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1 **Title: Targeting Survivin with YM155 (sepantronium bromide): A novel therapeutic strategy for paediatric acute myeloid leukemia.**

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Abstract

Despite aggressive chemotherapy, approximately one third of children with acute myeloid leukemia (AML) relapse. More effective treatments are urgently needed. Survivin is an inhibitor-of-apoptosis protein with key roles in regulating cell division, proliferation and apoptosis. Furthermore, high expression of survivin has been associated with poor clinical outcome in AML. The survivin suppressant YM155 (sepantronium bromide) has pre-clinical activity against a range of solid cancers and leukemias, although data in AML is limited. Therefore, we undertook a comprehensive pre-clinical evaluation of YM155 in paediatric AML. YM155 potently inhibited cell viability in a diverse panel of AML cell lines. All paediatric cell lines were particularly sensitive, with a median IC_{50} of 0.038 µM. Cell-cycle analyses demonstrated concentration-dependent increases in a sub-G1 population with YM155 treatment, suggestive of apoptosis that was subsequently confirmed by an increase in annexin-V positivity. YM155-mediated apoptosis was confirmed across a panel of 7 diagnostic bone marrow samples from children with AML. Consistent with the proposed mechanism of action, YM155 treatment was associated with down-regulation of survivin mRNA and protein expression and induction of DNA damage.

These data suggest that YM155-mediated inhibition of survivin is a potentially beneficial therapeutic strategy for AML, particularly pediatric disease, and warrants further evaluation.

Introduction

Acute myeloid leukemia (AML) is a clonal disorder of haematopoietic stem cells. It is clinically and genetically heterogeneous, typified by the accumulation of somatic mutations or aberrations altering the normal cellular functions of proliferation, differentiation and self-renewal [1]. Resistance to conventional chemotherapy remains a major challenge in AML patients with up to 30% of adults and 17% of children failing to achieve complete remission (CR) [1, 2]. Furthermore, the prognosis for AML patients with refractory disease is exceptionally poor, where only one third of patients achieve remission with second-line chemotherapy [3]. It is clear that novel therapeutic strategies are needed, particularly for those patients with primary refractory disease [2, 4].

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and regulates mitosis and apoptosis [5]. Furthermore, Survivin participates in a chromosomal passenger protein complex with Borealin and Inner Centromere Protein (INCENP) that is essential for chromosome condensation, spindle assembly and microtubule-kinetochore interactions during chromosome segregation and cytokinesis [6]. Survivin protein is encoded by the *BIRC5* gene and in many normal adult tissues the Survivin transcript is expressed at low or undetectable levels [7]. In contrast, the *BIRC5* gene is one of the most frequently amplified transcripts in cancer [7]. There are several mechanisms of Survivin upregulation in cancer including amplification of the *BIRC5* locus [8], hypomethylation of the *BIRC5* promoter [9], and enhanced promoter activity [5]. Importantly, the upregulation of Survivin in cancer occurs independently of the cell-cycle [5]. This suggests that the anti-apoptotic function of Survivin may be enhanced in cancer cells compared to normal cells rather than modulation of the mitotic regulatory function [5]. Hyperactivation of kinase signalling cascades, for example the PI3K, MAPK or STAT3 pathways, in addition to particular tumour microenvironments such as hypoxia and angiogenesis, induce an upregulation of Survivin expression in many malignant diseases including neuroblastoma, breast, lung, pancreatic, colon [5] and haematological malignancies [10]. Importantly, there is a strong correlation between Survivin expression and adverse prognosis, whereby a decreased overall survival rate, increased relapse rate and enhanced metastatic rate have been observed in several malignant diseases in patients with increased Survivin expression [5, 8, 11-14]. A specific role for Survivin in the promotion and maintenance of leukaemogenesis was recently identified in an *in vivo* transgenic

61 mouse model whereby Survivin overexpressing mice developed haematological malignancies at a faster rate with shorter latency than
62 control mice [15]. Furthermore, Survivin is highly expressed in CD34+/38- AML [10] and CML progenitors [16, 17] compared to normal
63 CD34+ and peripheral blood mononuclear cells suggesting that targeting Survivin may be a novel therapeutic strategy in eliminating
64 putative leukemic stem cells.

65 Targeted therapies to inhibit Survivin expression have been utilised in an experimental setting including antisense oligonucleotides or
66 siRNA, vaccination strategies and small molecule inhibitors with promising results to date. All three methodologies have reached clinical
67 trials including the utilisation of the small molecule inhibitor YM155 (Sepantronium Bromide) for the treatment of solid tumours and
68 lymphoma [18-32]. Despite the growing body of literature defining the role of Survivin in leukaemogenesis, there is a paucity of pre-clinical
69 data for YM155 in AML, particularly paediatric disease. Therefore, we undertook a comprehensive preclinical evaluation of YM155 in AML
70 and show for the first time that YM155 has potent *in vitro* efficacy in AML, inducing apoptosis at low nanomolar concentrations.
71 Apoptosis correlated to a reduction in Survivin protein but not gene expression. The induction of cell death was also associated with a
72 concentration-dependent increase in γ-H2AX phosphorylation, indicative of activation of the DNA damage response. We propose that
73 YM155 is an effective small molecule that warrants further investigation in AML and may be of particular benefit in paediatrics.

74 **Materials and Methods**

75 **Cell culture and reagents**

76 All human AML cell lines were purchased directly from recognised repositories; Kasumi-1, MV4-11, CMK, AML-193, M-07e, HL-60, ML-2,
77 OCI-AML3, ME-1 and HEL from DSMZ (Braunschweig, Germany) and THP-1 from ATCC (Manassas, USA). The DSMZ and ATCC authenticate
78 all human cell lines by DNA-typing and confirm species of origin by PCR-analysis. Working stocks for the experiments described in this
79 study were prepared immediately after the initial thawing of stock cells from DSMZ or ATCC. THP-1, Kasumi-1, MV-411, CMK, HL-60, ML-2,
80 ME-1 and HEL cells were maintained in RPMI 1640 (Life Technologies, Victoria, Australia); AML-193 and M-07e in IMDM (Life
81 Technologies) supplemented with 2ng/mL and 10ng/mL of huGMCSF (Peprotech, NJ, USA), respectively and OCI-AML3 in MEMα (Life
82 Technologies). All cell lines were supplemented with 10% foetal calf serum except M-07e cells that are supplemented with 20% FCS (FCS;
83 Life Technologies).

84 Human bone marrow samples from 8 patients with AML were obtained from the Queensland Children's Tumour Bank. Studies were
85 approved by Institutional Human Research Ethics Committees and consent given by all patients. Mononuclear cells were isolated by Ficoll-
86 Hypaque density-gradient centrifugation, washed, and resuspended at $5x10^5$ cells/mL in culture media (IMDM; Life Technologies)
87 supplemented with 0.5% FCS and 1% penicillin/streptomycin. The mean percentage of bone marrow blasts in the primary samples was
88 80% (± 4.9%, range 64-93%).

89 **Compounds**

90 YM155 (Sepantronium Bromide), cytarabine (cytosine arabinoside) and daunorubicin hydrochloride (daunorubicin) were obtained from
91 Selleck chemicals (Houston, USA). All compounds were dissolved in DMSO and stored at -20°C.

