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Bullseye: Targeting Cancer Stem Cells to Improve the Treatment of Gliomas by Repurposing Disulfiram

J TRISCOTT, M.R PAMBID, AND S.E. DUNN

Experimental Medicine Program, Department of Pediatrics, University of British Columbia, Vancouver BC V5Z 4H4,

Corresponding author: Sandra E. Dunn, University of British Columbia, 3082 – 950 West 28th Ave, Vancouver, BC, Canada, V5Z 4H4, telephone: 604-875-2000 ext 6015, Email: sedunn@mail.ubc.ca; Corresponding author: Sandra E. Dunn, Ph.D. email: sedunn@mail.ubc.ca, telephone: 604-875-2000 ext 6015

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ABSTRACT

Cancer stem cells (CSCs) are thought to be at the root of cancer recurrence because they resist conventional therapies and subsequently re-initiate tumour cell growth. Thus targeting CSCs could be the bullseye to successful cancer therapeutics in the future. Brain tumours are one of the most challenging types of cancer to treat where the median survival following the initial diagnosis is 12-18 months. Among the different types of brain tumours, glioblastoma (GBM) is the most common type and remains extremely difficult to treat. Despite surgery, radiation and chemotherapy, most patients develop refractory disease. Temozolomide (TMZ) is a chemotherapy used to treat GBM however resistance develops in most patients. The underlying mechanisms for TMZ resistance (TMZ-resistant) involve the expression of DNA repair gene MGMT (O(6)-methylguanine-DNA methyltransferase). The CSC genes such as Sox-2, BMI-1 and more recently YB-1 also play a role in resistance. In order to develop novel therapies for GBM, libraries of small interfering RNAs and off-patent drugs have been screened. Over the past few years, several independent laboratories identified disulfiram (DSF) as an off-patent drug that kills GBM CSCs. Reportedly DSF has several modes of action including its ability to inhibit aldehyde dehydrogenases, E3 ligase, Polo-Like Kinase 1 (PLK1) and NFkB. Due to the fact that GBM is a disease of heterogeneity, chemotherapy with multi-targeting properties may be the way of the future. In broader terms, DSF kills CSCs from a range of other cancer types further supporting the idea of repurposing it for "target practice". STEM CELLS *2014; 00:000–000*

INTRODUCTION

Brain tumours: General overview and need for new therapies

Brain tumours are difficult to treat in general given their location and the lack of targeted therapies. In adults, glioblastoma (GBM) is the most common type of brain tumour, and while they do occur in children to a lesser extent, most patients are faced with living ~12-18 months after diagnosis. There are several characteristics of GBM that hinder therapeutic development. These include a heterogeneous morphology and the presence of subpopulations of cancer stem-like cells (CSC) that appear undifferentiated, have a functional capacity for self-renewal and are drug resistant [1–3]. Evidence of CSCs in pediatric tumours questions whether these may also be responsible for the initial formation of these malignancies [4]. There are several genes associated with GBM CSCs including CD44 [5], CD133 [1], Nanog, Oct4, Sox-2, Mushashi, BMI-1, and more recently the transcription and translation factor YB-1 (Y-box binding protein-1) [6].

Maximal safe resection and radiation therapy are used in the treatment of GBM. As well, temozolomide (TMZ), has been incorporated into standard care procedures with the establishment of the Stupp protocol [7, 8]. With a reported 1.9% 5-year survival rate for patients treated with radiotherapy alone, the addition of TMZ with radiotherapy only increased 5-year survival to 9.8% of patients [8]. Unfortunately, the toxicity of TMZ is often not well tolerated by patients, and the number

