



# $\alpha$ -lipoic acid induces apoptosis in human colon cancer cells by increasing mitochondrial respiration with a concomitant $O_2^{\cdot-}$ -generation

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The antioxidant  $\alpha$ -lipoic acid (ALA) has been shown to affect a variety of biological processes associated with oxidative stress including cancer. We determined in HT-29 human colon cancer cells whether ALA is able to affect apoptosis, as an important parameter disregulated in tumour development. Exposure of cells to ALA or its reduced form dihydrolipoic acid (DHLA) for 24 h dose dependently increased caspase-3-like activity and was associated with DNA-fragmentation. DHLA but not ALA was able to scavenge cytosolic  $O_2^{\cdot-}$  in HT-29 cells whereas both compounds increased  $O_2^{\cdot-}$ -generation inside mitochondria. Increased mitochondrial  $O_2^{\cdot-}$ -production was preceded by an increased influx of lactate or pyruvate into mitochondria and resulted in the down-regulation of the anti-apoptotic protein bcl-X<sub>L</sub>. Mitochondrial  $O_2^{\cdot-}$ -generation and apoptosis induced by ALA and DHLA could be prevented by the  $O_2^{\cdot-}$ -scavenger benzoquinone. Moreover, when the lactate/pyruvate transporter was inhibited by 5-nitro-2-(3-phenylpropylamino) benzoate, ALA- and DHLA-induced mitochondrial ROS-production and apoptosis were blocked. In contrast to HT-29 cells, no apoptosis was observed in non-transformed human colonocytes in response to ALA or DHLA addition. In conclusion, our study provides evidence that ALA and DHLA can effectively induce apoptosis in human colon cancer cells by a prooxidant mechanism that is initiated by an increased uptake of oxidizable substrates into mitochondria.

**Keywords:** HT-29 human colon cancer cells; mitochondrial apoptosis pathway; monocarboxylate transporter; superoxide anion generation.

**Abbreviations:** Ac-DEVD-AMC, acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine; ALA, Alpha-lipoic acid; CHAPS, 3-[(cholamidopropyl)-dimethyl-ammonium]-1-propane-sulfonate; CLSM, Confocal laser scanning microscopy; DTT, dithiothreitol; GAP-DH, glyceraldehyd-3-phosphate dehydrogenase; ModEM, modified Eagle medium; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate; PDH, pyruvate-dehydrogenase; proxyl fluorescamine,

5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one, potassium salt; ROS, reactive oxygen species.

## Introduction

Oxidative stress is suggested to play a causative role in different degenerative diseases such as cancer.<sup>1–3</sup> Dietary or endogenous antioxidants do interfere with these processes<sup>3–5</sup> and amongst those alpha-lipoic acid (ALA) has recently gained considerable attention.<sup>6–11</sup> ALA has been reported to have beneficial effects in patients with advanced cancers by increasing the glutathione peroxidase activity and by reducing oxidative stress.<sup>7,8</sup> On a molecular basis ALA and DHLA (dihydrolipoic acid) were shown to alter the expression of the protooncogene c-fos when induced by 12-O-tetradecanoyl-phorbol-13 acetate (TPA)<sup>9</sup> and TPA effects were found to be associated with the production of  $O_2^{\cdot-}$ .<sup>10</sup> Whereas DHLA reduced the expression of c-fos, ALA increased expression<sup>9</sup> and it was suggested that the suppression of c-fos expression is due to the capability of DHLA but not of ALA to scavenge  $O_2^{\cdot-}$ .<sup>11</sup>

Apoptosis is another mechanism in which the presence of reactive oxygen species (ROS) plays an important role and apoptosis is generally impaired in cancer cells.<sup>12</sup> We previously showed in HT-29 colon cancer cells that apoptosis induction by flavone is associated with a high rate of mitochondrial  $O_2^{\cdot-}$  production and that scavenging mitochondrial ROS prevents apoptosis to occur.<sup>13,14</sup> That apoptosis induction by flavone is specific for tumor cells was demonstrated by a lack of flavone effects in non-transformed colonocytes which could be explained by a higher antioxidant status of these cells.<sup>15</sup> Similar findings were obtained with ALA. It was shown to trigger apoptosis in human cancer cell lines while inducing a reversible cell-cycle arrest but failed to induce apoptosis in non-transformed cell lines.<sup>16</sup> Moreover, ALA could potentiate apoptosis in human leukemia cells and based on its antioxidant properties it was suggested that ALA may

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promote a reducing environment that is required for the activation of caspases.<sup>17</sup>

Whereas ALA in leukemia cells predominantly showed proapoptotic effects, in neurons<sup>18</sup> and in hepatocytes<sup>19</sup> ALA appears to protect cells from apoptosis by its antioxidative properties. So, depending on the cellular background, ALA possesses quite different biological activities in tumor cells and non-transformed cells. Since we demonstrated that scavenging of mitochondrial  $O_2^-$  by antioxidants such as ascorbic acid or benzoquinone blocks drug-induced caspase-activation and apoptosis induction in colon cancer cells<sup>20</sup> we investigated whether ALA and its reduced form DHLA can similarly affect apoptosis in HT-29 colon cancer cells. Caspase activation and nuclear fragmentation served as early and late apoptosis markers, respectively. Changes in the level of the apoptosis relevant bcl-X<sub>L</sub> protein were determined by Western-blotting and confocal microscopy was used to determine the role of  $O_2^-$  in the apoptotic response of the cells. To assess whether apoptosis execution by ALA or DHLA is specific for colonic tumor cells, the non-transformed human colonocyte cell line NCOL-1<sup>21</sup> served as a control.

