Extremely low-frequency magnetic fields significantly enhance the cytotoxicity of methotrexate and can reduce migration of cancer cell lines via transiently induced plasma membrane damage

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A B S T R A C T
Extra Low-Frequency Magnetic Fields (ELF-MFs) significantly enhance cellular uptake of methotrexate by inducing transient plasma membrane pores/damage. This enhanced ‘dose loading’ of methotrexate via the electromagnetically induced membrane pores leads to similar outcomes as the normal control while using significantly smaller therapeutic doses in vitro when compared to non-ELF-MF treated control. Approximately 10% of the typical therapeutic dose yielded similar results when used with ELF-MF. ELF-MFs increase PC12, THP-1 and HeLa proliferation in vitro (120% of the control). Analysis of adherent cells demonstrate significantly less migration towards an induced scratch injury (20 μm in 24 h when compared to a control). Our results suggest an important role for the use of ELF-MFs in the treatment of tumours that opens some new and exciting possibilities including using smaller therapeutic doses of chemotherapeutic agents and disrupting tumour metastasis.

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1. Background

One of the most significant challenges to medicine is the design and the delivery of therapeutic doses of drug that can be effectively targeted [1]. This is exemplified by many current strategies used for cancer therapy. A common technique to treat cancer is the use of chemotherapeutic drugs that are classified based upon their mode of action: alkylating agents, alkaloids, antitumour antibiotics, antimitabolites, topoisomerase inhibitors and antineoplastics [2]. These drugs have relatively short half-lives, typically between 30 min and 100 h, although this can vary [3]. Tumour cells have a limited time to engage with them and as a result sublethal anticancer drug regime(s) are delivered with insufficient lethal doses of the drug being taken up after administration [4]. The side effects of this type of therapy means that all body cells are liable to drug toxicity, leading to side effects such as peripheral neuropathy, hair shedding and damage to the immune system [5].

The role of biophysical techniques in cancer therapies have become increasingly important for the treatment and diagnosis of disease owing to significant innovations in the field of informatics coupled with new affordable hardware. This has led to important leaps in sustainable and rigorous treatments for disease in particular, with novel approaches to cancer [6].

Extremely low-frequency magnetic fields (ELF-MFs) have begun to capture the attention of researchers in the life sciences. They are charge carrying magnetic fields with a low density of magnetic flux or field density defined for this research as being in the μT range [7] and of a low frequency 10 – 300Hz [7]. They are emitted by electronic devices that use alternating currents. Alternating magnetic fields generated by alternating currents are used for driving automotive force and are characterised by a typical sinusoidal wave form moving from positive to negative and the rapid flipping of magnetic poles [8].

Magnetic fields carry charged particles such as electrons that can be used to move ‘heavy’ molecules, as for example with the Maxwell electromagnetic stress tensor on charged molecules [9]. Lines of magnetic force can penetrate through non-metallic media. When applied to cells, magnetic fields interact with charged cellular components such as phospholipids [10] and polar/charged proteins [11,12]. We have previously reported the induction of
transient membrane pores in cells, using ELF-MFs, that led to cellular/metabolic stress [7], with the influx of charged ions.

The role of magnetic fields and cancer progression has been reported with conflicting results [13,14] likely due to the diversity of tissue types studied responding differently to various strengths and/or frequencies of magnetic stimuli [15,16]. Studies conducted on cell lines derived from different tissues and organisms demonstrate numerous therapeutic or negligible effects [14,17]. Electromagnetic exposure of neuronal progenitor cells resulted in DNA damage and up-regulation of genes relating to programmed cell death [18,19]. These effects were transient and it was speculated that cellular mechanisms act to compensate for these damaging magnetic effects [14] to minimise them.

