The effects of monoclonal anti-CD47 on red cells, compatibility testing and transfusion requirements in refractory acute myeloid leukemia

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Abbreviations:

AML – acute myeloid leukemia

DAT – direct antiglobulin test

IAT – Indirect antiglobulin test

DTT – dithiothreitol

Hb – hemoglobin

RBCs – red blood cells

CTCAE- Common Terminology Criteria for Adverse Events

Abstract

Background:

CD47 is a novel therapeutic target in the treatment of solid organ and hematological malignancies. CD47 is also expressed on red blood cells. Here we report our experience of the red cell effects, impact on blood bank testing and transfusion management in a Phase I trial of the humanised anti-CD47 monoclonal antibody Hu5F9-G4 in relapsed or primary refractory acute myeloid leukemia (AML) (NCT02678338).

Patients & Methods:

19 patients with relapsed or primary refractory AML treated across 5 UK centres were included for analysis. Patients received escalating doses of Hu5F9-G4. Serial laboratory data were collected to evaluate impact on hemoglobin (Hb), markers of hemolysis (bilirubin, lactate dehydrogenase (LDH), reticulocyte count), transfusion requirements and blood compatibility testing.

Results:

A decline in Hb was observed with drug administration (median Hb change -1.0 g/dL, range 0.4 - 1.6) with associated increase in transfusion requirements. Patients responded to transfusion with a median Hb increment per unit of 1.0 g/dL. Red cell agglutination was seen in all cases without associated change in Hb, LDH, bilirubin or reticulocyte count. 9/19 (47%) of patients developed a newly positive antibody screen with a pan-agglutinin identified in plasma. Invalid ABO blood grouping occurred in 4/12 (33%) non-group O patients due to anomalous reactivity in the reverse ABO type results.

Conclusions:

Treatment with Hu5F9-G4 in patients with AML resulted in an Hb decline and increased transfusion requirements. Problems with ABO blood typing and compatibility testing were widely observed and should be expected by centers treating recipients of Hu5F9-G4.

Background

The transmembrane glycoprotein CD47 acts as a physiological 'don't-eat-me' signal, binding to macrophages to inhibit phagocytic clearance.(1, 2) CD47 expression levels on cancer cells is thought to correlate with their ability to escape immune surveillance and cell death.(3) Blockade of CD47 is a promising therapeutic strategy to trigger macrophage-mediated clearance of tumor cells in hematological and solid organ malignancies.(4, 5)

Hu5F9-G4 is a novel anti-CD47 monoclonal IgG4 antibody under evaluation in six studies in the U.S. and U.K.(6) Results from a Phase Ib study of HU5F9-G4 in combination with rituximab in relapsed/refractory B cell Non-Hodgkins Lymphoma (B-NHL) were recently published and demonstrated a well-tolerated safety profile with rare dose-limiting events, a suggested phase 2 dose of 30mg/kg and a 36% complete response rate.(7) Studies in progress with Hu5F9-G4 monotherapy include Phase 1 trials in advanced solid tumor malignancies (NCT02216409), and in relapsed/refractory acute myeloid leukaemia (AML) / high risk myelodysplasia (MDS) (NCT02678338). (8, 9) There are three further trials examining Hu5F9-G4 as combination therapy, with cetuximab in patients with solid tumours and advanced colorectal cancer (NCT02953782), with avelumab in ovarian cancer (NCT03558139) and with azacitidine in AML / MDS (MDS) (NCT03248479).(10)

CD47 is also highly expressed on red blood cells (RBCs) as a member of the Rh membrane complex, and the downregulation of CD47 alongside a conformational change is thought to trigger physiological clearance of ageing RBCs.(11) In mouse models, transfused CD47-/- RBCs are quickly cleared from the circulation of wild-type recipients.(12) Drug binding to CD47 on RBCs results in interference with standard serological techniques for blood compatibility testing. This type of effect is a recognized not only with CD47 but also with several novel agents, for example daratumumab (anti-CD38 monoclonal antibody).(13) This presents a significant challenge to the provision of compatible blood. Specific strategies may be used in daratumamab treated patients to prevent antibody binding such as dithiothreitol (DTT) treatment of reagent RBCs or the use of $F(ab')_2$ fragments to block drug binding to the CD38 epitope.(14, 15) For anti-CD47, use of multiple alloadsorptions and monoclonal Gamma-clone anti-IgG, which does not detect IgG4, have been proposed to help mitigate antibody interference.(16)

