SCIENTIFIC SECTION DESIGNATION: LYMPHOID NEOPLASIA

A TCR-mimic antibody to WT1 bypasses tyrosine kinase inhibitor resistance in human BCR-ABL+ leukemias.

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Key points:
1. This study shows the effective anti-cancer activity of a T-cell receptor mimic antibody targeting WT1 in resistant human Ph+ ALL.
2. In combination with tyrosine kinase inhibitors, ESKM can result in cure of Ph+ ALL in murine models.

Abstract
Acute and chronic leukemias, including CD34+ CML cells, demonstrate increased expression of the Wilms tumor gene 1 product (WT1), making WT1 an attractive therapeutic target. However, WT1 is a currently un-druggable, intracellular protein. ESKM is a human IgG1 T-cell receptor mimic monoclonal antibody directed to a 9 amino acid sequence of WT1 in the context of cell surface HLA-A*02. ESKM was therapeutically effective, alone and in combination with tyrosine kinase inhibitors (TKIs,) against Philadelphia chromosome positive acute leukemia in murine models, including a leukemia with the most common, pan-TKI, gate keeper resistance mutation, T315I. ESKM was superior to the first generation TKI, imatinib. Combination therapy with ESKM and TKIs was superior to either drug alone, capable of curing mice. ESKM showed no toxicity to human HLA-A*02:01+ stem cells under the conditions of this murine model. These features of ESKM make it a promising non-toxic therapeutic agent for sensitive and resistant Ph+ leukemias.

Introduction
Wilms tumor gene 1 protein (WT1) is a zinc finger transcription factor involved in the embryonic development of multiple organ systems including kidney, with restricted expression in adult tissues. The function of WT1 is complicated and unclear; it regulates a variety of proteins as a transcription factor, some as an activator and some as a repressor. Multiple cancers demonstrate significantly increased expression of WT1, including myelodysplastic syndromes, acute myeloblastic leukemias, acute lymphoblastic leukemias (ALL), chronic myelogenous leukemia (CML) and many solid tumors including mesotheliomas, ovarian cancer, and various gastrointestinal and central nervous system (CNS) tumors. Over-expression of WT1 has also been demonstrated in the CD34+ stem cells of patients with CML making it an attractive therapeutic target for eradication of this disease.

As an intracellular transcription factor, WT1 currently cannot be inhibited by small molecule drugs or directly targeted by antibody therapies. However, after processing within the
cell, peptides from WT1 protein are presented on the cell surface in the context of human leukocyte antigen (HLA) class I molecules. Previous work creating WT1 vaccines to stimulate human cytotoxic T-cells has identified key immunogenic peptide sequences of WT1 presented by HLA\(^{17}\); a number of new epitopes were recently discovered\(^{18}\). The widespread expression of this gene product in tumors, its involvement in oncogenesis, and its suppression in normal cells after birth\(^{19}\) make this a useful tumor marker and an ideal antigen target for cancer therapy\(^{20}\).

We have developed an antibody, ESKM, directed against the 9 amino acid peptide RMFPNAPYL (RMF), expressed in the context of HLA-A*02:01\(^{21,22}\). ESKM is a T-cell receptor mimic (TCRm) monoclonal human IgG1 in which the Fc portion has been modified by alternative glycosylation that results in stronger binding to effector cell activating Fc\(\gamma\) receptors and increased antibody-dependent cell cytotoxicity (ADCC)\(^{23}\). While T-cell based therapies have been attempted against WT1 expressing cancers, monoclonal TCRm antibodies have several advantages over vaccines, TCR constructs, and adaptive T-cells: ESKM can be produced and administered easily; it has greater potency, more predictable and simpler pharmacokinetics, and high efficacy. While several TCRm antibodies have been developed for other antigens\(^{24}\), none have entered human trials.