## Materials and methods

### Cell culture

HT-29 cells (passage 106) were provided by American Type Culture Collection and used between passage 150 and 200. HT-29 cells were cultured and passaged in RPMI-1640 supplemented with 10% FCS and 2 mM glutamine. Antibiotics added to the media were 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. NCOL-1 cells (passage 50) were a kind gift of Prof. Clifford W. Deveney and Dr. Michael J. Rutten, School of Medicine, Oregon Health Sciences University, Oregon, USA. Cells were cultured and passaged in DMEM/Hepes/glutamine supplemented with 10% FCS, MEM amino acids, BME vitamin solution and 1 nM human recombinant epidermal growth factor. Antibiotics added to the media were 200 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 12.5  $\mu$ g/ml gentamicin and 1  $\mu$ g/ml fungizone. All cultures were maintained in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Cells were passaged at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA (all materials for cell culture were from Invitrogen, Karlsruhe, Germany).

### Detection of apoptosis

Caspase-3-like activity was measured as described previously,<sup>13</sup> based on the method of Nicholson *et al.*<sup>22</sup> In brief, HT-29 cells were seeded at a density of  $5 \times 10^5$  per well onto 6-well plates (Renner, Dannstadt, Germany)

and allowed to adhere for 24 h. Cells were then exposed for the times indicated to the test compounds. Subsequently, cells were trypsinized, cell numbers were determined and then the cells were centrifuged at 2500 g for 10 min. Cytosolic extracts were prepared by adding 750  $\mu$ l of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10  $\mu$ g/ml pepstatin A, 20  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin and 10 mM HEPES/KOH, pH 7.4, to each pellet and homogenizing by 10 strokes. The homogenate was centrifuged at  $100.000 \times g$  at 4°C for 30 min and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC (Calbiochem, Bad Soden, Germany) at a final concentration of 20  $\mu$ M. Cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using a fluorescence microtiter plate reader (Fluoroskan Ascent, Labsystems).

Nuclear fragmentation as a late marker of apoptosis was determined by staining of DNA with Hoechst 33258 (Sigma, Deisenhofen; Germany). HT-29 cells ( $3 \times 10^4$ ) were grown on glass slides placed into Quadriperm wells (Merck, Darmstadt, Germany) and then incubated with the test compounds for 24 h. Thereafter, cells were washed with PBS, allowed to air-dry for 30 min and then fixed with 2% paraformaldehyd prior to staining with 1  $\mu$ g/ml Hoechst 33258 and visualization under an inverted fluorescence microscope (Leica DMIRBE). Photographs were made from at least three independent cell batches and apoptotic cells were determined according to the number of cells displaying chromatin condensation and nuclear fragmentation versus total cell counts.

### $O_2^-$ -detection

For detection of  $O_2^-$  inside HT-29 colonocytes the cells were loaded with 50  $\mu$ M proxylfluorescamine (Bioprobes, Leiden, The Netherlands) for 2 h. 200  $\mu$ M cysteine were added to the incubation media to yield an increase in the emission of proxylfluorescamine fluorescence due to the reduction of the fluorophore nitroxide to its corresponding hydroxylamine in the presence of superoxide.<sup>23</sup> Subsequently to the loading with fluorophore cells were washed free of medium with a modified Krebs-buffer, containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.3 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM glucose, 200  $\mu$ M cysteine and 10 mM Hepes/Tris, pH 7.4.  $O_2^-$ -generation inside HT-29 cells was achieved by the addition of 50  $\mu$ M camptothecin. The amount of  $O_2^-$  in HT-29 cells was followed over 3 h by measuring the fluorescence of proxylfluorescamine at 460 nm after excitation at 390 nm using the fluorescence microtiter plate reader.

$O_2^-$  in mitochondria of HT-29 cells were visualized by confocal laser scanning microscopy (CLSM) using a TCS

SP2 microscope (Leica). For staining of mitochondria, cells were grown on glass slides placed into Quadriperm wells and loaded with 500 nM MitoTracker Red CMXRos (Bio-probes) for the last 30 min of incubation. For detection of  $O_2^-$ , cells were loaded with 50  $\mu$ M proxylfluorescamine for the last 2 h of incubation.  $O_2^-$  were detected after excitation with the UV-laser at emissions of 440–480 nm and mitochondria were detected after excitation at 543 nm at emissions of 590–650 nm, respectively. The fluorescence ratios of proxylfluorescamine over MitoTracker were determined for the mitochondrial areas only using the Leica Confocal Software, Version 2.5.

### Uptake of fluorescein into mitochondria

CLSM was used to measure uptake of fluorescein into mitochondria of HT-29 cells. Therefore, cells were grown on glass slides placed into Quadriperm wells prior to staining of mitochondria for 40 min with 500 nM MitoTracker Red CMXRos. Subsequently, cells were lysed with 20  $\mu$ g/ml saponine and then incubated for 5 min in the modified Krebs buffer containing 50  $\mu$ M fluorescein. Cells were fixed by 50% acetone/50% methanol for 10 min on ice and viewed under the confocal laser scanning microscope with excitations at 488 and 543 nm and emissions at 500–530 nm and 590–650 for the detection of fluorescein and mitochondria, respectively. Uptake of fluorescein into mitochondria was assessed based on the fluorescence ratio of fluorescein over MitoTracker at the area of mitochondria only using the Leica Confocal Software.

