Biological activity of tumor-treating fields in preclinical glioma models

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Glioblastoma is the most common and aggressive form of intrinsic brain tumor with a very poor prognosis. Thus, novel therapeutic approaches are urgently needed. Tumor-treating fields (TTFields) may represent such a novel treatment option. The aim of this study was to investigate the effects of TTFields on glioma cells, as well as the functional characterization of the underlying mechanisms. Here, we assessed the anti-glioma activity of TTFields in several preclinical models. Applying TTFields resulted in the induction of cell death in a frequency- and intensity-dependent manner in long-term glioma cell lines, as well as glioma-initiating cells. Cell death occurred in the absence of caspase activation, but involved autophagy and necroptosis. Severe alterations in cell cycle progression and aberrant mitotic features, such as poly- and micronucleation, preceded the induction of cell death. Furthermore, exposure to TTFields led to reduced migration and invasion, which are both biological hallmarks of glioma cells. The combination of TTFields with irradiation or the alkylating agent, temozolomide (TMZ), resulted in additive or synergistic effects, and the O6-methyl-guanine DNA methyltransferase status did not influence the efficacy of TTFields. Importantly, TMZ-resistant glioma cells were responsive to TTFields application, highlighting the clinical potential of this therapeutic approach. In summary, our results indicate that TTFields induce autophagy, as well as necroptosis and hamper the migration and invasiveness of glioma cells. These findings may allow for a more detailed clinical evaluation of TTFields beyond the clinical data available so far.

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TTFields induce cell death in an intensity- and frequency-dependent manner in human glioma cells. The human long-term glioma cell (LTC) LN-18 or LN-229 or the human...
Tumor-treating fields in gliomas

M Silginer et al

Next, we aimed at assessing the independent manner. TTFields-induced cell death occurs in a caspase-dependent manner (Figure 1c). Treatment to a variable degree in terms of cell death induction composed of LTC and GIC, was sensitive to TTFields (Figure 1b). Importantly, the whole panel of glioma cell lines, also in the GIC lines ZH-161 and T-325 in an intensity-dependent manner (Figure 1b, bottom, Supplementary Figure 1b). Importantly, the effects on migration and invasion exceeded those predicted from the loss of viable cells at the same time (Supplementary Figure 3a). These data were further corroborated using a scratch wound-healing assay, which showed reduced migration and growth toward the center of the gap of TTField-exposed LN-18 or LN-229 cells (Figure 4c). Importantly, TTFields also interfered with the migration of the GIC lines T-325 and ZH-161, which are thought to be of particular importance for tumorigenesis and tumor recurrence (Figure 4d). Again, the effects on migration of GIC were more prominent than those expected from loss of viability (Supplementary Figure 3b).

TTFields act in an additive or synergistic manner with irradiation or TMZ. As radiation therapy and alkylating chemotherapy with TMZ are part of the standard treatment for glioblastoma patients, we assessed the activity of TTFields in combination with these treatment modalities. In acute cytotoxicity assays, mostly additive effects were observed in LN-18 cells when TTFields were combined with TMZ or irradiation, whereas synergistic effects were seen in LN-229, T-325 or ZH-161 cells by either combination (Figure 5a). Analyses of clonogenic survival in limiting dilution assays revealed synergistic effects of TTFields in combination with irradiation in LN-18, as well as T-325 cells, and with TMZ in LN-229 and ZH-161 cells (Figure 5b). Hence, depending on the cell line, the combination approach and the read-out, the effects may be additive or synergistic, however, never antagonistic in the models studied here.

