BIOCHEMICAL PHARMACOLOGY 74 (2007) 1713–1726

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Mechanisms underlying the anticancer activities of the angucycline landomycin E

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ARTICLE INFO

Article history:
Received 12 June 2007
Accepted 21 August 2007

Keywords:
Landomycin E
Antibiotic
Adriamycin
Anticancer activity
Apoptosis
Multidrug resistance

ABSTRACT

Anthracyline antibiotics, produced by *Streptomyces* sp., still rank among the most efficient anticancer drugs in clinical use. Aim of this study was to gain deeper insight into the anticancer properties of the anthracycline-related angucycline landomycin E (LE). The impact of LE on nuclear morphology was assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining in the human carcinoma cell model KB-3-1. LE treatment led to the appearance of typical morphological signs of programmed cell death like cell shrinkage, chromatin condensation and formation of apoptotic bodies. Apoptotic cell death induced by LE was further characterised by caspase (substrate) cleavage and intense mitochondrial membrane depolarisation (JC-1 and rhodamine 123 staining) already after 1 h drug incubation. Moreover, incubation with LE led to reduced intracellular ATP pools suggesting LE-induced apoptotic cell death as a consequence of rapid mitochondrial damage. Furthermore, LE treatment led to profound generation of intracellular oxidative stress, indicated by radical scavenger pre-treatment and dichlorofluorescin diacetate (DCF-DA) staining experiments. Since chemoresistance is a common problem in cancer therapy, we also investigated the influence of ABCB1 (P-glycoprotein, P-gp), ABCC1 (multidrug resistance-related protein, MRP1) and ABCG2 (breast cancer resistance protein, BCRP) overexpression on the anticancer activity of LE. Compared to anthracyclines, cytotoxic activity of LE was only weakly reduced by P-gp and MRP1 overexpression. Moreover, BCRP expression had no influence on LE anticancer activity. In summary, LE exerts anticancer activity via potent induction of apoptosis and has promising anticancer activity even against multidrug resistant (MDR) cells. Taken together, these data suggest further development of LE as a new anticancer drug.

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Abbreviations: ABC, ATP-binding cassette; ADR, adriamycin (doxorubicin); BCRP, breast cancer resistance protein (ABCG2); BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DNR, daunomycin; GI_{50}, growth inhibition 50%; FACS, fluorescence-activated cell sorting; LE, landomycin E; JC-1, 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; MDR, multidrug resistance; MX, mitoxanthrone; MRP, multidrug resistance-related protein (ABCC); NAC, *N*-acetyl-L-cysteine; PARP, poly(ADP-ribosyl) polymerase; PBS, phosphate-buffered saline; P-gp, P-glycoprotein (ABCB1); PI, propidium iodide; Rh123, rhodamine 123; ROS, reactive oxygen species; TGI, total growth inhibition; Topo, topoisomerase; VP, verapamil.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.08.026

1. Introduction

New specific antibiotics are in scope of interest because of their potential anticancer activity [2]. Landomycin E (LE) is a novel representative of the angucycline antibiotics produced by *Streptomyces globisporus* strain 1912 [3]. Angucyclines contain a benz[*a*]anthraquinone framework with varying degree of insaturation and oxygenation. Thus, this group of antibiotics is, after tetracyclines and anthracyclines, the third class of natural antibiotics featuring a carbotetracyclic skeleton and displays a broad spectrum of biological activities including antitumor, antifungal and antiviral properties [6,7]. The landomycins belong to a subgroup of the angucycline family, characterised by a linear oligosaccharide conjugated to the angular carbotetracyclic quinone. The cytostatic properties of particular members of the landomycin family were shown to depend on the length of the oligosaccharide chain [7]. In case of LE, this conjugated oligosaccharide chain consists of the sequence α-L-rhodinose-(1 → 3)-β-D-olivose-(1 → 4)-β-D-olivose (Fig. 1). LE antitumor action was already demonstrated towards Guerin rat carcinoma [8], and several human cell lines [9]. Although, it was already suggested that, like most chemotherapeutic drugs, LE acts via apoptosis induction in tumor cells [9], the molecular mechanisms underlying its antineoplastic effect are still unknown.

Moreover, it is unclear whether classical resistance mechanisms might limit the use of LE. One of the most common reasons for the resistance to anthracyclines [10] is the overexpression of membrane-located efflux pumps of the ATP-binding cassette family (ABC-transporters) [11,12]. These proteins transport drugs out of the cell before they are able to reach their intracellular targets. Cells overexpressing ABC-transporters are termed multidrug resistant (MDR) because they are insensitive not only against one drug (e.g. anthracyclines) but against diverse chemotherapeutics, even with unrelated modes of action. Most prominent in mediating MDR with respect to antineoplastic drugs are members of three ABC-transporter subfamilies: ABCB including the well-known drug-transporter P-glycoprotein (ABCB1; P-gp), ABCC comprising the nine multidrug resistance proteins (MRP1–9) and ABCG harbouring the breast cancer resistance protein (ABCG2, BCRP) [11,12].

Here, we present novel data on the mechanisms of LE growth inhibiting and cytotoxic activities executed towards a wide range of tumor cell lines. The anticancer activity of LE was shown to be based on oxidative stress and induction of apoptosis via mitochondrial damage. Moreover, in contrast to anthracyclines, P-gp and MRP1 overexpression had only a minor protective effect towards LE. BCRP was found to have no impact on LE anticancer activity. Taken together, LE possesses promising potential for treatment of tumors which are known to be chemoresistant.

2. Material and methods

2.1. Drugs

Landonycin E (95% purity according to TCL analysis) was prepared as already described [3,8] at D.K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kyiv. Briefly, the LE-producing *S. globisporus* 1912 (strain 3-1) was cultured for 48 h at 28 °C in a medium consisting of soybean meal 2% and glucose 2% (pH 7.2). The ethyl acetate from the extract of the culture filtrate was evaporated under vacuum and dry organic sediment was dissolved in MeOH. LE was purified by chromatography on a silica gel (CH_3Cl–MeOH, 85:15) column (2.5 cm × 100 cm, MeOH) to yield LE (200 mg/l). Final purification by gel filtration on Sephadex LH-20 (MeOH) gave a pure red-orange amorphous LE powder. This powder was dissolved in ethanol and stored in hermetic flask at 4 °C. The main characteristics of LE (color, Rf, spectra of absorption and antibiotic activity) were stable during 3-month keeping. For the experiments LE was diluted in culture medium at the concentrations indicated (final ethanol concentration was always below 1%, v/v). Verapamil was purchased from Abbott (Vienna, Austria), gefitinib (Iressa) from Astra Zeneca and imatinib (Gleevec) from Novartis (Basel, Switzerland). All other substances were supplied from Sigma–Aldrich (St. Louis, USA). All solutions were freshly prepared before usage.

2.2. Cell culture

The following human cell lines and their chemoresistant sublines were used in this study: the epidermal carcinoma-derived cell line KB-3-1 and its P-gp-overexpressing subline KBC-1 (generously donated by W. Shen, Bethesda, USA) [1], the promyelocytic leukemia cell line HL60 and its MRP1-overexpressing subline HL60/adr and P-gp-overexpressing subline HL60/vinc (M. Center, Kansas State University, USA) [13], the breast adenocarcinoma cell line MDA-MB-231 with its BCRP-transfected subclone MDA-MB-231/bcrp (D. Ross, University of Maryland, Greenbaum Cancer Centre, USA) [4], the colon carcinoma cell line HCT116 (ATCC) and the non-small cell lung cancer cell lines A549 (ATCC) and SW1573 with its MRP1- and LRP-overexpressing subline 2R120 and its P-gp-overexpressing subline 2R160 (H. Broxterman, Department of Medical Oncology, Free University Hospital, Amsterdam, The Netherlands) [5]. HCT116 cells were grown in McCoy's culture medium and SW1573 cell lines in DMEM. All other cell lines were grown in RMPI 1640 supplemented with 10% fetal calf serum.

