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Letter
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Discovery of Novel Indoleamine 2,3-Dioxygenase 1 (IDO1) and Histone Deacetylase (HDAC) Dual Inhibitors

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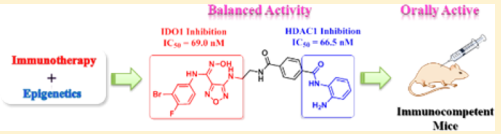
Supporting Information

ABSTRACT: In order to take advantage of both immunotherapeutic and epigenetic antitumor agents, the first generation of dual indoleamine 2,3-dioxygenase 1 (IDO1) and histone deacetylase (HDAC) inhibitors were designed. The highly active dual inhibitor **10** showed excellent and balanced activity against both IDO1 (IC_{50} = 69.0 nM) and HDAC1 (IC_{50} = 66.5 nM), whose dual targeting mechanisms were validated in cancer cells. Compound **10** had good pharmacokinetic profiles as an orally active antitumor agent and significantly reduced the L-kynurenine level in plasma. In particular, it showed excellent *in vivo* antitumor efficacy in the murine LLC tumor model with low toxicity. This proof-of-concept study provided a novel strategy for cancer treatment. Compound **10** represents a promising lead compound for the development of novel antitumor agents and can also be used as a valuable probe to clarify the relationships and mechanisms between cancer immunotherapy and epigenetics.



KEYWORDS: *IDO1, HDAC, dual inhibitors, cancer immunotherapy, epigenetics, antitumor efficacy*

In the past two decades, immune checkpoint therapy has achieved important clinical advances for the treatment of cancer.[1] Rather than targeting the tumor cells directly, cancer immunotherapy acts by activating T cells to enhance patients' native immune response. The immune checkpoint cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibody ipilimumab and programmed death (PD-1) antibodies pembrolizumab and nivolumab were approved by U.S. Food and Drug Administration (FDA).[1,2] As compared to antibody drugs, small-molecules for cancer immunotherapy have remarkable advantages, such as oral administration, access to intracellular targets, and greater drug exposure within the tumor microenvironment. Therefore, there is an urgent need to develop small molecules to modulate the immune system and fight against cancer.[3]

Indoleamine 2,3-dioxygenase 1 (IDO1), an extrahepatic heme-containing dioxygenase, is capable of catalyzing the conversion of L-tryptophan (Trp) to *N*-formylkynurenine (NFK) in the first rate-limiting step of the kynurenine pathway (KP).[4,5] NFK is then metabolized to L-kynurenine (Kyn) and subsequent bioactive metabolites.[4] The tryptophan depletion results in inhibiting the proliferation of T lymphocytes, which are sensitive to low Trp levels. The production of KP metabolites can enhance immune tolerance by activating the aryl hydrocarbon receptor (AhR). Both of them contribute to the immunosuppressed state of the tumor microenvironment.[6,7] In addition, numerous evidence indicated that elevated levels of IDO1 expression in both tumor cells and antigen-presenting cells were correlated with poor prognosis and reduced survival.[8,9] Given the important role in tumor immune escape, IDO1 represents a valuable therapeutic target in cancer immunotherapy. A number of potential small-molecule inhibitors of IDO1 have been disclosed, among which D-1-MT (**1**), INCB024360 (**2**), and GDC-0919 (**3**) have entered clinical trials (Figure 1).[10−12]

IDO1 inhibitors control and eradicate the growth of tumor cells by enhancing antitumor immune responses. However, a number of preclinical studies revealed that IDO1 inhibitors only exhibited moderate antitumor activity when used as single agents.[8,13] Indeed, preclinical and clinical data indicated that IDO1 inhibitors are generally developed as combination therapies with cytotoxic antitumor agents, radiotherapy, therapeutic vaccination, and PD-1 antibodies.[8,13,14] For example, compound **2**, an orally active and competitive IDO1 inhibitor, is currently evaluated in phase III clinical trials for the treatment of multiple tumor types in combination with immune checkpoint inhibitors.[15] Despite the synergistic effects observed by the combination of IDO1 inhibitors with other therapies, drug combination strategies are always limited by complex pharmacokinetics and drug−drug interactions.[16] To overcome the problem, designing a single agent that simultaneously

