In Vitro Drug Response and Efflux Transporters Associated with Drug Resistance in Pediatric High Grade Glioma and Diffuse Intrinsic Pontine Glioma

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Abstract

Pediatric high-grade gliomas (pHGG), including diffuse intrinsic pontine gliomas (DIPG), are the leading cause of cancer-related death in children. While it is clear that surgery (if possible), and radiotherapy are beneficial for treatment, the role of chemotherapy for these tumors is still unclear. Therefore, we performed an in vitro drug screen on primary glioma cells, including three DIPG cultures, to determine drug sensitivity of these tumors, without the possible confounding effect of insufficient drug delivery. This screen revealed a high in vitro cytotoxicity for melphalan, doxorubicin, mitoxantrone, and BCNU, and for the novel, targeted agents vandetanib and bortezomib in pHGG and DIPG cells. We subsequently determined the expression of the drug efflux transporters P-gp, BCRP1, and MRPI in glioma cultures and their corresponding tumor tissues. Results indicate the presence of P-gp, MRPI and BCRP1 in the tumor vasculature, and expression of MRPI in the glioma cells themselves. Our results show that pediatric glioma and DIPG tumors per se are not resistant to chemotherapy. Treatment failure observed in clinical trials, may rather be contributed to the presence of drug efflux transporters that constitute a first line of drug resistance located at the blood-brain barrier or other resistance mechanism. As such, we suggest that alternative ways of drug delivery may offer new possibilities for the treatment of pediatric high-grade glioma patients, and DIPG in particular.


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Introduction

Pediatric high grade glioma (pHGG) constitutes 15–20% of pediatric central nervous system tumors [1]. These aggressive tumors are difficult to treat, and are associated with an extremely poor prognosis. The extent of surgical resection is the most important clinical prognostic factor in these patients [2]. Together with radiotherapy, which is a standard component of postoperative management, a 2-year-survival rate of 10–30% for supratentorial HGG has been established [1]. Diffuse intrinsic pontine glioma (DIPG), an infiltrative tumor typically originating in the pons, does not qualify for surgical resection due to its delicate location. In DIPG radiotherapy prolongs progression free survival (PFS) and improves quality of life, yet, the median overall survival (OS) in these children is still only nine months [3]. Unfortunately, no chemotherapeutical regimens or alternative radiation options have successfully improved OS or PFS in children with HGG and DIPG [4,5]. These disappointing results emphasize the need to identify effective drugs. Therefore, we screened a heterogeneous group of primary pHGG cell cultures, including three DIPG cultures, for their sensitivity in vitro to different drugs.

To clarify whether a lack of clinical response results from tumor cell resistance or from poor drug delivery to the tumor cells, we also explored the mode of drug resistance in these tumors. In particular, we focused on one of the main mechanisms of drug resistance in the brain mediated by overexpression of ATP-binding cassette (ABC) transporters. Drug delivery to the brain is hampered by the presence of P-glycoprotein (P-gp, ABCB1), breast-cancer-resistance protein (BCRP, ABCG2), and multidrug-resistance-associated proteins (MRPs, ABCC1) [6]. Presence of these transporters on tumor cells or (peri)tumoral vasculature results in active efflux of chemotherapeutics by transmembrane transport, leading to a decrease in intracellular drug levels and subsequently a decrease in their cytotoxic activity [7–9].

Here we show that several classic chemotherapeutic drugs display a high cytotoxicity in primary pediatric glioma cell cultures in vitro, in physiologically relevant doses. Immunohistochemical staining on the corresponding tumor tissue indicates the presence of P-gp, MRPI, and BCRP1 on the tumor vasculature, while only...
MRP1 is also expressed in glioma cells. These findings suggest that the presence of all three major drug efflux pumps in the blood-brain-barrier (BBB) may form a first line of resistance of pHGG to treatment with classic chemotherapeutic drugs which by themselves are capable of inducing cytotoxicity in pediatric glioma cells. The drug transporters at the BBB impede drug delivery to the tumor site, while the presence of MRP1 protein in glioma cells themselves, may further protect the tumor cells from chemotherapy.

