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Chemical conjugation of muramyl dipeptide and paclitaxel to explore the combination of immunotherapy and chemotherapy for cancer

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Abstract Paclitaxel (Taxol®) conjugated to muramyl dipeptide (MDP) is described. Biological testing showed that the conjugation of MDP at 2′-*O*-paclitaxel (**2′-*O*-MTC-01**) not only has antitumor activity, but also have immunoenhancement capacity. Compared with paclitaxel or MDP alone or with a mixture of paclitaxel + MDP, **2′-*O*-MTC-01** significantly increases the production and expression of TNF-α and IL-12 from mouse peritoneal macrophages, which demonstrates a synergism of MDP and paclitaxel in one conjugated molecule.

Keywords Muramyl dipeptide · Paclitaxel · Chemical conjugation · Synergism · Immunotherapy · Chemotherapy · Cancer

Introduction

The major clinical challenge for cancer therapy recently remains the prevention or eradication of metastatic diseases. Activated macrophages *in vivo* are a possible immunotherapeutic route for the treatment of tumor metastases and particularly multiple-drug resistance [1, 2]. Macrophages activated by a liposome-encapsulated immunomodulator (MTP-PE, a muramyl dipeptide [MDP] derivative) have been shown clinically to exert tumoricidal activity [2]. This overcomes the cellular heterogeneity of tumors, which ultimately leads to a resistance to chemotherapy. However, the major limitation in the treatment of disseminated metastases by the systemic activation of macrophages appears to be the tumor burden. Thus, it is necessary to design a regimen that combines immunotherapeutic and chemotherapeutic cancer treatments.

Paclitaxel (Taxol®) is one of the most widely used chemotherapeutic agent and is active in many types of cancer [3]. Paclitaxel acts by inhibiting the assembly of tubulin into microtubules [4]. Recently, paclitaxel has also been proved to be the Toll-like receptor 4 (TLR4) ligand [5] and as a lipopolysaccharide (LPS) mimetic, targeting macrophages [6], it stimulates murine macrophage cells to produce tumor necrosis factor α (TNF-α) in both normal hosts and tumor-bearing hosts.

Muramyl dipeptide (MDP) is the minimal structure of the cell wall of Gram-positive and Gram-negative bacteria to elicit human immunological responses [7]. MDP and its analogs are NOD2 ligands [8–11]. And 6-*O*-Acylated MDP analogues were reported to be TLR2/TLR4 agonists [12]. A strong synergism [13–15] between MDP and LPS was observed that greatly increased the ability of LPS-activated macrophages stimulated with MDP to produce cytokines, including TNF-α [16, 17]. Therefore, we hypothesized that a synergism between MDP or its analogs and paclitaxel would occur when both of them encounter murine macrophages at the same time. Such a synergism may facilitate a novel treatment that effectively prevents tumor cell growth and metastasis.

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Therefore, the conjugates of MDP and paclitaxel (MTCs) were designed and synthesized in this paper to discover a molecule that combines chemotherapy and immunotherapy in the treatment of cancer (Fig. 1).

We have reported elsewhere a method for the solid-phase synthesis of MDP derivatives using Rink resin [18, 19]. Paclitaxel was strategically designed to couple to MDP on a solid support. The chosen linkage positions were based on the knowledge of the structure–activity relationships at three sites on paclitaxel: at the 3′-amino, 2′-hydroxyl, and 7-hydroxyl groups [20–22].

A number of analogues incorporate modifications at the side chain 3′-amino group. Replacement of the 3′-*N*-benzamide with a 3′-*N*-*t*-Boc moiety, such as in docetaxel or 10-acetyl

