Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 787–791

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Transfection Properties of Novel Non-Toxic Monocationic Lipids. Variation of Lipid Anchor, Spacer and Head Group Structure

Thomas Fichert, Anne Regelin and Ulrich Massing*

Tumor Biology Center, Department of Clinical Research, Breisacher Straße 117, 79106 Freiburg, Germany

Received 4 October 1999; accepted 8 February 2000

Abstract—This report describes the synthesis and the transfection properties of novel monocationic non-toxic lipids. We have carried out structural variations in all three units of the transfection lipid, the lipid anchor, the spacer moiety and the positively charged head group. Our results lead to the conclusion that systematic modification of structural subunits is a promising way to enhance the transfection efficiency. © 2000 Elsevier Science Ltd. All rights reserved.

Cationic lipid-mediated gene transfer is currently under investigation for gene therapy applications due to findings that these non-viral vectors represent a safe gene transfer strategy not causing immune responses. Various cationic lipids have been synthesized and shown to be able to mediate the transfer of DNA into cells in vitro.[1–5] Most of the lipids bear head groups containing one tertiary or quaternary amino group or a polyamine. Within the last years, effort has been made to synthesize more effective compounds. Up to now, no cationic lipid has been described which has transfection properties sufficient for the strong requirements for in vivo gene therapy application.

Here we describe the synthesis of novel cationic lipids containing cholesterol or 1,2-dimyristyloxypropan-3-ol as a lipid anchor, a tertiary or a quaternary amino group as a head group and various spacer moieties which differ in length and polarity (Fig. 1, Table 1).

Chemistry

The synthesis of all lipids[7] is shown in Figure 2. In general, the first step was to link the lipid anchor cholesterol (Chol) or 1,2-dimyristyloxypropan-3-ol (DMG) to various spacer moieties by esterification. We have chosen the acetyl, the carbonate and the succinyl spacer which differ not only in polarity but also in length.

The lipid anchor 1,2-dimyristyloxypropan-3-ol was prepared by a four-step reaction sequence[8] (**a**). The acetyl spacer was introduced by esterification of cholesterol or 1,2-dimyristyloxypropan-3-ol with chloroacetic anhydride. Alkylation of dimethyl amine using the respective chloroacetic esters **2** and **4** gave the compounds **3** and **5** bearing a tertiary amino group. Esterification of *N,N*-dimethylaminoethanol using cholesteryl chloroformiate led to **6**. After esterification of cholesterol with succinic anhydride the resulting cholesterol hemisuccinate was converted into the acid chloride[9] which was added to the respective tertiary amino compounds yielding the lipids **12**, **13**,[6] **14** and **15**. In addition, we have carried out some head group variations by alkylating the tertiary amino group using dimethylsulfate (to give the lipids **7**, **8** and **10**) or 2-iodoethanol (to give the lipids **9** and **11**).

Preparation of cationic liposomes

The respective cationic lipid and 1,2-dioleoyl-glycero-3-phosphoethanol-amine (DOPE, in a molar ratio of 1:1; in the case of lipids bearing the DMG anchor cholesterol was used as helper lipid) were dissolved in chloroform/methanol (2:1 v/v) and mixed in a glass vial. Dried lipid films were prepared by slowly removing the solvent under a nitrogen stream. HEPES buffer (pH 7.4) was added at a final cationic lipid concentration of 1.29 mM to rehydrate the lipids at rt, then the vial was sealed and sonicated for 2 min in a water bath sonicator. Liposome sizes determined by means of a submicron particle sizer were 50 to 200 nm depending on the respective lipid. Using lipids **2** and **4** no liposomes could be prepared.

