International Congress Series 1277 (2005) 85–94

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Application of nanoparticles for the delivery of drugs to the brain

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Abstract. The blood–brain barrier (BBB) represents an insurmountable obstacle for the delivery of a large number of drugs to the central nervous system (CNS). One of the possibilities to overcome this barrier is drug delivery to the brain using nanoparticles. Drugs that have been transported into the brain and led to significant pharmacological effects after intravenous injection using this carrier include the hexapeptide dalargin, the dipeptide kytorphin, loperamide, tubocurarine, doxorubicin, and the NMDA receptor antagonists MRZ 2/576 and MRZ 2/596. In order to achieve a significant transport across the blood–brain barrier the coating of the nanoparticles with polysorbate 80 (Tween® 80) or other polysorbates with 20 polyoxyethylene units was required. Other surfactants were less successful. The most promising results were obtained with doxorubicin for the treatment of brain tumours. Intravenous injection of polysorbate 80-coated nanoparticles loaded with doxorubicin (5 mg/kg) achieved very high brain levels of 6 μg/g brain tissue while all the controls, including uncoated nanoparticles and doxorubicin solutions mixed with polysorbate, did not reach the analytical detection limit of 0.1 μg/g. Moreover, experiments with the extremely aggressive glioblastoma 101/8 transplanted intracranially showed a long term survival for 6 months of up to 40% of the rats after intravenous injection of the polysorbate 80-coated nanoparticle preparation (3×1.5 mg/kg). The surviving animals were sacrificed after this time and showed total remission by histological investigation. Untreated controls died within 10–20 days, the animals in the doxorubicin control and uncoated doxorubicin nanoparticle groups died between 10–50 days. The mechanism of the drug transport across the blood–brain barrier with the nanoparticles appears to be endocytotic uptake by the brain capillary endothelial cells followed either by release of the drugs in these cells and diffusion into the brain or by transcytosis. After injection of the nanoparticles, apolipoprotein E (apo E) or apo B adsorb on the particle surface and then seem to promote the interaction with the LDL receptor followed by endocytotic uptake. The nanoparticles thus would mimic the uptake of naturally occurring lipoprotein particles. This hypothesis was supported by the achievement of an antinociceptive effect using dalargin-loaded poly(butyl cyanoacrylate) nanoparticles with adsorbed

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0531-5131/
doi:10.1016/j.ics.2005.02.014

apo E or loperamide-loaded albumin nanoparticles with covalently bound apo E. © 2005 Published by Elsevier B.V.

Keywords: Blood–brain barrier; BBB; Nanoparticles; Drug delivery; Apolipoprotein E

1. Introduction

The blood–brain barrier (BBB) represents an insurmountable barrier for the majority of drugs including anticancer agents, antibiotics, peptides and other oligo- and macromolecular drugs. The limited access of these drugs to the brain is due to the tight junctions between the endothelial cells lining the brain blood vessels as well as to the existence of very active drug transport systems, e.g. the so-called ABC-transporters (ATP-binding cassette transporters, such as Pgp) in the luminal cell membrane of these cells. The tight junctions prevent all paracellular drug transport whereas the ABC-transporters immediately transport drugs that had partitioned into the endothelial cell membranes back into the blood stream [1]. A number of strategies have been developed to overcome this problem including the employment of prodrugs, opening of the tight junctions using hyperosmolaric solutions, or drug carrier systems such as antibodies, liposomes, or nanoparticles [2–4].

Nanoparticles for pharmaceutical and chemical use are defined as polymeric particles made of natural or artificial polymers ranging in size between about 10 and 1000 nm (1 μm). Drugs may be bound in form of a solid solution or dispersion or be adsorbed to the surface or may be chemically attached [5]. The polymer that was used for the majority of nanoparticles employed for the transport of drugs across the BBB is poly(butyl cyanoacrylate) (Fig. 1). This polymer is about the most rapidly biodegrading artificial polymers [6,7]. As a consequence of this rapid degradation as well as due to the low molecular weights of the polymer in the nanoparticles [8,9] the polymer material is rapidly eliminated from the body, i.e. about 80% already after 24 h [10].