The current standard of care for chronic phase CML is treatment with tyrosine kinase inhibitors (TKIs), but this therapy is not curative, is extremely expensive and may be required life-long\(^{25}\). Variable compliance with long-term therapy, with approximately one third of patients stopping TKI’s altogether, and the development of mutations that provide resistance to TKIs, frequently results in treatment failure, which sometimes leads to accelerated phase or blast crisis\(^{26,27}\). Long-term use of TKIs used to suppress leukemia leads to a myriad of side effects, including pleural edema, effusions, pulmonary hypertension, sepsis, gastrointestinal problems and lethal cardiovascular events\(^{28,29}\). Furthermore, outside of stem cell transplantation (SCT), there is no effective therapy of CML in blast crisis or Philadelphia chromosome positive (Ph+) ALL\(^{30}\); treatment with TKI’s results in brief responses only. The presence of WT1 expression in CML and its progenitors allows us to test for the first time a curative strategy for this disease, by use of ESKM alone or in conjunction with TKIs. In this study, we found ESKM alone was a more effective therapy than TKI’s in murine models of human Ph+ ALL. Importantly, the efficacy of the antibody was not affected by the presence of the BCR-ABL “gatekeeper”
resistance mutation T315I that inactivates all the first and second generation FDA approved TKI’s in clinical use. In combination with TKIs, ESKM was capable of curing mice of Ph+ ALL.

Materials and methods:

ADCC

ADCC was evaluated by chromium release assay, incubating target cells in 50 µCi Cr\(^{51}\) for one hour before three washes, with the determined optimal ADCC time of 6 hours. Three effector to target (E:T) ratios were used (either 10:1, 30:1, and 100:1 or 25:1, 50:1, and 100:1). Effector PBMCs were derived from healthy donors by Ficoll density centrifugation, after obtaining informed consent on a Memorial Sloan Kettering IRB-approved protocol. This study was conducted in accordance with the Declaration of Helsinki.

BV173R Synthesis

The BV173R cell line was engineered to harbor the T315I BCR-ABL, utilizing a Clonetech pMSCV Puro vector with the T315I BCR-ABL gene, a kind gift from Charles Sawyers\(^31\). BV173-luc-gfp cells were transduced with the retroviral vector, selected and expanded in media with puromycin. The presence of the resistant T315I mutation was confirmed by PCR for the plasmid, as well as ABL sequencing of cDNA and genomic DNA in the resultant BV173R cell line.

Flow Cytometry and RT-PCR

Evaluation of ESKM binding to target cells and cell surface HLA-A*02:01 expression was done by direct flow cytometry. ESK was labeled with APC using the Innova Biosciences Lightning-Link® Allophycocyanin (APC) kit. BB7 (anti-HLA-A*02:01) was purchased from various vendors. Minimal residual disease of cell lines in mouse bone marrow was evaluated using custom made primers to the Firefly Luciferase gene with SYBR® Green using GAPDH as control.

Trials of ESKM with TKIs in vivo in mice

Trials of ESKM were done using xenograft models, in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice with IL2 gamma receptor knockout (NSG) mice\(^32\) engrafted with BV173 Ph+ leukemias. The BV173 and BV173R cell lines used for all in vivo studies stably expressed the firefly luciferase gene, and disseminated engraftment of
leukemia was confirmed 6 days after tail vein injection by BLI before treatments. Mouse research is approved by MSKCC IRB under protocol 96-11-044.

Preliminary drug dosing studies in NSG mice demonstrated the MTD of imatinib to be 50 mg/kg IP daily, with higher dosing resulting in diarrhea and severe toxicity. Dasatinib was also initially tested, with the MTD of 40 mg/kg (0.8 mg/mouse) resulting in no appreciable short-term toxicity. However, longer-term trials of dasatinib resulted in mouse sudden deaths, and the dose was therefore lowered for subsequent experiments to 20 mg/kg and then to 10 mg/kg to eliminate drug-related mortality. For mice receiving ponatinib, the initial published dose of 5 mg/kg showed inadequate leukemic affect without any evident health side effects, and therefore ponatinib doses were increased to 10 mg/kg. At this dose, toxicity was comparable to 50 mg/kg of imatinib, with intermittent diarrhea and poor growth.

