Published in final edited form as:

Cancer Immunol Immunother. 2012 December ; 61(12): 2261–2271. doi:10.1007/s00262-012-1286-5.

Phase II trial of hu14.18-IL2 for patients with metastatic melanoma

Mark R Albertini^{1,2,7,8}, Jacquelyn A. Hank^{1,3}, Brian Gadbow⁴, Jordan Kostlevy¹, Jennifer Haldeman¹, Heidi Schalch¹, Jacek Gan^{1,3}, KyungMann Kim^{1,5}, Jens Eickhoff^{1,5}, Stephen D. Gillies⁶, and Paul M. Sondel^{1,3,4}

¹University of Wisconsin Carbone Cancer Center, Madison, WI

²Department of Medicine, at the University of Wisconsin School of Medicine and Public Health, Madison, WI

³Department of Human Oncology, at the University of Wisconsin School of Medicine and Public Health, Madison, WI

⁴Department of Pediatrics, at the University of Wisconsin School of Medicine and Public Health, Madison, WI

⁵Department of Biostatistics, at the University of Wisconsin School of Medicine and Public Health, Madison, WI

⁶Provenance Biopharmaceuticals Inc. Waltham MA

⁷Medical Service, William S. Middleton Memorial Veterans Hospital, Madison, WI

Abstract

Phase I testing of the hu14.18-IL2 immunocytokine in melanoma patients (pts) showed immune activation, reversible toxicities, and a maximal tolerated dose of 7.5 mg/m²/day. In this phase II study, fourteen pts with measurable metastatic melanoma were scheduled to receive hu14.18-IL2 at 6 mg/m²/day as 4-hour intravenous infusions on days 1, 2 and 3 of each 28 day cycle. Pts with stable disease (SD) or regression following cycle 2 could receive 2 additional treatment cycles. The primary objective was to evaluate anti-tumor activity and response duration. Secondary objectives evaluated adverse events and immunologic activation. All pts received 2 cycles of treatment. One pt had a partial response (PR) [1 PR of 14 pts = response rate of 7.1%; confidence interval 0.2%–33.9%] and 4 pts had SD and received cycles 3 & 4. The PR and SD responses lasted 3–4 months. All toxicities were reversible and those resulting in dose reduction included grade 3 hypotension (2 pts) and grade 2 renal insufficiency with oliguria (1 pt). Pts had a peripheral blood lymphocytosis on day 8 and increased C-reactive protein. While one PR in 14 pts met protocol criteria to proceed to stage 2 and enter 16 additional pts, we suspended stage 2 due to limited availability of hu14.18-IL2 at that time and the brief duration of PR and SD. We conclude that subsequent testing of hu14.18-IL2 should involve melanoma patients with minimal residual

⁸**Correspondence:** Mark R. Albertini, K6-530 Clinical Science Center, 600 Highland Avenue, Madison, WI 53792, USA. Tel: + 1-608-263-0117; fax: + 1-608-265-8133. mralbert@wisc.edu.

Conflict of Interest Statement

The authors have the following financial or other conflicts of interests to disclose related to this publication: The commercial rights to hu14.18-IL2 Immunocytokine are now held by Apeiron-Biologics AG of Vienna Austria. They were licensed from Merck Serono of Darmstadt Germany. A separate (subsequent and ongoing) trial of hu14.18-IL2 in patients with melanoma, chaired by Dr. Mark Albertini and involving this clinical team, received partial per patient research support for components of the clinical study from Merck Serono. No direct support from Merck Serono was received regarding the study reported on here. Dr. Gillies has a patent related to hu14.18-IL2 that he has licensed to Merck Serono.

disease based on compelling preclinical data and the confirmed immune activation with some antitumor activity in this study.

Keywords

Immunocytokine; Advanced Melanoma; Immunotherapy; Interleukin-2; anti-ganglioside antibody; phase II clinical trial

Introduction

Treatment of metastatic melanoma patients with high-dose bolus interleukin-2 (IL2) results in durable anti-tumor benefit in some patients [1]. Prior studies reported objective response rates of 16%, complete response rates of 6%, and noted that metastatic patients who responded for more than 30 months after high dose IL-2 were unlikely to have subsequent disease recurrence [2]. Thus, improving IL-2 therapy has potential to significantly impact management of metastatic melanoma patients. One approach to improve efficacy of IL2-based treatment is to focus the cytolytic activity of activated NK cells to mediate increased tumor specific destruction [3,4]. This can be demonstrated *in vitro* with the addition of monoclonal antibodies (mAb) to facilitate antibody-dependent cell-mediated cytotoxicity (ADCC). This approach has been attempted in clinical trials of anti-ganglioside mAb combined with IL2 [5]. GD2 is a disialoganglioside expressed on tumors of neuroectodermal origin including melanoma, neuroblastoma and certain sarcomas. Expression on normal tissues is limited to the cerebellum, peripheral nerves, and very few other tissues. The relatively tumor selective expression of GD2 makes it a suitable target for mAb treatment. *In vitro*, anti-GD2 antibodies can mediate substantial ADCC and complement-dependent cytotoxicity (CDC) against GD2⁺ tumor target cells [4].

The original IgG3 murine anti-GD2 mAb 14.18 was developed by Dr. Ralph Reisfeld [6]. An IgG2a class switch variant of 14.18, called 14.G2a, was prepared in an attempt to mediate enhanced ADCC. This antibody has been tested as a single agent and in combination with IL2 in clinical trials. When antitumor activity was noted, a human/murine chimeric mAb ch14.18 was constructed using the murine variable genes of 14.18 and the human constant IgG1 and k genes, which are known to be effective at CDC and ADCC [7]. The ch14.18 antibody was tested in Phase I clinical trials alone and in combination with IL2 in the treatment of melanoma patients. In an effort to improve the immunologically-mediated antitumor activity, an immunocytokine (IC) composed of ch14.18 antibody and the cytokine IL2 was constructed by fusing a synthetic human IL2 gene sequence to the carboxyl end of the human Ig gene [8]. This ch14.18-IL2 IC has undergone extensive preclinical testing and has shown significant tumor reactivity and clinical potential [9–11]. Despite the previous observation of ADCC with anti-GD2 mAbs used in combination with soluble IL2, this mechanism is potentially limited by the number and type of available effector cells. Achieving ADCC depends upon the presence of effector cells with Fc receptors and upon the number of Fc receptors on the effector cells [12]. When NK cells with Fc receptors are activated and expanded with IL2 *in vivo*, they mediate dramatically augmented ADCC [4]. However, many of the activated NK cells circulating in cancer patients following *in vivo* treatment with IL2 do not have Fc receptors, in contrast to resting NK cells [13]. These Fc receptor negative, activated NK cells are more cytolytic in direct lytic assays not dependent on mAb and Fc receptors [13]. Thus, it would be advantageous to enable these activated NK cells to lyse tumor using a recognition mechanism that does not depend upon Fc receptors.

