Chapter 2
Results

2.1 Effects of CAP on Liquids

2.1.1 CAP Effects on Medium

First, the effects of CAP and its components on liquids were investigated. Among others, pH change during CAP application was verified (supplementary data S26). No significant change in pH was observed for treatment times up to 320 s in DMEM supplemented with 1 % FCS. Effects on FCS alone or in medium were not found (supplementary data S27). From publications and experiments previously carried out by our and other groups it is known that the height of the liquid has an effect on the CAP impact on the respective cells. To analyze the effects of CAP treated medium on glioma cells, LN18 cells were CAP treated for different time durations in 100 µl/well of a 96-well plate and medium was either filled up to 200 µl (Fig. 2.1a) or medium was removed completely after CAP treatment and fresh medium was added (Fig. 2.1b). Effects of respective CAP treatment on the proliferation of glioma cells were examined using the MTT assay. When the medium was filled up, the proliferation was strongly reduced independently of the CAP treatment time (Fig. 2.1a). In comparison, the medium change led to a dose dependent effect of CAP treatment on the proliferation of the cells (Fig. 2.1b).

2.1.2 Storage of Pre-Treated Medium Reduces the CAP Efficacy

To further distinguish the effects of CAP on medium, 100 µl/ 96-well DMEM was pre-treated with CAP and kept at 37 °C and 5 % CO_2 for 1 h, 12 h and 24 h, before applying solely the pre-treated medium to the cells. Effects of the pre-treated medium with incubation time on cell proliferation were compared to the treatment of cells with medium which was exposed to CAP immediately before

J. Köritzer, *Biophysical Effects of Cold Atmospheric Plasma on Glial Tumor Cells*, Springer Theses, DOI: 10.1007/978-3-319-06224-2_2,

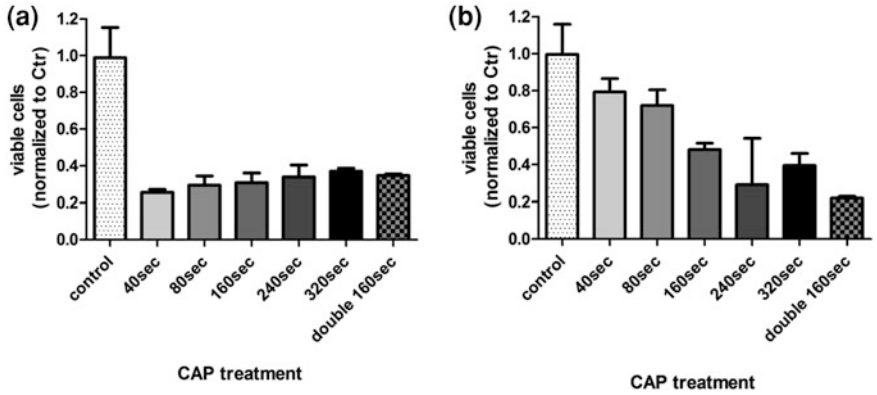

Fig. 2.1 CAP effects on medium. **a** LN18 glioma cells were CAP treated for the indicated times in medium and afterwards the well was filled up with fresh medium to double the amount of medium. MTT assay was performed 48 h later. **b** After CAP was applied on LN18 cells in medium, the medium was completely changed to fresh medium and cell viability was measured using a MTT assay. Double 160 s: twice applications of 160 s of CAP with a 1 min pause in between

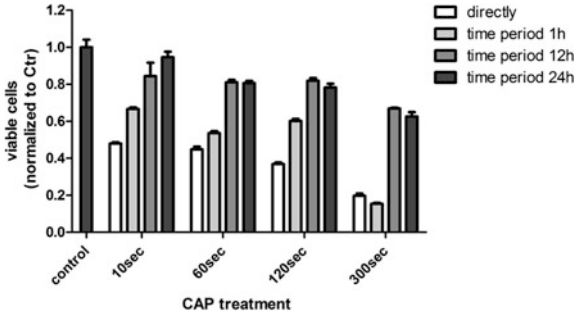

Fig. 2.2 Pre-treated medium is effective in reducing cell proliferation. DMEM supplemented with 1 % FCS was pre-treated with CAP without cells. Pre-treated medium was either immediately applied to LN18 cells ("directly"), or stored at 37 °C for 1 h, 12 h or 24 h and afterwards applied to the cells. Viability was detected using MTT assay

applying to the cells (Fig. 2.2). The results show that cell viability of glioma cells is reduced if the cells are incubated with pre-treated medium. Furthermore, the data confirms that CAP "activated" medium reveals effects on cancer cells upon storage, nevertheless reduced effects of CAP on the cell viability were observed for storage times of 12 h and 24 h.

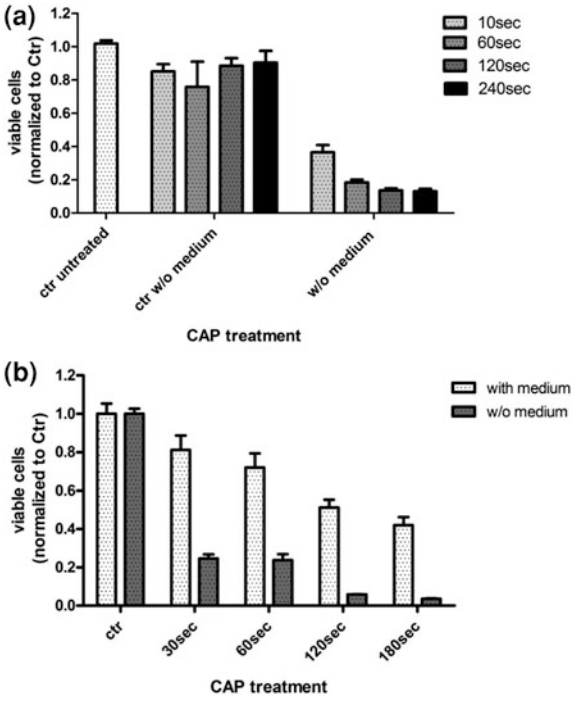

Fig. 2.3 **a** LN18 glioma cells were kept without medium to investigate differences in proliferation by only having a thin film of liquid on the cells for up to 240 s. Cells were CAP treated without medium for the indicated times and cell viability was measured 48 h afterwards. **b** LN229 cells were either CAP treated in 100 µl medium or treated while the medium was sucked off and fresh medium was added to the cells subsequently after treatment. Proliferation was detected using the MTT assay

