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No evident dose-response relationship between cellular ROS level and its cytotoxicity – a paradoxical issue in ROS-based cancer therapy

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Targeting cancer via ROS-based mechanism has been proposed as a radical therapeutic approach. Cancer cells exhibit higher endogenous oxidative stress than normal cells and pharmacological ROS insults via either enhancing ROS production or inhibiting ROS-scavenging activity can selectively kill cancer cells. In this study, we randomly chose 4 cancer cell lines and primary colon or rectal cancer cells from 4 patients to test the hypothesis and obtained following paradoxical results: while piperlongumin (PL) and β -phenylethyl isothiocyanate (PEITC), 2 well-defined ROS-based anticancer agents, induced an increase of cellular ROS and killed effectively the tested cells, lactic acidosis (LA), a common tumor environmental factor that plays multifaceted roles in promoting cancer progression, induced a much higher ROS level in the tested cancer cells than PL and PEITC, but spared them; L-buthionine sulfoximine (L-BSO, 20 μ M) depleted cellular GSH more effectively and increased higher ROS level than PL or PEITC but permitted progressive growth of the tested cancer cells. No evident dose-response relationship between cellular ROS level and cytotoxicity was observed. If ROS is the effector, it should obey the fundamental therapeutic principle – the dose-response relationship. This is a major concern.

Persistent high ROS level is a common biochemical feature of cancer cells^{1–3}. Cancer cells rely on moderate increase of cellular ROS, which plays multiple vital roles in cancer cell survival and proliferation, tumor angiogenesis and growth, cancer invasion and metastasis, and even resistance to therapy^{1,4–8}. On the other hand, the higher level ROS in cancer cells than that in normal cells may render the former more vulnerable to further ROS insults. The pharmacological approach on this basis was proposed a decade ago^{9,10}. The biological basis underlying this approach is straightforward: the higher endogenous ROS level in cancer cells than that in normal cells is the basis for therapeutic selectivity, pharmacologically active prooxidants, via either promoting ROS production or inhibiting ROS-scavenging systems, can increase ROS to lethal level preferentially in cancer cells, leading to irreversible oxidative damage and cell death. *Trachootham et al.*¹ outlined the biological basis of the therapeutic approach, the current status of the research in this field, and future perspectives. Piperlongumine (PL)^{11–13} and β -phenylethyl isothiocyanate (PEITC)^{14–18} are typical ROS-based anticancer agents with great potential in clinical application. So far, numerous agents targeting tumor ROS modulation have entered clinical trials^{1,19}.

However, after reviewing the previous publications in the field, we found that the fundamental basis of the therapy – the causative role of ROS in cancer cell death – is unclear, because it is not known if pharmacologically-induced ROS level is truly excessive or fatal to cancer cells. The evidence we could find in the previous publications to support this claim are: anticancer prooxidants can induce an increase of ROS concurrently with a cell death and antioxidants such as N-acetylcysteine (NAC) can antagonize prooxidant-induced ROS and attenuate cell death. The evidence is important but not enough, because one can argue based on the following questions: is there any agent with potency to augment ROS stronger than anticancer prooxidants but permit cell survival? Is it possible that antioxidants can antagonize prooxidant-induced ROS but cannot block cell death or antioxidants can block prooxidant-induced cell death but cannot antagonize ROS? More importantly, if ROS is the effector that kills or inhibit cancer cells, it should obey the therapeutic principle – dose-response relationship. Therefore, the antic-

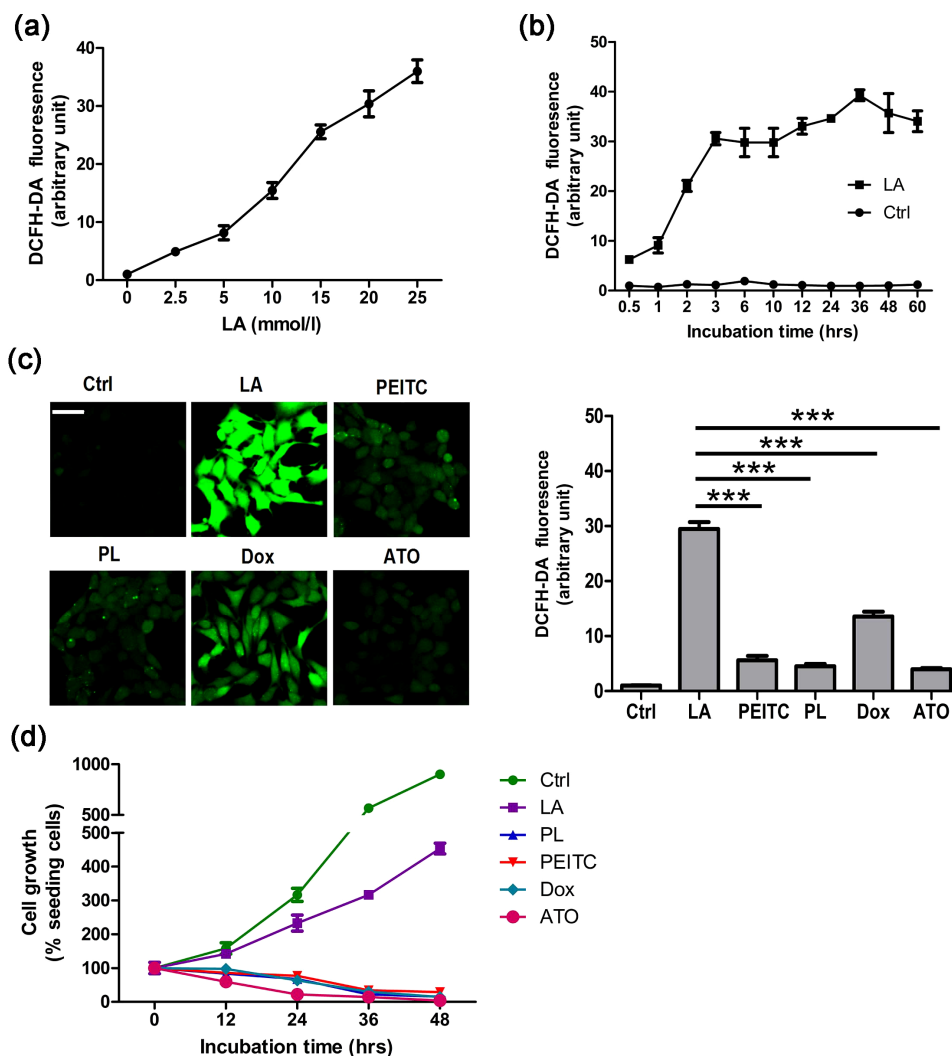


Figure 1 | LA is a more potent ROS inducer than PEITC, PL, Dox, or ATO but does not kill 4T1 cells. (a) 4T1 cells were incubated with LA (2.5–25 mM) for 4 hours, and cellular ROS was probed by DCFH-DA. (b) 4T1 cells were incubated with LA (20 mM), and at the indicated time points, cellular ROS was probed by DCFH-DA. (c) Comparison of cellular ROS induced by LA, PEITC, PL, Dox, and ATO. Cells were treated with LA (20 mM), PEITC (10 μ M), PL (10 μ M), Dox (10 μ g/ml), or ATO (10 μ M) for 4 hours, and intracellular ROS was probed by DCFH-DA. (d) 4T1 cells were incubated with LA (20 mM), PEITC (10 μ M), PL (10 μ M), Dox (10 μ g/ml) or ATO (10 μ M), at the indicated time points, cell counts were performed. Experiments are repeated at least 3 times. Scale bar = 50 μ m. *** $p < 0.001$.

ancer efficacy of pharmacologically active prooxidants should correlate with their potency to disable GSH antioxidant system and/or to augment ROS, and cell death rate should be proportional to cellular ROS level when it reaches toxic level or inversely proportional to cellular GSH level. These lines of evidence are essential but lacking.

