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Strategies increasing the effectiveness of temozolomide at various levels of anti-GBL therapy

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ABSTRACT

Glioblastoma (GBL) is the most common (60–70% of primary brain tumours) and the most malignant of the glial tumours. Although current therapies remain palliative, they have been proven to prolong overall survival. Within an optimal treatment regimen (incl. surgical resection, radiation therapy, and chemotherapy) temozolomide as the current anti-GBL first-line chemotherapeutic has increased the median overall survival to 14–15 months, and the percentage of patients alive at two years has been reported to rise from 10.4% to 26.5%. Though, the effectiveness of temozolomide chemotherapy is limited by the serious systemic, dose-related side effects. Therefore, the ponderation regarding novel treatment methods along with innovative formulations is crucial to emerging the therapeutic potential of the widely used drug simultaneously reducing the drawbacks of its use. Herein the complex temozolomide application restrictions present at different levels of therapy as well as, the currently proposed strategies aimed at reducing those limitations are demonstrated. Approaches increasing the efficacy of anti-GBL treatment are addressed. Our paper is focused on the most recent developments in the field of nano/biomaterials-based systems for temozolomide delivery and their functionalization towards more effective blood-brain-barrier crossing and/or tumour targeting. Appropriate designing accounting for the physical and chemical features of formulations along with distinct routes of administration is also discussed. In addition, considering the multiple resistance mechanisms, the molecular heterogeneity and the evolution of tumour the purposely selected delivery methods, the combined therapeutic approaches and specifically focused on GBL cells therapies are reviewed.

1. Introduction

Glioblastoma is adults' most common malignant central nervous system (CNS) tumour. According to the latest fifth edition of the World Health Organization (WHO) Classification of Tumours of the Central Nervous System published in 2021 [1], GBL is a Grade 4 brain tumour [2]. The abovementioned document presents major changes that advance the role of molecular diagnostics in CNS tumour classification. Moreover, the new tumour types and subtypes were introduced while the term glioblastoma multiforme (GBM) has been withdrawn. Nevertheless, the prepared review draws on the studies that were mainly published based on previous classifications therefore we have retained the abbreviation GBM for the original terminology used in the cited publications.

GBL arises from glial cells and has, among its most prominent characteristics, high invasiveness, and a strong tendency to recur after surgical resection [3]. Moreover, tumour's cells show a discrepancy in structure and morphology, they are infiltrative in nature, therefore, the therapeutic efficacy of a drug is only possible by providing its fairly targeted and in very high doses [4]. The current treatment strategies for GBL involve surgical resection, usually followed by combined radiochemotherapy [5]. Despite this multimodal approach, the median survival of glioblastoma patients who underwent maximum safe resection plus combined radiochemotherapy is limited to 16 – 19 months. Patients whose tumours display epigenetic silencing of the DNA repair enzyme *O*-methyl- guanine-methyltransferase are believed to experience better outcomes [6]. Considering the poor survival with currently approved treatments, new therapeutic options for GBL are of great importance. A significant challenge in glioblastoma treatment is to overcome the blood-brain barrier (BBB) in the CNS, which is the brain's own defence system. This protective barrier impedes drugs to affect brain cells, thus, oral, and intravenous routes for drug delivery to the

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brain are frequently inadequate. The circumvention of the BBB through straight intervention into insubstantial brain tissues can result in severe neurotoxicity and loss of brain key functionality [7]. **As a result, there is a need to design a more specific and noninvasive approach to target GBL.**

Temozolomide (TMZ) is currently first-line chemotherapy for glioblastoma treatment. TMZ has increased the median overall survival to 14.6 months, and the percentage of patients alive at two years has been reported to increase from 10.4% to 26.5% [8]. TMZ as one of the few pharmaceuticals is able to enter the brain parenchyma and exert a therapeutic effect. However, only a part of the dose administrated penetrates the BBB since the essential problem with TMZ is its low stability [9]. TMZ (8-carbamoyl-3-methylimidazo-[5,1-d]−1,2,3,5-tetrazin-4-(3 H)-one) is a prodrug, DNA-alkylating agent that under physiological conditions undergoes chemical degradation spontaneously. During the TMZ hydrolysis process, the active metabolite, the 5-(3-methyl-triazen-1-yl) imidazole-4-carboxamide (MTIC) is formed. This product is then rapidly degraded to inactive 5-aminoimidazole-4--carboxamide (AIC) and the methylating entity. MTIC is considered the major cause of toxicity of TMZ chemotherapy since it brings methylation of the *O*6 guanine position [10]. The strongly pH-dependent half-life time of this prodrug is less than 2 h under physiological conditions (pH = 7.4) and about 24 h in an acidic environment (pH<4 at 25°C). Thus, systemically administered TMZ may be degraded in the blood, resulting in the MTIC production, an active metabolite being unable to exceed the BBB. The formation of TMZ hydrolysis products before reaching the CNS decreases the therapeutic efficacy. Moreover, the short half-life time of TMZ requires multiple high doses exposing the patient to severe side effects including haematological toxicity, acute cardiomyopathy, oral ulceration, hepatotoxicity, and pneumocystis pneumonia [4]. **Therefore, the TMZ reformulation, using carriers or functionalization towards the more effective BBB crossing or employing various routes of TMZ administration could increase its concentration in the brain simultaneously reducing the risk of adverse systemic complications.**

2. Effective crossing of the blood-brain barrier (BBB)

2.1. Structural and functional aspects of the BBB

The central nervous system, composed of the encephalon and spinal cord, is the most complicated and essential part of the human body. On this account, the CNS needs to be isolated from the direct impact of the external environment to avoid sudden changes in the composition of cerebrospinal fluid (CSF) as well as the infiltration of harmful substances, including those circulating in the blood [11]. CNS protection is possible due to the existence of the blood-brain barrier (BBB), which represents the physical and biochemical border between blood vessels and nervous tissue. The BBB, in addition to its protective function, also plays an important role in providing a milieu suitable for the proper functioning of neurons, by maintaining homeostasis and regulating metabolite influx/efflux [12,13]. Therefore, the BBB is conceived as a highly selective semipermeable membrane, which impedes the transport of potentially noxious compounds to the brain but allows nutrients passage [14]. Within the CNS, there are other barriers like a blood-cerebrospinal fluid barrier (BCB) or CSF- brain barrier (CBB), however, their surface area is much smaller than the BBB which is the reason the BCB and CBB are often skipped.

The BBB structure (Fig. 1) consists of brain capillary endothelial cells (BCECs), pericytes, astrocytes, and basement membrane [12]. The former ones create a continuous layer as they are closely connected to each other with three types of junctions: tight junctions (TJs), adherent junctions (AJs), and gap junctions (GJs). However, the physical limitation of paracellular diffusion of water-soluble compounds into the brain stems mainly form a thick web of TJs [11,14]. On the surface of BCECs, embedded in the basal lamina, there are pericytes, i.e., macrophage-like cells, that regulate the blood flow through capillaries, TJs, AJs as well as transcytosis through the BBB [4]. Furthermore, astrocytes provide a connection between blood vessels and neurons and by modulating neurotransmitter concentration, support homeostasis maintenance [12].

Besides the physical barriers, exist certain mechanisms responsible for the passage of necessary substances such as amino acids or glucose into the brain and the outflow of the redundant ones from the CNS [12]. The physicochemical properties of various compounds affect the type of pathway that a concrete molecule is able to enter the brain [15]- the possible mechanisms of the BBB crossing are presented in the diagram below (Fig. 2).

Gases included in the blood (O_2, CO_2) and small lipid-soluble agents (e.g., ethanol, nicotine, steroid hormones, etc.) are able to enter the internal brain milieu by free diffusion through the lipoidal BCECs membranes (transcellular lipophilic pathway) [12,16]. Whereas, crossing the BBB in the case of hydrophilic compounds and macromolecules requires active transport pathways such as receptor-mediated transcytosis (RMT), adsorptive-mediated transcytosis (AMT) or carrier-mediated transcytosis (CMT). During RMT, the molecule binds to a specific site of the receptor located on the cell, what leads to endocytosis followed by transcytosis. Three of the receptors in the BBB are the most expressed: those for low-density lipoprotein (LDL), transferrin (Tf), and insulin [17]. On the other hand, cationic molecules can cross the BBB via the AMT mechanism, after electrostatic interaction with negatively charged membranes of BCECs [18]. Finally, numerous ions, as well as nutrients, use the CMT pathway for passage into the brain, for instance, glucose is carried by glucose transporter-1 (GLUT1) [11]. Next to the influx mechanisms, there are present efflux systems. They are represented by ATP-binding cassette transporters (ABCs), known as active efflux pumps. Transporters such as P-glycoprotein (P-gp) or multidrug resistance proteins (MRPs) are expressed on the endothelium and their function is to force chemical compounds that crossed the BBB to re-enter the bloodstream [19]. Numerous lipophilic pharmaceuticals are substrates for efflux pumps, thus the proteins affect the pharmacological drug's behaviour [20]. Although in CNS disorders the structure of the BBB may be disrupted and thus accelerate drug transportation, the problem emerges to be more complex as in the case of GBL tumour cells tend to migrate into deeper parts of the brain [21], where the membrane is completely intact [22].

2.2. The brain TMZ delivery approaches

Unfortunately, the BBB, due to its unique properties, hinders the effective treatment with virtually 98% of agents [23], since only

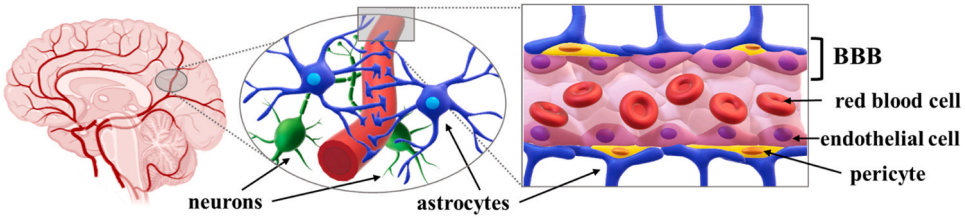

Fig. 1. The schematic diagram of the BBB structure.

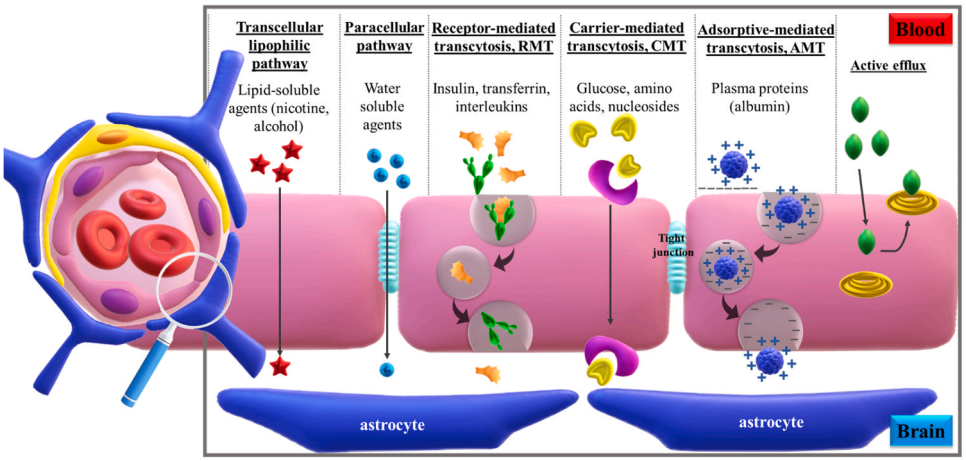

Fig. 2. Schematic representation of several transport types across the BBB.

lipid-soluble drugs with a molecular weight below 500 Da are able to reach pharmacologically significant concentrations in the CNS [24]. As was noted above, TMZ can enter the brain parenchyma and cause a therapeutic effect, however, only a partially applied dosage penetrates the BBB [9]. Because of the low stability, TMZ is degraded to the active metabolite (MTIC) being unable to exceed the BBB. It is believed that the prolonged TMZ half-life in the bloodstream may improve the passage of its molecules through the BBB, which would favour the greater accumulation in the brain parenchyma, thus increasing the chances of reaching the GBL. Achieving the aforementioned results would allow the dose of the chemotherapeutic agent to be reduced due to easier maintaining the drug concentration within the therapeutic window. Therefore, various strategies for TMZ delivery to the brain are employed, including chemical modification, encapsulation in different types of delivery systems as well as alternative ways of administration.

2.3. TMZ delivery platforms

So far, many types of nanoplatforms that can reach the brain have been evaluated. The use of carriers has a range of advantages, such as improving the bioavailability and biocompatibility of drugs, increasing their accumulation and thus therapeutic concentration at certain sites, and finally reducing side effects.

2.3.1. Polymer-based systems

Placing the molecules inside the polymeric capsule significantly reduces their enzymatic and hydrolytic degradation in the harsh organism environment. The longer circulation in the bloodstream results in a more effective targeting strategy. Poly(lactide-co-glycolic-acid) (PLGA) is one of the well-known representatives of copolymers in biomedical applications due to its biocompatibility, tuneable mechanical properties, and degradation rate. Ramalho et al. [25] showed that entrapping TMZ molecules inside PLGA nanoparticles (NP PLGA) was possible with good encapsulating efficiency ranging from $48 \pm 10\%$ for functionalized-PLGA NPs to $44 \pm 3\%$ for non-modified NPs. The functionalized PLGA carriers enhanced the drug's anticancer activity and changed its release time under *in vitro* conditions. Elsewhere, PLGA nanoparticles with TMZ obtained employing three different preparation procedures (single emulsion solvent evaporation, double emulsion solvent evaporation, and single emulsion solvent evaporation with TMZ saturated aqueous phase) exhibit poor drug loading (less than 5%) and unsatisfying TMZ encapsulation efficiency results (a few to a dozen or so percent depending on the procedure) [26].

