*Journal of Drug Targeting*, 2002 Vol. 10 (4), pp. 317–325

Taylor & Francis
healthsciences

# Apolipoprotein-mediated Transport of Nanoparticle-bound Drugs Across the Blood–Brain Barrier

JÖRG KREUTER[a,*], DMITRY SHAMENKOV[b], VALERY PETROV[b], PETER RAMGE[a,†], KLAUS CYCHUTEK[c], CLAUDIA KOCH-BRANDT[d] and RENAD ALYAUTDIN[b]

[a]*Institut für Pharmazeutische Technologie, Biozentrum, J.W. Goethe-Universität, Marie-Curie-Strasse 9, Frankfurt D-60439, Germany;* [b]*Department of Pharmacology of Moscow Medical Academy, Moscow, Russia;* [c]*Department of Medical Biotechnology, Paul-Ehrlich-Institut, Langen, Germany;* [d]*Institut für Biochemie, Johannes Gutenberg-Universität Mainz, Mainz, Germany*

*(Received 21 February 2001; In final form 21 May 2001)*

Recent studies have shown that drugs that are normally unable to cross the blood–brain barrier (BBB) following intravenous injection can be transported across this barrier by binding to poly(butyl cyanoacrylate) nanoparticles and coating with polysorbate 80. However, the mechanism of this transport so far was not known. In the present paper, the possible involvement of apolipoproteins in the transport of nanoparticle-bound drugs into the brain is investigated. Poly(butyl cyanoacrylate) nanoparticles loaded with the hexapeptide dalargin were coated with the apolipoproteins AII, B, CII, E, or J without or after precoating with polysorbate 80. In addition, loperamide-loaded nanoparticles were coated with apolipoprotein E alone or again after precoating with polysorbate 80. After intravenous injection to ICR mice the antinociceptive threshold was measured by the tail flick test. Furthermore, the antinociceptive threshold of polysorbate 80-coated dalargin-loaded nanoparticles was determined in ApoEtm1Unc and C57BL/6J mice. The results show that only dalargin or loperamide-loaded nanoparticles coated with polysorbate 80 and/or with apolipoprotein B or E were able to achieve an antinociceptive effect. This effect was significantly higher after polysorbate-precoating and apolipoprotein B or E-overcoating. With the apolipoprotein E-deficient ApoEtm1Unc mice the antinociceptive effect was considerably reduced in comparison to the C57BL/6J mice. These results suggest that apolipoproteins B and E are involved in the mediation of the transport of drugs bound to poly(butyl cyanoacrylate) nanoparticles across the BBB. Polysorbate 80-coated nanoparticles adsorb these apolipoproteins from the blood after injection and thus seem to mimic lipoprotein particles that could be taken up by the brain capillary endothelial cells via receptor-mediated endocytosis. Bound drugs then may be further transported into the brain by diffusion following release within the endothelial cells or, alternatively, by transcytosis.

*Keywords*: Apolipoprotein B; Apolipoprotein E; Nanoparticles; Blood–brain barrier; Drug delivery to the brain; Dalargin; Loperamide

## INTRODUCTION

The blood–brain barrier (BBB) represents an insurmountable obstacle for the entry of many drugs and blood-borne substances into the brain (Brightman, 1992; Begley, 1996; Davson and Segal, 1996). It is formed by tight junctions between the cerebral endothelial cells which abolish all aqueous paracellular diffusional pathways and by biochemical systems consisting of enzymes which specifically metabolise many drugs (Minn *et al.*, 2000) as well as of specific efflux mechanisms (P-glycoprotein; mdr, multi-drug resistance protein; MOAT, multiple organic anionic transporters), which transport many of the more lipophilic compounds out of the CNS. A number of studies have shown that drugs that normally cannot cross the BBB can be transported across this barrier into the brain and exhibit a pharmacological effect following intravenous injection by binding to poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80. Drugs that have successfully been transported into the brain using this carrier include the hexapeptide dalargin (Alyautdin *et al.*, 1995; Kreuter *et al.*, 1995; 1997; Schröder and

*Corresponding author. Tel.: +49-69-7982-9682. Fax: +49-69-7982-9694. E-mail: kreuter@em.uni-frankfurt.de
†Boots Healthcare Deutschland GmbH, Reinbek, Germany

ISSN 1061-186X print/ISSN 1029-2330 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10611860290031877

Sabel, 1996; Schroeder *et al.*, 1998a,b; Ramge *et al.*, 1999), the dipeptide kytorphin (Schroeder *et al.*, 1998a), loperamide (Alyautdin *et al.*, 1997), tubocurarine (Alyautdin *et al.*, 1998), the NMDA receptor antagonist MRZ 2/576 (Friese *et al.*, 2000), and doxorubicin (Gulyaev *et al.*, 1999; Gelperina *et al.*, 2000). The possibility of drug transport into the brain by nanoparticles opens up totally new perspectives for the treatment of diseases like brain tumours, Alzheimer's, and multiple sclerosis.

The mechanism of the transport enhancement into the brain mediated by the nanoparticles, however, still is not fully elucidated. A number of possibilities exist that could explain the drug delivery by nanoparticles across the BBB: First, an increased retention of the nanoparticles in the brain blood capillaries could occur combined with an adsorption to the capillary walls. This could create a higher concentration gradient that would enhance the transport across the endothelial cell layer and as a result the delivery to the brain. Second, a general surfactant effect characterized by a solubilisation of the endothelial cell membrane lipids could lead to membrane fluidisation and thus to an enhanced drug permeability through the BBB. Third, the nanoparticles could lead to an opening of the tight junctions between the endothelial cells. The drug could then permeate through the tight junctions in free form or together with the nanoparticles in bound form. Fourth, the nanoparticles may be endocytosed by the endothelial cells followed by the release of the drugs within these cells and delivery to the brain. Fifth, the nanoparticles with bound drugs could be transcytosed through the endothelial cell layer. Sixth, the polysorbate 80 used as the coating agent could inhibit the efflux system, especially P-glycoprotein (Pgp). All these mechanisms also could work in combinations.

