Cancer Letters 320 (2012) 104–110

Contents lists available at SciVerse ScienceDirect

Cancer Letters

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

Dasatinib synergizes with both cytotoxic and signal transduction inhibitors in heterogeneous breast cancer cell lines – Lessons for design of combination targeted therapy

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ARTICLE INFO

Article history:
Received 11 January 2012
Received in revised form 26 January 2012
Accepted 26 January 2012

Keywords:
Dasatinib
Combination index
Rapamycin
Molecular targeted therapy

ABSTRACT

Molecularly targeted therapies have emerged as the leading theme in cancer therapeutics. Multi-cytotoxic drug regimens have been highly successful, yet many studies in targeted therapeutics have centered on a single agent. We investigated whether the Src/Abl kinase inhibitor dasatinib displays synergy with other agents in molecularly heterogeneous breast cancer cell lines. MCF-7, SKBR-3, and MDA-MB-231 display different signaling and gene signatures profiles due to expression of the estrogen receptor, ErbB2, or neither. Cell proliferation was measured following treatment with dasatinib ± cytotoxic (paclitaxel, ixabepilone) or molecularly targeted agents (tamoxifen, rapamycin, sorafenib, pan PI3K inhibitor LY294002, and MEK/ERK inhibitor U0126). Dose-responses for single or combination drugs were calculated and analyzed by the Chou–Talalay method. The drugs with the greatest level of synergy with dasatinib were rapamycin, ixabepilone, and sorafenib, for the MDA-MB-231, MCF-7, and SK-BR-3 cell lines respectively. However, dasatinib synergized with both cytotoxic and molecularly targeted agents in all three molecularly heterogeneous breast cancer cell lines. These results suggest that effectiveness of rationally designed therapies may not entirely rest on precise identification of gene signatures or molecular profiling. Since a systems analysis that reveals emergent properties cannot be easily performed for each cancer case, multi-drug regimens in the near future will still involve empirical design.

1. Introduction

Combination cytotoxic therapy has produced cures in adult and pediatric leukemias, lymphomas, and solid tumors as well as long-standing disease control for a number of other cancers [1]. However, cytotoxic therapy may result in severe toxicity and compromise in quality of life. By being more selective and less toxic, molecularly targeted therapy provides a new paradigm in rational cancer therapy [2]. Three principles drive the development of molecularly targeted therapy. Firstly, tumor cells depend on or are "addicted" to the activities of an oncogene, which provides an "Achilles' heel" for a drug to target [3]. A second principle states that a combination of drugs is more effective than any single agent by preventing chemoresistance [4]. An emerging third principle advances that each cancer has its own signature [5]. The design of multi-drug regimens incorporating molecularly targeted agents amidst a growing range of novel therapeutics and in the context of unique cancer profiles poses a great challenge [6]. Also complicating the design of molecularly targeted therapy has been the revelations of previously unappreciated pathways, such as feedback upregulation of PI 3-kinase by rapamycin or activation of c-Raf by B-Raf inhibitors [7,8].

Dasatinib is an oral, Src/Abl tyrosine kinase inhibitor, first approved in 2006 by the Food and Drug Administration for use in patients with resistance or intolerance to prior therapy including imatinib in patients with Ph+ chronic myeloid leukemia (CML). Dasatinib also targets Src family kinases (SFK), which drives many different signaling pathways [9]. Aberrant SFK activity promotes the survival, proliferation, and metastases of many different human cancers, such as breast, colorectal, and prostate cancers [10]. We have previously reported that dasatinib inhibits cell progression by inducing G1 arrest and blocks migration in the highly invasive, triple-negative (ER–, PR–, Her2–) MDA-MB-231 breast cancer cell line [11]. By blocking the actions of either non-receptor or receptor tyrosine kinases, dasatinib exerts anti-cancer actions by promoting apoptosis or inhibiting proliferation, angiogenesis, invasion, or bone resorption [12].

Despite supportive preclinical data, two single agent phase II studies showed limited responses to dasatinib in patients with

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doi:10.1016/j.canlet.2012.01.039

Table 1
Characteristics of signaling molecules in MDA-MB-231, MCF-7, and SK-BR-3 cell lines.

