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Title Page

CC-115, a dual mTOR/DNA-PK inhibitor in clinical trial, is a substrate of ABCG2, a risk factor for CC-115 resistance

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Running Title Page

Running Title: ABCG2 contributes to CC-115 resistance

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Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia mutated related, DNA-PK, DNA-dependent protein kinase; mTOR, mammalian target of rapamycin; PIKK, PI3K-related kinase; FTC, fumitremorgin C; ABC, ATP-binding cassette. FASN, fatty acid synthase; NHEJ, non-homologous end joining.

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Abstract

CC-115, a triazole-containing compound, is a dual mTOR/DNA-PK inhibitor currently in clinical trials. To further develop this compound, we sought to investigate factors that may affect cellular response to CC-115. Previously it has been shown that fatty acid synthase (FASN) up-regulates DNA-PK activity and contributes to drug resistance, we hypothesized that FASN may affect cellular response to CC-115. Instead, we show that CC-115 is a substrate of ABCG2, a member of the ATP-binding cassette transporter superfamily and that ABCG2, not FASN, expression affects the potency of CC-115. ABCG2 overexpression significantly increases resistance to CC-115. Inhibiting ABCG2 function, using small molecule inhibitors, sensitizes cancer cells to CC-115. We also found that CC-115 may be a substrate of ABCB1, another known ABC protein contributing to drug resistance. These findings suggest that expression of ABC transporters, including ABCB1 and ABCG2, may affect the outcome in clinical trials testing CC-115. Additionally, the data indicate that ABC transporters may be used as markers for future precision use of CC-115.

Significance Statement

In this study, we report findings on potential mechanism of resistance to CC-115, a dual inhibitor of mTOR and DNA-PK currently in clinical trial. We show that CC-115 is a substrate of ABCG2 and can also be recognized by ABCB1, which contributes to CC-115 resistance. These findings provide novel information and potential guidance on future clinical testing of CC-115.

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Introduction

The PIKK family of serine/threonine kinases includes ATM, ATR, hSMG1, DNA-PK, and mTOR (Lovejoy and Cortez, 2009). These protein kinases regulate cellular response to DNA damage and to nutrients, controlling cell growth (Lovejoy and Cortez, 2009). DNA-PK in particular plays an important role in non-homologous end joining (NHEJ) repair of DNA double strand breaks (DBSs) (Hammel et al., 2010), while mTOR functions in regulating protein synthesis, cell proliferation and survival (Hung et al., 2012). Both DNA-PK and mTOR have been considered as targets for drug discovery.

CC-115, a triazole-containing compound, is a dual mTOR/DNA-PK inhibitor currently in clinical trials (Mortensen et al., 2015). Early studies showed that CC-115 was selective to mTOR and DNA-PK over other closely related PIKK family members (Tsuji et al., 2017). There are several ongoing clinical trials testing CC-115 in an array of different cancer types, including phase I trials in squamous cell carcinoma of head and neck, Ewing's osteosarcoma, chronic lymphocytic leukemia (CLL), and prostate cancer (Munster et al., 2016). There is also a phase II trial in glioblastoma. Early findings on CC-115 in CLL patients showed that 7 of 8 patients had a decrease in lymphadenopathy with 1 partial response and 3 partial responses with lymphocytosis (Thijssen et al., 2016). While these early results in CLL show promise for further development of CC-115, there is an observed variability in sensitivity. In fact, 1 of the 8 CLL patients showed no decrease in lymphadenopathy and may be resistant to CC-115. Clearly, resistance to CC-115 will likely be observed and there is a need to identify the mechanism of CC-115 resistance and to overcome this resistance for better development and clinical use of CC-115.

There are many known molecular mechanisms of drug resistance. In particular, ATP binding cassette (ABC) transporters such as ABCB1, ABCC1, and ABCG2 are well known in

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contributing to multidrug resistance by actively transporting drug substrates out of cells using ATP (Schinkel and Jonker, 2003). Recently, it has also been shown that fatty acid synthase (FASN) contributes to resistance of DNA-damaging drugs by increasing NHEJ repair of DNA damages via facilitating recruitment of Ku proteins and increasing DNA-PK activity (Wu et al., 2016a). Thus, it is possible that FASN may contribute to CC-115 resistance by increasing DNA-PK activity. In this study, we tested this hypothesis. However, we found that ABCG2, not FASN, contributes to CC-115 resistance by reducing CC-115 accumulation. Inhibiting ABCG2 using small molecule inhibitors was able to reverse CC-115 resistance. These findings suggest that CC-115 is a substrate of ABCG2 and ABCG2 expression likely will cause resistance to CC-115.

