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New muramyl dipeptide (MDP) mimics without the carbohydrate moiety zas potential adjuvant candidates for a therapeutic hepatitis B vaccine (HBV)

Nan Zhao [a,†], Yao Ma [a,†], Shengmei Zhang [a], Xin Fang [b], Zhenglun Liang [b], Gang Liu [a,*]

[a] Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, 2 Nanwei Rd., Xicheng District, Beijing 100050, PR China
[b] National Institute for the Control of Pharmaceutical and Biological Products, 2 Tiantan Xili Rd., Dongcheng District, Beijing 100050, PR China

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ABSTRACT

A series of new muramyl dipeptide (MDP) mimics were designed and synthesized via a solid-phase synthetic route. Their adjuvant activities were evaluated ex vivo for investigation of the synergism of the S_{28-39} peptide, which is an MHC class I binding epitope of recombinant hepatitis B surface antigen (HBsAg) for both humans and mice. Several compounds without the carbohydrate moiety exerted better adjuvanticity than the MDP-C that has been reported by our laboratory previously. A primary screening test revealed that compounds **6**, **14** and **16** exhibited stronger adjuvanticity compared with other MDP mimics.

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Hepatitis B virus (HBV) infection affects about 350 million people globally and is a leading cause of hepatocellular carcinoma and mortality.[1] Although the antiviral drugs currently available efficiently decrease HBV load in the human serum, they fail to eradicate infection because of the persistence of HBV covalently closed circular DNA (cccDNA) in hepatocytes and of the emergence of resistant viruses.[2] A vaccine designed to break tolerance and stimulate virus-specific T-cell responses in chronic HBV patients offers an interesting alternative therapeutic approach. The eradication of infected liver cells by both humoral and cytotoxic T-cell responses would be required for effective therapeutic vaccination.[3] However, alum-based vaccines can only be used to promote humoral and Th2-biased immune responses.[4] Therefore, the development of novel classes of adjuvant for an HBV therapeutic vaccine is an urgent clinical need.

The muramyl dipeptide (MDP, Fig. 1), which is the minimal immunologically active component of the peptidoglycan molecule of the bacterial cell wall,[5,6] is a potent adjuvant for antigens and induces both humoral and cell-mediated responses in animal models.[7] However, MDP yielded significant concomitant side effects in vivo, such as pyrogenicity,[8] poor penetration of cell membranes,[9] and rapid elimination,[10] which limit its use in clinical applications.

MDP-C (Fig. 1), which is a novel compound reported previously by our group, proved to be an apyrogenic, nonallergenic, and low-toxicity immunostimulator with potential for immunotherapeutic and prophylactic applications in diseases such as HBV and Severe Acute Respiratory Syndromes Coronavirus (SARS-CoV).[11,12] However, the structure of MDP-C is an integration with a muramic acid moiety, which renders the synthetic route difficult, costly, and time consuming. This Letter reported new MDP mimics (Fig. 2) in which the carbohydrate moiety was replaced by an aromatic group and which exhibited improved immunological activity.

The solid-phase synthetic route shown in Scheme 1 was employed for parallel preparation of novel MDP mimics. Rink Amide-AM resin was selected as the solid carrier. Fmoc-D-Glu-OtBu, different Fmoc-protected lipophilic amino acids, and *o*-nitro benzoic acid derivatives were assembled onto the resin successively under mild coupling conditions. The subsequent reduction of the aromatic nitro group in the presence of Tin(II) chloride and the reductive ammoniation steps were performed to produce resin-bound compounds successfully (f). Finally, the target products (g) were cleaved off the resin. Twenty new MDP mimics (Table 1) were obtained with a satisfied yield and were characterized fully using ^{1}H NMR and HR MS (TOF).

The ability of the new MDP mimics to enhance the antigenicity of the HBsAg MHC I restricted peptide (S_{28-39}: IPQSLDSWWTSL) was examined using an ELISPOT assay, to select promising compounds for further evaluation. BALB/c mice were immunized subcutaneously on day 0 and boosted on day 7 with S_{28-39} (100 μg in 100 μL of PBS) and the various MDP mimics (100 μg in 100 μL of PBS). MDP-C (100 μg in 100 μL of PBS) was used as a positive control. Mice were sacrificed 7 days after the last immunization. Splenocytes were collected and incubated with or without the

* Corresponding author. Tel.: +86 10 63167165; fax: +86 10 63165246.
E-mail address: gliu@imm.ac.cns (G. Liu).
† These authors contributed equally to this work.

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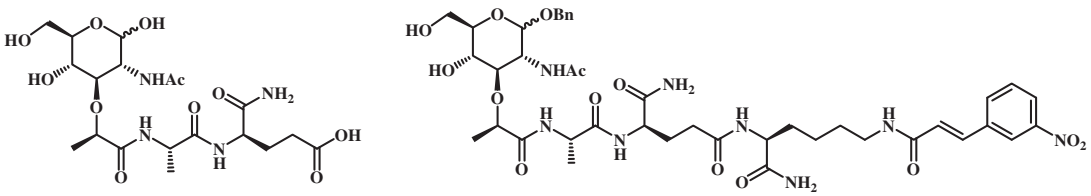


Figure 1. Structures of MDP and MDP-C.