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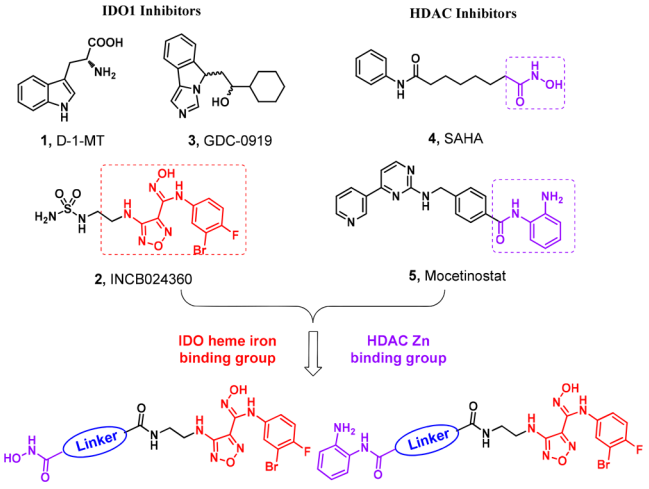


Figure 1. Chemical structures of IDO1 inhibitors and HDAC inhibitors and design of dual IDO1 and HDAC inhibitors.

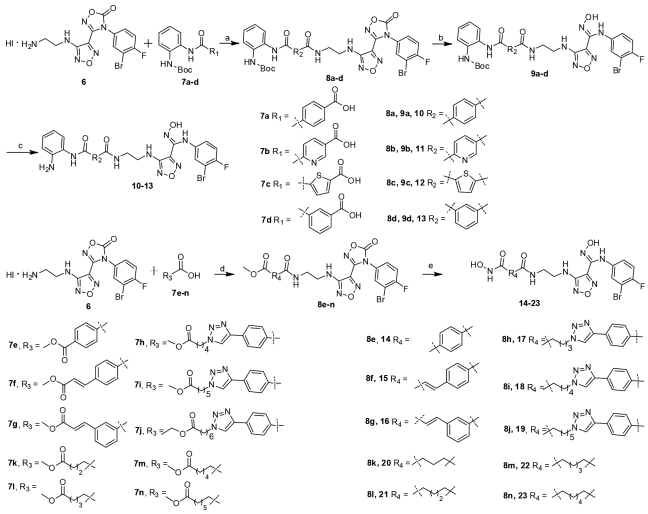
targets two or more synergistic mechanisms has attracted great interests.[17,18]

Histone deacetylases (HDACs) are a family of enzymes, which catalyze the deacetylation of the lysine residues at the amino terminal of histones.[19] HDAC inhibitors (HDACIs) can induce cell cycle arrest, differentiation, and apoptosis by blocking abnormal HDAC deacetylation.[20] Several HDACIs, such as SAHA (**4**) and Mocetinostat (**5**), have been approved for the treatment of various hematological malignancies.[21] Importantly, recent studies indicated that HDACIs could also improve tumor recognition and reverse immune suppression via various mechanisms.[22,23] Therefore, the discovery of dual IDO1 and HDAC inhibitors may provide a novel strategy for cancer treatment by taking advantages of both immunotherapeutic and epigenetic drugs. Herein, the first dual IDO1 and HDAC inhibitors were designed and evaluated. Interestingly, a highly potent inhibitor with balanced activity against IDO1/HDAC was successfully identified, which was orally active and showed excellent *in vivo* antitumor potency.