*Corresponding author. Fax: +49-761-206-2174; e-mail: umas@tumorbio.uni-freiburg.de

PII: S0960-894X(00)00099-8

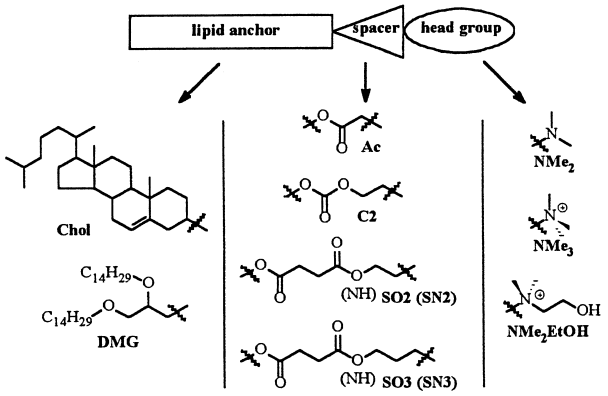

Figure 1. Structures of the synthesized monocationic lipids.

Table 1.

Comp.	Lipid anchor	Spacer	Head group
5	Chol	Ac	NMe_2
8	Chol	Ac	NMe_3
9	Chol	Ac	NMe_2EtOH
6	Chol	C2	NMe_2
10	Chol	C2	NMe_3
11	Chol	C2	NMe_2EtOH
12	Chol	SO2	NMe_2
13[6]	Chol	SN2	NMe_2
14	Chol	SO3	NMe_2
15	Chol	SN3	NMe_2
3	DMG	Ac	NMe_2
7	DMG	Ac	NMe_3

Lipoplex formation and cell transfection

Plasmid DNA (pCMVluc, containing a firefly luciferase gene driven by a CMV promoter) dissolved in HEPES buffer (pH 7.4) was added to eight different dilutions of each liposomal dispersion, finally leading to eight different lipid/DNA charge ratios (from 1:1 to 15:1) at a constant DNA concentration (125 ng/well, 96-well plate). The DNA and the liposome dispersion were gently mixed and allowed to stand for 1 h at rt to form lipid/DNA complexes (lipoplexes). Twenty-four hours prior to transfection, COS-7 cells, maintained in EMEM/10% fetal calf serum/1% penicillin-streptomycin at 37 °C/5% CO_2, were seeded in 96-well plates at 5000 cells per well. One hour before lipoplexes were given to the cells, 50% of the cell culture medium was removed from each well to be later replaced by an equal volume (90 μl) of lipoplex dispersion. All experiments were performed in triplicates, i.e., cells in three different wells were transfected by the same lipoplex dispersion. Cells were incubated for 4 h at 37 °C, then the medium was completely removed and replaced by fresh medium.

Luciferase and protein assay

Forty-six hours post transfection, cells were washed and lysed. Using equal volumes of the cell lysate, a reporter gene assay and a BCA protein assay were performed. Luciferase activity was determined by adding 80 μl of luciferin reagent (25 mM glycylglycine, 5 mM ATP, 0.2 mM luciferin) to 20 μl of the homogenized cell suspension in a white 96-well microtiterplate and by subsequently measuring the enzymatic activity over a period of 10 s in a microtiterplate luminometer. Luciferase activity was expressed as RLU/μg of protein and compared to that of DOTAP lipoplexes used as standard.

Discussion

Transfection profiles

The transfection efficiencies (TE) of all lipids, in dependence on the lipid/DNA charge ratios, are presented in Table 2. Using our lipids for transfection experiments with COS-7 cells, apparently no cytotoxic side effects could be observed. (This was concluded from the amount of cellular protein, determined after transfection, which is closely related to the cytotoxicity of the respective kind of lipid.) The ability of forming liposomes (bilayer structures) seems to be a crucial prerequisite for high TE which is illustrated by the low TE of the compounds **3** and **5**. These two lipids do not form liposomes and therefore show only marginal TE, an observation which is consistent with previous results (data not shown). For most lipids, the highest TE were obtained at lipid/DNA charge ratios of about 3:1. Such a typical transfection profile is depicted in Figure 3 (lipid **8**). As an exception, the transfection profiles of the compounds **7** and **9** are characterized by a different profile described in Figure 4 (lipid **7**). Using these two lipids no distinct maximum at the typical lipid/DNA charge ratio could be observed. Instead, TE rose steadily with increasing lipid/DNA charge ratio, leading to a rather monotonous profile. We suppose that the lipoplexes containing lipids **7** and **9** are taken up into the cell by a different mechanism, possibly by membrane fusion and not by endocytosis.