The novel research presented for the first time here investigates the potential of ELF-MFs to enhance the efficiency and potency of chemotherapy regimens. The research demonstrates an increase in the uptake of currently prescribed therapeutic doses of cytotoxic drugs in leukaemia (THP-1), pheocromocytoma (PC12) and cervix cancer (HeLa) cells. These cell lines were chosen to demonstrate the broad applicability of the technique used in this research to the broader scientific community. As a beneficial side effect, ELF-MFs are used to significantly inhibit the migration of adherent cancer cells in vitro. We propose a model for ELF-MF interaction with charged lipid components and the influx of membrane permeable and impermeable molecules.

2. Methods

2.1. Maintenance of cancer cell lines and stimulation with ELF-MF

THP-1, PC12 and HeLa were maintained as described previously in complete growth medium (RPMI 1640, 10% FCS, 1% penicillin/streptomycin) at 37 °C, 5% CO2. Prior to the cells were washed (× 2) with RPMI 1640 and suspended in CGM. The cells were assayed for their viability and number using ViaCount reagent on the Guava EasyCyte 8HT flow cytometer before being suspended at the required concentration (typically 5 × 10^5/mL) and dispensed into 12- or 24-well plates (Costar) as appropriate. 24 h after seeding the cells were then stimulated with 0.3 μM 6V A/C ELF-MFs for 30 min at 37 °C and cultured for 24/48 h upon which they were assayed using ViaCount (as before) to ascertain the cell number and viability.

2.2. ELF-MF effects cells treated with H2O2

THP-1 cells cultured in CGM were washed (× 2) by centrifugation at 160g for 5 min with RPMI 1640 and suspended at 5 × 10^5 cells/mL before being suspended in CGM and 1 mM H2O2. THP-1 monocytes were then stimulated with ELF-MFs (0.3 μT 6V A/C) for 30 min. The cells were immediately assayed for their viability using ViaCount reagent on a Guava EasyCyte 8HT flow cytometer. The ELF-MF stimulated cells were compared to H2O2 treated and untreated controls.

2.3. ELF-MF enhance the effects of methotrexate on THP-1 and PC12 cells

The cells were cultured in 24 well plate (Costar) at 1 × 10^5/mL supplemented with 10 μM methotrexate and subjected to 6V 10Hz ELF-MF field densities ranging between 0.1 and 0.6 μT. The viability was measured using ViaCount assay (confirmed using propidium iodide).

Cultured cells were washed and treated with Methotrexate 10 μM, 1 μM and 0.1 μM concentrations respectively and stimulated with 0.3 μT 6V A/C ELF-MFs for 30 min. The cells were cultured for 48 h with methotrexate, cells being assayed for viability and cell number at 24 h intervals.

THP-1 were cultured in complete growth media (CGM) containing 10% FBS RPMI and 1% PenStrep. Cells >95% viability were washed 2x by centrifugation 160g for 5 min and finally suspended in CGM at 1 × 10^6 cells/mL and dispensed into sterile 12 well plates (Costar). THP-1 cells were subjected to identical concentrations both with and without stimulation of ELF-MFs 0.3 μT 6V A/C. THP-1 within ELF-MFs were stimulated for 30 min at 37 °C and then incubated for 48 h at 37 °C in 5% CO2.

PC12 were grown in 12 well plates (Costar) to 1 × 10^6 cells/mL in CGM with >95% viability and supplemented with 1 μM, 0.1 μM and 0.01 μM concentrations of methotrexate for 48 h at 37 °C in 5% CO2. PC12 were then washed 2x with RPMI 1640 and removed by 0.25% Trypsin EDTA and washed in CGM to deactivate the trypsin before being analysed by flow cytometry using a ViaCount assay (according to manufacturer’s instructions) to assess both cellular proliferation and viability.