### Determination of lactate levels

Lactate levels in HT-29 cells were determined using an UV-test (Boehringer, Mannheim, Germany). Absorbance of NADH was measured using a multiwell-plate reader (Multiskan Ascent, Labsystems). For preparation of cytosolic extracts cells were trypsinized, cell numbers were determined and then the cells were centrifuged at 2500 g for 10 min. Cytosolic extracts were prepared by adding 750  $\mu$ l of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10  $\mu$ g/ml pepstatin A, 20  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin and 10 mM HEPES/KOH, pH 7.4, to each pellet and homogenizing by 10 strokes. The homogenate was centrifuged at  $100.000 \times g$  at 4°C for 30 min and the supernatant was used for lactate analysis.

### Immunoblotting

HT-29 cells were incubated in 6-well cell culture plates (Renner) with or without effectors for 24 h and scraped off in Laemmli equilibration buffer containing 50 mM

Tris, 100 mM DTT, 10% glycerin, 2% SDS and 0.1% bromphenol blue. Samples were centrifuged at 2500 g for 5 min and protein content in the supernatant was determined by the Bradford reaction (Bio-Rad, Munich, Germany). The samples were resolved by SDS-PAGE according to the method described by Schagger and von Jagow<sup>24</sup> and were electroblotted onto PVDF membranes (Roth, Karlsruhe, Germany). Control of protein transfer and identification of the molecular weight marker proteins was achieved by Ponceau Red staining. Thereafter, the blotting membranes were blocked for 1 h with TBST and then incubated with the primary antibody (anti-bcl-X<sub>L</sub>, sc-7195; anti-actin, sc-1615; Santa Cruz, Heidelberg, Germany) for 1 h in a 1:1000 dilution in TBST. Bound antibodies were detected after 1 h incubation with HRP-conjugated secondary reagents (sc-2020 for anti-actin and sc-2004 for bcl-X<sub>L</sub>; Santa Cruz) using the ECL western blotting detection reagents (Amersham, Freiburg, Germany) according to the manufacturer's instructions.

### Calculations and statistics

Variance analysis between groups was performed by One-way ANOVA and significance of differences between control and treated cells were determined by a Bonferroni's multiple comparison test (GraphPadPrism). For each variable at least 3 independent experiments were carried out. Data are given as the mean  $\pm$  SEM.

## Results

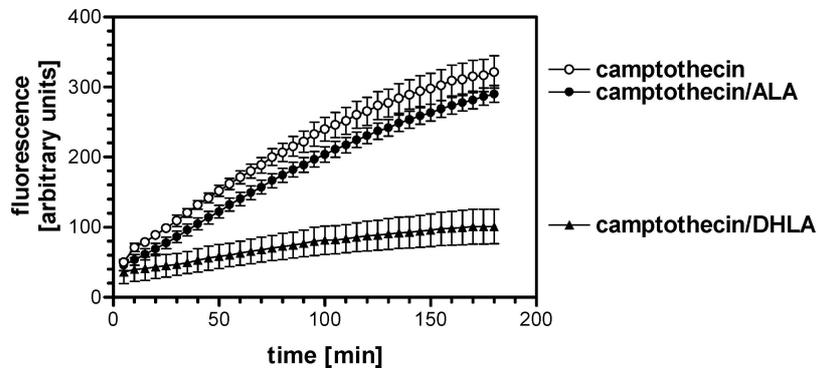
### DHLA but not ALA scavenges cytosolic $O_2^-$ in HT-29 cells

ALA and DHLA differ in their  $O_2^-$ -scavenging properties as shown in cell free systems.<sup>6,11</sup> For assessing their effects on  $O_2^-$ -levels in living cells, we loaded HT-29 cells with the  $O_2^-$ -sensitive dye proxylfluorescamine and generated  $O_2^-$  by the use of camptothecin (Figure 1). In cells exposed to camptothecin, fluorescence intensity increased over time and this increase was essentially blocked by the concomitant application of DHLA but not by ALA (Figure 1).

