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OXIDATIVE STRESS INVOLVEMENT IN CHEMICALLY-INDUCED DIFFERENTIATION OF K562 CELLS.

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Running title - Oxidative stress in K562 cells differentiation

Abstract - The erythroid differentiation of K562 cells could be achieved by exposure to several pharmacological agents including hemin, butyric acid (BA) and anthracycline antitumor drugs such as aclarubicin (ACLA) and doxorubicin (DOX). When used at subtoxic concentration, these drugs induce the overexpression of erythroid genes leading to hemoglobinization of cells. Since anthracyclines are known to generate oxidative damages, we intended to demonstrate the involvement of an oxidative stress in the chemically-induced differentiation process. The addition of antioxidants to anthracycline- and BA-induced cells decreased their growth and dramatically reduced the percentage of differentiated cells at day 3. Northern blot experiments showed that antioxidants also decreased the expression of erythroid genes and related transcription factors in induced cells. Moreover analyses of oxidative stress markers showed that a treatment with BA, ACLA and DOX lead to a decrease in reduced glutathione and antioxidant enzymes (glutathione peroxidase, glutathione reductase, CuZn superoxide dismutase (SOD) and catalase). In addition, DOX increased thiobarbituric acid reactants, and the MnSOD activity was decreased by BA and DOX. Finaly, the production of reactive oxygen species by differentiating agents was demonstrated using the dihydroethidium probe in a microspectrofluorometric assay. Altogether, these results strongly suggested the involvement of an oxidative stress, generated by BA or anthracyclines, as the first step turning on the irreversible differentiation process. Additionally these results also underlined the differences between BA, ACLA and DOX molecular mechanisms.

Keywords - Aclarubicin, Antioxidants, Butyric acid, Doxorubicin, Tumor cell differentiation, Hemin, Oxidative stress, K562 cells.

INTRODUCTION

Leukemic cells are generally characterized as maturation-arrested cells which remain in the proliferative pool and rapidly accumulate. Therefore, induction of tumor cells differentiation may provide an alternative or support to conventional cytotoxic chemotherapy of leukemia [1-3]. Abundant experimental evidence has demonstrated that chemically-unrelated agents, including retinoids, vitamin D, butyrate derivatives and anthracyclines, are able to overcome this differentiation block, shifting the cells from the proliferating into the differentiating pool [3-7].

Anthracycline antitumor drugs such as aclarubicin (ACLA) and doxorubicin (DOX), are widely used in conventional cancer chemotherapy of solid tumors and leukemia. While their cytotoxic effects are commonly accepted to be due to DNA-intercalation [8], additional targets have been recently proposed such as interaction with the nuclear enzymes DNA-topoisomerases [9, 10], alteration of the mitochondrial structure and function [11], and production of free radicals [12-14]. In addition, numerous *in vitro* and *in vivo* studies have demonstrated that anthracyclines are potent differentiation inducers of leukemic cells and solid tumors [6, 7].

We have used the human K562 cell line, originally derived from pleural effusion of a patient with chronic myeloid leukemia [15], to study the mechanisms of pharmacologically-induced differentiation of leukemic cells. Erythroid differentiation of K562 cells could be achieved by exposure to several pharmacological agents including hemin [16], butyric acid (BA) [17], and anthracyclines such as ACLA and DOX [6]. We have previously demonstrated the involvement of GATA-1 and NF-E2 transcription factors in the transcriptional activation of porphobilinogen deaminase (PBGD) and γ-

globin genes in BA- and ACLA-induced K562 cells [17-20]. In contrast, we have shown that DOX- and hemin-induced differentiation were independent of both GATA-1 and NF-E2 overexpression and erythroid promoters enhancement [19-21]. Nevertheless, no information is yet available concerning the early events or transduction pathways involved in the differentiation process induced by such drugs.

Since anthracyclines were previously reported to generate oxidative damages, particularly in cardiac tissue [22, 23], we intended here to demonstrate the involvement of an oxidative stress in the differentiation process triggered by ACLA and DOX. The effect of three chemically-unrelated antioxidants was investigated. Modifications in some cellular redox markers and oxidation of the fluorescent probe dihydroethidium were examined in order to detect the induction of an oxidative stress by ACLA and DOX. Results were compared to those obtained when using butyric acid, another differentiating agent.

Altogether, results reported here strongly suggested the involvement of an oxidative event in the anthracycline- and BA-triggered differentiation, and underlined the differences between ACLA- and DOX-molecular mechanisms.

MATERIALS AND METHODS

Chemicals

Culture medium (RPMI-1640) and fetal bovine serum were from Life Technologies (Saint Quentin en Yvelines, France). All other chemicals were purchased from Sigma (L'Isle d'Abeau Chesnes, France) unless otherwise stated and were of reagent or molecular biology grade. Differentiation inducers, including ACLA, DOX, BA and hemin, were

prepared as previously described [17, 24]. Quercetin and pyrolidine dithiocarbamate (PDTC) were solubilized in ethanol at 10 mM and 100 mM respectively, then PDTC was diluted to 10 mM in PBS, and both were stored at -20°C. The N-acetylcysteine (NAC) 1 M aqueous stock solution was buffered to pH 4.0 with sodium hydroxide and stored at -80°C.

Cell culture and differentiation

K562 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine, in a 5% CO_2 humidified atmosphere. Cells were treated with differentiation inducers (ACLA 20 nM, DOX 40 nM, BA 0.5 mM, hemin 30 µM) at the begining of exponential growth phase for 72 h in complete culture medium. In the case of short-term exposure, cells were treated with either ACLA 200 nM or DOX 1000 nM for 30 min in complete culture medium, then centrifuged (400 x g, 10 min, 4°C) and replaced in complete medium for additional 72 h. For H_2O_2 treatment, cells were incubated at 37°C for 30 min in phosphate buffered saline with various concentration of H_2O_2 (0.5 µM to 10 mM), then centrifuged and replaced in complete medium. Cell growth and viability were assessed at day 2 or 3 by direct counting of Trypan blue dye-excluding cells. Growth inhibition was calculated from: $\{[(C_n - C_0) - (T_n - T_0)] / (C_n - C_0) \times 100\}$, where C_n, C_0, T_n, T_0 represent the numbers of control (C) or treated (T) cells/ml at days 0 and n, respectively. The percentage of hemoglobin-producing cells was determined at day 2 or 3 by a benzidine staining method as previously described [24].

Analysis of gene expression

Erythroid RNA expression was determined by Northern blot analysis as previously described [17]. Briefly, total RNAs from either control, 20 nM ACLA-, or 0.5 mM BA-treated cells were separated in a denaturating 1% agarose gel and blotted onto a nylon membrane. Blots were hybridized with radiolabeled cDNAs according to standard procedures, and finally reprobed with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA from Clontech (Ozyme, Montigny le Bretoneux, France) as a control. The $^{A}\gamma$-globin, and GATA-1 human cDNAs were a kind gift from Dr. S. Ottolenghi (University of Milan, Italy). Human PBGD and murine p45 NF-E2 cDNAs were from Drs. P.-H. Roméo (INSERM U91, Créteil, France) and S.H. Orkin (Harvard Medical School, Boston, MA), respectively.

Assays for cellular redox markers

Cells were treated as described above and lysed in hypotonic Tris-HCl buffer (20 mM, pH 7.3) using a Potter tissue homogeneizer. For gluthatione and lipid peroxidation determinations, aliquots were taken from the whole homogeneized lysate. The other determinations were performed on the supernatant obtained after centrifugation of the lysate at 4°C for 10 min at 2665 x g.

Superoxide dismutase (SOD). Total SOD, MnSOD and CuZnSOD were determined using the pyrogallol assay following the procedure described by Marklund and Marklund [25], based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD. The specific CuZnSOD inhibition by KCN allowed the MnSOD determination in the same conditions. Results were expressed as SOD units per

mg of soluble cell protein measured according to the method of Lowry et al. [26], using bovine serum albumin as standard.

Glutathione peroxidase (GPx). GPx activity was determined by the modified method of Flohe and Günzler [27], using tert-butyl hydroperoxide, reduced glutathione and glutathione reductase. Glutathione peroxidase activity was expressed as international units per g of soluble protein (IU/g protein).

Glutathione reductase (GRase). GRase was measured by a method developed in our laboratory, derived from that described by Carlberg and Mannervik [28]. The principle is the measurement at 340 nm of NADPH consumption catalyzed by GRase. Results were given as IU per g of soluble protein.

