Gynecologic Oncology 135 (2014) 142–148

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Neratinib shows efficacy in the treatment of HER2/neu amplified uterine serous carcinoma *in vitro* and *in vivo*

Carlton L. Schwab [a], Diana P. English [a], Dana M. Roque [a], Stefania Bellone [a], Salvatore Lopez [b], Emiliano Cocco [a], Roberta Nicoletti [a], Thomas J. Rutherford [a], Peter E. Schwartz [a], Alessandro D. Santin [a,*]

[a] Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA
[b] Division of Gynecologic Oncology, University Campus Biomedico of Roma, Via Álvaro del Portillo, 21-00128 Roma, Rome, Italy

HIGHLIGHTS

- Neratinib, an irreversible small tyrosine kinase inhibitor, inhibits HER2/neu amplified USC proliferation, cell cycle progression and signaling *in vitro*.
- Neratinib inhibits HER2/neu amplified USC xenograft growth and improves overall survival *in vivo*.
- Clinical trials in carefully selected patients are warranted.

ARTICLE INFO

Article history:
Received 29 May 2014
Accepted 4 August 2014
Available online 12 August 2014

Keywords:
Neratinib
Small tyrosine kinase inhibitors
Uterine serous carcinoma
HER2/neu
ErbB inhibitors

ABSTRACT

Objectives. Uterine serous carcinoma (USC) represents an aggressive variant of endometrial cancer and accounts for a large proportion of deaths annually. HER2/neu amplification is associated with USC in approximately 30–35% of cases. The objective of this study was to determine the sensitivity of a panel of primary USC cell lines to the small tyrosine kinase inhibitor neratinib, an ErbB1 and HER2 inhibitor, both *in vitro* and *in vivo*.

Methods. HER2/neu amplification was determined by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) in 24 USC cell lines. Flow cytometry was used to determine the effects of neratinib on cell viability, cell cycle distribution and signaling *in vitro*. Mice harboring HER2/neu amplified xenografts were treated with neratinib to assess the efficacy of the drug *in vivo*.

Results. HER2/neu amplification was noted in 8/24 primary cell lines. Data regarding the efficacy of neratinib was determined using 4 HER2 amplified cell lines and 4 non-amplified cell lines with similar growth rates. Data revealed that cell lines with HER2/neu amplification were exquisitely more sensitive to neratinib compared to non-amplified cell lines (mean ± SEM IC_{50}: 0.011 μM ± 0.0008 *vs.* 0.312 μM ± 0.0456 $p < 0.0001$). Neratinib caused arrest in the G0/G1 phase of the cell cycle and resulted in decreased autophosphorylation of HER2 and activation of S6. Neratinib treated mice harboring xenografts of HER2/neu amplified USC showed delayed tumor growth and improved overall survival compared to vehicle ($p = 0.0019$).

Conclusions. Neratinib may be a potential treatment option for patients harboring HER2/neu amplified USC. Clinical trials for this subset of endometrial cancer patients are warranted.

Introduction

Endometrial cancer is the most common of gynecologic malignancies with an increasing number of cases diagnosed in the US annually. According to the American Cancer Society 52,630 new endometrial cancers will be diagnosed in 2014 and 8590 will die from the disease [1]. Fortunately most endometrial cancers are classified as endometrioid histotype, related to a hyperestrogenic state and diagnosed at an early stage [2]. The most deadly of endometrial carcinomas are classified as type 2 endometrial cancers. These tumors are typically associated with genetic alterations in tumor suppressor genes and proliferation pathways rather than being hormonally driven tumors [3]. While type 2 endometrial cancers make up the minority of newly diagnosed endometrial cancers (10%), they are responsible for an overwhelming number of deaths from the disease (40%) [2,4]. Within the type 2 endometrial cancer group uterine serous histology makes up the majority of cases. Current standard treatment of uterine serous carcinoma consists of primary staging surgery followed by cytotoxic chemotherapy with or without radiation therapy. The majority of patients treated in

* Corresponding author at: Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, Room 305 LSOG, 333 Cedar Street, PO Box 208063, New Haven, CT 06520, USA. Fax: +1 203 737 4339.
E-mail address: Alessandro.santin@yale.edu (A.D. Santin).

http://dx.doi.org/10.1016/j.ygyno.2014.08.006
0090-8258/

this way have a favorable initial response; however, 50% develop recurrent disease which is usually fatal [5]. As a result, new treatments options are being sought for uterine serous carcinoma.

Current research is focused on understanding the genetic alterations that occur in different tumors including USC through next generation sequencing. The genetic landscape for a number of uterine serous carcinomas has been published and may allow for direct targeting of altered pathways [6,7]. While these data have revealed a number of genetic alterations, which may be potential targets for therapy, targeting amplified driver pathways remains the most promising approach at this time.

A number of different tumors have been identified that rely on mutations or amplification of the ErbB family of receptors including HER2/neu [8–11]. This has led to the development of new small molecule tyrosine kinase inhibitors which selectively target these receptors in a variety of combinations. Previous reports suggest that a subset of uterine serous carcinomas is reliant upon HER2/neu amplification as a driver pathway [10]. Recent reports suggest that HER2/neu is amplified in approximately 33% of cases [12]. Overexpression of HER2/neu is associated with a worse prognosis and a more aggressive tumor phenotype in a number of cancers including USC [13–17]. When HER2/neu is amplified there is an increase in the number of receptors on the surface of tumor cells. This increase in expression leads to increased homo- and heterodimerization leading to proliferation of the tumor [18]. Neratinib (HKI 272), an irreversible inhibitor of ErbB1 and HER2/neu, was developed to inhibit activation of the signaling pathways brought about by receptor dimerization.

