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The first-in-Africa ex vivo drug sensitivity testing platform identifies novel drug combinations for South African leukaemia patient cohort

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In South Africa, leukemia remains a major health concern, posing significant challenges in treatment due to its varied subtypes. There is an unmet need for a testing pipeline that can identify drug effects on patient samples in an ex-vivo setting. Using the pilot study with South African patient samples, this paper reports the development of a drug-sensitivity testing pipeline for studying the drug effects in leukemia patient-derived cells. Forty-one (41) patients with Acute myeloid leukemia (AML) (n = 7), Chronic myelogenous leukemia (CML) (n = 30), and Chronic lymphocytic leukemia (CLL) (n = 4) were recruited for this study. Thirty (30) FDA-approved drugs were utilized for single drug sensitivity screening (DSS) on leukemia patient-derived cells with drug concentrations (1–1000 nM). The single DSS showed a distinct sensitivity pattern with different profiles among patients of the same subtype, confirming the need for precision therapy. This study observed irinotecan, used in solid tumour treatment, demonstrated efficacy in PBMCs in many patient samples compared to conventional leukemia drugs such as nilotinib. For drug combination studies, ten clinically relevant drugs were selected and tested based on the results of single drug sensitivity tests. This pilot study marks a crucial stride towards revolutionizing leukemia treatment in South Africa through an innovative ex vivo drug sensitivity testing platform. This pioneering initiative forms the basis for tailored and effective treatment options holding promise for more personalized treatment. Further exploration and validation of these findings could significantly contribute to cancer precision medicine efforts in South Africa.

Keywords Drug sensitivity test, Leukemia, Synergy, Full matrix combination

After cardiovascular diseases, cancer is the second most common cause of death and is a major burden of diseases worldwide[1,2]. In Sub-Saharan Africa, current data estimates that there will be 1 million cancer deaths per year by 2030[3]. Acute myeloid leukemia (AML), Chronic lymphocytic leukemia (CLL), and Chronic myelogenous leukemia (CML) are among the four major types of leukemia. AML, CML, and CLL are cancers of the bone marrow and blood. CLL is the only lymphoproliferative amongst the three leukaemia in developed countries but leukemia cases in Sub-Saharan Africa are under-estimated and under-reported[4,5]. While there are potentially increasing cancer survivors in developed countries such United States, and Scandinavian countries, South Africa faces increased cancer mortality with an estimated four thousand deaths due to leukemia every year (South African Cancer Registry 2022)[6]. Currently, there are very few research and clinical studies published on the South African leukemia patient cohort[7]. There is a critical need for efficient research and technology

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development in leukemia studies for South Africa to join the global trend with increased survival rates through improved treatment options for patients.

AML is an aggressive hematologic malignancy and is characterized by an uncontrolled proliferation of genetically altered immature myeloid cells, which progresses rapidly and heterogeneously without treatment[8]. CML is a myeloproliferative disorder and is often diagnosed in chronic phase[9]. Deep molecular remissions are expected with Tyrosine Kinase inhibitors such as nilotinib. Once it has transformed into acute leukaemia, treatment options are more limited, and the prognosis is guarded. It is characterized by a granulocytic cell line with increased proliferation and without loss of its differentiation capabilities. CLL, on the other hand, is a B-cell monoclonal disorder that progresses slowly and affects predominately older adults[10]. This leukemia is characterized by the progressive proliferation and accumulation of functionally incompetent mature lymphocytes. AML, CLL, and CML present significant treatment challenges, often leading to relapse and necessitating multiple lines of therapy. Further, it is crucial to have timely and precise interventions for leukemia management and improved survival rates[11]. This is mainly due to the association of gene mutations and drug resistance in leukemia. To address drug resistance in leukemia, several researchers from high-income countries have developed drug sensitivity and resistance testing (DSRT) to evaluate the drug sensitivity and drug combinations' effectiveness against the cancer cells[12,13]. Further, there's a notable absence of comprehensive comparative analysis regarding leukemia demographics and phenotypic characteristics in South Africa. This is primarily attributed to the lack of dedicated cancer registries specific to South Africa at present[10]. These cancers are understudied in South Africa, highlighting a critical gap that needs to be addressed to enhance patient outcomes. Thus, there is a major need for a drug testing platform in South Africa.