2.4. Migration assay

HeLa cells (>95% viability), cultured in CGM were grown to confluence in 6-well plates at 37 °C in 5% CO2. The adherent HeLa cells were ‘injured’ by scratching using a 200 μL pipette tip along the equator of the well causing a 1500 μm wide ‘clean scratch’, devoid of cells. The HeLa cells were washed × 2 in RPMI 1640 and suspended in 1 mL CGM. The injured HeLa cultures were viewed under bright field using a 1X81 Olympus microscope along the entire length of the scratch injury with a × 40 objective lens. The test wells were subjected to ELF-MFs as before and incubated at 37 °C for 30 min, as were the untreated control cells (not exposed to ELF-MF). All cells were further incubated at 37 °C (5% CO2) for a further 48 h.

After 24 h the cells were washed using RPMI and re-cultured in 1 mL CGM. The cells were photographed under bright field (as above). The scratch injury in each well was measured at 20 points that represented the smallest gap, at right angles along the entire scratch and the collected data was interpreted by GraphPad prism 7 statistical analysis. The control and test samples were then assessed for their viability and cell number using Guava Millipore ViaCount reagent (as before). The HeLa cells were washed using RPMI 1640 before 100 μL of 0.25% trypsin-EDTA was added to the wells and incubated for 3 min at 37 °C to detach the cells. Once detached the 0.25% trypsin-EDTA was deactivated by adding 900 μL of CGM and the cells were pelleted by centrifugation at 160g for 5 min. The HeLa cells were cultured in 1 mL RPMI 1640 and analysed using ViaCount reagent. After 48 h the procedure was repeated as at the 24 h time point.

Statistical analysis was performed using GraphPad prism 7 software. Independent samples (n = 20) t-test and Tukey’s post hoc were used to interpret the results. Significance stated p ≥ 0.05.

3. Results

The role(s) of ELF-MF in chemotherapy were investigated using both non-adherent and solid tumour models; THP-1 (monocytic leukaemia) and PC12 (neuroblastoma) respectively. Verification of the mechanisms first proposed in our previous publication [7] were confirmed, such that ELF-MF induces membrane damage whilst maintaining cell viability and increased cell proliferation over 24 h (Fig. 2A). The use of propidium iodide (PI) as a membrane exclusion dye demonstrates significant cell loading in THP-1 cells while stimulated with ELF-MF (~7.5%) *p ≤ 0.05. The stimulated cells took up significant levels of propidium iodide while exposed to ELF-MF, this immediately diminishes when removed from the...
Fig. 1. Alternating current (A/C) and direct current (D/C). (A) A/C current oscillate from positive to negative as a part of one complete oscillation, frequency (Hz) is the number of oscillations a wave has in 1 s (s). Amplitude (amp) is the size of the wave and voltage (v) is the potential difference across a circuit or electromotive force. An A/C current has a net neutral charge, D/C are only positive and therefore generate static magnetic fields. (B) Membrane charged and non-charged lipids are mixed, when A/C magnetic field is applied to the membrane then charged lipids align with the magnetic field.

Fig. 2. ELF-MFs stimulate increased proliferation of THP-1 and PC-12. (A) THP-1 stimulated with ELF-MF for 30 min (black) led to ~6 ± 1% uptake of PI. Membrane damage induced by ELF-MF are unstable: once THP-1 were removed from the stimulation by ELF-MF the membrane dye was unable to enter the cell (white). The results however demonstrate a consistent trend, an insignificant decrease in viability owing to ELF-MF damage, ≤1% for the duration of the experiment. (B) THP-1 cells were subjected with 0.3 μT ELF-MF for 30 min and their viability ascertained using ViaCount, either immediately afterwards (0 min) or after 30 min recovery period **p < 0.05 t-test. (C) THP-1 and PC-12 (1 × 10^5/mL) were exposed to ELF-MFs for 30 min and cultured at 37 °C 5% CO₂ for 24 h. The viability and cell number were quantified using Guava Millipore Viacount analysis. THP-1 cells showed both increased growth (white) ~11% higher than the control (black) and viability was maintained. PC-12 showed an increase in cell number (white) ~15% higher than the control (black) and viability was maintained. Cells were assayed using ViaCount and flow cytometry. *p ≤ 0.001 t-test.
electromagnetic field, PI continues to enter the cells (1–2%) (Fig. 2A) until their membrane integrity is fully restored 30 min later (Fig. 2B). This result is supported by our previous publication where we demonstrate that lysosomes fuse to the plasma membrane [7] restoring membrane integrity.

