In vivo effects of immunostimulating lipopeptides on mouse liver microsomal cytochromes P-450 and on paracetamol-induced toxicity

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Received 7 February 1989; accepted 16 May 1989

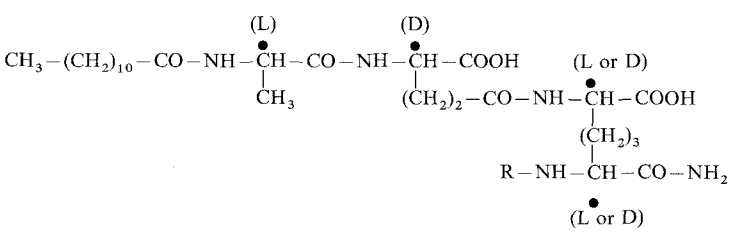
Summary. Immunomodulating lipopeptides lauroyl-L-Ala-γ-D-Glu-LL-A2pmNH2-Gly (RP 44.102) and lauroyl-L-Ala-γ-D-Glu-LL-A2pmNH2 (RP 56.142) were found to protect mice against the hepatotoxicity of paracetamol, which is due to cytochrome P-450 dependent formation of toxic metabolites and radicals. In fact they decreased the amount of hepatic microsomal cytochrome P-450, and the level of CCl_4-induced lipid peroxidation. In contrast lauroyl-L-Ala-γ-D-Glu-DD-A2pmNH2 (RP 53.204), which only differs by the configuration of the two chiral carbons of A2pm (diaminopimelic acid) and is not an immunomodulating agent, failed to protect against poisoning by paracetamol and had no effect on the level of hepatic cytochrome P-450 or the microsomal CCl_4-induced lipid peroxidation. This provides a clear connection between the immunostimulating properties of a compound and its effects on xenobiotic biotransformations.
Key words. Lipopeptides; immunomodulation; cytochrome P-450; lipid peroxidation.

The synthetic lipotetrapeptide, N2-(N-(N-lauroyl-L-alanyl)-γ-D-glutamyl) N6-(glycyl)-DD,LL-2,6-diaminopimelamic acid (lauroyl-L-Ala-γ-D-Glu-DD,LL-A2pm-NH2-Gly) is representative of a new family of substances exhibiting in vitro and in vivo immunopotentiating activities [11, 12, 25]. Experiments performed with the synthetic lauroyltetrapeptide (l-tetraP) isomers (fig. 1) containing either LL-A2pm (l-tetraP LLA2pm) or DD-A2pm (l-tetraP DDA2pm) led to the conclusion that only the compound with LL-A2pm is biologically active in vivo. Furthermore, the lauroyltripeptide (l-triP) lauroyl-L-Ala-γ-D-Glu-LL-A2pmNH2 was found to be as active as l-tetraP LLA2pm [5]. Both stimulate interleukin-1 production by murine macrophages and induce release of colony-stimulating factors (CSF) in the blood of mice, at the same doses as those which protect these animals against bacterial infections [4].

Several immunomodulators such as *C. parvum*, BCG, endotoxins, poly rI-rC and dextran sulfate have a marked effect on the hepatic metabolism of xenobiotics [2, 6]. They decrease the level of some hepatic cytochrome P-450 isozymes [9, 26]. This may explain, at least in part, their protective action against some toxic effects caused by compounds such as carbon tetrachloride [27] or paracetamol [19] which are known precursors of reactive electrophilic metabolites formed during cytochrome P-450-dependent reactions.

As these immunomodulators stimulate the secretion of immune factors, some of these factors, such as interferon [20], interleukin-1 [21] and soluble factors secreted by macrophages [15] or Kupffer cells [16] have been tested and were shown to be implicated in these alterations of hepatic drug metabolizing enzymes.

The present study was undertaken in order to determine whether the administration to mice of the immunostimulating lipopeptides l-tetraP LLA2pm and l-triP affects their hepatic cytochrome P-450 amounts and may lead to a protection against paracetamol-induced toxicity. A comparison of the effects of the non-immunostimulating lipotetrapeptide l-tetraP DDA2pm isomer, which only



R = NH_2–CH_2–CO– : l-tetraP LL or DD A2pm
R = H– : l-triP LL A2pm

Figure 1. Synthetic lipopeptides studied

differs by the configuration of the two chiral carbon atoms of the diaminopimelic acid, should allow one to confirm clearly the connection between the immunostimulating properties of a compound and its effects on xenobiotic biotransformations.