We would address these issues as described below.

Results and discussion

Lactic acidosis induces a much higher cellular ROS level than PEITC or PL but permits a progressive growth of the tested cancer cells. 4T1 cancer cells were treated with lactic acidosis (LA), PEITC, PL, doxorubicin (Dox), or arsenic trioxide (ATO). PEITC and PL increases ROS by depleting GSH^{11,14}, Dox enhances ROS production via quinone one-electron redox cycling²⁰, and ATO promotes ROS production via inhibiting mitochondrial respiratory chain²¹. LA, a common environmental factor of diverse cancers²², has been shown to induce a dramatic increase of ROS in endothelial cells²³.

LA dramatically increased cellular ROS level and maintained it in 4T1 cancer cells in a time course of 60 hours (Fig. 1a & b). The LA condition used in this study was within the physiological range of pH

and lactate levels in solid tumors²². LA increased ROS by ~30 folds, much higher than those induced by PEITC, PL, Dox, and ATO (Fig. 1c). Paradoxically, LA only slowed down the proliferation rate of 4T1 cancer cells (Fig. 1d, note that cells still kept a progressive growth), while the others killed the cells (Fig. 1d). Similar results were obtained by using Bcap37, Hela, and HepG2 cells (Supplementary Fig. S1–3). LA had no harmful effect on 4T1 cells and other cancer cells even in the long-term culture, instead, it conferred cancer cells (4T1, Bcap37, RKO, SGC7901) with resistance to metabolic stress, such as glucose deprivation²⁴.

L-BSO induces a severer oxidative stress than PEITC or PL but permits a progressive growth of the tested cancer cells. L-BSO is a classical γ -glutamylcysteine synthetase inhibitor²⁵ and it is probably the most widely used agent for depleting cellular GSH²⁶, the most important antioxidant in cells. L-BSO is also a ROS-based anticancer agent¹. We used concentration and time dependent assays to define a concentration of L-BSO (20 μ M) which depleted ~95% GSH (Fig. 2 a & b).

Then, we compared L-BSO (20 μ M) with PL or PEITC with respect to their activity to deplete GSH (Fig. 2c). L-BSO was more

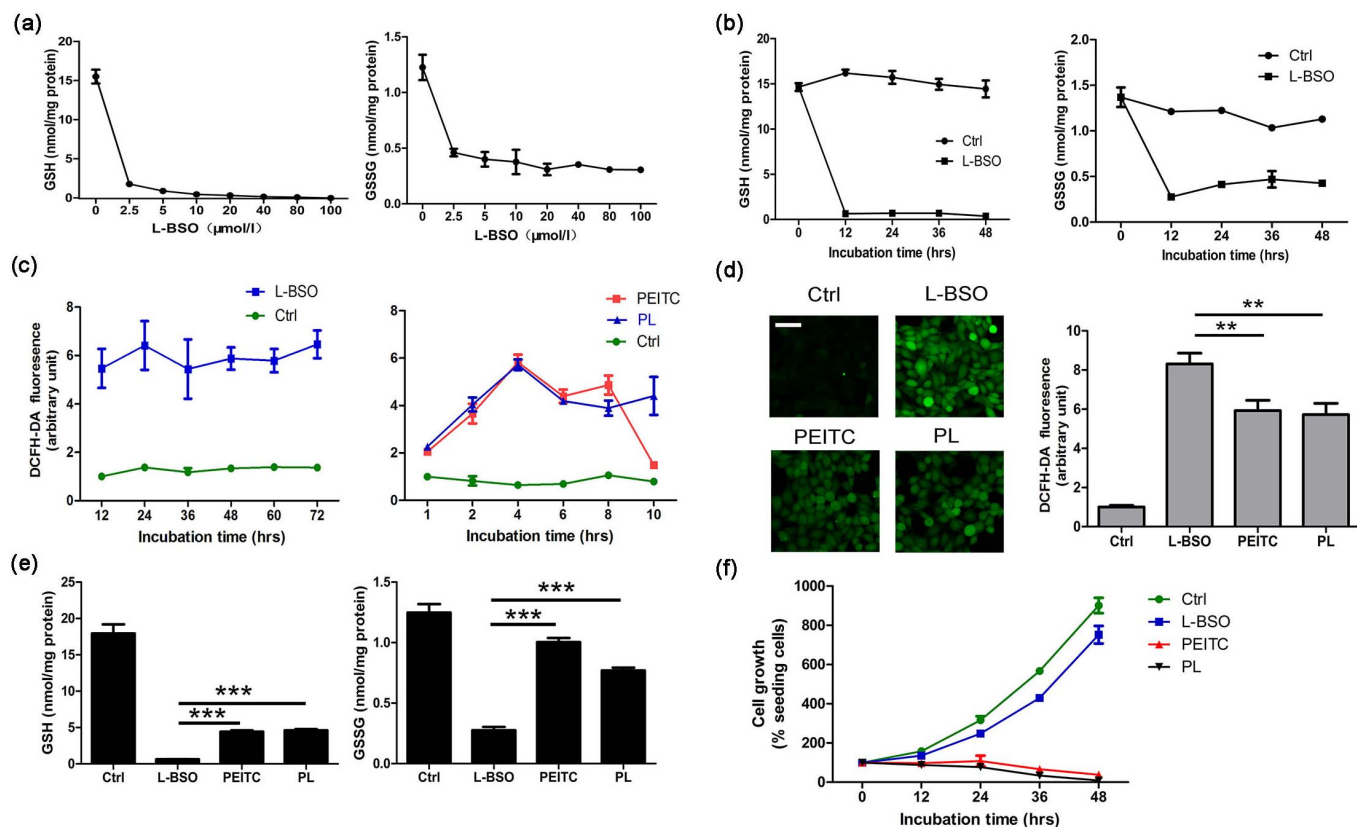


Figure 2 | L-BSO, a more potent oxidative agent than PEITC and PL, allows a progressive growth of 4T1 cells. (a) 4T1 cells were incubated with L-BSO (2.5–100 μM) for 48 hours, and cellular GSH and GSSG were assayed as described in materials and methods. (b) 4T1 cells were incubated with L-BSO (20 μM), at the indicated time points, cells were collected and subjected for GSH and GSSG quantitation. (c) Cells were incubated with L-BSO, PEITC, and PL at the indicated time points, cells were collected and cellular ROS was probed by DCFH-DA. (d & e) Cellular GSH and ROS change in cells treated with L-BSO, PEITC, and PL. Cells were treated with PEITC (10 μM) or PL (10 μM) for 4 hours, or treated with L-BSO (20 μM) for 12 hours, followed by ROS, GSH, and GSSG measurement, as described in Materials & Methods. (f) 4T1 cells were incubated with L-BSO (20 μM), PEITC (10 μM), or PL (10 μM), at the indicated time points, cell counts were performed. Experiments are repeated at least 3 times. Scale bar = 50 μm . ** p < 0.01, *** p < 0.001.

potent in augmenting ROS and depleting GSH than PL or PEITC. L-BSO induced a sustained high ROS level and sustained depletion of GSH, whereas PL and PEITC induced a transient elevation of ROS followed by a decline. The decline of ROS in PEITC-treated cells was due to the loss of cell viability. Then we did an experiment to directly compare ROS and GSH levels in cells treated with L-BSO, PL, or PEITC. Cells were divided into 4 groups, control, L-BSO, PL, and PEITC. L-BSO was added first. After 8-hour incubation, PL or PEITC was added. After another 4-hour incubation (because ROS peaks at the 4-hour point after PL and PEITC treatment [Fig. 2c], using this time point for comparison is appropriate.), cells were subjected for ROS and GSH measurement (Fig. 2d & e). L-BSO depleted GSH more efficiently than and induced a significantly higher ROS level than PL and PEITC. Unlike PEITC or PL, which killed 4T1 cells, L-BSO did not kill them (Fig. 2f). The results were reproduced by using Bcap37, HepG2, and Hela cells (Supplementary Fig. S1–3). Previously, Fath et al.²⁷ demonstrated that even 100 μM L-BSO does not kill A549 and H292 cancer cells. Miller et al showed that 20 μM BSO did not change the doubling time of PR4 cells, a ras-transformed NIH 3T3 subclones²⁸.

