Acta Neurochir (Wien) (2001) 143: 563–573

Acta Neurochirurgica

Printed in Austria

Tamoxifen Sensitivity-Testing of Glioblastomas: Comparison of in Vitro and in Vivo Results

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Summary

Background. Only less than half of the patients with malignant gliomas respond to a continuous high dose Tamoxifen (TAM) and/or Carboplatin (CP)-treatment. Therefore, a method for predicting the efficacy of TAM-treatment would be desirable.

Methods. Paralleling a clinical study, the predictive value of in vitro-sensitivity testing of TAM and TAM's metabolite 4-OH-TAM in primary cultures of tumour explants from 15 of a total of 50 patients was examined. Additionally, the influence of TAM, 4-OH-TAM, and CP on the proliferation of established glioblastoma cell lines and of those explanted from athymic nude mice and re-established in cell culture was investigated. Human glioblastomas xenotransplanted subcutaneously into athymic nude mice and subsequently treated with TAM and/or CP were examined in a parallel in vivo-study.

Findings. TAM-chemosensitivity-testing of glioblastomas failed to predict the clinical response to TAM-treatment in our patients and did not correlate with the in vivo-TAM-response of tumours xenotransplanted into nude mice. TAM's and 4-OH-TAM's ability to inhibit growth of various glioblastoma cell lines in vitro in very similar concentrations was shown to be a consistent phenomenon which seems to be independent of the in vivo response in either patients or mice as previous hosts. However, CP's antiproliferative effect on glioblastomas in vivo was paralleled by respective in vitro results. Whereas TAM showed to mediate its in vitro antiproliferative effect by inducing apoptosis in most cell lines examined, CP-treatment lead to necrosis of cells.

Interpretation. Combining the results obtained from our human and mouse studies, it has to be postulated that host factors other than the sensitivity to TAM of the individual cell, determine the efficacy of TAM-treatment in vivo.

Keywords: Glioma; tamoxifen; carboplatin; nude mice.

Introduction

Considering the available knowledge about the treatment of glioblastomas with tamoxifen (TAM) a response rate of approximately 30% may be expected [4, 6, 7, 8, 10, 11, 25, 26, 31, 36]. Carboplatin (CP)-therapy has shown to be successful in 40%–50% of glioblastoma patients [12, 24, 27, 38]. Both agents are burdened with considerable side effects, so a method for predicting the response is desirable in order to save non-responding patients from unnecessary risk.

In the past, in vitro sensitivity testing of tumour material obtained during surgery has become an accepted method for predicting the efficacy of the different therapeutic options in patients with various kinds of malignancies [2, 17, 20, 21, 22, 32]. As demonstrated in a cohort of 2300 patients with different malignomas, an in vitro predictive assay performed according to the cloning system (colony forming assay) [32] gives true positive results in 69% of patients and true negative results in 91%. This is equivalent with a test-sensitivity of 79% and a test-specificity of 86% [19]. However, the in vitro data obtained by different test methods used, show a variability which might explain inconclusive results by the subsequent clinical use of previously tested agents. As demonstrated by Wolff *et al.*, who retrospectively analysed the results of 1643 articles dealing with in vitro sensitivity testing, these discrepancies are caused by the various cell-culture methods used rather by any unreliability of in vitro experiments *per se* [42]. In addition they stated that the unsatisfactory and unreliable results (standard deviation of experiments exceed the average values) gained by the in vitro sensitivity testing of a few agents (bleomycin, cytosine arabinoside, methotrexate, vinblastine) seem to be due to further experimental variables specific for these agents and presently unknown.

Correlative studies of chemosensitivity-testing (methods used: colony forming assay, MTT-assay) and subsequent chemotherapeutic treatment in patients with malignant gliomas were performed for the

drugs ACNU, BCNU, CCNU, cisplatin, procarbazine, and vincristine [5, 21, 22, 33, 34, 42, 43]. In most of these studies, patients whose tumours shown to be sensitive to the respective drugs tested in vitro had significantly longer relapse free intervals and lived significantly longer after treatment with these drugs compared to patients whose tumours shown to be not sensitive. Due to too low numbers of patients no exact values for test-sensitivity and test-specificity for these correlative studies in glioma patients were given.

Accompanying a phase-ll-study investigating postoperative treatment with continuous TAM, CP, and radiation therapy in 50 glioblastoma patients [31], we undertook an in vitro testing of a cohort of 15 patients to test whether treatment success could be predicted in the cultured tumour material obtained during surgery. In order to interpret the latter results we compared the effects of TAM-treatment on the proliferation of glioblastoma cells established in athymic nude mice (in vivo model) with those growing in cell culture (in vitro model).

Methods and Patients

In Vitro Materials

From 15 patients belonging to our study group of 50 patients total [31] tumour material could be obtained for in vitro TAM-sensitivity testing. 10 of these patients underwent their first operation for glioblastoma and in 5 of them a tumour was removed, which had recurred in spite of the combined treatment with TAM, CP and radiotherapy. The histological diagnosis of glioblastoma multiforme was confirmed for all tumours. In addition, 11 established glioma cell lines were selected for studies: NCE G 28, NCE G 44, NCE G 55, NCE G 59, NCE G 112, NCE G 120, NCE G 122, NCE G 124, NCE G 130, NCE G 168 [41], and U 251, originally derived from glioblastomas. Also, low passage glioma cell lines of tumour-explants from nude mice (/nm) harbouring established xenotransplant lines NCE G 55, NCE G 122, and NCE G 124 were investigated.

In Vivo Materials

In order to establish human glioblastomas on nude mice, cells of the three glioma cell lines NCE G 55, NCE G 122, and NCE G 124, were injected subcutaneously into the flank of female nude mice (outbred strain nunuBalbC, age 4–5 weeks, initial body weight 20–25 g) which were housed according to routine procedures as described previously [29]. After the tumours had grown to volumes of 3–4 cm^3, animals were killed, the tumours explanted and sliced into 1–2 mm^3 pieces which were finally re-implanted on new mice. The different tumours were passaged on nude mice as follows: NCE G 55: 87×, NCE G 122: 60×, and NCE G 124: 72×.

