (**3i**), 4-*tert*-Bu (**3j**), 4-SO_2Me (**3p**), 4-COPh (**3q**), and 4-COOH (**3o**) were completely inactive; IDO disfavored bulky and hydrophilic substituents at this position. Further addition of 2-Cl to *S*-(4-chlorobenzyl)isothiourea **3d** resulted in sub-μM inhibition (**3r**); however, further addition of 3-Cl (**3s**) reduced activity. The SAR with respect to the position of the Cl atom of chlorophenethylisothioureas (**3t**–**3v**) was similar to that of chlorobenzylisothioureas, namely, 4-Cl (**3v**) was rather potent, but the inhibitory activity was substantially reduced. These results indicate that the halogen atom might play an important role in the interaction with IDO, which was recently found to make an important contribution to protein–ligand binding affinity,[23] and the distance from the isothiourea moiety is crucial for the inhibitory activity. The potent analogues are still small; they have an high ligand efficiency (LE)[24], that is, LE = 0.67 for compound **3r**.

All analogues were also evaluated for kynurenine production in human epithelial carcinoma A431 cells (Table 1).[25] In this cell line, there observed up-regulation of IDO and increased production of kynurenine by stimulation with interferon-γ (IFN-γ) (Fig. 1).[26] The potent IDO inhibitors displayed inhibition of kynurenine production with an SAR similar to that for IDO inhibition. Most of the compounds demonstrated slightly more potent activity in the A431 cell assay than in the IDO enzyme assay, as in a previous report.[20] This result was presumably due to the complexity of the IDO enzyme assay with a regeneration system of IDO in its active Fe^{2+} form, or due to the difference between recombinant IDO in the enzyme assay and endogenous IDO in the A431 cells. Furthermore, compound **3d** suppressed kynurenine production without reduction of IDO expression at 10 μM, which is higher concentration than IC_{50} value (Fig. 1).

Table 1
Inhibitory activity of benzylthioureas **3** and compounds **4**–**12**



Compound	R^1	*n*	R^2	HX	IC_{50}, μM	
					IDO	A431[a]
3a	H	1	SC(=NH)NH_2	HCl	61	>10
3b	2-Cl	1	SC(=NH)NH_2	HCl	10	4.8
3c	3-Cl	1	SC(=NH)NH_2	HCl	4.6	1.4
3d	4-Cl	1	SC(=NH)NH_2	HCl	2.2	0.6
3e	4-F	1	SC(=NH)NH_2	HCl	13	2.0
3f	4-Br	1	SC(=NH)NH_2	HBr	1.3	0.8
3g	4-Me	1	SC(=NH)NH_2	HCl	30	6.8
3h	4-Et	1	SC(=NH)NH_2	HCl	16	2.5
3i	4-*i*Pr	1	SC(=NH)NH_2	HBr	>100	>20
3j	4-*tert*-Bu	1	SC(=NH)NH_2	HBr	>100	>20
3k	4-CF_3	1	SC(=NH)NH_2	HCl	2.6	1.2
3l	4-OMe	1	SC(=NH)NH_2	HCl	52	10
3m	4-CN	1	SC(=NH)NH_2	HBr	19	2.6
3n	4-NO_2	1	SC(=NH)NH_2	HCl	11	2.2
3o	4-COOH	1	SC(=NH)NH_2	HCl	>100	>20
3p	4-SO_2Me	1	SC(=NH)NH_2	HBr	>100	>10
3q	4-COPh	1	SC(=NH)NH_2	HBr	>100	>20
3r	2,4-Cl_2	1	SC(=NH)NH_2	HBr	0.4	1.1
3s	3,4-Cl_2	1	SC(=NH)NH_2	HCl	17	7.3
3t	2-Cl	2	SC(=NH)NH_2	HBr	>100	>20
3u	3-Cl	2	SC(=NH)NH_2	HBr	>100	>20
3v	4-Cl	2	SC(=NH)NH_2	HBr	57	>20
4	4-Cl	1	SC(=NMe)NHMe	$HClO_4$	21	>20
5a	4-Cl	0	NHC(=NH)SMe	HI	>100	>20
5b	4-Cl	2	NHC(=NH)SMe	HI	>100	>20
6a	4-Cl	0	$NHCSNH_2$	None	>100	>20
6b	4-Cl	2	$NHCSNH_2$	None	100	>20
7	4-Cl	1	$NHCONH_2$	None	>100	>20
8a	4-Cl	1	NH_2	None	>100	>20
8b	4-Cl	2	NH_2	None	>100	>20
9	4-Cl	1	OH	None	>100	>20
10a	4-Cl	1	SH	None	3.2	1.1
10b	2-Cl	1	SH	None	1.2	15
10c	H	1	SH	None	1.7	13
10d	4-F	1	SH	None	1.8	1.9
10e	4-Me	1	SH	None	1.3	6.5
10f	4-OMe	1	SH	None	1.4	9.4
10g	2,4-Cl_2	1	SH	None	3.5	4.6
10h	3,4-Cl_2	1	SH	None	0.1	1.1
10i	4-Cl	0	SH	None	1.9	>20
11	4-Cl	0	SO_2Na	None	>100	>20
12	4-Cl	0	SO_3H	None	>100	>20

[a] Kynurenine production in A431 cells.

