

**Phase I/II Study of Trastuzumab in Combination with Everolimus (RAD001)
in Patients with HER2-Overexpressing Metastatic Breast Cancer That
Progressed on Trastuzumab-Based Therapy**

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Supplemental Appendix

Introduction

This appendix discusses the analysis of pharmacokinetics of the combination of everolimus and trastuzumab, as well as the utility of Positron Emission Tomography-Computed Tomography (PET-CT) imaging and circulating tumor cells (CTCs) in predicting clinical response in this trial.

Studies at our institution have found the utility of CTCs, in combination with PET/CT, may be useful in prediction of clinical outcome of metastatic breast cancer (MBC).^{1,2} In addition, studies have demonstrated that PET/CT imaging may have a role in the early prediction of response to therapy in the metastatic setting. Schwarz and colleagues found that, among 11 patients who underwent both PET imaging (after cycles 1 and 2 of chemotherapy) and conventional imaging (after cycles 3 and 6 of chemotherapy), PET evaluation was an accurate early predictor of early response.³ In addition, a UTMDACC retrospective study of 115 patients with MBC demonstrated that midtherapy (9 to 12 weeks after initiation of therapy) CTCs and PET/CTs predicted overall survival ($p < .001$ and $p = .001$, respectively).¹

Methods

Pharmacokinetic studies

Pharmacokinetic evaluation was identical at both institutions. In consenting patients, everolimus levels in whole blood were measured during cycle 1 for each dose level. Blood samples (2 mL) were drawn during the first day (accumulation phase) prior to treatment and at 0.5, 1, 2, 5, 8, 15, and 24 hours after initiation of treatment. A one-time blood sample was also drawn at steady-state on day 15; the sample could be collected at any time on day 15.

Analysis of Circulating Tumor Cells

Samples (three CellSave tubes) for assessment of circulating tumor cells (CTCs) were drawn before the everolimus dose was administered on day 1 of cycle 1, day 1 of cycle 5, and day 1 of cycle 9. All samples were analyzed at Brigham and Women's Hospital. The quantitation was performed using the CellSearch Epithelial Cell Kits. Briefly, 7.5 cc of peripheral blood was collected into CellSave tubes, maintained at room temperature, and processed within 72 hours of collection. Blood (7.5 cc) was added to an equal volume of "dilution buffer" and centrifuged at 800xg in a Beckman benchtop centrifuge (also at room temperature) for 10 minutes. Sample tubes were then placed in the CellSearch AutoPrep instrument where the cells were isolated, labeled with fluorescently-tagged antibodies, and returned in a MagneSt cartridge for enumeration. After isolation, cells were allowed to incubate at room temperature for 20 minutes in the MagneSt cartridge and then loaded into the Cell Tracks Analyzer. Each

sample was analyzed by the Analyzer II, and a gallery of images was presented to the user for final interpretation.

Samples were prepared for further nucleic acid analysis by 2 methods. When only one CTC tube was available, following enumeration as described above, the cells were transferred (with a glass pipet) to a 1.5 cc Eppendorf tube and placed in a rare earth magnet to pellet the cells. The buffer was removed and replaced with Allprotect (Quiagen). The samples were then frozen at -80 C.

When 2 CellSave tubes were available from a patient, one was processed as described in the paragraph above and the other was processed using the CellSearch Profile Kit. The Profile Kit used the same immunomagnetic methods to isolate the CTCs as the Epithelial Cell Kit (above) but it did not add any fluorescently-tagged antibodies. It was designed for users who wish to perform downstream applications (FISH, sequencing, RNA analysis, etc) directly on the isolated CTCs. The Profile kit returns the CTCs in a small volume of buffer. Similarly, the samples were then placed in a rare-earth magnet to pellet the cells and the buffer exchanged for Allprotect and the samples were stored at -80 C until processed.

Evaluation for Early Predictors of Response Utilizing Positron Emission

Tomography - Computed Tomography (PET-CT) Scans

In UTMDACC patients, PET-CT scans were obtained at baseline and 3 weeks after treatment initiation when possible, in order to determine if changes in FDG avidity may be used as early predictors of objective response. All studies

were performed and interpreted at UTMDACC, and the result of each PET-CT scan was reviewed by the UTMDACC principal investigator. A two-sided, two-sample pooled t-test was used to investigate changes in PET/CT standardized uptake value (3 weeks minus baseline) for responders vs. non-responders.

Results

Pharmacokinetics

Pharmacokinetic analysis was performed in seven patients. Based upon previous studies, it was expected that blood levels of everolimus would increase two-fold from first dose to steady state (day 15). A moderate increase in the range of 1.25-1.6 fold occurred for only five out of seven patients. For two patients, no accumulation was detected. One patient demonstrated high everolimus levels. Mean everolimus concentration at days 1 and 15 are depicted in figure 1 and 2.

