Cell Death and Differentiation (2006) 13, 166–169

www.nature.com/cdd

Letter to the Editor

Butylated hydroxyanisole is more than a reactive oxygen species scavenger

Cell Death and Differentiation (2006) **13,** 166–169. doi:10.1038/sj.cdd.4401746; published online 2 September 2005

Dear Editor,

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are two structurally related lipophilic compounds generally used as antioxidants (Figure 1a). These synthetic phenols can scavenge reactive oxygen species (ROS) by donating a labile hydrogen to oxygen radicals derived from fatty acids and other sources, leaving an oxidized phenolic ion stabilized by the inherent resonance of the benzene ring. The antioxidant properties of BHA are also derived from its capacity to increase the levels of liver glutathione and glutathione-*S*-transferase and the activity of hepatic cytosolic gamma-glutamylcysteine synthetase. The antioxidant asset of BHA is widely exploited in food industries.

The ROS-scavenging capacities of BHA and BHT are frequently used to argue that ROS have a role in certain signaling pathways. As both compounds decrease the levels of ROS and at the same time protect against TNF-induced necrosis of L929 fibrosarcoma cells, it was concluded that ROS, formed and acting in the hydrophobic environment of the inner mitochondrial membrane, mediate this cell death process.[1,2] The same rationale led to the conclusion that ROS were also involved in dsRNA-induced necrosis of L929 cells.[3] Nevertheless, here we show that although BHA and BHT differ only slightly in their ability to reduce the levels of ROS induced by TNF stimulation of L929 cells, BHA is clearly more antinecrotic than BHT in this necrotic cell death system (Figure 1b). Both BHA and BHT reduce ROS levels and necrosis of L929 cells after stimulation with dsRNA (Figure 1c). However, BHA apparently does not block dsRNA-induced cytotoxicity, but shifts cell death from necrosis to apoptosis, whereas BHT does not modulate cell death type (Figure 1d).[3] Together, these data imply that BHA possesses additional properties that make it a more potent antinecrotic agent than BHT.

The discrepancy between the ROS-scavenging and the antinecrotic activities of BHA and BHT might be attributed to the fact that BHA is more lipophilic and less sterically hindered than BHT. However, based on the literature, we surmised that BHA might affect mitochondrial complex activities. For example, it was shown that BHA can block cell respiration by inhibiting the activity of complex I (NADH-CoQ reductase), complex II (succinate-CoQ oxidoreductase) and complex III (cytochrome *c*-ubiquinole reductase).[4–7]

However, there is no uniformity in the data with respect to the source of mitochondria used to test the inhibitory effect (rat liver cells,[4] ascites tumor cells[8] or U937 cells[7]), the method used to score the effect (measurement of oxygen consumption[4] *versus* spectrophotometric analysis of complex activities[7]) and the amount of BHA used, which ranges from 250 μM to millimolar amounts in most studies. On the other hand, to the best of our knowledge, there is no data in the literature on the effects of BHT on mammalian respiratory complexes. Most studies describing BHT-dependent inhibition of mitochondrial respiration were performed in the protozoon *Trypanosoma cruzi*. Finally, BHA and BHT may also function as uncouplers of oxidative phosphorylation by rendering the inner mitochondrial membrane permeable to protons.

We decided to examine, in a single experimental setup, the possibility that BHA and BHT might have strikingly different cell death-protective capacities because they differ in their ability to interfere with the respiratory chain. We therefore used spectrophotometric analysis to study the effects of BHA and BHT on mitochondrial complex activity of isolated mitochondria from murine skeletal muscle, using known complex inhibitors as a reference. Both BHA and BHT were able to inhibit complex I activity in a concentration-dependent manner. However, BHA was clearly more effective than BHT, although less efficient than rotenone. Neither of the agents had a significant effect on the activities of complexes II, III and IV (Figure 2a). As complex I is a major ROS-producing site in the electron transport chain (ETC),[9] BHA and to a lesser extent BHT might not only scavenge ROS but also partially prevent their complex I-mediated production. This complex I-mediated ROS production after TNF and dsRNA treatment of L929 cells is confirmed by the fact that addition of rotenone also decreases the levels of ROS (Figure 2b). Therefore, these results suggest that mitochondrial respiration is involved in TNF- and dsRNA-induced necrotic signaling. These data are confirmed by the inhibition of both TNF- and dsRNA-induced necrotic cell death by the complex I inhibitor rotenone (Figure 2c)[10] and an *in vivo* study showing partial blockage of zVAD-fmk/TNF-induced hyperacute cardiovascular collapse, renal damage and death of mice after pretreatment with rotenone.[11]

