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Cancer Drug Resistance

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Temozolomide exposure and delivery barriers in glioma: cross-compartment pharmacokinetics and implications for resistance

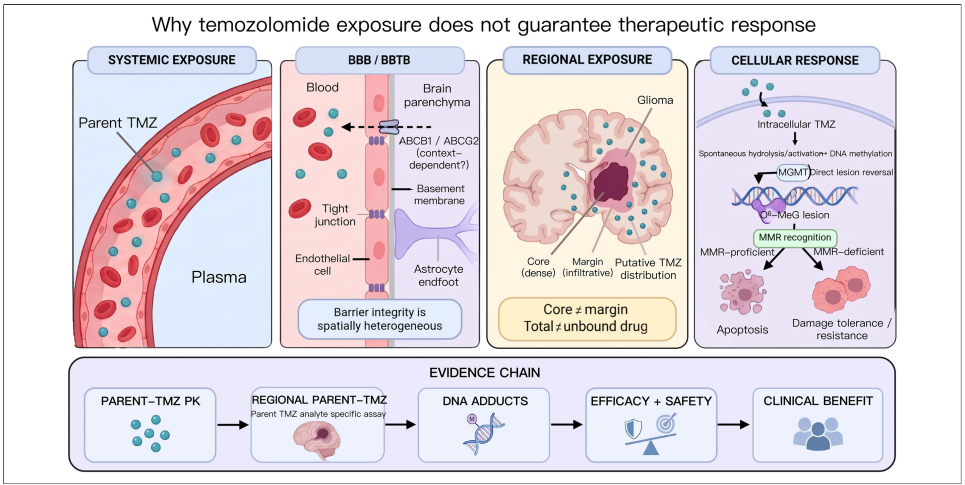
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Abstract

Temozolomide (TMZ) remains central to glioblastoma therapy, yet treatment failure reflects both pharmacodynamic resistance and incomplete exposure across relevant intracranial compartments. In this evidence-audited narrative review, we trace TMZ from the systemic circulation across the blood–brain and blood–brain tumor barriers to regional distribution, cellular uptake or export, intracellular retention, chemical activation, and DNA damage. Combined adenosine triphosphate (ATP)-binding cassette subfamily B member 1 (ABCB1)/ATP-binding cassette subfamily G member 2 (ABCG2) deficiency increases brain penetration in mice without changing systemic exposure, supporting a contribution from endothelial transport; however, evidence that individual tumor-cell transporters directly export TMZ is heterogeneous and includes negative findings. Transporter-mediated influx and vesicular partitioning further indicate that inadequate uptake or intracellular removal can be mistaken for classical efflux. Human cerebrospinal fluid and brain-interstitial studies demonstrate partial, compartment-dependent penetration, whereas focused-ultrasound

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studies support the feasibility of barrier opening. Survival outcomes from single-arm studies cannot be attributed to ultrasound without a randomized comparison. A focused full-text audit of 15 TMZ-containing delivery-platform studies identified extensive formulation and efficacy reporting but only three studies with direct analyte-specific measurements targeting parent TMZ in biological samples. These measurements were limited to plasma or whole blood and single-time-point total-tissue extracts of whole brain or brain tumor; none established regional unbound parent-TMZ pharmacokinetics. We propose a claim–evidence framework that separates direct transport and pharmacokinetic evidence from functional sensitization, association, delivery-concept, and negative evidence. Linking parent-drug exposure, regional distribution, pharmacodynamic competence, safety, and clinical feasibility can help determine when inadequate exposure is a modifiable component of glioma resistance.

INTRODUCTION

Glioblastoma treatment remains constrained by infiltrative growth, molecular heterogeneity, recurrence, and incomplete delivery of therapeutically active drugs to relevant intracranial compartments. Temozolomide (TMZ) resistance is often discussed in relation to O^6-methylguanine-DNA methyltransferase (MGMT), mismatch repair, glioma stem-like states, and adaptive signaling. Although these mechanisms are important, they do not, by themselves, establish whether an effective amount of TMZ reaches invasive tumor cells or whether a combination partner achieves complementary exposure in the same region.

A recurring problem is the use of "efflux" as an umbrella term for mechanistically different observations. Increased transporter expression, reduced fluorescent-probe retention, altered cholesterol export, improved nanoparticle uptake, and prolonged survival in a xenograft are not interchangeable demonstrations that a specific transporter directly exports TMZ. The distinction matters because an intervention can improve TMZ sensitivity through cell-cycle regulation, membrane remodeling, ferroptosis, or cellular uptake without increasing TMZ efflux or changing intracranial TMZ concentrations.

This narrative review focuses on exposure- and delivery-related contributors to TMZ treatment failure rather than providing an exhaustive account of all resistance mechanisms. Accordingly, the synthesis follows the pharmaceutical disposition pathway from circulation to tumor-cell DNA instead of grouping studies solely by transporter family or nanomaterial class. The organizing questions are as follows: Which anatomical barrier is involved? Which chemical entity was measured? Was transport demonstrated directly? Were systemic and regional brain exposures distinguished? Does the intervention alter a surrogate endpoint, parent-drug exposure, pharmacodynamic competence, or an authentic clinical endpoint? This analyte- and compartment-resolved approach distinguishes the present synthesis from broad reviews of DNA-repair pathways or nanocarrier classes. Figure 1 summarizes the claim–evidence classes used to interpret findings across compartments.

LITERATURE IDENTIFICATION AND EVIDENCE-AUDIT METHODS

Review design and scope

This work was designed as an evidence-audited narrative review with a structured literature-identification and evidence-assessment workflow. It was not prospectively registered and should not be interpreted as a formal systematic review or meta-analysis. The scope addressed four linked questions: whether TMZ itself undergoes measurable transport; where the relevant anatomical or cellular barrier is located; whether an intervention changes regional or intracellular exposure; and whether the evidence has progressed from experimental models to human pharmacokinetics (PK) or clinical feasibility. Studies of DNA repair, stemness, metabolism, ferroptosis, autophagy, and microenvironmental signaling were included when they helped distinguish altered drug response from altered drug delivery[1-4].

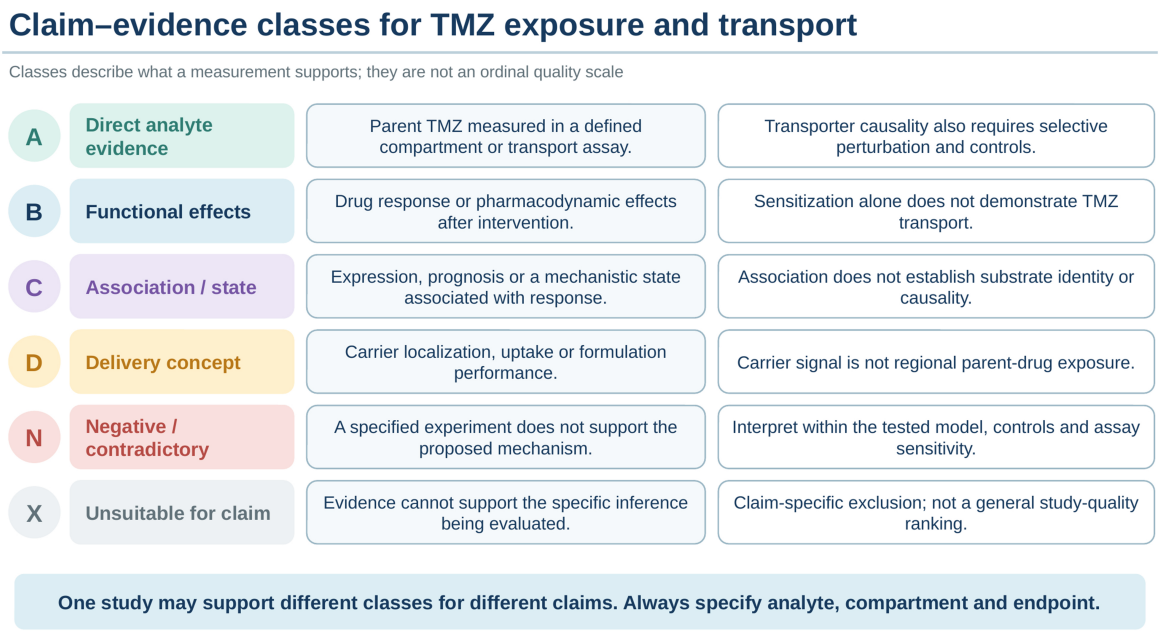


Figure 1. Claim–evidence classes for TMZ exposure and transport. Classes A, B, C, D, N and X describe the relationship between a specified claim and the available measurements, not an ordinal ranking of study quality. Direct parent-TMZ measurement does not by itself establish transporter causality. A study may support multiple classes for different claims. This figure was created by the authors using Python/python-pptx and LibreOffice. No AI-based image-generation tools or third-party graphical elements were used. TMZ: Temozolomide.

Search concepts, library consolidation, and full-text retrieval

PubMed- and Web of Science-derived records were consolidated in the prespecified Zotero collection "Literature Library for the Review of TMZ Efflux and Delivery in Glioma" and audited through 25 August 2026. Search concepts combined glioma or glioblastoma with TMZ and terms related to efflux, transporters, adenosine triphosphate (ATP)-binding cassette (ABC) proteins, blood–brain or blood–tumor barriers, PK, extracellular vesicles, nanoparticles, liposomes, intranasal administration, local delivery, and focused ultrasound. The searches were intentionally broad because identical terminology can describe different substrates. For example, cholesterol efflux, fluorescent-probe efflux, and TMZ efflux were retained for later mechanistic adjudication rather than excluded at the keyword stage.

The prespecified classification mapping contained 500 records, whereas the live Zotero collection snapshot contained 513 items. Of the mapped records, 475 matched uniquely to Zotero entries. Thirty-five valid PDFs were already present, leaving 440 uniquely matched records without a local PDF. DOI and open-access metadata were checked using OpenAlex, Europe PMC, publisher pages, and institutional repositories. Among the 440 initially missing records, 433 had a DOI and 246 were marked as open access. Automated retrieval produced 124 complete PDFs after seven truncated or structurally invalid downloads were excluded using PDF-header, terminal-EOF, and parser checks. This process yielded 159 library-linked full texts, of which 157 were text-extractable. Targeted recovery and lawful retrieval from authoritative web or publisher sources subsequently expanded the evidence-access set to 168 records. Because these routes included records that overlapped with earlier workflow states, the counts are not additive to the library-linked total.

