Peer-reviewed version available at Molecules 2019, 24, 4312; doi:10.3390/molecules24234312

Review

Nasal Drug Delivery of Anticancer Drugs for the Treatment of Glioblastoma: Preclinical and Clinical Trials

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Abstract: Glioblastoma (GBM) is the most lethal form of brain tumor, characterized by rapid growth and surrounding tissue invasion. The current standard treatment is surgery followed by radiotherapy, and concurrent chemotherapy, typically with temozolomide. Although extensive research has been performed over the past years to develop an effective therapeutic strategy for the treatment of GBM, efforts have not provided major improvements in the overall survival of patients with GBM. Thus, new therapeutic approaches are urgently needed. A major challenge in the development of therapies for central nervous system (CNS) disorders is overcoming the blood–brain barrier (BBB). In this context, the intranasal (IN) route of drug administration has been proposed as a non-invasive alternative route to directly targeting the CNS. In fact, this route of drug administration may bypass the blood-brain barrier and reduce systemic side effects. Recently, formulations have been developed to further enhance nose-to-brain transport, mainly with the use of nano-sized and nanostructured drug delivery systems. The focus of this review will be on the strategies developed to deliver a number of anticancer compounds for the treatment of GBM using the nasal administration. In particular, the specific properties of nanomedicines proposed for the nose-to-brain delivery will be critically evaluated. The number of preclinical and clinical data reviewed support the idea that nasal delivery of anticancer drugs might represent a breakthrough advancement in the fight against GBM.

Keywords: nasal delivery; glioblastoma multiforme; drug delivery; nanoparticles; nose-to-brain delivery; pre-clinical studies; clinical evaluation.

1. Introduction

Malignant brain tumors are a devastating disease with high morbidity and mortality in adults. In children they are the second leading cause of cancer related deaths [1,2]. Glioblastoma multiforme (GBM) is the most common and most lethal malignant primary brain tumor in adults. Moreover, it has a recurrence rate of more than 90% even after multimodal treatments that combine surgery and chemotherapy [3]. Based on the level of malignancy, the World Health Organization (WHO) classified GBM as grade IV tumor. GBM is the most invasive and aggressive type of glial tumors with

high malignancy grade. Primary GBM, i.e. arising without a known precursor, are the most common form of GBM (~90%) and tend to be more aggressive and generally affect older patients. Alternatively, secondary GBM develops slowly through progression from a lower-grade astrocytic tumor (WHO Grade II or III). They manifest in younger patients and carry a significantly more favorable prognosis. Histologically, primary and secondary GBM are indistinguishable, but they differ in their genetic and epigenetic profiles [4,5].

It is estimated that GBM has an incidence of 3.19 per 100,000 persons in the United States [6], with median survival time of around 7-15 months from the time of diagnosis [7]. Incidence rates of glioblastoma increase with age, with the highest rates in individuals aged between 75 and 84 years [6]. Patient survival at five years form diagnosis is lower than 5% [8]. The etiology of GBM is complex and has not been fully elucidated, but a mix of genetic and environmental factors is the most likely cause of the disease [9].

GBM is a malignancy extremely challenging to treat due to its highly invasive nature and current treatments are based on maximal surgical resection followed by radiotherapy and adjuvant chemotherapy [10]. Temozolomide (TMZ) is the standard chemotherapeutic agent for the treatment of GBM. This second-generation imidazotetrazinone derivative exerts its anticancer effect through DNA methylation [11]. TMZ is in fact a prodrug, which is spontaneously hydrolyzed into physiological pH to its alkylating metabolite 3-methyl-(triazen-1-yl)-imidazole-4-carboxamide (MTIC) [11]. TMZ is administered orally and leads to fewer side effects when compared with other chemotherapeutic agents administered parenterally [12]. Notwithstanding, its clinical effectiveness remains limited, since tumors rapidly develop resistance to the treatment [13]. In addition, several GBM cases may result intrinsically resistant to TMZ even with initial treatments. This inherent resistance is a consequence of various defense mechanisms such as expression of multi-drug resistance proteins and DNA repair systems impairment [14]. Extensive and complete surgical resection of GBM represents the most effective way to increase the survival of GBM patients [15]. However, the surgical intervention is very difficult and, in most cases, less than 90% of the tumor can be removed [16]. In fact, these tumors exhibit high degree of invasiveness and are often localized in important functional areas of the brain, including areas that are involved in the control of speech, motor functions and senses [17]. Furthermore, GBM is difficult to access for conventional drug therapy due to the presence of the blood–brain barrier (BBB) that limits the passage of molecules, like many anticancer drugs, from the bloodstream into the brain [18].

The restrictive nature of the BBB and low brain permeability to most drugs means that high doses must be administered in order to obtain therapeutic concentrations in the brain. Despite several strategies have been proposed to overcome these obstacles (e.g. oncolytic viruses, targeted therapies, immunotherapy, vaccines, etc.) brain delivery of therapeutic molecules against glioblastoma remains a challenge [19].

Nose-to-brain delivery has been proposed as a non-invasive direct access to the brain able to bypass the BBB and it is actively under investigation as an alternative administration route for the delivery of pharmaceutically active molecules potentially useful in a number of CNS disorders. In particular, the intranasal delivery route might represent a major breakthrough in treatment of GBM, offering an effective drug delivery approach for a number of innovative therapeutic strategies (Figure 1).

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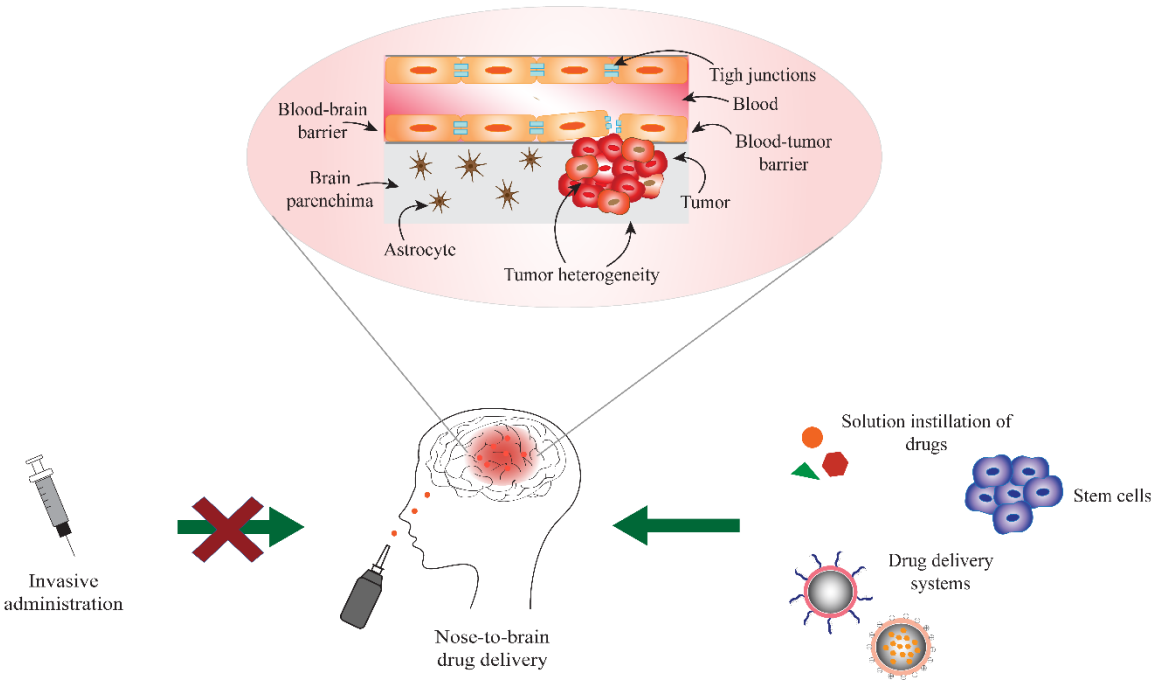

Figure 1. Obstacles and opportunities of drug delivery approaches for the treatment of GBM.

2. Blood-Brain and Blood-Tumor Barriers (BBB/BTB)

The protective blood-brain barrier (BBB) separates the CNS from bloodstream, exhibiting a highly selective permeability to preserve brain homeostasis and ensuring correct neuronal functioning of the brain. The BBB is a cellular barrier and its properties are mainly due to the presence of tight junctions (TJ) between the brain capillaries endothelial cells and to the surrounding cells, i.e. adjoining pericytes, astrocytes and microglia [20, 21]. The BBB prevents the passage of macromolecules as well as of undesirable toxic or infectious agents and selectively ensures the supply of essential nutrients and oxygen into the CNS, providing an adequate brain homeostasis [22, 23]. Along with defensive functions, the BBB also prevents the entry of xenobiotic drugs from the blood into the brain. The BBB is normally only permeable to small and lipophilic molecules, with molecular weight (Mw) lower than 400-500 Da [24]. In addition, the LogP required for an efficient transport across the BBB is estimated to be in the range between 1.5 and 2.7 [25]. Another important drug characteristic to ensure access to the CNS is low hydrogen-bonding potential [26].

In brain tumors, the tumoral microenviroment is distint in comparison to normal brain since the morphology, function and organization of BBB are affected. The result of this is the formation of the so called blood-tumor barrier (BTB) between brain tumor cells and capillary vessels [27, 28, 29]. In high-grade gliomas, as in glioblastoma, the BTB is formed of existing and newly formed blood vessels that contribute to the delivery of nutrients and oxygen to the tumor and facilitate glioma cell migration to other parts of the brain [30, 27]. The tumor expansion creates hypoxic areas that trigger the overexpression of vascular endothelial growth factor (VEGF) and consequently, the promotion of neoangiogenesis [31]. The neovascularization process commonly leads to the formation of abnormal vessels able to maintain the high metabolic activity of tumor cells. These vessels are characterized by increased fenestration or loss of tight junctions between endothelial cells. Furthermore vascular endothelial cells are overexpressing caveolae, have increased pinocytotic activity and are rich in mitochondria [31, 32, 33]. Notwhitstanding, BTB presents continuous fenestrated vessels with a defined pore size, precluding the entrance in brain tumor of hydrophilic compounds and large molecules [30, 34]. As a consequence, the most of antitumor agents are not delivered to brain tumors due to the presence of BTB [30, 34]. With the progress of brain tumor in late stages, the permeability of BTB can increase, since an impairment of BBB/BTB often occurs along with an intensification of

enhanced permeability and retention (EPR) effect, resulting in a tendency of large molecules and particles in nanoscale accumulate at the brain tumor site [32, 34].

