

**PHARMACOLOGICAL APPROACHES IN AN EXPERIMENTAL MODEL OF
NON-SMALL CELL LUNG CANCER: EFFECTS ON TUMOR BIOLOGY**

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ABSTRACT

Lung cancer (LC) remains the leading cause of cancer mortality worldwide, and non-small cell LC (NSCLC) represents 80% of all LC. Oxidative stress and inflammation, autophagy, ubiquitin-proteasome system, nuclear factor (NF)- κ B, and mitogen activated protein kinases (MAPK) participate in LC pathophysiology. Currently available treatment for LC is limited and *in vivo* models are lacking. We hypothesized that antioxidants and NF- κ B, MAPK, and proteasome inhibitors may exert an antitumoral response through attenuation of several key biological mechanisms that promote tumorigenesis and cancer cell growth. Body and tumor weights, oxidative stress, antioxidants, inflammation, NF- κ B p65 expression, fibulins, apoptosis, autophagy, tumor and stroma histology were evaluated in the subcutaneous tumor of LC (LP07 adenocarcinoma) BALB/c mice, with and without concomitant treatment with NF- κ B (sulfasalazine), MEK (U0126), and proteasome (bortezomib) inhibitors, and N-acetyl cysteine (NAC). Compared to LC control mice, in subcutaneous tumors, the four pharmacological agents reduced oxidative stress markers and tumor proliferation (ki-67). Inflammation and NF- κ B p65 expression were attenuated by NF- κ B and MAPK inhibitors, and the latter also enhanced apoptotic markers. Catalase was induced by the three inhibitors, while bortezomib also promoted superoxide dismutase expression. NF- κ B and MEK inhibitors significantly reduced tumor burden through several biological mechanisms that favored tumor degradation and attenuated tumor proliferation. These two pharmacological agents may enhance the anti-tumor activity of selectively targeted therapeutic strategies for LC. Proteasomal inhibition using bortezomib rather promotes tumor degradation, while treatment with antioxidants cannot be recommended. This experimental model supports the use of adjuvant drugs for the improvement of LC treatment.

Word count: 250

KEY WORDS: lung adenocarcinoma; therapeutic strategies; stromal structure; tumor proliferation; apoptosis; autophagy; redox imbalance; inflammation.

INTRODUCTION

Lung cancer (LC) remains the leading cause of cancer mortality worldwide. Non-small cell LC (NSCLC) represents 80% of all LC, and adenocarcinoma is the most common subtype [1-5]. The presence of underlying inflammatory conditions such as chronic obstructive pulmonary disease (COPD), which also represents a major cause of death in industrialized regions, increases the risk of LC in patients [1, 3, 5-7]. Despite recent progress using several therapeutic strategies, the five-year survival is still extremely poor in patients with LC (15-20% [1-3, 5-9]). In these patients, LC is usually diagnosed at advanced stages, as the disease may remain silent for several months or symptoms of underlying COPD may mask those of the neoplasm. Moreover, a major hindrance in the chemotherapy of LC is resistance to currently available drugs, establishing an appropriate selective therapy [1, 3, 6].

Oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of the former, may promote carcinogenesis by oxidation of specific targets that result in DNA mutations or in the activation of cellular processes that lead to neoplastic transformation [10]. In fact, it has been proposed that oxidative stress may drive a continuous process of DNA adducts and posttranslational modifications to proteins and lipids that favor growth and cell viability [11]. In keeping with, we also demonstrated that in patients with LC, oxidized DNA levels were increased in the blood, and redox imbalance and inflammation were predominant in their normal airway epithelium distant to the neoplasm [12]. More recently, it has been shown that antioxidant inhibition of SOD1 by the small molecule ATN-224 decreased tumor burden in mice, while it also induced cell death in different NSCLC cell lines [13].

Mitogen-activated protein kinases (MAPK) and nuclear factor (NF)- κ B, which are central regulators of gene expression, redox balance, and metabolism, have also been shown to play a major role in adaptive or maladaptive responses to cellular stress within several

tissues. Several strategies of drug combinations have been recently demonstrated to counteract the resistance developed by tumors to cancer therapies [14]. For instance, in NSCLC, the combination of MAPK inhibitors with other drugs was effective *in vitro* in attenuating resistance to chemotherapy [14]. NF- κ B also plays a key role in several biological processes that favor tumor growth such as cell proliferation, anti-apoptosis, inflammation, vascular regeneration, infiltration, and metastasis [15]. In fact, activation of NF- κ B is a characteristic feature of several cancer types including lung cancer [15]. Recently, lung adenocarcinoma cells were also shown to be resistant to NF- κ B inhibition through Akt phosphorylation [16].

