Research paper

Synthesis and biological evaluation of new berberine derivatives as cancer immunotherapy agents through targeting IDO1

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A B S T R A C T

To discover small-molecule cancer immunotherapy candidates through targeting Indoleamine 2,3-dioxygenase 1 (IDO1), twenty-five new berberine (BBR) derivatives defined with substituents on position 3 or 9 were synthesized and examined for repression of IFN-γ-induced IDO1 promoter activities. Structure–activity relationship (SAR) indicated that large volume groups at the 9-position might be beneficial for potency. Among them, compounds 2i, 2l, 2n, 2o and 8b exhibited increased activities, with inhibition rate of 71–90% compared with BBR. Their effects on IDO1 expression were further confirmed by protein level as well. Furthermore, compounds 2i and 2n exhibited anticancer activity by enhancing the specific lysis of NK cells to A549 through IDO1, but not cytotoxicity. Preliminary mechanism revealed that both of them inhibited IFN-γ-induced IDO1 expression through activating AMPK and subsequent inhibition of STAT1 phosphorylation. Therefore, compounds 2i and 2n have been selected as IDO1 modulators for small-molecule cancer immunotherapy for next investigation.

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1. Introduction

Over the past few years, the cancer immunotherapy has made great progress, which shows significant efficacy in human solid tumors, with several drugs approved by the FDA, such as programmed cell death protein 1 (PD-1) antibody Keytruda® and Opdivo® [1,2]. Clinical benefit of these antibodies as single agent, however, has been limited to some factors such as a subset of patients, high cost, difficulty to generate a decent amount and efficacy not for all tumor types. These limitations call for the development of rational combination strategies or small-molecule therapeutics aiming to extend therapeutic benefit to a broader range of patients [3]. Meanwhile, as promising immunotherapy candidates, Indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors including Epacadostat and Indoximod have already entered clinic trial. Especially, combination of small-molecule IDO inhibitors with PD-1 antibodies could significantly improve the objective response rate (ORR) in specific tumor treatment [4]. Phase I/II results showed that Epacadostat and Keytruda combination demonstrated an ORR of 53% (10 out of 19 patients) and a disease control rate of 74% (15 out of 19 patients) across multiple malignancies [5]. Since it is a single-chain catalytic enzyme with a well-defined biochemistry [6], IDO1 is considered to be an attractive target for small-molecule immunotherapeutics development. Therefore, it is a promising therapeutic strategy to develop small-molecule IDO1 inhibitors or modulators and provide new components for cancer immunotherapy combination.

A number of pathways are related to IDO1 expression [7], and IFN-γ is the major inducer of IDO1 expression. In an effort to discover and explore novel small-molecule immunotherapeutics through targeting IDO1, the high-throughput screening model on inhibition of IFN-γ-induced IDO1 promoter activity was then established in our laboratory. Given its inherent sensitivity, large signal dynamics, and simple set up, the reporter assay platform has been used as a high-throughput homogenous assay for screening IDO1 modulators in this study. Then, a library of natural products constructed in our lab was screened for their IDO1 regulating ability. Luckily, berberine (BBR, Fig. 1), as a Traditional Chinese Medicine used in China for decades against diarrhea, exhibited a moderate potency with inhibition rate of 17% at 10 μM [8–12]. The

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unique isoquinoline skeleton of BBR evokes our great interest to carry out structure–activity relationship (SAR) so as to discover small-molecule cancer-immunotherapy candidates targeting IDO1. Therefore, a series of novel BBR derivatives, including esters, amides and sulfonates on positions 3 and 9 as depicted in Fig. 1, were prepared and evaluated for their IDO1 effects, as well as primary mode of mechanism of the representative compounds.

2. Results and discussion

2.1. Synthesis

A total of twenty-five new BBR derivatives were designed and semi-synthesized as displayed in Schemes 1–3, taking commercial available BBR, palmatine (PMT) or jatrorrhizine (JTH) as the starting material respectively. As shown in Scheme 1, BBR esters 2a–q and sulfonate 3a were obtained by esterification and sulfonation of compound 1 using previously reported methods [13,14]. The key intermediate 4 was prepared with 2,4-dimethoxyaniline as the nucleophilic reagent as well as the solvent. Another key intermediate 5 with a free amine group was then acquired, and HCl/CH3OH was selected to remove 2,4-dimethoxybenzyl in 80% yield. The desired products 6a–c were obtained by amidation with corresponding acyl chloride using pyridine as the base with the yields of 33–37%.

As described in Scheme 2, compounds 8a and 8b were prepared through de-methylation and esterification from PMT using the procedures reported previously in overall yields of 25–33%. Similarly, as depicted in Scheme 3, the desired products 9a and 9b were obtained via esterification of JTH by 33–34% yields. All the final products were purified via flash column chromatography using CH3OH/CH2Cl2 as the gradient eluent (see Table 1).

