# *Leishmania donovani* in hamsters: Stimulation of non-specific resistance by some novel glycopeptides and impact on therapeutic efficacy

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*Summary.* Several glycopeptides structurally related to muramyl dipeptide (MDP) have been synthesized and evaluated for their ability to stimulate the non-specific resistance of hamsters against *L. donovani* infection. These compounds have been named CDRI compounds. The synthetic procedure used for compounds 86/448 and 84/212 is described.

MDP and its synthetic congeners were administered as immunostimulants at a prophylactic dose of 3 mg/kg at two weeks interval. The challenge infection ($1 \times 10^7$ amastigotes i.c./hamster) was given in between two doses of the compounds. One of the glycopeptides, CDRI comp. 86/448, has been found to be significantly more potent than MDP, effecting 92% inhibition of the challenge dose, whereas MDP produced only 26.5% inhibition. The effect of comp. 86/448 lasted until day 7 of challenge. The efficacy of sodium stibogluconate was appreciably improved in hamsters treated with comp. 86/448.

*Key words.* Hamster; *L. donovani*; CDRI comp. 86/448; MDP; non-specific resistance; stibanate.

Visceral leishmaniasis (V.L.) is a chronic and infectious disease which often becomes epidemic and leads to a heavy loss of human lives in many parts of the world. The causative organism of this disease, *Leishmania donovani*, is a flagellated protozoan parasite and resides within the macrophages of the RE system as an obligate parasite. The currently available drugs for the treatment of V.L. are only of limited value, because of their inherent toxicity[1,2]. Although they have been found to be effective in reducing the mortality to a considerable extent, it is not always possible to achieve a complete cure. The situation gets particularly complicated because of a severe impairment of the immune system of the infected host[3–6]. In order to overcome this problem, several attempts have been made to potentiate the immune system with the help of known immunostimulants. In the process, considerable success has been reported in the case of experimental leishmaniasis of mice and hamsters[6–10].

As part of a programme aimed at developing novel, chemically well-defined and clinically acceptable immunomodulators, several glycopeptides with a close structural resemblance to muramyl dipeptide (MDP) were designed and synthesized at the Central Drug Research Institute (CDRI) during the last few years. Five compounds, in which local conformational constraint was introduced by replacing the Ala residue of MDP with $N^{\alpha}$-methyl, $C^{\alpha}$-methyl and dehydro amino acids, were tested for their immunopotentiating properties in hamsters infected with *L. donovani*. The synthetic strategy, and the biological activity of these conformationally constrained analogues, Ac*nor*Mur-MeAla-D-Glu-$NH_2$ (84/212), Ac*nor* Mur-MeAla-D-Glu(NH.$C_8H_{17}$)-$NH_2$ (84/246), AcMur-$\Delta$Ala-D-Glu-$NH_2$ (86/247), AcMur-Aib-D-Glu-$NH_2$ (84/248) and Ac*nor*Mur-MeVal-D-Glu-$NH_2$ (86/448), are presented in this communication. Our results suggest that the MDP analogue, N-acetyl*nor*muramyl-L-N-methylvalyl-D-isoglutamine (CDRI comp. 86/448), exerts significant immunostimulatory effects and enhances the resistance of hamsters to infection.

## *Materials and methods*

*Parasite and host.* The strain of *L. donovani* (Man/IN/80/Dd8) was obtained from P.C.C. Garnham, Imperial College, London, in 1981. Since then, the parasites have been maintained in male golden hamsters (*Mesocricetus auratus*) of 40–45 g body weight through serial passages (amastigote to amastigote). Promastigotes were cultured in NNN medium with RPMI-1640 medium as an overlay. Promastigotes were removed from the culture medium, washed thrice in Locke's solution and counted in a haemocytometer. Appropriate inoculum suspensions were prepared for experimentation.

Golden hamsters obtained from the CDRI colony served as the host. These were provided with standard rodent diet (Hindustan Lever, Bombay) and water ad libitum.

*Synthesis of the glycopeptides.* All the glycopeptides were synthesized by coupling the required protected carbohydrate derivatives with appropriate dipeptide amines using DCC-HOB t or the mixed anhydride (MA) method and subsequent cleavage of the protecting groups by catalytic hydrogenation. The synthesis of protected carbohydrate moieties, 1-α-O-benzyl, 2-acetamido, 4,6-O-benzylidine-3-O-carboxyethyl-2-deoxy-α-D-glucopyranoside and 1-α-O-benzyl-2-acetamido, 4,6-O-benzylidine-3-O-carboxymethyl-2-deoxy-α-D-glucopyranoside, was achieved by the procedure described earlier[11–13].