Eligibility, evidence selection, and classification

Records were prioritized when they addressed at least one prespecified compartment: systemic-to-cerebrospinal fluid (CSF) or systemic-to-brain PK; blood–brain barrier (BBB)/blood–brain tumor barrier (BBTB) transport; tumor-cell influx or efflux; vesicular or intracellular drug redistribution;

mechanism-informed delivery; or clinical translation. Corrigenda were linked to their parent studies rather than treated as independent evidence. Studies of another drug were retained only when they demonstrated combination-partner exposure mismatch or validated a barrier model; they were not used to infer TMZ substrate status. Two structurally invalid PDFs were excluded from full-text inference.

Claims were classified according to the measured analyte, anatomical compartment, intervention specificity, and endpoint. This claim–evidence framework evaluates what each study can support; it is neither a validated risk-of-bias instrument nor a clinical certainty-of-evidence system. Class A denotes direct measurement targeting unlabeled parent TMZ in a defined biological compartment; transporter-specific claims additionally require causal perturbation and appropriate controls. Class B denotes functional, pharmacodynamic, or efficacy evidence, including cellular and animal endpoints, but does not by itself establish drug concentration or spatial distribution. Class C denotes expression, prognosis, or mechanistic association. Class D denotes formulation, carrier-localization, or other delivery-concept evidence without validated free-TMZ exposure in the target compartment. Class N captures negative or contradictory findings, and Class X identifies evidence unsuitable for independent mechanistic inference. One hundred and two references were selected for the journal-oriented synthesis using stratified coverage of pharmacology, BBB/BBTB biology, cellular transport, vesicles, the microenvironment, sensitization, nanodelivery, local or physical delivery, clinical translation, and methodology. Selection favored original studies, direct measurements, negative results, and human evidence; reviews were used to define context and identify convergent themes[5-8]. For the 15-study delivery-platform audit, the four co-first authors (Tianxiang Jiang, Zhaoxing Jia, Xiaokai Cen, and Yuhui Zhao) independently assigned claim–evidence classes. Classification differences were jointly adjudicated by consensus of both corresponding authors (Weiping Zhang and Xianming Lin). The final adjudicated classification comprised three A+B studies (R0002, R0173, and R0179) and twelve D+B studies. These classes describe the relationship between a particular claim and the supporting measurements; they do not provide an ordinal ranking of study quality. A study may support more than one class for different claims, and a negative finding is not inherently lower-quality evidence.

Structured evidence-audit flow and limitations

The verified workflow states described above provide a structured account of literature identification and evidence access. We do not present them as a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram because they describe library acquisition and evidence availability rather than a complete, reproducible sequence of systematic screening and exclusion decisions. Moreover, lack of prospective registration alone does not distinguish a narrative review from a systematic review. The counts therefore represent verified workflow states rather than reconstructed or estimated screening events. This terminology preserves auditability without implying compliance with formal systematic-review requirements.

FROM SYSTEMIC EXPOSURE TO TUMOR-CELL DNA DAMAGE

TMZ activity depends on systemic and regional exposure, cellular drug handling, chemical activation, and DNA-damage processing. The parent drug must reach the relevant tumor compartment, whereas chemical activation is not restricted to the intracellular space. The resulting DNA lesions and their processing by repair and stress-response pathways then influence cell fate. Interpretations of resistance should therefore identify the specific step and compartment measured.

Anatomical compartments are not interchangeable

Plasma, total brain, CSF, tumor core, invasive margin, and intracellular concentrations answer different pharmacological questions. Favorable plasma exposure does not establish adequate exposure within infiltrative tumor margins. Conversely, a higher total-tissue concentration does not necessarily indicate a larger pharmacologically available intracellular fraction.

Table 1. Human pharmacokinetic and barrier-opening evidence relevant to TMZ delivery

Evidence	Population/design	Compartment or intervention	Direct TMZ measurement	Main result	Interpretation and limitation
Ostermann *et al.*, 2004[10]	35 patients with newly diagnosed or recurrent malignant glioma	Plasma and CSF population PK	Yes, HPLC	CSF/plasma AUC approximately 20%	Demonstrates partial CNS penetration; CSF is not tumor tissue and exposure did not outperform cumulative dose as an outcome predictor
Portnow *et al.*, 2009[11]	9 enrolled; 7 evaluable paired profiles	Plasma and non-enhancing peritumoral brain interstitium	Yes, ICMD and LC–MS/MS	Brain-interstitium/plasma AUC 17.8%; brain Tmax approximately 2 h	Direct regional PK, but small diagnostically heterogeneous postoperative cohort
Jackson *et al.*, 2018[12]	Pilot study in recurrent high-grade glioma	Regadenoson plus paired plasma/brain microdialysis	Yes	Brain/plasma AUC remained approximately 18%-19%; no significant improvement	Clinically important negative barrier-opening result; dose and sample size limit generalization
Büsker *et al.*, 2022[13]	11 children receiving metronomic TMZ	Plasma and CSF population PK	Yes, HPLC	Model-estimated median CSF/plasma AUC 37%	Pediatric schedule-specific finding; prior radiotherapy, intraventricular treatment, sparse sampling, and no external validation
Mainprize *et al.*, 2019[14]	Presurgical phase I feasibility study; 5 patients, 4 receiving TMZ	MRgFUS; paired sonicated/unsonicated tissue	Yes, LC–MS	Only one TMZ-treated patient yielded a quantifiable paired tissue comparison, favoring sonicated tissue	Direct human tissue signal but no reproducible effect estimate or efficacy inference
Park *et al.*, 2020[15]	Six-patient uncontrolled one-year follow-up	Repeated MRgFUS during adjuvant TMZ	No regional TMZ PK	Repeated opening appeared feasible without procedure-related delayed complications	Supports feasibility only; cannot establish concentration increase or survival benefit
Woodworth *et al.*, 2025 (BT008NA)[16]	Multicenter open-label single-arm phase I/II; 34 patients	Perirsectional MB-FUS during monthly adjuvant TMZ	No spatial TMZ PK endpoint	Opening visualized in all treatments; median OS 31.3 months and PFS 13.5 months	Promising signal; nonrandomized design and non-protocol-prespecified external control preclude causal survival claims

TMZ: Temozolomide; CSF: cerebrospinal fluid; PK: pharmacokinetics; HPLC: high-performance liquid chromatography; AUC: area under the concentration-time curve; CNS: central nervous system; ICMD: intracerebral microdialysis; LC–MS/MS: liquid chromatography-tandem mass spectrometry; MRgFUS: magnetic-resonance-guided focused ultrasound; MB-FUS: microbubble-enhanced focused ultrasound; OS: overall survival; PFS: progression-free survival.

The OPARATIC phase I study illustrates the value of direct regional sampling in recurrent glioblastoma. Olaparib was detectable in all 71 tumor-core specimens from 27 patients and in all 21 tumor-margin specimens from 9 patients; reported median concentrations were 496 nM in the tumor core and 512.3 nM at the margin. However, the combination exacerbated TMZ-associated hematological toxicity and required intermittent scheduling[9]. These data support clinically meaningful evaluation of combination-partner penetration and tolerability. They do not constitute direct measurement of regional TMZ concentration and must not be described as such.

Human PK show partial and compartment-dependent penetration

Table 1 summarizes the human pharmacokinetic and barrier-opening evidence. Direct human measurements challenge the shorthand claim that TMZ simply "crosses the BBB well". In 35 patients with malignant glioma, population pharmacokinetic analysis showed that CSF exposure was approximately 20% of plasma exposure; after adjustment for dose effects, neither plasma nor CSF area under the concentration–time curve (AUC) predicted toxicity, survival, or progression-free survival better than cumulative dose[10]. CSF is a useful marker of central nervous system (CNS) access, but it is not equivalent to extracellular drug exposure in infiltrative tumor tissue.

Intracerebral microdialysis provides a more local measure of exposure. In a postoperative pilot study, paired samples from seven evaluable patients yielded mean plasma and brain-interstitial TMZ AUC values of 17.1 and 2.7 μg·h/mL, respectively. The mean brain-interstitium/plasma ratio was 17.8%, and the peak brain concentration occurred at approximately 2 h[11]. The small and diagnostically heterogeneous cohort limits outcome inference, but the study directly demonstrates that systemic exposure substantially exceeds extracellular exposure in non-enhancing peritumoral brain.

An attempted pharmacological opening of the barrier also produced informative negative evidence. In patients with recurrent high-grade glioma, standard-dose regadenoson did not significantly increase TMZ peak concentration, AUC, or the brain/plasma AUC ratio measured by microdialysis; ratios remained approximately 18%-19% with and without the intervention[12]. This contrasts with earlier rodent findings and illustrates why barrier-opening strategies require human regional pharmacokinetic confirmation rather than extrapolation from preclinical permeability data.

Age, schedule, and sampling compartment further affect interpretation. In 11 children receiving metronomic TMZ for malignant CNS tumors, the model-estimated median CSF/plasma AUC ratio was 37%[13]. All had prior cranial radiotherapy and concomitant intraventricular therapy; the sample was small, and CSF exposure cannot be equated with brain or tumor exposure. Collectively, these studies support partial CNS penetration with substantial contextual uncertainty, not uniform delivery to all glioma compartments.

Pharmacokinetic mismatch in combination therapy

Targeted agents considered for use with TMZ may face transport limitations different from those of TMZ itself. The cited studies characterize the brain distribution and CNS PK of the ATR inhibitors berzosertib and elimusertib[17,18]. They illustrate the need to evaluate a potential combination partner's CNS exposure separately; they do not establish TMZ transport, simultaneous target-site exposure during TMZ coadministration, or clinical efficacy of a TMZ-based combination.