Materials and Methods

Materials: Antibodies against ABCG2 (#MAB4146), DNA-PK (#PC127), and actin (# JLA-20) were from Millipore, Calbiochem, and Sigma, respectively. Antibodies against mTOR (#2983, 1:500), p-S6 (#2215, 1:1000), S6 (#2317, 1:200), p-S6K (#9234, 1:1000), S6K (#2708, 1:1000), p-AKT (#4051, 1:1000) were all from Cell Signaling (Danvers, MA). DNA-PKcs (#PIMA513238, 1:1000) was from Invitrogen (Carlsbad, CA). S6K and p-S6K antibodies can also detect p85. Actin (#JLA-20, 1:3000), Anti-mouse secondary (#A2554, 1:3000), and Anti-rabbit secondary (#A0545, 1:3000) were from Sigma (Saint Louis, MO). CC-115 (#S7891) and NU7441 (#S2638) were obtained from Selleckchem (Houston, TX) and fumitremorgin C (FTC) (#344847) was obtained from Millipore (Danvers, MA). PZ-39C8 was obtained from SPECS and as previously reported (Peng et al., 2009). G418 (#G5005) was purchased from TEKnova (Hollister, CA). Doxorubicin (#D1515) and thiazolyl blue tetrazolium bromide (#M5655) were from Sigma (Saint Louis, MO). Protein concentration assay kit and polyvinylidene difluoride (PVDF)

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membranes were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were of molecular biology grade from Fisher Scientific or Sigma.

Cell Lines: MCF7 and M3K cells were cultured in DMEM (#10-013-CV) (Corning, Manassas, VA) containing 10% fetal bovine serum (FBS) (#A3160601) (Life Technologies, Grand Island, NY) with supplementation of 5 µM doxorubicin for M3K cells as previously described (Liu et al., 2006). MCF7 cells with stable expression of ectopic FASN (MCF7/FASN) and M3K cells with stable knockdown of FASN (M3K/ShFASN) along with their respective control cells (MCF7/Vec and M3K/Scr) were established previously and cultured in DMEM containing 10% FBS and 600 µg/ml G418 as previously described (Liu et al., 2008). HEK293/ABCG2-venus and HEK293/Vec-venus was also established in a previous study and cultured in DMEM containing 10% FBS with 600 µg/ml G418 as previously described (Zhang et al., 2019). MCF7 cells with stable expression of ectopic ABCB1 (BC19) were established previously (Horton et al., 1998) and cultured similarly in DMEM containing 10% FBS with 0.1 µM Adriamycin.

To establish MCF7/ABCG2 cells with stable expression of ectopic ABCG2, MCF7 cells were transfected with pcDNA3.1 (+) harboring ABCG2 cDNA or with vector alone followed by selection with 600 µg/ml G418, cloned, and propagated under the same condition.

Survival assays: 500-4000 cells/well were plated in a 96-well plate and cultured overnight followed by treatment with increasing concentrations of CC-115 in the absence or presence of doxorobucin at its IC_{20} concentration for three days and cell survival was measured using methylene blue staining (Oliver et al., 1989) or MTT assay. For methylene blue assay, cells were first fixed with methanol for 30 min and stained with 1% (W/V) methylene blue in 10 mM borate buffer, pH 8.5, for 30 min. Excess dye was removed by aspiration and plates were washed 3 times with ddH_2O. The stains were then released using a 1:1 mixture of 100% ethanol and 0.1M HCl

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and followed by determination of OD_{650nm}. For MTT assay, cells were treated with 5 mg/ml thiazolyl blue tetrazolium bromide in PBS for 4 hrs at 37ºC and cells were collected by centrifugation. Finally, DMSO was added to solubilize cell-associated dye and $OD_{570\ nm}$ was determined.

Cell lysate preparation and Western blot. Cells were treated with the given concentration of CC-115 for 2 hrs and cell lysates were prepared as previously described (Liu et al., 2006) and separated by SDS-PAGE followed by transfer onto PVDF membranes. Western blot analysis was performed also exactly as we previously described (Xu et al., 2004; Xu et al., 2007). To test the effect of ABCG2 inhibition on CC-115 inhibition of mTOR, 1 μM C8 was added at the same time as CC-115 and incubated for 2 hrs before cell lysates were prepared.