For the synthesis of compounds 84/212 and 86/448, Z-D-Glu (ONBz1)-$NH_2$ (I) was obtained from Z-D-Glu-$NH_2$[14] by treatment with p-nitrobenzyl bromide in the presence of TEA. Removal of the Z group from (I) by HBr/AcOH treatment followed by coupling of the resulting amine with Z-MeAla[15] via the mixed anhydride method and with Boc-MeVal[16] by the DCC-HOB t procedure yielded Z-MeAla-D-Glu (ONBz1)-$NH_2$ (II) and

Boc-MeVal-D-Glu (ONBz1)-$NH_2$ (III), respectively. After the cleavage of the Z group from II and the Boc group from III by treatment with HBr/AcOH and $CH_2Cl_2$-TFA (1:1) respectively, the resulting dipeptide amines were condensed with 1-α-O-benzyl, 2-acetamido, 4-6-O-benzylidine-3-O-carboxymethyl-2-deoxy-α-D-glucopyranoside using the MA procedure, and the protected glycopeptides 1-α-O-benzyl-4,6-O-benzylidine-N-acetyl*nor*-Mur-MeAla-D-Glu(ONBz1)-$NH_2$ (IV) and 1-α-O-benzyl-4,6-O-benzylidine-N-acetyl*nor*Mur-MeVal-D-Glu-(ONBz1)-$NH_2$ (V) were obtained. Catalytic hydrogenation of IV and V in 60% AcOH over 10% Pd-C yielded the glycopeptides 84/212 and 86/448 which were characterized by reverse phase HPLC, $^{13}C$ NMR and elemental analysis. The glycopeptide 84/246 was also synthesized in a similar manner. Coupling of Z-MeAla with the amine obtained after HBr/AcOH treatment of Z-D-Glu-(NH $C_8H_{17}$)-$NH_2$ [17] by the MA procedure yielded Z-MeAla-D-Glu(NH $C_8H_{17}$)-$NH_2$ (VI). After the cleavage of the Z group from VI by catalytic hydrogenation, the resulting amine was coupled with 1-α-O-benzyl, 2-acetamido, 4,6-O-benzylidine-3-O-carboxymethyl-2-deoxy-α-D-glucopyranoside via the MA procedure to give 1-α-O-benzyl-4,6-O-benzylidene-N-acetyl*nor*Mur-MeAla-D-Glu (NH $C_8H_{17}$)-$NH_2$ (VII) which on catalytic hydrogenation in glacial AcOH afforded the glycopeptide 84/246. It was characterized by reverse phase HPLC, $^{13}C$ NMR, FDMS and elemental analysis.

The synthesis of glycopeptides 86/247 and 84/248 was accomplished as described in a recent communication [18].

*Formulation and immunopotentiation.* Five novel glycopeptides and muramyl dipeptide (MDP, Sigma Chemical Co., USA) were assessed for immunopotentiation. They were dissolved in chilled triple distilled water in the desired concentrations and administered to hamsters intraperitoneally within an hour of reconstitution.

*Experimental protocol. In vivo.* In one replicate, 3–5 animals were allocated for each test compound and an additional similarly constituted group which received placebo (D.W.) served as the control. The initial protocol for each test compound was the administration of 3 mg/kg × 2 on day −7, and repeat administration on day + 7 of chal-

Table 1. Prophylactic efficacy on novel glycopeptides in *L. donovani*-infected hamsters

