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Design and validation of lipidic carriers for drug delivery in brain cancer therapy

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To my mother, for making me curious

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CHAPTER 1

General introduction

1.1 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is a malignant brain tumor that was described for the first time in XIX century [1]. It is a IV grade adult-type diffuse glioma isocitrate dehydrogenase (IDH)-wild type, as per the revised classification of the World Health Organization (WHO) in June 2021 [2]. This classification gives more importance to molecular parameters, rather than immunohistochemical and histological features based on the previous classifications of 2016 and 2007 [3,4]. This change will provide several clinical implications about diagnosis and prognosis of tumors of central nervous system (CNS) and particularly GBM. Indeed, restricting the diagnosis only to IDH-wild type tumors will allow to gather more homogenous populations of patients in clinical trials and to provide more targeted therapies [5].

However, since this classification is very recent and needs more time to be discussed and absorbed by clinicians and scientists, in this PhD thesis GBM is going to be described considering also the previous histopathological and molecular classifications.

1.1.2 Overview

Primary malignant tumors of CNS have an incidence of 5-6 cases out of 100,000 people each year and 80% of these tumors is represented by malignant gliomas. Within them, GBM is the most common malignant primary brain tumor, and it shows a poor prognosis. In fact, the survival rate is about 30% in 2 years and 4-5% in 5 years, with a median lifespan of 15 months [6,7].

GBM is part of a larger group of brain tumors divided on the basis of which glial cell type is involved, that includes astrocytomas (astrocytes), oligodendrogliomas (oligodendrocytes) and ependymomas (ependymal cells) [8]. Since GBM originates from populations of astrocytes, according to this classification it is considered an astrocytoma. A further classification divides GBM into primary GBM and secondary GBM (Figure 1), which differ in origins, molecular features, distribution and prognosis [9]. Primary GBM represents 90% of all GBMs and it arises *de novo*. On the contrary, secondary GBM commonly originates from a pre-existing lower grade tumor. In addition, the mean age distribution of primary GBM is usually restricted to people older than 62 years with a predominance for males, while secondary GBM is diagnosed in younger patients (45 years) with a lower pronounced difference between males and females [10–13]. From a molecular point of view, the two types of tumors present very different genetic alterations [14]. In fact, primary GBM usually carries epidermal growth factor receptor (EGFR) mutation, amplification, or overexpression and secondary GBM typically shows mutations in tumoral protein 53 (*TP53)* gene [15]. Other genetic alterations in primary GBM include phosphatase and tensin homologue (PTEN) mutations and loss of chromosome 10, while in secondary GBM 19q loss is more common [10]. IDH1 mutations are likely to be present in 80% of secondary GBM [7] and this mutation has been associated to a better overall survival [16]. On the contrary, less than 10% of primary GBM presents a mutation in IDH1-2 [17,18], and from 2021 on only IDH-wild type tumors can be considered primary GBM (see above) [2].

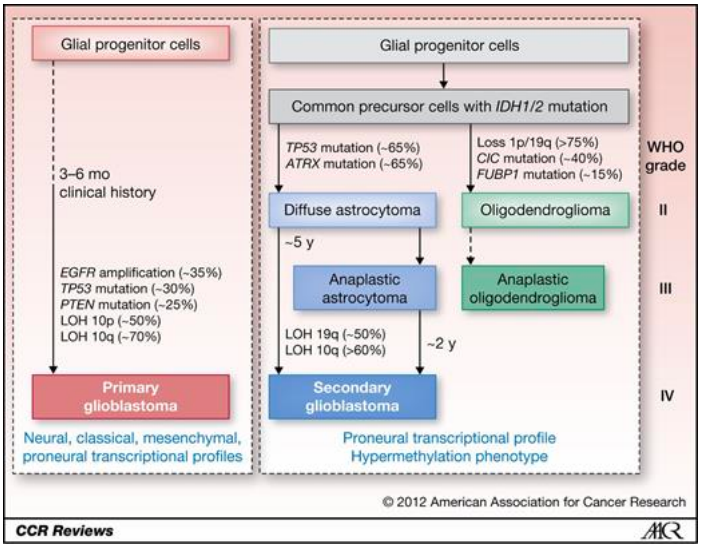

Figure 1. Genetic differences and distinct origins of several brain cancers, particularly primary and secondary GBM. From Ohgaki H. et al., 2013.

The anatomic localization of primary GBM lacking IDH1 mutation is not stationary and it may spread in every cortical area [19], depending on where the tumoral cells are located. Conversely, secondary GBM arises prevalently in the frontal lobe, in the same areas of lower grade astrocytomas or oligondendrogliomas from which it originates or shares common progenitor cells, respectively [9,20].

The symptoms of GBM differ considering the cerebral distribution of the tumor and they include seizures, weakness, language alteration, increased intracranial pressure, changes in mood or mental status, headaches, loss of vision and fatigue [21].

1.1.3 Molecular features

Within the last years, the progression of genomics, epigenetics and high-throughput screening techniques improved the comprehension of several tumoral mechanisms and pathways. This helped to better understand the features of tumors like GBM, where even mild differences may be crucial for diagnosis and prognosis. In 2010 Verhaak et al. identified a novel classification for GBM, which was divided into four subsets based on gene-expression: classical, mesenchymal, proneural and neural (Figure 2) [22]. The classical subtype was characterized by EGFR amplification, which was observed in 97% of cases and it was not common in the others. In addition, chromosome 7 amplification paired with chromosome 10 loss was found in all analyzed patients. EGFRvIII mutation was also frequent, while *TP53* mutation was not. Neurofibromin 1 (NF1) mutations, together with PTEN mutations, were typical of mesenchymal GBM, and a high expression of genes belonging to tumor necrosis factor (TNF) superfamily and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) was also frequent. As concerns proneural subtype, the two major alterations that were found implied the focal amplification of platelet-derived growth factor receptor A (*PDGFRA*) gene or point mutations in *IDH1* and *TP53*. Finally, neural subtype was characterized by the expression of several neural markers such as neurofilament light polypeptide (*NEFL*), synaptotagmin 1 (*SYT1*), gamma-aminobutyric acid type A receptor alpha 1 subunit (*GABRA1*). In addition, the Cancer Genome Atlas (TCGA) Research Network collected samples from 206 GBM patients to analyze the genetic abnormalities associated to GBM tumorigenesis.

It has been shown that the most part of GBM patients displayed mutations in tyrosine kinase receptor, *TP53* and retinoblastoma tumor suppressor (*RB*), providing interesting data about which frequent genomic alterations are required to drive the pathogenesis [22].

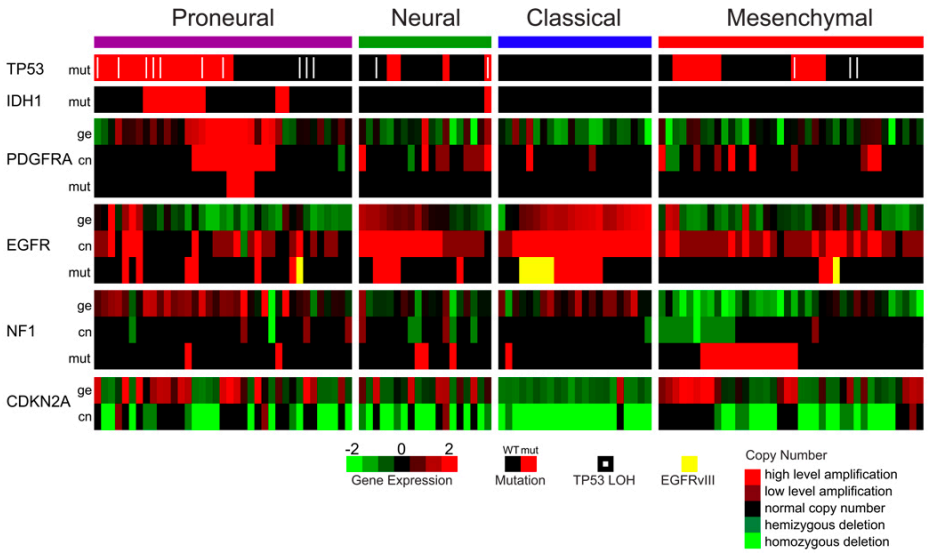

Figure 2. Gene expression and genomic alterations across glioblastoma subtypes. Mutations (mut) are indicated by a red cell, a white pipe indicates loss of heterozygosity, and a yellow cell indicates the presence of an EGFRvIII mutation. Copy number events are in bright green for homozygous deletions, green for hemizygous deletions, black for copy number neutral, red for low level amplification, and bright red for high level amplifications. A black cell indicates no detected alteration. From Verhaak R.G.W. et al., 2010.

In another work [23], Noushmehr H. and colleagues analyzed 272 GBM samples and they found several alterations concerning DNA methylation. Particularly, the glioma CpG-islands methylator phenotype (G-CIMP) was associated with secondary or recurrent GBM carrying *IDH1* mutations. The silenced genes were involved with tumor invasion pathways, markers of molecular matrix and mesenchyme. Interestingly, this G-CIMP phenotype was prevalently found in proneural tumors subtype and in lower grade brain tumors. These types of tumors were associated with a better prognosis and survival in respect to other types, even though these patients often do not benefit of aggressive therapies, which are more indicated for classic and mesenchymal GBM [14,24]. However, these genetic characteristics could be used to further improve and refine the diagnosis basing on molecular phenotypes and the development of targeted therapies for distinct groups of patients.

In 2016 the WHO proposed a series of diagnostic terms with the purpose to integrate histopathological, cytoarchitectural and molecular data (like *IDH1* mutations) of CNS tumors in a four-grades scale (Figure 3) that also included not precisely defined entities [25,26]. Diffuse gliomas, which could be distinguished in astrocytomas, oligodendrogliomas and mixed tumors, were categorized as grade II (low-grade astrocytomas or oligodendrogliomas) and grade III (anaplastic astrocytomas or oligodendrogliomas). GBM was classified as a IV grade astrocytoma (the most aggressive) and other gliomas like ependymomas, that displayed limited growth characteristics, were scaled until grade III [27].

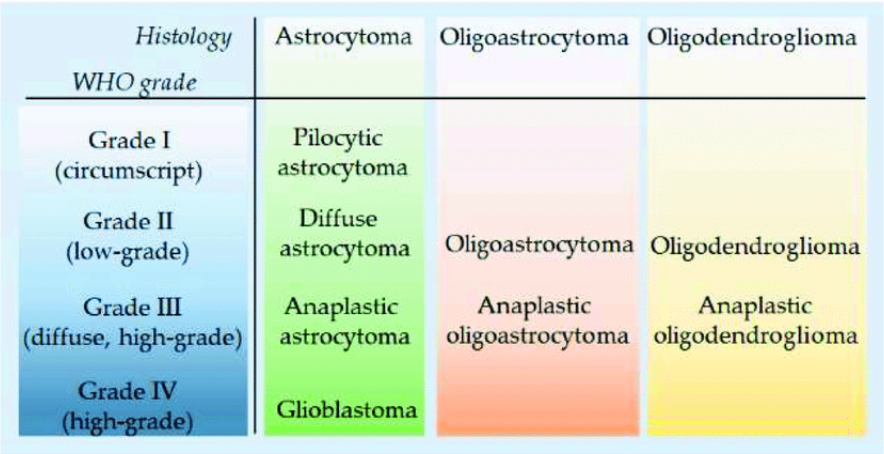

Figure 3. The 2016 WHO classification of diffuse gliomas. From Arcella A. et al., 2020.

The innovation in this classification, included the "NOS designation", indicating all those CNS tumors with insufficient information or without a precise definition to be classified in other groups, for example tumors with uncertain genetic markers or carrying lesions without showing genetic alterations. Regarding GBM, the mutations in *IDH1*/*IDH2* were considered crucial to distinguish this tumor from other astrocytomas, like diffuse or anaplastic astrocytoma [28,29], to include in the same classification entities sharing the same prognostic features. Following the same logic, some variants subtypes of GBM were identified to strongly improve the diagnosis and the choice of treatments [30]. Epigenetic modifications emerged to have a key role in GBM pathogenesis and prognosis. Methylation of DNA repair protein O6-methylguanine-DNA-methyltrasferase (MGMT) has been shown to be a biomarker that indicates a better prognosis [31–33].

Indeed, patients carrying MGMT promoter methylation displayed longer overall survival (OS), even though in a large meta-analysis a significant improve in progression-free survival (PFS) was not demonstrated [34]. MGMT promoter methylation leads to gene silencing and decrease of protein expression, and this produces benefits for GBM treatment with alkylating agents such as temozolomide (TMZ, see below). In fact, the decrease of protein expression potentially rescues TMZ-resistance phenotype and promotes cell death after therapy [35,36]. Prognosis seems to be also influenced by gender, as demonstrated by the study of Franceschi E. et al. in which they found a strong improve in survival when MGMT methylated phenotype was associated to female patients in comparison to male patients [37]. Another common epigenetic modification in GBM is represented by histone modifying enzymes such as histone acetyltrasferase (HATs), histone deacetylase (HDACs), lysine/arginine methyltrasferase, lysine demethylase, SUMOylating and ubiquitinating enzymes. HATs are ubiquitous enzymes that have the ability to acetylate lysine residues on NH_2-terminal of histone tails using acetil-CoA as a coenzyme [38–40]. This modification is usually associated to a better accessibility on chromatin by transcription complex, leading to an increase in transcription and subsequent protein expression. However, specific HATs can acetylate also non-histonic targets, such as cytoplasmic protein α-tubulin [41]. Aberrant regulation or expression of HATs were associated to several cellular pathways including DNA repair and chromatin integrity [42], cell cycle [43,44] and self-renewal [45].

In addition, several diseases were associated to HATs deregulation, such as Rubinstein-Taybi syndrome [46], amyotrophic lateral sclerosis [47] and cancer [48–51]. In GBM, there is evidence that the upregulation of specific HATs like KAT6A is crucial for glioma formation, making this protein a potential target for therapy [52]. HDACs are correlated to tumorigenesis as well (Figure 4) [53,54]. Contrarily to HATs, HDACs remove acetyl groups from histonic and non-histonic proteins with consequent silencing of transcription and at least eighteen enzymes with this function were identified [55,56].

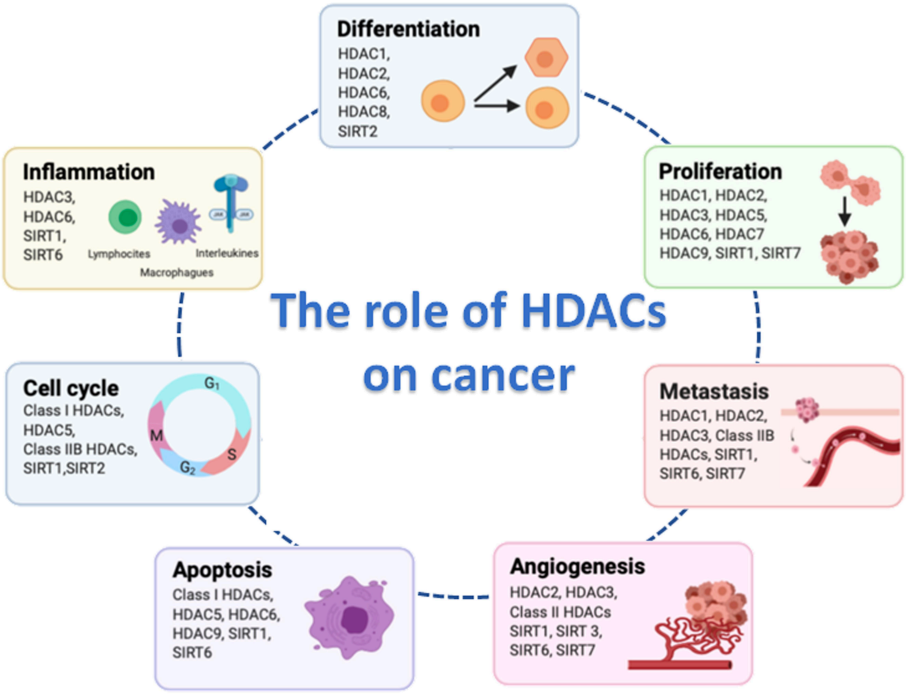

Figure 4. Pathways in which histone deacetylases (HDACs) are involved. From Hontecillas-Prieto et al., 2020.

The HDACs superfamily includes four classes of enzymes (Figure 5), whose classification is based on their homology to the correspondent yeast protein [57]. Among the members of these classes, HDACs can either be nuclear (HDAC1), cytoplasmic (HDAC6) or can shuttle between the two compartments (HDAC4). HDAC7 was found to be present also in mitochondria [58].

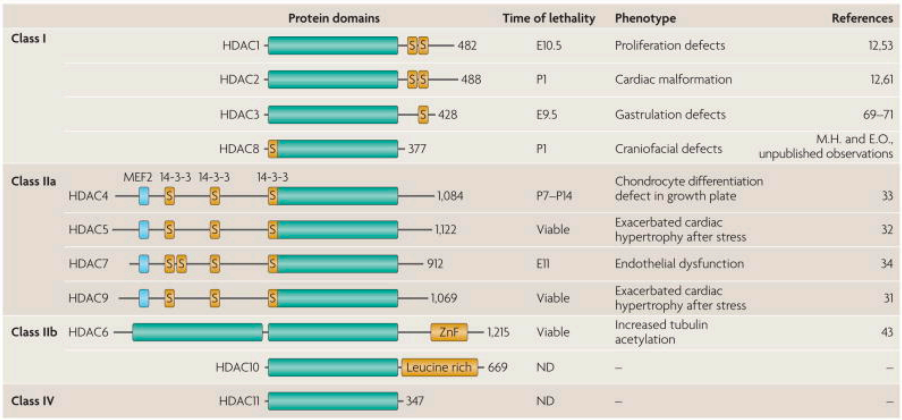

Figure 5. The histone deacetylase (HDAC) superfamily, showing protein domains, loss-of-function phenotypes in mice and time point of lethality of the knockouts. Green rectangles indicate the conserved HDAC domain; numbers following the HDAC domain indicate the number of amino acids. Myocyte enhancer factor 2 (MEF2)-binding sites are marked by a blue square, and binding sites for the 14-3-3 chaperone protein are also shown. E, embryonic day; ND, not determined; P, days postnatal; S, serine phosphorylation sites; ZnF, zinc finger. From Haberland M. et al., 2009.

Class III HDACs, or sirtuins, are considered an exception: unlike the other HDACs they are not zinc-dependent but NAD^+-dependent and their activity has been correlated to aging processes and metabolic health [59], as well as glioma [60].

As concerns the link between HDACs superfamily and GBM, there is a lot of evidence that overexpression, mutations or aberrant activity of these enzymes are crucial for its pathogenesis and prognosis [53,61]. This is the reason why many different drugs, known as HDACs inhibitors (HDACis), were developed to counteract the pathology. As a matter of fact, HDACis were proven to display several anticancer activities (Figure 6) involving multiple cellular pathways including metabolic reprogramming [62], cell differentiation [63–65], apoptosis [66] and angiogenesis [67]. In addition, both pan-HDACis and selective-HDACis have been used in combination with other anticancer drugs, some of which are in clinical trials, including alkylating agents, proteasome inhibitors [68], radiotherapy [69], other epigenetic drugs and immunotherapy [70], in order to enhance their effects [71,72].

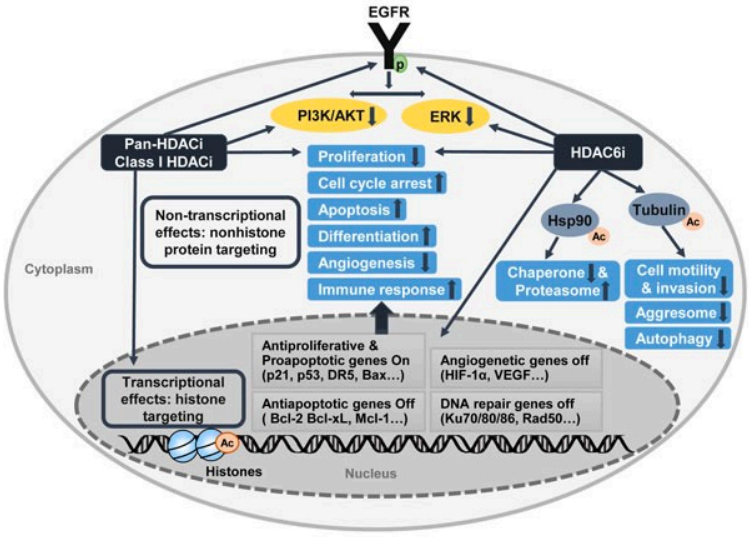

Figure 6. Antitumoral activitiy of HDACs inhibitors. From Dong Hoon L. et al., 2017.

Lysine/arginine methyltransferase are enzymes able to transfer one or more methyl residues on histonic proteins to regulate the transcription process [73] and histone hypermethylation was correlated to tumor progression and malignancy in GBM [74].

Histone demethylation is another epigenetic modification mediated by histone demethylases. An overexpression of these enzymes was described in GBM cells [75], together with mutations in genes coding for histonic proteins leading to DNA hypomethylation [76]. Finally, ubiquitination and SUMOylation are processes regulated by a similar signaling cascade, that differ because an ubiquitinated protein is recognized by proteasome and degraded, while a SUMOylated protein remains stable [77]. Both modifications were associated to GBM proliferation, invasion and poor prognosis [78–80].

1.1.4 Heterogeneity and intercellular communication in GBM

GBM displays a high grade of heterogeneity, even in different areas of the same tumor. This particular feature is due both to the presence of GBM stem cells (GSCs) and to the tumor microenvironment (TME). GSCs, like other stem cells, are characterized by self-renewal, persistent proliferation, capacity to differentiate in multiple lineages (multipotency) and peculiar marker expression [81]. Several markers were proposed to unequivocally define GSCs using FACS analysis and targeting, including CD133 [82,83] and L1CAM [84], although many of them are shared with normal stem cells. In any case, GSCs should be able not only to reproduce the original tumor when transplanted *in vivo* [85] but they should also recapitulate the tumor heterogeneity [86].

In addition, GSCs can adapt to different TEMs and can communicate and interact both with the differentiated cancer cells and with the cells and structures within the extracellular matrix (ECM) or within the healthy tissues. These include neurons, immune cells, fibroblast, vasculature and structural proteins of ECM [86]. In this regard, GSCs are subject to a cellular hierarchy that depends on one of the three major TEMs (or niche) they belong, which activates different cellular pathways inside the GSCs.

The first TEM is called “perivascular niche”, where the endothelial cells promote the stemness phenotype of GSCs by secreting paracrine factors and chemokines and GSCs secrete proangiogenic factors [87,88]. Here the endothelial cells also promote the overexpression of matrix metalloproteinase 9 (MMP-9) that is a mediator of tumor invasion [89]. The second microenvironment, the hypoxic and necrotic niche, supports GBM resistance to chemo- and radiotherapy and recurrence. GSCs metabolism appears to be driven by glycolysis and mitochondrial NIX-mediated mitophagy seems to promote cells survival in these restricted conditions [90,91]. The last microenvironment is represented by the invasive niche. Here, the possibility of relapse reaches its maximum potential, since migrating GSCs cannot be resected during surgery. The migrating phenotype is obtained by the expression of markers like L1CAM [84,92]. GSCs invade other cerebral areas and spread around the healthy tissues using integrins and cadherins cleaved by MMP-2 and MMP-9 available in the ECM [92,93].

The heterogeneity of GSCs subpopulations and the peculiarity of their ability to adapt in different TEMs require the development of sophisticated *in vitro* and *in vivo* models able to reproduce the complexity of GBM with an acceptable grade of reliability. The recurrence rate after surgery is beyond 80% in 5 years [31]. Only 20-30% of these tumors is accessible to surgery and the survival after diagnosis is about 15 months. Because of the infiltrative nature of tumor-initiating cells and GSCs, a new tumor grows with extensive necrotic areas and the pharmacologic treatment is largely ineffective [94,95].

As already explained, TME is a complex network of cells within the ECM, where cancer cells proliferate and acquire the invading phenotype. Nevertheless, resident or infiltrating cells can play a pivotal role in immune surveillance by recognizing newly formed tumor cells and eliminating them [96]. Because of this intrinsic characteristic of TME, immunotherapy has been proposed as a therapeutic approach against cancer, with the help of nanomedicine (see Chapter 2). Unfortunately, GSCs can down-regulate immune recognition signals like toll-like receptor 4 (TLR-4) [97] and recruit tumor-associated macrophages (TAMs) to sustain GBM progression by secreting multiple factor such as migration inhibitory factor (MIF) [98]. The cross-talk between tumor cells and cells in the TME can be also mediated by extracellular vesicles (EVs) like exosomes, microvesicles, apoptotic bodies and large oncosomes (Figure 7) [99]. Indeed, EVs play a critical role in intercellular communication by delivering proteins, lipids and other cargos to promote invasion, angiogenesis and drug resistance [100].

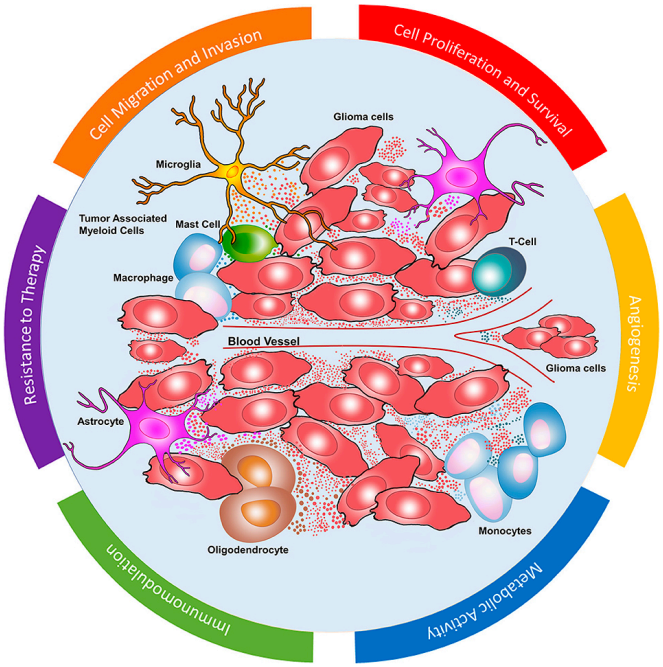

Figure 7. GBM microenvironment and dynamic EVs mediate communication between glioma cells and stromal cells including monocytes, macrophages, mast cells, microglia, T cells, astrocytes, and oligodendrocytes. From Balaj L. et al., 2020.

Beyond EVs, intercellular communication takes place also through secretion, gap junctions, tunneling nanotubes (TnTs) and microtubes (TmTs) [101]. Secreted factors are already known as signaling molecules and include interleukin 6 (IL-6), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) [102]. However, TnTs and TmTs seem to play a key role in intercellular communication, and they could be exploited in GBM therapy and drug delivery (see Chapter 3).

Briefly, TnTs and TmTs are open, transient, membranous channels made of actin that can connect near or distant cells and exchange non-secretable materials. These protrusions can have different length, thickness and each cell can produce more than one channel [103–105]. It has been shown that GBM cells uses TnTs and TmTs to help and rescue chemo- and radiotherapy damaged cells providing structural components or even organelles [106].

