A Pilot Pharmacologic Biomarker Study in HLA-Haploidentical Hematopoietic Cell Transplant Recipients

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Abstract

PURPOSE—Eleven patients diagnosed with various hematologic malignancies receiving an HLA-haploidentical hematopoietic cell transplant (HCT) participated in an ancillary biomarker trial. The goal of the trial was to evaluate potential pharmacologic biomarkers pertinent to the conditioning regimen (fludarabine monophosphate (fludarabine) and cyclophosphamide (CY)) or postgrafting immunosuppression (CY and mycophenolate mofetil (MMF)) in these patients.

METHODS—We characterized the interpatient variability of nine pharmacologic biomarkers. The biomarkers evaluated were relevant to fludarabine (i.e., area under the curve (AUC) of 2-fluoro-ara-A or F-ara-A); CY (i.e., AUCs of CY and four of its metabolites); and MMF (i.e., total mycophenolic acid (MPA) AUC, unbound MPA AUC, and inosine monophosphate dehydrogenase (IMPDH) activity).

RESULTS—Interpatient variability in the pharmacologic biomarkers was high. Among those related to HCT conditioning, the interpatient variability ranged from 1.5-fold (CY AUC) to 4.0-fold (AUC of carboxyethlphosphoramide mustard, a metabolite of CY). Among biomarkers evaluated as part of postgrafting immunosuppression, the interpatient variability ranged from 1.7-fold (CY AUC) to 4.9-fold (IMPDH area under the effect curve). There was a moderate correlation (R^2=0.441) of within-patient 4-hydroxycyclophosphamide formation clearance.

CONCLUSIONS—Considerable interpatient variability exists in the pharmacokinetic and drug-specific biomarkers potentially relevant to clinical outcomes in HLA-haploidentical HCT recipients. Pharmacodynamic studies are warranted to optimize the conditioning regimen and postgrafting immunosuppression administered to HLA-haploidentical HCT recipients.

Keywords

fludarabine; cyclophosphamide; mycophenolate mofetil; IMPDH; hematopoietic cell transplant; biomarkers; pharmacokinetics

INTRODUCTION

While hematopoietic cell transplantation (HCT) offers the possibility of a lasting remission to patients with hematologic malignancies and disorders, its curative potential has

traditionally been limited to those patients with a closely matched donor. As many as a third of Caucasian patients in need of HCT are unable to find a suitably matched donor; patients from racial or ethnic minorities face even greater difficulties in finding an appropriate donor[1]. Additionally, unrelated donor searches are costly and time-consuming, with the risk that disease progression will outpace the search process[1]. Since an overwhelming majority of patients of all ethnic backgrounds have at least one readily available HLA-haploidentical donor, broadening the scope of HCT to include this type of donor grafts is clearly desirable. Unfortunately, initial attempts to expand HCT to patients with HLA-mismatched donors had historically been unsuccessful, characterized by increased risk of graft failure, severe graft-versus-host disease (GVHD), and significant non-relapse mortality[1].

The addition of post-transplant cyclophosphamide (CY) to postgrafting immunosuppression, used in conjunction with a calcineurin inhibitor and mycophenolate mofetil (MMF), appears to have overcome these barriers. The combination of reduced-intensity conditioning, an HLA-haploidentical donor graft, and postgrafting immunosuppression that includes CY has led to acceptable rates of GVHD and improved survival[2]. It is desirable, however, to further lower rates of graft rejection, grades II-IV GVHD (currently 2% and 32%, respectively) and relapse [3]. We hypothesize the interpatient variability in the outcomes of an HLA-haploidentical HCT is associated with pharmacokinetic and drug-specific pharmacodynamic variability. Thus, we sought to characterize the interpatient variability in the pharmacokinetics and drug-specific pharmacodynamics of the various medications administered as part of the conditioning regimen (CY and fludarabine) and postgrafting immunosuppression (CY and MMF), as shown in Figure 1. Since the pharmacodynamics of these drugs differ between HCT conditioning regimens[4] and graft sources[5, 6], we sought to characterize the interpatient variability of nine biomarkers previously associated with clinical outcomes in HCT patients to determine the feasibility of pharmacodynamic studies in larger patient populations.

METHODS

Patient Selection

Eleven patients were enrolled onto this ancillary biomarker study between June 2010 and November 2011; participation did not influence the HCT procedure. Participants were eligible for this study if they were 18 years of age or older and receiving conditioning that included fludarabine, an HLA-haploidentical graft, and postgrafting immunosuppression that included post-transplant CY and MMF. Patients were ineligible if their hemoglobin was less than 8g/dL, if they could not speak or read English, if they had been diagnosed with an immunodeficiency disorder (including HIV), if they were pregnant or breastfeeding, if their life expectancy was severely limited by diseases other than malignancy, or if their conditioning regimen or post-graft immunosuppression included a radiolabeled monoclonal antibody. Among the 22 patients receiving an HLA-haploidentical donor HCT, 16 were eligible to participate in this ancillary biomarker study. Among eligible patients, eleven (69%) agreed to participate and eight participants (73%) complied with all the blood sampling (Figure 1). This ancillary study was registered on clinicaltrials.gov with identifier NCT01141959; patients' treatment protocols were registered with identifiers NCT01028716, NCT01008462, and NCT00789776. This research was conducted with the approval of the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board, and written consent was obtained from all participants prior to study conduct.

