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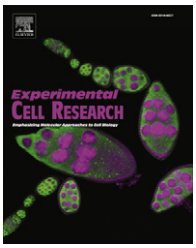

Research Article

A targeted enzyme approach to sensitization of tyrosine kinase inhibitor-resistant breast cancer cells

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ABSTRACT

Gefitinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) of potential use in patients with breast cancer. Unfortunately, in clinical studies, gefitinib is often ineffective indicating that resistance to EGFR inhibitors may be a common occurrence in cancer of the breast. EGFR has been shown to be overexpressed in breast cancer, and in particular remains hyperphosphorylated in cell lines such as MDA-MB-468 that are resistant to EGFR inhibitors. Here, we investigate the cause of this sustained phosphorylation and the molecular basis for the ineffectiveness of gefitinib. We show that reactive oxygen species (ROS), known to damage cellular macromolecules and to modulate signaling cascades in a variety of human diseases including cancers, appear to play a critical role in mediating EGFR TKI-resistance. Furthermore, elimination of these ROS through use of a cell-penetrating catalase derivative sensitizes the cells to gefitinib. These results suggest a new approach for the treatment of TKI-resistant breast cancer patients specifically, the targeting of ROS and attendant downstream oxidative stress and their effects on signaling cascades.

Introduction

Epidermal growth factor receptors (EGFR) are part of the ErbB family (or epidermal growth factor family) composed of receptor tyrosine kinases [1,2]. EGFR is activated by binding of its ligand, epidermal growth factor (EGF), leading to dimerization of the receptor and stimulation of intracellular kinase activity. Upon dimerization, autophosphorylation occurs, eliciting downstream signaling cascades involved in cell survival and increased cellular proliferation [1,2]. In some breast cancers, EGFR is overexpressed, although tyrosine kinase inhibitors targeting EGFR (EGFR TKIs) have yet to demonstrate uniform efficacy in the clinic [3,4]. Interestingly, in breast cancer cell lines resistant to EGFR TKIs, EGFR-phosphorylation is often maintained at high levels in the presence of the TKI [5], indicating that EGFR may continue to signal to promote cell growth and survival. This is supported by evidence that targeted knockdown of EGFR expression can decrease growth and survival of EGFR TKI-resistant breast cancer cells [6].

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Mechanisms of EGFR inhibitor resistance in all cancers are the focus of many researchers and specific insights have been realized [7]. The most common mutation known to occur in EGFR is a truncation resulting in a deletion of exons 2–7; the resultant molecule is known as EGFRvIII. Importantly, this mutation occurs in 86% of gliomas and is thought to mediate resistance to EGFR inhibitors [8]. Resistance mechanisms involve upregulation of parallel signaling tyrosine kinase receptors including insulin like growth factor receptor (IGF-1R), vascular endothelial growth factor (VEGFR), and Met as well as the constitutive activation of downstream molecules including Akt and MAPK (reviewed in [7]). These EGFR TKI resistance pathways circumvent EGFR TKIs by the activation of additional signaling pathways.

An additional mechanism to explain the somewhat disappointing overall response of breast tumors to EGFR TKIs is the possibility that the EGFR still has the ability to signal in the absence of innate EGFR kinase activity. Indeed, it has been clearly demonstrated that additional tyrosine kinases can phosphorylate the EGFR independent of ligand. For example, c-Src phosphorylates a kinase-impaired EGFR on its autophosphorylation sites as well as several additional tyrosines [9,10]. In addition, another EGFR family member, HER2, also has the ability to phosphorylate the EGFR on its autophosphorylation sites [11]. Furthermore, it has been shown that a point mutation in the HER2 kinase domain results in constitutive phosphorylation of EGFR in the presence of EGFR TKIs [12]. These data indicate that, even without the EGFR kinase activity, phosphorylation of the EGFR may occur to promote cell growth and survival pathways.

The EGFR TKI-resistance seen in certain cancer cells may result from an ROS-induced inactivation of an EGFR-associated tyrosine phosphatase [13]. In this context, a role for ROS and accompanying oxidative stress in the initiation and progression of breast cancer has been suggested and variously supported in a number of studies [14–16]. Oncogenes with tyrosine kinase activity, including HER2 in breast cancer cells, can induce the upregulation of cellular/oncogenic ROS and influence the metabolic transformation of cancer cells [17]. Furthermore, the cancer cell redox state can affect the function of oncogenic kinases [14].

