

# Mitochondrial formation of hydrogen peroxide is causally linked to the antimycin A-mediated prevention of *tert*-butylhydroperoxide-induced U937 cell death

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**Abstract** Antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), both of which bind to the same site of complex III, prevented U937 cell killing promoted by *tert*-butylhydroperoxide (tB-OOH). This cytoprotection was not directly caused by inhibition of electron transport or reduced formation of tB-OOH-derived toxic species, but rather appeared to be the consequence of a mechanism involving mitochondrial formation of hydrogen peroxide. Ubisemiquinone was most likely the electron donor allowing the formation of superoxides and, as a consequence, of hydrogen peroxide.

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**Key words:** Antimycin A; 2-heptyl-4-hydroxyquinoline N-oxide; Complex III; *tert*-butylhydroperoxide; Cytotoxicity

## 1. Introduction

The organic hydroperoxide *tert*-butylhydroperoxide (tB-OOH) generates reactive intermediates within the cell which subsequently damage an array of biomolecules including lipids [1], thiols [2,3], DNA [4–8] and proteins [9]. As a consequence, these cells lose their ability to generate ATP [10–13] and to control cation homeostasis [14,15] and redox equilibrium [16]. Although the exact mechanism of the lethal response is still unknown, mitochondrial damage and the formation of permeability transition pores are currently considered to be the final events leading to cell death after treatment with tB-OOH [11,13,17]. It is therefore important to identify the nature and the sequence of the upstream events leading to pore opening and then to cell death. In order to do so, however, it is first necessary to identify the tB-OOH-derived cytotoxic intermediates and the subcellular site(s) in which these species are being formed. Previous studies [18,19] reported that inhibitors of cytochrome P-450 reduce the formation of tB-OOH-derived radical species. Other studies [7,20] showed that the oxygen- and carbon-centered radical species derived from tB-OOH are at least partially generated at the level of mitochondria.

The results presented in this paper extend our previous findings indicating that a high concentration of antimycin A reduced the formation of these radicals and that this effect

was associated with a small reduction in the cytotoxic response [7]. We herein report that the lethal effects promoted by tB-OOH are prevented by concentrations of antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) resulting in inhibition of oxygen consumption. The cytoprotective effect, however, is not a direct consequence of inhibition of oxygen consumption or mitochondrial reductive decomposition of tB-OOH. As previously observed for the potentiation of tB-OOH-induced DNA cleavage [8], inhibition of cell death appears to be mediated by the enforced mitochondrial formation of hydrogen peroxide resulting from dismutation of superoxide anions generated in a reaction in which ubisemiquinone serves as electron donor.

## 2. Materials and methods

### 2.1. Cell culture and treatments

U937 cells were grown in RPMI 1640 culture medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Seralab Ltd., Sussex, UK), penicillin (50 units/ml) and streptomycin (50 µg/ml), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) in an atmosphere of 95% air-5% CO<sub>2</sub>. Respiration-deficient U937 cells were isolated by culturing the cells in RPMI medium containing 110 µg/ml pyruvate, 5 µg/ml uridine and 400 ng/ml ethidium bromide or 50 µg/ml chloramphenicol for 6 days with medium changes every 2 days. Under these conditions, these cells were unable to consume oxygen in response to glucose or pyruvate.

A stock solution of tB-OOH was freshly prepared in saline A (0.14 M NaCl, 5 mM KCl, 4 mM NaHCO<sub>3</sub>, 5 mM glucose). Metyrapone and  $\alpha$ -phenyl- $\alpha$ -propylbenzeneacetic acid 2-[diethylamino] ethyl ester (SKF 525A) were dissolved in distilled water. Antimycin A, HQNO and myxothiazol were dissolved in 95% ethanol. At the treatment stage the final ethanol concentration was never higher than 0.05% and, under these conditions, was neither toxic nor did it affect the cytotoxic properties of tB-OOH. Treatments were performed in saline A and the cell density was  $2 \times 10^5$  cells/ml.

### 2.2. Cytotoxicity assay

After the treatments, the cells were washed with saline A and resuspended in pre-warmed culture medium, plated into 35-mm tissue culture dishes and incubated at 37°C for 6 h. Cytotoxicity was determined using the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue and the cells were counted using a haemocytometer. Results are expressed as the percentage of dead cells calculated as the ratio of stained cells vs. the total number of cells.