THP-1 and PC12 cancer models exposed to ELF-MF have the same membrane damage mechanisms leading to an immediate uptake in membrane exclusion dye PI. THP-1 cells stimulated with ELF-MFs and allowed to proliferate in culture for 24 h had a population ~11 ± 1% significantly larger than the un-stimulated control cells. PC12 that were stimulated with ELF-MFs and similarly allowed to proliferate for 24 h (as before) had a significant 15 ± 1% larger population than the un-stimulated control PC12 cells (Fig. 2C). The viability of both cell types remained normal, with no significant difference.

Using the membrane permeable analogue apoptotic inducer [20] H2O2 as a control demonstrated that THP-1 underwent a significant loss of viability ~67.2 ± 5% after a 30 min treatment (Fig. 3A). This phenomenon is well documented. When co-stimulated with 0.3 μT ELF-MF for the same duration, the cell viability significantly decreased to ~49.1 ± 3.6% (Fig. 3A). These results suggest that ELF MF stimulation facilitates H2O2-dependent cell death in cancer cells as its effect was enhanced nearly two-fold. These results were obtained using Guava Viacount and suggest that the cell death mechanism was apoptotic. We acknowledge that oxidative stress may account for decreased viability however this enhanced drop in viability was not observed in ELF-MF untreated cells.

Analysis of the effects of ELF-MFs on the viability and proliferation of THP-1 monocytes as a model for leukaemia was characterised by a dose dependent response across the field densities examined as described before [7]. This was used as an analogue of for Leukaemia. The magnetic field density (μT) was verified using an EMF tester gauss electromagnetic field meter TES-1390. The data confirms our previous finding [7] that 0.3 μT offers the most consistent and conservative decreases in viability ~21% and as a result a decrease in the cell population of ~1 × 10^4/mL or 10% (Fig. 3B). The differences between field densities were not statistically significant but were highly reproducible and indicated that ELF-MF stimulation does effect membrane integrity.

Using a MTX titration to establish the model our results show that adding ELF-MF to 10 μM methotrexate significantly reduces the viability of THP-1 cells, compared to treatment with methotrexate alone (Fig. 4A). In keeping with this observation, the combination of methotrexate and ELF-MF significantly increased the activity of methotrexate alone in PC12 cells (Fig. 4B). Notably this effect was apparent at even lower concentrations, compared to THP-1 cells.

HeLa cells were grown to confluence (~1 × 10^5/mL) in 12 well plates and the given a 1500 μm scratch along the equator of the
confluent culture and immediately washed with RPMI. Together with control cells not treated with ELF-MF cells were incubated for 48 h at 37 °C in 5% CO₂ and the scratch measured at 24 h intervals. HeLa (non-control) were stimulated with 0.3 μM 6 V A/C ELF-MFs for 30 min at 37 °C and 5% CO₂ and then removed from the ELF-MF and incubated at 37 °C and 5% CO₂ for 48 h, the scratch measured at 24 h intervals. Control (non-ELF-MF stimulated) and scratched HeLa cells showed significant cell migration and proliferation into the scratch by 24 h and by 48 h the scratch had significantly shrunk (Fig. 5A). However, HeLa treated with 0.3 μM 6 V A/C ELF-MFs showed little migration at 24 h and by 48 h although the scratch has shrunk, the edge of the scratch is still visible and ‘clean’ the measurements for the scratch were made along its entire length at multiple points (Fig. 5A).