The system composed of PLGA core functionalized with poly (ethylene) glycol (PEG) as an agent responsible for NPs longer bloodstream circulation, and folic acid (FOL) in the role of targeting molecule for folate receptors on the BBB is another group of polymeric TMZ delivery structures. However, it was found that the optimization of the process of creating the planned TMZ formulation is a big challenge because the commonly used methods of polymeric particle synthesis (emulsion solvent evaporation or nanoprecipitation method) did not allow for obtaining satisfactory carriers with a non-hydrolysed form of the drug or effective encapsulation. Since TMZ is not hydrolytically stable, the conditions of its encapsulation need to be adjusted to prevent the degradation process, and higher drug loading values seem to be limited by the TMZ solubility in the solvent used during the carrier's preparation [27].

PEG is the polymer that enhances the nanocarriers' pharmacokinetics thanks to its ability to associate with water molecules around, which makes the system modified with PEG molecules invisible to plasma proteins [28]. Functionalization with PEG provides steric stability, protein-repelling surface, and longer half-life, ergo, it is a commonly used element in many drug delivery systems (DDSs), also for TMZ [27,29]. On the other hand, considering the PEG disadvantages like the presence of anti-PEG antibodies in some patients [30], Xu et al. [31] devised a drug delivery system based on micelles composed of poly (2-ethyl-2-oxazoline) (PEtOz) and TMZ conjugates (PEtOz-TMZ). In this approach, the stability of TMZ at physiological pH was improved, and the half-life increased from approximately 1.1 h for unmodified TMZ to nearly 14 h for the tested micelles. *In vivo* study demonstrated the extension of medical product circulation time in mice bodies. Moreover, the comparison of drug distribution in the main tissues (heart, liver, spleen, lung, kidney, the brain) for the three formulations: TMZ, and PEG system with TMZ and PEtOz-TMZ micelles indicated significant differences. The greatest concentration in the brain was revealed for PEtOz-TMZ formulation. Further, the content of alkylating agent in the tumour was much higher than in the surrounding healthy brain parenchyma. The obtained data suggest that the combination of TMZ and PEtOz not only passages through the BBB but also is able to accumulate in the GBL. Another promising polymer in the field of biomaterials dedicated to CNS is zwitterionic poly (2-methacryloyloxyethyl phosphorylcholine) (polyMPC). TMZ conjugated with polyMPC (polyMPC-TMZ) has gained greater stability against hydrolytic degradation as well as better solubility in the aqueous environment. Importantly, polyMPC-TMZ is a prodrug that can be successfully incorporated into other systems, e.g., by copolymerization [32,33]. One more example of polymeric-based TMZ nanocarriers are formulations composed of chitosan combined with carboxy-enriched polylactic acid. It was revealed that these amphiphilic nanoparticles successfully inhibit the hydrolysis of entrapped TMZ molecules under simulated physiological conditions. *In vitro* tests carried out on the mouse embryonic fibroblast continuous (ATCC CRL-1658™ NIH/3T3) cell line showed that the encapsulation of

TMZ in the developed carriers prolonged its cytotoxic effect [34].

Dendrimers have been also developed as nanostructures capable of reaching the CNS, however, their application is limited due to possible toxic effects [35]. Although, combining polyamidoamine (PAMAM) dendrimers with chitosan (Fig. 3**A**) proved that the obtained nano-transporters cause lower hemolytic toxicity than unmodified PAMAM structures (Fig. 3**C**). The tested systems showed prolonged release of TMZ molecules as well as better accumulation in the brain after intra-peritoneal injection, confirming effective transport through the BBB. Nevertheless, the concentration of the drug in other organs such as the heart, liver, and kidney was also higher in the case of carriers compared to the free TMZ dispersion (Fig. 3**B**) [36].

2.3.2. *Lipid-based systems*

Liposomes, the artificially constructed vesicles of phospholipid bi-layers, are willingly tested as nanocarriers of medicines delivered into the brain mainly due to their excellent biocompatibility, a structure similar to this present in the organism cell membranes, ability to biodegradation, low toxicity as well as simplicity of preparation. Lipo-somes are capable of encapsulating hydrophilic drugs in their inner aqueous compartments as well as hydrophobic compounds in the bilayer lipid membranes [37]. The main obstacle is their poor stability in physiological conditions, which may result in the premature/early leakage of a transported substance or vesicles' clearance into the liver or spleen. These limitations resulted from the rapid degradation, aggre-gation, fusion and significant opsonisation by serum proteins and changes in the physicochemical properties of carriers caused by them [38]. For successful liposomal formulations application in GBL therapy, two main strategies are used. The purpose of one is to enhance the stability of lipid structures and to escape the reticuloendothelial system (RES), while in another approach the vesicles are functionalized towards a better affinity to the diseased/targeted tissue.

Gabay et al. [39] proposed a liposomal drug delivery system uni-versal for 3 substances with anti-cancer properties, such as TMZ, doxorubicin (DOX), and curcumin (COX). The carriers with sizes 111–137 nm were made of cholesterol-ovine wool, L-α-phosphatidic acid sodium salt, L-α-phosphatidylcholine, and a short peptide, a five amino acid sequence (RERMS). This active domain for amyloid pre-cursor protein (APP) expressed on the BBB was used to enhance the penetration efficiency of the above-mentioned pharmaceuticals through the BBB *in vitro* model (Fig. 4**A**). Targeted systems containing 3 types of pharmaceuticals enabled to achieve of significantly higher survival rates (71% for CUR, 62% for TMZ, and 45% for DOX) compared to untreated control in the case of mice with injected U87 GBL cells (Fig. 4**B**). Similar results were obtained with the counterparts of the unmodified drugs.

In another work, formulation consisted of TMZ entrapped in the nearly neutral (−3.03 mV) PEGylated lipid vehicles size of 121 nm to examine their effectiveness during convection-enhanced delivery (CED) was employed. Although the differences during the treatment in both liposomal and free temozolomide formulations were not statistically relevant, the authors observed a significant decrease in oedema in rats after the application of TMZ in the nanocarriers (Fig. 4**C**) [40]. Such reports are especially valuable because the oedema present in the brain complicates the course of GBL therapy. An alternative approach to actively targeting liposomes is to design their properties in such a way that, when introduced into the body, they naturally acquire the features that will allow them to reach the CNS. Proteins present in the biological fluid and modifying the nanomaterial surface determine the interactions between cells and nanoparticles (the protein layer provides changes in the zeta potential of nanomaterials, their size and even shape). This process is called the formation of a protein/biomolecular corona and is specific to the nanoparticles' type. Several studies have shown that merely a change in the phospholipid bilayer composition of liposomes determines a different protein corona pattern. After *ex vivo* incubation with human plasma, on the surface of liposomes consisting of DOTAP adsorb mainly proteins responsible for the blood coagulation process, on DOPC liposomes immunoglobulins and lipoproteins, and on DOPG li-posomes mainly lipoproteins [41]. Arcella et al. synthesized the four various binary liposomal formulations for TMZ differing in lipids composition and investigated their affinity to specific biomolecules in the blood plasma [42]. They demonstrated that liposomes' surface charge is a crucial factor while 'biomolecular corona' formation (Fig. 5**A**). It was revealed that among the various plasma proteins, the apolipoproteins bind specific lipoprotein receptors (scavenger receptor class B, type I (SR-B) and low-density lipoprotein receptor (LDLR) that are present in brain microvascular endothelial cells found in the BBB. The selected system (CL2) composed of dioleoylphosphatidylethanol-amine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) with the known library of proteins present on the surface of the liposomal carrier after their incubation in human plasma (CL2-BC)) were tested using *in vitro* BBB model, i.e., human umbilical vein endo-thelial cells (HUVECs). Authors have shown that the cationic liposomes containing naturally bonded biomolecules exhibit better efficiency of TMZ action towards HUVECs (Fig. 5**B**) [42].

A slightly different point of view on the potential lipid-based struc-tures for brain delivery systems that demonstrated prolonged drug

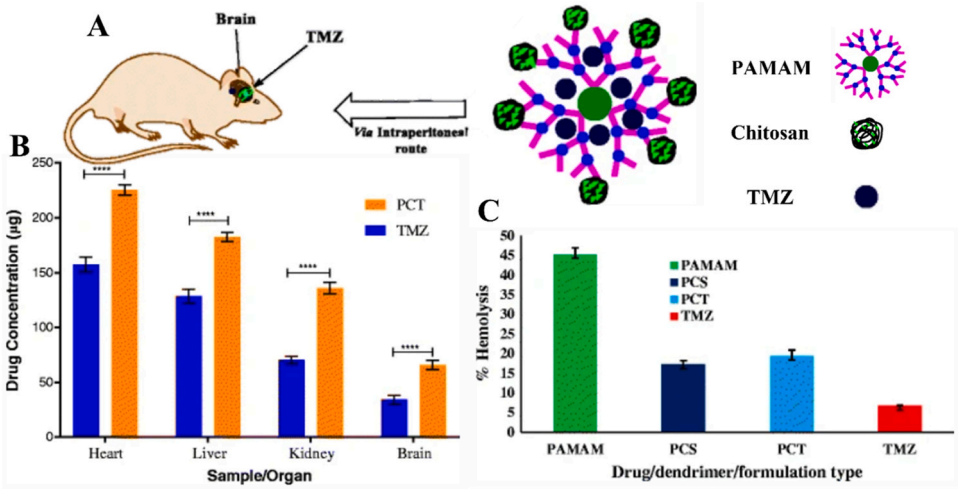

Fig. 3. (A) Schematically depicted delivery of TMZ via PAMAM-CS conjugate. (B) Data presenting distribution of tested formulation in various organs versus TMZ. (C) Results for red blood cells hemolysis study for PAMAM-Polyamidoamine, PCS- PAMAM-chitosan conjugate, PCT- PAMAM-chitosan conjugate loaded with TMZ [36].

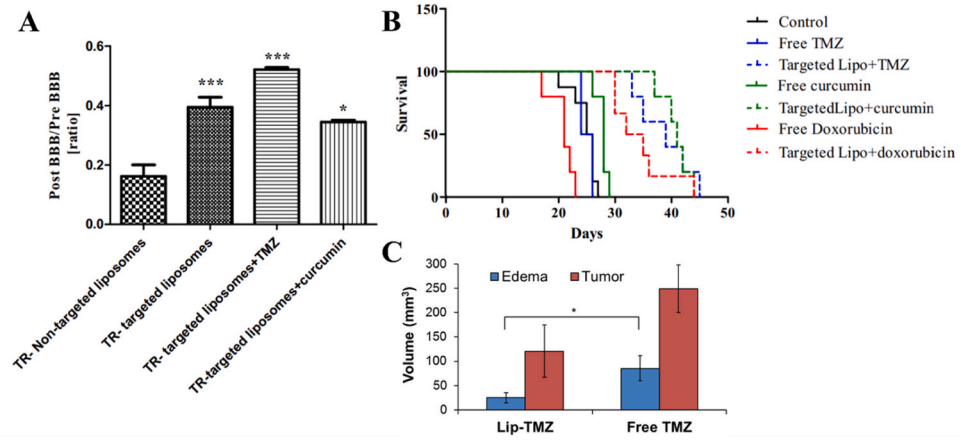

Fig. 4. (A) The ratio of fluorescence signals of Texas-Red post-BBB to pre-BBB. Fluorescently labelled liposomes were exposed to the BBB *in vitro* model for 24 h. Statistically significant differences were determined after the one-way analysis of variance test followed by Dunnett's test * **$p < 0.0001$, *$p < 0.05$ compared to non-targeted liposomes. (B) Results of survival rate studies for SCID mice model after various treatment schemes [39]. (C) Analysis of volumes of tumour and oedema for rats treated with TMZ-encapsulated in liposomes and free drug (1 mg/mL). Data obtained from MRI examination (contrast-enhanced T1-weighted) [40].

