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Targeted drug delivery to C6 glioma by transferrin-coupled liposomes

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Abstract: Recent advances in liposome technology have shown promise relative to the introduction of chemotherapeutic agents with reduced toxicity, extended longevity, and potential for cell-specific targeting. In this study we report the engineering of a liposomal delivery system for the chemotherapeutic drug doxorubicin. The system was targeted specifically to C6 glioma *in vitro* by coupling transferrin to the distal ends of liposomal polyethylene glycol (PEG) chains. The transferrin receptor is overexpressed on glioma, with the extent of overexpression correlated to the severity of the tumor. Significantly increased gliomal doxorubicin uptake was achieved by drug encapsulation within transferrin-coupled liposomes compared to other liposome populations. Doxorubicin encapsulated within transferrin-coupled liposomes exhibited 70% of free doxorubicin uptake as compared to 54, 14, and 34% for non-PEG, PEG, and albumin-coupled PEG liposomes, respectively. Competitive binding assays support the receptor-mediated mechanism of targeting. The addition of one μ*M* free transferrin reduced the uptake of doxorubicin encapsulated within transferrin-coupled liposomes by 30%. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 51, 10–14, 2000.

Key words: PEG liposome; targeted drug delivery; C6 glioma; transferrin receptor; doxorubicin

INTRODUCTION

Liposomes are becoming increasingly attractive as drug delivery vehicles for improving current methods of cancer chemotherapy. Compared to unassisted methods of drug delivery, drugs encapsulated within liposomes show reduced toxicity, increased circulation time, and the potential for specific receptor-mediated targeting of a tumor.[1–3] Previous difficulties with rapid liposomal clearance and uptake by cells of the mononuclear phagocytic system (MPS) have been overcome largely by the incorporation of a polyethylene glycol (PEG) derivatized polymer coating into the liposome membrane.[4–6] The presence of PEG surface chains has been shown to reduce the binding of plasma proteins and thus largely to prevent recognition and uptake by the MPS. Targeting tumor antigens through ligands bound to the liposome surface is sterically at odds with the addition of PEG; however coupling ligands to the distal ends of PEG chains enables both efficient targeting and prolonged circulation times.[7,8]

Until completely tumor-specific antigens can be found and isolated, the relative overexpression of certain surface receptors offers the potential for favorable targeting of a tumor over its surrounding normal tissue.[9] Cellular transferrin receptor (TfR) currently shows promise as a site for receptor-mediated targeting of glioma. The density of cellular TfR is correlated with the extent of cell growth and division. Neoplastic cells ordinarily divide faster than normal cells and

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consequently express more TfR than their surroundings. This discrepancy is even more appreciable in the stable environment of the brain. The extent and diffuseness of TfR expression in glioma has been shown to be significantly greater than in normal brain tissue, with expression linked to the severity of tumor.[10]

In this study we report the design of a PEG liposome delivery vehicle for the chemotherapeutic agent doxorubicin. We demonstrate that coupling transferrin distally to the liposomal PEG chains enables preferential drug uptake by C6 glioma *in vitro.* A mechanism of TfR-mediated targeting is established, and the potential benefits of this delivery system for chemotherapy of glioma *in vivo* are discussed.

MATERIALS AND METHODS

Materials

Cholesterol (CHOL), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylethanolamine (DSPE), and PEG2000-DSPE were obtained from Avanti Polar Lipids (Birmingham, Alabama). Doxorubicin, transferrin (Tf), albumin (Alb), HOOC-PEG-COOH (MW = 3350), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dicyclohexylcarbodiimide (DCDI), Kieselgel 60, and Sepharose CL-4B were obtained from Sigma (St. Louis, Missouri). N-hydroxysulfosuccinimide (S-NHS) was obtained from Pierce. β-BODIPY® 500/510 C_{12}-HPC, a fluorescently tagged phosphatidylcholine, was obtained from Molecular Probes (Eugene, Oregon).