Besides being an inhibitor of electron transport and a radical scavenger, BHA was also reported to inhibit lipoxygenases (LOXs).[12] These constitute a family of monomeric nonheme, nonsulfur iron dioxygenases that catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides. The main substrate of LOXs is arachidonic acid, either in its esterified or free form depending on the type of enzyme. Lipid peroxidation by LOXs can also cause permeabilization of

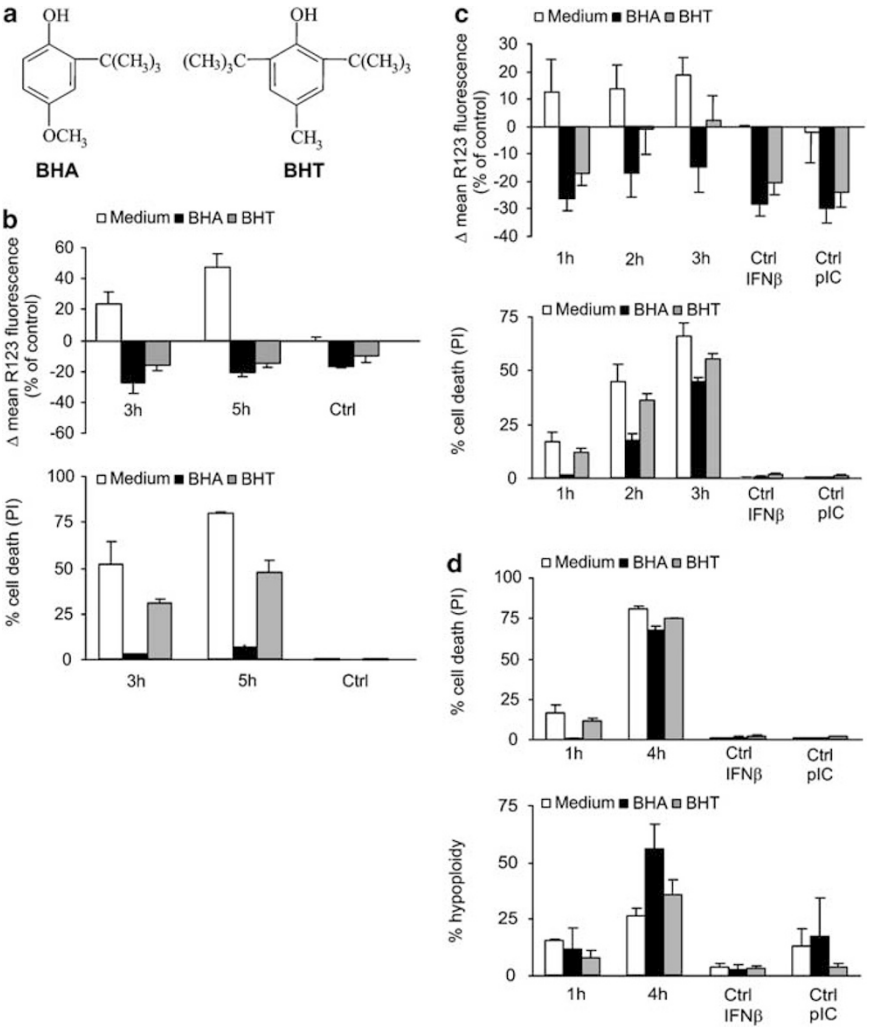

Figure 1 (**a**) Structure of BHA and BHT. (**b–d**) L929sAhFas cells were pretreated with medium (white bars), 100 μM BHA (black bars) or 100 μM BHT (gray bars) for 30 min. Finally, 10 000 IU/ml mTNF-α + 10 mM LiCl were added in each condition for the indicated durations. Control (Ctrl) cells were only treated with LiCl (**b**). In parallel, L929sAhFas cells were treated with 200 IU/ml mIFN-β for 24 h,[3] followed by medium (white bars), 100 μM BHA (black bars) or 100 μM BHT (gray bars) for 30 min. Finally 2.5 μg/ml dsRNA (pIC) was added for the indicated durations. Control (Ctrl) cells were only treated with IFN-β or dsRNA, respectively (**c** and **d**). DNA degradation was expressed as the percentage of cells with hypoploid DNA levels measured by PI (30 μM) staining after permeabilization by freezing and thawing (**d**). Cells were analyzed by flow cytometry. Activation of ROS production was determined by measuring the conversion of DHR123 to R123 in PI-negative cells. Bars represent mean values from three experiments, relative to time zero (set to 100%). Loss of membrane integrity as a measure for cell death was determined as the fraction of PI positive cells. Note that BHA inhibited death induced by TNF, but only caused a delay in death induced by IFN-β + dsRNA. Cells however died by apoptosis as shown by a higher level of hypoploid DNA. The histograms represent an average of three independent experiments with error bars indicating standard error of the mean (S.E.M)

