CANCER

Antioxidants can increase melanoma metastasis in mice

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Antioxidants in the diet and supplements are widely used to protect against cancer, but clinical trials with antioxidants do not support this concept. Some trials show that antioxidants actually increase cancer risk and a study in mice showed that antioxidants accelerate the progression of primary lung tumors. However, little is known about the impact of antioxidant supplementation on the progression of other types of cancer, including malignant melanoma. We show that administration of *N*-acetylcysteine (NAC) increases lymph node metastases in an endogenous mouse model of malignant melanoma but has no impact on the number and size of primary tumors. Similarly, NAC and the soluble vitamin E analog Trolox markedly increased the migration and invasive properties of human malignant melanoma cells but did not affect their proliferation. Both antioxidants increased the ratio between reduced and oxidized glutathione in melanoma cells and in lymph node metastases, and the increased migration depended on new glutathione synthesis. Furthermore, both NAC and Trolox increased the activation of the small guanosine triphosphatase (GTPase) RHOA, and blocking downstream RHOA signaling abolished antioxidant-induced migration. These results demonstrate that antioxidants and the glutathione system play a previously unappreciated role in malignant melanoma progression.

INTRODUCTION

Research into the role of reactive oxygen species (ROS) and effects of antioxidant supplementation in cancer is fraught with controversy and conflicting results. On one hand, antioxidants are widely used by healthy people and cancer patients as a strategy to fight cancer (*1*–*3*). On the other hand, clinical trials with antioxidants have produced conflicting results: some trials show that antioxidants actually increase cancer risks (*4*–*6*). Moreover, the antioxidants *N*-acetylcysteine (NAC) and vitamin E increase the proliferation of human lung cancer cells and tumor growth in mice with B-RAF– and K-RAS–induced lung cancer by reducing ROS, DNA damage, and p53 (*7*). These results suggest that lung tumor cells proliferate faster when the amounts of ROS are low, and this may be accomplished by dietary antioxidants or by mutations that activate an endogenous ROS defense system coordinated by NRF2/KEAP1 (*8*).

There is little information, however, on the effects of antioxidants on the progression of other cancer types, including malignant melanoma—a cancer known to be sensitive to changes in redox status (*9*–*11*). One possibility is that melanoma cells, like lung cancer cells, proliferate faster. Another possibility is that antioxidants inhibit melanoma cell migration and invasion because ROS may promote these processes (*12*–*14*). Here, we used a mouse model of endogenous malignant melanoma and a panel of human malignant melanoma cell lines to evaluate these possibilities.

RESULTS

In vivo effects of NAC on melanoma progression

To define the effects of antioxidant supplementation on malignant melanoma progression, we administered NAC in the drinking water to mice harboring a conditional oncogenic *Braf* allele, conditional knockout alleles for *Pten*, and a tyrosinase-*Cre* transgene (designated *BPT* mice) (*15*). NAC administration had no impact on the number and size of primary tumors in *BPT* mice (Fig. 1, A and B, and table S1), and the time points at which NAC-treated and control mice were sacrificed because of primary tumor burden did not differ (median, 83 and 82 days, respectively; $P > 0.05$). Immunohistochemical analyses revealed that expression of melanoma marker S100B and stem cell marker nestin was similar in primary tumors of NAC-treated and control mice, as were the numbers of proliferating cells, indicated by Ki-67 staining (fig. S1, A and B, and table S1). Surprisingly, NAC administration doubled the number of lymph node metastases and increased lung metastases to a lesser extent (Fig. 1, C and D, and tables S1 and S2). Lymph node metastases from NAC-treated and control mice were both black and similar in size, as judged by visual inspection and caliper measurements, but metastases from NAC-treated mice contained higher proportions of S100B- and nestin-positive melanoma cells (Fig. 1, E to G, fig. S2A, and tables S1 and S2). The higher proportion of melanoma cells in metastases of NAC-treated mice was not caused by increased proliferation because Ki-67 staining was similar to that in the controls (fig. S2B and table S1). Thus, NAC increased the number of lymph node metastases and the proportion of tumor cells in each lymph node. To determine whether NAC affects the redox state in tumors, we quantified amounts of reduced (GSH) and oxidized (GSSG) glutathione and found similar amounts of GSH and GSH/GSSG ratios in primary tumors of NAC-treated and control mice (Fig. 1H and tables S1 and S3). However, the GSH amount and GSH/GSSG ratio in lymph node metastases were markedly higher in NAC-treated mice (Fig. 1H and tables S1 to S3). In untreated mice, the amounts of GSH and GSH/GSSG ratio tended to be lower in metastases than in primary tumors (Fig. 1H and tables S1 to S3). Thus, the increase in metastasis correlated with the expected pharmacologic effects of NAC. To rule out the possibility that NAC was unable to access the primary tumors, we stained them with an antibody to 8-oxoguanine, which detects ROS-induced DNA damage, and found reduced amounts in tumors of NAC-treated mice (fig. S2C and tables S1 and S2).