The OPARATIC study similarly showed that olaparib reached both tumor core and margin, while clinically important hematological toxicity constrained scheduling[9]. Co-delivery platforms, brain-penetrant analogs, and schedule optimization should therefore be evaluated using paired exposure-response data for each agent. Future studies should establish whether both compounds reach the same tumor compartment at biologically meaningful concentrations rather than infer synchronization from administered dose or carrier colocalization[19-21].

Chemical instability, sampling design, and the meaning of exposure

TMZ is orally absorbed and undergoes spontaneous, pH-dependent conversion to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) and subsequent methylating species[22]. This chemistry makes sample stabilization, collection timing, and analytical specificity central to interpretation. A single tissue concentration may reflect vascular content, extracellular drug, intracellular drug, or degradation during handling. CSF can support population modeling but is separated from tumor extracellular fluid by distinct interfaces; total-brain homogenate averages vascular, extracellular, and intracellular pools; and contrast-enhancing core samples may overrepresent regions with barrier disruption. For this reason, brain/plasma or CSF/plasma AUC ratios, paired regional sampling, and microdialysis provide complementary rather than interchangeable evidence[10-13].

The human studies reviewed here converge on partial CNS penetration but do not establish a universal exposure threshold. Adult CSF and non-enhancing brain-interstitial AUC values were approximately one-fifth of plasma exposure, whereas a small pediatric metronomic cohort yielded a higher model-estimated

CSF fraction[10-13]. Differences in age, dose schedule, prior irradiation, intraventricular therapy, sampling intensity, and model assumptions can plausibly explain part of this variation. The key translational question is therefore not whether TMZ "crosses the BBB", but whether sufficient unbound drug reaches infiltrative tumor cells with a molecular context permissive for TMZ-induced cytotoxicity for a sufficient duration.

DNA-repair context determines whether increased exposure is useful

TMZ exposure and DNA-damage processing are distinct determinants of response. MGMT directly removes O^6-methylguanine lesions, reducing the substrate available for subsequent cytotoxic processing. When these lesions persist, replication generates mismatches that can engage the mismatch-repair (MMR) machinery; loss of MMR can therefore promote TMZ tolerance despite adequate drug delivery. Base-excision repair (BER) processes other TMZ-associated methylated bases and influences responses to combinations targeting DNA-damage repair. Readers seeking broader coverage of these pathways are referred to the resistance review in Ref.[2].

Consequently, repair proficiency should not be treated as a single binary marker: MGMT-mediated lesion removal and MMR-dependent damage processing can have different implications for TMZ sensitivity. A delivery intervention should be assessed alongside MGMT and MMR status and lesion-linked pharmacodynamic endpoints. Higher parent-drug exposure without the expected DNA-damage response warrants investigation of downstream tolerance; a stronger damage response without higher exposure may indicate sensitization rather than improved delivery. Increased delivery alone should not be assumed to overcome every molecular form of resistance. Combination studies must also assess normal-tissue toxicity, as illustrated by the hematological constraints in the olaparib–TMZ study[9].

ENDOTHELIAL TRANSPORT AT THE BLOOD–BRAIN AND BLOOD–BRAIN TUMOR BARRIERS

ABCB1 and ABCG2 provide the strongest compartment-specific anchor

The most relevant mechanistic anchor is the investigation of TMZ penetration in wild-type and transporter-deficient mice. Combined deletion of Abcb1a/b and Abcg2 increased brain penetration of TMZ by approximately 1.5-fold without changing systemic exposure, indicating that endothelial transporter activity can restrict intracranial TMZ access[23]. The study incorporated *in vitro* transport experiments, *in vivo* pharmacokinetic comparisons, and antitumor evaluation, making it substantially stronger than an expression-only association.

The magnitude of the reported change also deserves careful interpretation. A measurable increase in brain exposure supports a transport contribution, but it does not imply complete exclusion of TMZ from the brain or demonstrate that transporter blockade alone will reverse clinical resistance. Genetic deletion in mice, pharmacological inhibition in patients, and delivery across heterogeneous human tumor margins involve different assumptions and risks.

Barrier heterogeneity complicates extrapolation

The BBTB is spatially heterogeneous. Contrast enhancement, disrupted vasculature, perivascular abnormalities, and infiltrative margins do not necessarily represent a single pharmacological compartment. Delivery strategies should therefore be evaluated against region-specific questions: Does the treatment cross relatively intact endothelial barriers? Does it accumulate only in a permeable core? Does it reach disseminated cells beyond radiographic enhancement?

Existing nanoparticle and liposomal studies provide multiple candidate approaches, but their reported endpoints vary widely[24-26]. Carrier signal within the brain is not equivalent to free TMZ concentration at the

malignant-cell target. Where available, direct quantification of TMZ and its relevant pharmacological products should take precedence over imaging of the carrier alone.

Models of the BBB/BBTB determine what can be concluded

Monolayer permeability assays, transporter-overexpressing cells, multicellular BBB models, orthotopic xenografts, genetically engineered animals, and surgically obtained human samples address different questions. A human *in vitro* BBTB model can reveal endothelial–tumor interactions and screen relative permeability, but it cannot reproduce regional perfusion, immune-cell effects, intracranial pressure, or drug clearance[27]. Orthotopic models improve anatomical relevance but still vary in vascular phenotype and transporter expression. Tumor control in a subcutaneous model is particularly weak evidence of brain delivery because the model omits the barrier that the formulation is intended to overcome.

Barrier permeability is also not uniformly undesirable. Excessive or nonspecific opening may increase edema, hemorrhage, inflammatory signaling, or normal-brain exposure. Strategies targeting tumor endothelium or adhesion molecules may improve regional delivery, but therapeutic interpretation requires matched measurement of vascular effects and drug exposure[28]. The effects of adenosine-A2A activation were species- and model-dependent. In glioma-bearing mice, regadenoson did not significantly change plasma TMZ or brain:plasma and tumor:plasma concentration ratios. In GSC827-bearing rats, it increased the tumor:plasma TMZ concentration ratio by 25.1% without changing the brain:plasma ratio or improving survival with repeated TMZ treatment[29]. In patients with recurrent high-grade glioma, standard-dose regadenoson did not significantly increase TMZ peak concentration, AUC, or the brain:plasma AUC ratio in non-enhancing peritumoral tissue[12]. These discordant findings support species-aware preclinical interpretation and early human regional pharmacokinetic endpoints.

Efflux inhibition *vs.* barrier bypass

Direct ATP-binding cassette subfamily B member 1 (ABCB1)/ATP-binding cassette subfamily G member 2 (ABCG2) inhibition is conceptually different from packaging TMZ in a carrier, using a prodrug, opening the barrier physically, or delivering drug locally. Inhibition attempts to change transporter-mediated flux but can also alter systemic disposition and protective transport in healthy organs. Barrier bypass may avoid a specific transporter yet introduce reticuloendothelial clearance, incomplete drug release, or regional deposition. The approximately 1.5-fold increase produced by combined ABCB1/ABCG2 disruption in mice defines a measurable contribution, not a guarantee that systemic transporter inhibition will provide an adequate therapeutic index[23]. Mechanism-informed selection should ask whether the observed exposure deficit is large enough, spatially relevant, and safely modifiable.

TUMOR-CELL TRANSPORT: DIRECT EFFLUX, UPTAKE, AND NONTRANSPORT FUNCTIONS

Direct transport requires direct measurement

A transporter should be implicated in TMZ efflux only when the experimental design establishes that transport, accumulation, or exposure of TMZ itself changes after a transporter-specific intervention. Bidirectional transport assays, transporter-knockout or knockdown systems, appropriately controlled inhibitors, intracellular TMZ measurements, and brain-to-plasma comparisons provide stronger evidence than changes in mRNA abundance or fluorescence-probe efflux alone.

Studies using rhodamine 123, Hoechst dyes, calcein, or related substrates can establish transporter activity but do not automatically identify TMZ as the transported substrate. Similarly, reduced viability after combining a transporter inhibitor with TMZ may reflect pathway crosstalk or altered apoptosis rather than direct changes in TMZ transport.

Negative evidence challenges a uniform P-glycoprotein narrative

One glioblastoma cell-line investigation evaluated MGMT, MMR factors, P-glycoprotein, and CD133 under TMZ exposure and concluded that P-glycoprotein did not appear to participate in TMZ resistance in the models studied[30]. This does not negate endothelial ABCB1/ABCG2 effects in genetically modified mice. Instead, it illustrates that endothelial transport at the BBB and tumor-cell resistance in a particular culture system cannot be assumed to share the same mechanism.

TMZ can also alter endothelial P-glycoprotein expression and fluorescent-probe transport through Wnt-related signaling[31]. This is evidence that TMZ regulates a transporter-associated state; it is not, by itself, proof that the same transporter directly exports TMZ. Distinguishing modulation of transporter expression from direct substrate transport avoids circular reasoning.

The same distinction applies to glioblastoma stem-like cells. TMZ exposure reduced Wnt3a signaling, ABCB1 transcription, P-glycoprotein abundance, and transport activity in these cells[32]. The resulting chemosensitization was demonstrated with established P-glycoprotein substrates such as doxorubicin, vinblastine, and etoposide. The study therefore supports TMZ-mediated remodeling of a multidrug-resistance phenotype, but does not directly demonstrate P-glycoprotein transport of TMZ.

MRP1/ABCC1 effects depend on the substrate and biological context

Multidrug resistance protein 1 (MRP1) is frequently discussed as a multidrug-resistance transporter, but the available glioblastoma evidence is not uniformly supportive of direct TMZ transport. In a study of primary and recurrent glioblastoma models, pharmacological inhibition with MK571 or specific MRP1 knockdown did not alter TMZ drug response[33]. This negative observation must be reported alongside studies suggesting ATP-binding cassette subfamily C member 1 (ABCC1)-associated resistance or redox adaptation.

ABCC1-related ferroptosis studies introduce a different mechanism: nuclear factor erythroid 2-related factor 2 (NRF2)-associated ABCC1 activity can contribute to glutathione handling and susceptibility to ferroptotic stress[34]. Such evidence may be important for designing combination therapy, but glutathione export is not synonymous with TMZ export. The transported molecule, downstream death pathway, and relevant experimental endpoint must be specified.