Influence of the lipid anchor

In this study cholesterol was chosen as a lipid anchor because it represents a naturally occurring structure which should be biodegradable and therefore non-toxic. There are some hints that cholesterol is also responsible for enhanced TE[10–12] which is consistent with our results: Compound **7** (containing the 1,2-dimyristyloxypropan-3-ol anchor) and compound **8** (containing the cholesterol anchor) have the acetyl spacer and the quaternary ammonium head group in common. Comparing the transfection results of these two compounds which differ in their apolar subunits, the cholesterol derivative **8** shows a higher TE than the DMG derivative **7** (see Figs. 3 and 4).

Influence of the spacer moiety

The TE was greatly influenced by the spacer moiety due to the inductive effect of the ester group on the amino group and due to its polarity and length. The chemical structure of the spacer influences the protonation state of the tertiary amino head group[13] and therefore the transfection properties of the lipid. From our results we conclude that the acetyl spacer seems to decrease the pK of the tertiary amine due to its strong inductive effect

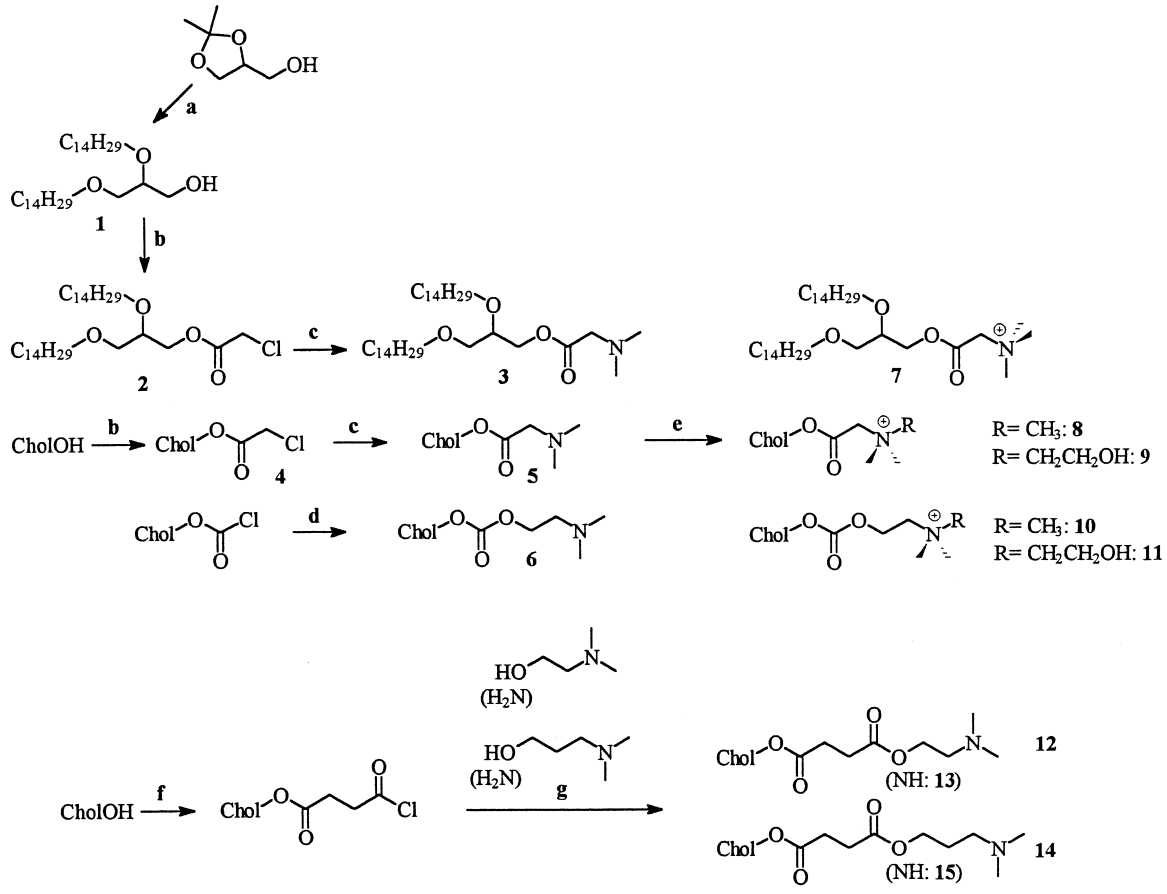

Figure 2. Synthesis scheme: (**a**) 1: BnCl, *t*-BuOK, THF, rt; 2: 2 N HCl; 3: $C_{14}H_{29}Br$, *t*-BuOK, toluene, reflux; 4: H_2, Pd-C, THF:MeOH (1:1); 69% over all 4 steps; (**b**) $(ClCH_2CO)_2O$, NEt_3, H_2CCl_2, 96% (**2**), 97% (**4**); (**c**) $HNMe_2$, EtOH, 32% (**3**), 53% (**5**); (**d**) $HOCH_2CH_2NH_2$, NEt_3, DMAP, H_2CCl_2, 60%; (**e**) $(MeO)_2SO_2$, acetone, 40% (**7**), 72% (**8**), 88% (**10**); ICH_2CH_2OH, acetone, 70% (**9**), 49% (**11**); (**f**) 1: succinic anhydride, NEt_3, DMAP, ethyl acetate; 2: $SOCl_2$, toluene, 80 °C; (**g**) the respective amino compound, NEt_3, H_2CCl_2, 62% (**12**), 52% (**13**), 42% (**14**), 64% (**15**).

