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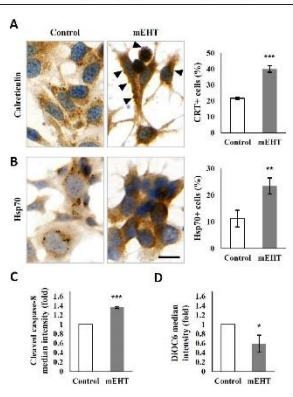
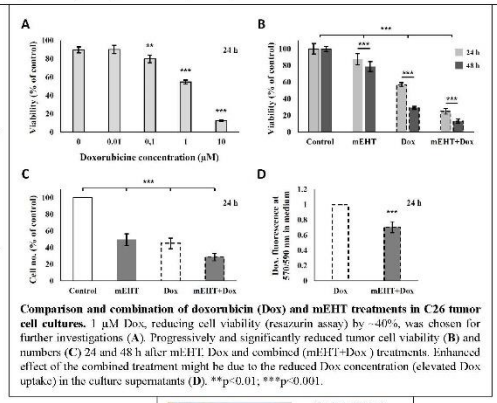
Introduction: Modulated electro-hyperthermia (mEHT, tradename: oncothermia), a non-invasive complementary treatment to radio- or chemotherapy, can induce a selective tumor damage by itself based on cell stress and heat shock at ~42°C. Here we studied the molecular background of mEHT tumor destruction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

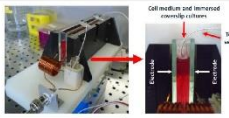
Materials and methods: Coverslip cultures of C26 mouse colorectal adenocarcinoma cell line were treated with mEHT at 42°C (2x60 min with 120 min breaks) either alone or in combination with the topoisomerase inhibitor and DNA-intercalating 1 μM doxorubicin (mEHT+Dox). Post-treatment stress response, cell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



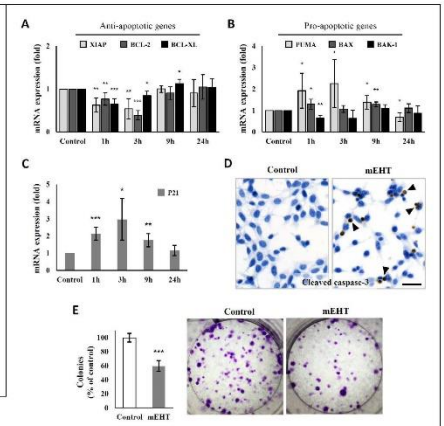
Signs of significant cell stress in C26 tumor cells 24 hours after mEHT treatment. Cytosolic release and cell membrane translocation of calreticulin with positive membrane blebs (arrowheads) (**A**). Elevated cytoplasmic Hsp70 reaction released from paranuclear vesicles (**B**). Scale bar: 20 μm. Significant increase of cleaved caspase-8 levels (**C**) and reduction of DiOC6 uptake by mitochondrial membranes (**D**) measured using flow cytometry indicate the induction of both the intrinsic and the extrinsic programmed cell death pathways, respectively. *p<0.05; **p<0.01; ***p<0.001.



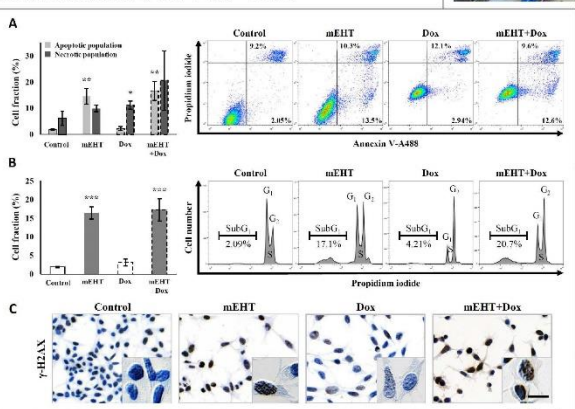
Comparison and combination of doxorubicin (Dox) and mEHT treatments in C26 tumor cell cultures. 1 μM Dox, reducing cell viability (resazurin assay) by ~40%, was chosen for further investigations (**A**). Progressively and significantly reduced tumor cell viability (**B**) and numbers (**C**) 24 and 48 h after mEHT, Dox and combined (mEHT+Dox) treatments. Enhanced effect of the combined treatment might be due to the reduced Dox concentration (elevated Dox uptake) in the culture supernatants (**D**). **p<0.01; ***p<0.001.



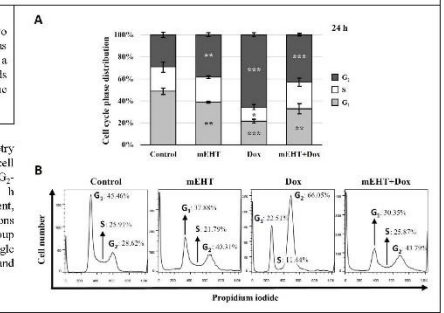
The *in vitro* applicator includes two plan-parallel copper condenser plates as the electrodes. Between the electrodes a medium containing chamber stands where the coverslip cultures and the thermo-sensors were put.



