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Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways

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ABSTRACT **Methylene blue (MB) has been used clinically for about a century to treat numerous ailments. We show that MB and other diaminophenothiazines extend the life span of human IMR90 fibroblasts in tissue culture by >20 population doubling (PDLs). MB delays senescence at nM levels in IMR90 by enhancing mitochondrial function. MB increases mitochondrial complex IV by 30%, enhances cellular oxygen consumption by 37–70%, increases heme synthesis, and reverses premature senescence caused by H_2O_2 or cadmium. MB also induces phase-2 antioxidant enzymes in hepG2 cells. Flavin-dependent enzymes are known to use NAD(P)H to reduce MB to leucomethylene blue (MBH_2), whereas cytochrome *c* reoxidizes MBH_2 to MB. Experiments on lysates from rat liver mitochondria suggest the ratio MB/cytochrome *c* is important for the protective actions of MB. We propose that the cellular senescence delay caused by MB is due to cycling between MB and MBH_2 in mitochondria, which may partly explain the increase in specific mitochondrial activities. Cycling of MB between oxidized and reduced forms may block oxidant production by mitochondria. Mitochondrial dysfunction and oxidative stress are thought to be key aberrations that lead to cellular senescence and aging. MB may be useful to delay mitochondrial dysfunction with aging and the decrease in complex IV in Alzheimer disease.—Atamna, H., Nguyen, A., Schultz, C., Boyle, K., Newberry, J., Kato, H., Ames, B. N. Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. *FASEB J.* 22, 000–000 (2008)**

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SENESCENCE IN PRIMARY human fibroblasts (IMR90 cells) can be delayed by several mitochondrial protective compounds: carnosine (1), aminoguanidine (2), and N-t-butyl hydroxylamine (NtBHA) (3, 4), whereas classic antioxidants appear not to delay senescence (5, 6). We chose to test the ability of methylene blue (MB) to delay senescence, because of its unique properties; MB readily cycles between oxidized and reduced states (7), binds heme (8), modulates the activity of soluble guanylate cyclase (9), interacts with the mitochondria (10, 11), acts as a radioprotector (12), and is an antioxidant (13). These properties overlap with those of NtBHA, the most potent agent we previously found to delay senescence in IMR90 cells (3, 4, 14–18). We show here that MB at nanomolar levels is over a thousand times more active in protecting mitochondria and delaying senescence than NtBHA.

MB is a diaminophenothiazine that has been in clinical use for approximately 100 years to treat a variety of ailments. MB treats congenital and poison-induced methemoglobinemia; prevents the side effects of chemotherapy (19, 20), and treats septic shock (21). The dose of MB usually used in clinical settings is between 1 and 2 mg/kg/day (22); signs of toxicity start at higher levels (>7.5 mg/kg/day) (23). There is weak evidence that chronic MB extends the life span of mice at 2.5 mg/kg/day (24).

The therapeutic potential of MB also has been demonstrated in models for specific ailments. MB protects against endotoxin-induced lung injury, bacterial lipopolysaccharide-induced fever (25, 26), cyclosporine injury to the kidney (27), doxorubicin injury to the heart (28), and streptozotocin injury to the pancreas (29). MB also protects from ischemic-reperfusion injury (30) and enhances β-oxidation of long-chain fatty acids (31). Administering MB *in vivo* appears to benefit the central nervous system and cognitive function; MB has anxiolytic properties (32, 33), improves cognitive function in rats, and increases cytochrome *c* oxidase (complex IV) activity (34), protects from methylmalonate-induced seizures (35), and protects from the cognitive decline inflicted by inhibitors of complex IV (36).

High doses of MB cause toxicity, such as the formation of Heinz bodies in erythrocytes in humans (37) and impaired hematological parameters in rodents (24). Although MB has a variety of clinical uses, high doses should also be avoided in G6PDH deficiency. When exposed to UV light, MB can cause oxidative damage to isolated DNA, though toxicity is expected to be minimal in humans (38) because it requires high exposure to UV, and most of the MB *in vivo* is reduced

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to leucomethylene blue (MBH_2), which has no photodynamic activity (11).

MB has a redox potential of 11 mV (39). Because its redox potential is close to zero, MB is very efficient in cycling between oxidized and reduced forms by suitable redox centers and reducing agents such as those in the mitochondria. MB is efficiently reduced by NAD(P)H-dependent dehydrogenases to form the colorless MBH_2. MB loses the blue color on reduction due to the disappearance of the absorbance bands at 600 and 666 nm. MBH_2 can readily reoxidize to MB by O_2 if suitable electron acceptors such as cytochrome *c* (40) or other heme proteins (13) are absent.

MB is soluble in water and also can dissolve in organic solvents (41). Electron delocalization in MB results in a partial positive charge located on both nitrogen and sulfur atoms, which may increase the permeability of MB through membranes. The lipid solubility of MBH_2 is higher than for MB; thus, both forms enter the mitochondria (11), in addition to other intracellular compartments (such as lysosomes) (42).

In this study, we demonstrate that MB delays cellular senescence of normal human fibroblasts (IMR90) at both 5 and 20% oxygen. MB increases oxygen consumption, heme synthesis, and resistance to oxidative stress and cadmium. MB also increases the cellular content of cytochrome *c* oxidase (complex IV) relative to the other mitochondrial respiratory complexes. MB also induces phase-2 antioxidant defense enzymes. Experiments on lysates from mitochondria suggest that MB recycles between oxidized and reduced forms through interactions with specific mitochondrial electron carriers, which may contribute to its antisenescence action.

MATERIALS AND METHODS

Measuring the effect of MB on cellular senescence

Normal human lung fibroblasts (IMR90) are an *in vitro* model for cellular senescence (43). Cells are started in culture as young cells with a low population doubling level (PDL) and allowed to increase in PDL until senescence (high PDL), when the cells stop dividing but remain metabolically active. Cells were obtained from the Coriell Institute for Medical Research at a PDL of 9.0. PDL was calculated as $\log_2 (D/D_o)$, where D and D_o are defined as the density of cells at the time of harvesting and seeding, respectively. Stock cultures were grown in 100 mm Corning tissue culture dishes containing 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS). Stock cultures were split once a week when near confluent, and PDL was calculated as described (4). MB (Fluka, Buchs, Switzerland) was prepared in PBS and added to the growth medium at final concentrations of 10, 100, or 1000 nM. The medium was changed after 4 days with the addition of fresh MB. The cultures were split at the 7th day from seeding and reseeded with fresh medium supplemented with MB. Other diaminophenothiazines (thionione, 1,9-dimethyl-methylene blue) were tested as described above. PDL was calculated, and the cultures were seeded again in fresh medium in triplicate for each condition as described (4). Additional cells from each splitting cycle were collected and used for measuring oxygen consumption or heme synthesis, or stored at –20°C for additional analysis.