of clinically available compounds that are capable of crossing the blood-brain barrier (BBB) are limited. As well, additional modalities of TMZ-resistant such as O(6)-methylguanine-DNA methyltransferase (MGMT) expression further complicate the problem. This repair enzyme can remove the alkyl groups added by TMZ to the O6 position of guanine in DNA, therefore preventing interstrand cross-linking and block TMZ-induced apoptosis of proliferative cells [9, 10]. Other proteins that have received less attention, such as YB-1, also convey TMZ resistance [11], and its expression was linked to maintaining GBM in a stem cell state [6]. Inactivating YB-1 with siRNA suppresses the growth of TMZ-resistant cells *in vitro* and *in vivo* [11]. Of note, YB-1 is not highly expressed in murine normal brain tissues at high levels except in the subventricular zone where stem cells reside [6]. Likewise, Faury reported ~4-fold increase in its expression in pediatric GBM as compared to normal brain tissue [12]. The challenge in targeting YB-1 directly is that it is a transcription factor and therefore not easily inhibited directly with small molecules. Given the preponderance of TMZ resistance in patients, new agents are urgently needed to improve patient outcomes. While developing better therapies can take decades of research and testing, we question whether off-patent compounds currently exist that can be repurposed to target CSCs.

C. Repurposing DSF for brain tumour treatment

Disulfiram (DSF), also known as Antabuse®, has been used for treatment of substance abuse and in addiction studies [13, 14]. Initially, the compound had been used in the process of rubber manufacturing. In 1937, it was discovered that factory workers, who were regularly exposed to DSF, would experience flu-like symptoms when they ingested alcohol [14]. The first clinical trials for use of DSF as an anti-alcoholic treatment began in 1948, and it has been used in patients for over 60 years [13]. With the more recent discovery of a stem cell population in cancer, scientists are once again finding new purposes for DSF. Currently, there are two ongoing clinical trials for DSF in GBM (www.clinicaltrails.gov, identifiers NCT01907165 and NCT01777919) (Table 1). There are no pediatric brain tumour trials with DSF reported, however, this would be a logical next step pending positive outcomes from GBM trials in adults.

We and other groups as well report that DSF inhibits the growth of glioma cell lines and blocks self-renewal. DSF importantly inhibits the growth of TMZ-resistant cells isolated from patients [15, 16]. To illustrate this point, we compared TMZ to DSF in two primary GBM isolates (Figure 1A). While TMZ had no effect on the growth of these cells, they were highly sensitive to DSF. Importantly, DSF is effective regardless of MGMT expression because ABT011 cells express high levels of MGMT while ABT015 cells express low levels of this enzyme (data not shown).

In T98G GBM cells that are TMZ-resistant, Paranipe et al. recently reported that DSF down-regulates MGMT in xenografts implanted subcutaneously [17]. They suggest that DSF could, therefore, be used to treat gliomas because it crosses the BBB however they did not perform intracranial injections of T98G cells. However, Choi et al. recently published an elegant study in atypical teratoid rhabdoid tumours (AT/RT) that demonstrated DSF crosses the BBB in mice and can reduce AT/RT CSCs [18]. AT/RT are a rare yet deadly type of pediatric brain tumour where improved therapies are most certainly needed. Of note, they reported that DSF reduced ALDH *in vitro* by ~75% and in tumours. It also inhibited EdU incorporation and tumour cell proliferation based on Ki67 staining. AT/RT CSCs were more sensitive to DSF than clinically used drugs such as ifosfamide (IFO). Likewise, IFO had no survival benefit in mice whereas DSF did prolong survival. There were no adverse effects of DSF reported in the mice. Thus, DSF is promising for the treatment of brain tumours because it crosses the BBB and suppresses the growth of brain tumours yet additional studies are needed to understand how widespread the effect will be.

D. Potential mechanisms of DSF anti-cancer activity

DSF could be a way to hit the "Bullseye" given the fact that it not only kills CSCs, but seems to do so by targeting multiple pathways operative in these refractory cells (Figure 1B). DSF is most widely known as an inhibitor of aldehyde dehydrogenase (ALDH). ALDH is a family of metabolic enzymes that catalyze the oxidation of aldehydes, which are toxic products of alcohol metabolism [19]. A relationship between high ALDH activity and stem cell behavior prompted the use of an ALDH based fluorescence assay, Aldefluor®, to identify undifferentiated populations both within cancer and normal tissues [20–22]. In cancer studies, high ALDH expressing cells have been associated with enhanced xenograft tumour formation in mice and chemotherapeutic resistance [23, 24]. ALDH enzyme activity is thought to be involved with cell detoxification, and Aldefluor® active cells are associated with resistance to cisplatin, docetaxel, and doxorubicin [25].