The results pose a paradox. Although PEITC, PL, and L-BSO all increase ROS via depleting GSH^{11,14}, the fate of the cells are opposite. More confounding is that L-BSO, the more potent one to deplete cellular GSH than PL or PEITC does not kill the tested cancer cells.

H₂O₂ induces a higher oxidative stress than PEITC or PL but permits cell growth. Exogenous H₂O₂ is often used to study the effect of

oxidative stress on cancer cells. We compared the oxidative stress induced by H₂O₂ (0.1 mM) with those induced by PEITC or PL. 0.1 mM H₂O₂ exerted a severer oxidative stress on 4T1 cells than PEITC, PL (Fig. 3a–c), note that hydroxyl free radical, the most toxic free radical and the decomposed product from H₂O₂, is ~2 folds higher than those induced by PEITC or PL. Unlike PL or PEITC, which killed 4T1 cells, 0.1 mM H₂O₂ allowed progressive growth of 4T1 cells (Fig. 3d). Similar results were obtained using Bcap37, Hela, and HepG2 cells (Supplementary Fig. S1–3).

100 μM H₂O₂ is a very high concentration. It is not known if this concentration of H₂O₂ in vivo could be generated and maintained.

LA-induced ROS contains highly reactive species whose concentrations are significantly higher than those induced by anticancer prooxidants. ROS is composed of many species, some of which are more chemically reactive than hence more toxic than others, e.g., hydroxyl free radical is much more reactive than superoxide. Because the quantity of ROS induced by LA is far higher than that induced by PEITC or PL but does not kill cancer cells, we assumed that the composition of ROS induced by anticancer prooxidants might be different from and more deadly than that by LA. We determined several reactive species using available probes (Fig. 4a). DCFH-DA fluorescent signals largely reflected the concentrations of NO₂[•] and CO₃^{•-}, which were much higher in cells exposed to LA than to PEITC or PL. HPF signal (•OH or ONOO⁻ level) was significantly higher in cells exposed to LA than those exposed to PL, PEITC, or ATO. LA induced an elevation of extra-mitochondria

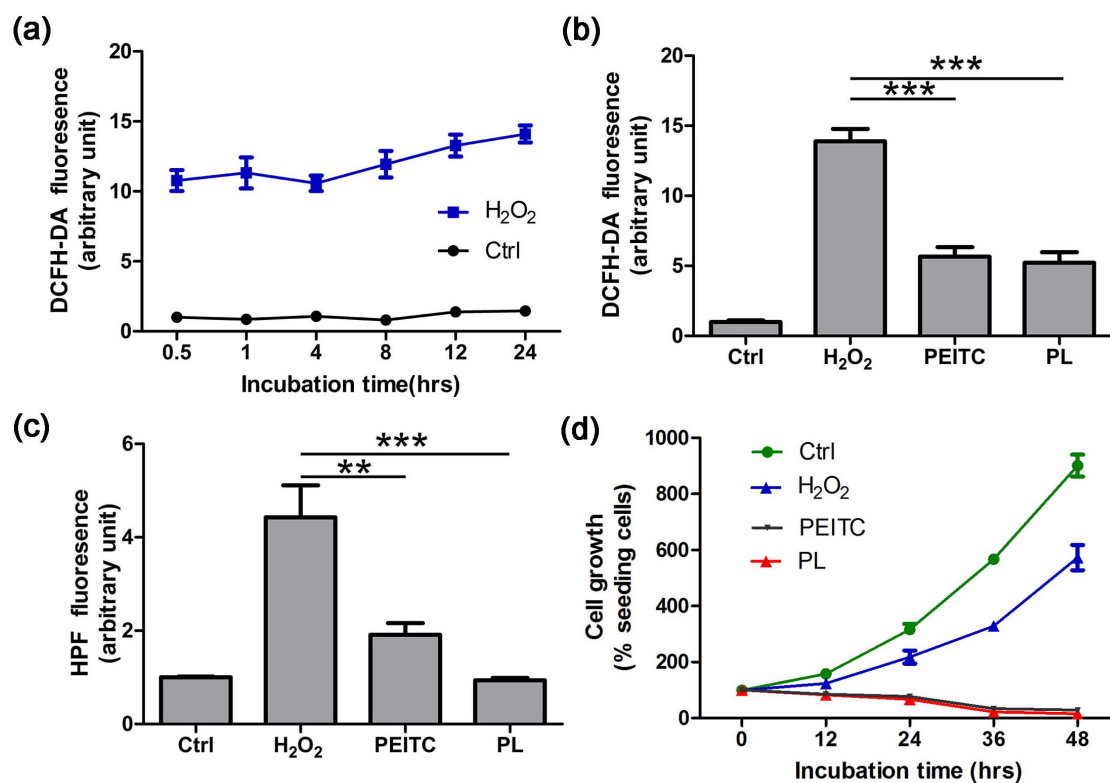


Figure 3 | Hydrogen peroxide induces a severe oxidative stress which does not kill 4T1 cells. (a) Time course of ROS in 4T1 cells treated with 0.1 mM hydrogen peroxide. (b & c) ROS levels (probed by DCFH-DA and HPF) in 4T1 cells treated with 0.1 mM hydrogen peroxide, 10 μ M PEITC, or 10 μ M PL. (d) 4T1 cell growth in the absence or presence of 0.1 mM hydrogen peroxide, 10 μ M PEITC, or 10 μ M PL. ** $p < 0.01$, *** $p < 0.001$. Data were repeated at least 3 times.