All tyrosine kinase inhibitors, including imatinib, dasatinib and ponatinib, and plerixafor were purchased from Selleckchem or Fisher Scientific. The plerixafor stock solution was made in PBS at 200 µg/ml and stored at room temperature. Mice received 1 mg/kg (20 µg per mouse) daily by IP injection, as utilized in previous studies. No toxicity was noted during therapy. Prior to use in vivo, stock solutions of TKIs were prepared in DMSO, with the scheduled dose of TKI in 50 µL DMSO per mouse. All TKIs and ESKM treatments were administered intraperitoneally at these schedules: ESKM 100 µg twice weekly and TKIs daily. Male NSG mice, 6-8 weeks old, were purchased from Jackson Labs. Three million leukemic cells (BV173 or BV173R) were injected per mouse by tail vein on day 0. Luciferin/luciferase BLI was then performed on day 6, with therapy commencing immediately after confirmation of equivalent levels of disseminated leukemia by imaging. IVIS 200 and IVIS Spectrum machines were used for BLI.

**CD34+ Cell Transplantation**

CD34+ cells were obtained by leukapheresis from two healthy donors as part of a harvest for sibling SCT on an MSKCC IRB approved protocol. The leukapheresis product was purified by the CliniMACS® system by the Memorial Hospital clinical stem cell lab. Twenty NSG mice, aged 6-8 weeks, were irradiated with 320 cGy on day -1, and received 3 x 10^6 CD34+ cells per mouse on day 0 by dorsal penile vein injection. Ten mice received cells from patient A and ten from patient B. These two cohorts were then divided, with five mice from each group treated with ESKM 100 µg IP twice weekly starting on day +6 for 4 weeks, and the other five mice
receiving the same dosing schedule of IgG isotype control. No specific supportive therapy or antibiotics were provided.

Results:

ESKM therapy of Ph+ ALL in NSG mice

Imatinib is a first generation TKI that has been in longest use in humans for the treatment of CML. BV173 is a Ph+ HLA-A*02:01+ leukemia cell line. Bioluminescent imaging (BLI) was used to document disseminated engraftment of luciferase marked BV173 Ph+ ALL in \textit{NOD SCID gamma} (NSG) mice 6 days after tail vein injection. Mice were then split into four groups of equal mean tumor burden and treated with: no therapy, imatinib only, ESKM only, a combination of imatinib and ESKM. After 5 weeks of treatment, ESKM therapy was significantly superior to imatinib therapy (78% vs 52 % tumor growth reduction, p<0.01) (Figure 1A). The combination of ESKM and imatinib was significantly better than either imatinib or ESKM alone (p<0.001), reducing tumor growth by 94% and possibly stabilizing progression of disease at that low level (Figure 1B). Bone marrows (BM) from all four groups were evaluated for HLA expression and ESKM binding immediately after completion of therapy (Figure 1C), with no group showing elimination of BV173 cells.

ESKM Therapy with the second generation TKI, dasatinib

Dasatinib is a second generation TKI with far greater potency than imatinib in humans and in animal models. Using the same mouse model as described above, dasatinib administered at 40 mg/kg IP daily in concurrent combination with ESKM resulted in the clinical and molecular cure of 3 out of 4 mice (Figure 2A-B), confirmed by BM qPCR 3 weeks after the end of therapy (supplementary Table 1, MRD <0.001%). No cures were seen in mice treated with either drug alone. The one mouse treated with both ESKM and dasatinib that relapsed, first developed recurrence of disease in the CNS on day 27 (Figure 2C). Because of their large size, antibodies do not enter the CNS. At this dasatinib dose, all mice receiving the TKI became ill about 1 week into therapy, with one mouse fatality on day 8 of treatment. All dasatinib treated mice sustained temporary BLI remissions, but those not treated with ESKM relapsed 2-3 weeks after cessation of therapy.
To determine whether combination therapy with TKI’s and ESKM could be optimized by sequential therapies rather than concurrent treatment, BV173 engrafted mice were treated with dasatinib at a lower dose of 20 mg/kg alone for 18 of planned 21 days. By this point, 4 mice receiving dasatinib died, and the remaining 11 mice were randomized to receive further treatment: 6 received ESKM, and 5 received no additional therapy. Four weeks of ESKM therapy did delay relapse (Figure 2D), but no mice were cured, and ESKM therapy did not result in complete remission as measured by BLI at the end of therapy (Figure 2E).