Cell culture

The rat C6 glioma cell line was purchased from American Type Culture Collection (Rockville, Maryland). The cells were maintained in Dulbecco's modified Eagle medium (Life Technologies, Rockville, Maryland) containing 10% fetal calf serum and 1% penicillin/streptomycin. C6 glioma cells were seeded in 33-mm culture dishes at 5×10^5 cells/well 40 h prior to incubation with free and liposomal doxorubicin. Cell incubation was performed at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of liposomes

DSPE-PEG-COOH was synthesized to facilitate protein–liposome binding according to methods described previously.[11] In brief, 25 mg of HOOC-PEG-COOH were combined with 0.8 mg of DCDI in 2 mL of chloroform and stirred for 48 h under N_2. DSPE (2.77 mg) and 1.4 μL of triethylamine then were added in 2 mL of chloroform, and the resulting mixture was acidified by the addition of 5 mL of chloroform and 4 mL of 0.02*M* phosphate/0.02*M* citrate buffer (pH 5.5) with vigorous shaking. The aqueous phase was removed by low-speed centrifugation and the organic phase dried over anhydrous sodium sulfate. DSPE-PEG-COOH was isolated by passage though a silica gel (Kieselgel 60) column with 50-mL chloroform washings containing successively 0, 10, 20, 30, and 50% methanol. The elutants were analyzed using thin-layer chromatography on silica gel plates developed with chloroform/methanol/water (65:25:4 v/v). The presence of DSPE-PEG-COOH was identified by iodine vapor absorption.

DSPC/CHOL/DSPE (2:1:0.2 molar) (O), O-PEG, and O-PEG-COOH liposomes were prepared by dissolving 13.75 mg of lipid in 2 mL of chloroform. The chloroform was evaporated and the lipid mixture resuspended in 2 mL of 400 m*M* citrate/5 m*M* phosphate buffer (pH 4.0) with vortexing. After five cycles of freezing and thawing, the resulting lipid suspension was extruded nine times through a 100-nm membrane at 65°C using an Avanti Mini-Extruder (Avanti Polar Lipids). The average liposome diameter was determined by dynamic light scattering (Nicomp, Santa Barbara, California).

Tf and Alb were coupled to the distal carboxyl ends of O-PEG-COOH liposome populations using methods similar to those described previously.[12] Briefly, 1 mL of phosphate-buffered saline (PBS; pH 7.5) and 360 μL each of 0.25*M* of EDC and 0.25*M* of S-NHS in water were added to 10 μmol of lipid in 1 mL of extrusion buffer. The mixture was allowed to incubate for 10 min at room temperature before neutralization to pH 7.5 with 1*M* NaOH. Tf or Alb was then added in equal molar amounts, and the protein–liposome mixture was gently stirred for 8 h at 4°C. Unbound protein was removed by passing liposome populations through a Sepharose CL-4B column pre-equilibrated with PBS. Peak liposome elutions were identified visually by a marked change in turbidity. Free protein eluted in subsequent fractions and was quantified by protein assay (Bio-Rad, Hercules, California). Phospholipid content was quantified by fluorescent monitoring of elutions after the addition of a small amount of fluorophore [β-BODIPY® 500/510 C_{12}-HPC] to the lipid mixture. The number of liposomes was determined using an estimation of the number of phospholipid molecules per liposome through methods published previously.[13] The average number of protein molecules per liposome then could be calculated.

Doxorubicin was loaded into the liposomes across a pH gradient of the inner extrusion buffer (pH 4.0) and the outer column buffer (pH 7.5).[14] Liposome solutions were warmed to 60°C before doxorubicin was added in a 0.2:1 (wt/wt) drug/lipid ratio. The mixtures were incubated for 15 min at 60°C with periodic vortexing. The loading efficiency was determined by monitoring liposomal doxorubicin content at 485 nm after separation over a Sepharose CL-4B column.[15]

