Can in-vitro chemoresponse assays help find new treatment regimens for malignant gliomas?

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Various in-vitro chemosensitivity and resistance assays (CSRAs) have been demonstrated to be helpful decision aids for non-neurological tumors. Here, we evaluated the performance characteristics of two CSRAs for glioblastoma (GB) cells. The chemoresponse of fresh GB cells from 30 patients was studied *in vitro* using the ATP tumor chemoresponse assay and the chemotherapy resistance assay (CTR-Test). Both assay platforms provided comparable results. Of seven different chemotherapeutic drugs and drug combinations tested *in vitro*, treosulfan plus cytarabine (TARA) was the most effective, followed by nimustine (ACNU) plus teniposide (VM26) and temozolomide (TMZ). Whereas ACNU/VM26 and TMZ have proven their clinical value for malignant gliomas in large randomized studies, TARA has not been successful in newly diagnosed gliomas. This seeming discrepancy between *in vitro* and clinical result might be explained by the pharmacological behavior of treosulfan. Our results show reasonable agreement between two cell-based CSRAs. They appear to confirm the clinical effectiveness of drugs used in GB treatment as long as pharmacological preconditions such as overcoming the blood–brain barrier are properly considered. *Anti-Cancer Drugs* 25:375–384 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Various in-vitro chemoresponse assays or chemosensitivity and resistance assays (CSRAs) have been developed and applied since the 1950s [1–5]. However, only after the introduction of microtiter technology could the laborious and time-consuming handling typical of the assays in the early days of the technique be overcome. Meanwhile, quantifiable semiautomated methods exist that render these assays feasible for clinical application [6,7].

Many publications have demonstrated the predictive value of these assays [8–12]. For various tumor entities such as lung cancer [13], melanoma [14], and malignant glioma [15], correlations between cell-based and gene expression assays could be demonstrated. In Japan, CSRAs are officially approved as 'advanced clinical medicine' [16]. In other parts of the world, however, they are still considered research tools [17,18].

Here, we present response data of glioblastoma (GB) cells to two CSRAs, which substantiate that these assays might be helpful clinical decision aids that deserve more attention.

Materials and methods

Tumor tissue specimens

From August 2008 to January 2013, a total of 33 GB specimens were collected from 30 patients undergoing resection of malignant glioma at the Department of Neurosurgery of the RWTH Aachen University Hospital. In three cases, not only samples of the newly diagnosed tumor but also of the first recurrence were collected for chemoresponse analysis. All patients provided written informed consent for in-vitro testing of their tumor tissue before surgery. The study protocol was approved by the local ethics committee (EK-158/07) as part of trial number DRKS00000264 registered with the German Clinical Trials Register.

Each specimen was reviewed by two neuropathologists (K.N. and J.W.) and classified according to the current WHO classification of tumors of the central nervous system [19].

When the diagnosis glioma grade IV was confirmed, samples of at least 0.5 g were sent in RPMI medium by carrier to the test laboratory Lance (Bonn, Germany) for the ATP tumor chemoresponse assay (ATP-TCA). In 11 cases, the samples were split and additionally sent to a second laboratory (TherapySelect, Heidelberg, Germany) to perform a chemoresponse assay called 'chemotherapy resistance test' (CTR-Test). Arrival of the tumor tissue at the test laboratory was within 24 h after surgical resection in all cases.

MGMT methylation status

MGMT gene silencing was determined by methylation-specific PCR. DNA was isolated from the control blood samples and – in most cases – from unfixed tumor tissue

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using the QIAamp DNA Mini Kit, and in a few cases from formalin-fixed, paraffin-embedded tumor tissue using the QIAamp DNA FFPE Tissue Kit. The Epitect Bisulfite Kit was used for bisulfite treatment of the DNA and the modified DNA was amplified using the Epitect MSP-Kit with the primers described in Hegi *et al.* [20]. All kits were purchased from Qiagen (Hilden, Germany). Fragment analysis was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

ATP-TCA

Tissue processing and cell culture were performed at Lance according to a published protocol [6].

Briefly, tumor tissue was dissociated to produce a cell suspension. The cells were washed and purified by centrifugation to remove debris. Viability was measured by trypan blue exclusion. Viable cells were resuspended in 96-well plates at a density of 7500 cells/well. In three cases, parallel samples were also prepared with 22 500 cells. Each drug was tested in duplicate at six test drug concentrations (TDC). The TDC levels were chosen so that the reference concentration (TDC100) corresponded to the peak plasma range found in human blood (Table 1). The whole set covered the concentration range from twice (TDC200) to 1/16th (TDC6.25) the reference concentration in two-fold dilutions. Negative medium controls and positive controls with maximum ATP inhibition complemented the test sets. After 5–6 days at 37°C, the cells were lysed, and ATP was measured in the luciferin–luciferase reaction.