In the Phase Ib study of Hu5F9-G4 in relapsed/refractory B-NHL, anemia occurred in ~42% of patients. This was mitigated by the use of a priming dose of 1 mg/kg to enable clearance of older RBCs and a compensatory reticulocytosis to occur before commencing a maintenance dose.(7) Binding of Hu5F9-G4 was postulated to unmask prophagocytic signals in ageing red cells, leading to homeostatic clearance.(7) In mouse models, a priming dose of 5F9 triggers clearance of a subset of RBCs and results in near complete loss of CD47 expression on the red cell surface.(17) In non-human primate studies of Hu5F9-G4, the use of an initial low priming dose followed by higher maintenance doses prevented the anemia from being dose-limiting.(6) A reduction in Hb was noticed

from the first dose and recovered over 15-32 days, with robust reticulocyte responses. Pre-clinical study findings alongside the known anti-CD38 mAb effect on antibody screens led us to hypothesize that Hu5F9-G4 binding to circulating RBC may target RBC for clearance and confound blood type and antibody testing.

The primary objectives of the study were to analyse the occurrence and severity of anemia, the presence and extent of any blood grouping or compatibility testing problems, the transfusion requirements on treatment, and any adverse events associated with transfusion. The secondary objectives were to identify any practical approaches to mitigate Hu5F9-G4 RBC antibody coating interference and enable safe compatibility testing.

Methods

19 patients were recruited at five U.K. centers as part of the Camellia study, a phase I dose escalation trial of the humanized Anti-CD47 monoclonal antibody Hu5F9-G4 in hematological malignancies (NCT02678338). Patients were sequentially allocated to one of five escalating dose cohorts of Hu5F9-G4 (Supplementary Table 1). The dose of Hu5F9-G4 ranged from 0.1 mg/kg to 30.0 mg/kg. Doses were given twice weekly by intravenous infusion.

Serial hematological and biochemical testing was performed through the course of the study. For each patient, laboratory testing of hemoglobin, markers of hemolysis (haptoglobin, bilirubin, lactate dehydrogenase (LDH), reticulocyte count), direct antiglobulin (DAT), blood smear agglutination, ABO typing and antibody screening was performed at baseline and twice weekly (on treatment days 1, 4, 8, 11, 15, 18, 22, 25 and further if remained on study). Samples for DAT, ABO blood group and antibody screen were taken immediately pre-dose, and all other samples were taken both pre-and post-dose (4 hrs +/- 30mins post infusion). Tests were performed in accordance with local laboratory protocols. Blood film agglutination was reported locally and reviewed centrally, and graded from 0-4 as follows: 0 (0-9% agglutination), 1 (10-19% agglutination), 2 (20-50% agglutination), 3 (51-75% agglutination) and 4 (>75% agglutination), in line with the CTCAE version 4.03 for Thrombotic Thrombocytopenic Purpura and Haemolytic Uremic Syndrome.(18)

Serologic testing was performed by standard methods. DAT was completed using BioRad column agglutination technology. ABO typing was performed by forward typing with anti-A, anti-B, and reverse typing with A1/B RBCs using immediate spin technique in a microtitre plate on the Immucor Neo platform; or by gel column agglutination technology (Grifols' DG gel cards, Diamed Gelstation). Antibody screening was performed using the Immucor Capture Ready Screen R technology on the Neo platform; or by gel column agglutination technology (Grifols). Antibody identification was performed using a range of techniques including Capture R technology, column agglutination technology and tube IAT. Allo-adsorptions were performed with papain-, ficin-, DTT-, trypsin- and chloroquine- treated RBCs.

Red cell typing was performed at initial patient screening. Patients transfused in the three months prior to entering the study underwent red cell genotyping for D, C, E, Kell, Kidd, Duffy, and MNS. In patients not transfused within the three months prior to entering the study, phenotyping for these antigens was performed. Transfusion requirements in the three months prior to study entry and all further transfusions after study entry were ascertained by contacting local hospital blood banks. Patients were transfused to a target Hb of 10 g/dL in an initial cohort of patients and then subsequently 8 g/dL as recommended by the trial protocol.

Summary statistics such as median and ranges were presented and compared between groups using the Mann Whitney U test. A p value of < 0.05 was considered significant. All tests were two-sided.

Statistical analyses were performed using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 24. Armonk, NY: IBM Corp).

