Syntheses and Characterization of Liposome-incorporated Adamantyl Aminoguanidines

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ARTICLE TYPE

Syntheses and Characterization of Liposome-incorporated Adamantyl Aminoguanidines

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A series of mono and bis-aminoguanidinium adamantane derivatives has been synthesized and incorporated into liposomes. They combine two biomedically significant molecules, the adamantane moiety and the guanidinium group. The adamantane moiety possesses the membrane compatible features while the cationic guanidinium subunit was recognized as a favourable structural feature for binding to complementary molecules comprising phosphate groups. The liposome formulations of adamantyl aminoguanidines were characterized and it was shown that the entrapment efficiency of examined compounds is significant. In addition, it was demonstrated that liposomes with incorporated adamantyl aminoguanidines effectively recognized the complementary liposomes *via* the phosphate group. These results indicate that adamantane derivatives bearing guanidinium groups might be versatile tools for biomedical application, from studies of molecular recognition processes to usage in drug formulation and cell targeting.

Introduction

Over the past 40 years the adamantane structural motif has been used in medicinal chemistry[1] and several adamantane-containing compounds are in current clinical use.[2] This is due to specific chemical and physical properties of adamantane that, among others, include lipophilicity, bulkiness, rigidity, and low strain energy.[3,4] Today, adamantane is usually incorporated into molecules that already show some biological activity, as this can often lead to improvement of pharmacological properties of these systems.[5] The reason for the enhancement of bioactivity probably lies in the overall increase of lipophilicity of the whole molecule due to the adamantane subunit. That in turn has a consequence of a higher solubility of these compounds in blood plasma and their easier crossing of the cell membranes.[6]

On the other hand, guanidine-containing derivatives represent a very important class of biologically active compounds, not only as antimicrobial agents but also as compounds (*e.g.* dendritic guanidines) which are used in the intracellular delivery of biologically significant cargos.[7,8] Previously, it was shown that cationic lipids, bis-guanidinium-spermidine-cholesterol (BGSC) and bis-guanidinium-trencholesterol (BGTC) derivatives alone or incorporated in DOPE liposomes exhibit high transfection activity.[9] The most efficient transfection agents are dendrimers such as polyethylenimine (PEI) but they show undesirable dose-dependent cytotoxicity effect.[10] Recently, it was demonstrated that the polycationic adamantane-based dendrons of different generations display high cellular uptake without triggering cytotoxicity.[11] These compounds possess adamantane scaffolds and peripheral ammonium or guanidinium groups linked with a short ethylene glycol chain. Among other nonviral vectors, cationic liposomes are recognized as excellent synthetic, nonviral vectors for gene transfer studies and gene therapy applications.[12,13] Considerable progress has been made in several areas towards their development and understanding of the mechanism of their action.[14] In addition to the drug delivery properties, liposomes have been recognized as an artificial model membrane system and have been used for different biochemical and biophysical studies and are well described.[15] The most important advantage of using liposomes as model membrane systems is that they can incorporate structurally different lipophilic molecules into the lipid bilayer and thus create a number of artificial membrane structures. The studies of model liposomal systems have provided a useful approach to understanding the complex processes in biological systems. Molecular recognition at the environment provided by the phospholipid bilayer interface plays an important role in biology and is subject of intense investigations.[16] Furthermore, the investigation of hydrophilic cell-penetrating peptides and their ability to translocate across a lipid membrane has shown that transduction of arginine-rich cell-penetrating peptides is enabled by interactions of guanidinium group with phosphate groups of membrane's phospholipids.[17-19]

Our previous studies of chemical syntheses and characterization of adamantane derivatives of biologically active peptidoglycan fragments[20,21] have shown their strong interaction with cell membranes and the important role in stimulating particular immunological mechanisms. In the continuation of our study dealing with the synthesis of adamantane derivatives with a guanidine subunit[22,23] and investigations of liposomes as drug delivery systems as well as a model membrane,[24] we prepared a

series of adamantyl aminoguanidines **1-6** (Fig. 1) and characterized their liposome formulations. Herein we report on the synthesis and a detailed study of the molecular symmetry, as well as liposome encapsulation of synthesized compounds and characterization of such formulations.

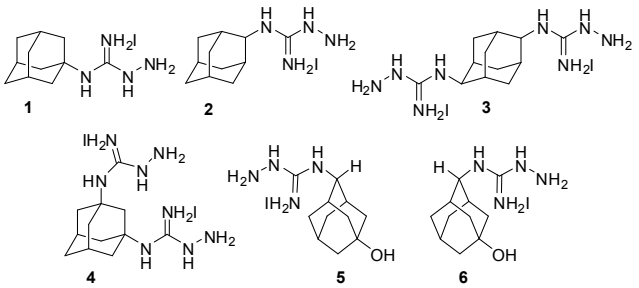
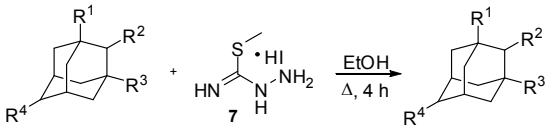


Fig. 1 The structure of adamantyl aminoguanidines **1-6**

Results and discussion

Synthesis and computational results

The adamantyl aminoguanidines **1-6** were synthesized in very good yields by condensation of *S*-methylisothiosemicarbazide hydroiodide **7** with the corresponding aminoadamantanes **8-13** (Sheme 1). All the obtained compounds were fully characterized by spectroscopic methods.