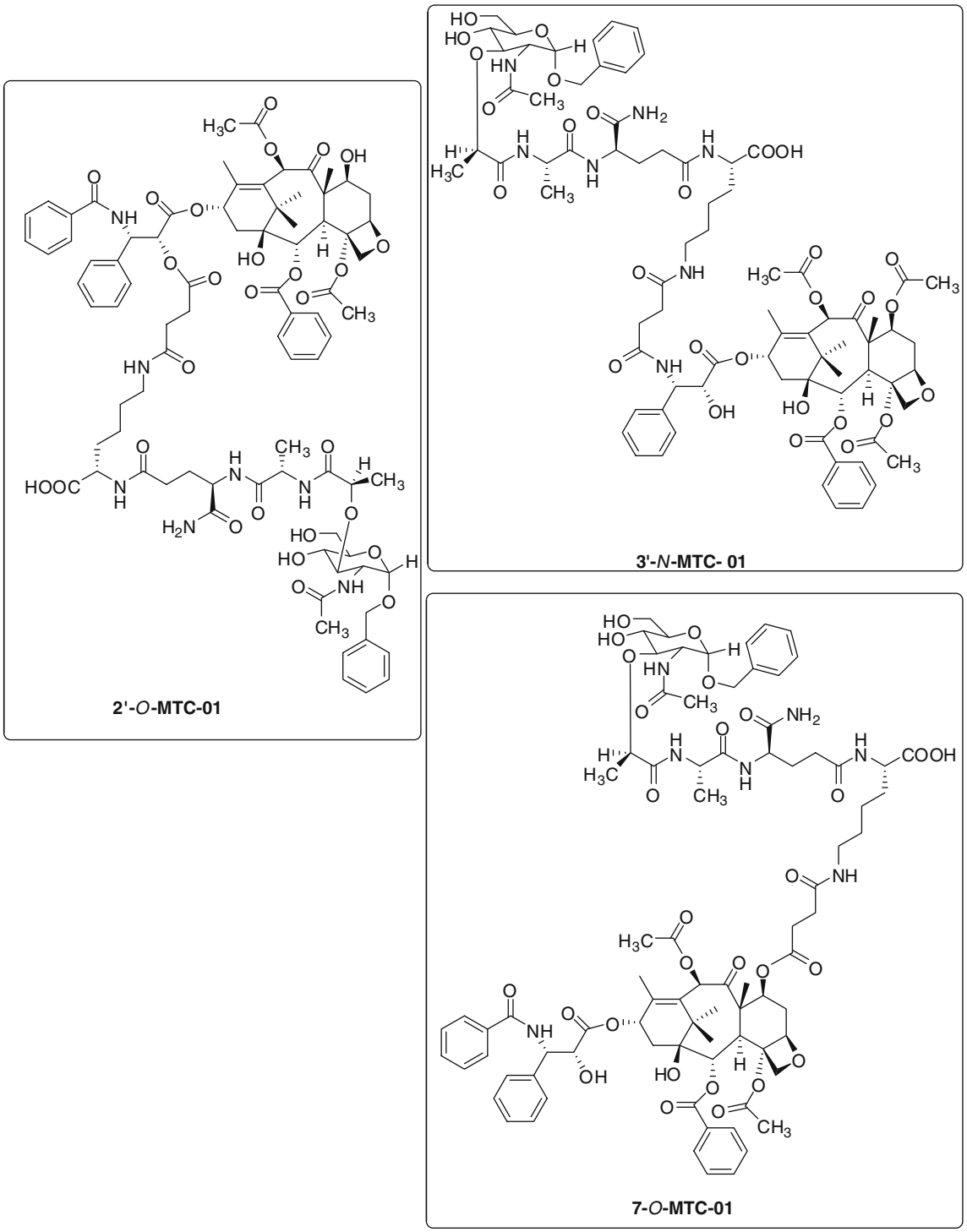


Fig. 1 Molecular structures of three different MDP and paclitaxel conjugates

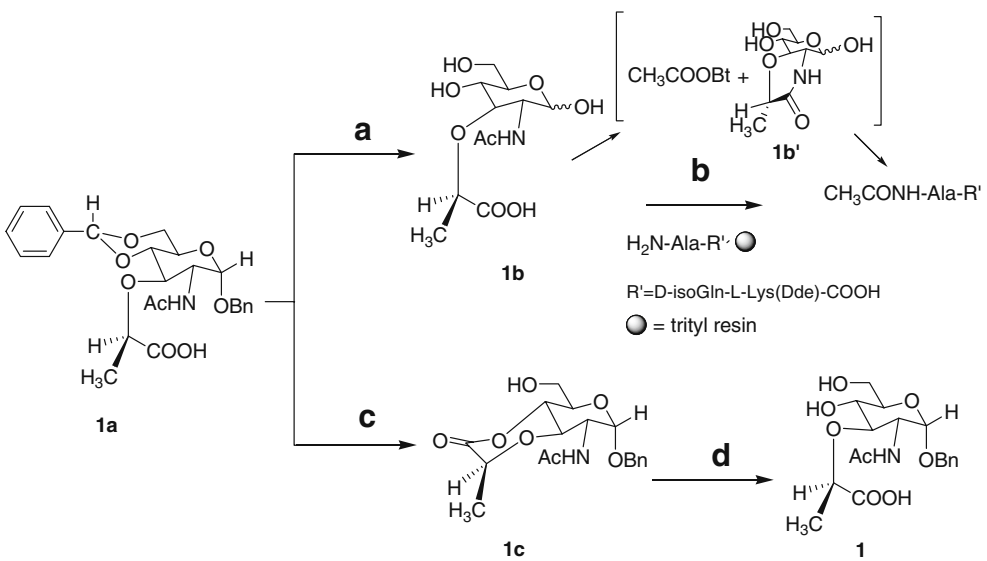
docetaxel, has generally resulted in increased cytotoxic potency [23, 24]. These previous observations suggest that the 3′-*N* group might be a suitable position at which to conjugate MDP. Therefore, the initial conjugation strategy of this study was to synthesize **3′-*N*-MTC-01** (Fig. 1).

Because paclitaxel is sensitive to trifluoroacetyl acid (TFA) and hydrogenation conditions, such as H_2/Pd/C, those are used to deprotect the 4, 6-*O*-benzylidene and 1-*O*-benzyl groups of the protected MDP, all the protected groups of the sugar moiety are best removed before the conjugation of paclitaxel and MDP. We initially produced fully unprotected compound **1b** from **1a** through general hydrogenation using H_2/Pd/C. However, when performance of the standard acylation of free amino group of tripeptide [Ala-D-isoGln-L-Lys(Dde)] on trityl resin, a byproduct (acetylated compound, m/e 553, $[M+H]^+$) was observed with a equal ratio (1:1 in HPLC area, UV detector under 214 nm wavelength) to the anticipated compound (acylated by **1b**, m/e 786, $[M+H]^+$) that was analyzed by LC-MS/MS system. It was supposed that CH_3COOBt, which quickly acetylated the above tripeptide, was generated through a *in situ* activation of **1b** in the presence of diisopropylcarbodiimide (DIC) and hydroxybenzotriazol (HOBt) and inner molecular neucleophilic substitution by AcNH that formed a inactive **1b'**(Scheme 1, up route).

