Activation of HGF/c-Met pathway contributes to the reactive oxygen species generation and motility of small cell lung cancer cells

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Activation of HGF/c-Met pathway contributes to the reactive oxygen species generation and motility of small cell lung cancer cells. Am J Physiol Lung Cell Mol Physiol 292: L1488–L1494, 2007. First published February 23, 2007; doi:10.1152/ajplung.00147.2006.—Small cell lung cancer (SCLC) is a difficult disease to treat and sometimes overexpression or mutation of c-Met receptor tyrosine kinase. The effects of c-Met/ hepatocyte growth factor (c-Met/HGF, ligand for c-Met) on activation of reactive oxygen species (ROS) was determined. HGF stimulation of c-Met-overexpressing H69 SCLC cells (40 ng/ml, 15 min) resulted in an increase of ROS, measured with fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) or dihydroethidine (DHE) but not in c-Met-null H446 cells. ROS was increased in juxtamembrane (JM)-mutated variants (R988C and T1010I) of c-Met compared with wild-type c-Met-expressing cells. ROS was significantly inhibited by preincubation of SCLC cells with pyrrolidine dithiocarbamate (PDTC, 100 μM) and/or SU11274 (small molecule c-Met tyrosine kinase inhibitor, 2 μM) for 3 h. PDTC and SU11274 also abrogated the HGF proliferative signal and cell motility in a cooperative fashion. H2O2 treatment of SCLC cells (over 15 min) led to phosphorylation of c-Met receptor tyrosine kinase and further upregulated downstream phosphorylation of phospho-AKT, ERK1/2, and paxillin in a dose-dependent manner (125 μM to 500 μM). c-Met is an important target in lung cancer, and the pathways responsible for ROS generation together may provide novel therapeutic intervention.

LUNG CANCER is one of the most common fatal malignancies in the developed world. Small cell lung cancer (SCLC), which constitutes 16% of all lung neoplasm and is different from non-small cell lung cancer (NSCLC) types in that it has neuroendocrine features, grows more rapidly, spreads earlier, is more responsive to chemotherapy and radiotherapy, and has a lower cure rate (4). SCLC metastasizes rapidly to distant sites within the body and is most often discovered after it has spread extensively. Only 2% of patients with extensive stage disease and 12% of patients with limited stage disease treated with currently available therapy survive 5 or more years (7).

New therapeutic strategies are urgently needed, and these will most likely result from a better understanding of the biological and biochemical events associated with SCLC. In SCLC patients, the high level of oxidative stress, expressed as a spontaneous generation of hydrogen peroxide in tumor tissue, was associated with clinical progression of the tumor’s stage (35). However, despite the positive correlation between increased generation of reactive oxygen species (ROS) and the invasion of cancer, the specific mechanisms by which antioxidants act to suppress cancer development through ROS is unknown.

Hepatocyte growth factor (HGF) has multiple biological effects on a wide variety of cells, including mitogenic, morphogenic, and anti-apoptotic activities (2, 3, 34). The receptor for HGF is c-Met, a proto-oncogene product consisting of an α-chain of 50 kDa and a β-chain of 145 kDa. Overexpression and mutation of the c-Met receptor has been well described in various cancer tissue samples as well as cell lines (11, 13, 15, 21, 23, 26). We have recently reported overexpression of c-Met and missense mutations and alternative splicing isoforms of c-Met in SCLC. The juxtamembrane (JM) domain mutations of c-Met were shown to have activating phenotype with increased cell motility and tumorigenesis (23). Currently, several ATP binding small molecules against c-Met are in the pipeline to treat various cancers (6).

ROS such as hydrogen peroxide (H2O2), superoxide (O2−), and hydroxyl radical (HO·) are generated from cells modulated by various growth factors, cytokines, hormones, and stress factors. In excess, ROS and their byproducts that are capable of causing oxidative damage may be cytotoxic to cells. However, moderate levels of ROS have been shown to regulate cellular functions such as gene expression and signal transduction (19). Stimulation of tyrosine kinase activity by ROS has been described before (30). For example, ionizing radiation and H2O2, agents that induce oxidative stress, have been demonstrated to induce tyrosine phosphorylation events and activate downstream kinases such as protein kinase C, p56, LCK, and SHC (10, 25). Miura et al. (24) reported that ROS induce the expression of hepatocyte growth factor (HGF) genes and stimulate the autocrine action of HGF in invading ascites hepatoma cells.