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Effects of NAC and Trolox on cultured human melanoma cells

We next defined the effects of NAC on cultured melanoma cells. Consistent with the in vivo findings, NAC administration did not affect proliferation of seven human malignant melanoma cell lines but increased their migration and invasive properties; administration of Trolox, a structurally unrelated antioxidant and vitamin E analog, produced similar results (Fig. 2, A and B, fig. S3, and tables S1 and S2). NAC and Trolox increased the GSH/GSSG ratio in melanoma cells; the ratios were normalized by the simultaneous administration of L-BSO, an inhibitor of de novo glutathione synthesis (Fig. 2C and tables S1 to S3). Moreover,

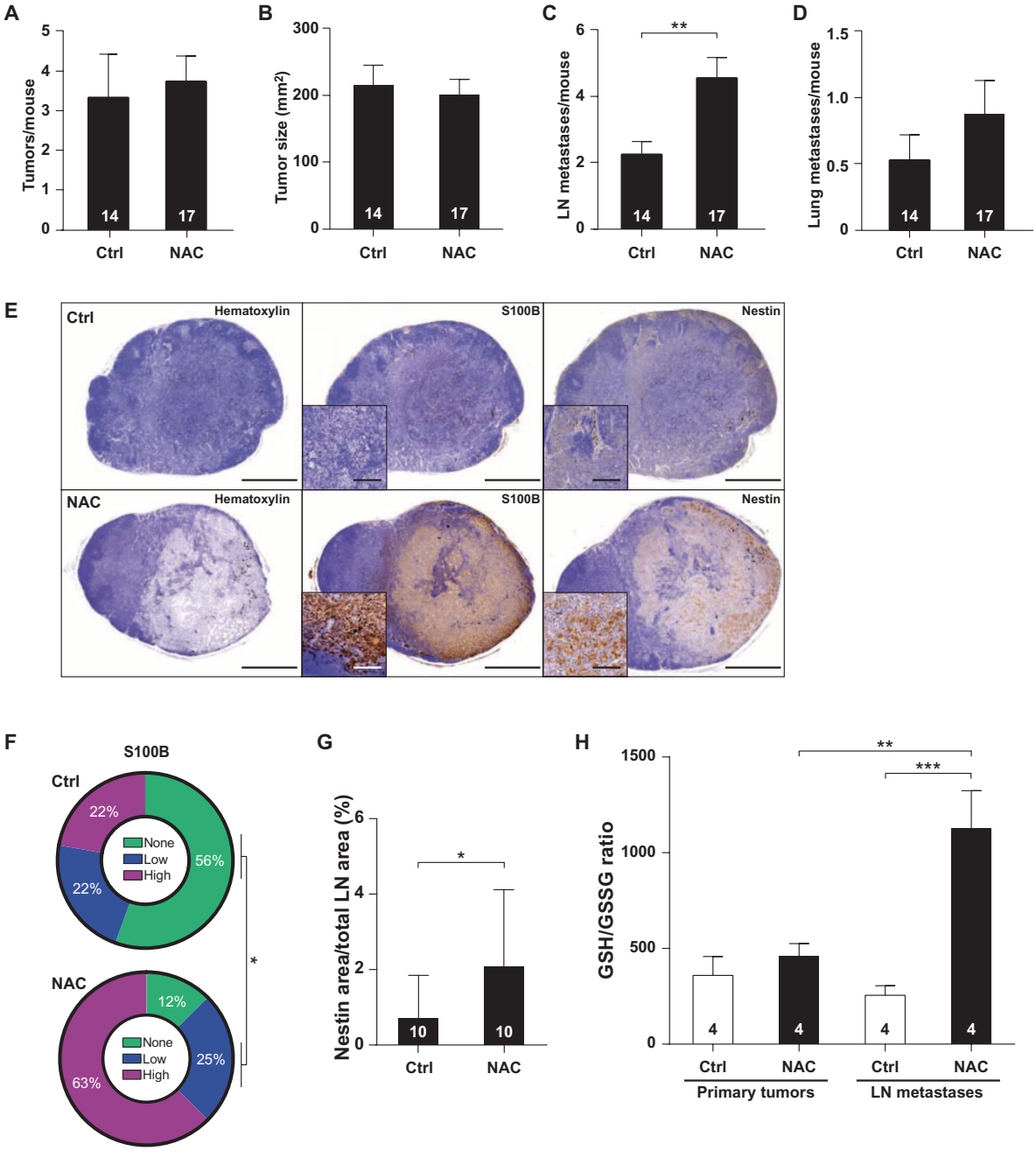

Fig. 1. Administration of NAC increases metastasis in mice with malignant melanoma. NAC was administered in the drinking water to newly weaned mice with melanocyte-specific $BRAF^{V600E}$ expression and *Pten* inactivation (*BPT* mice). (**A** and **B**) Number (A) and size (B) of primary tumors in *BPT* mice. (**C** and **D**) Number of lymph node (LN) (C) and lung (D) metastases per mouse. Data are from two independent experiments. (**E**) Histology and immunohistochemical detection of S100B and nestin in lymph node metastases of control (Ctrl; top panel) and NAC-treated (bottom panel) *BPT* mice. Higher-magnification insets show areas with typical staining; brown color indicates positive staining. (**F** and **G**) Quantification of S100B (F) and nestin (G) staining in lymph nodes ($n = 10$ mice; one lymph node per mouse). (**H**) Redox state assessed by GSH/GSSG ratios in lysates of primary tumors and lymph node metastases. Data are means of triplicate analyses. Scale bars, 2 mm; 100 μm (insets). Numbers in bars indicate *n*. $^*P < 0.05$; $^{**}P < 0.01$.

