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1 Title

2 The contradictory role of Febuxostat in ABCG2 expression and potentiating Hypericin-mediated photodynamic
3 therapy in colorectal cancers

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22 **Abbreviations:** PDT – Photodynamic Therapy, CRC – Colorectal cancers, HYP – Hypericin, FBX - Febuxostat

24 Abstract

25 Photodynamic Therapy (PDT) is an emerging method to treat colorectal cancers (CRC). Hypericin (HYP) is an
26 effective mediator of PDT and the ABCG2 inhibitor, Febuxostat (FBX) could augment PDT. HT29 and HEK293
27 cells showed light dependant cytotoxic response to PDT in both 2D and 3D cell models. FBX co-treatment was
28 not found to improve PDT cytotoxicity. Next, ABCG2 protein expression was observed in HT29 but not in
29 HEK293 cells. However, *ABCG2* gene expression analysis did not support protein expression results as *ABCG2*
30 gene expression results were found to be higher in HEK293 cells. Although HYP treatment was found to
31 significantly reduce *ABCG2* gene expression levels in both cell lines, FBX treatment partially restored *ABCG2*
32 gene expression. Our findings indicate that FBX co-treatment may not be suitable for augmenting HYP-mediated
33 PDT in CRC but could potentially be useful for other applications.

35 1. Introduction

36 Colorectal cancer (CRC) accounts for 10% of all cancer diagnoses and cancer-related deaths worldwide[1,2].
37 Surgery with curative intent is the cornerstone in treating CRC with 5-fluorouracil based systemic chemotherapy
38 often administered in the adjuvant and palliative settings. However, mounting clinical evidence suggest the
39 beneficial impact of traditional chemotherapies to be short-lived in CRC, with incomplete tumour eradication and
40 disease recurrence contributing to poor outcomes of treatments[3,4]. There is a growing trend in oncological
41 research, exploring ways in which the management and treatment of CRC can be improved, leading to better
42 patient outcomes. Such research includes the use of CRC-targeting biologics, inorganic nanomedicines and re-
43 evaluating current treatment regimes, switching from adjuvant to neoadjuvant treatments, in an effort to improve
44 outcomes[5–7].

46 One particular method in treating CRC, showing great promise is photodynamic therapy (PDT). PDT is a
47 minimally invasive treatment modality that has garnered much attention in the past decades as an alternative, next
48 generation method for treating solid cancers[8]. This treatment method involves the administration of a non-toxic
49 photosensitising agent followed by a period of incubation. Light of a specific wavelength, typically in the UV-Vis
50 and near-infrared region of the electromagnetic spectrum, is applied and in the presence of atmospheric oxygen,
51 results in the production of hydroxyl radical and reactive oxygen species. This in turn leads to cellular toxicity in
52 cancers and peritumoural destruction in the tumour microenvironment, specifically loss of tumour vasculature and
53 activation of innate immunity leading the starvation of tumour cells from vital oxygen and nutrients and a mounted
54 immunogenic cytotoxicity[8–12].

55
56 Hypericin (HYP), a polycyclic aromatic naphthodianthrone and primary constituent of the St. John's wort
57 flowering plant (*Hypericum perforatum*) has long been evaluated as a potent photosensitising agent for PDT in
58 many different cancers[13]. In our previous studies, we successfully demonstrated HYP to be an effective mediator
59 of PDT (HYP-PDT), for treating CRC *in vitro* and *in vivo*[14,15]. In our previous study, we found HYP to be a
60 substrate for the ATP-binding cassette transporter and drug efflux protein, ABCG2[14,16]. We successfully found
61 ABCG2 to contribute to HYP-mediated PDT resistance in CRC and the small molecule ABCG2 inhibitor, Ko143,
62 can be used in conjunction with HYP-mediated PDT to improve sensitivity. The suggestion of HYP as a
63 substrate of ABCG2 and a subject of the transmembrane protein's drug efflux activity has also been confirmed by
64 other studies[17].