2.1.3 Treatment of Glioma Cells in Minimized Amounts of Medium

To decline of the CAP effects on liquids and to resemble the situation in the patient, LN18 glioma cells were treated with only a thin film of liquid covering them (referred to as w/o medium). Keeping the cells with only a thin film of medium for time durations of up to 240 s did not significantly change the viability of the cells (Fig. 2.3a). Upon treatment between 10 s and 240 s with CAP w/o medium, a strong reduction of cell viability was achieved when normalized to the control which was kept w/o medium for the indicated times. Henceforth the treatment with CAP was performed after sucking off the medium from the cells followed by addition of fresh medium immediately after the treatment. Comparison of the effects of CAP on the viability of LN229 (Fig. 2.3b) and LN18 (data not shown) glioma cells indicated that CAP was effective in reducing the proliferation either for treatment with or without medium, nonetheless the CAP effect was less pronounced when treated in medium (Fig. 2.3b).

2.2 Effects of CAP on TMZ-Resistant and -Sensitive Glioma Cell Lines

2.2.1 CAP Treatment Inhibits Cell Proliferation in MGMT Positive and MGMT Negative Glioma Cell Lines

Several tumor biology studies identified the prognostic role of O6-methylguanine-DNA methyltransferase (MGMT) status in patients with newly diagnosed glioblastoma. Thus, newly diagnosed glioblastoma can be stratified into two prognostic groups based upon *MGMT* promoter methylation status. Promoter methylation of the *MGMT* gene predicts benefit from chemotherapy with the standard chemotherapeutic temozolomide (TMZ), whereas patients with an unmethylated promoter status do not benefit from chemotherapy. The expression of the MGMT protein of the cell lines U87MG, LN229, LN18 and T98G under standard culture conditions was illustrated by antibody detection (Fig. 2.4a). GAPDH served as a loading control. The cell lines U87MG and LN229 that do not express MGMT (MGMT negative, favorable) and the cell lines LN18 and T98G that express MGMT (MGMT positive, unfavorable) were treated with TMZ for 3 days consecutively and cell viability was measured afterwards (Fig. 2.4b). Treatment with TMZ was able to reduce the viability of the MGMT negative cell lines by about 20–25 %, whereas the viability of the MGMT positive cell lines was only reduced to a minor content (T98G) or remained unreduced (LN18). These MGMT positive and MGMT negative cell lines were CAP treated with one single application and viability was detected 48 h later (Fig. 2.4c). A dose dependent inhibition of proliferation by CAP treatment for all cell lines was observed. Treatment times of 60 s and longer achieved a significant reduction of proliferation in the resistant as well as in the sensitive cell lines.

2.2.2 Induction of Apoptosis is Present to a Minor Extent After CAP Treatment

The perceived reduction of proliferation in all tested cell lines was conducted by minor induction of apoptosis. Immunoblotting of protein lysates gained from LN18 (MGMT positive, unfavorable) (Fig. 2.5) and LN229 (MGMT negative, favorable) (supplementary data S28) cells with antibodies against yH2AX, cleaved PARP1 (Fig. 2.5a), Caspase 3 (Fig. 2.5b) and Caspase 9 (Fig. 2.5c) as marker for DNA damage was performed. Cleavage of PARP1 was detected 48 h and 72 h post-treatment for 120 s of CAP treatment and longer, but not after 4 h and 12 h (data not shown). Phosphorylation of H2AX was demonstrated for a treatment time of 180 s after 72 h, but not after 4 h, 12 h (data not shown) and 48 h. Cleavage of Caspase 9 was observed to a minor extent after treatment for 120 s and 180 s only after 48 h, while cleavage of Caspase 3 was detected at none of the

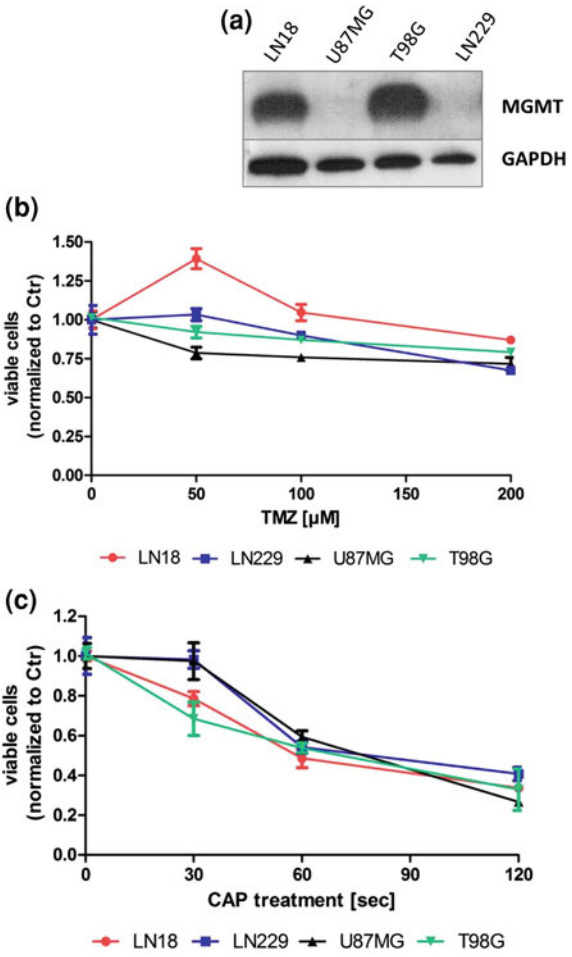

Fig. 2.4 MGMT promoter methylation predicts response to chemotherapy but does not affect the response to CAP. **a** Immunoblotting of cell lysates for the expression of MGMT protein under normal culture conditions. GAPDH served as a loading control. **b** TMZ was administered in several concentrations to glioma cells for 3 days consecutively and cell viability was detected. **c** Glioma cells were CAP treated once for 30–120 s and viability was detected 48 h afterwards