### 2.3. Oxygen consumption

U937 cells were washed once in saline A and then resuspended in the same medium at a density of  $1 \times 10^7$  cells/ml. Oxygen consumption was measured using a Y.S.I. oxygraph equipped with a Clark electrode (model 5300, Yellow Springs Instruments Co., Yellow Springs, OH, USA). The cell suspension (3 ml) was transferred to the polarographic cell and the oxygen levels were monitored for 3 min under constant stirring (basal respiration). Each of the different compounds (see Sections 3 and 4) was then added and respiration was again

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**Abbreviations:** tB-OOH, *tert*-butylhydroperoxide; HQNO, 2-heptyl-4-hydroxyquinoline N-oxide; SKF 525A,  $\alpha$ -phenyl- $\alpha$ -propylbenzeneacetic acid 2-[diethylamino] ethyl ester; SSF, strand scission factor

measured for 3 min. The rate of oxygen utilization was calculated as described in [21].

### 3. Results

#### 3.1. Complex III inhibitor-mediated prevention of tB-OOH-induced cytotoxicity

U937 cell killing induced by a 1 mM concentration of tB-OOH was prevented by antimycin A and HQNO (Fig. 1A–B), which are known to inhibit complex III at the level of the same site [22]. In contrast, the complex III inhibitor myxothiazol – which binds to a site different from that of antimycin A and HQNO [23] – did not affect the toxicity elicited by tB-OOH (Fig. 1C). The cytoprotection afforded by antimycin A and HQNO was concentration-dependent and apparent over the same dose-range in which inhibition of oxygen consumption was also observed (Table 1). Increasing the concentrations of antimycin A above 0.5–1  $\mu$ M, however, resulted in a progressive appearance of cell killing (Fig. 1A). The results illustrated in Fig. 2 indicate that low levels of antimycin A promote death in respiration-proficient cells treated with the combination 50  $\mu$ M HQNO/1 mM tB-OOH as well as in respiration-deficient cells treated with 1 mM tB-OOH. The toxicity curves resulting from these treatments were basically superimposable regardless of whether the respiration-deficient phenotype was induced by growth in chloramphenicol or ethidium bromide. It is also important to note that the combination HQNO/tB-OOH, or the hydroperoxide alone, was not toxic for respiration-proficient (Figs. 2 and 1B) or -deficient (Fig. 2) cells, respectively.

Lastly, we demonstrated that, under the experimental conditions utilized in this study, the mode of cell death caused by tB-OOH was necrosis and that its prevention by antimycin A, or HQNO, was not associated with the onset of apoptosis (not shown). Indeed, the morphological alterations induced by 1 mM tB-OOH were mainly represented by swelling of the cells followed by loss of nuclear material, with no evidence of chromatin condensation or fragmentation. Conventional

Table 1

The effect of antimycin A, HQNO, myxothiazol, metyrapone or SKF 525A on U937 cell oxygen consumption

Treatment ( $\mu$ M)	nmol O <sub>2</sub> /min/10 <sup>7</sup> cells
–	12.83
Antimycin A	
0.1	5.59
0.3	3.31
0.5	1.37
HQNO	
1	7.84
3	3.91
10	0.92
Myxothiazol	
0.5	4.64
1	2.76
10	0.67
Metyrapone	
500	12.94
SKF 525A	
75	12.49

The cells were rinsed with saline A and then analyzed for oxygen consumption as described in Section 2. The rate of oxygen consumption was monitored for 3 min at the beginning of the experiment (basal respiration) and after addition of the drugs. Results represent the mean of two separate experiments.

