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Discovery of IDO1 and DNA Dual Targeting Antitumor Agents

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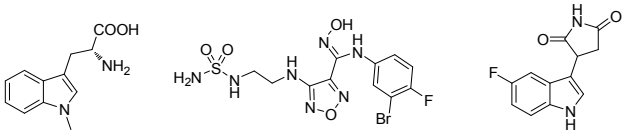
The development of small molecules for cancer immunotherapy is highly challenging and indoleamine 2,3-dioxygenase 1 (IDO1) represents a promising target. Inspired by the synergistic effects between IDO1 inhibitors and traditional antitumor chemotherapeutics, the first orally active dual IDO1 and DNA targeting agents were designed by the pharmacophore fusion strategy. The bifunctional hybrids exhibited enhanced IDO1 enzyme inhibitory activity and *in vitro* cytotoxicity as compared to IDO1 inhibitor 1-methyl-tryptophan and DNA alkylating agent melphalan. In a murine LLC tumor model, the dual targeting agents demonstrated excellent antitumor efficacy, highlighting the advantages of this novel design strategy to improve the efficacy of small molecule cancer immunotherapy.

Immune checkpoint therapy has become a new weapon for clinical treatment of cancer.[1] The approach does not aim to attack particular targets in the tumor cells, but rather to affect T cells to enhance antitumor immune responses.[1] Currently, there are a number of immune checkpoint inhibitors under clinical investigation, such as programmed death (PD-1), cytotoxic T-lymphocyte antigen CTLA, T-cell immunoglobulin and mucin 3 domain (TIM3), and indoleamine 2,3-dioxygenase 1 (IDO1). Three immune checkpoint antibodies (ipilimumab, pembrolizumab and nivolumab) have been approved for the treatment of melanoma and achieved great success in clinic.[1, 2] As compared to antibodies, the small molecule immunotherapy has remarkable advantages such as oral administration and suitable pharmacokinetic profiles. IDO1 is a monomeric heme-containing enzyme which catalyzes the degradation of *L*-tryptophan (Trp) in the initial rate-limiting step of the kynurenine (Kyn) pathway.[3, 4] The depletion of Trp can impede T lymphocytes proliferation and increase their susceptibility to apoptosis.[5, 6] Subsequently, accumulation of toxicity metabolites, including Kyn, kynurenic acid, 3-hydroxy-kynurenine, and quinolinic acid, can also increase the apoptosis of both T helper 1 (T_H1) lymphocytes and natural killer (NK) cells.[7, 8] Ultimately, the combined effects of the two mechanisms result in cancer immune escape in tumor microenvironment.

Numerous studies indicated that low IDO1 activity was observed in most tissues.[9] In contrast, increased IDO1 expression was found in various cancer types, such as colorectal cancer, pancreatic cancer, non-small cell lung cancer and glioblastoma.[4, 10] Moreover, up-regulation of IDO1 in tumor cells is correlated with poor prognosis and reduced survival.[11-13] Thus, IDO1 has attracted great interests as a potential cancer immunotherapy target and a number of IDO1 inhibitors have been reported.[14-17] Five of them, namely indoximod (D-1-MT, **1**), INCB024360 (**2**), PF-06840003 (**3**), GDC-0919 (structure not disclosed) and F001287 (structure not disclosed), are in the stage of clinical trials (**Fig. 1**). The racemic 1-methyl-tryptophan (**4**, 1-MT) was first identified as a competitive IDO1 inhibitor in 1991.[18] Interestingly, the *R*-enantiomer (D-1-MT) displayed less IDO1 inhibitory activity but higher *in vivo* antitumor activity than the *S*-enantiomer (L-1-MT).[19] Although the detailed mechanisms of D-1-MT are still poorly understood, it is now evaluated as a clinical candidate in combination with traditional cancer chemotherapies.[20]



1 Indoximod **2** INCB024360 **3** PF-06840003

Fig. 1 Chemical structures of IDO1 inhibitors in clinical trials.

