

Endoplasmic reticulum stress mediates resistance to BCL-2 inhibitor in uveal melanoma cells

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1 **Endoplasmic reticulum stress mediates resistance to BCL-2 inhibitor in uveal**
2 **melanoma cells**
3

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19 Running title: ABT-263 induces apoptosis in uveal melanoma cells
20
21

22 **Abstract**

23

24 To address unmet clinical need for uveal melanomas, we assessed the effects of
25 BH3-mimetic molecules, the ABT family, known to exert pro-apoptotic activities in
26 cancer cells.

27 Our results uncovered that ABT-263 (Navitoclax), a potent and orally bioavailable
28 BCL-2 family inhibitor, induced antiproliferative effects in metastatic human uveal
29 melanoma cells through cell cycle arrest at the G0/G1 phase, loss of mitochondrial
30 membrane potential, and subsequently apoptotic cell death monitored by caspase
31 activation and poly-ADP ribose polymerase cleavage. ABT-263-mediated reduction
32 in tumor growth was also observed *in vivo*. We observed in some cells that ABT-263
33 treatment mounted a pro-survival response through activation of the ER stress
34 signaling pathway. Blocking the PERK signaling pathway increased the pro-apoptotic
35 ABT-263 effect. We thus uncovered a resistance mechanism in uveal melanoma
36 cells mediated by activation of endoplasmic reticulum stress pathway. Therefore, our
37 study identifies ABT-263 as a valid therapeutic option for patients suffering from
38 uveal melanoma.

39

40 **Introduction**

41

42 Uveal melanoma is the most common primary intraocular malignancy in adult
43 population^{1,2}. Despite enucleation or radiotherapy of the primary lesion, metastases
44 develop in 50% of patients, mainly to the liver. These metastases are remarkably
45 refractory to conventional chemotherapies, immunotherapy with checkpoint inhibitors
46 and external radiotherapy^{3,4}. The median survival of patients who develop liver
47 metastasis is reported to be 4 to 15 months, and the one-year survival rate is
48 estimated to be 10-15%⁴. This highlights an urgent need for an efficient treatment.

49 Defective apoptosis, which contributes to sustained cell survival, is a major causative
50 factor in the development and progression of cancer. The ability of a cell to undergo
51 apoptosis is governed by members of the BCL-2 protein family that are grouped into
52 three sub-families based on the number of BH (BCL-2 Homology) domains they
53 share (BH1-4). They can be anti-apoptotic (e.g., BCL-2, BCL-XL, MCL-1) or pro-
54 apoptotic (e.g., BAX, BID, BIM, NOXA)⁵, among which some of them only contain the
55 BH3 domain⁶.

56 BCL-2 can exert its anti-apoptotic function by sequestering the activator BH3-only
57 proteins or through direct interaction with apoptosis-activating factors such as BAX
58 (BCL-2 associated-X-protein) and BAK (BCL-2 homologous antagonist/killer),
59 thereby modulating mitochondrial cytochrome c release. The release of cytochrome c
60 subsequently leads to caspase 9 activation and to apoptosome formation, which
61 activates the other caspases (caspases 3-7), ending in cell apoptosis.

62 Overexpression of the pro-survival BCL-2 family members is commonly associated
63 with cancer⁷. Such deregulations can be exploited by chemotherapeutic strategies,
64 such as the BH3-mimetic drugs, which inhibit the antiapoptotic proteins by occupying
65 their BH3-binding groove, to counteract the apoptotic blocks, and halt tumor
66 progression^{8,9}. Several BH3 mimetics, including ABT-737, ABT-263 (Navitoclax) and
67 ABT-199 (Venetoclax) have been developed as cancer therapeutics¹⁰. ABT-263
68 (Navitoclax), an orally available derivative of ABT-737, was tested as a single agent
69 in phase I/II for the treatment of different solid and haematological malignancies, yet
70 side effects such as thrombocytopenia have been reported^{9,11,12}. ABT-199 is an oral
71 second-generation BH3 mimetic that inhibits BCL-2, with much less activity against
72 BCL-xL. It is the first BH3 mimetic drug approved by the US Food and Drug
73 Administration for the treatment of some leukemias and lymphomas¹³.

