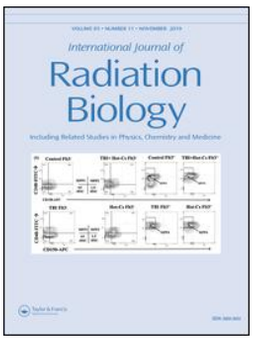

International Journal of Radiation Biology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/irab20

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To cite this article: Narasimha Kumar Karanam & Michael D. Story (2020): An overview of potential novel mechanisms of action underlying Tumor Treating Fields-induced cancer cell death and their clinical implications, International Journal of Radiation Biology, DOI: 10.1080/09553002.2020.1837984

To link to this article: https://doi.org/10.1080/09553002.2020.1837984

Published online: 29 Oct 2020.

INTERNATIONAL JOURNAL OF RADIATION BIOLOGY
https://doi.org/10.1080/09553002.2020.1837984

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REVIEW

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An overview of potential novel mechanisms of action underlying Tumor Treating Fields-induced cancer cell death and their clinical implications

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ABSTRACT

Traditional cancer therapy choices for clinicians are surgery, chemotherapy, radiation and immune therapy which are used either standalone therapies or in various combinations. Other physical modalities beyond ionizing radiation include photodynamic therapy and heating and the more recent approach referred to as Tumor Treating Fields (TTFields). TTFields are intermediate frequency, low-intensity, alternating electric fields that are applied to tumor regions and cells using noninvasive arrays. TTFields have revolutionized the treatment of newly diagnosed and recurrent glioblastoma (GBM) and unresectable and locally advanced malignant pleural mesothelioma (MPM). TTFields are thought to kill tumor cells predominantly by disrupting mitosis; however it has been shown that TTFields increase efficacy of different classes of drugs, which directly target mitosis, replication stress and DNA damage pathways. Hence, a detailed understanding of TTFields' mechanisms of action is needed to use this therapy effectively in the clinic. Recent findings implicate TTFields' role in different important pathways such as DNA damage response and replication stress, ER stress, membrane permeability, autophagy, and immune response. This review focuses on potentially novel mechanisms of TTFields anti-tumor action and their implications in completed and ongoing clinical trials and pre-clinical studies. Moreover, the review discusses advantages and strategies using chemotherapy agents and radiation therapy in combination with TTFields for future clinical use.

ARTICLE HISTORY
Received 5 August 2020
Revised 23 September 2020
Accepted 12 October 2020

KEYWORDS
TTFields; GBM; DNA damage; replications tress; novel combination therapy

Introduction

Cancer is one of the deadliest diseases as it caused 8.8 million deaths in 2015 according to the World Health Organization statistics. Standard cancer treatment options, such as surgery, chemotherapy, radiation therapy, and immunotherapy (Morgensztern and Govindan 2010; Morgensztern et al. 2010), are commonly used in the clinic, either as standalone therapies or in various combinations. However, despite this multitude of options, survival rates for patients with advanced stage cancers are very low (www.cancer.net). The dual specter of poor prognosis and an unfavorable therapeutic index calls for novel therapeutic interventions and combined therapy modality options to improve overall survival rates in patients. Hence, the cancer research field remains dynamic and is ever evolving to improve existing therapies and discover new modalities of cancer therapy.

Emergence of Tumor Treating Fields (TTFields) as a new physical modality of cancer therapy

The scientific community has shown an increasing interest in the biological effect of external electrical fields on cells. Grosse and Schwan (1992) showed that steady state transmembrane voltage can be induced in spherical cells by an external alternating field. Polarization induced by alternating current (AC) may affect cells in a frequency-dependent manner by orienting, deforming, and moving them. Low frequencies below 1 kHz can stimulate nerve, muscle, heart, and other tissues through membrane depolarization. Stimulatory effects gradually decrease when the frequency of the alternating electric fields increases above 1 kHz, because the response time of the cells' excitable processes is too slow to follow the higher frequency. Higher frequency fields above 1 MHz generate heat due to dielectric loss to disrupted membranes and can cause electroporation and cell death, depending on the field strength (Markx 2008). Consequently, frequencies commonly used in medical treatments for radio frequency tumor ablation are in the high MHz or GHz range (Figure 1).

Intermediate frequency electric fields alternate too quickly to cause tissue stimulation, and they generate minimal heat. Initially, intermediate frequency AC electric fields (KHz to MHz range) were thought to have no meaningful biological effects. The composition of biological molecules that contain positive and negative charges renders them dipolar the moment that alternating electric fields are applied. Because

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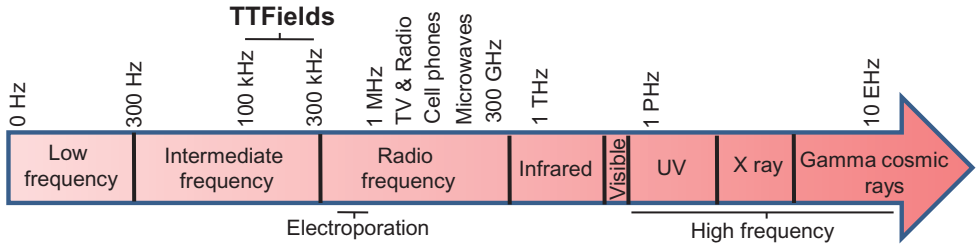

Figure 1. Frequency ranges of different applications across different frequency ranges. TTFields frequency falls in intermediate frequency range as indicated. Electroporation and more popular appliances such as TV, radio, cell phone and microwaves uses radio frequency range waves. Ionizing radiation frequency falls in the higher frequency range.

of this, it was hypothesized that, with precise spatial and temporal alignment, alternating electric fields at intermediate frequencies can disrupt cells undergoing mitosis. A decade ago, it was shown that electric fields in the intermediate frequency range of 100–500 KHz have an anti-mitotic effect (Kirson et al. 2004, 2007). This finding led to developing TTFields to selectively destroy cancer cells, which have a higher mitotic index than normal cells (Wenger et al. 2015). The advent of TTFields has revolutionized the treatment of solid, therapy-resistant primary and recurrent tumors (Giladi, Schneiderman, et al. 2014; Vymazal and Wong 2014; Wong et al. 2014; Inui et al. 2016). TTFields neither stimulate nerves/muscle, nor generate heat because of their relatively high frequency range and low intensity (Davies et al. 2013).

