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

Marcin Luty1, Edyta Kwieceń1, Magdalena Firlej2, Anna Łabędź-Masłowska1, Milena Paw1, Zbigniew Madeja1 and Jarosław Czyż1,2

1Department of Cell Biology and 2Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

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

Received: 16 March, 2016; revised: 24 April, 2016; accepted: 06 May, 2016; available on-line: 08 July, 2016

INTRODUCTION

Mitoxantrone (1,4-dihydroxy-5,8-bis(2-hydroxyethylamino)ethylaminoanthraquinone; 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 vivo and in vitro (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; Sharif-Brizzi, 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 (Sharif-Brizzi et al., 2015). The Walker-256 cells reflect the phenotypic diversity of carcinosarcoma because non-adenherent, blebbing and lamellipodia-forming (LC), “mesenchymal” Walker-256 lineages have been propagated (Sroka et al., 2015 and at the XLIII Winter School of Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Zakopane, 2016. Abbreviations: CC, curcumin; MTX, mitoxantrone.
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” (lamellipodia-forming (LC)) sub-line of rat carcinosarcoma Walker-256 cells (Sroka et al., 2002) was cultured under standard conditions (37°C, 5% CO₂) 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. The viability of the 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 was observed 24 hours after their administration (Fig. 2B), however, the less pronounced effects of MTX/CC on 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.

**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...
Mitoxantrone and curcumin in carcinosarcoma therapy

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. 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.

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 0.1 nM MTX and/or CC for 72 h and subjected to viability tests. Concomitantly, apoptotic cells were visualized with Hoechst33342 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 50000 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 effect 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.
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 surviving 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 (Mapeourang 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 (nano-emulsion, 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.

Acknowledgement

The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education.
This work was financially supported by the “Student Research Project” sponsored by the Faculty of Biochemistry, Biophysics and Biotechnology of Jagiellonian University (to M. Luty) and the Polish National Science Centre (grant 2015/17/B/NZ3/01040 to J. Czyż).

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

Alem HB, AlNoury MK (2014) Management of spindle cell carcinoma. Eur Neurol Int J Hematol Oncol Stem Cell Res Cancer Res 64: 401

Koczurkiewicz P, Podolak I, Wójcik KA, Galanty A, Madeja Z, Michalak M, Limtrakul P, Nantasri M, Czyż J (2013) Presence of both mesenchymal and carcinomaous features in an in-vitro model of ovarian carcinoma derived from patients’ ascites fluid. Int J Hematol Oncol Stem Cell Res 6: 01791.x.

Kanthy R, Senger JL (2011) Uterine carcinosarcoma: malignant mixed tumour-like: a review with special emphasis on the controversies in management. Ohelät Gynol Int 2011: 470795.

Karp JE, Garrett-Mayer E, Estey EH, Rudek MA, Smith BD, Greer JM, Dye DM, Mackey K, Doty KS, Goren SD, Levine MJ, McClellan MA, Carraway HE, Pratz KW, Gladstone DE, Showel MM, Othus M, Dorcy KS, Gore SD, Levis MJ, McDevitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ, Steward WP (2004) Phase I clinical trial of mitoxantrone in the treatment of advanced non-squamous carcinoma of the cervix (A phase II trial of the gynecologic oncology group). Cancer Res 64: 1118–1124.

Mapoonporn S, Pirschakarn P, Yodkeeree S, Ovatlarnporn C, Sakorn N, Limtrakul P (2016) Chemosensitizing effects of synthetic curcumin analogs on human multi-drug resistance leukemia cells. Chemico-Biological Interactions 244: 140–148. http://doi.org/10.1016/j.chembioint.2015.12.001.

Mehrabani D, Farjam M, Geramizadeh B, Tanideh N, Amini M, Panjehshahin MR. (2015) The Healing effect of curcumin on burn wounds in rat. World J Plast Surg 4: 29–35.

Mass HB, Bundy BN, Homesley HD, Wilbanks G (1987) Mitoxantrone in the treatment of advanced non-squamous carcinoma of the cervix (A phase II trial of the gyn/Onc Colorectal oncology group). Int J Nanomedicine 23: 199–202.