2. Nanoparticle preparation

The nanoparticles used for brain delivery were manufactured using butyl cyanoacrylate monomer. This monomer was added to an HCl solution with a pH below 3 in order to avoid too rapid polymerisation. Dextran 70,000, 1%, was added as a stabilizer. The drugs were added during or after polymerisation. The polymerisation was terminated after 2.5–4 h by neutralisation with NaOH. The material then was used after preparation or was lyophilised

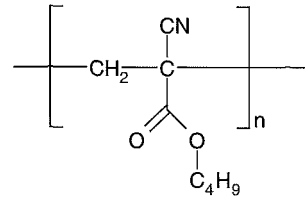

Fig. 1. Structure of poly(butyl cyanoacrylate).

Table 1
Antinociceptive effects induced in male ICR mice (20–22 g) in the tail-flick test 45 min after intravenous injection of dalargin or of excipients in free form or in combination with nanoparticles to mice (n=5) determined by percentage (mean±standard deviation) of the maximally possible effect (MPE)

Group		%MPE
1	Suspension of empty nanoparticles (200 mg/kg)	0.75±3.0
2	Polysorbate 80 solution (1%, 200 mg/kg)	12.0±3.1
3	Dalargin (solution 10 mg/ml, 10 mg/kg)	9.3±8.7
4	Dalargin (10 mg/kg) + polysorbate 80 (1%, 200 mg/kg)	7.8±2.3
5	Dalargin (10 mg/kg) + empty nanoparticles (200 mg/kg)	1.5±5.4
6	Dalargin (10 mg/kg) + empty nanoparticles (200 mg/kg) + polysorbate 80 (200 mg/kg)	12.5±2.0
7	Dalargin-loaded nanoparticles (10 mg/kg)	3.7±1.1
8	Polysorbate 80-coated and dalargin-loaded nanoparticles (2.5 mg/kg)	11.6±9.7
9	Polysorbate 80-coated and dalargin-loaded nanoparticles (5 mg/kg)	36.8±21.5*
10	Polysorbate 80-coated and dalargin-loaded nanoparticles (7.5 mg/kg)	51.8±20.2*

(Adapted from Kreuter et al. [2]).
* (p<0.05).

using 3% mannitol as a cryoprotector. In the case of doxorubicin as the drug these lyophilised products were stable at least for 2 years. The reconstituted or freshly prepared nanoparticles were then injected after addition of and incubation in 1% polysorbate 80 (Tween® 80) or other surfactants.

3. Drug transport across the blood–brain barrier using nanoparticles

The first drugs that was transported across the BBB using nanoparticles was dalargin. Dalargin is a hexapeptide Leu-enkephalin analogue with the sequence Tyr-D-Ala-Gly-Phe-Leu-Arg [2,11]. It possesses opioid activity after direct intraventricular injection into the brain, but since it cannot cross the BBB it does not induce a central antinociceptive (analgesic) effect after intravenous injection. After adsorption of the dalargin to the nanoparticles and overcoating with polysorbate 80 intravenous injection led to a dose and time dependent antinociceptive effect in the tail-flick as well as in the hot plate tests [2,11–14], whereas all controls including a solution of dalargin, a solution of polysorbate 80, a suspension of poly (butyl cyanoacrylate) nanoparticles, a mixture of dalargin with polysorbate 80, dalargin with nanoparticles or a mixture of all three components, dalargin, polysorbate 80, and nanoparticles, mixed immediately before injection, as well as dalargin bound to nanoparticles without polysorbate 80 overcoating exhibited no effect (Table 1).

Table 2
Drugs delivered across the blood–brain barrier with nanoparticles

Kytorphin (dipeptide)
Loperamide
Tubocurarine
MRZ 2/576 and MRZ 2/596 (NMDA-receptor antagonists)
Doxorubicin

Other drugs that have been transported across the BBB using the polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles are listed in Table 2 [2,3].