Uptake of free and liposomal doxorubicin by C6 glioma

Cellular uptake through receptor-mediated targeting was determined using strategies similar to those published before.[16] After the culturing of C6 glioma cells for 40 h, the

culture medium was replaced with 100 μg/mL of free or liposomal doxorubicin diluted in fresh culture medium. Liposomal doxorubicin included doxorubicin entrapped in O, O-PEG, O-PEG-Alb, and O-PEG-Tf populations. One μ*M* free Tf was added to the culture medium with O-PEG-Tf liposomes for competitive binding assays. After 2 h of incubation, the cells were washed three times with PBS to remove extracellular doxorubicin and liposomes. Cells were then detached from the culture wells and solubilized in 2 mL of PBS containing 1% Triton-X 100. Quantification of cellular doxorubicin content was performed using a spectrofluorometer (ISS Inc, Champaign, Illinois; 475 nm excitation/580 nm emission) utilizing the inherent fluorescent properties of doxorubicin.

RESULTS

Characterization of liposomes

Free doxorubicin and four liposome-encapsulated doxorubicin populations were used in this study: (a) free doxorubicin; (b) DSPC/CHOL/DSPE (2:1:0.2 molar) (O); (c) O-PEG; (d) O-PEG-Alb; and (e) O-PEG-Tf, as shown schematically in Figure 1. DSPE-PEG-COOH was synthesized to promote protein–liposome coupling, and its presence was detected in the 20% and 30% methanol effluents during purification. During fabrication, liposome populations were extruded through a 100-nm filter membrane, yielding an average liposomal diameter of 122 nm, as measured by light scattering. Alb and Tf were conjugated in molar equivalents to the distal ends of O-PEG-COOH liposomes, and uncoupled protein was removed by passage through a Sepharose CL-4B column pre-equilibrated in PBS. The number of phospholipid molecules in 122 nm diameter liposomes was estimated to be 1.25×10^5 molecules/liposome. Assuming equal distribution of coupled proteins among liposomes, the average number of Tf molecules per liposome used in targeting was 96. Doxorubicin was loaded in a 0.2/1 (wt/wt) drug/lipid ratio across a pH gradient, with approximately 90% of the drug being successfully encapsulated.

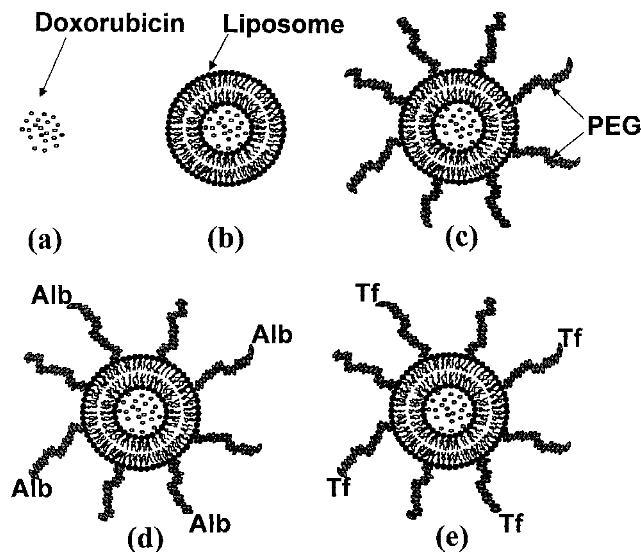

Figure 1. Schematic of doxorubicin delivery systems. (a) Free doxorubicin; (b) doxorubicin encapsulated within liposomes consisting of DSPC/CHOL/DSPE (2:1:0.2 molar); (c) doxorubicin encapsulated within liposomes containing polyethylene glycol (PEG) chains; (d) doxorubicin encapsulated within PEG-liposomes coupled to albumin (Alb); (e) doxorubicin encapsulated within PEG-liposomes coupled to transferrin (Tf).

Comparative uptake of free and encapsulated doxorubicin

Free and encapsulated doxorubicin were incubated with C6 glioma for 2 h before removal of the medium and solubilization of the cells in 1% Triton-X 100. Quantification of cellular doxorubicin content was performed using a fluorescence spectrophotometer after solubilization of the exposed cells. Doxorubicin uptake for different liposome populations is shown as a percentage of free doxorubicin uptake in Figure 2.

