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RESEARCH PAPER

Molecular Adjuvants Based on Nonpyrogenic Lipophilic Derivatives of norAbuMDP/GMDP Formulated in Nanoliposomes: Stimulation of Innate and Adaptive Immunity

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ABSTRACT

Purpose The aim of this work was to demonstrate an immunostimulatory and adjuvant effect of new apyrogenic lipophilic derivatives of norAbuMDP and norAbuGMDP formulated in nanoliposomes.

Methods Nanoliposomes and metallochelating nanoliposomes were prepared by lipid film hydration and extrusion methods. The structure of the liposomal formulation was studied by electron microscopy, AF microscopy, and dynamic light scattering. Sublethal and lethal γ-irradiation mice models were used to demonstrate stimulation of innate immune system. Recombinant Hsp90 antigen (*Candida albicans*) bound onto metallochelating nanoliposomes was used for immunisation of mice to demonstrate adjuvant activities of tested compounds.

Results Safety and stimulation of innate and adaptive immunity were demonstrated on rabbits and mice. The liposomal formulation of norAbuMDP/GMDP was apyrogenic in rabbit test and lacking any side effect *in vivo*. Recovery of bone marrow after sublethal γ-irradiation as well as increased survival of mice after lethal irradiation was demonstrated. Enhancement of specific immune response was demonstrated for some derivatives incorporated in metallochelating nanoliposomes with recombinant Hsp90 protein antigen.

Conclusions Liposomal formulations of new lipophilic derivatives of norAbuMDP/GMDP proved themselves as promising adjuvants for recombinant vaccines as well as immunomodulators for stimulation of innate immunity and bone-marrow recovery after chemo/radio therapy of cancer.

KEY WORDS Adjuvant · Bone-marrow radioprotection · Liposome · Muramyl dipeptide · Vaccine

ABBREVIATIONS

AFM	Atomic force microscopy
APC	Antigen presenting cell
CFA	Complete Freund's adjuvant
CMI	Cell-mediated immunity

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DLS	Dynamic light scattering
DOGS-NTA-Ni	1,2-Dioleoyl-sn-Glycero-3-[[N (5-Amino-1-Carboxypentyl) iminodiAcetic Acid] Succinyl] (Nickel Salt)
EM	Electron microscopy
EPC	Egg phosphatidylcholine
GM-CFC	Granulocyte-monocyte colony-forming cells
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
MDP	Muramyl dipeptide
MTP-PE	Muramyl tripeptide phosphatidylethanolamine
MT01 – MT08	Lipophilic derivatives based on norAbuMDP and norAbuGMDP
NALP3	NACHT LRR and PYD domains-containing protein 3
NOD	nucleotide-binding oligomerization domain
norAbuMDP	NorMurNAc-L-Abu-D-iGln
norAbuGMDP	NorAbu-glucosaminylmuramyl dipeptide
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
rHsp90	Recombinant Heat shock protein 90
SEC	Size exclusion chromatography
TLR	Toll-like receptor

INTRODUCTION

The immunostimulatory or adjuvant-like activity of the peptidoglycan component of bacterial cell walls has been known for a long time. The minimal structural element of peptidoglycan with adjuvant and immunomodulatory activity is muramyl dipeptide (MDP) [1]. Regrettably, a broader use of MDPs in medicine has been limited by some undesirable side reactions including fever, nausea, hyper- or hypotension, thrombocytolysis, local necrosis, and haemorrhages, development of granuloma at the injection site, and induction of autoimmune diseases [2, 3]. More than one thousand MDP derivatives have been synthesized in the search for powerful stimulants of innate immunity (against cancer, bacterial and viral infections [4–6]) and of adjuvants enhancing specific antibody responses plus specific cell-mediated immunity (CMI) [2, 7–9].

In spite of the enormous effort of many scientists and the pharmaceutical industry, only one derivative has recently reached the stage of clinical application. However, the side effects were not suppressed. Mifamurtide (liposomal muramyl tripeptide phosphatidylethanolamine, MTP-PE) is a non-specific immunomodulator, which can enhance the effects of the standard chemotherapy of osteosarcoma patients, but is not free of severe side effects [10, 11]. On the other hand, new lipophilic analogues based on norMurNAc-L-Abu-D-iGln (norAbuMDP) [12, 13] have been synthesized (Fig. 1), and compared with MDP, these analogues appear to exhibit immunostimulatory activities with suppressed pyrogenity. With respect to intended application of these analogues, we recently selected liposomes as a platform delivery technology for the construction of immunomodulatory preparations and liposome-based vaccines [14–16].

In this work, we now summarize our long-term effort to design and synthesise effective and safe synthetic lipophilic norAbu-MDP and norAbu-glucosaminylmuramyl dipeptide (GMDP) based immunomodulators for formulation into two types of liposomes for the construction of recombinant vaccines and preparations applicable to stimulate innate immunity.

MATERIALS AND METHODS

General

Egg phosphatidylcholine (purity of 99%), 1,2-Dioleoyl-sn-Glycero-3-[[N (5-Amino-1-Carboxypentyl) iminodiAcetic Acid] Succinyl] (Nickel Salt) (DOGS-NTA-Ni) lipids were purchased from Avanti Polar Lipids (Birmingham, AL). Membrane filter Anotop 10 (pore size of 20 nm) and Anotop 10 LC (pore size of 0.2 μm) were purchased from Whatman (Maidstone, UK). All other chemicals were from Sigma (St. Louis, MO), unless else specified.

Characterization of Liposomes by Transmission and Scanning Electron Microscopy

The structure of the liposomes was determined using Philips Morgagni transmission electron microscope (EM Philips 208 S, MORGAGNI software, FEI, CZ). All samples were negatively stained with 2% (w/w) ammonium molybdate (pH 6.8). Scanning electron microscopy was performed on Hitachi SU 8010 (Hitachi, Japan). Samples were coated by Pt/Pd in the Cressington sputter coater 208HR (Cressington Scientific Instruments Ltd. UK) and observed at the magnification of 600 000, 15 kV.

Atomic Force Microscopy (AFM)

The topography of the liposomes and proteoliposomes was investigated by atomic force microscopy. The AFM measurements were performed with NTEGRA Prima NT MDT system (Ireland) under ambient conditions. The tip–sample surface interaction monitored the van der Waals force between the tip and the surface; this may be either the short-range repulsive force (in the contact mode) or the longer-range attractive force (in the non-contact tapping mode). The AFM measurements of the metallochelation liposomes and