To determine the effect of H_2O_2 on the replicative life span, cells were first seeded with fresh medium, with or without MB (see above), for a week. Next, cells grown with or without MB were each split into two additional groups and then either *1)* treated with 10 or 20 μM H_2O_2 or *2)* incubated without H_2O_2 as described previously (4). A similar experiment was used to test protection against cadmium (1 μg/ml).

Measuring the effect of MB on heme synthesis in IMR90 cells

The effect of MB on heme synthesis was tested using cells grown in media supplemented with MB for at least a week. Heme synthesis was measured in IMR90 cells using iron (^{59}Fe), as described previously (44). Total protein content in the sample was measured and used for normalization of heme synthesis and iron uptake.

Measuring the effect of MB on the rate of oxygen consumption of IMR90 cells

Oxygen consumption was measured at 30°C in intact cells using a Clark-type oxygen electrode. The cellular respiration medium was DMEM supplemented with 40 mM HEPES (DMEM/HEPES). HEPES was included to prevent the change in pH that usually occurs during incubation. The oxygen electrode was calibrated before the start of the experiment using sodium dithionite. The cells were harvested, collected by centrifugation, resuspended into 300 μl of DMEM/HEPES (prewarmed to 30°C), and added to the 2 ml DMEM/HEPES already in the chamber. The difference between the rates of O_2 consumption in the presence and absence of cells was calculated and used to determine the nanomoles of O_2 consumed per minute per milligram of protein. Protein content was determined using Bio-Rad (Hercules, CA, USA) protein reagent.

Isolating mitochondria from rat liver to study the interaction with MB

The Children's Hospital Oakland Research Institute Animal Care and Use Committee approved the use of animals for isolating mitochondria. Male Fisher 344 rats (Simonsen, Gilroy, CA, USA) were allowed *ad libitum* access to standard Purina rodent chow. For mitochondrial isolation, the rats were fasted overnight, anesthetized with ether, and sacrificed. The liver was resected and placed in ice-cold mitochondrial isolation buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, and 1 mM EDTA, pH 7. The liver was homogenized immediately, and the mitochondrial fraction was isolated by differential centrifugation, as described previously (3). Mitochondrial protein was stored at −80°C and quantified using Bio-Rad protein reagent.

We dissolved isolated mitochondria in 0.5% Triton-X100 for spectrophotometric and fluorometric end points in order to allow the metabolites used (*e.g.*, NADH and cytochrome *c*) to access the active sites of the mitochondrial enzymes. We measured the effect of MB on the oxidation of NADH by the mitochondrial lysate. Mitochondrial proteins (234 μg) in 30 μl were added to 900 μl PBS followed by 20 μM Q_2 (an analog of coenzyme Q), rotenone, and 100 μM NADH. The oxidation of NADH was measured at 340 nm. The rate of NADH oxidation was measured using the millimolar extinction coefficient of 6.8 for NADH. The effect of 10 μM MB on NADH oxidation by the mitochondrial lysate was measured as de-

scribed above. The direct oxidation of NADH by MB was measured in the reaction components described above except that 30 μl PBS were added to the reaction instead of the lysate.

To test whether the oxidation of NADH by the mitochondrial lysate is associated with production of oxidants, we used 2′,7′-dichlorodihydrofluorescein (DCFH; Invitrogen, Carlsbad, CA, USA). We added 10 μM DCFH to the reaction system similar to the one described above. Oxidants (*e.g.*, $^{\bullet}OH$ radical) convert the nonfluorescent DCFH to highly fluorescent DCF (Ex, 488 nm, and Em, 530 nm). The effect of MB on the NADH-dependent production of oxidants by the mitochondrial lysate was tested by three different concentrations of MB (0.1, 1, and 10 μM). In a similar reaction system, we tested whether cytochrome *c* affects the MB-induced increase in oxidant production that we found at 10 μM. Cytochrome *c* was added at 10, 50, or 100 μM final concentration.

Measuring the effect of MB on specific subunits of mitochondrial electron transport complexes (ETCs) using Western blot analysis

Frozen MB-treated cells or controls were thawed, and lysate was prepared into 1% Tween-20, protease inhibitor cocktail (Sigma), and sonicated in ice. Cellular proteins (50–100 μg) were resolved in 15% SDS-PAGE, transferred to PVDF membrane, blocked with 5% milk proteins, and incubated with specific antibodies for selected subunits of the mitochondrial ETC I, III, and IV. For complex IV, we used subunit II (COX-II). For complex III, we used subunit CorI, and for complex I, we used ND39. The protein bands were visualized using goat anti-mouse secondary antibody conjugated to HRP and chemiluminescence and exposure to imaging film. Quantification of the protein bands in Western blot analysis was performed by densitometry analysis of bands detected on the film using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measuring the effect of MB on induction of thioredoxin reductase 1 (TrxR1) and NAD(P)H quinone oxidoreductase (NQO1) in HepG2 cells

HepG2 cells were cultured and maintained in minimum essential medium (MEM) containing 10% FBS, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). HepG2 cells were seeded in 24-well plates at 1×10^5 cells/well. After 24 h, the cells were transiently transfected with 1 μg pGL3 vector (Promega, Madison, WI, USA) bearing human TrxR1 promoter region or rat NQO1 promoter region, for which we are grateful to K. J. Hintze (45), and 0.04 μg pRL-SV40, an expression vector for normalization (Promega). Transfection was performed using LipofectAmine 2000, according to the manufacturer's instructions (Invitrogen). After 24 h of transfection, HepG2 cells were incubated with media containing MB or L-sulforaphane at a final concentration of 10–1000 nM for 48 h. The cells were washed with PBS and lysed. Luciferase assays were performed using the dual-luciferase reporter assay system (Promega), following the manufacturer's protocol. Luminescence was measured on a luminometer (TD20/20; Turner Designs, Sunnyvale, CA, USA). To normalize data, all values are reported as the ratio of firefly luminescence (pGL3, experimental plasmid) to renilla luminescence (pRL-SV40, noninducible plasmid).