| Sl. No. | Compound | Dose mg/kg | Groups | Parasite burden mean with range (no. of animals) | Percent inhibition mean ± SE | Significance 'U' & 'D' test |
|---|---|---|---|---|---|---|
| 1. | 84/212 | 3 × 2 | Exp. | 31.25 (7) (11–96) | 39.02 ± 11.4 | NS |
| | | | Control | 38.10 (17) (17–100) | | |
| 2. | 84/246 | 3 × 2 | Exp. | 41.3 (8) (13–90) | 23.98 ± 10.15 | NS |
| | | | Control | 38.1 (17) (17–100) | | |
| 3. | 86/247 | 3 × 2 | Exp. | 18.38 (7) (8–45) | 18.38 ± 9.6 | NS |
| | | | Control | 55.8 (17) (17–100) | | |
| 4. | 84/248 | 3 × 2 | Exp. | 43.5 (7) (20–70) | 10.98 ± 6.2 | NS |
| | | | Control | 38.1 (17) (17–100) | | |
| 5. | 86/448 | 6 × 1 | Exp. | 19.15 (13) (9–44) | 65.5 ± 4.8 | ** |
| | | | Control | 50.82 (17) (15–109) | | |
| | | 3 × 2 | Exp. | 7.15 (33) (0–25) | 91.9 ± 1.13 | ** |
| | | | Control | 60.4 (33) (10–250) | | |
| | | 1.5 × 2 | Exp. | 32.18 (11) (12–55) | 19.1 ± 6.5 | ** |
| | | | Control | 38.14 (7) (15–50) | | |
| 6. | MDP | 6 × 1 | Exp. | 34.17 (6) (13–57) | 27.7 ± 13.25 | NS |
| | | | Control | 43.50 (8) (9–100) | | |
| | | 3 × 2 | Exp. | 27.32 (22) (7–54) | 26.5 ± 5.1 | NS |
| | | | Control | 34.39 (28) (14–100) | | |

NS, Not significant; ** $p < 0.001$.

Table 2. Effect of comp. 86/448 on the chemotherapeutic response to sodium stibogluconate

| Drugs and dose | Groups | Parasite burden with range (No. of amastigotes/ 100 cell nuclei) Initial (on day 25–30 p.i.) | Final (on day 40–45 p.i.) | Percent inhibition ± SE | Difference in percent inhibition |
|---|---|---|---|---|---|
| Comp. 86/448 (3 mg/kg × 2) (d−7, +7) | Exp. (Stibanate therapy 10 mg/kg × 5) | 6.0 (6)* (1–22) | 11.6 (6) (3–20) | 84.1 ± 2.5 | |
| | Control | 7.6 (6) (0–22) | 72.3 (6) (4–130) | | + 20.5 ($p < 0.05$) |
| Untreated | Exp. | 29.5 (6) (9–66) | 48.6 (6) (28–98) | 63.3 ± 6.5 | |
| | Control | 17.0 (7) (4–31) | 89.2 (7) (60–150) | | |

* No. of animals

lenge infection with $1 \times 10^7$ amastigote per hamster (on day 0).

The drug effect was assessed on 25–30 days after infection by spleen biopsy[19,20].

One compound (86/448), which showed considerable immunopotentiation, was examined using different schedules. MDP was used as a reference drug.

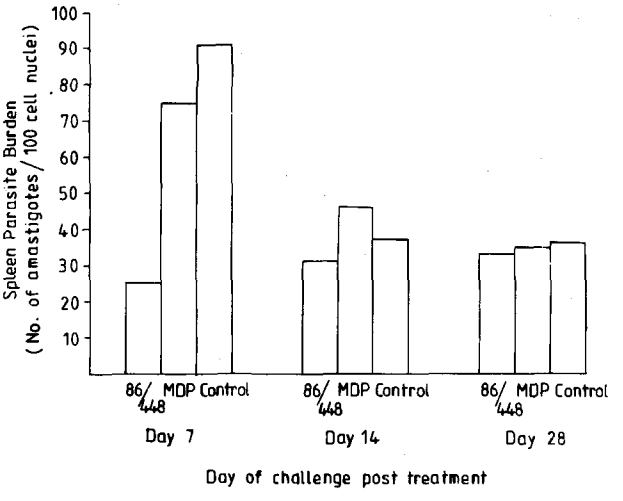
*Combination therapy.* Twenty hamsters were equally divided into two groups. Hamsters of group I were given comp. 86/448 according to the schedule described above. Half of these animals were treated with sodium stibogluconate (Stibanate®; Gluconate India Ltd) (10 mg/kg × 5) on day 25–35 p.i. and the other half served as an untreated control. The parasite assessment, on autopsy, was done on day 7 post-treatment, as described above. Group II, which received no immunomodulator but only stibanate, was used to compare the additive effect of immunomodulators.

