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Lclet 4 enhances pro-apoptotic and anti-invasive effects of mitoxantrone on human prostate cancer cells — *in vitro* study*

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Triterpene saponosides are widely distributed plant secondary metabolites characterized by relatively low systemic cytotoxicity and a range of biological activities. These include anti-inflammatory, antimicrobial, vasoprotective and antitumor properties. In particular, the ability of saponins to enhance the cytotoxicity of chemotherapeutic drugs opened perspectives for their application in combined cancer chemotherapy. Here, we used human prostate cancer DU-145 cells as an *in vitro* model to elucidate the synergy of the interactions between biological activities of an oleanane type 13β,28-epoxy triterpene saponoside (Lclet 4) and mitoxantrone, which is a cytostatic drug commonly used in prostate cancer therapy. No cytotoxic or pro-apoptotic effect of Lclet 4 and mitoxantrone administered at the concentrations between 0.05 and 0.1 μg/ml could be seen. In contrast, cocktails of these agents exerted synergistic pro-apoptotic effects, accompanied by the activation of the caspase 3/7 system. This effect was paralleled by attenuating effects of Lclet 4/mitoxantrone cocktails on the invasive potential, metalloproteinase expression and motility of DU-145 cells. Multifaceted and additive effects of Lclet 4 and mitoxantrone on basic cellular traits crucial for prostate cancer progression indicate that the combined application of both agents at systemically neutral concentrations may provide the basis for new promising strategies of prostate cancer chemotherapy.

Key words: triterpene saponosides (Lclet 4); mitoxantrone; combined therapy; prostate cancer; apoptosis; invasiveness.

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INTRODUCTION

Prostate cancer is one of the most commonly diagnosed tumors in men and the second leading cause of cancer mortality in Europe and USA (Arcangeli *et al.,* 2012). At early stages, it can be treated with surgery and androgen deprivation therapy, but no efficient curative therapy exists against its progressed, hormone refractory and malignant form (Crawford & Connor, 2009). Moreover, currently available therapeutical approaches evoke a number of adverse effects, which increase the morbidity and mortality rates of the patients (Ichikava *et al.,* 2005; Hurria *et al.,* 2011). For instance, this problem concerns the application of mitoxantrone, a widely used cytostatic and anti- neoplastic drug (Bouchet *et al.,* 2010). Elderly patients treated with mitoxantrone-based therapy usually suffer from acute and/or delayed bone marrow depression, cardiomyopathy and mucositis (Fleming *et al.,* 2012), which considerably limit its benefits.

A promising possibility of reducing the adverse effects of prostate cancer chemotherapy without attenuation of its efficiency is provided by a combined application of chemotherapeutic agents and plant phytochemicals characterized by anti-cancer activity, such as saponosides (Kim *et al.,* 2010). Till recently, saponosides were predominantly used as adjuvants that enhance the effect of vaccines (Liu *et al.,* 2012). In cancer therapy, they were applied in combination with immunotoxins to increase the selectivity of the immunotoxin against cancer cells and to inure normal cells to its cytotoxic effect (Weng *et al.,* 2012). On the other hand, direct pro-apoptotic, anti-angiogenic, cytostatic and anti-invasive activity of saponosides has been demonstrated (Galanty *et al.,* 2008; Chen *et al.,* 2012; Kim *et al.,* 2012; Podolak *et al.,* 2013). They can enhance the effect of commonly used chemotherapeutic agents, suggesting possibilites for lowering their therapeutic doses and reducing the possible side-effects of chemotherapy The observations of therapeutic efficacy of the combinations of some natural products from Traditional Chinese Medicine with standard chemotherapeutic agents (Efferth *et al.,* 2002; Hofman *et al.,* 2012) warrant intensive research on the anti-cancer activity of saponosides and on the mechanisms of their interference with chemotherapeutics.

In vitro models of prostate cancer were previously applied to identify the determinants of prostate cancer invasion (Djamgoz *et al.,* 2001, Miękus *et al.,* 2005). They also facilitate the elucidation of pro-apoptotic and anti-invasive activities of phytochemicals in strictly controlled conditions (Czernik *et al.,* 2008 Wybieralska *et al.,*2011; Gawlik-Dziki *et al.,* 2012, Gawlik-Dziki *et al.*, 2013). In the present study, we investigated the synergy of pro-apoptotic and anti-invasive effects of a newly identified triterpene saponoside (referred to as Lclet 4) and mitoxantrone (MTX) in human prostate DU145 cell populations. Lclet 4 is an oleanane type 13β,28-epoxy triterpene saponoside isolated from *Lysimachia clethroides* (Duby), which contains a branched sugar chain (3-O-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→2)-

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Abbreviations: FBS, fetal bovine serum, Lclet 4, triterpene saponin 3-O-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-]-α-L-arabinopyranosyl-protoprimulagenin, LDH, lactate dehydrogenase, MTX, mitoxantrone, PI, propidium iodide.

