

1 **Title: Multiple Myeloma: Combination Therapy of BET Proteolysis Targeting Chimeric
2 Molecule with CDK9 Inhibitor**

3 Su-Lin Lim^{*1¶}, Liang Xu^{2¶}, Bing-Chen Han¹, Pavithra Shyamsunder², Wee-Joo Chng², H. Phillip
4 Koeffler¹⁻²

5 ¹ Cedars Sinai Medical Center, Los Angeles, California, United States of America

6 ² Cancer Science Institute of Singapore, National University of Singapore, Singapore.

7 * Corresponding author

8 Email address: sulin_lim_86@hotmail.com

9 ¶These authors contributed equally to this work.

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21 **Abstract:**

22 Cyclin Dependent Kinase 9 (CDK9) associates with Bromodomain and Extra-Terminal Domain
23 (BET) proteins to promote transcriptional elongation by phosphorylation of serine 2 of RNAP II
24 C-terminal domain. We examined the therapeutic potential of selective CDK9 inhibitors (AZD
25 4573 and MC180295) against human multiple myeloma cells *in vitro*. Short-hairpin RNA silencing
26 of CDK9 in Multiple Myeloma (MM) cell lines reduced cell viability compared to control cells
27 showing the dependency of MM cells on CDK9. In order to explore synergy with the CDK9
28 inhibitor, proteolysis targeting chimeric molecule (PROTAC) ARV 825 was added. This latter
29 drug causes ubiquitination of BET proteins resulting in their rapid and efficient degradation.
30 Combination treatment of MM cells with ARV 825 and AZD 4573 markedly reduced their protein
31 expression of BRD 2, BRD 4, MYC and phosphorylated RNA pol II as compared to each single
32 agent alone. Combination treatment synergistically inhibited multiple myeloma cells both *in vitro*
33 and *in vivo* with insignificant weight loss. The combination also resulted in marked increase of
34 apoptotic cells at low dose compared to single agent alone. Taken together, our studies show for
35 the first time that the combination of a BET PROTAC (ARV 825) plus AZD 4573 (CDK9 inhibitor)
36 is effective against MM cells.

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42 **Introduction:**

43 Multiple myeloma (MM) is a clonal plasma cell malignancy. It is the second most common
44 hematologic malignancy in United States ¹. Despite advances in treatment such as proteasome
45 inhibitors and immunomodulatory drugs, the disease remains incurable. Bromodomain and Extra-
46 Terminal Domain (BET) family is composed of BRD-2, -3, -4 and -T. They facilitates
47 transcriptional activation by RNA polymerase II (RNAP II)². ARV 825 (Arvinas, Inc) is a hetero-
48 bifunctional molecule composed of a Bromodomain binding moiety (OTX 015) joined to
49 pomalidomide. Pomalidomide binds to an intracellular E3 ubiquitin ligase, cereblon (CRBN);
50 OTX 015 brings the complex to the BET molecules. This variety of inhibitor is called PROTAC
51 (Proteolysis Targeting Chimeric molecules) which in this case causes ubiquitination of BET
52 proteins resulting in rapid and efficient degradation of these proteins³. BET PROTAC ARV 825
53 inhibits the proliferation of MM cells both in vitro and in vivo ^{4,5}.

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55 Cyclin Dependent Kinase 9 (CDK9) is the kinase subunit of the positive transcription elongation
56 factor b (P-TEFb) that associates with BET proteins which promotes transcriptional elongation by
57 phosphorylation of serine 2 of RNAPII C-terminal domain (CTD)⁶. CDK9 has a major 42kDa and
58 a minor 55kDa isoform. The 55kDa isoform is at an upstream transcriptional start site of the 42
59 kDa protein. Both are expressed in human cancer cell lines and in normal tissues ⁷. The 42 kDa
60 isoform is localized diffusely in the nucleoplasm, whereas the 55 kDa accumulates in the
61 nucleolus⁸. CDK9 has been shown to play an important role in controlling global transcription,
62 including expression of genes regulated by super-enhancers, such as MYC, MCL-1 and cyclin
63 D1⁹. MCL-1 and MYC are critical for proliferation of MM cells, often causing resistance to drugs

64 and producing relapse in these patients ^{10,11}. Therefore, CDK9 may represent a druggable target in
65 myeloma having dysregulated MYC expression ^{12,13}.

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67 Inhibition of both CDK9 and BRD 4 has been reported synergistically to induce growth arrest and
68 apoptosis of cancer cells including MM ^{14,15}. Previously studied CDK inhibitors (eg. Flavopiridol
69 and SNS-032) are not selective to CDK9, inhibiting other CDKs and enzymes. Their lack of
70 selectivity and decreased potency may contribute to many adverse effects in clinical trials ⁸.
71 Therefore, selective inhibitors of CDK9 are needed to prevent the undesirable off-target effects
72 and to enhance potency.

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74 AZD 4573 is highly potent against CDK9 (<3 nM IC₅₀) and selective (>10 fold) against CDK9.
75 The drug results in caspase activation and loss of viability across a diverse set of hematological
76 cancers including MM ¹⁶. MC180295 is also a highly selective CDK9 inhibitor (> 22 fold, IC₅₀ =
77 5 nM) that has broad anti-cancer activity in vitro and in vivo ¹⁷. In this study, we noted that AZD
78 4573 and MC180295 *in vitro* inhibited the viability of MM cells. We also showed that AZD 4573
79 is synergistic with ARV 825 in inducing apoptosis and inhibiting MM cell proliferation both *in*
80 *vitro* and *in vivo*.

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87 **Materials and Methods:**

88 **Cell Culture**

89 Human MM cell lines: KMS11, KMS28, KMS18, KMS12, MM1S, MM1R, H929, 8226,
90 8226 LR5 and 8226 P100V were kind gifts from Dr. W.J. Chng (Cancer Science Institute of
91 Singapore, Singapore) and KMS11 res and MM1S res were generous gifts from Dr. A.K. Stewart
92 (Mayo Clinic, Arizona). All cell lines were cultured and maintained in RPMI1640 with 10% fetal
93 bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5%
94 CO₂. The 8226 LR5 cells were maintained in 10 nM Melphalan, the 8226 P100V cells were
95 cultured with 100 nM bortezomib for 2 days every 2 weeks.

96

97 **Cell Proliferation Assay**

98 Twenty thousand cells were seeded in 96-well plates followed by drug treatment. After 72 h culture,
99 10 µl of MTT (2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the
100 wells and cultured at 37°C for an additional 4 h followed by addition of 100 µl stop solution (10%
101 Sodium Dodecyl Sulphate). Plates were measured with a spectrophotometer at 570 nm absorbance.
102 IC₅₀ values were calculated using Graph Pad Prism.

103

104 **Annexin V and Propidium Iodide (Annexin V-PI) Apoptosis Analysis**

105 Cells were treated with different concentrations of ARV 825 for 48 h. Staining was performed
106 using Apoptosis Detection Kit II (BD Biosciences, USA). Cells were harvested and washed twice

107 with phosphate-buffered saline (PBS, Life technologies, USA), suspended in 1X binding buffer
108 with 5 μ l of FITC conjugated Annexin V and 5 μ l of PI for 15 min in the dark at room temperature.
109 Samples were analyzed using flow cytometric analysis (Sony SA3800). Cells positive for Annexin
110 and PI were defined as apoptotic cells.

111

112 **Cell Cycle Analysis**

113 Cells were treated with different concentrations of AZD 4573 (24 h), fixed with 70% chilled
114 ethanol, washed with PBS two times and stained with PI solution [40 μ g/ml PI, Triton X-100 (1%),
115 20 μ g/ml DNase-free RNase A in PBS] for 30 min at 37 °C in the dark followed by flow cytometric
116 analysis (Sony SA3800).

117

118 **Drug Combination Study**

119 Results from MTT assays with different combinations of ARV 825 and AZD 4573 were evaluated
120 by CompuSyn¹⁸(ComboSyn, Inc, Paramus, NJ). A combination index (CI) plot is a Fa-CI plot in
121 which CI<1, =1, >1 indicate synergism, additive and antagonism, respectively. Fa: fraction of
122 proliferation inhibition by the drug.

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126 **Reagents and Antibodies**

127 ARV 825 was developed by the C.M. Crew's laboratory (Department of Chemistry, Yale
128 University, New Haven, CT, USA). We obtained the drug from Chemietek (Indianapolis, IN,
129 USA). MC180295 was a generous gift from Dr. H.H. Zhang and J.P. Issa (Temple University,
130 Philadelphia). We obtained AZD 4573 from MedChemExpress (New Jersey, USA). For *in vitro*
131 administration, ARV 825, AZD 4573 and MC180295 were dissolved in dimethyl sulfoxide
132 (Sigma-Aldrich) (10 mM) and stored at -80°C. List of antibodies and inhibitors is present in S1
133 Table.