It is well known that the DNA repair protein O6-methylguanine DNA methyltransferase (MGMT) influences the antitumor activity of the alkylating agent, TMZ, in vivo and in vitro.11,12 Thus, we determined a possible impact of the cells’ MGMT status on their sensitivity to TTFields. To this end, we used genetically engineered cells with modulated MGMT expression. LN-18 cells with a silenced MGMT gene or LN-229 cells overexpressing MGMT displayed similar sensitivity to TTFields as their wild-type counterparts. Thus, TTField-induced cell death seems to be independent from the MGMT status (Figure 6a, Supplementary Figure 3c). Virtually all patients relapse or progress during or after TMZ therapy, which encouraged us to analyze whether TTFields exert antitumor activity against TMZ-resistant glioma cells, which had been generated by repeated exposure to TMZ.13
Figure 1  TTFields induce cell death in an intensity- and frequency-dependent manner in human glioma cells. (a) LN-18, LN-229, T-325 or ZH-161 cells were left untreated or exposed to TTFields (TTF; 2 V/cm) at the indicated frequencies for 48 h. Viable cell counts were obtained using trypan blue exclusion (*P < 0.05). (b) The human LTC LN-18 or LN-229 (top) or T-325 or ZH-161 GIC (bottom) were exposed to increasing intensities of TTFields as indicated for 48 h (LTC) or 72 h (GIC). Cell death was assessed by annexin V/PI staining. (c) The LTC LN-428, LN-319, A172, U87MG, T98G or LN-308 (top) and the GIC T-269, S-24 or ZH-305 (bottom) were exposed to TTFields (2 V/cm) or not for 48 or 72 h, respectively, and subsequently analyzed for cell death by annexin V/PI staining.
TMZ-resistant LN-18 or LN-229 cells were similarly sensitive to TTF-field-induced cell death as their parental counterparts (Figure 6b), which does not point to mechanisms of cross-resistance.

**Discussion**

Glioblastoma is a highly lethal brain tumor with a median overall survival of approximately 16 months within clinical
trials. Since 2005, the standard of care for newly diagnosed glioblastoma has been maximal safe tumor resection, followed by irradiation with concomitant and maintenance chemotherapy with TMZ. However, the survival benefit conferred by TMZ is largely restricted to patients with tumors that harbor a methylation of the MGMT gene promoter.12,14 TTFields represent a novel treatment approach that has already been approved by FDA for newly diagnosed, as well as recurrent glioblastoma based on the results of two phase III trials.7,8 However, there is a mismatch between the encouraging clinical data and the biological effects of TTFields on tumor cells, which have only been poorly understood.

Here we demonstrate that TTFields potently induce cell death in all glioma cell lines. Importantly, TTFields treatment displayed strong activity against glioma cells with stem-like properties, too, which is of particular interest because of the potential contribution of this cell population to tumor recurrence and therapy resistance (Figure 1).15,16 Glioblastomas are characterized by extensive heterogeneity at a cellular and molecular level.17,18 Owing to advances in single-cell technology it became clear that each tumor contains multiple distinct populations of tumor cells with variation in the expression of a range of transcriptional programs, including oncogenic signaling, proliferation, immune response and hypoxia.19 Hence, inter- and intratumoral heterogeneity may contribute to the failure of targeted therapeutic treatment strategies, which greatly depend on the activity of certain molecular pathways.20

As TTFields are a physical rather than chemical treatment modality, they may be effective over a wider range of tumors with heterogeneous characteristics.5 The antitumor activity of TTFields may depend on parameters like the cell size that may determine the optimal frequency to induce cell death or the cell’s doubling time as TTFields is believed to act mainly during cellular division.5,21 Accordingly, GIC may be less responsive to TTFields than other tumor cells because of slower proliferation and higher heterogeneity in cell size.

In contrast to a report suggesting that HCT-116 colon cancer cells undergo apoptosis upon TTFields treatment,22 we did not observe caspase activation, a hallmark of apoptosis, in the cell lines tested in this study. However, our experiments revealed an important role for autophagy and necroptosis in TTField-induced cell death (Figure 2). Electron microscopy further supported the induction of autophagy, whereas no cellular changes typically associated with apoptosis were detected. Thus, the type of cell death induced upon TTFields treatment may differ between tumor entities and cell lines. In line with other studies, we observed an increased number of nuclear aberrations in TTField-treated cells that finally result in cell death (Figure 3).22,23 Moreover, we noticed an accumulation of nuclear actin filaments, which has been attributed a consequence of cellular stress and may have a protective role.24