In this study, the modulation of ROS with c-Met activation by HGF and inhibition with a novel specific c-Met tyrosine kinase inhibitor, SU11274, was determined. The reverse was also investigated; i.e., modulation of c-Met activation or inhibition was determined with upregulation or downregulation of ROS. Finally, we show that modulating ROS and c-Met leads to alterations in cell viability and cell motility of SCLC cells.

MATERIALS AND METHODS

Reagents and drugs. Human recombinant HGF was purchased from Calbiochem (Cambridge, MA), and fetal bovine serum (FBS) was from Gemini Bioproducts (Woodland, CA). Cell culture media,
penicillin, and streptomycin were obtained from Cellgro (Boehringer Ingelheim, Heidelberg, Germany). Antiphosphotyrosine (4G10) antibody was obtained from Upstate Biotechnology (Lake Placid, NY). The PI3K-p85 antibody, polyclonal phosphorylation site-specific c-Met (Y1230/1234/1235), paxillin (Y31), and ERK1/2(T185/Y187) antibodies were obtained from Biosource International (Camarillo, CA). Antibody against p-AKT (S473) was obtained from Cell Signaling Technology (Beverly, MA). Anti-mouse or rabbit IgG, horse-

**Fig. 1.** Hepatocyte growth factor (HGF) induces intracellular reactive oxygen species (ROS) generation in c-Met-overexpressing small cell lung cancer (SCLC) cells (H69). H69 (A and B) and H446 (C and D) cells were starved for 16 h and incubated in the presence/absence of 40 ng/ml HGF. Changes in intracellular ROS were measured by two FL detection systems (FL1-H and FL3-H) using the redox-sensitive dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA; A and C) and dihydroethidine (DHE; B and D). H69 cells had robust expression of c-Met, whereas H446 cells had very minimal to no expression of c-Met. E: equal loading of lysate is shown by the 85-kDa subunit of phosphatidylinositol 3-kinase (PI3K) immunoblot.

**Fig. 2.** Juxtamembrane (JM) domain mutation of c-Met leads to alteration in ROS levels. A: the gene structure of c-Met is illustrated schematically here in the context of the functional domains; SEMA, semaphorin; PSI, plescin, semaphorin, integrin; IPT, immunoglobulin-like regions in plescins and transcription factors; TM, transmembrane. B: expression of c-Met receptor in BaF3 cells with wild-type (Wt) c-Met or its JM-mutated variants (R988C.Met and T1010I.Met) was examined by immunoblotting using polyclonal c-MET (c12) antibody; shown is also a negative control. ROS production was measured using fluorescence probe DCFH-DA by FACScan analysis for BaF3 cells transfected with wild-type c-Met, R988C.Met and T1010I.Met. C: relative 2′,7′-dichlorofluorescein (DCF) florescence (%) was calculated in mutant R988C and T1010I cells compared with wild-type c-Met, then plotted to show the change of ROS levels in mutants (n = 3, *P < 0.01). D: histogram is representative of three independent experiments.
Fig. 3. Pyrrolidine dithiocarbamate (PDTC) and SU11274 alter HGF-induced cell viability. H69 cells that had been starved for 24 h with 0.5% BSA containing RPMI-1640 medium and cultured in the absence or the presence of HGF (40 ng/ml), HGF (40 ng/ml) + PDTC (10 μM), HGF (40 ng/ml) + SU11274 (1 μM), and HGF (40 ng/ml) + PDTC (10 μM) + SU11274 (1 μM) for 24–48 h. Cell viability was measured using trypan blue dye exclusion method (n = 3, *P < 0.001).

