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Regular paper

Curcumin augments the cytostatic and anti-invasive effects of mitoxantrone on carcinosarcoma cells *in vitro**

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Numerous adverse effects limit the applicability of mitoxantrone for the treatment of drug-resistant tumors, including carcinosarcoma. Here, we estimated the additive effects of mitoxantrone and curcumin, a plant-derived biomolecule isolated from *Curcuma longa*, on the neoplastic and invasive potential of carcinosarcoma cells *in vitro*. Curcumin augmented the cytostatic, cytotoxic and anti-invasive effects of mitoxantrone on the Walker-256 cells. It also strengthened the inhibitory effects of mitoxantrone on the motility of drug-resistant Walker-256 cells that had retained viability after a long-term mitoxantrone/curcumin treatment. Thus, curcumin reduces the effective doses of mitoxantrone and augments its interference with the invasive potential of drug-resistant carcinosarcoma cells.

Key words: carcinosarcoma; mitoxantrone; curcumin; apoptosis; motility

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INTRODUCTION

Mitoxantrone (1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino)ethylamino]-anthraquinone; MTX, Fig. 1A) is a derivative of anthraquinone, which inhibits the topoisomerase II activity. It also intercalates into DNA, thus interfering with the proliferation of cancer cells. Apart from cytostatic effects on cancer cells, MTX interferes with the physiology of normal cells, such as macrophages and lymphocytes (Kamm *et al.*, 2014). This results in liver dysfunctions, urinary tract infections, pneumonia and other respiratory tract inflammations (Rivera *et al.*, 2013). Collectively, the adverse effects of MTX impose restrictions on its therapeutic doses and limit the efficiency of MTX for the treatment of drug-resistant tumors. These restrictions can be overcome by combined therapies based on the concomitant application of cytostatic drug(s) and non-toxic biomolecules that sensitize tumor cells to cytostatics (Karp *et al.*, 2012; Koczurkiewicz *et al.*, 2013; Zhao *et al.*, 2015).

Numerous biological activities and a low systemic toxicity of curcumin (((1E, 6E)-1,7-bis (4-hydroxy-3-methoxyphenyl) hepta-1,6-dien-3,5-dione); CC; Fig. 1B) suggested the suitability of this biomolecule for a combined tumor therapy (Wang, 2013; Negi *et al.*, 2014). CC is abundant in *Curcuma longa* (turmeric) which, due to its anti-inflammatory, antibacterial and cytoprotective activity, is commonly used in traditional Indian medicine (Liu & Huang, 2012; Prasad *et al.*, 2014; Santos *et al.*, 2015). For instance, it exerts cytoprotective effects on hepatocytes and inhibits the proliferation of breast, lung and prostate cancer cells, being potentially responsible for a relatively low incidence of these tumors in India (Ide *et al.*, 2010; Chen *et al.*, 2014; Mehrabani *et al.*, 2015). CC also interferes with the progression of advanced colon cancers in the absence of any serious side effects (Sharma *et al.*, 2004) and increases the sensitivity of tumor cells to radiotherapy *in vitro* and *in vivo* (Shehzad *et al.*, 2013; Qian *et al.*, 2015). These observations open perspectives for the application of CC as an adjuvant that would reduce the effective doses and adverse effects of MTX, thus overcoming the restrictions of MTX application in tumor treatment.

Till now, MTX has been used in the treatment of breast, cervical and prostate cancers (Szwed, 2014). However, it has been reported as relatively ineffective against carcinosarcoma (Muss *et al.*, 1997, Kanthan & Senger, 2011). Carcinosarcomas occur most frequently in the female genital tract (5% of all female genital system cancers), where the clinical 5-year survival rate of patients is only about 50% (Bigby *et al.*, 2005; Shariftabrizi, *et al.*, 2015), although they may also develop in the bronchi (Carcano *et al.*, 2012) and in the prostate (Furlan *et al.*, 2013). Doxorubicin derivatives, cisplatin and paclitaxel are commonly applied in the carcinosarcoma chemotherapy (Kanthan & Senger, 2011), in combination with surgical intervention and radiotherapy (Alem *et al.*, 2014, Bigby *et al.*, 2005). However, the effectiveness of these agents decreases during the treatment of distant metastases. We presumed that MTX could still be considered in the treatment of patients with disseminated carcinosarcoma, provided that the MTX effects on tumor cells are strengthened by a concomitant administration of an adjuvant, such as CC.

