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Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

Effects of liposomes with polyisoprenoids, potential drug carriers, on the cardiovascular and excretory system in rats

Olga Gawrys^a, Marta Polkowska^a, Malwina Roszkowska-Chojecka^a, Katarzyna Gawarecka^b, Tadeusz Chojnacki^b, Ewa Swiezewska^b, Marek Masnyk^c, Marek Chmielewski^c, Janina Rafałowska^d, Elżbieta Kompanowska-Jeziarska^{a,*}

^a Department of Renal and Body Fluid Physiology, M. Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

^b Department of Lipid Biochemistry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

^c Institute of Organic Chemistry, Polish Academy of Sciences, Warsaw, Poland

^d Department of Experimental and Clinical Neuropathology, M. Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

ARTICLE INFO

Article history:

Received 4 April 2013

Received in revised form 3 September 2013

Accepted 12 September 2013

Available online 3 March 2014

Keywords:

Liposomes

Drug delivery

Polyisoprenoid alcohols

Renal toxicity

Renal morphology

ABSTRACT

Background: The unpredictable side effects of a majority currently used drugs are the substantial issue, in which patients and physicians are forced to deal with. Augmenting the therapeutic efficacy of drugs may prove more fruitful than searching for the new ones. Since recent studies show that new cationic derivatives of polyisoprenoid alcohols (APrens) might exhibit augmenting properties, we intend to use them as a component of liposomal drug carriers. In this study we investigate if these compounds do not *per se* cause untoward effects on the living organism.

Methods: Male Sprague–Dawley rats received for four weeks daily injections (0.5 ml sc) of liposomes built of dioleoyl phosphatidylethanolamine (DOPE), liposomes built of DOPE and APren-7 (ratio 10:1) or water solvent. Weekly, rats were observed in metabolic cages (24 h); blood and urine were sampled for analysis; body weight (BW) and systolic blood pressure (SBP) were determined. After chronic experiment, kidneys and heart were harvested for histological and morphometric analysis.

Results: The 4-week BW increments were in the range of 97 ± 4 to $102 \pm 4\%$, intergroup differences were not significant. Microalbuminuria was the lowest in the group receiving liposomes with APren-7 (0.22 ± 0.03 mg/day). Water and food intake, plasma and urine parameters were similar in all groups.

Conclusions: Newly designed liposomes containing APren-7 did not affect functions of the excretory and cardiovascular systems, and renal morphology; therefore we find them suitable as a component of liposomal drug carriers.

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Introduction

A majority of currently used drugs display, in addition to the therapeutic action, definite side effects which are often severe and unpredictable; the difference between the therapeutic and

harmful dose may be quite small. This may necessitate monitoring drug concentration in the patient's serum, which is cumbersome and increases the cost of treatment [9]. In light of the recent literature of the subject, a search for substances which augment the therapeutic efficacy of existing drugs may prove equally fruitful as the development of the new ones. Drug delivery systems (DDS) which include many different conjugates, such as polymeric micelles, liposomes, hybrid carriers and polyplexes (complexes of the polymer–DNA type) [1,4] may provide better penetration of drugs across biological membranes which results in faster access to the interior of cells and, in general, to areas distant from the lumen of blood vessels. Currently, liposomal formulations which facilitate the penetration through biological membranes are widely used as carriers of pharmacologically active substances [24]. Cationic lipids of various structures have been suggested to enhance the

Abbreviations: APren, cationic derivative of polyisoprenoid alcohol; BW, body weight; DDS, drug delivery systems; DOPE, dioleoyl phosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; Ht, haematocrit; LV/BW, left ventricular – to – body weight ratio; NO, nitric oxide; NO_x, excretion of nitric oxide metabolites; PE, phosphatidylethanolamine; P_{Na}, plasma sodium concentration; P_{osm}, plasma osmolality; SBP, systolic blood pressure; sc, subcutaneously; UAE, urinary albumin excretion; U_K, concentration of potassium in urine; U_{Na}, concentration of sodium in urine; U_{osm}, urine osmolality.

* Corresponding author.

E-mail address: ellakomp@gmail.com (E. Kompanowska-Jeziarska).

<http://dx.doi.org/10.1016/j.pharep.2013.09.009>

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efficacy of liposomal drug and nucleic acids delivery [14,21]. Recent studies show that cationic derivative of polyprenol tentatively named APren, new semi-synthetic derivative of polyisoprenoid alcohol, might be successfully used as component of liposomal formulas used for lipofection [18].