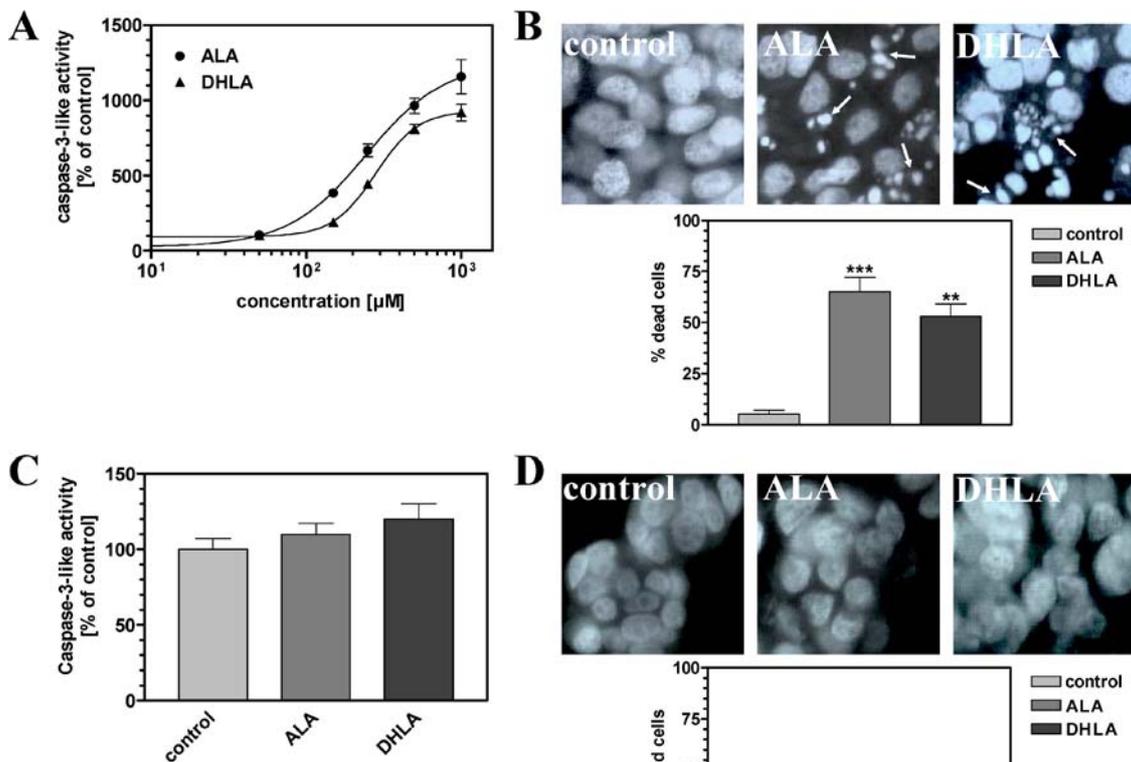
### ALA and DHLA induce apoptosis in HT-29 cells but not in NCOL-1 cells

Caspase-3-like activity reached maximal levels after 24 h of exposure of HT-29 cells to both, ALA and DHLA, and the caspase-3 activity was dose-dependently increased by both compounds (Figure 2A). Whereas ALA caused a maximal stimulation of caspase-3-like activity of 12-fold over controls, the DHLA-mediated increase was only

**Figure 1.**  $\alpha$ -dihydrolipoic acid (DHLA) but not  $\alpha$ -lipoic acid (ALA) scavenge  $O_2^{\cdot-}$  inside HT-29 cells. Cells were loaded with the  $O_2^{\cdot-}$ -sensitive fluorochrome proxylfluorescamine as described in the Methods section and then incubated with 50  $\mu$ M camptothecin either alone or in the presence of either 1 mM DHLA or 1 mM ALA. Fluorescence of proxylfluorescamine was followed over 3 h using the microtiter plate reader.



**Figure 2.** Effects of  $\alpha$ -lipoic acid (ALA) and  $\alpha$ -dihydrolipoic acid (DHLA) on apoptosis in HT-29 cells. (A) Caspase-3-like activity was assessed in HT-29 cells incubated for 24 h in the absence or the presence of increasing concentrations of ALA and DHLA by determining the cleavage rate of Ac-DEVD-AMC. Caspase-3-like activity of cells treated with medium alone was set as 100%. (B) Effects of ALA and DHLA on nuclear fragmentation (arrows) assessed after 24 h by Hoechst 33258 staining in HT-29 cells treated with medium alone (control) or with 1 mM ALA, or with 1 mM DHLA using an inverted fluorescence microscope. The percentage of cells displaying signs of chromatin condensation and DNA-fragmentation is given in the lower panel \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  versus the control. (C) Caspase-3-like activity in NCOL-1 cells as determined at 24 h of incubation with medium alone (control), 1 mM ALA or 1 mM DHLA. (D) No nuclear fragmentation was visible in NCOL-1 cells incubated for 24 h with medium alone (control), 1 mM ALA or 1 mM DHLA.



9-fold. The corresponding EC<sub>50</sub>-values for this activation were around 250 μM for both compounds (Figure 2A). The further execution of apoptosis beyond activation of caspase-3 was demonstrated by a pronounced chromatin condensation and nuclear fragmentation and final cell death in 50–70% of the cells exposed to ALA or DHLA (Figure 2B). Although NCOL-1 cells, representing non-transformed human colonocytes, were previously shown to be very sensitive to classical apoptosis-inducing agents such as camptothecin,<sup>15</sup> they did not respond with an increase in caspase-3-like activity (Figure 2C) or with nuclear fragmentation (Figure 2D) when exposed to ALA or DHLA.