Catalase (CAT). CAT activity was determined by the method described by Beers and Sizer [29] which follows the disappearance of hydrogen peroxide at 240 nm. The reaction rate was related to the amount of catalase present in the mixture. Results were expressed as IU per mg of soluble protein.

Glutathione determination. Samples of whole lysates were deproteinized by adding an aqueous solution of metaphosporic acid 6%. The mixture was centrifuged for 10 min at 2665 x g at 4°C. Total glutathione (GSHt) was determined by the method described by Akerboom and Sies [30], slightly modified [31] and based on the spectrophotometric evaluation of the reduction rate of 5,5'-dithiobis-2-nitrobenzoic acid into 5-thio-2-nitrobenzoate. Values were determined by comparing the reduction rate against a standard curve of glutathione. Oxidized glutathione (GSSG) was determined under the same conditions after adjusting pH with ethanolamine and trapping of reduced gluthatione (GSH) with 3-vinyl pyridine added to the sample. Glutathione level (total and oxidized) was expressed as µmol per g of total cell protein measured according to Shopsis and Mackay [32].

Lipid peroxidation. Lipid peroxidation was evaluated by thiobarbituric acid reactants (TBARs) determination, using a 1,1,3,3-tetraethoxypropane calibration curve, as previously described by Richard et al. [33]. Results were expressed as µmol of TBARs per g of total protein.

Statistics. Samples were assayed at least 3 times for each determination, and results were given as mean ± SEM. One-way ANOVA with Newman-Keuls post-hoc comparison was used for statistical significance with $p \leq 0.05$ or $p \leq 0.01$.

Microspectrofluorometric assay of reactive oxygen species (ROS) production.

Confocal laser scanning microspectrofluorometry was applied to the acquisition and analysis of X-Y emission spectra from a confocal section within a single living cell treated with fluorescent probe. The microspectrofluorometer M51 (Dilor, Lille, France) was coupled to an ionised argon laser 2065 (Spectra Physics, Les Ulis, France), and a 2 µW laser beam at 365 nm (dihydroethidium excitation) was used. The optical microscope (Olympus BH-2, Tokyo, Japan) was equipped with a 100X phase contrast water-immersion objective (Olympus). The thickness of the optical section was controlled by opening a square pinhole from 50 to 1200 µm. For intracellular measurements, the pinhole size was fixed to a diameter of 200 µm. The fluorescence emission was spectrally dispersed by diffraction grating (300 grooves mm^{-1}) and was analysed with an air-peltier-cooled CCD detector (Wright, Stonehouse, UK) supplied by an 1125 x 298 pixel sensor element.

Cells treated for 1 or 24 h in the presence of differentiating agents were centrifuged and incubated with 2 µM of dihydroethidium (λ_{em} 420 nm) for 10 min, washed, and then analysed by microspectrofluorometry. 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. For each determination, about 80 individual cells per experiment were analysed, and results are given as mean ± SEM of 2 independent experiments.

RESULTS

Inhibition of K562 cell hemoglobinization by antioxidants

Cells were treated for 72 h with differentiation inducers (ACLA 20 nM, DOX 40 nM, BA 0.5 mM or hemin 30 µM) in the presence of NAC. In ACLA- and BA-induced cells, the percentage of differentiated cells was reduced by approximatively 50% in the presence of 1 mM NAC and felt to control level with 10 mM NAC (Fig. 1A). Total inhibition of cell differentiation was observed with 15 mM and 20 mM NAC in DOX- and hemin-treated cells, respectively (Fig. 1A). In the same way, ACLA- and BA-induced differentiation were abolished in the presence of 1 µM PDTC, whereas 5 µM were required in the case of DOX (Fig. 1B). In contrast, hemin-induced differentiation was poorly affected by PDTC (Fig. 1B).

As shown in Fig. 1C, 10 µM quercetin reduced by approximatively 50% the differentiation of ACLA-, BA- and DOX-treated cells. A return to basal level of benzidine-positive cells was obtained in the presence of 50 µM quercetin in DOX-treated cells, or 75 µM in ACLA- and BA- treated cells (Fig. 1C). Hemin-induced differentiation was only slightly affected by quercetin treatment (Fig. 1C).

A short-term exposure protocol was also conducted using higher doses of ACLA and DOX, which have been previously reported to gave similar differentiation and growth

inhibitory effects in K562 cells [24]. The percentages of differentiated (i.e. benzidine-positive) cells at day 3 following 30 min treatment with 200 nM ACLA or 1000 nM DOX were 34 ± 3 % and 29 ± 3% respectively, and felt to control level (< 3%) in the presence of either NAC 10 mM, PDTC 1 µM or quercetin 100 µM.

Hence, all three antioxidants dramatically reduced the percentage of differentiated cells in ACLA-, DOX- or BA-induced cells. In contrast, hemin-induced differentiation was not significantly affected by PDTC and quercetin, although decreased with high doses of NAC.

Effect of antioxidant-treatment on cell growth and viability

Since chemically-induced differentiation is accompagnied by a reduction or arrest of cell growth [6, 17, 24], except for hemin [16], we have investigated the effect of antioxidants on this parameter. The cell growth inhibtion was determined at 72 h of culture in the presence or absence of the indicated drugs. Results showed that NAC reduced the growth of control and hemin-treated cells by 50% at 10 mM, and slightly enhanced the growth-inhibitory effect of the other inducers (Fig. 2A). In the presence of 1 µM PDTC the growth of control cells was reduced by 70%, and the growth-inhibitory effect of inducers was markedly increased (Fig. 2B). Quercetin also inhibited the growth of control cells and enhanced the growth-inhibitory effect of inducers (Fig. 2C), except for DOX. In addition the cell viability was not affected by NAC, PDTC and quercetin in the concentration ranges used.

Then, although the differentiating effect of each inducer was inhibited, antioxidants did not restore the proliferating potential of the cells and rather enhanced the cell growth inhibition. This was probably due to their own growth-inhibitory, but not cytotoxic, effect.

Induction of differentiation by H_2O_2

Hydrogen peroxide was used to investigate the differentiating effect of oxidative stress in K562 cells. Despite a marked cytotoxicity, 100 µM of H_2O_2 applied for 30 minutes induced 38% of benzidine-positive cells and 97% of growth inhibition at day 3.

Inhibition of erythroid RNA expression by PDTC and quercetin

In contrast with DOX- and hemin-, both ACLA- and BA-induced differentiation involved the overexpression of erythroid genes such as γ-globin, PBGD, and transcription factors NF-E2 and GATA-1 [17-20]. Therefore, Northern blot experiments were done to study the effect of antioxidants on erythroid RNA expression in both 20 nM ACLA- and 0.5 mM BA-induced K562 cells. As shown in Fig. 3, the level of γ-globin and PBGD RNAs, as well as RNAs for NF-E2 and GATA-1 erythroid transcription factors, were increased in both ACLA- and BA-induced cells and reduced near to the control levels in the presence of 1 µM PDTC. Similar results could be observed in the presence of 10 mM NAC (Data not shown).

Despite quercetin (100 µM) induced a slight increase of γ-globin, NF-E2 and GATA-1 RNAs in control cells, without apparition of benzidine-positive cells, it also inhibited the overexpression of erythroid RNAs in both ACLA- and BA-induced cells (Fig. 3). Nevertheless, the inhibitory effect of quercetin was lower than that of PDTC.

These results suggest the involvement of an early oxidative event, which precedes the drug-induced elevation of NF-E2 and GATA-1 transcription factors RNA levels and overexpression of erythroid genes.

Effect of anthracycline- and BA-treatment on cellular redox markers

In order to evidence some oxidative change in K562 cells following induction of differentiation, the measurement of several redox markers was done in ACLA-, DOX- and BA-treated cells. As shown in Fig. 4A, the GSHt level was markedly increased after 24 h of treatment with either 20 nM ACLA, 40 nM DOX or 0.5 mM BA, and then decreased significantly below the control level to be minimal at 72 h. Nevertheless, the amplitude of these variations was weaker in the case of BA than for DOX and ACLA. Detailed study of both oxidized and reduced forms of intracellular glutathione showed that GSSG level was significantly ($p < 0.01$) augmented at 24 h (0.87 ± 0.01, 1.17 ± 0.13, 0.81 ± 0.06 µmol/g protein in ACLA-, DOX, and BA-treated cells, respectively, versus 0.37 ± 0.07 µmol/g protein in control cells). Afterwards, the GSSG level declined to control level by 72 h (Data not shown). Therefore, the 24 h increase in GSHt level was mainly the fact of increased GSSG level, while the later diminution of GSHt reflected a reduced GSH level in either ACLA-, DOX-, and BA-treated cells. In the same way, high dose of DOX in a short-term exposure protocol (1000 nM, 30 min) significantly decreased the GSHt, and more precisely GSH, level at 72 h (Fig. 4A). By contrast, any changes in GSHt, GSSG, or GSH could be observed with ACLA (200 nM, 30 min) at this belated time-point.