Results

Tumor and Patient Characteristics

Primary cell cultures were established from tumor material from nine children, aged one week to 11 years, diagnosed with high grade glioma (Table 1). In six patients, the tumor was located supratentorially, among these were five glioblastoma multiforme (GBMs), one anaplastic astrocytoma (AA), and one anaplastic oligodendroglioma. Among the GBMs was one rare case of giant cell GBM. Also, three DIPGs were obtained with different histological grading, including a GBM, an AA WHO grade III, and one diffuse fibrillary astrocytoma. In most cases resections were performed before initiation of therapy, except for one DIPG patient, from whom autopsy material was retrieved approximately two hours after death (VUMC-DIPG-01). Malignancy of the established cell lines was confirmed by determining the chromosomal aberrations, either by array CGH or by classical karyotyping. Apart from VUMC-HGG-06 and VUMC-HGG-07, all cell cultures showed abnormalities, varying from small deletions in VUMC-HGG-02 and -03, to extensive chromosomal changes in VUMC-DIPG-A. Within the VUMC-DIPG-A culture, diverse chromosomal aberrations were identified.

Chemoresponsiveness of Primary Pediatric High Grade Glioma Cultures

In order to determine the sensitivity of primary pHGG cultures to a series of drugs, a small chemical screen was designed, which included conventional chemotherapeutic drugs of a variety of classes, (anthracyclins, alkylating agents, topoisomerase I/II inhibitors, nitrosureas, mitotic inhibitors, anti-metabolites), and small molecule inhibitors targeting specific proteins that are often upregulated in pHGG or DIPG (Figure 1S, Material and Methods S1). Drug concentrations used were based on IC50 values reported for adult malignant glioma cell lines (Table 2 and 3). The effect of the compounds on cell survival was tested at least four times for each primary culture. Robustness of the assay was assessed by calculating the coefficient of variation (CV = 9.4 ± 3.7) and the Z’ factor for each cell line. Except for VUMC-DIPG-01, all Z’ factors indicated a powerful assay (Z’ = 0.6 ± 0.09). In addition, drug sensitivity of primary human astrocytes was measured, to determine the therapeutic window for the various drugs. As shown in Figure 1A, cell cultures VUMC-HGG-01, and VUMC-DIPG-A were relatively sensitive, with <50% cell survival in response to more than half of the chemotherapeutics. In contrast, VUMC-HGG-05 was the most resistant cell line of the panel, remaining unaffected by the majority of compounds. BrdU incorporation studies showed that this resistance or sensitivity was not correlated to proliferation index (Figure S2, Material and Methods S1). The only drug capable of inducing significant toxicity in VUMC-HGG-05 was melphalan. In general, melphalan, the anthracyclines doxorubicine and mitoxantrone, and BCNU most effectively affected cell survival, followed by etoposide, thiopeta, and carboplatin. Among the novel drugs, bortezomib had a significant anti-glioma effect; in six out of nine cultures, exposure to 100 nM bortezomib resulted in >50% reduction in cell survival. Bosutinib, dasatinib, sorafenib, and olaparib (either as monotherapy or in combination with irradiation), had an effect in approximately 4-5 cell cultures. In VUMC-HGG-05, VUMC-HGG-06, and VUMC-HGG-07 the effect of olaparib increased when combined with irradiation. Although the drug dose ranges in our study overlapped with published IC50 values in cancer cell lines, no or little effect was observed with erlotinib, everolimus, panobinostat, and SB431542 (Figure 1B).

Expression of Drug Efflux Transporters in pHGG

In order to determine whether ABC transporters play a role in the responses of our cell cultures, we assessed the presence of the main ABC transporter proteins P-gp (ABCB1), MRP1 (ABCC1) and BCRP1 (ABCG2). Therefore, Western blotting experiments were performed on the pHGG cell cultures (Figure 2). MRP1 was detected in seven out of nine primary glioma cultures, with a variable intensity (2 low, 5 high expression). High MRP1-expression was detected in VUMC-HGG-01, VUMC-HGG-07, VUMC-DIPG-A, and VUMC-DIPG-B. All cultured were negative for P-gp, and in all except VUMC-HGG-01, BCRP1 was also absent. The MCF7/P-gp and MCF/BCRP1 cell lines were used as a positive control for P-gp, and BCRP1 [10,11], and the 2000/MRP1 cell line for MRP1 [12]. Several bands on the BCRP1 blot appeared at a molecular weight which was higher than expected (220 kD instead of 72 kD) (data not shown). These bands were present in the cell lysates of all glioma cultures, and were of pronounced intensity in the six supratentorial pHGG cultures.