Glioblastoma is characterized by highly infiltrative growth, which precludes complete surgical resection and thus contributes to its aggressive phenotype.25 Upon exposure to TTFields, we found aberrant changes in the shape of glioma cells (Figure 3), which may indicate altered cell motility.26 Accordingly, we observed reduced migration and invasion of TTField-treated glioma cells (Figure 4). In line with these data, reduced metastatic spread of solid tumors to the lungs by TTFields treatment has been reported and may be a result of impaired formation of microtubule-based processes in migrating cells.3,26,27

The combination of TTFields with irradiation or TMZ showed additive or synergistic effects supporting the clinical combination of these treatment modalities as done for TMZ in one trial (Figure 5).28 Similarly, it has been reported that combining TTFields with drugs, such as paclitaxel or doxorubicin, may result in reduced tumor cell proliferation and viability in preclinical models.28,29 In our experiments, altering MGMT expression did not influence the efficacy of TTFields, and TMZ-resistant glioma cells remained responsive to TTFields treatment suggesting that the mechanisms of resistance toward TMZ, which may involve MGMT but also other mediators13 do not overlap with a putative resistance to TTFields (Figure 6). Accordingly, TTFields may be particularly attractive for the majority of glioblastoma patients with tumors that are unlikely to benefit from TMZ treatment because of the unmethylated MGMT promoter. These findings are in line with data demonstrating that TTFields are also active against mitoxantrone- or doxorubicin-resistant breast cancer cells that overexpress ABC transporters. Moreover, it was shown that TTFields re-sensitizes drug-resistant tumor cells to chemotherapeutic agents, a finding that was interpreted as TTField-induced changes on the integrity of the cytoskeleton and microtubules, as well as mitochondria distribution.29

In summary, TTFields represent a novel therapeutic approach that has entered late-stage clinical development in glioblastoma patients. Our data set reveals that TTFields induce autophagy and necroptosis and interfere with the migration and invasion of glioma cells in vitro. The additive or synergistic effects of TTFields in combination with irradiation or TMZ, as well as their ability to induce cell death in TMZ-resistant glioma cells may represent a biological rationale for the further clinical evaluation of TTFields in glioma patients.

**Materials and Methods**

**Cells and reagents.** The human LTC lines LN-18, LN-229, LN-428, LN-319, A172 and LN-308 were kindly provided by Dr. N de Tribolet (Lausanne, Switzerland)