Statistical analysis

Graphing and statistical analysis using *t*-tests, Mann-Whitney nonparametric tests, or one-way ANOVA were performed using Prism 4.0 software (GraphPad, San Diego, CA, USA). Significance was defined as the 95% confidence interval when $P < 0.05$.

RESULTS

MB delays the senescence of normal human fibroblasts (IMR90)

The life span of IMR90 cells in tissue culture at 20% O_2 maintained with MB was extended by >20 PDL relative to controls (**Fig. 1*A***), indicating a delay of cellular senescence. Although MB delays senescence at 10 nM, the most effective concentration of MB among those tested was 100 nM, which is 1000-fold less than NtBHA (4). Senescence delay by MB and NtBHA was additive (**Table 1**); however, the efficiency decreased when both agents were used at their optimal concentrations.

MB also delays senescence under conditions of normoxic (5%) O_2 (Fig. 1*B*). At 20% O_2, 1 μM MB was less effective in delaying cell senescence than the 10 or 100 nM MB (Fig. 1*A*); however, under 5% oxygen, 1 μM MB was nearly as efficient as the 100 nM MB (Fig. 1*B*). MB also delayed cell senescence when administered at any PDL (Fig. 1*C*); however, the efficiency decreased as the starting PDL increased.

MB is a tetramethylated thionine. Thus, we tested whether thionine would also delay senescence. Thionine delayed cell senescence, though at higher concentrations than MB (1 μM). We also tested compounds with chemical structures similar to MB: 1,9-dimethyl-methylene blue, Toluildine blue O, and Celestin Blue; they did not have antisenescence activity (data not shown).

MB protects IMR90 cells against oxidative stress and exposure to cadmium

IMR90 cells were maintained in medium containing 10 and 20 μM/ml H_2O_2 or 1 μg/ml cadmium. The concentrations of cadmium or H_2O_2 used resulted in slow and progressive toxicity to the cells, evident by the decline in the gain in PDL per week (**Fig. 2**). MB (100 nM) prevented the reduction in PDL per week caused by H_2O_2 and cadmium (Fig. 2*A*, *B*).

MB increases oxygen consumption by IMR90 cells

IMR90 cells maintained with MB significantly increased the rate of oxygen consumption by 70%. (In a different set of experiments, MB significantly increased O_2 consumption by 37%.) Control cells consumed 5.7 ± 1.2 nmol O_2/min/mg protein, whereas MB treated cells consumed 9.74 ± 1.95 nmol O_2/min/mg protein (mean±SD, $n=3$, $P<0.04$, unpaired *t*-test). MB (100 nM) added to the control cells (*i.e.*, cells grown without MB) during the O_2 consumption measurement did not affect O_2 consumption, indicating that MB itself did not account for the increase in O_2 consumption. Thus,

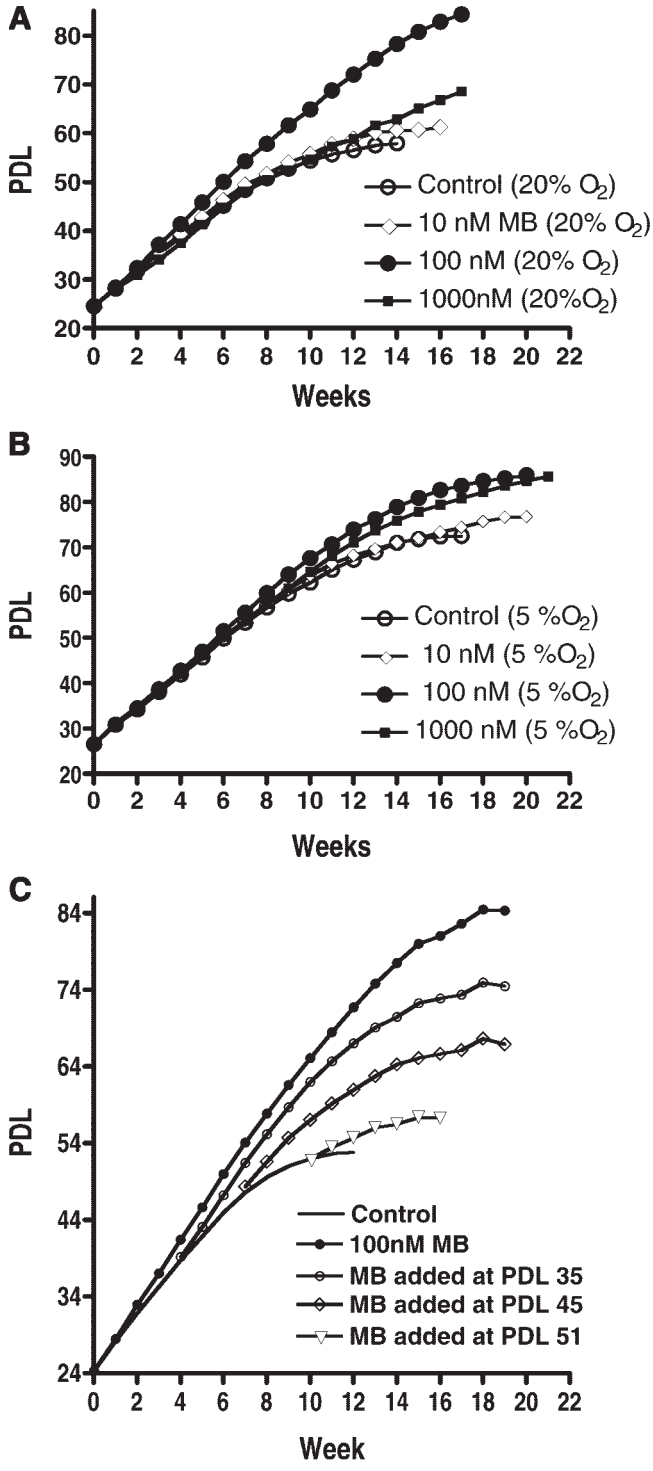


Figure 1. The effect of methylene blue (MB) on senescence of human lung fibroblasts (IMR90). Young IMR90 cells (PDL 23–25) were cultivated continuously in triplicate with 0, 10, 100, and 1000 nM MB and harvested every week; then the cells were counted and the PDLs were calculated. Cells (0.5 million) were used to seed new dishes as described in Materials and Methods: under atmospheric oxygen (20% O_2) (*A*) and under normoxia (5% O_2) (*B*). Each time point is the average of triplicates. One representative experiment out of eight is shown; MB delay of cellular senescence in IMR90 cells when administered at different PDLs (*C*). One representative experiment out of three is shown.

the increase in O_2 consumption is most likely due to cellular factors altered by growth in MB.