The GPx activity was markedly reduced in cells treated by DOX 40 nM, and totaly shut down at 72 h (Fig. 4B). High dose of DOX (1000 nM, 30 min) caused a decrease in GPx activity similar to that observed at 48 h with 40 nM DOX (Fig. 4B). Total inhibition of GPx occured earlier, at 24 h, in ACLA- and BA-treated cells. At the 72 h time-point GPx activity returned to control level in 20 nM ACLA-treated cells, or was further increased in 200 nM ACLA- and BA-treated cells (Fig. 4B). In addition, the GRase activity was decreased by all differentiating treatments and did not changed with time

between 24 and 72 h. Nevertheless, GRase activity was minimal in 20 nM ACLA-treated cells at 48 and 72 h (Fig. 4C).

As an indicator of lipid peroxydation, the TBARs level was increased 72 h after treatment of cells by ACLA 200 nM and DOX 1000 nM for 30 min (Fig. 5A). It was also increased in 40 nM DOX-treated cells at 24 and 72h, whereas reduced at 48 h (Fig. 5A). By contrast, the TBARs level was decreased in 20 nM ACLA-treated cells (all time-points) and BA-treated cells (24 and 48 h) as shown in Fig. 5A.

Total SOD and CAT activities were significantly decreased by 20 nM ACLA, 40 nM DOX and 0,5 mM BA treatments with roughly no significative difference between 24, 48 and, 72 h time-points (Fig. 5B,C). Nevertheless, ACLA only modified CuZnSOD activity while DOX also strongly decreased MnSOD activity at 72 h (Table 1). In BA-treated cells, the MnSOD activity was progressively decreased with time, while CuZnSOD activity, which was minimal at 24 h, slightly increased (Table 1). High dose of DOX (100 nM) and ACLA (200 nM) did not affect CAT activity and, only ACLA induced a slight decrease in SODt (Fig. 5B, C) attributed to CuZnSOD inhibition (Table 1).

Altogether these results showed that both DOX and ACLA (40 and 20 nM respectively) affect the redox status of the cells as revealed by a transient increase in GSSG, and a decrease in GSH level and GPx, GRase, CuZnSOD and CAT activities. Nevertheless, only DOX (40 nM, 72 h) induced an increase in TBARs level and a decrease in MnSOD activity. In the case of high doses in short term exposure protocol, DOX (1000 nM) gave essentialy the same results than with 40 nM dose, whereas ACLA (200 nM) produced no (CAT and GPx activities) or opposites (GSHt and TBARs levels) effects in comparison with ACLA 20 nM. Nevertheless, it would be noted that these measurements were done 72 h after treatment and reversible effects, expected for an intracellular messenger role of redox modifications, could not be observed. In comparison with

anthracyclines, BA treatment provoked roughly the sames changes than 20 nM ACLA, excepted the BA-induced progressive inhibition of MnSOD.

Production of ROS in anthracycline- and BA-induced K562 cells.

Finaly, the production of ROS in ACLA-, DOX- and BA-induced cells was examined by using dihydroethidium as a fluorescent probe in a microspectrofluorometric assay. Figure 6 showed that treatment with either ACLA, DOX or BA for 1 h increased the 640 nm band fluorescence by at least 2 fold, demonstrating the oxidation of dihydroethidium by ROS. Surprisingly, this ROS production was slightly higher with ACLA 20 nM, DOX 40 nM and BA 0.5 mM than with ACLA 200 nM and DOX 1000 nM. In addition, this ROS production disappeared at 24 h (Fig. 6).

These results are consistent with the generation of ROS by anthracycline and BA, and are in favour of oxidative stress involvement in the earliest step of the chemically-induced differentiation process.

DISCUSSION

Erythroid differentiation mechanisms are extremely complex and involve multiple regulatory events depending on the inducer used. Detailed pathways remain unknown and no common explanation is available for the effects of the great number of drugs, including several known antineoplastic agents, inducing erythroid differentiation of K562 human erythroleukemia cells [4-8].

Among the inducers used, DOX and ACLA have been clearly shown to produce free radicals such as semi-quinone radicals and ROS during their metabolization in cells

[12-14]. Nevertheless, the relationship between ROS and differentiation has been poorly studied. Some results were obtained on HL-60 human promyelocytic leukemia cells [34] and the involvement of Fenton reaction products in differentiation induction of K562 cells by Ara-C, ADP-Fe^{2+} and H_2O_2 was suggested [35]. Our present work also indicates that radical steps are involved in the case of irreversible induction of differentiation by BA, DOX and ACLA.

Oxygen free radicals are implicated in numerous processes such as carcinogenesis or inflammation [36-38]. These reactive species may damage directly or indirectly nucleic acids, proteins or membrane phospholipids, a mitogen effect was demonstrated in several studies. However these compounds are also produced at physiological levels and are involved in normal processes such as cell division and probably differentiation. In fact, a hypothesis has been proposed, assuming that part of the $OH^{\cdot}$ free radicals derived from H_2O_2 by iron-induced heterolysis may play a physiologically important role in cell growth and maturation [39]. The most important fact to consider is the balance between free radicals and antioxidant systems which are commonly used as markers of cellular redox status. Numerous constitutive and/or inducible systems serve to defend the cells against oxidative stress. The main antioxidant enzymes are CAT, seleno-dependant GPx, CuZn SOD, Mn SOD and GRase. Among non enzymatic systems, glutathione plays a key role as free radicals scavenger and detoxifying system.

Hemin was chosen as a non oxidative-stress-related [40] and reversible [16] inducer. Treatment of K562 cells with hemin induced differentiation and related intracellular modifications such as increase in heat shock gene expression and thioredoxin expression, without any reduction of glutathione level, suggesting that oxidative stress was not involved in this process [40]. Our results showed that hemin did not act on cell growth and that among the antioxidants used, only NAC at high dose inhibited hemin-induced

differentiation. NAC is supposed to act either by entering the cellular glutathione cycle, increasing the pool of reduced glutathione after metabolisation, or independently of changes in GSH amount or redox state [41]. Therefore, NAC may be involved in direct interactions either with hemin or at other steps of heme synthesis induced by hemin, for example inhibition of expression or activity of δ-aminolevulinic acid synthetase or δ-aminolevulinic acid dehydratase or reactivation of heme oxygenase [16]. The metal chelators PDTC and quercetin have also no effect on hemin-induced erythroid differentiation, indicating that the mechanism of hemin is linked neither to a radical process nor to the availability of iron.

Short chain fatty acids, such as butyrate and propionate, are effective suppressors of colorectal cancer. In such cell type, these compounds have been shown to increase cellular peroxide generation, suggesting the role of ROS in regulating cell growth [42]. Indeed, butyrate and propionate are substrates of the β-oxidation and can increase basal superoxide generation at mitochondrial level by entering the general oxidative metabolism of the cell. In our study, BA-induced oxidative stress was evidenced by changes in GSSG and GSH levels, the decrease in cellular antioxidant enzymes activities (CAT, GPx, GRase, CuZnSOD and particularily mitochondrial MnSOD), and by the oxidation of the dihydroethidium probe. Addition of NAC, PDTC or quercetin considerably decreased the differentiation suggesting that the ROS production was involved in this process. Moreover, the fact that antioxydants reduced the overexpression of the transcription factors GATA-1 and NF-E2 mRNAs to control levels, and that major changes in redox sytems occured at 24 h, indicated that ROS may be involved in the earliest steps of the differentiation process.

Anthracyclines, for example ACLA and DOX, have been shown to induce the *in vitro* differentiation of various malignant cells including leukemic cells [6, 7], solid tumors

such as neuroblastoma cells [43], and melanoma cells [44]. Concerning leukemic cells, several studies were conducted to try to elucidate the molecular bases of the differentiating effect of ACLA and DOX. In erythroleukemic K562 cells, we previously demonstrated that the drugs affected differently the balance between growth and differentiation. By contrast to DOX, ACLA did not require a total arrest of cell growth to achieve its optimal differentiating effect [24]. In addition, these antitumor drugs induced hemoglobin synthesis by affecting erythroid gene expression in different ways [19-21], and discrepancies in their differentiating effects were also reported in human leukemic HL60 cells [45, 46].

