Cancer Chemother Pharmacol (2004) 54: 537–545
DOI 10.1007/s00280-004-0845-7

ORIGINAL ARTICLE

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Synergistic killing of human leukemia cells by antioxidants and trichostatin A

Received: 1 February 2004 / Accepted: 19 April 2004 / Published online: 10 July 2004
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Abstract *Purpose*: Antioxidants and trichostatin A (TSA) are promising anticancer drugs, and are capable of enhancing the neoplastic toxicity of other chemicals that exert anticancer activity via different mechanisms. Since antioxidants and TSA (the specific inhibitor of histone deacetylase) are believed to combat cancer via different mechanisms, we sought to determine whether combining them would improve their anticancer activity in human leukemia cells (HL-60). *Materials and methods*: HL-60 cells were treated with antioxidants (ascorbic acid, AA and *N*-acetyl-cysteine, NAC), TSA or their combination, and cell proliferation arrest, lactate dehydrogenase (LDH) release and cell viability were measured as indicators of cell damage. Accumulation of reactive oxygen species (ROS) and the acetylation of histones were also measured. *Results*: The cytotoxicity of AA, NAC and TSA increased in a time- and dose-dependent manner. AA (1, 2 and 4 m*M*) and NAC (0.2, 0.5 and 1 m*M*) were able to diminish ROS generation but showed no influence on histone acetylation in HL-60 cells. In contrast, TSA (20, 50, 100 and 200 n*M*) did not inhibit ROS generation but significantly increased histone acetylation, indicating a possible role for both scavenging ROS and increasing histone acetylation in the induction of cell death in HL-60 cells. This conclusion was further confirmed by the finding that the combination of antioxidant and TSA not only diminished ROS generation, but also increased histone acetylation, and hence showed greater cytotoxicity in HL-60 cells than either component alone. *Conclusions*: Our findings show that combining antioxidants and TSA can enhance their neoplastic toxicity at least in human leukemia HL-60 cells, providing a new approach to the design of chemotherapy strategies and the development of anticancer drugs.

Keywords Cytotoxicity · Reactive oxygen species · Histone acetylation · Ascorbic acid · *N*-acetyl-cysteine · Trichostatin A

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Introduction

Cancer therapy has improved in recent years owing to the better understanding of the mechanisms of cell death and the development of better-designed chemotherapy strategies. Since reactive oxygen species (ROS) are critically involved in the different stages of carcinogenesis [1], antioxidants have long been used for the treatment of cancer, especially in combination with other anticancer drugs [2–15]. Despite the theoretical concern that antioxidant therapies interfere with chemotherapy and radiation by lowering oxidative damage [8, 16–18], evidence supporting this mechanism is currently lacking [13]. In contrast, it is well known that antioxidants enhance the antitumor effects of chemotherapy in vitro and in vivo [2, 3, 8, 12, 15, 18]. There is also evidence that antioxidants act as therapeutic biologic response modifiers and are able to directly induce differentiation and apoptosis in already established neoplastic cells [4, 5, 12, 14–16].

Recent studies have shown that histone hypoacetylation plays an important role in gene silencing and carcinogenesis [19–21]. Specifically, histone acetylation contributes to the formation of a transcriptionally competent environment by ''opening'' chromatin, and permits access of transcription factors to DNA [22, 23]. Conversely, histone deacetylation contributes to a

"closed" chromatin state and transcriptional repression. The histone acetylation–deacetylation balance favors hypoacetylation in tumor cells [19], while it is accurately maintained through a balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme activities in normal cells [19, 20]. The regulation of the transcription of a particular gene is dependent on the status of histone acetylation in close proximity to the target gene [19, 20, 22]. Hypoacetylation in tumor cells may selectively disrupt the transcriptional initiation of genes for differentiation and apoptosis, such as p53, APC, $p14^{ARF}$, and K-ras [24]. Thus increasing the acetylation of histones in tumor cells through inhibiting the activity of HDAC has been suggested as an efficient strategy to combat cancer, and hence HDAC inhibitors are thought to be promising as single anticancer agents or in combination therapies [25–31]. Among the HDAC inhibitors, trichostatin A (TSA) has been well studied and has been found to exhibit cytotoxicity in different tumor cell lines [27–30].

Considering that antioxidants and TSA exert their anticancer activity through different mechanisms, combining them may significantly improve their cytotoxicity to neoplastic cells. To address this hypothesis, the cytotoxicity of antioxidants, TSA and their combinations was studied in human leukemia HL-60 cells, and their effects on ROS generation and histone acetylation were also evaluated. Both antioxidants and TSA showed cytotoxicity against HL-60 cells, and their combinations showed significantly greater cytotoxicity. These results suggest a new approach (simultaneously diminishing ROS generation and increasing histone acetylation) to designing a chemotherapy strategy at least for human leukemia.

Material and methods

Reagents

Ascorbic acid (AA), *N*-acetyl-cysteine (NAC), TSA, trypsin and trypan blue were purchased from Sigma (Sigma, St Louis, Mo.), RPMI-1640 was purchased from Gibco (Santa Clara, Calif.). All other reagents are of analytical grade.

Cell culture and treatment

Human leukemia cells (HL-60) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (equivalent to 100 U/ml and 100 mg/ml, respectively) at 37°C in a humidified atmosphere containing 5% CO_2. After culturing the cells (1×10^5 cells/ml) for 24 h, the culture medium was replaced with new medium containing AA, NAC and TSA as indicated, after that, the different cultures were replaced with fresh medium containing the corresponding reagents every 24 h.

Determination of cell proliferation and viability

Cells at 1×10^5/ml were cultured for 24 h, then treated with AA, NAC and/or TSA at the indicated concentrations. Three dishes for each treatment were collected every 8 h during the first 2 days, and the total and dead cells were counted using the trypan blue staining exclusion method under a phase-contrast microscope.

Measurement of lactate dehydrogenase (LDH)

LDH release was measured in a 100-μl aliquot of cellular suspension using an assay that monitored the decrease in absorbance at 340 nm during the reduction of pyruvate [32]. LDH release is expressed as a percentage in relation to the total LDH released from cells treated with 10% Triton X-100.