To further explore the role of the various ABC transporters in pHGG, and to assess their presence in the (peri)tumoral vasculature, immunohistochemistry was performed on tumor tissue sections of the patients corresponding to the cell cultures (Figure 3). A semi-quantitative analysis of the sections was performed to distinguish between tumor cells and endothelial cells of the BBB (Table 4). P-gp was absent in the glioma cell membranes in most tumor sections, but showed a moderate expression in the tumor vasculature in half of the patients. MRP1 was expressed in both the glioma cells, and the tumor vasculature in most sections. Staining of BCRP1 in glioma cells was mostly negative or weak, while the microvasculature showed intense staining in the majority of the sections. Representative pictures are given in Figure 3.

Discussion

Here we show that primary cultures derived from pediatric glioma patients, are sensitive to a number of traditional chemotherapeutics as well as to novel, targeted therapeutics. Moreover, - to our knowledge - we provide the first data on drug sensitivity screening of primary DIPG cultures, suggesting that DIPG per se is not necessarily resistant to chemotherapy.

Although there is a substantial heterogeneity in tumor origin and genetic aberrations, a number of drugs show high cytotoxicity in most cell cultures. Generally, the classical, active chemotherapeutics can be divided into two classes: topoisomerase II inhibitors (mitoxantrone, doxorubicin, and etoposide), and alkylating agents (melphalan, BCNU, carboplatin, and thiopeta). We found that melphalan was the only drug to induce significant cell death in all primary cultures, even in the most resistant glioma culture, which was a giant cell glioblastoma. However, the efficacy of melphalan in pediatric gliomas has not been assessed so it is unknown whether sufficient drug levels can be reached locally in these children. Testing in in vivo glioma models will show whether melphalan has the potential to produce toxicity at levels that can
### Table 1. Tumor and patient characteristics corresponding with the primary cell cultures.

<table>
<thead>
<tr>
<th>VUMC-</th>
<th>Sex</th>
<th>Age</th>
<th>Histology</th>
<th>Location</th>
<th>Surgery</th>
<th>Radiotherapy</th>
<th>Chemotherapy</th>
<th>Survival</th>
<th>Classical karyotype</th>
<th>Deduced copynumber karyotype[^73] (array results)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGG-02</td>
<td>f</td>
<td>0</td>
<td>GBM</td>
<td>right frontal lobe</td>
<td>STR</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0</td>
<td>46,XX [10]</td>
<td>dup(10)(q11.2q12)</td>
</tr>
<tr>
<td>HGG-03</td>
<td>f</td>
<td>1</td>
<td>GBM</td>
<td>left hemisphere</td>
<td>GTR</td>
<td>–</td>
<td>valproic acid</td>
<td>6</td>
<td>46,XX [20]</td>
<td>del(10)(q11.2q12)</td>
</tr>
<tr>
<td>HGG-05</td>
<td>f</td>
<td>11</td>
<td>GC GBM</td>
<td>right frontal lobe</td>
<td>STR</td>
<td>60 Gy</td>
<td>vincristin, carboplatin, cyclophosphamide, etoposide</td>
<td>12</td>
<td>46,XX(17)(q10 or add(17)(p?) [2]/40,XX, –6, –13, add(18)(p11.72), –19, –20, 21,add(21)(p11.21)[1]/46,XX [16])</td>
<td>arr(1–22,X)x2</td>
</tr>
<tr>
<td>HGG-06</td>
<td>f</td>
<td>5</td>
<td>AO</td>
<td>left hemisphere</td>
<td>STR</td>
<td>54 Gy</td>
<td>procarbazine, CCNU, vincristine</td>
<td>84**</td>
<td>46,XX [13]</td>
<td>arr(1–22,X)x2</td>
</tr>
<tr>
<td>HGG-07</td>
<td>f</td>
<td>8</td>
<td>GBM</td>
<td>thalamus</td>
<td>GTR</td>
<td>–</td>
<td>CCNU, vincristine, prednisone</td>
<td>24</td>
<td>46,XX [10]</td>
<td>arr(1–22,X)x2</td>
</tr>
<tr>
<td>DIPG-01</td>
<td>m</td>
<td>5</td>
<td>GBM</td>
<td>pons</td>
<td>autopsy</td>
<td>45 Gy*</td>
<td>temozolomide</td>
<td>9</td>
<td>46,XY,del(4)(p12) [3]</td>
<td>–</td>
</tr>
</tbody>
</table>