Generation of TTFields

Clinical generation of TTFields using the NovoTTF system

The FDA approved Optune (NovoCure), a TTFields-generating transducer array, for treating recurrent and newly diagnosed GBM in combination with temozolomide, and unresectable and advanced malignant pleural mesothelioma (MPM) in combination with platinum-based chemotherapy. Novocure Inc. developed a TTFields-generating first generation Optune device called the NovoTTF 100 A system, which is portable, can be used at home or work and which only minimally impacts normal daily activities. The second generation NovoTTF 200 A system, which is lighter and more compact than the first generation system, was approved by the FDA for clinical use in 2016. The NovoTTF 200 A system mainly consists of two components: (1) the electric field generator and (2) insulated transducer arrays. The transducer arrays are directly applied to bare skin to produce two perpendicular electric fields that alternate 200,000 times per second between positive and negative polarity (a frequency of 200 kHz) when treating glioblastoma (GBM) and 150,000 times per second (a frequency of 150 kHz) when treating malignant pleural mesothelioma (MPM). Continuous daily use of TTFields therapy for more than 18 hours (>75% of the time) and optimal placement of the transducer arrays properly are critical for good clinical benefit. The NovoTAL software program derives the optimal orientation of transducer arrays to deliver the highest intensity of TTFields to the site of the tumor. Mild to moderate scalp irritation and headache are the most common adverse effects related to using the system (Benson 2018).

Pre-clinical TTFields generation using the Inovitro system

The Inovitro™ system (NovoCure Ltd, Haifa, Israel) is used to apply TTFields to cultured cells. TTFields are generated using two pairs of electrodes placed perpendicularly on the outer walls of a ceramic Petri dish. Petri dishes containing trays are connected to an electric field generator, which generates low intensity electric fields at the desired frequencies in the medium. The orientation of the TTFields is rotated 90° every second, thus covering the majority of the orientation axes of cell divisions. The plate temperature is maintained at 37° C by placement in a refrigerated 19° C incubator to offset the heat generated by the inovitro system. The temperature is continuously monitored by two thermistors (Omega Engineering, Stamford, CT, USA) attached to the ceramic dish walls. Cells are grown on a cover slip inside the ceramic Petri dish (NovoCure Ltd, Haifa, Israel) and for exposure to TTFields for the times desired.

TTFields induced mechanisms of action

TTFields exposure leads to mitotic aberrations

Although several hypotheses have been proposed to explain the mechanistic basis of TTFields' anti-cancer effects, interfering with mitosis was the first mechanism of action identified. TTFields treatment generates intracellular heterogeneity that induces a dielectrophoretic movement of polar molecules such as tubulin and septin toward the region of higher field intensity, thereby affecting tubulin polymerization, septin localization and cytokinesis (Gonzalez and Remcho 2005). Due to their high mitotic index TTFields specifically target cancer cells, thus effectively sparing their normal counterparts. Dividing hematopoietic cells are unaffected because the surrounding muscle and bone create interference (Stupp et al. 2015).

TTFields inhibit human and rodent tumor cell proliferation and induce cell death (Giladi, Schneiderman, et al. 2015) by preventing the proper formation of the mitotic spindle apparatus and activating the mitotic spindle checkpoint (Kirson et al. 2004, 2007). This leads to instability of plasma membrane and blebbing that disrupts cytokinesis, eventually result in abnormal chromosome segregation, cell