Results

Patient diagnoses, characteristics and previous treatments are summarized in Table 1. The median age at diagnosis was 71 years (range 30-82 years), with a median age at randomization of 72 years (range 31-86 years). 16 patients (84%) had relapsed disease, and 3 (16%) had primary refractory disease. All patients had received prior treatment, with a median of 1 previous lines of therapy (range 1-5). 11 patients (58%) of patients had received previous induction chemotherapy with Daunorubicin and Cytarabine. Median time-on-study was 35 days (range 20-354).

Hu5F9-G4-induced anemia

All patients (19/19; 100%) experienced a drop in Hb between the pre- and post-Hu5F9-G4 blood draws. In general, the largest fall in Hb was associated with the first dose of Hu5F9-G4, with lesser degrees of change seen with subsequent doses. The median Hb decline following any dose of Hu5F9-G4 per patient across the course of the study was 1.0 g/dL (range 0.4 - 1.6) (Figure 1). In 3 patients (16%), the Hb decreased post- Hu5F9-G4 by ≥ 3 g/dL. The maximum observed Hb drop was 5.2 g/dL, which occurred after the first dose in a patient in dose cohort 3. There was no clear relationship between dose of Hu5F9-G4 and decrease in Hb.

There was evidence of RBC agglutination in 19/19 patients (100%) on post-dose blood smear examination. RBC agglutination persisted until the next blood draw (i.e. pre-dose) in 17/19 (89%) patients. The maximum grade of RBC agglutination was greater post-dose (median 2, range 1-3) than pre-subsequent dose (median 1, range 0-3, $p<0.05$). No consistent clinical sequalae were associated with the presence of hemagglutination on the peripheral blood smear.

18/19 (95%) patients developed a newly positive DAT on-study. Of these 18 patients, 12 patients (67%) had a positive DAT after the first dose prior to delivery of the second dose. The DAT remained positive in 12/18 (67%) at the end of treatment. In 8/18 (44%), the DAT was only weakly positive. In all instances, the DATs were positive with polyspecific antihuman globulin reagents and with monoclonal anti-IgG, and negative with monoclonal anti-C3d.

Despite evidence of RBC agglutination and DAT positivity, there was no consistent laboratory evidence of hemolysis. Specifically, there was no concomitant peak in bilirubin or LDH. (Figure 2) Patients did not mount a compensatory reticulocytosis, with reticulocyte counts never exceeding the normal range. The median peak reticulocyte count, at point of maximal Hb drop, was 1.6% (range 0.4-4).

Transfusion requirements on Hu5F9-G4

14/19 patients (68%) had received at least one RBC transfusion in the 30 days prior to the study start date. 18/19 patients (95%) were transfused after initiation of Hu5F9-G4. There was a significant increase in the 30-day transfusion requirement on-trial compared to the 30 days prior to trial start date: median 2 RBC units (range 0-7) pre-trial vs 6 RBC units (range 0-15) on-trial, $p<0.05$ (Table 2, Figure 3). It should be noted that the first cohort of patients were transfused to a higher hemoglobin threshold (Hb of 10 g/dL) than typical standard of care (Hb 8 g/dL) due to physician preference, which is a contributing factor to the increase in transfusion requirements.

The increase in transfused RBCs supported a median Hb of 9.6 g/dL (range 7.9-1.1 g/dL) on-trial, compared to 9.0 g/dL (range 6.1-12.5 g/dL) pre-trial. Overall, there was no statistically significant difference in Hb pre- vs on-trial ($p=0.91$). There was no clear evidence of a Hu5F9-G4 dose effect

impacting on transfusion requirements. Patients responded to transfusion with a median Hb increment per unit of RBC of 1.0 g/dL which was maintained until the subsequent dose of Hu5F9-G4.

Hu5F9-G4-related compatibility testing issues

Hu5F9-G4 has previously been reported to confound ABO blood group typing.(19, 20) To identify potential issues with ABO typing after initiation of Hu5F9-G4, a group and screen was conducted on each patient twice weekly. Discrepancy between forward and reverse typing, or the presence of new alloantibodies, was reported and characterized by the testing laboratory.