The proteasome inhibitor bortezomib, which is commonly used in the treatment of multiple myeloma and mantle cell lymphoma, has scarce clinical applicability as a single drug against solid tumors including LC [17]. Nevertheless, bortezomib has been shown to sensitize cancer cells to apoptosis partly via caspase activation [18]. In a recent investigation [19], the combination of bortezomib with inducible caspase-9 delivered by mesenchymal stromal cells enhanced the anti-tumor activity in human NSCLC cell lines.

On this basis, we hypothesized that antioxidants and NF- κ B, MAPK, and proteasome inhibitors may exert an antitumoral response through attenuation of several key biological mechanisms that promote tumorigenesis and cancer cell growth. Thus, key biological events such as cell proliferation, redox balance, inflammation, apoptosis, and autophagy were analyzed in the subcutaneous tumors of lung adenocarcinoma cells in BALB/c mice in response to several pharmacological agents that directly influence tumor growth and survival, namely the antioxidant N-acetyl cysteine (NAC), and the inhibitors of MAPK (MEK), NF- κ B, and proteasome pathways: U0126, sulfasalazine, and bortezomib, respectively. The current model has been previously validated [20-24], and is the only approach to obtain sufficient material to warrant the reliability of the biological determinations in the tumors,

since they would have not been possibly analyzed in the lung micrometastasis of the experimental mice.

METHODS

(Detailed methodologies are provided in the electronic supporting information).

Animal experiments

Tumor. The LP07 cell line derives from the P07 lung tumor, which spontaneously arose in the lungs of BALB/c mice[20]. This cell line was obtained *in vitro*, after different passages of a P07 primary culture in previous studies[20-22]. LP07 cell line shares identical characteristics with the P07 tumor regarding both lung tumor incidence and histology [20-22]. Moreover, one month after tumor transplantation, all animals developed lung metastasis and spleen enlargement without affecting other organs [20-23, 25].

Experimental design. Female BALB/c mice (2 months old, 20 g average weight) were purchased from *Harlan Interfauna Ibérica SL* (Barcelona, Spain). The rodents were maintained with a regular 12:12 h light-dark cycle and had free access to water and food. LC was induced by inoculation of mice subcutaneously with 4×10^5 of LP07 viable cells in 0.2 mL of Minimal Essential Medium (MEM) in the left flank on day 1. All pharmacological agents were administered from day 15 post-inoculation of the LP07 cells on to the end of the study protocol on day 30.

Mice were assigned to the different experimental groups (n=7, all groups): 1) non-treated LC control, inoculation of LP07 tumor cells in the left flank, and animals bearing the LP07 tumor (4×10^5 cells), which received concomitant treatment with the different pharmacological agents that were grouped as follows: 2) LC-antioxidant: mice were treated orally with 0.05ml (0.5g/kg) NAC/48h (Pharmazam, Barcelona, Spain). Group 3) LC-Proteasome inhibitor: mice were inoculated into the tail vein with 0.1 ml (0.15 mg/Kg) bortezomib, a proteasome inhibitor, (Velcade, Millenium Pharmaceuticals, Cambridge, MA, USA) every 6 days[26]; 3)

LC-MAPK inhibitor: LC mice were inoculated intraperitoneally with 0.1 mL (30 mg/Kg)/48h of U0126 (selective MEK inhibitor), a highly selective inhibitor of ERK1 and ERK2 proteins of the MAPK pathway, (Selleck chemicals, Houston, TX, USA)[27]; 4) LC-NF- κ B inhibitor: LC mice were inoculated intraperitoneally with 0.3 mL (200 mg/Kg) sulfasalazine/48h, a NF- κ B inhibitor, (Pfizer, Madrid, Spain)[28]. These experimental groups were studied for a period of one month. All animal experiments were carried out in the animal facilities at *Parc de Recerca Biomèdica de Barcelona* (PRBB, Spain). This was a controlled study designed in accordance with the ethical regulations on animal experimentation (EU 2010/63 CEE, *Real Decreto* 53/2013 BOE 34, Spain) at *Parc de Recerca Biomèdica de Barcelona* (PRBB) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986). Our study was approved by the Animal Research Committee at *Parc de Recerca Biomèdica de Barcelona* (protocol number EBP-09-1228AE).

In vivo measurements in the mice

Body weight and food intake were determined every day during all the study period. Moreover, food and water were supplied ad libitum for the complete duration of the study protocol in the tumor-bearing mice. The positron emission tomography (PET) was conducted in the LC group on days 13 and 20 in order to verify the tumor progression, as shown in previous studies[23, 25]. Tumor sizes were extracted and weighed out on day 30 right after the sacrifice of the animals (Figure E1).