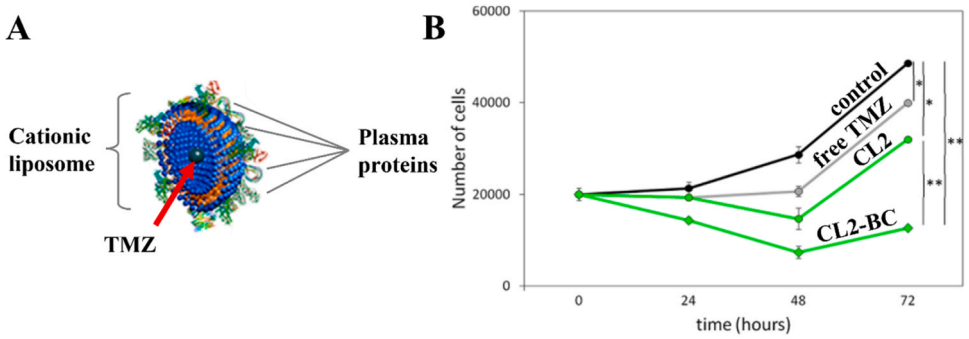

Fig. 5. (A) A biomolecular corona (BC) formed on TMZ-loaded cationic liposomes (CLs). (B) Improved efficacy of anticancer therapy obtained for surrounded by BC CLs with TMZ (CL2 −BC) compared to free TMZ and TMZ- loaded carriers [42].

release (approximately 12–20 h for different formulations compared to 6 h for free medicine) and better TMZ entrapment efficiency was presented by Waghule and co-workers [43]. In this paper, nanocarriers in the form of proliposomes and lipid crystal nanoparticles (LCNPs) to improve TMZ bioavailability and prolong its circulation time were proposed. Stability studies at physiological pH proved the formulations to inhibit drug degradation (68% in the case of proliposomes and 77% for LCNPs). Particles of the first type, proliposomes, are powders that form lipid vehicles after their introduction to an aqueous medium. These structures contain water-soluble solid carriers like, for instance, mannitol, coated with phospholipids. Their advantage over conventional liposomes is enhanced stability and persistent properties associated with way of storage, thus longer shelf-life. Moreover, proliposomes can be obtained during organic solvent-free synthesis [44]. On the other hand, the LCNPs form three-dimensional structures in aqueous conditions, which can hold hydrophilic and hydrophobic pharmaceuticals. Interestingly, these particles besides favourable features of conventional liposomes, are thermodynamically stable and show the potential of controlled release, multi-drug loading as well as improved drug entrapment efficiency [45]. Additionally, the authors pointed out that the employed preparation process of LCNPs could be scaled-up to industrial applications.

Carriers made of solid physiologically tolerated lipid components, namely solid lipid nanoparticles (SLNs) pose an interesting strategy among colloidal drug delivery systems. The SLNs feature themselves with qualities characteristic of liposomes or emulsions and polymeric nanoparticles. They can be described as biodegradable and biocompatible structures with low systemic toxicity and cytotoxicity, capable of sustained release of hydrophilic, as well as hydrophobic agents that can be sterilized and obtained in large-scale production. However, two of the most popular synthesis procedures of SLNs such as high-pressure homogenization and solvent evaporation method are energy-intensive processes. Carriers formed with solid at room and human body temperature lipids are able to improve crossing the more resistant barriers like the BBB and further enhance the drug bioavailability by changing its solubility [46,47]. Solid lipid matrix mainly composed of lecithin and stearic acid presented the prolonged TMZ *in vitro* release in comparison with free drug solution (85.2% of TMZ was released after 1 h for unmodified medicine while for the system with SLNs it was 17.6%) [48]. Moreover, *in vivo* tests carried out on rabbits demonstrated the ability of the system to cross the BBB and an enhanced accumulation in the brain. The formulation revealed low entrapment efficiency- nearly 1% and loading drug approximately 59%. Nevertheless, the TMZ distribution, although more favourable than for currently used TMZ intravenous therapy, was not significantly reduced in other tissues like the heart, liver, lungs, or spleen [48].

In order to verify which type of particles are the most promising in the aspect of TMZ delivery systems, Qu et al. [49] have synthesized three kinds of carriers loaded with temozolomide. Studied systems involved: PLGA polymeric nanoparticles (T-PNPs), solid lipid nanoparticles (T-SLNs) made of stearic acid and soya lectin, and nanostructured lipid carriers (T-NLCs) composed of both solid and liquid lipids. All of the developed formulations were characterized by particles with a size of approx. 100 nm, low polydispersity, and the high encapsulation efficiency of TMZ at the level of 80% in each system. T-SLN and T-NLC had a positive zeta potential of approx. 40 and 30 mV, respectively, while the value of this parameter in the case of T-PNP reached nearly - 30 mV. T-PNP and T-SLN formulations showed similar drug loading (~10%) whereas for T-NLC it was halved. Stability tests performed by incubation of the formulations in foetal bovine serum (FBS) demonstrated the 24-hour stability of all prepared systems. *In vivo*, antitumour efficiency studies carried out on BALB/c nude mice showed that each system can

inhibit the tumour growth when compared with control (by 85% for T-NLCs, 59% for T-SLNs, 45% for T-PNPs, and by 27% in the case of free TMZ solution (T-SOL)). A similar tendency was observed during *in vitro* cytotoxicity tests with U87MG cells. Importantly, all the tested formulations exhibited sustained TMZ release behaviour. The release profile emerged to be the slowest, the most favourable for T-PNPs, then for T-NLCs, and the fastest for T-SLNs.

2.3.3. Drug carriers for CNS – important parameters

While carrier designing, several different factors that affect the more effective BBB infiltration and further *in vivo* bioreactivity should be considered, namely: size, shape, lipophilicity, charge as well as flexibility.

The carrier size must be adjusted to achieve the best encapsulation efficiency and maximize the ability to cross the BBB. Completely small particles (a few nm) undergo renal clearance right after their intravenous administration and can be toxic to normal tissues. On the other hand, large structures, bigger than 200 nm, will not be able to enter the CNS [50]. The circulation time of nanocarriers is size-dependent - the long half-life of small particles in the bloodstream significantly raises the possibility of reaching the desirable site. The optimal size of the systems designed to deliver drugs into the brain is considered in the range from several dozen to less than 200 nm since such carriers can cross the BBB as well as have the chance to locate in the neoplastic tissue using the enhanced permeation and retention (EPR) effect [51]. The EPR phenomenon concerns objects (particles, macromolecules, carriers) of a certain size (several dozen to about 200 nm) that accumulate in tumour tissue because of the greater fenestrations existing between endothelial cells building vessels in diseased tissue [52].

Typically sizes of nanoplatforms designed to cross the BBB do not exceed 200 nm, however, in the case of lipid structures, there is a need for compromise between stability, drug encapsulation efficiency and biological performance. Smaller lipid structures, 50–200 nm, have been found to be more stable, showing prolonged circulation time and exhibiting increased cellular uptake [53]. Unfortunately, their loading capacity is rather limited, and they often undergo burst release. On the other hand, the bigger ones- above 300 nm provide higher drug encapsulation efficiency as well as a sustained release profile [54]. Although, these carriers tend to agglomerate due to a lack of enough physical stabilisation. In the literature, one can find plenty of lipid carriers' examples [55–57] in the diameter up to 300–400 nm that still can cross the BBB using distinct crossing paths and additionally, the ability to deformability [55]. Another parameter having an influence on the cellular uptake and biodistribution of the carriers in controlled drug delivery systems is their shape. Polystyrene (PS) nanoparticles in two shapes: rods and spheres modified with antibodies directed to TfR (transferrin receptors) expressed on the surface of the BBB endothelium showed that the rod-like system exhibited increased accumulation compared to the latter counterpart formulation (Fig. 6**B**). However, rod-like structures emerged specific assembling in lungs tissue too [41]. Comparable results were found for silica coated gold nanorods, containing rabies virus glycoprotein (RVG-PEG-AnNRs@SiO2), that enables bypassing the BBB and reaching the CNS via neuronal pathway due to interactions with the expressed on neuronal cells nicotinic acetylcholine receptor (AchR). It was revealed that the spherical counterparts (RVG-PEG-AnNPs@SiO2) with similar volumetric dimensions showed less effective cellular uptake than nanorods (Fig. 6**C**) [58].

More articles have confirmed the trend that non-spherical particles exhibit higher internalization rates when considering different cells. It was postulated that the effect may be related to particles' curvature. Structures with a reduced value of this parameter in comparison with spherical particles could display a greater likelihood of making bonds with receptors present on the surface of the endothelium (Fig. 6**A**) [60]. However, spherical particles are still the most extensively explored since formulations of other shapes are more difficult to synthesize.

It was revealed that lipophilicity is a key property when reaching the CNS. Together with the particle size, it determines the transport path of the system to achieve brain parenchyma. Small (<400 Da) and lipid-soluble structures can easily diffuse through the BBB endothelial cells. Although, the more lipophilic particle, the better transport, increasing this property may lead to molecule efflux by P-gp pump, MRP, or BCRP. On the other hand, it has been proved that higher lipophilicity escalates protein adsorption on the particle's surface, which leads to the formation of the 'biomolecular corona'. This effect can reverse the surface properties of nanoparticles and further limit prefigure of the system's behaviour *in vivo*. Literature provides two solutions to this situation, namely utilizing compounds like PEG to hide the nanoparticles from plasma proteins or, on the contrary- testing the interactions of such structures in simulated fluids to build a useful strategy to make particles naturally altered in the organism [61,62]. As it was mentioned, small hydrophilic compounds (for example glucose or amino acids) need protein transporters and the larger ones use specific receptors during RMT [19,63]. In addition, hydrophobic particles are marked by short half-lives (seconds to minutes) *in vivo* as they are rapidly recognized by the reticuloendothelial system and removed [61].

The carriers' surface charge is an important parameter affecting their ability to penetrate the BBB as well as influencing their circulation lifetime. The positively charged particles have a chance to infiltrate the

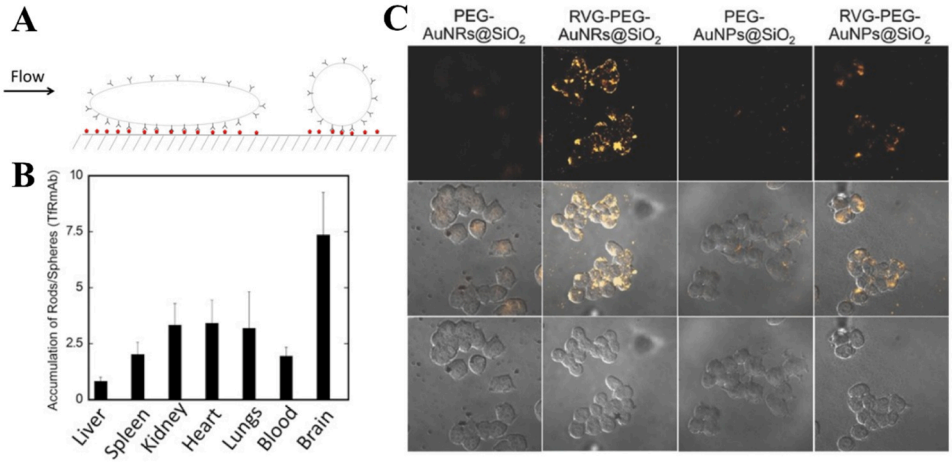

Fig. 6. Shape effect. (A) A diagram of particles in two shapes exhibiting different surface avidity under flow conditions [41]. (B) Accumulation of the tested structures in various organs presented as a ratio of TfR-mAb–coated rods to their counterparts in the shape of spheres) [59]. (C) Cellular uptake by N2a cells of structures like PEG-AuNRs@SiO2, RVG-PEG-AuNRs@SiO2, PEG-AuNPs@SiO2, and RVG-PEG-AuNPs@SiO2 (0.1×10^{-3} m) 4 h incubation [58].

CNS due to transcytosis through the polarized membranes of the blood vessel epithelium in the brain, however, a toxic effect may occur. In contrast, neutral or negatively charged platforms have a reduced affinity for plasma protein adsorption compared to cationic particles and can therefore circulate in the blood longer. Moreover, in the case of high concentrations of either positively, or negatively charged, the integrity of the BBB can be violated [64]. Nevertheless, utilizing cationic systems that demonstrate a higher accumulation and uptake in tumour angiogenic endothelium and sites of chronic inflammation in contrast to normal vasculature tissue seems to be just [65].

Nowak et al. noted that flexibility could affect the passage through the brain endothelium. They found the connection between carrier physical features and crucial steps in transit across the BBB, such as particle adhesion to the endothelial cells and their further uptake. It was revealed that the transport rate through the membrane is faster for stiff rather than for soft spheres as well as the association with endothelial cells is significantly lower for soft nanocarriers compared to their stiff counterparts [66]. Those results are in line with our previous findings associated with cellular uptake of silicone-stabilized liposomes, where we postulated that the endocytosis of rigid nanoparticles is more effective than for the soft ones [67].

2.4. Transport mechanisms through the BBB

2.4.1. Receptor-mediated transcytosis (RMT)

By adjusting the properties of therapeutic formulations, it is possible to devise strategies with the ability to regulate their entry into the brain via specific transport mechanisms. Natural pathway through the BBB via RMT was extensively applied in the past, most often in systems with targeting molecules directed to lactoferrin, transferrin, insulin, or LDL receptors. The mentioned specific receptors are highly expressed on the BBB endothelium membrane since they are responsible for large molecules' transit vital for the proper functioning of the brain. The interest of lactoferrin (Lf), a natural cationic glycoprotein belonging to the transferrin family, is validated by its ability to make bonds with both, transferrin receptors in the BBB, as well as Lf membrane internalization receptors (LfRs), present in the highly proliferating cells (in a tumour and on cerebral endothelium surface) [68]. Lactoferrin was utilized in many different systems for CNS drug delivery as a coating for silica [69] or solid lipid nanoparticles [70], magnetic nanocarriers [71], liposomes [72], dendrimers [73] and formulations composed of biodegradable polymers [74–76]. Sonali et al. [77] proposed TMZ-loaded nanoparticles made of lactoferrin (TMZ-LfNP). *In vitro* experiments confirmed the specific role of Lf in that formulation in the cellular uptake of a cell line, which exhibits high LfR expression. Thereby, a better accumulation of the drug was observed in cells while using the TMZ-LfNP system in comparison to the free pharmaceutic. Utilizing TMZ-LfNP favourable TMZ release kinetics was demonstrated. The concentration of drug delivered through LfNP rose gradually up to 4 h after application and remained at a constant level up to 24 h while the free TMZ concentration reached a peak after 1 h, decreased up to 5 h and then achieved approximately 4-fold lower values than in TMZ-LfNP at the same time. Transcytosis across the BBB was estimated based on the tissues' confocal images of mice treated with fluorescein-marked lactoferrin nanoparticles (FL-LfNPs). Fluorescence was detected mainly in sections of the brain and to a lesser extent in the liver as in both types of cell tissues the LfR is expressed. No signal in the case of lung, kidney, and spleen slides stated for lack of LfNP accumulation in those tissues, thus confirming the selective character of transport via the RMT mechanism into the brain.