92 **Cell proliferation, cell cycle and apoptosis assays**

93 Human AML cell lines (2 x 10^4 cells/well) were seeded in a 96-well plate with appropriate factors and the indicated concentrations of
94 compound for 72 hours. Assays were plated in quadruplicate and repeated at least three times. Proliferation was assessed using a
95 resazurin reduction assay (CellTiter-Blue™, Promega, WI, USA). The concentration of compound that reduced cell viability by 50% (IC_{50})
96 was determined using non-linear regression with variable slope after normalising fluorescence to untreated cellular controls. For
97 combination assays, cells were treated at fixed a concentration of YM155 (0.25 x IC_{50}) with or without Cytarabine or daunorubicin (0.01,
98 0.1, or 1μM) for 72h. For cell cycle analysis, cells (2 ×10^5 cells/mL) were seeded in 24-well plates with appropriate factors and the indicated
99 compound concentrations for 24, 48 or 72h. Assayed cells were fixed in 1 mL 70% ethanol at 4°C. Cells were washed in PBS then incubated
100 with 40 μg/mL propidium iodide (PI; Sigma Aldrich, MO, USA) and 250 μg/mL RNase (Sigma Aldrich) for 30 minutes at 37°C. Samples were
101 analyzed on an LSRII Fortessa™ flow cytometer (BD Biosciences, NJ, USA), and histograms were fitted for cell cycle ratios using BD
102 FACSDiva (BD Biosciences). For assessment of $H2AX^{Ser319}$ phosphorylation (γ-H2AX), control cells were exposed to 30 minutes of UV
103 exposure, where plates were placed within 5cm of a UV light with the plate lid off.

104 Apoptosis was measured using the annexin-V FITC apoptosis detection kit according to manufacturer's instructions (BD Biosciences). AML
105 cell lines were seeded at 2 x 10^5 cells/mL and primary AML mononuclear cells were seeded at 5 x 10^5 cells/mL and treated with indicated
106 concentrations of compound for 72 or 24h respectively. Samples were stained for annexin-V FITC (BioLegend, CA, USA) and PI and
107 analysed on an LSRII Fortessa™ flow cytometer. Data were acquired using the BD FACSDiva software and analysed with FlowJo software.

108 **Immunoblotting**

109 Cells were washed in ice-cold PBS and lysed in a buffer solution (120mM NaCl, 50mM Tris-HCl pH7.4, 1% Triton X-100, 10mM NaF, 10mM
110 EDTA and supplemented with 0.1% protease inhibitor (Sigma Aldrich)). Total protein (10μg) was separated by SDS-PAGE and transferred
111 onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, England). Antibodies used for immunoblotting were anti-Survivin and
112 anti-GAPDH (Cell Signalling Technology, Inc., MA, USA). Membranes were incubated with IRDye® 680LT or IRDye 800CW conjugated
113 secondary antibodies and protein-antibody complexes visualised by an infrared imaging system. Images were captured with the Odyssey
114 (LI-COR Biosciences, NE, USA) detection system.

115 **Flow cytometric analysis**

116 Survivin expression was assessed by flow cytometric analysis (FACS) to validate immunoblot results. Briefly, a minimum of 1x10^5 cells were
117 fixed in 3.7% formaldehyde/PBS for 10 minutes at room temperature, permeabilized in ice-cold methanol then incubated at -20°C
118 overnight. Cells were blocked in 5% BSA/PBS for 10 minutes, incubated with either 1:1,000 anti-Survivin or matched IgG isotype control
119 antibody for 1 hour, then with 1:1,000 Alexa-Flour-680 conjugated anti-Rabbit secondary antibody (Life Technologies Inc.) for 1 hour.
120 Phosphorylated $H2AX^{Ser319}$ (γ-H2AX) and total H2AX were assessed by FACS. Following treatment, cells were fixed in 1.5%
121 formaldehyde/PBS for 10 minutes at room temperature, permeabilized in ice-cold methanol and incubated at -20°C overnight. Cells were
122 washed in 0.5% BSA/PBS/0.02% sodium azide and incubated with either 1:100 anti- γ-H2AX, H2AX (Cell Signalling Technology, Inc.) or
123 matched IgG isotype control antibody for 1 hour. Samples were then stained with 1:1000 Alexa-Flour-488 conjugated anti-Rabbit
124 secondary antibody (Life Technologies Inc.) for 1 hour. Samples were analysed on an LSRII Fortessa™ flow cytometer. Data were acquired
125 using the BD FACSDiva software and analysed with FlowJo software.

126 **Real-time quantitative PCR**

127 Total RNA was extracted from AML cell line pellets containing 1 × 10^6 cells using the RNeasy mini kit and QIAShredder (Qiagen, Hilden,
128 Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesised from 500 ng of RNA. For
129 quantitative, real-time PCR (qRT-PCR) analysis, expression of Survivin (*BIRC5*) was analysed using a commercially available TAQMAN assay
130 (Hs03063352_s1) and the ViiA™ 7 Real-Time PCR Sequence Detector System (Applied Biosystems, CA, USA). The comparative Ct method
131 (ΔΔCt) for relative quantification of gene expression was used for analysis with *RPS18* gene expression used as an internal control.

132 **Combination treatments**

133 MV4-11 and Kasumi-1 cells (2x10^5cells/mL) were seeded in 96 well plates and treated with YM155 in combination with either cytarabine
134 or daunorubicin. Variable concentrations of each compound were used, relative to their single-agent IC_{50} against each cell line. Assays
135 were plated in quadruplicate and repeated at least three times. Proliferation was assessed using a resazurin reduction assay. The
136 percentage of inhibition of proliferation was normalised to DMSO-treated controls.

137 **Statistical analysis**

138 Statistical significance of differences between samples was assessed using a Student's *t*-test. IC_{50} values were calculated using non-linear
139 fit of transformed data normalized to the DMSO-treated control samples. Values shown are the mean ± SEM. All analyses were performed
140 using GraphPad Prism 6 software (GraphPad Software Inc, CA, USA).

141

142 **Results**

143 **YM155 inhibits *in vitro* proliferation of AML cell lines**

144 To determine the *in vitro* efficacy of YM155 in AML, we utilised a panel of 5 adult and 6 paediatric AML cell lines, representative of the
145 common cytogenetic and molecular subtypes observed in AML patients (Table 1), and examined the effects of treatment on cell
146 proliferation. The IC_{50} values for YM155 against all AML cell lines were in the low nanomolar range (Table 1). When compared to the
147 conventional chemotherapeutic cytarabine, YM155 showed significantly greater efficacy in 4/6 paediatric AML cell lines and 2/5 adult
148 lines. Interestingly, YM155 was significantly more potent than cytarabine in all three *MLL*-rearranged AML cell lines (MV4-11, THP-1 and
149 ML-2) including the MV4-11 cell line that co-expresses a *FLT3* internal tandem duplication (*FLT3*-ITD) mutation.

150 **YM155 induces apoptotic cell death**

151 Previous studies have suggested that survivin's tumorigenic properties are a result of an increase in its anti-apoptotic activity, rather than
152 its role in the cell cycle given that in cancer cells Survivin expression remains consistent throughout the cycle [33]. In order to investigate
153 whether YM155 has an effect on the cell cycle of paediatric AML cell lines, we performed cell cycle analyses following both time (24, 48 or
154 72h) and concentration (0-1µM) sensitive treatment. YM155 treatment resulted in a significant increase in the sub-G1 population of 4/6
155 paediatric AML cell lines (THP-1, AML-193, Kasumi-1 and MV4-11) compared to DMSO treated controls, suggesting induction of cell death
156 (Figure 1). Sub-G1 accumulation was observed in both a time- (Figure 1b) and concentration-dependent manner (Figure 1c). In order to

157 establish the mechanism of cell death observed in cell cycle analysis, we utilised an annexin-V assay. In all cell lines, 72h YM155 treatment
158 induced a significant concentration-dependent increase in annexin-V+ cells at both 0.1µM and 1µM (Figure 2b). Taken together, these
159 data indicate that YM155 induces cell death through an apoptotic mechanism. Importantly, YM155 treatment induced apoptosis to a
160 similar level as the conventional cytotoxic Daunorubicin (Figure 2b).

