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**Effects of an off-label combination treatment of
three repurposed drugs on selected molecular
and biological features of patient-derived
glioblastoma cells in vitro**

Dissertation

submitted for the degree of Doktor der Medizin (Dr. med.) at the
Medical Faculty of Ulm University

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2022

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Day doctorate awarded: 18.01.2024

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Abbreviations

ACNU	nimustine
AE	adverse event
BBB	blood-brain barrier
BCNU	carmustine
Bcl-2	B-cell lymphoma-2
Bcl-xl	B-cell lymphoma-extra large
B27	vitamin B27 without vitamin A
CCNU	lomustine
CD133	cluster of differentiation 133
CNS	central nervous system
CO ₂	carbon dioxide
CSC	cancer stem cell(s)
CSF	colony-stimulating factors
CSF	cerebrospinal fluid
CSF-1	colony stimulating factor-1
CUSP9	Coordinated Undermining of Survival Paths with 9 repurposed drugs
CUSP9v3	Coordinated Undermining of Survival Paths with 9 repurposed drugs, version 3
DAPI	4',6-diamidin-2-phenylindol
DC	differentiated cell(s)
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF4E	eukaryotic translation initiation factor 4E
ELISA	enzyme-linked immunosorbent assay

EMA	European Medicines Agency
EMT	epidermal-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FDA	food and drug administration
FGF	fibroblast growth factor
G35	Glioblastoma 35
G38	Glioblastoma 38
G40	Glioblastoma 40
GB	glioblastoma
G-CSF	granulocyte colony-stimulating factor
GF	growth factor
GM-CSF	granulocyte monocyte-colony-stimulating factor
GSC	glioma stem cell(s)
Gy	Gray
h	hour(s)
HCV	hepatitis C virus
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HIV	human immunodeficiency virus
IC ₅₀	half-maximal inhibitory concentration
IDH	isocitrate dehydrogenase
IGF-1	insulin-like growth factor-1
IL-1 β	interleukin-1 beta
IL-8	interleukin-8
IMPDH	inosine monophosphate dehydrogenase
INF- α	interferon-alpha
IRS-1	insulin receptor substrate-1
LDL	low-density lipoprotein
M	molar
μ g	microgram
μ l	microliter

μM	micromolar
μm	micrometer
ml	milliliter
MET	mesenchymal-to-epithelial transition
MGMT	methylguanine methyltransferase
MMP-2	matrix-metalloproteinase-2
MMP-9	matrix-metalloproteinase-9
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEFL	neurofilament light polypeptide
NF1	neurofibromin 1
NLR	neutrophil- to-lymphocyte ratio
OS	overall survival
PD-1	programmed death-1
PDGF	platelet-derived growth factor
PFS	progression-free survival
PI	propidium iodide
PI3K	phosphoinositid 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PPAR	peroxisome proliferator-activated receptor
PTEN	phosphatase and tensin homolog
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
RSV	respiratory syncycial virus
RT	room temperature
SC	stem cell(s)
SSPE	subacute sclerosing panencephalitis
STAT-3	signal transducer and activator of transcription-3
streptavidin-PE	streptavidin-phycoerythrin

TGF- β	transforming growth factor- β
TMB	tumor mutational burden
TME	tumor microenvironment
TMZ	temozolomide
TNF- α	tumor necrosis factor-alpha
TP53	tumor protein 53
TG	triglyceride
v/v	volume per volume
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

1 Introduction

1.1 Primary CNS tumors

Among all malignancies, primary tumors of the central nervous system (CNS) have one of the poorest prognoses and are almost always fatal. Even though CNS tumors are only the eighth most common cancer in adults older than 40 years, they are more common in younger patients. For young adults between 15 and 39 years, CNS malignancies are the third most common cancer and for patients younger than 14 years, brain tumors are the most common cancer site [76, 110].

Brain tumors and other type of tumors of the nervous system, including malignant and non-malignant entities, have an average incidence of 26.6 per 100,000 inhabitants in women and an incidence of 22.4 per 100,000 inhabitants in men [74]. Non-metastatic, primary, CNS tumors can be categorized into extra-axial where the most frequent histological type is meningioma (37,1%), a tumor where cells derive from the meninges of the brain, and intra-axial tumors [110]. Malignant intra-axial and intrinsic brain tumors can further be categorized into tumors of the neuroepithelial tissue, gliomas, where astrocytomas, deriving from astrocytes, form the majority. 60% of all malignant intrinsic brain tumors are counted to astrocytomas [114], other entities of gliomas are oligodendroglioma, ependymoma or oligo-astrocytoma [56]. Astrocytomas can be divided into four sub-groups according to the World Health Organization (WHO) classification.

1.2 Glioblastoma

Glioblastoma in general is the third most common brain tumor (14.7%), counting malignant and non-malignant entities [110]. Looking only at malignant brain and CNS tumors, glioblastoma almost make-up the half of them (47.7%), followed by other astrocytomas (16.6%). For glioblastoma itself, the incidence is 3.21 per 100,000 [110]. Interestingly, glioblastoma occurs 1.6 times more often in men than in women [74].

According to the 2007 classification of the WHO, glioblastoma is graded as IV, which comprises the most malignant group tumors [89]. Pilocytic astrocytoma, for

example, is classified as grade I, with grade I tumors exhibiting low proliferation and the potential of cure by surgical resection only [89].

In 2016, the WHO revised their 2007 classification to include not only histological features, but also molecular characteristics like IDH mutation status or presence of 1p/19q co-deletion when differentiating primary brain tumors [90]. The employment of genetic and epigenetic molecular investigations has now become a standard in the diagnosis of gliomas.

When looking at the clinical picture, presenting symptoms are often based on the decompensated expansion of the tumor mass, leading to signs of increased intracranial pressure, including headache, vomiting and nausea while specific symptoms depend on the affected cerebral region and may comprise with both transient or stable neurologic and motoric deficits. Epileptic seizures may be the result of infiltrative tumor growth and/or perifocal edema [21]. Magnetic resonance imaging is essential for diagnosis and follow-up, and typically shows a non-enhancing necrotic glioblastoma core with a garland-like contrast enhancement at the outer layer representing vital invasive tumor cells. The contrast enhancement requires a leaking of the blood-brain barrier (BBB). The tumor characteristically exhibits no sharp demarcation. [7] Perifocal edema is typically present and intratumoral hemorrhages may be identified [139]. Despite advances in magnetic resonance imaging (MRI) technology, it is usually unavoidable to obtain a definitive diagnosis via histologic and molecular analysis. Glioblastoma grows highly invasive and is characterized by microvascular proliferation and necrotic and mitotic bodies, which distinguishes it from other astrocytomas [89, 139, 167].

Today's first-line treatment consists of safe maximum surgical resection [33, 170], which aims to remove the greatest possible part of the tumor mass while preventing surgical induced neurological damage. This is followed by adjuvant radiation, with a fractionated dose of typically 1.8 Gy (~2 Gy) applied five days a week, usually from Monday to Friday. Over a period of up to six weeks this means a total dose of 60 Gy. The radiation is directed at the resection cavity and includes a safety margin of 1 to 2.5 cm.

During and after radiotherapy, a concomitant and adjuvant chemotherapy with temozolomide (TMZ) is started according to the "Stupp protocol" [33, 143, 144, 147]. Historically, GB was classified into two different types, namely primary and secondary GB. Whereas primary GB, IDH-wildtype, occurs mainly in older patients

(mean age: 55 years) and develops as a new neoplasm, secondary GB, IDH-mutant GB, occurs much earlier in life (mean age: 40 years) and derives from lower-grade diffuse astrocytomas. [106, 167] Secondary GB account for ~5% of all GB and are forming the minor part [105].

This division is now obsolete and subtypes of glioblastoma are nowadays distinguished in genetic differences according to the WHO classification of 2016 [90].

Genetic alterations are found in both types, while the majority, IDH-wildtype, of GB have an epidermal growth factor receptor (EGFR) overexpression, phosphatase and tensin homolog (PTEN) mutation and a loss of chromosome 10, IDH-mutant GB is mainly marked by isocitrate dehydrogenase 1 (IDH1) mutations, tumor protein 53 (TP53) mutations and 19q loss. [105, 112, 154].

IDH1 and IDH2 mutations are mainly seen in GB developing from lower-grade gliomas (WHO grade II and III) and are considered as a favorable prognostic factor. Overall survival of patients with GB and a mutation of IDH1 or IDH2 is 31 months, compared to wild-type IDH1 with an overall survival of 15 months it is significantly higher [175].

The median survival rate with debulking surgery, concomitant radiochemotherapy and adjuvant chemotherapy with TMZ is 14.6 to 16.7 months, compared to 12.1 months with radiotherapy alone [25, 146, 147] and 5-year survival is only 5.6% [76].

1.2.1 Temozolomide and MGMT

Since TMZ proved in a clinical trial that it conveyed a survival benefit of 2.5 months compared to radiotherapy alone [147], it became part of the standard of care for patients with glioblastoma. Temozolomide is an oral alkylating agent which methylates deoxyribonucleic acid (DNA) guanine residues to O-6-methylguanine, resulting in DNA damage, cell cycle arrest and potentially apoptosis [144].

However, methylated DNA can be repaired by base excision or DNA mismatch repair mechanisms. The DNA repair enzyme O6-methylguanine methyltransferase (MGMT) can be found in various tumors including GB.

If the MGMT promotor is not methylated, MGMT protein can be synthesized and thus repair DNA damage, which had been previously caused by alkylating agents like TMZ or nitrosurea-based chemotherapy like carmustine and lomustine [147].

In contrary if the MGMT promotor region is methylated, alkylating agents can inflict DNA damage without a repairing mechanism [23].

Stupp *et al.* have shown in their work, that the patient group with methylated MGMT status had a higher median survival rate compared to the group with non-methylated MGMT and that the methylation status was the strongest prognostic factor for survival [145]. In the literature, a certain cut-off value for non-methylated and methylated MGMT promoter is widely discussed but is relevant in the decision process whether to treat a patient with an alkylating agent or not. Various works suggest a MGMT methylation cut-off value of 9% [38, 116] while Brigliadori *et al.* describe a prognostic benefit only at a cut-off value of 30% or higher with a median overall survival (OS) of 33.1 months compared to 17.4 months [13].

1.2.2 Tumor recurrence

As described above, survival rates with GB are very limited. This is partly due to the highly invasive growth of glioblastoma itself as surgery can never achieve a total resection. In 95% of the cases, the tumor recurs in a radius of two to three centimeters around the resection cavity. Tumor cells can even be found in the contralateral hemisphere [47].

This characteristic behavior of GB cells to migrate through brain tissue is attributed to epithelial-to-mesenchymal transition (EMT), a complex process where epithelial cells lose their adherent character and transform into a mesenchymal morphology, in order to migrate into joining tissue [4, 86].

Normal cells undergo EMT during wound healing and chronic inflammation, but the same process occurs in tumor cells identically. During EMT, tumor cells lose their cell adhesion and are more motile, less mitotic and less sensitive for chemotherapy and radiation [86]. Thus neoplastic cells are able to disseminate in the whole brain. Cells can also perform the reverse, mesenchymal-to-epithelial transition (MET) to acquire a less invasive and more proliferative behavior [68]. Both transitions are fluid and follow the "Go or Grow" principle. But nevertheless, both states are inevitable for the cells for metastasis and colony formation at a newly metastatic spot [18].

Additionally, the malignant cells exhibit a highly invasive character which allows them to infiltrate surrounding tissue and perivascular niches [4], a driving force for cell invasion is the expression of matrix-metalloproteinase-2 (MMP-2), transforming

growth factor- β (TGF- β) or the colony stimulating factor-1 (CSF-1) [28]. Recent work has shown that irradiation might increase the migration of tumor stem cells and accordingly, radiotherapy might induce EMT in brain tumors, making them more invasive and resistant towards current chemotherapeutic options [49, 53].

Another reason for limited therapeutic success is due to the fact that there is only a small selection of chemotherapeutic agents that have shown both activity against GB cells and ability to cross the BBB *i.e.*, small lipophilic molecules [144].

Furthermore, GB shows characteristic molecular heterogeneity which defines different subclasses that can be divided into proneural, neural, classical and mesenchymal. For example, IDH1/2 mutations are associated with chromosome 1p/19q co-deletions and TP53 mutation, and these are classified as the proneural type; the classical type is associated with EGFR amplification whereas the mesenchymal subtype shows loss of neurofibromin 1 (NF1) [111]. The neural subtype expresses neuron markers like neurofilament light polypeptide (NEFL) [161]. Interestingly, Sottoriva *et al.* showed that one or more subtypes can appear in a single tumor [141]. This fact makes GB exceptionally difficult to treat, especially when bearing in mind that all these different molecular targets should be addressed by one therapy.

Over the decades, the multitude of experimental approaches has led to a better understanding of the tumor microenvironment and different cancer pathways. Theoretically, the immunosuppressive behavior of glioblastoma cells and their ability to communicate with systems like the bone marrow might play a key role in understanding and treating GB [36], and this concept will later be discussed in this work.

1.2.3 Tumor stem cells

Growing evidence suggests that solid tumors as well as leukaemias arise from a small population of tumor stem cells that have the potential for self-renewal and differentiation [48, 136]. Cancer stem cells (CSC) are seen as the driving force behind resistance to therapy [75]. Furthermore, recent data suggest that there may be a direct connection between EMT and migrating CSC [9] and that cellular stress induction, for example via chemo- or radiotherapy, can induce an increase in CSC [48].

The cells of origin for GB are neural stem cells [15, 122]. Looking closer at the behavior of glioblastoma stem cells, as well as "differentiated" GB cells, Schneider *et al.* investigated the differences between these two types, using G35, G38 and G40 primary tumor cells in our laboratory. Their work suggests that the transformation from stem cells to differentiated cells is fluent but can also be reversed depending on their microenvironment, which in the experimental case was stem cell culture medium versus adherent cell culture medium, respectively. The tumors that arise from xenografted differentiated cells and stem cells are both positive for stem cell markers like Sox-2 and Nestin and show similar characteristics in terms of size and biological behavior [130].

1.2.4 Signaling pathways and genetic alterations

As mentioned above, IDH-wildtype and IDH-mutant GB differ in their genetic alterations. Both, activation of cellular proto-oncogenes like EGFR and platelet derived growth factor (PDGF) as well as inactivation of tumor suppressor genes like TP53 and PTEN are involved in the development of GB. Loss of heterozygosity (LOH) on chromosome 10 is the most frequent aberration in both GB types [105–107], PTEN mutation and EGFR overexpression are also very common [106, 167]. EGFR gene amplification occurs almost exclusively in IDH-wildtype GB and is present in approximately of all cases [39]. TP53 is often mutated in IDH-mutated glioblastoma [90]. Unsurprisingly, this same mutation is also seen in WHO grade II and grade III astrocytomas, the less malignant precursors of GB before and during malignant transformation [88, 105, 106].

In GB, like in other cancers, different activated signaling pathways are the key to cell survival and resistance towards apoptosis.

The PI3K/Akt/mTOR pathway is one of the most commonly altered pathways in GB [78] and is affected in 88% of all GB cases [154]. Overexpression of EGFR and the inactivation of PTEN, which normally acts as an inhibitor of the phosphoinositid 3-kinase (PI3K) pathway, result in overactivation of PI3K/Akt/mTOR.

In basic terms, PI3K is activated *via* different growth factor receptors, which are either activated from specific growth factors or constitutively active, e.g. as a result of mutation.

PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-trisphosphate (PIP₃). PIP₃ in turn activates the

serine/threonine kinase Akt (protein kinase B, PKB), resulting in inhibition of apoptosis and increased cell proliferation [107].

1.2.5 Second-line treatment

After recurrence of GB, treatment options are limited. Patients can either re-undergo surgery or radiation which is not feasible in every case, depending on the patient's condition, recurrent tumor location and invasiveness, total radiation dose applied and time period since last radiation. The main second-line treatment is chemotherapy with only a few anticancer drugs approved.

As mentioned above, TMZ is the current chemotherapeutic standard of care for GB. Other alkylating agents are nitrosurea, for example lomustine (CCNU), carmustine (BCNU) or nimustine (ACNU). In a phase III randomized study, Chang *et al.* compared the effects of nitrosurea combined with radiotherapy with that of TMZ and radiotherapy on patients with anaplastic astrocytoma (WHO grade III). The study showed that nitrosurea combined with radiotherapy did not improve the OS compared to TMZ combined with radiotherapy, but that the toxicity was significantly higher with nitrosurea [20].

Another option for the second-line treatment of GB are antivascular agents like bevacizumab, a recombinant humanized monoclonal antibody against the vascular endothelial growth factor (VEGF) receptor. Bevacizumab is approved by the European Medicines Agency (EMA) for use in colorectal, lung, ovarian, renal, and breast cancer and is also a treatment option for cases of highly vascularized glioblastoma [44, 144, 150].

The phase II BELOB trial (a randomized phase II study on bevacizumab versus bevacizumab plus lomustine versus lomustine single agent in recurrent glioblastoma) compared the effect of bevacizumab in combination with lomustine against both single agents. The results showed an OS benefit for the combination treatment compared to the single agents (8 months of median OS for bevacizumab and lomustine, compared to 12 months for the combination therapy) [150].

Three years later in 2017, Wick *et al.* published the results of their randomized phase III clinical study where the combination of bevacizumab and lomustine was compared to single lomustine treatment. The combined treatment did not confirm a survival benefit (8.6 months median OS for lomustine and 9.1 months for the combination), however progression-free survival (PFS) was 2.7 months longer in

the combination group (1.5 months for lomustine and 4.2 months for the dual treatment) [169].

The new understanding of genetic alterations and molecular tumorigenesis of GB led to an intensified development of inhibitors for targeting essential signaling pathways. In this context, the overactivated pathways of overexpressed and amplified receptor tyrosine kinases like EGFR and PDGF as well as the aberrant pathways of mTOR and PI3K-Akt are of the most interest [133, 144, 167].

While a great extent of research has focused on inhibiting the latter pathway, the results have not been very conclusive, with Idelalisib being the only PI3K inhibitor so far that was approved in the U.S. and Europe for anti-cancer therapy [178].

EGFR-targeted therapy originally seemed very promising, but so far EGFR inhibition has not achieved a real breakthrough in the treatment of GB [3, 100, 118].

Despite various clinical studies with tyrosine kinase inhibitors (TKI), none of them are currently approved for GB treatment. There may be different explanations for TKI resistance like the lack of penetrating the BBB or the adaption of GB cells to the blockage of one receptor kinase by upregulating other receptors or pathways [72].

1.2.6 Immunotherapeutic approaches

Immunomodulatory drugs and anti-GB vaccines, are under development as well [44, 97, 133]. One key element in the mounting an immune response against tumor cells are CD8+ T-cells that recognize tumor antigens *via* human leucocyte antigen (HLA)-I presentation, after interaction with which the tumor cell can effectively be targeted [97]. One approach to activation of the immune system is represented by immunomodulatory drugs like checkpoint inhibitors. A phase III clinical trial where patients with recurrent GB treated with either the checkpoint inhibitor nivolumab (a programmed death-1 (PD-1) antibody) or bevacizumab showed similar median OS of 9.8 months with nivolumab *versus* 10 months with bevacizumab [119]. The failure of checkpoint inhibitors might be explained on one hand by the immunosuppressive characteristics of the tumor microenvironment (TME) [165], on the other hand by the low tumor mutational burden (TMB) of GB. Studies showed a correlation between high TMB and a better response rate in various cancers [123, 176] but the authors pointed out that this especially does not apply for gliomas [123].

Another clinical trial examined the survival and immune response in patients with recurrent GB after adjuvant or neoadjuvant treatment with the PD-1 antibody

pembrolizumab. The median OS was significantly higher in the neoadjuvant arm (13.7 months) compared to the adjuvant group (7.5 months). Additionally, the neoadjuvant use of pembrolizumab was associated with increased CD8+ T cell infiltration in the tumor [24].

A different approach to creating an active immune response are vaccines [174]. An international phase III trial evaluated the OS of patients with newly diagnosed GB who received rindopepimut, a vaccine targeting the epidermal growth factor receptor class III variant (EGFRvIII). As a result of a deletion-mutation involving exons 2-7 of the extracellular receptor domain, EGFRvIII is constitutively (*i.e.*, ligand-independently) activated. With a median OS of 20.1 months *versus* 20 months (control group), no survival benefit by the vaccination was detected [166]. Gliovac (ERC1671), a vaccine that combines cells and lysates from autologous and allogeneic tumors from three different donors, elicited biological response in a first clinical trial with nine patients with recurrent, incompletely resected and treatment-resistant GB. A raise in the observed survival of 5 months was detected [126]. Currently, a phase II clinical trial is investigating the effect of Gliovac combined with the granulocyte-monocyte-colony-stimulating factor (GM-CSF) as an injection together with cyclophosphamide and bevacizumab against a placebo vaccine injection, oral control for cyclophosphamide and bevacizumab in 84 patients (NCT01903330). Other vaccination approaches exist such as the combination of autologous tumor proteins with autologous dendritic cells or tumor stem cell vaccines, where autologous dendritic cells are loaded with stem cell antigens [174]. Cytomegalovirus (CMV) is found to be present in over 90% of GB patients [6]. Antiviral therapy showed no effect on GB, therefore the vaccine PEP-CMV is now under investigation (NCT03299309).

1.3 Drug repurposing

The development of new anticancer pharmaceuticals is a main part of cancer research. The time between the first experiments with a candidate compound and until the approval of a new drug is approximately 12 years [103] and development costs vary between 161 to 1800 million dollar per drug [138]. Especially for oncologic drugs, prices are enormous. In general, cancer drug approval has decreased in the last years both by the U.S. food and drug administration (FDA) and the EMA [138].

Due to these reasons, drug repurposing, also called drug repositioning, has gained increasing attention in recent years. The advantage of repositioning older drugs is mainly due to the fact that the drugs are already approved for other medical indications, which means that their entire safety profiles are well-known. Importantly, repurposed drugs which may have pharmacological effects that can be exploited in a new treatment indication. This strategy has worked for a lot of drugs that are in use today. A prominent example is the use of sildenafil (Viagra), which was originally developed against arterial hypertension and angina pectoris, in erectile dysfunction. Among repurposed non-oncological drugs for the treatment of cancer, thalidomide is well-known and was originally approved to treat morning sickness of pregnant women. Tragically, the drug was found to cause malformations and birth defects in new-borns, leading to its withdrawal from the market. After subsequent research discovered potential anticancer activity of thalidomide, the drug gained FDA approval for multiple myeloma treatment in 2006 and was authorized by the EMA in 2008 [134].