Sacrifice and sample collection

At day 30 post-inoculation of LP07 tumor cells or MEM (control group), animals from all experimental groups were sacrificed (under anesthesia, 0.1 mL of sodium pentobarbital, 60 mg/Kg, intraperitoneal injection) to obtain the subcutaneous tumors. The pedal and blink reflexes of each mouse were evaluated to check total anesthetic depth. As soon as it was

reached, the subcutaneous tumor was obtained from each animal. In all mice, tumor weights were determined using a high-precision scale.

Importantly, control non-LC mice were not used for the purpose of the current study, as the investigation was exclusively focused on the analyses of the molecular and cellular events within the adenocarcinoma lung tumors (subcutaneous) of the mice. Therefore, LC non-treated mice were the actual controls in the present investigation.

Frozen tumors were used for enzyme-linked immunosorbent assay (ELISA) and immunoblotting techniques, while paraffin-embedded tumors were used for immunohistochemistry experiments and for the quantification of the number of apoptotic nuclei using the Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay.

Tumor biology analyses

All the tumor biology analyses were conducted blind in the same laboratory, at *IMIM-Hospital del Mar*, in Barcelona.

Cytokine Enzyme-linked Immunosorbent Assay (ELISA). Protein levels of the cytokines TNF-alpha and IL-1beta were quantified in tumor homogenates of all mice groups using specific sandwich ELISA kits (RayBiotech, Norcross, GA, USA) as previously described[29-31].

Immunoblotting of 1D electrophoresis. Protein levels of the different molecular markers explored in the current investigation were evaluated according to methodologies published elsewhere[30-34]. The following specific primary antibodies were used to detect the different molecular markers: total protein carbonylation (OxyBlot Protein Oxidation Detection Kit, Chemicon International Inc., Temecula, CA, USA), total protein nitration (anti-3-nitrotyrosine antibody, Invitrogen, Eugene, Oregon, USA), malondialdehyde protein adducts (anti-MDA antibody, Academic Bio-Medical Company, Inc. Houston, TX, USA), catalase (anti-catalase antibody, Calbiochem, Darmstadt, Germany), superoxide dismutase (SOD)

(anti-SOD2 antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), SOD1 (anti-SOD1 antibody, Santa Cruz), p-62 (anti-p62/SQSTM1 antibody, Sigma-Aldrich), beclin-1 (anti-Beclin 1 antibody, Santa Cruz), LC3B (anti-LC3B antibody, Cell Signaling), fibulin-3 (anti-fib-3 antibody, Santa Cruz), fibulin-5 (anti-fib-5 antibody, Santa Cruz), Bax (anti-Bax antibody, Santa Cruz), bcl-2 (anti-bcl-2, antibody Santa Cruz) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, anti-GAPDH antibody, Santa Cruz). To validate equal protein loading among various lanes, SDS-PAGE gels were stained with Coomassie Blue and the glycolytic enzyme GAPDH was used as the protein loading controls in all the immunoblots (Figure E2).

Immunohistochemistry. In the tumor specimens from all groups, inflammatory and vascular markers were identified on the three-micrometer tumor paraffin-embedded sections using immunohistochemical procedures as previously described in our group[23, 25]. In these experiments the following antibodies were used: COX-2 (anti-COX-2 antibody, Santa Cruz), NF- κ B (p65) (anti-NF- κ B (p65), Santa Cruz), ki-67 (anti-ki67 antibody, Millipore Iberica, CA, USA), Bax (anti-Bax antibody, Santa Cruz), fibulin-5 (anti-fib-5 antibody, Santa Cruz), E-cadherin (anti-E-Cadh, Transduction Laboratories), fibronectin-1 (anti-Fn1, DAKO, Glostrup, Denmark), S100A4 (anti-S100A4/FSP1, Millipore, Darmstadt, Germany), Snail1 (anti-Snail1,[35]). For the E-cadherin, fibronectin, S100A4, and Snail1 staining, tumor cross-sections were deparaffinized and antigen retrieval was carried out by digesting with Proteinase K (Fibronectin1), or heating in a pressure cooker in Tris buffer pH 9 (Snail1) or Citrate buffer, pH 6 (E-Cadherin, S100A4) following previously published procedures[36]. The number of positively stained nuclei for Ki-67 (a marker of cell proliferation) was also counted in tumors from all animal groups. Data are expressed as the percentage of positively-stained nuclei in each group of mice. The morphology of the subcutaneous tumors (to identify

the malignant cells, vessels, the stroma and other structures) was also analyzed using hematoxylin-eosin staining following standard procedures.

Quantification of immunohistochemical signals using histo-scores. The intensities of COX-2 and NF- κ B (p65) staining were scored using methodologies similar to those previously published[37].

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using immunohistochemistry. Apoptotic nuclei were identified in paraffin-embedded sections of tumor specimens, through the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (In Situ Cell Death Detection Kit, POD, Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions and according to methodologies previously published[23].