74 Alternatively, BCL-2 family members are contained in other multiprotein complexes at
75 the endoplasmic reticulum (ER) that are involved in the control of diverse cellular
76 processes including calcium homeostasis and autophagy to regulate the switch
77 between adaptive and proapoptotic phases under stress. Increasing evidence
78 indicates that a functional activity of BCL-2 on ER protects mitochondria under
79 diverse circumstances¹⁴.

80 In primary uveal melanoma, expression of BCL-2 is significantly higher compared to
81 normal ocular structures, or choroidal melanocytes^{5,15}, suggesting that it may be
82 involved in the development and progression of these lesions. However, effects of
83 ABT drugs have not been assessed.

84 In this study, we showed *in vitro* and *in vivo* that ABT-263 has antiproliferative and
85 proapoptotic activities in uveal melanoma cells derived from primary tumors and
86 metastases. We demonstrated that the effect of ABT-263 in some cells is
87 accompanied with the activation of the ER stress response pathway that exerted a
88 cytoprotective effect. Blocking ER stress enhanced ABT-263 efficacy.

89

90 **Material and methods**

91

92 **Cell cultures and reagents**

93 Human uveal melanoma cell lines and short-term cultures derived from different
94 patients with metastatic malignant melanoma cells were grown in DMEM
95 supplemented with 7% FBS at 37°C in a humidified atmosphere containing 5% CO₂.
96 Lipofectamine™ RNAiMAX and opti-MEM medium were purchased from Invitrogen
97 (San Diego, CA, USA). ABT-263 was obtained from Euromedex and qVD from
98 Clinisciences.

99

100 **Western blot assays**

101 Western blotting was performed as previously described^{16,17}. Briefly, cell lysates (30
102 µg) were separated using SDS-PAGE, transferred onto a PVDF membrane and
103 subsequently exposed to the appropriate antibodies. Antibodies to BCL-2 (ms-123-
104 P0) was from neomarker, to caspase 3 (#610323) was from BD, to BAX (#8429) was
105 from sigma, to PARP (#9542), BCL-XL (#2762), CASPASE 9 (#9502), PUMA
106 (#4976), CHOP (#2895), PERK (#5683), Phospho-PERK (#3179), IRE1α (#3294),
107 BIP (#3177), were from Cell Signaling Technology Inc, to phospho-IRE1α (#NB100-
108 2323) was from Novus, to MCL1 (#sc-819), to NOXA (#sc-56169), and HSP90 (#sc-
109 13119) were from Santa Cruz biotechnology. The proteins were visualized using the
110 ECL system (Amersham). The western blots shown are representative of at least 3
111 independent experiments.

112

113 **Cell death analysis by flow cytometry**

114 Cells were seeded at a density of 100 000 cells/well, in 6-well plate and treated with
115 ABT-263 for indicated time. Cells were harvested using accutase enzyme, washed
116 twice with ice-cold phosphate-buffered saline, resuspended in a buffer (Hepes
117 250mM, NaCl 150mM, KCl 5mM, MgCl₂ 2mM, CaCl₂ 2mM) with DAPI (1µg/ml) and
118 Annexin V- Alexa Fluor 647 conjugate (1/100) and incubated for 15 minutes at room
119 temperature (25°C) in the dark. Samples were immediately analyzed by a flow
120 cytometer (MACS QUANT) using a laser at 405 nm excitation with a bandpass filter
121 at 425 nm and 475 nm for DAPI detection and a laser at 635 nm excitation with a
122 bandpass filter at 650 nm and 665 nm for Alexa Fluor 647 dye. Annexin and DAPI
123 mono- or double positive cells were counted as dead cells.

124

125

126 **Colony formation assay**

127 Human uveal melanoma cells were seeded onto 6-well plates. The cells were
128 subsequently placed in a 37°C, 5% CO₂ incubator. Colonies of cells were grown
129 before being stained with 0.04% crystal violet/2% ethanol in PBS for 30 min.
130 Photographs of the stained colonies were captured. The colony formation assay was
131 performed in duplicate.