1.4 G-CSF and GM-CSF

Colony-stimulating factors (CSF) are a group of cytokines that are essential for survival, formation and differentiation of hematopoietic progenitor cells. The granulocyte-colony-stimulating factor (G-CSF) stimulates the maturation of granulocytes. On the other hand, GM-CSF is necessary for the differentiation from myeloid progenitor cells to macrophages and granulocytes [30, 94].

Recombinant G-CSF, like filgrastim and lenograstim, is therapeutically used for the prophylaxis and treatment of chemotherapy-induced neutropenia [32].

In the brain, G-CSF is expressed by neurons and is upregulated in a stroke model. G-CSF is able to cross the BBB and has a neuroprotective effect in ischemic stroke by protection against apoptosis [124, 128, 129].

Furthermore, G-CSF and GM-CSF are not only inducing the maturation of granulocytes but are also having effects on the differentiation of neural stem cells within the process of neurogenesis [59, 77, 85, 115, 163].

When looking at the secretion of normal astrocytes, G(M)-CSF are both expressed *in vitro* without stimulation [22], with the stimulation of the cytokines tumor necrosis

factor-alpha (TNF- α) and interleukin-1 beta (IL-1 β) both of their secretion is even upregulated [2, 22, 82].

The expression of G(M)-CSF as well as their associated receptors, has been shown in several GB cell lines. [31, 98, 102, 156, 164]

1.4.1 The role of G(M)-CSF in Glioblastoma

In 1996, Pei *et al.* showed the invasive character of G-CSF-producing human pulmonary adenocarcinomas compared to their non-producing counterparts [113]. Investigating the role of G(M)-CSF in GB, *in vitro* data demonstrated that cells that were treated with G-CSF showed increasing viability along with a higher proliferation rate and that the autocrine growth regulation was activated *via* the two factors [98, 164]. Furthermore, the work of Wang *et al.* proved that G-CSF treatment increased cellular invasion and migration and that an anti-G-CSF receptor antibody reversed the effects of G-CSF on invasion and migration [164].

In various solid tumors including gliomas, high levels of G(M)-CSF were associated with poorer prognosis [40, 60, 101] by inducing anti-apoptotic and pro-angiogenic signaling, mainly *via* activation of signal transducer and activator of transcription 3 (STAT3) and increasing expression of VEGF [59, 60, 120].

STAT3 is a transcription factor that can be activated by different cytokines and growth factors. As soon as it is activated, STAT3 induces the expression of different genes. Firstly, STAT3 activates the proto-oncogenes B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xl) which are inhibitors of apoptosis. Secondly, invasion and migration of GB cells are enhanced by upregulating the expression of pro-invasive factors like MMP-2 and MMP-9. Thirdly, STAT3 promotes angiogenesis by expression of VEGF [10, 59, 164].

Kast *et al.* reviewed eight independent clinical studies which demonstrated relative lymphocytopenia in peripheral blood samples of GB patients with a consecutive increase of the neutrophil-to-lymphocyte ratio (NLR) and an increasing fraction of neutrophils [64].

In a meta-analysis of 100 studies the NLR was examined as a putative prognostic factor in different solid tumors. A higher NLR correlated with lower OS and the prognostic effect was most significant in mesothelioma, pancreatic cancer and renal cell carcinoma. Templeton *et al.* thus suggested the NLR as a possible prognostic biomarker [153]. The same approach was taken three years later where the OS of

glioma patients in view of the NLR was analyzed. Like in the previous meta-analysis, OS was poorer with higher NLR but only when looking at the preoperative data. The postoperative NLR was not a predictor for clinical outcome [177]. Neutrophilia occurs for the majority of GB patients and is also considered a negative prognostic factor [117, 125]. Increased neutrophils and decreased lymphocytes define the immunosuppressive state in glioblastoma patients.

In addition, radiation, chemotherapy and the common use of dexamethasone in brain tumor patients result in iatrogenic aggravation of immunosuppression, especially in terms of lymphopenia [64]. Neutrophils specifically contribute to VEGF delivery to tumors, thus driving angiogenesis [67, 152]. Kast *et al.* have confirmed VEGF-laden neutrophil adhesion in and around glioblastoma venules [67].

In vitro and *in vivo* data suggest, that G-CSF can directly induce VEGF secretion of neutrophils, and G-CSF-treated mice had higher plasma levels of VEGF [108]. The cytokine interleukin-8 (IL-8) attracts neutrophils and induces their migration [66] while IL-8 itself may directly promote angiogenesis in GB [11].

Glioblastoma cells are known to secrete IL-8 [22] and along with the secretion of G(M)-CSF, the tumor directs VEGF delivery to itself to induce neoangiogenesis, thus supporting tumor growth.

In summary, GB itself and today's standard of care jointly induce an immunosuppressive state, allowing the tumor to escape immunosurveillance. Furthermore, angiogenesis is driven by G-CSF or stimulated by neutrophils, and cell proliferation is propelled by G(M)-CSF directly. In addition, G(M)-CSF lead to a more invasive and motile cellular phenotype. To may overcome the specific attributes described in this section, three different repurposed drugs, ribavirin, dapsone and fenofibrate might have the potential to lower G(M)-CSF levels [64].

1.5 Ribavirin

The guanosine analogue ribavirin is mainly used as an antiviral agent against hepatitis C virus (HCV) infection, and is usually combined with pegylated interferon since the 1990s, where it is used to enhance the efficacy of interferon-alpha (INF- α) therapy. Besides its use for the treatment of respiratory syncycial virus (RSV) infection and lassa fever, ribavirin is active against RNA and DNA viruses [16, 42].

Steady state is reached after a few weeks, and the usual ribavirin plasma concentration is in the range of 2.0 - 2.5 µg/ml. [137]

The main side effects of this antiviral agent are nausea and hemolytic anemia [148], and ribavirin is contraindicated in pregnancy because of its teratogenic effect, which is why contraception is required in all women with childbearing potential [16]. In combination with INF, neutropenia may occur as an adverse event (AE), but this is perceived as interferon-related [132, 148].

Despite the long clinical use of ribavirin, its antiviral action remains barely understood. A few proposed mechanisms are inhibition of HCV polymerase, inhibition of inosine monophosphate dehydrogenase (IMPDH) and important for anticancer therapy, antagonistic effects against the eukaryotic translation initiation factor 4E (eIF4E), resulting in an inhibition of mRNA capping. [16, 42, 64]

The oncogene eIF4E is upregulated in 30% of all human neoplasms including ALL, CML and subtypes of AML.

Further anti-tumor effects by inhibiting eIF4E and MMP-3 and MMP-9 have been described in lymphoma, metastasis breast cancer and hepatocellular carcinoma evolving in hepatitis C patients. [16]

Ribavirin has shown the most promising effect in leukemia as reviewed by Casaos *et al.* [16].

In addition to all the malignant diseases mentioned above, anti-glioma effects were also shown in several studies, which are discussed below.

In 2014, the first study demonstrated variably growth-inhibitory effects of ribavirin on seven different malignant glioma cell lines. Because not all cell lines responded to the growth inhibition equally, the authors suggested a differentiation into ribavirin-effective and ribavirin-resistant cell lines [104].

Two studies published in 2017, both investigating the effects of ribavirin on glioma-cell lines, described similar results [54, 162]. One of these studies showed apoptotic potential and induction of autophagy by inhibition of IMPDH as well as suppression of the PI3K/Akt/mTOR pathway. The authors pointed out that when autophagy was inhibited by bafilomycin, the cells were more vulnerable to ribavirin-induced apoptosis. These results indicate a protective role of autophagy in case of cellular stress [54].

In a study by Volpin *et al.*, ribavirin's effects on migration and adhesion were investigated *in vitro*. Furthermore, inhibition of tumor growth with either single ribavirin or combined with TMZ and irradiation was observed *in vivo* [162].

The results showed that ribavirin enhanced the effect of TMZ and irradiation and reduced both migration and adhesion *in vitro*, in an orthotopic mouse model, the tumor mass decreased [162].

Ribavirin has the potential to cross the BBB, as seen in patients with human immunodeficiency virus (HIV) infection and subacute sclerosing panencephalitis (SSPE). Here, 74% of the average plasma concentration are reached in the cerebrospinal fluid (CSF). All these features render ribavirin a potential candidate for drug repurposing in GB [16]. While neutropenia may occurs as an AE in patients treated with ribavirin and INF- α , this is mainly attributable to the suppressive effect of INF- α on cytokines like G(M)-CSF [87, 151].

1.6 Dapsone

The antimicrobial function of the sulfone agent dapsone was first recognized in 1937. Dapsone is also effective as an anti-inflammatory drug and has antimicrobial/antiprotozoal properties [173]. Dapsone's main side effects result from toxicity towards red and white blood cells and the skin. Hemolysis, methemoglobinemia and agranulocytosis are frequently seen and AE like rash, pruritus and erythema multiforme have been reported [173].

Because of its anti-inflammatory characteristics, dapsone is widely used in the field of dermatology *i.e.* in dermatitis herpetiformis or bullous pemphigoid and for topical treatment of acne vulgaris. Furthermore, dapsone is used in lepra patients and serves as a prophylactic agent against malaria and *Pneumocystis jirovecii* pneumonia [26, 41].

Dapsone acts by blocking neutrophilic invasion into inflamed tissues and reduces chemotaxis of neutrophils and their adhesion to vessel walls [26, 64, 155].

Neutrophils are attracted by an IL-8 gradient [71]. IL-8 is usually produced from endothelial cells, monocytes and macrophages. Macrophages are highly present in the brain and in case of a lesion, including brain tumors, they migrate into the damaged area. The same applies for microglia, *i.e.* cells in the CNS that are derived from the monocyte/macrophage lineage. Microglia secrete IL-8 as well and

additionally, macrophages and microglia both express IL-1 and TNF- α which may induce a secondary IL-8 release in tumor cells [11]. IL-8 is physiologically expressed by CNS neurons and endothelial cells of brain venules [66].

When staining glioblastoma tissue, accumulation of IL-8 is seen particularly in necrotic and perivascular areas. In patients whose tissues display more intense staining of IL-8, higher vascularization and shorter survival are seen [66].

Dapsone is an oral agent with 86% bioavailability [173]. It can cross the BBB and is detected in the CSF with a concentration of about 73% when compared to plasma concentration [121] and in keratocyte cultures, dapsone reduces their IL-8 secretion [127]. Moreover, dapsone reduces the neutrophil adhesion to epidermal biopsy sites in healthy donors [96].

In glioblastoma primary cell cultures as well as in the cell line U87, Karpel-Massler *et al.* have shown that dapsone and its derivatives D2-D5 potently reduce IL-8 secretion. This work demonstrates the anti-migratory effect of dapsone as well as anchorage-independent growth reduction *in vitro* [61].

Because IL-8 and neutrophil migration and adhesion seem to play an important role in the aggressiveness, invasiveness and neovascularization of glioblastoma, dapsone might be a potential drug available to counteract these particular characteristics of GB cells.

1.7 Fenofibrate

Fenofibrate is a member of the fibrate drug family. There are different derivatives such as fenofibrate, clofibrate, gemfibrozil, bezafibrate and ciprofibrate. Initially, those compounds were produced by the chemical industry as insecticides but after exposure in farm workers, the drugs elicited unexpected reductions of serum lipids. Thus, fenofibrate was approved as a lipid-lowering agent in 1974 [135].

Treatment with fibrates leads to an increase in high-density lipoproteins (HDL), a high decrease of triglycerides (TG) and a modest reduction of low-density lipoproteins (LDL). The target of fibrates is the nuclear peroxisome proliferator-activated receptor (PPAR), a receptor which plays a role in the regulation of the cellular energy balance [135].

Fenofibrate is a prodrug and is hydrolyzed in the liver into its active metabolite fenofibric acid [17], which is an agonist of the PPAR- α receptor. *Via* the regulation

of gene expression, this receptor controls the metabolism of fatty acids and lipoproteins in multiple organs like the kidney, the liver or muscles [135].

In vitro, fenofibrate showed a dose-dependent reduction of IL-1 β induced secretion of G(M)-CSF by human bronchial epithelial cells and similarly decreased IL-1 β -induced IL-8 secretion. [142]

In a mouse model, fenofibrate was able to cross the BBB and showed a protective effect in ischemic stroke when used preventively [34].

There are a few studies examining the effects of PPAR- α agonists in high-grade gliomas. In human cell lines, fenofibrate exerted anti-proliferative effects on p53-mutant and non-mutant cell lines [8, 51]. In higher concentrations, fenofibrate was pro-apoptotic and furthermore, inhibited stem cell invasion and diminished the stem cell marker cluster of differentiation 133 (CD133). However, the observed anti-glioma effects of fenofibrate appeared to be PPAR- α receptor-independent [8]. Wilk *et al.* hypothesized that fenofibrate's effects on glioma cell lines are both PPAR- α -dependent and independent [172].

Apart from effects on the PPAR- α receptor, fenofibrate showed interference with the insulin-like growth factor-1 (IGF-1) signaling in medulloblastoma and glioma cell lines [37, 157]. Fenofibrate reduced cell motility and decreased the activation of IGF-IR signaling cascade by downregulating phosphorylation of Akt, extracellular signal-regulated kinase (ERK) and insulin receptor substrate-1 (IRS-1) [37].

1.8 Aim of the study

For several decades, extensive research including the development of new treatment options for glioblastoma has not led to a clinical breakthrough.

New strategies are needed to address tumor heterogeneity and resistance for which recently developed drugs have not proven successful.

Drug repurposing appears to be as a viable avenue to be explored in order to fill this gap. In addition, combining different drugs is reasonable when aiming to target the multiplicity of active pathways that are simultaneously upregulated in GB.

The goal of this work is to examine the effects of three drugs, *i.e.*, ribavirin, dapson and fenofibrate, on different biological properties of patient-derived GB cells *in vitro*. All these drugs are approved and marked for other indications than GB so that their hypothetical clinical development against GB would constitute off-label use at this time.

Specifically, the drugs' effects on metabolic activity, cell count, apoptosis, migration and secretion were explored in primary tumor material using concentrations that clinically achievable by oral administration. Special emphasis was to be given to the analysis of drug combination effects *in vitro*.

2 Materials and Methods

2.1 Materials

2.1.1 Cell populations

Tumor stem cells were isolated from neurosurgical specimens obtained in the Department of Neurosurgery, Ulm University Hospital, Ulm, Germany. Written consent was obtained before surgery.

This study was approved by the Institutional Review Board at Ulm University (reference no.: 162/10).

The cell populations G35, G38 and G40 were used for this project. The corresponding primary tumors had been neuropathologically characterized as glioblastoma (WHO grade IV).

“G” stands for “Glioblastoma”, “35”, “38” and “40” are the numbers of consecutive glioblastoma samples in the laboratory.

2.1.2 Stem cell medium and supplements

Dulbecco's modified Eagle's medium F-12 (DMEM-F12)	Life Technologies, Paisley, UK
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1% penicillin/streptomycin	Life Technologies, Grand Island, USA
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Added immediately before use:

0,01% epidermal growth factor (EGF)	Biomol GmbH, Hamburg, Germany
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0,02% fibroblast growth factor (FGF)	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
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2% vitamin B27 without vitamin A (B27)	Life Technologies, Grand Island, USA
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2.1.3 Medium and supplements for differentiated cells

Dulbecco's modified Eagle's medium (DMEM)	Life Technologies, Paisley, UK
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10% Fetal Calf Serum (FCS)	Life Technologies, Grand Island, USA
2,5% 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)	Biochrom AG, Berlin, Germany
1% Penicillin/Streptomycin	Life Technologies, Grand Island, USA
Trypsin/Ethylenediaminetetraacetic acid (EDTA) solution	Biochrom
Dulbecco's Phosphate-Buffered Saline (DPBS)	Life Technologies, Paisley, UK
<u>Cell count:</u>	
Trypan blue	Sigma-Aldrich, Steinheim, Germany
Neubauer cell counting chamber	Optik Labor, Lancing, United Kingdom

2.1.4 Chemotherapeutic agents and reagents

Ribavirin (R)	
Dapsone (D)	
Fenofibrate (F)	
granulocyte colony-stimulating factor (G-CSF)	
Temozolomide (TMZ)	Sigma-Aldrich
Dimethylsulfoxide (DMSO)	Sigma-Aldrich
Aqua ad iniectabilia	Fresenius Kabi, Bad Homburg, Germany

2.1.5 MTT Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder	Sigma-Aldrich
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Roswell Park Memorial Institute (RPMI)
Medium 1640 (1x) without L-glutamine,
Phenol Red
Isopropyl alcohol

Life Technologies, Paisley, UK

VWR, Leuven, Belgium

Appliances:

Plate Reader EL800

BioTek, Highland Park, USA

2.1.6 Determination of cell number (CASY)

CASYton solution

Roche, Mannheim, Germany

CASYton tubes

Innovatis, Reutlingen, Germany

CASY clean

Innovatis

CASY1 DT

Innovatis

2.1.7 Determination of specific apoptosis (FACS)

FACS tubes

Sarstedt, Nümbrecht, Germany

FACS flow

BD Biosciences, San José, USA

FACS clean

BD Biosciences

FACS rinse

BD Biosciences

FACS calibur

Becton Dickinson, Heidelberg,
Germany

Nicoletti buffer:

50 µg/ml propidium iodide (PI)

Sigma-Aldrich, Hamburg,
Germany

Triton X-100

Sigma-Aldrich

Sodium citrate dihydrate

AppliChem, Darmstadt,
Germany

2.1.8 Transwell Assay

Fibronectin

Roche, Mannheim, Germany

Transwell-24 System

Corning Inc., Corning, USA

Microscope slides

VWR, Radnor, USA

Cover slips	Menzel-Gläser, Braunschweig, Germany
4',6-diamidino-2-phenylindole (DAPI)	
Methanol	Sigma-Aldrich, Steinheim, Germany
Vectashield mounting medium	Vector Laboratories, Burlingame, USA

2.1.9 Bioplex Assay

Magnetic Luminex Assay	R&D Systems, Abingdon, UK
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2.1.10 Consumables

TC Flask T75, Standard, Ventilation Cap (red)	Sarstedt, Nümbrecht, Germany
TC Flask T75, Suspension, Ventilation Cap (green)	Sarstedt
TC Flask T25, Standard, Ventilation Cap (red)	Sarstedt
TC Flask T25, Suspension, Ventilation Cap (green)	Sarstedt
96-well tissue culture plate	Sarstedt
96-well non-tissue culture plate	Sarstedt
24-well tissue culture plate	Sarstedt
24-well non-tissue culture plate	Sarstedt
12-well tissue culture plate	Sarstedt
12-well non-tissue culture plate	Sarstedt
Serological pipettes (5 ml, 10 ml, 25 ml)	Sarstedt
Combitips advanced (1 ml, 2,5 ml, 5 ml, 10 ml)	Eppendorf, Hamburg, Germany
SafeSeal tubes (0,5 ml, 1,5 ml, 2 ml)	Sarstedt
Tubes (15 ml, 50 ml)	Sarstedt
Pipette tips	Sarstedt

2.1.11 Appliances

Lamina flow hood, Hera Safe KS	Thermo Scientific, Langenselbold, Germany
Incubator Hera Cell, Heraeus	Thermo Scientific, Schwerte, Germany
Water bath W12	Medingen, Arnsdorf, Germany
Heraeus Multifuge X3 FR	Thermo Scientific, Schwerte, Germany
Centrifuge 5417R	Eppendorf
Microscope CK30	Olympus, Hamburg, Germany
Microscope AX70	Olympus
Microscope BZ-9000	Keyence, Osaka, Japan
CC-12 microscope camera	Soft Imaging System GmbH, Münster, Germany
Analytical balance BP221S	Sartorius, Göttingen, Germany
Pipettes (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf
Multipette M4	Eppendorf
Pipetboy acu	Integra Biosciences, Zizers, Swiss
VacuSafe	Integra Biosciences
MS2 Minishaker	IKA
REAX 1d	Heidolph, Schwabach, Germany
BioPlex-200 System	Bio-Rad Laboratories, Hercules, USA

2.1.12 Software

Gen5	BioTek Instruments, Winooski, USA
Cell [^] F	Olympus
CellQuest Pro	Becton Dickinson
BZ-II Viewer	Keyence
BZ-II Analyzer	Keyence

2.2 Methods

2.2.1 Cell culture

In this work, the cultured cell populations G38, G35 and G40 were used as suspension stem cells or adherent "differentiated" cells, depending on culture conditions. To allow expression of the stem cell phenotype, cells were maintained in serum-free DMEM/F12 with 1 % penicillin/streptomycin. The growth factors (GF) B27, EGF and FGF were immediately added before cell seeding into non-tissue cell culture flasks. These cells were grown in neurospheres suspension while conversely, the same stem cells were "differentiated" DMEM medium supplemented with 10% FCS, in a tissue cell culture flask, resulting in an adherent phenotype. Cells were cultured in T75 cell culture flasks at 37°C with 5% CO₂ and 96% humidity.

2.2.1.1 Stem cells

The suspension cells, also referred as stem cells 35 (SC35), stem cells 38 (SC38) and stem cells 40 (SC40), were passaged once or twice a week, depending on the cell density which was evaluated under the microscope. The 20-ml cell suspension of the whole T75 flask was transferred into a 50 ml tube which was then centrifuged at 1300 revolutions per minute (rpm) at room temperature (RT) for 5 min. Next, the viable cells were resuspended in fresh stem cell medium and a certain fraction was transferred into new T75 flasks which contained 20 ml of fresh medium.

2.2.1.2 Differentiated cells

Adherent cells, referred as differentiated cells 35 (DC35), differentiated cells 38 (DC38) and differentiated cells 40 (DC40), were routinely passaged twice a week. For passaging, the medium was removed and the cells were carefully washed with 10 ml DPBS which was removed after washing. Subsequently, 4 ml trypsin/EDTA were added to the cells, followed by 5 minutes of incubation. During this period, the cells were detaching from the bottom of the cell culture flask and exhibited a round morphology under the microscope. After incubation, the cells were resuspended and a certain fraction of the suspension was transferred into a fresh T75 flask which had been pre-filled with 20 ml of fresh DMEM with 1% penicillin/streptomycin and

2.5% HEPES buffer. Adherent cells were used for experiments after passaging them between two to ten times.