3. Nose-to-brain drug delivery

The choice for the treatment and average patient survival depends on the glioma type, size, location and grade [35, 36]. In some cases the median survival can be extended by the addition of an adjuvant chemotherapy (TMZ) to the radiotherapy (RT) [37,38]. Stupp et al. (2005) found that the median survival of patients receiving TMZ in addition to RT was 14.6 months as compared with 12.1 months among those who were assigned to RT alone [39]. However, despite the benefits, the treatment with TMZ can entail in some immediate side effects such as nausea, vomiting, lymphopenia, neutropenia, thrombocytopenia, fatigue, disturbed sleep and depression [40,41,42]. Furthermore, the risk of neurocognitive impairment is increased when radiotherapy is administered to the whole brain and even more when the chemotherapy is associated to RT [43, 44, 45].

Approaches to overcome physiological barriers and limitations to access the human central nervous system (CNS) include the exploitation of ways suitable for the direct administration of the drug to the brain. This can be done by intraventricular, intrathecal or nasal administration [46]. The intrathecal administration requires some risky surgical procedures and the drugs administered can present limited distribution throughout the cerebralspinal fluid (CSF) pathways. The results of better distribution into the CSF can be obtained by the intraventricular administration, but this type of administration requires the implant of drug release controlling reservoirs. Furthermore some severe side effects can occur when drugs are administrated intrathecally and intraventricularly including meningitis, arachnoiditis, and focal neurologic injury. Moreover, if in presence of CSF flow abnormalities, these approaches will result in the potential increase of drug-related toxicities because of the restricted volume of distribution [47, 48]. As a consequence, these invasive brain administration approaches appear to be applicable only to a limited number of selected patients.

The intranasal (IN) route of administration appears as an alternative route of delivery of drugs to the CNS able to bypass the BBB. In fact, several evidences have been provided in the scientific literature supporting the claim that drugs can reach de CNS after administration into the nasal cavity [49,50,51]. In order to obtain an efficient formulation that allows access to the CNS through the intranasal route, it is essential to understand the mechanism of transport of the compounds through this route, the anatomy of the nervous system and the pathophysiology of the disease, as well as experimental parameters [52]. In the next paragraphs, the anatomical organization of the nasal cavity will be briefly discussed, in particular the structures that are necessary for understanding nose-to-brain transport, since excellent descriptions can be found in many review papers and textbooks [53].

Anatomically, the nose presents two cavities limited by a septal and a lateral wall dominated by the turbinates, structures responsible for the temperature regulation and humidification of the inspired air [54]. The innervation of the human nasal cavity can be divided into sensory and olfactory. Sensory innervation consists of the first and second divisions of the trigeminal nerve (ophthalmic nerve and maxillary nerve), the olfactory innervation is ascribed to the olfactory nerve [55, 56]. The nasal cavity can be divided in three regions: the vestibular region, the respiratory region and the olfactory region. The vestibular region, located in the frontal part of the nasal cavity is followed by the respiratory region that presents approximately 130 cm^2 of area and characterized by the sensory/trigeminal innervation. In humans this is the largest region and can reach up to 80-90% of the nasal cavity [57]. The respiratory epithelium is responsible for covering the nasal conchae (bone projections of the lateral walls of the nasal cavity) and the paranasal sinuses (cavities in the bones of the face that communicate with the nasal cavity) [58]. Another important region of the nasal cavity is the olfactory region, that in humans represents approximately 10% of the nasal cavity surface area. This region is located in the upper part of the nasal fossa, below the cribriform lamina of the ethmoid bone and is innervated by the olfactory nerve [54, 59, 60]. Olfactory cells are bipolar unmyelinated neurons that present dendrites with terminations protruding above the surface of the nasal mucosa interspaced between supporting cells and an axon extending through the connective tissue towards

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the olfactory bulb loated in the CNS [61]. The constant replacement of olfactory receptor neurons, make the olfactory mucosa relatively "leaky" and thereby making possible the nose-to-brain transport [62]. The molecular weight and the hydrophilic/lipophilic nature of the drug directly influence the absorption of the drugs through the nasal route. Poor bioavailability after nasal administration is generally observed for drugs with a molecular weight greater than 1 kDa [63]. Lipophilic compounds having a molecular weight lower than 1 kDa can present bioavailabilities close to 100%, i.e. similar to what can be obtained after intravenous administration [64].

After the administration into the nasal cavity of a formulation, firstly occurs the mucociliary clearance in the vestibular region [65]. Afterwards the formulation moves to the posterior region of the nasal cavity in direction to the respiratory and olfactory region. Therefore, the transport to the brain of the drug or of the formulation itself can happen by five different pathways: the olfactory nerve, the lymphatic, the trigeminal nerve, the cerebrospinal fluid and the vascular pathways. Depending of the nature of the drug, the characteristics of the formulation and the physiological conditions the nose-to-brain transport can occur via a single route or through a combination of pathways mentioned above [66]. The substances can then move towards lamina propria and the brain by two different mechanisms, the intracellular and the extracellular transport mechanism. Once at the lamina propria, substances follow the nerve channel created by a glial cell type, the olfactory ensheathing cells that cover the non-myelinated axons, cross the cribriform plate and enter into the CSF and olfactory bulb (Figure 2).

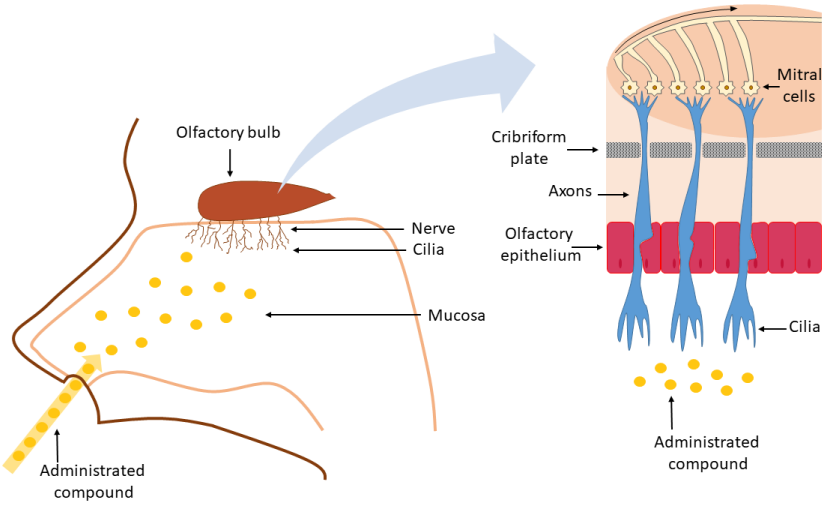

Figure 2. Structures involved in nose-to-brain transport by the olfactory pathway.

From the CSF, substances are distributed throughout the brain via bulk flow after being mixed with the interstitial fluid. They are also rapidly distributed throughout the CNS via perivascular transport [52, 67]. The olfactory nerve pathway begins in the receptor neurons located in the olfactory mucosa and is responsible for capturing odors and transmitting the information to the CNS, being a direct CNS connection to the external environment, the olfactory nerve innervates the nasal olfactory epithelium and terminates in the olfactory bulb [23, 68]. Through the olfactory pathway, the permeation of the compounds to the nervous system occurs along or within the neurons present at the olfactory epithelium [69]. In the case of intraneuronal axonal transport, the compounds are internalized in the olfactory epithelial neurons and then they are conveyed to the olfactory bulb, thus enabling the compounds to be further distributed to the rest of the brain [70]. In the intracellular form of transport, generally preferred by substances with an hydrodynamic radius above 20 nm, permeation can happen through endocytosis primarily to sensory olfactory neurons and subsequent intraneuronal transport to the olfactory bulb, or through transcellular transport to the cells of the lamina propria [49, 71].

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However, the intraneuronal axonal transport is usually quite slow, with times of delivery to the CNS ranging from hours to several days. The extracellular transport involves the absorption of the compounds across the nasal epithelium and the extracellular diffusion associated with bundles of nerves with consequent migration to the cranial compartment [72]. In fact, the perineural spaces of the cranial nerves, as in the case of the olfactory and trigeminal nerves, seem to allow communication with the cerebrospinal fluid of the subarachnoid space, allowing a rapid access route for the molecules absorbed across the nasal mucosa to reach the CNS [70].

The direct nose-to-brain transport can also occur by the trigeminal nerve, which innervates the respiratory and olfactory epithelium of the nasal and allows the access of compounds to the caudal and rostral sections of the brain after intranasal administration [73, 74]. However, it is important to point out that generally it is not possible to determinate a specific/exclusive way how molecules/peptides access the CNS after the nasal administration because this access can occurs simultaneously by multiple pathways [66]. In fact, in parallel with these pathways, other mechanisms can provide access to the CNS from the nasal cavity such as the nasal and the brain lymphatic systems that participate to the CSF and CNS interstitial spaces drainage by bulk flow mechanisms trough perivascular channels surrounding blood vessels [66, 75]. Another way that compounds can use to penetrate into the CNS from the nasal respiratory region, though in an indirect way, is via the vascular pathway. In fact, nasal blood vessels presents continuous but fenestrated endothelia enabling the small molecule passage and the delivery to the brain by distribution across the BBB [74, 76].