132

133 **mRNA preparation and real-time/quantitative PCR**

134 The mRNA was isolated using TRIzol (Invitrogen) according to a standard procedure.
135 QRT-PCR was performed using SYBR® Green I (Eurogentec, Seraing, Belgium) and
136 Multiscribe Reverse Transcriptase (Applied Biosystems) and subsequently monitored
137 using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster
138 City, CA). The detection of the SB34 gene was used to normalize the results. Spliced
139 Xbp1 primers were previously reported¹⁸. Primer sequences for each cDNA were
140 designed using either Primer Express Software (Applied Biosystems) or qPrimer
141 depot (<http://primerdepot.nci.nih.gov>), and these sequences are available upon
142 request.

143

144 **Animal experimentation**

145 Animal experiments were performed in accordance with French law and approved by
146 a local institutional ethical committee. The animals were maintained in a temperature-
147 controlled facility (22°C) on a 12-h light/dark cycle and provided free access to food
148 (standard laboratory chow diet from UAR, Epinay-S/Orge, France). Human Mel270
149 uvealmelanoma cells were subcutaneously inoculated into 8-week-old female,
150 immune-deficient, athymic, nude FOXN1^{nu} mice (Harlan Laboratory). When the
151 tumors became palpable (0.1-0.2 cm³), the mice received an intraperitoneal injection
152 of ABT-263 (50 mg/kg), dissolved in 10% DMSO 6 times per week. Control mice
153 were injected with DMSO alone. The growth tumor curves were determined after
154 measuring the tumor volume using the equation $V=(L \times W^2)/2$. At the end of the
155 experiment, the mice were euthanized by cervical dislocation.

156

157 **Statistical analysis**

158 The data are presented as the means + SD and analyzed using a two-way ANOVA
159 or two-sided t-test with Graph Pad Prism. The difference between both conditions
160 was statistically significant at p-value <0.05.

161 **Results**

162

163 **ABT-263 triggers apoptotic cell death of uveal melanoma cells.**

164 We first conducted experiments to assess the effect of three ABT drugs, ABT-199,
165 ABT-737 and ABT-263 on uveal melanoma cell proliferation using four human uveal
166 melanoma cell lines. Mel270¹⁹, 92.1²⁰ and OMM2.5¹⁹ were not sensitive to ABT-199,
167 and OMM1²¹ showed a moderate proliferation inhibition (about 15%) (Fig.1a).
168 Whereas Mel270 and 92.1 were also highly resistant to ABT-737 and ABT-263, both
169 drugs strongly reduced proliferation of OMM1 and OMM2.5 cells. Accordingly, cell
170 death was higher in OMM1 and OMM2.5 cells compared to Mel270 and 92.1 cells
171 (Fig. 1b). Thus, ABT-263 and the related ABT-737 gave similar and superior results
172 to ABT-199. Since ABT-263 is used in clinical trials, we decided to pursue with ABT-
173 263 for subsequent studies.

174 In agreement with the previous observations, in a colony formation assay, the OMM1
175 and OMM2.5 cell lines were more sensitive than Mel270 and 92.1 cell lines to the
176 long-term growth inhibitory activity of ABT-263 (Fig. 1c).

177 Western blot indicated that the effect of ABT-263 was associated with cell cycle
178 arrest in Mel270 cells, as shown by a reduced CDK4 expression and an increase in
179 the cell cycle inhibitors p27 and p21 (Supplementary Fig. 1).

180 We next assessed the ability of ABT-263 to induce apoptosis in the cell line panel.
181 ABT-263 cytotoxicity as measured by Annexin V/DAPI staining revealed that OMM1
182 and OMM2.5 uveal melanoma cells were much more sensitive than Mel270 and 92.1
183 uveal melanoma cell lines, inducing 80% versus 20-35% cell death, respectively (Fig.
184 2a). The pan-caspase inhibitor qVD triggered a protective effect in all cell lines.
185 Additionally, ABT-263 induced a time-dependent decrease of the full-length PARP
186 protein, and caspase 3 zymogen (Fig. 2b) in the different cell lines. The
187 disappearance of total poly ADP-ribose polymerase (PARP) and of the zymogenic
188 form of caspase 3 appeared weaker in Mel270 and 92.1 cells compared to OMM1
189 and OMM2.5 cells.