CC-115 accumulation assay. Cells were harvested, washed twice with warm PBS, and incubated with or without ABCG2 inhibitors for 15 min at 37ºC. Then, 10 μM CC-115 or DMSO control was added to the cells followed by incubation at 37ºC for 30 min with shaking every 10 min. Cells were then washed with PBS 2 times and subjected to analysis using flow cytometry with an excitation at 405 nm and emission at 421 nm.

Statistical Analysis. Statistical analysis for all experiments was run using Prism Graphpad. Results are presented as mean ± SD. IC_{50} values were calculated from 3 biological replicates which were each run in triplicate. Western blots were run on 4 different sample preps and accumulation assays were run in triplicate. Two-tailed T-test was used for comparing two means and ANOVA using the Tukey post hoc test for comparing a group of mean values.

Results

FASN does not contribute to cellular resistance to DNA-PK inhibitors. To investigate the potential mechanisms of CC-115 resistance, we first took advantage of MCF7/AdVp3000 (M3K)

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cell line, which was selected for Adriamycin resistance. M3K cells harbor multiple mechanisms of drug resistance, including overexpression of FASN (Liu et al., 2008), and were tested for their response to CC-115 in comparison with the parental MCF7 cells. As shown in Figure 1A-B, M3K cells are much more resistant than MCF7 cells to CC-115 with IC_{50} of ~2.2 µM and ~0.05 µM, respectively.

Previously, it has been shown that CC-115 inhibits DNA-PK (Tsuji et al., 2017) and that FASN increases DNA-PK activity in M3K cells (Wu et al., 2016b). Thus, it is possible that FASN overexpression in M3K cells may contribute to CC-115 resistance. To test this possibility, we took advantage of MCF7 cells with stable overexpression of ectopic FASN (MCF7/FASN) and M3K cells with stable FASN knockdown (M3K/ShFASN) (see Figure 1C) and tested their response to CC-115 in comparison with their respective control cells (MCF7/Vec and M3K/Scr). As shown in Figure 1A and Supplemental Figure S1, ectopic FASN overexpression or FASN knockdown did not influence cellular response to CC-115. Thus, FASN may not contribute to CC-115 resistance via its DNA-PK inhibitory activity.

To ensure that DNA-PK inhibition is not part of the resistance due to FASN expression, we tested a DNA-PK selective inhibitor, NU7441. As shown in Figure 1D and supplemental Figure S2, alteration of FASN expression did not change cellular response to NU7441 albeit M3K cells are slightly more resistant to NU7441 than the parental MCF7 cells. Due to DNA-PK activation in response to DNA damage it was also tested if FASN causes resistance to DNA-PK inhibition in the presence of DNA damages. As shown in supplemental Figure S3, there was no change in cellular response to NU7441 in the presence of doxorubicin at its IC_{20} concentration. There was also no significant difference in cellular response to CC-115 in the presence of doxorubicin at its IC_{20} concentration although there may be a trend towards resistance (supplemental Figure S3).

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Because CC-115 is a dual mTOR/DNA-PK inhibitor, we next tested if its inhibition of mTOR pathway was impaired in the drug resistant M3K cells. As shown in Figure 1E, CC-115 inhibition of constitutive activation of S6K and phosphorylation of S6, downstream targets of mTOR, were significantly impaired in M3K compared with MCF7 cells. Based on these findings, we conclude that FASN does not play a role in DNA-PK inhibitor resistance.

CC-115 accumulation is reduced in the drug resistant M3K cells. To further investigate the molecular mechanism of CC-115 resistance, we tested the possible involvement of ABCG2, which is also overexpressed in M3K cells (Liu et al., 2005) (see also Figure 2A), by first determining the accumulation of CC-115 in M3K vs MCF7 cells. As shown in supplemental Figure S4, CC-115 is auto-fluorescent with an emission wavelength at 421 nm, which was used to monitor its intracellular accumulation. As shown in Figure 2B-C, CC-115 accumulation is much lower in M3K cells with ~80% reduction as compared to MCF7 cells.

Sensitization of M3K cells to CC-115 by inhibiting ABCG2. To determine if ABCG2 is responsible for the reduced CC-115 accumulation in M3K cells, we tested the effect of ABCG2 specific inhibitors, C8 and FTC (Peng et al., 2009; Rabindran et al., 2000), on CC-115 accumulation in M3K cells. As shown in Figure 3A-B, both C8 and FTC dose-dependently increased CC-115 accumulation in M3K cells. Both C8 and FTC also significantly reversed CC-115 resistance of M3K cells (Figure 3C-D). Furthermore, inhibiting ABCG2 with C8 sensitized M3K cells to CC-115 inhibition of mTOR activation of S6K (Figure 3E).

