

RESEARCH ARTICLE

Engineering a biocompatible 3D bone marrow niche model for drug screening in the *KMT2A*-rearranged acute myeloid leukaemia cell line MOLM-13

Lauren Hope^{1*}, Alvaro Sanchez-Rubio², Catherine Berry², Manuel Salmeron-Sanchez^{2,3,4}, Mhairi Copland¹, and Helen Wheadon¹

¹Paul O’Gorman Leukaemia Research Centre, School of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom

²Centre for the Cellular Microenvironment, School of Molecular Biosciences, University of Glasgow, Glasgow, United Kingdom

³Institute for Bioengineering of Catalonia, The Barcelona Institute for Science and Technology, Barcelona, Spain

⁴Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain

*Corresponding author:

Lauren Hope
(Lauren.Hope@glasgow.ac.uk)

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Abstract

Three-dimensional (3D) bioprinting technology has the potential to fabricate highly reproducible, advanced biocompatible *in vitro* 3D models. It has been used successfully to emulate aspects of the bone marrow microenvironment to study leukaemia, including acute myeloid leukaemia (AML). AML is an aggressive haematological cancer which arises due to the accumulation of genetic mutations in haematopoietic stem and progenitor cells within the bone marrow, leading to the production of leukaemic stem cells (LSCs). Interactions between LSCs and bone marrow stroma induce LSC quiescence, protecting them from chemotherapy. In this study, we engineered a 3D-bioprinted co-culture bone marrow model containing MOLM-13 AML cells in co-culture with two main supportive cell types: bone marrow stromal cells (HS5) and osteoblast-like cells (CAL-72). This system was used to explore the efficacy of the cyclin-dependent kinase 2/9 inhibitor fadraciclib alone and in combination with current AML therapies. Characterisation of alginate/gelatin hydrogels revealed a highly porous matrix structure, with a hydrogel stiffness within the range of human bone marrow. These hydrogels sustained MOLM-13 cells and HS5 spheroid viability for up to seven days, highlighting the suitability of this system to model bone marrow. Next, the efficacy of fadraciclib alone and in combination with current chemotherapies was assessed in two-dimensional (2D) culture and in the final 3D-bioprinted model. These data revealed that fadraciclib induced MOLM-13 cell death in both 2D and 3D culture, with a reduced level of cell death observed in 3D culture. Together, these data reveal a reproducible human bone marrow niche system, with potential for screening of novel single and combination therapies.

Keywords: 3D bioprinting; Additive manufacturing; Bioengineering; Leukaemia; Bone marrow; Chemotherapy; Hydrogels; Spheroids

1. Introduction

Acute myeloid leukaemia (AML) is the most common acute leukaemia in adults, with an average worldwide incidence rate of 1.73 cases per 100,000 people; incidence increases with age.¹⁻³ AML arises due to the acquisition of genetic mutations within haematopoietic stem and progenitor cells within the bone marrow, which can lead to the development of leukaemic stem cells (LSCs).⁴ LSCs interact with bone marrow stromal cells such as mesenchymal stem cells (MSCs), fibroblasts and osteoblasts, which can result in LSC quiescence, as well as remodelling of the bone marrow niche to support LSCs at the expense of haematopoietic stem and progenitor cells.⁵⁻¹⁰ Consequently, these interactions protect LSCs from chemotherapy, leading to treatment refractoriness and relapse.¹¹ Understanding and overcoming this protective microenvironment is critical for improving AML treatment.

Preclinical AML studies rely on two-dimensional (2D) human cell studies, genetically modified animal models, and patient-derived xenograft models in immunocompromised mice. However, 2D cell culture is far removed from the *in vivo* environment, resulting in differences between *in vivo* and *in vitro* systems, including differences in gene expression and cell behaviour.¹² Animal models provide a complete multi-organ system to study; however, there are many physiological and genetic differences between these animals and leukaemia patients, and primary leukaemia cells display inconsistent engraftment in mice, varying significantly with AML subtype.¹³ Three-dimensional (3D) *in vitro* bone marrow niche systems have gained popularity due to their closer similarity to the human *in vivo* environment compared to animal models and 2D culture models.¹⁴⁻¹⁹ Hydrogels are frequently used components of these systems. Hydrogels are polymers made from synthetic or naturally occurring materials that have been crosslinked to form a hydrophilic gel.²⁰⁻²² Hydrogels provide a 3D environment more analogous to the *in vivo* extracellular matrix compared to traditional 2D culture.^{20,23,24} Additionally, aspects such as diffusion kinetics are more similar to *in vivo* tissue compared to 2D culture.²⁵ Polymers used to make hydrogels can often be adapted for 3D bioprinting to make scaffolds that are more complex, yet highly reproducible and cost-effective.²⁶

Recently, 3D bioprinting has emerged as a technique with the potential to produce high-throughput, highly reproducible systems for tissue engineering,²⁷ disease modelling^{28,29} and drug screening.^{30,31} 3D bioprinting involves embedding one or more cell types within a biomaterial, such as a hydrogel, resulting in a bioink which can then be used to print structures containing these cells. Previously, 3D bioprinting has been utilised

to produce models which emulate the bone marrow microenvironment in healthy haematopoiesis,³² as well as haematological malignancies including AML,¹⁴ chronic lymphocytic leukaemia^{33,34} and multiple myeloma.³⁵ Together, this highlights that 3D bioprinting has the potential to manufacture reproducible humanised bone marrow models for the study of AML.

However, the stresses exerted on the cells through the printing process, such as shear stress, tension and compression forces, can affect cell viability.³⁶ Hence, the choice of hydrogel is influenced by its gelation, crosslinking capabilities and viscosity, as it is important to select a gel that will print well and will protect the cells against these stresses.³⁷ Alginate (Alg)/gelatin (Gel) hydrogels are frequently used as bioinks due to their good printability, good cell viability post-printing, and tuneable stiffness.^{20,26,38,39} Alginate is bioinert;^{40,41} however, it can be combined with gelatin, which contains cell adhesion sites and therefore improves cell viability.⁴² Gelatin alone is liquid at physiological temperature (37 °C);⁴³ therefore, the addition of alginate provides structural advantages and makes Alg/Gel suitable for 3D bioprinting.⁴⁴ Despite the wide use of Alg/Gel as a bioink,^{20,26,44} it has not been used to develop an AML model, highlighting a key area for further research.

Therefore, the aim of this study was to use Alg/Gel bioinks to develop a reproducible humanised 3D-bioprinted AML model, incorporating selected components of the bone marrow microenvironment. Our system is optimised for printing directly into a 96-well plate. This model was then used to explore the efficacy of a novel cyclin-dependent kinase (CDK) 2-and-9 inhibitor known as fadraciclib. Previous studies evaluating the efficacy of fadraciclib in AML cell lines, primary AML samples and *in vivo* studies have revealed that fadraciclib is an effective and potent inhibitor of AML cells, and combines synergistically with current AML therapies such as azacitidine, cytarabine and venetoclax.^{45,46} Here, our model was used to assess fadraciclib alone and in combination with current AML therapies: azacitidine, cytarabine, and venetoclax.

2. Materials and methods

2.1. Alginate/gelatin hydrogel preparation

Sodium alginate (Sigma Aldrich, United States of America [USA]) and gelatin type A (Sigma Aldrich, USA) powders were used to make 1.5× hydrogel stocks of 1%Alg/8%Gel, 1.5%Alg/8%Gel, and 2%Alg/8%Gel. Under sterile conditions, alginate was added to phosphate-buffered saline, and the bottle was incubated at 37 °C, mixing occasionally. When the Alg had dissolved, Gel was added under sterile conditions. The gel mixture was incubated at

37 °C, mixing occasionally, until the gelatin powder had fully dissolved. Alg/Gel hydrogel stocks were stored at 4 °C until use.

Prior to use, gel stocks were incubated at 37 °C for at least one hour. In cell-free experiments, the stock was diluted in Roswell Park Memorial Institute 1640 (RPMI; Gibco, USA) to make a 1× gel solution. In cell-based experiments, the gel was diluted by one-sixth in cell-specific media (RPMI or 50:50 RPMI/Dulbecco's Modified Eagle Medium [DMEM; Gibco, USA]), then diluted by a further sixth using media containing cells (MOLM-13: final concentration of 2×10^6 cells/mL of hydrogel; HS5 spheroids: final concentration of 3,500–4,000 spheroids/mL of hydrogel depending on spheroid yield). Alg/Gel hydrogels were crosslinked in a 150 mM calcium chloride (dissolved in distilled water; Sigma-Aldrich, USA) solution for 8–10 minutes. The gels were rinsed twice with media and incubated at 37 °C.