Nine commercially available drugs were used as single agents or combinations of up to three compounds (Table 2). The results were expressed as %inhibition at concentration c, and a sum index (SI) to express response was determined as $SI = 600 - \Sigma_i \mathrm{Inhib}(c_i)$ with $c_i = 200, 100, 50, 25, 12.5$, and 6.25% TDC. An SI of 600 corresponds to complete resistance and an SI of 0 to full inhibition.

CTR-Test

Tissue processing and cell culture were performed at TherapySelect, according to a published protocol [21]. In summary, tissue was mechanically and enzymatically disrupted until spheroids could be isolated. The tumor cells were plated at a density of 10 000 cells/well, drugs were added, and the microplates were incubated for 72 h. Tritiated thymidine (^{3}H-thymidine) was added and incubation was continued for another 48 h. Cells were then harvested onto glass fiber filters and the uptake of the radioactive thymidine into DNA was measured by scintillation counting. Cultures without drug served as negative controls and cells exposed to lethal doses of cisplatin as positive controls. Results were reported as extreme drug resistance (EDR) when proliferation was 1 SD above the median population density, low drug resistance (LDR) when proliferation was 1 SD below the median, and intermediate drug resistance (IDR) when proliferation was between the SD limits. Limits for EDR, LDR, and IDR were determined by a Bayesian algorithm, unique for each agent tested [22].

The final concentrations for the single-drug assays were as cited in Table 1. For dual drug combinations, the agents were used in half the final concentration of the single-drug assays.

Table 2 Rationale for the choice of the chemotherapeutic drugs applied to the CSRAs in the present study

Name (abbreviation)	Rationale
Temozolomide (TMZ)	Present chemotherapeutic treatment standard for GB
Nimustine/ACNU + teniposide/ VM26 (ATEN)	According to randomized trial (NOA-1) equally effective as TMZ
Procarbazine + lomustine/ CCNU + vincristine (PCV)	Until recently, a frequently used combination for malignant gliomas
Vincristine (VCR)	Component of PCV, to serve as consistency control
Topotecan (TPT)	Same compound class as irinotecan but presumably improved crossing of BBB
Temozolomide + nimustine/ ACNU (TA)	New combination of the most successful compounds against GB
Treosulfan + cytarabine (TARA)	Successful for extracranial multiresistant cancers such as malignant melanoma; new combination for brain tumors

ACNU, aminomethylpyrimidinylmethylchloroethylnitrosourea; BBB, blood–brain barrier; CCNU, chloroethylcyclohexylnitrosourea; CSRA, chemosensitivity and resistance assay; GB, glioblastoma.

Table 1 Chemotherapeutic drugs tested against GB in chemoresponse assays

Name (abbreviation)	Chemical class/mechanism of action	TDC100 (μmol/l)	CTR (μmol/l)
Cytarabine (ARAC)	Nucleoside analog/DNA mismatch	82	4
Lomustine (CCNU)	Nitrosourea/direct alkylation	14	–
Nimustine (ACNU)	Nitrosourea/direct alkylation	18	–
Procarbazine (PCB)	Methylhydrazine/indirect alkylation	18	–
Temozolomide (TMZ)	Tetrazine/indirect alkylation	258[a]	760
Teniposide (VM26)	Epipodophyllotoxin/topoisomerase II inhibitor	30	–
Topotecan (TPT)	Alkaloid/topoisomerase I inhibitor	2	0.2
Treosulfan (TREO)	Dimethylsulfonate/indirect alkylation	180	18
Vincristine (VCR)	Alkaloid/microtubule inhibitor	1	0.5

TDC100 corresponds to 100% test drug concentration for the ATP-TCA [6]. CTR stands for the final test concentration in the CTR-Test.
ACNU, aminomethylpyrimidinylmethylchloroethylnitrosourea; CCNU, chloroethylcyclohexylnitrosourea; CTR, chemotherapy resistance; GBs, glioblastomas; TCA, tumor chemoresponse assay; TDC, test drug concentration.
[a]Concentration of active compound MTIC is 2% of TMZ.

Statistical analysis

Continuous data are reported as means and corresponding SDs. One-factor repeated measures analysis of variance (rmANOVA) was used to assess differences between the tested chemotherapeutic drugs in terms of relative inhibition expressed as SI. The rmANOVA model included SI as dependent variable and chemotherapeutic drug as grouping factor. To account for the correlation between the measurements, an unstructured covariance structure was fitted to the data. To explore the validity of the findings, several sensitivity analyses were performed, including successive removal of patients with GB variants in an rmANOVA model. The exclusion of samples from patients with GB variants (for each variant and in combination) does not alter the findings.

