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New Biocompatible Cationic Amphiphiles Derivative from Glycine Betaine: A Novel Family of Efficient Nonviral Gene Transfer Agents

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With the aim of developing new efficient agents for transfecting of eukaryotic cells we have designed and synthesized a novel family of cationic lipid vectors derived from glycine betaine. In this study we present three novel molecules differing by the length of their aliphatic chains (R=12,R=14,R=16). The lyotropic properties of these cationic lipids have been determined, and their transfection efficiency on different cell lines evaluated, using a luminescent assay. Two of these compounds, GB14 and GB12 are efficient *in vitro* experiments. Cytoxicity evaluation of these new molecules showed promising results with a low cytotoxicity, especially when co-lipids were included in the formulation. These compounds represent a new family of gene transfer vectors which display good transfection efficiency and low toxicity, possibly due to the natural properties of glycine betaine. These compounds have great potential for the future development of *in vivo* gene transfer protocols.

The field of non viral vector-mediated gene therapy, and particularly the use of cationic lipids has made a great strides since the initial report by Felgner *et al* (1) in 1987 to their use in the world's first human gene therapy clinical trial by Nabel *et al* in 1992 (2). Cationic lipids have been shown to be an interesting alternative to viral vector-mediated gene delivery. Cationic lipids can be defined as comprising of three principal structural motifs: a cationic polynucleotide binding domain a hydrophobic domain and a linker or spacer arm that bridges the headgroup with the lipid anchor.

The chemical structure of each moiety dictates the biophysical properties exhibited by the cationic lipid. Structural modifications may therefore result in significant changes in gene delivery efficiency. Numerous studies have tried to establish general principles for the design of efficient cationic lipids for DNA transfection (3,4,5,6,7). However, most cationic lipids are not metabolized and accumulation may cause toxic cell effects. Therefore, there is a need for more efficient, less toxic and biodegradable cationic lipids. We have designed a novel family of biocompatible amphiphiles (GB compounds) which are characterized by: (i) a positively charged head group derived from the naturally occurring glycine betaine; (ii) a hydrophobic domain composed of two fatty acid esters; and (iii) a glycerol-type spacer which is linked to the polar head group lipophilic chains by readily metabolizable peptide and ester bonds, respectively. These molecules are characterized by their low toxicity *in vitro* and one might except a good biodegradability. Transfection activity was evaluated using a reporter gene *in vitro* on two types of cell lines: adherent and non-adherent. The transfection results are shown in comparison with those observed using the commercially available compound :Lipofectin (DOTMA/DOPE, GIBCO-BRL). Serum resistance of the GB compounds was tested on different cell lines and it was shown that the inhibitory effect of serum on lipofection could be overcome by including co-lipid in the cationic liposome formulation. Using two of these GB compounds, superior transfection activity was obtained compared with Lipofectin even in presence of serum.

MATERIALS AND METHODS

Cell-culture. K562 cells, and HT29 cells were maintained in RPMI-1640 medium or DMEM respectively and supplemented with 10% fetal calf serum (FCS), 0.2 mM glutamine, 100 U/ml of penicillin, 100 U/ml of streptomycin and 1% fungizone. All cells were maintained in 5% Co2 and at 37°C.

Plasmids. The plasmids used was pCMVLacZ, containing the LacZ gene encoding β-galactosidase under the control of the cytomegalovirus (CMV) promoter and pCMVLuc (a gift of O. Feugeas, Strasbourg) encoding luciferase protein.