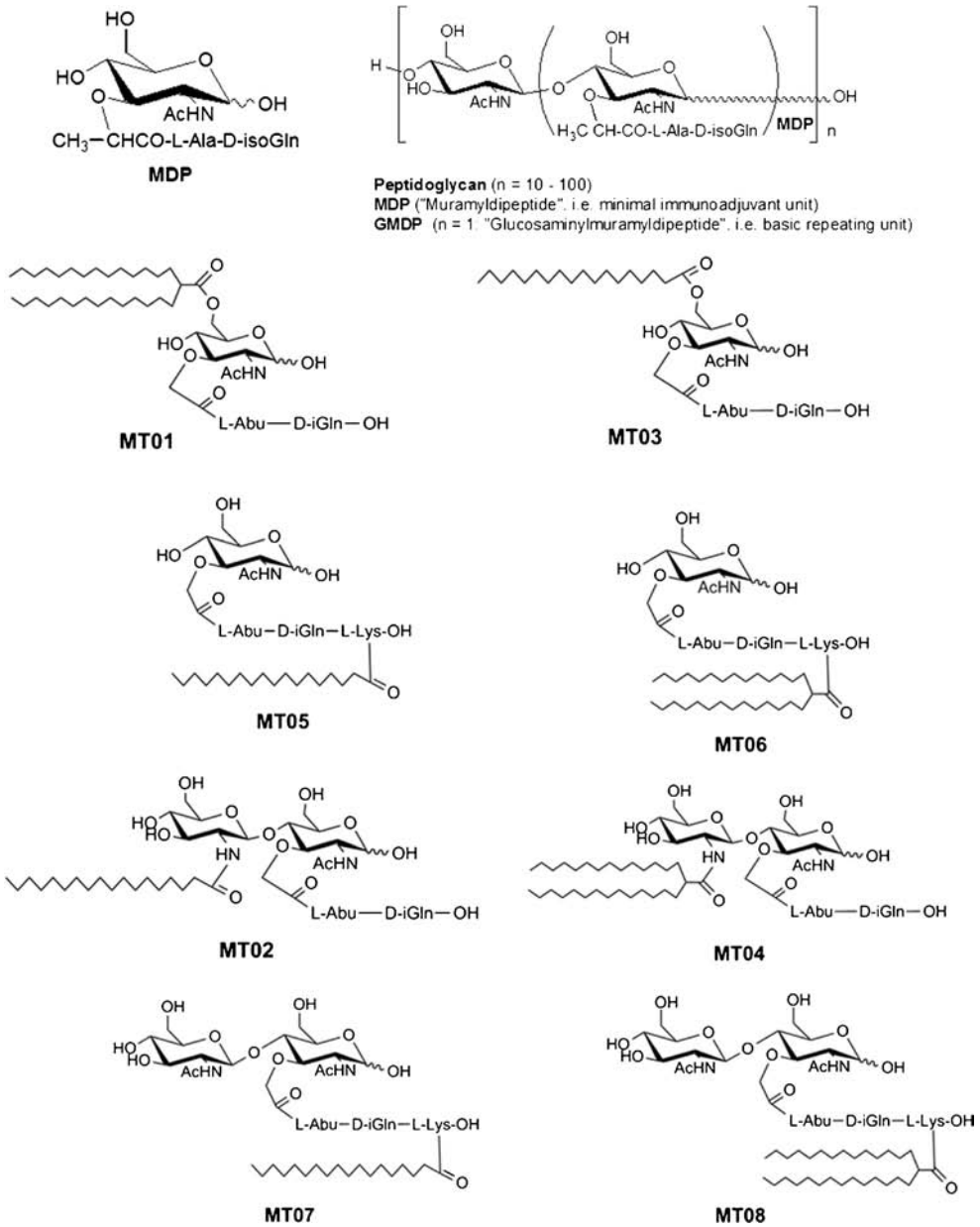

Fig. 1 Structural formulae of peptidoglycan, MDP, GMDP, and norAbuMDP/GMDP derivatives.

proteoliposomes were performed using the tapping mode. Each sample was scanned under the soft CSG 10 type of probe. The tapping mode consisted of oscillating the cantilever at its resonance frequency (14–28 kHz) and light "tapping" the tip on the surface during scanning.

Syntheses and Characterization of MDP Analogues

The syntheses of lipophilic derivatives based on nor-AbuMDP and nor-AbuGMDP (coded as MT01 – MT08) and the confirmation of their structures are described in patent cooperation treaty (PCT) by Ledvina and co-workers (Ledvina M, Turánek J, Miller AD, Hipler K. US8653049 B2, EP 2271661 B3) (Supp. info section D). The structural formulae are presented in Fig. 1.

Recombinant Antigen rHsp90

Recombinant (r) Hsp90 antigen was prepared using cDNA from a clinical isolate of *C. albicans* as reported elsewhere [17]. cDNA was used for the construction of prokaryote expression vector allowing the expression of rHsp90 both C'- and N'-terminally fused with His-tag in *E. coli*. Endotoxin was removed by successive two-phase separation with Triton X-114 as described elsewhere [18]. The entire procedure was repeated until the endotoxin level was below 0.25 EU

per 1 mg of protein (measured by Limulus amebocyte lysate according to FDA guidelines). Purity of the protein was above 95% (SDS PAGE).

Preparation and Characterization of Liposomes

Liposomes were prepared by the method based on hydration of a lipid film followed by extrusion through 0.2 and 0.1 μm polycarbonate filters in an analogous way to that described previously [19]. The hand-operated device Mini-Extruder (Avanti Polar Lipids) was used for the extrusion of small volumes of liposomes (up to 1 ml). Large volumes of the liposomes were extruded by means of a high-pressure cell (Millipore, Billerica, MA) linked with FPLC instrument (Pharmacia, Uppsala, Sweden). Liposomes with immunomodulators were composed of EPC/POPG/immunomodulator at the molar ratio of 76:19:5 mol%.

Metallochelating nanoliposome-based rHsp90 vaccine was prepared and characterized as recently described by our group [16, 20].

Characterization of Micelles and Liposomes by DLS, AFM and EM

The size distribution, zeta-potential and structures of various liposomal preparations were analysed by dynamic lighst scattering (DLS), Atomic force microscopy (AFM) and electron microscopy (EM) (supplementary material, section A, B).

Test of Pyrogenity and Side Effects

Standard pyrogenity test on rabbits was used to prove the safety of liposomal adjuvants. The test was carried out in the authorized laboratory ITEST Plus s.r.o. (Bílé Vchýnice 10, 533 13 Vápno u Přelouče, Czech Republic) according to the Czech Pharmacopoeia 2005. Details are described in the part C of supporting information.

Stimulation of Hemopoiesis *in Vivo* in Sublethally Irradiated Mice

Administration of Tested Compounds Prior to Sub-Lethal Irradiation

ICR mice (female, age of 3 months, 3–4 per group) were stimulated by *s.c.* administration of a tested preparations (volume of 200 μl, 100 nmol per dose) 24 h prior to sublethal γ-irradiation (6 Gy, ^{60}Co γ-ray source Chisostat (Chirana Ltd., Czech Republic) at the dose of 0.285 Gy/min). After 13 days, the mice were sacrificed and the recovery of granulocyte-monocyte colony-forming cells (GM-CFC) in femur was assayed by counting of colonies (CFC) grown up after cultivation in medium [21].

Administration of Tested Compounds after Sub-Lethal Irradiation

The tested preparations (volume of 200 μl, 100 nmol per dose) were administered 24 h after the sublethal γ-irradiation (6 Gy) and the effect was evaluated according to the same experimental protocol as described above. The statistical significance of the change of hemopoietic parameters was calculated by one-way analysis of variance. The Newman-Keuls test for the comparison of all pairs of columns and the Dunnett test for the comparison of all columns with the control column were used in the analysis.

Non-irradiated mice served as a control for normal GM-CFC count in femur. PBS and plain liposomes were used as controls for spontaneous recovery. Liposomal MDP was used as a reference standard.

Protection of Mice Against γ-Irradiation-Induced Death

Particular preparations (volume of 200 μl, 100 nmol per dose) were applied to ICR mice (female, the age of 3 months, 10 mice per group) by *s.c.* route 24 h prior to irradiation (radiation dose of 10 Gy). PBS and plain liposomes served as negative controls, free and liposomal muramyl dipeptide (MDP) served as positive controls. The methodological details of the model were published by Turanek and Kašná [21, 22].