Currently, few cancer diagnostic methods support clinicians in selecting suitable next-line treatment options[14]. Ex vivo drug sensitivity test-based precision medicine approaches have been demonstrated to have the capability to predict various cancer-type specific drug sensitivities[15]. In leukemia treatment, most of the drug combinations currently available are predominantly tested in Caucasian patient cohorts. Hence, there is an unmet need for a test to identify drug combinations for South African patient cohorts. Several research groups have developed ex vivo drug sensitivity testing pipelines for various cancers like leukemia[16], multiple myeloma[17,18], and other solid tumors[19]. However, such drug sensitivity pipelines are predominantly established using Western countries' patient cohorts[20]. Furthermore, these drug-sensitivity testing pipelines are implemented to address the unmet medical need for Western countries' cancer patient cohorts[21]. Some of the examples of the drug sensitivity test pipelines applied for leukemia in high-income countries include ex vivo drug-sensitivity profiling using Norwegian acute myeloid leukemia (AML) patient samples[22], Swedish Chronic Lymphocytic Leukemia (CLL) patient samples[23], Finnish AML and Chronic Myeloid Leukemia (CML) patient samples[24,25] and USA AML and CML patient samples[26]. Some of the main advantages of implementing a drug sensitivity testing platform are its ability to identify new combination therapies and its ability to support a drug repurposing approach. Furthermore, the ex vivo drug sensitivity testing platform is of major benefit to patients in tailoring the treatment regime[27]. There has been a reported case study that showed that drug sensitivity testing can support personalized AML treatment selection in Finland[28]. A similar approach can be applied to addressing the unmet medical need for South African leukemia patients. This would be of major benefit to the South African leukemia patient cohort, the platform would enable to understand the drug resistance in cancer cells which is also linked to genetic mutation in the African cohort. There are several studies report the different genetic diversity in the African leukemia patient cohort compared to the Western patient cohort[29–31]. Therefore, there is a critical need to develop and implement ex vivo drug sensitivity tests for South African cancer patient cohorts enabling a more global view of the technology application[32].

In a resource-limited environment such as South Africa, an ex vivo drug sensitivity test would enable effective rationale to suggest unique drug combinations for leukemia and support individualized treatment options for South African patients with a shorter time frame and potentially reduced cost. The larger purpose of the ex vivo drug sensitivity test would be to apply to all types of cancer cells both liquid biopsy and solid tumour. From the South African patient cohort. The objective of this study is to demonstrate the establishment of an ex vivo drug sensitivity testing platform for testing South African Leukemia patient samples. This paper reports the development of an ex vivo drug sensitivity testing platform that can be used as a pre-clinical tool to assess the utility of a panel of clinically approved leukemic drugs and two drug combinations for synergistic effects in South African patient samples. This platform can potentially identify effective single drugs and drug combinations against leukemia patient-derived cells.

Material and methods

Ethics and study approval

Blood samples from AML CLL and CML patients either at diagnosis or relapse were obtained from the Chris Hani Baragwanath Academic Hospital and the Donald Gordon Medical Centre, Johannesburg, South Africa from 2021 to 2022. All experimental protocols conducted in this study were approved through the Medical and Health Research Ethics clearance committee from the University of Witwatersrand, (WITS) Johannesburg (M200307) and the Council for Scientific and Industrial Research CSIR (Ref:318/2020). The written informed consent was obtained from all participants. Patient clinical data are listed in Supplementary Tables S1 and S2. All methods reported in this article were performed in accordance with the Declaration of Helsinki guidelines and regulations.

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood within 6 h of sample collection using the Ficoll density gradient method as previously reported[33]. Cryopreservation of PMBCs in liquid nitrogen and thawing of the samples for experimental procedures were done as reported previously[34].

Revival and culturing of patient samples

Vials containing patient samples were revived by thawing and gentle agitation in a 37 °C water bath for 2 min. After thawing, the vial underwent prompt decontamination with 70% ethanol, achieved through dipping or spraying. The cells were then carefully resuspending in 15 mL of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM) (Whitesci) supplemented with 10% of heat-inactivated FBS and 1% of Penicillin–Streptomycin (Sigma) antibiotics, after which centrifugation was performed at 300 g for 10 min. Upon removal of the supernatant, 10 mL of fresh medium was added to resuspend the cell pellet. The cell suspension was transferred to a tissue culture flask or 96-well plate.

Culturing of HeLa cells and K562 cells

For culturing of HeLa cells, the flask was incubated at 37 °C with 5% CO_2, with regular monitoring conducted to assess confluency. Trypsinization was performed at 70–90% confluency for further splitting and passaging of HeLa cells. For culturing of K562 cells, the flask was incubated at 37 °C with 5% CO_2, with regular monitoring conducted to assess confluency. The Trypan Blue exclusion method was used to perform the cell viability test and cell counting was performed using the hemacytometer or automated cell counter system.

Selection and printing of drugs for single and double drug combinations

Thirty approved drugs relevant to CLL, CML, and AML were selected and tested for single-drug efficacy at 4 concentrations ranging from 1 to 1000 nm (Table S3). The protocol for preparing the master plate, source plate, and final drug printing is detailed in Supplementary Methods SM1 (Suppl. Figure 1). Drug combinations (n = 5 double combinations) were selected based on clinical relevance to CLL, CML, and AML (Table S3) and tested on 41 patient samples. Anchored drug combinations were designed with a primary drug tested at 10 nm concentration. The primary drug was combined with secondary drugs with a concentration range of 1–1000 nm. 3 by 3 concentrations (1 to 100 nm) of two drugs were used for the full matrix drug sensitivity test. The drugs selected for the full matrix drug combination are listed in Table S4.