134

135 **Western Blot Analysis**

136 Cellular lysates were prepared using M-PER mammalian protein extraction reagent (Thermo
137 Scientific, Rockford, USA) containing 1X protease cocktail inhibitor (Roche, Switzerland). After
138 20 min incubation on ice, lysates were centrifuged (14,000g, 30 min, 4°C). Total protein
139 concentrations were measured by Pierce Coomassie Plus (Bradford) assay kit (Thermo Fisher
140 Scientific). Twenty micrograms of protein were loaded per lane on SDS-PAGE gel and resolved
141 at 90 voltages, followed by transfer to PVDF (Millipore, Massachusetts). Membranes were
142 blocked with 5% non-fat milk and incubated with antibodies.

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146 **Lentiviral Production and Silencing of CDK9**

147 shRNA targeting CDK9 was cloned into pLKO.1 lentiviral vector (Sequence: Forward:
148 CCGGGTTCGACTTCTGCGAGCATGACTCGAGTCATGCTCGCAGAAGTCGAACCTTTG
149 Reverse:AATTCAAAAAGTCGACTTCTGCGAGCATGACTCGAGTCATGCTCGCAGAA
150 GTCGAC. Luciferase vector was purchased from Addgene (plasmid #17477). Recombinant
151 lentiviral vector and packaging vector (pCMV-dR8.9 and pMD2.G-VSVG) were cotransfected
152 into 293 FT cells using polyethylenimine (PEI) according to the manufacturer's instructions. Virus
153 supernatants were harvested at 48h and 72h after transfection, and placed through a 0.45 μ m filter.
154 8226 and KMS28 cells (1 X 10^6 per well) were seeded in 6-well plates. Cells were transduced with
155 lentiviral vectors in the presence of 8 μ g/ml polybrene (Sigma-Aldrich) for 24 h. Stable cell lines
156 were selected with puromycin.

157

158 **In Vivo Xenografts**

159 *In vivo* studies were performed with a protocol approved by the Institutional Animal Care and Use
160 Committee at Cedars Sinai Medical Center. To access the *in vivo* activity of ARV 825, KMS11
161 expressing luciferase (KMS11^{LUC}) were injected into lateral tail vein of SCID-Beige mice. Mice
162 were monitored for 7 days and imaged by Xenogen IVIS spectrum (PerkinElmer, Massachusetts)
163 camera to document engraftment before treatment was initiated. At 7 days after mice were injected
164 with cells, they were randomly divided into four groups (5 mice in each group) [vehicle (5%
165 Kolliphor® HS15), 5 mg/kg of ARV 825 (intraperitoneal injection daily for 28 days), 10 mg/kg
166 of AZD 4573 (intraperitoneal injection, twice a day with 2 h interval for two consecutive
167 days/week for 4 weeks) and combination of ARV 825 and AZD 4573]. Tumor burden in each

168 treatment group was monitored daily and imaged weekly by Xenogen camera for 28 days. The
169 mice were then euthanized within 24 hours after the end of experiment. No mice died before the
170 end of experiment. For euthanasia, the mice received isoflurane overdose followed by cervical
171 dislocation. All research personnel in mice study were trained for animal care and welfare
172 according to IACUC protocol.

173

174 **Statistical Analysis**

175 For *in vitro* and *in vivo* experiments, the statistical significance of difference between two groups
176 used two-tailed student t-test and two-way ANOVA. Asterisks in the figures represent significant
177 differences between experimental groups in comparison to controls (* $p < 0.01$, ** $p < 0.001$,
178 *** $p < 0.0001$). Data points in figures represent means \pm SD (standard deviation).

179

180 **Results:**

181 **CDK9 inhibitors (AZD 4573 and MC180295) decreased cellular 182 proliferation of MM cells.**

183 AZD 4573 and MC180295 (CDK9 inhibitors) in a dose-dependent manner were tested against a
184 panel of 12 human MM cell lines (KMS11, MM1R, KMS12BM, H929, KMS18, 8226 LR5,
185 MM1S, KMS11 res, 8226, KMS28, 8226 P100V, MM1S res) using an *in vitro* proliferation assay
186 (MTT, 72 h). Cell lines included melphalan-resistant (8226 LR5), steroid-resistant (MM1R),
187 bortezomib-resistant (8226 P100V) and lenalidomide-resistant (KMS11res and MM1Sres) cell

188 lines. Some of the cell lines have cytogenetics associated with a poor prognosis [e.g. t(4:14):
189 KMS11, KMS28, H929; t(14:16): MM1S, 8226]. All MM cell lines were sensitive to AZD 4573
190 with IC_{50} ranging from 8 – 60 nM (Fig 1A). MM1S, MM1Sres, MM1R and KMS11 cell lines (IC_{50}
191 = 8 nM) were most sensitive to AZD 4573; whereas 8226 P100V was a relatively more resistant
192 cell line (IC_{50} = 70 nM) (S2 Table). The data showed that AZD 4573 was effective even if the cells
193 were resistant to either melphalan, lenalidomide, steroid, bortezomib or they had a cytogenetically
194 unfavorable chromosome. MC180295 was not as potent as AZD 4573 against MM cell lines with
195 IC_{50} ranging from 260 nM to >1000 nM (Fig 1B). H929 was the most sensitive (IC_{50} = 260 nM),
196 whereas 8226 P100V was the relatively resistant cell line to MC180295 (IC_{50} > 1000 nM) (S2
197 Table).

198

199 **Figure 1: CDK9 inhibitors: anti-proliferative activities against MM cells.** (A) Twelve MM
200 cell lines were cultured with AZD 4573 (1 nM-62.5 nM, 72 h). Growth inhibition was measured
201 by MTT assays. Results are mean \pm SD, N=3. IC_{50} s are shown in Supplementary Table 2. (B) MM
202 cells treated with MC180295 (1 nM-1,000 nM, 72 h). Growth inhibition was measured by MTT
203 assays. Results are mean \pm SD, N=3. IC_{50} s are shown in S2 Table.

204

205 **Silencing of CDK9 reduced cell proliferation and viability of MM
206 cells**

207 To examine the dependency of MM cells on CDK9 expression, we performed short hairpin RNA
208 (shRNA)-mediated silencing of CDK9 in KMS28 and 8226 cell lines. RT-qPCR and western blot

209 analysis confirmed the successful silencing of CDK9 using shRNA in these cells (Figs 2A-B, left
210 upper and lower panel). Silencing CDK9 in 8226 and KMS28 cell lines reduced cell viability
211 compared to control cells (MTT, 72 h) (Figs 2A-B, right panel).

212

213 **Figure 2: shRNA mediated silencing of CDK9 decreased proliferation of MM cells.** (A) Levels
214 of CDK9 mRNA (Left, upper panel) and protein (Left, lower panel) after shRNA-mediated
215 silencing of 8226 cells. Cell proliferation assay (MTT) after shRNA silencing of 8226 cells (right
216 panel). (B) Levels of CDK9 mRNA (left, upper panel) and protein (left, lower panel) after shRNA-
217 mediated silencing of CDK9 in KMS28 cell line. Cell proliferation assays (MTT) after shRNA
218 silencing of CDK9 in KMS28 cell line (right panel). Results are mean \pm SD, N=3.

219

220 **ARV 825 and AZD 4573 showed synergistic growth inhibitory
221 activity against MM cell lines**

222 The IC₅₀ of ARV 825 against KMS11, 8226 and KMS28 MM cells are 9 nM, 84 nM and 137 nM,
223 respectively (S3 Table). Combination of ARV 825 and AZD 4573 treatment for 72h showed
224 synergistic growth inhibitory activity against these MM cells (Combination Index < 1) (Fig 3).
225 The combination index analysis is shown in S4 Table.

226

227 **Figure 3: Combination index plot of ARV 825 with AZD 4573 against MM cells.** Synergistic
228 growth inhibition of KMS11, KMS28 and 8226 cells when ARV 825 and AZD 4573 are combined
229 (72 h, MTT assay). CI < 1, CI = 1 and CI > 1 represent synergism, additive, and antagonism

230 respectively of the combination of the two compounds. Values of Combination Index analysis are
231 shown in S4 Table.

