Ibrutinib is not an effective drug in primografts of TCF3-PBX1

Cesca van de Vena,b, Aurélie Boeree a,b, Femke Stalpers a,b, C. Michel Zwaana,c, Monique L. Den Boera,b,c

Aim: The Bruton’s tyrosine kinase (BTK) inhibitor ibrutinib (PCI-32765) is effective in patients with multiple myeloma, non-Hodgkin lymphoma and chronic lymphoblastic leukemia. We previously showed that primary cells of children with TCF3-PBX1 positive B-cell precursor acute lymphoblastic leukemia (BCP-ALL) express BTK and are sensitive to ibrutinib in vitro. However, preclinical studies in mice are lacking that justify clinical implementation.

Methods: Immunocompromised NSG mice were engrafted with a luciferase-positive TCF3-PBX1 leukemic cell line or primary leukemic cells and treated with ibrutinib or placebo. Additionally, primary cells were exposed in vitro to 4 main induction drugs as monotherapy and in combination with ibrutinib.

Results: Treatment with ibrutinib of mice engrafted with a TCF3-PBX1 cell line, TCF3-PBX1 positive or TCF3-PBX1 negative primary leukemic cells did not result in prolonged life span compared to placebo treated mice. In vitro sensitivity to ibrutinib was unaltered in leukemic cells obtained from engrafted mice compared to the original material. However, ibrutinib treatment did not affect leukemic cell viability and tumor outgrowth, nor could lymphocytosis be detected. ibrutinib was biologically active, since hCD19+ cells harvested from ibrutinib treated mice had no detectable levels of phospho-BTK at tyrosine 223 (pBTK Y223), whereas pBTK Y223 was still detectable in placebo treated cases. In combination tests, we noticed an antagonistic effect of ibrutinib on vincristine sensitivity, which was not observed for prednisolone, L-asparaginase and daunorubicin.

Conclusions: We conclude that ibrutinib is not the precision medicine of choice for TCF3-PBX1 positive BCP-ALL.

Original research

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Conclusions: We conclude that ibrutinib is not the precision medicine of choice for TCF3-PBX1 positive BCP-ALL.
the survival of treated mice nor reduced tumor burden in mice engrafted with leukemic cell lines and with primary patients’ cells. Moreover, we provide evidence that ibrutinib has an antagonistic effect on vincristine in vitro, suggesting a combination of both therapeutics in the clinic is not desirable.

**Material and methods**

**Primary leukemic cells**

Primary human BCP-ALL cells were isolated from bone marrow and/or peripheral blood from children (1–18 years) with BCP-ALL at diagnosis. Written informed consent was obtained from parents or guardians to use excess of diagnostic material for research purposes, as approved by the institutional review board and in accordance with the Declaration of Helsinki. Mononuclear cells were isolated and processed as described previously [20]. When necessary, samples were enriched towards >90% leukemic cells by depletion of non-leukemic cells using immunomagnetic beads [21]. Cytogenetic subtype was characterized using established diagnostic workflows, including tests for the recurrent translocations ETV6-RUNX1, BCR-ABL1, KMT2A-AF4, TCF3-PBX1, and high hyperdiploidy [22]. Patient characteristics were provided by the central study center of DCOG, The Hague, the Netherlands. Leukemic cells were stored frozen in liquid nitrogen at 50–100 million cells per vial. Upon thawing a vial for an experiment, the percentage of dead cells was determined using a Trypan blue staining and percentage of leukemic cells was determined by May-Grünwald Giemsa staining.

**Cell lines**

The human acute lymphoblastic leukemia cell line RCH-ACV was purchased from the Leibniz Institute DSMZ-German Collection of Micro-organisms and Cell lines (DSMZ, Braunschweig, Germany) and cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum (Bodinco BV, Alkmaar, Netherlands), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.125 μg/mL fungizone (Life Technologies, Bleiswijk, Netherlands).

In the identity of the cell line was verified by DNA fingerprinting by short tandem repeat method (STR) at purchase and throughout culture for consistency.