To verify this notion, we estimated the dose-dependent effects of MTX, CC and of their cocktails on the Walker-256 cells. Carcinosarcomas are comprised of morphologically heterogeneous cells that exhibit both, epithelioid and sarcomatoid properties (Shariftabrizi *et al.*, 2015). The Walker-256 cells reflect the phenotypic diversity of carcinosarcoma because non-adherent, blebbing and lammellipodia-forming (LC), "mesenchymal" Walker-256 lineages have been propagated (Sroka *et al.*,

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Abbreviations: CC, curcumin; MTX, mitoxantrone

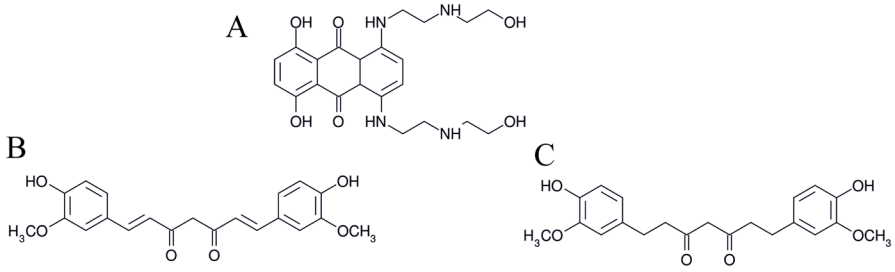

Figure 1. Chemical structure of mitoxantrone (A), curcumin (B) and tetrahydrocurcumin (C).

2002). Due to their relatively strong adhesion and prominent motility, we used LC_Walker-256 cells as a tool for the analysis of additive anti-tumorigenic activities of MTX and CC in relation to the phenotypic heterogeneity of carcinosarcoma. To comprehensively assess these relationships, we focused on selective short-term and long-term effects of both biomolecules on drug-sensitive and drug-resistant cell sub-populations.

MATERIALS AND METHODS

Curcumin (CC) and mitoxantrone (MTX) treatment of the Walker-256 cells. "Mesenchymal" (lammellipodia-forming (LC)) sub-line of rat carcinosarcoma Walker-256 cells (Sroka *et al*., 2002) was cultured under standard conditions (37°C, 5% CO_2) in the RPMI medium supplemented with 5% FBS and antibiotics. For endpoint experiments, media supplemented with CC or MTX alone or with curcumin/MTX cocktails, at the concentrations ranging from 0.1 nM to 100 nM (MTX) and 1.25 μM or 2.5 μM (CC), were added after 24 h-long cell pre-incubation in the culture medium.

Analyses of cell motility and cytoskeleton. Cell movement was analyzed with an inverted Leica DMI6000B microscope equipped with IMC optics, at 37ºC in 5% CO_2. Cells were seeded into 12-well plates at a density of 500 (control and 0.1 nM MTX; 72 h variant) or 5000 cells/cm² (other variants). Velocity of cell movement (total length of cell trajectory/time of recording; μm/h) and velocity of cell displacement (i.e. the direct distance from the starting point to the cells' final position/time of recording; μm/h) were quantified with the Hiro program. For the visualization of cytoskeleton, the cells were fixed with 3.7% formaldehyde, Triton-solubilized, stained with mouse anti-vinculin IgG and counterstained with Alexa 488-conjugated goat anti-mouse IgG, TRITC-conjugated phalloidin and Hoechst33258 (all from Sigma). Image acquisition was performed with a Leica DMI6000B microscope (Leica Microsystems, Wetzlar, Germany; Daniel-Wójcik *et al*., 2008).