2.3. Cytotoxicity assays and National Cancer Institute (NCI) cell line screen

Cells were plated (2×10^3 cells for KB line, and 5×10^3 cells for HL60 line) in 100 μl per well in 96-well plates, and allowed to attach for 24 h. Drugs were added in another 100 μl culture medium and cells were exposed for 72 h. The proportion of viable cells was determined by EZ4U assay according to the manufacturer's recommendations (EZ4U, Biomedica, Vienna, Austria). Cytotoxicity was expressed as IC_{50} values calculated from full dose–response curves (drug concentration including 50% reduction in cell survival comparing to the control cultured in parallel without drug).

The methods used for the NCI 60 cell line anticancer drug screen have been described elsewhere [14]. Mean graphs were prepared from the appropriate data for each compound, and COMPARE correlation analyses were performed as described

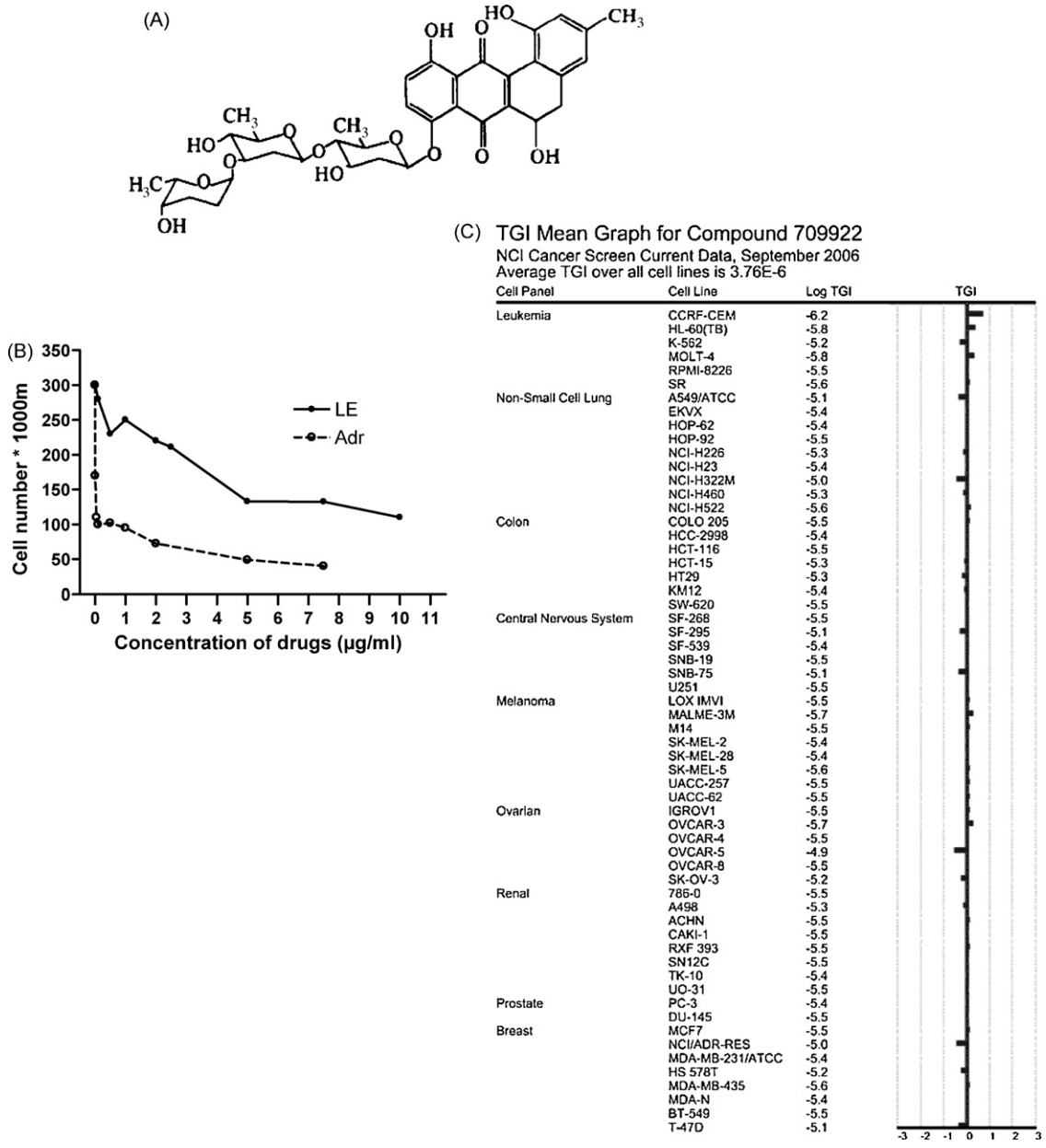

Fig. 1 – Chemical structure and cytotoxic activity of LE (A). (B) Effects of LE and ADR on viability of KB-3-1 cells were determined by trypan blue exclusion test following 72 h drug exposure. (C) Antiproliferative activity of LE in the National Cancer Institute anticancer screen. A graph of mean TGI profiles is shown. The bar scale is logarithmic. A bar extending to the right or to the left demonstrates enhanced or reduced sensitivity, respectively, of the cell line against LE as compared to the average sensitivity of all cell lines tested.

previously [15]. A detailed description is available at http://dtp.nci.nih.gov.

2.4. *DAPI staining*

After 24 h drug treatment, KB-3-1 cells were harvested, washed in phosphate-buffered saline (PBS) and cytospins were prepared. After fixation with a 1:1 methanol/acetone solution, slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) containing antifade solution (Vector Laboratories, Inc., USA). Nuclear morphology of cells was examined using a Laica DMRXA fluorescence microscope (Laica Mikroskopie and System, Wetzlar, Germany) equipped with appropriate epi-fluorescence filters and a COHU charge-coupled device camera. Duplicate slides were prepared for each cell type/treatment group and 300–500 cells were counted for each sample.

2.5. *Western blot analyses*

After 24 h drug treatment, protein extracts of KB-3-1 were prepared and Western blot analyses performed, as described

[16]. The following antibodies were used: Apoptosis Sampler Kit (Cell Signaling Technology, Beverly, MA)—anti-PARP (dilution 1:1000), anti-caspase 3 (dilution 1:500), anti-cleaved caspase 3 (dilution 1:1000), anti-caspase 7 (dilution 1:1000) and anti-cleaved caspase 7 (dilution 1:1000). All antibodies are polyclonal rabbit. For loading control β-actin monoclonal mouse AC-15 (Sigma, USA, dilution 1:1000) was used. All secondary peroxidase-labeled antibodies were purchased from Santa Cruz Biotechnology and used at working dilution of 1:10,000.

2.6. Cell cycle analyses

KB3-1, A549 or HL60 cells (2×10^5) were seeded into six-well plates and allowed to recover for 24 h. After recovery, cells were exposed another 24 h to 0.1–10 μg/ml LE or adriamycin (ADR). Control and drug-treated cells were collected, washed with PBS, fixed in 70% ice-cold ethanol, and stored at 4 °C. To determine cell cycle distribution, cells were transferred into PBS, incubated with RNAse A (10 μg/ml) for 30 min at 37 °C, followed by 30 min treatment with 5 μg/ml propidium iodide. Fluorescence levels were analysed by flow cytometry using FACS Calibur (Becton Dickinson, Palo Alto, CA). The resulting DNA histograms were quantified using Cell Quest Pro software (Becton Dickinson and Company, New York, USA).

