Reduction-Sensitive Lipopolyamines as a Novel Nonviral Gene Delivery System for Modulated Release of DNA with Improved Transgene Expression

Gerardo Byk,*,‡ Barbara Wetzer,† Marc Frederic,† Catherine Dubertret,† Bruno Pitard,† Gabrielle Jaslin,‡ and Daniel Scherman†

Laboratory of Peptidomimetics and Genetic Chemistry, Department of Chemistry, Bar Ilan University, 52900-Ramat Gan, Israel, and UMR 7001, Laboratoire de Chimie Bioorganique et de Biotechnologie Moléculaire et Cellulaire, Ecole Nationale Supérieure de Chimie Paris/Centre National de la Recherche Scientifique/Aventis Pharma, Centre de la Recherche de Vitry-Alfortville, 13 Quai Jules Guesde, B.P. 14, 94403 Vitry sur Seine, France

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We have designed and synthesized original cationic lipids for modulated release of DNA from cationic lipid/DNA complexes. Our rationale was that modulated degradation of the lipids during or after penetration into the cell could improve the trafficking of DNA to the nucleus resulting in increased transgene expression. The new reduction-sensitive lipopolyamines (RSL) harbor a disulfide bridge within different positions in the backbone of the lipids as biosensitive function. A useful synthetic method was developed to obtain, with very good yields and reproducibility, unsymmetrical disulfide-bridged molecules, starting from symmetrical disulfides and thiols. The new lipopolyamines are good candidates as carriers of therapeutic genes for in vivo gene delivery. To optimize the transfection efficiency in these novel series, we have carried out structure−activity relationship studies by placing the disulfide bridge at different positions in the backbone of the cationic lipid and by systematic variation of lipid chain length. Results indicate that the transfection level can be modulated as a function of the location of the disulfide bridge in the molecule. We suggest that an early release of DNA during or after penetration into the cell, probably promoted by reduction of a disulfide bridge placed between the polyamine and the lipid, implies a total loss of transfection efficiency. On the other hand, proper modulation of DNA release by inserting the disulfide bridge between one lipid chain and the rest of the molecule brings about increased transfection efficiency as compared to previously described nondegradable lipopolyamine analogues. Finally, preliminary physicochemical characterization of the complexes demonstrates that DNA release from complexes can be modulated as a function of the surrounding reducing conditions of the complexes and of the localization of the disulfide bridge within the lipopolyamine. Our results suggest that RSL is a promising new approach for gene delivery.

Introduction

The development of new gene delivery technologies is a prerequisite as research moves toward the application of gene therapy in humans. In the last 10 years, a large number of nonviral gene delivery systems with high in vitro transfection efficiency have been described.[1−18] Although a number of nonviral gene delivery systems displayed significant in vivo gene expression, none of them has reached the level of viral-mediated gene expression.[19]

Studies on the intracellular fate of cationic lipid/DNA complexes have revealed that cationic lipid-mediated gene delivery is still a rather inefficient process.[20] Although a great deal of work is still needed, it is clear that the intracellular disassembling of cationic lipid/DNA complexes and nuclear uptake of plasmid DNA are avenues for improvement.[21] In previous studies using fluorescent cationic lipids/DNA complexes, we suggested that penetration into the cell cytoplasm is not the main limiting barrier for plasmid expression. In these fluorescence microscopy studies, we observed morphological differences between transfecting and nontransfecting particles in the cytoplasm. However, no correlation could be established between the amount of internalized complexes and gene expression. Indeed, using physicochemical methods,[22] the complexes were generally observed in the cytoplasm of all cells, while gene expression was observed in only about 5−12% of cells. In other studies, fluorescent plasmid could not be detected in the nucleus after cationic lipid-mediated gene transfer. It is thus likely that only a few copies (or even a single copy) of the plasmid reach the nucleus after penetration across the nuclear envelope barrier and that lack of detection results from the limited sensitivity of fluorescence microscopy to detect single or a few copies of the labeled plasmid. Finally, it was observed in double-labeling experiments that plasmid and lipids are mostly detected associated together. [23−26]

Taken together, our conclusion and working hypothesis is that an important limiting step in nonviral gene delivery and transgene expression is the release of DNA from cationic lipid complexes. This rationale prompted us to design and synthesize a new nonviral gene delivery system able to modulate the release of plasmid DNA from the complexes, after their penetration into cyto-

* To whom correspondence should be addressed. Tel: (972)-3-5318325. Fax: (972)-3-5351250. E-mail: bykger@mail.biu.ac.il.
‡ Bar Ilan University.
† Centre de la Recherche de Vitry-Alfortville.

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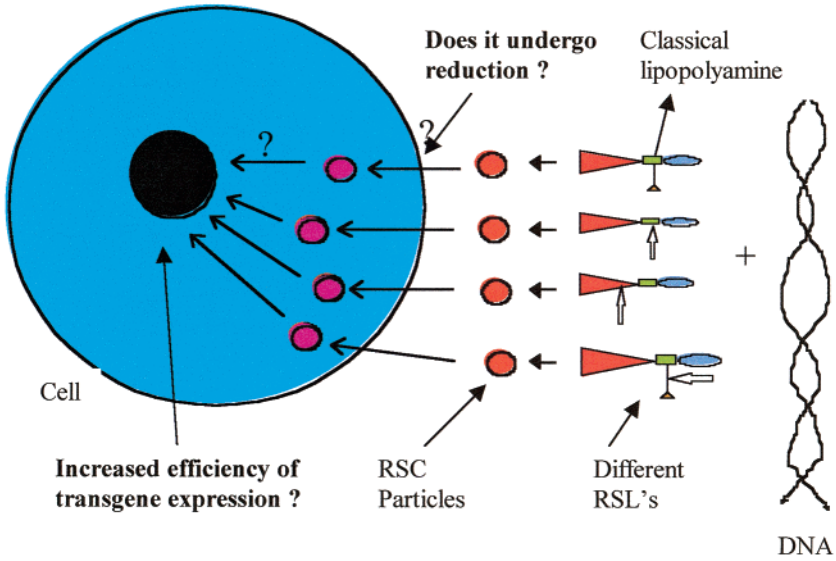

Figure 1. Schematic model of disulfide-bridged lipid (RSL) and complex (RSC): full arrows, disulfide bridge location within the RSL; blue spheres, polyamine; green boxes, linker; red triangles, fatty chains; orange triangles, side chain entity.

plasm.[27] This modulation might bring about a consequent increase in transgene expression.

We define modulation of DNA release as the ability of a nonviral transfection agent to enhance or decrease the expression of a formulated transgene. This can be obtained by inserting a biodegradable group in different positions of the lipid, provided that the biodegradable group does not interfere with the formation of the complexes with DNA and does not react with polyamines or other reactive functional groups present in the cationic lipid/DNA complexes. In previous studies, other groups have introduced ester functions into quaternary ammonium lipid/DNA complexes, to obtain lipids with reduced cytotoxicity and for an eventual accelerated release of DNA from complexes by the action of esterases (see a review in ref 19). This strategy cannot be easily applied to lipopolyamines, since the ester groups react with the free amines before or during the formation of the complex with DNA. In the present study we propose to use a disulfide bridge as the biodegradable group.[27] Disulfide-bridged molecules are relatively stable under atmospheric conditions and are not reactive toward polyamines or other functions present in the lipopolyamine/DNA complexes. On the other hand, one can expect the breaking of this disulfide bond in the reducing environment of cytoplasm.

So far no general mechanism of reduction in cells has been reported. Nevertheless several biological mechanisms, such as diphterietoxin and ricin cytotoxicity or sinbis virus membrane fusion, that depend on reduction by cellular components have been described.[28−30] In this context the importance of freely accessible thiol groups on the cell surface for disulfide reduction was shown. Moreover parts of the Golgi apparatus seem to play a role on reduction reactions in the cell. High cytosolic glutathione concentration, that can reach 10 mM in the liver, is also reported to affect intracellular reduction.[31]

We have called these systems RSL for **r**eduction-**s**ensitive **l**ipids and RSC for **r**eduction-**s**ensitive **c**omplexes formed between RSL and DNA. We have established a model series of RSL's, to answer several questions raised by this novel approach. First: Do RSL's

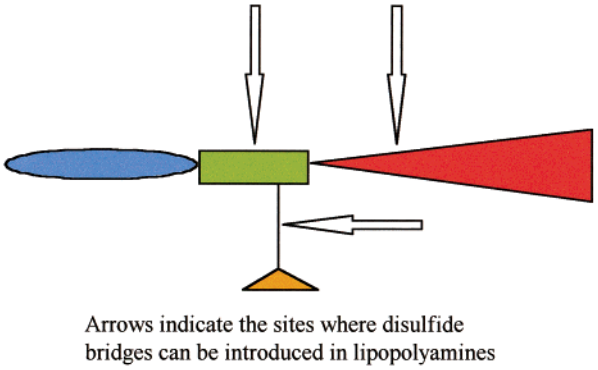

Figure 2. Design of lipopolyamines harboring disulfide bridges at different positions within lipopolyamine: blue spheres, polyamine; green boxes, linker; red triangles, fatty chains; orange triangles, side chain entity.

form complexes with DNA (RSC) similarly to classical lipopolyamines? Second: Is it possible to obtain increased transfection efficiency using such RSC's? (See Figure 1.) These questions will be answered in the next sections of this article.