In order to examine the potential link between cellular ROS and EGFR TKI-resistance, we employed a targeted antioxidant enzyme, CAT-SKL, to reduce oxidant levels in breast cancer cell line models expressing high levels of ROS and EGFR. Our results indicate that CAT-SKL sensitizes previously resistant MDA-MB-468 cells to the anti-cancer effects of the EGFR-targeted TKI, gefitinib. EGFR inhibitors are of great potential value as therapies in breast cancer models; resistance presents a major challenge to this treatment modality. CAT-SKL, a hydrogen peroxide (H_2O_2) metabolizing protein therapeutic shown to be effective in a number of *in vitro* and pre-clinical indications [18–20], thus represents a potentially powerful new means of combating this devastating disease.

Materials and methods

Cell culture

SUM149 cells were maintained in Ham's F-12 supplemented with 5% FBS, 1 μg/mL hydrocortisone, and 5 μg/mL insulin. MDA-MB-468 cells were grown in DMEM supplemented with 10% FBS. Both cell lines were cultured with 2.5 μg/mL amphotericin B and 25 μg/mL gentamicin. For vehicle control treated cells, dimethyl sulfoxide was added at 0.1% final concentration.

SDS-PAGE/immunoblotting

Lysates were prepared from the indicated cells in CHAPS lysis buffer (10 mM CHAPS, 50 mM Tris, pH 8.0, 150 mM NaCl, and 2 mM EDTA with 10 μM NaOVa and 1X protease inhibitor cocktail (EMD Biosciences, Philadelphia, PA)). For immunoblotting, 10 μg of protein lysates were separated by SDS-PAGE and transferred to Immobilon P. Membranes were blocked in either 5% nonfat dry milk or 5% BSA for 1 h at 25 °C. Primary antibodies utilized in this study include: anti-EGFR and anti-pY1068 EGFR from Cell Signaling (Danvers, MA); anti-pMAPK and anti-actin from Sigma (St. Louis, MO); and anti-pTyrosine from Invitrogen (Carlsbad, CA). Anti-mouse and anti-rabbit IgG-HRP was used from Cell Signaling and enhanced chemiluminescence (ECL) reagents were from GE Healthcare Life Sciences (Piscataway, NJ). Experiments were repeated at least three times.

Dot blot analyses were performed with streptavidin alkaline-phosphatase (1:1000) or anti-catalase antibodies (1:4000) and goat anti-rabbit-alkaline-phosphatase (1:5000) and developed with the NBT/BCIP color development substrate (Thermo Scientific). Protein transduction was performed as follows; 100 nM of biotinylated CAT-SKL was added for 0, 1, 2, and 4 h, cells were washed with PBS, and harvested directly into standard 2X sample buffer. Proteins were separated by 10% SDS-PAGE, and then transferred to a nitrocellulose membrane and blocked for 1 h in 5% nonfat dry milk in Tween-containing, tris buffered saline. Membranes were probed with anti-streptavidin alkaline phosphatase (1:1000) and developed with NBT/BCIP.

Biotinylation of CAT-SKL

CAT-SKL containing an 11 arginine peptide transduction domain and a modified peroxisomal targeting signal was expressed, purified, and biotinylated as described in [18,20–22].

Cell viability and cell growth assays

For testing cell viability, two approaches were employed: enzymatic assays that measure metabolic activity and a dye-exclusion assay that distinguishes live from dead cells. For the generation of IC_{50} curves, 1000 MDA-MB-468 or SUM149 cells/well were incubated for 72 h with various concentrations of gefitinib (dissolved in dimethyl sulfoxide). At this point, cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), as per manufacturer's protocol. For the sensitivity to H_2O_2 experiments, cells were plated at 400,000 cells/well, pretreated with CAT-SKL (1 μM) for 4 h, and challenged with H_2O_2 (1 mM) for 2 h. MDA-MB-468 cell viability was determined using the water soluble tetrazolium cell proliferation assay (WST-1) from Millipore following the manufacturer's protocol. For the latter, 200,000 cells, pretreated with CAT-SKL and challenged with H_2O_2 as described above, were incubated with 0.2% trypan blue (final concentration) for 3–5 min and the percentage of non-viable (blue) cells were determined microscopically with a hemocytometer.

Growth assays were performed by plating MDA-MB-468 or SUM149 cells in triplicate in 6-well plates at 35,000 cells/well