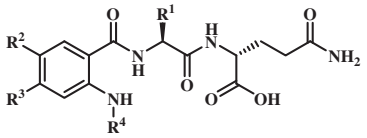


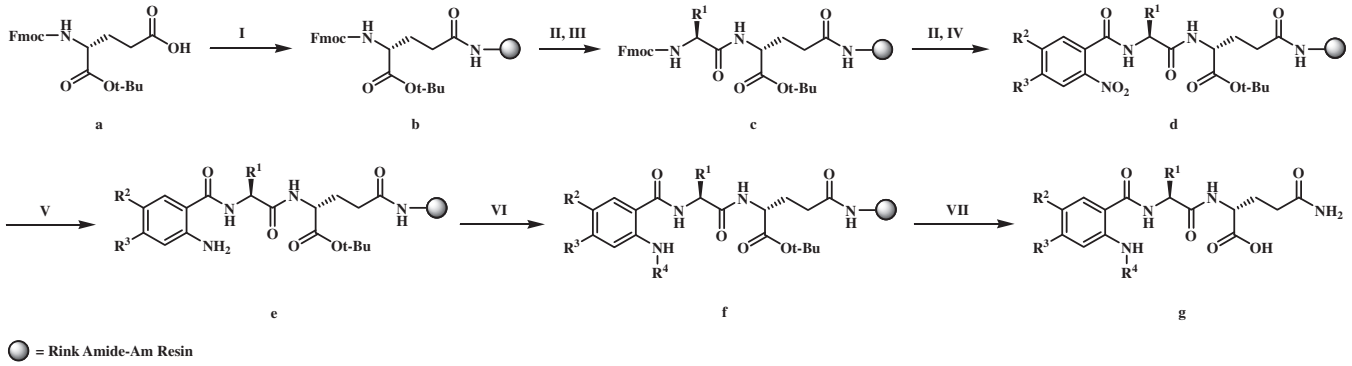
Figure 2. Structural diversity of MDP mimics.

HBsAg MHC I restricted peptide S_{28-39} (20 μg/mL) in ELISPOT plates. The difference in the number of IFN-γ-secreting cells obtained in the presence or absence of HBsAg peptide S_{28-39} stimulation was considered as an indicator of the HBsAg-specific cellular response. As illustrated in Table 1 and 70% of MDP mimics synergized the HBsAg peptide S_{28-39} to produce IFN-γ at significantly higher levels ex vivo compared with S_{28-39} alone. Among all the compounds tested, five (**1**, **2**, **6**, **14** and **16**) exhibited significantly better activity compared with MDP-C. Among them, compounds **6**, **14**, and **16** had the strongest adjuvanticity, as assessed using this test.

Analysis of structure–activity relations (SAR) revealed that the methyl and benzyl groups were more efficient R^1 groups regarding the enhancement of the immunological activity of MDP mimics (e.g., **6**, and **14**). Specially, the comparison of compounds **4**, **6** and **19** showed that increasing the lipophilicity of R^1 decreased the adjuvanticity, meanwhile, compound **4** basically lost its activity possibly due to the influence of isopropyl group in terms of increased lipophilicity or steric factors. The adjuvanticity of MDP mimics (e.g., **6** and **14**) was the strongest when R^2 was a chloro atom. Regarding the R^3 group, it seems that replacement with an electron-donating group, such as a methoxyl group, was unable to contribute significantly to the activity of the compound (e.g., **7**, **8**, **13**, **18** and **20**). The electron-withdrawing group was not included in this Letter. Obviously, R^4 is a critical factor that contributes to the immunological adjuvant activity of the whole molecule. MDP mimics exhibited the strongest adjuvanticity when R^4 was a phenylethyl group (e.g., **6**, **14** and **16**). It is worthy of note that derivation of aromatic amino group (R^4) of compound **15** using phenylethyl group to generate compound **16** resulted in extremely remarkable improvement of activity. Further introduction of a strong electron-withdrawing group, such as a nitro group or fluorine atom, onto phenylethyl group at R^4 doesn't lead to an apparent improvement in activity compared with the control group (e.g., **9**, **11** and **17**). Similarly, it also doesn't demonstrate dramatic effects on the activity for derivation of the aromatic amino group with certain unsaturated or saturated carbon chains (R^4, e.g., **18** and **20**).