β-D-glucopyranosyl-(1→4)-]-α-L-arabinopyranosyl-protoprimulagenin A). Our preliminary studies have shown a prostate cancer cell-specific pro-apoptotic effect of Lclet 4 (unpublished data). In the present study, we demonstrate that it can dramatically reduce the therapeutic doses of MTX in prostate cancer treatment.

MATERIALS AND METHODS

Plant material and Lclet 4 isolation. Underground parts of *Lysimachia clethroides* (Duby) were collected from The Garden of Medicinal Plants, Jagiellonian University, Cracow, Poland. Voucher specimen KFg/2010016 is deposited at the Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland.

The Lclet 4 compound was isolated according to the procedure described in Podolak *et al.* (2013). Air-dried plant material (150 g) was extracted with CHCl3 (400 mL × 3) followed by MeOH with 0.5% pyridine (400 mL × 4). The combined extract was concentrated in vacuo, dissolved in water and exhaustively eluted with n-BuOH. The n-BuOH-soluble fraction was subjected to silica gel column chromatography (Merck Kieselgel 60; 70–230 mesh) using $CHCl_3$/MeOH/H_2O 23:12:2 and fractions were combined on the basis of TLC. Pooled saponin-containing fractions were subjected to repeated preparative TLC (commercially precoated silica gel G plates; Analtech, 500 microns) using $CHCl_3$/MeOH/H2O 23:12:2; 8:7:1 as solvents. Bands were removed from plates after spraying with water, the silica was extracted with MeOH and solvent was evaporated in vacuo to yield Lclet 4 (86 mg). Its identity was confirmed based on chemical and spectral characterization using FAB MS and 1D and 2D NMR experiments (Podolak *et al.* 2013) as well as on comparison with literature data (Bloor, 1994; Hegde *et al.,* 1995).

Cells and cell culture. Human prostate cancer cell line DU-145 and the normal PNT2 prostate cells were used in the study. The cells were cultured in standard conditions (37°C, 5% CO2) in DMEM- F12 medium (DU-145) or in RPMI (PNT2), supplemented with 10% FBS.

Lclet 4 and MTX treatment. Cells were seeded into the culture dishes at the densities given below and incubated for 24 hours. Afterwards, the culture medium was replaced for the new one, supplemented with Lclet 4 or MTX alone or with Lclet 4/MTX cocktails. The tested concentrations of both compounds ranged from 0.05 μg/ml to 5 μg/ml for viability tests and from 0.05 to 0.1μg/ml for other endpoint experiments.

Viability and Lactate Dehydrogenase cytotoxicity tests. For viability tests, the cells were seeded into 24-well plates (Corning) at a density of 1×10^4 cells/cm^2, were cultivated in the presence of Lclet 4, MTX (0.05 μg/ml to 5 μg/ml) for 24 hours and their viability determined by the fluoresceine diacetate and ethidium bromide test (Yang *et al.*, 1998). At least 200 cells were analyzed for each condition.

For Lactate Dehydrogenase (LDH) cytotoxicity tests (Clonetech) DU-145 cells were seeded into 96-well plate (Corning) at 1×10^4 cells/cm^2, incubated for 24 hours and treated with the agents for the next 24 hours. The plates were then centrifuged at $250 \times g$ for 10 minutes and 100 μl of the supernatant was removed carefully from each well and transferred into the corresponding wells of an optically clear 96-well flat-bottom plate. Next, 100 μl of freshly prepared Reaction Mixture was added to each well and incubated in darkness for up to 30 minutes at room temperature. Absorbance of the samples was measured at 492 nm using a multi-well plate reader (TECAN). Cytotoxicity was measured as (%) = (Triplicate Absorbance-Low control/High control-Low control) ×100. Three independent experiments were performed for each condition.

Analysis of apoptosis. DU-145 cells were seeded into a Corning flask at a density of 1×10^4 cells/cm^2, incubated for 24 hours and treated with the agents for the next 24 hours. Then, the cells were washed with PBS without Ca^{2+} and Mg^{2+} ions, trypsinized, centrifuged and counted with a Bürker's chamber. After washing with cold PBS, the cells were resuspended in Binding Buffer (Becton Dickinson) at the final concentration of 1×10^6 cells/ml. Next, 100 μl of the cell suspension (1×10^5 cells/ml) was transferred to a 5 ml culture tube, followed by addition of Annexin V/PI solution (Becton Dickinson) and Binding Buffer. Flow cytometric analyses (BD LSR II) were performed according to the manufacturer's protocol. 50 000 cells were analyzed in each experiment. Three independent experiments were performed for each condition.

For the analyses of caspase 3/7 activity DU-145 cells were seeded into a 96-well plate at a density of 3×10^3 cells/cm^2. After 24 hours the cells were treated with Lclet 4 and MTX alone or with Lclet 4/MTX cocktails for the next 24 hours. Caspase-Glo3/7 Reagent (Promega) was added (100 μl) to each well of a white-walled 96-well plate, containing 100μl of culture medium. Plates were gently agitated using a plate shake 300–500 rpm for 30 seconds, and incubated at room temperature for 30 minutes. The sample luminescence was measured in a plate-reading luminometer (TECAN) according to manufacturer's instructions. For each condition three experiments were performed.