All participants were adults receiving HCT from HLA-haploidentical donors to treat high-risk hematological malignancies; the majority of participants were enrolled on NCT01028716. The stem cell source was bone marrow for three participants (27%) and

peripheral blood stem cells (PBSC) for the remaining eight participants. One participant was enrolled on a phase I/II protocol and received an additional infusion of donor natural killer cells one week after the initial stem cell infusion in a trial to boost the graft versus tumor effect and to enhance early immune reconstitution after HCT[7].

Method of HCT

All participants received the same reduced-intensity conditioning regimen prior to infusion of their HLA-haploidentical donor graft. Conditioning consisted of the following: 1) CY, 14.5mg/kg per day, administered on days −6 and −5 (29 mg/kg total); 2) fludarabine monophosphate (fludarabine), $30mg/m^2$ per day, administered on days −6 through −2 (150 mg/m^2 total); 3) one 200cGy fraction of total body irradiation (TBI) administered on day −1. Intravenous (IV) CY was dosed according to adjusted ideal body weight (AIBW), where AIBW = ideal weight + 0.25(actual weight - ideal weight). Ideal weight was calculated as follows: for men, ideal weight = 50kg + (2.3kg/inch of height over 5ft); for women, ideal weight = 45.5kg + (2.3kg/inch of height over 5ft). Fludarabine was dosed by body surface area (BSA), which was calculated using actual weight. CY was administered over 60 minutes (min); fludarabine was administered over 30 or 60 min, according to treatment protocol. Likewise, the order of administration of CY and fludarabine varied depending on the treatment protocol.

Post-grafting immunosuppression for all patients consisted of CY, MMF and tacrolimus. All patients were administered CY 50 mg/kg per day as a 60 min infusion. Three patients received one dose of post-transplant CY on day +3 (50 mg/kg total) and the remaining eight patients received two doses of post-transplant CY on days +3 and +4 (100 mg/kg total). The addition of the second dose of post-transplant CY has been shown to reduce chronic, but not acute, GVHD.[2] MMF and tacrolimus were initiated the day after the final post-transplant CY dose. MMF was given orally every 8 hour (hr) at a dose of 15 mg/kg based on AIBW and rounded to the nearest 250mg increment. In patients who could not tolerate oral MMF, the IV equivalent was given instead at a one-to-one substitution. All patients began tacrolimus therapy at a dose of 1 mg IV daily or 1 mg PO every 12 hr. Tacrolimus doses were adjusted to reach target trough concentrations of 5–15 ng/mL, based on patients' treatment protocols. All patients also received daily G-CSF, beginning one day after the last dose of post-transplant CY, until the third consecutive day with an absolute neutrophil count greater than 0.5×10^9/L. One patient received an infusion of donor natural killer cells on day +7, as per treatment protocol (NCT00789776).

Supportive care, including antiemetics, antifungals, and antibiotics, was similar among all patients and followed FHCRC standard practice guidelines; three patients received corticosteroids during conditioning. On the days that pretransplant CY was administered, nine of eleven patients received 2-mercaptoethane sulfonate (mesna) for uroepithelial prophylaxis at a dose equivalent to that of CY. With post-transplant CY, all patients received an equivalent dose of mesna. All patients received antimicrobial prophylaxis against *Pneumocystis jiroveci*, *Candida albicans*, and herpes viruses.

Patients were monitored at least weekly for cytomegalovirus reactivation and treated pre-emptively with ganciclovir or foscarnet as needed. Patients were considered to have attained neutrophil engraftment on the first of three consecutive days with an absolute neutrophil count 0.5×10^9/L. Platelet engraftment was defined as the first of seven days, in the absence of transfusion, with a platelet count 20×10^6/L. GVHD was diagnosed according to established criteria[5, 8]. Relapse was assessed according to treatment protocols or as clinically necessary.

HCT conditioning biomarkers

2-fluoro-ara-A (F-ara-A) pharmacokinetics were evaluated as follows: on day −6, three blood samples were collected to evaluate the area under the concentration-time curve (AUC) of F-ara-A. On days −5 through −2, one blood sample was collected immediately before the start of each fludarabine infusion. On day −1, one blood sample was collected 24hr after the start of the final dose of fludarabine. The samples used to determine the AUC of F-ara-A were collected immediately at the end of the infusion, five minutes after the end of the infusion, 90 min after the start of the infusion, and 24 hr after the start of the infusion[9]. Our earlier experience from a similar ancillary biomarker study (clinicaltrials.gov #NCT00764829) was that this shorter sample schedule improved patient participation due to its shorter duration than the optimal schedule (i.e., 0.583, 1.5, 6.5, and 24 hr). The shorter sample schedule had an acceptable scaled mean squared error of 13.5 compared to 12.3 for the optimal schedule.[9] F-ara-A concentrations were quantified as previously described[9], with the following modifications: 100μL plasma was combined with 50μL methanol, 20μL clofarabine (internal standard), and 100μL acetonitrile. The mixture was vortexed and centrifuged. The supernatant was analyzed using high-performance liquid chromatography/ tandem mass spectroscopy (LC/MS/MS) running a gradient mobile phase of 0.1% formic acid and methanol through an Agilent Zorbax SB-C18 column (Agilent, Santa Clara, CA), monitoring m/z 304 (internal standard) and 286 (F-Ara-A). The assay's dynamic range was 0.1 to 5.0μg/mL, and the interday precision was less than 10%. The AUCs were calculated using post-hoc Bayesian estimates from NONMEM version 7.2 (Icon Development Solutions, Ellicott City, MD). We did not assess the accumulation rate of the active metabolite fludarabine triphosphate (F-ara-ATP) in $CD4^+$ and $CD8^+$ T-lymphocytes due to concerns regarding participant safety[10]. Specifically, the assessed biomarkers required 165 to 230mL of blood drawn over four weeks. Considering that F-ara-ATP requires an additional 60 mL blood draw, we chose not to assess F-ara-ATP after extensive discussions with the IRB.