Polyprenols are linear polymers built of 5 up to 150 isoprene units. Their postulated cellular functions comprise involvement in cell response to environmental stress [26]. It is known that polyprenols and their phosphorylated derivatives increase the permeability and fluidity of model membranes and intensify the fusion of model membranes [26]. A new type of semi-synthetic cationic polyisoprenoid derivatives has been obtained recently. Taking advantage of their lipofecting properties [18] new drug carriers based on liposomes containing these derivatives of polyisoprenoid alcohols were designed. From among various naturally occurring polyprenols, the one containing seven isoprene units was subjected to chemical modifications to obtain heptaprenyltrimethylammonium iodide (APren-7), to be used as a component of liposomal drug carrier. Our aim was to investigate if this compound does not *per se* cause untoward effects on the living organism.

Among mammalian organs and tissues the kidneys are known to be particularly sensitive to drug-dependent toxicity which may damage the renal glomeruli and various structures and tissues located in the renal medulla, such as local tubule fragments, interstitium and vasa recta. The vulnerability of the medulla obviously depends on the usual process of concentration of numerous substances, including various drugs, which occurs in this region. In this study we focused on potential changes in kidney structure and function but also examined some indices of general health and behaviour (e.g. weight gain, activity) as well as selected status indices of the cardiovascular system.

The impact of APren-7 on living organisms was studied in male Sprague–Dawley rats; animals aged 5–6 weeks were used to determine, in addition, the possible influence of APren-7 on the development and maturation. Effects of subcutaneous injections of liposomes containing APren-7 were compared with those of liposomes built exclusively of commonly used dioleoyl phosphatidylethanolamine (DOPE), and those seen in rats receiving water solvent for liposome solutions.

Materials and methods

Preparation of heptaprenyltrimethylammonium iodide (APren-7)

This compound was obtained by modification of polyisoprenoid alcohols derived from birch wood following the procedure described earlier [18].

Preparation of liposomal suspensions for injection

Liposomal suspensions were prepared using lipid film hydration protocol. Briefly, to obtain *classical liposomes* (L), 55 μ l of a solution of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Sigma) in chloroform (90 mg/ml) was dried by stream of nitrogen gas to form a thin film on the bottom of the glass tube. The film was further dried by exsiccation under reduced pressure, overnight. Then water was added to hydrate the film and multilamellar liposomes were formed by vortexing each tube for at least 8 min. The obtained suspension was extruded few times through a 100 nm membrane to obtain unilamellar liposomes (LiposoFast extruder, Avestin Europe GmbH, Mannheim, Germany).

To obtain *liposomes with APren-7* (L + P), 50 μ l of a solution of DOPE in chloroform (90 mg/ml) and 25 μ l of a solution of APren-7 in chloroform (18 mg/ml) were mixed together (DOPE:APren-7 molar ratio 10:1) and then the procedure of further preparation was as described above.

Experimental animals

The experimental procedures were approved by the IV Ethical Committee, Warsaw. Male Sprague–Dawley rats, weighing 154 ± 3 g at the start of experiments, were fed *ad libitum* a standard diet (STD, 0.25% Na w/w, SSNIFF GmbH, Soest, Germany) and had free access to drinking water during the whole experiment. The animals were accustomed to the housing and measurement procedures during the week preceding the experiments. This was done to eliminate the stress associated with immobilization needed to measure systolic blood pressure (SBP); such stress is known to increase SBP *per se*.

Experimental protocols and measurements

During four weeks three groups of rats received daily injections (0.5 ml, *sc*) of freshly prepared solutions of classical liposomes (L, $n = 13$), liposomes with APren-7 (L + P, $n = 13$, 12 mg/kg), or water solvent (W, $n = 12$). At one-week intervals rats were placed for 24 h in metabolic cages (Tecniplast S.p.A. Buguggiate, Italy) to measure food and water intake, the weight of faeces, and urine volume and osmolality (U_{osm}), as well as the concentration of sodium (U_{Na}), potassium (U_K), albumin and nitric oxide metabolites. Also determined were body weight (BW) and SBP (tail-cuff method, Coda System, Kent Scientific Corporation, Connecticut, USA); blood was sampled for plasma osmolality (P_{osm}), plasma sodium concentration (P_{Na}) and haematocrit (Ht). In the end of the experiment all animals were anesthetized with intraperitoneal sodium thiopental (Sandoz GmbH, Kundl, Austria), 100 mg/kg, and the samples of the kidneys (for morphometric and histologic studies) and of the heart (for morphometric examination only) were harvested.