2.2. Pharmacological evaluation

2.2.1. SAR for down-regulated IFN-γ-induced IDO1 promoter activity

Thus, we screened all the newly-synthesized BBR analogues for their IDO1 regulating abilities by IDO1 promoter reporter assay. A549 cells were transfected with a pGL4-IDO1-luc vector and the IDO1 regulating abilities by IDO1 promoter reporter assay. A549 cells were transfected with a pGL4-IDO1-luc vector and the IDO1 promoter activity, and their activities almost vanished Xiang-g. Wang, et al., Synthesis and biological evaluation of new berberine derivatives as cancer immunotherapy agents through targeting IDO1, European Journal of Medicinal Chemistry (2017), https://doi.org/10.1016/j.ejmech.2017.10.078
treated with the indicated compounds with or without IFN-γ for 16 h. Cells were washed and then NK cells were co-cultured with A549 cells at 10:1, NK killing activity was assessed by LDH assay (Fig. 5A) and cell impedance assay (Fig. 5B), respectively. As shown in Fig. 5A, compounds 2i, 2n and 8b significantly enhanced the specific lysis of NK cells to A549 cells. In consideration of the cytotoxic effects of the three compounds, we choose compounds 2i and 2n to assess its effects on killing activity of human NK cells toward A549 cells by cell impedance assay. 1-Methyl-D-tryptophan (1-MT) was used as a positive IDO1 inhibitor. As shown in Fig. 5B, significant reduction in the cell index of A549 cells was observed following compounds 2i and 2n treatment compared with BBR, and compound 2i exhibited stronger activity than 1-MT.

2.2.5. BBR analogues inhibited IFN-γ-induced STAT1 transcription activity by inhibiting STAT1 phosphorylation and activating AMPK

IFN-γ-induced IDO1 expression involves the activation of signal transducer and activator or transcription 1 (STAT1), which can be activated via tyrosine phosphorylation by Janus kinases 1 (JAK1). Activated STAT1 homodimerizes and translocates into the nucleus, where it binds to and activates IFN-γ-responsive specific promoters of IDO1 [15,16]. Considering that STAT1 plays a critical role in IFN-γ-induced IDO1 expression, preliminary mechanism study was carried out to verify if the ten selected compounds work through STAT1 pathway. STAT1 phosphorylation at residue Tyr701 is required for acting as an active transcription factor. Therefore, we asked whether BBR analogues interfered with STAT1 phosphorylation. The expression level of STAT1 and phosphorylation of STAT1 Tyr701 were detected by western blot. As shown in Fig. 6, stimulation of cells with IFN-γ alone resulted in a rapid increase in Tyr701 phosphorylation of STAT1, and this increase was remarkably inhibited by the treatment of compounds 2e, 2f, 2i, 2n, 2o and 8b respectively.

Recent studies have shown that adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) activation suppresses STAT signaling [17,18]. AMPK kinase (AMPKK), an upstream kinase of AMPK, phosphorylates threonine 172 of the AMPK subunit α. Activated AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC) [19,20]. To assess whether compounds 2e, 2f, 2i, 2n, 2o and 8b-induced STAT1 inactivation through AMPK pathway, AMPK activation was determined by assessing ACC phosphorylation on Ser79 and AMPK phosphorylation on Thr172 [21,22]. Western blot results (Fig. 7) demonstrated that compounds 2i, 2n and 8b significantly increased IFN-γ-induced ACC and AMPK phosphorylation. Compounds 2i, 2n and 8b induced AMPK activation and STAT1 dephosphorylation. Considering compounds 2i, 2n and 8b markedly suppressed IFN-γ-induced IDO1 promoter activities, which demonstrated that they inhibited IFN-γ-induced IDO1 promoter activity by activating AMPK and subsequent inhibition of STAT1 phosphorylation, indicating these compounds had great potential as immunotherapy agents targeting inhibition of IDO1 at the transcriptional level.
Table 1
Structures of all the newly synthesized BBR derivatives.

| No  | R     | No  | R     | No. | R     |
|-----|-------|-----|-------|-----|-------|
| 2a  |       | 2j  |       | 6a  |       |
| 2b  |       | 2k  |       | 6b  |       |
| 2c  |       | 2l  |       | 6c  |       |
| 2d  |       | 2m  |       | 8a  |       |
| 2e  |       | 2n  |       | 8b  |       |
| 2f  |       | 2o  |       | 9a  |       |
| 2g  |       | 2p  |       | 9b  |       |
| 2h  |       | 2q  |       | BBR|       |
| 2i  |       | 3a  |       |     |       |

Fig. 2. Inhibition effect of BBR analogues on IFN-γ-induced IDO1 promoter activity. A549 cells were transfected with a pGL4-IDO1-luc vector and the pRL-CMV vector as an internal control, followed by pretreatment with 10 μmol/L of the indicated compounds for 2 h and stimulation with or without IFN-γ for 24 h. The level of luciferase activity was measured and normalized to Renilla luciferase activity. #p < 0.001 compared with untreated control group; *p < 0.05, **p < 0.01, ***p < 0.001 compared with the IFN-γ group.
Fig. 3. BBR analogues inhibited IFN-γ-induced IDO1 expression. A549 cells were pre-treated with 10 μmol/L of the indicated compounds for 2 h, followed by IFN-γ (5 ng/mL) treatment for 24 h. The expression levels of IDO1 were measured by western blot. D: DMSO. GAPDH served as the loading control. **p < 0.01 compared with the IFN-γ group.