65
66 Recent developments in cancer and disease modelling has identified 3D spheroidal cell cultures as better advanced
67 pre-clinical models that recapitulate the characteristics of *in vivo* pathologies and mimic the tumour
68 microenvironment[18,19]. We previously identified that 3D CRC models showed increased ABCG2 protein
69 expression, as compared to their 2D monolayered counterparts. This was especially prevalent in the outer layers
70 of cells in 3D models embedded with the viable rim, where the highly proliferative cells had increased expression
71 that reduced in the inner central cells mass[14]. This led to 3D models being more resistant to PDT, as compared
72 to 2D models, owed to the mechanism and action of ABCG2 and further highlights the importance of the dynamic
73 tumour characteristics captured in 3D models. With the inherent limitations of 2D cell cultures in drug discovery,
74 hindering their applicability in extrapolating results for clinical translation, the advantages of 3D spheroids have
75 propelled them to being adopted as suitable models for bridging the gap between cell cultures and animal models
76 and effective drug screening[20].

77
78 Here in this study, we will explore the use of the potent ABCG2 inhibitor, febuxostat (FBX) to improve PDT in
79 CRC. Previous studies have highlighted FBX to be more pharmacologically potent than Ko143 and we will
80 explore the impact of FBX in HYP-mediated PDT[21,22]. We will also be the first to report the impact of FBX,
81 HYP and PDT on the expression of the *ABCG2* gene.

82 83 2. Materials and Methods

84 2.1. Photosensitiser and ABCG2 inhibitor

85 Hypericin (extracted from *Hypericum perforatum*) was obtained from Sigma-Aldrich (Gillingham, UK) and
86 prepared as a 100µM stock solution in ethanol. 1mL aliquots of the stock solution were stored in the dark.
87 Febuxostat was obtained from Sigma-Aldrich and prepared as a 1mM stock solution in Dimethyl sulfoxide
88 (DMSO) and stored in the dark at room temperature.

89 90 2.2. Cell cultures

91 Human colorectal adenocarcinoma (HT29) and human embryonic kidney (HEK293) cell lines were obtained from
92 the European Collection of Authenticated Cell Cultures (Salisbury, UK). Cell lines were cultured in Roswell Park
93 Memorial Institute (RPMI) 1640 Medium plus GlutaMAX™ (Thermo Fisher Scientific, Altrincham, UK)
94 supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Sigma-Aldrich, Gillingham, UK). Cell cultures were
95 incubated and maintained at 37°C/5% CO₂/95% relative humidity. Upon 80–90% confluency, cell cultures were
96 washed with sterile Phosphate Buffered Saline (PBS, Thermo Fisher) and incubated for 5 mins with 0.05% (v/v)
97 trypsin and 0.5% (v/v) ethylenediaminetetraacetic acid (EDTA, Thermo Fisher) in PBS. Cell medium containing
98 10% (v/v) FBS was added to trypsinised cells and the cell suspensions centrifuged at 400g for 5 mins. The
99 supernatant was discarded, and the pelleted cells resuspended in fresh medium, seeded into tissue culture flasks
100 (Corning Inc., New York, USA) and grown to 80–90% confluence for experiments.

101 102 2.3. 2D monolayer and 3D spheroidal cell culturing

103 For 2D monolayer cell culturing, cells were washed, trypsinised, centrifuged and resuspended. 1000 cells were
104 seeded per well into 96-well cell culture plates (Thermo Fisher) and incubated for 4 days. For 3D spheroidal cell
105 culturing, cells in suspension were seeded into Nunclon™ Sphera™ 96-Well, Nunclon Sphera-Treated, U-Shaped-
106 Bottom Microplates (Thermo Fisher) at 500 cells per well for 4 days until 3D spheroid cultures had formed.

107 108 2.4. Photodynamic Therapy

109 HT29 and HEK293 cells were modelled into 2D monolayer and 3D spheroidal cell cultures as described above.
110 Cells were then treated with varying concentrations of HYP (0 - 200nM) and incubated for 24h in the dark. Cell
111 cultures were then washed and subjected to photoactivating light treatment as described below. Cell cultures were
112 then placed back into incubation in the dark. After 24h, cell viability was assessed. For 2D cell cultures, cell
113 viability was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Thermo
114 Fisher) assay as per the manufacturer's protocol. For 3D spheroidal cell cultures, spheroids were co-stained with
115 Hoechst 33342 (0.1mg/ml, Thermo Fisher) and Propidium Iodide (1mg/ml, Thermo Fisher) for 1h and imaged
116 using the EVOS™ FL imaging system (Thermo Fisher).

