International Journal of Anatomy and Physiology ISSN: 2326-7275 Vol. 6 (4), pp. 001-006, April, 2017. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Anti glioma effect of doxorubicin loaded liposomes modified with angiopep-2

Mei Danyu[1,2], Gao Huile[1,2], Gong Wei[3], Pang Zhiqing[1,2], Jiang Xinguo[1,2]* and Chen Jun[1,2]

[1]School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai, 201203, China.
[2]Key Laboratory of Smart Drug Delivery, Ministry of Education and PLA, 826 Zhangheng Road, Shanghai 201203, China.
[3]Academy of Military Medical Sciences, Beijing 100853, China.

Accepted 28 August, 2016

The low-density lipoprotein receptor-related protein (LRP) was highly expressed in blood brain barrier and glioma cells. Angiopep-2 was a new and effective ligand of LRP. Herein, we present a novel brain targeting delivery system: doxorubicin (DOX) loaded angiopep-2 modified liposome (AL). The particle size of AL was 102 nm and the zeta potential was -9.8 mV, which was stable and suitable for brain targeting delivery. In the anti glioma study, AL group had the smallest average tumor volume, the strongest tumor apoptosis, gain more weight after treatment and longer median survival time than that of other groups. This proved that Angiopep- 2 modified doxorubicin liposomes had certain glioma targeting therapeutic effects and lower toxicity.

Key words: Angiopep-2, liposome, glioma, targeted drug delivery.

INTRODUCTION

Glioma is the most frequent brain cancers that accounts for about 46% of intracranial tumors with a risk of 30~100 per million (Jain et al., 2007). Surgical excision is the primary choice in its clinical treatment. But because of its infiltration into normal brain tissue and the specificity of its growing locations (Ong et al., 2009), the tumor usually cannot be removed completely, and the treatment always has to be supplemented with radiotherapy and chemotherapy in order to kill the rest part of the tumor (Genc et al., 2011). However, the average survival expectancy is 14.6 months and the overall 5 year survival rate for glioma is 9.8% (Goellner et al., 2011). When glioma is in its early stage, the blood-brain barrier (BBB) surrounded the tumor is still functionally complete. With the progression of the disease, it begins to generate a large number of tumor angiogenesis, and the vascular endothelial cell gap goes up to 50 to 300 nm (Bulnes et al., 2009). Supplemented treatments against the margin of the tumor where usually maintains after tumor resection and also where BBB still has its function, is significantly important to the prognosis of glioma treatment.

Functional group determines the active targeting ability of drug delivery systems such as liposome to penetrate membrane through the corresponding receptor or vector. Different transporters and receptors presented at the BBB have been described as playing important roles in maintaining the integrity of the BBB and brain homeostasis (Pardridge, 2007). Among them, the low-density lipoprotein receptor-related protein (LRP) has been reported to possess the ability to mediate transport of ligands across endothelial cells of the BBB. LRP is a member of the low-density lipoprotein receptor family (Bell et al., 2007), that can bind numerous ligands, including proteinases, proteinase-inhibitor complexes,

*Corresponding author. E-mail: docma96@sina.com. Tel: +86-551-2283114. Fax: +86-551-2283409.

Abbreviations: AL, DOX loaded angiopep-2 modified liposome; **BBB,** blood-brain barrier; **DOX,** doxorubicin; **DPPC,** 1,2-dipalmitoyl -sn-glycéro-3-phosphatidylcholine; **EE,** encapsulation efficiency; HSPC, hydrogenated soybean phosphatidylcholine; **LRP,** low-density lipoprotein receptor-related protein; **NL,** DOX loaded liposome; **PEG-DSPE-MAL,** 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene-glycol)-2000]; **TEM,** transmission electron microscope, **TUNEL,** terminal deoxynucleotide transferase dUTP nick end labeling.