Materials and methods

The lauroyltetrapeptides l-tetraP LLA2pm (RP 44.102), l-tetraP DDA2pm (RP 53.204) and the lauroyltripeptide l-triP (RP 56.142) were synthesized by the Rhône-Poulenc Santé laboratories (Vitry/Seine, France).

Treatment of animals. 7–9-week-old female NMRI mice (Iffa Credo, St Germain L'Arbresle, France), were daily injected intraperitoneally (i.p.) for various periods with different doses of lipopeptide dissolved in saline. Control mice received saline only. Unless otherwise stated, food was withdrawn during 3 days (fasting mice) before the treatment.

In vitro experiments. Preparation of microsomes: the liver of each sacrificed animal was immediately removed and perfused with ice-cold saline solution. Hepatic microsomes were prepared as previously described[1]. Briefly, the liver was homogenized in ice-cold 50 mM TRIS-HCl buffer, pH 7.4, containing 0.2 M saccharose and 1 mM EDTA and centrifuged for 20 min at 10,000 × g and 4 °C; the supernatant was centrifuged for 1 h, at 105,000 × g and 4 °C and the microsomal pellet, suspended in ice-cold 100 mM sodium pyrophosphate, pH 7.4, was centrifuged under the same conditions; the final pellet was homogenized in 1 or 1.5 ml of ice-cold 0.1 M potassium phosphate, pH 7.4, containing 20% glycerol and stored at −80 °C until enzymatic determinations.

Determination of cytochromes P-450 and b_5: they were characterized and quantified by spectrophotometric methods[14] and correlated to protein levels determined according to Lowry[8].

Production of malondialdehyde (MDA) by liver microsomes: 1 mg of microsomal proteins was added to 1 ml of 0.5 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA; four assays were performed: 1) without any further additive, i.e. with NADPH alone; 2) with addition of lipopeptide; 3) with addition of 5 mM CCl_4 and 4) with addition of 5 mM CCl_4 and lipopeptide; assay mixtures were incubated for 10 min at 37 °C.

For chemically induced lipid peroxidation, 1 mg microsomal protein was added to 0.5 mM ascorbic acid in 0.1 M potassium phosphate buffer, pH 7.4 and incubation was initiated by addition of 0.05 mM ferrous sulfate. The amount of thiobarbituric acid-reacting substances, i.e. MDA, produced in each incubated solution was measured as previously described[7].

In vivo experiments. Protection of mice against a lethal dose of paracetamol[24] *by treatment with the lipopeptides*: Three days fasting mice, treated with lipopeptide as described above, were i.p. injected with 600 mg/kg of paracetamol (lethal dose of the drug).

In vivo amounts of exhaled ethane: immediately after poisoning, mice were placed into a 1-l chamber connected to a hydrocarbon-free oxygen reservoir. A 5-ml air sample was taken after 5 min, 1 h, 2 h and 3 h and was injected into a Packard gas chromatograph equipped with a flame ionisation detector and a carbosieve G column (Supelco, Bellefonte, Penn. USA) (1.50 m × 3 mm) at 150 °C; the carrier gas helium flowed at 30 ml per min. The ethane content of the air sample was determined by comparison with known amounts of ethane[10].

Survival of animals: 4 h after paracetamol poisoning the animals received food and were observed twice daily for 3 days; after this time no mortality was observed for more than one month. Statistical evaluation of survival was determined according to the 'Logrank test'[17].