2.7. Mitochondrial membrane potential measurement

Breakdown of ΔΨm was determined by FACS analyses using JC-1 (5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetra-ethylbenzimidazol-carbocyanine iodide), which allows to detect changes in the mitochondrial membrane potential (ΔΨm). For this purpose the Mitochondrial Membrane Potential Detection Kit (Stratagene, La Jolla, CA, USA) was used, as described in the manufacturer's instruction. 10^6 adherent KB-3-1 or HL60 cells were treated for 1 or 24 h with the tested drugs. After trypsinisation and PBS washing, cells were incubated for 10 min in freshly prepared JC-1 solution (10 μg/ml in culture medium) at 37 °C. Spare dye was removed by PBS washing and cell-associated fluorescence was measured with FACS.

2.8. N-acetyl cysteine (NAC) assay

KB-3-1 cells (2×10^4 ml^{-1}) were seeded in 100 μl per well in 96-well plates and allowed to recover for 24 h. After 30 min preincubation with 50 μl NAC solution (1 and 2 mM in culture medium), drugs (LE, ADR, or bleomycin) were added in another 50 μl. After 72 h exposure, the proportion of viable cells was determined by EZ4U assay.

2.9. DNA gel electrophoresis

Selective extraction of degraded DNA was performed as described previously [17]. Briefly, after drug treatment, cells were collected by centrifugation and fixed in 70% ice-cold ethanol. After centrifugation, cell pellets were incubated in phosphate citrate buffer (192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid (pH 7.8)) on shaker for 30 min at room temperature. After another centrifugation, the supernatants were treated with 3 μl of 0.25% Nonidet NP-40 (dissolved in water) and 3 μl RNase A (1 mg/ml also in water). After 30 min incubation at 37 °C, 3 μl of proteinase K (1 mg/ml in water; Boehringer Mannheim, Indianapolis, USA) was added and the extract incubated for an additional 30 min at 37 °C. Subsequently, 12 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) were added. Horizontal gel electrophoresis (2% agarose containing ethidium bromide (0.5 μg/ml)) was performed in Tris–borate–EDTA buffer (TBE, pH 8.0) at 60 V for 3 h. DNA was visualized under UV light and photographed.

2.10. DNA intercalation assay

Intercalation of the tested drugs into salmon sperm DNA (Sigma) was determined using the methyl green assay as described [16]. Incubation times with test drugs were reduced to 2 h.

2.11. [^{3}H]-thymidine incorporation assay

KB-3-1 (5×10^4 cells/ml) were seeded in a 96-well plate, and treated 24 h later with LE for another 24 h. Medium was replaced by a 2 nM [^{3}H]-thymidine solution (diluted in full culture medium; radioactivity: 25 ci/mM). After 1 h incubation at 37 °C, cells were washed three times with PBS. Cell lysates were prepared and the radioactivity determined as described [16].

2.12. Measurement of intracellular oxidants

2′,7′-dichlorofluorescin diacetate (DCF-DA) was used to detect the intracellular production of free radicals [18]. Stock solutions of 33.4 mM in DMSO were stored at −20 °C. HL60 and KB-3-1 cells (2.5×10^5 cells per sample in phenol-free Hanks balanced salt solutions (HBSS)) were treated with the test compounds for 1 h at 37 °C, followed by 1 h exposure to DCF-DA. Mean fluorescence intensity was measured by FACS.

2.13. Rhodamine 123 accumulation studies

Rhodamine 123 (Rh123) accumulation assays were performed as previously described [19]. Briefly, 5×10^5 KB-3-1 cells were preincubated with LE for 30 min at 37 °C followed by incubation with Rh123 (0.25 mg/ml). After 60 min exposure, fluorescence of Rh123 was collected through a 530/30 nm bandpass filter by FACS.

2.14. ATP measurement

Intracellular ATP levels were measured using the ViaLight® MDA Plus Cytotoxicity and Cell Proliferation BioAssay Kit (Cambrex, Verires, Belgium) according to the manufacturer's recommendations.

2.15. Statistical analysis

Generally, experiments were performed in triplicate and repeated at least three times. Significance of the difference was assessed by Student's *t*-test. The level of significance was set at 0.05. Statistical interpretation of obtained densitometric

data was done with Microcal Origin (Microcal Software, Inc., Northampton, MA, USA).

3. Results

3.1. Cytotoxicity of LE in vitro

Comparable to already published data [9], the LE sensitivity of the tumor cell lines used in this work was in the low micromolar range with IC_{50} values of 0.76 μg/ml for MDA-MB-231, 1.87 μg/ml for HL60 and 4.3 μg/ml for KB-3-1 cells. In all experiments, LE was found to be significantly less toxic as compared to ADR, which exerts its anticancer activity generally in the low nanomolar range (Fig. 1B).

Additionally, the antiproliferative effects of LE were evaluated against a panel of 60 cell lines as part of the in vitro anticancer-screening services provided by the NCI. A detailed description of the protocol is available at http://dtp.nci.nih.gov. In the NCI panel, LE had an overall mean growth inhibition 50% (GI_{50}) at 1 μg/ml (=1.48 μM) and a total growth inhibition (TGI) at 2.69 μg/ml (=3.76 μM). The TGI concentrations of LE for the individual cell lines are presented in Fig. 1C.

3.2. Characterisation of LE-induced cell death

In a first approach to characterise its anticancer activity, the impact of LE on nuclear morphology was assessed in KB-3-1 cells by DAPI staining. LE treatment led to typical signs of apoptosis, i.e. chromatin condensation and fragmentation of nuclei into apoptotic bodies (Fig. 2A). Already after 24 h incubation with 1 μg/ml LE apoptotic features were observable in more than 20% of the cells. Further increasing LE concentrations led to enhanced apoptosis as indicated by a higher percentage of cells with apoptotic and shrinked/pygnotic nuclei (Fig. 2B). Moreover, already treatment with 1 μg/ml LE reduced mitotic cell population drastically from 14% to 2.5%. In the remaining mitoses aberrant mitotic figures (e.g. incomplete segregation and uneven distribution of chromosomal material) were found (Fig. 2C). Mitotic failure is known to cause aneuploidy and subsequent cell death. This might explain the increased apoptotic proportion at 1 μg/ml LE although the percentage of non-apoptotic nuclei remained constant.

LE was already demonstrated to induce DNA-fragmentation, a typical hallmark of apoptosis, in L1240 cells [9] by DNA laddering on agarose gels. After 24 h treatment with 5 μg/ml LE, DNA fragmentation was also detectable in KB-3-1 cells (Fig. 2D) but to a lesser extend than in L1210. This is probably due to an enhanced sensitivity of the leukemic cells to LE (IC_{50} 1.75 μg/ml for L1210 versus 4.3 μg/ml LE for KB-3-1). Moreover, KB-3-1 cell lacked the typical fragmentation pattern, which indicted that this cell line might have a reduced ability to activate endonucleases necessary for the further DNA fragmentation [20].

Apoptosis is characterised by a well-organized sequence of cellular events, resulting in the activation of the caspase cascade. Caspases are proteases which, once activated, cleave enzymes and proteins essential to cell vitality, leading to further progressing of apoptosis. In order to further characterise LE-induced cell death, the caspase-mediated cleavage of caspase 3, caspase 7 and the caspase substrate poly(ADP-ribosyl) polymerase (PARP) was analysed by Western blotting (Fig. 2E). After 24 h incubation with LE, cleavage products of caspase 3 (17 kDa) and caspase 7 (20 kDa) were easily detectable. Moreover, a dose-dependent increase of cleaved PARP was shown, as indicated by the appearance of 89 and 24 kDa fragments.