and certain apoE- and lipoprotein lipase-enriched lipoproteins (Ke et al., 2009). Furthermore, LRP could mediate the cellular internalization of the ligands and their transport across the BBB (Bell et al., 2007; Ito et al., 2006; Shibata et al., 2000). A new peptide, angiopep-2 (TFFYGGSRGKRNNFKTEEY, Mw 2.4 kDa), is one of the peptides that derived from the Kunitz domain, possessing a higher brain penetration capability than other proteins, such as transferrin, which has been demonstrated using both an in vitro model of the BBB and in situ brain perfusion in mice (Demeule et al., 2008; Ke et al., 2009; Shao et al., 2010; Van Rooy et al., 2011). Research shows that angiopep- 2 has affinity with the LRP which was highly expressed on the BBB endothelial cells and glioma cells (Chung and Wasan, 2004; Demeule et al., 2008; Maletinska et al., 2000; Shen et al., 2010).

The objective of this study was trying to establish a novel brain targeting delivery system, angiopep-2 modified liposome. To evaluate the effect of this delivery system, the doxorubicin (DOX) was employed as a model drug, then the pharmacokinetics and anti glioma effect of DOX loaded angiopep-2 modified liposome (AL) were evaluated.

MATERIALS AND METHODS

1,2-Dipalmitoyl-sn-glycéro-3-phosphatidylcholine (DPPC) was purchased from Northern Lipids Inc (Canada). Hydrogenated soybean phosphatidylcholine (HSPC) was obtained from Degussa Corporation (Germany). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine -N-[maleimide (polyethylene-glycol)-2000] (PEG-DSPE-MAL) was obtained from Nanocs International Inc. (USA). Angiopep-2 was synthesized by Chinese Peptide Company (China). Doxorubicin hydrochloride was obtained from Zhejiang Hisun Pharmaceutical Co. Ltd. (China).

ICR mice (male, 4-5 weeks, 20 to 22 g) were purchased from the Department of Experimental Animals, Fudan University, and maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

Optimization of DOX liposome

To achieve maximum encapsulation efficiency and appropriate particle size, several formulations with different phospholipid composition were designed and evaluated. pH gradient method was used to encapsulate the drug as described previously. The drug/lipid ratio was 1:25 and the theoretical drug concentration was 2 mg/ml.

Preparation of DOX liposomes

AL was prepared by the modified reverse phase evaporation method. DPPC, PEG-DSPE and PEG-DSPE- MAL (90:10:2, w/w/w) were dissolved in chloroform and evaporated to dryness. The dried lipid films were hydrated in citrate buffer (pH 4.0). The suspensions then were extruded through polycarbonate membranes (pore size 100 nm) to obtain drug free liposomes with pH adjusted to 7.2. Angiopep-2 was added into the suspensions and incubated at room temperature for overnight. Then doxorubicin was added into the suspension and incubated for 30 min at 35°C. The production was applied to ultrafiltration to remove the free DOX. DOX loaded liposome (NL) was prepared as above without the angiopep-2 conjugation procedure.

Characterization of NL and AL

The morphological examination of liposomes was observed by transmission electron microscope (TEM) (H-600, Hitachi, Japan). The particle size and zeta potential of the NL and AL were determined by dynamic light scattering using a Zeta Potential/Particle Sizer NICOMP TM 380 ZLS (PSS.NICOMP PARTICLE SIZE SYSTEM, Santa Barbara, USA).

To evaluate the encapsulation efficiency (EE), NL and AL were dissolved in methanol while the encapsulated DOX determined by high performance liquid chromatography (HPLC). 20 l of diluted sample was injected in the system. The HPLC system (Dual Absorbance Detector, Binary HPLC Pump, Waters, USA) consisted of a pump and a UV detector (233 nm). With a reversed phase C18 ((Agela Venusil MP C18, 4.6 x 250 mm, 5 m) column, the mobile phase was methanol: 10 mM phosphate buffer (75:25, pH 7.4) with a flow rate of 1.0 ml/min.

Anti glioma study

Glioma-bearing mice (6 days after the tumor planting surgery) were randomly assigned to one of the following four treatment groups (n = 20 per group): physiological saline, free DOX (2 mg/kg), NL (2 mg/kg), AL (2 mg/kg). Mice were dosed on day 2, 5 and 8 through tail vein, and all drugs were diluted with physiological saline.