Cyclophosphamide pharmacokinetics were obtained with each CY dose. For patients treated in the outpatient setting, samples were collected at 1(end of infusion), 2, 4, 8, and 24hr from the start of the infusion. For patients who had been admitted for inpatient care, samples were collected at 1 (end of infusion), 2, 4, 8, 16, 20, and 24 hr from the start of the infusion[11]. When applicable, the 24hr samples were collected before the start of the following dose. Samples were placed at 4 C within five minutes of collection and were processed within 18 hr. Concentrations of CY,4-hydroxycyclophosphamide (4HCY), carboxyethylphosphoramide mustard (CEPM), deschloroethylcyclophosphamide (DCCY), and 4-oxocyclophosphamide (KetoCY) were measured as previously reported[12], except using LC/MS/MS. The dynamic range of the assays of CY and its metabolites were as follows: CY, 0.96 to 192μM; 4HCY, 0.125 to 25μM; CEPM 0.043 to 17μM; DCCY 0.063 to 25μM; KetoCY 0.022 to 9.1μM. All assays had an interday precision less than 10%. The AUCs were estimated using noncompartmental modeling with SAS (SAS, Version 9.2, Cary, NC)[13, 14].

Fludarabine and cyclophosphamide pharmacodynamics were assessed by two blood samples, collected before and after conditioning administration to evaluate circulating $CD4^+$ and $CD8^+$ T-cell counts. The first sample was collected on day −6 before administration of either CY or fludarabine. The second was collected on day −1, 24hr after the start of the final dose of fludarabine and before TBI. Samples were assayed by a single-platform method using BD Trucount tubes (BD Biosciences, San Jose, CA) and Beckman Coulter Cytostat Tetra One monoclonal antibody reagent with a Coulter FC-500 flow cytometer running Beckman Coulter CXP software (all Beckman Coulter, Brea, CA). CD45/SS gating was used to identify lymphocytes and distinguish $CD4^+$ and $CD8^+$ T-cell populations in peripheral blood.

Post-transplant immunosuppression biomarkers

Cyclophosphamide pharmacokinetics were evaluated with each post-transplant CY dose, using the methods described above in the conditioning regimen biomarker section.

Two MMF biomarkers were evaluated, specifically MPA pharmacokinetics and a MMF-specific pharmacodynamic marker of IMPDH activity. The pharmacokinetics of MPA were measured on days +7 and +21. On each day, five blood samples were collected for measurement of the total and free MPA AUCs. For patients on oral MMF, samples were collected immediately before the dose and 1.25, 2, 3, and 4 hr after the dose[15]. For patients on IV MMF, samples were collected at 2 (end of infusion), 2.5, 3, 4, and 6 hr from the start of the two-hour infusion[16]. Total plasma MPA was measured by LC/MS as previously described[5]. Free MPA was determined by equilibrium dialysis using the Pierce RED (Rapid Equilibrium Dialysis) Device (Thermo Fisher Scientific, Waltham, MA). AUCs were estimated using noncompartmental modeling with SAS (SAS, Version 9.2, Cary, NC).

IMPDH activity in peripheral blood mononuclear cells was assessed on two occasions. Pre-transplant baseline (i.e., before MMF administration) IMPDH activity was determined with a sample collected on day −6, concurrent with the first F-ara-A pharmacokinetic blood draw. Post-transplant IMPDH activity was measured on day +21; samples were collected at the same times as the MPA pharmacokinetic draws, above.

Blood samples were stored at 4 °C until processing. Peripheral blood mononuclear cells (PMNC) were isolated within 6 hr of collection. PMNC were isolated by diluting blood in PBS at a 1:1 v:v ratio, then layering the mixture on Ficoll. The height ratio of Ficoll to diluted whole blood sample was 3:4. This suspension was then centrifuged at 298 g for 30 min at 22 °C. PMNC were collected from the interface and diluted with PBS to a volume of 10 mL to wash, then centrifuged at 405 g for 15 min at 22 °C. To facilitate cell counting and limit the variability in cell concentration, all but 1.1 mL of the supernatant was removed. The PMNC pellet was resuspended in the remaining PBS, and 1.0 mL of the PBS-cell slurry was transferred to a 2 mL tube. White blood cell counts in this sample were quantitated using a Horiba Diagnostics ABX Micro 60 (requires <10 μL) (Irving, CA). Following cell counting, the sample was centrifuged at 325 g for 10 min at room temperature. From the microcentrifuge tube, 920 μL of the supernatant was removed; distilled water was added to adjust the cell concentration to 0.5×10^7 cells/mL lysate. The cells were subsequently stored at −80°C until incubation. After thawing, insoluble fragments of disrupted cells were removed by centrifugation at 16000 g for 2 min. The supernatant was used for IMPDH activity assay.