radish peroxidase-linked secondary antibodies were obtained from Invitrogen (Carlsbad, CA). c-Met (c12) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was acquired from Acros Organics (Morris Plains, NJ). Dihydroethidine (DHE) was obtained from Molecular Probes (Eugene, OR). SU11274 was obtained from Pfizer (San Diego, CA). Hydrogen peroxide (H2O2), pyrrolidine dithiocarbamate (PDTC), β-actin monoclonal antibody, and all other chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Cell lines and cell culture. The SCLC cell lines (NCI-H69, H446) and the murine pre-B IL-3-dependent BaF3 cell line were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI-1640 medium containing 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified, 5% CO2 atmosphere. BaF3 cells were cultured in RPMI-1640 medium containing 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified, 5% CO2 atmosphere. BaF3 cells were cultured in the presence of 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified, 5% CO2 atmosphere.

Detection of ROS. For intracellular ROS measurement, 5 × 10^5 cells were incubated for 15 min at 37°C with the oxidation-sensitive fluorescent probe DCFH-DA (5 μM), or DHE (2 μM), followed by FACScan (Becton-Dickinson) analysis (33) of cells were imaged with an Olympus IMT-2 inverted phase/epifluorescence microscope. Fluorescence intensity was measured using a cooled slow-scanning PC-controlled camera (Hamamatsu, Hamamatsu City, Japan) coupled with ImagePro Plus software for the quantification of fluorescent changes. ROS was measured by measuring changes in fluorescence resulting from intracellular probe oxidation as described previously (31, 32). The probe DCFH-DA (5 μM) is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within cells. Hydrogen peroxide or low-molecular-weight peroxides produced by cells oxidizes DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus increases in DCFH oxidation to DCF (i.e., increases in DCF fluorescence) suggest ROS generation.

Immunoblot analysis. Cells were harvested and lysed in lysis buffer [20 mM HEPES-NaOH (pH 7.2), 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM Na3VO4, leupeptin (5 μg/ml), aprotinin (5 μg/ml), and 1 mM phenylmethylsulfonyl fluoride]. The lysates were incubated on ice for 20 min and then centrifuged for 5 min at 10,000 g. Protein concentration of the supernatant was quantified using the Bio-Rad detergent-compatible protein assay kit with BSA as standard. Equal amounts of cellular protein were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. The membranes were first blocked for 1 h in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% nonfat dried milk, and then incubated with the appropriate primary antibody with appropriate dilution in TBS containing 0.1% Tween 20 and 3% nonfat dried milk overnight at 4°C. Nitrocellulose membranes were washed three times in wash buffer (TBS and 0.1% Tween 20). Primary antibody was detected using horseradish peroxidase-linked goat anti-mouse or goat anti-rabbit IgG antibodies and visualized with the ECL detection system (Amersham) on autoradiography films. Immunoblot experiments were performed at least three times.

Analysis of cell motility by time-lapse video microscopy. H69 cells were plated on cell culture dishes and placed into a temperature-controlled chamber at 37°C in an atmosphere of 5% CO2. The cells were examined by time-lapse video microscopy (TLVM) as described previously (17), using an Olympus IX81 or Axiovert inverted microscope equipped with cooled CCD cameras in growth factor-deprived media with the absence or presence of HGF, and HGF with 3-h pretreatment of PDTC and/or SU11274; pictures were taken at 0–2 h

Fig. 4. PDTC and SU11274 decrease HGF-induced intracellular level of ROS in responsive SCLC cell lines. H69 cells were serum-starved for 16 h and pretreated with PDTC and/or SU11274, and changes in intracellular ROS were measured in the absence or the presence of HGF (40 ng/ml) after 60 min. SCLC (H69) cells were exposed for 2 h to HGF, HGF+PDTC, HGF+SU11274, HGF+SU11274+PDTC, and relative fluorescence was measured in these cells, and control cells and plotted to show the ROS kinetics. PDTC+SU11274 completely attenuated HGF-induced ROS generation at 60 min (n = 3, #P = 0.02, *P < 0.001).
at 1-min intervals. Cell movement and changing shapes were analyzed using NIH Image 1 analysis program and MetaMorph (Universal Imaging) from TLVM pictures taken at 1-min intervals over 2 h; images of the per-pixel intensity standard deviation (SD) were created with false colors. Membrane movements create intensity changes that in turn produce greater SD values at each pixel that changed. Warmer color indicates greater SD and movements.