BV173 in NSG mice was next treated with the maximally tolerated dose (MTD) of concurrent dasatinib and ESKM. Combination therapy reduced leukemia, while the mice treated with dasatinib alone doubled their leukemic burden (Figure 3A). Cells were analyzed by flow cytometry upon completion of therapy showing no change in antigen expression (Figure 3B). No leukemia was seen by BLI at the end of therapy in mice that received both dasatinib and ESKM (Figure 3C). The mice treated with combination therapy were followed with BLI scans until Day 58. Mouse #2 died on day 31, likely from dasatinib toxicity, and interestingly, the other four mice all showed focal tumor relapse but not recurrent leukemia (Figure 3D). This striking pattern of ALL relapse as discrete tumors (Figure 3E) is unusual and was only seen with the combination therapy.

**Therapy of T315I Resistant Ph+ Leukemia Therapy**

BV173R (TKI pan-resistant T315I mutant) leukemia xenografted NSG mice were initially treated with ESKM, imatinib, dasatinib and concurrent combinations of imatinib plus ESKM and dasatinib plus ESKM. Only 3 weeks of therapy were given, as the leukemia proved resistant to the first and second generation TKIs. In contrast, ESKM retained full and equivalent efficacy against the resistant BV173R leukemia compared to TKI sensitive leukemia BV173 (Figure 4A) as quantified by luciferase imaging during treatment. These findings are consistent with in vitro studies indicating that in ADCC assays, ESKM is as active against BV173R as it is against BV173 (Figure 4B). Dasatinib provided only modest slowing of leukemic growth with leukemic burden rising five-fold over the same time period. The combination of ESKM and dasatinib showed substantial reduction in leukemia burden, though no mice were cured. As in the prior experiments with BV173 treated with the combination ESKM plus dasatinib, relapse was lymphoid tumors rather than diffuse leukemia (Figure 4C). The lack of ESKM efficacy on
BV173 growing as primary subcutaneous lymphomas was documented, suggesting that these cells escape by lack of penetration of the few NSG effector cells that circulate (supplementary Figure 1). How this form of escape will be manifested in humans is uncertain as the NSG mice lack NK cells.

NSG mice engrafted with this T315I Ph+ resistant leukemia were then treated with the third generation drug, ponatinib. Leukemic regression was seen in all ponatinib treated mice, with negative BLI showing no disease at the end of therapy on day 34 (supplementary Figure 2A). Mice treated with ponatinib monotherapy subsequently developed systemic leukemic relapse within two weeks after end of treatment, while those treated with the combination of ESKM and ponatinib developed either focal lymphoid relapse (3/5), or had no evidence of disease (2/5) 4 weeks after completion of therapy (supplementary Figure 2B). These results were confirmed by repeating the experiment with a complete 4-week course of ponatinib 10 mg/kg alone and in combination with ESKM (Figure 4D). Mice were followed for 4 weeks after end of therapy (Figure 4E), at which time their tumors were evaluated by resection and BLI (Figure 4F; supplementary Figure 3A-B). Of note, ponatinib at 10 mg/kg was fairly toxic (diarrhea, poor weight gain), and while all treated mice survived 4 weeks of therapy, 4 of the 20 mice treated in these experiments died of late side effects.