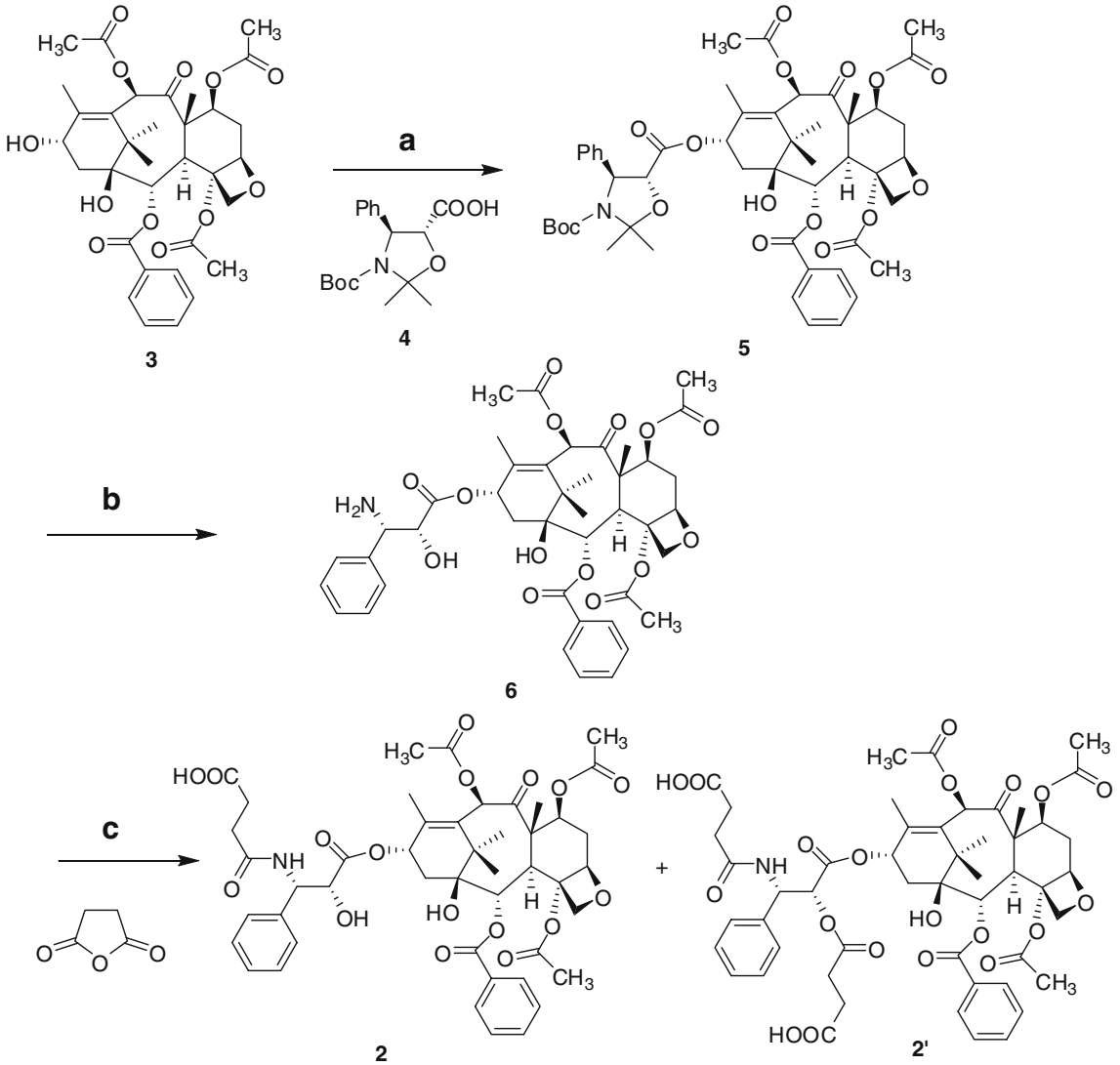
The results of previous studies [25–27] have indicated that modification of the remaining 1-*O*-benzyl group of muramic acid not only does not alter its biological activities, but also introduces a hydrophobic group, which is advantageous in its penetration of the cell membranes of macrophages. Therefore, an alternative approach was investigated experimentally in which the 4,6-*O*-benzylidene group of compound **1a** was removed by treatment with 90% TFA in water. However, under such strong acidic conditions, an inner cyclized ester, compound **1c**, was produced. Thus, saponification in the presence of 1.0 M LiOH and then acidification with 1.0 M HCl was used to produce **1** (Scheme 1, down route).

The projected solid-phase synthesis of target compound **3′-*N*-MTC-01** required the preparation of the advanced intermediate **2** (Scheme 2) from commercially available 10-deacetyl-baccatin III (**3**). We herein employed Bourzat's approach's strategy [28] to obtain **2**. To this end, **3**, protected by regioselective synthesis at C-7 and C-10 as an acetate, was then coupled with (4*S*, 5*R*)-*N*-Boc-2, 2-dimethyl-4-phenyl-5-oxazolidinecarboxylic acid (**4**) under DIC/*N*,*N*-dimethylaminopyridine (DMAP) to yield **5**. Subsequent deprotection with formic acid produced amino alcohol **6**. The intermediate **6** was then acetylated with succinic anhydride in CH_2Cl_2 for 4 h at room temperature. After purification by HPLC, **2** was produced with a 60% overall yield, and the 10% byproduct **2′**.

2-Chlorotrityl chloride resin was selected as the solid support because its ultra-acid sensitivity is advantageous, ensuring safe cleavage of the anticipated compound **3′-*N*-MTC-01** from the resin under mild conditions of 10% HOAc in dichloromethane (DCM). Because accessing building block to the target compound is subject to steric hindrance, the resin loading was decreased to 0.1 mmol/g. An amount of Fmoc-Lys(Dde)-OH corresponding to 0.1 mmol/g of resin was first reacted with 2-chlorotrityl chloride resin overnight at room temperature. Excess trityl chloride was then quenched and capped by the addition of DCM:MeOH:*N*,*N*-diisopropylethylamine (DIPEA) (17:2:1, v:v:v). After removal of the Fmoc protective group with 20% piperidine in DMF, the resin-bound **3′-*N*-MTC-01** (**11**) was achieved through the sequential



Scheme 1 Synthesis route of **1** and **1b**. Reagents and conditions: (a) H_2, Pd/C, r.t., 24 h; (b) DIC, HOBt, DMF, r.t., 3 h; (c) CF_3COOH/H_2O, r.t., 2 h; (d) 1 N LiOH, r.t., 1 h; 1 N HCl



Scheme 2 Synthesis route of compound **2**. Reagents and conditions: (a) DIC, DMAP, toluene, 80°C, 3 h; (b) 95% HCOOH, 0°C, overnight; (c) succinic anhydride (1.5 equiv.), CH_2Cl_2, DIPEA