2.2.2 Cell counting and determination of cell concentration

For cell counting, cells were centrifugated and resuspended. Thereafter, 20 µl of cell suspension were vortexed and added to 60 µl trypan blue. Next, 10 µl of the mixed suspension were transferred into a Neubauer improved cell counting chamber. As trypan blue only dyes viable cells, these cells were counted under the microscope and the cell concentration was determined according to the following equation:

$$\frac{\text{Counted cells in 4 quadrants}}{4} * 4 (\text{dilution factor}) * 10^4 = \frac{\text{Cell number}}{\text{ml}}$$

2.2.3 Treatment of the cells

Cells were treated with different drugs in this work. According to the clinically relevant plasma and/or CSF concentrations of the substances found in the literature, the following concentrations were used for every experiment:

- Ribavirin: 2 µg/ml
- Dapsone: 1 µg/ml
- Fenofibrate: 4 µg/ml

Dapsone and fenofibrate were dissolved in DMSO and ribavirin was soluble in distilled water. The stock concentrations were 20 mg/ml for ribavirin, 20 mg/ml for dapsone and 30 mg/ml for fenofibrate.

The controls for the experiment consisted of the agent's solvents. The same amount of solvent needed the highest concentration of the respective substance were used in the MTT assay, in all following assays the equal concentrations as in the clinically relevant plasma concentration were used.

For FACS and CASY experiments, a stock solution of 100 mM TMZ dissolved in DMSO and a final concentration of 100 µM (19.4 µg/ml) TMZ were used.

2.2.4 MTT Assay

2.2.4.1 Single treatment

In every assay, G35, G38 and G40 SCs and DCs were seeded in a density of 1×10^4 cells/cm² on 96-well plates. Non-tissue plates were used for SCs and tissue plates for DCs. For SCs, 50 μ l of the cell suspension were seeded while for DCs 100 μ l were dispensed in the respective culture medium. The plates were incubated at 37°C overnight, and 24 h later the cells were exposed to ribavirin, dapsone and fenofibrate. Cells were treated with single dosing. The ranges are listed below.

Table 1: Concentration range of the compounds used for the in-vitro experiments in this work. The average plasma concentration of each substance is bold-faced.

Ribavirin (μ g/ml)	Dapsone (μ g/ml)	Fenofibrate (μ g/ml)
0.0002	0.0001	0.0004
0.002	0.001	0.004
0.02	0.01	0.04
0.2	0.1	0.4
2	1	4
20	10	40
200	100	400
1000	500	1000

For the SCs, 50 μ l of the respective medium with the corresponding amounts of the substances were added to the cells. In contrast, the standard medium was completely removed and 100 μ l of drug-containing medium were added to the DCs. Since all substances in this assay had very high concentrations, we assumed that the control group with the solvent DMSO showed cytotoxic effects due to the high concentrations of DMSO itself. To avoid false effects in the control group we additionally used untreated medium as a "real" control group.

After incubating the cells for 24 h, 48 h, 96 h and 144 h, the metabolic activity of the cells was determined.

At the time points mentioned, 20 μ l MTT solution with a concentration of 10 mg/ml MTT powder were added to the SCs. The medium of the DCs was removed and

100 µl of MTT working solution, consisting of 20% MTT solution and 80% RPMI medium, were added to the cells. Before measuring the cells' metabolic activity, incubation was continued for another 3 h. Afterwards, 100 µl isopropanol were pipetted into each well. To stir the cells, the wells were resuspended and the plates were put on a shaker for 30 min. Finally, the optical density was measured at a wave length of 550 nm using an EL800 plate reader. The yellow-colored MTT can only be reduced by viable and metabolically active cells to an insoluble dark-blue colored formazan. The color change can then be quantitatively recorded by a plate reader.

2.2.4.2 Combination treatment

For the combination experiments, ribavirin, dapson and fenofibrate were used in their clinically relevant plasma concentrations which were 2 µg/ml for ribavirin, 1 µg/ml for dapson and 4 µg/ml for fenofibrate.

All substances were used alone as well as in dual and triple combinations, and only SC35 and DC35 were used for this experimental part.

The same procedure was employed as described in the above section, except that measurements were performed after 24 h and 144 h of drug exposure, respectively.

2.2.4.3 DMSO treatment

To compare the effects of the substances to the cytotoxic effect of the single solvent DMSO in the same concentration, MTT Assays were performed, using a selected section of DMSO concentrations which corresponded to the DMSO concentration in the solved drugs of the previously performed MTT Assays.

SC35 and DC35 cells were used and measurements were taken after 144 h.

The following concentration ranges were used:

Table 2: Concentration range of DMSO (dimethylsulfoxide)

DMSO (µg/ml)	
0.2	0.4
2	4
20	40
200	400

2.2.5 Determination of cell numbers (CASY)

For the determination of viable cell numbers, SC35 and DC35 were seeded into 12-well plates with a density of 1×10^4 cells/cm². For SCs, 1000 µl of cell suspension were put in each well while for DCs, the double amount was used. The cells were incubated for 24 h and afterwards the following substances and their combinations were added:

Table 3: Concentrations of drugs and solvent control used for CASY and FACS.
DMSO (dimethylsulfoxide), TMZ (temozolomide), R (ribavirin), D (dapson), F (fenofibrate)

DMSO / control group	4 µg/ml
TMZ	19.4 µg/ml
R + D + F	2 µg/ml + 1 µg/ml + 4 µg/ml
R + D + F + TMZ	2 µg/ml + 1 µg/ml + 4 µg/ml + 19.4 µg/ml

1000 µl of the respective medium and dissolved substances were added to the SCs whereas the DC medium was removed and 2000 µl of the specific medium with the substances was subjoined to the cells. Measurements started after 24 h and 144 h. At the indicated time point, 100 µl of each SC's well were added to a prepared tube, which had been filled with 10 ml CASY ton solution.

In contrast, the DC medium was removed and 500 µl of Trypsin/EDTA were added to each well. After incubating for 5 min, the detached cells were resuspended in 1.5 ml DPBS. One-hundred µl of the cell suspension were mixed with CASY ton solution in a 1:100 volume per volume (v/v) ratio.

By exposing the cells to a voltage field, only viable cells with an intact plasma membrane were counted by CASY1 DT.

2.2.6 Determination of specific apoptosis (FACS)

For the Nicoletti assay, SC35 and DC35 cells were seeded in a density of 1×10^4 cells/cm² in a 12-well plate. The same set-up as described for CASY was chosen to compare the viability of treated and untreated cells with respect to specific apoptosis. After 24 h and 144 h, the supernatant of the adherent cells was transferred into FACS tubes, and 200 µl of Trypsin/EDTA was used to detach the

cells. After incubating the plates for 5 min, the cells were resuspended with 400 µl of DPBS and added to their respective supernatant.

At the same timepoint, the stem cell medium was aspirated and transferred into FACS tubes.

All FACS tubes, either containing SCs or DCs, were centrifuged for 5 min at 4°C at 1800 rpm. Supernatants were carefully aspirated and the cell pellets were mixed with 100 µl of Nicoletti buffer. Before measurements started, the resuspended cells were incubated at 4°C for 15 min.

Flow cytometry is used to detect cells in different phases of the cell cycle. Initially, the cells were permeabilized with Triton X and afterwards the DNA was stained with the fluorescent propidium iodide (PI). These substances are components of the Nicoletti buffer. The signal of PI was measured with the FACSCalibur flow cytometer using the CellQuest Software. The signal intensity correlates with the different stages of the cell cycle of viable cells and is lower in apoptotic cells, due to DNA fragmentation and degradation.

DNA fragments leave the cells through the cell membrane defects caused by Triton X, those fragments can further be detected with the FACS.

After FACS measurements, specific apoptosis rates were calculated with following formula:

$$\text{specific apoptosis [\%]} = \frac{\text{induced apoptosis [\%]} - \text{spontaneous apoptosis [\%]}}{100 - \text{spontaneous apoptosis [\%]}} * 100$$

2.2.7 Transwell Assay

2.2.7.1 Different experimental conditions with precedent drug exposure

For the Transwell Assay, SC35, DC35, DC38 and DC40 cells were used.

In this experimental setting, SC35 and DC35 cells were seeded in a density of 1×10^4 cells/cm² in T75 flasks 24 h prior to their transfer into the Transwell inserts. The plates with the Transwell inserts had been freshly prepared and coated with fibronectin.

The fibronectin stock was diluted 1:100 v/v with DPBS. Six-hundred µl of the working solution were pipetted into the lower compartment and 100 µl were dispensed into the upper compartment. The plates were then incubated at 37°C for 2 h.

Subsequently, the plates were washed with DPBS three times.

Meanwhile, the seeded cells in the T75 flasks were treated with the combination of all three substances. The same was performed with DMSO as a control, followed by incubation at 37°C for 1 h.

After 1 h, the suspension cells were removed, centrifuged and resuspended with 5 ml fresh medium. The medium of the adherent cells was removed and cells were detached with 3 ml Trypsin/EDTA and resuspended with 6 ml fresh DMEM.

The cells of the 4 different flasks were counted again and then seeded in a density of 1×10^4 cells/50µl/insert.

To create a stimulus for the cells to migrate through the membrane, a concentration gradient between the upper and the lower compartment was created in two experimental groups, using growth factors as an attractant. These groups were compared to the two following experimental groups without a gradient between the upper and the lower compartment. Following concentrations were used:

Table 4: Concentration gradients for SC35 in the precedent drug exposure Transwell Assay. 0% refer to medium without growth factors. GF% refer to the usual growth factor concentration in the medium also used in cell culture conditions.

SC (stem cells), GF (growth factor), UC (upper compartment), LC (lower compartment)

SC35			
no gradient		gradient	
0% GF (UC) - 0% GF (LC)	GF% (UC) - GF% (LC)	0% GF (UC) - GF% (LC)	0% GF (UC) - supernatant (LC)

Table 5: Concentration gradients for DC35 in the precedent drug exposure Transwell Assay. 0% refer to medium without FCS. 10% refer to the usual FCS concentration in the medium also used in cell culture conditions.

DC (differentiated cells), GF (growth factor), UC (upper compartment), LC (lower compartment)
FCS (fetal calf serum)

DC35			
no gradient		gradient	
0% FCS (UC) - 0% FCS (LC)	10% FCS (UC) - 10% FCS (LC)	0% FCS (UC) - 10% FCS (LC)	0% FCS (UC) - supernatant (LC)

In the first two experimental groups, no gradient was generated, either by adding the DC growth factor FCS or the stem cell growth factors B27, EGF and FGF in both compartments equally or by just adding medium without (0%) growth factors.

In the last two experimental groups, a gradient was created. First without (0%) growth factors in the upper compartment and 10% FCS in the DC medium or B27, EGF and FGF for the SC medium in the lower compartment.

The second gradient was created by putting 0% growth factors in the upper compartment and supernatant from the respective previously cultured cells in the lower compartment. The supernatant hypothetically contains growth factors or other factors secreted by the tumor cells. The plates were then incubated at 37°C for 18 h.

After 18 h, each insert was wiped with a cotton swab to remove the rest of the cells in the upper compartment. Subsequently the medium was removed and 600 µl and 100 µl of DPBS, respectively, were added to the lower and upper compartment. These steps were repeated twice. After washing, the membranes of the inserts were stained for 5 min with 4',6-Diamidin-2-phenylindol (DAPI), diluted 1:2000 v/v with methanol .

Mounting medium was then put on microscope slides, the membranes were cut out with a scalpel and put onto the mounting medium. Cover slips were pressed on the microscope slides and sealed with nail polish.

The dried slides were examined under the fluorescent microscope and the cells were counted with the BZ-II Viewer and BZ-II Analyzer.

2.2.7.2 Equal experimental conditions with continuous drug exposure

To compare the previous results with a continuous drug exposure, we started a second Transwell Assay. Because results of the first experiment showed that quantitatively DCs were more likely to migrate through the membrane, we only used DC35, DC38 and DC40 cells in the second experimental setting. In this set-up, the cells were not treated with the substances 1 h prior to seeding, but ribavirin, dapsone and fenofibrate were added to the medium of both the upper and lower compartment. The same cell number (1×10^4 cells/50µl/insert) was used as previously described (section 2.2.7.1).

Only the following concentration gradient was used:

Table 6: Combined FCS concentration gradient for DC35, DC38 and DC40 in the continuous drug exposure Transwell Assay.

DC (differentiated cells), UC (upper compartment), LC (lower compartment), FCS (fetal calf serum)

DC35	DC38	DC40
1.5% FCS (UC) - 10% FCS (LC)		

Following another protocol, this time, the fibronectin coating was performed 24 h before cell seeding, and the plates were stored at 4°C prior to use. On the next day, the plates were washed according to the protocol in the first setting. After cell seeding, the plates were incubated at 37°C for 24 h.

Subsequently, washing, staining and counting procedure was performed as previously described (section 2.2.7.1). Because the cell count of the first results showed a high standard deviation when only evaluating one membrane, this time, three membranes, instead of only one, were counted and the means of all triple counts were calculated.

2.2.7.3 Transwell Assay with G-CSF

For the third Transwell Assay, SC35 and DC35 cells were used.

First, the Transwell inserts were coated with fibronectin and incubated at 37°C for two hours as described in section 2.2.7.1. The cells were not treated with any substances, and the seeding was performed as previously described (sections 2.2.7.1 and 2.2.7.2).

The assay examined two conditions: the first condition served as the control with no growth factors in both the lower and the upper compartment. The second condition used G-CSF as a chemoattractant in the lower compartment. No further growth factors were added to any compartment.

G-CSF was dissolved in distilled water with a stock concentration of 0.1 mg/ml and for the assay, a concentration of 60 pg/ml was used.

The plates were incubated at 37°C for 18 h and subsequently, the previously described procedure (section 2.2.7.1) was performed for staining and counting the transmigrated cells.

2.2.8 Bioplex Assay

2.2.8.1 Preparation of the supernatant

The assay works similarly to an enzyme-linked immunosorbent assay (ELISA). It uses beads that are coated with analyte-specific antibodies. The beads are binding the analyte, when adding biotinylated antibodies, they form an antigen-antibody complex with the analyte and the beads. Streptavidin-phycoerythrin (streptavidin-PE) is binding to the biotinylated antibodies, and its signal can further be quantified using the Bio-Plex 200 Analyzer.

The purpose of this test is the detection of the secretion profile in different cell populations of our primary tumor material, more specifically the detection of G-CSF in treated and untreated cells at different timepoints.

Prior to starting the Bioplex assay, cultured cells' supernatant had to be collected. Therefore, DC35, DC38 and DC40 were seeded in a density of 1×10^4 cells/cm² in T25 flasks. For each cell line, six T25 flasks were used. Three timepoints, *i.e.*, 24 h, 48 h and 72 h, were examined in both treated and untreated cells.

After the cell seeding, a combination of ribavirin (2 µg/ml), dapson (1 µg/ml) and fenofibrate (4 µg/ml) was added to the 24-h, 48-h and 72-h incubation flasks. Separately, DMSO was added to the three control flasks in a concentration corresponding to that obtained with fenofibrate.

At the first 24-h timepoint, the supernatants of each cell line were aspirated and 2 ml of it were collected tubes. The tubes were then stored at -20°C until the later use. Subsequently, 1 ml of Trypsin/EDTA was added to each T25 flask, and the cells were incubated at 37°C for 5 min.

Afterwards the detached cells were resuspended with 2 ml DMEM.

Using the Neubauer improved cell counting chamber, cells were then counted under the microscope. The two remaining flasks for the timepoints of 48 h and 72 h were once again treated with the drug combination with the previously used concentration; the control flasks were treated with DMSO.

For the last two timepoints, the procedures were repeated as described above.

2.2.8.2 Performing the Assay

All reagents of the assay were prepared according to the manufacturer's recommendations.

50 µl of the standards and the 1:1 diluted samples were added to each well of the 96-well plate. Fifty µl of the diluted microparticle suspension were added to each well and the plate was incubated on a shaker at 800 rpm and RT for 2 h.

Next, the plate was washed three times with a washing buffer. Afterwards, 50 µl of the diluted biotin-antibody solution was pipetted into each well. The plate was then covered and incubated on the shaker at 800 rpm at RT for 1 h. After that, the washing procedure was repeated. Subsequently, 50 µl of the diluted streptavidin-PE were added to each well prior to incubation on the shaker at RT for 30 min. The washing was repeated, and 100 µl of the washing buffer were added to each well. The plate was incubated for 2 min on the shaker before starting the reading with the Bio-Rad 200 Analyzer, which detects the PE-derived signal via flow cytometry.

3 Results

3.1 Effects of monotherapy with ribavirin, dapsone and fenofibrate on the metabolic activity of SCs and DCs

To measure the cells' metabolic ability, MTT Assays were performed. Drugs were used as single treatments and four different timepoints were examined with six different cell cultures. G35, G38 and G40 were used both as stem cells as well as differentiated cells, depending on culture conditions.

For every assay, three independent experiments were performed, and the mean values and standard deviations are graphically shown in the figures below.

For experimental planning, the clinically achievable concentrations in plasma and/or CSF were retrieved from the medical literature for each of the three drugs. It may be speculated that the intact BBB may reduce drug concentrations achievable in brain tissue.

The following concentrations were found for each agent:

- **Ribavirin:** 2 to 2.5 µg/ml in plasma [137], and 0.8 to 2.5 µg/ml in the CSF [55]. A concentration of **2 µg/ml** was used in this work.
- **Dapsone:** 0.84 to 1.52 µg/ml in plasma [46] and an average of 0.84 µg/ml in the CSF [121]. A concentration of **1 µg/ml** was used in this work.
- **Fenofibrate:** 4.42 µg/ml in plasma [52]. A concentration of **4 µg/ml** was used in this work.
- **Temozolomide:** 0.1 to 20 µg/ml in plasma [73] and 0.16 to 1.93 µg/ml in CSF [109]. A concentration of **19.4 µg/ml** (100 µM) was used in this work.

For the single MTT measurements, a wide range of concentrations was examined, using the plasma concentrations above as "center values" and going up and down one power of ten. Three different cell populations were examined with this assay, *i.e.*, G35, G38 and G40. The stem cells were then compared to their corresponding differentiated cells. The first MTT assay examined all three drugs, their biological effects on a relatively broad spectrum of cells and their effectiveness in different concentrations.

In most of the figures presented below, data were normalized to the respective controls which had been equated to 1 (100%).

3.1.1 Ribavirin

In general, differentiated cells are more sensitive to ribavirin than stem cells. But DC40 behaved similar to treated stem cells.

Ribavirin was dissolved in H₂O, therefore H₂O was added as a control.

In all three differentiated cell lines, a reduction of the metabolic activity is seen following exposure to 20 µg/ml and after 24 h and further decreased dose-dependently. The activity continued to reduce after 48 h and 96 h but mostly increased again after 144 h. The H₂O-treated group showed no effect.

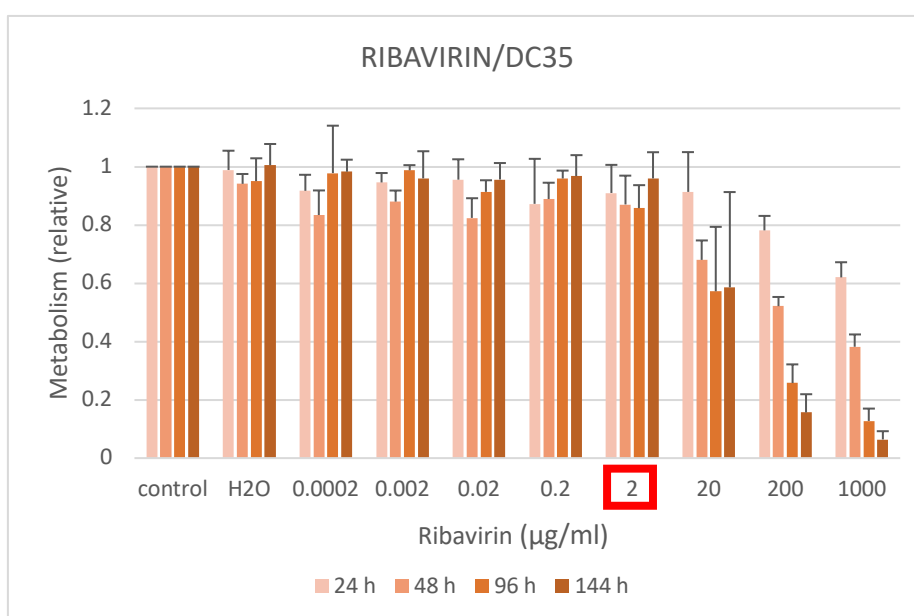


Figure 1: Effects of ribavirin on the metabolic activity of DC35. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours)

In DC35, a small effect was seen in the reported clinically relevant concentration of 2 µg/ml. At 96 h, the metabolic activity decreased by about 15%. At a concentration of 20 µg/ml, a reduction of 9% to 43% was seen at all four different timepoints, and at a concentration of 200 µg/ml and 1000 µg/ml, the metabolic activity was only 15% and 6% after 144 h (Figure 1).

DC38 behaved similarly to DC35 but may be a bit more sensitive. The highest reduction in the clinically relevant concentration was 22% after 96 h. One order of magnitude higher, the greatest reduction was again seen after 96 h with 52% less

metabolic activity. The two highest concentrations, 200 µg/ml and 1000 µg/ml, led to a reduction of 88% and 96% after 144 h (Figure 2).

When looking at DC40, the cells were not sensitive to the treatment until a concentration of 20 µg/ml. At this concentration, the highest reduction, 27%, was seen like in the other two differentiated cell lines after 96 h. At the two higher concentrations, the metabolism of the cells was reduced to 14% and 5%, again measured after 144 h (Figure 3).