2.2. Oscillatory rheology

Hydrogels were produced as described above and cast into 15-mm-diameter polydimethylsiloxane moulds (Silicone elastomer curing agent and elastomer base; SYLGARD™ 184, Dow Inc., USA). After crosslinking, the gels were incubated in culture medium at 37 °C in 5% CO₂ for 1 hour, 1 day, or 2 days before measurements were taken. Rheology was completed using a modular compact rheometer (MCR 302, Anton Paar, Germany) using a 15-mm parallel plate with the platform heated to 37 °C to match physiological temperature. Strain sweeps were completed to identify the linear viscoelastic region at an angular frequency of 10 rad/s and a normal force of 0.5–1 N. Loss factor (ratio of loss modulus to storage modulus) was calculated using Equation 1, and the Young's modulus (E) was calculated using Equation 2:

$$\tan\delta = G''/G' \quad (1)$$

$$E = G' \times 2(1 + \nu) \quad (2)$$

where G'' is the loss modulus, G' is the storage modulus, and ν is Poisson's ratio, which was assumed to be 0.5 for hydrogels. Measurements were performed at a strain of 0.01–0.1% and an angular frequency of 10 rad/s.

2.3. Scanning electron microscopy

The gels were cast as described and incubated in culture medium for 1 hour or 2 days. Gels were dehydrated by submerging in liquid nitrogen for approximately 30–60 seconds, then freeze-drying at –80 °C for approximately 24 hours at 0 bar (FreeZone 12L Console Freeze Dryer, Labconco, USA). Freeze-dried gels were sliced and coated

with gold-palladium prior to microscopy. Scanning electron microscopy (SEM) was performed using JSM IT 100 (JEOL, Japan) running at 10 kV, and images were captured at 30×, 300×, and 2,000× magnification using INTOUCH Scope software version 1.05. These images were analysed using Fiji (versions 1.54h-t, NIH, USA). Approximate pore areas were calculated using Equation 3:

$$Area_{\text{Ellipse}} = \pi ab \quad (3)$$

where a represents the radius of the semi-major axis and b represents the radius of the semi-minor axis.

2.4. Cell lines

All cells were cultured at 37 °C in 5% CO₂, in culture medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) (all Gibco, USA). The human AML MOLM-13 cell line (DSMZ, Germany), which harbours the *KMT2A-AF9* fusion gene,⁴⁷ was cultured in RPMI medium with 20% fetal bovine serum (FBS; Gibco, USA). HS5 bone marrow fibroblast cells (ATCC, USA) were cultured in DMEM with 10% FBS (Gibco, USA) and 20% HS5-conditioned medium. CAL-72 osteosarcoma cells (ATCC, USA) were cultured in DMEM supplemented with insulin–transferrin–selenium (ITS; 1×; Gibco, USA) and maintained on Nunclon plates (Thermo Fisher Scientific, USA) to promote cell adhesion.

2.5. Generation of HS5 spheroids using AggreWell™ plates

GFP-HS5 spheroids were generated using 24-well (1,200 microwells) low-adherence AggreWell™ plates (Stem Cell Technologies, Canada) according to manufacturer's instructions. GFP-HS5 cells were seeded at a density of 100 cells/spheroid, and the AggreWell™ plate was incubated at 37 °C for 24 hours to allow spheroid formation. Spheroids were harvested by gently resuspending each well and passing the contents through a 37 µm strainer to remove single cells (average spheroid diameter: 86 µm).

2.6. 3D bioprinting of alginate/gelatin hydrogels

The hydrogel 2%Alg8%Gel containing MOLM-13 cells and HS5 spheroids was loaded into the cartridge (Nordson Engineering Fluid Dispensing, USA), and a 22G needle (Nordson Engineering Fluid Dispensing, USA) was added to the tip of the cartridge. The cartridge was then loaded into the 3D bioprinter (BIOX6, CELLINK, Sweden), which maintained the cartridge at 37 °C until printing. The constructs were designed using DNA Studio V3.3.2+7 (CELLINK, Sweden). Bioprinted gels were directly cast into a 96-well plate containing 200 µL of 150 mM calcium chloride solution per well, with one hydrogel per well. The

printing pressure was 8–12 kPa, with a dispensing time of 1.25 seconds per droplet of gel. The gels were incubated at 37 °C for 24 hours prior to drug treatment.

For the final 3D model, CAL-72 cells were seeded into a 96-well Nunclon™ Delta-treated plate (Thermo Fisher Scientific, USA) and incubated for 24 hours prior to printing. 3D bioprinting was completed as outlined above. Following the production of bioprinted gels, the medium covering the CAL-72 cells was aspirated and replaced with 50:50 media supplemented with ITS. Bioprinted gels were then transferred into the wells containing CAL-72 cells. Gels were then incubated at 37 °C for 24 hours prior to drug treatment.

2.7. Viability staining

NucBlue® Live stain (Ex/Em: 360/460 nm) from the ReadyProbes™ Cell Viability Imaging Kit (R37605, Thermo Fisher, USA) and ethidium homodimer-1 (Ex/Em: 517/617 nm) from the LIVE/DEAD™ Viability Kit (Thermo Fisher, USA) were added to warm cell-specific media as per manufacturer's instructions, and then hydrogels were submerged in the mixture. The plate was incubated at 37 °C for 15–30 minutes, protected from light. Gels were imaged using the EVOS M7000 Imager (Invitrogen, USA) or the Zeiss Axiovert (Axio Observer 7 Inverted Microscope, Zeiss, Germany). Images were processed using Fiji and QuPath (version 0.7.0; <https://qupath.github.io/>), whereby the number of live (NucBlue®-positive) and the number of dead (ethidium homodimer-1-positive) MOLM-13 cells were counted, and the average MOLM-13 cell viability was calculated in Microsoft Excel (Microsoft Corporation, USA).

2.8. Drug treatment of MOLM-13 cells in 2D monoculture

For cell treatments, 10 mM stocks of fadraciclib (CYC-065, Cyclacel, United Kingdom) and azacitidine (Selleck Chem, United Kingdom) were prepared in dimethyl sulfoxide (DMSO) and stored at –20 °C and –80 °C, respectively. Venetoclax stocks (10 mM; Selleck Chem, United Kingdom) were prepared in DMSO and stored at –80 °C, with 1 mM stocks stored at –20 °C. 10 mM stocks of cytarabine (Sigma-Aldrich, USA) were prepared in water and stored at –20 °C. Stocks were diluted in RPMI before treatment of MOLM-13 cells. The cells were incubated with chemotherapy treatments at 37 °C for 48 hours before the completion of downstream experiments.

2.9. Drug treatment of MOLM-13 cells in 2D co-culture with HS5 bone marrow fibroblasts

HS5 cells were seeded at 3.125×10^4 cells/cm² in six-well plates (Greiner Bio, Austria) and cultured for 24 hours

before MOLM-13 cell seeding and drug treatment were completed (see above). Co-cultured cells were maintained in 20% RPMI and 10% DMEM with 20% HS5-conditioned media (in a 50:50 ratio).

2.10. Annexin V/DAPI staining of MOLM-13 cells

In co-culture experiments, surface marker staining was performed first to distinguish MOLM-13 cells (CD33⁺ CD73⁻) from HS5 cells (CD33⁻ CD73⁺) and CAL-72 (CD33⁻ CD73⁻). Cells were then stained in Hank's balanced salt solution with calcium and magnesium, containing Annexin V antibody (556419, BD Biosciences, USA) and DAPI stain (564907, BD Biosciences, USA), and incubated at room temperature, protected from light, for 15–20 minutes before flow cytometry analysis (BD FACS Canto™ II, BD Biosciences, USA). Flow cytometry data were analysed using FlowJo software.