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From a mechanistic point of view, IDO1 inhibitors can kill cancer cells by restoring immune recognition and enhancing T cell immune responses.[21] As a single agent, IDO1 inhibitors generally exhibited modest antitumor activities in preclinical studies.[4, 22] Therefore, the combination of IDO1 inhibitor-based immunomodulation with chemotherapy, radiotherapy and/or immunotherapy, may be a promising strategy against an array of human cancers.[23, 24] Numerous studies have suggested that the efficacy of traditional cancer chemotherapies were improved by concomitant administration of an IDO1 inhibitor.[22, 25] In mouse tumor models, 1-MT displayed remarkable synergism with various chemotherapeutic (*e.g.* platinum compounds, taxane derivatives and cyclophosphamide) without increased toxicity.[22, 26]

The alkylating antitumor agents, including cyclophosphamide, chlorambucil and melphalan, are widely used as first line treatment for various cancers in clinic.[27-29] On the basis of the synergistic antitumor effects between IDO1 inhibitors and nitrogen mustards, we envisioned that dual IDO1 and DNA targeting agents might take advantages of immune checkpoint therapy and chemotherapy, which could enhance the anticancer efficacy and reduce systemic toxicity.

The anticancer ability and systemic toxicity of melphalan (**5**) is ascribed to the extent of its interstrand cross-linking with DNA. In consideration of low toxicity of aromatic nitrogen mustard, we introduced the alkylating pharmacophore *N*,*N*-bis(2-chloroethyl)-amine to the C1- or C2- position of 1-MT (**4**) using the pharmacophore fusion strategy (**Fig. 2**). As a result, two novel hybrids of 1-MT-bearing nitrogen mustards were designed and synthesized, which showed potent *in vivo* antitumor activity.

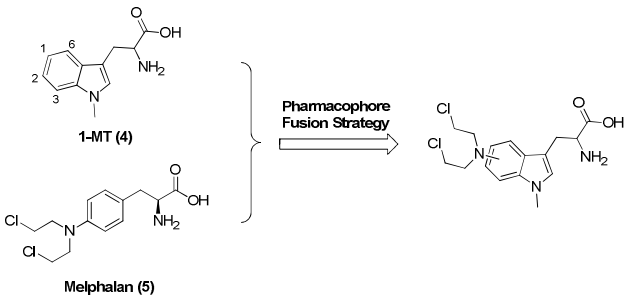
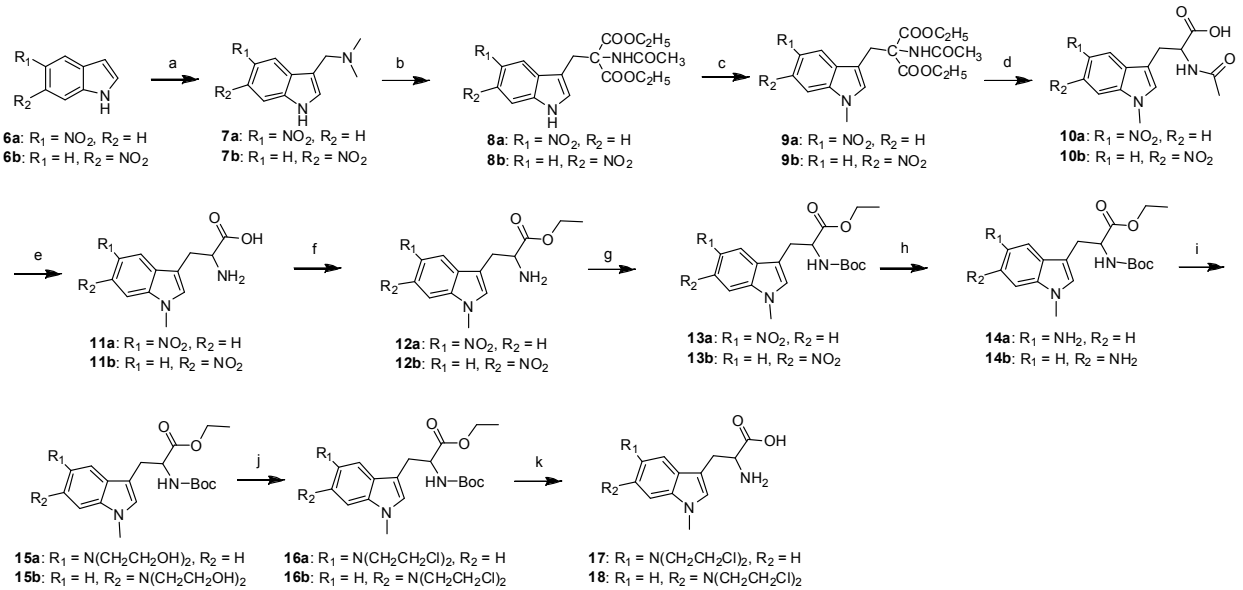


Fig. 2 Design of dual IDO1- and DNA- targeting agents.