IMPDH activity in lysed lymphocytes was determined from the conversion of inosine-monophosphate (IMP) to xanthosine 5 -monophosphate (XMP) according to a procedure adapted from Glander *et al.*[17] and Daxecker *et al*[18]. For each incubation a fresh reaction mixture was prepared from stock solutions: 1.6 mL NaH_2PO_4 (120 mmol/L, stored at 4 °C), 1.6 mL KCl (300 mmol/L, stored at 4 °C), 0.8 mL IMP (6.0 mmol/L, stored at −20 °C), 0.8 mL NAD (4.5 mmol/L, made fresh each day); the pH was adjusted to 7.4 using 1M NaOH, and the total volume brought to 5.2 mL with deionized water. From this reaction mixture 130 μL was used. The incubation was carried out in a 37 °C shaking water bath. The reaction was started via the addition of 130 μL of reaction mixture to the 50 μL pre warmed (5 min) cell lysate (standardized concentration of 0.5×10^7 cells/mL). After 2.5 hr, the enzymatic reaction was terminated by the addition of 1250 μL methanol. Internal standard (20 μL of approximately 130 pmol/μL 8-Bromo adenosine 5 -monphosphate (BMP) in deionized water) was then added to each incubate via repeating syringe, followed by centrifugation at 16000 g for 10 min. The supernatant was then transferred to 12 × 75

disposable culture tubes and evaporated to dryness under a stream of air at 37°C. The residue was dissolved in 75μL of deionized water and 5μL were injected on the HPLC-MS.

The HPLC separation was performed on an Agilent HPLC/MS series 1100 system equipped with a thermostated autosampler (Agilent Technologies, Palo Alto, CA). Agilent ChemStation (version B.01.03) was used for instrument control. Separation was achieved using a Thermo Scientific Hypercarb column (2.0mm × 100mm × 5μ, part no. 35005-102130, Thermo Scientific, Bellefonte, PA). The mobile phase consisted of acetonitrile and 0.1 M ammonium acetate adjusted to pH 8.5 with ammonium hydroxide. A gradient system was used starting at 5% acetonitrile for 0.5 min, increasing to 30% at 4 min, held at 30% until 5 min, then returning to 5% at 5.1 min. The total run time was 10 min. The injector was maintained at 4 °C. The injection volume was 5 μL. The column thermostat was set to 30.0 °C and the solvent flow was maintained at 0.3 mL/min.

An Agilent G1946D MSD (Agilent Technologies, Palo Alto, CA) API-ES in positive ion mode was used. The temperature of the drying gas (N_2) was maintained at 350 °C at a flow of 11 L/min. The nebulizing pressure was 35 psi; the capillary voltage was 2400 V, and the fragmentor voltage was 100 V. The MSD was run in the SIM mode. Monitored ions included m/z 365 for the (M+H$^+$) ion of xanthosine monophosphate, m/z 348 for the (M+H$^+$) ion of adenosine 5 -monophosphate, and m/z 426 for the (M+H$^+$) ion of BMP, the internal standard. The MSD conditions for quantification were as described above; the fragmentor and capillary voltage were optimized under analytical conditions with Chemstation FIA software. During analysis, eluent before 3 min and after 7.5 min was diverted to waste. Typical retention times were 4.2 min for IMP, 5.5 min for XMP, and 6.9 min for BMP (Supplemental Figure 1). The area under the effect curves (AUECs) on day 21 were estimated using noncompartmental modeling with SAS (SAS, Version 9.2, Cary, NC).

RESULTS

Patient Population

Eleven patients were enrolled, of whom six were female and five were male. The median age of participants was 50.8 years (range 21.8 – 68.4). Five patients were treated for Non-Hodgkin lymphoma, three patients for Hodgkin lymphoma, and three patients for myelodysplastic syndrome (MDS) and/or acute myelogenous leukemia (AML). All patients had HLA-haploidentical related donors - six donors were patients' children, three were parents, and two were siblings. The median number of graft-versus-host mismatches was five (range: 3–5); the median number of host-versus-graft mismatches was also five (range: 2–5). Patient characteristics are summarized in Table 1.

Clinical Outcomes

All patients attained neutrophil engraftment. The median (range) day of neutrophil engraftment, defined as the first of three consecutive days with an absolute neutrophil count 0.5×10^9/L, was day +15 (11 – 20). Nine participants attained platelet engraftment at a median (range) of day +15 (12 – 40) days post-transplant; platelet engraftment was defined as the first of seven consecutive days with a platelet count 20×10^6/L without transfusion support. Eight patients (73%) developed acute GVHD at a median (range) of 29 (15 – 97) days post-transplant. All patients had grade II GVHD (i.e., no grade 1, 3 or 4 GVHD was observed) and were treated with corticosteroids, as well as continuation of tacrolimus and MMF. Of the nine participants who were cytomegalovirus (CMV) positive prior to HCT, eight (89%) participants reactivated CMV. Six participants are currently alive, with a median follow-up of 19 months (range 14–29) post-transplant, one of whom relapsed on day + 534 and is in CR after undergoing additional treatment Two participants died of relapse

(days +46 and +85). Three participants died of non-relapse mortality (Table 3): one of pneumonia (day +126), one of multi-organ failure with GVHD (day +259), and one of acute respiratory distress syndrome (day +265); all three were on immunosuppression at the time of death.

Pharmacokinetic and pharmacodynamic biomarkers

Considerable interpatient variability was seen in both pre- and post-transplant biomarkers, as listed in Tables 2 and 3. The majority of participants had a substantive decline in their circulating $CD4^+$ or $CD8^+$ cells with 10 of the 11 participants having a greater than 90% decline (with 100% decline meaning no circulating $CD4^+$ or $CD8^+$ cells found). The percent decline varied 3.3-fold and 1.1-fold, respectively. The majority of patients experienced an increase in the ratio of $CD4^+$ to $CD8^+$ cells after conditioning: the pre-transplant median (range) $CD4^+/CD8^+$ ratio was 0.6 (0.12 – 2.5, N=11), while the post-transplant median (range) was 1.5 (0 – 6.2, N=10). Fludarabine AUC_{0-24hr} varied by a factor of 2.1 (maximum/minimum). Among F-ara-A trough (pre-dose) concentrations, the median within-patient coefficient of variation (the standard deviation divided by the average) was 16% (range 9–36%) and the within-patient variability was 1.5-fold (range 1.2- to 2.6-fold).