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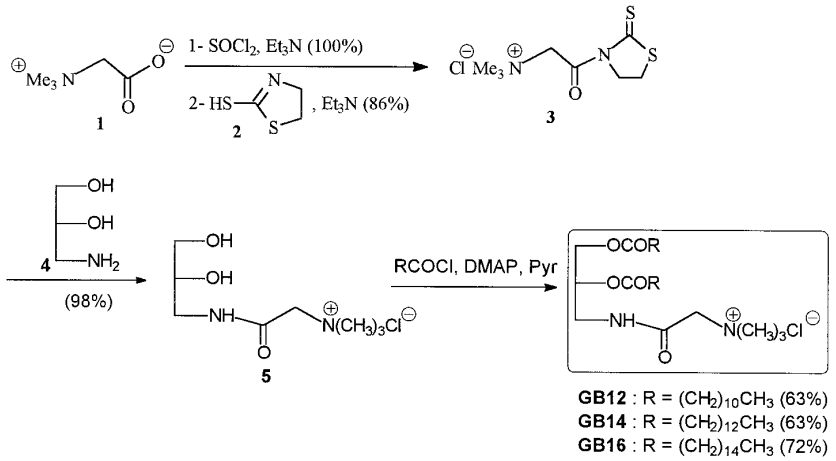

FIG. 1. Synthesis of cationic amphiphiles GB12, GB14 and GB16.

Cationic lipids. Commercialy available compound :Lipofectin (DOTMA/DOPE, GIBCO-BRL) and GB compounds (see syntheses in results section, Figure 1) were used.

Lipoplex preparation. Each of the GB compounds was prepared alone or in combination with the neutral lipid DOPE (Sigma, Saint Quentin Fallavier, France) as described elsewhere (8, 9).

Determination of lyotropic liquid crystalline properties. The lyotropic liquid crystalline properties were determined by thermal polarized light microscopy using a Zeiss Universal polarizing transmitted light microscope equipped with a Mettler FP82 microfurnace in conjunction with a FP80 Central Processor. Investigations into the phase identification and determination of phase transition temperature were carried out by simply running a small amount of water onto a crystalline sample sandwiched between a cover-slip and a slide. The lyotropic phase textures were observed in the polarizing microscope at room temperature by allowing crystals of the materials to dissolve in water and upon heating in order to measure the phase transition temperatures.

Transfection protocol. Transfection activity of the cationic lipid: DNA complexes *in vitro* was assessed using K562, HT29, CFPAC-1 and CFT1 cell lines. Cells were seeded onto a 96-well tissue culture plate at 20000 per well (16 wells per lipid tested). Adherent cells (HT29, CFPAC-1, CFT1) were seeded 24 hours before transfection and incubated overnight in a humidified 5% CO2 atmosphere at 37°C. Non adherent cells (K562) were seeded one hour before transfection. Transfection of the cells was performed as described by Felgner *et al* (3) with the following modifications. Appropriate amounts of the cationic lipids and the plasmid vector pCMVβGal in OptiMEM were complexed and 100μl were added to each well. After 2.5 hours at 37°C, the cells were supplemented with 200μl of appropriate growth medium. Following a further 72 hours at 37°C, the cells were assayed for β-Gal expression using a chemiluminescent assay. Using this transfection protocol it was possible to compare transfection activity of 8 different charges ratio of the lipoplex. This charge ratio ranged from 0.22 to 13.97.

Determination of charge ratio of lipoplex. The charge ratio was theoretically calculated as mole ratio of GB compound (one positive charge per molecule) or Lipofectin (one positive charge per molecule) to nucleotide residue (average MW 330).

Determination of cell toxicity. The relative cytotoxicity of the different lipid:DNA complexes were determined as the number of cells surviving the transfection experiment measured using a chemiluminescent assay: CYTOLITE assay (Pakard) as specified by the manufacturer. 24 hours before the assay, the cells were plated in a 96-wells plate (25000 cells per well). Cells were treated for transfection as described above and incubated for an additional 48 hour period. After this time the cytotoxicity assay was carried out as specified by the manufacture. The amount of relative light units (RLU) formed was proportional to the number of living cells. Non transfected cells were used non transfected cells.

Luminescent detection of β-galactosidase. β-galactosidase activity was measured with a luminescent β-galactosidase detection kit (Clontech). Assays were carried out as described by the manufacturer. The results were expressed in TRLU (Total Relative Light Unit obtained with the 16 wells) or in RLU (Relative Light Unit obtained with 1 well).