*In vitro.* Peritoneal macrophages of normal hamsters, and of those treated with comp. 86/448 (6 mg/kg × 2) 7 days before, were harvested[21]. Macrophages ($1 \times 10^6$) were co-cultured with $1 \times 10^7$ promastigotes per tube in Leighton tubes containing coverslips, and containing RPMI medium with 10% foetal calf serum (GIBCO) under 5% $CO_2$ for 48 h[22]. After incubation, cover slips were removed, air dried, fixed in methanol and stained with Giemsa for microscopic examination. Assessment of infection was made by counting the percentage of infected macrophages and the average number of amastigotes per macrophage in experimental and control groups.

*Data analysis.* Because of existence of heterogeneity of variables the comparisons among experimental groups in vivo were made by the Fisher Behran 'D'-test and the Mann Whitney U-test.

## *Results*

*In vivo.* The data on immunopotentiation are presented in table 1. Comp. 86/448 showed marked superiority over MDP and other compounds. A single dose of 6 mg/kg given on day −7, or the 2 doses each of 3 mg/kg administered on day −7 and on day + 7 of infection, significantly reduced the parasitic load (91.9%). The most promising effect was obtained when comp. 86/448 was administered on day −7 of challenge. Administration of the compound on earlier days (day −14 or −28 of infection) had no effect (fig.). MDP was found to be much inferior in both the sets of experiments. Stibanate showed a better effect ( + 20% difference, $p < 0.05$) in hamsters receiving comp. 86/448 than in the group which received no immunomodulators (table 2).




Effect of comp. 86/448 and MDP at different time intervals in *L. donovani*-infected hamsters.

Table 3. In vitro susceptibility of peritoneal macrophages of comp. 86/448 treated hamsters to *L. donovani* infection

| Groups | Percentage of infected macrophages | Average number of amastigotes/ 100 macrophages |
|---|---|---|
| Comp. 86/448 | 11.0 (4)* ± 5.2 | 7.2 ± 4.2 (4) |
| Control | 55.8 (5) ± 19.42 | 70 ± 16.9 (5) |

* No. of experiments.

*In vitro*. The in vitro observations matched with those made in vivo (table 3). Only 11% of the macrophages obtained from comp. 86/448-treated hamsters were found to be infected on co-culturing. The parasitized macrophages had only 7.2 amastigotes per cell.
The infection rate in normal macrophages was 55.0% and each cell harboured on average 70 amastigotes.

*Discussion*

Chemotherapeutic management of V.L. requires intense and prolonged treatment with overtly toxic drugs. Under the circumstances, any auxiliary therapeutic measure which allows reduction in the amount of drug needed would be welcome. Among various factors which enhance the activity of drugs, immunity and immunostimulation are of considerable importance. With this view we used immunomodulators to make the host partially refractory to infection. Among the compounds tested, comp. 86/448 provided considerable immunostimulation, which is evident from the low parasite burden in animals treated with it. The experiments were designed in hamsters for which, unlike Balb/c mice, the chemotherapeutic extrapolation correlates well with the human situation[6]. Since hamsters exhibit wide variations in their reaction to *Leishmania* infection, and in addition there are seasonal fluctuations[23], the data were analysed using the 'D' and 'U' tests which to a great extent circumvent such variation.
Comp. 86/448, in which the muramic acid residue of MDP has been replaced by *nor*muramic acid and Ala with MeVal, was found to stimulate both humoral and CMI responses to a significantly higher extent than MDP (CDRI patent application no. NF/78/89 dated 5.9.89). This compound is the result of a systematic investigation of the structure-function relationship of MDP which was undertaken with the objective of getting more potent synthetic congeners that would be devoid of the toxic side effects of the parent substance, such as pyrogenicity and arthritogenicity. Since conformationally rigid analogues are generally expected to possess enhanced specificity of action due to enhanced conformational integrity, and are more stable to enzymatic degradation in the biophase[24-27], we decided to induce conformational constraint through peptide backbone modifications, i.e. the introduction of MeAle, MeVal, Aib and $\Delta$Ala in place of the Ala residue of MDP. It is obvious from the results of our study that replacement of Ala by MeVal, which may restrict the conformational freedom four-fold, leads to a congener (86/448) which exhibits the highest order of immunostimulant activity. Substitution by MeAla at the same position, which is known to restrict the conformational freedom only two-fold, results in a less active molecule (86/212). The corresponding hydrophobic analogue (84/246) exhibits a still lower order of activity; its activity is comparable to that of MDP. The highly rigid congeners of MDP (comp. 86/247 and 84/248) are nearly half as active as the parent molecule. It is noteworthy that the introduction of *nor*muramic acid in place of a muramic acid residue in the glycopeptides reported in this paper has led to compounds which are nonpyrogenic[28]. This finding is in agreement with earlier reports concerning the activity of *nor*MDP[29,30].
Comp. 86/448 was administered at 3 mg/kg × 2, given on day −7 and on day + 7 p.i., and similar immunostimulation to that produced by 86/448 was observed in *L. donovani* infected hamsters. A total of 6 mg/kg of the compound was sufficient for achieving maximum immunopotentiation, whether given once or in divided doses. Higher amounts did not enhance the degree of immunostimulation. Thus, prophylactically, 6 mg/kg × 1 was considered suitable for the second set of experiments. Further, the widening of the interval between sensitization and challenge caused a proportional waning of immunostimulation. The in vitro results corroborate the observations in the in vivo experiments.
The hamsters treated with comp. 86/448 responded better to stibanate therapy than the untreated animals. It is well documented that host immunity significantly adds to the therapeutic efficacy of the drug[31-33]. Misra et al.[34] have also demonstrated that each parasite requires a specific amount of drug for its elimination/killing. The superior action of stibanate observed in comp. 86/448-treated animals could be due to a low parasitic burden. MDP appears to be quite inferior to comp. 86/448 in terms of percentage inhibition of parasites. For optimum results, MDP has to be administered very near to challenge infection[6,35]. When MDP was administered 7 days before challenge (d-7), no immunostimulation in treated animals was observed. This is in agreement with the findings of Adinolfi et al.[6], Parant et al.[36], and Lederer[37], who failed to register any immunostimulation when MDP was administered only before challenge infection. Because of its weak immunopotentiation and pyrogenicity[37], MDP was not employed in combination therapy. The glycopeptide 86/448 thus appears to be promising from the point of view of its clinical exploitation as a potent immunostimulant. An exhaustive study dealing with different treatment protocols is now needed, in order to achieve absolute/near absolute cure of visceral leishmaniasis.