organelle and plasma membranes.[13] As oxidative rancidity is mediated by increased LOX activity, the LOX-inhibiting activity of BHA may contribute to its action as a food preservative. To determine whether the LOX-inhibiting activity affects cell death, we compared the influence of two LOX inhibitors, nordihydroguaiaretic acid (NDGA, a general LOX inhibitor) and AA861 (a 5-LOX specific inhibitor), with the effect of BHA. In parallel, we investigated the effect of inhibiting phospholipases (PLA_2), because they are responsible for the liberation of arachidonic acid, the main substrate of LOXs, from the sn-2 position of phospholipids, and are therefore considered upstream components of the LOX signaling pathway. For this, we used a general PLA_2 inhibitor, methyl-arachidonyl fluorophosphonate (MAFP), and a specific calcium-independent PLA_2 ($iPLA_2$) inhibitor, bromoenol lactone (BEL). All of the LOX and PLA_2 inhibitors we tested inhibited cytotoxicity. However, the effect was less profound than that observed with BHA (Figure 3a). All inhibitors with the exception of MAFP, which increased basal ROS production, also markedly decreased the levels of ROS. This suggests that some LOXs and PLA_2 may play a role in signaling to or execution of necrotic cell death also by increasing the production of ROS (Figure 3b). Interestingly, it had been shown that treatment of L929 cells with TNF leads to the activation of PLA_2, whereas overexpression of Ca^{2+}-dependent PLA_2 ($cPLA_2$) sensitizes TNF-resistant L929 variants to TNF-induced cytotoxicity,[14,15] confirming that $cPLA_2$ contributes to this necrotic signaling.