mentioned time points. Additionally, DNA damage including single strand breaks (ssb), double strand breaks (dsb) and cross linking sites at the level of single cells was observable by the comet assay to a minor content 1 h after CAP treatment in LN229 cells, but was repaired 24 h afterwards (supplementary data S29). Taken these results into account, apoptosis was induced in glioma by CAP treatment only to a minor extent, primarily in the first hour(s) after application of CAP.

2.2.3 Cytolysis is Only Induced by Long CAP Treatment Durations

Measurement of released lactate dehydrogenase (LDH) in the medium for the detection of cytolysis was performed 2 h, 24 h and 48 h after CAP treatment

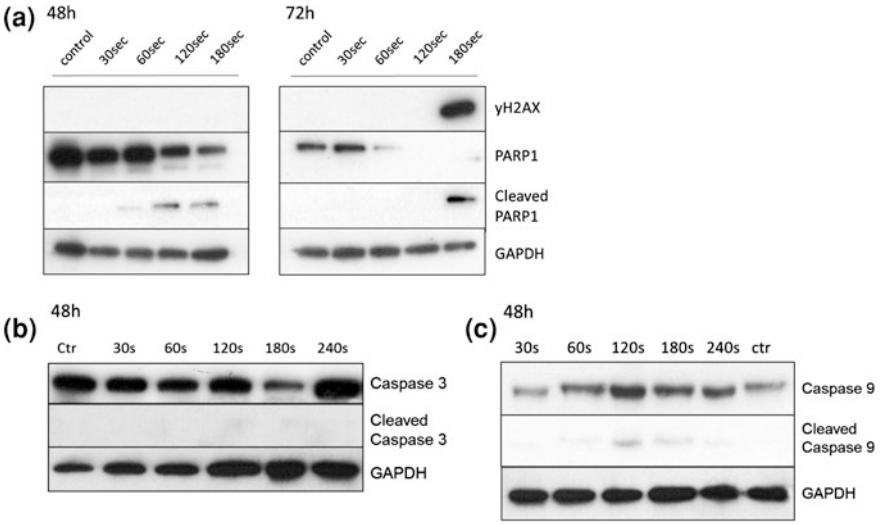

Fig. 2.5 Severe apoptosis is not an early feature of CAP treatment in glioma cells. **a** Immunoblotting of LN18 cell lysates taken 48 h and 72 h after CAP treatment with antibodies for PARP1, cleaved PARP1 and γH2AX. GAPDH served as a loading control. **b** Caspase 3 full length was detectable by immunoblotting in LN18 cell lysates 48 h after CAP treatment but not the cleaved and therefore activated Caspase 3. **c** Cleavage of Caspase 9 as a marker for induction of apoptosis was not found in LN18 cell lysates after CAP treatment

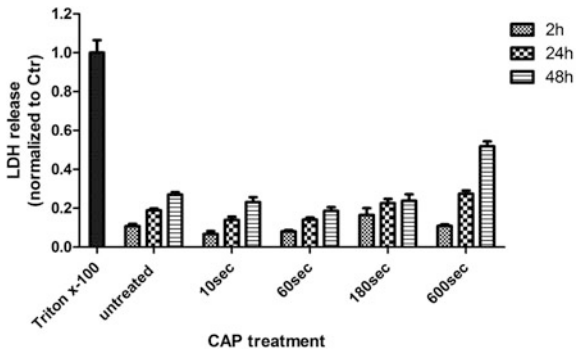

Fig. 2.6 Cytolysis is not promoted by CAP treatment in LN18 glioma cells. The release of LDH from the cells in the medium 2 h, 24 h and 48 h after CAP exposure up to 600 s was assessed. Treatment with 1 % Triton x-100 served as a positive control

(Fig. 2.6). This assay is based on measurement of LDH which is a stable enzyme normally found in the cytosol of all cells but is rapidly released into the supernatant upon damage of the plasma membrane. Treatment of cells with 1 % Triton x-100 served as a positive control. No increased release of LDH after treatment for up to 180 s was displayed, whereas treatment for 10 min resulted in about 50 % increase of released LDH in the medium.