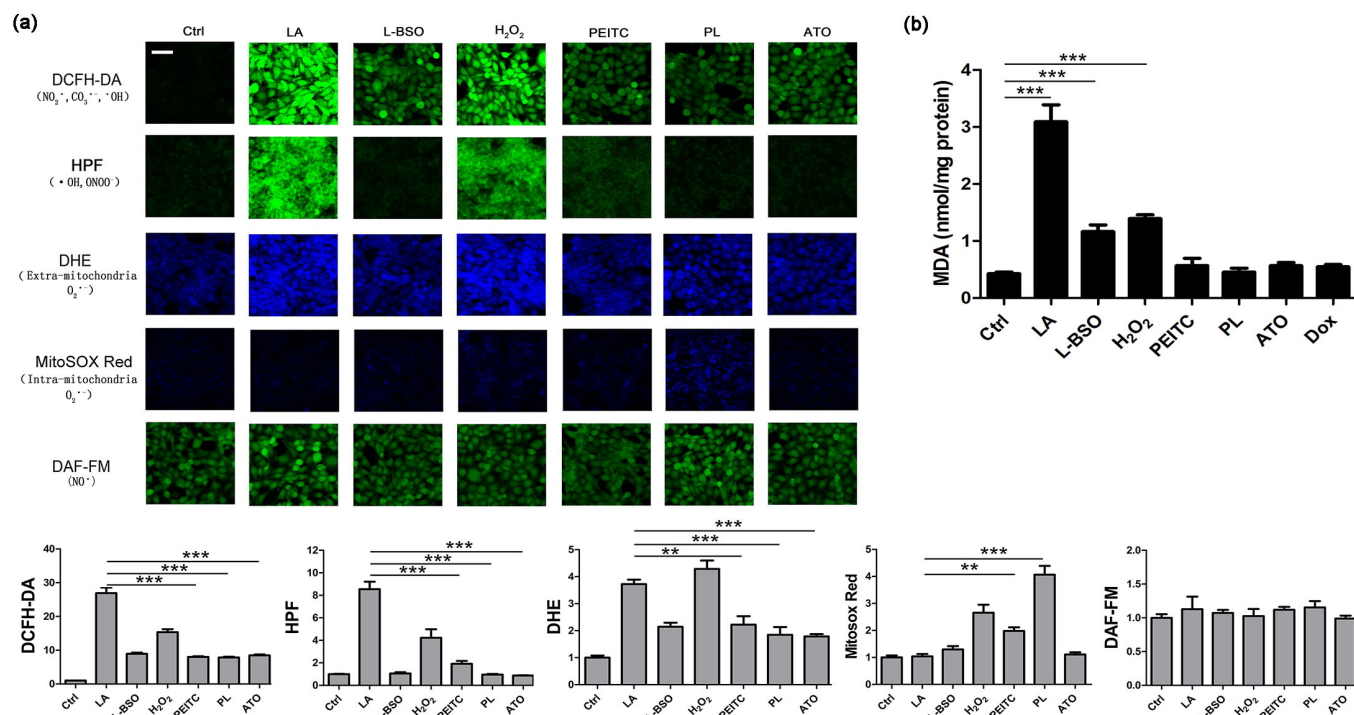


Figure 4 | ROS species and malonaldehyde in 4T1 cells treated by LA, L-BSO, H₂O₂, PEITC, PL, ATO, or Dox. (a) The composition of ROS in 4T1 cells. (b) MDA content in 4T1 cells. ROS levels were measured by 5 probes and MDA were measured as described in Materials and Methods. Experiments are repeated 3 times. Scale bar = 50 μ m. ** $p < 0.01$, *** $p < 0.001$.

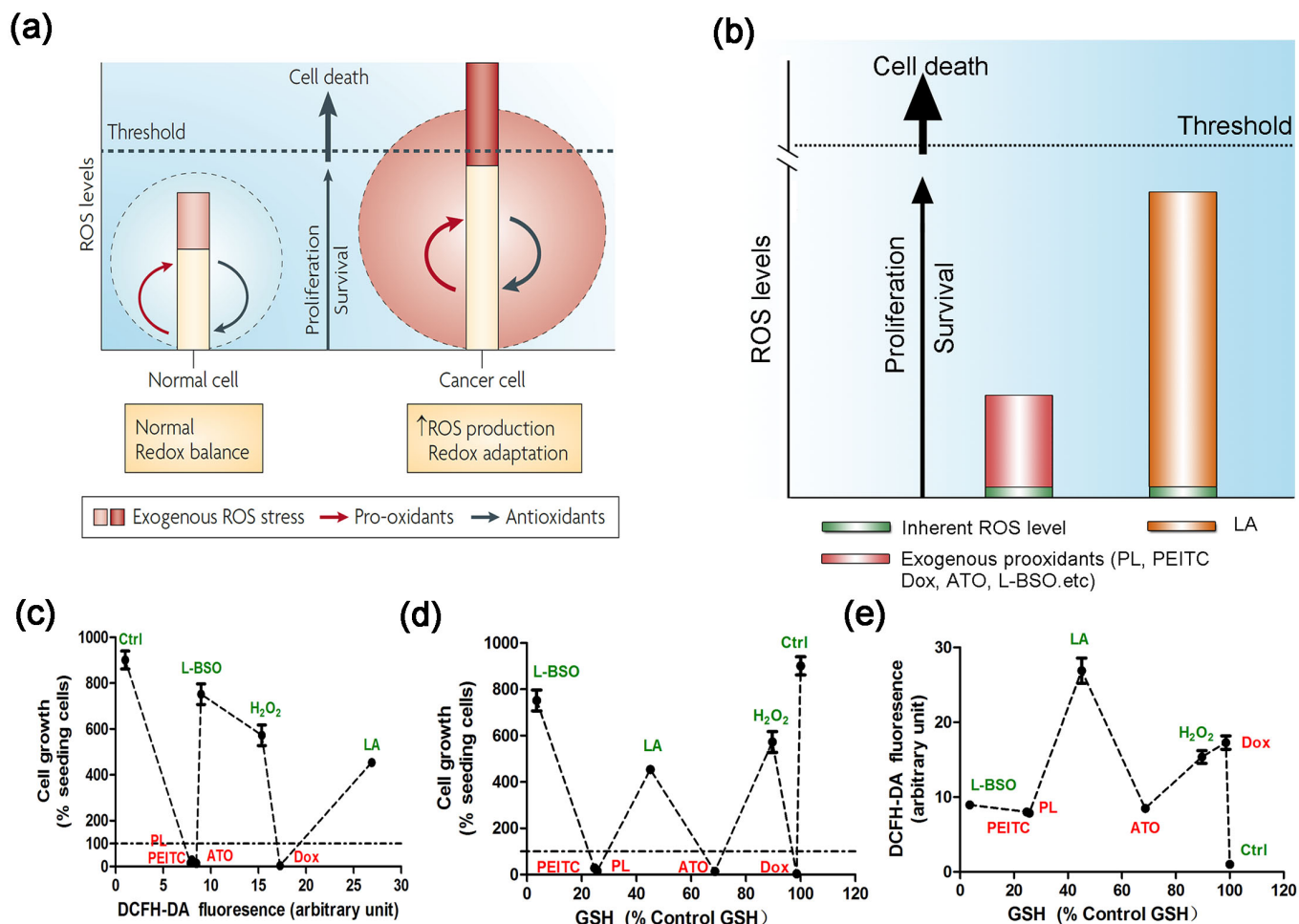


Figure 5 | No evident dose-response relationship between cellular ROS level and cell growth inhibition or death. (a) The current model of ROS-based therapy (copied from Ref. 1) – ROS modulation as the biological basis for therapeutic selectivity. Cancer cells show higher inherent ROS levels than normal cells. This difference is the basis for therapeutic selectivity. The exogenous ROS insults would kill cancer cells by increasing intracellular ROS to lethal concentration but spare normal cells. Note that it is on the premise that the basal ROS level in cancer cells is significant and near the lethal threshold. This is the basis for the feasibility of this therapy. (b) We show that the basal ROS level in cancer cells is negligible, and even pharmacologically active prooxidant-induced ROS is far below that induced by LA, which does not kill 4T1 cells. (c & d) 4T1 cell death or cell growth inhibition is irrelevant with the potency of exogenous insults to augment ROS or to deplete GSH. (e) Cellular GSH level is not inversely proportional to ROS level. Except L-BSO treatment, in which cellular ROS and GSH levels were determined 12 hours after L-BSO exposure, ROS and GSH levels in cells exposed to other agents were measured 4 hours after treatment. Cell counts were performed 48 hours after treatment.

superoxide levels (probed by DHE), but PL increased intramitochondria superoxide (probed by MitoSOX Red) by ~3 folds higher than LA. This difference might account for toxicity of PL, but confounded to interpret the toxicity of PEITC or ATO, which showed similar toxicity as but much lower activity to augment intramitochondrial superoxide than PL. Nitric oxide levels (DAF-FM signal) were comparable with each other. We repeated the experiments using Bcap 37, HeLa, and HepG2 cells and obtained similar results (Supplementary Fig. S4–6).