Time lapse-monitoring of movement of individual cells. Cell movement was observed with an inverted Leica DMI6000B microscope with IMC optics, at 37ºC and 5% CO_2 atmosphere. DU-145 cells were seeded into 6-well plates at a density of 1×10^4 cells/cm^2, the medium was replaced with new one containing the agents and time-lapse recording of cell movements was immediately performed. The cell trajectories were constructed from 88 subsequent centroid positions recorded over 480 minutes at 15 minute time intervals. They were presented in circular diagrams with the starting point of each trajectory situated at the plot center. The parameters characterizing cell locomotion were calculated as described previously (Djamgoz *et al.,* 2001; Madeja *et al.*, 2001). For each data point measured, 50 cells were analyzed. Pre-apoptotic and apoptotic cells were discriminated by morphology and their trajectories not considered for statistical analysis. Three independent experiments were performed for each condition.

Fluorescence staining. DU-145 cells were stained with mouse monoclonal antibody against human α-tubulin (Sigma-Aldrich) or mouse monoclonal antibody against human cytochrome *c* (Pharmingen) followed by Alexa Fluor 488 goat anti-mouse IgG (clone A11001, Sigma- Aldrich) and counterstained with Phallodin-TRITC (Sigma-Aldrich). Visualization of specimens mounted in polyvinyl alcohol (Mowiol; Sigma-Aldrich) was performed with a Leica DMI6000B microscope and images were acquired with a DFC360FX CCD camera.

Matrix metalloproteinase expression activity. The activities of MMP-2 and MMP-9 were assayed by gelatin zymography. Briefly, subconfluent DU-145 cells were incubated in control serum-free medium, the