For the tumor growth study, 4 mice of each group were killed on day 14 by cervical vertebra dislocation, and their tumors were immediately harvested, measured, and analyzed using terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay.

The remaining mice in each treatment group were used for survival analysis. For the life span study, the experiment was ended on day 45. For each treatment group, 8 mice were used for daily monitoring of weight loss in order to analysis the physiological status of the mice and study the toxicity of test preparation.

Statistical analysis

Data were expressed as the means with 95% confidence intervals. Statistical tests were performed with the Student t test, the Wilcoxon signed rank test, or the Mann-Whitney test. When differences were detected, the Wilcoxon signed rank test was used to test for pairwise differences between treatment groups (SPSS software, version 12.0, SPSS Inc) and the Mann-Whitney test was used to determine the difference between independent sample groups (SPSS software, version 12.0, SPSS Inc). Survival was assessed with the Kaplan-Meier method. For all tests, P values less than 0.05 were considered to be statistically significant. All statistical tests were two-sided.

RESULTS

Formulation of doxorubicin liposomes

The formulation that contained DPPC as the main

Table 1. Influence of the different phospholipids ratio on the average particle size and encapsulation efficiency of the liposomes.

Formulation	Ratio	Particle size(mean ± SD, nm)	EE (%)
D1 DPPC/ PEG-DSPE /DSPE-PEG-MAL	90:10:0.5	100±2.1	98.6
D2 DPPC/ PEG-DSPE /DSPE-PEG-MAL	90:10:1	102±2.8	99.1
D3 DPPC/ PEG-DSPE /DSPE-PEG-MAL	90:10:2	99±1.7	99.2
H HSPC/PEG-DSPE /DSPE-PEG-MAL	90:10:2	145±5.6	81.3

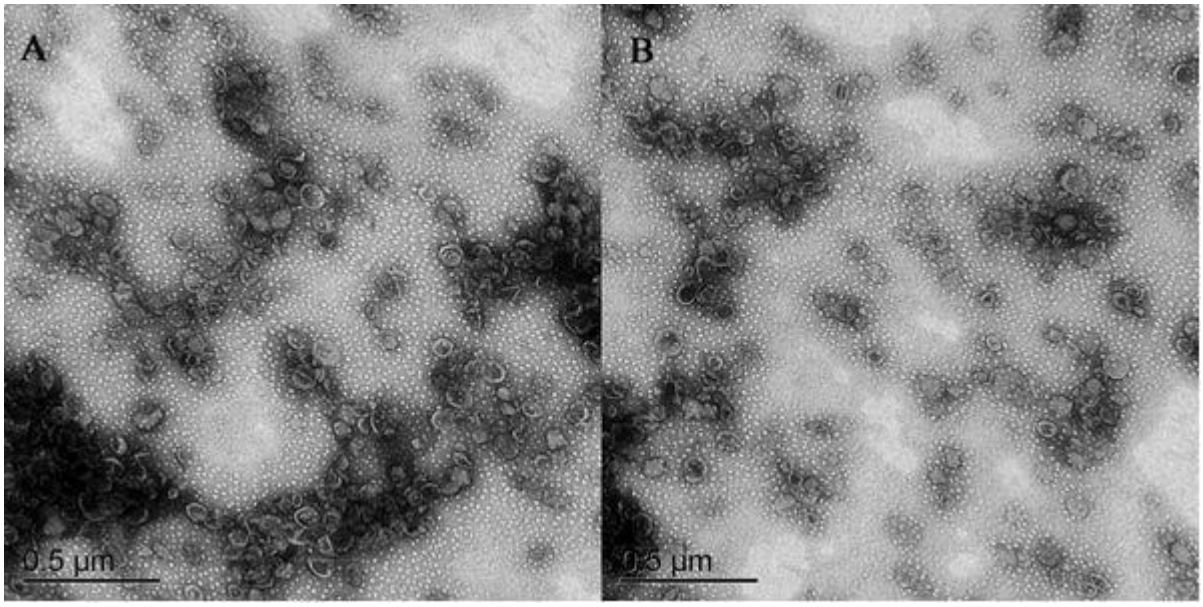

Figure 1. Transmission electron micrograph of AL (A) and NL (B).

prescription was denoted by D1, D2, D3, and that contained HSPC as the main prescription was denoted by H (Table 1). The results showed that liposomes of D1, D2 and D3 formulation had smaller size and higher EE than those of H. The size and EE of the former were 100 nm and 99% respectively, which addressed the requirements of the study.