4. Chemotherapy with polysorbate 80-coated nanoparticles

Brain tumours, especially glioblastomas, belong to the most malignant and aggressive tumours and typically lead to the death of the patients about half a year after diagnosis. Due to their rapid proliferation, diffuse growth, and invasion into distant brain areas in addition to extensive cerebral edema and high levels of angiogenesis, surgical operative techniques, radiotherapy, and chemotherapy so far largely remained ineffective [15]. Most anticancer agents cannot cross the BBB and due to the very short diffusional range for drugs in the brain, distant brain areas cannot be reached after local injection or implantation. Doxorubicin is one of the most important anticancer drugs, but it also normally cannot cross the BBB and, therefore, is not used against brain tumours. After binding to poly(butyl cyanoacrylate) nanoparticles and coating with polysorbate 80 very considerable doxorubicin concentrations (6 μg/g) were detected in the brain after intravenous injection at a level of 5 mg/kg doxorubicin to rats, whereas with all three control preparations, i.e. doxorubicin solution in saline, doxorubicin solution in saline plus polysorbate 80, doxorubicin bound to nanoparticles without polysorbate-coating, the concentrations were below the detection limit of 0.1 μg/g [16]. Interestingly, the differences in plasma concentrations between the four preparations were not large, although sometimes statistically significant. Moreover, as already observed by Couvreur et al. [17], both nanoparticle preparations extremely decreased the heart concentrations of doxorubicin and yielded levels below the detection limit after 2 h. This finding is very important because the use of doxorubicin is limited by its high heart toxicity.

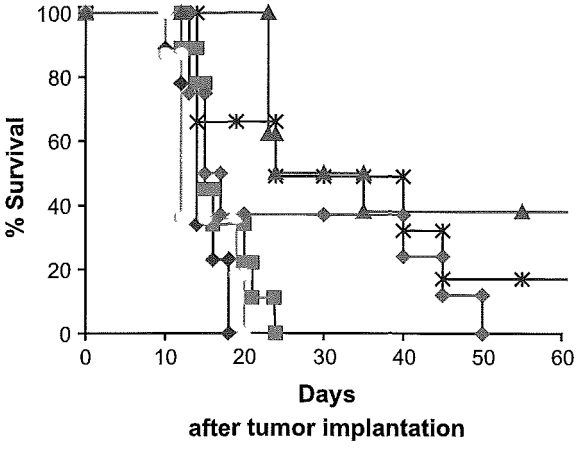

Fig. 2. Survival of rats with an intracranially transplanted glioblastoma 101/8 after intravenous injection of doxorubicin (1.5 mg/kg on days 2, 5, and 8 after tumour transplantation using the following preparations: ♦ (left line) untreated controls, ● empty nanoparticles coated with polysorbate 80, ■ doxorubicin in saline, ♦ (right line) doxorubicin-loaded nanoparticles, * doxorubicin in polysorbate 80, ▲ doxorubicin-loaded nanoparticles coated with polysorbate 80.

In rats with intracranially transplanted glioblastomas 101/8 (Fig. 2) that typically kill the rats within 10–20 days, intravenous injection of these doxorubicin-loaded polysorbate 80-coated nanoparticles led to 20–50% cure in different experimental runs. The dose schedule in these experiments was rather conservative, i.e. 3×1.5 mg/kg. Cure was proven by histology following sacrifice of these animals after 6 months. In the 7 control groups only one other rat survived (doxorubicin in saline plus polysorbate 80) [15] (Fig. 2).

5. Toxicity

The acute toxicity after intravenous injection in normal and tumour-bearing rats of uncoated and polysorbate 80-coated doxorubicin-loaded nanoparticles was similar to that of the doxorubicin solution in saline [18]. The weight loss, another indication of toxicity, even was lower with both nanoparticle preparations and also after addition of the polysorbate to the doxorubicin solution. In addition, also the gastrointestinal and lung toxicity of doxorubicin appeared to be reduced in preliminary experiments by the nanoparticles, and no indication of neurotoxicity was visible after 12 days and half a year [15].