MB increases the level of mitochondrial cytochrome *c* oxidase (complex IV) in IMR90 cells

Complex IV was evaluated by measuring its core subunit II. The level of subunit II increased by ~30% relative to ETC I and III in cells maintained with 100 nM MB as compared to the control cells (**Fig. 3**), indicting selective increase in complex IV. On the other hand, the level of complex IV was undetectable when 1 μM MB was used to treat the cells (Fig. 3), which was totally reversible on removal of MB from the medium (data not shown). The level of subunit ND39 (complex I) or subunit core I (complex III) MB were not affected by MB (Fig. 3).

MB increases heme synthesis by IMR90 cells

IMR90 cells that were maintained with 100 nM MB showed an increased rate of heme synthesis, whereas 1 μM MB lowered the rate of heme synthesis by 50% compared to the control cells (**Fig. 4*A***). Consistent with the role of iron in heme synthesis, iron uptake by IMR90 paralleled the effect of MB on the rate of heme synthesis. IMR90 cells that were maintained with 100 nM MB showed a suggestive increase in iron uptake ($P = 0.08$), whereas IMR90 cells that were maintained in 1 μM MB showed a significant decrease in iron uptake (Fig. 4*B*).

MB:cytochrome *c* ratio is important for blocking the production of oxidants by mitochondria isolated from rat liver

NADH reduces MB to MBH_2, albeit at a very slow rate, whereas the presence of cellular dehydrogenases accelerates the NADH-dependent reduction of MB (46). Mitochondrial lysate accelerated the oxidation of NADH by 17-fold, whereas the addition of 10 μM MB to the lysate accelerated NADH oxidation by more than 57-fold (**Table 2**). NADH dehydrogenase of complex I is the major NADH-consuming enzyme in the mitochondrial lysate and is likely to contribute to NADH oxidation by MB.

Incubating the mitochondrial lysate with NADH at atmospheric O_2 (20%) also leads to an increase in the fluorescence of DCFH, indicating the oxidation to DCF by NADH-dependent production of oxidants (left section of **Fig. 5**). Lowering the O_2 of the reaction buffer to 5% (by bubbling with argon) decreases the NADH-dependent oxidant production to background levels (right section of Fig. 5). Subsequently, MB was added to the mitochondrial lysate at 0.1, 1, or 10 μM to assay the NADH-dependent production of oxidants. MB increases the oxidation of DCFH at 1 and 10 μM MB, but not at 100 nM (Fig. 5).

The MB-dependent increase in oxidant production at the high concentration of MB could be due to MBH_2

TABLE 1. *Effect of various concentrations of MB, NtBHA, and their combination on the PDL of IMR90 cells grown in air*

Effect	Control	10 μM NtBHA	100 μM NtBHA	10 nM MB	100 nM MB	10 nM MB +10 μM NtBHA	10 nM MB +100 μM NtBHA	100 nM MB +10 μM NtBHA	100 nMB +100 μM NtBHA
Senescence	51.7	58.72	77.4	56.03	73.24	64.4	75.01	82.2	80.98
Gain	–	Δ7.02	Δ25.7	Δ4.33	Δ21.54	Δ12.7	Δ23.31	Δ30.05	Δ29.28
Expected gain	–	–	–	–	–	(11.35)	(30.03)	(28.56)	(47.24)

First row shows the PDL at which the cells senesced. Second row shows the gain in PDL induced by the specific treatment; this is calculated by subtracting the PDL of the control (51.7). Third row shows the expected gain in PDL due to the combined treatments calculated by adding the gain in PDL of each individual treatment from the respective concentration.Δ Indicates the gain in PDL found experimentally. The expected gains in PDL are in parenthesis and calculated from adding the gain from each single treatment.

auto-oxidization by O_2 to form a superoxide radical. Because it is known that MBH_2 preferentially reduces cytochrome *c* over O_2 (40), we suspected that the levels of cytochrome *c* in the mitochondrial lysate become limiting in the presence of 1 or 10 μM MB. Therefore, we tested whether cytochrome *c* added in excess to the mitochondrial lysate could block the production of oxidants by 10 μM MB. We found that adding cytochrome *c* to the mitochondrial lysate did, in fact, eliminate the MB-dependent increase in the oxidation of DCFH (**Fig. 6**). The effect of cytochrome *c* is likely not mediated by the ability of cytochrome *c* to scavenge superoxide radical, as has been previously demonstrated (13, 30, 40).

