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Inhibiting myosin light chain kinase retards the growth of mammary and prostate cancer cells

Lian-Zhi Gu[a,d], *Wen-Yang Hu*[a,d], *Nenad Antic*[a], *Rajendra Mehta*[b], *Jerrold R. Turner*[c], *Primal de Lanerolle*[a,*]

[a]*Department of Physiology and Biophysics, University of Illinois at Chicago, College of Medicine, 835 South Wolcott Ave, Chicago, IL 60612-7342, United States*
[b]*Illinois Institute of Technology Research Institute, Chicago, IL, United States*
[c]*Department of Pathology, The University of Chicago, Chicago, IL, United States*

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ABSTRACT

We have previously shown that ML-7, which inhibits myosin light chain kinase (MLCK), induces apoptosis in transformed and non-transformed cells. We have extended these studies and found that ML-7 stimulates the ability of etoposide to induce apoptosis in Mm5MT mouse mammary adenocarcinoma cells and Mat-Ly-Lu rat prostate cancer cells in vitro. ML-7 was also found to have a chemopreventive effect using an in vitro mouse mammary organ culture model. In vivo experiments demonstrated that ML-7 retards the growth of mammary tumours in mice and prostate tumours in rats. Moreover, ML-7 significantly stimulates the ability of etoposide to prevent the growth of established mammary tumours in mice and prostate tumours in rats. These results provide evidence for the efficacy of ML-7 as an adjuvant to etoposide in these models and warrants further development.

1. Introduction

Increased cell division and motility are hallmarks of cancer. Preventing the proliferation and migration of cancer cells are major objectives when treating cancer. The cytoskeleton plays a central role in cell division and motility. The organization of the cytoskeleton is determined primarily by tension generated by actin and myosin II. The actin-myosin II interaction in smooth muscle and non-muscle cells (in contrast to highly differentiated striated muscle cells) is regulated by the phosphorylation of a specific serine residue (ser 19) on the 20 kD light chain of myosin II.[1] This reaction, catalyzed by myosin light chain kinase (MLCK), stimulates the actin-activated, Mg^{2++}-dependent ATPase activity of myosin II.[1] Many laboratories have shown that MLC_{20} phosphorylation (MLC-P) and de-phosphorylation (MLC-DP) are required for smooth muscle contraction/relaxation[1] and to mediate a host of other cellular responses,[2,3] including cell motility[4] and cytokinesis,[5] the final step in cell division.

The role of the cytoskeleton in apoptosis or programmed cell death is poorly understood. Apoptosis is a carefully regulated cellular event that involves multiple signalling pathways.[6] One of the signature features of apoptosis is a dramatic reorganization or disruption of the cytoskeleton. The integrity of the cytoskeleton is determined mainly by

* *Corresponding author:* Tel.: +1 312 996 6430; fax: +1 312 996 1414.
E-mail address: primal@uic.edu (P. de Lanerolle).
[d] These authors contributed equally to this work.
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actin and myosin II as described above. While actin and actin-binding proteins[7,8] have been implicated in mediating these cytoskeletal changes, the role of myosin II in apoptosis is poorly understood.

We have investigated the kinetics of MLC-P during apoptosis.[9] In agreement with others,[10–12] we found an initial increase in MLC-P. However, this initial increase is followed by MLC_{20} dephosphorylation when transformed and non-transformed cells were treated with agents that induce apoptosis.[9] Other experiments showed that treating with KT5926 and ML-7, two different inhibitors of MLCK,[13,14] resulted in dose-dependent decreases in MLC-P and corresponding increases in cell death. In addition, MLC-DP precedes caspase activation and the onset of apoptosis and pre-treatment with z-VAD-fmk, a cell permeable caspase inhibitor,[15] protected cells subsequently treated with ML-7.

In this study, we used a mouse mammary cancer model and a rat prostate cancer model to investigate the anticancer effects of ML-7. Breast cancer is the most common malignancy among American women and prostate cancer is one of the leading malignancies resulting in a high degree of morbidity and mortality in men. ML-7 [1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazapine hydrochloride] is a cell-permeable, potent and selective inhibitor of MLCK ($K_i = 0.3\ \mu M$) that inhibits protein kinase A ($K_i = 21\ \mu M$) and protein kinase C ($K_i = 42\ \mu M$) at higher concentrations.[16] We have found that ML-7 induces apoptosis by itself and stimulates the ability of etoposide, an anticancer agent that is used clinically,[17] to induce apoptosis in mammary and prostate cancer cells in vitro. ML-7 also stimulates the ability of etoposide to prevent the growth of mammary and prostate tumours in mice and rats, respectively. Our data suggest that ML-7 may have a potent adjuvant effect when given in combination with a clinically used anticancer agent.