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235 **CDK9 inhibitor AZD 4573 downregulated phosphorylation of ser2
236 pol II CTD, MCL-1 and MYC protein.**

237 We evaluated the protein expression of BRD 2, BRD 3, BRD 4, phosphorylated ser2 pol II carboxy
238 terminal domain (Pol II CTD), total RNA polymerase II, MCL-1 and MYC in KMS11 and 8226
239 cells after treatment with ARV 825 and AZD 4573 either as a single agent or in combination (7 h,
240 ARV 825 [KMS11 (20 nM; 40 nM); 8226 (100 nM; 200 nM)], AZD 4573 [KMS11 (20 nM; 40
241 nM); 8226 (60 nM; 120 nM)].

242

243 Protein levels of phosphorylated RNA pol II, MCL-1 and MYC decreased significantly after
244 KMS11 and 8226 cells were treated with AZD 4573 [KMS11 (20 nM; 40 nM); 8226 (60 nM; 120
245 nM, 7 h) whereas the total RNA pol II was not affected. In contrast, after ARV 825 treatment
246 [KMS11 (20 nM; 40 nM); 8226 (100 nM; 200 nM), 7 h] of KMS11 and 8226 cells, protein
247 expression of BRD 2, BRD 3, BRD 4 and MYC reduced, but did not affect the protein expression
248 of MCL-1 and phosphorylated RNA pol II (Figs 4A-B). Combination treatment (7 h) of ARV 825
249 [KMS11 (20 nM; 40 nM); 8226 (100 nM; 200 nM)] and AZD 4573 [KMS11 (20 nM; 40 nM);
250 8226 (60 nM; 120 nM)] of KMS11 and 8226 cells markedly reduced their protein expression of

251 BRD 2, BRD 4, MYC and phosphorylated RNA pol II as compare to single agents alone (Figs 4A-
252 B).

253

254

255 **Figure 4: Effect of ARV 825 and AZD 4573 on protein expression of BRD 2, BRD 3, BRD 4;**
256 **phosphorylated Ser 2 RNA pol II; total RNA pol II; MCL-1 and MYC in MM cells.** (A)
257 KMS11 cells were treated with ARV 825 (20 nM and 40 nM), AZD 4573 (20 nM and 40 nM) and
258 their combination [ARV 825 + AZD 4573 (20 nM + 20 nM; 40 nM + 40 nM, respectively) for 7
259 h; and protein expression was examined by western blot (GAPDH, internal control). (B) 8226 cells
260 were treated with ARV 825 (100 nM and 200 nM), AZD 4573 (60 nM and 120 nM) and their
261 combination [ARV 825 + AZD 4573 (100 nM + 60 nM; 200 nM + 120 nM, respectively) for 7 h;
262 and protein expression was examined by western blot.

263

264 **AZD 4573 and ARV 825 combination markedly induced apoptosis in**
265 **MM cells**

266 Flow cytometric analysis of KMS11 and 8226 MM cells showed a marked increase in the
267 percentage of apoptotic cells after treatment with combination of ARV 825 and AZD 4573 for 48
268 h compare to control cells. Either ARV 825 (2.5 nM) or AZD 4573 (2.5 nM) alone produced 26%
269 and 13% apoptotic KMS11 cells, respectively; but their combination (2.5 nM + 2.5 nM) led to 67%
270 apoptotic cells. Similarly, 8226 cells treated with either ARV 825 (20 nM) or AZD 4573 (10 nM)

271 alone led to 14% and 20% of apoptotic cells, respectively; but their combination (20 nM + 10 nM)
272 led to 71% of apoptotic cells (Fig 5A).

273

274 Cell cycle analysis of MM cells was performed in the presence of various concentrations of AZD
275 4573 for 24 h compare to control cells. AZD 4573 only produced a minimal dose-dependent
276 increase in G1 phase, decrease S phase and G2/M phase in KMS11 and 8226 cell lines (Fig 5B).

277

278 **Figure 5: Apoptosis and cell cycle analysis of MM cells after treatment with ARV 825 and/or**
279 **AZD 4573.** (A) Apoptosis: KMS11 and 8226 MM cells were treated with of ARV 825 (2.5 nM;
280 20 nM), AZD 4573 (2.5 nM; 10 nM) and their combination (2.5 nM + 2.5 nM; 20 nM + 10 nM,
281 respectively) for 48 h, stained with annexin V-FITC and PI, and analyzed by flow cytometry.
282 Histograms represent percentage of apoptotic cells. Mean \pm SD of three independent experiments.
283 (B) Cell cycle: KMS11 and 8226 MM cells were treated for 24 h with either AZD 4573 (2.5-5 nM
284 or 5-10 nM, 24 h), respectively or diluent control (DMSO), stained with propidium iodide (PI) and
285 analyzed by flow cytometry. Histograms showed proportion of cells in different phases of cell
286 cycle. Representative of three independent experiments. *p \leq 0.01; **p \leq 0.001; ***p \leq 0.0001
287 for ARV 825 vs. control.

288

289 **Combination of AZD 4573 and ARV 825 inhibited MM cells in vivo**

290 Anti-proliferative effect of either ARV 825 or AZD 4573 alone or their combination was examined
291 *in vivo* against MM xenografts growing in SCID Beige mice. One week after injection, the MM
292 cells were observed by bioluminescence imaging; after which, mice (n=5 per group) were

293 randomly assigned to receive ARV 825 (5 mg/kg, IP daily), AZD 4573 (10 mg/kg, IP, twice a day
294 with 2 h interval for two consecutive days/week) or combination treatment for a total duration of
295 28 days. Control mice received vehicle alone. Bioluminescence was measured at days 0, 7, 14, 21,
296 28. Combination of ARV 825 and AZD 4573 significantly ($P < 0.001$) slowed tumor growth in
297 experimental mice compared to single agent alone as measured by bioluminescence (Figs 6A-B)
298 at days 21 and 28. ARV 825 and AZD 4573 alone or in combination did not affect either the normal
299 activity or the weight (loss < 10%) of the mice (S1 Fig).

300

301 **Figure 6: AZD 4573 acts synergistically with ARV 825 in inhibiting MM cells In vivo. (A)**
302 Whole-body bioluminescence images of SCID-beige mice after intravenous injection with
303 KMS11^{LUC} cells followed 7 days later by treatment with either ARV 825 (5 mg/kg IP daily for 28
304 days) or AZD 4573 (10 mg/kg, IP, twice a day with 2 h interval for two consecutive days/week for
305 4 weeks) as well as a combination of both drugs and vehicle control alone. (B) Tumor burden as
306 measured by bioluminescence in SCID-beige mice after intravenous injection with KMS11^{LUC}
307 cells. Data represent mean \pm SD ($N = 5$ per group). * $p \leq 0.01$, ** $p \leq 0.001$, *** $p \leq 0.0001$.

308

309 **Discussion:**

310 Management of multiple myeloma remains challenging especially relapse/refractory MM despite
311 major advancement in treatment. Therefore, new targeted therapies are urgently required. BET
312 PROTAC and CDK9 inhibitors have shown promising results in preclinical studies against MM
313 ^{4,5,19,20}. However, the limited clinical efficiency of CDK9 inhibitors due to side-effects and dose-
314 limiting toxicities have prevented these drugs from receiving FDA approval. Hence, the need for

315 better CDK9 inhibitors ⁸. In addition, combination therapies targeting multiple survival pathways
316 may minimize adverse effects by reducing dosage and improving outcomes. Furthermore,
317 targeting BRD 4 when also targeting CDK9 is able to block the compensatory increase in
318 expression of MYC ¹⁵.

319 AZD 4573 is a selective CDK9 inhibitor that led to dose- and time- dependent decrease in
320 phosphorylated ser 2 RNAP II and loss of MCL1 mRNA and protein; also, the in vivo efficacy of
321 the drug has been reported in multiple hematological tumors ¹⁶. It is currently in phase I clinical
322 trials for treatment of hematological malignancies ²¹. MC180295 is a novel CDK9 inhibitor that is
323 more selective against CDK9 than AZD 4573. However, MC180295 was not as potent as AZD
324 4573 against MM cell lines. AZD4573 markedly decreased growth of almost all the MM cells
325 having resistance to standard drugs. We performed shRNA mediated silencing of CDK9 against
326 MM cells and found it paralleled the drugs' activity to inhibit proliferation and viability of MM
327 cells.

328

329 In this study, we also demonstrated that combination of the BET PROTAC ARV 825 and the
330 selective CDK9 inhibitor AZD 4573 synergistically caused significant growth inhibition of
331 myeloma cells both in vitro and in an orthotopic xenograft model. Also, flow cytometric analysis
332 demonstrated that low dose combination of ARV 825 and AZD 4573, as compare to single agent,
333 induced enhanced apoptosis. These lower drug concentrations will minimize unwanted off target
334 activity or side-effects. We observed only minimal G1 cell cycle arrest in KMS11 and 8226 cells
335 after treatment with AZD 4573 suggesting that AZD 4573 probably does not inhibit the cell cycle
336 as a mechanism of cell kill but it does block transcriptional elongation ⁸.