Table 2. Transfection efficiencies of the monocationic lipids depending on different lipid/DNA charge ratios[a]

Lipid/DNA	Transfection efficiencies (mean ± SD) [$\times 10^2$ RLU/μg protein]											
Charge ratio	**3**[b]	**5**[b]	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1:1	33 ± 5	69 ± 11	405 ± 32	42 ± 9	170 ± 28	43 ± 9	485 ± 102	173 ± 23	140 ± 10	170 ± 30	80 ± 18	128 ± 32
3:1	80 ± 7	40 ± 7	930 ± 117	41 ± 3	866 ± 195	48 ± 16	972 ± 144	832 ± 75	270 ± 29	411 ± 57	92 ± 18	373 ± 18
5:1	55 ± 4	15 ± 1	766 ± 97	100 ± 13	201 ± 7	185 ± 33	772 ± 32	255 ± 9	122 ± 21	941 ± 69	92 ± 11	225 ± 36
7:1	6 ± 1	17 ± 6	418 ± 41	151 ± 25	53 ± 9	265 ± 21	454 ± 27	105 ± 13	106 ± 9	1089 ± 42	89 ± 2	148 ± 17
9:1	17 ± 5	20 ± 2	248 ± 5	177 ± 4	43 ± 5	296 ± 45	114 ± 6	87 ± 10	96 ± 5	981 ± 58	91 ± 6	98 ± 8
11:1	10 ± 2	31 ± 11	152 ± 12	187 ± 10	28 ± 2	311 ± 40	36 ± 6	81 ± 7	75 ± 2	754 ± 89	60 ± 8	88 ± 22
13:1	25 ± 4	21 ± 12	109 ± 24	294 ± 26	20 ± 2	300 ± 16	22 ± 2	50 ± 9	72 ± 9	543 ± 69	61 ± 8	79 ± 14
15:1	6 ± 1	34 ± 5	74 ± 6	314 ± 29	17 ± 4	310 ± 30	20 ± 1	53 ± 9	67 ± 9	365 ± 14	47 ± 18	80 ± 6

[a]Transfection efficiency of DOTAP at a lipid/DNA charge ratio of 2.5:1 was 415 ± 89 [$\times 10^2$ RLU/μg protein].
[b]These compounds do not form liposomes.

caused by the ester group: The lipids **3** and **5** are obviously not easily protonated because of the inductive effect of the ester group and exhibited only low TE. In contrast, lipid **6**, where the tertiary amino group is linked with cholesterol by a longer hydrocarbon chain and where the pK is apparently not affected by an inductive effect, led to high TE.

