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Investigation of soft long chain quaternary ammonium compounds as co-factors to enhance *in vitro* gene delivery

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The effect of soft long chain quaternary ammonium antibacterial agents on the *in vitro* gene delivery of a luciferase plasmid to COS-1 cell lines was investigated. Low concentrations of these compounds could be used to enhance gene delivery with Lipofectamine Plus™.

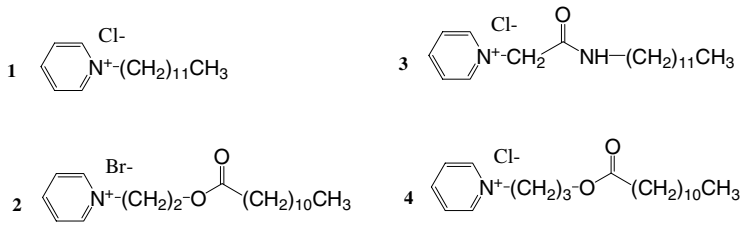
Long chain quaternary ammonium antibacterial agents, such as cetyl pyridium chloride and benzalkonium chloride, are widely used as disinfectants. These compounds are slowly metabolized compounds which can be relatively toxic and persistent in the environment. "Soft" analogues of these compounds are degraded relatively rapidly via a hydrolytic pathway and are therefore less toxic and safer for the environment than their "hard" analogues (Bodor et al. 1980). Investigation of the structure activity relationship of soft long chain quaternary ammonium compounds have shown that moderately labile and highly active compounds can be obtained (Thorsteinsson et al. 2003a; Loftsson et al. 2005).

Cationic lipids, forming cationic liposomes (Mahato et al. 1997) and poly-cationic polymers (Kabanov 1999), have been used as non-viral gene delivery systems. These agents form condensed complexes with plasmid DNA and promote endocytosis and endosomal escape of the condensed DNA. The components of these gene delivery systems share some structural features with long chain quaternary ammonium disinfectants, such as positive charge and, in the case of cationic lipids, amphiphilic properties. It is therefore of some interest to investigate the utility of these compounds and the soft analogues in non-viral gene delivery.

Four compounds were investigated in this study; cetyl pyridinum chloride (**1**) and three soft long chain quaternary ammonium compounds (**2**–**4**) (Table). Investigation of the toxicity towards COS-1 cell lines showed that **1** was highly toxic at 10 μg/ml with complete cell death after 24 h incubation. The soft compounds were less toxic to the cell lines. Complete cell deaths was only observed at 1000 μg/ml for all compounds. Complete cell deaths was also observed at 100 μg/ml concentrations for compounds **2** and **3**. No cell death was observed with compound **4** at concentrations of 100 μg/ml and below.

The ability of cationic compounds to form a complex and condense anionic DNA was investigated. The complexation was determined from the effect of these compounds on the electrophoretic mobility of DNA plasmid in an agarose gel (Ruponen et al. 1999). In general, compounds **1**–**4** showed poor ability to form complex with DNA. Compounds **1** and **3** had some effect on the mobility of DNA at relatively high cation/anion charge (+/−) ratios. When the +/− ratio was 30 or higher only a weak band for the free plasmid was observed (data not shown). How-

Table: Long chain quaternary ammonium compounds used in the study: structure, degradation rate and cellular toxicity



Half life (at 60 °C, pH 7)*

	1	2	3	4
$t_{1/2}$ (h)	>100	3	4	6

Cell death of COS-1 cells after 24 h incubation

	1	2	3	4
10^3 μg/ml	+++	+++	+++	+++
10^2 μg/ml	+++	+++	+++	−
10^1 μg/ml	+++	+	++	−
1 μg/ml	+	+	+	−
10^{-1} μg/ml	−	+	+	−
10^{-2} μg/ml	−	+	+	−
10^{-3} μg/ml	−	−	−	−

* Conditions for the degradation rate studies have previously been reported (Thorsteinsson et al. 2003a)
** +++ Complete cell death. ++ Significant cell death. + Some cell death − no cell death.