Despite the numerous advantages, the nose-to-brain drug delivery can be limited some aspects related to the intranasal administration, such as low bioavailability of peptides and proteins due to enzymatic degradation, high clearance from the nasal cavity due to mucociliary transport and other restrictions determined by the anatomy of the nasal cavity (small administration volume, limited surface area of the olfactory mucosa, mucus barrier). In terms of enzymatic degradation, it can occur at the lumen of the nasal cavity or during transit across the epithelial barriers due to the presence of exo-peptidases such as mono- and diaminopeptidases that can cleave peptides at their N and C terminal and endo-peptidases which can attack internal peptide bonds [77]. The mucus present in the upper respiratory region act as a physical and chemical barrier entrapping particles and molecules. The mucus is than drained from the nasal cavity into the pharynx through ciliary movement to be swallowed or expectorated [58]. Notwithstanding these limitations, Quintana and collaborators [78] reported results from a clinical trial which investigated the intranasal delivery of oxytocin (OT) to 16 male health adults. Treatments were divided into two different doses intravenous OT 8 and 24 intranasal units (IU) or 1IU intravenous and placebo with a period of at least 6 days between treatments to prevent potential carryover and/or practice effects. Blood samples were collected to determinate the peripheral levels of OT, cross-reactive vasopressin (AVP) and cortisol. All the treatments produced similar plasma OT increases compared with placebo. The data suggested that OT delivered intranasally using a Breath Powered bi-directional device reaches the brain and influences social cognition, whereas IV administered OT, which similarly increased plasma OT concentration, did not, providing support for a direct nose-to-brain effect, independent of blood absorption, of low-dose OT [78].

4. Drugs for GBM Treatment Administered Intranasally

Several studies have been conducted to determine the best treatment of GBM via the intranasal approach, using monotherapies or drug combinations including natural and/or synthetic compounds. Below are listed some studies that were conducted for this purpose trying to develop an effective way to treat this aggressive brain tumor.

Natural compounds such curcumin (CC), a polyphenolic extracted from the rhizomes of the *Curcuma longa* that presents anti-oxidant and anti-inflammatory characteristics, are interesting for the treatment of cancer and neurodegenerative disorders and have been proposed for the treatment of

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GBM. The anticancer activity of CC occurs because of its ability to reduce the expression of E3 ubiquitin ligase NEDD4, a neuronal precursor responsible for substrate recognition implicated in cancer development, and Notch1 and pAKT (cancer signaling pathways), leading to glioma cell growth inhibition, apoptosis and suppression of migration and invasion [79, 80]. Mukherjee and collaborators [81] used the IN route to deliver curcumin (CC) coupled to a glioblastoma specific antibody (CD68 Ag). The targeted CC-CD68 Ag conjugate was administered intranasally to mice in which glioma GL261 cells were implanted in the brain. Ten days after GL261 cells implantation, male adults C57BL/6 mice had CC-CD68 Ab solution in PBS administered intranasally every 72 h while another group of animals received a solution of a commercially available curcumin phytosome (CCP) by IP injection every 72 h. Both the intranasal delivery of CC-CD68 Ab conjugate and the IP injection of CCP cause elimination GL261 brain tumor, confirming that the CD68 Ab could be delivered to the brain via the IN route and confirming that CD68 Ab presented a targeted therapeutic effect after IN delivery. Seventy percent of the animals that received CC-CD68Ab IN and sixty percent of the CCP-treated IP were still alive at day 90, while all control group animals, i.e. vehicle-treated mice were already dead at that time. The results show that appropriately delivered CC not only rescues 50–60% of the GBM model animals, but also prolongs the life of the treated mice. In the same study, it was also observed a marked induction and activation of microglial NF-kB and STAT1 (transcription factors), that function in coordination to cause the induction of nitric oxide synthase (iNOS), and consequently tumor regression. Therefore, the findings in this study indicate that delivered CC can directly kill GBM cells and also repolarize tumor-associated microglial cells (TAMs) to the tumoricidal state [81].

Another natural compound, the anthranoid 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein) exhibits anti-inflammatory, antioxidant, antifibrosis, neuroprotective and antitumor activities [82, 83]. The antitumor activity is attributed to the inhibition of MAPK, PI3K-AKT and HIF-1 signaling pathways and the down-regulation of VEGF signaling pathway [83, 84]. Blacher and colleagues [85], aiming at demonstrating that the inhibition of the ectoenzyme CD38 in tumor microenvironment can attenuate glioma progression, conducted a study using a syngeneic mouse glioma progression model. The animals, C57BL/6J wild-type (WT) and CD38-deficient C57BL/6J (CD38-/-) mice, were pretreated with vehicle or rhein by nasal administration. Rhein is a highly water-soluble salt form of rhein. After 24 h, glioma cells (GL261) were intracranially injected into the brains of the mice and the administration of vehicle or rhein was carried three times per week over 22 days. The researchers found that the rhein is capable to inhibit the CD38 enzymatic activity, reducing the microglia activation that support the progression of the tumor. In fact, the IN administration of rhein into WT mice significantly inhibited glioma progression suggesting that CD38 is a therapeutic target in the tumor microenvironment and that small-molecule inhibitors of CD38 may serve as a useful approach to treat glioma. Furthermore, computed tomography (CT) images of the mice brains showed that WT and Cd38-/- mice treated intranasally with rhein had the volume of the tumor reduced; however, this effect was significantly higher in WT mice compared to Cd38-/- (reduction of 74 and 19% respectively on day 22), demonstrating that rhein inhibits glioma progression and that this effect is mainly CD38 dependent. With this study, it was possible to conclude that the IN administration is an effective drug delivery route to the CNS and that the rhein has a therapeutic potential to treat glioblastoma [85]. These data additionally support the possibility of access the brain from the nasal cavity and demonstrate that compounds can be directed to the CNS to be effective in the treatment of GBM even in monotherapy.

Other studies involve the use of compounds in association. The study performed by Shingaki and coworkers evaluated the direct brain uptake from the nasal cavity of a model drug, 5-fluorouracil (5-FU) and whether the inhibition of cerebrospinal fluid (CSF) secretion by choroid plexus could lead to increased drug concentration in the brain [86]. 5-FU is a fluoropyrimidine widely used in the treatment of malignant tumors, such as breast, skin, colorectal and neck [87]. This uracil pyrimidine analog is an antimetabolite drug that can inhibit the thymidylate synthase (TS) enzyme and perform a miss-incorporation of fluoronucleotides into RNA and DNA, leading to cytotoxicity and cell death

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[88, 89, 90]. In this study, 5-FU was infused intravenously or perfused nasally in the presence and absence of intravenous administration of acetazolamide (AZA) in male Wistar rats. In groups of co-treatment, AZA (25 mg/kg) was injected 15 min before starting the nasal perfusion of 5-FU. AZA is an inhibitor of the secretion of cerebrospinal fluid (CSF) by choroid plexus epithelial cells. In these cells the CSF secretion is linked to the active transport of Na+ ions and AZA is significantly decreasing the activity of the Na/K ATPase [91]. The study demonstrated that the IV administration of AZA was able to enhance the CSF concentration of nasally administered 5-FU by 200–300% compared to that obtained by 5-FU nasal perfusion but in absence pre-treatment with AZA. AZA enhancement of nose-to-brain drug transport was obtained by decreasing the CSF secretion from the choroid plexus and thus sustaining the concentration of the nasally applied drug in the CSF [86]. These results further demonstrated that 5-FU is capable to access the brain through the nasal administration allowing the conclusion that the co-administration of active compounds to treat neurological diseases with drugs that can decrease the CSF secretion from the choroid plexus could be an interesting alternative to the treatment of diseases into the brain, like GBM, because it permit to enhance the concentrations of the active compounds into the brain.

In another study, the same group studied a similar effect by nasal and intraperitoneal administration of methotrexate (MTX) in male Wistar rats [92]. MTX is a folic acid antagonist that inhibits the enzyme dihydrofolate reductase having a therapeutic effect on a wide range of cancer types [93]. MTX presents a low penetration across the BBB, which limits its therapeutic use for GBM treatment by the oral route [94]. In the study MTX was administrated nasally using sodium carboxymethyl cellulose (CMC) to enhance the nasal residence time of the formulation, and acetazolamide (AZA) was administrated orally 30 min before the nasal administration of MTX as a co-therapy. The brain uptakes of tritium labelled MTX after IN administration of a formulation containing CMC and after IP administration were evaluated by blood samples and analysis of the cerebral cortex. To evaluate the results after repeated administrations, MTX was administered for five days with the interval of two days from each treatment. The results showed that 15 min after IN administration the amount of MTX quantified in CSF was higher than in the plasma, indicating the significant direct transport of MTX from the nasal cavity to the CSF. In contrast, after IP administration a higher concentration was obtained in plasma compared to those obtained in the CSF. At the same time, the effect of oral administration of AZA 30 min before the nasal administration of MTX was evaluated and it was found that the co-treatment increased by 195% the concentration of MTX in CSF [92]. The study demonstrated that the IN administration is a promising route of the administration of drugs directed towards brain diseases and that AZA can enhance the amount of MTX in the CSF in agreement with the results obtained with 5-FU [86, 92].

In another study, MTX was loaded in chitosan microspheres with the view of a nasal administration. The microspheres were produced by spray-drying technique using chitosan with different molecular weights to promote the nose-to-brain delivery of the MTX. The animals received MTX by IV injection or MTX was administered intranasally using a drug solution or MTX-loaded chitosan microspheres. The study demonstrated a higher concentration of MTX in rat brain tissues after IN administration of the MTX-loaded chitosan microspheres compared to the MTX solution, while MTX could not be detected in rat brain sections after the IV administration. The fact that MTX-loaded chitosan microspheres showed a higher nose-to-brain transport, as compared to MTX aqueous solution after nasal administration, was attributed to the presence of chitosan. Indeed, chitosan is considered a safe mucoadhesive polymer that could effectively improve nose-to-brain transport of hydrophilic drug like MTX through intranasal administration [95].