7/19 patients were blood group O. In the 12 non-group O patients, discordant results from forward and reverse typing was seen in 4/12 (33%). In these four cases, extra plasma reactivity occurred in the reverse typing on samples sent after doses of Hu5F9-G4. Forward typing was not affected. Allo-adsorption techniques were applied to all 4 samples, and successfully resolved interference in 3/4 cases. Patient-level data from antibody screen testing is shown in Table 3. During the study, 9/19 (47%) patients developed a new positive antibody screen, which was a pan-agglutinin. One patient had a known anti-E antibody resulting in a positive antibody screen. The median time to positive antibody screen from first dose of Hu5F9-G4 was 4 days (range 1-114). Standard serologic methods failed to eliminate pan-reactive antibodies in 8/9 (89%). Treatment with DTT, chloroquine, papain, ficin, or trypsin was also attempted but failed to prevent pan-agglutination. Patients with a pan-agglutinin required matching of donor blood to the patients' genotype to provide RBCs for transfusion. There were no significant consequences to patient care and all patients were safely transfused with no clinical complications.

Discussion

Hu5F9-G4 is an attractive novel therapy for the treatment of both solid organ and hematological malignancies. As it transitions toward phase II clinical trials, the data in this study indicate potentially clinically important effects on RBCs and compatibility testing in the blood bank.

In this Phase 1 study of Hu5F9-G4 for AML, all patients had decreases in Hb post-dose requiring RBC transfusion. Although all patients were asymptomatic, in 3 patients (23%) the Hb decrease post-Hu5F9-G4 was ≥ 3 g/dL. 18/19 patients developed DAT positivity and 19/19 had demonstrable blood film agglutination after treatment. CD47-deficient mice have previously been reported to develop profound autoimmune hemolytic anemia due to enhanced opsonization of RBCs.(21) In our patient cohort we found no laboratory evidence of hemolysis, although this appears to be the likeliest mechanism for anemia, mainly through phagocytosis of RBCs. Alternatively, IgG4 antibodies (as Hu5F9-G4) have been described to cause anemia without significant hemolysis.(22) Increasing transfusion requirements in the relapsed/refractory AML patient population may also occur secondary to disease progression, sepsis or bleeding, and higher hemoglobin thresholds, but the timing of the Hb drop between the pre- and post-dose blood draws implicates Hu5F9-G4. At the current doses used in this study, there was no clear evidence of a dose effect, as a reduction in Hb was seen across all cohorts. While a priming-and-maintenance strategy may prove useful in this patient cohort, there

are likely to be significant differences in marrow reserve between a relapsed/refractory AML population and the published B-NHL patient population.(7) Ongoing marrow failure and lack of marrow reserve likely accounts for the absence of a compensatory reticulocytosis in the face of anemia described here.

Clinicians and laboratories testing samples from patients on Hu5F9-G4 should be aware of potential problems with blood compatibility testing. Newly positive antibody screens were seen in ~50% of patients, with pan-reactive antibodies, and interference with ABO typing occurring in ~33% of non-group O patients. Multiple RBC alloadsorptions and/or the use of monoclonal Gamma-clone anti-IgG (which does not detect IgG4) may help limit interference with compatibility testing.(16) In due course, use of an anti-idiotype antibody against Hu5F9-G4 on patient serum samples could further reduce this interference.

Widespread use of Hu5F9-G4 would present challenges to transfusion services, and clear protocols and laboratory procedures need to be developed to mitigate interference with blood typing and compatibility testing. For example, baseline ABO and D and antibody screen plus RBC phenotyping/genotyping for all patients for treatment with Hu5F9-G4 is essential for the identification of compatible blood. In addition, patients may not be suitable for electronic crossmatching due to concurrent invalid ABO blood typing results, and this should be highlighted on their blood bank records. CD47 antibody interference may preclude compatibility testing by IAT consequently, blood units may need to be issued by “emergency release” overrides and documented in the transfusion service exception log to ensure compliance with regulatory and accreditation requirements.

There are intrinsic limitations to this study. Our findings are derived from a small, first-in-man safety and dose-finding Phase I study. The results may be specific only to the relapsed/refractory AML population. These patients have often been previously transfused, may have pre-extant antibodies and may be susceptible to anemia due to bone marrow failure. Methods and reagents used in laboratory testing varied between centres, reflecting the variation in results observed. Transfusion occurred by clinical need and physician decision. While the threshold for transfusion specified by the trial protocol was 80g/L, in practice, records demonstrated that a more liberal transfusion threshold was adopted in the early stages of the trial, likely due to physician preference in managing their pat’ent's supportive care. From a mechanistic perspective, whether the pan-agglutinin identified is Hu5F9-G4 (binding to CD47) could not be directly demonstrated.