Fig. 4. Effects of BBR analogues on A549 cell viability. A549 cells were treated with 10 μmol/L of the indicated compounds for 24 h, cell toxicity was measured by MTT assay. Control cells were treated with 0.5% (v/v) DMSO. The results are presented as means ± standard error and represent three individual experiments. *p < 0.05; **p < 0.01 compared with the untreated control group.

3. Conclusions

Taking IDO1 as the target, a series of novel BBR derivatives including esters, amides and sulfonates on different positions were designed, prepared and examined for their activity for suppression of IFN-γ-induced IDO1 promoter expression. SAR analysis indicated that large volume substituent at the 9-position might be beneficial for enhancing the potency. Among them, compounds 2f, 2i, 2n, 2o and 8b exhibited increased potency with inhibitory rate of 71–90% compared with BBR. Their activities were further confirmed by protein level. Furthermore, compounds 2i and 2n exhibited their anticancer activity by enhancing the specific lysis of NK cells to A549 cells, as through targeting IDO1, but not cytotoxicity. Preliminary mechanism revealed that compounds 2i and 2n inhibited IFN-γ-induced IDO1 expression through activating AMPK and subsequent inhibition of STAT1 phosphorylation. Thus, the results provided powerful information on further strategic optimization, and compounds 2i and 2n have been selected as promising IDO1 modulators for cancer immunotherapy for next investigation.

4. Experimental section

4.1. General

Melting point (mp) was obtained with CXM-300 melting point apparatus and uncorrected. The 1H NMR spectra was performed on a Varian Inova 500 or 600 MHz spectrometer (Varian, San Francisco, CA) and 13C NMR on a Bruker Avance III 500 or 600 spectrometer with Me4Si as the internal standard, all the samples were dissolved in DMSO-d6 before testing. ESI high-resolution mass spectra (HRMS) was recorded on an Autospec Ultima-TOF mass spectrometer (Micromass UK Ltd, Manchester, UK). Flash chromatography was performed on CombiblackRF 200 (Teledyne, Nebraska, USA), particle size 0.038 mm. Antibodies against IDO1, STAT1, phospho-STAT1 (Thr701), AMPKα, phospho-AMPK (Thr172) and phospho-ACC (Ser79) were purchased from Cell Signaling Technology (Danvers, MA, USA). MTT, α-Tubulin, GAPDH and β-actin antibody were obtained from Sigma (St. Louis, MO, USA). 1-MT (1-Methyl-L-tryptophan, IDO1 inhibitor) was purchased from Selchkem (Shanghai, China).

4.2. Chemistry

4.2.1. General synthesis procedure for synthesis of compounds 2a–q and 3a

BBR (3.71 g, 10 mmol) was heated at 195–210 °C for 10–15 min under vacuum (30–40 mmHg) to afford the black oil, which was acidified with ethanol/concentrated HCl (95:5). The solvent was removed by evaporation, the residue was collected and then purified by flash chromatography over silica gel using CH2Cl2/CH3OH as the gradient eluent, affording the title compound 1 (2.85 g, 80%) as a yellow solid.

To a stirred solution of 1 (100 mg, 0.28 mmol) in anhydrous CH3CN, triethylamine (175 μL, 1.26 mmol) was added and heated at 70 °C. Then the ROCIC/HSO4Cl (11:1.2 eq) was added and stirred for 5–6 h. The mixture was cooled to precipitate precipitate, filtered and washed with CH2Cl2 to afford compounds 2a–q and 3a. Compounds 2a–g, 2i, 2n–q and 3a were gained following the same procedure using purchased acyl chloride or sulfuryl chloride as material. Compounds 2h, 2j–m were gained by the same procedure using purchased acid which was reflux in SOCl2 to afford acyl chloride.