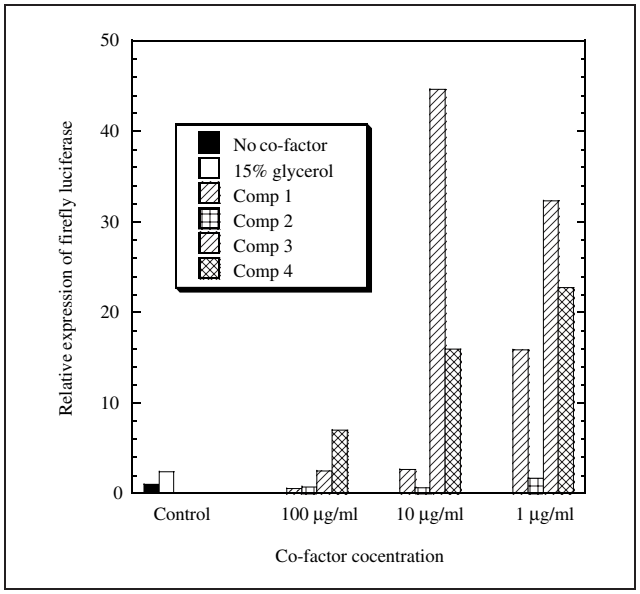


Fig.: Relative expression of firefly luciferase after 3 h transfection of COS-1 cells with lipofectamine plus followed by short incubation with the co-factor

ever at charge ratios below 30 there was no effect on the mobility of DNA and strong band for the free plasmid was observed. DEAE-dextran was used as a control compound in these investigations. This polymer is commonly used to condense DNA. DEAE-dextran formed a strong complex with DNA and no band for free plasmid was observed when the +/− ratio was 0.3 or higher.

Transfection experiments with compounds **1**–**4** and pGL3-Control Vector plasmid were also consistent with weak complexes formation. The plasmid was incubated with compounds **1**–**4** prior to application to the COS-1 cells but this did not affect the expression of luciferase, which was monitored with the Promega Dual-Lucifease Reporter Assay System (data not shown). Lipofectamine Plus™ is a commercial DNA complexing and condensing agent that is used for *in vitro* transfection. Incubation with Lipofectamine Plus™ resulted in effective transfection of the COS-1 cells and a 10^3 increase in the expression of luciferase relative to the background level for untreated cells.

Glycerol is commonly used to increase transfection efficacy and this effect is thought to be partially due to enhanced release of DNA into the cytoplasm (Wagner 1998). Long chain quaternary ammonium compounds are also membrane active agents, which can penetrate and affect the barrier function of cell membranes. This property to increase the permeability of the cell membranes is the basis for the antibacterial effect and it has also been utilized in drug delivery applications (Thorsteinsson et al. 2003b). Thus it is possible that this type of compounds can have similar effects, as glycerol, to enhance transfection efficacy in gene-delivery.

The Fig. shows the relative expression of luciferase when the 3 h transfection with Lipofectamine Plus™ was followed by a brief incubation with compounds **1**–**4** or 15% glycerol. All the compounds are highly toxic at 1000 μg/ml and no expression of luciferase was therefore observed after brief incubation at this concentration. At lower concentrations compounds **3** and **4** promoted transfection of the cells. There was a 45 fold increase in the relative expression at 10 μg/ml of **3** and a 23 fold increase at 1 μg/ml of **4**. Incubation with 10 and 1 μg/ml of **1** also had a positive effect with 2.7 and 16 fold increase in the expression levels, respectively. Compound **2** did not enhance delivery. Glycerol enhanced the expression 2.4 fold.

These results indicate that long chain quaternary ammonium antibacterial agents could be used as co-factors to enhance the efficiency of gene delivery *in vitro*. In the present investigation the best results were obtained with soft compound **3**. A significant increase in expression was also obtained with the least toxic compound **4**.

Experimental

1. Materials

Cetyl pyridinum chloride (**1**) and DEAE-dextran were obtained for Sigma. Firefly luciferase plasmid (pGL3-Control Vector) and renilla luciferase plasmid were obtained form Promega (USA) Synthesis of compounds **2**–**4** has previously been described (Thorsteinsson et al. 2003a).

2. Investigation of cytotoxicity

Cells from COS-1 cells were seeded at about 1.5×10^5 cells per well in 24-well dishes (Nunclon™). Thousand μg/ml solutions of compounds **1**–**4** where prepared in Dulbecco's medium, containing glutamax-1 with 4500 mg/L glucose and pyridoxine. These solutions were serially diluted into the same medium onto the wells with 0.5 ml per well. Cell were then incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 and controlled humidity in a Stericult 200 incubator (Forma Scientific). The cells were then stained with tryptohan blue and the number of viable cells adhering to the surface was counted under microscope (Leica MP530). Wells were the cells had been incubated in pure medium were used as reference.

3. DNA complexes

Compounds **1**, **2**, **3**, **4** and DEAE-dextran were dissolved in H_2O and mixed with 1 μg plasmid. The +/− ratios ranged from 0.03 to 300 for each of the compounds. The mixtures were incubated at room temperature for 45 min to form the complex. The DNA complex samples were loaded onto 0.8% agarose gels in, pH 8, Tris-borate EDTA buffer. After electrophoreses for 30–60 min at 75–100 V (Bio-Rad power pac 200) the gels were stained with ethidium bromide and scanned on an Image station 440 CF (Kodak digital science™). The free 5.256 bp plasmid appeared as strong band at the given position, which could be identified by the markers. When a complex was formed the mobility of the DNA was inhibited and the plasmid could not be drawn into the gel form the starting position. In this case no band or a very weak band appeared at 5.256 bp position.