It is now well established that several anticancer drugs, among which doxorubicin and aclarubicin, are metabolically activated to free radical states (semi-quinone). These species are themselves very reactive and interact with molecular oxygen to generate toxic, partially reduced oxygen species (superoxide, hydrogen peroxide and hydroxyl radical) [12-14]. At least concerning DOX, the involvement of iron in these processes was demonstrated [47]. In turn, the species produced are able to interact with the antioxidant defense systems [48] and a decrease in intracellular enzymes activities (SOD, CAT, Grase or GPx) and in GSH level after DOX treatment was previously reported [49, 50].

In this work, evidences were obtained concerning the modification of intracellular redox state after cell treatment by ACLA and DOX. In both cases, antioxidants inhibited the differentiation of cells (at lower doses for ACLA), and analysis of the intracellular antioxidant markers indicated a decrease in CAT, CuZn SOD, GRase and GPx activities and in GSH level. Nevertheless some discrepancies could be observed such as lipid peroxidation and MnSOD activity which were differently affected by DOX and ACLA. Finaly, ROS production was demonstrated in both ACLA- and DOX-treated cells by using the dihydroethidium probe. These results are in favour of the production of ROS by

anthracyclines and involvement of free radicals during the earliest steps of the differentiation process. But they also confirm that some differences exist which may be linked to the subcellular level of metabolization of DOX and ACLA, the global quantity and relative proportions of the radical species produced. ROS seem to act early via the specific transcription factors GATA-1 and NF-E2 as indicated by the inhibition obtained with antioxidant in the case of ACLA, or via other factors able to induce overexpression of γ globin gene, in the case of DOX. It would be noted that ACLA effect on cellular antioxidant systems resembles BA effects and contrast with DOX, remaining the previously observed similarities between ACLA and BA transcriptional mechanisms in contrast with DOX post-transcriptional differentiation process [17-21].

In conclusion, these results showed the generation of an oxidative stress following BA- and anthracycline-treatment and strongly suggested the involvement of this ROS synthesis as the first event in the BA- and anthracycline-triggered differentiation process. In addition, hemin-induced differentiation was not inhibited by antioxidants showing that stress may be sometimes not required in the erythroid differentiation process. Furthermore, the differences observed between BA, ACLA and DOX effects on cellular redox systems underline the complexity of the mechanisms involved in the induction of erythroid differentiation.

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ABBREVIATIONS

ACLA - aclarubicin

BA - butyric acid

CAT - catalase

DOX - doxorubicin

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GPx - glutathione peroxidase

GRase - glutathione reductase

GSH - reduced glutathione

GSHt - total glutathione

GSSG - oxidized glutathione

NAC - N-acetylcysteine

PBGD - porphobilinogen deaminase

PDTC - pyrolidine-dithiocarbamate

ROS - reactive oxygen species

SOD - superoxide dismutase

TBARs - thiobarbituric acid reactants

REFERENCES

[1] Beere, H.M.; Hickman, J.A. Differentiation: a suitable strategy for cancer chemotherapy? *Anti-cancer Drug Design* **8:**299-322, 1993.

[2] Fenaux, P.; Chastang, C.; Chomiennne, C.; Degos, L. Tretinoin with chemotherapy in newly diagnosed acute promyelocytic leukaemia. *Lancet* **343:**1033, 1994.

[3] Reiss, M.; Gamba-Vitalo, C.; Sartorelli, A.C. Induction of tumor cell differentiation as a therapeutic approach: preclinical models for hematopoietic and solid neoplasms. *Cancer Treat. Rep.* **70:**201-218, 1986.

[4] Degos, L.; Dombret, H.; Chomienne, C.; Daniel, M.T.; Micléa, J.M.; Chastang, C.; Castaigne, S.; Fenaux, P. All-*trans*-retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia. *Blood* **85:**2643-2653, 1995.

[5] Newmark, H.L.; Young, C.W. (1995) Butyrate and phenylacetate as differentiating agents: practical problems and opportunities, *J. Cell. Biochem. Supp.* **22:**247-253, 1995.

[6] Jeannesson, P.; Lahlil, R.; Chénais, B.; Devy, L.; Gillet, R.; Aries, A.; Morceau, F.; Trentesaux, C. Anthracyclines as tumor cell differentiating agents: effects on the regulation of erythroid gene expression. *Leuk. Lymphoma* **26:**575-587, 1997.

[7] Casazza, A.M. Anthracyclines as inducers of tumor cell differentiation. In: Lown, J.W. ed. *Bioactive Molecules*. Amsterdam: Elsevier; 1988:715-734.

[8] Gabbay, E.J.; Grier, D.; Fingerle, R.E.; Reimer ,R.; Levy, R.; Pearce, S.W.; Wilson, W.D. Interaction specificity of the anthracyclines with deoxyribonucleic acid. *Biochemistry* **15:**2062-2069, 1976.

[9] Buhl Jensen, P.; Sandhal Sorensen, B.; Sehested, M.; Demant, E.J.F.; Kjeldsen, E.; Friche, E.; Hoi Hansen, H. Different modes of anthracycline interaction with topoisomerase II. *Biochem. Pharmacol.* **45:**2025-2035, 1993.

[10] Bridewell, D.J.A.; Finlay, G.J.; Baguley, B.C. Differential actions of aclarubicin and doxorubicin: the role of topoisomerase I. *Oncology Res.* **9:**535-542, 1997.

[11] Papadopoulou, L.C.; Tsiftsoglou, A.S. Mitochondrial cytochrome *c* oxidase as a target site for daunomycin in K-562 cells and heart tissue. *Cancer Res.* **53:**1072-1078, 1993.

[12] Doroshow, J.H. Anthracycline antibiotic-stimulated superoxide, hydrogen peroxide, and hydroxyl radical production by NADH dehydrogenase. *Cancer Res.* **43:**4543-4551, 1983.

[13] Powis, G. Free radical formation by antitumor quinones. *Free Radic. Biol. Med.* **6:**63-101, 1989.

[14] Keizer, H.G.; Pinedo, H.M.; Schuurhuis, G.J.; Joenje, H. Doxorubicin (adriamycin): a critical review of free radical-dependent mechanisms of cytotoxicity. *Pharmacol. Ther.* **47:**219-231, 1990.

[15] Lozzio, C.B.; Lozzio, B.B. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood* **45:**321-334, 1975.

[16] Dean, A.; Erard, F.; Schneider, A.B.; Schechter, A.N. Induction of hemoglobin accumulation in human K562 cells by hemin is reversible. *Science* **212:**459-461, 1981.

[17] Chénais, B.; Molle, I.; Trentesaux, C.; Jeannesson, P. Time-course of butyric acid-induced differentiation in human K562 leukemic cell line: rapid increase in γ-globin, porphobilinogen deaminase and NF-E2 mRNA levels. *Leukemia* **11:**1575-1579, 1997.

[18] Chénais, B. Requirement of GATA-1 and p45 NF-E2 protein expression in butyric acid-induced differentiation. *Biochem. Biophys. Res. Commun.***253:**883-886, 1998.

[19] Morceau, F.; Chénais, B.; Gillet, R.; Jardillier, J.C.; Jeannesson, P.; Trentesaux, C. Transcriptional and posttranscriptional regulation of erythroid gene expression in anthracycline-induced differentiation of human erythroleukemic cells. *Cell Growth Differ.* **7:**1023-1029, 1996.

[20] Morceau, F.; Aries, A.; Lahlil, R.; Devy, L.; Jardillier, J.C.; Jeannesson, P.; Trentesaux ,C. Evidence for distinct regulation processes in the aclacinomycin and doxorubicin-mediated differentiation of human erythroleukemic cells. *Biochem. Pharmacol.* **51:**839-845, 1996.

[21] Aries, A.; Trentesaux, C.; Ottolenghi, S.; Jardillier, J.C.; Jeannesson, P.; Doubeikovski, A. Activation of erythroid specific promoters during anthracycline-induced differentiation of K562 cells. *Blood* **87:**2885-2890, 1996.