6. Mechanism of nanoparticle-mediated drug transport to the brain

A number of possibilities exist that could explain the mechanism of the delivery of the above mentioned substances across the blood–brain barrier [3,4]:

1. An increased retention of the nanoparticles in the brain blood capillaries 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.
2. A general surfactant effect characterized by a solubilisation of the endothelial cell membrane lipids that would lead to membrane fluidisation and an enhanced drug permeability through the blood–brain barrier.
3. 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.
4. The nanoparticles may be endocytosed by the endothelial cells followed by the release of the drugs within these cells and delivery to the brain.
5. The nanoparticles with bound drugs could be transcytosed through the endothelial cell layer.
6. 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 it was shown that also nanoparticles overcoated with polysorbate 20, 40, 60, or 80 adsorb apolipoprotein E, whereas a number of other surfactants do not adsorb this lipoprotein [19]. It was also shown that only these four polysorbates but not the other surfactants induced an antinociceptive effect with dalargin in the tail-flick model in mice [20]. In addition, coating of the dalargin-loaded nanoparticles with

apolipoprotein E or B without polysorbate 80 also induced the antinociceptive effect although this effect was lower than with polysorbate 80 alone [21]. However, the antinociceptive effect was even higher when the nanoparticles were first coated with polysorbate 80 and then overcoated with apolipoprotein E or B. From these results it was concluded that due to the polysorbate coating the nanoparticles adsorb apolipoprotein E and/or B from the blood steam after intravenous injection [15,21]. They thus would mimic natural lipoprotein particles which could interact with the LDL receptor family located in the brain capillary endothelial cells followed by endocytotic uptake. Consequently, in apolipoprotein E-deficient knock-out mice the antinociceptive effects with dalargin-loaded polysorbate 80-coated nanoparticles were reduced by about 50%. In this scenario the polysorbate-coated nanoparticles would act as Trojan horses that would transport the drugs into the endothelial cells. Extensive endocytotic brain capillary endothelial cell uptake was already shown in in vitro cell cultures of human, bovine, porcine, rat and mouse origin [22]. After the endocytosis two mechanisms are possible: one is transcytosis into the brain, a mechanism suggested for LDL transport by Cecchelli et al. [23]. The other mechanism is the simple release of the drug within the endothelial cells and diffusion into the brain. Since the ABC-transporters are mainly located in the luminal membrane this diffusion into the brain would not be obstructed.

7. Conclusions

- Polysorbate 80-coated nanoparticles (DOX-NP+PS) represent a very promising preparation for the delivery of drugs across the blood–brain barrier.
- A high incidence of tumour cure was observed in the extremely aggressive glioblastoma 101/8 with polysorbate 80-coated doxorubicin nanoparticles.
- Histologically there were no indications of neurotoxicity.

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Discussion

Whittle

What was the sensitivity in vitro to doxorubicin of this glioma cell line?

Kreuter

We did have difficulties in cultivating this cell line in vitro. The tumour is kept deep-frozen and then has to be transfected from animal to animal. It is not preserved in vitro cell lines.

Lage

Is there any doxorubicin accumulation in other organs, for example the liver?

Kreuter

There are always very high levels of doxorubicin in the liver. With the nanoparticles, the increase is about 20–25%, but the pathology does not seem to be severe. Actually, it's not worse than with free doxorubicin. What we also saw is that the intestinal toxicology, the intestinal bleeding, is reduced. Actually, we always find that in other organs, even in the liver and in the spleen, the toxicology is reduced by binding to nanoparticles.

Galla

Do you have any idea why the Apo B or the Apo E is binding to the surface of the nanoparticles? Was that just lucky? Would it have worked if there had been absolutely no albumin?

Kreuter

Well, I think we were lucky to find that these apolipoproteins bind to the nanoparticle surfaces after coating with polysorbate 80, because, initially, if I had known all the data better, I would have suggested coating with poloxamine-908, and it would not have worked. I think it was pure coincidence as it very often happens. I remember myself telling my boss, 25 years ago, when he suggested using nanoparticles for delivery drugs across the blood–brain barrier, that this was the stupidest idea I've ever heard.

Galla

But have you checked that, because if the Apo proteins are on the surface you could easily detect that.

Kreuter

To detect this, a couple of years after our initial experiments, a method to wash down proteins from the nanoparticle surface was used in collaboration with Prof. Rainer Müller's laboratory in Berlin. After washing the proteins were subjected to 2D-PAGE and were identified and semi-quantified by this method.