The dual IDO1 and HDAC inhibitors were designed by a pharmacophore fusion strategy. IDO1 inhibitor **2** and HDACIs **4**[24] and **5**[25] were used as the templates for drug design. Binding mode analysis of the docked conformation of compound **2** (Figure S1 in Supporting Information) revealed that the oxygen of the hydroxyamidine bound to heme iron in the active site (pocket A) of IDO1, which was identified as a crucial functional group for IDO1 inhibition.[26] The aminoethyl-sulfamide substituent projected out of the active site toward solvent (pocket B),[26] which could be modified without impairing IDO1 binding affinity. Thus, the zinc binding functional group (hydroxamic acid or benzamide) that is essential for HDAC inhibition can be introduced on the IDO heme binding scaffold directly or via a proper spacer (Figure 1). As a result, a series of novel IDO1 and HDAC dual targeting molecules were designed, synthesized, and assayed.

The preparation of target compounds **10**–**23** are shown in Scheme 1. Amidation of protected and substituted carboxylic acids **7a**–**d** with hydroiodide salt intermediate **6**, using HATU (1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate) as the coupling reagent, led to intermediates **8a**–**d**. After alkaline cleavage with NaOH and removal of Boc protecting group with trifluoroacetic acid, benzamide analogues **10**–**13** were

Scheme 1. Synthesis of Compounds 10–23[a]



[a]Reagents and conditions: (a) HATU, DIPEA, DMF, rt, overnight, yield 49–59%; (b) NaOH, MeOH, rt, 2 h, yield 71–85%; (c) CF_3COOH, DCM, 40 °C, 3 h, yield 62–77%; (d) HATU, DIPEA, DMF, rt, overnight, yield 53–85%; (e) NH_2OH-HCl, KOH, MeOH, rt, 2 h, yield 43–83%.

obtained. Amide condensation of intermediate **6** with 4-(methoxycarbonyl)benzoic acid **7e**, 2-methoxycarbonylvinyl benzoic acids **7f**–**g**, 4-phenyl-1*H*-1,2,3-triazol carboxylic acids **7h**–**j**, or aliphatic carboxylic acids **7k**–**n** gave esters **8e**–**n**, which were converted to corresponding hydroxamic acids **14**–**23** using hydroxylamine hydrochloride in the presence of KOH. Intermediates **6** and **7a**–**n** were prepared as outlined in Schemes S1 and S2 (Supporting Information).

Initially, HDAC1 and IDO1 were used as the primary assays with compounds **2** and **4** as the reference drugs. Then, pan-HDAC activity assay was performed for a representative compound to investigate whether it is a pan-HDAC inhibitor or a selective inhibitor. As shown in Table 1, the designed compounds generally showed potent inhibitory activity against both targets. The linkers between HDAC1 Zn binding group and IDO1 heme binding scaffold played an important role in enzyme inhibition. Compound **10** containing the benzamide-*N*-phenylamine group showed excellent activity toward both IDO1 (IC_{50} = 69.0 nM) and HDAC1 (IC_{50} = 66.5 nM). Replacement of the phenyl group in compound **10** to pyridine (compound **11**) and thiophene (compound **12**) or changing the position of amide (compound **13**) resulted in the decreased activity against both targets. When the phenylamine group was replaced by hydroxamic acid, compound **14** showed decreased activity against both targets. Interestingly, the insertion of a double bond between the hydroxamic acid and phenyl group in compound **15** led to the improved activity against both targets. Compounds **15** (IC_{50} = 46.2 nM) and **16** (IC_{50} = 70.5 nM) were highly active against HDAC1. In particular, compound **16** was the most active IDO1 inhibitor (IC_{50} = 27.0 nM), which was about 3-fold more potent than compound **2**. Triazole is widely used as the linker in HDAC-based multitargeting antitumor agents.[27,28] Our results also showed that the 1,2,3-triazol derivatives **17**–**19** with the increasing of the carbon chain length exhibited better inhibitory activities against HDAC1, which were consistent with the docking results (Table S1 and Figure S2 in Supporting Information). The order of influence of carbon chain length on HDAC1 inhibition