Another study proposed the nasal delivery of temozolomide (TMZ) [96]. TMZ is efficiently absorbed after oral administration and is available in capsules. Additionally, TMZ has shown good penetration across the BBB and a low toxicity profile [97]. However, the increase in survival for the multimodal treatment with TMZ and radiotherapy is only 2.5 months compared with radiotherapy alone and studies suggest that 60–75% of patients with GBM present no clinical benefit from treatment with TMZ [98]. Based on these data, a rat model bearing orthotopic C6 glioma xenografts

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was used to study the therapeutic effect of IN administration of TMZ in order to exploit the brain-targeting properties of this delivery route. In fact, IN administration of TMZ was proposed to limit the systemic exposure to the drug and thus reduce the toxic effects on the healthy organs. The animals were treated with saline solution or with TMZ by three different administration routes, IV, oral or IN, and the tumor size, rat survival time and pathological changes were observed during the 40 days of the experiment. Magnetic resonance imaging showed a significant reduction in the volume of glioma xenografts in the IN TMZ group compared to all the other groups including controls ($p<0.05$). Analysis of proliferating cell nuclear antigen (PCNA) and tumor cell apoptosis obtained by immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay demonstrated that the animals treated by the IN route presented the lowest expression of PCNA and the highest tumor cell apoptosis rate. The median survival time of the C6 glioma-bearing rats was also significantly longer in the intranasal TMZ group when compared to the other three groups. The control animals treated with saline solution survived 20 days, the animals treated with TMZ oral 21.5 days, TMZ intravenous 19 days, while animals treated with TMZ intranasally survived 31 days showing that the IN administration of TMZ promotes an improvement in the survival time[96]. The results presented in this study allow concluding that the intranasal TMZ administration can suppress the growth of C6 glioma *in vivo* and may serve as an effective strategy for glioma treatment.

A solution of TMZ in DMSO was also tested by IN administration in nude mice xenograft models carrying human glioblastoma tumors generated from the human glioma stem cell lines TG16, TG1N and TG20 by Pineda and co-authors [99]. The human glioma cell lines TG16, TG1N and TG20 were administered by intrastriatal injection to ten-week-old female Swiss nu/nu mice. One month after graft, anesthetized mice received IN 10 μL of TMZ or vehicle; this procedure was repeated three times a week during two weeks. The TMZ administered intranasally delayed tumor growth and significantly extended the lifespan of mice engrafted with TG16 and TG1N cells, but presented no effects on the tumors generated by TG20 cells that are resistant to TMZ *in vitro*. The presented results demonstrated that the intranasal route should be further considered as an option for TMZ delivery into the brain to treat intrastriatal brain tumors [99].

These studies taken together demonstrate that the intranasal administration of anticancer drugs can bring benefits in the treatment of GBM and that the intranasal route of administration may allow a direct access of the drugs to the brain serving as an effective strategy for glioblastoma treatment. However, meaningful comparative studies between intranasal and other administration routes (oral or parenteral) should be always duly conducted to conclusively highlight the potential clinical benefits of using the nose-to-brain delivery over more traditional but well-established administrations.

4.1 Clinical Trials on the Use of Intranasal Perillyl Alcohol for Glioblastoma Treatment

Perillyl alcohol (POH) is a natural compound belonging to the group of hydroxylated monoterpenes found in many kinds of essential oils (peppermint, spearmint, cherries and others)[100]. The amphipathic character of POH makes it readily soluble in biological membranes and capable to modulate the lipid bilayer of gliomas cells, leading to an effective POH delivery into these cells [101]. A post translational Ras inhibition effect has been suggested by some studies as the main mechanism of anticancer action of POH, however it is not observed in others, thus the action of POH is often described as pleiotropic, affecting different cell growth regulation processes [102].

Thirteen clinical studies were conducted using POH delivered orally to cancer patients (ovarian, prostate, breast, colorectal and pancreatic cancer) to establish safety and efficacy of this molecule [103]. POH was dosed in capsules along with soybean oil, and the dose regimen included dozens of capsules per day per patient. However, no significant therapeutic response was observed and the trials were halted before reaching Phase 3. In subsequent studies, the focus was shifter on the use of the intranasal route for the delivery for POH and, although an excellent review has been recently

published on this specific topic [102], here we will briefly summarized the results of the clinical trials conducted on GBM patients.

To date, POH is the only therapeutic agent intended to cancer treatment that reached clinical trials Phases 1 and 2, which employ intranasal route, although studies use an inhalation protocol, which may not involve the nose-to-brain delivery mechanism solely. Clinical trials have consistently showed the safety and tolerability of POH administered by the nasal route for up to 8 years besides positive therapeutic responses in some cases [102, 104, 105]. The first clinical trial carried in Brazil enrolled 37 patients with recurrent malignant glioma, including 29 with glioblastoma aging from 38 to 62 years old. POH was administered by inhalation 4 times a day at concentration of 0.3% (v/v) to receive total daily dose of 220 mg. After 6 months, 14 patients with GBM showed partial response (1 patient) or stable disease parameters (13 patients), suggesting some antitumor activity for POH [106]. A following study included 141 patients with recurrent glioblastoma divided into a treatment group including 83 patients with recurrent primary GBM and 6 with secondary GBM receiving POH and a control group with 52 patients receiving supportive care. The treatment consisted of inhalation of POH 4 times per day to reach a total daily dose of 440 mg. The results showed a significant increase in survival between the POH treated groups over control group, between patients with secondary GBM over patients with primary GBM and between patients with tumor at deep site (thalamus, basal ganglia) over those with tumor at lobar region. Later, a 4 years study with a cohort of 198 patients with recurrent malignant glioma (151 with primary GBM and 38 with secondary GBM) was conducted using again a protocol of inhalation of POH 4 times a day but adopting a higher dosing compared to previous studies (533.6 mg/day). Patients with secondary GBM had a significant increase in survival time compared to patients with primary GBM, confirming the results of previous studies, but most importantly, 19% of patients enrolled in this trial remained in clinical remission after 4 years under exclusive POH inhalation treatment [104]. Santos and colleagues recently reported a study that combined inhalation of POH (55mg 4 times per day) with a ketogenic diet (KD) for three months. In the context of cancer therapy, some authors argue that the KD is viewed as a metabolic therapy and consists of a high-fat, low-carbohydrate with adequate amounts of protein, promoting a specific metabolic state that is characterized by increased ketone body levels and low glucose levels in the blood [102]. Data showed that 88% of patients that followed this treatment showed partial responses and stable disease parameters at the end of the study [107].

Encouraged by the positive results observed in the clinical trials carried out in Brazil, a synthetic GMP grade POH (NEO100) is now under Phase 1/2A clinical trials in U.S.A. sponsored by Neonc Technologies, Inc. (NCT02704858) [103]. These studies were started in 2016 and are still recruiting patients with recurrent glioblastoma. Treatment protocol will follow that adopted in previous trials, with a regimen of POH inhaled 4 times a day over a period of 6 months. Four dosing levels will be studied: 96 mg, 144 mg, 192 mg and 288 mg per inhalation in order to determine the maximum tolerated dose (MTD). A total of 25 patients will be treated at the MTD and pharmacokinetic studies will be conducted during Phase 1 at the first dosing and after first dose of the third cycle [103]. The study is expected to be concluded in October 2020 and no partial results were made available to date.

5. Drug Delivery Systems for Nose-to-Brain Delivery in Glioblastoma Therapy

Several therapies that apply novel drug delivery systems are under investigation for the treatment of GBM. Recently, nanoparticles (NP) have received significant attention due to several advantages they offer over conventional therapy, such as, for example, their ability in some cases to carry drugs across the BBB [108, 109]. Furthermore, these systems offer a controlled drug release, which potentially would allow decreasing the frequency of administrations [110]. Moreover, nanoparticles are expected to improve the drug physicochemical stability and increase the biological availability [111, 112].

The application of NP for the enhancement of drug delivery directly from the nasal cavity to the brain is demonstrating great potential. The encapsulation of drugs into NP can overcome problems of IN administration (e.g., the poor capacity of penetration through the nasal mucosa, the rapid

462 mucociliary clearance, and the enzymatic degradation) and thus enhance the nose-to-brain drug
463 delivery [113]. The small diameter of the NPs also allows them to be transcellularly transported to
464 the brain more effectively [61]. Besides, NP may offer improved drug delivery to the brain since they
465 can prevent extracellular transport by P-glycoprotein (P-gp) efflux proteins localized in the olfactory
466 epithelium and the endothelial cells that surround the olfactory bulb [114, 115]. Additionally, the
467 nanocarriers may also have their functionalized surface with specific ligands to transport agents even
468 more effectively through the BBB [116]. The NPs that have been mainly studied for nose-to-brain
469 delivery are chitosan nanoparticles, polymeric nanoparticles, liposomes, solid lipid nanoparticles,
470 nanoemulsions, micelles and nanoplexes among others. The main features of the NPs specifically
471 designed for GBM therapy by IN route in recent years and under pre-clinical stages of development
472 are summarized in Table 1.

473 **Table 1.** Characteristics and pre-clinical findings in the last 10 years using nanocarriers administered by the intranasal route for GBM therapy.