Statistical analysis. Statistical significance was tested using statistics software 15.0 (Chicago, IL). For comparisons between means of two groups, Student’s t-test was used. For comparing means among more than two groups, one-way ANOVA was used. A value of $P < 0.05$ was regarded statistically significant. All values were expressed as mean ± SE.

RESULTS

HGF induces ROS generation in c-Met-overexpressing SCLC cells. Intracellular generation of ROS in c-Met-overexpressing H69 and nonexpressing H446 cells was determined using DCFH-DA and DHE by flow cytometry with HGF. Stimulation of c-Met-overexpressing SCLC cells (H69) with HGF (40 ng/ml, 15 min) resulted in elevation of 13 ± 8% ($n = 3, P < 0.001$) and a 39 ± 3% ($n = 3, P < 0.001$) increase in ROS, measured with fluorescent probe DCFH-DA and DHE, respectively. Moreover, there was no marked increase in the level of ROS generation in c-Met-null H446 (SCLC) cells with HGF (Fig. 1).

Mutations of c-Met alter ROS generation. Since JM mutations (R988C and T1010I) are found in SCLC, we examined the ROS level in BaF3 cell lines that were transfected with either the wild-type c-Met or its JM-mutated variants (R988C and T1010I), using fluorescence indicator DCFH-DA by FACScan analysis. Equal expression of c-Met in two JM mutant c-MET-transfected cell lines (R988C.Met-BaF3, and T1010I.Met-BaF3) and a wild-type c-MET-transfected cell line (Wt-Met-BaF3) was confirmed by immunoprecipitation with c-Met and thereafter immunoblotting. The characteristics of these cell lines were previously described (23). Intracellular ROS levels were significantly elevated in R988C (41 ± 4%) and T1010I (24 ± 7%) compared with wild-type c-Met-transfected BaF3 cells (Fig. 2).

PDTC and SU11274 inhibit SCLC cell proliferation in response to HGF. HGF leads to a proliferative response in H69 SCLC cells. Treatment of H69 cells with a scavenger of ROS such as PDTC (10 μM), or c-Met inhibitor SU11274 (2.5 μM), significantly inhibited the proliferative response of H69 cells to 40 ng/ml HGF and also decreased cell viability within the 24-h and 48-h incubation periods. Results are shown as percentages calculated from trypan blue exclusion. PDTC had an inhibitory effect on cell growth of H69 cells even in the presence of HGF. Even though PDTC or SU11274 individually led to decreased viability of H69 cells, the combination led to further decrease in viability (Fig. 3).

PDTC and SU11274 inhibit HGF-induced ROS in SCLC cells. The levels of ROS in H69 cells with or without PDTC (ROS scavenger, 100 μM) and/or SU11274 (2 μM) treatment were analyzed by epifluorescence microscope using the fluorescence probe DHE for response with HGF (40 ng/ml) at various time exposures. The relative fluorescence in a field of ~500 cells was measured at each time point. The addition of PDTC and SU11274 results in a further decrease in ROS levels (Fig. 4).
PDTC and SU11274 completely attenuated HGF-induced ROS generation at 60 min (Fig. 4).

H$_2$O$_2$ mediates c-Met tyrosine phosphorylation and downstream signal transduction. To investigate whether H$_2$O$_2$ could serve as a mediator of HGF-induced c-Met phosphorylation and its downstream signaling, we tested the effect of exogenous H$_2$O$_2$ on c-Met phosphorylation. We observed a significant increase in c-Met phosphorylation as well as the activation of other downstream signaling molecules of c-Met, such as AKT, ERK1/2, and paxillin, when the cells were incubated with H$_2$O$_2$ in a dose- and time-dependent manner similar to HGF. To compare and determine the biochemical consequences of c-Met kinase activation by HGF and H$_2$O$_2$ in SCLC cells, changes in tyrosine phosphorylation of cellular proteins were evaluated. In H$_2$O$_2$-treated cell lysates, compared with HGF-treated cell lysates, tyrosine phosphorylation of a set of unique and overlapping proteins was observed. In particular, there was a significant tyrosine phosphorylation of 120- to 130-, and 145-, and 175-kDa protein bands that appeared in both the HGF- and H$_2$O$_2$-treated SCLC cell lysates. Total c-Met expression was not affected by any of these treatments (Fig. 5).