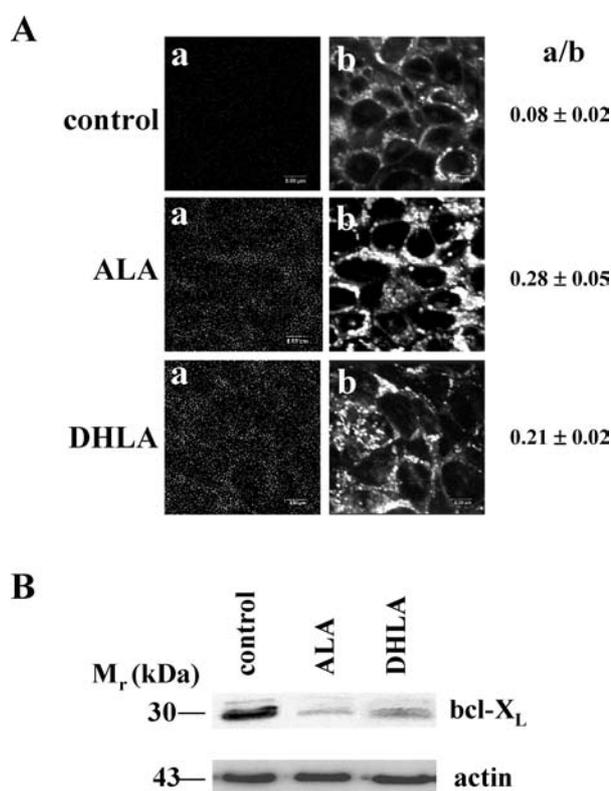
### ALA and DHLA increase mitochondrial O<sub>2</sub><sup>-</sup> production with a concomitant down-regulation of bcl-X<sub>L</sub>

Although ALA and DHLA clearly differed in their ability to scavenge cytosolic O<sub>2</sub><sup>-</sup> in HT-29 cells (see Figure 1), both led to a markedly increased mitochondrial O<sub>2</sub><sup>-</sup>-production (Figure 3A) and a concomitant down-regulation of the anti-apoptotic protein bcl-X<sub>L</sub> (Figure 3B). The prime role of mitochondrial O<sub>2</sub><sup>-</sup>-generation for the pro-apoptotic effects of ALA and DHLA was confirmed by the use of the O<sub>2</sub><sup>-</sup>-scavenger benzoquinone, that blocked the generation of O<sub>2</sub><sup>-</sup> (Figure 4A), the down-regulation of bcl-X<sub>L</sub> (Figure 4B) and finally also apoptosis execution (Figures 4C and D) in HT-29 cells exposed to 1 mM ALA or 1 mM DHLA, respectively.

### ALA and DHLA increase mitochondrial uptake of lactate or pyruvate

We previously observed that increased O<sub>2</sub><sup>-</sup>-generation in mitochondria of HT-29 cells is associated with an enhanced influx of lactate and/or pyruvate into mitochondria mediated by a mitochondrial monocarboxylate transporter.<sup>25</sup> We therefore tested whether ALA and DHLA are also capable to enhance mitochondrial monocarboxylate influx that in turn could increase mitochondrial respiration and ROS-production. Fluorescein can serve as a fluorescent probe to visualize monocarboxylate uptake into mitochondria.<sup>25</sup> As shown in Figure 5A, cells exposed to ALA and DHLA showed an increased uptake of fluorescein into mitochondria exceeding this in control cells by 5–6-fold. ALA-induced increased fluorescein uptake was blocked by the addition of either lactate or by the monocarboxylate transport inhibitor 5-nitro-2-(3-phenylpropyl-amino)benzoate (NPPB) (Figure 5A). Similarly, DHLA-stimulated fluorescein uptake into mitochondria was inhibited by lactate and NPPB (data not shown). Both, ALA and DHLA caused a time-dependent

**Figure 3.** ALA and DHLA increase the generation of O<sub>2</sub><sup>-</sup> in mitochondria and reduce the protein levels of bcl-X<sub>L</sub>. (A) HT-29 cells were exposed to medium alone (control), or to 1 mM ALA or 1 mM DHLA for 6 h. Cells were loaded with prooxylfluorescamine for the detection of O<sub>2</sub><sup>-</sup>: (a) in combination with MitoTracker for the visualisation of mitochondria (b). The fluorescence ratios of a over b were determined for the mitochondrial areas only. (B) Cells were incubated for 24 h as described under (A) and subsequently bcl-X<sub>L</sub> and actin (used as a control) were detected in the homogenate by immunostaining after blotting.