Statistical analyses

The normality of the study variables was explored using the Shapiro-Wilk test and histograms. Results are presented as median and interquartile ranges in the tables and as standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values in the graphs. Comparisons of the different variables between LC mice treated with each pharmacological agent and the non-treated LC mice were assessed using the non-parametric U Mann-Whitney test. Spearman's correlation coefficients were used to assess the relationships between the different study variables. Statistical significance was established at $P \leq 0.05$.

RESULTS

Characteristics of the tumor pathology

The implantation of the LP07 cells induced the development of a subcutaneous tumor with morphological characteristics compatible with lung adenocarcinoma in all experimental mice. As illustrated in Figure E1, a subcutaneous tumor grew in the flank of mice in all experimental groups and were detected 30 days post-inoculation of the tumor cells. Hematoxylin-eosin staining showed a differentiated tumor, with areas of necrosis (Figure 1A) and high levels of apoptosis (Figure 1B). Malignant cells grew in solid nests, and showed important nuclear pleomorphism, atypia, and large numbers of mitosis (Figure 1D, red arrow). Blood vessels were also observed (Figure 1C), as well as numerous fibroblasts (Figure 1D, thick arrow) in the tumor stroma (Figure 1D, thin arrow). Moreover, metastatic foci of adenocarcinoma were also detected in the mouse lungs (Figure 1E).

Immunohistochemical analyses of the adenocarcinoma tumors confirmed the expression of the epithelial marker E-cadherin in the membrane of tumor cells (Figure E3A). Stromal fibroblasts deposited abundant fibronectin (Figure E3B) and also expressed two markers that are characteristic of cancer-associated fibroblasts, Snail1 and S100A4 (Figures E3C and E3D, respectively).

Physiologic characteristics of mice

As shown in Table 1, at the end of the study period (day 30), the percentage of body weight gain was negative in LC mice. In animals receiving concomitant treatment with either MAPK or NF- κ B inhibitors, but not with the antioxidant or the proteasome inhibitor, body weight significantly increased compared to LC non-treated mice (Table 1). At the end of the study period, subcutaneous tumor sizes, as measured by weight, were significantly reduced in response to treatment with proteasome (13%), MAPK (60%), and NF- κ B (21%) inhibitors compared to non-treated LC rodents (Table 1).

Interestingly, treatment of LC mice with both MEK and NF- κ B inhibitors induced a significant reduction in tumor cell proliferation, as measured by the percentage of Ki-67 positively stained nuclei, compared to non-treated LC control rodents (Figure E4 and Table 1). Treatment with antioxidant or the proteasome inhibitor did not have any significant effect on tumor proliferation in the mice (Figure E4 and Table 1). Moreover, the following correlations were found between cell proliferation rates and tumor weights in non-treated LC mice, ($r=0.771$, $p=0.072$), in animals treated with the antioxidant ($r=0.812$, $p=0.050$), bortezomib ($r=0.429$, $p=0.397$), and with MAPK and NF- κ B inhibitors ($r=0.029$, $p=0.957$ and $r=-0.500$, $p=0.391$, respectively).

NF- κ B (p65) expression

Staining for p65 was significantly reduced in LC animals treated with either proteasome, MEK, or NF- κ B inhibitors (histoscores 3 and 4) (Figure E5 and Table 2).

Redox balance markers

Oxidative stress markers. Importantly, antioxidant and the proteasome, MEK and NF- κ B inhibitors induced a significant decline in total levels of protein tyrosine nitration and MDA-protein adducts in the tumors compared to non-treated LC mice (Figures 2A and 2B, and Figure E6, respectively). Moreover, levels of reactive carbonyls were decreased only in LC rodents treated with the antioxidant when compared to non-treated LC mice (Figures 2C and Figure E6).

Antioxidant enzymes. Compared to non-treated LC animals, protein levels of catalase were significantly greater in response to proteasome, MEK and NF- κ B inhibitors (Figure 3A and Figure E7). Furthermore, protein content of SOD1 and SOD2 exhibited a significant rise in LC mice treated with the proteasome inhibitor when compared to non-treated LC rodents (Figures 3B and 3C, respectively, and Figure E7).

Inflammatory markers

Inflammatory cytokines. Protein levels of IL-1beta were significantly increased in the subcutaneous tumor of LC mice treated with MAPK inhibitor compared to non-treated LC animals, while treatment with the antioxidant and the proteasome or NF- κ B inhibitors did not induce any significant effect (Table 3). Additionally, protein expression of the cytokine TNF-alpha did not show any statistically significant difference among the study groups (Table 3). Furthermore, a significant direct correlation was found between TNF-alpha and MDA-protein adduct levels in non-treated LC animals ($r=0.900$, $p=0.037$). However, this correlation was not significant in LC mice treated with the antioxidant ($r=0.600$, $p=0.285$) or with the inhibitors of proteasome ($r=-0.300$, $p=0.624$), MAPK ($r=0.200$, $p=0.747$) or NF- κ B ($r=0.000$, $p=1.000$).