Measurement of intracellular ROS generation

The levels of intracellular ROS were determined in terms of the alteration of fluorescence resulting from oxidation of 29,79-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, Ore.) [33]. DCFH-DA was dissolved in DMSO to a final concentration of 20 m*M* before use. For the measurement of ROS, cells were incubated with 10 μ*M* DCFH-DA at 37°C for 30 min, then the excess DCFH-DA was washed with RPMI-1640 medium prior to treatment with AA, NAC and/or TSA for the indicated times. The intensity of fluorescence was determined by flow cytometry (Becton Dickinson), with an excitation filter of 485 nm and an emission filter of 535 nm. The ROS levels were calculated as the ratio mean intensity of exposed cells to that of unexposed cells.

Histone purification and histone acetylation assay

Cells were plated at a density of 2×10^5/ml, exposed to AA, NAC and/or TSA as indicated in the presence of 10 μCi/ml [^{3}H]acetate (5.0 Ci/mmol) for the indicated times. Preparation of histones from HL-60 cells was done according to the method described by Cousens et al. [34] with the following modifications. The washed cells were suspended in lysis buffer [34] containing TSA (100 ng/ml) and PMSF (1 m*M*). After pipetting up and down 20 times, the nuclei were washed three times in the lysis buffer and once in 10 m*M* Tris and 13 m*M* EDTA (pH 7.4). The histones were extracted from the pellet in 0.4 *N* H_2SO_4. After centrifugation, the histones in the supernatant were collected by cold acetone precipitation, air-dried, then suspended in 4 *M* urea and stored at −20°C until use. ^{3}H-Labelled histones were determined by liquid scintillation counting.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA post-hoc Bonferroni). *P* values less than 0.05, 0.01, and 0.001 are denoted *, **, and ***, respectively.

Results

Cytotoxicity of antioxidants and TSA in HL-60 cells

Treating cells with AA, NAC or TSA resulted in a concentration-dependent and time-dependent arrest in the proliferation of HL-60 cells (Fig. 1). Under the same conditions, LDH leakage, an indicator of plasmatic membrane damage [35], increased (Fig. 2), while the viability of cells significantly decreased (Fig. 3), proving the cytotoxicity of AA, NAC and TSA in HL-60 cells.

Effect of antioxidants, TSA and their combinations on the ROS generation and histone acetylation

To evaluate ROS generation in our system, HL-60 cells preloaded with DCFH-DA, commonly used to detect the generation of ROS in cells [33] were exposed to AA, NAC, TSA or the indicated combinations for 8 h.

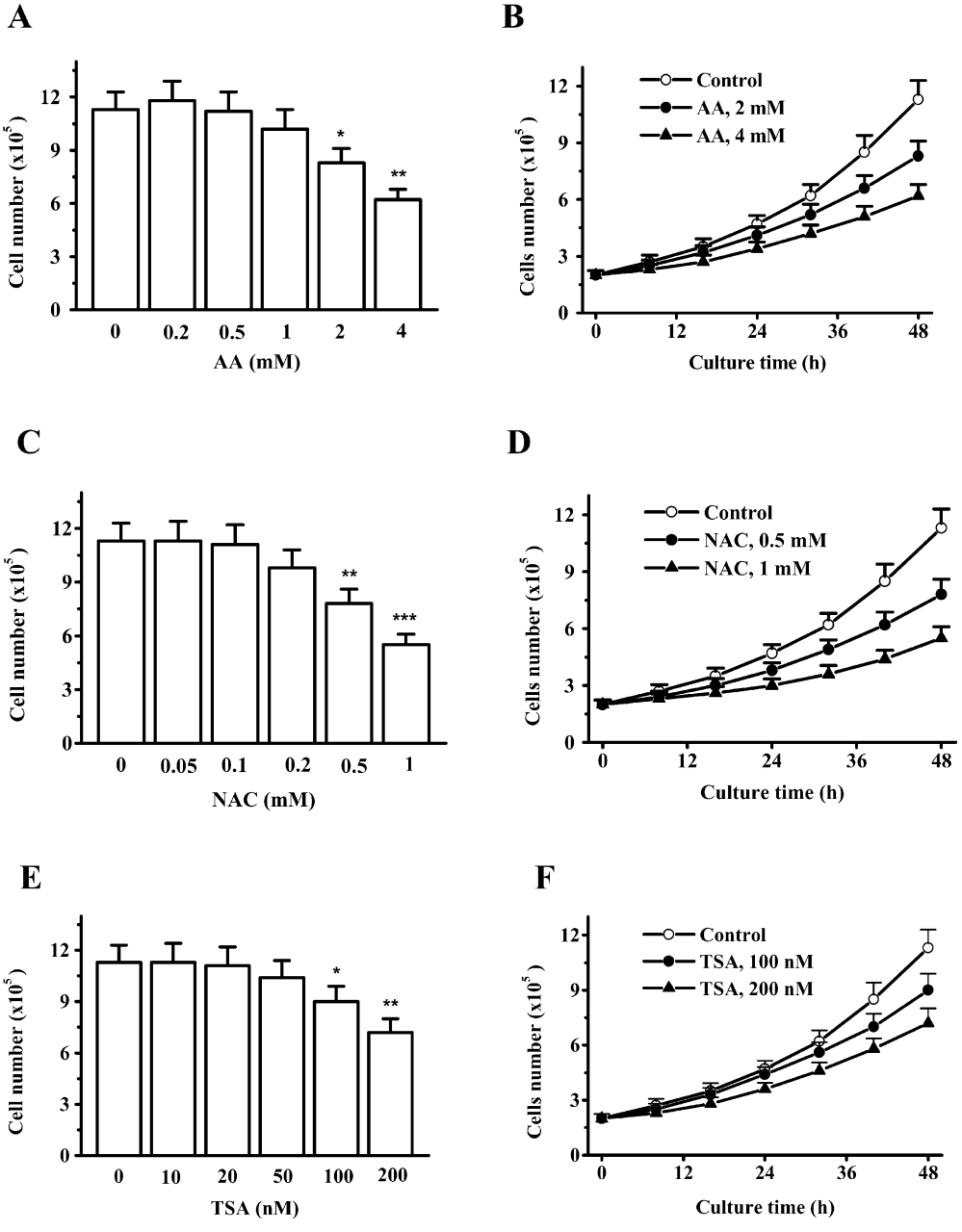

Fig. 1a–f Effects of AA, NAC and TSA on the proliferation of HL-60 cells. Cells were incubated with different concentrations of AA, NAC and TSA for 48 h (**a**, **c**, **e**) or with the indicated concentrations of AA, NAC and TSA for different times (**b**, **d**, **f**). The means ± SD of three parallel experiments are indicated, $n = 3\times3$ cultures per condition; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs the control group