To detect, whether the LE-induced apoptotic cell death is executed by involving the mitochondrial pathway, the fluorescent cationic dye JC-1 was used to detect the mitochondrial permeability transition. The collapse of the mitochondrial membrane potential is an early step in the induction of apoptosis by the intrinsic pathway [21]. In healthy, non-apoptotic cells the dye accumulates and aggregates within the mitochondria, resulting in bright red staining. In apoptotic cells, due to the collapse of the membrane potential, JC-1 cannot accumulate within the mitochondria and remains in the cytoplasm in its green-fluorescent monomeric form. In Fig. 3A, FACS analyses of JC-1-stained HL60, KB-3-1 and HCT116 cells treated with LE for 24 h are shown. While, after treatment with 2 μg/ml LE, 14% of the leukemic HL60 cells had a collapsed membrane potential, 4 μg/ml LE led to mitochondrial damage in nearly all cells (92%). Also in case of KB-3-1 and HCT116 cells LE had a very profound effect on the mitochondrial membrane potential leading to almost complete depolarisation at 10 μg/ml. Unexpectedly, LE disturbed the mitochondrial potential in KB-3-1 cells even stronger after 1 h compared to 24 h exposition (Fig. 2B). In contrast, after 1 h ADR treatment of equimolar concentrations only 24% of the cells (20 μg/ml ADR) showed mitochondrial depolarisation increasing to a maximum of 54% after 24 h treatment. To confirm that LE affects the integrity of the mitochondria after 1 h, the fluorescent cationic dye Rh123 was used in addition. This compound is known to accumulate based on the required transmembrane potential only into intact mitochondria [22]. Treatment with LE led to a dose-dependent decrease in the Rh123 accumulation (Fig. 3C), with an 18% reduction induced by 1 μg/ml LE rising to 72% reduction when 10 μg/ml LE were used. Up to 50 ng/ml ADR showed no effect on Rh123 accumulation. Further ADR escalation was not possible due to the autofluorescence of ADR which was strongly interfering with Rh123 detection at concentrations >0.5 μg/ml (data not shown). Next, the effect of LE on intracellular ATP levels was tested. LE treatment led to a significant decline in ATP pools in KB-3-1 cells suggesting disrupted energy metabolism (Fig. 3D).

3.3. LE and radical-induced DNA damage

Intercalation into DNA is believed to be one of the underlying mechanisms necessary for cytotoxic activity of anthracyclines [2,23]. In order to analyse whether LE, comparable to ADR, intercalates into double-stranded DNA, methyl green competition assays were performed (Fig. 4A). Up to 50 μg/ml LE no significant indication for intercalation was observed while ADR was highly active already at 10 μg/ml.

Most anthracyclines are reported to induce under certain circumstances oxidative stress via generation of reactive

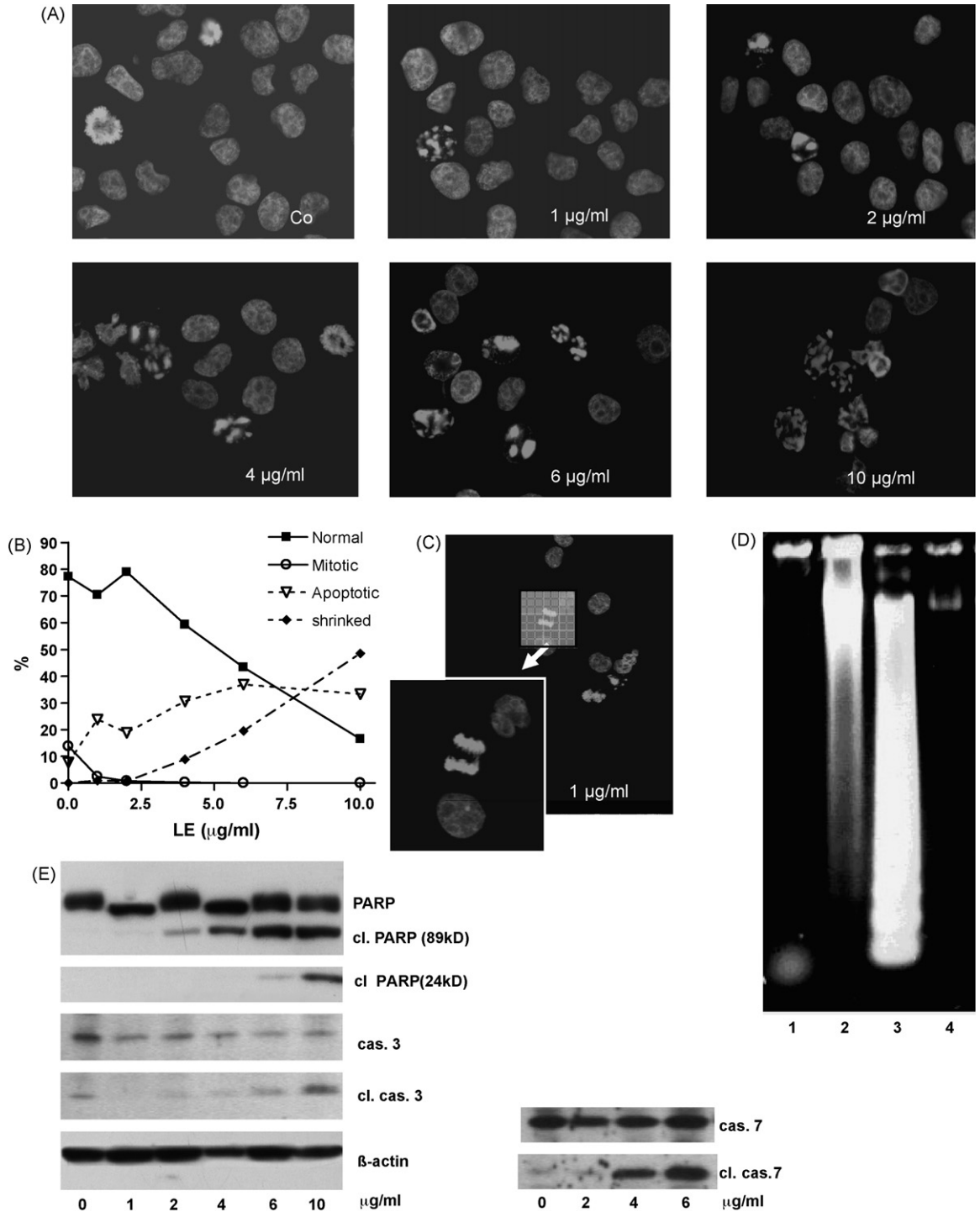

Fig. 2 – Induction of apoptosis in KB-3-1 cells after LE treatment. (A) Induction of apoptosis in KB-3-1 cells was determined after treatment with LE for 24 h. Staining of nuclei by DAPI is shown in untreated controls and cells treated with the indicated drug concentrations. (B) Morphological features of 300–500 nuclei of at least two slides for each concentration were analysed by DAPI staining. Percentages of normal, mitotic, apoptotic and shrinked/pygnotic nuclei at the indicated concentrations are shown. (C) A representative cell with an aberrant mitotic figure induced in KB-3-1 cells after 24 h treatment with LE is depicted. (D) Agarose gel electrophoresis of DNA extracted from cells treated for 24 h with LE was performed. Lane 1: KB-3-1 control; lane 2: KB-3-1 treated with 5 µg/ml LE; lane 3: L1210 treated with 5 µg/ml LE; lane 4: L1210 control. (E) Caspase-induced cleavage of caspase 3, caspase 7 and PARP in KB-3-1 cells after 24 h treatment with LE was determined via Western blot. Antibodies used are described under Section 2.