117

118 2.5. *ABCG2* protein inhibition

119 For ABCG2 protein inhibition, 2D and 3D cell cultures were treated with varying concentrations of FBX (0 –
120 100nM) for 2h prior to HYP and light treatment.

121

122 2.6. *Light treatment*

123 Cell culture plates were placed on top of the diffuser surface of a light-radiating device and treated with total light
124 dose of 1J/cm². Light treatment lasted for 72 mins and 28 secs at 0.23mW/cm². The light radiating device
125 comprised of a series of HLMP-EL3B-WXKDD Amber LEDs (Avago Technologies, California, USA), with peak
126 wavelength of 594 nm and an internal fan to prevent overheating.

127

128 2.7. *ABCG2* immunofluorescence

129 Sterile glass coverslips were placed into 6-well plates and cells were seeded at a density of 1x10⁶ cells per well.
130 Plates were incubated for 24h to allow the cells to adhere to the coverslips. Cells were then washed with PBS and
131 fixed in 4% paraformaldehyde for 30 mins. Fixed cells were washed with PBS and blocked with 0.5% skimmed
132 milk solution. Cells were then incubated with primary anti-BCRP antibody (1:50, BXP-21) (Millipore, Watford,
133 UK) overnight at 4°C. Cells were then washed with PBS and incubated with secondary Alexa Fluor 488-
134 conjugated antibody (1:300, Thermo Fisher) and 5µg/mL Hoechst 33342 (Thermo Fisher) for 1hr at room
135 temperature. Coverslips were then washed, mounted onto glass slides and imaged using a Zeiss Axio Imager Z1
136 fluorescent microscope (Carl Zeiss Ltd, Cambridge, UK).

137

138 2.8. *ABCG2* gene expression

139 HT29 and HEK293 cells were seeded at 2x10⁵ cells per well in 6-well plates for 24h. Cells were then subjected
140 to the following conditions: 1) Cell media culturing only, 2) HYP (175nM) treatment only for 24h [HYP ONLY],
141 3) FBX (100nM) treatment only for 24h [FBX ONLY], 4) HYP-mediated PDT as described above [HYP PDT]
142 and 5) HYP-mediated PDT with FBX treatment as described above [HYP+FBX PDT]. Cells were then washed,
143 collected from plates and RNA was extracted using the Monarch® Total RNA Miniprep Kit (New England Biolabs,
144 Hitchin, UK) according to the manufacturers protocol. ProtoScript® cDNA synthesis was performed using the
145 First Strand cDNA Synthesis Kit (New England Biolabs). qPCR was performed using TaqMan® Gene Expression
146 Assays (Thermo Fisher Scientific). *GAPDH* gene was used as endogenous controls. *ABCG2* gene Assay ID =
147 Hs01053790_m1. *GAPDH* Assay ID = Hs99999905_m1.

148

149 2.9. *Image processing and Statistical Analyses*

150 Microscope images were processed using the Fiji (ImageJ, National Institutes of Health, Maryland, USA)
151 software. One-way ANOVA and Student's *t*-test were used to perform statistical analysis using GraphPad Prism
152 10 (GraphPad Software, Inc., California, USA). *p*<0.05 was considered to be statistically significant. Data are
153 presented as the mean ± standard deviation.

154

155 3. Results

156 3.1. *Light and Hypericin dose dependant PDT induced cytotoxicity*

157 HT29 and HEK293 cells were treated with varying concentrations of HYP and either kept in the dark or subjected
158 to light irradiation. Both HT29 and HEK293 cells demonstrated light and HYP dose dependant reduction in cell
159 viability (100nM HYP; HT29 light = 57% vs. HT29 dark = 103% (*p*=0.0001) and HEK293 light = 12% vs.
160 HEK293 dark = 80% (*p*<0.0001) (Figures 1A & 1B). As the IC₅₀ value could not be achieved in HT29 cells using
161 0 - 100nM HYP, higher concentrations of HYP (100nM – 200nM) were administered to HT29 (Figure 1C) and
162 HEK293 (Figure 1D) cells. As expected, significant differences in cell viability were observed in both cell cultures

163 kept in the dark and treated with light (light vs. dark, HT29 ($p=0.0005$) and HEK293 ($p=0.0081$)). IC_{50} for HT29
164 was found to be at 100nM (Figure 1C). This was also confirmed in 3D spheroidal models of HT29 and HEK293
165 cell cultures, as indicated by the presence of dead cells in light and HYP treated HT29 and HEK293 spheroids,
166 illustrated by the uptake of propidium iodide in the spheroids, not seen in spheroids kept in the dark (Figures 1E
167 and 1F).