**Lentiviral transduction**

RCH-ACV cells were made to express luciferase by transduction of the FUW-Luc-mCherry-puro vector (obtained from Dr. A. Kung, DFCI, Boston). The vector was transfected in HEK293T cells, together with psPAX2 (Addgene plasma 12260; Addgene, Cambridge, MA, USA) and pMD2.G (Addgene plasmid 12259) using X-tremeGene 9 DNA transfection reagent (Roche, Woerden, The Netherlands). Virus was collected on the second and third day after transfection and concentrated by ultracentrifugation at 32,000 rpm, 4 °C for 2 h (Optima L-90K Ultracentrifuge; Beckman Coulter, Woerden, The Netherlands). Concentrated virus was aliquoted and stored at −80 °C. RCH-ACV cells were transduced by spin-infection. Positive cells were selected by puromycin resistance and verified by DNA fingerprint. Luciferase and mCherry expression were confirmed by a luminescence assay (E1500; Promega, Leiden, The Netherlands) and flow cytometry respectively.

**Ex vivo drug resistance assay**

The ex vivo cytotoxicity of all therapeutics was determined using the MTT assay. The assay conditions were essentially the same as described before [23]. Concentration of the therapeutics ranged from: Ibrutinib 0.08–40 μM (Janssen, Leiden, The Netherlands), daunorubicin 0.002–2 μg/mL (Cerubidine; Rhone Poulenc Rorer, Amstelveen, The Netherlands), prednisolone disodium phosphate 0.06–250 μg/mL (Bula pharmaceutical Products, Uitgeest, The Netherlands), vincristine 0.05–50 μg/mL ( Oncovin; Eli Lilly, Nieuwegein, The Netherlands), L-asparaginase 0.003–10 IU/mL (Medac; Medac, Hamburg, Germany). All tests were performed in 96-well round-bottomed microculture plates, cells were exposed to ten different concentrations of ibrutinib, six different concentrations of the other four drugs, or to culture medium only. For the synergy tests, cells were exposed to six concentrations of daunorubicin, prednisolone, vincristine or L-asparaginase with or without 4 μM Ibrutinib. After 4 days of culture at 37 °C and 5% CO2, 50 μg [3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to each well and incubated for 6 h at 37 °C. The formed formazan molecules (by viable cells only) were dissolved in acidified isopropanol (0.04 M HCl) and the quantity of formazan was measured spectrophotometrically at 562 nm. The optical density (OD) is linearly related to the number of viable cells in each well [23]. The LC50 value represents the drug concentration which killed 50% of the cells compared with the number of cells that were present in the control wells without drug. Primary patient samples of all eleven patients were tested, but six were not informative.

**Xenografts and in vivo ibrutinib treatment**

Primary leukemic cells were injected intra-femoral in 7–12 weeks old female NOD.Cg-Prkdcs1Id2r2tpy1-WjI/Sij1 (NSG) mice (Charles River, Wilmington, Massachusetts, USA; eight NSG mice per patient, later divided over placebo and ibrutinib treatment groups), as approved by the animal ethics committee (EMC 3117). Xenografted mice were monitored for successful engraftment using weight and human CD19-positivity in the peripheral blood (Brilliant Violet 421™ anti-human CD19 Antibody, Biolegend, San Diego, California, United States) was determined by flow cytometry using a MACSQuant analyzer (Miltenyi Biotec, Leiden, The Netherlands).

Treatment of mice engrafted with primary leukemic cells started at a percentage of human CD19 in the blood >1%, with daily intraperitoneal injections of 12 mg/kg ibrutinib in 50% polyethylene glycol (PEG 400, PanReac AppliChem, Barcelona, Spain) for the ibrutinib group or 50% PEG for the placebo group. Mice were treated for a maximum of 12 weeks. Treatment with a higher dose of ibrutinib (25 mg/kg) caused high mortality within 24 h in engrafted NSG mice, and was therefore not further investigated.

Luciferase-positive RCH-ACV cells were engrafted similar to the procedure used for primary leukemic cells. Xenografted mice were monitored for human CD19-positivity in the peripheral blood and bioluminescence determined by intravenous injection of luciferin (RediLect D-Luciferin Bioluminescent Substrate, Perkin-Elmer, Waltham, Massachusetts, United States) and subsequent measurement of released photons using the IVIS in vivo imaging system 200 (Xenogen, now: Perkin-Elmer). Treatment with 12 mg/kg ibrutinib or placebo started 7 days after engraftment until overt leukemia was reached.

After treatment xenografted mice were sacrificed and blood plasma was collected by centrifugation of peripheral blood obtained via heart puncture. Additionally, cells were harvested from bone marrow, spleen and peripheral blood. Cells were assessed for human CD19 percentage by flow cytometry, viable frozen or processed for protein isolation.