8 R^1=NH_2, R^2=R^3=R^4=H
9 R^2=NH_2, R^1=R^3=R^4=H
10 R^2=R^4=NH_2, R^1=R^3=H
11 R^1=R^3=NH_2, R^2=R^4=H
12 R^4=*E*-NH_2, R^1=OH, R^2=R^3=H
13 R^4=*Z*-NH_2, R^1=OH, R^2=R^3=H

1 R^1=NHC(NH_2I)$NHNH_2$, R^2=R^3=R^4=H
2 R^2=NHC(NH_2I)$NHNH_2$, R^1=R^3=R^4=H
3 R^2=R^4=NHC(NH_2I)$NHNH_2$, R^1=R^3=H
4 R^1=R^3=NHC(NH_2I)$NHNH_2$, R^2=R^4=H
5 R^4=*E*-NHC(NH_2I)$NHNH_2$, R^1=OH, R^2=R^3=H
6 R^4=*Z*-NHC(NH_2I)$NHNH_2$, R^1=OH, R^2=R^3=H

Sheme 1 Synthesis of adamantyl aminoguanidines **1-6**

To better understand the structure–activity relationship (SAR) of adamantyl aminoguanidines **1-6** the quantum chemical calculations were performed to ascertain the geometry of these derivatives. The results of these studies are summarized in Table 1. Although these aminoguanidines are not diastereoisomers, they can nevertheless be regarded as constitutional pairs. This is why the energy difference between the pair members can be compared. The first pair comprises of **1** and **2** and the second of **3** and **4**. The third pair consists of **5** and **6** but these derivatives can also be viewed as *E*/*Z* isomers.

Table 1 Enthalpies and relative enthalpies of stationary points for aminoguanidines **1-6** optimized at the B3LYP/6-311+G(d,p) level of theory

Compound	*H*(298 K) / a.u.	H_{rel} / kcal mol^{-1}
1	-650.45882	-0.2[a]
2	-650.45845	0.0[a]
3	-910.26719	-6.3[b]
4	-910.25715	0.0[b]
5	-725.69856	0.0[c]
6	-725.69991	-0.8[c]

[a] comparison of **1** and **2**; [b] comparison of **3** and **4**; [c] comparison of **5** and **6**

Monoguanidines **1** and **2** have almost identical enthalpies, the difference being only 0.2 kcal mol^{-1}, as is expected for compounds that are chemically so similar. In both of these structures there are no significant sterical hindrances between the adamantane skeleton and the aminoguanidine group. Based on the calculated enthalpies it can be concluded that almost no energetically disfavouring interaction between the guanidine and the polycyclic carrier exists in compounds **1** and **2**.

On the other hand, constitutional pair **3** and **4** shows a markedly different energy profile. The 2,6-disubstituted bisaminoguanidine **3** is significantly less thermodynamically stable when compared to the 1,3-derivative **4**. The difference amounts to 6.3 kcal mol^{-1}, although both of these geometries have their guanidine groups oriented away from each other (Fig. 2) and no interference is expected. We hypothesize that the reason for this lies in the transannular effect of the adamantane 2,6-substitution pattern. It is known that when this type of substitution is present in the adamantane molecule, a transannular interaction "through space" takes place.[25,26] In other words, a homoconjugation through the polycyclic skeleton and the increase of electron density around sp^2 carbon atoms occurs. When two double bonds are present in the 2,6-positions, one of them influences another, sometimes even to the extent that it suppresses the chemical reactivity of the second group. In the case of compound **3**, this type of transannular interaction energetically destabilizes the molecule and is responsible for the high enthalpy value that was calculated.

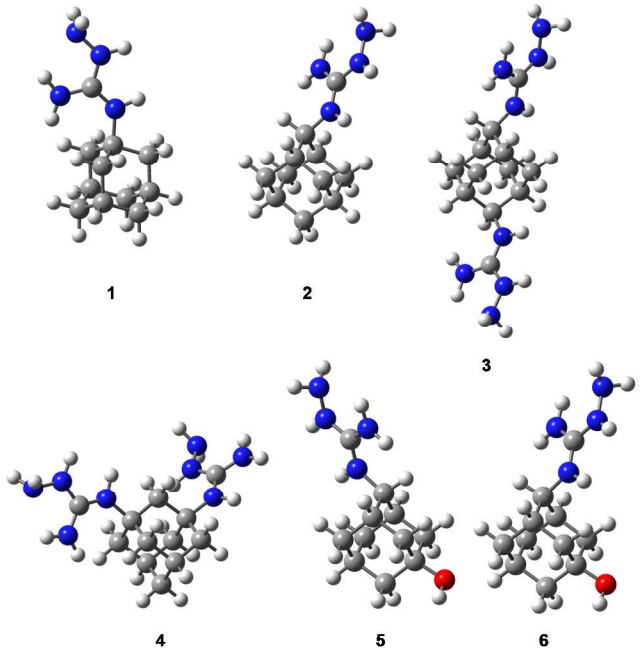


Fig. 2 Structures of aminoguanidines **1-6** optimized by B3LYP/6-311+G(d,p) level of theory

When comparing the *E*/*Z* isomers **5** and **6** one can notice that,

somewhat unexpectedly, the enthalpy difference between them is not as substantial and amounts to only 0.8 kcal mol^{-1} in favour of the *E* isomer **5**. The possible reason for this is the fact that both of the isomers possess enough steric freedom to orient as favourably as possible and therefore the effect of the hydroxy group on the aminoguanidine moiety is not so severe.

