Delivery of Loperamide Across the Blood-Brain Barrier with Polysorbate 80-Coated Polybutylcyanoacrylate Nanoparticles

Renad N. Alyautdin,[1] Valery E. Petrov,[1] Klaus Langer,[2] Achim Berthold,[2] Dimitry A. Kharkevich,[1] and Jörg Kreuter[2,3]

Received September 18, 1996; accepted December 24, 1996

Purpose. The possibility of using polysorbate 80-coated nanoparticles for the delivery of the water insoluble opioid agonist loperamide across the blood-brain barrier was investigated. The analgesic effect after i.v. injection of the preparations was used to indicate drug transport through this barrier.

Methods. Loperamide was incorporated into PBCA nanoparticles. Drug-containing nanoparticles were coated with polysorbate 80 and injected intravenously into mice. Analgesia was then measured by the tail-flick test.

Results. Intravenous injection of the particulate formulation resulted in a long and significant analgesic effect. A polysorbate 80 loperamide solution induced a much less pronounced and very short analgesia. Uncoated nanoparticles loaded with loperamide were unable to produce analgesia.

Conclusions. Polysorbate 80-coated PBCA nanoparticles loaded with loperamide enabled the transport of loperamide to the brain.

KEY WORDS: loperamide; nanoparticles; polysorbate 80; drug delivery; brain.

INTRODUCTION

The coating of nanoparticles with surfactants offers the possibility to alter the body distribution of this carrier system after intravenous injection. Coating with polysorbate 80 not only can lead to higher brain concentrations after intravenous injection (1) but also can increase the uptake of nanoparticles into cultivated bovine brain blood vessel endothelial cells (2). Using polybutylcyanoacrylate (PBCA) nanoparticles overcoated with polysorbate 80 it was shown to be possible to transport the water-soluble leu-enkephaline analogue and opioid agonist dalargin across the blood-brain barrier (BBB) (3,4). This was shown by the induction of analgesia after intravenous injection to mice. Uncoated nanoparticles loaded with dalargin or the simple mixture of the three components, nanoparticles, drug, and surfactant without allowing the sorptive binding of drug and surfactant yielded no effect. Preliminary data indicated that overcoating with polysorbate 80 induced the endocytotic uptake of the particles by the endothelial cells lining the blood vessels in the brain, followed by the delivery of the active agent to brain tissue.

The objective of the present study was to determine if the unprecedented result of our earlier study with dalargin was a singular event or if other drugs may also be transported across the blood-brain barrier in a similar way. For this purpose loperamide was chosen because, like dalargin, it produces central pharmacological effects but is unable to pass through the blood-brain barrier. However it differs totally from dalargin in terms of its chemical and physico-chemical character. Loperamide is a poorly water-soluble opioid agonist, which stimulates the μ-and δ-opioid receptors of the guinea pig ileum and murine vas deference in vitro (5). In vivo, neither subcutaneous nor intraperitoneal injection of loperamide in a 10% propylenglycol solution produced analgesia in the tail-flick test or Straub-effect (6). For this reason, it was assumed that, under usual conditions, loperamide is unable to penetrate the blood-brain barrier. In the present experiments, loperamide was entrapped in a polymer matrix of PBCA nanoparticles which were then coated with polysorbate 80. The ability of this system to enable passage of this drug across the blood-brain barrier and to produce an analgesic effect after intravenous injection into mice was then investigated.

MATERIALS AND METHODS

Animals

Male ICR mice 20–22 g (Central animal laboratory of Russian Medical Academy of Science, Moscow, Russia) were used for the in vivo study. Water and standard laboratory chow were freely available to the animals.

Drugs and Reagents

n-Butyl-2-cyanoacrylate (n-BCA) (Sichel-Werke, Hannover, Germany) was used as the monomer. Sodium sulfate, ethanol 96%, 0.1 N HCl, and 1.0 N NaOH were obtained from Merck (Darmstadt, Germany). Poloxamer 188 was purchased from Erbslöh (Düsseldorf, Germany). Polysorbate 80 was obtained from Atlas Chemicals (Essen, Germany). Loperamide was a gift from Janssen (Beerse, Belgium). All reagents were of analytical grade and were used without further purification.