Recently, Kreuter *et al.* (1997) observed that besides polysorbate 80, coating of the nanoparticles with polysorbate 20, 40, or 60 also enabled an antinociceptive effect after i.v. injection of dalargin nanoparticles, while other surfactants such as poloxamers and poloxamines were unable to achieve such an effect. At the same time, Lück (1997) found that apolipoprotein E (apo E) was adsorbed on the surface of the polysorbate 20, 40, 60 or 80-coated nanoparticles after their incubation for 5 min in human citrate-stabilised plasma at 37°C. The particles were separated from the serum by centrifugation and the adsorbed plasma proteins desorbed and analysed by two dimensional polyacrylamide gel electrophoresis (2-D PAGE). Again an apo E adsorption was detected only after coating with polysorbate 20, 40, 60, or 80, whereas no apo E adsorption resulted after incubation of uncoated nanoparticles or after coating with poloxamers 338, 407, Cremophor® EL, or Cremophor® RH40. These results suggest that apo E may be involved in the mediation of the drug transport into the brain by the polysorbate-coated nanoparticles. On the other hand, another lipoprotein; apo J, was previously shown to facilitate the uptake of sAβ1–40 across the blood–brain and the blood–cerebrospinal fluid barrier by a receptor-mediated process (Zlokovic *et al.*, 1996). In order to investigate the involvement of these apolipoproteins in the transport of drugs bound to nanoparticles into the brain, we adsorbed apo E, apo J, as well as other apolipoproteins on uncoated and on polysorbate 80-precoated dalargin-loaded nanoparticles and investigated the antinociceptive effects by the tail flick test after intravenous injection to mice. In addition, we measured this effect in apo E-deficient ApoEtm1Unc mice and compared it to that in C57BL/6J mice from which these apo E-deficient mice were derived.

## MATERIALS AND METHODS

### Materials

Butylcyano acrylate monomer was obtained from Sichelwerke (Hannover, Germany), dalargin from Bachem (Heidelberg, Germany), polysorbate 80 from ICI Chemikalien (Essen, Germany), dextran 70,000 and D(+)-glucose from Fluka (Buchs, Switzerland), and apolipoproteins AII, B, CII, and E from Calbiochem (Bad Soden/Ts, Germany). Loperamide was provided by Dainippon (Osaka, Japan). Apolipoprotein J was manufactured as described below. All other chemicals including 0.01N hydrochloric acid and 1N sodium hydroxide solution were from E. Merck (Darmstadt, Germany).

### Production of Apolipoprotein J

MDCK cells (ATCC-CCL-43) were grown to confluency and then incubated for 72 h in serum free medium (Taub *et al.*, 1979). The apolipoprotein J enriched medium was collected and floating cells removed by a 10 min centrifugation at 3000*g*. The supernatant was passed over a wheat germ agglutinin column and bound apo J was eluted at 20 mM Tris pH 7.5, 500 mM NaCl, and 400 mM *N*-acetylglucosamine. Proteins in the eluate were concentrated by ammonium sulphate precipitation. The precipitate was dissolved in 20 mM Tris pH 7.5 and 100 mM NaCl, and passed over a Sephacryl S 200 column. Proteins in the eluted fractions were analysed by SDS-PAGE (Gerace *et al.*, 1982). Fractions enriched in Apo J and devoid of detectable impurities were pooled.

### Preparation of Poly(butyl Cyanoacrylate) Nanoparticles

Poly(butyl cyanoacrylate) (PBCA) nanoparticles were produced as described before (Alyautdin *et al.*, 1995). Specifically, 1% (V/V) butyl cyanoacrylate monomer was added slowly dropwise to a 1% (W/V) solution of dextran 70,000 in 0.01N hydrochloric acid. This mixture was stirred for 4 h with a magnetic stirrer to perform the polymerisation. After this time the polymerisation was completed by neutralisation of the mixture with 1N sodium hydroxide. After filtration through a G3 glass filter

TABLE I Mean percentage of maximally possible effect (% MPE) and standard deviation (SD) of nociceptive threshold after i.v. injection of dalargin-loaded (7.5 mg/kg) apolipoprotein-coated poly(butyl cyanoacrylate) nanoparticles in mice determined by the tail-flick test

| | % MPE (mean ± SD) | | | | |
|---|---|---|---|---|---|
| Preparation | 15 min | 30 min | 45 min | 60 min | 90 min |
| Empty PBCA nanoparticles | 3.8 ± 3.3 | 1.5 ± 9.0 | 0.75 ± 3.2 | 3.9 ± 4.3 | −2.0 ± 9.8 |
| Dalargin solution | 2.3 ± 4.6 | 10.0 ± 9.8 | 9.3 ± 2.8 | 4.7 ± 5.1 | 2.0 ± 6.1 |
| Mixture of dalargin and polysorbate 80 | 4.8 ± 1.7 | 8.3 ± 2.3 | 7.8 ± 2.3 | 6.1 ± 4.2 | 6.6 ± 2.6 |
| Dalargin-loaded PBCA nanoparticles | 5.7 ± 5.1 | 5.0 ± 9.4 | 4.7 ± 9.1 | 6.9 ± 11.1 | 3.8 ± 9.1 |
| Apolipoproteins coated onto the surface of dalargin-loaded PBCA nanoparticles | | | | | |
| Apo AII | 5.29 ± 2.00 | 2.97 ± 6.28 | 5.98 ± 7.64 | 5.94 ± 7.26 | 9.44 ± 12.5 |
| Apo B | 6.76 ± 5.26 | 25.17 ± 4.31* | 37.74 ± 6.61* | 27.1 ± 8.82 | 17.54 ± 8.17* |
| Apo CII | 8.39 ± 2.19 | 7.19 ± 3.64 | 3.65 ± 5.67 | 7.26 ± 5.18 | 1.14 ± 7.67 |
| Apo E | 38.8 ± 13.7* | 36.08 ± 11.63* | 29.7 ± 5.57* | 19.59 ± 7.47 | 2.03 ± 6.49 |
| Apo J | 3.32 ± 2.58 | 5.84 ± 15.86 | 10.89 ± 8.64 | 13.73 ± 13.56 | 5.00 ± 5.00 |
| Dalargin-loaded polysorbate 80-coated PBCA nanoparticles | 35.2 ± 5.8 | 50.4 ± 4.1 | 49.5 ± 4.5 | 36.5 ± 13.7 | 7.1 ± 6.3 |
| Apolipoproteins coated onto the surface of dalargin loaded polysorbate 80-pre-coated PBCA nanoparticles | | | | | |
| Apo AII | 1.98 ± 9.56 | 0.5 ± 10.58 | 12.81 ± 16.8 | 18.29 ± 21.81 | 48.8 ± 13.24† |
| Apo B | 30.87 ± 19.43 | 74.68 ± 15.81† | 58.71 ± 8.03† | 45.09 ± 18.55 | 25.51 ± 16.44 |
| Apo CII | 7.76 ± 2.56 | 22.24 ± 9.36 | 49.48 ± 10.88 | 16.19 ± 16.55 | 3.72 ± 8.58 |
| Apo E | 61.39 ± 8.59† | 62.09 ± 6.91† | 64.52 ± 13.98 | 62.33 ± 11.82† | 51.73 ± 12.9† |
| Apo J | 18.49 ± 27.2 | 53.37 ± 28.69 | 51.51 ± 16.68 | 36.33 ± 19.73 | 19.39 ± 19.1 |

* Statistically significant difference ($2p > 0.05$) compared to dalargin-loaded PBCA uncoated nanoparticles.
† Statistically significant difference ($2p > 0.05$) compared to dalargin-loaded polysorbate 80-coated PBCA nanoparticles.