Entrapment of adamantyl aminoguanidines in liposomes

The liposomes were prepared by a modified thin film method. The examined compounds were dissolved in methanol and added to the lipids in the chloroform solution. After rotary evaporation of the organic solvent the remaining lipid film was rehydrated by phosphate buffer. In this manner the lipophilic compounds could be encapsulated in liposomes. We have previously shown that this modification of the method has a significant impact on encapsulation efficiency of adamantylpeptides into multilamelar liposomes.[27] Adamantyl moiety has been shown to possess membrane compatible features. On the other hand, the guanidinium group also presents an interesting feature; with phosphate it forms anion characteristic pairs of parallel zwitterionic hydrogen bonds, which provide binding strength by their charge and structural organization. Therefore, for the adamantyl aminoguanidines **1**-**6** two different ways of their incorporation into liposomes could be expected, the compounds could be placed in the water interior of liposomes as well as incorporated into the lipid bilayer due to the lipophilic character of the adamantyl moiety. The ability of adamantane to be incorporated into the lipid core of the bilayer, despite the presence of a polar substituent in the molecule, was described in the literature and proven with X-ray diffraction study and EPR spectroscopy.[28] Our results of the overall entrapment efficiency obtained for aminoguanidines **1**-**6** are presented in Table 2. The efficiency depends on the structural characteristics of examined compounds and the higher efficiency was obtained for mono-aminoguanidines **1** and **2**. Bis-aminoguanidine compound **4** has a lower entrapment efficiency, while the OH-groups in molecules **5** and **6** further decrease the entrapment. The entrapment of compound **3**, 2,6-bis-aminoguanidine derivative, was the lowest. This is understandable considering the steric requirements of molecule **3** when the lipid bilayers are formed. In compound **3** the guanidinium groups are on opposite sides of the adamantane moiety and that hinders its accommodation into the hydrophobic core of the lipid bilayer. The relative lipophilicity for examined compounds was estimated by calculation of average CLogP values (Table 2). The calculated CLogP correlate with the obtained values for entrapment efficiency for each adamantyl aminoguanidine compounds except for compound **4**. More lipophilic compounds (higher CLogP) have the higher entrapment efficiency. The monoguanidine compounds **1** and **2** are the most lipophilic and therefore have the highest entrapment efficiency. The bis-aminoguanidines **3** and **4** have similar calculated lipophilicity but they differ in the entrapment efficiency. The discrepancy between calculated CLogP and the entrapment efficiency for compound **4** demonstrates the importance of stereochemical arrangement of substituents on incorporation of the adamantane molecule into the liposomes. These results have demonstrated that structural characteristics of the adamantyl aminoguanidines and their ability to incorporate into the lipid bilayer directly affect the physicochemical properties of the prepared liposomes. Several papers from C. Paleos' group were dealing with physicochemical study of the molecular recognition processes of complementary liposomes as well as guanidinylated dendrimers and liposomes.[29,30] The phosphate group was incorporated in the bilayer using dihexadecyl phosphate, while the guanidinium moiety was introduced in the membrane through the incorporation of various guanidinium lipids. It was demonstrated that molecular recognition of the guanidinium and phosphate moieties located at the membrane surface of the liposomes was affected by the anchoring ability of the lipophilic part of the molecule. It should be noted that previously mentioned bis-guanidinium-spermidine-cholesterol (BGSC) and bis-guanidinium-trencholesterol (BGTC) derivatives explore the cholesterol molecule as an anchor in lipid bilayers. The results presented here are in line with our previously published paper[24] where we showed that the adamantyl moiety could also be used as an efficient anchor in the lipid bilayer for surface modified liposomes.

Table 2. Entrapment efficiency of adamantyl aminoguanidines in liposomes and calculated CLogP for compound **1**-**6**.[a]

Compound	Entrapment (%)	CLogP[b]
1	57.15 ± 2.35	-0.71
2	51.81 ± 3.45	-0.86
3	18.20 ± 5.45	-3.88
4	43.23 ± 3.62	-3.60
5	36.18 ± 4.81	-1.92
6	31.51 ± 6.38	-1.92

[a] the results are expressed as an average value ± standard deviation (SD) of three separate experiments; [b] public domain software ALOGPS 2.1 (*http://www.vcclab.org/lab/alogps/*)

Dynamic light scattering technique has not shown significant differences in the size of liposomes between specific formulations of examined compounds. The liposomes have been prepared by sequential extrusion of the multilamellar vesicles through polycarbonate membranes of 400 and 200 nm. The size distribution was sharp and the average diameter of prepared liposomes was approximately 170 nm while the polydispersity index was about 0.47. The mean size of empty liposomes was very similar to the adamantyl aminoguanidine liposomes and was 185 nm. On the other hand, the zeta-potential of liposomes with encapsulated adamantyl aminoguanidines compared to empty liposomes is slightly increased to positive zeta-potential, Table 3. Since the zeta-potential reflects the surface charge of liposomes, it may be assumed that the guanidinium groups of incorporated adamantyl aminoguanidine compounds are exposed on the liposomal surface and contribute to the electrophoretic mobility of liposomes, *i.e.* to zeta-potential.