The AUCs of CY and its metabolites varied widely both before and after transplant. During conditioning, CY AUC_{0-48hr} varied 1.6-fold, while the AUCs of CY's metabolites were more widely distributed with a 3.1-fold range for 4HCY AUCs, a 4.0-fold range for CEPM AUCs, a 3.9-fold range for DCCY AUCs, and a 2.6-fold range for KetoCY AUCs. Similar variation was seen in post-transplant CY. Among patients receiving two doses of post-transplant CY, CY AUC_{0-48hr} had a 1.7-fold variation. In this setting, the metabolites of CY varied as follows: 2.0-fold for 4HCY, 3.2-fold for CEPM, 3.0-fold for DCCY, and 2.1-fold for KetoCY. Both pre- and post-transplant formation clearances of 4HCY and DCCY (the AUC of the metabolite divided by the AUC of the parent compound) showed great variation. The pre-transplant formation clearance for 4HCY varied 3.8-fold, while the DCCY formation clearance varied 5.4-fold. Post-transplant, there was 5.3-fold variability in the formation clearance of 4HCY and 4.1-fold variability for DCCY. Notably, the formation clearance of 4HCY was similar in six of the 11 participants after the lower (14.5 mg/kg), pre-transplant dose to that after the higher (50 mg/kg), post-transplant CY dose (Figure 2). There was a moderate correlation (R^2=0.441) of within-patient 4HCY formation clearances. As expected, the maximum plasma concentrations (C_{max}) increased from the first dose of pre-transplant CY (14.5 mg/kg) to the first dose of post-transplant CY (50 mg/kg). The median (range) C_{max} for pre-transplant CY and 4HCY, respectively, were 91μM (75–113) and 1.4μM (1.0 – 6.2). Post-transplant, the median (range) C_{max} for CY and 4HCY were 304μM (262 – 375) and 9.7μM (3.6 – 18.8), respectively. Three of the 11 participants declined the day +21 blood draws, which were used to estimate the MPA AUC and IMPDH activity. The AUC_{0-8hr} of total MPA varied widely but consistently; there was a 3.7-fold difference on both days +7 and +21. Free MPA AUC_{0-8hr} was similarly variable, showing 3.0-fold variation on day +7 and 2.8-fold variation on day +21. The variability in pre-and post-transplant IMPDH activity was, however, much greater. Before transplant, there was a 3.0-fold difference in the recipients' baseline (i.e., before MMF) IMPDH activity (Table 2). After transplant, there was 4.9-fold variation in the IMPDH AUEC (Table 3).

DISCUSSION

The key findings from this pilot study are: 1) ancillary biomarker studies seeking to characterize the pharmacokinetics and drug-specific pharmacodynamics of several medications used for HLA-haploidentical HCT are feasible; 2) there is substantial interpatient variability in these potential biomarkers with conventional body weight or BSA-based dosing. The majority (69%) of eligible patients agreed to participate, and most

participants (79%) completed all the blood sampling. Despite these encouraging participation rates, the study population is insufficient to evaluate whether potential drug-specific biomarkers are associated with clinical outcomes. As shown in Table 4, an adequately powered study would need 25–50 participants, which could be easily achieved through multi-center studies, to evaluate the pharmacodynamics in HLA-haploidentical HCT recipients. Such prospective studies are needed to determine whether personalized dosing of the conditioning regimen or post-grafting immunosuppression can improve survival. A complete biomarker analysis from 50 participants, would require 100 patients, assuming rates of participation (69%) and blood sampling compliance (Figure 1, 73%) similar to what we observed. Acute GVHD occurred in most (72%) of the patients, including the three participants who died of non-relapse mortality by day 265 post-HCT. Each of these three participants was taking immunosuppressants at the time of death. The wide interpatient variability in the potential biomarkers, combined with the prevalence of acute GVHD and non-relapse mortality raises the question of whether further biomarker studies could identify ways to personalize the HCT procedure to improve patient outcomes. Biomarkers, once validated, hold great promise to guide treatment selection (e.g., gene expression profiling for breast cancer)[19] or personalization of chemotherapy dosing (e.g., kinetics-based busulfan dosing[4] or genetics-based dosing of mercaptopurine)[19, 20]. However, the potential need for large sample sizes to draw definitive conclusions about infrequent toxicities[21] is a challenge for biomarker studies in patients with cancer. We sought to characterize multiple biomarkers for two reasons. First, often a single biomarker does not sufficiently explain the clinical outcomes in a patient population (e.g., estrogen receptor, HER-2 are used in breast cancer treatment[19, 21]). Second, quantitative and systems pharmacology (QSP), an evolving discipline which seeks to combine experimental research with computational modeling, presents a possible avenue to more quickly identify the optimal conditioning regimen and/or postgrafting immunosuppression in HLA-haploidentical HCT recipients. QSP studies are designed to advance the discovery, development, and clinical use of therapeutic drugs. As the use of alternative graft sources continues to increase, multi-center studies should take a QSP approach to evaluate various biomarkers and their clinical relevance. In this setting, QSP studies are necessary: their novel biochemical, pathway-oriented, and physiological approaches complement substantive investments in genomics, with the goal of understanding drug-target interactions and pathophysiology[22].