Proliferation, cell cycle and apoptosis assays. Cells were seeded into 24- (proliferation assays), 12- (viability tests) or 6-well plates (cell-cycle and apoptosis tests; Corning) at the density of 5×10^3 cells/cm² and cultivated in a culture medium for 24 hours. Thereafter, a medium containing MTX (0.1–100 nM) and/or CC (1.25 or 2.5 μM) was administered for 72 hours; the cells were then harvested, re-suspended in the original culture medium and counted with the Coulter counter (Beckman). Their viability was determined by the fluorescein diacetate/ethidium bromide assays using a Leica DMI6000B microscope in the epifluorescence mode. At least 200 cells were analyzed for each condition. For DNA analyses, cells were stained in plates with Hoechst33242 (Sigma, 1 μg/ml) and analyzed as above. Alternatively, suspended and EtOH-fixed (70% at –20ºC) cells were stained with propidium iodide (PI; 50 μg/ml) in the presence of RNAseA. For analyses of apoptosis, cells were subjected to AnnexinV/propidium iodide staining according to the manufacturer's protocol (BD Pharmingen). Flow cytometric analyses of DNA content and detection of apoptotic cells was performed with BD LSR Fortessa X-20 FACS (Koczurkiewicz *et al*., 2013).

RESULTS AND DISCUSSION

MTX and CC cooperatively inhibit the motility of LC_Walker-256 cells

Time-lapse analyses of short-term effects of mitoxantrone (MTX; 0.1–1.6nM) and curcumin (CC; 1.25 and 2.5 μM) demonstrated slight additive effects of both agents on motility of the LC_Walker-256 cells. MTX and CC interfered with this parameter in a dose-dependent manner. This effect was illustrated by the reduction of an averaged cell displacement rate to about 55% and 50% of control observed in the presence of 1.6 nM MTX and 2.5 μM CC, respectively (Fig. 2A). CC/MTX cocktails inhibited the LC_Walker-256 cell motility to the levels observed in the presence of CC or MTX administered alone (at low and high MTX concentrations, respectively), whereas an additive effect of CC could be observed in the presence of 1.6 nM MTX. Cell motility is crucial for cancer invasion; therefore these effects can illustrate anti-invasive effects of both agents exerted at the concentrations corresponding to those observed in the sera of rats after MTX/CC intake (Prasad *et al*., 2014). The lack of true synergy of MTX/CC activity might result from their converging effects on the signaling pathways that regulate cell motility. Actually, NF-κB-, PI3K/Akt- and small G protein-dependent pathways are involved in the regulation of cell motility and both agents have been shown to interfere with their activity (Limtrakul *et al*., 2007; Lin *et al*., 2010; Bidaud-Meynard *et al*., 2013; Chen *et al*., 2014; Seo *et al*., 2014). However, the less pronounced effects of MTX/CC on cell motility observed 24 hours after their administration (Fig. 2B) attracted our attention to the selective effects of both agents, resulting from phenotypic heterogeneity of the LC_Walker-256 cells.

Curcumin augments long-term cytostatic effects of MTX on LC_Walker-256 cells

Long-term cytostatic and cytotoxic effects of MTX and CC confirmed the heterogeneous sensitivity of the LC_Walker-256 cells to the MTX/CC treatment. CC had no effect on the WC-256 viability when administered alone, but a relatively strong and dose-dependent cytotoxic effect of MTX on the LC_Walker-256 cells was observed