4.2.1.1. 2,3-Methylenedioxy-9-((2,5-difluorobenzoyl)oxy)-10-methoxyprotoberberine chloride (2n). Compound 1 (100 mg, 0.28 mmol) was treated with 2,5-difluorobenzoyl chloride (39 μL, 0.31 mmol) according to the general procedure to give the desired product 2n as a yellow solid, yield: 43%; Mp: 184–186 °C (dec.); 1H NMR (500 MHz) δ 10.07 (s, 1H), 9.14 (s, 1H), 8.37 (d, J = 9.3 Hz, 1H), 8.30 (d, J = 9.3 Hz, 1H), 8.10 (s, 1H), 7.85 (s, 1H), 7.84–7.77 (m, 1H), 7.67–7.59 (m, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.92 (t, J = 6.4 Hz, 2H), 4.06 (s, 3H), 3.22 (t, J = 6.4 Hz, 2H); 13C NMR (126 MHz) δ 160.1, 159.5, 157.6, 150.9, 150.7, 148.4, 145.2, 138.9, 133.6, 133.4, 131.5, 127.9, 126.5, 124.3, 121.6, 121.3, 120.0, 120.2, 119.4, 118.2, 109.1, 106.2, 102.8, 58.03, 56.0, 26.8; HRMS: calcd for C32H31F3NO5Cl [M − Cl]−
4.2.1.2. 2,3-Methylenedioxy-9-((2,3,4-trifluorobenzoyl)oxy)-10-methoxyprotoberberine chloride (2b).

Compound 1 (100 mg, 0.28 mmol) was treated with 2,3,4-trifluorobenzoyl chloride (40 μL, 0.31 mmol) according to the general procedure to give the desired product 2b as a yellow solid, yield: 36%; Mp: 186–188°C (dec.); 1H NMR (500 MHz) δ 10.06 (s, 1H), 9.13 (s, 1H), 8.37 (d, J = 9.2 Hz, 1H), 8.30 (d, J = 9.2 Hz, 1H), 8.22–8.12 (m, 1H), 7.85 (s, 1H), 7.75–7.63 (m, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.91 (t, J = 6.3 Hz, 2H), 4.06 (s, 3H), 3.22 (t, J = 6.3 Hz, 2H); 13C NMR (126 MHz) δ 160.0, 154.7, 152.1, 150.9, 150.7, 148.4, 145.1, 140.5, 139.0, 133.6, 133.3, 131.5, 128.6, 128.0, 126.5, 121.6, 121.3, 121.0, 115.2, 114.2, 109.1, 106.2, 102.8, 58.0, 56.0, 26.8; HRMS: calcd for C26H17F3NO5Cl [M+Cl]⁺ 480.1053, found 480.1058.

Fig. 5. BBR analogues induce killing activity of human NK cells toward A549 cells. A549 cells were pre-treated with the indicated compounds with or without IFN-γ (10 ng/mL) for 16 h. Cells were washed and then NK cells were co-cultured with A549 cells at 10:1 in RPMI 1640 plus 10% FBS. (A): After 4 h, the specific lysis of NK cells to A549 cells was determined by LDH releasing assay. All of the experiments were performed in triplicated and the results were calculated as means ± standard error. # p < 0.05 compared with untreated control group; * p < 0.05 compared with the IFN-γ group. (B): A549 cells were freshly plated in 96 wells and the killing activity of human NK cells toward A549 cells was measured by a cell impedance assay. Data was analyzed using the RTCA Software 1.2 program (Roche Diagnostics). All data is presented as the mean (n = 2) normalized cellular index ± SEM over time.

Fig. 6. BBR analogues suppressed IFN-γ-induced STAT1 phosphorylation. A549 cells were pre-treated with 10 μmol/L of the indicated compounds for 2 h, followed by IFN-γ (5 ng/mL) treatment for 24 h. The expression level of STAT1 and phosphorylation of STAT1 (Tyr701) were measured by western blot using corresponding antibodies. D: DMSO. GAPDH served as the loading control. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the IFN-γ group.
Fig. 7. BBR analogues induces AMPK activation. A549 cells were pre-treated with 10 μmol/L of the indicated compounds for 2 h, followed by IFN-γ (5 ng/mL) treatment for 24 h. The expression level of phosphor-ACC (Ser79), AMPK and phosphor-AMPK (Thr172) were measured by western blot using corresponding antibodies. D: DMSO. GAPDH served as the loading control. **p < 0.01, ***p < 0.001 compared with the IFN-γ group.

0.28 mmol) was treated with 6-fluoropyridine-3-carbonyl chloride (54 μmol, 0.34 mmol) according to the general procedure to give the desired product 2c as a yellow solid, 39%; Mp: 187–197 °C (dec.); 1H NMR (500 MHz) δ 9.96 (s, 1H), 9.03 (s, 1H), 8.30 (d, J = 5.4 Hz, 1H), 8.23 (d, J = 8.8 Hz, 1H), 7.79 (s, 1H), 7.09 (s, 1H), 6.16 (s, 2H), 4.91 (t, J = 6.3 Hz, 2H), 4.13 (s, 2H), 3.98–3.94 (m, 2H), 3.41 (s, 3H), 3.22 (t, J = 6.3 Hz, 3H); 13C NMR (151 MHz) δ 166.2, 161.7, 151.3, 150.8, 150.5, 148.2, 144.9, 144.8, 138.8, 133.4, 133.2, 131.3, 127.7, 126.4, 123.5, 121.4, 121.1, 120.8, 111.0, 108.9, 106.0, 102.6, 57.8, 55.8, 26.6; HRMS: calcd for C25H18FN2O5Cl [M - Cl]+ 445.1194, found 445.1201.