Compounds **12**–**15** contain the succinyl spacer which is longer and more polar than the acetyl or the carbonate spacer. Compounds **13** and **15**, characterized by a more polar amide linkage, led to higher TE than the lipids **12** and **14**, in which the amide function is replaced by a less polar ester function. Elongation of the distance between the amino function and the spacer moiety from two methylene units (lipids **12** and **13**) to three methylene units (lipids **14** and **15**) seems to have a negative effect on the TE.

Influence of the head group

We have also investigated the influence of quaternization of the tertiary amino head group on the TE. Quaternization of the amino group of lipids **3** and **5** was

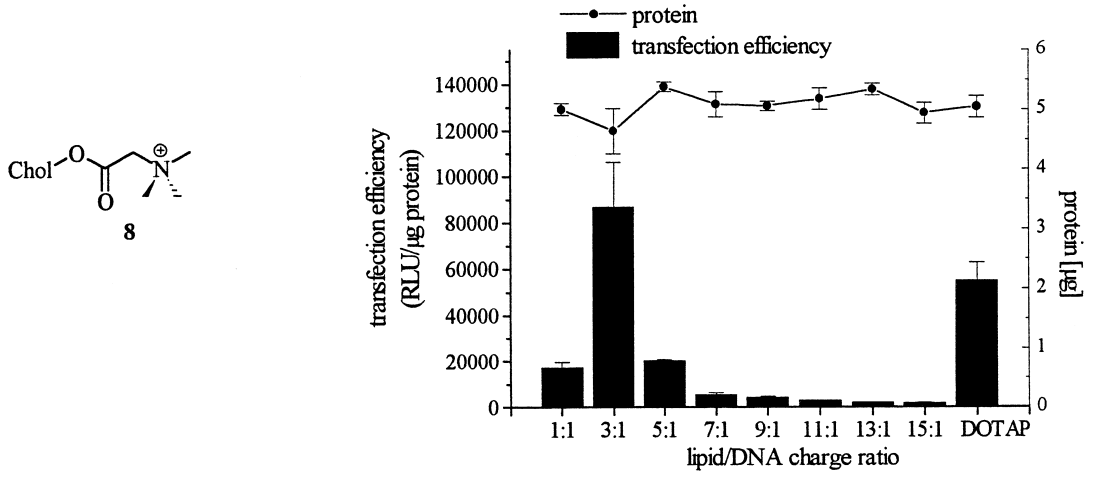

Figure 3. Transfection profile of compound **8**.

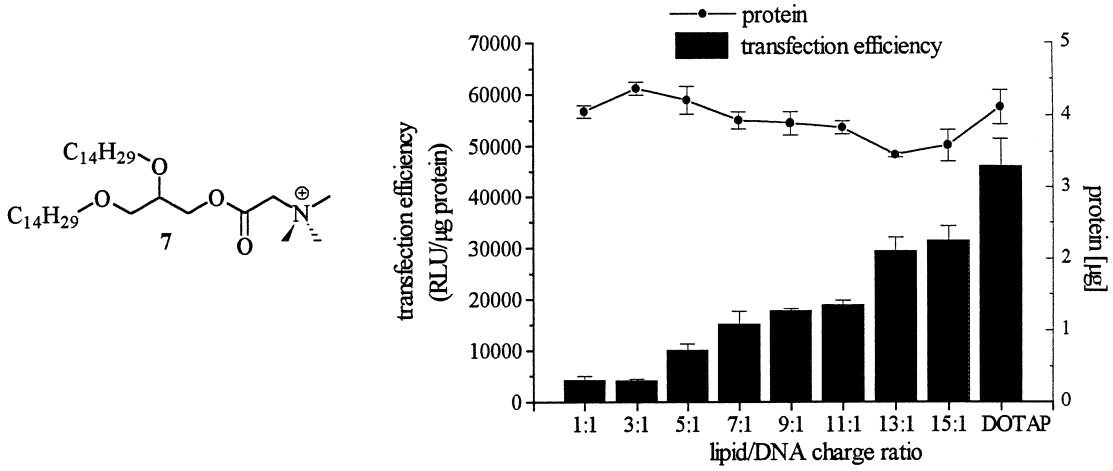

Figure 4. Transfection profile of compound **7**.

