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**Free radical production and labile iron pool decrease triggered by subtoxic
concentration of aclarubicin in human leukemia cell lines**

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Keywords - Aclarubicin, differentiation, HL-60, K562, labile iron pool, oxidative stress.

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Abstract

Aclarubicin (ACLA), which belongs to the anthracycline class of antineoplastic agents, has been demonstrated as a differentiating agent for a broad range of human solid tumors and leukemia. By using dihydroethidium as a fluorescent probe, we show the ability of subtoxic (i.e. differentiating) concentration of ACLA to generate reactive oxygen species in both K562 and HL-60 leukemia cell lines. Besides, we have used a calcein-based spectrofluorimetric assay to determine the influence of ACLA-treatment on the cellular labile iron pool (LIP). In both cell lines, the LIP level was markedly decreased in the presence of ACLA. Nevertheless, whereas ACLA-induced differentiation was obviously ROS-dependent, the LIP decrease was rather ROS-independent.

Abbreviations: ACLA, aclarubicin; calcein-AM, calcein-acetoxymethyl-ester; IRE, iron responsive element; IRP, iron regulatory protein; LIP, labile iron pool; NAC, N-acetylcysteine; NBT, nitroblue-tetrazolium; PDTC, pyrrolidone-dithiocarbamate, ROS, reactive oxygen species

1. Introduction

Aclarubicin (ACLA) belongs to the anthracycline class of antineoplastic agents widely used in conventional cancer chemotherapy of solid tumor and leukemia [1]. While anthracyclines cytotoxic effects are commonly accepted to be due to DNA-intercalation and interaction with the nuclear enzymes DNA-topoisomerases [2], additional targets have been proposed such as alteration of the mitochondrial function [3], and production of free radicals [4-6]. In addition, numerous *in vitro* and *in vivo* studies have demonstrated that anthracycline, when used at subtoxic concentrations, are potent differentiation inducers of solid tumors and leukemia [7, 8]. Particularly, ACLA induces differentiation of K562 and HL-60 human leukemic cell lines [8, 9]. It should be noticed that the subtoxic concentration of ACLA used in differentiation studies was 50-200 times lower than the plasmatic concentration found in patients [10].

The ability of anthracyclines to produce free radicals, or reactive oxygen species (ROS), in cells or tissues has been demonstrated with high doses of doxorubicin, as commonly used in chemotherapy [4-6]. Then, oxidative damages consecutive to anthracycline treatment has been thought to explain the high cardiotoxicity of these drugs [11, 12]. Furthermore, our recent studies have suggested the involvement of oxidative stress in the anthracycline-triggered differentiation of leukemic cells. In these studies, subtoxic concentrations of ACLA were used to induce the erythroid differentiation of the human K562 cell line, and antioxidants were shown to inhibit the ACLA effect [13]. These results supported the involvement of ROS production in the mechanism of action of ACLA, even at very low concentration.

The same redox properties that allow iron to be a functional enzymatic cofactor also make this metal a key participant in oxygen-mediated toxicity as a catalysor of the Fenton reaction [14]. Then, a role for iron has been suggested in the cardiotoxicity of

anthracyclines and the influence of iron addition or depletion on anthracycline effects has been investigated [15]. In addition, a putative effect of anthracycline on iron metabolism has been recently questioned [16].

All aspects of intracellular iron homeostasis are mirrored in the so-called labile iron pool (LIP), a low-molecular-weight pool of weakly chelated iron that rapidly transits through the cell and constitutes a real crossroads of metabolic pathways of iron-containing compounds. It has been claimed that LIP is a cellular source of iron participating in the Fenton reaction [17] as well as a sensor for the iron responsive element (IRE) -iron regulatory protein (IRP) system [18, 19]. It seems that under physiologic conditions, a constitutive level of LIP is midway between the cellular need for iron and the hazard of excessive generation of OH radical, the most potent oxidant encountered in biological systems. Any depletion or rise in the LIP may result either in impairment of synthesis of iron-containing proteins or in cell injury by pro-oxidants, as this has been clearly demonstrated in erythroid cells overexpressing H-ferritin [20] or exposed to transferrin [17].