In summary, this study indicates that Hu5F9-G4 may have clinically relevant effects on RBCs, transfusion requirements, and laboratory compatibility testing. With careful monitoring of Hb and informed compatibility testing, recipients of Hu5F9-G4 can nonetheless be safely transfused.

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Tables

Table 1: Patient characteristics (n=19)

Diagnosis	N (%)
AML with myelodysplasia-related changes	10 (53%)
Therapy-related AML	2 (11%)
AML without maturation	4 (21%)
AML NOS	2 (11%)
AML with recurrent genetic abnormalities	1 (5%)
Disease status	
Relapsed refractory disease	8 (42%)
Relapsed disease	8 (42%)
Primary refractory disease	3 (16%)
Age at diagnosis (median, range)	71 (30-82)
Age at randomisation (median, range)	72 (31-86)
Previous lines of treatment (median, range)	1 (1-5)
Previous treatment	
Azacitidine	14 (74%)
Daunorubicin + Cytarabine	9 (47%)
Daunorubicin + Cytarabine + Myelotarg	2 (11%)
Cytarabine	7 (37%)
FLAG-IDA	3 (16%)
Clinical trial (Ravva study; NCT01617226)	2 (11%)
Amsacrine + Cytarabine	1 (8%)
Fludarabine + Cytarabine + G-CSF	1 (5%)

*NOS= not otherwise specified, G-CSF: granulocyte-colony stimulating factor, FLAG-IDA: fludarabine, cytarabine, G-CSF, idarubicin

Table 2: Transfusion requirements and hemoglobin level on Hu5F9-G4

	Prior to trial	**On-trial**	**p-value**
30-day transfusion requirement (median number of RBC units; range)	2 (0-7)	6 (0-15)	0.0002
Median Hb (g/dL; range)	9.0 (6.1-12.5)	9.6 (7.9-10.7)	0.91

Table 3: Compatibility testing issues with Hu5F9-G4

Patient	Dose cohort	Positive Ab screen	Time from 1st Hu5F9-G4 dose to positive Ab screen in days#	Ab identified	Transfusion strategy
1	1	No*	NA		
2	1	Yes (pre-dated study entry)	0	Anti-E	E negative
3	1	No*	NA		
4	2	No*	NA		
5	2	Yes	1	Pan-agglutinin	Group O, genotype matched
6	2	Yes	21	Pan-agglutinin	Genotype matched
7	3	Yes (weak)	114	Pan-agglutinin	Genotype matched
8	3	Yes	1	Pan-agglutinin	Genotype matched
9	3	No*	NA		
10	4	No*	NA		
11	4	No*	NA		
12	4	Yes	2	Pan-agglutinin	Genotype matched
13	4	Yes	1	Pan-agglutinin	Phenotype- & crossmatched
14	5	No*	NA		
15	5	No*	NA		
16	5	No*	NA		
17	5	Yes	7	Pan-agglutinin	Phenotype- & crossmatched
18	5	Yes	4	Pan-agglutinin	Phenotype- & crossmatched
19	5	Yes	7	Pan-agglutinin	Phenotype- & crossmatched

#Samples taken pre-dose *Negative antibody screen results may be due to use of a reagent that does not detect IgG4. NA= non-applicable, Ab= antibody