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169
170

171 3.2. *The impact of Febuxostat on PDT mediated cytotoxicity*

172 FBX has previously been identified to inhibit the ABCG2 protein, which in turn has been also previously been
173 shown to augment PDT[22–24]. To further explore this, HT29 and HEK293 were co-treated with 100nM FBX
174 and varying concentrations of HYP. As expected, light treatment induced HYP photoactivation and cytotoxicity
175 (200nM HYP & 100nM FBX; HT29 light = 37% vs. HT29 dark = 100% ($p=0.0069$) and HEK293 light = 11% vs.
176 HEK293 dark = 105% ($p<0.0001$) (Figures 2A & 2B). This was further confirmed in 3D spheroidal models of
177 HT29 and HEK293 (Figure S1). However, similar to that observed in Figure 1C, the IC_{50} value in HT29 cells with
178 the addition of 100nM FBX remained at 100nM (Figure 2A). Furthermore, when cells were co-treated with 200nM
179 HYP and varying concentrations of FBX, light dependant cytotoxicity was observed (200nM HYP & 100nM
180 FBX; HT29 light = 44% vs. HT29 dark = 104% ($p=0.0017$) and HEK293 light = 14% vs. HEK293 dark = 109%
181 ($p=0.0007$) (Figures 2C & 2D) and additionally observed in 3D spheroidal models (Figure S2). However, no
182 further reduction in cell viability was observed with increasing concentration of FBX (difference in cell viability
183 in light treated cultures between 25nM and 100nM FBX - HT29: 2% ($p=0.78$) and HEK293: 3% ($p=0.32$)).
184

185

186 3.3. *ABCG2 protein and gene expression in HT29 and HEK293 cells*

187 To explore the impact of ABCG2 in HT29 and HEK293, we performed protein expression analysis and found the
188 ABCG2 protein to be exclusively expressed in HT29 cells, as indicated by the fluorescent staining of ABCG2,
189 which was not observed in HEK293 cells (Figure 3). We further investigated *ABCG2* gene expression and found
190 *ABCG2* gene expression to be 27% higher in HEK293, as compared to HT29 cells ($p=0.04$) contradicting ABCG2
191 protein expression (Figure 4A). Next, we evaluated the impact of HYP and FBX treatment on *ABCG2* gene
192 expression (Figure 4B). HYP treatment was found to reduce *ABCG2* gene expression in HT29 by 0.83-fold
193 ($p<0.0001$) and 0.84-fold ($p=0.0021$) in HEK293 cells. Whereas treatment with the ABCG2 inhibitor, FBX,
194 reduced *ABCG2* gene expression levels by 0.62-fold ($p<0.0001$) and 0.12-fold ($p=0.67$) in HT29 and HEK293
195 cells respectively. When HT29 cells were further subjected to light treatment, administering PDT and evaluated
196 for *ABCG2* gene expression levels, a 0.92-fold ($p<0.0001$) reduction was observed in HYP+PDT and 0.21-fold
197 ($p=0.006$) reduction in HYP+FBX+PDT.

198

199 4. Discussion

200 This study evaluated the impact of FBX as an inhibitor of the ABCG2 protein and modulating HYP-mediated
201 PDT. This study builds upon our previous study in modulating ABCG2 protein to potentiate HYP-mediated PDT
202 in CRC cell models[14]. Previous evaluations identified FBX to be a potent inhibitor of the ABCG2 protein, which
203 we were not able to corroborate in this study[25]. FBX co-treatment did not improve HYP mediated PDT as
204 evidenced through no synergistic increase in cytotoxicity in HT29 and HEK293 cells. An interesting finding was
205 that independent to HYP-mediated PDT cell death, we found FBX treatment did not induce any cytotoxicity,
206 especially in the CRC cell line, HT29 (Figure 2). This is unlike the study by Abdel-Aziz *et al.* who found FBX
207 treatment alone to reduce the growth of benign prostatic hyperplasia *in vivo* in rats[26]. We are the first to report
208 the administration of FBX in CRC and found it to not induce any cytotoxicity with or without PDT co-treatment.
209 The findings from our study are contradictory to other studies that found FBX to augment PDT in
210 glioblastomas[23,24]. This warrants further investigation into the differences between different types of cancers
211 to elucidate and identify particular types of cancer which may respond and benefit from FBX and PDT co-
212 treatment.