Therefore, we intended here to compare the production of ROS in both K562 and HL-60 cell lines treated with subtoxic concentration of ACLA, and to determine the effect of ACLA on intracellular LIP level. By using spectrofluorimetric assays, we show that ACLA induces ROS generation and a decrease of LIP in both K562 and HL-60 leukemia cell lines. Nevertheless, whereas ACLA-induced differentiation was ROS-dependent, the LIP was rather ROS independent.

2. Materials and methods

2.1 Chemicals

Culture medium (RPMI-1640 with Glutamax) and fetal bovine serum were from Life Technologies (Saint-Quentin-en-Yvelines, France). Calcein-acetoxymethyl-ester (Calcein-AM) was purchased from Molecular Probes (Eugene, OR). All other chemicals, including the fluorescent probe dihydroethidium, N-acetylcysteine (NAC), pyrrolidine-dithiocarbamate (PDTC) and ACLA were purchased from Sigma (L'Isle-d'Abeau-Chesnes, France).

2.2 Cell culture and induction of differentiation

The K562 and HL-60 cell lines were cultured in RPMI-1640 medium with Glutamax supplemented with heat-inactivated fetal bovine serum (10% and 15%, respectively), in a 5% CO₂ humidified atmosphere at 37°C. To induce differentiation, cells were treated for 72 h with 25 nM ACLA at the beginning of exponential growth phase. In the case of short-term exposure, cells were incubated with 250 nM ACLA in the presence, or absence, of antioxidants (NAC, 5 mM or PDTC, 10 µM) for 1 h in complete medium, then centrifuged (400 x g, 10 min, 4°C), washed, and replaced in complete medium for additional 72 h. Cell growth and viability were assessed at day 3 by direct counting of Trypan blue dye-excluding cells. The % of erythroid differentiation in the K562 cell line, and the % of granulocytic differentiation of HL-60 cells were assayed by a benzidine staining method and by the nitroblue-tetrazolium (NBT)-test, respectively [13, 10].

2.3 Spectrofluorimetric assay of ROS production.

Cells treated for 1 to 24 h in the presence of 25 nM ACLA, and untreated control cells, were centrifuged and incubated with 2 μ M of dihydroethidium (λ_{ex} 360 nm, λ_{em} 420 nm) for 10 min, washed with phosphate-buffered saline, and then analysed by spectrofluorometry in RPMI-1640 medium without phenol red. In the presence of ROS, dihydroethidium is oxidized to ethidium and fluoresces in red (λ_{em} 640 nm). The 640 nm / 420 nm fluorescence intensity ratio permits to evaluate the production of ROS in living cells. Raw data were normalized with respect to control value and results expressed as the fold increase of 640/420 fluorescence intensity ratio. Results are given as mean $\pm$ SD of 3 independent experiments.

2.4 Spectrofluorimetric determination of LIP

LIP was determined by spectrofluorimetry as previously described [17] with slight modifications. Briefly, after indicated treatment, $2 \cdot 10^6$ cells were resuspended in 2 ml prewarmed buffer (20 mM HEPES, pH 7.3, 150 mM NaCl, 1% bovine serum albumin), and were incubated with 50 nM calcein-AM for 15 min at 37°C. After centrifugation and washing, cells were resuspended in the same buffer, and the calcein fluorescence intensity was recorded as a function of time. A stable baseline signal was recorded for 60 sec before the addition of 100 μ M SIH, a highly permeant iron chelator, which caused a rise in fluorescence corresponding to the level of LIP. Calibration and calculation of LIP concentration were made as described by Epsztejn *et al.* [17]. The excitation and the maximal emission wavelengths were 483 nm and 505 nm, respectively.

3. Results

3.1 Differentiating effect of ACLA

A subtoxic concentration of ACLA (25 nM) induced erythroid and granulocytic differentiation of K562 and HL-60 cell lines, respectively. In both cases, maximal differentiation was achieved within 3 days with a marked growth inhibition but without cytotoxicity (Table 1). Although a long term exposure to 25 nM ACLA (i.e. 72 h) was more convenient, the exposure period could be reduced to 24 h without affecting the percentage of differentiated cells at day 3 (data not shown). Furthermore, a 1 h pulse with a ten fold higher concentration of ACLA (250 nM) was sufficient to induce significant differentiation of K562 and HL-60 cells (Table 2). These results indicated that the differentiation process was engaged irreversibly in the first hour of ACLA treatment.

In addition, differentiation was markedly inhibited by the presence of antioxidants (either NAC or PDTC) during the 1 h pulse of ACLA (Table 2), suggesting the involvement of ROS as a signal for ACLA-induced differentiation.