Analytical procedures

Urine volume was determined by gravimetric method, plasma and urine osmolality by freezing point depression (Osmomat[®] 030 M, Gonotec, Berlin, Germany), sodium and potassium concentration by flame photometry (PFP7/C, Jenway Ltd, Stone, UK), nitrites/nitrates using nitric oxide (total) detection kit (Enzo Life Sciences Inc., New York, USA), and albumin content using UAE immunoperoxidase assay for determination of albumin in rat samples (Immunology Consultants Laboratory, Inc., Portland, USA).

Statistics

Data are presented as means \pm SEM. The significance of changes was evaluated by multivariate analysis of variance (ANOVA) with repeated measurements, followed by Newman–Keuls *post hoc* test (STATISTICA, version 10.0, StatSoft Inc.). The 4-week increments in the parameters measured were compared between groups by one-way ANOVA. The level of statistical significance was set at of $p < 0.05$.

Results

Body weight profiles over 4 weeks are shown in Fig. 1. The increase of the body mass was almost parallel for all three groups of animals analyzed. When expressed as per cent of the starting weight value (unlike in Fig. 1, where absolute values are given), the curves for L, L + P and W groups were superimposable. The 4-week BW increments were 109 ± 4 , 97 ± 4 and 102 ± 4 % in the respective groups (intergroup differences not significant).

Water and food intake, faeces weight, and urine volume, total solute, sodium and potassium excretion in four consecutive weeks are presented in Table 1. For most of the parameters measured the starting values (week 0) tended to be slightly higher in animals

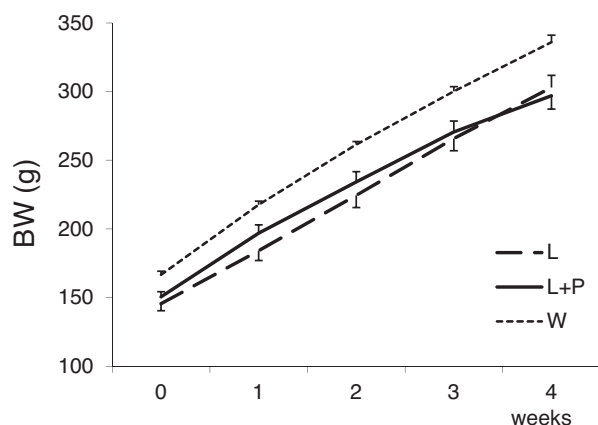


Fig. 1. Four-week profiles of body weight (BW) of rats receiving: L – liposomes built of DOPE ($n = 13$), L + P – liposomes built of DOPE and APren-7 ($n = 13$), W – water ($n = 12$).

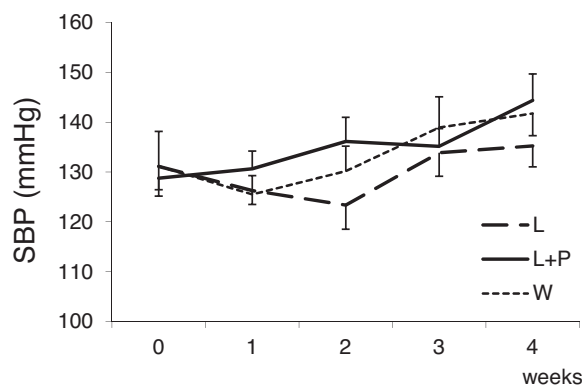


Fig. 2. Four-week profiles of systolic blood pressure (SBP) in rats receiving: L – liposomes built of DOPE ($n = 8$), L + P – liposomes built of DOPE and APren-7 ($n = 8$), W – water ($n = 8$).

receiving water solvent compared to those of the two liposome treated groups and the differences were maintained throughout the 4-week observation period. The intergroup differences in total 4-week increments in water intake and urine volume deserve attention. The increment (Δ) of water intake was in the two liposome treated groups distinctly lower than in the W group; the difference was significant for the L + P group only (last column of Table 1). On the other hand, the increment of urine volume in liposome treated groups was higher than in the W group (difference significant for the L group).