2.11. Quantitative reverse transcription polymerase chain reaction

First, MOLM-13 cells (CD33⁺ CD73⁻) were isolated from an HS5 (CD33⁻ CD73⁺) co-culture using fluorescence-activated cell sorting based on cell surface markers. RNA was extracted using either the RNEasy Micro kit (74004, Qiagen, the Netherlands) or the Arcturus PicoPure RNA extraction kit (KIT0204, Thermo Fisher Scientific, USA). Complementary DNA was generated using the High-Capacity cDNA Reverse Transcription kit (4368814, Thermo Fisher Scientific, USA). Sample target amplification was completed on all samples, followed by multiplex quantitative reverse transcription polymerase chain reaction (qRT-PCR) using the Fluidigm Biomark system (Biomark X9, USA). Primer sequences can be found in Table S2. The $2^{-\Delta\Delta C_t}$ method was used to determine the expression of selected target genes.⁴⁸ The average threshold cycle (Ct) of the housekeeping genes (*GUSB*, *ATP5B*, *ENOX2*, *TYW1*, *TBP*, *B2M*) was subtracted from the average Ct of each target gene to obtain ΔC_t . This value was then subtracted from the No Drug Control (NDC) average ΔC_t value ($\Delta\Delta C_t$), and the fold change was calculated using $2^{-\Delta\Delta C_t}$.

2.12. Statistical analyses

Unless otherwise stated, all data are presented as the average of three replicates with standard deviation as error bars. Raw data were processed using Microsoft Excel, and then statistical analyses were performed using Prism 10 (GraphPad, USA). Shapiro–Wilk normality testing was completed to assess the distribution of the data. Parametric tests included *t*-tests, one-way ANOVAs with multiple comparisons and two-way ANOVAs with multiple comparisons. Mann–Whitney *U* tests and Kruskal–

Wallis test with Dunn's multiple comparisons post-test were performed on datasets which were not normally distributed. Significance was depicted using asterisks as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, and **** $p \leq 0.0001$. Analysis was completed within and between groups; non-significance is indicated via the absence of asterisks. Heat maps representing the multiplex qRT-PCR results were produced using Prism 10. The $2^{-\Delta\Delta C_t}$ method was used to calculate fold change relative to NDC, then fold changes were $\log_{10}$ transformed to produce the values included in the heatmap.

3. Results

3.1. Characterisation and optimisation of alginate/gelatin hydrogels for use in a 3D-bioprinted acute myeloid leukaemia model

Bone marrow is reported to have a stiffness ranging 0.3–50 kPa,^{49,50} with the central marrow being the softest region (0.1–1 kPa) and the endosteal niche being the stiffest (25–50 kPa). Rheology was used to assess the viscoelastic properties and stiffness of hand-cast 1%Alg8%Gel, 1.5%Alg8%Gel, and 2%Alg8%Gel hydrogels, with and without MOLM-13 AML cells (Figures 1, S1, and S2). Except for the 1%Alg 8%Gel cell-laden condition, the Young's modulus significantly decreased with time in culture (Figure 1A–D). The Young's moduli of the 1%Alg8%Gel and 2%Alg8%Gel hydrogels decreased from 3.0 and 10.7 kPa on day 0 to 1.0 and 3.4 kPa, respectively, by day 2. On day 0, significant differences were found between the Young's moduli of cell-laden and cell-free conditions for 1% and 1.5%Alg8%Gel, with the cell-free hydrogels displaying a higher Young's modulus compared to cell-laden (Figure 1A–D). However, at later timepoints, there was no significant difference in Young's modulus between cell-free and cell-laden hydrogels (Figure 1A–D). The 2%Alg8%Gel hydrogels were selected for future experiments as this hydrogel composition exhibited the highest Young's modulus and therefore may be more suitable for emulating the stiffer endosteal bone marrow niche.

Bone marrow is highly porous; therefore, materials which aim to emulate this environment must also be suitably porous. SEM was therefore used to elucidate the microarchitecture of 2%Alg8%Gel hydrogels over two days in culture (Figure 1E–I). The average pore area after two days had significantly increased compared to day 0, and the standard deviation had also increased (Figure 1I). This indicates that the ions within the media could have disrupted the calcium crosslinking, resulting in increased pore size and increased heterogeneity of the hydrogel structure. This concurs with the rheology data, which revealed a decrease in Young's modulus after two days in

culture, potentially due to the increased pore size as the hydrogel degrades.²⁶ Previous work has also highlighted that these hydrogels initially swell when immersed in media but then begin to degrade as the ions in the media interact with the ionic hydrogel network.⁵¹ Next, the ability of small molecules to diffuse through the hydrogels was assessed by immersing the hydrogels in media containing nanoparticles conjugated to FITC (Figure S3). Here, it was observed that small molecules could diffuse through the network within two hours, with optimal diffusion after 24 hours.

To further determine whether 2%Alg8%Gel hydrogels were suitable as a bone marrow model, MOLM-13 cell and HS5 spheroid viability were assessed. MOLM-13 is an AML cell line which displays a *KMT2A* rearrangement. Previous research has shown that it is sensitive to fadaciliclib,⁴⁵ making it ideal to test on this proof-of-concept AML bone marrow model.^{45,46} Spheroids were selected instead of 2D adherent cell monolayers because spheroids display increased deposition of extracellular matrix proteins compared to 2D culture, as well as more relevant oxygen, nutrient and cytokine gradients compared to 2D monolayers.^{52,53} There were a few significant differences observed between MOLM-13 cells which had been cast within hand-cast and 3D-bioprinted hydrogels, indicating that bioprinting does not negatively impact MOLM-13 viability (Figure S4C and S4D). Additionally, similar results were observed with HS5 spheroids, which remained over 85% viable over seven days (Figure S4F). No significant differences were observed between MOLM-13 in monoculture and co-culture with HS5 spheroids in the hydrogels (Figure S4E).

MOLM-13 cells within 3D-bioprinted hydrogels exhibited a cell viability of approximately 70–75% at day 0, and there was no significant decrease in viability across all timepoints for the monoculture condition (Figure S4B, S4C, and S4E). Aside from day 7, there were no significant differences between the monoculture and co-culture conditions; however, through F-actin staining, there was a significant increase in MOLM-13 cluster size in monoculture compared to co-culture, particularly at later timepoints (Figure S5A, S5B, S5G, and S5H). Clustering of leukaemia cells has previously been observed in hydrogels.^{26,54,55} This indicates that the hydrogel supports the viability and proliferation of MOLM-13 cells. Spheroids establish oxygen, nutrient, and cytokine gradients more analogous to those found *in vivo*; therefore, this reduced clustering indicates that the presence of HS5 spheroids may be reducing the rate of MOLM-13 proliferation, hence reducing the cluster size. HS5 spheroids in both hand-cast and 3D-bioprinted conditions also remained round throughout all timepoints, indicating that the cells within the spheroid do not migrate into the surrounding hydrogel