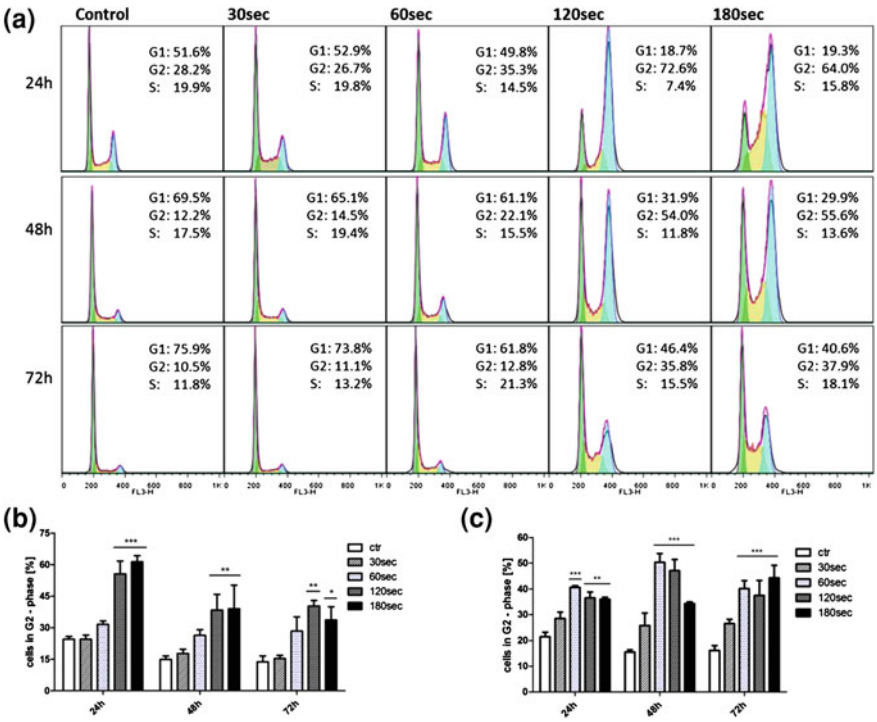

Fig. 2.7 CAP treatment induces G2/M phase cell cycle arrest in glioma cells. **a** Representative cell cycle distribution of U87MG glioma cells after CAP treatment. Treatment was performed only with a thin film of liquid covering the cells. Cell cycle distribution was analyzed using flow cytometry. Similar results were observed for the LN229 (MGMT negative) and LN18 (MGMT positive) cells (supplementary results S5). **b** Statistical significances of the observed arrest in the G2/M phase in U87MG and **c** in LN18 cells. P-value *** <0.001

2.2.4 Strong Induction of Cell Cycle Arrest in MGMT Positive and MGMT Negative Glioma Cells by CAP Treatment

Based on these findings, we assumed that CAP has an influence on the regulation of the cell cycle progression. Thus, cell cycle progression of the MGMT positive and MGMT negative cell lines was analyzed 24 h, 48 h and 72 h after CAP exposure (Fig. 2.7 and supplementary data S30). Treatment times of 120 s and longer resulted in a significant arrest in the G2/M phase of the cell cycle in the U87MG (Fig. 2.7a), LN229 and T98G (supplementary data S30B and S30C) cells. A factor two to four higher amount of cells in the G2/M phase was found for the CAP treated cells compared to the control cells. For the LN18 cell line with an unfavorable MGMT status, the arrest was achieved after treatment for 60 s (supplementary data S30A). The arrest achieved in the tested cell lines U87MG,

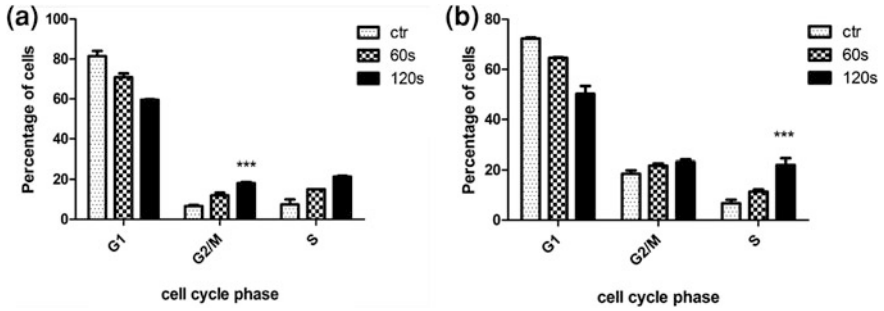

Fig. 2.8 Extended cell cycle arrest was observed post-treatment with CAP. **a** LN18 and **b** U87MG cells were CAP treated for the indicated times and cell cycle progression was examined 7 days afterwards. P-value <0.001

LN18 and LN229 persisted for at least 72 h and was independent of the MGMT status. Statistical analysis revealed a significant arrest in the U87MG cells (Fig. 2.7b) after 120 s and longer, whereas the cell cycle progression of the LN18 cells (Fig. 2.7c) and LN229 cells (supplementary data S30D) was significantly inhibited after 60 s and longer of CAP treatment.

2.2.5 Induction of Cell Cycle Arrest by CAP Application is a Long Lasting Effect in Glioma Cells

Long term investigation of the cell cycle progression in LN18 and U87MG cells was conducted 7 days post-treatment, as an extended duration of the CAP effects on cell cycle regulation would represent a benefit regarding the application in a combined therapy with chemotherapeutics. Therefore, LN18 and U87MG cells were CAP treated once without medium and cultured for 7 days. Cell cycle analysis was performed using flow cytometry. A significant cell cycle arrest in G2/M phase 7 days post-treatment was demonstrated in the resistant cell line LN18 by CAP treatment for 120 s (Fig. 2.8a). Noticeably, the sensitive cell line U87MG featured a significant arrest 7 days post-treatment by 120 s of CAP application in the S phase of the cell cycle (Fig. 2.8b).

2.2.6 CAP Reduces the Clonogenic Potential of Glioma Cells

The ability of cells to form clones after treatment with the TMZ or CAP was verified (Fig. 2.9). Therefore, glioma cells were either TMZ or CAP treated and 24 h later 150 cells were seeded and allowed to form colonies over 12 days.