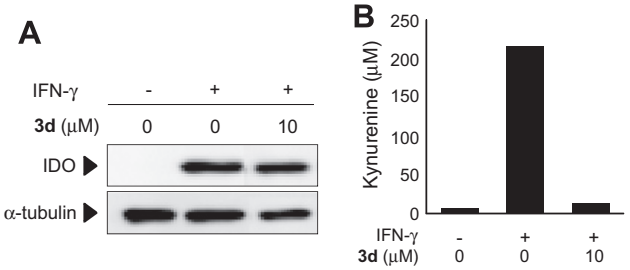


Figure 1. Effects of compound **3d** on IDO expression and kynurenine production in A431 cells stimulated with IFN-γ. (A) Western blot analysis of IDO in cell lysate from A431 cells. (B) Kynurenine concentration in culture medium.

Next, modification of the isothiourea moiety was investigated (Table 1). Bis-methylation of the N atoms (**4**) of isothiourea reduced inhibitory activity. *N*-4-Chlorophenyl (**5a**) and *N*-4-chlorophenethyl (**5b**) isothioureas were completely inactive. Also, replacement with thioureido (**6a** and **6b**), ureido (**7**), amino (**8a**

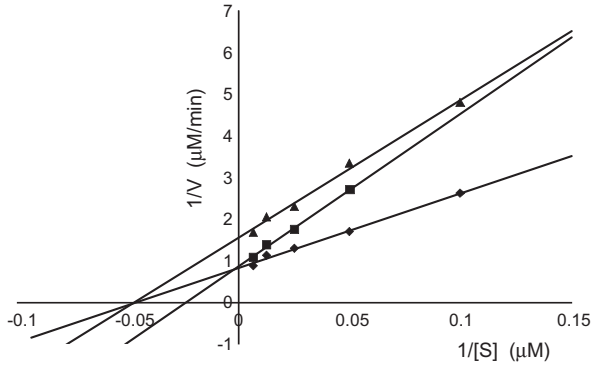


Figure 2. Steady-state kinetic analysis of recombinant human IDO by Lineweaver–Burk plot. ♦ No inhibition, ■ L-1-MT (100 μM), and ▲ compound **3d** (1 μM).

and **8b**) and hydroxyl (**9**) groups resulted in total loss of activity. On the contrary, the benzylthiols (**10a**–**10h**) showed potent inhibitory activity both for IDO and kynurenine production in A431 cells. With respect to the substituents on the phenyl ring, the SARs differed from those of the benzylisothioureas **3**; 4-Cl (**10a**) was also a potent inhibitor, and 2-Cl (**10b**), H (**10c**), 4-F (**10d**), 4-Me (**10e**), and 4-OMe (**10f**) retained similarly potent inhibitory activity, unlike the corresponding benzylisothioureas **3**. For incorporation of an additional Cl atom (**10g** and **10h**), 2,4-Cl_2 (**10g**) was equipotent to the parent 4-Cl (**10a**), and 3,4-Cl_2 (**10h**) exhibited the most potent IDO inhibition (IC_{50} = 0.1 μM), in contrast with the corresponding isothioureas (**3r** and **3s**). 4-Chlorobenzenethiol (**10i**), which did not include the methylene chain of 4-chlorobenzylthiol (**10a**), exhibited similarly potent inhibitory activity for IDO; however, no cellular inhibition of kynurenine production in A431 cells was observed. This might be due to cellular penetration issues caused by different physicochemical properties such as the stronger pK_a value of the benzenethiol. Analogues with SO_2Na (**11**) and SO_3H (**12**), which are oxidized forms of SH, were completely inactive.