[^73]: f: female, m: male, AA: anaplastic astrocytoma, GBM: glioblastoma, GC GBM: giant cell glioblastoma multiforme, AO: anaplastic oligodendroglioma, DA2: diffuse fibrillary astrocytoma, GTR: gross total resection, STR: subtotal resection, PR: partial resection. Chemotherapy was administered adjuvant to resection and/or radiotherapy in all treated patients.

[^8]: hypofractionated radiotherapy 15 fractions of 3 Gy.

[^9]: Survivor, n.a.: does not apply.
Table 2. Classical drugs used in this study.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Substrate for BCRP</th>
<th>Concentrations used in this study</th>
<th>Published in vitro IC50 in malignant glioma</th>
<th>Published peak plasma concentration in children</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU</td>
<td>Alkylating agent</td>
<td>+</td>
<td>100 uM, 1 mM</td>
<td>155 uM (12.7 ug/mL) [47]</td>
<td>not reported</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200 uM, 2.6 mM</td>
<td>173 uM (14.6 ug/mL) [44]</td>
<td>479 uM (273 ug/mL) [45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>500 uM, 5 mM</td>
<td>31 uM (115 ug/mL) [51]</td>
<td>89 uM [52]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100 uM, 10 mM</td>
<td>not reported</td>
<td>8.3-38.9 uM [55]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 uM, 400 uM</td>
<td>not reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100 uM, 1 mM</td>
<td>not reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>500 uM, 5 mM</td>
<td>not reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25 uM, 250 uM</td>
<td>not reported</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100 uM, 1 mM</td>
<td>not reported</td>
<td></td>
</tr>
<tr>
<td>Carboplatin</td>
<td>Alkylating agent</td>
<td>+</td>
<td>200 uM, 2.6 mM</td>
<td>500 uM, 5 mM</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Topoisomerase II</td>
<td>+</td>
<td>100 uM, 10 mM</td>
<td>20 uM, 200 uM</td>
<td></td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase II</td>
<td>+</td>
<td>100 uM, 10 mM</td>
<td>20 uM, 200 uM</td>
<td></td>
</tr>
<tr>
<td>Melphalan</td>
<td>Alkylating agent</td>
<td>+</td>
<td>20 uM, 200 uM</td>
<td>20 uM, 200 uM</td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Alkylating agent</td>
<td>+</td>
<td>100 uM, 1 mM</td>
<td>20 uM, 200 uM</td>
<td></td>
</tr>
<tr>
<td>Procarbazine</td>
<td>Alkylating agent</td>
<td>+</td>
<td>200 uM, 2.6 mM</td>
<td>20 uM, 200 uM</td>
<td></td>
</tr>
<tr>
<td>Temozolomide</td>
<td>Alkylating agent</td>
<td>+</td>
<td>100 uM, 1 mM</td>
<td>20 uM, 200 uM</td>
<td></td>
</tr>
<tr>
<td>Thiotepa</td>
<td>Alkylating agent</td>
<td>+</td>
<td>200 uM, 2.6 mM</td>
<td>20 uM, 200 uM</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>Topoisomerase I</td>
<td>+</td>
<td>100 uM, 10 mM</td>
<td>1 uM, 10 mM</td>
<td></td>
</tr>
</tbody>
</table>

The drug sensitivity of pediatric high-grade glioma in cell cultures is limited due to the expression of drug efflux transporters, which can limit the therapeutic efficacy of chemotherapy. In this study, we investigated the role of drug efflux transporters in pediatric glioma cultures and compared their expression to that in adult glioma cultures. The results suggest that pediatric glioma cultures may be more sensitive to certain drugs, such as temozolomide, compared to adult glioma cultures. However, the lack of P-glycoprotein (MDR1) expression in pediatric glioma cultures limits the effectiveness of drugs that rely on P-glycoprotein for efflux. Further studies are needed to explore the potential of targeted therapies for pediatric glioma, taking into account the specific characteristics of these tumors.
contain P-gp activity, and although ABCB1 gene expression has been reported in adult HGG [31], this was not always confirmed by immunohistochemical staining [32]. Together, these results suggest that the role of P-gp in pediatric glioma patients is limited to the BBB, and that an anti-tumor effect of therapeutic agents that are substrate for P-gp is not ruled out when these barriers are bypassed by intratumoral administration. Indeed, a significant effect of doxorubicin, the most important therapeutic agents that are substrate for P-gp, is observed in our drug screen.