Van Tellingen

Coming back to the tumour model that you used, you said that you were able to identify whether that is truly glioma or not. Of course, that is very crucial to these experiments. Do you have any other indications that there is at least a blood–brain barrier present in those tumours? Most of the experimental glioma models grow in a very expansive fashion, which makes them relatively easy to hit and that's probably the reason why all our previous studies with drugs in mice work so well and why the reaction is so poor in humans.

Kreuter

Well, we know that with Evan's blue the blood–brain barrier opens after a couple of days, exactly after 6 days. This tumour model works very precisely. In the controls there was no effect. That shows that nanoparticles can add on to what the normal drugs can do.

Van Tellingen

What do you mean by the controls?

Kreuter

The controls were doxorubicin solution, doxorubicin solution in polysorbate 80, and nanoparticles without polysorbate 80.

Van Tellingen

Although there are, of course, many instances where this has also been noticed for extra-cranial tumours where there is increased accumulation. Those tumours are obviously not protected by any blood–brain barrier. In that case, also the nanoparticles or the lyposomes or any other formulation seem to work better in mice at least, not in humans but in mice, than does the free drug formulation.

Kreuter

Yes, definitely, there are some effects in these peripheral tumours—called the EPR effect. This may contribute here too, but, in our case, we would have expected that the

uncoated nanoparticles loaded with doxorubicin also would be of some effect. Uncoated nanoparticles show this EPR effect in peripheral tumours, but not in our case.

Van Tellingen

Were you able to grow these tumours on other sites besides the brain?

Kreuter

We never did that.

Gaillard

I think you have shown fairly effectively that the formulations with nanoparticles do work. Now I'm wondering what happened to the NMDA receptor antagonists. Are they still alive or in development?

Kreuter

No. They are not alive, unfortunately, because the drugs themselves showed some toxicity, their development was discontinued.

Gaillard

To continue on that, do you have any suggestions about further development of drugs that can be used in the clinic? Do you think it will be feasible?

Kreuter

I think that there are a number of drugs that could benefit from our technology, but there are a number of drugs that do not bind to these particles. Not every drug binds to every type of nanoparticles.

McQuaid

Have you any evidence for invasiveness of the tumour in other areas of the brain? And was the drug delivery able to cope with that, because that would be the key thing in human high grade gliomas?

Kreuter

In contrast to other tumours we also have looked at, such as RG-2 and 9L, which, in our hands, grow very nodular, the gliobalsoma 101/8 grows rather squamous. Dr. Geiger, who is supposed to be one of the best brain pathologists in Germany, said that this is a very good model because it grows very similarly to what she had observed in human tumours, and that it shows the invasive growth and all these things that are very typical of human glioblastomas. Our thinking and hope is that since we can also go across the intact blood–brain barrier, we can treat or kill these cells growing behind it.

Fricker

I have a question concerning the toxicity or potential toxicity of the polymerised cyanoacrylate particles. When you say that these carrier systems can be applied for a variety of drugs, I understand it's for tumour therapy, but how does it look with chronic administration? What actually happens to the cyanoacrylate? Acrylic acid is highly neurotoxic, and what happens to the polymer in the brain?

Kreuter

This polymer is eliminated rather rapidly from the brain. I don't think you can use it for drugs that have to be permanently administered. If you have to administer every day, I don't think that you can use these particular systems, but this is always the problem with any carrier because you need an excess of carrier. Concerning the degradation, we have shown with mass spectrometry as well as with gel permeation chromatography that the molecular weight is around 3000, and what happens is that with the esterases in the body

the ester is cleaved away and then you have the polymer acid, which is water-soluble and has a molecular weight that can be calculated to be around 2000.

De Boer

Can you say something about the distribution of those nanoparticles in the body?

Kreuter

Well, they go in a high percentage into the liver and the spleen because they are taken up by macrophages. A long time ago it was shown that they go into the lysosomes of such cells. The amount in the brain is not that high. It seems to be enough to get the drugs there. The heart is avoided, which is, of course, good for drugs like doxorubicin or epirubicin.