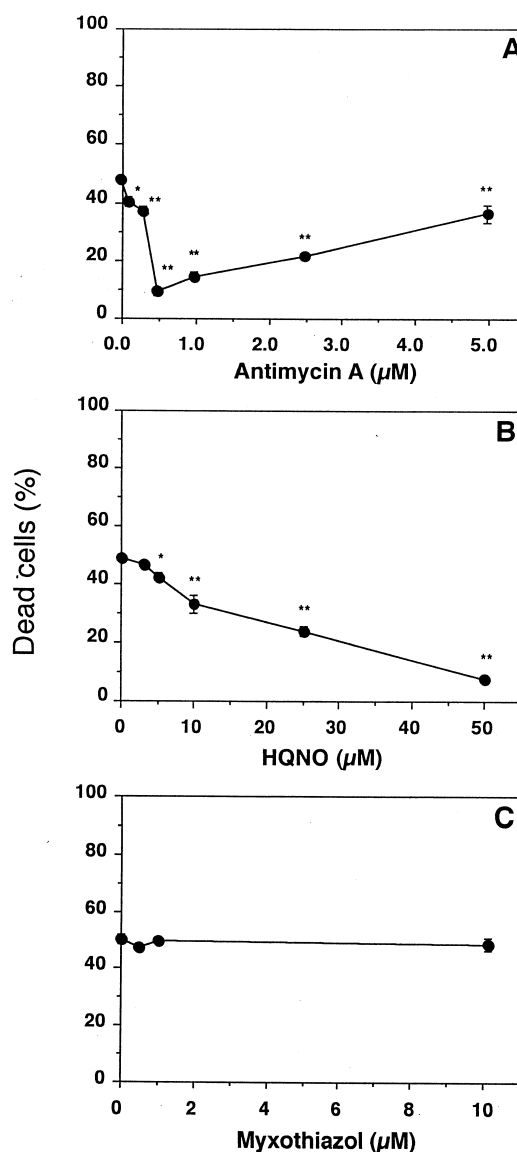


Fig. 1. The effect of antimycin A, HQNO or myxothiazol on U937 cell killing induced by tB-OOH. U937 cells ( $2 \times 10^5$ /ml) were exposed to 1 mM tB-OOH for 30 min in saline A after a 5 min pre-incubation with increasing concentrations of antimycin A (A), HQNO (B) or myxothiazol (C). After treatments, the cells were rinsed and post-incubated for 6 h in drug-free culture medium and then analyzed for cytotoxicity (see Section 2). Results represent the mean  $\pm$  S.E.M. calculated from three experiments, each performed in duplicate. \* $P < 0.01$  and \*\* $P < 0.001$  vs. treatment with 1 mM tB-OOH alone (unpaired  $t$ -test).

electrophoresis revealed that this treatment did not result in the formation of internucleosomal DNA cleavage. Pulsed field gel electrophoresis of the DNA from these cells indicated the absence of discrete or 50 kilobase paired DNA fragments which are produced during apoptotic cell death [24], but rather generated the smeared fragments which are indicative of necrotic cell death [25]. In marked contrast, most of the cells treated with the cocktail antimycin A/tB-OOH displayed normal morphology and DNA fragmentation was not detected using both conventional and pulsed field gel electrophoresis.

Morphological and biochemical analyses revealed the ab-

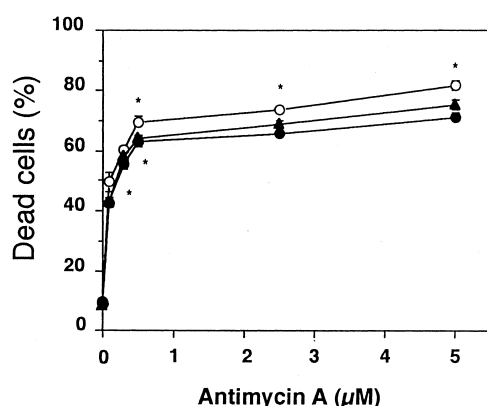


Fig. 2. The effect of antimycin A on cell killing induced by tB-OOH in respiration-proficient HQNO-supplemented cells or in respiration-deficient cells. Cells made respiration-deficient by growth in either ethidium bromide (closed circles) or chloramphenicol (closed triangles) were exposed to 1 mM tB-OOH for 30 min in saline A after a 5 min pre-incubation with increasing concentrations of antimycin A. Respiration-proficient cells were treated for 5 min with 50  $\mu$ M HQNO, for an additional 5 min with increasing concentrations of antimycin A and then for 30 min with 1 mM tB-OOH (open circles). After treatments, the cells were rinsed and post-incubated for 6 h in drug-free culture medium and then analyzed for cytotoxicity. Results represent the mean  $\pm$  S.E.M. calculated from 3–4 experiments, each performed in duplicate. \* $P$  < 0.0002 vs. treatment with 1 mM tB-OOH in HQNO-supplemented respiration-proficient cells or treatment with 1 mM tB-OOH in respiration-deficient cells (unpaired  $t$ -test).

sence of apoptosis in respiration-deficient cells treated with 1 mM tB-OOH.