190 A dose-dependent cleavage of PARP, caspase 9 and caspase 3 was also observed
191 in all cell lines (Fig. 2c). Cleavage of caspase 3 was detected earlier in the OMM1
192 metastatic cells than in Mel270 cells. As confirmation that cell death was due to
193 apoptosis, pretreatment with the pan caspase inhibitor qVD prevented both PARP
194 and caspase cleavage (Fig. 2c).

195

196 Protein immunoblot revealed that the expression level of the main anti-apoptotic
197 BCL-2 subfamily members (BCL-2, MCL1, BCL-xL) was higher in OMM1 cell line
198 compared to Mel270 cells (Fig. 2d). After 24h of treatment, the only noticeable
199 change in primary cells was the increase in pro-apoptotic NOXA level (Fig. 2d). In
200 OMM1 cells, but not in Mel270 cells, due to significant cell death, ABT-263 effect was
201 accompanied by a reduced expression of pro-survival (BCL-xL, MCL1), and pro-
202 apoptotic (PUMA, BAX) members (Fig. 2d). BCL-2 exhibited no change in response
203 to ABT-263.

204 BH3-only proteins control the mitochondrial pathway to apoptosis. We therefore
205 measured the mitochondrial membrane potential by tetramethylrhodamine ethyl ester
206 (TMRE) staining. ABT-263 dose-dependently reduced the mitochondrial membrane
207 potential in both Mel270 and OMM1 cells (Fig. 2e). Interestingly, while qVD treatment
208 did not prevent mitochondrial membrane depolarization in Mel270 cells
209 (Supplementary fig. 2a), it blocked this process following ABT-263 exposure in
210 OMM1 cells (Supplementary fig. 2b), suggesting that different mechanisms of cell
211 death induction operate in these the two cell lines.

212 Altogether, these data demonstrated that ABT-263-induced cell death via apoptosis
213 in uveal melanoma cells. Notably, OMM1 and OMM2.5 melanoma cells appeared
214 more sensitive to ABT-263 effects than Mel270 and 92.1 cells.

215

216 **ABT-263 Prevents Growth of Human Uveal Melanoma Tumor Xenografts**

217 Experiments were next conducted *in vivo* to investigate the antineoplastic effect of
218 ABT-263. For this purpose, the Mel270 and OMM1 human uveal melanoma cells
219 were engrafted subcutaneously on the flank of nude mouse, which were
220 subsequently treated with ABT-263 or with its vehicle.

221 ABT-263 was found to be partially effective in the group of established Mel270
222 xenografts (Fig. 3a) whereas it induced marked tumor growth inhibition of metastatic
223 OMM1 xenografts, leading in mice to complete tumor shrinkage (Fig. 3b). Excised
224 tumors in the ABT-263 group weighted significantly less than those in the control
225 group (Fig. 3c-d). There was no significant difference in body weight between mice
226 treated with vehicle or ABT-263, indicating no toxicity issues caused by ABT-263 *in*
227 *vivo*. Thus, ABT-263 proved to be highly efficient in killing metastatic uveal
228 melanoma cells and not only prevented tumor growth *in vitro* but also *in vivo*.

229

230 **ABT-263 induces endoplasmic reticulum (ER) stress**

231 BCL-2 protein family is mainly known for its anti-apoptotic role operating at the
232 mitochondria level, yet it is also recognized for its role in the endoplasmic reticulum
233 (ER)²². Since our different cell lines showed variable sensitivity to ABT-263, we
234 decided to investigate whether these cell lines also differed in the effect of ABT-263
235 on the ER pathway.

236 ER stress leads to unfolded protein response (UPR) which is sensed by the ER
237 chaperone protein glucose regulated protein 78 (GRP78/also called BIP) and
238 activation of three known ER resident proteins: inositol requiring protein-1 (IRE1),
239 protein kinase RNA-like ER kinase (PERK), and activating transcription factor-6
240 (ATF6), and then converge to drive the expression of C/EBP homologous protein
241 (CHOP)²³.