Gap junctions are made of connexin hexamers and their arrangement can create channels with distinct properties. These structures are mainly used by tumor cells to transfer small molecules and ions [107]. Since gap junctions are fundamental for cell-to-cell communication, connexin is usually overexpressed during tumorigenesis, especially in TMZ-resistant cells [108].

In conclusion, unraveling the mechanisms underlying the complex network of interactions among cells appears to be cardinal to increase knowledge and improve the therapeutic approach to this tumor.

1.1.5 Diagnosis and treatment

GBM is a serious clinical issue and within the last 20 years, some progress was made to improve the diagnostic and prognostic criteria. Magnetic resonance imaging (MRI) is commonly used for initial diagnosis and the clarification of tumor grade assessment before surgery (Figure 8). This assessment is required because surgical resection is not indicated for every CNS malignancy and it can help to choose the appropriate section for biopsy to define the tumor grade [109]. The biopsy is commonly established using formaldehyde-fixed and paraffine-embedded tissue sections.

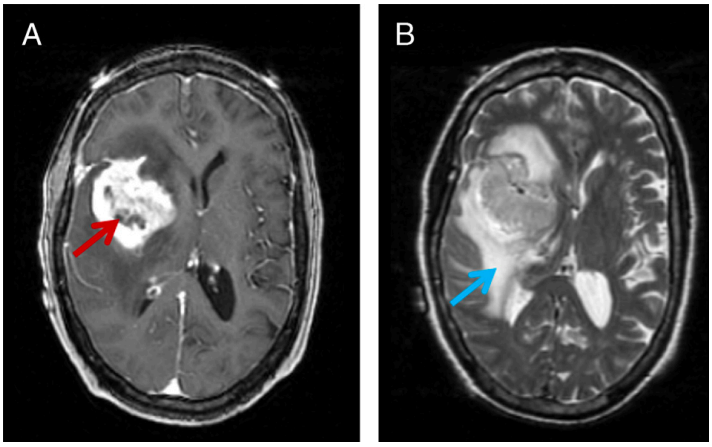

Figure 8. Example of a newly diagnosed glioblastoma. **A**, T1-weighted image with gadolinium enhancement: note the large, heterogeneously contrast-enhancing mass with irregular areas of central necrosis (red arrow). **B**, T2-weighted image, demonstrating the mass as well as the significant surrounding edema (blue arrow). From Clarke J.L. et al., 2012.

There are no known risk factor for GBM, with the exception of ionizing radiations exposure and some genetic syndromes [110]. In addition, the association with genes of susceptibility seems to be present but weak [111], given to the high genetic heterogeneity. This also limits the clinical application of prognostic biomarkers, like IDH1 [112], protein death ligand 1 (PD-L1) [113] and MGMT promoter methylation [34]. However, circulating tumor-derived nucleic acid in fluid samples can be analyzed through liquid biopsy to identify novel biomarkers and stratify patients basing on prognosis [114]. The current standard care for GBM was established by Roger Stupp in 2005 [115] and it is still used to treat patients together with surgical resection. The protocol consists in radiotherapy and daily oral administration of TMZ until the last day of radiotherapy, plus six cycles of adjuvant TMZ.

TMZ (3-methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) derives from imidazotetrazine and its lipophilic nature allows the blood-brain barrier (BBB) crossing from blood. It is well tolerated, with relatively acceptable side effects [116].

After administration, TMZ is converted to 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC), the active metabolite. MTIC can methylate the guanine at O6 and N7 and adenine at N3 in DNA or RNA structure, altering replication and translation and generating apoptosis [116,117]. TMZ-resistance, provided by the expression of enzymes like MGMT, limits its use as a chemotherapeutic agent. There is also some evidence about the induction of hypermutation induce by TMZ itself in low-grade gliomas, which contribute to get the patients' prognosis worse [118].

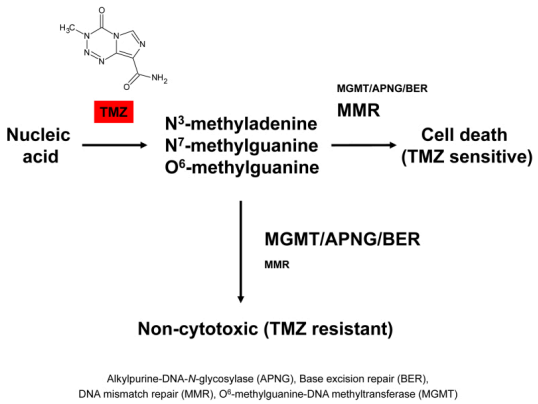

Figure 9. Mechanism of Temozolomide and Temozolomide resistance. Temozolomide (TMZ) modifies DNA or RNA at N7 and O6 sites on guanine and the N3 on adenine by the addition of methyl groups. The methylated sites can remain mutated, be fixed by DNA mismatch repair (MMR), be removed by base excision repair (BER) by the action of a DNA glycosylase such as, alkylpurine-DNA-N-glycosylase (APNG), or deakylated by the action of a demethylating enzyme such as O6-methylguanine methyltransferase (MGMT). Cells are TMZ sensitive when MMR is expressed and active. When MGMT, APNG, and BER proteins are expressed, GBM cells are resistant to TMZ. From Lee S.Y., 2016.

Beyond TMZ, the other drug approved by Federal Drug Administration (FDA) for the treatment of GBM is the humanized monoclonal antibody Bevacizumab. This antibody exerts an anti-VEGF-A activity, and it was developed because VEGF-A is 30-fold overexpressed in higher-grade gliomas and it is a marker of worse prognosis. VEGF-A promotes proliferation and survival of endothelial cells, resulting in angiogenesis [102,119]. It has been shown that Bevacizumab reduces tumor growth and cells viability but in a large phase II clinical trial it did not provide a significant benefit in terms of overall survival [120]. Moreover, it has been demonstrated that tumor cells can become resistant to Bevacizumab as well, using VEGF-independent angiogenetic pathway [121]. Other emerging treatments for GBM include immunotherapy, oncolytic viruses, small molecule inhibitors and HDACis (see Chapter 4) [122].

1.2 Nanomedicine

Nanomedicine is a field of study of medicine that implies the application of nanotechnologies in human health. The National Nanotechnology Initiative (NNI) gave a definition of nanotechnology, which has to maintain a size in nanometer scale and this property must provide a significative advantage [123]. In medicine, these nanomaterials are devices with a dimension in the range of nanometers and include drug delivery vehicles, diagnostic agents, imaging tools (nanoparticles contrast agents), nanosensors and other medical devices [124].

In medicine and biology, the term “nanoparticles” (NPs) is commonly used to describe pharmaceutical drug carriers with potential application both in diagnostic and therapy [125].

1.2.1 Nanoparticles in medicine

NPs are useful and versatile tools with many applications in medicine. They may be functionalized or multi-functionalized on their surface using peptides, small molecules, and antibodies to improve the targeting and drug delivery towards cells or biological structures and to efficiently cross biological barriers (like the BBB). In addition, NPs have the potential to increase the solubility and the stability of encapsulated drug, thus improving the safety profile and the efficacy of medicines prolonging the circulation time [126].

The number of NPs in literature is countless, but despite the great efforts there are a few formulations approved for clinical use. This may be in consequence of NPs that result to be non-biocompatible or non-biodegradable or of a failure in translatability between animals and humans [127].

NPs can be divided into two large groups: organic and inorganic, based on the material they are made of. Among organic NPs the most used are lipid-based NPs (i.e. solid-lipid NPs (SLNs) and liposomes), polymer-based NPs and nanoemulsions. Inorganic NPs include metallic NPs (iron oxide, gold and silver NPs) and carbon-based NPs [125,128]. A summary of the most commonly used NPs is shown in Table 1.

Type of nanoparticle	Typical size range/nm	Structure and properties
Inorganic		
Metals (Au, Ag, Cu)	5–250	■ Easy to synthesize over a broad range of sizes and shapes (*e.g.* spheres, rods, core–shells); robust and functionalizable *via* thiol-metal chemistry ■ Surface plasmon resonance; surface enhanced Raman scattering
Iron oxides	5–200	■ Typically magnetite ($M_xFe_{3-x}O_4$, M = Mn, Ni, Co, Fe) or maghemite (Fe_2O_3) ■ Ferromagnetic or superparamagnetic properties
Quantum dots	3–30	■ Typically II–VI or III–V chalcogenides synthesized as core–shell or alloy nanocrystalline colloids (*e.g.* CdSe/ZnS, $CdTe_{1-x}Se_x$) ■ Bright, photostable fluorophores with broad absorption and narrow emission; large two-photon cross section; FRET-donors
Silica	3–100	■ Biodegradable; available also in micro- or mesoporous form for encapsulation of dyes and drugs; easily derivatizable with different surface chemistries using silanes
Layered double hydroxide	50–200	■ $Mg_6Al_2(CO_3)(OH)_{16}{\cdot}4H_2O$ ■ Biocompatible and biodegradable in mildly acidic environments; high drug loading capacity
Calcium phosphate	10–100	■ $Ca_5(PO_4)_3OH$ ■ Biodegradable and biocompatible; can be doped with lanthanides or organic fluorophores
Organic		
Liposomes	Multilayer: 500–5000 Unilayer: 100–500	■ Spherical self-closed structures composed of one of more concentric phospholipid bi-layers ■ Biocompatible, can entrap both hydrophobic and hydrophilic moieties; protects payload from external environment ■ Size and surface functionality can be tuned by adding new ingredients to the lipid mixture prior to synthesis
Polymer micelles	20–200	■ Self-assembled spherical micelles composed of amphiphilic block co- or tri-polymers containing a hydrophobic core and a hydrophilic corona ■ Hydrophobic payload can be entrapped in the core ■ Geometry and functionality can be modularly controlled *via* the length and composition of the polymer blocks; can be biodegradable
Polymer nanoparticles	50–300	■ Linear polymers with payload conjugated to the sidechain; precipitated into colloidal nanoparticles in solution ■ Controllable size, surface functionality by adjusting polymer length, composition, and synthesis conditions; can be biodegradable
Dendrimers	2–10	■ Radially hyperbranched polymers with regular repeat units ■ High structural and chemical homogeneity; high ligand density and payload capacity per particle; controlled biodegradation ■ Common dendrimers for biological applications: polyether, polyester, PAMAM
Carbon nanotubes	d = 0.5–3 l = 10 nm to several centimetres	■ Single or multi-layered graphene sheets rolled into concentric cylinders ■ NIR-photoluminescence, strong resonance Raman scattering effects; directional conductivity, high tensile strength ■ Water-soluble through covalent chemical modification or non-covalent adsorption; ability to translocate cellular membranes *via* non-endocytosis mechanisms
Viral nanoparticles	25–150	■ Self-assembled protein cages with multivalent surface functionalities ■ Natural ability to internalize and unpack payload within cells

Table 1. NPs used in biomedical research. From Chou et al., 2011.

SLNs are spherical NPs composed of lipids that are solid at room temperature and they can be loaded with lipophilic drugs within their core. SLNs are biocompatible and biodegradable and can be easily functionalized for targeting.

Polymeric NPs are commonly made of poly lactic-glycolic acid (PLGA) or chitosan and they are biocompatible and biodegradable. These NPs are used as drug carriers and for controlled release of drugs and some formulations were approved by the FDA for clinical uses [129].

Nanoemulsions are colloidal dispersions used as drug carriers for molecules with limited water solubility and they are composed of a dispersion of oil nanodroplets in water or water nanodroplets in oil. These compositions are able to protect the drug from degradation, allowing parenteral and transdermal administration. Their fields of application include anti-cancer drugs and vaccines [130]. Iron oxide, gold and silver NPs (also known as inorganic NPs) conjugate a metal core with a biocompatible polymer. These types of NPs are indicated for diagnostic, therapeutic and radiotherapeutic purposes and silver NPs also got some attention as biosensors, antimicrobial coatings and as parts of biomedical devices. However, their toxicity profile needs to be improved [131–133]. Carbon-based NPs, for example carbon nanotubes (CNTs), were suggested for use in gene therapy, drug delivery and imaging, due to their high stability and high drug loading capacity. Unfortunately, their safety profile and toxicity were highlighted in various works, especially during chronic exposure [134,135].

NPs have been studied for decades due to their unique characteristics and the possibility to modify their architecture, composition and properties (Figure 10). In particular, the possibility to load drugs and contrast agents improves the pharmacokinetic and pharmacodynamic profile of embedded molecules, increases their solubility, stability and circulation time, and provides a reduction of toxicity and side effects improving the active tissue targeting [136].

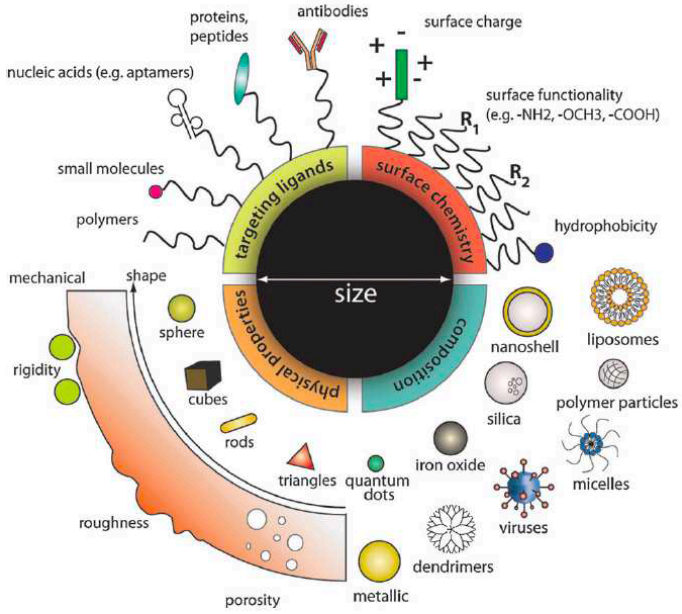

Figure 10. Nanoparticles for biomedical applications. Nanoparticles can be made of different materials with different physical and chemical properties and functionalized with several ligands for biological targeting. Flexibility in design enables researchers to tailor nanoparticle for specific applications as contrast agents, drug delivery vehicles, and therapeutics. From Chou et al., 2011.

The administration of NPs, depending on the composition and properties of the single formulation, can be parenteral, oral, ocular, transdermal or inhalational [137]. Their pharmacokinetics and pharmacodynamics properties are mainly affected by size, shape and charge. The size is crucial for NPs biomedical application, since it influences several factors such as plasma half-life, immune system recognition, opsonization and extravasation.

Moreover, NPs with a diameter <5 nm are subject to renal clearance, while clearance of bigger NPs (diameter >200 nm) is provided by liver and spleen.

Therefore, NPs with a size around 100-150 nm are the most investigated for biomedical use, since they have a prolonged circulation time [138,139]. The shape of NPs influences their adhesion and interaction with biological structures (i.e. vasculature and biological barriers). Several shapes were proposed to improve these features: spherical, discoidal, rods etc [128,140]. Finally, also the surface charge affects plasma half-life, diffusion properties and accumulation in targeted tissue. In particular, NPs neutrally or negatively charged have a lower rate of non-specific cellular uptake and lower interactions with serum proteins that provide a faster elimination, in comparison to positively charged NPs [139,140]. Serum proteins can also adsorb on NPs surface, creating a layer known as protein corona, which increase the elimination rate and the untargeted. There are some strategies to overcome this issue, for example functionalizing the NPs with polyethylene glycol (PEG) [141]. This molecule has the peculiarity to interact with water, in order to create a shell that protect the NP from opsonization and elimination [142]. It is worth of mention that under specific conditions, protein corona formation can be exploited as target strategy for NPs delivery [143,144].

1.2.2 Liposomes

Liposomes are lipid-based NPs, described for the first time in 1965 by Bangham A.D. and colleagues [145]. The composition of liposomes consists in amphiphilic lipids (hydrophilic head and hydrophobic tail) forming a bilayer that surrounds an inner aqueous core and their form is generally spherical. Various lipids can be used to produce liposomes, and they can be either cationic, neutral or anionic, depending on the specific need.

Cationic lipids increase cellular internalization due to the attraction between the liposome and the negatively charged plasma membranes, and lysosome uptake and degradation. Examples of cationic lipids are DOTMA (2,3-dioleoyl-propyl)-trimethylamine bromide) and DOTAP ((2,3-dioleoyl-propyl)-trimethylamine). Neutral lipids exhibit lower toxicity and help the stabilization of liposomes membranes.

The most common are cholesterol (Chol), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and their variants such as DSPE (1,2-distearoylsn-glycero-3-phosphoethanolamine) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine). Finally, anionic lipids like phosphatidylserine (PS) provide a great stability in solution and low aggregation [146–148]. The choice of lipid composition is critical also because they influence the permeability, the fluidity and the rigidity of the structure. Liposomes can either possess a single or multiple bilayers (Figure 11) (*lamellae*) and based on this feature three group can be distinguished: 1) small unilamellar vesicles (SUV) are formed by a single bilayer and range from 20 to 100 nm; 2) large unilamellar vesicles (LUV) have a single bilayer as well but they have a larger size than SUV, from 100 to 250 nm; 3) multilamellar vesicles (MLV) are made of several bilayers separated by single layers of water and they are generally larger than SUV and LUV, ranging from 1 to 5 μm. The number of bilayers and the size, influence the amount of drug that can be encapsulated and the behavior *in vivo* [149].

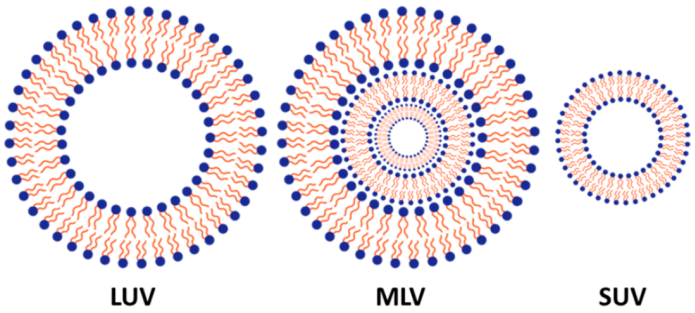

Figure 11. Liposomes can be classified as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV). From Gonzalez Gomez A. et al., 2020.

The synthesis of liposomes can be performed with several methods, divided into two main classes: 1) in hydration methods, lipid powders or cakes are directly dissolved in water or lipid films are deposited on a substrate before hydration with an aqueous solution (thin-film hydration or Bargham method) sometimes in presence of an electric field (electroformation); 2) in Bulk methods, lipids are dissolved in an organic solvent that is then replaced by an aqueous solution [150]. The preparation may include other steps of preparation and purification, such as sonication, freeze/thaw cycles and extrusion in order to control the number of *lamellae* and the size. The choice of the most appropriate preparation method depends on the characteristics of drugs and liposomes, the application of the formulation, the reproducibility of the method and the toxicity of the individual components [151].

The characterization of liposomes is performed by analyzing the following parameters: 1) size and polydispersity, that determine the volume of the aqueous core (influencing the encapsulation of the drug), their metabolism *in vivo* and the homogeneity of the sample. Size and polydispersity can be measured using dynamic light scattering (DLS), electron microscopy and size-exclusion chromatography. 2) ζ-potential is a parameter that can indicate the surface charge of colloidal particles, thus providing an estimate of stability and NPs properties. 3) Lamellarity is the number of lipid bilayers of the liposomes, and it can be measured using cryogenic TEM or small-angle X-ray scattering. 4) Encapsulation efficiency (EE) indicates the percentage of drug that is entrapped inside the aqueous core (hydrophilic drugs) or the lipid bilayer (hydrophobic drugs) in respect to the total drug used during the preparation. The external drug can be purified by dialysis, ultracentrifugation or ultrafiltration, while the internal drug is measured using detergents or solvents to release the liposome content. EE% is calculated as difference of external and internal drug on total drug. 5) Cargo release provides information about the stability of the final formulation, specifying if an acceptable amount of drug is retained inside the liposomes after selected periods of time. Dialysis is usually the best method for measuring this parameter [149]. Since liposomes are biodegradable, biocompatible, versatile and with low immunogenicity they are one of the most used NPs in research and medicine, with several FDA approved formulations (Figure 12) [152].

Figure 12. Therapeutic areas covered by liposome-based products. From Bulbake U. et al., 2017.

1.2.3 Drug delivery of liposomes

In order to be effective, NPs need to reach the organ of interest (drug targeting) and then the encapsulated drug must be released to exert its pharmacological effect (drug delivery) [153]. In addition, the drug needs to be bioavailable within its therapeutic window and for enough time [154]. A major problem that must be overcome after liposomes administration is the clearance from blood circulation. A very common strategy is the functionalization of surface with polyethylene glycol (PEG), which increase plasma half-life making the liposomes stealth for serum proteins [141,155,156]. The surface functionalization can be performed using a variety of molecules to target different organs and that includes aptamers, antibodies, proteins, peptides, small molecules and carbohydrates [157].

However, once targeted, the drug may need to be delivered inside the cells to exert its effect. Hydrophobic small molecules passively diffuse across the membranes, while larger and hydrophilic drugs cannot. One strategy is the formulation of liposomes with fusogenic lipids able to fuse with plasma membrane or lipids with pH-dependent disruption [158,159]. Another strategy includes the functionalization with molecules that can bind to membrane receptors to perform receptor-mediated endocytosis [160–162]. CNS drug delivery is more difficult compared to other organs, due to the presence of BBB and the intrinsic cellular organization in brain. These issues led to the failure of many treatments for brain diseases. The BBB is a semi-permeable barrier whose main function is to segregate the brain from its external environment (blood circulation) in order to protect the organ and preserve homeostasis [163]. The anatomical structure of BBB (Figure 13) is formed by a monolayer of microvascular endothelial cells forming a capillary wall that makes contact with pericytes and astrocytes, composing the so-called neurovascular unit (NVU). In addition, vascular (internal) and parenchymal (external) basement membranes provide structural support and further protection from external substances acting as second barrier [164].

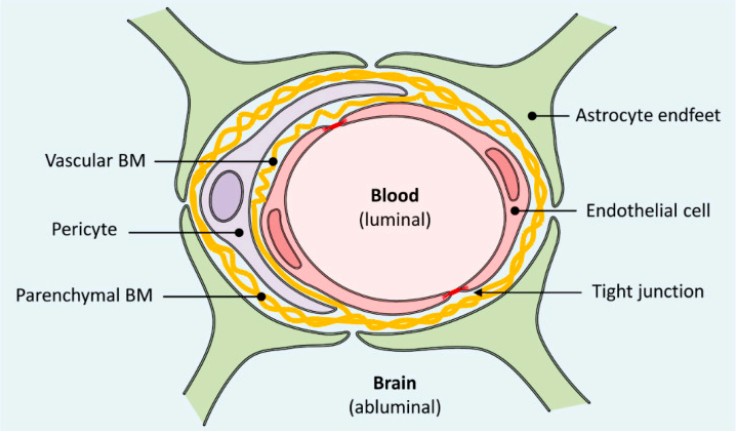

Figure 13. Anatomical structure of the blood–brain barrier (BBB). The wall of all brain capillaries is formed by a thin monolayer of specialized brain microvascular endothelial cells joined together by tight junctions, which act as a physical, transport and metabolic barrier. They are surrounded by a vascular basement membrane (BM), pericytes, a parenchymal BM and astrocyte endfeet, all of which directly or indirectly contribute to the barrier function of the BBB. From Neumaier F. et al., 2021.

The BBB endothelial cells, unlike other endothelial cells, do not possess fenestrations and show low levels of non-specific pinocytosis. Moreover, there is a high content of efflux transporters and they are closely connected by tight junctions (TJs) formed by integral transmembrane proteins like occludins, zonula occludens proteins and claudins anchored to cytoplasmic cytoskeleton. Adherens junctions (AJs), like TJs, maintain the integrity and tightness of BBB and are composed of cadherins, junctional adhesion molecules (JAMAs) and platelet endothelial cell adhesion molecules (PECAM1) (Figure 14) [165]. This results in a very high selectiveness and low permeability towards polar molecules that do not bind to specific transporters [166–168].

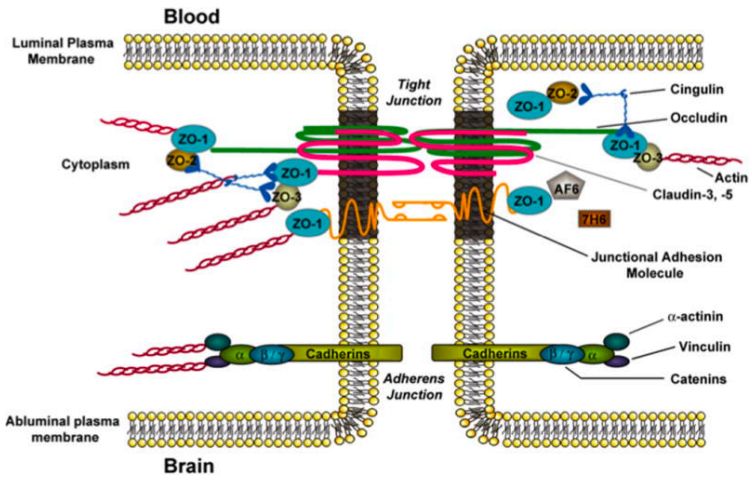

Figure 14. Basic molecular organization of tight junction protein complexes at the blood-brain barrier. From Ronaldson P.T. et al., 2012.

As mentioned, the selectivity of BBB towards external molecules is very high. Nevertheless, a few groups of substances can cross the cellular barrier by 1) simple diffusion (small hydrophobic molecules, water or gases); 2) carrier-mediated transport or CMT (vitamins, hormones, organic ions); 3) absorptive-mediated transcytosis or AMT (cationic proteins and oligonucleotides); 4) receptor-mediated transcytosis or RMT (insulin, transferrin and apolipoproteins). Only small hydrophobic drugs smaller than 400-600 Da can cross the BBB by simple diffusion, while other drugs and NPs need a specific transporter [169,170].

Liposomes have been suggested to be useful as a treatment for brain diseases that cause disruption of BBB, creating small fenestration in vascular endothelium and allowing the passage of small unfunctionalized liposomes [171,172].

However, conjugation with ligands capable of entering through CMT is the best strategy to provide an efficient brain delivery. Transferrin, Apolipoprotein E and insulin are two of the most used ligands because their receptors are highly expressed on BBB [173,174].

Another valid strategy for brain delivery is the functionalization of liposomes surface with antibodies or antibody fragments (immunoliposomes). These NPs can be also used to provide an efficient targeting to cells inside the brain [175]. Examples of immunoliposomes include functionalization with anti-EGFR [176], anti-insulin receptor [177,178] and anti-MPB (myelin basic protein) [179].

1.3 Nanomedicine for GBM therapy

The tumor resection, in the context of GBM therapy, does not display a curative character and radio- and chemotherapy are necessary to provide an appropriate treatment. However, drugs often possess limitations associated to non-specific targeting and biodistribution or fast metabolism [180]. That is why in the last years researchers focused on finding novel or alternative treatment strategies and perfectioning drug delivery systems to improve GBM therapy.