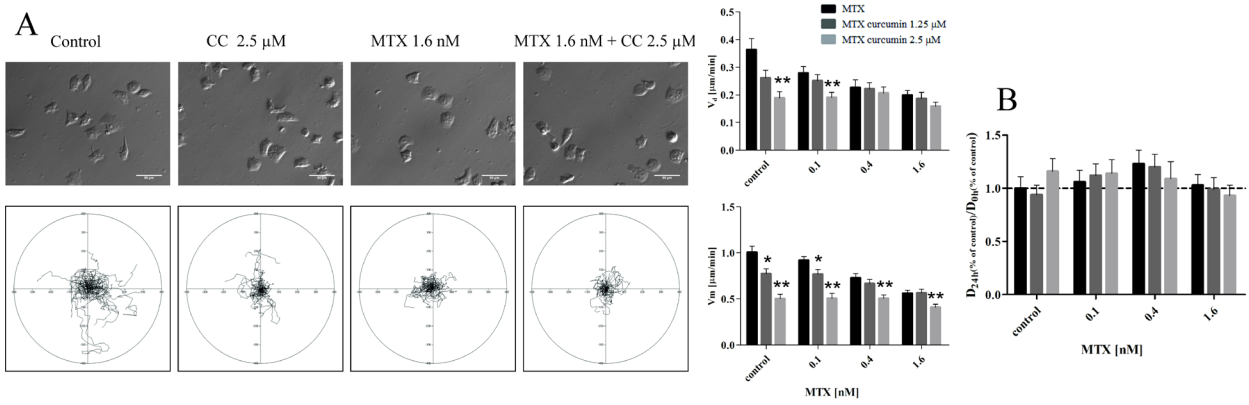

Figure 2. Curcumin and mitoxantrone cooperatively inhibit the LC_Walker-256 cell motility.
Movement of the LC_Walker-256 cells was registered immediately after the administration of media supplemented with MTX and/or CC. At least 50 cell trajectories (360 min at 5 min intervals) were drawn for each condition and presented in circular diagrams with the starting point of each trajectory situated at the plot center. Pictures show representative areas of data acquisition. Column charts summarize the effect of both agents on velocity of cell movement (V_m) and displacement (V_d), respectively. (**B**) Cells were cultivated in the medium supplemented with MTX and/or CC for 24 hours and their averaged displacement rates were compared to those observed immediately after drug administration (see **A**). Statistical significance was estimated with the non-parametric Mann-Whitney test (vs. control; **P*≤0.05; ***P*≤0.01). Error bars represent SEM. Scale bar: 25 μm. Results are representative of 3 independent experiments. Note the partial recovery of the cells from the inhibitory effect of MTX/CC on cell motility after their long-term incubation in the presence of MTX and CC.

(Fig. 3A). 2.5 μM CC slightly decreased the viability of the WC-256 cells in the presence of 0.1 nM MTX and augmented the cytotoxic effect of 0.4 nM MTX, whereas 1.25 μM CC also increased the effect of 0.4 nM MTX. These effects were correlated with the pro-apoptotic activity of MTX, illustrated by micronuclei tests and DNA content staining analyses (Fig. 3B). Fragmentation of cell nuclei and abundant AnnexinV-staining were accompanied by considerable fractions of propidium iodide (PI)-stained cells in the MTX-treated LC_Walker-256 populations, which were further increased by CC (Fig. 3C). Noteworthy, about 10% of viable (Fig. 3A), non-apoptotic cells (Fig. 3C), could still be observed after a long-term incubation in the presence of the highest MTX/CC concentrations. A considerable fraction of G_2/M cells in the MTX/CC-treated LC_Walker-256 populations indicates that a G_2-specific apoptosis is induced by both agents and suggests that some cells retain their proliferative activity under such conditions (Fig. 3B). These observations confirm that CC could be applied to reduce the effective doses of MTX in carcinosarcoma treatment, as previously suggested for MTX- and doxorubicin-based prostate, lung and liver cancer therapy (Koczurkiewicz *et al.*, 2013; Zhao *et al.*, 2015). However, such a combined treatment may still result in the propagation of drug-resistant LC_Walker-256 cell sub-population(s).