ABCG2 overexpression reduces CC-115 accumulation in both HEK293 and MCF7 cells. To further determine the role of ABCG2 in CC-115 efflux and resistance, we took advantage of HEK293 and MCF7 cells stably transfected with ABCG2 (HEK293/ABCG2-venus, which contains a venus tag and MCF7/ABCG2, which does not) and their respective control cells

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transfected with vector (HEK293/Vec, venus tagged, and MCF7/Vec) (see Figure 4A). Using these cells, we first determined CC-115 accumulation as described above. As shown in Figure 4B-C, accumulation of CC-115 in HEK293/ABCG2-venus and MCF7/ABCG2 cells was significantly reduced compared with their respective control cells. We next determined their response to CC-115 in a survival assay. As shown in Figure 4D-E, the CC-115 IC_{50} was significantly increased in both HEK293/ABCG2-venus and MCF7/ABCG2 compared with their respective control cells. The CC-115 inhibition of the mTOR activation of S6K, as indicated by the level of phosphorylated S6K and S6, was also reduced by ABCG2 overexpression in these cells (Figure 4F). We also tested the effect of CC-115 on mTORC2 by determining its effect on /AKT phosphorylation. As shown in supplemental Figure S5A, CC-115 inhibited AKT phosphorylation and ABCG2 overexpression reduced CC-115 inhibition of AKT phosphorylation. Thus, CC-115 likely inhibits both mTORC1 and mTORC2, which can be reduced by ABCG2 expression.

To validate the role of ABCG2 in CC-115 resistance and accumulation, we determined if inhibiting the function of ABCG2 could reverse the ectopic ABCG2-induced resistance and accumulation reduction of CC-115. As shown in Figure 5A-C, both C8 and FTC significantly reversed ABCG2-induced CC-115 accumulation reduction. Moreover, both C8 and FTC significantly reduced CC-115 IC_{50} in HEK293/ABCG2-venus cells (Figure 5D-E). Consistently, C8 was able to sensitize HEK293/ABCG2-venus cells to CC-115 inhibition of mTORC1 pathway, as indicated by S6K and S6 phosphorylation (Figure 5F), and mTORC2, as indicated by AKT phosphorylation (supplemental Figure S5B).

Role of ABCB1 in CC-115 resistance. It is known that ABC transporters involved in drug resistance have overlapping substrates and, thus, we next tested if other ABC transporters such as ABCB1 may also contribute to CC-115 resistance. For this purpose, we took advantage of MCF7

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cells that overexpress ectopic ABCB1 (BC19 cells) and tested CC-115 accumulation and resistance compared with vector-transfected MCF7 cells (MCF7/Vec) (Figure 6A). As shown in Figure 6B-C, CC-115 accumulation is significantly reduced in BC19 compared with the control MCF7/Vec cells. The IC_{50} of CC-115 was also significantly increased in BC19 cells compared with the MCF7/Vec cells (Figure 6D-E). When cells were treated with increasing concentrations of CC-115 there was a slight change in S6K and S6 phosphorylation when compared to MCF7/Vec cells (Figure 6F). Thus, ABCB1, in addition to ABCG2, may also contribute to CC-115 resistance by reducing its intracellular accumulation.

Discussion

In this study, we showed that ABCG2 expression plays a major role in CC-115 resistance by reducing its intracellular accumulation and its inhibition of mTOR pathway. ABCB1, to a lesser extent, may also contribute to CC-115 resistance via a similar mechanism. Clearly, CC-115 may be a substrate of multiple ABC transporters and reduction in clinical response is expected in patients that express any of these ABC transporters.

Based on these findings, it is tempting to speculate that ABCG2 and ABCB1 may be utilized as markers to stratify patients into treatment groups for future clinical trials. The observed variability in CLL patient response in a past trial may possibly be due to different expression of ABCG2 or ABCB1. Previously, it has been shown that ABCG2 and ABCB1 are expressed in leukemia patients and their expression influences the outcome of these patients who were subjected to chemotherapy (Mo and Zhang, 2012; Svirnovski et al., 2009). Currently, there is an ongoing clinical trial of CC-115 for glioblastoma. However, it is also well known that ABCG2 and ABCB1 are both highly expressed in the blood brain barrier (Loscher and Potschka, 2005), which may affect the outcome of this trial.