Characterization of NL and AL

The TEM of NL and AL showed that they were spherical particles (Figure 1). The average size of NL was 100.5±5.5 nm, the zeta potential was - 7.6±4.5 mV. The conjugation with angiopep-2 was only slightly affected these parameters, which were 102.0±3.7 nm and - 8.9±3.1 mV respectively. The EE of both NL and AL were higher than 99%. The negative or neutral liposomes provide more effective barrier to plasma macromolecular protein adsorption and are easy to resuspend in blood (Mobed and Chang, 1998), which indicated the AL and NL may content long blood circulated time.

Anti glioma study

Glioma volume

After treatments, the mice glioma volume of the control group, DOX group, NL group and AL group were 68.63±30.80, 36.47±12.00, 11.93±4.21 and 5.39±4.27 mm^3, respectively. The tumor volume of AL group was significantly smaller than that of all the other groups.

TUNEL detection of apoptosis

TUNEL-positive nuclei marked mainly located in the "nuclear condensation" and were round or oval brown clumps, the typical apoptotic chromatin are crescent or bean-shaped stain. TUNEL results showed that AL group (Figure 2D) was expressed the most significant distribution of brown granules, mainly on the peripheral of tumor necrosis. The control group (Figure 2A) has the least expression of the brown clumps compared with the

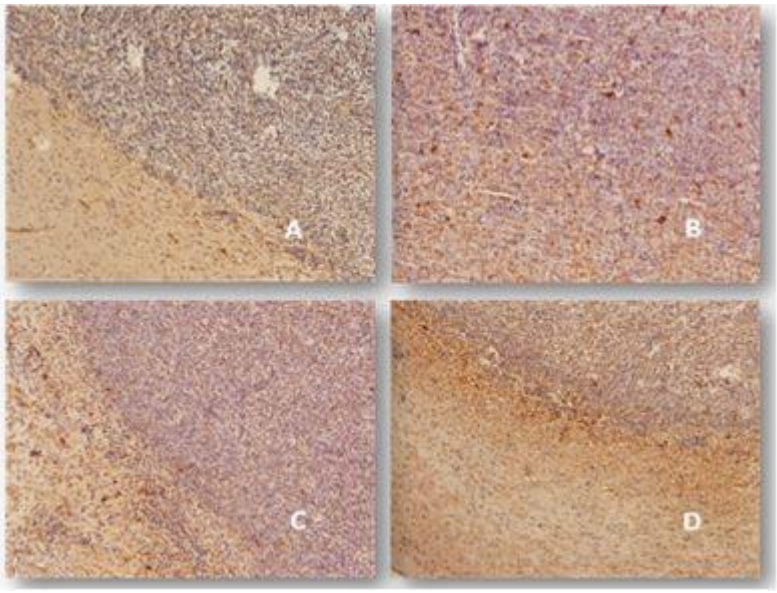

Figure 2. TUNEL results of four therapeutic groups; (A) was control group; (B) was DOX group; (C) and (D) were NL and AL therapeutic groups, respectively.