Negi N, Prakash P, Gupta ML, Mohapatra TM (2014) Possible role of curcumin as an efflux pump inhibitor in multi drug resistant clinical isolates of Pseudomonas aeruginosa. J Clin Diagn Res 8: 2–11. http:// doi.org/10.3774/jcdr.2014.8329.4965.

Prasad S, Tyagi PK, Aggarwal BB (2014) Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer Res Treat 46: 2–18. http://doi.org/10.4143/ert.2014.12.

Qian Y, Ma J, Guo X, Sun J, Yu Y, Cao B, Zhang L, Ding X, Huang J, Shaof J (2015) Curcumin enhances the radiosensitivity of U87 cells by inducing DUSP-2 up-regulation. Cell Physiol Biochem 35: 132–141. http://doi.org/10.1155/2013/790790.

Rivera VM, Jeffery DR, Weinstock-Guttman B, Bock D, Dangond F (2013) Results from the 5-year, phase IV RENEW (Registry to Evaluate Novel Antracyclines in Worsening Multiple Sclerosis) study. BMC Neurology 13: 80. http://doi.org/10.1186/1471-2373-13-80.

Santos AM, Lopes M, Oalcastro M, Gato IV, Lorentz S, Pereira T, Seixas E, Machado J, Guerreiro AS (2015) Curcumin enhances gastric inflammation induced by Helicobacter pylori infection in a mouse model. Nutrients 7: 306–320. http://doi.org/10.3390/nu7010036.

Sarkar FH, Li Y (2006) Using chemopreventive agents to enhance the efficacy of cancer therapy. Cancer Res 66: 3547–3551. http://doi.org/10.1158/0008-5472.CAN-05-4526.

Seo BR, Min KJ, Cho IJ, Kim SC, Kwon TK (2014) Curcumin significantly enhances dual PI3K/Akt and mTOR inhibitor NPV-BEZ235-induced apoptosis in human renal carcinoma caki cells through down-regulation of p53-dependent Bel-2 expression and inhibition of Mcl-1 protein stability. PLoS One 9, http://doi.org/10.1371/journal.pone.0095588.

Sharif-Tehrani A, Pellicer A, Adelhuth A, Venditti CA, Samuelson R, Shahabi S (2015) Presence of both mesenchymal and carcinosarcomaous features in an in-vitro model of ovarian carcinoma derived from patients’ ascites fluid. Int J Hematol Oncol Stem Cell Res 6: 3–6. http://doi.org/10.1159/000400562.

Shehzad A, Park JW, Lee J, Lee YS (2013) Curcumin induces radio-sensitivity of in vitro and in vivo cancer models by modulating pre-mRNA processing factor 4 (Pmrf). Chemico-biological interactions 206: 394–402. http://doi.org/10.1016/j.chembioint.2013.10.007.

Sokra J, von Gunten M, Dunn GA, Keller HU (2002) Phenotype modulation in non-adherent and adherent sublines of Walker carcinosarcoma cells: the role of cell-substratum contacts and microtubules in controlling cell shape, locomotion and cytoskeletal structure. Int J Biochem Cell Biol 34: 882–899.

Szewd, M (2014) Mitoxantrone – an anthraquinone antibiotic with anti-tumor activity applied for the treatment of multiple sclerosis. Protagy Fm Med Dose (Online) 68: 198–208.

Wang BL, Shen YM, Zhang QQ, Li YL, Luo M, Liu Z, Li Y, Qian ZY, Gao X, Shi HS (2015) Codeelivery of curcumin and doxorubicin by MPEG-PLA-PEG nanoparticles results in improved efficacy of systemically administered chemotherapy in mice with lung cancer. Int J Nanomedicine 10: 3521–3531.

Zhao X, Chen Q, Liu W, Li Y, Tang H, Liu X, Yang X (2015) Codeelivery of doxorubicin and curcumin with lipid nanoparticles results in improved efficacy of chemotherapy in liver cancer. Int J Nanomedicine 10: 257–270. http://doi.org/10.2147/ijnm.57332.