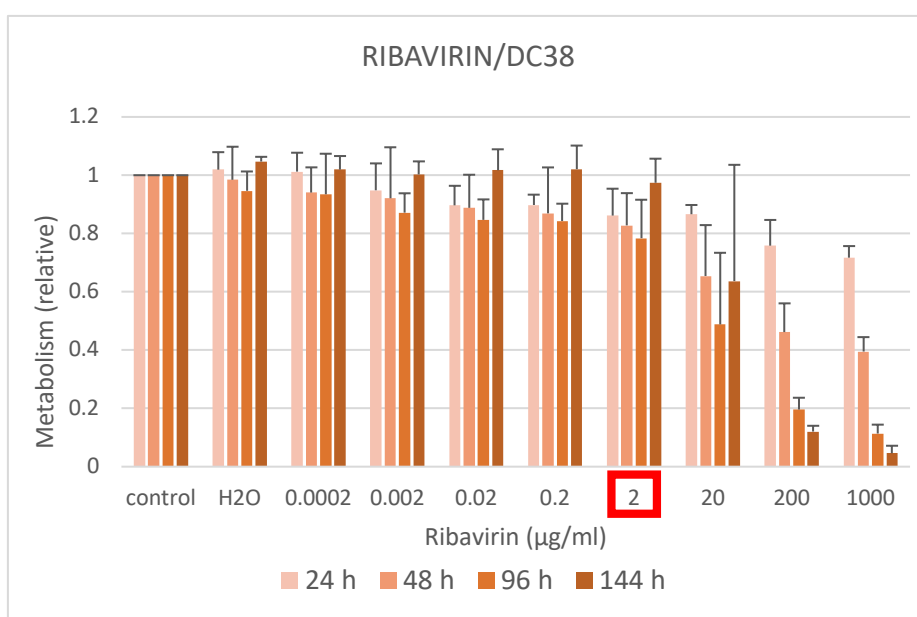


Figure 2: Effects of ribavirin on the metabolic activity of DC38. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours)

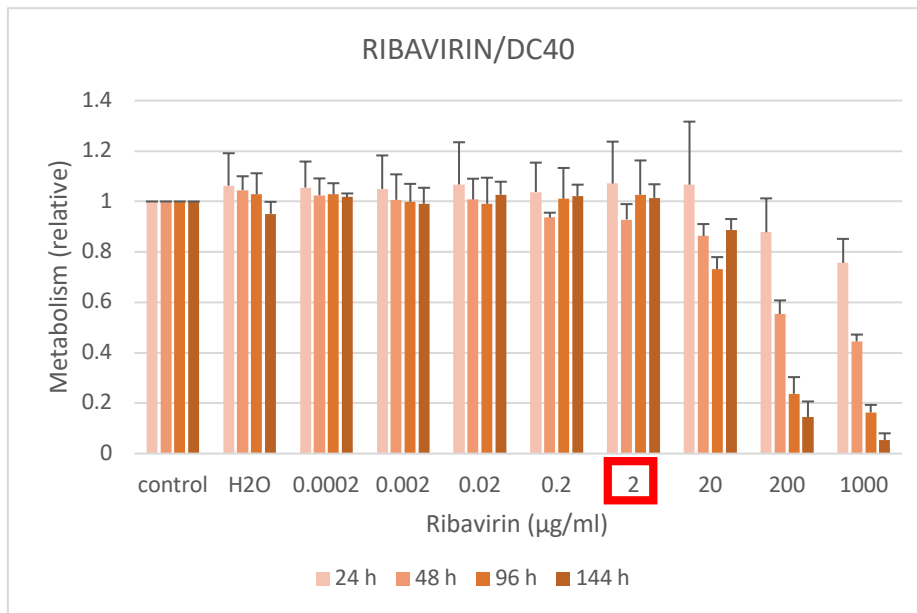


Figure 3: Effects of ribavirin on the metabolic activity of DC40. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours)

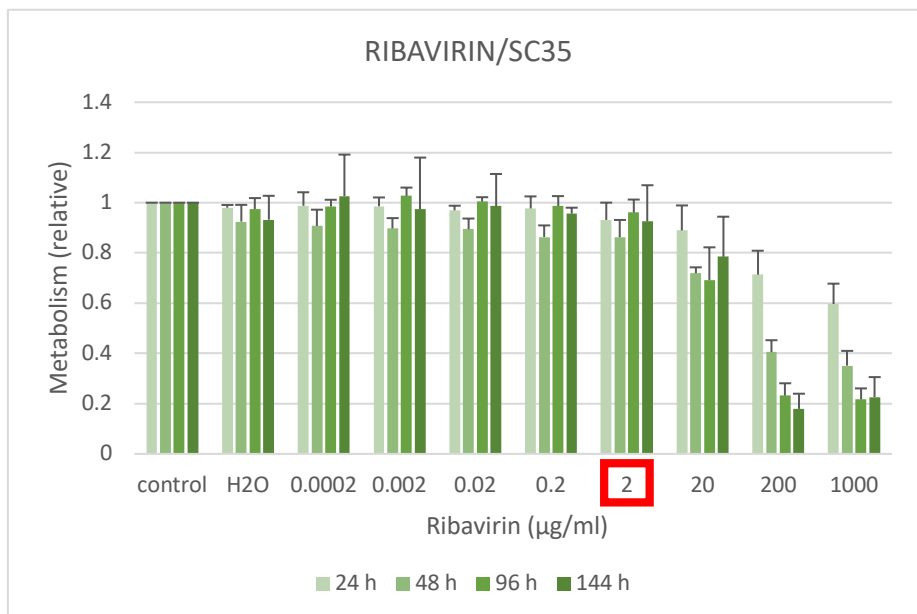


Figure 4: Effects of ribavirin on the metabolic activity of SC35. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours)

In all three stem cell lines, the most sensitive timepoint in the clinically relevant concentration group was after 48 h, where they showed a slightly reduced effect on metabolism. All SCs were less sensitive to the treatment than the differentiated cells. Even at the highest ribavirin concentrations, they did not reach the amount of reduction in metabolism as seen in differentiated cells.

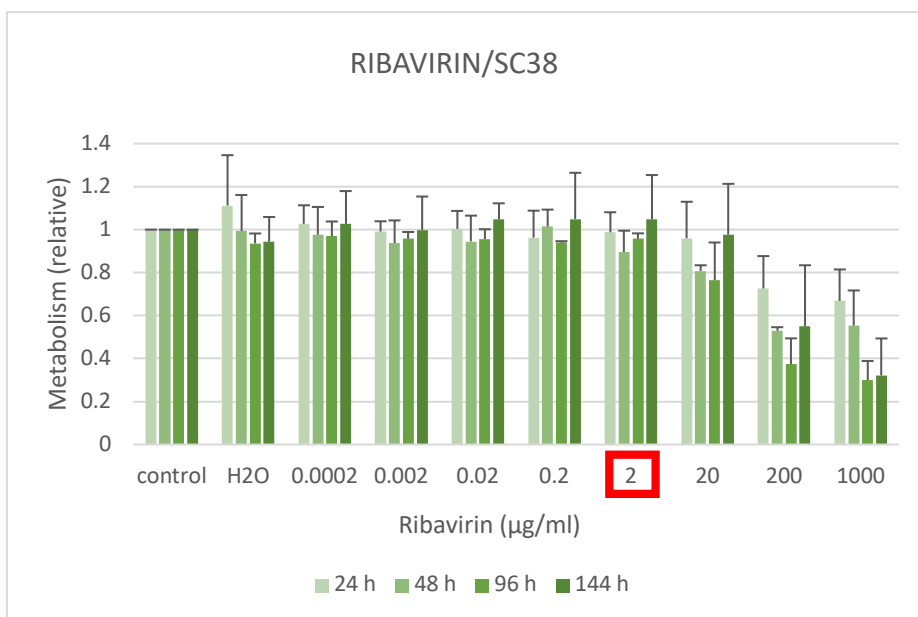


Figure 5: Effects of ribavirin on the metabolic activity of SC38. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours)

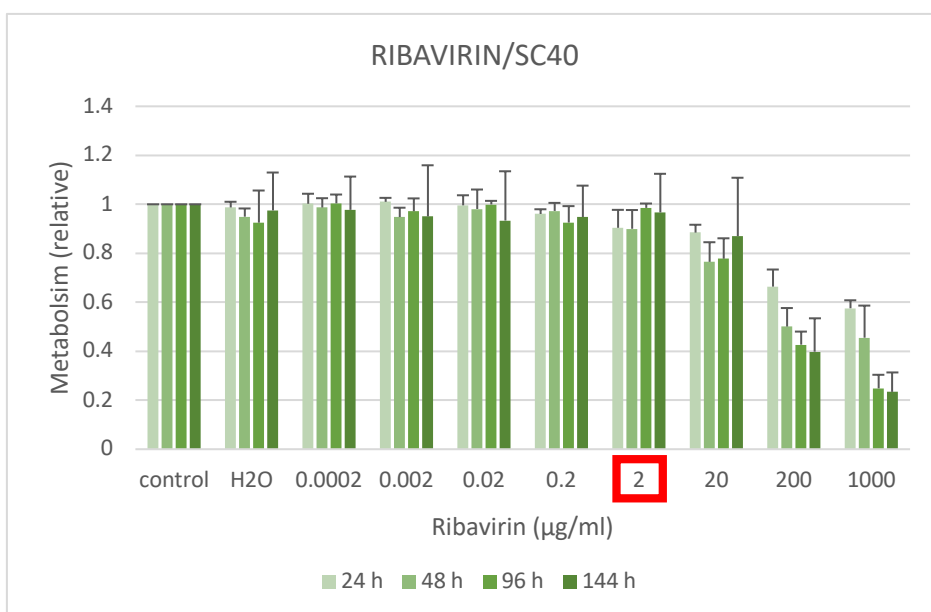


Figure 6: Effects of ribavirin on the metabolic activity of SC40. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours)

In SC35, the metabolic activity was reduced by about 14% after 48 h and was further decreased at higher ribavirin concentrations. At the two highest concentrations, the metabolic activity was reduced to 17% and 22% (Figure 4). SC38 were less sensitive to ribavirin than SC35; at a concentration of 2 µg/ml, the metabolic activity

decreased approximately about 11% and was not reduced as much as in SC35 at higher concentrations.

In the next-higher concentration, as seen in Figure 5, the metabolic activity ranged between 76-97%. After 144 h at the two highest concentrations, the metabolism of the cells was reduced to 55% and 32%, respectively.

In SC40, the reduction of metabolic activity amounted to only 10% after 24 h at a concentration of 2 µg/ml, 24% after 48 h and a concentration of 20 µg/ml, while the remaining metabolism of the cells after 144 h at a concentration of 200 µg/ml and 1000 µg/ml was 39% and 23%, respectively (Figure 6).

3.1.2 Dapsone

Next, the effect of a single treatment with dapsone was further studied.

Again, all three cell populations were used as adherent and suspension cells. As DMSO was the solvent of dapsone, DMSO was used as control corresponding to the highest corresponding concentration of dapsone (500 µg/ml).

In those high-concentration ranges one can see the cytotoxic effect of DMSO. DC38 were the most sensitive cells regarding DMSO their remaining metabolic activity was only 17% (Figure 8) compared to SC38 which were not equally sensitive and had a preserved metabolic activity of 61% after 144 h (Figure 11).

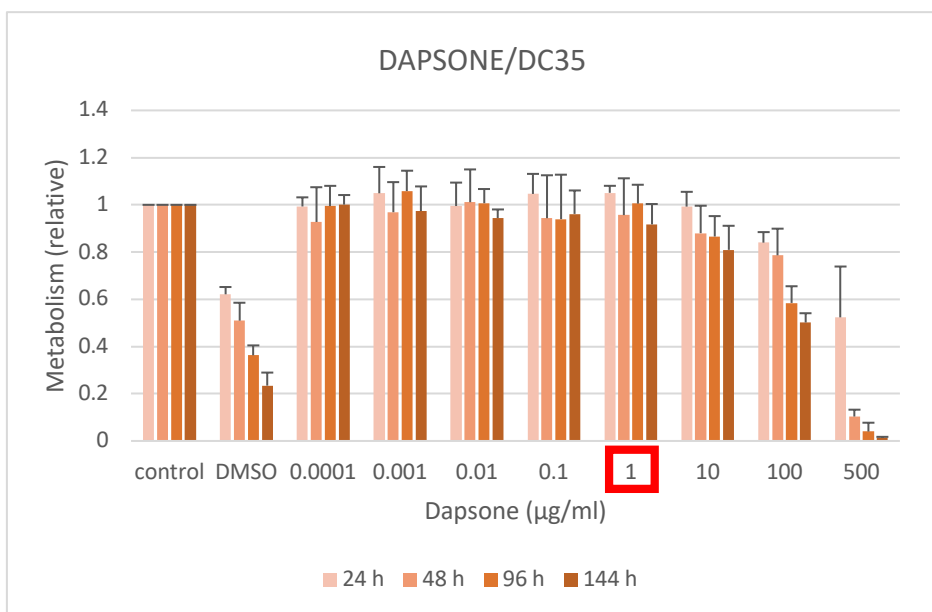


Figure 7: Effects of dapsone on the metabolic activity of DC35. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide)

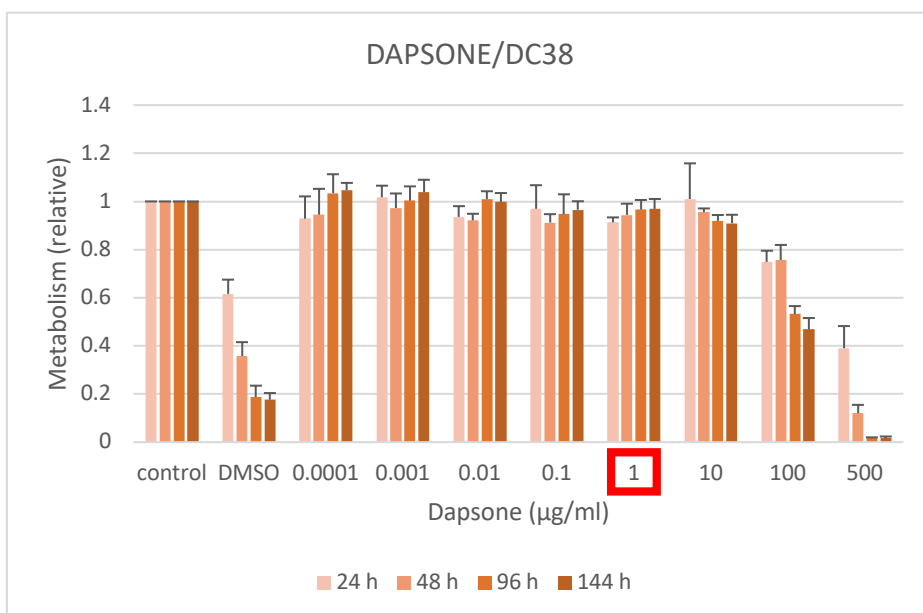


Figure 8: Effects of dapsone on the metabolic activity of DC38. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide)

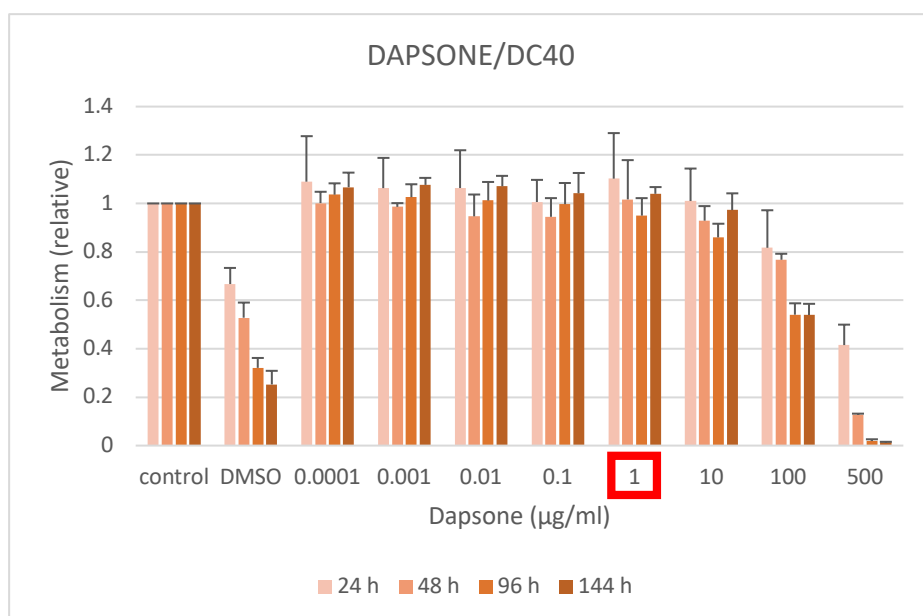


Figure 9: Effects of dapsone on the metabolic activity of DC40. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide)

Until a concentration of 10 µg/ml there were no measurable effects on the metabolic activity of the cells. At a concentration of 1 µg/ml, the viability decreased by only about 9% in DC35 and DC38 (Figure 7, 8) and even at a concentration ten times higher, the greatest reduction was seen in DC35 (20%) after 144 h. DC38 and DC40 only decreased their metabolic activity by around 10% and 15% either after 96 h or 144 h (Figure 8, 9). In the next-higher concentration (100 µg/ml), the respectively antimetabolic effect in all three differentiated cells ranged between 54% and 47% after 144 h and was highest in DC38 (54%). In the highest concentration of 500 µg/ml, the cells had no metabolic activity left; after 24 h, the activity of the cells ranged between 40% and 50% in all three differentiated cell lines and further decreased to just 1% after 144 h (Figure 7, 8, 9). SC35 only reduced their metabolic activity by about 7% at a concentration of 100 µg/ml and did not respond to any lower concentration. Surprisingly, at a concentration of 500 µg/ml, their metabolic activity reduced drastically; after 24 h it was at 89% and reached 13% after 144 h (Figure 10). Dapsone proved to be more effective in SC38 than in SC35. At a concentration of 1 µg/ml, 10 µg/ml and 100 µg/ml the reduction of metabolism was between 10% and 20%, and the residual activity further decreased by 80% after 144 h at a concentration of 500 µg/ml (Figure 11). In SC40 we had no notable reduction

in the lowest six concentrations while at 100 µg/ml there was only a reduction of 18% after 96 h. But at the highest concentration, the remaining metabolic activity decreased to 15% (Figure 12).

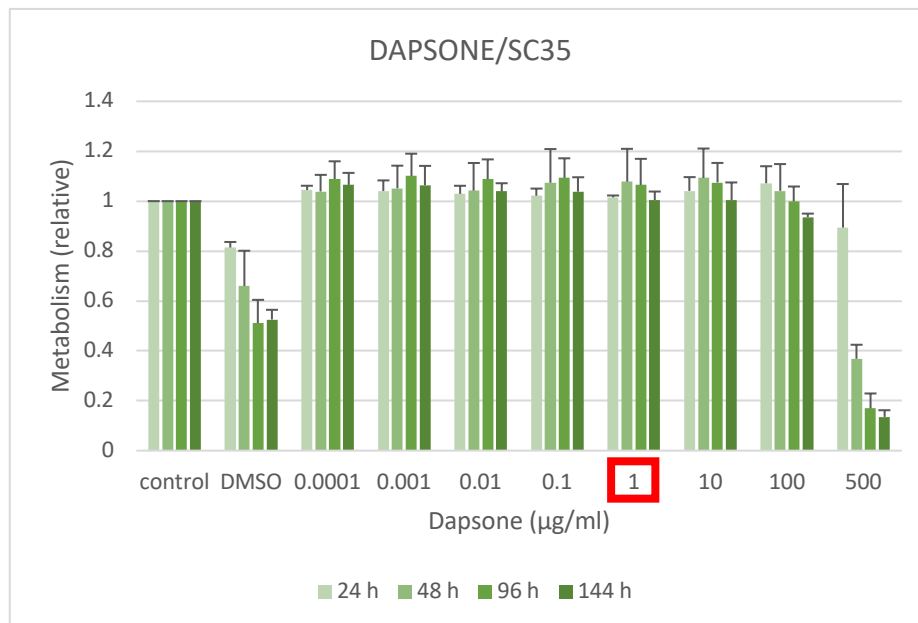


Figure 10: Effect of dapsone on the metabolic activity of SC35. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours), DMSO (dimethylsulfoxide)

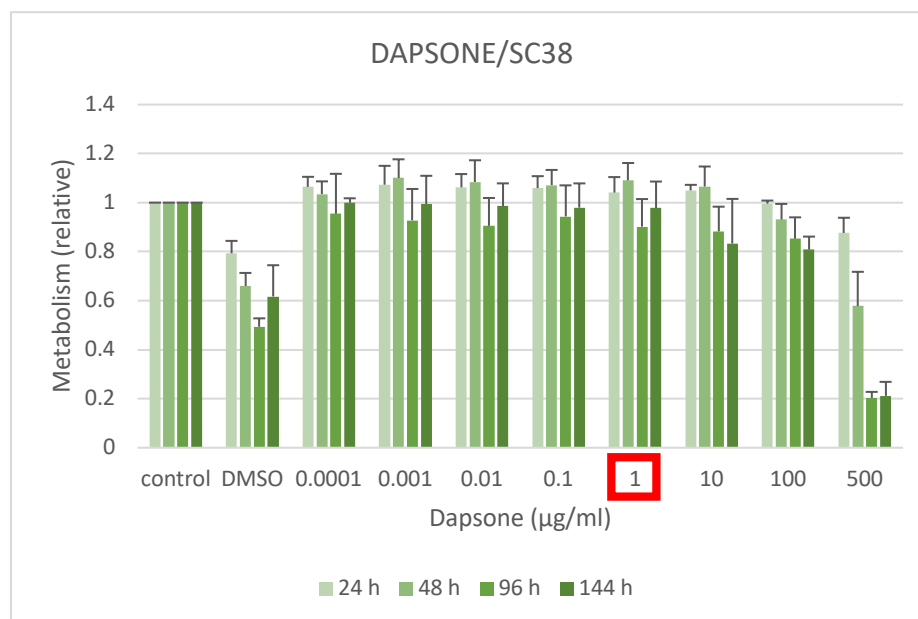


Figure 11: Effect of dapsone on the metabolic activity of SC38. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours), DMSO (dimethylsulfoxide)

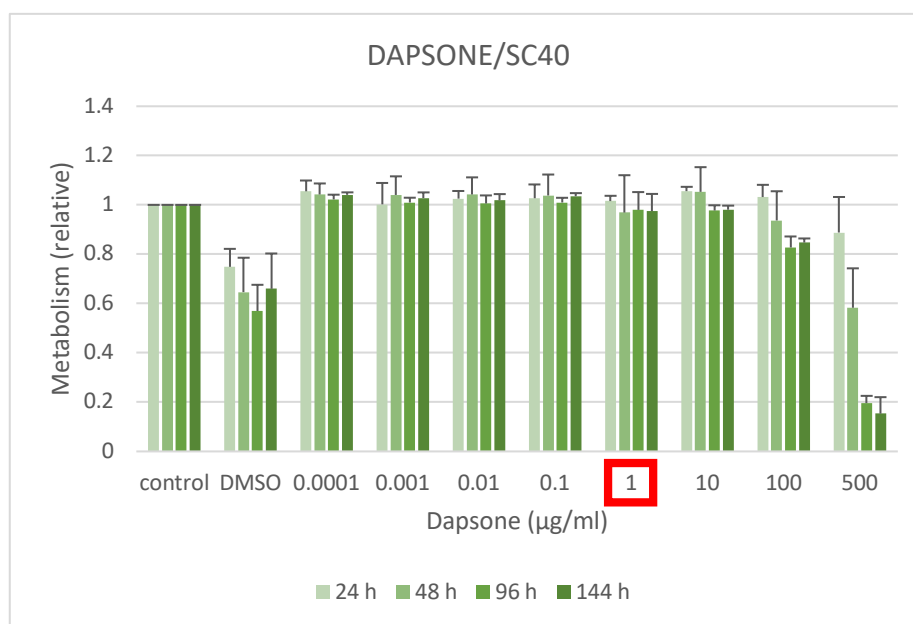


Figure 12: Effect of dapsone on the metabolic activity of SC40. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours), DMSO (dimethylsulfoxide)

3.1.3 Fenofibrate

Finally, the single use of fenofibrate and its effect on G35, G38 and G40 cells was examined. Results of this experiment were largely similar to those of the previous two drugs and again, differentiated cells were slightly more sensitive to the treatment than their corresponding stem cells.