### 3.2. Cytochrome P-450 inhibitor-mediated prevention of tB-OOH-induced cytotoxicity

The effect of inhibitors of cytochrome P-450 on U937 cell killing induced by 1 mM tB-OOH was investigated. As illustrated in Fig. 3, metyrapone as well as SKF 525A efficiently prevented the toxic response evoked by the hydroperoxide. The cytoprotection afforded by these inhibitors was concentration-dependent and the lethal response evoked by the hydroperoxide was abolished by 250  $\mu$ M metyrapone or 75  $\mu$ M SKF 525A. It is important to note that, under the experimental conditions utilized in this study, neither of these two inhibitors produced significant effects on oxygen consumption (Table 1).

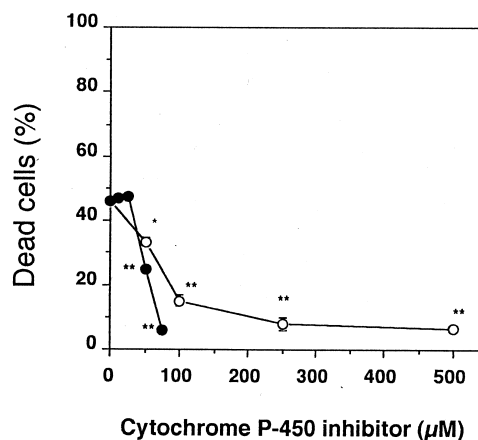


Fig. 3. The effect of metyrapone or SKF 525A on U937 cell killing induced by tB-OOH. U937 cells ( $2 \times 10^5$ /ml) were exposed to 1 mM tB-OOH for 30 min in saline A after a 5 min pre-incubation with increasing concentrations of either metyrapone (open circles) or SKF 525A (closed circles). After treatments, the cells were rinsed and post-incubated for 6 h in drug-free culture medium and then analyzed for cytotoxicity (see Section 2). Results represent the mean  $\pm$  S.E.M. calculated from three experiments, each performed in duplicate. \* $P$  < 0.01 and \*\* $P$  < 0.001 vs. treatment with 1 mM tB-OOH alone (unpaired  $t$ -test).

### 3.3. The complex III inhibitor-mediated prevention of tB-OOH-induced cytotoxicity is causally linked to enforced formation of hydrogen peroxide

The possibility that enforced formation of hydrogen peroxide in complex III-inhibited cells is responsible for the reduced tB-OOH-induced toxic response was investigated. Myxothiazol inhibits the electron flow from the reduced coenzyme Q to cytochrome  $c_1$  and therefore prevents the formation of ubisemiquinone [26]. Since ubisemiquinone is most likely the electron donor in antimycin A (or HQNO)-treated cells [8], we investigated the effect of myxothiazol on the antimycin A-mediated prevention of the tB-OOH-induced cytotoxic response. The cells were therefore sequentially treated with 5  $\mu$ M myxothiazol, 0.5  $\mu$ M antimycin A and 1 mM tB-OOH. As illustrated in Table 2, preventing the formation of ubisemiquinone with myxothiazol abolished the cytoprotective effects of antimycin A.

The results reported in Table 2 also indicate that the toxicity of tB-OOH was prevented by a nontoxic level of reagent hydrogen peroxide (500  $\mu$ M) or by cyclosporin A (0.5  $\mu$ M).

