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Introduction: Glioblastoma (GB) is one of the most challenging central nervous system (CNS) tumors in treatment options and response, urging the development of novel management strategies. The anti-alcoholism drug, disulfiram (DS), has a potential anticancer activity, and its complex mechanism of action is assumed to be well exploited against the heterogeneous GB.

Area covered: Through a systematic literature review about repositioning DS to GB treatment, an evaluation of the clinical, pharmacological, and formulation strategies is provided to specify the challenges of drug delivery and thus to advance its clinical translation. From 6 databases, 35 articles were selected, including case report (1); clinical trials (3); original articles mainly representing in vitro and preclinical pharmacological data, and 10 dealing with technological approaches.

Expert opinion: The repositioning of DS in GB treatment is facing drug and tumor-associated limitations due to the oral drug's low bioavailability, unwanted metabolism, and inefficient delivery to brain-tumor tissue. Development strategies using molecular encapsulation of DS and the parenteral dosage forms improve the anticancer pharmacology of the drug. The development of optimized drug delivery systems (DDS) shows promise for the clinical translation of DS into GB adjuvant therapy.

Keywords: anticancer activity; bioavailability; brain tumor; disulfiram; drug delivery systems; drug repositioning; glioblastoma, formulation development

1. Introduction

1.1 Epidemiology, pathogenesis and treatment of GB

Malignant brain and other CNS tumors account for a small proportion, approximately 1% of all invasive cancer cases, but are the most commonly diagnosed solid tumor in children and adolescents and the leading cause of cancer death among males aged <40 years and females aged <20 years [1].

GB is a quite heterogeneous and undifferentiated type of brain tumor, characterized by diffuse invasiveness, high recurrence rate and low survival rate [2,3]. It

is also the most common malignant primary tumor of CNS, accounting for 14.5% of all CNS tumors and 48.6% of malignant brain tumors [4].

By the World Health Organization (WHO) classification of CNS tumors, it is categorized as grade IV, which is the most severe category, relatively resistant to therapy and correspondingly with poor prognosis [5]. The median overall survival (OS) of GB patients is at only 15 months [3,4]. The incidence of it varies from 3.19 to 4.17 cases per 100,000 person-years, depending on the reports [4]. The risk of being diagnosed with this type of brain tumor increases with age and is significantly more common in men [3,4].

The tumor presents as a relatively large, irregularly shaped heterogeneous mass, characterized by multifocal necrosis, increased mitotic activity and proliferation of vascular endothelial cells. It is usually located in the white matter of the cerebral hemispheres and surrounded by vasogenic edema [3,6].

An important aspect of the pathogenesis of GB is that malignant transformation results from the sequential accumulation of genetic alterations and abnormal regulation of growth factor signaling pathways [3]. The 5^{th} edition of the WHO classification, published in 2021, incorporates numerous molecular changes with clinicopathologic utility that are important for the most accurate classification of CNS neoplasms [7]. According to this, the key diagnostic genes, molecules, pathways, and/or combinations in GB are isocitrate dehydrogenase (IDH) -wildtype status, telomerase reverse transcriptase (TERT) promoter mutation, chromosomes 7 gain and 10 loss, endothelial growth factor receptor (EGFR) amplification.

The complex genetic background and the limited permeability of the blood-brain barrier (BBB) contribute to the increased tumor resistance [3,6]. The presence of glioma stem cells (GSC) show resistance to radiotherapy (RT) via preferential activation of DNA-damage-response pathways; and to alkylating agent-based chemotherapy via O6-

methylguanine-DNA methyltransferase (MGMT), the inhibition of apoptosis and the up-regulation of multidrug resistance genes [3,6]. The MGMT and IDH status have a prognostic value, the methylation of MGMT promoter (negative expression of MGMT) is associated with a favorable outcome and with sensitivity to alkylating agents [3], similarly, the mutation of IDH is associated with better survival [3].

Despite, the growing tumor molecular knowledge, which provides visions for the improvement of existing therapeutic strategies and the development of a new paradigm for the management of this deadly malignancy [8], the current treatment of GB is limited and unspecific [3].

The "gold standard" treatment consists primarily of surgery, RT, and concomitant or adjuvant chemotherapy [3]. The resection is maximal with neurological function maintenance [8], but due to the infiltrating tumor growth relapse may occur in the margin of the original lesion [8]. The RT is associated with risk factors, like neuronal damage and radio-resistance of some tumors due to the presence of GSC. The only standard chemotherapy is the orally administrated alkylating agent, temozolomide (TMZ), which ultimately has just lightly increased the survival of patients, confronted by the expression of MGMT [8,9]. There is no commonly accepted standard of care for recurrent GB, when most of the patients are ineligible for re-operation or re-irradiation [10]. In case of TMZ-resistance, carmustine (alkylating agent) and bevacizumab (anti-angiogenic monoclonal antibody) can be administered, however, they have a significant side effect profile, are less effective, and presuppose invasive use (implantable, intravenous) [6].

Due to the lack of effective treatment options, the survival for GB is lagging behind; the 5-year survival is less than 6%, which is the lowest long-term survival rate of malignant brain tumors, drawing attention to the unmet need for successful target inhibition and drug delivery strategies [11]. Therefore, hundreds of clinical trials are

currently underway in GB, with various candidates at different stages of development, hoping for a breakthrough. Rajaratnam et al. [6], published a comprehensive list of these competent therapeutic agents for GB. Considered as a rare disease, in GB treatment the growing intention to repurpose previously approved non-chemotherapeutic agents with potential anticancer activity offers the advantage of time- and cost-effective development and clinical translation [12].

1.2 Original indication and repositioning of DS

The anti-alcoholism drug, DS or tetraethylthiuram disulfide, used in clinics for 70 years, interferes with the metabolism of ethanol, and irreversibly inhibits the activity of aldehyde dehydrogenase (ALDH), by competing with nicotinamide adenine dinucleotide at the cysteine (Cys) residue in the active site of the enzyme [13,14]. ALDH is responsible for the oxidation of acetaldehyde into acetate, and thereby elicits excessive accumulation of acetaldehyde, leading to distressing symptoms like dyspnea, tachycardia, hypotension, and headache [14]. Based upon this ethyl alcohol-DS interaction, DS was proposed for the treatment of chronic alcoholism [15]. However, this is the current approved indication of DS, but firstly in 1930s, it found a medicinal use as antiparasitic (scabicide, vermicide) agent [15-17].

Due to its prominence safety and tolerability profile, in the last 100 years extensive investigations have been carried out to explore other biomedical and pharmacological effects [18], e.g. in cocaine dependence [19], obesity [20], intraocular pressure [21], bacterial, fungal and viral infections [16,22-29] and human cancers such as melanoma, non-small cell lung cancer, liver cancer, breast cancer, prostate cancer, pancreatic cancer, head and neck squamous cell carcinoma, atypical teratoid/rhabdoid tumors and GB [30,31].

High expression of ALDH is a functional marker of cancer stem cells (CSC) and is believed to be involved in maintaining the progenitor cell phenotype [32]. The inhibition of ALDH is therefore considered an attractive approach to tackle GB, by blocking GSC division to non-stem daughter cells and inhibiting stem cell derived tumor-mass regeneration after primary resection [33]. This first hypothesis, introducing DS for enhancing GB treatment due to ALDH inhibition of GSCs is from 2009 [32]; and in the same year, *in vitro* was demonstrated that DS and copper (Cu) increases sensitivity to cytotoxic drugs by blocking nuclear factor kappa B (NF-κB) activity and increasing levels of intracellular reactive oxygen species (ROS) in GB [34]. DS is mentioned in context of GB in the last decade, however, its anti-cancer activity dates back to 1970s, when the total resolution of metastasis was spotted in a female alcoholism patient with breast cancer [14,30]. Since 1990s, cumulative evidence has been revealing the tumor-inhibiting effect of DS [35] and its metabolites [36]. According to ClinicalTrials.gov, 22 clinical trials were carried out to explore the antitumor potential of DS in various cancers [14], including in majority GB. In 2013 is suggested to use as an adjunct to the Stupp Protocol [37]. Recently, Zou et al. [38], performed a pathway enrichment analysis, suggesting, that alcoholism may share a common pathway with glioma, thus DS may have a proven effect in glioma treatment.

The great clinical interest and the promising results from preclinical trials led our multidisciplinary team to evaluate the current status of DS in GB through a systematic literature review, emphasizing the clinical relevance. However, there are precious reviews about DS use in the field of cancer treatment [14,18,30,35,39-43] and gliomas [44], but none of them evaluated the challenges of repositioning DS for the treatment of this high-grade tumor from both pharmacological and technological point of view; therefore, the

focused overview covered in this critical review is desired in the hope of advancing its clinical translation.