Transferrin receptor (TfR) plays an important role in iron supply to cells, so the introduction of endogenous transferrin or antibodies directed against the TfR to the delivery system may enable its transport via RMT. Although, the use of Tf could be limited due to the high concentration of this protein in plasma [63]. The active targeting strategies for TfR have been realised by appropriate functionalisation of TMZ delivery systems like PLGA nanoparticles with the monoclonal antibody (OX26 type) [25] or PAMAM dendrimers conjugated with PEG and thiolated Tf [78].

The low-density lipoprotein receptors (LDLRs) family is also present on the membrane of brain vessels' endothelium. The role of LDLR is to bind LDLs, which carry cholesterol particles and transport them to cells through receptor-mediated endocytosis. In this way, the level of cholesterol in the blood is regulated and the material necessary for the synthesis of the cell membrane is provided [79]. The most commonly used molecules targeting these receptors are apolipoproteins (Apo), especially ApoA and ApoE [80]. Interestingly, the functionalization of nanoparticles with Apo is often coupled with their previous modification with polysorbate 80. In this strategy, nanoparticles are coated with the mentioned surfactant since its molecules can be associated covalently with apolipoproteins present in the bloodstream and further interact with LDLRs [81]. Applying the above approach to polybutylcyanoacrylate (PBCA) polymeric capsules to carry TMZ allowed for more effective brain targeting *in vivo* compared to the unmodified drug [82].

Another way to reduce the dose of TMZ and thus minimize side effects can be combination therapy. The synergistic activity of the two agents was observed in better survival outcomes during *in vivo* experiments employing therapy with both the peptide-drug conjugates (PDCs: M1-RGD-PTX), and TMZ [83]. The PDC was composed of paclitaxel (PTX), GBL targeting RGD motif (the tripeptide arginine-glycine-aspartate), and a peptide part (M1) responsible for endocytosis by a low-density lipoprotein receptor-related protein-1 (LRP1) receptors expressed both on the BBB as well as glioma cells. Furthermore, the novel peptide M1 (order of amino acids: TFYGGRPKRNNFLRGIR) selected after sequencing seems to have a wide range of applications in the treatment of CNS disorders as a universal BBB-penetrating vector. It was also reported that peptides from the Kunitz domain (the active protein domain responsible for proteases inhibition) such as Angiopeps exhibit better brain penetration ability in comparison to other molecules like large hydrophilic proteins: Tf, Lf, and LDL [84]. Angiopep-2 (An2) that poses a ligand to LRP1 enhanced the permeability for immunoliposomes with TMZ [85].

2.4.2. Adsorptive-mediated transcytosis (AMT)

The AMT mechanism was also employed as a transport route across the BBB for positively charged particles. To AMT-based formulations belong cationized proteins (albumin, immunoglobulin G (IgG)), basic oligopeptides (cell-penetrating peptides (CPPs)), as well as functionalized transporters, for instance, liposomes or nanoparticles [86]. The properly modified drugs or carriers that convey their molecules can induce electrostatic interactions with negatively charged domains on the brain capillary endothelium membrane. Important advantages of AMT over RMT or CMT pathways are the lack of such a strict size/stereochemical conformation limitation (both small molecules and larger peptides can be transported) and the better availability of sites used during AMT [87]. This strategy seems to be potentially useful for the delivery of pharmaceutics in high concentrations. Nevertheless, AMT does not belong to processes specific to the BBB, thus it may lead to undesired pharmaceutics accumulation in other tissues. Moreover, some reports revealed that positively charged formulations are toxic [64] as they force the endothelium membrane to intensive folding causing cell necrosis.

2.4.3. Carrier-mediated transcytosis (CMT)

The specific protein carriers expressed on the endothelial cells of brain blood vessels pose another way to overcome BBB. The key protein transporters in CMT include glucose transporter (GLUT), organic anion transporting polypeptides (OATPs), L-type amino acid transporters (LATs), and monocarboxylate transporters (MCTs). In nature, this mechanism is used to supply nutrients to CNS, though certain drugs can also succumb to CMT as long as they are sufficiently similar to the substrates of the expressed transporters [87].

Targeting GLUT transporters is one of the well-known drug delivery pathways via CMT. An increase of permeability was successfully achieved thanks to the formation of glycosyl conjugate of the antidepressant, 7-chlorokynurenic acid, which in the unmodified version showed no ability to cross the BBB [88]. Elsewhere, polymeric micelles formed by an association of PEG-based block ionomers with an opposite charge, modified with multiple glucose molecules presented the successful reaching of CNS [89]. The CMT route is size limited, thus for instance glycosylation of chlorambucil did not bring the expected outcomes because of the large size of the chemotherapeutic molecules [90]. In case of TMZ-based therapies against GBL, this type of transport is rather employed for tumour targeting and often to inhibit the above-mentioned transporters overexpressed in glioblastoma tissue. Nevertheless, combining pharmaceuticals molecules with substances that use a transporter to cross the BBB could be a reasonable strategy for DDS addressed to CNS.

However, designing a medium aimed at transport via CMT has some limitations. Since the therapeutic agent in the appropriate form for this mechanism competes with natural carrier substrates, thus it may lead to nutrient deficiencies. Additionally, the development of a biologically active, suitable system itself seems challenging because the CMT is sensitive to various physicochemical properties of ligands [87].

2.4.4. The BBB disruption

Disrupting the TJs of the brain's endothelium belongs to the less frequently used strategies to enhance the effectiveness of crossing the blood-brain barrier. The use of stimuli, whether physical or chemical, has some potential to loosen the BBB and thus increase its permeability. The aforementioned effect was achieved, inter alia, by employing osmotic shock, ultrasounds, or activation of appropriate receptors. It was found that the outcomes of TMZ chemotherapy were improved in *in vivo* studies in rats when the drug administration was preceded by acoustic cavitation, which resulted in higher BBB permeability [91]. Mannitol is the best-known agent for the BBB opening, however, when efficiently used intra- arterial, its high dosages caused the toxic effect related to acute dehydration and shrinking of endothelial cells, which eliminates clinical trials [92,93]. Nevertheless, studies carried out by Choi and co-workers have shown that the combined treatment of TMZ and mannitol leads to better penetration of the BBB than in the case of monotherapy with any of these drugs, which gives hope for decreasing the amount of the therapeutics thus reducing side effects [93]. When used together at doses appropriate for clinical trials, both compounds show suppression of TJs-forming proteins. *In vivo* tests confirmed the increased membrane permeability, and *in vitro* experiments with the use of fluorescent markers revealed that the presented therapeutic strategy could be employed when introducing larger molecules into the CNS. Overall there are several reasons why TJs permeability is rarely manipulated, the most important being: high invasiveness, risk of tumour spreading, increased probability of harmful substances penetration, and high costs [87,94].

3. The importance of the TMZ route of administration

The complete removal of the neoplastic tissue is often impossible due to the high risk of damage of the eloquent areas of the brain or extensive infiltration of the glioma. Therefore, the resection is followed by complementary therapy. In order to obtain positive results of GBL treatment, it is crucial to reach and maintain the concentration of the active form of medicine at the tumour site for a sufficiently long time. If cancer cells are sensitive to a chemotherapeutic agent, the efficacy of the used drug depends on its concentration and is reduced by the toxicity induced to non-target tissues [95]. The proper drug exposition is determined by chemotherapeutic distribution, metabolism, absorption as well as elimination [96]. The type of administration influences pharmacokinetics to some extent and thus the therapeutic effect. Therefore, it is crucial to choose the optimal route of drug administration (Fig. 7) and also take into account while drug delivery system designing [87].

3.1. The oral administration

Currently, TMZ is the most commonly administered orally, sold under the brand name Temodar or Temodal [97]. Oral intake of TMZ is easy, quick, and convenient, which results directly from the drug in the form of capsules. Nevertheless, the multiplicity of biological processes

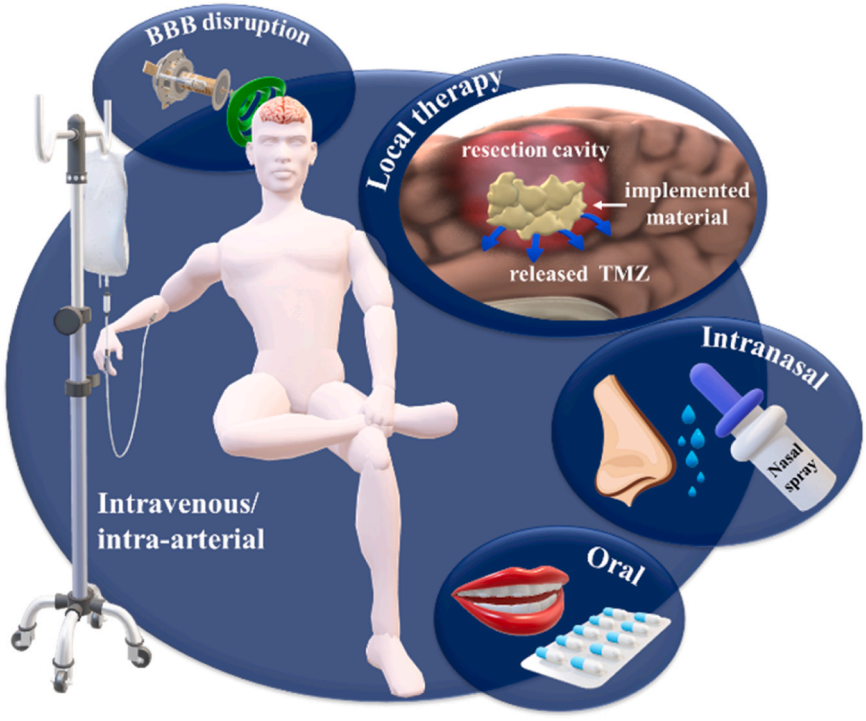

Fig. 7. The schematic routes of drug administration in GBL therapy.

involved may account for the variable pharmaceutical absorption and individual pre-systemic metabolism in the gut and liver for the different bioavailability [98]. In order to achieve a therapeutic effect in the brain a significant drug's amount is necessary since 25–35% of TMZ reaches the CNS after an orally-administered daily dosage of this agent (150 mg/m^2) [99]. Unfortunately, it is associated with a greater risk of side effects of therapy. On the other hand, a low chemotherapeutic concentration at the target site may affect drug tolerance and reduce its therapeutic effect [100]. Besides, there is a need to reformulate temozolomide tablets as a change in their colour is observed during storage, which is also indicative of the degradation of the prodrug, reducing its efficacy [97]. Unfortunately, taking tablets is a problem for a certain group of people who have conditions significantly affecting the absorption of TMZ. For instance, patients may suffer from impaired gastrointestinal functioning, obstruction, persistent nausea, or oesophageal dysfunction [101].

3.2. The intravenous / intra-arterial dosing

Another approved method of TMZ administration is the intravenous (IV) dosing of prodrug preparations. In pharmacokinetics analyses including 19 subjects with primary central nervous system malignancies, it was demonstrated that exposure to a 90-minute intravenous infusion is equivalent to equal oral TMZ dosage [101]. The greatest advantage of IV infusion is the ability to control plasma levels of the drug to fit individual patient's needs. Moreover, the duration of infusion may be maintained, interrupted, or terminated when necessary [102]. Due to other factors to which the neurotherapeutic agent is exposed after intravenous administration, in order to achieve the best therapeutic effect, it should show resistance to enzymatic degradation and phagocytosis, solubility in the bloodstream and the ability to overcome the barriers between systemic circulation and the CNS [87]. A large part of the developed systems for TMZ (also implied in this study) include modifications aimed at increasing the effectiveness of chemotherapy by improving the stability of the preparation in physiological conditions, exceeding the BBB, and even targeting the neoplastic tissue.

The requirements for intra-arterial (IA) and intravenous (IV) delivery formulations are similar in terms of the environment in which they are introduced into the body. The relevant difference between those two approaches is the higher initial pharmaceutical concentration in the CNS after IA administration. To improve the effectiveness of such a strategy, it is used in conjunction with agents that increase BBB permeability [96,103]. Experiments in nude rats showed that the median survival of the animals with the chemosensitive rat brain tumour model was 25.5 days, 25.5 days, and 33 days for oral, intravenous, and intra-arterial administration respectively, while the control result was 17.5 days. Furthermore, TMZ applied alone or with the BBB disruption exhibited enhanced medicine delivery in the brain around the tumour. Unfortunately, there is a concern that the IA route may lead to neurotoxicity and is not completely safe [103].