Free doxorubicin exhibited the greatest degree of cellular uptake during the incubation period. O-PEG and O-PEG-Alb liposome populations showed 14% and 34% of free doxorubicin uptake, respectively. O liposomes exhibited 54% of free doxorubicin uptake; however the lack of a PEG coating has been shown to greatly decrease liposomal stability *in vivo.* O-PEG-Tf

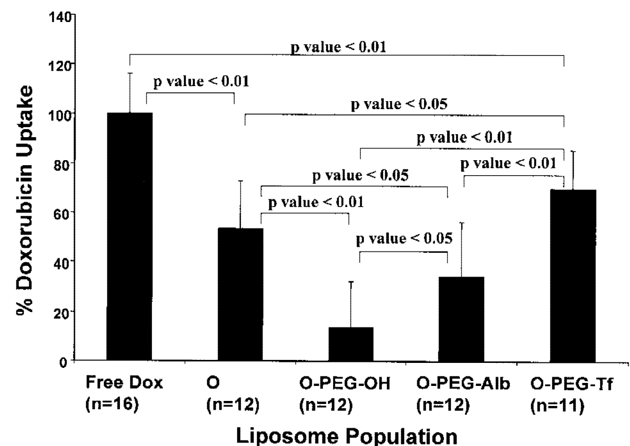

Figure 2. Cellular doxorubicin uptake after 2 h of incubation as quantified by fluorescence at 475 nm excitation/580 nm emission. The uptake values of liposomal doxorubicin populations are expressed as a percentage of free doxorubicin uptake. The error bars denote standard deviation. O: liposomes consisting of DSPC/CHOL/DSPE (2:1:0.2 molar); PEG: polyethylene glycol; Tf: transferrin; Alb: albumin.

liposomes enabled 70% of free doxorubicin uptake, the highest among liposome populations in this study. In competitive binding assays, the uptake from O-PEG-Tf was diminished by 30% through the simultaneous addition of one μM free Tf, as shown in Figure 3.

DISCUSSION

The anti-neoplastic effects of doxorubicin have proven effective against a wide range of human cancers. Problems with cardiotoxicity and low tumor accumulation in the clinical use of free doxorubicin have led to research involving liposomes as drug-delivery vehicles. In this study, we compare the C6 gliomal accumulation of free doxorubicin to accumulation of doxorubicin encapsulated in modified liposomal populations *in vitro.* Free doxorubicin showed the greatest uptake during incubation; however, the previously mentioned difficulties make this a poor choice for *in vivo* application.

Doxorubicin encapsulation within liposomes lacking PEG chains allowed a 292% increase in uptake by glioma compared to liposomes of similar composition