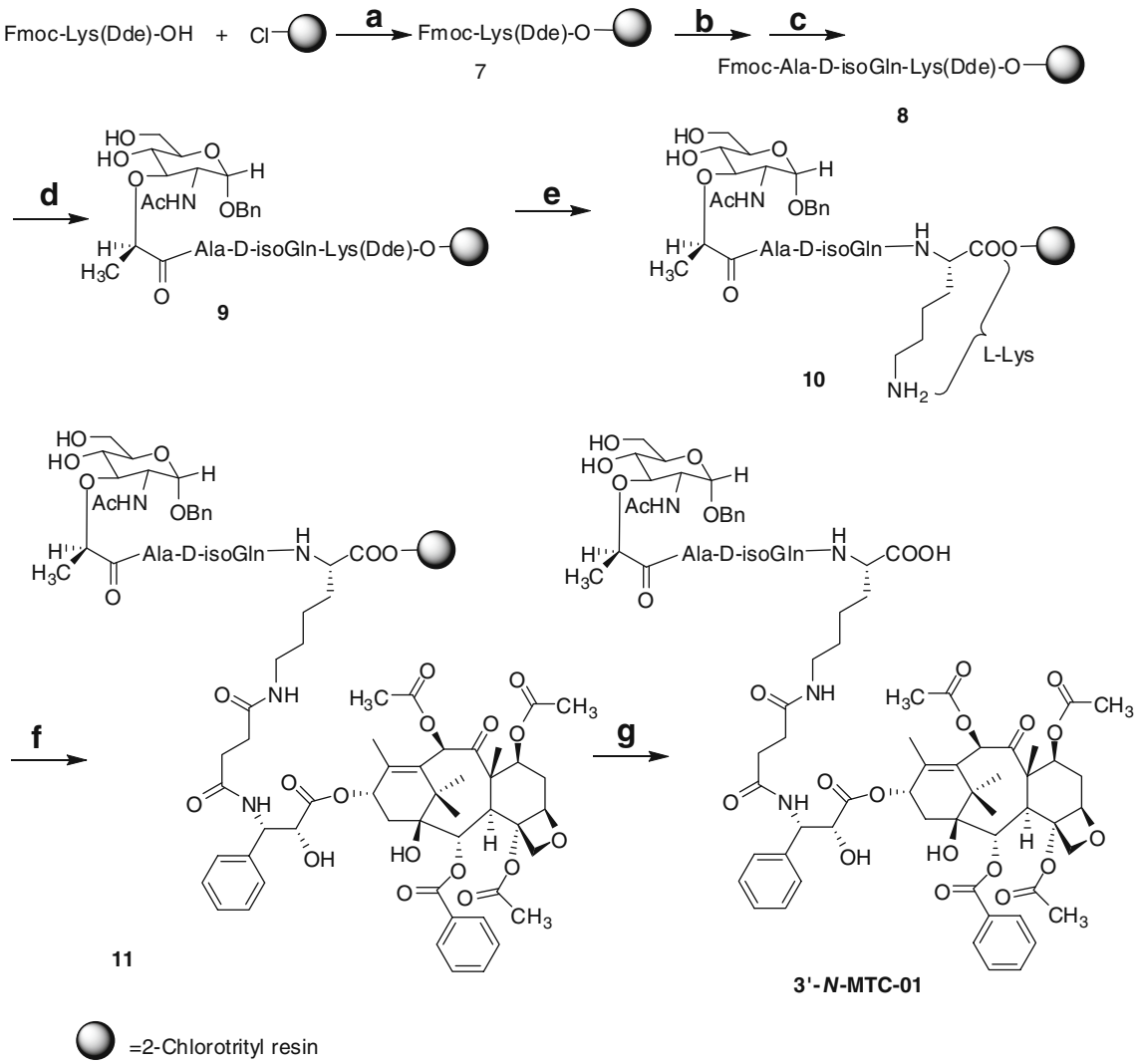
assembly of the pre-made building blocks as outlined in Scheme 3.

The final **3′-*N*-MTC-01** was obtained when **11** was treated with 10% HOAc in DCM (v:v) for 2 h at room temperature. Its purity was assessed by HPLC at a UV wavelength of 214 nm. Further studies of the structure by electronic spray ionization–mass spectrometry (ESI-MS) and by one- and two-dimensional NMR indicated that this synthesis yielded the desired taxoid **3′-*N*-MTC-01** at a purity of 95%.

The conjugates at C-2′ or C-7 of paclitaxel with MDP, **2′-*O*-MTC-01** and **7-*O*-MTC-01**, respectively, were also successfully synthesized by coupling **10** with intermediate **12** or **13** respectively [29, 30], as outlined in Scheme 4.

The *in vitro* biological activities of the conjugates were initially evaluated in three tumor cell lines: a human breast cancer cell line (MCF-7), a human cervical cancer cell line (HeLa), and a human skin cancer cell line (A431). The compound conjugated through C-2′ of paclitaxel (**2′-*O*-MTC-01**) was the most potent and **7-*O*-MTC-01** displayed much weaker inhibition (data not shown). However, the activity of **3′-*N*-MTC-01** against tumor cell growth was totally lost. We determined experimentally that **3′-*N*-MTC-01** does not trigger murine macrophages to induce detectable TNF-α and NO in the presence of IFN-γ or bind to microtubules in macrophages at a concentration up to 30 μmol (data not shown). Obviously, conjugation of MDP through the 3′-*N* of paclitaxel significantly altered binding affinity of paclitaxel for both microtubules and Toll-like receptors. **2′-*O*-MTC-01** was then further tested against a wide spectrum cell lines. Table 1 lists the results, which indicate that **2′-*O*-MTC-01** retains its cytotoxicity against most tumor cell lines, although its activity was slightly decreased.

We then investigated whether **2′-*O*-MTC-01** induces the expression of immunostimulatory molecules in murine



Scheme 3 Synthesis route of anticipated conjugate **3′-*N*-MTC-01**. Reagents and conditions: (a) CH_2Cl_2, DIPEA, r.t., overnight; (b) 20% piperidine/DMF; Fmoc-D-isoGln-OH, DIC, HOSu, DMF, r.t., 3 h; (c) 20% piperidine/DMF; Fmoc-L-Ala-OH, DIC, HOSu, DMF, r.t., 3 h; (d) 20% piperidine/DMF; **1**, DIC, HOSu, DMF, r.t., 3 h; (e) 3% NH_2-NH_2/DMF, 3 min, twice; (f) **2**, DIC, HOSu, DMF, r.t., overnight; (g) 10% HOAc/CH_2Cl_2, r.t., 2 h