RESULTS

GB Compounds Synthesis

Cationic amphiphiles are generally synthesized by standard quaternization of tertiary amines (10). In order to avoid toxic methylating reagents, an attractive alternative introduces the ammonium group directly from a natural amino-acid, i.e. glycine betaine **1.** The second part of the target GB-amphiphiles is composed of a diacyl glycerolipid derivative. The glyceryl moiety was obtained according to a multistep procedure that involved solketal as a starting material (11). This product is available in a racemic or in an optically pure form. However, racemic but unprotected 3-amino-1,2-propanediol 6 was preferred because the cationic head group could be introduced prior to the aliphatic chains, thus limiting the number of chemical and purification steps (Figure 1).

Owing to its low reactivity, glycine betaine **1** is not commonly used as a chemical reagent. Nevertheless, this nucleophilic substrate could be converted into a reactive electrophilic derivative **3.** Reaction of **1** with thionyl chloride (12,13) quantitatively bound the corresponding acyl chloride which was then, without any further purification, treated with 2-thiazoline-2-thiol **2** (14,15) to yield the stable crystalline and activated *N*-acyl thiazolidine-2-thione **3.** Commercially available 3-amino-1,2-propanediol **4** was quantitatively *N*-acylated

TABLE 1

Mesophase Domains for GB Compounds

Compound	n	Mesophase at room temperature	Phase transition temperature H_{II}-$L\alpha_{\square\square}$(°C)
GB12	10	Lamellar L_α	—
GB14	12	Hexagonal H_{II}	56.6
GB16	14	Hexagonal H_{II}	54.7

by reagent **3** to give the cationic diol **5** under mild conditions. The target cationic amphiphiles GB12, 14, 16 were obtained in a second step using lauroyl, myristoyl or palmitoyl chloride, respectively, in pyridine and in the presence of 4-dimethyl-aminopyridine (DMAP). The 2-thiazoline-2-thiol by-product was easily removed by column chromatography. GB12, 14, 16 were finally purified by recrystallization from ethyl acetate-chloroform and isolated in 63-72% yields. Therefore, this method represents a rapid access to cationic glycerolipid compounds derived from natural glycine betaine. Additionally, it is noteworthy that GB-derivatives could be produced on a multigram scale with a low overall cost.

Lyotropic Properties of Cationic Lipids

Investigations into the lyotropic phases were carried out for all three compounds GB12, GB14 and GB16 in distilled water. The observation of the solvation process under the polarizing microscope indicated that all derivatives exhibited lyotropic behaviour. The lauroyl derivative GB12 exhibited a lamellar mesophase at room temperature and no change in the phase texture was observed upon heating up to 80°C. Conversely, compounds GB14 and GB16 having longer acyl chains which increased their hydrophobic volume, induced a conical molecular geometry, favoring the formation of an inverted hexagonal phase H_{II} at room temperature. However, a phase transition temperature was observed around 55°C corresponding to a hexagonal to lamellar phase transition for both compounds (Table 1).

These results may be useful for correlating transfection efficiencies with mesomorphic properties of cationic carriers at physiological temperature (37°C).

Transfection Efficiency on Different Cell Lines

First, the ability of our new compounds to mediate gene transfer in cell lines was assessed. To determine if these new compounds exhibited the same transfection activity in different cell lines, two types of cell lines non adherent (K562) or adherent (HT29) were used. The cells were transfected using a vector containing a LacZ reporter gene under the control of the CMV promoter. Most currently produced cationic lipid reagents contain a mixture of a cationic lipid and a neutral lipid (DOPE). The transfection activities of the three cationic amphiphilic compounds formulated in either presence or absence of DOPE (1:1, w:w) were compared with the results obtained using a commercially available cationic lipids formulation :Lipofectin, three days after transfection using a luminescent βgalactosidase detection assay (Figure 2). Using the TRLU criterion, the best transfection activities were obtained with the GB compounds rather than the commercial formulation, irrespective of the cell line used. In K562 cell line (Figure 2) the transfection activity was higher for all the cationic amphiphile lipids when DOPE was not added in the formulation. Using adherent cell line (Figure 2) , HT29 the results were increased by adding DOPE in the formulation. Similar results have been previously reported with other cationic lipids (6,8,9). It is important to note that for the K562 cell line, the best transfection activity was obtained using the GB14 compound, whereas the HT29 adherent cell line showed the best results after transfection with the