Immunization and Adjuvants

Mice were divided into 8 groups, each of 5 animals, and immunized by two (priming, booster) *i.d.* applications of rHsp90 (20 μg per dose, vaccines formulated in apyrogenic PBS (phosphate buffered saline, pH 7.2) incorporated in metallochelation liposomes with different MT adjuvants) as indicated in Table I. The volume of each dose was 50 μl, the interval between priming and booster was 14 days. Complete Freund's adjuvant (CFA) and aluminium hydroxide (Bioveta, Ivanovice na Hané, Czech Republic) were used as adjuvant controls.

The animal experiments were approved by The Ethics Committees (Veterinary Research Institute, Brno and Palacky University, Olomouc) and were done according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals (Czech Society for Laboratory Animal Science).

rHsp90-Specific Antibody Response

rHsp90-specific serum antibody levels were determined by ELISA. Sera were collected from the experimental mice 1 day before boosting (post prime) and 14 days after boosting (post boost) by tail-vein incision. For individual sera, all assays were performed in triplicates. 96-well microplates (Nalge Nunc International, Rochester, NY) were coated with 100 μl of 1 μg/ml rHsp90. Bound antibodies were detected with

Table I Composition of rHsp90 Immunization Protocols Tested in this Experiment

Groups of 5 mice	Composition per one dose (50 μl)				
	rHsp90 (μg)	AlOH (μl)	Liposome (total lipid, μg)	CFA (μl)	MDP derivate (μg)
PBS (control)	–	–	–	–	–
rHsp90	20	–	–	–	–
Lip-Ni-rHsp90	20	–	35	–	–
rHsp90 AlOH	20	12.5	–	–	–
rHsp90 CFA	20	–	–	25	–
Lip-Ni-rHsp90 MT01	20	–	35	–	2.07
Lip-Ni-rHsp90 MT02	20	–	35	–	2.05
Lip-Ni-rHsp90 MT03	20	–	35	–	1.69
Lip-Ni-rHsp90 MT04	20	–	35	–	2.4
Lip-Ni-rHsp90 MT05	20	–	35	–	1.98
Lip-Ni-rHsp90 MT06	20	–	35	–	2.54
Lip-Ni-rHsp90 MT07	20	–	35	–	2.41
Lip-Ni-rHsp90 MDP	20	–	35	–	1.6

The dose of MDP derivatives is equivalent to 2.2 nmol per mouse

CFA and AlOH (Bioveta, Ivanovice na Hané, Czech Republic) were used as positive controls. The antigen formulation was prepared according to manufacturer's instructions. The FCA formulation was prepared by mixing the antigen with CFA (50/50 v/v), the Alum formulation was prepared by mixing the antigen with Alum (75/25 v/v). MDP, MT01 to MT07 were added in equimolar amounts, 9.3 nmol per dose. The liposomal formulations contained EPC/POPG/DOGS-NTA-Ni (76/19/5 mol%) or EPC/POPG/DOGS-NTA-Ni/MDP or MT01 to MT07 adjuvants (71/19/5/5 mol%)

horseradish peroxidase-labelled goat anti-mouse IgG + IgM + IgA (Ig total*) (MP Biomedicals, Solon, OH), goat anti-mouse IgG1 (MP Biomedicals, Solon, OH), goat anti-mouse IgG2a (Bethyl laboratories, Montgomery, TX), rabbit anti-mouse IgG2b (MP Biomedicals), or goat anti-mouse IgG3 (Bethyl laboratories), (MP Biomedicals, Solon, OH) and developed with O-phenylenediamine plus H_2O_2 substrate. The reaction was stopped with 1 M sulphuric acid and the absorbance was read at 490 nm. For antigen-specific ELISA, the results were expressed as mean end-point titre plus SD using Gen5 Software (release 2, BioTek Instruments, Vermont, USA).

RESULTS

Test of Pyrogenity and Safety of norAbuMDP/GMDP and its Lipohilic Analogues

Intravenous Administration

The pyrogenity of free analogues was tested. In comparison to MDP, norAbuMDP exerted a suppressed pyrogenicty owing to the structural changes in the molecule Table II. However, at higher doses (200 and 1,000 nmol per kg), this compound was proved to be pyrogenic similarly to MDP. Contrary to norAbuMDP, norAbuGMDP as well as the lipophilic analogues of both parent compounds were completely non-pyrogenic, even at higher doses (Table II).

Subcutaneous Administration

Pyrogenity of liposomal formulations of MDP and lipophilic derivatives was tested. A standard pyrogenity test (modified for *s.c.* application) was performed with rabbits. It showed that both free and liposomal MDP were pyrogenic. Sum + ΔT_{max} (3 rabbits per group) was 2.7°C and 2.9°C, respectively. Sum + ΔT_{max} for plain liposomes was 0.2°C. Both liposomal and free norAbuMDP, norAbuGMDP as well as their lipophilic derivatives MT were within the range of sum + ΔT_{max} of 0.4 – 0.6°C. Hence, these analogues were classified as non-pyrogenic (Table II). Pyrogenity data obtained after *i.v.* and *s.c.* application of the tested compounds are in a good accordance. No adverse or toxic effects were observed for norAbuMDP, norAbuGMDP and their lipophilic derivatives MT in rabbits during a two-week period.

Recovery of Hemopoiesis (GM-CFS in femur) in Sublethally Irradiated Mice (6 Gy)

Lipophilic MDP Analogues Administered 24 h Prior to Irradiation

Compared with controls at the 13th day, all the tested compounds improved the recovery of hemopoiesis. MT02 improved the recovery process only slightly, MT04 resulted in nearly 75% of normal level and MT05, MT06, MT07, and MT08 induced recovery to normal level comparable to the level of non-irradiated control. The effect was comparable to liposomal MDP (Fig. 2a). In contrast, free MDP affected the recovery only negligibly (*results not shown*).

Lipophilic MDP Analogues Administered 24 h After Irradiation

Compared with controls at the 13th day, all the tested compounds improved the recovery of hemopoiesis. MT02 and MT07 surpassed liposomal MDP and induced recovery to normal level (comparable with non-irradiated controls, C $p<0.01$). MT04, MT05, MT06, and MT08 resulted in about 50 – 60% of normal level. The effect was comparable to liposomal MDP (Fig. 2b).

Survival Curves of Lethally Irradiated mice Stimulated by Various Liposomal Preparations of Lipophilic Analogues of MDP

As for sub-lethal irradiation applied after priming the mice with the test analogues or controls, the best performance was found with analogue MT05, followed by the performance of a

Table II Pyrogenity Tests on Rabbits

Tested compouds											
subcutaneous application	liposomes	lip. MDP	Free MDP	lip. MT01	lip.MT02	lip.MT03	lip.MT04	lip.MT05	lip.MT06	lip.MT07	lip.MT08
+ΔTmax [°C]	0.2	2.7	2.9	0.5	0.7	0.3	0.7	0.5	0.4	0.6	0.8
Intravenous applicatio		free MDP, 40 nmol/kg	free MDP, 200 nmol/kg	free MDP, 1,000 nmol/kg	norAbu MDP, 40 nmol/kg	norAbu MDP 200 nmol/kg	norAbu MDP 1000 nmol/kg	norAbu GMDP, 40 nmol/kg	norAbu GMDP, 200 nmol/kg	norAbu GMDP, 1000 nmol/kg	
+ΔTmax [°C]		1.8	2.8	3.6	0.8	3.5	3.4	0.5	0.6	0.6	

A preparation was evaluated as nonpyrogenic, if the Sum + ΔTmax < 1.15°C (3 rabbits/group).
liposomal molecular adjuvants MT were applied at dose of 100 nmol/kg

"middle field" of other analogues. Overall protective effects were found in the case of MT01, MT02, MT05, MT06, MT07, and liposomal MDP (30–60% survivors). However, neither analogue MT04 nor free MDP showed any protective effects (Fig. 3).