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It is noteworthy that the resistance of M3K cells to CC-115 compared with the parental MCF7 cells is ~50 folds while the resistance of MCF7/ABCG2 cells to CC-115 compared with MCF7/Vec cells is only ~2 fold. This discrepancy may be due to the difference in the expression level of ABCG2 (see supplemental Figure S6). This is also supported by the fact that ABCG2 overexpression in HEK293 cells, which express also a much higher level of ABCG2 than MCF7/ABCG2 cells (Figure S6), induced a much larger (~10 fold) CC-115 resistance compared with HEK293/Vec cells. It is also possible that ABCG2 in M3K cells has a gain of function in increased activity of transporting CC-115 due to its mutation of Arg^{482} to Thr^{482} (Alqawi et al., 2004). Lastly, M3K cells are known to harbor other mechanisms of drug resistance (Liu et al., 2008; Liu et al., 2006; Liu et al., 2005; Wu et al., 2016a) that may also contribute to CC-115 resistance in M3K cells.

It is also noteworthy that although ABCB1 expression causes resistance to CC-115, reduces intracellular accumulation of CC-115 and CC-115 inhibition of mTOR pathway, these effects appear to be less than that of ABCG2. The reason for this difference is unknown. However, it is possible that CC-115 has higher affinity to ABCG2 than to ABCB1. It is also possible that the expression level of ABCB1 is less than ABCG2 as discussed above for ABCG2 in different expressing host cells. These possibilities remain to be tested in future studies.

Despite the fact that FASN up-regulates DNA-PK activity (Wu et al., 2016a), FASN overexpression does not appear to contribute to cellular resistance to the DNA-PK inhibitory activity of CC-115 or a DNA-PK selective inhibitor, NU7441. Interestingly, we have shown that FASN overexpression causes resistance to DNA-damaging drugs (Liu et al., 2013) and inhibitors of PARP1 (manuscript submitted), a mediator of FASN-induced up-regulation of DNA-PK activity (Wu et al., 2016a). Although it remains to be determined, the failure of FASN

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overexpression in causing resistance to DNA-PK inhibition may be due to the fact that FASN overexpression only leads to an increase in activity and not in expression of DNA-PK, which may be insufficient to overcome the effect of DNA-PK inhibition.

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Authorship contributions

Participated in research design: Beebe, Zhang

Conducted experiments: Beebe

Performed data analysis: Beebe, Zhang.

Wrote or contributed to the writing of the manuscript: Beebe, Zhang

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Legends for Figures

Figure 1. FASN does not contribute to DNA-PK resistance. A) IC_{50} of CC-115 in different cell lines as determined from dose-dependent survival curves using methylene blue assay. B) Dose-dependent survival curve of MCF7 and M3K cells in the presence of CC-115. C) FASN expression in MCF7/Vec, MCF7/FASN, MCF7, M3K, M3K/Scr, and, M3K/shFASN cell lines. D) IC_{50} of the DNA-PK inhibitor Nu7441 as determined from dose-dependent survival curve using methylene blue assay. E) CC-115 inhibition of mTOR, monitored by phosphorylation of S6 and S6K (lower band) as determined using Western blot analyses. Note that p85 was also detected by S6K and pS6K antibodies. Statistical analysis using ANOVA comparing all groups was used in panels A and E followed by Tukey post hoc test (n=3; *p<0.05=, **p<0.01, ***p<0.001). DNA-PK=DNA-PKcs.

Figure 2. Identification of ABCG2 as a resistance factor for CC-115. A) Expression of ABCG2 in MCF7 and M3K cells as determined using Western blot analysis. B) and C) CC-115 accumulation in MCF7 and M3K cells determined using FACS analysis. T-test was used to compare CC-115 accumulation (B) in different cells (n=3; *p<0.05=, **p<0.01, ***p<0.001).

Figure 3. Inhibition of ABCG2 and reversal of CC-115 resistance. A) and B) CC-115 accumulation in M3K cells in the presence of ABCG2 inhibitors C8 and FTC as determined using FACS analysis. C) and D) CC-115 IC_{50} in and representative dose-dependent survival curves of M3K cells in the absence or presence of 1μM ABCG2 inhibitor C8 or FTC as determined using methylene blue assay. E) CC-115 inhibition of mTOR activation in the absence or presence of 1μM ABCG2 inhibitor C8 as determined by monitoring phosphorylation of S6 and S6K using Western blot analysis. Note that p85 was also detected by S6K and pS6K antibodies. ANOVA

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statistical analysis was used to compare CC-115 accumulation (A) and IC_{50} (C) in different cells (n=3-4; *p<0.05, **p<0.01, ***p<0.001). DNA-PK=DNA-PKcs.