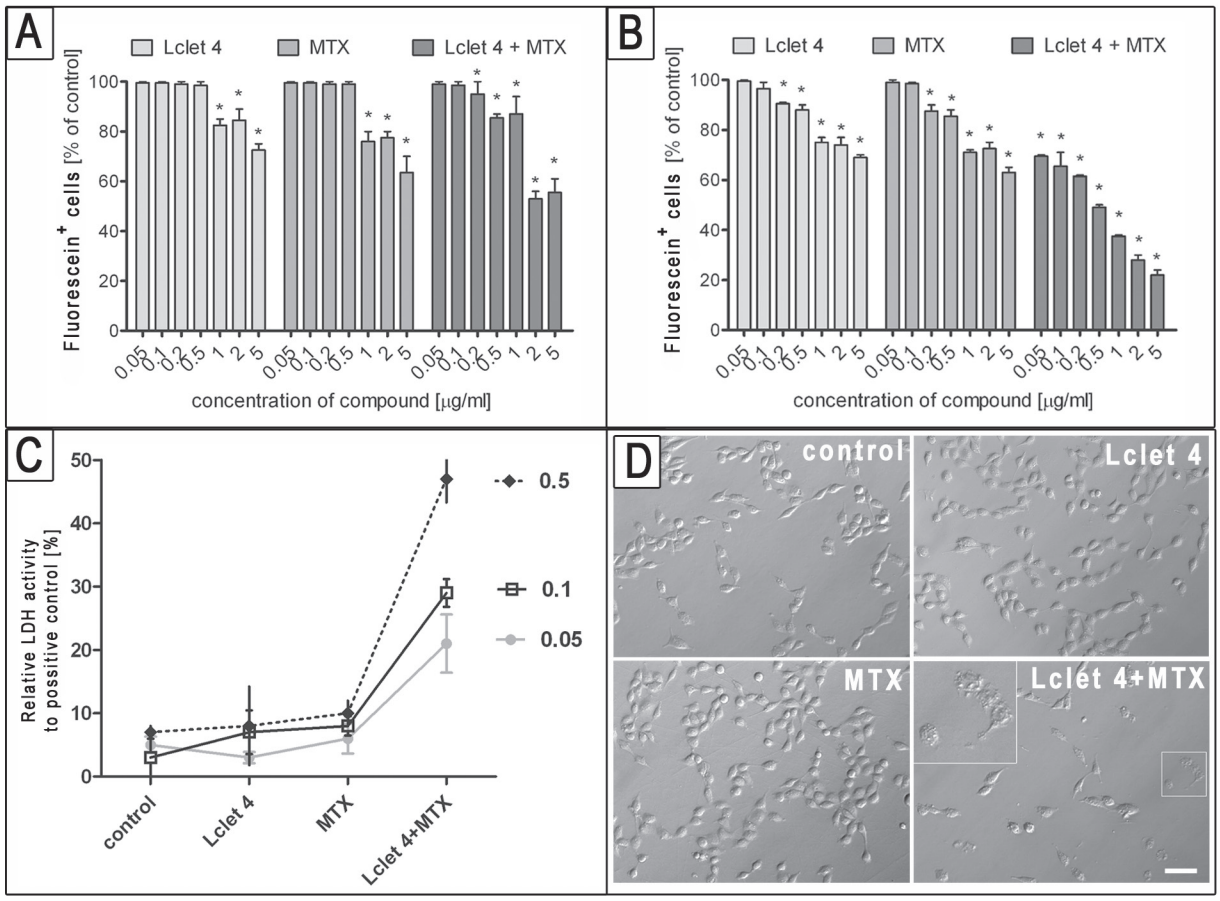

Figure 1. Additive cytotoxic effect of Lclet 4 and MTX on DU-145 cells.
Neither Lclet 4 nor MTX exerted any effect on the viability of PNT2 (**A**) and DU-145 cells (**B, C**) when administered at the concentrations between 0.05 and 0.1 μg/ml. Decreased fraction of viable DU-145 but not PNT2 cells in the presence of the Lclet 4/MTX cocktail (0.05–0.1 μg/ml) was paralleled by changes in the morphology of adherent DU-145 cells (**D**). Bar=20 μm. PNT2 (**A**) and DU-145 (**B, C**) cells were cultured in medium supplemented with 10% FBS in the presence of Lclet 4 or MTX (in concentrations ranging from 0.05 to 5 μg/ml) or in the presence of both compounds. After 24 hours of incubation cell viability was determined by the fluoresceine diacetate and ethidium bromide test (**A–B**) and by LDH cytotoxicity test (**C**). Values represent means ± S.E.M. of the total number of viable cells (%) in comparison to vehicle control in three independent experiments (**A, B**). (*) Statistically significant *versus* control at $p < 0.05$.

medium supplemented with Lclet 4 alone, or with the Lclet 4/MTX cocktail for 24 hours. Then the supernatants were collected, mixed with loading buffer and subjected to 10% SDS-polyacrylamide gel containing 0.1% gelatin (Sigma Aldrich, Cat. no G8150). Electrophoresis was performed at 70 V for 15 minutes and 130 V for 120 minutes. Gels were then washed with washing buffer (2% Triton X-100) at room temperature to remove SDS, followed by incubation at 37°C in reaction buffer (Tris base, Tris HCl, $CaCl_2$). After 24 hours the gels were stained with Comassie Blue R-250 for 1 hour and destained with destaining solution (20% methanol, 10% acetic acid, 70% ddH_2O). The relative optical density (ROD) of the signal bands was estimated using the ImageJ 1.45s software.

Transmigration experiments. DU-145 cells were seeded into chambers containing "naked" microporous membranes (Corning, pore diameter: 8 μm, membrane diameter: 6.5 mm) at a density of 1×10^4, or membranes covered with Matrigel (1:8; Sigma Aldrich) at a density of 2×10^4 cells/cm^2 and allowed to transmigrate in the absence or presence of the agents. The cells which transmigrated during 24 hours (naked microporous membranes) or 72 hours (Matrigel-coated membranes) were stained with bis-benzimide (Hoechst 33342-Sigma Aldrich) and counted. Three independent experiments were performed for each condition.

Statistical methods. Each variable was expressed as the mean (± S.E.M.). The statistical significance was determined using the Student's *t*-test or the non-parametric Mann-Whitney U-test (motility tests), with $p < 0.05$ considered to indicate significant differences. For morphological and fluorescent staining evaluation, at least 20 microscopic fields of view were analyzed.

RESULTS

Synergistic effects of Lclet 4 and MTX on the viability of DU-145 cells

Cell viability assays were first performed to examine the specificity and synergy of the effects of Lclet 4 and MTX on the viability of prostate cells. Diacetate fluoresceine/ethidium bromide exclusion tests demonstrated that neither Lclet 4, MTX nor Lclet 4/MTX cocktails affected the viability of PNT2 cells, when administrated at concentration of up to 1 g/