337

338 We found that in MM cells AZD 4573 decreased phosphorylation of RNAP II, decreased anti-
339 apoptotic proteins (MCL-1 and MYC), whereas ARV 825 degraded BET proteins and decreased
340 expression of MYC. Combining both inhibitors synergistically inhibited cell growth of MM cells.
341 Prior studies showed that inhibition of CDK9 paradoxically increased expression of MYC¹⁵. This
342 did not occur with our drug combination. Our therapeutic targeting of MYC is important in MM
343 because of the importance of this protein causing progression of MM. In summary, our studies
344 showed for the first time that the combination of a BET PROTAC (ARV 825) plus AZD 4573
345 (CDK9 inhibitor) is effective against MM.

346

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349 inhibitor.

350

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403 randomized study to assess the safety, tolerability, pharmacokinetics, and preliminary
404 antitumor activity of AZD4573, a potent and selective CDK9 inhibitor, in subjects with
405 relapsed or refractory hematological malignancies. *J Clin Oncol.* 2018;36(15)

406

407 **Supporting Information**

408 **S1 Table. List of antibodies**

List of antibodies	
Antibodies	Manufacturer
BRD 2	Cell Signaling Technology, 5848
BRD 3	Proteintech, 11859-1-AP
BRD 4	Cell Signaling Technology, 13440
CDK 9	Cell Signaling Technology, 2316T
MCL 1	Cell Signaling Technology, 5453
Phosphor- Rpb1 CTD (ser2)	Cell Signaling Technology, 13499
Rpb1 CTD	Cell Signaling Technology, 2629
List of inhibitors	
Inhibitors	Manufacturer
AZD 4573	MedChemExpress, Catalog no. HY-112088

Bortezomib	Selleckchem Catalog no. S1013
Melphalan	Selleckchem Catalog no. S8266

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411 **S2 Table. IC50s of AZD 4573 against MM cells, 72 h**

Cell lines	AZD 4573 IC50s ± SD (nM), 72 h
MM1S (14:16)	8 ± 0.6
KMS11 (4:14)	8 ± 0.1
MM1S res (lenalidomide resistant)	8 ± 0.4
MM1R (steroid resistant)	8 ± 3.0
KMS11 res (lenalidomide resistant)	9 ± 1.1
KMS18	12 ± 1.1
KMS28 (4:14)	17 ± 1.4
KMS12BM	21 ± 2.1
H929	20 ± 0.3
8226 LR5 (Melphalan resistant)	22 ± 3.1
8226 (14:16)	23 ± 3.2
8226 P100V (bortezomib resistant)	70 ± 4.4
Cell lines	MC180295 IC50s ± SD (nM), 72 h
H929	260 ± 20
KMS11	280 ± 23
KMS18	330 ± 24

KMS11 res (lenalidomide resistant)	330 ± 40
MM1S	340 ± 42
MM1S res (lenalidomide resistant)	340 ± 42
MM1R (steroid resistant)	350 ± 31
8226	460 ± 43
8226 LR5 (Melphalan resistant)	520 ± 22
KMS28	510 ± 14
KMS12BM	700 ± 9
8226 P100V (bortezomib resistant)	>1000

412

413 **S3 Table. IC50s of ARV 825 against MM cells, 72 h**

Cell lines	ARV 825 IC50 ± SD (nM), 72 h
KMS11	9 ± 1.9
MM1R (Steroid resistant)	10 ± 1.8
KMS12BM	11 ± 1.3
MM1S	11 ± 1.8
H929	16 ± 1.6
KMS18	17 ± 1.1
8226 LR 5 (Melphalan resistant)	20 ± 1.9
KMS11 res (Lenalidomide resistant)	70 ± 1.4

U266	71 ± 1.8
8226	84 ± 1.4
KMS28BM	137 ± 1.1
8226 P100V (Bortezomib resistant)	500 ± 0.6
MM1S res (Lenalidomide resistant)	>500

414

415 **S4 Table. Combination Index.** Combination index of AZD 4573 synergistic with ARV 825 (CI
416 < 1, CI = 1 and CI > 1 represent synergism, additive and antagonism, respectively)

KMS11 (cell line)			
	ARV 825		
	2.5 nM	5 nM	10 nM
AZD 4573			
2.5 nM	0.75	0.71	0.82
5 nM	0.83	0.85	1.1

8226 (cell line)			
	ARV 825		
	40 nM	80 nM	160 nM
AZD 4573			
12.5 nM	0.38	0.36	0.40
25 nM	0.63	0.66	0.71

KMS28 (cell line)			
	ARV 825		
	50 nM	100 nM	200 nM
AZD 4573			
7.5 nM	0.61	0.65	0.62
15 nM	0.64	0.66	0.64

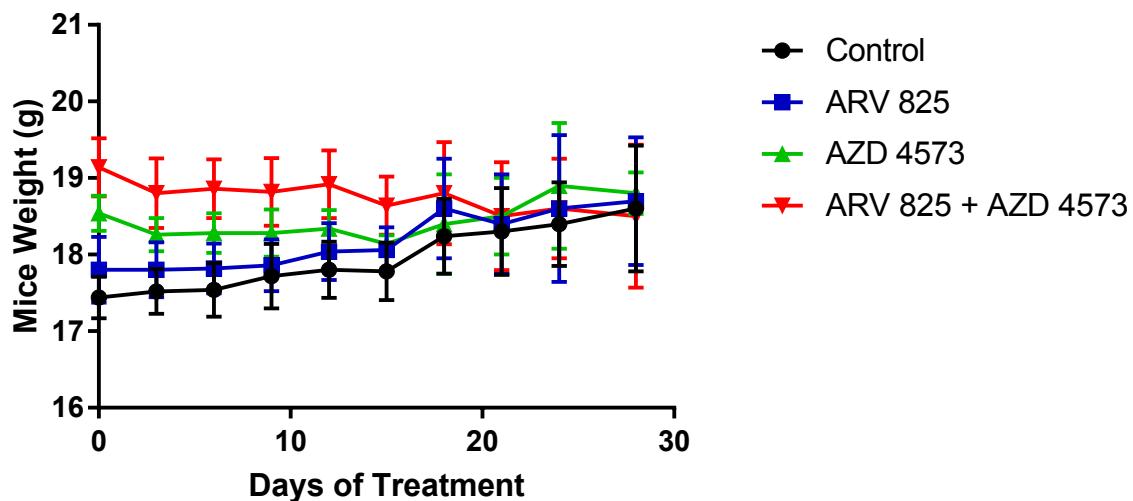
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424 **S1 Fig. Mice weight after treatment.** Comparison of weight of mice after treatment with ARV 425 825 (5 mg/kg IP daily for 28 days), AZD 4573 (10 mg/kg, IP, twice a day with 2 h interval for 426 two consecutive days/week for 4 weeks), combination of both drugs or diluent control. Mean \pm SD of 427 5 mice in each group.