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We then explored the expression of ABCG2 protein and in agreement with previous studies, found ABCG2
transmembrane protein expression to be present in HT29 cells. However, we did not find FBX to inhibit ABCG2
protein activity and further improve PDT activity. However, ABCG2 protein expression data did not correlate with
ABCG2 gene expression data, where gene expression was found to be higher in HEK293 cells than in HT29 cells

217 (Figures 3 & 4). Interestingly, HYP treatment and HYP-mediated PDT was found to reduce the expression of
218 *ABCG2* gene. To explore this further, Englund *et al.* (2007) reported a clinical study, investigating the expression
219 levels of different efflux transport proteins, including *ABCG2*, in patients with inflammatory bowel disease
220 (ulcerative colitis, UC)[27]. The study found *ABCG2* protein to be strongly reduced in UC patients and the colonic
221 epithelium expression of *ABCG2* protein was diminished in inflamed tissue. Following these findings, a
222 subsequent study by Gutmann *et al.* (2008) also identified reduced expression of *ABCG2* protein in patients with
223 active UC[28]. Both studies confirm that the state of inflammation in the bowel and release of pro-inflammatory
224 cytokines contributes to the reduction of *ABCG2* expression[29]. The resulting phototoxicity, oxidative stress and
225 damage to cells induced by PDT, causes the plasma membrane to leak intracellular components into the tumour
226 microenvironment. This includes the release of damage-associated molecular patterns (DAMPs), cytokines and
227 inflammatory mediators as a pro-inflammatory response to the cellular damage and immune activation[30].
228 Hence, it could be suggested that reduced *ABCG2* protein expression is a consequence of post-PDT inflammation
229 and phototoxic effects.

230

231 Conversely, in this study we found the addition of FBX to increase *ABCG2* gene expression. This was also seen
232 in HT29 cells treated with HYP-mediated PDT, whereby FBX co-treatment increased *ABCG2* gene expression
233 levels. To our knowledge, the influence of FBX on *ABCG2* gene expression has previously not been reported.
234 However, the overexpression of *ABCG2* has commonly been observed in different forms of solid cancers, when
235 treated with drugs such as mitoxantrone, topotecan, flavopiridol and imatinib[31–36]. It has been postulated that
236 the drug-induced *ABCG2* overexpression is independent of P-glycoprotein and Multidrug Resistance Protein
237 expression and may be a result of alternative gene activation mechanisms including alternative promoter usage,
238 demethylation of the *ABCG2* promoter, histone modification and PI3K/Akt signalling[31].

239

240 Tumour Lysis Syndrome (TLS) is the term given to a pathological condition, as a result of an oncometabolic
241 emergency caused by rapid cell death during cancer treatments and characterised by the abrupt rise of serum uric
242 acid levels[37]. FBX has recently emerged as an effective prophylaxis for TLS, through reducing uric acid levels
243 and adverse toxicities[38–40]. Looking beyond the scope of augmenting treatments via *ABCG2* inhibition, the
244 role of FBX in phototherapies should not be to improve PDT through increased intracellular accumulation of HYP
245 or other *ABCG2*-substrate photosensitisers, but rather improve patients' quality of life through treating PDT
246 associated TLS.

247

248 5. Conclusion

249 In summary, this study explores the impact of FBX and HYP-mediated co-treatment in treating CRC through
250 modulating *ABCG2* activity. As expected, HYP was found to successfully induce light dependant cytotoxicity in
251 both the CRC cell line, HT29 and embryonic kidney cell line, HEK293, as owed to the nature of HYP
252 accumulating in metabolically active cells. However, the additional co-treatment with FBX did not prove to add
253 any benefit in augmenting HYP-mediated PDT cytotoxicity. Furthermore, FBX treatment alone did not elicit any
254 cytotoxic response. *ABCG2* protein and gene expression analysis illustrated protein expression in HT29 cells,
255 which was not observed in HEK293 cells. This was contradictory to gene expression analysis that found HEK293
256 to possess higher *ABCG2* gene expression. Furthermore, as a noted inhibitor of the *ABCG2* protein, FBX
257 treatment was found to increase *ABCG2* gene expression in both cell lines which may in part be the result of an
258 intricate complex signalling mechanism, that cells employ to upregulate *ABCG2* activity in response to the
259 presence of xenobiotics. Although our study did not evidently highlight FBX to synergistically improve PDT in
260 CRC *in vitro*, it does suggest further exploration of FBX in the clinical setting and given in adjunct to PDT, to
261 help minimise adverse toxicities such as TLS.