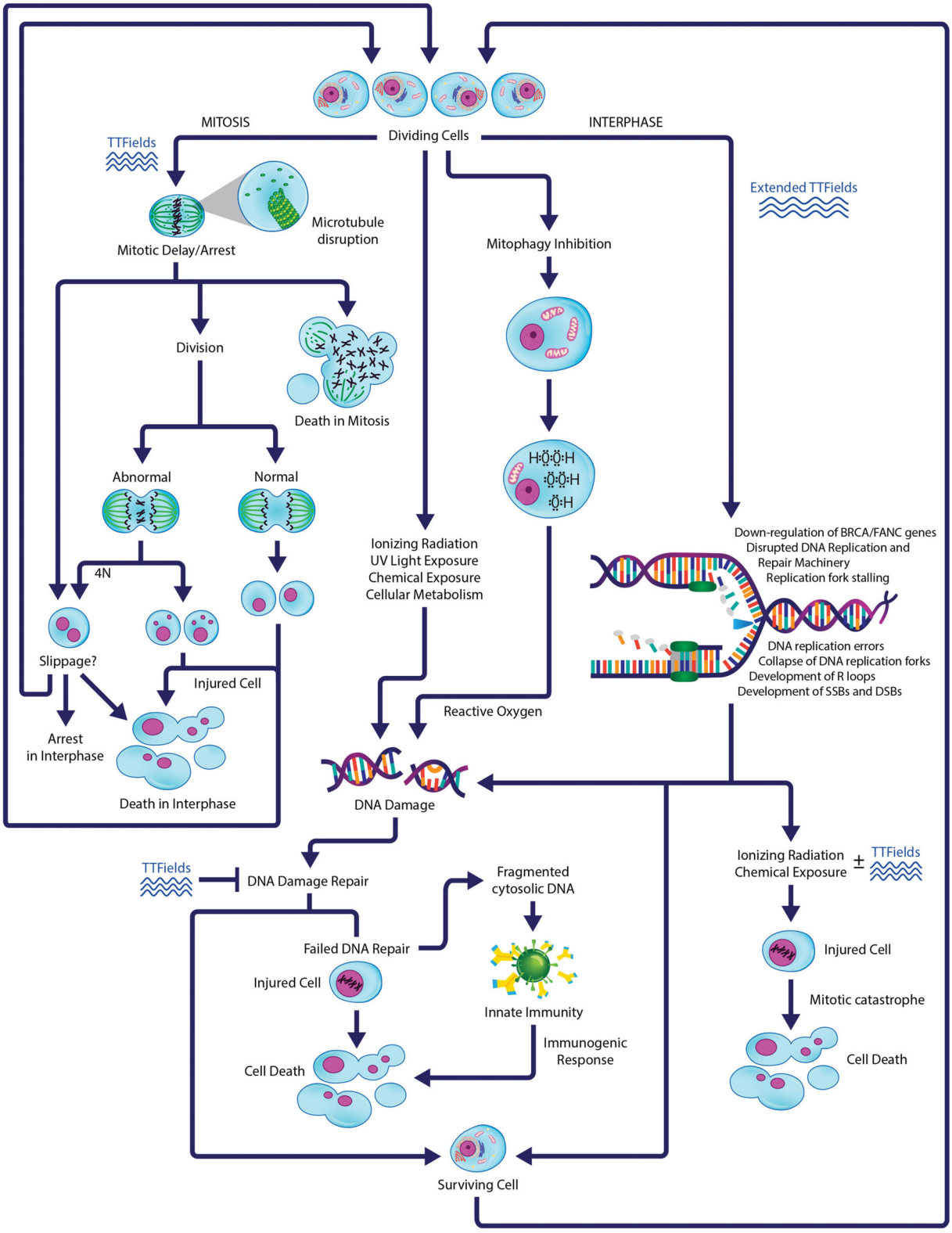

Figure 2. Schematic drawing of the TTFields influence on key events of mitosis and DNA damage, replication stress pathways in cancer cells. TTFields exposure affects mitosis process by increasing mislocalization of septins, mitotic spindle disruption and interfering with tubulin polymerization, which results abnormal cell division and chromosome segregation thereby leading to mitotic catastrophe and cell death. FA pathway genes expression decreases under TTFields treatment which are implicated in DNA damage repair and replication fork stabilization processes. Because of improper response to ongoing high replication stress and DNA damage it eventually lead to cell death. Surviving cells undergo prolonged TTFields exposure. UV: Ultra Violet; DSBs: Double Strand Breaks; SSBs: Single Strand Breaks; BRCA: BReast CAncer; FANC: Fanconi Anemia Complementation Group.

cycle arrest, and injured cell production; these cells subsequently undergo cell death/apoptosis (Gera et al. 2015). Earlier it was shown that sensitivity to TTFields treatment is p53 status dependent (Gera et al. 2015); but recent results suggest that TTFields treatment induced biological effects are independent of p53 status (Giladi, Schneiderman, et al.

2015; Voloshin et al. 2016; Karanam et al. 2017). Katsir et al. (2020) conducted a meta-analysis of 41 different cancer cell lines including GBM, lung, ovarian, colorectal, pancreas and breast etc., by correlating the genetic background with TTFields response in terms of cytotoxicity and clonogenicity for all cell lines. They determined that the TTFields response in this large panel of tumor cell lines was independent of p53 mutation status.

TTFields inhibits DNA damage repair and induces replication stress

The increased efficacy of drugs affecting mitosis and spindle assembly checkpoint in combination with TTFields, identified in pre-clinical studies and clinical studies, can be explained by the established role of TTFields in mitosis, as mentioned earlier. However, TTFields in combination with other major drug classes such as pemetrexed, doxorubicin, temozolamide (TMZ), gemcitabine, and platinum-based compounds (Schneiderman et al. 2010; Giladi, Schneiderman, et al. 2014; Giladi, Weinberg, et al. 2014; Giladi, Lee, et al. 2015; Voloshin et al. 2016; Kessler et al. 2018), which primarily affect DNA damage and replication stress pathways, also showed improved efficacy. These results suggest that TTFields not only intervenes in the mitosis process but also affects other major pathways, which cumulatively contribute to the TTFields anti-tumor effect. Kim et al. (2016) showed increased γ-H2AX foci which is a marker of DNA damage upon TTFields treatment but did not provide a mechanistic explanation for their observation. Karanam et al. (2017) examined TTFields treatment-induced gene expression changes in a set of NSCLC cells, and a provided mechanistic reasoning behind TTFields-induced DNA damage. TTFields treatment decreases Fanconi Anemia (FA) pathway gene expression, which plays an important role in DNA damage and repair, may contribute to TTFields-induced cell death. TTFields exposure results in increased DNA damage and delay DNA repair kinetics over time after ionizing radiation (IR) exposure. TTFields treatment alone increased the frequency of chromatid type aberrations and number of γ-H2AX foci, besides slowing the repair kinetics of double-strand breaks (DSBs) induced by IR. Karanam et al. proposed that TTFields treatment generates a conditional vulnerability, BRCAness (Turner et al. 2004), due to the downregulation of the BRCA1/2 genes. Giladi et al. (2017) also described that TTFields exposure slowed the repair kinetics of radiation- or chemo agents-induced DNA damage.

Interestingly, TTFields exposure in and of itself was shown to produce γ-H2AX foci, which is a marker of DNA damage as well as a marker for stalled replication forks, suggesting that TTFields not only delay DNA damage repair, but also induces replication stress. TTFields treatment downregulates the expression of *MCM6* and *MCM10* genes, essential components of the DNA replication complex and members of the FA pathway genes, leading to an elevated number of chromatid type aberrations. Furthermore, as part of the induction of replication stress, there is a decrease in the length of newly synthesized DNA and an increase in R-loop formation (Karanam et al. 2018, 2019). Mitosis and DNA damage pathways are tightly regulated through feedback mechanisms. By monitoring temporal gene expression changes associated with regulators of mitosis and DNA damage pathways, Karanam et al. showed that mitotic aberrations and DNA damage events while certainly linked to one another likely also occur independent of each other. These results established the role of TTFields in DNA damage repair and replication stress pathways. Key events in mitosis and DNA damage and replication stress pathways that are affected by TTFields are shown schematically in Figure 2.