The results demonstrated that the levels of $\cdot\text{OH}$, ONOO^- and $\text{CO}_3^{\cdot-}$ induced by LA were significantly higher than that by other treatments (Fig. 4a). It should be noted that $\cdot\text{OH}$ is the most reactive free radical identified so far, and ONOO^- and $\text{CO}_3^{\cdot-}$ are highly reactive³⁰. Therefore, LA induced-ROS is quantitatively higher and qualitatively more toxic than ROS-based anticancer drug -induced one in the tested cancer cells, but paradoxically, it does not kill the tested cancer cells.

ROS versus MDA. In order to confirm if ROS induced by LA, PEITC, PL, Dox, or ATO exert oxidative stress in 4T1 cancer cells, we measured malondialdehyde (MDA), a product generated from

lipid peroxidation. Indeed, these treatments caused an increased MDA generation, LA caused a highest MDA production (Fig. 4b), followed by H₂O₂, L-BSO. MDA Levels in cells treated by PEITC, PL, ATO, and Dox were comparable with each other and were marginally increased in comparison to control. The results further suggest that LA induced a higher oxidative stress than PL, PEITC, ATO, and Dox in 4T1 cells.

The effect of LA and prooxidants on antioxidative enzymes. Apart from GSH, we measured the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) in the cell lysate derived from 4T1 cells treated with LA, L-BSO, H₂O₂, PEITC, PL, Dox, or ATO. The treatment, except PEITC, an inhibitor of GR³¹, did not significantly alter the activity of these antioxidant enzymes (Supplementary Fig. S7) in this cell line.

No evident dose-response relationship between cellular ROS level and cell growth inhibition or death. We would further discuss the rationale of ROS-based therapy. According to the prevailing hypothesis (Fig. 5a)³², there is a toxic threshold of ROS. In cancer

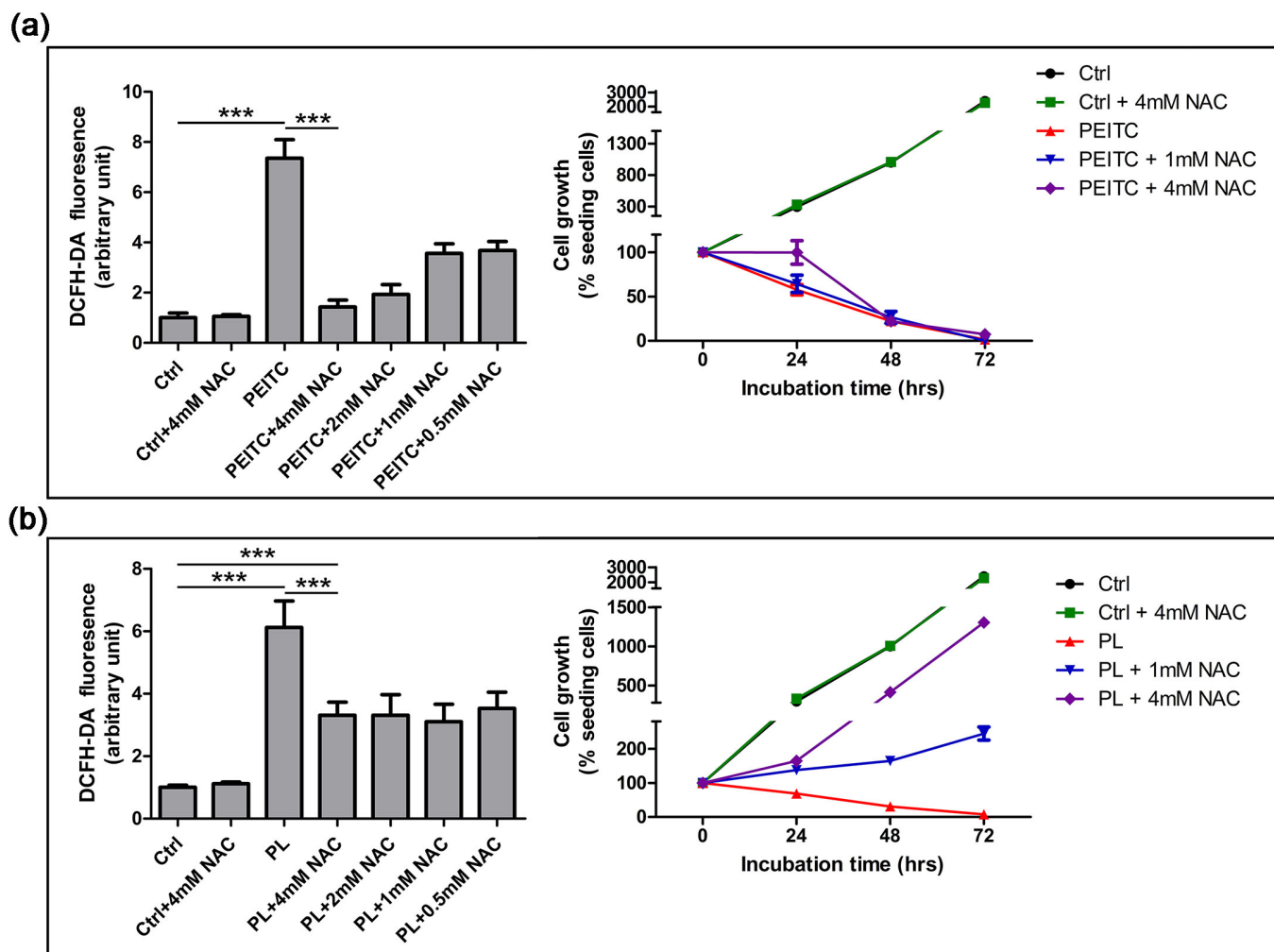


Figure 6 | NAC effect on PEITC- or PL-induced 4T1 cell death. (a) NAC fully reverses PEITC-induced ROS to basal level but does not rescue cell death. Left panel shows ROS levels (probed by DCFH-DA) in 4T1 cells treated with 10 μ M PEITC in the presence or absence of NAC. Right panel shows 4T1 cell growth inhibition (lower growth rate) or death. (b) NAC fully rescues PL-induced 4T1 cell death but only partially reverses ROS basal. 4T1 Cells were treated with 10 μ M PL in the presence or absence of NAC. Cell counts were done at the indicated time intervals. ** $p < 0.01$, *** $p < 0.001$. Experiments were performed for 3 times.

cells, ROS production and scavenging system are both upregulated. The upregulated ROS scavenging capacity in cancer cells keeps ROS level below the toxic threshold. Kinetically, although ROS flux in cancer cells is high, ROS level or readout is below the toxic threshold. The redox balance is intricately maintained in cancer cells. On the other hand, because ROS flux in cancer cells is high, redox balance is vulnerable to pharmacological intervention either via inhibiting ROS scavenging or enhancing ROS production. When the redox balance is disturbed, ROS production overwhelms ROS scavenging, high ROS flux would result in ROS accumulation to toxic threshold. When ROS level reaches death threshold, it can not only cause irreversible oxidative damage but also trigger death signaling. Prooxidants such as PL and PEITC kill cancer cells via elevating ROS to toxic threshold^{11,14}. Moreover, the higher basal ROS level in cancer cells than that in normal cells lays the basis for the therapeutic selectivity, i.e., cancer cells, but not normal cells, are sensitive to further ROS insults, such that exogenous ROS inducer can selectively eliminate cancer cells.