161 **YM155 down-regulates Survivin expression in paediatric AML cell lines**

162 To determine whether the functional effects of YM155 observed in AML cell lines induced downregulation of Survivin we firstly examined
163 Survivin protein expression following treatment (0-1µM; 72h). Immunoblot and FACS analyses revealed that YM155 induced a
164 concentration-dependant reduction in Survivin expression in all 6 paediatric AML cell lines (Figure 3a-c). At 1µM YM155 all cell lines
165 displayed a reduction in Survivin expression, however a decrease in GAPDH levels was also observed at this high concentration in THP-1,
166 CMK and AML-193. This is most likely due to the extensive level of cell death and breakdown that occurs after 72h of YM155 treatment.
167 Previous reports suggest that YM155 blocks Survivin transcription via inhibition of the *BIRC5* gene promoter [34]. In order to establish
168 whether the reduction in Survivin protein expression was due to direct inhibition of gene transcription, we performed qRT-PCR for *BIRC5*
169 in the paediatric AML cell lines. *BIRC5* gene expression was significantly decreased with 0.1 and 1µM YM155 treatment relative to the
170 control gene *RPS18* across the panel of paediatric AML cell lines (Figure 3d). This finding supports the mechanism of action of YM155 as an
171 inhibitor of *BIRC5* gene promoter activation.

172 **YM155 induces a DNA damage response**

173 Recent studies have demonstrated that YM155 induces a DNA damage response in a panel of solid tumour cell lines [35] but not chronic
174 lymphocytic leukemia (CLL) cells [36]. Therefore, we assessed the panel of paediatric AML cell lines for induction of γ-H2AX. Interestingly,
175 only MV4-11 and M07e cells exhibited a significant concentration-dependant increase in γ-H2AX with YM155 treatment compared to
176 DMSO controls (Figure 4). MV4-11 were the most sensitive to DNA damage induction with a mean 6.7 (±0.2)-fold induction of γ-H2AX
177 relative to H2AX expression and normalised to DMSO controls ($p=0.0002$) following 1µM YM155 treatment (Figure 4E). The conventional
178 cytotoxic compounds cytarabine (Ara-C; 1µM) and daunorubicin (1µM), and 30 minutes of direct UV exposure also induced γ-H2AX
179 expression in these cell lines (Figure 4), however not to the same extent as YM155. This suggests that YM155 may not only mediate AML
180 cell death by affecting Survivin expression, but also by inducing DNA damage in cells expressing certain molecular backgrounds. Kasumi-1
181 cells also exhibited and increase in γ-H2AX relative to H2AX expression, however did not reach significance. Given that CMK, AML193 and
182 THP-1 cells did not exhibit DNA damage response activation with YM155, cytotoxic or UV treatment suggests they are inherently resistant
183 to γ-H2AX mediated DNA damage from multiple sources.

184 **YM155 increases the cytotoxicity of conventional chemotherapy in AML cells**

185 Since conventional treatment of AML is based on intensive use of cytarabine and anthracyclines, we set out to establish whether YM155 in
186 combination with these drugs would increase their anti-leukemic effects. MV4-11 and Kasumi-1 cells were co-cultured with cytarabine or
187 daunorubicin in the presence or absence of YM155 for 72h and proliferation measured. Combination treatment was more effective at
188 inhibiting the proliferation of both MV4-11 and Kasumi-1 cells compared to either compound alone (Figure 5).

189 **YM155 induces cell death of AML patient blasts**

190 To determine if primary human AML cells are sensitive to YM155, paediatric AML blasts from 8 patients with diverse cytogenetic and
191 molecular characteristics (including 1 with acute promyelocytic leukemia, selected due to the presence of a dual *FLT3*-ITD-D835 mutation;
192 Table 2) were treated with increasing concentrations of YM155 (0-1µM) for 24h. Cell death was assessed by annexin-V/PI staining. YM155
193 treatment induced a concentration-dependent increase in annexin-V+ cells (Figure 6A) and concomitant reduction in the percentage of
194 live viable cells (annexin-V- / PI-; Figure 6B). Of note, four of the five most sensitive AML patient samples had *FLT3* mutations (Table 2).
195 Internal duplication of the FLT3 tyrosine kinase gene (*FLT3*-ITD) is a frequent event in AML and is associated with higher relapse rates and
196 reduced survival [37]. When grouped according to *FLT3* status, there appeared to be a trend toward greater sensitivity of those patients
197 expressing *FLT3*-ITD compared to *FLT3*-WT patient blasts at all concentrations of YM155 however this did not reach significance (Figure 6C
198 and D).

199 **Discussion**

200 This study has shown that the small molecule Survivin inhibitor YM155 is efficacious for the inhibition of paediatric AML cell growth *in*
201 *vitro.* Treatment of AML cell lines with nanomolar concentrations of YM155 lead to inhibition of proliferation and accumulation of a sub-
202 G1 population due to an induction of apoptotic cell death, in both a time and concentration dependent manner. Importantly, the efficacy
203 of YM155 was not limited to established AML cell lines, with primary samples from paediatric patients with AML also displaying
204 therapeutic sensitivity. Inhibition of proliferation and induction of apoptosis was associated with downregulation of Survivin protein and
205 gene expression. Induction of the DNA damage response as measured by γ-H2AX expression was also evident in a subset of cell lines.

206 Survivin is a small inhibitor-of-apoptosis protein involved in regulating apoptosis, the cell cycle and the cellular stress response. The
207 overexpression and nodal characteristics of Survivin including resistance to apoptosis, metastasis, circumvention of cell cycle checkpoints
208 and resistance to chemotherapy make it an attractive therapeutic target for a range of human cancers [5]. Several strategies have been
209 utilised to suppress Survivin expression and concomitantly target its cancer promoting networks including antisense oligonucleotides [5,
210 38-40] and the small molecule YM155 [5, 34]. YM155 was reported to bind to the Survivin promoter, modulating gene expression in
211 human prostate tumour models [34]. This supports observations in our *in vitro* paediatric AML model whereby the mRNA expression was
212 reduced with YM155 treatment (0.1, 1µM; 72h), and was associated with a concomitant reduction in Survivin protein expression; a finding
213 validated by both immunoblotting and flow cytometric analyses. Taken together, these data suggest that YM155 regulates Survivin
214 expression at the transcriptional level in AML cells.