242 ABT-263-mediated ER stress was analyzed in the cell lines panel using detection of
243 BIP, ATF4 (PERK signaling) and CHOP as surrogates for an ER stress response. In
244 both Mel270 and 92.1 cells, ABT-263 enhanced ATF4 and CHOP as well as BIP
245 level (Fig. 4a). In contrast, induction of ATF4 and CHOP by ABT-263 was hardly
246 detectable in OMM1 and OMM2.5 human uveal melanoma cells, and BIP was not
247 detected in OMM1 cells. We also did not detect PERK and IRE1 α phosphorylation
248 (Thy980 and Ser724 respectively) in OMM1 and OMM2.5 cell lines treated with ABT-
249 263 (data not shown).

250 Of note, in contrast to ABT-263, tunicamycin, another ER stress inducer, was able to
251 stimulate CHOP in OMM1, thus ruling out that the ER stress pathway is deficient in
252 OMM1 and OMM2.5 cell lines (Supplementary Fig. 3). Furthermore, XBP1 splicing
253 was significantly higher following ABT-263 exposure (Fig. 4b).

254 Kinetics analysis in Mel270 primary uveal melanoma cells revealed that the protein
255 level of BIP was enhanced following treatment with ABT-263 (Fig. 4c). PERK and
256 EIF2 α were phosphorylated (Thy980 and Ser51 respectively) and ATF4 increased.
257 We also observed IRE1 α phosphorylation and an increase in ATF6 expression and
258 cleavage. There was also a significant upregulation of the very downstream ER
259 stress effector CHOP (Fig. 4c). Treatment with the pan caspase inhibitor qVD did not
260 impact the ABT-263 mediated change in ER molecules. These data indicate that in

261 Mel270 uveal melanoma cells, ABT-263 activated all branches of UPR, and that UPR
262 operated upstream of the apoptotic pathway.

263 Altogether, our data indicate that ABT-263 elicits activation of the ER stress pathway
264 in Mel270 and 92.1 uveal melanoma cells, while there are signs of defective
265 activation of this pathway in OMM1 and OMM2.5 cells.

266

267 **ER stress protects from ABT-263-induced apoptosis**

268 Chemotherapy regimens were previously found to cause ER stress and to activate
269 UPR, which paradoxically is a process that prevents apoptosis of the cancer cells
270 and promotes their survival.

271 To delineate the role of ER stress pathway activation in ABT-263 effects, we used
272 genetic or pharmacological intervention to target these pathways. Although ATF6
273 increases XBP1 expression, the spliced form of XBP1 that activates the UPR
274 efficiently is mediated by IRE1 α ²⁴. We were therefore focusing on PERK and IRE1 α ,
275 which are the two main branches.

276 We first analysed siRNA against IRE1 α . Whereas it efficiently reduced IRE1 α
277 expression in Mel270 and 92.1 cells, it did not enhance cell death mediated by ABT-
278 263 treatment (Supplementary Fig. 4a-b). Further, although OMM1 and OMM2.5
279 cells were more sensitive to ABT-263, no additive effect was observed when ABT-
280 263 was combined with siRNA against IRE1 α (Supplementary Fig. 4b). siRNA
281 against PERK efficiently inhibited PERK mRNA level as shown by QPCR in both 92.1
282 and Mel270 cells (Figure 5A and Supplementary Figure 5A). Inhibition of total PERK
283 that translated into inhibition of its phosphorylation was also confirmed by western
284 blot analysis in 92.1 cells (Fig. 5b). PERK inhibition also impaired EIF2 α activation as
285 revealed by the loss of EIF2 α phosphorylation in both basal and ABT-263-treated
286 92.1 cells (Fig. 5c). To reveal the effect of combined ABT-263 and PERK down-
287 regulation, we decreased the ABT-263 concentration to 3 μ M, which did not induce
288 cell death in 92.1 cells (Fig. 1a-b). Whereas ABT-263 treatment or PERK inhibition
289 exhibited a relatively low effect on PARP cleavage and cell death when used alone,
290 the combination significantly increased the effect of ABT-263 and its killing efficacy,
291 illustrated by PARP cleavage and an increase in cell death (Fig. 5b-c). This same
292 holds true for Mel270 cells (Supplementary Fig. 5b-c). We also tested the effect of
293 GSK2606414, a cell-permeable PERK inhibitor. GSK2606414 treatment reduced
294 CHOP and ATF4 accumulation, demonstrating its efficacy in Mel270 (Supplementary

295 Fig. 5d). Whereas GSK2606414 alone displayed no effect on cell viability, the effect
296 of ABT-263 was improved when combined with GSK2606414 (Fig. 5e and
297 Supplementary Fig. 5e), consistent with data obtained with PERK siRNA. Altogether,
298 inhibition of anti-apoptotic BCL-2 proteins by ABT-263 induces a protective feedback
299 response in 92.1 and Mel270 uveal melanoma cells, via induction of the ER stress
300 response that can be prevented with PERK inhibitor.