Drug sensitivity testing of patient samples

Ten thousand cells in 90 µL per well were seeded into the drug-printed 96-well plate. Finally, the plate was incubated at 37 °C with 5% CO_2 for 48 h. For cell viability measurements, 10 ul of PrestoBlue™ Cell Viability Reagent (10X stock solution) was added directly to each well-containing cell in the culture medium, excluding the media-only well. Subsequently, the plate was incubated for 10 min at 37 °C for 48 h. Fluorescence readings were then conducted on the Tecan Infinite F5000 plate reader, with excitation at 535 nm (25 nm bandwidth) and emission at 615 nm (10 nm bandwidth).

Drug sensitivity testing data analysis

The drug sensitivity score values were calculated by uploading the raw fluorescence data along with the control data (DMSO and BzCl) in the Breeze open-source pipeline[35]. Here the DMSO (negative control) and BzCl (positive control) are normalized to 100 and 1 respectively. The drug sensitivity score, LC50, and area under the curve (AUC) were calculated for single and double drug combinations. Clustering (unsupervised) of DSS values for single drugs on a leukemia patient cohort was plotted using the NG-CHM Builder heatmap tool[36] using the Euclidean distance and Ward linkage method. Synergy in drug combinations was calculated by uploading raw data into the Synergy-finder open-source pipeline[37]. The Bliss prediction model was applied for synergy analyses[38] and visualized using the SYNERGYFINDER open-source pipeline[39].

Results

Drug sensitivity testing of CLL, CML, and AML patient-derived cells

A customized leukemia drug library (Table S3 and S4) was used to perform single and double-drug combination screening. Prestoblue-based viability readouts were recorded at 48 h to quantify and interpret drug efficacy using drug sensitivity scores (DSS). To validate the reproducibility, drug sensitivity testing using 30 single drugs (Supplementary Table S3) was performed on the HeLa and K562 cell lines (Suppl. Figure 2 and 3). 30 single drugs were selected based on the drugs that are given for treatment in the current cohort and clinical trial elsewhere along with drugs that are tested for drug sensitivity testing for leukemia previously[40]. High reproducibility for biological replicates was observed from a drug sensitivity test on the HeLa cell line ($R^2 = 0.986$) and K562 leukemia cell line ($R^2 = 0.9639$). The leukemia cell viability showed 90% viability up to 48 h and 72 h with 50% viability (data not shown). Ex vivo drug sensitivity test for leukemia patient-derived cells (annotation of patient samples are shown in Suppl. Table S5) was then performed (Fig. 1 and Suppl. Figure 4).

To identify effective drugs for leukemia patient samples, 30 drugs at four concentrations (1–1000 nm) were tested on 40 patient-derived cells and 1 healthy donor-derived cells. Previously, it has been reported that drug sensitivity score (DSS) is robust in capturing drug potency/efficacy[41] and hence, well suited for downstream analysis of the drug sensitivity screening data (Fig. 2a). Drug sensitivity score (DSS) is calculated using the integral (I) response for the concentration range that is more than the given minimum activity level A_{min} along with its slope at IC_{50} together with the top and bottom response asymptotes (R_{max} and R_{min}). A DSS score of more than 20 is considered high, while a DSS score less than 5 is considered low. Selective DSS is calculated by subtracting the DSS of patient samples with the DSS of healthy donors. Differential drug responses as DSS were evaluated using heatmap cluster analysis. Clustering in drug response for all patient samples with both DSS and selective DSS was observed with the red label representing high sensitivity to the drugs (Figs. 2b–e, and 3). Two drugs and two patient clusters were identified. Bortezomib, 5-azacytidine, chlorambucil, vinblastine, and gemcitabine were observed to be the most effective compared to healthy samples, consistent with relevance in AML, CML, and CLL (red clusters in Fig. 3). Second drug clusters include doxorubicin, imiquimod, fludarabine, nilotinib,