215 Although YM155 was originally considered a small molecule inhibitor of Survivin promoter activity, recent studies have revealed that
216 YM155 treatment results in other functional effects including DNA damage [35]. Glaros *et. al.* reported that YM155 had broad potency
217 against the NCI-60 cell line panel with IC_{50} values in the nanomolar range, attributable to an induction of γ-H2AX and pKAP1 expression.
218 Importantly, the activation of this alternative cellular pathway occurred at concentrations lower than those required to inhibit Survivin
219 protein expression in PC3 prostate cancer cells [35]. This suggests that DNA damage preceded Survivin inhibition, at least in PC3 cells, and
220 contributed to cell death earlier and more potently than Survivin downregulation. Further evidence for the function of YM155 as a DNA
221 damaging agent arose when the authors compared YM155 to three well characterised DNA damaging agents (chromomyocin A3,

222 bisantrene HCl and actinomyocin D) and found significant concordance between the compounds (R=0.864, 0.705 and 0.689 respectively)
223 [35]. Similarly, an earlier study identified downregulation of the Bcl-2 family member, Mcl-1, as a mediator of YM155-induced apoptosis in
224 a wide range of established cancer cell lines [41]. Mcl-1 is a pro-survival protein that protects cells from stimuli-mediated cell death
225 resulting from activation of the intrinsic apoptotic pathway. YM155 treatment was shown to downregulate Mcl-1 expression in a time- and
226 concentration-dependent manner [41]. Importantly, downregulation of Mcl-1 was independent of, and preceded the downregulation of
227 Survivin suggesting that Survivin downregulation is not an isolated mechanism of action of YM155 and may not be the initiating apoptotic
228 event. These studies support our observations of an induction of γ-H2AX expression in parallel with a downregulation of Survivin,
229 suggestive of a dual function of YM155. However, this effect was only observed in 2 of 6 paediatric AML cell lines (MV4-11 and M07e).
230 Similarly, the cytotoxic chemotherapeutic drugs cytarabine and daunorubicin both induced a DNA damage response in MV4-11 and M07e
231 cells *in vitro,* further supporting the notion that YM155 may act primarily as a cytotoxic DNA-damaging agent, rather than a specific
232 Survivin inhibitor. The lack of DNA damage response activation following all treatments including UV exposure in the CMK, AML-193, and
233 THP-1 cells suggests that these cells may be particularly resistant to DNA damaging stimuli, and alternative mechanisms of action of
234 YM155 such as its *BIRC5* promotor binding activity likely account for the nanomolar sensitivity of these cell lines. The accumulation of a
235 sub-G1 population in cells in cell cycle assays, induction of γ-H2AX phosphorylation in selected cell lines and induction of annexin V
236 positivity suggests that YM155 sensitivity may induce cell death through a number of mechanisms including mitotic catastrophe, DNA
237 damage and apoptosis.

238 Patient samples positive for *FLT3*-ITD were particularly sensitive to YM155 *in vitro*, consistent with the low-nanomolar IC_{50} demonstrated
239 for the *FLT3*-ITD+ cell line, MV4-11. Although there was no statistical significance between *FLT3*-ITD+ and *FLT3*-WT+ samples, the trend
240 raises the possibility of a differential effect in *FLT3*-ITD+ AML; a larger cohort of *FLT3*-ITD+ and *FLT3*-WT samples is needed to confirm this.
241 Previous studies support these findings; it has been reported that Survivin plays a critical role in proliferation of AML cells harbouring *FLT3*-
242 ITD and resistance to FLT3 kinase inhibition. Survivin was over-expressed in pre-clinical models of FLT3-inhibitor resistance and was
243 identified as a downstream transcription target of the FLT3-STAT pathway [42]. Survivin expression and STAT activation were both
244 increased when MV4-11 cells were cultured long-term with the FLT3 inhibitor ABT-869. Sensitivity to ABT-869 in these resistant MV4-11
245 cells was restored by silencing Survivin with shRNA. Furthermore, Survivin over-expression in transfected parent MV4-11 cells, conferred
246 resistance to FLT3 inhibition [42]. Survivin expression has also been shown to be higher in *FLT3*-ITD transfected Ba/F3 cells and *FLT3*-ITD+
247 primary samples compared to FLT3-WT samples [43]. Furthermore, studies using Survivin knock-out mice suggest that it regulates *FLT3*-
248 ITD driven proliferation [43]. Taken together, this evidence suggests that YM155 may be of particular benefit to patients with *FLT3*-ITD+
249 AML. Importantly, 2 primary patient samples harbouring *FLT3*-ITD and a secondary tyrosine kinase domain (TKD) mutation at D835 were
250 sensitive to YM155. Dual *FLT3*-ITD-TKD mutations have recently been identified as a mechanism of FLT3 inhibitor resistance [4, 37, 44-46],
251 suggesting that YM155 may offer a therapeutic option for this subset of patients and further investigation is warranted.

252 Early phase clinical trials of YM155 have thus far shown that YM155 is generally well tolerated and clinical responses have been seen in a
253 range of tumour types [47]. Given the very short half-life of YM155, most trials have thus far assessed continuous 7 day infusions.
254 Liposomal formulations of YM155 are being developed, which may facilitate weekly bolus dosing [48]. Although the exact mechanism of
255 action of YM155 as a specific Survivin suppressant is debated, it is acknowledged that YM155 may remain a valuable anti-cancer agent
256 [47]. Importantly, our data shows that *in vitro* combination of YM155 with daunorubicin and cytarabine increases the effectiveness of

257 these clinically relevant chemotherapeutic agents against AML cell lines. These data are of importance to clinical trial design in AML, since
258 cytarabine and anthracyclines form the backbone of upfront therapy and low dose cytarabine is commonly used in combination for early-
259 phase trials of novel therapeutics.

260 The preclinical data presented here demonstrate for the first time that AML may be a malignancy with particular sensitivity to YM155.
261 Whether YM155 functions primarily through the inhibition of Survivin gene expression or other mechanisms, such as DNA damage,
262 remains unclear. However, given the urgent need for novel therapeutic approaches in AML, the potent *in vitro* efficacy demonstrated here
263 and the relative safety profile from recent trials in other malignancies, clinical evaluation of YM155 in AML is warranted. It will be
264 important to ensure that the design of such clinical trials allows for expansion cohorts of subtypes such as *FLT3*-ITD, if initial clinical
265 sensitivity reflects the preclinical data reported here. Furthermore, the paucity of novel agents available for children with AML demands
266 that adult phase I trials are promptly followed by paediatric trials once the adult safety profile in acute leukemia is established.

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Tables

Table 1. YM155 inhibits *in vitro* proliferation of AML cell lines at nanomolar concentrations

				IC_{50} Ratio		
	Cell Line Cytogenetic/Molecular Characteristics	**FAB**	**YM155 IC50[1] (µM)±SEM**	**YM155:Daun**	**YM155:Cyt**	***P* value (YM155:Cyt)**
Paediatric						
Kasumi-1	t(8;21)(q22;q22) *RUNX1-CBFA2T1; c-KIT* mut (N822K), *TP53*(R248Q)	M2	0.009±0.0009	0.447	0.086	**<0.05**
M-07e	t(11;21)(p11;p13), add(13)(p13), add(22)(p13); *CREBBP*(Q2208H)	M7	0.040±0.013	5.263	2.385	0.337
THP-1	t(9;11)(p21;q23) *MLL-AF9; TP53*(R174fs*3), *NRAS*(G12D)	M5	0.051±0.013	1.308	0.005	**<0.005**
CMK	Myeloid leukaemia associated with Down Syndrome. Complex; *TP53*(D49H and M133K), *CDKN2A*(M1_*157del), *JAK3*(A572V), *GATA1*(E2fs*37)	M7	0.053±0.009	0.676	0.123	**<0.001**
MV4-11	t(4;11)(q21;q23) *MLL-AF4; FLT3*-ITD	M5	0.055±0.028	7.205	0.024	**<0.001**
AML-193	Complex karyotype with 4% polyploidy	M5	0.462±0.060	5.775	0.108	0.052
Adult						
HL-60	Complex Karyotype; *CDKN2A*(R80* and P135L), *NRAS*(Q61L).	M2	0.001±0.0002	0.058	0.002	0.702
ML-2	t(6;11)(q27;q23) *MLL-AF6; CREBBP*(L1090*), *NOTCH1*(P2514fs*4), *KRAS*(A146T), *CDKN2A*(M1_*157del)	M4	0.009±0.002	1.848	0.157	**<0.05**
OCI/AML3	Hyperdiploid(+1, +5, +8, der(1)t(1;18)(p11;q11), i(5p), del(13)(q13q21), dup(17)(q21q25); *DNMT3A*(R882C), *NPM1*(W288fs*12)	M4	0.011±0.002	0.522	0.023	0.391
HEL	Hypertriploid with 2.3% polyploidy; *JAK2*(V617F), *TP53*(M133K), *CDKN2A*(M1_*157del)	M6	0.559±0.038	4.050	6.803	**<0.05**
ME-1	inv(16)(p13q22), del(17)(p12p13), +8	M4eo	0.684±0.179	2.280	1.745	0.265