Multiple biomarkers related to fludarabine and CY in the HCT conditioning regimen were evaluated. Because of its ability to decrease lymphocyte counts, fludarabine has become an essential part of many reduced-intensity HCT conditioning regimens. After administration, nucleotidases rapidly dephosphorylate fludarabine to F-ara-A[23] and subsequently form F-ara-ATP intracellularly. The F-ara-A AUCs from this study are consistent with those from patients conditioned with fludarabine (30–40 mg/m^2) and 50 mg/kg cyclophosphamide (median AUC 5.0μg×h/mL, range 2.0 to 11.0) [24]. Intracellular accumulation of F-ara-ATP is similar between CD4$^+$ and CD8$^+$ isolated from HCT recipients[10]. Notably, we observed similar decreases in circulating CD4$^+$ and CD8$^+$ cells (Table 2). Fludarabine administration leads to a marked prolonged reduction in circulating CD4$^+$ and CD8$^+$ cells, but the immediate effects of its administration upon these cells has yet to be described[25]. *In vitro* data regarding the effects of fludarabine upon the proportion of CD4$^+$ vs. CD8$^+$ cells undergoing apoptosis are contradictory and suggest that CD4$^+$ cells are similarly[26] or less susceptible[27] to fludarabine than CD8$^+$ cells. The immediate effects of fludarabine upon recipients' circulating CD4$^+$ and CD8$^+$ cell counts may influence the ratio of CD4$^+$ to CD8$^+$ cells which affects their sensitivity to TBI *in vitro*[28]. In turn, recipient T-cell suppression may be a critical determinant of engraftment[29] and long-term immune function[30]. As shown in Table 2, there was considerable variability in the decline in circulating CD4$^+$ cell counts but less variability in CD8$^+$ cell counts.

With fludarabine and TBI, CY is also an integral component of this reduced-intensity conditioning regimen. To our knowledge, we are the first to evaluate the pharmacokinetics of post-transplant CY. CY is a prodrug with multiple metabolites, of which 4HCY formation is critical because it is transported intracellularly and subsequently broken down to phosphoramide mustard, which covalently cross-links DNA. The growth of bone marrow progenitor[31, 32], CFU-GM[33], natural killer[34], and T-[35, 34] cells are suppressed *ex-vivo* by 4HCY in a concentration-dependent manner. The current method of dosing CY by body weight leads to considerable interpatient variability in the AUCs of CY, 4HCY, and additional metabolites (Tables 2 and 3), which is consistent with our data in patients receiving CY 120 mg/kg administered with TBI (CY/TBI) or targeted busulfan (^{T}BU/CY) [4]. Variability in the exposure to CY and/or its metabolites may account for interpatient differences in the efficacy and toxicity of post-transplant CY. The AUCs of CY and/or its metabolites are biomarkers for the efficacy and toxicity of the CY/TBI conditioning regimen[11]. However, the concentration-effect relationships of CY vary between different conditioning regimens, as the AUCs of CY and its metabolites are not associated with clinical outcomes in patients conditioned with the ^{T}BU/CY regimen[4]. Similarly, pharmacodynamics of MPA differ based on graft source when MMF is a component of post-grafting immunosuppression[5, 6]. The pharmacokinetics and pharmacodynamics of CY have yet to be characterized in patients receiving HLA-haploidentical HCT. The use of post-transplant CY has lead to acceptable outcomes after receipt of an HLA-haploidentical donor graft[2, 3]. Thus, a pharmacokinetic study in a homogenous patient group receiving post transplant CY is necessary to determine the clinical relevance of the pharmacokinetic variability of CY and its metabolites. Unfortunately, the number of haploidentical HCT performed at our single center, despite our encouraging accrual rate (69%), did not provide a large enough sample to conduct such a pharmacodynamic analysis. To our knowledge, this study is novel as it is the first to characterize the pharmacokinetic variability of post-transplant CY.

Because CY was administered on two separate occasions, we could characterize the within-patient variability of 4HCY formation clearance, which is the 4HCY AUC divided by the CY AUC. As shown in Figure 2, six of the 11 participants had a consistent 4HCY formation clearance, which suggests that a test dose of CY may be possible to determine individual-specific 4HCY AUC. One participant, however, had a lower 4HCY formation clearance after the pre-transplant CY dose. Furthermore, four participants had a greater 4HCY formation clearance after the lower (14.5 mg/kg) pre-transplant CY dose compared to the higher (50 mg/kg) post-transplant CY dose. The latter group qualitatively agrees with finding in 16 breast cancer patients that the extent of bioactivation is greater with lower dose CY (500 mg/m^{2}, equivalent to 12.5 mg/kg) compared to myeloablative dose CY (100 mg/kg)[36, 37]. No drug interactions were identified that could have affected the 4HCY formation clearance in this latter group (Supplemental Table 1). Of note, although CY autoinduces 4HCY formation[38], this is not expected to influence the between-dose comparison for two reasons. First, eight days elapsed between the last pre-transplant CY dose and the first post-transplant CY dose. Since the usual half-life of CY is 3–4 hr, there should be no residual CY at the time of post-transplant CY administration. Second, if autoinduction was a factor, 4HCY formation clearance would be expected to be higher with post-transplant CY which only occurred in one of the 11 participants (Figure 2). Notably, although concurrent medications did change, few CY-drug interactions were possible (Supplemental Table 1).