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Table 1. HDAC1 and IDO1 Inhibitory and Antiproliferative Activities of the Target Compounds

compds	HDAC1 IC_{50} (nM)[a]	IDO-1 IC_{50} (nM)[a] or % inhibition at 1 μM	LLC (IC_{50}, μM)	CT-26 (IC_{50}, μM)	A549 (IC_{50}, μM)	HCT-116 (IC_{50}, μM)	HT-29 (IC_{50}, μM)
2	NT[b]	77.8 ± 6.4	>100	>100	>100	>100	>100
4	14.4 ± 3.8	NT[b]	9.68 ± 0.15	5.97 ± 0.85	2.63 ± 0.54	3.07 ± 0.55	1.78 ± 0.89
10	66.5 ± 3.3	69.0 ± 7.1	17.62 ± 1.06	59.84 ± 8.51	16.73 ± 2.12	5.12 ± 0.43	11.71 ± 1.54
11	604.4 ± 44.2	260.3 ± 13.5	15.13 ± 1.85	23.3 ± 4.35	20.65 ± 5.66	6.36 ± 1.25	12.24 ± 3.11
12	1429.5 ± 325.6	76%	18.34 ± 4.21	38.82 ± 7.71	14.52 ± 3.27	7.12 ± 1.72	20.26 ± 6.07
13	632.7 ± 53.8	79%	31.38 ± 5.67	25.51 ± 4.35	27.76 ± 7.73	16.18 ± 3.12	46.42 ± 9.43
14	262.4 ± 16.7	95%	>100	>100	89.79 ± 6.99	28.08 ± 6.44	68.01 ± 7.19
15	46.2 ± 5.9	167.9 ± 8.7	21.64 ± 2.16	12.79 ± 3.13	25.56 ± 6.84	5.89 ± 0.54	14.15 ± 2.01
16	70.5 ± 3.8	27.0 ± 3.5	53.30 ± 8.97	38.94 ± 7.56	41.66 ± 8.46	12.44 ± 2.53	23.31 ± 4.12
17	894.8 ± 93.6	88%	>100	>100	>100	37.53 ± 7.59	>100
18	66.5 ± 4.9	87%	>100	>100	>100	>100	>100
19	23.5 ± 1.5	209.6 ± 18.7	35.95 ± 7.66	90.18 ± 5.85	45.48 ± 10.55	29.44 ± 8.12	23.88 ± 2.73
20	121.1 ± 9.1	133.0 ± 14.1	>100	>100	>100	80.24 ± 8.46	>100
21	308.1 ± 10.5	122.5 ± 10.5	>100	>100	>100	85.37 ± 7.91	>100
22	9.2 ± 0.08	113.4 ± 11.2	90.11 ± 13.05	95.46 ± 20.28	40.66 ± 9.56	17.46 ± 5.61	28.74 ± 4.84
23	47.7 ± 5.3	139.8 ± 13.3	56.58 ± 5.68	97.45 ± 15.34	36.03 ± 8.95	4.70 ± 0.38	14.88 ± 3.09

[a]IC_{50} values are the mean of at least three independent assays, presented as mean ± SD. [b]NT = not tested.

was five carbons (**22**) > six carbons (**23**) > three carbons (**20**) > four carbons (**21**). Notably, compound **22** was the most potent HDAC1 inhibitor (IC_{50} = 9 nM). However, no improvement of the IDO1 inhibitory was observed for these compounds with a long linker.

Given the potent enzyme inhibitory activities, we further evaluated the antiproliferative activities of the IDO1/HDAC dual inhibitors against LLC (lewis lung cancer), CT-26 (mouse colon cancer), A549 (human lung cancer), HCT-116 (human colon cancer), and HT-29 (human colon cancer) cell lines by the CCK8 (Cell Counting Kit-8) assay. Compounds **2** and **4** were used as the positive controls. As shown in Table 1, HDAC inhibitor **4** was active in the low micromolar range, whereas compound **2** was inactive against the five solid tumor cell lines because IDO1 inhibitors do not destroy tumor cells directly. Generally, the target compounds showed modest to good antitumor activities. Among the tested cell lines, the dual inhibitors were more active against the HCT-116 cell line. Particularly, compounds **10** (IC_{50} = 5.12 μM), **15** (IC_{50} = 5.89 μM), and **23** (IC_{50} = 4.70 μM) exhibited comparable antitumor activity to compound **4** (IC_{50} = 3.07 μM) in the HCT-116 cell line.