2. Methods

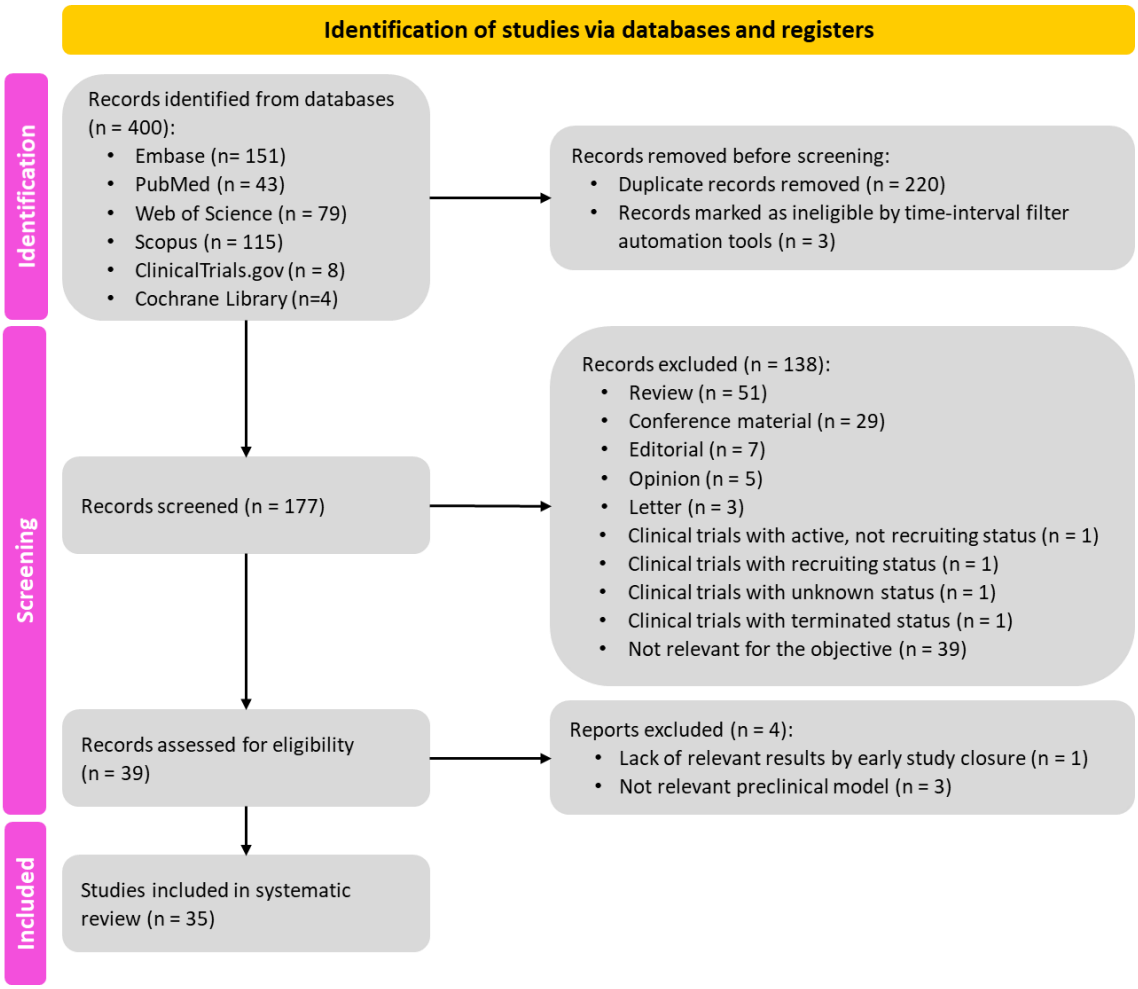
Data sources were obtained from PubMed, Web of Science, Scopus, Embase, Cochrane Library, and ClinicalTrials.gov databases, using the combinations of the terms: disulfiram "AND" glioblastoma. The interrogated time interval was from 2009 to 2022 July, according to the date of publication of one of the first articles, which hypothesized the efficacy of DS in GB treatment, described by Kast et al. [32]. The eligibility system of the hits was built up by determining the inclusion and exclusion criteria. Original articles, publishing results of a registered clinical trial, were considered together, and noted as duplications. Data sources were selected according to the perspectives of this review, focusing on the clinical, pharmacological and formulation development evaluation of DS in GB treatment. **Table 1** represents the detailed data selection procedure. This review was compiled following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [45]. **Figure 1** illustrates the most relevant data for synthesis of the results.

Table 1. Summary of data selection procedure.

Data source selection	
Included sources	• Original articles • Case reports • Completed controlled clinical trials
Excluded sources	• Reviews • Opinions • Editorial articles • Conference abstracts • Withdrawn articles • Clinical trials with recruiting; active, not recruiting; terminated; suspended; unknown status; or with a lack of relevant results
Perspectives of data selection	

Clinical evaluation	• Study phase • Aim of trial • Study design • Intervention scheme • DS dose and administration strategy • Number of participants • Age and gender • Results and the outcome measures (OS and PFS)
Pharmacological evaluation	• Mechanism of action • Pharmacokinetics' data
Formulation development evaluation	• Route of administration • Formulation strategies • Excipient type and role • Results and clinical relevance

1 Abbreviations: OS=overall survival; PFS=progression-free survival



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3 **Figure 1.** PRISMA-2020 flow diagram showing relevant articles included in the

4 systematic review.

3. Results and Discussion

3.1 The repositioning status of DS in GB: from in vitro studies to clinical trials

3.1.1 In vitro and preclinical experiments

The number of original articles published in this field shows a growing interest in DS repositioning for GB treatment with the first original article from 2012. In total, 400 records were identified from 6 databases and after application of the eligibility criteria mentioned under methods, 35 articles were included (**Supplementary material**).

The majority of the included studies are based on early phase experiments: 1 article with in-silico, 15 articles with in vitro, and 13 articles with preclinical experiments, using allograft or xenograft GB models.

The primary focus in the retrieved literature is on the pharmacological aspects of DS, and a few articles (10) deal with technological challenges. Multiple *in vitro* and preclinical studies have demonstrated the promising anticancer effects of DS [12]; the multi-targeted anti-GB mechanism of action rely on ALDH-, MGMT-, NF-κB-, proteasome inhibition, increased intracellular ROS generation, and on a combination of these actions [46]. While, the novel technological approaches are generally based on the design of parenteral formulations with favorable pharmacokinetics (PK) for DS delivery, but none of them reached the clinical phase stage.

In general, the *in vitro* evaluations have been undertaken using two-dimensional (2D) culture, however, it is increasingly appreciated that such models are ill-equipped to reproduce the multifaceted characteristics of GB [47], in contrast with 3D culture systems, which can better mimic a natural tumor mass [48]. In addition, the established cell lines were shown to be less representative for GB tumors, as failing to recapitulate the phenotype and harboring non-parental genotypic mutations [49]. Given the growing understanding of GB biology, the discovery of GSCs, and their role in tumor formation

and therapeutic resistance, the GB research tendency is turning more towards patient-derived cells GSCs and xenografts [49]. Therefore, new *in vitro* 3D cell culture systems have special role in testing compounds using models developed directly from patient tumor samples and primary cell cultures from GBs [50,51], improving the translation toward the human situation.

3.1.2 Clinical trials

On *ClinicalTrials.gov* 8 studies were found, 4 with completed status, and of these, 1 trial (NCT02678975) was excluded, because of the early study closure with a lack of relevant results.

The human evidence is represented by phase I/II controlled trials (NCT01907165, NCT03034135, NCT02770378) together with 1 case report [46]. The controlled trials are non-randomized, using DS in combination with current standards of care (NCT01907165, NCT03034135) or similarly with TMZ, but in a multi-combinational therapy, namely "Coordinated Undermining of Survival Paths with 9 repurposed non-oncological drugs" (CUSP9) (NCT02770378). Moreover, Halatsch et al. [52], published the use of the CUSP9 approach on 8 patients with heavily pre-treated recurrent GB. They were ineligible for re-resection, more cytotoxic chemotherapy or clinical trial participation. In total 70 GB patients received DS in their treatment strategy, of these 61 patients were followed under controlled conditions.

DS commercially is available via oral administration, in 200-500 mg doses [13], thus in all the trials was taken *per os* in a daily dose range between 240-1000 mg, with (NCT01907165, NCT03034135) or without (NCT01907165, NCT02770378) Cu supplementation [10,12,53,54]. The maximum tolerable dose (MTD) with adjuvant TMZ was 500mg [12,53] and the toxicity and pharmacodynamic (PD) effects of DS were similar with or without concurrent Cu [53].

There is a lack of randomized studies and none of the existent trials has as primary objective to determine the survival data; however, these data were measured during the experiments, which are comparable with the survival rate of GB. **Table 2** summarizes the details of the included clinical trials.

According to *in vitro* and animal experiments, the addition of DS could enhance the current treatment strategy, due to its unspecific antitumor activity, promoting cell death, sensitizing tumor cells to RT and reversing chemotherapy resistance, [41,54]. However, the completed clinical trials had unsatisfactory results, not supporting the promising preclinical data [12,37,53-56]. The reason for the discrepancy between these is multifactorial, can be attributed to the drug`s poor solubility, instability, low bioavailability, the rapid, unwanted metabolism, the poor delivery efficiency to tumor tissue [14,57], and also to the weak representativity of the available GB models [49].

Antitumor activity of DS is still needed to be improved in clinical [14], as the orally administered drug does not reach the desired effects in the tumor area. The well-known PD and PK of DS from alcohol dependence treatment cannot be entirely extended to GB; the metabolic mechanism of oral DS in the liver explains the success in anti-alcoholism, but fails to achieve the same efficacy in clinical cancer treatment [14].

Table 2. Summary of clinical trials showing the clinical relevance of DS use for GB treatment.

Ref.	NCT	P	Study design	Aim	Intervention	DS dose	MoA	Nr. P.	A&G	Results	Survival data	
											OS	*PFS*
Huang et al. **[12]**	NCT01907165	I	Non-randomized, open-label, single-arm	• Safety, MTD, DLT • Preliminary efficacy of DS in combination with adjuvant TMZ • Proteasome inhibition	DS + TMZ	500 or 1000 mg	PO 1x	12	≥18Y F+M	• MTD=500 mg • DLT=1000 mg • Acceptable safety profile • Limited proteasome inhibition	*12.1 months (95 % CI 4.9–24.5)	*5.4 months (95 % CI 0–17.3)
Huang et al. **[53]**	NCT01907165	I	Non-randomized, open-label, single-arm	• Toxicity and PD data of DS with and without Cu combined with adjuvant TMZ	DS + TMZ + Cu	500 mg	PO 1x	18	≥18Y F+M	• Addition of Cu to DS did not increase toxicity • Limited proteasome inhibition	*14.0 months (95% CI 8.3–19.6)	*4.5 months (95% CI 0.8–8.2)
Huang et al. **[54]**	NCT03034135	II	Non-randomized, open-label,	• Potential effectiveness of DS + Cu to re-sensitize	DS + TMZ + Cu	80 mg	PO 3x	21	≥18Y F+M	• Tolerable • Low clinical benefit	*7.1 months (95% CI: 5.8–8.5)	*1.7 months (95% CI: 1.4–1.9)

			single-arm	recurrent GB to TMZ							
Halatsch et al. [10]	NCT 0277 0378	I / II	Non-randomized, open-label, single-arm	• Treatment resistance avoidance with multiple drug combination • Safety	$CUSP_{V3}$ + TMZ	250 mg	PO 1x or 2x	10	≥18Y F+M	• Potential positive effect • Under careful monitoring is safe	**50% (95% CI, 27–93%).	**50% (95% CI, 27–93%).