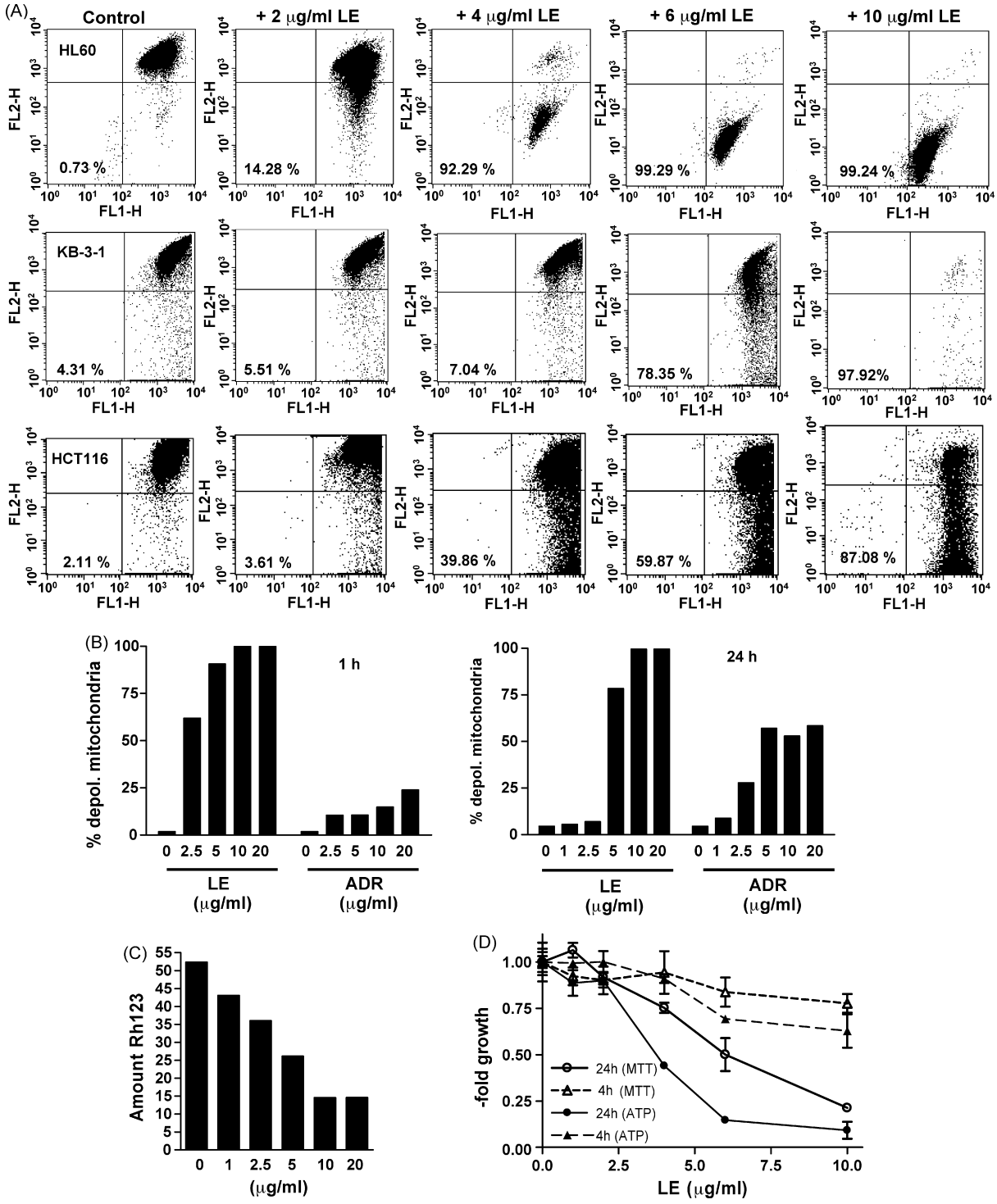

Fig. 3 – Characterisation of LE-induced apoptosis. (A) Loss of mitochondrial membrane potential after 24 h treatment with LE was determined by JC-1 staining. Increase of the green fluorescent apoptotic (FL-1) populations of HL60, KB-3-1 and HCT116 cells at the indicated drug concentrations (cells in the lower right field) are indicated (30,000 events were analysed in total per group). (B) Percentages of KB-3-1 cells with depolarised mitochondria (JC-1 staining) after treatment with equimolar concentrations of LE or ADR were determined after 1 and 24 h treatment. (C) Rh123 accumulation in KB-3-1 cells was assessed after 1 h incubation with the indicated LE concentrations by FACS analysis. For this purpose 20,000 events were analysed. (D) After the indicated incubation periods with LE, ATP levels and vitality of KB-3-1 cells were determined as described under Section 2. Values given are means ± S.D. from at least three independent experiments.