The *in vivo* activated NK cells have augmented expression of the IL2 receptor beta (IL2R- β) [14] and demonstrate a dramatic *in vitro* response to IL2 [15]. Furthermore, IL2R-bearing T cells that do not specifically recognize these tumors with their TCRs should still be responsive to IL2. Thus, it would be advantageous to activate these IL2 receptors with a molecule that will bridge the NK cells and T cells to tumor and activate lytic interactions. This is the proposed function of the ch14.18-IL2 IC. This molecule utilizes the 14.18 anti-GD2 antibody-mediated recognition component to bind to tumor cells, the Fc component to bind to cells expressing Fc receptors, and the IL2 component to activate lytic cells expressing IL2 receptors. Despite the substantial human component of the chimeric ch14.18 antibody, several patients treated with this antibody developed strong anti-idiotypic antibody responses. The dose-limiting-toxicities (DLT) also included allergic symptoms (angioedema) in some patients, potentially related to the murine component of this chimeric antibody [16, 17]. Hu14.18-IL2 was thus developed to maintain the immunologic activity of the ch14.18-IL2 IC molecule and decrease IC-related human anti-chimeric antibody (HACA) responses and allergic reactions. S. Gillies and colleagues utilized the same technology used to create ch14.18-IL2 [8] for the construction of hu14.18-IL2. The hu14.18 Ab portion contains only the complementarity-determining regions (CDR) of the murine variable chains, grafted into the intact human IgG₁ molecule, which has an IL2 molecule at each carboxy terminus of the IgG₁ heavy chains. Our Phase I clinical trial of hu14.18-IL2 in adults with melanoma showed that hu14.18-IL2 is clinically safe and well-tolerated at doses (0.8 – 7.5 mg/m²) that induce immunologic activation [18]. A total of 33 patients with melanoma were enrolled to establish the maximal tolerated dose (MTD). Patients were administered hu14.18-IL2 at one of the following dose levels: 0.8, 1.6, 3.2, 4.8, 6.0 or 7.5 mg/m²/day. Hu14.18-IL2 was administered as a 4-hour IV infusion over 3 consecutive days during the first week of each course. The dose of 7.5 mg/m²/day was found to be the MTD, as 2 of 6 subjects showed reversible dose-limiting toxicity at this dose level. These included hypoxia, hypotension and elevations of AST and ALT [18].

The primary objective of this phase II study of hu14.18-IL2 in advanced melanoma patients was to evaluate clinical anti-tumor activity and duration of response at one dose level beneath the previously determined MTD. Secondary objectives included evaluating adverse events and immunologic activation. The hu14.18-IL2 dose for this Phase II trial was 6 mg/m² and was one dose below the MTD of 7.5 mg/m² established in our Phase I trial [18]. The dose of 6.0 rather than the 7.5 mg/m²/d was chosen, as the MTD determination in the phase I study was based on DLT events in the first course of treatment. However, most patients in this study were expected to be receiving 4 courses of treatment. As some patients in the adult and pediatric phase I studies did have episodes of DLT in subsequent courses of treatment not necessarily seen in the first course, it was determined that starting at the 6.0 mg/m²/day dose would be more likely to enable patients to continue on treatment without requiring dose reductions.

Materials and Methods

Clinical Protocol

Patients—Fourteen patients were enrolled and all patients provided written informed consent. Enrollment required patients to have histologically confirmed malignant melanoma that was considered surgically and medically incurable and was measurable by clinical assessments or imaging. All patients needed to have adequate hematologic parameters (Total WBC 3500/mm³, platelets 100,000/mm³, and hemoglobin 10.0 gm/dl), adequate liver function (AST/ALT <2-times normal and a total bilirubin <2.0 mg/dl), and adequate renal function (Serum creatinine <2.0 mg/dl or a creatinine clearance of 60 ml/minute). All patients had an Eastern Cooperative Oncology Group performance status of 0 or 1. Patients

with a history of cardiac disease, age ≥ 65 years old, or with significant risk factors for coronary artery disease were required to complete an exercise radionuclide scan with no evidence of myocardial ischemia or heart failure and have normal pulmonary function. Criteria for patient exclusion included exclusion of patients who had previously received corticosteroids or other immunosuppressive therapy within 2 weeks before study entry. Patients with CNS metastases were also excluded. Patients who previously received mAbs for any reason and had detectable antibody (over background) to hu14.18 were excluded. Patients with a history of diabetes mellitus that required systemic therapy within the past 3 months were excluded, as this therapy may alter blood glucose levels. Patients with HIV or Hepatitis B surface antigen carrier state or with clinical evidence of hepatitis were ineligible. Patients who had received any (standard or experimental) systemic therapy for stage IV disease were excluded.

Hu14.18-IL2—The hu14.18-IL2 was supplied collaboratively by the NCI-Biological Resources Branch (NCI-BRB, Frederick, MD) as well as EMD Pharmaceuticals (Durham, NC) and Merck Serono (Darmstadt, Germany). Preclinical evaluation has shown that 1 mg of the fusion protein contains approximately 3×10^6 U of IL-2 (based on a proliferative assay with IL-2 responsive Tf-1 beta cells) and approximately 0.8 mg of the hu14.18 mAb [19].