Rational Design of Disulfide-Containing Lipopolyamines

Our working hypothesis was that lipopolyamines harboring disulfide bridges in their backbone would form normal complexes with DNA and that during penetration into the cell, which can be considered as a reducing medium (plasma membrane, cytoplasmic reductases), DNA/lipid complexes could be reduced and disrupted and DNA could be released from these complexes. This reduction/disruption process would potentially affect the transfection efficiency of the complexes.

Lipopolyamines are composed of four elements: (1) a lipid usually harboring two fatty chains (red triangle in Figure 2), (2) a cationic entity composed of polyamines, quaternary ammonium salts, amidinium, etc. (blue circle in Figure 2), (3) a spacer that links between the lipid and the cationic entity (green box in Figure 2), and (4) a side chain entity able to target the lipid to specific

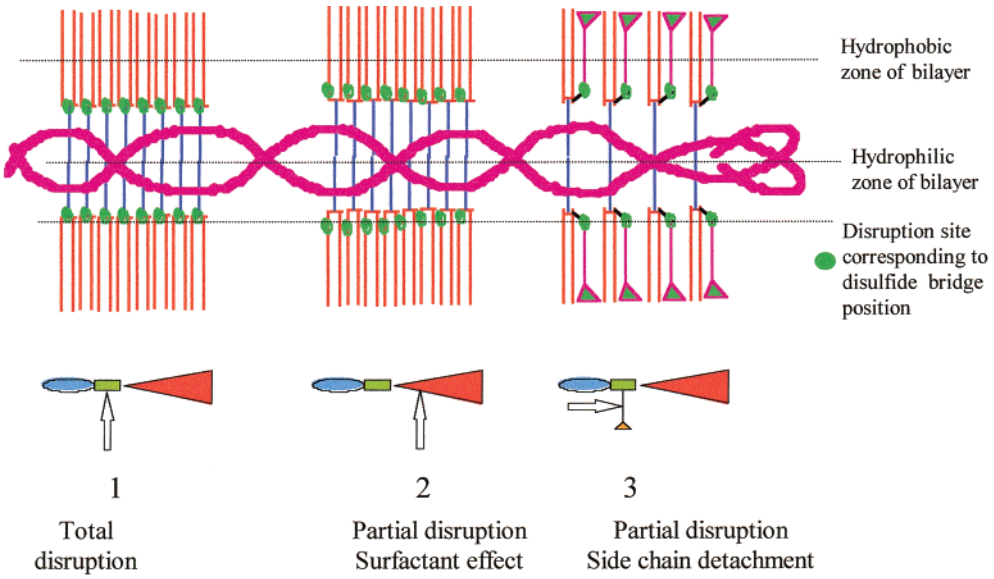

Figure 3. Predicted different effects of disulfide bridge reduction as a function of its location within the lipopolyamine: green circles, disruption site induced by disulfide bridge reduction.

tissues or for introduction of fluorescent probes as a tool for localization studies (orange triangle in Figure 2). The disulfide bridge can be introduced as a spacer or handle between each of the different elements of the cationic lipid.

Thus, if the disulfide bridge is inserted in the green zone, a total separation of the cationic (blue) and lipid (red) entities would be obtained after penetration and reduction in the cytoplasm (see 1 in Figure 3). Introduction of the disulfide bridge between one of the fatty chains and the rest of the cationic lipid in the red zone would transform the lipid structure with two fatty chains into a surfactant or detergent harboring a single fatty chain (see 2 in Figure 3). Finally, introduction of the disulfide bridge between the side chain entity and the backbone of the cationic lipid (zone orange) would induce the separation of the side chain entity from the lipid, and the lipid structure will not be essentially disrupted, but the targeting side chain entity will be separated from the lipid (see 3 in Figure 3). Each modification will affect differently the stability of the DNA/cationic lipid complexes and DNA delivery to the nucleus. Consequently, the final expression of the transgene will likely be also affected by these modifications.

During preparation of this article, some cationic lipids harboring a disulfide bridge between the cationic entity and the lipid (related to 1 in Figure 3) have been proposed by others as gene delivery systems.[32−34] However, none of them contained disulfide bridges in other positions of the cationic lipid. Our results will be analyzed and compared to those lipids in the next section, and extensive physicochemical considerations will be discussed in a separate article.[35]

Results and Discussion

Chemistry. The synthesis of unsymmetrical disulfide-bridged molecules of the type $R_1-S-S-R_2$ has not been extensively studied up to now. In early works, unsymmetrical disulfides were obtained by direct treatment of a symmetrical disulfide $R_1-S-S-R_1$ with an excess of a thiol R_2SH under basic conditions.[36−37] All the newer methods are tedious as they necessitate the synthesis of intermediate active sulfide ethers or active disulfides.[38−41] Therefore, we have adapted and extended the early procedure[36] to the synthesis of unsymmetrically substituted disulfide scaffolds suitable for the synthesis of cationic lipids. To introduce the disulfide bridge in the different positions of the cationic lipid, it was necessary to design and synthesize two different scaffolds bearing disulfide bridges (products **1** and **3** in Figure 4) and to use protected amino acid cystine as the third scaffold (product **2** in Figure 4) and as starting material for the synthesis of scaffold **3**. The scaffolds were constructed starting from the corresponding symmetrical disulfides.

The disulfide scaffolds were reacted with the corresponding monofunctionalized polyamines and lipids, as previously reported,[10] to obtain a variety of cationic lipids containing disulfides in different positions. Briefly, the acidic component (scaffold **1**, **2**, or **3**) was reacted with dialkylamines or monoalkylamines using BOP reagent (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) in dichloromethane. The coupling was followed by cleavage of the amino-protecting group *tert*-butoxycarbonyl with trifluoroacetic acid and further coupling with N-protected monofunctionalized polyamines obtained as previously described using BOP reagent. The final products were purified using preparative HPLC and characterized by analytical HPLC, NMR, and mass spectroscopy. The products obtained, grouped by scaffold families, are shown in Figure 5.

Physicochemical Characterization of Complexes. We have used RPR128522 as a model for the physicochemical characterization of complexes. Extensive studies using other lipopolyamines will be disclosed elsewhere.[35]

We performed a primary characterization of the lipopolyamine/DNA complex. In an agarose gel electrophoresis experiment, retardation of DNA migration paired previously described results obtained for nonreducible lipopolyamine analogues.[10] Subsequently, fluorescence experiments were performed by exposing

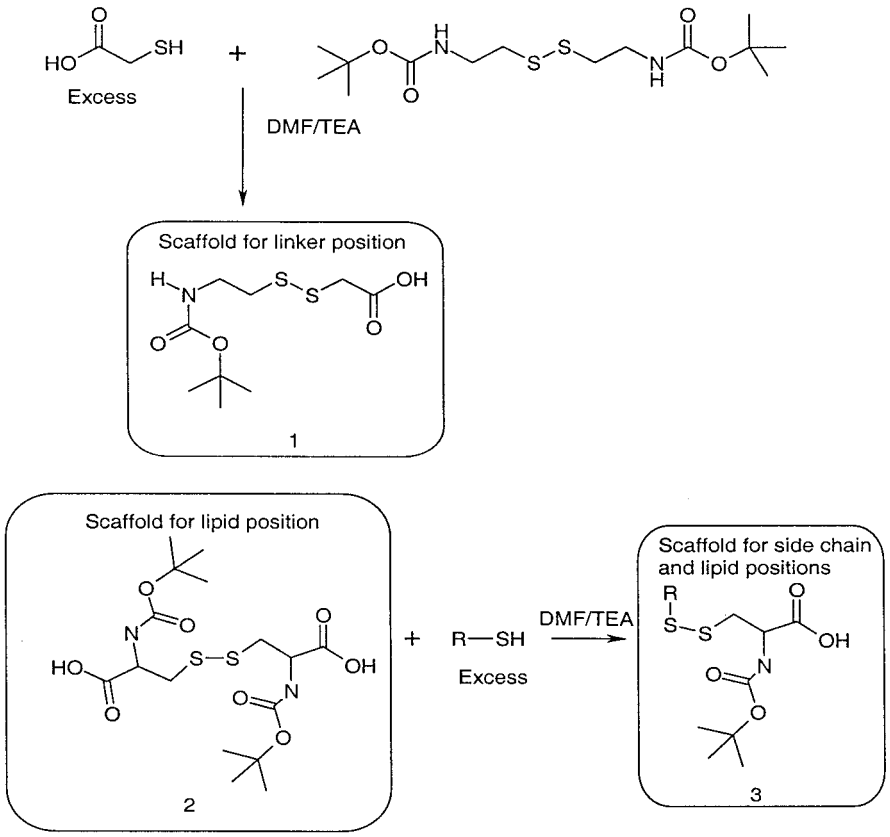

Figure 4. Scaffolds for the synthesis of disulfide-bridged lipopolyamines.