262

263 Declarations

264 The authors declare no conflict of interest.

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271 Authors Contributions

272 D.G.J. and M.I.K. created the research project and conceptualised the study. A.K. performed the experiments.
273 T.M. and E.H. provided technical and experimental supervision. A.K., M.I.K. and J.L.P. analysed the results.
274 M.I.K. wrote and edited the manuscript. All authors approve of the final manuscript.

275

276 Data Availability

277 Data will be made available on request.

278

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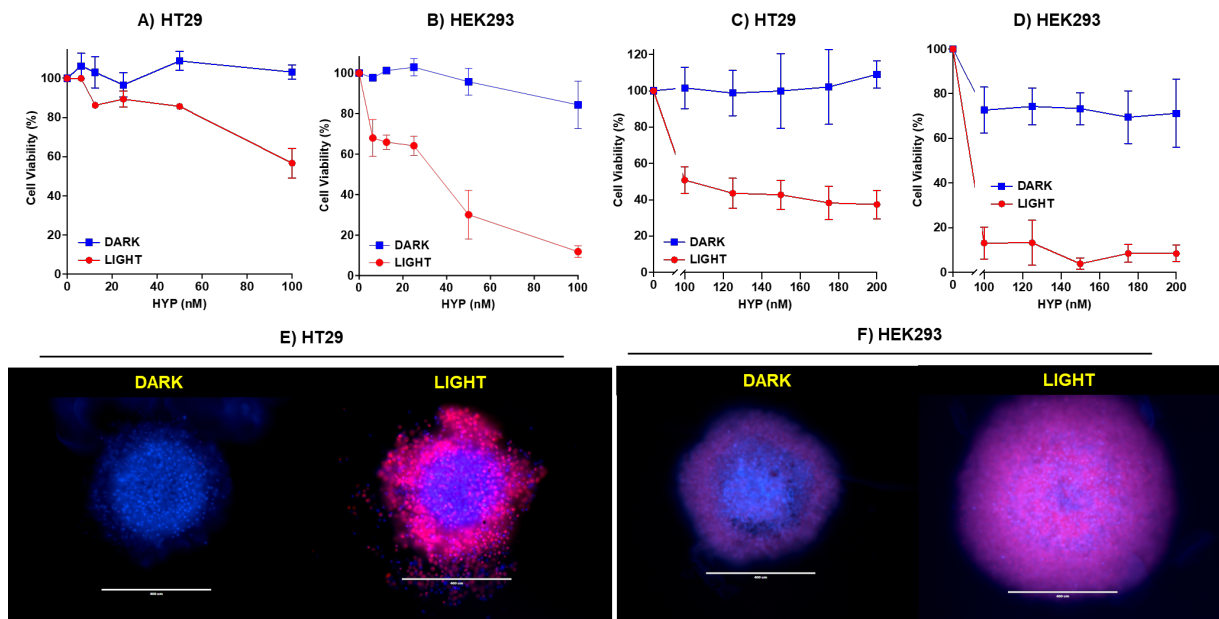
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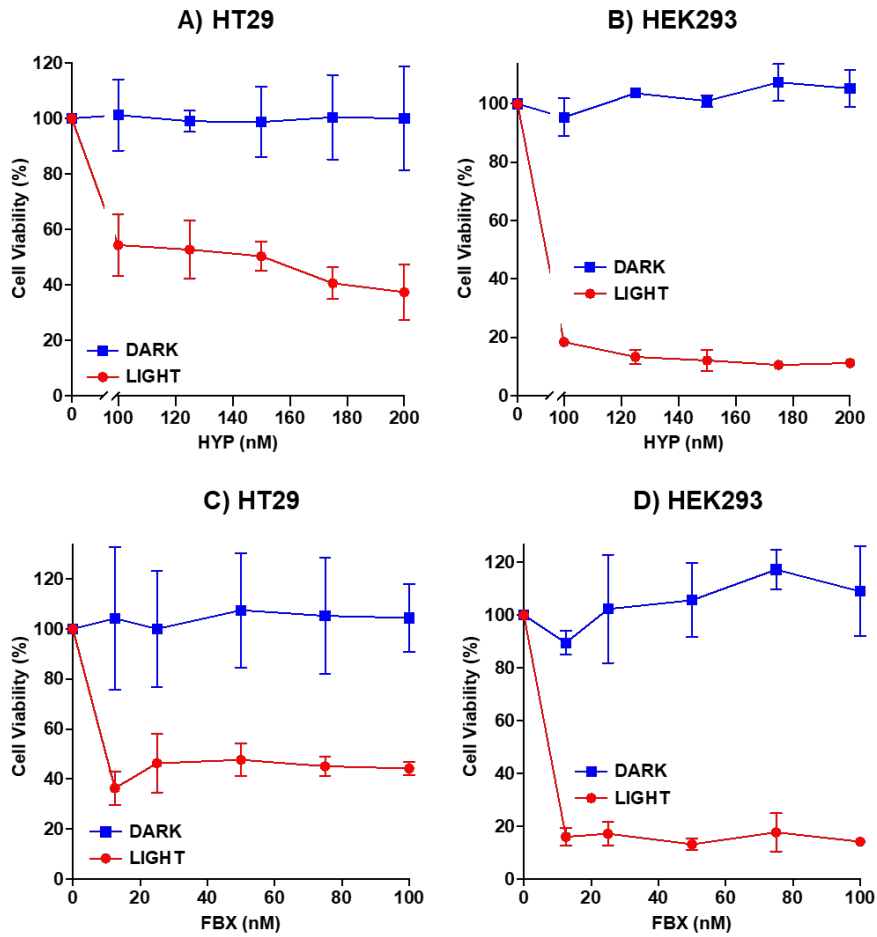
388 Figures – all figures to be printed in colour
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Figure 1. Hypericin mediated PDT in 2D and 3D cell models of HT29 and HEK293