PDTC and SU11274 inhibit HGF-induced cell motility and morphological changes in SCLC cells. We examined the effects of pretreatment of PDTC and/or SU11274 on HGF-stimulated SCLC cell motility. HGF-induced cell motility were significantly inhibited in the drug-treated cells. Morphological changes including shape and formation of filopodia were observed (data not shown) in response to HGF, whereas the combination of PDTC and/or SU11274 pretreatment diminished these processes to levels obtained in control. PDTC and SU11274 reduced HGF (100 ng/ml, 4 h), stimulated motility of SCLC cells, and achieved more suppression of HGF activity in individual cells than cell clusters (Fig. 6).

PDTC and SU11274 inhibit HGF-induced tyrosine phosphorylation of cellular proteins in SCLC cells. To determine the biochemical consequences of c-Met inhibition by PDTC and/or SU11274 in SCLC (H69) cells, changes in tyrosine phosphorylation of cellular proteins were evaluated. In SCLC cells stimulated with HGF, a set of unique tyrosine phosphorylated proteins was observed, with prominent tyrosine phosphorylation of c-Met. Pretreatment of H69 cells with PDTC and SU11274 reduced tyrosine phosphorylation of a number of proteins in response to HGF and phosphorylation of c-Met. Expression of total c-Met was not affected by any of these treatments (Fig. 7).

DISCUSSION

Novel targeted therapies are desperately needed in the treatment of SCLC. HGF is the ligand for c-Met that is produced in a juxtacrine/paracrine fashion in SCLC. The stromal cells contain a considerable amount of HGF, whereas, HGF is not produced by the lung cancer cells. In this study, we identified cross-modulation of the c-Met receptor tyrosine kinase and ROS. Upon stimulation of SCLC cells with HGF, there was upregulation of ROS, enhanced proliferation and cell motility, and enhanced phosphorylation of c-Met and other downstream targets. Previously, we had identified that c-Met can be mu-

![Fig. 6. PDTC and SU11274 inhibit HGF-induced cell motility and shape in H69 cells. Pretreated (PDTC and SU11274) H69 cells were observed by time-lapse video microscopy with or without 40 ng/ml HGF. The pictures were taken every 1 min, the response in control or pretreated cells is shown and was analyzed as described in MATERIALS AND METHODS. Membrane movements create intensity changes that in turn produce greater standard deviation (SD) at each pixel that changed. Warmer color indicates greater SD and movements. Cell motility increased in the presence of HGF. Warmer color with HGF stimulation indicates that motility and cell shape was increased dramatically. A: note the decreased cell motility of the c-Met-expressing H69 cells with pretreatment of PDTC and SU11274. B: the position of cell was measured, tracked every 1 min using the NIH Image J and MetaMorph (Universal Imaging) programs, and plotted to show the trace of change in cell shape and motility (n = 3, P < 0.01).](www.ajplung.org)
Peroxynitrate (ONOO⁻) results in the generation of H₂O₂. Reduction of H₂O₂ in the presence of Fe³⁺ through the Fenton reaction. ROS levels can be quenched not only by enzymes, antioxidants, and sulfhydryl groups, but also by reacting with cellular molecules such as lipids and DNA bases. Both O₂⁻ and HO are radicals with unpaired electrons that have the potential to cause cellular damage. SCLC is directly secondary to cigarette smoking and toxicity secondary to generated ROS (27). A delicate balance between oxidants and the protective effects of the intracellular and extracellular oxidant defense system are abrogated in SCLC. We have found that ROS is generated by HGF in SCLC. ROS may act as second messengers to regulate activities of redox-sensitive enzymes, including protein kinases and protein phosphatases. Of particular interest is the fact that protein tyrosine phosphatases (PTPases) are highly sensitive to oxidation due to the presence of a critical thiol group. It is likely that, in addition to PTPases, there are other redox-sensitive molecules that can be regulated by ROS. For example, oxidation of Cys118 in RAS is known to activate its GTPase activity (20). Overexpression of the O₂⁻-generating NADPH oxidize Mox1 in NIH 3T3 fibroblasts increases cell growth and induces tumors in athymic mice (29). It would now be useful to investigate the activity of oxidant and antioxidant enzymes in the context of HGF/c-Met activation. There are several mechanisms of ROS production through the small GTPases such as Rac, and this should also be investigated in the future.