Negative findings should be interpreted at the level of the tested model and endpoint. Failure to alter TMZ cytotoxicity after MRP1 perturbation[33] does not directly measure TMZ flux, while the cell-line P-glycoprotein result[30] addresses a different compartment from endothelial ABCB1/ABCG2 disruption[23]. Possible explanations for discordance include differences in transporter abundance or membrane localization, incomplete inhibition, alternative transport routes, TMZ instability, and a cytotoxicity endpoint dominated by DNA-repair status. These are testable explanations, not established causes of the reported null results. Paired parent-TMZ measurements, confirmation of target perturbation, positive-control substrates, and genetic rescue would distinguish a true lack of transport contribution from an insensitive or confounded readout.

ABCB5 and ABCA1 may influence resistance without establishing direct TMZ efflux

Targeting ATP-binding cassette subfamily B member 5 (ABCB5) sensitized glioblastoma to TMZ-associated apoptosis and reduced tumor growth in xenograft models, with a proposed contribution from cell-cycle-checkpoint regulation[35]. These data support a functional therapeutic relationship, but they do not alone establish direct ABCB5-mediated TMZ transport.

ATP-binding cassette subfamily A member 1 (ABCA1)-associated work links resistance to glioma stem-like enrichment, immune-state changes, cholesterol regulation, and altered treatment sensitivity[36,37]. These mechanisms are biologically plausible but should not be collapsed into a single claim of direct TMZ export. An additional methodological hazard is that "AUC" in some ABCA1 papers refers to a diagnostic receiver-operating-characteristic curve rather than pharmacokinetic area under a drug-concentration curve[37].

GLUT3 reveals the importance of TMZ influx

Recent work identified glucose transporter 3 (GLUT3)-associated uptake of TMZ using uptake assays, transporter perturbation, binding-related analysis, site-directed mutagenesis, and xenograft experiments[38]. Increased GLUT3 expression was associated with increased TMZ uptake and chemosensitivity. This evidence concerns influx, not efflux.

The finding has conceptual implications: effective intracellular exposure reflects the balance between entry and removal. A strategy that enhances influx may improve TMZ activity even when it does not inhibit an efflux transporter. Future studies should evaluate bidirectional flux and avoid treating every transporter-associated phenotype as drug export. An L-type amino acid transporter 1 (LAT1)-utilizing TMZ amino-acid prodrug further illustrates that increasing transporter-mediated accumulation does not necessarily overcome DNA repair-mediated chemoresistance[39].

Adaptive resistance networks can mimic transport phenotypes

TMZ-resistant cells frequently acquire migration, side-population, stem-like, metabolic, and stress-tolerance phenotypes. These states can coincide with transporter expression without being caused by direct TMZ export. Rat C6 resistance selection increased migration and side-population features, while lysine demethylase 5A (KDM5A), protein neddylation, carbonic anhydrase XII, transient receptor potential cation channel subfamily C member 5 (TRPC5)-nuclear factor of activated T cells 3 (NFATc3)-P-glycoprotein signaling, and Rho-associated coiled-coil containing protein kinase 2 (ROCK2)/ABCG2 have each been linked to TMZ response in specific models[40-45]. Such studies identify intervention points, but the causal chain must be decomposed into transporter abundance, measured substrate, intracellular TMZ, DNA damage, repair, and survival.

Membrane composition further changes apparent drug response. Polyunsaturated fatty acids, lipid rafts, and cholesterol handling can alter oxidative stress, receptor organization, apoptosis, and membrane protein function[46,47]. These mechanisms may change net TMZ efficacy even when they do not directly change TMZ flux. Similarly, ATP-binding cassette subfamily C member 3 (ABCC3)-mediated protection of immune cells from chemotherapy concerns immune-cell substrate handling and cannot be transferred automatically to glioma-cell TMZ export[48]. A compartment-aware review should preserve these biologically useful findings while refusing substrate substitution.

Uptake, intracellular retention, and DNA-damage competence form a net-exposure system

GLUT3-mediated uptake provides the clearest counterexample to an efflux-only model: increased entry can enhance chemosensitivity even if outward transport is unchanged. After entry, intracellular retention may be affected by vesicular partitioning, while lesion persistence depends on MGMT, mismatch repair, BER, homologous-recombination-associated responses, autophagy, and cell-cycle checkpoints. Ibudilast, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)-directed small interfering RNA (siRNA), the connexin 43 mimetic peptide (alphaCT1), bone morphogenetic protein 7 (BMP7), and radiosensitizer strategies improve response through different points in this network[49-53]. Their therapeutic value does not require relabeling them as efflux inhibitors.

Autophagy is especially context-dependent. Phosphoinositide 3-kinase (PI3K) inhibition and direct autophagy inhibition have increased TMZ-associated apoptosis or ferroptosis in experimental systems, while other adaptive programs may use autophagy for survival[54-56]. Ferroptosis-oriented approaches similarly interact with NRF2, glutathione export, lipid peroxidation, and stemness[57]. The practical implication is that intracellular TMZ concentration and downstream lesion competence should be measured together whenever a sensitizer is proposed to restore delivery.

VESICLES, MEMBRANE REMODELING, AND INTRACELLULAR REDISTRIBUTION

Extracellular vesicles and microvesicles can transfer resistance-associated proteins, nucleic acids, and signaling states between glioma and stromal cells. Studies involving exosome-directed delivery and ADP-ribosylation factor-like protein 13B (ARL13B)-associated microvesicles support a role for vesicular biology in TMZ-related resistance networks[58,59]. However, a vesicle-associated resistance phenotype does not establish that vesicles physically export pharmacologically meaningful amounts of TMZ.

In TMZ-resistant TBD-0220 cells, Hong *et al.* reported that EPIC-1042 co-treatment approximately doubled intracellular TMZ and reduced the TMZ signal in the isolated small-extracellular-vesicle fraction[60]. Supplementary Figure 3A and B in the original publication by Hong *et al.*[60] display the multiple-reaction monitoring (MRM) transition 195.1 to 138.0, supporting an analyte-directed measurement rather than a vesicle-count proxy. These observations support altered drug partitioning associated with the intervention, but do not alone establish intravesicular localization or the fraction of total TMZ exported. Concurrent effects on poly(ADP-ribose) polymerase 1 (PARP1) degradation, BER, MGMT recruitment, and autophagy also preclude attributing sensitization exclusively to inhibited vesicular export.

Membrane cholesterol and lipid rafts offer another important example. Ginsenoside-associated studies report altered cholesterol efflux, lipid-raft distribution, and TMZ sensitivity[47]. Here, "efflux" refers primarily to cholesterol handling. The appropriate interpretation is membrane-state modulation of treatment response unless intracellular or vesicular TMZ measurements establish otherwise.

Therefore, vesicular mechanisms should be separated into at least three categories: transfer of resistance-associated information, redistribution or sequestration of cellular material, and quantitatively demonstrated removal of TMZ itself. Only the third category supports a direct drug-export claim.

Vesicles as carriers of resistance information

Glioblastoma-derived extracellular vesicles transfer proteins, microRNAs, long RNAs, and metabolic signals between tumor subclones and the microenvironment. Reviews and experimental studies support roles in therapy adaptation, immune modulation, angiogenesis, and stem-like-state maintenance[61-63]. Engineered exosomes can also be used in the opposite direction - as delivery vehicles for siRNA, gene-editing components, or drug combinations[58,64,65]. These two functions should not be conflated: natural vesicle-mediated resistance communication does not prove removal of TMZ, and engineered-vesicle uptake does not establish inhibition of endogenous export.

Direct drug partitioning is a higher evidentiary bar

The supplementary methods for the EPIC-1042 study describe methanol extraction of cell and small extracellular vesicle (sEV) pellets, followed by liquid chromatography–mass spectrometry (LC–MS) analysis of TMZ[60]. The sEV preparation included a phosphate-buffered saline (PBS) wash and repeated ultracentrifugation; cell counting and cell-number-normalized vesicle protein and particle measurements were also reported. These procedures should not be confused with a demonstrated normalization scheme for the TMZ assay itself. The supplied supplement does not explicitly document TMZ-specific calibration,

internal-standard correction, extraction recovery, stability validation, or free-drug carryover controls. Its acidified extraction and internal-standard procedure concerns tissue EPIC-1042, not pellet TMZ. Thus, the reported drug measurements support a transport-related hypothesis, while quantitative vesicular mass balance and analytical specificity against degradation products remain insufficiently documented. ARL13B-associated resistance transfer should likewise be distinguished from direct measurement of drug cargo.

MECHANISM-INFORMED DELIVERY STRATEGIES

Match the intervention to the limiting barrier

The existing corpus contains substantial nanoparticle and liposomal literature, but a mechanism-informed review should organize these studies by the barrier they claim to address rather than listing carrier compositions. The central distinction is whether a platform improves endothelial passage, regional tumor distribution, tumor-cell uptake, intracellular retention, combination-drug synchronization, or local exposure.

Nanoparticle co-delivery of TMZ and ligustilide, for example, is relevant because the combination was designed around brain targeting and transporter-associated barrier modulation[25]. Magnet-guided TMZ-containing nanoparticles address an alternative delivery concept[26]. These approaches remain preclinical and should be compared using matched controls, direct TMZ measurements, appropriate orthotopic models, and safety endpoints.

Distinguish sensitization from restored drug delivery

Sensitization can result from altered DNA repair, ferroptosis, peptide signaling, microRNA regulation, autophagy, or cell-cycle adaptation[50-52]. These interventions may be therapeutically important even when they do not improve brain delivery or reduce TMZ export. Their value should be categorized according to the demonstrated mechanism, not forced into an efflux framework.

Local, intranasal, and physical delivery approaches

Intranasal TMZ-containing formulations and ultrasound-activated treatment platforms offer potential ways to bypass or modify conventional anatomical barriers[66,67]. However, evidence remains dependent on model design. A strong translational assessment should specify the administered dose, anatomical distribution, direct TMZ concentration, reproducibility, local toxicity, and whether treatment reaches infiltrative regions.