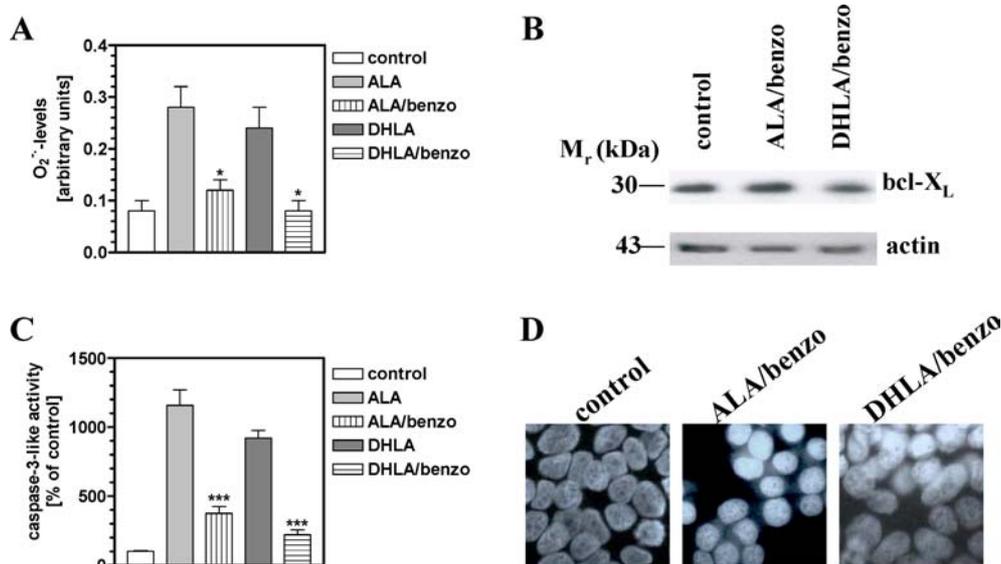


reduction in cytosolic lactate levels (Figure 5B) suggesting that both compounds foster the uptake of monocarboxylates such as lactate or pyruvate into mitochondria of HT-29 cells. NPPB not only prevented the increased flux of substrates into mitochondria but also reduced the generation of mitochondrial O<sub>2</sub><sup>-</sup> (Figure 6A), inhibited caspase-3 activation (Figure 6B) and prevented fragmentation of DNA (Figure 6C) in the presence of ALA or DHLA.

## Discussion

*α*-lipoic acid (ALA) and its reduced form *α*-dihydrolipoic acid (DHLA) are known to react with ROS<sup>6</sup> and have been shown to be beneficial in a number of oxidative stress models, such as ischemia-reperfusion injury,<sup>26,27</sup> diabetes<sup>28,29</sup> or neurodegeneration.<sup>30,31</sup> In cancer cells ROS also play a crucial role in cell growth and apoptosis. However, the

**Figure 4.** The  $O_2^{\cdot-}$ -scavenger benzoquinone inhibits ALA- and DHLA-induced apoptosis in HT-29 cells. Cells were incubated with medium alone (control) or with 1 mM ALA or 1 mM DHLA in the absence or the presence of 10  $\mu$ M benzoquinone (benzo). (A) Levels of  $O_2^{\cdot-}$  were determined after 6 h of incubation based on the fluorescence ratios of the  $O_2^{\cdot-}$ -sensitive prooxylfluorescamine over fluorescence of MitoTracker. The fluorescence ratios were determined for the mitochondrial areas only. \*  $P < 0.05$  versus cells treated with ALA or DHLA alone. (B) Determination of bcl-X<sub>L</sub> and actin levels by Western blotting of proteins isolated from cells exposed to compounds for 24 h. (C) Caspase-3-like activity in cells determined after 24 h of incubation with the test compounds. \*\*\*  $P < 0.001$  versus cells treated with ALA or DHLA alone. (D) Nuclear fragmentation determined by staining of fixed cells with Hoechst 33258. Percentages of apoptotic cells did not differ significantly between the control and ALA or DHLA treated cells, respectively. Benzoquinone alone did not affect any of the parameters measured (not shown).

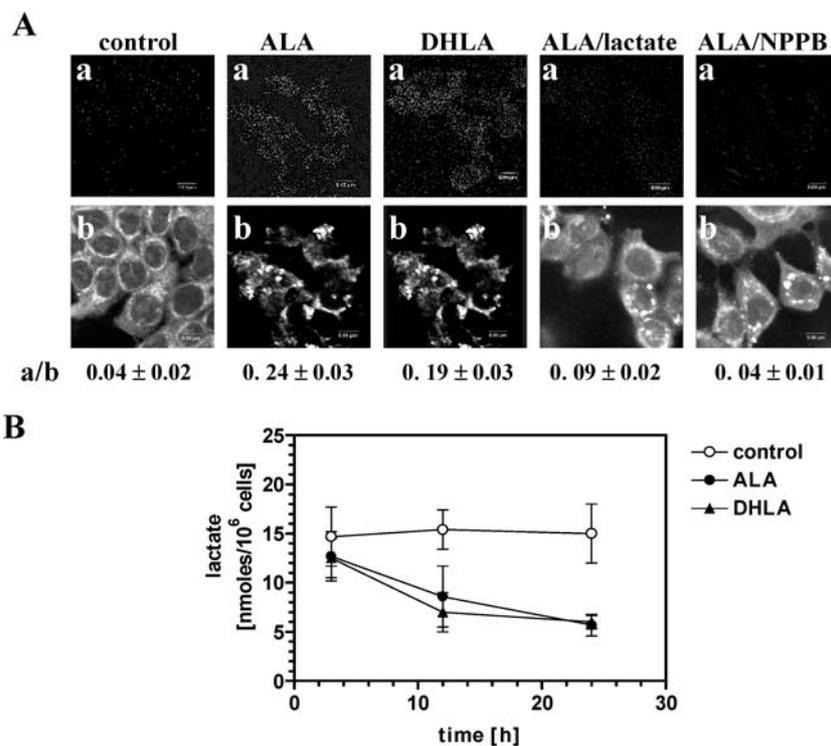


role of ROS in cancer development and progression might be that of a double-edged sword. ROS can initiate cell transformation by causing base alterations leading to mutations during DNA replication whereas in already transformed cells ROS play an important role in the initiation and execution of apoptosis.<sup>32-34</sup> In this context, the up-regulation of a variety of anti-apoptotic proteins that act as cellular antioxidants, such as certain members of the bcl-2 family, allows cancer cells to escape apoptosis.<sup>35-37</sup> The balance of ROS and antioxidant levels therefore critically determines apoptosis in cancer cells and overcoming the antioxidative defense systems by accelerating ROS production could promote apoptosis.