There is precedence in the literature for production of ROS in the context of receptor activation. H₂O₂ induces epidermal growth factor receptor (EGFR) tyrosine phosphorylation in intact cells as well as in membranes of A549 lung epithelial cells (12). We identify here that c-Met phosphorylation can occur with ROS stimulation, and c-Met activation with HGF leads to a number of biological and biochemical changes. c-Met has been implicated in cell proliferation, cell motility, invasion, metastasis, and angiogenesis (8). c-Met can synergize with other receptors as well as downstream signal transduction pathways.

It can also be mutated in a number of solid tumors. The majority of mutations have been identified in the tyrosine kinase domain, especially in hereditary papillary renal cell carcinoma and head and neck cancer. Our studies have shown that in SCLC, NSCLC, and mesothelioma there were no mutations in the tyrosine kinase domain, but alterations occurred in the JM domain and the semaphorin domain. The JM domain serves as a negative regulator of the tyrosine kinase domain (14, 22, 23). It is believed that having a mutation in the JM domain alters the tyrosine kinase domain activity. This could occur through the potential production of ROS as we have shown here.

c-Met and ROS have dramatic effects on biological functions such as cell motility. SCLC cells have a unique ability to migrate, since this tumor is highly metastatic. SCLC cells also move as a cluster rather than individual cells. In this study, modulation of c-Met and/or ROS led to alterations in cell motility. It is possible that this occurs through actin binding molecules such as the focal adhesion proteins. Paxillin is a focal adhesion protein that is involved intimately in cell motility and association with small GTPases such as Rho. We show that paxillin is phosphorylated in response to ROS and c-Met activation. It would now be useful to determine the role of focal adhesion proteins such as paxillin in c-Met/ROS-induced cell motility. In particular, in certain conditions, paxillin has been shown to be cleaved or translocated to the...

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**Fig. 7.** PDTC and SU11274 inhibit HGF-induced activation of cell signaling proteins. H69 cells were pretreated with 10 μM PDTC for 3 h, or 1 μM SU11274 for 12 h, or a combination of PDTC and SU11274, and followed by HGF (40 ng/ml) stimulation, after which cell lysates were immunoblotted and analyzed for their phosphotyrosine content with antiphosphotyrosine antibody (4G10). B: the same membrane was stripped and reprobed for phospho-c-Met (Y1230/1234/1235). C: protein loading was monitored by using antibody against total c-Met.

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nucleus (5). It would be of further interest to determine the localization of paxillin in the context of modulation of c-Met and ROS.

The clinical relevance of c-Met inhibition has not yet been determined. There are several pharmaceutical companies that are in the process of developing specific c-Met tyrosine kinase inhibitors, antibodies against c-Met or HGF, or competitive peptides. We have recently evaluated two specific inhibitors, SU11274 and PHA665752, in lung cancer. As an example, SU11274 leads to differential decrease in cell viability of various non-SCLC cell lines. Also, small interfering RNA (i.e., siRNA) gene silencing has been used to show the importance of c-Met in lung cancer. Since SCLC is a difficult disease to treat and novel therapies have repeatedly failed in clinical trials, if the relevance of c-Met inhibition would come to clinical fruition, it would be important to combine it with other therapeutic strategies such as antioxidant treatment.

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