Drug	Type of nanocarrier	Surface Modification	Preparation Method	Size (nm)	Zeta potential (mV)	*In vivo* model	Ref.
Ecto-5′-nucleotidase (CD73)	Nanoemulsion	-	Microfluidization	262.7 ± 12.8	+ 3.5 ± 3.0	C6 rat glioma	[117]
Teriflunomide	Microemulsion	-	Progressive aqueous phase titration	22.81 ± 0.48	−22.62 ± 1.1	-	[118]
Melatonin	Polymeric NPs (PCL)[a]	-	Nanoprecipitation	166.7 ± 6.3	−34.0 ± 5.2	-	[119]
Temozolomide	Polymeric NPs (PLGA)[b]	Anti-EPHA3	Emulsion-solvent evaporation	125 to 146	-21 to +23	C6 rat glioma	[120]
Kaempferol	Nanoemulsion	Chitosan	High-pressure homogenization	180.53 ± 4.90 (coated) 145.07 ± 4.91 (uncoated)	+26.09 ± 2.67 (coated) −18.10 ± 2.55 (uncoated)	-	[121]
Farnesyl thiosalicylic acid	Hybrid nanoparticles	-	Emulsion sonication	164.3 ± 10.3	−12.0 ± 1.3	RG2 rat glioma	[122]
Curcumin	Microemulsion	-	Oil titration method	< 20	~+ 10	-	[123]
Curcumin	Nanostructured Lipid Carriers	-	High pressure homogenization	146.8	−21.4 ± 1.87	-	[124]
siRNA	Chitosan nanoparticles	-	Ionic gelation	141 ± 5	+32	GL261 tumor bearing mice	[125]

siRNA siRNA + TMZ or immunotherapy	Chitosan nanoparticles	-	Ionic gelation	141	+32	GL261 tumor bearing mice	[126]
Methotrexate	Polymeric nanodispersion (PLA)[c]	-	Emulsion/Solvent evaporation	351 ± 13.4	+25.1 ± 1.2	-	[127]
Carboplatin	Polymeric nanoparticles (PCL)[a]	-	Double emulsion/solvent evaporation	311.6 ± 4.7	-16.3 ± 3.7	-	[128]
BMP4 plasmid DNA	Polymeric nanoparticles (PBAE)[d]	-	Self-assembly	218 ± 7	+17 ± 1	U87 rat glioma	[129]
Camptothecin	Polymer micelles (MPEG-PCL)[e]	Tat	Self-assembly	88.5 ± 20.2	+10.4 ± 2.84	C6 rat glioma	[130]
siRaf-1 / Camptothecin	Polymer micelles (MPEG-PCL)[e]	Tat	Self-assembly	60 to 200	-2.86 to 15.9	C6 rat glioma	[131]

[a] Poly(ε-caprolactone)

[b] Poly(lactic-co-glycolic acid)

[c] Poly-(lactic acid)

[d] Poly(beta-amino ester)

[e] Methoxy[poly(ethylene glycol)]-b-[poly(ε-caprolactone)] amphiphilic block copolymers

Encapsulation of drugs into nanocarriers has enhanced the therapeutic potential of a wide variety of molecules in view of the treatment of GBM. Among several bioactive compounds, many researchers have shown interest in the nanoencapsulation of curcumin. In fact, NPs are able to overcome a number of limitations related to this natural compound, such as low solubility, low oral bioavailability and low capacity to cross the BBB [132, 133].

In this context, Madane and Mahajan developed a nanostructured lipid carrier (NLC) system for curcumin (CC) using hot high-pressure homogenization [124]. Curcumin showed a biphasic release pattern from NLC formulations, initially showing a burst release of approximately 25% followed by a sustained release up to 24 h. Moreover, an *ex vivo* permeability study carried out using Franz diffusion cells showed greater drug permeability across the sheep nasal mucosa of curcumin formulated in NLC system compared to the free drug suspension. The *in vitro* cytotoxicity studies using astrocytoma-glioblastoma cell line (U-373 MG) showed IC50 values of 9.8 ng/mL for the nanoformulation and 13.6 ng/mL for the positive control (adrenomycin), demonstrating the potential effectiveness of CC-NLCs against the glioblastoma. The results of biodistribution studies in Wistar rats showed higher drug concentration in the animal brain after of IN administration of NLCs than free drug suspension. The C_{max} was 5.4321 ± 2.098 ng/g (t_{max} 180 min) for the free drug and was 8.6201 ± 8.182 ng/g (t_{max} 120 min) for curcumin-loaded NLC [124].

In another study, Shinde *et al.* investigated the brain bioavailability and efficacy *in vitro* of curcumin-loaded microemulsions (ME) after nasal and intravenous administration to rats [123]. The drug delivery system proposed consisted of microemulsion formulated with curcumin and docosahexaenoic acid (DHA), which in addition of improving the curcumin bioavailability, also has antitumor effects by itself. In fact, the results of *in vitro* cytotoxicity studies showed a synergistic effect of CC with DHA formulated in a ME against the U-87 MG glioblastoma cell line. The IC50 value was 3.7 ± 0.2 ng/mL for curcumin-loaded DHA-ME, 502.7 ± 24.6 ng/mL for CC-ME while it was 747.8 ± 53.0 ng/mL for a simple CC solution, thus confirming the synergistic effect of CC and DHA in the microemulsion. It was suggested by the authors that the anticancer activity of DHA could be due to its natural affinity to the neuronal cells and due to DHA capacity to induce lipid peroxidation. Additionally, the combination of curcumin with DHA and its subsequent encapsulation in ME increased the distribution to the brain after IV and IN administration in healthy rats. Curcumin brain concentrations following IN administration were strikingly higher compared to IV administration, especially in the case of the MEs. In particular, the brain targeting efficiency (DTE) and direct transport percentage (DTP) calculated for the curcumin-loaded DHA-ME were 1615.429% and 97%, respectively [123].

Colombo and collaborators investigated the brain biodistribution and antitumor efficacy of nanoemulsions containing kaempferol (KPF) prepared by high-pressure homogenization with and without chitosan [121]. KPF is a natural flavonol found in several species of edible plants (berries, broccoli, apples, grapes, cabbage and beans) and medicinal plants (*Ginkgo biloba*, *Rosmarinus officinalis*, *Aloe vera*, *Centella asiatica*, *Hypericum perforatum*) [134]. This compound has shown antioxidant, anti-inflammatory and anti-tumor activities [135]. Despite its excellent properties, it is a drug with low solubility and low oral bioavailability [136]. As a consequence, KPF is not approved by the FDA and there are no pharmaceutical formulations available in the market containing this natural compound. However, when formulated in a nanoemulsion coated with chitosan, the amount of KPF permeating across pig nasal mucosa in *ex vivo* diffusion studies using Franz diffusion cells increased significantly. Furthermore, during *in vitro* experiments, the formulation coated with chitosan reduced C6 glioma cell viability through induction of apoptosis to a greater extent than either unencapsulated KPF or a chitosan-free nanoemulsion loaded with KPF. The IC50 values of the formulation coated with chitosan was about 20-fold smaller than free KPF. *In vivo* studies in Wistar rats indicated a significant increase in brain uptake after IN administration in comparison to the control KPF solution. The formulation coated with chitosan enhanced significantly the amount of drug reaching the brain. The KPF brain concentration detected after nasal administration of chitosan coated KPF-loaded nanoemulsion was in fact 5-and 4.5-fold higher than that obtained using the free drug solution and KPF-loaded nanoemulsion without chitosan, respectively. The increased KPF concentration in the

brain was not only attributed to the IN administration, but also to the mucoadhesive properties and efficient permeation enhancement provided by chitosan [137, 138].

Polymeric nanoparticles represent a versatile formulation and demonstrated great potential in drug delivery. Recently, some authors worked on melatonin-loaded poly(ε-caprolactone) (PCL) nanoparticles (MLT-NP) for intranasal administration [119]. Melatonin (MLT) is an indolic hormone synthesized and secreted by the pineal gland, acting in the regulation of the circadian cycle [139]. Synthetic melatonin is marketed as a dietary supplement. Therefore, MLT is not officially approved by the FDA for any specific therapeutic indication. However, there are several studies showing its action as antioxidant, antitumor, immune system modulator and neuroprotective agent [140. 141]. Its therapeutic use however is limited by its short half-life, low oral bioavailability, poor solubility and extensive first-pass metabolism that limit the drug's ability to reach therapeutic concentrations [142]. MLT-NP were characterized by an average size of 166.7 ± 6.3 nm and 51% encapsulation efficiency and showed controlled release of MLT from the nanoparticles (71.2% release in 48 h). The formulation demonstrated strong activity against U-87 MG glioblastoma cell line, resulting in IC50 ~2500 fold lower than that of the free MLT. Moreover, selective cytotoxicity effects of MLT-NP by tumor cell line was demonstrated, since at low doses of MLT-NP no cytotoxic effect was observed against MRC-5 pulmonary human fibroblasts. After the nasal administration to rats, fluorescence tomography images evidenced rapid and direct translocation of nanoparticles from nasal cavity to the brain. The *in vivo* pharmacokinetic study was conducted on male Wistar rat and the result shows a significant increase in brain uptake of the MLT when MLT-NP were administered. Moreover, 0.5 h after administration, the percentage of administered MLT-NP in the brain was ~9 and ~18 fold higher than that of obtained using an MLT suspension administered intranasally and orally, respectively [119].

In a similar study, another group developed a polymeric NP formulation of carboplatin (CPC) using the biodegradable polymer poly(ε-caprolactone) [128]. Carboplatin (CP) is an antineoplastic drug belonging to the class of platinum-based alkylating agents and is widely used to treat various forms of cancer. However, development of resistance, systemic toxicity and rapid blood clearance are common problems related to carboplatin use in oncology clinical practice [143]. CP is available as a solution (Paraplatin®, Bristol-Meyers Squibb) for IV administration. For the production of the polymeric nanoparticles, polyvinyl alcohol (PVA) was selected as the emulsifying agent as it provided nanocarriers with lower particle size and maximal entrapment efficiency avoiding particle aggregation. The *in vitro* drug release studies showed that the drug was released from the NP with a biphasic pattern characterized by an initial burst followed by a prolonged sustained release due to a non-Fickian diffusion. Permeation studies across sheep nasal mucosa provided data similar to *in vitro* release studies. *In vitro* cytotoxicity on LN229 GBM cells showed an enhancement in cytotoxicity by CPCs only for long incubation times (96 h). *In situ* nasal perfusion studies conducted in Wistar rats with two CPC containing different amount of PVA demonstrated that both formulations showed progressive nasal absorption of carboplatin with time. Indeed, CP nanoencapsulated showed better nasal absorption compared to free drug, indicated by the smaller amount of CP detected on the perfusate after IN administration [128].

Another group worked on lipid-PEG-PLGA hybrid nanoparticles (HNP) for intranasal delivery of FTA with the aim to increase the brain-targeting efficacy of farnesyl thiosalicylic acid [122]. Farnesyl thiosalicylic acid, also known as Salirasib, is a synthetic derivative of salicylic acid. FTA is a potent and specific inhibitor of Ras proteins, which are found in most malignant tumors [144]. However, FTA presents poor oral bioavailability and is not able to cross the BBB at effective concentrations [145]. HNP were produced by emulsion sonication method and showed particle size of around 160 nm and negative surface charge (-12 mV).