TTFields upregulate autophagy and induce immunogenic cell death

TTFields-treated C57BL/6 mice inoculated with malignant melanoma cells and New Zealand rabbits implanted with VX-2 kidney tumors developed a lower number of lung metastases per tumor cross-section than controls (Kirson, Giladi, et al. 2009). A mononuclear cell infiltration was observed around and within metastases, and the extent of this cell infiltration was more profound in TTFields-treated animals. Immunohistochemical staining for lymphocyte subsets revealed that TTFields treatment induced a significantly higher CD4, CD8, and CD45 T cell count than controls, suggesting a T cell-mediated immune response in rabbits. Interestingly, an abundant intra-tumoral cell infiltration was observed though most of the immune cell infiltration was seen in the peri-tumoral location (Kirson, Giladi, et al. 2009). Post-hoc analysis of a phase III clinical trial comparing TTFields vs best physician's choice (BPC; Stupp et al. 2012) provided an opportunity to study the effect of dexamethasone, an anti-inflammatory and immunosuppressive drug. Patients who received a lower dose of dexamethasone ($<$4.1 mg/day) in combination with TTFields exhibited better overall survival (OS) than patients who received a higher dose of dexamethasone ($>$4.1 mg/day) in combination with TTFields. These results support the role of immune competence in the effectiveness of TTFields treatment. In addition, a significant correlation between overall survival and T-lymphocyte counts was observed in patients treated with TTFields in combination with dexamethasone (Wong et al. 2015). In support of a potential enhanced immune response in tumors, TTFields-treated cells showed sign of endoplasmic reticulum (ER) stress leading to calreticulin translocation to the cell surface, and to the release of chromatin binding protein HMGB1 and ATP. TTFields treatment stimulates phagocytosis by dendritic cells (DCs) and maturation of DCs under co-culture conditions (Voloshin et al. 2018). All of these results together suggest that TTFields treatment induces a T-cell mediated anti-tumor immune response.

Cells exposed to TTFields undergo autophagy and necroptosis-mediated cell death associated with increased numbers of autophagosomes, dilated ER, and abnormal mitochondrial structures (Silginer et al. 2017). TTFields treatment was shown to increase cellular granularity by accumulating larger acidic lysosomal pools. TTFields exposure increases the number of autophagosomes and

upregulates autophagy in GBM cells (Shteingauz et al. 2018). Interestingly, TTFields-induced autophagy depends on AMPK activation and inhibition of this pathway increases cell susceptibility to treatment. These results suggest that glioma cells upregulate autophagy when exposed to TTFields as a survival mechanism, rendering the cells resistant to therapy. Therefore, inhibiting autophagy could be exploited from a therapeutic standpoint.

TTFields increase cancer cell membrane permeability and activates calcium channels

Chang et al. (2018) recently showed that exposure to TTFields increase the number and size of holes on GBM cancer cell membranes. Exposure to TTFields not only makes GBM cells more permeable to small substances, as small as 4 kDa, but also more permeable to substances as large as 20 kDa, but not greater than 50 kDa. Interestingly, this phenomenon was not observed in normal human primary dermal fibroblasts (PCS-201). Moreover, this effect can be modulated with the duration of cell membrane permeability dependent upon the length of TTFields exposure. Increased cancer cell permeability may have clinical implications such as increased uptake of chemotherapeutic agents which would be especially important when considering the potential to open up the blood–brain barrier in the treatment of GBM (Salvador et al. 2020).

TTFields exposure was also shown to induce calcium signals in a dose-dependent manner by activating L-type calcium channels (CACNA1C) in GBM cells (Neuhaus et al. 2019). Those results suggest that the pharmacological blockade of calcium channels with agents like benidipine and nifedipine may augment the effects of TTFields exposure. Summary of important findings are listed in Table 1.

Clinical trials

Completed clinical trials in GBM

Two clinical trials for GBM, EF-11 and EF-14, have been completed to date. The EF-11 trial was conducted in patients with recurrent GBM with OS as a primary end point (Stupp et al. 2012). The efficacy of TTFields as a monotherapy (median OS 6.6 months) was similar to that of the best physician's choice (BPC) arm (median OS 6.0 months). However, TTFields therapy exhibited less frequent systemic toxicities and much better quality of life compared to BPC therapy. Post-hoc analysis revealed that patients whose compliance was $\geq$75%, that is a minimum of 18 hours per day, achieved a median OS of 7.7 months. For patients whose compliance was less than 75%, that is less than 18 hours per day, the median OS was only 4.5 months (Kanner et al. 2014; Vymazal and Wong 2014).

The EF-14 clinical trial compared TTFields with adjuvant TMZ and TMZ monotherapies in patients with newly diagnosed GBM with progression free survival (PFS) and median OS as primary end points (Stupp et al. 2015). The median OS for patients treated with TTFields plus TMZ was significantly higher (19.6 months in intent to treat population and 20.5 months in as per-protocol population) than that for patients treated with TMZ monotherapy (16.6 months in intent to treat population and 15.5 months in as per-protocol population). Although TTFields treatment did not cause any systemic toxicities relative to chemotherapy alone, mild to moderate skin irritation was observed in 43% and severe skin reactions in 2% of patients. These TTFields-associated dermatological toxicities may be managed prophylactically (Lukas et al. 2017). Recent mature data from the EF-14 clinical trial showed a significantly higher median OS in patients treated with TTFields plus TMZ (median OS 20.9 months) than in patients treated with TMZ alone (median OS 16.0 months; Stupp et al. 2017).

STELLAR clinical trial in MPM

TTFields were recently approved for treatment of unresectable locally advanced or metastatic malignant pleural mesothelioma (MPM). 80 patients were recruited to the STELLAR phase 2 clinical trial to assess the efficacy of TTFields in combination with standard of care chemotherapy, pemetrexed plus cisplatin or carboplatin (Ceresoli et al. 2018). In this trial, patients treated with TTFields plus pemetrexed and either cisplatin or carboplatin responded better (median OS of 18.2 months and median PFS of 7.6 months) when compared to historical control data from patients treated with pemetrexed and either cisplatin or carboplatin (median OS of 12.1 months and median PFS of 5.7 months). No serious adverse effects were reported besides mild to moderate skin irritation in 46% of patients and grade 3 skin irritations in 5% of patients.