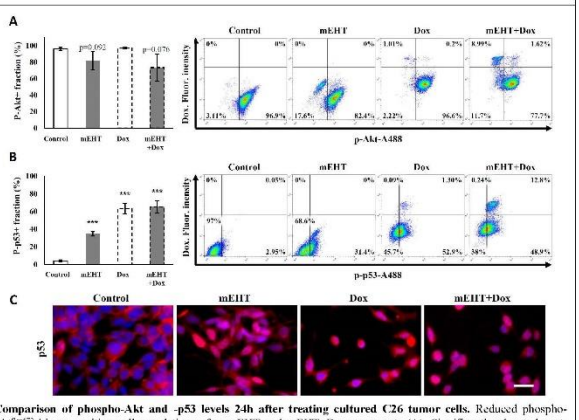
Expression of apoptosis regulation related genes in C26 tumor cells after mEHT treatment. Significant reduction of the anti-apoptotic XIAP, BCL-2, BCL-XL mRNA levels 1- and 3-hours post-treatment (**A**). Elevated pro-apoptotic PUMA mRNA levels at 1, 3 and 9 hours, and BAX levels at 1 and 9 hours after mEHT (**B**). Similarly increased temporal pattern of P21 mRNA levels to that of PUMA (**C**). In line with the apoptosis-promoting mRNA profile, cleaved caspase-3 protein expression (arrowheads) was significantly elevated 24 h after treatment as tested with immunocytochemistry (**D**). Scale bar: 100 μm. Significant reduction of colony-forming tumor progenitor-cell populations 10 days after mEHT treatment (**E**). *p<0.05; **p<0.01; ***p<0.001.



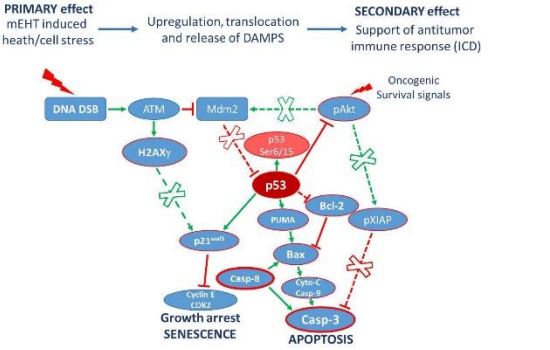
Comparison of the ratio of cell and DNA damage 24h after treating cultured C26 tumor cells. Significantly elevated apoptotic cell fractions after mEHT and necrotic cell fractions after Dox treatments and their additive, merged effect after combination (mEHT+Dox) therapy (**A**). Significant increase in subG$_1$ phase cell fractions both after mEHT and combined treatments refer to the apoptosis-related DNA damage (**B**), where DNA double strand breaks were indicated by upregulated H2AXγ granular positivity (brown) in cell nuclei using immunocytochemistry (**C**). Scale bar is 100 μM on the larger FOVs. *p<0.05; **p<0.01; ***p<0.001.



Summary graph (**A**) of flow cytometry analysis (**B**) of the treatment related cell cycle fractions. Significant but less G$_2$-phase cell cycle arrest seen 24 h after mEHT than after Dox treatment, where the sizes of G$_1$ and G$_2$ populations in the combined (mEHT+Dox) group show about the averages of the single treatments. *p<0.05, **p<0.01 and ***p<0.001.



Comparison of phospho-Akt and -p53 levels 24h after treating cultured C26 tumor cells. Reduced phospho-Akt^{Ser473} kinase positive cell populations after mEHT and mEHT+Dox treatments (**A**). Significantly elevated post-treatment phospho-p53^{Ser15} levels showing the same highest values after Dox and the combined treatments (**B**). Increased cytoplasmic to nuclear relocalization of phospho-p53^{Ser15} indicated p53 activation (**C**). Scale bar: 100 μM. ***p<0.001.



Conclusion: Here we introduce an *in vitro* mEHT treatment model of C26 mouse colorectal cancer useful for feasibility studies of the molecular background of combining mEHT with other treatment modalities, starting with Dox chemotherapy. Our results show that mEHT monotherapy can induce irreversible cell stress both through caspase-dependent apoptosis and p21^{waf1} mediated cell cycle arrest, which are likely to be driven by p53 activation. Elevated phospho-p53^{Ser15} and reduced phospho-Akt^{Ser473} are known to promote p53 escape from Mdm2 control. In combination, mEHT seems to promote the uptake and significantly potentiate tumor destruction and control by Dox through merging efficient apoptosis, necrosis and cell cycle arrest induction by mEHT and Dox.

As a validation of this *in vitro* model the release of damage associated molecular pattern (DAMP) signals from damaged cells were also observed like earlier in our *in vivo* studies on C26 tumor allografts. DAMPs may promote the accumulation of T-cells and antigen presenting cells contributing to a secondary immune-mediated tumor cell death. This model can serve for pilot testing of mEHT combinations prior to comprehensive investigations of allografted C26 cells using immune competent animals.

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