2. Materials and methods

2.1. Cell culture

The Mm5MT mouse mammary adenocarcinoma cancer cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 μg/ml streptomycin. The Mat-Ly-Lu (MLL) sub-line of the Dunning R-3327 prostate adenocarcinoma (graciously provided by B. Lokeshwar, University of Miami)[18] was maintained in RPMI1640 medium supplemented with 10% FBS, 250 nM dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. Apoptosis assays

Mm5MT or MLL cells (200,000 cells per well) were seeded in 6-well dishes one day before drug treatment and cultured as described above. On the day of the experiment, media was changed to DMEM supplemented with 0.5% FBS and the cells were treated with drugs as defined by the specific experimental protocol. ML-7 (Biomol, Plymouth Meeting, PA) was incubated with cells for 16 h when used alone. When used in combination with etoposide, ML-7 was added to cells 2 h before adding the indicated concentration of etoposide (Calbiochem, La Jolla, CA) and the cells were incubated with ML-7 and etoposide for an additional 16 h. The cells were then treated with trypsin, washed twice with cold PBS and re-suspended in 100 μl of buffer containing 10 mM Hepes, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$ (binding buffer). Then, 5 μl of FITC-conjugated annexin V (Pharmingen, San Diego, CA) and 10 μl of propidium iodide (PI) (50 μg/ml) were added and cells were incubated in the dark at room temperature for 15 min. Next, 400 μl of binding buffer was added per sample and the cells were analyzed cytoflourometrically using a Coulter Epics Elite ESP flow cytometer (Ex: 488 nm, Em: 585 nm). At least 10,000 cells were counted per analysis and cells that stained positive for annexin V and PI were judged to be apoptotic.

2.3. Chemoprevention assay

Mammary glands obtained from young Balb/c mice that are exposed to 7,12-dimethylbenz(*a*)anhracene (DMBA) for 24 h in culture to form precancerous lesions in 24 days. The procedure has been successfully used to determine efficacy of chemopreventive agents and is described in detail elsewhere.[19] Briefly, 70 mammary glands from 35 Balb/c mice were divided into seven groups of 10 glands each and incubated in serum-free medium containing insulin, prolactin (5 μg/ml each), aldosterone and hydrocortisone (1 μg/ml each) for 10 days. DMBA (2 μg/ml) was included in the medium for 24 h on day 3. The glands were incubated for an additional 14 days in the absence of hormones except insulin. This allows the regression of the normal mammary alveolar structures whereas the precancerous mammary alveolar lesions (MAL) acquire altered hormonal responsiveness and do not regress under these conditions. Chemopreventive agents were included in the medium during the first 10 days. The glands were fixed in formalin and stained with alum carmine and evaluated for MAL. Percent inhibition was calculated by comparing the incidence in the control glands with the treated groups.

2.4. Urea/glycerol gel-immunoblotting

Mm5MT or MLL cancer cells in 6-well plates were treated with ML-7 (10 or 5 μM, respectively) or etoposide (30 μM) or combination of two at indicated concentrations for 16 h. MLC-P in cancer cells was then quantified by the urea/glycerol gel-immunoblotting method.[9] Briefly, cancer cells were fixed in 10% trichloroacetic acid (TCA) containing 10 mM dithiothreitol (DTT). Cell pellets were washed four times with acetone and protein was extracted by dissolving in buffer containing 9 M urea, 10 mM DTT and 20 mM Tris, pH 8.0. The unphosphorylated and phosphorylated forms of MLC_{20} were separated using urea/glycerol PAGE, transferred to nitrocellulose and probed with an affinity purified antibody to MLC_{20}. This antibody recognizes the unphosphorylated and phosphorylated forms of MLC_{20}. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).[9]

2.5. Tumour models and drug treatment

Mm5MT cells grown in culture were harvested immediately before injection into syngeneic MMTV-C3H/HeN mice. Cells were washed to remove serum and 10^6 cells were resuspended in 100 μl of serum-free DMEM. Healthy, MMTV-free female mice (14–20 weeks old) were anesthetized with ether and 10^6 cells were injected subcutaneously into the right flank. The mice were randomly divided into four groups of five mice, each, and treated with vehicle, ML-7, etoposide or ML-7 plus etoposide. Drug administration was started 1 week after the cells were injected. To deliver ML-7, a small horizontal incision was made in the interscapular area and a 200 μl osmotic pump (Alzet, Cupertino, CA) filled with either 27 mM ML-7 in 50% DMSO or 50% DMSO (vehicle control) was implanted and the wound closed. These pumps have a release rate of 0.25 μl/h and released drug at this rate for 4 weeks. When given, 25 mg/kg etoposide was injected intraperitoneally on the first 3 days of every week for 4 weeks (days 7–9, 14–16, 21–23 and 28–30).[20] The mice were sacrificed with ether after 4 weeks of drug administration and tumours were removed, weighed and processed for analysis.

MLL cells grown in culture as described above were harvested and washed in serum-free Hank's buffer. The cells were suspended in 500 μl serum-free Hank's and 10^6 cells were injected subcutaneously into the right flank of 12-week old male Copenhagen rats anesthetized with ether. The cells were allowed to grow and drug treatment was started 5 days after inoculation when the rats had developed palpable tumours. The rats were randomly divided into four groups, five in each group. The rats received injections of ML-7 or vehicle via the jugular vein every 4 days for 2 weeks. ML-7 was used at the dose of 35 mg/kg. Etoposide was injected IP at the maximum tolerant dose of 50 mg/m^2 daily.[21] The rats were sacrificed with ether 14 days after the start of drug treatment. The tumours were removed, weighed and processed as described below.

All animal procedures were performed with prior UIC Animal Care Committee approval.

2.6. Analysis of tumours

The excised tumours were gently patted dry and weighed using a Mettler digital balance. Tumours were then sectioned into 2 mm slices, fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin. Five micron sections demonstrating the entire surface were stained by hematoxylin and eosin and examined by a surgical pathologist (J.R.T.) blinded to the experimental conditions. Photomicrographs documenting the entire section were collected and areas of necrotic and viable tumour were determined using the manual tracing tools within MetaMorph 6.2 (Universal Imaging Corporation, Downingtown, PA).