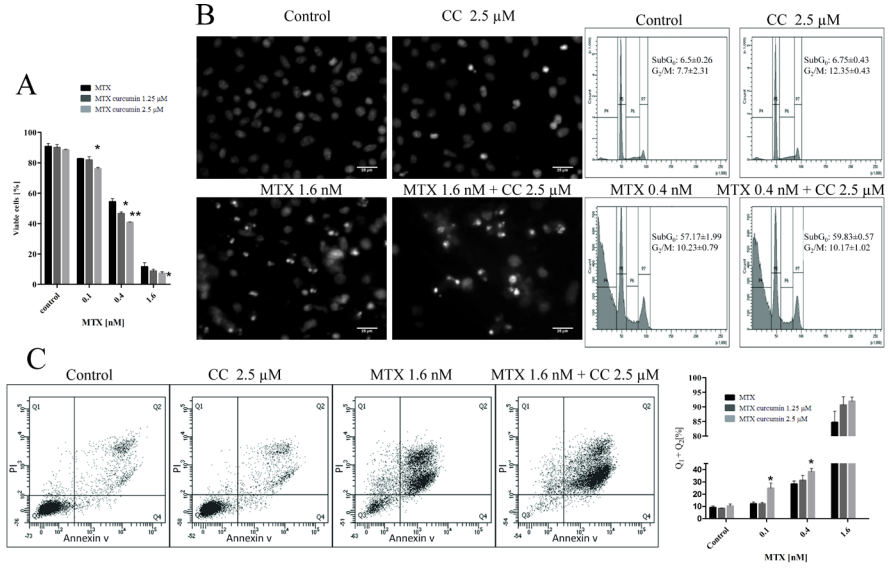

Figure 3. Curcumin and mitoxantrone exert additive cytotoxic and pro-apoptotic effects on the carcinosarcoma LC_Walker-256 cells.
(**A**) Cells were cultivated in the presence of MTX and/or CC for 72 h and subjected to viability tests. Concomitantly, apoptotic cells were visualized with Hoechst33242 and analyzed by FACS-assisted analyses of DNA content (**B**) or identified by AnnexinV/PI tests (C). Column charts show relative numbers of living (A) and propidium iodide-positive (Q1+Q2) cells (**C**). Dot-plots show an AnnexinV/PI staining pattern. At least 50 000 cells were counted for each experiment. Statistical significance was estimated with the Student's *t*-test (*vs.* control; **P*≤0.05; ***P*≤0.01). Error bars represent SEM. Scale bar: 25 μm. Results are representative of 3 independent experiments. Note the additive effects of CC and MTX (0.1–0.4 nM), which are accompanied by the presence of non-apoptotic cells after a long-term MTX/CC treatment.

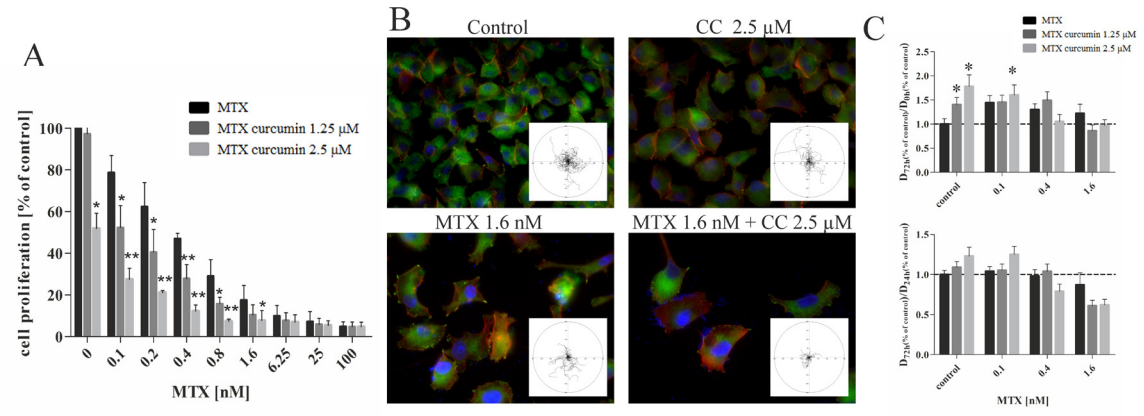

Figure 4. Long-term MTX/CC treatment facilitates the expansion of drug-resistant LC_Walker-256 cell sub-population(s).
(**A**) 72 hour-long cultivation of the LC_Walker-256 cells in the presence of MTX and CC was followed by the calculation of their relative numbers with a Coulter counter (control=100%). (**B**) Cells were treated with MTX/CC as in A, fixed and stained against vinculin and actin for fluorescence microscopy analyses of their morphology and cytoskeleton architecture. Alternatively, cells were cultivated in the medium supplemented with MTX and/or CC for 72 hours and their movement was registered with a time-lapse videomicroscopy (inserts) and compared to that observed immediately after the drug administration or after 24 h-long treatment (**C**). Statistical significance was estimated with the non-parametric Mann-Whitney test (*vs.* control; **P*≤0.05; ***P*≤0.01). Error bars represent SEM. Scale bar: 25 μm. Results are representative of 3 independent experiments. Note that a small population of the LC_Walker-256 cells, which had survived a long-term (72 h) MTX/CC treatment, displays a considerably higher motility than after the short-term (0–24 h) MTX/CC regimens. Its motility is inhibited in the presence of 1.6nM MTX/CC cocktails.

