# Development of artificial bone marrow fiber scaffolds to study resistance to antileukemia agents

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## Acknowledgment

To the editor:

The mechanisms for resistance in the chronic myeloid leukemia (CML) patients with no *BCR-ABL1* kinase domain mutations are unknown (1). Similar situations can be observed in other myeloid malignancies such as AML, in which patients may develop resistance to agents targeting molecular drivers, such as aberration of the *FLT3* gene, via mechanisms that are equally poorly understood (2). Many lines of evidence support the presence of persistent low-levels of quiescent leukaemia stem cells (LSCs) residing in the bone marrow (BM) niche and surviving independent of their tyrosine kinase activity, possibly through interaction with the microenvironment. There is also *in vitro* evidence that the interaction of leukaemia cells with an artificial scaffold in three-dimensional (3D) culture causes resistance to chemotherapeutic agents (3). 2D cell-based assays have limited value in predicting long-term clinical response to anti-cancer drugs (4) due in particular to absence of the extracellular matrix (5).

To address this experimental limitation, attempts have recently been made to produce 3D cultures (6). Polymeric fibers have been applied extensively in the manufacture of scaffolds for 3D culture because of their unique characteristics such as high surface area to volume ratio, high rate of absorption and low density. We hypothesized that a 3D culture using a fiber-based synthetic scaffold designed to mimic the BM microenvironment might provide an optimal microenvironment for proliferation of primary leukaemia cells (7). Pressurized gyration is a simple and cost effective technique which can generate fibers at high production rate with control of surface features such as roughness and porosity (8), therefore it was used for development of 3D Poly(methylmethacrylate)- hydroxyapatite (PMMA-HA) fiber scaffold in this study (*Supplementary information for method*). Scanning electron microscopy (SEM) images of the PMMA-HA fibers (Figure 1) reveal a rough surface containing nanopores and HA nanoparticles (Figure S1). Composite fibers were continuous and cylindrical in shape without any bead-on-string morphology indicating that the solution and process parameters were correctly tuned during the gyration process (Figure 1, S2). To demonstrate that these fibers are not toxic to hematological cells, leukaemia cells from an AML patient were cultured in the PMMA-HA scaffolds (*method described in supplementary information*). Microscopic observation revealed that these cells proliferated in the presence of the scaffold and concentrated around the fibers (Figure S3).

To investigate whether this culture was capable of recapitulating *in vivo* imatinib resistance, K562 cells were cultured in the presence or absence of the lowest inhibitory dose of imatinib (0.5 µM) in 2D and 3D culture for 72 hours followed by viability and proliferation assessment (*Supplementary information for method*). Imatinib-induced inhibition was more significant in 2D (70% reduction) compared to 3D culture (56% reduction; $p$=0.04) (Figure 2A). To investigate if the protective effect of PMMA-HA based 3D culture is specific against imatinib; the response of other leukemia cells to antileukemia agents was investigated. More live AML-derived HL60 cells were observed in the 3D culture after treatment with 10 µM doxorubicin (3) compared to 2D culture (8% v 4%, respectively). This difference did not reach significance ($p$=0.07), but suggested a trend toward more protection by scaffold (Figure 2B).

To further mimic the BM microenvironment, which has cellular and matrix components, the PMMA-HA-based culture was modified via the addition of HS-5 cells, a stromal cell line derived from human BM (*Supplementary information for method*). The co-culture of HS-5 and K562 cells has been reported to reduce the sensitivity of K562 cells to the inhibitory effect of imatinib due to the release of cytokines activating alternative survival signaling pathways (9). We found a reduced sensitivity of co-cultured K562 cells to imatinib, compared to those cultured alone (47% reduction versus 70% reduction; $p$=0.006) (Figure 2A). Similarly, co-culture of HL60 cells with HS-5 showed a trend toward reduced doxorubicin sensitivity compared to cells cultured alone, although this did not reach significance ($p$=0.055; Figure 2B). When added to the PMMA-HA-based culture, HS-5 cells appeared to be attracted to the scaffold (Figure S4A) as observed with K562 (Figure S4B) and AML cells. We then investigated whether the combination of scaffold with HS-5 stromal cells had any additive effect on the sensitivity of the K562 and HL60 cells to antileukemia agents. HL60 or K562 were added to the HS-5 3D culture followed by the addition of doxorubicin or imatinib 24h later. 72 hours after adding the drugs the live cells were counted. The combination of HS-5 cells and scaffold further reduced the sensitivity of K562 cells to imatinib compared to co-culture with HS-5 in 2D (30% versus 47% reduction; $p$=0.006) and to 3D scaffold without HS-5 (30% versus 44% reduction; $p$=0.008). The same experiment with HL60 treated with doxorubicin showed a similar trend although this did not reach significance (Figure 2B).

In summary, proliferation and concentration of primary AML, K562, HL60 and HS-5 cells around the fibers of this scaffold supported the suitability of this matrix for studying leukaemia cells. The induced relative resistance to either imatinib or doxorubicin supported the notion that the 3D structure of the BM protects cells against chemotherapeutic agents. The effect might be because the cells have not had the same degree of exposure to the drugs due to being shielded in the fibers where there may be a lower drug concentration, or alternatively because attaching to the fibers reduces cells' dependence on the targeted oncoprotein. Combining the PMMA-HA with HS-5 cells enhanced similarity to the BM microenvironment by encompassing all of its basic components: scaffold, stromal cells and cytokines, the latter secreted by HS-5 (10). This 3D model that we termed advanced PMMA-HA 3D culture fostered an increased level of resistance to imatinib compared to either 2D culture or PMMA-HA culture, with the stromal cells contributing a larger proportion of the phenotype. In this study, we have demonstrated that advanced PMMA-HA-based 3D culture recapitulates the resistance to targeted therapy observed in a subset of leukaemia cells *in vivo*. The system may therefore permit the design of therapies capable of circumventing the protective role of the microenvironment, allowing complete, rather than transient, cure from disease.

## Figures Legends

**Figure 1**. SEM images of PMMA-HA fibers. *A*: This is the SEM image of the PMMA-HA fibers. *B* and *C* show the pores on the surface and a cross section of the fibers respectively at higher magnification.

**Figure 2**. The effect of imatinib and doxorubicin on proliferation of the leukaemia cells in 2D and 3D cultures. *Figure A* shows the percentage of the viable K562 cells following 72 hours of treatment with 0.5 µM imatinib compared to untreated control for 2D, scaffold, HS-5 cells and combined scaffold and HS-5 cells conditions. *Figure B* shows the same experiment as 3A but for HL60 cells treated with doxorubicin.

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