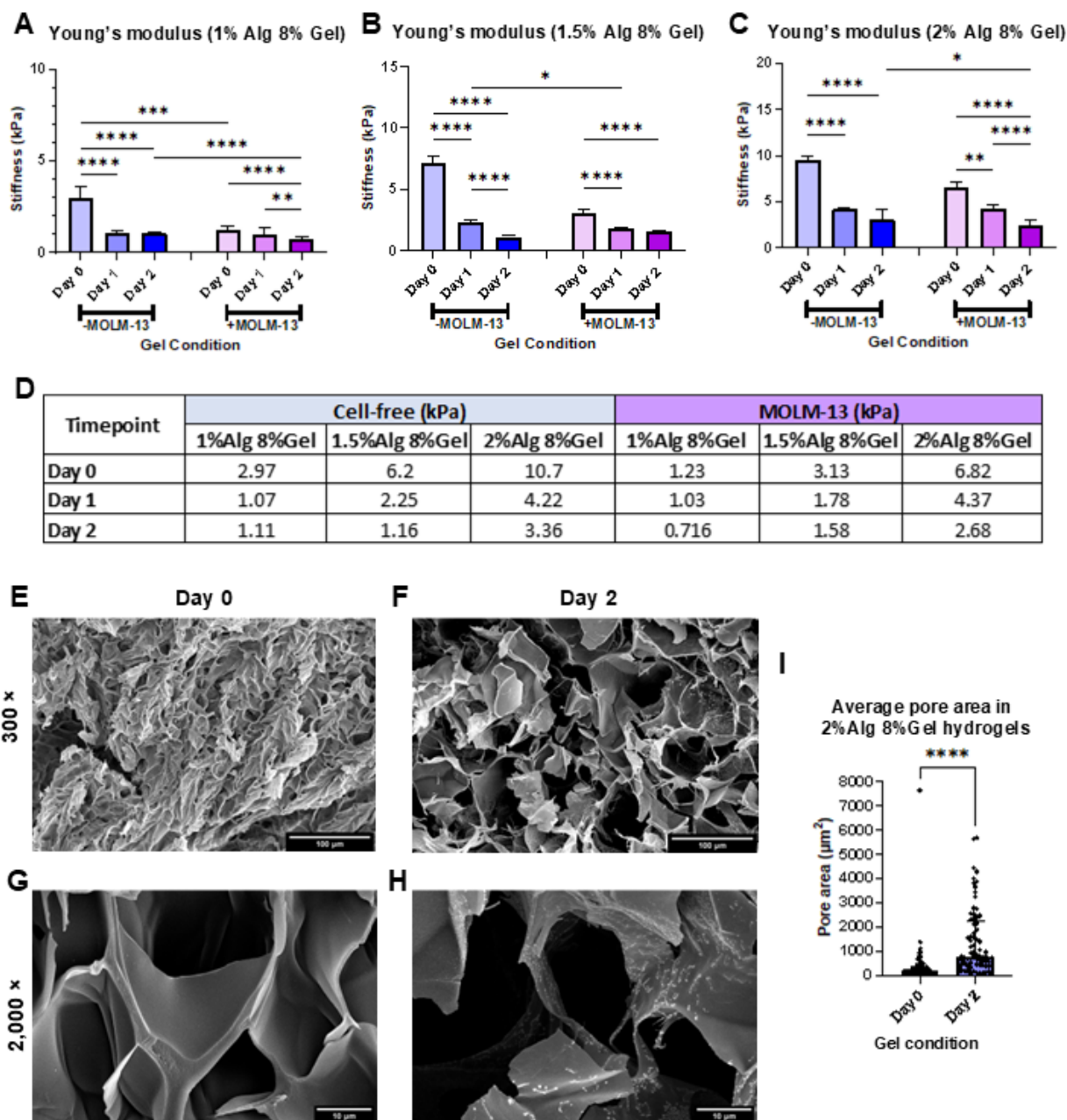


Figure 1. Characterisation of Alg/Gel hydrogel mechanical and structural properties. (A–C) Young's modulus calculated using the storage modulus (Equation 2) for (A) 1%Alg8%Gel; (B) 1.5%Alg8%Gel; (C) 2%Alg8%Gel. Results were compared using a Kruskal–Wallis test with Dunn's multiple corrections post-tests. (D) Average Young's modulus for each condition (kPa). (E–H) Representative scanning electron microscopy images of 2%Alg8%Gel hydrogels. (E) Day 0. Scale bar: 100 µm; magnification: 300×; (F) Day 2. Scale bar: 10 µm; magnification: 2,000×; (G) Day 0. Scale bar: 100 µm; magnification: 300×; (H) Day 2. Scale bar: 10 µm; magnification: 2000×. (I) Mean pore area calculated using Equation 3. Results were compared using the Mann–Whitney *U* test. Significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$. Bars represent the mean of three technical replicates, with error bars showing the standard deviation. Preliminary data were obtained from Ref.²⁶ (rheology data for cell-free Alg/Gel hydrogels and SEM images and pore-area calculations for day 0).

matrix and that 3D bioprinting does not disrupt HS5 spheroid morphology (Figure S5A, S5B, S5E, and S5F). Overall, these results show that 2%Alg8%Gel hydrogels

can support MOLM-13 cells and HS5 spheroids for up to seven days, and that the 3D bioprinting process does not affect cell viability or spheroid morphology.

3.2. Fadraciclib–azacitidine-induced apoptosis in 2D MOLM-13 cell culture

To assess the final 3D-bioprinted model, single and combination treatments using selected chemotherapies were first completed in 2D MOLM-13 monoculture and HS5 co-culture (Figure 2A). In this study, HS5 cells were selected as they exhibit similar expression patterns and surface marker expression to MSCs and can enhance the survival of leukaemia blasts *in vitro*,^{56–60} with reduced batch-to-batch variation compared to MSCs.^{61,62}

For this assessment of our 2D and 3D models, three drugs were selected in combination with fadraciclib based on previous work:⁴⁵ azacitidine, a hypomethylating agent;⁶³ cytarabine, which prevents DNA replication,⁶⁴ and venetoclax, which is a B-cell lymphoma 2 inhibitor.⁶⁵ All three of these are current chemotherapies which have been utilised effectively as combination therapies in AML.^{66,67} Previous work revealed a promising therapeutic window through the combination therapy of fadraciclib with azacitidine.⁴⁵ First, the half-maximal effective concentration (IC₅₀) for each drug was determined at 24, 48, and 72 hours (Figures S6, S7A–S7D, S8A–S8D, and S9A–S9D; Table S1). Fadraciclib was then combined with the selected chemotherapies at a range of concentrations (0.5× IC₅₀, 1× IC₅₀, and 2× IC₅₀) to determine optimal concentrations and timepoints (Figures S7E, S7F, S8E, S8F, S9E, and S9F). Here, it was revealed that the optimal time point for drug treatment was 48 hours; therefore, for future experiments, this time point was selected. MOLM-13 cells in monoculture and co-culture were treated with the 48-hour IC₅₀s of fadraciclib (350 nM) and azacitidine (1,600 nM) as single and combination therapies (Figure S10A–C). Annexin V/DAPI and trypan blue staining indicated that the combination therapy induced more MOLM-13 cell death compared to either single drug treatment. This was observed to a lesser extent with co-culture, with fewer cells progressing to late apoptosis, indicating a protective effect by the HS5 cells (Figure 2B and 2C). For example, in the monoculture fadraciclib/azacitidine combination-treated condition, 4.8×10^4 cells (36%) were in late apoptosis, whilst only 2.13×10^4 combination-treated cells in HS5 co-culture (12.1%) had entered late apoptosis, with most of the cells remaining viable or in early apoptosis (Figure 2B and 2C). Similar results were obtained for fadraciclib combined with other selected AML therapies, cytarabine and venetoclax (Figures 2C and S10D–S10I).

This protective effect was further assessed using multiplex qRT-PCR. Almost global downregulation of genes within 2D monoculture MOLM-13 cells and upregulation of several genes in 2D MOLM-13/HS5 co-culture was observed (Figure 2D). Genes affected by

fadraciclib either directly or indirectly, which include *CDK2*, *CDK9*, *MCL1*, *HOXA9*, and *MEIS1*, were downregulated in monoculture but upregulated in HS5 co-culture (Figure 2E). Genes relating to HS5-mediated protection of MOLM-13 cells were also examined (Figure 2F). *SOX9* displayed significantly increased expression in co-cultured MOLM-13 cells compared to monoculture. *SOX9* encodes a transcription factor which has been associated with cell proliferation,^{68,69} cell cycle regulation,^{69,70} and stem cell homeostasis.⁷¹ Transcription of *SOX9* is stimulated by interleukin (IL)-6, which is secreted by HS5 cells.^{72,73} Together, this indicates that upregulation of *SOX9* in MOLM-13/HS5 co-culture could be induced by cytokines released from the HS5 cells and could show that *SOX9* has a role in self-renewal and maintenance of MOLM-13 cells, as observed in other cancers.^{74–76} Similar trends were also observed with the cytarabine- and venetoclax-treated conditions (Figure S11).