To clarify whether the inhibition in cell growth is associated with apoptosis, HCT-116 cells were treated with DMSO or various concentrations of compounds **10**, **15**, and **23** for 48 h. The cells were stained with Annexin-V and propidium iodide (PI), and the apoptotic ratio was determined by flow cytometry. As shown in Figure 2A,B, the percentage of apoptotic cells for compound **15** was 26.7% (5 μM), 45.4% (10 μM), and 61.3% (20 μM), respectively. The ability of compound **15** to induce apoptosis was stronger than that of compounds **10** (18.8%, 29.9%, and 48.9%) and **23** (25.5%, 31.1%, and 44.5%). These results demonstrated that dual IDO1 and HDAC inhibitors induced HCT116 cells apoptosis in a dose-dependent manner.

To investigate the effect of compounds on the various phases of cell cycle, HCT116 cells were treated with 10 μM of compounds **10**, **15**, and **23** for the indicated time interval and analyzed by flow cytometry (Figure 2C,D). Compared with the DMSO control, the cell cycle showed all the compounds arrested HCT116 mainly in G2/M phase. Compound **10** also

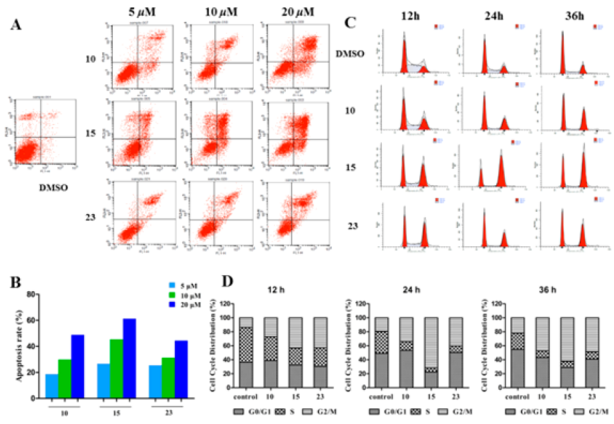

Figure 2. Compounds **10**, **15**, and **23** induce cell apoptosis and cycle arrest. (A,B) Apoptotic index analysis at different concentrations in HCT116 cells. (C,D) Time-dependent effects on cell cycle progression. The cell cycle and the proportions are shown in each phase of HCT116 cells treated with or without compounds **10**, **15**, and **23**.

induced the increase of G2/M-phase cells (27.2% to 47.4%) in a time-dependent manner. Compound **10**, with the balanced inhibitory activity against HDAC1 (IC_{50} = 66.5 nM, Figure 3A) and IDO1 (IC_{50} = 69.0 nM, Figure 3B) and excellent apoptosis inducing activity, was selected for cellular mechanism and *in vivo* studies.

In order to evaluate the IDO1 inhibitory activity of compound **10** under the cellular environment (EC_{50}), a HeLa cell-based assay[29] measuring the Kyn was performed (Figure 3C). Moreover, its cytotoxic activity (LC_{50}) against HeLa cells was also assayed (Figure 3D). Compound **10** had an EC_{50} and LC_{50} value of 0.41 and 24.77 μM, respectively, with a LC_{50}/EC_{50} ratio of 60.4. These results suggested that compound **10** effectively inhibited IDO1 activity in HeLa cells and that the cell-based IDO1 activity was not caused by the cytotoxicity. In order to investigate whether compound **10** inhibits HDAC in cancer cells, we evaluated its effect on the acetylation of histone H3 using the Western blot assay. After the incubation with compound **10** or compound **4** in HCT116 cells for 12 h, the acetylation level of histone 3 was elevated in a dose-dependent

