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Original Contribution

# Vorinostat synergizes with EGFR inhibitors in NSCLC cells by increasing ROS via up-regulation of the major mitochondrial porin VDAC1 and modulation of the c-Myc-NRF2-KEAP1 pathway

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ABSTRACT

In non-small-cell lung cancer (NSCLC) patients, the activation of alternative pathways contributes to the limited efficacy of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib. The present study examines a panel of EGFR wild-type, K-Ras mutated, NSCLC lines, which were all intrinsically resistant to EGFR-TKIs, and demonstrates that the histone deacetylase inhibitor vorinostat can improve the therapeutic efficacy of gefitinib or erlotinib, inducing strong synergistic antiproliferative and pro-apoptotic effects that are paralleled by reactive oxygen species accumulation and by increased DNA damage. By knockdown experiments, we suggested that the up-regulation of voltage-dependent anion-selective channel protein 1 (VDAC1), the major mitochondrial porin of the outer mitochondrial membrane, which was induced by vorinostat and further increased by the combination, could be functionally involved in oxidative stress-dependent apoptosis. Significantly, we also observed the attenuation of the expression of both the enzyme hexokinase1, a negative VDAC1 regulator, and the anti-apoptotic porin VDAC2, only in the combination setting, suggesting convergent mechanisms that enhanced mitochondria-dependent apoptosis by targeting VDAC protein functions. Furthermore, the prosurvival capacities of the cells were also inhibited by the combination treatments, as shown by complete pAKT deactivation, increased GSK3β expression, and c-Myc down-regulation. Finally, we observed that the combination treatment of vorinostat and either of the EGFR-TKIs induced the down-regulation of the c-Myc-regulated nuclear factor erythroid 2-related factor 2 (NRF2) transcription factor and the up-regulation of the NRF2 repressor Kelch-like ECH-associated protein 1 regulator (KEAP1). These two genes are crucial for the redox stress response, often dysfunctional in NSCLC, and involved in EGFR-TKI resistance. Taken together, these results are the first to demonstrate that altering redox homeostasis is a new mechanism underlying the observed synergism between vorinostat and EGFR TKIs in NSCLC.



## 1. Introduction

Non-small-cell lung cancer (NSCLC), which accounts for approximately 85% of all cases of lung cancer, represents the leading cause of cancer deaths worldwide in both men and women [1,2]. Because 80–90% of NSCLC patients have advanced stages of this disease at diagnosis, the overall survival after standard treatment with platinum-based chemotherapy, radiation, and/or surgery remains less than 12 months. To improve response rates and overall survival, several drugs targeting specific cancer-related alterations have been developed in the last decade [1,2].

Epidermal growth factor receptor (EGFR), a member of the ErbB

*Abbreviations:* AREs, Antioxidant response elements; CI, Combination index; DRI, Dose reduction index; EGFR, Epidermal growth factor receptor; EMT, Epithelial-to-mesenchymal transition; GSK3β, Glycogen synthase kinase 3β; HDACi, Histone deacetylase inhibitor; HK1, Hexokinase1; KEAP1, Kelch-like ECH-associated protein 1 regulator; NAC, N-Acetyl cysteine; NRF2, Nuclear factor-erythroid 2-related factor 2; NSCLC, Non-small-cell lung cancer; PI3K, Phosphatidylinositol 3-kinase; ROS, Reactive oxygen species; SCCHN, Squamous cell carcinoma of head and neck; SCLC, Small cell lung cancer; TKI, Tyrosine kinase inhibitors; VDAC, Voltage-dependent anion-selective channel protein

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family of transmembrane receptors, is involved in the development and progression of several human cancers, including NSCLC. Indeed, two EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, represent the first examples of targeted agents approved for the treatment of NSCLC. However, despite impressive clinical success, particularly in patients harboring specific somatic activating EGFR gene mutations in their cancer cells, most patients, if not all, eventually experience relapse because of acquired drug resistance after an initial response. Multiple mechanisms seem to concur in the establishment of spontaneous or acquired resistance to EGFR-TKI, with the development of secondary mutations within the EGFR kinase domain, particularly the T790M mutation, the amplification of cMET, or the induction of epithelial-to-mesenchymal transition (EMT) as the most common mechanisms described [2–8]. The complex interplay between EGFR and other members of the ErbB receptor family is another key element that may determine the susceptibility of tumor cells to EGFR-targeting agents [8–10]. To overcome these mechanisms of resistance, different strategies based on combinations of gefitinib or erlotinib with several different agents have been developed; however, the results have been disappointing [11].

Patients with lung cancer have displayed altered expression and/or somatic mutations in two crucial genes involved in the response to redox stress, the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) and Kelch-like ECH-associated protein 1 (KEAP1), which is a repressor protein that binds to NRF2, retaining NRF2 in the cytoplasm and promoting its degradation by the ubiquitin proteasome pathway [12–14]. The NRF2-KEAP1 pathway is the major regulator of cytoprotective responses to oxidative stress. In detail, under normal homeostatic conditions, low levels of NRF2 are maintained by KEAP1-Cullin3 E3 ubiquitin ligase, which mediate NRF2 ubiquitination and proteasomal degradation. In contrast, upon oxidative stress, KEAP1-mediated inhibition is altered, resulting in NRF2 stabilization and nuclear accumulation and binding of antioxidant response elements (AREs) of target cytoprotective genes [13]. Notably, in malignant cells, NRF2 activity provides a growth advantage by increasing cancer chemoresistance and by enhancing tumor cell growth; thus, NRF2 expression is associated with poor patient prognosis [13,14]. Interestingly, beyond its role in the development of chemo- or radioresistance [13,14], recent publications have demonstrated that NRF2 is an additional cause of resistance to EGFR-TKI [15,16], suggesting that regulators of the oxidative stress pathway may represent novel targets in NSCLC to circumvent resistance to anti-ErbB receptors.