Cell Culture, Tumour Cell Growth Assays

Cells were grown as monolayer cultures in Earle minimal essential medium (MEM) with 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM natriumpyruvate, 2.5 μg/mL amphotericin (Fungizone®), 40 μg/mL gentamicin, and passaged depending on growth characteristics by using 0.25% trypsin [41].

The influence of TAM, the TAM-metabolite 4-OH-Tamoxifen (both obtained from Sigma, Deisenhofen, Germany), and Carboplatin (Bristol, Munich, Germany) on cell proliferation was examined by performing 7–8 days growth assays. 2,500 cells of the established cell lines and of the tumours obtained from the nude mice were seeded in Earle MEM into flat bottom 96 well plates (Greiner, Frickenhausen, Germany) and incubated at 37 °C in 5% CO_2 humidified air. For the experiments with primary cultures of study patients' tumours as a modification, 48 well plates (CorningCostar, Bodenheim, Germany) and concentrations of 10,000 cells per well were used to take into account that primary cells like to be grown at higher densities. 24 hours after seeding the cells, drugs were added. TAM was dissolved in dimethyl sulfoxide, DMSO, (Sigma, Deisenhofen, Germany) and 4-OH-TAM in ethanol. CP (only used for the experiments with the tumours obtained from the nude mice) was diluted in Earle MEM. Concentrations and length of treatment (2 days or continuously for 8 days) were used as indicated in the figures. Starting 24–48 hours after seeding, cells were fixed in 1% glutaraldehyde and stained with crystal violet at daily (96 well plates) or 2–3 days intervals (48 well plates). The colour from stained nuclei was solubilized in 10% sodium dodecylsulfate (SDS) and cell growth was quantified by reading the absorbance at 540 nm (Bio-Tek Instruments, Inc., Winooski, VT, USA). All measurements represent the mean of duplicate (48 well plates) or triplicate (96 well plates) determinations.

In Vivo Experiments with Nude Mice

For in vivo experiments on nude mice, the tumour bearing animals were distributed randomly to 4 groups undergoing different treatment modalities: (1) treatment with CP only, (2) treatment with TAM only, (3) combined treatment with CP and TAM, and (4) no treatment (negative control). Each treatment group consisted of 8 animals. (1) CP (50 mg/kg body weight) was given to the nude mice by intraperitoneal injections 7, 10, and 13 days after tumour implantation [14]. (2) TAM-treatment was started 7 days after tumour implantation by using long-acting TAM-depot tablets (Innovative Research, Toledo, OH, USA) which were implanted subcutaneously between the shoulders of the animals [16]. These tablets contain 5 mg TAM which is released over a period of 60 days (80–85 μg/d). This treatment is equivalent to a daily TAM-dose of 2.8 mg/kg body weight. (3) Combined treatment of CP and TAM was performed by giving both drugs in concentrations and time intervals as indicated above for the respective monotherapies. (4) Untreated nude mice harbouring transplanted gliomas served as negative controls. The influence of treatment on tumour growth was determined by measuring the diameter and subsequently calculating the volume of the transplanted tumours at regular time intervals [1]. 35–46 days after transplantation the animals were sacrificed and the tumours explanted for final evaluation.

Histopathology of Xenotransplanted Tumours

The excised tumour tissues was either fixed in 4% formalin and processed for paraffin embedding or fixed in Tissue-Tek (Sakura, Zoeterwoude, Netherlands), subsequently cooled down in 2-butylisopropanol (Fluka, Neu-Ulm, Germany) and finally frozen in liquid nitrogen. Paraffin embedded tumours were sectioned according to a routine procedure and slices were stained with hematoxylin-eosin, Gomori, Masson-Goldner, and van Gieson (elastica). By using a cryostat, frozen tumour blocks were sectioned to slices measuring 4–6 μm and processed for immunohistochemistry which

was performed according either to the peroxidase/antiperoxidase (PAP)-staining method or to the immunofluorescence-staining method, both indicated by the manufacturer (Dakopatts, Hamburg, Germany). Antibodies against the proliferation-associated antigen Ki 67 (Mib 1) (Dianova, Hamburg, Germany), and the von Willebrand factor (factor VIII) (Boeringer, Mannheim, Germany) were used at dilutions of 1:20, and 1:400 respectively. The extent of neovascularization was detected by examining the anti-factor VIII-immunostained slices according to the method of Weidner [39].

Assay to Detect Apoptosis and Necrosis as Mechanisms of Cell Death

To distinguish between apoptosis and necrosis as two possible mechanisms leading to cell death the photometric enzyme-immunoassay Cell Death Detection ELISA^{Plus} (Boehringer, Mannheim, Germany) was used. It determines cytoplasmic histone-associated DNA-fragments quantitatively [3]. In this assay the intracellular enrichment of mono- and oligonucleosomes which occur after induction of endogenous endonucleases is due to the fact that in apoptosis DNA-degradation occurs several hours before plasma membrane breakdown. In contrast, necrotic cell death results in early release of fragmented DNA into the culture supernatant. 20,000 cells each of the 5 different glioblastoma cell lines NCE G 28, NCE G 44, NCE G 112, NCE G 120 and NCE G 130 were seeded into 96 well plates and cells were allowed to adhere for 24 hours. Cultures were rinsed and treatment was started by adding TAM or CP in concentrations indicated in the figures. After incubation for 4 hours cultures were centrifuged at 200× g and culture supernatants were collected. Cells were lysed and 20 µl of lysate or 20 µl of corresponding supernatant were used in Cell Death Detection ELISA^{Plus} according to the manufacture's instructions. A specific enrichment factor of mono- and oligonucleosomes released into the cytoplasm is calculated by absorbance of treated sample divided by absorbance of the corresponding untreated control. Data represent the mean of duplicate determination.