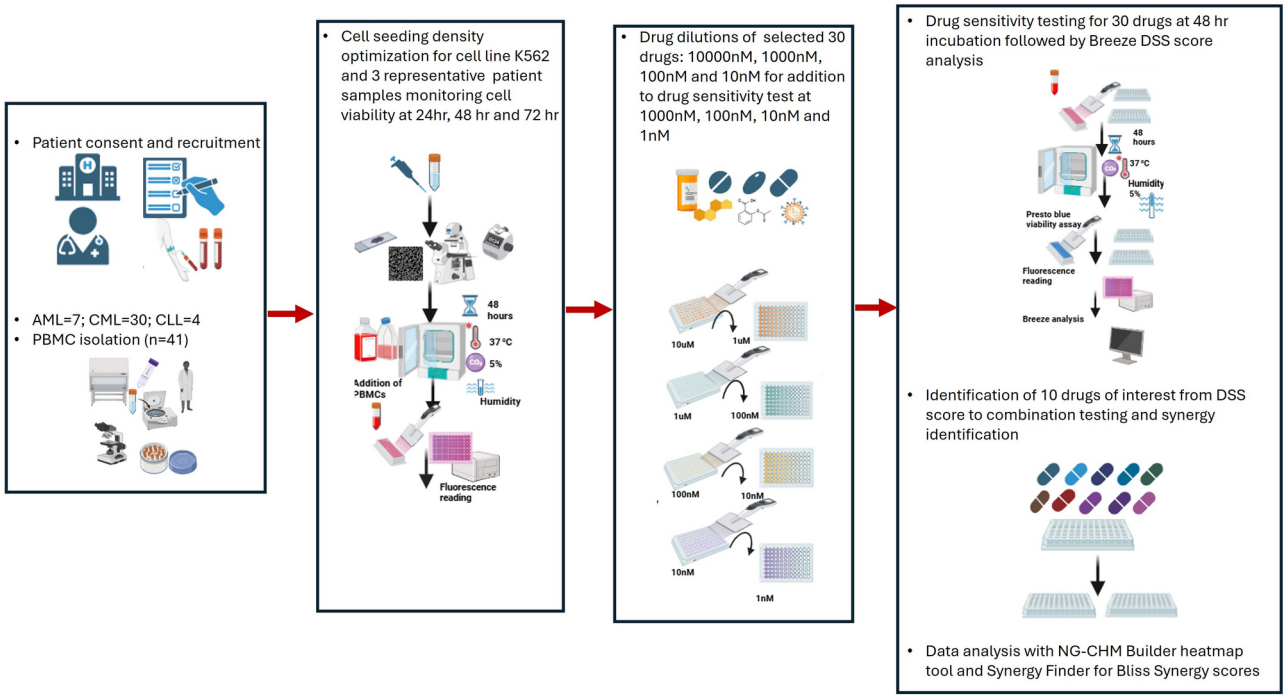

Fig. 1. Drug sensitivity testing workflow.

everolimus, and irinotecan which displayed more variable drug efficacy across the patient samples (blue clusters in Fig. 3). There is no obvious association between patient clinical data and patient DSS clusters. These results confirm the previously published results that compared the drug effects between Norwegian patient samples and healthy donor[42]. Previously, it has been reported that bortezomib is effective in the treatment of hematologic malignancies such as AML and ALL[43–45]. 5-azacytidine has been reported to have enhanced the efficacy of several chemotherapeutic drugs in AML[46]. There have been reports on chlorambucil with the honokiol (HN) as carrier being more selective in inhibiting lymphocytic leukemia (LL) cell survival compared to PBMCs from healthy donors[47]. Vinblastine has been reported to induce NOXA and acutely sensitize CLL cells[48]. Gemcitabine has also been previously reported to be a potent drugs for tumors and for pediatric AML[49,50]. Doxorubicin, imiquimod, fludarabine, nilotinib, everolimus, and irinotecan have been reported to have varying effects on Leukemia cells[51–53].

Leukemia treatments are mainly based on the multi-drug combination treatment regime[54–59]. The synergistic effects of leukemia drugs on leukemia patient-derived cells are understudied. Furthermore, there are no literature studies on the synergistic effects in the South African leukemia patient cohort. Due to the limited patient material available, the effects of double-drug combinations were tested using the primary drug at 10 nm concentration with a secondary drug at four concentrations (1–1000 nm) for 48-h incubation (Table S4). The top 9 drugs with high average DSS scores (Fig. 2a) and 5-azacytidine (used for patient treatment in the study cohort) were selected for drug combination experiments. A heatmap was developed using the DSS values of 40 patient samples for anchored drug combinations. Drug combinations of nilotinib with irinotecan, fludarabine with cladribine, and dasatinib with bortezomib were effective in at least 30 patient samples when compared to the effects observed in single drugs. Heatmap analysis for anchored drug combinations indicated that leukemia patient-derived cells were collectively more sensitive to combination treatment (Fig. 4). Synergistic effects for drug combinations (fludarabine with cladribine and nilotinib with irinotecan) in CML04 samples were observed (Fig. 5a and b). Bliss independence model was used to analyze the combination data for both anchored and full matrix drug combinations. The bliss independence model is the most common model used for combination data analysis[60].

Full matrix drug combination studies for Nilotinib with Irinotecan and, fludarabine with cladribine were performed on at least three patient samples (AML04, CML04, and BH30). The synergy score was calculated using the Bliss independence model where a score less than 0 is considered antagonistic effects, a score of 0 is considered additive effects and a score of more than 0 is considered synergistic. In the synergy plot, the red colour represents synergistic effects and the green colour represents antagonistic effects. High synergistic effects in CML04 for at least double drug combinations (fludarabine with cladribine and nilotinib with irinotecan) were observed. The Bliss synergy score for fludarabine and cladribine drug combinations was 30.2, while nilotinib and irinotecan combinations were 19.62 respectively (Fig. 6a and b). Previously, there was a study comparing the cladribine- and fludarabine-based chemotherapy in relapsed/refractory AML that demonstrated differential survival outcomes for the cladribine-fludarabine combination[61]. There have also been reports of synergistic effects of fludarabine and cladribine against lymphoid malignancies with increased severe hematological toxicity[62]. In the case of nilotinib and irinotecan drug combination results, there has been a report of synergistic effects of