Table 2  
The effect of myxothiazol on the antimycin A-mediated prevention of the tB-OOH-induced cytotoxicity

Treatment	Cell death (%)
1 mM tB-OOH	57.8 $\pm$ 5.7
1 mM tB-OOH+5 $\mu$ M myxothiazol	60.3 $\pm$ 2.2
1 mM tB-OOH+5 $\mu$ M antimycin A	10.2 $\pm$ 0.5*
1 mM tB-OOH+5 $\mu$ M myxothiazol+0.5 $\mu$ M antimycin A	61.9 $\pm$ 4.5
1 mM tB-OOH+500 $\mu$ M H <sub>2</sub> O <sub>2</sub>	18.5 $\pm$ 1.8*
1 mM tB-OOH+0.5 $\mu$ M cyclosporin A	8.1 $\pm$ 1.1*

The cells were treated for 30 min with tB-OOH in the absence or presence of antimycin A and/or myxothiazol. The sequence of addition of these compounds was the same as that detailed in the legend to Fig. 2. Also shown is the effect of cyclosporin A or H<sub>2</sub>O<sub>2</sub> on the toxicity promoted by the hydroperoxide. Cyclosporin A or H<sub>2</sub>O<sub>2</sub> was given to the cultures 5 min prior to addition of tB-OOH. After treatments, the cells were rinsed and post-incubated for 6 h in drug-free culture medium and then analyzed for cytotoxicity (see Section 2). Antimycin A, myxothiazol, cyclosporin A and H<sub>2</sub>O<sub>2</sub> were not toxic when given alone to the cultures. Results represent the mean  $\pm$  S.E.M. calculated from three experiments, each performed in duplicate and were significantly different from those for cytotoxicity generated by the hydroperoxide alone at \* $P$  < 0.002 (unpaired  $t$ -test).

#### 4. Discussion

The results presented in this study indicate that the complex III inhibitors antimycin A and HQNO (Fig. 1A–B) – but not myxothiazol (Fig. 1C) – prevent the necrotic response induced by tB-OOH. Prevention of necrosis was not associated with a switch to apoptosis (not shown) and was apparent over the same dose-range in which inhibition of oxygen consumption was also observed (Table 1). The effect of antimycin A, however, was biphasic in nature since nanomolar levels of this inhibitor effectively reduced the toxic response elicited by the hydroperoxide, whereas under the same conditions a progressive appearance of dead cells was observed using micromolar concentrations of antimycin A (Fig. 1A). While the exact mechanism mediating this effect of antimycin A remains to be elucidated, two lines of evidence suggest that the onset of the lethal response was caused by toxic species resulting from the interaction of tB-OOH (or tB-OOH-derived radical species) with the fraction of antimycin A not bound to complex III. First, cytotoxicity was detected in cells pre-treated with a concentration of HQNO abolishing oxygen consumption (and possibly saturating the complex III binding sites) and then exposed to very low levels of antimycin A and 1 mM tB-OOH (Fig. 2). Under these conditions HQNO prevented the toxicity of tB-OOH and the addition of antimycin A, at concentrations which are cytoprotective in the absence of HQNO, promoted cell death. Secondly, cells made respiration-deficient by growth in either chloramphenicol or ethidium bromide were on the one hand resistant [27] to the toxicity induced by 1 mM tB-OOH (which results in approx. 50% cell death in respiration-proficient cells), but on the other hand were efficiently killed by the hydroperoxide associated with nanomolar levels of antimycin A (Fig. 2). Thus, the same concentrations of antimycin A which were cytoprotective in respiration-proficient cells promoted cell death in respiration-deficient cells challenged with a subtoxic level of tB-OOH.

These results therefore indicate that two stoichiometric inhibitors of complex III abolish the lethal effects of tB-OOH and provide an explanation for the small and transient protective effect previously observed using 5  $\mu$ M antimycin A [7]. Although these effects were observed at the same concentrations producing inhibition of oxygen utilization, the possibility that interruption of electron transport in the respiratory chain is causally linked to cytoprotection is unlikely since, at levels abolishing oxygen utilization (Table 1), the complex III inhibitor myxothiazol – which binds to a site different from that of antimycin A and HQNO [23] – did not affect the toxicity elicited by tB-OOH (Fig. 1C).

Taken together, the results thus far discussed indicate that inhibition of oxygen consumption does not in and of itself reduce the toxicity promoted by tB-OOH. Rather, it would appear that cell killing is prevented by the binding of antimycin A or HQNO to their specific sites in complex III.