TABLE II Mean percentage of maximally possible effect (% MPE) and standard deviation (SD) of nociceptive threshold after i.v. injection of loperamide-loaded (3.6 mg/kg) and/or apolipoprotein E-coated poly(buty cyanoacrylate) nanoparticles in mice determined by the tail-flick test

| | % MPE (mean ± SD) | | | | |
|---|---|---|---|---|---|
| Preparation | 15 min | 45 min | 90 min | 120 min | 180 min |
| Loperamide-loaded PBCA PBCA nanoparticles | 4.2 ± 13.6 | 3.3 ± 12.1 | 4.9 ± 6.9 | 1.4 ± 10.0 | 2.2 ± 6.2 |
| Loperamide-loaded PBCA PBCA nanoparticles coated with apo E | 28.9 ± 23.8* | 56.1 ± 23.4* | 38.7 ± 21.1* | 25.8 ± 42.1 | 37.9 ± 34.0* |
| Loperamide-loaded PBCA nanoparticles coated with polysorbate 80 | 83.7 ± 22.3 | 100 | 36.2 ± 20.3 | 40.2 ± 32.9 | 22.5 ± 19.2 |
| Loperamide-loaded PBCA nanoparticles pre-coated polysorbate 80 and coated with apo E | 93.2 ± 12.8 | 100 | 84.2 ± 15.9† | 57.5 ± 10.02 | 30.3 ± 23.1 |

* Statistically significant difference ($2p < 0.05$) compared to loperamide-loaded uncoated PBCA nanoparticles.
† Statistically significant difference ($2p > 0.05$) compared to loperamide-loaded polysorbate 80-coated PBCA nanoparticles.

(Schott AG, Mainz, Germany) the particles were separated from residual monomers by threefold centrifugation (L8-60M, Beckman, Hannover, Germany) at 90,000*g* for 1 h and washing with distilled water after resuspension by ultra sonication. The particle diameters were determined by dynamic light scattering (photon correlation spectroscopy, PCS) using a digital correlator BI-2030 (Brookhaven Instruments Corporation, Holtsville, New York, USA). An average diameter of 300 nm was found with a polydispersity index of 0.177 before lyophilisation. The particles were stored after addition of 100% D(+)-glucose and lyophilisation in a LYOVAC GT2 (Leybold AG, Köln, Germany).

The lyophilised PBCA nanoparticles were resuspended in phosphate buffer saline under constant stirring. The concentration of PBCA nanoparticles was 20 mg/ml. After this 0.75 mg/ml or 1.0 mg/ml dalargin was added and the mixture was stirred at 400 rpm for 4 h.

### *Polysorbate 80-coated Nanoparticles*

After addition of dalargin and stirring for 4 h, 1% (v/v) polysorbate 80 was added and the mixture further stirred for 30 min.

### *Apolipoprotein-coated Nanoparticles*

After addition of dalargin and stirring for 4 h, apolipoprotein AII, B, CII, E or J (12.5 μg/ml) were added and the mixture further stirred for 1 h.

### *Polysorbate 80-coated and Apolipoprotein-overcoated Nanoparticles*

Dalargin-loaded and polysorbate 80-coated nanoparticles were prepared as described above. Then apolipoproteins AII, B, CII, E, or J (12.5 μg/ml) were added and the mixture further stirred for 1 h.

### *Loperamide-containing PBCA Nanoparticles*

Loperamide-containing PBCA nanoparticles were prepared according to a previously published technique (Alyautdin *et al.*, 1997). Briefly, poloxamer 188 and sodium sulphate were dissolved at a concentration of 1% (m/v) in a mixture of 10 ml ethanol 96% and 10 ml 0.1N HCl. Loperamide then was added at a concentration of 0.1%. While stirring, 200 μl n-BCA was added dropwise to the solution. The mixture then was stirred for 4 h at room temperature with a magnetic stirrer at 400 rpm. After adjusting the pH of the suspension to 6.0 ± 0.5 with 1N NaOH, stirring was continued for an additional hour to complete the reaction. The ethanol was then removed using a rotatory evaporator (Büchi RE 111, Büchi, Flawil, Switzerland). This nanoparticles preparation was divided into four batches. Apolipoprotein E (12.5 μg/ml) was added to one of these batches and this suspension further stirred for 1 h. Additionally, to two of the batches polysorbate 80 was added to give a final concentration of 1% (v/v) polysorbate 80 and stirred at 400 rpm for 30 min. The polysorbate 80-containing nanoparticles suspension was either used as such or after further addition of apolipoprotein E (12.5 μg/ml) as described above.

## Animal Testing

The animal experiments were performed in accordance to the Russian Guidelines for Animal Experiments and authorised by the Russian Ministry of Health (1045-73 and 52-F3-24.04.95) or the German Tierschutzgesetz and the Allgemeine Verwaltungsvorschrift zur Durchführung des Tierschutzgesetzes and were authorised by the Regierungspräsident Darmstadt (II 25.3-19 c 20/15-F 95/07).

### *Antinociceptive Effects in Mice After Injection of Apolipoprotein-coated Nanoparticles*

Male ICR mice 20–22 g were obtained from the Centre of Oncology of Russian Academy of Medical Sciences, Moscow, Russia, and were kept at an ambient temperature (22 ± 3°C) with a 12 h light and a 12 h dark cycle (light at 6.00 h). Water and standard laboratory feed were freely available.