To model the endosteal niche, an osteoblastic component is required; therefore, CAL-72 cells were selected. CAL-72 is an osteosarcoma cell line that resembles primary osteoblastic cells more closely than other osteosarcoma cell lines.⁷⁷ Previous studies have revealed that CAL-72 co-culture with primary AML blasts results in increased blast proliferation and increased secretion of angiogenic factors such as IL-8, angiopoietin-1, and vascular endothelial growth factor.^{78–80} Initial 2D co-culture experiments were conducted using MOLM-13 and CAL-72 (Figure 3A). Absolute MOLM-13 cell counts decreased upon treatment with fadraciclib and azacitidine, with a further significant decrease in combination therapy (Figures 3B and S12). Correspondingly, a large proportion of drug-treated cells progressed toward early and late apoptosis (Figure 3B and 3C). These results indicated that the treatments remained effective at inducing apoptosis of MOLM-13 cells despite the presence of the CAL-72 monolayer. Overall cell counts appeared to be lower, even in the NDC (72.12×10^4 viable cells in monoculture compared to 43.06×10^4 cells), which shows reduced cell proliferation in co-culture and therefore contrasts with previous findings.^{78–80}

3.3. Greater chemoprotection of acute myeloid leukaemia cells in the 3D-bioprinted model than in the 2D monoculture and co-culture models

MOLM-13 cells were then bioprinted within 2%Alg8%Gel hydrogels with or without the presence of HS5 spheroids (Figure 4A). Spheroids were selected due to their more physiologically relevant oxygen, nutrient, and cytokine gradients compared to 2D monolayers.^{52,53} Bioprinted hydrogels containing MOLM-13 cells in monoculture or in HS5 spheroid co-culture were treated with fadraciclib alone

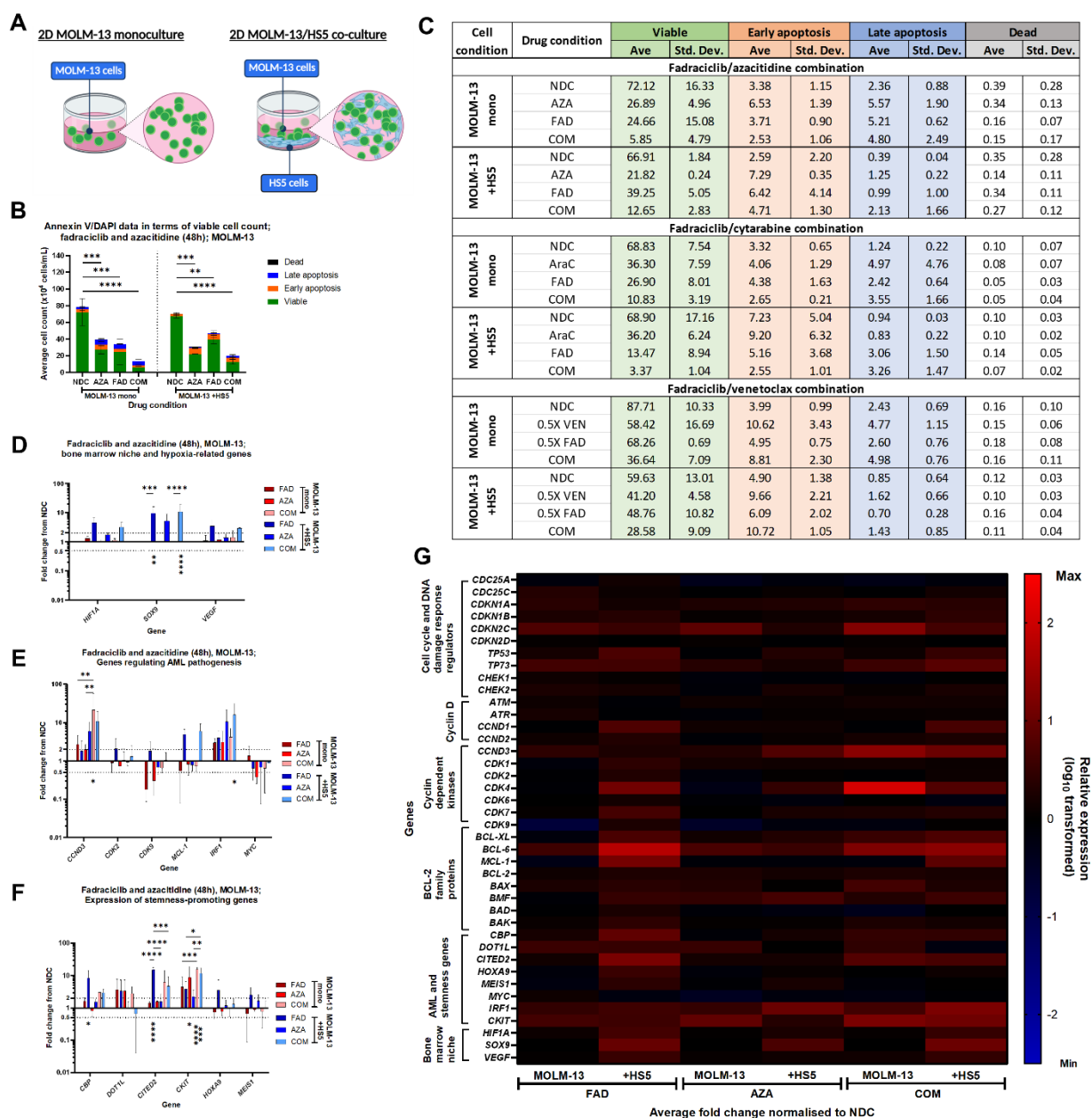


Figure 2. Efficacy of fadraciclib and azacitidine in 2D monoculture and HS5 co-culture. (A) A schematic of MOLM-13 cells in 2D monoculture and co-culture. Created in BioRender. Hope, L. (2026) <https://BioRender.com/4hj9gpi>. (B) Annexin V/DAPI data expressed in terms of cell counts for MOLM-13 cells treated with fadraciclib (FAD), azacitidine (AZA) or combination (COM) therapy in monoculture (MOLM-13 Mono) (left) and co-culture (MOLM-13 +HS5) (right). (C) A table outlining the average number of cells in each stage of apoptosis for the three combination-therapy datasets in monoculture and co-culture; cell counts are expressed as $\times 10^4$ cells; (D) Heat map displaying differences in gene expression for FAD/AZA treated MOLM-13 cells in monoculture and HS5 co-culture. Blue: downregulated compared to No Drug Control (NDC); red: upregulated compared to NDC. (E) Bar chart of selected genes affected by drug treatment. Two-way ANOVAs with multiple comparisons were used to detect significant differences between conditions in (E) and (F). Significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$. Bars represent the mean of three independent biological replicates, with error bars showing the standard deviation. Analysis was completed within and between groups; non-significance is indicated via the absence of asterisks. Asterisks below the x-axis indicate a significant difference compared to NDC.

and in combination with the selected chemotherapies. Significant differences were observed between NDC (76%

viability in monoculture and co-culture) and all treated conditions (ranging from 57.5% to 62% viability); however,