Applied tests were two-sided, and resulting *P*-values less than an α level of 0.05 were considered to indicate statistical significance. In the case of post-hoc comparisons, Bonferroni correction was applied. All statistical analyses were performed using SAS statistical software, V9.2 (SAS Institute Inc., Cary, North Carolina, USA) under Windows XP.

Results

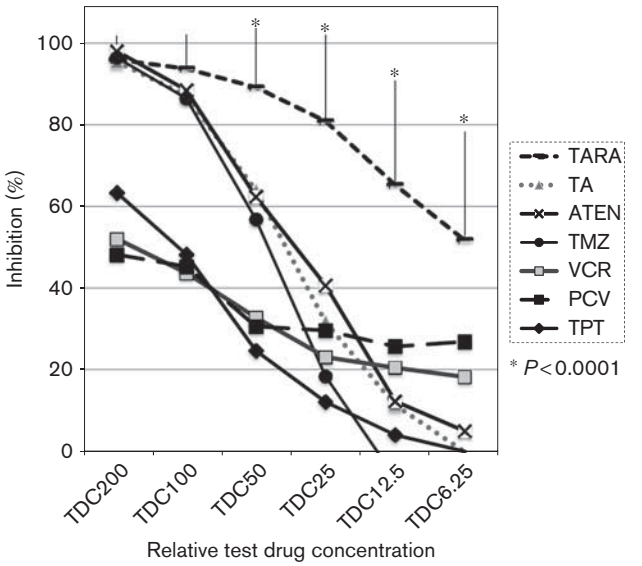
Histological grading of the 30 newly diagnosed tumor samples and three recurrences revealed 23 'classical' GBs. Four GB samples contained foci of oligodendroglial components, one met the criteria of gliosarcoma, one was a giant cell GB, and one was a secondary GB. Statistical analysis for all the results presented here was performed with or without the GB variants to ensure that they would not affect the outcome.

The rationale for choosing the various drugs and drug combinations is summarized in Table 2.

ATP-TCA

GB specimens from surgical resections were exposed to six different concentrations of the solutes in Table 2. All assays were available for evaluation.

Fig. 1



Inhibition profile of glioblastomas to various test drugs in the ATP-TCA. The total number of tumors was 30 for TARA, ATEN, VCR, and TPT; 29 for TMZ; 24 for PCV; and 23 for TA, respectively. For TARA, the positive SDs are shown as vertical bars. The asterisks indicate *P*-values less than 0.0001 of the TARA curve in relation to corresponding concentrations in all other curves. The abbreviations of the drugs are TARA, treosulfan + cytarabine (▬); TA, temozolomide + ACNU (▲); ATEN, ACNU + teniposide (X); TMZ, temozolomide (●); VCR, vincristine (□); PCV, procarbazine + CCNU + vincristine (■); TPT, topotecan (◆). ACNU, aminomethylpyrimidinylmethylchloroethylnitrosourea; CCNU, chloroethylcyclohexylnitrosourea; TCA, tumor chemoresponse assay; TDC, test drug concentration.

Table 3 Intrapatient variability of the ATP assay

	A			
Comparator	Resistant	Weakly sensitive	Intermediate sensitive	Highly sensitive
B (7500 cells)				
Resistant	**5**	1		
Weakly sensitive		**4**		
Intermediate sensitive			**3**	1
Highly sensitive				**7**
C (22 500 cells)				
Resistant	**2**	2		
Weakly sensitive			1	1
Intermediate sensitive		1	**1**	
Highly sensitive				**6**
D (first recurrence)				
Resistant	**10**	2	1	
Weakly sensitive	1	**2**		
Intermediate sensitive	1	1		
Highly sensitive		2		**1**
Identical assay results	**17**	**6**	**4**	**14**

From five tumors, parallel samples were submitted to the seven test drugs of the ATP assay listed in Table 2 to determine the degree of variation (A vs. B and C, respectively). In three cases, cells from the newly diagnosed glioblastoma and the first recurrence were submitted to the ATP assay (A vs. D).
Bold values indicate identical assay results (e.g. A resistant vs. B resistant or A highly sensitive vs. C highly sensitive, etc.).

From five tumors, two parallel samples each were submitted to the seven drug sets to study the reproducibility and robustness of the assay. Twenty-eight of the 35 resulting tests (80%) yielded identical results (Table 3). This included nine pairs where one of the assays comprised voluntarily three times the standard number of tumor cells (22 500 vs. 7500 cells/well) to check for possible sampling effects.

The mean inhibition profile of all the GBs tested in the ATP-TCA is illustrated in Fig. 1. Procarbazine–CCNU–vincristine (PCV), vincristine (VCR), and topotecan (TPT) had by far the lowest inhibitory effect on the tumors. Even at the highest dose (TDC200), growth inhibition hardly reached the 60% level. The four remaining drug sets were able to induce nearly complete inhibition at the highest dose level and still at least 80% at the reference concentration TDC100. This difference was significant ($P < 0.0001$) as compared with PCV, VCR, and TPT. Treosulfan plus cytarabine (TARA) exhibited a very favorable dose gradient with a relatively flat curve, illustrating growth inhibition already at moderate concentration levels. ACNU/teniposide (ATEN) and temozolomide alone (TMZ) or in combination with ACNU (TA) revealed sigmoid curves spanning the whole inhibition range from 100 to 0%.