1.3.1 Drug delivery across the BBB

As highlighted in paragraph 1, the heterogeneity and complexity of GBM is an obstacle to the success of current therapies. In this circumstances, the use of nanomedicine has gained attention as a possible valuable tool to improve diagnosis, treatment and prognosis of GBM (Figure 15) [181].

In particular, the BBB provides a crucial impediment to overcome, since relevant clinical evidence showed that there are several tumor regions with an intact barrier. Indeed, drugs with insufficient BBB permeability do not reach the tumor cells, bringing the therapy to failure [182]. One of the strategies applied was to modify the physico-chemical properties of NPs, by coating negatively-charged doxorubicin-loaded poly(butyl cyanoacrylate) particles with surfactant polysorbate 80 [183]. It has been shown that this modification increased the rate of transcytosis across the BBB and increased the amount of doxorubicin in the brain parenchyma. Another strategy to efficiently improve the crossing of BBB is to exploit the receptor-mediated endo- transcytosis. In 2013, Gao H. et al. used an interleukin-13-derive peptide, called IL-13p, which displayed unprecedented cell-penetrating properties across the endothelial cells of the BBB. Moreover, IL-13p bound the tumor-specific receptor IL13Rα2, providing not only an increase in brain delivery, but also an efficient tumor targeting [184]. Another common targeting peptide is transferrin receptor (TfR), since it is highly expressed on BBB [185], but it is expressed on glioma cells as well [186]. Anti-transferrin receptor monoclonal antibodies were used to perform both brain and tumor increased uptake [187]. Bi-functionalization of liposomes with chlorotoxin (CLTX) and ApoE-derived peptide mApoE has shown to be beneficial for boosting NPs uptake across the BBB and simultaneously allowing the delivery of doxorubicin to cells [188]. Combined strategies may be used as well, as shown by Yang F.Y. et al. by coupling pulsed high-intensity focused ultrasound (HIFU) and doxorubicin-loaded liposomes functionalized with human atherosclerotic plaque-specific peptide (AP-1).

Pulsed HIFU was used to disrupt the BBB of mice, allowing a better brain penetration of NPs. This combination of treatments led to inhibition of tumor growth and increase of overall survival [189]. Emerging evidence has shown that the need to overcome several barriers so that the therapy efficiently completes its pharmacological activity, require multifunctional systems able to target different receptors in different tissues. These multifunctional systems, implying NPs coating, surface functionalization and cross-linked peptides, have been used both to deliver drugs like doxorubicin [190,191] or other chemotherapies [192], or even to deliver nucleic acids [193].

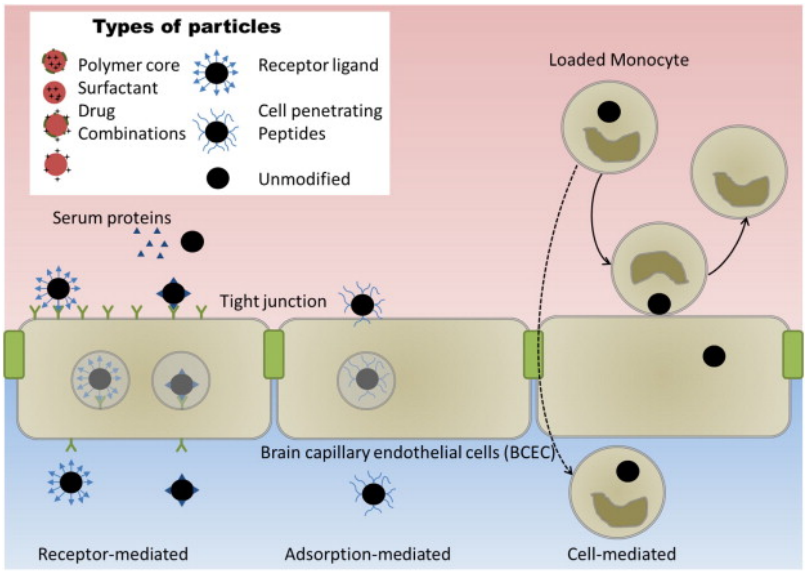

Figure 15. Strategies for NPs crossing of the BBB. From Mallapragada S.K. et al., 2014.

1.3.2 Immunotherapy

Within the last years, immunotherapy became a powerful strategy against cancer. Patients' immune system can recognize and eliminate malignant cells *per se*, but an intrinsic characteristic of many tumors is the so-called immunoediting, which includes the elimination of immune response, the equilibrium with immune cells and the escape from immune system [194]. Cancer immunotherapy is a wide and challenging field that may be performed using a large number of different tools such as immune checkpoints (ICs) inhibitors (ICIs), engineered T cells and vaccines [195]. The demand for specific targeting, and the need to reach an adequate pharmacological effect in the tumor site, made possible a very close relationship between cancer immunotherapy and nanomedicine. The progress made in this field is deepen in Chapter 2.

The most important ICs are Protein Death 1 (PD-1) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). PD-1 is expressed by T-cells in response to several stimuli like inflammation and tumor cells inhibit the immune response by expressing its inhibitory ligand PD-L1. This mechanism of cancer-mediated inhibition is called "tumor escape" [196]. CTLA-4 is another co-inhibitory receptor of T-cells expressed by $CD4^+$ T regulatory (T_{reg}) cells [197]. PD-1 or CTLA-4 blockade was performed in several clinical trials for cancer therapy. About GBM, an anti-PD-1 mAb (Nivolumab) was used alone or in association with an anti-CTLA-4 mAb (Ipilimumab) in a phase I study, showing that the treatment with Nivolumab alone was better tolerated than the combination therapy [198].

The phase III study did not show an improve in OS in patients and the trial was prematurely interrupted [199]. However, PD-1 and CTLA-4 blockade, together with the research of prognostic markers, remain a subject of interest for scientists and there are pre-clinical [200–202] and clinical studies [203] with alternating results.

Chimeric Antigen Receptor T cells (CAR T) are engineered T cells that were shown to be very beneficial in the treatment of several cancers. In CAR T method, patient's own T cells are collected and modified to express CARs specific for the tumor cell [204]. CAR T cells were applied in several pre-clinical studies in GBM. Choe J.H. and colleagues used a synNotch receptor, which is an engineered receptor able to induce a transcriptional output after recognizing its cognate antigen, against EGFRvIII or MOG (myelin oligodendrocyte glycoprotein). SynNotch CAR T cells were used in mice bearing patient-derived xenograft and a high anti-tumor efficacy has been shown for both antigens, together with specificity and persistence of T cells against GBM [205]. CAR T cells directed to other antigens were proven to be effective in the treatment of GBM, like CAR T cells targeting CLTX receptor. Also in this case, the treatment showed high specificity and tumor regression in orthotopic xenograft GBM models [206].

Tumor-specific antigens (neoantigens) originate from DNA mutations within the cancer cells and they are specific for the disease [207]. Given this premise, neoantigen vaccines respond to the need to boost the immune system against the cancer, generating an adequate immune response [195].

Keskin D.B. et al. [208] used a multi-epitope neoantigen vaccine in newly diagnosed GBM patients after surgical resection and conventional therapy. Vaccination induced circulating neoantigen-specific T cells responses, particularly in patients who did not receive dexamethasone as part of the therapy. This phase I/Ib trial as well as other studies [209,210] demonstrated that these types of vaccines could be beneficial even in relatively *cold* tumors like GBM.

1.4 Scope of the thesis

GBM is a boundless and challenging subject of study, with countless approaches and intensive research to find novel strategies for diagnosis and treatment. Therapy resistance and immune escape are critical issues to consider during each study aimed to highlight unraveled pathways or to investigate novel drugs and drug delivery procedures. Nanomedicine turned out to be a versatile field of study, with many applications in cancer therapy and particularly in GBM therapy, where the crossing of barriers and the specific targeting are mandatory.

These points were taken into consideration in the following sections. In Chapter 2 two years of progresses in nanomedicine applied to GBM immunotherapy were collected and discussed, particularly focusing on non-spherical and biomimetic NPs and on nanovectors for the delivery of nucleic acids.

In Chapter 3 the discussion is focused on formation of TNTs in GBM and on possible strategies to use these peculiar structures as a vehicle for drug delivery systems.

Finally, the paper reported in Chapter 4 investigated liposomes containing pan-HDACi Givinostat, used in 2D and 3D *in vitro* models to test the efficacy for GBM therapy. Givinostat-liposomes pharmacokinetics was also assessed in a pilot experiment on healthy mice to compare the improve of plasma half-life and brain penetration in comparison to the free drug.

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CHAPTER 2

An update of nanoparticle-based approaches for glioblastoma multiforme immunotherapy

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Review

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2.1 Abstract

Glioblastoma multiforme is a serious medical issue in the brain oncology field due to its aggressiveness and recurrence. Immunotherapy has emerged as a valid approach to counteract the growth and metastatization of glioblastoma multiforme. Among the different innovative approaches investigated, nanoparticles gain attention because of their versatility which is key in allowing precise targeting of brain tumors and increasing targeted drug delivery to the brain, thus minimizing adverse effects. This article reviews the progress made in this field over the past 2 years, focusing on nonspherical and biomimetic particles and on vectors for the delivery of nucleic acids. However, challenges still need to be addressed, considering the improvement of the particles passage across the blood–meningeal barrier and/or the blood–brain barrier, promoting the clinical translatability of these approaches.

2.2 Introduction to the clinical issue

Glioblastoma multiforme (GBM) is the most common malignant tumor of the CNS, representing about 65% of all primary CNS malignancies [1] and 82% of cases of malignant glioma [2]. Despite this, the incidence (3.1/100,000 per year) is low in comparison with other non-neural cancers and it is more frequent in people older than 75 years [3]. The 2007 WHO classification of tumors of CNS split gliomas into four grades of aggressiveness, and GBM and its variants were classified as grade IV tumors [4]. GBMs can either start from normal brain cells or develop from an existing low-grade astrocytoma.

Indeed, GBM is also defined as a grade IV astrocytoma. From a molecular point of view, malignant gliomas are highly heterogeneous tumors [5]. They may be divided into four molecular subclasses (classical, mesenchymal, proneural and neural) based on the transcriptional pathways and the mutations of several genes, including isocitrate dehydrogenase, EGFR, tumor protein 53 and NF-κB [6]. Within the definition of GBM, it is possible to distinguish primary and secondary GBMs, which develop at different ages, carry specific molecular alterations and differ in terms of histology, localization, grade of necrosis and metastatization, responsiveness to therapies and clinical outcome. This is the reason why many studies suggest that primary and secondary GBM should be considered as different tumor entities [7]. GBMs is usually diagnosed using CT scan, MRI scan or tissue biopsy, or a combination of the three. The current standard of care involves surgery followed by radiotherapy, with concomitant cycles of temozolomide chemotherapy [8]. High-dose steroids may also reduce symptoms, like brain swelling, but GBM usually recurs after some time. Without treatment, the median survival is about 3 months from diagnosis, while treated patients' life expectancy is 12–15 months. Unfortunately, less than 3–7% of treated patients survive over 5 years following diagnosis [9]. The tumor recurrence is attributable to heterogeneity, therapy resistance, high angiogenesis and the high invasiveness of GBM stem cells.

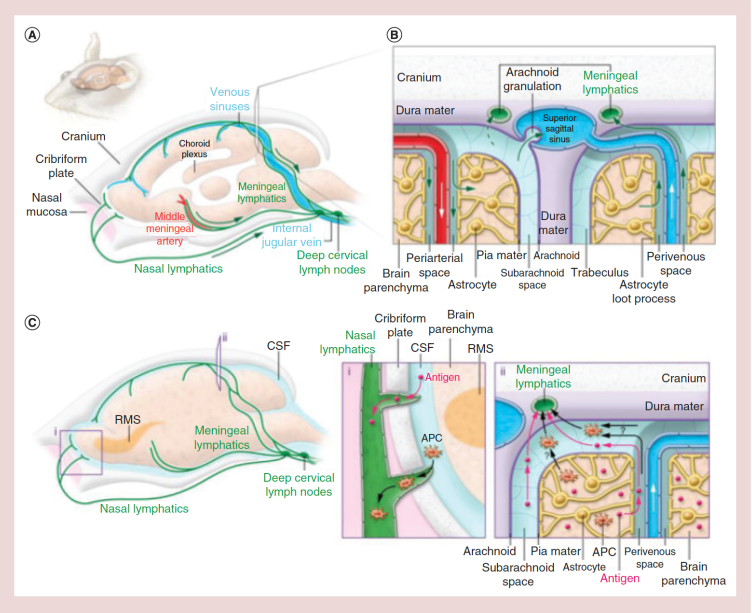

Figure 1. Illustration of the presumptive network of vessels in cerebral glymphatic system and lymphatic drainage, together with cerebrospinal fluid and interstitial fluid circulations across the brain. APC: Antigen-presenting cells; CSF: Cerebrospinal fluid; RMS: Rostral migratory stream. Reproduced with permission from [18], American Society for Clinical Investigation (2017).

2.3 The immune system in the brain

The CNS has been traditionally considered an immune privileged system because it lacks a classical lymphatic system and because of the difficulty in starting a destructive T-cell response from parenchyma [10]. However, recent findings have shown that the presence of a functional meningeal system (located in the *dura mater*) allows a flow of molecules and immune cells into the deep cervical lymph nodes [11].

The structures that express all the molecular hallmarks of lymphatic endothelial cells were deeply analyzed by Iliff et al. in 2012 [12], and by Louveau et al. in 2015 [13], and now they are commonly known as the 'glymphatic system'. The exact extent of the network (Figure 1, reproduced from Louveau et al.) is still unknown [14] but further analysis revealed a complex system of perivascular tunnels, basement membranes [15] and astroglial cells that allow the continuous exchange of cerebrospinal fluid and interstitial fluid along the periarterial space, including macromolecules and solutes [16]. Moreover, other studies showed a specific paravascular compartment for small lipid transport and glial communication signaling [17].

Originally, CNS immune privilege was partly attributed to the lack of a classical lymphatic system, although allografts that established an immune response in peripheral organs were able to maintain and potentiate that response when implanted into brain parenchyma [18]. Brain parenchyma and meningeal compartment are very different in terms of properties, starting from the fact that the blood–meningeal barrier is more permissive than the blood–brain barrier (BBB), which is why immune cells are free to circulate only within meningeal spaces under physiological conditions [19]. Despite that, Shechter et al. proposed a model in which selective barriers such as the BBB or blood–testis barrier do not represent static structures only able to segregate immune cells outside the organs but, rather, they are permissive gates that regulate the passage of cells under specific conditions, for example in some particular phenotypes. In this context, the privilege of CNS is not the power of exclusion, but the ability to build an effective communication with the active immune system [20].

In fact, recent studies demonstrate that antigens can drain from the brain to deep cervical lymph nodes, via meningeal lymphatic vasculature, throughout an internal recirculation mechanism involving cerebrospinal fluid and interstitial fluid in order to initiate the immune response [14]. However, since the response is slow, there is a need for a large amount of antigen or a secondary signaling to trigger this response. Alternatively the cervical lymph nodes may have the property to modulate the immune response to CNS antigens either toward tolerance or reactivity [18].

2.4 GBM immunotherapy

The tumor microenvironment (TME) is a complex network of extracellular matrix factors and cells, in which tumor cells can resist, proliferate and invade healthy tissues. The immune response is a crucial factor for communication between GBM and TME and tumor progression. It has been reported that nascent tumor cells can be eliminated by the host immune system based on both innate and adaptive immunity, opening the possibility to approach GBM by immunotherapies [21]. Immunotherapy has gained a lot of consideration in the last years and has become one of the most valid choices for cancer treatment, since it represents a better approach to prevent metastatization and recurrence in respect to conventional drugs. Immunotherapy includes the use of vaccines, oncolytic viral therapies, immune checkpoint inhibitors (ICIs) and chimeric antigen receptor T-cell (CAR-T) therapy [22].

Immune checkpoints, such as cytotoxic T lymphocyte-associated protein 4, protein death 1 (PD-1) and protein death ligand 1 (PD-L1) are negative modulators of T-cell activation which is why they have recently been the targets of drugs in clinical practice [10]. In GBM, cancer cells are able to escape immune surveillance through changes in receptor expression. For this reason, ICIs have become an intriguing subject to explore. PD-L1 is present in a variable subset of GBMs (between 2 and 88%) and its higher expression has shown to correlate with a poor prognosis [23]. Some studies have demonstrated that tumor-infiltrating $CD8^+$ T lymphocytes and intratumoral TH_1-type molecules are associated with positive therapeutic outcome by blockading PD-1 and PD-L1. However, therapies with an antibody targeting PD-1 (anti-PD-1) displayed response rates from 17 to 21%, probably due to the tumor heterogeneity and to the fact that immune responses and GBM are not caused by a single immune cell or checkpoint, but by multiple more complex interactions [24]. Indeed, cocktails of ICIs have demonstrated, in preclinical or clinical studies, a huge activity alone or in combination with traditional therapies, increasing overall survival and providing a good safety profile [22]. This opens the possibility to further guide the individualized treatment of patients by generating personalized medicines.

2.5 Innovative approaches: nanoparticles

Existing cancer immunotherapies have limited clinical benefits because of side effects (e.g., autoimmune diseases) and because cancer antigens are often not effectively delivered to immune cells.

In particular, for solid tumors, like GBM, immunotherapy is less effective than in lymphoma due to the difficulty of immune cell penetration within the abnormally grown extracellular matrix. In addition, the TME is a hostile environment for immune response because of the presence immune-suppressive factors (e.g., tumor-secreted cytokines). These limitations can be overcome by using nanoparticles (NPs) for cancer immunotherapy. NPs are small structures, with a size range between 1 and 100 nm, made by either inorganic, polymeric or organic materials which can be loaded with drugs [25]. Their physical and chemical properties make them attractive for many uses, especially in the medical field [26], where they have been successfully applied to treat several diseases, cancer included [27]. An overview of NPs exploited for drug delivery in cancer therapy is shown in Figure 2 (reproduced from Sun et al.) [25].

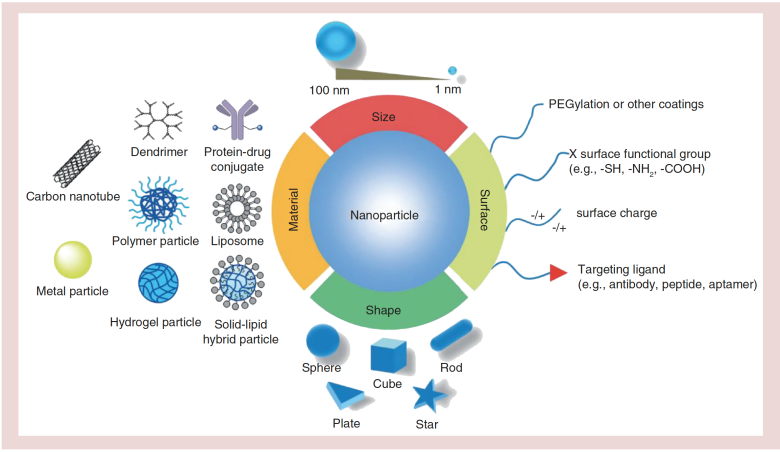

Figure 2. General overview of the main types of nanoparticles applied in cancer therapy and their possible functionalizations. Reproduced with permission from [25], WILEY-VCH Verlag GmbH & Co. KGaA.

To achieve effective immunotherapy, tumor antigens need to reach the lymph nodes in an efficient way, so that the immune response against cancer can start. Thus, NPs represent the best-characterized delivery vehicle to make sure tumor antigens reach lymph nodes. A specially functionalized NP can induce, inhibit or alter the innate immune system, for example by inducing cytokine production, activating downregulation mechanisms or immunosuppressing immune cells [28–31]. Beyond tumor antigens, NPs can efficiently deliver adjuvants to antigen-presenting cells situated in the lymph nodes, allowing antigen presentation. Accordingly, NP-based immunotherapy can provide a long-lasting vaccine effect and a wide range of immune responses as well, contrarily to conventional immunotherapy. However, because of the heterogeneity and complexity of brain diseases, GBM included, each NP has to be efficiently designed both to overcome the BBB (even if it is disrupted in some disease conditions and brain areas) and to specifically target cancer cells. However, these issues are difficult to be achieved due to the absence or low density of BBB-ligands and cancer-specific receptors. In fact, the choice of the tumor-specific ligand to be targeted by NPs strongly depends on its level of expression, rate of recycling, cellular localization and then its accessibility. All these factors are important in NP design because they determine the efficacy and safety of NPs themselves [32]. The challenge for the future will be to associate the diagnosis with the therapy in order to identify the targeted ligand that is overexpress in diseases tissue and then design the NP early [33–35].

2.6 2018-2020: what's new?

Non-spherical NPs & biomimetics for drug delivery

Although liposomes (spherical NPs) are one of the most used NPs in nanomedicine, nonspherical NPs (like discoidal particles, nanorods and filamentous particle) seem to display better performances in tumor treatment in terms of their ability to avoid the uptake by macrophages in organs and vessels, cellular uptake and biodistribution and their ability to cross biological barriers, the BBB included [36,37]. In one study, verteporfin (VP), a benzoporphin-derived small molecule, was encapsulated in a micellar vehicle composed in poly(ethylene glycol)-poly(-amino ester)-poly(ethylene glycol) (PEG-PBAE-PEG) in order to improve stealthness and to avoid protein corona formation [38]. VP was chosen since it has been shown that this molecule can interfere with GBM cell growth and proliferation [39]. Contrarily to spherical micelles containing VP, drug delivery mediated by filamentous micelles containing VP (fVPs) was proven to induce specific cytotoxicity on GBM cells, saving normal human astrocytes. Moreover, it has been shown by the same research group [40], that fVPs better avoid macrophage uptake *in vitro*, thus prolonging their circulation in the blood. Interestingly, a single treatment with fVPs in an ectopic xenograft tumor model has shown more than double the accumulation within the tumor in respect to the treatment with spherical micelles containing VP, demonstrating that nonspherical NPs can be a better tool for drug delivery [38]. The cellular uptake and targeting properties of NPs are essential to maximize their specificity toward target tissues.

In comparison with spherical NPs, nonspherical ones display a stronger targeting avidity due to their ability to form a higher number of multivalent interactions between the ligand on the NP's surface and the target molecule on the tumor cell surface [41], GBM included [42]. Moreover, thanks to the intrinsic properties of nonspherical NPs (high aspect ratio and prolonged lifetime in the blood circulation), the extravasation rate, the penetration capacity and the margination effect within solid tumors are improved when compared with those of spherical ones. All these features, together with size, can make nonspherical NPs better in terms of crossing the BBB, as suggested by Dal Magro et al. who demonstrated that discoidal NPs can be more efficiently transcytosed across the BBB *in vitro*, in respect to their spherical counterparts [37]. This issue is of a particular relevance for the GBM treatment. Taken together, these peculiarities allow more efficient drug delivery and probably for this reason, the immune response results are increased, even if this is an indirect effect of NPs shape. Another strategy to improve the tumor-targeting ability is the design of biomimetic NPs, which are small structures that imitate the characteristics of biological entities physiologically present in human body [43]. They are widely used as a valid tool for cancer nanomedicine. HDLs are heterogeneous particles naturally in the form of nanodiscs, involved in reverse cholesterol transport [44]. They may be used for drug-delivery purposes, providing accumulation and diffusion through the tumor because of their small size. In a study of 2019, therapeutic drugs were used to elicit tumor cell death and anti-glioma immunity [45].

In this study, Kadiyala et al. set up HDL-mimicking nanodiscs conjugated to CpG (a TLR9 ligand expressed by several immune cells able to trigger immune rejection) and loaded with docetaxel (DTX), a chemotherapeutic agent [45]. DTX-sHDL-CpG nanodiscs were demonstrated to trigger antitumor $CD8^+$ T cell-mediated immunity and to develop a long-term immunological memory; in fact, treated mice remained tumor-free when the contralateral hemisphere was injected a second time with the tumor cells. Among biomimetic NPs, extracellular vesicles (EVs), originating from the endosomal system are considered good candidates for intercellular communication and exchange [46], since they contain proteins from their cell type of origin. Zhu et al. have evaluated the antitumor activity and the tumor-targeting ability of EVs derived from natural killer (NK) cells pre-exposed to IL-15, so called NK-EVsIL-15, in comparison with EVs isolated from naïve NK cells (NK-EVs) [47]. It is known that IL-15 is able to improve survival and activation of NK cells [48]. NK-EVsIL-15 were demonstrated to express more cytotoxic proteins such as perforin and FasL than NK-EVs. Further, the accumulation of NK-EVsIL-15 in the GBM area *in vivo* was double that of NK-EVs. Moreover, the antitumor effect was improved, but it was not stable after the interruption of the treatment. The same group explored the antitumor activity of exosome-mimetic (EMs) vesicles derived from NK cells (NK-EMs) *in vitro* and *in vivo* in a xenograft mouse tumor model of GBM [49]. NK-EMs were prepared by disrupting NK cells through serial extrusions using nanosized filters. NK-EMs combine the characteristics of cells and exosomes and their cytotoxicity in various cancer cell lines, including GBM, was shown to be superior.

On the contrary, exosomes directly derived from NK cells (NK-Exo) come from NK cells culture medium and they do not show the same characteristics. Furthermore, EMs were shown to efficiently cross the BBB and to provide tumor targetability and cytotoxicity in a GBM xenograft model. Another biomimetic delivery system is an albumin-based structure for the co-delivery of disulfiram (a copper chelating agent) and the macrophage modulator regorafenib. This structure has been used to simultaneously target glioma cells and protumor M2 macrophages [50]. In this case, albumin can target SPARC proteins, which are overexpressed on tumor cells and on tumor-associated blood vessels (i.e., on endothelial cells). Moreover, in the study, transferrin receptor-binding peptide T12 was used to enhance traversal of the BBB and uptake into glioma cells. This approach was demonstrated to induce cytotoxic T lymphocyte immune responses and the suppression of protumor M2 macrophages. Notably, a combination of chemotherapy and immunotherapy has shown to improve the treatment outcomes [51].

Table 1. Different types of nanoparticles that may be used to deliver nucleic acid for cancer therapy.

Type of Nanoparticle	Advantages	Disadvantages	Ref.
Lipid-based	Allow efficient condensation and encapsulation of negatively charged molecules such as nucleic acids	Cannot actively reach the tumor or the tumor microenvironment without functionalization	[56]
Natural, semi-synthetic or synthetic polymer-based	Can bind nucleic acids through electrostatic forces and are efficient in gene delivery	Polymers can be easily inactivated by opsonization or fibrinogen in the circulatory system. To avoid this, polyethylene glycol, RGD peptide or other functional groups must be added	[54,56]
Gold	Easy to prepare and stable. They can be efficiently functionalized and conjugated	May show high cytotoxicity	[54]
Cyclodextrin-based	Water soluble and capable of forming complexes with nucleic acids	Have low stability and a tendency to aggregate and fall apart upon dilution	[55]
Chitosan	Show low immunogenicity and high biodegradability and biocompatibility	Have low solubility at physiological pH and cannot easily release drugs in a controlled manner	[55,56]

RGD: Arginylglycylaspartic acid peptide.