MTX/CC cocktails inhibit the motility of drug-resistant LC_Walker-256 cells

To unequivocally confirm the selective effects of MTX/CC treatment on the LC_Walker-256 cells, we further analyzed growth curves of the MTX/CC-treated LC_Walker-256 cells and estimated the motile activity of drug-resistant cells. Long-term treatment of the LC_Walker-256 cells with MTX/CC cocktails exerted a considerably stronger effect on cell proliferation than these agents administered alone. This finding confirms the reducing effect of CC on the effective doses of MTX. Interestingly, a considerable fraction (ca. 10%) of adherent cells could again be seen in the presence of 100nM MTX, even though CC considerably reduced their numbers (Fig. 4A). This observation is consistent with the data obtained in the viability, cell cycle and apoptosis assays (see Fig. 3), and confirms that a single LC_Walker-256 may be resistant to both agents. Actually, these cells retained morphology and cytoskeleton architecture similar to the control cells, although increased numbers of vinculin-positive focal adhesions were seen in the MTX-treated cells, whereas this effect was not observed in the presence of the MTX/CC cocktails (Fig. 4B). Drug-resistant LC_Walker-256 cells were considerably more motile than the cells analyzed immediately after the MTX/CC administration (Fig. 4C, cf. Fig. 2). Whereas the role of MTX/CC effects on MDR mechanisms remains to be elucidated (Mapoung *et al.*, 2016), our data demonstrate the phenotypic heterogeneity of the LC_Walker-256 sub-populations and show that the MTX/CC treatment leads to the selection of drug-resistant cells. This observation remains in contrast to the effects of tetrahydrocurcumin (Fig. 1C) that has been shown to attenuate tumor cell resistance to MTX (Limtrakul *et al.*, 2007). It indicates that CC does not completely eliminate the expansion of MTX-resistant cells. On the other hand, a considerable inhibition of cell motility was still observed after a long-term incubation of the LC_Walker-256 cells in the presence of 1.6 nM MTX/2.5 μM CC cocktail (Fig. 4B, C). Our data indicate that CC not only reduces the effective cytotoxic doses of MTX, but also affects the invasive potential of MTX-resistant cells. Noteworthy, 2.5 μM CC can exceed the serum concentrations observed in humans after CC uptake, therefore alternative routes of CC administration and/or application of the formulated curcumin (nanoemulsion, nanocapsules etc.) may be necessary to achieve its full systemic activity (Prasad *et al.*, 2014).

SUMMARY AND OUTCOME

An approach based on a combined application of cytostatic drugs and phytochemicals is a promising tool in the therapy of drug-resistant tumors. Plant-derived biomolecules (such as curcumin) display a relatively low systemic toxicity and can sensitize tumor cells to extrinsic cytostatic cues. Therefore, they can be applied to augment the bioactivity of chemotherapeutic drugs, thus reducing their effective doses and potentially prolonging the expected life-span and the patient standard of living (Sarkar *et al.*, 2006). The augmenting effects of CC on carcinosarcoma cell sensitivity to MTX, along with anti-inflammatory and hepatoprotective activity of CC (El-Bahr 2014; Santos *et al.*, 2015), indicate a potential of the MTX/CC cocktails for elaboration of a "gold standard" or at least of the 2nd line of chemotherapeutic approaches against carcinosarcoma. Anti-angiogenic activity of CC (Gong *et al.*, 2013; Li *et al.*, 2005) may also counteract the pro-angiogenic MTX effects. Therefore, this biomolecule could also be used as an adjuvant in metronomic MTX-based anti-carcinosarcoma approaches. CC does not completely eliminate the expansion of MTX-resistant cells, however it exerts additive inhibitory effects on their motility. It may indicate that CC could slow down the tumor progression that results from the chemotherapy-induced microevolution of drug-resistant, invasive tumor cell sub-populations.

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Conflicts of interest

The authors declare no conflict of interest.

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