Early clinical focused-ultrasound studies provide a useful hierarchy of endpoints. In a five-patient presurgical feasibility study, four patients received oral TMZ before magnetic resonance (MR)-guided focused-ultrasound barrier opening; LC–MS was planned for paired sonicated and unsonicated tissue[14]. The barrier opened radiographically and the procedure was tolerated, but limited resectable tissue allowed quantitative TMZ comparison in only one patient. In that patient, TMZ was higher in sonicated than unsonicated peritumoral tissue. This is direct human tissue evidence, but a single quantifiable pair cannot establish the magnitude, reproducibility, or clinical benefit of enhanced delivery.

A subsequent six-patient study applied repeated barrier opening during adjuvant TMZ and reported no immediate or delayed procedure-related complications at one year[15]. Five patients completed six cycles, but the uncontrolled sample was too small to estimate efficacy, and the study did not demonstrate that higher TMZ concentration mediated the observed outcomes. Its appropriate evidentiary role is repeated-procedure feasibility rather than proof of survival benefit.

The multicenter BT008NA phase 1/2 study substantially strengthened the safety and implementation evidence[16]. Thirty-four participants received periresectional microbubble-enhanced focused ultrasound during monthly adjuvant TMZ; barrier opening was visualized in all treatments, median overall survival was

31.3 months, and median progression-free survival was 13.5 months. However, the trial was open-label and single-arm, and its external control analysis was not protocol-prespecified. It therefore supports feasibility and a promising efficacy signal, not causal proof that focused ultrasound improves survival. A randomized comparison and spatial TMZ pharmacokinetic substudy remain necessary.

Thus, physical barrier opening is further along clinically than most TMZ nanocarriers, but the evidence chain remains incomplete: radiographic opening is established, repeated treatment appears feasible, and one highly limited tissue comparison suggests increased TMZ delivery; reproducible regional PK and randomized clinical benefit have not yet been demonstrated[14-16]. Table 2 summarizes representative TMZ-containing delivery platforms and distinguishes formulation and efficacy endpoints from direct parent-drug exposure measurements.

Nanocarriers should be compared by pharmacological function, not material novelty

The corpus contains liposomes, polymeric particles, lipid-polymer hybrids, magnetic particles, self-microemulsifying drug delivery systems (SMEDDS), exosome-based systems, and surface-engineered dual-drug carriers. Across these platforms, the clinically meaningful functions are limited and comparable: protection from premature degradation, altered systemic disposition, endothelial passage, receptor-mediated uptake, regional guidance, controlled release, and synchronized delivery of a sensitizer. Liposomal and nanoparticle reviews show broad design flexibility, but also recurring gaps in free-drug measurement, infiltrative-margin exposure, manufacturing reproducibility, and long-term toxicity[24,72-75].

Several examples illustrate why matched controls are essential. TMZ/ligustilide and TMZ/ferucarbotran formulations combine carrier effects with a pharmacological partner or magnetic guidance[25,26]. PEGylated TMZ/O^6-benzylguanine systems and p53-gene nanoparticles combine delivery with DNA-repair or tumor-suppressor modulation[76,77]. Surface-engineered dual-drug liposomes and SMEDDS formulations add targeting or metabolic modification[78,79]. Without free-TMZ PK, empty-carrier controls, non-targeted carrier controls, and free-combination controls, improved survival cannot be attributed to barrier crossing rather than altered dose, partner activity, or nonspecific retention.

A targeted full-text audit of 15 representative TMZ-containing delivery studies exposed a consistent endpoint hierarchy [Table 2 and Supplementary Table 1]. These studies were purposively selected to span major platform classes, administration routes, model levels, and exposure endpoints, and each had an accessible source sufficient for study-level verification. The set was designed for comparative evidence auditing rather than exhaustive enumeration and should not be interpreted as including all eligible TMZ-delivery studies. Formulation reporting was often detailed: representative systems ranged from approximately 25 nm lipid nanocarriers to approximately 210 nm polymeric or inorganic constructs, with reported TMZ encapsulation efficiencies spanning about 20% to 98%. A transferrin-functionalized liposome contained 2.9 wt% TMZ with 48.3% encapsulation efficiency[68], while an intranasal anti-EphA3 formulation contained a TMZ hexadecyl-ester prodrug at 16.81 wt% with 93.58% encapsulation efficiency[66]. Such values establish formulation feasibility, but they are not interchangeable with target-compartment exposure.

Three of the 15 audited platform studies reported direct analyte-specific measurements targeting parent TMZ in biological samples, although each had important limitations[19,25,70]. The TMZ/ligustilide PLGA-mPEG study used LC–MS to measure plasma TMZ PK and a single total-brain concentration at 3 h. After intravenous administration of a TMZ-equivalent dose of 5 mg/kg in rats, plasma $AUC_{0-\infty}$ increased from 10.061 to 44.953 μg·h/mL, and half-life increased from 1.758 to 2.853 h[25]. The biomimetic TMZ/OTX015 study reported high-performance liquid chromatography (HPLC)-based TMZ measurements in whole blood and total-tissue extracts at 8 h, including 7.22% of the injected dose per gram of tumor for the targeted

Table 2. Representative TMZ-containing delivery platforms and the endpoints used to infer delivery

Platform (reference)	Formulation anchor	Model/intervention	Efficacy or exposure anchor	Measured analyte	Evidence-supported interpretation	Principal limitation
PLGA-mPEG TMZ/ligustilide nanoparticles[25]	117.6 ± 0.7 nm; ζ -26.5 ± 0.4 mV	SD rats; intravenous TMZ-equivalent dose 5 mg/kg	Plasma TMZ $AUC_{0-\infty}$ 44.953 *vs.* 10.061 μg·h/mL and t1/2 2.853 *vs.* 1.758 h *vs.* free TMZ; total-brain measurement at 3 h	Parent TMZ by LC–MS	Direct systemic parent-TMZ PK with a single-point total-brain delivery signal	Single total-brain time point; no unbound, tumor-margin or intracellular PK
Transferrin-functionalized PEG liposomes containing TMZ/JQ1[68]	137 nm; PDI 0.17; ζ -12.1 mV; TMZ EE 48.3%, DL 2.9 wt%	Orthotopic U87MG and GL261; *n* = 8/arm	Marked suppression of day-7 tumor bioluminescence with targeted dual-loaded particles	Carrier imaging and efficacy endpoints	Receptor-targeted carrier transport and combination efficacy	No regional free parent-TMZ PK; efficacy cannot be assigned solely to TMZ delivery
Anti-PD-L1-scFv TMZ lipid nanocarrier[69]	24.8 ± 1.1 nm; PDI 0.408 ± 0.068; ζ +1.2 ± 0.4 mV; TMZ loading 7%	Orthotopic syngeneic GL261; *n* = 5/arm	Survival was prolonged relative to controls after three low-dose administrations; the exact median was not retained because the reported 47-day value conflicts with termination of the experiment on day 45 while one animal remained alive	Carrier/formulation and efficacy endpoints	Targeted nanocarrier feasibility and functional efficacy	Long-term safety and systemic biodistribution not assessed; no regional parent-TMZ PK
ApoE-RBC-membrane biomimetic TMZ/OTX015 nanomedicine[19]	TMZ loading 6.7%; OTX015 loading 7.6%	Orthotopic immunocompetent primary/recurrent GL261	Whole-blood TMZ profile and 8-h total-tissue extraction by HPLC; tumor value 7.22% injected dose/g for the targeted combination; recurrent-model median survival 58.5 days *vs.* 28 days TMZ-carrier and 20 days PBS	Parent-TMZ-targeted HPLC in whole blood and total tissue extracts; carrier signal and efficacy	Limited direct total-tissue exposure evidence plus strong orthotopic combination efficacy	Single 8-h total-tissue sampling; no unbound or regional parent-TMZ PK; OTX pharmacodynamics confound efficacy attribution
Liposomal TMZ plus ultrasound-mediated BBB opening[70]	148.13 ± 2.66 nm; PDI 0.23 ± 0.05; ζ -23.14 ± 0.50 mV; EE 52%	Orthotopic GBM plus ultrasound BBB opening	Plasma TMZ measurements and a 2-h total brain-tumor extract measurement by HPLC–UV; tumor suppression and survival benefit were also reported	Parent-TMZ-targeted HPLC–UV in plasma and total brain-tumor extract; efficacy endpoints	Limited direct total-tissue exposure evidence supporting liposomal TMZ with physical BBB opening	Single 2-h total-tissue measurement; no unbound or spatially resolved comparison between opened and unopened regions
Transferrin/pH-responsive TMZ-siEGFR carrier[71]	80.29 ± 3.14 nm; intravenous TMZ 3 mg/kg plus siEGFR 1 mg/kg	Orthotopic U87; *n* = 10/arm	Median survival 64.5 days targeted *vs.* 48 days nontargeted, 36.5 days free TMZ and 25.5 days PBS	Cy5-labeled TMZ signal and efficacy	Targeted co-delivery efficacy and preliminary tolerability	Label signal is not validated free parent-TMZ PK

Intranasal anti-EphA3 TMZ16e-prodrug PLGA nanoparticles[66]	135.1 ± 2.4 nm; PDI 0.085 ± 0.037; ζ -28.65 ± 1.20 mV; EE 93.58% ± 1.39%; DL 16.81% ± 1.17%	Orthotopic T98G; intranasal dosing	Median survival 41 days targeted *vs.* 32 days untargeted and 24 days saline	TMZ16e prodrug formulation, coumarin uptake and efficacy	Intranasal targeted prodrug feasibility and survival benefit	Prodrug and fluorescent-marker results cannot be reported as parent-TMZ PK

Interpretive rule: carrier localization, fluorescent uptake, tumor regression and survival are informative but non-equivalent endpoints. A strong delivery claim requires direct measurement of parent or free TMZ in the target intracranial compartment. TMZ: temozolomide; PLGA: poly(lactic-co-glycolic acid); mPEG: methoxy-polyethylene glycol; ζ: zeta potential; SD: Sprague–Dawley; AUC: area under the concentration–time curve; LC–MS: liquid chromatography–mass spectrometry; PK: pharmacokinetics; JQ1: bromodomain and extra-terminal domain inhibitor JQ1; PDI: polydispersity index; EE: encapsulation efficiency; DL: drug loading; PD-L1: programmed death-ligand 1; scFv: single-chain variable fragment; ApoE: apolipoprotein E; RBC: red blood cell; OTX: OTX015, a bromodomain and extra-terminal domain inhibitor; HPLC: high-performance liquid chromatography; PBS: phosphate-buffered saline; BBB: blood–brain barrier; GBM: glioblastoma; UV: ultraviolet; siEGFR: small interfering RNA targeting epidermal growth factor receptor; EphA3: ephrin type-A receptor 3.

combination nanomedicine[19]. The ultrasound/liposomal study used HPLC–ultraviolet (UV) detection for plasma measurements and a measurement in a total brain-tumor extract at 2 h[70]. These studies support limited systemic or total-tissue exposure claims, but none determined unbound concentration–time profiles, spatial distribution across enhancing and infiltrative regions, or intracellular parent-TMZ availability.