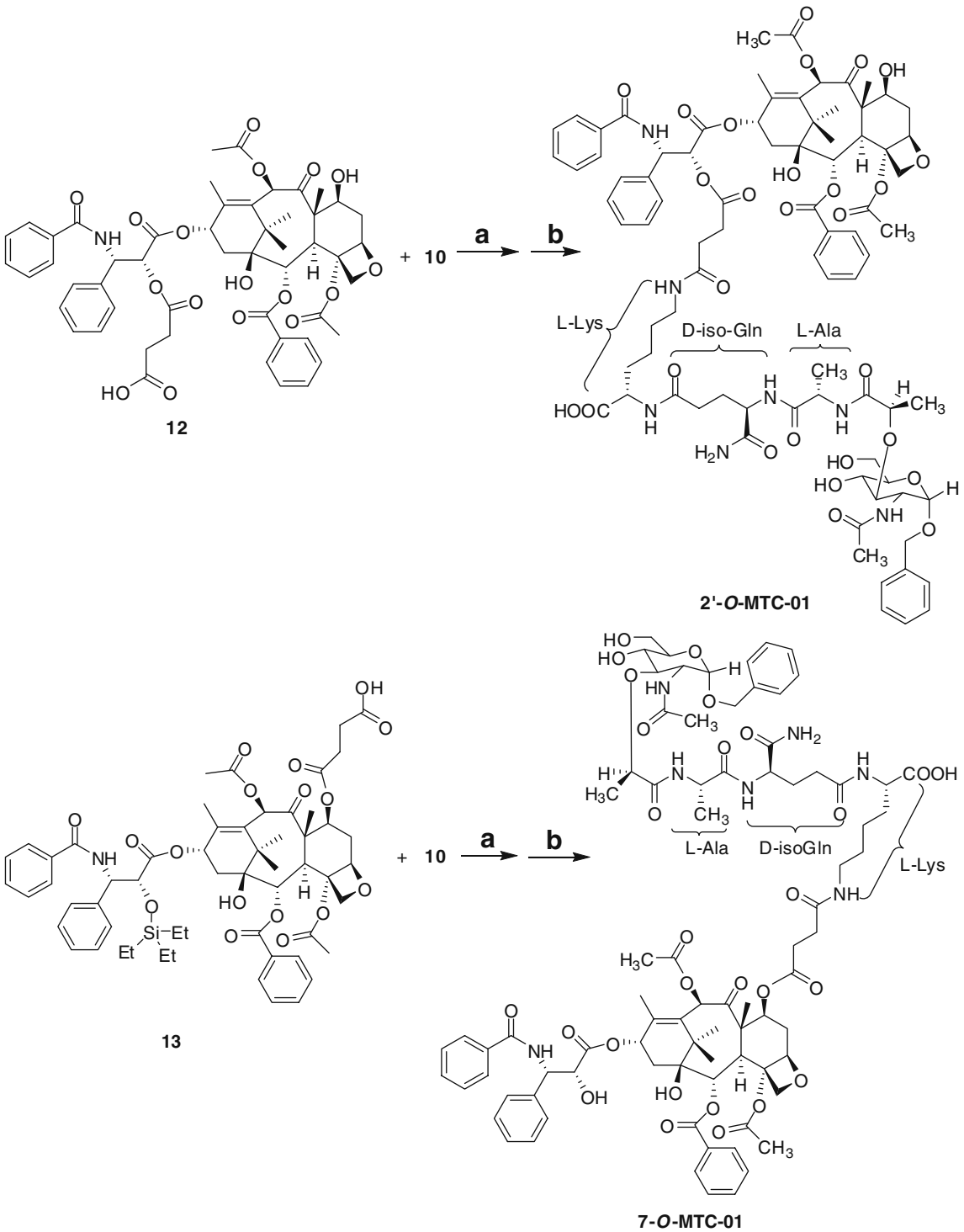
peritoneal macrophages (Table 2). MDP or paclitaxel alone induced TNF-α and IL-12 synthesis by macrophages. The additive expression was observed, when macrophages were stimulated with a mixture of MDP and paclitaxel. Most interestingly, a significant dose-dependent synergism of TNF-α and IL-12 productions was induced when murine peritoneal macrophages were treated by **2′-*O*-MTC-01**.

To determine whether **2′-*O*-MTC-01** could transcriptionally regulate cytokine production, the levels of TNF-α and IL-12 mRNAs were measured using reverse transcription-polymerization chain reaction (RT-PCR) technology. The results are illustrated in Fig. 2 and indicate that **2′-*O*-MTC-01** significantly up-regulated the mRNA expression of these cytokines, particularly at a concentration of 5.0 μM or higher.

When MDP was conjugated to paclitaxel, the water solubility of the preferred conjugate **2′-*O*-MTC-01** was analyzed at an equilibrium concentration at room temperature using HPLC measurement. The results showed that the conjugate is more water-soluble (about 200 times) than paclitaxel. This physical property of **2′-*O*-MTC-01** might be useful for drug formulation preparation.

Conclusion

A method has been developed for the efficient solid-phase synthesis of conjugates of MDP and paclitaxel at 3′-*N*, 2′-*O* and 7-*O* positions on paclitaxel. The conjugation of MDP to the 2′-C position of paclitaxel forms a compound (**2′-*O*-MTC-01**) that is about 200 times more water-soluble than paclitaxel, with antitumor activity *in vitro*. The observation also proves a synergism between



Scheme 4 Synthesis route of target conjugates **2′-*O*-MTC-01** and **7-*O*-MTC-01**. Reagents and Conditions: (a) DIC, HOSu, DMF, r.t., overnight; (b) 10% HOAc/CH_2Cl_2, r.t., 2 h

the effects of MDP and paclitaxel. This study demonstrates that paclitaxel and MDP conjugates are potentially amenable to further development into anticancer drugs with immunotherapeutic and chemotherapeutic properties.

Experimental section

^1H and ^{13}C NMR spectra were measured on a Varian Mercury-300, Mercury-400 NMR spectrometer (Palo Alto, CA) and on a Varian Inova-500 NMR spectrometer (Palo

Table 1 Growth inhibitory effects of **2′-*O*-MTC-01** and paclitaxel on cultured cancer cells and normal human embryo lung fibroblast cells (IC_{50} nmol/L)

Tumor cell line	Paclitaxel	2′-O-MTC-01	Tumor cell line	Paclitaxel	2′-O-MTC-01
KB	0.3	1.3	KeTr3	19.0	24.0
HeLa	0.7	3.0	HCT-8	22	38
BGC-823	1.1	2.4	A431	1.2	1.6
A2780	1.8	5.9	BEL-7402	220	170
MCF-7	1.9	3.0	HELF	280	320
PC3M	1.9	14.0			

Human cancer cell lines: *KB* (head and neck cancer), *HeLa* (cervical cancer), *BGC-823* (stomach cancer), *A2780* (ovarian cancer), *MCF-7* (breast cancer), *PC3M* (prostate cancer), *KeTr3* (renal cancer), *HCT-8* (colon cancer), *BEL-7402* (hepatic cancer), and *A431* (skin cancer); and a human embryo lung fibroblast cell line (HELF). *IC_{50} values were determined by MTT assay with eight drug concentrations in quadruplicates as described in experimental section.

Alto, CA), respectively (using tetramethylsilane as the internal standard). IR spectra were recorded on a Nicolet Impact 400 (San Jose, CA). The correct molecular weights were determined on an automatic ThermoFinnigan LCQ-Advantage MS/MS analysis system (San Jose, CA), equipped with a Gilson 322 pump, Gilson UV/vis-152 detector, Gilson 215 liquid handler (Lewis Center), and a effluent splitter with a 5-cm pheminax C18 column (5-μm). The eluent was a mixture of acetonitrile and water containing 0.05% TFA, with a linear gradient from 5:95 v/v acetonitrile/H_2O to 100:0 v/v acetonitrile/H_2O over 5 min at 1 mL/min flow rate. The 5% fluent was split into the MS system. Melting points were measured using a Yanaco MP 500 C microscope (Uji-city, Japan) and were uncorrected.

