Modulated electro-hyperthermia promotes doxorubicin cytotoxicity in a C26 colorectal carcinoma cell line model

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**Objective:** Modulated electro-hyperthermia (mEHT), a non-invasive complementary treatment to radio- or chemotherapy, can also induce a selective tumor damage by itself based on cell stress and heat shock at ~42°C. Here we studied the molecular background of mEHT tumor destruction and its combination with doxorubicin treatment using an *in vitro* model.

**Methods:** Coverslip cultures of C26 mouse colorectal adenocarcinoma cell were treated with mEHT at 42°C (2x60 min with 120 min breaks) either alone or in combination with the topoisomerase inhibitor and DNA-intercalating 1 M doxorubicin (mEHT+Dox). Post-treatment stress response, cell death, apoptosis and proliferation related markers were detected using immunocytochemistry; complemented with resazurin viability assay, qPCR, flow-cytometry and clonogenic assay compared to non- (Ctrl) and doxorubicin (Dox)-treated control cultures.

**Result:** Modulated EHT induced the significant release of hsp70 and calreticulin proteins 24 h after treatment and reduced the tumor stem-cell related colonies 10-days post-treatment. Early (1-3h) after the significant decrease of the anti-apoptotic XIAP, BCL-2 and BCL-XL, and the elevation of the pro-apoptotic BAX and PUMA mRNA levels was detected. P21 transcripts were also significantly increased between the 1-9th h. From 24 to 48 h the progressive reduction of cell viability was seen accompanied by the occurrence of cleaved-caspase-3 positive tumor cells which was further augmented in combination with Dox. In line with this, mEHT caused major apoptotic cell death, which was significantly enhanced after combined mEHT+Dox treatment, while Dox alone dominantly caused necrosis. After 24h the nuclear phospho-p53(Ser15) protein levels were also significantly increased in all treated groups, while phospho-Akt(Ser473) levels were reduced but only in the mEHT and mEHT+Dox groups.

**Conclusion:** mEHT induced cell stress caused caspase-dependent programmed cell-death and inhibition of tumor-cell proliferation, possibly linked to p53 activated p21^{waf1} upregulation and the concomitant reduction of active Akt protein, which could normally inhibit p53 functions. This mEHT induced mechanism could potentiate the cytotoxic effect doxorubicin in C26 colorectal cancer cells.

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Introduction: Modulated electro-hyperthermia (mEHT, temozolomide-temozolomide), a non-invasive, proprietary treatment to radiotherapy, can induce a selective tumor damage by heat triggered on cell lines and animals. In this study, the molecular background of mEHT tumor destruction and its combination with doxorubicin treatment was investigated, using the colorectal tumor cell line C26.

Molecular and methods: Cultured colonies of C26 murine colorectal carcinoma cell line were treated with mEHT at 45°C (240 min) with 120 min breaks after 60 s of microwaves in combination with the topoisomerase inhibitor and DNA-interstrand cross linker, doxorubicin (doxorubicin + mEHT). Post-treatment stress response, cell death, apoptosis, and proliferation related markers were detected using immunochemistry, complemented with immunofluorescence assays, qPCR, flow cytometry and deoxyribose more or less compared to non-treated (Control) and doxorubicin (Dox)-treated control cultures.

Significant cell stress in C26 tumor cells 24 hours after mEHT treatment. (A) Western-blot and western immunofluorescence analysis of phosphorylated mitogen-activated protein kinases (MAPKs) (MAPK1) (B) and elevated intracellular p53 protein expression in doxorubicin-sensitive conditions. (C) Cell death was increased significantly in both the estrogen and mEHT treated groups compared to the control group. (D) Increased expression of p53-mRNA in the estrogen and mEHT treated groups compared to the control group.

Expression of apoptosis specific protein related genes in C26 tumor cells after mEHT treatment. (A) Significant induction of the proapoptotic BAX, BAX, BAX3, BAX4, BAX6, and BAX9 genes by treatment. (B) Significantly increased expression of the antiapoptotic BCL2 genes by treatment. (C) Significant induction of the proapoptotic BAX, BAX, BAX3, BAX4, BAX6, and BAX9 genes by treatment. (D) Significant induction of the proapoptotic BAX, BAX, BAX3, BAX4, BAX6, and BAX9 genes by treatment.

Conclusions: Here we introduce an in vitro mEHT treatment model of C26 colorectal cancer cell line useful for studying the molecular background of combining mEHT with other treatment modalities, showing less chemotherapy. Our results show that mEHT monotherapy can induce irreversible cell death through compensatory mechanisms of apoptosis and mEHT-mediated cell death in vitro, which are likely to be driven by p53 activation. Phosphorylated p53 and reduced phospho-AKT are known to promote p53 escape from Mdm2 control. In conclusion, mEHT seems to promote the ultimate and significant tumor destruction and to delay further through targeting efficient apoptosis, autophagy and cell cycle arrest induction by mEHT. A validation of this in vitro model the release of damage-mitochondrial proteins (DMM) signals the damage induced cells were also observed earlier in in vitro studies on C26 tumor lines. DMMs may prevent the accumulation of TDFs and autophagic p53 cells. Contributing to a secondary immunologically tumor cell death. This model can serve for pilot testing of mEHT combinations prior to comprehensive interventions in colorectal C26 cells using immune competent hosts.

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