The mice were divided into 16 groups of 5 mice. The mice obtained 200 μl of one of the following preparations into the tail vein (Table I): Group 1 PBCA nanoparticle

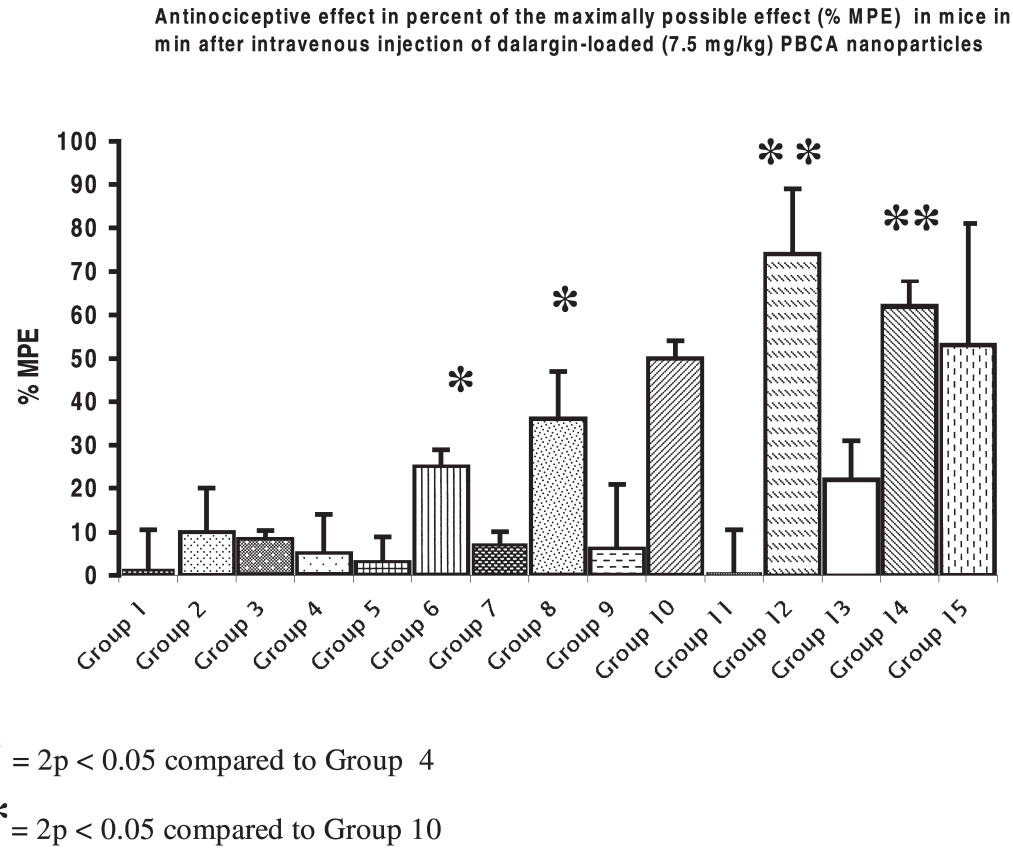


FIGURE 1 Antinociceptive effects [% MPE] in mice ($n = 5$/group) 30 min after intravenous injection of one of the following preparations: Group 1 – empty PBCA nanoparticles (control 1); group 2 – dalargin solution (control 2); group 3 – mixture of dalargin and polysorbate 80 (control 3); group 4 – dalargin-loaded PBCA nanoparticles (control 4); group 5 – dalargin-loaded PBCA nanoparticles coated with apo AII; group 6 – dalargin-loaded PBCA nanoparticles coated with apo B; group 7 – dalargin-loaded PBCA nanoparticles coated with apo CII; group 8 – dalargin-loaded PBCA nanoparticles coated with apo E; group 9 – dalargin-loaded PBCA nanoparticles coated with apo J; group 10 – dalargin-loaded PBCA nanoparticles coated with polysorbate 80 (control 5); group 11 – dalargin-loaded PBCA nanoparticles precoated with polysorbate 80 and overcoated with apo AII; group 12 – dalargin-loaded PBCA nanoparticles precoated with polysorbate 80 and overcoated with apo B; group 13 – dalargin-loaded PBCA nanoparticles precoated with polysorbate 80 and overcoated with apo CII; group 14 – dalargin-loaded PBCA nanoparticles precoated with polysorbate 80 and overcoated with apo E; group 15 – dalargin-loaded PBCA nanoparticles precoated with polysorbate 80 and overcoated with apo J.

suspension without drug; group 2 dalargin (7.5 mg/kg) in PBS solution; group 3 a mixture of polysorbate with dalargin (7.5 mg/kg) in PBS; group 4 dalargin (7.5 mg/kg) bound to PBCA nanoparticles: Groups 5–9 received dalargin (7.5 mg/kg) bound to PBCA nanoparticles coated by apolipoproteins (12.5 μg/kg) AII (group 5), B (group 6), CII (group 7), E (group 8), or J (group 9). Group 10 received dalargin (7.5 mg/kg) bound to PBCA nanoparticles coated with polysorbate 80. Groups 11–15 were treated with dalargin (7.5 mg/kg) bound to PBCA nanoparticles (7.5 mg/kg) which were precoated with polysorbate 80 as in group 10 but additionally overcoated with apolipoproteins AII (group 11), B (group 12), CII (group 13), E (group 14), or J (group 15).

In another experiment with loperamide (Table II), 20 mice were divided into four groups of five mice each. The mice obtained 200 μl of one of the following preparations into the tail vein: Loperamide (3.6 mg/kg) loaded nanoparticles (group 1), loperamide (3.6 mg/kg) loaded nanoparticles, coated with apo E (group 2), loperamide (3.6 mg/kg) loaded nanoparticles, coated with polysorbate 80 (group 3), loperamide (3.6 mg/kg) loaded nanoparticles, coated with polysorbate 80 and overcoated with apo E (group 4).

The nociceptive threshold was measured using the tail flick test (Mod. 33 Tail Flick Analgesia Meter, Iitic Inc., Woodland Hills, CA., USA). Antinociceptive procedures were carried out according to a previously published technique (Alyautdin *et al.*, 1995). Tail flick latency was tested 15, 30, 45, 60 and 90 min after injection. The response latencies were converted to percent maximal possible effect (MPE, mean ± standard deviation) using Eq.1:

$$\%\ \mathrm{MPE} = \frac{\text{post drug latency} - \text{pre drug latency}}{\text{cut off time} - \text{pre drug latency}} \times 100 \quad (1)$$

### *Antinociceptive Effects in Apolipoprotein E-deficient (ApoEtm1Unc) Mice*

Apolipoprotein E-deficient (ApoEtm1Unc) mice as well as C57BL/6J mice, from which the ApoEtm1Unc mice were derived, were obtained from Charles River WIGA (Sulzfeld, Germany) and treated as described above. Both