Statistical Analysis

IC50 values were determined by linear regression analysis. In the in vivo and in vitro experiments, the results of the different treatment groups were compared by using student's t-test. To correlate the results of in vitro testing in low passage glioma cell lines with the response to therapy in the respective patients (time interval until tumour-recurrence, survival time, response according to the criteria of response used in our study [31]), Pearson Product Moment correlation was used in the case of numeric values and Spearman Rank Order correlation in the case of ranked or ordered values. In order to examine clinical discrepancies between the subset of 15 patients used for the present in vitro study and the entire study group of 50 patients [31], the clinical parameters known to influence the prognosis of glioblastoma-patients (age, extent of tumour resection, postoperative Karnofsky Performance Score (KPS) 4 weeks after inclusion to study) were correlated to the "time to progression", "survival time" and "response to therapy" by using the same test methods.

Results

In Vitro Testing of TAM-Sensitivity in Primary Cultures of Study Patients' Tumours

Tumour material of 15 patients belonging to the study group of our phase II study investigating a postoperative treatment with TAM, CP and radiotherapy for glioblastoma, was tested for TAM-sensitivity in vitro. As demonstrated in Table 1, no relation between the results of the in vitro testing and the clinical response to treatment or the prognosis of the patients

Table 1. *In Vitro Testing of TAM- and 4-OH-TAM-Sensitivity in 15 Glioblastoma-Patients Included in our Phase II-Study (Postoperative Treatment with TAM, CP and Radiotherapy). Primary Cultures of Tumours Originated from Tissue Obtained at the Initial Operation for Glioblastoma in 10 Patients and from Tissue of Recurrent Tumours in 5 Patients*

Pat no.	Age	f/m	Type of tumour	IC 50 TAM (µM)	IC 50 4-OH-TAM (µM)	Re-sponse to therapy	Time to progression (weeks)	Survival (weeks)
02	26	f	1st recurrence	8,0	n. d.	yes	66	113
03	35	f	1st recurrence	8,1	n. d.	yes	94	150
13	58	m	1st recurrence	11,8	n. d.	no	30	70
13	58	m	2nd recurrence	8,3	n. d.	no	30	70
14	35	m	1st recurrence	9,9	n. d.	no	31	55
14	35	m	2nd recurrence	6	6,9	no	31	55
15	46	f	original tumour	11,5	n. d.	no	19	42
17	61	f	original tumour	>20*	n. d.	yes	120	122
18	55	m	1st recurrence	7,8	>12,5	no	23	47
19	57	m	original tumour	10,1	n. d.	no	10	25
21	55	m	original tumour	11,4	n. d.	no	28	84
23	58	m	original tumour	6,3	n. d.	no	21	41
25	57	f	original tumour	8,7	n. d.	no	4	14
26	56	m	original tumour	8,2	8,1	no	9	92
30	58	m	original tumour	10.6	>12,5	no	34	91
31	60	m	original tumour	9,8	>12,5	yes	59	119
36	55	f	original tumour	10,3	n. d.	no	38	78

* At determination of this value, a methodological mistake could not be excluded definitely. Therefore, this value was omitted from statistical analysis. *n. d.* Not determined.

Table 2. *IC 50-Values of TAM and Factors Influencing Prognosis of Glioblastoma Patients in 15 Study Patients Whose Tumours were Examined by Using in Vitro Sensitivity Testing*

	Time to progression	Survival time	Response to therapy
IC-50-values of primary tumours	r = 0.293 p = 0.444		r = 0.261 p = 0.446
IC-50-values of recurrent tumours		r = −0.372 p = 0.538	r = −0.289 p = 0.683
Age	r = −0.238 p = 0.394	r = −0.272 p = 0.326	r = 0,018 p = 0.944
Extent of tumour resection	r = 0.520 *p = 0.045*	r = 0.627 *p = 0.012*	r = 0.241 p = 0.374
Karnofsky Performance Score	r = 0.318 p = 0.248	r = 0.613 *p = 0.015*	r = 0.018 p = 0.944

r Correlation coefficient.

could be observed. Even tumours, which had recurred in spite of continuous high-dose TAM-treatment, did not show a decreased sensitivity to TAM. These results were confirmed statistically by using Pearson Product Moment correlation and Spearman Rank Order correlation (Table 2). As in the entire study group of 50 patients, the prognosis of this subset of 15 patients whose tumours were examined in vitro, was shown to be influenced by the extent of tumour resection and by the postoperative KPS, but not by the age of the patients [31].

With one exception, TAM-concentrations necessary to achieve a 50% growth inhibition (IC50) in the primary cultures of the investigated tumours ranged between 6 μM and 11.8 μM. This reflects a physiologically therapeutic range, which has been shown to be reached in TAM-treatment of breast cancer patients and glioblastoma patients [13, 23].

Characteristics of TAM's Anti-Proliferative Effect on Established Glioma Cell Lines

Tumour cell growth assays in 6 established glioma cell lines NCE G 55, NCE G 59, NCE G 122, NCE G 124, NCE G 168, and U 251 demonstrate the ability of TAM and its metabolite 4-OH-TAM to inhibit proliferation in a dose dependent manner (Fig. 1). The concentrations of TAM, leading to a 50% inhibition of growth (IC50) in the different cell lines varied in a