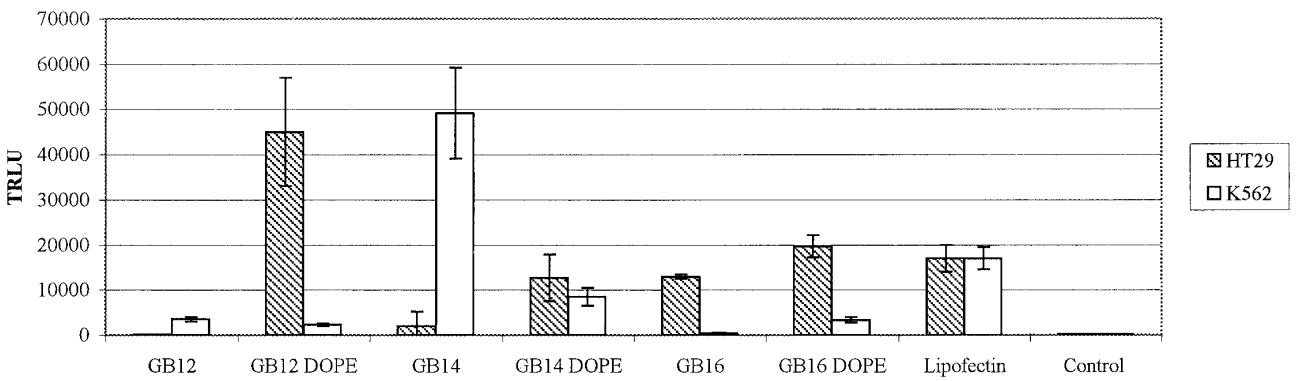

FIG. 2. Transfection efficiency of GB compounds on K562 and HT29 cells. Comparison of the transfection efficiency in K562 non-adherent cells and adherent HT29 cells of GB compounds and Lipofectin, a commercially available lipid reagent for DNA delivery. The cells were transfected in a 96-wells plate as described in Materials and Methods section. Cells transfected with free DNA were used as control. βgal activity was measured 2 days after transfection. Values represent the means of at least 3 different experiments.

TABLE 2

Evaluation of the Influence of Serum on the GB Compounds Transfection Efficiency Using the Optimal Well RLU Criterion

	Without serum		With serum	
Compounds	Optimal well RLU	R	Optimal well RLU	R
GB12	400	1,75	210	0,87
GB12 DOPE	11025	0,87	9500	0,87
GB12 CHOL	4500	0,87	3000	0,87
GB14	800	1,59	850	0,8
GB14 DOPE	6000	0,8	7345	0,8
GB14 CHOL	3000	0,8	3886	0,8
GB16	300	1,47	200	1,47
GB16 DOPE	3250	0,73	4375	0,73
GB16 CHOL	1000	0,73	1135	0,73
Lipofectin	900	0,37	1100	0,74
Control	12		13	

Note. For each TRLU value the corresponding calculated charge ratio between lipid and pDNA was reported. HT29 cells were transfected as described in Materials and Methods section. Not transfected cells were used as a control. Results shown are average values from 3 experiments.

GB12 compound (formulated with DOPE). Similar results were obtained using two other adherent cell lines; CFT1 and CFPAC (data not shown).