Cyclooxygenase-2. In the tumor of non-treated LC mice, treatment with both MAPK and NF- κ B inhibitors, but not NAC or bortezomib, induced a significant decrease in COX-2 intensity staining as indicated by histoscore 3, at the expense of a significant rise in histoscore 1, representing the lowest intensity of COX-2 immunohistochemical expression in these samples (Table 3 and Figure E8).

Cell adhesion an invasion markers

Compared to non-treated LC animals, protein content of fibulin-5 was significantly reduced in response to the antioxidant NAC, while no differences were observed in response to proteasome, MAPK or NF- κ B inhibition (Figure 4A and Figures E9A and E9B). Fibulin-3 protein levels did not differ among any of the study groups (Figure 4B and Figure E9C).

Apoptosis markers

Treatment with either proteasome or MAPK inhibitors, but not with the antioxidant or NF- κ B inhibitor, induced a significant rise in Bax protein content in the tumors compared to non-treated LC rodents (Figure 5A and Figures E10A and E10B). Protein content of Bcl-2 did not

significantly differ among the study groups (Figure 5B and Figure E10C). Furthermore, proportions of TUNEL positively stained nuclei were significantly increased in the subcutaneous tumor of mice treated with proteasome, MAPK, and NF- κ B inhibitors compared to non-treated LC mice (Figure 5C and Figure E10D).

Autophagy markers

Protein levels of the autophagy system beclin-1 and p62 did not significantly differ among the study groups (Figures 6A, 6B, and Figure E11). Compared to non-treated LC animals, protein levels of LC3I and LC3II were significantly reduced in response to the antioxidant (Figures 7A, 7B, and E11). Furthermore, total LC3 protein content, as measured by the ratio of LC3-II to LC3-I was also significantly decreased in LC mice treated with NAC, but not with the other inhibitors compared to non-treated LC mice (Figures 7C and E11).

DISCUSSION

The experimental model used in the present study has been well-validated in previous investigations [20-25] and has enabled us the analysis of markers of tumor growth and degradation in response to several pharmacological agents in mice bearing the LP07 lung adenocarcinoma. Moreover, as a novelty, specific markers of cancer-associated fibroblasts have also been identified such as Snail1 and S100A4 [38, 39].

Administration of the MEK inhibitor resulted in a significant reduction in the tumor size of the treated animals. Importantly, total body weight gain was significantly improved in these mice, thus leading to the conclusion that ERK pathway inhibition also induces beneficial effects on cancer cachexia as already demonstrated in a previous investigation from our group[23]. ERK signaling pathway has been shown to participate in tumor-acquired resistance to chemotherapy [14] and promotes cell survival and proliferation, thus favoring tumor growth. Lung tumors become resistant to single-agent inhibition of the primary driver

oncogene as well as to single-agent inhibition of the acquired “bypass track” as downstream signaling activity is maintained. Therefore, inhibition of the latter pathway together with that of the primary driver oncogene led to tumor cell death and cell cycle arrest in lung [40, 41] and breast cancer [42]. In the current study, ERK pathway inhibition also resulted in a significant decrease in levels of p65 expression, oxidative stress markers, inflammatory parameters, and tumor proliferation, while concomitantly induced a rise in apoptosis levels as measured by the proportions of TUNEL-apoptotic nuclei and bax levels, and in the content of the antioxidant catalase. Taken together, these findings suggest that ERK signaling is likely to play a major role in cancer development and growth in LC, and that its inhibition clearly reduced tumor burden through several molecular mechanisms. In fact, tumor cells were shown to grow faster in response to MAPK inhibition [43].

Another relevant finding in the investigation was the substantial reduction in tumor growth induced by inhibition of NF- κ B signaling in the mice. Furthermore, tumor proliferation was also significantly decreased (20%) in these animals, suggesting that NF- κ B is likely to play a major role in promoting cell proliferation and survival in these tumors. In fact, activation of NF- κ B signaling pathway is a characteristic feature in cancer including LC [15, 16]. In this regard, resistance to NF- κ B inhibition of lung adenocarcinoma cells involved Akt activation and both pathways were shown to promote survival of the cancer cells [16]. In another study, cell cycle arrest was also observed in response to several pharmacological inhibitors including NF- κ B pathway [44]. In the present investigation, treatment with sulfasalazine induced a decrease in the expression of p65, COX-2 (the score representing the highest intensity), and oxidative stress markers (protein tyrosine nitration and MDA-protein adducts), while it also favored a rise in catalase content and apoptosis in the tumors. These findings suggest that the reduction in tumor burden achieved by NF- κ B inhibition was likely mediated by decreased oxidative stress and inflammation levels within the cancer cells.