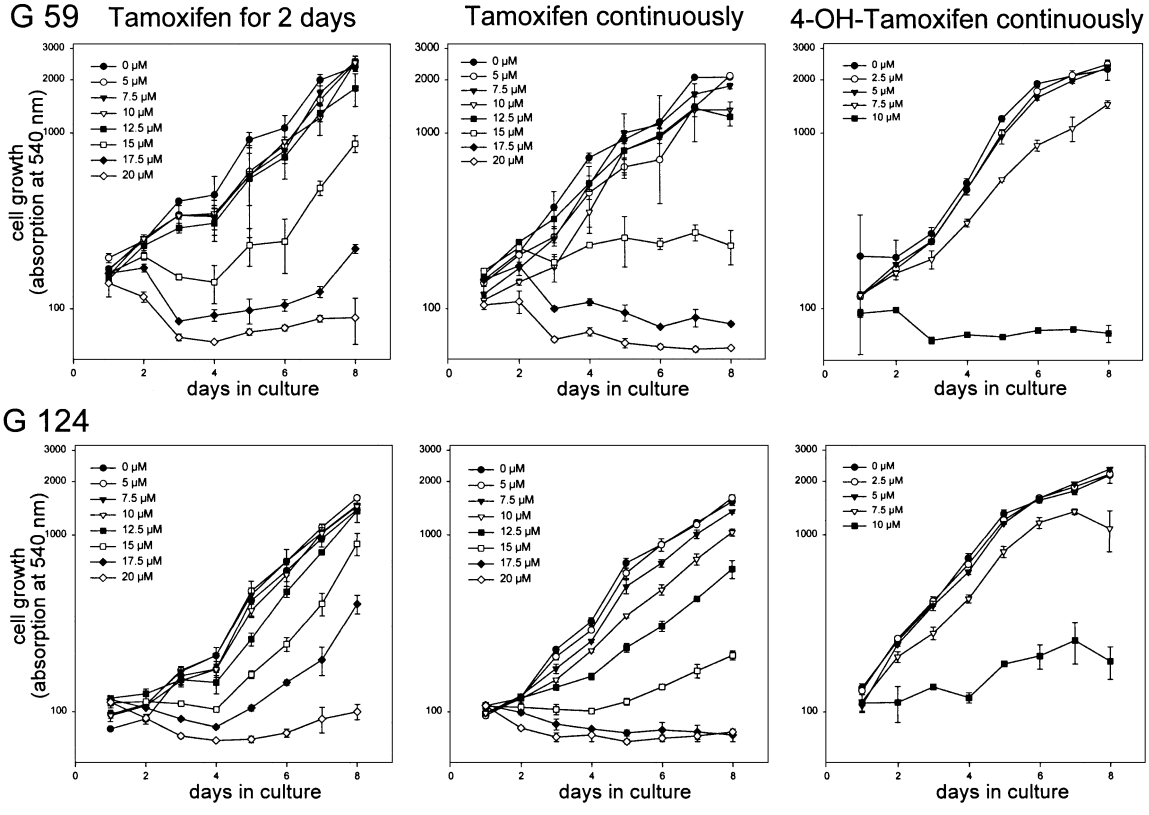

Fig. 1. Tumour cell growth assays of established glioma cell lines show a dose-dependent inhibition of proliferation by TAM in two different treatment modes and by 4-OH-TAM. Data represent the mean of triplicate determinations, error bars = S.D

Table 3. *IC 50-Values of TAM in two Different Treatment Modes and of 4-OH-TAM in 6 Established Glioma Cell Lines*

Cell line	IC 50, Tamoxifen for 2 days	IC 50, Tamoxifen continuously	IC 50, 4-OH-Tamoxifen continuously
G 55	11.6 μM	9.8 μM	6.2 μM
G 59	13.1 μM	11.2 μM	7.1 μM
G 122	12.4 μM	10.2 μM	6.5 μM
G 124	14.2 μM	10.6 μM	6.9 μM
G 168	11.3 μM	9.7 μM	5.9 μM
U 251	12.5 μM	11.2 μM	7.1 μM

narrow range between 9.7 μM and 11.2 μM if cells were treated with the drug during the whole experiment and between 11.3 μM and 14.2 μM if the drug was removed after the first 2 days of the experiment (Table 3). This latter mode of treatment allowed the cells to recover from TAM-concentrations, which they otherwise did not survive on the continuous treatment mode (NCE G 59 and NCE G 124: 17,5 μM, NCE G 55, NCE G 122, NCE G 168, and U 251: 15 μM). As shown in the experiments with 4-OH-TAM, this TAM-metabolite has a 30%–40% higher antiproliferative potential than TAM itself.

To test whether TAM and CP trigger apoptotic cell death an ELISA^{TM} was used. Following TAM treatment a dose dependent increase of DNA fragmentation indicating the cell death mechanism of apoptosis is found in 3 of 5 cell lines investigated (NCE G 44, NCE G 112, NCE G 120). In the cell line NCE G 130 TAM-treatment resulted in a dose-dependent release of fragmented DNA into the supernatant indicating necrosis as the main mechanism of cell death in these cells. In cell line NCE G 28, cell death after TAM-treatment followed the mechanisms of apoptosis and necrosis to an even extent (Fig. 2). In contrast to TAM, CP-treatment of the same 5 cell lines only resulted in necrosis.