Results

It has been shown that the in vivo effects of some immunostimulating agents on xenobiotic-metabolizing enzymes could lead to a protection against the hepatotoxic effects of CCl_4 or paracetamol and, in particular, to a decrease of the intense lipid peroxidation induced in vitro or in vivo by these two compounds[19,27]. In this study, two kinds of experiments were performed to determine the effects of the administration of the immunostimulating lipopeptides to mice: 1) ex vivo experiments on mouse liver microsomes including measurement of total cytochrome P-540 and a study of the ability of these microsomes to induce lipid peroxidation in the presence of CCl_4 and NADPH, a cytochrome P-450 dependent reaction; 2) in vivo experiments on mice treated with a lethal dose of paracetamol, including a measure of exhaled ethane as an index of in vivo lipid peroxidation and a measure of the survival of mice pretreated with the lipopeptides before paracetamol administration.

Ex vivo experiments. Treatment of normally nourished NMRI mice for 3 days with 10 mg per kg of l-tetraP LLA2pm and l-triP led to a marked decrease (about 33%) of the level of liver microsomal cytochrome P-450 but had no significant effect on cytochrome b_5; the depletion of cytochrome P-450 was correlated with the decrease of the cytochrome P-450/cytochrome b_5 ratio (table 1). Moreover, liver microsomes from mice treated with l-tetraP LLA2pm and l-triP exhibited a lower ability to produce MDA during lipid peroxidation induced by activation of CCl_4. It is noteworthy that the low level of lipid peroxidation induced by addition of NADPH alone to microsomes was not modified in microsomes from mice treated with the lipopeptides (fig. 2). Interestingly, the l-tetraP DDA2pm isomer had no significant influence on the amount of microsomal cytochrome P-450 nor on CCl_4-induced MDA production.

Since intoxication with paracetamol and survival studies were done with fasting mice, the effect of lipopeptides was also studied on fasting mice for 3 days before administration of lipopeptides. It was already known that starvation induced a decrease of liver glutathione, an in-

Table 1. Amounts of cytochrome P-450 and cytochrome b_5 in liver microsomes of NMRI mice treated with lipopeptides at a dose of 10 mg/kg.

Treatment	Number of animals	Cytochromes P-450 (nmole/mg prot.)	b_5	P-450/b_5 ratio
Nourished				
Saline	6	1.53 ± 0.10	0.68 ± 0.05	2.26
l-tri P	6	1.01 ± 0.15	0.63 ± 0.07	1.59
l-tetraP LLA2pm	6	1.00 ± 0.12	0.65 ± 0.05	1.52
l-tetraP DDA2pm	6	1.37 ± 0.14	0.74 ± 0.09	1.85
Fasting				
Saline	10	1.58 ± 0.07	0.69 ± 0.07	2.29
l-tri P	6	0.90 ± 0.02	0.62 ± 0.09	1.46
l-tetraP LLA2pm	10	0.89 ± 0.10	0.55 ± 0.06	1.62
l-tetraP DDA2pm	10	1.31 ± 0.07	0.59 ± 0.07	2.22

Underlined values are significantly different from control values (mean values: nmole/mg of protein ± SEM, Student-Fisher's t-test, $p < 0.02$ [23].)

Table 2. Influence of dose-dependent treatment of fasting mice with immunomodulating lipopeptides (3 days) on the level of their liver microsomal cytochromes P-450.

Dose (mg/kg)	l-tetraP LLA2pm P-450 (nmole/mg prot.)	l-triP P-450 (nmole/mg prot.)
Saline (a)	1.38 ± 0.07	1.38 ± 0.07
0.01	1.38 ± 0.04	1.50 ± 0.09
0.1	0.96 ± 0.08	1.06 ± 0.09
Saline (b)	2.07 ± 0.20	1.96 ± 0.07
0.5	0.95 ± 0.14	1.00 ± 0.06
1	0.97 ± 0.10	0.98 ± 0.10
5	1.00 ± 0.16	1.12 ± 0.01
10	1.22 ± 0.04	1.13 ± 0.15

Underlined values are significantly different from control values. (mean values of 3 animals/dose, nmole/mg of protein ± SEM, Student-Fisher's t-test, $p < 0.05$). (a) 10-week-old mice, (b) 7-week-old mice.