performed to permanently fix a positive charge yielding the lipids **7** and **8** which both show increased TE. This can be explained by the modified molecular shape after quaternization allowing the formation of liposomes. In contrast, lipid **6** (tertiary amino group, protonated at pH 7.4) and lipid **10** (quaternized amino group) show no essential differences in their liposomal sizes (data not shown) and in their TE. Quaternization of lipids **5** and **6** by introducing the more polar hydroxyethyl group[14] (instead of the methyl group) leading to the lipids **9** and **11** also results in considerable TE.

In summary, novel non-toxic monocationic lipids have been synthesized and have been tested for their application as gene transfer vectors into cells. The spacer moiety and the head group structure were widely varied and could therefore be optimized. Our studies reveal some very promising candidates which do not induce any cytotoxicity in experiments using COS-7 cells. Compared to the standard lipid DOTAP, we could observe considerable TE. From our point of view, cholesterol as lipid anchor has not only the advantage of good biodegradability but it is also responsible, as counterpart to the polar head group, for enhanced TE.

Based on the promising results of this study it is of special interest to synthesize and analyse also 2-fold and 3-fold cationic lipids based on cholesterol and various kinds of spacers discussed in this paper because polycationic head groups are known as a further crucial element to increase TE.

Acknowledgement

This work was supported by a research fellowship of the "Hermann-Schlosser-Stiftung".

References and Notes

1. Miller, A. D. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1768.
2. Byk, G.; Dubertret, C.; Escriou, V.; Frederic, M.; Jaslin, G.; Rangara, R.; Pitard, B.; Crouzet, J.; Wils, P.; Schwartz, B.; Scherman, D. *J. Med. Chem.* **1998**, *41*, 224.
3. Ren, T.; Liu, D. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1247.
4. Nazih, A.; Cordier, Y.; Bischoff, R.; Kolbe, H. V. J.; Heissler, D. *Tetrahedron Lett.* **1999**, *40*, 8089.

5. Bhattacharya, S.; Dilepp, P. V. *Tetrahedron Lett*. **1999**, *40*, 8167.
6. Preparation for the first time described in: Takeuchi, K.; Ishihara, M.; Kawaura, C.; Noji, M.; Furuno, T.; Nakanishi, M. *FEBS Lett*. **1996**, *397*, 207.
7. All compounds reported gave spectral data according to assigned structures.
8. Preparation based on: Ren, T.; Liu, D. *Tetrahedron Lett*. **1999**, *40*, 209.
9. Kley, J. T.; Fichert, Th.; Massing, U. *Chem. Mon*. **1998**, *129*, 319.
10. Cooper, R. G.; Etheridge, C. J.; Stewart, L.; Marshall, J. M.; Rudginsky, S.; Cheng, S. H.; Miller, A. D. *Chem. Eur. J*. **1998**, *4*, 137.
11. Lee, E. R.; Marshall, J.; Siegel, C. S.; Jiang, C.; Yew, N. S.; Nichols, M. R.; Nietupski, J. B.; Ziegler, R. J.; Lane, M. B.; Wang, K. X.; Wan, N. C.; Schuele, R. K.; Harris, D. J.; Smith, A. E.; Cheng, S. H. *Hum. Gene Ther*. **1996**, *7*, 1701.
12. Obika, S.; Yu, W.; Shimoyama, A.; Uneda, T.; Miyashita, K.; Doi, T.; Imanishi, T. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1817.
13. Bottega, R.; Epand, R. M. *Biochemistry* **1992**, *31*, 9025.
14. Banerjee, R.; Das, P. K.; Srilakshmi, G. V.; Chaudhuri, A.; Rao, N. M. *J. Med. Chem*. **1999**, *42*, 4292.