Apoptosis induction in the human colorectal cancer cell line HT-29 is strictly related to the generation of  $O_2^{\cdot-}$  in mitochondria that is followed by a down-regulation of the mitochondrial anti-apoptotic protein bcl-X<sub>L</sub> and by caspase-3 activation.<sup>14</sup> Scavengers of  $O_2^{\cdot-}$ , such as NO, ascorbic acid or benzoquinone, were able to block drug-induced down-regulation of bcl-X<sub>L</sub> and consequently prevented apoptosis to occur.<sup>14,20</sup> Here we determined whether and by which mechanisms the antioxidants ALA and DHLA do affect apoptosis in HT-29 cells. As DHLA but not ALA was reported to efficiently scavenge  $O_2^{\cdot-}$  in cell-free systems,<sup>6,11</sup> we expected both compounds to have different effects on apoptosis in our cell model.

Interestingly, ALA as well as DHLA could induce apoptosis in HT-29 cells almost to the same degree, despite their differing  $O_2^{\cdot-}$ -scavenging activities inside HT-29 cells. Whereas cytosolic  $O_2^{\cdot-}$ -generation induced by camptothecin was markedly reduced in the presence of DHLA, ALA failed to prevent ROS production. However, when  $O_2^{\cdot-}$ -generation in mitochondria was determined, ALA but also DHLA increased the production of this ROS. This indicates that either the generation of  $O_2^{\cdot-}$  in mitochondria induced by DHLA is higher than is  $O_2^{\cdot-}$ -scavenging activity or that DHLA does not reach the compartment of mitochondrial  $O_2^{\cdot-}$ -production. The margin between these apparent opposing effects of ROS-production and ROS-scavenging seems to be above 100  $\mu$ M since at lower concentrations of DHLA no apoptosis-induction was observed. That mitochondrial  $O_2^{\cdot-}$  liberation and the subsequent down-regulation of bcl-X<sub>L</sub> is crucial for apoptosis induction and execution by DHLA and ALA was shown by use of benzoquinone, an  $O_2^{\cdot-}$ -scavenger, that blocked all DHLA- or ALA-mediated effects. The most plausible explanation for the enhanced mitochondrial  $O_2^{\cdot-}$ -production in the presence of ALA or DHLA is that the flow of reduction equivalents through the respiratory chain is increased. We previously demonstrated that the rate of apoptosis in HT-29 cells is closely linked to an accelerated

**Figure 5.** ALA and DHLA increase fluorescein/lactate uptake into mitochondria. (A) Uptake of fluorescein served as a marker for monocarboxylate carrier mediated transport activity and was studied by CLSM. Mitochondria of HT-29 cells were stained using MitoTracker as described in the Methods section and thereafter cells were lysed with 20 μg/ml saponine and incubated for 5 min with medium alone containing 50 μM fluorescein (control) or containing in addition 1 mM ALA, 1 mM DHLA or combinations of either 1 mM ALA and 10 mM lactate or 1 mM ALA and 250 μM NPPB. Mitochondrial uptake of fluorescein was assessed based on the ratio of fluorescein to MitoTracker Red CMXRos fluorescence presented as a over b only in mitochondria. (B) Lactate levels in the cytosol of HT-29 cells were determined enzymatically after 3, 12 and 24 h of incubation of cells with medium alone (control) or with 1 mM ALA or 1 mM DHLA as described in the Methods section.