Orthotopic studies frequently yielded substantial efficacy signals without analyte-resolved PK[19,69-71]. Targeted co-delivery platforms prolonged survival or suppressed bioluminescence, and an intranasal anti-EphA3 TMZ-ester formulation extended median survival from 24 days with saline to 41 days[66]. However, these outcomes combined carrier targeting, altered chemistry, sensitizing cargo, or physical barrier manipulation. Fluorescent carrier signals, coumarin uptake, Cy5-labeled drug signals, and magnetic contrast should therefore be reported as distinct endpoints rather than collectively described as increased TMZ delivery. The audit supports a two-tier presentation: direct parent-TMZ PK and rigorously controlled orthotopic studies in the main evidence table, with *in vitro* formulation-only studies in the Supplementary Materials.

Intranasal, local, and ultrasound-assisted approaches address different spatial failures

Intranasal delivery aims to reduce dependence on systemic BBB passage through olfactory and trigeminal routes. TMZ ester nanoparticles and thermosensitive nanoemulsion gels show preclinical feasibility, while broader intranasal reviews emphasize deposition, mucociliary clearance, enzymatic degradation, and device-dependent dosing[66,80,81]. Human translation requires absolute bioavailability, regional brain distribution, nasal toxicity, and comparisons with standard oral TMZ - not merely a higher brain/plasma ratio after unequal doses.

Local depots and resection-cavity systems can generate high concentrations near the surgical bed, but diffusion through brain extracellular space is limited and recurrence often arises beyond the cavity. Their evaluation should include release kinetics under physiological conditions, active drug stability, penetration distance, wound healing, neurotoxicity, and distant infiltrative disease. Ultrasound-activated TiO_2 systems and focused-ultrasound-assisted nanoparticle or gene delivery add spatial control but involve distinct mechanisms: acoustic activation of a material is not the same as microbubble-mediated BBB opening[67,82].

A minimum evidence package for delivery claims

For a preclinical delivery platform to support a strong claim, five elements should be present: physicochemical and release characterization; matched free-drug and non-targeted controls; plasma and regional brain/tumor PK of TMZ itself; efficacy in an orthotopic and preferably heterogeneous model; and systemic plus neurological safety. Carrier fluorescence may document localization but cannot replace

active-drug quantification. Total-tissue TMZ should be complemented by unbound or extracellular measurements when possible. Studies of nitrosylcobalamin, beta-elemene combinations, natural-product adjuvants, and platinum nanoformulations demonstrate the diversity of potential partners, but also the need to distinguish partner PK from TMZ PK[83-86].

A translationally credible platform must also address scale-up, batch variability, sterilization, storage, route-specific devices, and whether the proposed targeting ligand remains accessible in protein-rich biological fluids. These considerations are often peripheral in efficacy-focused animal studies but central to Q1-level assessment of drug-delivery readiness.

Natural products and multi-component interventions require unusually strong controls

Curcumin, beta-elemene, ginsenosides, polyunsaturated fatty acids, and other natural-product-derived interventions are frequently paired with TMZ because they can influence oxidative stress, membrane organization, apoptosis, autophagy, inflammatory signaling, or drug metabolism. Nanocurcumin and traditional-medicine delivery reviews illustrate how formulation can improve solubility or apparent brain availability while simultaneously introducing multiple active constituents[87-89]. These studies can generate useful hypotheses, but they are especially vulnerable to mechanism inflation: a reduction in viability may reflect intrinsic cytotoxicity, altered TMZ chemistry, general membrane injury, or a genuine sensitizing interaction.

At minimum, these experiments should include concentration-matched monotherapy arms, formal interaction analysis, stability testing, vehicle controls, and measurement of intracellular TMZ or DNA adducts when a delivery mechanism is claimed. Botanical extracts require chemical standardization and batch documentation. If the intervention is incorporated into a carrier, the design must additionally separate the effects of carrier, natural product, TMZ, free combination, and loaded combination. Without this factorial structure, claims of transporter inhibition or restored BBB delivery remain speculative even when tumor suppression is statistically significant.

Delivery of non-TMZ partners can still inform the platform - within limits

Proteasome inhibitors, platinum agents, gene therapies, and radiosensitizers face many of the same anatomical barriers as TMZ and can inform platform engineering[90-92]. Their studies are valuable for evaluating particle size, surface chemistry, protein corona, endothelial transcytosis, release behavior, and regional toxicity. They cannot, however, be counted as TMZ PK evidence. A platform validated with a highly lipophilic or transporter-sensitive partner may behave differently when loaded with TMZ, whose small size, spontaneous activation, and dosing schedule impose distinct formulation constraints.

This distinction is particularly important for co-delivery. If only the partner is retained by the carrier while TMZ releases rapidly, nominal co-encapsulation does not guarantee synchronized tumor exposure. Conversely, a carrier optimized to stabilize TMZ could delay activation or reduce the free fraction available for tumor-cell uptake. Future formulations should report drug-specific loading, release, degradation, and regional PK for every cargo rather than a single carrier-level biodistribution curve.

CLINICAL TRANSLATION: FROM DISTRIBUTION SIGNALS TO PATIENT BENEFIT

Human regional PK should precede efficacy claims

The adult CSF, postoperative microdialysis, regadenoson, pediatric CSF, and presurgical focused-ultrasound studies demonstrate that informative TMZ PK can be embedded in small clinical protocols[10-14]. Their combined lesson is methodological: collect paired plasma and regional samples, stabilize TMZ immediately, record the anatomical sampling location, and separate feasibility endpoints from therapeutic outcomes.

Contrast enhancement confirms permeability change but not drug concentration; tissue detection confirms access but not unbound intracellular exposure; and a favorable ratio does not identify the concentration required for tumor-cell killing.

Sampling the non-enhancing margin is particularly important because this compartment contains infiltrative cells protected by a relatively intact barrier. Liquid-biopsy and sono-liquid-biopsy approaches may provide serial biomarkers, but they do not replace regional drug PK[93]. Biomarker release after focused ultrasound can support monitoring and biological sampling, yet must be validated against tumor burden, treatment effect, and tissue molecular state.

Focused ultrasound is promising but remains an evolving intervention

The clinical focused-ultrasound sequence has progressed from single-session feasibility to repeated treatment and a multicenter phase I/II experience[14-16]. The 2019 presurgical study provided the only direct paired human-tissue TMZ evidence, but only one treated patient yielded quantifiable paired TMZ samples. The six-patient repeated-opening study supported short-term feasibility without a concentration endpoint. BT008NA then demonstrated implementation across sites and reported encouraging survival, but its open-label single-arm design, technology evolution, and non-protocol-prespecified external control prevent a randomized causal conclusion.

The next decisive study should combine a randomized design with an embedded PK cohort. Key variables include timing between oral TMZ and sonication, microbubble dose, acoustic emissions, opening volume, location relative to the resection cavity and infiltrative pathways, plasma exposure, paired tissue or microdialysis TMZ, hemorrhage-sensitive magnetic resonance imaging (MRI), steroid use, MGMT status, and subsequent treatment. Clinical benefit should be evaluated alongside - not instead of - evidence that the intervention increases TMZ exposure in the intended compartment.

Patient selection and safety may determine net benefit

MGMT status, prior therapy, tumor location, vascular phenotype, extent of resection, steroid exposure, and hematological reserve can modify both expected benefit and risk. Barrier opening or transporter inhibition may be most rational when inadequate exposure is a dominant limitation and tumor cells remain capable of responding to TMZ-induced lesions. Conversely, increasing exposure may add toxicity without benefit in repair-proficient or profoundly resistant disease. Combination-partner studies and radiosensitizer development reinforce the need to align delivery with molecular susceptibility[94-97].

A clinically useful framework should therefore guide patient selection using both delivery and response biomarkers. Imaging permeability, regional PK, circulating or CSF biomarkers, and tumor repair-state markers can be combined prospectively. The goal is not maximal brain exposure in every patient, but a favorable therapeutic index in the subgroup for whom delivery is limiting and downstream TMZ response remains recoverable.

A FRAMEWORK FOR CLAIM–EVIDENCE CLASSIFICATION

For transporter and delivery claims, we propose six categories:

1. Class A: direct analyte-resolved evidence. Parent TMZ is quantified in a defined compartment or transport assay. Evidence for a specific transporter additionally requires transporter-specific perturbation and appropriate controls; direct drug measurement alone does not establish transporter causality.

2. Class B: functional exposure or pharmacodynamic evidence. An intervention changes brain exposure, intracellular drug-associated responses, or efficacy in animals, but the direct substrate mechanism remains incompletely resolved.

3. Class C: expression, prognosis, or clinical association. Transporter abundance correlates with resistance, subtype, or outcome without direct transport measurement.

4. Class D: delivery-concept evidence. A carrier or treatment improves a surrogate endpoint without proving increased target-compartment TMZ exposure.