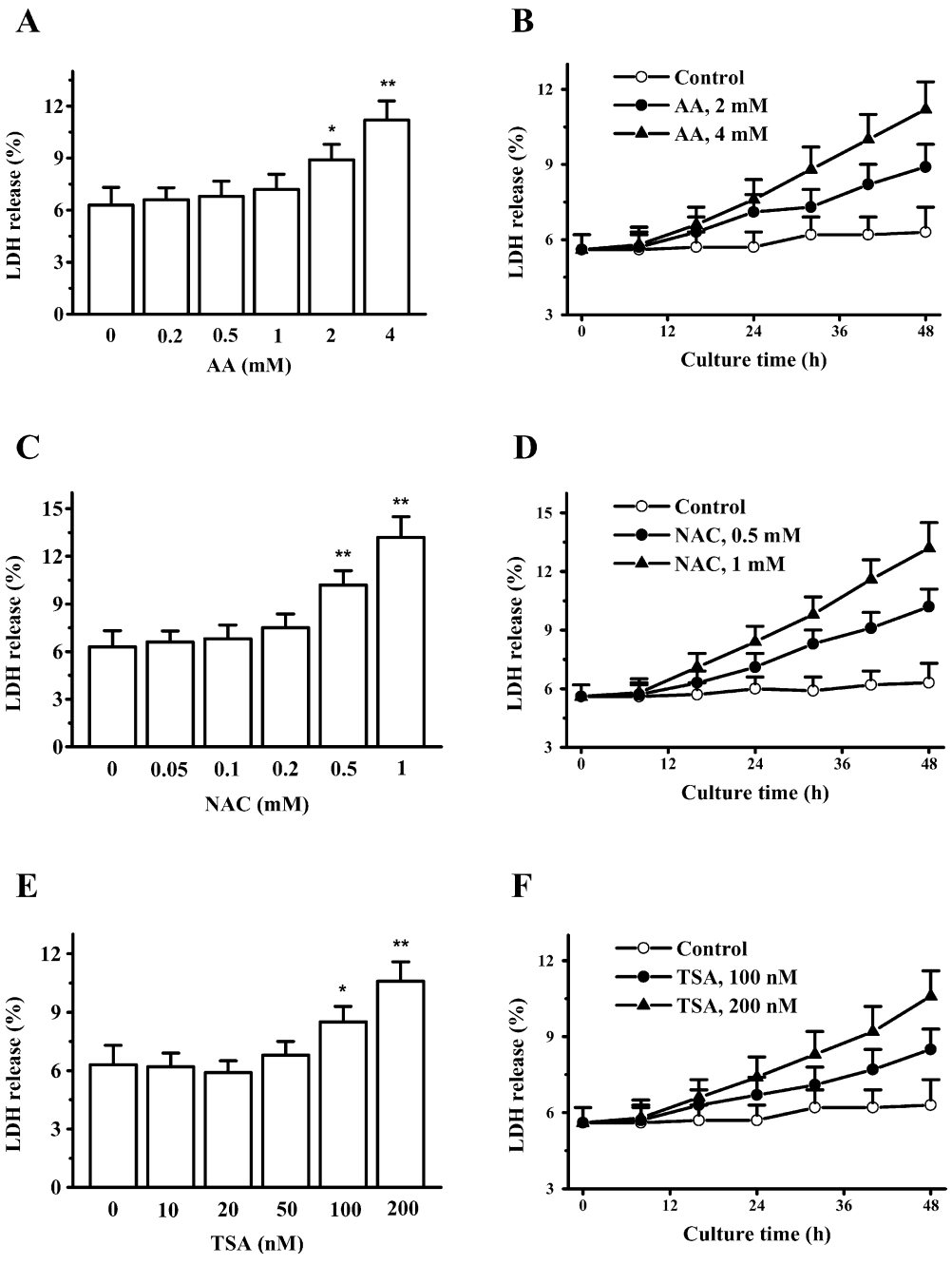

Fig. 2a–f Effects of AA, NAC and TSA on LDH release in HL-60 cells. Cells were incubated with different concentrations of AA, NAC and TSA for 48 h (**a**, **c**, **e**) or with the indicated concentrations of AA, NAC and TSA for different times (**b**, **d**, **f**). Means ± SD of three parallel experiments are indicated, $n = 3\times3$ cultures per condition; $^{*}P < 0.05$, $^{**}P < 0.01$, vs the control group

AA and NAC treatment dose-dependently diminished ROS generation (Fig. 4a, b), while TSA showed no effect on ROS accumulation in HL-60 cells (Fig. 4c). The combination of antioxidants and TSA significantly attenuated ROS generation (Fig. 4d, e), indicating that the combination of antioxidants with TSA did not weaken the ROS scavenging activity of antioxidants. At the same time, the state of histone acetylation was also evaluated in differently treated cells. AA and NAC had no obvious effect on the acetylation of histones (Fig. 5a, b), while TSA significantly increased histone acetylation in HL-60 cells in a dose-dependent manner (Fig. 5c). Similar to the results shown in Fig. 4, the combination of antioxidant and TSA significantly increased histone acetylation (Fig. 5d, e), indicating that the effect of TSA in increasing histone acetylation was not weakened by combining it with an antioxidant. In other words, the combinations possessed both the characteristic of antioxidants and the activity of TSA. Considering the importance of scavenging ROS and increasing histone acetylation in the cancer therapy, these results suggest that combining an antioxidant with TSA may improve the anticancer activity of each. To test this hypothesis, the cytotoxicities of the antioxidants and TSA in combination in HL-60 cells were determined.