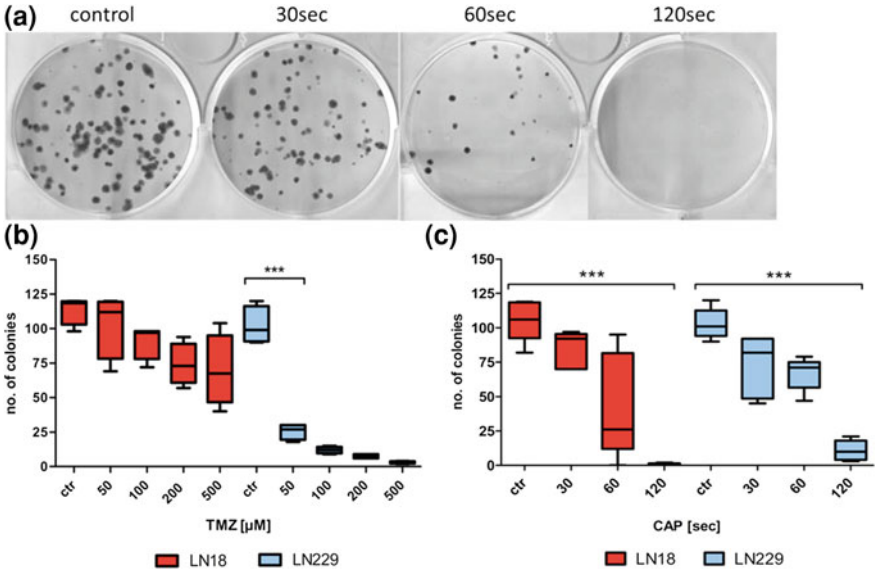

Fig. 2.9 Reduced clonogenic capacity of glioma cells treated with TMZ or CAP. Glioma cells were either TMZ or CAP treated and 24 h later 150 cells/well were seeded on a 6-well plate. Colonies formed after 12 days were stained and counted. P values *** <0.001. **a** Picture of the LN18 (MGMT positive, unfavorable) cells treated with CAP for 30 s, 60 s and 120 s. Afterwards the formed colonies were stained. **b** Results of the colony formation assay for MGMT positive and negative glioma cells (LN18 and LN229) treated with TMZ with concentrations of up to 500 µM. **c** CAP treatment followed by the colony formation assay in either MGMT positive or MGMT negative cells

Treatment with TMZ was able to reduce the clonogenicity in LN18 cells (MGMT positive, unfavorable) only to a minor content up to concentrations of 500 µM (Fig. 2.9b). The MGMT negative cell line (favorable) LN229 was sensitive to treatment with 50 µM TMZ, resulting in a significant reduction of clonogenicity. In contrast, a significantly reduced clonogenicity was found after CAP treatment for both cell lines independent of their MGMT status (Fig. 2.9c). CAP treatment of 120 s strikingly culminated in a complete loss of the clonogenic capacity of the LN18 cells.

2.2.7 CAP Application Impairs Migration of Glioma Cells

To further address the effects of CAP application on glioma cells, the migration ability after CAP treatment in LN18 and LN229 cells was investigated. A gap was originated by a cell culture insert and cells were CAP treated. Migration of the cells was monitored for 48 h under the microscope.

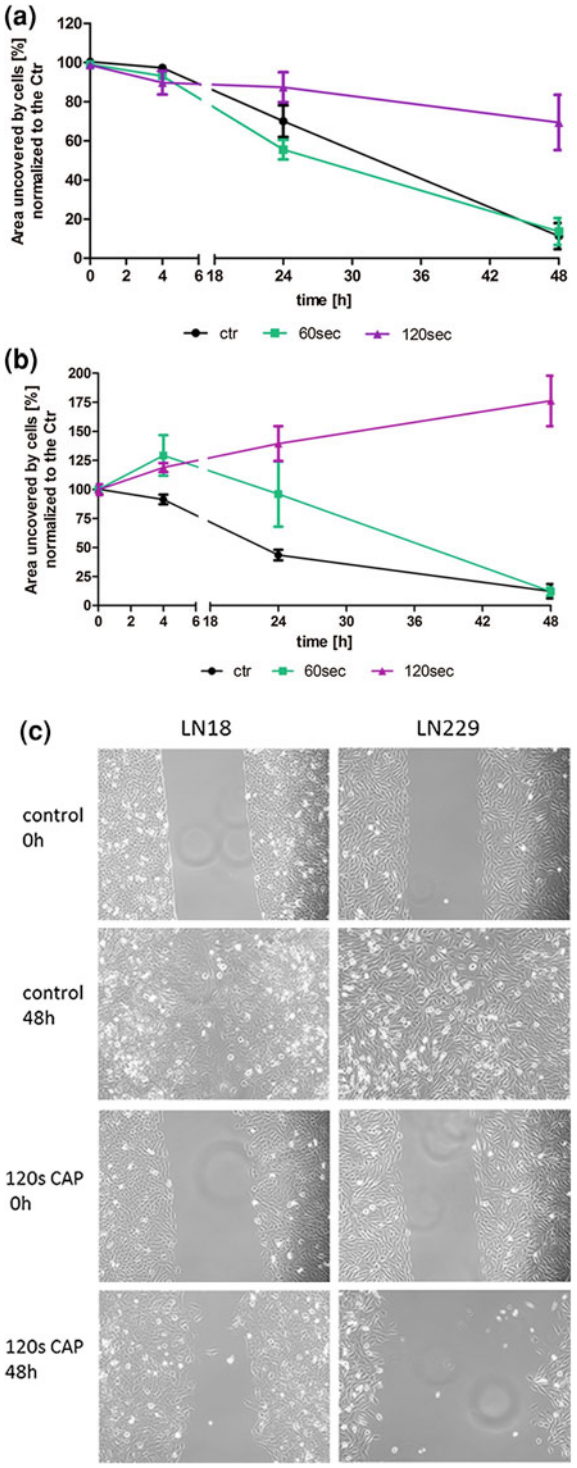

Fig. 2.10 Inhibition of migration in glioma cells after CAP treatment. **a** LN18 cells and **b** LN229 cells were CAP treated and migration of the cells was monitored over 48 h. Gap size was measured after 4 h, 24 h and 48 h. **c** Pictures illustrating the gap directly after the treatment in CAP treated cells or in control cells, compared to migration in CAP treated and control cells after 48 h

Migration was reduced in LN18 cells (Fig. 2.10a) after 120 s of CAP, but not after 60 s of treatment. A noticeable enlargement of the gap was observed 4 h after CAP treatment for both 60 and 120 s in the LN229 cell line (Fig. 2.10b). Strikingly, the gap was even more distinct 24 h and 48 h post-treatment for 120 s possibly due to de-attachment of cells. Migration of LN229 cells with 60 s of CAP treatment recovered after 48 h. Reduced migration rate and de-attachment of cells was visible in LN18 and LN229 cells which were CAP treated for 120 s on the pictures taken directly after CAP treatment (0 h) and after 48 h of incubation after treatment (Fig. 2.10c).