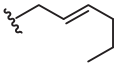
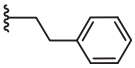
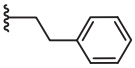
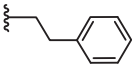
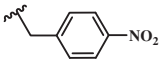
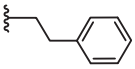
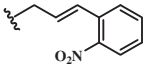
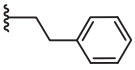
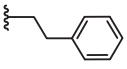
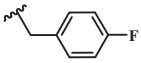
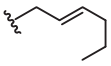
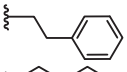
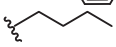
The new MDP mimics were composed of three building blocks. D-Gln was used to replace D-isoGln, to guarantee the pharmacophore configuration. The lipophilic amino acids at the N terminus of D-Gln, such as L-Val, L-Ala, L-Phe, L-Leu, and L-Ile, were then selected to assist the penetration of the cell membrane by the compounds. Herein, we first introduced *o*-amino benzoic acid or its derivatives to replace the muramic acid moiety, which greatly simplified the synthetic route and significantly improved the adjuvanticity of MDP ex vivo, in synergy with the HBsAg S_{28-39} peptide.

Previous studies of the SARs of MDP, MDP derivatives and MDP analogues revealed that the immunostimulating activity was specifically connected with the L-Ala-D-isoGln pharmacophore of the molecules. Only very limited variations are allowed regarding amino acid type (e.g., L-Ala to L-Val, D-isoGln to D-Gln) are allowed, but not regarding configuration changes (e.g., D-isoGln to L-isoGln).[13–15] This study further confirmed the necessity of the D configuration of glutamine, however, hints that D-Gln is the basic requirement of MDP mimics to guarantee its adjuvanticity. Most interestingly, the muramic acid moiety was fully replaced by an aromatic moiety for the first time, which completely changed the chemophysical properties of the molecule, from hydrophilicity to hydrophobicity. Obviously, this diversification prompts us to synthesize and characterize additional adjuvants in the near future.



Scheme 1. Reagents and conditions: (a) HOBt, DIC, and DMF, rt, 3 h; (b) 20% piperidine/DMF, rt, 20 min, two times; (c) Fmoc-protected amino acids, HOBt, DIC, and DMF, rt, 3 h; (d) *o*-nitro benzoic acid derivatives, HOBt, DIC, and DMF, rt, 3 h; (e) 2 M $SnCl_2$, NMM, and DMF, rt, 12 h; (f) organic aldehyde, NaH_3BCN, AcOH, and DMF; 40 °C, 36 h; (g) 95% TFA/H_2O, rt, 1 h.

Table 1
SAR investigation of twenty new MDP mimics

Compd.	R^1	R^2	R^3	R^4	IFN-γ SFCs/4 × 10^5 splenocytes[a]
1		Cl	H	H	212.5 ± 19[c,d]
2		H	H	H	224.0 ± 24[c,d]
3		H	H		164.0 ± 26[b]
4		Cl	H		14.3 ± 7
5		Cl	H	H	122.5 ± 15
6		Cl	H		316.0 ± 20[c,d]
7		CH_3O	CH_3O	H	186.8 ± 47[b]
8		CH_3O	CH_3O		185.8 ± 49[b]
9		H	H		102.0 ± 37
10		H	H		173.3 ± 21[c]
11		H	H		116.3 ± 22
12		H	H	H	139.5 ± 9[b]
13		CH_3O	CH_3O	H	144.3 ± 30
14		Cl	H		317.0 ± 46[c,d]
15		H	H	H	31.8 ± 11
16		H	H		304.3 ± 17[c,d]
17		H	H		130.3 ± 10[b]
18		CH_3O	CH_3O		117.8 ± 9
19		Cl	H		125.3 ± 9[b]
20		CH_3O	CH_3O		125.3 ± 29

[a] HBsAg-specific IFN-γ ELISPOT responses after immunization with various MDP mimics + HBsAg MHC I restricted peptide S_{28-39}. BALB/c mice were immunized subcutaneously on day 0 and boosted on day 7 with S_{28-39} (100 μg in 100 μL of PBS) and the various MDP mimics (100 μg in 100 μL of PBS), or S_{28-39} alone. MDP-C was used as a positive control. Mice were sacrificed 7 days after the last immunization and splenocytes were collected. Isolated splenocytes were tested for HBsAg MHC I restricted peptide S_{28-39}-specific IFN-γ secretion using the ELISPOT assay. Data were expressed as mean ± SEM.
[b] p <0.05 versus S_{28-39} alone.
[c] p <0.01 versus S_{28-39} alone.
[d] p <0.01 versus MDP-C.

In summary, we have designed and synthesized a class of new MDP mimics that are composed of three building blocks and include the replacement of the carbohydrate with an aromatic moiety. This was easily performed via the solid-phase synthetic route. All 20 compounds synthesized were primarily evaluated ex vivo through the investigation of the synergism between new MDP mimics and the S_{28-39} peptide. Compounds **6**, **14**, and **16** were the MDP mimics that exhibited the relatively strongest adjuvanticity. Since MDP and its mimics were reported to be NOD2 ligands[16–19] or TLR2/TLR4 agonists,[20] compounds **6**, **14**, and **16** may also act on NOD2 or TLR2/TLR4 signaling. Further research is needed to identify the target.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.056.

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