Materials All reagents and solvent, unless otherwise specified, were of commercial grade. The chemicals were purchased from Aldrich Co. and Sigma (Milwaukee, WI), and purified before use by standard methods. THF was freshly distilled under sodium metal and benzophenone. DCM and DMF were also distilled immediately prior to use. 2-ChloroTrityl chloride resin (100–200 mesh, 1.05 mmol/g) was purchased from Chem-Impex International, Inc (Wood dale, IL).

Benzyl 2-actamido-4, 6-*O*-benzylidene-2-deoxy-3-*O*-[R-1-(methoxycarbonyl) ethyl]-α-D-glucopyranoside **1a** was prepared using procedures previously reported [31].

Synthesis of Benzyl-2-actamido-2-deoxy-3-*O*-(*R*-2-propionic acid)-α-*D*-glucopyranoside (1)

With strong stirring, 5 mL of 90% CF_3COOH in water was added to 188.4 mg (0.4 mmol) of **1a**. After 8 h at room temperature, the solvent was treated with 20 mL of 1.0 N LiOH for 1 h. The resulting cloudy solution was carefully adjusted to pH 3 with the addition of 35 mL freshly prepared 1.0 M HCl. The resulting suspension was filtered and the filtrate was concentrated under reduced pressure. The residue was purified on a ODS reversed phase column with acetonitrile (ACN)/water. The fractions of 20–30% (ACN in water) were collected and lyophilized. A white solid was gained. The analysis of this material by LC-MS and ^1H NMR indicated the right compound **1** in 86% yield (136.7 mg).

Table 2 Effects of **2′-O-MTC-01** on the production of immunotherapeutic indicators by mouse peritoneal macrophages (mean ± SD; $n=3$)

Compounds		TNF-α Mean ± S.D. (pg/mL)	IL-12 Mean ± S.D. (pg/mL)	MHC II (positive ratio%)	CD54 (positive ratio%)
Control		10.8±1.1	262±2	2.95±0.03	0.79±0.003
Paclitaxel (5.00 μM)		30.7±2.4*	530±33*	4.50±0.02*	3.27±0.06*
MDP (5.00 μM)		40.2±2.9*	486±22*	3.64±0.04*	2.73±0.02*
MDP (5.00 μM)+ Paclitaxel (5.00 μM)		91.0±3.2	676±49*	4.89±0.02*	3.6±0.05*
2′-*O*-MTC-01	10.00 μM	664.8±4.4*	1790±64*	4.58±0.48*	4.57±0.02*
	5.00 μM	537.0±5.3*, **	1592±73*, **	5.51±0.03*, **	4.57±0.01*, **
	1.00 μM	245.5±3.1*	900±80*	4.58±0.02*	4.13±0.02*
	0.10 μM	87.9±4.3*	356±56*	4.01±0.02*	3.13±0.02*

*$P<0.01$ *vs.* control
**$P<0.01$ *vs.* (paclitaxel + MDP) group.

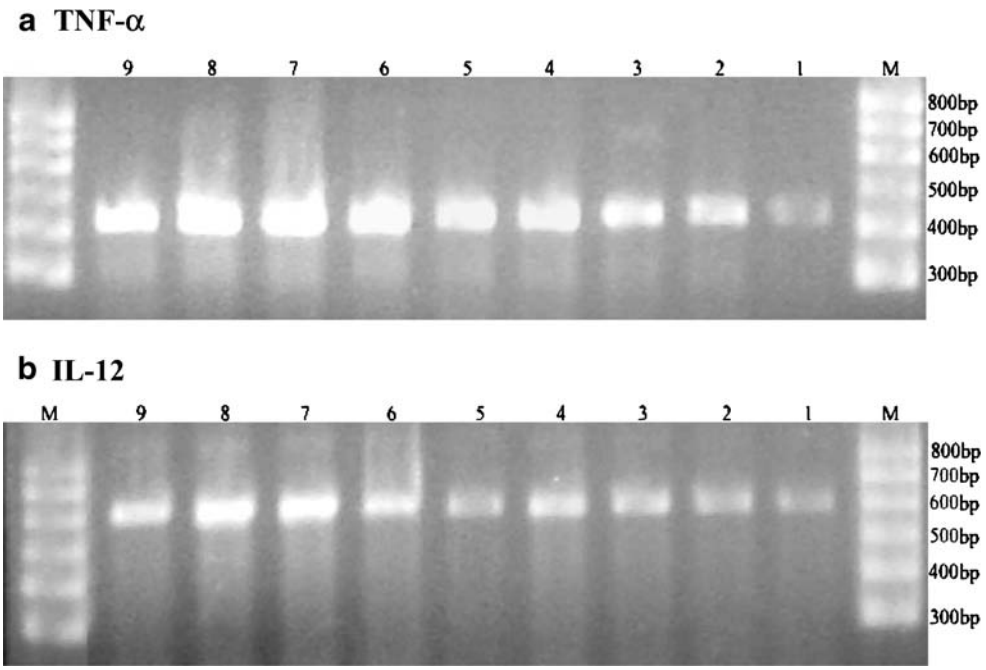


Fig. 2 Levels of TNF-α and IL-12 mRNAs in mouse peritoneal macrophages treated with different compounds. Mouse peritoneal macrophages were incubated with compounds at concentrations of 0.1–10 μmol/L for 10 h. mRNA levels of TNF-α (A) and IL-12 (B) were detected by RT-PCR with specific primers. The amplified cDNAs were resolved on 1% (w/v) agarose gel and visualized with ethidium bromide. Lane M: DNA marker; lane 1: untreated control cells; lane 2: MDP (5.00 μM); lane 3: paclitaxel (5.00 μM); lane 4: MDP (5.00 μM) + paclitaxel (5.00 μM); lane 5: **2′-*O*-MTC-01** (0.10 μM); lane 6: **2′-*O*-MTC-01** (1.00 μM); lane 7: **2′-*O*-MTC-01** (5.00 μM); lane 8: **2′-*O*-MTC-01** (100 μM); lane 9: positive control

^{1}H NMR of compound **1** (300 MHz, DMSO-d_6):

δ (ppm): 8.12 (brs, 1H, 2-NHAc), 7.27–7.37 (m, 5H, Ph), 4.99 (brs, 1H, H-1), 4.66 (d, *J*=12.6 Hz, 1H, 1-PhCH_2O), 4.42 (d, *J*=12.6 Hz, 1H, 1-PhCH_2O), 4.50 (q, *J*=7.0 Hz, 1H, α-H), 3.64 (d, *J*=10.5 Hz, 1H, 6-H), 3.50–3.33 (m, 5H), 1.85 (s, 3H, 2-Ac), 1.30 (d, *J*=7.2 Hz, 3H, CH_3). HRMS: $[M+H]^+$ 384.1658 (calcd, C18H26NO8, 384.1652, ppM error = 1.3182).