2.3 Combined Treatment with CAP and Chemotherapy

2.3.1 Concomitant Treatment with CAP and TMZ has a Synergistic Effect on Cell Viability in MGMT Positive and MGMT Negative Cells

To investigate the effects of a combined therapy consisting of CAP and the chemotherapeutic TMZ in glioma cells, both sensitive as well as both resistant cell lines were CAP treated once followed by a consecutively TMZ application for 3 days. Proliferation of the cells was quantified using the MTT assay. Combined treatment with CAP and TMZ leads to a significant stronger inhibition of proliferation in the sensitive U87MG (Fig. 2.11a) and LN229 (supplementary data S31) cells, as well as the resistant LN18 (Fig. 2.11b) and T98G (supplementary data S31) cells compared to separate treatment with CAP or TMZ alone. Furthermore, combined treatment with low dose of TMZ (50 μM) and short treatment times of CAP (30 s in U87MG and LN18, 60 s in LN229 and T98G cells) caused significant higher suppression of cellular growth as compared to a high dose of TMZ (100 μM) alone. As displayed in Fig. 2.11, combined therapy revealed a synergistic effect on cell viability in the treated TMZ-sensitive and -resistant glioma cells. Especially low dose TMZ (50 μM) combined with short CAP treatment times (30 s) clearly showed a synergistic effect rather than an additive effect.

2.3.2 Combined Therapy Induces Prolonged G2/M Phase Cell Cycle Arrest

The LN18 and T98G cell lines, which are known to be fairly resistant to therapy with TMZ, were exposed to various TMZ concentrations repeatedly (Fig. 2.12 and supplementary data S32). Remarkably, only the highest concentration of 500 μM was able to induce a G2/M phase arrest. In contrast, combined treatment of CAP for 60 s (single treatment) and TMZ (50 μM, 100 μM, 200 μM for 3 days

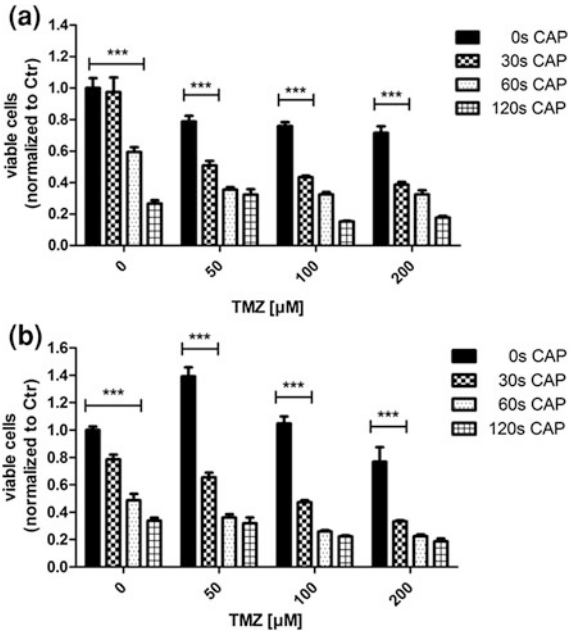

Fig. 2.11 Concomitant therapy with TMZ and CAP re-sensitizes glioma cells. **a** U87MG cells were CAP treated once and TMZ was applied consecutively afterwards for 3 days. MTT assay for evaluating the cell viability was performed at day four. Controls were kept without medium and/or DMSO treated. **b** The same approach was carried out for LN18 cells, which were CAP treated once and TMZ was applied afterwards consecutively for 3 days. P-value *** <0.001

consecutively) indicated a significant cell cycle arrest (Fig. 2.12a). Especially a combination of 60 s CAP and 100 µM TMZ or 200 µM TMZ, respectively, turned out to be strongly effective in inhibition of the cell cycle progression (Fig. 2.12b).

2.4 CAP Displays Cell Selectivity Towards Tumor Cells

2.4.1 Cell Cycle Distribution in Primary Astrocytes

As CAP treatment was effective in glioma cells, the influence of CAP on healthy, non-tumorous cells and brain tissue was investigated. Therefore, primary astrocytes were isolated from newborn mice and exposed to CAP similar to the tumor cells before (Fig. 2.13). Treatment occurred with only a thin film of medium covering the cells. The experiment was performed twice. Using flow cytometry the cell cycle progression was determined 48 h after CAP treatment. Primary astrocytes did not respond to CAP application with a cell cycle arrest in the G2/M phase, even not when treated for up to 180 s. A shift towards the S phase was observable for CAP times of 180 s, but not for shorter treatment times (Fig. 2.13). These results indicate that CAP treatment induces different effects in non-tumorous compared to the effects induced in tumor cells. Furthermore, the treatment time needed to induce a change on cell cycle progression in non-tumorous cells is longer than in tumor cells, suggesting a ‘therapeutic window’ for the CAP application.