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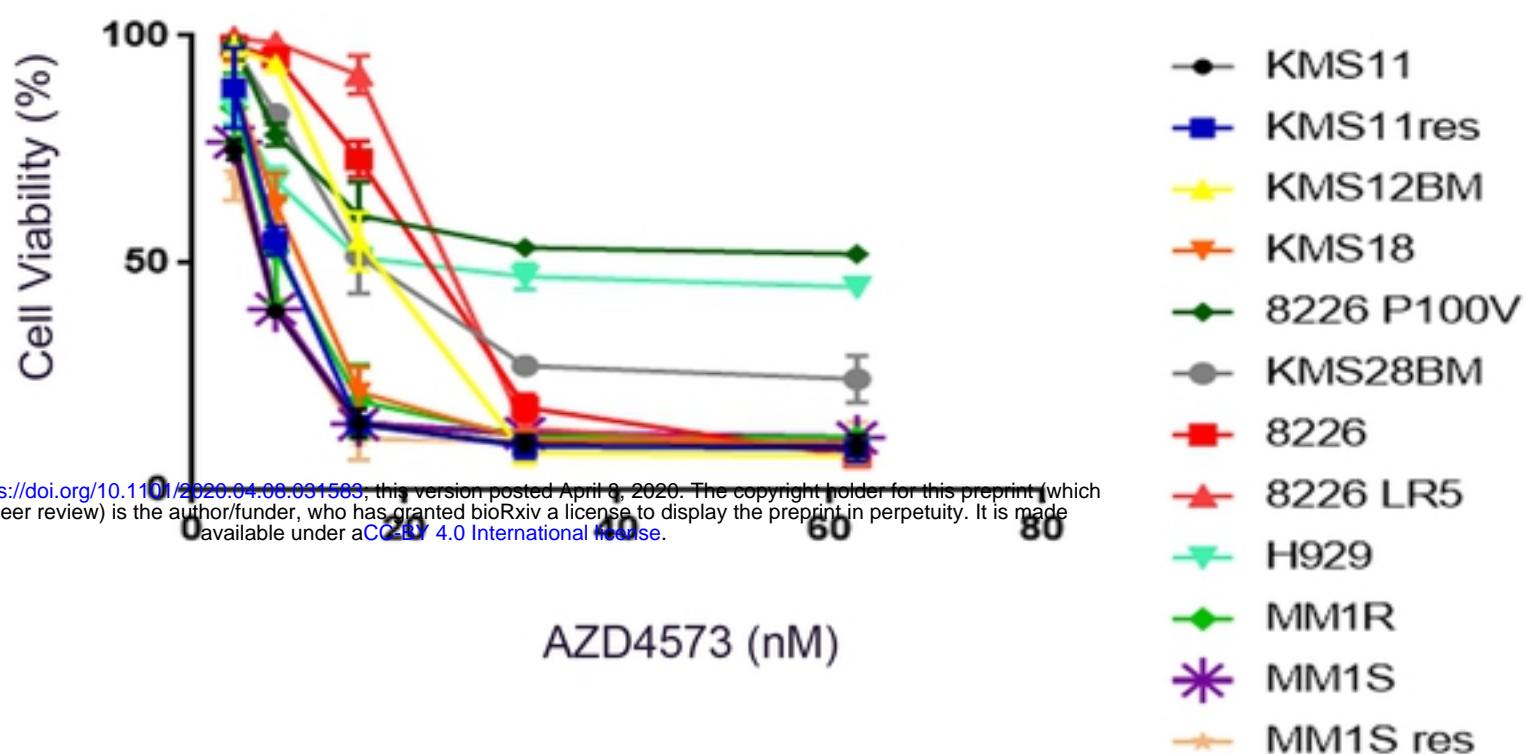
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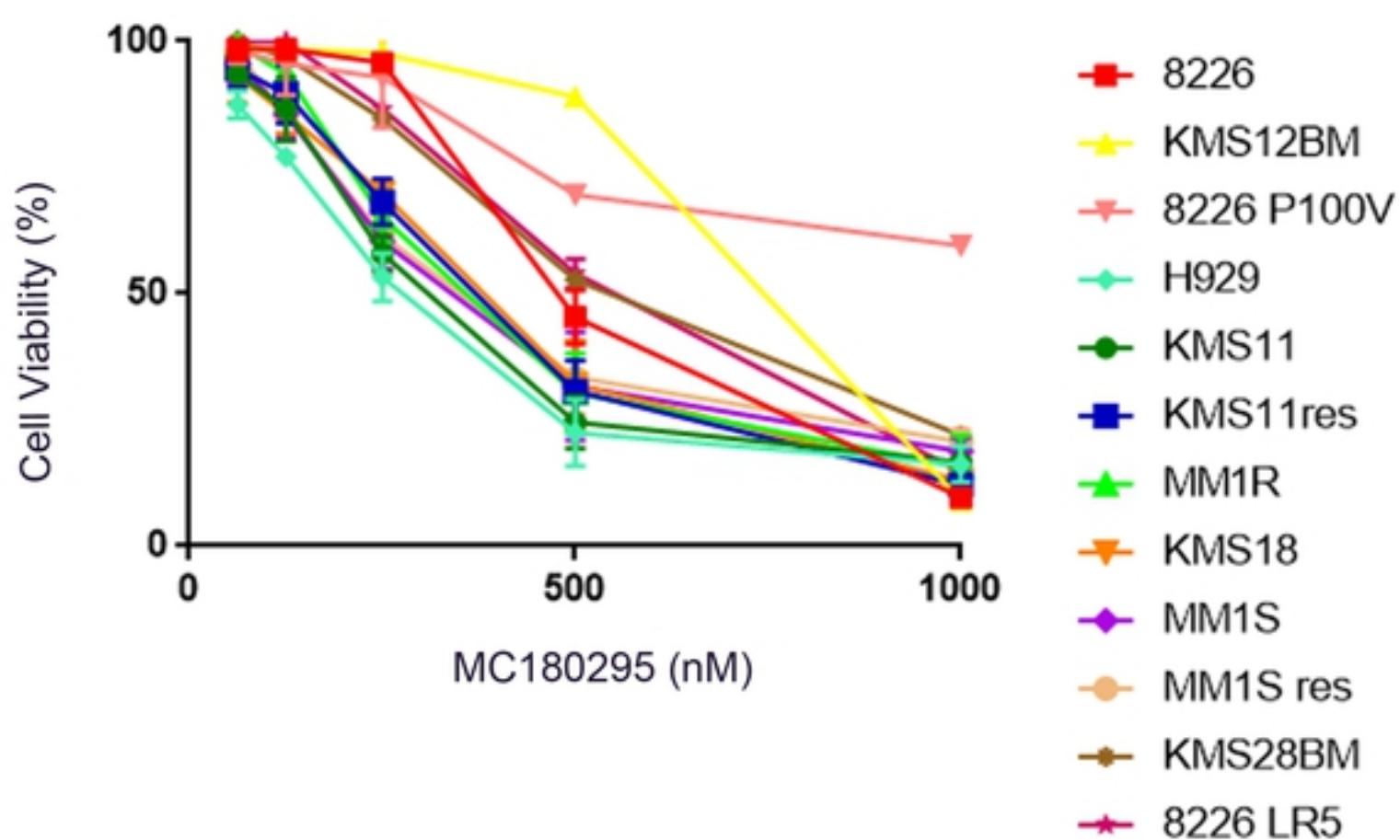
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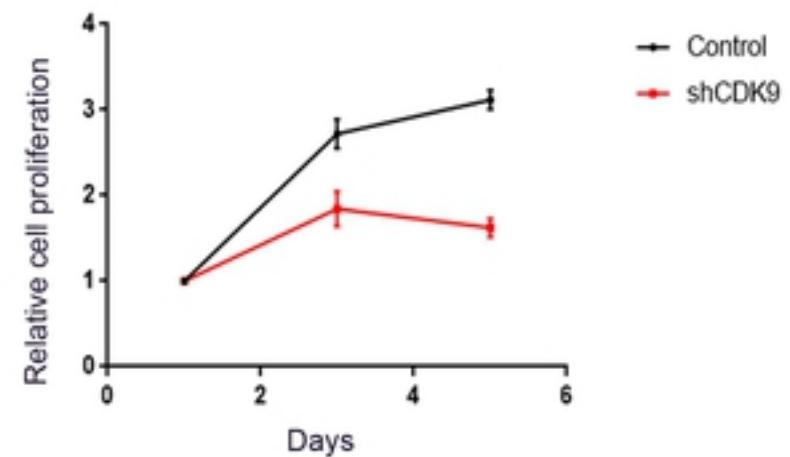
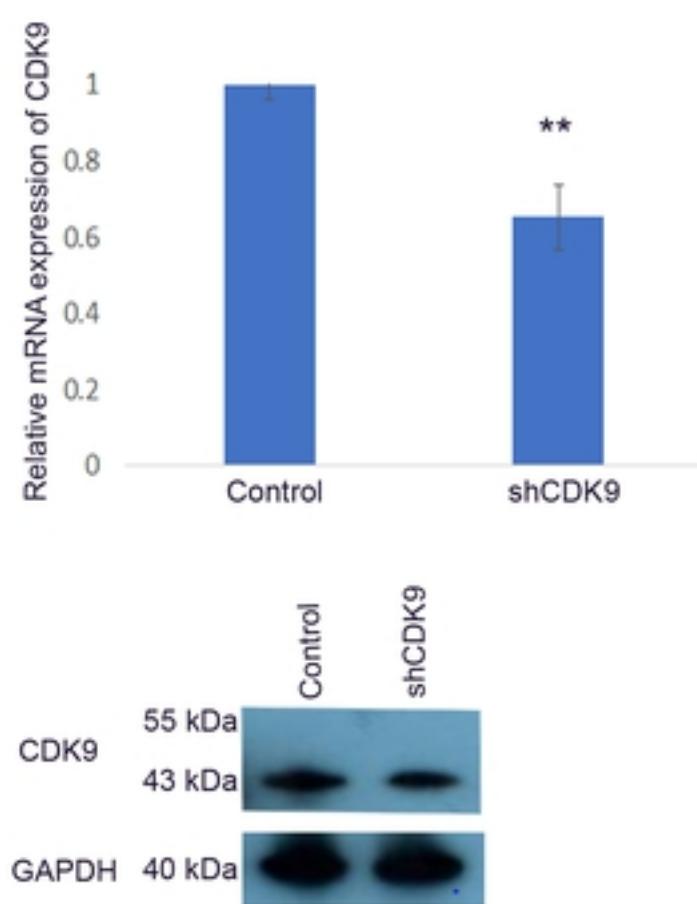