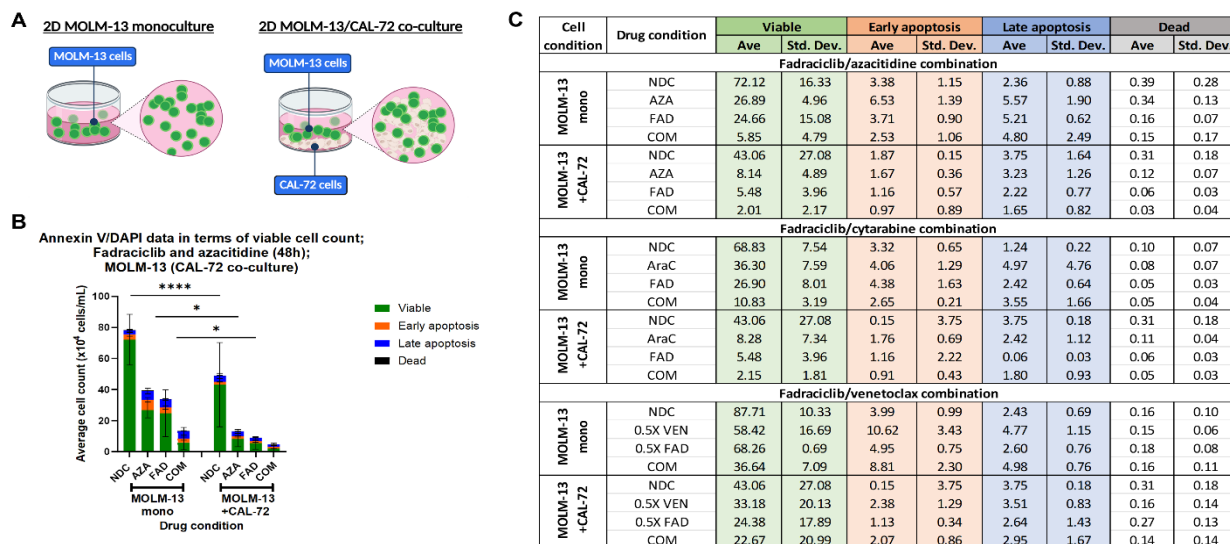


Figure 3. Assessment of fadracilicb and azacitidine efficacy in 2D CAL-72 co-culture. (A) A schematic of MOLM-13 cells in 2D monoculture and CAL-72 co-culture. Created in BioRender. Hope, L. (2026) <https://BioRender.com/nggmc79>. (B) Annexin V/DAPI data expressed in terms of cell counts for MOLM-13 cells treated with fadracilicb (FAD), azacitidine (AZA), or combination (COM) therapy in monoculture (left) and CAL-72 co-culture (right). (C) A table outlining the average number of cells in each stage of apoptosis for all three combination therapy datasets, in monoculture and CAL-72 co-culture. Significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Bars represent the mean of three independent biological replicates, with error bars showing the standard deviation. Analysis was completed within and between groups; therefore, non-significance is shown via the absence of asterisks.

no significant differences were seen between the single and combination therapies (Figure S13).

A CAL-72 cell monolayer was then cultured with bioprinted hydrogels containing MOLM-13 alone and MOLM-13 in co-culture with HS5 spheroids (Figure 5A). These cells were added as a monolayer instead of within the hydrogel system to mimic the endosteal localisation of osteoblast-like cells, which typically line bone surfaces.⁵¹ No significant differences were found between MOLM-13 cells in monoculture and HS5 co-culture in any treatment condition (Figures 5B and S14), similar to results obtained without the presence of CAL-72 cells (Figure 4B). Interestingly, MOLM-13 cells within the final tri-culture model displayed a decrease in viability of 5% when compared to azacitidine alone and 8% when compared to fadracilicb alone (Figure 5B). Although the decrease was not statistically significant, even modest changes in viability may be biologically relevant and should be explored in future studies.⁸¹

Clustering of MOLM-13 cells was observed in 3D-bioprinted hydrogels (Figures 4C and 5C), which could have led to increased cell numbers and therefore could influence the percentage of viable cells recorded. Therefore, the reduced clustering of MOLM-13 cells in

HS5 spheroid co-culture could indicate that the HS5 cells are slowing the proliferation of MOLM-13 cells, creating a more quiescent-like phenotype, similar to the results obtained in 2D co-culture. However, the results obtained with and without the CAL-72 monolayer were not significantly different, which indicates that the addition of the CAL-72 monolayer may not have added protection to the MOLM-13 cells compared to HS5 spheroids alone (Figure S15). This could be due to a lack of direct cell-cell contact, as found in AML and other leukaemias.^{57,82}

Finally, the data obtained from the 3D niche systems were compared to the 2D monoculture and co-culture data (Figure S15). In the 2D systems, the MOLM-13 viability in the NDC conditions was slightly higher than those in the 3D hydrogel systems; however, there were larger decreases in cell viability for each single treatment arm and a more prominent decrease in combination arms in the 2D cultures (Figures 2B, 2C, 3B, 3C, and S15) when compared to those observed in 3D culture (Figures 4B, 4C, 5B, 5C, and S15). Correspondingly, this resulted in a higher normalised MOLM-13 cell viability in the 3D systems compared to the 2D systems (Figure S15B, S15D, and S15F). Together, this indicates that the 3D culture system confers a greater protective effect on the MOLM-13 cells compared to 2D culture.

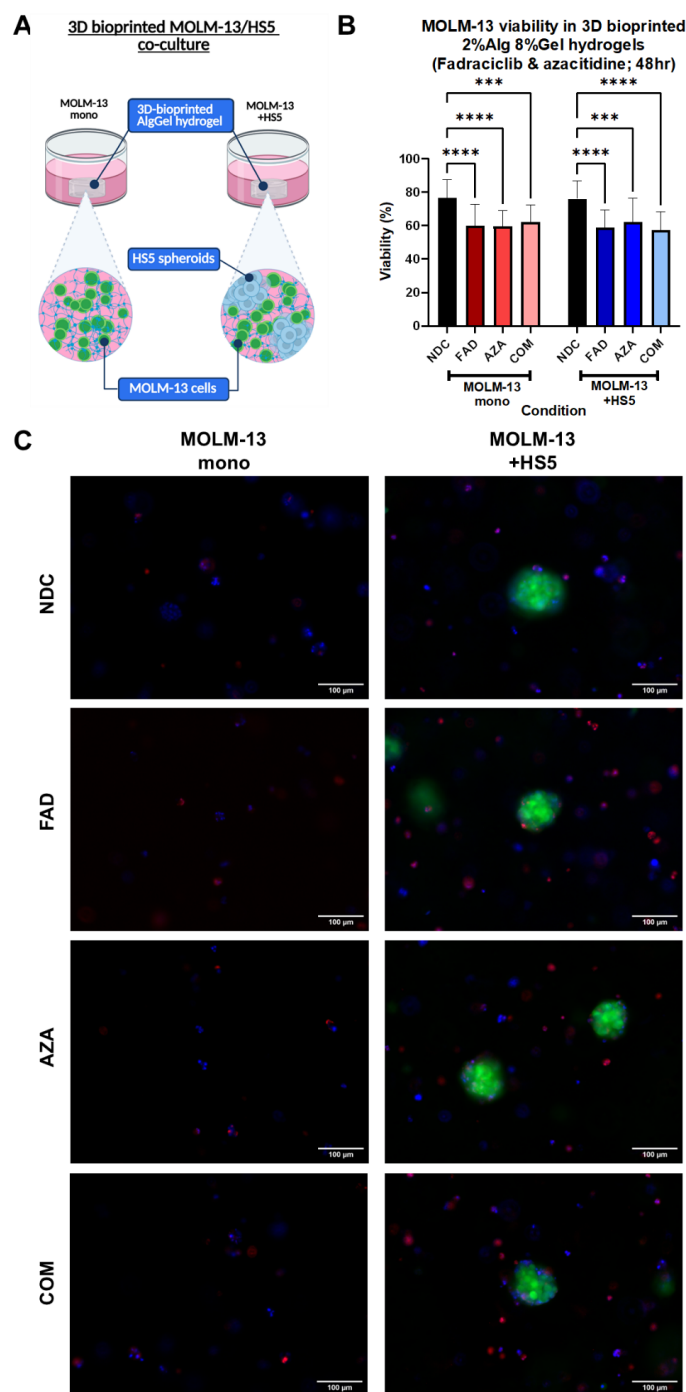


Figure 4. MOLM-13 cells in monoculture and HS5 spheroid co-culture within 3D-bioprinted 2%Alg8%Gel hydrogels, treated with fadraciclib and azacitidine. (A) Schematic of 3D-bioprinted MOLM-13/HS5 model. Created in BioRender. Hope, L. (2026) <https://BioRender.com/128bdxn>. (B) MOLM-13 cell viability within the 3D-bioprinted hydrogels. One-way ANOVAs with multiple comparisons were used to find significant differences between conditions. (C) Representative images of 3D-bioprinted MOLM-13 cells in monoculture (MOLM-13 Mono) and HS5 co-culture (MOLM-13 + HS5). Images taken at 20× using the M7000 Imager (scale bar: 100 µm). Green: HS5 cells (GFP-HS5 cells); blue: live cells; red: dead cells. Significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$. Bars represent the mean of three biological replicates from independent experiments, each of which had three technical replicates, with error bars showing the standard deviation. Analysis was completed within and between groups; therefore, non-significance is indicated via the absence of asterisks.

Abbreviations: AZA: Azacitidine; COM: Combination; FAD: Fadraciclib; NDC: No Drug Control.

