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Rapid generation of hydrogen peroxide contributes to the complex cell death induction by the angucycline antibiotic landomycin E

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Abstract

Landomycin E (LE) is an angucycline antibiotic produced by *Streptomyces globisporus*. Previously, we have shown a broad anticancer activity of LE which is, in contrast to the structurally related and clinically used anthracycline doxorubicin (Dx), only mildly affected by multidrug resistance-mediated drug efflux. In the present study, cellular and molecular mechanisms underlying the anticancer activity of landomycin E towards Jurkat T-cell leukemia cells were dissected focusing on the involvement of radical oxygen species (ROS). LE-induced apoptosis distinctly differed in several aspects from the one induced by Dx. Rapid generation of hydrogen peroxide already at one hour drug exposure was observed in case of LE but not found before 24h for Dx. In contrast, Dx but not LE induced production of superoxide radicals. Mitochondrial damage, as revealed by JC-1 staining, was weakly enhanced already at 3h LE treatment and increased significantly with time. Accordingly, activation of the intrinsic apoptosis pathway initiator caspase-9 was not detectable before 12h exposure. In contrast, cleavage of the down-stream caspase substrate PARP-1 was clearly induced already at the three hour time point. Out of all caspases tested, only activation of effector caspase-7 was induced at this early time points paralleling the LE-induced oxidative burst. Accordingly, this massive cleavage of caspase-7 at early time points was inhibitable by the radical scavenger N-acetylcysteine (NAC). Additionally, only simultaneous inhibition of multiple caspases reduced LE-induced apoptosis. Both, a specific H₂O₂ and an OH⁻ scavenger (catalase and mannitol, respectively) effectively decreased LE-induced ROS production, but only partially inhibited LE-induced apoptosis. In contrast, NAC efficiently blocked both parameters. Summarizing, rapid H₂O₂ generation and a complex caspase activation pattern contribute to the antileukemic effects of LE. As superoxide generation is

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considered as the main cardiotoxic mechanism of Dx, LE might represent a promising drug candidate for further (pre)clinical development.

Keywords

ANTICANCER DRUGS; REACTIVE OXYGEN SPECIES; HYDROGEN PEROXIDE; SUPEROXIDE RADICALS; N-ACETYLCYSTEINE; LANDOMYCINE; APOPTOSIS; MULTI-DRUG RESISTANCE

Introduction

The anthracycline family of polyketide antibiotics has been one of the most intensely studied classes of microbial natural products for development of antimicrobial and antitumor agents. Daunorubicin and doxorubicin (Dx) were the first anthracyclines approved as anticancer drugs in 1967 and 1974, respectively, and since then they have been among the most widely used cancer chemotherapeutic agents worldwide. Despite their impressive potential, the usefulness of both compounds is hampered e.g. by several acute and long-term side effects. The most severe is cumulative cardiotoxicity [1], since it limits the dosage that can be prescribed. Recent studies of this phenomenon revealed that the main source of anthracycline cardiotoxicity is the production of superoxide radicals which are later transformed to highly toxic hydroxyl radicals in the presence of Fe^{2+} ions (Fenton reaction) [2]. In addition, the emergence of multi-drug resistance in cancer cells upon long-term treatment poses serious limitations for the use of anthracyclines in clinical oncology [3]. Thus, the search for other anthracycline analogs which are less toxic and less affected by drug resistance mechanisms remains a crucial task of systemic cancer therapy research. Soil-dwelling gram-positive actinobacteria, especially members of the genus *Streptomyces*, became the main source of novel anthracycline analogues.

The angucyclines are the largest group of polycyclic aromatic polyketides produced by *Streptomyces* with currently more than 120 described derivatives [4, 5]. The most characteristic feature of angucyclines is their uniquely shaped benz[a]anthracene tetracyclic framework with an angularly condensed ring [6]. This “curved” aglycon distinguishes angucyclines from other aromatic polyketides and is at least partially responsible for the wider range of bioactivities of angucyclines compared to, for instance, anthracyclines and tetracyclines [5]. Although the group is rich in chemical scaffolds and various biological activities, predominantly antitumor and antibacterial, none of these compounds has been developed to clinical use so far mostly due to toxicity or solubility issues [4, 5, 7]. Landomycins are the most auspicious group of angucyclines possessing strong antineoplastic potential. All natural landomycins identified to date share the same aglycon (landomycinone) and vary in their oligosaccharide chain, a linear glycosidic chain containing only di- and trideoxysugars (β -D-olivose and α -L-rhodinose) [8]. They show broad range of activity against many cancer cell lines, with the general tendency that compounds with longer saccharide chains show enhanced potency [9, 10].

The best-investigated compound, landomycin A, containing a hexasaccharide side chain, has so far been shown to be the most potent congener. It has been extensively tested by the

National Cancer Institute (USA) towards the 60 selected human cancer cell line panel and particularly prostate cancer models [11, 12]. In contrast to many clinically useful drugs of similar structure, like the anthracyclines and chromomycins, landomycins do not bind directly to DNA [6, 13, 14]. Landomycin E (LE) is a novel representative of landomycins synthesized by *Streptomyces globisporus* strain 1912 growing in a soy-bean culture medium [15, 16]. It contains three saccharide residues (α -L-rhodinose-(1 $\rightarrow$ 3)- β -D-olivose-(1 $\rightarrow$ 4)- β -D-olivose) conjugated to an angular tetracyclic quinone. Potent antitumor activity of LE was demonstrated against various tumor cell lines *in vitro* [17] and Guerin carcinoma in rats *in vivo* [18]. LE is widely unaffected by resistance to doxorubicin, vincristine and colchicine in cancer cells based on overexpression of various types of ABC-transporters (ABCB1, ABCC1, ABCG2) [13, 14]. Pre-treatment with the ROS scavenger N-acetylcysteine (NAC) significantly lowered LE cytotoxicity towards KB-3-1 carcinoma cells [14] which suggests possible ROS involvement in LE-induced apoptosis in tumor cells. However, the exact molecular mechanisms underlying the antineoplastic effect of LE are still not fully elucidated.

