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

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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 S28–39 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.

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-o-Glu-OrBu, 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 1H NMR and HR MS (TOF).

The ability of the new MDP mimics to enhance the antigenicity of the HBsAg MHC I restricted peptide (S28–39: 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 S28–39 (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
HBsAg MHC I restricted peptide S28–39 (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 S28–39 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 S28–39 to produce IFN-γ at significantly higher levels ex vivo compared with S28–39 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 R1 groups regarding higher levels ex vivo compared with MDP-C. Among them, compounds 6, 14, and 16 showed that increasing the lipophilicity of R1 decreased the adjuvanticity of MDP ex vivo, in synergy with the HBsAg S28–39 peptide. The lipophilic amino acids at the N terminus improved the synthetic route and significantly improved the adjuvanticity of MDP mimics ex vivo, in synergy with the HBsAg S28–39 peptide.

MDP mimics exhibited the strongest adjuvanticity when R4 was a phenoylethyl group (e.g., 6, 14 and 16). It is worthy of note that derivation of aromatic amino group (R4) 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 R4 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 (R4, e.g., 18 and 20).

The new MDP mimics were composed of three building blocks. d-Glu was used to replace d-isoGlu, to guarantee the pharmacophore configuration. The lipophilic amino acids at the N terminus of d-Glu, 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 S28–39 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-isoGlu to d-Glu) are allowed, but not regarding configuration changes (e.g., d-isoGlu to l-isoGlu). This study further confirmed the necessity of the d configuration of glutamine, however, hints that d-Glu 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) HOBr, DIC, and DMF, rt, 3 h; (b) 20% piperidine/DMF, rt, 20 min, two times; (c) Fmoc-protected amino acids, HOBr, DIC, and DMF, rt, 3 h; (d) o-nitro benzoic acid derivatives, HOBr, DIC, and DMF, rt, 3 h; (e) 2 M SnCl2, NMM, and DMF, rt, 12 h; (f) organic aldehyde, NaH₂BCN, AcOH, and DMF; 40 °C, 36 h; (g) 95% TFA/H₂O, rt, 1 h.
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 S28–39 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 ligands16–19 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.

Table 1
SAR investigation of twenty new MDP mimics

| Compd. | R1 | R2 | R3 | R4 | IFN-γ SFCs/4 × 10^5 splenocytes |
|--------|----|----|----|----|-------------------------------|
| 1      | Cl | H  | H  | H  | 212.5 ± 19^c,d                |
| 2      | H  | H  | H  | H  | 224.0 ± 24^c,d                |
| 3      | H  | H  | H  | H  | 164.0 ± 26^b                  |
| 4      | Cl | H  | H  | H  | 14.3 ± 7                      |
| 5      | Cl | H  | H  | H  | 122.5 ± 7                     |
| 6      | Cl | H  | H  | H  | 316.0 ± 20^c,d                |
| 7      | CH2O| CH2O| H  | H  | 186.8 ± 47^d                  |
| 8      | CH2O| CH2O| H  | H  | 185.8 ± 49^b                  |
| 9      | H  | H  | H  | H  | 102.0 ± 37                    |
| 10     | H  | H  | H  | H  | 173.3 ± 21^c                  |
| 11     | H  | H  | H  | H  | 116.3 ± 22                    |
| 12     | H  | H  | H  | H  | 139.5 ± 9^b                   |
| 13     | CH2O| CH2O| H  | H  | 144.3 ± 30                    |
| 14     | Cl | H  | H  | H  | 317.0 ± 46^c,d                |
| 15     | H  | H  | H  | H  | 31.8 ± 11                     |
| 16     | H  | H  | H  | H  | 304.3 ± 17^c                   |
| 17     | H  | H  | H  | H  | 130.3 ± 10^b                  |
| 18     | CH2O| CH2O| H  | H  | 117.8 ± 9                     |
| 19     | Cl | H  | H  | H  | 125.3 ± 9^b                    |
| 20     | CH2O| CH2O| H  | H  | 125.3 ± 29                    |

^a HBsAg-specific IFN-γ ELISPOT responses after immunization with various MDP mimics + HBsAg MHC I restricted peptide S28–39. BALB/c mice were immunized subcutaneously on day 0 and boosted on day 7 with S28–39 (100 μg in 100 μL of PBS) and the various MDP mimics (100 μg in 100 μL of PBS), or S28–39 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 S28–39-specific IFN-γ secretion using the ELISPOT assay. Data were expressed as mean ± SEM.
^b p <0.05 versus S28–39 alone.
^c p <0.01 versus S28–39 alone.
^d p <0.01 versus MDP-C.
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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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