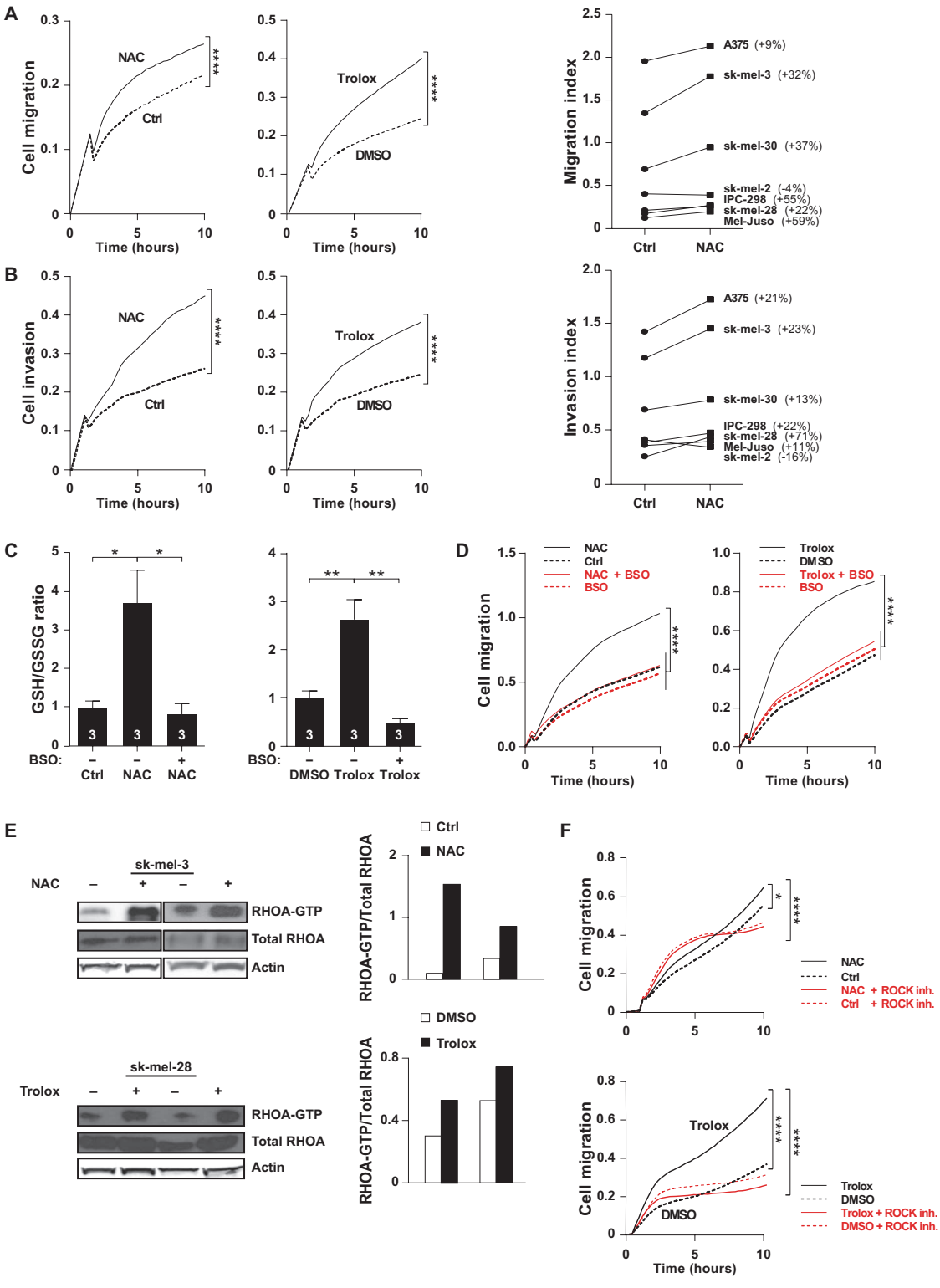

Fig. 2. NAC and Trolox increase migration and invasive properties of human malignant melanoma cells. (**A** and **B**) Real-time analyses of cell migration (A) and invasion (B) of melanoma cell line sk-mel-28 in medium supplemented with NAC (left panels) or Trolox (middle panels); data are means of quadruplicate analyses. Right panels: Migration and invasion indices at the 10-hour time point from real-time analyses of these parameters in seven melanoma cell lines incubated with control medium or medium supplemented with NAC; data are means of four to eight replicates per cell line in two to three independent experiments. The mean percent change in migration and invasion in response to NAC is shown in parentheses. (**C**) Left panel: GSH/GSSG ratios in sk-mel-28 incubated for 10 hours in control medium and in medium supplemented with NAC or NAC + BSO (buthionine sulfoximine; an inhibitor of de novo glutathione synthesis). Right panel: GSH/GSSG ratios in melanoma cells incubated for 10 hours in control medium [dimethyl sulfoxide (DMSO)] and in medium supplemented with Trolox or Trolox + BSO. Numbers in bars indicate *n*. (**D**) Real-time analyses of sk-mel-28 migration in response to NAC, BSO, and NAC + BSO (left panel) and Trolox, BSO, and Trolox + BSO (right panel); data are means of quadruplicate analyses. (**E**) Left panels: Western blots of GTP-bound and total forms of RHOA in lysates of melanoma cells incubated with antioxidants for 2 (lanes 1 and 2) and 4 hours (lanes 3 and 4). Actin was the loading control. Right panels: Ratios of guanosine 5′-triphosphate (GTP)–bound and total RHOA in response to NAC and Trolox. (**F**) Effects of a ROCK inhibitor (Y27632) on NAC- and Trolox-induced migration of sk-mel-28 cells. Data are means of quadruplicate analyses. Similar results were observed in three independent experiments. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

BSO administration prevented NAC- and Trolox-induced increase in melanoma cell migration (Fig. 2D and tables S1 and S2), demonstrating that glutathione synthesis is required for the effect. The antioxidants did not affect basal amounts of cellular ROS, as determined by fluorescence-activated cell sorting analyses with a redox-sensitive probe and the use of a genetically encoded biosensor (fig. S4 and table S1).