NPs for the delivery of nucleic acids

Gene therapy involves the use of nucleic acids (DNA or RNA) to treat, cure or prevent disorders using different tools such as naked oligonucleotides, viral and nonviral vectors [52]. Although the idea is simple, in theory, it is actually subject to multiple factors which make it very complex. For brain diseases, the most commonly used viral vectors are adeno-associated viruses and lentiviruses, while the most commonly used nonviral vectors are naked plasmid DNA and complexes with polymers or cationic lipids [53]. An overview of some nonviral NPs for the delivery of nucleic acids is shown in Table 1 [54–56]. In a recent study, *in vitro*-transcribed mRNA was formulated into an injectable therapeutic with the purpose to reprogram tumor-associated macrophages (TAMs) [57]. In fact, M1 macrophages are known as antitumoral cells, while M2 macrophages act as protumoral cells [58]. *In vitro*-transcribed mRNAs can efficiently target genes useful to shift the macrophages phenotype from M2-like toM1-like, thus promoting their genetic reconfiguration. These nucleic acids (two mRNAs encoding interferon regulatory factor 5 and an interferon regulatory factor 5 kinase were encapsulated into biodegradable polymeric PBAE NPs providing a reduction of tumor progression in glioma mouse models. In 2018 and 2019, Kim et al. improved the efficacy of ICIs in GBM by restoring p53 functions through the delivery of TP53 gene via a novel encapsulating plasmid cationic liposome (SGT-53) [59,60]. These nonviral vectors were functionalized with a single chain antibody fragment recognizing transferrin receptor that is more expressed by cancer cells than healthy ones, thus enhancing the targeting performance of the NPs.

The treatment was performed in co-administration with anti-PD-1 antibody both *in vitro* and *in vivo* in syngeneic mouse models of GBM. Restoration of p53 function improved the anti-PD-1 response, modified the TME by increasing lymphocytes tumor-infiltration and shifted the macrophage phenotype from M2 to M1. The reason why many patients develop a resistance to ICIs is because GBM is basically an immunologically 'cold' tumor, meaning there is a limited presence of tumor-infiltrating lymphocytes. However, as Baratta noticed, this kind of tumors can be successfully infiltrated by antigen-specific T cells that have the potential to kill cancer cells and to turn the 'cold' tumor into a 'hot' one, for example by using personalized vaccines and combined treatments [61]. This idea has been pursued through the use of plant virus-like particles derived from Cowpea mosaic virus to promote an *in situ* vaccine immunotherapy against malignant glioma by triggering antitumor responses [62]. In this study, brain injection of Cowpea mosaic virus in syngeneic glioma mouse models provided a significant increase in infiltration of $CD8^+$ T cells, effector memory $CD8^+$ T cells and NKT cells (unique innate T cells that express markers for T and NK cells) [63]. Beyond mRNAs, other nucleic acids can be delivered into tumor cells. A siRNA is a short double-stranded RNA from 21 to 23 nucleotides length, which contains a sequence of mRNA (sense strand) and its complement (antisense active strand) [64]. siRNAs can inhibit complementary post-transcriptional mRNAs by forming RNA-induced silencing complexes. Together with the RNase III enzyme dicer, these can promote endonucleolytic cleavage of target mRNA strands [65]. It has been shown that the incorporation of disulfide bonds in PBAE polymer NPs is able to ameliorate siRNA delivery in patient-derived

GBM cells in co-culture with brain capillary endothelial cells. This modification promotes triggered release of siRNA into the intracellular space and has reduced risk of cytotoxicity. In a mouse model of GBM, these NPs have been proven to be safe, although further investigation about efficacy is needed [66]. In a study of 2019, five siRNAs were included in a single type of PBAE-based NP (R646 siRNA NPs) to knockdown different genes known to promote proliferation and migration (YAP1, NKCC1, survivin, Robo1 and EGFR) in GBM cells [67]. These siRNAs were included in the PBAE-based NPs at a very low dose. For this reason, the authors could not test the efficacy, but instead they focused their attention on safety and specificity for GBM cells over healthy cells. Thus, although further investigations are needed, this kind of delivery system seems to be promising. Moreover, in tumor mouse models a reduction of tumor burden over the time was seen after siRNA-NP administration. At last, an alternative nonviral gene delivery agent is represented by solid lipid NPs (SLNs). These are colloidal nanocarriers made up of a high melting fat matrix and a monolayer of phospholipids acting as a surfactant [68]. iRGD, a cyclic peptide with high affinity to vascular endothelial cells αv integrins, can be conjugated to SLNs embedding siRNAs against EGFR and PD-L1 in *in vitro* and *in vivo* models of GBM [69]. Furthermore, it was shown that a short burst of radiation therapy was able to improve the tumor uptake of SLNs. This delivery system provided activation of the immune response, inhibition of tumor growth and an increase in mouse survival.

Other NP-based strategies

Possible strategies to design different NPs are virtually boundless. Sometimes NP design is aimed to compensate a lack of response from free drugs, for example when they are not able to diffuse inside the target cells because they are polar molecules. On the other hand, the design of a particular NP may be made to provide a better response from drugs. For example, many patients with solid cancers do not respond to CAR-T cells therapies [70]. This is because solid cancers are able to suppress T-cell functions by secreting inhibitory factors into their TME [71]. Many studies have tried to remedy this issue: one strategy was presented by Zhang et al. in 2018 by designing liposomes containing a drug cocktail of PI-3065/7DW8-5 (a PI3K inhibitor and an indirect immunostimulator of natural killer T [NKT] cells, respectively) followed by engineered CAR-T cells infusion. This can block suppressor cells within the TME and at the same time can stimulate key antitumor immune cells [72]. These NPs were proven to be effective *in vitro* and *in vivo* in a mouse model of glioma, transiently resetting TME, and they might be a valid approach for clinical trials. Immunosuppression of GBM does not involve only T cells, but also TAMs and myeloid-derived suppressor cells. This is often due to PD-1/PD-L1 pathway, which promotes, for example, the immunosuppressive mechanism of TAMs to counteract the antitumor activity of T cells [73]. Since PD-L1 is often overexpressed on GBM cells, Zhang et al. proposed the functionalization of a lipid NP with anti-PD-L1 antibodies in order to effectively deliver dinaciclib (a CDK5 inhibitor) to tumor-associated myeloid cells (TAMCs, which include myeloid-derived suppressor cells and TAMs) [74].

Combined treatment of mice with radiotherapy that elicits upregulation of PD-L1, enhanced the targeting efficiency of these lipid NPs and provided a better delivery of dinaciclib to TAMCs. This approach led to increased cytotoxicity and depletion of immunosuppressive TAMCs, benefitting tumor-bearing mice. At last, a device formed by nanodiamonds with surface functionalization of polyglycerol loaded with doxorubicin (Nano-DOX) has been developed to reprogram immunosuppressive TME of GBM and to induce anticancer immune response [75]. Activation of autophagy, instead of apoptosis, was confirmed in Nano-DOX-treated GBM cells and in xenograft models. Moreover, dendritic cells-stimulated T-cell activation was shown both *in vitro* and *in vivo*.

2.7 Conclusions & future perspectives

In the last 2 years, many strategies to improve targeted drug delivery via NPs have been proposed. However, further studies and insights are needed in the most part of published papers, some proposals are very promising, especially from the clinical translatability point of view. Biomimetics, for example, seems to be a valid tool because of their intrinsic characteristics, including replicating already existing cellular structures, thus increasing their safeness. As a matter of fact, the side effects of these treatments are remarkably reduced and there is in theory the possibility to create countless variants of these NPs. Likewise, nonspherical NPs provide better performance in cell interactions and in crossing biological barriers. Consequently, this finding can provide better strategies to pursue in order to improve drug delivery.

Combined treatments and NPs functionalization can serve multiple needs for different cellular types and even known drugs can be reused with different tools or for a different purpose. This highlights the possibility, by exploiting nanomedicine, to design tailor-made treatments for each patient, thus corroborating precision and personalized medicine at the same time. The major challenge of next years will be to bypass the defects of these novel approaches and eventually to promote the translatability of therapies. In this context, even if there is the possibility to generate very innovative and complex supermolecular NPs, the simplicity and biomimicry in NP design seems to be more successful in the context of scale-up and clinics.

Author contributions

Each author has contributed to the written text. L Taiarol read and evaluated the references and wrote the manuscript. F Re conceived the original idea, supervised the work and reviewed each version of the manuscript. B Formicola and RD Magro contributed to the revision of the manuscript. S Sesana reviewed the final version and handled the graphic layout. All authors provided critical feedback.

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CHAPTER 3

The 3.0 cell communication: new insights in the usefulness of Tunneling Nanotubes for glioblastoma treatment

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Review

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3.1 Simple summary

Communication between cells helps tumors acquire resistance to chemotherapy and makes the struggle against cancer more challenging. Tunneling nanotubes (TnTs) are long channels able to connect both nearby and distant cells, contributing to a more malignant phenotype. This finding might be useful in designing novel strategies of drug delivery exploiting these systems of connection. This would be particularly important to reach tumor niches, where glioblastoma stem cells proliferate and provoke immune escape, thereby increasing metastatic potential and tumor recurrence a few months after surgical resection of the primary mass. Along with the direct inhibition of TnT formation, TnT analysis, and targeting strategies might be useful in providing innovative tools for the treatment of this tumor.

3.2 Abstract

Glioblastoma (GBM) is a particularly challenging brain tumor characterized by a heterogeneous, complex, and multicellular microenvironment, which represents a strategic network for treatment escape. Furthermore, the presence of GBM stem cells (GSCs) seems to contribute to GBM recurrence after surgery, and chemo- and/or radiotherapy. In this context, intercellular communication modalities play key roles in driving GBM therapy resistance. The presence of tunneling nanotubes (TnTs), long membranous open-ended channels connecting distant cells, has been observed in several types of cancer, where they emerge to steer a more malignant phenotype.

Here, we discuss the current knowledge about the formation of TnTs between different cellular types in the GBM microenvironment and their potential role in tumor progression and recurrence. Particularly, we highlight two prospective strategies targeting TnTs as possible therapeutics: (i) the inhibition of TnT formation and (ii) a boost in drug delivery between cells through these channels. The latter may require future studies to design drug delivery systems that are exchangeable through TnTs, thus allowing for access to distant tumor niches that are involved in tumor immune escape, maintenance of GSC plasticity, and increases in metastatic potential.

3.3 Introduction

Glioma is the most common primary tumor of the central nervous system, with an annual incidence of approximately 6 cases per 100,000 individuals worldwide and with approximately 50% of them being classified as glioblastoma (GBM). GBM is the most aggressive form of glioma, with a median lifespan from time of diagnosis to death of approximately 15 months. Based on its histological appearance, GBM has been traditionally classified as an astrocytoma, though the precise cell type from which the disease originates is still a controversial issue. Some experts argue that the GBM origin is a subpopulation of neural stem cells, while others claim that it derives from the transformation of differentiated astrocytes [1]. Regardless, little progress has been made in GBM therapy, with no change in the standard of care for almost 20 years [2]. The current therapeutic approach for newly diagnosed GBM patients is based on surgery, followed by temozolomide chemotherapy and radiotherapy in combination with corticosteroids.

In addition, in 2015, a noninvasive technique based on the application of alternating electrical fields (tumor treating fields, TTF) was approved as an adjuvant therapy for newly diagnosed GBM [3]. GBM is assigned WHO grade IV [4], and recently, the classification has been refined, with diagnosis based not only on histology but also on several molecular markers such as isocitrate dehydrogenase (IDH) and epidermal growth factor receptor (EGFR) [5]. Unlike other cancers, GBM remains confined in the brain without any systemic spread [6]. However, almost every GBM recurs, and recurrent tumors are chemotherapy-resistant, with higher invasiveness and aggressiveness compared with the original tumor [7–9]. Consequently, there is no standard treatment for recurrent GBM, partially due to poor biological knowledge of the disease [10,11]. Recent studies on tumor heterogeneity suggest that residual tumor cells after whole total tumor resection share only 60–80% of their mutations with the primary tumor and differ significantly in terms of gene expression profile, microenvironment, and extent of immune cell infiltration [12,13]. Additionally, cancer stem cells play a pivotal role in GBM recurrence, though there is no generally accepted definition of them within GBM and how they specifically contribute to therapy resistance and tumor recurrence has not been clarified. This review describes the main data currently available about the communication modalities between the different cells composing the GBM environment, focusing on tunneling nanotubes (TnTs), which are described in detail. Finally, an overview of potential therapeutic approaches based on TnTs is presented.

3.4 Main cell types interacting in glioblastoma microenvironment

The GBM mass consists not only of a heterogeneous population of cancer cells but also of a variety of resident and infiltrating host cells, secreted factors, and extracellular matrix components, which collectively create the tumor microenvironment (TME). The TME is a complex network of signals and trafficking able to regulate tumor growth and invasiveness, angiogenesis, and chemotherapy resistance and to modulate immune response, drug delivery, and therapeutic responses [14,15]. Consequently, innovative therapeutic approaches should be designed considering the GBM microenvironment. However, there is currently limited understanding of cell communication between tumor and non-tumor cell types in the TME. In this section, we provide an overview about the main cells composing the GBM TME, which can communicate between them.

Tumor immune cells

Increasing evidence has revealed that infiltrating immune cells and other stromal components in the TME, of which the proportions vary according to cancer stage, are associated with the prognosis of GBM. Maintenance of the TME is one of the crucial factors influencing local immune dysfunction, which plays a critical role in GBM-induced immunosuppression [6]. This milieu in turn leads to immunotherapeutic treatment failure [1]. The immunosuppressive TME is mainly caused by recruited immunosuppressive cells [13], tumor-derived immunosuppressive factors [14], overexpressed immune checkpoints [16], and GBM cell epigenetics that silence HLA molecules [17].

Microglia cells and tumor-associated macrophages (TAMs) are tissue-resident cells (comprising 15% of the TME) and bone marrow-derived macrophages (comprising 85% of the TME) that may regulate tumorigenesis and are collectively known as glioblastoma associated microglia and macrophages (GAMs) [18]. There are two main TAMs subtypes, known as M1 and M2. The former is capable of antitumor activity, promoting cytotoxic and inflammatory effects; on the contrary, the latter subtype is pro-tumoral, with its anti-inflammatory activity switching off the host immune response against GBM. These cell types are considered important targets for GBM therapy [19]. Along with TAMs, $CD4^+$ T and $CD8^+$ T cells are capable of influencing tumorigenesis by receiving inhibitory signals from other TME cells and cancer cells, which lead to immune exhaustion and tumor tolerance [20]. A major mechanism leading to tumor immune-tolerance is activation of the PD-1/PD-L1 pathway. GBM cells massively express programmed cell death-ligand 1 (PD-L1) and consequently bind more programmed cell death protein 1 (PD-1) on T cells. This ligand–receptor interaction inhibits the differentiation of T cells into T effectors and promotes the switch towards other phenotypes such as T regulatory (Tr_{egs}) and T exhausted cells. This phenomenon prevents the tumor rejection usually mediated by $CD8^+$ lymphocytes [21–23]. T_{regs} are potent immunosuppressive cells that cause GBM immune escape. T_{regs} do not exist in normal human brain tissue, but a large number of immunosuppressive T_{regs} have been found in the GBM TME [24].

It has been also suggested that, in brain tumors, dendritic cells (DCs) recognize and present tumor-derived antigens inside the brain tissue or in the draining lymphoid stations in order to boost a T effector cell response against cancer cells [25,26]. These cells are normally not present in the healthy brain parenchyma. However, during tumorigenesis, they can reach the brain tissue via afferent lymphatic vessels and/or endothelial venules [27–29]. Drainage of tumor antigens into cervical lymph nodes has been observed in animal models via the glymphatic system [30,31]. The glymphatic system is a functional meningeal system located in the *dura mater*, which allows for the passage of molecules and immune cells into the deep cervical lymph nodes [32–34], where internal recirculation mechanisms involve the cerebrospinal fluid and interstitial fluid [16]. Moreover, cervical lymph nodes may have the property to modulate the immune response to tumor antigens toward either tolerance or reactivity [35]. Even if the specific role of DCs in the GBM environment is not yet elucidated, it is accepted that they have a pivotal role in antitumor immunity [36–38].

Glioblastoma stem cells

Several subtypes of GBM have been defined on the basis of different molecular alterations and gene expression patterns. Verhaak et al. described four GBM molecular subtypes: classical, proneural, mesenchymal, and neural [39]. These subtypes might arise from multiple stem cell-like populations through distinct differentiation pathways. GBM stem cells (GSCs) are defined as a quiescent subpopulation of cancer cells that have high self-renewing abilities, clonal tumor initiation capacity, and long-term repopulation potential [40].

In addition, the contribution of GSCs to GBM malignancy is widely accepted, as these cells can recreate the tumor mass after surgery. GSCs can be classified as proneural (PN) or mesenchymal (MES) subtypes because of their gene expression profiles and distinct biological characteristics [41]. GSCs are located in different niches, specific protective TME regions in GBM tumors where they can preferentially interact with specific cell types. For example, GSCs in the perivascular niche can mutually communicate with endothelial cells that secrete soluble cues, thus supporting the GSCs' self-renewal. In return, GSCs release vascular endothelial growth factor (VEGF) and stromal-derived factor 1, thus promoting angiogenesis [42,43]. In addition, GSCs transdifferentiate into pericytes and contribute to the vascular structure [44,45]. Another phenomenon, called "vasculogenic mimicry", takes place in GBM, where GSCs differentiate into endothelial-like cells, forming vessel-like structures. These structures are able to supply the tumor cells with nutrients and oxygen [46,47]. GSCs can also communicate with immune cells, promoting the establishment of a suppressive TME and thus allowing for tumor immune escape and progression [44]. In return, GAMs promote GSCs metabolic pathways to gain energy [48]. Furthermore, GSCs directly regulate immune cells, leading to the activation of T_{regs}, the inhibition of cytotoxic T cell proliferation, and the induction of cytotoxic T cell apoptosis [40,49]. In summary, GSCs present in these niches preserve their phenotypic plasticity, protect themselves from the immune system, facilitate GBM metastasis, and are resistant to commonly employed cancer therapies. This is one of the main reasons why targeted therapy has not demonstrated efficacy in phase 3 clinical trials against GBM so far.

Other brain cells

GBMs are among the most vascularized solid tumors found in humans, and blood vessels play a key role in supporting tumor progression [50], especially the endothelial cells (ECs) that are closely associated with GSCs [45]. Therefore, hindering communication between the endothelium and GSCs might represent a strategy to hamper GSCs survival. GSCs proliferation and invasiveness are also supported by the presence of aberrant tumor-derived vasculature that is usually associated with a higher degree of malignancy and a poor prognosis. Thus, high vascularization together with the vasculogenic mimicry contribute to the failure of antiangiogenic therapies against GBM [51–53]. Astrocytes, essential components in the structure and function of the blood–brain barrier (BBB), have been shown to support tumor angiogenesis via multiple mechanisms including secretion of angiogenic and growth factors, such as VEGF, and protein carriers, such as insulin and albumin. Astrocytes surrounding GBM commonly undergo functional and phenotypical changes through astrogliosis, a process in which reactive astrocytes secrete a large number of soluble factors that promote GBM invasiveness, proliferation, and migration [54]. In turn, tumor cells suppress p53 expression in astrocytes, thus promoting GBM cell survival through modulation of the extracellular matrix composition [55,56]. In addition, neuronal-GBM cell interactions have recently emerged as an important factor in tumor growth, as neuronal activity promotes tumor progression by inducing the release of brain-derived neurotrophic factor (BDNF) [57], neuroligin-3 [58], and dopamine [59].

Furthermore, GBM cells can increase local neuronal excitability, a mechanism that is responsible for the presence of seizures in GBM patients. In addition, this last event fosters the excitability-dependent secretion of mitogens, generating a vicious cycle to sustain the GBM microenvironment [60].

3.5 Cell communication modalities

The high complexity of TME aroused huge scientific interest about the communication modalities between cells in GBM. Understanding this communication could open new avenues for therapy design. The GBM progression and invasion of the brain involves multiple communication strategies between the cells that compose the TMEs, for instance, secretion of soluble factors such as chemokines and cytokines, extracellular vesicles (EVs; including exosomes and microvesicles), and direct cell–cell contact (gap junctions, nanotubes, and microtubes) [56]. Gap junctions are involved in the exchange and transfer of small molecules such as Ca^{2+}, ATP, and metabolites between two adjacent cells [61]. These clusters of intercellular channels are composed of connexins, the core proteins in gap junctions, in which connexin43 (Cx43) is expressed by human cerebral microvascular ECs (hCMEC), astrocytes, and GBM cells. High levels of this protein enable the formation of multicellular networks and allow cell-to-cell communication via calcium waves. An increase in Cx43 expression in GBM-initiating cells has been associated with increased invasiveness [61–63]. Connexins may also increase metastatic cell growth in GBM and resistance to standard therapies [64–66].

Despite the evidence of connexins role in malignant conditions, gap junctions are fundamental in tissue homeostasis, regulation of cell growth, and differentiation, making their targeting challenging without inducing toxic side effects [67]. EVs are membrane-bound vesicles secreted by all cells into the extracellular space in both physiological and pathological conditions, thus representing a powerful strategy for intercellular communication. Accordingly to their biogenesis, size, release pathways, cargo, and function, EVs can be divided into a wide range of vesicle types including exosomes (50–200 nm), microvesicles (>100 nm–1 μm), apoptotic bodies (50 nm–2 μm), and large oncosomes (>1 μm) [68,69]. EVs are exploited by cells for the transport of transcription factors, lipids, proteins, enzymes, and several metabolites, thus influencing the physiology and the phenotype of the receiving cell. The presence of EVs in almost every tissue, brain included, opens an entirely new perspective on cellular communication in GBM. Cancer cells usually exhibit higher EVs release compared with their healthy counterparts. Moreover, EVs belonging to glioma cells contain altered and different cargos than normal glial cells, resulting in the transfer of oncogenic activity. In the context of GBM, tumor-derived EVs are able to change the phenotype of normal cells to promote angiogenesis, tumor cell invasion, immune suppression, and altered metabolic regulation. Furthermore, EVs are able to switch to a normal cell phenotype and to promote angiogenesis, tumor cells invasion, immune suppression, and altered metabolism [70–72]. Pace K.R. et al. reported that exosomes decorated with L1 protein, which are normally involved in neuronal development, are upregulated in many types of cancers, such as GBM.

This increase may affect proliferation and cell motility and may promote tumor invasiveness *in vitro* [73]. Similarly, Lane R. et al. suggested that GBM subtypes could be distinguished based on EVs cargo, thus proposing the existence of subtype-specific EVs-associated biomarkers that are also involved in the regulation of tumor aggressiveness and recurrence. The diagnostic identification of such biomarkers would be helpful to develop personalized treatments for GBM patients [74]. Moreover, EVs are also involved in the recruitment, activation, or suppression of an innate immune system, particularly by mediating immunosuppressing mechanisms such as the induction of immunosuppressive monocytes without directly inhibiting T cell PD-L1 expression [75].

3.6 Tunneling Nanotubes

The communication mechanisms between cells in a tumor matrix remain poorly understood, especially for cells that are distant from each other. In the last two decades, experimental evidence from different research groups proved the existence of thin membranous tubes that interconnect cells called tunneling nanotubes (TnTs), tumor microtubes (TMs), or membrane bridges.

TnT discovery, characteristics and classification

TnTs are dynamic connections between two or more cells, which act as a route for cell-to-cell communication. Discovered in PC12 cells by Rustom et al. in 2004, TnTs have been defined as open-ended channels mediating membrane continuity between two or more cells over short to long distances [76].

TnTs are heterogeneous, transient, highly dynamic and sensitive nanotubular structures that connect cells by creating a complex network [76]. Unlike filopodia, TnTs are not branched and are suspended above the matrix [77]. Connecting distances up to 120 μm, TnTs can be classified as two types: (1) short (100–200 nm), thin (≤0.7 μm), and dynamic type I nanotubes are made of actin and formed by cells within their surroundings to make cell–cell contact; (2) long (1 μm), thick (≥0.7 μm), and more stable type II nanotubes are made of tubulin and cytokeratin filaments and formed by the detachment of two cells that are already connected [78–80] (Figure 1). Type II TnTs transfer cellular cargo between neighboring cells. Interestingly, it has been shown that different classes of TnTs exist even within a single cell type [81,82].

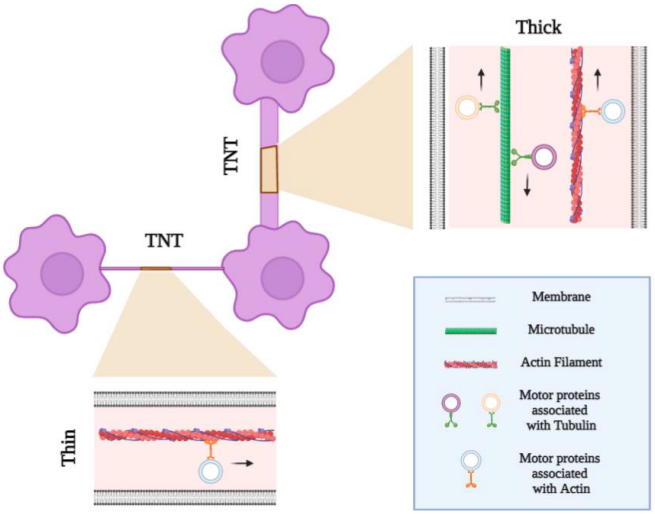

Figure 1. Tunneling nanotube (TnT) structures. TnTs are heterogeneous, transient, and highly dynamic nanotubular structures that connect cells over short or long distances. TnTs can be classified as two types: (1) short (100–200 nm), thin (≤0.7 μm), and dynamic type I nanotubes are made of actin and formed by cells exploring their surroundings to make cell–cell contact; (2) long (1 μm), thick (≥0.7 μm), and more stable type II nanotubes are made of tubulin and actin filaments. TnTs transport cellular organelles such as mitochondria and lysosomes, as well as viruses, intra-cellular vesicles, and electrical signals. The mechanism of cargo transport can be unidirectional or bidirectional. Type I nanotubes (actin-based) are characterized by a unidirectional transport mechanism in which cellular components are anchored and driven by the directional actin polymerization at one end. The hallmark of type II nanotubes is bidirectional delivery, in which cargo is moved by microtubule-based molecular motor proteins.

TnTs can have different lifetimes ranging from a few minutes (T cells) to hours (PC12 and NRK cells). These differences may reflect the existence of different subclasses of TnT-like structures supporting the experimental heterogeneity seen in TnT visualization [77]. These long membranous nanotubular structures have been identified *in vitro* in diverse cell types including neuronal cells, epithelial cells, almost all immune cells, and tumor cells [76,83,84].