2.3-Methylenedioxy-9-((2-ethylbutanoyl)oxy)-10-methoxyprotoberberine chloride (2f). Compound 1 (100 mg, 0.28 mmol) was treated with 2-ethylbutanoyl chloride (42 μL, 0.31 mmol) according to the general procedure to give the desired product 2f as a yellow solid, yield: 27%; Mp: 200–201 °C (dec.); 1H NMR (500 MHz) δ 9.72 (s, 1H), 9.09 (s, 1H), 8.30 (d, J = 9.2 Hz, 1H), 8.23 (d, J = 9.2 Hz, 1H), 7.83 (s, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.96 (t, J = 6.3 Hz, 2H), 4.03 (s, 3H), 3.22 (t, J = 6.3 Hz, 2H), 2.85 (p, J = 6.6 Hz, 1H), 1.93–1.70 (m, 4H), 1.06 (t, J = 7.4 Hz, 6H); 13C NMR (126 MHz) δ 173.2, 150.9, 150.6, 148.3, 144.7, 143.7, 134.2, 133.6, 131.3, 131.5, 127.3, 126.5, 121.7, 121.3, 121.0, 106.2, 102.8, 57.7, 56.2, 47.6, 26.8, 24.0 (2), 11.8 (2); HRMS: calcd for C25H26NO5Cl2 [M - Cl] + 420.1806, found 420.1816.

2.3-Methylenedioxy-9-((3,5,5-trimethylhexanoyl)oxy)-10-methoxyprotoberberine chloride (2g). Compound 1 (100 mg, 0.28 mmol) was treated with 3,5,5-trimethylhexanoyl chloride (65 μL, 0.34 mmol) according to the general procedure to give the desired product 2g as a yellow solid, 29%; Mp: 206–207 °C (dec.); 1H NMR (500 MHz) δ 9.97 (s, 1H), 9.07 (s, 1H), 8.30 (d, J = 9.2 Hz, 1H), 8.22 (d, J = 9.2 Hz, 1H), 7.83 (s, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.97 (t, J = 6.3 Hz, 2H), 4.03 (s, 3H), 3.23 (t, J = 6.3 Hz, 2H), 2.90 (dd, J = 15.6, 5.8 Hz, 1H), 2.69 (dd, J = 15.6, 8.1 Hz, 1H), 2.22–2.12 (m, J = 6.8 Hz, 1H), 1.45 (dd, J = 14.0, 4.1 Hz, 1H), 1.25 (dd, J = 14.0, 6.6 Hz, 1H), 1.14 (d, J = 6.6 Hz, 3H), 0.97 (s, 3H); 13C NMR (126 MHz) δ 170.5, 151.0, 150.6, 145.1, 138.7, 134.2, 133.6, 131.5, 127.3, 126.5, 121.8, 121.2, 121.0, 106.2, 102.8, 57.8, 55.9, 50.6, 43.5, 31.45, 30.6 (3), 27.3, 26.8, 22.8; HRMS: calcd for C28H32NO5Cl [M - Cl] + 462.2275, found 462.2285.
4.2.1.2. 2.3-Methylenedioxy-9-((1-methylocyclopropane-1-carbonyl)oxy)-10-methoxy protoberberine chloride (2h). Compound 1 (100 mg, 0.28 mmol) was treated with 1-methylocyclopropane-1-carbonyl acid (40 mg, 0.34 mmol) according to the general procedure to give the desired product 2h as a brown solid, yield: 20%; Mp: 184 – 186°C (dec); 1H NMR (500 MHz) δ 9.78 (s, 1H), 9.07 (s, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.20 (d, J = 9.2 Hz, 1H), 7.82 (s, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.98 (t, J = 6.2 Hz, 2H), 4.04 (s, 3H), 3.22 (t, J = 6.2 Hz, 2H). 13C NMR (126 MHz) δ 173.6, 150.8, 148.5, 134.8, 138.8, 134.4, 133.7, 130.6, 126.5, 125.5, 121.9, 120.6, 120.3, 107.9, 105.2, 102.3, 56.1, 55.9, 45.7, 40.7, 39.8, 370, 319, 28.8, 26.6, 24.2; HRMS: calcld for C27H26NO3Cl [M – Cl]1 − 444.1806, found 444.1809.