Consequently, the main aim of the present study was to dissect in more detail the role of ROS in the anti-leukemic activity of LE as compared to Dx and to elucidate the involved cell death pathways.

Materials and methods

Materials

LE-overproducing *Streptomyces globisporus* 1912 strain was obtained in the laboratory of Prof. B. Matselyukh (D.K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kyiv). LE (99.5% purity, according to HPLC data) was prepared in the laboratory of Prof. J. Rohr (University of Kentucky, USA) and dissolved in absolute ethanol to obtain a 4 mg/ml stock solution. Doxorubicin hydrochloride was obtained from Pfizer (New York, NY). Catalase (C9322), catalase polyethylene glycol (C4963), superoxide dismutase (SOD; S7571), N-acetylcysteine (NAC; A7250), diphenyleneiodonium chloride (D2926) and D-mannitol (M4125) were purchased from Sigma-Aldrich (St. Louis, MO). Caspase inhibitors Ac-DEVD-CHO (caspase-3/7 dual reversible inhibitor, ALX-260-030), Ac-IETD-CHO (caspase-8 reversible inhibitor, ALX-260-043), Ac-LEHD-CHO (caspase-9 reversible inhibitor, ALX-260-079) and z-VAD-fmk (ALX-260-020) were purchased from Enzo Life Sciences (Farmingdale, NY). Chemical formulas for LE and Dx are depicted in Suppl. Fig. 1.

Cell culture and treatments

Jurkat human T-leukemia cells were obtained from ATCC. Cells were cultured in RPMI medium, supplemented with 10% fetal calf serum (Sigma-Aldrich), 50 μ g/ml streptomycin (Sigma-Aldrich), 50 units/ml penicillin (Sigma-Aldrich) in 5% CO₂-containing humidified atmosphere at 37°C. For experiments cells were seeded into 24-well tissue culture plates (Greiner Bio-one, Germany). Short-term (24h) cytotoxic effect of antitumor drugs was studied under the Evolution 300 Trino microscope (Delta Optical, Poland) after cell staining with trypan blue dye (0.1%).

Catalase (15 mg/ml, equivalent to 45 kU/ml) was dissolved in 50 mM potassium phosphate buffer, pH 7.0, while SOD (1000 U/ml) was dissolved in 0.1 M potassium phosphate, pH 7.5, catalase polyethylene glycol was dissolved in sterile water (1 mg/ml, equivalent to 40 kU/ml), and NAC and D-mannitol were dissolved in 1x phosphate buffered saline (PBS). Antioxidants were added to cell culture 30 min before addition of anticancer drugs, and final concentration of catalase was 1000 U/ml, SOD 50 U/ml, D-mannitol 40 mM, and NAC 1 mM. Diphenyleneiodonium chloride was dissolved in DMSO to obtain 10 mM stock solution, which was dissolved in PBS (final concentration 5 μ M) and added to cell culture 30 min before addition of anticancer drugs. Caspase inhibitors Ac-DEVD-CHO (caspase-3/7 dual reversible inhibitor, ALX-260-030), Ac-IETD-CHO (caspase-8 reversible inhibitor, ALX-260-043), Ac-LEHD-CHO (caspase-9 reversible inhibitor, ALX-260-079) and z-VAD-fmk (ALX-260-020) were dissolved in DMSO to prepare 20 mM stock solutions. Stock solutions of aforementioned reagents were dissolved in PBS before addition to cell culture. Caspase inhibitors (final concentration 50 μ M) were added to cell culture 1h before addition of anticancer drugs.

For long-term (72h) cytotoxicity assays, Jurkat cells were plated (5×10^3) in 100 μ l per well in 96-well plates, and allowed to grow for 24 h. Drugs were added in another 100 μ l 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 IC₅₀ values calculated from full dose-response curves (drug concentration including 50% reduction in cell survival comparing to the control cultured in parallel without drug).

Flow cytometric assays

Analyses were performed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) and Summit v3.1 software (Cytomation, Inc., Fort Collins, CO). Jurkat T-leukemia cells were treated as indicated in the figures. For cell death analyses, cells were stained with Annexin-V-FITC (or Annexin-V-APC) and propidium iodide (PI) using an apoptosis detection kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. In detail, 24 h after the addition of LE doses at LC₇₅ (6 μ M) and LC₉₀ (8 μ M) Jurkat cells were centrifuged at 2000 rpm, washed twice with 1x PBS, and incubated for 15 min in Annexin V-binding buffer (BD Biosciences, San Jose, CA) containing 1/50 volume of FITC- or APC-conjugated Annexin V solution and PI (50 μ g/ml). Then samples were diluted 2 times in Annexin V-binding buffer (BD Biosciences, San Jose, CA) and immediately measured on FL1/FL2 (FITC-PI) or FL4/FL2 (APC-PI) channels of FACScalibur flow cytometer (BD Biosciences, San Jose, CA).