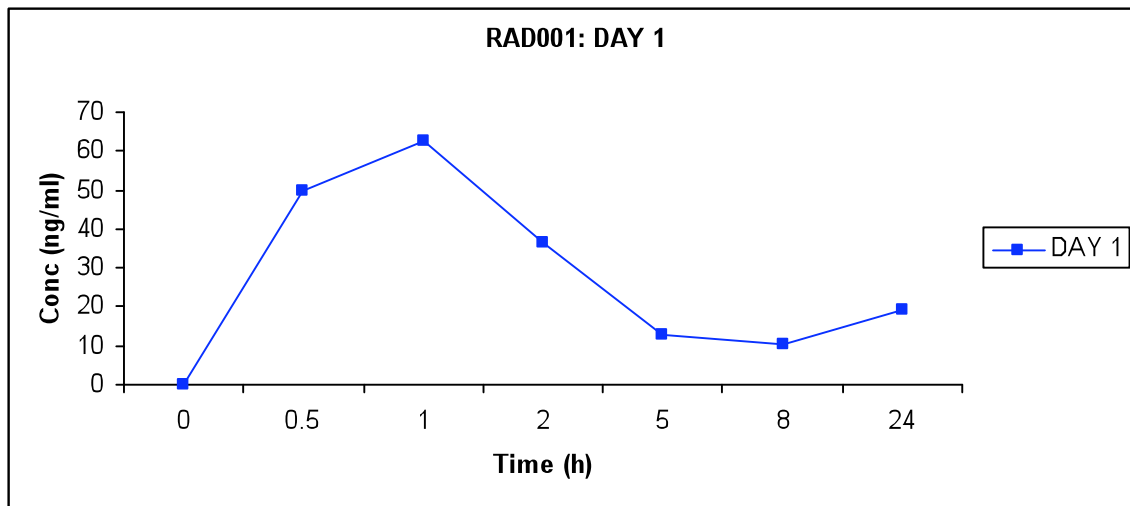


Figure 1. Mean concentration versus time on day 1.

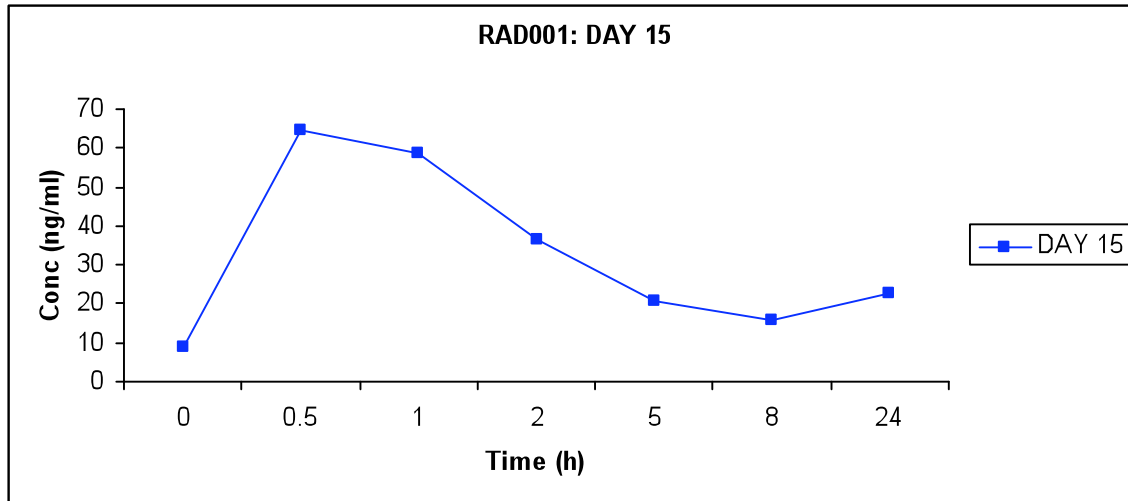


Figure 2. Mean concentration versus time on day 2.

Subsequently, the everolimus exposure in the presence of trastuzumab in this study was compared to a previous study of patients who received everolimus alone.^{4,5} These data suggest that trastuzumab does not have significant influence on the pharmacokinetic profile of everolimus.

Analysis of PET/CT findings

Among the nine patients who underwent PET-CT scans at baseline and at week 3, there were 3 responders and 6 non-responders. There was a trend ($p=.06$) towards a correlation between early PET-CT response and clinical outcome (PR/pSD). Specifically, responders demonstrated a mean decline in -standard uptake value (SUV) by PET-CT of -4.1040 (95% CI, -5.72 to 0.96), while non-responders had a mean change in SUV of -1.73 (95% CI, -5.39 to 0.77).

However, this subgroup analysis did not have adequate power to demonstrate a statistically significant difference.