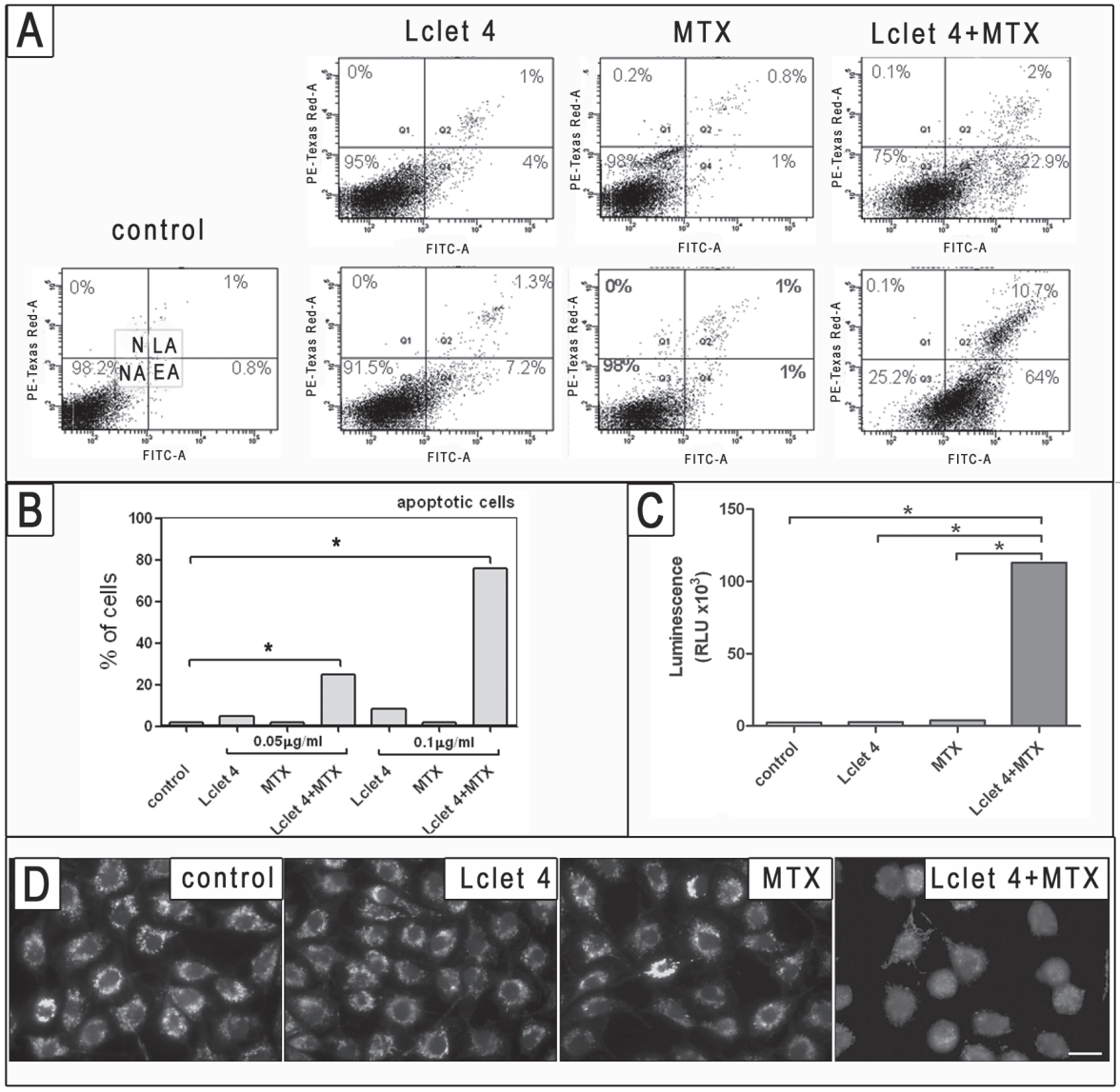

Figure 2. Lclet 4 and MTX induced apoptotic response in DU-145 cells is accompanied by up-regulation of caspase 3/7 and release of cytochrome *c* from mitochondria.
DU-145 cells were cultured in medium supplemented with 10% FBS in the presence of Lclet 4 (0.05 or 0.1 µg/ml) or MTX (0.05 or 0.1 µg/ml), or in the presence of Lclet 4/MTX cocktails. After 24 hours the cells were stained with Annexin V/PI for FACSs analyses. Dot-plots of Annexin V/ PI (FITC-A/PE-Texas Red A) — stained cells (**A**) were quantified and the results presented as percent of apoptotic (early-apoptotic (EA) + late-apoptotic (LA)) cells (**B**). Activity of caspase 3/7 was determined using luminescence method (**C**). Values represent means ± S.E.M. in arbitrary units (relative luminescence units — RLU) of three independent experiments; (*) statistically significant *versus* control at $p < 0.05$ (**B**) and $p < 0.001$ (**C**). Intracellular localization of cytochrome *c* was visualized by immunofluorescence staining performed 24 hours after administration of the agents (D). Bar=20 µm.

ml of each agent (Fig. 1A). In contrast, both agents exerted significant cytotoxic effects on DU-145 cells at the concentrations between 0.2 and 5 µg/ml (Fig. 1B). Only minute cytotoxic effects were observed when Lclet 4 and MTX were separately administered at the concentrations between 0.05 and 0.1 µg/ml. In contrast, their combined administration at these concentrations significantly attenuated DU-145 cell viability (Fig. 1B). For instance, 0.1 µg/ml of Lclet 4 and MTX slightly reduced the fraction of fluoresceine$^+$/EB$^-$ cells to 97 and 99%, respectively, whereas the percentage of fluoresceine$^+$ cells decreased to 61% in the presence of both agents. In order to confirm the results of diacetate fluoresceine/ethidium bromide exclusion test, LDH assays were conducted. These tests revealed considerably higher cytotoxicity of Lclet 4/MTX cocktails than separately administrated agents, especially at higher concentrations (Fig. 1C). Accordingly, the prolonged incubation of DU-145 cells with the cocktail of Lclet 4 and MTX (0.05 µg/ml) resulted in the appearance of detached cells, which displayed numerous blebs reminiscent of apoptotic bodies (Fig. 1D). No such effect could be observed in the presence of each agent when administered alone. These data indicate that the combination of Lclet 4 and MTX applied at non-cytotoxic concentrations effectively induces an apoptotic response of DU-145 cells.