2.7. TUNEL staining

Tumour sections were deparaffinized and rehydrated according to standard protocol. Tissue sections were permeabilized by placing slides in 10 mM citrate buffer (pH 6.0) and applying 350 W microwave irradiation for 5 min. Tissue sections were then stained with TMR Red-labelled terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) enzyme reagent using the In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Indianapolis, IN) as described by the manufacturer. Sections stained with the labelling solution without the terminal transferase was used as negative control. Tissue sections were finally mounted using Vectashield containing DAPI and examined using a Zeiss LSM 510 laser confocal microscope.

2.8. Statistical analysis

Data are presented as means ± SE. The level of the significance of differences between means was evaluated by Student's *t*-test or χ^2 analysis, and values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. ML-7 induces apoptosis in mammary and prostate cancer cells

We have previously demonstrated that ML-7 induces apoptosis in smooth muscle cells.[9] To determine if ML-7 has a similar effect on cancer cells, Mm5MT mouse mammary cancer cells and MLL rat prostate cancer cells were treated with varying concentrations of ML-7 for 16 h. The cells were collected and apoptosis was quantified as described in Section 2. Fig. 1 shows that ML-7 induced a dose-dependent increase in apoptotic cells in both Mm5MT and MLL cells.

3.2. ML-7 has a chemopreventive effect in an in vitro mammary cancer model

Mammary glands obtained from young Balb/c mice were exposed to DMBA as described in Methods and MAL formation was monitored. The inhibition of MAL formation has previously been successfully used as a parameter to judge the possible efficacy of chemopreventive agents.[19] In the present study, we evaluated effects of etoposide and ML-7 at 0.1, 1.0 and 10.0 μM concentrations on the development of MAL in organ culture. As shown in Fig. 2, etoposide inhibited the incidence of lesion formation at 1 and 10 μM concentration by 40–46% compared to control. Etoposide at 0.1 μM, however, did not affect MAL formation. ML-7 suppressed the development of MAL by 40% even at 0.1 μM and further reduced it to 58% of control at 1 μM. The differences observed between 0.1 and 10 μM ML-7 were not statistically different. However the inhibition of 58% at 1 μM compared to a 70% incidence in the control glands (7/10 glands positive) was significant.

3.3. ML-7 stimulates the ability of etoposide to induce apoptosis in Mm5MT cells

The combined effects of ML-7 and etoposide to induce apoptosis in mouse Mm5MT mammary cancer cells was determined by pre-treating cells with vehicle or 10 μM ML-

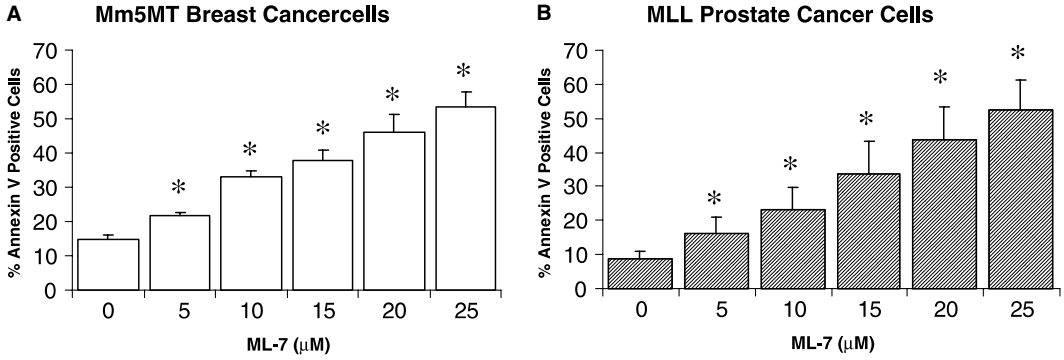

Fig. 1 – (A) ML-7 induces apoptosis in Mm5MT mammary cancer cells and (B) MLL prostate cancer cells. Mm5MT or MLL cells were treated with vehicle (0) or increasing concentrations (5–25 μM) of ML-7 for 16 h. The cells were collected and apoptosis was quantified by FACS analysis. The annexin V and PI positive cells as a percent of total cells, at each concentration of ML-7, are shown (N = 4). *P < 0.05 compared to control.

7 for 2 h. Varying concentrations of etoposide (1–1000 μM) were added and apoptosis was quantified 16 h later. ML-7 (10 μM), by itself, significantly increased apoptosis (0 etoposide, Fig. 3), consistent with the data in Fig. 1A. ML-7 also significantly increased the ability of etoposide to induce apoptosis (Fig. 3). A curve-fitting program (Cricket Graph) showed that the concentrations of etoposide required for inducing apoptosis in 50% of the cells was 25.4 μM plus ML-7; and 572 μM minus ML-7. Urea/glycerol gel-immunoblotting showed that both 10 μM ML-7 and 30 μM etoposide decreased MLC-P in Mm5MT cells and that the combination of the two drugs almost completely eliminated MLC-P.