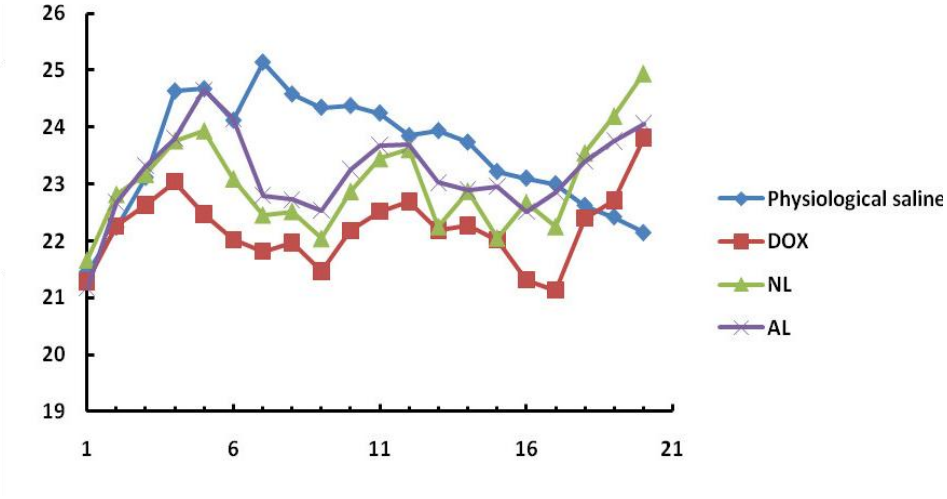

Figure 3. The change of weight in 20 days of different experimental groups of mice (n = 8).

other three groups. AL group showed the most significant tumor cell apoptosis, indicating that AL can effectively deliver the drug into the brain and inhibit the proliferation of cancer cells.

Change of weight

The mice average weight change in 20 days of different experimental groups (Figure 3) showed that mice average weight of DOX, NL, AL groups fluctuated with the administrations of drug in day 2, day 5 and day 8, while the mice weight of saline group gain normally. During the treatment period (day 2 to 8), the body weight of control group, DOX group, NL group and AL group was increased 13.47, 0.86, 1.75 and 6.43% respectively. These indicated that free DOX has certain toxicity and NL was less toxic than DOX solution. The average weight of the saline group rose at the beginning, and was much higher than the other groups. However, later with the development of the glioma, it decreased and was much lower than the other groups on day 20. This suggested

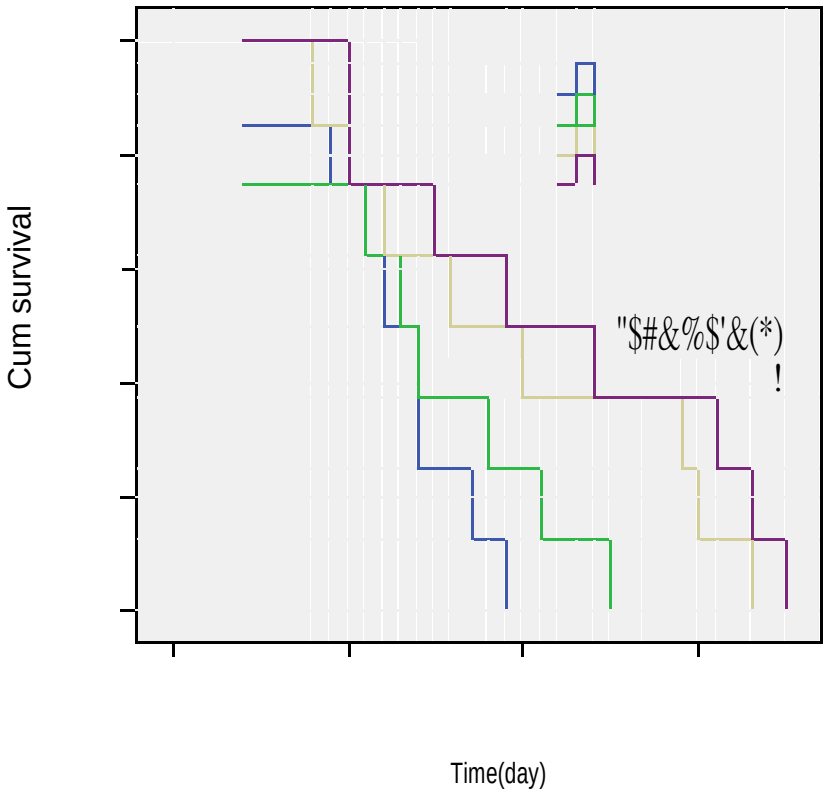

Figure 4. Survival (Kaplan-Meier plot) of four groups mice with intracranially transplanted C6 glioma.

Table 2. Median survival time for glioma implanted mice of different therapeutic groups.

