


RESEARCH

Open Access



Liposomal formulations of carboplatin injected by convection-enhanced delivery increases the median survival time of F98 glioma bearing rats

Minghan Shi¹, Malathi Anantha^{2,3}, Mohamed Wehbe^{2,3}, Marcel B. Bally^{2,3}, David Fortin⁴, Laurent-Olivier Roy⁵, Gabriel Charest⁶, Maxime Richer⁷, Benoit Paquette^{6*}  and Léon Sanche⁶

Abstract

Background: Effectiveness of chemotherapy for treating glioblastoma (GBM) brain tumors is hampered by the blood–brain barrier which limits the entry into the brain of most drugs from the blood. To bypass this barrier, convection-enhanced delivery (CED) was proposed to directly inject drugs in tumor. However, the benefit of CED may be hampered when drugs diffuse outside the tumor to then induce neurotoxicity. Encapsulation of drugs into liposome aims at increasing tumor cells specificity and reduces neurotoxicity. However, the most appropriate liposomal formulation to inject drugs into brain tumor by CED still remains to be determined. In this study, four liposomal carboplatin formulations were prepared and tested *in vitro* on F98 glioma cells and in Fischer rats carrying F98 tumor implanted in the brain. Impact of pegylation on liposomal surface and relevance of positive or negative charge were assessed.

Results: The cationic non-pegylated (L1) and pegylated (L2) liposomes greatly improved the toxicity of carboplatin *in vitro* compared to free carboplatin, whereas only a modest improvement and even a reduction of efficiency were measured with the anionic non-pegylated (L3) and the pegylated (L4) liposomes. Conversely, only the L4 liposome significantly increased the median survival time of Fisher rats implanted with the F98 tumor, compared to free carboplatin. Neurotoxicity assays performed with the empty L4 liposome showed that the lipid components of L4 were not toxic. These results suggest that the positive charge on liposomes L1 and L2, which is known to promote binding to cell membrane, facilitates carboplatin accumulation in cancer cells explaining their higher efficacy *in vitro*. Conversely, negatively charged and pegylated liposome (L4) seems to diffuse over a larger distance in the tumor, and consequently significantly increased the median survival time of the animals.

Conclusions: Selection of the best liposomal formulation based on *in vitro* studies or animal model can result in contradictory conclusions. The negatively charged and pegylated liposome (L4) which was the less efficient formulation *in vitro* showed the best therapeutic effect in animal model of GBM. These results support that relevant animal model of GBM must be considered to determine the optimal physicochemical properties of liposomal formulations.

Keywords: Brain tumor, Carboplatin, Convection-enhanced delivery, Glioblastoma, Liposome

*Correspondence: Benoit.Paquette@USherbrooke.ca

⁶ Center for Research in Radiotherapy, Department of Nuclear Medicine and Radiobiology, Université de Sherbrooke, Sherbrooke, QC, Canada
Full list of author information is available at the end of the article



Background

The efficacy of chemotherapeutic drugs to treat glioblastoma (GBM) is hampered by the blood–brain barrier (BBB), which limits drug accumulation in tumor cells [1]. Recently, chemotherapy drug delivery by convection-enhanced delivery (CED) [2] has attracted considerable attention. For example, CED of a liposomal formulation of irinotecan resulted in a better survival time than intravenous (i.v.) injection of the same drug in a GBM mouse model [3]. The efficiency of platinum compounds injected by CED was compared to different routes of delivery [i.v., intra-arterial (i.a.), or i.a. plus blood-brain barrier disruption] for their concomitant effect with radiotherapy in animal models of brain tumors. These results showed that CED of free platinum-based drugs increased the uptake of platinum in tumor and improved the median survival time of F98 glioma-bearing Fischer rats when combined with radiation [4–8]. Amongst the platinum compounds tested (carboplatin, cisplatin and oxaliplatin), carboplatin exhibited the lowest toxicity while providing the best survival benefits. In addition, induction of DNA damage by low energy secondary electrons produced by radiation, was greater when using carboplatin compared to cisplatin, suggesting that carboplatin is a better radiosensitizer [9]. These studies suggest that carboplatin is the best candidate among the approved platinum drugs for treatment of human brain tumors. In addition, a phase I clinical trial has shown that carboplatin delivered by CED is feasible and safe [10, 11].

Although CED increases the accumulation of carboplatin in tumor, its concentration decreases rapidly. For example, initially injected into the tumor at 600 $\mu\text{g/g}$ of tissue, the concentration of carboplatin decreased to only 16 $\mu\text{g/g}$ tissue in 24 h [4]. To improve its retention time in tumor and consequently increase the survival time of animals, liposomal formulations of carboplatin were suggested [12–14]. A negative or positive charge is needed on the liposomes to facilitate binding to charged cell

membranes and to prevent clustering, which reduces their availability. However, the effects of liposome size, charge, and formulation on drug distribution volume after CED, still need to be optimized [15–20]. In our study, four different liposomal formulations of carboplatin were assessed: (1) cationic liposomes without pegylation (PEG) (L1); (2) cationic liposomes with pegylation (L2); (3) anionic liposomes without pegylation (L3); and (4) anionic liposomes with pegylation (L4). The addition of PEG to cationic liposomes should reduce their interaction with the negatively charged cell surface [21]. The distribution volume of pegylated liposomes is therefore expected to be increased, although the internalizing efficiency in F98 glioma cells may be reduced [18, 22, 23]. Since drug-free cationic liposomes could be neurotoxic, empty anionic liposomes with and without pegylation were also assessed [18, 24]. The in vitro toxicity of these liposomal carboplatin formulations was measured, as well as their maximum in vivo tolerated dose (MTD) and antitumor efficacy in a F98 glioma rat model.

Results

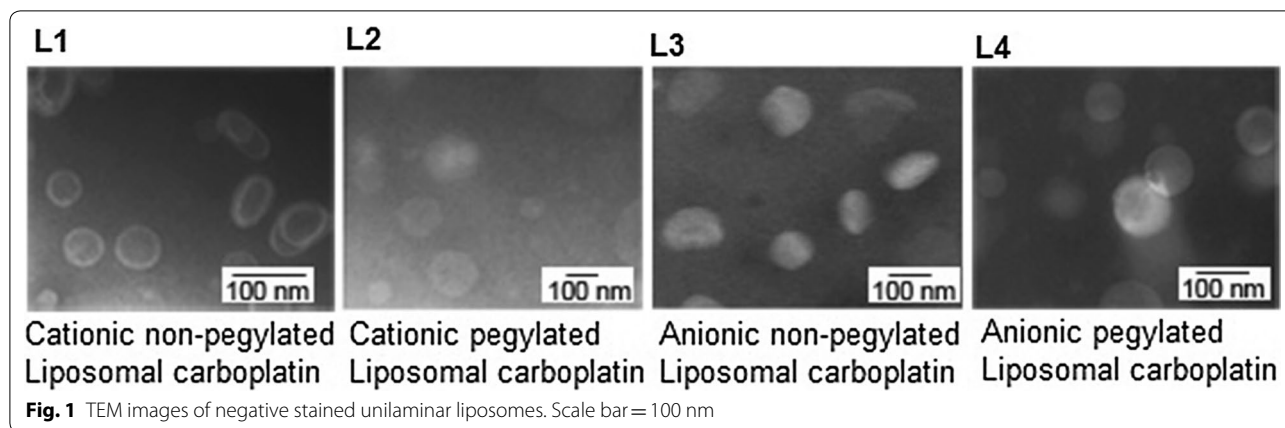
Characterization of liposomal carboplatin

The 4 types of liposomal carboplatin are detailed in Table 1: L1 = cationic non-pegylated liposomal carboplatin; L2 = cationic pegylated liposomal carboplatin; L3 = anionic non-pegylated liposomal carboplatin; and L4 = anionic pegylated liposomal carboplatin. The negative or positive charge was confirmed by measuring Zeta potentials and ranged between -48.0 and 55.9 mV, consistent with values reported for similar liposomal formulations [25, 26]. Addition of 4.8% pegylation has only slightly reduced the Zeta potential values, as reported by Qin et al. [27]. Images of liposomes were captured under TEM with negative stain (Fig. 1). Unilaminar liposomes with sizes ranging from 57.4 to 85.6 nm were measured (Table 1).