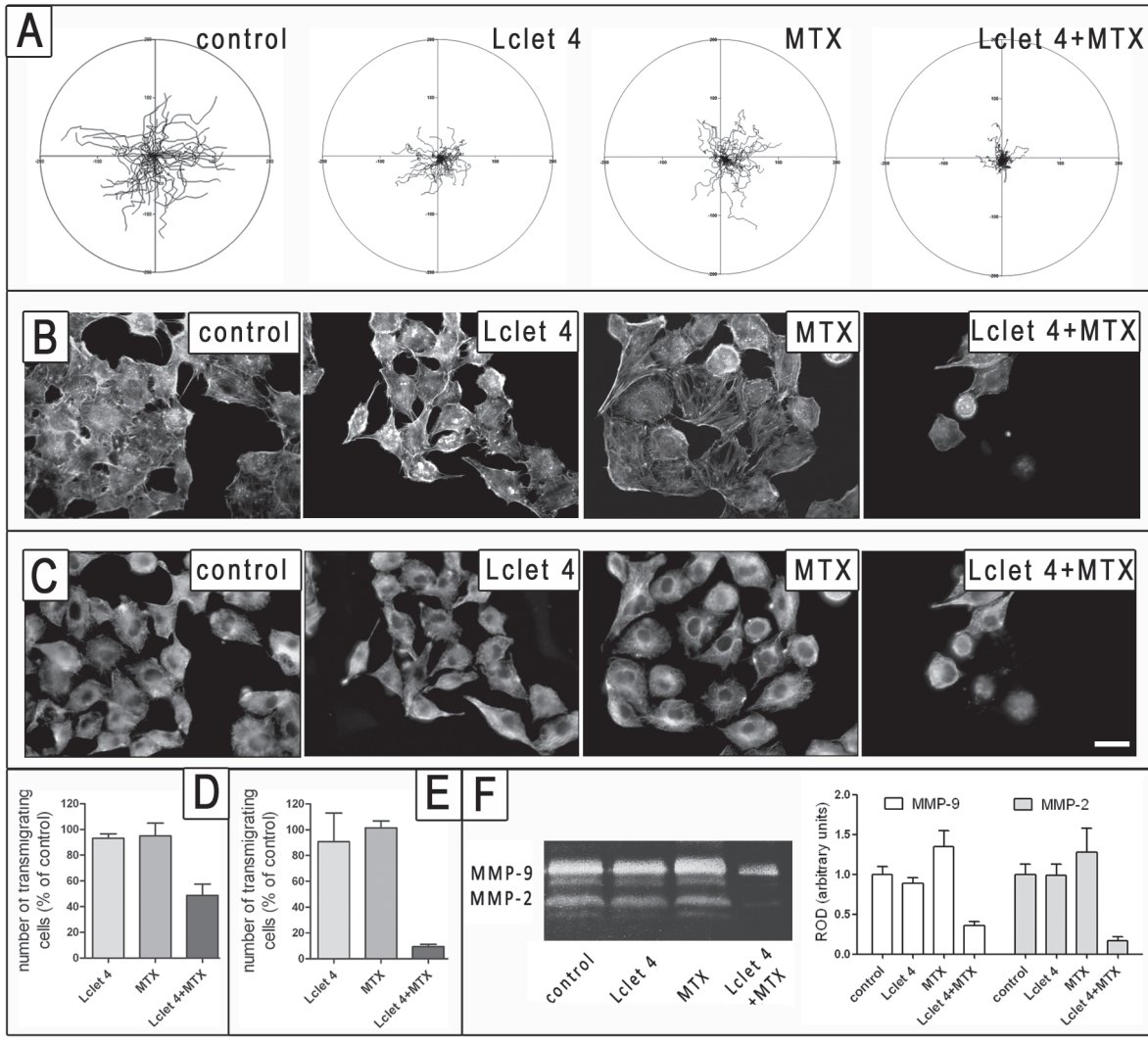

Figure 3. Attenuating effect of Lclet 4/MTX cocktails on the motility and MMP expression in DU-145 cells
DU-145 cells were cultured in medium supplemented with 10% FBS in the presence of Lclet 4 (0.05 μg/ml), MTX (0.05 μg/ml) or Lclet 4/MTX cocktails. Time lapse analyses of single DU-145 cell movement were performed after 24 hours of incubation (**A**), paralleled by immunocytochemical analysis of actin cytoskeleton (**B**) and microtubule organization (**C**). Bar=20 μm. Transmigration of DU-145 cells through "naked" (**D**) and matrigel-coated microporous membranes (**E**) was analyzed, 24 hours (**D**) and 72 hours (**E**) after cell seeding in the presence of agents. Matrix metalloproteinase (MMP-2 and MMP-9) secretion was analyzed by gelatin zymography after 24 hours of incubation with the tested compounds. (**F**). Values represent means ± S.E.M. in arbitrary units (relative optical density ROD) after 24 hours of incubation.