301 Discussion

302

303 Metastatic uveal melanoma represents a major clinical challenge since, thus far,
304 there is no efficient systemic treatment. Uveal melanomas are genetically different
305 from cutaneous melanomas and are highly resistant to the therapeutic options used
306 to treat cutaneous melanomas. One of the main mechanisms for cancer cells
307 escaping apoptosis induction is through over-expression or anti-apoptotic proteins,
308 such as the BCL-2-protein family, of which BCL-2 is the prototype.

309 Certain malignancies, mostly leukemias and lymphomas, appear addicted to a single
310 pro-survival protein (mainly BCL-2). In line with that, BCL-2 transgenic mice
311 exclusively develop hematopoietic malignancies²⁵.

312 BCL-2 was also previously shown to be overexpressed in uveal melanoma compared
313 to normal tissue^{26,27}. Previous studies showed that inhibition of BCL-2 alone by
314 antisense oligonucleotides caused cell death of uveal melanoma cells and that
315 reduction in BCL-2 through the use of miR-182 suppressed the *in vitro* and *in vivo*
316 growth of uveal melanoma cells²⁸. Zeaxanthin, a carotenoid pigment, has also been
317 shown to decrease uveal melanoma cell viability through BCL-2 inhibition²⁹.

318 However, the survival of solid tumors, is often safeguarded by multiple pro-survival
319 BCL-2 family members¹⁰. Thus, these tumors require drugs targeting both BCL-2 and
320 BCL-xL¹⁰. Consistent with this notion, our results showed that ABT-199 (mainly
321 directed against BCL-2) lacked efficiency in killing uveal melanoma cells. In contrast,
322 we showed that ABT-263 which targets BCL-2 and BCL-xL has anti-proliferative and
323 pro-apoptotic activities in uveal melanoma cell lines. Noteworthy, in another study,
324 monotherapy with S44563-2, another BCL-2/BCL-xL inhibitor, exhibited a limited
325 effect on uveal melanoma cells; however, the killing efficacy of S44563-2 was
326 improved when combined with fotemustine²⁷. Differences in the mechanisms of drug
327 action may explained the discrepancies in ABT-263 and S44563-2 effects. Another
328 explanation might be that the level of BCL-2 and BCL-xL expression varied in the
329 cells selected in these two different studies and thereby influenced their response to
330 ABT-263 and S44563-2.

331 The BCL-2 protein family has extensively been studied for their role in mitochondrial
332 apoptosis. However, the BCL-2 family seems to play a crucial role also in the
333 endoplasmic reticulum and on the crosstalk with the mitochondria, operating as a
334 stress rheostat³⁰. We observed that ABT-263 caused endoplasmic reticulum (ER)

335 stress and activated the unfolded protein response (UPR) in primary melanoma cells,
336 whereas in metastatic cells, ABT-263 elicited only a partial activation of the UPR.
337 Although BIP increase following ABT-263 treatment was easily seen in Mel270, 92.1
338 and OMM2.5 uveal melanoma cells, BIP was hardly detected in OMM1 cells.
339 Increased BIP expression reflects the intensity of the UPR activation. Furthermore,
340 CHOP and ATF4 elevation were not detected in OMM1 and OMM2.5 cell lines upon
341 ABT-263 treatment. However, tunicamycin (a known ER stress inducer), enhanced
342 CHOP levels (Supplementary figure 3), thereby indicating that this pathway is
343 functional in the metastatic cells and that the lack of ER stress stimulation by ABT-
344 263 is specific.