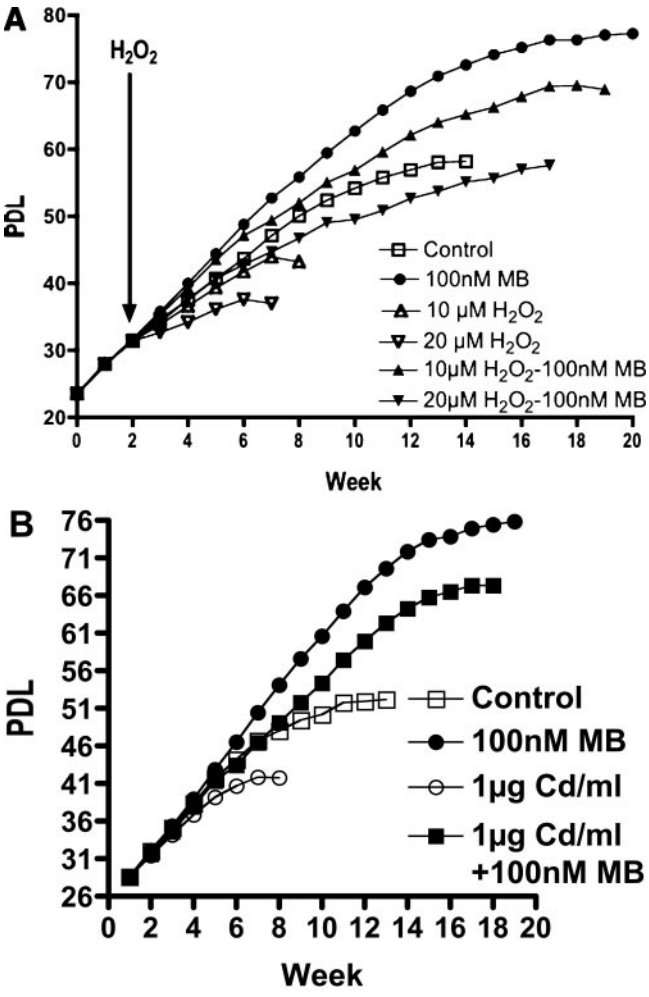


Figure 2. MB protects IMR90 cells from the toxicity of H_2O_2 and cadmium. Cells were grown for a week in the presence of MB before the start of the treatment with 10 or 20 μM H_2O_2 (*A*), and 1 μg/ml cadmium (*B*), as described in Materials and Methods. Every week, the cells were harvested, counted, and reseeded with or without 100 nM MB, and the respective treatment with H_2O_2 or cadmium. One representative experiment out of two is shown.

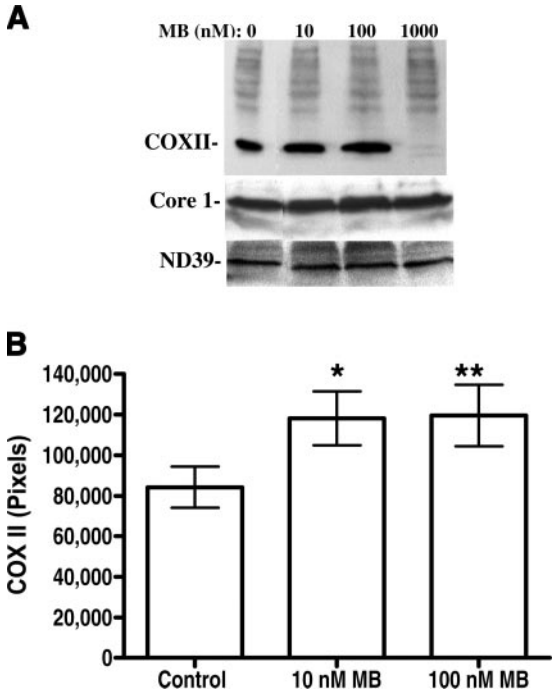


Figure 3. MB increases the level of subunit COX II of mitochondrial complex IV. IMR90 cells were grown in the presence of 10, 100, and 1000 nM MB for at least a week. *A)* Proteins from the cells were separated using SDS-PAGE and immunoblotted for subunit COX II of mitochondrial complex IV, core 1 of complex III, and ND39 of complex I. One representative experiment out of five is shown. *B)* The effect of MB on the band density of COX II. The density of the immunoblot bands was measured using National Institutes of Health ImageJ software. Data from seven independent experiments were pooled for final analysis (mean ± SE; $*P<0.05$, $**P<0.001$ (ANOVA, post hoc Friedman test).

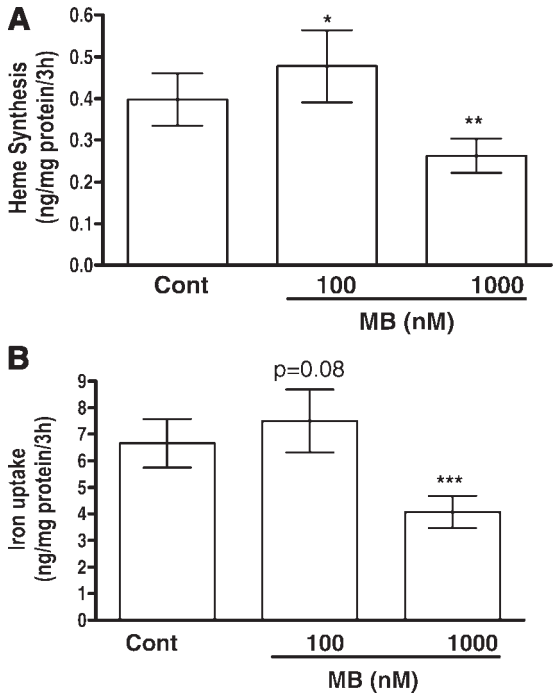


Figure 4. The effect of MB on the rate of heme synthesis and iron accumulation in IMR 90 cells. Cells treated with 100 and 1000 nM MB for at least a week, and controls were harvested and used to measure heme synthesis as described in Materials and Methods. *A)* Data on heme synthesis; $*P < 0.01$ $**P < 0.005$. *B)* Data on iron accumulation; $***P < 0.0005$ (paired *t* test). Data are means ± SE and are an average of 4 different experiments.

MB induces the phase-2 defense enzymes TrxR1 and NQO1 in HepG2 cells

MB dose-dependently increased the luminescence in HepG2 cells transfected with luciferase reporter gene constructs for TrxR1 (**Fig. 7*A***) and NQO1 (Fig. 7*B*), suggesting the ability of MB to increase the transcription of TrxR1 and NQO1. The patterns of induction were similar for Trx1 and NQO1 using concentrations between 10 and 1000 nM of MB. We also found a similar effect of L-sulforaphane on Trx1 and NQO1 transcription, which was consistent with previous findings (45). Although L-sulforaphane induced Trx1 and NQO1 in hepG2 cells, as did MB, it did not extend the life span of the IMR 90 cells when tested at concentrations ranging between 1 nM and 10 μM (data not shown).

TABLE 2. *Effect of MB on the oxidation of NADH with or without the mitochondrial lysate*

Incubation	Average ± SD	*n*
NADH + MB	1.77 ± 0.59	2
Mitochondria + NADH	30.7 ± 0.94*	3
Mitochondria + NADH + MB	101.5 ± 3.16*	3

*$P < 0.0001$, nmol/min/mg protein.