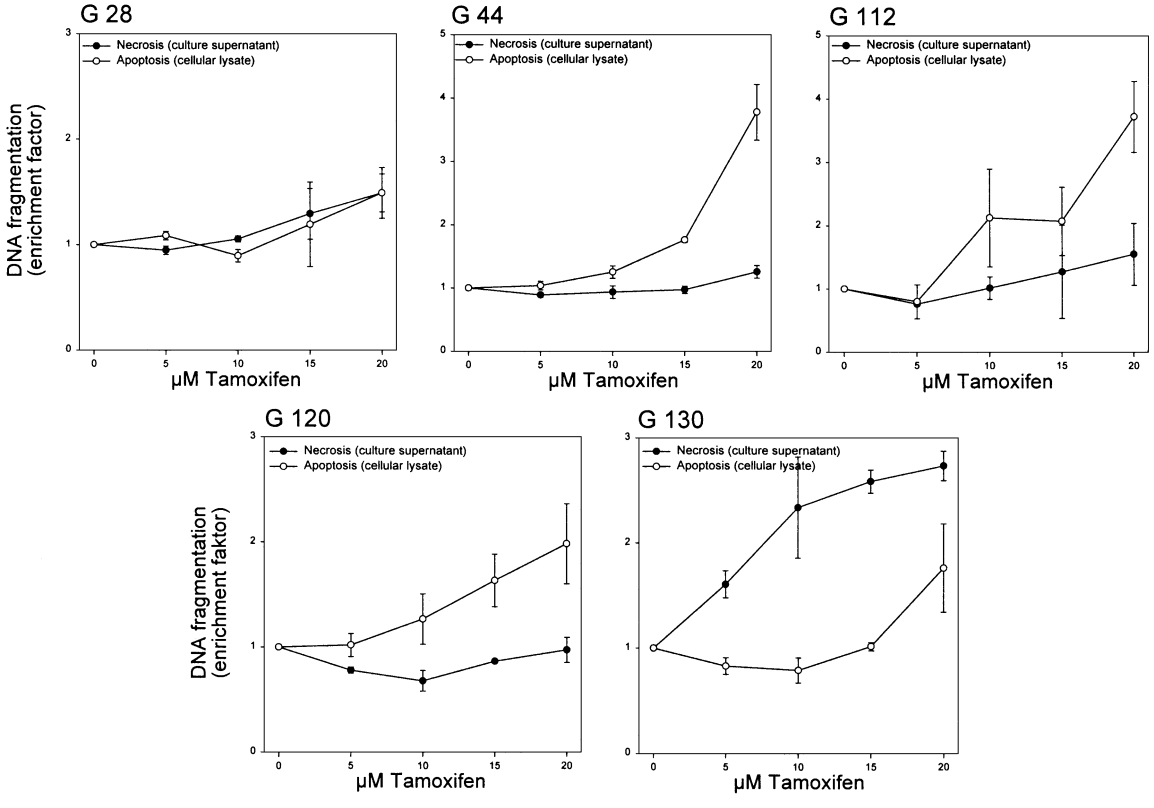

Fig. 2. Determination of the mechanism of cell death occurring after treatment with TAM in increasing concentrations. In three of the five cell lines examined, accumulation of fragmented DNA was found to be within the cellular lysate indicating apoptosis as the underlying mechanism of cell death (NCE G 44, NCE G 112, NCE G 120). In one cell line TAM's antiproliferative action led to necrosis indicated by an increase of DNA fragments within the cellular supernatant (NCE G 130). One cell line followed both mechanisms of cell death to an even extent (NCE G 28). Data represent the mean of duplicate determinations, error bars = S.D

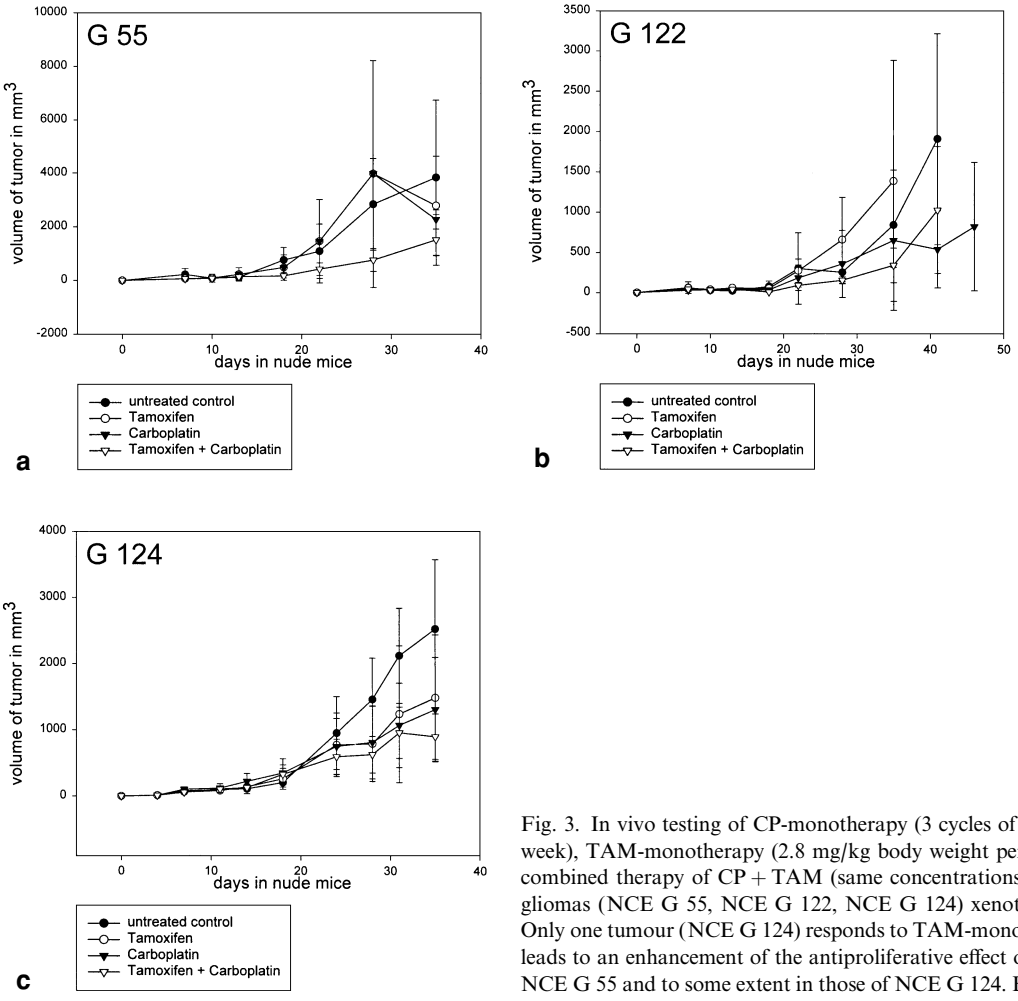

Fig. 3. In vivo testing of CP-monotherapy (3 cycles of 50 mg/kg body weight per week), TAM-monotherapy (2.8 mg/kg body weight per day for 60 days), and the combined therapy of CP + TAM (same concentrations as in monotherapies) in 3 gliomas (NCE G 55, NCE G 122, NCE G 124) xenotransplanted on nude mice. Only one tumour (NCE G 124) responds to TAM-monotherapy. Addition of TAM leads to an enhancement of the antiproliferative effect of CP in xenotransplants of NCE G 55 and to some extent in those of NCE G 124. Error bars = S.D

In Vivo – Effects of CP, TAM, and CP + TAM on Gliomas Established in Nude Mice

In the in vivo model of glioblastoma cell lines xenotransplanted on athymic nude mice, single treatment with TAM did not result in an inhibition of growth in all three xenotransplanted tumours. Only TAM-treated NCE G 124 xenotransplants showed some slower growth than untreated control tumours. This difference nearly reached statistical significance (student's t-test: $p = 0.09$). In contrast, CP-monotherapy resulted in a growth inhibition in all xenotransplanted gliomas compared with negative controls, reaching a level of statistical significance in two tumours (student's t-tests, NCE G 55: $p = 0.041$, NCE G 122: $p = 0.014$, NCE G 124: $p = 0.067$). Again, the combined treatment of TAM + CP led to a significant inhibition of growth in all 3 tumours investigated compared with negative controls (student's t-tests, NCE G 55: $p = 0.003$, NCE G 122: $p = 0.019$, NCE G 124: $p = 0.008$). However, only xenotransplants of NCE G 55 and to some extent those of NCE G 124 but not the tumours of NCE G 122 showed a higher inhibition of their growth by the combined treatment of CP + TAM when compared with CP-monotherapy. These latter differences did not reach statistical significance (Fig. 3).