Table 1 Properties of the liposomal formulation of carboplatin

ID	Chemical	Lipids molar ratio	Properties	Zeta potential (mV)	Method	Size (nm)	mg carboplatin mg lipids
L1	DPPC:DC-Chol	1:1	cationic	52.1 ± 8.84	REV	60.3 ± 21.1	0.050
L1'	DPPC:DC-Chol	1:1	cationic	55.9 ± 9.38	REV	57.4 ± 16.1	–
L1''	DPPC:DC-Chol	1:1	cationic	38.9 ± 12.9	Hydration	77.7 ± 21.0	–
L2	DPPC:DC-Chol: PEG2000 PE	10:11:1	cationic + PEG	45.1 ± 12.6	REV	71.3 ± 17.8	0.032
L3	DSPC:DSPG:Chol	7:2:1	anionic	-48.0 ± 15.0	Hydration	72.7 ± 24.3	0.096
L3'	DSPC:DSPG:Chol	7:2:1	anionic	-47.3 ± 14.6	Hydration	83.6 ± 17.2	–
L4	DPPC:Chol:PEG2000 PE	10:11:1	anionic + PEG	-35.8 ± 5.43	REV	85.6 ± 19.9	0.32
L4'	DPPC:Chol:PEG2000 PE	10:11:1	anionic + PEG	-33.0 ± 6.27	REV	77.4 ± 24.3	–

PEG pegylated liposomes, REV reverse-phase evaporation method, Hydration hydration method



For the cationic liposomal formulations L1 and L2, concentrations of 0.050 and 0.032 mg carboplatin per mg lipids were obtained. A greater accumulation of carboplatin was measured in the anionic formulation L3, which reached 0.096 mg per mg lipids. It is noteworthy that the liposomes L4 were by far the best at encapsulating carboplatin, reaching a concentration of 0.32 mg per mg lipids (Table 1).

In vitro toxicity of drug-free liposomes and liposomal carboplatin

Toxicity associated with the different liposomal formulations on the F98 cells were determined (Fig. 2, Table 2). The 2 cationic liposomal carboplatin showed higher toxicity than the anionic formulations. A LD50 of 0.169 μM carboplatin was measured with the non-pegylated liposomal carboplatin L1. Encapsulation of carboplatin in this cationic liposomal formulation decreased its efficiency, leading to a LD50 80 times higher than that of free carboplatin (LD50 = 13.6 μM). The drug-free cationic non-pegylated liposome L1' showed some toxicity for the F98

cells, with an LD50 of 6.07 μM in lipid equivalent, which corresponds to about 3 times more lipids then used for the carboplatin formulation L1 (Fig. 2a, Table 2). To determine whether this toxicity was caused by the preparation method, the same liposomal formulation was prepared by the hydration method (L1''). The toxicity of liposomes L1'' (LD50 of 8.08 μM total lipids) on the F98 cells, was similar to that measured with L1'. At the LD50 of liposomal carboplatin L1, the total lipids concentration was 1.98 μM , which corresponded to about 3.6 times less lipids than at the LD50 concentrations of formulations L1' and L1''. Therefore, while it cannot be excluded that a part of the toxicity measured with the carboplatin formulation L1 is due its lipid components, the toxic effect on the F98 cells was mainly caused by carboplatin.

The second cationic formulation L2 was prepared by adding ~5 mol% PEG2000 PE (Table 1). This cationic pegylated liposome exhibited a slightly, but not significantly, lower positive charge, which was associated with the use of the anionic PEG2000 PE. It is noteworthy that the addition of PEG-modified lipids improved

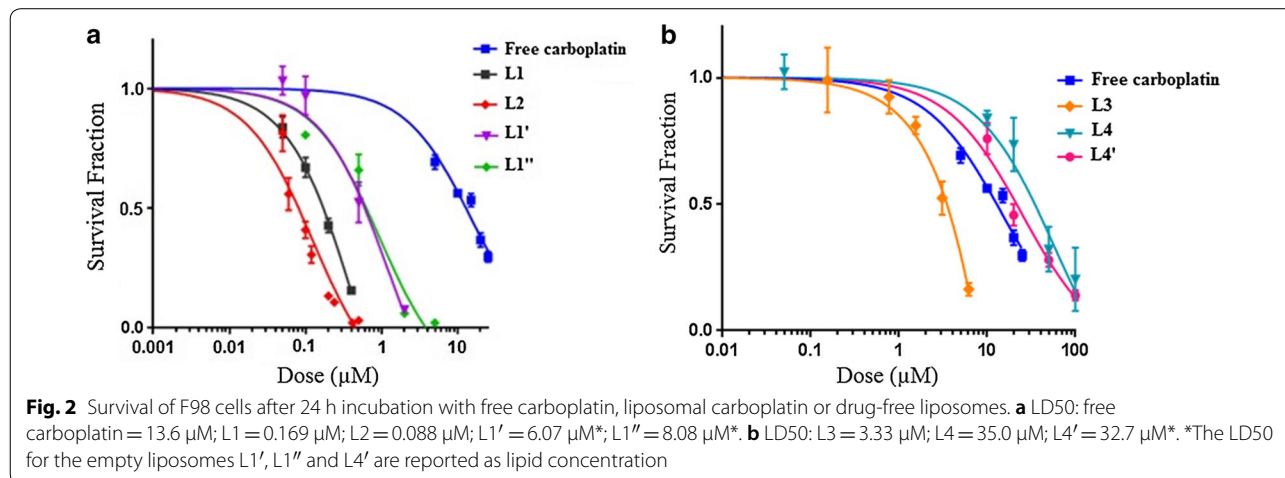


Table 2 LD50 and cellular uptake of free carboplatin and liposomal formulations of carboplatin

ID	Drugs	LD50		Cellular uptake ^a (ng Pt/10 ⁶ cells)	Cellular uptake normalized for LD50 (ng Pt/10 ⁶ cells/ μ M LD50)
		Carboplatin (μ M)	Lipids (μ M)		
	Dextrose	–	–	–	–
	Carboplatin	13.6	–	12.8 \pm 1.2	0.94
L1	Liposomal carboplatin	0.169	1.98	36.8 \pm 8.0	218.75
L1'	Hollow liposomes	–	6.07	–	–
L1''	Hollow liposomes	–	8.08	–	–
L2	Liposomal carboplatin	0.088	1.03	1.9 \pm 0.1	21.64
L3	Liposomal carboplatin	3.33	17.0	8.7 \pm 2.1	2.61
L3'	Hollow liposomes	–	> 509	–	–
L4	Liposomal carboplatin	35.0	30.8	26.5 \pm 1.9	0.76
L4'	Hollow liposomes	–	32.7	–	–

^a Cell uptake of carboplatin (Pt) measured after 24 h incubation at the concentration corresponding to the LD50 of each drug

the anti-cancer potential of carboplatin encapsulated in this liposomal formulation, as shown by its lower LD50 (0.088 μ M) compared to 0.169 μ M for the L1 liposome.