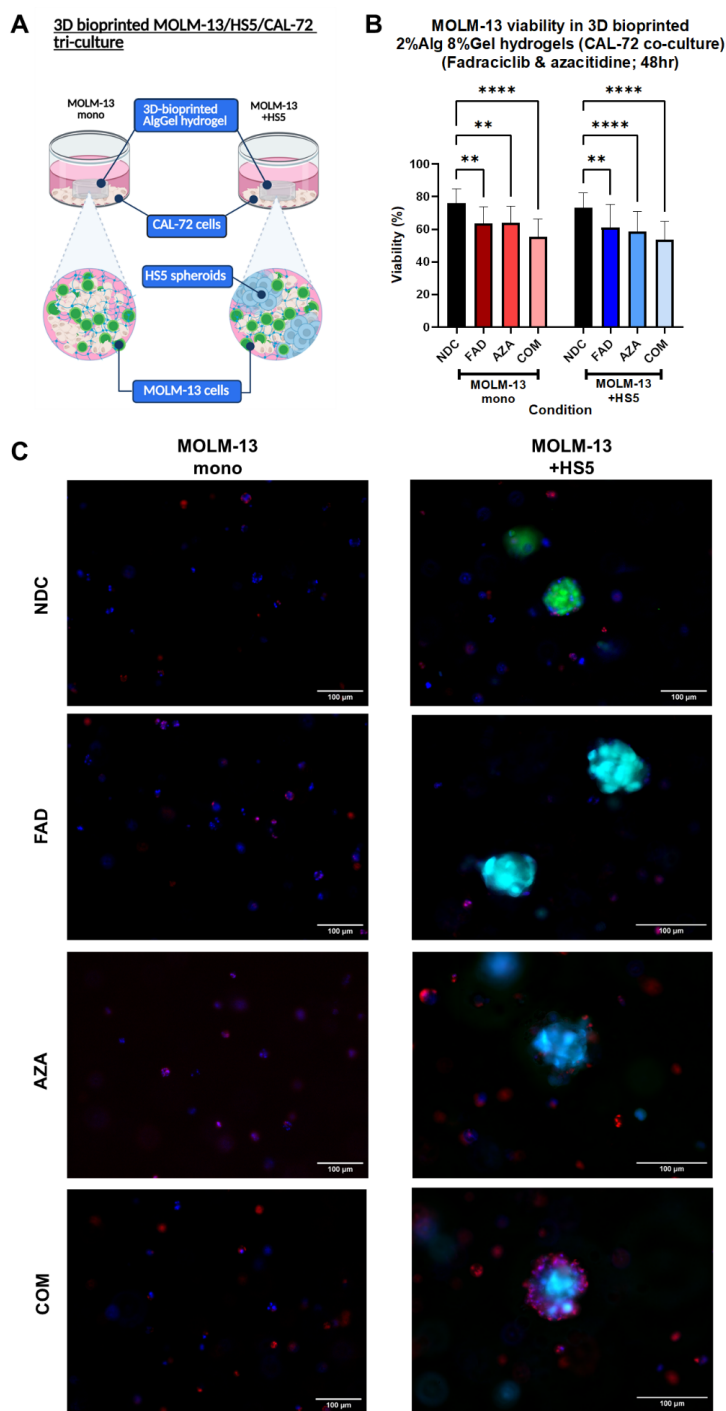


Figure 5. MOLM-13 cell viability within the final 3D-bioprinted model following chemotherapy treatment. A: Schematic of 3D-bioprinted MOLM-13/HS5 model with CAL-72 monolayer. Created in BioRender. Hope, L. (2026) <https://BioRender.com/o9ahyli>. (B) MOLM-13 cell viability within the final 3D-bioprinted model. (C) Representative images of 3D-bioprinted MOLM-13 cells in monoculture and HS5 co-culture within the final model. Images taken at 20 $\times$ using the M7000 Imager (scale bar 100 μ m). Green: HS5 cells (GFP-HS5 cells); blue: live cells; red: dead cells. One-way ANOVAs with multiple comparisons were used to detect significant differences between conditions. Significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$. Bars represent the average of three independent biological replicates, each with three technical replicates, with standard deviation shown as error bars. Analysis was completed within and between groups; non-significance is indicated via the absence of asterisks. Abbreviations: AZA: Azacitidine; COM: Combination; FAD: Fadraciclib; NDC: No Drug Control.

4. Discussion

Three-dimensional bioprinting has emerged as a novel high-throughput technology for generating reproducible bone marrow models for several blood cancer types.^{14,28,34,83} Alg/Gel hydrogels were selected as they have previously been shown to display high cell viability and good printability.^{20,26,38,39,84,85} SEM revealed that 2%Alg8%Gel hydrogels have a highly porous structure, with increasing pore size over time (Figure 1E–G). This increased pore size was reflected in a reduction in Young's modulus (Figure 1A–D). Interestingly, the presence of MOLM-13 cells did not significantly affect the rheological properties of the hydrogels (Figure 1A–D). This could be because MOLM-13 cells are unable to degrade the surrounding matrix, as there are no cell-cleavable sites on the alginate polymers. Regardless, the stiffness of 2%Alg8%Gel hydrogels remained within the range of bone marrow stiffness,^{49,50} highlighting its utility as a bone marrow model. MOLM-13 cell viability did not differ significantly between hand-cast and 3D-bioprinted hydrogels, indicating that the hydrogel matrix in which the cells were embedded protected them from the shear forces and other stressors experienced during the printing process.^{86,87} These data are consistent with previous research, showing that there were no significant differences in viability between hand-cast and 3D-bioprinted Alg/Gel hydrogels containing adipose-derived stem cells.⁸⁸ Staining of F-actin and cell nuclei showed that MOLM-13 cells formed clusters within the hydrogel over time, thus indicating they were able to proliferate. This mirrors previous studies culturing leukaemia cells within hydrogels,^{26,54,55} and highlights that bioprinting does not induce significant adverse effects on MOLM-13 cells. Together, this indicates that 2%Alg8%Gel hydrogels support the viability and proliferation of MOLM-13 cells within this hydrogel matrix. Further, HS5 spheroids maintained their shape and did not migrate into the hydrogel matrix, which could be because alginate lacks cell adhesion motifs and MMP-degradable sites required to promote the migration of HS5 cells out of the spheroid.^{40,41} Cell spreading has been observed in hydrogel compounds with more cell binding motifs and MMP-cleavable sites.^{26,89,90} Altogether, these data highlight the potential for 2%Alg8%Gel hydrogels as the basis of this biocompatible bone marrow model.

Next, we determined the efficacy of our 3D-bioprinted bone marrow model for drug screening. In 2D culture, significant differences in MOLM-13 viability were observed between single-treated and combination-treated arms, whereas no significant differences were observed between these arms in 3D culture (Figure S15). This reflects that whilst the therapies were effective at inducing

apoptosis of MOLM-13 cells, the combination therapy was less effective in 3D compared to 2D cultures. Therefore, the bone marrow niche model developed appears to provide a higher level of chemoprotection compared to 2D culture. This increased drug resistance in 3D models compared to 2D has been observed in several previous bone marrow *in vitro* models,^{34,91} with resistance increased further when stromal cells such as MSCs are added to the 3D model.^{14,16} Bray *et al.*¹⁶ developed a 3D *in vitro* tri-culture model, revealing that AML cell lines and primary samples displayed increased resistance to chemotherapies in 3D culture compared to 2D, with this resistance increasing further in 3D co-culture with MSCs and endothelial cells. Treatment with a combination of chemotherapy drugs at clinically relevant concentrations resulted in a vast reduction in cell viability, similar to our findings, whereby 3D culture reduced sensitivity to chemotherapy. Sbrana *et al.*³⁴ developed a successful 3D-bioprinted bone marrow model for modelling CLL using CELLINK bioinks, which could be cultured for up to 28 days. However, they did not include stromal cells, which could have strengthened their model. In a separate study, Alhattab *et al.*¹⁴ developed a 3D-bioprinted AML model comprising AML cells with endothelial cells and MSCs cultured within a peptide hydrogel, and found increased chemoresistance and AML cell quiescence in 3D culture compared to 2D culture. In 3D culture, several genes associated with AML cell survival were upregulated, including *HIF1A*, *CCND1*, and *MYC*. This further highlights the advantages associated with 3D culture over 2D culture: in 3D culture, AML cells survive in an environment which is closer to *in vivo* conditions, and this affects their gene expression, proliferation and viability.⁹² As a result, this impacts the chemoresistance of AML cells, which could lead to more clinically translatable results compared to 2D culture. However, their hydrogels were made in-house, whilst alginate and gelatin are highly characterised and commercially available.^{20,26}

To represent the bone marrow endosteal niche, CAL-72 osteosarcoma cells were included in the model. CAL-72 displays many characteristics reminiscent of human primary osteoblastic cells and is frequently utilised as an osteoblastic model.^{77,93} On this basis, they were selected as part of the model. However, few significant differences were observed in 3D cultured MOLM-13 cells with and without the CAL-72 monolayer (Figure 5). This could be because AML cells require cell–cell contact to be fully protected from chemotherapeutic drugs. Previous studies using leukaemia cells cultured with stromal cells separated using Transwell inserts have indicated that direct cell–cell contact is necessary for increasing leukaemia cell chemoresistance.^{57,82} Therefore, the addition of CAL-72 cells as a monolayer may not be as effective as including them within the 3D hydrogel network.