RHO family proteins RHOA and RAC1 mediate cytoskeletal changes during cell migration and invasion, and recent studies indicate that they are regulated by the cell's redox status through direct modification of residues involved in nucleotide binding (*16*–*18*). We therefore determined whether antioxidants affect the activation of RHOA and RAC1. The amounts of GTP-bound RHOA were higher in melanoma cells incubated with NAC and Trolox compared to control; GTP loading of RAC1 was not consistently altered by antioxidants (Fig. 2E, fig. S5A, and table S1). Moreover, inhibiting RHOA downstream signaling with an inhibitor of RHOA-associated kinase (ROCK) prevented antioxidant-induced migration but had relatively little impact on basal migration (Fig. 2F and tables S1 and S2). Melanoma invasive phenotypes are also regulated by microphthalmia-associated transcription factor (MITF), c-Jun N-terminal kinase (JNK), and factors associated with epithelial-mesenchymal transition (*19*–*21*). However, none of these factors were consistently altered by antioxidant administration in melanoma cells (figs. S5, B and C, and S6 and table S1).

DISCUSSION

Melanoma has steadily increased in incidence and lethality over several decades (*22*), and identifying factors that affect metastasis would be valuable. Here, antioxidant supplementation was found to increase metastasis in mice with endogenous malignant melanoma and increase the invasive behavior of human melanoma cells by boosting GSH concentrations and activating RHOA.

NAC administration increased the number of lymph node metastases and the proportion of tumor cells in each metastasis but had no impact on the number and size of primary tumors. A potential explanation for this result is that tumor cells that have left the primary tumor experience oxidative stress that limits their ability to migrate, invade, and form metastases, and NAC helps them overcome this limitation. This reasoning is supported by three findings. First, the amounts of GSH and GSH/GSSG ratios tended to be lower in metastases than in primary tumors of untreated mice. Second, NAC increased these values in metastases but not in primary tumors. Third, NAC increased migration and invasive abilities of cultured human melanoma cells. Moreover, a previous study showed that the amounts of GSH in the B16 mouse melanoma cell line were higher in a clone with high metastatic potential than in a clone with low metastatic potential (*23*).

NAC and Trolox have distinct chemical and physical properties. NAC is a hydrophilic precursor of cysteine and GSH (*24*); Trolox is a soluble analog of vitamin E, a lipophilic peroxyl radical scavenger (*25*). NAC and Trolox nevertheless produced markedly similar biochemical and phenotypic effects on human melanoma cells, and the effects of both antioxidants were blocked by an inhibitor of GSH synthesis. The latter result was expected in the case of NAC but less evident in the case of Trolox. Trolox could potentially boost GSH concentrations by scavenging ROS that would otherwise convert GSH to GSSG or by interacting with enzymes in glutathione pathways (*26*, *27*). Although the similar effects of NAC and Trolox suggest a common mechanism of increased GSH synthesis and RHOA activation, we cannot rule out the possibility that distinct mechanisms underlie their effects.

Another limitation of this study is that we do not yet know whether Trolox or vitamin E would increase metastasis in vivo, although it would not be far-fetched to hypothesize that they would. In a previous study, NAC and Trolox produced similar effects on human lung cancer cell proliferation, and NAC and vitamin E produced similar transcriptional profiles in mouse lung tumors and highly overlapping effects on tumor growth and survival (*7*).