Group	Median survival time	Standard error	95% confidence interval	
			Lower bound	Upper bound
Saline	22	1.41	19.23	24.77
DOX	23	2.12	18.84	27.16
NL	26	5.66	14.91	37.09
AL	29	6.36	16.53	41.47

that treatment groups had better living quality than the saline group during late stage of treatment.

Survival analysis

Life-span extension treated with different formulations were showed diverse curve (Figure 4) . By log-rank test (Table 2). The median survival time of AL group and NL group was significantly longer than that of saline group and DOX group. Although the median survival time of AL group was longer than that of NL group, there was no significant difference. In the other hand, the curve of DOX group was almost similar with salin group, which indicated the tocixity of DOX covered its therapy effect.

DISCUSSION AND CONCLUSION

Chemotherapy is widely used for brain glioma treatment; however, the outcome continues to be unsatisfactory (Gupta and Torchilin, 2007). Increasing the concentration of anticancer drug in the tumor side may improve the therapy effect, but due to the aggressive growth of glioma and the existence of BBB, even if partly destroyed, intracranial drug delivery may result in unexpected side effects (Zhan et al., 2010). Brain targeting ligands modified nanocarriers are one of promising strategies for glioma therapy and gain more and more attention.

Ligands are very important for the targeting effect. Most of them, such as cyclic RGD, folic acid, only could be recognized by BBB or tumor (Sanguino et al., 2008; Zhan

et al., 2010), which may not suit for brain glioma therapy for the potential distribution of chemotherapeutic agents into the normal brain tissues. Fortunately, there are still several ligands that could be recognized by both. One is angiopep-2, which could be recognized by LRP1 that highly expressed in both BBB and glioma (Demeule et al., 2008). This is also the reason that we select angiopep-2 as targeting ligand for glioma therapy.

In this study, glioma- bearing mice model was constructed by intracranial injection of C6 cells. This method is similar with glioma removal surgery in the way that they both in some degree damaged the BBB and leaved survived tumor cells in the surgical site. The volume of glioma was often used for evaluating the chemotherapeutic effect. In our study, after 2 weeks treatment, the average glioma volume of DOX, NL and AL was only 53.1, 17.4 and 7.8% of control group. These were constant with the pharmacokinetic study. Free of angiopep-2, NL could not precisely target to the glioma, which resulted in the comparatively lower chemotherapeutic effect. The TUNEL results were also showed that AL could induce the strongest apoptosis of tumor cells. In the survival experiment, the results were almost the same. The DOX was showed slightly effect on prolonging the median survival time while NL and AL could significantly prolong the median survival time, which confirmed the better therapeutic effect of NL and AL. In the other hand, AL should contain better therapeutic effect compared with NL, but the results were not promising. AL was only slight prolong the median survival time but there was no significant difference between AL and NL.

The tumor volume and TUNEL detection were directly proofs for the anti glioma effect of AL and NL, which indicated the AL observed better effect, while the median survival time was a result of not only the glioma growth but also several other reasons. In the other hand, the dose of DOX may be higher than necessary that NL group had achieved the best treatment concentration, although AL could further increase the concentration, it resulted none. The toxicity could be reflected by the body weight change. Administration of free DOX could significantly reduce the body weight increase, while NL and AL were much slighter. These supported that NL and AL could be target to the brain and glioma and reduce the systemic toxicity.

In conclusion, we have developed a new brain targeting system that is effective for glioma treatment and lower toxiferous for system administration.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (973 Program, 2007CB935802), the National Key Program of Pharmaceutical Creation and Development (2009ZX09310-006) and National Natural Science Foundation of China (81072592).

REFERENCES

Bell RD, Sagare AP, Friedman AE, Bedi GS, Holtzman DM, Deane R, Zlokovic BV (2007). Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. J Cereb. Blood Flow Metab., 27: 909-918.

Bulnes S, Bilbao J, Lafuente JV (2009). Microvascular adaptive changes in experimental endogenous brain gliomas. Histol. Histopathol., 24: 693-706.

Chung NS, Wasan KM (2004). Potential role of the low-density lipoprotein receptor family as mediators of cellular drug uptake. Adv. Drug Deliv. Rev., 56: 1315-1334.