Study Design—This was a two-stage study design. Initially 14 patients were to be enrolled and receive hu14.18-IL2 on days 1, 2, and 3 of each course of therapy as a 4-hour continuous intravenous infusion at a daily dose of 6 mg/m²/day. Patients could receive a maximum of four 28-day courses of treatment at the same dose level, provided that there was no dose limiting toxicity (DLT) or significant non-DLT toxicity. The protocol allowed for 2 dose modifications for DLT or significant non-DLT toxicity: the first a 50% dose reduction to 3 mg/m²/day, and the second an additional 50% dose reduction to 1.5 mg/m²/day. Missed doses were not made up, and all dose reductions were permanent. If there were no responses in these 14 subjects (either complete or partial) the study would be closed due to lack of clinical efficacy. If a response was seen (complete or partial) in at least one subject, an additional 16 subjects could be enrolled depending on the availability of the hu14.18-IL2. If insufficient clinical material were available at that time, after discussion with the NCI-BRB (the producer of the hu14.18-IL2) and EMD (the licensor for hu14.18-IL2 and MCRADA holder with the NCI, at the time of this study), then the study would be closed to accrual. If sufficient material were available, however, 16 additional patients could be enrolled at the same dose level. If four or more of the 30 subjects had objective responses, the protocol regimen would be considered to have sufficient clinical activity to warrant further study. All eligible subjects who receive any administration of hu14.18-IL2 are considered evaluable for efficacy and safety.

Toxicity Grading and Dose Modifications—The definition of dose limiting toxicity (DLT) was defined as Grade 3 or 4 toxicity, using the NCI Common Terminology Criteria Adverse Events (CTCAE), version 3.0, except for the following which are known side effects of IL-2 and hu14.18-IL2 therapy: Grade 3 pain, Grade 3 nausea and vomiting, Grade 3 fever (Temp >40°C), Grade 3 skin toxicity, Grade 3 metabolic/laboratory toxicity of hyponatremia, hyperglycemia, or hypophosphatemia in the absence of CNS symptoms, Grade 3 sensory or motor peripheral (non-cranial) neuropathy if transient and reversing within 3 days of completion of hu14.18-IL2, Grade 3 hematologic toxicity (or grade 4 lymphopenia—a known transient marker of immune activation), Grade 3 infusional reactions lasting less than 24 hours, Grade 3 fatigue or ECOG performance status change that resolves in ≤ 1 week, and Grade 3 infection that resolves in ≤ 1 week. These toxicities, if seen, were graded and recorded but not used as criteria for determining DLTs as they were expected,

based on observations of hu14.18-IL2 given to 33 adults with melanoma, administered by the same schedule at similar doses to that being tested in this trial [18].

All grade 4 toxicities, with the exception of transient lymphopenia, were considered DLT and required stopping of treatment. If this toxicity was related to the hu14.18-IL2 the patient was removed from the study without further treatment. If the Grade 4 toxicity was not related to the hu14.18-IL2 and resolved to baseline in less than 3 days, treatment was continued at 50% of the daily dose from the previous course. All other DLT and significant non-DLT toxicity had therapy held, and resumed at 50% of the initial dose once the toxicity resolved. Missed doses were not given at a later date. If toxicity again required treatment cessation, the dose was reduced to 25% of the initial dose. If treatment at this reduced dose resulted in recurrence of the DLT or significant non-DLT toxicity, treatment was permanently discontinued.

Response Criteria—Serial clinical measurements of tumor size were performed by standard radiologic and physical exam measurements, appropriate for each patient's clinical status. Tumor measurements were scheduled to take place after every 2 courses of treatment and at the completion of protocol treatment. The clinical response to treatment was determined at each of the disease status evaluation time points. The NCI Response Evaluation Criteria in Solid Tumors (RECIST) was used. A complete response was defined as a disappearance of all target lesions. A partial response was defined as at least a 30% decrease in the sum of the longest diameters of target lesions. Progressive disease was defined as at least a 20% increased in the sum of the longest diameters of the target lesions, taking as reference the longest diameters recorded at baseline. Stable disease was defined as neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease.

Immunologic Monitoring—Serum samples were obtained from all patients to measure hu14.18-IL-2 levels, anti-hu14.18-IL2 antibodies, and soluble IL2 receptor α levels. Serum samples were drawn at baseline and on Days 1,3,4, 8, and 29. Blood samples were also collected from selected patients, at times shown, to evaluate C-reactive protein (CRP) levels and functional NK and ADCC assays. All assays shown (except CRP) involve ELISA methods or in vitro cell-mediated cytotoxicity assays as previously published [16, 18, 20–22]. The anti-idiotypic bridge assay was considered positive for samples with O.D. > 0.6; borderline for samples with O.D. from 0.3 – 0.6, and negative for samples with O.D. < 0.3 [22]. The anti-idiotypic binding inhibition assay was considered positive for samples showing >28% inhibition [22]. The CRP assay was performed by the UW Hospitals and Clinics clinical lab, and the other lab assays used to evaluate DLTs per the CTCAE 3.0 criteria were standard clinical lab assays.

Statistical Analysis—Treatment effects were assessed as the change in parameter varies from baseline to various times during treatment. The clinical response to treatment was determined at each of the disease status evaluation points. Serum IC level, changes in absolute lymphocyte count from baseline to C1D3 and C1D8, and changes in CRP from baseline to C1D3 and C1D4 between those with best response of PD and PR/SD and sIL2R levels and absolute CRP levels at C1D4 and C2D4 between patients with and without DLTs were compared using two-sample Wilcoxon rank sum test. Changes in peripheral blood lymphocyte counts from baseline to days 3 and 8 for both cycle 1 and 2 were evaluated using a paired t-test. All p-values are two-sided, and levels of $P < 0.05$ were considered as statistically significant. Data analysis was conducted using SAS software version 9.2 (SAS Institute, Cary, NC).

Results

Patient Characteristics

Fourteen patients were entered into this study and their pretreatment characteristics are outlined in Table 1. Eight of the 14 patients were men, the median age was 60 years, and all patients were ECOG performance status 0 or 1. Five patients had received previous adjuvant immunotherapy and 1 had received previous adjuvant biochemotherapy. Thirteen of the patients had a primary melanoma site on the skin, and one patient did not have a documented primary site. The most common sites of metastatic disease were lung (11 patients), liver (9 patients) and lymph node or other soft tissues (9 patients).