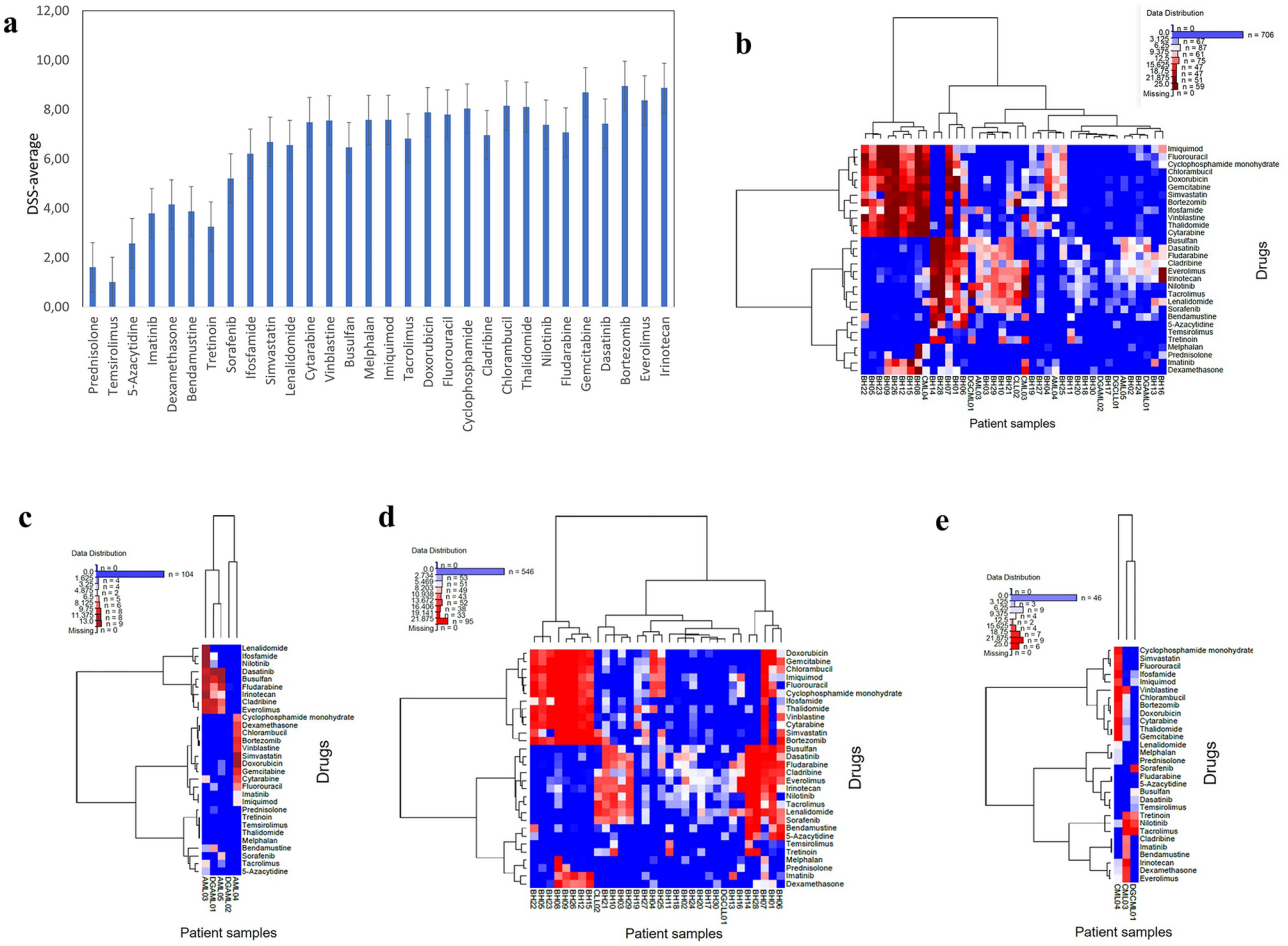

Fig. 2. (**a**) Average DSS scores for all the 30 drugs tested in 40 patient samples. (**b**) Heatmap in the single drug sensitivity screening analysis of the patient samples. Here, samples annotated as AML, and DGAML represent AML; samples annotated as BH and DGCLL represent CLL; and samples annotated as CML and DGCML represent CML. (**c**) Heatmap for the single drug senstivity screening on AML patient samples. (**d**) Heatmap for the single drug senstivity screening on CLL patient samples. (**e**) Heatmap for the single drug senstivity screening on CML patient samples.

irinotecan with Axitinib both in vitro and in vivo concerning anticancer activity[63]. Axitinib and nilotinib are both tyrosine kinase inhibitors that target signaling pathways. Although there are synergistic effects with kinase inhibitors and irinotecan, there are reports of significant toxicity in clinical use[64]. Thus, using the full matrix experimental results, the platform can assess double drug combinations and their synergistic effects in an ex vivo drug sensitivity test setting environment. However, there is an additional need for research validation on identifying the toxicity level in the effects of identified drug combinations in the clinical setting.