Demeule M, Currie JC, Bertrand Y, Che C, Nguyen T, Regina A, Gabathuler R, Castaigne JP, Beliveau R (2008). Involvement of the low-density lipoprotein receptor-related protein in the transcytosis of the brain delivery vector angiopep-2. J. Neurochem., 106: 1534-1544.

Demeule M, Regina A, Che C, Poirier J, Nguyen T, Gabathuler R, Castaigne JP, Beliveau R (2008). Identification and design of peptides as a new drug delivery system for the brain. J. Pharmacol. Exp. Ther., 324: 1064-1072.

Genc DB, Canpolat C, Berrak SG (2011). Clinical features and management of carboplatin-related hypersensitivity reactions in pediatric low-grade glioma. Support. Care Cancer.

Goellner EM, Grimme B, Brown AR, Lin YC, Wang XH, Sugrue KF, Mitchell L, Trivedi RN, Tang JB, Sobol RW (2011). Overcoming Temozolomide Resistance in Glioblastoma via Dual Inhibition of NAD+ Biosynthesis and Base Excision Repair. Cancer Res., 71: 2308-2317.

Gupta B, Torchilin VP (2007). Monoclonal antibody 2C5-modified doxorubicin-loaded liposomes with significantly enhanced therapeutic activity against intracranial human brain U-87 MG tumor xenografts in nude mice. Cancer Immunol. Immunother., 56: 1215-1223.

Ito S, Ohtsuki S, Terasaki T (2006). Functional characterization of the brain-to-blood efflux clearance of human amyloid-beta peptide (1-40) across the rat blood-brain barrier. Neurosci. Res., 56: 246-252.

Jain RK, di Tomaso E, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT (2007). Angiogenesis in brain tumours. Nat. Rev. Neurosci., p. 8.

Ke W, Shao K, Huang R, Han L, Liu Y, Li J, Kuang Y, Ye L, Lou J, Jiang C (2009). Gene delivery targeted to the brain using an Angiopep-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. Biomaterials, 30: 6976-6985.

Maletinska L, Blakely EA, Bjornstad KA, Deen DF, Knoff LJ, Forte TM (2000). Human glioblastoma cell lines: Levels of low-density lipoprotein receptor and low-density lipoprotein receptor-related protein. Cancer Res., 60: 2300-2303.

Ong BY, Ranganath SH, Lee LY, Lu F, Lee HS, Sahinidis NV, Wang CH (2009). Paclitaxel delivery from PLGA foams for controlled release in post-surgical chemotherapy against glioblastoma multiforme. Biomaterials, 30: 3189-3196.

Pardridge WM (2007). Drug targeting to the brain. Pharm. Res., 24: 1733-1744.

Sanguino A, Lopez-Berestein G, Sood AK (2008). Strategies for *in vivo* siRNA delivery in cancer. Mini Rev. Med. Chem., 8: 248-255.

Shao K, Huang R, Li J, Han L, Ye L, Lou J, Jiang C (2010). Angiopep-2 modified PE-PEG based polymeric micelles for amphotericin B delivery targeted to the brain. J. Control Release, 147: 118-126.

Shen J, Zhan C, Xie C, Meng Q, Gu B, Li C, Zhang Y, Lu W (2010). Poly (ethylene glycol)-block-poly (d,l-lactide acid) micelles anchored with angiopep-2 for brain-targeting delivery. J. Drug Target.

Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, Holtzman DM, Miller CA, Strickland DK, Ghiso J, Zlokovic BV (2000). Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. J. Clin. Invest., 106: 1489-1499.

van Rooy I, Mastrobattista E, Storm G, Hennink WE, Schiffelers RM (2011). Comparison of five different targeting ligands to enhance accumulation of liposomes into the brain. J. Control Release, 150: 30-36.

Zhan C, Gu B, Xie C, Li J, Liu Y, Lu W (2010). Cyclic RGD conjugated poly(ethylene glycol)-co-poly(lactic acid) micelle enhances paclitaxel anti-glioblastoma effect. J. Control Release, 143: 136-142.