Compound **3d** was selected for further kinetic studies. As shown in Figure 2, the Lineweaver–Burk plot was found to be consistent with an noncompetitive inhibition mode, unlike exiguamine A derivatives with uncompetitive inhibition[18] or 4-amino-1,2,5-oxadiazole-3-carboximidamide with competitive inhibition.[20] In this experiment, L-1-MT exhibited competitive inhibition that was the same as reported in the literature.[27]

The distinct SARs between benzylisothioureas **3** and benzylthiols **10** might be due to differing binding modes in the heme region at the active site of IDO, since noncompetitive inhibitors could also bind at alternative pockets around the active site of IDO.[28,29] Given the well-known metal coordinating properties of SH, it is likely that the SH moiety of benzylthiol chelates to the heme iron. In similar fashion, the isothiourea may bind to heme itself. Alternatively, the isothiourea could be a good bidentate acceptor of carboxylic acid. Since there are propionic acid in the heme structure, and some amino acid residues of carboxylic acid such as Asp274 and Glu171 around the active site,[30] we speculate that the isothiourea moiety interacts with such carboxylic acid. This speculation is supported by the reduction in IDO inhibition for bis-methylated *S*-isothioureas (**4**) and *N*-isothioureas (**5a** and **5b**). Further analysis to clarify the molecular interaction of those compounds with IDO is in progress.

In conclusion, we have identified the benzylisothiourea analogues **3** as novel noncompetitive IDO inhibitors by our screening campaign, derivatization and kinetic analysis. The 4-Cl/Br atom on the phenyl ring and the distance from the isothiourea moiety were important for potent IDO inhibition. Replacements of the isothiourea moiety afforded potent benzylthiol analogues with SARs that were distinct from those of the benzylisothioureas. Most of the potent IDO inhibitors also suppressed kynurenine production in A431 cells, with the exception of benzenethiol analogue **10i**. Combined with their excellent ligand efficiencies, benzylthioureas could potentially serve as lead compounds for further drug design such as structure-based drug design.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.025.

References and notes

1. Takikawa, O. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 12.
2. Munn, D. H.; Mellor, A. L. *J. Clin. Invest.* **2007**, *117*, 1147.
3. Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841.
4. Botting, N. P. *Chem. Soc. Rev.* **1995**, *24*, 401.
5. Sono, M.; Hayaishi, O. *Biochem. Rev.* **1980**, *50*, 173.
6. Uyttenhove, C.; Pilotte, L.; Theate, I.; Stroobant, V.; Colau, D.; Parmentier, N.; Boon, T.; van den Eynde, B. *Nat. Med.* **2003**, *9*, 1269.
7. Guillemin, G. J.; Brew, B. J.; Noonan, C. E.; Takikawa, O.; Cullen, K. M. *Neuropath. Appl. Neurobiol.* **2005**, *31*, 395.
8. Fujigaki, H.; Saito, K. *Int. Congr. Ser.* **2007**, *1304*, 314.
9. Potula, R.; Poluektova, L.; Knipe, B.; Chrastil, J.; Heilman, D.; Dou, H.; Takikawa, O.; Munn, D. H.; Gendelman, H. E.; Persidsky, Y. *Blood* **2005**, *106*, 2382.
10. Munn, D. H.; Shafizadeh, E.; Attwood, J. T.; Bondarev, I.; Pashine, A.; Mellor, A. L. *J. Exp. Med.* **1999**, *189*, 1363.
11. Okamoto, A.; Nikaido, T.; Ochiai, K.; Takakura, S.; Saito, M.; Aoki, Y.; Ishii, N.; Yanaihara, N.; Yamada, K.; Takikawa, O.; Kawaguchi, R.; Isonishi, S.; Tanaka, T.; Urashima, M. *Clin. Cancer Res.* **2005**, *11*, 6030.
12. Brandacher, G.; Perathoner, A.; Ladurner, R.; Schneeberger, S.; Orbist, P.; Winkler, C.; Werner, E. R.; Werner-Felmayer, G.; Weiss, H. G.; Gobel, G.; Margreiter, R.; Konigsrainer, A.; Fuchs, D.; Amberger, A. *Clin. Cancer Res.* **2006**, *12*, 1144.
13. Cady, S. G.; Sono, M. *Arch. Biochem. Biophys.* **1991**, *291*, 326.
14. Peterson, A. C.; Migawa, M. T.; Martin, M. J.; Hamaker, L. K.; Czerwinski, K. M.; Zhang, W.; Arend, R. A.; Fisette, P. L.; Ozaki, Y.; Will, J. A.; Brown, R. R.; Cook, J. M. *Med. Chem. Res.* **1994**, *3*, 531.
15. Muller, A. J.; Scherle, P. A. *Nat. Rev. Cancer* **2006**, *6*, 613.
16. Gaspari, P.; Banerjee, T.; Malachowski, W. P.; Muller, A. J.; Prendergast, G. C.; DuHadaway, J.; Bennett, S.; Donovan, A. M. *J. Med. Chem.* **2006**, *49*, 684.
17. Kumar, S.; Malachowski, W. P.; DuHadaway, J. B.; LaLonde, J. M.; Carroll, P. J.; Jaller, D.; Metz, R.; Prendergast, G. C.; Muller, A. J. *J. Med. Chem.* **2008**, *51*, 1706.
18. Carr, G.; Chung, M. K. W.; Mauk, A. G.; Andersen, R. J. *J. Med. Chem.* **2008**, *51*, 2634.
19. Kumar, S.; Jaller, D.; Patel, B.; LaLonde, J. M.; DuHadaway, J. B.; Malachowski, W. P.; Prendergast, G. C.; Muller, A. J. *J. Med. Chem.* **2008**, *51*, 4968.
20. Yue, E. W.; Douty, B.; Wayland, B.; Bower, M.; Liu, X.; Leffet, L.; Wang, Q.; Bowman, K. J.; Hansbury, M. J.; Liu, C.; Wei, M.; Li, Y.; Wynn, R.; Burn, T. C.; Koblish, H. K.; Fridman, J. S.; Metcalf, B.; Scherle, P. A.; Combs, A. P. *J. Med. Chem.* **2009**, *52*, 7364.
21. Zheng, X.; Koropatnick, J.; Li, M.; Zhang, X.; Ling, F.; Ren, X.; Hao, X.; Sun, H.; Vladau, C.; Franek, J. A.; Febg, B.; Urquhart, B. L.; Zhong, R.; Freeman, D. J.; Garcia, B.; Min, W.-P. *J. Immunol.* **2006**, *177*, 5639.
22. IDO activity was determined as follow. In brief, the standard reaction mixture (200 μL) contained 50 mM KPB (pH 6.5), 20 mM ascorbic acid (neutralized with NaOH and HCl), 100 μg/mL catalase, 10 μM methylene blue, 200 μM L-tryptophan, 5 nM recombinant human IDO, and DMSO solution of the compound (4 μL). The reaction was carried out at 37 °C for 60 min and stopped by the addition of 40 μL of 30% (w/v) CCl_3COOH. After heating at 50 °C for 15 min, the reaction mixture was centrifuged at 15000*g* for 5 min. The supernatant (150 μL) was transferred into a well of a 96-well microplate and mixed with 150 μL of 2% (w/v) *p*-dimethylaminobenzaldehyde in acetic acid. The yellow pigment derived from kynurenine was measured at 490 nm using a SPECTRAmax M5SK microplate reader (Molecular Devices).
23. Matter, H.; Nazaré, M.; Güssregen, S.; Will, D. W.; Schreuder, H.; Bauer, A.; Urmann, M.; Ritter, K.; Wagner, M.; Wehner, V. *Angew. Chem., Int. Ed.* **2009**, *48*, 2911.
24. Hopkins, A. L.; Groom, C. R.; Hopkins, A. A. *Drug Discovery Today* **2004**, *9*, 430.