Table 3. Zeta-potential of liposome formulations of the adamantyl aminoguanidines.[a]

Liposome formulations of compound	Zeta potential / mV
Empty liposomes	2.84 ± 0.40
1	3.49 ± 0.43
2	3.25 ± 0.27
3	N/A[b]

4	4.41 ± 0.35
5	3.7 ± 0.27
6	3.69 ± 0.36

[a] the results are expressed as an average value ± standard deviation (SD) of three separate experiments; [b] not applicable

Interaction of adamantyl aminoguanidines with liposomes

Interaction of multilamellar liposomes composed of phosphatydylcholine, cholesterol and encapsulated carboxyfluorescein with adamantyl aminoguanidines was carried out in order to define the stability of the liposome bilayer in the presence of the examined compounds. The liposomes were mixed and incubated with a particular adamantyl aminoguanidine for 12 h. The supernatants were separated by ultracentrifugation and the concentration of carboxyfluorescein was measured. It was shown that the leakage of carboxyfluorescein was increased up to 15 % when the liposomes were mixed and incubated with adamantyl aminoguanidines. This interaction of adamantyl aminoguanidines with the lipid bilayer of phosphatydylcholine:cholesterol liposomes could be ascribed mainly to the adamantane moiety of the molecule. The phosphatidylcholine headgroup contains a positively charged choline group and negatively charged phosphate and carbonyl groups, although it is a neutral molecule as a whole. These observations suggest that the synthesized compounds could interact with the lipid bilayer with hydrophobic interactions as well, and not only with electrostatic interactions. This is in agreement with the proposed interaction mechanism of guanidinylated dendrimers with the liposomal membrane where the guanidinium group first adheres on liposomal membrane following their interaction with the negatively charged phosphate group. After the charge neutralization of the adhered dendrimers they become less polar and therefore cross easily the hydrophobic bilayer.[31]

Interaction of complementary liposomes

In order to confirm that the guanidinium groups are exposed on the liposome surface the interactions with complementary liposomes were tested. The interaction of liposomes composed of phosphatydylcholine, cholesterol and encapsulated adamantyl aminoguanidine **1** with complementary liposomes composed of phosphatydylcholine, cholesterol, dicetylphosphate and encapsulated carboxyfluorescein took place spontaneously. It was expected that if the guanidinium groups are on the surface they could recognize the phosphate groups on the complementary liposomes. The dynamic light scattering has revealed that the average sizes of liposomes were increased up to 650 nm and a small fraction of aggregates has appeared (Fig. 3). The spectrophotometric analysis of supernatants of the liposome mixtures revealed that there is no leakage of carboxyfluorescein. This is in line with the published phenomena that the guanidinylated liposomes form the multicompartment structure and aggregate with complementary liposomes bearing a phosphate group on the surface.[32]

In parallel, the multilamellar liposomes composed of phosphatydylcholine, cholesterol, and encapsulated carboxyfluorescein were incubated with negatively charged liposomes composed of phosphatydylcholine, cholesterol and dicetylphosphate. There was no change in the mean size of mixed liposomes nor was the leakage of carboxyfluorescein observed. From these results it can be concluded that the initial adhesion and aggregation of the liposomes occurred due to the presence of a recognizable guanidinium/phosphate pair on the liposomes surface, (Fig. 4). The process of liposome aggregation did not cause significant disruption of the bilayer and no leaking of carboxyfluorescein was observed.

The explanation of how the guanidinium group could protrude from the liposome interface, facilitating therefore the interaction with the complementary phosphate group, is based on our previously published results regarding the incorporation of mannosylated adamantyltripeptides in liposomes.[24] We have demonstrated that the mannosylated adamantyltripeptides are incorporated into the lipid bilayer of liposome due to the adamantane moiety while the mannose is exposed on liposome surface. Using the atomic force microscopy it has been shown that the mannosylated adamantyltripeptides are grouped into "domains" inside the lipid bilayer. The same assumption could be applied to adamantyl aminoguanidine molecules, and then it is possible that a certain number of guanidine molecules would be available for recognition of the complementary phosphate group on the other vesicle.

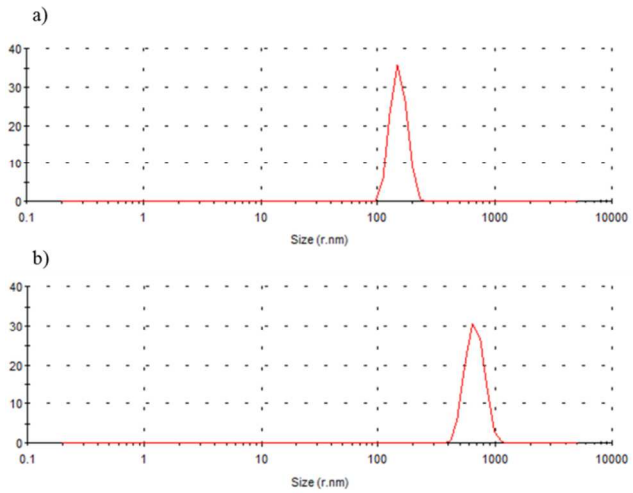


Fig. 3 Particle size distribution of multilamellar liposomes composed of phosphatydylcholine, cholesterol and encapsulated adamantyl aminoguanidines: a) before the complementary liposomes were added; b) after the complementary liposomes were added