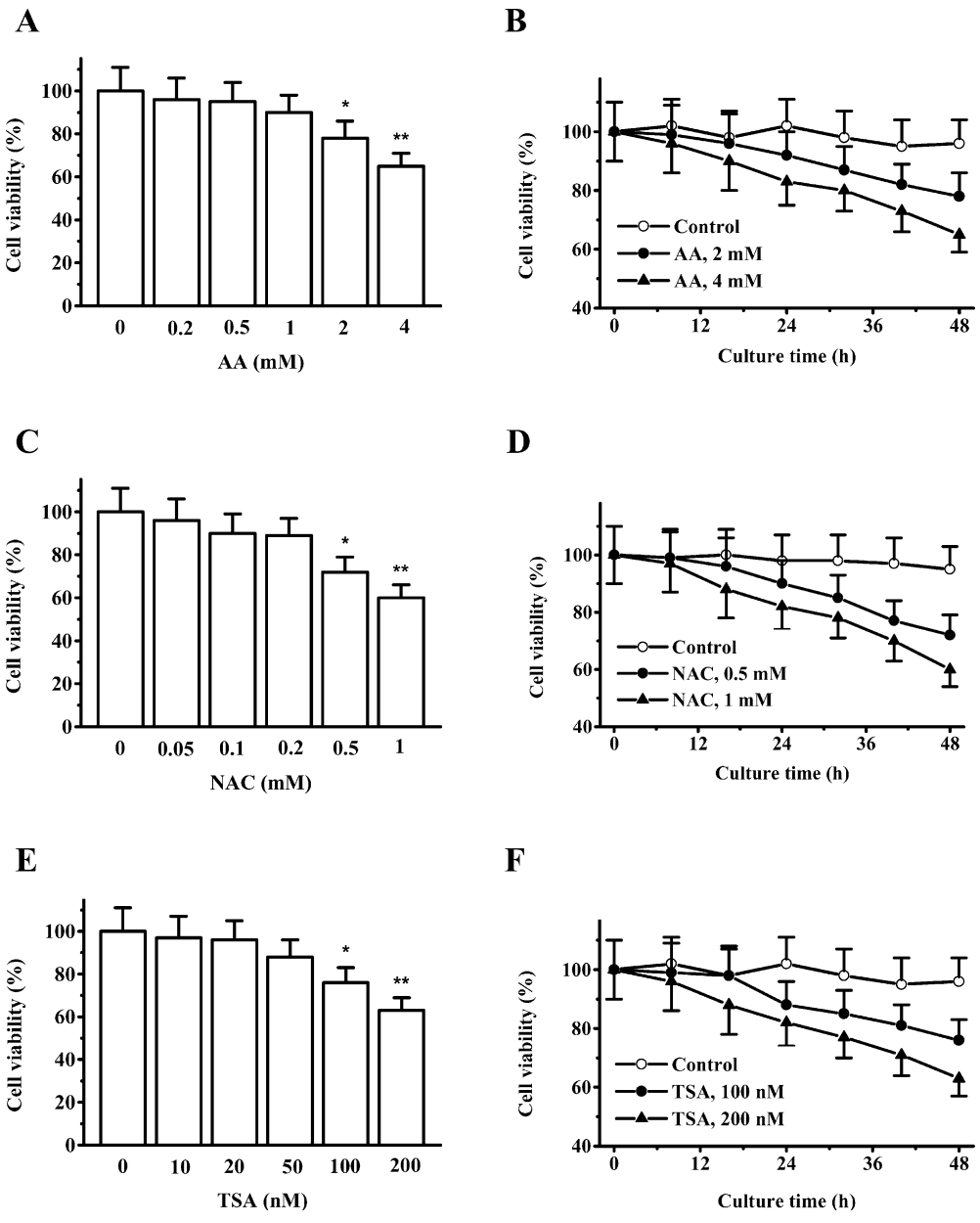

Fig. 3a–f Effects of AA, NAC and TSA on the viability of HL-60 cells. Cells were incubated with different concentrations of AA, NAC and TSA for 48 h (**a**, **c**, **e**) or with the indicated concentrations of AA, NAC and TSA for different times (**b**, **d**, **f**). Means ± SD of three parallel experiments are indicated, $n = 3\times3$ cultures per condition; $^{*}P < 0.05$, $^{**}P < 0.01$, vs the control group

The cytotoxicities of antioxidants and TSA in HL-60 cells are improved in combination

The effects of antioxidants and TSA on proliferation arrest (Fig. 6a, b), LDH release (Fig. 6c, d) and cell death induction (Fig. 6e, f) were significantly improved in combination. Further analysis indicated that antioxidants and TSA were synergistic in their anticancer activity. For example, 1 m*M* AA, 100 n*M* TSA and the combination of 1 m*M* AA and 100 n*M* TSA resulted in inhibition of cell proliferation of about 9.7%, 20.4% and 45.1%, respectively, increases in LDH release of about 14.3%, 34.9% and 93.7%, respectively, and induction of cell death of about 10%, 24% and 50%, respectively. In addition, although neither 1 m*M* AA nor 50 n*M* TSA showed obvious effects on proliferation arrest, LDH release or cell viability, their combination significantly induced these processes.