Preparation of Nanoparticles

The preparation of polybutylcyanoacrylate (PBCA) nanoparticles was performed according to a previously published method of emulsion polymerization (7). The preparation method was modified by the use of a water/ethanol mixture as the polymerization medium (8). Both stabilizers, poloxamer 188 and sodium sulfate, were dissolved at a concentration of 1% (m/v) in a mixture of 10 ml ethanol 96% and 10 ml 0.1 N HCl. Loperamide then was added at a concentration of 0.1% (m/v). 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 1.0 N NaOH, stirring was

[1] Department of Pharmacology, Sechenov Medical Academy, B. Pirogovskaja st., 2/6, Moscow 119881, Russia.
[2] Institute of Pharmaceutical Technology, Johann Wolfgang Goethe-University, Marie-Curie-Str. 9, D-60439 Frankfurt am Main, Germany.
[3] To whom correspondence should be addressed.

ABBREVIATIONS: BBB, blood-brain barrier; CNS, central nervous system; PBCA, polybutylcyanoacrylate.

0724-8741/97/0300-0325$12.50/0

continued an additional hour in order to complete the reaction, after which the suspension was filtered through a glass filter (G3, Schott, Germany). The ethanol was then removed using a rotary evaporator (Büchi RE 111, Büchi, Flawil, CH) at 40°C under vacuum until about 10 ml aqueous suspension of the nanoparticles remained. After addition of 500 mg mannitol the aqueous suspension was lyophilised in a Lyovac GT2 freeze-dryer (Leybold Heraeus, Hürth, Germany) for 24 h under vacuum ($2 \cdot 10^{-3}$ bar).

For the production of the nanoparticle preparations for the group 1–3 studies, 10, 15, and 20 mg quantities of the freeze-dried product, containing about 1.8% (m/m) loperamide, were homogeneously dispersed in 1.0 ml of phosphate buffered saline (PBS) by ultrasonication for 5 min. Polysorbate 80 was added at a concentration of 1% (m/v) followed by an incubation period of 30 min.

Determination of Loperamide Loading

Before freeze-drying of the nanoparticle suspension an aliquot was transferred to a Beckman Airfuge® ultracentrifuge (Beckman Instruments, Palo Alto, USA) and centrifuged at a mean relative centrifugal field (RCF) of 90000 g for 1 h. An aliquot of the supernatant was diluted with 0.1 N HCl and analyzed for unbound drug by means of UV spectrophotometry (Hitachi U-3000, Berkshire, UK) at a wavelength of 259.0 nm against a reference solution which had been prepared and treated the same way.

Particle Size Measurement

After preparation of the nanoparticles, the particle size was measured by photon correlation spectroscopy (PCS) (9,10). The measurements were performed using a BI-200SM goniometer (Brookhaven Instruments Corp., Holtsville, NY, USA) equipped with a 30 mW helium-neon-laser and connected to a BI-2030AT correlator. Prior to the measurement, the suspensions were diluted with filtered water (Millex-GS 0.22 μm filter unit, Millipore, Molsheim, France). All measurements were taken at a scattering angle of 90°. Particle sizes were calculated as effective diameters (11). The width of the size distributions were characterized by the polydispersity index.

Animal Testing

The mice were divided into 6 groups with 6 animals per group (Table I). Groups 1–3 were treated with different doses of drug containing polysorbate 80-coated nanoparticles (1.8, 2.7, 3.6 $mg \cdot kg^{-1}$ loperamide). Group 4 was treated with loperamide containing nanoparticles at a loperamide concentration of 3.6 $mg \cdot kg^{-1}$, without the addition of polysorbate 80. Group 5 was treated with loperamide (3.6 $mg \cdot kg^{-1}$) in an aqueous solution of 1% polysorbate 80. Group 6 was a simple mixture of empty nanoparticles with 1% polysorbate 80 and loperamide without any further treatment. All the preparations used for the animal study were prepared with phosphate buffered saline. Each mouse was injected with 0.2 ml of one of the above preparations via the tail vein.

Nociceptive Testing

Nociceptive threshold was measured using the tail-flick test (Mod. 33 Tail-Flick Analgesia Meter, Iitic Inc., Woodland Hills, USA). The tail-flick test was evoked by placing the mouse's tail over a slit onto which a quartz projection bulb was focused, and the time for tail withdrawal was recorded. To prevent tail tissue damage, the experiments were truncated after 10s if no response was evoked (cut off time). This time point was considered to indicate complete analgesia. For each animal, the tail-flick latency was determined before dosing of any preparation (= pre drug latency). Tail-flick latencies were measured at 15, 45, 60, and 90 min after dosing. An additional test after 5 min was performed with groups 3, 5, and 6. In group 3, the tail-flick test was performed over a period of 210 min when analgesic effect ceased.