Breakdown of Ψ_m mitochondrial membrane potential was determined by FACS analysis using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). For this purpose, the Mitochondrial Membrane Potential Detection Kit (Stratagene, La Jolla, CA, USA) was used as described in the manufacturer's instruction. Briefly, 10^6 Jurkat cells were treated for 1, 3, 6, 12 and 24h with the tested drugs. After PBS washing, cells were incubated for 10 min in freshly prepared JC-1 solution (10 mg/ml in culture medium) at 37°C. Spare dye was removed by PBS washing and cell-associated fluorescence was

measured with FACS. Rhodamine 123 (Rh123) accumulation assays were performed as previously described [19]. Briefly, 5×10^5 Jurkat cells were preincubated with LE for 30 min at 37°C followed by incubation with Rh123 (0.25 mg/ml). After 1, 3, 6, 12, 24h exposure, fluorescence of Rh123 was collected through a 530/30 nm bandpass filter by FACS. Cellular ROS contents were measured by incubating control or drug-treated cells with fluorescent dyes dihydrodichlorofluorescein diacetate (DCFDA, H_2O_2 -specific) and dihydroethidium (DHE, $O_2^{\cdot -}$ -specific) in concentrations of 10 μ M at 37°C for 30 min. After incubation with the dyes, cells were washed with PBS and immediately analyzed at FL1 (DCFDA) or FL2 channel (DHE) of FACSCalibur flow cytometer (BD Biosciences, San Jose, CA)

Western blotting

Western blot analysis was used to evaluate expression of proteins involved in cell cycle signaling pathways under the treatment of Jurkat T-cells with LE at various time points (1, 3, 6, 9, 12, 24h). The cells were washed twice on ice with PBS before the addition of lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1% Trasylol, 1 mM PMSF), 20 μ l per 10^6 cells, vortexed, and centrifuged. Protein concentration in supernatants was measured, as described [20]. Then the appropriate volume of 5x Laemmli buffer (10% SDS, 10% 2-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, 250 mM Tris-HCl, pH 6.8) was added and samples were heated for 5 min in boiling water and subjected to Western-blot analysis. 30 μ g of protein of each cell sample was loaded onto 12% polyacrylamide gel. After electrophoresis (4 h at 0.02 A), proteins were transferred onto nitrocellulose membranes (GE Healthcare Bio-Sciences, Marlborough, MA) using Mini Trans-Blot Cell (Bio-Rad Laboratories, Inc, Hercules, CA) at 90 V for 1.5 h. Incubation of membranes in 5% milk solution for 1h at 37°C was used to block nonspecific binding. Membrane was incubated with monoclonal rabbit antibodies raised against cleaved caspase-3, procaspase-6, cleaved caspase-6, cleaved caspase-7, Bid (Cell Signaling Technology, Inc., Danvers, MA), AIF (sc-5586), Bcl-X_{L/S} (sc-634) (Santa Cruz Biotechnology, Inc., Dallas, TX), caspase-9 (BD 556585) (BD Biosciences, San Jose, CA), β -actin (Sigma-Aldrich, St. Louis, MO), monoclonal goat antibodies against procaspase-3 (sc-1226) (Santa Cruz Biotechnology, Inc., Dallas, TX) and monoclonal mouse antibodies against PARP-1 (BD 556434), FADD (BD 556402), c-IAP-1 (BD 556533), Mcl-1 (BD 559027), caspase-2 (BD 611022), caspase-8 (BD 551242) (BD Biosciences, San Jose, CA), caspase-10 (M059-3) (MBL International Corporation, Woburn, MA) for 12 h at 4°C at slow shaking. Dilution for primary antibodies was 1:1000 in 5% BSA, 0.1% PBS-Tween, except for antibodies against β -actin (1:5000), as recommended by supplier. After incubation with primary antibody, the membrane was washed three times for 5 min in 1x PBS with 0.1% Tween 20, and then incubated in 1:5000 dilution of secondary anti-rabbit IgG horseradish peroxidase-linked antibody (GE Healthcare Bio-Sciences, Marlborough, MA) for 1h at room temperature. The membrane was washed 3 times for 5 min with PBS-Tween 20, and proteins that bound antibodies were visualized by incubation of the membrane for 1 min in ECL buffer (1.25 mM luminol (Sigma-Aldrich, St. Louis, MO), 2.72 mM cumaric acid (Sigma-Aldrich, St. Louis, MO) and 0.01% H_2O_2 in 0.1 M Tris-HCl (pH 8.5), and exhibition for 10–15 min to an X-ray film (Fujifilm, Tokyo, Japan). Relative amounts of protein in the electrophoretic bands was quantified by using Gel-Pro Image Analysis software (Media

Cybernetics, Rockville, MD). Protein normalization was conducted for β -actin level in the same samples.

Statistical analyses

Where indicated, statistical comparisons were conducted using Student's t test. Statistical significance was set at $P = 0.05$.