Treatment Summary and Anti-tumor effects

The treatment summary of patients in this study is shown in Table 2. All 14 patients received at least 2 cycles of hu14.18-IL2. Five patients were eligible for four cycles of therapy because of stable disease (four patients) or partial response (one patient). The other 9 patients had objective disease progression at the initial assessment of clinical response following cycle 2. The duration of stable disease or partial response was measured in months starting from the date of the clinical response assessment following cycle 2. The duration of stable disease ranged from 3–4 months, and the duration of the one partial response was 3.5 months. Three patients required dose reductions, and a modified dose of hu 14.18-IL2 was administered in 6 of the 38 treatment cycles.

Toxicities

Toxicities requiring dose reductions are shown in Table 3a and include hypotension refractory to brief intravenous fluids (2 patients) and grade 2 renal insufficiency with oliguria (1 patient). All three of these patients were able to receive the remainder of planned hu14.18-IL2 infusions with a 50% dose reduction. The anticipated IL-2 constitutional symptoms (fever, chills, fatigue, pruritis, and myalgias) were seen in the majority of patients treated on this clinical trial. Two patients had severe fatigue and decline in performance status, but returned to baseline after the conclusion of therapy. Several patients experienced pain associated with infusion of hu14.18-IL2 and required opioids for adequate pain control. All treatment-associated toxicities experienced by patients in this study were reversible.

The Grade 3 and 4 laboratory toxicities experienced by patients in this study are outlined in Table 3b. The only grade 4 laboratory toxicity was a transient grade 4 lymphopenia associated with hu14.18-IL2 infusions and was similar to what we previously reported with hu14.18-IL2 infusions [18]. All grade 3 laboratory changes were reversible and did not require a dose reduction. Many patients had transient grade 1 and 2 laboratory changes that were similar to those reported in our prior phase I study of hu14.18-IL2 [18].

Immunologic Results/Correlative Studies

Hu14.18-IL2 levels—Analysis of serum IC for all patients showed a mean value of 1.42 ug/ml on C1D1 and 1.38 ug/ml on C1D3. The slightly decreased level of IC on Day 3 (compared to Day1) is expected and was also seen in a separate Children's Oncology Group study of patients with neuroblastoma [20]. No correlation was found between peak IC level on Day 1 and any toxicity parameter or clinical response. The peak level of IC on Day 1 did not differ significantly between patients who had progressive disease and those that had stable disease or a partial response (Table 4a). There was also no association between peak IC level and the following parameters: absolute drop in lymphocyte count from baseline to the nadir (day 3), absolute rise in lymphocyte count from baseline to the lymphocyte

rebound seen on day 8, increase in soluble IL2 receptor levels, or change in CRP (not shown).

Immune activation—Figure 1 shows immune activation as measured in a ^{51}Cr release assay. Killing of M21 melanoma cells was examined in the presence of serum taken from each of the 14 patients just before or just after the 4 hour IC infusion on Day-1 of course 1. This assay was done in the presence or absence of PBMCs from a healthy donor. The serum from the patient was not heat treated, to enable endogenous complement to mediate killing if an appropriate antibody was present. The first column in figure 1 shows that virtually no killing is mediated against M21 melanoma cells by pre-infusion serum from these 14 patients. Addition of PBMCs from a healthy donor (column 2 in Fig. 1) does not induce detectable cytotoxicity. In contrast, when serum obtained just after the IC infusion was tested in this assay, in the absence of PBMCs (column 3 in Fig. 1) 6 of the 14 patients showed detectable tumor cell killing. This indicates that sufficient anti tumor antibody (the IC infused over the prior 4 hrs) and complement was present in the serum from those 6 patients to enable complement mediated tumor cell lysis, that was not observed with the pre-treatment serum sample. More importantly, for all 14 patients, when healthy PBMCs were added as effector cells to the serum obtained after the 4 hr infusion, an increase in killing was seen over that mediated by those same effector cells with the patients' pre-treatment serum (comparing columns 2 and 4 in Fig. 1), documenting the acquisition of conditions able to induce ADCC. The fact that these same effector cells do not induce any detectable killing when the source of serum is obtained from these same 14 patients immediately before the IC infusion (again, compare columns 2 and 4 in Fig. 1), indicates that the infusion of the IC into these patients has enabled their serum to mediate ADCC, using the circulating IC (detected by ELISA and shown in table 4a). Furthermore, the addition of these PBMCs to the 4-hr serum sample caused an increase in killing over that observed by this same serum without effectors, for all 14 patients (compare columns 3 and 4). This demonstrates that the killing observed in the presence of PBMCs is greater than that which can be mediated via complement with this same serum sample, and thus reflects ADCC.

Figure 2 shows the lymphopenia that was anticipated on Day 3 of each course that was followed by a lymphocytosis, as previously described [18, 20]. The magnitude of the drop in absolute lymphocyte count (ALC) on day 3 was not different for the 9 patients with PD than for the 4 evaluated patients with SD/PR (Table 4b). Neither was the rebound increase in ALC seen on day 8 (Table 4b).

As previously seen, we observed a significant increase in soluble IL2 receptor (sIL2R) levels in all courses from baseline to day 3 ($p < 0.001$ for both courses 1 and 2) [18, 20]. Table 4c shows the increase in sIL2R levels was not different for those 3 patients with DLT versus those 11 without. Similarly, there was no difference in sIL2R level for the 5 with PR or SD vs. the 9 patients with progressive disease (not shown). CRP levels are a common clinical laboratory parameter for immune activation. As expected [20], we found this parameter was increased by treatment with hu14.18-IL2 (as detected on the 3rd and 4th day of each course). As this lab assay was not required initially for all patients, we do not have a complete data set of values for all patients at all time points. Even so, the increased values over baseline are seen in Tables 4d and 4e. Table 4d shows that there is no difference in the CRP response for the patients with PD vs. those with SD/PR. It is of interest that the trend was for CRP levels on day 4 of both course 1 and course 2 to be higher for the patients with DLT than those without (Table 4e; $p = 0.08$), consistent with the DLT being due to the immune activation (likely from the IL2 component of the IC).

