

Inhibition of Breast Cancer Growth with the Combination of Lapatinib and an ADAM Protease Inhibitor

Lois Witters¹, Peggy Scherle², Steven Friedman², John Redman², Jordan Fridman², Eian Caulder², and Allan Lipton¹

¹Department of Medicine, The Milton S. Hershey Medical Center/Penn State University College of Medicine, Hershey, PA; ²Incyte Corp., Wilmington, DE

Introduction

The ErbB family of receptors, ErbB1 (EGFR) and ErbB2 (HER-2/neu), plays a significant role in the proliferation of many human cancers. Many breast tumors overexpress these receptors and this correlates with poor prognosis and resistance to therapy. Although ErbB targeted therapies have demonstrated clinical efficacy, not all HER-2/neu or EGFR positive tumors respond and many that respond initially develop resistance, presenting the need for alternative therapies. Proteolytic cleavage of both ErbB ligands and receptors has been shown to be a critical event resulting in pathway activation. This cleavage is necessary for the generation of soluble, functionally active forms of the ligands and in the case of HER-2/neu, results in a shed extracellular domain (ECD) and a membrane bound fragment (p95) containing a kinase domain with significant constitutive activity. Both ErbB ligand and HER-2/neu cleavage have been shown to be mediated by the ADAM (a disintegrin and metalloproteinase) family of zinc-dependent proteases. We examined the effect of combining a lapatinib-like dual EGFR and HER-2/neu tyrosine kinase inhibitor (GW2974) with an ADAM protease inhibitor (INCB3619, Incyte Corp.) (Fig. 1) on the growth of human breast cancer.

Methods

Growth Assay:

MCF-7 human breast cancer cells and HER-2/neu transfected MCF-7 cells were plated in 24 well plates at 20,000 cells/well. After an overnight incubation to allow attachment at 37°C in 5% CO₂, the lapatinib-like compound (GW2974) and the sheddase inhibitor (INCB3619) were added at various concentrations, alone or in combination. After an additional 3 day incubation, cell growth was determined using the colorimetric MTT tetrazolium dye assay. Cells treated with corresponding amounts of DMSO (the vehicle for both GW2974 and INCB3619) were used as controls. All concentrations were done in triplicate and the medians were used for final data analysis. Isobologram analysis was performed to determine the effect from the combination.

HER-2/neu Shedding Experiment:

HER-2/neu-transfected MCF-7 cells were plated in 75 cm² flasks at a concentration of 750,000 cells per flask. After an overnight incubation to allow attachment, INCB3619 or the corresponding amount of DMSO (control) was added and an additional 3 days incubation was done. The supernatant was then centrifuged to remove cellular debris and measurement of HER-2/neu shedding was performed using a HER-2/neu ELISA kit specific for the ectodomain of human HER-2/neu (Oncogene Science). The supernatant was incubated in microwell plates to allow binding of the antigen by the capture antibody immobilized on the surface of the wells. The immobilized antigen reacted with a detector antibody. The antigen/detector antibody was measured by binding it with a streptavidin/horseradish peroxidase conjugate which catalyzed the conversion of the chromogenic substrate o-phenylenediamine (OPD) into a colored product. Quantitation of HER-2/neu was performed by reading the absorbance at 490 nm on a microplate reader.

Amphiregulin Shedding Experiment:

MCF-7 cells were seeded at 20,000 cells/well in 96 well plates in DMEM + 10% fetal bovine serum and allowed to adhere overnight in 5% CO₂ at 37°C. The following morning, the cells were pretreated with various concentrations of INCB3619 (100 nM = 10 μM) for 10 minutes. As a control, cells were treated with corresponding concentrations of DMSO. The cells were then stimulated with 1 μM PMA (Calbiochem) for 2 hours, supernatants harvested and assayed for amphiregulin levels using an amphiregulin specific ELISA (R&D Systems) according to the manufacturer's instructions.

Animal Studies:

When mice were 7 to 8 weeks old, each mouse was inoculated with 1 x 10⁶ BT-474 cells in 0.2 ml of medium subcutaneously. The treatments were started when the tumor size reached approximately 200 mm³. INCB007639 was dosed with mini-osmotic pumps (Alzet, Cupertino, CA) implanted subcutaneously and lapatinib was administered orally (PO) twice daily. Tumor sizes were measured twice weekly in two dimensions using a caliper, and the volume is presented in mm³ using the formula: V = 0.5a × b², where a and b are the long and short diameters of the tumor, respectively. Tumor growth delay was measured as time (days) for the treated group to reach an arbitrary tumor size of 1000 mm³. Responses were designated as Complete Remission (CR) when tumor volume decreased in size to the point of being undetectable (< 3 mm x 3 mm) and as Partial Remission (PR) when tumor volume decreased to < 50% of its starting volume.