MMF is another component of postgrafting immunosuppression. MPA, MMF's active metabolite, is a selective, reversible, and noncompetitive inhibitor of IMPDH, which is involved in the *de novo* pathway of purine synthesis in T- and B-lymphocytes[39, 40]. Other investigators have found associations between mycophenolic acid AUC or predose

concentrations and clinical outcomes in patients receiving fludarabine/CY conditioning regimens and umbilical cord blood grafts[6]. To our knowledge, no one has evaluated MPA pharmacodynamics in HLA-haploidentical HCT recipients. We sought to characterize IMPDH activity, based on recent data from renal transplant patients reporting that high recipient IMPDH activity is associated with a higher risk of graft rejection[41]. Additional data from renal transplant patients shows considerable variability in IMPDH inhibition at a fixed MPA concentration. Of note, IMPDH had the greatest interpatient variability in this pilot study and would be an obvious candidate biomarker for future studies.

In summary, this pilot study demonstrates the feasibility of evaluating multiple biomarkers in HCT recipients treated in the ambulatory clinic. Adequately powered (Table 4) multi-center studies should be conducted to evaluate the clinical relevance of these biomarkers in HLA-haploidentical HCT recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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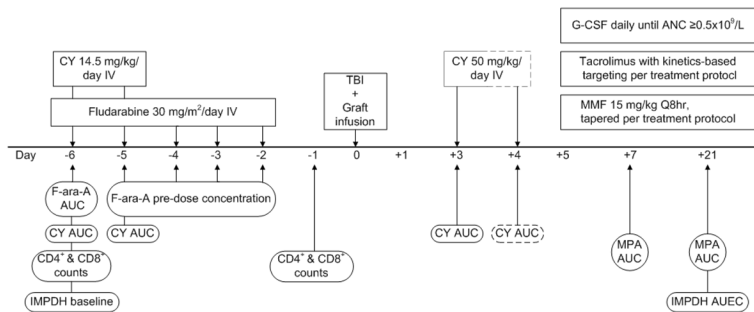
Figure 1.
Schema of pharmacologically-based biomarkers. Abbreviations: AUC: area under the curve; AUEC: area under the effect curve; CY: cyclophosphamide, includes CY and four of its metabolites; fludarabine: fludarabine monophosphate; IMPDH: inosine monophosphate dehydrogenase; MMF: mycophenolate mofetil; MPA: mycophenolic acid, includes both total and unbound MPA; TBI: total body irradiation

Figure 2.
Comparison of 4HCY formation clearances (4HCY AUC/CY AUC) from first doses of CY before and after transplant.

Table 1

Patient characteristics[a]

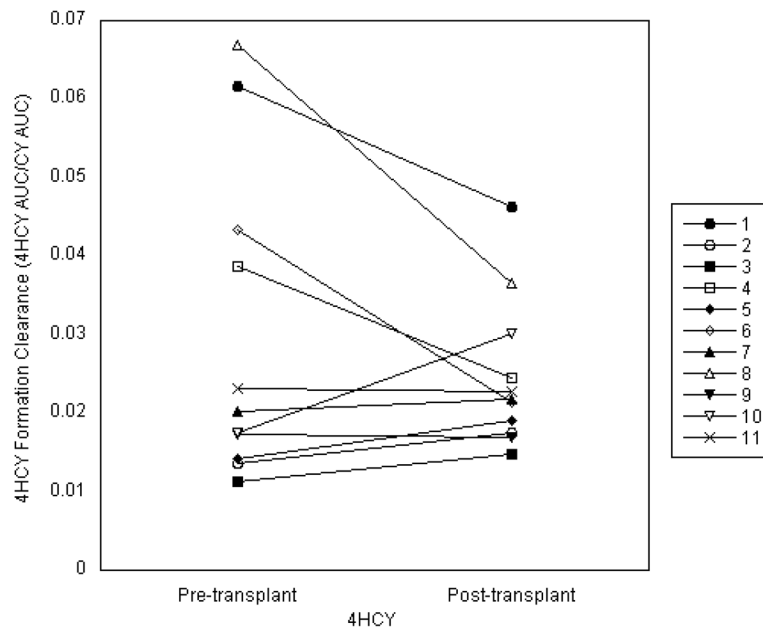|	N[b] (%)
Age (yr)	50 (21 – 68)
Male sex	5 (45%)
AIBW (kg)	64.6 (51.1 – 82.8)
Donor relationship	
Mother	2 (18%)
Father	1 (9%)
Brother	2 (18%)
Daughter	3 (27%)
Son	3 (27%)
Disease	
Non-Hodgkin lymphoma	5 (45%)
Hodgkin lymphoma	3 (27%)
Acute myeloid leukemia/myelodysplastic syndrome (MDS)	3 (27%)
Cytomegalovirus serostatus	
Recipient +/Donor −	5 (45%)
Recipient +/Donor +	4 (36%)
Recipient −/Donor −	1 (9%)
Recipient −/Donor +	1 (9%)
Graft source	
Peripheral blood stem cells	8 (73%)
Bone marrow	3 (27%)
Post-transplant CY[c]	
One dose (Day +3)	3
Two doses (Days +3 and +4)	8

[a] All patients received the same conditioning regimen prior to HCT;

[b] data are presented as median (range) or number (%);

[c] all patients received tacrolimus and MMF. One patient's second dose of post-transplant CY was delayed by 24hr because the patient was not clinically stable.