4.2.1.3. 2,3-Methylenedioxy-9-((noradamantine-1-carbonyloxy)-10-methoxy protoberberine chloride (2m). Compound 1 (100 mg, 0.28 mmol) was treated with 3-noradamanatecarboxylic acid (56 mg, 0.34 mmol) according to the general procedure to give the desired product 2m as a yellow solid, yield: 36%; Mp: 201 – 203°C (dec); 1H NMR (500 MHz) δ 9.62 (s, 1H), 9.08 (s, 1H), 8.29 (d, J = 9.2 Hz, 1H), 8.22 (d, J = 9.2 Hz, 1H), 7.84 (s, 1H), 7.12 (s, 1H), 6.19 (s, 2H), 4.98 (t, J = 6.3 Hz, 2H), 4.03 (s, 3H), 3.22 (t, J = 6.3 Hz, 2H). 13C NMR (126 MHz) δ 174.8, 150.8, 150.5, 144.8, 148.3, 138.6, 134.8, 133.6, 131.6, 127.2, 126.5, 121.7, 121.3, 121.0, 109.1, 106.2, 105.2, 102.8, 56.0, 55.9, 44.7, 47.2 (2), 44.4, 43.8 (2), 376 (2), 34.7, 26.8; HRMS: calcd for C28H26NO3Cl [M – Cl]1 − 470.1692, found 470.1696.

4.2.1.4. 2,3-Methylenedioxy-9-((adamantyl-1-carboxyl)-oxy)-10-methoxy protoberberine chloride (2n). Compound 1 (100 mg, 0.28 mmol) was treated with 2-(1-adamantyl)acetyl chloride (66 mg, 0.31 mmol) according to the general procedure to give the desired product 2n as a yellow solid, yield: 43%; Mp: 188 – 190°C (dec); 1H NMR (500 MHz) δ 9.96 (s, 1H), 9.07 (s, 1H), 8.30 (d, J = 9.2 Hz, 1H), 8.21 (d, J = 9.2 Hz, 1H), 7.82 (s, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.96 (t, J = 6.3 Hz, 2H), 4.05 (s, 3H), 3.82 – 3.73 (m, 1H), 3.22 (t, J = 6.3 Hz, 2H). 2.50 – 2.35 (m, 4H), 2.16 – 2.04 (m, 1H), 2.02 – 1.90 (m, 1H); 2H NMR (126 MHz) δ 172.9, 151.0, 150.6, 148.3, 145.1, 138.7, 134.3, 133.5, 131.5, 127.2, 126.4, 121.8, 121.0, 109.1, 106.2, 102.8, 57.9, 56.0, 37.7, 26.8, 5.24 (2), 18.6; HRMS: calcd for C32H28NO3Cl [M – Cl]1 − 498.2275, found 498.2278.

4.2.1.5. 2,3-Methylenedioxy-9-((3-bromoadamantine-1-carbonyl)oxy)-10-methoxy protoberberine chloride (2o). Compound 1 (100 mg, 0.28 mmol) was treated with 3-bromoadaman-1-carbonyl chloride (94 mg, 0.34 mmol) according to the general procedure to give the desired product 2o as a yellow solid, yield: 39%; Mp: 191 – 193°C (dec); 1H NMR (500 MHz) δ 9.63 (s, 1H), 9.08 (s, 1H), 8.29 (d, J = 9.2 Hz, 1H), 8.22 (d, J = 9.2 Hz, 1H), 7.83 (s, 1H), 7.12 (s, 1H), 6.19 (s, 2H), 4.99 (t, J = 6.3 Hz, 2H), 4.03 (s, 3H), 3.23 (t, J = 6.3 Hz, 2H), 2.59 (s, 2H), 2.07 – 1.98 (m, 3H), 1.84 – 1.77 (m, 6H), 1.78 – 1.63 (m, 6H); 13C NMR (126 MHz) δ 168.8, 150.9, 150.6, 148.3, 145.1, 138.7, 134.2, 133.6, 131.5, 127.3, 126.5, 121.8, 121.0, 109.1, 106.2, 105.2, 102.8, 57.7, 55.9, 48.7, 41.2 (2), 370 (3), 33.3, 28.7 (3), 26.8; HRMS: calcd for C32H28BrNO3Cl [M – Cl]1 − 562.1224, found 562.1240.