Results

NAC protects leukemia cells from LE- but not from Dx-induced cell death

Previously we have shown that the cytotoxic activity of LE can be significantly inhibited by pre-treatment of target cells with the ROS scavenger NAC [14] indicating involvement of ROS in LE-induced cancer cell death. However, the exact molecular mechanisms of these processes have not been completely elucidated yet. As ROS are short living molecules, we compared the impact of NAC on the anti-leukemic potential of LE and Dx at both early (24h) and late (72h) time points (Fig. 1). In the short exposure experiment, 1 mM NAC effectively inhibited the cytotoxic activities of LE over the entire tested concentration range restoring the viable cell population to 75–95% of the untreated control. In contrast, in case of Dx only an insignificant trend towards a protective effect of NAC was observed. At 72h exposure, these differences became even more distinct with NAC completely eliminating the anticancer activity of LE opposed to a complete lack of impact in case of Dx-induced effects. Based on these data, sub-LC₅₀ (2 μ M and 4 μ M), LC₇₅ (6 μ M) and LC₉₀ (8 μ M) doses of LE were used in our further studies. Dx doses, used for comparison, were 0.5 μ M (LC₇₅) and 1 μ M (LC₉₀).

LE in contrast to Dx leads to early hydrogen peroxide but not superoxide anion production

To further investigate the role of ROS in LE-induced cell death, time-dependent ROS production was measured in LE- and Dx-treated Jurkat cells by flow cytometry. The ROS indicator fluorescent dyes DCFDA and DHE were used to distinguish between H₂O₂ and O₂⁻, respectively. LE led to rapid increase of hydrogen peroxide level in a concentration-dependent manner (2.5- and 5-fold increase at 2 μ M and 4 μ M, respectively) peaking already at 1h exposure (Fig. 2). H₂O₂ stimulation by LE followed a biphasic pattern with reduced but still significant upregulation at 3 h and 6 h followed by a second peak at 12 h and again mild attenuation at 24 h. NAC distinctly blocked H₂O₂ production in response to LE over the whole incubation time. Dx treatment, in contrast to LE, did not result in considerable upregulation of H₂O₂ level up to 12h exposure time. However, at longer exposure times (12h, 24h), H₂O₂ production was moderately (up to 2.5-fold) increased also by Dx. NAC did not inhibit hydrogen peroxide production by Dx.

In contrast to H₂O₂, LE rather reduced (up to 3-fold) than increased superoxide radicals production at early time points (1–3 h), while O₂⁻ levels were slightly but insignificantly increased as compared to the control at longer exposure (12–24 h) (Supplemental Fig. 2). Dx strongly induced superoxide radical production with a peak activity at 24 h exposure. NAC had no inhibitory impact on this process.

There are several cellular sources of hydrogen peroxide, but mitochondrial NADPH dehydrogenases and membrane-bound NADH oxidases are considered central ones [21], [22]. To address the role of these enzymes in LE-mediated H₂O₂ production, we investigated the impact of diphenylene iodonium (DPI) – a broad spectrum inhibitor of NADPH oxidases [23] – with LE (Fig. 2B). However, co-incubation with DPI unexpectedly led to increased ROS production in combination with LE (Fig. 2B). Thus, we concluded that neither mitochondrial NADPH dehydrogenases nor membrane-bound NADPH oxidases seem to be involved in LE-induced oxidative burst.

LE causes earlier mitochondrial damage and membrane depolarization as compared to Dx

Mitochondria are known to be one of the main sources of ROS in the cells. Thus, the impact of LE and Dx on the status of mitochondria was comparatively investigated by rhodamine-123 accumulation and JC-1 staining followed by FACS analyses. Rhodamine-123 accumulation data suggested that LE led to dose-dependent mitochondrial damage already after 1h exposure (Supplemental Fig. 3). LE-mediated reduction of Rh-123 fluorescence was dose-dependent and peaked at 6 h (reduction at 2 μ M and 4 μ M by 36% and 58%, respectively) followed by partial recovery until 24 h exposure time. In contrast to LE, Dx, had no impact on mitochondrial integrity at early time points (from 1h to 12h), while after 24h massive mitochondrial damage occurred (55% and 66% at 0.5 and 1 μ M, respectively). In line with the DCF-DA and viability data, LE-induced mitochondrial damage was partially recovered by NAC, although this effect was much weaker as compared to the impact in cell viability assays (compare Fig. 1). In contrast, NAC did not protect from Dx-induced mitochondrial damage.

As a second parameter of mitochondrial integrity, the mitochondrial membrane potential was followed by JC-1 staining (Fig. 3). Weak signs of mitochondrial membrane depolarization by LE were observed only at 3h, reaching a peak at 12h exposure (21% of depolarized mitochondria for LE 2 μ M and 42% for LE 4 μ M) followed by partially recovery after 24h. Dx-induced mitochondrial membrane depolarization occurred later and was relatively weak up to 12 h followed by a massive effect at 24h (41% for 0.5 μ M and 67% for 1 μ M Dx).