In conclusion, all three major ABC transporters are active at the BBB, impeding the delivery of chemotherapeutics to the tumor site. There are higher levels of P-gp and BCRP1 in the endothelial cells of the tumor vasculature than in the glioma cells themselves, which is in line with findings in adult glioma [36,37]. Targeting P-gp and BCRP1 in the vasculature might therefore enhance the permeability of its substrates, not only in tumor cells but also across tumoral microvessels. Based on these results, it is not likely that substrates for MRP1 will target pediatric glioma in a satisfying manner, even when using local delivery, unless MRP1 function is inhibited.

Most inhibitors of ABC transporter activity developed thus far have considerable toxic side effects, and therefore have failed to significantly improve chemotherapeutic efficacy in patients [38]. Interestingly, tyrosine kinase inhibitors (TKIs), which are considered among the most promising agents for HGGs, were reported to antagonize ABC-transporter-related drug resistance [39–41]. Therefore, combinations of TKIs, with established chemotherapeutic agents, could hold promise in overcoming drug resistance, although single-agent TKIs did not show much anti-tumor activity in our screen.

Potential limitations of the study are the limited sample size, and the design of the customized drug screen. The drug targets, concentrations, and ABC efflux transporter substrate specificity for novel, targeted drugs.

Table 3. Novel drugs used in this study.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Target</th>
<th>Substrate for MRP1</th>
<th>Substrate for P-gp</th>
<th>Substrate for BCRP</th>
<th>Concentrations used in this study</th>
<th>Published in vitro IC_{50} in human cancer cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>26S proteasome</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>10 nM, 100 nM</td>
<td>28.9–48.2 nM (glioma) [63]</td>
</tr>
<tr>
<td>Bosutinib</td>
<td>c-Abl, Src, HDAC</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>1 uM, 10 uM</td>
<td>1.3 (melanoma) [64], 5.7 uM (melanoma) [65]</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>BCR/ABL, c-KIT, Src, Ephrin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100 nM, 1 uM</td>
<td>210 nM–1.5 uM (glioma) [66]</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>EGFR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5 uM, 50 uM</td>
<td>9 uM (glioma) [67]</td>
</tr>
<tr>
<td>Everolimus</td>
<td>mTOR</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100 nM, 1 uM</td>
<td>271 nM (glioma) [68]</td>
</tr>
<tr>
<td>Olaparib</td>
<td>PARP</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>1 uM, 10 uM</td>
<td>1.42–7.43 uM (glioma) [69]</td>
</tr>
<tr>
<td>Panobinostat</td>
<td>HDAC</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>50 nM, 500 nM</td>
<td>not reported</td>
</tr>
<tr>
<td>SB431542</td>
<td>TGFβ, Alk</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>1 uM, 10 uM</td>
<td>0.1–10 uM (dose range used in vitro studies) [70]</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>Raf, PDGFR, VEGFR, c-Kit</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>2.5 uM, 25 uM</td>
<td>5–20 uM (glioma) [71]</td>
</tr>
<tr>
<td>Temsirolimus</td>
<td>mTOR</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1 uM, 10 uM</td>
<td>1.4 uM (variety) [72]</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>EGFR, VEGFR, Ret</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>10 uM, 100 uM</td>
<td>10 uM [73]</td>
</tr>
</tbody>
</table>

Drug targets, concentrations, and ABC efflux transporter substrate specificity for novel, targeted drugs. doi:10.1371/journal.pone.0061512.t003
concentrations used in the present study were mainly based on information obtained in adult glioma cell lines, which could bias our results in both directions. The purpose of our screen was to identify potential effective anti-glioma therapeutics, rather than to exclude therapeutics for future protocols. Therefore, it is important to emphasize that therapeutics that were not considered effective in this experimental design, should not necessarily be considered ineffective in pediatric glioma without further exploration, as higher doses might prove successful. When aiming for translation to the clinical situation, in vivo experiments with HGG and DIPG xenograft models will be crucial to further elucidate the role of the drug efflux transporters in relation to drug resistance.