In has been recently shown that neuronal-like cells are able to form TnT-like structures, which are implicated in the cell-to-cell spreading of Tau aggregates, thereby worsening the progression of Alzheimer's disease and other tauopathies [85]. Multiple studies have shown the capability of some epithelial and mesenchymal cancer cell types to form very long TnTs and TMs that are involved not only in the increase in malignancy but also in the formation of the tumor ecosystem connecting different cells types between them [86,87]. Although clearly demonstrated *in vitro*, the presence of TnTs in mammalian tissues *in vivo* has initially been questioned because of technical limits that made their detection arduous. The identification of TnTs in the mammalian cornea has provided the first evidence of membrane nanotubes *in vivo* [88], opening the possibility to removing the word "like" from the structures observed *in vivo*, which are currently carefully indicated as "TnT-like structures" [89–91].

TnTs formation

TnTs are transient structures that can originate by two mechanisms: *de novo* or via cell dislodgments [92]. *De novo* generation is an actin driven process whereby filopodia-like protrusions elongate via actin polymerization, probably initiated by Rho GTPases, until they reach a target cell. The resulting physical contact then results in membrane fusion [76]. The cell dislodgement mechanism occurs when two migrating cells that are initially in contact separate, thereby generating a nanotube. This mechanism has been observed in T cells, macrophages, and natural killer cells [81]. In this process, the time of contact is essential; a transient contact of less than 4 min hardly gives rise to TnTs [93].

Studies have mainly focused on well-known molecular machinery involved in blocked/reduced/activated TnT formation, such as filamentous actin (F-Actin) [94], M-Sec, RalA GTPase, LST1, myosin Va and X [76], and Cx43 [89] and plays a key role in defining the biology, the mechanisms, and the forces involved in TnT dynamics. Moreover, I-Bar lipid raft proteins [95] are required to generate membrane curvature during TnT formation as adhesion molecules such as N-cadherin and β–catenin [96] are fundamental in TnT guidance and initiation. Several research groups have demonstrated the presence of connexin and gap junction channels in TnTs, opening a long-range gap junctional communication mediated by the TnTs processes [97], probably because the two communication systems evolved to complement each other in a coordinating cell-to-cell communication. Different cancer cell models exhibit spontaneous formation of TnTs when cultured *in vitro* [76,98–100], while epithelial HBEC-3 cells, which are non-tumorigenic, rarely form TnTs when cultured *in vitro* [98]. Contrarily, the exposure of cell cultures to stress conditions can induce the formation of TnTs. In 2011, Wang et al. demonstrated that H_2O_2 exposure or starvation induced by serum depletion can produce TnTs in rat hippocampal astrocytes and neurons [101]. Moreover, TnT formation correlates with different stress factors such as infection [102], inflammation [82,103], hypoxia [104], ultraviolet exposure [105], X-ray exposure [106], and particle radiation exposure [107]. The role of TnTs after irradiation is poorly characterized. However, in 2015, Osswald et al. showed the protecting effect of TnTs after X-ray radiation, while in 2019, Reindl et al. demonstrated a decrease in connections after α-particle irradiation.

TnT function, biological effects, and mechanisms of transport

Evidence indicates that TnTs play a role in intercellular exchanges of signal clues, molecules, organelles, and pathogens, implicating them in a diverse array of physiological functions and pathological events. In particular, TnTs allow for the exchange of mitochondria [108], lysosomes [109], vesicles [110], proteins [111], viruses [112,113], and miRNAs [114] between connected cells. Furthermore, multiple studies have described the role of TnTs in the intercellular exchange of Ca^{2+} signals between distant cells, suggesting a continuity of membrane and cytosol of TnTs, and active gap junction channels [115]. Different communication systems may coordinate, interact, and develop to complement each other, as demonstrated by long-range transmission of inositol triphosphate mediated by TnTs through a gap junction-dependent mechanism [79,115]. Armulik et al. have described the communication between pericytes exploiting TnTs, thus confirming the essential role of these branched cells in regulation and brain homeostasis [116]. Triple immunostaining confocal microscopy analysis revealed pericytes as the main source of TnTs during neuro-angiogenesis, both in the early phases in physiological conditions and in tumor state [79]. Other than exerting physiological functions, TnTs are involved in cancer development, in reprogramming of malignant cells, and in alterations of the TME. In certain cases, TnTs can promote invasiveness and protection of cancer cells from cytotoxic drugs. It has been shown that the mitochondrial transfer between leukemic cells via TnTs increases chemotherapy resistance [117].

Desir s. et al. have demonstrated that in colon cancer cells, TnTs transfer KRAS oncogene, resulting in a heterogeneous distribution of mutant KRAS that profoundly modulates the TME and subsequent tumor progression [118]. Similarly, diffusion of the DNA repair enzyme O6-alkylguanine DNA alkyltransferase (MGMT) through TnTs mediates the protection of GBM cells against temozolomide [67]. In other conditions, TnTs may promote drug distribution between target cells, highlighting their potential beneficial properties for therapy [119]. An intriguing note to clarify is the mechanism of cargo transport that could be unidirectional. It may be, for instance, that actin and myosin drive transport, in which cellular components are anchored and driven by the directional actin polymerization at one end. Alternatively, it may be bidirectional from TnTs containing microtubules, in which cargo is moved by microtubule-based molecular motors (kinesin/dynein-mechanism) [120]. In both mechanisms, ATP is required, as they are active transports [81]. Another active cytoskeleton-independent transport (vesicular dilatation or "gondolas") is present in both type I and type II TnTs. The two mechanisms of transport differ in dilatation along the tubes, speed, and direction [78].

3.7 Tunneling Nanotubes in glioblastoma

Formation of TnTs in GBM

TnTs have been reported in several *in vitro*, *ex vivo*, and *in vivo* tumor models of GBM. The presence of TnTs has been demonstrated and characterized *in vitro* in cultures of C6 glioma cells, U87MG cells, U251 cells, primary human-derived brain tumor cells, and patient-derived GBM stem cells [15,121].

In terms of functional analysis, Civita et al. demonstrated the trafficking of mitochondria from astrocytes to GBM cells via F-actin TnT structures using both 2D and 3D *in vitro* GBM co-culture models, indicating that contact communication between non-neoplastic astrocytes and tumor cells may occur [122]. Interestingly, we recently showed that TnTs formed by healthy astrocytes or by GBM cells display structural differences in terms of length and thickness, which reflect different transport efficiencies [123]. A recent paper showed that irradiated U87 GBM cells quickly establish a network of cell-to-cell connections with high TnT content in comparison with non-irradiated cells, suggesting that the TnT formation may be also a consequence of treatment [124]. *In vivo*, in a syngeneic astrocytoma mouse model, it has been shown that many tumor cells extend ultra-long membrane protrusions and use TMs and TnTs as routes for brain invasion, proliferation, and interconnection over long distances [106]. In general, cancer cells exploit this physical connection to exchange material between themselves or with the cells present in TME (Figure 2A). *In vitro* and *in vivo* studies published by Errede et al. also revealed the existence of brain pericyte-derived TnTs that appear to be involved in exploring the surrounding microenvironment, searching for and connecting with targeted vessels, and contributing to the pathological angiogenesis in GBM. In particular, the results revealed that TnTs are formed both among different pericytes and between pericytes and other brain cells, especially ECs. Moreover, since GBM is a highly vascularized tumor [125], the exchange of molecular messages and/or organelles through pericytes and ECs by TnTs may contribute to the tumor spreading [79].

In terms of *ex vivo* work, Pinto et al. have shown for the first time that GSCs obtained from GBM patients were able to form TnTs and TMs in culture and were able to exchange organelles. These cells were obtained from the infiltrative tumor niche, which is responsible for GBM recurrence [121], suggesting that TnTs could be involved in long-range cell-to-cell communication in GBM (Figure 2B).

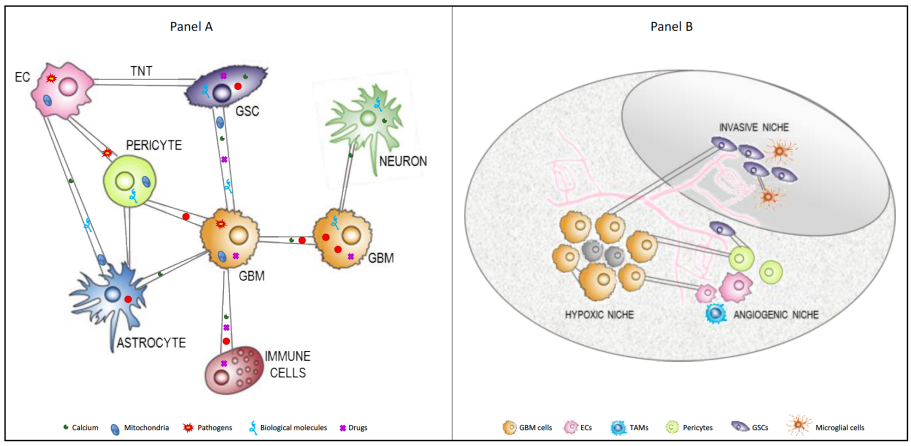

Figure 2. TnTs between cells composing the GBM TME. (A) TnTs are long membranous open-ended channels connecting cancer cells between them or with other cells composing the TME. They are dynamic and sensitive structures of different lengths and thicknesses able to allow for the interchange of organelles, vesicles, macromolecules, and pathogens between connected cells. (B) GBM and GSCs are embedded in a heterogeneous TME, which is composed of diverse stromal cells, including vascular cells, infiltrating and resident immune cells, and other non-neoplastic glial cell types, but they are also compartmentalized in distinct brain areas, called niches. These niches regulate metabolic needs, immune surveillance, survival, invasion, and the progression of GBM. TnTs are able to physically connect these cells even if located in different niches. Abbreviations: TnT, tunneling nanotube; TME, tumor microenvironment; EC, endothelial cells; GSCs, glioblastoma stem cells; GBM, glioblastoma; TAMs, tumor-associated macrophages.

Role of TnTs in GBM

TnT cell-to-cell communication allows tumor cells to acquire new abilities, such as enhanced plasticity, migratory phenotypes, angiogenic ability, and therapy resistance, which can contribute to cancer aggressiveness, invasiveness, and recurrence [67,126]. In this context, Valdebenito et al. have shown that TnTs mediate the chemo- and radiotherapy resistance of GBM by exchanging MGMT protein from resistant to sensitive cells, suggesting a role of TnTs in promoting a more malignant phenotype [67]. Moreover, MGMT proteins diffuse long distances, suggesting that TnTs can be formed with GBM cells located far away from the primary tumor. In co-culture experiments, astrocytes surrounding U87 GBM cells enhanced TnT formation toward tumor cells and exploited this physical connection to transfer undamaged mitochondria, useful molecules, or energy substrates to GBM cells in order to modulate cell behavior and response to cytotoxic agents [122]. A continuously growing body of evidence suggests that immune cells, both brain-resident and infiltrated from the periphery, play key roles in GBM progression and invasiveness. To the best of our knowledge, there is only one study reporting that mast cells (MCs), perivascular immunomodulatory cells, may form functional TnTs *in vitro* to communicate among themselves and with U251 GBM cells. This communication allowed for the bidirectional transfer of mitochondria and secretory granules [127]. In addition, the same authors speculate that TnTs provide a way for MCs to “alert” other cell types with a specificity that is not present when the mediators are secreted into the tissue microenvironment.

Despite recent studies on TnTs in GBM, many aspects of their function, biology, and mechanism of formation are still poorly understood and the progression of science in this important field is useful in developing new therapeutic strategies in pathological conditions.

3.8 TnTs as a novel strategy to enhance tumor drug delivery

TnTs are involved in the multistep process of cancer development from tumorigenesis to treatment resistance. Therefore, the scientific community is committed to characterize these communication systems for therapeutic purposes. Two possible strategies could be pursued: the pharmacological inhibition of TnT formation (Figure 3A) or the exploitation of TnTs as drug delivery channels (Figure 3B).

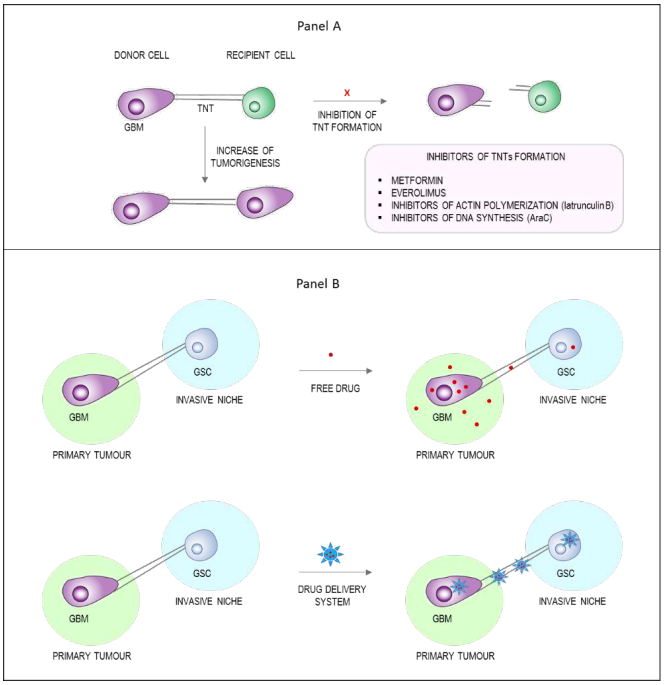

Figure 3. Potential therapeutic approaches based on TnTs. TnTs have been observed in several types of cancer, where they emerge to steer a more malignant phenotype thanks to the exchange of intracellular materials. (A) The design of molecules able to inhibit the formation of TnTs (i.e., inhibitors of actin polymerization, inhibitors of DNA synthesis, or inhibitors of mTOR pathway) may be a new therapeutic opportunity. (B) Since TnTs are long membrane nanotubes connecting distant cells, they can represent an exciting opportunity to deliver drugs or drug delivery systems in order to target inaccessible brain regions where GSCs are hidden and unreachable. Abbreviations: TnTs, tunneling nanotubes; GSCs, glioblastoma stem cells; GBM, glioblastoma.

Inhibition of TnTs formation

The discovery of TnTs biomarkers could be useful to generate targeting molecules that effectively inhibit TnTs formation. Currently, the only two TnTs markers known are actin and M-Sec (also referred to as tumor-necrosis factor alpha-induced protein 2), which are, unfortunately, not specific for TnTs [128].

The pharmacological targeting of TnTs *in vitro* has been performed using several molecules (reviewed in [15]), from inhibitors of actin polymerization to inhibitors of DNA synthesis, with positive results in terms of inhibiting formation or trafficking along TnTs.

However, the clinical relevance of this approach has yet to be determined. In 2016, Desir et al. evaluated metformin and everolimus, two drugs that have received FDA approval for GBM treatment as TnT formation inhibitors in ovarian cancer cells. The results revealed that these compounds were able to interfere with TnT development by acting on the mammalian target of rapamycin (mTOR) pathway [104]. These data are in agreement with previous results obtained by Lou et al. [86], where the TnT formation in human mesothelioma cells was inhibited by metformin and everolimus. However, *in vivo* preclinical data about the applicability of these mTOR inhibitors are not available yet.

Exploiting TnTs as drug delivery channels

A more creative alternative would be the exploitation of these membranous cellular structures as a novel Trojan horse strategy to strengthen tumor drug delivery. Consequently, the exchange of drug delivery systems between cells composing the TME via TnTs comes as an interesting and advantageous opportunity to reach tumor cells, which usually escape current therapies.

Moreover, exploiting the capability of TnTs to make physical contact with even distant cells indicates that they could be used to reach metastatic cells located in different tumor niches.

In the context of GBM therapy, different chemotherapeutics have been encapsulated in nanosystems (e.g., nanoparticles, Box 1) to facilitate BBB crossing and/or to enhance their brain penetration and tumor targeting, thereby reducing side effects and improving drug concentration in the TME. A plethora of multifunctional nanoparticles have been synthetized and characterized as potential devices for GBM therapy (reviewed in [23,129]). Among them, liposomes and polymeric nanoparticles are the most promising in terms of versatility and biocompatibility. Among the different ligands used to functionalize these particles, proteins, peptides, aptamers, and small molecules are the most widely employed to promote active BBB crossing and GBM targeting of nanosystems.

BOX 1. Nanoparticle for non-invasive brain drug delivery

Nanoparticulate systems comprise a wide range of carriers such as lipid-based nanoparticles, polymer and dendrimer nanocarriers, metallic and inorganic nanoparticles. They differ in several features, like size, shape, porosity, chemical composition and surface properties (charge and functionality). Surface chemistry can affect not only the cellular uptake and the distribution of nanoparticles, but also their ability to cross biological barriers, like BBB, and the pharmacokinetics properties. Nanoparticles have been investigated as drug delivery platforms for many years with the approval of the first nanoformulation for cancer therapy (Doxil® and Marqibo®) more than 20 years ago. However, now there are few approved nanoparticle-based therapeutics and none for the treatment of brain disorders. The reason is the difficulty in the design of nanoparticles with an optimal combination of long half-life, BBB crossing and drug payload.

Some studies related to the ability of different cells to interchange nanoparticles through TnTs are available [130]. It has been shown that fluorescently labelled silica nanoparticles can be transferred between tumor cells by TnTs [131]. Franco S et al. demonstrated the intercellular trafficking of mesoporus silica nanoparticles along TnTs and TMs between macrophages and cancer cells *in vitro* and *in vivo* [132]. A recent paper from our group reported the exchange of multifunctional liposomes between human GBM cells and healthy astrocytes *in vitro*. Interestingly, the TnT-mediated transport of liposomes was more efficient between tumor cells compared with healthy astrocytes [123]. This highlights the structural differences in TnTs formed between tumor and healthy cells, which reflect a different rate of material exchange, which can be used to improve the precision of treatments. These findings support the exploitation of TnTs for cell-to-cell transfer of drug delivery systems to maximize treatment efficacy and efficiency.

Insights and TnTs research outlook

The adoption of TnTs as therapeutic or prognostic targets is an attractive approach. At least two alternatives have been proposed: (1) the identification of specific markers in order to design molecules that inhibit TnTs formation between cells in the TME and (2) the enhancement of drug delivery between cells by exploiting these channels. In this context, we foresee that TnTs are useful drug-delivery channels for cancer therapy, as they facilitate the intercellular distribution of the drug (or drug delivery systems) between close and distant cells that are not sufficiently targeted upon simple drug diffusion in the brain parenchyma.

Although sustained by few experimental data thus far, this approach is innovative and intriguing and further investigation is warranted. In the field of GBM treatment, GSCs represent a challenge due to their resistance to commonly employed anticancer drugs. Moreover, this issue is worsened by the localization of these cells in tumor niches, often far away from the tumor mass. Therefore, the combination of long TnTs formation from cancer cells that colonize normal tissue [133] and their intercellular transport ability makes their targeting a valuable approach for the prevention and treatment of GBM recurrence. In addition, the possibility of TnTs formation between different cell types, e.g., between tumor and immune cells, and the structural differences between TnTs formed by tumor vs. healthy cells could be exploited to boost the precision of nanocarrier delivery.

3.9 Conclusions

This review explores the heterogeneity of the GBM environment, with a focus on the intercellular communication between different cell types, which facilitates biological diversity within the tumor and rapidly evolving TME. We explain the emerging role of TnTs and outline the perceived current limitations in the field that must be addressed before pharmacological targeting of TnTs can become a clinical reality. A key requirement is the standardization of the terminology surrounding and definition of TnTs as well as the development of appropriate tools suitable for the detection and characterization of these structures *in vivo*. We conclude that TnTs provide a new and intriguing avenue to target the key cellular players in GBM with nanoparticles and their incorporated drugs.

Author contributions

Conceptualization, F.R.; bibliographic research, L.T., B.F., A.D. and M.C.; writing—original draft preparation, L.T., S.F., F.V., R.D.M. and A.D.; writing—review and editing, L.T., G.S., A.R., M.K. and F.R.; supervision, administration and funding acquisition F.R. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 4

Givinostat-Liposomes: anti-tumor effect on 2D and 3D glioblastoma models and pharmacokinetics

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Article

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4.1 Simple summary

Glioblastoma is the most common malignant brain tumor with a high grade of recurrence, invasiveness, and aggressiveness. Currently, there are no curative treatments; therefore, the discovery of novel molecules with anti-tumor activity or suitable drug delivery systems are important research topics. The aim of the present study was to investigate the anti-tumor activity of Givinostat, a pan-HDAC inhibitor, and to design an appropriate liposomal formulation to improve its pharmacokinetics profile and brain delivery. The present work demonstrates that the incorporation of Givinostat in liposomes composed of cholesterol and sphingomyelin improves its *in vivo* half-life and increases the amount of drug reaching the brain in a mouse model. Furthermore, this formulation preserves the anti-tumor activity of glioblastoma in 2D and 3D *in vitro* models. These features make liposome-Givinostat formulations potential candidates for glioblastoma therapy.

4.2 Abstract

Glioblastoma is the most common and aggressive brain tumor, associated with poor prognosis and survival, representing a challenging medical issue for neurooncologists. Dysregulation of histone-modifying enzymes (HDACs) is commonly identified in many tumors and has been linked to cancer proliferation, changes in metabolism, and drug resistance. These findings led to the development of HDAC inhibitors, which are limited by their narrow therapeutic index. In this work, we provide the proof of concept for a delivery system that can improve the *in vivo* half-life and increase the brain delivery of Givinostat, a pan-HDAC inhibitor.

Here, 150-nm-sized liposomes composed of cholesterol and sphingomyelin with or without surface decoration with mApoE peptide, inhibited human glioblastoma cell growth in 2D and 3D models by inducing a time- and dose-dependent reduction in cell viability, reduction in the receptors involved in cholesterol metabolism (from -25% to -75% of protein levels), and reduction in HDAC activity (-25% within 30 min). In addition, liposome-Givinostat formulations showed a 2.5-fold increase in the drug half-life in the bloodstream and a 6-fold increase in the amount of drug entering the brain in healthy mice, without any signs of overt toxicity. These features make liposomes loaded with Givinostat valuable as potential candidates for glioblastoma therapy.

4.3 Introduction

Histone acetylation and deacetylation dynamically affect DNA structure, leading to the activation or suppression of gene transcription. These processes are mediated by two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Genes whose expression is affected by histone acetylation changes are frequently involved in the control of cell cycle progression, differentiation, and apoptosis [1,2].

HDACs are nuclear and/or cytosolic enzymes divided into four classes based on their homology to yeast proteins: class I, II, and IV HDACs are zinc-dependent hydrolases while class III HDACs, the sirtuins, couple lysine deacetylation to NAD hydrolysis.

Epigenetic dysregulation of histone-modifying enzymes is commonly found in many tumors and has been linked to cancer proliferation, changes in metabolism, drug resistance, migration, angiogenesis, and escape from the immune system [3,4]. This theme has inspired researchers to develop different classes of HDAC inhibitors (HDACis), some of which are in clinical trials for the treatment of tumors [5,6]. Among HDACis, Givinostat (ITF2357) is a potent pan-HDAC inhibitor that was first described by Leoni F. et al. in 2005 [7]. It has completed phase II clinical trials for polycythaemia vera [8] and is presently being evaluated in a phase III trial for Duchenne muscular dystrophy. The achievement of this advanced drug development phase was determined by the superior tolerability of Givinostat with respect to other already approved HDACis [9]. Pre-clinical data indicate the potential anti-tumor activity of Givinostat on solid tumors, including brain tumors [10–12]. Among them, glioblastoma multiforme (GBM, a grade IV astrocytoma) is the most common malignant brain tumor and is responsible for 46.1% of all primary malignant brain tumors [13]. This tumor displays aberrant expression and/or defective activity of HDACs, which have been linked to tumorigenesis [14]. The current standard care for GBM is based on Stupp's protocol, which includes radiotherapy and a concomitant treatment with temozolomide (TMZ) chemotherapy after surgical resection of the primary tumor mass [15]. Unfortunately, GBM shows a high grade of recurrence mainly attributable to GBM stem cells (GSCs) [16], which is the reason for the median lifespan from the time of diagnosis to death of approximately 15 months [17].

Since there are no curative treatments for GBM and the prognosis is poor, finding novel molecules with antitumor activity, or developing suitable delivery systems for already existing drugs are important research topics. The anti-proliferative and pro-apoptotic efficacy of Givinostat has been demonstrated on GSCs [18]. Together with its ability to revert the transformed phenotype, anti-cancer efficacy has also been shown in *in vivo* models of GBM [12]. Nevertheless, the ability of Givinostat to cross the blood-brain barrier (BBB) and reach the brain parenchyma at therapeutic doses have never been directly demonstrated but only deduced from the downstream effects of its administration [12]. Moreover, the use of novel drug delivery systems to improve its pharmacokinetics and the therapeutic index and reduce its side effects, such as thrombocytopenia, has not been investigated yet. Among the different drug delivery systems available, liposomes are the most used for the transport of a variety of anti-cancer agents directly to tumors, including GBM. Liposomes offer many advantages, including synthetic flexibility, biodegradability, biocompatibility, low immunogenicity, and toxicity. Accordingly, several liposomal formulations have been approved by the United States Food and Drug Administration (FDA). Moreover, ligand attachment to the surface of liposomes has facilitated active targeting and subsequent improved therapeutic efficacy of different chemotherapeutic drugs [19–21]. In this context, we evaluated the pharmacokinetics of Givinostat and its metabolites in a healthy animal model. Additionally, we designed an appropriate liposomal formulation to improve the drug half-life in the systemic circulation and enhance its brain delivery.

Moreover, the efficacy of Givinostat after incorporation in liposomes was investigated in 2D and 3D *in vitro* models composed of human GBM cells, and further studies were performed to determine their mechanism of action and potential use for GBM treatment.

4.4 Materials and methods

4.4.1 Materials

Givinostat (ITF2357; [6-diethylaminomethyl) naphtalen-2-yl] methyl N-[4-(hydroxycarbamoyl) phenyl] carbamate) was synthetized and characterized by Italfarmaco S.p.A. Free Givinostat stock solution was prepared by diluting the powder in DMSO at a concentration of 2 mM and stored at -20 °C until use. Cholesterol (Chol) was purchased from Sigma-Aldrich (Milano, Italy). 1,2-Distearoylsn-glycero-3-phospho-ethanolamine-N[maleimide(polyethyleneglycol)-2000] (mal-PEG-DSPE) and sphingomyelin from bovine brain (Sm) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA).

4.4.2 Animals

Five-week-old healthy Swiss CD1 mice (25–30 g of body weight) were purchased from Envigo (Italy). The animals were housed under a 12-h light/dark cycle in a controlled environment (22±2 °C with a relative humidity of 55±10%) in the institutional animal facility with ad libitum access to food and water. Animal care and husbandry were conducted in conformity with the institutional guidelines in compliance with national (d.lgs. 26/2014, Gazzetta Ufficiale della Repubblica Italiana, n. 61, 14 March 2014) and international laws and policies

(European Union directive 2010/63/UE; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). The procedures were authorized by the Italian Ministry of Health (Protocol FB7CC.5.EXT.39, 28 September 2021, authorization number 433/2016-PR).