5. Class N: negative or contradictory evidence. Inhibition, knockdown, or expression analysis fails to support the proposed transport mechanism.

6. Class X: evidence unsuitable for independent mechanistic inference. Examples include corrigenda treated as original papers, unrelated drug substrates, nonpharmacokinetic AUC metrics, and unreadable full-text files.

The distinction between Class A and Class B is especially important. An improvement in survival or chemosensitivity can justify further investigation but should not be translated automatically into a claim that TMZ is a direct substrate of a particular transporter. Table 3 applies these classes to the principal transporter, uptake, vesicle, and membrane-remodeling mechanisms discussed in this review.

TRANSLATIONAL PRIORITIES

First, studies should quantify TMZ itself using validated analytical methods in plasma, relevant brain regions, tumor core, infiltrative margins, and, where feasible, tumor cells. Second, transporter interventions require appropriate genetic controls and separation of endothelial from tumor-cell effects. Third, combination studies must quantify both TMZ and its partner to identify intracranial exposure mismatch. Fourth, delivery systems should be evaluated in orthotopic, heterogeneous, and clinically relevant models. Carrier fluorescence, total brain concentration, and subcutaneous xenograft suppression are insufficient substitutes for active drug exposure in infiltrative glioma. Fifth, clinical studies should incorporate safety and scheduling considerations. The OPARATIC experience shows that promising intratumoral penetration can coexist with clinically important hematological toxicity[9].

Finally, contradictory findings should be integrated rather than excluded. A balanced synthesis can accommodate transporter-mediated BBB effects, absent MRP1-dependent TMZ sensitization in selected cell systems, nontransport roles of ABCB5, and increased GLUT3-mediated TMZ uptake[23,33,35,38]. Together, these observations support a net-flux and compartment-specific model rather than a single universal efflux mechanism.

Standardization priorities for transport experiments

Transporter studies should report the exact cell background, transporter abundance and localization, barrier integrity, direction of flux, recovery or mass balance, TMZ stability, and whether the analytical method distinguishes parent TMZ from degradation products. Genetic loss and rescue are preferable to inhibitor-only inference because many inhibitors have off-target effects and can change membrane or metabolic states. Known transporter substrates remain useful positive controls, but their movement cannot substitute for direct TMZ quantification. When a transporter intervention changes cytotoxicity without changing intracellular TMZ, downstream signaling should be investigated before a transport mechanism is proposed.

Table 3. Claim–evidence classification for transporter- and vesicle-associated TMZ mechanisms

Candidate mechanism	Anatomical/cellular compartment	TMZ itself measured?	Principal evidence	Class	Calibrated interpretation	Main limitation
ABCB1/ABCG2[23]	BBB endothelium	Yes	Combined Abcb1a/b-Abcg2 deletion increased mouse brain penetration approximately 1.5-fold without changing systemic exposure; elacridar produced a similar effect	A	Strongest direct evidence that endothelial transport restricts TMZ brain exposure	Mouse genetics/pharmacological inhibition; modest effect size; clinical blockade not established
P-glycoprotein in glioblastoma cell lines[30]	Tumor cells	No	P-glycoprotein expression did not appear to explain TMZ resistance in the tested models	N	Negative, model-specific evidence against a uniform tumor-cell P-glycoprotein mechanism	Does not exclude endothelial transport or effects in other tumor states
TMZ-Wnt3-ABCB1 regulation[31,32]	BBB cells and glioblastoma stem-like cells	No; transporter activity assessed with other substrates	TMZ reduced Wnt signaling, ABCB1/P-glycoprotein abundance, and probe or known-substrate transport	B/C	TMZ remodels a multidrug-resistance state	Does not show P-glycoprotein-mediated TMZ transport
MRP1/ABCC1[33]	Primary and recurrent glioblastoma models	No direct TMZ flux	MK571 or MRP1 knockdown failed to alter TMZ response	N	Important negative evidence for direct MRP1-dependent TMZ resistance in these systems	Substrate-, model-, and intervention-specific
NRF2-ABCC1 axis[34]	TMZ-resistant glioblastoma cells	No; glutathione/redox endpoints	High NRF2/ABCC1 state altered glutathione handling and ferroptosis susceptibility	B/C	Relevant combination vulnerability, not direct TMZ efflux	Transported substrate is mainly glutathione-related material
ABCB5[35]	Glioblastoma cells and xenografts	No	ABCB5 targeting enhanced TMZ-associated apoptosis and reduced tumor growth, with cell-cycle-checkpoint involvement	B	Functional sensitization with plausible nontransport mechanisms	Direct TMZ transport not demonstrated
ABCA1[36,37]	Glioma stem-like, cholesterol, and immune-state models	No	ABCA1-associated expression and perturbation correlated with resistance phenotypes	C	Cholesterol/state regulation associated with TMZ response	Expression and diagnostic ROC AUC must not be mistaken for TMZ PK
GLUT3[38]	Glioblastoma cells and xenografts	Yes, uptake-focused	Transporter perturbation and uptake experiments supported increased TMZ entry and chemosensitivity	A for influx	Direct uptake evidence; demonstrates that net exposure depends on influx as well as removal	Does not address efflux or endothelial delivery
sEVs/PTRF-Cavin1[60]	TMZ-resistant glioblastoma cells	Yes; reported TMZ LC–MS/MRM	EPIC-1042 approximately doubled intracellular TMZ and reduced TMZ in the small-EV fraction	A/B (claim-specific)	Drug-partitioning evidence; intravesicular localization not established	TMZ assay validation and normalization incompletely specified; multiple mechanisms
Cholesterol efflux/lipid rafts[47]	TMZ-resistant glioblastoma cells	No	Ginsenosides altered cholesterol efflux, raft distribution, and TMZ sensitivity	C	Membrane-state sensitization	"Efflux" refers to cholesterol, not demonstrated TMZ export

Classes: A, direct analyte-resolved evidence; B, functional exposure or pharmacodynamic evidence; C, expression or mechanistic association; N, negative or contradictory evidence. TMZ: Temozolomide; ABCB1: ATP-binding cassette subfamily B member 1; ABCG2: ATP-binding cassette subfamily G member 2; BBB: blood–brain barrier; MRP1: multidrug resistance protein 1; ABCC1: ATP-binding cassette subfamily C member 1; NRF2: nuclear factor erythroid 2-related factor 2; ABCB5: ATP-binding cassette subfamily B member 5; ABCA1: ATP-binding cassette subfamily A member 1; ROC: receiver operating characteristic; AUC: area under the concentration–time curve; PK: pharmacokinetics; GLUT3: glucose transporter 3; sEVs: small extracellular vesicles; LC–MS: liquid chromatography-mass spectrometry; MRM: multiple-reaction monitoring.

The same rigor applies to vesicles. Investigators should distinguish total extracellular TMZ, vesicle-associated TMZ, protein-bound or precipitated drug, and contamination introduced during isolation. Reporting

particle number without drug mass cannot establish pharmacologically meaningful export. MicroRNA and stem-cell studies show that resistance can spread through regulatory information and cell-state transitions independently of drug removal [98,99]. Gene-directed combinations such as TMZ with siHOXB9 likewise require separation of gene-silencing effects from altered drug exposure[100].

Standardization priorities for animal delivery studies

Orthotopic models should be favored over subcutaneous tumors, with explicit reporting of tumor location, volume, vascular phenotype, MGMT status, immune context, sex, and randomization. Pharmacokinetic sampling should include plasma, contralateral brain, enhancing tumor, and infiltrative margin when technically feasible. A single terminal concentration cannot define exposure; serial AUC, unbound fraction, or microdialysis data are more informative. Efficacy studies should use clinically interpretable schedules and report neurological toxicity, hematology, body weight, organ pathology, and survival with prespecified humane endpoints.

For nanosystems, batch-level properties should be measured in the dosing medium, not only in water. Protein-corona formation, aggregation, sterilization, storage stability, and drug leakage can alter *in vivo* behavior. Studies should disclose whether an apparent brain signal derives from the intact carrier, released fluorophore, vascular retention, or parenchymal drug. Syntheses of ferroptosis-based nanotherapeutics emphasize the appeal of combining delivery with redox vulnerability but also reveal heterogeneity in models and endpoints that limits cross-study comparison[101].

Clinical trial priorities and decision gates

Early clinical studies should define a mechanistic success threshold before examining efficacy. For focused ultrasound, this could combine a prespecified increase in regional TMZ exposure with acceptable hemorrhage and edema rates. For transporter inhibition, it could require a brain/plasma change without clinically meaningful systemic PK alteration. For intranasal or local delivery, it should include absolute dose recovery and evidence that drug reaches disease beyond the administration site. If the mechanistic threshold is not met, a survival signal in a small uncontrolled study should trigger reassessment rather than escalation.

Later trials should stratify or adjust for MGMT promoter methylation, isocitrate dehydrogenase (IDH) status, extent of resection, corticosteroid use, performance status, and subsequent therapies. Imaging, tissue PK, and liquid biomarkers should be scheduled to distinguish true progression, pseudoprogression, and treatment-induced permeability changes. Reviews of future TMZ strategies consistently emphasize combination therapy, but clinical development will improve only when each combination has a falsifiable delivery hypothesis and a biomarker showing that the intended barrier was altered[102].

What would change clinical practice?

A practice-changing result would link four levels of evidence in the same program: validated regional TMZ measurement; demonstration that the intervention changes that exposure; confirmation that tumors retain downstream TMZ susceptibility; and randomized improvement in a patient-centered endpoint with acceptable toxicity. No current approach fully satisfies all four levels. The evidence is strongest for the existence of partial human CNS penetration, experimental ABCB1/ABCG2 restriction, and technical feasibility of focused-ultrasound opening. It is weaker for routine systemic transporter inhibition, clinically effective vesicle-export blockade, and most nanocarrier claims at infiltrative margins.

This staged interpretation avoids two opposite errors: dismissing delivery because oral TMZ reaches CSF, and attributing all resistance to poor delivery despite dominant DNA-repair or cell-state mechanisms. The appropriate clinical question is patient- and compartment-specific: is inadequate exposure a modifiable bottleneck in a tumor that can still respond when exposure is increased?