Structure of rHsp90 Metallochelating Liposomes

Structures of metallochelating liposomes and proteoliposomes were studied by means of size exclusion chromatography (SEC), transmission electron microscopy (TEM) and AFM microscopy. All these methods clearly showed the molecules of rHsp90 bound onto the surface of liposomes (Fig. 4) leading to an increase in the hydrodynamic radii of the liposomes as confirmed by dynamic light scattering (*results not shown*).

Induction of Adaptive Immunity

Immune Response

Candida rHsp90 with various lipophilic analogues of norAbuMDP and norAbuGMDP formulated into liposomes was tested for immunogenicity after intradermal administration in mice. End-point titers of Hsp90-specific antibodies (Ig total) were measured by ELISA (Fig. 5a). As a positive control, non-liposomal formulations of rHsp90 with Alum (aluminium hydroxide) or complete Freund's adjuvant (CFA) were tested in separate groups of mice. Also rHsp90 was tested without adjuvant present either as soluble protein or in the form of a proteoliposome (Lip-Ni-rHsp90). The highest Ig titres were obtained after the second immunization (post boost) with rHsp90 plus Alum or CFA. Both approaches elicited detectable antibodies even after the first immunization (post prime). Among the tested MDP analogues, the strongest Hsp90-specific antibody response was detected for MT03, and a rather lesser response for MT07, MT06, and MT05. The effect of other MDP analogues was comparable to rHsp90 or Lip-Ni-rHsp90. These compounds, especially MT02, MT04 and MT01, even showed some suppression of the elicited specific response. In the group of mice immunized with Lip-Ni-rHsp90 plus MDP, only moderate Hsp90-specific antibody responses were elicited. It should be emphasized that no side effects were recorded post administration of norAbuMDP, norAbuGMDP, or analogues (MT01 - MT07) formulated into rHsp90 proteoliposomes. In contrast, serious local inflammation, induration, ulceration and/or necrosis was found typically in animal skin at sites of CFA applications (whether or not co-administered with rHsp90 antigen protein, data not shown) while administration of rHsp90 plus MDP adjuvant led to local irritation in mice.

Furthermore, the effect of particular MDP analogues on the magnitude of rHsp90-specific IgG isotype titers (IgG1,

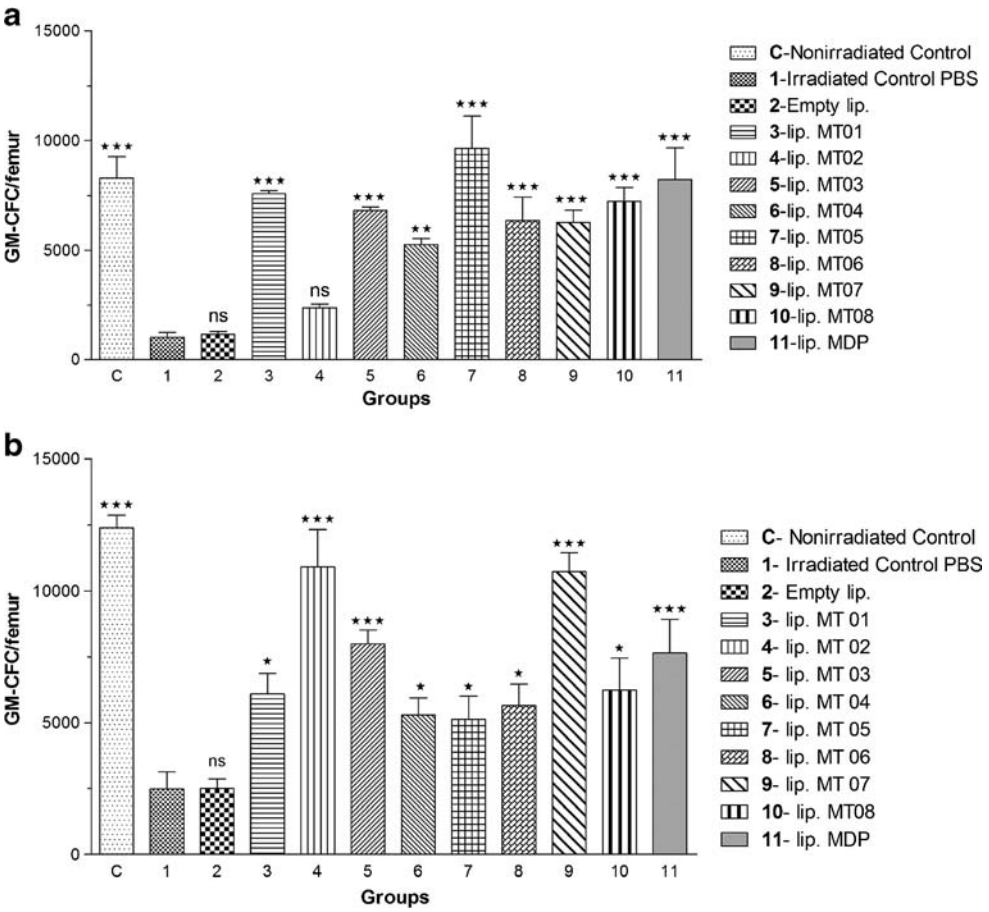

Fig. 2 Recovery of hemopoiesis (GM-CFC in femur) in sublethally irradiated mice (6 Gy). ICR mice (female, age of 3 months) were stimulated by s.c. application of the liposomal derivatives MT0X (200 μl, 100 nmol per dose) 24 h before (Fig. 2 A) or after (Fig. 2 B) sublethal γ-irradiation (6 Gy). The mice were sacrified at the 13th day after the irradiation and the recovery of GM-CFC in femur was assayed by counting of colonies (CFC) formed after the cultivation in medium. Non-irradiated mice were used as a control for normal GM-CFC count in femur. PBS and empty liposomes were used as controls for spontaneous recovery. Liposomal MDP was the positive reference standard.

IgG2a, IgG2b and IgG3) was measured by ELISA to assess the Th1/Th2-type polarization (Fig. 5b). For each of the tested norAbuMDP and norAbuGMDP analogues, substantially different Hsp90-specific antibody titers in IgG2a and IgG2b (Th1) *versus* IgG1 (Th2) isotypes or IgG3 isotype were detected. Data illustrate that some MDP analogues were optimal in eliciting Th1-type rHsp90-specific immune responses and others in eliciting Th2-type responses. It should be emphasized that Lip-Ni-rHsp90 formulated with MDP analogues MT03, MT07 and to a lesser extent MT06, MT05, and MT02 exhibited the capacity to elicit substantial rHsp90-specific titers in IgG2a and IgG2b isotypes indicating Th1 polarization of the specific immune response. CFA with rHsp90 elicited IgG2a and IgG2b antibodies even more effectively, however, due to its unacceptable adverse effects it was removed from comparison. On the other hand, Alum as