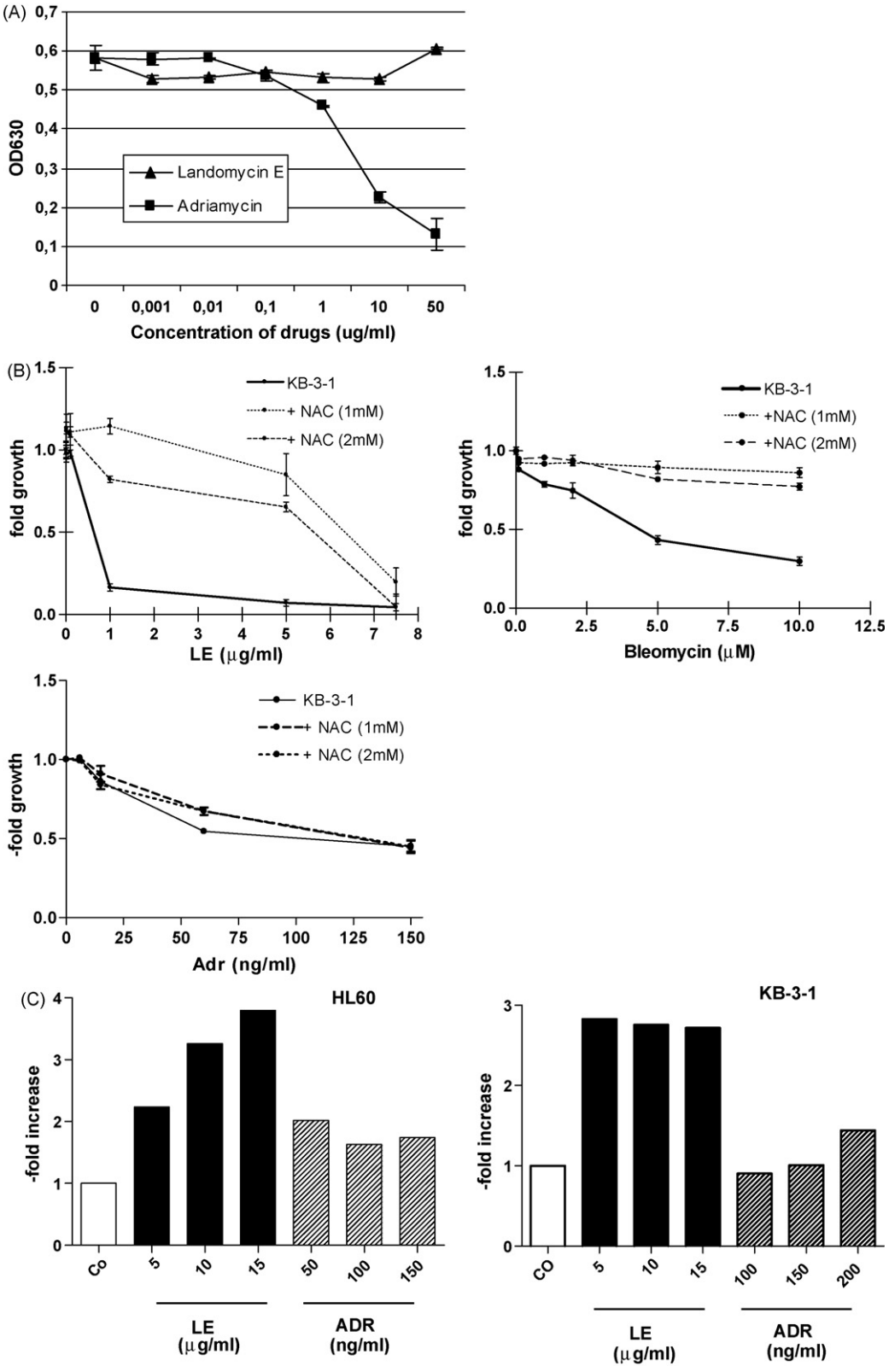

Fig. 4 – DNA intercalation and radical-induced DNA damage by LE. (A) DNA intercalation was determined by methyl green assay using salmon sperm DNA (10 mg/ml) treated for 2 h with the indicated drug concentrations. Absorbance was measured at 642 nm. Dose–response curves derived from two independent experiments in triplicates are shown. (B) Effects of 30 min pre-treatment with the radical scavenger NAC on the anticancer activity of LE, bleomycin (positive control) or ADR analysed after 72 h drug exposure by EZ4U kit. (C) Production of intracellular ROS by 1 h incubation with LE and ADR was determined in HL60 and KB-3-1 cells using the ROS indicator DCF-DA. Fluorescence was measured by flow cytometry. One representative experiment out of three delivering comparable results is shown.

oxygen species (ROS) [23]. The known radical scavenger N-acetyl cysteine was used to indicate whether ROS might be involved in the anticancer activity of LE. Thirty minutes NAC pre-treatment showed significant protective effect against the cytotoxic activity of LE in KB-3-1 cells (Fig. 4B). This protective effect was even stronger than against bleomycin which was used as positive control in this experimental setting. In contrast, NAC had only a weak protective effect against ADR treatment with toxic concentrations in the nanomolar range. To test, whether the ROS induction by LE is caused by extra- or intracellular free radicals, the cell permeable dye DCF-DA whose metabolite becomes highly fluorescent after oxidation was used. After 1 h incubation, LE treatment led to a significant and dose-dependent increase (up to 4-fold) of intracellular fluorescence determined by FACS analysis (Fig. 4C), while ADR treatment at comparable cytotoxic concentrations had only a minor impact (~2-fold).

3.4. *Impact of LE on DNA synthesis and cell cycle distribution*

The effect of LE on DNA synthesis was determined by [^{3}H]-thymidine incorporation assays. LE treatment reduced [^{3}H]-thymidine incorporation to less than 50% in KB-3-1 cells (Fig. 5A) already at 1 μg/ml (24 h incubation) indicating that LE has a strong impact on DNA synthesis. Similar effects were

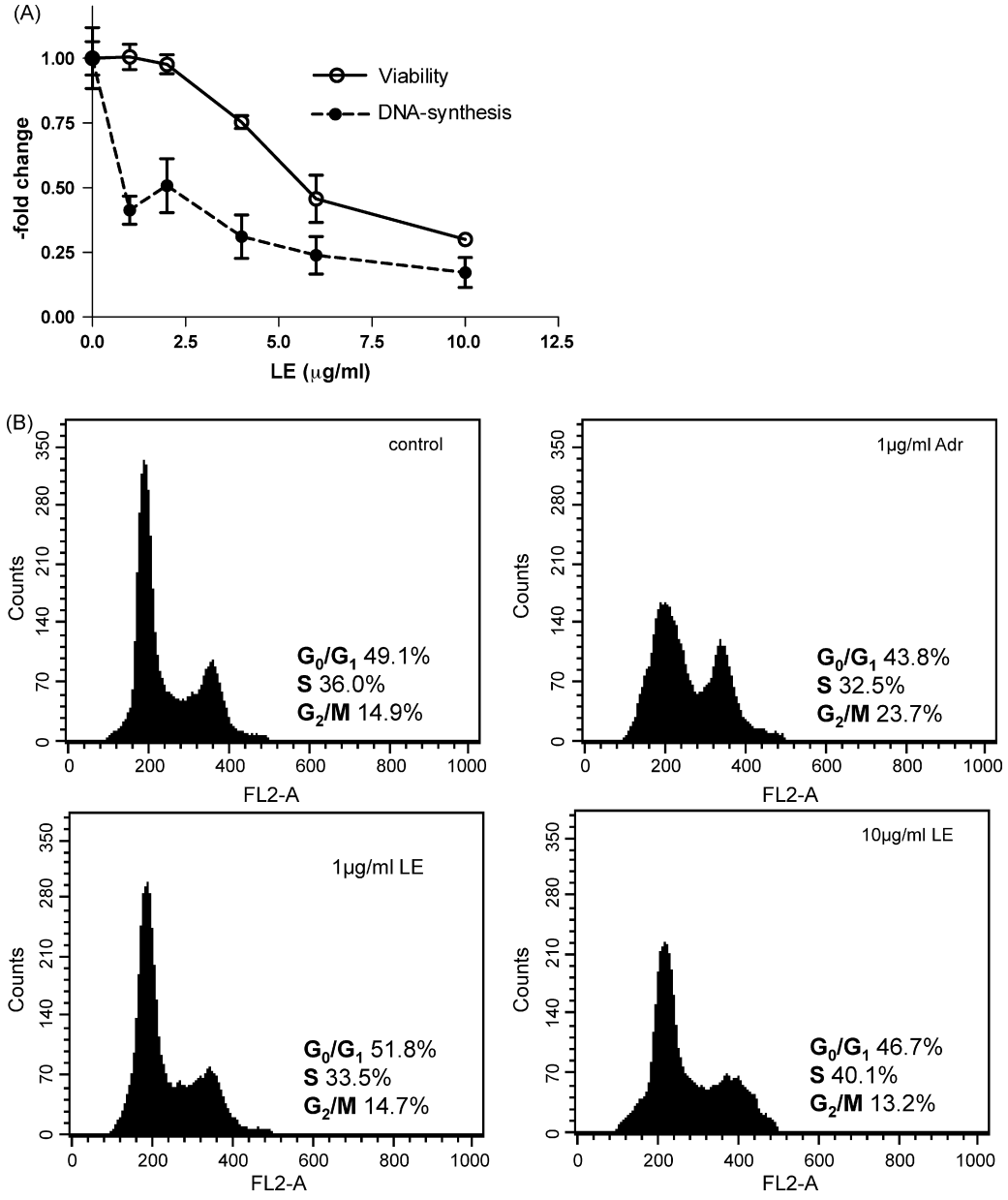

Fig. 5 – Impact of LE on DNA synthesis and cell cycle. (A) DNA synthesis and cell viability of KB-3-1 cells were determined by [^{3}H]-thymidine incorporation and MTT assay, respectively, after 24 h treatment with LE at the indicated concentrations. (B) Cell cycle analysis was done by flow cytometry determining the DNA content of PI-stained KB-3-1 cells after 24 h treatment with the indicated drug concentrations. Percentages of 25,000 cells in G_0/G_1, S and G_2/M phases of cell cycle were calculated by Cell Quest Software.