RPR128522/DNA complexes to ethidium bromide, whose fluorescence increases upon intercalation between DNA base pairs. The fluorescence intensity of each sample is depicted in Figure 6. A large and very sharp decrease of the fluorescence intensity was observed at charge ratios ranging from 0 to 1, as previously shown for a classical lipopolyamine analogue, such as RPR120535.[10,13] The fluorescence signal remained low for lipid/DNA complexes whose charge ratios were over 1. Therefore, plasmid DNA is accessible to ethidium bromide for charge ratios ranging from 0 to 1, while over these ratios the plasmid was inaccessible to ethidium bromide intercalation indicating optimal DNA compaction. Additionally, we studied the effect of the charge ratio on the size of the formed particles. Results in Figure 6 show that over an amine/phosphate charge ratio of 4, small particles of about 100 nm in diameter are obtained. On the other hand, for an amine/phosphate ratio between 1 and 3, very large particles are detected, which likely reflected an aggregated state of DNA/lipid complexes, as already observed with the nonreducible analogue RPR120535. Together, these results suggest that DNA is likely in a compacted state similar to that obtained for classical nonreducible lipopolyamines.

Residual fluorescence and agarose gel retardation experiments were then performed in the presence or absence of a reducing agent. In Figure 7A it is observed that fluorescence due to the ethidium bromide/DNA complex is maintained in the presence of the reducing agent DTT, while the charge ratio ± is increased. However, fluorescence is lost in the absence of DTT as usualy observed for nonreducible cationic lipids. In Figure 7B is shown the gel retardation of RSC in the presence of increasing concentrations of DTT, a strong reducing agent. Increasing concentrations of DTT bring about disassembling of the complexes and normal migration of uncomplexed DNA in lanes e and f. Therefore, disassembling of RSC's during or after penetration into cell compartments is likely to happen if a reducing media surrounds the complexes.

In Vitro Biological Activity. 1. Structure−Activity Relationship Studies. The effect of the disulfide bridge location on the expression of a marker transgene in vitro was then studied.

The products were tested on HepG2 and HeLa cells in vitro using different amine/phosphate charge ratios in order to determine the ideal transfection efficiency of each product. Additionally, products were tested in the presence of 10% fetal calf serum (FCS) as a model for in vivo conditions. The transfection efficiency of the products was compared to that of RPR120535 which we have previously reported[10] as being a lipopolyamine with high transfection efficacy as compared to commercially available products (results are disclosed in Figure 8 for HepG2 cells and Figure 9 for HeLa cells).

2. Introduction of a Bioreducible Disulfide Bridge Between the Fatty Chains and the Polyamine Induces a Loss of Transfection Ability. The first interesting result in Figures 8 and 9 is that the introduction of the disulfide bridge between the polyamine and the fatty chains in RPR128522 results in a total (HepG2 cells) or substantial (HeLa cells) loss of transfection ability at all charge ratios, as compared to the nonreducible counterpart RPR120535. It should be noted that RPR128522 forms multilamellar complexes with DNA and displays similar compaction characteristics as nonreducible lipopolyamines like RPR120535. The complex is stable and remains intact as long as it

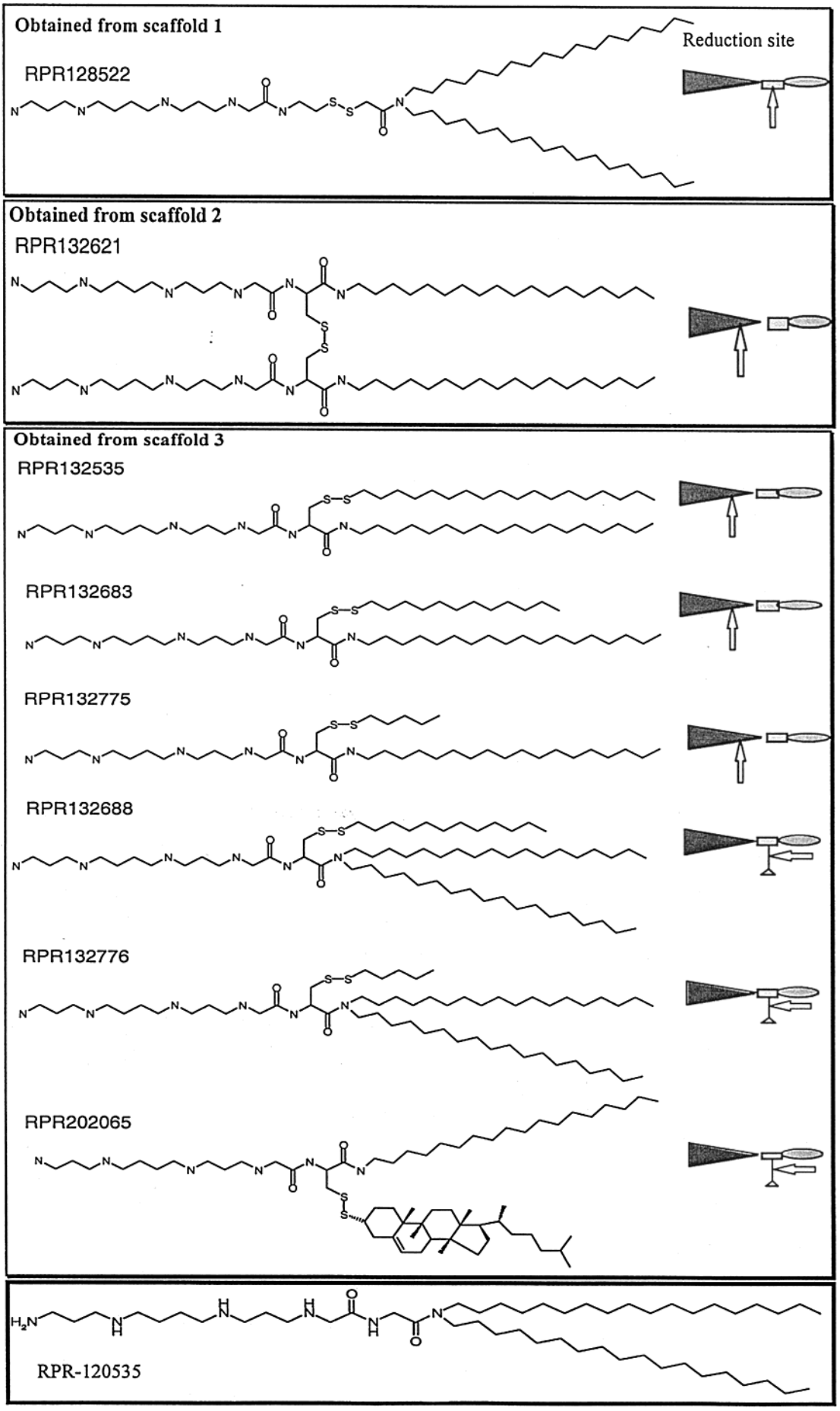

Figure 5. RSL's obtained in this work grouped as scaffold families.