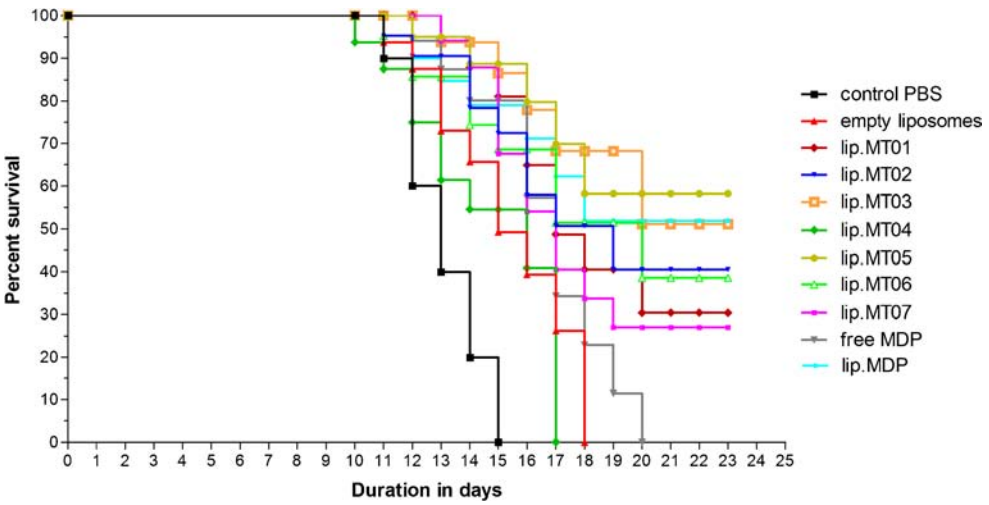

Fig. 3 Survival curves of irradiated mice stimulated by various liposomal preparations of lipophilic analogues of MDP. The mice of ICR strain, female, the age of 3 months. The preparations were applied s.c. 24 h before γ-irradiation (10 Gy).

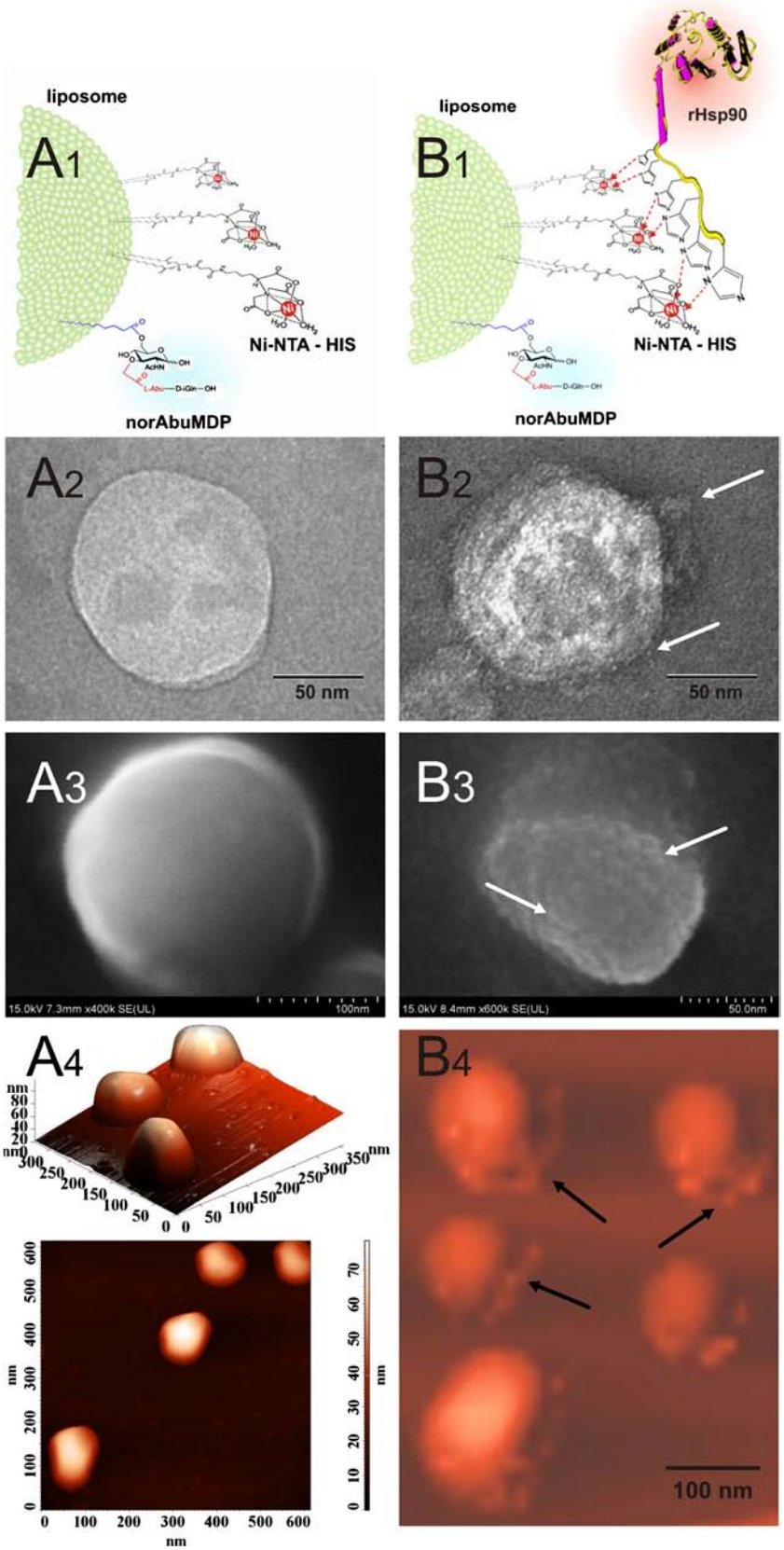

Fig. 4 Structure of metallochelating liposomes and proteoliposomes revealed by TEM, SEM and AFM. (**a**1) Schematic presentation of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. (**a**2) TEM photograph of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. (**a**3) SEM photograph of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. (**a**4) AFM photograph of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. (**b**1) Schematic presentation of a metallochelating liposome (**a**1) with rHsp90 bound via metallochelating bond. (**b**2) TEM photograph of a metallochelating liposome with rHsp90 bound via metallochelating bond. (**b**3) SEM photograph of a metallochelating liposome with rHsp90 bound via metallochelating bond. (**b**4) AFM photograph of a metallochelating liposome with rHsp90 bound via metallochelating bond. The arrows mark the molecules of liposomal surface-bound rHsp90.