DMSO was added as a control since it was the solvent of fenofibrate. Here again, it was used with amount corresponding to the highest concentration of fenofibrate, 1000 µg/ml.

The cytotoxic reduction of DMSO was, on average, 90% of all differentiated cell lines, compared to the mean reduction of fenofibrate which was 66% in all three stem cell lines after 144 h.

In the clinically relevant concentration of 4 µg/ml, the highest reduction of cell viability was seen in DC38 and DC35 with a reductive effect of 15% (Figure 14) and 14% (Figure 13), respectively both after 48 h. In DC40, no real effect occurred, as their viability decreased by only about 7% after 96 h (Figure 15).

With the next-higher concentration, the greatest effect was seen in DC35, which reduced their metabolic activity by about 56% after 48 h, followed by DC40 with a reduction of 42% after 24 h and DC38 with 37% reduction after 48 h. In the two highest concentrations of 400 µg/ml and 1000 µg/ml, DC35 decreased their metabolic activity to 6% and 1% after 144 h, while DC38 and DC40 reduced their metabolic activity to 20% and 1% after 144 h (Figure 13, Figure 14, Figure 15).

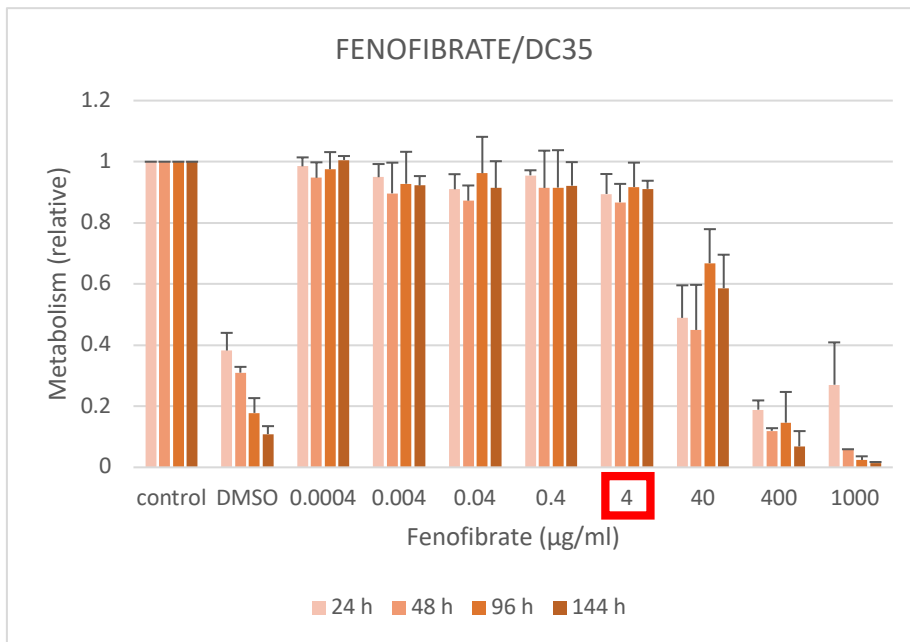


Figure 13: Effect of fenofibrate on the metabolic activity of DC35. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide)

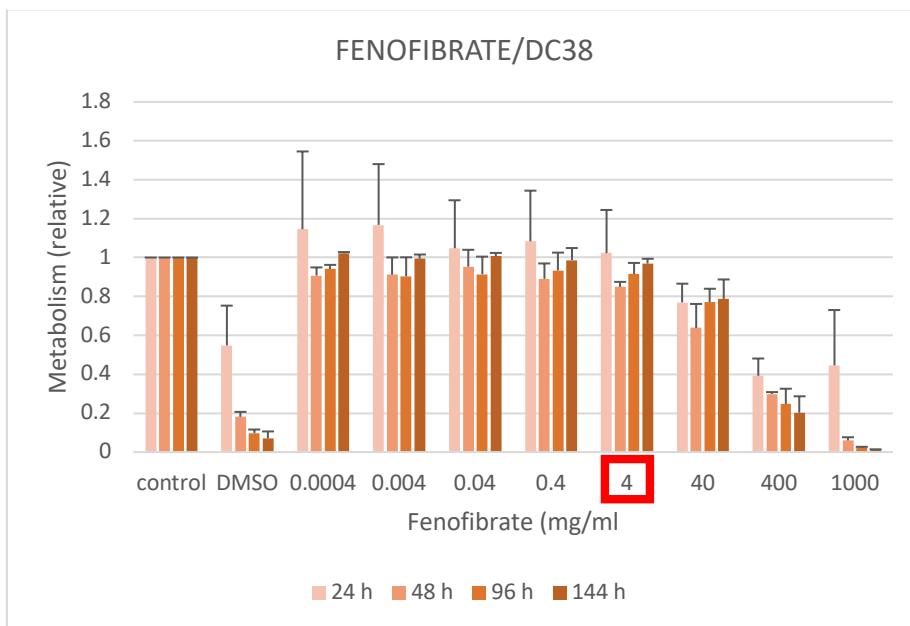


Figure 14: Effect of fenofibrate on the metabolic activity of DC38. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide)

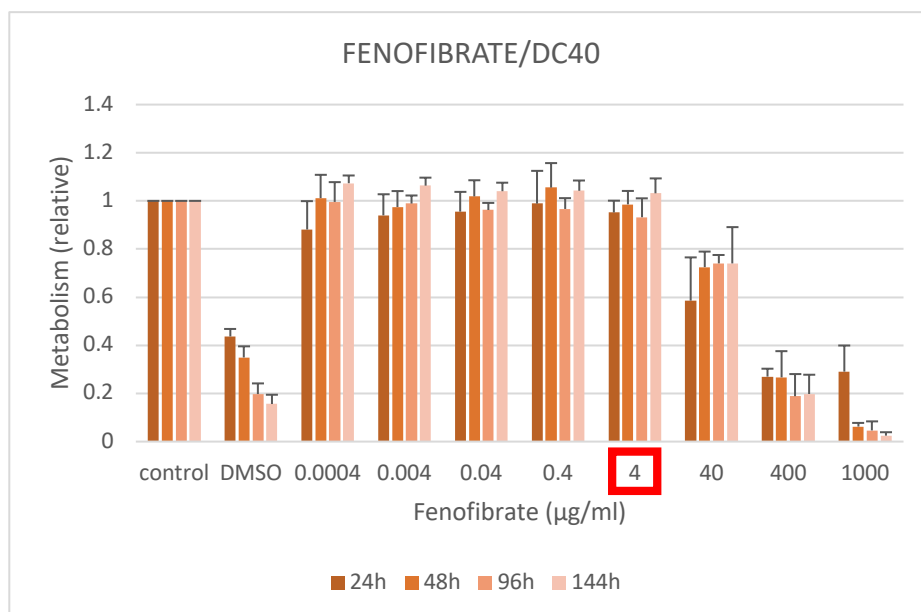


Figure 15: Effect of fenofibrate on the metabolic activity of DC40. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide)

In SC38 (Figure 17), SC35 (Figure 16) and SC40 (Figure 18), 90%, 92% and 97% of the cells were still metabolically active after 48 h at a concentration of 4 µg/ml. The activity decreased rapidly with the next-higher concentration (40 µg/ml) to about 43% in SC35 after 144 h, 71% in SC38 and 42% in SC40 after 144 h.

After 144 h, in the two highest concentrations of 400 µg/ml and 1000 µg/ml, the cell metabolism of SC35 was reduced to 29% and 17%.

When looking at the other two stem cell lines under the same conditions, SC38 decreased their metabolic activity to 42% and 25% after 144 h and SC40 to 34% and 23% (Figure 17 and Figure 18).

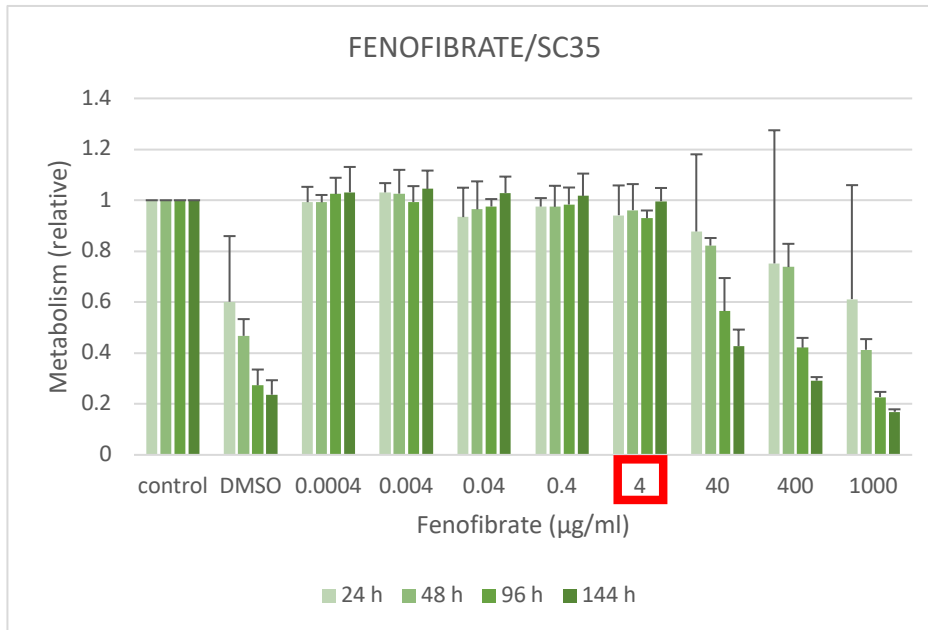


Figure 16: Effect of fenofibrate on the metabolic activity of SC35. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours), DMSO (dimethylsulfoxide)

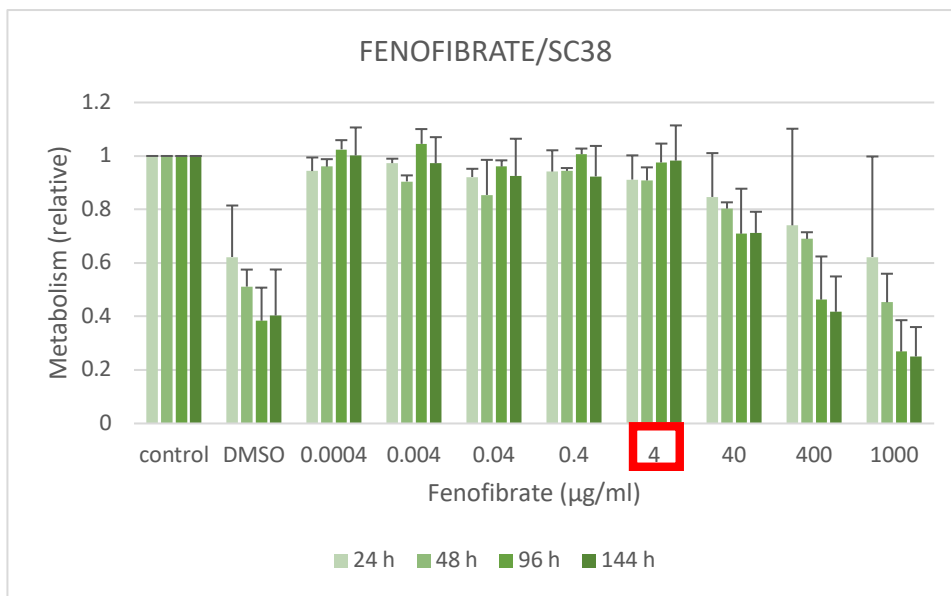


Figure 17: Effect of fenofibrate on the metabolic activity of SC38. Cells were treated with ascending concentrations of ribavirin and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours), DMSO (dimethylsulfoxide)

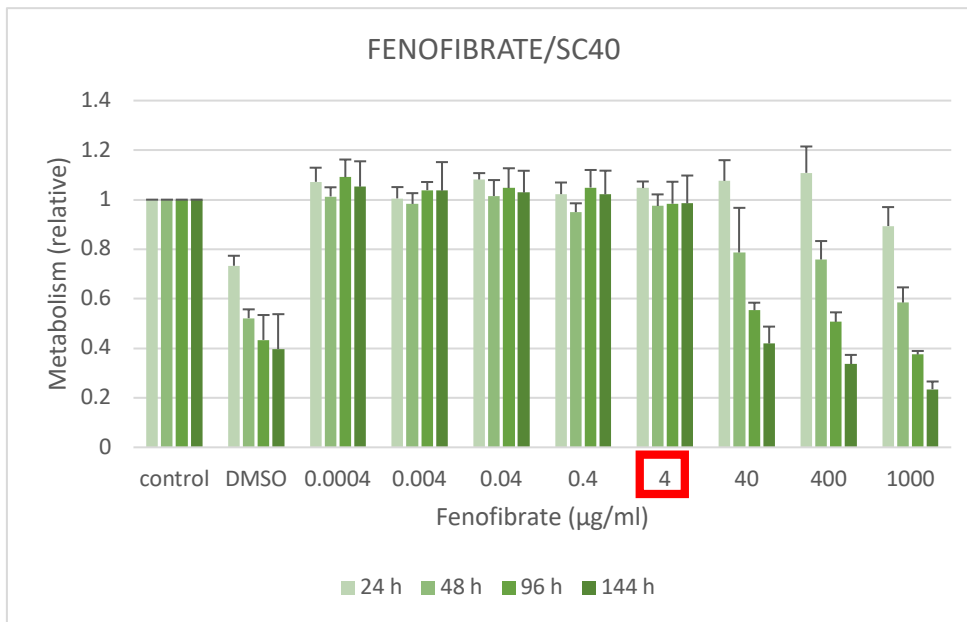


Figure 18: Effect of fenofibrate on the metabolic activity of SC40. Cells were treated with ascending concentrations of fenofibrate and metabolism was measured after 24 h, 48 h, 96 h and 144 h; the clinically relevant concentration is highlighted. SC (stem cells), h (hours), DMSO (dimethylsulfoxide)

To summarize the results of the MTT assay, we first saw that, generally speaking, glioblastoma stem cells are more resistant to treatment than differentiated glioblastoma cells.

Furthermore, one can say that all cells behaved and responded differently to the indicated periods of exposure and concentrations used, but most of them adhered to the rule that during the four measurements, the cells seemed to "recover" after 144 h. First, their metabolism decreased but it later rose again. This was most notably seen in DCs and in lower concentrations.

However, in the desired concentration, which was the clinically relevant concentration and the periods of exposure that we used, no pronounced effect of drug exposure could be observed in three independent experiments.

3.2 Effects of combination treatment with ribavirin, dapsone and fenofibrate on the metabolic activity of SCs and DCs

After the single-drug treatments, further MTT analyses were performed on the combination of all three drugs. First, all three medications were again used alone, than in double and at last in triple combination. This time, only the clinically relevant concentration of each drug was applied in the different groups.

In the previous MTT assays, G35 and G38 were more sensitive to the treatments than G40. For the current "combination MTT", G35 cells were used as both SC35 and DC35 phenotypes. After cell seeding, the assay was performed at 24 h and 144 h. The solvents, DMSO and H₂O, were added to two control groups, given that ribavirin was dissolved in H₂O and dapsone and fenofibrate in DMSO. Both control groups were treated with a DMSO concentration of 4 µg/ml, since this corresponded to the maximum required for the dissolution of fenofibrate. In both SC35 and DC35, no measurable effect was seen with the specific concentrations at the two indicated time points. In DC35, the reduction of metabolic activity varied between 4% and 11% in the seven treated groups after 24 h and seemed to increase again at the 144 h time point (Figure 19).

In SC35, the decrease was even less with the only slight change happening after 144 h with the triple combination (9%) (Figure 20). However, when weighing the results in both cell populations against the standard deviation, there was no noticeable effect left.

So we can conclude with reasonable that in the experimental system used here, ribavirin, dapsone and fenofibrate have no effect on human glioblastoma cell metabolism in the clinically relevant concentrations after 24 h and 144 h of exposure, either as single agents or in combination.

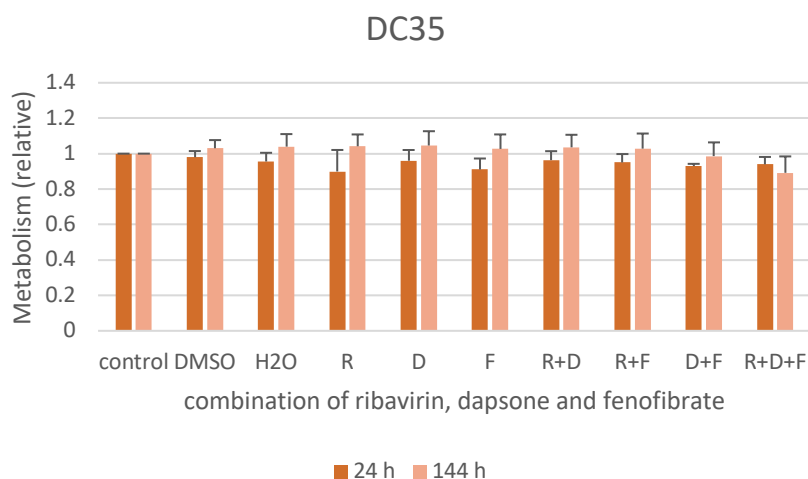


Figure 19: Effect of the combination of ribavirin, dapsone and fenofibrate on the metabolic activity of DC35. Cells were treated with the clinically relevant concentrations of 2 µg/ml ribavirin, 1 µg/ml dapsone and 4 µg/ml fenofibrate in single, dual and triple combination and were analyzed 24 h and 144 h after drug exposure. DC (differentiated cells), h (hours), R (ribavirin), D (dapsone), F (fenofibrate), DMSO (dimethylsulfoxide)

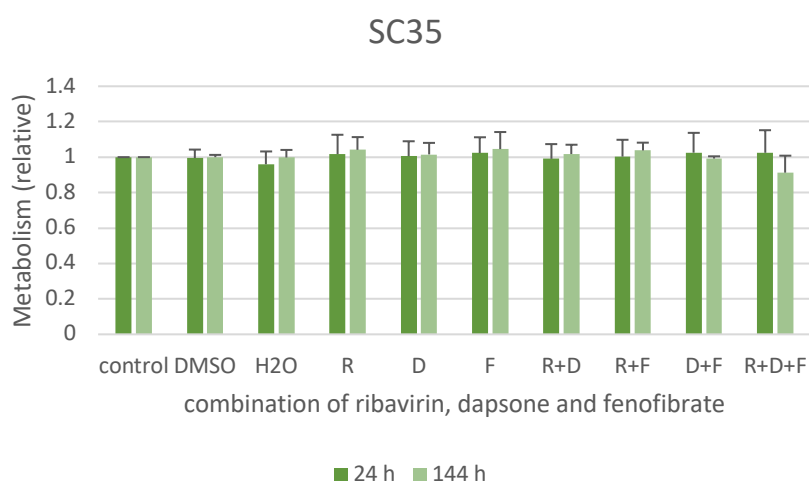


Figure 20: Effect of the combination of ribavirin, dapsone and fenofibrate on the metabolic activity of SC35. Cells were treated with the clinically relevant concentrations of 2 µg/ml ribavirin, 1 µg/ml dapsone and 4 µg/ml fenofibrate in single, dual and triple combination and were analyzed 24 h and 144 h after drug exposure. SC (stem cells), h (hours), R (ribavirin), D (dapsone), F (fenofibrate), DMSO (dimethylsulfoxide)

3.3 Effects of DMSO on the metabolic activity of SCs and DCs

Because DMSO shows cytotoxic effects in higher concentrations (Figure 1 to 18), it was important to ensure that a possible effect of the drugs was not indeed due to the cytotoxicity of the solvent itself. Therefore, separate MTT assays were performed using different concentrations of DMSO.

These MTT assays confirmed that DMSO did not exert damaging effects on G35, neither in suspension nor in adherent form, in concentrations that corresponded to the respective clinically relevant drug concentration or one concentration rank higher (see Materials and Methods). Only in a three digit [$\mu\text{g/ml}$] concentration, a biological effect was seen on DC35 which reduced their metabolism to 72% and 50% at concentrations of 200 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$, respectively.

The findings are presented in Figure 21.