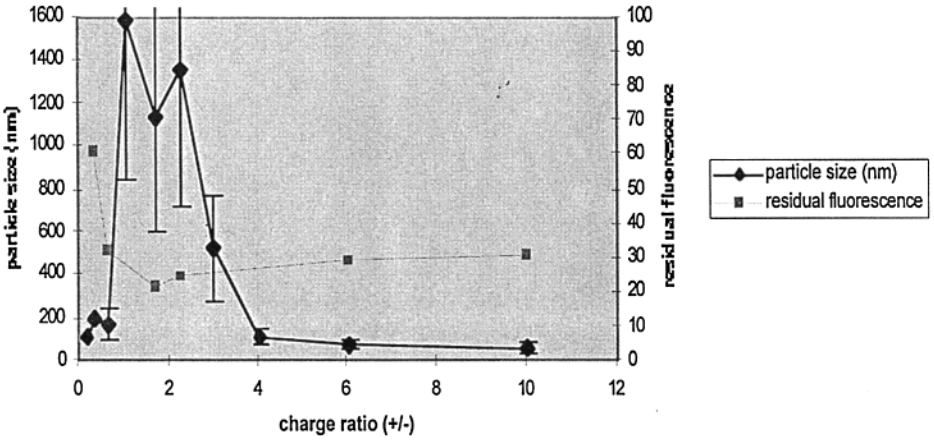

Figure 6. Particle size, measured by dynamic light scattering (■), and residual DNA fluorescence (◆) of RPR128522/DNA complexes at different charge ratios.

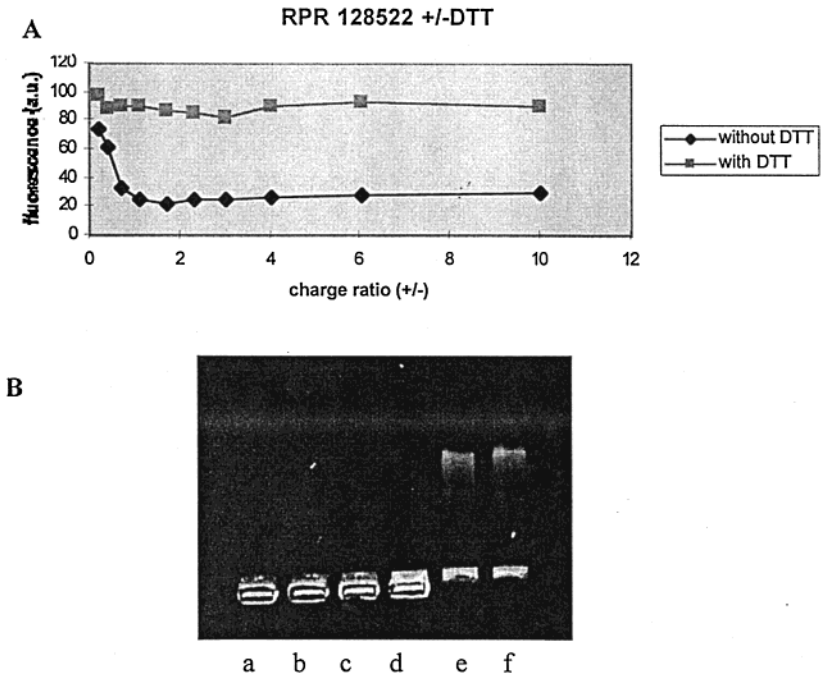

Figure 7. (A) EtBr fluorescence measurements before (◆) and after (■) incubation with DTT, to assess DNA release from RPR128522/DNA complexes at different charge ratios. (B) Influence of DTT concentration on DNA release. RPR128522/DNA complexes at charge ratio 6 were incubated with 0, 0.1, 0.2, 1, and 5 mM DTT, corresponding to lanes a−e, respectively.

is maintained under physiological conditions. Treatment of the complexes with the reducing agent DTT brings about a total disruption of the characteristic multilamellar transfecting particles (see Physicochemical Characterization above). We suggest that this cleavage implies an early release and degradation of the DNA during or after penetration into the cell, which drastically impairs gene transfer. Recently reported cationic lipids bearing a disulfide bridge similar to that in RPR128522 had to be co-formulated with DOPE (dioleoylphosphatidylethanolamine) in order to obtain significant transfection activity.[32−34] Co-formulation with DOPE probably either allows maintainance of lipid bilayers after reduction of the disulfide bridge or prevents extra- or intracellular early reduction, therefore preventing a total disruption of the particles. In the present work, we used RPR128522 without DOPE coformulation and obtained no significant transfection activity. In a separate article to be published elsewhere,[35] we have shown, accordingly with results obtained by others,[32−34] that transfection activity can be partially recovered by formulating RPR128522 with DOPE.

3. Introduction of a Reduction-Sensitive Disulfide Bridge Between One Fatty Alkyl Chain and the Lipid Leads to Increased Transgene Expression. Most of the RSC's displayed higher transfection activity as compared to nonreducible RPR120535 for HepG2. One exception was product RPR132775, which displayed poor transfection efficiency on both cell lines. This could be expected, as the asymmetry in the fatty alkyl chains likely affects the formation of multilamellar structures. The nonreducible isoster of this lipid also displays poor transfection efficiency (data is shown in a separate article[35]). In HeLa cells this relationship could not be established.

4. Introduction of a Reduction-Sensitive Disulfide Bridge Between One Fatty Alkyl Chain and the Lipid Leads to Decreased Transgene Expression When Transfection Is Performed in the Pres-

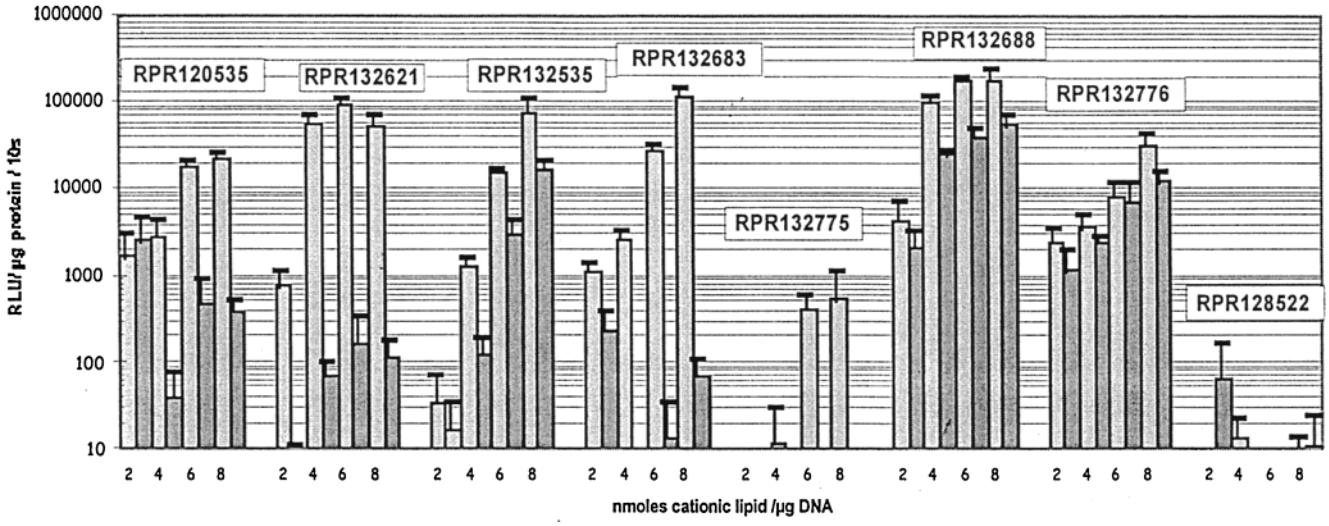

Figure 8. Expression of luciferase in human HepG2 cells transfected with lipopolyamines bearing disulfide bridges at different positions using various charge ratios and in the presence (shaded bars) or absence (open bars) of serum. For details see the Experimental Section.