[1]IC_{50}; is defined as the concentration of drug that reduces cell viability by 50%, calculated by non-linear regression. The IC_{50} ratio was calculated by dividing the IC_{50} of YM155 by the IC_{50} of Daunorubicin or Cytarabine. P-value determined by students t-test.
Abbrev: Daun; daunorubicin, Cyt; cytarabine, FAB; French-American-British classification, SEM; standard error of the mean.

Table 2. Patient characteristics

Patient ID	Age at Dx (years)	Sex	Diagnosis	Cytogenetics	% Bone Marrow Blasts	RANK[δ] According to Annexin V+	FLT3 Status
QBQAWJB	3.8	F	AML	Complex karyotype with t(4;16), -7, -18	90	1	ITD and D835
QJBFWJQ	4.8	F	AML	Interstitial deletion of short arm of one chromosome 3 (bands p13->p21).	60	2	ITD
QBQFLJB	10.8	M	APL	t(15;17)	72	3	ITD and D835
QQQLJJB	1.3	M	AML	inv(16) (p13q22)	64	4	
QBBJDJQ	10.6	F	AML	Complex karyotype with t(6;9)(p23;q34)	93	5	ITD
QJTQDJQ	4.7	F	AML	t(8;21)(q22;q22)	76	7	
QBQWTJB	7.8	F	AML	t(9;11)(p22;q23)	93	8	
QTBJLJB	15.5	M	AML	t(9;22)(q34;q11.2) and unbalanced t(1;11)(q21;q23)*	92	9	

[δ]Patients ranked according to sensitivity (1 highest sensitivity, 9 least sensitivity) to YM155 (1µM;24h) measured by percent of Annexin-V+ cells by FACS analysis. *This sample was acute ambiguous lineage leukemia, with populations consistent with B-cell and myeloid leukemia. The t(9;22) resulted in an e1a2 BCR/ABL1 transcript. The t(1;11) was unbalanced with no rearrangement of the MLL gene.
Abbrev: ID; identification, Dx; diagnosis, F; female, M; male, AML; acute myeloid leukemia, APL; acute promyelocytic leukemia.

400 **Figure Legends**

401 **Figure 1. YM155 induces Sub-G1 accumulation of AML cells.** A) Representative histogram of accumulation of a sub-G1 population of THP1
402 cells treated with DMSO or 1µM YM155 for 72h. B) AML cell lines were grown in the presence of YM155 (1µM) for indicated times. C) AML
403 cell lines were grown in the indicated concentrations of YM155 for 72h. Propidium iodide was used to assess DNA content by flow
404 cytometric analysis. *Columns*; mean percent of cells in Sub-G1, *bars;* SEM. *p<0.05, **p<0.01, Students t-test compared to DMSO controls.

405 **Figure 2. YM155 induces apoptosis in AML cells.** A) Representative dot plot of apoptosis induction showing THP1 cells treated with DMSO,
406 1µM Daunorubicin or 1µM YM155 for 72h. B) AML cell lines were grown in the presence of YM155 (1µM) for indicated times. B) AML cell
407 lines were treated with indicated concentrations of Daunorubicin or YM155 for 72h. Levels of apoptosis were measured by an annexin-V
408 apoptosis assay. Percent apoptosis was defined as all cells expressing Annexin V positivity (addition of quadrant 2 and quadrant 4 of dot
409 plot). *Points*, mean of at least 3 independent experiments; *bars*, SEM. *p<0.05. Students t-test relative to DMSO control.

410 **Figure 3. YM155 induces downregulation of Survivin in AML cells.** Survivin expression was assessed qualitatively by A) immunoblot and B)
411 quantitatively by flow cytometry. GAPDH was used as a loading control. C) Flow cytometry data represents all paediatric cell lines, the bars
412 denote mean±SEM D) RT-PCR was used to analyse the expression of *BIRC5* gene expression in paediatric AML cell lines. ΔCT is *BIRC5*
413 normalised to *RSP18* relative to DMSO control. *Points:* mean of 3 independent experiments, *bars*: SEM. *p<0.05, ***p<0.005 compared to
414 control. Immunoblots are representative of at least 3 independent experiments.

415 **Figure 4. YM155 induces DNA damage.** A) CMK, B) Mo7e, C) AML-193, D) Kasumi-1, E) MV4-11 and F) THP-1 cells were incubated in
416 increasing concentrations of YM155, Ara-C (1µM) or Daunorubicin (1µM) for 72h. Following 72h treatment, half the DMSO control cells
417 were subject to 30minutes of direct UV exposure. The level of $pH2AX^{Ser319}$ and total H2AX was determined by immunofluorescence and
418 FACS analysis. Data was analysed by dividing the percentage of $\gamma H2AX^{Ser319}$ positive cells by the percentage of total H2AX positive cells and
419 normalising to DMSO controls. *Column*, mean of 3 independent experiments; *Bars*, SEM. *p<0.05,**p<0.01, ***p<0.005. Students *t*-test
420 relative to DMSO control.

421 **Figure 5. YM155 enhances the anti-proliferative effect of chemotherapy.** A) MV411 or B) Kasumi 1 cells were incubated with YM155 (0.25
422 x IC_{50}) in the presence or absence of i) 0.5 x IC_{50} or ii) 1 x IC_{50} of Daunorubicin or iii) 0.5 x IC_{50} or iv) 1 x IC_{50} of Cytarabine for 72h. Following
423 72h treatment, cells were subject to a resazurin reduction assay. Percentages were normalised to DMSO controls. *Column*, mean of 3
424 independent experiments; *Bars*, SEM. *p<0.05, **p<0.01, ***p<0.005. Students *t*-test. Similar trends were observed with 0.5 x and 1 x IC_{50}
425 of YM155 in combination with Daunorubicin or Cytarabine (data not shown).

426 **Figure 6. YM155 induces apoptosis of primary paediatric AML blasts.** AML mononuclear cells were incubated in increasing concentrations
427 of YM155 for 24h. The level of apoptosis was determine by an annexin V apoptosis assay and all cells negative for annexin V or PI staining
428 were deemed live cells whilst cells positive for annexin V were deemed apoptotic. A) Representative dot plot of AML patient mononuclear
429 cells treated with DMSO, 1µM Daunorubicin or 1µM YM155 showing induction of apoptosis. B) The ratio of annexin V+ cells significantly
430 increased with increasing YM155 concentration. B) The percentage of live cells significantly decreased with increasing concentrations of
431 YM155. *FLT3*-ITD+ blasts are sensitive to C) induction of apoptosis and D) reduced viability compared to *FLT3* wild-type (*FLT3*-WT) patient
432 blasts. *Points*, individual patient values; *Column*, mean (n=4); *Bars*, SEM. *p<0.05, **p<0.01, ***p<0.005. Students t-test relative to DMSO
433 control.