The synthetic route of hybrid compounds **17** and **18** is outlined in **Scheme 1**. Starting from commercially available 1- and 2-nitro-1*H*-indole **6a** and **6b**, they were transformed to 5- and 6-nitrogramine **7a** and **7b** by the Mannich reaction, which reacted with diethyl acetamidomalonate in the presence of NaOH and toluene to give **8a** and **8b**. Intermediates **10a** and **10b** were obtained by monomethylation of **8a** and **8b**, affording **9a** and **9b**, which were subsequently saponifed and decarboxylated under the alkaline condition. Then, after hydrolysis under the acid condition, 5- and 6-nitro-1-methyl-tryptophan **11a** and **11b** were obtained. Followed by esterification, Boc protection and catalytic reduction, intermediates **14a** and **14b** were afforded, which were reacted with excessive ethylene oxide, and subsequently treated with $POCl_3$ in the presence of $CHCl_3$ to give nitrogen mustards **16a** and **16b**. Finally, deprotection and hydrolysis of **16a** and **16b** with 2M HCl under the reflux condition gave the target compounds **17** and **18**.

Scheme 1. Synthesis of compounds **17** and **18**[a]



[a]Reagents and Conditions: (a) Dimethylamine, formaldehyde, AcOH, 30 min, 0 °C; 3 d, rt; 15% aq. NaOH solution, 0 °C, yield 73%; (b) Diethyl acetamidomalonate, NaOH, PhMe, 110 °C, 10 h, yield 64%; (c) MeI, NaH, DMF, rt, overnight, yield 77%; (d) 4% aq. NaOH solution, EtOH, N_2, 76 °C, 6 h; (e) 6M aq. HCl solution, reflux, 10 h, two steps yield 76%; (f) $SOCl_2$, EtOH, reflux, 6 h, yield 93%; (g) $(Boc)_2O$, Et_3N, DCM, rt, overnight, yield 95%; (h) Pd/C, H_2, Ethyl acetate, rt, overnight; (i) Ethylene oxide, MeOH, rt, overnight, two steps yield 95%; (j) $POCl_3$, $CHCl_3$, 60 °C, 3 h; (k) 2M aq. HCl solution, reflux, 2.5 h, two steps yield 73-80%.

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Initially, we investigated their inhibitory efficacy against purified IDO1 enzyme (**Table 1)**. Compound **4** was a poorly active IDO1 inhibitor with an IC_{50} value of 667 μM. Interestingly, after the introduction of the nitrogen mustard substitution on the indole C5- or C6- position, the IDO1 inhibitory activity was significantly improved. Compounds **17** (IC_{50} = 14 μM) and **18** (IC_{50} = 227 μM) was 47.6–fold and 2.7–fold more active than compound **4**, respectively.

Furthermore, the hybrids as well as melphalan and 1-MT were screened for *in vitro* cytotoxicity against three cancer cell lines (LLC lewis lung cancer, CT-26 mouse colon cancer, and HCT-116 human colon cancer) with a constitutive IDO1 expression by the standard CCK8 assay. As shown in **Table 1**, compound **4** was nontoxic to the three cell lines even at a concentration of 500 μM. For LLC and HCT-116 cell lines, compounds **17** and **18** possessed stronger inhibitory efficacy than compound **5**. Compound **17** exhibited the most potent antiproliferative activity against all the cell lines with the IC_{50} values ranging from 23.7 to 47.2 μM. Thus, the nitrogen mustard substitution on 1-MT was beneficial for enhancing both IDO1 inhibitory activity and *in vitro* cytotoxicity.

Table 1. IDO1 inhibitory activities and *in vitro* antitumor activities of compounds **17** and **18** (IC_{50}, μM)[a]

Compds	rhIDO-1	LLC	CT-26	HCT116
4	667 ± 34	>500	>500	>500
5	NT	>100	21.7 ± 3.7	>100
17	14 ± 1.3	23.7 ± 3.6	37.5 ± 5.1	47.2 ± 4.6
18	227 ± 23	63.9 ± 6.2	88.5 ± 7.4	73.5 ± 8.3

[a]IC_{50} values are the mean of at least three independent assays, presented as mean ± SD.

DNA damage induced by nitrogen mustards is known to arrest the cell cycle progression mainly at the G2/M phase. To assess whether the suppression of LLC cells growth by compounds **17** and **18** is caused by a cell-cycle effect, DNA cell cycle analysis was performed (**Fig. 3**). After treating with compounds **17** (10 μM) and **18** (30 μM) for 24 h, the percentage of cells in G2 fraction increased from 7.24% to 75.37% and 79.02%, respectively, with a concomitant decrease of cells in other phases of the cell cycle.