3.2 ACLA-triggered ROS production

Whether ACLA induced oxidative stress was investigated by using the fluorescent probe dihydroethidium. ACLA-treated cells were then incubated in the presence of 2 μ M dihydroethidium for 10 min and analysed by spectrofluorimetry. Into the cells, the blue fluorescence (λ_{em} 420 nm) of dihydroethidium probe is shifted to red (λ_{em} 640 nm) when oxidized in the presence of ROS. Then, the 640 nm / 420 nm fluorescence intensity ratio permits to evaluate the production of ROS in cells, and allow a comparison between the two cell lines. As evidenced by the increased fluorescence intensity ratio, ROS production was observed in both K562 and HL-60

cell lines after one hour of treatment with 25 nM ACLA (Fig. 1). Nevertheless, this ROS production was higher in the K562 cell line (Fig. 1). The fluorescence intensity ratio diminished in the case of longer ACLA-treatment, suggesting that ROS production occurred in the first hour of ACLA-treatment and then declined with time (data not shown).

3.3 ACLA-induced decrease of LIP level

The incidence of subtoxic ACLA-treatment on LIP level was investigated in K562 and HL-60 cell lines by using a calcein-based spectrofluorimetric assay. Despite LIP level was about 2.5 fold higher in the K562 pre-erythroid cell line (Fig. 2A) than in the HL-60 cells (Fig. 2B), the results depicted in Figure 2 indicates that treatment with 25 nM ACLA for 18 h strongly reduced the LIP level in both cell lines. Nevertheless, the ACLA-induced LIP decrease was more marked in the HL-60 cell line (60% of decrease) than in K562 cells (31%) after 18h of incubation. This ACLA-mediated LIP decrease could be observed as soon as 8 h after ACLA-treatment but in a lesser extent (27% and 18% of decrease in HL-60 and K562 cells, respectively). Furthermore, the presence of the antioxidant NAC during the 18 h of ACLA treatment did not affect the reduction of LIP (Fig. 2). In both cell lines, the extent of 25 nM ACLA effect was roughly the same as did the well known iron chelator deferoxamine at the concentration of 150 μ M, suggesting that ACLA is a potent iron depletory agent (Fig. 2).

4. Discussion

It is now well established that several anticancer drugs, among which the anthracyclines ACLA and doxorubicin, are metabolically activated to free radical states (semi-quinone). These species are themselves very reactive and interact with molecular oxygen to generate partially reduced oxygen species (superoxide, hydrogen peroxide and hydroxyl radical) [4-6]. In the case of doxorubicin, the involvement of iron in these processes was demonstrated [21]. In turn, the species produced are able to interact with the antioxidant defense systems [22] and a decrease in intracellular enzymes activities (superoxide dismutase, catalase, glutathion reductase or glutathion peroxidase) and in reduced glutathion level after DOX treatment was previously reported [23, 24].

Our present results obviously indicate that ACLA-induced differentiation was dependent from ROS production. This further support previous data obtained in the K562 and HL-60 cell lines with various inducers, including anthracyclines, butyric acid, Ara-C, ADP-Fe²⁺ and H₂O₂ [13, 25, 26]. In the present case of ACLA-induced differentiation, such a ROS generation will probably stimulates gene specific expression through redox sensitive transcription factors (NF-κB, AP-1, NF-E2...), the involvement of which has already been demonstrated, at least in the erythroid differentiation of K562 cells [13].

Iron metabolism is often linked with ROS production, therefore it appeared important to evaluate the influence of anthracycline treatment on the intracellular iron metabolism, and particularly the LIP level. Our present results clearly show that LIP was decreased in ACLA-treated cells. Nevertheless, the ACLA-induced fall of LIP appeared to be independent of ROS production. The fact that NAC did not prevent the LIP decrease whereas it efficiently reduced cell differentiation suggest that LIP decrease was not a consequence of ACLA-induced differentiation. By contrast, the

ACLA-induced decrease of LIP could rather be thought as a cause of differentiation. Indeed, a role for iron in the monocytic differentiation has been recently reported [27, 28].