[22] Nowak, D.; Drzewoski, J. Anthracycline-induced oxidative stress - its role in the development of cardiac damage. *Cancer J.* **9:**296-303, 1996.

[23] Singal, P.K.; Iliskovic, N.; Li, T.; Kumar, D. Adriamycin cardiomyopathy: pathophysiology and prevention. *FASEB J.* **11:**931-936, 1997.

[24] Ngo-Nyoung, M.; Trentesaux, C.; Aries, A.; Carpentier, Y.; Jardillier, J.C.; Gorisse, M.C.; Jeannesson, P. Effect of aclacinomycin-doxorubicin association on differentiation and growth of human erythroleukemic K562 cells. *Anticancer Res.* **14:**1203-1208, 1994.

[25] Marklund, S.; Marklund, G. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay of superoxide dismutase. *Eur. J. Biochem.* **47:**469-474, 1974.

[26] Lowry, D.H.; Rosebrough, N.J.; Farra, A.; Randall, R.J. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193:**265-275, 1951.

[27] Flohe, L.; Günzler, W.A. Assays of glutathione peroxidase. *Methods Enzymol.* **105:**114-121, 1984.

[28] Carlberg, I.; Mannervik, B. Glutathione reductase. *Methods Enzymol.* **113:**484-490, 1985.

[29] Beers, B.; Sizer, W. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195:**133-139, 1952.

[30] Akerboom, T.P.; Sies, H. Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological samples. *Methods Enzymol.* **77:**373-382, 1981.

[31] Emonet, N.; Leccia, M.T.; Favier, A.; Beani, J.C.; Richard, M.J. Thiols and selenium: protective effect on human skin fibroblasts exposed to UVA radiation. *J. Photochem. Photobiol. B* **40:**84-90, 1997.

[32] Shopsis, C.; Mackay, G.J. Semi automated assay for cell culture. *Anal. Biochem.* **140:**104-107, 1984.

[33] Richard, M.J.; Portal, B.; Meo, J.; Coudray, C.; Hadjian, A.; Favier, A. Malondialdehyde kit evaluated for determining plasma and lipoprotein fractions that react with thiobarbituric acid. *Clin. Chem.* **38:**704-709, 1992.

[34] Nagy, K.; Pasti, G.; Bene, L.; Zs-Nagy, I. Induction of granulocytic maturation of HL-60 human leukemia cells by free radicals: a hypothesis of cell differentiation involving hydroxyl radicals. *Free. Radic. Res. Comm.* **19:**1-6, 1993.

[35] Nagy, K.; Pasti, G.; Bene, L.; Zs-Nagy, I. Involvement of Fenton reaction products in differentiation induction of K562 human leukemia cells. *Leukemia Res.* **19:**203-212, 1995.

[36] McCord, J.M. Oxygen-derived free radicals. *New Horiz.* **1:**70-76, 1993.

[37] Buechter, D.D. Free radicals and oxygen toxicity. *Pharm. Res.* **5:**253-260, 1988.

[38] McCord, J.M. Roles of superoxide in inflammation and ischemic shock. In: Sorenson, J.R.J., ed. *Inflammatory diseases and copper*. Totowa, New Jersey USA: Humana Press; 1982:255-266.

[39] Zs-Nagy, I. A proposal for reconsideration of the role of oxygen free radicals in cell differentiation and aging. *Ann. N.Y. Acad. Sci.* **673:**142-150, 1992.

[40] Leppä, S.; Pirkala, L.; Chow, S.C.; Eriksson, J.E.; Sistonen, L. Thioredoxin is transcriptionally induced upon activation of heat shock factor 2. *J. Biol. Chem.* **272:**30400-30404, 1997.

[41] Jones, D.P.; Maellaro, E.; Jiang, S.; Slater, A.F.; Orrenius, S. Effects of N-acetyl-L-cysteine on T-cell apoptosis are not mediated by increased cellular glutathione. *Immunol. Lett.* **45:**205-209, 1995.

[42] Giardina, C.; Inan, M.S. Nonsteroidal anti-inflammatory drugs, short-chain fatty acids, and reactive oxygen metabolism in human colorectal cancer cells. *Biochim. Biophys. Acta* **1401:**277-288, 1998.

[43] Rocchi, P.; Ferreri, A.M.; Simone, G.; Prodi, G. Epirubicin-induced differentiation of human neuroblastoma cells in vitro. *Anticancer Res.* **7:**247-250, 1987.

[44] Supino, R.; Mariani, M.; Colombo, A.; Prosperi, E.; Croce, A.C.; Bottiroli, G. Comparative studies on the effects of doxorubicin and differentiation inducing agents on B16 melanoma cells. *Eur. J. Cancer* **28A:**778-783, 1992.

[45] Wilson, R.G.; Feit, S.; Vossugh, S.; Randolph, V. Aclacinomycin A and adriamycin induction of differentiation of HL-60 and ML-1 cells. *Proc. Am. Assoc. Cancer Res.* **27:**749, 1986.

[46] Stöcker, U.; Schaefer, A.; Maquardt, H. DMSO-like rapid decrease in c-myc and c-myb mRNA levels and induction of differentiation in HL-60 cells by the anthracycline antitumor antibiotic aclarubicin. *Leukemia* **9:**146-154, 1995.

[47] Sinha, B.K.; Trush, M.A.; Kennedy, K.A.; Minnaugh, E.G. Enzymatic activation and binding of adriamycin to nuclear DNA. *Cancer Res.* **44:**2892-2896, 1984.

[48] Pigeolet, E.; Corbisier, P.; Houbion, A.; Lambert, D.; Michiels, C.; Raes, M.; Zachary, M.D.; Remacle, J. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. *Mech. Ageing Dev.* **51:**283-297, 1990.

[49] Julicher, R.H.; Sterrenberg, L.; Bast, A.; Riksen, R.O.; Koomen, J.M.; Noordhoek, J. The role of lipid peroxidation in acute doxorubicin-induced cardiotoxicity as studied in rat isolated heart. *J. Pharm. Pharmacol.* **38:**277-282, 1986.

[50] Sazuka, Y.; Tanizawa, H.; Takino, Y. Effect of adriamycin on the activities of superoxide dismutase, glutathione peroxidase and catalase in tissues of mice. *Jpn. J. Cancer Res.* **80:**89-94, 1989.

LEGENDS TO FIGURES

Fig. 1. Inhibition of K562 cell differentiation by antioxidants. The percentage of benzidine-positive cells was determined after 3 days in either control cells or 20 nM ACLA-, 40 nM DOX-, 0.5 mM BA- or 30 µM hemin-induced cells in the presence of various concentrations of NAC (A), PDTC (B) and quercetin (C). Data are the mean of three independent experiments and SEM were below 10%.

Fig. 2. Increase of K562 cell growth inhibition by antioxidants. The percentage of cell growth inhibition was determined after 3 days in either control cells, or 20 nM ACLA-, 40 nM DOX-, 0.5 mM BA- or 30 µM hemin-induced cells in the presence of various concentrations of NAC (A), PDTC (B) and quercetin (C). Data are the mean of three independent experiments and SEM were below 10%.

Fig. 3. Effect of the antioxidants PDTC and quercetin on erythroid RNA expression. Northern blot analysis was performed with total RNA from 3 days-cultured cells. Control cells (lanes 1, 3, 5 and 7), 0.5 mM BA- (lanes 2, 4, 6 and 8) and 20 nM ACLA- (lanes 9-12) induced cells were treated, or not, with either 100 µM quercetin (lanes 1, 2 and 9) or 1 µM PDTC (lanes 7, 8 and 12). The γ-globin and PBGD blots were performed separately from the same RNA extracts, dehybridized and reprobed with either NF-E2 or GATA-1 cDNA respectively. Finally membranes were blotted with GAPDH probe. Data from a typical experiment representative of three.

Fig. 4. Effect of the differentiation inducers BA, ACLA and DOX on GSHt level (A), GPx (B) and GRase (C) activities. Exponentially growing cells were treated or not (Control), as indicated and GSHt level, GPx and GRase activities were determined after 24, 48 or 72 h as described in Materials and Methods. Data are the mean ± SEM of three independent experiments. One-way ANOVA with Newman-Keuls post-hoc comparison was used for statistical significance with * $p \leq 0.05$ or ** $p \leq 0.01$.