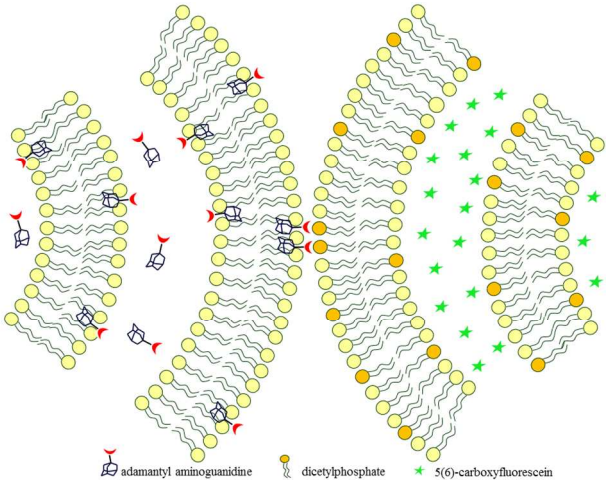


Fig. 4 Schematic representation of adamantyl aminoguanidine liposome interaction with a complementary liposome

To visualize the structural features of the formed liposome aggregates the phase–contrast optical microscopy was used. From images obtained by optical microscope it can be seen that the interaction of adamantyl aminoguanidine liposomes with complementary liposomes has resulted in a formation of multicompartment structures and aggregates (Fig. 5). The observed multicompartment structures and aggregates are similar to the structures obtained by recognition of the complementary liposomes previously described in the literature.[32,33] The studied systems of the complementary liposomes are different not only in groups that are recognized on the surface of liposomes, but also in the composition and size of liposomes used. It has been shown that the selective adhesion and membrane fusion processes between the vesicles bearing complementary molecular recognition groups depend on their size and surface concentration of the recognition groups.[33] Our presented images obtained by optical microscope and the increase in particles size after the interactions of adamantyl aminoguanidine liposomes with complementary liposomes bearing phosphate groups are reliable proofs of the surface vesicle recognition.

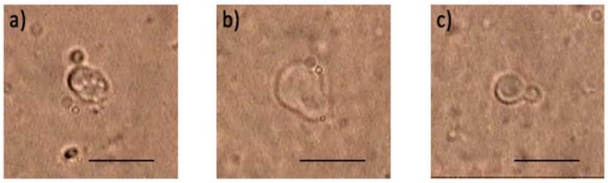


Fig. 5 Phase–contrast optical microscopy images of liposomal aggregates and multicompartment structure following the mixing of adamantyl aminoguandine:PC:Chol liposomes with complementary liposomes of PC:Chol:DHP. a) and b) different multicompartment or aggregate liposomes; c) fused vesicles; all images are of the same scale. The bar in the right corner indicates 10 μm.

These observations certainly require further research but it should be pointed out that the obtained results are a valuable contribution to the design and synthesis of a series of new derivatives that structurally belong to the already described family of potent biologically active compounds. Also, the contribution to the structural investigations of the surface modified liposomes by adamantyl aminoguanidines capable of effective intracellular delivery of biologically significant cargos was considerable. It should be emphasized that liposomes have some very attractive biological properties including biocompatibility, biodegradability and non-immunogenicity and therefore they have been widely investigated for different biomedical applications.

Besides our description of the liposome formulation of adamantyl aminoguanidines as the effective drug delivery system, this class of compounds, comprising the lipophilic part and the guanidinium group in their structure, incorporated in the liposome are described as very promising vectors for gene transfection into the eukaryotic cells[9]. In the continuation of our study dealing with enzyme inhibition by adamantane derivatives with a guanidine subunit[22], we have also conducted a preliminary inhibition study with the adamantyl aminoguanidines incorporated in the liposome to see if such a formulation could affect the inhibitory potential towards butyrylcholinesterase. It is known that the microenvironment at or near the membrane surface may modulate the activities of enzymes located in this region.[34] The recent scientific studies are aimed at designing selective, potent and well-tolerated inhibitors of both acetyl and butyryl cholinesterases in order to determine which enzymes need to be targeted for maximum effect in treating neurodegenerative deseases such as Azheimer's disease.[35] Acetylcholinesterase from a number of sources is always found in association with membranes, and the activity of the enzyme appears to be influenced by this association.[36] Several papers described the liposome-formulated butyrylcholinesterase and it was found that encapsulated enzyme retain its activity, moreover the increase of the enzyme activity was found.[37] Our preliminary investigation has shown that liposome formulations of compounds **2**, **3** and **6** affect the inhibitory potential of newly synthesized adamantyl aminoguanidines towards butyrylcholinesterase (Supplementary Information, Table S3). The preliminary results justify further research of possible biological activity of synthesized compounds.

Conclusions

The present study has described the synthesis of a series of mono and bis-aminoguanidinium adamantane derivatives **1-6** containing two biomedically attractive compounds, adamantane and guanidine. The newly synthesized compounds combine the membrane compatible features of adamantane moiety and favourable structural features of the cationic, guanidinium subunit. We have shown that the adamantyl aminoguanidines were successfully encapsulated into multilamellar liposomes and that the liposome stability and permeability was affected by the presence of synthesized adamantyl aminoguanidines. Our results clearly show that adamantane derivatives with guanidinium polar head groups incorporated into the lipid bilayer effectively recognized the complementary liposomes bearing the phosphate group on their surface. It has been established that the adamantyl moiety can serve as a molecular anchor in the lipid bilayer while the polar guanidinium head is exposed on the liposome surface.