The induction of apoptosis by the two compounds was further evaluated using an annexin V-FITC/propidium iodide (PI) binding assay. The percentages of apoptotic LLC cells were determined by flow cytometry. As shown in **Fig. 3**, compounds **17** and **18** induced LLC cells apoptosis in a concentration-dependent manner. After exposure to the various concentrations of **17** and **18** for 24 h, the percentages of apoptotic cells were 38.3% (30 μM for **17**), 55.6% (50 μM for **17**), 39.0% (80 μM for **18**) and 48.9% (100 μM for **18**), respectively.

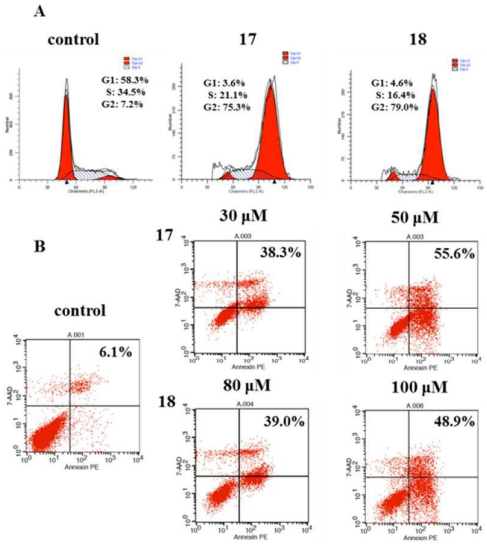

Fig. 3 Effects of compounds **17** and **18** on cell cycle (A) and cell apoptosis (B).

To investigate whether dual IDO1 and DNA targeting agents were effective *in vivo*, immunocompetent mice bearing IDO1-expressing LLC tumors were treated with 1-MT-coupled nitrogen mustards. The model was widely used to evaluate the antitumor efficacy of 1-MT.[30] Female C57BL/C mice were randomized into five groups (n = 6), and orally received the vehicle (control), compound **17** (20 mg/kg, qd), compound **18** (20 mg/kg, qd), compound **4** (400 mg/kg, bid) or compound **5** (20 mg/kg, qd) treatments. As shown in **Fig. 4A**, both compounds **17** and **18** demonstrated excellent antitumor efficacy with tumor growth inhibition (TGI) values of 63.8% and 73.5%, respectively, which was significantly more potent than compound **4** (TGI = 24.2%) and compound **5** (TGI =36.5%). Moreover, compounds **17** and **18** exhibited potent antitumor activity without significant effects on the body weight (**Fig. 4B**). Also it should be noted that compound **18** showed better *in vivo* antitumor potency than compound **17**, although it was less active *in vitro*, indicating that they might have different pharmacokinetic properties.

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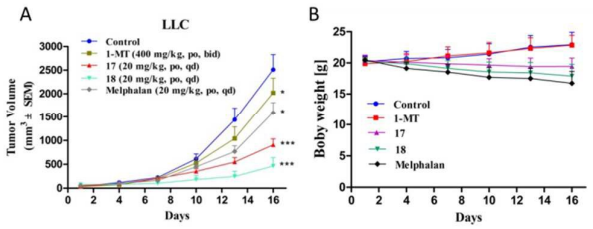

Fig. 4 Antitumor efficacy of compounds **17** and **18** in murine LLC cancer model. A, mean tumor volumes (mm^3) ± SEM (n = 6 mice/group) are shown from the initiation of dosing (~100 mm^3). Data are presented as the mean ± SEM; n = 6 mice per group: (*) $P < 0.05$, (***) $P < 0.001$, versus control group, determined with Student's t test. B, body weights were measured three times per week and data are presented as the mean (g) ± SD.

In summary, to improve the antitumor efficacy of small molecule cancer immunotherapy, dual IDO1 and DNA targeting agents were designed by pharmacophore fusion of IDO1 inhibitor 1-MT and DNA alkylating nitrogen mustard. The two bifunctional anticancer agents showed higher IDO1 inhibitory potency than 1-MT, and better antiproliferative activities than melphalan. They could induce cell death by apoptosis with G2 cell cycle arrest. Particularly, hybrids **17** and **18** were orally active and exhibited significantly higher antitumor potency than 1-MT and melphalan. Taken together, our findings highlighted the advantages of the development of small molecules simultaneously targeting IDO1 and DNA to improve the therapeutic effects of immune-chemotherapy. Further lead optimization as well as asymmetric synthesis of the isomers is in progress in our lab.

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Conflicts of interest

There are no conflicts to declare.

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