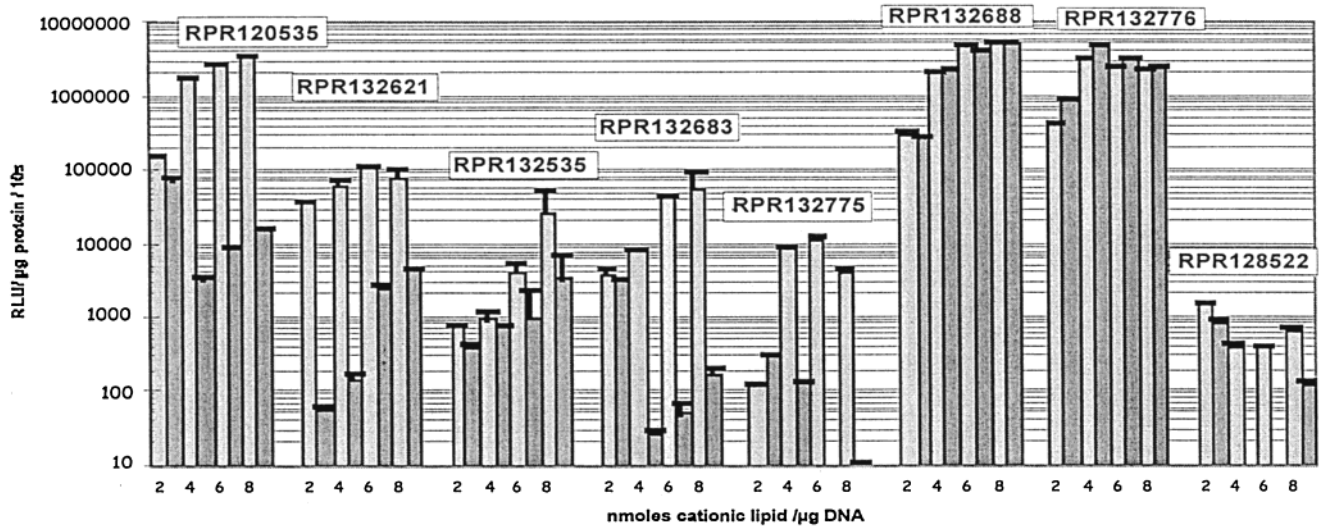

Figure 9. Expression of luciferase in human HeLa cells transfected with lipopolyamines bearing disulfide bridges at different positions using various charge ratios and in the presence (shaded bars) or absence (open bars) of serum. For details see the Experimental Section.

ence of Fetal Calf Serum. For the series of RPR132-621, RPR132535, RPR132683, and RPR132775, transfection efficiency in both cell lines was dramatically affected by the presence of FCS. The inhibitory effect increases as fatty chain length decreased from 18 carbons in RPR132535 to 5 carbons in RPR132775 (this correlation is better observed for HepG2 cells). On the basis of these results, we speculate that FCS affects the complex structure and that the inhibitory effect is not especially related to the disulfide bridges but to the hydrophobicity of the lipids. This interesting effect will be considered in a separate report.

5. Introduction of a Reduction-Sensitive Disulfide Bridge Between a Side Chain Entity and the Lipid Leads to Increased Transgene Expression in the Presence or Absence of Fetal Calf Serum. The introduction of the disulfide bridge between the lipopolyamine and side chain entity, in this case a third hydrophobic fatty chain of variable length (12 carbons in RPR132688 and 5 carbons in 132776), resulted in a significant increase of transfection efficiency especially in HepG2 cells, as compared to the classical lipid RPR120535. The transfection efficacy was maintained in the presence of FCS. It should be pointed out that the only difference between these two lipopolyamines and RPR120535 is the presence of a third fatty chain as a side chain entity. In previous work, we have shown that the introduction of a fatty chain as a side chain entity resulted in decreased transfection efficiency in HeLa cells, as compared to RPR120535.[10] Therefore, the introduction of the reduction-sensitive group between a third lipid chain and the lipopolyamine, which increased transfection efficiency, appears to play a crucial role for optimal gene delivery. Reduction of this side chain entity in RPR132688 and RPR132776 transforms the lipids into one common lipid similar to RPR120535. Thus, the increased transfection efficiency of the lipids (10-fold increase for RPR132688 and 5-fold for RPR132-776 in HepG2 cells) necessarily results from a disruption caused by the reducible side chain entity. This disruption effect was higher for RPR132688 and better observed in HepG2 cells.

Conclusions

The cellular disassembling of lipopolyamine/DNA complexes is one of the main avenues for improvement of transgene expression. Here, we have developed a new and versatile nonviral gene delivery system for controlled formulation and release of DNA for gene delivery. The new chemical approach is based on the use of a disulfide bridge as a reduction-sensitive group in lipopolyamines. We have established that the location of the disulfide bridge affects gene expression mediated by the lipopolyamine. Thus, DNA delivery and transgene expression can be modulated by proper introduction of the disulfide bridge at different positions in the lipopolyamine. Gene expression is significantly improved if the disulfide bridge is inserted between one lipid chain and the rest of the lipopolyamine. On the other hand, when the disulfide bridge is placed at the linker position, a total loss of gene expression is observed for HepG2 cells or substantial loss for HeLa cells. Preliminary physicochemical characterization of RSC's demonstrates that the new RSL's behave similarly to classical lipopolyamines, as long as a reducting agent is not in contact with the particles. Treatment of the particles with reducting agent promotes a fast disruption and, in some of the cases, decomplexation of DNA from the particles.

The extensive physicochemical characterization of the complexes has been done and will be presented in a separated article.[35]

We are currently studying the in vivo gene delivery using the new RSL's. The intracellular disassembling of the RSC's is also being studied by introduction of molecular probes and use of confocal microscopy. Our results strongly support and extend the scope of recently out coming data obtained for a limited type of transfection agents bearing disulfide bonds between the cationic entity and the lipid. Our extensive DNA controlled-release approach represents a new way for obtaining increased transgene expression using nonviral gene delivery systems.

Experimental Section

Materials and Methods. Triethylamine (TEA), diisopropylethylamine (DIEA), diooctadecylamine and *N*,*N*′-di-Boc-cystine were purchased from Fluka. Amylamine, octadecylamine, pentanethiol, dodecanethiol, octadecanethiol and thiocholesterol were purchased from Aldrich. BOP reagent was purchased from Neosystem. All solvents were analytically pure grade from Prolabo and were used without further purification. The synthesis of Boc-$NH(CH_2)_3N$(Boc)-$(CH_2)_4N$(Boc)-$(CH_2)_3$-N(Boc)-CH_2COOH was described in a previous article.[11] NMR and MS were carried out at the Analysis Department of Rhône Poulenc Rorer, Vitry sur Seine. ^1H NMR spectra were recorded on Brucker 300, 400 and 600 MHz spectrometers. Samples were dissolved in $CDCl_3$ or DMSO. Chemical shifts are in ppm relative to TMS internal standard. MS were carried out on a VG autospec by LSIMS technique equipped with a cesium canon; the matrix was a mixture of glycerol and thioglycerol or nitrobenzyl alcohol (FABMS) and a Perkin-Elmer Sciex API (III) MS. Analytical HPLC was performed on a Merck-HITACHI gradient pump equipped with a AS-2000A autosampler, a L-6200A intelligent pump, a UV−vis detector L-4000 with tuneable wavelength set at 220 nm and a column BU-300 aquapore butyl 7 μm, 300 Å, 300 × 4.6 mm from Applied Biosystems. Analysis of products was carried out using a gradient (H_2O (0.1% TFA)/MeCN (0.08% TFA): 3 min [40/60], 3−20 min [0/100], 20−35 min [0/100]) and a flow rate of 1.0 mL/min. Preparative HPLC was performed on a Gilson gradient system equipped with two Gilson 305 intelligent pumps, a UV−vis Gilson 119 detector with double channel set at 220 and 254 nm and a fraction collector Gilson 202 and a column C4 214TP1022 from Vydac. Purification of products was carried out using a gradient (H_2O (0.1% TFA)/MeCN (0.08% TFA): 10 min [70/30], 10−80 min [0/100], 80−120 min [0/100]) and a flow rate of 18 mL/min. Thin-layer chromatography was performed on silica gel plates (60 F254; Merck) and the spots were revealed by UV (254 and 366 nm), a spray of ninhydrin solution (0.1% in MeOH) or a spray of fluorescamine solution (0.04% in acetone). Flash chromatography was performed on silica gel (60-Merck). We have previously shown[10] that the presence of polyamines in the cationic lipids makes elementary analysis inadequate as a purity criteria. The polyamines are highly hygroscopic, and their salt degree is ambiguous because of the polyamines. As proof of our findings, we have performed a complete analysis of the comparation product RPR120535, including MS, IR, and ^1H NMR recorded with an Avance DMX 600 Brucker (2D DQF COSY and HOHAHA methods, $t = 80$ ms) and ^{13}C NMR (heteronuclear 2D techniques HMQC and HMBC with a delay of 50 ms in the reverse mode). Thus, we could unambiguously confirm the proposed structure, although the elementary analysis was not within the ±0.4% purity criteria.[10]

BocNHCys[S-S-$(CH_2)_4CH_3$]-OH (1). To a stirred solution of *N*,*N*′-di-Boc-cystine (3 g, 6.81 mmol) in DMF (20 mL) were added TEA (8.1 mL, 58.1 mmol) and pentanethiol (0.86 mL, 6.81 mmol) portionwise. The mixture was stirred at room temperature for 2 h. TEA was removed from the solution under reduced pressure and the DMF was acidified with $KHSO_4$ (0.5 M solution, 300 mL). The precipitate was extracted with $CHCl_3$ (3 × 100 mL). The combined organic extracts were dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The crude product dissolved in Et_2O (100 mL) was extracted with saturated $NaHCO_3$ solution (3 × 50 mL). The combined aqueous phase was acidified with $KHSO_4$ (0.5 M solution) to pH 3 (350 mL). The precipitate was extracted with $CHCl_3$ (3 × 100 mL) which was further washed with brine (2 × 50 mL), dried over $MgSO_4$ and evaporated to dryness. Chromatography of the residue on silica gel in $CHCl_3$/MeOH (9/1 v/v) gave a white powder 0.745 g (2.31 mmol, yield 34%) of the expected product **1**: TLC $R_f = 0.63$ ($CHCl_3$/MeOH, 9/1); $MH^+ = 324$.