345 Thus, most likely, OMM1 and OMM2.5 cells are more sensitive because of partial
346 UPR activation that is not able to counteract the ABT-263 killing effect. It will be
347 interesting to assess whether activation of UPR was employed by uveal melanoma
348 cells used in the previous studies^{28,29,31}. In this context, the UPR pathway may be a
349 powerful novel target to improve drug efficacy in the treatment of these uveal
350 melanoma tumors.

351 Among the three UPR branches, two converge towards IRE1 α . Indeed, ATF6
352 increases *XBP1* mRNA expression while IRE1 α mediates its splicing, resulting in the
353 translation of a spliced active form of XBP1 (XBP1s). The PERK-EIF2 α axis
354 enhances ATF4. Both XBP1s and ATF4 function as transcription factors that regulate
355 a wide range of genes, which plays a crucial role in cell adaptation to stress
356 conditions^{32,33}.

357 Our results indicate that the protective effect mounted by Mel270 and 92.1 uveal
358 melanoma cells in response to ABT-263 specifically involved the PERK/EIF2 α /ATF4
359 signaling cascade. Indeed, in contrast to IRE1 α inhibition that did not change the
360 effect of ABT-263, the combination of ABT-263 with PERK inhibition synergistically
361 reduced the survival rate of primary uveal melanoma cells.

362 Mel270 and 92.1 which are primary cells appeared more resistant to ABT-263 killing
363 activity than OMM1 and OMM2.5 that are metastatic cells. Interestingly, following
364 ABT-263 treatment, which targets both BCL-2 and BCL-xL, we did not observe in
365 Mel270 and OMM1 cells a compensatory increase in the other anti-apoptotic
366 proteins, ruling out the possibility that a change in the anti-apoptotic protein level
367 causes the different sensitivity of the cell lines to ABT-263.

368 The difference in sensitivity of primary and metastatic cells may also reflect the
369 addiction of the selected cell lines to pro-survival BCL-2 family members.
370 Another explanation could be that the uveal melanoma cell lines did not retain the
371 major features of the original tissue. Indeed, we showed that ABT-263 was able to
372 efficiently kill primary uveal melanoma cells that we freshly isolated from a human
373 biopsy (Supplementary Figure 6). We are aware that a higher number of cell lines
374 should be tested to firmly conclude on the response of primary versus metastatic
375 cells to ABT-263 effect. Nevertheless, independently of the tumor stage, we
376 uncovered a resistance mechanism in uveal melanoma cells mediated by activation
377 of endoplasmic reticulum stress pathway. In such context, expression level of ER
378 stress effectors could represent both marker of ABT-263 response and therapeutic
379 targets. Therefore, inhibition of anti-apoptotic BCL-2 proteins by ABT-263 alone or in
380 combination with an ER stress inhibitor represents a potential therapeutic strategy in
381 uveal melanoma treatment.
382

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384

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393

394 **Competing interests**

395

396 The authors declare no conflicts of interest.

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398

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484

485

486 **Legends**

487

488 **Figure 1: Effect of ABT drugs on uveal melanoma cells.** **a.** Viability test (XTT)
489 after exposure of a panel of uveal melanoma cell lines to BH3 mimetics drugs for
490 48h. Results are represented as means +SD of a minimum of three independent
491 experiments, **P-value<0.01, ***P-value<0.001. **b.** FACS analyses of Annexin
492 V/DAPI double staining in a panel of uveal melanoma cells indicate dead (early, late
493 apoptosis and necrosis in light grey) cells after exposure to BH3 mimetics drugs for
494 48h. Results are represented as means +SD of minimum three independent
495 experiments, *P-value<0.05, **P-value<0.01, ***Pvalue<0.001. **c.** Colony formation
496 assay of primary and metastatic uveal melanoma cells exposed to ABT-263 3 μ M and
497 5 μ M. Colonies were stained with crystal violet after 7 days (representative
498 micrographs are shown).