The anionic liposome L3 was made with 20 mol% DSPG, an anionic lipid that is known to be a suitable replacement for PEG-modified lipids in terms of reducing surface–surface association, which can lead to liposome aggregation and/or liposome–cell interactions [27, 28]. The LD50 of this formulation prepared without carboplatin (L3') was more than 500 μ M total lipids, making it by far the least toxic of the cationic formulations tested. When carboplatin was encapsulated in this anionic formulation L3, a large improvement in LD50 was measured, reaching 3.33 μ M carboplatin, which corresponded to 17.0 μ M total lipids. These results also indicate that the toxicity of L3 on F98 cells was due solely to the carboplatin. Furthermore, the L3 formulation was about 4-times more efficient than free carboplatin (LD50 = 13.6 μ M).

For the last formulation L4, DC-Chol was replaced by cholesterol to remove the positive charge of the liposomes, while PEG2000 PE was included to obtain a negative Zeta potential (Table 1). These L4' anionic liposomes without carboplatin shown a LD50 of 32.7 μ M which was more than five times less toxic than the cationic liposomes without carboplatin L1' and L1''. However, encapsulation of carboplatin in the anionic pegylated formulation L4 was not effective for treating the F98 cells since its LD50 was 35.0 μ M, which corresponded to 30.8 μ M of lipids used to make the liposomes. These results suggested that the toxicity of the L4 may be caused by the lipids used to make them. Moreover, the efficiency of the L4 liposome was 10.5 to 400 times lower than the other liposomal formulations of carboplatin (Table 2).

Cellular accumulation of liposomal formulations carboplatin

Drug delivery to cancer cells can be affected by the presence of negative or positive charge and also of PEG on the liposome surface [18]. The ability of our liposomal formulations to deliver carboplatin to the F98 cells was therefore determined experimentally. Cellular carboplatin concentration was normalized to take account of the LD50 of each liposomal formulation. The cationic non-pegylated L1 increased by more than 230 times the carboplatin concentration in the F98 cells relative to free carboplatin. The addition of PEG on the cationic liposome (L2) decreased by 10 times the concentration of carboplatin in the F98 cells relative to the non-pegylated L1, but it still resulted in a 23 times higher accumulation than free carboplatin. On the other hand, only a marginal improvement of carboplatin uptake was measured with the anionic liposome L3, while the pegylated anionic liposome L4 didn't lead to any improvement relative to free carboplatin.

Median survival time (MeST) of F98 glioma bearing rats treated with liposomal carboplatin

The MTD of cationic non-pegylated liposomal carboplatin (L1, 10 μ g) and the pegylated one (L2, \geq 18 μ g, maximum injectable dose) were lower than achieved with free carboplatin (25 μ g), which showed that these cationic formulations couldn't reduce the toxicity of carboplatin on Fisher rats (Table 3). Conversely, the anionic liposomes successfully reduced the toxicity of carboplatin. The liposomal carboplatin L3 and the pegylated form L4 exhibited a MTD of > 38.7 μ g and 50 μ g, respectively.

Their anti-tumor efficiency were then assessed at their respective MTD, or at the maximum injectable dose for L2 and L3, in Fischer rat bearing the F98

Table 3 Median survival time of F98 glioma bearing Fischer rats

ID	Drugs	MTD (µg)	MeST (days)	Range
	Dextrose	–	23.5	20–25
	Carboplatin	25	38.5	31–47
L1	Liposomal carboplatin	10	35.0	31–42
L1'	Hollow liposomes	–	22.5	22–23
L1''	Hollow liposomes	–	–	–
L2	Liposomal carboplatin	18 ^a	29.0	25–32
L3	Liposomal carboplatin	38.7 ^a	31.0	28–34
L3'	Hollow liposomes	–	–	–
L4	Liposomal carboplatin	50	49.5	31–71
L4'	Hollow liposomes	–	25	22–26

LD50 lethal dose for 50% of the cells, MeST Median survival time, MTD maximum tolerated dose

^a Maximal concentration that can be injected

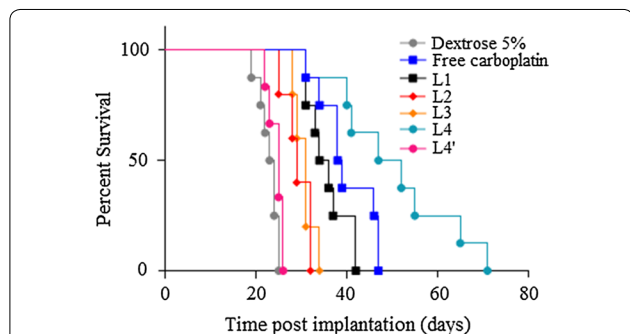


Fig. 3 Kaplan–Meier survival curve of F98 glioma bearing rats treated with CED of different carboplatin formulations. Cationic liposomes L1 and L2 improved median survival times (35 days for non-pegylated L1, 29 days for pegylated L2) when compared to control (dextrose 5%) (23.5 days), but shorter or equivalent survival time when compared to free carboplatin (38.5 days). Anionic pegylated liposomal carboplatin L4 offered the best median survival time (49.5 days) and showed a better median survival time than free carboplatin. Injection with the empty anionic pegylated liposome L4' didn't improved the median survival time (25 days) which was similar to animals injected with dextrose 5%. Log Rank test *p* value compared to 5% dextrose: L1, *p* < 0.0001; L2, *p* = 0.0018; L3, *p* = 0.0003; L4, *p* < 0.0001; L4', *p* = 0.447 (*p* = 0.0001, when compared to L4), free carboplatin, *p* < 0.0001

GBM tumor (Fig. 3, Table 3) to comply with clinical practice. Free carboplatin increased animal survival time by 15 days with respect to the untreated group (carboplatin = 38.5 days; untreated group = 23.5 days, *p* < 0.0001). Encapsulation of carboplatin in the liposomes L1 didn't significantly increase the MeST, which reached 35 days (*p* value, free carboplatin vs L1 = 0.08). The addition of PEG was no benefit since a reduction in anti-tumor activity relative to free

carboplatin was measured for the cationic pegylated liposomal L2, as shown by a shorter MeST of 29 days (*p* value, free carboplatin vs L2 = 0.0015). The anionic non-pegylated liposome L3 was no better with a MeST of 31 days (*p* value, L2 vs L3 = 0.6313). It is noteworthy that L2 and L3 were not assessed at their MTD but instead at their maximum injectable dose.

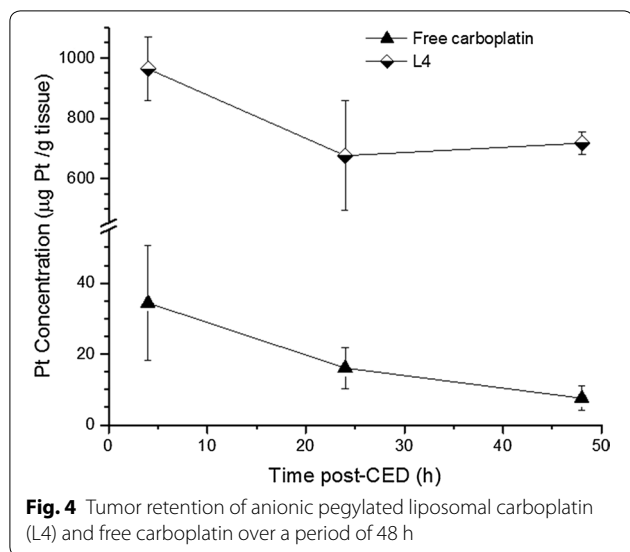
The highest anti-tumor efficiency was observed with the anionic pegylated liposomal carboplatin L4, with a MeST of 49.5 days, which is significantly better than that achieved with free carboplatin (*p* = 0.0335), and the cationic liposome L1 (*p* = 0.0008). The empty liposomes L4' (carboplatin free) showed a similar MeST to that of the untreated group, which suggests that the lipid formulation of L4 was not itself toxic to tumor cells.