Results

- Treatment of MCF-7 cells with various concentrations of INCB3619 dose-dependently blocked amphiregulin (an EGFR ligand) shedding with >80% inhibition observed at the 10 μM concentration (Fig. 2).
- Treatment of HER-2/neu-transfected MCF-7 cells with 10 μM INCB3619 blocked HER-2/neu shedding by 41% (Fig. 3).
- Addition of the INCB3619 sheddase inhibitor (at doses that gave minimal growth inhibition as a single agent) to a lapatinib-like dual inhibitor of EGFR and HER-2/neu resulted in a synergistic inhibitory effect in MCF-7 and HER-2/neu-transfected MCF-7 human breast cancer cells (Fig. 4) as confirmed by isobologram analysis (Table 1).
- Treatment of HER-2/neu positive BT474-SC1 human breast cancer xenografts with INCB7839 (an INCB3619 analogue currently in clinical trials) or lapatinib alone resulted in a decrease in mean tumor volume. Treatment with the combination of INCB7839 and lapatinib resulted in complete prevention of the increase in mean tumor volume (Fig. 5).

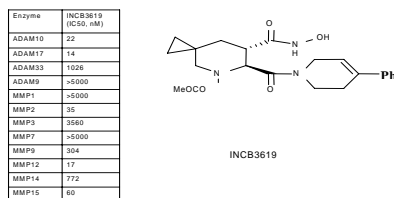


Figure 1. Enzymatic profile and structure of INCB3619.

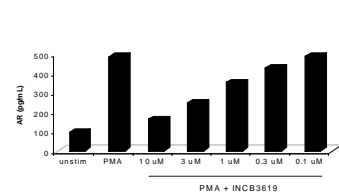


Figure 2. Amphiregulin levels measured in the supernatant of MCF-7 human breast cancer cells exposed to INCB3619.

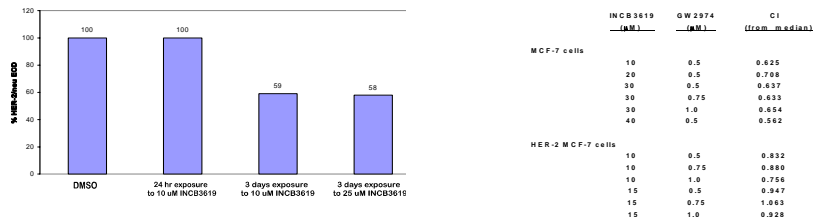


Figure 3. Percentage of HER-2/neu ectodomain measured in the supernatant of HER-2/neu-transfected MCF-7 human breast cancer cells exposed to INCB3619.

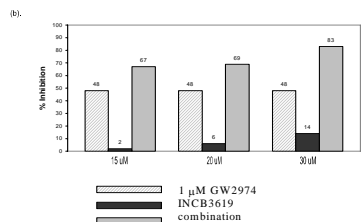
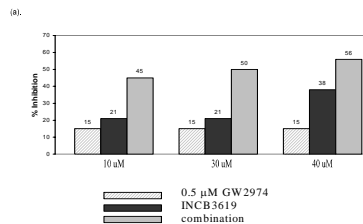


Figure 4. Percentage of growth inhibition in (a) MCF-7 and (b) HER-2/neu-transfected MCF-7 human breast cancer cells exposed to the combination of INCB3619 and GW2974.

	INCB3619 (μM)	GW2974 (μM)	C1 (I _{max} × a ₁ × a ₂)
MCF-7 cells	10	0.5	0.625
	20	0.5	0.704
	30	0.5	0.637
	30	0.75	0.633
	30	1.0	0.654
HER-2 MCF-7 cells	10	0.5	0.552
	10	0.75	0.880
	10	1.0	0.756
	15	0.5	0.947
	15	0.75	1.063
	15	1.0	0.928

Table 1. Antiproliferative activity of the combination of GW2974 and INCB3619 in human breast cancer cells using the multiple drug-effect equation of the Chou-Talalay method of isobologram analysis. C1 (combination index) > 1 = antagonistic effect, C1 = 1 = additive effect, C1 < 1 = synergistic effect.

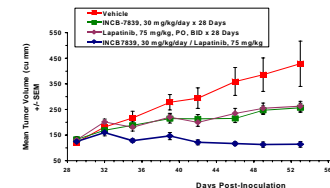


Figure 5. The effects of INCB7839, alone and in combination with lapatinib, on the growth of a BT474-SC1 human breast cancer xenograft.

Conclusions

- Sheddase is an enzymatic activity required for the cleavage of ErbB ligands and receptors. The INCB3619 ADAM protease inhibitor which inhibits sheddase activity can reduce shedding of both HER-2/neu and EGFR ligands.
- Addition of a lapatinib-like dual inhibitor of EGFR and HER-2/neu to an ADAM protease inhibitor results in synergistic *in vitro* growth inhibition of human breast cancer cells.
- The combination of a dual EGFR/HER-2/neu tyrosine kinase inhibitor and an ADAM protease inhibitor can completely prevent an increase in mean tumor volume in HER-2/neu positive human breast cancer xenografts.