As some of the drugs that display a high toxicity in cultures were never found to be beneficial in clinical trials, the understanding of drug resistance in pHGG is essential for the development of efficient therapies. Our data suggest that the presence of drug efflux pumps in the blood-tumor barrier may constitute an important first line of resistance of pHGG to such treatment, and the efficacy may be further impaired by expression of a subset of these, as well as other drug resistance proteins in the glioma cells themselves. Possible approaches to overcome these mechanisms of resistances are direct intratumoral drug delivery, such as convection enhanced delivery (CED), and the use of drugs that are not substrate for these drug transporters.

Materials and Methods

Processing of Tumor Material and Cell Culture

Single cell cultures were established from biopsy samples derived from pediatric glioblastoma multiforme, anaplastic astrocytoma, anaplastic oligodendroglioma and diffuse intrinsic pontine glioma (DIPG) or from DIPG autopsy samples. Informed consent was obtained according to institutionally-approved protocols. Tumor pieces were collected into DMEM (Dulbecco’s Modified Eagles Medium, PAA Laboratories GmbH, Pasching, Austria) and washed twice with PBS to remove blood clots. Samples were sliced into small (3–5 mm³) pieces and either mechanically dissociated by filtering through a cell strainer (BD Falcon Biosciences, Bedford,
USA), or dissociated by incubation with Accutase (PAA Laboratories GmbH, Pasching, Austria). Single cells were seeded in DMEM-F12, constituted with stable glutamine, 10% fetal bovine serum (PerBio Science Nederland B.V., Etten-Leur, The Netherlands), 1% penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria), and 0.5% sodium pyruvate. For primary astrocytes 15% fetal bovine serum was used. Cells were grown at 37°C in a 5% CO2 humified atmosphere.

Karyotyping and Comparative Genomic Hybridization (CGH)

Malignancy of the established cell lines was confirmed by determining the chromosomal aberrations, either by classical karyotyping, or by array CGH. For cytogenetic analysis, cells were harvested according to standard cytogenetic techniques. Briefly, cells were treated with demecolcine (final concentration 0.4 μg/ml; Sigma) for 2 hours, trypsinized, treated with a 75 mM KCl hypotonic solution and finally fixed using methanol:acetic acid (3:1). Metaphase spreads were prepared by dropping the fixed cells on glass slides. Karyotyping was performed on GTG banded metaphase cells. Metaphase spreads were observed using a Zeiss Axioskop 20 microscope. Images were captured using a Cytovision imaging system (Leica-microsystems, United Kingdom). For array CGH, genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega) and hybridized to NimbleGen Human CGH 3×720 K Whole-Genome Tiling v2.0 Arrays (Roche Diagnostics) according to the manufacturer’s instructions. Data analyses were performed using the Nexus 5.0 software.

Drug Screen

The primary pediatric glioma- and astrocyte cultures were exposed to a customized chemical screen, consisting of 21 chemotherapeutic drugs, at dose ranges that were based on published in vitro IC50s in malignant glioma (Table 2 and 3). Fifteen hundred cells were seeded per well in 96-well tissue culture plates. Cells in each well were treated with a different compound at two different concentrations. For treatment with temozolomide and olaparib, treatment was done with paired 96-well plates, where one plate was exposed to 3 Gray in a Gammacell® 220 Research Irradiator (MDS Nordion, Canada) after 24 hours of incubation, and the other plate was a non-irradiated control. After 96 hours cell survival was assessed using the Acumen X3 laser cytometer (TTO Labtech, UK), using 300 mM of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) as read-out. Results were analyzed using Acumen Explorer software, calculating the survival percentage for each compound tested in the assay. Each experiment was performed at least four times. A coefficient of variation (CV) and Z’ factor were calculated to assess the reproducibility and robustness of the small molecule screens, where CV = SD/μ and Z’ = 1 – (3σe + 3σc)/Iμe−μc−I [42].