Table 1 – Used MDR cell models

Parental lines	Sublines	Overexpressed MDR proteins	Method of selection	Tissue
KB 3-1	KBC-1 [1]	P-gp	Grown in ↑ concentrations of colchicine	Cervix carcinoma
HL60	HL60/adr [13]	MRP1	Grown in ↑ concentrations of ADR	Leukemia
	HL60/vinc [13]	P-gp	Grown in ↑ concentrations of vincristine	
MDA-MB-231	MDA-MB-231/bcrp [4]	BCRP	Transfection and selection (geneticin)	Breast cancer
SW1573	2R120 [5]	MRP1, LRP	Grown in ↑ concentrations of ADR	NSCLC
	2R160 [5]	P-gp	Grown in ↑ concentrations of ADR	
A549		MRP1, MRP2, BCRP, LRP	Intrinsic resistance	NSCLC

observed in A549 lung cancer cells after LE treatment (data not shown). To characterise whether LE also effects the cell cycle distribution, PI-stained cells were analysed by flow cytometry. Unexpectedly and in contrast to ADR which strongly arrests cells in G_2/M phase, LE had no significant change on cell cycle distribution neither of KB-3-1 (Fig. 5B), nor of A549, HCT116 and HL60 cells (data not shown).

3.5. *Impact of P-gp, MRP1 or BCRP overexpression on the anticancer activity of LE*

To test whether the expression of diverse ABC-transporters has a protective influence against LE anticancer activity, sensitivity of parental cell lines compared to their chemoresistant sublines expressing defined resistance mechanisms was investigated (Table 1).

Both MRP1 and P-gp expressions were able to moderately but significantly reduce the cytotoxic activity of LE (Table 2). In general, this led to about 2-fold enhanced IC_{50} values for highly P-gp and MRP1-overexpressing sublines (highest HL60/adr with 2.5-fold and lowest KBC-1 with 1.5-fold resistance against LE). As expected, all P-gp- and MRP1-overexpressing sublines tested were profoundly resistant against ADR or daunomycin (DNR) (Table 2). In case of BCRP, MDA-MB-231/bcrp cells were comparably sensitive as the respective parental vector control line. The known BCRP substrate anthracycline mitoxanthrone (MX) was used as positive control in experiments with these cells (Table 2).

3.6. *Influence of ABC-transporter modulation on LE cytotoxicity*

Several substances, e.g. verapamil (VP) are known to inhibit ABC-transporter-mediated drug efflux. As expected, 10 μM VP were highly effective at restoring sensitivity against the known P-gp and MRP1 substrate DNR in 2R160 and 2R120 cells (Fig. 6A). However, it had only a weak sensitising effect towards LE. In case of BCRP, the co-incubation with the known modulators imatinib and gefitinib (10 μM) had no impact on the cytotoxicity of LE, while it potently restored MX sensitivity (Fig. 6B).

4. Discussion

Up to now anthracyclines rank among the most effective anticancer drugs in clinical therapy [2]. Unfortunately, the use of these compounds is often limited due to resistance development [10–12] and unwanted side effects [2]. Consequently, the search for a ''better'' anthracycline is still an important issue. LE is an antibiotic, angucyclic compound produced by *S. globisporus* [3] with promising anticancer

Table 2 – Cytotoxic activity of LE against various cell models at 72 h treatment

Cell line	-Fold resistance against ADR	-Fold resistance against LE[a]	IC_{50} (μg/ml)	
			Mean[b]	±S.D.
HL60	–	–	2.48	0.03
HL60/adr	80 [1]	2.5***	6.31	0.02
HL60/vinc	140 [1]	1.8***	4.51	0.04
KB-3-1	–		6.5	0.04
KBC-1	162 [1]	1.5***	9.58	0.04
MDA-MB-231	–	–	0.79	0.01
MDA-MB-231/bcrp	>1000 [4]	1.0	0.77	0.03
SW1573	–		2.80	0.02
2R120	20 [5]	2.0***	5.56	0.02
2R160	25 [5]	1.9***	5.26	0.01
A459	–	–	1.72	0.01

[a] Differences in LE sensitivity calculated by dividing IC_{50} values of the MDR subline by those of the parental cell lines.
[b] IC_{50} were calculated from whole dose response curves. Values given are means ± S.D. of one representative experiment out of three, performed in triplicates.
*** Significantly different from parental cell line ($P < 0.001$).

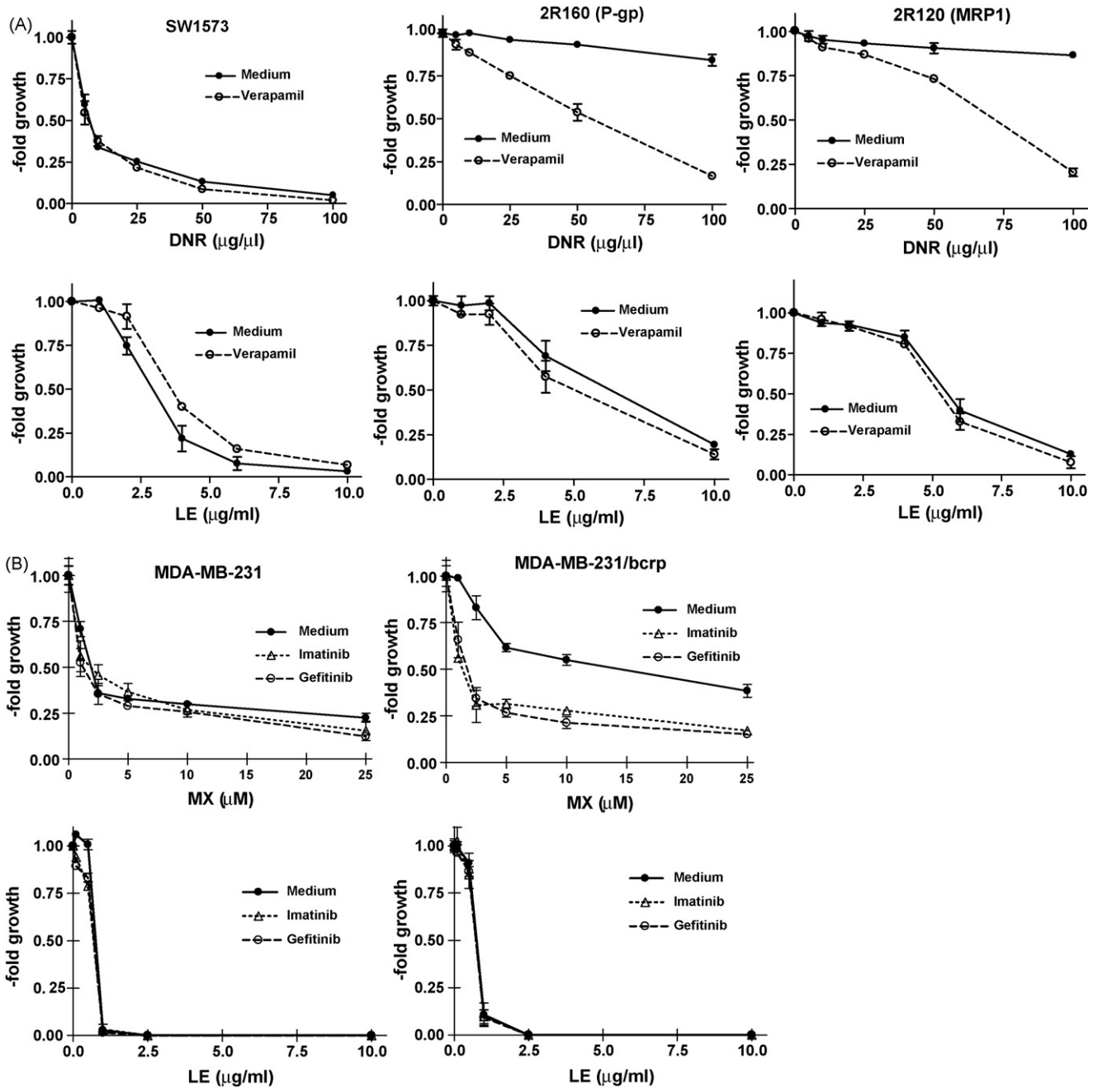

Fig. 6 – Modulation of ABC-transporter-mediated resistance against LE. (A) SW1573 cells and the P-gp- and MRP1-overexpressing sublines 2R160 and 2R120, respectively, were incubated for 72 h with increasing concentrations of LE in combination with the P-gp/MRP1 modulator verapamil (VP, 10 μM). Sensitising effects of VP against DNR was used as positive controls. Values given are means ± S.D. from at least three independent experiments performed in triplicate. (B) Sensitisation of MDA-MB-231 cells in comparison with BCRP-transfected MDA-MB-231/bcrp cells against LE or MX by co-incubation with the known BCRP substrates gefitinib and imatinib (10 μM) was determined by MTT assays. Values given are means ± S.D. from at least three independent experiments performed in triplicate.