Specific ROS scavengers differentially modulate LE-induced DCFDA fluorescence and apoptosis

LE distinctly induced DCFDA but not DHE fluorescence at short exposure times. DCFDA is considered to be the indicator of diverse ROS (e.g., hydrogen peroxide, hydroxyl radicals, peroxynitrite radicals and others) [24]. Consequently, an increase in DCFDA fluorescence does not allow identifying the exact type of ROS detected. NAC belongs to thiol-type ROS scavengers and is known to block also other types of ROS in addition to the ones derived from H₂O₂ [25]. Thus, to allow a more precise identification of the ROS induced by short-term LE exposure, specific scavengers of hydrogen peroxide (catalase), hydroxyl radicals (mannitol) and superoxide radicals (SOD) were used (Table 1 and Fig. 4). To avoid interference with the growth-supporting effects of antioxidants [26], higher (namely, LC₇₅ and LC₉₀) doses of LE were used in this experiment. As expected, these massively cytotoxic concentrations led to higher DCFDA fluorescence (6-fold and 7.4-fold increase, respectively) compared to sub-LC₅₀ doses (compare Fig. 2). Co-incubation with catalase

significantly decreased LE-induced DCFDA fluorescence, thus confirming a crucial role of hydrogen peroxide in LE-induced oxidative burst. Mannitol (OH^- scavenger) exerted a weaker effect, thus indicating a more secondary role of hydroxyl radicals in this process. Finally, SOD had a very weak effect on DCFDA fluorescence at all studied time points indicating no contribution of superoxide radicals in LE-induced ROS burst. Surprisingly, combination of catalase and mannitol had almost no synergistic effect, while simultaneous addition of catalase, mannitol and SOD (CMS) exerted a massive ROS-scavenging effect even exceeding the one of NAC at least at 1h exposure (Table 1 and Fig. 4A). Noteworthy, the enzyme catalase is too big (60 kD) to enter the cell by diffusion. Consequently, co-incubation with catalase allows predominant extracellular scavenging of hydrogen peroxide. To further estimate the possible cellular sources of LE-induced hydrogen peroxide, the impact of catalase was compared with that of a cell-permeable catalase polyethylene glycol conjugate in the DCFDA assay (Fig. 4B). Interestingly, the inhibition of ROS-production by intracellular PEGylated catalase tended to be less potent as compared to extracellular catalase and did not reach statistical significance (Fig. 4B). Thus, the source of the LE-induced hydrogen peroxide boost seems to be located either extracellularly or in close proximity to the cell membrane, but definitely not in the mitochondria. However, based on the DPI assay (compare Fig. 2B) plasma membrane-bound NADPH oxidases can be excluded as major players in this process.

As a next step, the impact of ROS scavengers on the apoptosis-inducing effects of LE was investigated using annexin V/PI double staining (LE at 6 μM in Fig. 5 as well as controls and LE at 8 μM in supplemental Fig. 4). Surprisingly, despite strong hydrogen peroxide scavenging activity, catalase exerted only a minor protective effect at the 8 μM dose (reduction of AnV (+) cells < 10%). The same holds true for mannitol, while the impact of SOD was negligible. In contrast, NAC massively reduced apoptosis induction (from 42.3% to 17.0% at 6 μM LE). Combination of catalase, mannitol and SOD exerted a synergistic protective effect (decrease of AnV (+) cell number from 42.3% to 28.7%), but was still distinctly lower compared to effect of NAC alone. Similar effects were observed for IC₉₀ (8 μM) dose of LE (supplemental Fig. 4). Together this indicates that ROS play a significant but not dominant role in LE-induced apoptosis and NAC is able to protect cells by additional, probably ROS-independent effects.

LE induces a unique death signaling pathway with activation of effector caspase-7 preceding mitochondrial damage

ROS-producing anticancer drugs frequently induce apoptosis via the intrinsic, mitochondrial pathway. However considering the relatively weak protection of ROS scavengers and comparably minor mitochondrial depolarization by LE, we hypothesized on other cell death induction mechanisms inhibitable by NAC. Thus, the LE-mediated activation of several initiator and effector apoptotic proteins involved in various types of apoptosis (ER-induced, mitochondria-mediated) was analyzed (Fig. 6). Unexpectedly, activation of initiator caspase-9, involved in mitochondria-mediated apoptosis, was not detectable before 9h of LE treatment and this process was not inhibited but rather enhanced by NAC. Also activation of several other initiator caspases involved in receptor-mediated (caspases-8, -10) or ER-mediated (caspase-2) apoptosis was either not observable or occurred not prior to 12h–24h

LE exposure. Activation of the main effector caspase-3, involved in crucial steps of cell death, was detectable from 12h LE treatment onwards. In contrast to these initiator caspases, NAC-inhibitable activation of effector caspase-7 was readily observable already at 3h LE treatment accompanied by distinct PARP-1 cleavage (Fig. 6). Interestingly, NAC inhibited caspase-7 activation only at 3h and 6h, while later on this process becomes NAC-insensitive correlating well with the impact of NAC on LE-induced ROS production (compare Fig. 2). These data indicate that rapid hydrogen peroxide production by LE treatment might be involved in activation of caspase-7 while activation of caspase-9 might be a secondary event. In contrast, Dx-induced apoptosis followed the classical sequence by induction of caspase-9 and subsequent activation of effector caspases-3/7 (supplemental Fig. 5).

To further dissect the role of caspases in LE-induced Jurkat cell death, the impact of specific inhibitors of caspase-3/7 (Ac-DEVD-CHO), caspase-8 (Ac-IETD-CHO), caspase-9 (Ac-LEHD-CHO) and the pan-caspase inhibitor z-VAD-fmk were compared. Only the dual caspase-3/7 inhibitor led to a minor decrease in the number of AnV (+) cells, while inhibition of either caspase-8 or caspase-9 alone did not reduce the cytotoxic activity of LE (Fig. 7). However, simultaneous addition of Ac-DEVD-CHO, Ac-IETD-CHO and Ac-LEHD-CHO exerted synergistic activities distinctly decreasing the number of AnV (+) cells. Pre-incubation of Jurkat T-cells with pan-caspase inhibitor z-VAD-fmk massively reduced the number of apoptotic cells, clearly indicating on crucial role of obviously several caspases in execution of LE-induced cell death.