Discussion

Both antioxidants and TSA are promising anticancer drugs, and are capable of improving the efficiency of chemotherapy when combined with other anticancer drugs [2, 3, 8, 12, 15, 18, 29–31], but as far as we know, no reports are currently available on the anticancer effects of antioxidants and TSA in combination. Considering the important role of ROS generation and histone hypoacetylation in the process of carcinogenesis [1, 19–21], to evaluate the neoplastic toxicity of antioxidants and TSA in combination would probably provide

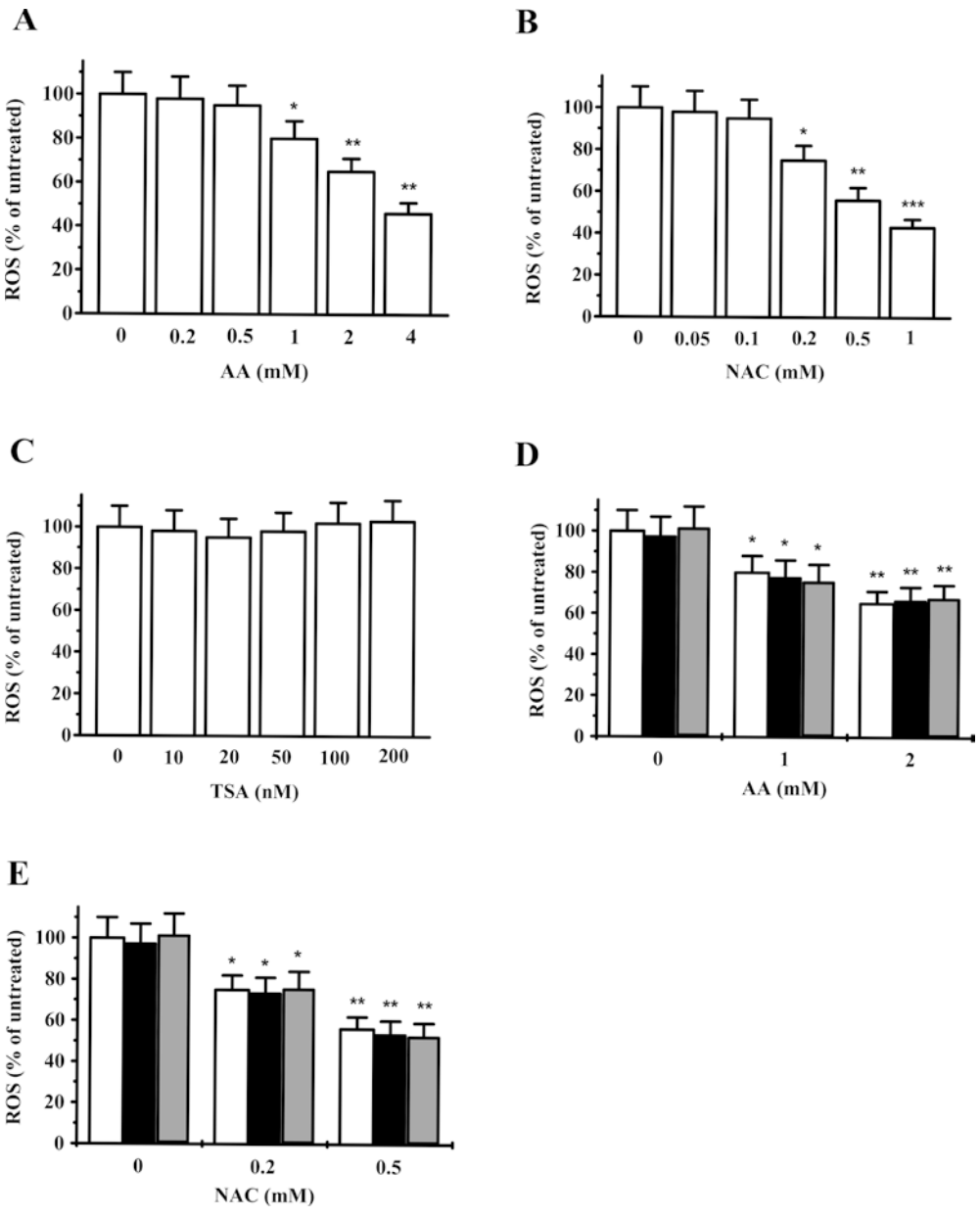

Fig. 4a–e Effects of antioxidants, TSA and their combinations on ROS generation. **a–c** Relative ROS generation in HL-60 cells exposed to the indicated agents for 8 h. **d**, **e** Cells were treated with different concentrations of antioxidants in the absence (*white bars*) or presence of TSA at 50 n*M* (*black bars*) and 100 n*M* (*gray bars*). The data presented are means ± SD of six independent measurements; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs the control group

new approaches to the design of chemotherapy strategies and anticancer chemicals.

AA is an essential nutrient involved in many biochemical functions, and its biochemical roles are closely related to its antioxidant activity. One of the most studied and, until recently, most controversial therapies is the use of high-dose AA [6, 7, 10, 36, 37]. Recent work has increased the understanding of the activity of AA, and has shown that AA at doses many times over the RDA is preferentially cytotoxic to neoplastic cells [2, 6–8]. At doses in the gram range, it has been demonstrated to increase survival time of patients with malignancies [6, 7, 10]. Thus AA was selected as a suitable antioxidant in this study. Another antioxidant selected was NAC, which has been used successfully for investigating the role of ROS in numerous biological and pathological processes [38]. Because both AA and TSA have been found to be cytotoxic in human leukemia cells [3, 30, 39], human leukemia HL-60 cells were used as model cells.

Treating HL-60 cells with AA, NAC or TSA resulted in a dose-dependent and time-dependent increase in cell proliferation arrest, LDH release and cell death, proving the cytotoxicity of AA, NAC and TSA in human leukemia cells. As expected, both AA and NAC significantly scavenged ROS generation, while TSA dramatically increased histone acetylation in HL-60 cells. A certain amount of ROS has been proven to be beneficial and necessary for the survival and proliferation of mammalian cells [40–43]. Thus the anticancer activity of AA and NAC is probably due to their efficient scavenging of ROS necessary for the survival of leukemia cells. In contrast, the anticancer activity of TSA clearly