The drug cyclophosphamide is a fundamental chemotherapeutic in many pediatric brain tumour treatment protocols. High levels of ALDH have been directly shown to intervene with cyclophosphamide metabolism and decomposition making this a potential mechanism for chemotherapeutic resistance [26, 27]. The ALDH1a1 isoform had previously thought to have the strongest association with the CSC phenotype. For example, Marcato et al. (2011) suggest expression of ALDH1a3 to have greater CSC correlation and prognostic importance compared to ALDH1a1 in breast cancer [28]. With a family of 19 total ALDH isoforms it is difficult to pinpoint complete functional independence due to redundancy in activity.

There are additional anticancer properties of DSF. For example, it also suppresses the proteasome and NFkB pathways [29–33]. More specifically, DSF suppresses ubiquitin E3 ligase activity [34]. In the body, the DSF molecule is converted into a smaller metabolite called diethyldithiocarbamate. This metabolite has been shown to chelate into complexes when in combination with copper or zinc ions. These complexes are suggested to inhibit proteasome activity and elevate radical oxygen species (ROS) [33]. Under this premise, many cancer studies use DSF in combination with copper [33, 35, 36]. While some studies report increased efficacy of DSF in combination copper in killing cancer cells, this increased copper-mediated cytotoxicity is apparent in normal cells as well [18]. Choi et al. (2014) discuss the potential danger of using additional copper and zinc in treatment regimens, as they are teratogenic, and could result in developmental defects. It is crucial that potential metal ion toxicities are considered in the design of clinical studies.

DSF has also been shown to impinge on epigenetic pathways. It is suggested that DSF contains functional groups that are extremely thiol reactive, and this chemistry is effective in blocking the active site of certain enzymes. In prostate cancer, DSF can act as a DNA demethylating agent through inhibition of DNA methyltransferase 1 (DNMT1) [37]. Aberrant methylation in cancer genomes can potentiate overexpression of oncogenes or inhibit the expression of tumour suppressors, therefore, targeting epigenetic controls allows reprogramming of cell pathways. More recent studies on the fusion protein NUP98-PHF23 shows DSF treatment can reduce its chromatin modifying potential and induce cell death in acute myeloid leukemia. Recently, transcriptional availability of CSC signature genes, such as *Hoxa, Hoxb* and *Meis1*, is blocked by DSF [38]. These observations further exemplify the anti-CSC activity of DSF.

Interestingly, treatment of primary GBM cells with DSF *in vitro* reduced the expression of kinases such as PLK1 protein and mRNA [16]. The exact mechanism driving DSF induced PLK1 down-regulation still requires further investigation. However, these findings suggest DSF to be capable of targeting aggressive PLK1 high cell populations, which may be responsible for driving tumour relapse. Out of all the cancer pathways affected by DSF, PLK1 is the one that stands out as an interesting molecular target because it is a well-established drug candidate for cancer.

E. Repurposing DSF for other cancers

DSF was identified in several studies as an agent that inhibits CSCs for cancers of the breast, ovary, pancreas, lung and blood [15, 16, 39, 40]. A recent study reported that the liposomal packaging of DSF (Lipo-DSF) inhibited breast cancer CSCs in part by disrupting the NFkB pathway [29]. Hypoxia rendered the cells resistant to chemotherapy and expanded the CSCs population as defined by the markers CD24, CD44, Oct4, Sox2 and Nanog. However, DSF blocked the hypoxia induced CSC expansion. Of note, *in vivo* they combined Lipo-DSF (a novel liposomal formulation) with Copper and demonstrated that it suppressed the growth of MDA-MB-231 cells. Lipo-DSF also reduced the ALDH+ CSC population in mice. Importantly, the treatment did not have any obvious adverse effects on the major vital organs based on histopathological evaluations. DSF has also been formulated into micelles where this new delivery system reduced the metastatic potential in the 4T1 model of breast cancer [41]. In both instances, they show that DSF inhibits breast cancer cells that are refractory to conventional therapies because MDA-MB-231 and 4T1 cells are reportedly resistant to chemotherapies. Thus, this introduces two examples where altering the formulation of DSF could improve drug delivery. Therefore, it is reasonable to consider that changes to the formulation of DSF could further improve its delivery to the brain.