The ability of anthracyclines to irreversibly chelate iron could explain the decrease of LIP (and the production of ROS) [29]. In addition, the anthracycline doxorubicin (DOX) has been very recently reported to modulate the activity of iron regulatory proteins (IRPs). From one hand, the alcohol metabolite of DOX converted the cytoplasmic aconitase into its RNA binding form IRP-1 [30]. From the other hand, DOX produce ROS which inactivate both IRP-1 and IRP-2 [31]. But the consequences of the modulation of IRPs by DOX has not been assessed at the level of LIP. Then, elucidating the exact mechanism by which ACLA affects LIP level requires further investigations.

In view of our biological findings, clinical trials which associate ACLA, especially at very low dose, with other therapeutic treatment (chemotherapeutic agents or ionising radiation) should continue to be proposed. Indeed, nanomolar concentrations of aclarubicin are able to enhance the differentiation effect induced by 1 μ M doxorubicin in leukemic cells [32]. Enhancement of radiation-induced cell killing and DNA double-strand breaks in human colon tumour cells was also observed in presence of nanomolar concentrations of ACLA [33]. Another study from the same group has demonstrated that repair of radiation-induced DNA double-strand breaks decreased with cellular differentiation [34]. Taking into account that ROS production is induced by subtoxic doses of ACLA, we can suggest that ROS should enhance radiation-induced DNA double-strand breaks via induction of cellular differentiation. In addition, the LIP decrease we described here could serve as an additional explanation for the antiproliferative and pro-apoptotic effects of ACLA. Indeed, iron depletion has been reported to inhibit DNA synthesis at the level of ribonucleotide reductase [35] and,

apoptosis has been observed in response to a decrease of intracellular iron level and in synergy with anticancer drug [36-38]. By contrast, the addition of hemin has a protective effect against DOX-induced apoptosis [39].

To conclude, the use of very low dose of ACLA should reduced its cardiotoxicity and nevertheless allowed its antiproliferative and pro-apoptotic effects probably through ROS production and LIP decrease.

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Table 1. Differentiation, cell growth inhibition and viability of ACLA-treated cells.

Cell line	Differentiation ^a	Cell growth inhibition ^a	Viability ^a
K562	52 ± 4 %	56 ± 2 %	≥ 95 %
HL-60	73 ± 5 %	59 ± 2 %	≥ 95 %

^aAfter 3 days of culture in the presence of 25 nM ACLA, the % of differentiation was determined as the % of benzidine- and NBT-positive cells for K562 and HL-60 cell lines, respectively. Cell growth inhibition and viability were determined by direct counting of the Trypan-blue dye excluding cells. Results are the mean ± S.D. of 10 independent experiments.

Table 2. Cell differentiation induced by a 1 h pulse of ACLA, and inhibition by antioxidants

Treatment ^a	% of differentiation at day 3 ^b	
	K562	HL-60
None	3.0 ± 1.5	2.0 ± 1.7
ACLA	34.1 ± 2.9	74.3 ± 3.8
ACLA + NAC	2.0 ± 1.1	10.7 ± 6.4
ACLA + PDTC	1.5 ± 1.2	4.7 ± 2.5

^a Cells were incubated for 1 h with 250 nM ACLA in the presence, or absence, of 5 mM NAC or 10 μ M PDTC, then centrifuged, washed, and replaced in culture medium for additional 72 h. ^b The % of differentiation was determined as the % of benzidine- and NBT-positive cells for K562 and HL-60 cell lines, respectively. Data are the mean $\pm$ S.D. of three independent experiments.

FIGURE LEGENDS

Figure 1. ROS production in ACLA-treated cells. K562 and HL-60 cells were treated for 1 h with 25 nM ACLA before incubation in the presence of 2 μ M dihydroethidium for 10 min. The 640/420 nm fluorescence ratio was then determined and results were expressed as fold increase with respect to fluorescence level in untreated cells (hatched line). Results are the mean $\pm$ S.D. of three independent experiments.

Figure 2. LIP level in control and ACLA-treated cells. K562 (A) and HL-60 (B) cells were treated for 18 h with 25 nM ACLA, in the presence or absence of 5 mM NAC. Then, cells were incubated with 5 μ M calcein-AM for 30 min. The 505 nm fluorescence was then measured and LIP level was calculated as indicated in the Materials and Methods section. Results are the mean $\pm$ S.D. of three independent experiments. Statistical significance was tested with one-way ANOVA with post-hoc Student-Newman-Keuls comparison; columns with different letters significantly differ from each other ($p < 0.01$).

Fig. 1