**Protein isolation and western blot analysis**

Frozen cell pellets of primary leukemic cells, RCH-ACV and harvested xenografted cells were lysated using lysis buffer with protease and phosphatase inhibitors. Protein concentration was determined by the BCA method (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 25 μg lystate was loaded on 10%-mini-PROTEAN TGX precast protein gels (Biorad, Veenendaal, The Netherlands) and transferred onto nitrocellulose membranes (Bio-rad). Primary antibody incubation was performed according to manufacturer’s protocol. Anti-BTK (#85475) and Anti-phospho-BTK-Tyr223 (#50825) were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). Anti-β-actin (ab6276) was obtained from Abcam (Cambridge, UK). Blots were incubated with secondary antibodies (IRDye 680 CW or 800 CW-labeled anti-rabbit and IRDye 680 CW or 800 CW-labeled anti-mouse;
Results

Outgrowth of RCH-ACV in vivo remains unaltered by ibrutinib treatment

To explore the potency of ibrutinib in vivo, we generated murine xenografts with the human TCF3-PBX1 positive cell line RCH-ACV. RCH-ACV cells were sensitive to ibrutinib in vitro with an IC50 of 2.8 μM (95% CI, 2.2–3.7 μM, Fig. 1A). The cell line was made transgenic for mCherry and luciferase and retained sensitivity to ibrutinib (IC50 of 1.16 μM, 95% CI: 1.2–2.7 μM, Fig. 1A). This cell line was injected directly into the femur of NOD.Cg-Prkdcscid Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice. Intrafemoral injection resulted in a swift engraftment in the bone marrow. RCH-ACV rapidly populated the mouse, typically spread to the liver, but not the spleen, and mice died around 25 days after engraftment due to hepatic complications. Due to this swift spread and short life span, we started daily treatment with 12 mg/kg ibrutinib or placebo through intraperitoneal injection 7 days after intrafemoral injection, even before human CD19 positive (hCD19<sup>+</sup>)}
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A. TCF3-PBX1 positive

B. TCF3-PBX1 negative

C. TCF3-PBX1 positive

D. TCF3-PBX1 negative

E. TCF3-PBX1 positive

F. TCF3-PBX1 negative

G. % survival relative to control

Ibrutinib concentration (in μM)

(caption on next page)
cells could be detected in the peripheral blood (Fig. 1B), however isolated cells from the liver died immediately after isolation for both ibrutinib and placebo treated mice, and thus could not be used for further analysis.

During treatment, all mice were monitored weekly for the presence of hCD19+ cells in peripheral blood by flow cytometry. Although ibrutinib causes lymphocytosis in CLT patients [24], we could detect only very low percentage of human cells in the blood during treatment, with no significant difference between ibrutinib and placebo treated mice at any of the measured time points (Fig. 1B).

Tumor load over time could be detected by bioluminescence, since engrafted RCH-ACV cells were transgenic for luciferase. Mice were monitored twice per week, but no significant difference between ibrutinib and placebo treated groups could be identified at any of the timepoints (Fig. 1C, Suppl Fig. 1A). Furthermore, no significant difference in tumor load between ibrutinib and placebo treated mice could be detected in harvested organs (Fig. 1D). Viability of the hCD19+ cells in the different organs remained unaltered (Fig. 1E), as did the viability of murine hematopoietic mCD45+ cells (Suppl Fig. 1B), suggesting ibrutinib did not cause cell death in human cells in vivo nor affected healthy cells of the host.

Treatment with ibrutinib did not result in a prolonged life span for xenografted mice (median survival time after treatment start (MST): 13.5 days for 12 mg/kg ibrutinib) compared to placebo (MST: 13 days, p = 0.74, Fig. 1F).

**Ibrutinib does not prevent outgrowth of primary pediatric leukemia in vivo**

To elucidate if ibrutinib may successfully contribute to the cure of patients with TCF3-PBX1 positive BCP-ALL and to exclude possible bias of cell line models, we engrafted leukemic cells from four patients with newly diagnosed TCF3-PBX1 positive BCP-ALL (BCP-ALL #1-4, Suppl Table S1) and two TCF3-PBX1 negative BCP-ALL (BCP-ALL #5&6) into NSG mice.