Lclet 4 and MTX synergistically induce apoptosis in DU-145 cells

Flow cytometry analyses of Annexin V/PI cells were further performed to confirm the apoptotic response of DU-145 cells evoked by combined Lclet 4/MTX treatment. When administered alone at the concentrations of 0.05 and 0.1 μg/ml, both agents induced only minute apoptotic response of DU-145 cells, whereas their combination increased the fraction of Annexin V+ cells up to 25% (0.05 μg/ml) and 75% (0.1 μg/ml; Fig. 2A summarized in B). The analyses of caspase 3/7 activity in treated cells (Fig. 2C) confirmed the synergistic character of pro-apoptotic effects of Lclet 4 and MTX in DU-145 populations. A significantly increased activity of both enzymes in DU-145 cells treated with the Lclet 4/MTX cocktails was observed. Accordingly, the synergistic pro-apoptotic effect of both agents was confirmed by the analyses of cytochrome *c* release from DU-145 mitochondria, which was observed in the Lclet 4/MTX cocktail-treated cells (Fig. 2D).

Synergistic effect of Lclet 4/MTX cocktails on the invasive potential of DU-145 cells

We further analyzed the effect of Lclet 4/MTX cocktails on DU-145 motility on planar substrata. Both agents significantly inhibited non-apoptotic DU-145 translocations when administered alone at the concentrations of 0.05 and 0.1 μg/ml. The magnitude of this attenuation was the highest in the presence of the Lclet 4/MTX cocktail (Fig. 3A, Table 1). The effects of Lclet 4/MTX on DU-145 motility were paralleled by changes in cytoskeleton architecture, i.e. partial disintegration

Table 1. Effect of Lclet 4 and MTX on the motility of DU-145 cells
DU-145 cells were cultured in medium supplemented with 10% FBS in the presence of Lclet 4 (0.05 μg/ml), mitoxantrone (0.05 μg/ml) alone or in the presence of both compounds for 24 hours. Results statistically significant *vs* Lclet 4 (*) and *vs* mitoxantrone (#); $p < 0.05$

Movement parameters	DU-145			
	control	Lclet 4	MTX	Lclet 4 + MTX
Total length of cell trajectory [μm]	140.9 ± 5.04	123.25 ±3.1	156 ±3	112.4 ±6.3(*)(#)
Average speed of cell movement [μm/min]	0.336 ± 0.012	0.205 ±0.01	0.260 ±0.03	0.187 ±0.02(*)(#)
Total length of cell displacement [μm]	92.3 ± 4.6	50 ±3.25	64.2 ±4.2	17.5 ±2.7(*)(#)
Average rate of cell displacement [μm/min]	0.220 ± 0.011	0.083 ±0.005	0.107 ±0.008	0.029 ±0.001(*)(#)
Coefficient of movement efficiency (CME)	0.655 ± 0.022	0.416 ±0.02	0.415 ±0.02	0.156 ±0.01(*)(#)

of microfilament bundles (Fig. 3B) and microtubules (Fig. 3C), most prominent in cells treated with the Lclet 4/MTX cocktail. Importantly, in contrast to apoptosis induction, all the analyzed cells responded to the Lclet 4/MTX cocktail with an inhibition of motility, whereas apoptosis was observed only in a fraction of DU-145 cells. To directly address the anti-invasive effects of both agents, we further examined the transmembrane migration efficiency of DU-145 cells (Fig. 3D, E). A relatively strong inhibition of DU-145 transmigration through "naked" microporous membranes was seen in the presence of the Lclet 4/MTX cocktail (Fig 3D). It was paralleled by a synergistic inhibitory effect of both agents on the transmigration of DU-145 cells through Matrigel-coated microporous filters (Fig 3E). Notably, a distinct synergistic effect of Lclet 4 and MTX on the activity of metalloproteinases was also observed. No significant differences in MMP-2/MMP-9 activity were observed in DU-145 populations treated with Lclet 4 or MTX alone for 24 hours (Fig. 3F). In contrast, a considerable inhibition of their activity could be seen in DU-145 populations treated with the Lclet 4/MTX cocktail. These data indicate that the cocktail of both agents administered individually at non- cytotoxic concentrations may interfere with prostate cancer invasion through the combined effect on cell motility and the ability of prostate cancer cells to degrade local tissues.

DISCUSSION

Current strategies of prostate cancer treatment should not only keep in focus prolonged longevity but also the life quality of the affected patients. The combined therapy based on the application of bioactive plant compounds, such as saponins, with well known chemotherapeutic agents may help to attenuate prostate cancer progression without evoking undesired adverse effects. Combinations of therapeutic drugs with different modes of anti-cancer activity can allow for lowering their concentrations and for reduction of systemic toxicity (Berenbaum, 1989; Greco *et al.,* 1995). Here, we demonstrated that the administration of *Lysymachia sp.* saponin — Lclet 4, augments the cytostatic and anti-invasive potential of mitoxantrone - an agent commonly used in prostate cancer therapy. These data indicate that Lclet 4/MTX-based combined therapy may allow for decreasing the effective MTX concentrations, thus reducing its adverse effects.