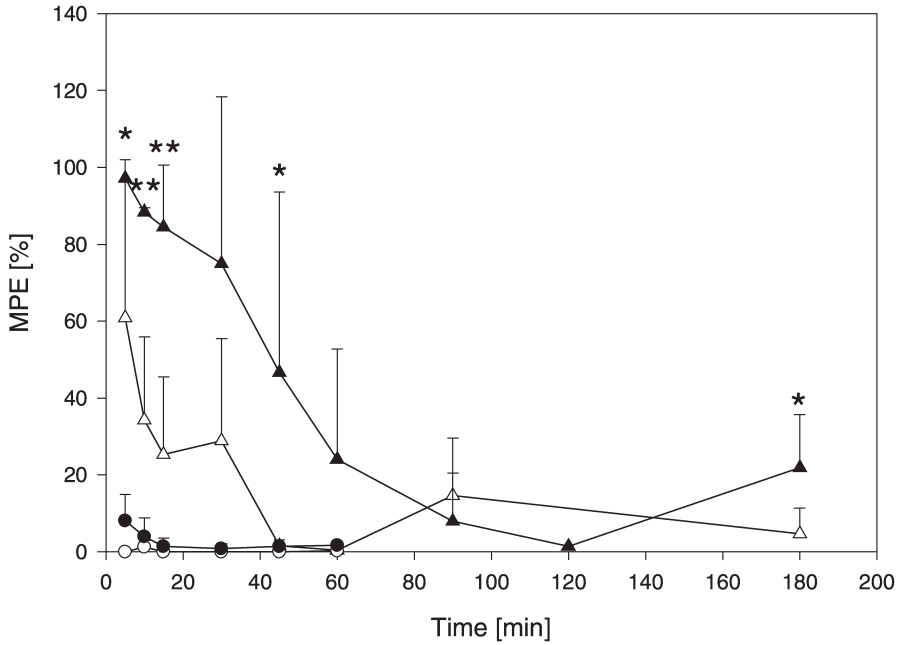


FIGURE 2 Antinociceptive effects [% MPE] in apolipoprotein E-deficient (ApoEtm1Unc) and C57BL/6J mice ($n = 6$/group) after intravenous injection of one of the following preparations: ○ dalargin solution in PBS (ApoEtm1Unc mice); •dalargin solution in PBS (C57BL/6J mice); △ dalargin-loaded nanoparticles coated with polysorbate 80 (ApoEtm1Unc mice); ▲ dalargin-loaded nanoparticles coated with polysorbate 80 (C57BL/6J mice). Statistical difference for polysorbate 80 coated nanoparticles between ApoEtm1Unc mice and C57BL/6J mice: $=2p < 0.05$, $= 2p < 0.01$.

types of mice were divided into two groups of six animals and injected intravenously into the tail vein with 10 mg/kg of dalargin either dissolved in PBS or adsorbed to PBCA nanoparticles coated with 1% (v/v) polysorbate 80 (Fig. 2). The antinociceptive reaction in [% MPE] was determined with a Tail-Flick-Testgerät (Ugo Basile, Comerio Varese, Italy) as described above.

The results were analysed using student's *t*-test.

## RESULTS

The antinociceptive responses of the mice after i.v. injection using apolipoprotein-coated nanoparticles or control preparations are listed in Table I. Figure 1 provides a comparison of the results after 30 min. Among the preparations without polysorbate 80-coating including the controls (empty PBCA nanoparticles, dalargin solution in PBS, dalargin solution in PBS plus polysorbate 80, dalargin-loaded nanoparticles without polysorbate 80) only apolipoprotein B and E-coated nanoparticles yielded a significant ($2p < 0.05$) analgesic effect. This effect, however, was lower than after polysorbate 80-coating alone. After polysorbate 80-precoating, a statistically significant antinociceptive response compared to the dalargin solution was observable with all apolipoproteins. In the case of apolipoprotein AII (apo AII) this effect appeared delayed and was significant only after 90 min. Apolipoproteins B and E (apo B and apo E) showed a rapid onset of the antinociceptive reaction and, moreover, even statistically higher effects ($2p < 0.05$) than polysorbate 80 alone. With apo E this effect remained high for the entire observation period.

The same tendency was observable with loperamide (Table II). Loperamide-loaded nanoparticles without coating were not able to achieve any antinociceptive effect. Apo E-coated nanoparticles induced statistically significant results compared to this group between 15 and 90 min ($2p < 0.05$). Much higher effects were obtained after polysorbate 80-coating or additional apo E-overcoating. Apo E-overcoating achieved a prolonged antinociception even compared to polysorbate 80-coating alone. The difference between these two preparations lasted to the end of the observation period, i.e. for over 180 min, and led to a statistically significant difference ($2p < 0.05$) at 90 min.

The antinociceptive reactions in C57BL/6J mice appeared more rapidly than in the ICR mice obtained from the Centre of Oncology of Russian Academy of Medical Sciences (Fig. 2) used in the above experiments. However, they were similar in extent to the latter mice. Moreover, the extent and the rapid onset of the reactions in the C57BL/6J mice were similar to those observed by other authors (Schröder and Sabel, 1996; Schroeder *et al.*, 1998a,b; Ramge *et al.*, 1999) and to those observed with ICR mice obtained by Harlan–Winkelman (Borchen, Germany; data not shown). With the ApoEtm1Unc mice the antinociceptive reaction was lower and disappeared much more rapidly, i.e. between 30 and 45 min. The difference between C57BL/6J and ApoEtm1Unc mice was statistically significant up to 45 min ($2p < 0.05$; except after 30 min) and again after 180 min.

## DISCUSSION

These results (Table I and II, and Fig. 1) clearly show that apolipoprotein B or E (apo B or apo E) are able to mediate the delivery of the analgesic hexapeptide dalargin or the analgesic opioid loperamide across the BBB after binding to nanoparticles and intravenous injection: An antinociceptive effect with dalargin-loaded PBCA nanoparticles was observed after coating of the nanoparticles with these apolipoproteins alone as well as after overcoating following polysorbate 80 precoating. In the case of coating with apo B or E alone this effect was slightly lower or similar to previously observed effects with polysorbate 80 alone (Alyautdin *et al.*, 1995; Kreuter *et al.*, 1995; 1997; Schröder and Sabel, 1996; Schroeder *et al.*, 1998a,b; Ramge *et al.*, 1999). It was even higher if apo B or E-coating followed polysorbate 80-precoating. In contrast, other apolipoproteins including AII, CII or J were not able to induce these effects with nanoparticles without precoating with polysorbate 80. Consequently, it can be assumed that the antinociceptive effects observed with the latter apolipoproteins after precoating with polysorbate 80 were due to the presence of polysorbate 80. The observed slower onset of the antinociceptive effects with these apolipoproteins, especially apo AII, may be an indication that at least partial desorption of the latter apolipoproteins from the polysorbate 80-precoated nanoparticles was necessary to enable a dalargin transport into the brain.