4. Transfection experiment

Cells from COS-1 cell lines were seeded at about 1.5×10^5 cells per well in 24-well dishes (Nunclon™). In the case of compounds **1**, **2**, **3** and **4** the DNA complexes were prepared by adding 1 μg of the firefly luciferase plasmid and 0.1 μg of renilla luciferase plasmid to 100 μl cell of growth medium solutions containing 1–10 μg/ml of the cationic compound. The solutions were incubated for 30–60 min to promote complexation before it was added onto the cells.

The Lipofectamine Plus™-DNA complex was prepared as prescribed in the product information. Briefly, 4 ml of the Plus solution was added to 25 ml serum free medium containing 0.4 μg of the DNA firefly plasmid and 0.04 μg of renilla plasmid and allowed to stand for 15 min. One ml of the Lipofectamine reagent solution was added to 25 ml of serum free medium. This solution was mixed with the DNA solution and the mixture was incubated for 15 min before it was added to the cells.

The cell growth medium was changed to a serum free medium on COS-1 cells, which had been grown in multi-well dishes (Nunclon™) for one day. One hundred μl of the DNA-complex solution was added to each well. The cells were then incubated for 3 h in a Stericult 200 incubator. In the case where 15% glycerol or compounds **1**, **2**, **3** or **4** were used as co-factors to promote delivery the medium was removed and after the incubation the solution of the co-factor was added and allowed to stand for 1.5 min before it was diluted with phosphate buffer saline (PBS) and removed. Normal medium was then added and the cells were kept in the incubator for 48–72 h. The cells were washed with PBS and lysed with 5× diluted Promega lysis buffer. After 15 min incubation, 20 μl samples were drawn from each well and transferred to a 96 well microplate. The Promega Dual-Luciferase Reporter Assay System was used to determine the firefly and renilla luciferase activity and the bioluminescence was measured in an Applied Biosystems TR717 (USA) microplate reader. The firefly luciferase and the renilla luciferase activities were generally consistent. However the Renilla luciferase activity was much lower and more variable. Therefore only the relative firefly luciferase activity is reported.

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Peripheral anti-hyperalgesia by oxcarbazepine: involvement of adenosine A_1 receptors

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In this study we determined whether oxcarbazepine (OXC) could produce local peripheral antinociceptive effects in a rat model of inflammatory hyperalgesia, and whether adenosine receptors were involved. When coadministered with the pro-inflammatory compound concanavalin A, OXC (1000–3000 nmol/paw) caused a significant dose- and time-dependent anti-hyperalgesia. Caffeine (1000–1500 nmol/paw), a nonselective adenosine receptor antagonist, as well as 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (10–30 nmol/paw), a selective A_1 receptor antagonist, coadministered with OXC, significantly depressed its anti-hyperalgesic effect. Drugs injected into the contralateral hind paw did not produce significant effects. These results indicate that OXC produces local peripheral anti-hyperalgesic effects, which is mediated *via* peripheral A_1 receptors.

Oxcarbazepine (OXC), a relatively novel anticonvulsant drug, has been used in neuropathic pain treatment (Carrazana and Mikoshiba 2003). Recently, it has been shown that it has anti-hyperalgesic activity in animal models of inflammatory pain (Kiguchi et al. 2004; Tomić et al. 2004). However, the sites and mechanisms of analgesic actions of OXC are not fully understood. Beside a blockade of ion currents (Kiguchi et al. 2001; Ambrosio et al. 2002), there is an evidence indicating that some receptors are also involved in analgesic action of OXC. We have previously shown that systemic OXC reversed the mechanical hyperalgesia of an inflamed rat paw, and that this effect is mediated *via* A_1 receptors (Tomić et al. 2004). It is well known that activation of both central and peripheral A_1 receptors inhibits pain in rodents (Sawynok 1998). The interaction of OXC with central adenosine receptors has been demonstrated in receptor binding studies (Marangos et al. 1983; Fujiwara et al. 1986). However, the ability of OXC to produce a local peripheral anti-hyperalgesic effect has not been evaluated before. Moreover, it remained unknown whether peripheral adenosine receptors are involved in the OXC-induced antinociception. In this study, we determined (1) the effects of locally administered OXC on Con A-induced inflammatory hyperalgesia in the rat and (2) the effects of caffeine, a nonselective A_1