Table I. Experimental Groups

Groups #	Preparation	Loperamide concentration [$mg \cdot kg^{-1}$]	Polysorbate 80 [%]	Incubation [min]
1	NP overcoated with P80	1.8	1.0	30
2	NP overcoated with P80	2.7	1.0	30
3	NP overcoated with P80	3.6	1.0	30
4	uncoated NP	3.6	—	—
5	solution	3.6	1.0	30
6	simple mixture	3.6	1.0	—[a]

Note: NP - PBCA nanoparticles; P80 - polysorbate 80. Simple mixture—mixture of three components: nanoparticles, drugs, polysorbate 80.

[a] Empty nanoparticles were added without further incubation.

Statistics

Six animals per group were used in all groups. The tail-flick response latencies were recorded and converted to percent maximal possible effect (MPE) using equation 1 and are presented as mean ± standard deviation (sd).

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

The statistical comparison between pre- and post-drug treatment was carried out with a paired Student's t-test.

RESULTS AND DISCUSSION

Polybutylcyanoacrylate (PBCA) nanoparticles were prepared by emulsion polymerisation in the presence of the opioid agonist loperamide. After the preparation step, the nanoparticles showed an effective diameter of 290 nm and a narrow size distribution (polydispersity 0.08). About 47% of the loperamide in the preparation was bound to the nanoparticles. The rest was present in free form and was not removed for the animal experiments. A separation of the bound from the free loperamide was not performed because a new equilibrium between both forms of the drug would have been formed over a time frame of several hours. The concentrations listed reflect the total amount of loperamide in the preparations.

The results of the nociceptive testing showed that loperamide-containing polysorbate 80-coated nanoparticles evoked

Table II. Mean Percentage of MPE and Standard Deviation (sd) After Intravenous Injection into Mice (n = 6)

Group #	%MPE (mean ± sd)			
	15 min	45 min	60 min	90 min
1	30.5 ± 25.8[a]	56.1 ± 21.8[b]	56.4 ± 20.3[b]	19.8 ± 25.5
2	27.6 ± 23.1[a]	84.7 ± 24.5[b]	74.2 ± 37.4[b]	56.5 ± 43.4[b]
3	100 ± 0.0[b]	98.5 ± 3.8[b]	87.8 ± 18.8[b]	73.0 ± 45.1[b]
4	0.2 ± 11.8[b]	6.1 ± 11.9	−4.7 ± 10.1	−0.2 ± 9.0
5	58.5 ± 25.7	13.7 ± 25.9	2.0 ± 6.7	0.1 ± 8.9
6	46.1 ± 34.2	6.2 ± 26.4	−7.0 ± 17.9	−3.9 ± 4.4

Note: Statistically significant difference from the corresponding reference (loperamide solution #5): [a]P = 0.05; [b]P = 0.01.

a dose-dependent analgesic effect in mice after intravenous injection (Table II). Peak effects in groups 1 and 2 (MPE 56% and 85%) were obtained 45 min after administration. At the highest concentration of the carrier system (group 3), complete analgesia was achieved within 15 min of dosing. Even at 5 min after dosing, a high level of analgesia was achieved in group 3 (MPE 99.7%) and a typical Straub-effect was observed. In this group, analgesia measurements were continued up to 210 min, at which time no further analgesia was detectable.