4.2.1.6. 2,3-Methylenedioxy-9-((1R,2R)-2-phenylcyclopropene-1-carbonyl)-oxy)-10-methoxy protoberberine chloride (2p). Compound 1 (100 mg, 0.28 mmol) was treated with (1R,2R)-2-phenylcyclopropane-1-carbonyl chloride (45 μL, 0.31 mmol) according to the general procedure to give the desired product 2p as a brown solid, yield: 34%; Mp: 172 – 174°C (dec); 1H NMR (500 MHz) δ 9.95 (s, 1H), 9.09 (s, 1H), 8.30 (d, J = 9.2 Hz, 1H), 8.23 (d, J = 9.2 Hz, 1H), 7.83 (s, 1H), 7.41 – 7.32 (m, 4H), 7.32 – 7.25 (m, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.96 (t, J = 6.4 Hz, 2H), 4.06 (s, 3H), 3.22 (t, J = 6.3 Hz, 2H), 2.85 – 2.79 (m, 1H). 2.45 – 2.39 (m, 1H). 1.84 – 1.77 (m, 1H); 13C NMR (126 MHz) δ 170.8, 150.9, 150.6, 148.8, 145.1, 140.1, 138.9, 138.7, 135.1, 131.5, 129.2 (2), 127.4, 127.4, 126.9 (2), 126.5, 121.7, 121.3, 121.0, 109.1, 106.2, 102.8, 58.0, 56.0, 50.9, 45.8 (2), 45.8 (2), 34.7, 32.0 (2), 26.8; HRMS: calcd for C30H32NO3Cl [M – Cl]1 − 484.1806, found 484.1809.
4.2.1.18. 2,3-Methylenedioxy-9-(1-methyl-1H-imidazole-4-yl)sulfonoyl-10-methoxyprotoberberine chloride (3a). Compound 3a (100 mg, 0.28 mmol) was treated with 1-methyl-1H-imidazole-4-sulfonyl chloride (45 mg, 0.31 mmol) according to the general procedure to give the desired product as a yellow solid; yield: 82%; Mp: 181–183 °C (dec.); 1H NMR (500 MHz) δ 9.54 (s, 1H), 9.07 (s, 1H), 8.30 (d, J = 9.2 Hz, 1H), 8.25 (d, J = 9.2 Hz, 1H), 8.22 (s, 1H), 8.05 (s, 1H), 7.82 (s, 1H), 7.13 (s, 1H), 6.19 (s, 2H), 4.88 (t, J = 6.3 Hz, 2H), 3.95 (s, 3H), 3.77 (s, 3H), 3.21 (t, J = 6.3 Hz, 2H); 13C NMR (126 MHz) δ 152.7, 150.6, 148.2, 144.7, 141.6, 138.8, 133.9, 133.7, 132.1, 131.3, 129.6, 128.6, 126.8, 122.0, 121.2, 120.7, 108.9, 106.0, 102.7, 57.7, 56.1, 34.5, 26.7; HRMS: calcd for C23H23NO3SCl [M − Cl]− 466.1076, found 466.1073.

4.2.2. General synthesis procedure for synthesis of compounds 6a–c

The solution of BBR (7.4 g, 20 mmol) in 2,4-dimethoxybenzylamine (15 mL, 78 mmol) was stirred at 120 °C for 6–8 h. The mixture was cooled to room temperature and washed with acetone (3 × 50 mL) to remove the remainingamine. The residue was purified by flash chromatography over silica gel using CH2Cl2/CH3OH (96.5:3.5) as the gradient eluent to afford compound 4 (3.5 g, 37%). Mp: 239–240 °C (Dec.); 1H NMR (500 MHz) δ 9.98 (s, 1H), 8.73 (s, 1H), 7.88 (d, J = 8.8 Hz, 1H), 7.77 (s, 1H), 7.52 (d, J = 8.8 Hz, 1H), 7.14 (d, J = 8.3 Hz, 1H), 7.09 (s, 1H), 6.52 (d, J = 2.2 Hz, 1H), 6.44 (t, J = 6.5 Hz, 1H), 6.42–6.39 (m, 1H), 6.17 (s, 2H), 4.80 (t, J = 6.2 Hz, 2H), 4.66 (d, J = 6.3 Hz, 2H), 3.87 (t, J = 5.3 Hz, 2H), 3.77 (s, 3H), 3.71 (s, 3H), 3.21 (t, J = 6.3 Hz, 2H); 13C NMR (126 MHz) δ 160.5, 153.8, 150.1, 148.2, 141.8, 141.7, 137.5, 136.3, 133.5, 130.4, 130.5, 128.1, 121.3, 120.5, 120.3, 118.0, 107.7, 109.1, 105.9, 104.8, 102.6, 98.9, 57.5, 56.0, 55.8, 55.6, 47.0, 27.3; HRMS: calcd for C23H22N2O3Cl [M − Cl]− 417.1941, found 417.1919.

Compound 4 (3 g, 6.4 mmol) was dissolved in CH3OH, and hydrochloric acid 3 mL was added. The mixture was stirred for 5–6 h, filtered, and washed with 80% ethanol to afford compound 5 (1.8 g, 80%). Mp: 212–214 °C (Dec.); 1H NMR (500 MHz) δ 10.19 (s, 1H), 8.64 (s, 1H), 7.84 (d, J = 8.6 Hz, 1H), 7.76 (s, 1H), 7.32 (d, J = 8.6 Hz, 1H), 7.08 (s, 1H), 6.69 (s, 2H), 6.16 (s, 2H), 4.70 (t, J = 6.3 Hz, 2H), 3.98 (s, 3H), 3.20 (t, J = 6.3 Hz, 2H); 13C NMR (126 MHz) δ 149.8, 148.0, 147.0, 143.9, 138.1, 135.4, 132.1, 130.4, 123.0, 121.2, 119.8, 113.7, 113.3, 108.9, 105.6, 102.4, 56.9, 55.1, 27.2; HRMS: calcd for C24H21N2O3Cl [M − Cl]− 432.1237, found 432.1235.