On the other hand, we came to a different point of view (Fig. 5b). First, if the endogenous ROS level in cancer cells is the biological basis for therapeutic selectivity, its concentration would be close to lethal threshold as depicted in Fig. 5a. However, we show that the endogenous ROS level is virtually negligible, as compared with LA-induced one. Second, although

it is believed that pharmacologically-induced ROS level is greater than the toxic threshold^{11,14}, we show that it is much lower than that induced by LA, which permit progressive growth. Thus, the toxic threshold of ROS is a key issue unresolved, at least in the tested cancer cells.

More importantly, according to the therapeutic principle, a therapeutic drug must show a dose-response relationship. If ROS is the effector, cancer cells shall respond to ROS in a dose-dependent manner. Thus, cell growth inhibition or cell death should be inversely proportional to cellular ROS level when it reaches toxic level. If PEITC or PL-induced cellular ROS level is taken as the reference of toxic threshold, the higher ROS concentrations would be more toxic. We plotted the cell (4T1, Bcap37, Hela, HepG2) growth against ROS levels or GSH level, and ROS levels against GSH level. The plots showed that growth inhibition or cell death was not associated with the potency of agents to augment ROS (Fig. 5c, Supplementary Fig. S8) or to deplete GSH (Fig. 5d, Supplementary Fig. S8), e.g., LA, the most potent ROS-inducer, allowed a rapid cell proliferation, in sharp contrast to much weak ROS-inducers PEITC, PL, ATO, or DOX, which killed ~71–94% of seeding cells. ROS level was not inversely proportional to GSH level (Fig. 5e, Supplementary Fig. S8). Taken together, there is no evident dose-response relationship between cellular ROS level and cell death or cell proliferation in the tested cells.

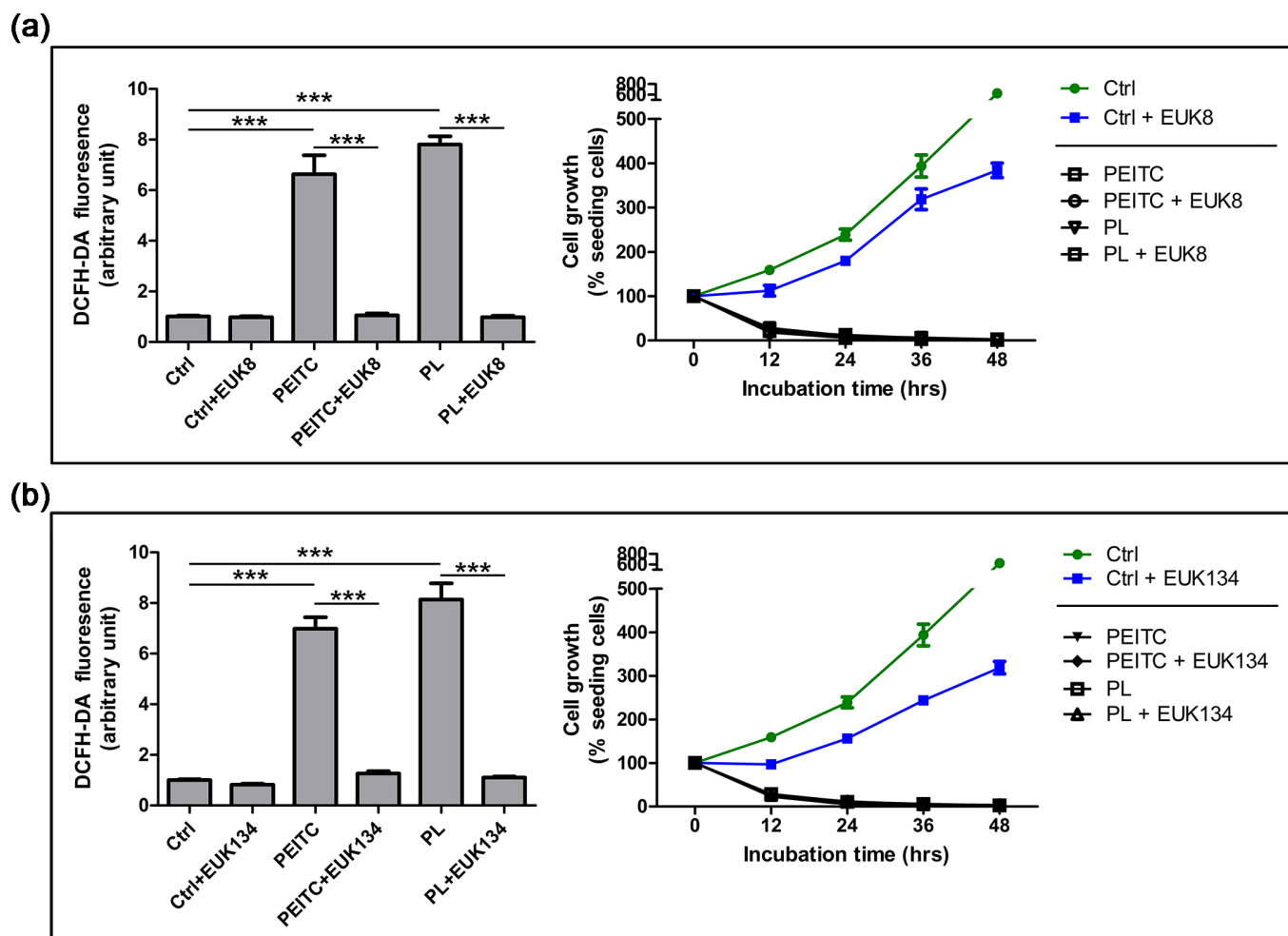


Figure 7 | SOD and catalase mimetics fully reverse PEITC- or PL-induced ROS to basal level but does not rescue 4T1 cell death. 4T1 cells were treated with 10 μ M PEITC or PL in the presence or absence of EUK8 or EUK134. Cell counts were done at the indicated time intervals. *** $p < 0.001$. Experiments were performed for 3 times.

The effects of N-acetylcysteine on prooxidant-induced cell death and ROS are complicated.

NAC exhibited a concentration-dependent inhibition on PEITC-induced ROS in 4T1 cells (Fig. 6a). Although NAC at 4 mM fully reversed PEITC-induced ROS to basal level, it only delayed but did not block PEITC-induced death, as the viable cell numbers with or without NAC ultimately were the same (Fig. 6a).

Similarly, 4 mM NAC could completely reverse Dox- or ATO-induced ROS to basal level but could not block cell death (Supplementary Fig. S9 & 10).

Unlike its effect on PEITC, NAC exhibited a weak inhibition on PL-induced ROS increase (Fig. 6b). There was no evident concentration-dependent effect of NAC on PL-induced ROS. Interestingly, NAC at 1 mM blocked PL-induced cell death, NAC at 4 mM abolished the hazardous effect of PL (Fig. 6b).

Thus, NAC completely reversed ROS induced by PEITC, Dox, or ATO to basal level but did not block cell death; on the other hand, NAC only partially inhibited PL-induced ROS but completely blocked PL-induced cell death. We obtained consistent results by using Bcap 37, Hela, and HepG2 cells (Supplementary Fig. S11–14). The results suggested that prooxidant-induced ROS was not tightly linked with the death of 4T1, Bcap37, Hela, and HepG2.