Influence of the Co-lipid on the Inhibitory Effect of Serum on Lipofection

Most currently produced cationic lipid reagents contain a mixture of a cationic lipid and a neutral lipid. The transfection activities of the three amphiphilic cationic GB compounds formulated in the presence or absence of DOPE or cholesterol (in ratio 1:1) were compared 72 after the transfection. The effect of serum on lipofection was also tested. Ht29 cells were lipofected in 96-wells plates using a pCMVLacZ plasmid as described above. The results obtained are presented in Table 2. For each GB compound (formulated with or without co-lipid) the optimal well RLU and the corresponding lipoplex charge ratio in this well were observed after lipofection with and without serum. The transfection activity of the GB compounds was increased by the inclusion of a co-lipid in the cationic lipid formulation, in presence or absence of serum. In every cases the results obtained with Dope were superior to those observed using cholesterol as co-lipid in the formulation. The transfection activities decreased in presence of serum when the GB compounds were formulated without co-lipid. With the GB16 and GB14 compounds, the inhibitory effect of serum on lipofection was overcome by including co-lipid in the formulation. With GB12, a high transfection activity was always found when co-lipid was included even in presence of serum. Using GB compounds formulated with co-lipid produced the best results when negatively charged DNA/lipid complexes (R=0.8, R=0.73) were used. The optimal charge ratio lipoplex (R) used was the same in presence or absence of serum. The transfection activities of the GB formulation without co-lipid decreased in presence of serum irrespective the compound used.

Analysis of the in Vitro Cytotoxicity of the GB Compounds

The cytotoxicity of the GB compounds were evaluated using the same procedure as described above. HT29 cells (25000 per well) were seeded and lipofected, with the cells then assayed for cytotoxicity using a luminescent assay 72 hours after transfection (see Materials and Methods). This assay determined the number of viable cells (Figure 3). Only very low toxicity levels were observed when cells were lipofected in presence of serum, regardless of the lipoplex used, with cells growing as well as untreated control. Both in absence or presence of serum, the GB compounds formulated without co-lipid were more cytotoxic than those formulated with co-lipid especially with GB12. For each GB compound, the relative cytotoxicity observed 72 hours after the lipofection was lower than that obtained using the commercial reagent, Lipofectin.

DISCUSSION

Our aim was to study the feasibility of glycinebetaine amphiphile derivative (GB compounds) for DNA gene transfer *in vitro*. First, the transfection efficiency of the GB/DNA complexes targeted to several types of cell lines were evaluated using a LacZ reporter gene. In both K562 and HT29 cell lines, the results obtained were higher than those observed using commercially available lipid cationic agents. But it was neither the same GB compound (GB12/GB14) nor the same formulation (with or without DOPE) which gave the best transfection activity on the 2 cell lines. Several authors (3,16) have reported that the inclusion of DOPE in the cationic lipid formulation increases transfection activity by enhancing mixing and fusion of liposomes and cell membranes. DOPE is not always required to obtain efficient transfection (6,8). Observations concerning cell type-specific transfection efficiencies have already been reported (17). Fasbender suggested that (17),whilst the reasons for cell type-specific lipid effects have not yet been established, the cell surface composition or specific tissue uptake mechanism of lipoplexes could be important to explain variations in transfection efficiencies. Reports about structure/activity of cationic lipids (3,4,5,6,7) have not yet established precise rules for predicting specific cell line or tissue transfection efficiency. Therefore, screen-