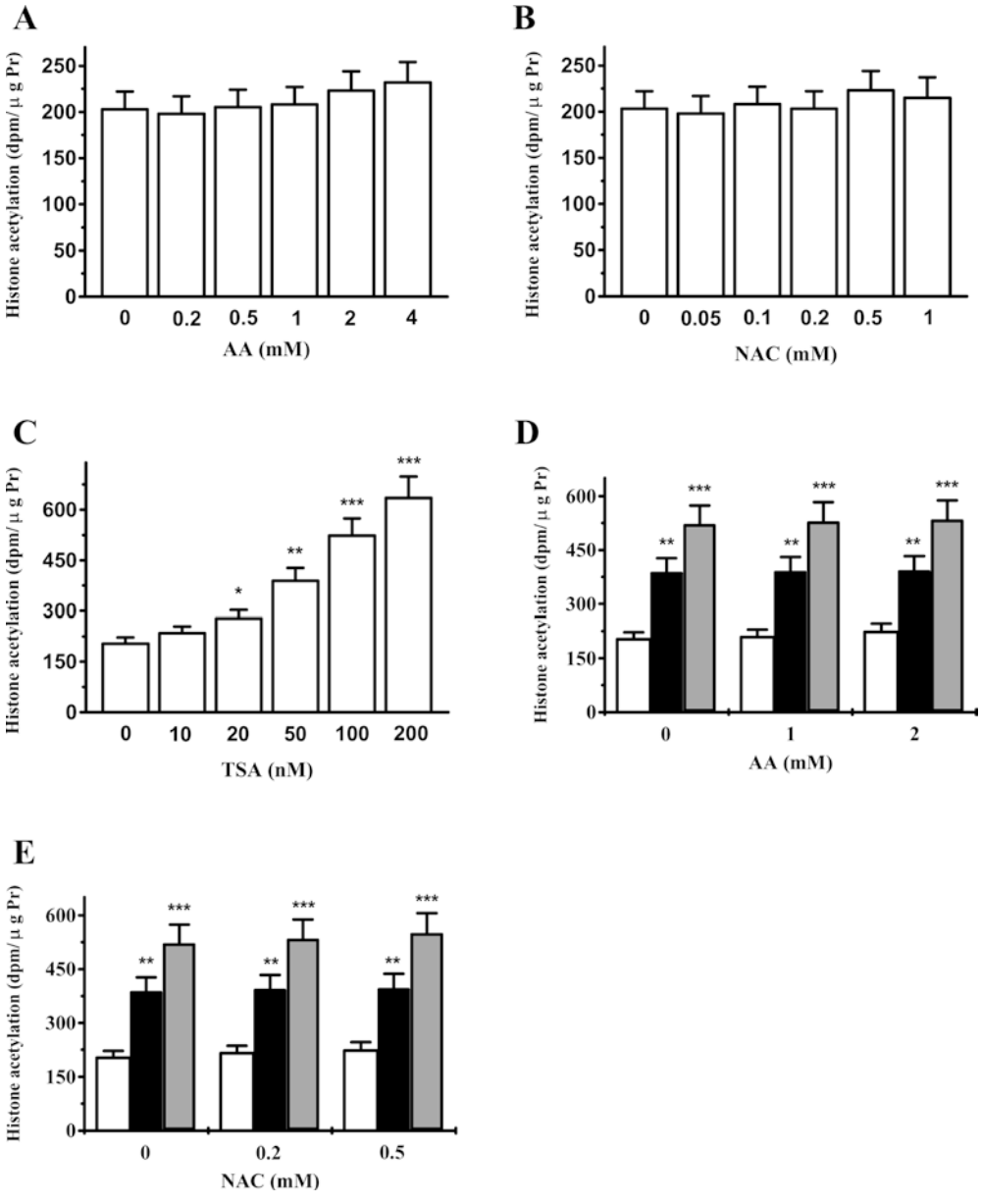

Fig. 5a–e Effects of antioxidants, TSA and their combinations on acetylation of histones. In the presence of 10 μCi/ml [^{3}H]acetate, HL-60 cells were incubated with the indicated agents for 8 h. The radioactivities of the [^{3}H]acetylated histones isolated from the cells were determined by liquid scintillation counting. **d**, **e** Cells were treated with different concentrations of antioxidants in the absence (*white bars*) or presence of TSA at 50 n*M* (*black bars*) and 100 n*M* (*gray bars*). The data presented are means ± SD of three parallel experiments, $n = 3\times3$ cultures per condition; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs the control group

cannot be explained by its ROS scavenging activity, since it does not scavenge ROS at all. Consistent with previous reports [26–30], TSA may induce the death of leukemia cells through increasing histone acetylation. As we have noted, the dose-dependent cytotoxicities of antioxidants and TSA were measured after cells were treated for 48 h, while ROS generation and histone acetylation were detected after treatment for 8 h, mainly because the significant cell death caused by treating cells for 48 h may inevitably result in a decrease in ROS generation and histone acetylation, and hence influence the evaluation of the effect of antioxidants and TSA, while treating cells for 8 h did not lead to obvious cell death.

Both antioxidants and TSA not only possess obvious anticancer activity [4, 5, 12, 14–16, 26–30], but can also improve the neoplastic toxicity of many chemicals that combat cancers through different mechanisms [2, 3, 8, 12, 15, 18, 31]. Since the antioxidants and TSA exhibited their anticancer activity in our system through scavenging ROS and increasing histone acetylation, respectively, and the ROS scavenging activity of the antioxidants and the activity of TSA in increasing histone acetylation were not weakened when they were combined (Figs. 4 and 5), their combination should show significantly improved anticancer activity. As we expected, the anticancer activity of the combinations was indeed improved, confirming that both scavenging ROS and increasing histone acetylation play important roles in the induction of HL-60 cell death. Although further studies are needed to clarify the mechanisms involved in the cytotoxicity of antioxidants, TSA and their combinations in human leukemia cells, and the nature of antioxidants and TSA-induced cell death, we