BocNHCys[S-S-$(CH_2)_{17}CH_3$]-OH (2). To a stirred solution of *N*,*N*′-di-Boc-cystine (1.32 g, 3 mmol) in DMF (20 mL) was added to TEA (3.5 mL, 25 mmol) and octadecanethiol (0.88 g, 3 mmol) portionwise. The mixture was heated to 40 °C and stirred for 2 h. TEA was removed from the solution under reduced pressure and DMF was acidified with $KHSO_4$ (0.5 M, 300 mL). The product was extracted with $CHCl_3$ (3 × 100 mL). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The crude product dissolved in Et_2O (100 mL) was washed with saturated $NaHCO_3$ (3 × 50 mL), $KHSO_4$ (0.5 M, 2 × 100 mL) and brine (2 × 50 mL), dried over $MgSO_4$ and evaporated to dryness. The pure compound **2** (0.287 g, 0.567 mmol, yield 19%) was obtained after crystallization from petroleum ether: TLC $R_f = 0.67$ ($CHCl_3$/MeOH, 9/1); HPLC $t_R = 17.80$ min; $MH^+ = 506$.

BocNHCys[S-S-cholesteryl]-OH (3). The title compound was prepared similarly to product **2** but using thiocholesterol (1 g, 2.47 mmol) and *N*,*N*′-di-Boc-cystine (1.09 g, 2.47 mmol). After chromatography of the residue on silica gel in $CHCl_3$/MeOH (9/1 v/v) gave a 0.088 g (0.141 mmol, yield 5.8%) of **3**: TLC $R_f = 0.59$ ($CHCl_3$/MeOH, 9:1); HPLC $t_R = 19.16$ min; $MH^+ = 622$.

BocNHCys[S-S-$(CH_2)_{11}CH_3$]-OH (4). The title compound was prepared similarly to product **2** but at room temperature using 1-dodecanethiol (1.66 mL, 6.81 mmol) and *N*,*N*′-di-Boc-cystine (3 g, 6.81 mmol) in 40% yield (1.15 g): TLC $R_f = 0.69$ ($CHCl_3$/MeOH, 9:1); HPLC $t_R = 13.23$ min; $MH^+ = 422$.

BocNHCys[S-S-$(CH_2)_4CH_3$]-NH$(CH_2)_{17}CH_3$ (5). To a stirred solution of product **1** (0.37 g, 1.15 mmol) in CH_2Cl_2 (10 mL) were added octadecylamine (0.34 g, 1.15 mmol), DIEA (0.5 mL, 2.86 mmol) and BOP reagent (0.56 g, 1.27 mmol) at room

temperature. The reaction mixture was left for 2 h. The solvent was evaporated under vacuum and the crude product dissolved in $CHCl_3$ (100 mL), washed with $KHSO_4$ (0.5 M, 3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL) and brine (2 × 50 mL). The organic phase was dried over $MgSO_4$ and concentrated to yield 0.386 g (0.672 mmol, yield 59%) of yellow honey-like product **5**: TLC $R_f = 0.91$ ($CHCl_3$/MeOH, 9:1); HPLC $t_R = 18.31$ min; $MH^+ = 576$.

BocNHCys[S-S-$(CH_2)_4CH_3$]-N[$(CH_2)_{17}CH_3$]$_2$ (6). The title compound was prepared from product **1** (0.37 g, 1.15 mmol) and dioctadecylamine (0.6 g, 1.14 mmol) by an analogous procedure to that described in preparation of **5** to give 0.607 g (yield 64%) of white honey-like product **6:** TLC $R_f = 0.90$ ($CHCl_3$/MeOH, 9:1); HPLC $t_R = 25.96$ min; $MH^+ = 827$.

BocNHCys[S-S-$(CH_2)_{17}CH_3$]-NH$(CH_2)_{17}CH_3$ (7). The title compound was prepared from product **2** (0.28 g, 0.554 mmol) and octadecylamine (0.166 g, 0.554 mmol) by an analogous procedure to that described in preparation of **5**, to give 0.409 g (yield 97%) of white powder product **7**: TLC $R_f = 0.89$ ($CHCl_3$/MeOH, 9:1); HPLC $t_R = 24.84$ min; $MH^+ = 757$.

BocNHCys[S-S-$(CH_2)_{11}CH_3$]-NH$(CH_2)_{17}CH_3$ (8). The title compound was prepared from product **4** (0.3 g, 0.713 mmol) and octadecylamine (0.21 g, 0.701 mmol) by an analogous procedure to that described in preparation of product **5** to give 0.412 g (yield 86%) of white powder **8**: TLC $R_f = 0.90$ ($CHCl_3$/MeOH, 9:1); HPLC $t_R = 22.22$ min.

BocNHCys[S-S-$(CH_2)_{11}CH_3$]-N[$(CH_2)_{17}CH_3$]$_2$ (9). The title compound was prepared from product **4** (0.278 g, 0.660 mmol) and dioctadecylamine (0.316 g, 0.6 mmol) by an analogous procedure to that described in preparation of product **5** to give 0.517 g (yield 85%) of white powder **9**: TLC $R_f = 0.90$ ($CHCl_3$/MeOH, 9:1); $MH^+ = 925$.

BocNHCys[S-S-cholesteryl]-NH$(CH_2)_{17}CH_3$ (10). The title compound was prepared from product **3** (0.3 g, 0.483 mmol) and octadecylamine (0.144 g, 0.481 mmol) by an analogous procedure to that described in preparation of product **5** to give 0.420 g (yield 99%) of white powder **10**: TLC $R_f = 0.88$ ($CHCl_3$/MeOH, 9:1); HPLC $t_R = 26.98$ min. The product was used without further analysis.

RPR132775 (11). The Boc-protected product **5** (0.37 g, 0.645 mmol) was deprotected using TFA (10 mL) for 1.5 h. TFA was evaporated under vacuum. The crude product was dissolved in CH_2Cl_2 (10 mL). To the stirred solution were added DIEA (0.56 mL, 3.34 mmol), BocNH-$(CH_2)_3$-NBoc-$(CH_2)_4$-NBoc-$(CH_2)_3$-NBoc-CH_2CO_2H (0.42 g, 0.636 mmol) and BOP (0.32 g,0.724 mmol). The reaction mixture was left at room temperature 1.5 h. The solvent was evaporated under vacuum and the crude product dissolved in $CHCl_3$ (100 mL) and washed with $KHSO_4$ (0.5 M solution, 3 × 50 mL), saturated $NaHCO_3$ solution (3 × 50 mL) and brine (2 × 50 mL). The organic phase was dried over $MgSO_4$ and concentrated. The final Boc-protected product was deprotected using TFA (10 mL) for 1 h. The crude compound was purified by HPLC to afford after freeze-dry 0.109 g (yield 14%) of product **11**: HPLC $t_R = 8.70$ min; ^{1}H NMR (250 MHz, $(CD_3)_2SO$-d_6) δ 0.86 and 0.88 (2 t, $J = 7.5$ Hz, 6H: CH_3); from 1.15 to 1.50 (m, 34H: CH_2); 1.42 (m: CH_2); from 1.55 to 1.75 (m, 2H: CH_2); 1.65 (broad band, 4H: CH_2 of butyl); from 1.85 to 2.10 (m, 4H: CH_2 of propyls); 2.76 (t, J = 7 Hz, 2H: SCH_2); from 2.85 to 3.20 (m, 16H: NCH_2 of butyl − NCH_2 of propyls − CH_2S of cysteine and $CONCH_2$); 3.80 (broad band, 2H: NCH_2CON); 4.60 (m, 1H: CONCHCON of cysteine); 8.27 (t, $J = 5.5$ Hz, 1H: CONH); 8.90 (d, $J = 8$ Hz, 1H: CONH of cysteine); 7.96−8.80 and 9.10 (3 broad bands: NH and NH_2); MH^+= 717.