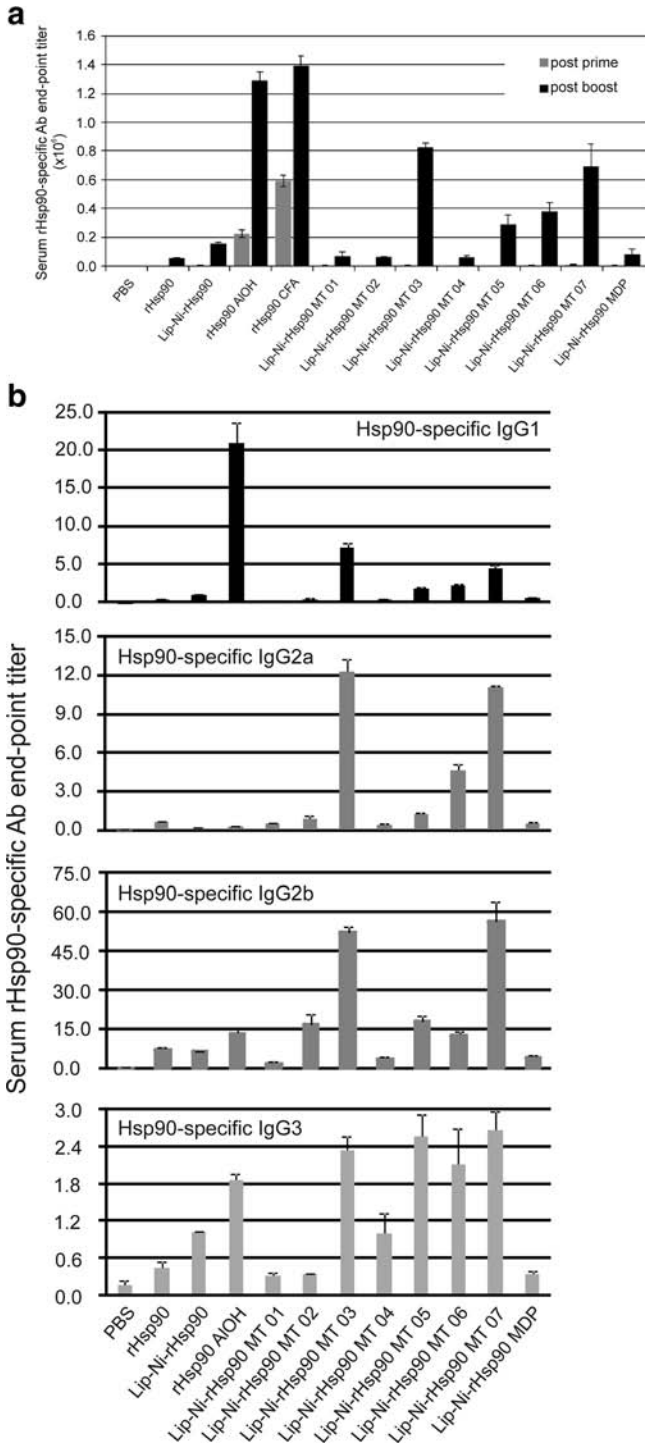

Fig. 5 Antibody responce towards rHsp90 antigen formulated in various experimental vaccines. (**a**) ELISA titres of specific antibodies against rHsp90 induced by various MDP analogues. Experimental mice (5 per group) were immunized by intradermal administration of various formulations of rHsp90 as indicated in Table I. PBS was administered to the negative control group. The rHsp90 represents free soluble rHsp90; Lip-Ni-rHsp90 represents metallochelating liposomes with bound rHsp90; rHsp90 AlOH represents free soluble rHsp90 with Alum; rHsp90 CFA represents free soluble rHsp90 in 50% Complete Freund's adjuvant; Lip-Ni-rHsp90 MT01, Lip-Ni-rHsp90 MT02, Lip-Ni-rHsp90 MT03, Lip-Ni-rHsp90 MT04, Lip-Ni-rHsp90 MT05, Lip-Ni-rHsp90 MT06, and Lip-Ni-rHsp90 MT07 represent metallochelating liposomes with rHsp90 and a respective lipophilic nor-AbuMDP or nor-AbuGMDP analogues of MDP; Lip-Ni-rHsp90 MDP represents metallochelating liposomes with rHsp90 and free MDP. Hsp90-specific IgG + IgM + IgA antibody (Ig total) titres were measured 1 day before the second immunization (post prime) and 14 days after the second immunization (post boost) by ELISA in triplicates. The results are expressed as the mean end-point titre and SD for each experimental group. (**b**) Effect of MDP analogues on Hsp90-specific mouse IgG isotypes after immunization. Experimental mice (5 per group) were immunized by intradermal administration of various formulations of rHsp90 as indicated in Table I. PBS was administered to the negative control group. The rHsp90 represents free soluble rHsp90; Lip-Ni-rHsp90 represents metallochelating liposomes with bound rHsp90; rHsp90 AlOH represents free soluble rHsp90 with Alum; rHsp90 CFA represents free soluble rHsp90 in 50% Complete Freund's adjuvant; Lip-Ni-rHsp90 MT01, Lip-Ni-rHsp90 MT02, Lip-Ni-rHsp90 MT03, Lip-Ni-rHsp90 MT04, Lip-Ni-rHsp90 MT05, Lip-Ni-rHsp90 MT06, and Lip-Ni-rHsp90 MT07 represent metallochelating liposomes with rHsp90 and a respective lipophilic nor-AbuMDP or nor-AbuGMDP analogue of MDP. The Hsp90-specific IgG1, IgG2a, IgG2b, and IgG3 antibody titres were measured 14 days after the second immunization (post boosting) by ELISA. The results are expressed as the mean end-point titres and SD for each experimental group.

the only clinically applicable adjuvant was ineffective in eliciting both IgG2a and IgG2b isotypes. For eliciting IgG1, the most effective immunization was Lip-Ni-rHsp90 with MT03 and MT07 and to a lesser extent MT05 and MT06. Furthermore, IgG3 rHsp90-specific titers were effectively elicited by Lip-Ni-rHsp90 formulated with MT03, MT05, MT06, and MT07 and to a lesser extent with MT04. The IgG3 titers obtained were comparable with those detected for rHsp90 plus Alum.

Moreover, no toxic effects scored by the test of general toxicity (Berlin test: including motoric disorder, respiratory problems, apathy, horrent fur, behavioral changes and loss of body mass) were recorded in mice treated with the experimental vaccines containing norAbuMDP or norAbuGMDP lipophilic derivatives. The test was complemented by dissection of the euthanized animals and inspection of their organs (weighing, microscopic observation of morphological changes) (*results not shown*).

DISCUSSION

Several MDP analogues and related compounds such as stearoyl-MDP derivatives, MTP-PE, FK565, FK156, and RP40639, murabutide and MDP (thr) have been reported to stimulate the host innate immune system against bacterial infections in experimental models [23, 24].

MDP-Lys (L18) (Romurtide) appeared on the market in Japan, and was widely applied to cancer patients, previously treated by radiation therapy, in order to restore white blood cells. The most common side effect of Romurtide administration was fever that was controlled by antipyretics. Other side effects, such as local reactions at the injection site, were transient [25, 26].

Another MDP analogue (MTP-PE, Mifamurtide) was approved for combined chemotherapy and immunotherapy of osteosarcoma. As shown in clinical trials, administration of MTP-PE was associated with hypersensitivity reactions along with pleural and pericardial effusions, seizures, and muscle spasms. Severe hearing loss occured in 12% of the Mifamurtide-treated patients *versus* 7% of other patients experiencing a pure chemotherapy regime [11]. The side effects mentioned above are tolerable for treatment of cancer but not for the application as adjuvants in prophylactic vaccines. This is the reason why no MDP analogue has been approved as an adjuvant yet.

Pharmaceutical Formulation of Lipophilic Analogues

The main goal in preparing various MDP-based therapeutic liposome formulations was to target adjuvants to enter relevant immune cells like dendritic cells or macrophages and combine with intracellular receptors. The lipophilic modification of MDP and its analogues represents a common approach for the improvement of their pharmacokinetics. On the other hand, the addition of a lipophilic moiety to hydrophilic glycopeptide core results in the creation of a surfactant-like molecular structure. Therefore, the formation of micelles and surfactant-related cytotoxicity must be taken in account.

All synthesized compounds of MT-series are able to form micelles (supplementary material Fig. 1S). Stearoyl derivatives form small well-defined micelles of the size of about 6–10 nm, while branched B30-modified fatty-acid analogues formed larger structures with a bimodal size distribution. Owing to the large lipophilic moiety, no sign of *in vitro* toxicity or haemolitic acitivity (rabbit red blood cell haemolytic test) was observed when mouse and human dendritic cells or peripheral blood monocytes, T and B lymphocytes were tested (*data not shown*).

Lipid micelles are metastable structures and their interaction with biological milieu and cells can hardly be predicted. Therefore, we focused on the application of multifunctional liposomes, that represent a well established, much more stable platform for the formulation and inclusion of hydrophobic or hydrophilic drugs and for the construction of vaccines [20, 27, 28] which we further modified to reach adjuvant-antigen multifunctionality. No haemolitic activities of liposomal formulations were observed *in vitro* in absence or presence of rabbit sera.