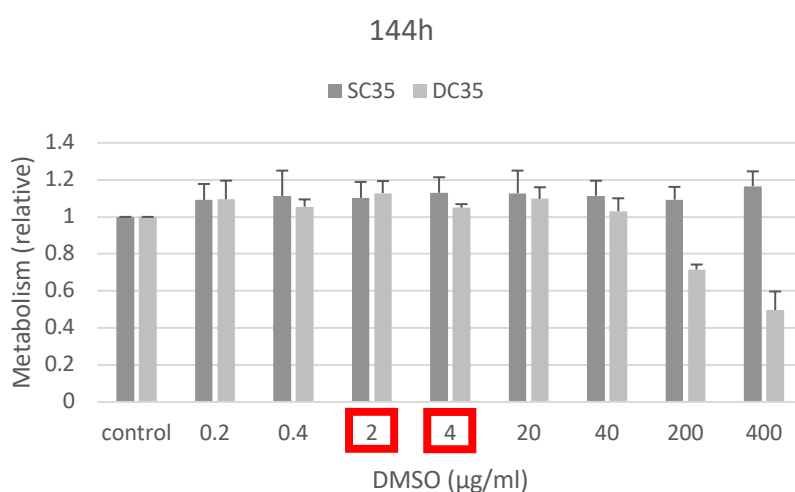


Figure 21: concentration-dependent effects of DMSO on the metabolic activity of SC35 and DC35. Cells were treated with increasing concentrations of DMSO and were analyzed 144 h after drug exposure; the clinically relevant plasma concentration of ribavirin (2 $\mu\text{g/ml}$) and fenofibrate (4 $\mu\text{g/ml}$) are highlighted by red frames.

SC (stem cells), DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide)

3.4 Effects of the triple drug combination alone and with augmentation by temozolomide on the cell counts of SCs and DCs

The MTT assay results described so far indicated that all three drugs had no real impact on the metabolism of human glioblastoma SCs and DCs.

These results prompted the question whether a combination of ribavirin, dapsone and fenofibrate might influence cellular proliferation as determined by the cell counts of SC35 and DC35.

Langhans *et al.* have shown that primary cell populations used in our laboratory are sensitive to TMZ, which induced apoptosis and decreased cell counts [78].

In this work, as presented in Figure 22, TMZ was used as a single treatment as well as in combination with the other three drugs, in order to have an established positive control for which the certainty to effect proliferation was already proved by our working group. As shown by the previous MTT assay, results already described in this work, DMSO had no effect on the cells in the relevant concentration which was thus used as the control here as well as in all experiments reported hereafter.

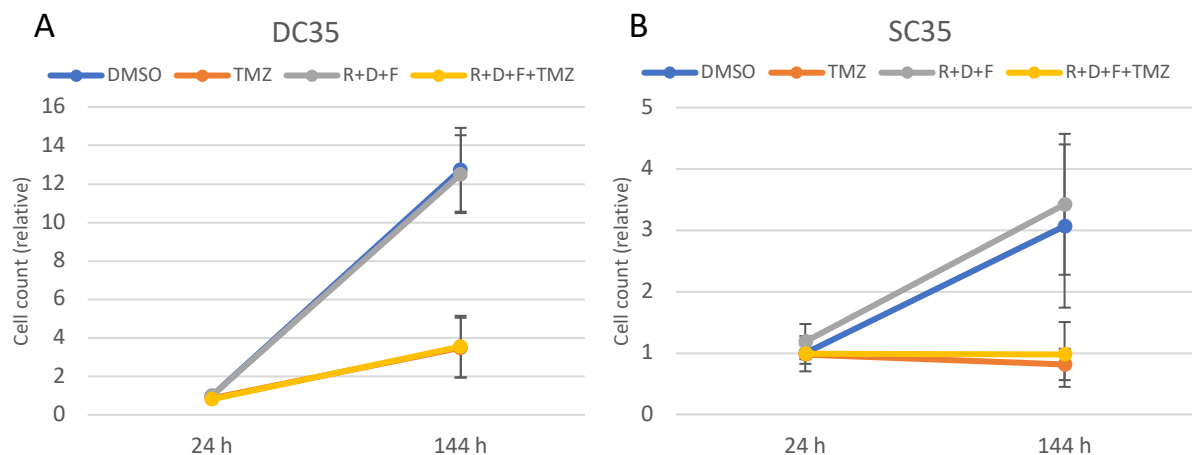


Figure 22: Effects of ribavirin, dapsone, fenofibrate and temozolomide on the cell count of DC35 and SC35.
A) DC35 and B) SC35 were treated with concentrations of 2 $\mu\text{g/ml}$ ribavirin, 1 $\mu\text{g/ml}$ dapsone, 4 $\mu\text{g/ml}$ fenofibrate and 19.4 $\mu\text{g/ml}$ of temozolomide in single, triple or quadruple combination and were analyzed 24 h and 144 h after drug exposure. Data were normalized relative to the DMSO-treated control. SC (stem cells), DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide), R (ribavirin), D (dapsone), F (fenofibrate), TMZ (temozolomide)

Schneider *et al.* have analyzed the differences between stem cells and their corresponding differentiated cells and demonstrated the growth behavior of the different phenotypes [130]. Their results match with the data reported here which generally showed a higher proliferation rate in DC35 than in SC35.

After 144 h, the cell count in DC35 was four times higher than in SC35 while effects of the triple combination of ribavirin, dapsone and fenofibrate did not significantly differ from the control group in both SC35 and DC35 (Figure 22).

With single TMZ treatment, cell numbers of SC35 did not change after 144 h compared to the 24 h time point (Figure 22B) and behaved similarly upon exposure to the quadruple combination which included TMZ and the other three drugs.

The same effects were observed in DC35 where the cell counts in the two treatment groups are even more alike. This statement applies to single treatment with TMZ compared to four-fold treatment and to triple treatment compared to control. The two respective groups are so similar that both graphs overlapped without a noticeable difference (Figure 22A).

In conclusion, the only effect detectable in this experiment can be ascribed to temozolomide. Cell counts were not influenced by the combination of ribavirin, dapsone and fenofibrate compared to control.

3.5 Effects of the triple drug combination alone and with augmentation by temozolomide on apoptosis induction in SCs and DCs

From past data, it is known that chemotherapy with temozolomide activates apoptosis in primary tumor cells, here in G40 SCs and DCs [78].

To further investigate whether the triple drug combination could also induce apoptosis, flow cytometric analysis was performed. Temozolomide was added as a single drug and in four-fold combination as in the previous CASY cell count experiment (section 3.4).

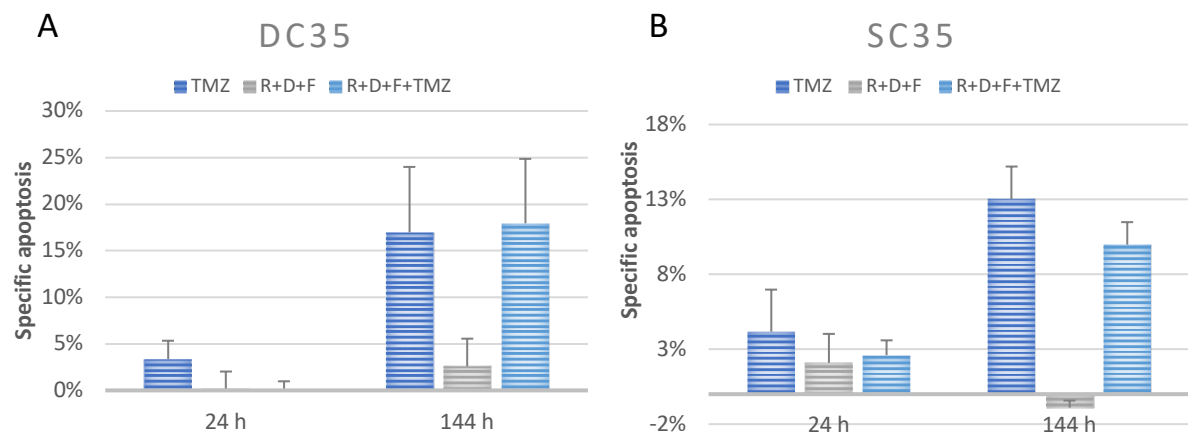


Figure 23: Effects of ribavirin, dapson, fenofibrate and temozolomide on specific apoptosis of DC35 and SC35. A) DC35 and B) SC35 were treated with a concentration of 2 $\mu\text{g/ml}$ ribavirin, 1 $\mu\text{g/ml}$ dapson, 4 $\mu\text{g/ml}$ fenofibrate and 19.4 $\mu\text{g/ml}$ of temozolomide in single, triple or quadruple combination and were analyzed 24 h and 144 h after drug exposure. Data were normalized relative to the spontaneous apoptosis rate of DMSO-treated control cells.

SC (stem cells), DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide), R (ribavirin), D (dapson), F (fenofibrate), TMZ (temozolomide)

The results from the CASY experiment were corroborated in this experiment. We only observed an effect with single TMZ and the quadruple combination treatment. In both, SCs and DCs the specific apoptosis rate after 24 h ranged from 0% to 4% in all three groups and increased up to ~18% in DC35 which was similar in the single TMZ and the four-fold combination after 144 h (Figure 23A).

In SC35, the specific apoptosis rate rose up to 13% with TMZ alone and increased to 10% with ribavirin, dapson and fenofibrate combined with TMZ at the second time point (Figure 23B).

Here again, the induction of apoptosis was only due to TMZ. None of the other three drugs, ribavirin, dapson and fenofibrate, influenced apoptosis rate of the examined human GB stem cells or differentiated cells.

Because ribavirin, dapson and fenofibrate had no effect on induction of apoptosis in SCs and DCs, no decrease of the cell count in any of the examined cell lines occurred. On the other hand, we confirmed that TMZ induced apoptosis, thus the cell counts decreased correspondingly to the positive control.

3.6 Effects of a combination of ribavirin, dapson and fenofibrate on migration and invasion of SCs and DCs

As mentioned in the introduction, past work has indicated that some of the three drugs have a reductive effect on the migrating potential of glioblastoma tumor cells [64].

To further understand how the triple drug combination influences the migration of patient-derived tumor cells, a Transwell assay was performed using fibronectin for coating. as the membranes of the Transwell inserts are perforated with 8- μ m pores, the cells from the upper compartment can migrate through the pores towards the lower side of the membrane and may then be stained and counted.

Fibronectin is a component of the extracellular matrix and its function in the assay is to close the micropores in the membrane so that prior to the migration process, the tumor cells first have to pass through fibronectin, therefore the invasive potential of the cells can be also examined.

A gradient, as specified in the Material and Methods section 2.2.7, between the upper and the lower compartment is necessary to create a stimulus for the cells to migrate. Besides, gravity also plays an artificial role, causing cells to settle onto the upper side of the membrane. Nevertheless, cells have to invade the fibronectin-coated membranes and migrate through the pores *via* chemotaxis to the bottom side and only those cells are counted. This excludes cells which do not fully complete this complex, active process.

3.6.1 Precedent drug exposure of SC35 and DC35 cells

In this experimental setting, four different methods were examined. The performance of the assay and the exact composition of the gradients is described in the Material and Methods section 2.2.7.

In the two groups with no gradient, the cells behaved and migrated very differently in all three experiments, resulting in a rather wide range of standard deviation.

In DC35, using a 0% to 0% FCS gradient, the triple drug combination seems to inhibit the migration potential by about 22%. In contrast, with the same gradient resulting from 10% FCS in the upper compartment and 10% FCS in the lower compartment, the treated cells migrated one third more than their respective control cells. In the next group, with a gradient from 0% to 10% FCS, the drugs elicited a

small increase (~10%) of migration. When looking at the last gradient, the ribavirin, dapson and fenofibrate-treated cells were inhibited by approximately 37% in their migration potential compared to control cells (Figure 24A).

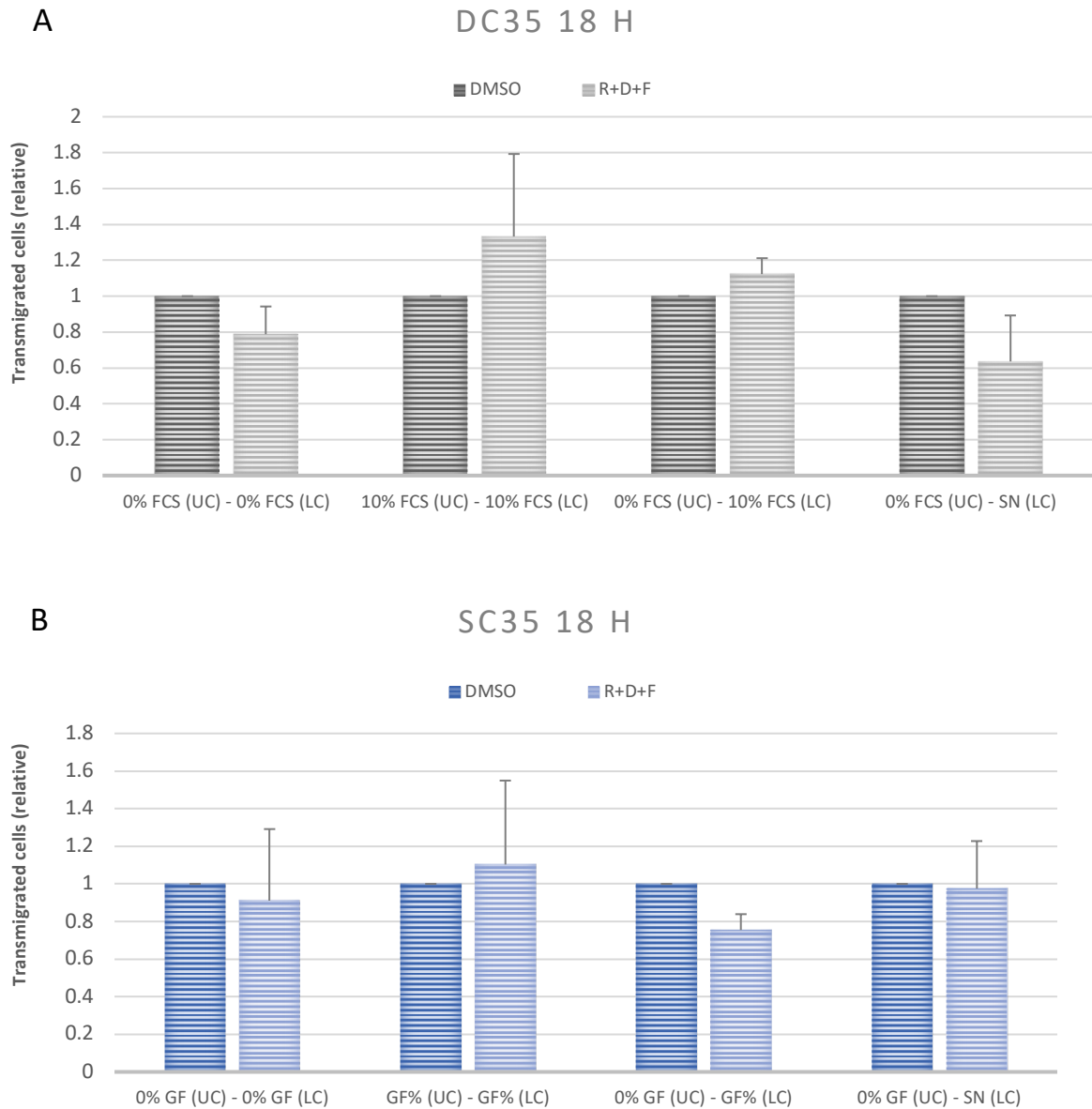


Figure 24: Effects of ribavirin, dapson and fenofibrate on the migration of DC35 and SC35 human GB cells. **A)** DC35 and **B)** SC35 were incubated with either DMSO (control) or ribavirin (R), dapson (D) and fenofibrate (F) 1 h prior to seeding. Cells were seeded in a density of 1×10^4 /insert and were analyzed after 18 h. Four different gradients were created. **A)** 0% refer to medium without FCS, 10% refer to the usual FCS concentration in the medium also used in cell culture conditions. **B)** 0% refers to medium without GF, GF% refers to the usual GF concentration in the medium also used in cell culture conditions Data were normalized to the DMSO-treated control
SC (stem cells), DC (differentiated cells), GB (glioblastoma), h (hours), DMSO (dimethylsulfoxide), R (ribavirin), D (dapson), F (fenofibrate), FCS (fetal calf serum), GF (growth factor), SN (supernatant), UC (upper compartment), LC (lower compartment)

SC35 cells did not show any response to treatment in the first two experimental settings with no gradient between the two compartments and the gradient with supernatant in the lower compartment. The only effect occurred in the third setting with growth factor between 0% (upper compartment) and the usual amount of growth factors, used in cell culture conditions, in the lower compartment. Under these conditions, a 25% reduction of migration was observed compared to control (Figure 24B).

Taken together, under certain conditions, the invasive and migratory capacities of SC35 and DC35 human GB cells can be reduced by treatment with a drug combination composed of ribavirin, dapson and fenofibrate in clinically relevant concentrations.

3.6.2 Continuous drug exposure of DC35, DC38 and DC40 cells

To further investigate whether only one treatment condition shows the same effect in various cell populations, the Transwell assay was repeated. This time the anti-migratory effect was examined only in DCs and the method was modified. All inserts were coated with fibronectin prior to the start of each experiment. Cells were allowed to invade and migrate for 24 h instead of 18 h and the same concentration gradient was applied to the different cells. In this assay, DCs were treated with continuous drug exposure in the upper and lower compartment of the Transwell insert.

Using this method and time point, no anti-migratory effect would be observed in all three differentiated cell types (Figure 25). Thus, the combination of all three drugs had no effect on the migration of the cells even when added to the medium in both compartments prior to starting the assay.

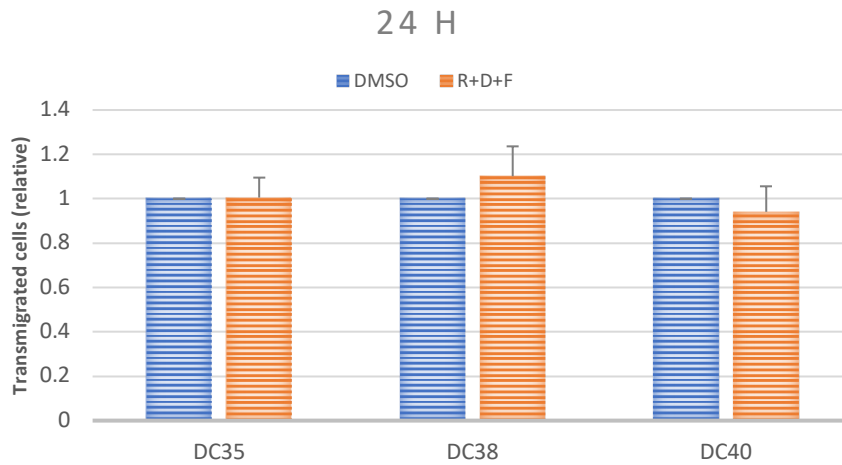


Figure 25: The effects of ribavirin, dapsone and fenofibrate on the migration of DC35, DC38 and DC40 human GB cells. Cells were seeded in a density of 1×10^4 /insert and were analyzed after 24 h. Ribavirin (R), dapsone (D) and fenofibrate (F) were added to the culture medium of the upper and the lower compartment, prior to seeding the cells. A gradient of 1.5% FCS in the upper compartment versus 10% FCS in the lower compartment was created. Data were normalized to the DMSO-treated control cells. DC (differentiated cells), h (hours), DMSO (dimethylsulfoxide), R (ribavirin), D (dapsone), F (fenofibrate), FCS (fetal calf serum)

3.7 G-CSF as a migratory stimulus in untreated SCs and DCs

To investigate whether G-CSF could be a chemoattractant for glioblastoma cells and may lead to a higher migration rate in the experimental setting used in this thesis, Transwell assays were performed using untreated SC35 and untreated DC35 with G-CSF added in a concentration of 60 pg/ml as the only migration incentive to the lower compartment. The control had no supply in any compartment, neither G-CSF nor any other growth factors like FCS, B27, EGF or FGF.

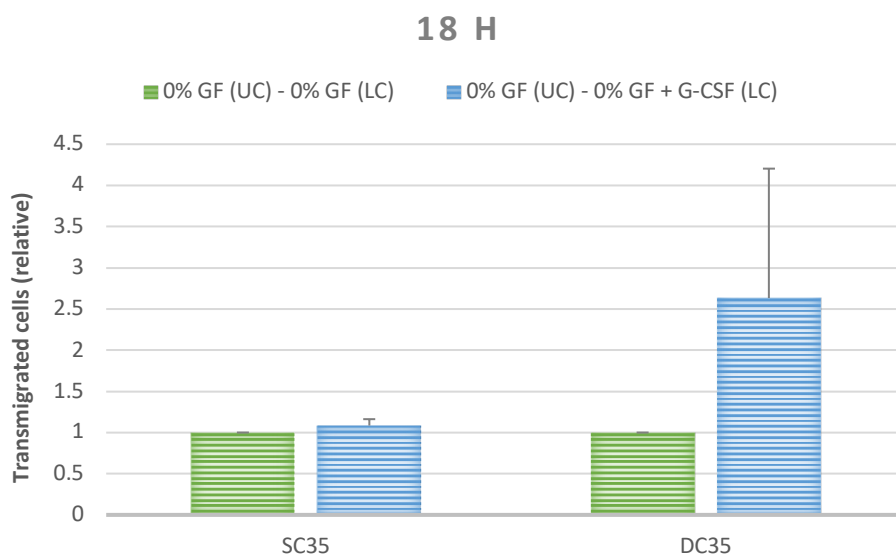


Figure 26: The effect of G-CSF on the migration of SC35 and DC35 human GB cells. Cells were seeded in a density of 1×10^4 /insert and were analyzed after 18 h. G-CSF was added to the medium in the lower compartment which contained no other growth factors. 0% refers to 0% GF in the medium. Data were normalized to the control cells without G-CSF. SC (stem cells), DC (differentiated cells), h (hours), G-CSF (granulocyte colony-stimulating factor), GF (growth factor), UC (upper compartment), LC (lower compartment)

Figure 26 displays a great difference between the behavior of SC35 and DC35 cells. While SC35 remained unaffected by the addition of G-CSF compared to control cells, the migration of DC35 was 2.6-fold enhanced by G-CSF.

It has to be kept in mind that SC35 are rather insensitive to deprivation of growth factors while DC35 tend to show a more pronounced biological response in terms of inhibition of migration. Microscopic images of the whole membrane demonstrate that difference quite clearly. Figure 27 and Figure 28: the SCs, unaffected by treatment, infiltrated the whole membrane whereas DCs migrated only at the margin of the membrane.