Previous studies have raised the possibility that antioxidants might inhibit migration and invasion of melanoma cells and other cancer cells in culture (*12*–*14*), and others showed that antioxidants reduce tumor burden in mice with ultraviolet (UV)–induced skin cancer (*28*, *29*). Although our results conflict with these studies, we studied different antioxidants, cell lines, and mouse models. These factors likely contribute to conflicting results of ROS studies in cancer. Indeed, one of the studies on UV-induced skin cancer also showed that a different antioxidant increases, rather than reduces, skin tumor burden (*28*). Another factor that clearly affects the outcome of antioxidant-cancer studies is whether they are designed to evaluate effects on tumor initiation or tumor progression (*30*–*32*). Here, we evaluated the impact of NAC supplementation on the progression of malignant melanoma, and our finding of increased metastasis suggests that cancer patients should use antioxidant supplements with caution.

MATERIALS AND METHODS

Study design

The aim of the study was to define the effects of antioxidant supplementation on tumor progression and metastasis in mice with endogenous malignant melanoma and the proliferation, migration, and invasion of malignant melanoma cell lines. For in vivo experiments, melanomas were induced with tamoxifen in the skin of newborn mice; littermate mice were randomized to NAC and control groups at weaning. The mice were sacrificed when moribund because of primary tumor burden. Primary tumors and metastases were evaluated by macro- and microscopic techniques; tumor cell markers, cell proliferation, and GSH/GSSG ratios were monitored with routine histology, immunohistochemistry, and enzyme-linked immunosorbent assays. The effects of antioxidants on melanoma cells were evaluated by cellular assays, GSH and GSSG content, Western blotting, and TaqMan analyses.

Mice and tumor initiation

Two-day-old *Braf*$^{CA/+}$*Pten*$^{fl/fl}$Tyr-*Cre*$^{+/0}$ mice (designated *BPT*) were painted with 4-hydroxytamoxifen (T5648, Sigma) on the flank skin to activate BRAF^{600E} expression and inactivate PTEN expression in melanocytes, as described (*15*). At 3 weeks of age, when small nevi were visible on the skin, littermate mice were randomized to cages with regular drinking water or water supplemented with NAC (1 g/liter, ≥99% purity; 616-91-1, Sigma). The NAC water was changed every week; the pH of the solution was 3.1 ± 0.14, and the temperature was 23.3 ± 0.8°C. The NAC dose (1 g/liter) is standard in mouse studies and corresponds to 114 to 229 mg/kg body weight for an adult male mouse. After compensating for the faster drug metabolism in mice compared to humans, these doses correspond to 665 to 1330 mg/day in a human weighing 70 kg, which is within the range of doses recommended for supplement and pharmacological use. The mice were sacrificed when they became

listless because of primary tumor burden or when primary tumors ulcerated. *BPT* mice at autopsy exhibited black lymph node metastases. The mice had a mixed genetic background (129SV and C57BL/6) and ate a standard mouse chow (R34, Lantmännen). Mouse experiments were approved by the Research Animal Ethics Committee in Gothenburg.

Histology and immunohistochemical analyses

Tissues were fixed in paraformaldehyde and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. Immunohistochemical analyses were performed as described (*33*). The sections were incubated with antibodies recognizing Ki-67 (RTU, RM-9106-R7, Thermo Scientific), S100B (1:1000, ab52642, Abcam), nestin (1:100, ab134017, Abcam), and 8-oxoguanine (1:500, ab64548, Abcam) and then processed with the VECTASTAIN Elite ABC Kit (PK6101) and the DAB Peroxidase Substrate Kit (SK4100, Vector Laboratories). Histological slides were scanned with a MIRAX SCAN microscope with MIRAX Control software (Zeiss). Pathology and staining were quantified with TissueMorph software and the TISSUEalign module to exclude cellular areas negative for tumor markers (Visiopharm Integrator System version 5.0.2.1158).