1 Abbreviations: 1x=once daily, 2x=twice daily, 3x=three times a day, A&G=age and gender, Cu=copper, $CUSP_{V3}$=Coordinated Undermining of Survival Paths
2 combining 9 repurposed non-oncological drugs (aprepitant, auranofin, captopril, celecoxib, DS, itraconazole, minocycline, ritonavir sertraline) with
3 metronomic temozolomide—version 3, DS=disulfiram, DLT=dose-limiting toxicity of DS, F=female, GB=glioblastoma, M=male, M+F=both genders
4 represented, MoA=method of administration, MTD=maximum tolerated dose of DS, NCT=registration number of clinical trial on *ClinicalTrials.gov*, Nr.
5 P.=number of enrolled patients, OS=overall survival, P=phase, PD=pharmacodynamics, PO=per oral, PFS=progression free survival, TMZ=temozolomide,
6 Y=years
7 Notes: *Median survival data measured from the initiation of DS therapy, **Survival rate measured from the initiation of CUSP9 therapy

3.1.3 Combinational therapies with DS to treat GB

DS is a non-chemotherapeutic anticancer agent and may play adjuvant role in GB treatment, therefore, its inclusion in multi-combinational strategies is an increasingly studied therapeutic direction, as single agent therapies will never be enough to stop or reduce recurrence in GB treatment, but un-specific combinations have the potential to be effective while also reducing recurrence [58]; thus the combination drug regimens are proposed to overcome the heterogenic nature of GB tumors [59] (**Table 3**).

The current standard of care for GB is already considered a multimodal treatment strategy, and the addition of DS is proposed as an adjunct to the Stupp protocol due to its radio/chemo-sensitization effect [32,37], however there are contradictory results too. Zirjacks et al. [31] did not observe a TMZ-sensitizing effect of DS; quite the contrary, TMZ attenuated the inhibitory effect of DS on clonogenic survival, interfering with triggered lethal pathways. Moreover, diethyldithiocarbamate (DDC), one of the pharmaco-active metabolites of DS, is one of the most effective *in vitro* and *in vivo* radioprotective agents (87). Interestingly, when Strømme et al. [60], studied in vivo the radioprotective effect of DDC and DS, they found, that only DDC possesses this characteristic, as the free thiol from the metabolism of DDC exerts its protective action, which is not present in a significant amount during DS metabolism, however the radioprotective effects of DS in normal cells and RT-sensitizing activity in tumor cells still require a full investigation [61]. Therefore, the addition of DS to the Stupp protocol should be re-evaluated, the possible interactions with RT and TMZ need to be clarified to design the future inclusion of DS in the standard therapy.

The underlying rationale of Cu supplementation is based on the idea that its presence could further increase the antitumor efficacy of DS; enhancing the cytotoxic efficacy of the metal-chelator drug [30,62], however, its addition to DS adjuvant therapy

is a debatable therapeutic strategy in the overviewed literature (**Table 4**). Cu plays a role in both the PD and PK of DS (See: Chapter 3.2 *"Pharmacology of DS in GB"*); and in GB, its level is typically elevated, moreover, the high levels correlate with the occurrence, development, recurrence, and invasion of tumors [14]. Thus, the Cu-dependent cytotoxicity of DS and the modified Cu levels in cancer cells may enable DS to specifically target the tumor [30,62]. The complex of DS and Cu, bis(diethyldithiocarbamate)-copper [$Cu(DDC)_2$], is proposed to be the decisive metabolite for tumor suppressing effects [14], as cellular uptake of it causes an increase in Cu level which provokes massive induction of ROS, leading to DNA damage, proteasome dysfunction and apoptosis [62]. This consideration led to the addition of Cu to DS in mechanistic experiments [31,33,55,57,63-67], however, it is unclear whether such *in vitro* mechanism might be translated to *in vivo* [62]. In clinical trials, the additional Cu does not significantly influence the drug`s efficacy and tolerability (NCT0190716, NCT03034135, NCT02770378). Therefore, the anticancer activity of DS in combination with Cu observed *in vitro* should be treated with caution before translating in human situation [68].

On the idea that multiple drug treatments can target different pathways to enhance the efficacy of treatment and ultimately to improve the prognosis of GB patients [59], the CUSP9 protocol was designed. This GB therapeutic strategy is a poly-pharmaceutical, multitargeting approach, which combines drugs already approved for non-oncological indications to address the intra- and inter-tumoral heterogeneity and to allow for fast clinical translation [10,52,69] potentially. The protocol, including DS in intervention scheme, shows positive preclinical and clinical outcomes [10,69] (**Table 3**). Based on similar principles, multidrug adjuvant cancer treatment (MDACT) strategy is hypnotized to be efficient in GB, combining 6 repurposed drugs which also includes DS [70].

The molecular heterogeneity of GB is linked to differences in survival and treatment response, in accordance the development of personalized treatments is desirable [58]. Specific multi-targeting combinations could be considered as personalized treatment strategies; therefore Garrett et al. [58], identified eight genes that could be used for the characterization of GB and according to this set up personalized, significantly more effective anti-GB drug combinations, e.g. DS with Cu, irinotecan, and pitavastatin, which resulted in a high response rate for five different GB samples (**Table 3**).

The multimodal drug treatment in GB seems to become a new tendency in the management strategy; but the clinical benefit is still ambiguous. Over the toxicity profile of multi-drug treatments and the increased number of interactions, another disadvantage is the complicated administration plan, the intake instructions of different medicines may be hard to follow, and consequently, patient adherence and therapy effectiveness will be reduced.

Table 3. Combinations therapy for GB therapy, containing DS.

Combination strategy	Study type	Observations	Ref.
Stupp Protocol, DS, Cu	Phase I/II	Promising in preclinical trials, but no positive result in clinical use.	[12,53,54]
TMZ, CUSP9		The early development stage also included Cu-gluconate, but since DS chelates Cu in the stomach even without adding exogenous Cu, it has been deleted.	[10]
TMZ, DS, carbenoxolone	*In vitro* + *In vivo*	Inhibition of two distinct interactions between GB and TIME: stress-induced cell-matrix adhesion (DS) and gap junction mediated cell-cell communication (carbenoxolone).	[71]
Regorafenib, DS, Cu		DS and Cu complex combination was found to have a synergistic effect with regorafenib on the tumor associated macrophage polarization, “re-educating” the protumor towards antitumor TAM.	[72]
Honokiol, DS, Cu		DS + Cu present synergistic effect with honokiol (the main active compound in the Chinese herb Hou-Pu) in remodeling TIME.	[73]

Galunisertib, DS		DS sensitizes a therapeutic-resistant GB to the TGF-β receptor inhibitor, galunisertib, while ALDH activity positively correlates with TGF-β-induced mesenchymal properties in GB.	[74]
CUSP9, ABT263 (navitoclax)	*In vitro*	CUSP9 reduced to a very low dosage sensitizes for intrinsic apoptosis and induces mostly synergistic cell death when combined with the ABT263, which restores the pro-apoptotic cellular phenotype, promoting death of cancer cells.	[75]
RT, DS, Cu, metformin		An early phase I CT, using the same combination was terminated due to problems with including patients (NCT03151772).	[76]
Gemcitabine, DS, Cu		DS + Cu enhances the cytotoxicity of gemcitabine on GB stem-like cells due to by induction of ROS and inhibition of both ALDH and the NF-κB pathway.	[63]
Irinotecan, pitavastatin, DS, Cu		The combination targets at least 8 growth-promoting and cell-signaling pathways: topoisomerase, autophagy via the LC3, mevalonate synthesis, proteasome, ALDH, PLK-1, MGMT and NF-κB.	[58,59]

Abbreviations: ALDH=aldehyde dehydrogenase, CSC=cancer stem cells, Cu=copper, CUSP9=Coordinated undermining of survival paths with 9 drugs (aprepitant, auranofin, captopril, celecoxib, DS, itraconazole, minocycline, ritonavir and sertraline), CT=clinical trial, DS=disulfiram, GB=glioblastoma, LC3=light chain 3, MGMT=O6-methylguanine-DNA-methyltransferase, NF-κB=nuclear factor-kappa B, PLK-1=polo-like kinase, ROS=reactive oxygen species, RT=radiotherapy, Stupp protocol=current standard therapy used for newly diagnosed GB, composed by maximal surgical resection, followed by RT (60 Gy in 30 fractions for 6 weeks) plus concomitant TMZ (75 mg/m^2/day for 6 weeks) and then six maintenance cycles of TMZ (150–200 mg/m^2/day for the first 5 days of a 28-day cycle), TAM=tumor associated macrophage, TIME=tumor immune-microenvironment, TGF-β=transforming growth factor beta, TMZ=temozolomide, TNF-α=tumor necrosis factor-alfa

3.2 Pharmacology of DS in GB

Previous reviews have summarized the current state of knowledge about the tumoricidal activity of DS [14,40,41]. Comprehensive mechanisms of action of DS are proposed and many molecular biological targets were identified, however, exact pathway in cancer therapy is not yet fully understood and very little is known about the activity on brain

tumors [14,33]. Therefore, this review is focusing on DS effects studied on glioma models, the mechanisms of metal chelation and protein inhibition. In order to reveal the outcome shift between the preclinical and clinical trials a PK overview is given, highlighting the supposed bioavailability and safety in GB from a clinically relevant perspective.