B



A

8226



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B

KMS28

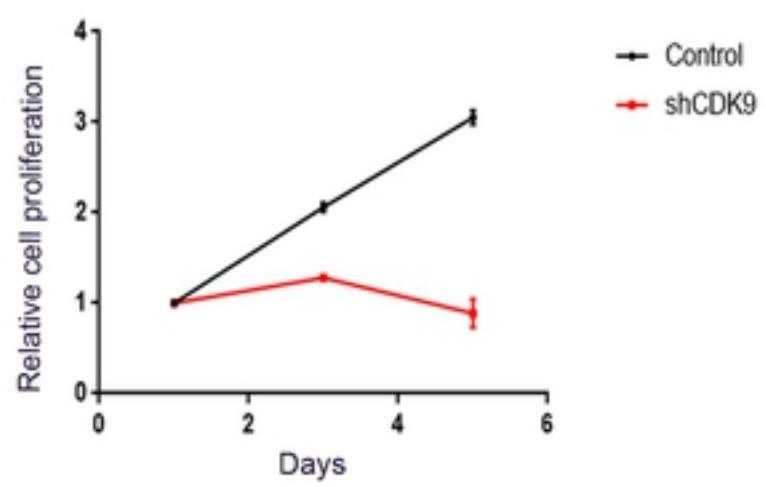
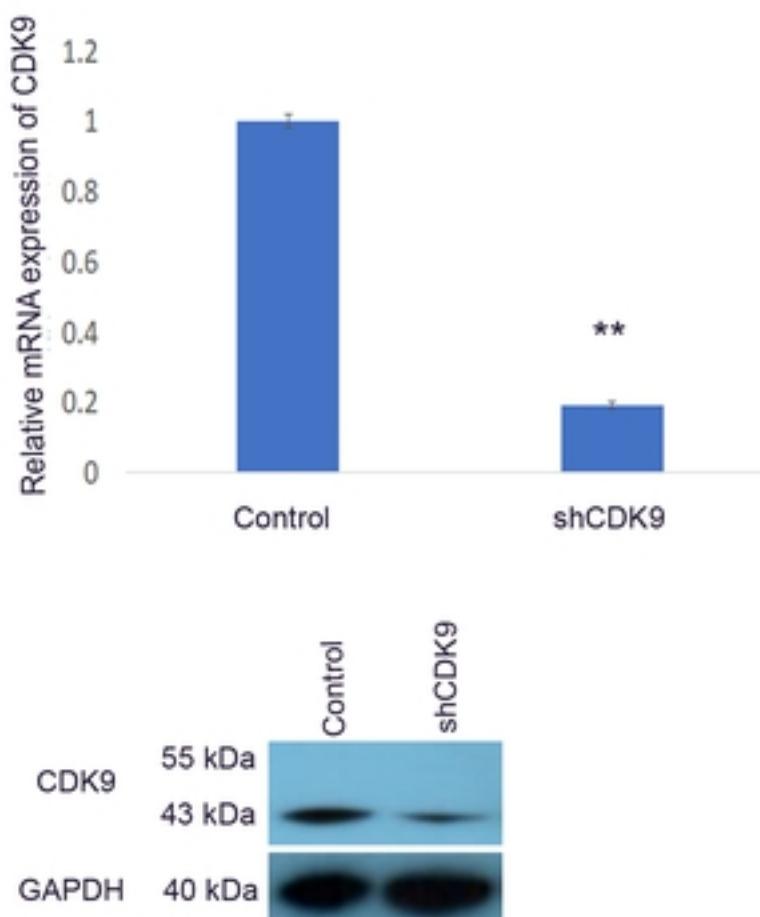
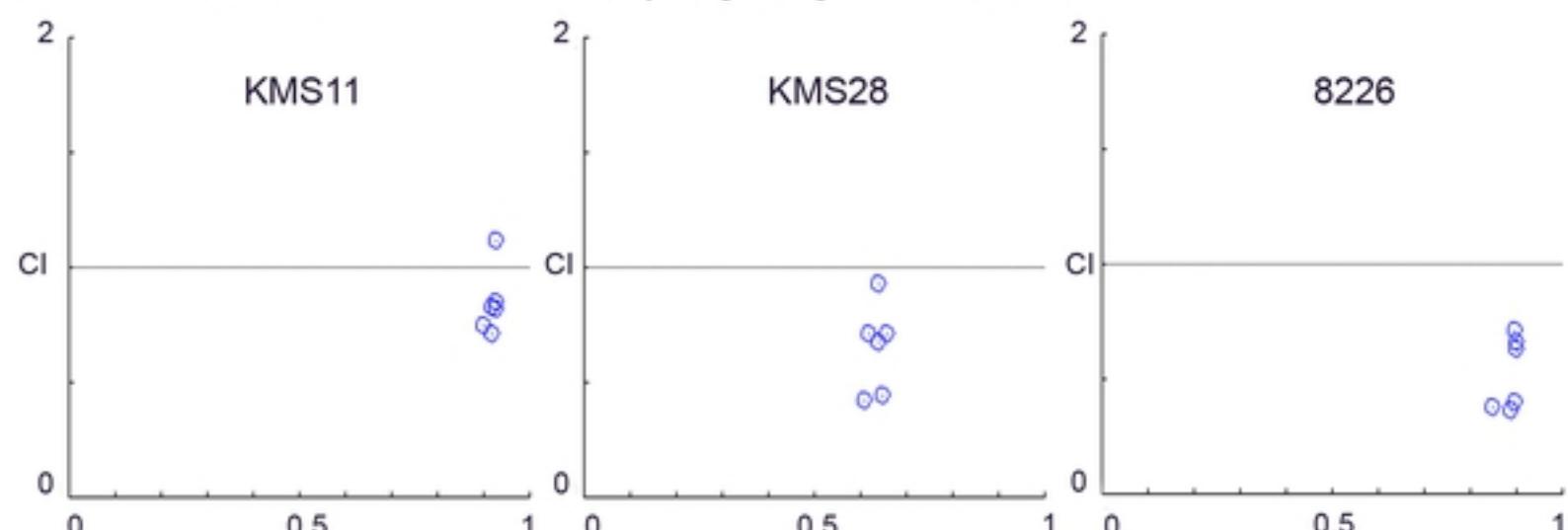


Fig 2

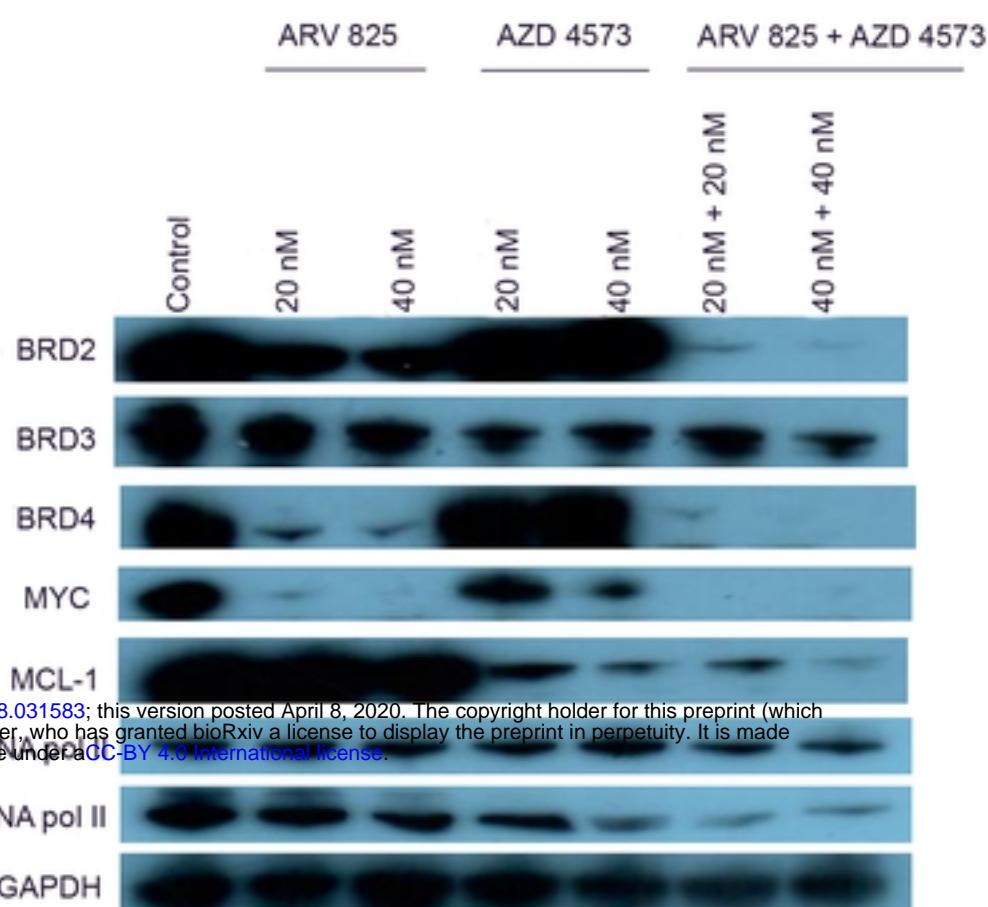
Combination Index: AZD4573 + ARV825, synergistic growth inhibition