Furthermore, these results reveal that the liposome-incorporated adamantane derivatives bearing guanidinium groups might be versatile tools for various applications, from studies of molecular recognition processes to usage in drug formulation and cell targeting.

Experimental section

All the solvents used for the synthesis were obtained commercially and used as such and *S*-methylisothiosemicarbazide hydroiodide was prepared according to the literature procedure.[38] L-α-Phosphatidylcholine, type XI-E: from fresh egg yolk (egg-PC) and cholesterol from porcine liver (CHL) were from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade from Merck (Darmstadt, Germany). A daily supply of water was obtained from Millipore Simplicity–Personal ultra-pure water system (Bedford, MA, USA). Chemicals for buffers and solutions were from Kemika (Zagreb, Croatia), unless stated otherwise. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-300 Spectrometer at 300 MHz. All NMR spectra were measured in DMSO-d_6 using tetramethylsilane as a reference. IR spectra were recorded on a FT-IR ABB Bomem MB 102 spectrophotometer. MALDI-TOF MS spectra in reflection mode were obtained on an Applied Biosystems Voyager DE STR instrument (Foster City, CA). ESI MS spectra were obtained on an Agilent Technologies 6410 Triple Quadrupole Mass Spectrometer (Palo Alto, CA) operating in a positive ion mode. Melting points were obtained using an Original Kofler Mikroheitztisch apparatus (Reichert, Wien) and are uncorrected. The liposome suspensions were centrifuged in the Beckman model L5-65, ultracentrifuge (Beckman Coulter TM, USA) for 1 hr at 300 000 x g or in the Eppendorf centrifuge 5810 R. After centrifugation the supernatants were separated from the pellets and submitted to spectrophotometric analysis. All spectrophotometric measurements were performed on Multiskan Spectrum, Thermo Fisher Scientific, (Waltham, MA, USA).

General procedure for the synthesis of adamantyl aminoguanidine hydroiodides

The respective amine (1 equivalent) was dissolved in ethanol (10 mL) and then *S*-methylisothiosemicarbazide hydroiodide **7** (1 equivalent) was added in the solution in one portion. The reaction mixture was refluxed for 4 h and after cooling the solvent was evaporated yielding the crude product. The obtained solid was purified by washing with ether/ethanol solvent mixture and the pure product was isolated.

1-(3-Aminoguanidino)adamantane hydroiodide (1).
The condensation of 1-aminoadamantane **8**[39] (378 mg, 2.5 mmol) and *S*-methylisothiosemicarbazide hydroiodide **7** (583 mg, 2.5 mmol) yielded compound **1** (712 mg, 85 %). m.p. 224-226 °C. IR (KBr): ν_{max} = 3435 (br), 3024 (m), 2922 (s), 2853 (m), 1637 (m), 1362 (m) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H = 1.51-1.69 (m, 6H, Ad-CH_2), 1.72-1.76 (m, 6H, Ad-CH_2), 2.08 (br s, 3H, Ad-C*H*), 5.25 (s, 1H, guan.-N*H*), 5.46-7.65 (guan.-N*H*) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ_C = 28.2 (3C, Ad-*C*H), 34.9 (3C, Ad-*C*H_2), 40.1 (3C, Ad-*C*H_2), 50.8 (1C, Ad-*C*$_q$), 150.6 (guan.-*C*$_q$) ppm. MS (ESI) calcd. for $[C_{10}H_{17}N+H]^+$ 152.1, found 152.1.[40]

2-(3-Aminoguanidino)adamantane hydroiodide (2).
The condensation of 2-aminoadamantane **9**[41] (378 mg, 2.5 mmol) and *S*-methylisothiosemicarbazide hydroiodide **7** (583 mg, 2.5 mmol) yielded compound **2** (628 mg, 75 %). m.p. 231-233 °C. IR (KBr): ν_{max} = 3397 (br), 3021 (br), 2916 (s), 2854 (m), 1655 (m), 1566 (m), 1489 (m), 1470 (m), 1393 (w) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H = 1.50-1.61 (m, 2H, Ad-CH_2), 1.70 (br s, 2H, Ad-CH_2), 1.73-1.77 (m, 2H, Ad-CH_2), 1.78-1.88 (m, 4H, Ad-CH_2), 1.90-1.98 (m, 4H, Ad-C*H*), 3.32 (s, 1H, Ad-C*H*), 5.41 (s, 1H, guan.-N*H*), 5.92-8.51 (guan.-N*H*) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ_C = 26.1 (1C, Ad-*C*H), 26.2 (1C, Ad-*C*H), 29.3 (2C, Ad-*C*H_2), 30.0 (2C, Ad-*C*H), 35.9 (2C, Ad-*C*H_2), 36.6 (1C, Ad-*C*H_2), 54.5 (1C, Ad-*C*H), 150.2 (guan.-*C*$_q$) ppm. HRMS (MALDI) calcd. for $[C_{11}H_{20}N_4+H]^+$ 209.1761, found 209.1769.