3.3. Intranasal administration

Intranasal (IN) administration is an alternative route of drug delivery to the central nervous system. The nose-to-brain route has attracted considerable attention since it is a noninvasive and simple approach characterized by high bioavailability and the short onset of action that can avoid the blood-brain barrier (BBB) together with systemic side effects. It was reported that active agents can be delivered through the nasal cavity via the trigeminal and olfactory nerves and their permeation depends on both the features of drugs and carrier. In this approach, the nasal cavity's large surface for absorption (160 cm^2) and rich vascular submucosa can facilitate pharmaceutics assimilation in a convenient way for a patient - in the form of a commonly used nasal spray. Nevertheless, the formulation needs to be specially designed to cope with issues such as nasal epithelium low pH, mucociliary clearance, or degradation by nasal peptidases and proteases. The fast absorption may lead to an increase in drug concentration in the brain (within minutes) and accelerates its onset of action. Moreover, the IN type of application reduces the systemic blood circulation time significantly and avoids first-pass elimination in the liver [94,104]. However, many factors are limiting the permeation of drug/drug carriers via this route and the crucial ones are low metabolic stability, the short residence time in the mucous layer and the high rate of mucociliary clearance [105]. Compounds that may be termed "penetration enhancers" include surfactants, phospholipids as well as cationic polymers [105]. Effective methods of extending the retention time in the nasal cavity are increasing the viscosity of the preparation or using gel forms of drugs. The lipophilic nature, biocompatibility, biodegradability, and relatively high stability along with the small size and tuneable encapsulation efficiency make the nanostructured lipid carriers a popular nasal drug delivery system. The results obtained by Khan et al. [106] for lipid nanoparticles confirmed their ability to enter the brain after intranasal administration. During the optimization of the system, relationships between the concentration of solid lipid, the ratio of liquid lipid to total lipid, the concentration of surfactant, and the time of sonication were determined. It has been observed that increasing the content of liquid lipids leads to greater efficiency of temozolomide encapsulation. Likewise, surfactant concentration also affects EE and drug release. *In vitro* tests revealed more favourable release kinetics of TMZ trapped in nanostructured lipid carriers (TMZ-NLCs) compared to free drug dispersion (TMZ-disp.) (Fig. 8**A**).

As part of an *in vivo* study in Wistar rats, three approaches were tested (Fig. 8**B**). One group of animals was dosed intranasally with an optimized lipid nanoparticles formulation (TMZ-NLC-opt), in the second group TMZ-disp. Was administered in the same way, and TMZ-disp. Was injected via the tail vein in the third group. The estimated ratio of the drug concentration in the brain to the plasma revealed that the IN administration ensures the effective transport of nanoparticles to the brain and allows for better results compared to the unmodified drug.

It was reported that incorporating chitosan as a gelling agent in the lipid delivery system of temozolomide by the intranasal route is a promising approach. The higher formulation viscosity, prolonged drug release, and improved uptake efficiency in the brain were demonstrated. Chitosan is a biocompatible, biodegradable polymer that exhibits mucoadhesive properties which prolong the contact time of the formulation with the nasal mucosa. Moreover, the interaction of the positively charged amino groups of the biopolymer with the negatively charged sialic acid residues of the cell membranes enables the modulation of tight junctions, which in this case leads to their temporary opening and thus increases the drug penetration capacity [107]. Histopathological examinations carried out on a porcine nasal mucosa model showed no irritating effect [108].

Effective methods of extending the retention time in the nasal cavity are increasing the viscosity of the materials or using gel-like forms of drugs. Various biocompatible systems for the intranasal delivery of temozolomide were nanoemulsion and nanoemulsion gelling in situ. It was disclosed that the thermosensitive formulations enriched with poloxamer derivatives introduced into the body change their physical properties and undergo the gelling process. The obtained drug carrier provides a prolonged duration in the application site, which allows for the longer release of active substance molecules from nanodroplets what was evidenced *in vivo*. The results of gamma scintigraphy of radiolabelled TMZ present in both nanoemulsions showed high drug accumulation in the brains of Wistar rats after nasal administration [109].

3.4. The local delivery

Other strategies, such as intracerebroventricular, intrathecal or intraparenchymal, injections/infusions provide drug transport straight to the brain, overcoming the BBB. As almost all types of administration have to overcome many obstacles in the body before they reach the CNS,

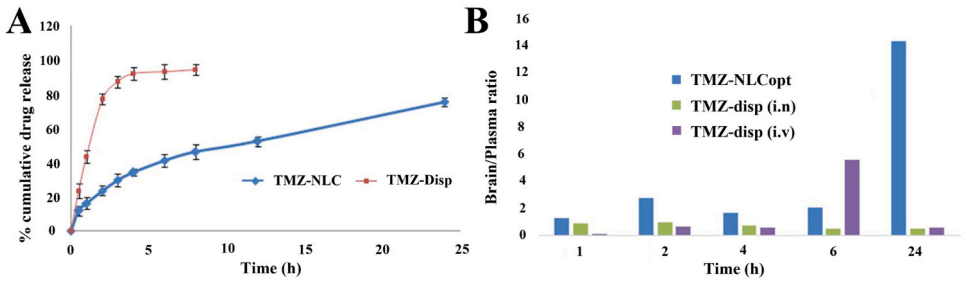

Fig. 8. (A) TMZ release profile from TMZ-NLC-opt as well as TMZ-disp. (B) *In vivo* study of the drug distribution presented as brain/plasma ratio after administration of TMZ-NLCopt (i.n.), TMZ-disp (i.n.), or TMZ-disp (i.v.) [106].

the direct introduction of the pharmaceuticals into the parenchyma of the brain appears to be a reasonable approach, especially because of the fact that in the 80–90% of cases, the recurrence occurs approximately 2 cm from the resection site [110]. However, the mentioned methods are invasive as opposed to oral, intravenous or IN routes. There is a concern that local therapy is more likely to develop oedema, seizures, or healing disorders [110]. Nevertheless, these types of administration should allow for the direct contact of the preparation with diseased tissues as well as the application of a lower dose of the drug, which reduces the problem of systemic toxicity. Unfortunately, injection of fluid or infusion into small ventricles causes an increase in intracranial pressure and, as a consequence, may lead to haemorrhage, neurotoxicity, or infection [111]. It should be also emphasized that there is a risk of obstruction, clots or mechanical failure when administering drugs for topical therapy using common catheters or, less frequently, pumps [110, 112]. Several studies have found the implementation/injection of drug delivery devices just after resection to be exploitable as an element of complementary therapy in the GBL postsurgical treatment. The application of the sustained release system directly to the site of excised tissue enables the continuous delivery of the active substance to the surrounding tissues in order to destroy any remaining diseased cells. The limitations of this approach include the necessary large operating cavity and the inability to refill it. Additionally, after the insertion of the implant, the kinetics of drug release cannot be changed [113].

The only clinically approved to date implantable therapeutic formulation for GBL therapy is Gliadel®, a material based on biodegradable polymer wafers of poly[bis(p-carboxyphenoxy propane) sebacic acid] and loaded with the anticancer drug, carmustine (BCNU) [95]. The active substance of Gliadel BCNU (3.8% *w*/w) is mixed with the polyanhydride polymer in a dichloromethane solution where after solvent evaporation, the formed powder is compressed into wafers. Gliadel® has been shown to increase overall survival in patients with recurrent GBL as well as in patients with newly diagnosed GBL [114]. The success of Gliadel® mostly results from the unique features of this route of delivery: systemic effects are minimized, while local drug delivery is increased. The release time, based on *in vitro* tests, is up to 21 days, with most of the therapeutic being released after 3–4 days, and the whole system degrades within 6–8 weeks [115,116]. Though, it was reported that Gliadel® suffers from the "sink effect" – the drug is being released from wafers and washed away into systemic circulation due to excessive diffusion of the drug.

Promising results of adjuvant therapy were obtained in *in vivo* studies using a system releasing BCNU and TMZ simultaneously, from the brain-biocompatible PLGA wafers. The median lifetime of animals implanted with the 9 L gliosarcoma treated with a wafer containing BCNU or TMZ or after oral administration of TMZ was 15, 19, and 18 days, respectively, while in the case of the binary wafer grafting it was 28 days. It is worth mentioning that 25% of the animals from the group treated with the last system lived longer than 120 days. In the tested material, to extend the release of TMZ over time, the drug was twice-coated with a layer of polymer (PLGA) before it was placed in the wafer made from the same compound. This strategy was found to be crucial as it provides slower TMZ release when compared to uncoated TMZ placed directly into the PLGA wafer [117]. It is considered that the slower release in the initial days of the experiment can be attributed to the presence of hydrophobic lactic acid in the copolymer, whereby water adsorption on the wafers was limited. In order to verify whether there are differences in the kinetics of TMZ release from various polymeric systems, a comparative analysis was performed between wafers made of PCL-LA, PSA, or PLGA. The first two materials released 90% of the entrapped drug within 1 day, while PLGA was able to gradually lose TMZ molecules over 35 days. Moreover, the authors suggest that PSA may not have been the best choice for this type of material (wafer) as its compression was not as convenient compared to PLGA and PSA [117].

It is believed that materials useful in implantable therapy are biocompatible systems from two groups- biodegradable, that release the active agent while their decomposition or non-biodegradable, the matrix of which remains intact after the drug has been expended [110], however, most of the research examples concern degradable structures. Hydrogels show great potential in biomedical applications as, in addition to protecting the entrapped drug against premature degradation or adverse environmental factors, their properties can be adjusted in a wide range. Systems based on copolymers seem particularly easily tuneable because by changing the molar ratios of the used compounds, adjusting molecules of different hydrophilicity and chain length, one can manipulate biocompatibility, degradation rate, mechanical properties, or release kinetics [118].

High potential in terms of implantable materials releasing TMZ into the brain was demonstrated by a photopolymerizable hydrogel based on polyethylene glycol dimethacrylate (PEG-DMA) (Fig. 9**A**). The proposed drug-free system did not induce significant cell apoptosis in the brain parenchyma, neither after implantation nor after photopolymerization, which indicates its good tolerability in brain tissue. *In vivo* cytotoxicity tests performed in mice showed a better antitumor efficacy of the hydrogel material (TMZ hydrogel) compared to the intravenously administered therapeutic agent (TMZ IV). The results indicate that the tested system led to a decrease in tumour mass and even to its complete disappearance in two animals (Fig. 9**B**). Interestingly, cells that underwent apoptosis after application of the TMZ hydrogel were in the central part of the tumour (green colour in Fig. 9**C**). The authors speculate that this is due to the heterogeneity of GBL and its heterogeneous vascularity. Another instance of a hydrogel system for local administration was presented by Sayiner et al. [119]. They found that biodegradable material in the form of temozolomide-loaded PLGA nanoparticles placed in a thermo-reversible gel (Pluronic® F-127) ensured more favourable drug release kinetics. It was reported that TMZ-loaded nanoparticles exhibited burst release within 4 h and then reached a plateau for 72 h while 93% of TMZ entrapped in bare hydrogel matrix was released after 8 h and the rest for 3 days. Whereas the tested system composed of hydrogel loaded PLGA nanoparticles with TMZ released 10% of the drug for 12 h from the application, reaching the plateau in 60 days.

It was noted that the kinetics of drug release from biodegradable polymer systems depends, among others, on the rate of their degradation and the nature of erosion- bulk or surface. The latter type is more

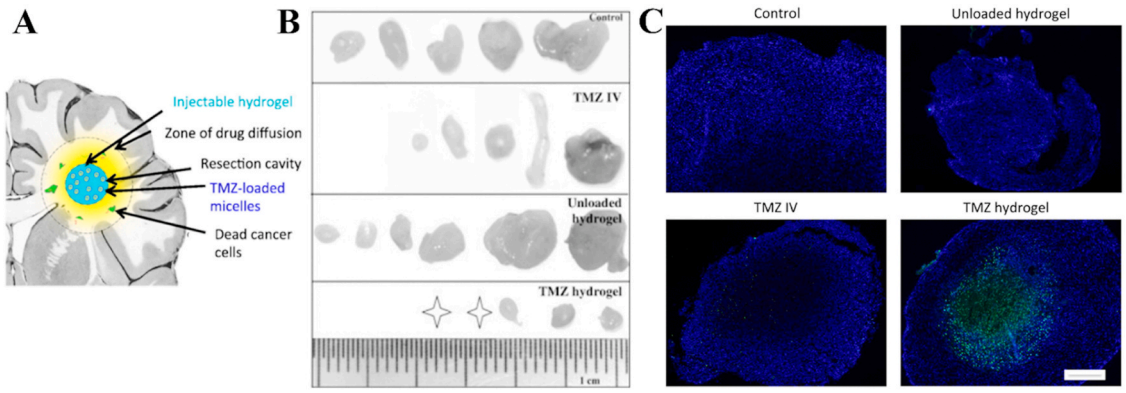

Fig. 9. (A) Concept of local treatment based on hydrogel containing TMZ loaded into the polymeric micelles. (B) Images of tumours taken after 1 week after implantation (n = 5–7). Stars indicate complete tumour regression. (C) U87MG cell apoptosis as a result of M-TMZ/PEG-DMA hydrogel. Tumours were removed 7 days after treatment and immunohistochemistry was performed. The tumour section was treated with TUNEL (green). To label the nuclei, DAPI staining (blue) was used. Scale bar = 1 mm [92].

predictable and therefore more appropriate in DDS [110]. To control the course of degradation of bioresorbable PLGA, optimization of the structure of copolymer fibres of this polymer was carried out using the electrospinning method. The systems with the addition of either ε-caprolactone (ε-Cap), influencing the degradation time and mechanical properties, or trimethylene carbonate (TMC), increasing the share of surface material degradation were analysed.