In these experiments apo E induced slightly higher antinociceptive effects than apo B. For this reason, experiments also were performed with apo E using loperamide as the antinociceptive agent (Table II) and, additionally, experiments with dalargin in apo E-deficient ApoEtm1Unc mice (Fig. 2). With loperamide the same trend as with dalargin was observed in the normal (ICR) mice. In the apo E-deficient ApoEtm1Unc mice a reduced antinociceptive reaction was obtained in comparison to C57BL/6J mice from which the ApoEtm1Unc mice were derived. Both results further underline the special role of apo E in mediating the delivery of nanoparticle-bound dalargin or loperamide across the BBB. Apo E was previously shown to be adsorbed on the surface of polysorbate-coated nanoparticles after *in vitro* incubation in human plasma whereas no adsorption occurred without coating or after coating of the nanoparticles with a number of other surfactants (Lück, 1997). These latter preparations also were not able to transport sufficient dalargin across the BBB to induce an antinociceptive effect after i.v. injection (Kreuter *et al.*, 1997). In the present investigation, coating of the nanoparticles with apo B or apo E alone yielded lower antinociceptive effects in the mice than polysorbate 80-coating alone. This also can be explained by the finding of Lück (1997). He observed that after incubation of nanoparticles coated with apo E alone in the plasma, the apo E was replaced to a significant extent by other plasma components. Hence it can be concluded that the polysorbates act as an anchor for apo B and apo E.

As mentioned in the introduction, endocytosis by the brain capillary endothelial cells, followed by the release of the drugs within these cells and delivery to the brain, is one of the possible mechanism that could explain the observed pharmacological effects after i.v. injection of dalargin, loperamide, and other drugs. A rapid endocytotic uptake of the polysorbate 80-coated but almost no uptake of uncoated nanoparticles was observed in cultured mouse, rat, bovine, and human brain capillary endothelial cells (Ramge 1998; Ramge *et al.*, 2000; Kreuter and Alyautdin, 2000). In addition, fluorescent and electron microscopical pictures taken 45 min after i.v. injection of polysorbate 80-coated FITC-dextran labelled PBCA nanoparticles to mice also indicate an uptake by the brain endothelial cells (Kreuter *et al.*, 1995), although it has to be kept in mind that electron microscopy can be prone to artefacts. The results of the present and Lück's study (Lück, 1997] indicate that after i.v. injection apo E and/or apo B are anchored by the polysorbate on the surface of the nanoparticles coated with this substance. Apolipoproteins B and E both bind to lipoprotein receptors on the surface of cells (Brown and Goldstein, 1986; Knott *et al.*, 1986; Yang *et al.*, 1986; Mahley, 1988; Wilson *et al.*, 1991). Furthermore, the brain is equipped with a self-sufficient transport system for maintaining cholesterol and lipid homeostasis. The presence of LDL receptor has been demonstrated in rat and monkey brains (Dehouck *et al.*, 1997). These LDL receptors are also present in the brain capillary endothelial cells (Méresse *et al.*, 1989; 1991; Dehouck *et al.*, 1994; 1997). All members of the LDL receptor family bind apo E on their extracellular domains (Willnow *et al.*, 1999). Additionally LRP, megalin, and ApoER 2 have been shown to be expressed in the CNS (Kim *et al.*, 1996; Ji *et al.*, 1998; Willnow *et al.*, 1999). Consequently, the present results combined with the earlier findings of Kreuter *et al.* (1997) and Lück (1997) indicate that the nanoparticles which are coated with apo B or E appear to mimic lipoprotein particles and thus are able to interact with members of the LDL receptor family followed by their endocytotic uptake. The assumption of LDL receptor family-mediated uptake by the brain capillary endothelium is further supported by other *in vitro* findings: Ramge *et al.* (2000) observed that the uptake of the polysorbate 80-coated nanoparticles was inhibited by cytochalasin B, a phagocytic uptake inhibitor, but not by colchicin, a pinocytic uptake inhibitor, indicating that phagocytosis and not pinocytosis is involved in the uptake of nanoparticles. More importantly, pre-incubation of the brain endothelial cells with lipoprotein-deficient fetal calf serum for 22 h led to a significant increase in the *in vitro* nanoparticle uptake. The pre-incubation for 22 h with the lipoprotein-deficient serum probably starved the cells so that they were more susceptible for the reception and uptake of lipoprotein particles. Remaining small amounts of lipoprotein in the deficient fetal calf serum then may have augmented the interaction of the nanoparticles with the lipoprotein receptors, resulting in the observed

increase in uptake. Taken together, these results are a strong indication of lipoprotein receptor-mediated brain capillary endothelial cell uptake of the polysorbate 80-coated nanoparticles. This, however, does not imply that apolipoproteins E or B themselves are taken up together with the nanoparticles; it is more likely that the apolipoproteins are facilitators of the interaction with the endothelial cells.

Following this uptake the drug could be released and would be able to reach the brain interior by diffusion. Since the drugs already would have crossed the luminal membranes in which the very efficient efflux pumps (P-glycoprotein; mdr, MOAT) are mainly contained, the drugs would be less prone to the action of these pumps. Moreover, polysorbate 80 was shown to be able to inhibit P-glycoprotein (Woodcock *et al.*, 1992; Nerurkar *et al.*, 1996). Hence, inhibition of this efflux pump located in the brain blood vessel endothelial cell could further enhance the nanoparticle-mediated transport of drugs to the brain. Polysorbate 80 alone, added to the drug solutions without nanoparticles has no effect (Alyautdin *et al.*, 1995; Kreuter *et al.*, 1995). However, it is possible that the surfactant may be delivered to the brain endothelial cells more efficiently if it is adsorbed to the nanoparticles, thus augmenting drug transport into the brain. At present the significance of the inhibition of the efflux pumps by polysorbate 80 during nanoparticle-mediated drug delivery to the brain is not known. Nevertheless, although impediment of the efflux system by polysorbate 80 may contribute to the brain drug delivery by nanoparticles, the results of the present study indicate that endocytotic uptake seems to play a much more significant role. This argument is further supported by the observation that in the pharmacokinetic study with doxorubicin (Gulyaev *et al.*, 1999) in contrast to other organs significant brain concentrations were only obtained after 2–4 h. Such a delayed response seems to be a reflection of time consuming processes, such as endocytosis.