DSF is also promising for pancreatic cancer. Again, the suggested mechanism relates to triggering the proteasome pathway leading to degradation of NFkB [42]. Likewise, DSF inhibited the growth of chronic lymphocytic leukemia cells through a similar mechanism [43]. As well, DSF had little effect on peripheral blood mononuclear cells at doses that are clinically achievable in patients [43]. A potential application of DSF is also reported in hepatocellular carcinomas where it reportedly reduces CSCs by disrupting the p38 MAPK pathway [44]. Considered together, there is gaining momentum that DSF inhibits CSCs in a wide-range of cancers placing it in a unique category as a potential anticancer agent.

Metastases are a major problem in many types of cancers. Bone metastases are a particular problem with sarcomas. Greco et al. reported that ALDH is present in bone metastases from patients with sarcomas [45]. In a small number of cell-based models they were also able to show that DSF suppressed the growth of cells that had metastasized to the lymph nodes and lungs [45]. Consistent with this study, DSF was identified in a large screen for anti-metastatic agents using a model of fibrosarcoma [46]. Given that 90% of all cancer deaths are due to metastases it is encouraging that DSF could provide some benefit in cancers that have spread.

F. Concluding remarks

In oncology, kinase inhibitors such as those that block PLK1 are attractive for many reasons, but their downside can be dose-limiting toxicities. The main side effect is often neutropenia. DSF, on the other hand, is not commonly associated with neutropenia suggesting that its mode of action has a better safety profile. Despite the safety profile of DSF that has been put to practice for decades, researchers are attempting to establish which dosing schedule and chemotherapeutic combination will deliver the greatest response from tumour cells. The only adverse side effect reported is hepatotoxicity when DSF is prescribed at high doses. Since the exact mechanism behind the potent efficacy of DSF on

tumour cells *in vitro* remains vague, the question of whether additional copper supplements are necessary for efficacy still needs exploration.

Treatment of malignant brain tumours offers unique challenges due to the sensitive nature of neural tissues, the BBB, and CSC subpopulations. While current standard of care regimens like TMZ are often ineffective and can be hard for patients to tolerate, there are limited options for clinicians to offer patients. DSF provides a means to deliver a multi-targeting agent that kills CSCs. Other advantages of DSF include that it is inexpensive, accessible worldwide, and has potential efficacy against the chemo-resistant CSC population. DSF may offer hope for pediatric cases that are in dire need for novel treatments that reduce adverse side effects. Fighting cancer with what we already have may help pave the way for future targeted therapeutic options. CSCs are positioned as the bullseye for developing advanced cancer therapeutics in the next decade.

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Author Contributions

J.T.: conception and design, collection and assembly of data, manuscript writing, final approval of manuscript; M.R.P.: collection and assembly of data, final approval of manuscript; S.E.D.: conception and design, manuscript writing, final approval of manuscript.