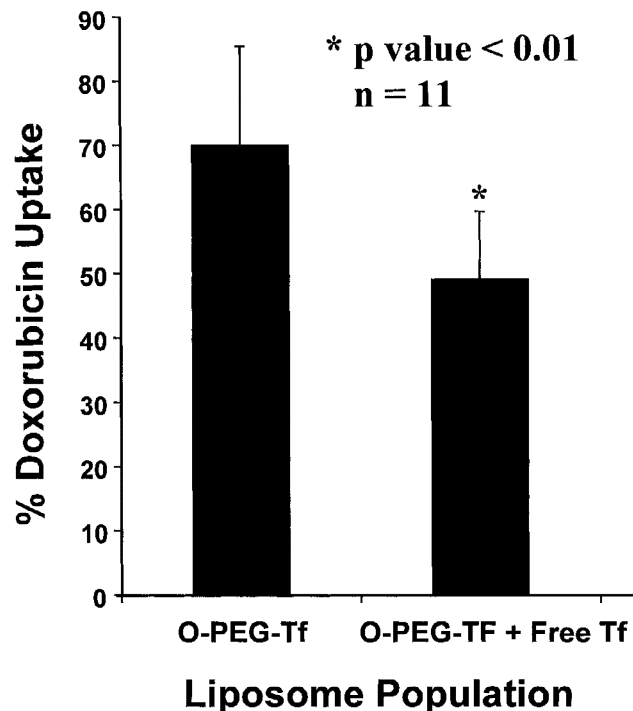

Figure 3. Cellular doxorubicin uptake after 2 h of incubation during competitive binding assays. The uptake values of liposomal doxorubicin populations are expressed as a percentage of free doxorubicin uptake. The error bars denote standard deviation. The addition of one μM free transferrin (Tf) inhibited the cellular uptake of doxorubicin encapsulated within Tf-coupled liposomes by 30%. O: liposomes consisting of DSPC/CHOL/DSPE (2:1:0.2 molar); PEG: polyethylene glycol.

containing PEG. While PEG chains appear sterically unsuited for tumor uptake, the incorporation of PEG into the liposome membrane has been shown to increase circulation times dramatically and to decrease drug leakage within circulation.[17] Ligands were coupled to the distal ends of the PEG chains in an attempt to increase uptake through receptor-mediated targeting while maintaining PEG stability. Tf was used as a targeting agent for glioma TfR, and Alb was used as a similar molecular weight protein control. While an average of 96 Tf molecules per liposome was used for targeting, the optimal *in vivo* ratio of Tf to liposome has not been established. A previous study demonstrated an optimal range of 30–75 targeting groups/liposome for a targeting antibody.[12] Increasing the coupling ratio within and beyond the optimized range did not increase targeted cellular uptake, but it did result in reduced liposome blood circulation times and increased liver uptake. Differences in targeting agents and mechanisms of cellular uptake make the direct comparison of related studies difficult; the optimal ratio of Tf to liposomes for balancing the need for sufficient targeting uptake with maintaining prolonged circulation is best addressed through subsequent *in vivo* experimentation.

The liposomal coupling of Alb provided a 150% increase in uptake over PEG liposome populations not coupled to protein. This increase is likely a result of the PEG terminal carboxylation process used to aid in protein binding (see Methods) rather than the coupling of Alb itself. Experiments involving carboxyl PEG liposomes not coupled with protein (data not shown) demonstrated similar cellular uptake to Alb-coupled liposomes. Tf-coupled liposomes showed a 412% increase over noncarboxylated PEG-liposomal uptake and a 104% increase over Alb-coupled liposomal uptake, indicating that the increased efficacy of these liposomes is due to TfR targeting rather than to PEG modification or the addition of nonspecific proteins. Evidence for the mechanism of receptor-mediated targeting was further supported by competitive binding assays. The addition of one μM free Tf to the incubation medium decreased drug uptake within Tf-coupled liposomes by 30%. Competition by free Tf therefore may play a large role in the efficacy of this system *in vivo.*

The use of Tf-coupled liposomes for the treatment of glioma is also advantageous in view of the role of TfR in normal brain tissue. TfR is found primarily on capillary endothelial cells and is involved in iron transport across the blood–brain barrier (BBB).[18] Though the role of endothelial TfR in the transcytosis of Tf across the BBB is still controversial,[19–23] liposomes may be assisted across the BBB through Tf coupling. The effectiveness of transport could be dependent on the method of administration; a previous study has shown that Tf–protein complexes are able to cross the

BBB successfully when administered through the carotid artery but not when injected intravenously.[24]

CONCLUSIONS

We have demonstrated that Tf-coupled liposomes are able to achieve preferential receptor-mediated targeting of C6 glioma *in vitro.* The suitability of liposomes for drug delivery, the overexpression of TfR on glioma, and the potential for TfR-mediated transport across the BBB make this system attractive to complement and improve existing treatment methods for glioma. Further investigation and optimization of this system would be best addressed through *in vivo* experimentation.