Groups 4 to 6 (Table II) served as controls. Due to the low solubility of loperamide, it was not possible to produce a simple aqueous solution of this drug in water, saline, or phosphate buffered saline alone. Polysorbate 80 in a concentration of 1% enabled the preparation of a micellar solution of this drug (group 5). This preparation yielded a maximal analgesic effect 5 min after injection (MPE 68.0%) followed by a rapid decrease. No effect was observed at 45 min or later. Uncoated nanoparticles containing loperamide (group 4) failed to produce any significant analgesic effect. A simple mixture of loperamide with empty PBCA-nanoparticles and polysorbate 80 (group 6) injected immediately after mixing produced an effect that was not statistically different from the polysorbate-containing loperamide solution. These results are in excellent agreement with earlier results obtained with dalargin (3,4). In those experiments, the nanoparticles were not able to induce any analgesic effect without polysorbate 80-overcoating or if a simple mixture of nanoparticles, drug, and polysorbate was injected. Only nanoparticles with bound dalargin and additional polysorbate 80-coating yielded a statistically significant and pharmacologically useful effect. On the other hand, and in contrast to dalargin, the present experiments with loperamide indicated that it was possible to achieve a slight, rapid, and rapidly disappearing analgesia after solubilisation of the drug in a 1% polysorbate micellar solution, even though loperamide is considered to be unable to cross the blood-brain barrier. For this reason, it must be concluded that the inability of loperamide to cross the BBB under usual dosing conditions is in part a solubility problem. It has been already shown that solubilisation of methotrexate by polysorbate leads to an increased brain uptake (12,13). It may also be argued that polysorbate 80 enhances the uptake of drugs by interaction with and fluidization of endothelial cell membranes. However, in the methotrexate study, no such effects were found (13). In addition, our own work (in progress) with other surfactants has demonstrated that these surfactants did not facilitate an analgesic effect. A simple surfactant-related membrane fluidization, therefore, is unlikely to be the cause of the observed analgesia obtained with loperamide.

The results of our study also show that the administration of nanoparticles containing loperamide and overcoated with polysorbate 80 led to a much higher and considerably longer analgesic effect at the same drug concentration (Fig. 1) than was achieved in the control experiments. The mechanism of transport across the BBB of the nanoparticle formulation of loperamide is unlikely to consist of a simple diffusion process. This is supported by the observation that in the earlier experiments with the relatively hydrophilic dalargin, no analgesic effects were observed when it was administered either in an aqueous or in a polysorbate 80 solution. After binding to nanoparticles and coating with polysorbate 80, the time frame and the development of analgesia was similar for both the hydrophilic peptide dalargin and the lipophilic opiate loperamide. Consequently, it is proposed that both are transported by a similar uptake mechanism. As mentioned in the introduction, earlier (4) as well as unpublished recent experiments indicate that uptake of nanoparticles by endocytosis by the endothelial cells lining the brain blood vessels can occur after they are coated with polysorbate 80 and it appears that endocytosis is the relevant transport mechanism in this case. On the other hand, analgesia was observed within 5 min of administration of loperamide in the nanoparticle preparation. At this time point, there is no significant difference from the effect seen after administra-

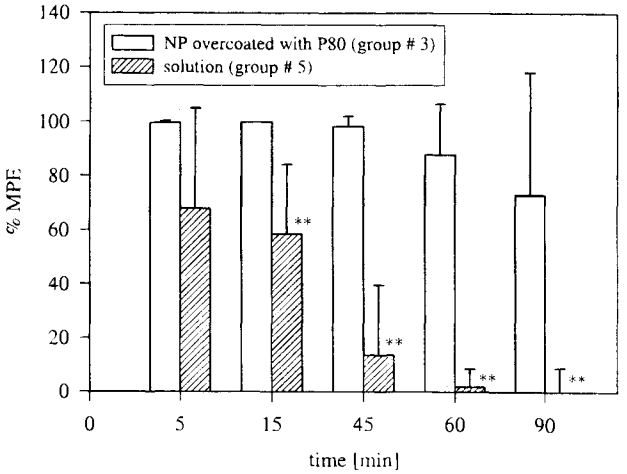

Fig. 1. Analgesic effect of polysorbate 80-coated and loperamide (3.6 mg/kg)-loaded PBCA nanoparticles (L-NP) and of a micellar solution of loperamide (3.6 mg/kg) in 1% solution of polysorbate 80 (L-solution). Statistically significant difference from the corresponding reference (loperamide solution #5): *P = 0.05, **P = 0.01.

tion of loperamide in solution form. Only at longer times was the analgesic effect significantly greater with the nanoparticle preparation. The short term effect is probably attributable to the significant fraction of unbound loperamide solubilized in polysorbate rather than endocytic uptake of nanoparticles.