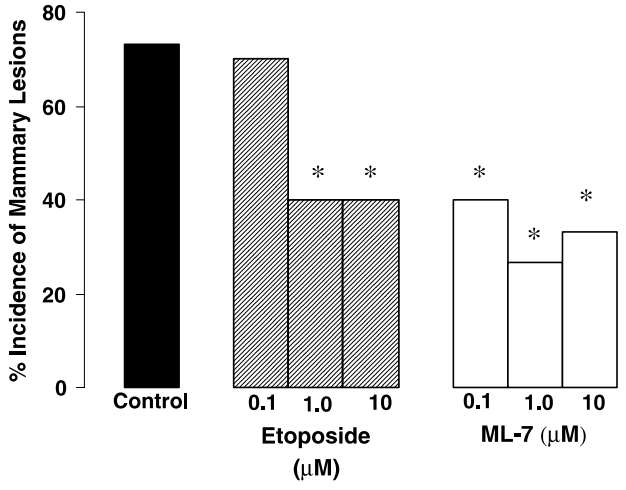

Fig. 2 – Chemopreventive effect of ML-7 in mouse mammary gland organ culture. Mammary glands from Balb/c mice were treated as described in Methods and the effects of etoposide and ML-7 on preventing MAL formation was quantified. ML-7 prevents MAL formation at 0.1 μM while a similar level of inhibition requires a 10× higher concentration of etoposide. Percentage inhibition was calculated by comparing the incidence in the control glands with the treated groups. Results were subjected to χ^2 analysis. *P < 0.05 compared to control.

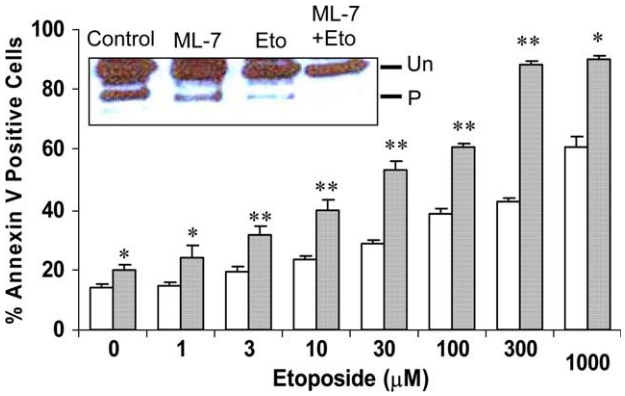

Fig. 3 – ML-7 stimulates the ability of etoposide to induce apoptosis in Mm5MT cells in vitro. Mm5MT cells were pretreated with vehicle (open bars) or 10 μM ML-7 (stippled bars) for 2 h prior to adding the indicated concentrations of etoposide. Cells were collected 16 h after adding etoposide and apoptosis was quantified by FACS analysis. The annexin V and PI positive cells as a percentage of total cells, at each concentration of etoposide, are shown (N = 4, *P < 0.05, **P < 0.001 vs. etoposide alone). (Inset) Mm5MT cells were treated with vehicle (control), 10 μM ML-7, 30 μM etoposide or 10 μM ML-7 and 30 μM etoposide. MLC-P was measured by urea/glycerol gel-immunoblotting as described in Methods. Un and P identify unphosphorylated and phosphorylated MLC_{20}, respectively. Note the decrease in the phosphorylated band in the treated groups compared to the control. This experiment was repeated four times and the data from a representative experiment is shown.

3.4. *ML-7 has an additive tumouricidal effect with etoposide on mammary cancer in mice*

To investigate the anticancer activity of ML-7 in vivo, Mm5MT cells were injected into the right flanks of female mice. The tumours were allowed to grow for 1 week, which was sufficient time to develop palpable tumours. The mice were then treated with the various combinations of drugs for 4 weeks.

Physical examination of the mice showed that the mice treated with vehicle, ML-7 or etoposide alone tolerated these drugs without visible signs of discomfort (see Section 4). ML-7 and etoposide both decreased tumour growth but only the etoposide effect was statistically significant ($P < 0.05$) compared to mice receiving vehicle. Importantly, the combination of ML-7 and etoposide dramatically reduced tumour growth compared to mice receiving vehicle (88.5% inhibition of tumour growth, $P < 0.001$) and to mice receiving etoposide alone ($P < 0.05$) (Fig. 4).

3.5. *Histological evaluation of mammary tumours*

Histological analysis revealed significant necrosis within control, ML-7-treated, etoposide-treated, and ML-7 plus etoposide-treated mice. Blinded examination of photomicrographs and quantification of areas of necrotic and viable tumour (Section 2) revealed significantly less viable tumour area in mice treated with etoposide and ML-7 (Fig. 5A). It was apparent on further examination that the distribution of necrosis in control, etoposide treated and ML-7-treated mice was predominantly confined to the center of the tumour. This pattern is typically seen in rapidly growing tumours. Away from these central areas, small foci of apoptosis were apparent but adjacent tumour was viable, with intact cell adhesions, as evident by tumour cell cohesion, and readily identifiable mitotic figures (Fig. 5B). In contrast, necrosis in tumours of mice treated with ML-7 plus etoposide was distributed in a predominantly perivascular pattern (Fig. 5B). This pattern was clearly distinguishable from that seen in the other tumours and suggests that necrosis may have been induced by blood-borne agents, i.e., ML-7 and etoposide. Individual cells within these areas of necrosis were characterized by dense eosinophilic cytoplasm and shrunken fragmented nuclei, a morphology typical of apoptosis. Cells adjacent to these areas, that were not frankly necrotic, generally showed early signs of cell death, including dis-cohesion, vacuolization and absence of mitoses. Thus, the in vivo synergy between ML-7 and etoposide causes a pattern of tumour necrosis consistent with the enhanced apoptosis observed in vitro.