Fig. 5. Effect of the differentiation inducers BA, ACLA and DOX on TBARs level (A), SOD (B) and CAT (C) activities. Exponentially growing cells were treated, or not (Control), as indicated and TBARs level, SOD and CAT activities were determined after 24, 48 or 72 h as described in Materials and Methods. Data are the mean ± SEM of three independent experiments. One-way ANOVA with Newman-Keuls post-hoc comparison was used for statistical significance with * $p \leq 0.05$ or ** $p \leq 0.01$.

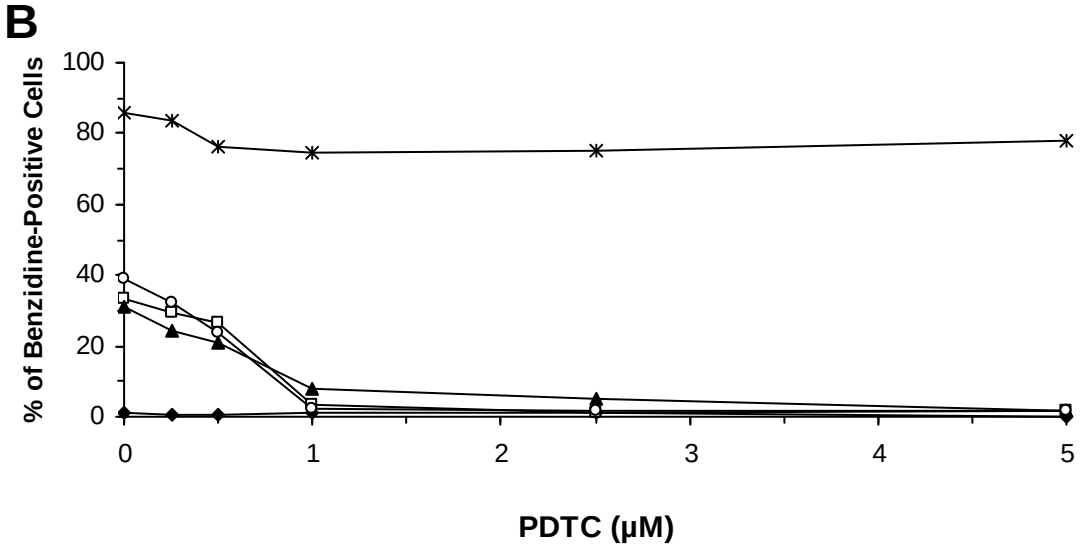
Fig. 6. Microspectrofluorometric analysis of ROS production. Cells were treated, or not (Control), with either ACLA 20 nM, ACLA 200 nM, DOX 40 nM, DOX 1000 nM or BA 0.5 mM for 1 or 24 h and incubated with 2 µM dihydroethidium for 10 min, washed and then analysed by confocal laser scanning microspectrofluorometry. The production of ROS was evaluated by the 640 nm (oxydized) / 420 nm fluorescence intensity ratio. Data are the mean ± SEM of two independent experiments.

Table 1. Effect of BA and anthracycline treatment on CuZnSOD and MnSOD activities

Treatment [a]	CuZnSOD activity (IU/mg protein)			MnSOD activity (IU/mg protein)		
	24 h	48 h	72 h	24 h	48 h	72 h
Control			9.40 ± 0.70 [b]			1.95 ± 0.20
ACLA 20 nM	5.28 ± 0.13 [c]	4.30 ± 0.40 [c]	5.40 ± 1.08 [c]	2.11 ± 0.11	1.85 ± 0.16	1.97 ± 0.20
ACLA 200 nM			5.63 ± 1.62 [c]			2.10 ± 0.22
DOX 40 nM	7.17 ± 0.11 [c]	7.02 ± 0.51 [c]	6.78 ± 0.31 [c]	1.92 ± 0.17	2.16 ± 0.09	0.84 ± 0.01 [c]
DOX 1000 nM			8.98 ± 0.75			2.34 ± 0.18
BA 0,5 mM	4.81 ± 0.31 [c]	5.61 ± 0.10 [c]	6.46 ± 1.26 [c]	2.01 ± 0.10	1.77 ± 0.08	1.03 ± 0.01 [c]

[a] Cells were treated with differentiating agents and SOD activities were determined at the indicated time as described in Materials and Methods. [b] Results are the mean ± SEM of three independent experiments. [c] Value significantly different from control as assayed by one-way ANOVA with Newman-Keuls post-hoc comparison ($p \leq 0.05$).

Figure 1


B
% of Benzidine-Positive Cells
100
80
60
40
20
0
0
1
2
3
4
5
PDTC (µM)

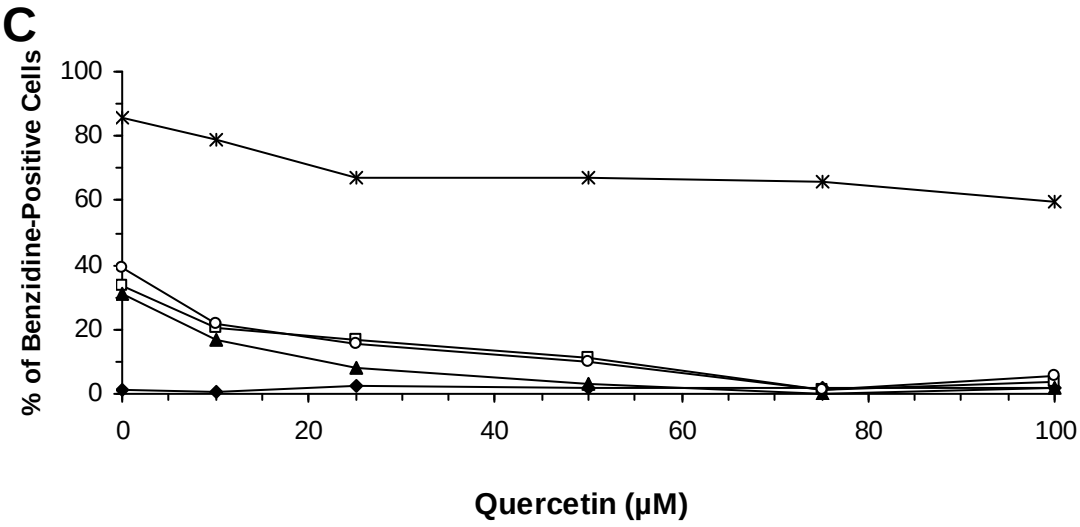

C
% of Benzidine-Positive Cells
100
80
60
40
20
0
0
20
40
60
80
100
Quercetin (µM)