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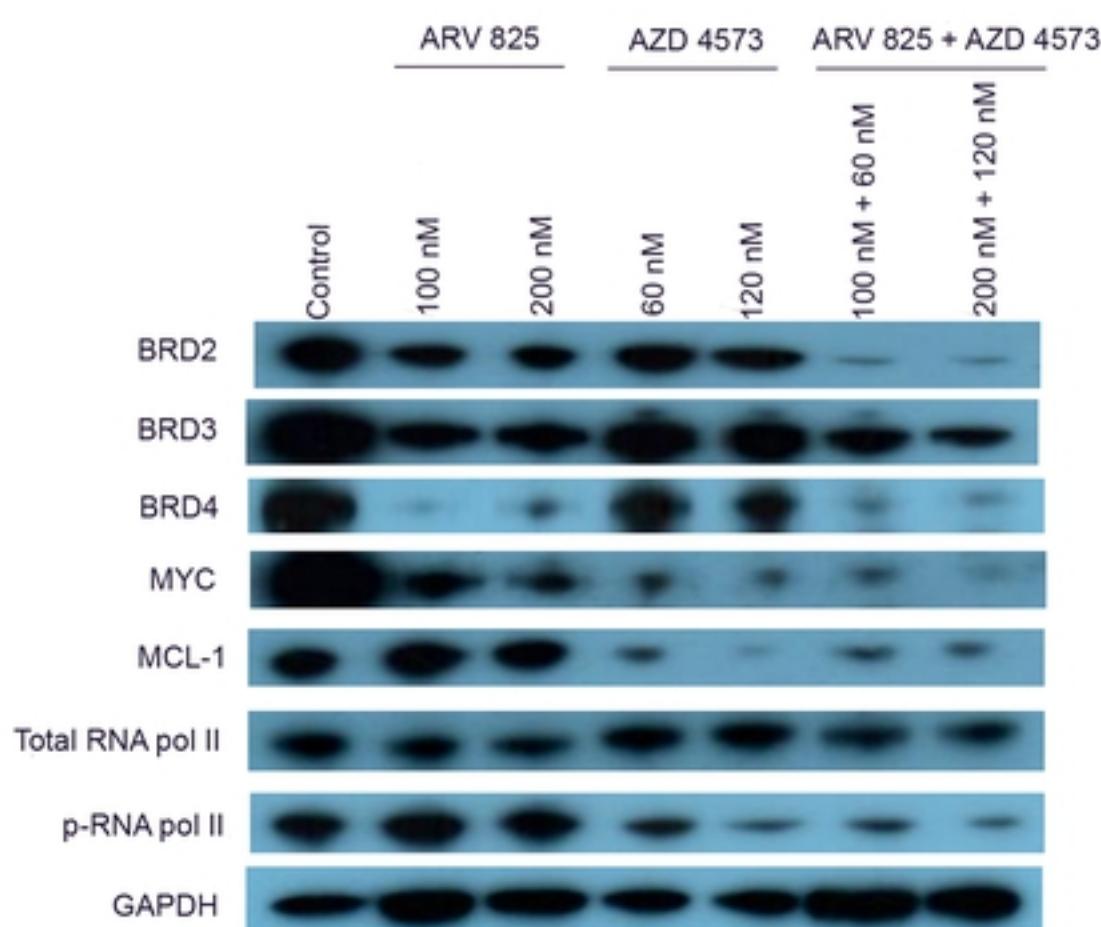
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KMS11



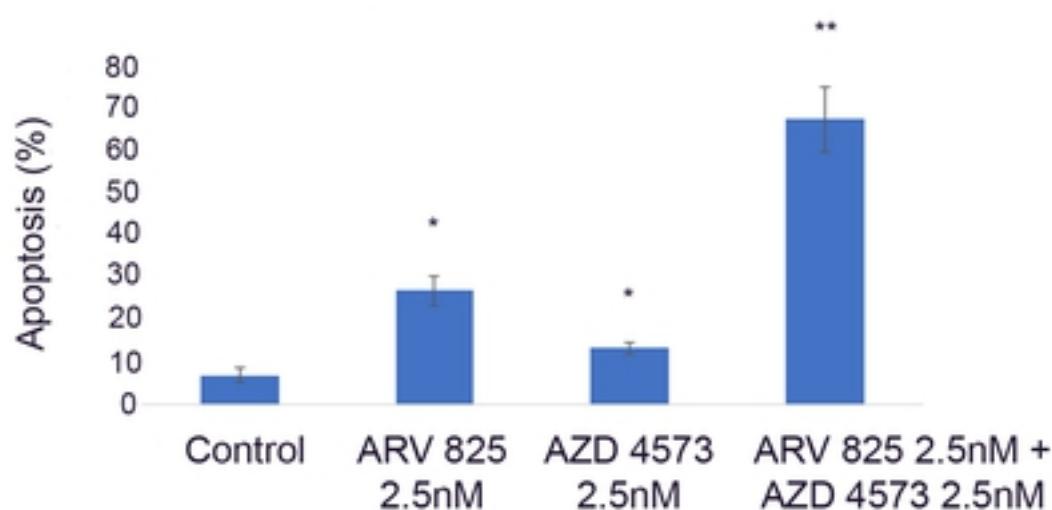
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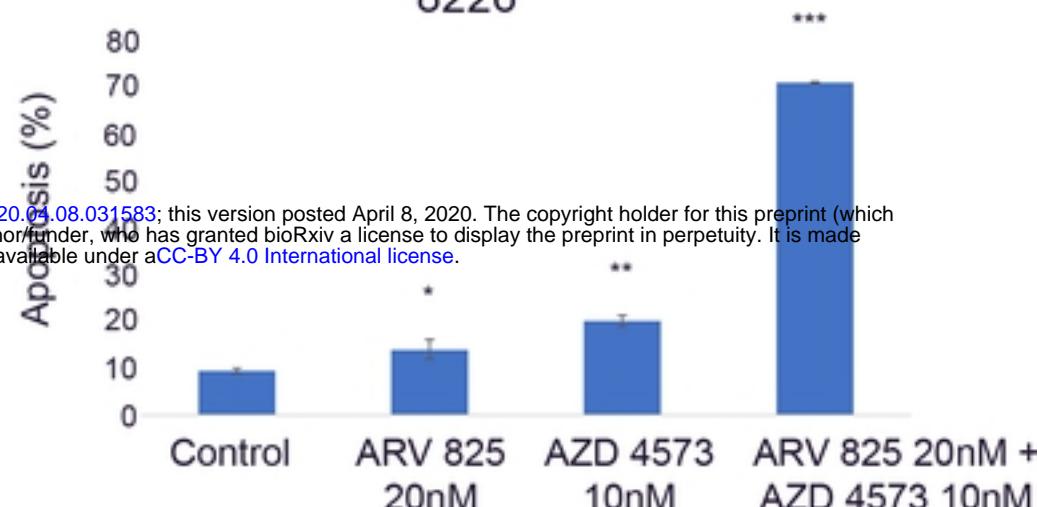