To a stirred solution of 5 (100 mg, 0.28 mmol) and pyridine (100 µL, 1.24 mmol) in anhydrous CH2Cl2 (5 mL), the ROCOCI (2×3 eq) was added, and refluxed for 10–12 h. The solvent was removed by evaporation and purified by flash chromatography over silica gel using CH2Cl2/CH3OH (95:5) as the gradient eluent to give 6a–c.
129.4, 127.1, 126.5, 121.5, 121.0, 119.5, 111.9, 109.4, 57.9, 56.8, 56.5, 56.5, 41.6, 38.9 (2), 36.5 (2), 27.9 (2), 26.5; HRMS: calcd for C13H24NO3Cl [M – Cl]− 500.2432, found 500.2432.

4.2.3.2. 2,3,10-trimethoxy-9-(2-(adamantan-1-yl)acetoxy)protoberberine chloride (8b). Compound 7 (100 mg, 0.29 mmol) was treated with 2-(1-adamantyl)acetyl chloride (66 mg, 0.31 mmol) according to the general procedure to give the desired product 8b as a brown solid, yield: 33%; Mp: 142–143 °C (dec.).

4.3. Cell culture and western blot analysis

A549 human lung cancer cell lines were procured from ATCC. The cells were cultured in DME/F-12 (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate, and incubated at 37 °C in a humidified atmosphere with 5% CO2. Activated NK cells were purchased from Stemcell Technologies (Vancouver, BC, Canada).

Western blot was performed as described previously [23]. Briefly, A549 cells were washed with PBS and lysed in M2 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium pyrophosphate, 5 mM NaF, 1 mM Na3VO4, 0.5% Triton X-100, and 1 mM DTT) supplemented with protease inhibitor cocktail (Sigma P8340). Proteins were separated by SDS-PAGE and were electrically transferred to a PVDF membrane. The membrane was probed with the appropriate primary antibody and with a HRP-conjugated secondary antibody. Blots were visualized by Tanon 5200 system (Tanon, Shanghai, China).

4.4. MTT assay

The effect of the indicated compounds on the cell viability of A549 cells was evaluated using the MTT assay [24]. Briefly, Cells were seeded (5 × 103/well) into 96-well plate and the investigated compounds were added at indicated concentrations for 48 h. Next, MTT solution at a concentration of 5 mg/mL was added to each well. After subsequent 4 h, the culture medium was removed and formazan crystals were dissolved with 150 μl DMSO. Finally, the absorbance was measured at 570 nm using a microplate reader (Multiskan FC, Thermo, USA).

4.5. IDO1 promoter activity assay

A549 cells were co-transfected with pGL4-IDO1-luc with the pRL-CMV plasmid using the Viggofect transfection reagent (Beijing, China) as instructed by the manufacturers. After 24 h of transfection, cells were pretreated with the indicated compounds (10 μmol/L) for 2 h and then stimulated with or without IFN-γ (10 ng/mL) for 24 h. Following IFN-γ treatment, the cells were lysed, and the luciferase activity was determined using the luciferase reporter assay system (Promega, Madison, CA, USA) according to manufacturer’s protocols. The luciferase activity values were normalized to the expression of the Renilla luciferase, and presented as the percentages of luciferase activity.

4.6. LDH release assay

Cytokitoytic human NK cells against A549 cells was assessed with LDH release assay, as previously described [25]. Briefly, A549 cells were freshly plated at 5 × 103/well in 96-well plates and pretreated with the indicated compounds (10 μmol/L) for 2 h and then stimulated with IFN-γ (10 ng/mL) for 16 h. A549 cells were washed and then cocultured with NK cells at 1:10 in triplicate in RPMI 1640 plus 10% FBS. Four hours later, cytotoxicity assay was conducted using non-radioactive lactate dehydrogenase (LDH) release using a cytotoxicity detection kit (CytoTox 96, Promega, Madison, WI, USA) as the manufacturer’s instructions. Spontaneous release and maximum release were determined by incubating target cells without effector cells in medium alone or in 0.5% NP40, respectively. The percent cytotoxicity was calculated as follows: (experimental release-spontaneous release)/(maximum release-spontaneous release) × 100%.

4.7. Cell impedance assay

A549 cells were freshly plated at 5 × 103/well in E16 plates (Roche Diagnostics, Basel, Switzerland). The plate was connected to an xCELLigence RTCA SP instrument (Roche Diagnostics) within a humidified cell culture incubator. After 4 h incubation and A549 cells were treated with the indicated compounds (10 μmol/L) with IFN-γ (10 ng/mL). After 16 h, A549 cells were washed and cocultured with NK cells at 1:10 in triplicate in RPMI 1640 plus 10% FBS. Data was analyzed using the RTCA Software 1.2 program (Roche Diagnostics). All data is presented as the mean normalized cellular index ± SEM over time.
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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2017.10.078.

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