Figures

Figure 1

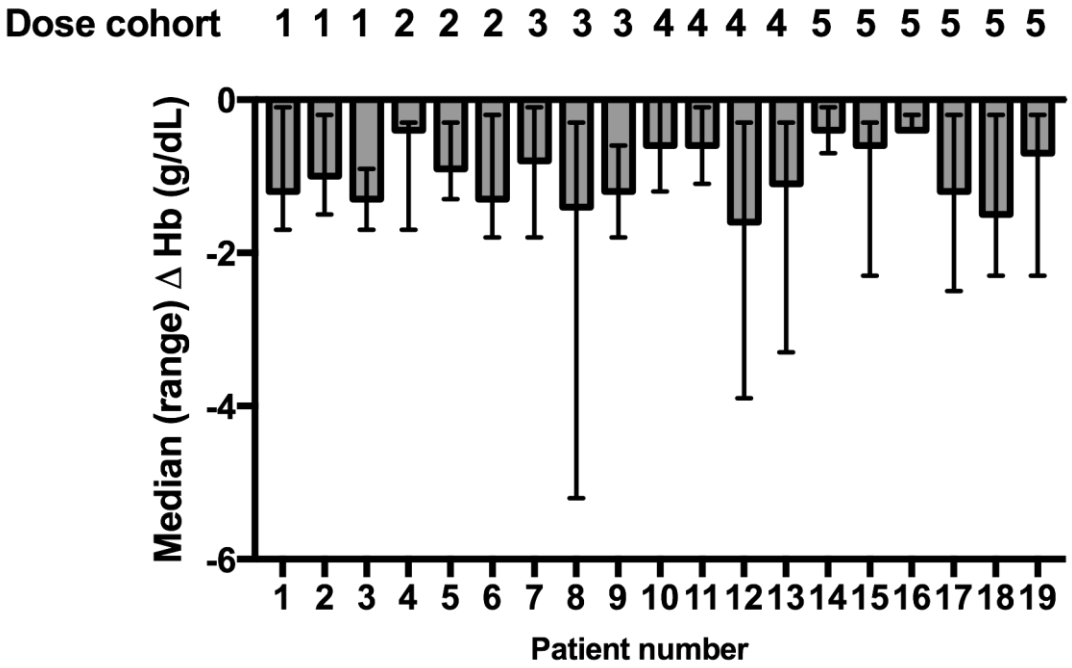

Figure 1: Change in hemoglobin following Hu5F9-G4 by dose cohort. The median and range of drop in hemoglobin following Hu5F9-G4 is shown for each patient between pre-dose and post-dose blood draws (~ 4 hour interval).

Figure 2

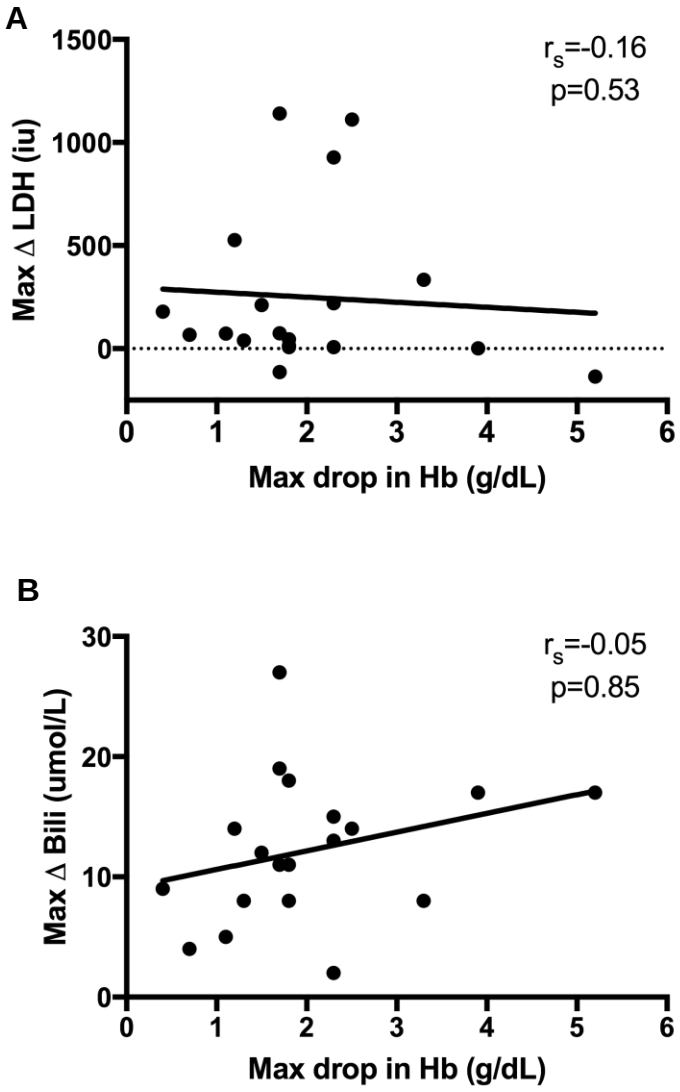

Figure 2: Lack of hemolysis with Hu5F9-G4. The maximum drop in hemoglobin following Hu5F9-G4 is shown for each patient, from pre- to post-drug blood draws, alongside the maximum change in LDH (A) and the maximum change in bilirubin (B). The largest falls in Hb were not accompanied by a concomitant rise in LDH and bilirubin to suggest hemolysis as the mechanism of anemia and the Spearman's rank correlation coefficient (r_s) demonstrates no evidence of correlation.