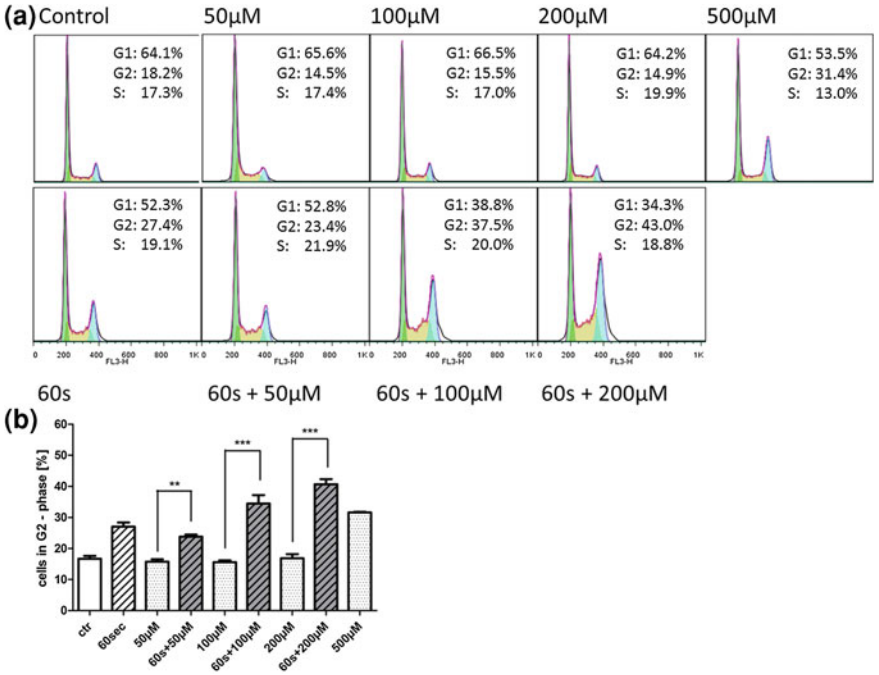

Fig. 2.12 TMZ resistant cells respond with cell cycle arrest to concomitant treatment. **a** LN18 glioma cells were TMZ treated for 3 days consecutively with 50 µM, 100 µM, 200 µM and 500 µM and cell cycle analysis was performed afterwards. In comparison, CAP treatment for 60 s was applied once to LN18 glioma cells, followed by TMZ treatment with 50 µM, 100 µM and 200 µM for 3 days consecutively. Cell cycle distribution was determined afterwards. **b** Analysis of the percentage of cells in the G2/M phase after treatment with TMZ and combined treatment with CAP. P-value *** <0.001

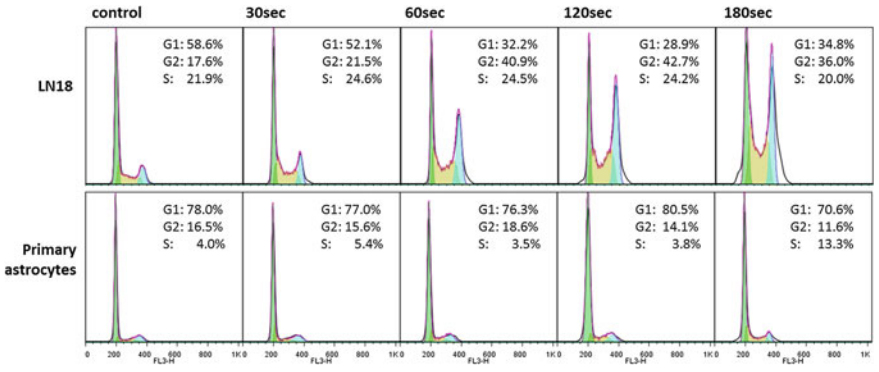

Fig. 2.13 CAP demonstrates cell selective properties. Primary astrocytes isolated from newborn mice were CAP treated and cell cycle progression was analyzed 48 h later. In comparison the cell cycle distribution for CAP treated LN18 glioma cells is shown

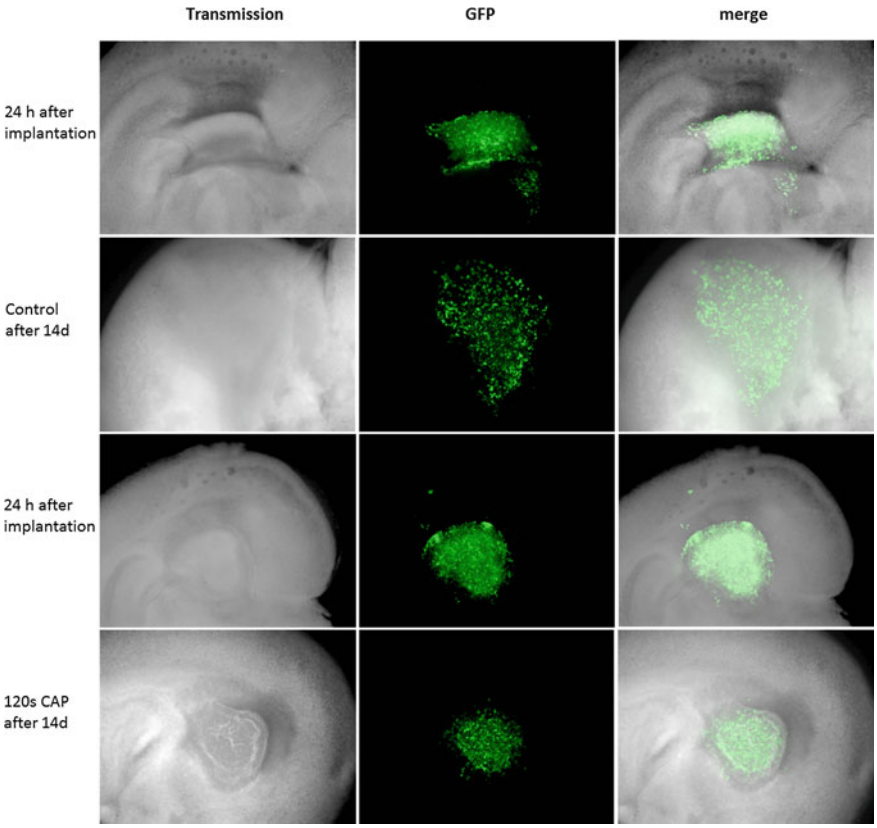

Fig. 2.14 CAP treatment of LN18pEGFP cells in a murine brain slice model. LN18 cells expressing EGFP were implanted in freshly prepared brain slices from newborn mice. CAP was applied 24 h after implantation of the cells for four times within 48 h and cells were monitored over 14 days

