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Research paper

Canertinib induces ototoxicity in three preclinical models

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ABSTRACT

Neuregulin-1 (NRG1) ligand and its epidermal growth factor receptor (EGFR)/ERBB family regulate normal cellular proliferation and differentiation in many tissues including the cochlea. Aberrant NRG1 and ERBB signaling cause significant hearing impairment in mice. Dysregulation of the same signaling pathway in humans is involved in certain types of cancers such as breast cancer or non-small cell lung cancer (NSCLC). A new irreversible pan-ERBB inhibitor, canertinib, has been tested in clinical trials for the treatment of refractory NSCLC. Its possible ototoxicity was unknown. In this study, a significant dose-dependent canertinib ototoxicity was observed in a zebrafish model. Canertinib ototoxicity was further confirmed in two mouse models with different genetic backgrounds. The data strongly suggested an evolutionally preserved ERBB molecular mechanism underlying canertinib ototoxicity. Thus, these results imply that clinical monitoring of hearing loss should be considered for clinical testing of canertinib or other pan-ERBB inhibitors.

1. Introduction

Lung cancer is the number one cause of cancer mortality globally with few screening and treatment options available (Jemal et al., 2011). Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases (Jemal et al., 2010).

Advanced NSCLCs (Stage IIIb and IV) are treated with cisplatin or combination therapies; however, the five-year survival rate is under 5% (de Castria et al., 2013). Cisplatin, as first-line treatment of many solid tumors, has been extensively studied for its ototoxicity (Lobarinas et al., 2013; Harrison et al., 2014), however, its effectiveness against NSCLC is often subverted (Lipp and Hartmann, 2005; Yorgason et al., 2006; Schacht et al., 2012). Recent research into novel anti-NSCLC drugs has focused on inhibition of the epidermal growth factor receptors (EGFRs/ERBB), which belong to the receptor family of tyrosine kinases (RTKs).

RTK inhibitors, which block ERBB receptors, present novel and exciting therapies for NSCLC (Cowan-Jacob, 2006; Pytel et al., 2009; Awada et al., 2012; Ou, 2012; de Castria et al., 2013; Majem and Pallares, 2013). Most ERBB family members have three domains: an extracellular N-terminus responsible for ligand binding, a transmembrane hydrophobic region, and a conserved intracellular domain with a ligand-activated tyrosine kinase on the C-terminal end (Allen et al., 2003). The ERBB family of receptors includes ERBB1 (EGFR), ERBB2 (Her-2/neu), ERBB3 (Her-3), and ERBB4 (Her-4) (Janne et al., 2007). Aberrant signaling of ERBB family members (Hynes and Stern, 1994; Leung et al., 1997) plays an important role in many cancers including breast (Sainsbury et al., 1985), ovarian (Meden and Kuhn, 1997), prostate (Schwartz et al., 1999), gastric (Hirono et al., 1995), laryngeal (Almadori et al., 1999), and indicate a more aggressive cancer phenotype (Klijn et al., 1994; Zinner et al.,

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2007). ERBB1 is overexpressed in the majority of NSCLC and is found frequently in lung squamous cell carcinoma (57–100%) whereas ERBB2 is found in lung adenocarcinomas (33%).

Gefitinib and erlotinib are first generation EGFR RTK inhibitors that result in 10–20% lung tumor suppression but do not inhibit other ERBB family members (Wakeling et al., 2002; Barbacci et al., 2003). After treatment with first generation RTK inhibitors, 50% of patients have a secondary missense T790 mutation resulting in acquired resistance due to steric hindrance with a bulkier methionine (Pao et al., 2005; Shepherd et al., 2005; Engelman and Janne, 2008; Sequist et al., 2011; Majem and Pallares, 2013). Although no preclinical studies of their ototoxicity, one clinical case was reported with erlotinib ototoxicity (Koutras et al., 2008). The success of this first generation inhibitors coupled with the known acquired resistance has resulted in a need for new RTK inhibitor therapies with more sustained effects than gefitinib and erlotinib (Han et al., 2008; Butts et al., 2010; Mukherjea et al., 2011; Allen et al., 2012; Yang et al., 2013; Agustoni et al., 2014). Second and third generation ERBB inhibitors are irreversible, have higher binding affinities, inhibit multiple ERBB family members, and have better activity against T790 mutation. Therefore, these new inhibitors, such as canertinib, may provide additional clinical benefits by functioning as a pan-ERBB inhibitor (Fry, 2000; Slichenmyer et al., 2001; Allen et al., 2003; Janne et al., 2007). Although adverse effects such as diarrhea, asthenia, stomatitis, and persistent rash were observed in canertinib clinical trials (Janne et al., 2007), no clinical studies of ototoxicity for these new inhibitors are reported so far.