In addition, we tested the combination of the caspase inhibitors and ROS scavengers (catalase, SOD, mannitol) on the apoptotic potential of LE. Unexpectedly, the combination of Ac-DEVD-CHO, Ac-IETD-CHO and Ac-LEHD-CHO with the scavengers demonstrated additive to synergistic protective effects against LE (supplemental Figure 6). In contrast, addition of the scavengers to the pan-caspase inhibitor resulted in only marginal increase of the protective effect. Thus, simultaneous inhibition of ROS production and caspases-3,8,9 strongly reduced the anticancer activity of LE. These data suggest a complex interplay of ROS generation and caspase activation in LE-induced apoptosis.

4. Discussion

ROS in the context of cell biology comprise a group of small molecules with significant oxidative activity including highly reactive free oxygen radicals, such as superoxide anion [$O_2^{\cdot-}$] and the hydroxyl radical [$OH^{\cdot}$], as well as stable 'diffusable' non-radical oxidants (e.g. hydrogen peroxide [H_2O_2]) [27]. The impacts of ROS on cell biology are ambivalent. On the one hand, they have been implicated in the regulation of essential cellular functions, including cell cycle, proliferation and apoptosis based on their function as second messengers in signal transduction. On the other hand, ROS overproduction can lead to oxidative damage of key cellular components and induction of cell death. This implicates that in healthy organs the physiological levels of ROS have to be strictly controlled. Malignant tissues, however, are characterized by deregulated ROS homeostasis and, consequently, cancer cell survival depends on massive upregulation of antioxidant systems like glutathione and ROS-metabolizing enzymes. This protective barrier allows cancer cell survival despite massive redox deregulation and even permits utilization of specific ROS-

mediated signals to support malignant progression [28]. Nevertheless, based on these high ROS levels cancer cells are hypersensitive for further redox deregulation by several anticancer compounds. Consequently, the pharmacological generation of enhanced oxidative stress in cancer tissues, also termed “oxidation therapy”, is an active area of targeted therapy development [29]. Additionally, although several anticancer drugs of both synthetic and natural origin induce apoptosis in tumor cells by mechanisms involving ROS [30], the exact factors of ROS generation and consequences have been rarely worked out in detail [29, 31]. However, the dissection of ROS production in frame of systemic cancer therapy is critical as they might not only be a mode-of-action for anticancer drugs but also a main cause of even dose-limiting adverse effects. In that respect, anthracyclines are particularly problematic: while the role of ROS in their in vitro and in vivo anticancer effects is questionable [32, 33], the contribution of superoxide in cardiotoxicity, as major side effect, is beyond doubt [34]. In accordance, we were unable to protect KB-3-1 cervix cancer cells against Dx by application of the broad ROS scavenger NAC, while the anticancer activity of LE was completely blocked [14].

Based on this observations, we hypothesized that LE in contrast to Dx might represent a novel strategy for anticancer “oxidation therapy”. Using Jurkat leukemic cells, we demonstrate in the present study that the apoptosis-inducing mechanism of LE is indeed involving a complex temporal sequence of ROS generation and caspase activation. This ROS-involving cell death cascade is completely different from the cytotoxic effects induced by the related and clinically used anthracycline drug Dx. In the leukemic Jurkat cell model, the cytotoxic effect of Dx was insensitive to NAC application, while LE activity was massively inhibited. Unexpectedly, hydrogen peroxide production by LE exposure was rapidly and massively induced within 1h followed by a steep decline and a second lower peak at 12h–24h. However, only a tendency towards increased superoxide anion levels was detectable at 12h. For Dx, in contrast, generation of superoxide radicals was detectable culminating at 24 h exposure together with mitochondrial damage. Accordingly, the late upregulation of DCFDA staining by Dx supports secondary generation of H₂O₂ from superoxide radicals via SOD, while an additional generation of hydroxyl radicals in a Fenton reaction is feasible [35]. Interestingly, the maximum level of mitochondrial depolarization by LE treatment was observed in parallel to the comparably late superoxide anions production, suggesting that these two events might be mitochondria-derived. Accordingly, the second peak of H₂O₂ generation during LE exposure might be explainable by conversion of mitochondria-derived superoxide anions by SOD to hydrogen peroxide as part of cellular ROS detoxification processes [36]. Together these data would suggest that the classical mitochondria-mediated ROS-derived cell death pathway might not initiate but only support LE-induced apoptosis at later time points. This is also confirmed by the activation of initiator caspase-9 and caspase-3 in Western blot analysis and by the necessity for combining caspase 3/7 inhibitor with at least a caspase 9 and caspase 8 inhibitors to significantly reduce LE-mediated apoptosis. In case of Dx, neither mitochondrial depolarization nor apoptosis induction was sensitive to NAC. This suggests that superoxide radicals do not play a major role in the anticancer activity of Dx.