activity in vitro [9] and in vivo [8]. It has already been suggested that LE exerts its anticancer activity through induction of programmed cell death [9]. In this study, data on LE-mediated cell death were extended by demonstrating several characteristics of programmed cell death like cell shrinkage, chromatin condensation, and apoptotic body formation in DAPI staining. Additionally, LE treatment led to massive and rapid permeabilization of the outer mitochondrial membrane, an important step to trigger programmed cell death [21]. Since LE also induced caspase-dependent cleavage of PARP, this points together with the mitochondrial membrane depolarisation towards apoptosis execution via the ''intrinsic pathway'' [21]. Interestingly, Rh123 and JC-1 staining experiments revealed a very rapid disruptive effect of LE towards the mitochondrial

integrity which was accompanied by reduction of the ATP pools. This suggests that LE treatment induces mitochondrial dysfunctions comparable to the one reported for ADR [2,24], however, at distinctly higher potency. Interaction of LE with redox processes of the respiratory chain might be the source for the oxidative stress detected after LE treatment. The precise elucidation of the underlying molecular processes is currently aim of ongoing studies in our laboratory.

In addition to induction of apoptosis, our studies revealed a reduction of DNA synthesis ([^{3}H]-thymidine incorporation) at all tested LE concentrations which was not accompanied by changes in cell cycle distribution irrespective of the cellular p53 status. Similar effects on DNA synthesis were also reported for landomycin A in case of proliferating smooth muscle cells [25]. However in contrast to landomycin E, this drug inhibited cell cycle progression after synchronisation in G_0/G_1 phase. It was not evaluated whether treatment with landomycin A induced changes in cell cycle distribution in logarithmically growing cells. The lack of altered cell cycle distribution by LE treatment is in strong contrast to data on anthracycline drugs leading in most cases and also in our hands to a profound G_2/M arrest probably due to inhibition of topoisomerase I and/or II (topo I/II) [2,23,26]. Thus, our data suggest that LE in contrast to clinically used anthracyclines does not inhibit topo II. This is supported by the fact that, in contrast to ADR, LE has no or only very weak DNA intercalation properties which would be necessary for topo II inhibition [27]. This is not unexpected since LE lacks planar structure because of the sterical repulsion between the hydroxyl group at the fourth ring and the carbonyl oxygen at the second ring which would occur in case of planarity. Due to the presence of two sp^3 carbon atoms in the third ring, this repulsion can be diminished by structural twisting which consequently interrupts the planarity.

Another explanation for the lack of cell cycle check point arrest in case of LE might be the strong oxidative stress induced by this drug and the profound disruption of the mitochondrial membrane potential and depletion of ATP pools. This might lead to stop in cell cycle progression irrespective of any activated cell cycle checkpoints. Comparably, Davies [28] reported that at H_2O_2 concentrations at the border to cytotoxicity fibroblasts undergo permanent, checkpoint-independent growth arrest still performing most essential cell functions but never entering proliferation again. Accordingly, although primarily regarded as a topo II poison, numerous cellular effects of ADR are mediated through generation of ROS. In our hands NAC pre-treatment significantly but relatively weakly reduced the antiproliferative effects of ADR while in contrast the effects on LE were almost completely reversed at lower drug concentrations as also seen in case of the ROS-generating drug bleomycin. This suggests that the cytotoxic activity of LE is primarily mediated by ROS-induced processes. As in some studies the cardiotoxicity of ADR has been linked to ROS induction, it needs to be evaluated in animal studies whether LE treatment leads to comparable side effects. However it has to be kept in mind, that beside ADR the anticancer activity of many successful chemotherapeutics like bleomycin or even thalidomide are at least partly based on the production of free radicals [29]. Most of these drugs lack cardiotoxicity suggesting that production of ROS is not sufficient to induce this adverse effect of anthracyclines like ADR and DNR [29,30].

Among the most prominent limitation factors for the clinical use of anthracyclines is the rapid development of tumor cell resistance. Overexpression of most MDR-mediating ABC transporters including P-gp, MRP1 and BCRP [10–12] leads to decreased cellular anthracycline accumulation and thus drastically reduced cytotoxicity. Unexpectedly and in contrast to ADR, DNR or MX, our results suggest that LE is only a weak substrate for P-gp and MRP1, and is not transported by BCRP. Numerous researchers have worked on identifying chemical modifications of anthracyclines that would decrease drug efflux by P-gp [31–35]. Beck and Qian [36] proposed that P-gp substrate drugs often contain at least two planar groups and a tertiary nitrogen charged at physiological pH. Regarding ADR, distinct modifications of the sugar moiety [34] (often regarding the nitrogen group [31–33]) were in the focus of interest. Results of these approaches suggest that neutral charge and enhanced lipophilicity prevent anthracyclines from being transported by P-gp. Regarding MRP1, efflux of ADR was also suggested to be, beside the necessary co-transport of glutathione, dependent on the drug lipophilicity and its cationic charge [37–39]. Since, LE lacks the respective sugar moiety containing the tertiary nitrogen, the drug is very likely not protonatable, thus staying in neutral charge. This is probably the explanation why LE partly eludes extrusion by P-gp and MRP1. Moreover, these data indirectly suggest that LE is, comparable to ADR [39], not conjugated to glutathione. Regarding BCRP, the data on structural requirement for functional interaction of the substrates with BCRP is limited and yet not fully understood [40]. BCRP is capable of transporting a wide range of substrates ranging from chemotherapeutic agents to organic anion conjugates [40]. At least in case of the camptothecin analogues, polarity seems to be important for the recognition by BCRP [40]. Interestingly, anthracyclines are not transported by wild-type BCRP but are substrates of the polymorphic BCRP mutants R482G and R482T only [4,40]. The cell line we used in this study was transfected with a BCRP plasmid, which has the R482T mutation [4,41] and has been shown to confer resistance against ADR and DNR [4]. In contrast, we found that this plasmid did not render cells resistant to LE. Which of the structural differences between LE and anthracyclines is responsible for this enhanced activity in BCRP-overexpressing cells remains speculative but again the lack of charge might have an impact.

In conclusion, LE is a promising representative of a new angucycline class with potent anticancer activity especially against drug-resistant cancer cells overexpressing ABC-transporters. Additionally, further investigations of the interactions of ABC-transporters with LE might help to gain more insights into the structural requirements leading to recognition of anthracyclines by these efflux pumps.

Acknowledgements

We are indebted to Vera Bachinger and Maria Eisenbauer for the skilful handling of cell culture, Elisabeth Rabensteiner, Rosa-Maria Weiss and Christian Balcarek for competent

technical assistance, Irene Herbacek for FACS analysis, and the NCI for delivering the cell panel screening data. This work was supported by the Austrian-Ukrainian Cooperation for Science, Education and Culture, Vienna-Lviv.

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