The NRG1-ERBB signaling pathway plays a significant role in the development and mature function of the inner ear. In the cochlea, *NRG1* is expressed in spiral ganglion neurons (SGNs) (Morley et al., 1998; Bao et al., 2003, 2004); whereas hair cells, Schwann cells, and supporting cells express *ERBB* (Morley, 1998; Hansen et al., 2001; Zhang et al., 2002; Hume et al., 2003). Cochlear innervation is abnormal in *NRG1* and *ERBB2* null mice and these adult mice have progressive hearing loss (Lee et al., 1995; Meyer and Birchmeier, 1995; Fritzsch et al., 1997; Morley, 1998; Adlkofer and Lai, 2000; Chen et al., 2003; Fritzsch et al., 2004; Hellard et al., 2004; Stankovic et al., 2004; Morris et al., 2006). One chemical compound blocking ERBB signaling could lead to progressive hearing loss in mature female pigmented guinea pigs (Watanabe et al., 2010). Since canertinib can block ERBB receptors, we first assessed possible hair cell toxicity of canertinib in a zebrafish model, and then in two mouse models (C57BL/6J and CBA/CaJ) by auditory brainstem recording (ABR) thresholds, distortion product otoacoustic emissions (DPOAE), and hair cell counts. Canertinib resulted in significant ototoxicity in all three preclinical animal models.

2. Materials and methods

2.1. Zebrafish studies

Zebrafish (*Danio rerio*) embryos were produced by paired matings of adult fish maintained at the University of Washington zebrafish facility. We used AB wild-type zebrafish strains. Embryos were maintained in fish embryo media (EM; 1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 μM NaCl, and 500 μM $NaHCO_3$ in dH_2O) at a density of 50 animals per 100 mm^2 Petri dish and kept in an incubator at 28.5 °C. At 4 days post-fertilization (dpf), larvae were fed live paramecia. All zebrafish procedures described were approved by the University of Washington Animal Care and Use Committee.

Free-swimming 5 dpf zebrafish larvae were transferred into a 48 well plate at a density of 10–12 fish per well using a wide-bore glass pipette. Fish were then treated with variable doses of canertinib (0–500 μM) for one hour. After canertinib exposure, larvae were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma–Aldrich) and then fixed for 1 h in 4% paraformaldehyde (PFA) at room temperature. After fixation in PFA, larvae were rinsed in PBS and then incubated in blocking solution [1% Triton-X, 5% normal goat serum (NGS) in PBS] for 1–2 h at room temperature. Larvae were then incubated overnight at 4 °C in anti-parvalbumin primary antibody (monoclonal, 1:400 in 1% Triton-X, 1% NGS in PBS; Millipore) to label hair cells. After primary antibody labeling, larvae were rinsed in 1% Triton-X in PBS and then incubated for 2–4 h at room temperature in Alexa 488 goat anti-mouse fluorescent antibody solution (1:500 in 1% Triton-X, 1% NGS in PBS; Invitrogen) secondary antibody. The larvae were then rinsed and mounted between two coverslips in Fluoromount-G (Southern Biotech) for imaging. A Zeiss Axioplan II microscope using a FITC filter set at a final magnification of 200× was used to count hair cells from the SO1, SO2, O1, and OC1 neuromasts (Raible and Kruse, 2000). Results are presented as the mean hair cell survival as a percentage of the control group treated. Error bars in figures indicate ±1 standard error (SE).

2.2. Mouse studies

All experiments in mice were approved by the Animal Studies Committee at Washington University in St. Louis. The dosages used in the clinical studies varied based on which group the individuals were in: 1) 50 mg per day for 21 consecutive days; 2) 150 mg per day for 21 consecutive days; 3) 450 mg per day for 14 days followed by 7 days off. Dosages were subsequently titrated on an individual basis based on the National Cancer Institute Common Toxicity Criteria version 2 with the most common side effects being rash and diarrhea. However, no serum studies in humans were performed so a direct comparison between human effect and mouse effect was not possible. Furthermore, drug pharmacokinetics is vastly difference between humans and rodents, with a much faster rate in rodents for most of drugs. Therefore, our testing dosage in mice with ototoxicity could be much lower if compared to the clinical dosages in humans. This study started with a total of 40 C57BL/6J and 24 CBA/CaJ mice for preliminary dosage studies, which showed lethality with 120 mg/kg/day and higher dosages, but showed safe at 30 and 60 mg/kg/day with no loss of body weight after a 14-day treatment. We thus selected 30 mg/kg/day for this study. The data presented here were from 15C57BL/6J and 13 CBA/CaJ mice, at two months old, purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

2.3. Drug application

Animals were subjected to one protocol with two arms, a control arm under which saline was administered and a treatment arm wherein canertinib was administered. The volume of saline was 0.1 ml/kg/day and the dose of canertinib (dissolved in saline) was 30 mg/kg/day. Canertinib was obtained from LC laboratories product number C-1201. All injections were administered intraperitoneally (i.p). ABR and DPOAEs were taken prior to and after each cycle of the drug administration. In clinical human studies, one common treatment cycle was 450 mg/day for 14 days followed by 7 days off. Accordingly, here, one cycle consisted of two weeks of daily i.p. injections followed by one week when ABRs and DPOAEs were assessed (Diagram 1).

2.4. ABR recording

ABR thresholds were obtained as described previously (Ohlemiller et al., 2000a, b; Bao et al., 2005, Bao et al., 2013).