2.4.2 Treatment of LN18pEGFP in Organotypic Brain Slice Cultures

To further explore the impact of CAP on cell selectivity, LN18pEGFP tumor cells were implanted in organotopic brain slices cultures (OSCs) from newborn mice and cultured for 14 days. CAP was applied twice 24 h and 48 h after implantation. Tumor progression was monitored via fluorescence before and 14 days after CAP treatment (Fig. 2.14 and supplementary data S34). LN18pEGFP revealed similar proliferation behavior towards CAP as LN18 wt cells did in vitro (supplementary data S33). Tracking of the LN18 cells via GFP fluorescence revealed that the tumor cells were migrated through the OSCs up to a depth of 80–160 μM in the slides after 14 days. CAP treated OSCs seem to express a reduced fluorescence

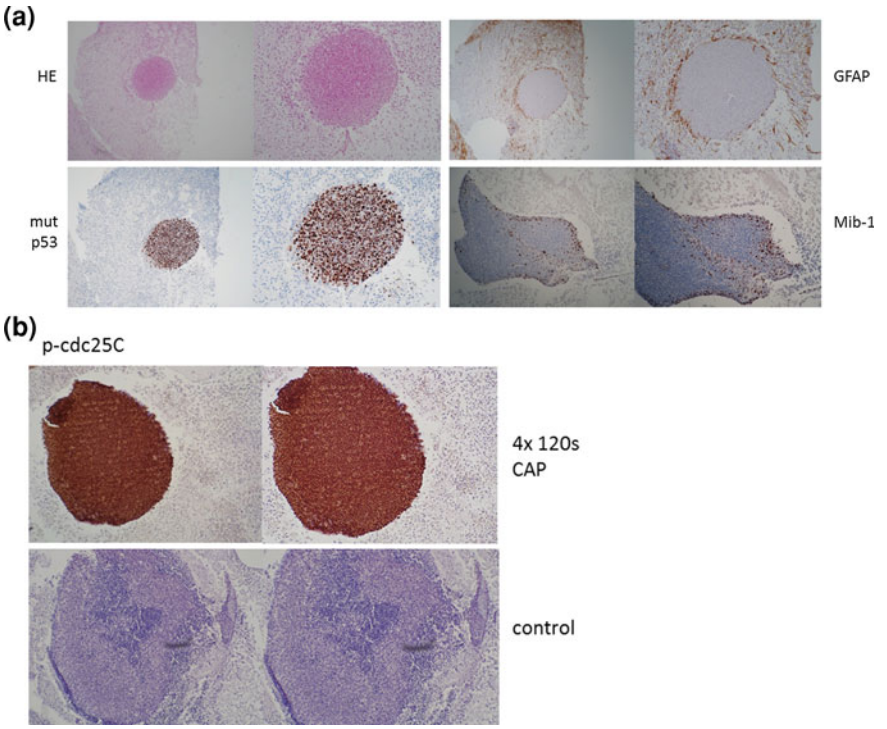

Fig. 2.15 Staining of the OSC revealed tumor formation after implantation of LN18pEGFP glioma cells. **a** Immunohistological staining of OSCs with hemotoxylin-eosin (HE), mut p53, GFAP and Mib-1. **b** The activated checkpoint kinase Chk1 can inactivate Cdc25C via phosphorylation at Ser216, thereby blocking the transition into mitosis. The control slice presented no phosphorylation of Chk1

area for treatment times between 60 s and 180 s, even so CAP treatment did not diminish fluorescence in vitro (data not shown). There was a remarkable morphological change on the surface of the tissue 14 days after CAP application exclusively in the tumor area, even though CAP was applied overall onto the OSCs (Fig. 2.14).

For immunohistological analyzes of effects on brain tissue and tumor cells slices were fixed 14 days after four times of CAP application and stained with hematoxylin-eosin (HE), mut p53, Glial fibrillary acidic protein (GFAP) and Mib-1 (Fig. 2.15a). A precise discrimination between normal tissue and tumor cells can be made based upon these staining. p53 is mutated in LN18pEGFP cells, whereas non-tumorous cells express wt p53. GFAP is exclusively expressed in astrocytes in the central nervous system, and is not present in LN18 tumor cells (ATCC® CRL-2610™). The proliferation marker Mib-1 (also known as Ki-67) is solely express in the periphery of the tumor, demonstrating a marginal growth of the tumor.

2.4.3 Staining for p-Cdc25c Reveals Activation of the ATM/ATR Signaling Pathway by CAP Application in OSCs

OSCs were stained for the phosphorylation of Cdc25c (Fig. 2.15b), a key regulator of the cell cycle. Cdc25c plays a role in promoting progression from G2 phase to mitosis. The checkpoint kinases Chk1 and Chk2 phosphorylate Cdc25c at Ser216 in response to DNA damage or unreplicated DNA, thus delaying progression of the cell cycle to provide time to repair the damaged DNA or to complete replication. Slices that were CAP treated four times for 120 s demonstrated phosphorylation of the Cdc25c phosphatase in the tumor, whereas phosphorylation was not detectable in the tumor of the untreated control slices. Notably, the phosphorylation at Ser216 does not occur in mouse cells as mouse Cdc25c does not have an equivalent to this residue.

http://www.springer.com/978-3-319-06223-5

Biophysical Effects of Cold Atmospheric Plasma on Glial Tumor Cells

Köritzer, J.

2014, XIV, 74 p. 38 illus., 12 illus. in color., Hardcover
ISBN: 978-3-319-06223-5