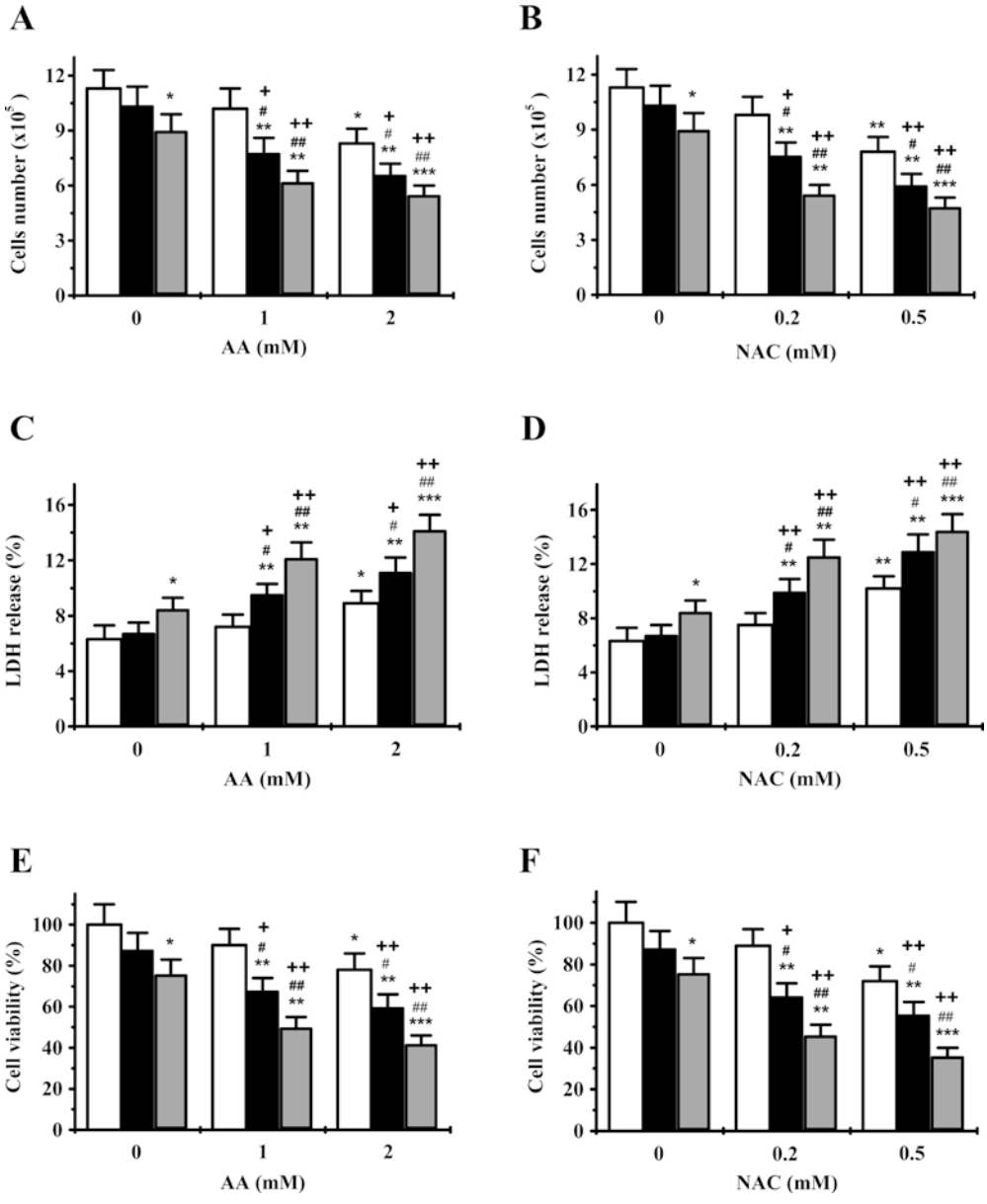

Fig. 6a–f Effect of the combination of antioxidants and TSA on their cytotoxicity. Cells were treated for 48 h in the absence (*white bars*), or presence of TSA at 50 n*M* (*black bars*) and 100 n*M* (*gray bars*), then cell proliferation, LDH leakage and cell viability were measured as described in Materials and methods. The data presented are means ± SD of three parallel experiments, $n = 3 \times 3$ cultures per conditions; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs the group treated with the corresponding AA or NAC; $^{+}P < 0.05$, $^{++}P < 0.01$, vs the group treated with 50 or 100 n*M* TSA alone

proved the synergistic anticancer activity of antioxidants and TSA in human leukemia cells.

In summary, we conclude that both scavenging ROS and increasing histone acetylation can lead to the death of HL-60 cells, and hence both antioxidants and HDAC inhibitors, such as TSA, are suitable agents for chemotherapy of human leukemia cells. Moreover, combining antioxidants and HDAC inhibitors, especially TSA, produced significantly improved anticancer activity, providing a new approach to the design of efficient chemotherapy strategies and better anticancer drugs.

References

1. Deshpande SS, Irani K (2002) Oxidant signalling in carcinogenesis: a commentary. Hum Exp Toxicol 21:63–64
2. Park CH, Amare M, Savin MA, Hoogstraten B (1980) Growth suppression of human leukemic cells in vitro by L-ascorbic acid. Cancer Res 40:1062–1065
3. Park CH, Kimler BF (1991) Growth modulation of human leukemic, preleukemic, and myeloma progenitor cells by L-ascorbic acid. Am J Clin Nutr 54:1241S–1246S
4. Kang J, Shi Y, Zheng R (1999) Effects of ascorbic acid on human hepatoma cell proliferation and redifferentiation. Acta Pharmacol Sin 20:1019–1024
5. Kang J, Shi Y, Zheng R (2000) Effects of ascorbic acid and DL-alpha-tocopherol on human hepatoma cell proliferation and redifferentiation. Acta Pharmacol Sin 21:348–352
6. Riordan NH, Riordan HD, Meng YL, Jackson JA (1995) Intravenous ascorbate as a tumor cytotoxic chemotherapeutic agent. Med Hypotheses 44:207–213
7. Riordan NH, Riordan HD, Casciari JP (2000) Clinical and experimental experiences with intravenous vitamin C. J Orthomol Med 5:201–213
8. Prasad KN, Hernandez C, Edwards-Prasad J, Nelson J, Borus T, Robinson WA (1994) Modification of the effect of tamoxifen, cisplatin, DTIC and interferon-2b on human melanoma