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**Figure 2** TTFields induce cell death via autophagy. (a) LN-18, LN-229 or ZH-161 cells were left untreated or exposed to TTFields (TFF; 3 V/cm), staurosporine (Stauro, 1 μM), zVAD-fmk (10 μM) or combinations thereof for 6 h. DEVD-amc cleaving activity was determined fluorometrically (**P < 0.01**). (b) Whole-cell lysates of LN-18, LN-229 or ZH-161 cells, untreated or exposed to TFF (3 V/cm) or staurosporine (1 μM) for 48 h, were analyzed for full-length and cleaved caspase-3 and Lc3A/B protein levels. Actin was used as a loading control. (c) LN-18 or ZH-161 cells were left untreated or exposed to TTFields (TFF; 3 V/cm) or Stauro (1 μM) for 48 h and then monitored by electron microscopy. Autophagosomes are indicated with white arrows, white arrowheads point to mitochondria with swollen matrices, the white star to the dilated endoplasmatic reticulum, black arrows to membrane blebs and black arrowheads to condensed DNA along the nucleus (scale bar, 2 μm). (d) LN-18 or ZH-161 cells were treated with 3-methyladenine (3-MA, 1 mM) for 60 min followed by TTFields (2 V/cm) or not for 72 h. Cell death was assessed by annexin V/PI staining. (e) LN-18 or ZH-161 cells were treated with necrotatin-1 (Nec-1; 100 μM) for 60 min followed by TTFields (3 V/cm) or not for 72 h. Cell death was assessed by annexin V/PI staining.
Figure 3  TTFields interfere with cell cycle progression. (a) LN-18 or LN-229 cells were exposed to TTFields (2 V/cm) for 24 or 48 h as indicated. Cell cycle analysis was performed by flow cytometry and cell cycle distribution is shown in bar graphs, as well as flow cytometry profiles. (b) LN-18 cells were left untreated or exposed to TTFields (2 V/cm) for 24 h. Actin (phalloidin staining, green), beta III tubulin (red) and nuclei (DAPI, blue) were analyzed by immunofluorescence. Different magnifications are shown. An isotype control for anti-beta III tubulin is included at the bottom. Accumulations of nuclear actin are indicated with arrows, arrowheads point to altered cell shape and stars to nuclear aberrations (scale bar, 10 μm)
Figure 4  TTFields reduce migration and invasion of glioma cells. (a and b) LN-18 or LN-229 cells were left untreated or exposed to TTFields (2 V/cm) for 24 h. Subsequently, modified migration/matrigel invasion Boyden chamber assays were performed to analyze migration (a) or invasion (b). Data are expressed as mean cells per field of vision (FoV) and exemplary photographs of migrated or invaded LN-18 or LN-229 cells are shown (scale bar, 100 μm). (c) A scratch wound-healing assay was performed during which LN-18 or LN-229 cells were left untreated or exposed to TTFields (2 V/cm). After 24 h, the gap distance was imaged on a microscope and representative images are shown (scale bar, 100 μm). (d) T-325 or ZH-161 cells were left untreated or exposed to TTFields (2 V/cm) for 24 h. Subsequently, a modified migration Boyden chamber assay was performed. Data are expressed as mean cells per FoV and exemplary photographs of migrated cells preexposed to TTFields or not are shown (scale bar, 100 μm). (**P<0.01)
Tumor-treating fields act synergistically with irradiation or TMZ to reduce acute and clonogenic survival. (a) LN-18, LN-229, T-325 or ZH-161 cells were left untreated or irradiated (LN-18, LN-229, 3 Gy; T-325 and ZH-161, 5 Gy) followed or not by TTF-fields (2 V/cm) for 24 h (left panel). Alternatively, the cells were exposed to DMSO control or TMZ (LN-18, 100 μM; LN-229, 5 μM; T-325, 200 μM; ZH-161, 25 μM) paralleled by TTF-fields exposure (2 V/cm) for 24 h or not (right panel). After another 48 h, viable cells were counted by Trypan blue exclusion test (*P<0.05; **P<0.01, relative to control cells). (b) Cells, treated as in a, were seeded at the indicated densities and clonogenic survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 14 days.

**Figure 5** TTF-fields treatment. To study the effects of TTF-fields on cell cultures in vitro, we used the inovitro system, a laboratory research device provided by Novocure (Haifa, Israel). A cell suspension containing 20,000 cells in 200 μl medium was seeded as a drop in the center of a glass coverslip (20 mm diameter; Thermo Scientific, Waltham, MA, USA) within a ceramic dish. After attachment of the cells,
2 ml of medium were added and the ceramic dishes were placed onto a base plate connected to two pairs of electrodes perpendicularly to each other and linked to a sinusoid function generator and an amplifier in order to generate alternating electric fields. The electric field intensities are expressed in V/cm root mean square.

Migration and invasion. A cell suspension containing 40 000 or 80 000 viable cells pretreated with TTFields or not for 24 h before migration or invasion, respectively, was added to the upper well of transwell migration inserts or to BD BioCoatTM MatrigelTM invasion chambers (pore size: 8 μm, BD Biosciences, Franklin Lakes, NJ, USA). In the lower well, 700 μl of NIH-3T3 cell-derived conditioned medium were used as a chemoattractant. After 16 h at 37 °C and 5% CO2, the cells were fixed in ice-cold methanol for 10 min and stained with Mayer’s alum hematoxylin for 60 min. Inserts were mounted in glass slides and nine fields per sample were counted on a microscope.