Other ongoing clinical trials

Several clinical trials are currently being conducted in different anatomic settings (Wang et al. 2019), including the advanced stage trials described below.

The LUNAR phase II clinical trial was conducted in 41 patients with inoperable stage IIIB and IV NSCLC who had tumor progression after at least one line of chemotherapy (pemetrexed). An overall median survival of 13.4 months was reported in patients treated with TTFields plus chemotherapy, with only device-related adverse events such as mild to moderate contact dermatitis (Pless et al. 2011). The LUNAR pivotal phase III clinical trial with an expected enrollment of 534 patients is ongoing to assess the efficacy of TTFields in combination with the immune checkpoint inhibitor anti-PD-1 or docetaxel in patients with advanced inoperable stage IV NSCLC (Weinberg et al. 2019).

The PANOVA phase II clinical trial was conducted in 40 patients with newly diagnosed locally advanced or metastatic PDAC either with TTFields + gemcitabine or TTFields + gemcitabine and nab-paclitaxel. The median PFS was 8 months and the median OS was 14.9 months in patients who were treated with TTFields plus gemcitabine. The median PFS was 12.7 months and the median OS was not reached in patients who had received TTFields + gemcitabine and nab-paclitaxel (Rivera et al.

Table 1. Summary of recent important findings regarding the mechanism of TTFields' cell killing for different cancer cell lines.

Study	Journal and year	Cancer type	Outcome of study
Neuhas, E. et al.	Cancers 2019	Human GBM cell lines	TTFields activate L type calcium channels (CACNA1C); calcium channels inhibition augment TTFields effects.
Shteingauz, A. et al.	Cell Death and Disease 2018	Human GBM, rat glioma cell lines	TTFields treatment upregulates AMPK-dependent autophagy, which serves as a survival mechanism
Chang, E. et al.	Cell Death Discovery 2018	human GBM and PCS-201 cell lines	TTFields reversibly increase membrane hole size and number there by permeability.
Jo, Y. et al.	Cell Death Discovery 2018	Melanoma – Patient-derived primary cells and melanoma cell lines	TTFields cause selective damage to cancer cells, but spare normal cells. TTFields treatment does not damage normal tissue in mice.
Kessler, A. et al.	Cell Death Discovery 2018	GBM – human GBM cell lines	TTFields' anti-proliferative effect is enhanced by combination with spindle assembly checkpoint inhibitor MPS1-IN-3.
Giladi, M. et al.	Rad Oncol 2017	GBM – human GBM cell lines and mouse model	TTFields enhance RT efficacy in GBM cell lines by impairing repair of IR- or chemical-induced DNA damage. Phantom model studies showed an increase in the received dose just below the arrays. Mouse model studies revealed no significant difference in body weight loss and increase in inflammation, edema, hemorrhage, and fibrosis in groups irradiated with or without transducer arrays placed on the skin.
Karanam, N.K. et al.	Cell Death and Disease 2017	NSCLC – NSCLC cell lines	TTFields slow down IR-induced DNA damage repair kinetics by downregulating the BRCA1 pathway. TTFields synergistically enhance IR-induced cell killing. First study to describe changes in TTFields-induced gene expression.
Voloshin, T. et al.	Int J of Cancer 2016	Ovarian cancer – Ovarian cancer cell lines and mouse model	TTFields enhance the efficacy of paclitaxel in ovarian cancer cells and mice.
Kim, E.H. et al.	Oncotarget 2016	GBM – human GBM cell lines	TTFields increase DNA damage; the effect of TTFields combined with IR is synergistic. TTFields combined with IR synergistically suppress cell migration and invasion.
Inui, T. et al.	Anti Cancer Res 2016	Case report – NSCLC patient treated with GcMAF, SDT and TTFields	Combination of GcMAF immunotherapy, SDT, and ozone therapy with TTFields can be used for NSCLC patients with few adverse effects.
Giladi, M. et al.	Scientific Rep 2015	Ovarian, Lung, Pancreas, Cervical, Breast, and GBM cancer cell lines	TTFields decrease polymerization of microtubules and induce formation of multinuclear cells and chromosome aneuploidy. TTFields treatment's efficacy depends on cell lines' doubling time and treatment duration.
Gera, N. et al.	PLOS One 2014	Breast, Cervical and Colon cancer cell lines	TTFields inhibit septin localization to the anaphase spindle midline and cytokinetic furrow, and cause aberrant mitotic exit, depending on p53 mutational status.
Giladi, M. et al.	Seminars in Oncology 2014	NSCLC – NSCLC cell lines and mouse models, LLC1 cells, and KLN205-T1 injected into C57BL6	TTFields provide additive efficacy benefit when combined with chemotherapy agents, i.e. pemetrexed, cisplatin and paclitaxel, both *in vitro* and *in vivo*.
Giladi, M. et al.	Pancreatology 2013	Pancreatic cancer cell lines and hamster pancreatic adenocarcinoma PC-1.0 cells injected into syrian hamsters	TTFields increase cell volume and decrease clonogenicity of pancreatic cancer cells. TTFields show an additive effect when combined with chemotherapy agents, i.e. gemcitabine, 5FU, irinotecan, and panclitaxel, *in vitro* and *in vivo*.
Schneiderman, R.S. et al.	BMC Cancer 2010	MDR cell lines – ovarian, breast cancer cell lines	TTFields alone and in combination with chemotherapy agents, i.e. doxorubicin and paclitaxel, significantly reduce MDR cells' viability.
Kirson, E. et al.	BMC Med Physics 2009	Breast, glioma cell lines and VX2 kidney tumor model in rabbits and patient data	TTFields increase efficacy and sensitivity of chemotherapy agents, i.e. paclitaxel, doxorubicin, and cyclophosphamide, and DTIC in breast and glioma cancer cell lines as well as in VX2 kidney tumor animal model in rabbits. TTFields in combination with temozolomide significantly increase OS and PFS in 20 GBM patients.
Kirson, E. et al.	Clin Exp Metastasis 2009	Melanoma lung metastasis model in mice and VX2 kidney tumor model in rabbits	TTFields significantly decrease tumor volume and metastatic spread of solid tumors to the lung *in vivo*.
Kirson, E. et al.	PNAS 2007	GBM, breast, NSCLC, melanoma cancer cell lines, B16F1 mice melanoma and intracranial F-98 rat glioma models, and patient data	TTFields reduce proliferation of breast, GBM, NSCLC, and melanoma cell lines. Rat glioma model experiment suggests that TTFields decrease tumor growth. Ten patients receiving TTFields showed better OS and PFS than historical controls, with a few adverse effects.
Kirson, E. et al.	Cancer Res 2004	Human (melanoma, glioma, lung, prostrate, breast) and rodent tumor cell lines, and melanoma (B16F1 in C57BL/6) and colon (CT-26 in BALB/c) cancer syngeneic mouse models	TTFields treatment decreases various cancer cell lines' growth rate through disruption of mitosis. TTFields decrease tumor growth volume in mice without damaging surrounding normal tissue.