3.2.1 PDs of DS

3.2.1.1 Mechanism of action

Analyzing the whole spectrum of biological interactions of DS, the main activities from which the anticancer mechanism can be derived are metal chelation and protein inhibition. The structure-effect relationship is determined by the sulfur content of the symmetric molecule, from which, during its decomposition, free thiol groups form. These enable it to form chelate complexes with metal ions (Cu^{2+}, Zn^{2+}), modifying the intracellular trace element-dependent processes and to participate in thiocarbamate-thiol type reactions with free thiol groups of proteins and enzymes (**Figure 2**), inducing inhibitory effect (e.g. inhibition of ALDH family of enzymes or MGMT).

The chemical metal-chelating effect makes DS in clinics act as an ionophore, which chelates metal ions in the extracellular space then transport them via biological membranes and releases them into the intracellular space [30,62]. According to this ability, its anticancer activity overlaps with that of its major metabolite, DDC, and its complex with Cu, $Cu(DDC)_2$, as DS, a symmetrical disulfide molecule, at physiological pH is better than 99% ionized to two molecules of the free thiol group containing DDC [77]. The dissociation of DS into DDC is induced by the presence of Cu, compounds with free sulfhydryl (e.g., reduced glutathione, proteins), acidic environment, heating etc., thus the easy reducibility of DS is required for any discussion of its biological actions, as the

interconvertibility affects their *in vivo* pharmacological activities [77]. Both molecules (DS, DDC) are unstable in acid medium (up to pH 7.0) and forms more stable complexes with heavy metal ions [15,40]. The lipophilic complex with Cu, $Cu(DDC)_2$, accumulates Cu and causes increased level, overcoming the Cu-transporter-controlled regulation of intracellular Cu homoeostasis [63]. This provokes enormous release of ROS (e.g. H_2O_2, hydroxyl radicals) arising from Fenton chemistry and Haber–Weiss reaction [14], that have a vast range of effects, including the induction of apoptosis, DNA damage, and dysfunction of proteasomes. The Cu and DS co-mediated impairment of redox homeostasis is most probably the reason for the observed pleiotropic actions [31], consequently, $Cu(DDC)_2$ is considered as the active, potent anticancer ingredient [14,40,57,78]. This reaction underlies the Cu-dependent anti-GB (**Table 4**) and generally the anticancer activity of DS [40], such as proteasome inhibition [12,33,53,55], leading to the accumulation of poly-ubiquitinated proteins and cytotoxic protein aggregates, which results in the inhibition of cell-cycle progression and subsequent apoptosis [40]. However, it is unclear whether such in vitro mechanism might be translated to in vivo [79]. In *in vitro* assays, on the addition of the Cu^{2+} ions to the media, the cells are exposed to rapid transformation of DS; the mixture immediately results in a highly oxidized intermediate, bis(dialkyliminium)-tetrathiolane dication (Bitt-4^{2+}), and Cu^{1+}, followed by subsequent spontaneous decomposition of small amount of DS to its anionic chelate form DDC, which on further redox reaction with Cu^{2+} and forms a stable complex $Cu(DDC)_2$ with the massive release of ROS [14,40]. The oxidation reactions are relatively rapid and thus may be highly cytotoxic, therefore, the induction of apoptosis in tumor cells by a Cu^{2+} and DS cocktail *in vivo* is difficult to envisage as it is probably not caused by a discrete $Cu(DDC)_2$ complex but rather is due to a reaction [68]. This mechanism is even

more hardly achievable *in vivo*, considering the poor bioavailability of DS and the bimodal cytotoxicity [62].

The protein inhibitory activity of DS is due to its ability to complex the metals of metalloenzymes (carboxylesterase and cholinesterase), or to react with enzyme sulfhydryl groups (i.e. reacts and conjugates with the protein-bound nucleophilic Cys) [64,80] (**Figure 2**). DS and its metabolites form mixed disulfide bridges with a critical Cys (Cys302) near the active site region of ALDH to inactivate the enzyme [64]. The inactivated enzyme may, but need not have the DS moiety bound to it covalently, reaction may occur if a second, suitably positioned vicinal thiol group is present on the enzyme and such a sequence of reactions occur with the cytosolic ALDH [80]. Due this mechanism of ALDH inhibition, DS found medical use in chronic alcoholism treatment and has a potential to be repurposed in recurrent GB treatment, as ALDH is also a decisive enzyme for the stemness of GSCs, responsible for tumor relapse, metastasis and RT- and chemo-resistance [14,32]. Similarly, evidence showed that active site Cys from MGMT (Cys145), critical for DNA repair, was the sole site of DS modification in the enzyme [64]. MGMT is a unique antimutagenic DNA repair protein, removing the mutagenic O6-alkyl groups from guanines, and thus confers resistance to alkylating agents in brain tumors [64]. Therefore, DS, as a MGMT protein modulator, could serve as an adjuvant drug for chemotherapy sensitivity maintenance.

Just in case of alcoholism treatment, in GB treatment the active metabolites contributes to the anticancer activity of DS (**Figure 3**), however S-methylation during its metabolism masks the Cu-chelating functional thiol group and completely abolishes the Cu-dependent cytotoxicity [14], as the intact thiol group in their structure is essential and indispensable for them to chelate divalent transition metal ions [40]. In contrast, the molecular mechanism of ALDH inhibition is mediated also by S-methylated and

subsequent P450-catalyzed oxidation metabolic products of DS [15,78,80,81], however different metabolites of DS inactivate different isozymes of ALDH [19]. DS itself inhibits ALDH1A1 (cytosolic ALDH subfamily playing a pivotal role in embryogenesis and development by mediating retinoic acid signaling, and also related with various properties of CSC, tumor growth and carcinogenesis) more potently that it does ALDH2 (mitochondrial ALDH subtype, crucial for alcohol metabolism), due to the fact that the hydrophobic tunnel in the enzyme's architecture through which the substrate enters is larger in ALDH1 and therefore capable of accommodating DS, a bulky molecule, more effectively [81]. DS and diethylmonothiocarbamate methyl ester sulfoxide and sulfone inhibit both the cytosolic and mitochondrial isoforms of ALDH [82]. DS, possessing strong inhibitory effect on ALDH1A1 [46], was suggested to be investigated as adjunct in GB treatment, as this cytoplasmatic isoform of ALDH is mentioned as a novel CSC marker in human GB [46].

DS, DDC and $Cu(DDC)_2$ are interconvertible *in vivo* but administered separately, they behave differently [60,83,84], the combination of DS and Cu does not have the identical molecular mechanisms to $Cu(DDC)_2$ nor does the simple additive effect of DS and Cu [14]. It should be highlighted that DS and DDC differ in their properties and mode of action [80]. DS inhibits chiefly by reacting with thiol groups of proteins, thereby producing mixed disulfides and releasing DDC as a by-product of the reaction [80]. DDC acts chiefly as a metal ion chelator and a thiol, which can inhibit enzyme action by complexing metals in the active site, or by scavenging free radicals that may be necessary for a reaction [80].

Zirjacks et al. [31] concluded, that the tumoricidal actions of DS seem to be mediated rather by its Cu-overloading than its ALDH-inhibiting function, and the majority of mechanistic studies focus on the cytotoxicity-inducing effect of DS, despite

the more significant DS affinity to proteins, than DDC. However, there is growing literature, suggesting that the anti-GB properties of DS do not rely solely on a single activity, rather all of them contributes to its anticancer mechanism [55]. Consequently, the mechanism of action of DS is presumably non-specific multipotent tumor suppressing activity, as it has a pleiotropic effect on the cell cycle by disrupting the trace element balance, inducing apoptosis and inhibiting various enzymes involved in tumor survival.

Table 4. Anti-GB mechanisms of action of DS and Cu-dependence.

The role of DS in GB therapy	Mechanism of action	Cu-dependence	References
Cytotoxicity	ROS generation	+	[58,63]
	Proteasome inhibition	+/-	[12,33,53,55]
	Ferroptosis induction	0	[85]
TIME modulation	NF-κB inhibition	+	[57,63,71,86,87]
	Angiogenesis inhibition	+	[65]
	ROS generation	+	[72]
	Crippling valosin-containing protein/p97 segregase adaptor NPL4	+	[73,88]
Targeting CSC	ALDH inhibition	+/-	[33,46,55,63,64,89]
RT enhancement	DNA damage promoting	+	[31,55,56,76,85]
Chemotherapy enhancement	MGMT inhibition	-	[46,64]
	PLK-1 expression inhibition	-	[90]
	DNA repair pathways suppression	+	[55]
	ROS generation	+	[72]

Abbreviations: ALDH=aldehyde dehydrogenase, CSC=cancer stem cells, Cu=copper, DS=disulfiram, GB=glioblastoma, MGMT=O6-methylguanine-DNA-methyltransferase, NF-κB=nuclear factor-kappa B, PLK-1=polo-like kinase, RT=radiotherapy, ROS= reactive oxygen species, TAM=tumor associated macrophage, TIME=tumor immune-microenvironment

Notes: (+): Copper dependent mechanism, (-): Copper independent mechanism, (+/-): Divergent results in literature, (0): Copper dependence was not studied