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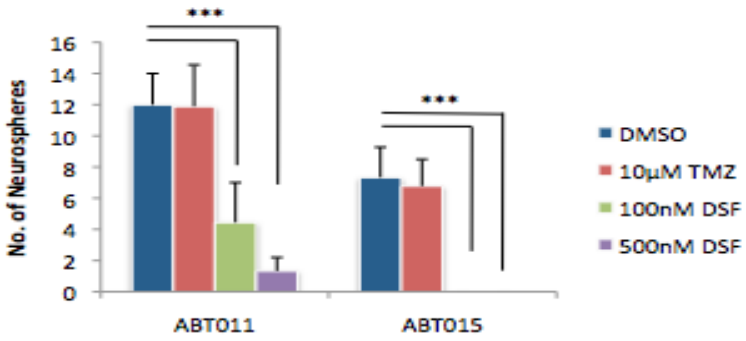
Figure 1. Targeting CSCs with DSF. (A) ABT011 and ABT015 are two primary GBM cell lines propagated in neurosphere conditions that enriches for CSC populations. High doses of 10uM TMZ had no effect on neurosphere growth. Conversely, low doses of DSF at 100nM and 500nM had great efficacy in eliminating cell growth (***signifies statistical significance p<0.001). (B) Infographic depicting some of the reported targets of DSF. These are key molecules in CSC regulatory pathways may be used to target different cancer types with drug resistance and heterogeneity.

A.



B.

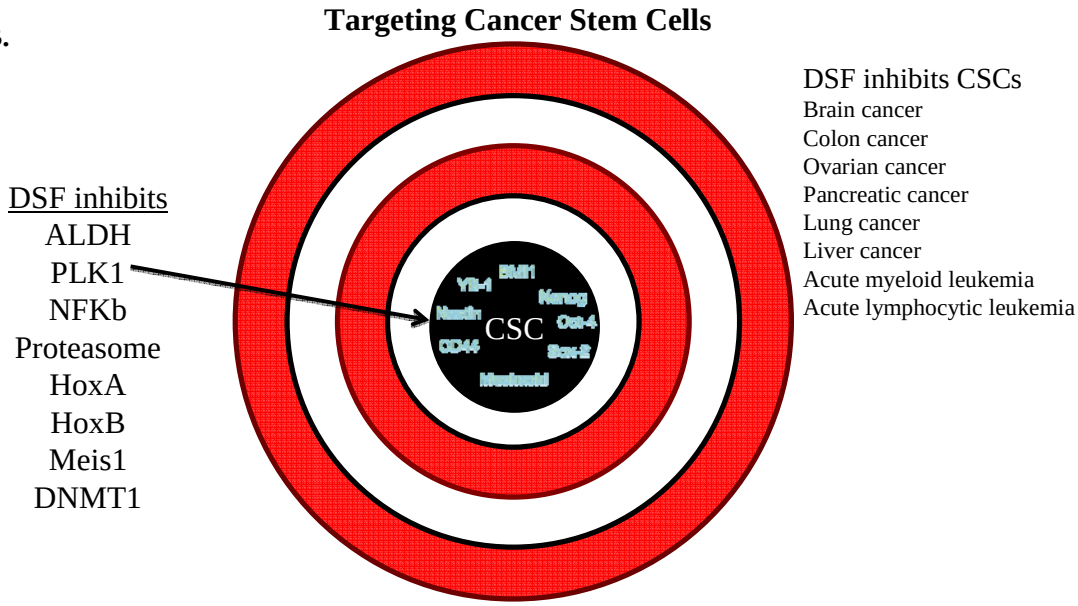


Table 1.Clinical trials involving DSF in cancer

Clinical Trial Identifier	Disease	Study Phase	Sponsor	Enrollment	Status	Start/Completion Dates
NCT00742911	Advanced Solid malignancy with liver metastasis	Phase I	University of Utah	21	Completed	July 2008-March 2013
NCT00571116	Metastatic Melanoma	Phase I	University of California, Irvine	15	Terminated	September 2006-August 2012
NCT00256230	Metastatic Melanoma	Phase I/II	University of California, Irvine	7	Completed	January 2002-August 2007
NCT01777919	Glioblastoma	Phase II	Olympion Medical Center	TBD	Not active yet	September 2015-Septembr 2018
NCT00312819	NSCLC	Phase II/III	Hadassah Medical Organization	60	Completed	March 2006-December 2009
NCT01118741	Prostate Cancer	Phase I/II	Johns Hopkins University	19	Completed	May 2010-June 2012
NCT01907165	Glioblastoma	Phase II	Washington University School of Medicine	TBD	Active	October 2013- December 2017

*Identifiers in reference to www.clinicaltrials.gov online database
TBD: to be determined