Table 2. Summary of completed and ongoing important clinical trials incorporating TTFields with different combination therapies.

Clinical trial name	Disease	Combination therapy agent	Reference
EF-11 (NCT00379470)	**Recurrent GBM**	**Best standard of care**	Stupp et al. (2012)
EF-14 (NCT00916409)	**Newly diagnosed GBM**	**Temozolomide**	Stupp et al. (2017)
STELLAR (NCT02397928)	**Unresectable locally advanced or metastatic malignant mesothelioma**	**Pemetrxed and cisplatin or carboplatin**	Ceresoli et al. (2018)
LUNAR phase III (NCT02973789)	Inoperable stage IV NSCLC	Anti-PD1 or docetaxel	Weinberg et al. (2019)
PANOVA phase III (NCT01971281)	Locally advanced unresectable pancreatic adenocarcinoma	Gemcitabine and nab-paclitaxel	Rivera et al. (2019)
INNOVATE phase III (NCT03940196)	Platinum resistant ovarian cancer	Paclitaxel	Vergote et al. (2018)
METIS phase III (NCT02831959)	1–10 newly diagnosed brain metastasis from NSCLC	Best standard of care	Mehta et al. (2019)
HEPANOVA phase III (NCT 03606590)	Locally advanced liver cancer	Sorafenib	Grosu et al. (2020)
TRIDENT (NCT03869242)	Newly diagnosed GBM	Concomitant radiation therapy and temozolomide	Shi et al. (2020)
PriCo TTF Phasel/II	Newly diagnosed GBM	Prior and concomitant radiation therapy and temozolomide	Glas et al. (2018)

Completed clinical trials are marked bold. GBM: Glioblastoma; NSCLC: Non-small cell lung cancer.

2019). The PANOVA-3 trial is a pivotal phase 3 clinical trial with an expected enrollment of 556 patients will assess the efficacy of TTFields in combination with standard of care gemcitabine and nab-paclitaxel in newly diagnosed, locally advanced, unresectable pancreatic adenocarcinoma.

The INNOVATE phase II single arm clinical trial tested the safety and efficacy of TTFields in combination with paclitaxel given weekly in 31 patients with recurrent and platinum-resistant ovarian cancer. The median PFS was 8.9 months whereas the median OS was not reached (Vergote et al. 2018). The INNOVATE-III trial is a pivotal randomized phase III clinical trial that tests the efficacy and safety of TTFields in combination with paclitaxel in patients with platinum resistant ovarian cancer.

The METIS trial is a pivotal phase III clinical trial that assesses the efficacy of TTFields in combination with standard of care in patients with 1–10 newly diagnosed brain metastases from NSCLC (Mehta et al. 2019).

HEPANOVA is a prospective phase II clinical trial in which the overall response rate of TTFields is tested along with the standard of care, sorafenib, in patients who were recently diagnosed with locally advanced liver cancer (Grosu et al. 2020).

TRIDENT is an ongoing international phase III clinical trial which is intended to compare the efficacy of standard radiation therapy plus temozolomide with the triple combination of radiation therapy and temozolomide plus concomitant TTFields in newly diagnosed GBM patients (Shi et al. 2020).

PriCoTTF trial is a phase I/II clinical trial, which will evaluate the safety and efficacy of TTFields initiated prior and concomitant to combined radiation and temozolomide therapy in newly diagnosed GBM patients (Glas et al. 2018).

A list of completed and ongoing clinical trials in different cancer settings incorporating TTFields with respective combination therapies are provided in Table 2.

Rational application of TTFields in combination therapies

Targeting mitosis

Anti-mitotic agents are highly selective and effective because the loss of cell cycle control is a hallmark of cancer. Mitosis is a complex and elaborate process, but it is also the shortest and most fragile phase of the cell cycle. The whole cell cycle is tightly regulated through several checkpoints to ensure elimination of mitotically defective and severely damaged cells by triggering mitotic catastrophe and apoptotic cell death or senescence processes. Several studies have reported that TTFields exposure results in the accumulation of cells in the G2/M phase of the cell cycle, suggesting that the G2/M checkpoint may be triggered to prevent cells from prematurely entering mitosis. The major cell cycle control mechanism in mitosis is the spindle assembly checkpoint (SAC), which will induce prolonged mitotic arrest to assure that accurate chromosomal segregation takes place. Giladi, Schneiderman, et al. (2015) observed such prolonged mitotic arrest in cells exposed to TTFields and Kessler et al. (2018) recently showed that TTFields increase the efficacy of the SAC check point inhibitor MPS1-IN-3 in GBM cells.

Microtubule targeting agents (MTAs) disrupt microtubule (MT) dynamics and induce prolonged mitotic arrest that can eventually lead to cell death. There are two classes of MTAs: (1) microtubule stabilizing agents such as paclitaxel and docetaxel; and (2) microtubule destabilizing agents such as vincristine and vinblastine. TTFields exposure increases the depolymerized microtubule fraction, suggesting a disruption of the mitotic spindle assembly apparatus (Giladi, Schneiderman, et al. 2015). TTFields treatment in combination with paclitaxel or doxorubicin increased cell killing in multi-drug resistant (MDR) cancer cells without elevating the intracellular concentration of the drugs (Schneiderman et al. 2010); TTFields decreased cellular proliferation and survival, and increased sensitivity of taxol, in a hamster model of pancreatic cancer (Giladi, Schneiderman, et al. 2014); and TTFields decreased cellular proliferation and increased the cell killing potency of pemetrexed, cisplatin, and paclitaxel in NSCLC cells both in vitro and in vivo (Kirson, Schneiderman, et al. 2009; Giladi, Weinberg, et al. 2014).