Cell line	ER, PR expression	Her2 amplification	PI3K mutation	PTEN mutation	Ras mutation	Raf mutation
MDA-MB-231	–	–	–	+	+	–
MCF-7	+	–	+	–	–	–
SK-BR-3	–	+	–	–	–	–

advanced Her2-positive, hormone-receptor-positive, or triple negative breast cancers [13,14]. We hypothesized that combining a specific molecular targeted drug with dasatinib can enhance efficacy in inhibiting cell growth of a specific breast cancer cell line according to the molecular profile of the given breast cancer cell type. To test this hypothesis, we evaluated dasatinib-containing regimens on breast cancer cell lines with different molecular profiles. Breast cancer cells may be distinguished by the presence or absence of estrogen receptor (ER), progesterone receptor (PR), or ErbB2 (Her2). We studied three cell lines with different receptor profiles: MDA-MB-231 (ER–, PR–, Her2–), MCF-7 (ER+, PR+, Her2–), and SK-BR-3 (ER–, PR–, Her2+). These three cell lines also possess different oncogene mutations (Table 1). MDA-MB-231 cells are highly sensitive to dasatinib, while MCF-7 cells are moderately sensitive and SK-BR-3 cells are resistant to dasatinib [11,15]. These cell lines display different genetic [16,17], epigenetic [18], and protein [19] expression patterns as well as single drug response profiles [20]. Surprisingly, our results showed that synergy between dasatinib and both cytotoxic and molecularly targeted agents were found in all cell lines. These results suggest that molecularly targeted agents, such as the multi-kinase inhibitor dasatinib, can have a broader role in cancer therapeutics and that the design of clinical trials of combination therapies will remain empiric.

2. Materials and methods

2.1. Cell culture

MDA-MB-231 and MCF-7 cells were cultured in MEM media (Invitrogen) supplemented with 10% FBS, 2 mM glutamine (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, and 10 mM HEPES buffer solution. SK-BR-3 cells were cultured in DMEM media (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in 37 °C in 5% CO_2. The cell lines were validated by short tandem repeat DNA fingerprinting, as reported elsewhere [21].

2.2. Cell proliferation assay

Dose-responses were measured through MTT assay (ATCC #30-1010K) following the manufacturer's instructions. Cells were seeded at a density of 3500 cells per well in 96-well plates for 24 h prior to treatment in growth medium with 10% FCS. After 24, 48, or 72 h of incubation with the indicated drugs or compounds, an MTT assay was performed with absorbance measured at 600 nm using a microplate reader (FLUOstar OPTIMA). Data analysis was performed with Excel software (Microsoft, Redmond, WA). Surface response models were generated using MATLAB software (The MathWorks, Inc., Natick, MA).

2.3. Cell cycle distribution

Cells were plated 5×10^5 cells in 100 mm dishes for 24 h prior to treatment in growth medium containing 10% FCS. Drugs were administered for 48 h. Cells were detached from the dish surface through trypsinization, fixed with 70% cold EtOH, and stored at −20 °C. Cells were stained with 100 μg/ml RNase A (Sigma), 10 μg/ml propidium iodide (Sigma), and 0.1% Triton X-100 in PBS for 30 min at room temperature. Fluorescence was measured using a flow cytometer (BD LSR II). Cell cycle distribution was analyzed with FlowJo software (TreeStar, San Carlos, CA).

2.4. Mathematical analysis

The dose-response of the drug combinations were modeled using the Median-Effect Equation, a generalized equation unifying the Hill, Scatchard, Michaelis–Menten, and Hasselbalch equations to succinctly describe the dose-effect relationship [22]. The Median-Effect Equation provides a way to assess the dose-response of a substance in a given population of cells:

$$\frac{f_a}{f_u} = \left(\frac{D}{IC_{50}}\right)^m, \tag{1}$$

where D is the dose, f_a is fraction affected, f_u is fraction unaffected, IC_{50} is median-effect dose, and m is the slope or kinetic order. Taking the logarithm converts this equation into a linear form that can be easily applied to linear regression analysis:

$$\log(f_a/f_u) = m\log(D) - m\log(\mathrm{IC}_{50}). \tag{2}$$

From obtaining a small collection of data points and using regression analysis, IC_{50} values can be accurately and rapidly interpolated from dose-response curves modeled using the Median-Effect Equation.