Systolic blood pressure (SBP) slightly increased in each group during four weeks of observation: in group L from 131 ± 3 to 135 ± 3 mmHg, in L + P from 129 ± 4 to 144 ± 5 mmHg, and in W from 131 ± 7 to 142 ± 4 mmHg (non-significant, Fig. 2). The variability of SBP data was relatively high and there were no significant within-group or inter-group differences.

Plasma osmolality, sodium concentration and blood haematocrit did not show significant changes over time (within groups) or between experimental groups (Table 2).

Urinary albumin excretion (UAE) measurements before treatments and in the last week of observation (Fig. 3) disclosed a significant increase in W group only; in the fourth week UAE in this group was significantly higher than in groups L and L + P.

The excretion of nitric oxide metabolites (nitrites/nitrates, NO_x) measured before and in the fourth week of treatments disclosed no significant within-group (time dependent) or inter-group differences (Fig. 4).

Left ventricular – to – body weight ratio (LV/BW), measured in each animal at the end of the experiments, was the highest in the L group and significantly different from that in the W but not in the L + P group (Fig. 5). The values for L and L + P groups were not significantly different.

Table 1

Four-week profiles of daily water and food intake, urine volume, total solute, sodium and potassium excretion in the three groups.

	Group	Weeks					Δ 0-4
		0	1	2	3	4	
Water intake (ml/24h)	L	21 ± 1	23 ± 2	23 ± 1	25 ± 1	27 ± 2	6 ± 2
	L+P	24 ± 1	25 ± 1	27 ± 1	26 ± 2	29 ± 2	4 ± 2*
	W	23 ± 1	27 ± 1	29 ± 1	30 ± 1	37 ± 3	13 ± 3
Food intake (g/24h)	L	14.2 ± 0.8	16.3 ± 1.0	17.2 ± 0.8	18.5 ± 0.5	19.1 ± 0.9	6.2 ± 1.1
	L+P	16.0 ± 0.7	16.9 ± 1.0	17.9 ± 1.7	16.2 ± 0.7	19.9 ± 1.0	3.9 ± 1.2
	W	17.2 ± 0.5	18.8 ± 0.7	20.2 ± 1.3	22.3 ± 0.7	21.4 ± 0.9	4.3 ± 1.0
Faeces weight (g/24)	L	5.1 ± 0.4	7.2 ± 0.8	6.7 ± 0.8	7.8 ± 0.7	7.2 ± 0.7	2.1 ± 0.8
	L+P	5.6 ± 0.5	7.4 ± 0.7	7.1 ± 0.6	7.4 ± 0.6	9.2 ± 0.6	3.6 ± 0.8
	W	6.8 ± 0.5	8.5 ± 0.5	8.4 ± 0.7	9.0 ± 0.6	10.7 ± 0.6	3.9 ± 0.8
Urine volume (ml/24h)	L	5.1 ± 0.4	7.9 ± 0.8	7.3 ± 0.7	9.0 ± 0.6	10.9 ± 0.6	5.8 ± 0.5*
	L+P	6.1 ± 0.7	8.1 ± 0.4	9.0 ± 0.6	9.1 ± 1.0	12.1 ± 0.9	6.0 ± 1.1
	W	7.0 ± 0.5	9.0 ± 0.6	8.4 ± 0.6	10.0 ± 0.6	11.0 ± 0.5	3.9 ± 0.6
Total solutes (mosm/24h)	L	12.5 ± 0.6	17.0 ± 0.9	16.7 ± 0.8	20.0 ± 0.7	22.0 ± 0.8	9.5 ± 1.1
	L+P	12.8 ± 1.0	17.1 ± 0.5	17.5 ± 0.6	18.1 ± 0.7	21.8 ± 0.7	9.0 ± 1.5
	W	16.1 ± 0.5	20.1 ± 0.4	20.5 ± 1.0	22.7 ± 0.9	24.3 ± 0.5	8.2 ± 0.9
Na^+ (mmol/24h)	L	0.7 ± 0.0	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	0.7 ± 0.1
	L+P	0.5 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	0.7 ± 0.1
	W	0.9 ± 0.0	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	0.7 ± 0.1
K^+ (mmol/24h)	L	3.1 ± 0.2	4.0 ± 0.2	3.5 ± 0.2	4.3 ± 0.1	4.6 ± 0.2	1.5 ± 0.2
	L+P	3.2 ± 0.3	3.8 ± 0.1	3.6 ± 0.1	3.8 ± 0.2	4.2 ± 0.1	1.0 ± 0.3
	W	4.0 ± 0.1	4.8 ± 0.1	4.7 ± 0.2	4.7 ± 0.2	4.9 ± 0.2	1.0 ± 0.2

Values are means ± SEM; L – rats receiving liposomes ($n = 13$), L + P – liposomes with APren-7 ($n = 13$), W – water solvent ($n = 12$).