Application of saponins has previously been proposed for combined therapy of a range of cancers, including drug resistant colon cancer and leukemia (Li *et al.,* 2011; Wink *et al*., 2012). For instance, digitonin (saponin isolated from *Digitalis sp.*) synergistically enhanced the cytotoxicity towards MCF-7 and Caco-2 cells, when administered in combination with thymol, EGCC or glaucin (Eid *et al.,* 2012). Similarly, Panax notoginseng saponins enhanced the cytotoxicity of cisplatin (Yu *et al.,* 2012). Our data showed that the cytotoxic and pro-apoptotic activity of Lclet 4/MTX cocktails was considerably higher than that of each agent administered alone. Comparative analyses of prostate cancer and normal cell reactions to Lclet 4/MTX cocktails showed their selective cytostatic and pro-apoptotic effects on cancer cells and the lack of adverse

effects on normal cell growth. Previously, green tea polyphenols were shown to enhance the growth inhibitory effect of cyclooxygenase-2 inhibitor on human prostate cancer cells *in vitro* an *in vivo* (Adhami *et al.,* 2007). Similarly, combined application of resveratrol or propolis with vinorelbine reinforced DU-145 cell growth inhibition (Scifo *et al*., 2006). Our findings demonstrate the potential of the Lclet 4/MTX-based therapeutic regimen for systemically neutral, palliative prostate cancer treatment.

Proapoptotic activity is a major goal in clinical cancer research aimed at identifying compounds that would selectively affect cancer cells (Hickman, 2002; Wang *et al*., 2007; Fuchs *et al*., 2009; Kim *et al.,* 2010; Chen *et al.,* 2012). In only a few reports was saponin activity considered in terms of inhibition of cancer progression. For instance, ginsenoside Rb2 was shown to inhibit tumor associated angiogenesis after intravenous inoculation of B16-BL6 melanoma cells and to suppress lung metastases (Mohizuki *et al.,* 1995), whereas ginsenoside Rg3

augmented anti-angiogenic effects of gemcitabine (Liu *et al.,* 2009). Our report is perhaps the first to demonstrate the synergy of the anti-invasive activities of saponins and cytostatic drugs on prostate cancer cells. Not surprisingly, the pronounced inhibition of prostate cancer cell movement in the presence of the Lclet 4/MTX cocktail was accompanied by changes in cytoskeleton architecture of DU-145 cells. Together with their influence on DU-145 transmigration and metalloproteinase activity, these changes comprehensively illustrate the multifaceted action of Lclet 4 and MTX on DU-145 invasiveness. It is premature to speculate whether cytoskeletal rearrangements result from the direct effect of both agents on cytoskeletal proteins. However, the inhibition of MMP-2 and MMP-9 activity was previously found to suppress the metastatic potential of prostate cancer cells (Itoh & Nagase, 2002; Chien *et al*., 2010). Conceivably, synergistic effects of Lclet 4 and MTX on MMP-2 and MMP-9 activity predominantly account for the attenuation of DU-145 invasive potential.

A strong pro-apoptotic and anti-invasive activity of Lclet 4/MTX cocktails at concentrations at which the individual components were non-toxic, demonstrates that such a combined treatment can be advantageous in malignancies that are irresponsive to either treatment alone. Furthermore, synergistic activation of the caspase3/7 system and the inhibition of MMP-2 and MMP-9 activity by the Lclet 4/MTX cocktail was accompanied by its synergetic effects on cell motility. Notably, inhibition of motility was observed in virtually all treated cells, regardless of their further apoptosis-related fate. This indicates that the pro-apoptotic effects of the Lclet 4/MTX cocktail are independent of its anti-invasive activity on DU-145 cells. The synergy of Lclet 4 and MTX effects may rely on their independent interference with intracellular signalling systems determining cancer cell viability and invasiveness. Importantly, neither Lclet 4 nor MTX affected MMP-2/9 and caspase 3/7 function in DU-145 cells when administered alone. Accordingly, Lclet 4 may help to amplify weak signals produced by MTX and/or circumvent the drug resistance. Saponosides were shown to modulate multiple drug resistance (MDR) in tumor cells, thus Lclet 4 can sensitize DU-145 cells to MTX in this way (Wink *et al*., 2012). In general, the elucidation of the structure-activity relationship of different saponins in combination with conventional drugs is much more challenging than the studies of saponins alone.

When extrapolated to *in vivo* situation, our observations suggest that Lclet 4/MTX cocktails may, through the converging effects, interfere with the efficiency of prostate cancer metastatic cascade. Combined, although independent, pro-apoptotic and anti- invasive effects account for their activity. Whereas the mechanistic aspects of this convergence remain elusive, our observations expand the knowledge about a wide range of saponin activities. Further detailed studies on this topic may result in elaboration of efficient saponin-based tumor therapies in the future.

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