General procedure for synthesis of 7-acetyl-3′-debenzoyl-3′-*N*-succinyl-Taxol (2)

To a solution of 0.267 g (0.425 mmol) of commercial available 7-acetyl-baccatin III (**3**) in 20 mL of anhydrous toluene added 0.6 g (1.87 mmol) 4, 0.53 mL (3.4 mmol) DIC and 26 mg (0.21 mmol) of DMAP. The mixture was stirred for 2 h at 80°C. After filtration and evaporation of toluene under reduced pressure, the residue was purified by an ODS reversed column with water/methanol eluents. Two hundred forty-one milligrams of **5** was obtained in a yield of 61% (m. p. 149–150°C) [32]. A solution of 5 mL of 95% HCOOH was added at 0°C to 0.125 g (0.134 mmol) of **5**. After 18 h, 10 mL of DCM was added at 0–10°C. The resulting solution was adjusted to pH 6 with the addition of saturated $NaHCO_3$ solution and extracted twice with 30 mL of DCM. The organic layer was washed twice with 30 mL of water and then concentrated to achieve 98 mg of product **6**. A solution of 0.3 g (0.38 mmol) of **6**, 0.057 g (0.57 mmol) of succinic anhydride, and 0.065 mL (0.38 mmol) of DIPEA in 5 mL of DCM was stirred at room temperature for 4 h and detected, using HPLC, by the disappearance of **6**. Then the solution was evaporated to dryness in a vacuum. The residue was treated with 10 mL of water, stirred for 20 min, and filtered. The precipitate was dissolved in methanol and finally purified by HPLC on a semiprepared C18 column (Vydac). After lyophilization, the fine crystals were collected. This yielded 0.2 g of **2** (60%).

^{1} H NMR of compound **2** (500 MHz, $CDCl_3$):

δ (ppm): 8.11 (d, *J*=8.0 Hz, 2H), 7.606 (t, *J*=8.0 Hz, 1H),7.51 (t, *J*=7.5 Hz, 2H), 7.40–7.42 (m, 5H, 3’-Ph), 6.43 (d, *J*=9 Hz, 1H, 3’-NH), 6.31 (t, 1H, H-13β), 6.23 (s, 1H, H-10α), 5.68 (d, *J*=7 Hz, 1H, H-3’), 5.63 (d, *J*=7.5 Hz, 1H, H-2), 5.53–5.57 (dd, *J*=11, 7.5 Hz, 1H, H-7), 4.95 (d, *J*=9 Hz, 1H, H-5), 4.69(s, 1H, 2’-OH), 4.30 (d, *J*=8.0 Hz, 1H, H-20α), 4.20(d, *J*=8.5 Hz, 1H, H-20β), 3.92 (d, *J*= 7.0 Hz, 1H, H-3), 2.56–2.60 (m, 2H, CH_2, H-14), 2.40–2.44 (m, 2H, CH_2, H-6), 2.44 (s, 3H, OAc), 2.18 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.91 (s, 3H, 18-Me), 1.81 (s, 3H, 19-Me), 1.28 (s, 3H, 17-Me), 1.11 (s, 3H, 16-Me). ESI-MS: 892.8 $[M+H]^+$.

General coupling procedure for the synthesis of conjugate 3′-*N*-MTC-01

Fmoc-Lys(Dde)-OH (0.1 equiv. relative to the load for 2-chlorotrityl resin) and DIPEA (4.0 equiv. relative to carboxylic acid) were dissolved in dry DCM. The resin then was added and gently stirred for 30 min at room temperature. At the end of this time period, the resin was washed with 3 × DCM:MeOH:DIPEA (17:2:1), 3 × DCM, 2 × DMF, and 2 × DCM, respectively. The loaded resin

next was treated with 20% piperidine/DMF, to remove Fmoc, twice for 10 and 20 min, respectively, followed by thorough washing with DMF, MeOH, and DCM, respectively, three times of each. Repeating the Fmoc deprotection, washing, Fmoc-D-isoGln-OH, Fmoc-Ala-OH, and part protected muramic acid (**1**) sequence coupling steps, muramyl dipeptide with 4,6-hydroxyl unprotected-sugar moiety was successfully assembled onto resin. The Dde group of lysine then was removed with 2% hydrazine in DMF twice for 3 min each. After that, the resin was thoroughly washed again by a sequence of DMF, MeOH, and DCM 3 times of each, which offered resin-bound **10**. Intermediate **2** was finally coupled onto the side chain of lysine under conditions of 1.5 equiv of DIC/HOSu/DMF, which led to the resin-bound taxoid **3′-*N*-MTC-01** (**11**). Target compound **3′-*N*-MTC-01** was cleaved off the resin using 10% HOAc/DCM for 2 h at room temperature. The cocktail cleavage solution was concentrated under reduced pressure. The white residual powder was dissolved in methanol for LC-MS analysis. The gradient was buffer B (5–95%), used for 5 min on an RP C18 column (4.6 μm×50 mm). Buffer A: 0.05% TFA/H_2O; buffer B: 0.05%TFA/ACN. Electronic spray ionization MS (ESI-MS) was carried out on a ThermoFinnigan, LCQ-Advantage that offered the correct molecular weight. The purity of the crude compound was judged by a UV detector at 214 nm wavelength, to be over 95%, which was directly characterized by NMR spectroscopic methods without purification.

3′-*N*-MTC-01: ESI-MS: 1584.4 $[M+H]^+$.

General coupling procedure for the synthesis of conjugate **2′-*O*-MTC-01**

Compound **12** (2′-Succinyltaxol) was accomplished by following Deutsch's procedure [29].