**Effects in vitro of ESKM and TKIs against Ph+ ALL**

The striking activity of ESKM in combination with the two TKI’s led us to ask whether there was a mechanistic interaction of the TKI’s on the ability of ESKM to kill target cells through its known cytotoxic mode of action, ADCC. Chromium release ($^{51}$Cr) assay was used to evaluate ESKM directed human peripheral blood mononuclear cell (PBMC) driven ADCC in vitro against BV173. The addition of ESKM resulted in 21-29% ADCC across multiple experiments, using the effector to target ratio (E:T) of 100:1 (Figure 5A). Control IgG1 isotype was not cytotoxic. ADCC was also measured in presence of imatinib and dasatinib. Imatinib had no effect on ADCC at concentrations up to 10 µM (Figure 5B), at ten times the peak concentrations of this drug in humans. However, dasatinib showed a profound inhibition on ADCC in repeated experiments, with complete inhibition of PBMC mediated ADCC at concentration of 100 nM (Figure 5C), the peak concentration of drug in humans.
We also examined whether TKIs might affect expression of the WT1/HLA epitope, ESKM binding and hence, efficacy. This issue was interrogated both in vitro and in vivo. BV173 cells were grown in culture with imatinib (1 µM), dasatinib (100 nM) and ponatinib (100 nM), in the approximate peak concentrations achieved in vivo by these drugs. Consistent with previously reported studies, exposure of Ph+ cells to high doses of TKIs resulted in significant downregulation of HLA expression and a mild decrease of WT1 (supplementary Figure 4). However, this downregulation of HLA was seen in vitro by culturing cells in the prolonged presence of a constant peak dose of TKIs, conditions that are not physiologic.

To evaluate whether TK1 treatment could also decrease HLA or RMF cell surface expression in vivo and thereby contribute to ESKM resistance, BV173 leukemia was extracted immediately following termination of therapy from the imatinib treated mice BM (Figure 1B) and from the livers of mice treated with dasatinib 10 mg/kg (Figure 3C). Five weeks of therapy in vivo with imatinib alone, ESKM alone, or combination of both drugs, did not alter HLA surface expression on CD19+ cells as measured by flow cytometry (supplementary Figure 5A). A small decrease in ESK median binding was seen in cells treated with imatinib (Figure 1C), though it could not be discerned on the flow histogram (supplementary Figure 5B). There was no difference in HLA or ESK surface binding between BV173 cells obtained from the livers of mice treated with 25 days of dasatinib as compared to vehicle control (DMSO), though both had equally decreased HLA expression compared to freshly cultured BV173 (Figure 3B).

Leukemia Escape Mechanisms

BV173 leukemia treated with monotherapy for up to 5 weeks with ESKM monotherapy was noted to overgrow in the BM. To evaluate whether BM stromal cells were protective against TCRm therapy, NSG mice engrafted with BV173 were treated for 4 weeks with a combination of ESKM and the CXCR4 inhibitor plerixafor to inhibit binding of the BV173 cells to the stroma. Treatment with plerixafor was associated with reproducible but not statistically significant reduction in BV173 growth. A concomitant reduction of tumor growth with plerixafor and ESKM treatment was also observed, but the difference in growth inhibition was not significant (supplementary Figure 6A-B).

We also evaluated whether alterations in HLA expression or presentation of WT1 peptide as measured by ESK binding could alter sensitivity to ESKM mediated ADCC in the presence or
absence of TKIs. To do this we evaluated ADCC against fresh leukemic blasts from a patient with blast crisis CML. This leukemia was selected for its having low expression of HLA-A*02:01 (supplementary Figure 7A: 7 fold shift vs 30-40 fold shift for BV173) and low ESK binding (supplementary Figure 7B). ADCC with ESKM was background level, compared to 21-29% for BV173 (supplementary Figure 7C). As expected, in vivo, ESKM alone had no efficacy against this CML with low HLA expression and ESK binding. However, BV173 did not show significant losses of antigen expression that would account for relapse (Figure 3B, supplementary Figure 5B).