Anti-hu14.18-IL2 antibody—In previous studies [20, 21] we have shown that some patients develop antibodies to the idiotypic determining region of the IC; these antibodies

are measured by a bridging assay or a binding inhibition assay [22]. In this phase II study of 14 evaluable patients, 8 patients developed a “positive” anti-idiotypic antibody against hu14.18-IL2 based on the bridging assay and 13 developed a detectable anti-idiotypic antibody based on the “binding inhibition” assay. The development of these anti-IC antibodies was not associated (statistically) with the peak level of IC, for these 13 patients in this study (not shown). Furthermore, there was no significant effect of this anti-idiotypic antibody response on the *in vivo* level of hu14.18-IL2. Specifically, there was no significant association of the level of anti-idiotypic antibody developed after course-1 ($p = 0.06$ for the bridge assay and 0.6 for the binding inhibition assay) with any detectable decrease in peak hu14.18-IL2 level seen on day-1 of course-2 (compared to the level seen on day-1 of course-1). This lack of a significant association is in contrast to the significant drop in hu14.18-IL2 levels from course-1 day-1 to course-2 day-1 for those patients with positive anti-idiotypic responses (in the “binding inhibition” ($p = 0.002$) assay) in our past phase I trials (where most patients received lower IC doses) [22].

Discussion

New insights are critically needed to achieve durable benefit for patients with metastatic melanoma, as this cancer is usually incurable once metastatic to distant sites. Recent advances provide new options for metastatic melanoma patients. Ipilimumab and vemurafenib were approved in 2011 based on their proven benefit of improving the survival of some patients with metastatic melanoma [23–25]. While vemurafenib can induce complete or partial tumor regression in up to 80% of metastatic melanoma patients with the V600E BRAF mutation, those responses are of limited duration for the overwhelming majority of treated patients [25]. Response rates with Ipilimumab are only in the 10–20% range, but those responses can be durable for years [23, 24]. Therapy with high-dose bolus interleukin-2 also provides durable benefit for a minority of metastatic melanoma patients [7]. The clinical challenge remains identifying treatments that can achieve durable benefit for a greater number of metastatic melanoma patients.

This protocol examined the treatment of advanced melanoma patients with hu14.18-IL2 at one dose level beneath the previously determined MTD as 4-hour IV infusions over 3 consecutive days during the first week of each 28-day course of therapy. Treatment with hu14.18-IL2 at this dose and schedule (6 mg/m²/d for three consecutive days) is well tolerated and has toxicities that are reversible and readily managed. Of the 14 patients that were treated, only 3 patients required dose reductions due to toxicities. It is of interest that the three patients who required dose reductions due to treatment-associated toxicities had either stable disease (2 patients) or a partial response (1 patient) that lasted up to 4 months. We considered the hypothesis that these patient toxicities were due to a more pronounced activation of their immune system. However, comparing laboratory parameters of immune activation including sIL2R, CRP, and absolute change in lymphocyte count between patients with PD vs SD/PR did not reveal significant differences. Immune activation was found in all treated patients as evidenced by rebound lymphocytosis, elevated CRP, increased sIL2R levels and ADCC. Thus, general measures of immune activation did not predict PR or SD for patients on this study.

The objective response rate for unselected advanced melanoma patients receiving hu14.18-IL2 at 6 mg/m²/day, one dose level beneath the MTD, was 7.1% (confidence interval 0.2%–33.9%). Only one patient of the 14 enrolled had an objective partial response. While the confidence interval for this response rate is wide, none of the patients in this trial had durable benefit, as the longest duration for either stable disease or patient response was only 4 months. Due to this fact combined with the limited supply of the hu14.18-IL2 at that time, we concluded that there was not sufficient evidence to pursue treatment of unselected

advanced melanoma patients with this dose and schedule of hu14.18-IL2, and we cancelled plans for stage 2 of this study.

In our phase I trials of hu14.18-IL2 [18, 20] we found that development of a detectible antibody response against the hu14.18-IL2 IC was associated with a significant decrease in the peak IC level seen in subsequent courses [22]. Such a relationship was seen in 11 melanoma patients receiving 2 courses of IC in the phase I trial [18]. In contrast, in this present study of 14 melanoma patients, there was no statistically significant influence of anti-IC antibody on the levels of IC detected in serum in subsequent courses. We hypothesize that even though we can detect anti-IC antibody in some IC-treated patients, the “neutralizing” effect of such antibody is not sufficient to influence the detected serum levels of IC for patients receiving the higher dose of IC received in this phase II trial (compared to the lower doses of IC used for most patients in the Phase I melanoma trial) [18]. Recent in vitro data from our lab simulating the in vivo concentrations of IC and anti-IC antibody in the sera of these patients are consistent with this conclusion [Hank et al, manuscript in preparation].

In contrast to the observed limited activity of hu14.18-IL2 for patients with advanced melanoma seen in this phase II study, there have been encouraging data for this agent in the treatment of children with advanced (relapsed or refractory) neuroblastoma. Clear antitumor activity has been observed in our phase II trial [21]. However, in that phase II trial, antitumor activity was not observed in children with bulky disease (measurable by MRI or CT scans), but was only seen in children with “less bulky” disease; neuroblastoma evaluable only by bone marrow histology or ¹²³I-MIBG scintigraphy [21]. These clinical data, showing greater antitumor activity in the setting of less bulky disease, were consistent with our preclinical data demonstrating greater activity of hu14.18-IL2 in mice with smaller amounts of tumor at the time of treatment [26]. As our preclinical data [26] and our clinical data from our study of children with neuroblastoma both suggest that hu14.18-IL2 may demonstrate greater efficacy in the minimal residual disease state, we are now studying the administration of hu14.18-IL2 in adult patients with recurrent stage III or IV melanoma who can be rendered free of all known disease with surgical resection.

As our preclinical data indicated that a mechanism for IC-induced antitumor efficacy was via NK cells mediating ADCC [27, 28], we also evaluated the influence of receptors on cells of the innate immune system for their potential involvement in the antitumor activity seen in our phase II neuroblastoma trial [21]. Our clinical data demonstrated that individuals with favorable killer immunoglobulin-like receptor (KIR) and KIR-ligand genotypes were more likely to benefit from IC treatment [28]. In addition there was a trend toward benefit for individuals with favorable Fc receptor genotype (for CD32) [28]. Further evaluation of KIR/KIR-ligand and FcR genotypes may be helpful in future studies of this hu14.18-IL2 in melanoma, and of other immunotherapies that act, in part, via ADCC.