HeLa migration into the wound site was measured at 24 h and 48 h (Fig. 5B). Surprisingly, control (non-ELF-MF treated) HeLa cells migrated into the wound and over 48 h the scratch healed ~68% (480 μm scratch) however when exposed to 0.3 μM 6 V A/C ELF-MF treated HeLa had migrated less over 48 h and the scratch healed ~56% (650 μm scratch) (Fig. 5A and B). The ELF-MF stimulated HeLa proliferated ~10.1% more than the control HeLa over 48 h, maintaining a high viability ~97% as seen with THP-1 and PC12 (Fig. 5C and Fig. 2C).

THP-1 treated with methotrexate and ELF-MFs lead to significant loss of viable cells (Fig. 5A), those results were obtained using 0.3 μM 6 V A/C ELF-MF, THP-1 were significantly affected by varying ELF-MF density (Fig. 2A and C) leading to a plateau of viability loss as field density increased. However, reproducing the experiment (Fig. 2A) and supplementing the media with 10 μM methotrexate produced results with no significant differences at all magnetic field densities tested. Nevertheless THP-1 subjected to 0.3 μM 6 V A/C ELF-MFs lead to ~1 x 10⁷/mL lower viable cells than the other densities tested (Fig. 3B) and ~2% lower population viability (Fig. 3C).

4. Discussion

The research presented in this paper demonstrates for the first time that ELF-MF (administered to cell lines in the μT range) may offer a key contribution in the treatment of cancer. ELF-MF significantly enhanced the in vitro effects, most likely the uptake of the chemotherapeutic methotrexate by these cells, (Figs. 2A and 4A). Charged molecules, in this instance membrane charged lipids can align with lines of magnetic force [10] that results in the formation of transient membrane pores or damage that is large enough to allow the passage of medium sized molecules where the magnetic field density is sufficient [7].

Using H₂O₂ as a model for cytotoxicity (Fig. 3A), the membrane offered some impedance to membrane permeable drugs [21]. H₂O₂ caused significant decrease in cell viability and this effect was enhanced by using H₂O₂ in conjunction with 0.3 μM 6 V A/C ELF-MF (Fig. 2A). This effect is likely multifactorial as H₂O₂ entered via induced membrane pores and produced superoxide’s as a result of ELF-MF stimulation.

THP-1 and PC12 were used as models for leukemic and solid mass cancers respectively. PC12 cell line as a model for neuronal tumour or an inaccessible solid mass tumour, the PC12 cells were not differentiated into neuromes for this purpose.

It was hypothesised that cytotoxic drugs’ death inducing potential would be enhanced when used in conjunction with ELF-MFs [22,23]. Methotrexate is a membrane permeable antifolate that ultimately blocks the synthesis of thymidine [21]. It was proposed that membrane pores could lead to increased cell loading of the drug by uptake through ELF-MF induced membrane pores along a concentration gradient (Fig. 1B). Indeed, it was found that ELF-MFs significantly enhanced the potency of cytotoxic drugs (Fig. 4A and B). Using the same concentration of methotrexate but stimulating with ELF-MFs there was a ~25% decrease in viable THP-1 and ~18% decrease in viable PC12. Methotrexate dose was reduced by 90% (1 μM) when stimulated with ELF-MF yielded statistically similar results to unstimulated therapeutic dose (10 μM) in vitro (Fig. 4).

We hypothesise that removal of the magnetic field closes the induced membrane damage leaving the enhanced concentration of methotrexate trapped within the cell (Fig. 2B). When used in conjunction with 0.3 μT 6 V A/C ELF-MFs, the potency of MTX is significantly enhanced, so much so that 1/10th dosage in the presence of ELF-MFs produced lower, but approximating that of the control regimen (no ELF-MF, 10 μM methotrexate) and therefore 10 μM methotrexate with ELF-MF produces significant effects in both lowering viability and cell number. Furthermore a 1/100 dose with ELF-MF also produces acceptable chemotherapeutic effects. These findings provide a promising model for further development as an enhancement of current cancer chemotherapy. This method allows a larger bio-available dose of methotrexate to enter the cell, so much so, that smaller therapeutic doses such as 1 μM can be administered with the added benefit of achieving larger intracellular concentrations.