434

435 **Figures.**

Figure 1

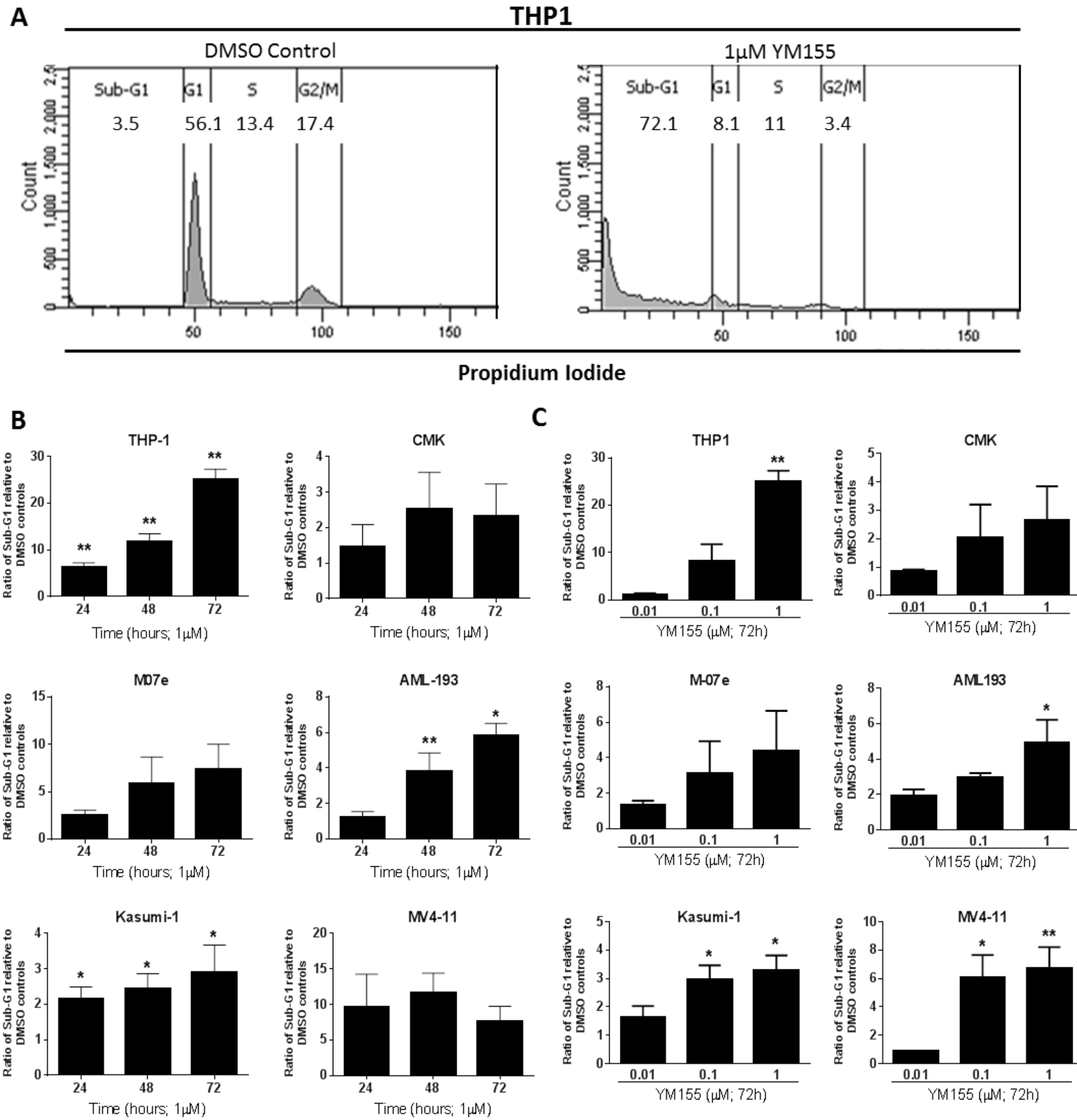

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Figure 2

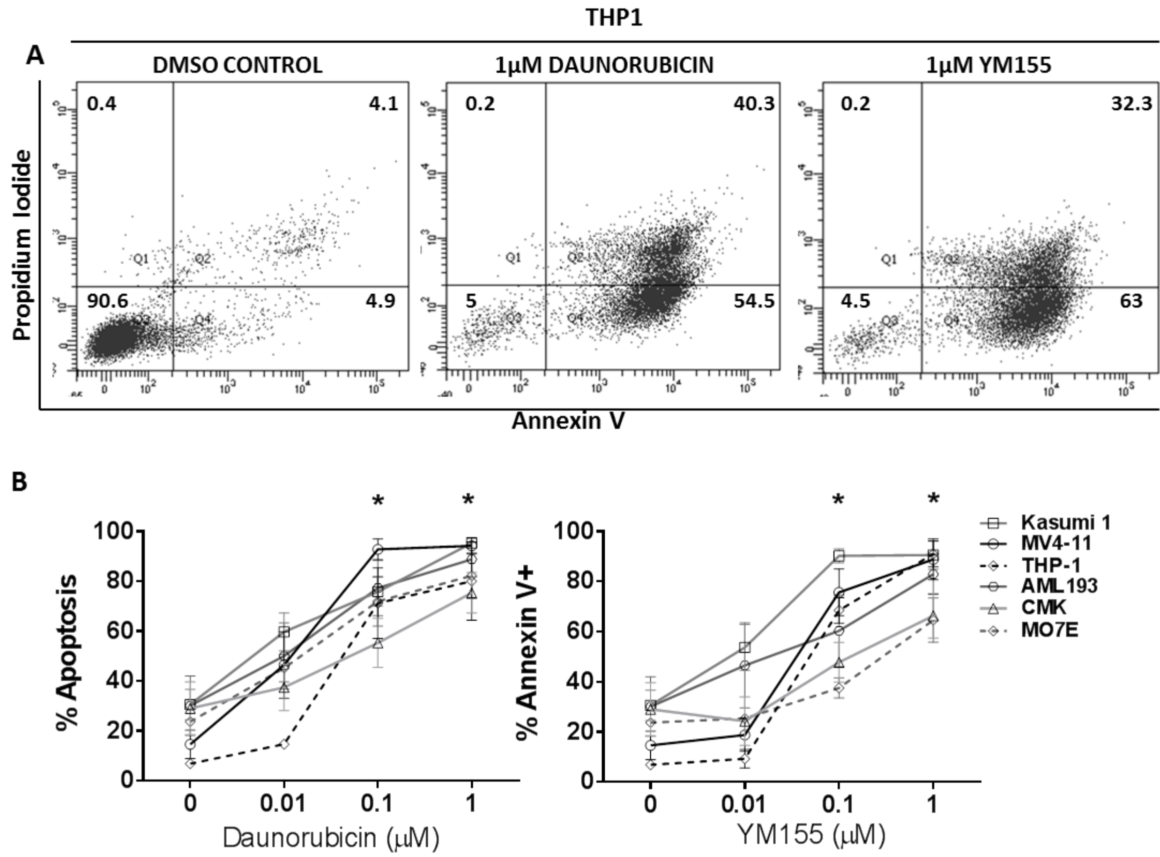

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Figure 3