The current study did not require histological demonstration of GD2 expression for study eligibility. It is possible that GD2 expression by melanoma patients in this study was less than anticipated. We are testing GD2 expression of resected melanoma in the current study of hu14.18-IL2 for patients with recurrent stage III or stage IV melanoma who can be rendered free of all known disease with surgical resection. In addition, the demonstrated immunological activation of hu14.18-IL2 with manageable toxicities suggests the potential for combination treatments of advanced melanoma patients with hu14.18-IL2 and other agents. Concepts that merit consideration include the combination of hu14.18-IL2 with experimental anti-melanoma vaccines, combination of hu14.18-IL2 with other immunotherapies such as Ipilimumab, and combination of hu14.18-IL2 with targeted therapies such vemurafenib. In addition, our preclinical data demonstrate more potent local

and systemic effects can be obtained when hu14.18-IL2 is given as an intratumoral injection [29]. As other immunotherapies are being applied via intratumoral administration for testing in melanoma [30], we are working towards a future clinical trial of hu14.18-IL2 as intratumoral therapy in patients with melanoma.

Acknowledgments

We thank Barrett P. Wagner for technical assistance and Laddie Johnson for assistance with manuscript preparation. We also thank the nurse on the UW CTSC for outstanding nursing care and for clinical trial support.

Grant Sponsors: This material was supported by National Institutes of Health Grants CA032685, CA87025, and grants from the Midwest Athletes for Childhood Cancer Fund, the Crowdaddy Foundation; CTSC grant (GM067386); grant P30 CA014520 from the National Cancer Institute; the Gretchen and Andrew Dawes Melanoma Research Fund; Ann's Hope Foundation; the Jay Van Sloan Memorial from the Steve Leuthold Family; and the Tim Eagle Memorial.

References

1. Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, Abrams J, Sznol M, Parkinson D, Hawkins M, Paradise C, Kunkel L, Rosenberg SA. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol*. 1999; 17:2105–2116. [PubMed: 10561265]
2. Atkins MB, Kunkel L, Sznol M, Rosenberg SA. High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update. *Cancer J Sci Am*. 2000; 6(Suppl 1):S11–S14. [PubMed: 10685652]
3. Harel W, Shau H, Hadley CG, Morgan AC Jr, Reisfeld RA, Cheresch DA, Mitchell MS. Increased lysis of melanoma by in vivo-elicited human lymphokine-activated killer cells after addition of antiganglioside antibodies in vitro. *Cancer Res*. 1990; 50:6311–6315. [PubMed: 2400994]
4. Hank JA, Robinson RR, Surfus J, Mueller BM, Reisfeld RA, Cheung NK, Sondel PM. Augmentation of antibody dependent cell mediated cytotoxicity following in vivo therapy with recombinant interleukin 2. *Cancer Res*. 1990; 50:5234–5239. [PubMed: 2386933]
5. Bajorin DF, Chapman PB, Wong G, Coit DG, Kunicka J, Dimaggio J, Cordon-Cardo C, Urmacher C, Dantes L, Templeton MA, et al. Phase I evaluation of a combination of monoclonal antibody R24 and interleukin 2 in patients with metastatic melanoma. *Cancer Res*. 1990; 50:7490–7495. [PubMed: 2253196]
6. Mujoo K, Cheresch DA, Yang HM, Reisfeld RA. Disialoganglioside GD2 on human neuroblastoma cells: target antigen for monoclonal antibody-mediated cytotoxicity and suppression of tumor growth. *Cancer Res*. 1987; 47:1098–1104. [PubMed: 3100030]
7. Gillies SD, Lo KM, Wesolowski J. High-level expression of chimeric antibodies using adapted cDNA variable region cassettes. *J Immunol Methods*. 1989; 125:191–202. [PubMed: 2514231]
8. Gillies SD, Reilly EB, Lo KM, Reisfeld RA. Antibody-targeted interleukin 2 stimulates T-cell killing of autologous tumor cells. *Proc Natl Acad Sci U S A*. 1992; 89:1428–1432. [PubMed: 1741398]
9. Becker JC, Pancook JD, Gillies SD, Furukawa K, Reisfeld RA. T cell-mediated eradication of murine metastatic melanoma induced by targeted interleukin 2 therapy. *J Exp Med*. 1996; 183:2361–2366. [PubMed: 8642346]
10. Lode HN, Xiang R, Varki NM, Dolman CS, Gillies SD, Reisfeld RA. Targeted interleukin-2 therapy for spontaneous neuroblastoma metastases to bone marrow. *J Natl Cancer Inst*. 1997; 89:1586–1594. [PubMed: 9362156]
11. Lode HN, Xiang R, Dreier T, Varki NM, Gillies SD, Reisfeld RA. Natural killer cell-mediated eradication of neuroblastoma metastases to bone marrow by targeted interleukin-2 therapy. *Blood*. 1998; 91:1706–1715. [PubMed: 9473237]
12. Berke, G. The functions and mechanisms of action of cytolytic lymphocytes. In: Paul, WE., editor. *Fundamental Immunology*. New York, NY: Raven Press; 1993. p. 965-1014.