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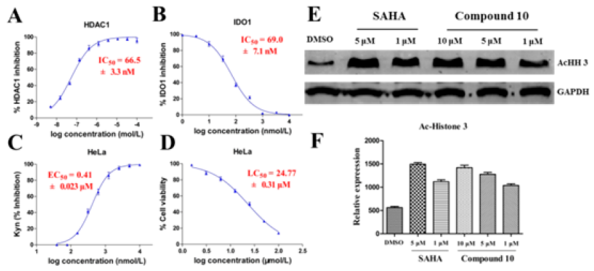

Figure 3. Compound **10** inhibits IDO1 and HDAC1 *in vitro*. (A,B) Concentration−effect curves for IDO1 and HDAC1 enzyme inhibitory activity of compound **10**. (C) Compound **10** effectively inhibits Kyn production in IFN-γ treated human HeLa cells. (D) Cytotoxic effect of compound **10** on HeLa cells. (E) Western blot analysis of acetylated histone H3 after 12 h treatment with compound **10** in HCT116 cells. (F) Quantification of HDAC inhibitory responses by densitometry. Data points represent a mean ± SD of biological triplicates from a representative experiment.

manner (Figure 3E,F), indicating that it could inhibit HDAC in cells. To explore the inhibitory activity toward other HDAC isoforms, compound **10** was tested for its inhibitory activity of HDAC2, HDAC3, HDAC4, HDAC6, and HDAC8 (Table S2 in Supporting Information). Compound **10** displayed nanomolar activity against HDAC2 (IC_{50} = 179 nM), HDAC3 (IC_{50} = 45 nM), and HDAC6 (IC_{50} = 70 nM). The results indicated that compound **10** was a pan-HDAC inhibitor at the molecular level. In male mouse liver microsome, compound **10** had a half-life of 48.37 min with apparent clearance of 0.0287 mL/min/mg (Figure S2 in Supporting Information).

Inspired by the excellent IDO1/HDAC inhibition of compound **10**, it was subjected for *in vivo* pharmacokinetic evaluation in male Sprague−Dawley (SD) rats. Compound **10** was administered in a single intravenous (iv) dose of 2 mg/kg or an oral dose of 100 mg/kg, and the main pharmacokinetic parameters are listed in Table 2. It demonstrated good oral drug exposure ($AUC_{(0\text{-inf})}$ = 33534 ± 225 ng/mL × h) with oral bioavailability of 18%, suggesting that oral administration would be a suitable dosing route for further pharmacodynamic study (Table 2). Inhibition of IDO1 activity *in vivo* could reduce the plasma Kyn levels. We observed that treatment of wild-type female C57BL/6 mice with compound **10** (100 mg/kg) significantly decreased the plasma Kyn levels between 3−8 h (Figure 4). Similarly, compound **2** decreased plasma Kyn levels within 1 h and the levels remained at least 50% of suppression at 8 h. Taken together, the results indicated that compound **10** was an orally active agent and able to effectively suppress tryptophan catabolism in mice. Moreover, compound **10** was stable in the PBS buffer after 24 h at room temperature, but its water solubility (<0.01 g/L) remains to be improved.

Compound **10** was further investigated for its *in vivo* antitumor efficacy in a LLC tumor growth model in immunocompetent mice. Compound **10** (100 mg/kg, bid), compound **2** (100 mg/kg, bid), and compound **4** (100 mg/kg, qd) were dosed orally by gavage for 14 consecutive days. As shown in Figure 5, treatment with compound **10** showed good

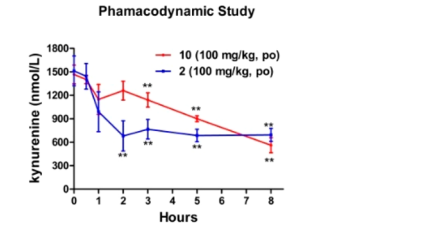

Figure 4. Compound **10** efficiently suppresses Kyn in wild-type mice. Wild-type female C57BL/6 mice were orally administrated compound **10** or **2** at 100 mg/kg, and blood was harvested at the indicated times. Average values from the plasma for three mice (±SD), analyzed for Kyn levels by LC/MS/MS, are shown. All data between 0.5 and 8 h for effects in wild-type mice are statistically significant (**, $P < 0.01$).