The *in vitro* cytotoxicity after 24 h showed that the hybrid nanocarriers significantly decreased rat glioma-2 (RG2) cells viability of ~60%, compared to only ~13% obtained using free FTA treatment. Furthermore, cytotoxicity studies towards healthy cells evaluated using L929 mouse fibroblasts evidenced a significant toxic effect for free drug treatment, whereas FTA-loaded HNP did not show significant toxicity. For the *in vivo* studied RG2 cells were implanted unilaterally into the right

striatum of female Wistar rats. After 10 days, glioma bearing rats received single dose treatment or 5 repeated doses of HNP (500 μM/20 μL) or free FTA via IN or IV administration. Data showed that tumor area shown by MRI analysis was decreased by 57.3% and 31.0% compared to controls for single IV or IN doses of HNP, respectively. Both IV and IN administrations of free drug and blank nanocarriers had no significant effect *in vivo*. After a treatment period of 5 days, the IN administration of the nanocarrier achieved a significant decrease of 55.7% in tumor area, similar to that observed by IV administration of the same formulation (Fig. 3A). This result was corroborated by the *in vivo* distribution studies that indicated that after IN and IV administration of HNP, the percentage of the FTA dose reaching the brain was similar (Fig. 3B). However, after IN administration the highest accumulation of NPs was detected in the olfactory bulb, whereas following IV administration the nanocarrier caused a high accumulation of FTA in the spleen and liver (Fig. 3B) [122].

Recently, several researchers have proposed the inclusion of nanoformulations within mucoadhesive gelling systems for nasal administration in order to enhance the nasal residence time and reduce the mucociliary clearance [146, 147]. For example, Jain and collaborators developed an innovative MTX formulation for GBM by encapsulating the drug into polymeric PLA nanoparticles (MTX-NP) and including poloxamer 188 in combination with Carbopol 934 in the formulation to obtain a thermosensitive hydrogel [127]. Using a mucoadhesiveness testing apparatus, it was demonstrated that MTX-NP formulation mucoadhesivity correlates with the amount of Carbopol 934 included. *In vivo* studies carried out using male Wistar rats indicated that combination of the *in situ* gelling system and nanoparticles resulted in an increase of MTX in the brain when compared to data obtained with MTX solution. The pharmacokinetic parameters demonstrated increase in area under the plasma concentration–time curve (AUC) for the drug when administered through the nasal route compared to the administration through the IV route. Moreover, PLA methotrexate nanoparticles enhanced the maximum drug concentration (C_{max}) and AUC 1.5 times as compared to the control MTX solution administered by nasal route [127].

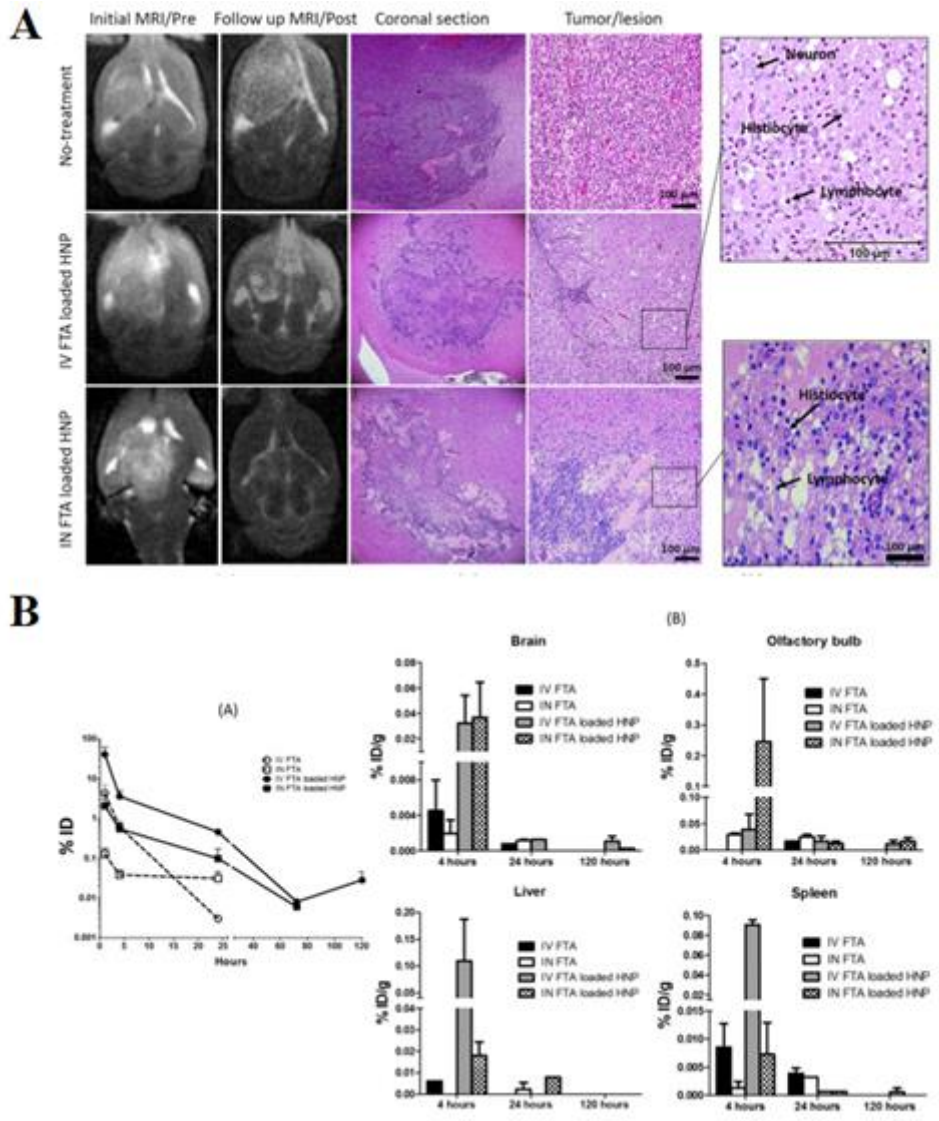

Figure 3. Initial/pre-treatment and follow up/post-treatment MRI images of rat brains from non-treated or after repetitive treatments with IV or IN FTA-loaded HNP formulations and their corresponding coronal brain sections stained with H&E are shown (Panel A). In the coronal brain sections, the upper panels show a dense tumor area in the right striatum of non-treated rats whereas the middle and lower panels show cellular re-organization of tumor cells after treatment with IV FTA-loaded HNPs or IN FTA-loaded HNPs, respectively. Presence of inflammatory response is shown by the abundant presence of histiocytes and lymphocytes. Biodistribution study of the formulations in healthy rats (Panel B). A) Plasma FTA concentration versus time profile is represented for the treatment formulations. B) The distribution of FTA in the brain, olfactory bulb, liver and spleen of healthy rats after 4, 24 and 120 h of formulation administration (reproduced with permission from [122].

On a similar note, Gadhave and his team worked on microemulsion (ME) and mucoadhesive hydrogel (MME) for intranasal delivery of teriflunomide (TFM) with the aim to increase the brain delivery TFM [118]. TFM is a selective and reversible inhibitor of the mitochondrial enzyme dihydroorotate dehydrogenase necessary for the *de novo* synthesis of pyrimidine nucleotides [148]. The TFM was approved by the FDA in September 2012 for the treatment of adults with multiple sclerosis and is available as a tablet for oral administration (Aubagio®, Sanofi-Aventis). However, has been reported that oral administration of TFM should be performed with caution because of the high risk of severe liver injury [149]. Recent studies have demonstrated its action as an antitumor agent in breast cancer [150], glioblastoma [118], prostate cancer [151] and lung cancer [152].

The development and optimization of TFM-MME were performed using a Box-Behnken design of experiments. The optimized formulations were formulated by using the mixture of mucoadhesive agents HPMC K4M (0.3%) and Poloxamer 407 (17%). In the cytotoxicity assay carried out in human U-87 MG glioblastoma cell line the authors used carmustine as positive control. After 48 h of treatment, the cell viability was reduced to 38.5% and 37.8% at 160 µg/mL for carmustine and TFM-MME, respectively, indicating that cytotoxicity profiles against glioma cells were comparable. The *in vivo* biodistribution study in Swiss Albino mice was assessed by gamma scintigraphy *via* ^{99m}Tc labeling of the particles. The TFM-MME formulation showed enhanced brain accumulation (C_{max} 0.62% RA/g) with a direct transport percentage (DTP) of 99.2% and a brain targeting efficiency (DTE) of 359% when compared with the intravenous TFM-ME. However, the *in vitro* and *in vivo* studies did not include the free TFM controls as comparators with the proposed microemulsions. The *in vivo* safety of TFM-ME and TFM-MME was evaluated in toxicological studies carried out using male Wistar rats receiving daily administrations for 28 days. TFM-MME formulation did not reflect any changes in liver or kidney biomarkers, hematology, and histopathological examination at low and medium doses. Although these formulations demonstrated to be safe for nasal administration, the study needs more robust *in vitro* and *in vivo* investigations to demonstrate the efficacy of the TFM-ME and TFM-MME to treatment of GBM [118].