Combination index (CI) values were calculated through the Chou–Talalay method [22]. The CI provides a quantitative value for synergy and is given by:

$$\mathrm{CI}_{A+B} = \frac{D_{A|A+B}}{D_A} + \frac{D_{B|A+B}}{D_B}, \tag{3}$$

for two mutually exclusive drugs. CI values less than 1.0 indicate synergy, with values closer to zero representing increasing synergy.

A surface response model provides a drug response landscape between two drugs and is useful in predicting the effects of a combination of drugs at various ratios. In this study, drug combinations were performed in a 1-to-1 ratio. Since a 1-to-1 ratio of drugs may not generally be the most efficacious combination, response surface models were generated to simulate the dose-response at all ratios of a two drug combination. The drug response landscapes were modeled using the following equation [23]:

$$E = \frac{E_{\max} \times \left(\frac{D_A}{\mathrm{IC}_{50A}} + \frac{D_B}{\mathrm{IC}_{50B}} + \alpha + \frac{D_A}{\mathrm{IC}_{50A}} + \frac{D_B}{\mathrm{IC}_{50B}}\right)^n}{\left(\frac{D_A}{\mathrm{IC}_{50A}} + \frac{D_B}{\mathrm{IC}_{50B}} + \alpha + \frac{D_A}{\mathrm{IC}_{50A}} + \frac{D_B}{\mathrm{IC}_{50B}}\right)^n + 1}. \tag{4}$$

The maximal effect is represented by E_{max}, which is 1 since ideally 100% of the cells become affected with increasing drug concentration. The coefficient α is a factor for the interaction between the two drugs, with $\alpha > 0$ indicating synergy, $\alpha = 0$ additivity, and $\alpha < 0$ antagonism. This equation assumes that the response of each drug has the same kinetic order, n, which measures the steepness of the effect. However, each drug in actuality possesses a different kinetic order. Therefore, the model will provide a more accurate representation of the interaction between drugs with relatively similar kinetic orders. Matlab code for generating surface response plots is provided in Supplemental text.

3. Results

To test our hypothesis, we chose to study the effects of dasatinib in combination with cytotoxic drugs or molecularly targeted agents on three breast cancer cell lines, which are well-characterized in the variability in their gene expression, protein expression, and cancer phenotype [24,25]. Single agent dose-response and combination dose-responses were experimentally derived and are shown in Figs. 1 and 2. Table 2 summarizes the calculated IC_{50} values for single agents. Not surprisingly, the cytotoxic drug paclitaxel was the most potent for all three cell lines studied. The IC_{50} and CI values of combinations with dasatinib are shown in Table 3. The combination of dasatinib and rapamycin was observed to be strongly synergistic in both MDA-MB-231 and MCF-7 cell lines.

3.1. Effects of dasatinib-based treatment on ER+ breast cancer cells

Derived from invasive breast ductal carcinoma, MCF-7 cells display estrogen and progesterone receptors [26]. MCF-7 cells were found to be moderately sensitive to dasatinib with an IC_{50} of 2100 nM. In combinations with other drugs, dasatinib was most synergistic with the cytotoxic molecules ixabepilone and paclitaxel with CI of 0.19 and 0.21 respectively. The molecularly targeted agents sorafenib, PI3K inhibitor, and MEK/ERK inhibitor were only slightly synergistic when combined with dasatinib (Figs. 2 and 3).

MCF-7 cells will respond to estrogen with cell proliferation. As a result, the estrogen receptor antagonist tamoxifen was expected to impose a large inhibitory growth response. However, the observed IC_{50} value for single agent tamoxifen was large (IC_{50} = 9900 nM) and had only a marginal inhibitory effect in the proliferation of MCF-7 cells. This observation can be attributed to the absence of the hormone estrogen in our cultured growth media. Therefore, tamoxifen was essentially inhibiting a receptor for a hormone that was nonexistent in the surrounding environment. The fact that synergy was observed in the combination of