Accumulation of carboplatin in brain tumor

The ability of L4 liposomes, which achieved the best therapeutic outcomes in the F98 model, to deliver carboplatin in brain tumor by CED was determined. The amount of carboplatin in tumor was quantified at 4, 24 and 48 h after CED by ICP-MS. Free-carboplatin injected at its MTD was rapidly eliminated (Fig. 4). Four hours after its injection, the carboplatin concentration in tumor reached only 34.4 µg/g tissue. This rapid elimination of carboplatin was largely prevented by its encapsulation into the liposome L4 which allowed a concentration of 963.7 µg/g tissue to be maintained at 4 h after CED (Fig. 4). Furthermore, the concentration of carboplatin in tumor has decreased by only 1.3-fold at 48 h after CED, reaching 719.1 µg/g tumor. These results indicate that the anionic liposomal formulation L4 favors the accumulation and retention of carboplatin in the brain tumor F98.

Toxicity of empty liposome L4'

The empty liposome L4 (named L4') was administrated by CED to assess the contribution of its lipid components in the improvement of MeST in rat implanted with the F98 tumor, and also in tumor-free animals to assess its neurotoxicity. The liposome L4' was injected at a lipid concentration equivalent to that of the MTD of the liposome L4. In animal implanted with the F98 tumor, the liposome L4' didn't modify the MeST which shows that this empty liposome had no therapeutic effect on the tumor cells (Table 3, Fig. 3). Fifty days post-CED in tumor-free animals, no signs of neurotoxicity were observed. Histopathological analyses showed that the empty liposome L4' didn't affect brain structures (Fig. 5). These neurotoxic analyzes were not performed with the other liposomal formulations since they did not improve the MeST compared to that of free carboplatin.



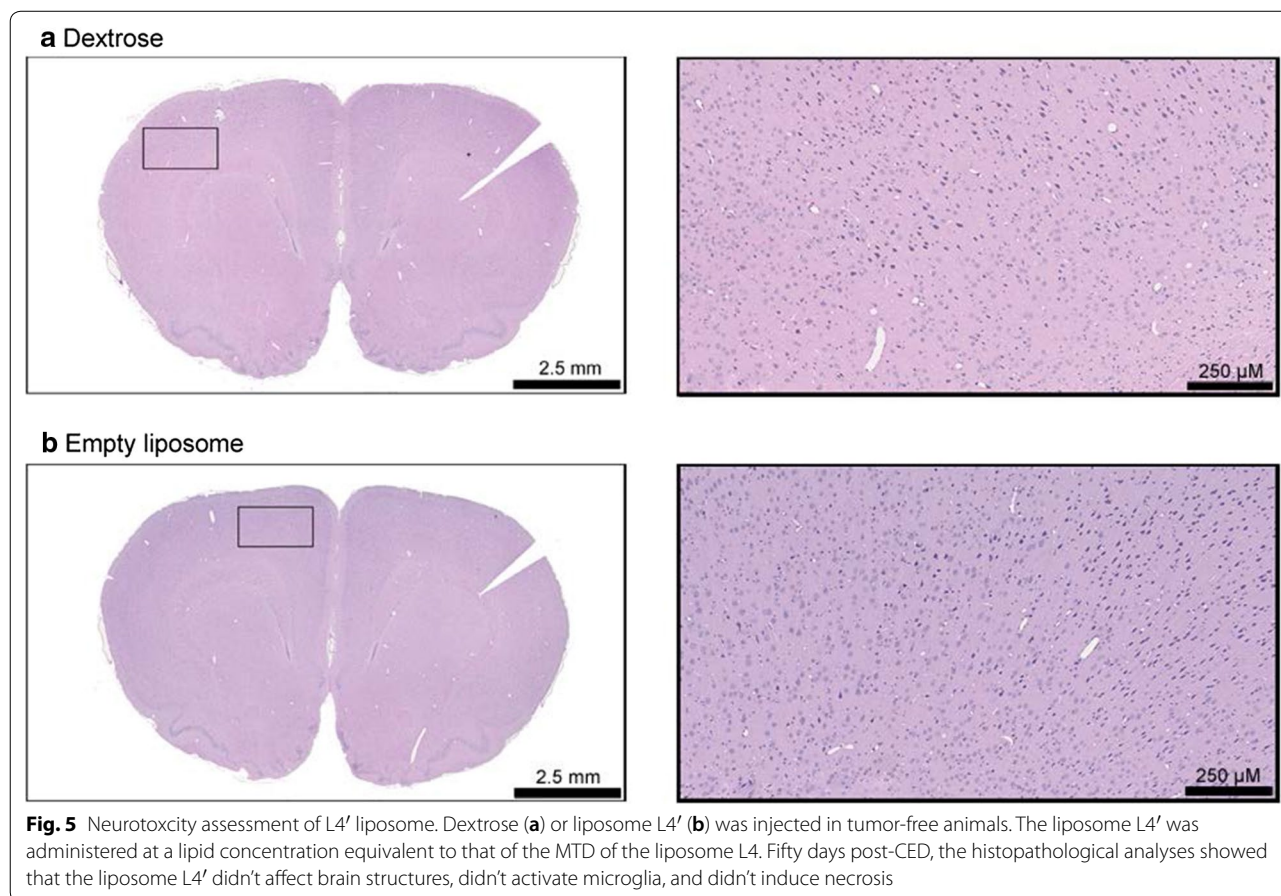
Discussion

To overcome this obstacle of BBB, platinum-based drugs have been injected directly into the tumor by CED in

animals bearing a glioblastoma [4, 6–8, 29]. The pressure gradient created by CED should significantly increase the drug distribution volume in the brain allowing higher concentrations to be attained over longer distances [28].

Carboplatin was chosen for our study, because it is better tolerated than cisplatin and oxaliplatin, and produces the highest therapeutic benefit in GBM animal models [4–8]. However, one shortcoming of carboplatin delivery via CED is its rapid clearance from the extracellular compartment. To overcome this limitation, liposomal formulations were developed to enhance tumor retention and reduce neurotoxicity. Physicochemical properties of liposomes can influence their ability to retain a drug, bind to target cells, and spread through a tumor [28, 30, 31]. In our study, the effects of charge and PEG on four liposomal formulations of carboplatin were assessed from: (i) a cationic non-pegylated formulation (L1), (ii) a cationic pegylated formulation (L2), (iii) a anionic non-pegylated formulation (L3), and (iv) a anionic pegylated formulation (L4).

Bypassing the BBB with a direct injection into the tumor enables the concentration of carboplatin to exceed that obtainable by i.v. injection. Using the same animal



model of GBM, systemic injection of liposomal formulations of cisplatin or oxalyplatin led to only 0.32 $\mu\text{g/g}$ tissue and 0.14 $\mu\text{g/g}$ tissue of these platinum derivatives in tumor [5], which corresponds to 3000 to 7000 times lower concentrations than obtained after CED injection with our liposome L4. These results also suggest that liposome L4 administrated by CED may circumvent carboplatin resistance in cancer cells.