RPR132535 (12). The title compound was prepared from product **7** (0.4 g, 0.53 mmol) by an analogous procedure to that described in preparation of **11**, to give 0.224 g (yield 31%) of **12**: HPLC $t_R = 15.63$ min; ^{1}H NMR (400 MHz, $(CD_3)_2SO$-d_6) δ 0.89 (t, $J = 7.5$ Hz, 6H: CH_3); from 1.15 to 1.45 (m, 60H: CH_2); 1.42 (m, 2H: CH_2); from 1.55 to 1.70 (m, 2H: CH_2); 1.66 (broad band, 4H: CH_2 of butyl); from 1.85 to 2.05 (m, 4H: CH_2 of propyls); 2.76 (t, $J = 7.5$ Hz, 2H: SCH_2); from 2.85 to 3.10 (m, 14H: NCH_2 of butyl − NCH_2 of propyls − 1H of $CONCH_2$ and 1H of CH_2S of cysteine); 3.10 (dd, $J = 13.5$ and 6 Hz, 1H: the other H of CH_2S of cysteine); 3.18 (m, 1H: the other H of $CONCH_2$); 3.82 (limit AB, 2H: NCH_2CON); 4.60 (m, 1H: CONCHCON of cysteine); 8.27 (t, $J = 5.5$ Hz, 1H: CONH); 8.90 (d, $J = 8.5$ Hz, 1H: CONH of cysteine); 7.95−8.82 and 9.07 (3 broad bands: NH and NH_2); MH^+= 899.

RPR132683 (13). The title compound was prepared from **9** (0.40 g, 0.598 mmol) by an analogous procedure to that described in preparation of **11**, to give 0.201 g (yield 26.5%) of **13**: HPLC $t_R = 12.36$ min; ^{1}H NMR (400 MHz, $(CD_3)_2SO$-d_6) δ 0.90 (t, $J = 7.5$ Hz, 6H: CH_3); from 1.15 to 1.50 (m, 48H: CH_2); 1.43 (m: CH_2); from 1.55 to 1.70 (m, 2H: CH_2); 1.65 (broad band, 4H: CH_2 of butyl); from 1.85 to 2.05 (m, 4H: CH_2 of propyls); 2.76 (t, $J = 7.5$ Hz, 2H: SCH_2); from 2.80 to 3.05 (m, 14H: NCH_2 of butyl − NCH_2 of propyls − 1H of $CONCH_2$ and 1H of CH_2S of cysteine); 3.11 (dd, $J = 13.5$ and 6 Hz, 1H: the other H of CH_2S of cysteine); 3.17 (m, 1H: the other H of $CONCH_2$); 3.83 (limit AB, 2H: NCH_2CON); 4.60 (m, 1H: CONCHCON of cysteine); 8.25 (t, $J = 5.5$ Hz, 1H: CONH); 8.99 (d, $J = 8.5$ Hz, 1H: CONH of cysteine); 7.96−8.84 and 9.09 (3 broad bands: NH and NH_2); MH^+= 815.

RPR202065 (14). The title compound was prepared from **10** (0.420 g, 0.48 mmol) by an analogous procedure to that described in preparation of **11** to give 0.040 g (yield 5.6%) of **14**: HPLC $t_R = 16.59$ min; ^{1}H NMR (400 MHz, $(CD_3)_2SO$-d_6, at a temperature of 383 K) δ 0.74 and 1.05 (2 s, 3H each: CH_3 in 18 and CH_3 in 19 of cholesteryl); from 0.80 to 0.95 (m, 12H: CH_3 and CH_3 in 26 − CH_3 in 27 and CH_3 in 21 of cholesteryl); 1.77 (m, 4H: CH_2 of butyl); from 1.85 to 2.10 (m, 4H: CH_2 of propyls); from 2.90 to 3.25 (m, 16H: NCH_2 of butyl − NCH_2 of propyls − CH_2S of cysteine and $CONCH_2$); 3.63 (limit AB, 2H: NCH_2CON); 4.61 (m, 1H: CONCHCON of cysteine); 5.39 (m, 1H: CH in 6 of cholesteryl); 7.69 (m, 1H: CONH); 8.25 (broad band, 1H: CONH of cysteine); for the other protons of this molecule, the signals are between 0.60 and 3.00 ppm; MH^+= 1015.

RPR132688 (15). The title compound was prepared from **9** (0.517 g, 0.56 mmol) by an analogous procedure to that described in preparation of **11** to give 0.330 g (yield 39%) of **15**: HPLC $t_R = 19.75$ min; ^{1}H NMR (400 MHz, $(CD_3)_2SO$-d_6) δ 0.87 (t, $J = 7.5$ Hz, 9H: CH_3); from 1.15 to 1.50 (m, 78H: CH_2); 1.47 (m, 2H: CH_2); from 1.50 to 1.70 (m, 4H: CH_2); 1.68 (broad band, 4H: CH_2 of butyl); from 1.85 to 2.10 (m, 4H: CH_2 of propyls); 2.77 (t, $J = 7.5$ Hz, 2H: SCH_2); 2.80 (m, 1H: 1H of CH_2S of cysteine); from 2.70 to 3.50 (m, 15H: NCH_2 of butyl − NCH_2 of propyls − the other H of CH_2S of cysteine and $CONCH_2$); 3.80 (broad s, 2H: NCH_2CON); 5.05 (m, 1H: CONCHCON of cysteine); 9.07 (d, $J = 8$ Hz, 1H: CONH of cysteine); from 7.75 to 8.20 and from 8.65 to 9.25 (2 broad bands: NH and NH_2); MH^+= 1067.

RPR132776 (16). The title compound was prepared from product **6** (0.6 g, 0.726 mmol) by an analogous procedure to that described in preparation of product **11**, to give 0.115 g (yield 11%) of **16**: HPLC $t_R = 17.79$ min; ^{1}H NMR (400 MHz, $(CD_3)_2SO$-d_6) δ 0.90 (m, 9H: CH_3); from 1.15 to 1.50 (m, 64H: CH_2); 1.47 (m, 2H: CH_2); from 1.55 to 1.75 (m, 4H: CH_2); 1.65 (broad band, 4H: CH_2 of butyl); from 1.85 to 2.05 (m, 4H: CH_2 of propyls); from 2.70 to 2.85 (m, 1H: 1H of CH_2S of cysteine); 2.78 (m, 2H: SCH_2); from 2.85 to 3.50 (m, 17H: NCH_2 of butyl − NCH_2 of propyls − the other H of CH_2S of cysteine and $CONCH_2$); 3.80 (broad s, 2H: NCH_2CON); 5.07 (m, 1H: CONCHCON of cysteine); 9.05 (d, $J = 8$ Hz, 1H: CONH of cysteine); 7.95−8.85 and from 8.90 to 9.15 (respectively 2 broad bands and very broad band: NH and NH_2); MH^+= 969.

{**[NHBoc][CONH$(CH_2)_{17}CH_3$]CHCH-S-**}$_2$ **(17).** To a stirred solution of *N*,*N*′-di-Boc-cystine (0.25 g, 0.57 mmol) in $CHCl_3$ (15 mL) were added octadecylamine (0.3 g, 1 mmol), DIEA (1 mL, 5.66 mmol) and BOP reagent (0.55 g, 1.24 mmol) at room temperature. The reaction mixture left for 2 h. The solvent was evaporated under vacuum and the crude product dissolved in AcOEt (100 mL) and washed with $KHSO_4$ (0.5 M, 3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL) and brine (2 × 50 mL). The organic phase was dried over $MgSO_4$ and concentrated to yield 0.326 g (0.346 mmol, yield 61%) of white powder **17**: TLC $R_f = 0.94$ ($CHCl_3$/MeOH, 9:1).