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# Pentaploidy in hybrid salamanders demonstrates enhanced tolerance of multiple chromosome sets

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*Summary.* Breeding populations of unisexual hybrid salamanders (genus *Ambystoma*) are dominated by allotriploid (3n) and allotetraploid (4n) forms, however, sexually mature allopentaploids (5n) may also occur. These are the first known naturally-occurring pentaploid vertebrates, and their genesis differs from that of previously studied autopentaploid urodeles induced or observed in the laboratory. The latter always suffered severely deleterious effects in development, and could not attain sexual maturity.
*Key words.* Vertebrates; unisexuality; allopentaploidy; *Ambystoma.*

Cytogenetically, pentaploidy is difficult to establish in vertebrates, and unlikely to be maintained due to meiotic irregularities[1,2]. Developmental and physiological constraints in pentaploids can lead to arrested or abnormal development at any life-history stage[1,2], resulting in drastically reduced individual survival. When physical, physiological or behavioral debilities have been over-ridden in the laboratory in order to study development in pentaploid urodeles, sexual maturation is the most recognizably affected ontogenetic trait[1,3]. Thus, the presence of adult pentaploid females in breeding aggregations of hybrid salamanders is of some significance. Here we describe the discovery of these first known naturally-occurring pentaploid vertebrates, and discuss the relevance of their genesis compared to laboratory pentaploids.

*Materials and methods*

During April of 1988 and 1989, we collected salamanders of the *Ambystoma laterale-jeffersonianum* complex[4] during breeding migrations to a pond in Haliburton, Ontario, Canada. In addition to *A. laterale*, a range of all-female hybrid biotypes of *A. laterale-jeffersonianum* constitute a large segment of the local breeding aggregate. In order to determine ploidy ratios, we initially separated *A. laterale* (2n) from hybrids (3n, 4n) based on morphological criteria[5]. Because ploidy classes within hybrids cannot be determined through visual inspection, ploidy analysis was carried out using flow-cytometry[6]. Individual salamanders were anesthetized in a weak solution of tricaine methanosulfate (MS 222), a few µl of blood collected from a toe-clip, and the animals released. Blood was frozen and stored following standard methods[6]. Nuclear DNA content of erythrocytes was measured[6], using propidium iodide (PI) as the nuclei stain. Samples were analyzed in an Argon-ion laser flow cytometer (EPICS V Flow Cytometer, Coulter Electronics, Hialeah, Fla). For each sample, 1000–10,000 nuclei were examined and histograms were accumulated by the MDADS computer