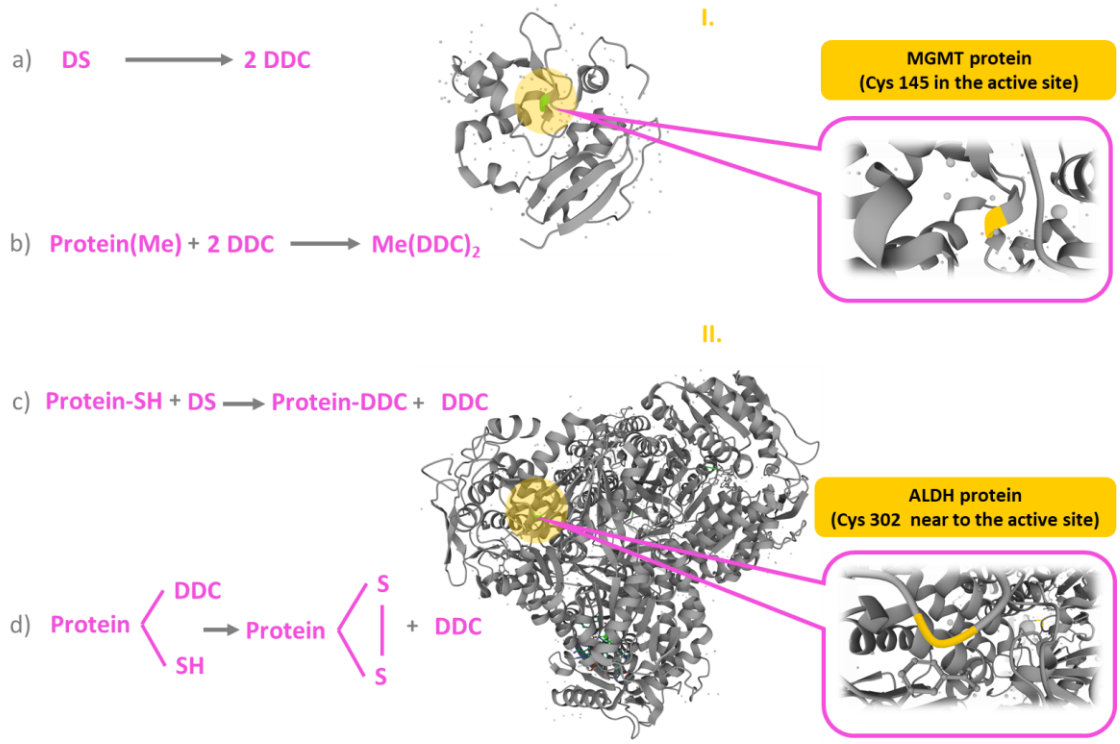


Figure 2. DS metal protein inhibitory activity and the target proteins in GB: MGMT (I), ALDH (II).

Abbreviations: ALDH=aldehyde dehydrogenase, DDC=diethyldithiocarbamate, DS=disulfiram, Cys=cysteine, Me=metal, MGMT= O6-methylguanine-DNA-methyltransferase, Protein(Me)= protein with metal co-factor in its structure, -SH=thiol group -S-S-=disulfide bond.

Notes: DS interacts with proteins in different ways, binding to Cys residue in the active site or near the active site of a protein, modifying its function (c), or chelating the co-factor metal component (a, b). The inactivated enzyme may, but need not have the DS moiety bound to it covalently, (d) reaction may occur if a second, suitably positioned vicinal thiol group is present on the enzyme and such a sequence of reactions occur with the cytosolic ALDH [80]. Origin of protein molecules: https://www.uniprot.org/

3.2.1.2 Toxicity and side effects

The most serious side effects of DS include hepatitis, hepatotoxicity, psychosis, seizures, peripheral neuropathy and optic neuritis [19]. DS inhibits the levels of the cerebrospinal dopamine-β-hydroxylase at high doses; and the low activity of the enzyme correlates with DS sensitivity, leading to a transient psychotic state [19]. This cerebrospinal enzymatic

dysfunction could threaten the safety of DS in GB, especially in formulations, which target directly the brain. However, DS-related neurological toxicities are difficult to distinguish from tumor effects [12]. In GB treatment, neurological symptoms such as ataxia, delirium, dizziness, nausea, and neuropathy can occur, especially after prolonged administration of DS. These adverse effects are mostly self-limited or may be improved by dose reduction [53]. A possible ethanol interaction also can cause serious side effects in patients with weak condition after RT or chemotherapy, therefore the concomitant use of alcohol-containing medicinal (e.g., cough syrups, elixirs) or non-medicinal products during DS therapy requires caution [19].

The most common adverse events related to DS and Cu co-administration were nausea/vomiting [54], which is a risky side effect because it can lead to a loss of oral chemotherapeutic agent dose.

DS was developed and mainly used in adolescent and adult populations [17], though, it was evaluated to target malignant brain tumors affecting the child population, e.g., as a radiosensitizer against atypical teratoid/rhabdoid tumor [91] and as a degradation inducer of the oncoprotein mixed-lineage leukemia against pediatric glioma [92]. Therefore, tolerability and safety data on pediatrics are additionally needed for age-appropriate dosage [17].

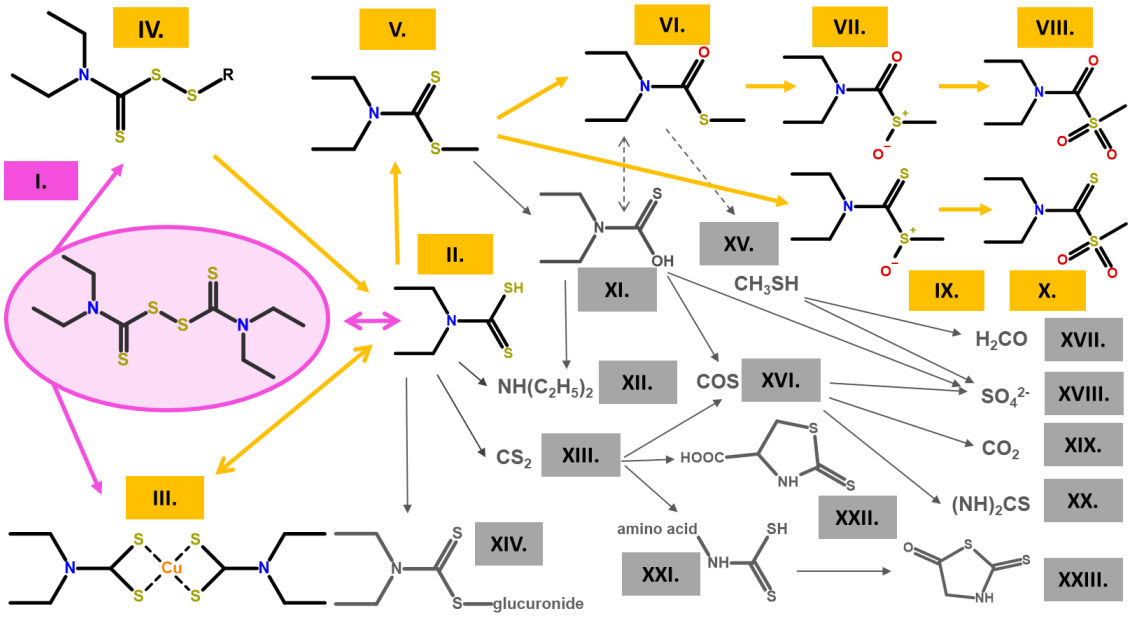
3.2.3 PKs of DS

Orally administered DS is absorbed rapidly but incompletely, in 70-90%. In the strongly acidic juice of the stomach, DS is decomposed to DDC, which is a highly polar and hydrophilic, forming chelate complex with Cu, $Cu(DDC)_2$ [15,19,31,78]. DS and the more stable $Cu(DDC)_2$ are lipophilic, thus the absorption along the entire length of the upper gastrointestinal tract is not restricted to the parent drug but also includes the metal-complex [15,19,31,78]. After distribution across the gastrointestinal mucosa into blood

of portal circulation, the erythrocytic glutathione reductase may split the $Cu(DDC)_2$ complexes into DDC monomers which form mixed disulfides with free thiols of proteins. DS entering the blood may be alternatively reduced by a reaction with serum albumin to DDC and mixed disulfide of DDC with serum albumin [14,78]. DS is tightly bound to plasma proteins, thus preventing the distribution and metabolism [93]. Reaching the liver, DS and DDC are rapidly metabolized and degraded [14,78]. DDC is detoxified by rapid glucuronidation and renal excretion or is decomposed into diethyl-amine and carbon disulfide, which are excreted or exhaled [31]. The rapid degradation of DDC occurs spontaneously at acidic pH, and it can take place in the stomach after ingestion [78]. DS undergoes further bio-transformations, including S-methylation, and S-oxidation, forming compounds with strong inhibitory activity on mitochondrial ALDH (e.g. methyl ester of DDC) [14,15,31,57,78,94]. The detailed metabolic fate of DS is summarized on **Figure 3.** The amount of DS excreted in feces varies from 10% to 30%, the metabolites are mainly excreted via the kidney, lungs and feces too [19].

DS and its metabolites are uniformly distributed throughout the body in various tissues [19], and the brain consistently reveals the least detectable amounts of DS and its metabolites [15], according to the results obtained with radio-labelled DS [60,93]. The enzymes and the redox systems necessary for the biotransformation of DS are present in the blood, liver, and probably most other tissues, hence metabolism of these compounds is likely to occur, to a varying extent, at many sites, so presumably in the brain as well [78]. There is limited knowledge about the exact metabolism of DS in the CNS, metabolites of ^{35}S-labelled DS, such as methyl ester of DDC, glucuronide of DDC, inorganic sulphate, and carbon disulfide, appear in the brain after *i.p.* administration [14,57,78,93,95]. Gunasekaran et al. [95], demonstrated that DS penetrates CNS, and dimethyl sulfoxide increases the entry into the brain, opening reversible the BBB;

however, in general, the content of DS in the brain was quickly reduced with time. Thus, the small lipophilic molecule even if it does manage to diffuse across the BBB it can very quickly diffuse back making it difficult to obtain constant drug levels at the site of action [86], therefore the therapeutically achievable concentrations in the brain might be low. Zirjacks et al. [31] described, that the interstitial concentrations of DS and metabolites in the brain are in equilibrium with the unbound free plasma pool of these compounds, thus the interstitial brain concentrations of DS and metabolites can be expected to be far below 1 µM [31,54]. The main metabolites are lipophilic or highly reactive, and the overwhelming majority of them can be expected to bind to serum albumin, profoundly lowering their free plasma concentrations [31]. Sub-micromolar IC_{50} values indicate potent tumoricidal effects of DS *in vitro* [31,33,55,90]. However, Skaga et al. [69] observed that the marked inhibitory effect of DS is at plasma concentrations well above what could be considered clinically achievable and also the disappointing outcome of clinical trials upon oral DS, does not support the promising results of *in vitro* experiments [31].