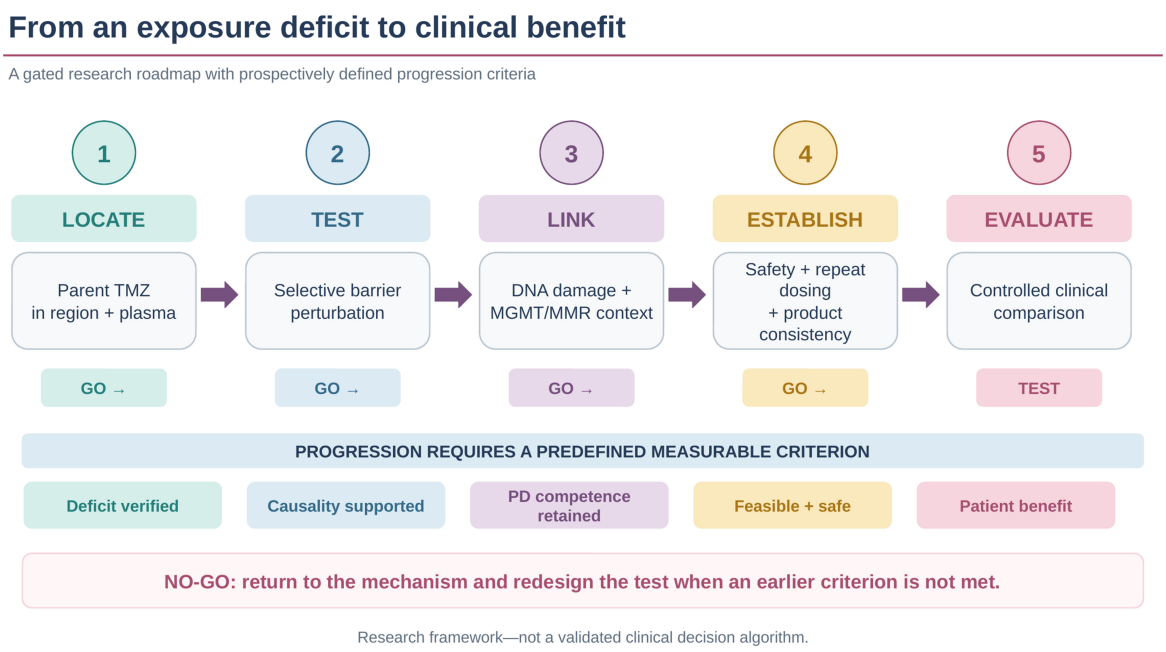


Figure 2. Proposed research roadmap from an exposure deficit to clinical benefit. Each step links a mechanistic question to a measurable endpoint. MGMT and MMR are considered separately. Progression criteria require prospective definition and validation; this framework does not prescribe patient treatment. This figure was created by the authors using Python/python-pptx and LibreOffice. No AI-based image-generation tools or third-party graphical elements were used. MGMT: O^6-Methylguanine-DNA methyltransferase; MMR: mismatch-repair; TMZ: temozolomide.

A testable roadmap from exposure deficit to clinical benefit

We propose a sequence of linked research decisions rather than a universal delivery platform. First, establish whether inadequate parent-TMZ exposure is present in the compartment of interest using a stability-controlled assay and paired systemic sampling. Second, test the implicated barrier with a selective intervention and matched controls. Third, determine whether any exposure gain produces the expected DNA-damage response in the relevant MGMT/MMR context. Fourth, establish reproducibility, manufacturing or device feasibility, regional safety, and combination scheduling before controlled clinical evaluation. Failure at an earlier step should prompt reassessment of the mechanism rather than attribution of a later efficacy signal to improved delivery. Figure 2 summarizes this proposed roadmap; it is a research framework, not a validated clinical decision algorithm.

Figure 3 integrates these translational priorities with the underlying exposure barriers and DNA-damage pathways, emphasizing why improved delivery and reversal of cellular resistance are not interchangeable outcomes.

CONCLUSIONS

TMZ disposition and treatment response are constrained across multiple anatomical and cellular compartments, but transporter-associated phenotypes should not be equated with direct TMZ export. The strongest evidence supports an experimental contribution of endothelial ABCB1/ABCG2 to brain exposure; evidence from tumor cells is more heterogeneous and includes negative findings, alternative substrates, nontransport signaling, and uptake-dependent sensitivity. Across the audited delivery studies, formulation innovation has outpaced analyte-resolved pharmacology: carrier characterization, localization, uptake, and

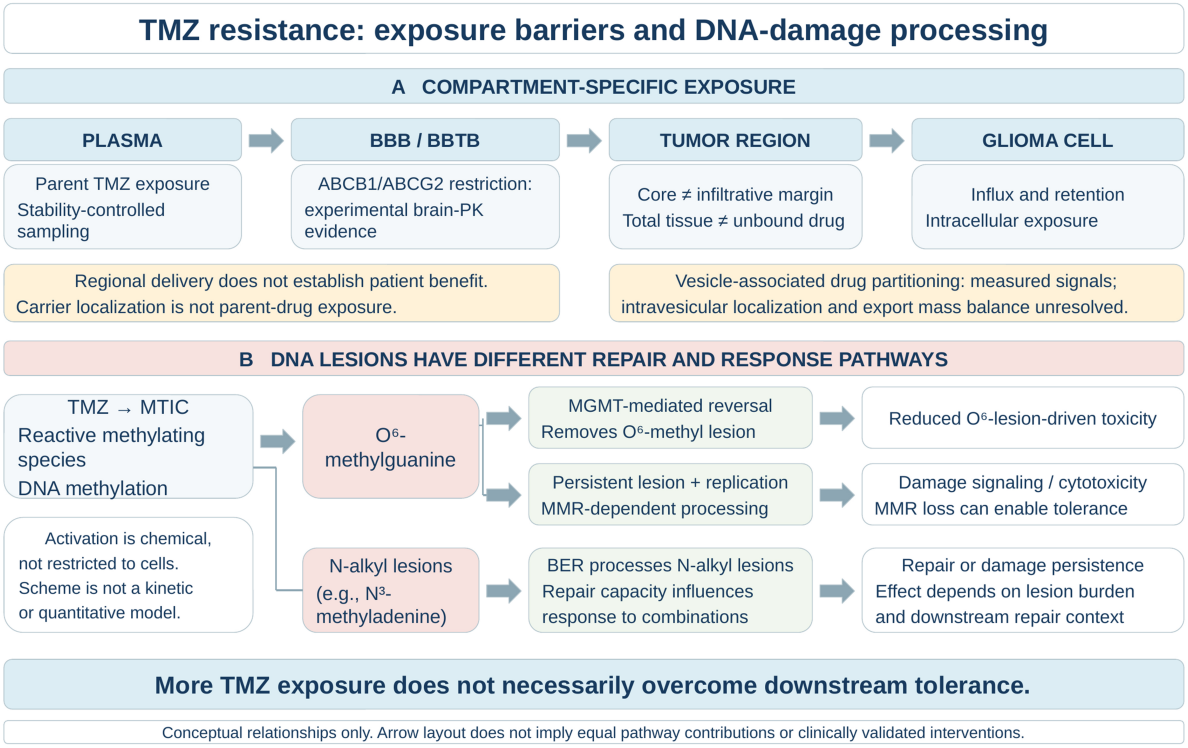


Figure 3. Exposure barriers and DNA-damage processing jointly shape the response to TMZ. (A) Compartment-specific exposure must be distinguished from carrier localization and clinical efficacy; vesicle-associated drug signals alone do not establish intravesicular localization or export mass balance; (B) Chemical activation generates methylating species and distinct DNA lesions. MGMT-mediated removal of O^6-methylguanine and MMR-dependent processing of persistent lesions have different implications for response, whereas BER processes N-alkyl lesions. The diagram summarizes the mechanisms discussed in "DNA-repair context determines whether increased exposure is useful", "Endothelial transport at the blood–brain and blood–brain tumor barriers", "Tumor-cell transport: direct efflux, uptake, and nontransport functions", and "Vesicles, membrane remodeling, and intracellular redistribution", as well as in Table 3[2,23,60]; it is neither a quantitative kinetic model nor a validated treatment algorithm. This figure was created by the authors using Python/python-pptx and LibreOffice. No AI-based image-generation tools or third-party graphical elements were used. TMZ: Temozolomide; MGMT: O^6-methylguanine-DNA methyltransferase; MMR: mismatch-repair; BER: base-excision repair; BBB: blood–brain barrier; BBTB: blood–brain tumor barrier; ABCB1: ATP-binding cassette subfamily B member 1; ABCG2: ATP-binding cassette subfamily G member 2; PK: pharmacokinetics; MTIC: 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide.

efficacy are common, whereas direct parent-TMZ exposure measurements in the relevant intracranial compartment remain exceptional. In particular, survival outcomes in single-arm focused-ultrasound studies cannot establish an ultrasound-attributable benefit without a randomized comparator.

The principal translational gap is therefore an endpoint mismatch between what is engineered and what is measured. Future programs should link anatomical compartment, analyte identity, parent or unbound drug exposure, pharmacodynamic response, safety, and clinical feasibility within a single evidence chain. This standard would prevent carrier signals from being mistaken for TMZ delivery and prioritize platforms that measurably improve exposure at infiltrative, pharmacologically responsive tumor sites.

DECLARATIONS

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Supervision, project administration and funding acquisition: Zhang W, Lin X
All authors approved the submitted version and agree to be accountable for the accuracy and integrity of the work.

Availability of data and materials

Supplementary Table 1 provides the structured study-level audit of 15 representative TMZ-containing delivery-platform studies. No new patient-level dataset was generated.

AI and AI-assisted tools statement

During preparation of this manuscript, the authors used OpenAI (version GPT-5.6, released July 9, 2026). for English grammar checking, formatting assistance, and consistency checks. The authors reviewed and revised all AI-assisted text, independently verified the cited sources and scientific interpretations, and take full responsibility for the final manuscript and figures.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Supplementary Materials

Supplementary Materials

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