Figure 2. Western blot for detection of P-gp, MRP1, and BCRP1 in pHGG cultures. MW represents approximate molecular weight of these proteins, as indicated at the right. The pHGG lanes were loaded with 20 μg of protein, the lanes with positive controls were loaded with 5 μg of protein. doi:10.1371/journal.pone.0061512.g002
Cell Extraction and Western Blotting

Cell cultures were washed twice in PBS, and cell-free extracts were made by resuspension in lysis buffer (50% Tris/\(\text{HCl}\) pH7.6, 25% \(\text{MiliQ}\), 20% glycerol, 4% Protease Inhibitor Cocktail, 0,5% 1 M Dithiotreitol (DTT), 0,5% NP-40) and sonification. The samples were then centrifuged at 13000 rpm for 10 minutes at 4°C, and the supernatants were subsequently used for Western blotting. Equal amounts of protein were loaded on a 4–12% gradient SDS polyacrylamide gel and transferred to PVDF membranes (Millipore, Amsterdam, The Netherlands). The membranes were washed, blocked with 5% milk, and incubated overnight with an 1:50 diluted antibody against P-gp/\(\text{ABCB1}\) (MDR1/clone JSB-1 mab4120, Chemicon, Millipore, Temecula, California), BCRP/\(\text{ABCG2}\) (BXP-21 Ab3380, Abcam, Cambridge, United Kingdom), MRP1/\(\text{ABCC1}\) (ABCC1 A10 HPA002380, Sigma-Aldrich), or with an 1:15,000 diluted antibody directed against \(\beta\)-Actin (Millipore, MAB1501R). After several washes the membranes were incubated with anti-mouse or anti-rabbit IgG HRP (Dako, Glostrup, Denmark) in a dilution of 1:3,000. Antibody binding was detected using Enhanced Chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Immunohistochemistry

Paraffin-embedded tissue was sliced into 5\(\mu\)m sections, deparaffinized and rehydrated by washing in xylene and ethanol series. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase in methanol for 30 minutes. The sections were washed and heated in citrate buffer pH6, and gradually cooled to room temperature. After a PBS wash, the sections that were stained for P-gp were blocked with 5% normal goat serum (NGS) in PBS for thirty minutes prior to primary antibody incubation. Sections were incubated for one hour at room temperature with an 1:20 diluted antibody against P-gp (MDR1/clone JSB-1 mab4120, Chemicon, Millipore, Temecula, California), an 1:40 diluted antibody against BCRP1 (BXP-21 Ab3380, Abcam, Cambridge, United Kingdom), or an 1:75 diluted antibody against MRP1 (ABCC1 A10 HPA002380, Sigma-Aldrich). For the detection of the primary antibody, EnVision anti-mouse/anti-rabbit (ImmunoLogic, Duiven, The Netherlands) was used according the manufacturer’s instructions. Peroxidase activity was detected using 3,3-diaminobenzidine-Tetrachloride (DAB) (Sigma, USA) in 0.1% hydrogen peroxide. All sections were counterstained with haematoxylin. Protein expression was scored for quantity (percentage of cells, ranging from 0 = 0, 1 = <20%, 2 = 20–40%, 3 = 40–60%, 4 = 60–80%, to 5 = 80–100%), and intensity (0 = absent, 1 = low, 2 = moderate, 3 = high). Using these data, an immunohistochemistry score (IHS) was calculated by multiplying the quantity and staining intensity scores. IHS: 0 = negative, 1–3 = weak, 4–9 = moderate, 10–15 = strong. Images were made using a Zeiss Axioskop microscope (HBO100W/Z), equipped with a Canon digital camera (Canon PowerShot A460, Canon Inc., Tokyo, Japan); imaging software is Canon Utilities, ZoomBrowser Ex. 5.7, Canon Inc, Nort Ryde, Australia.
Table 4. Immunohistochemistry scores of P-gp, Mrp1, and Bcrp1 in tumor tissue sections.

<table>
<thead>
<tr>
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<th>ABCB1/Mrp1</th>
<th>ABCB2/Bcrp1</th>
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<td>Tumor cells</td>
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Material and Methods S1 Supplementary Material and Methods.

(DOCX)

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Author Contributions

Conceived and designed the experiments: EH DV GJ DN WPV PW. Performed the experiments: VH LW DB MJ. Analyzed the data: SV EH DV MJ. Contributed reagents/materials/analysis tools: MJ DV DN. Wrote the paper: SV EH DV WP PW GJ.