Figure 2

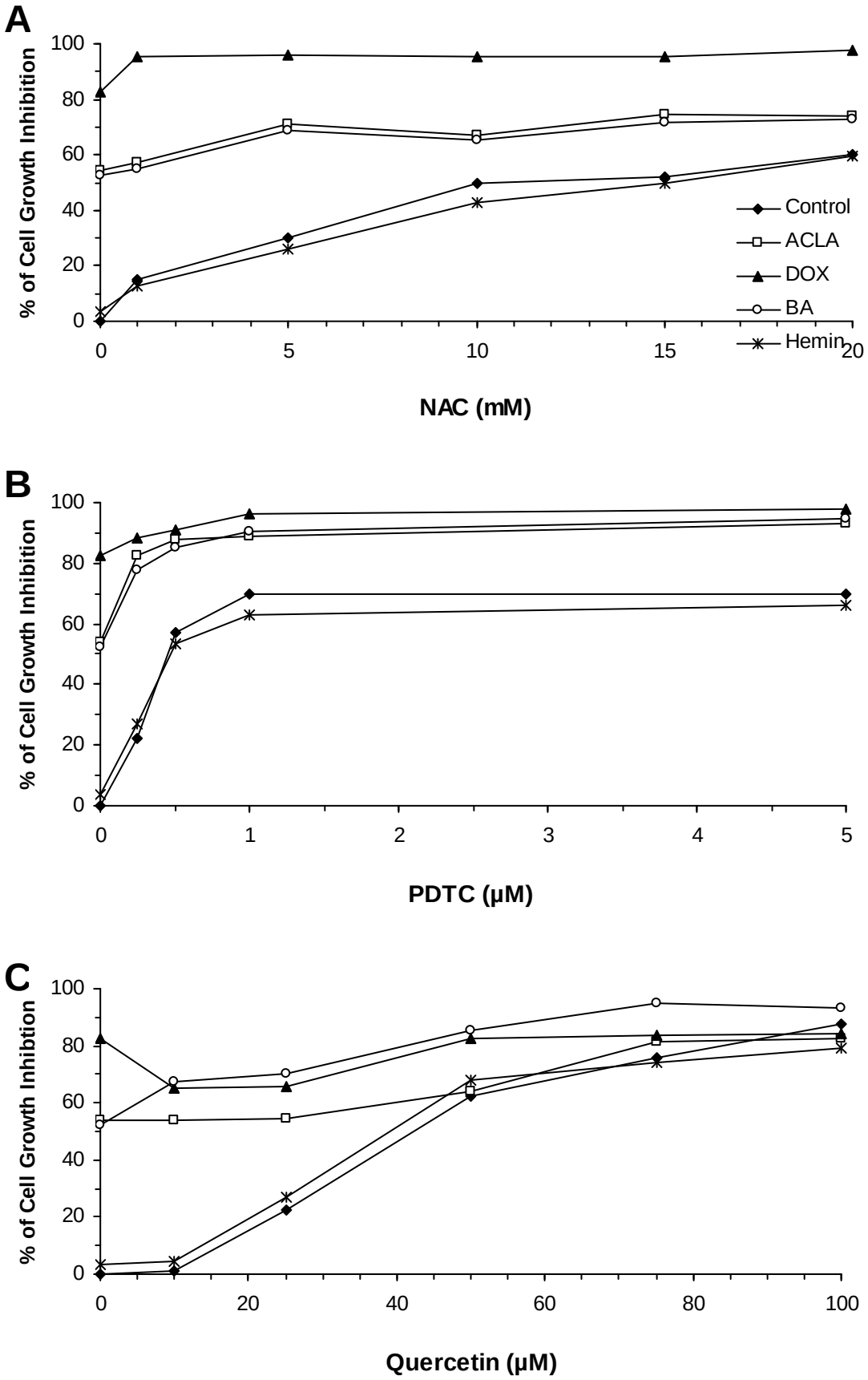

A
% of Cell Growth Inhibition
100
80
60
40
20
0
0
5
10
15
20
NAC (mM)
Control
ACLA
DOX
BA
Hemin
B
% of Cell Growth Inhibition
100
80
60
40
20
0
0
1
2
3
4
5
PDTC (µM)
C
% of Cell Growth Inhibition
100
80
60
40
20
0
0
20
40
60
80
100
Quercetin (µM)

Figure 3

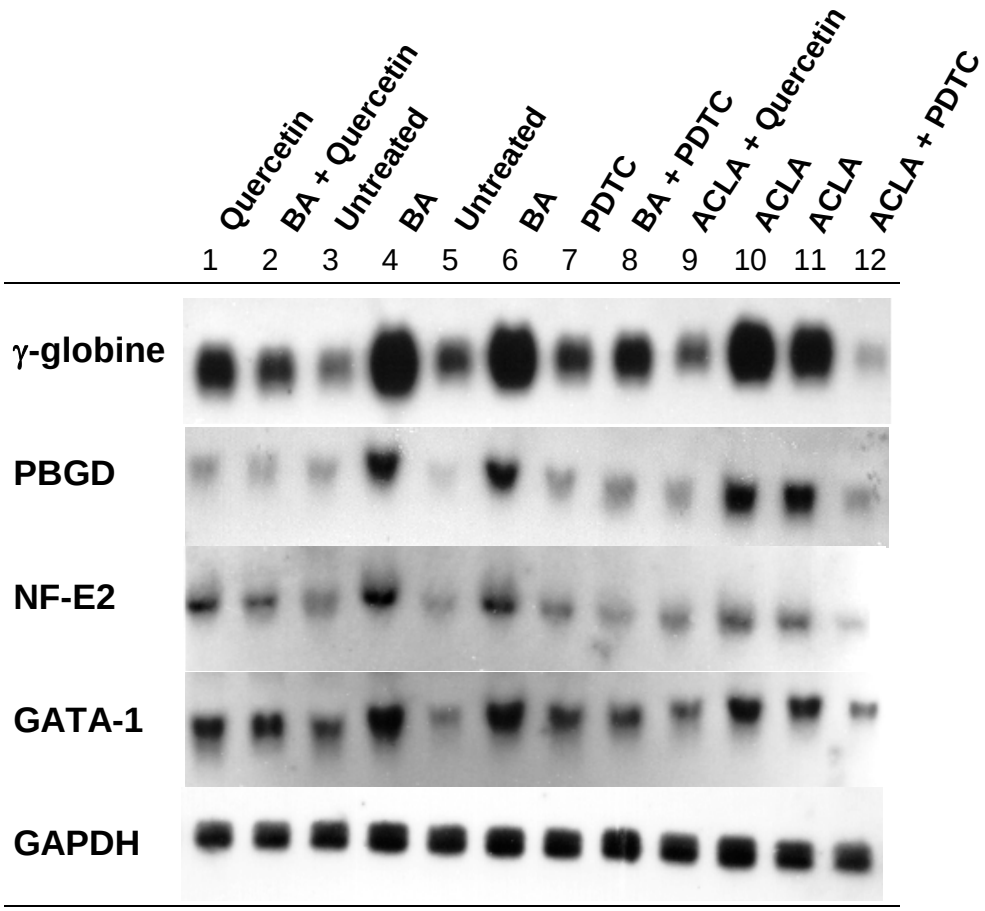

Quercetin
BA + Quercetin
Untreated
BA
Untreated
BA
PDTC
BA + PDTC
ACLA + Quercetin
ACLA
ACLA
ACLA + PDTC
1 2 3 4 5 6 7 8 9 10 11 12
γ-globine
PBGD
NF-E2
GATA-1
GAPDH