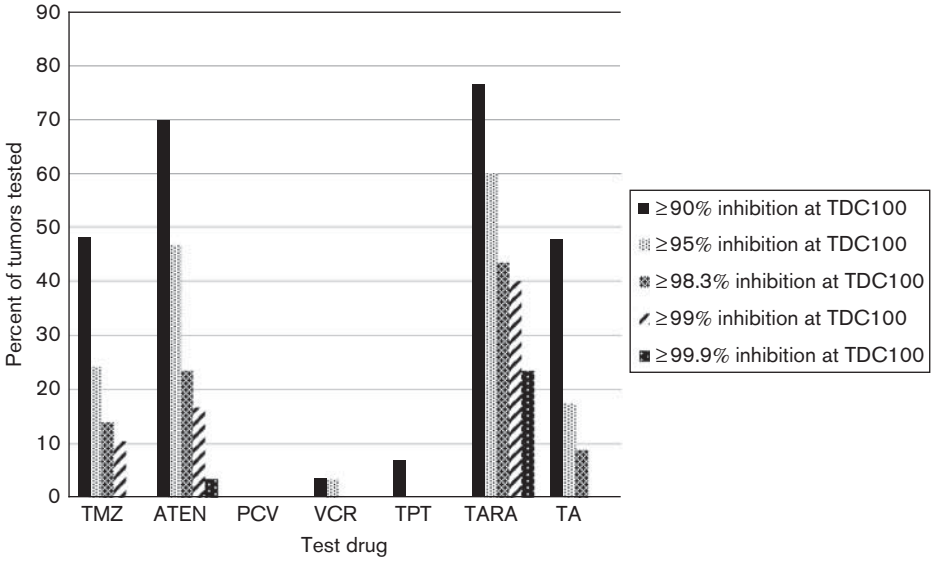
Except for ATEN and TA ($P = 0.1748$) or PCV and VCR ($P = 0.0814$), the differences in inhibition were significant (PCV and TMZ: $P = 0.0101$, TPT and VCR: $P = 0.0005$, all other drug combinations: $P < 0.0001$). When inhibition at individual concentration levels rather than inhibition as a whole was compared, ATEN was significantly more effective than TMZ at the lower TDCs (TDC25: $P < 0.0152$ and TDC12.5: $P < 0.0001$) and TARA was significantly more effective than all other agents at $\leq$ TDC50 ($P < 0.0001$).

We further determined how many tumors demonstrated at least 90% inhibition at TDC100 and four higher threshold values up to 99.9% (Fig. 2). All five levels were most often achieved with TARA (23, 18, 13, 12, and seven of 30 cases, respectively). Of the other agents, only ATEN achieved the highest level of inhibition, but only in one case. PCV was the least effective. It did not cause growth inhibition of at least 90% in any of the 24 tumors tested.

The relative inhibition was also expressed as SI, a measure for the overall growth inhibition. The mean index of all samples tested was lowest for TARA (Fig. 3). This difference was statistically significant in comparison with all other agents ($P < 0.0001$). The SI of ATEN was also significantly lower than that of TMZ ($P = 0.0004$), PCV ($P < 0.0001$), TPT ($P < 0.0001$), and VCR ($P < 0.0001$), even when the gliosarcoma and the GBs with divergent patterns of differentiation were excluded from the analysis.

In three patients, we could study the behavior of the primary tumor and the first recurrence. Of the 21 individual ATP assay samples, 13 yielded identical results (62%, cf. Table 3). In four cases, the recurrent tumor showed higher sensitivity and for three test drugs the recurrent tumor was less sensitive. It turned out that two

Fig. 2



Response profile of glioblastomas to various test drugs in ATP-TCA. Number of tumors and abbreviations of the drugs are as in Fig. 1. TDC100, 100% relative test drug concentration. TCA, tumor chemoresponse assay; TDC, test drug concentration.