The proteasome inhibitor bortezomib also induced a significant reduction in tumor size in the treated animals. Nevertheless, tumor proliferation rates did not significantly differ from levels detected in the tumors of the non-treated mice. Importantly, body weight gain did not improve in the mice treated with the proteasome inhibitor compared to non-treated animals. The discrepancy between the reduction in tumor size and the lack of an improvement in body weight in the tumor-bearing mice receiving bortezomib was also observed in a previous study aimed to identify the effects of the proteasome inhibitor on muscle mass loss [23]. Expression of NF- κ B p65 as measured by histo-scores representing the greatest expression was decreased in the tumors of mice treated with bortezomib. This is in line with previous knowledge in which proteasomal degradation of inhibitor I κ B α was blocked by bortezomib, thus hampering NF- κ B activity in bone marrow stromal cells [45].

Interestingly, levels of protein tyrosine nitration and MDA-protein adducts were significantly decreased in the tumors of mice treated with bortezomib, while protein levels of the antioxidant enzymes SOD1, SOD2, and catalase were significantly increased in the tumor lesions of the same animals. The latter findings are in line with those reported in a previous investigation, in which a rise in SOD2 protein levels was also detected in limb muscles of LC cachectic mice treated with bortezomib [23]. Nonetheless, SOD2 levels increased in response to enhanced NF- κ B activity in cultured myocytes [46]. Differences in cancer cell types and experimental models may account for the discrepant results found among studies regarding SOD induction in response to NF- κ B activation and inhibition. Importantly, the rise in the expression of proapoptotic markers bax and TUNEL-positively stained nuclei detected in the tumors in response to treatment with bortezomib may partly account for the reduction in tumor size observed in those animals. Taken together, these findings suggest that the proteasome inhibitor mainly favors tumor degradation rather than attenuating tumor growth.

Treatment of the mice with the antioxidant NAC did not significantly modify tumor size or growth or body weight. As expected levels of oxidative stress as measured by protein tyrosine nitration, MDA-protein adducts and protein carbonylation were decreased in tumors of the mice treated with the antioxidant, while no differences were seen in levels of any the antioxidants analyzed in the study. Expression levels of p65, COX-2, and apoptosis markers in the tumors were not modified by NAC treatment. However, protein levels of fibulin-5 and the autophagy marker LC3 exhibited a significant decline in the tumors of the mice receiving treatment with the antioxidant. The fibulins are a family of extracellular matrix glycoproteins characterized by repeats of epidermal growth factor (EGF)-like domains that may promote or suppress tumors[47]. For instance, silencing of fibulin-5 expression induced lung tumor invasion and metastasis through ERK pathway [48], whereas fibulin-3 blocked epithelial-to-mesenchymal transition and self-renewal of LC stem cells, thus offering a potential therapeutic target in lung tumor cells [49]. In the current investigation, the reduction seen in fibulin-5 and autophagy expression may account for the lack of improvements seen in tumor size or body weight in the animals. On this basis, antioxidants do not seem to induce beneficial effects on lung cancer at least in this experimental model.

Study limitations

A limitation refers to tumor weight as the only measurement of tumor size available in the study, which was obtained at the time of the sacrifice of the animals. However, we do not believe that this limitation may hinder the study results as mice from all five experimental groups have been exposed to identical experimental conditions and tumor burden has been defined on the basis of tumor weight in all groups at the end of the study period (30 days).

Another limitation has to do with the potential applicability of these results in clinical settings of patients with LC. As MEK and NF- κ B inhibitors may also exert their effects on other tissues such as bones, joints, gastrointestinal system, liver, and muscles care should be

taken when prescribing these drugs in combination with therapeutic targets with the aim of enhancing anti-tumor activity [42, 50-53]. Anyhow, results obtained from this experimental model support the use of adjuvant drugs for the improvement of LC treatment as it is the case in other cancer types.

Conclusions

We conclude from the current findings that NF- κ B and MEK inhibitors significantly reduce tumor burden through several biological mechanisms including a decline in oxidative stress and inflammatory events, which probably promote tumor degradation and tumor cell cycle arrest. These pharmacological agents may enhance the anti-tumor activity of selectively targeted therapeutic strategies for NSCLC. Proteasomal inhibition induced tumor apoptosis rather than attenuation of tumor growth, while the use of antioxidants cannot be recommended.

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FIGURE LEGENDS

Figure 1: Representative histological preparations in which the most relevant features of the mouse adenocarcinoma are shown.