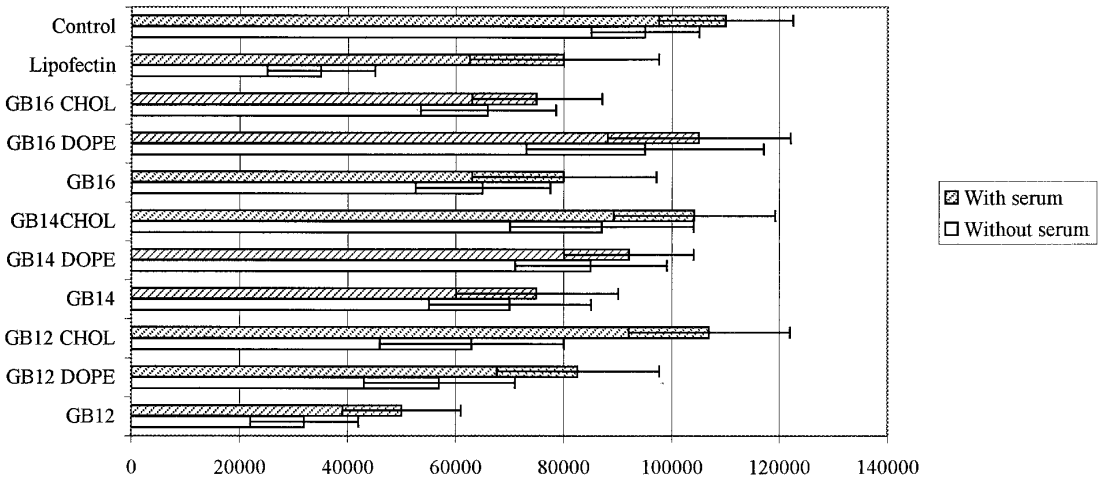

FIG. 3. Determination of cytotoxicity of the GB compounds. The toxicities given here are expressed by the number of cells surviving after the transfection experiment using the most efficient lipid/DNA ratio (see Table 2). HT29 cells were transfected as described in Materials and Methods section. Viable cell numbers were determined using a chemiluminescent assay (cytolyte Packard). Untransfected cells were used as a control. Results shown are average values from 2 experiments.

ing assays on different cell lines or tissue are still necessary to carry out.

Although the hexagonal inverted phase structures have been described as transfection enhancing structures (18) the higher transfection efficiency on HT29 cell line was observed using the GB12 compound which is in a lamellar phase structure at 37°C. Our results are in agreement with those observed by Balasubramanian (4), who stated that cationic lipids Tc is not predictive of transfection efficacy. It is important to note that we have determined the Tc of the cationic lipid alone, and as addition of DNA on cationic lipids results in profound change in the aggregate morphology (19), the Tc of the cationic lipid may not be indicative of the Tc of the lipoplex.

Decreasing of transfection efficiency of cationic lipids in presence serum could be one explanation of their low activity *in vivo*, and consequently limits their *in vivo* applications (1). New cationic lipids (GS2888) (20) and new formulations of DNA and cationic lipids (21) have been synthesized to resolve this problem. It has been demonstrated that in addition to their high transfection activity in the absence of serum, GB formulations are found to be equally or more active in the presence of serum when co-lipid is included. It was recently described (22) that inclusion of cholesterol in DOTAP transfection complexes increased the delivery of DNA to cells *in vitro* in the presence of serum. Here, DOPE was more effective than cholesterol on HT29 cell line in overcoming the inhibitory effect of serum on lipofection. This suggests that the neutral co-lipid may play a major role in cationic lipid mediated gene transfer, particularly in presence of serum. However, as reported by Fasbender *et al* (17), the development of lipid mediated gene transfer must consider the neutral co-lipid in the context of the specific target cell. Recently, Yang (23) reported that the inhibitory effect of serum on lipofection can be overcome by increasing the charge ratio of the cationic lipid/ DNA complex. Here, increasing the charge ratio did not improve the transfection activity of the GB formulations in presence of serum. In presence or absence of serum, it was the same ratio which gave the higher result. In addition, our results showed that there was no direct relationship between transfection efficiency and net positive charge of the complex, the highest transfection efficiency being obtained using a negatively charge complex. Only a theoretically calculated charge ratio was reported here, this observation needs to be confirmed by determination of the zeta potential of the complexes.

In general, *in vitro* studies have shown that cationic lipids exhibit significant toxicity. Thus, at higher concentrations of cationic lipid, specific transfection is high but cells are lost, whilst at lower lipid concentrations both toxicity and transfection are minimal (24). The toxicity of some of the commercially available cationic lipids, such as Lipofectin, has been attributed to their non-natural, non-biodegradable nature (1,25). Much effort has recently been devoted to resolving this toxicity problem. For example novel pyridinium surfactants for non toxic *in vitro* delivery and cationic lipid derived from sphingosine have recently been synthesized (26,27). We suggest that the natural properties of glycine betaine could explain the low toxicity of GB formulations. The relative cytotoxicity of the GB12 compound could be explained by its short acyl chain length.

In summary, we have shown that transfection efficiency with low toxicity can be obtained in vitro using glycine betaine amphiphile derivatives. Their transfec-

tion efficiency and low economical cost argue that this compounds would be good candidates for gene therapy experiments both *in vitro* and *in vivo.*

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