* $p < 0.05$ vs. W group.

Table 2
Four-week profiles of plasma osmolality and sodium concentration and blood haematocrit.

	Group	Weeks				
		0	1	2	3	4
Plasma osmolality (mosm/l)	L	302 ± 3	299 ± 2	300 ± 2	306 ± 1	306 ± 1
	L+P	304 ± 2	301 ± 2	306 ± 2	310 ± 2	310 ± 2
	W	300 ± 1	302 ± 2	307 ± 1	309 ± 2	307 ± 3
Plasma sodium concentration (mmol/l)	L	136 ± 2	139 ± 2	133 ± 2	132 ± 2	133 ± 1
	L+P	130 ± 1	136 ± 1	132 ± 2	137 ± 3	129 ± 2
	W	132 ± 3	134 ± 2	135 ± 2	131 ± 2	135 ± 2
Haematocrit (%)	L	40 ± 1	41 ± 1	43 ± 0	44 ± 1	43 ± 1
	L+P	40 ± 0	41 ± 1	42 ± 1	44 ± 1	42 ± 1
	W	40 ± 1	41 ± 1	44 ± 1	44 ± 1	44 ± 1

Values are means ± SEM; rats receiving: L – liposomes (n = 13), L+P – liposomes with APren-7 (n = 13), W – water solvent (n = 12).

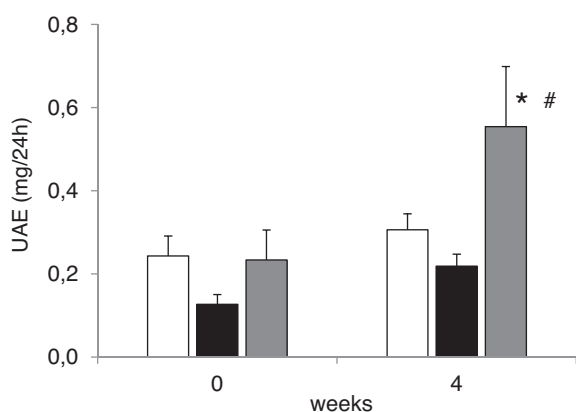


Fig. 3. Urinary albumin excretion (UAE, means ± SEM) before and after 4 weeks of observation in rats receiving: □ L – liposomes (n = 7), ■ L+P – liposomes with APren-7 (n = 7), ▒ W – water solvent (n = 6); *p < 0.05 vs. day “0”; #p < 0.05 vs. concurrent values in L and L+P groups.

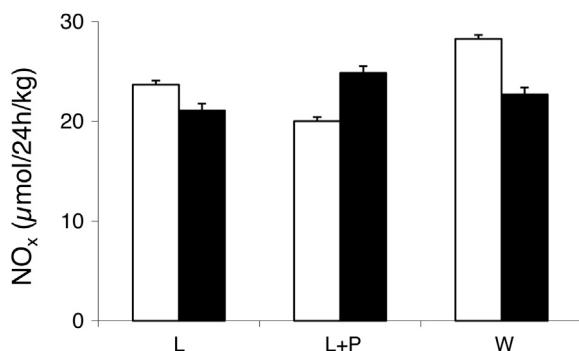


Fig. 4. Urinary excretion of nitric oxide metabolites (U_{NOx-V} , means ± SEM) □ before and ■ after four weeks of observation in rats receiving: L – liposomes (n = 7), L+P – liposomes with APren-7 (n = 6), W – water solvent (n = 6).

Renal cortical and medullary tissue were harvested at the end of experiments and the slices were stained with haematoxylin–eosin (Fig. 6). There were no explicit signs of tissue damage in any experimental group. Nor were there any inter-group differences in the microscopic images of the renal cortex and medulla.