cells in culture by a mixture of vitamins. Nutr Cancer 22:233–245
9. Prasad KN, Kumar A, Kochupillai V, Cole WC (1999) High doses of multiple antioxidant vitamins: essential ingredients in improving the efficacy of standard cancer therapy. J Am Coll Nutr 18:13–25
10. Cameron E, Pauling L (1976) Supplemental ascorbate in the supportive treatment of cancer: prolongation of survival times in terminal human cancer. Proc Natl Acad Sci U S A 73:3685–3689
11. Drisko JA, Chapman J, Hunter VJ (2003) The use of antioxidant therapies during chemotherapy. Gynecol Oncol 88:434–439
12. Conklin KA (2000) Dietary antioxidants during cancer chemotherapy: impact on chemotherapeutic effectiveness and development of side effects. Nutr Cancer 37:1–18
13. McCall MR, Frei B (1999) Can antioxidant vitamins materially reduce oxidative damage in humans? Free Radic Biol Med 26:1034–1053
14. Prasad KN, Cole W, Hoveland P (1998) Cancer prevention studies: past, present, and future. Nutrition 14:197–210
15. Chinery R, Brockman JA, Peeler MO, Shyr Y, Beauchamp RD, Coffey RJ (1997) Antioxidants enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer: a p53-independent induction of p21 WAF1/CIP1 via C/EBPβ. Nat Med 3:1233–1241
16. Weijl NI, Cleton FJ, Osanto S (1997) Free radicals and antioxidants in chemotherapy induced toxicity. Cancer Treat Rev 23:209–240
17. Labriola D, Livingston R (1999) Possible interactions between dietary antioxidants and chemotherapy. Oncology 13:1003–1012
18. Salganik RI, Albright CD, Rodgers J, Kim J, Zeisel SH, Sivashinskiy MS, Van Dyke TA (2000) Dietary antioxidant depletion: enhancement of tumor apoptosis and inhibition of brain tumor growth in transgenic mice. Carcinogenesis 21:909–914
19. Archer SY, Hodin RA (1999) Histones acetylation and cancer. Curr Opin Gene Dev 9:171–174
20. Klochendler-Yeivin A, Yaniv M (2001) Chromatin modifiers and tumor suppression. Biochim Biophys Acta 1551:M1–M10
21. Lehrmann H, Pritchard LL, Harel-Bellan A (2002) Histone acetyltransferases and deacetylases in the control of cell proliferation and differentiation. Adv Cancer Res 86:41–65
22. Fry CJ, Peterson CL (2002) Unlocking the gates to gene expression. Science 295:1847–1848
23. Grunstein M (1997) Histone acetylation in chromatin structure and transcription. Nature 389:349–352
24. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG (2001) Aberrant pattern of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 10:687–692
25. Marks PA, Richon VM, Rifkind RA (2000) Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J Natl Cancer Inst 92:1210–1216
26. Henderson C, Brancolini C (2003) Apoptotic pathways activated by histone deacetylase inhibitors: implications for the drug-resistant phenotype. Drug Resist Updat 6:247–256
27. Yamashita Y, Shimada M, Harimoto N, Rikimaru T, Shirabe K, Tanaka S, Sugimachi K (2003) Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. Int J Cancer 103:572–576
28. Donadelli M, Costanzo C, Faggioli L, Scupoli MT, Moore PS, Bassi C, Scarpa A, Palmieri M (2003) Trichostatin A, an inhibitor of histone deacetylases, strongly suppresses growth of pancreatic adenocarcinoma cells. Mol Carcinog 38:59–69
29. Rosato RR, Grant S (2003) Histone deacetylase inhibitors in cancer therapy. Cancer Biol Ther 2:30–37
30. Marks PA, Richon VM, Breslow R, Rifkind RA (2001) Histone deacetylase inhibitors as new cancer drugs. Curr Opin Oncol 13:477–483
31. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 63:7291–7300
32. Wroblewski F, La Due J (1995) Lactic dehydrogenase activity in blood. Proc Soc Exp Biol Med 90:210–213
33. LeBel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 29,79-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5:227–231
34. Cousens LS, Gallwitz D, Alberts BM (1979) Different accessibilities in chromatin to histone acetylase. J Biol Chem 254:1716–1723
35. Del Raso N (1992) In vitro methods for assessing chemical or drug toxicity and metabolism in primary hepatocytes. In: Watson RR (ed) In vitro methods of toxicology. CRC, Boca Raton, pp 176–201
36. Creagan ET, Moertel CG, O'Fallon JR, Schutt AJ, O'Connell MJ, Rubin J, Frytak S (1979) Failure of high dose vitamin C (ascorbic acid) to benefit patients with advanced cancer: a controlled trial. N Engl J Med 301:687–690
37. Moertel CG, Fleming TR, Creagan ET, Rubin J, O'Connell MJ, Ames MM (1985) High dose vitamin C versus placebo in the treatment of patients with advanced cancer who had had no prior chemotherapy: a randomized double-blind comparison. N Engl J Med 312:137–141
38. Zafarullah M, Li WQ, Sylvester J, Ahmad M (2003) Molecular mechanisms of *N*-acetylcysteine actions. Cell Mol Life Sci 60:6–20
39. He L, Tolentino T, Grayson P, Zhong S, Warrell RP, Rifkind RA, Marks PA, Richon VM, Pandolfi PP (2001) Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. J Clin Invest 108:1321–1330
40. Lee YS, Jin DQ, Park SH, Han SY, Kim HS, Jeong TC, Huh K, Kim JA (2002) 2,3,7,8-Tetrachlorobenzo-*p*-dioxin inhibits proliferation of SK-N-SH human neuronal cells through decreased production of reactive oxygen species. Free Radic Res 36:1283–1289
41. Gorlach A, Kietzmann T, Hess J (2002) Redox signaling through NADPH oxidases: involvement in vascular proliferation and coagulation. Ann N Y Acad Sci 973:505–507
42. Burdon RH (1995) Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med 18:775–794
43. Kang J, Zheng R (2004) Dose-dependent regulation of superoxide anion on the proliferation, differentiation, apoptosis and necrosis of human hepatoma cells: the role of intracellular Ca^{2+}. Redox Rep 9:37–48