Our results seem to be contradictory to but in fact are generally consistent with previous reports. Trachootham et al.¹⁴ showed that NAC (3 mM) fully reversed PEITC-induced ROS and significantly

attenuated PEITC-induced death in a time course of 5 hours. As they did not show data of longer time points, it was not known if NAC delayed or blocked PEITC-induced cell death. We showed that NAC only delayed but did not block PEITC-induced cell death. Raj et al.¹¹ showed that NAC (3 mM) fully reverse PL-induced ROS to basal level in EJ cells and completely blocked cell death. We also observed that 4 mM NAC completely blocked PL-induced cell death (Fig. 6b). The only discrepancy was the inhibitory effect of NAC on PL-induced ROS: while Raj et al. demonstrated that NAC fully inhibited PL-induced ROS, we showed that NAC only partially reversed PL-induced ROS (Fig. 6b). The results were consistent using 4 different cell lines, as described above. Therefore, the third party to perform the same experiments would be the proper way to resolve the discrepancy.

SOD and catalase mimetics reverse PL- or PEITC-induced ROS but do not rescue cell death.

We further used SOD and catalase mimetics (EUK8 & EUK134) to intervene the action of PL or PEITC on cancer cells. Although EUK8 or EUK134 could fully reverse PL- or PEITC-induced ROS, they did not rescue cell death (Fig. 7). The results convey the same implication as NAC experiments.

LA induces a much higher ROS in primary human colon or rectal cancer cells than anticancer prooxidants but does not kill them. Because primary cancer cells are different from cancer cell lines, it is

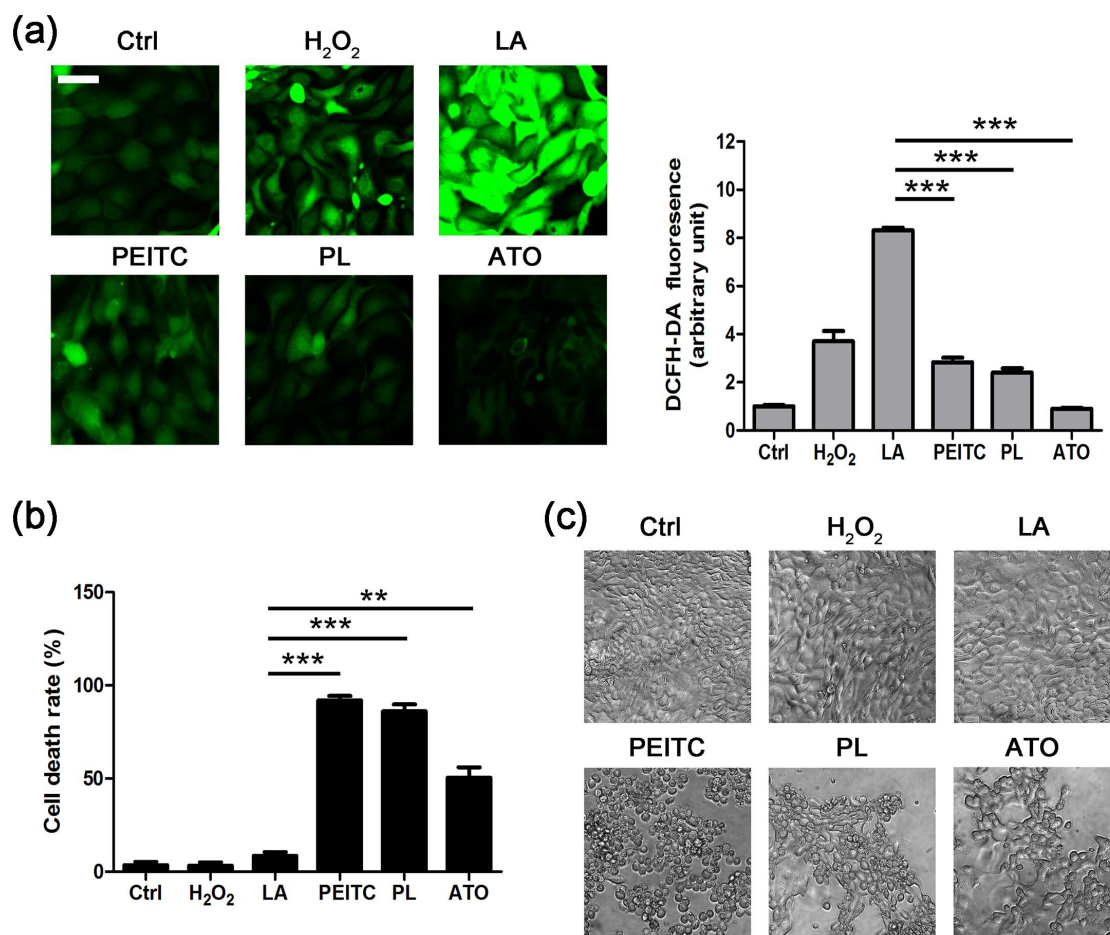


Figure 8 | LA induces a significantly higher ROS than PL, PEITC, and ATO but does not kill primary human colon cancer cells. (a) Comparison of cellular ROS induced by hydrogen peroxide, LA, PEITC, PL, or ATO. Cells were treated with hydrogen peroxide (100 μ M), LA (20 mM), PEITC (10 μ M), PL (10 μ M), or ATO (10 μ M) for 4 hours, followed by cellular ROS measurement, as reflected by DCFH-DA signals. (b) Cells were treated with hydrogen peroxide (100 μ M), LA (20 mM), PEITC (10 μ M), PL (10 μ M), or ATO (10 μ M) for 24 hours, subjected for cell counts and death rate calculation. (c) Representative photos of cells 24 hours after treatment. Scale bar = 50 μ m. ** $p < 0.01$, *** $p < 0.001$.

necessary to test the effect of anticancer prooxidants and LA on primary cancer cells. The primary human colon cancer cells from a patient were resistant to hydrogen peroxide (Fig. 8). Although LA was obvious the most powerful ROS inducer in the tested compounds, it did not kill cancer cells (Fig. 8). The much weaker ROS-inducers PEITC, PL, ATO (Fig. 8) were highly cytotoxic. The effects of LA, PEITC, PL, hydrogen peroxide, and ATO on primary colon or rectal cancer cells from 4 patients were consistent (Fig. 8 & Supplementary Fig. S15). Thus, the results of primary human colon and rectal cancer cells are virtually the same as those of cancer cell lines: while the much stronger ROS inducer LA does not kill the tested primary cancer cells, the much weaker ROS inducer PEITC, PL, or ATO effectively killed them.

Concluding remark. Using randomly chosen cancer cells cell lines and primary human colon and rectal cancer cells, we show that there is no evident dose-response relationship between ROS and cell killing or cell growth inhibition. Dose-response relationship is a basic therapeutic principle, which ROS therapeutics also should obey, if ROS is the effector.

Another important point is the function of LA-induced ROS. In this study, we show that LA is a potent ROS inducer in all the tested cancer cell lines and primary human colon and rectal cancer cells. Previous studies from many laboratories have confirmed that LA is a common environmental factor²² in diverse solid tumors and played multifaceted roles in promoting cancer progression including cancer

cell metabolism^{33,34} and survival^{24,35}, cancer cell chromosomal instability³⁶, tumor angiogenesis^{22,23}, and cancer metastasis^{37,38}. Clinical studies demonstrated that high level of lactate was a strong prognostic indicator of increased metastasis and poor overall survival^{22,39–44}. We would ask if there is relationship between LA-induced ROS and the events listed above. Vegran et al suggested that LA-induced ROS may involve in tumor angiogenesis²³.