3.6. *ML-7 induces apoptosis in prostate cancer cells and has tumouricidal effects on rat prostate cancer*

To determine if ML-7 stimulates the ability of etoposide to induce apoptosis and retard tumour growth widely, we determined the effects of ML-7 and etoposide on MLL pros-

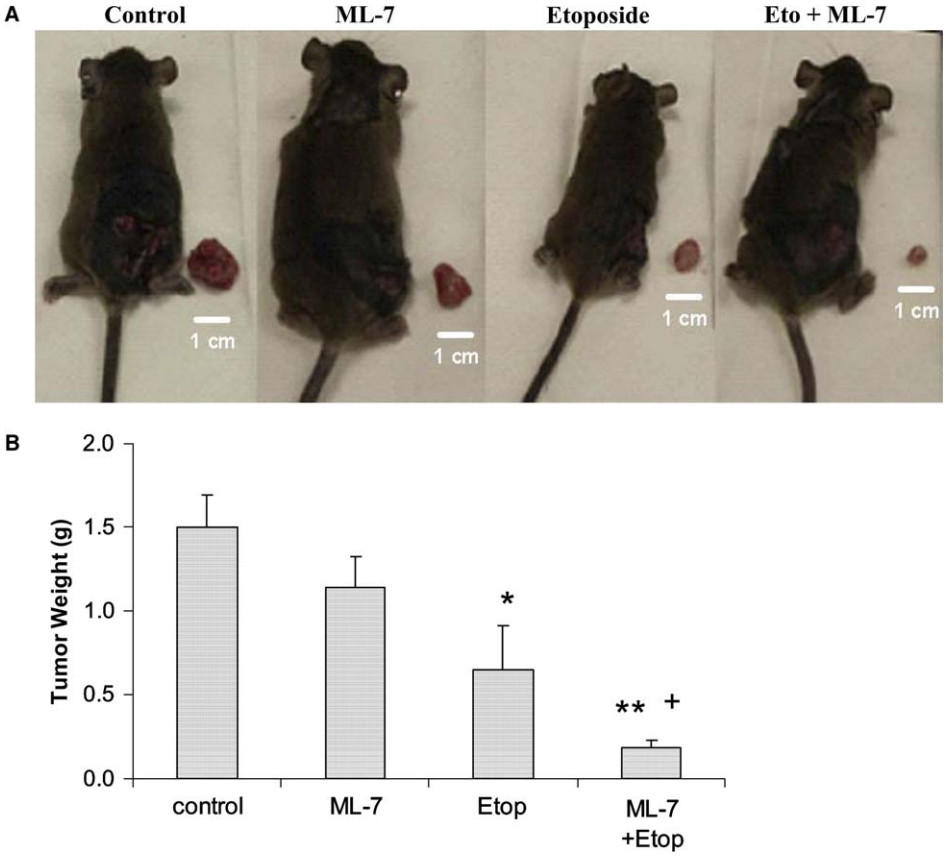

Fig. 4 – ML-7 and etoposide have a potent, additive tumouricidal effect on mammary tumours. Female MMTV/C3H/HeN mice were inoculated with Mm5MT cells as described in Methods. Drug treatment was started 7 days later when the mice had developed palpable tumours. The mice were sacrificed after 28 days of drug treatment. (A) Tumours removed from representative mice in each treatment group and a ruler is included as a size reference. (B) shows the means ± SE for tumour weight in each group ($N = 5$, $^{*}P < 0.05$, $^{**}P < 0.001$ vs. vehicle control, $^{+}P < 0.05$ vs. etoposide alone).