Top, medium, and bottom panels images of representative details observed in the representative areas of the hematoxylin-eosin staining in the subcutaneous tumor of LC mice. A differentiated tumor, with areas of necrosis (panel A) and apoptosis (panel B) can be seen. Blood vessels were also observed (panel C) as well as a dense tumor stroma (thick arrows) containing a large number of fibroblasts (thin arrows) (panel D). Moreover, metastatic foci were observed in all lungs obtained from all animals (panel E).

Figure 2: Graphical representation of the measurements of oxidative stress markers within the tumors in the different groups of mice.

A) Optical densities in the box plots are expressed as the ratio of the optical densities of total protein tyrosine nitration to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Total protein tyrosine nitration, was significantly decreased in the subcutaneous tumor of LC mice treated with either the antioxidant or any of the inhibitors (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant, * $p < 0.05$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

B) Optical densities in the box plots are expressed as the ratio of the optical densities of total malondialdehyde (MDA)-protein adducts to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and

whiskers at minimum and maximum values are depicted. Total MDA-protein adducts, were significantly decreased in the subcutaneous tumor of LC animals treated with either the antioxidant or any of the inhibitors (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: ** $p < 0.01$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

C) Optical densities in the box plots are expressed as the ratio of the optical densities of total protein carbonylation to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Total protein carbonylation showed a significant decline in LC mice treated with the antioxidant (N=7) compared to non-treated LC animals (N=7). Statistical significance is as follows: n.s., non-significant and *** $p < 0.001$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

Figure 3: Graphical representation of the measurements of antioxidant markers within the tumors in the different groups of mice.

A) Optical densities in the box plots are expressed as the ratio of the optical densities of catalase protein content to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Catalase content was significantly increased in the subcutaneous tumor of LC mice treated with proteasome (N=7), MAPK (N=7) or NF- κ B (N=7) inhibitors compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant, * $p < 0.05$ and *** $p < 0.001$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC.

- B)** Optical densities in the box plots are expressed as the ratio of the optical densities of SOD-1 protein content to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. SOD-1 content was significantly greater in the subcutaneous tumor of LC mice treated with the proteasome inhibitor (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant, * $p < 0.05$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.
- C)** Optical densities in the box plots are expressed as the ratio of the optical densities of SOD-2 protein content to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. SOD-2 protein content was significantly increased in the subcutaneous tumor of LC mice treated with the proteasome inhibitor (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant, ** $p < 0.05$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

Figure 4: Graphical representation of the measurements of fibulins within the tumors in the different groups of mice.

- A)** Optical densities in the box plots are expressed as the ratio of the optical densities of fibulin-5 protein content to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Fibulin-5 protein content was significantly decreased in the subcutaneous tumor of LC mice treated with the antioxidant (N=7) compared to non-treated LC rodents (N=7). Statistical significance is

as follows: n.s., non-significant, $*p < 0.05$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

B) Optical densities in the box plots are expressed as the ratio of the optical densities of fibulin-3 protein content to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Fibulin-3 protein content did not significantly differ among the study groups (N=7). Statistical significance is as follows: n.s., non-significant between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

Figure 5: Graphical representation of the measurements of apoptosis markers within the tumors in the different groups of mice.

A) Optical densities in the box plots are expressed as the ratio of the optical densities of bax protein content to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Bax protein content was greater in the subcutaneous tumor of LC mice treated with the proteasome (N=7) and the MAPK inhibitors (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant, $*p < 0.05$ and $**p < 0.01$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

B) Optical densities in the box plots are expressed as the ratio of the optical densities of bcl-2 protein content to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Bcl-2 protein content did not significantly differ among the study groups (N=7). Statistical significance is as follows:

n.s., non-significant between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

- C) Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values of total TUNEL positive nuclei are depicted. Total TUNEL positive nuclei were increased in the subcutaneous tumor of LC mice treated with the proteasome, MAPK and NF- κ B inhibitor (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC.

Figure 6: Graphical representation of the measurements of autophagy markers within the tumors in the different groups of mice.

- A) Optical densities in the box plots are expressed as the ratio of the optical densities of the autophagy protein beclin-1 to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. Beclin-1 protein content did not significantly differ among the study groups (N=7). Statistical significance is as follows: n.s., non-significant between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.
- B) Optical densities in the box plots are expressed as the ratio of the optical densities of the autophagy protein p-62 to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. P-62 protein content did not significantly differ among the study groups (N=7). Statistical significance is as follows: n.s., non-significant between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC.

Figure 7: Graphical representation of the measurements of autophagy markers within the tumors in the different groups of mice.