Upon histological examination, the xenotransplanted tumours revealed all characteristics of a malignant tumour. Within the first 14 days after transplantation, the tumours were surrounded by a fibrous capsule. After this time interval the tumours started to infiltrate the adjacent tissue. Whereas the peripheral margin of the tumours were characterised by a high rate of vascularization, and vital and highly proliferative tumour cells, the centre of the tumours revealed larger areas of necrosis. By immunhistochemical staining with the MIB-1 antibody the xenotransplants showed MIB-indices of 48% (NCE G 55),

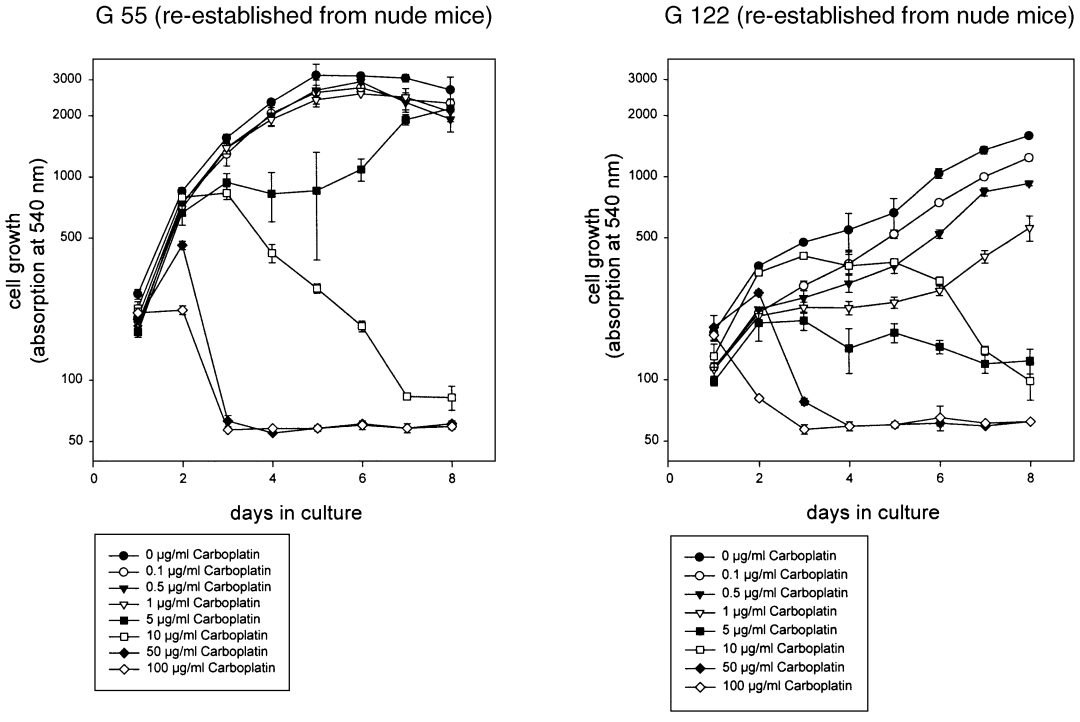

Fig. 4. Tumor cell growth assays of low passage glioma cell lines derived from xenotransplants on nude mice (NCE G 55/nm, NCE G 122/nm) show a dose-dependent inhibition of proliferation by CP. The IC50 ranges between 2 mg/l and 5.2 mg/l. Data represent the mean of triplicate determinations, error bars = S.D

42% (NCE G 122), and 46% (NCE G 124). Compared with untreated control tumours the different forms of in vivo treatment (TAM, CP, CP + TAM) did not lead to alterations of MIB-indices of xenotransplants. Anti-factor VIII immunostaining for detection of neovascularization according to Weidner's method was performed in xenotransplants of tumours NCE G 122 and NCE G 124. Whereas either form of treatment did not influence the density of capillaries in xenotransplants of NCE G 122, TAM-treatment was associated with an increased vascularization in the xenotransplanted tumour NCE G 124 (data not shown). This result reached statistical significance (student's t-test: $p < 0.025$).

In Vitro – Inhibitory Effect of CP, TAM, and CP + TAM on Low Passage Cell Cultures of Gliomas Established in Nude Mice

Paralleling the in vivo results, the growth of all 3 primary cultures of gliomas re-established from nude mice, were inhibited by CP in a dose dependent manner. The concentrations leading to a 50%-inhibition of proliferation (IC50) varied between 2 mg/l in NCE G-122/nm, 2.8 mg/l in NCE G 124/nm and 5.2 mg/l in NCE G 55/nm. CP-concentrations of 5 mg/l (NCE G 122/nm, NCE G 124/nm) and 10 mg/l (NCE G 55/nm) were not survived by the cells (Fig. 4).

Growth assays testing the influence of TAM (continuous treatment mode) on the proliferation of primary cultures of the re-established glioma cell lines NCE G 55/nm, NCE G 122/nm and NCE G 124/nm revealed similar results as obtained for the established cell lines before xenotransplantation on nude mice (Table 3). The respective IC50 values were slightly lower compared with the corresponding cell lines not xenotransplanted to athymic nude mice before: NCE G 55/nm: 9.3 µM, NCE G 122/nm: 9.6 µM, and NCE G 124/nm: 10.5 µM. This result is shown to be in clear contrast to the in vivo result.