Stimulation of Innate and Adaptive Immunity, Mechanism (s) of Action

The mechanism (s) of action of MDP and its analogues are not fully understood. Therefore, *in vivo* immunological models are the best endpoint for demonstration of immunostimulatory and adjuvant acitivities of tested complex preparations.

We tested series of lipophilic norAbu-MDP and norAbu-GMDP analogues formulated into liposomes for the restoration of haemopoiesis in sublethally and lethally γ-irradiated mice. These models address several aspects of stimulation of innate immunity at the level of haemopoiesis, stimulation of immune cells (e.g. macrophages) and tissue regeneration. Therefore this *in vivo* model represents perfect end point for testing of such a complex event as is stimulation of innate imunity. The capability of the tested analogues to stimulate regeneration of bone marrow was demonstrated by determination of GM-CFC regeneration 13 days after sublethal γ-irradiation of mice. Most of the tested derivatives stimulated GM-CFC regeneration to a level equivalent with non γ-irradiated control mice. The effect was comparable to or better than that obtained with liposome formulated MDP (Fig. 2). Control γ-irradiated non-treated mice exhibited signs of a deep bone-marrow haematopoietic depression. Interestingly, some analogues differed in their ability to stimulate the regeneration of bone marrow after γ-irradiation dependent on the time schedule of administration (pre- or post-γ-irradiation regime). In the post irradiation regime the highest efficacy was exhibited by formulations containing MT-02 and MT-07. This could be of interest with respect to possible combination with drugs causing leukopenia (e.g., cytostatics) or in the case of radiation therapy, or even post-irradiation therapy of accidentally irradiated persons.

The *in vivo* model of lethally γ-irradiated mice proves the potential of MDP analogues to induce complex protective and regenerative mechanisms including effective and long-lasting activation of macrophages that prevent spread of septicemia from injured intestines in the early days post-irradiation. New findings about the expression of various peptidoglycan structural unit receptors like NOD-1 and NOD-2 in several cell types suggest that the activation of γδ T-lymphocytes could be one of the mechanisms responsible for protection of intestinal mucosa [29]. Recruitment and differentiation of mesenchymal stem cells [30] and activation of hepatocytes [31] are other events leading to protection and regeneration of injured intestine mucosa and induction of innate immunity. Together with the restoration of haemopoiesis, these processes are responsible for the increased survival of lethally irradiated mice pretreated with immunomodulators in liposome formulations [21].

On the other hand, unlike liposomal formulations, free hydrophilic MDP molecules are not effective *in vivo* at tested doses (Fig. 3). This is because of several factors including pharmaco-kinetic effects such as short-half-life effects owing to rapid clearance, dilution, lack of significant occurence at the site of interest, serum protein binding and enzymatic degradation. These unfavourable factors can be limited by appropriate means such as formulation with liposomes. After *s.c.* application, liposomes can penetrate through the extracellular matrix and can reach lymph nodes via lymphatic vessels [32].

Owing to the small size, liposomes are not completely retained in lymph nodes. Instead, they spread via the bloodstream and thereby reach the bone-morrow tissue. After passing through endothelial cell fenestrations, several cell populations could be targeted [32].

At the cellular and molecular level, the first evidence of the existence of a specific intracellular receptor for MDP was reported in 1989 by Tenu and colleagues [33]. Recent studies reveal NOD1, NOD2, and cryopyrin (NALP3) as possible intracellular receptors for peptidoglycan-(PGN-) derived units such as MDP (NOD2, NALP3) and D-γ-glutamyl-*meso*-DAP (iE-DAP), which can either be generated by a degradation of PGN in lysosomes or secreted by bacteria during replication [34, 35].

The mechanisms by which MDP or its analoges are able to cross the host's cell membrane to stimulate NOD1 and NOD2 remain incompletely understood. The same holds true for mechanisms responsible for the transport of peptidoglycan fragmets like MDP from phagosome into the cytoplasm. Both NOD1 and NOD2 are highly expressed in antigen presenting cells (APCs) such as monocytes, macrophages, and dendritic cells [36, 37]. Several recent studies identified NOD2 expression also in other haematopoietic lines like B-lymphocytes [38] or T-cells [39]. In addition, NOD1 is expressed in many epithelial cell subsets, whereas NOD2 seems to be more restricted to specialized cell types such as Paneth cells in the small intestine. NOD2 expression is potently induced by TLR ligands including LPS and by inflammatory mediators such as TNF-α, IFN-γ, and IL-17 in other non-haematopoietic tissues. Nevertheless it is not known whether the intracellular receptors for MDP can directly interact also with lipophilic analogues or if the lipophilic moiety has to be cleaved-off first. In the latter case, the character of the lipophilic moiety and its chemical linkage to the glycopeptides affects the biological activity by changing the pharmacokinetics at the tissue and intracellular level (Fig. 6). Our data support these considerations and these aspects are of interest for future research.

Immunogenicity of rHsp90 Mounted by Metallochelation to Liposomes in the Presence and Absence of norAbuMDP/GMPD Analogues

It is supposed that the activation of inflammasome is the basis for the potent adjuvant effect of MDP and its analogues [40]. The mounting of recombinant antigen proteins (such as rHsp90) on liposomes by metallochelation represent an interesting new system for the formulation of weak recombinant antigens with a His-Tag anchor [15, 16]. In the present work we tested the adjuvant effect of lipophilic MDP derivatives during immunization with recombinant *Candida* Hsp90 antigen that is a target for specific antibodies during systemic candidiasis [17, 41]. The IgG2a and IgG2b antibody response associated with Th1 dominance was elicited by the immunization with metallochelating rHsp90 liposomes containing MT03, MT07, or MT06 adjuvants. Such antibodies can act by opsonization, neutralization of extracellular virulence factors, inhibition of the *Candida* adherence to host tissues, inhibition of the yeast- to-mycelium transition, and by direct fungicidal activity [41].

Safety of norAbuMDP and norAbuGMDP Analogues

The main achievement of our approach based on structural changes in MDP (Fig. 1) was suppression of pyrogenity (Table II) and suppression of related flu like syndromes. Moreover, no side effects on rabbits (including skin lesions at the site of application) were observed during a 1-month period after the application. NorAbuMDP and especially norAbuGMDP analogues were shown apyrogenic at doses far in excess of those appropriate for a clinical application (e.g. as adjuvants in vaccines).

In this study, none of the tested analogue exhibited any toxic effects. This is in a good accordance with our previous experience with various animal species like mice, guinea pigs, goats, calves and pigs, which have been tested in our laboratory [21, 42–44].

At present, in addition to the immunostimulatory effect of MT03, MT07 and to a lesser extent MT06, leading to dominance in Th1 responses specific to *C. albicans* rHsp90, liposomal formulations of MT05 and MT06 are in testing as adjuvants for veterinary recombinant vaccines. No side effects have so far been observed after *s.c.* or *i.m.* application of experimental recombinant vaccine against boreliosis in dog puppies or cats (unpublished data). These results are promising for the intended application in human vaccines.

Potential Toxicity of Nickel

Toxicity of nickel is broadly discussed topic. With respect to metallochelating liposomes, it should be stressed, that the problem is quite different form toxicity on inhaled nickel oxide nanoparticles, which are accumulated in lungs of workers in metall industry and this form of nickel is responsible for chronic inflamation and lung cancer.

For the general population, the predominant route of exposure to nickel is through food intake. Nickel intake in the United States ranges between 69 and 162 μg/day for adults (>18 years of age) (http://rais.ornl.gov/tox/profiles/nickel_and_nickel_compounds_f_V1.html.) The estimated dose of nickel in vaccine represents only 1–5% of daily income.