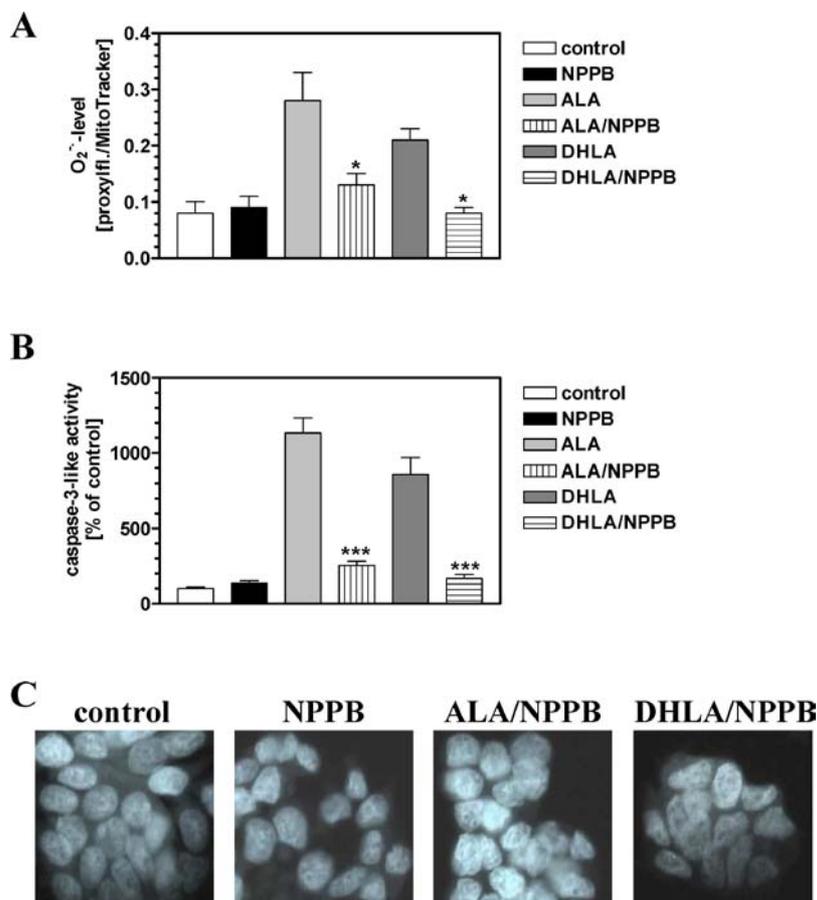


mitochondrial O<sub>2</sub><sup>-</sup>-generation when lactate or pyruvate uptake into mitochondria and therefore substrate availability for oxidative metabolism is increased.<sup>25</sup> A low rate of oxidation of pyruvate is characteristic for almost all cancer cells<sup>38</sup> and consequently lactate is the prime metabolic end product of glucose-dependent energy metabolism.<sup>39</sup> We here show that ALA and DHLA treatment of HT-29 cells increases substantially the activity of the mitochondrial monocarboxylate transporter as assessed by accumulation of the reporter molecule fluorescein and that this uptake pathway is shared by lactate. Using 5-nitro-2-(3-phenylpropylamino) benzoate, a potent inhibitor of monocarboxylate transporters,<sup>40</sup> blocked the mitochondrial uptake of lactate/pyruvate in the presence of ALA and DHLA but also prevented the increased production of mitochondrial O<sub>2</sub><sup>-</sup> and blocked apoptosis.

Although all findings suggest this to represent the most plausible mechanisms for apoptosis induction by ALA and DHLA, the compounds might also affect mitochondrial pyruvate-dehydrogenase (PDH) activities and thereby alter metabolism. A progressive decrease in PDH activity has been shown to occur during transformation from liver

cells to highly dedifferentiated hepatoma cells,<sup>41</sup> whereas higher PDH activities have been reported in tumorigenic than in non-tumorigenic cells.<sup>42</sup> Since DHLA is an essential cofactor for PDH activity, it could affect pyruvate oxidation by increasing the flux through the PDH complex.<sup>43</sup> The regeneration of DHLA from ALA however could in the presence of sufficient redox equivalents also allow ALA to promote an increased flow of pyruvate through the PDH pathway. In any case, a higher substrate availability by increased influx of pyruvate or lactate into mitochondria would be required for PDH-dependent shuttling of acetyl-CoA into citric acid cycle and for promoting oxidative metabolism and this appears to take place when colon cancer cells are exposed to ALA or DHLA. Human non-transformed colonocytes, in contrast to HT-29 cells, did not respond with apoptosis to the exposure to ALA or DHLA. This might be due either to the higher antioxidative capacity of NCOL-1 cells<sup>15</sup> or due to the lack of a blockade of mitochondrial pyruvate oxidation that seems to be a characteristic of transformed cells which minimizes oxidative stress during intense DNA-replication and phases of a high biosynthetic activity.<sup>44</sup>

**Figure 6.** The monocarboxylate transport inhibitor NPPB blocks ALA and DHLA induced generation of  $O_2^-$ , caspase-3 activation and DNA-fragmentation. (A) Generation of  $O_2^-$  was assessed after 6 h of incubation of HT-29 cells with medium alone (control), with 250  $\mu$ M NPPB, 1 mM ALA, 1 mM DHLA or combinations of ALA or DHLA with NPPB. The fluorescence ratios of  $O_2^-$ -sensitive proxylfluorescamine to fluorescence of MitoTracker was determined over mitochondrial areas. \*  $P < 0.05$  versus cells treated with ALA or DHLA alone. (B) Caspase-3-like activity was determined after 24 h of incubation with the compounds indicated in (A) according to the cleavage of the fluorogenic caspase substrate Ac-DEVD-AMC. \*\*\*  $P < 0.001$  versus cells treated with ALA or DHLA alone. (C) DNA-fragmentation and chromatin condensation was determined after 24 h of exposure of cells to the test compounds by staining of fixed HT-29 cells with Hoechst 33258.



## Conclusion

In conclusion, our studies demonstrate that the antioxidants ALA and DHLA do effectively induce apoptosis in HT-29 human colon cancer cells via an increased ROS production in mitochondria. Although at least DHLA acts as a potent  $O_2^-$ -scavenger in the cytosol, both compounds increase substantially mitochondrial  $O_2^-$ -production that can obviously not be quenched or detoxified. The underlying mechanism seems to be an enhancement of the uptake of monocarboxylates (pyruvate/lactate) from glycolysis into mitochondria followed by their oxidation in the citric acid cycle with increased delivery of reduction equivalents to the respiratory chain which in turn drastically increases mitochondrial  $O_2^-$ -production. This high  $O_2^-$ -burden appears to overcome the intrinsically high an-

tioxidative capacity of antiapoptotic proteins and allows apoptosis in tumor cells to be executed.

## Acknowledgments

The authors greatly acknowledge the expert technical assistance of Mrs. Margot Siebler and Mrs. Beate Rauscher.

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