Resin-bound **10** was made as described above. After the resin was thoroughly washed again by a sequence of DMF, MeOH, and DCM 3 times each, intermediate **12** was coupled onto the side chain of lysine under conditions of 1.5 equiv of DIC/HOSu/DMF, which led to the resin-bound taxoid **2′-*O*-MTC-01**. Target compound **2′-*O*-MTC-01** was cleaved off the resin using 10% HOAc/DCM for 2 h at room temperature. The cocktail cleavage solution was concentrated under reduced pressure. The white residual powder was dissolved in methanol for LC-MS analysis. The gradient was buffer B (5–95%), used for 5 min on an RP C18 column (4.6 μm×50 mm). Buffer A: 0.05% TFA/H_2O; buffer B: 0.05%TFA/acetonitrile. Electronic spray ionization MS (ESI-MS) was carried out on a ThermoFinnigan, LCQ-Advantage that offered the correct molecular weight. The purity of the crude compound was judged by a UV detector at 214 nm wavelength, to be over 95%, which was directly characterized by NMR spectroscopic methods without purification.

ESI-MS: 1646.3 $[M+H]^+$.

General coupling procedure for the synthesis of conjugate 7-*O*-MTC-01

Compound **13** (2′-Triethylsilyl-7-Succinyltaxol) has been successfully synthesized following literature with slightly modifications [30]. Protect at 2′-OH, which is normally the most reactive, and only when protected, reaction proceeds selectively at the C-7 hydroxyl group.

Resin-bound **10** was made as described above. After the resin was thoroughly washed again by a sequence of DMF, MeOH, and DCM 3 times each, intermediate **13** (1.5 equiv. relative to the load for **10**) was coupled onto the side chain of lysine under conditions of 1.5 equiv of DIC/HOSu/DMF, which led to the resin-bound taxoid **7-*O*-MTC-01**. Target compound **7-*O*-MTC-01** was cleaved off the resin using 10% HOAc/DCM for 2 h at room temperature. The cocktail cleavage solution was concentrated under reduced pressure. The white residual powder was dissolved in methanol for LC-MS analysis. The gradient was buffer B (5–95%), used for 5 min on an RP C18 column (4.6 μm×50 mm). Buffer A: 0.05% TFA/H_2O; buffer B: 0.05%TFA/ACN. Electronic spray ionization MS (ESI-MS) was carried out on a ThermoFinnigan, LCQ-Advantage that offered the correct molecular weight. The purity of the crude compound was judged by a UV detector at 214 nm wavelength, to be over 90%, which was directly characterized by NMR spectroscopic methods without purification.

ESI-MS: 1646.4 $[M+H]^+$.

General procedures of biological assay

Reagents Lipopolysaccharide (LPS, E coli 055:B5). Muramyl dipeptide (MDP) was synthesized in our laboratory. Taxol was a gift from Professor Qicheng Fang. ELISA kits for murine recombined TNF-α and IL-12 were from R&D systems. TRIzol reagent and M-MLV reverse transcriptase were from GIBCO-BRL. Taq DNA polymerase was from TaKaRa and T4 polynucleotide kinase and Poly(dI-dC) were from Amersham Pharmacia Biotech.

Animals Male C57BL-6J mice (H-2b, 17 g±1 g, 6–7 weeks old) were from the Experimental Animal Center, Chinese Academy of Medical Sciences & Peking Union Medical College (SPF, certificate No. SCXK 11-00-0006). All animals were housed in groups under 12 h regime (lights on from 7:00 h to 19:00 h) at 23±2°C prior to the experiments, and were given standard laboratory chow and tap water *ad libitum*.

Cell Culture The human cancer cell lines KB (head and neck cancer), HeLa (cervical cancer), BGC-823 (stomach

cancer), A2780 (ovarian cancer), MCF-7 (breast cancer), PC3M (prostate cancer), KeTr3 (renal cancer), HCT-8 (colon cancer), BEL-7402 (hepatic cancer), and a human embryo lung fibroblast cell HELF, were maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and 50 units/mL of both penicillin and streptomycin, in a humidified 5% CO_2/air atmosphere at 37°C.

Cytotoxicity Assay MTT assay was used to determine the cytotoxicity of drugs. Briefly, Logarithmic cells were plated in the 96-well plates at concentration of 1,200–1,500/100 μL per well. Drugs at final concentrations of 0.1 pmol/L to 100 μmol/L were added with quadruplicates of each concentration after 24 h. The cells were incubated further at 37°C for 72 h, the medium was aspirated, and 100 μL MTT of 0.5 mg/mL in medium was added. After 4 h incubation, the medium was aspirated and 100 μL DMSO was added to solubilize the formazan crystals. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650 nm. The dose–response curves were fitted with Sigma Plot and IC_{50}s were determined.

Isolation of murine peritoneal macrophages Murine peritoneal macrophages were harvested from male C57BL-6J mice 3 days after the injection (ip) of brewer thioglycollate medium (50 mL·kg^{-1} body weight), washed once in D-Hank's buffer and resuspended in complete RPMI-1640 medium. Peritoneal macrophages were seeded at densities of 1.2×10^6 cells/mL in 24 wells plate and allowed to adhere for 2–3 h in a 5% CO_2 humidified atmosphere. The non-adherent cells were removed by washing the plates twice with prewarmed medium.

ELISA kits for cytokine determination Adherent peritoneal macrophages (1.2×10^6 cells/mL) were treated with test compound with or without the LPS (1 μg/mL) for 24 h, and cell supernatants were collected and levels of TNF-α and IL-12 was measured by a procedure of commercial ELISA kit.

RT-PCR for cytokine and surface molecular gene expression The total RNA was extracted from 5.0×106 macrophages treated with different concentrations of testing compound for 10 h. Cultured macrophages were washed and the RNA was extracted with the TRIzol reagent according to the recommendation of the manufacturer. First strain cDNA was synthesized from equal amount of total RNA with M-MLV reverse transcriptase and random hexamer. Genes were amplified by PCR using sense and anti-sense primers of TNF-α, IL-12 p40 as described before with some modifications. Primers were as follows: TNF-α: sense: AAAAGATGG GGGGCTTCCAGAACTC, anti-sense: AGATAGCAAATCGGCTGACGGTGTG; IL-12p40: sense: AAACAGT GAACCTCACCTGTGACAC, anti-sense: TTCATCTGCAAGTTCTTG GGCG; PCR annealing temperature: TNF-α: 58°C; IL-12: 56°C. Semi-quantitative RT-PCR was performed using GAPDH as an internal control to normalize gene expression for the PCR templates. The PCR products were studied on a 1.0% agarose gel and the amplified bands were visualized after staining with ethidium bromide.

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Supporting Information Available Characterization data for new compounds **3′-*N*-MTC-01**, **2′-*O*-MTC-01**, **7-*O*-MTC-01** and key intermediates of **12**, **13**. This material is available free of charge via the Internet.

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