13. Weil-Hillman G, Fisch P, Prieve AF, Sosman JA, Hank JA, Sondel PM. Lymphokine-activated killer activity induced by in vivo interleukin 2 therapy: predominant role for lymphocytes with increased expression of CD2 and leu19 antigens but negative expression of CD16 antigens. *Cancer Res.* 1989; 49:3680–3688. [PubMed: 2471587]
14. Voss SD, Robb RJ, Weil-Hillman G, Hank JA, Sugamura K, Tsudo M, Sondel PM. Increased expression of the interleukin 2 (IL-2) receptor beta chain (p70) on CD56+ natural killer cells after in vivo IL-2 therapy: p70 expression does not alone predict the level of intermediate affinity IL-2 binding. *J Exp Med.* 1990; 172:1101–1114. [PubMed: 1698909]
15. Sondel PM, Kohler PC, Hank JA, Moore KH, Rosenthal NS, Sosman JA, Bechhofer R, Storer B. Clinical and immunological effects of recombinant interleukin 2 given by repetitive weekly cycles to patients with cancer. *Cancer Res.* 1988; 48:2561–2567. [PubMed: 3258545]
16. Albertini MR, Gan J, Jaeger P, Hank JA, Storer B, Schell K, Rivest T, Surfus J, Reisfeld RA, Schiller JH, Sondel PM. Systemic interleukin-2 modulates the anti-idiotypic response to chimeric anti-GD2 antibody in patients with melanoma. *J Immunother Emphasis Tumor Immunol.* 1996; 19:278–295. [PubMed: 8877722]
17. Saleh MN, Khazaeli MB, Wheeler RH, Allen L, Tilden AB, Grizzle W, Reisfeld RA, Yu AL, Gillies SD, LoBuglio AF. Phase I trial of the chimeric anti-GD2 monoclonal antibody ch14.18 in patients with malignant melanoma. *Hum Antibodies Hybridomas.* 1992; 3:19–24. [PubMed: 1576319]
18. King DM, Albertini MR, Schalch H, Hank JA, Gan J, Surfus J, Mahvi D, Schiller JH, Warner T, Kim K, Eickhoff J, Kendra K, Reisfeld R, Gillies SD, Sondel P. Phase I clinical trial of the immunocytokine EMD 273063 in melanoma patients. *J Clin Oncol.* 2004; 22:4463–4473. [PubMed: 15483010]
19. Hank JA, Surfus JE, Gan J, Jaeger P, Gillies SD, Reisfeld RA, Sondel PM. Activation of human effector cells by a tumor reactive recombinant anti-ganglioside GD2 interleukin-2 fusion protein (ch14.18-IL2). *Clin Cancer Res.* 1996; 2:1951–1959. [PubMed: 9816154]
20. Osenga KL, Hank JA, Albertini MR, Gan J, Sternberg AG, Eickhoff J, Seeger RC, Matthay KK, Reynolds CP, Twist C, Krailo M, Adamson PC, Reisfeld RA, Gillies SD, Sondel PM. A phase I clinical trial of the hu14.18-IL2 (EMD 273063) as a treatment for children with refractory or recurrent neuroblastoma and melanoma: a study of the Children's Oncology Group. *Clin Cancer Res.* 2006; 12:1750–1759. [PubMed: 16551859]
21. Shusterman S, London WB, Gillies SD, Hank JA, Voss SD, Seeger RC, Reynolds CP, Kimball J, Albertini MR, Wagner B, Gan J, Eickhoff J, DeSantes KB, Cohn SL, Hecht T, Gadbar B, Reisfeld RA, Maris JM, Sondel PM. Antitumor activity of hu14.18-IL2 in patients with relapsed/refractory neuroblastoma: a Children's Oncology Group (COG) phase II study. *J Clin Oncol.* 2010; 28:4969–4975. [PubMed: 20921469]
22. Hank JA, Gan J, Ryu H, Ostendorf A, Stauder MC, Sternberg A, Albertini M, Lo KM, Gillies SD, Eickhoff J, Sondel PM. Immunogenicity of the hu14.18-IL2 immunocytokine molecule in adults with melanoma and children with neuroblastoma. *Clin Cancer Res.* 2009; 15:5923–5930. [PubMed: 19737959]
23. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urbaniak WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med.* 2010; 363:711–723. [PubMed: 20525992]
24. Robert C, Thomas L, Bondarenko I, O'Day S, M DJ, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, Davidson N, Richards J, Maio M, Hauschild A, Miller WH Jr, Gascon P, Lotem M, Harmankaya K, Ibrahim R, Francis S, Chen TT, Humphrey R, Hoos A, Wolchok JD. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med.* 2011; 364:2517–2526. [PubMed: 21639810]
25. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'Dwyer PJ, Lee RJ, Grippo JF, Nolop K, Chapman PB. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med.* 2010; 363:809–819. [PubMed: 20818844]
26. Neal ZC, Yang JC, Rakhmilevich AL, Buhtoiarov I, Lum HE, Imboden M, Hank JA, Lode HN, Reisfeld RA, Gillies SD, Sondel PM. Enhanced activity of hu14.18-IL2 immunocytokine against

- the murine NXS2 neuroblastoma when combined with IL2 therapy. *Clinical Cancer Research*. 2004; 10:4839–4847. [PubMed: 15269160]
27. Neal ZC, Imboden M, Rakhmilevich AL, Kim KM, Hank JA, Surfus J, Dixon JR, Lode HN, Reisfeld RA, Gillies SD, Sondel PM. NXS2 murine neuroblastomas express increased levels of MHC class I antigens upon recurrence following NK-dependent immunotherapy. *Cancer Immunology and Immunotherapy*. 2004; 53:41–52. [PubMed: 14504825]
28. Delgado DC, Hank JA, Kolesar J, Lorentzen D, Gan J, Seo S, Kim KM, Shusterman S, Gillies SD, Reisfeld RA, Yang R, Gadbar B, DeSantes KD, London WB, Seeger RC, Maris J, Sondel PM. Genotypes of NK Cell KIR Receptors, Their Ligands, and Fcγ Receptors in the Response of Neuroblastoma Patients to Hu14.18-IL2 Immunotherapy. *Cancer Research*. 2010; 70:9554–9661. [PubMed: 20935224]
29. Johnson EE, Lum HD, Rakhmilevich AL, Schmidt BE, Furlong M, Buhtoiarov IN, Hank JA, Raubitschek A, Colcher D, Reisfeld RA, Gillies SD, Sondel PM. Intratumoral Immunocytokine Treatment Results in Enhanced Antitumor Effects. *Cancer Imm and Immunother*. 2008; 57:1891–1902.
30. Triozzi PL, Tuthill RJ, Borden E. Re-inventing intratumoral immunotherapy for melanoma. *Immunotherapy*. 2011; 3:653–671. [PubMed: 21554094]

Figure 1. Antibody-dependent cell-mediated cytotoxicity (ADCC) with hu14.18-IL2 in patient serum. The M21 human melanoma target (% cytotoxicity; effector:target ratio of 50:1) was evaluated with (+) and without (–) effector PBMC from a healthy control donor in medium containing patient serum obtained pretreatment (pre) or after the initial 4 hour hu14.18-IL2 infusion (4 hr).

Figure 2.