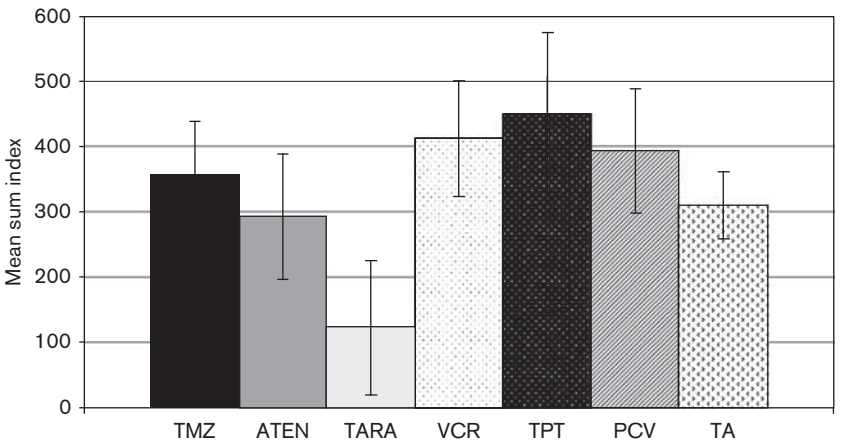
of the three cases with reduced sensitivity were for TMZ, the drug that all patients had received as first-line chemotherapy (data not shown).

CTR-Test

A total of 15 tissue samples of 11 tumors were subjected to the CTR assay in parallel to the ATP-TCA. Only seven of these were evaluable for all agents. The choice of test drugs could not be fully matched to the ATP-TCA because at the time of the study not all agents were validated for the CTR-Test. The following drugs were applied for comparison with the ATP assay: TMZ, TPT, VCR, and TARA.

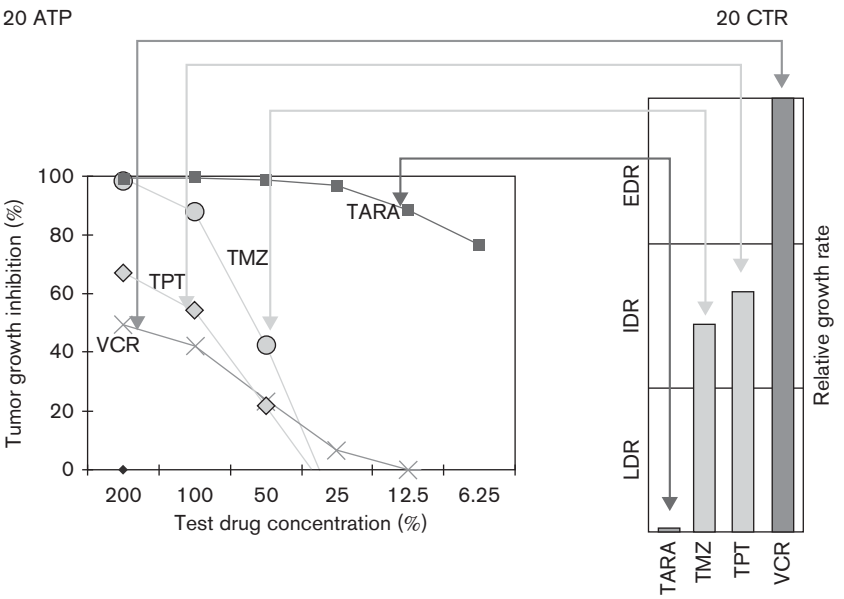
In Fig. 4, an example of a CTR-Test result is shown in comparison with the respective ATP assay. In this case,

Fig. 3



Summary of ATP-TCA results. The sum index (SI) is a measure for the overall growth inhibition; $SI = 600 - \Sigma_i Inhib(c_i)$ with $c_i = 200, 100, 50, 25, 12.5, 6.25\%$ TDC; the lower the value of SI, the higher the inhibition. Number of tumors and abbreviations of the drugs are as in Fig. 1. Mean values and SDs are provided in Table 5. TCA, tumor chemoresponse assay; TDC, test drug concentration.

Fig. 4



Comparison of ATP-TCA and CTR-Test results. Example for glioblastoma #20. Abbreviations of the drugs are as in Fig. 1. CTR, chemotherapy resistance; EDR, extreme drug resistance; IDR, intermediate drug resistance; LDR, low drug resistance; TCA, tumor chemoresponse assay.

Table 4 Comparison of ATP-TCA and CTR-Test results

Sample number	TMZ	TARA	VCR	TPT
ATP				
20	2	1	3	4
21	3	1	4	2
22	3	1	2	4
24	4	1	2	3
27	2	1	3	4
28	2	1	4	3
30	2	1	4	3
Mean rank	2.6	1.0	3.1	3.3
CTR				
20	2	1	4	3
21	3	1	4	2
22	1	2	3	4
24	3	1	4	2
27	2	1	4	3
28	2	1	3	4
30	2	1	4	3
Mean rank	2.3	1.1	3.6	3.0

Rank 1 was assigned to the lowest sum index for ATP-TCA and to the lowest resistance level in the case of the CTR assay, respectively. Ranks 2, 3, and 4 were assigned accordingly to the next higher indices.
CTR, chemotherapy resistance; TARA, treosulfan + cytarabine; TCA, tumor chemoresponse assay; TMZ, temozolomide; TPT, topotecan; VCR, vincristine.

the tumor displayed EDR for VCR, IDR for TMZ and TPT, and LDR for TARA.