- A) Optical densities in the box plots are expressed as the ratio of the optical densities of the autophagy protein LC3-I to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. LC3-I protein content showed a reduction in the subcutaneous tumor of LC mice treated with the antioxidant (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant and * $p < 0.05$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.
- B) Optical densities in the box plots are expressed as the ratio of the optical densities of the autophagy protein LC3-II to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. LC3-II protein content was decreased in the subcutaneous tumor of LC mice treated with the antioxidant (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant and *** $p < 0.001$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.
- C) Optical densities in the box plots are expressed as the ratio of the optical densities of the total LC3-II/LC3-I to those of GAPDH. Standard box plots with median (25th and 75th percentiles) and whiskers at minimum and maximum values are depicted. LC3-II/LC3-I protein content expressed a reduction in the subcutaneous tumor of LC mice treated with the antioxidant (N=7) compared to non-treated LC rodents (N=7). Statistical significance is as follows: n.s., non-significant and ** $p < 0.01$ between any group of mice

with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

Table 1. Body weights, tumor size and growth in mice from all experimental groups at the end of the study period (30 days).

	LC				
	Non-treated LC (N=6)	Antioxidant (N=6)	Proteasome inhibitor (N=6)	MEK inhibitor (N=6)	NF-κB inhibitor (N=6)
Body weight gain (%)	-6.44 (13.98)	-12.97 (18.33), n.s.	-13.02 (15.5), n.s.	+0.81 (7), **	+0.97 (9.62), **
Subcutaneous tumor weight (g)	1.42 (0.76)	1.60 (0.57), n.s.	1.24 (1) *	0.57 (0.74) ****	1.11 (0.72) *
Subcutaneous tumor weight with respect to non-treated LC mice (%)	NA	+13%, n.s	-13% *	-60% ****	-21% *
Ki-67 positively stained nuclei (%)	85 (3)	85 (3), n.s.	86 (2), n.s.	78 (6), **	68 (4), **

Variables are presented as median (interquartile ranges).

Statistical significance: n.s.: non-significant, *: $p \leq 0.05$ **: $p \leq 0.01$, and ****: $p \leq 0.001$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

Definition of abbreviations: NA: not applicable.

Table 2. Histochemical scores corresponding to NFκB (p65) immunohistochemical expression of the subcutaneous tumor in mice from all the experimental groups.

	LC				
	Non-treated LC (N=6)	Antioxidant (N=6)	Proteasome inhibitor (N=6)	MEK inhibitor (N=6)	NF-κB inhibitor (N=6)
NF-κB (p65), Histoscore 0, %	0 (1)	0 (1), n.s.	0 (1), n.s.	0 (0), n.s.	1 (1), n.s.
NF-κB (p65), Histoscore 1, %	0 (1)	0 (0), n.s.	52 (52), *	13 (28), **	3 (5), **
NF-κB (p65), Histoscore 2, %	2 (2)	13 (36), n.s.	42 (21), *	99 (76), **	71 (35), **
NF-κB (p65), Histoscore 3, %	104 (64)	151 (171), n.s.	54 (57), *	42 (48), *	115 (62), n.s.
NF-κB (p65), Histoscore 4, %	242 (113)	168 (263), n.s.	2 (119), **	6 (15), **	79 (76), **

Variables are presented as median (interquartile ranges).

Statistical significance: n.s.: non-significant, *: $p \leq 0.05$ and **: $p \leq 0.01$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.

Table 3. Expression of inflammatory markers in the subcutaneous tumor of mice from all the experimental groups.

	Non-treated LC (N=6)	LC			
		Antioxidant (N=6)	Proteasome inhibitor (N=6)	MEK inhibitor (N=6)	NF- κ B inhibitor (N=6)
IL-1 β , pg/mL	793 (173)	756 (262), n.s.	972 (592), n.s.	1274 (414) **	941 (244), n.s.
TNF- α , pg/mL	1474 (394)	2814 (366), n.s.	1291 (1280), n.s.	2965 (417), n.s.	2672 (337), n.s.
COX-2, Histoscore 0, %	0.08 (1)	0.08 (0.4), n.s.	0.08 (0.5), n.s.	0.80 (2), n.s.	0.08 (0.6), n.s.
COX-2, Histoscore 1, %	14 (20)	23 (14), n.s.	19 (29), n.s.	44 (20)**	53 (33)*
COX-2, Histoscore 2, %	74 (59)	73 (33), n.s.	66 (36), n.s.	71 (41), n.s.	93 (52), n.s.
COX-2, Histoscore 3, %	167 (103)	123 (67), n.s.	155 (115), n.s.	15 (75), *	0 (6), **

Variables are presented as median (interquartile ranges).

Statistical significance: n.s.: non-significant, *: $p \leq 0.05$ and **: $p \leq 0.01$ between any group of mice with LC treated with the inhibitors or the antioxidant and non-treated LC animals.