Activity of ESKM against normal hematopoietic cells

While the potent therapeutic effects in mice appear promising, cytotoxicity against normal hematopoietic cells is a potential risk. Previously we showed that ESKM does not affect hematopoietic stem cell numbers or frequencies, LSK cells, or mature WBC subpopulations in HLA-A*02:01 transgenic mice that express WT1. ESKM was investigated for cross reactivity against healthy CD34+ human hematopoietic stem cells by evaluating ESKM vs IgG isotype control therapy of mice transplanted with human HLA-A*02:01 CD34+ cells. Two mice died from early irradiation toxicity, and on days +45-46, eight mice died from SCT across all four groups, all attributable to late radiation effects. The remaining mice (3 ESKM treated and 6 IgG treated) were submitted to pathology for BM and organ evaluation. These mice were found to have anemia with good reticulocytosis, suggesting severe anemia as the cause of death on days +45-46. There was no histologic difference between the BM of mice treated with ESKM compared to those who received isotype control (Figure 6A). Importantly, human CD45+ staining showed 20-60% engraftment in all mice (Figure 6B). No toxicity was observed secondary to ESKM therapy in the BM or in any of the mouse organs by pathology compared to IgG controls.

Discussion:

Compared to TKIs, ESKM is more potent, has a longer half-life and is not susceptible to tumor escape by gatekeeper mutations. ESKM is also a therapeutically effective antibody in mouse models of other human leukemias and solid tumors. However, as monotherapy, the antibody was unable to achieve cures in these models. In combination, TKIs and ESKM did not
exhibit enhanced toxicity, had at least an additive therapeutic effect and resulted in molecular cures of Ph+ ALL in mice. BM relapse of leukemia post treatment may reflect inadequate effector cells rather than loss of antigen, or local stromal cell protection, since RMF/HLA-A*02:01 was still expressed on leukemia cells at the end of therapy and reducing leukemic cell adherence to stromal cells with plerixafor did not significantly increase antileukemic activity. This hypothesis is further supported by the consistent and efficient leukemic cell clearance in the liver, where there are abundant Kupffer cells for ADCC (Figure 1B) and not in the BM, which lack many effectors in these NSG mice.

The data support a more generally accepted hypothesis that mAb therapies would likely benefit from a second agent to decrease cancer burden and keep cancer growth rates low, thereby fostering more effective ADCC in vivo at increased E:T ratios. Thus, patients with CML in remission on TKIs may be ideal candidates for ESKM treatment, since they have minimal residual leukemic burden and slow cancer growth. The main mechanism of escape of ALL for ESKM monotherapy seemed to be inadequate effector function. Surprisingly, the combination therapy with dasatinib or ponatinib resulted in lymphoid tumor relapse, which is exceedingly rare in CML in humans. We speculate that a lack of effector cells infiltrating the leukemic tumor or the reduced ESKM penetration into the lymphoid tumors may account for this observation.

An important advantage to TCRm antibody therapy was its efficacy against pan-resistant Ph+ leukemias. First and second generation TKI’s are not effective against leukemias that carry the BCR-ABL T315I resistance mutation. Such mutations occur in 12-15% of screened patients with CML and are a source of relapse and failure of treatment40,41. While a third generation TKI, ponatinib, is effective in the presence of this mutation, its use is currently restricted because of severe vascular toxicity. Therefore, there is an urgent need for alternative therapies in patients with these mutations. ESKM works through an entirely different mechanism than the TKI’s, and our experiments demonstrated equal efficacy of the TCRm mediated ADCC against both TKI sensitive BV173 and its resistant mutant BV173R in vitro and in vivo. Combining ESKM with another TKI could result in faster remissions and possible cure, which may allow a shorter course of these expensive, potentially toxic and non-curative drugs.