HeLa were selected for scratch injury studies for their ease of culturing and relatively fast doubling time. Using a scratch injury model to investigate the effects of ELF-MF on cellular migration we demonstrated a reduced capacity for these cells to ‘heal’ (Fig. 5A and B). The induced scratch was 1500 μm at 0 h. At 48 h the control (non-ELF-MF treated) cells had reduced the size of the scratch to...
However the ELF-MF treated cells demonstrated significantly impeded migration, the scratch only reduced to ~850 μm. However the ELF-MF treated cells demonstrated significantly impeded migration, the scratch only reduced to ~850 μm.

Analysis by flow cytometry confirmed that the ELF-MF stimulated cells had a ~15% larger population but the cells had not migrated into the scratch. They proliferated much more, whereas un-stimulated cells migrated relatively quickly but did not proliferate to such an extent (Fig. 5A, B and C). The mechanism by which migration was inhibited was not elucidated, however A/C ELF-MFs...
have been reported to change cell migration velocity [24] by
damage of cell polarisation [25]. ELF-MFs significantly
enhanced the proliferative potential of the cell lines studied (Fig. 2C and 4C)
[26–28]. Furthermore, prolonged exposure of low frequency
pulsed magnetic fields caused permanent change in body tissues
[29].

Cell migration uses pseudopodia extending from the anterior of
the cell [30,31]. We speculate therefore that membrane damage
may have inhibited the formation of lipid rafts that lead to pseudo-
podia formation.

Other groups have however published conflicting results, that
magnetic therapy can increase wound healing through migration as
well as proliferation using 1 GHz ELF-MFs on fibroblasts [32].
Higher levels of ELF-MFs slow proliferation but increases differ-
entiation of osteoblasts [33]. DNA damage was not observed how-
ever nuclear pore formation was detected by propidium iodide
binding to DNA [7,16]. It is reasonable to assume that studies on the
ELF-MF report conflicting results as the potential biological effects
will vary between cell types and the strength, density, amplitude and
duration of the electromagnetic treatment [34].

The main limitation of this study were that the results were not
confirmed in vivo however the use of the three chosen cell lines
would partially address the applicability of the use of ELF-MFs to
enhance the effects of therapeutic doses of MTX in tumours. The
research did not fully identify the mode of action of the enhanced
effects of chemotherapeutic agents on cancer cells however did
identify the likelihood of enhanced uptake via induced membrane
damage [7]. We have previously shown that ELF-MFs can affect the
cell by inducing apoptosis [7]. Further research would identify
whether ELF-MFs generated super-oxides in vitro/in vivo. However
the research presented offers a firm foundation offering exciting
further research, including testing ELF-MF on various chemother-
apeutic and non-chemotherapeutic agents.

The exciting potential for this therapy is clear and easily appli-
cable. ELF-MFs of the correct charge and magnetic field density can
be targeted to cellular clusters or tissue in conjunction with
significantly smaller therapeutic doses of methotrexate. This
approach would minimise the cytotoxic side effects while
enhancing its delivery into targeted cancer cells. The beneficial
additional effect of potentially impeding cancer metastasis would
reduce the spread of cancer. The capacity of ELF-MF to enhance the
proliferation of the studied cell lines could support the treatment of
cancer further by creating a diminished pool of ‘local’ resources and
nutrients for the cells to consume. The targeted approach for drug
use with ELF-MFs reducing cell mobility would ‘buy’ time for ELF-
MF/cytotoxic drug combination therapy to diminish cancer cell
spread and growth. A repeated regime of methotrexate treatment
in conjunction with focused and directional ELF-MFs could result in
a greater degree of targeted cell death and an improved prognosis
and greatly diminished side effects from chemotherapy for the patient(s).

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Declaration of competing interest

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