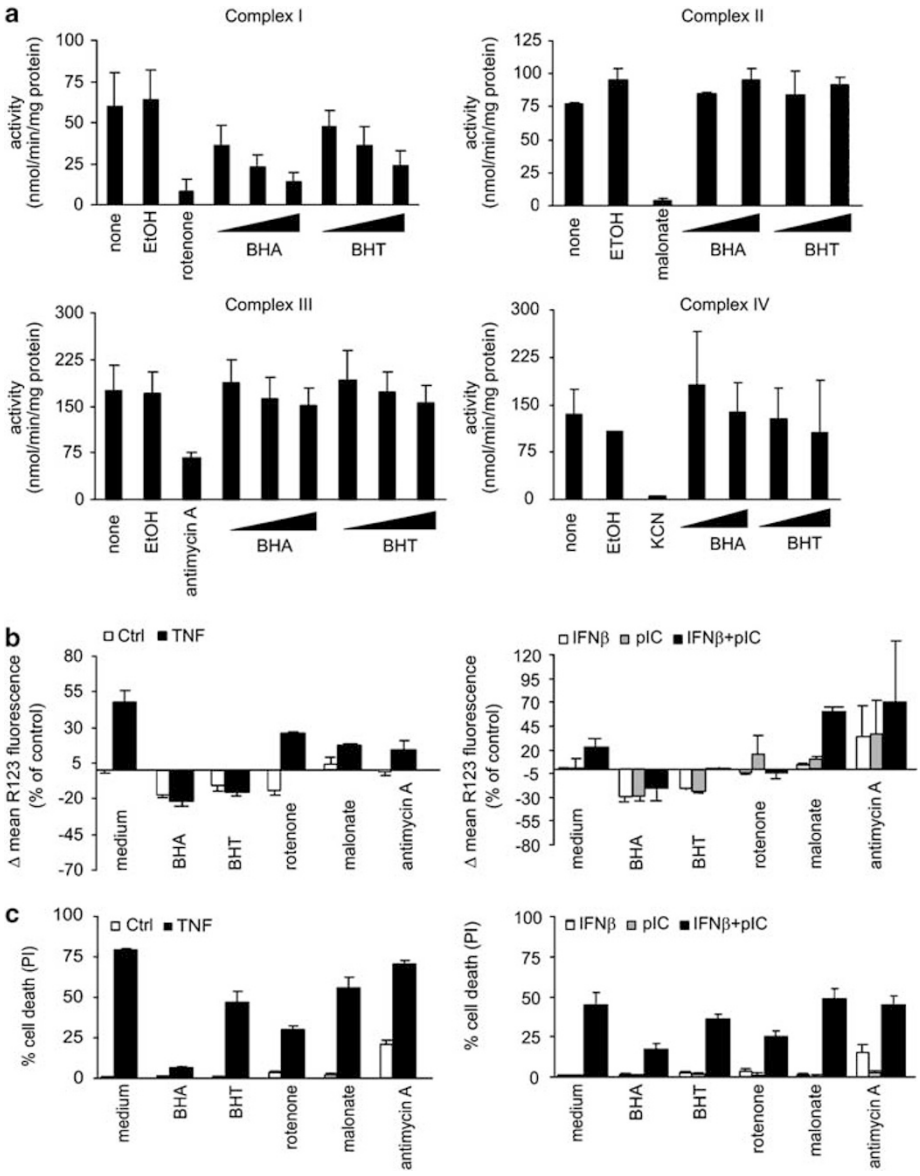

Figure 2 (**a**) For each experiment, the activities of the ETC complexes were determined spectrophotometrically using 10 independent murine skeletal muscle samples (for a detailed description of the Materials and Methods see Supplementary Information). Heavy membrane fractions were either left untreated or else treated with 100, 200 and 400 μM BHA or BHT (complexes I and III); 100 and 200 μM BHA or BHT (complexes II and IV). In all, 10 μM rotenone (CI), 5 mM malonate (CII), 1 μM antimycin A (CIII) and 0.6 mM KCN (CIV) were included as positive controls for inhibition of the activities of complexes I, II, III and IV, respectively. Results are corrected for mitochondrial input as determined by citrate synthase activity and are presented as the average activity of ETC complexes; error bars indicate S.E.M. (**b** and **c**) L929sAhFas cells were pretreated during 30 min with medium, 100 μM BHA, 100 μM BHT, 25 μM rotenone, 5 mM malonate or 50 μM antimycin A. Finally, 10 000 IU/ml mTNF-α + 10 mM LiCl were added for 5 h in each condition. Control (Ctrl) cells were only treated with LiCl (left panels). Cells were treated with 200 IU/ml mIFN-β for 24 h, followed by medium, 100 μM BHA, 100 μM BHT, 25 μM rotenone, 5 mM malonate or 50 μM antimycin A for 30 min. Finally, 2.5 μg/ml dsRNA (pIC) was added for 2 h. Control cells were only treated with IFN-β or dsRNA, respectively (right panels). Cell death (**c**) and ROS production (**b**) were determined by FACS as the fraction of cells that are PI positive and the conversion of DHR123 to R123 by PI negative cells, respectively. Histograms show an average of two independent experiments with error bars indicating S.E.M. Results are representative for six independent experiments

PLA_2 has also been identified as another source of ROS in a study examining TNF-induced caspase-independent cell death *in vivo*.[11]

The ROS-scavenging capacity of BHA has often been used to argue that ROS play a role in certain signaling pathways and in necrotic cell death in particular. Our results, however, emphasize the importance of considering all properties of BHA. We show that the strong antinecrotic effect of BHA reflects not only its ROS scavenging property but also its ability to inhibit complex I and LOXs. Moreover, our data suggest that deregulation of the function of mitochondrial complex I and activation of a PLA_2/LOX pathway contribute to TNF- and dsRNA-induced ROS production, and consequently to necrotic cell death.