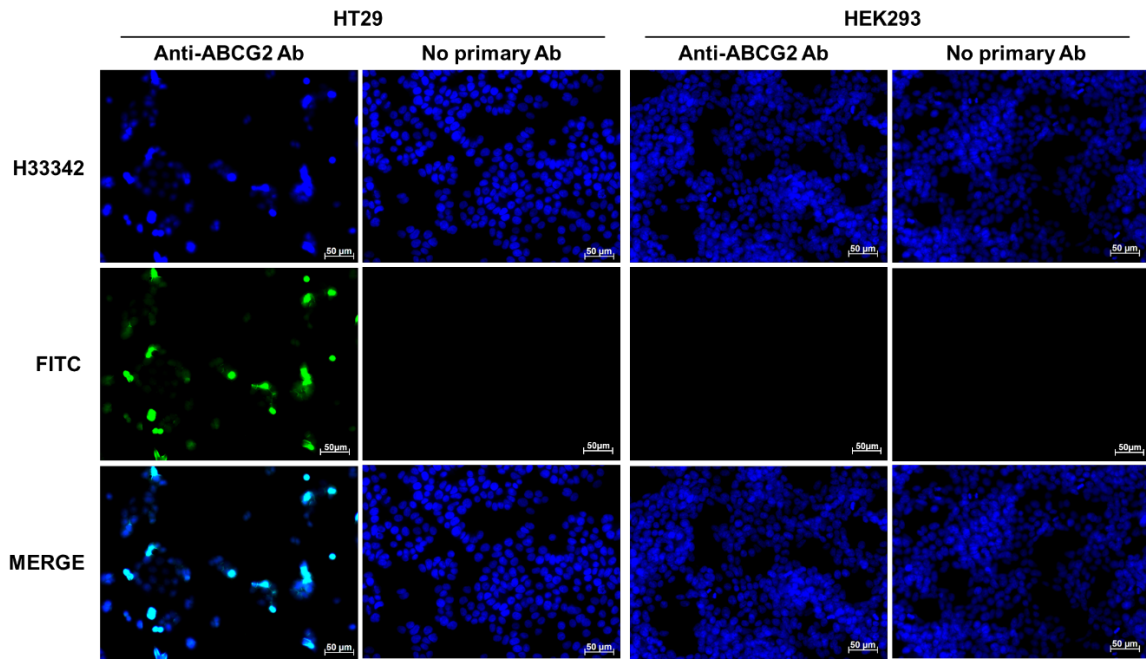
A) 2D HT29 and B) 2D HEK293 monolayered cell cultures were treated with 0-100nM of Hypericin for 24h. C) 2D HT29 and D) 2D HEK293 cell cultures were also treated with higher 0-200nM of Hypericin for 24h. Hypericin treated cultures were then subjected to a light treatment dose of 1J/cm² and cell viability was assessed after 24h. 3D spheroidal cell cultures of HT29 and HEK293 were treated with 200nM Hypericin for 24h and then kept in the dark or treated with light. E) 3D HT29 kept in the dark and treated with light. F) 3D HEK293 kept in the dark and treated with light. Cell viability in 3D models was assessed after 24h via fluorescent imaging. Blue = Hoechst 33342 and pink/red = Propidium Iodide. Scalebar = 400μm. Data are shown relative to control treated cells and represent means with SD of 3 independent experiments.