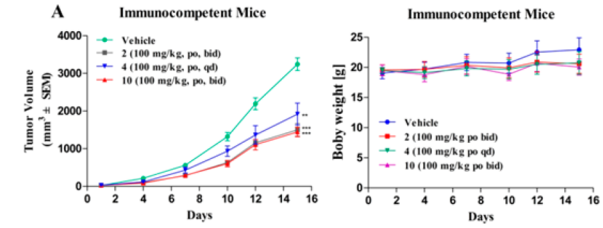

Figure 5. Antitumor efficacy of compound **10** in murine LLC tumor model. (A) Mean tumor volumes (mm^3) ± SEM (n = 6 mice/group) are shown from the initiation of dosing (~100 mm^3). The statistical difference was determined by Student's t test. **$P < 0.01$, ***$P < 0.001$ compared with the control group. (B) Body weights were measured three times per week, and data are presented as the mean (g) ± SD.

tumor growth control (TGI = 56.0%) without significant changes in body, which was comparable to compound **2** (TGI = 54.3%) and superior to compound **4** (TGI = 41.4%). From the pharmacokinetic data, compound **2** achieved higher exposure (AUC 70.2 μM·h vs 60.0 μM·h, calculated from ng/mL·h) and a longer half-life (4.5 h vs 3.4 h) than compound **10**, which may explain why compound **10** did not exhibit higher *in vivo* antitumor effect than compound **2**. Thus, further structural optimization of dual IDO1/HDAC inhibitor **10** is required to improve the *in vivo* antitumor efficacy.

In summary, the first dual IDO1/HDAC inhibitors were designed and evaluated. Compound **10**, a highly active dual inhibitor, was successfully identified, which showed balanced activity against both IDO1 and HDAC1. It significantly induced the apoptosis in the HCT116 cells with a G2/M cell cycle arrest. Compound **10** had acceptable pharmacokinetic profiles as an orally active antitumor agent and significantly reduced Kyn levels in plasma over an 8 h period. It showed good *in vivo* antitumor efficacy in the murine LLC tumor model with low toxicity. This proof-of-concept study provided a novel strategy for the development of novel antitumor agents. Compound **10**

Table 2. Pharmacokinetic Profiles of Compound 10 in Sprague−Dawley Rats[a]

compds	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{(0\text{-inf})}$ (ng/mL·h)	F (%)
2 (Po)	5560 ± 967	0.8	4.5 ± 0.8	30747 ± 785	
10 (Po)	5765 ± 1276	2.0	3.4 ± 0.1	33534 ± 225	18
10 (Iv)	12039 ± 1762	0.083	1.4 ± 0.05	3646 ± 201	

[a]Data are presented as mean ± SD.

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represents a promising lead compound for drug development. It can also be used as a valuable probe to clarify the relationships and mechanisms between cancer immunotherapy and epigenetics. Also, it should be noted that structural optimization of compound **10** and the synergistic effects of dual IDO1/HDAC inhibitors with immune checkpoint inhibitors (e.g., PD-1 antibodies) remain to be further investigated. Such studies are in progress in our lab.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00487.

Chemical synthesis and structural characterization of the target compounds; protocols of biological assays (PDF)

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Author Contributions

‖K.F., G.D., and Y.L. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed death; IDO1, indoleamine 2,3-dioxygenase 1; Kyn, L-kynurenine; Trp, L-tryptophan; NFK, *N*-formylkynurenine; AhR, aryl hydrocarbon recepto; HDACs, histone deacetylases; TGI, tumor growth inhibition; HATU, 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate; DIPEA, *N*,*N*-diisopropylethylamine; PBS, phosphate buffer saline

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