Our group has focused its interest on histone deacetylase inhibitors (HDACis), a class of antitumor agents that are able to affect multiple genes and pathways based on the functions of the epigenetic enzymes they regulate. In particular, we and many others have demonstrated the synergistic antitumor activity of HDACis in combination with many structurally diverse conventional and targeted anticancer drugs, highlighting the role of reactive oxygen species (ROS) generation as a critical mechanism by which HDACis exert their lethality and synergize with different agents [17–19]. The mechanism by which this effect occurs involves alterations in the expression of redox-related genes and/or mitochondrial homeostasis [19–22].

Using squamous cell carcinoma of head and neck (SCCHN) cells, we recently demonstrated that the clinically approved HDACi vorinostat differentially regulates ErbB receptor expression, and this may enhance the antiproliferative and pro-apoptotic effects of an EGFR-TKI such as gefitinib [23]. Using a proteomic approach, we further investigated the interaction between vorinostat and gefitinib in a mesenchymal gefitinib-resistant cell line and demonstrated the modulation of several proteins that regulate mitochondrial homeostasis, including the up-regulation of voltage-dependent anion-selective channel protein 1 (VDAC1) expression [24]. VDAC1 is the most abundant isoform of the VDAC family, a class of mitochondrial porins, and represents a major component of the outer mitochondrial membrane that regulates the diffusion of large metabolites and ATP flux, depending on the mitochondrial membrane potential [25]. VDAC1 in the open state induces apoptosis by inducing the translocation of the pro-apoptotic protein bax, the release of cytochrome c, and the activation of caspase cascade [25,26].

In this study, we investigated a combinatorial strategy based on the use of vorinostat to increase the therapeutic efficacy of gefitinib or erlotinib in a panel of NSCLC cell lines. We demonstrated that vorinostat, in combination with either EGFR-TKI, induced synergistic antiproliferative and pro-apoptotic effects. We also provided evidence that the mechanism underlying the synergistic interaction between the two classes of agents is related to altering mitochondrial homeostasis by modulating the expression and function of VDAC proteins and of a c-Myc-NRF2-KEAP1 pathway.

## 2. Materials and methods

### 2.1. Materials

Gefitinib (ZD1839, Iressa) was provided by AstraZeneca Pharmaceuticals (Macclesfield, UK), while erlotinib (Tarceva) was provided by LC Laboratories (Boston, MA, USA), and vorinostat (Zolinza) was provided by Merck & Co., Inc. (Rahway, NJ, USA). N-Acetylcysteine was provided by Sigma Aldrich (St. Louis, MO, USA).

Stock solutions of gefitinib were prepared in polyethylene glycol and then diluted to appropriate concentrations in culture medium before being added to the cells. Stock solutions of erlotinib or vorinostat were prepared in dimethyl sulfoxide and then diluted to appropriate concentrations in culture medium before being added to the cells.

The following primary antibodies were purchased: EGFR, phospho-EGFR, ErbB2, phospho-ErbB2, phospho-ErbB3, phospho-p44/42 ERK, p44/42 ERK, phospho-AKT, AKT, cleaved Caspase 3, and c-Myc antibodies from Cell Signaling Technology, Inc. (Boston, MA, USA); ErbB3, bax, cytochrome c, hexokinase1 (HK1), KEAP1, VDAC1, VDAC2, NRF2, glycogen synthase kinase 3β (GSK3β), and γ-tubulin antibodies from Santa Cruz Biotechnology, Inc. (San Jose, CA, USA); E-cadherin antibody from Abcam (Cambridge, England); and $\gamma H_2AX$ antibody from Millipore (Billerica, MA, USA).

Secondary anti-rabbit, anti-goat, and anti-mouse antibodies were purchased from Abcam (Cambridge, UK). Enhanced chemiluminescence (ECL) immunodetection reagents were obtained from GE Healthcare (Milan, Italy).

All media, serum, antibiotics, and glutamine were obtained from Cambrex Bio Science (Verviers, Belgium). Fetal bovine serum (FBS) was purchased from Gibco. Sulforhodamine B (SRB) was obtained from ICN Biomedicals (Irvine, CA, USA).

### 2.2. Cell culture and cell viability assay

The NSCLC cell lines A549, H460, and Calu1 were purchased from American Type Culture Collection (Rockville, MD, USA) and were all authenticated by LGC Standards (Sesto San Giovanni, MI, Italy). O11 primary tumor cell culture derived from a malignant pleural effusion of a NSCLC patient was kindly provided by Dr. R. Mancini [27].

All cell lines were grown in the appropriate medium (DMEM for A549 and Calu1 and RPMI for H460 and O11 cells) supplemented with 10% FBS, penicillin (50 units/ml), streptomycin (500 μg/ml), and 2 mM glutamine, in a humidified atmosphere of