One strategy to improve brain tumor accumulation of drug delivery systems is the functionalization of the surface of nanocarriers with targeting moieties. Ephrin type-A receptor 3 (EPHA3) is a membrane-associated receptor overexpressed in the stroma and vasculature of gliomas [153]. Chu and co-authors developed PLGA nanoparticles functionalized with anti-EPHA3 antibodies for direct nose-to-brain delivery of temozolomide butyl ester (TBE) [120]. Nanoparticles loaded with TMZ were prepared by emulsion-solvent evaporation method and subsequently coated with N-trimethylated chitosan (TMC) and their surface functionalized with anti-EPHA3 antibodies. The drug release studies showed a sustained release of TMZ from the nanoparticles up to 48 h. The results of a cytotoxicity assay on C6 cells and of nanoparticles cellular uptake demonstrated that the anti-EPHA3 functionalization could enhance GBM targeting increasing the cytotoxic effect of the drug. Furthermore, the fluorescence distribution and anti-glioma efficacy in glioma-bearing rats confirmed the enhanced anti-glioma effects were attributed to the nanoparticles surface modification. Anti-EPHA3 functionalized nanoparticles increased the median animal survival by 1.37-fold compared to non-targeted nanoparticles. Overall, the author concluded that anti-EPHA3 modified PLGA nanoparticles might potentially serve as a nose-to-brain drug carrier for the treatment of GBM [120].

Galectin 1 (Gal-1) is a protein over-expressed in GBM and highly associated with tumor progression [154]. The knockdown of Gal-1 using small interfering RNA (siRNA) administration has shown promising results in GBM. Van Woensel and collaborators recently developed chitosan nanoparticles loaded with a Gal-1 siRNA for nasal delivery to treat GBM [125]. Gal-1 siRNA loaded chitosan NPs were formed spontaneously by direct complexation due the electric interaction of positively charged chitosan and negatively charged siRNAs, resulting in successfully encapsulating siRNAs in the nanoparticles and protecting them from RNases. The NPs strongly adhered to the nasal mucosa and the siRNAs were detectable up to 8 h after administration, compared to free siRNA which showed only weak adhesion. This was attributed to the mucoadhesive properties of chitosan that allowed the nanoparticles to overcome mucosal clearance in the nasal cavity and improve the retention time [138]. In addition, the encapsulated siRNAs were effectively transported to the glioma cells from the nasal cavity since a strong reduction in Gal-1 expression was observed. There was also a reduction in the vascular diameter of the tumor microenvironment in the GL261mice brain tumor model [125].

In a subsequent study, the same group showed that Gal-1 knockdown obtained through nasal administration of chitosan nanoparticles loaded with a Gal-1 siRNA displays synergistic effects with TMZ oral treatment and immunotherapy with dendritic cell (DC) vaccination or programmed cell death protein-1 (PD-1) blockade via IP administration, suggesting the possibility of combination

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therapy. The intranasal delivery of Gal-1 siRNA induced a remarkable switch in the tumor micro-environment cellular composition, reducing macrophage polarization from M1 (pro-inflammatory) to M2 (anti-inflammatory) and inhibiting recruitment of monocytic myeloid derived suppressor cells during GBM progression. Furthermore, the results demonstrated that the median survival increased from 32 days in TMZ treated mice, to 53 days in mice treated with TMZ orally and nasally with chitosan nanoparticles loaded with a Gal-1 siRNA. The prophylactic vaccination model showed that the combining DC vaccine with chitosan nanoparticles loaded with a Gal-1 siRNA administered IN also increased the median survival to 53 days. Similarly, the concomitant IN administration of chitosan nanoparticles loaded with a Gal-1 siRNA improved the therapeutic effect of anti-PD-1 antibodies, and increased the median survival to 51.5 days when compared to control groups (17.5 and 30 days for untreated mice and only anti-PD-1, respectively) [126].

Kanazawa et al performed a comparative study between methoxy[poly(ethylene glycol)]-*b*-[poly(ε-caprolactone)] (MPEG-PCL) polymer micelles and trans-activator of transcription (TAT) modified MPEG-PCL micelles [155]. TAT is a cell-penetrating peptide (CPP) derived from human immunodeficiency virus type 1 (HIV-1) containing a protein transduction domain that can induce endocytosis [156]. Polymer micelles were prepared by the self-assembly method exploiting the amphiphilic properties of the block copolymer. The use of micelles modified with TAT and loaded with a model drug, i.e. coumarin, showed an enhancement of direct IN brain delivery [155]. Furthermore, was investigated the effect of particle size (100, 200, 300 and 600 nm) on brain distribution after IN administration to glioma C6 cells-bearing rats. The coumarin concentrations in the brain administered with 100 nm micelles were significantly higher than in rat brain administered with 600 nm. Interestingly, the drug concentrations in the left side of the brain were higher than those in the right (non-inoculated side) [155].

In a later study from the same group, camptothecin was encapsulated in TAT-modified micelles and administered directly by IN route in rats. Camptothecin (CPT), a quinolone alkaloid, is an inhibitor of the nuclear enzyme DNA-topoisomerase I, which relieves DNA torsional strain by inducing reversible single-stranded breaks [130]. This naturally occurring alkaloid is extracted from the bark of the Chinese tree, *Camptotheca acuminata* [157]. Even though CPT has shown interesting antitumor activity, its clinical use is limited by extremely low solubility, poor stability and systemic toxicity. In fact, although initial clinical trials had shown strong antitumor activity CPT was discontinued during Phase II trials in 1972. CPT caused severe and unpredictable adverse effects including myelosuppression, vomiting, diarrhea and severe hemorrhagic cystic disease [158]. An *in vitro* cytotoxicity study in C6 glioma cells indicated the CPT-loaded MPEG-PCL-TAT micelles showed higher cytotoxicity than CPT-loaded MPEG-PCL "naked" micelles. *In vivo*, compared to unmodified micelles, TAT-modified micelles significantly increased median survival time of rats bearing intracranial glioma tumors. After 7 days of nasal treatment with the simple CPT solution, body weight was significantly reduced compared to untreated rats, indicating severe systemic toxicity. In contrast, CPT-loaded MPEG-PCL or CPT-loaded MPEG-PCL-TAT did not cause significant changes in total body weight, suggesting that micellar formulations were effective in reducing the systemic toxicity of the drug [130].

This approach has also been studied to improve the co-administration of siRNA to the brain [131]. The MPEG-PCL-TAT micelles were loaded with anti-rat Raf-1 siRNA (siRaf-1) and camptothecin (CPT) and evaluated for their brain uptake efficiency on a C6 glioma model (Fig. 4A). Compared to IV delivery the IN delivered MPEG-PCL-TAT significantly enhanced the nucleic acid concentration in rats brain (Fig. 4C). As shown in Fig. 4B and D, significant inhibition of tumor growth *in vitro* and *in vivo* was demonstrated. This was attributed to the combined effects of the CPT and the Raf-1 gene silencing of siRaf-1 in glioma tissues [131].

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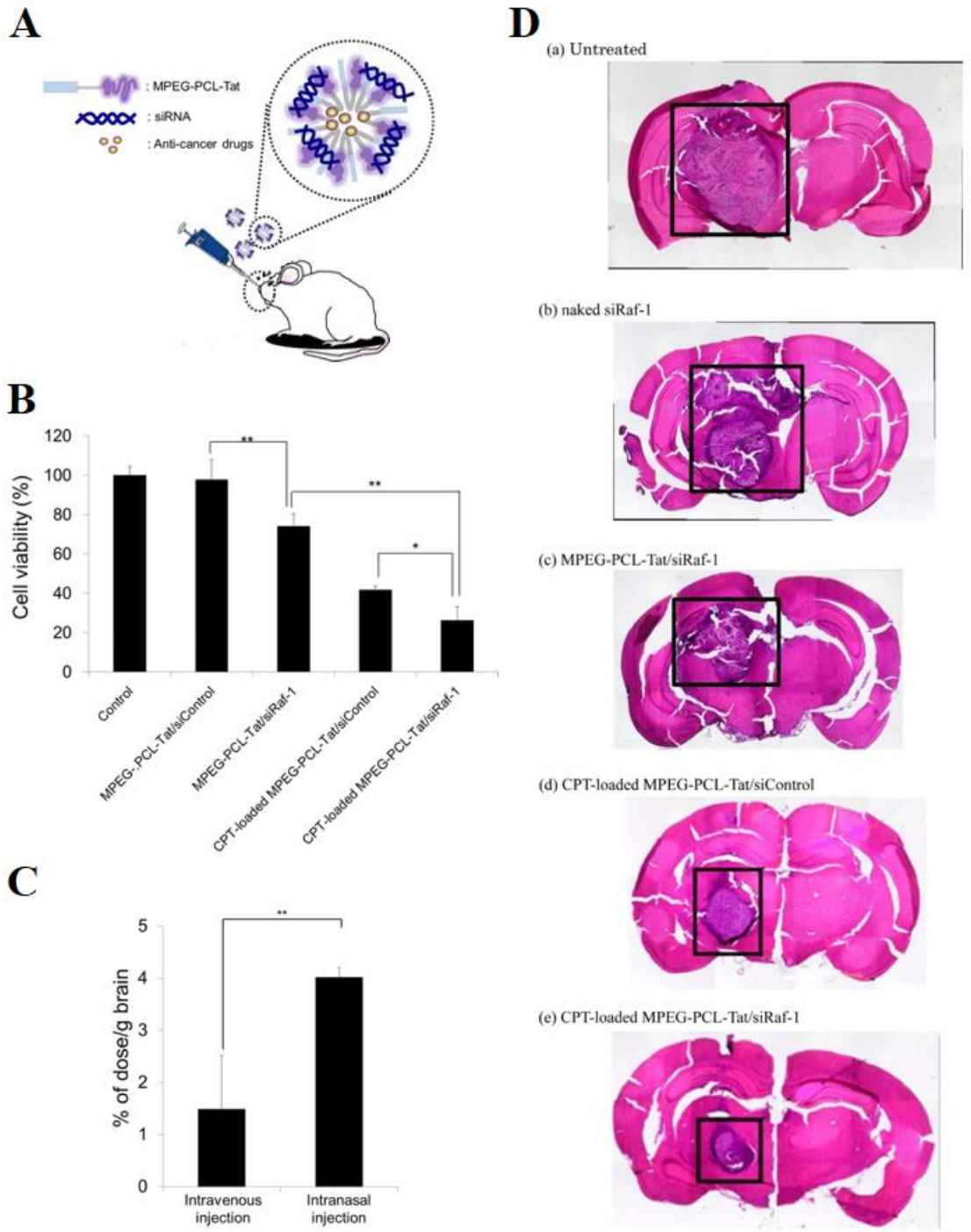

Figure 4. Efficacy in vitro and in vivo of cell-penetrating peptide-modified micelles. (A) Illustrative model for CPT-loaded MPEG-PCL-Tat/siRaf-1. (B) In vitro cytotoxicity (WST-8 assay) in C6 glioma cells transfected with CPT-loaded MPEG-PCL-Tat/siRaf-1 complexes. (C) Distribution of siRNA in brain tissue after intravenous or IN administration of MPEG-PCL-Tat/siRNA complex. Rats were killed after the administration of siRNA/MPEG-PCL-Tat complex (20 μg as siRNA), and each brain was enucleated. (D) Images of HE-stained brain tissue in intracranial C6 glioma-bearing rats after IN administration of siRaf-1 complexed with camptothecin-loaded micelles. After 2 weeks, tissues were taken from untreated rats (a) and rats treated with naked siRaf-1 (b), MPEG-PCL-Tat/siRaf-1 complex (c), CPT-loaded MPEG-PCL-Tat/siControl (d) and CPT-loaded MPEG-PCL-Tat/siRaf-1(e) (* $P<0.05$, ** $P<0.01$) (adapted with permission from [131]. Copyright 2014 American Chemical Society).