Compounds targeting GB due their copper-chelating activity	I., II., III.
Compounds targeting GB due their protein inhibitory activity by binding to active site Cys	I., IV. →X.
Inactive compounds	XI.→ XXIII.

Figure 3. The metabolic fate of DS and the presupposed activity of its metabolites in GB.

Abbreviations: I.= Disulfiram, II.= Diethyldithiocarbamate, III.= Bis(diethyldithiocarbamate)-copper, IV.= Mixed disulfides with protein sulfhydryl groups, V.= Diethyldithiocarbamate methyl ester, VI.= Diethylmonothiocarbamate methyl ester, VII.= Diethylmonothiocarbamate methyl ester sulfoxide, VIII.= Diethylmonothiocarbamate methyl ester sulfone, IX.= Diethyldithiocarbamate methyl ester sulfoxide, X.= Diethyldithiocarbamate methyl ester sulfone, XI.= Diethylmonothiocarbamate, XII.= Diethylamine, , XIII.= Carbon disulphide, XIV.= Diethyldithiocarbamoyl-S-glucuronide, XV.= Methanethiol, XVI.= Carbonyl sulphide, XVII.= Formaldehyde, XVIII.= Sulphate, XIX.= Carbon dioxide, XX.= Thiourea, XXI.= Amino acid dithiocarbamate, XXII.= Thiazolidine-2-thione-4-carboxylic acid, XXIII.= 2-thio-S-thiazolidinon, Cys= cysteine, GB= glioblastoma

3.3 Formulation development of DS intended to treat GB

In chronic alcoholism treatment, the only approved dosage form of DS are tablets, effervescent forms show increased bioavailability, and the enteric-coated tablets improve the transport of intact DDC through the stomach into the alkaline part of the small

intestine, increasing the stability of DS and DDC [19]. There were technological endeavor for the development of implants, which was attractive in the sense that it provided a long-term treatment for the non-complying patient; however, in 1950s, the inadequate understanding of the physicochemical characteristics of implants, the highly variable PK properties and disposition of DS have made such an approach to treatment of alcoholism of equivocal value [15]. Similarly, the repositioning strategy of DS into GB treatment challenging, due to the tumor- and drug-related limitations.

The difficulty of handling GB can be explained by its infiltrative characteristic, the limitations of BBB permeability and the development of resistance over time due to complex alternative signaling pathways [3].

DS falls into class II of the Biopharmaceutical Classification System (BCS), with low solubility and high tissue permeability, thus its bioavailability is solubility dependent [15,66,96], and it is unstable in acidic, oxidizing, reducing and high-temperature conditions, leading to challenging pharmaceutical formulation development [97].

In GB treatment, the clinical trials with orally administered DS, the repurposed drug show un-satisfactory anticancer efficacy, mainly explainable by: its poor solubility, instability under physiological conditions in gastric acid and blood, its rapid unwanted metabolism, the first-pass effect, and the presence of BBB, affecting the bioavailability, the achievable therapeutic concentrations and target tissue accumulation [14].

Recognizing the delivery limitations, the focus of GB research turned also on technological issues to overcome the multiple biological barriers and to realize a maximum brain tumor accumulation and minimum off-target delivery of DS [14,59]. Innovative DDCs show a promise to improve the anticancer repositioning of DS and the clinical translation. Therefore, in this section the formulation strategies of DS are highlighted to target GB.

The identified approaches use DS in a molecularly encapsulated form, embedded in lipid emulsion [65,67,97], polymers [57,86,98] or in cyclodextrin inclusion complex [66]; and drug delivery strategies for brain targeting were parenteral, including invasive (e.g., BBB disruption, injection, implantation), non-invasive (e.g. intranasal drug delivery) and ligand-mediated drug delivery methods [67] (**Table 5**). None of them reached the clinical phase stage, therefore to demonstrate their superiority in comparison with oral administration, the achievable PK profile should be evaluated in the future. **Figure 4** summarizes the advantages and disadvantages of the locally applicable, injectable and intranasal formulations of DS intended to use in GB.

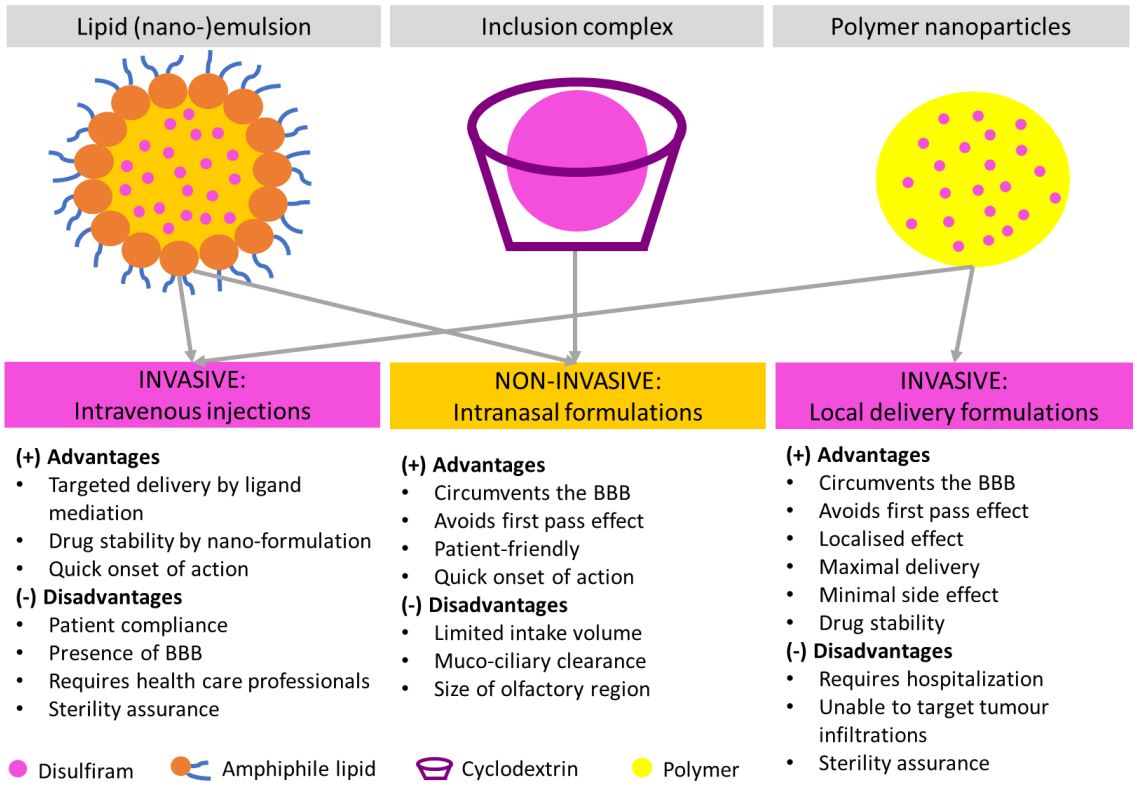


Figure 4. Parenteral formulations of DS intended to treat GB: advantages and disadvantages

Abbreviations: BBB=blood-brain barrier

Notes: Molecular drug-encapsulation strategies: DS-loaded lipid emulsion, cyclodextrin inclusion complexes and polymers. Parenteral dosage forms of DS in GB: intravenous, intranasal and local delivery.

3.3.1 Intranasal formulations

The nose-to-brain pathway is a non-invasively and patient-friendly local administration with a quick onset of action by not only circumventing the BBB but also avoiding the hepatic first-pass effect [67]. Intranasal administration can result in the liberation of more drugs into the brain, reducing the peripheral distribution and the systemic side effects [67]. Drug transport from the nasal cavity to the brain occurs mainly through three pathways, including the olfactory nerve pathway, the trigeminal nerve pathway, and the indirect trans-BBB pathway [66]. The olfactory region is the only site in the human body where the nerve system exists directly contacting with the surrounding environment [66]. Qu et al. [66,67], designed two intranasal formulations of DS, one of them is a nano-emulsion in situ gel formulation [67], and the other is a solution of DS embedded in hydroxypropyl-β-cyclodextrin inclusion complex [66]. The nano-gelling system improved mucosal absorption by overcoming issues of fast drug mucociliary clearance [67]. The hydrophilic cyclodextrin derivative was employed to enhance the solubility and absorption of the drug [66]. To compare the oral, intravenous and intranasal administration routes, the researchers tested *in vivo* the brain-targeting efficacy of the inclusion complex via these pathways and confirmed the superiority of the intranasal administration. However, these formulations present limitations, such as different PK/PD profiles from the known data of oral administration [66]. The difference between the olfactory region size can influence the result transferability from preclinical to clinical situations, as in humans this region is only approximately 10% of the area, in rodents which are mainly used for intranasal administration studies, the olfactory region can make up to 50% of the total area [99]. Hence, in-depth PK, pharmacology and toxicology human studies are needed to be performed in the future to identify the optimal dosage for effective GB therapy with nose-to-brain delivery [66]. Despite the limitations, this is the only parenteral and non-invasive route, which is promising to deliver DS into the brain

tumor. In addition, intranasal administration may be particularly beneficial for cancer patients who experience frequent nausea/vomiting. Considering the new tendency for poly-medication strategies, the nasal formulation of DS may be suitable, as does not overload the oral route. DS is a promising pediatric anti-glioma agent too; and due to its favorable adverse effect profile, with further investigation on child population, an intranasal formulation may be disseminated in pediatric use, especially as this age category is less exposed to alcohol consumption, the interaction that causes the most frequent side effect.