Discussion

Over the past decade, liposomal drug delivery systems were used by many researchers and various applications were reported, such as targeted gene therapy [11,25], transfollicular [15] or

ophthalmic [20] drug delivery. In many cases DOPE (dioleoyl phosphatidylethanolamine), an analogue of the native PE with a defined fatty acid composition, was used in liposomal formulations. Due to its fusogenic properties DOPE is considered as valuable component of the lipofecting formulas. DOPE is not considered as toxic *per se*, however it has been shown to enhance the toxicity of various cationic lipids tested in the cell-line models since the replacement of DOPE by dipalmitoylphosphatidylcholine (DPPC) significantly reduced liposome toxicity towards macrophages [6]. Subsequently, new derivative of polyisoprenoid alcohol, the APren, was investigated in cell-line model [18] as potential component of liposomal drug carriers. A literature search shows that possible untoward effects of these compounds have never been tested before, therefore in the present study we address this issue, focusing on the impact of APren on some functional and morphologic features of the cardiovascular and renal excretory system of normal Sprague–Dawley rats.

All studied animals were in good health and displayed normal physical activity. In general, the parameters measured in the two groups receiving liposomes (the classical liposomes built of DOPE and the newly designed liposomes with DOPE and APren-7) were more similar to each other than to the group (W) receiving water solvent. There were no differences between groups regarding food intake, the renal excretion of total solute, sodium and potassium, or plasma osmolality and sodium concentration, and blood haematocrit.

It should be noted that the apparent between-group difference in absolute values of BW (Fig. 1) depended on higher baseline BW in the W group, obviously a matter of imperfect randomization of animals. Overall, the differences between groups were small and

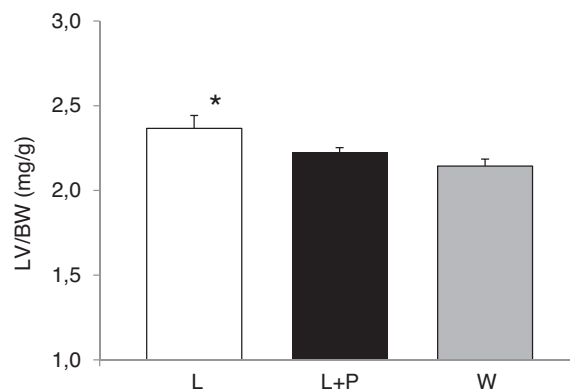


Fig. 5. Left ventricular weight to body weight ratio after 4 weeks of observation rats receiving: □ L – liposomes (n = 13), ■ L+P – liposomes with APren-7 (n = 13), ▒ W – water solvent (n = 16); *p < 0.05 vs. W (one-way ANOVA followed by Newman–Keuls *post hoc* test).

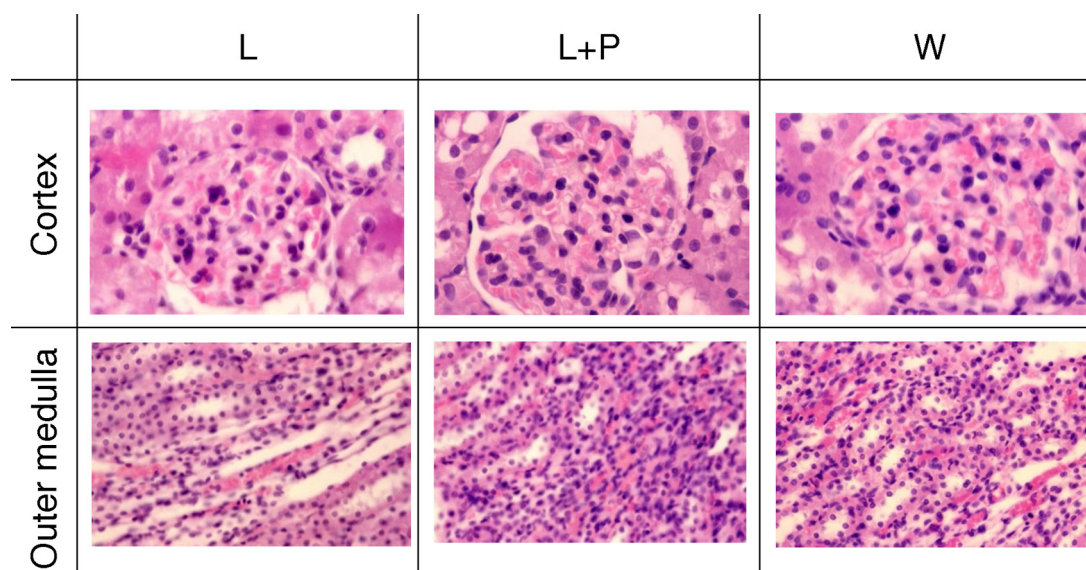


Fig. 6. Renal tissue slices (haematoxylin–eosin); L – liposomes, L + P – liposomes with APren-7, W – water solvent; magnification in the cortex 200 \times ; in the outer medulla 100 \times .

the values for L, L + P and W groups expressed as per cent of the starting weight were almost identical. According to Maitani et al. [19] the liposomes composed of dipalmitoylphosphatidylcholine (DPPC) do not cause any changes *per se* in the rat's body weight after subcutaneous injection. To the best of our knowledge physiological effects of any PE analogue treatment has never been tested in animals before.