25. Kynurenine production in A431 cells was determined as follows. In brief, A431 cells (2.0×10^5 cells/mL) were seeded in a 96-well culture plate (100 μL/well) and grown overnight. Serial DMSO dilutions of compounds in a total volume of 100 μL culture medium including tryptophan and human IFN-γ (5 ng/mL final concentration) per well were added into wells containing the cells. After an additional 24 h of incubation, 200 μL/well of a mixed solution of 7% (v/v) aqueous CCl_3COOH and 2% (w/v) *p*-dimethylaminobenzaldehyde in acetic acid (2:5) was added into each well. The yellow color derived from kynurenine was measured at 460 nm using a SPECTRAmax M5SK microplate reader (Molecular Devices).
26. A431 cells were precultured for 24 h. The medium was replaced with fresh medium and the cells were stimulated with or without IFN-γ (5 ng/ml) and the compound **3d** was treated for 24 h. After the treatment, culture media and cell extracts were collected, IDO and α-tubulin were detected by western blot analysis.
27. Hou, D.-Y.; Muller, A. J.; Sharma, M. D.; DuHadaway, J.; Banerjee, T.; Johnson, M.; Mellor, A. L.; Prendergast, G. C.; Munn, D. H. *Cancer Res.* **2007**, *67*, 792.
28. Lu, C.; Lin, Y.; Yeh, S.-R. *J. Am. Chem. Soc.* **2009**, *131*, 12866.
29. Sono, M.; Cady, S. G. *Biochemistry* **1989**, *28*, 5392.
30. Sugimoto, H.; Oda, S.-i.; Otsuki, T.; Hino, T.; Yoshida, T.; Shiro, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 2611.