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Figure 2. Hypericin mediated PDT with Febuxostat co-treatment

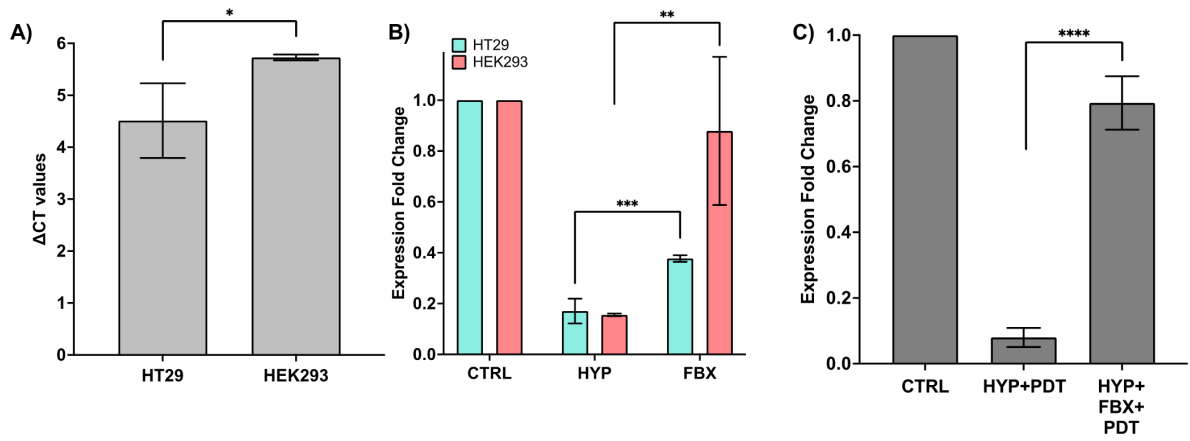
A) HT29 and B) HEK293 cells were co-treated with 100nM Febuxostat and varying concentrations of Hypericin for 2h and 24h respectively. C) HT29 and D) HEK293 cells were co-treated with varying concentrations of Febuxostat and 200nM Hypericin for 2h and 24h respectively. For both conditions, cells were subjected to a light treatment dose of 1J/cm² and cell viability was assessed after 24h. Data are shown relative to control treated cells and represent means with SD of 3 independent experiments.



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Figure 3. ABCG2 protein expression in HT29 and HEK293 cells

HT29 and HEK293 cells were fixed and stained with primary anti-ABCG2 followed by secondary Alexa Fluor 488 fluorescent antibodies (Ab). No primary Ab cultures did not receive primary anti-ABCG2 Ab incubation. Hoechst 33342 (H333342) cell nuclei staining is blue and ABCG2 protein staining is green. Scalebar = 50µm.



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Figure 4. *ABCG2* gene expression in HT29 and HEK293 cells under different treatment conditions

A) *ABCG2* gene expression in HT29 and HEK293 cells. B) HT29 and HEK293 cells were treated with Hypericin or Febuxostat for 24h and 2h respectively and qPCR was performed to quantify *ABCG2* gene expression levels. C) HT29 cells were subjected to Hypericin mediated PDT or Hypericin mediated PDT with Febuxostat co-treatment and *ABCG2* gene expression levels were quantified. Data are shown relative to control treated cells and represent means with SD of 3 technical replicates. * $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$ **** $p < 0.0001$.