Two series of nanowovens (A and B) with two co-polymer types (PLACap and PLAGATMC) were formed (Fig. 10**A**) and high encapsulation efficiency was obtained during the synthesis of both kinds of bioresorbable mats (nearly 90% for all systems). TMZ has been evenly distributed within the PLACap or PLAGATMC fibre in series A, whereas in series B, the fibres of series A act as an interior of the core-shell fibres that were covered with the additional layer of the same polymer. *In vitro* release studies (Fig. 10**B** and **C**), regarding materials from the A series demonstrated sustained release of small TMZ amounts (11% and 21% after 15 weeks for PLACap and PLAGATMC, respectively). On the other hand, in the case of coaxial fibres (series B), the drug release profile can be divided into 3 stages: slow diffusion of drug molecules, accelerated loosing and saturation. The latter series appears to be promising during therapy against glioblastoma since the patients after resection should maintain a two to four-week gap to have a chance for wound healing [120,121].

4. GBL therapeutic approaches

Targeted delivery to cancer cells can be achieved through active or passive transport by focusing on specific tumour features like low extracellular pH of its microenvironment, unique characteristics of blood vessels, as well as abnormalities at the cellular level such as altered overexpression, faulty apoptotic mechanisms, or changed molecular targets. To drive a system to the desired site, the formulation of any type (chemical, physical, or biological) must be properly functionalized, with or without any carriers applied [57].

4.1. *Targeting glioblastoma cells*

The concept of target therapy was introduced by dr. Paul Ehrlich and the specifically designed agents, intended only for the desired site were called “magic bullets” [57]. The selective character of this type of drug delivery is accomplished by choosing the receptors that are overexpressed in the case of diseased cells in contrast to the normal ones. Although TMZ itself has the ability to cross the BBB, its distribution within the brain is not favourable, as TMZ does not exhibit tumour-targeted capabilities. Most often, certain modifications with the targeting ligands ensure binding to a specific receptor on the membrane of the targeted cell, facilitating endocytosis.

The clever idea of enriching the composition of liposomes containing TMZ and decorated with ApoE, a target ligand with an affinity for LDL receptors, increases the likelihood of both crossing the BBB as well as

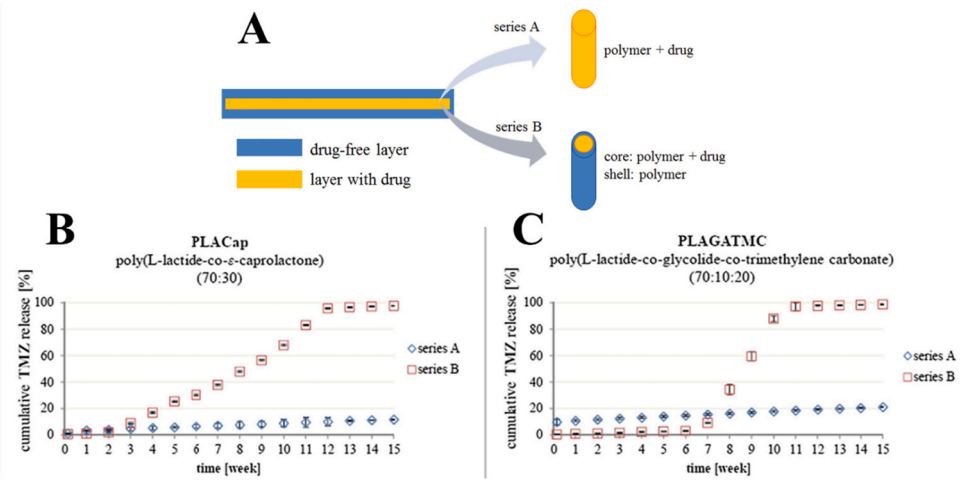

Fig. 10. (A) Scheme of layer nonwovens along with two types of fibres – A and B. Release profile of TMZ from PLACap (B) and PLAGATMC (C) [120].

tumour accumulation [80]. Liu et al., presented a similar approach, in which elevated expression of the glucose transporter 1 (GLUT1) has been utilized twice (in BBB and GBL) to enhance the GBL cellular uptake of formulation co-delivering of TMZ along with reprogramming drug resistance RNA fragment, siPD-L1 [122].

The ephrin type-A receptor 3 (EphA3) was used by Wang et al. [123] in the role of the GBL-targeted site as its overexpression has been noted in the case of tumour-initiating cells [124], where it is associated with neoplastic cells proliferation and tumour angiogenesis [125]. The developed intranasal formulation was based on the temozolomide-conjugated gold nanoparticles that were functionalized with antibodies against the EphA3 receptor, anti-EphA3 (Anti--EphA3-TMZ@GNPs). This type of carriers was selected due to its unique features such as low toxicity, biocompatibility, ease of modification and size control [126]. The tumour-targeted carriers exhibited improved cellular uptake along with increased toxicity toward the GBL model *in vivo*. The median survival of rats bearing glioma treated with Anti-EphA3-TMZ@GNPs achieved the highest value of 42 days

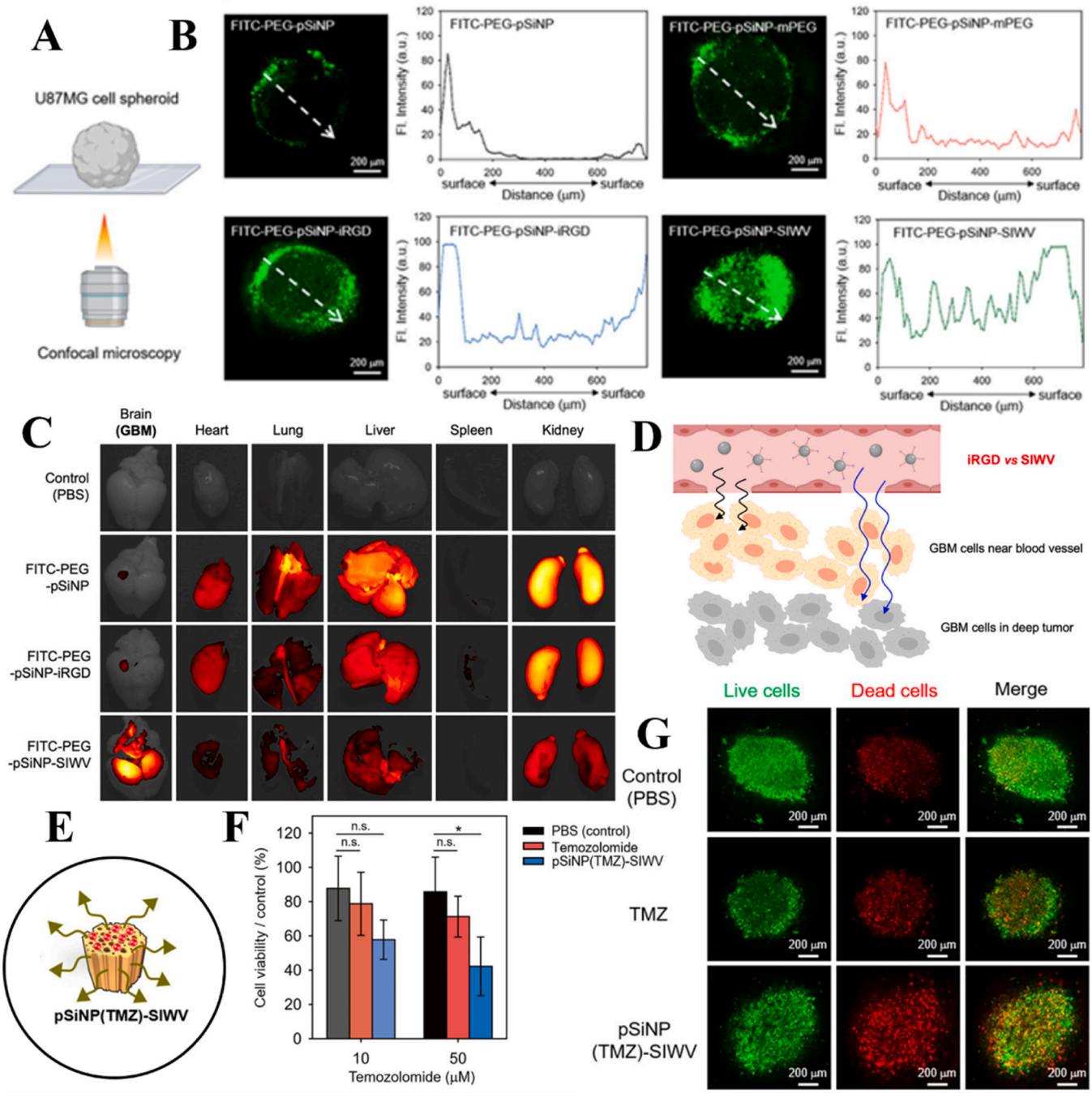

Fig. 11. Results obtained for the series of materials, presenting the role of proper carriers' functionalization in their further tumour-deep penetration [133]. (A) Scheme of fluorescence method used for examination of the penetration ability of tested systems in the case of the 3D bioprinted with U87MG cells model, (B) stacked fluorescence images (FTIC channel) of U87MG spheroid after treatment with different types of carriers (100 μg/mL) (48 h, 37^{O}C), (C) biodistribution of nano-formulations in some organs of GBM mice obtained after 3 h of carriers' circulation, (D) the main aim of the article- evaluation of deep penetration efficiency of a tumour by specifically targeted platforms (with iRGD vs. SIWV peptide), (E) illustration of TMZ-loaded porous silicon structures functionalized with SIWV peptide, (F) cell viability results for the free drug as well as pSiNP(TMZ)-SIWV for U87MG spheroids, (G) staining live (with calcein-AM) and dead (with ethidium homodium homodimer-1) cells on the 3D spheroid model after treatment with TMZ (50 μM) and pSiNP(TMZ)-SIWV (with applied TMZ concentration of 50 μM).

compared to 17, 20 and 30 days for three other groups, in which the saline, temozolomide, or temozolomide attached to gold nanoparticles were applied, respectively [123].

Chlorotoxin (CTX), a scorpion toxin, poses another ligand that has targets in GBL cells, such as up-regulated matrix metalloproteinase-2 (MMP-2)[127], ClC-3 chloride ion channels [128], as well as some different proteins [129]. Since it is proved the CTX is able to specifically binding to glioma [129], this targeting molecule has been used as a part of nanoformulation composed of particles made of chitosan-TMZ core and biotin-neutravidin- Alexa Fluor 647-CTX shell [130]. The DNA-alkylating agent entrapped in those carriers exhibited a longer half-life (13.4 h) compared to free TMZ (1.8 h) at physiological pH. *In vitro* experiments performed on three cell lines (U-118 MG, SF767, and GBM6) showed that IC_{50} has been significantly reduced for carriers functionalised with CTX (86.5, 66.0, 119.8 for three cell lines, respectively) in contrast to non-targeted counterparts (273.9, 771.4, 222,0) or TMZ itself (302.3, 789.1, 686.1), which directly proves the better therapeutic efficiency of the CTX-enriched formulation. Another group demonstrated chlorotoxin-decorated structures in the form of an M13 bacteriophage, showing selective accumulation in glioblastoma tumours *in vivo,* which may play a role in both gliomas imaging and as carriers in anti-GBL therapy [131]. The advantage of carriers, in the form of resistant to many solvents and a wide range of temperatures, the M13 bacteriophages, that are the filamentous protein nanoplatforms, is their naturally uniform size, but above all the ease of fusion of peptide sequences, leading to the desired functionalization of the capsid surface [132].

A further challenging aspect when it comes to effective antiglioblastoma therapy is the limited capability of delivery systems to tumour-deep penetration. Kang et al. [133] prepared several types of carriers based on porous silicon nanoparticles (pSiNPs) incorporating polyethylene glycol (PEG) in order to examine their tumour penetration abilities (Fig. 11**D**). The authors functionalized the pSiNPs with either mPEG, iRGD peptide (cyclic peptide made of nine amino acids with RGD motif), or SIWV (Ser-Ile-Trp-Val) tetra-peptide, forming FITC-PEG-pSiNP-mPEG, FITC-PEG-pSiNP-iRGD and FITC-PEG-pSiNP-SIWV, respectively. In the first formulation, PEGylation has been proposed to prolong blood circulation time and drive carriers to the tumour via the EPR effect, next, the iRGD, is a targeting peptide that binds to neuropilin-1 (NRP-1) receptors overexpressed in the case of various tumours and the latter, SIWV, exhibits GBL specificity during caveolin/lipid raft-dependent endocytosis[134,135]. The synthesized platforms contained FTIC (fluorescein isothiocyanate) as a fluorescence agent necessary to track the location of carriers in the tested models. Penetration efficiency was evaluated using 3D bioprinted with spheroid U87MG cells (Fig. 11**A**). The analysis showed a higher fluorescence intensity in the case of targeted formulations, especially for FITC-PEG-pSiNP-SIWV, suggesting the deepest penetration (Fig. 11**B**). Next, the results of *in vivo* particles' biodistribution experiments in the GBM xenograft mouse model demonstrated the most favourable brain accumulation after treatment with the formulation containing the SIWV peptide (Fig. 11**C**). Therefore, the system functionalised with SIWV was selected for further use as TMZ carriers, pSiNP(TMZ)-SIWV (Fig. 11 **E**). Higher toxicity was observed for specifically designed formulation in comparison with free TMZ in the 3D model (Fig. 11**F** and **G**), probably due to enhanced deep penetration of GBM by pSiNP(TMZ)-SIWV.