499

500 **Figure 2: ABT-263 induces apoptosis of uveal melanoma cells.** **a.** FACS
501 analyses of Annexin V/DAPI double staining in a panel of uveal melanoma cells
502 indicate alive (white) or dead (early, late apoptosis and necrosis in light grey) cells
503 after exposure to ABT-263 5 μ M for 48h in the absence or presence of qVD-OPh 20
504 μ M. Results are represented as means +SD of three independent experiments, *P-
505 value<0.05, ***P-value<0.001. **b.** Western blot analysis of PARP and caspase 3 in
506 Mel270, 92.1, OMM2.5 and OMM1 uveal melanoma cells exposed to ABT-263 5 μ M
507 for 24h and 48h. Detection of HSP90 serves as a loading control. Representative
508 immunoblots are shown. **c.** Western blot analysis of PARP, caspase 9 and caspase 3
509 human primary Mel270 and metastatic OMM1 uveal melanoma cells exposed to
510 ABT-263 3 μ M or 5 μ M for 48h in the absence or presence of qVD-OPh 20 μ M.
511 Detection of HSP90 serves as a loading control. Representative immunoblots are
512 shown. **d.** Western blot analysis of pro- and anti-apoptotic members of the BCL-2
513 family proteins in Mel270 and OMM1 uveal melanoma cells that were left untreated
514 or treated with ABT-263 3 μ M or 5 μ M for 24 hrs. HSP90 was used as a loading
515 control. **e.** Detection of the mitochondrial membrane potential using TMRE staining in
516 Mel270 and OMM1 uveal melanoma cells exposed to ABT-263 3 μ M or 5 μ M for 48h.
517 Percent of TMRE negative cells is shown. ***P-value<0.001.

518

519 **Figure 3. ABT-263 impairs tumor growth in vivo.** **a.** Mel270 and **b.** OMM1 uveal
520 melanoma cells were subcutaneously engrafted into athymic nude mice (n=8 per
521 group). Once the tumours reached 100mm³, mice were treated daily with ABT- 263.
522 The red arrow indicates the start of the treatment. The growth tumor curves were
523 determined by measuring the tumor volume using the equation $V = (L \times W \times W)/2$,
524 where V is tumor volume, W is tumor width, L is tumor length. Results are presented
525 as mean (\pm SEM) tumor volumes (mm³). **P-value< 0.01, ***P-value<0.001 are from
526 2-way anova test in ABT-263 treated versus vehicle at each point. **c-d.** The weight of
527 the sub-cutaneous tumor from control (Ct) and ABT-263-treated mice is shown. *P-
528 value<0.05, **P-value< 0.01.

529

530 **Figure 4: ABT-263 induces ER stress and activation of the unfolded protein**
531 **response.** **a.** Western blot of ER stress molecules in uveal melanoma cells treated
532 with ABT-263 5 μ M for 24 h and 48 h. HSP90 is used as a loading control.
533 Representative western blots are shown. **b.** XBP1 splicing in uveal melanoma cells
534 exposed to ABT- 263 5 μ M for 15h. *P-value<0.05, **P-value< 0.01. **c.** Kinetic
535 analysis of ER stress proteins in Mel270 cells treated with ABT-263 5 μ M in the
536 absence or presence of qVD-Oph 20 μ M. HSP90 is used as a loading control.
537 Representative western blots are shown.

538

539 **Figure 5: PERK dampens ABT-263 killing activity.** **a.** Q-PCR analysis of PERK
540 level in 92.1 uveal melanoma cells treated with control (siCtl) or PERK (siPERK)
541 siRNA for 48 h. ***P-value< 0.001. **b.** Western blot analysis to PERK and P-PERK in
542 92.1 cells treated with control (siCtl) or PERP (siPERK) siRNA. HSP90 is used as a
543 loading control. **c.** 92.1 uveal melanoma cells treated with control (siCtl) or PERK
544 (siPERK) siRNA for 48 h before being exposed to ABT-263 3 μ M for 48 h.
545 Representative western blot to P-EIF2 α , total EIF2 α and PARP are shown. HSP90
546 serves as a loading control. **d.** 92.1 cells were treated as in (B). FACS analyses of
547 Annexin V/DAPI double staining in uveal melanoma cells indicate alive (white) or
548 dead (early, late apoptosis and necrosis in light grey) cells. ***P-value< 0.001. **e.** 92.1
549 cells were treated as in (B). FACS analyses of Annexin V/DAPI double staining in
550 uveal melanoma cells indicate alive (white) or dead (early, late apoptosis and
551 necrosis in light grey) cells. ***P-value< 0.001.