In liposomes, Ni+2 is complexed in the metallochelating ligand in metallochelating lipids. This complex is extremly stable, therefore leakage of Ni ions is very slow (basic characteristic generally known from metalloaffinity chromatrography)

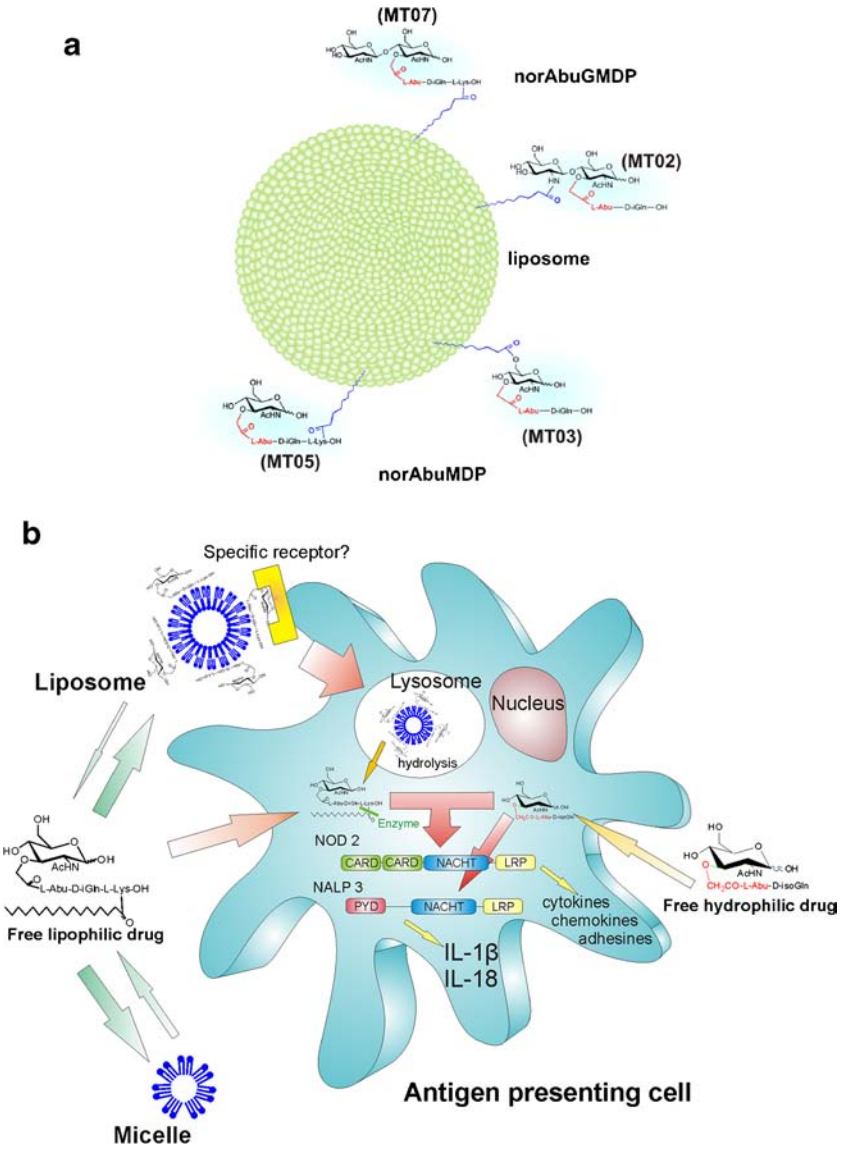

Fig. 6 Schematic structure of norAbuMDP/GMDP nanoliposomes and their pharmacokinetic pathways. (**a**) Schematic presentation of nanoliposome with various MT derivatives incorporated into bilayer. Various modifications of the norAbuMDP/GMDP molecule by lipidic residues affect the exposition of sugar- and peptide-moieties on the liposomal surface. Depending on the concentration of particular analogues or their mixture in the bilayer, new molecular patterns can be formed on the liposomal surface. These molecullar patterns can be recognized by cell-membrane receptors. (**b**) Schematic presentation of pharmacokinetic pathways of liposomal and free MDP analogues at cellular and intracellular levels. A free hydrophilic drug (e.g., MDP) has difficulties to cross the cell membrane and thus, it is difficult to reach an efficient intracellular concentration *in vivo*. The hydrophobized analogues of MDP form micelles whose pathways are difficult to be predicted owing to the complexity of the interactions with the components of biological milieu. Direct interaction with the cell membrane and penetration into cytoplasm is supposed to occour. The liposomal formulation of both hydrophilic and lipophilic derivatives of MDP is relatively stable in biological milieu and endocytozed by dendritic cells. In comparison to the free drug, liposomal formulations significantly increase the intracellular concentration of MDP analogues. Lysozomal and cytoplasmatic enzymes cleave the ester-bond-linking glycopeptide part to a hydrophobic residue. The importance of this pathway has not been recognized and fully understood yet. Nevertheless, it is reasonable to suppose that various derivatives will differ in their sensitivity to the enzymatic hydrolysis. Moreover, the mechanism of MDP-based activation of NOD and NALP3 receptors has not been precisely described and it is a hot issue. A question of a special relevance is, whether the hydrophobic residue of some derivatives has to be cleaved off to release the active glycopeptide molecule. The complexity of these processes is reflected in the variances in the effects of particular analogues having the same glycopeptide core but different position and structure of the hydrophobic residue.

and local long lasting increase of free Ni+2 is not the case. Moreover soluble nickel ions are not accumulated in the body, clearence is rapid and at least in animals nickel is essencial element. We have not seen any adverese effect on metallochelating liposomes containing nickel applied into several animal species. Our data are in very good accordance with the data from a Phase I Open-label Study of the Safety and Immunogenicity of Escalating Doses of Lipovaxin-MM (NCT01052142) (http://clinicaltrials.gov/ct2/show/NCT01052142?term=

NCT01052142&rank=1) regarding testing of anticancer vaccine based on metallochelating liposomal platform.

CONCLUSIONS

New lipophilic derivatives of norAbuMDP/GMDP proved themselves as promising molecular adjuvants for recombinant vaccines as well as immunomodulators for the stimulation of innate immunity and bone-marrow recovery after chemo/radio therapy of cancer. No side effects like pyrogenicity or local irritation (e.g. necrosis or ulceration) were observed in mice and rabbits. Liposome-based formulations were demonstrated to be an effective and safe application form for the tested lipophilic compounds.

At present clinical evaluation of a liposomal recombinant vaccine against Lyme boreliosis adjuvanted with lipophilic derivatives of norAbuMDP/GMDP is running in dogs and cats. Safety and induction of immune response has been demonstrated and data is in a good agreement with that published in this paper.

ACKNOWLEDGMENTS AND DISCLOSURES

By this paper we would like to revere the memory of deceased MUDr. Antonín Vacek, CSc., who pioneered the field of the effect of γ-rays on the immune system and haematopoiesis. His scientific work contributed to the cosmic flight of spaceships with human crew and was awarded by NASA and Russian space agency within the programme Intercosmos. Moreover, we would like to revere the memory of Prof. Antonín Holý, deceased in 2012, who pioneered the field of modern antiviral drugs. The development of new norAbuMDP/GMDP analogues would not have been possible without his long-lasting support to this project.

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Conflict of Interest The authors have declared that no conflict of interest exists. I certify that this manuscript, or any part of it, has not been published and will not be submitted elsewhere for publication while being considered by the journal Pharmaceutical Research.

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