Albumin appears to be a promising choice for carriers material because, in addition to passive delivery via the EPR effect, this protein has a high affinity for glioma-specific plasma membrane glycoproteins (such as gp60) [136]. Moreover, overexpression of the albumin-binding SPARC receptors (secreted protein, acidic and rich in cysteine) may promote cellular uptake and subsequent accumulation of albumin particles in the tumour interstitium [137]. In two cell lines, namely glioblastoma multiforme cells (GL261) and glioblastoma stem cells (BL6), albumin nanoparticles loaded with temozolomide acid (TMZA) showed cytotoxicity similar to that of the free drug. Both cell types express SPARC; however, BL6 exhibits higher up-regulation. These findings are consistent with an increase in albumin carrier cellular uptake after 24 h of incubation for the BL6 cell line [138].

Exosomes, being an example of biological carriers, natural phospholipid structures, which exhibit a better ability to the BBB penetration than artificial capsules, were used in co-therapy of TMZ and dihydrotanshinone (DHT), as a drug resistance reversing agent. Vesicles isolated from the glioma-261 (GL261) cell line were subjected to ultrasound to remove their contents, including genetic material. Due to the transmembrane proteins (CD9, CD63, and CD81) left on the surface of the carriers, the exosomes had a high ability to target the tumour, which was proven *in vivo*. Caveolin-mediated endocytosis and pinocytosis are believed to be the two main pathways for the uptake of the exosomes presented[139]. Among other target sites for TMZ delivery formulations that would target glioblastoma lactoferrin receptors [77,140] and transferrin receptors [141] have also been used.

4.2. Genetic mechanism-specific strategies

Depending on the origin, the glioblastoma can be a primary (*de novo* developed) or secondary (arose through the progression of a lower-grade glioma) type of tumour. Both clinical presentations have different molecular correlations. In the case of, more common, primary GBL (pGBL), amplification/overexpression of the Epidermal Growth Factor Receptor (EGFR), as well as mouse double minute 2 (MDM2) protein is often observed. Furthermore, pGBL can also be characterised by the deletion of two antioncogenic genes p16 (responsible for cell proliferation controlled by inhibition of the kinases CDK4 and CDK6) and PTEN (implied in the signalling trail, regulating cell division), as well as loss of heterozygosity on chromosome 10 [142,143]. Secondary glioblastomas (sGBL) are frequently associated with mutations in TP53, the tumour suppressor gene, isocitrate dehydrogenase 1 (IDH1) or less commonly mutation in IDH2 [3]. Determination of IDH status, which is a therapeutic biomarker, may indicate the effects of treatment directed against it.

The synergistic action of TMZ and the brain-penetrating antidepressant drug, fluoxetine was disclosed towards GBL with EGFR amplification [144], being recognised in 40–50% of GBLs [145,146]. EGFRvIII, the mutated form of the EGFR is more oncogenic than its unchanged version. The EGFRvIII activity affects the cells' viability, mobility, proliferation, their invasiveness as well as resistance [143].

The authors identified sphingomyelin phosphodiesterase 1 (SMPD1) as a target in GBL. The SMPD1 is responsible for the regulation of sphingomyelin to ceramide (Cer) conversion [147]. Inhibition of SMPD1 enzymatic activity using fluoxetine led to gathering sphingomyelin, reducing the number of EGFR receptors present on the lipid rafts domains and cancerous cells' surface. *In vivo* experiments in the GBM39 orthotopic model (intracranial GBL xenograft models with EGFRvII with nude mice) demonstrated that applying the fluoxetine at a clinically approved dose along with TMZ can significantly enhance the cytotoxic effect (Fig. 12**A**), prolong survival (Fig. 12**B**) and suppresses the GBL recurrence. Animals after combination therapy with 5 mg/kg TMZ had more than doubled survival when compared to the monotherapy, and in 20 mg/kg TMZ co-therapy with fluoxetine, six of eight animals did not show any recurrence after 5 months.

Among other ligands applied to obtain the TMZ delivery system directed to EGFR was found: panitumumab in the formulation composed of PLGA nanoparticles [148], 2-((4-(3-chloro-4-fluorophenyl)amino)−7-(3-morpholinopropoxy)quinazoline-6-yl)oxy) acetic acid (CFMQ) exposed in chitosan coated PLGA capsules [149], bispecific monoclonal antibodies in bacterially derived nanocells containing microRNA (responsible for changing the activity of signalling pathways in GBL cells used with TMZ during combination therapy) [150] or cetuximab in the case of polymeric carriers [151]. Furthermore, the reactivation of p53 in GBL, that is, the suppressor protein, which can stop tumour growth appears to be an intriguing strategy to improve TMZ action. Kim et al. [152] used this approach in studies on a system of cationic liposomes that selectively targeted glioblastoma in a mouse model due to functionalisation with a single-chain antibody fragment that targets highly

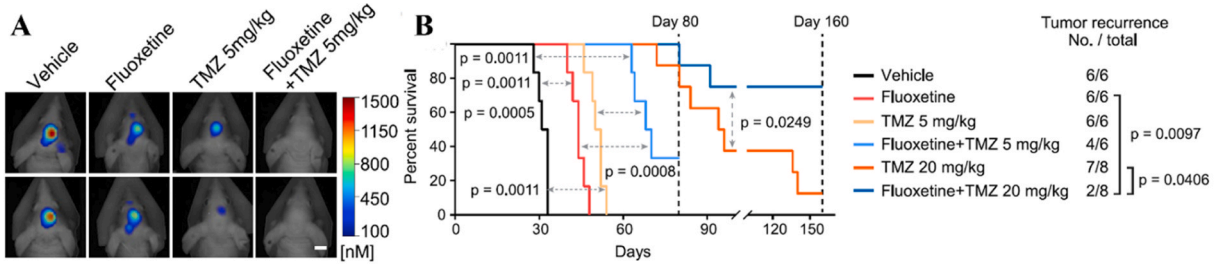

Fig. 12. The synergistic effect of TMZ and fluoxetine was proved during *in vivo* experiments [144]. (A) Images of tumour after 5 weeks of treatment. (B) Survival analysis of nude mice bearing a GBM39 orthotopic xenograft.

expressed transferrin receptors in tumour cells. In another work the therapeutic effectiveness of TMZ was improved for the brain implant, aiming at the simultaneous release of TMZ and an inhibitor for Nek1 (NIMA-related kinase 1). The formulation in the form of electrospun microfibers with polyvinyl alcohol (PVA) containing an inhibitor, in which stearic acid-based particles loaded with TMZ were embedded, was tested *in vitro* and *in vivo*. The results confirm that Nek1 inhibition has the potential to become a promising oncotarget during co-delivery with TMZ, as it promotes cancer cell growth and chemoresistance in GBL [153].

4.3. *TMZ-resistance fighting*

Unfortunately, anti-GBL therapy with TMZ is often associated with the development of resistance to the drug (some resistance degree is present in 60–75% of patients). Therefore the planned outcomes of the chemotherapy are not achieved and the GBL recurrence occurs [154]. High heterogeneity is a major obstacle in the fight against GBL at the cellular and molecular levels [143]. Targeting only one signalling pathway generally proves ineffective, since the proliferation of glioblastoma cells is generated by a range of mechanisms that are simultaneously affected [5]. It is important to keep in mind that resistance to TMZ can emerge from a variety of ways, the most documented of which is MGMT overexpression. Further, due to improper signal transduction brought on by mutations in the genes encoding the intracellular phosphatidylinositide 3-kinase/ protein kinase B/ mammalian target of rapamycin, (PI3K/AKT/mTOR) pathway kinases, which control cell differentiation, survival, and proliferation, there is an increase in GBL resistance [155]. In some patients with GBL, the MET gene has hyperexpression, which is associated with glioblastoma recurrence during tumour cell invasion and migration [156]. Less commonly, mutations in the TERT promoter, BRAF, or fusion of genes expressing NTRK that activate the oncogenic TRK route are seen [157,158].

To address the abovementioned issue certain approaches, embracing co-delivery of some inhibitors or more specific functionalisation have been proposed to sensitise GBL to TMZ. Overexpression of O6-methylguanine-DNA-methyltransferase (MGMT) in tumour cells poses the primary cause of resistance to TMZ. DNA methylation at the O^6 position of guanine generated by TMZ can be removed by the MGMT protein, invalidating the drug's action. The methylation status of the MGMT promoter is one of the most important prognostic factors for GBL treatment. Patients with methylated MGMT promoter exhibited a better response to treatment with alkylating agents such as TMZ [159].

Carriers in the form of exosomes were used by Liang and co-workers [160] for the combined delivery of TMZ along with O^6 -benzylguanine (BG) to improve anti-glioma therapy. Since the TMZ action can be reversed by the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), also known as MGMT, making tumour cells resistant to the alkylating agent, BG was used as the AGT inhibitor. In order to enhance tumour-targeting capability (Fig. 13**A**), the exosomes were properly functionalised with a specific ligand for highly expressed in the case of both the BBB and GBL cells receptor LRP-1, angiopep-2 (An2). Furthermore, considering CD133, a transmembrane glycoprotein, which is overexpressed in glioblastoma stem cells (GSCs), the surface of the exosomes was enriched with the CD133 RNA aptamer (Apt). Dual-targeted structures loaded with TMZ, and BG (EXO-An2-Apt-TMZ, BG) (Fig. 13**B**) inhibited the proliferation of U87MG as well as GSCs. *In vivo* biodistribution tests of the fluorescently labelled exosomes (Fig. 13**C**) performed using the U87MG-bearing nude mouse model showed significant differences between the location of the structures modified with two targeting ligands (EXO-An2-Apt) compared to bare exosomes (EXO) or those with An2 only (EXO-An2). The former ones, EXO-An2-Apt, exhibited nearly two times higher fluorescence signal in the brain, indicating facilitated BBB permeation and further, more favourable accumulation at the desired site [160].

The research group whose work on gold nanoparticles functionalised with antibodies for the EphA3 receptor was referred to in one of the above sections used their formulation in synergistic chemophotothermal therapy (GNPs-PPTT) (Fig. 14**A**) [161]. The authors point out that structures capable of exhibiting a localised plasmonic effect, such as gold nanoparticles with a size of about 40–50 nm, after irradiation with a laser-emitting (near-infrared) NIR, can convert the absorbed radiation into heat more effectively than dye particles absorbing in this range. Injected into the tail vein, the gold nanoparticles-based formulation targeted at GBL cells thanks to EphA3 antibodies (anti-EpHA3) and containing TMZ after irradiation with the NIR laser (anti--EphA3-TMZ@GNPs+Laser) allowed a significant reduction in tumour volume in mice implanted with TMZ resistant glioblastoma cells, T98G compared to control groups - with no irradiation (anti--EphA3-TMZ@GNPs), TMZ alone and after no treatment (Saline) (Fig. 14**B**). In addition, *in vitro* tests on the T98G cell line showed that GNP-PPTT can lead to a reduction in drug resistance associated with overexpression of MGMT by up-regulating p53 activation, which probably inhibits the expression of the MGMT promoter (Fig. 14**C**).

In addition to TMZ chemotherapy, radiation is one of the several treatment plans for GBL. The efficiency of the latter may be diminished in the tumour microenvironment, where oxygen levels are lower than in normal brain tissue as a result of the high oxygen consumption by the tumour cells' rapid growth. The radiation resistance is nearly three times higher in hypoxic zones [162]. Xie and co-workers [163] developed the nanoplatform to improve chemo-radiotherapy. The authors created a formulation composed of several elements including TMZ, metronidazole (Met) as radio sensitizer and siMGMT (small interfering O6-methylguanine-DNA-methyltransferase RNA) responsible for down-regulation of MGMT encapsulated in polymer-modified liposomes (Fig. 15**A**). It was confirmed *in vitro* using TMZ-resistant U87MG cell line that the system caused enhanced the downregulation (65.4%) of MGMT under hypoxia and 40.9% applying normoxic conditions in contrast with levels for PBS and free siMGMT groups. The developed formulation allowed the synergistic enhancement of therapeutic outcomes during *in vivo* experiments of the simultaneous TMZ chemotherapy and radiation (RDPP(Met)/TMZ/siMGMT +RT) when compared to other groups with no MGMT silencing part (RDPP(Met)/TMZ/NCsiRNA+RT), free TMZ and radiation (TMZ+RT), carriers without TMZ nor siMGMT (RDPP

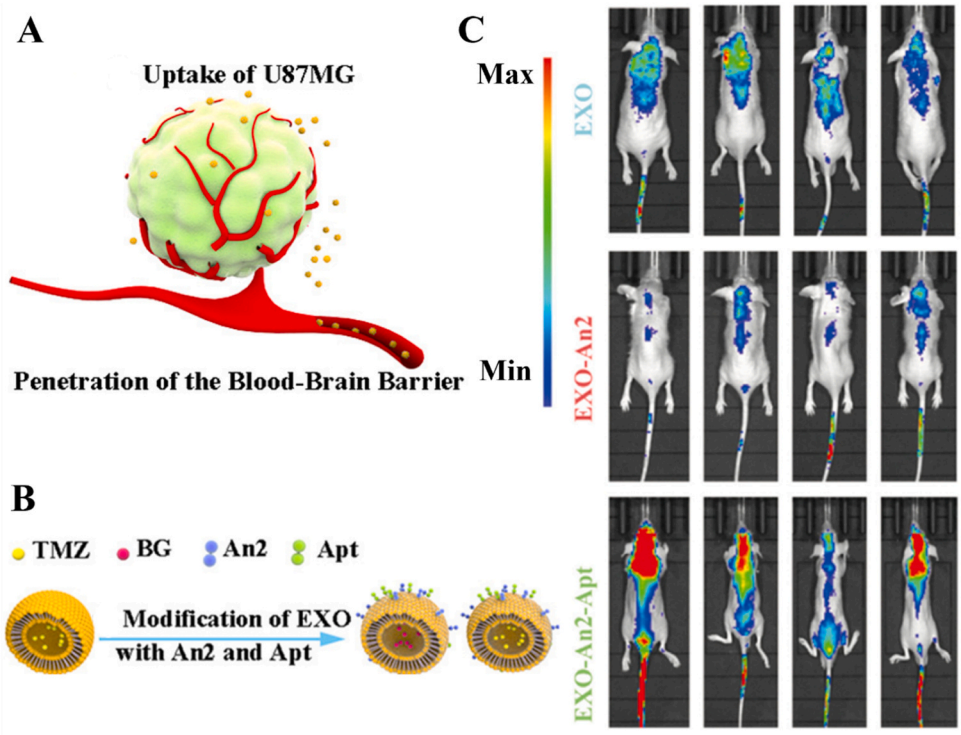

Fig. 13. (A) The action of the formulation, (B) Procedure of exosome functionalization, (C *In vitro* viability test for U87MG cells after various treatment schemes. (D) Results for U87MG cell viability when incubated with free TMZ, free TMZ, and free BG (30 μM), Exo-An2-Apt-TMZ, BG (30 μM) at different TMZ concentrations (0, 50, 100, 150, and 200 μM). (E) The distribution of DiD-labelled crafted exosomes *in vivo*. The exosomes were DiD-labeled before being injected into the mice model via the tail vein [160].