3.3.2 Intravenous formulations

To improve the stability in the bloodstream of DS intravenous lipid emulsion with lecithin was prepared and optimized by Chen et al. [97], demonstrating, that lecithin containing more PE caused more degradation of DS due to its higher peroxidation, but Lipoid S100® was optimal for the formulation. This formulation was evaluated *in vivo* by Li et al. [65], indicating the anti-angiogenic activity of DS in combination with oral Cu. As the intact sulfhydryl group is essential for the reaction between DS and Cu, Kannappan et al. [57], have shown that using a poly (lactic-co-glycolic acid) (PLGA) nano-delivery system the sulfhydryl group can be protected and the anticancer efficacy of DS can be assured.

3.3.3 Local delivery formulations

Stereotactic injections have been used to deliver chemotherapeutic drugs directly into brain tumors, however, these injected liquids may distribute irregularly and be cleared quickly from the tumor site [86]. McConville et al. [86], have investigated the development of injectable drug delivery depots to provide extended drug release with polymer millirods, which are gels that solidify upon injection into the tumor as well as micro and nanoparticle (NP) formulations. DS containing PLGA millirods could be

placed directly into a tumor, using stereotactic surgery, alleviating the reliance on drug diffusion into the tumor and thus restricted penetration, intending to reduce its size before surgical removal or to reduce the size of inoperable tumors with the potential of making them operable; and could also be placed around a tumor reducing proliferation. Implantable DDCs are localized treatment alternatives overcoming the issues associated with the BBB, these could provide long-term sustained release of drugs directly to the site of the tumor, reduce the dose of drug needed to provide a therapeutic effect and minimize the systemic side effects due to the avoidance of systemic circulation[86], moreover offer increased drug stability as it remains in the delivery device until released [98]. Similarly, Zembko et al. [98] developed the DS-loaded-PLGA biodegradable wafers. Implants can be disadvantageous in extreme cases, when the infiltration of GB is extent or when the tumor bulk is completely absent [71]. The unfocused nature of this disease makes localized treatment, e.g., maximal safe surgery or locally delivered drugs ineffective [71].

3.3.4 Ligand mediated drug delivery and BBB disruption

Many receptors or transporters, expressed on BBB, were chosen as targets for enhanced drug delivery to the brain, formulations with ligand mediation could facilitate the drug accumulation into gliomas [100]. Zhao et al. [72] developed a dual-targeting biomimetic co-delivery intravenous treatment, which improves anticancer efficacy and also PK profiles of the used drugs. Albumin NPs were loaded with DS and Cu complex combined with regorafenib, and modified with dual ligands, a transferrin receptor-binding peptide T12 and mannose, which efficiently passed through the BBB via the nutrient transporters [72]. The honokiol and DS/Cu co-delivery liposome system designed by Zheng et al. [73], was modified with α7 nicotinic acetylcholine receptor-binding peptide to target and to treat GB via remodeling tumor-immune microenvironment (TIME). Lan et al. [100]

developed transferrin-modified DS-loaded copper sulfide (CuS) nano-complex attended to intravenous injection and enhanced its delivery by ultrasound-targeted microbubble destruction (UTMD). UTMD could induce transient and reversible separation of endothelial tight junctions in the presence of microbubbles by the cavitation effect, allowing the extravasation of drugs for enhanced brain delivery [100]. To achieve an efficacious delivery of DS into the brain this formulation used a combinational strategy of nano-formulation, ligand-mediation and BBB disruption. These methods used DS as a prime material and pre-treated with Cu, forming the metal complex [72,73,100]. Consequently, the final product contained $Cu(DDC)_2$ and the antitumor effect was supported by the formed complex [100].

3.3.5 Combinational formulations of DS

In the formulation strategies of DS, the addition of Cu was also present, in most of the *in vivo* studies it was administered separately via oral route (only one case represented the reversed administration: oral DS + intravenous Cu [101]) or was co-formulated with DS. Considering the poor bioavailability of both compounds (DS, Cu), in case of administration on different routes may not achieve the enhanced anticancer effect on the tumor site. A rationale administration of Cu might not cause for concern adverse effects [14], but it is potentially a highly toxic element [62] and cancer patients do not have Cu deficiency. Direct supplements could result in severe disorders due to the non-selective distribution of Cu in the body, therefore specific dosages need to be verified by preclinical and clinical trials [41] and it is important to explore DDSs for the supply of exogenous Cu [100]. Limitations with multi-combinational therapies exist, such as the presence of the BBB, rapid systemic drug degradation, high systemic doses from each drug, the burden of the intraoral route, the *per os* administration of chemotherapeutics and adjuvants may increase the chance of vomiting, leading to unsuspected dose loss, etc.

[58,59]. These could be improved by technological approaches, e.g. with convection enhanced-, or local delivery, such as the development of implantable devices [58].

Table 5. Summary of the parenteral delivery strategies of DS to target GB.

Administration	Formulation strategy	Excipients and their role	Study type	Results	Ref.
Intranasal	DS-loaded nano-emulsion in situ gel	• **Ethyl oleate**: oil phase • **Tween 80**: emulsifier • **Transcutol® HP**: co-emulsifier • **DSPE-PEG 2000**: lipid, stabilizer • **DGG**: ion-sensitive in situ gelling agent • **Water:** aqueous phase	*In vitro* + *In vivo*	Suitable particle size and zeta potential, high solubility and safety, sustained release, effective GB growth inhibition.	[67]
	DS embedded in HP-β-CD inclusion complex	• **HP-β-CD:** solubilizer and stabilizer		Improved solubility, effective GB growth inhibition, superior efficacy compared to oral and intravenous administrations.	[66]
Intravenous	DS-loaded lipid emulsion	• **Oleic acid:** pH modifier, oil phase • **Lecithin types with different PC and PE content:** lipid, emulsifying agent • **MCT:** oil phase, solvent • **Pluronic F68®:** stabilizer, aqueous phase • **Glycerol:** aqueous phase, co-solvent • **Water:** aqueous phase, solvent	*In vitro* + *In vivo*	Improved chemical stability of DS in blood, reduced contact of drug with plasma-proteins by enclosing DS in oil.	[65,97]

	DS-loaded PLGA NP	• **PLGA:** drug nano-delivery system, biodegradable and biocompatible co-polymer, stabilizer		DS`s sulfhydryl group protection, prolonged release and improved anticancer efficacy.	[57]
Local delivery	DS-loaded PLGA NP (stereotactic injectable millirod)	• **PLGA:** drug nano-delivery system, millirod forming agent, biodegradable and biocompatible co-polymer, stabilizer	*In vitro*	Manufacturing technique: HME and IM. Improved stability and the same cytotoxicity as the unprocessed DS.	[86]
	DS-loaded PLGA NP (implantable wafer)	• **PLGA:** drug nano-delivery system, wafer forming agent, biodegradable and biocompatible co-polymer, stabilizer		Manufacturing technique: compression/ solvent casting/ heat compression moulding. The solvent casting technique underperformed in both drug stability and cytotoxicity assuring, the others had similar cytotoxicity to the unprocessed DS.	[98]

Abbreviations: DGG= deacetylated gellan gum, DS=disulfiram, DSPE=distearoyl phosphoethanolamine, GB=glioblastoma, HP-β-CD=hydroxypropyl-β-cyclodextrin, HME=hot-melt extrusion, IM=injection moulding, MCT= medium-chain triglyceride, NP=nanoparticles, PC=phosphatidylcholine, PE=phosphatidylethanol-amine PEG=polyethylene glycol, PLGA= poly lactic–co-glycolic acid, Pluronic F68= poloxamer188, S100= commercial lecithin.

Notes: Several strategies were excluded from this table, due to the following reasons: Cu was formulated instead of DS to enhance its efficacy [101], DS was co-formulated with other active ingredients (regorafenib [72] and honokiol [73]), and the final product contained the active metabolite of DS [100].

4. Conclusions

The limitations of the standard of care urge the development of novel therapeutic strategies, and due to the presence of intra- and intertumoral heterogeneity, targeted therapies fail to tackle GB. Drug repositioning is a novel emerged strategy in oncology, and compared with the new therapeutic molecule invention, is a more economical and time-efficient way with reliable biosafety [35]. DS, used for alcoholism therapy, is a potential adjuvant non-chemotherapeutic, unspecific anticancer agent, as the complexity of the mechanism of action of DS is thought to be well exploited against the heterogenous GB. Although the anticancer activity of DS is not fully understood [14,44], DS is considered as multipotent drug with pleiotropic effects on the cell cycle due to Cu-chelating property and Cu-dependent proteasome inhibition; and with inhibitory effects on various enzymes involved in tumor survival, e.g. ALDH associated with GSC regulation and MGMT related to chemotherapy sensitivity. The administration of combinations of repurposed drugs that target different growth promoting pathways of high-grade gliomas have the potential to be translated into the clinic as a novel treatment strategy [59]. Presenting a favorable adverse effect profile and just few interactions, DS could be applied in PD combinations next to chemotherapeutic agents, producing complementary tumor-suppressing activities and chemotherapy sensitizing effect. The role of endogenous Cu in the pharmacology of DS is indisputable, but exogenous supplementation is already pushing the benefit-risk boundary. The administration of DS in combination therapies need to be designed to maximize the benefit of its addition and minimize the risk of adversely affecting the primary anti-GB treatment; therefore, patient-friend, non-invasive strategies are preferred. The clinical interest in the introduction of DS in GB therapy has highlighted the drug- and the brain tumor-related limitations of oral administration, such as poor bioavailability and low tumor targeting efficacy,

therefore the development of parenteral formulations, containing DS in a molecularly encapsulated form, intend to gain ground in the drug delivery improving technological approaches. To the best of our knowledge, this is the first complete review of the use of DS in GB that summarizes both clinical results and technological approaches to delivery.