After distribution of the liposomes through the tumor by CED, the restricted extracellular space may improve their retention. In our study, encapsulation of carboplatin in pegylated liposome L4 has increased by 28 times the concentration of this platinum derivative in F98 tumor, relative to free carboplatin. The clearance rate between the period of 4 h to 48 h post-CED was also significantly reduced with the liposome L4. The concentration of free carboplatin decreased by 4.5 times, while those of liposome L4 went down by only a factor 1.3. Using the same animal model, a similarly rapid elimination of free carboplatin after its injection directly in the tumor was reported, as only 10.4 $\mu\text{g/g}$ tissue was measured immediately after CED [8].

When a drug is injected directly into the brain, minimizing its neurotoxicity becomes a major issue. Drug encapsulation into liposomes was thus initially proposed to reduce the neurotoxicity [18, 26, 32]. However, some liposomal formulation can be very neurotoxic. The negatively charged LipoplatinTM and its empty version without cisplatin were highly neurotoxic when given by CED in rat brain resulting in death immediately following or within a few days after administration [26]. Intracranial injection of liposomes without drug, composed of lecithin–cholesterol–dicetyl phosphate (net negative charge) or lecithin–cholesterol–stearylamine (net positive charge) induced epileptic seizures and some deaths due to respiratory failure immediately after injection, and a subsequent widespread tissue necrosis [33]. Assessed in tumor free animals, our cationic liposomal formulation L1 also failed to reduce the neurotoxicity as its MTD was even lower than free carboplatin. The MTD of the pegylated version L2, couldn't be assessed as the intracranial injection was limited by maximal injectable volume. The 2 negatively charged liposomes L3 and the pegylated L4 were better tolerated as their MTD was about 2-times higher than that of free carboplatin. The neurotoxicity could be caused either by the lipid composition of the liposome, or be associated to the release of carboplatin from the liposomes into the brain. Consequently, the potential neurotoxicity of the empty liposomes L4 (named L4') was assessed at a lipid concentration equivalent to that of the MTD of the liposomes L4. Fifty days post-CED, no signs of neurotoxicity were observed.

Histopathological analyses showed that the empty liposomes L4' didn't affect brain structures. In animal implanted with the F98 tumor, the liposome L4' didn't modify the MeST of the animals, which shows that this empty liposomal formulation had no therapeutic effect on the tumor cells nor significant neurotoxicity.

Another benefit of adding PEG is to increase the stability of drug encapsulation in liposomes, which can potentially reduce its toxicity to non-target cells [34–36]. Although a significant improvement is already measured with only 4% PEG, a much higher stability can be achieved with 8% PEG and more [35]. Since our liposomes contained 4.8% PEG, this addition may have contributed to reduce carboplatin toxicity for cerebral parenchyma in our rat model.

Lipid composition, charge, pegylation, and diameter of liposomes and also the chemical characteristics of a drug affect its loading efficiency into liposomes [37]. With our liposomal formulations, the highest encapsulation of carboplatin was obtained with the cationic pegylated formulation L4, which reached a concentration similar to that previously reported by Zhang et al. [38]. Our results also support the hypothesis that higher drug loading can be obtained with positive liposome compared to those with a negative charge [39, 40], which can be further increased by adding PEG [40].

Carboplatin and its analogues cisplatin and oxalyplatin use the copper transporter Ctr1 to cross the cell membrane. Resistance to cisplatin has been associated with a reduction in the efficacy of this receptor [41]. Liposomes are taken up by the tumor cells either via phagocytosis or by direct fusion with the cell membrane. These two mechanisms enable a 10- to 400-fold higher intracellular uptake of platinum drugs in tumor cells compared to normal cells, by bypassing the carboplatin resistance of tumor cells [42]. It was also shown that the uptake of cationic liposomes by cancer cells was about 20-fold higher than for anionic liposomes [43]. We have measured a similar enhancement of carboplatin accumulation in vitro in the F98 cells. The highest accumulations of carboplatin were obtained with the cationic liposomes L1 and L2, while a modest increase was measured with the anionic liposomes L3. Pegylation of the anionic liposome reduced the net negative charge of the liposome L4, and this modification seems to have reduced the interaction between the liposome and the cancer cells, since no improvement of carboplatin accumulation in the F98 cells occurred. It was previously suggested that PEG can reduce the uptake of liposome by cells, because the binding of these polymers to cell membranes decreases the cell surface hydrophobicity, which reduces the fluid phase of endocytic process [44], and consequently compromises the internalization of liposome [18, 31].

The relative effectiveness of our liposomes to increase carboplatin accumulation corresponds exactly to their ability to eliminate the F98 cells *in vitro*, the best one being the best the L1 formulation. Conversely, the liposome L4 that couldn't improve the LD50 of carboplatin *in vitro*, was surprisingly the only liposome formulation to increase significantly the MeST of rats implanted with the F98 tumor.

We hypothesize that the higher therapeutic efficiency of our pegylated anionic liposome L4 compared to the cationic liposomes L1 and L2 was caused by a larger distribution volume in the tumor. Indeed, Mackay et al. have reported that their anionic 80 nm liposomes travelled for longer distances as they obtained a penetration radius of about 0.8 mm from the CED injection site, compared to less than 0.2 mm for their cationic liposomes [18]. Similar observations were made by Nie et al. who reported that their cationic liposomes were more readily taken up by cells, both *in vitro* and *in vivo*, because of the interaction with the negatively-charged cell membrane [45], which can thus contribute to reduce the distribution volume of cationic liposomes.

When delivered by CED, pegylated liposomes could travel for longer distances than non-pegylated liposomes. Presumably, steric shielding caused by PEG reduces the rate of liposome binding to cells in the brain during CED [18]. However, MacKay et al. have reported that a significant quantity of their pegylated and non-pegylated cationic liposomes were located near the infusion cannula used for CED [18]. The tumor cells near the injection site were thus exposed to a higher concentration of carboplatin than those located farther within the tumor. This non-uniform distribution of carboplatin could explain why our cationic liposomes did not significantly improve MeST of the animals.

Conclusions

The *in vitro* and *in vivo* behaviors of our cationic and anionic liposomes and their therapeutic efficiency varied drastically. Cationic liposomes are known to bind more efficiently to the cell membrane, facilitating carboplatin accumulation in tumor cells and increasing their therapeutic efficacy *in vitro*. In the animal model of GBM, this property of cationic liposomes didn't significantly improve the MeST. Conversely, the anionic and pegylated liposomes can diffuse over a larger distance in the tumor, probably resulting in a more uniform distribution of carboplatin. Supporting this hypothesis, our anionic pegylated liposome L4 was the best to improve the MeST, and the encapsulation of carboplatin in this liposome had also the advantage of increasing its concentration in the tumor, reducing its clearance rate and reducing neurotoxicity relative to free carboplatin. With these properties

and an anti-tumor efficiency significantly better than that achieved with free carboplatin, intratumor injection of liposomal carboplatin L4 should be considered as a promising replacement for pure carboplatin in the chemotherapeutic treatment of GBM tumors.

Methods

Chemicals

Carboplatin was purchased from Hospira (Montreal, Canada) and Hande Tech Development Co (Houston, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (Chol), 3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2000 PE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-distearoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DSPG) were obtained from Avanti Polar Lipids Inc (Alabaster, USA).