When comparing the mean absolute cell numbers among control groups, migration of SC35 was about 6 times enhanced over DC35. Expressed in numbers, out of 10,000 seeded SC35, a mean of 4,711 (47%) cells migrated through the membrane compared to only 787 (18%) DC35 with the same initial value of seeded cells.

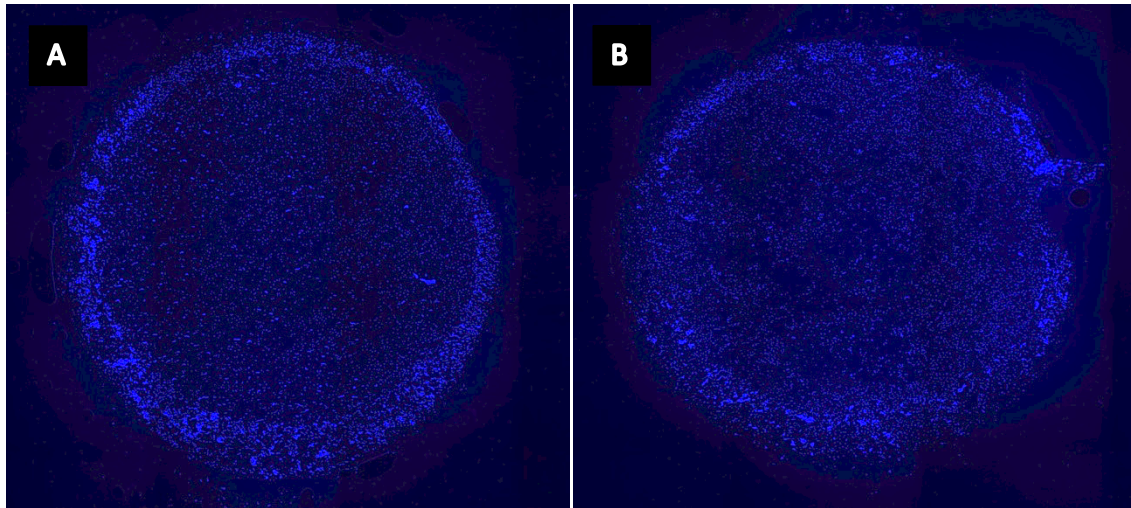


Figure 27: Gradient without (A) and with G-CSF (B): **SC35** after migration through the membrane. Cells were stained with DAPI and counted under the microscope.
SC (stem cells), G-CSF (granulocyte colony-stimulating factor), DAPI (4',6-diamidin-2-phenylindol)

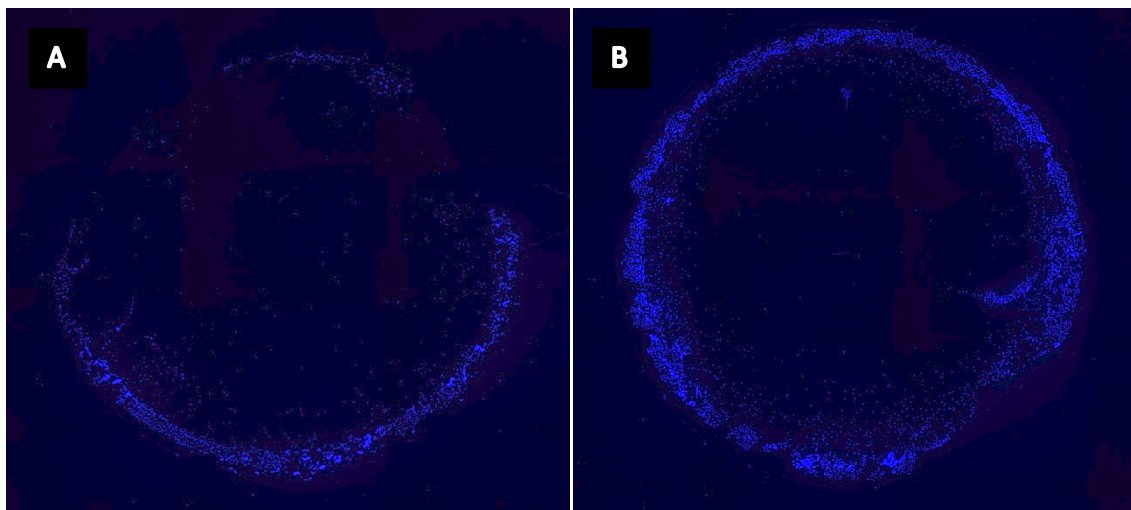


Figure 28: Gradient without (A) and with G-CSF (B): **DC35** after migration through the membrane. Cells were stained with DAPI and counted under the microscope
DC (differentiated cells), G-CSF (granulocyte colony-stimulating factor), DAPI (4',6-diamidin-2-phenylindol)

3.8 G-CSF expression of treated and untreated DCs

Using a Human Magnetic Luminex Assay, the G-CSF secretion capacity of all three differentiated cell lines, DC35, DC38 and DC40, was examined at three different time points (24 h, 48 h, 72 h), the performance of the assay is specified in the Material and Methods section 2.2.8. This time only differentiated cell lines were used because we assumed that this cell type represents the majority of G-CSF-secreting cells in a primary tumor mass. The cells were either treated with a combination of ribavirin, dapsone and fenofibrate or DMSO as the control.

This experiment was performed to corroborate that glioblastoma cells from patient-derived tumor material established in our laboratory secrete G-CSF. Furthermore, we wanted to examine our working hypothesis that all three drugs may inhibit or reduce the capacity of the cells to secrete G-CSF.

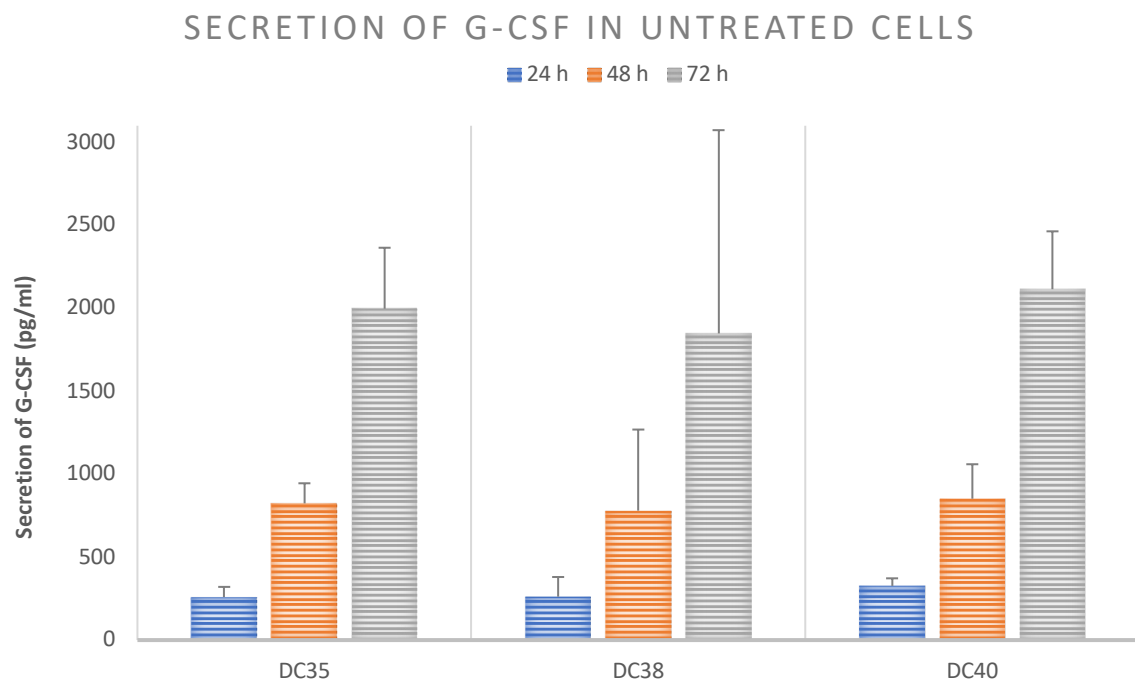


Figure 29: Levels of G-CSF secreted by untreated DC35, DC38 and DC40 human GB cells over time. Cells were seeded in a density of 1×10^4 cells/cm², the supernatant of the cells was collected 24 h, 48 h and 72 h after seeding.
DC (differentiated cells), GB (glioblastoma), h (hours), G-CSF (granulocyte colony-stimulating factor)

Figure 29 presents the levels of G-CSF secreted by untreated differentiated human GB cells at the three indicated time points. All three cell lines behaved similarly regarding the amount of the secreted G-CSF level.

The cells were seeded in a density of 1×10^4 cells/cm², after 24 h, all three differentiated cell lines secreted about 300 pg/ml G-CSF. At the second time point, the value was three times higher and ranged between 780 pg/ml and 850 pg/ml. At the last time point, G-CSF levels were 2.4-times higher than at the second time point, reaching 2000 pg/ml.

When comparing treated and non-treated cell lines DC35, DC38 and DC40 showed no significant reduction of G-CSF secretion at any time point examined in this work (Figure 30).

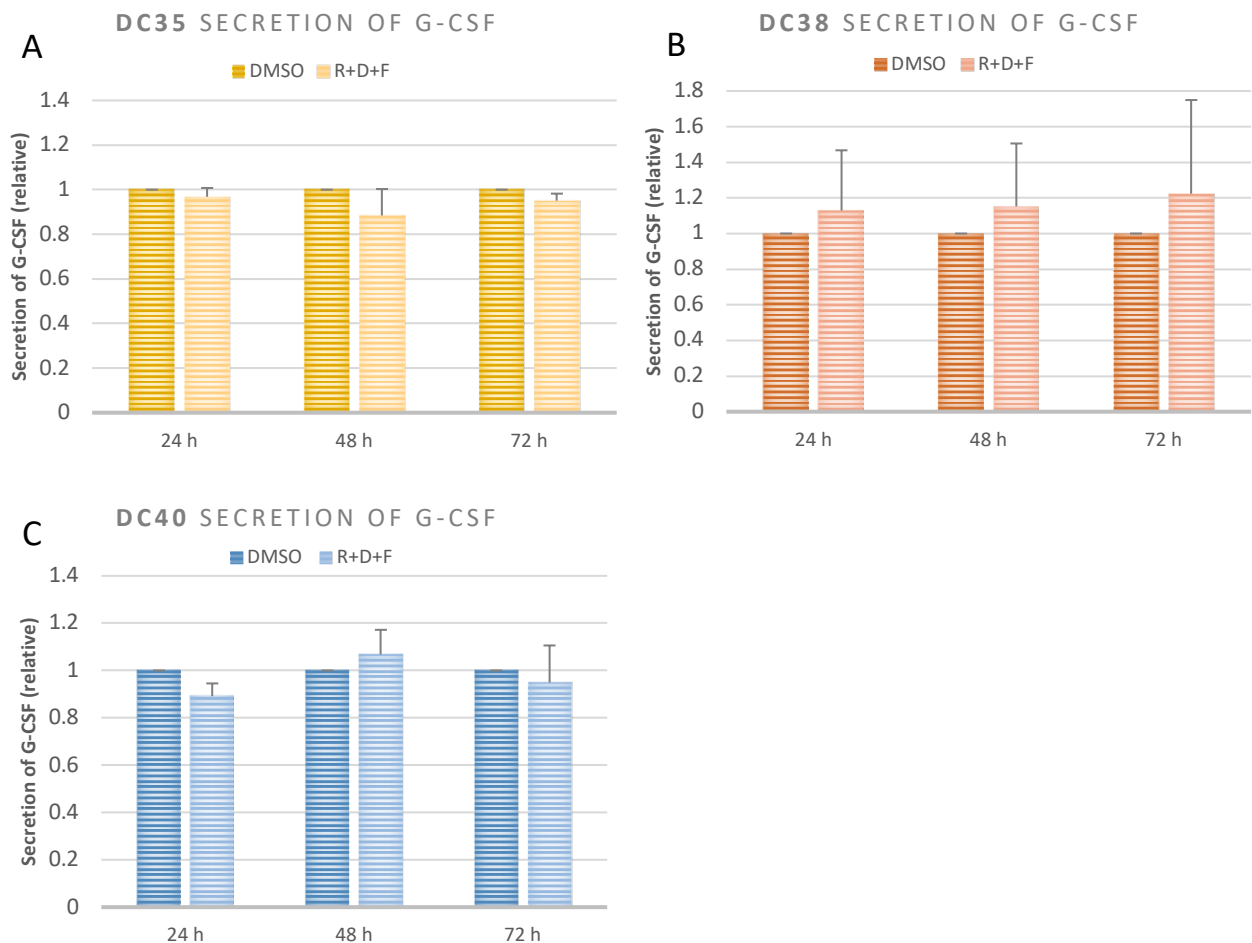


Figure 30: Effects of ribavirin, dapsone and fenofibrate on the secretion of G-CSF in differentiated human GB cells. Cells were seeded in a density of 1×10^4 cells/cm², after 24 h, 48 h and 72 h the secretion of G-CSF was measured in A) DC35, B) DC38, C) DC40. Data were normalized to the DMSO-treated control cells. DC (differentiated cells), GB (glioblastoma), h (hours), DMSO (dimethylsulfoxide), R (ribavirin), D (dapsone), F (fenofibrate), G-CSF (granulocyte colony-stimulating factor)

The highest G-CSF reduction (12%) due to drug treatment occurred in DC35 after 48 h (Figure 28A). The drug combination decreased G-CSF secreted by DC40 by about 11% after 24 h (Figure 28B).

DC38 showed a tendency towards increased G-CSF secretion upon treatment, though in DC38 we also observed the highest standard deviation within all three experimental series reflected in Figure 27.

In summary, all of the differentiated cell lines produced high amounts of G-CSF but contrary to the working hypothesis, ribavirin, dapsone and fenofibrate had no effect on G-CSF secretion of adherent cells at the examined time points.

4 Discussion

4.1 Development of the working hypothesis

The focus of this body of research was an investigation of three older drugs, ribavirin, dapsone and fenofibrate, which have shown significant anti-tumor effects as reported in the scientific literature. All of them are approved for their respective indications but none of them have ever been approved for repurposing in anti-cancer therapy.

Because no data was available on how the patient-derived tumor cultures would respond to the drugs, the first experiment used the MTT assay to measure the metabolic activity of the treated cells. During this approach, a range of different concentrations and the single use of all three drugs were examined.

Since the beginning of this work, focus was on *in vitro* experiments which at the same time should use clinically relevant conditions as much as possible. Therefore the research focused specifically on one concentration, namely the concentration determined to be clinically achievable in humans. When a drug is orally administered and metabolized, its concentration in plasma and CSF can be measured. The maximum concentration which can be achieved in plasma or CSF under normal conditions is available in the literature and was used for all individual experiments [45, 46, 52, 55, 137]. The idea of combining all three drugs stems from the fact that none of them, via single use, could achieve high enough concentrations in plasma or CSF to achieve anti-tumor effects but possibly in combination.

The grounding framework to this study was a review from Kast *et al.* (2017) which summarized the potential role of ribavirin, dapsone and fenofibrate in an off-label anti-glioblastoma treatment [64].

4.2 *In vitro* results and extrapolation to the clinic

In vitro investigations form the major part of modern research. Moreover, *in vitro* work marks the first step before further expansion to animal testing and application in humans. To obtain meaningful results, experimental conditions in cell culture are chosen that are reasonably close to the ones in living species, although many differences between the systems remain.

Incubators for the cell cultures normally apply a constant temperature of 37°C, which corresponds to the human body temperature, and the air is supplemented with 5% of carbon dioxide (CO₂) to maintain a physiological pH in the cell culture medium. In order to provide an environment that serves energy for the cells to survive and to complete the cell cycle, the cells are cultivated in aforementioned special media which are supplemented with glucose, lipids and amino acids. The different media for adherent and suspension cells are then further supplemented with selected growth factors or the fetal calf serum (FCS) which contains an array of placental hormones [91]. However, the representation of the *in vitro* situation by *in vitro* cultivation of tumor cells is principally limited. When looking at electrolyte and glucose levels in humans and comparing them to the levels in commonly used non-supplemented media like DMEM or RPMI, the values differ greatly. Glucose for example is four-times higher in DMEM than it physiologically occurs in humans [93]. Hormonal regulation, e.g. through changes in body temperature or circadian rhythm, happens several times during day and night and cannot be easily replicated in a cell culture model [91]. Yet, likely among the most profound differences between *in vivo* and *in vitro* conditions is the human immune system and immune response that is not modeled *in vitro*.

Another aspect of the limited *in vivo* representation of *in vitro* data lies in the fact that, in cell culture, no limit exists regarding drug concentrations which may be increased far beyond any clinical relevance. This especially applies to patients with brain tumors where the BBB typically further limits drug delivery and target tissue concentration. When taking a closer look at already published work, notable biological effects, for example in tumor cells, are often reported for high drug concentrations that are not attainable in humans.

Still, a central aim of gaining information from laboratory work is to obtain a better understanding of biological effects in tumor cells, possibly enabling meaningful research in *in vitro* systems and finally in humans. If *in vitro* treatment applies unrealistically high drug concentrations from the clinical point of view, the large biological effects observed may not be translationally applicable.

To avoid this effect and to obtain clinically relevant results only drug concentrations achievable in humans were used in this work.

4.2.1 The controversy of using FCS in cell culture

The addition of FCS to cell culture media is both widely practiced, and highly controversial. FCS contains a complex mixture of hormones, vitamins, growth factors, transport proteins, lipids and enzymes which are essential factors for cell metabolism and cell growth. There is reasonable ethical critique towards the exploitation of FCS; approximately 500,000 liters per year are sold which means that over a million of bovine fetuses are used for FCS collection, mostly under suffering conditions. The serum is taken from fetuses of pregnant cows sent to slaughter and is either collected directly by puncture of the heart or the jugular or umbilical vein [14]. But not only ethical concerns pertain to the use of fetal animal serum, there are also qualitative and quantitative issues. Firstly, the exact composition regarding the various factors is rather unknown and secondly, a batch-to-batch variability exists, depending on geographical conditions and the timepoints of harvest [158]. Thirdly, the usage of animal products like FCS can confer contaminations with *mycoplasma*, viruses, bacteria or fungi [14] which may affect *in vitro* results and cell culture *per se*.

With regard to all uncertainty associated with fetal sera, alternative solutions like cultivation of the cells in serum-free media or the use of serum substitutes should be considered [50].

4.2.2 Patient-derived primary tumor material

In the 1950s, the scientific cultivation of the first brain tumors began and prospered in the 1960s. In the same decade, the widely used cell line U87MG was formally established but researchers from the university of Uppsala realized that this cell line was of unknown origin and exhibited many differences to the original tumor [79].

Until today, considerable parts of the *in vitro* glioblastoma research rely on this cell line and other commercial cell lines like A172, T98G or U251. As these popular cell lines were initiated as adherent cells about 50 years ago, there is growing evidence that they may not be sufficiently representatives of primary CNS tumors [80, 83]. Besides, research has shown that primary tumor material which is cultivated in serum-free medium, supplemented with growth factors like EGF and FGF, remains closer to the gene expression profile and the biological behavior of patient-derived primary tumors [80].

Serum-cultured adherent cell lines showed genomic alterations which were not found in the corresponding primary tumors [79].

The G35, G38 and G40 cell lines which were used in this work were directly isolated from patient-derived tumor samples and cultured under serum-free conditions.

Suspension cells that are growing in neurospheres and are cultured in B27-, EGF- and FGF-containing medium, are stem cell-like and can be differentiated by letting them adhere on plastic while using a different medium that is supplemented with FCS instead of B27, EGF and FGF. When passaging adherent cells various times, DCs lose their specific characteristics and undergo artificial mutation as mentioned previously. For that reason, the differentiated cells in this work were only used from passages 2-10 and were thereafter renewed again from stem cells.

4.3 Overcoming the blood-brain barrier

The first evidence of the existence of the BBB was demonstrated by Paul Ehrlich in 1885, when he injected a dye into peripheral blood and later described that all organs except the CNS were stained [168]. In congruence with the findings made over 135 years ago we nowadays know that the BBB works as a filter system to prevent the entry of pathogens and toxins but allows nutrients or immune cells like lymphocytes to cross it. The BBB consists of four different layers which form a tight unit. The inner layer is formed by the endothelium which is in contact with the peripheral blood and regulates the intake of molecules. Subsequent to the inner layer we find the basement membrane which is surrounded by the endothelium on one side and pericytes on the other side. The basement membrane is composed from both of them. The endfeet of astrocytes form the last component; they show the ability to regulate the water and electrolyte balance and are essential for an intact barrier [131, 168]. There are different mechanisms for transportation of compounds into the CNS. Generally the BBB is permeable to gases and lipid molecules that can pass *via* diffusion, and electrolytes *via* specific ion channels. Greater proteins can cross in a receptor-mediated fashion [131]. There exist different strategies for drug delivery for example nanoparticles, active transporters in the BBB, viral vectors etc. but drug uptake through the BBB into the CNS is definitely a limiting factor [35].

Drugs that are able to penetrate into brain tissue show a high lipophilicity, other determining factors are molecular size and ionization [43, 159]. Ribavirin was assumed to show little potential to cross the BBB due to its highly hydrophilic character [57], the drug is water-soluble as confirmed by our work. In contrast, Colombo *et al.* demonstrated intraparenchymal uptake of ribavirin (after intravenous administration) which was even higher than concentrations obtained after intranasal application. The authors suggested a possible active transport mechanism underlying this observation [27].

Dapsone has a low molecular weight and is highly lipophilic, therefore it should penetrate the BBB well. Actually, it was shown that when administering the drug orally, therapeutically relevant concentrations for *Pneumocystis carinii* pneumonia prophylaxis can be achieved in CSF [45, 121].

Fenofibrate also shows a high lipophilicity which makes it difficult to achieve sufficient plasma levels when administering the drug orally [84]. At the same time, fenofibrate should be biochemically suited to overcome the BBB. Unfortunately, no data exist on how fenofibrate penetrates the BBB or concentrates in CSF. Despite the lack of exact pharmacokinetic information, all three mentioned drugs should be reasonable candidates for the treatment of CNS malignancies in view of their putative therapeutic availability in brain tissue.