The early oxidative burst by LE was accompanied by an NAC-sensitive and, hence, obviously ROS-dependent activation of effector caspase-7 at 3h treatment. NAC was able to

significantly attenuate at least partly all events including early ROS and caspase-7 activation and LE-mediated mitochondrial damage as well as cell death induction. Together, this data would suggest a central role of H_2O_2 in the mode-of-action of LE. However, inhibition of hydrogen peroxide production by catalase and/or by mannitol (scavenger of hydroxyl radicals) only moderately (by 15–20%) inhibited apoptosis induction by LE, while NAC (as thiol-type ROS scavenger) demonstrated distinctly stronger protective effects (80% apoptosis inhibition). Joint combination of catalase, mannitol and SOD demonstrated an additive protective effect (about 30%) which, however, was surprisingly still weaker as compared to NAC. This indicates that during different stages of LE-derived apoptosis all three types of ROS (H_2O_2 , $OH^\cdot$, $O_2^{\cdot-}$) seem to participate. However, their production does not seem to be the sole mechanism of cell death induction by LE. The high efficiency of NAC in blocking LE-induced ROS production and apoptosis might be explained either by scavenging of other ROS species not hit by catalase, mannitol or SOD or, more likely, by ROS-independent functions of this thiol-containing antioxidant (e.g., potential formation of covalent adducts between NAC and LE). These possibilities are currently addressed in ongoing investigations.

However, the question remains where and how LE-mediated ROS generation takes place. The main physiological sources in mammalian cells are 1) mitochondrial electron transport chain (ETC)-derived electrons reacting with molecular oxygen to produce ROS or 2) cytosolic or membranous enzymes including besides the NADPH oxidases (NOX) and xanthine oxidase also metabolic enzymes like cytochrome P450, lipoxygenase, and cyclooxygenase [37, 38]. Hydrogen peroxide is a relatively stable molecule, which can be easily transferred through various cellular membranes by aquaporins [39], in contrast to superoxide anions, which cannot exit the organelles where they have been produced due to their negative charge [36]. For localization of possible source of LE-induced hydrogen peroxide, extracellular and PEGylated (intracellular) catalase were employed. Surprisingly, the efficiency of PEGylated (intracellular) catalase was significantly weaker compared to extracellular catalase to inhibit LE-induced hydrogen peroxide production. This clearly indicates that the early oxidative burst by LE is mitochondria-independent and occurs obviously at or close to the plasma membrane. The major enzyme located there, which could be responsible for H_2O_2 generation, is the membrane-bound NADPH oxidase [22]. However, pre-treatment of Jurkat T-cells with its inhibitor diphenylene iodonium (DPI) even enhanced H_2O_2 levels in response to LE. Thus, membrane-bound NADPH oxidase does not seem to play crucial role in LE-induced ROS generation. However, there are also several cytosolic proteins involved in the reduction of quinone-containing drugs – namely, NAD(P)H:quinone oxidoreductase1 (NQO1) and NRH:quinone oxidoreductase2 (NQO2) [40, 41]. NQO1 has been shown to be the main target of β -Lapachone – novel anticancer ortho-quinone drug, which realizes its cytotoxic activity via ROS production [42], while NQO2 was found to be associated with specific hydrogen peroxide production, induced by vitamin K3 (menadione), possessing the same para-quinone core, as LE [43]. Interestingly, also in that cases exogenous catalase was shown to decrease both ROS production and toxic effects of β -lapachone and menadione [44] [45], indicating on fact that H_2O_2 production by NQO1 and NQO2 takes place in close proximity to plasma membrane. Taking these facts

into consideration, we are currently testing a role of NQO1 or NQO2 in LE-induced ROS production.

However not only concerning the involvement of ROS, but also with regard to the involved cell death machinery LE-induced cytotoxicity seems to follow unconventional patterns. Surprisingly, we found that activation of the effector caspase-7 by LE preceded activation of other initiator caspases. This effect might be caused by direct oxidative modification of this enzyme by hydrogen peroxide, which is known to oxidize methionine residues and thiol groups of cysteines. This might result in a change in the redox status of the proteins and either can activate or inactivate the respective activity depending on H₂O₂ concentration [46]. It has been already demonstrated, that low concentrations of hydrogen peroxide (50 µM) are rapidly consumed by Jurkat T-cells already within 30 min, but are enough to activate effector but not activator caspases (measured by DEVD-AMC cleavage) with 3h delay, while higher doses of H₂O₂ (>200 µM) block caspase activation shifting apoptotic towards necrotic cell death [21]. Hence, LE seems to exert similar activity as low doses of H₂O₂ (see Fig. 2, Fig. 4) – in particular, activation of caspase-7 immediately after drug exposure. LE indeed induces caspase-dependent apoptosis, as pan-caspase inhibitor z-VAD-fmk almost completely blocked LE-induced cell death. However, caspases-3,8,9 seem to play minor roles here, as use of specific caspase-3/7 inhibitor Ac-DEVD-CHO led to a weak decrease in number of AnV (+) cells, while the caspase-8 inhibitor Ac-IETD-CHO, surprisingly, even tended to enhance the cytotoxic activity of LE. However, combined application of Ac-DEVD-CHO, Ac-IETD-CHO and Ac-LEHD-CHO led to prominent apoptosis inhibition, thus indicating that these caspases obviously cooperate during LE-induced apoptosis. Moreover, pre-treatment of Jurkat T-cells with ROS scavengers and caspase-3,8,9 inhibitors also exerted cumulative effect. Thus, both ROS and caspases-3,8,9 exert significant and indispensable impacts on LE-induced apoptosis. However, this combination is still less effective compared to with the pan-caspase inhibitor z-VAD-fmk (16,85%), indicating on important role of other caspases (e.g., caspase-7) in LE-induced apoptosis.