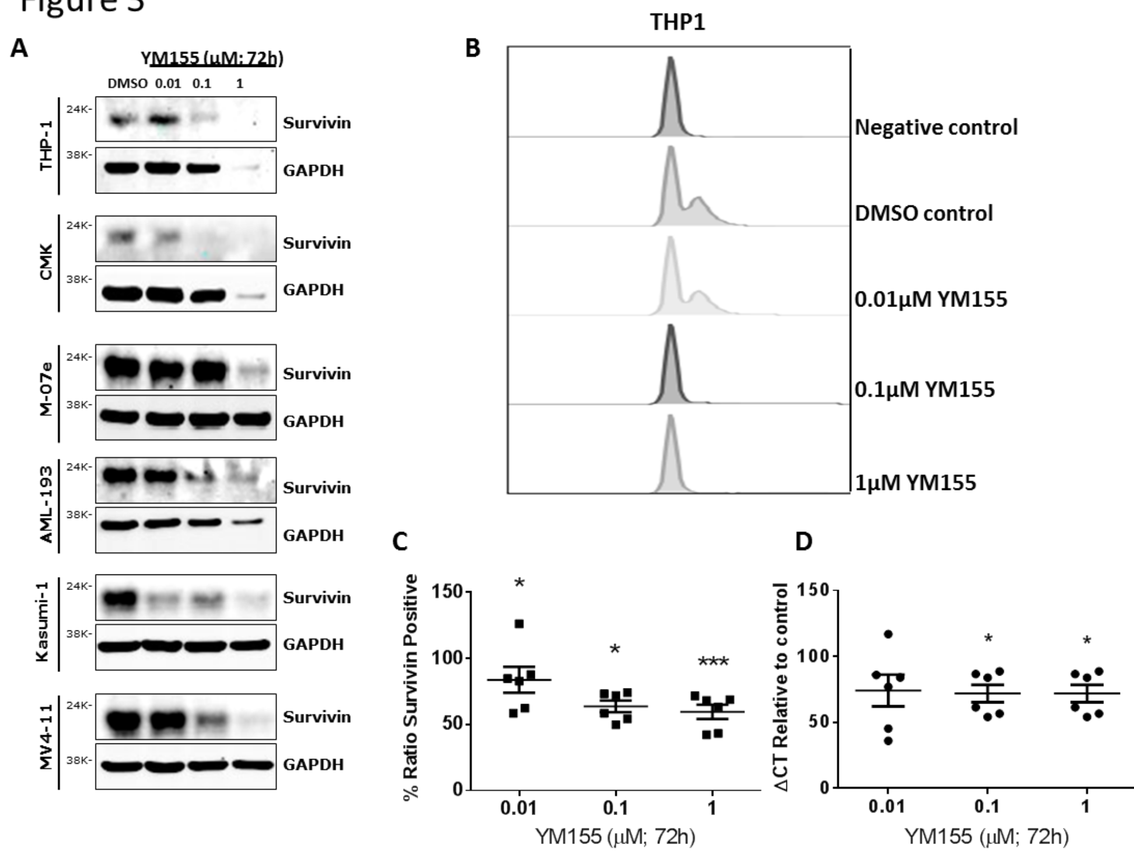

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Figure 4

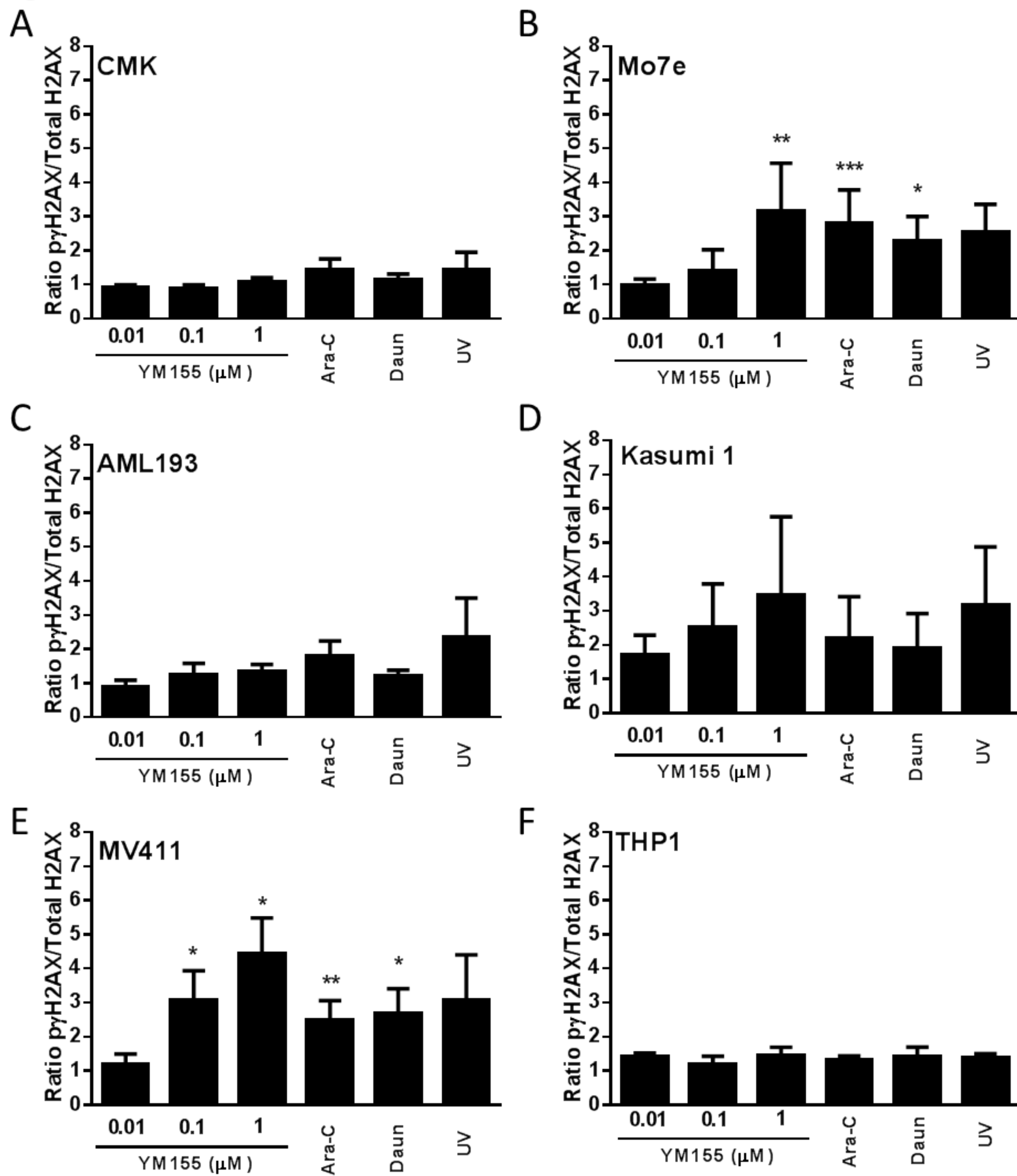

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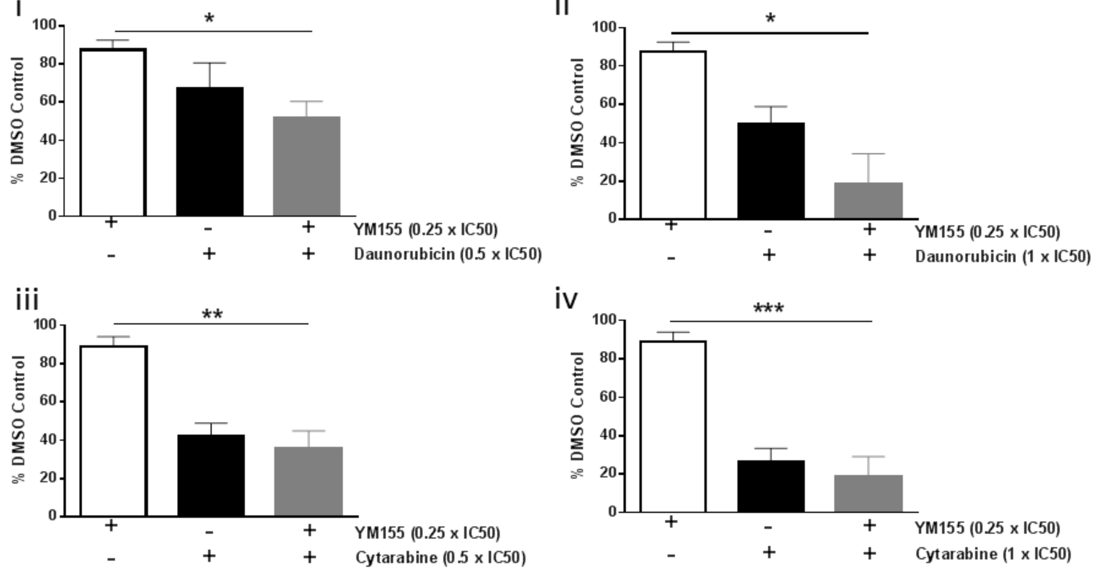
Figure 5

A