A

KMS11

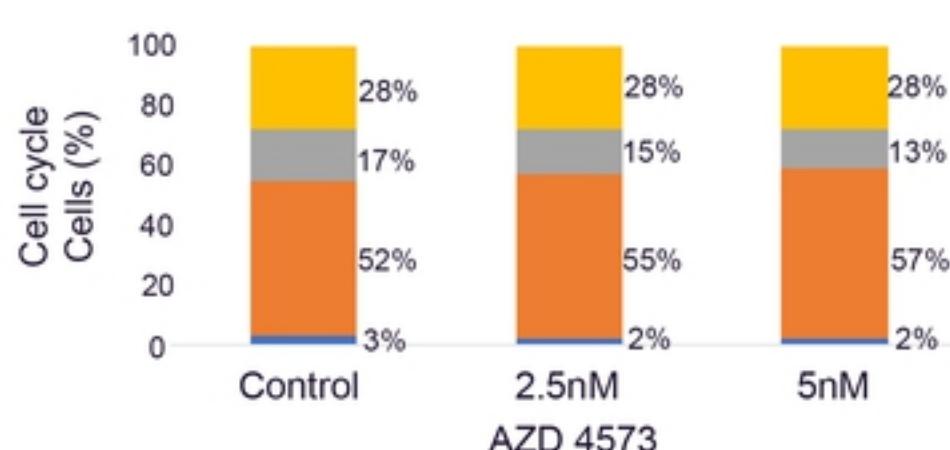


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B

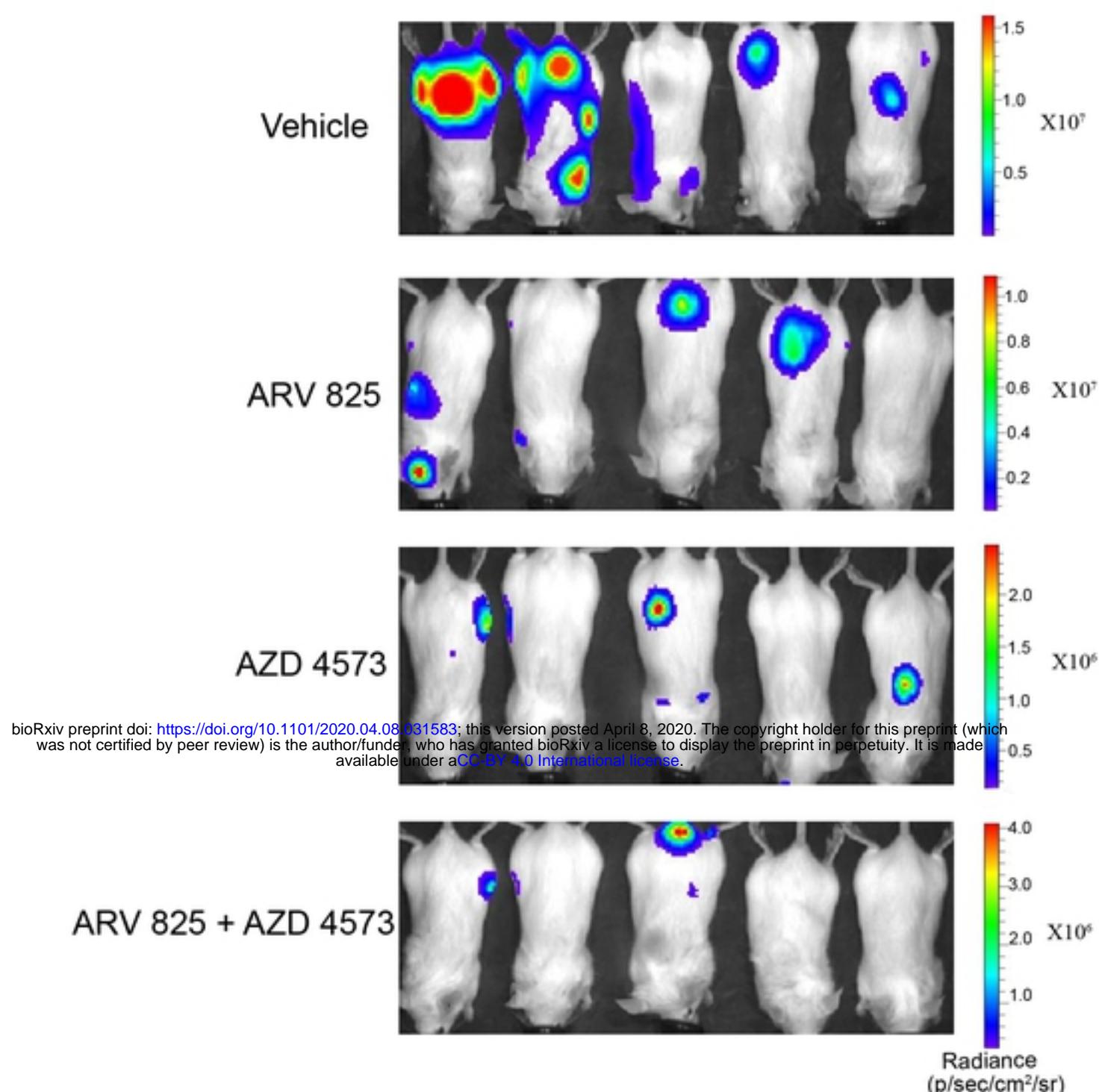
KMS11



8226



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B

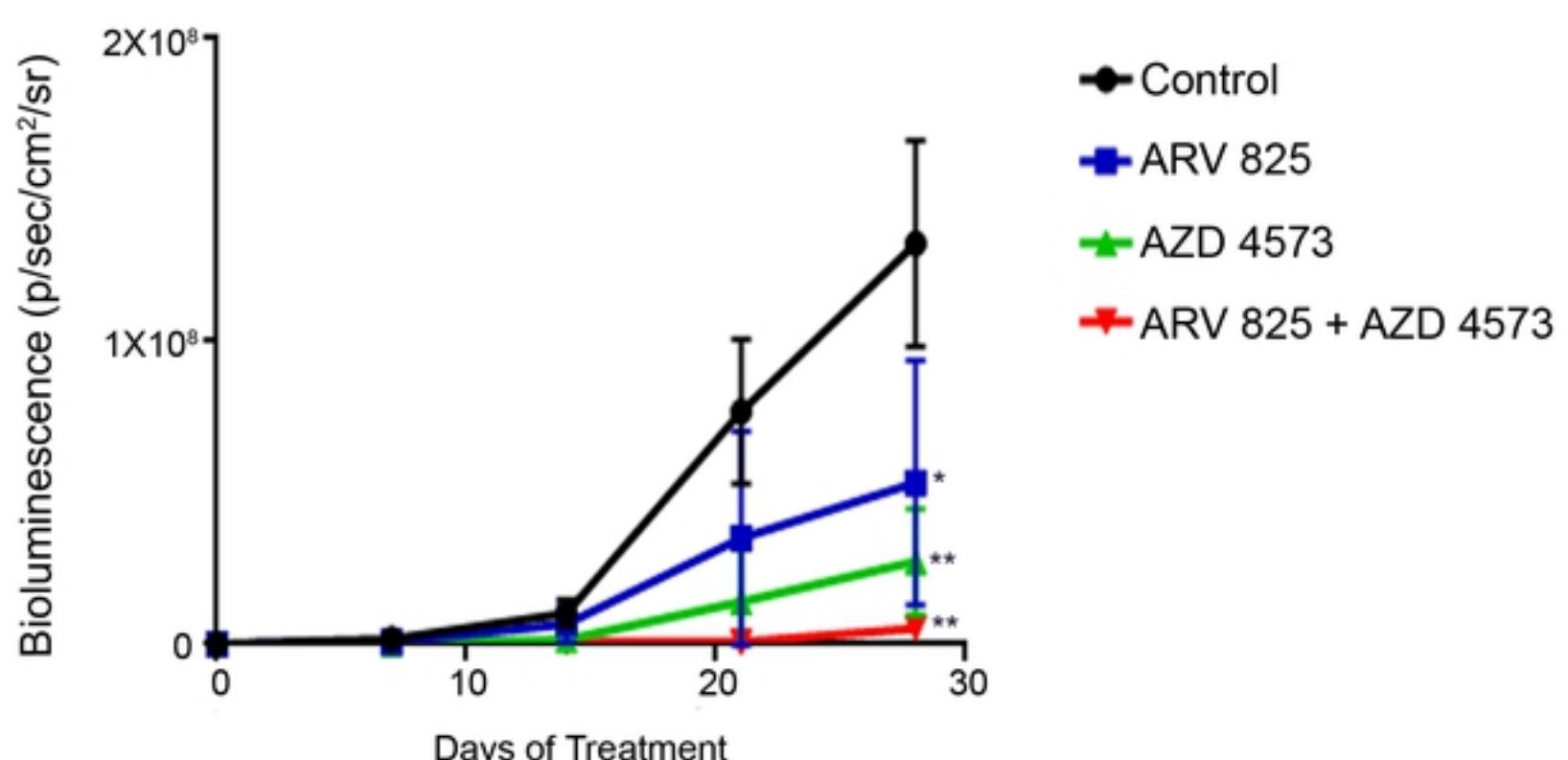


Fig 6