4.4.3 Preparation and physico-chemical characterization of liposomes

Marqibo®-like small unilamellar liposomes were formulated. Liposomes composed of Chol/Sm/mal-PEG-DSPE (48.75/48.75/2.5 molar ratio) [22], combined with 1 mol% BODIPY^{TM}-Sm for CLS experiments, were prepared by the extrusion procedure. Briefly, lipids were mixed in $CHCl_3$/CH_3OH (2:1, v/v) and dried under a gentle stream of nitrogen followed by a vacuum pump for 3 h to remove organic solvent. The resulting lipid film was rehydrated in 62.5 mM sucrose octasulfate-ammonium salt (SOS-AS) solution (pH 4.5) for 1 h at 65 °C, vortexed, and then extruded through 200- and 80-nm polycarbonate membrane filters at 60±4 °C under 20 bar nitrogen pressure. Liposomes were then dialyzed against 10% sucrose (pH 5.5) at RT for 48 h [23] using Dialysis membrane Spectra/Por® 1, 6–8K MWCO (Spectrum Medical Devices, CA). Givinostat was dissolved in 65 °C water at a concentration of 6 mg/mL for 1 h. Drug loading was carried out by adding 2 mg/mL of Givinostat to 20 mM (total lipids) liposomes and the pH was adjusted to 4.0 with HCl. The mixture was incubated at 65 °C for 1 h. Unencapsulated drug was removed by Amicon®Ultra 10 kDa Protein Purification and Concentration Filters (Merck, Darmstadt, Germany).

The yield of Givinostat encapsulation was calculated by measuring the OD_{265nm} of the unencapsulated drug compared to the total drug loaded into the liposomes preparation using a calibration curve for free Givinostat dissolved in water at 65 °C. Lipid recovery was estimated by Stewart assay [24]. The encapsulation efficiency (EE%) and drug-to-lipid mass ratio (D/L, μg/μg) were calculated as described [25]. After purification, sample solutions were adjusted to pH 7.0 by adding PBS and stored at 4 °C until use. The liposome surface was functionalized with mApoE peptide (CWGLRKLRKRLLR, Karebay Biochem, Monmouth Junction, NJ, USA), exploiting the thiol–maleimide coupling reaction, as described [26]. Unfunctionalized liposome preparations were named LIP-GIV while mApoE-functionalized liposome preparations were named LIP/m-GIV. The morphology of the liposomes was characterized by cryo-EM as follows: 3 μL of the aqueous solution was placed on a porous carbon supporting TEM grid (Plano, Wetzlar, Germany, type S147-4), blotted for 2 s, and plunged into liquid ethane at -165 °C using a Gatan (Pleasanton, CA, USA) CP3 plunge freezer. The vitrified sample was transferred under liquid nitrogen to a Gatan model 914 cryo-TEM holder. Bright-field TEM imaging was performed at -170 °C and 200 kV accelerating voltage using a JEOL (Tokio, Japan) JEM-2100 LaB6 transmission electron microscope equipped with a Gatan Orius SC1000 CCD camera operating under low-dose conditions. Size, polydispersity index (PDI), and ζ-potential were analyzed using the dynamic light scattering (DLS) technique and interferometric Doppler velocimetry (Brookhaven Instruments Corporation, Holtsville, NY, USA equipped with ZetaPALS device) as previously described [26].

Stability was measured by following the size, PDI, and ζ-potential and drug release for three weeks. The amount of drug released from the liposomes was determined by measuring OD_{265nm} of the free Givinostat fraction, collected after sample centrifugation (Amicon®Ultra 10 kDa).

4.4.4 Pharmacokinetics and brain penetration

Givinostat 7.5 mg/kg, free (dissolved in 5% DMSO and 95% PEG400/H_2O: 1/1) or encapsulated in liposomes (dissolved in PBS), was administered by intravenous (i.v.) injection into 5-week-old healthy Swiss CD1 mice (n = 72, 3 mice/time point). Mice were sacrificed at different time points up to 48 h after the injection and blood and brain were harvested. Blood was collected from the cava vein in tubes coated with Li-heparin anticoagulant and centrifuged at +4 °C, 3000 g for 10 min to obtain the plasma. After blood collection, brain was harvested, washed in saline, dried on absorbent paper, weighed, and placed into appropriate tubes. All samples were analyzed for their Givinostat content using the LC-MS/MS method.

4.4.5 LC-MS/MS analysis

Stock solution of Givinostat, ITF2374, ITF2375 (metabolites), and internal standard ITF2400 were prepared in ACN/water 1:1 at 1 mg/mL. Working solutions were prepared by sequential dilution in water:ACN 8:2. Brains were homogenized in 20 mM ammonium formate buffer (1 g/5 mL). In total, 45 µL of blank plasma or brain homogenates was added to 200 µL of ACN containing ITF2400 at 25 ng/mL and acted as internal standards. Samples were vortexed for 3 min and centrifuged for 10 min at 5 °C at 500 rpm.

Samples were transferred into a 96-well plate, dried under nitrogen flow, and resuspended in 200 μL 0.1% FA H_2O/ACN (75:25). After vortexing, samples were injected into LC-MS/MS. Samples were analyzed on a UPLC Acquity (Waters, Milford, MA, USA) coupled with an API 3200 Triple Quadrupole (ABSciex). Mobile phases were water and ACN with 0.1% FA on a Kinetex 2.6 μm C18 100 A 75x3 mm (Phenomenex, Torrance, CA, USA). Analytes were quantified in MRM ESI positive mode. MRM transitions for qualification and quantification and MS parameters are reported in Table S1. Representative chromatograms of blank and LLOQ of Givinostat and metabolites in plasma and brain are reported in Figure S1. The Givinostat analytical ranges were as follows: plasma 2.5–4000 ng/mL; brain 0.5–2000 ng/mL; the ITF2374 analytical ranges were as follows: plasma 0.1–1000 ng/mL and brain homogenate 0.5–500 ng/mL; the ITF2375 analytical ranges were as follows: plasma 0.5–1000 ng/mL and brain homogenate 0.5–500 ng/mL. The calibration curves are reported in Figure S3.

4.4.6 Pharmacokinetics analysis

Pharmacokinetic parameters were calculated using Excel Add in (PK Solver 2.0, Excel 2007Microsoft add in). AUCs were calculated using an NCA by the linear trapezoidal rule, and a uniform weight was performed as a first approach. Graphical concentration–time curves were produced after Log transformation. The ke was estimated from the terminal part of the log-concentration–time plot including at least three data points excluding the C_{max}.

4.4.7 Cell lines

Gli36ΔEGFR-2 and U87-MG were used as GBM *in vitro* models. Gli36ΔEGFR [27,28], carrying the EGFRvIII mutation, was made resistant to TMZ after 1 month of *in vitro* exposure to 50 μM TMZ [29]. These cells were selected because the TMZ sensitivity was repeatedly tested *in vitro* and *in vivo* in orthotopic GBM models [30]. Both Gli36ΔEGFR-2 and U87-MG were maintained in Dulbecco's Modified Eagle Medium (DMEM) High Glucose w/o sodium pyruvate (ECM0101L, Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, ECS0180L, Euroclone, Milan, Italy), 4 mM L-glutamine (ECB3000D, Euroclone, Milan, Italy), and 100 U ml^{-1} penicillin/streptomycin (P/S) (ECB3001B, Euroclone, Milan, Italy). Normal human astrocytes (NHAs, CC-2565, Lonza, Basel, Switzerland) were used as healthy astrocytes and maintained in AGM™ Astrocyte Growth Medium BulletKit™ (CC-3186, Lonza) as per the manufacturer's protocol. Human cerebral microvascular endothelial cells (hCMECs), provided by Dr. S. Bourdoulous (Institut Cochin, Inserm, Paris, France), were used as a model of brain endothelial cells and cultured as reported in the literature [31]. Human umbilical vein endothelial cells (HUVECs, purchased from Lonza) were used as a model of peripheral endothelium and maintained in an Endothelial Cell Basal medium EGM™ SingleQuots™ Kit (CC-4133, Lonza) as per the manufacturer's protocol. All cell lines were maintained at 37 °C with 5% CO_2 and saturated humidity.

4.4.8 Cell viability assay and targeting efficacy of liposomes

The effect of free or encapsulated Givinostat was assessed by the MTT assay. Cells were seeded in 96-well plates at a density of $2x10^4$ (Gli36ΔEGFR-2 and NHA) or $3x10^4$ (hCMEC/D3 and HUVECs) cells/well. Different doses of Givinostat, ranging from 0.1 to 20 μM, free or encapsulated in liposomes, were added to the culture medium for up to 72 h. Culture medium alone or added with DMSO or EtOH or unloaded liposomes (0.153 mM) were used as controls. At the designated times, the assay was performed as per the manufacturer's protocol and absorbance was measured at 570 nm using a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). Results are presented as the mean of three independent experiments±SD. The IC_{50} mean value at 24 h was calculated basing on the relative viability values and concentrations using linear regression analysis provided by GraphPad Prism 8. The targeting efficacy of LIP/m-GIV vs. LIP-GIV was evaluated by fluorescent techniques. Gli36ΔEGFR-2 cells were seeded in a 96-well Cell Carrier Ultra plate (Perkin Elmer) at a density of $2.0x10^4$ cells/well. The actin cytoskeleton was labeled with CellMask™ Deep Red Actin Tracking Stain (1:1000) for 30 min. After washing with PBS, cells were incubated with Hoechst for 8 min as per the manufacturer's protocol and then cells were washed again. Finally, cells were treated with fluorescent-labeled LIP or LIP/m (400 nM total lipids) for 15 min. Images were acquired using the Operetta CLS High Content Analysis System (Perkin Elmer, Waltham, MA, USA) equipped with 40x water objective and standard instrument filters as per the manufacturer's protocol using the live imaging tool.

Quantitative analysis was performed by measuring the ratio between the fluorescence intensities in cell medium and cell lysates. Measurements were performed using a Spectrofluorometer FP-8500, Jasco, Tokyo, Japan.

4.4.9 Caspase-3 activity by immunofluorescence

An increase in cleaved Caspase-3 was determined by immunofluorescence microscopy and images were acquired using the Operetta CLS High Content Analysis System (Perkin Elmer) equipped with 40x water objective and standard instrument filters as per the manufacturer's protocol. Gli36ΔEGFR-2 cells were seeded on a rat tail collagen I-coated 96-well Cell Carrier Ultra plate (Perkin Elmer) at a density of $1.5x10^4$ cells/well. Cells were treated with Givinostat for 48 h, washed with PBS, and fixed with 100 µl of 4% (v/v) formaldehyde for 15 min at RT. Then, cells were permeabilized with 0.5% Triton X-100 in PBS (v/v) for 5 min at RT and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. Cleaved Caspase-3 was stained using Alexa Fluor® 488-conjugated anti-caspase-3 antibody (0.75 µg/mL in 3% BSA in TBS) (9669, ThermoFisher, Waltham, MA, USA) overnight at 4 °C. Actin cytoskeleton was stained with Phalloidin AlexaFluor® 633 (1:100 in PBS) (Invitrogen, Waltham, MA, USA) for 1 h at RT. Nuclei were stained with DAPI (1:1000 in PBS) (ThermoFisher) for 10 min at RT. Quantitative measurements of Caspase-3 accumulation after treatment were calculated as histograms' intensity of the green channel images, fixing the threshold to >800 a.u.

4.4.10 Evaluation of HDACs activity by fluorescence assay

Gli36ΔEGFR-2 cells were seeded in a 6-well plate at a density of $3x10^5$ cells/well, treated with Givinostat 0.5 μM, and lysed at different time points (10 and 30 min). Cell fractions (nucleus and cytoplasm) were extracted using an NE-PER™ Nuclear and Cytoplasmatic Extraction Kit (cat. no. 78835, ThermoFisher) as per the manufacturer's protocol. The reliability of cell fractions was assessed by Western blot, using GAPDH and Histone H3 primary antibodies (Figure S3). Then, the activity of HDACs was evaluated with an HDAC Activity Assay Kit (Fluorometric) (Ab156064, Abcam, Cambridge, UK) as per the manufacturer's protocol. Data were obtained using a microplate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany) with continuous measuring every minute for 1 h at 380/460 nm.

4.4.11 Immunoblot analysis

Cells were seeded in a 6-well plate at a density of $3x10^5$ cell/well and treated with Givinostat (0.25, 0.5 and 1 μM) for 48 h. Whole cell lysates were obtained by washing cells twice in cold PBS and harvesting in 60 μL of radioimmunoprecipitation assay (RIPA) buffer (cat. no. 89901, ThermoFisher) supplemented with 1% of protease and phosphatase inhibitor cocktail (cat. no. 78446, ThermoFisher). Whole cell lysates were quantified using a BCA Protein Assay Kit (cat. No. 23227, ThermoFisher) and separated by electrophoresis through precast gels (NuPAGE™ 4–12% Bis-Tris, 1.0 mm, Mini Protein Gel 10 or 15-wells, cat. No. NP0321 and NP0323, ThermoFisher). P

roteins were transferred to nitrocellulose membranes using iBlot™ Transfer Stack (cat. No. IB301002, ThermoFisher) and membranes

were blocked either in 5% milk or in 5% BSA in TBS with 0.1% Tween-20 (TBST) for 1 h. Membranes were then incubated overnight at 4 °C with the following primary antibodies: Acetyl-α-Tubulin (5335, 1:1000), α-Tubulin (2144, 1:1000), Histone H3 (9715, 1:1000) and β-Tubulin (2146, 1:1000) purchased from CST; β-Actin (MA5-15739, 1:5000), GAPDH (MA1-16757, 1:5000), VLDLR (MA5-24790, 1:1000), ABCA1 (PA1-16789, 1:500), LRP1 (MA1-27198, 1:500), and LDLR (PA5-22976, 1:1000) purchased from ThermoFisher. Membranes were incubated with secondary anti-rabbit antibody (A0545, 1:5000, Merck) or anti-mouse antibody (G21040, 1:20,000, Invitrogen) for 1 h at RT. Bands were detected using Immobilion ECL Ultra Western HRP Substrate (WBULS0100, Merck) under chemiluminescence using an Amersham Imager 600 (Cytiva, Marlborough MA, USA). Quantifications were made using ImageLab Software Version 6.1 (Bio-Rad www.biorad.com).

4.4.12 3D-bioprinted GBM models

The 3D-bioprinted GBM model was generated as already reported [32]. Briefly, a hybrid ink based on gelatin (GE-MF) and chitosan (CH-MF) was generated by Diels Alder crosslinking with maleimido-star-PEG (PEG-Star-MA). GE-MF (66 mg) and CH-MF (34 mg) were dissolved in 1.5 mL of PBS at 37 °C and vortexed until complete dissolution. PEGStar-MA (5 mg) was dissolved in 0.5 mL of PBS at RT, added to the GE-CH hybrid solution, and mixed. The GE-CH solution was left for 30 min under UV-light for further sterilization and 2 h at 37 °C to obtain partial network formation of the hydrogel solution.

U87-MG or Gli36ΔEGFR-2 cells (700 rpm centrifuge) ($2x10^5$/mL) in complete medium were added to the GE-CH solution (5%, 2 mL) and transferred into a 5 mL bioprinter syringe. Each sample was bioprinted as a cylinder on 35-mm Petri TC dishes using a 22 G nozzle with a 0.41 mm diameter at 50 KPa. After printing, cells were maintained at 37 °C with 5% CO_2. The culture media were refreshed every 2 days.

4.4.13 Drug testing and cell viability in 3D bioprinted models

Drug testing in 3D-bioprinted models was performed to test the effect of the following samples on Gli36ΔEGFR-2 and U87-MG viability: (1) empty liposomes as controls; (2) LIPGIV; and (3) LIP/m-GIV. Cells were treated with 1 μM Givinostat at days 1 and 7 in MEM culture medium (2 mL per 35-mm dish) for 24 h. The cell viability in the 3D-bioprinted constructs after treatments was evaluated using a LIVE/DEADTM viability/cytotoxicity kit (Invitrogen), following the manufacturer's instructions. In total, 1 mL LIVE/DEAD stock solution was added to each bioprinted construct. After 50 min of incubation at 37 °C, the stained bioprinted models were washed three times with PBS before image acquisition. Imaging analysis was performed with a confocal microscopy 10x or 20x Ph objective. Nuclei were stained using DAPI (1:1000 in PBS) (ThermoFisher) for 10 min at RT; living cells and dead cells were stained using calcein and EthD provided by the kit. Cell viability was calculated as ((number of green/red stained cells/number of total cells) x100) using Fiji ImageJ Software [33]. Cell viability was also evaluated by Alamar assay to estimate the viability and/or mortality.

In total, 200 μL of Alamar Blue solution (10% final volume) was added to each bioprinted sample and incubated for about 2 h. Absorbance was read at 570 nm at t0 (2 h of incubation), 24, and 48 h [34]. Results are presented as mean of five independent experiments±SD.

4.4.14 Statistical analysis

Statistical analysis was performed with GraphPad Prism 8, using the following tests: Two-way ANOVA, one-way ANOVA, unpaired t test, Sidak's multiple comparisons test, and Tukey's multiple comparisons test. Statistical significance was considered at $p < 0.05$.

4.5 Results and discussion

4.5.1 Liposomes improved the pharmacokinetics profile of Givinostat

Liposomes composed of cholesterol/sphingomyelin/DSPE-PEG-mal, embedding Givinostat and surface functionalized with mApoE (Figure 1A), were prepared using the lipid film hydration method followed by extrusion, and were characterized by DLS. The results (Table 1, Figure S4) showed that liposomes had a uniform size distribution (PDI < 0.2), with a diameter <200 nm. The ζ-potential measurement showed that the net surface charge of liposomes was negative. This suggests that the dispersions are stable and not prone to aggregation. A slight increase in the size (+8%) was detected after surface functionalization with mApoE. These parameters are indicative of homogenous samples with a stable profile. The EE% was 84±11% and 92±4% while the D/L (μg/μg) was 0.27±0.15 and 0.52±0.35 for LIP-GIV and LIP/m-GIV, respectively (n = 8). A representative cryo-EM image of the liposomes is shown in Figure 1B.

The image reveals spherical, unilamellar vesicles homogeneously distributed in vitreous ice, with diameters ranging from 30 to 100 nm. Black dots on the top and inside the vesicles indicate a high loading efficiency of the drug. The stability of liposomes was determined by following the size, PDI, ζ-potential, and drug release over three weeks. Results showed that the size (Figure 1C) and PDI (Figure 1D) of the liposomes as measured by DLS did not undergo significant changes. The ζ-potential and drug release remained <-20 mV and <0.9% (Figure 1E), respectively, for both formulations. We next investigated the effect of the liposome preparations on endothelial cell viability using the MTT assay on hCMEC/D3 and HUVEC cell lines as models of brain and peripheral endothelium, respectively (Figure S5). The cell viability after treatment with LIP-GIV or LIP/m-GIV was >50% for all the conditions tested, similar to the free drug. Moreover, 50% mortality was reached only at the highest dose (20 μM) of liposomes-Givinostat on hCMEC/D3 cells. Considering that, in other studies, the pharmacological effect of Givinostat on GBM cells was obtained at doses ranging between 0.25 and 5 μM [12], liposome preparations containing that concentration of drug can be considered harmless for endothelia. This is in accordance with the results obtained by Milan M. et al. about the potential protective effect of Givinostat on blood vessels from apoptosis [35].

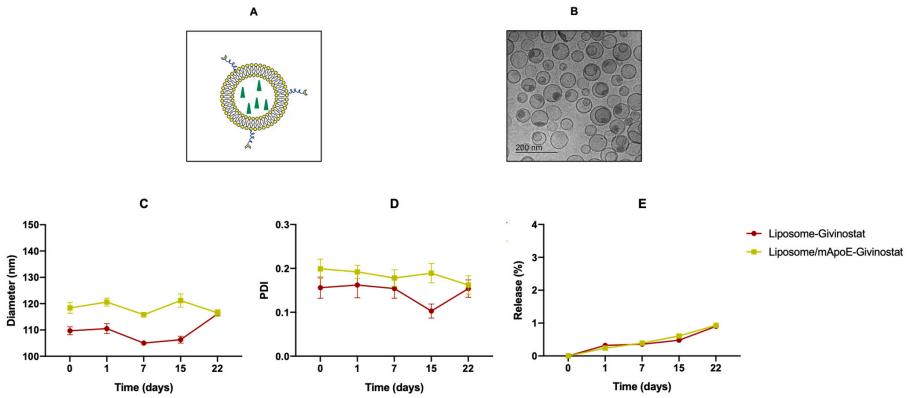

Figure 1. **(A)** Representative drawing showing a liposome loaded with Givinostat (green triangle) and functionalized with mApoE. **(B)** Cryo-EM image of drug-loaded vesicles with diameters ranging from 30 to 100 nm. Black dots on top and inside the vesicles indicate a high drug loading efficiency. **(C–E)** The diameter, polydispersity index, and drug release of LIP-GIV and LIP/m-GIV were measured around a period of three weeks. Data are presented as the mean of at least three independent experiments±SD.

Table 1. Physico-chemical characterization of LIP-GIV and LIP/m-GIV formulations at T0.

Liposomal Formulation	Diameter (nm) ± SD	PDI ± SD	ζ-Potential ± SD
LIP-GIV	109.7 ± 1.5	0.156 ± 0.024	−22.13 ± 1.51
LIP/m-GIV	118.4 ± 2.1	0.199 ± 0.022	−25.84 ± 1.87

The pharmacokinetics parameters of the free compound embedded in liposomes were measured in healthy animals. The mean plasma concentrations of Givinostat, after a single i.v. administration, are shown in Figure 2A (all concentrations are available in Table S2). LIP-GIV and LIP/m-GIV extended the half-life of free Givinostat (t1/2 = 1.7 h) to 5.1 and 3.8 h, respectively. At 6 h, the concentrations of LIP preparations in plasma were approximately 15,000 ng/mL.

In contrast, free Givinostat was rapidly removed from the circulation and could not be detected 24 h after i.v. administration. Liposomal formulations of Givinostat remained in the blood circulation up to 48 h post-injection and showed delayed plasma clearance, in comparison to the free drug. A strong increase in plasma exposure (AUC_{0-t}) ($AUC_{Givinostat}$ = 478 ng/mL h; $AUC_{LIP-GIV}$ = 276,825 ng/mL h; $AUC_{LIP/m-GIV}$ = 249,919 ng/mL h) was detected for LIP formulations, indicating lower plasma clearance (CL) was exhibited by LIP formulations compared to free Givinostat ($CL_{Givinostat}$ = 15,417 mL/kg*h, $CL_{LIP-GIV}$ = 27 mL/kg*h, and $CL_{LIP/m-GIV}$ = 30 mL/kg*h). In addition, an increase in the mean residence time (MRT_{0-t}) was observed with LIP ($MRT_{Givinostat}$ = 0.6 h; $MRT_{LIP-GIV}$ = 7 h; $MRT_{LIP/m-GIV}$ = 6.6 h). The volume of distribution of free Givinostat is 10.8 L/kg, largely exceeding the total body water, while the volume of distribution of the liposome formulations is significantly lower (~0.2 L/kg). The increase observed for the AUC and MRT values could be due to the liposomal composition. Specifically, PEGylation is a feature that may confer stealth properties to liposomes. These results agree with other published data showing the ability of liposomes to enhance drug stability and its persistence in plasma by limiting the adsorption of blood components onto their surface [36]. Taken together, these data are similar to those previously described for liposomal formulations of Vincristine [37].

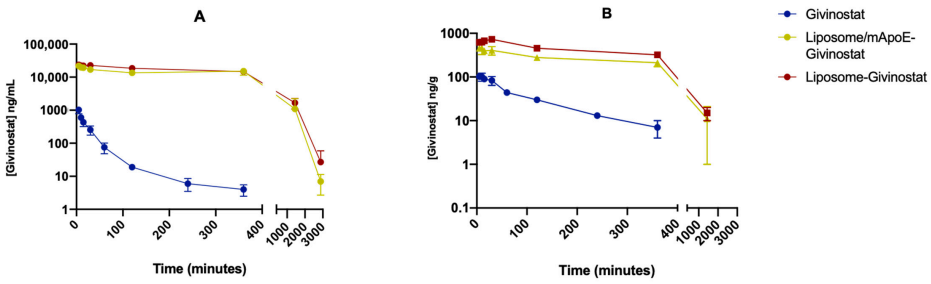

Figure 2. Concentration of Givinostat detected in plasma and brain after i.v. administration of free Givinostat, LIP-GIV, or LIP/m-GIV. **(A)** Plasma concentration of Givinostat from 5 min to 48 h post-injection. **(B)** Brain concentration of Givinostat from 5 min to 48 h post-injection. Data were obtained through the LC-MS/MS method.

The amount of Givinostat measured in the brain is shown in Figure 2B (all concentrations are available in Table S3), suggesting that the encapsulation of Givinostat in liposomes improved drug delivery to the brain. Both liposomal formulations led to a brain concentration of Givinostat of approximately 270 ng/g, 6 h after dosing. In contrast, the administration of Givinostat in the PEG/DMSO vehicle led to a brain tissue concentration of only 7 ng/g at the same time point, likely due to its high clearance rate from the systemic circulation. Then, 24 h after administration, Givinostat levels were below detection limits in the PEG/DMSO vehicle group while 15 ng/g of Givinostat was found in the brain of animals that received the liposomal formulations. The liposomal formulations showed a strong increase in the total exposure (AUC) of Givinostat that reached the brain, being much higher than the free drug (ratio from 20 to 30 times). On the other hand, the AUC_{brain}/AUC_{plasma} ratio for free Givinostat was 0.372, higher than liposomal formulations ratios (0.020 for LIP-GIV and 0.014 for LIP/m-GIV).

As Givinostat is a hydrophobic small molecule (~400 Da), it is able to cross the BBB by simple diffusion, unlike liposome formulations, which cross the BBB through endo/transcytosis [38]. In accordance with the results obtained for Vincristine entrapped in liposomes [37], we showed that the incorporation of Givinostat in liposomes should improve the therapeutic index by increasing the duration of drug exposure to the target tissue. *In vivo*, Givinostat gives rise to two main metabolites, deriving from the biotransformation of the hydroxamic acid mediated by different enzymes: the hydroxamate moiety may be hydrolyzed into a carboxylic acid (ITF2375) or reduced into an amide (ITF2374) [39]. The metabolites inhibit HDACs at concentrations from three to five orders of magnitude higher than Givinostat's and, in preclinical models, they do not contribute to its efficacy [40]. ITF2375 was the most abundant metabolite in plasma, in comparison to the ITF2374 compound (Figure S6, panels A,B and Table S2), as reported for free Givinostat [40]. In contrast, ITF2374 levels in the brain were slightly higher (Figure S6, panels C,D and Table S3). Accordingly, the ratio between ITF2374 and ITF2375 after 1 h of Givinostat administration was 0.18 and 5.6 in the plasma and brain, respectively. This difference could be due to the different enzymatic expression in the brain and peripheral tissues, or to the diverse grade of brain penetration of the two metabolites, but this hypothesis needs to be confirmed. The difference detected at the starting point between free and liposome-encapsulated Givinostat was not observed for the metabolites. This could be explained considering that only the free Givinostat fraction is metabolizable as it is accessible to enzymes, unlike when the drug is incapsulated in liposomes.