Six of the seven GBs evaluable demonstrated least resistance to TARA (Table 4) as compared with TMZ, VCR, and TPT. Only two of the tumors were classified as IDR whereas the other five fell into the LDR category.

TMZ ranked second in the CTR assay with three tumors in the LDR and four in the IDR group. The least effective agent was VCR, with four of seven tumors expressing EDR.

Discussion

The response of GB cells to various chemotherapeutic drugs was studied by established in-vitro chemoresponse assays. We used the ATP-TCA and in several cases the CTR-Test in comparison. In retrospective analyses, the ATP-TCA demonstrated a predictive accuracy of 70–90% for drug sensitivity and 90–100% for prediction of clinical resistance [4,23]. The CTR-Test is optimized for negative prediction or as drug exclusion test. Drugs that showed extreme resistance in this assay were found to be inactive *in vivo* in at least 95% of the cases [21].

Cell-based versus molecular CSRA

Rather than applying a genetic or protein marker assay to determine chemoresponse of GB, we opted for two cell-based assays because the current understanding of the cellular chemoresistance mechanisms is still insufficient to model all aspects involved. Single genes most often fall short [24–26]. However, gene signatures or profiles might also be of limited value for a heterogeneous tumor [27]. Therefore, it seems acceptable to adhere to the cell-based approach considering the cell as a 'black box' where inhibition of growth or cell death is the result of all active mechanisms of resistance. This pragmatic strategy does not require knowledge of the exact mechanisms of action. It is sufficient to know which concentration levels are reached *in vivo* and whether the drug of interest is able to reach the target cells, for example, by crossing the blood–brain barrier (BBB). This latter aspect is also a relevant precondition for molecular assays, as is the fact that either approach provides only a momentary snapshot of the chemoresponse.

ATP-TCA

In accordance with findings by Andreotti *et al.* [4], reproducibility of the ATP assay was $\sim \pm 20\%$. Variations greater than $\pm 20\%$ occurred only at TDC less than or equal to 25% or between different patients (Fig. 1). When the ranking rather than the numerical values was compared, 90% of the parallel samples yielded identical chemoresponse levels. Even in the case of the 14 samples having three times the cell number of the standard assay (22 500 vs. 7500 cells/well), nine yielded identical results (Table 3). Four assays demonstrated the next lowest sensitivity level as compared with the standard set-up, which would be expected if one assumes an increasing number of resistant cells with increasing tumor cell load. In only one case (7%), the assay with the three-fold cell number showed slightly higher sensitivity than the standard assay, which could be due to sample inhomogeneity. Overall, the ATP assay demonstrated robust and consistent results after repeated sampling of GB tumor tissue and within a wide range of tumor cells submitted.

GBs showed major differences in the response to the solutes tested. Of the drugs that have already been investigated for GB in phase II/III trials (TMZ, ATEN, PCV, VCR, and TPT), ATEN showed the best inhibition profile, followed by TMZ. VCR, TPT, and PCV followed on a much lower level (Fig. 1). TARA yielded the best profile of all drug sets, reaching close to 60% inhibition even at the lowest drug concentration. The outcome of the new combination TA lay between those of ATEN and TMZ.

A similar ranking of the drugs was obtained when the degree of inhibition at TDC100 was compared (Fig. 2). We reasoned that the growth reduction at the end of a full set of six cycles of chemotherapy would have to be at least 50% to be clinically notable. If one considers the in-vitro assay as a single cycle of chemotherapy, one would need at least a reduction in cell survival of 90% in the assay to achieve such an effect ($0.9^6 = 53\%$). Further, we determined which percentage of tumor samples reached 95, 98.3, 99, and 99.9% growth reduction theoretically corresponding to inhibition effects after six cycles of 74, 90, 94, and 99%, respectively.

Among the clinically established drugs, ATEN showed the highest cytotoxic effect under our test conditions, with 70% of the tumors inhibited in cell growth by