Mean peripheral blood lymphocyte count (cell number per mcl $\pm$ SD) at the indicated protocol day for the 14 patients in this study. Timepoints are pretreatment (Pre) and at the indicated cycle (C) and day (D) of that cycle. Changes from Pre were significant for C1D3, C1D8, C2D3, C2D26 ($p < 0.001$) and C1D26 ($p = 0.011$)

Table 1

Patient characteristics (N=14)

Characteristics		Number of Patients [*]
Sex	Male	8
	Female	6
Site of Primary disease	Skin	13
	Unknown	1
Prior Therapy	Surgery	13
	Immunotherapy	5
	Biochemotherapy	1
	Radiation Therapy	3
Sites of Disease	Lymph node, subcutaneous, and other soft tissues	9
	Lung	11
	Liver	9
	Other visceral metastases (spleen, pancreas, adrenal)	5
	Bone	1
AJCC Disease classification ¹	M1a	0
	M1b	2
	M1c	12
Performance Status ²	0	13
	1	1

^{*} Median age, 60; range 21 to 78

¹ American Joint Committee on Cancer

² Eastern Cooperative Oncology Group Performance Status

\$watermark-text

\$watermark-text

\$watermark-text

Treatment Summary

Table 2

Patient # n=14	Number of Treatment cycles	Dose Reductions (# required)	Clinical Response after cycle 2	Clinical Response after cycle 4	Duration of Best Response ¹
1	2	0	Progression		
2	2	0	Progression		
3	2	0	Progression		
4	2	0	Progression		
5	4	1	Stable Disease	Stable Disease	4 months
6	4	0	Stable Disease	Stable Disease	3 months
7	4	1	Stable Disease	Stable Disease	4 months
8	2	0	Progression		
9	2	0	Progression		
10	4	0	Stable Disease	Stable Disease	4 months
11	2	0	Progression		
12	2	0	Progression		
13	4	1	Partial Response	Partial Response	3.5 months
14	2	0	Progression		
Totals:	38 ²	3	Partial Response, n=1 Stable Disease, n=4 Progression, n=9	Partial Response, n=1 Stable Disease, n=4	

¹Duration of best response was measured in months from the time the response was detected after course 2 to the time of objective disease progression.

²Modified dose was given in 6 of the 38 treatment cycles.

Table 3

Toxicities and Laboratory changes observed during treatment

a. Toxicities Requiring Dose Reduction	
Patient # (n=3)	Toxicity
5 ¹	Hypotension refractory to brief IV fluids
7 ²	Grade 2 renal insufficiency with oliguria
13 ³	Hypotension refractory to brief IV fluids

b. Treatment Related Severe Laboratory Changes^{1,2}		
Toxicity	Grade 3 Toxicity³	Grade 4 Toxicity³
	N	N
Lymphopenia	4	9
Thrombocytopenia	3	0
PT/PTT	1 ⁴	0
Hypophosphatemia	5	0
Hyponatremia	1	0
Hypokalemia	1	0
Hypocalcemia	1	0
Hyperglycemia	2	0
Hypermagnesemia	1	0

¹ Cycle 4 day 3 therapy partially administered at 50% dose reduction. Infusion stopped after 15 minutes due to recurrent hypotension.

² Cycle 3 day 3 therapy held. Cycle 4 therapy administered at 50% dose reduction.

³ Cycle 2 day 2 therapy held. Cycle 2 day 3 and cycles 3 and 4 therapy administered at 50% dose reduction.

¹ All toxicities were reversible

² The majority of patients had transient grade 1 and 2 laboratory changes including neutropenia, anemia, thrombocytopenia, hyperbilirubinemia, elevated AST, hypophosphatemia, and hyperglycemia

³ Table provides the highest grade for each indicated toxicity

⁴ Unrelated to protocol therapy

\$watermark-text

\$watermark-text

\$watermark-text

Table 4

Laboratory Correlative Studies

a. Serum IC level by best response					
	Best response PD		Best Response SD or PR		
	N	Mean mcg/ml (SD)	N	Mean mcg/ml (SD)	p-value ¹
C1D1	9	1303.3 (447.6)	5	16316 (460.5)	0.217
C1D3	9	1130.7 (1003.6)	5	1371.8 (550.6)	0.632
C2D1	9	1399.1 (405.3)	5	1350.4 (480.9)	0.842
C2D3	9	1279.9 (820.0)	5	1399.8 (650.3)	0.786

b. Change in Absolute Lymphocyte Count (cells/mcl) from baseline to C1D3 or C1D4 by best response					
	Best response PD			Best Response SD, PR, or CR	
	N	Mean (SD)	p-value ¹	N	Mean (SD)
Baseline to C1D3	9	-1162 (598)	<0.001	4 ³	-1561 (455)
Baseline to C1D8	9	1851(964)	<0.001	4 ³	2362(1548)
					p-value ²
					0.006
					0.055
					0.476

c. Comparison of sIL2R levels between patients with DLTs vs no DLTs				
	DLT		No DLT	
	N	Mean pg/ml (SD)	N	Mean pg/ml(SD)
C1D4	3	6173(2286)	11	6595(2123)
C2D4	3	8098(2803)	11	7186(1884)
				p-value ¹
				1.0
				0.6

d. Change in CRP (mg/dL) from baseline to C1D3 or C1D4 by best response					
	Best response PD			Best Response SD or PR	
	N	Mean (SD)	p-value ¹	N	Mean (SD)
Baseline to C1D3	9	11.2(4.4)	<0.001	4	11.3(5.7)
Baseline to C1D4	3	10.3(3.1)	0.028	3	14.3 (6.0)
					p-value ²
					0.03
					0.054
					0.363

e. Comparison of absolute CRP levels (mg/dL) between patients with DLTs vs no DLTs					
	DLT		No DLT		
	N	Mean (SD)	N	Mean (SD)	p-value ¹
C1D4	2	19.5 (6.36)	5	10.6 (2.41)	0.081
C2D4	2	18.0 (1.41)	5	11.0 (2.45)	0.079

- /

non-parametric Wilcoxon Rank Sum test (two-tailed) between response groups
- /

paired t-test for evaluating change from baseline within groups
- 2

non-parametric Wilcoxon Rank Sum test (two-tailed) between response groups
- 3

4 pts, rather than 5 pts, in this group as one patient had sample obtained at incorrect time.
- /

non-parametric Wilcoxon Rank Sum test (two-tailed) between response groups
- /

paired t-test for evaluating changes from baseline within groups
- 2

non-parametric Wilcoxon Rank Sum test (two-tailed) between response groups
- /

non-parametric Wilcoxon Rank Sum test (two-tailed)