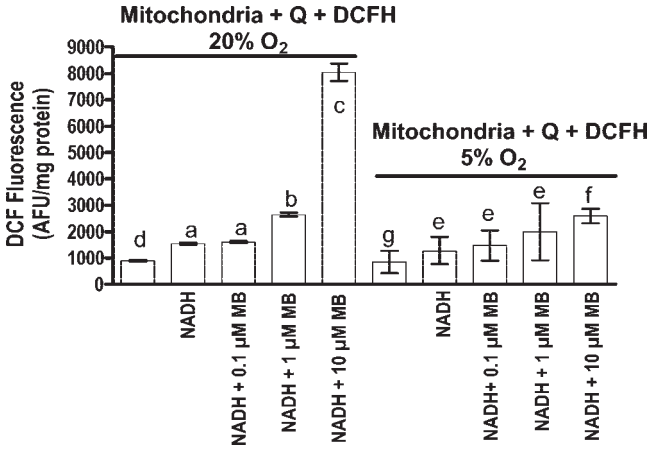


Figure 5. The effect of MB on the NADH-dependent oxidation of DCFH by the mitochondrial lysate at atmospheric (20%) and normoxic (5%) oxygen. Mitochondria were isolated from rat liver, and lysates were prepared as described in Materials and Methods. Where indicated, coenzyme Q_2 (Q), NADH, or MB was added to the mitochondrial lysate. The oxidation of DCFH, as an indicator for the production of oxidants, was measured by the fluorescence of DCF (Ex, 488 nm; Em, 530 nm). The experiments were performed at 20 and 5% oxygen, as labeled in the figure. Data shown are the means ± SD and are an average of 3 different experiments. Means without a common letter differ, $P < 0.0001$ (ANOVA, Bonferroni's test).

DISCUSSION

MB has various additional metabolic activities when administered at high concentrations, including an antioxidative activity (26, 47), inhibition of activated sGC (48), and inhibition of NOS (49). The concentrations of MB that we used in the current study are in the nanomolar range. Mitochondrial dysfunction, oxida-

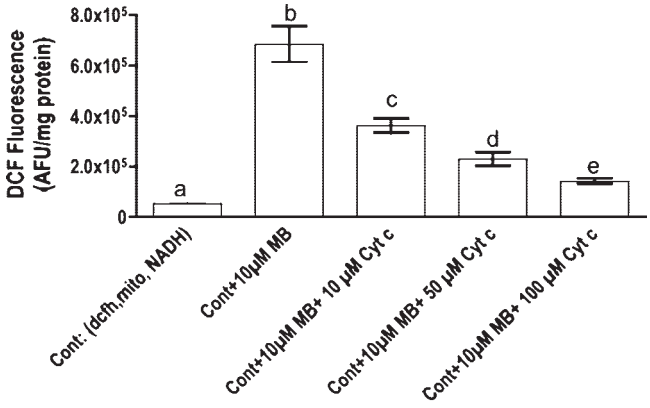


Figure 6. The effect of cytochrome *c* on the MB-dependent increases in the oxidation of DCFH by the mitochondrial lysate. The effect of MB on the production of oxidants by NADH-supplemented mitochondrial lysate at atmospheric oxygen is shown. Oxidant production was measured by the increase in the fluorescence resulting from oxidation of DCFH to DCF. The effect of increasing concentrations of cytochrome *c* (10, 50, 100 μM) on oxidant production is also shown. Data shown are the means ± SD of three different experiments. $***P < 0.0001$ (ANOVA, Bonferroni's test).

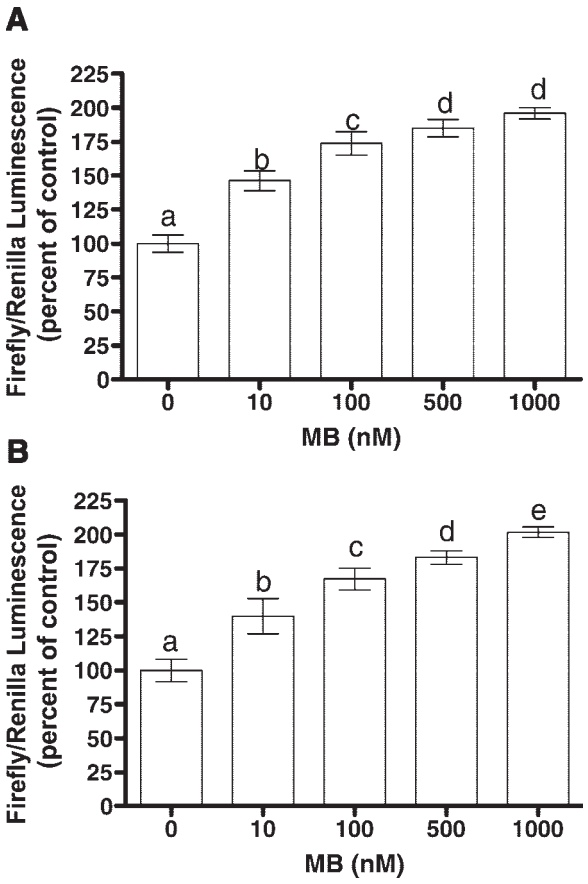


Figure 7. Induction of human thioredoxin reductase (*A*) or rat quinone reductase (*B*) by MB in HepG2 cells. HepG2 cells were transiently transfected with vector bearing promoter region of human TrxR1 (*A*) or rat NQO1 (*B*). Transfected HepG2 cells were incubated with MB at increasing concentrations for 48 h. Luciferase assays were performed using the dual-luciferase reporter assay system in cell lysates; luminescence data are reported as the ratio of firefly luminescence (pGL3, experimental plasmid) to renilla luminescence (pRL-SV40, noninducible plasmid), as described in Materials and Methods. Values are means ± SD; $n = 3$. Means without a common letter differ, $P < 0.05$ (one way ANOVA, Bartlett's test for equal variances).

tive damage to macromolecules, and telomere shortening are proposed mechanisms contributing to cellular senescence and aging. However, a complete understanding of the antisenescence mechanism of MB is possible only if each one of these mechanisms is studied. In this study, we concentrated on the interaction of MB with specific factors related to the mitochondria, as well as the unique redox activity of MB.