While these data strongly support the effectiveness of combining TKIs with ESKM, there are limitations. The patients must be HLA-A*02+, and have leukemias expressing WT1. Leukemias with reduced HLA-A*02 expression may be resistant to ESKM. Additionally, low
WT1 expression, or inadequate concentrations of effector cells such as in a leukemia packed bone marrow, may also limit cytotoxic activity of this TCRm antibody. Importantly, despite its potent activity against Ph+ ALL, ESKM had no discernable effect on normal human BM progenitor cells within these model systems. Because of its specificity for the leukemia cells, ESKM should cause minimal toxicity. In addition, this antibody could be evaluated in human clinical trials during the peri-stem cell transplant time frame.

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Conflict of interest statement: L.D., T.D., D.A.S. are inventors of intellectual property that is owned by MSKCC and for which a license has been obtained. None own equity or are paid by the licensee or receive grants. S.Y. and C.L. are employed by and have an equity stake in Eureka, which co-owns the intellectual property described above.

Contribution: L.D., E.J.B., T.D., C.L. and D.A.S. designed the study. R.J.O. and D.A.S. coordinated the study. L.D. conducted all experiments and analysis with assistance from D.P, E.J.B. and A.S. BV173R cell line was synthesized by D.P. ESKM was synthesized and supplied by S.Y. and C.L. L.D. drafted the manuscript, which was subsequently revised by all co-authors.

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References


Figure 1. BV173 engrafted xenograft NSG mouse model treated with imatinib at MTD (50 mg/kg, 1 mg/mouse IP daily) and ESKM at optimal efficacious dose (100 µg IP twice weekly). Mice received 5 weeks of ESKM, imatinib, combination therapy, or no therapy (control). Error bars show 5th and 95th percentiles. (A) Leukemic growth as measured by luciferase bioluminescent imaging for each of the four groups of mice. (B) End of therapy (5 weeks of therapy) image for each of the four groups of mice. (C) Three out of five mice where randomly chosen from each of the four groups for BM harvest and cells were evaluated by flow cytometry. Median staining by flow of the live hCD19+ cells (confirmed BV173) evaluated for BB7 (HLA-A*02:01) and ESK binding.

Figure 2. BV173 engrafted xenograft NSG mouse model treated with dasatinib at doses above MTD. Error bars show 5th and 95th percentiles. (A) Leukemic growth measured by BLI in mice treated with high dose dasatinib therapy (40 mg/kg x 8 days, then lowered to 20 mg/kg x3 days for a total 11 days of treatment and then stopped altogether secondary to high toxicity), ESKM only for 4 weeks, combination therapy and control (no therapy). (B) BLI on Day 55, 3 weeks after end of therapy. Mouse #1 in dasatinib with ESKM combination died from dasatinib toxicity on Day 13, and mouse #4 appears to have relapsed in CNS. (C) High dose dasatinib plus ESKM group BLI images, showing progression of relapse in mouse #4. (D) Leukemic growth measured by BLI in mice treated with dasatinib therapy at 20 mg/kg IP daily given x18 days, then stopped for high toxicity. ESKM was given to six mice (day 18 to day 42). All mice relapsed, though ESKM mice relapsed significantly slower, and lifespan was longer x1 week. Last point on each line pertains to when mice were sacrificed due to illness. (E) BLI at the end of ESKM therapy (day 42). Two cages received ESKM following discontinuation of dasatinib (Dasatinib→ESKM 1 and 2) and compared to one cage (5 mice) that received no additional therapy. ESKM salvaged mice have substantially less tumor burden then dasatinib only mice, though all mice eventually relapsed.
Figure 3. BV173 engrafted in NSG mice, treated with dasatinib 10 mg/kg daily ± ESKM 100 µg twice weekly for 25 days, beginning on day 6. Error bars show 5th and 95th percentiles. (A) Logarithmic tumor growth curve as measured by BLI. (B) Control and dasatinib-alone mice were sacrificed on day 30 for evaluation of ESKM binding and HLA-A*02:01 expression by flow cytometry. BV173 cells were harvested from the livers of all NSG mice immediately after cessation of therapy. Live hCD19+ cells (confirmed BV173) were evaluated for BB7 (HLA-A*02:01) and ESK staining, and compared to BV173 in culture. (C) BLI imaging from day 27, showing persistence of leukemia at end of therapy in dasatinib treated mice and eradication of leukemic signal in mice receiving combination therapy. (D) BLI image of dasatinib + ESKM group on day 55, 24 days after cessation of therapy, showing focal lymphoid relapse. (E) Dasatinib + ESKM treated mice dissected and re-imaged individually to localize tumor. Organs were removed and imaged separately for precise tumor localization. Mouse 1: primary tumor in lymph node, with small liver metastasis detected. Mouse 3: Primary tumor in stomach with focal liver metastasis and small renal met. Mouse 4: tumor seen in left upper quadrant, but unable to isolate by organ excision. Mouse 4: Diffuse right lung tumor, unable to visualize right upper quadrant tumor seen in prior imaging.