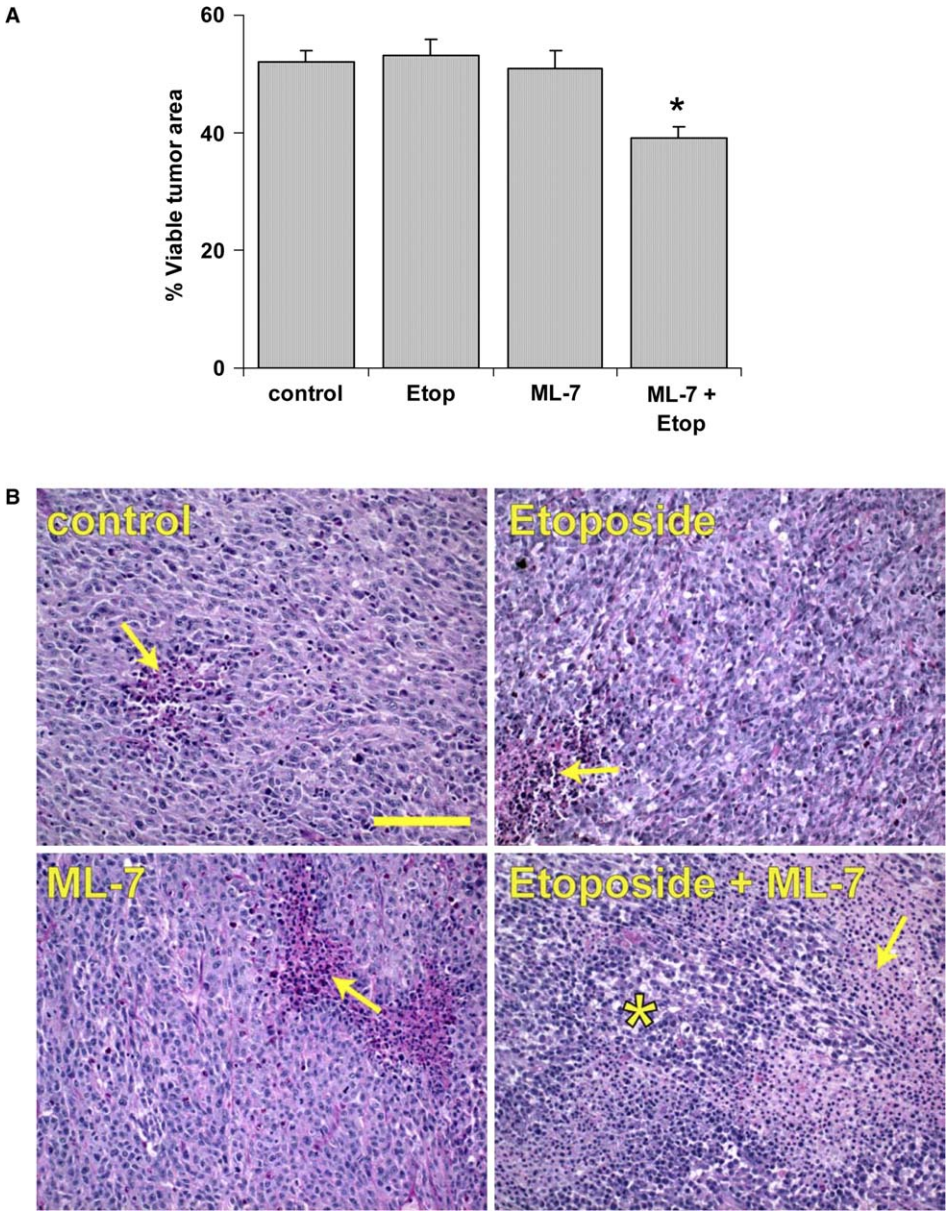

Fig. 5 – ML-7 and etoposide synergize to enhance tumour necrosis in vivo. (A) Photomicrographs were analyzed in blinded fashion for areas of necrotic and viable tumour and quantified using manual tracing tools within MetaMorph 6.2. Only the tumours from mice treated with both etoposide and ML-7 showed a significant decrease in viable tumour area compared to control ($N = 5$, $^{*}P < 0.05$). (B) Representative medium power images are shown of tumours from control, etoposide-treated, ML-7-treated, and etoposide plus ML-7-treated mice, as indicated. Limited areas of necrosis are present in tumours from etoposide- and ML-7-treated mice (arrow), but adjacent tumour is viable and mitotic figures are easily found. In contrast, larger areas of necrosis are present in tumours from etoposide plus ML-7-treated mice (arrow) and adjacent viable tumour show signs of impending apoptosis, including incohesion (asterisk). Bar = 100 μm.

tate cancer cells. In this case, we pre-treated MLL cells grown in culture with 5 μM ML-7 before adding varying concentrations of etoposide from 1 to 1000 μM. ML-7 significantly increased the apoptotic effect of etoposide when compared with cells treated with etoposide alone, and decreased the concentration required for inducing apoptosis in 50% of the cells from 376 μM (no ML-7) to 68 μM (with ML-7, Fig. 6). Urea/glycerol gel-immunoblotting showed that 5 μM ML-7 decreased MLC-P and that 30 μM etoposide resulted in a smaller decrease in MLC-P in MLL cells. When used together, MLC-P was decreased to a level comparable to ML-7 alone (Fig. 6, inset).

To test the anticancer effect of ML-7 in a rat prostate cancer model, ML-7 or vehicle was administered by intrajugular injection. Etoposide was administered by intraperitoneal injection. As with the mice, the rats appeared to tolerate the

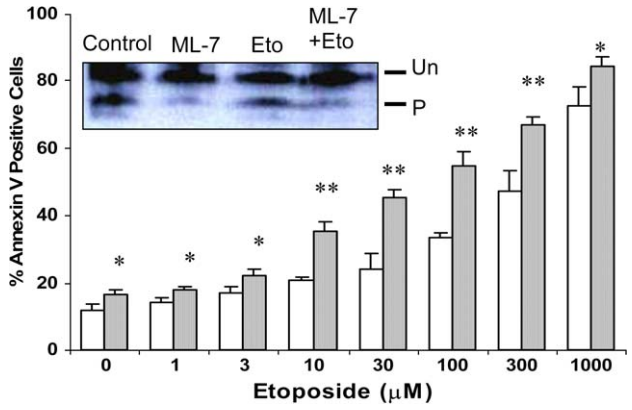

Fig. 6 – ML-7 stimulates the ability of etoposide to induce apoptosis in MLL cells in vitro. MLL cells were pretreated with vehicle (open bars) or 5 μM ML-7 (stippled bars) for 2 h prior to adding the indicated concentrations of etoposide. Cells were collected 16 h after adding etoposide and apoptosis was quantified by FACS analysis. The annexin V and PI positive cells as a percentage of total cells, at each concentration of etoposide, are shown (N = 4, *P < 0.05 and **P < 0.01 vs. etoposide alone). (Inset) MLL cells were treated with vehicle (control), 5 μM ML-7, 30 μM etoposide or 5 μM ML-7 and 30 μM etoposide. MLC-P was measured by urea/glycerol gel-immunoblotting as described in Methods. Un and P identify unphosphorylated and phosphorylated MLC_{20}, respectively. ML-7 alone and with 30 μM etoposide, resulted in a substantial decrease in the phosphorylated MLC_{20} band where as etoposide resulted in a smaller decrease in MLC-P. This experiment was repeated four times and the data from a representative experiment is shown.

individual drugs or vehicle without obvious discomfort. Rats receiving both ML-7 and etoposide, however, appeared to be more lethargic and lost on average 15% of their initial body weight. ML-7 or etoposide alone significantly inhibited the prostate tumour growth and decreased tumour weight by 29.6% and 43.3%, respectively ($P < 0.05$ vs. vehicle control). The combination ML-7 and etoposide further retarded tumour growth and decreased tumour weight by 79.1% compared to the vehicle control ($P < 0.001$ vs. vehicle control) (Fig. 7).