We have previously demonstrated that NtBHA was highly efficient in delaying the senescence of IMR90 cells (3, 4). MB is even more efficient in delaying cellular senescence of IMR90 cells maintained either in 5% (normoxia) or 20% (air) O_2 (Fig. 1*A, B*). MB also delays senescence when added to the cells at any PDL during the life span; however, the efficiency declines as the starting PDL increases (Fig. 1*C*). The metabolic activity of the mitochondria increased in response to MB, as measured by increases in oxygen consumption, complex IV (Fig. 3), and heme synthesis (Fig. 4), all of which are key mitochondrial metabolic pathways. Mitochondrial dysfunction occurs during cellular senescence. H_2O_2 causes oxidative stress to a variety of cellular components, whereas the toxic effects of cadmium are seen in mitochondria (50). Both toxins accelerated cellular senescence. MB increased the tolerance of IMR 90 cells to both hydrogen peroxide (Fig. 2*A*) and cadmium (Fig. 2*B*).

TrxR1 and NQO1 are two of 292 phase-2 enzymes elevated by Keap1/Nrf2, including GSH biosynthetic enzymes. The transient induction of phase-2 enzymes by alkylating and oxidizing agents increases cellular resistance to alkylating agents and oxidative stress (51, 52). Oxidation or alkylation of specific thiols in Keap1 increases the nuclear localization of Nrf2, which, in turn, induces phase-2 enzymes. MB induces TrxR1 and NQO1 in hepG2 cells, likely by transient oxidation of Keap1 on first exposure to MB (53). The effect of MB on phase-2 enzymes may contribute to the increased resistance to H_2O_2 and cadmium that we observed in IMR90 cells (Fig. 2). The role of the induction of phase-2 enzymes in the senescence delay by MB is not yet clear. Normal cells (IMR90) were used for the study on life span, while a transformed cell line (hepG2) was used for the induction of phase-2 enzymes; therefore, comparisons are uncertain. MB's effect on life span may be explained in part by the metabolic enhancement of mitochondria by MB.

Intracellular MB is likely to cycle between the oxidized (MB) and the reduced (MBH_2) forms. Although numerous NAD(P)H-dependent enzymes can reduce MB to MBH_2, cytochrome *c* in the mitochondria and methemoglobin (in the red blood cells) are the only heme proteins reported to reoxidize MBH_2 to MB (13, 40), which may also auto-oxidize with O_2 under specific circumstances (see below). Thus, MBH_2 may increase the rate of the reduction of cytochrome *c* over and above the normal enzymatic reduction of cytochrome *c* by complex III in the ETC. Cytochrome *c* is an electron carrier from complex III to complex IV (*i.e.*, cytochrome *c* is the substrate for complex IV). Complex IV catalyzes the electron transfer from reduced cytochrome *c* to O_2 to form H_2O. Thus, increased reduction of cytochrome *c* by MBH_2 (13, 40) could explain in part the increase in complex IV that occurs in the presence of 100 nM MB (Fig. 3). The signaling mechanism that mediates this increase in complex IV by MB needs to be clarified.

Adequate assembly and activity of complex IV depends on heme-*a* (54), which exists only in complex IV. Thus, the increase in the level of complex IV requires an increase in heme, which is the precursor for heme-*a*. The increase in the rate in heme synthesis that we observed at 100 nM MB could provide the cells with the heme-*a* to support the assembly of complex IV. The increases in heme synthesis can also support metabolic functions other than complex IV. Close to 95% of the intracellular O_2 is consumed by the activity of complex IV. Consistent with the increase in complex IV, we also observed an increase in O_2 consumption in cells maintained in 100

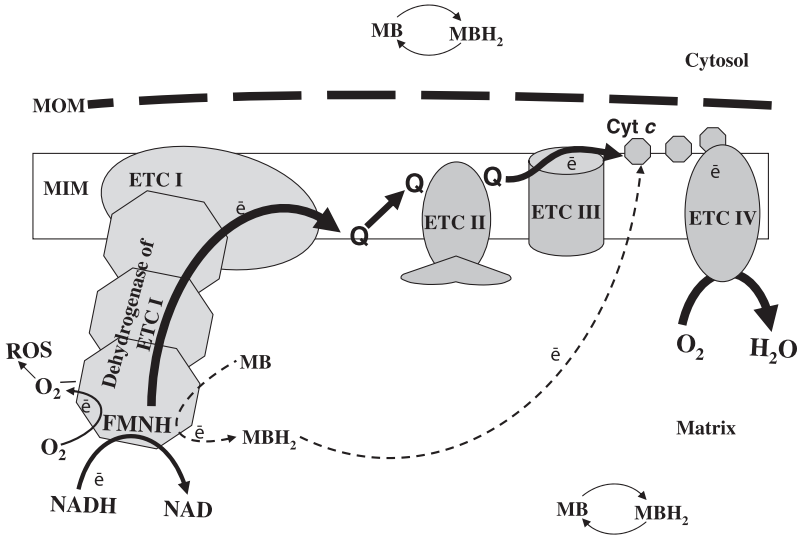
nM MB. Although the increase in complex IV reflects an increase in the mitochondrial activity, it is also likely to cause a decrease in the intracellular steady-state concentration of O_2, which may decrease oxidant production.

The level of complex IV and the rate of heme synthesis decreased at 1 μM MB, but increased at 100 nM MB. This similar pattern of response to 100 nM and 1μM MB by complex IV and heme synthesis suggests that both effects are linked as proposed above. The mechanism of the decrease in complex IV and in heme synthesis inflicted by 1 μM MB, however, is not clear. The loss of complex IV at 1 μM MB was totally reversible on removal of MB from the medium (data not shown). The adverse effects seen by the 1 μM MB on cellular senescence in air (20%) were not observed in 5% O_2 (Fig. 1*B*), suggesting that the effect of MB on cellular senescence and complex IV depends on the intracellular concentration of O_2. The relevance of these observations to the mechanism of senescence delay by MB is discussed below.