To mimic the clinical situation as well as possible, treatment with 12 mg/kg ibrutinib or placebo started when percentage of hCD19+ cells in the peripheral blood exceeded 1%. Treatment with 12 mg/kg ibrutinib did not result in a prolonged life span for mice engrafted with primary TCF3-PBX1 positive BCP-ALL cells compared to placebo control (Fig. 2A). In contrast, in mice engrafted with one out of two TCF3-PBX1 negative we observed a prolonged lifespan of 15 days (BCP-ALL #6: MST: 57.5 days, Fig. 2B) compared to placebo control (MST: 42.5 days, p = 0.03), while this was not observed in BCP-ALL #5. Strikingly, responsiveness of patient #6 to ibrutinib is not BTK dependent, since cells of neither patient #5 nor patient #6 have high total BTK or detectable levels of phosphorylated BTK (Fig. 2B).

Similar to the results with RCH-ACV, no signs of lymphocytosis were detected in 12 mg/kg ibrutinib treated mice compared to placebo (Fig. 2C+D), nor any differences in tumor load or viability of hCD19+ or mCD45+ cells in harvested organs for the TCF3-PBX1 positive ALL or TCF3-PBX1 negative ALL (Fig. 2E+F, Suppl Fig. 2).

However, engrafted primary TCF3-PBX1 positive cells were confirmed to retain sensitivity to ibrutinib in vivo when isolated from the mouse after successful engraftment and cytotoxicity was similar between engrafted cells and the original sample from patients (Fig. 2G), suggesting that the intrinsic response to ibrutinib was not affected in these cells.

**Ibrutinib is biologically active in NSG mice**

The biological activity of ibrutinib in our in vivo model, was confirmed in hCD19+ cells harvested from treated mice for inhibition of BTK. TCF3-PBX1 positive hCD19+ cells harvested from 12 mg/kg ibrutinib treated mice have no detectable levels above background of phospho-BTK at tyrosine 223 (pBTK Y223), whereas pBTK was still detectable in placebo treated cases (Fig. 3A, p < 0.0001). Total BTK remained unaltered in both ibrutinib and placebo treated samples (Fig. 3A, p = 0.56). As a consequence, the ratio between pBTK Y223 and total BTK was significantly lower in TCF3-PBX1 positive hCD19+ cells isolated from ibrutinib treated mice compared to those of placebo treated mice (Fig. 3A, p = 0.0099). In the TCF3-PBX1 negative cells, no differences in total BTK levels or ratio between pBTK and total BTK could be detected between ibrutinib and placebo treated mice, while levels of pBTK Y223 did not reach above background in both treatment groups (Fig. 3B, p = 0.24, p = 0.88 and p = 0.999 respectively).

**Ibrutinib antagonizes vincristine in vitro**

Finally, we examined if ibrutinib could enhance in vitro functionality of four main chemotherapeutics currently used to treat children with BCP-ALL. In four out of five primary patient cells we found an increase in survival of cells when treated with a combination of ibrutinib and vincristine, compared to monotherapy for either drug (Fig. 4A). Antagonism between ibrutinib and vincristine was seen in both TCF3-PBX1 positive and negative samples and was independent of BTK levels.

No marked changes in cell survival were found in combining ibrutinib with prednisolone, daunorubicin or L-asparaginase (Fig. 4B–D), except for a marked sensitizing effect of ibrutinib on L-asparaginase in one B-other case (BCP-ALL #9).

**Discussion**

Personalized therapies, specifically designed to target oncogenic pathways unaffected in healthy cells, may be of great added value to standard chemotherapy for children with BCP-ALL. These new agents could be key to prevent outgrowth of residual cells and/or salvage therapy for relapsed cases. Additionally, they might be able to replace or warrant a reduction of highly toxic therapeutics currently used, decreasing side effects. Ibrutinib has been hailed to be such a new agent; well tolerated, minimal side effects and targeting a molecule almost exclusively activated in the cancerous B-cell.

Although a very good response to ibrutinib is observed in adult patients with CLL [17–19] and results have been promising in vitro with pediatric BCP-ALL [12], here we show that ibrutinib does not prevent the outgrowth of primary BCP-ALL cells in vivo. Furthermore, ibrutinib was not efficacious in mice engrafted with TCF3-PBX1 positive nor TCF3-PBX1 negative leukemia. This lack of anti-tumor efficacy could not be attributed to a lack of ibrutinib activity in mice, since the pBTK levels in leukemic cells of treated mice were clearly reduced compared to those in the placebo treated group.