Furthermore, TUNEL staining showed more apoptotic cells in sections from rats receiving ML-7 or etoposide compared to vehicle control. Importantly, the combination of ML-7 and etoposide further increased the number of apoptotic cells (Fig. 8). Quantification of the TUNEL positive nuclei in 300 cells from randomly chosen fields in each group showed that 19.2%, 40.6%, 35.8% and 66.7% of the nuclei were TUNEL positive in control, ML-7-treated, etoposide-treated, and ML-7 plus etoposide-treated tumours, respectively.

4. Discussion

Tumour growth is characterized by an elevated mitotic index in cancer cells. Cytokinesis, the final step in mitosis, requires an actin-myosin II dependent contractile event.[5,6,22] This interaction in smooth muscle and non-muscle cells is regulated by MLC_{20} phosphorylation.[1–4] Consequently, changes in MLC_{20} phosphorylation should affect the ability of cells to divide. In support of this, microinjecting[23] or expressing[24] the active fragment of MLCK prolongs the cell cycle. We have also shown that a number of agents that induce apoptosis result in MLC_{20} dephosphorylation and that MLC_{20} dephosphorylation precedes caspase activation.[9] In addition, it has been shown that agents or procedures that destabilize the cytoskeleton also result in apoptosis.[7,8,25] Based on these data, we have proposed that the physical characteristics of the cytoskeleton are involved in as yet unknown way in determining cell fate.[9] This in turn suggests that cytoskeletal disrupters could be used to prevent cell proliferation.

The cytoskeleton, however, has not generally been targeted in treating cancer. All cells contain a cytoskeleton and it is difficult to visualize how cytoskeletal disrupters could only affect proliferating cells. Nevertheless, taxol, which affects the microtubule cytoskeleton has been effective in treating advanced breast cancer.[26,27] Recent studies have also suggested that MLCK inhibitors could be useful as anti-cancer agents. Because MLC_{20} phosphorylation is required for cell motility,[28,29] papers describing the use of MLCK inhibitors as an anti-cancer agent have focused on preventing cancer cell migration.[30,31] Inhibiting MLCK blocks cancer cell invasion and adhesion in vitro[31] and Tohtong and colleagues have shown that MLCK inhibitors inhibited the invasion of MLL cells by reducing their motile characteristics.[32] In contrast, the in vivo effects of inhibiting MLCK on cancer cell proliferation and tumour growth are poorly understood.

Therefore, we used both in vitro and in vivo models of mammary and prostate cancer to investigate the role of MLCK and MLC_{20} phosphorylation in determining cell fate. The data presented above demonstrate that inhibiting MLCK induces apoptosis in mammary and prostate cancer cells and inhibits the growth of mammary and prostate tumours in mice and rats. The in vivo effects of ML-7 apparently involve inducing apoptosis in tumour cells. In agreement with previous work,[9] our data demonstrate that inhibiting MLCK with ML-7 results in apoptosis in two types of cancer cells (Fig. 1). Moreover, we have shown that ML-7 significantly increases the number of apoptotic cells in mammary tumours. The data presented above also demonstrate the ML-7 has a significant chemopreventive effect at a 10-fold lower concentration than etoposide (Fig. 2). Lastly, we have also shown that the combination of ML-7 and etoposide are more effective in producing apoptosis in prostate tumours than either compound alone (Fig. 8). When considered in light of data showing that ML-7 inhibits tumour invasion,[30–32] it appears that ML-7 can prevent the growth of primary tumours and possibly inhibit metastatic colonization by cancer cells.

Our data also demonstrate that ML-7 has a significant adjuvant effect on etoposide with respect to inducing apoptosis in vitro and preventing tumour growth in vivo. Evaluation of novel anticancer agents and combination therapies has led to the realization that synergism between chemo-