2,6-Bis-(3-aminoguanidino)adamantane dihydroiodide (3).
The condensation of 2,6-diaminoadamantane **10**[42] (83 mg, 0.5 mmol) and *S*-methylisothiosemicarbazide hydroiodide **7** (233 mg, 1 mmol) yielded compound **3** (145 mg, 54 %). m.p. >300 °C. IR (KBr): ν_{max} = 3405 (br), 3345 (br), 3132 (br), 2922 (m), 1646 (s), 1602 (m), 1449 (m), 1305 (w) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H = 1.64 (d, 2H, *J* = 13.6 Hz, Ad-C*H*), 1.80-1.98 (m, 8H, Ad-CH_2), 2.08 (d, 2H, *J* = 13.6 Hz, Ad-C*H*), 3.35 (br s, 2H, Ad-C*H*), 5.54 (s, 2H, guan.-N*H*), 6.62-8.76 (guan.-N*H*) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ_C = 22.2 (1C, Ad-*C*H_2), 28.5 (2C, Ad-*C*H_2), 28.7 (2C, Ad-*C*H), 28.9 (2C, Ad-*C*H), 34.8 (1C, Ad-*C*H_2), 53.9 (2C, Ad-*C*H), 149.9 (guan.-*C*$_q$) ppm. MS (ESI) calcd. for $[C_{12}H_{24}N_8+H]^+$ 281.2, found 281.1.[40]

1,3-Bis-(3-aminoguanidino)adamantane dihydroiodide (4).
The condensation of 1,3-diaminoadamantane **11**[43,44] (125 mg, 0.75 mmol) and *S*-methylisothiosemicarbazide hydroiodide **7** (350 mg, 1.5 mmol) yielded compound **4** (321 mg, 80 %). m.p. >300 °C. IR (KBr): ν_{max} = 3402 (br), 2934 (s), 2887 (m), 1655 (m), 1494 (w), 1358 (m) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H = 1.53 (br s, 2H, Ad-C*H*), 1.64-1.76 (m, 8H, Ad-CH_2), 1.84 (br s, 2H, Ad-CH_2), 2.29 (br s, 2H, Ad-CH_2), 5.46 (s, 2H, guan.-N*H*), 6.15-6.85 (guan.-N*H*) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ_C = 28.3 (2C, Ad-*C*H), 33.1 (1C, Ad-*C*H_2), 38.5 (4C, Ad-*C*H_2), 43.0 (1C, Ad-*C*H_2), 51.6 (2C, Ad-*C*$_q$), 150.1 (guan.-*C*$_q$) ppm. MS (ESI) calcd. for $[C_{12}H_{24}N_8+H]^+$ 281.2, found 281.2.[40]

***E*-2-(3-Aminoguanidino)-5-hydroxyadamantane hydroiodide (5).**
The condensation of *E*-2-amino-5-hydroxyadamantane **12**[45] (334 mg, 2 mmol) and *S*-methylisothiosemicarbazide hydroiodide **7** (467 mg, 2 mmol) yielded compound **5** (493 mg, 70 %). m.p. 235-237 °C. IR (KBr): ν_{max} = 3387 (br), 3112 (m), 2916 (s), 2865 (m), 1592 (m), 1506 (s), 1399 (m), 1099 (s) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H = 1.38 (d, 2H, *J* = 12.9 Hz, Ad-CH_2), 1.58-1.72 (m, 6H, Ad-CH_2), 1.84 (d, 2H, *J* = 12.9 Hz, Ad-CH_2), 1.99-2.09 (m, 3H, Ad-C*H*), 3.27 (s, 1H, Ad-C*H*), 5.41 (s, 1H, guan.-N*H*), 5.73-7.53 (guan.-N*H*) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ_C = 28.4 (2C, Ad-*C*H_2), 28.8 (1C, Ad-*C*H), 32.1 (2C, Ad-*C*H), 43.6 (2C, Ad-*C*H_2), 45.0 (1C, Ad-*C*H_2), 53.9 (1C, Ad-*C*H), 65.0 (1C, Ad-*C*$_q$), 150.3 (guan.-*C*$_q$) ppm. HRMS (MALDI) calcd. for $[C_{11}H_{20}N_4O+H]^+$ 225.1710, found 225.1707.

***Z*-2-(3-Aminoguanidino)-5-hydroxyadamantane hydroiodide (6).**

The condensation of *Z*-2-amino-5-hydroxyadamantane **13**[45] (334 mg, 2 mmol) and *S*-methylisothiosemicarbazide hydroiodide **7** (467 mg, 2 mmol) yielded compound **6** (521 mg, 74 %). m.p. 232-234 °C. IR (KBr): ν_{max} = 3360 (br), 2925 (m), 1660 (m), 1626 (m), 1385 (s), 1091 (m) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ_H = 1.45 (d, 2H, *J* = 12.8 Hz, Ad-CH_2), 1.55-1.66 (m, 6H, Ad-CH_2), 1.81 (d, 2H, *J* = 12.8 Hz, Ad-CH_2), 1.98-2.03 (m, 1H, Ad-C*H*), 2.13 (br s, 2H, Ad-C*H*), 3.17 (s, 1H, Ad-C*H*), 5.58 (s, 1H, guan.-N*H*), 6.88-8.61 (guan.-N*H*) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ_C = 28.7 (1C, Ad-*C*H), 32.9 (2C, Ad-*C*H), 34.8 (2C, Ad-*C*H_2), 37.6 (2C, Ad-*C*H_2), 45.3 (1C, Ad-*C*H_2), 53.3 (1C, Ad-*C*H), 65.2 (1C, Ad-C_q), 149.9 (guan.-C_q) ppm. HRMS (MALDI) calcd. for $[C_{11}H_{20}N_4O+H]^+$ 225.1710, found 225.1715.