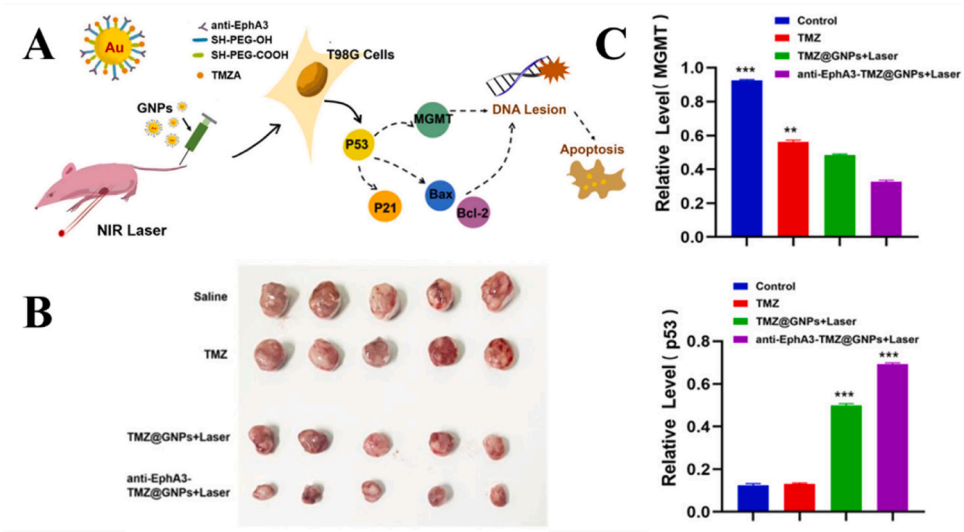

Fig. 14. (A) Scheme of GBL-targeted gold nanoparticles for synergistic chemophotothermal therapy (B) Images of tumour volume obtained from mouse model with implemented TMZ-resistant T98G cells after one-week treatment. (C) Results for MGMT and p53 levels in T98G cells after 72 h of treatment with different preparations [161].

(Met) +RT), radiation alone (RT) or PBS as a control. (Fig. 15**B**). This method achieved simultaneous radiotherapy sensitization, as well as, inhibited proliferation of GBL tumour cells, resulting in prolonged median survival in the U87MG tumour-bearing mice model.

Along with the TMZ chemotherapy, the enhanced activation of Akt, a serine/threonine kinase in the Akt pathway has been observed [164, 165]. The higher levels of the Akt promote a malignant phenotype, the uncontrolled growth of neoplastic tissue, and the avoidance of cancerous cells' apoptosis, facilitating tumour invasion [166,167]. Apoptosis induced by TMZ becomes attenuated by Akt [164] thus, the kinase activity appears to be an attractive target increasing the effectiveness of TMZ therapy [167]. It was reported that Akt is phosphorylated by the inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKBKE). Xiong et al. [168] apply amlexanox, the drug proved to pose an inhibitor to IKBKE, for developing the combined treatment with TMZ. The authors showed that amlexanox enhanced the GBL sensitivity to TMZ *in vivo* as well as *in vitro* using two cell lines- U87 MG and primary GBL cells.

It was found that the macrophage migration inhibitory factor (MIF) exhibits up-regulation in TMZ-resistant cells. This cytokine can be released from the exosomes secreted from cells with higher malignancy, inducing resistance in recipient cells. MIF appears to promote tumour proliferation via the PI3K/AKT signalling pathway; however, IOS-1 as a MIF inhibitor can reverse the cytokine action *in vivo*, exposing the

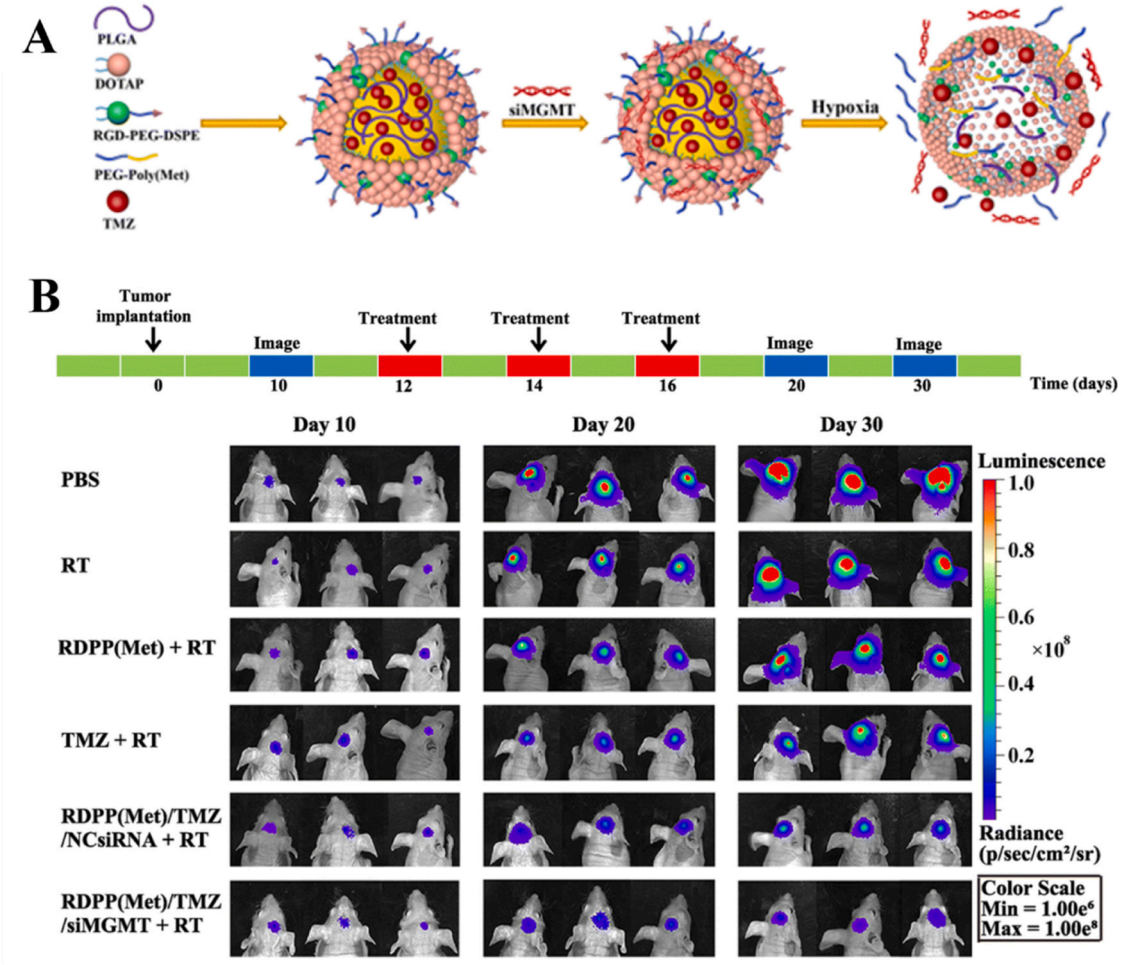

Fig. 15. (A) Illustration of the RDPP(Met)/TMZ/siMGMT formulation. (B) Changes in bioluminescent signal from U87MG-Luci tumour-bearing mice models at different time points – 10, 20 and 30 days from animals in different treatment groups [163].

tumour to chemotherapy, which makes the MIF the promising molecular target of TMZ-resistant therapy [169]. Elsewhere, after co-treatment with tubeimoside-I, two TMZ-resistant glioma cell lines, T98G and U118 MG, responded better to TMZ treatment. In this case, both MGMT expression and the PI3K/Akt/mTOR/NF-B pathway were inhibited at the same time[170]. Among other compounds, suppression of resistance to TMZ associated with the PI3K/Akt kinases pathway salvianolic acid A [171], cobalt chloride [172], GLS2 glutaminase [173], or the PI3K inhibitors such as XH30 [174] PX-866 (wortmannin analogue) [175], were discovered recently.

Furthermore, tumour tissues that are not removed during resection include glioblastoma stem cells (GSCs), which are resistant to chemoradiation and therefore support tumour regeneration and, as a result, are the main cause of recurrence [176,177]. Analysis of samples taken from glioma patients showed high levels of O-acetyl-GD2 ganglioside (OAcGD2) in GSCs and was subsequently identified as a target for the monoclonal antibody 8B6 immunotherapy by Fleurence et al. [178]. The use of the 8B6 antibody led to the sensitisation of glioblastoma cells to TMZ, noted as an improvement in therapeutic effects in the group where both agents were administered compared to monotherapy with either TMZ or 8B6. A scanning electron microscopy observation of cancer cells revealed that the enhanced effect of the methylating agent may be correlated with the increased permeability caused by the formation of pores in tumour cells after the application of 8B6. It is worth recalling that antibody molecules cannot cross the blood-brain barrier, so appropriate functionalisation or administration that bypasses BBB seems to be a necessity.

Adrenomedullin (ADM) expression was unearthed to be up-regulated in TMZ-resistant cell lines (also for GSCs.). ADM stimulates the proliferation of GBL cells in a malignant environment and has the potential to affect the regulation of the PI3K/Akt pathway and Bcl-2, preventing tumour cells from apoptosis. MicroRNAs (miRNAs) were used therapeutically to suppress ADM. It was hypothesized that miR-1297 will bind with the target mRNA and control the desired gene expression. Dual therapy with TMZ and miR-1297 resulted in a knockdown of ADM. Furthermore, *in vivo* studies using a mouse model showed that miR-1297 combined with TMZ can sensitise glioma cells, reducing tumour volume and mass [179]. It should be underlined that multidrug resistance (MDR), which can be intrinsic or acquired, leads to a high level of chemoresistance in GBL and subsequent therapy failure [180]. The MDR mechanisms operate at various stages of therapy, starting from preventing TMZ from entering GBL cells, and increasing the efflux of cytostatic molecules that have already managed to do so, by activating detoxification pathways, as well as inhibiting apoptosis, to triggering DNA repair processes [177]. This is a critical issue, however, beyond the scope of the current review.

5. Conclusions

Glioblastoma is a biologically heterogeneous and highly complex neoplasia that represents a major challenge for neurooncology. Considering the poor survival with currently approved treatments, new therapeutic options for GBL are of great importance. A significant challenge in treating GBL is to overcome the BBB' restrictions that make

it difficult for drugs to enter the CNS and reach a sufficient effective concentration. The circumvention of the BBB through straight intervention into insubstantial brain tissues can cause severe neurotoxicity and loss of brain key functionality. As a result, there is a need to design a more specific and non-invasive approach to target GBL. As it was discussed different strategies for drug delivery to the brain are employed, including chemical modification of drugs, their encapsulation in various types of DDS as well as alternative ways of administration. It is believed that the prolonged TMZ half-life time in the bloodstream would improve the passage of its molecules through the BBB, which would favour the greater accumulation in the brain, and thus increasing the chances of reaching the GBL cells. Therefore, the TMZ reformulation, using carriers or functionalization towards the more effective BBB crossing or employing various routes of TMZ administration that could increase its concentration in the brain while reducing the adverse systemic complications seem to be good therapeutic targets worthy of researchers' consideration and efforts. Moreover, the multiple resistance mechanisms, the molecular heterogeneity and evolution of tumour tissues as well as the complexity of their microenvironment represent the limitations that must be undoubtedly taken into account while designing the systems for GBL treatment. Hence, the combined therapeutic strategies, the targeted therapies and purposely selected delivery methods, glioma stem cell inhibition, as well as the use of approaches that modulate BBB permeability are prospective goals of research and development directions for GBL treatment. Finally, as discussed above, single-target therapy very often induces recurrence and then resistance to the original treatment. To address this issue the detection of biomarkers throughout the management of glioma patients and the multi-antigen targeting implementation represent the promising approach for precise and personalized GBL therapy.

CRediT authorship contribution statement

Aleksandra Krajcer: Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Ewelina Grzywna**: Conceptualization, Writing – review & editing, Supervision. **Joanna Lewandowska-Łańcucka**: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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