Discussion

Ex vivo culturing of CLL, CML, and AML patient-derived cells is reported to be challenging to establish and implement in the laboratory setting. To implement ex vivo DSS testing, it is recommended to establish ex vivo culturing of leukemia patient samples and perform ex vivo DSS testing using a clinically relevant drug list for the limited number of leukemia (CLL, CML, and AML) cells available. This paper demonstrates the applicability of a drug sensitivity testing pipeline that can be used to identify synergy-linked drug sensitivities in South African AML, CLL, and CML patient cohorts. Ex vivo platform can identify the previously reported effective single drugs such as nilotinib and irinotecan for leukemia treatment in South African patient samples[65,66]. Furthermore, synergistic effects were identified in some patient samples for drug combinations such as nilotinib and irinotecan, fludarabine, and cladribine.

However, there are limitations to this platform that would include the need to generate more data from various patient samples to correlate ex vivo drug sensitivity testing with clinical responses. Another major limitation is the use of peripheral blood samples for drug sensitivity testing. Particularly in the case of AML, the percentage of blast cells in peripheral blood samples compared to bone marrow will be different. Further, there would also be a need for a high blast count for ex-vivo drug sensitivity testing on AML. High blast count can be achieved through the use of enriched bone marrow aspirate for drug sensitivity testing. Currently, this platform has not

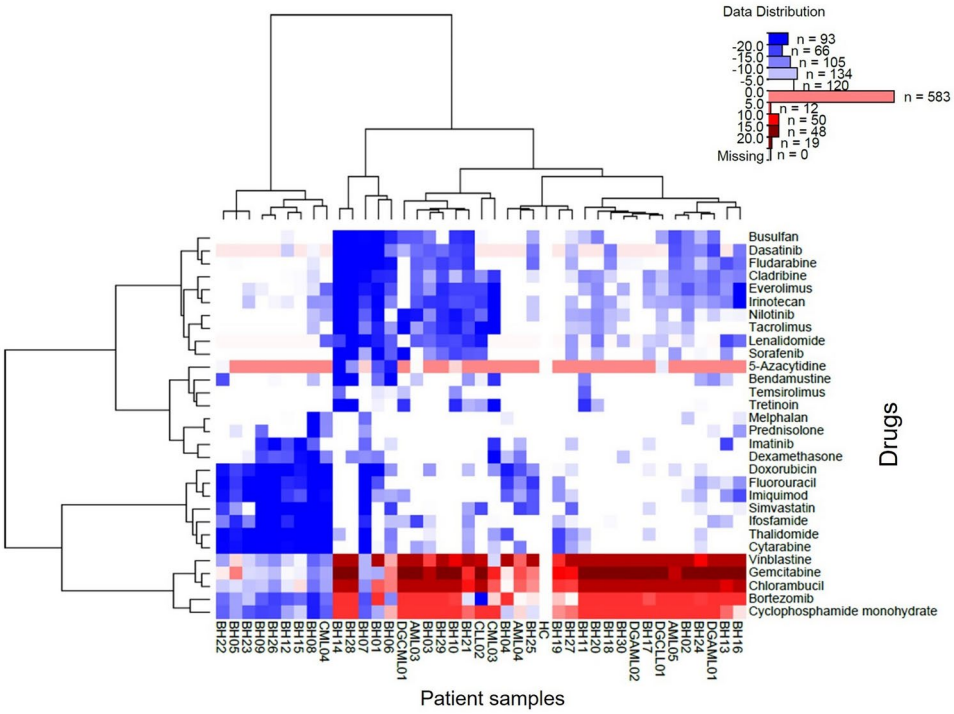

Fig. 3. Heatmap for selective DSS for single drug screening of 40 samples against the healthy donor samples.