Computational details

All calculations were performed by Gaussian03 program package.[46] Geometrical optimization of the structures and verification of the minima were done at the B3LYP/6-311+G(d,p) level of theory.[47-50] Enthalpies were obtained by correcting electronic energies for unscaled zero-point vibrational energy and work term (Ew = RT) as implemented in Gaussian03. All energies are given in kcal mol^{-1} (1 kcal mol^{-1} = 4.184 kJ mol^{-1}) and the structures at the stationary points were visualized by GaussView 5.[51]

Liposome preparation

Multilamellar liposomes were prepared by modified thin lipid films following previously described methods.[27] Briefly, egg-phosphatidylcholine, and cholesterol (total mass of lipid was 5 mg, giving a molar ratio of 7:5) were dissolved in 2 ml chloroform:ethanol (1:1). 1 ml of 5 mM methanol solution of examined adamantane-aminoguanidine compounds was added to the lipids in the chloroform:ethanol solution. After rotary evaporation of the solvent the remaining lipid film was dried in vacuum for an hour and then dispersed by gentle hand shaking in 0.5 mL phosphate buffer, concentration 10 mM and pH 7.4. The liposome suspension was left overnight at 4°C to swell and stabilize. Liposomes were separated from non-encapsulated material by ultracentrifugation. The liposome pellets obtained after centrifugation were resuspended in 0.5 mL phosphate buffer and liposome size was reduced by sequential extrusion of the multilamellar vesicles through polycarbonate membranes of 400 and 200 nm using the 0.5 mL extruder (LiposoFast, Avestin Inc., Canada).

Determination of the entrapment efficiency

The entrapment efficiency of examined adamantyl aminoguanidine compounds was determined spectrophotometrically in the supernatants following ultracentrifugation. The standard curves of examined compounds were constructed for each compound prior to the analyses of the supernatants from the liposome preparations. The concentrations of 0.0125-0.2 mg/mL were used for standard curves. The supernatant was 100 x diluted and absorption at 240 nm was measured. The amount of the entrapped solute was then calculated by subtracting the obtained value from the total amount of the particular compound used for liposome preparation and entrapment efficiency expressed as the percentage of the starting amount.

Dynamic light scattering and zeta-potential measurements

The size distribution and zeta potential of the liposomes was measured using Zetasizer Nano ZS (Malvern, UK) equipped with green laser (532 nm). Intensity of scattered light was detected at the angle of 173°. All measurements were conducted at 25 °C. All data processing was done by the Zetasizer software 6.20 (Malvern instruments). The size of liposomes is expressed as average diameter (z-average) that is obtained from the Zetasizer Nano software, which calculates it from the signal intensity. Each sample was measured ten times and the results were expressed as the average value.

Carboxyfluorescein assay

a) Interaction of adamantyl aminoguanidines with liposomes

Liposomes of egg-phosphatydylcholine and cholesterol, 10 mg of lipids per mL of phosphate buffer, molar ratio 7:5, with 5(6)-carboxyfluorescein were prepared according to the procedure described above. The solution of 2 mM 5(6)-carboxyfluorescein in 1 mM phosphate buffer, pH 7.4 was used for rehydration of lipid film. After the rehydration the liposomes were extruded through the polycarbonate membrane with 200 nm pore size. The 100 µl of liposomes were mixed with 100 µl of 5 mM phosphate buffer solution of examined compounds and after 12 h the liposomes were centrifuged in order to separate the supernatants from pellets. The concentration of carboxyfluorescein in supernatants was determined spectrophotometrically.

b) Interaction of complementary liposomes

The liposomes of egg-phosphatydylcholine and cholesterol with encapsulated adamantyl aminoguanidine **1** were prepared as described above. The complementary liposomes of egg-phosphatydylcholine, cholesterol and dicetylphosphate, molar ratio 7:5:1, with encapsulated 5(6)-carboxyfluorescein were prepared in the same way. After the removal of nonencapsulated adamantyl aminoguanidine and 5(6)-carboxyfluorescein by ultracentrifugation, the pellets were resuspended in phosphate buffer and 100 µl of each liposome suspensions were mixed. After 12 h the liposomes were ultracentrifuged and supernatants as well as liposome pellets were analyzed. The concentrations of 5(6)-carboxyfluorescein in supernatants were determined spectrophotometrically. The structural features of liposomal dispersions were investigated by optical microscopy and dynamic light scattering. The formed aggregates were visualized by Leica DMLS optical microscope equipped with Sony ExWave HAD camera.

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† Electronic Supplementary Information (ESI) available: NMR spectra of aminoguanidines **1-6**, UV spectra and spectroscopic characteristics of **1-6** in acetonitrile, Cartesian coordinates of B3LYP geometry optimized models of **1-6**, and enzyme inhibition measurement details. See DOI: 10.1039/b000000x/

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