4.4 Review of the findings

Essentially we wanted to investigate the impact of three repurposed drugs on different cellular measures i.e. apoptosis, cell count, metabolic activity and migration, which are all relevant parameters for the biology and treatment response of GB. As described in the results part, we did not observe effects on apoptosis, viability and proliferation in our primary glioblastoma tumor material when using clinically relevant concentrations of all three drugs in single or triple use. However, it appears that G-CSF has an effect on DCs and their invasive character and is highly expressed by this cell type.

4.5 Existing literature and applicability to our primary tumor material

4.5.1 Single treatments with ribavirin, dapsone and fenofibrate and their effects on cell metabolism

During the first experiments, which consisted of MTT assays, a wide range of drug concentrations was examined to gain an understanding of how the cells respond to the three drugs when using them separately. The purpose was to obtain a dose-response curve. Even if no measurable effects were observed with the desired concentrations, we attempted to investigate potential synergism when using all three drugs together.

In 2014, a first study explored the effect of the antiviral agent ribavirin on glioma cell lines. This work indicated a dose-dependent growth inhibition of glioma cell lines when treated with ribavirin at a concentration of 0.1 to 1000 μM [104].

Additionally, Ogino *et al.* determined the half-maximal inhibitory concentration (IC_{50}) of ribavirin after 72 h in seven different cell lines which had a range between 28 μM in the most sensitive cell line AM38 and 664 μM in the least responsive cell line U138 MG [104]. Three years later, two studies showed similar results that indicated induction of apoptosis and autophagy [54] and growth inhibition [162]. Isakovic *et al.* showed a dose-dependent reduction of viability of U251 cells with the first reductive effects apparent at a concentration of 100 μM , where the cells reduced their viability by about 25% [54].

Comparing the results above to the concentrations used in this work (2 $\mu\text{g/ml}$ corresponds to 8.2 μM) in the MTT assay one could see first biological effects at a concentration of 20 $\mu\text{g/ml}$ (82 μM) in all three cell populations, adherent and suspension cells, with the reduction of viability ranging between 25% and 50% (Figure 1-6). Apart from influencing cell viability, previously published work reported that ribavirin induces autophagy in a much higher concentration of 400 μM [54].

In 2016 Volpin *et al.* mainly used 30 μM *in vitro* in their research and showed reductive effects on cell viability in human malignant glioma cell lines, rat glioma cell lines and human GB stem-like cells. Furthermore, they described enhancement of the cytotoxic effects of TMZ and irradiation. To confirm the growth-inhibitory effects of ribavirin, they corroborated their results in both rats and mice. Rats were injected with rat gliosarcoma tumor cells and immunodeficient mice with human GB stem-like cells. The median OS of ribavirin-treated rats was 15 days compared to control

animals with 12 days. Median OS of immunodeficient mice which received ribavirin was 19 days and that by their respective controls 12 days. Rats were injected with 10 mg/kg ribavirin and mice with 100 mg/kg ribavirin [162]. Even though inhibitory effects were shown, all of the publications mentioned above used at least 3.7 times higher (30 μ M) concentrations than we did.

Very little information exist about the effect of dapsone on glioblastoma cells. While in theory some work suggests a potential rationale of dapsone [62, 66, 67, 160], there has been little experimental proof yet.

In 2017, Karpel-Massler *et al.* from the Department of Neurosurgery at Ulm University Hospital, published first results on how the glioblastoma cell line U87MG as well as adherent cells of G38 (which were also used in this work) respond to treatment with dapsone or chemically modified derivatives of dapsone (D2-D5) [61]. There, concentrations between 10 μ M and 100 μ M were used, compared to 1 μ g/ml (4 μ M) which was used in this work. This means that the lowest concentration used in that setting was 2.5 times higher than the one used in our experiments. Only slight effects could be achieved with dapsone treatment in the MTT assays performed in our research. At a concentration of 10 μ g/ml (40 μ M) there was a small reduction between 0% and 20% in all three cell populations, *i.e.* G35, G38 and G40 (Figure 7-12). The data from Karpel-Massler *et al.* did not reveal relevant effects of dapsone or its derivatives in the conducted MTT assays [61], which is consistent with our data. Additionally, dapsone seemed to inhibit IL-8 secretion when treating the cells with concentrations of 25 μ M, 50 μ M and 100 μ M [61]. In this work, the secretion of IL-8 was not examined so there is no available data for comparison. Probably the most significant result of the preceding work from Karpel-Massler *et al.* is the inhibitory effect of dapsone on cell motility [61].

In regards to fenofibrate, the *in vitro* effects of fenofibrate treatment were already examined [8, 37, 51, 172].

Fenofibrate showed potential anti-proliferative effects in a time- and dose-dependent manner. In a minimum concentration of 50 μ M, the viability of U87MG cells decreased by about 50% after 96 h [51]. In the work reported by Binello *et al.* in 2014, the cell viability of fenofibrate-treated cells decreased by about 50% to 60%, even at a concentration as low as 25 μ M, in the four cell lines U87MG, U343MG, U351MG and T98. U87MG cells decreased their metabolism by about 50% compared to control cells at a fenofibrate concentration of 50 μ M, which is consistent

with the work of Han *et al.* in 2015 [8]. DC35 and DC38 are the only cells which showed a slight viability reduction of 15% at a fenofibrate concentration of 4 µg/ml (11 µM) (Figure 13, Figure 14). At a concentration of 40 µg/ml (110 µM), the viability of all six cell populations decreased by 30% to 60% (Figure 13-18) which was a much smaller effect than that reported for other cell lines where the viability, at a concentration of 100 µM, practically diminished [8]. Interestingly, Han *et al.* described reductive effects of between 40% to 70% at a concentration of 100 µM in U87MG cells which is not consistent to the results of Binello *et al.* [8, 51]. Both authors used different methods to measure cell viability: Han *et al.* used the WST-8 assay and Binello *et al.* performed the MTT assay as we did in our work.

4.5.2 Combination treatments with ribavirin, dapsone and fenofibrate and effects on apoptosis and cell count

For all further experiments, only the clinically relevant concentrations were used as we intended to employ only the triple combination in all subsequent measurements, after MTT assays had been conducted with each single drug. The two experiments aimed at detecting apoptosis and cell count were performed in parallel to obtain exact comparisons between induction of apoptosis and decrease of cell counts. When comparing existing data from the literature with the results achieved in this project, one should be aware that the effects here were not associated with one single drug but with the combination of all three, whereas the literature so far only reports results for single uses of one drug. There is no exact analogy as this combination of drugs was never investigated before.

In the literature, two works suggest that fenofibrate induces apoptosis at a concentration of 50 µM [8, 171]. Ogino *et al.* in 2014 observed no apoptotic effect of ribavirin [104] while Volpin *et al.* in 2016 described apoptosis induction at a ribavirin concentration of 30 µM [162]. No data exist about the effects of dapsone on apoptosis in glioma cell lines. In the current work, no effects on apoptosis were seen in G35 cells with the combination of all three compounds at the indicated time points. The only pro-apoptotic effects were due to TMZ (Figure 23). With the triple drug combination we had no effect on apoptosis and also no effect on the cell count of G35 cells, neither with adherent monolayer nor in suspension. Here again, the only decrease was provoked by TMZ (Figure 22).

Taken together, these findings appear to be in stark contrast to the published literature. However, all three individual compounds were used in higher concentrations according to the literature than they were in this work.

4.5.3 Inhibition of migration by the combination treatment

In a scratch wound assay and an adhesion assay, ribavirin showed reductive effects on migration and adhesion in a concentration of 30 μ M [162].

In terms of dapsone, our results showed a dose-dependent reduction of migration in the Transwell assay that started at a concentration of 10 μ M and increased at 50 μ M and 100 μ M. U87MG and DC38 cells decreased their ability to migrate through the membranes in the Transwell assay about 20% and 35% , respectively when treated with the lowest concentration of 10 μ M. Interestingly, DC38 primary tumor cells were more sensitive than the commercially available U87MG cell line [61]. Two works examined the effects of fenofibrate on cell motility and migration. In a scratch wound assay, fenofibrate showed reductive effects on cell motility of T89G and LN-229 cells. Additionally, the migratory potential of the cell line LN-229 was effectively reduced in a Transwell assay. For both experiments, a concentration of 50 μ M was used [37]. Even at a lower concentration of 25 μ M, fenofibrate seemed to inhibit cell migration of glioma stem cells (GSC) in a Transwell assay and diminished the specific stem cell marker CD133.

In the Transwell assays performed in this work, the inserts were coated with fibronectin, a component of the extracellular matrix. This assay analyzes two components of motility, first, by the ability of the cells to invade fibronectin and secondly, cellular migration through the pores of the membrane.

In the first experiments one could see an anti-migratory effect under certain conditions in DC35 and SC35 cells when pre-treating them with a drug combination of ribavirin, dapsone and fenofibrate. Different concentration gradients were applied during migration of cells through the pores. With no gradient between the two compartments we could not determine any results, but if a gradient between no growth factors and a standard amount of growth factors existed, treated SC35 cells migrated 25% less than their corresponding controls (Figure 24B). By using different gradients, we aimed to investigate how the treatment with the three drugs may influence the cells in case of deprivation of growth factors, or when exposing them to their own supernatant or medium supplemented with a standard amount of growth

factors. If the concept of G(M)-CSF-producing glioblastoma cells was to be confirmed in the primary tumor material as it had been described in the literature [31, 98, 102, 156, 164], one would expect a secretion of G(M)-CSF into the medium that accordingly could be detected in the supernatant. As already mentioned in the introduction, all three drugs may reduce the secretion of G(M)-CSF. Therefore we had hypothesized to see an anti-migratory effect in the group where G(M)-CSF in the supernatant might have acted as a chemoattractant. Indeed, we observed a strong effect in DC35 cells with a reduction of 37% compared to the respective controls (Figure 24A). Obviously, stem cells were not affected in their invasion (Figure 24B). In a second approach, the method was modified and only differentiated cells were used. The cells were not treated with the drugs prior to the seeding, but ribavirin, dapsone and fenofibrate were added to the culture medium in the upper and lower compartment. This time, only one gradient existed with a standard amount of growth factors. Surprisingly, the drug combination under study had no effect on DC35, DC38 and DC40 cells in this specific setting (Figure 25).

In summary, ribavirin, dapsone and fenofibrate show potential anti-migratory effects in stem cells and differentiated cells under certain conditions.

Although the Transwell assay is a commonly used assay to measure the invasion and migration of cells, this method is nevertheless prone to errors during execution or the final counting of the stained cells.

4.5.4 High expression of G-CSF by differentiated cells

Almost 30 years ago, Nitta *et al.* showed an unstimulated high expression of G-CSF in U87MG and a lower expression in U138MG cell lines. The secretion was even higher when stimulating the cell lines with TNF- α and IL-1 β ; under these pre-treating conditions, other cell lines like U251 and A172 showed a similar expression profile as untreated U87MG cells. Regarding GM-CSF, a low level of the cytokine was detected in all six different cell lines, including U87MG, U138MG, U251 and A172. U87MG expressed both cytokines, but G-CSF was present 200 times higher than GM-CSF, 2000 pg/ml compared to 10 pg/ml [102]. The data of a Japanese working group confirmed these findings regarding the G-CSF secretion of the untreated U87MG cell line [70]. The work from Mueller *et al.* in 1999 studied the G-CSF expression in 22 recurrent human gliomas. The tumor cells were cultivated after surgically removed and corresponded to WHO grades II to IV. This groups showed,

that all of their glioma-derived samples expressed G-CSF, GM-CSF and/or their receptors. They then suggested a correlation between the co-expression of the cytokine, its receptor and the tumor stage. The expression of only G-CSF and the GM-CSF receptor occurred in some low-grade gliomas whereas the expression of both G-CSF and the G-CSF receptor was found in low- and high-grade gliomas; the concomitant expression of GM-CSF and the GM-CSF receptor was only found in grade IV glioblastoma [98].

While in this work we only focused on the single cytokine G-CSF, our corresponding results share the findings above with all of our tested primary tumor material expressing high levels of G-CSF as measured with the Bioplex assay, a similar method to an ELISA.

Figure 29 shows the native amount of G-CSF secreted by three different adherent cell lines, which is about 2000 pg/ml after 72 h without the stimulation of TNF- α , IL-1 β or the treatment of any drug. Interestingly, all three different cell populations showed a similar expression profile of G-CSF over time.

4.5.5 Absence of inhibitory effects on G-CSF expression by ribavirin, dapsone and fenofibrate

In the review of Kast *et al.* in 2017, the virostatic ribavirin, the antibiotic dapsone and the lipid-lowering agent fenofibrate were described as potential drugs for repurposing in glioblastoma. All of them are supposed to effect the synthesis of G-CSF and/or GM-CSF, so the proposed theory of this work. Dapsone should decrease IL-8 secretion and the attraction of neutrophils [64]. A few studies exist that examined the anti-glioma effects of all three drugs.

The work on ribavirin covered different aspects of the drug's anti-glioma activity, for example apoptosis, cell growth, motility etc., but none of the previous experiments examined the potential inhibition of cytokines, especially G-CSF [54, 104, 162].

The same applies to the two other drugs dapsone and fenofibrate [37, 61, 157].

Only Karpel-Massler *et al.* proved the ability of dapsone and its derivatives to decrease IL-8 expression, but this work also did not include any experiments regarding the anti-G(M)-CSF effects of dapsone [61].

To the best of knowledge, this work is the first to examine a potential inhibition of G-CSF by a combination of the three drugs ribavirin, dapsone and fenofibrate. As presented in Figure 30, the supernatants of three cell populations in their

differentiated phenotypes (DC35, DC38 and DC40) were examined. Clearly, no inhibition of G-CSF expression occurred when treating the cells with a combination of the three drugs in a clinically relevant concentrations. Because we initially had no experience regarding the timepoint at which the cells would reach the G-CSF peak secretion and as to when the drugs would possibly yield detectable effects, we chose three constant timepoints for all experiments, i.e., 24 h, 48 h and 72 h. As can be seen in Figure 29, high amounts of G-CSF were detected, indicating that measurements were performed in the right time slot. As indicated in Figure 30, no differences between treated and untreated DCs were detected as the amount of secreted G-CSF remained constant. This result finally disapproves our working hypothesis.

4.6 G-CSF as a chemoattractant in DC35 cells

G-CSF can be therapeutically used for the treatments of chemotherapy- or radiotherapy-induced side effects such as neutropenia, that can often lead into febrile neutropenia [95].

European and U.S. guidelines suggest prophylaxis in patients at high risk to develop febrile neutropenia by starting G-CSF treatment in parallel to chemotherapy [1, 140]. Specifically in Germany, G-CSF should not be administered in patients with afebrile neutropenia without other risk factors but here the guideline advocates G-CSF treatment for patients at higher risk [58]. Several malignant tumors, for example melanomas, various bladder cancers or types of lung cancer are producing G-CSF and/or GM-CSF [19, 92, 99].

In the Introduction of this work, it was pointed out that high expression of G-CSF is associated with a poorer clinical outcome and a higher progression rate in various tumors, including gliomas [40, 60, 101]. Also in meningiomas, G-CSF has a high influence on proliferation and vascularization. Meningiomas are slowly growing, usually benign intracranial tumors that are typically not invading the surrounding brain tissue. The risk of local recurrence correlates with the extent of resection and is also dependent on histological markers of the tumor. In rare cases, meningioma cells are also able to undergo malignant transformation. Braun *et al.* were the first group that explored the link between G(M)-CSF secretion and tumor proliferation in meningiomas. They concluded that the endogenous expression of G-CSF and GM-

CSF strongly correlates with the proliferation rate of the tumor and that G-CSF might lead to a higher vascularization [12].

In glioblastoma, *in vitro* data suggested that the treatment with G-CSF may lead to a higher proliferation rate and increased migration and invasion of glioma cells [164]. The typical plasma level of G-CSF in healthy humans is about 30 pg/ml [29, 69, 81, 149].

Wang *et al.* analyzed the G-CSF and G-CSF receptor expression in different glioma samples. In their work, they used concentrations of 10 pg/ml, 50 pg/ml, 100 pg/ml and 200 pg/ml for their experiments and obtained dose-dependent effects. For most of the assays, like the Transwell assay, a concentration of 100 pg/ml was chosen [164]. For our G-CSF Transwell assay, we decided to use the double amount of the normal plasma concentration which was 60 pg/ml. In the Transwell assay SC35 cells were not affected or allured by G-CSF and behaved like the control cells (Figure 26).

In stark contrast, DC35 cells that were attracted with G-CSF migrated on average 2.6 times more often through the membranes.

In conclusion, G-CSF acted as a strong attractant for differentiated cells to migrate while no such effect on stem cells was detected. Additionally, growth factor starvation had a great inhibitory effect on the migration of differentiated glioblastoma cells in contrast to glioblastoma stem cells which are not strongly affected.

Keeping in mind that G-CSF is a strong growth factor for various tumor cells and has been shown to increase invasion and motility of glioblastoma cells *in vitro*, the use of G-CSF in tumor patients should be avoided when not absolutely necessary. In this work we showed that patient-derived glioblastoma cells may be attracted by this specific cytokine and may increase their migratory activity when stimulated with G-CSF.

4.7 Drug repurposing in the context of CUSP9v3

As discussed in the Introduction, drug repurposing and off-label use play a fundamental role in clinical research and are considered more and more frequently a treatment strategy in multiple fields.

In 2013, Marc-Eric Halatsch and Richard Kast devised a protocol for drug repurposing in patients with recurrent glioblastoma called Coordinated Undermining of Survival Paths with 9 repurposed drugs (CUSP9) [63].

While the original CUSP9 combination consisted of nine already marketed drugs, the concept was slightly modified to become CUSP9* one year later which comprised aprepitant, artesunate, auranofin, captopril, celecoxib, disulfiram, itraconazole, ritonavir and sertraline [65]. All nine drugs are non-oncological drugs approved for different diseases. When combining them, different pathways in glioblastoma are blocked simultaneously as the drugs partially show synergistic effects [65].

In 2018, Basso *et al.* listed drugs potentially useful for drug repurposing in glioblastoma, and all nine drugs from CUSP9 were featured in this publication [5]. From 2016 to 2018, a first clinical trial started in the Department of Neurosurgery at Ulm University Hospital, including 10 patients over a period of two years where CUSP9v3 was first applied in patients (NCT02770378). The only change compared to CUSP9* consisted of the replacement of artesunate by minocycline.

The nine-drug cocktail was combined with daily low-dose temozolomide, and preliminary results showed that 50% of all patients with glioblastoma recurrence upon study entry remained free of progression after 12 months of treatment. The tolerability was good to moderate with frequent individually targeted dose reductions.

CUSP9v3 showed potential signals of activity in this group of patients but to potentially improve the combination, drugs with anti-migratory and anti-invasive effects appear to be on priority.

To further determine if the drugs ribavirin, dapsone and fenofibrate could potentially enhance CUSP9, the experiments described in this thesis were designed. As reviewed by Kast *et al.*, all of these drugs had demonstrated potential anti-migratory effects or growth inhibition in GB [64].

5 Summary

Glioblastoma belongs to the most difficult to treat cancers with a fatal prognosis and a median overall survival of 14.6 to 16.7 months.

Despite the availability of different second-line treatments, the disease almost invariably recurs and progresses after the initial treatment options such as safe maximal resection, radiation and chemotherapy are exploited. The motile and invasive properties of glioblastoma cells make this tumor especially hard to conquer. The off-label use of otherwise approved and marketed drugs plays an important role in many fields of medicine today. Although the off-label use of drugs for other indications, drug repurposing, is generally a safe and cheap therapeutic option, it is rarely applied in cancer therapy and little is known about the potential of drugs in glioblastoma.

Three older drugs - ribavirin, dapsone and fenofibrate - have been attributed with anti-tumor effects in some *in vitro* studies. This work is the first to examine the effects of a triple combination of these drugs on patient-derived glioblastoma cells. In contrast to preceding research, only clinically achievable blood concentrations of the drugs were used in all experiments. Disproving the working hypothesis, the findings revealed no effects on apoptosis, viability, proliferation or migration of the glioblastoma cells when exposing them to single compounds or the triple combination of ribavirin, dapsone and fenofibrate.

Instead, this work proved that patient-derived tumor material, especially the differentiated glioblastoma phenotype, produces a high amount of G-CSF (granulocyte colony-stimulating factor), a cytokine which normally stimulates the maturation of granulocytes. In glioblastoma, G-CSF is suspected to create an immunosuppressive state in affected patients and thus to protect the survival of the tumor itself. But here again, the tested drugs either alone or in combination, did not inhibit the secretion of G-CSF sufficiently. Because the drugs, studied in this work, were not eliciting the originally supposed impact on glioblastoma cells in the clinically relevant concentrations, the search for other drug candidates continues. Furthermore, the complex role of G-CSF in the glioblastoma biology is poorly understood and more research is warranted to elucidate the potential effects of these oncological co-treatments on glioblastoma and its microenvironment.

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Acknowledgements

Looking back on the process of writing this dissertation I would like to point out that I am exceptionally thankful to Prof. Dr. Marc-Eric Halatsch for giving me the opportunity to work with him. His patience, dedication and support fundamentally helped me to create this work over the past few years. Through his guiding expertise and acumen this dissertation has always had clear objectives and a purposeful investigative approach.

I would also like to express my dearest thanks to PD Dr. Mike-Andrew Westhoff for introducing me to the world of research and making my time at the laboratory an unforgettable experience. When I look back, I think of countless hours of learning about glioblastoma as well as gathering both trivial and non-trivial knowledge such as the history of the Pitcairn Islands or different kinds of cancer of animals. Thank you for giving me the guidance and help I needed but also for leaving me the space to create my own critical thinking.

I want to especially thank Prof. Dr. Klaus-Michael Debatin for allowing me to conduct my work in his laboratory as well as Andrea Schuster for introducing me into the practical work of the laboratory and offering me assistance throughout the different stages.

For her kindness and support during weekends and time-off work as well as for letting me conduct a part of my work in her laboratory, I would like to particularly give my thanks to Dr. Shaoxia Zhou.

Last but not least, I would like to thank Patricia Kattner, Verena Herbener, Christel Payer, Annika Dwucet and Daniela Zerrinius who made this journey a very special and rememberable time. Thank you for all the funny hours, coffee times in the laboratory and memorable Christmas parties.

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