No significant difference in the pharmacokinetics and brain penetration between LIPGIV and LIP/m-GIV was found. Indeed, both preparations were able to increase C_{max} in the brain of about 6-fold over free Givinostat (~700 and ~500 vs. ~100 ng/g, respectively). However, it is important to note that in previous work, we demonstrated an increase in mApoE receptors on both BBB and GSCs after GBM mice irradiation [41]. This information should play in favor of using LIP/m-GIV to increase the delivery of Givinostat to cancer cells in GBM *in vivo* models. Nevertheless, both liposomal formulations are valid tools to deliver Givinostat to the brain and to increase its persistence in plasma. However, further studies on tumor-bearing animal models are needed because the presence of a tumor can impact on the circulation time and biodistribution of liposomes, as shown for polymersomes [42].

4.5.2 Givinostat embedded in liposomes maintained its anti-tumor activity in 2D and 3D in vitro models

Givinostat has shown anti-cancer activity on various tumor cell lines [7,43,44], but the literature concerning GBM is scarce. We evaluated the cytotoxic activity of Givinostat on the Gli36ΔEGFR-2 cell line, expressing the EGFRvIII variant that is present on up to 54% of cells isolated from GBM patients (mean = 28–30%); it is one of the most frequent genetic aberrations associated with GBM (Figure S7) [45–47]. Thus, this cell line represents an appropriate *in vitro* model to study the disease. In parallel, the effect of Givinostat was also tested on NHA cells, used as healthy controls. Gli36ΔEGFR-2 and NHA cell lines were first treated with the free drug and the cell viability was determined using the MTT assay (Figure 3).

The toxicity of Givinostat was both dose- and time-dependent, and the drug seemed to exhibit a natural selectivity for cancer cells versus healthy cells, which was maintained up to the dose of 2.5 μM and until 72 h of treatment, even if the differences were slight. However, the NHA cell viability was equal to or higher than 100% only after 24 h of treatment with Givinostat doses ranging between 0.1 and 10 μM. It is possible to speculate that this selectivity is also maintained *in vivo*. After a single i.v. injection, Givinostat embedded in liposomes was still present in the brain after 24 h while it was undetectable after 48 h. If the drug selectivity *in vivo* was confirmed in future studies, Givinostat should be able to induce GBM cells' mortality without affecting healthy cells after the first administration. The selective cytotoxicity of HDACis on transformed cells has been described by others and several mechanisms have been invoked, such as differences in cell cycle checkpoints [48] or higher concentrations of reactive oxygen species (ROS) in cancer cells treated with HDACis. In fact, Bolden et al. [49] demonstrated that cancer cells accumulate ROS more easily compared to healthy cells after treatment with the HDACi Vorinostat. Moreover, previous reports [50,51] have noted that the different epigenetic regulations and gene expressions in cancer cells might be correlated to the cancer-selective cytotoxicity of HDACis. The mean calculated IC_{50} in Gli36ΔEGFR-2 cells after 24 h of treatment was 0.75 μM. Considering that the IC_{50} for TMZ on the U87-MG and T98G cell lines is in the range of 100–500 μM [52], we speculate that Givinostat could be a promising adjuvant or alternative chemotherapeutic drug, especially in TMZ-resistant cells.

To evaluate if Givinostat retains its cytotoxic activity after incorporation in liposomes, we performed an MTT assay on Gli36ΔEGFR-2 cells after treatment with LIP-GIV and LIP/m-GIV. Three low doses were chosen, considering the improved blood half-life and brain uptake of the drug incorporated in liposomes (see above). The results (Figure 4) showed that the encapsulation of the drug in liposomes did not affect its anti-tumor action, preserving the dose- and time-dependent cytotoxic activity. However, it is noteworthy that only LIP-GIV reduced free Givinostat activity at the highest dose and time tested ($p = 0.0042$). In addition, it is important to point out that considering the increased plasma half-life of liposome formulations in comparison to the free drug and the low toxicity on endothelial cells, an enhancement of the anti-tumor efficacy by increasing the tumor drug deposition and a reduction in side effects might be expected [53].

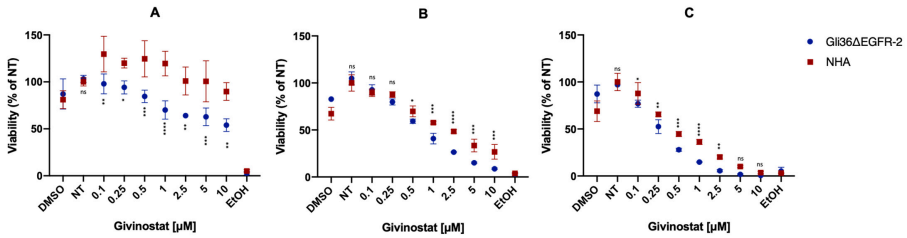

Figure 3. Evaluation of the cytotoxicity on Gli36DEGFR-2 and NHA cell lines treated with free Givinostat for 24 **(A)**, 48 **(B)**, or 72 h **(C)**. Control DMSO was given in equivalent microliters than highest dose of the inhibitor. Half an hour before the assay, three wells were pre-treated with 100% EtOH to provide a near 100% mortality as a control. NT were established as controls at 100% viability. Each graph is the result of three independent experiments. Ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, two-way ANOVA, Sidak's multiple comparisons test.

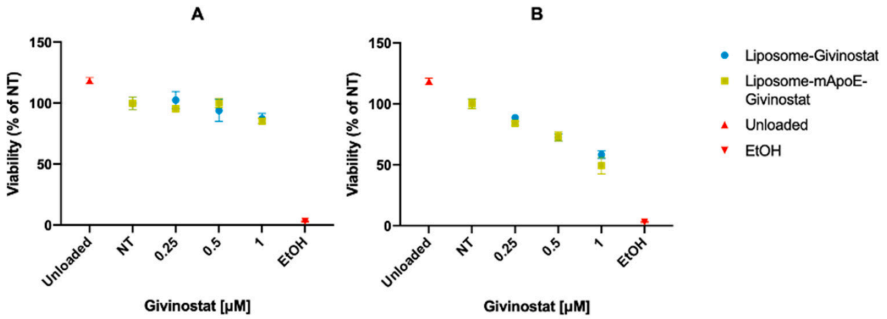

Figure 4. Evaluation of the cytotoxicity on the Gli36DEGFR-cell line treated with LIP-GIV or LIP/m-GIV for 24 **(A)** or 48 h **(B)**. The "unloaded" sample represents the unfunctionalized liposome without any drug loaded (lipid concentration ~8 μM, same lipid concentration as the 1 μM dose). Half an hour before the assay, three wells were pre-treated with 100% EtOH to provide a near 100% mortality as a control. NT were established as controls at 100% viability. Each graph is the result of three independent experiments±SD.

We also investigated if the liposomal formulations maintained the HDAC inhibitory effect of Givinostat in Gli36ΔEGFR-2 cells. We demonstrated that the activity of both cytosolic and nuclear HDACs was reduced after only 10 min of treatment with 0.5 μM LIP/m-GIV, reducing the activity by 25% after 30 min of treatment (Figure 5, panels A,B). This indicates that liposomal formulations are internalized inside cells within a few minutes, facilitating rapid action by Givinostat on HDACs. As a reminder, HDACs1-3 are exclusively nuclear while the others are either mostly cytoplasmic (HDAC6 and 10) or shuttle between the cytoplasm and nucleus [54,55]. Accordingly, we detected a strong dose-dependent increase in α-tubulin acetylation in treated Gli36ΔEGFR-2 (Figure 5C).

Of note, cytosolic HDAC6 is a microtubule-associated protein whose task is to deacetylate non-histonic proteins such as α-tubulin [56], thus explaining the increase in its acetylation induced by the treatment with LIP/m-GIV.

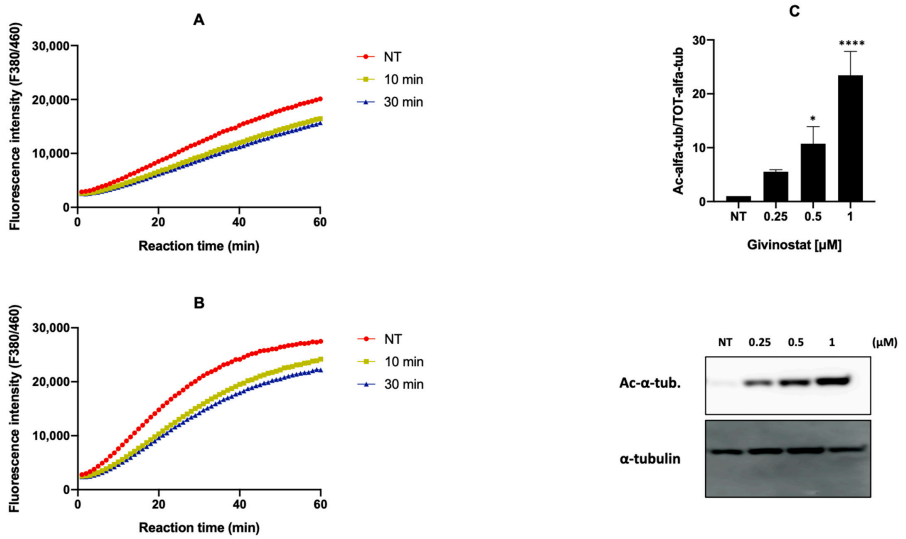

Figure 5. Percentage of HDACs activity in the Gli36DEGFR-2 cell line treated with LIP/m-GIV 0.5 μM in the cytoplasm **(A)** and nucleus **(B)**. After each selected time, cells were lysed, and cell fractions were extracted. HDAC activity was established through an HDAC activity assay based on ligand fluorescence. **(C)** The effect of LIP/m-GIV on α-tubulin acetylation. WB showing the protein levels of acetylated-α-tubulin (Ac-α-tub.) and total α-tubulin in Gli36DEGFR-2 cells without treatment (NT) or treatment with LIP/m-GIV in various concentrations. The graph shows the quantifications of Ac-α-tub. in three independent experiments. Total α-tubulin was used as a normalization protein. *, $p < 0.05$; ****, $p < 0.0001$, one-way ANOVA, Tukey's multiple comparisons test.

Traditionally, anti-cancer drugs have been evaluated in conventional 2D cell culture systems that poorly mimic the complexity and heterogeneity of *in vivo* tumors, which usually grow in 3D [57].

The limitations of 2D *in vitro* models include the absence of the GBM microenvironment (especially ECM components), very different culture conditions reported in the literature, and unphysiological oxygen levels, beyond the loss of the intrinsic *in vivo* heterogeneity of the tumor [58,59]. Although *in vivo* studies remain a fundamental step in cancer research, animals often do not represent a realistic model of GBM when human xenograft or orthotopic transplants are used. In fact, they are different than the original niche, do not show an infiltrative nature as human GBM does, and immunomodulatory therapies cannot be tested [60,61]. Moreover, mice do not exhibit endothelial proliferation and, more importantly, when xenograft transplantations are performed through subcutaneous injection, the local microenvironment is very different from the brain microenvironment, resulting in a lack of tumor growth [62]. Given these premises, several 3D *in vitro* models have been developed as a surrogate or a complementary approach to classic ones for evaluating drug efficacy [63]. They represent a valid compromise between the lack of complexity and heterogeneity of 2D *in vitro* models and the claims emerging from *in vivo* GBM models [64]. Although some limitations still need to be overcome [65], tumor cells (GBM cells included) grown in a 3D scaffold better recapitulate the features of patient-derived cells, in comparison to 2D culture conditions [66,67]. Accordingly, we tested the ability of LIP-GIV and LIP/m-GIV to affect the viability of 3D-bioprinted constructs generated using U87-MG and Gli36ΔEGFR-2 cells. Seven days after printing, 3D-bioprinted cells were treated for different times with liposomes and inhibition of cell growth was measured using the Alamar and LIVE/DEAD assays.

Results showed that both LIP-GIV and LIP/m-GIV were able to affect the viability of both U87-MG and Gli36ΔEGFR-2 cells (≥50% mortality) after 24 h of treatment in a 3D-printed model, as detected by the Alamar assay (Figure 6).

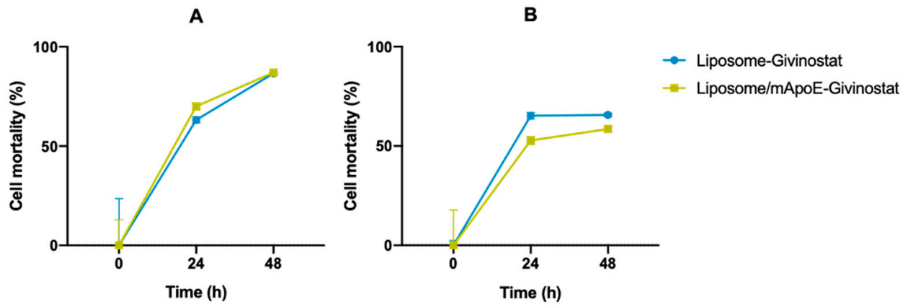

Figure 6. Alamar blue assay on bioprinted U87-MG **(A)** or Gli36DEGFR-2 **(B)** gelatin-chitosan hydrogels after liposome administration. Alamar blue solution (10% final volume) was added to each sample and incubated for approximately 2 h. Absorbance was read at 570 nm at the selected timepoints. Empty liposomes were employed as a positive control. Results are presented as five independent experiments±SD. All data were normalized with the positive control absorbance values obtained.

These data were also confirmed by the images obtained following the LIVE/DEAD assay (Figure 7), where > 50% cell mortality was detected after 24 h of treatment for both liposome formulations tested. Quantifications (Table 2) showed only mild and non-significant differences between the two cell lines used. This could be due to the different profile of liposome endocytosis. However, these results confirm those obtained in 2D models and demonstrate that LIP-GIV and LIP/m-GIV are also able to target GBM cells in a model with a complex ECM-like network and a 3D structural organization.

The functionalization of liposomes with mApoE was originally performed to promote their ability to reach the brain through the BBB in pathological conditions after mouse irradiation, as already shown in animal models [41,68,69]. In addition, this functionalization could also be exploited to increase the target selectivity towards tumor cells, because it has been shown that GBM cell lines overexpress low-density lipoprotein receptor (LDLR), very low-density lipoprotein receptor (VLDLR), and low-density lipoprotein receptor-related protein 1 (LRP1), to which mApoE binds [70,71]. Indeed, increased cellular uptake of mApoE-liposomes was also detected for Gli36ΔEGFR-2 (Figure S8). Nevertheless, our results showed that there is no difference in the cell viability between LIP-GIV and LIP/m-GIV (Figure 4). For this reason, we investigated the levels of LDLR, VLDLR, and LRP1 in Gli36ΔEGFR-2 cells. Interestingly, the results demonstrated that all three receptors were significantly reduced after treatment with Givinostat (Figure 8, panels A–C). These results can explain the comparable effect between functionalized and non-functionalized liposomes on Gli36ΔEGFR-2 viability. Moreover, to the best of our knowledge, the effect of Givinostat on these three receptors has never been shown and these findings increase the understanding of its mechanism of action. This additional feature may enhance the anti-tumor activity of Givinostat because the reduction in LDLR, VLDLR, and LRP1 levels can limit GBM survival by decreasing the uptake of lipoproteins, thus altering cell lipid metabolism. Their overexpression seems to be related to cancer progression; in fact, GBM cells are incapable of *de novo* cholesterol synthesis and their survival depends on cholesterol uptake by LDLRs [72].

Moreover, LRP1 expression has been linked to GBM cell migration and tumor invasion because it induces the expression of metalloproteases 2 and 9 via an ERK-dependent signaling pathway [73].

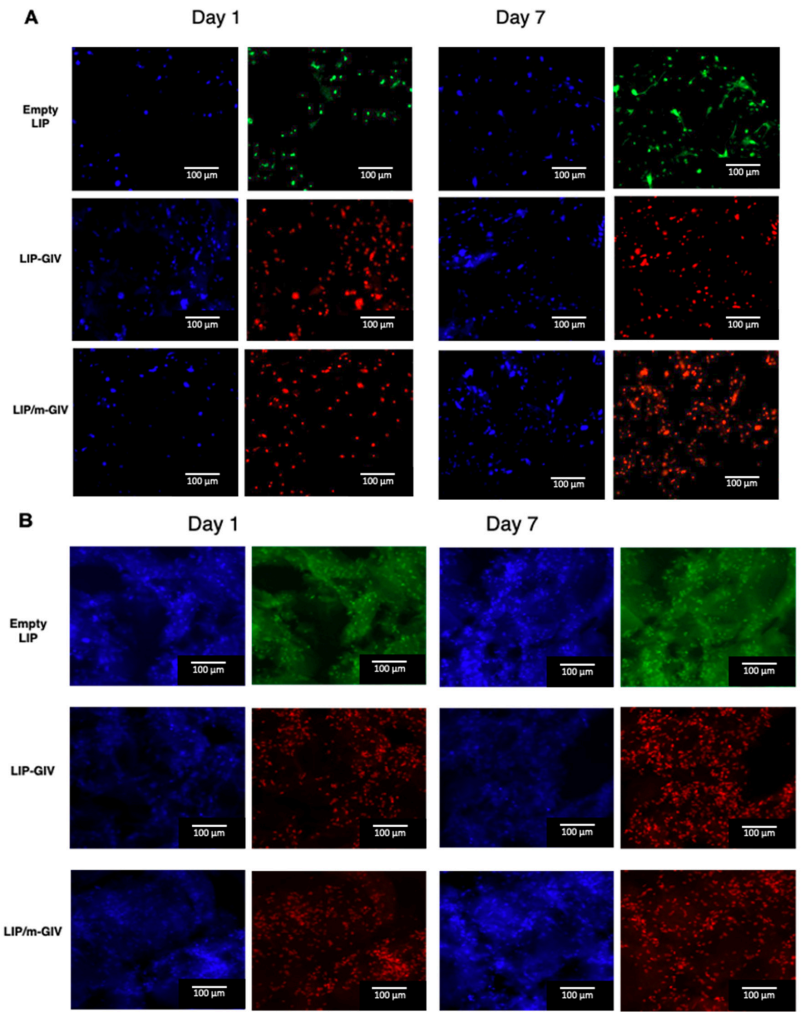

Figure 7. LIVE/DEAD microscopy images on day 1 and 7 after printing. U87-MG **(A)** or Gli36ΔEGFR-2 **(B)** cell lines were bioprinted with gelatin-chitosan hydrogel and cultured for 1 and 7 days. Then, empty liposomes, LIP-GIV, or LIP/m-GIV were administered for 24 h. Blue, DAPI (nuclei); red, EthD (dead cells); green, calcein (living cells). Cell viability was calculated as ((number of green-red stained cells/number of total cells) × 100) using Fiji ImageJ software.

Table 2. Cell mortality (% of control±SD) in 3D-bioprinted models after treatment with LIP-GIV or LIP/m-GIV.

Liposomal Formulation	Day 1	Day 7
LIP-GIV (Gli36ΔEGFR-2)	75 ± 13	78 ± 12
LIP/m-GIV (Gli36ΔEGFR-2)	66 ± 22	77 ± 16
LIP-GIV (U87-MG)	81 ± 11	76 ± 3
LIP/m-GIV (U87-MG)	89 ± 24	91 ± 3

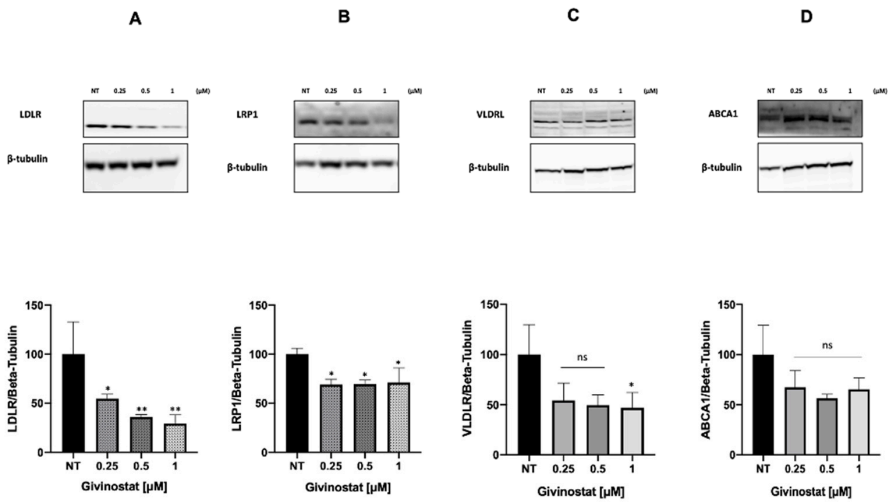

Figure 8. WB representing the effect of LIP-GIV on the expression of LDLR **(A)**, LRP1 **(B)**, VLDLR **(C)**, and ABCA1 **(D)** receptors in Gli36ΔEGFR-2 after 24 h, with quantitative graphs. Each graph is the result of three independent experiments. β-tubulin was used as a normalization protein. Ns, not significant; *, $p < 0.05$; **, $p < 0.01$, one-way ANOVA, Tukey's multiple comparisons test.

In addition, we investigated the expression of ATP-binding cassette protein A1 (ABCA1), which is involved in cholesterol efflux from astrocytes [71,74]. As shown in Figure 8D, there is a non-significant trend towards a decrease in ABCA1 expression after treatment with Givinostat.

This might be expected as a compensatory mechanism due to the parallel decrease in LDLR, VLDLR, and LRP1 protein expression. In other words, it is reasonable to assume that the decreased cholesterol uptake may stimulate GBM cells to mildly limit their efflux and preserve cholesterol storage. However, it is important to highlight that the ability of Givinostat to reduce ABCA1 levels could also be a potential strategy for the treatment of TMZ-resistant GBM because it has been shown that TMZ efflux is controlled by ABCA1 activity [75]. Therefore, co-administration of Givinostat could potentiate the TMZ efficacy in TMZ-resistant GBM cells. These assumptions need further investigation. Another point to consider is that the reduction in LDLR expression induces substantial apoptosis in U87EGFRvIII cells, as reported by Villa G.R. et al. [76]. To investigate whether this process also occurs in Gli36ΔEGFR-2 cells after Givinostat treatment, the expression of cleaved Caspase-3 was evaluated by immunofluorescence. As shown in Figure S9, the fluorescence associated with cleaved Caspase-3 was detected after 48 h of treatment with Givinostat in a dose-dependent manner. Similar results at the same timepoints have been obtained on human lymphoblastic leukemia [44], supporting the hypothesis that Givinostat acts as an apoptosis-inducing drug. However, in other published data on human sarcoma [77], Givinostat induced apoptosis after 72 h of treatment and exhibited a tumor-selective pro-apoptotic activity that was prolonged over time. This issue deserves further investigations.

4.6 Conclusions

GBM is the most common malignant and lethal primary brain tumor. Herein, using 2D and 3D *in vitro* models, we showed that the pan-HDAC inhibitor Givinostat embedded in liposomes counteracts GBM cell growth by inducing: (1) a dose- and time-dependent reduction in cell viability; (2) a reduction in LDLR, LRP1, and VLDLR protein receptors; (3) a mild reduction in ABCA1 levels; and (4) an increase in cleaved Caspase-3. In addition, the incorporation of Givinostat in liposomes increased the drug half-life in the bloodstream and the amount of drug entering the brain in healthy animal models for both preparations tested. Thus, LIP-GIV and LIP/m-GIV, by acting as a cytotoxic drug that is able to cross the BBB, could be considered as a potential approach against GBM. However, additional pre-clinical studies need to be performed to make this liposomal product applicable in this field.

Author contributions

Conceptualization: F.R. and L.T.; formal analysis: L.T., C.B., S.S., M.K. (Marcelo Kravicz), F.V., S.V., M.K. (Marcus Koch), F.B., L.M., E.P., V.A.C. and C.M.; data curation: G.D., R.M.M., L.R., C.S., L.T. and F.R.; writing—original draft preparation: F.R. and L.T.; writing—review and editing: L.T., G.D., M.K. (Marcus Koch), R.M.M., L.R., E.P., V.A.C., M.K. (Marcelo Kravicz), C.M., C.S. and F.R.; visualization: L.T., C.B., S.S., M.K. (Marcus Koch), F.B., L.R. and F.R.; supervision: G.D., C.S. and F.R.; project administration: F.R.; funding acquisition: F.R. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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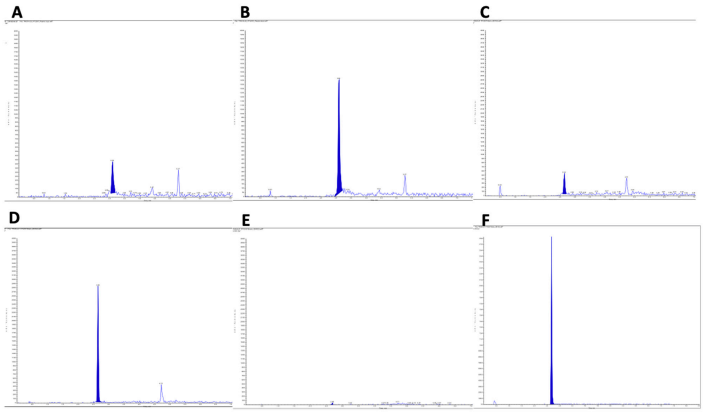
4.8 Supplementary materials

Table S1. MRM transitions for qualification and quantification and MS parameters of Givinostat and its metabolites.