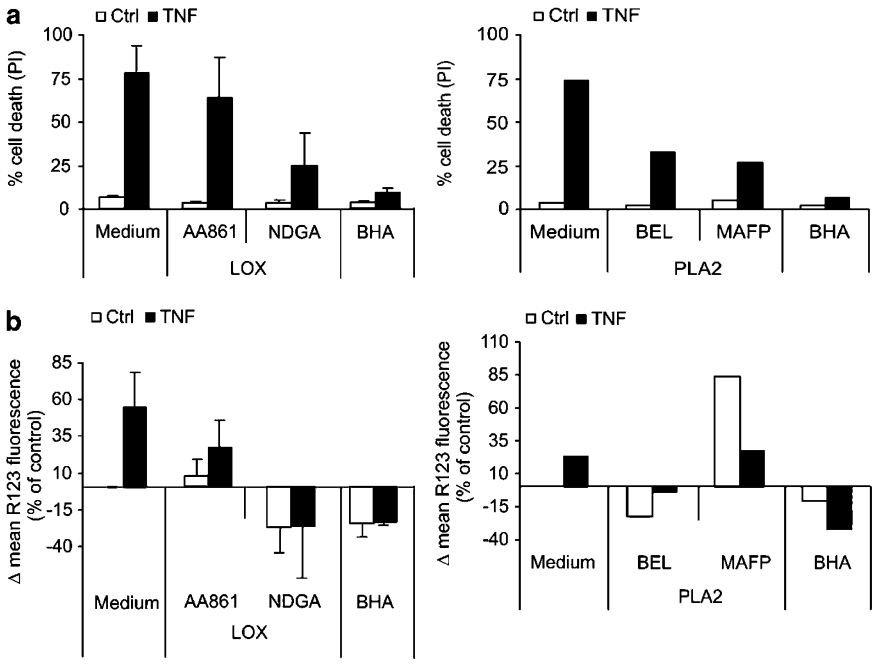

Figure 3 (**a** and **b**) L929sAhFas cells were either pretreated for 30 min with medium, 100 μM BHA and 50 μM AA861 or 100 μM NDGA (left panels) and 100 μM MAFP or 30 μM BEL (right panels), and then treated with either 10 mM LiCl (Ctrl, white bars) or 10 000 IU/ml mTNF-α + 10 mM LiCl (black bars). Cell death (**a**) and ROS production (**b**) were determined by FACS as the fraction of cells that are PI positive and the conversion of DHR123 to R123 by PI negative cells, respectively. Histograms in the left panels represent an average of three independent experiments with error bars indicating S.E.M. Histograms in the right panels are representative of three independent experiments

Acknowledgements

We thank Amin Bredan for editorial help. This work was supported in part by the Interuniversitaire Attractiepolen V (IUAP-P5/12-120C1402), the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (Grant 3G.0006.01), an EC-RTD Grant (QLG1-CT-1999-00739), a UGent-cofinancing EU Project (011C0300) and GOA Project (12050502). Furthermore, research in the unit of Peter Vandenabeele is supported by the Belgian Federation against Cancer. N Festjens was supported by a grant from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) and IUAP-P5/12-120C1402, X Saelens by the 'Biotech Fonds' and GOA Project (12050502), Michael Kalai was paid by the EC-RTD Grant and Ann Meeus by the FWO Grant 3G.0006.01 and by the Ghent University.

N Festjens[1], M Kalai[1,2], J Smet[3], A Meeus[1], R Van Coster[3], X Saelens[1,4] and P Vandenabeele[*,1]*

[1] Molecular Signalling and Cell Death Unit, Department for Molecular Biomedical Research, Ghent University and Flemish Interuniversity Institute for Biotechnology (VIB), Technologiepark 927, Ghent 9052, Belgium
[2] Laboratory of Cellular Microbiology, Unit of Molecular Microbiology, Institute Pasteur of Brussels, Engelandstraat 642, Brussels 1180, Belgium
[3] Department of Pediatrics, Division of Pediatric Neurology and Metabolism, Ghent University Hospital, De Pintelaan 185, Ghent 9000, Belgium
[4] Molecular Virology Unit, Department for Molecular Biomedical Research, Ghent University and VIB, Technologiepark 927, Ghent 9052, Belgium
[*] Corresponding author: P Vandenabeele, Molecular Signalling and Cell Death Unit, Department for Molecular Biomedical Research, Ghent University and Flemish Interuniversity Institute for Biotechnology (VIB), Fiers-Schell-Van Montagu Building, Technologiepark 927, Ghent (Zwijnaarde) 9052, Belgium. Tel: + 32-9-33-13-760; Fax: + 32-9-33-13-609; E-mail: peter.vandenabeele@dmbr.UGent.be

1. Matthews N *et al.* (1987) Immunology 62: 153–155
2. Goossens V *et al.* (1995) Proc. Natl. Acad. Sci. USA 92: 8115–8119
3. Kalai M *et al.* (2002) Cell Death Differ. 9: 981–994
4. Ferreira J (1990) Biochem. Pharmacol. 40: 677–684
5. Uslu R and Bonavida B (1996) Cancer 77: 725–732
6. Nakagawa Y *et al.* (1994) Eur. J. Pharmacol. 270: 341–348
7. Okubo T *et al.* (2004) Biol. Pharm. Bull. 27: 295–302
8. Fones E *et al.* (1989) Biochem. Pharmacol. 38: 3443–3451
9. Barja G (1999) J. Bioenerg. Biomembr. 31: 347–366
10. Goossens V *et al.* (1999) Antioxidants Redox Signal. 1: 285–295
11. Cauwels A *et al.* (2003) Nat. Immunol. 4: 387–393
12. Slapke J *et al.* (1983) Biomed. Biochim. Acta 42: 1309–1318
13. Van Leyen K *et al.* (1998) Nature 395: 392–395
14. Suffys P *et al.* (1991) Eur. J. Biochem. 195: 465–475
15. Hayakawa M *et al.* (1993) J. Biol. Chem. 268: 11290–11295

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)