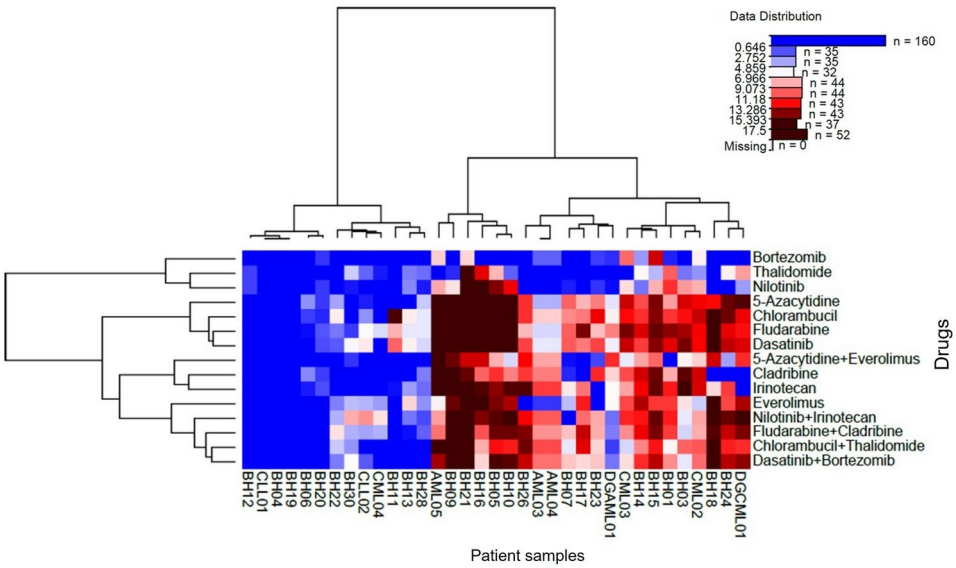

Fig. 4. Heatmap for double drug combination DSS score of 40 leukemia patients.

yet been optimized for bone marrow aspirate analysis. Ex vivo drug synergy testing can be used as the selection method that can select drug combinations for patient samples as a pre-clinical tool. As there is a need for such ex vivo drug sensitivity testing along with drug combination selection for leukemia patient samples, particularly in South Africa, this platform would be the foundation for further developing this technology for other cancers and developing strategies to identify possible effective drug combinations that can be further leveraged for n = 1 clinical trials. Furthermore, this platform would support the expansion of the methodology for testing drug sensitivity of the proliferating cancer cells from the South African patient cohort as demonstrated previously using the Norwegian leukemia patient samples[42].

The impact of this study would be the establishment of a First-in-Africa drug sensitivity test which can be further expanded to all types of cancer (blood cancer, liquid biopsy, and solid tumors). From a future perspective, the platform will be applied for drug sensitivity testing on solid tumors such as ovarian, uterine, and cervical

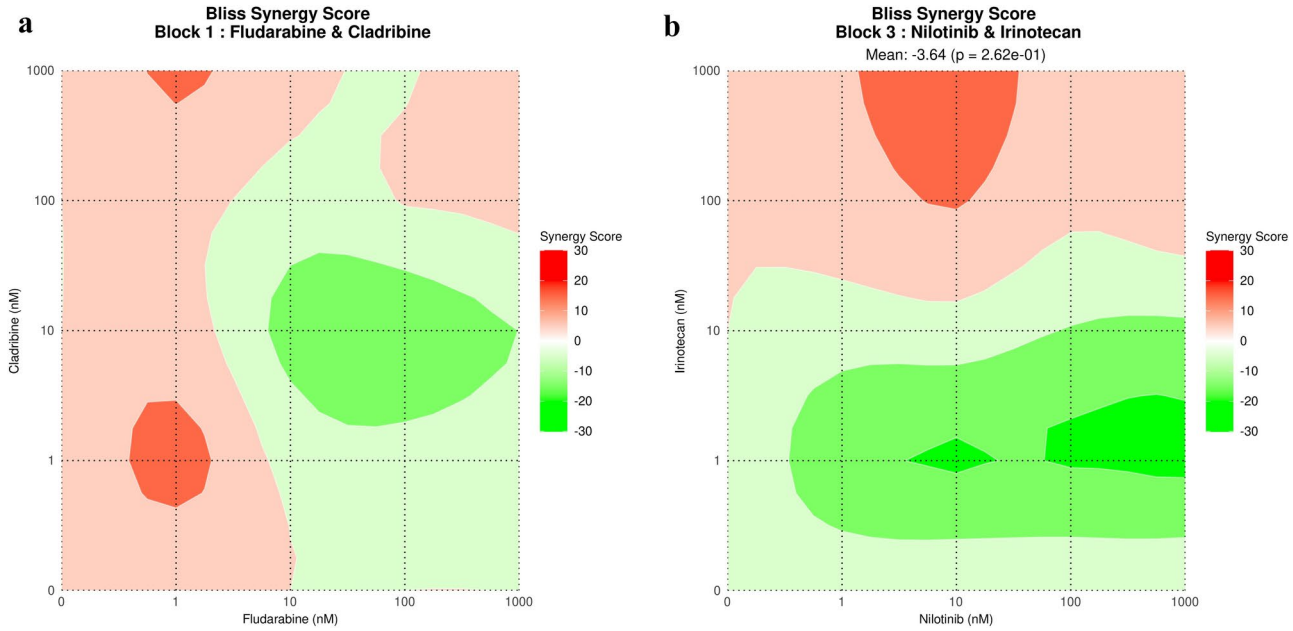

Fig. 5. Predicted bliss synergy results for anchored drug combination for CML04; (**a**) Fludarabine and Cladribine and (**b**) Nilotinib and Irinotecan.

cancer. The platform will be optimized to perform drug sensitivity testing using 2D and 3D spheroid cancer cells. The drug sensitivity testing will also be further developed to perform multi-drug combinations (three and four drugs) studies on the cancer cells. Using the results that will be generated in the coming years, the platform will be submitted for FDA and South African Health Products Regulatory Authority (SAPHRA) approval with the proceedings for clinical trial as a medical device.