Cell culture and analyses of proliferation, migration, and invasion

Human malignant melanoma cell lines (sk-mel-2, sk-mel-3, sk-mel-28, and A375 from the American Type Culture Collection; sk-mel-30, Mel-Juso, and IPC-298 from the German Collection of Microorganisms and Cell Culture) were cultured in DMEM (Dulbecco's modified Eagle's medium) GlutaMAX High Glucose (4.5 g/liter, 10569-010) supplemented with 10% fetal bovine serum (10270-106) and 1% penicillin/streptomycin (15070-063, Thermo Fisher). Proliferation assays were carried out by seeding 1×10^4 cells per well on 12-well plates; for each data point, the cells were trypsinized and viable cells were counted in a ViCell-XR cell counter (Beckman Coulter). NAC (≥99% purity; A7250) and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; 53101-49-8, Sigma) were used at concentrations of 200 and 20 μM, respectively. Water was used as vehicle control for NAC; DMSO was the control used for Trolox. Real-time analysis of proliferation was assessed by seeding 1×10^4 cells per well in E-plates (05 469 830 001, ACEA Biosciences) with control medium or medium supplemented with NAC or Trolox; the plates were then monitored for 48 hours with the xCELLigence Real Time Cell analyzer (ACEA Biosciences). Migration assays were carried out by plating 4×10^4 cells per well in CIM plates (05 665 817 001, ACEA Biosciences) with the assigned treatment; for invasion assays (defined as vertical migration through a layer of Matrigel), the CIM plates were precoated with a 1:40 dilution of Matrigel; the plates were monitored for 10 hours in the xCELLigence system. The xCELLigence system uses microelectronic biosensors in real time to measure impedance changes caused by proliferating cells. For migration experiments, cells seeded in serum-free medium in an upper chamber move through a microporous membrane and are detected by biosensors at the bottom of a lower chamber containing 10% serum in the medium. For invasion experiments, the membrane was precoated with Matrigel. The electrode impedance is displayed as a proliferation, migration, or invasion index. BSO (sc-200824, Santa Cruz Biotechnology) and the ROCK inhibitor Y27632 (Sigma) were used at concentrations of 1 mM and 10 μM, respectively.

Glutathione assays

GSH and GSSG were analyzed with the Bioxytech GSH/GSSG-412 kit (OxisResearch) in lysates of melanoma cells, primary melanomas, and lymph node metastases. Tissues and cells were snap-frozen in liquid nitrogen, stored at –80°C, and later homogenized with a TissueLyser II (85300, Qiagen). Samples were then prepared and analyzed according to the manufacturer's protocol.

Western blots and RHO protein activation assays

Cell lysates (10 to 20 μg) were resolved on 4 to 12% bis-tris gels (Bolt, Life Technologies), transferred to nitrocellulose membranes, and incubated with antibodies as described (*34*). Primary antibodies recognized phospho–histone H2AX (Ser^{139}; clone JBW301, Merck), total p53 (FL-393; sc-6243, Santa Cruz Biotechnology), MITF (C5; ab12039, Abcam), phospho-p53 (Ser^{15}, 9284), phospho–SAPK (stress-activated protein kinase)/JNK (Thr^{183}/Tyr^{185}; clone 81E11, 4668), total SAPK/JNK (9252), E-cadherin (clone 24E10, 3195), N-cadherin (clone D4R1H, 13116), Snail (clone C15D3, 3879), Slug (clone C19G7, 9585), ß-catenin (clone D10A8, 8480), vimentin (clone D21H3, 5741, Cell Signaling Technology), and ß-tubulin (926-42211, Li-Cor). Secondary antibodies were anti-mouse IRDye 680RD (926-68072) and anti-rabbit 680RD (926-68071, Li-Cor). Amounts of RHOA- and RAC1-GTP were analyzed with the Rho Activation Assay Biochem Kit (BK036, Cytoskeleton) and the Active Rac1 Pull-Down and Detection Kit (16118, Thermo Scientific), respectively. Protein bands were detected on a Li-Cor Odyssey Imager and analyzed with Image Studio Lite (version 5.0, Li-Cor).