Overall, the 3D-bioprinted AML model developed here effectively models certain aspects of the 3D leukaemic bone marrow microenvironment, such as high porosity, mechanical properties and reduced sensitivity to chemotherapy. Additionally, it is optimised with a standard 96-well assay format, and in general shows low variation between biological replicates (Figure S16). This model, in conjunction with current disease detection and stratification methods, could provide a method of assessing chemotherapies before administering them to AML patients. Next-generation sequencing is often completed on patients' blood using a selected panel of genes commonly associated with myeloid disorders.^{94,95} These data are then used to inform the subsequent treatment plan; however, the initial treatments may not always be effective, which can delay effective treatment and patient recovery. Potentially, our bone marrow niche system could reduce the time needed to identify the most suitable treatments. Administering selected targeted therapies to 3D-bioprinted hydrogels containing cells from the patient could highlight which are more effective, and these could translate to improved patient recovery. If this is effective, this system could be adapted for use in other haematological and solid disorders.^{20,87,96-98} However, it is

important to note that our model is a proof of concept and has been utilised only with one cell line. Further validation using other cell lines and primary cells would be necessary before progressing toward preclinical testing.

Finally, this system could be adapted to model other diseases and conditions. Previously, Alg/Gel hydrogels have been selected to model various cancers, including non-small cell lung cancer⁸⁷ and breast cancer.²⁰ This is likely due in part to the tuneable stiffnesses previously assessed using these hydrogels²⁶ and within this present study (Figure 1A–D), indicating that our model could be adapted to model other haematological and solid cancers. Additionally, these hydrogels are highly porous (Figure 1E–I), permitting the perfusion of media and chemotherapies (Figures 6 and S3). Finally, the spheroids maintain their structure for at least seven days in culture and remain viable (Figures S4 and S5). This highlights that this hydrogel formulation maintains a spheroid structure due to the lack of cell-cleavable sites. Many hydrogels, which are derived from natural sources, such as collagen, contain cleavable sites which are useful when studying biological processes such as migration, but do not maintain spheroid integrity whilst supporting cell viability; Alg/Gel bioinks are more suitable (Figures S4 and S5).

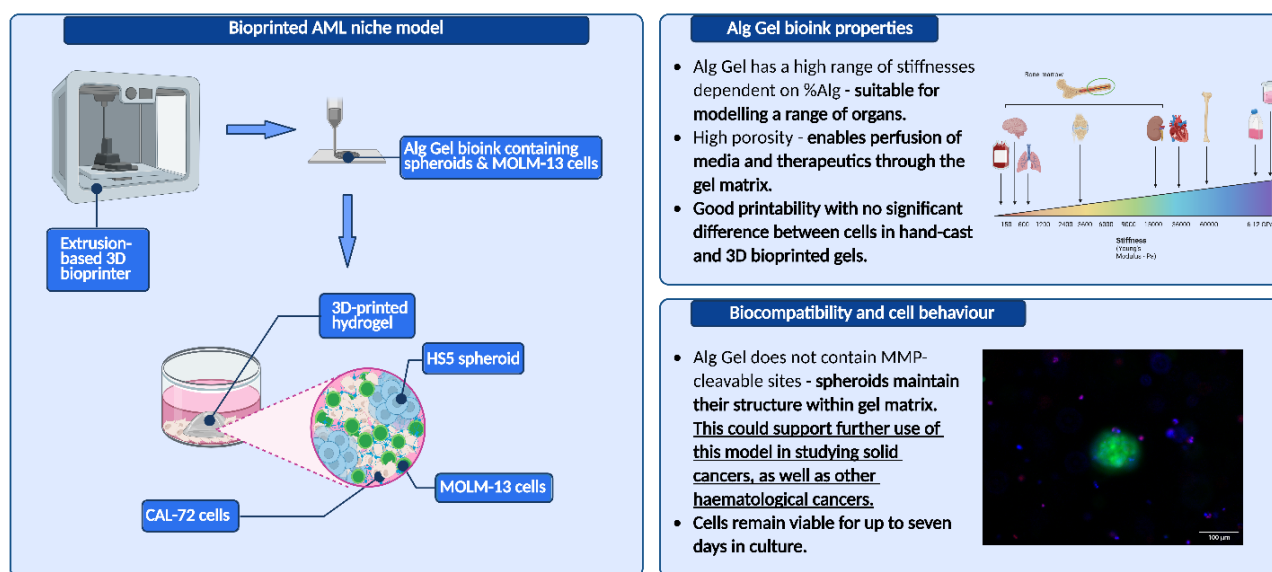


Figure 6. Description of this current model and an outline of features which could prove beneficial for modelling of other organs. The organ stiffness diagram was adapted from Ref.⁹⁹ with additional information taken from.^{49,50} Image of viability-stained MOLM-13 co-culture with GFP-HS5 spheroids within 2%Alg8%Gel hydrogel was taken using the Zeiss Axiovert microscope at 20× magnification (scale bar: 100 μm). Created in BioRender. Hope, L. (2026) <https://BioRender.com/njux9hk>.

5. Conclusion

In this study, a 3D-bioprinted bone marrow niche

model was produced, demonstrating a chemoprotective effect on MOLM-13 AML cells. This model comprised MOLM-13 cells and HS5 spheroids within a 2%Alg8%Gel

hydrogel, co-cultured with a CAL-72 cell monolayer. Whilst 3D-bioprinted AML models have previously been developed,¹⁴ this is the first 3D-bioprinted bone marrow model to use alginate/gelatin composite hydrogels, with an endosteal niche component. Greater viability following administration of novel therapeutic combinations was observed in the 3D model compared to 2D liquid culture, therefore producing results which more closely replicate human *in vivo* conditions compared to 2D culture. This model is also cost-effective, reproducible, and optimised for a standard 96-well plate format, and could potentially provide a robust model for drug screening in AML.

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Conflict of interest

Mhairi Copland has received research funding from Cyclacel and Incyte, is/has been an advisory board member for Novartis, Incyte, Ascentage, Jazz Pharmaceuticals, Pfizer, Azurity, Crossbow, Servier and Mendus, and has received honoraria from Astellas, Novartis, Incyte, Pfizer, Janssen and Jazz Pharmaceuticals. The other authors have no conflicts of interest to disclose.

Author contributions

Conceptualization: All authors

Formal analysis: Lauren Hope

Funding acquisition: Helen Wheadon, Mhairi Copland, Catherine Berry, Manuel Salmeron-Sanchez

Investigation: Lauren Hope, Alvaro Sanchez-Rubio

Methodology: Lauren Hope, Alvaro Sanchez-Rubio

Supervision: Helen Wheadon, Mhairi Copland, Catherine Berry, Manuel Salmeron-Sanchez

Writing—original draft: Lauren Hope

Writing—review & editing: Helen Wheadon, Mhairi

Copland, Catherine Berry, Alvaro Sanchez-Rubio

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data supporting this study can be found within the Results and Supplementary File, with further data available upon request. Preliminary data from **Figure 1** was originally published in Ref.,²⁶ and a more extensive data set, which included this data reanalysed with additional data added (presence of MOLM-13 cells in hydrogels used for rheology, and day 2 SEM analysis and images).

Further disclosure

Some of the data were presented as a poster at the European School of Haematology 6th International Conference, *Acute Myeloid Leukaemia “Molecular and Translational”: Advances in Biology and Treatment*, held in Estoril, Portugal, October 29–31, 2023.

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