References

1. Kim S. Liposomes as carriers of cancer chemotherapy. Current status and future prospects. Drugs 1993;46:618–638.
2. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171–199.
3. Allen TM. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol Sci 1994;15:215–220.
4. Maruyama K, Yuda T, Okamoto A, Kojima S, Suginaka A, Iwatsuru M. Prolonged circulation time *in vivo* of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). Biochim Biophys Acta 1992;1128:44–49.
5. Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov Jr AA, Trubetskoy VS, Herron JN, Gentry CA. Poly(ethylene glycol) on the liposome surface: On the mechanism of polymer-coated liposome longevity. Biochim Biophys Acta 1994;1195:11–20.
6. Allen TM, Hansen C, Martin F, Redemann C, Yau–Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo.* Biochim Biophys Acta 1991;1066:29–36.
7. Allen TM, Brandeis E, Hansen CB, Kao GY, Zalipsky S. A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. [Published erratum appears in Biochim Biophys Acta 1995; 1240(2): 285.] Biochim Biophys Acta 1995;1237:99–108.
8. Blume G, Cevc G, Crommelin MD, Bakker–Woudenberg IA, Kluft C, Storm G. Specific targeting with poly(ethylene glycol)-modified liposomes: Coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. Biochim Biophys Acta 1993;1149:180–184.
9. Kurpad SN, Zhao XG, Wikstrand CJ, Batra SK, McLendon RE, Bigner DD. Tumor antigens in astrocytic gliomas. Glia 1995;15: 244–256.
10. Recht L, Torres CO, Smith TW, Raso V, Griffin TW. Transferrin receptor in normal and neoplastic brain tissue: Implications for brain-tumor immunotherapy. [See comments.] J Neurosurg 1990;72:941–945.
11. Kung VT, Redemann CT. Synthesis of carboxyacyl derivatives of phosphatidylethanolamine and use as an efficient method for conjugation of protein to liposomes. Biochim Biophys Acta 1986;862:435–439.
12. Maruyama K, Takizawa T, Yuda T, Kennel SJ, Huang L, Iwatsuru M. Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. Biochim Biophys Acta 1995;1234:74–80.
13. Enoch HG, Strittmatter P. Formation and properties of 1000-A-diameter, single-bilayer phospholipid vesicles. Proc Natl Acad Sci USA 1979;76:145–149.
14. Mayer LD, Bally MB, Cullis PR. Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. Biochim Biophys Acta 1986;857:123–126.
15. Mayer LD, Bally MB, Hope MJ, Cullis PR. Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane potential. Biochim Biophys Acta 1985;816:294–302.
16. Lee RJ, Low PS. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro.* Biochim Biophys Acta 1995;1233:134–144.
17. Gabizon A, Martin F. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumours. Drugs 1997;54:15–21.
18. Jefferies WA, Brandon MR, Hunt SV, Williams AF, Gatter KC, Mason DY. Transferrin receptor on endothelium of brain capillaries. Nature 1984;312:162–163.
19. Roberts R, Sandra A, Siek GC, Lucas JJ, Fine RE. Studies of the mechanism of iron transport across the blood–brain barrier. Ann Neurol 1992;32:S43–S50.
20. Morris CM, Keith AB, Edwardson JA, Pullen RG. Uptake and distribution of iron and transferrin in the adult rat brain. J Neurochem 1992;59:300–306.
21. Skarlatos S, Yoshikawa T, Pardridge WM. Transport of [125I]transferrin through the rat blood–brain barrier. Brain Res 1995;683:164–171.
22. Descamps L, Dehouck MP, Torpier G, Cecchelli R. Receptor-mediated transcytosis of transferrin through blood–brain barrier endothelial cells. Am J Physiol 1996;270:H1149–H1158.
23. van Gelder W, Cleton–Soeteman MI, Huijskes–Heins MI, van Run PR, van Eijk HG. Transcytosis of 6.6-nm gold-labeled transferrin: An ultrastructural study in cultured porcine blood–brain barrier endothelial cells. Brain Res 1997;746:105–116.
24. Broadwell RD, Baker–Cairns BJ, Friden PM, Oliver C, Villegas JC. Transcytosis of protein through the mammalian cerebral epithelium and endothelium. III. Receptor-mediated transcytosis through the blood–brain barrier of blood-borne transferrin and antibody against the transferrin receptor. Exp Neurol 1996; 142:47–65.