Preparation of liposomal carboplatin

The liposomal formulations of carboplatin identified as cationic non-pegylated (L1), cationic pegylated (L2), and anionic pegylated (L4), and also the carboplatin-free liposomes cationic non-pegylated (L1'), and anionic pegylated (L4') were prepared by the reverse phase evaporation method [46]. Briefly, DPPC:DC-Chol (1:1 mol) (for cationic non-pegylated liposomes) or DPPC:DC-Chol/Chol:PEG2000 PE (10:11:1 mol) (for cationic/anionic pegylated liposomes) were co-dissolved in chloroform:isopropyl ether (1:1 vol) solvent. Then, 2 mL of 27 mM carboplatin or 5% dextrose (for empty liposomes) was added and sonicated for 5 min. The organic solvent was removed under reduced pressure for 15 min by a rotary evaporator. Rotary evaporation was then continued for 1 h. Then, unencapsulated carboplatin was removed by PD-10 desalting column containing Sephadex™ G-25 Medium (GE Healthcare Bio-Sciences Corp, Piscataway, USA).

Anionic non-pegylated liposomal carboplatin (L3) and carboplatin-free anionic non-pegylated liposomes (L3') were prepared by the hydration method through dissolving DSPC:DSPG:Chol (7:2:1 mol) in chloroform:methanol:water (50:10:1 vol). Lipids were then dried under a stream of nitrogen gas to form a thin film. Subsequently, the lipid film was subjected to low vacuum to remove any residual solvent. Lipid films were then hydrated by vortexing in the presence of hot (70 °C) SH buffer (300 mM sucrose, 20 mM HEPES, pH 7.5) with or without carboplatin (100 mM). The resulting lipid-buffer suspension was then extruded 10 times through stacked 100 nm and 80 nm polycarbonate filters at 70 °C (Northern Lipids Inc., BC, Canada). The external buffer was then exchanged with SH buffer at room temperature by a

G-50 Sepadex™ column. The concentration of carboplatin was determined by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Scientific XSERIES 2, Thermo Fisher Scientific Inc.) [32].

As an additional control, empty cationic non-pegylated liposomes (L1'') were also prepared according to the hydration method by dissolving DPPC:DC-Chol (1:1 mol) in chloroform:isopropyl ether (1:1 vol) solvent. Lipids were then dried under reduced pressure by a rotary evaporator to form a thin film. Lipid film was then hydrated by vortexing in the presence of 2 mL 5% dextrose. The resulting lipid-buffer suspension was frozen and thawed for five times, and then extruded 10 times through stacked 100 nm and 80 nm polycarbonate filters at 50 °C (Northern Lipids Inc., BC, Canada).

Characterization of liposomal carboplatin

The size and appearance of liposomes containing carboplatin were characterized with a Hitachi H-7500 transmission electron microscopy (TEM) operating at 80 kV. Samples were negative stained by mixing liposomal carboplatin with ammonium heptamolybdate and 2% of uranyl acetate. Charge on the liposomes was confirmed by measuring the Zeta-potential with a zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Phospholipids were quantified by choline oxidase-DAOS method following the protocol of Wako Phospholipids C (Wako Diagnostics Wako Chemicals USA, Inc., Richmond, USA).

Cell lines and animal model

F98 rat glioblastoma cells were purchased from American Type Culture Collection. Male Fischer rats of 210–225 g were purchased from Charles River Laboratories (Saint-Constant, Canada). The experimental animal protocol was approved by the institutional ethical committee and complied with the regulations of the Canadian Council on Animal Care (protocol # 329-13B).

Cytotoxicity study of carboplatin and its liposomal formulations in F98 cells

Cytotoxicity was assessed using a clonogenic assay as previously described [47]. Briefly, 400 cells were plated in 6-well plate, and 24 h later carboplatin or its liposomal formulations in FBS-free DMEM medium were added. After an additional 24 h, the drug-containing medium was removed, the cells were washed, and new DMEM medium enriched with 10% FBS, was added. Seven days later the colonies were stained with 0.1% crystal violet, and counted. The median lethal dose (LD50) for each liposomal formulation of carboplatin was then determined.

Cellular uptake of carboplatin and its liposomal formulations in F98 cells

F98 cells were incubated with carboplatin or one of the liposomal formulations at their respective LD50 for 24 h in FBS-free DMEM medium, and then washed three times with PBS, harvested, and counted. Cells were digested in 70% HNO₃ and 30% H₂O₂, followed by quantification for platinum with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (ELAN DRC-II, PerkinElmer) [32]. Data were expressed as ng of platinum per 10⁶ cells.

F98 cells implantation in Fischer rat brain

As described previously [48], the rat's head was mounted on a stereotaxic frame, an incision was made in the middle of the head and the bregma exposed. A burr hole was made with a dental drill, 3 mm to the right, 1 mm anterior of the bregma. Five microliter of FBS-free DMEM containing 10,000 cells was injected 6 mm deep into the burr hole over 5 min at a constant flow. Then, the needle was slowly withdrawn over 1 min and the burr was sealed by bone wax. Finally, the incision was sutured and smeared with antibiotic cream.

CED procedure

A solution of free carboplatin or liposomal formulations (10 µL in 5% dextrose) was injected by CED 10 days after tumor cell implantation with a microinfusion pump (World Precision Instruments) a rate of 0.5 µL/min for 20 min using a Hamilton syringe connected to a 33 Ga needle [4].

Determination of MTD for the liposomal carboplatin

The MTD of liposomal carboplatin was determined in Fisher rats by the traditional 3 + 3 design, which consists of dose escalation and de-escalation based on the number of dose limiting toxicities observed in a cohort of 3 animals [49]. Rats were followed during 10 days after CED. Those that were unable to feed or groom, or lethargic were considered to be moribund and were euthanized.

Assessment of median survival time

To comply with clinical practice, anti-tumor efficiencies were determined by animal survival assays performed at MTD. Ten days after F98 tumor implantation, each liposomal formulation and free carboplatin were injected by CED at their respective MTD. Animals were daily monitored which included weight measurement, mobility, coordination, loss of self-grooming (periocular secretion accumulation) and landing ability. They were euthanized when one of the monitored indexes reached a score of 1/10 or when they lost > 30% of their initial weight.

Tumor uptake of free carboplatin and the anionic pegylated liposomal formulation L4

Free carboplatin or anionic pegylated liposomal carboplatin (L4) was delivered at their respective MTD by CED. Animals ($n=3$) were euthanized at 4, 24 or 48 h after CED. Blood was evacuated by intra-cardiac infusion of 4% paraformaldehyde, and then the brain was extracted. Using a brain matrix, a slice of the brain was obtained from a millimeter before the tumor cell implantation point to a millimeter afterwards. Tumor and normal tissue around it were isolated, weighed, and digested in 70% HNO_3 and 30% H_2O_2 . The concentrations of platinum were analyzed by ICP-MS [32].

Histopathology

Euthanasia was carried out by exsanguination and intra-cardiac perfusion of 30 ml formaldehyde 4% for histological analysis. Brain specimens were removed and kept in formaldehyde for 48 h prior to be cut in the coronal plane using a brain matrix (taking the implantation needle mark on the cortex as a reference point for the slicing), and finally embedded into paraffin. The blocks were cut into 5 μm thick slides and stained with haematoxylin and eosin.

Statistical analysis

Results were analyzed by a Student's t test to compare two treatments together and by ANOVA for more than two groups. The median survival times were determined by the Quartile method before doing Kaplan–Meier survival curves which were analyzed by Log-Rank test with GraphPad Prism software. A p value < 0.05 was considered as statistically significant.

Abbreviations

BBB: blood–brain barrier; CED: convection-enhanced delivery; Chol: cholesterol; DC-Chol: 3 β -[N-(N,N-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPG: 1,2-distearoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt); GBM: glioblastoma; i.a.: intra-arterial; ICP-MS: inductively coupled plasma mass spectrometry; i.v.: intravenous; L1: cationic liposomes without pegylation; L2: cationic liposomes with pegylation; L3: anionic liposomes without pegylation; L4: anionic liposomes with pegylation; LD50: median lethal dose; MeST: median survival time; MTD: maximum in vivo tolerated dose; PEG: pegylation; PEG2000 PE: 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; TEM: transmission electron microscopy.