RPR132621 (18). The Boc-protected product **17** (0.326 g, 0.346 mmol) was deprotected using TFA (10 mL) for 1.5 h. TFA was evaporated under vacuum. The crude product was dissolved in CH_2Cl_2 (20 mL). To the stirred solution were added DIEA (0. 6 mL, 3.34 mmol), BocNH-$(CH_2)_3$-NBoc-$(CH_2)_4$-NBoc-$(CH_2)_3$-NBoc-CH_2CO_2H (0.46 g, 0.697 mmol) and BOP (0.38 g, 0.86 mmol). The reaction mixture was left at room temperature 1.5 h. The solvent was evaporated under vacuum and the crude product dissolved in $CHCl_3$ (100 mL) and washed with $KHSO_4$ (0.5 M,3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL) and brine (2 × 50 mL). The organic phase was dried over $MgSO_4$ and concentrated. The final Boc-protected product was deprotected using TFA (10 mL) for 1 h. The crude compound was purified by HPLC to afford after freeze-dry 0.211 g (yield 28%) of **18**: HPLC t_R =10.55 min; ^{1}H NMR (400 MHz, $(CD_3)_2SO$-d_6) δ 0.91 (t, J = 7.5 Hz, 6H: CH_3); from 1.10 to 1.40 (m, 60H: CH_2); 1.43 (m, 4H: CH_2); 1.64 (broad band, 8H: CH_2 of butyls); from 1.85 to 2.10 (m, 8H: CH_2 of propyls); from 2.80 to 3.15 (m, 32H: NCH_2 of butyls − NCH_2 of propyls − $CONCH_2$ and CH_2S of cysteines); 3.84 (broad band, 4H: NCH_2CON); 4.60 (m, 2H: CONCHCON of cysteines); 8.27 (broad band, 2H: CONH); 8.95 (broad band, 2H: CONH of cysteines); 7.97−8.87 and 9.15 (3 broad bands: NH and NH_2); MH^+= 1227.

BocNH-CH_2CH_2-S-S-CH_2COOH (19). To a stirred solution of *N*,*N*′-di-Boc-cystamine (1.2 g, 3.18 mmol) in $CHCl_3$ (20 mL) were added TEA (2.7 mL, 20 mmol) and H_2COOH (0.27 mL, 3.18 mmol) portionwise. The mixture was stirred at room temperature for 2 h. TEA was removed from the solution with $KHSO_4$ (0.5 M, 3 × 20 mL). The organic layer was dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The crude product dissolved in Et_2O (100 mL) and was extracted with saturated $NaHCO_3$ solution (3 × 50 mL). The combined aqueous phase was washed with Et_2O and acidified with $KHSO_4$ (0.5 M) to pH 3 (200 mL). The precipitate was extracted with $CHCl_3$ (3 × 80 mL) which was further washed with brine (2 × 50 mL), dried over $MgSO_4$ and evaporated to dryness. 0.32 g, (yield 31%) of the expected product **19** were obtained: TLC R_f = 0.25 ($CHCl_3$/MeOH, 9/1); MH^+ = 268.

RPR128522 (20). Product **19** (0.29 g, 1.1 mmol), dioctadecylamine (0.522 g, 1.1 mmol) and TEA (0.7 mL, 6 mmol) were dissolved in $CHCl_3$ (10 mL). BOP reagent was then add (0.5 M, 3 × 30 mL), with saturated $NaHCO_3$ solution (3 × 30 mL) and the organic layer was dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The intermediate lipid (0.7 g, yield 90%) was used without further purification: MH^+ = 771. The intermediate (0.35 g, 0.435 mmol) was deprotected using 10 mL TFA during 1 h at room temperature. TFA was evaporated under vacuum and the product was dissolved in DMF (10 mL). TEA was added until the pH of the solution was basic (about 0.36 mL, 2 mmol). This was followed by the addition of BocNH-$(CH_2)_3$-NBoc-$(CH_2)_4$-NBoc-$(CH_2)_3$-NBoc-CH_2CO_2H (0.209 g, 0.44 mmol) and BOP reagent (0.221 g, 0.5 mmol). The solution was left for 2 h until fluorescamine test was negative. 100 mL of $KHSO_4$ (0.5 M) was then added and the product was extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed with $KHSO_4$ (0.5 M, 3 × 30 mL), saturated $NaHCO_3$ solution (3 × 30 mL), dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give 0.465 g of the protected intermediate (yield 81%). The product was deprotected with 10 mL TFA during 1 h. TFA was evaporated and the final product 20 was purified by HPLC to afford after freeze-dry 0.25 g (yield 50%) of pure RPR-128522: HPLC t_R= 15.73 min; ^{1}H NMR (400 MHz, $(CD_3)_2SO$-d_6 with a few drops of CD_3COOD-d_4, at a temperature of 343 K) δ 0.88 (t, J = 6.5 Hz, 6H: CH_3); from 1.20 to 1.40 (mt, 60H: CH_2); 1.52 (broad band, 4H: CH_2); 1.70 (mt, 4H: $(CH_2)_2$ of butyl); from 1.90 to 2.10 (mt, 4H: CH_2 of propyls); from 2.85 to 3.10 (mt, 12H: NCH_2 of butyl and NCH_2 of propyls); 2.90 (t, J = 7 Hz, 2H: CH_2S); 3.28 (broad band, 4H: NCH_2); 3.50 (t, J = 7 Hz, 2H: CH_2NCO); 3.68 and 3.76 (2 broad s, 2H each: NCH_2CON and $CONCH_2S$); MH^+ = 913.

Cell Culture and Transfection. HeLa and HepG2 cells were incubated at 37 °C in a 5% CO_2/air incubator. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) and HepG2 cells were grown in Eagle's minimum essential medium (MEM). Both media were completed with the addition of 2 mM L-glutamine, 1% MEM nonessential amino acid solution ×100, 50 units/mL penicillin, 50 μg/mL streptomycin and 10% (v/v) fetal bovine serum (FBS). All media and additives were obtained from Gibco/BRL Life Technologies (Gaithersburg, MD).

Twenty-four hours before transfection, 500 μL of cellular suspensions was seeded into 24-well culture plates at a concentration of 1 × 10^5/mL for HeLa cells and 2 × 10^5/mL for HepG2 cells. These dilutions showed approximately 80% confluency after 36 h. Before transfection wells were rinsed twice and subsequently incubated with 500 μL of medium without FBS. DNA/lipid complex solutions (50μL) containing 0.5 μg of plasmid DNA (containing the luciferase gene under the dependency of the cytomegalovirus CMV) in 75 mM NaCl were added to each well. Two hours after transfection, the serum-free medium was supplemented with 10% (v/v) FBS. Cells were grown for 36 h at 37 °C in 5% CO_2.

Luciferase Assay. Luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI). Cell-layers were rinsed twice with 500 μL PBS and lysed with 250 μL Promega cell culture lysis reagent for 30 min at 20 °C. After 5 min of centrifugation at 12000*g* and 4 °C aliquots of 10 μL supernatant were measured for luciferase activity using the Promega luciferase substrate. Luminometric measurements were performed with a Lumat LB9501 luminometer from EG, Berthold (Evry, France) or a Victor^2 1420 multilabel counter (Wallac, Turku, Finland). Luciferase activity was quantified by integration over 10 s (Lumat LB9501) or 1 s (Victor^2) and expressed as relative light units (RLU) or counts per second (CPS), respectively. To compare RLU and CPS values, measurements of 500 identical samples of different expression levels were carried out with both luminometers. A factor of 10.8 was found to convert CPS results into equivalents of RLU values. To determine the luciferase activity per μg protein, the protein concentration of the lysate was obtained using a Pierce BCA assay kit (Rockford, IL).

DNA/Lipid Complex Formation and Characterization and DNA Release. DNA/lipid complexes were prepared by mixing equal volumes of cationic lipid at different concentrations with plasmid DNA solution at a constant concentration. Charge ratios are expressed as moles of positive charges per moles of negative charges.[10,13] The resultant DNA concentration was 10 μg/mL, the final NaCl concentration 75 mM. The size of particules was determined by dynamic light scattering using a Coulter N4 Plus particle analyzer (Coulter, Amherst, MA). Measurements were performed at 20 °C, at an angle of 90° and a DNA concentration of 10 μg/mL. Results were obtained from unimodal analysis. The binding of cationic lipid to DNA was shown by the exclusion of ethidium bromide (EtBr; Euromedex, Strasbourg, France) from DNA. 4 μg EtBr was added to 1 mL of complexes in 75 mM NaCl. The residual fluorescence was measured with a Jobin-Yvon Spex Fluoromax-2 spectrofluorometer at a DNA concentration of 10 μg/mL at 20 °C at excitation at 260 nm and emission detecton at 560 nm (Instruments SA, Edison, NJ). Values are expressed in arbitrary fluorescence units.

To detect DNA release due to disulfide lipid reduction, dithiothreitol (DTT; Sigma), final concentration 5 mM, was added to lipid/DNA complexes of different charge ratios and they were incubated at 37 °C for 12 h. Alternatively, different amounts of DTT were added to complexes containing 1 μg DNA (charge ratio 6) and after an incubation time of 20 min at room temperature, gel electrophoresis (0.8% agarose) was performed. Final DTT concentrations: 0, 0.1, 0.2, 1.0, 5.0 mM.

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