The in vitro and in vivo data described above provide the rationale for the combination therapies being tested in the INNOVATE-3, PANOVA, and LUNAR clinical trials where MTAs such as paclitaxel, docetaxel, and nab-paclitaxel are being used.

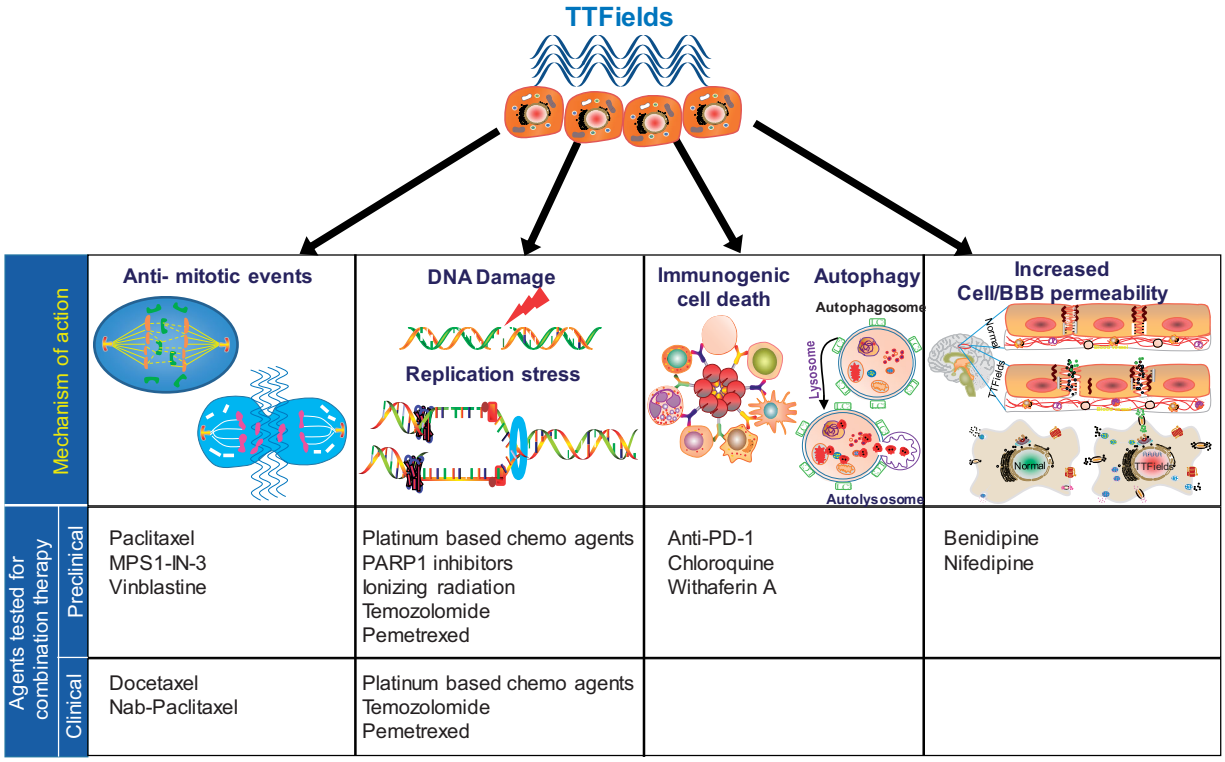

Figure 3. Summary of different molecular mechanisms underlying TTFields biological action. Different TTFields' mechanisms of action that are implicated in various combination therapies together with chemotherapeutic agents tested in pre-clinical and clinical settings. BBB: Blood Brain Barrier; MPS1-IN-3: inhibitor of MonoPolar Spindle 1; PARP1: Poly ADP Ribose Polymerase 1; PD1: Programmed cell Death protein 1.

Targeting DNA damage and replication stress pathways

Genome instability due to defects in the DNA damage response (DDR) is another hallmark of cancer. Exogenous and endogenous factors cause constant stress to the mammalian genome, and the failure of protective mechanisms leads to genomic instability. Faults in the DNA replication process during S phase leads to mutations or DNA replication blockage that in turn leads to DNA damage. This effect slows DNA synthesis and/or causes replication fork stalling/collapse is called replication stress. Many commonly used cancer chemotherapeutic agents target replication stress, which is thought to be the primary cause of genome instability (Gaillard et al. 2015). Cancer cells maintain unrestrained proliferation by keeping low to mild levels of replication stress with defective DDR and loss of cell cycle checkpoints. Normal cells maintain genome stability through the coordinated actions of DDR and cell cycle checkpoints. Defects in DDR and mild to low levels of replication stress are unique to cancer cells (Zhang et al. 2016) and, therefore, can be therapeutically exploited.

To target TTFields-induced replication stress, a combination of chemotherapy drugs with TTFields, which can further increase replication stress was tested. Platinum compounds (cisplatin) are known to generate DNA inter- and intra-strand crosslinks between nucleotide residues (Wang and Lippard 2005; Fu et al. 2012). The intra-strand crosslinks occur on same strand cause DNA lesions in the template strand, and the inter-strand crosslinks which occur between opposite strands lead to defects in DNA unwinding, which is the first essential replication step (Deans and West 2011; Sale et al. 2012). TTFields synergistically enhances cisplatin NSCLC cell killing when the treatments are combined, probably because TTFields inhibit the repair of DNA crosslinks produced by cisplatin exposure (Karanam et al. 2018). Dysfunction of BRCA genes predispose cells to chemo agents that target single-strand break (SSB) repair pathways, such as PARP inhibitors, result in 'synthetic lethality' (Kaelin 2005). TTFields synergistically enhance the efficacy of the PARP inhibitor olaparib and IR individually, and the triple combination further increases the synergy of cell killing (Karanam et al. 2018).