It is also possible that after endocytotic uptake the particles may be transcytosed through the brain blood vessel endothelial cells. *In vitro* transcytosis of LDL across the BBB was observed in the Cecchelli-Model by Dehouck *et al.* (1997). This process was totally blocked by the C7 monoclonal antibody that is known to interact with the LDL receptor. Furthermore, cholesterol depletion upregulated the expression of the LDL receptor in this model. Using the same *in vitro* model, transcytosis of dipalmitoyl phosphatidyl choline and cholesterol coated ionic crosslinked malto-dextrin nanoparticles of a size of about 60 nm was observed by Fenart *et al.* (1999). Hence, it is possible that the polysorbate-coated poly(butyl cyanoacrylate) nanoparticles used in the present study also can be transcytosed.

In summary, the above results support *in vitro* findings indicating that poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80 or other polysorbates adsorb apolipoprotein B and/or E after injection into the blood stream (Lück, 1997). The polysorbates act mainly as an anchor for the apolipoproteins. The apolipoprotein-overcoated nanoparticles thus would mimic lipoprotein particles and could interact with and then be taken up by the brain capillary endothelial cells via receptor-mediated endocytosis. In this scenario, nanoparticles would act as Trojan Horses for bound drugs. The drug then may be further transported into the brain by diffusion following release within the endothelial cells or, alternatively, by transcytosis. This, however, does not imply that apolipoproteins E or B themselves are taken up together with the nanoparticles. The apolipoproteins also merely could facilitate the interaction of the particles with the endothelial cells. The polysorbates may also augment drug transport by impediment of P-glycoprotein. In any case nanoparticles are necessary for the observed drug delivery to the brain since polysorbate 80 alone is unable to promote transport of these drugs (Alyautdin *et al.*, 1995; Kreuter *et al.*, 1995). In conclusion, nanoparticle-facilitated drug delivery is a promising new therapeutic strategy for the treatment of severe diseases of the brain and of the central nervous system.

## *Acknowledgements*

This work was supported by the DFG (Deutsche Forschungsgemeinschaft, Bonn, Germany) within the "Graduiertenkolleg Arzneimittelforschung und-entwicklung" at the Fachbereich für Biochemie, Pharmazie und Lebensmittelchemie of the Universität Frankfurt/M., Germany and travel grants for R. Alyautdin and by INTAS (International Association for the promotion of cooperation with scientists from the New Independent States of the former Soviet Union), Brussels, Belgium (grant 00-838).

## *References*

Alyautdin, R., Gothier, D., Petrov, V., Kharkevich, D. and Kreuter, J. (1995) "Analgesic activity of the hexapeptide dalargin adsorbed on the surface of polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles", *Eur. J. Pharm. Biopharm.* **41**, 44–48.

Alyautdin, R.N., Petrov, V.E., Langer, K., Berthold, A., Kharkevich, D.A. and Kreuter, J. (1997) "Delivery of loperamide across the blood–brain barrier with poly-sorbate 80-coated polybutylcyanoacrylate nanoparticles", *Pharm. Res.* **14**, 325–328.

Alyautdin, R.N., Tezikov, E.B., Ramge, P., Kharkevich, D.A., Begley, D.J. and Kreuter, J. (1998) "Significant entry of tubocurarine into the brain of rats by absorption to polysorbate 80-coated polybutylcyanoacrylate nanoparticles: an *in situ* brain perfusion study", *J. Microencapsul.* **15**, 67–74.

Begley, D. (1996) "The blood–brain barrier: Principles for targeting peptides and drugs to the central nervous system", *J. Pharm. Pharmacol.* **48**, 136–146.

Brightman, M. (1992) "Ultrastructure of the brain endothelium", In: Bradbury, M.W., ed, *Physiology and Pharmacology of the Blood–Brain Barrier* Handbook of Experimental Pharmacology 103, (Springer, Berlin), pp 1–22.

Brown, M.S. and Goldstein, J.L. (1986) "A receptor-mediated pathway for cholesterol homeostasis", *Science* **232**, 34–47.

Davson, H. and Segal, M. (1996) *Physiology of the CSF and Blood–Brain Barrier* (CRC Press, Boca Raton), pp 1–192.

Dehouck, B., Dehouck, M.P., Fruchart, J.C. and Cecchelli, R. (1994) "Upregulation of the low density lipoprotein receptor at the blood–

brain barrier: intercommunications between brain capillary endothelial cells and astrocytes", *J. Cell Biol.* **126**, 465–473.

Dehouck, B., Fenart, L., Dehouck, M.-P., Pierce, A., Torpier, G. and Cecchelli, R. (1997) "A new function for the LDL receptor: transcytosis of LDL across the blood–brain barrier", *J. Cell Biol.* **138**, 877–889.

Fenart, L., Casanova, A., Dehouck, B., Duhem, C., Slupek, S., Cecchelli, R. and Betbeder, D. (1999) "Evaluation of effect of charge and lipid coating on ability of 60-nm nanoparticles to cross an *in vitro* model of the blood–brain barrier", *J. Pharmacol. Exp. Therap.* **291**, 1017–1022.

Friese, A., Seiler, E., Quack, G., Lorenz, B. and Kreuter, J. (2000) "Enhancement of the duration of the anticonvulsive activity of a novel NMDA receptor antagonist using poly(butylcyanoacylate) nanoparticles as a parenteral controlled release delivery system", *Eur. J. Pharm. Biopharm.* **49**, 103–109.

Gerace, L., Ottaviano, Y. and Kondor-Koch, C. (1982) "Identification of a major polypeptide of the nuclear pore complex", *J. Cell Biol.* **95**, 826–837.

Gulyaev, A.E., Gelperina, S.E., Skidan, I.N., Antropov, A.S., Kivman G., Ya and Kreuter, J. (1999) "Significant transport of doxorubicin into the brain with polysorbate 80-coated nanoparticles", *Pharm. Res.* **16**, 1564–1569.

Gelperina, S.E., Smirnova, Z.S., Khalanskiy, A.S., Skidan, I.N., Bobruskin, A.I., Kreuter, J. (2000). "Chemotherapy of brain tumours using doxorubicin bound to polysorbate 80-coated nanoparticles." *Proc. 3rd World Meeting APV/APGI, Berlin, 3/6 April 2000,* pp. 441–442.