The oxidation of NADH by MB is accelerated by more than 50-fold in the presence of mitochondrial lysate (Table 2), consistent with the enzymatic reduction of MB by NAD(P)H. The NADH dehydrogenase activity of complex I is a possible catalyst for the oxidation of NADH by MB in the mitochondrial lysate. Interestingly, only a minor decline in the absorbance of MB at 666 nm occurred in the mitochondrial lysate and NADH. Such a minor decline was unexpected, and probably due to MBH_2 being quickly reoxidized to MB in the presence of mitochondrial lysate. Potential candidates responsible for reoxidizing MBH_2 are cytochrome *c* or O_2. Reoxidation of MBH_2 by O_2 produces oxidants. In an attempt to find whether cytochrome *c* or O_2 (13, 40) reoxidizes MBH_2 in the mitochondrial lysate, we used DCFH to examine whether the oxidation of MBH_2 in the mitochondrial lysate produces oxidants. We found that adding only NADH to the mitochondrial lysate at atmospheric O_2 (≈250 μM O_2) in the absence of MB almost doubled the oxidation of DCFH, suggesting an NADH-dependent increase in the production of oxidants, which was moderate (Fig. 5). Adding MB at 0.1, 1, and 10 μM to the mitochondrial lysate and NADH increases oxidants production by 60% and 500% at 1 μM and 10 μM MB, respectively (Fig. 5), whereas 0.1 μM MB did not increase DCFH oxidation. To test the contribution of O_2 to this phenomenon, we lowered the concentration of O_2 in the reaction buffer to 5% (≈62.5 μM). Low concentrations of O_2 eliminated the MB-dependent production of oxidants seen at 1 μM MB and dramatically decreased oxidant production at 10 μM MB (Fig. 5). Because at 5% O_2 the reaction buffer still contains 60- and 6-fold excess of O_2 over 1 μM and 10 μM MB, respectively, and yet oxidant production by MB dramatically decreases, we suspected cytochrome *c* in the mitochondrial lysate is involved in preventing the oxidant production by MB, based on previous work by McCord and Fridovich and others (13, 40).

MBH_2 is known to preferentially and directly reduce cytochrome *c* even in the presence of molecular oxygen, bypassing the production of superoxide radical (13, 40). Cytochrome *c* added to the mitochondrial lysate, NADH, rotenone, and 10 μM MB significantly eliminated the production of oxidants (Fig. 6). We interpreted this effect of cytochrome *c* on oxidants to be due to the transfer of electrons from MBH_2 to the excess cytochrome *c* rather than to O_2, preventing the formation of superoxide radical. These findings also suggest that a high ratio between cytochrome *c* and MB (or cytochrome *c*-complex IV/MB) is essential for preventing the autooxidation of MBH_2 inside the cell. Consistently, no increase in the oxidation of DCFH in the mitochondrial lysate was seen at 100 nM MB (Fig. 5), suggesting that the endogenous cytochrome *c* present in the lyaste is enough to compete with O_2 on the 100 nM MBH_2. Thus, the antisenescence action of MB at 100 nM (in air) and 1 μM (in 5% O_2) in IMR 90 cells might be explained, in part, by an optimal ratio (cytochrome *c* + complex IV)/MB/O_2 under these conditions.

NAD(P)H-dependent dehydrogenases (*e.g.*, NADH-dehydrogenase of complex I) can reduce artificial electron acceptors other than MB (*e.g.*, phenazine



Scheme 1. The sequence of reduction-oxidation events of MB proposed for the electron transport complexes (ETC I, II, III, and IV). MB is proposed to be reduced to MBH_2 by the NADH-dehydrogenase of complex I, thus competing with O_2 and preventing the formation of oxidants. Subsequently, MBH_2 is reoxidized by heme proteins such as cytochrome c (cyt *c*) and by ETC IV. Similar reactions may occur in other redox-active proteins, as discussed in the text. MB, oxidized MB; MBH_2, reduced MB; Q, coenzyme Q.

methosulfate) or O_2 to produce superoxide radical (55). Thus, we propose that MBH_2 and MB serve as electron carriers between several dehydrogenases and heme-proteins (*e.g.*, cytochrome *c*, **Scheme 1**). Complex IV, in turn, recycles the reduced cytochrome *c*. If this cycling of MB occurs within the mitochondrial ETC, we predict an inhibition of the production of superoxide radical by MB competing with O_2 at the site of free radical production at NADH-dehydrogenase component of complex I (55). The significance of cytochrome *c* in lowering the production of oxidants has been previously demonstrated (56). NADH-dehydrogenase of complex I faces the matrix; thus, part of the MBH_2 will be formed in the mitochondrial matrix in addition to the cytosol. The hydrophobicity of MBH_2 increases on reduction (53, 57, 58), thus increasing the chances that MBH_2 crosses the mitochondrial inner membrane and reaches cytochrome *c* and complex IV (Scheme 1).

A similar model for recycling of MB was also proposed for xanthine oxidase (XOD) (30). XOD, a flavo-enzyme, reduces O_2 to form superoxide radical. Enzymatic reduction of MB by XOD *in vitro* has been previously shown to prevent the production of superoxide radical by XOD and protect from postischemic injury in slices from rat liver. The product, MBH_2, has been proposed to produce H_2O_2 rather than superoxide radical, which was considered an advantage as H_2O_2 can readily be eliminated by catalase and glutathione peroxidase (30). Our data suggest that in the presence of a suitable physiological electron carrier (*e.g.*, heme proteins (13, 40, 53), oxidants will not be formed. We believe that other physiological electron carriers, in addition to cytochrome *c*, can recycle MB.

A promising strategy to prevent oxidative damage is to block the production of free radicals rather than intercepting them postformation. We propose that MB blocks the production of free radicals by recycling within the ETC. The effects of MB on complex IV and mitochondrial metabolic pathways may provide protection against a variety of pathological conditions, toxic agents, and age-related mitochondrial associated neurodegeneration. Complex IV decay is a key mitochondrial dysfunction in AD. It is shown here that MB increases complex IV *in vitro*. We have replicated these findings *in vivo* (unpublished data). Thus, MB, which is known to enter the brain, may be useful in AD. Complex I decay has primarily been implicated in Parkinson disease (PD). The experiments here also suggest that MB may also be useful for PD. FJ

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