Table 2

Pre-transplant pharmacologic biomarkers

Patient #	Cyclophosphamide AUC$_{0-48hr}$ (μM×hr)					F-ara-A AUC (μM×hr)	% Decline in Circulating T-cells		Recipient IMPDH (pmol×10⁶ cells/hr)
	CY	4HCY	CEPM	DCCY	KetoCY		CD4⁺ cells	CD8⁺ cells	
1	1303	84.8	155	57.8	83.5	17.7	97	100	649
2	1623	34.0	140	134	53.1	17.7	--[a]	--[a]	588
3	1590	28.0	59.1	123	31.6	13.2	97	96	660
4	1541	51.6	65.2	83.4	33.5	15.5	94	100	858
5	1594	27.1	98.7	52.0	35.9	16.4	93	98	1118
6	1190	43.0	51.2	149	44.1	11.8	100	100	983
7	1447	40.0	86.7	51.0	45.1	12.8	96	95	1260
8	1125	64.7	116	199	58.8	18.6	96	100	1214
9	1451	34.8	124	115	53.1	21.5	100	100	424
10	1637	35.0	122	167	57.3	18.8	100	100	1140
11	1055	31.2	38.5	156	--[b]	10.4	30	88	820
Median (range)	1451 (1055 – 1637)	35.0 (27.1 – 84.8)	98.7 (38.5 – 155)	123 (51.0 – 199)	49.1 (31.6 – 83.5)	16.4 (10.4 – 21.5)	96 (30 – 100)	100 (88 – 100)	858 (424 – 1260)
Fold range	1.6	3.1	4.0	3.9	2.6	2.1	3.3	1.1	3.0

[a] Post-transplant circulating T-cell counts not collected for this patient;

[b] KetoCY AUC not evaluable in this patient.

Table 3

Post-transplant pharmacologic biomarkers and clinical outcomes

Patient	Cyclophosphamide (μM×hr)					Total MPA AUC$_{0-8hr}$ (μg/mL×hr)		Free MPA AUC$_{0-8hr}$ (ng/mL×hr)		IMPDH AUEC (pmol × 10^6 cells)	Outcome (days)	Length of follow-up (months)
	CY	4HCY	CEPM	DCCY	KetoCY	Day +7	Day +21	Day +7	Day +21			
One CY dose (day +3); AUC$_{0-24hr}$												
3	2760	41.0	80.5	223	56.0	20.23	12.74	327	161	5676	Deceased; relapse (85)	
4	2663	65.2	133	215	69.8	35.35	25.18	469	305	4858	Relapsed (534), now in CR	25
10	1932	58.3	142	227	79.2	12.90	9.25	170	141	4039	Deceased; relapse (46)	
Two CY doses (days +3 and +4); AUC$_{0-48hr}$												
1	3839	228	520	297	184	21.60	15.55	237	208	1532		29
2	4796	174	489	508	158	9.83	8.51	158	145	5781	Deceased; ARDS[a], renal failure; on ISP[b] (265)	
5	4295	116	321	211	89.0	23.18	15.25	432	235	1171	Deceased; pneumonia; on ISP[b] (126)	
6	3812	121	160	555	95.5	12.09	--[c]	154	--	--		23
7	3685	134	228	258	122	9.46	11.46	192	179	4377	Deceased; MOF with GVHD; on ISP[b,d] (259)	
8	3123	120	269	635	111	12.20	--[c]	238	--	--		16
9	3425	35.7[e]	408	283	132	10.50	6.77	181	108	1930		15
11	2754	150	161	423	169	10.44	--[c]	196	--	--		14
Median (range)[f]	3748 (2754–4796)	134 (116–228)	295 (160–520)	360 (211–635)	127 (89.0–184)	12.20 (9.46–35.35)	12.10 (6.77–25.18)	196 (154–469)	170 (108–305)	4208 (1171–5781)		
Fold range[f]	1.7	2.0	3.2	3.0	2.1	3.7	3.7	3.0	2.8	4.9		

[a] Acute respiratory distress syndrome;

[b] Immunosuppression; pt was on immunosuppressants at time of death;

[c] Patients 6, 8, and 11 declined to participate in the second of the two MMF pharmacokinetic sampling days;

[d] Multi-organ failure with graft versus host disease;

[e] 4HCY pharmacokinetics only collected with first dose of CY; due to delay in second dose, 4HCY derivatizing agent was no longer viable at time of sample collection;

[f] Median and fold range values include only those patients receiving two doses of post-transplant CY.

Table 4

Power calculations showing feasibility of adequate participant accrual to conduct a meaningful pharmacodynamic analysis in HLA-haploidentical recipients

Potential primary endpoint	Historical rate[3]	Expected event rate					Power	
		Entire group	Lowest AUC group[a]	Highest AUC group[a]	Effect size		N=25	N=50
2-year progression free survival	35% [b]	35%	50%	20%	30%		0.33	0.61
		35%	60%	10%	50%		0.98	0.99
1-year progression free survival	48%	48%	70%	25%	45%		0.61	0.92
		48%	80%	15%	65%		0.95	0.99
1-year overall survival	62%	62%	90%	34%	56%		0.86	0.99
1 year cumulative incidence of relapse	45%	45%	10%	80%	70%		0.90	0.99
		45%	20%	70%	50%		0.72	0.97
Non-relapse mortality	7%	7%	1%	15%	14%		0.24	0.44

[a] Hypothesized event rate between lowest AUC group (i.e., < median) and highest AUC group (i.e., median). Effect size is the event rate in lowest AUC group minus the event rate in the highest AUC group;

[b] event rate per BMT CTN version 1.0