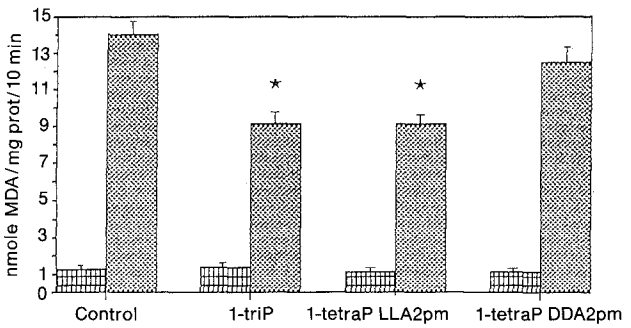


Figure 2. Ex vivo formation of MDA by hepatic microsomal preparations from normally nourished mice treated with lipopeptides. Mice were treated for three days with saline (control), l-triP, L-tetraP LLA2pm or l-tetraP DDA2pm (10 mg/kg, i.p.). 1 mg hepatic microsomal protein was incubated with NADPH alone (basal) or NADPH + 5 mM CCl_4 (NADPH + CCl_4) for 10 min at 37 °C. Mean values of 3 independent determinations (nmole/mg of protein ± SEM). * Significantly different from control values ($p < 0.02$).
▦ Basal; ▨ NADPH + CCl_4.

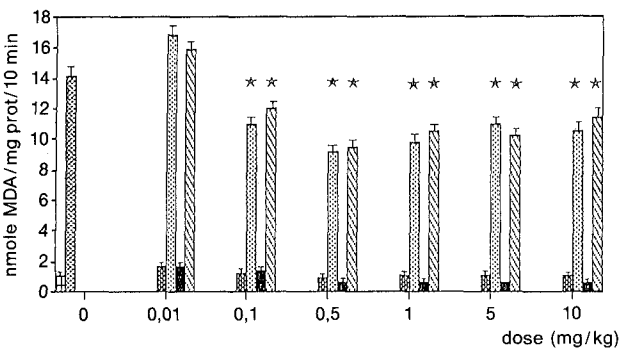


Figure 3. Dose-dependent ex vivo formation of MDA by hepatic microsomal preparations from fasting mice treated with lipopeptides. Fasting mice were treated for three days with lipopeptides (0.01 to 10 mg/kg, i.p.). 1 mg hepatic microsomal protein was incubated with NADPH alone (basal) or NADPH + 5 mM CCl_4 (CCl_4) for 10 min at 37 °C. Mean values of 3 independent determinations (nmole/mg of protein ± SEM). * Significantly different from control values ($p < 0.02$). ▦ Control basal; ▨ Control, CCl_4; ▩ l-triP basal; ▨ l-triP, CCl_4; ■ l-tetraP LLA2pm basal; ▨ l-tetraP LLA2pm, CCl_4.

crease of lipid peroxidation produced by paracetamol and a decrease of the lethal dose of this compound [24]. Thus, the use of fasting mice allowed us to obtain toxic effects with much lower doses of paracetamol. As shown in table 1, treatment with l-tetraP LLA2pm and l-triP led to a clear decrease (about 40%) of the liver cytochrome P-450 level and of the cytochrome P-450/cytochrome b_5 ratio. The non immunomodulating isomer l-tetraP DDA2pm had a slight but not significant effect on the cytochrome P-450 level and failed to modify the cytochrome P-450/cytochrome b_5 ratio.

It is noteworthy that neither the weight of liver nor the global amount of liver microsomal proteins was modified by treatment of fasting mice with the three lipopeptides (data not shown).

Dose-dependence was studied with l-tetraP LLA2pm and l-triP. Treatment of mice with 0.01 mg/kg of l-tetraP LLA2pm or l-triP had no effect on the level of their microsomal cytochrome P-450 (table 2) nor on the production of MDA by their liver microsomes in the presence of CCl_4 (fig. 3). A clear decrease of the cytochrome P-450 level and of the CCl_4-induced formation of MDA was observed already with 0.1 mg/kg. Maximal effect was obtained with doses between 0.5 and 5 mg/kg.

Whatever the dose, basal production of MDA induced by addition of NADPH alone was not modified, pointing out the effect of treatment by lipopeptides on CCl_4-induced but not on basal lipid peroxidation.