Authors' contributions

All authors have contributed to analyzing the results and writing the manuscript. Design, preparation and characterization of the liposomal formulations were done by MS, MA, MW, MB, BP and LS. Design of the in vitro assays and those with the animal model were done by MS, DF, BP. All the in vitro assays were performed by MS, while MS, LOR and GC have collaborated for the animal experiments. The neuropathological analyses of rat brain was done by MR. All authors read and approved the final manuscript.

Author details

¹ Department of Radiation Oncology, The Second Affiliated Hospital of Zhejiang University, School of Medicine, Hangzhou, China. ² Experimental Therapeutics, British Columbia Cancer Agency, Vancouver, BC, Canada. ³ Centre for Drug Research and Development, Vancouver, BC, Canada. ⁴ Department of Surgery, Division of Neurosurgery, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, QC, Canada. ⁵ Department of Pharmacology, Université de Sherbrooke, Sherbrooke, QC, Canada. ⁶ Center for Research in Radiotherapy, Department of Nuclear Medicine and Radiobiology, Université de Sherbrooke, Sherbrooke, QC, Canada. ⁷ Department of Pathology, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, QC, Canada.

Acknowledgements

We would like to Dr. AD Bass for his helpful suggestions and corrections.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Consent for publication

All authors agreed to submit this study.

Ethics approval

The experimental protocol was approved by the institutional ethical committee and complied with the regulations of the Canadian Council on Animal Care (protocol # 329-13B).

Funding

Statement of funding: This work was supported by Canadian Institutes of Health Research (grant # MOP 81356 and MOP 102611, LS) and Canadian Cancer Society Research Institute (INNOV14-1 Award # 702491, MBB). David Fortin, Léon Sanche, Maxime Richer and Benoit Paquette are members of the Centre de Recherche du CHUS supported by the Fonds de la Recherche en Santé Québec en Santé.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 23 August 2018 Accepted: 24 September 2018

Published online: 05 October 2018

References

- Miranda A, Blanco-Prieto M, Sousa J, Pais A, Vitorino C. Breaching barriers in glioblastoma. Part I: molecular pathways and novel treatment approaches. *Int J Pharm*. 2017;531:372–88.
- Bobo RH, Laske DW, Akbasak A, Morrison PF, Dedrick RL, Oldfield EH. Convection-enhanced delivery of macromolecules in the brain. *Proc Natl Acad Sci USA*. 1994;91:2076–80.
- Chen PY, Ozawa T, Drummond DC, Kalra A, Fitzgerald JB, Kirpotin DB, Wei KC, Butowski N, Prados MD, Berger MS, et al. Comparing routes of delivery for nanoliposomal irinotecan shows superior anti-tumor activity of local administration in treating intracranial glioblastoma xenografts. *Neuro Oncol*. 2013;15:189–97.
- Shi M, Fortin D, Sanche L, Paquette B. Convection-enhancement delivery of platinum-based drugs and Lipoplatin(TM) to optimize the concomitant effect with radiotherapy in F98 glioma rat model. *Invest New Drugs*. 2015;33:555–63.
- Charest G, Sanche L, Fortin D, Mathieu D, Paquette B. Optimization of the route of platinum drugs administration to optimize the concomitant treatment with radiotherapy for glioblastoma implanted in the Fischer rat brain. *J Neurooncol*. 2013;115:365–73.
- Biston MC, Joubert A, Adam JF, Elleaume H, Bohic S, Charvet AM, Esteve F, Foray N, Balosso J. Cure of Fisher rats bearing radioresistant