5. Expert Opinion

There are many old drugs with reported new treatment potential due to *in vitro* screened bioactivity, however it is still a large challenge for their clinical translation for drug repositioning, as there is a huge result-deviation gap between *in vitro* tests to *in vivo* efficacy [35]. This is also the situation, seen in case of oral DS, where the outcomes of clinical trials do not support the results obtained in preclinical and *in vitro* experiments. The observed incongruence suggests that repositioning of DS needs to be reached from both clinical and technological perspectives. The known pharmacology from alcoholism treatment of oral DS cannot transfer to GB management without addressing the drug delivery. The *in vivo* fate of a drug needs to be tailored for delivery to a new target for a new indication, which in this case is a heterogenic, diffuse, infiltrative CNS tumor, protected by BBB and tumor-brain barrier [35]. The clinical evidence suggests that the insoluble and unstable DS has poor bioavailability in case of GB, the rapid, unwanted metabolism after oral intake, the first pass effect and the presence of BBB limits its accumulation in the tumor tissue. The technological endeavors to encapsulate DS via polymers, lipids or cyclodextrins and to develop parenteral formulations with favorable PK profile shows promise for the effective application of DS, ensuring drug solubility, stability and accurate delivery into CNS. Therefore, the clinical translation of this adjuvant drug into GB therapy can only be achieved with optimized DDS that overcome the poor bioavailability and low tumor-mass targeting efficacy [14,44]. Comparing the non-oral formulation approaches, the local [59] or the nose-to-brain route are promising

drug delivery options for DS, as they avoid the first pass effect, bypass the BBB, and reduce the high systemic doses, achieving therapeutic levels at the target brain-tumor site. However, the repositioning of the anti-alcohol-abuse drug into GB requires further PD/PK studies in the future. The presented status of DS in GB suggests that in the near future, innovative drug formulation strategies, such as nanotechnology, will play a prominent role in GB management; in particular, non-invasive delivery systems seem promising in improving the treatment of a hard-to-treat cancer. DS could play an adjuvant role in newly diagnosed and recurrent GB treatment, enhancing the standard management protocol by RT- and chemo-sensitizing effect and suppressing the tumor mass, thus inhibiting the cancer progression. To prove the efficacy of DS in GB, randomized trials and comparative experiments demonstrating the superiority of novel pharmaceutical forms of DS, should be conducted. Furthermore, the models and methods for in vitro and preclinical studies should be carefully selected, thus minimizing translational failure.

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Declaration of interest

The authors report there are no competing interests to declare.

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Tables and footnotes

Table 1. Summary of data selection procedure

Abbreviations: OS=overall survival; PFS=progression-free survival

Table 2. Summary of clinical trials showing the clinical relevance of DS use for GB treatment.

Abbreviations: 1x=once daily, 2x=twice daily, 3x=three times a day, A&G=age and gender, Cu=copper, $CUSP_{V3}$=Coordinated Undermining of Survival Paths combining 9 repurposed non-oncological drugs (aprepitant, auranofin, captopril, celecoxib, DS, itraconazole, minocycline, ritonavir sertraline) with metronomic temozolomide—version 3, DS=disulfiram, DLT=dose-limiting toxicity, F=female, GB=glioblastoma, M=male, M+F=both genders represented, MoA=method of administration, MTD=maximum tolerated dose, NCT=registration number of clinical trial on *ClinicalTrials.gov*, Nr. P.=number of enrolled patients, OS=overall survival, P=phase, PD=pharmacodynamics, PO=per oral, PFS=progression free survival, TMZ=temozolomide, Y=years

Notes: *Median survival data measured from the initiation of DS therapy, **Survival rate measured from the initiation of CUSP9 therapy

Table 3. Combinations therapy for GB therapy, containing DS.

Abbreviations: ALDH=aldehyde dehydrogenase, CSC=cancer stem cells, Cu=copper, CUSP9=Coordinated undermining of survival paths with 9 drugs (aprepitant, auranofin, captopril, celecoxib, DS, itraconazole, minocycline, ritonavir and sertraline), CT=clinical trial, DS=disulfiram, GB=glioblastoma, LC3=light chain 3, MGMT=O6-methylguanine-DNA-methyltransferase, NF-κB=nuclear factor-kappa B, PLK-1=polo-like kinase, ROS=reactive oxygen species, RT=radiotherapy, Stupp protocol=current standard therapy used for newly diagnosed GB, composed by maximal surgical resection, followed by RT (60 Gy in 30 fractions for 6 weeks) plus concomitant TMZ (75 mg/m^2/day for 6 weeks) and then six maintenance cycles of TMZ (150–200 mg/m^2/day for the first 5 days of a 28-day cycle), TAM=tumor associated macrophage, TIME=tumor immune-microenvironment, TGF-β=transforming growth factor beta, TMZ=temozolomide, TNF-α=tumor necrosis factor-alfa

Table 4. Anti-GB mechanisms of action of DS and Cu-dependence.

Abbreviations: ALDH=aldehyde dehydrogenase, CSC=cancer stem cells, Cu=copper, DS=disulfiram, GB=glioblastoma, MGMT=O6-methylguanine-DNA-methyltransferase,

NF-κB=nuclear factor-kappa B, PLK-1=polo-like kinase, RT=radiotherapy, ROS= reactive oxygen species, TAM=tumor associated macrophage, TIME=tumor immune-microenvironment

Notes: (+): Copper dependent mechanism, (-): Copper independent mechanism, (+/-): Divergent results in literature, (0): Copper dependence was not studied

Table 5. Summary of the parenteral delivery strategies of DS to target GB.

Abbreviations: DGG= deacetylated gellan gum, DS=disulfiram, DSPE=distearoyl phosphoethanolamine, GB=glioblastoma, HP-β-CD=hydroxypropyl-β-cyclodextrin, HME=hot-melt extrusion, IM=injection moulding, MCT= medium-chain triglyceride, NP=nanoparticles, PC=phosphatidylcholine, PE=phosphatidylethanol-amine PEG=polyethylene glycol, PLGA= poly lactic–co-glycolic acid, Pluronic F68= poloxamer188, S100= commercial lecithin.

Notes: Several strategies were excluded from this table, due to the following reasons: Cu was formulated instead of DS to enhance its efficacy [101], DS was co-formulated with other active ingredients (regorafenib [72] and honokiol [73]), and the final product contained the active metabolite of DS [100].

Figures caption

Figure 1. PRISMA-2020 flow diagram showing relevant articles included in the systematic review.

Figure 2. DS protein inhibitory activity and the target proteins in GB: MGMT (I), ALDH (II).

Abbreviations: ALDH=aldehyde dehydrogenase, DDC=diethyldithiocarbamate, DS=disulfiram, Cys=cysteine, Me=metal, MGMT= O6-methylguanine-DNA-methyltransferase, Protein(Me)= protein with metal co-factor in its structure, -SH=thiol group -S-S-=disulfide bond.

Notes: DS interacts with proteins in different ways, binding to Cys residue in the active site or near the active site of a protein, modifying its function (c), or chelating the co-factor metal component (a, b). The inactivated enzyme may, but need not have the DS moiety bound to it covalently, (d) reaction may occur if a second, suitably positioned vicinal thiol group is present on the enzyme and such a sequence of reactions occur with the cytosolic ALDH [80]. Origin of protein molecules: *https://www.uniprot.org/*

Figure 3. The metabolic fate of DS and the presupposed activity of its metabolites in GB.

Abbreviations: I.= Disulfiram, II.= Diethyldithiocarbamate, III.= Bis(diethyldithiocarbamate)-copper, IV.= Mixed disulfides with protein sulfhydryl groups, V.= Diethyldithiocarbamate methyl ester, VI.= Diethylmonothiocarbamate methyl ester, VII.= Diethylmonothiocarbamate methyl ester sulfoxide, VIII.= Diethylmonothiocarbamate methyl ester sulfone, IX.= Diethyldithiocarbamate methyl ester sulfoxide, X.= Diethyldithiocarbamate methyl ester sulfone, XI.= Diethylmonothiocarbamate, XII.= Diethylamine, , XIII.= Carbon disulphide, XIV.= Diethyldithiocarbamoyl-S-glucuronide, XV.= Methanethiol, XVI.= Carbonyl sulphide, XVII.= Formaldehyde, XVIII.= Sulphate, XIX.= Carbon dioxide, XX.= Thiourea, XXI.= Amino acid dithiocarbamate, XXII.= Thiazolidine-2-thione-4-carboxylic acid, XXIII.= 2-thio-S-thiazolidinon, Cys= cysteine, GB= glioblastoma

Figure 4. Parenteral formulations of DS intended to treat GB: advantages and disadvantages

Abbreviations: BBB=blood-brain barrier

Notes: Molecular drug-encapsulation strategies: DS-loaded lipid emulsion, cyclodextrin inclusion complexes and polymers. Parenteral dosage forms of DS in GB: intravenous, intranasal and local delivery.

Article highlights

- Clinical relevancy of repositioning disulfiram for glioblastoma treatment
- Diverse anti-glioblastoma mechanism of disulfiram
- Pharmacokinetics and bioavailability of disulfiram in tumor treatment
- Formulation strategies of disulfiram to overcome delivery limitations to the brain

List of abbreviations

ALDH=aldehyde dehydrogenase

BCS= Biopharmaceutical Classification System

BBB=blood-brain barrier

CNS=central nervous system

CSC=cancer stem cells

Cu=copper

$Cu(DDC)_2$= bis(diethyldithiocarbamate)-copper

CUSP9= Coordinated Undermining of Survival Paths with 9 repurposed non-oncological drugs

Cys=cysteine

DDC= diethyldithiocarbamate

DDS=drug delivery system

DS=disulfiram

EGFR= endothelial growth factor receptor

GB=glioblastoma

GSC= glioma stem cells

IDH= isocitrate dehydrogenase

MGMT= O6-methylguanine-DNA-methyltransferase

NF-κB=nuclear factor-kappa B

NP=nanoparticles

OS=overall survival

PD= pharmacodynamics

PFS=progression free survival

PK= pharmacokinetics

PLGA=poly (lactic-co-glycolic acid)

PRISMA=Preferred Reporting Items for Systematic Reviews and Meta-Analyses

ROS=reactive oxygen species

RT=radiotherapy

TERT= telomerase reverse transcriptase

TMZ=temozolomide

WHO=World Health Organization