In vitro addition of lipopeptides (10^{-7} M–10^{-4} M) to liver microsomal preparations from untreated mice did not modify lipid peroxidation induced chemically (Fe-ascorbate system) or enzymatically (NADPH + CCl_4). This result showed that lipopeptides had no direct inhibitory effects on microsomal lipid peroxidation.

In vivo experiments. In the range of doses studied (0.5 to 10 mg/kg), l-tetraP LLA2pm and l-triP injected i.p. daily for 3 days before treatment of mice with a lethal dose of paracetamol led to a clear protection of these animals with up to 88% survival after administration of 5 mg/kg of l-tetraP (table 3). The maximum effect of l-triP (77%

Table 3. Effects of immunomodulating lipopeptide pretreatment on the lethality caused by paracetamol in mice and on the production of exhaled ethane.

Compound	Dose (mg/kg)	Survival of mice after 72 h Treated	Surviving (%)	Exhaled ethane (a) (% of control)
Saline	0	24	2 (8)	100
l-tetraP LLA2pm	10	23	14 (61)	29
	5	8	7 (88)	15
	1	14	6 (43)	14
l-tetraP DDA2pm	10	17	1 (6)	90
	5	6	0 (0)	nd
	1	6	0 (0)	nd
l-triP	10	27	14 (52)	42
	5	10	6 (60)	20
	1	22	17 (77)	11
	0.5	11	6 (55)	24

Animals were pretreated for 3 days with the lipopeptides and then treated with paracetamol the fourth day. Underlined values: significant number of surviving mice 72 h after poisoning with paracetamol of lipopeptide treated mice compared to saline treated animals (Logrank test, $p < 0.05$[17]). (a) mean values of 2 assays involving 2 mice each.

survival) was obtained with 1 mg/kg, and this compound still manifested a non-negligible activity at the lowest studied dose, 0.5 mg/kg.

With pretreatment only 24 h before paracetamol, using a dose of 10 mg/kg of l-tetraP LLA2pm and l-triP, about 50% survival was observed. No protection was observed, however, when mice were pretreated only 3 h before paracetamol injection.

The amount of exhaled ethane observed 3 h after administration of paracetamol to mice treated with different doses of both lipopeptides was significantly lower than that of control mice. With the most effective treatment for protection of mice (1 mg/kg of l-triP) ethane production was reduced to about 10%, corroborating the observed protective activity against the lethality of paracetamol. As for the survival assay, no influence of lipopeptides was established when they were injected 3 h before paracetamol. This inhibitory effect towards ethane formation appeared when the immunomodulating lipopeptides (10 mg/kg) were injected 24 h before paracetamol treatment; exhaled ethane decreased to 16% of the control values.

In the range of the studied doses, l-tetraP DDA2pm was totally inactive in the in vivo experiments of survival trial and production of exhaled ethane after paracetamol poisoning, as compared to control mice (table 3).

Discussion

The administration of a lethal dose of paracetamol to mice is responsible for a strong hepatotoxicity due to the formation of toxic metabolites and radicals dependent on cytochrome P-450 enzymes[13] and thus initiates lipid peroxidation, hepatic necrosis and then death[18].

The present data show that immunomodulating lipopeptides are able to protect mice against paracetamol toxicity. Using lipopeptide isomers, the relationship between their immunological properties and this protective effect was clearly shown; l-tetraP LLA2pm or l-triP, which are known to have immunopotentiating activities at the doses used[4,5], were also found to be active against paracetamol toxicity (significant survival up to 80% of mice) and led to a decrease of the in vivo ethane exhalation which can be used as an index of lipid peroxidation[24]. They also led to a decrease of the amounts of total hepatic microsomal cytochrome P-450 and of microsomal CCl_4-induced lipid peroxidation. In contrast, l-tetraP DDA2pm which only differs by the configuration of the two chiral carbons of A2pm, and which is not an immunomodulating agent, failed to protect against poisoning by paracetamol and had almost no effect on the hepatic cytochrome P-450 level and on the microsomal CCl_4-induced lipid peroxidation.