The proposed uptake mechanism, endocytosis by endothelial cells, had already been observed for the uptake of poloxamer 407-coated polystyrene nanoparticles into sinusoidal endothelial cells of the bone marrow in rabbits (14). In those studies, no uptake was observed by other endothelial cells or by sinusoidal macrophages of the bone marrow. This high selectivity suggests that the recognition and uptake by the bone marrow sinusoidal endothelial cells, and also in the case of the brain blood vessels endothelial cells, may be mediated via a plasma component (such as erythropoetin, transferrin, transcobalamin, etc.). Another possibility would be mediation by an endothelial derived factor which specifically adsorbs onto the surface of poloxamer 407- or polysorbate 80-coated nanoparticles and which exhibits its specificity for certain microdomains on the respective endothelial cell surface. These possibilities were already proposed by Porter et al. (14) and Moghimi and Patel (15). Blunk et al. (16) had previously demonstrated in vitro by 2-D gel electrophoresis that the adsorption pattern of plasma components differs significantly with the surfactant that is used to coat the nanoparticles. Poloxamer 407, which was shown previously to induce uptake of nanoparticles by the bone marrow sinusoidal endothelial cells, has also been shown to enhance the uptake of nanoparticles into bovine blood vessel endothelial in vitro. However, this effect was only significant at prolonged times, i.e., 6 hours (2). However, in contrast to the results with polysorbate 80, no analgesic activity was observed in vivo when poloxamer 407 was coated onto dalargin-loaded PBCA nanoparticles (recent unpublished results). The lack of in vivo effect with poloxamer 407 may in part be due to the long time required for this surfactant to induce endothelial cell uptake. On the other hand, it may also indicate that the formation of a plasma component coat is highly surfactant specific as discussed above.

In conclusion, the present results show that loperamide is transported across the BBB after binding to nanoparticles overcoated with polysorbate 80. These particles may also enable the transport of other drugs in a similar manner. Therefore, they hold promise as a brain and CNS delivery system for a variety of essential drugs, including peptides, that normally are unable to cross the BBB.

ACKNOWLEDGMENTS

This work was supported in part by the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany and The International Association of the Promotion of Cooperation with Scientists from the New Independent States of the Former Soviet Union (INTAS). We also would like to thank the Janssen Research Foundation (Beerse, Belgium) for the donation of loperamide.

REFERENCES

1. S. D. Tröster, U. Müller, and J. Kreuter. *Int. J. Pharm.* **61**:85–100 (1990).
2. G. Borchard, K. L. Audus, F. Shi and J. Kreuter. *Int. J. Pharm.* **110**:29–35 (1994).
3. R. Alyautdin, D. Gothier, V. Petrov, D. Kharkevich, and J. Kreuter. *Eur. J. Pharm. Biopharm.* **41**:44–48 (1995).
4. J. Kreuter, R. Alyautdin, D. Kharkevich, and A. Ivanov. *Brain Res.* **674**:171–174 (1995).
5. G. Giagnoni, L. Casiraghi, L. Senini, and D. Revel. *Life Sci.* **33**:315–318 (1983).
6. C. J. E. Niemegeers, F. M. Lenaerts, and P. A. Janssen. *Arzneim. Forsch. (Drug Res.)* **24**:1633–1653 (1974).
7. J. Kreuter. *Pharm. Acta Helv.* **58**:196–209 (1983).
8. P. H. Beck, J. Kreuter, E. G. Müller, and W. Schatton. *Eur. J. Pharm. Biopharm.* **40**:134–137 (1994).
9. J. Kreuter. *Int. J. Pharm.* **14**:43–58 (1983).
10. R. H. Müller, C. Lherm, J. Herbort, T. Blunk, and P. Couvreur. *Int. J. Pharm.* **84**:1–11 (1992).
11. N. De Jaeger, H. Demeyere, R. Finsy, R. Sneyers, J. Vanderdeelen, P. van der Meeren, and M. van Laethem. *Part. Part. Syst. Charact.* **8**:179–186 (1991).
12. M. N. Azmin, J. F. Stuart, K. C. Calman, and A. T. Florence. *Cancer Chemother. Pharmacol.* **9**:161–164 (1982).
13. M. N. Azmin, J. F. Stuart, and A. T. Florence. *Cancer Chemother. Pharmacol.* **14**:238–242 (1985).
14. C. J. H. Porter, S. M. Moghimi, L. Illum, and S. S. Davis. *FEBS Letters.* **305**:62–66 (1992).
15. S. M. Moghimi, and H. M. Patel. *Biochim. Biophys. Acta.* **948**:379–383 (1989).
16. T. Blunk, D. F. Hochstrasser, J.-C. Sanchez, B. W. Müller, and R. H. Müller. *Electrophoresis.* **14**:1382–1387 (1993).