The objective of this study was to understand the effects of neratinib (HKI-272) on cell viability, progression through the cell cycle and downstream signaling in uterine serous carcinomas with HER2/neu amplification both *in vitro* and *in vivo* using a preclinical animal model. This understanding may lead to potential therapies for patients harboring these aggressive and deadly tumors.

Materials and methods

Cell line establishment and analysis of HER2 expression

Patients were consented for tumor banking prior to proceeding to the operating room. At the time of frozen section a small portion of the tumor was obtained to establish primary cell lines. Cell lines were established in RPMI 1640 (Gibco, Life Technologies, Grand Island, NY) containing 10% FBS (Gemini, Woodland, CA), 1% Fungizone (Gibco, Life Technologies, Grand Island, NY) and 1% penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY). Once cultures were established as cell lines, they were used to test the efficacy of neratinib. Each primary tumor was assessed by pathology using FISH and IHC to determine HER2/neu expression as previously described [19]. Briefly, cell lines were classified as 0 if no HER2 staining was observed or 1+ if weak staining was noted in <10% of cells. Cell lines that showed moderate to strong staining in 10–30% of cells were considered 2+, while cell lines which demonstrated uniform intense staining in >30% of cells were considered 3+. Standard, clinically validated FISH assays were used to determine HER2/neu expression in primary cell lines as previously described by our group. The gene was considered amplified if the density score of the product was ≥2.

Drug

Neratinib (HKI-272) was obtained from EQ-Esteve. It was dissolved in DMSO to create a 10 mM stock solution. The stock solution was then diluted to make solutions in the following concentrations: 1 mM, 0.1 mM, 0.05 mM and 0.005 mM. When used *in vivo* neratinib was dissolved in purified water containing 0.5% methylcellulose (Sigma Life Sciences, St. Louis, MO) at a concentration of 8 mg/ml.

Determination of IC_{50}

The IC_{50} of each cell line was determined by harvesting cells from Petri dishes at 75% confluence and determining the number of cells available for culture by hemocytometer. The cells were then plated at a concentration of 20,000 cells/ml in a 6 well plate. The cells were allowed to incubate for 24 h and were subsequently treated with scalar amounts of neratinib ranging from 0.005 μM to 0.75 μM. Plated cultures treated with the drug were allowed to incubate over 72 h. The contents of each well were harvested in their entirety and stained with propidium iodide (Sigma Life Sciences, St. Louis, MO) (2 μl of 500 μg/ml stock solution in PBS with 1% azide and 2% fetal bovine serum). The cells were counted using flow cytometry. The number of viable cells in each well was normalized to the number of viable cells in the control well. The IC_{50} of each cell line was then determined by comparing the log base 10 of drug concentration in each well to the percentage of viable cells using a non-parametric 3 parameter regression. All IC_{50} data were calculated using Prism 5 software (GraphPad Prism Software Inc., San Diego, CA), and are presented as mean ± standard error of the mean. All experiments were completed in at least triplicate.

Cell cycle analysis

Cell cycle analysis was performed by harvesting cells at 75% confluence and plating them at a concentration of 20,000 cells/ml in a 6 well plate. The cells were allowed to incubate overnight and treated with scalar amounts of neratinib. The cells were allowed to incubate for 48 h after treatment. The entire contents of each well were harvested, washed and fixed in 70% ethanol for 30 min on ice. The cells were then washed with PBS three times and treated with 100 μl of ribonuclease in PBS at a concentration of 100 μg/ml at room temperature over 5 min. Propidium iodide, diluted to a concentration of 50 μg/ml in PBS, was then added to each sample to reach a final volume of 500 μl. Cell cycle was then read by flow cytometry and final analysis was performed with FlowJo (FlowJo, Ashland, OR). All data are expressed as mean ± standard deviation (mean ± SD).

Effects on HER2/neu and S6 phosphorylation

A representative cell line was selected from both the HER2 amplified and non-amplified groups. These cell lines were selected based upon their nearly identical doubling times. The cells were again harvested at 75% confluence and placed in 6 well plates at a concentration of 125,000 cells/ml. They were allowed to incubate for 24 h. The cells were then treated with 0.065 μM of neratinib. The cells were then allowed to incubate for 2, 4, 6, 8, 12, 14 and 16 h. The cells were harvested at each time point and assessed for HER2 phosphorylation and S6 phosphorylation. A dose–response experiment was performed at 4 h (phospho-HER2/neu) and at 12 h (phospho-S6) using 0.01, 0.065, and 0.133 μM of neratinib to evaluate maximal effects on HER2 and S6 phosphorylation respectively. When cells were collected for analysis they were washed with PBS and fixed with 4% formaldehyde for 10 min at 37 °C. The cells were then suspended in 90% methanol for 30 min on ice. They were washed and blocked in an incubation buffer, PBS + 0.5% BSA (Sigma Life Sciences, St. Louis, MO) for 10 min. The cells were then aliquoted into flow cytometry tubes and suspended in 100 μl of the incubation buffer. They were then labeled with phospho-HER2/neu-1221 antibody or phospho-S6 antibody (Cell Signaling Technologies, Danvers, MA) for 1 h on ice. They were washed two times with incubation buffer and suspended in 100 μl of the incubation buffer. A secondary FITC labeled antibody was place in each sample (Millipore). The samples were allowed to incubate for 30 min, washed with PBS, and suspended in PBS. They were then read by flow cytometry. The mean fluorescent intensity was then compared between treated and untreated control cells. All data are expressed as mean ± standard deviation.