In contrast to antioxidant compounds, lipopeptides l-tetraP and l-triP have no direct in vitro effect on microsomal enzymes involved in the formation of reactive metabolites, since addition of these products to microsomal preparations did not lead to a decrease of chemically or enzymatically induced lipid peroxidation. In addition, their maximum protective effect against paracetamol toxicity was obtained only after their administration 3 days before paracetamol, although a slight response occurred after 24 h with the highest dose (10 mg/kg). No effect was observed after treatment 3 h before paracetamol administration (data not shown).

The mechanism of this protective effect has not been elucidated so far. It could be mediated by the decrease of some hepatic cytochrome P-450 isozymes which are responsible for the formation of toxic paracetamol metabolites, as was found for the interferon-inducer Poly rI-rC by Renton and Dickson[19]. This decrease of some cytochrome P-450 isozymes could also be responsible for the inhibitory effects observed after lipopeptide administration on ex vivo NADPH and CCl_4-induced lipid peroxidation, which is dependent on CCl_4 activation into $CCl_3^{\cdot}$ by some cytochrome P-450 isozymes[23]. It is noteworthy that ex vivo microsomal lipid peroxidation induced by NADPH alone, which could be dependent on other P-450 isozymes, was found to be unaffected by lipopeptide administration. Electrophoretic profiles of hepatic microsomal fractions from animals treated with active lipopeptides suggested that at least one cytochrome P-450 isozyme was decreased (unpublished data). Recently, high doses of endotoxins were proved to cause marked depression or even suppression of certain cytochrome P-450 isozymes whereas low doses potentiated the expression of other isozymes such as 3-methylcholanthrene-induced P-450[22].

Lipopeptides could be implicated in the in vivo depletion of some isozymes of cytochrome P-450 either directly by modifying their regulation, or indirectly by factors secreted under their influence such as interleukin-1[4], which has been found to depress cytochrome P-450[21], or other factors secreted by macrophages[15] or Kupffer cells[16].

Acknowledgments. The authors thank Geneviève Bouillé for her excellent technical assistance, Chantal Guihenneuc and Virginie Lasserre (Laboratoire de statistique médicale, URA 842 C.N.R.S., UER Paris V) for their adaptation of statistical program of Logrank test, and Rhône-Poulenc for the gift of synthetic lipopeptides and financial support.

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0014-4754/89/090882-05$1.50 + 0.20/0

Noradrenergic control of ascorbic acid and glutathione concentrations in brown fat of cold-exposed rats

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Received 10 August 1988; accepted 16 May 1989

Summary. Ascorbic acid and glutathione concentrations increase in brown fat of cold-exposed rats. This phenomenon can be reproduced by noradrenaline or isoproterenol administration, and thus seems to be under sympathetic control. Histological study shows ascorbic acid storage in brown adipocyte nuclei.
Key words. Brown fat; cold exposure; ascorbic acid; gluthatione; noradrenaline; α-agonist; β-agonist.

In several mammalian species, exposure to cold triggers redevelopment [1] of brown adipose tissue (BAT), a tissue specialized in thermogenesis [2]. This so-called 'trophic response' is the cumulative result of various events, including increases in cell number, in mitochondrial mass per cell, and in the mitochondrial concentration of uncoupling protein (UCP), the protein responsible for the thermogenic capacity of BAT [2].

BAT trophic response is also characterized by an increase in ascorbic acid (AA) concentration [3, 4], the exact role of which has not been demonstrated [5], and an increase in reduced glutathione (GSH) concentration, for which a 'protective mechanism' against the peroxidative effect of AA has been put forward [4]. It has been suggested that AA is stored in the tissue nerve supply [5]. If the 'protective' hypothesis is correct, AA must be stored, at least in part, directly in brown adipocytes. The preliminary part of this work was thus an investigation of AA localization in BAT using a histological approach.

In rats, most of the events involved in the trophic response to cold are triggered and maintained by an increased stimulation of the tissue by noradrenaline (NA), through increased sympathetic tone [1, 6]. The second aim of the present study was to determine whether the rise of AA and GSH concentrations in BAT of cold-exposed rats is also due to noradrenergic stimulation. We used the technique utilized to show the control of UCP level by NA, i.e. chronic administration of NA by implanted pumps in rats kept at room temperature [7]. Since BAT has both α- and β-adrenoceptors [8], α- and β-agonists were tested in addition to the natural sympathetic mediator.