TaqMan gene expression analyses

RNA was isolated from melanoma cells with the RNeasy Plus Mini Kit (74136, Qiagen). For quantitative polymerase chain reaction (PCR) analyses, complementary DNA (cDNA) was synthesized with the iScript cDNA synthesis kit (170-889, Bio-Rad), and the expression of MITF, CREB1, TYRP1, DCT, POU3F2, ZEB2, EP300, MLANA, PAX3, PPARGC1, RAB27A, SOX10, TYR, *RPLP*, and *UBC* was analyzed by TaqMan quantitative reverse transcription PCR on an ABI 7900HT (Life Technologies) using probe sets Hs00231713, Hs01098278, Hs00914223, Hs01117294, Hs00194133, Hs00240950, Hs00271595, Hs00173304, Hs00608302, Hs00366918, Hs00165976, Hs00167051, and Hs00207691, respectively. *RPLP* and *UBC* were selected as reference genes after screening a panel of human reference genes (TATAA Biocenter) and analyzing variability with GenEx (version 6, MultiD).

ROS measurements

For flow cytometry analysis, cells were incubated with 2.5 μM CellROX Orange (C10443, Life Technologies) for 30 min at 37°C and then rinsed with phosphate-buffered saline and trypsinized. Five thousand events per condition were analyzed with the BD Accuri C6 (BD Biosciences). For positive control, cells were incubated for 1 hour with the prooxidants menadione (50 μM; M5625, Sigma-Aldrich) or H_2O_2 (100 μM; 216763, Sigma-Aldrich) before adding CellROX. Cellular H_2O_2 was measured with a redox biosensor: cells were transduced with pLPCX roGFP2-Orp1 (*35*) and stable clones were selected with puromycin (1 μg/ml; P8833, Sigma-Aldrich). Cells (10,000) were then seeded into black 96-well flat- and clear-bottom plates (3603, Corning). The next day, cells were incubated with antioxidants and analyzed with the Operetta High Content Screening System (PerkinElmer) using Alexa 488 and Sapphire filters (*35*). Fluorescence were recorded in >8 fields per well

(n = 6 to 8 wells per condition), and fluorescence ratios were calculated with the Harmony software (version 3.5.1, PerkinElmer).

Statistics

Values are presented as means ± SEM. GraphPad Prism software (version 6.03, $\alpha < 0.05$) was used for statistical analyses. χ^2 test was used for S100B staining, and Student's t test was used for all other histological analyses. Two-way analysis of variance (ANOVA) was used for cell proliferation, migration, and invasion; one-way ANOVA and Bonferroni's post hoc test were used for GSH/GSSG ratios; Student's t test was used for protein bands on Western blots; multiple unpaired Student's t test was used for gene expression; unpaired Student's t test was used for ROS amounts. Original data for all experiments are provided in table S1, and exact P values are presented in table S2.

SUPPLEMENTARY MATERIALS

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Fig. S1. NAC does not affect expression of melanoma markers and proliferation in primary tumors.

Fig. S2. NAC does not affect the size of lymph node metastases and the percentage of proliferating cells.

Fig. S3. NAC and Trolox do not affect proliferation of human malignant melanoma cells.

Fig. S4. NAC and Trolox do not reduce basal amounts of ROS in melanoma cells.

Fig. S5. NAC and Trolox do not affect RAC1 activation, MITF expression, or the expression of MITF target genes.

Fig. S6. Antioxidants do not affect DNA damage response, JNK signaling, and epithelial-mesenchymal transition.

Table S1. Original data (provided as an Excel file).

Table S2. Individual P values for all figures.

Table S3. Individual values for GSH, GSSG, and GSH/GSSG ratios in in vivo and in vitro assays.

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Editor's Summary

Another strike against antioxidants

Antioxidants are found in a variety of foods and dietary supplements and are frequently used with the goal of preventing cancer, but mounting evidence suggests that they may not be as beneficial as once thought. Clinical studies have shown mixed or no benefits, and other works demonstrated that antioxidants may accelerate the progression of lung cancer. Now, Le Gal *et al*. discovered that some common antioxidants increase the rate of melanoma cell migration and invasion and increase metastasis in a mouse model. These are early findings, and additional work will be required to confirm the generalizability of this observation. Nevertheless, the results suggest a need for caution in the use of antioxidants, especially for patients with existing cancer.

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