Scratch wound-healing assay. Cells were seeded on Thermanox coverslips (Thermo Scientific). At confluency, two straight lines in perpendicular direction were scratched into the monolayer using a 1 ml pipette tip. Cells were washed twice to remove detached cells and then grown in serum-containing medium for additional 24 h in the absence or presence of TTFields. The gap distance was imaged on a microscope.

Immunoblot analysis. Whole-cell lysates of LN-18 puro or MGMTsi, or LN-229 neo or MGMT cells were analyzed for MGMT expression by immunoblot. Actin was used as a control (top). The cells were exposed to TTFields (2 V/cm) or not for 48 h and analyzed for cell death by annexin V/PI staining. LN-18 parental or TMZ-resistant, or LN-229 parental or TMZ-resistant cells were exposed to TTFields (2 V/cm) or not. After 48 h cell death was analyzed by annexin V/PI staining

Viability assay. Cells, treated as indicated, were detached with accutase and resuspended in PBS. The number of viable cells was assessed by counting the cells with the Trypan blue dye exclusion method using a Vi-CELL Cell Viability Analyzer (Beckman Coulter, Nyon, Switzerland).

Clonogenic survival assay. The cells were pretreated as indicated and then seeded at the indicated densities in 96-well plates, followed by observation for 7–14 days. As a surrogate marker of viability, metabolic activity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (Sigma-Aldrich).

Annexin V/propidium iodide (PI) flow cytometry. For cell death analysis, cells were treated as indicated, followed by resuspension in annexin buffer (10 m M HEPES, 140 m M NaCl, 2.5 m M CaCl2, pH 7.4) and staining with Pacific blue-labeled annexin V (Biolegend, San Diego, CA, USA) and PI (Sigma-Aldrich) for 15 min at room temperature in the dark. Samples were analyzed by flow cytometry. Annexin V- or PI-positive cells were counted as dead cells (either apoptotic or necrotic), and the remaining cells were designated the surviving cell fraction.

Cell cycle analysis. Cells were fixed in ice-cold 70% ethanol for 1 h on ice, subsequently washed, stained with a solution containing 0.5 mg/ml PI, 1 mg/ml RNase A (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 30 min at 4 °C, washed and then analyzed by flow cytometry.

Caspase activity assay. Glioma cells, exposed to TTFields or not, were grown in phenol red-free medium. Subsequently, the cells were lysed and exposed to 12.5 μM of the fluorescent substrate DEVD-amc (Bachem AG, Bubendorf, Switzerland) for 1 h. Caspase activity was assessed using a Mithras microplate reader (Berthold Technologies, Bad Wildbad, Germany).

Immunofluorescence. Cells, grown on glass-coverslips, were exposed to TTFields or not for 24 h, and then fixed with 4% paraformaldehyde. Subsequently, the
Transmission electron microscopy. The cells including supernatant were fixed in a 2x fixation solution (2.5% glutaraldehyde and 1.6% formaldehyde in a 100 mM sodium cacodylate buffer, pH 7.4, final concentration), dehydrated in a graded ethanol series and embedded into Epon. Sections of 60 nm were imaged with a Tecnai Spirit transmission electron microscope (FEI, Hillsborough, OR, USA).

Statistical analysis. Data are presented as means and S.D. The experiments shown were commonly repeated three times. For some studies, representative experiments are shown. Analysis of significance was performed using a two-tailed Student’s t-test (Excel, Microsoft, Redmond, WA, USA) (*P<0.05, **P<0.01).

Conflict of Interest
MW has received research grants from Actelion, Alpinia Institute, Bayer, Isarna, MSD, Merck Serono, Piquor and Roche and honoraria for lectures or advisory board participation from Celldex, Isarna, Magforce, MSD, Merck Serono, Pfister, Roche and Teva. RS is the principal investigator for the pivotal clinical trials on TTFields in newly diagnosed and recurrent glioblastoma. RS served as an advisor to Novocure (non-remunerated), and has received travel assistance for trial associated activities, data review and data presentation at scientific meetings. PR has received honoraria for advisory boards and lectures from BMS, Roche, MSD, Novartis and Molecular Partners. MS declares no conflict of interest.

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