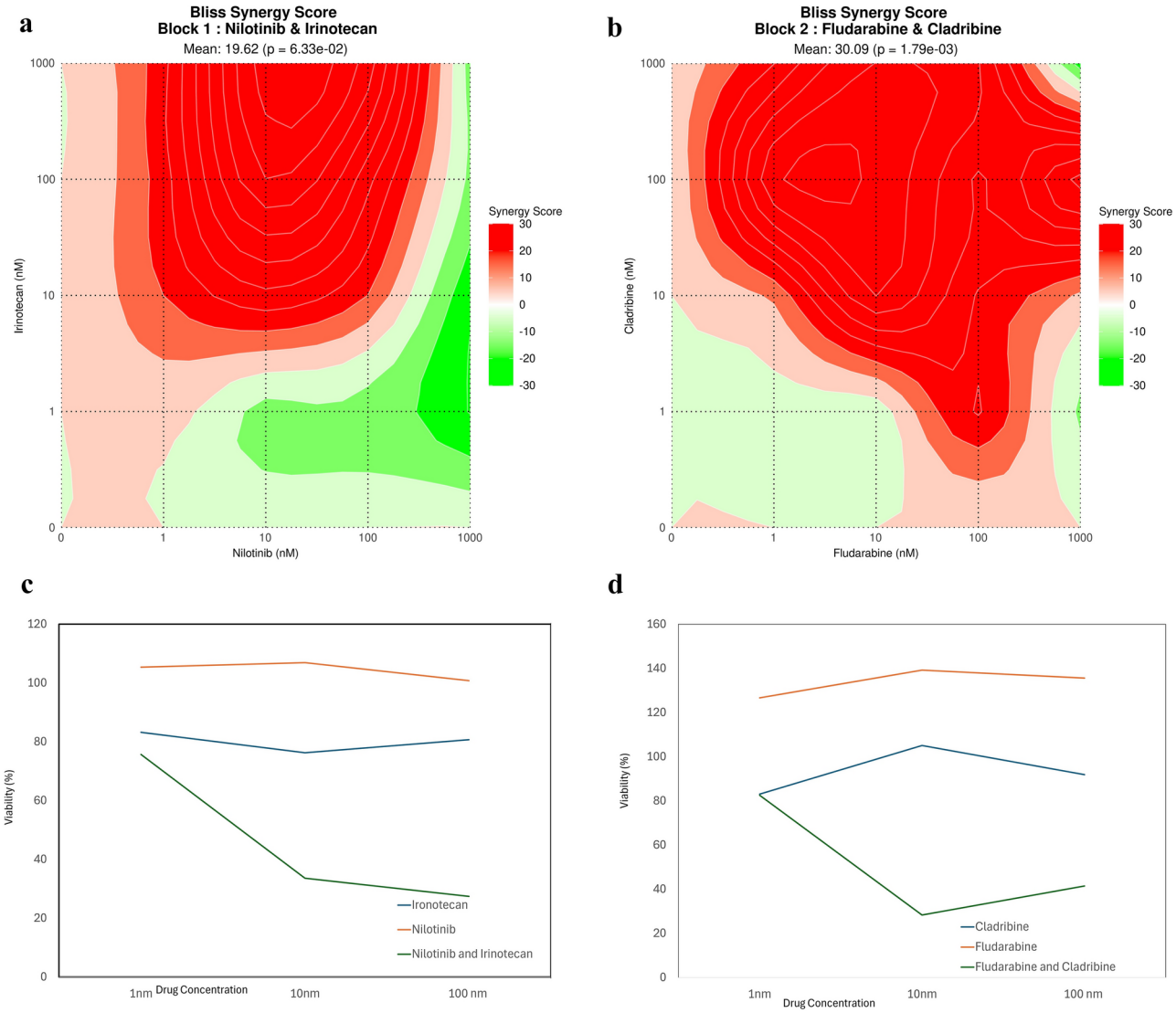

Fig. 6. Full matrix drug combinations for CML04. (**a**) Bliss synergy analysis for CML04 for drug combinations- Nilotinib and Irinotecan. (**b**) Bliss synergy analysis for CML04 for drug combinations- Fludarabine and Cladribine. (**c**) Concentration–response curve for single and drug combinations for Fludarabine and Cladribine; and (**d**) concentration–response curve for single and drug combinations for Nilotinib and Irinotecan.

Data availability

Datasets generated and analyzed in this study will be available from the corresponding author upon reasonable request.

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Author contributions

DBTGR designed the research. DBTGR and MMT designed the methodology. VLK performed the experiments with MMT, BM, and PD. VLK, and DBTGR, analyzed the data with inputs from MMT. DBTGR wrote the paper with inputs from MMT, BM, PD, PF, and EE. JDT and VPC contributed to the patient samples and clinical data. DBTGR, MMT, EE, and PNF co-supervised VLK. All authors read and commented on the paper.

Declarations

Competing interests

The author declares that they have no competing interests.

Additional information

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