The different potency of ibrutinib in CLL and BCP-ALL might be caused by an intrinsic difference between these two forms of leukemia. CLL cells are more differentiated compared to BCP-ALL, already having an activated mature BCR-signaling pathway. TCF3-PBX1+ BCP-ALL is
characterized by a VDJ rearranged heavy chain only and lack of further processing of the kappa and lambda light chain resulting in a differentiation arrest at the preBCR level [12]. In an in vivo model preBCR⁺ ALL cells may therefore need a different micro-environmental factor to expand than mature BCR⁺ CLL cells. This might cause a difference in cellular dependence on BTK signaling, explaining the difference in efficacy of ibrutinib in both cell types.

Exposure of different cell lines to various TKI inhibitors, showed all tested TCF3-PBX1 positive cell lines to be sensitive to ibrutinib in vitro [25]. Although treatment with 75 mg/kg resulted in a delay in outgrowth of leukemia, some marked differences are that a different mouse strain was used as well as a different TCF3-PBX1 positive cell line (ICN12). More importantly, ibrutinib has also previously been tested in vivo in a murine ALL model, using the same cell line as described in this study, RCH-ACV [26]. A crucial difference in experimental setup of both studies may account for the 3-days prolonged lifespan observed in the study of Kim et al. and the delayed outgrowth of ICN12 in the study of Geng et al. Ibrutinib has been reported to affect migration of ALL cells to the bone marrow by influencing CXCR4 signaling [27]. In the studies by Kim et al. and Geng et al., ibrutinib was given only 24 h after the injection of ALL cells, which may have inhibited CXCR4 and thus migration to the bone marrow (the preferred niche of BCP-ALL cells) thereby delaying the outgrowth of leukemic cells. However, patients with BCP-ALL present in clinic with a fully established leukemia in the bone marrow and inhibiting CXCR4 will not have a dramatic effect. We therefore started Ibrutinib treatment when the leukemia was fully settled. Our data show that ibrutinib as a single agent does not inhibit the expansion of leukemic cells once the BCP-ALL leukemia has homed to and settled in the bone marrow and extramedullary organs.

We also investigated whether ibrutinib might work synergistic to current chemotherapy. In this study we noticed a marked sensitizing effect only for L-asparaginase in one out of five tested patient samples, but not for the other drugs. Strikingly, we found an antagonistic effect between ibrutinib and vincristine in the majority of patient samples tested, for which the causative relationship remains to be determined.

In conclusion, our data show that monotherapy with ibrutinib does not delay outgrowth and is not effective in in vivo mouse models with a fully established human BCP-ALL. This opposes our observation that ibrutinib is cytotoxic to leukemic cells in monoculture, suggesting that the (mouse) leukemic niche protects against the cytotoxic effect of ibrutinib. The lack of strong synergy with 3 out of 4 tested induction drugs (prednisolone, daunorubicin and L-asparaginase) and impaired sensitivity to vincristine indicates that ibrutinib is not a preferred precision medicine in TCF3-PBX1 positive BCP-ALL despite target presence.

Author contributions statement

Cesca van de Ven: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. Aurélie Boeree: Software, Investigation, Data Curation, Writing - Review & Editing. Femke Stalpers: Validation, Investigation, Writing - Review & Editing. Michel Zwaan: Conceptualization, Methodology, Resources, Writing - Review &
Fig. 4. In vitro sensitivity to main therapeutics and ibrutinib in primary BCP-ALL samples. (A–D) In vitro sensitivity of five primary BCP-ALL samples measured in MTT assays to 0.78 μg/mL vincristine (A), 0.488 μg/mL prednisolone (B), 0.031 μg/mL daunorubicin (C) and 0.08 IU/mL asparaginase (D). Either as a single agent (black bars) or in combination with 4 μM ibrutinib (grey bars). Primary cells were TCF3-PBX1 positive (TP+, BCP-ALL #7 and #8), B-Other (BO, BCP-ALL #9) or BCR-ABL1-like (BA-like, BCP-ALL #10 and #11). Error and average survival relative to survival with no drug (for single agent) or survival with 4 μM ibrutinib (drug combination testing). Survival with 4 μM ibrutinib as a single agent ranged between 66 and 103% compared to survival of placebo treated cells.

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Appendix A. Supplementary data

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