Recently, Azambuja and co-workers developed a cationic nanoemulsions (NE) to delivery CD7 3siRNA for GBM treatment through intranasal route [117]. Ecto-5′-nucleotidase (CD73) regulates the

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extracellular adenosine monophosphate (AMP) and adenosine levels, which have been described as proliferation factor and drug resistance [159, 160]. Moreover, CD73 is overexpressed in GBM cells and its inhibition impairs tumor progression [161]. The cationic nanoemulsions were manufactured by microfluidization using lecithin, medium chain triglycerides and 1,2-dioleoyl-sn-glycero-3-trimethylammonium propane (DOTAP). The NE-siRNA CD73 were prepared by the adsorption of siRNA (different theoretical ratios of cationic lipids to siRNA) to blank formulations (ζ-potential + 32 mV).

In vitro studies using C6 glioma cells demonstrated that the NE-siRNA CD73 efficiently decrease cell viability after 48 h of treatment. On the other hand, NE-siRNA scramble used as control did not induce any alteration in C6 glioma cell viability. Additionally, cytotoxicity studies showed that the formulation is safe and does not produce any toxicity in rat primary astrocyte cultures. Interestingly, it was demonstrated that NE-siRNA CD73 was taken up by tumor cells both *in vitro* and *in vivo*, resulting in CD73 knockdown. The *in vivo* results in glioblastoma-bearing rats demonstrated that NE-siRNA CD73 treatment by IN administration significantly decreased glioma growth by 60% when compared to control groups (untreated and NE-siRNA scramble). Furthermore, NE-siRNA CD73 and NE-siRNA scramble treatment did not induce any systemic toxicity to glioblastoma-implanted rats [117].

5. Stem cells for Treatment of GBM

Stem cells have been proposed in recent years for glioma therapy [162, 163, 164, 165]. These cells have a tropism for brain tumoral tissue and a minimum tropism for normal neural cells [166, 167, 168, 169, 170]. Stem cells can be derived from multipotent stem cells such as mesenchymal stem cells (MSCs) and neuronal stem cells (NSCs) [171]. MSCs are hematopoietic stem cells and can be isolated from different tissue sources, such as adipose tissue or bone marrow, making them easier to isolate than NSCs [171, 172]. MSCs have the ability to self-renewal, to differentiate in specific functional cellular and immune-compatible nature [170]). MSCs isolated from human bone marrow [173], adipose tissue [174] and human umbilical cordon [175] have shown the potential to inhibit tumor cells growth. In particular, normal rat embryonic NSCs have been shown to significantly inhibit the survival, proliferation, invasion and migration of glioma cells [176].

The intranasal route is considered an interesting approach to administer stem cells. A study of Reitz and co-workers focused on the delivery of neural stem/progenitor cells (NSPCs) to target brain tumors after intranasal administration [177]. Intracerebral human (U87 and NCE-G55T2), and murine glioma cell-based (syngenic Gl261) glioblastoma models were used to evaluate the specific accumulation in mice brain of NSPCs. The NSPCs treatment initiated after ten days of tumor injection via intranasal administration. The histological analysis performed 5 days after the treatment demonstrated the presence of NSPCs in peritumoral and intratumoral areas of brain. The direct tropism was confirmed by absence of NSPCs in animal brains of control group. The distribution study showed that the cells entered in the brain tumor area 6 hours post-administration. The migration occurred initially (within 24 hours) via olfactory pathways, while later the cells migrated by microvasculature of nasal mucosa [177]. In a different study, it was demonstrated the nose-to-brain migration of MSCs delivered into the nasal cavity occurred via the olfactory and trigeminal pathways [178].

With the intent to exploit their brain tropism, stem cells have also been proposed as carriers to deliver cytotoxic agents. Dey and co-workers evaluated the ability of NSCs vehiculate the oncolytic virus (OV) CRAd-S-pK7 by intranasal administration [179]. CRAd-S-pK7 virus selectively infects tumor cells [180] and stem cells were able to efficiently deliver OV in various models of glioma [181, 182, 183]. In this study, NSCs were genetically modified without changes in their phenotype to verify the improvement the tumor tropism signaling. In two mice models of malignant glioma (GBM43 and GBM6 intracranial xenografts), the administration of NSCs by intranasal route extended the survival of CRAd-S-pK7 viruses in glioma tissue, attributed to an efficient migration of modified NSCs to the brain tissue and a successful delivery of CRAd-S-pK7 to tumoral site. Besides, the authors were able

to verify an extension of animal survival treated with NSCs vehiculating the oncolytic virus in association with radiotherapy (median survival benefit of 5 days) [179].

In another study, Balyasnikova and co-workers demonstrated that MSCs expressing TNF-related apoptosis-inducing ligand (TRAIL) were able to reach the tumoral tissue and to improve the median survival of irradiated mice with intracranial U87 glioma xenografts in comparison to non-irradiated and irradiated control mice [184]. TRAIL is an anticancer protein expressed and secreted by several stem cells, besides selectively promotes apoptosis in glioma cells with minimal effects on healthy cells [171]. The authors also verified the rapid MSCs delivery via the nasal cavity, with detection of MSCs in the animal brains already 2 hours after administration and their subsequent infiltration in the intracranial tumors.

Stem cells approach for GBM treatment has also been combined with nanotechnology. Mangraviti and co-workers developed a system combining polymeric nanoparticles and human adipose tissue derived MSCs to deliver bone morphogenetic protein 4 (BMP4) and evaluated the antitumor effect in a primary malignant glioma model [185]. Polymeric nanoparticles of poly(beta-amino ester)s demonstrated to be a good option for transfection of MSCs due to their favorable physicochemical characteristics: hydrodynamic diameter next to 220 nm, polydispersity index lower than 0.2 and positive zeta potential. Thus, MSCs transfected with polymeric nanoparticles to express BMP4 administered via intranasal route in rats significantly improved the survival of tumor bearing animals: 60% of treated rats survived up to 16 days after treatment with a 21.4% increase in median survival time over control group animals [185].

6. Conclusions

Glioblastoma multiforme is a devastating brain disease with an extremely poor prognosis. Usually, the oral route of administration is considered the most convenient for patients. However, for pharmacological GBM treatment is essential that drugs reach the brain in its bioactive form. Yet, the therapeutical agent has to overcome several biological barriers when administered orally, as enzymatic degradation, first-pass metabolism and the blood-brain barrier. At the moment, temozolomide is the standard chemotherapy agent employed at the clinic and it is administered orally. Intranasal route with focus on nose-to brain delivery of therapeutics to treat GBM present several advantages over the oral route. Here, we showed that different therapeutic agents (small organic molecules, biotech compounds, stem cells) are under investigation for GBM treatment by IN delivery. In terms of delivery systems, drugs entrapped into nanostructured carriers (nanoemulsions, microemulsions, polymeric nanoparticles) are the most employed approach by researchers for IN delivery. These formulations frequently have a functionalization at the surface of nanocarriers used to target receptors overexpressed in GBM facilitating the drug delivery. Nevertheless, most studies are currently only in a preclinical investigation, where successful results remain based on rodent models. Overall, the data obtained by in vivo pre-clinical models on these assessed reports suggest better biodistribution and improved the therapeutic effect of anticancer compound after IN delivery compared to respective controls. In addition, a long-term study carried in human with perillyl alcohol (POH) demonstrated patient compliance using this route of delivery over several years. Although there are still a restricted number of studies focused on IN delivery of anticancer compounds to treat GBM specifically, this strategy has a potential to be exploited and may lead to a new option for treatment of GBM patients in a near future. However, in order to overcome the promising status, it is urgent to start clinical trials. Furthermore, the IN route may be a feasible option as a route of delivery for new drugs that may be developed and also for drug repositioning arising from gene interaction networks for GBM.

Acknowledgments: Franciele Aline Bruinsmann, Adriana Raffin Pohlmann, Silvia Stanisçuaski Guterres and Fabio Sonvico, would like to acknowledge the Brazilian government as recipients of CNPq grants in the programs “Ciências sem Fronteiras” (BOLSA PESQUISADOR VISITANTE ESPECIAL—PVE 401196/2014-3) and “Produtividade em Pesquisa”. Gustavo Richter Vaz would like to acknowledge the Brazilian government as recipients of CAPES – Brazilian Federal Agency for Support and Evaluation of Graduate Education within the

Ministry of Education of Brazil in the program "Programa Doutorado-Sanduíche no Exterior" (PDSE - 88881.189309/2018-01). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, the INCT-NANOFARMA (FAPESP, Brazil) Grant #2014/50928-2, and (CNPq, Brazil) Grant # 465687/2014-8, and the PRONEX FAPERGS/CNPq 12/2014- Grant 16/2551-0000467-6.

Conflicts of Interest: The authors declare no conflict of interest.

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