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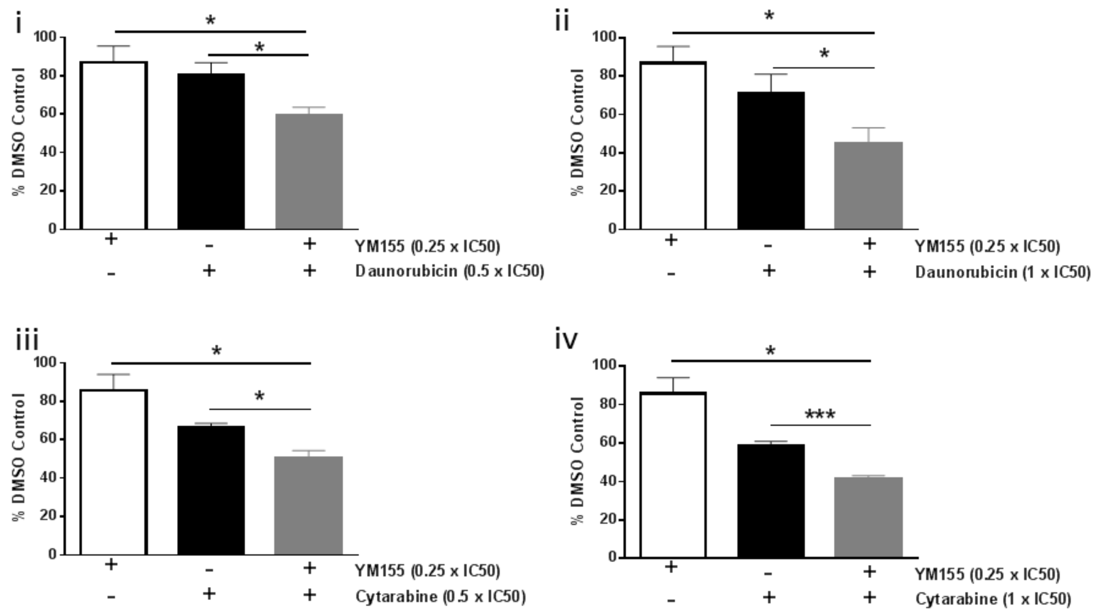

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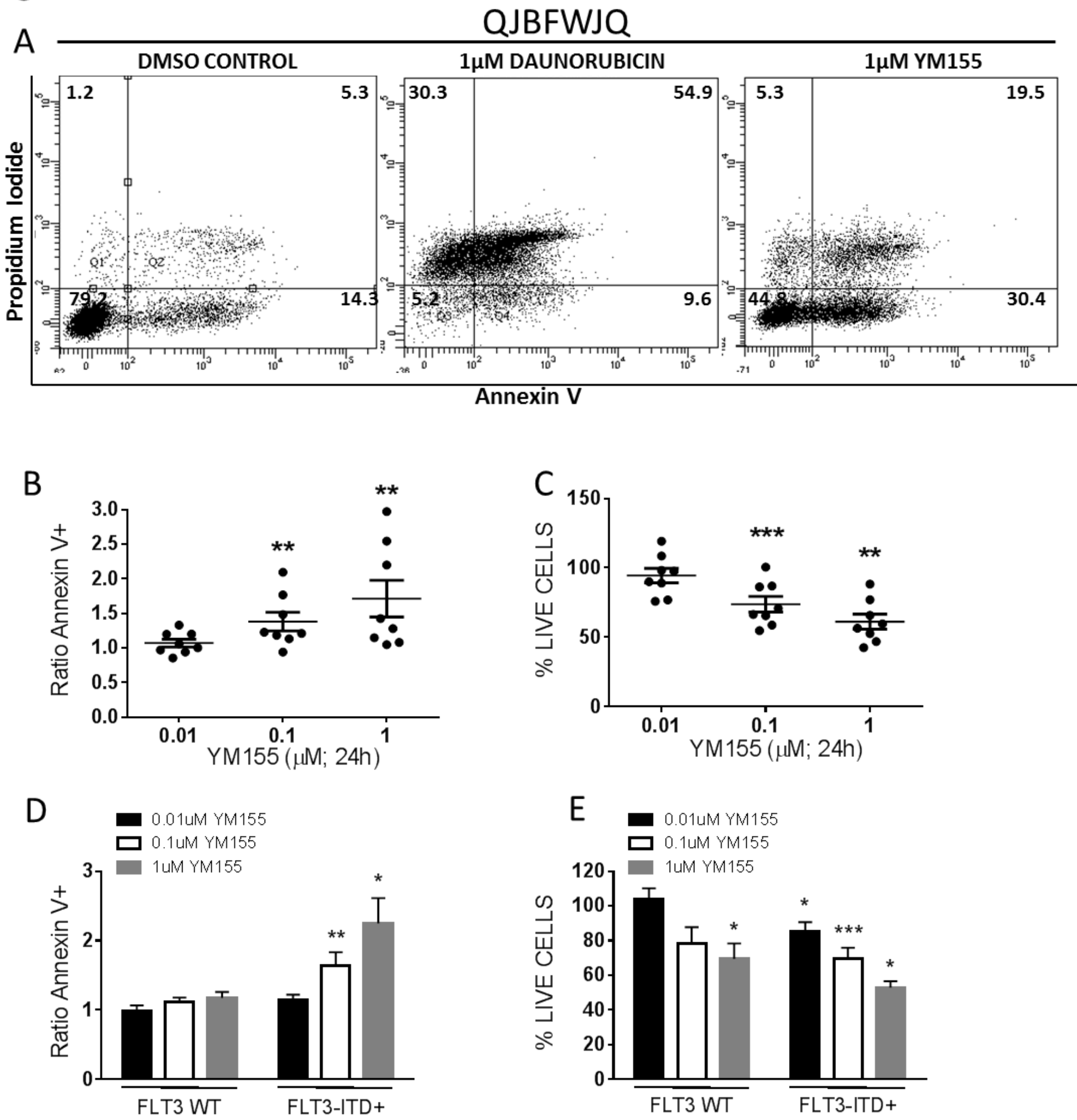
Figure 6



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