These are the concerns in ROS-based cancer therapy that requires attention.

Methods

Cancer cell lines. Murine breast cancer 4T1 cells, human breast cancer Bcap37 cells, human cervical cancer Hela cells, human liver cancer HepG2 were maintained in complete RPMI-1640 (Life Technologies) with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine.

Treatment of cancer cells with LA, hydrogen peroxide, L-BSO, PL, PEITC, Dox, or ATO. For cells treated with LA, cells were cultured in medium with LA, which was generated by adding pure lactic acid (Sigma-Aldrich, L1750) to the culture media to final lactate concentrations (2.5–20 mM) as described²⁴. For cells treated with hydrogen peroxide (Sigma-Aldrich, 323381), cells were cultured in the presence of 0.1 mM hydrogen peroxide. For cells treated with Dox and ATO, cells were cultured in medium containing Dox (10 μ g/ml), or ATO (10 μ M). For cells treated with PL and PEITC, cells were cultured in medium containing 10 μ M of PL or PEITC, as described^{11,14}. For cells treated with L-BSO (Sigma-Aldrich, B2515), cells were cultured in medium containing 2.5–100 μ M L-BSO.

Cell count. After treatment, cells were observed under microscope every day. After cells were cultured for 24, 48, and 72 hours, we observed that many cells were lysed in



the groups treated with PEITC, PL, ATO, or Dox. The viable cells were trypsinized and collected for cell counts using trypan blue exclusion assay.

Primary cancer cells. Human colon or rectal cancer biopsies were obtained from 4 colorectal cancer donors from the oncology department of the Second Affiliated Hospital of Zhejiang University School of Medicine, with patients' consent and approval of the hospital's Ethics Committee. All samples were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki). Cancer epithelial cells from surgical specimens were obtained according to the methods as described^{45–47}. Briefly, tissue sample was rinsed with PBS for three times. Fat, blood clots and connective tissues were removed from tumor tissues. Tissue sample was cut into small pieces (1-mm³) with scalpels, rinsed with complete DMEM medium twice. 3–5 pieces of tumor tissues were placed into one well of 12-well plate followed by addition of 500 μ L complete DMEM medium, and incubated in a humidified CO₂ incubator at 37°C. When the cells reached 70% confluency, cells were treated with LA (20 mM), PEITC (10 μ M), PL (10 μ M), ATO (10 μ M), or hydrogen peroxide (100 μ M). After 4-hour treatment, ROS levels in cells were probed with DCFH-DA. After 24-hour treatment, cell death rate was counted by trypan blue exclusion assay.

ROS measurement. ROS measurement is assayed by dichloro-dihydro-fluorescein diacetate (DCFH-DA, Sigma, D6883), dihydroethidium (DHE, Sigma, D7008), MitoSOXTM Red mitochondrial superoxide indicator (Invitrogen, M36008), 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM, Sigma, D2321), or hydroxyl radical and peroxynitrite sensor (HPF, Invitrogen, H36004), according to manufacturers' instruction. Briefly, cells were loaded with DCFH-DA (final concentration of 10 μ M for 30 minutes), DHE (final concentration of 5 μ M for 30 minutes), MitoSOXTM Red (final concentration of 2.5 μ M for 10 minutes), DAF-FM (final concentration of 10 μ M for 30 minutes), or HPF (final concentration of 10 μ M for 30 minutes), washed with ice cold HBSS (Hank's Balanced Salt Solution, pH 7.2), then observed under a Zeiss LSM710 laser confocal microscope (Carl Zeiss, Germany) equipped with Zen software to process the image.

Cellular MDA measurement. Cells were incubated with LA (20 mM), L-BSO (20 μ M), hydrogen peroxide (100 μ M), PEITC (10 μ M), PL (10 μ M), Dox (10 μ g/ml), or ATO (10 μ M) for 8 hrs. Cellular MDA were measured according to the previously described method using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), which is based on the principles in the previous report⁴⁸. Briefly, MDA reacts with thiobarbituric acid (TBA) at 90–100°C and acidic condition. The reaction yields a pink MDA-TBA conjugate, which was measured at 532 nm using a Multi-Mode Microplate Readers (Spectramax M5). The cellular MDA was expressed nmole/mg cellular protein.

Measurement of the activities of antioxidant enzymes. Cells were incubated with LA (20 mM), PEITC (10 μ M), PL (10 μ M), ATO (10 μ M), Dox (10 μ g/ml), or hydrogen peroxide (100 μ M) for 4 hrs, and L-BSO (20 μ M) for 12 hrs. Cellular extracts were prepared for the detection of total SOD, catalase, GR, and GPX, by corresponding kits (Beyotime Institute of Biotechnology, Shanghai, China), which are based on the principles in the previous reports. Briefly, SOD activity was measured according to its ability to inhibit the production of water-soluble formazan dye, as previously described by Sun, Y. *et al.*⁴⁹. The activity of catalase was determined by converting hydrogen peroxide into H₂O and O₂, as previously described by Goth.⁵⁰ The activity glutathione peroxidase (GPX) was determined by measuring the rate of oxidation of GSH to GSSG by the dismutation of cumene hydroperoxide (Cum-OOH), which is catalysed by GPX, as described by Flohe, L. *et al.*⁵¹. Activity of glutathione reductase is measured by rate of the NADPH-dependent reduction of GSSG to GSH, as previously described by Carlberg, I. *et al.*⁵².

Cellular GSH and GSSG measurement. Cells were cultured in the 6-well plate. When the culture reaches 70–80% confluency, cells were treated with L-BSO (20 μ M) for 12 hours, or treated with LA (20 mM), hydrogen peroxide (100 μ M), PEITC (10 μ M), PL (10 μ M), Dox (10 μ g/ml), or ATO (10 μ M) for 4 hours respectively. Cells were rinsed twice with 2 ml ice-cold Ca²⁺-/Mg²⁺-free PBS. Cells were collected by trypsinization and lysed in 0.2 ml of ice-cold extraction buffer (0.1% Triton-X and 0.6% sulfosalicylic acid in KPE) followed by 4 cycles of freezing and thawing (1 minute in liquid nitrogen and 2 min in water bath at 37°C). Cell lysate was centrifuged and supernatant was collected for GSH and GSSG measurement according to the method described by Rahman *et al.*⁵³. The GSH assay is based on the chemical conjugation of GSH with 5,5'-Dithiobis(2-nitrobenzoic acid)[DTNB] (Sigma, D-8130). Total glutathione was measured by firstly reducing oxidized glutathione using glutathione reductase (Sigma, G-3664) and β -NADPH (Sigma, N-7505) followed by conjugation with DTNB. To measure GSSG, GSH was firstly covalently reacted with 2-vinylpyridine (Aldrich, 132292), then GSSG was measured as described above. Pierce[®] BCA protein assay kit was used for protein determination. All samples were run in triplicates. GSH and GSSG levels were expressed in nmol/mg protein.

Statistics. All data were analyzed using the InStat software (GraphPad, CA, USA) and displayed as mean \pm SEM. Two-tailed Student's t-test was used for statistical analysis, and significance was defined as ***P < 0.001, **P < 0.01, *P < 0.05.

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Author contributions

X.H. conceived the concept, designed the study, and wrote the paper. C.Z., W.H. & H.W. conducted the experiments.

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