Figure 4

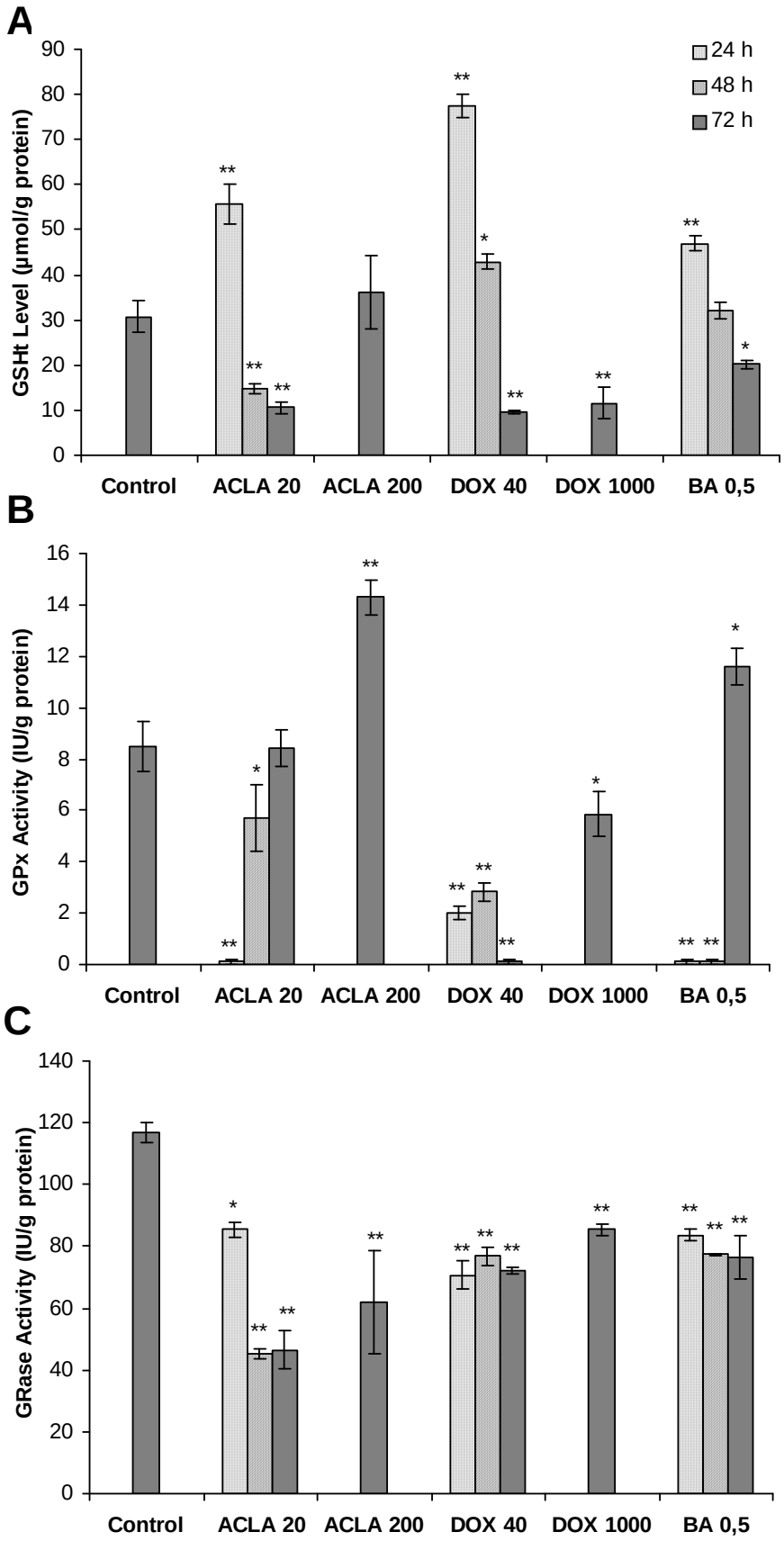

A
GSHt Level (µmol/g protein)
24 h
48 h
72 h
Control
ACLA 20
ACLA 200
DOX 40
DOX 1000
BA 0,5
B
GPx Activity (IU/g protein)
Control
ACLA 20
ACLA 200
DOX 40
DOX 1000
BA 0,5
C
GRase Activity (IU/g protein)
Control
ACLA 20
ACLA 200
DOX 40
DOX 1000
BA 0,5

Figure 5

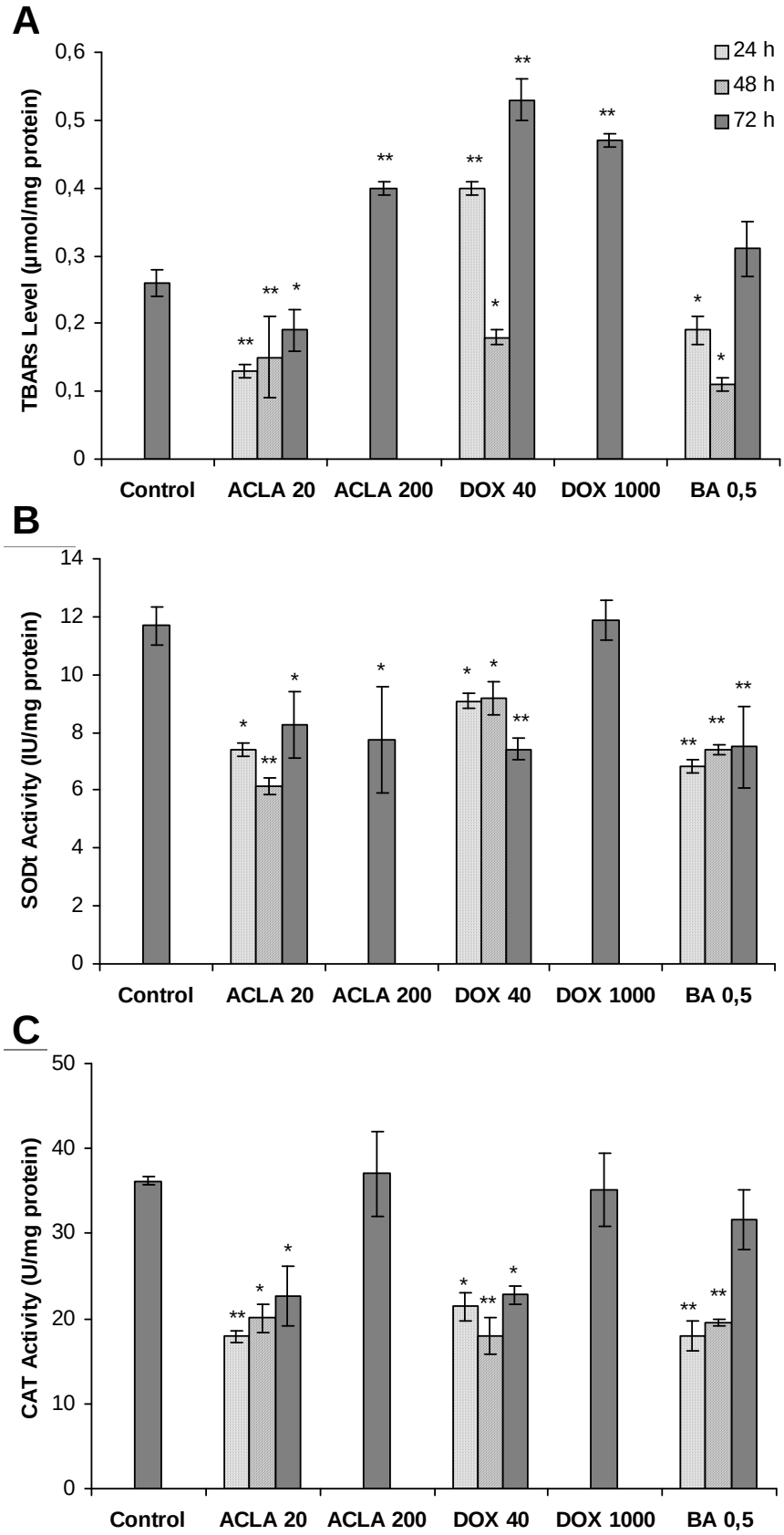
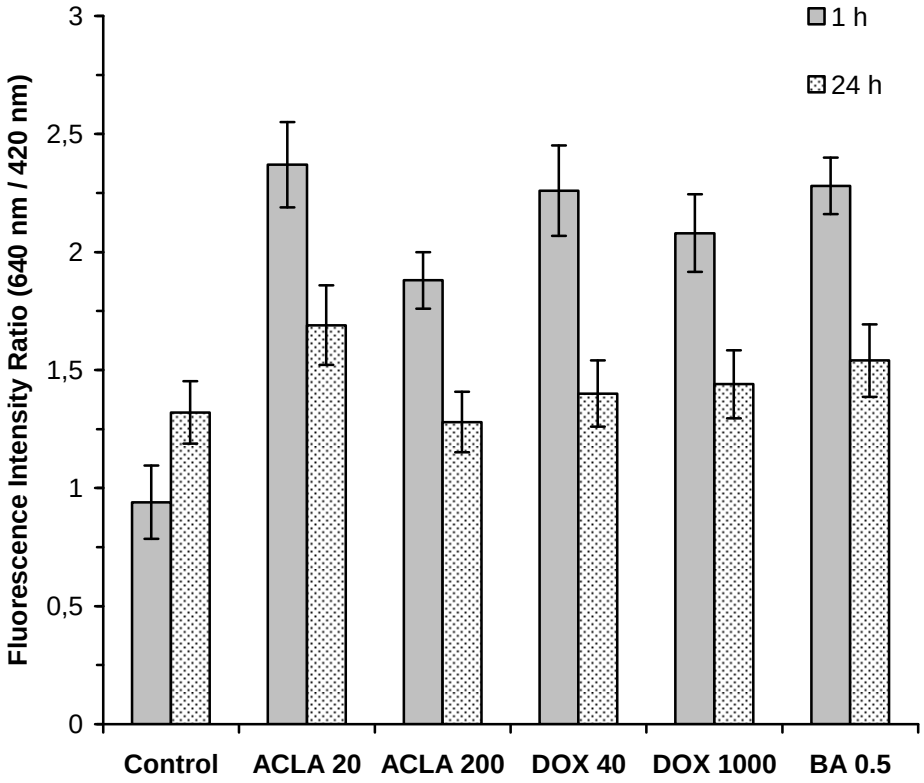

A
TBARs Level (µmol/mg protein)
0,6
0,5
0,4
0,3
0,2
0,1
0
24 h
48 h
72 h
Control
ACLA 20
ACLA 200
DOX 40
DOX 1000
BA 0,5
B
SODt Activity (IU/mg protein)
14
12
10
8
6
4
2
0
Control
ACLA 20
ACLA 200
DOX 40
DOX 1000
BA 0,5
C
CAT Activity (U/mg protein)
50
40
30
20
10
0
Control
ACLA 20
ACLA 200
DOX 40
DOX 1000
BA 0,5

Figure 6


1 h
24 h
Fluorescence Intensity Ratio (640 nm / 420 nm)
3
2,5
2
1,5
1
0,5
0
Control
ACLA 20
ACLA 200
DOX 40
DOX 1000
BA 0.5