Circulating Tumor Cell Analysis

In 21 consenting patients, circulating tumor cell (CTC) counts were measured at baseline and during treatment with everolimus and trastuzumab. Specifically, circulating tumor cells were drawn on days 1 and 8 of cycle 1, within 7 days of day 1 of cycle 2, with 7 days of day 1 of cycle 5, within 7 days of day 1 of every 4th cycle thereafter, and at the time the patient was taken off study. Among these patients, twenty patients had measurable CTC counts. One patient, at MDACC, had a baseline count of 0; her CTCs remained undetectable on six subsequent examinations, until the patient came off study after 336 days. Three patients (two at DFHCC and one at MDACC) only had baseline counts, and did not have further CTC counts. Figure 2 demonstrates the CTC counts of the remaining 17 patients who had at least 2 CTC counts, prior to starting treatment, and on day 1 of cycle 2. Four of the DFHCC patients also had CTC counts on day 8 of cycle 1. All patients demonstrated low CTC counts at baseline. CTC counts declined in almost all (16/17) patients with consecutive counts and did not correlate with clinical benefit. CTC counts did not rise toward the end of the study period, indicating that the CTC counts did not predict for disease progression.

Although PIK3CA mutational analysis was attempted on the CTCs, no PIK3CA mutations were found, likely due to lymphocyte contamination of the sample and lack of depth of sequencing.

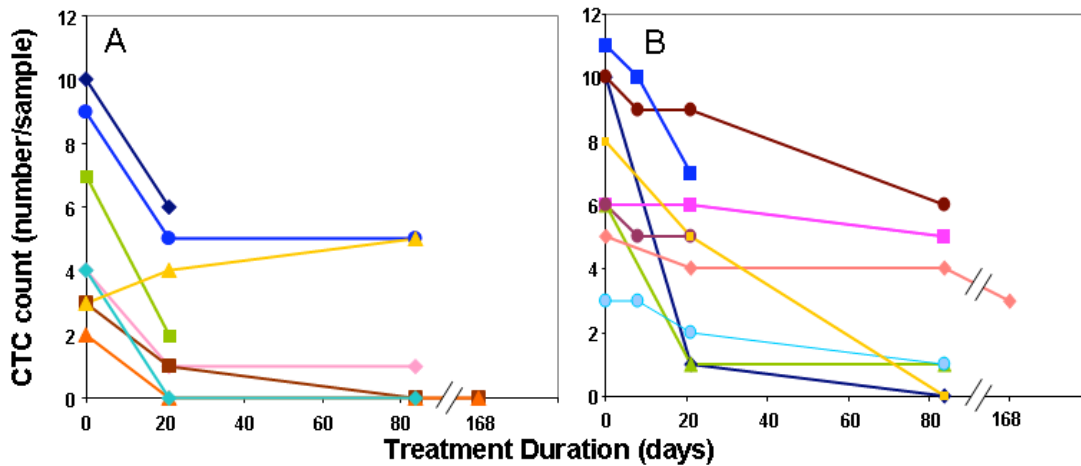


Figure 3: Circulating tumor cell counts at baseline during treatment with everolimus and trastuzumab. Twenty-one patients consented to the determination of CTCs, while one patient had undetectable CTC levels at baseline and on subsequent examinations. Three patients (2 at DFHCC and 1 at MDACC) only had baseline counts, and did not have further CTC counts. Displayed are the CTC counts of the remaining 17 patients who had at least 2 CTC counts, prior to starting treatment, and on day 1 of cycle 2. Four of the DFHCC patients also had CTC counts on day 8 of cycle 1. Results for patients at MDACC (A) and patients at DFHCC (B) are displayed separately.

Discussion

While the mean change in SUV in PET/CT did not result in a statistically significant correlation to clinical outcome, there was a trend towards a relationship between the two ($p=0.06$). As this subgroup contained only nine patients, the analysis did not have sufficient power to demonstrate a statistically significant difference, and a larger study would be necessary in order to

determine if there is PET/CT offers a role in early prediction of response in this population. Evaluation of the CTCs led to a set of interesting observations. CTCs decreased in all but one patient, but this decline occurred regardless of clinical benefit, and CTC counts did not rise at the time of progression. Thus, in this study, the use of CTC counts was not a proficient predictor of response to this regimen; however the sample size of this study precludes a definitive conclusion of the utility of CTCs in predicting response to this regimen. Finally, the mechanism of the decline in CTCs is unclear. Such a decrease may be due to everolimus' ability to increase clearance of tumor cells from the circulation or its ability to decrease mobilization of CTCs from the metastatic tumors. Another possibility is that prior trastuzumab therapy may have decreased expression of epithelial markers in CTCs, yielding them undetectable by the methodology used in this trial.

References

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