In principle, this binding may prevent the mitochondrial formation of tB-OOH-derived toxic radical species. This possibility is supported by the work of O'Donnel and Burkitt [20] which suggested that the mitochondrial electron-transport chain plays an important role in the reduction of tB-OOH to free radical(s) and, in particular, identified a species inhibitable by antimycin A. Using intact U937 cells, we also demonstrated that antimycin A reduces the formation of *tert*-butoxyl as well as methyl radicals [7].

In order to assess whether the complex III-mediated reduction of tB-OOH was critical for the onset of the cytotoxic response, we investigated the effect of inhibitors of cytochrome P-450, which were also reported to reduce the formation of tB-OOH-derived radical species [18,19]. Metirapone as well as SKF 525A efficiently prevented the U937 cell killing induced by 1 mM tB-OOH (Fig. 3) but did not affect oxygen consumption (Table 1). This finding on the one hand rules out the possibility that prevention of tB-OOH toxicity is due to nonspecific interaction of these inhibitors with the antimycin A binding site in complex III, and on the other hand supports the notion that cytoprotection can be dissociated from inhibition of electron transport (see above). The most important information emerging from these experiments, however, is that the mechanism whereby antimycin A and HQNO prevent the cell killing generated by tB-OOH cannot be entirely explained by prevention of the complex III-mediated reduction of tB-OOH.

Unlike cytotoxicity, the DNA cleavage generated by tB-OOH is enhanced by antimycin A and HQNO, and this response is mediated by enforced mitochondrial formation of hydrogen peroxide [8]. These inhibitors, by preventing the electron flow from reduced cytochrome  $b_{562}$  to oxidized coenzyme Q, promote the formation of superoxides and, as a consequence, of hydrogen peroxide. Although these experiments were performed using a nontoxic concentration of tB-OOH (200  $\mu$ M) we can however assume that the same events will occur in antimycin A/HQNO-supplemented cells exposed to lethal levels of the hydroperoxide. Thus, under conditions in which the complex III inhibitors prevent the toxicity of tB-OOH, a parallel accumulation of superoxides and hydrogen peroxides will most likely occur.

These results therefore suggest that hydrogen peroxide, and the resulting DNA single strand breakage, either does not play a significant role in tB-OOH-induced cell death or is involved in the antimycin A/HQNO-mediated protective response.

In the present study we report that myxothiazol, which prevents the formation of ubisemiquinone [26], abolished the cytoprotective effects of antimycin A (Table 2). Since the formation of ubisemiquinone is expected to promote formation of hydrogen peroxide, these results strongly suggest that the oxidant mediates the cytoprotection afforded by antimycin A in cells challenged with toxic levels of tB-OOH. The finding that a nontoxic level of reagent hydrogen peroxide reduced the cell killing induced by tB-OOH (Table 2) is consistent with this mechanism.

Finally, we have provided (Table 2) indirect experimental evidence that, under the conditions utilized in the present study, the formation of permeability transition pores mediates the toxicity evoked by tB-OOH since this response was prevented by cyclosporin A (0.5  $\mu$ M), an agent which binds to cyclophilin, thereby preventing pore opening [28].

Thus, our results are in apparent contradiction with the findings of Castilho et al. [17] indicating that in isolated mitochondria the permeability transition caused by tB-OOH is mediated by hydrogen peroxide. Using experimental conditions under which the formation of permeability transition pores was the final event leading to U937 cell death after treatment with tB-OOH (Table 2), we now report that mitochondrial formation of hydrogen peroxide is causally linked to the antimycin A-mediated prevention of the toxicity evoked

by tB-OOH. Although the mechanism(s) involved in these effects are currently unknown, it is possible that cytoprotection occurs as a result of mobilization of antioxidant defences [29].

In conclusion, the results presented in this study demonstrate that the binding of antimycin A, or HQNO, to complex III of tB-OOH-treated U937 cells leads to mitochondrial formation of hydrogen peroxide, which then mediates prevention of the toxic response evoked by the hydroperoxide. Experiments are in progress to elucidate the mechanism whereby low levels of hydrogen peroxide promote cytoprotection in cells injured with tB-OOH.

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