Figure 4. BV173R (with T315I mutation) treated with dasatinib 10 mg/kg and imatinib 50 mg/kg IP daily for 21 days with and without biweekly ESKM therapy (6 doses of ESKM). Error bars show 5th and 95th percentiles. (A) Exponential growth curves, from start of therapy (day 6). The blue bar shows duration of TKI therapy and the red lines are doses of ESKM. (B) The susceptibility of cell line BV173, resistant cell line BV173R (with BCR-ABL T315I mutation) to ESKM directed ADCC with human PBMCs and different effector to target ratios. (C) BLI imaging at the end of ESKM/TKI therapy (day 27). ESKM is superior to imatinib and dasatinib for treatment of resistant Ph+ ALL. (D) BLI of mice engrafted with BV173R treated with ponatinib 10 mg/kg alone and in combination with ESKM x 25 days (day 6 to day 30). Green bar shows duration of ponatinib therapy and red lines are doses of ESKM. (E) BLI image on day 57 of mice treated in panel (D), immediately prior to organ analysis for tumor location and MRD evaluation. (F) Ponatinib + ESKM mice from panel (D) were dissected and organs imaged for tumor localization. Mouse 1: No detectable disease on any imaging, possible cure. Mouse 2: gastrointestinal and bone marrow leukemia detected. Mouse 3: gastrointestinal, bone
marrow, and lung leukemia detected, with uncertain primary. Mouse 4: lymphoid tumor in the stomach, no other tumor detected. Mouse 5: Primary lymphoid tumor in the stomach, bone marrow leukemia detected.

**Figure 5. Effects of TKIs on ADCC.** (A) ADCC by Cr-51 assay with variable E:T ratios, using ESKM vs IgG1 isotype control. Effects of imatinib (B) and dasatinib (C) on ADCC. No significant effect on ADCC by imatinib was seen on human PBMC-mediated ADCC in concentrations as high as 10 µM. Dasatinib inhibited human PBMC-mediated ADCC starting at concentrations of 10 nM.

**Figure 6. Bone marrow pathology from NSG mice treated with four weeks of ESKM or isotype IgG control following human HLA-A*02:01 CD34+ transplant, compared to normal NSG mice.** (A) No differences were seen between ESKM- and IgG-treated mice, and both groups have increased immature myeloid and erythroid lineages compared to NSG mice. The transplanted groups also have lymphocytes, which NSG mice lack. Engraftment of human cells was the same in ESKM and IgG treated mice, ranging from 20-60%. Error bars show 5th and 95th percentiles. (B) Immunohistochemical staining of NSG mouse bone marrow transplanted with human CD34+ cells for hCD45. There was no significant difference between the mice treated with ESKM (top panels) and those treated with isotype control (bottom panels). Normal NSG mice did not stain for hCD45 (not shown).
A TCR-mimic antibody to WT1 bypasses tyrosine kinase inhibitor resistance in human BCR-ABL+ leukemias

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