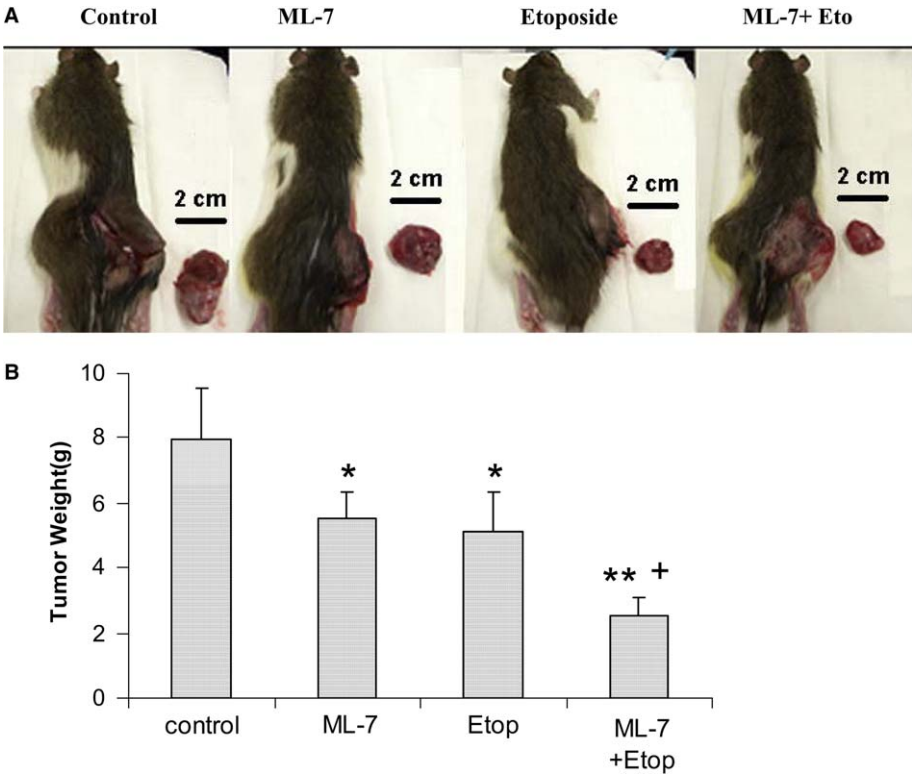

Fig. 7 – ML-7 and etoposide have a potent, additive tumouricidal effect on prostate tumours. Male Copenhagen rats were inoculated with MLL cells as described in Methods. Drug treatment was started 5 days later when the rats had developed palpable tumours. The rats were sacrificed after 14 days of drug treatment and tumour weight and body weight were recorded. (A) Tumours were removed from representative rats in each treatment and (B) shows the means ± SE for tumour weight in each group ($N = 5$, $^{*}P < 0.05$, $^{**}P < 0.001$ vs. vehicle control, $^{+}P < 0.05$ vs. etoposide alone).

therapy drugs with different mechanisms of action have increased response rates and improved outcomes for patients with cancer. For instance, etoposide, which blocks topoisomerase II and is used clinically to treat a number of solid tumours[17] has been shown to have a synergistic or additive effect on other drugs in treating cancer.[33–35] Our data demonstrate that ML-7 profoundly increases the ability of etoposide to induce apoptosis in vitro and prevent tumour growth in vivo. Mechanistically, it is possible that the combination of destabilizing the cytoskeleton and inhibiting topoisomerase II activity is highly toxic to cancer cells.

One of our major concerns in administering ML-7 to animals related to potential side effects of this drug. Because it inhibits MLCK, we were concerned that ML-7 would result in a profound systemic relaxation of smooth muscles that would be accompanied by a precipitous drop in blood pressure, respiratory distress or gastrointestinal problems. However, animals receiving ML-7 seemed to tolerate the drug without any obvious problems. They were active, there was no obvious loss of appetite, their breathing was not labored, they had no rectal bleeding and they put on weight. Blood pressure measurements on rats implanted with osmotic pumps containing 27 mM ML-7 have shown a decline in blood pressure during the first 2 weeks of therapy that stabilizes at a relatively normal blood pressure (Hu and de Lanerolle, unpublished). Although these data do not entirely eliminate the possibility of side effects, they demonstrate that ML-7, by itself, is tolerated without any obvious negative effects. However, rats receiving ML-7 via the jugular vein and etoposide IP lost more weight and visually did not appear to be as active as rats receiving either vehicle or ML-7. Their coats were also less shiny and they appeared to lose more fur. Additional experiments are clearly needed to determine the full toxicity of ML-7, especially when it is given as a bolus dose IV and in combination with other drugs.

In conclusion, we have shown that ML-7, which inhibits MLCK, results in apoptosis in cancer cells in vivo. We have also shown that ML-7 significantly increases the ability of etoposide, which is used clinically to treat solid tumours,[17] to induce apoptosis in cancer cells. These data further support the idea that inactivating myosin II by dephosphorylating MLC_{20} is an important part of the mechanism of apoptosis. Other experiments showed that ML-7 has a chemopreventive effect. In addition, we have shown that ML-7 possesses tumouricidal activity in vivo without any overt toxic side effects. Our data also demonstrate that ML-7 significantly increases the ability of etoposide to prevent the growth of mammary and prostate tumours. Thus, ML-7, alone or in combination with other drugs, may be a promising candidate for treating certain types of cancers.

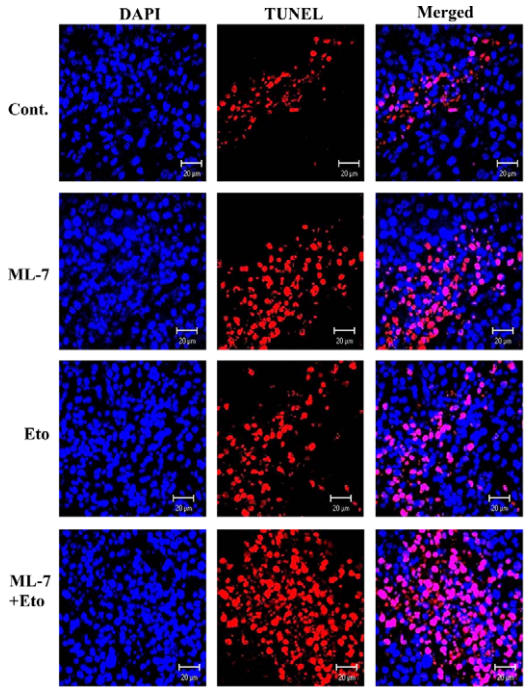

Fig. 8 – Tumours from untreated rats (control) and rats receiving ML-7 or etoposide (Eto) showed significantly higher numbers of TUNEL-positive cells than tumours removed from control rats. Tumours from rats receiving ML-7 and etoposide showed even higher levels of apoptosis. The pink color in right panel indicates the co-localization of the TUNEL and DAPI staining. Bar = 20 μm.

Conflict of interest statement

The authors state no financial or personal relationships with people or organizations that could have influenced this work.

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