Table 5 Association between ATP-TCA and the expression of the repair gene *MGMT*

	TMZ	ATEN	TARA	VCR	TPT	PCV	TA
Mean sum index	357.3	293.0	123.4	413.2	450.4	394.1	310.9
SD (overall)	94.2	93.4	91.9	101.6	116.9	103.2	55.2
Mean sum index	349.9	271.4	115.5	400.1	460.9	392.5	307.8
SD (m*MGMT*)	68.1	68.4	94.3	70.9	128.2	77.2	55.0
Mean sum index	375.0	317.5	137.8	412.9	450.1	395.4	313.2
SD (*MGMT*)	114.8	116.2	93.5	128.3	111.2	124.2	57.5
Mean growth inhibition at TDC100 (%)	86.5	88.5	93.8	43.7	48.2	45.2	87.1
SD (overall)	10.4	15.9	8.4	20.9	22.2	17.0	9.2
Mean growth inhibition at TDC100 (%)	88.3	91.0	95.7	46.6	50.1	45.1	88.7
SD (m*MGMT*)	8.4	13.8	6.7	13.7	23.8	14.1	8.7
Mean growth inhibition at TDC100 (%)	84.9	85.0	91.5	44.4	44.5	45.4	86.0
SD (*MGMT*)	11.7	18.7	10.0	26.1	21.0	19.7	9.8
# m*MGMT*	13	14	14	14	14	11	10
# *MGMT*	14	14	14	14	14	13	13

Mean sum index, mean growth inhibition at reference concentration (TDC100), and the respective SD are shown for all tumors and for those with methylated (m*MGMT*) or unmethylated (*MGMT*) repair gene, respectively.
ATEN, ACNU + teniposide; PCV, procarbazine + CCNU + vincristine; TA, temozolomide + ACNU; TARA, treosulfan + cytarabine; TCA, tumor chemoresponse assay; TDC, test drug concentration; TMZ, temozolomide; TPT, topotecan; VCR, vincristine.

at least 90% at TDC100. Three percent of the samples were inhibited by at least 99.9% at the same concentration.

Forty-eight percent of the GB samples tested with TMZ (14/29) responded with inhibition of survival by at least 90%. Growth inhibition at the highest level of 99.9% could not be achieved with TMZ at TDC100 for any of the tumor specimens examined.

All the tumor samples tested against PCV were also exposed to VCR to determine whether the cytotoxic effect of PCV was mainly due to the lipophilic alkylating agents PCB and CCNU or to the high-molecular microtubule inhibitor VCR, which hardly crosses the BBB [28,29]. It turned out that the survival curves after exposure to PCV were indeed very similar to those after VCR alone (Fig. 1).

Comparison of ATP-TCA and CTR-Test

One goal of the study was to determine whether the two CSRAs provided similar results that would lead to similar clinical recommendations. Therefore, we performed the ATP-TCA and the CTR-Test in parallel for 15 samples of 11 tumors in the two laboratories with the operators blinded to the results of the respective other. As some of the chemotherapeutic drugs that we had used in the ATP-TCA had not yet been validated for the CTR-Test, we could only compare the four solutes TMZ, VCR, TPT, and TARA (c.f. Fig. 4).

With only seven of the 15 samples evaluable, the CTR assay was considerably more error-prone for GB samples than the ATP assay, all samples of which were evaluable. Due to the different methodological evaluations and the limited number of parallel assays, we restricted data analysis to a qualitative ranking of the substances (Table 4).

In both assays, TARA showed by far the highest cytotoxic effect or the lowest resistance level. TMZ ranked second, and TPT and VCR were about equal on the third rank. Despite the fact that the CTR assay is primarily optimized to indicate drug resistance, whereas the ATP assay claims to predict chemosensitivity, the two assay platforms showed satisfying agreement.

Comparison between ATP-TCA and molecular markers

We compared the results of the ATP assay with the gene activity of the repair enzyme MGMT. Transcription of the *MGMT* gene is controlled by hypermethylation of the promoter region. The gene is silenced when methylated. Ignoring other mechanisms of resistance, methylation should lead to higher sensitivity to alkylating drugs because the enzyme is not available to repair resulting DNA adducts [30,31]. Though limited, our in-vitro results are in agreement with this hypothesis. There was a clear trend for a lower SI and hence less resistance when the *MGMT* gene was methylated. Only for TPT, which is a topoisomerase I inhibitor, was this the other way round. With the exception of PCV, the mean growth inhibition at reference drug concentration TDC100 also showed a tendency toward higher inhibition for tumors with methylated *MGMT* by about three to five percentage points (Table 5).

Comparison between ATP-TCA and clinical data

Our in-vitro results correspond well to published clinical data. Recently, a randomized trial using ACNU plus teniposide (NOA-1) was directly compared with EORTC trial 26981, which applied TMZ [32,33] and a trend for better results after treatment with ACNU plus teniposide was observed. Our ATP assay results confirm this advantage of ATEN in comparison with TMZ, as is illustrated by the higher frequency of tumors inhibited by at least 90% at reference concentration TDC100 (Fig. 2) and the lower SI of ATEN (Fig. 3).