Figure 3

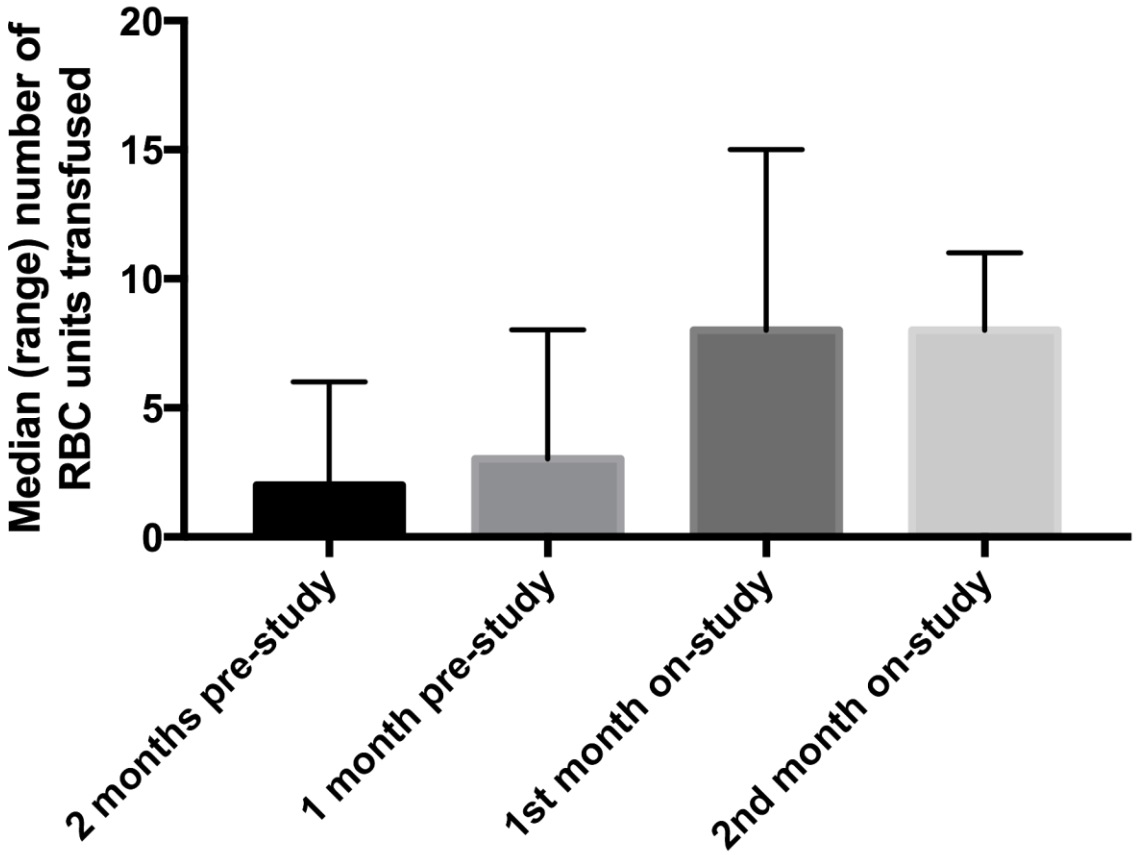

Figure 3: Transfusion requirements during the Camellia study. Median (range) of number of RBC units transfused per month before and after study start.

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Supplementary Data

Supplementary Table 1: Dosing schedule of Hu5F9-G4 in the Phase 1 Camellia study (mg/kg)

	Week 1		**Week 2**		**Week 3**		**Week 4**	
	Day 1	Day 4	Day 8	Day 11	Day 15	Day 18	Day 22	Day 25
Cohort 1 (n=3)	0.1	0.1	0.3	0.3	1.0	1.0	1.0	1.0
Cohort 2 (n=3)	0.3	0.3	1.0	1.0	3.0	3.0	3.0	3.0
Cohort 3 (n=3)	1.0	1.0	3.0	3.0	10.0	10.0	10.0	10.0
Cohort 4 (n=4)	1.0	1.0	10.0	10.0	15.0	15.0	15.0	15.0
Cohort 5 (n=6)	1.0	1.0	15.0	30.0	30.0	30.0	30.0	30.0