The profiles of water intake and urine excretion deserve some attention (Table 1), especially the observation that the four-week increment (Δ) of water intake was in the W group higher than in both liposome groups whereas the increment in urine output in the W group was the lowest. For some reason the liposome-treated rats were protected against any water retention showing a better balance of water intake and output compared to solvent-treated rats.

SBP tended to increase in all groups (Fig. 2), however, the changes were minor and physiologically meaningless. The increasing tendency may have been related to daily subcutaneous injections, blood sampling and the distress due to the tail cuff method of SBP measurement, which requires immobilization of animals; the prohypertensive role of such manoeuvres has been described before [12]. It is unlikely that the minor SBP changes could induce left ventricular hypertrophy, measured as LV/BW which is a sensitive index of hypertension or heart disease [7]. Nor did liposome treatment affect this index in some direct way: LV/BW values for all groups were in the normal range for normotensive Sprague–Dawley rats [10] and so were also the values of plasma osmolality, sodium concentration and blood haematocrit. On the whole, there were no signs indicating damage of the cardiovascular system.

Various drugs and active substances are known to cause a number of morphologic changes in renal structures, such as microvacuolization (vacuolar lesions) [23]; cell loss, tubular dilation or disruption of the brush border of proximal tubules [5], atrophy of glomeruli or patches of necrosis [22]. All these abnormalities are likely to impair renal function. However, inspection of renal tissue slices stained with haematoxylin–eosin did not reveal any damage that might be caused by administration of the tested substances; microscopic images of the renal cortex and medulla did not differ between groups.

Microalbuminuria (elevated urinary albumin excretion, UAE) is a good marker of kidney damage and malfunction, and is also considered as an early index of cardiovascular disease [8,27,30]. In the end of experiments the values (Fig. 3) were within the normal UAE range for healthy Sprague–Dawley rats (0–1 mg/day) [16,17],

which speaks against any kidney or cardiovascular system dysfunction. Since UAE significantly increased in the W but not in L or L + P group, one could speculate that liposomes possess some protective properties.

Nitric oxide (NO) is an important antioxidant signalling molecule, which plays a critical role in various biological processes, such as control of vascular tone, blood pressure, renal haemodynamic and excretion, as well as developmental functions [3,13]. The actual activity of NO, which is produced by nitric oxide synthases (NOS) in many tissues, can be estimated by measuring the excretion of its metabolites [28]. However, the excretion of nitrites/nitrates (NO_x) was almost stable throughout experiments and did not depend on the treatment applied (Fig. 5). Bentinger et al. [2] suggested that other derivatives of polyisoprenoids (epoxides) exhibit some beneficial activity, possibly by increasing the cell antioxidant capacity due to the stimulation of the biosynthesis of coenzyme Q, a molecule likely to protect nitric oxide against inactivation by free radicals [29]. However, in this study we found no indication of antioxidant properties of APren-7.

The newly designed liposomes containing APrens did not affect, after subcutaneous administration, the development and maturation, or the function of the cardiovascular and renal excretory system of normal Sprague–Dawley rats. Since, microalbuminuria was even lower in the two groups receiving liposomes compared to that receiving water solvent, one can speculate on some protective action of liposomes, a proposal which would require further investigation. In general, our results showed no explicit adverse influence of the newly designed liposomes. In particular, they were not found to cause any damage *per se* of the rats' cardiovascular or renal excretory system, therefore we find them suitable as a component of liposomal drug carriers.

Conflict of interest

All authors declare that there is no actual or potential conflict of interest.

Funding

This work was supported by the Polish National Cohesion Strategy innovative Economy [Grant No. UDA-POIG.01.03.01-14-036/09].

The author has been supported with a scholarship from the European Social Fund, Human Capital Operational Programme.

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