Our unfavorable in-vitro results for PCV are also in accordance with the findings of two randomized phase III trials for GB patients. Neither revealed a benefit of PCV

Table 6 Association between tumor characteristics, in-vitro result, and clinical outcome for 23 study patients

Variables	PFS (days)	OS (days)
All (23)	234	475
TMZ = 0 (6)	174	436
TMZ = 1 (14)	223	478
TMZ = 2 (2)	531	630
GTR (8)	313	551
SPR (12)	169	406
TMZ = 0 + GTR (2)	191	828
TMZ = 0 + SPR (4)	166	241
TMZ = 1 + GTR (4)	266	372
TMZ = 1 + SPR (10)	204	521
TMZ = 2 + GTR (2)	531	630
TMZ = 2 + SPR (0)	No data	No data

Mean progression-free survival (PFS) and overall survival (OS) are given in days. The number of patients is indicated in parentheses.
TMZ stands for the in-vitro result of the ATP-TCA for temozolomide, the drug that all patients received as first-line chemotherapy: 0 = resistant, 1 = weak sensitivity, 2 = intermediate sensitivity. GTR corresponds to gross-total resection showing no contrast enhancement in postoperative MRI, SPR, subtotal or partial resection. No subgroup reached significance level $P < 0.05$.

in progression-free survival (PFS) or overall survival in comparison with radiotherapy [34] or BCNU [35], respectively.

Glioma cells have been reported to express high levels of topoisomerase I [25]. Therefore, we were surprised by the low cytotoxicity of TPT to the tumors investigated in this study. However, in a phase II trial, Fisher *et al.* [36] did not observe any benefit for GB patients treated with TPT as compared with patients who had been irradiated only.

In our own small patient cohort, PFS and overall survival were longer when the ATP-TCA demonstrated higher sensitivity. This trend remained for PFS even when the degree of resection was considered. These results, however, did not reach statistical significance (Table 6).

Another interesting association between the chemoresponse *in vitro* and the clinical behavior observed deserves more attention. All patients received TMZ treatment independent of and blinded to the ATP assay result. However, when we compared the in-vitro result for TMZ to the number of treatment cycles that were administered, it seemed that patients whose tumors were resistant to TMZ *in vitro* underwent fewer cycles of TMZ than those with better in-vitro response. Only two of the six patients in the 'resistant' group (TMZ = 0) and seven of the 14 patients in the 'weak sensitivity' group (TMZ = 1) received more than half of the scheduled six cycles. The two patients in the intermediate sensitivity level (TMZ = 2), however, were exposed to five and six cycles of TMZ, respectively. A potential interpretation of this observation could be that patients whose tumor shows resistance to TMZ *in vitro* tolerate less of this drug or discontinue it early due to recurrence.

The disappointing clinical results of treosulfan – either administered alone [37]) or in combination with the nucleoside analog gemcitabine [38] – seem to be in clear contrast to our in-vitro results, where TARA yielded by far the best responses (cf. Figs 1–3).

However, there are several potential reasons why this combination, which has successfully been used previously for various extracranial tumors [39–41], was not successful in GB patients despite its positive response *in vitro*.

The most important question is whether the drugs are able to cross the BBB and reach the tumor. For gemcitabine, this is still being debated [42,43]. Appropriate and convincing pharmacokinetic data are currently missing. The related cytarabine reaches concentrations of ~5–10% of the plasma level in the cerebrospinal fluid after intravenous administration [44].

In a review by Kortmann *et al.* [45], treosulfan was listed as a chemotherapeutic drug with 'good' ability to cross the BBB. However, quantitative pharmacokinetic data relating to the central nervous system are lacking.

On the basis of its physicochemical characteristics, the diffusion of treosulfan through the brain capillaries into the brain tissue should be slow. Correspondingly, log BB, the ratio of a compound's concentration in brain tissue versus blood [46], is low ($\log BB = -2.5$). However, regarding the reactive metabolite diepoxybutane, the calculated partition coefficient is more favorable ($\log BB = -0.3$). As there are many examples where the calculated partition coefficient log BB does not correspond to the observed parameter, we submitted treosulfan to a BBB model consisting of porcine brain capillary endothelial cells to obtain additional information on the ability of treosulfan to enter the brain. It turned out that the amount crossing the BBB was more than one order of magnitude lower than for TMZ (these data will be published separately). In light of these observations, the disappointing clinical results with treosulfan are no longer surprising. However, the fact that GB cells respond rather strongly to treosulfan and the nucleoside analogs *in vitro* might argue for an attempt to improve the accessibility to the brain and tumor tissue, for example, by convection-enhanced delivery [47] or nanoparticulate transport methods [48,49]. Considering the poor prognosis of GB patients and the limited success of the present treatment standards, this could be a worthwhile venture.

Conclusion

Our results show reasonable agreement between the two cell-based CSRAs ATP-TCA and CTR-Test. Furthermore, they seem to confirm the clinical effectiveness of chemotherapeutic drugs used in GB therapy as long as pharmacological preconditions such as penetration of the BBB are properly considered. Under these conditions, CSRAs have the potential to support the development of new treatment regimens and deserve to be further evaluated in larger prospective clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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