By combining different concentrations of CP with TAM-concentrations of 2.5 µM (data not shown) and 5 µM which are not effective in TAM-monotherapy in vitro, no enhancement of growth-inhibition could be observed compared with the effect of the respective CP-concentrations without addition of TAM (Fig. 5).

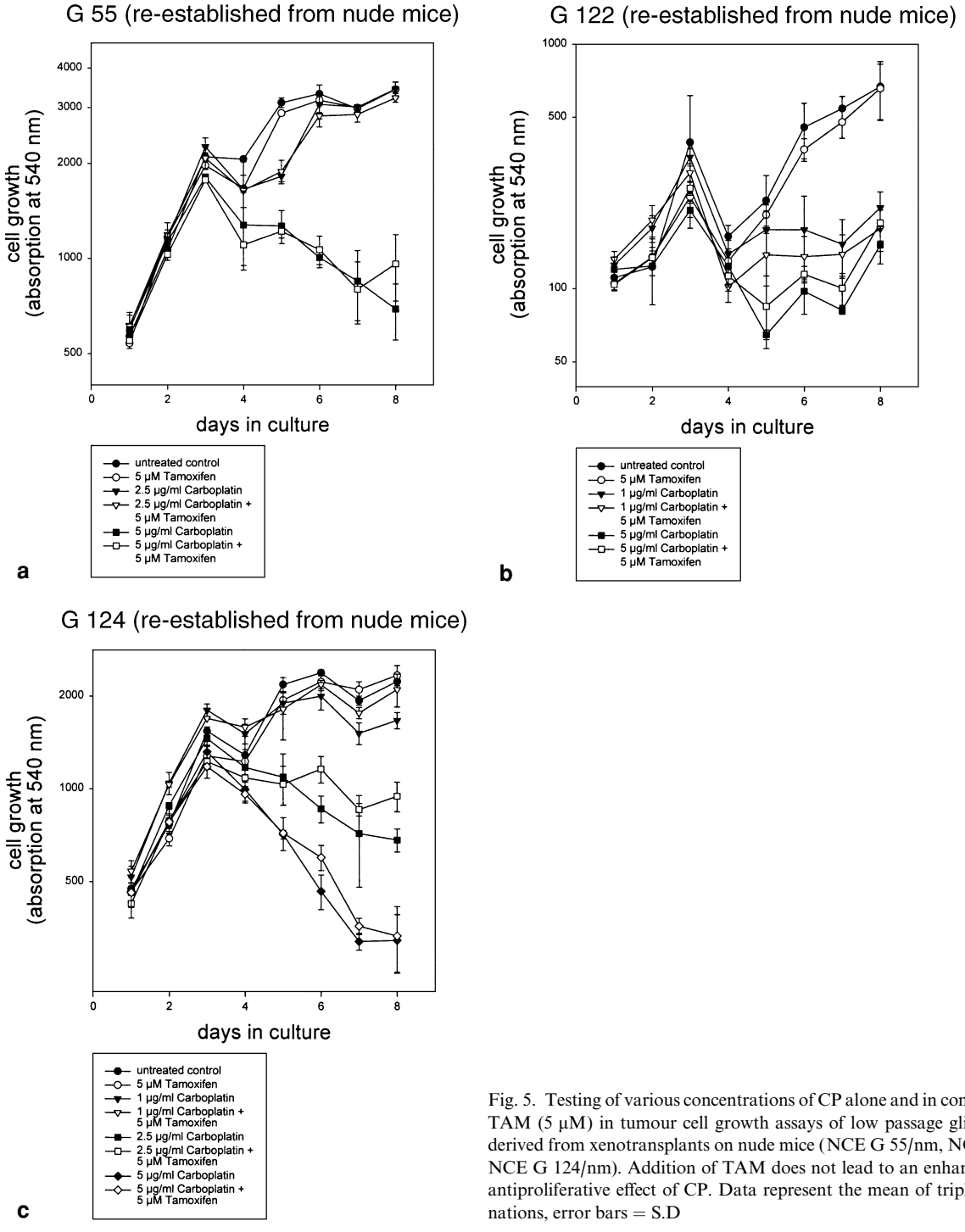

Fig. 5. Testing of various concentrations of CP alone and in combination with TAM (5 µM) in tumour cell growth assays of low passage glioma cell lines derived from xenotransplants on nude mice (NCE G 55/nm, NCE G 122/nm, NCE G 124/nm). Addition of TAM does not lead to an enhancement of the antiproliferative effect of CP. Data represent the mean of triplicate determinations, error bars = S.D

This result was obtained for all 3 low passage glioma cell lines tested (NCE G 55/nm, NCE G 122/nm, NCE G 124/nm) and revealed again a difference between the in vitro and in vivo model.

Discussion

Due to the interindividual different response to high-dose TAM therapy with an overall response rate of

approximately 30% [4, 6, 7, 8, 10, 11, 25, 26, 31, 36] the establishment of a predictive system prior to a planned TAM-therapy would be desirable. However, with the exception of one case report, no in vitro testing of tumour material for its sensitivity to TAM- and/or 4-OH-TAM-treatment and subsequent correlation to the clinical result of TAM-therapy is available so far [44].