By retrospectively examining a recently completed phase III clinical trial, Lu et al. (2019) showed that the triple combination therapy of bevacizumab, irinotecan, and temozolomide plus TTFields significantly improved the overall survival of patients with recurrent GBM. Irinotecan and temozolomide were found to increase replication stress in accordance with recent findings that suggested TTFields' role in DDR and replication stress. Moreover, these recent findings provide a rationale for added synergistic effects observed with chemotherapeutic agents such as irinotecan, doxorubicin, gemcitabine, 5-FU, cyclophosphamide and DTIC via increased replication stress when used in combination with TTFields (Giladi, Schneiderman, et al. 2014; Giladi, Weinberg, et al. 2014; Giladi, Lee, et al. 2015; Voloshin et al. 2016; Kessler et al. 2018). Increased replication stress may also have played a role in the recent STELLAR trial where TTFields, pemetrexed and cisplatin or carboplatin were combined to treat pleural mesothelioma. Here, overall survival was increased from 12.1 months to

18.2 months with no increase in systemic toxicity (Ceresoli et al. 2018).

Targeting DNA damage and repair after ionizing radiation (IR)

Therapeutic doses of ionizing radiation elicit complex cellular responses through several signaling pathways including DNA damage, mitotic catastrophe, apoptosis, autophagy, immune response and senescence (Maier et al. 2016). Because IR is known to primarily induce complex DNA damage, Karanam et al. (2017) studied its combinatory effect with TTFields and found that TTFields synergistically increase the cell killing ability of IR in NSCLC cells. Giladi et al. (2017) reported that TTFields treatment delays DNA damage repair caused by IR in glioma cells and in a rat model. Kim et al. (2016) showed that IR given before TTFields treatment also synergistically increases the cell killing effect, and also decreases migration and invasion in GBM cells. However newly identified mechanisms of TTFields' action led to the hypothesis that applying TTFields would first develop a conditional lethality environment, making cells more susceptible to agents such as IR or, in the case of BRCA1 downregulation, to PARP inhibition or cisplatin. Indeed, by delivering TTFields before IR treatment, Karanam et al. (2019) showed that IR was more effective than IR treatment before TTFields exposure. Moreover, TTFields application may be beneficial in cases where IR treatment cannot be applied due to the risk of local tissue toxicity. These results strongly suggest that using TTFields may be effective when given either before or concomitantly with IR.

Targeting immune modulation

Immunotherapy, one of the latest and rapidly advancing cancer therapy modalities, relies on augmenting tumor immunity using various strategies. Of all the different immunotherapies, the use of antibodies against immune checkpoint inhibitors (e.g. anti-CTLA4 and anti-PD-1) has been successful for some cancer patients and as a result, anti-checkpoint immune therapy has been approved by the FDA in a number of different settings. Commonly used to treat neurological symptoms caused by GBM, dexamethasone has been shown to affect patient antitumor immunity via global immunosuppression and a retrospective analysis of a phase III clinical trial revealed that the clinical efficacy of dexamethasone was increased when combined with TTFields. OS correlates included $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocyte counts (Wong et al. 2015). These data strongly suggest that TTFields-induced stimulation of antitumor immunity contributes to its therapeutic efficacy. Furthermore, it was recently shown that combining TTFields with the immune checkpoint inhibitor anti-PD-1 notably increased therapeutic efficacy by inducing autophagy and ER stress, resulting in immunogenic cell death (Voloshin et al. 2018) and that inhibiting autophagy using chloroquine was shown to enhance TTFields' anti-tumoral activity (Shteingauz et al. 2018). However, considering the double-edged sword of autophagy based upon the stage of cancer, autophagy inhibitors in combination with TTFields needs to be fully understood.

A summary of different molecular mechanisms of TTFields biological action and agents tested in preclinical and clinical settings are provided for in Figure 3.

Conclusions and future directions

TTFields are approved for the treatment of GBM and MPM but the fundamental mechanism of TTFields biological action is not known. One could speculate that because of the effect on tubulin due to the dipole moment generated by TTFields on mitotic cells, that the predominantly interphase effects described above could also be generated by altering the properties of key proteins based upon their charge or polarity. This might actually provide for changes in the activity of any number of proteins whose subsequent cascades of signaling are also altered leading to radiation or chemotherapy agent vulnerability and enhanced cell killing. Our current understanding of TTFields' mechanisms of action suggests that TTFields affect multiple pathways such as cell cycle, karyokinesis, the DNA damage response, DNA replication, and immune response, the identification of which are nearly all from in vitro experiments with little in vivo validation (Figure 3). Moreover, as a physical modality, as described above, TTFields may be comparable toionizing radiation in that they both induce more systemic effects that might render cancer cells more sensitive to different classes of drugs in combination therapy. TTFields' limited efficacy as a monotherapy in the clinic should be noted in this context (Stupp et al. 2012), however because of the vulnerabilities generated by TTFields exposure, with minimal adverse effects on normal cells or tissues, the potential for the use of TTFields as a neoadjuvant therapy is of paramount importance. Already, 'concommitant' application has revealed vulnerabilities that rationally explain the outcomes seen in combination therapies that can likely be enhanced if TTFields were used in advance and during radiation or chemotherapy treatments.

Acknowledgements

The authors thank Dr. Jonathan Feinberg and Dr. Damiana Chiavolini for editing the manuscript.

Disclosure statement

MDS was the recipient of a sponsored research agreement from Novocure Ltd. MDS is also the recipient of AACR- Novocure Tumor Treating Fields research grant. NKK is the recipient of AACR-Novocure Career development award for Tumor Treating Fields research. MDS and NKK received travel grants to attend conferences. NKK and MDS are listed as inventors on US patent applications entitled 'Treating tumors using Tumor Treating Fields combined with a PARP inhibitor' filed 28 March 2018 and which is pending final approval as well as a provisional patent entitled 'Combination therapeutics using Tumor Treating Fields' filed 23 March 2020.

Funding

This work was supported by American Association for Cancer Research.

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