Conclusion

LE exerts a unique mode of cell death induction due probably based on its specific quinine-containing structure. LE-induced apoptosis involves, besides other radicals, early H₂O₂ generation close to the cancer cell membrane leading to rapid caspase-7 activation. This effect cooperates with later depolarization of mitochondria and caspase-9/3 activation. The multi-targeted cell death pathway may explain the minor impact of drug resistance mechanisms on LE activity [13, 14]. Additionally, the lack of superoxide production anticipates absence or at least reduction of cardiotoxicity as compared to anthracyclines. Together these features suggest LE as a promising candidate for clinical development in systemic cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Dx	doxorubicin
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
DHE	dihydroethidium
LE	landomycin E
NAC	N-acetylcysteine
PI	propidium iodide
ROS	reactive oxygen species
SOD	superoxide dismutase

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Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Table 1

Impact of various ROS scavengers, alone, or in various combinations on production of hydrogen peroxide radicals by LE. Baseline level of hydrogen peroxide in control cells was considered 100%

Time, h	Control	+NAC	+Cat	+Man	+SOD	+Cat+SOD	+Cat+Man	+Man+SOD	+Cat+Man+SOD
1	99.1±8.6	88.3±4.8	38.3±5.5 ^{***}	96.7±6.9	131.0±2.4 [*]	52.3±5.7 ^{**}	51.8±6.5 ^{**}	120.0±8.6	54.4±3.9 ^{**}
3	101.3±13.1	80.6±2.9	68.0±9.7	67.4±2.4	80.8±5.8	71.1±1.3	66.5±14.1	65.3±7.0	64.3±8.1 [*]
6	102.9±15.00	85.4±7.7	77.8±15.2	67.4±2.4	79.8±1.0	79.5±7.1	77.1±22.9	60.5±11.8 [*]	70.9±12.6
12	106.6±11.9	213.0±12.1 ^{***}	126.4±2.3	126.4±2.3	140.9±7.6 [*]	109.4±2.0	115.5±8.7	111.4±5.6	103.0±9.6
24	104.34±14.98	233.0±4.2 ^{***}	156.8±2.8 ^{***}	119.72±0.00	109.44±1.97	138.33±4.98 [*]	143.31±7.39 ^{**}	91.42±1.64	140.78±2.53 [*]
	LE 6 μM	+NAC	+Cat	+Man	+SOD	+Cat+SOD	+Cat+Man	+Man+SOD	+Cat+Man+SOD
1	711.5±38.3 ^{**}	385.5±6.9 ^{▲▲▲}	269.4±14.5 ^{▲▲▲}	448.6±56.2 ^{▲▲▲}	542.8±19.5 ^{▲▲▲}	284.1±10.2 ^{▲▲▲}	270.4±57.5 ^{▲▲▲}	562.7±20.2 ^{▲▲▲}	224.3±39.9 ^{▲▲▲}
3	562.3±9.0 ^{***}	223.0±31.9 ^{▲▲▲}	275.4±29.6 ^{▲▲▲}	373.7±79.4 ^{▲▲▲}	495.9±8.9	294.3±9.8 ^{▲▲▲}	278.3±49.5 ^{▲▲▲}	437.4±15.7 [▲]	239.6±34.2 ^{▲▲▲}
6	230.1±24.7 [*]	123.5±19.8	162.2±28.9	180.7±32.2	198.1±0.0	157.0±8.5	169.2±15.2	191.2±6.9	141.3±12.7
12	198.2±7.1 ^{**}	184.5±6.6	168.5±3.0	191.2±6.9	174.7±3.1	145.9±2.6	154.1±5.5	143.3±8.5	138.2±9.7
24	352.3±13.3 ^{**}	241.5±4.3	283.9±0.0	305.3±11.0	274.0±9.8	294.3±0.0	269.0±4.8	259.5±4.7	233.0±4.2 [▲]
	LE 8 μM	+NAC	+Cat	+Man	+SOD	+Cat+SOD	+Cat+Man	+Man+SOD	+Cat+Man+SOD
1	739.5±66.3 ^{**}	563.8±40.5	322.0±5.8 ^{●●●}	524.7±37.7 ^{●●}	669.9±107.5	371.1±66.3 ^{●●●}	256.3±27.6 ^{●●●}	542.8±19.5 [●]	272.5±43.7 ^{●●●}
3	628.0±45.1 ^{**}	305.2±11.0 ^{●●●}	323.3±29.0 ^{●●●}	432.7±54.2 [●]	650.3±127.1	390.5±62.7 ^{●●}	290.2±26.0 ^{●●●}	486.9±9.0	290.5±61.8 ^{●●●}
6	259.5±4.7 ^{***}	154.9±16.7	174.9±9.4	202.5±18.2	255.5±18.4	194.9±10.5	177.9±6.4	209.4±11.3	178.9±19.2
12	241.5±4.4 ^{***}	202.0±10.9	187.7±3.4	232.9±4.2	245.8±16.4	174.7±3.1	205.4±12.5	237.1±0.0	184.4±14.9
24	352.5±12.7 ^{**}	269.0±4.8	340.1±12.2	358.7±6.5	340.7±24.5	316.4±11.4	333.8±6.0	322.0±5.8	278.9±5.0

* p < 0.05 relative to control,

** p < 0.01 relative to control,

*** p < 0.0001 relative to control, 2-way ANOVA, Bonferroni post-test;

▲ p < 0.05 relative to LE 6 μM,

▲▲ p < 0.01 relative to LE 6 μM,

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