Ji, Z.-S., Pitasi, R.E. and Mahley, R.W. (1998) "Differential cellular accumulation/retention of apolipoprotein E mediated by cell surface heparan sulphate proteoglycans", *J. Biol. Chem.* **273**, 13452–13460.

Kim, D.-H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H.-J., Suzuki, H., Kondo, H., Saeki, S. and Yamamoto, T. (1996) "Human apolipoprotein E receptor 2", *J. Biol. Chem.* **271**, 8373–8380.

Knott, T.J., Pease, R.J., Powell, L.M., Wallis, S.C., Rall, Jr, S.C., Innerarity, T.L., Blackhart, B., Taylor, W.H., Marcel, Y., Milnes, R., Johnson, D., Fuller, M., Lusis, A.J., McCarty, B.J., Mahley, R.W., Levy-Wilson, B. and Scott, J. (1986) "Complete protein sequence and identification of structural domains of human apolipoprotein B", *Nature* **323**, 734–738.

Kreuter, J. and Alyautdin, R.N. (2000) "Using nanoparticles to target drugs to the central nervous system", In: Begley, D.J., Bradbury, M.W. and Kreuter, J.J., eds, *The Blood–Brain Barrier and Drug Delivery to the CNS* (Marcel Dekker, New York), pp 205–223.

Kreuter, J., Alyautdin, R.N., Kharkevich, D.A. and Ivanov, A.A. (1995) "Passage of peptides through the blood–brain barrier with colloidal polymer particles (nanoparticles)", *Brain Res.* **674**, 171–174.

Kreuter, J., Petrov, V.E., Kharkevich, D.A. and Alyautdin, R.N. (1997) "Influence of the type of surfactant on the analgesic effects induced by the peptide dalargin after its delivery across the blood–brain barrier using surfactant-coated nanoparticles", *J. Control. Release* **49**, 81–87.

Lück, M., "Plasmaproteinadsorption als möglicher Schlüsselfaktor für eine kontrollierte Arzneistoffapplikation mit partikulären Trägern" PhD Thesis, Freie Universität (Berlin), pp 14–24.

Mahley, R.W. (1988) "Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology", *Science* **240**, 622–630.

Méresse, S., Dehouck, M.P., Delorme, P., Bensaid, M., Tauber, J.P., Delbart, C., Fruchard, J.C. and Cecchelli, R. (1989) "Bovine brain endothelial cells express tight junctions and monoamine oxidase acivity in long-term culture", *J. Neurochem.* **53**, 1363–1371.

Meresse, S., Dehouck, M.P., Delorme, P., Fruchart, J.C. and Ceccheli, R. (1991) "Lipoproteins and reconstituted blood–brain barrier", In: Wilson, G., Davis, S.S. and Illum, L., eds, *Pharmacological Applications of Cell and Tissue Culture to Drug Transport* (Plenum Press, New York).

Minn, A., El-Bachá, R.D.S., Bayol-Denizot, C., Lagrange, P. and Suleman, F.G. (2000) "Drug metabolism in the brain: Benefits and risks", In: Begley, D.J., Bradbury, M.W. and Kreuter, J., eds, *The Blood–Brain Barrier and Drug Delivery to the CNS* (Marcel Dekker, New York), pp 145–170.

Nerurkar, M.M., Burto, P.S. and Borchardt, R.T. (1996) "The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system", *Pharm. Res.* **13**, 528–534.

Ramge, P. (1998) *Untersuchungen zur Überwindung der Blut-Hirn-Schranke mit Hilfe von Nanopartikeln* (Shaker Verlag, Aachen), pp 99–110.

Ramge, P., Kreuter, J. and Lemmer, B. (1999) "Circadian phase-dependent antinociceptive reaction in mice after i.v. injection of dalargin-loaded nanoparticles determined by the hot-plate test and the tail-flick test", *Chronobiol. Int.* **17**, 767–777.

Ramge, P., Unger, R.E., Oltrogge, B., Zenker, D., Begley, D., Kreuter, J. and von Briesen, H. (2000) "Polysorbate-80 coating enhances uptake of polybutylcyanoacrylate (PBCA)-nanoparticles by human and bovine primary brain capillary endothelial cells", *Eur. J. Neurosci.* **12**, 1931–1940.

Schröder, U. and Sabel, B.A. (1996) "Nanoparticles, a drug carrier system to pass the blood–brain barrier, permit central analgesic effects of i.v. dalargin injections", *Brain Res.* **710**, 121–124.

Schroeder, U., Sommerfeld, P., Ulrich, S. and Sabel, B.A. (1998a) "Nanoparticle technology for delivery of drugs across the blood–brain barrier", *J. Pharm. Sci.* **87**, 1305–1307.

Schroeder, U., Sommerfeld, P. and Sabel, B.A. (1998b) "Efficacy of oral dalargin-loaded nanoparticle delivery across the blood–brain barrier", *Peptides* **19**, 777–780.

Taub, M., Chuman, L., Saier, M.H. and Sato, G. (1979) "Growth of Madin–Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serumfree medium", *Proc. Natl Acad. Sci. USA* **76**, 3338–3342.

Willnow, T.E., Nykjaer, A. and Herz, J. (1999) "Lipoprotein receptors: new roles for ancient proteins", *Nat. Cell Biol.* **1**, E157–E162.

Wilson, C., Wardell, M.R., Weisgraber, K.H., Mahley, R.W. and Agard, D.A. (1991) "Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E", *Science* **252**, 1817–1822.

Woodcock, D.M., Linsenmeyer, M.E., Chrojnowski, G., Kriegler, A.B., Nink, V., Webster, L.K. and Sawyer, W.H. (1992) "Reversal of multidrug resistance by surfactants", *Br. J. Cancer.* **66**, 62–68.

Yang, C.-Y., Chen, S.-H., Gianturco, S.H., Bradley, W.A., Sparrow, J.T., DeLooft, H., Rosseneu, M., Lee, F.-S., Gu, Z.-W., Gotto, A.M. and Chan, L. (1986) "Sequence, structure receptor-binding and internal repeats of human apolipoprotein B-100", *Nature* **323**, 738–742.

Zlokovic, B.V., Martel, C.L., Matsubara, E., McComb, J.G., Zheng, G., McCluskey, R.T., Frangione, B. and Ghiso, J. (1996) "Glycoprotein 330/megalin: Probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid β at the blood–brain and blood–cerebrospinal fluid barriers", *Proc. Natl Acad. Sci. USA* **93**, 4229–4234.