Compound	Parent Ion (Q1)	Product Ion (Q3)	Time	DP	EP	CE	CXP
ITF2357_1*	422.2	186.3	150	53	7.2	35	1
ITF2357_2	422.2	305.1	150	60	2.1	32.2	2
ITF2374_1*	406.2	170.2	150	47	4.2	41	1.4
ITF2374_3	406.2	333.3	150	47	5	28	4
ITF2375_1*	407.3	334.3	150	50	3.3	27	3
ITF2375_2	407.3	171.3	150	45	4.7	39	3.2
ITF2400_1*	436.2	186.4	150	59	3.2	32	1.4

T 550°C, Gas 1 50, Gas 2 50, CUR 25, CAD 2, IS 5500


A
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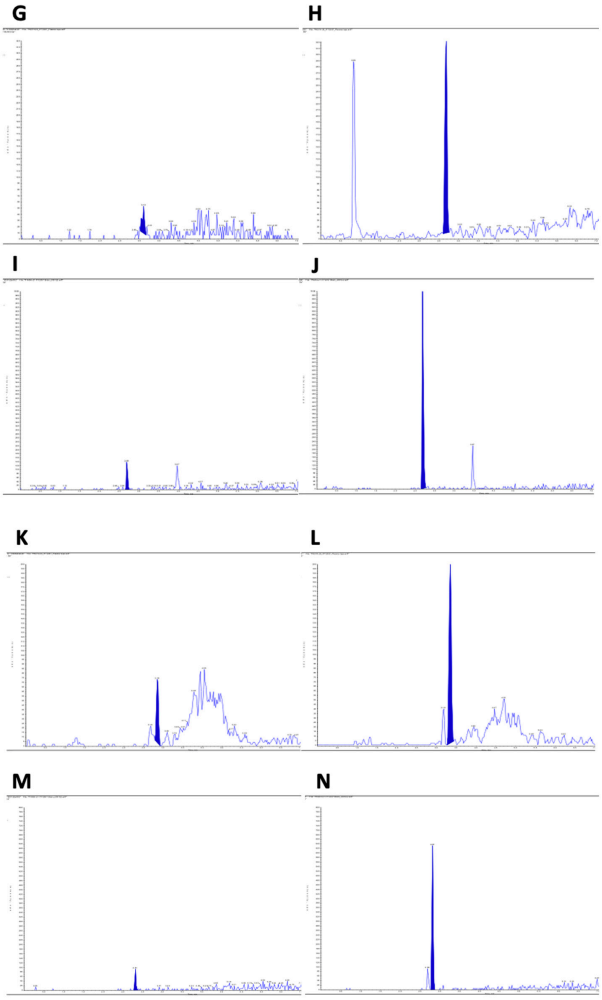
G
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Figure S1. **Panels A-F**: representative chromatograms of Givinostat MRM transition 422.2/186.3 amu. **(A)** Blank plasma; **(B)** LLOQ 2.5 ng/mL in plasma; **(C)** Blank brain homogenate; **(D)** LLOQ in brain homogenate 0.5 ng/mL; **(E)** Internal standard ITF2400 MRM transition 436.2/186.4 amu; **(F)** Representative chromatogram in plasma. **Panels G-J**: representative chromatograms of ITF2374 MRM transition 406.2/170.2 amu. **(G)** Blank plasma; **(H)** LLOQ in plasma; **(I)** Blank brain homogenate; **(J)** LLOQ in brain homogenate. **Panels K-N**: representative chromatograms of ITF2375 MRM transition 407.3/334.3. **(K)** Blank plasma; **(L)** LLOQ in plasma; **(M)** Blank brain homogenate; **(N)** LLOQ in brain homogenate.

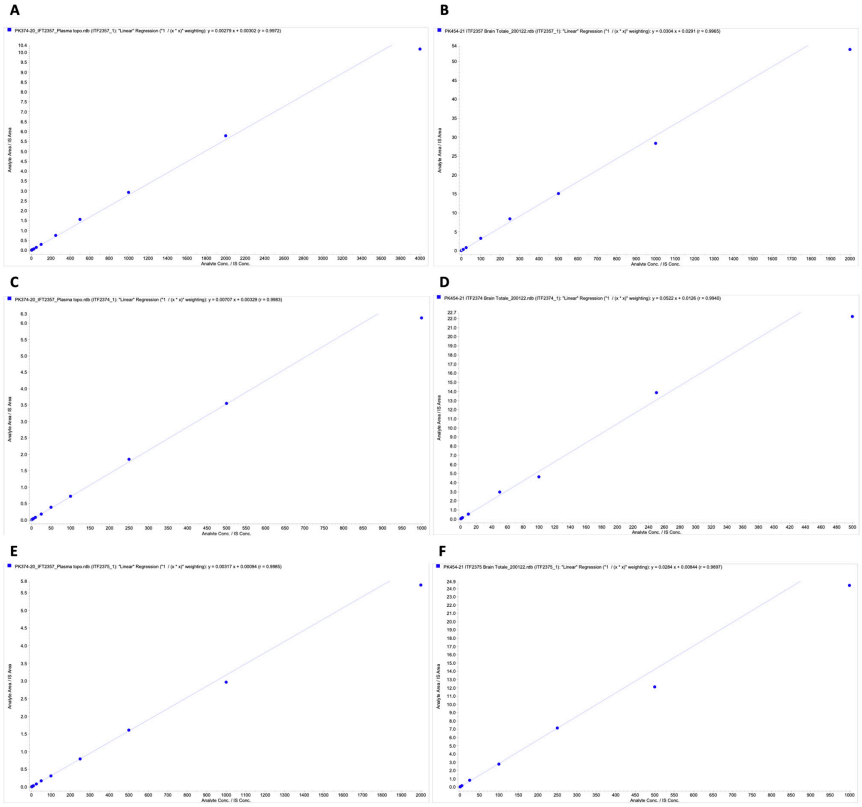

Figure S2. Panels **(A)** and **(B)**: Calibration curves for Givinostat in plasma (analytical range 2.5-4000 ng/mL) and brain homogenate (analytical range 0.5-2000 ng/mL). Panels **(C)** and **(D)**: Calibration curves for ITF2374 plasma (analytical range 0.1-1000 ng/mL) and in brain homogenate (analytical range 0.5-500 ng/mL). Panels **(E)** and **(F)**: Calibration curve for ITF2375 in plasma (analytical range 0.5-1000 ng/mL) and in brain homogenate (analytical range 0.5-500 ng/mL).

Figure S3. Reliability of cell fractions from Gli36ΔEGFR-2. The cytoplasm (C) and nucleus (N) were extracted after treatment with LIP/m-GIV using NE-PERTM Nuclear and Cytoplasmatic Extraction Kit. Reliability of cell fractions were assessed by WB using GAPDH and Histone H3 primary antibodies. As expected, GAPDH protein was predominantly expressed in cytoplasm, while Histone H3 was expressed in nucleus. These WB are representative images of non-treated controls.

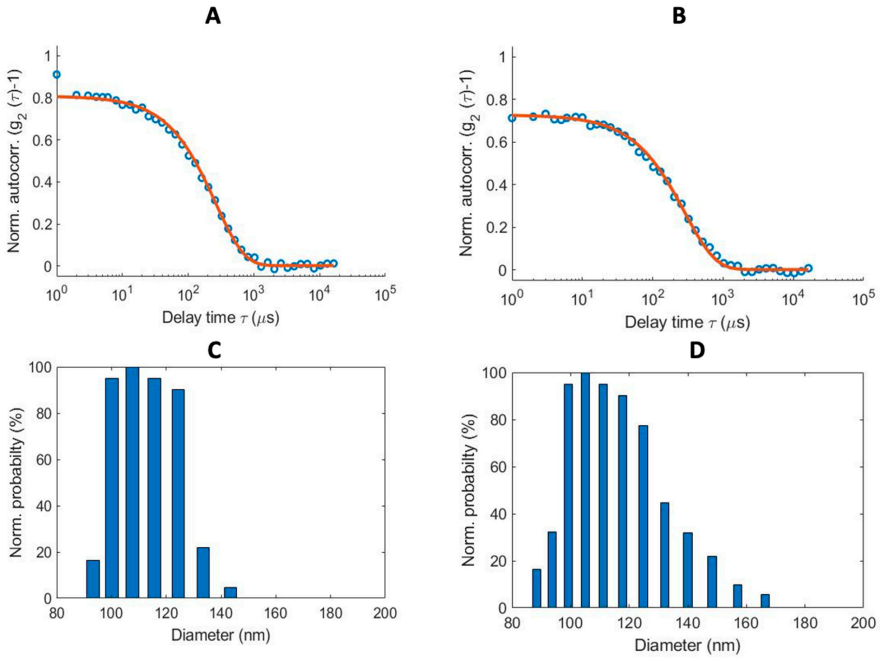

Figure S4. Representative autocorrelation functions with mono-exponential fit curve and CONTIN analysis of particle size distribution for LIP-GIV **(A-C)** and LIP/m-GIV **(B-D)** determined by DLS.

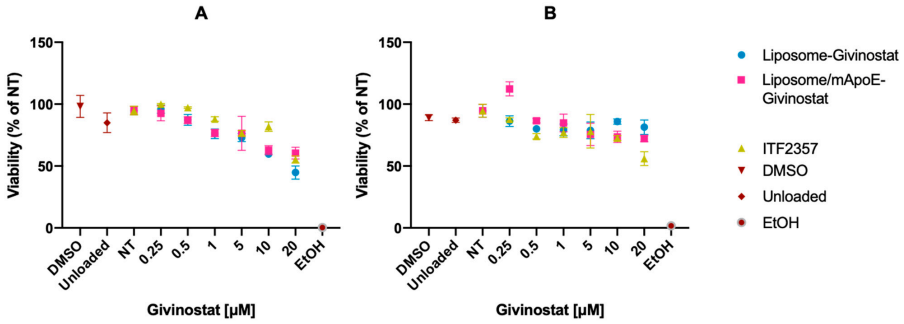

Figure S5. Evaluation of cytotoxicity on hCMEC **(A)** or Huvec **(B)** cell lines treated with LIPGIV, LIP/m-GIV or free Givinostat for 24 h. "Unloaded" sample represents the unfuctionalized liposome without any drug loaded and it was used at the same lipid concentration as 20 µM dose (lipid concentration = 0.153 mM). Control DMSO was given in equivalent microliters than the highest dose of the inhibitor. Half-an-hour before the assay, three wells were pre-treated with 100% EtOH to provide near 100% mortality as a control. NT were established as controls at 100% viability. Each graph is the result of three independent experiments ± SD.

Table S2. Plasma levels of Givinostat and its metabolites

Group A (Free Givinostat)			Group B (LIP-GIV)			Group C (LIP/m-GIV)	
Time (h)	Givinostat (ng/mL)	SD	Time (h)	Givinostat (ng/mL)	SD	Givinostat (ng/mL)	SD
0.083333	1028	241.6	0.083333	23652	3759.4	21926	1200.6
0.166667	597	37.5	0.166667	22209	2328.9	19780	2863.8
0.25	431	109.4	0.25	21988	1554.3	19281	1298.4
0.5	256	78.3	0.5	22607	4154.0	17081	2659.6
1	75	26.9	2	18655	3806.8	13569	2429.7
2	19	3.8	6	14721	2639.0	15171	3661.8
4	6	2.5	24	1664	578.9	1095	1143.5
6	4	1.5	48	27	31.5	7	4.3
Time (h)	ITF2374 (ng/mL)	SD	Time (h)	ITF2374 (ng/mL)	SD	ITF2374 (ng/mL)	SD
0.083333	93.3	7.6	0.083333	85.7	5.9	66.4	4.4
0.166667	135.3	13.4	0.166667	82.1	3.9	70.7	16.8
0.25	126.4	12.6	0.25	85.2	8.0	60.7	11.6
0.5	143.7	15.2	0.5	64.5	4.2	52.5	14.0
1	109.9	33.0	2	153.9	22.9	148.6	35.1
2	55.3	13.7	6	278.6	141.2	343.3	39.4
4	38.4	5.5	24	25.0	30.9	24.2	30.0
6	18.2	8.5	48	<LOQ	-	<LOQ	-
Time (h)	ITF2375 (ng/mL)	SD	Time (h)	ITF2375 (ng/mL)	SD	ITF2375 (ng/mL)	SD
0.083333	860.4	101.7	0.083333	736.5	43.4	557.7	40.1
0.166667	1509.6	158.5	0.166667	580.4	39.7	516.8	72.6
0.25	1611.4	112.0	0.25	558.9	38.7	390.6	32.8

0.5	1398.8	148.5	0.5	402.0	42.9	328.2	62.7
1	615.1	39.2	2	336.2	69.1	290.2	20.1
2	231.1	33.9	6	415.8	58.3	551.0	101.3
4	81.0	34.5	24	35.7	38.8	29.8	32.6
6	24.8	15.0	48	1.8	0.9	1.3	0.7

n=3, if not stated otherwise.

Table S3. Brain levels of Givinostat and its metabolites

Group A (Free Givinostat)			Group B (LIP-GIV)			Group C (LIP/m-GIV)	
Time (h)	**Givinostat (ng/g)**	**SD**	**Time (h)**	**Givinostat (ng/g)**	**SD**	**Givinostat (ng/g)**	**SD**
0.083333	102	2	0.083333	455	46	621	75
0.166667	101	21	0.166667	463	137	625	90
0.25	90	10	0.25	399	53	674	65
0.5	83	19	0.5	406	89	489	424
1	44	4	2	279	28	456	56
2	30	4	6	212	40	322	27
4	13	2	24	11	10	15	5
6	7	3	48	<LOQ		<LOQ	
Time (h)	**ITF2374 (ng/g)**	**SD**	**Time (h)**	**ITF2374 (ng/g)**	**SD**	**ITF2374 (ng/g)**	**SD**
0.083333	33.1	1.4	0.083333	28.5	11.4	22.6	11.2
0.166667	51.0	3.6	0.166667	30.9	11.7	25.7	11.9
0.25	43.2	1.8	0.25	31.0	6.7	22.5	0.6
0.5	64.8	12.0	0.5	25.7	8.9	14.6	3.1
1	55.7	14.1	2	22.3	6.5	20.9	3.8
2	28.7	3.8	6	2.6	1.2	26.5	3.1
4	16.2	1.0	24	2.6	1.2	5	5
6	6.7	3.2	48	<LOQ	3.2	<LOQ	-
Time (h)	**ITF2375 (ng/g)**	**SD**	**Time (h)**	**ITF2375 (ng/g)**	**SD**	**ITF2375 (ng/g)**	**SD**
0.083333	13.6	2.0	0.083333	13.6	2.0	4.2	2.8
0.166667	27.7	4.7	0.166667	27.7	4.7	2.9	0.2
0.25	25.4	4.8	0.25	25.4	4.8	2.8	0.6
0.5	25.8	7.5	0.5	25.8	7.5	<LOQ	
1	9.9	1.2	2	9.9	1.2	<LOQ	
2	3.5*	-	6	<LOQ		<LOQ	
4	0.3*	-	24	<LOQ		<LOQ	
6	4.3*	0.4	48	<LOQ		<LOQ	

*n=3, if not stated otherwise. *n=1*

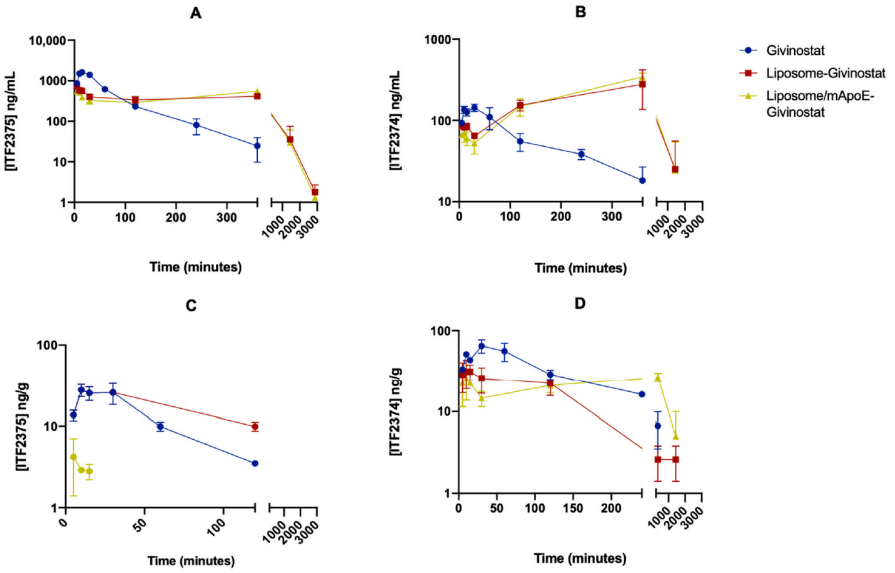

Figure S6. Distribution of Givinostat metabolites in brain and plasma after i.v. administration of free Givinostat, LIP-GIV or LIP/m-GIV. Panels **(A)** and **(B)** show the plasma distribution of ITF2375 and ITF2374 from 5 min to 48 h post-injection. Panels **(C)** and **(D)** show brain distribution of ITF2375 and ITF2374 from 5 min to 48 h post-injection. Data were obtained through LC-MS/MS method.

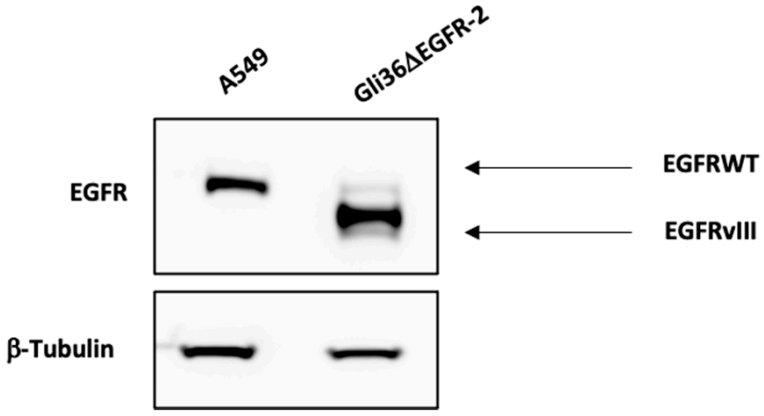

Figure S7. Expression of EGFR in A549 and Gli36ΔEGFR-2 cell lines. Protein levels were assessed by WB and equal loading was confirmed by β-tubulin antibody. EGFRWT (170 kDa) and EGFRvIII (140-155 kDa) are indicated by the arrows. Lung carcinoma A549 cell line was used as a representative control of a cell line carrying a WT expression of EGFR.

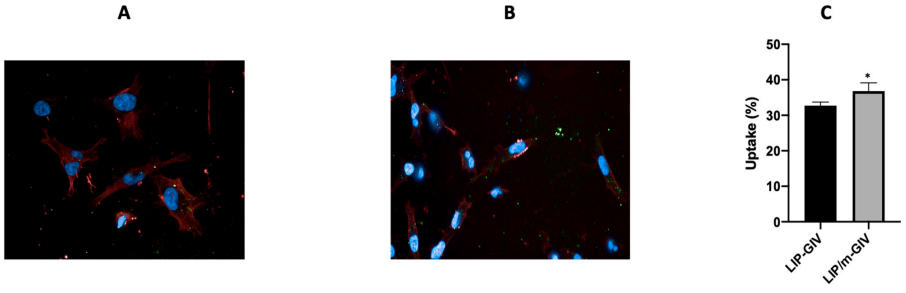

Figure S8. Targeting efficacy of fluorescent-labeled liposomes **(A)** and mApoE-liposomes **(B)** on Gli36ΔEGFR-2. Cells were stained with Hoechst (blue) and CellMask™ Deep Red Actin Tracking Stain (red). The liposomes fluorescence-associated is visible as green spots. Panel **(C)** shows the percentage of liposomes targeting efficacy. Results are presented as five independent experiments ± SD. *, $p < 0.05$, unpaired Student's t test.

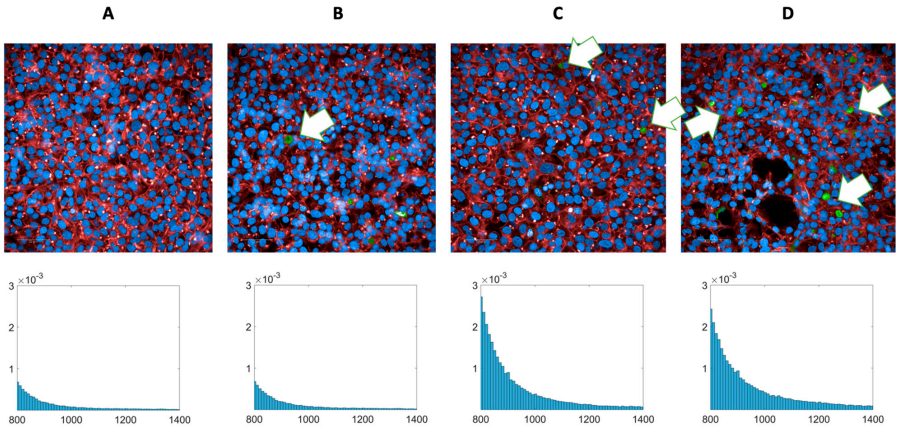

Figure S9. Increase of cleaved Caspase-3 in Gli36ΔEGFR-2 cells after treatment with Givinostat 0.25 µM **(B)**, 0.5 µM **(C)** or 1 µM **(D)**. **(A)** represents non-treated cells, used as a control. Cells were stained with Hoechst (blue), Phalloidin (red) and cleaved Caspase-3 (green). Immunofluorescence were performed using the Operetta CLS High Content Analysis System (Perkin Elmer). Arrows indicate green fluorescence. Results are presented as explicative images derived from two independent experiments. Quantitative measurements for Caspase-3 accumulation after treatment were presented as histograms showing intensities of the green channel images fixing the threshold >800 a.u.

CHAPTER 5

Summary, conclusions and future perspectives

5.1 Summary, conclusions and future perspectives

Glioblastoma multiforme (GBM, IV grade astrocytoma) is the most common and aggressive tumor of the central nervous system (CNS) and it is characterized by an infiltrative nature, high heterogeneity, aggressiveness, and recurrence. Current therapies display confined successes and the need to discover novel therapeutic agents or innovative drug delivery systems for targeted actions appears to be urgent [1,2].

The treatment failure may be attributable to several factors, including: 1) the complexity of the tumor microenvironment (TME); 2) the presence of GBM stem cells (GSCs), that easily resist to therapies and give rise to recurrency; 3) the selectivity of intact blood-brain barrier (BBB) that prevents molecules diffusion across the brain; 4) intercellular communication among cancer cells and GSCs through paracrine molecules or cellular structures like Tunneling Nanotubes (TnTs), that promote invasiveness, aggressiveness and TMZ resistance [3–7]. In this context, nanomedicine represents a suitable approach to administer small molecules with scarce brain penetration or insufficient specific targeting. Among existing nanoparticles (NPs), liposomes are biocompatible, biodegradable, low immunogenic and versatile tools that include the possibility of surface functionalization [8–10].

In thesis work, we proposed liposomes loaded with the pan-HDAC inhibitor (HDACi) Givinostat as potential therapeutic approach against GBM. Liposomes, unfunctionalized (LIP-GIV) or functionalized with mApoE peptide (LIP/m-GIV) to improve targeting efficacy, have been tested *in vitro* and *in vivo*.

Our results demonstrated that LIP-GIV and LIP/m-GIV are unilamellar, monodispersed, and homogenous NPs that remain stable over time. Our *in vivo* studies have shown a strong increase in plasma half-life and brain penetration of both preparations, compared to the free drug. The efficacy has been evaluated in 2D and 3D *in vitro* models, resulting in a dose- and time-dependent reduction of viability for every cell line used. In addition, the treatment with LIP-GIV and LIP/m-GIV increased the expression of cleaved Caspase-3 (indicating apoptosis) and decreased the expression of receptors related to cholesterol uptake, such as LDLR, LRP1 and VLDLR. This finding might explain one of the anti-tumor effects of Givinostat-Liposomes, depleting cancer cells of an essential cellular component. As a matter of fact, GBM cells are incapable of *de novo* synthesis of cholesterol and they depend on lipoproteins uptake.

Taken together, these results demonstrate the potential of Givinostat-Liposomes preparations in GBM therapy, even though further studies are needed to clarify the efficacy on tumor-bearing mouse models and other undiscovered anti-tumor effects on cancer cells. The incoming studies will focus on the improvement of liposomes preparations, for example investigating different functionalizations on NPs surface and testing the efficacy of other HDACis such as selective-HDAC6 inhibitor ITF3756. Plus, the exchange of liposomes among different cells through TnTs will be explored as a drug delivery strategy. Our work will also include the synthesis of other lipid-based NPs with high rate of brain penetration like discoidal NPs and solid-lipid NPs (SLNs).

In addition, since several HDACis have been successfully used as immunotherapeutic agents, together with anti-PD-1/PD-L1 mAbs, Givinostat could be a promising candidate to better investigate this kind of combined treatment, allowing to exploit both an anticancer and a immunotherapeutic effect for the treatment of GBM.

5.2 References

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5.3 Publications outside the thesis topic

I. Oxidative stress boosts the uptake of Cerium Oxide Nanoparticles by changing brain endothelium microvilli pattern

Roberta Dal Magro, Agostina Vitali, Stefano Fagioli, Alberto Casu, Andrea Falqui, Beatrice Formicola, Lorenzo Taiarol, Valeria Cassina, Claudia Adriana Marrano, Francesco Mantegazza, Umberto Anselmi-Tamburini, Patrizia Sommi, Francesca Re

Antioxidants (Basel). 2021 Feb 9;10(2):266. doi: 10.3390/antiox10020266.

Abstract

Vascular oxidative stress is considered a worsening factor in the progression of Alzheimer's disease (AD). Increased reactive oxygen species (ROS) levels promote the accumulation of amyloid-β peptide (Aβ), one of the main hallmarks of AD. In turn, Aβ is a potent inducer of oxidative stress. In early stages of AD, the concomitant action of oxidative stress and Aβ on brain capillary endothelial cells was observed to compromise the blood-brain barrier functionality. In this context, antioxidant compounds might provide therapeutic benefits. To this aim, we investigated the antioxidant activity of cerium oxide nanoparticles (CNP) in human cerebral microvascular endothelial cells (hCMEC/D3) exposed to Aβ oligomers. Treatment with CNP (13.9 ± 0.7 nm in diameter) restored basal ROS levels in hCMEC/D3 cells, both after acute or prolonged exposure to Aβ.

Moreover, we found that the extent of CNP uptake by hCMEC/D3 was +43% higher in the presence of Aβ. Scanning electron microscopy and western blot analysis suggested that changes in microvilli structures on the cell surface, under pro-oxidant stimuli (Aβ or H_2O_2), might be involved in the enhancement of CNP uptake. This finding opens the possibility to exploit the modulation of endothelial microvilli pattern to improve the uptake of anti-oxidant particles designed to counteract ROS-mediated cerebrovascular dysfunctions.

II. Reduced levels of ABCA1 transporter are responsible for the cholesterol efflux impairment in β-Amyloid-induced reactive astrocytes: potential rescue from biomimetic HDLs

Giulia Sierri, Roberta Dal Magro, Barbara Vergani, Biagio Eugenio Leone, Beatrice Formicola, Lorenzo Taiarol, Stefano Fagioli, Marcelo Kravicz, Lucio Tremolizzo, Laura Calabresi, Francesca Re

International Journal of Molecular Science. 2021 Dec 22;23(1):102. doi: 10.3390/ijms23010102.

Abstract

The cerebral synthesis of cholesterol is mainly handled by astrocytes, which are also responsible for apoproteins' synthesis and lipoproteins' assembly required for the cholesterol transport in the brain parenchyma. In Alzheimer disease (AD), these processes are impaired, likely because of the astrogliosis, a process characterized by morphological and functional changes in astrocytes.

Several ATP-binding cassette transporters expressed by brain cells are involved in the formation of nascent discoidal lipoproteins, but the effect of beta-amyloid (Aβ) assemblies on this process is not fully understood. In this study, we investigated how of Aβ1-42-induced astrogliosis affects the metabolism of cholesterol in vitro. We detected an impairment in the cholesterol efflux of reactive astrocytes attributable to reduced levels of ABCA1 transporters that could explain the decreased lipoproteins' levels detected in AD patients. To approach this issue, we designed biomimetic HDLs and evaluated their performance as cholesterol acceptors. The results demonstrated the ability of apoA-I nanodiscs to cross the blood-brain barrier in vitro and to promote the cholesterol efflux from astrocytes, making them suitable as a potential supportive treatment for AD to compensate the depletion of cerebral HDLs.

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