- F98 glioma treated with cis-platinum and irradiated with monochromatic synchrotron X-rays. *Cancer Res.* 2004;64:2317–23.
7. Rousseau J, Boudou C, Barth RF, Balosso J, Esteve F, Elleaume H. Enhanced survival and cure of F98 glioma-bearing rats following intracerebral delivery of carboplatin in combination with photon irradiation. *Clin Cancer Res.* 2007;13:5195–201.
 8. Yang W, Huo T, Barth RF, Gupta N, Weldon M, Grecula JC, Ross BD, Hoff BA, Chou TC, Rousseau J, Elleaume H. Convection enhanced delivery of carboplatin in combination with radiotherapy for the treatment of brain tumors. *J Neurooncol.* 2011;101:379–90.
 9. Rezaee M, Hunting DJ, Sanche L. New insights into the mechanism underlying the synergistic action of ionizing radiation with platinum chemotherapeutic drugs: the role of low-energy electrons. *Int J Radiat Oncol Biol Phys.* 2013;87:847–53.
 10. White E, Bienemann A, Pugh J, Castrique E, Wyatt M, Taylor H, Cox A, McLeod C, Gill S. An evaluation of the safety and feasibility of convection-enhanced delivery of carboplatin into the white matter as a potential treatment for high-grade glioma. *J Neurooncol.* 2012;108:77–88.
 11. Saito R, Tominaga T. Convection-enhanced delivery of therapeutics for malignant gliomas. *Neurol Med Chir (Tokyo).* 2017;57:8–16.
 12. Saito R, Krauze MT, Noble CO, Drummond DC, Kirpotin DB, Berger MS, Park JW, Bankiewicz KS. Convection-enhanced delivery of LS-TPT enables an effective, continuous, low-dose chemotherapy against malignant glioma xenograft model. *Neuro Oncol.* 2006;8:205–14.
 13. Krauze MT, Forsayeth J, Yin D, Bankiewicz KS. Convection-enhanced delivery of liposomes to primate brain. *Methods Enzymol.* 2009;465:349–62.
 14. French JT, Goins B, Saenz M, Li S, Garcia-Rojas X, Phillips WT, Otto RA, Bao A. Interventional therapy of head and neck cancer with lipid nanoparticle-carried rhenium 186 radionuclide. *J Vasc Interv Radiol.* 2010;21:1271–9.
 15. Chen MY, Lonsler RR, Morrison PF, Governale LS, Oldfield EH. Variables affecting convection-enhanced delivery to the striatum: a systematic examination of rate of infusion, cannula size, infusate concentration, and tissue-cannula sealing time. *J Neurosurg.* 1999;90:315–20.
 16. Mamot C, Nguyen JB, Pourdehnad M, Hadaczek P, Saito R, Bringas JR, Drummond DC, Hong K, Kirpotin DB, McKnight T, et al. Extensive distribution of liposomes in rodent brains and brain tumors following convection-enhanced delivery. *J Neurooncol.* 2004;68:1–9.
 17. Chen MY, Hoffer A, Morrison PF, Hamilton JF, Hughes J, Schlageter KS, Lee J, Kelly BR, Oldfield EH. Surface properties, more than size, limiting convective distribution of virus-sized particles and viruses in the central nervous system. *J Neurosurg.* 2005;103:311–9.
 18. MacKay JA, Deen DF, Szoka FC Jr. Distribution in brain of liposomes after convection enhanced delivery; modulation by particle charge, particle diameter, and presence of steric coating. *Brain Res.* 2005;1035:139–53.
 19. Saito R, Krauze MT, Noble CO, Tamas M, Drummond DC, Kirpotin DB, Berger MS, Park JW, Bankiewicz KS. Tissue affinity of the infusate affects the distribution volume during convection-enhanced delivery into rodent brains: implications for local drug delivery. *J Neurosci Methods.* 2006;154:225–32.
 20. Perlstein B, Ram Z, Daniels D, Ocherashvili A, Roth Y, Margel S, Mardor Y. Convection-enhanced delivery of maghemite nanoparticles: increased efficacy and MRI monitoring. *Neuro Oncol.* 2008;10:153–61.
 21. Natsume A, Mizuno M, Ryuke Y, Yoshida J. Antitumor effect and cellular immunity activation by murine interferon-beta gene transfer against intracerebral glioma in mouse. *Gene Ther.* 1999;6:1626–33.
 22. Kang B, Mackey MA, El-Sayed MA. Nuclear targeting of gold nanoparticles in cancer cells induces DNA damage, causing cytokinesis arrest and apoptosis. *J Am Chem Soc.* 2010;132:1517–9.
 23. Bellavance MA, Poirier MB, Fortin D. Uptake and intracellular release kinetics of liposome formulations in glioma cells. *Int J Pharm.* 2010;395:251–9.
 24. Grahm AY, Bankiewicz KS, Dugich-Djordjevic M, Bringas JR, Hadaczek P, Johnson GA, Eastman S, Luz M. Non-PEGylated liposomes for convection-enhanced delivery of topotecan and gadodiamide in malignant glioma: initial experience. *J Neurooncol.* 2009;95:185–97.
 25. Qin Y, Chen H, Yuan W, Kuai R, Zhang Q, Xie F, Zhang L, Zhang Z, Liu J, He Q. Liposome formulated with TAT-modified cholesterol for enhancing the brain delivery. *Int J Pharm.* 2011;419:85–95.
 26. Huo T, Barth RF, Yang W, Nakkula RJ, Koynova R, Tenchov B, Chaudhury AR, Agius L, Boulikas T, Elleaume H, Lee RJ. Preparation, biodistribution and neurotoxicity of liposomal cisplatin following convection enhanced delivery in normal and F98 glioma bearing rats. *PLoS ONE.* 2012;7:e48752.
 27. Heurtault B, Saulnier P, Pech B, Proust JE, Benoit JP. Physico-chemical stability of colloidal lipid particles. *Biomaterials.* 2003;24:4283–300.
 28. Allard E, Passirani C, Benoit JP. Convection-enhanced delivery of nanocarriers for the treatment of brain tumors. *Biomaterials.* 2009;30:2302–18.
 29. Tange Y, Kondo A, Egorin MJ, Mania-Farnell B, Danerlialis GM, Nakazaki H, Sredni ST, Rajaram V, Goldman S, Soares MB, Tomita T. Interstitial continuous infusion therapy in a malignant glioma model in rats. *Childs Nerv Syst.* 2009;25:655–62.
 30. Yingchoncharoen P, Kalinowski DS, Richardson DR. Lipid-based drug delivery systems in cancer therapy: what is available and what is yet to come. *Pharmacol Rev.* 2016;68:701–87.
 31. Li Y, Wang J, Gao Y, Zhu J, Wientjes MG, Au JL. Relationships between liposome properties, cell membrane binding, intracellular processing, and intracellular bioavailability. *AAPS J.* 2011;13:585–97.
 32. Charest G, Sanche L, Fortin D, Mathieu D, Paquette B. Glioblastoma treatment: bypassing the toxicity of platinum compounds by using liposomal formulation and increasing treatment efficiency with concomitant radiotherapy. *Int J Radiat Oncol Biol Phys.* 2012;84:244–9.
 33. Adams DH, Joyce G, Richardson VJ, Ryman BE, Wisniewski HM. Liposome toxicity in the mouse central nervous system. *J Neurol Sci.* 1977;31:173–9.
 34. Hu Y, Zhao Z, Harmon T, Pentel PR, Ehrlich M, Zhang C. Paradox of PEGylation in fabricating hybrid nanoparticle-based nicotine vaccines. *Biomaterials.* 2018;182:72–81.
 35. Piperoudi S, Fatouros D, Ioannou PV, Frederik P, Antimisiaris SG. Incorporation of PEG-lipids in arsonoliposomes results in formation of highly stable arsenic-containing vesicles. *Chem Phys Lipids.* 2006;139:96–106.
 36. Hong MS, Lim SJ, Lee MK, Kim YB, Kim CK. Prolonged blood circulation of methotrexate by modulation of liposomal composition. *Drug Deliv.* 2001;8:231–7.
 37. Swaminathan J, Gobbo OL, Tewes F, Healy AM, Ehrhardt C. Encapsulation into PEG-liposomes does not improve the bioavailability of pulmonary delivered salmon calcitonin. *J Aerosol Med Pulm Drug Deliv.* 2014;27:1–11.
 38. Zhang J, Huang C, Huang H. Antitumor and antimetastasis effects of carboplatin liposomes with polyethylene glycol-2000 on SGC-7901 gastric cell-bearing nude mice. *Oncol Lett.* 2014;8:2209–14.
 39. Akhtar A, Wang SX, Ghali L, Bell C, Wen X. Effective delivery of arsenic trioxide to hpv-positive cervical cancer cells using optimised liposomes: a size and charge study. *Int J Mol Sci.* 2018;19:1081.
 40. Haghirsadat F, Amoabediny G, Helder MN, Naderinezhad S, Sheikhha MH, Forouzanfar T, Zandieh-Doulabi B. A comprehensive mathematical model of drug release kinetics from nano-liposomes, derived from optimization studies of cationic PEGylated liposomal doxorubicin formulations for drug-gene delivery. *Artif Cells Nanomed Biotechnol.* 2018;46:169–77.
 41. Matsumoto S, Tanaka T, Kurokawa H, Matsuno K, Hayashida Y, Takahashi T. Effect of copper and role of the copper transporters ATP7A and CTR1 in intracellular accumulation of cisplatin. *Anticancer Res.* 2007;27:2209–16.
 42. Boulikas T. Molecular mechanisms of cisplatin and its liposomally encapsulated form, Lipoplatin™. Lipoplatin™ as a chemotherapy and antiangiogenesis drug. *Cancer Therapy.* 2007;5:349–76.
 43. Jansons VK, Weis P, Chen T, Redwood WR. In vitro interaction of L1210 cells with phospholipid vesicles. *Cancer Res.* 1978;38:530–5.
 44. Vertut-Doi A, Ishiwata H, Miyajima K. Binding and uptake of liposomes containing a poly(ethylene glycol) derivative of cholesterol (stealth liposomes) by the macrophage cell line J774: influence of PEG content and its molecular weight. *Biochim Biophys Acta.* 1996;1278:19–28.
 45. Nie Y, Ji L, Ding H, Xie L, Li L, He B, Wu Y, Gu Z. Cholesterol derivatives based charged liposomes for doxorubicin delivery: preparation, in vitro and in vivo characterization. *Theranostics.* 2012;2:1092–103.

46. Szoka F Jr, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci USA*. 1978;75:4194–8.
47. Tippayamontri T, Kotb R, Paquette B, Sanche L. Synergism in concomitant chemoradiotherapy of cisplatin and oxaliplatin and their liposomal formulation in the human colorectal cancer HCT116 model. *Anticancer Res*. 2012;32:4395–404.
48. Mathieu D, Lecomte R, Tsanaclis AM, Larouche A, Fortin D. Standardization and detailed characterization of the syngeneic Fischer/F98 glioma model. *Can J Neurol Sci*. 2007;34:296–306.
49. Korn EL, Midthune D, Chen TT, Rubinstein LV, Christian MC, Simon RM. A comparison of two phase I trial designs. *Stat Med*. 1994;13:1799–806.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