In contrast to this case-report, our in vitro data did not correlate with our clinical results [31]. However, by comparing our results of in vitro sensitivity testing of primary cultures with those obtained in tumour cell growth assays of either established glioblastoma cell lines or glioblastoma cell lines re-established after xenotransplantation on athymic nude mice a high similarity of the various IC50-values was seen. Whereas TAM-concentrations between 6 and 11.8 μM (continuous treatment mode) lead to a 50% inhibition of tumour growth in primary cultures of tumours explanted from our study patients, the respective IC50-values obtained in the experiments with established glioblastoma cell lines ranged between 9.7 μM and 11.2 μM and those obtained in the testing of glioblastoma cell lines re-established after xenotransplantation on athymic nude mice were in the range of 9.3 μM to 10.5 μM. These results show a good correlation with previous experiments investigating the antiproliferative action of TAM on established malignant glioma cell lines. In these studies IC50-values in the range between 1 μM and 20 μM were reported [4, 9, 30, 37, 40]. The results of our additional experiments examining the antiproliferative effect of TAM in a 2-day-treatment mode and that of 4-OH-TAM confirmed the above mentioned observations and allow the following conclusion: The growth of all glioblastoma cells, regardless of the number and type of passages which they had undergone before and regardless of the clinical response to TAM-treatment in their previous host (patient, nude mouse), can be dose-dependently inhibited in vitro. The TAM-concentrations necessary to lead to a 50% growth inhibition differ in such a small range, that the clinical observation of "TAM-response" and "non-response" seemed to be absent in the in vitro-model. Therefore an interindividual response to TAM-treatment seems to be an in vivo effect.

Our in vivo model of athymic nude mice approximates the clinical situation only to a certain extent (i.e. tumours were transplanted subcutaneously and not intracerebrally which causes a different pharmacokinetic situation; due to lack of T-lymphocytes the immunological situation of these mice is clearly different to patients [15]). However, the results obtained with it resembled more the clinical situation compared with our in vitro results. Paralleling the response rate of approximately 30% of patients treated with TAM [4, 6, 7, 8, 10, 11, 25, 26, 31, 36] only one (NCE G 124) out of 3 xenotransplanted tumours tested responded to a TAM-monotherapy. As the extent of anti-factor VIII-immunostaining has been shown to correlate with the extent of tissue-vascularization [35], our result of an increased anti-factor VIII-immunoreactivity of one xenotransplanted tumour (NCE G 124) after TAM-treatment might indicate TAM's ability to induce neovascularization in glioblastomas. Interestingly, the growth of only this tumour was inhibited by TAM-monotherapy. The increase of angiogenesis may produce an increased sensitivity to CP because of improved drug delivery. It might also explain the facilitation of multifocal tumour growth from small disseminated cell aggregates [30, 31].

After re-establishing the xenotransplanted tumours in cell culture again, the cells reacted to TAM-treatment like glioblastoma-cell-lines which had not undergone xenotransplantation to nude mice, i.e. distinguishing between tumours responding to TAM-therapy and those not responding was not possible any more. Therefore we suggest that this in vitro/in vivo – discrepancy might be related to a more basic mechanism which seems to be independent of the immunological state of the host and the location of the tumour.

The differences between the in vitro and the in vivo model observed at the TAM-experiments are in some contrast to our experiments investigating CP. In these experiments, CP constantly induced a dose-dependent growth inhibition of glioma cells regardless of the experimental model used. This good correlation between the in vitro- and in vivo-situation is paralleled by clinical studies describing a good predictive value of in vitro CP-sensitivity testing for the CP-treatment of patients with malignant gliomas [21, 42]. As further demonstrated in our experiments, the underlying mechanism of CP's antiproliferative action follows that of inducing necrosis as a sign of efficacy whereas that of TAM varied between induction of apoptosis (3 out of 5 cell lines), necrosis (1 out of 5 cell lines), or both of these mechanisms (1 out of 5 cell lines). In contrast to the necrosis the mechanism of apoptosis is considered to be rather complex and activated in cells, which are no longer able to follow certain cellular

programs and are unable to control their cell cycle [18]. It is possible that for TAM to be effective at inducing apoptosis, complex requirements and indirect effects need to be present and might explain the in vivo/in vitro difference. In how far the demonstrated variability of mechanisms by which TAM is inducing cell death corresponds to the observed different responses to TAM-treatment observed in vivo is unknown. The design of our experiments however cannot not give further information whether this correlation indicates a causal link.

An additional explanation for the observed inconstant response to TAM-therapy in vivo might be the already described interindividual different absorption and/or metabolization of TAM. As reported in studies determining plasma levels of TAM and its metabolites in glioma- and breast cancer-patients, the interindividual variation of values measured exceeded the factor 10 although patients were treated with the same daily dose of the drug [13, 23]. Measurements of TAM-concentrations in blood-samples of athymic nude mice have not been performed so far.

In conclusion, the demonstrated discrepancies between the in vitro and in vivo-situation by testing TAM's antiproliferative effect on glioblastomas showed that in vitro sensitivity testing of tumour material is not a useful method for predicting response to TAM in glioblastoma-patients. Moreover, the results of our clinical study [31] and of other in vitro experiments published recently [30], added one more aspect to the problem of predicting TAM-response in glioblastoma-patients: As explained in these two reports, we are now afraid that continuous high-dose TAM-therapy might lead to a selection of TAM-resistant glioma cell-subpopulations which are characterised by a highly migratory and aggressive growth pattern. Patients (33% in our study) whose tumours developed this kind of "TAM-resistance" were characterised by extremely rapidly growing multifocal tumour recurrences. Considering that a certain percentage of tumours initially responding to TAM-therapy may alter to tumours becoming resistant to this form of treatment, show the additional difficulty of predicting TAM-response in glioblastomas. In this respect we remain sceptical in how far the new method of proton magnetic resonance spectroscopic imaging which has been reported very recently to be a reliable predictor for TAM-response in patients with recurrent malignant gliomas [28], is also able to predict development of TAM-resistance.

Acknowledgment

M. G.'s work was in partial fulfilment of the requirements for the Doctor of Medicine, University of Hamburg. We thank Priv. Doz. Dr. C. Hagel (Dept. of Neuropathology, University Hospital Eppendorf, Hamburg, Germany) for his support in performing the statistical analysis of our data.

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Comment

The initial assumption that we need a method to predict the efficacy of Tamoxifen treatment is probably erroneous. I do not agree that less than half of the patients respond to Tamoxifen treatment. The real response rate with Tamoxifen (even at high dose) is probably less than 10%. Therefore, I am not convinced at all of the possible utility of a predictive assay. In addition, it is now clear that it is not possible to recapitulate in vitro, the complexity of the in vivo situation. In vitro assays to predict in vivo sensitivity to anticancer drugs have been almost abandoned, even for tumors for which we have highly active drugs.

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