Figure 4. Effect of ABCG2 overexpression on CC-115 accumulation and resistance. A) Western blot analysis of ABCG2 expression in HEK293/Vec vs HEK293/ABCG2-venus and MCF7/Vec vs MCF7/FASN cells. B) and C) CC-115 accumulation in HEK293/Vec vs HEK293/ABCG2-venus and MCF7/Vec vs MCF7/ABCG2 cells as determined using FACS. D) and E) CC-115 IC_{50} and representative dose-dependent survival curves of HEK293/Vec vs HEK293/ABCG2-venus and MCF7/Vec vs MCF7/ABCG2 cells as determined using methylene blue assay for HEK293 and MTT assay for MCF7 cells. F) CC-115 inhibition of mTOR activation in HEK293/Vec vs HEK293/ABCG2-venus and MCF7/Vec vs MCF7/ABCG2 cells as determined by monitoring phosphorylation of S6 and S6K using Western blot analysis. Note that p85 was also detected by S6K and pS6K antibodies. T-test was used to compare CC-115 accumulation (B) and IC_{50} (D) (n=3-4; *p<0.05, **p<0.01, ***p<0.001). DNA-PK=DNA-PKcs.

Figure 5. Inhibition of ABCG2 and reversal of CC-115 resistance. A-C) CC-115 accumulation in HEK293/Vec vs HEK293/ABCG2-venus cells in the absence or presence of ABCG2 inhibitors C8 and FTC as determined using FACS analysis. D) and E) CC-115 IC_{50} in and representative dose-dependent survival curves HEK293/ABCG2-venus cells in the absence and presence of inhibitor C8 or FTC as determined using methylene blue assay. F) CC-115 inhibition of mTOR activation in HEK293/ABCG2-venus cells in the absence or presence of ABCG2 inhibitor C8 as determined by monitoring phosphorylation of S6 and S6K using Western blot analysis. ANOVA was used for statistical analysis of CC-115 accumulation (A) and IC_{50} (C) (n=3-4; *p<0.05, **p<0.01, ***p<0.001). DNA-PK=DNA-PKcs.

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Figure 6. CC-115 is a substrate for ABCB1. A) Western blot analysis of ABCB1 expression in MCF7/ABCB1 and the control MCF7/Vec cells. B) and C) CC-115 accumulation in MCF7/Vec and MCF7/ABCB1 cells as determined using FACS analysis. D) and E) CC-115 IC_{50} in and representative survival curves of MCF7/ABCB1 and the control MCF7/Vec cells. F) Western blot analysis of ABCB1 effect on CC-115 inhibition of mTOR signaling in MCF7/Vec and MCF7/ABCG2 cells. Note that p85 was also detected using S6K and p-S6K antibodies. T-test was used for statistical analysis of CC-115 accumulation (C) and IC_{50} (E) (n=3; *p<0.05, **p<0.01, ***p<0.001).

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Figures

Figure 1

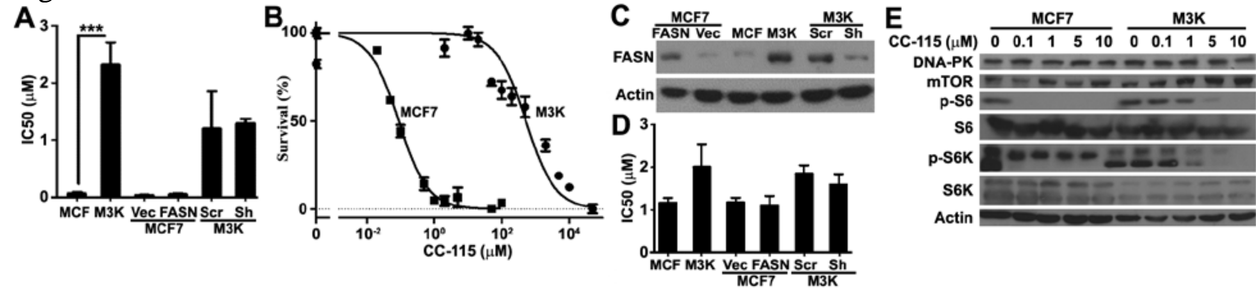

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Figure 2

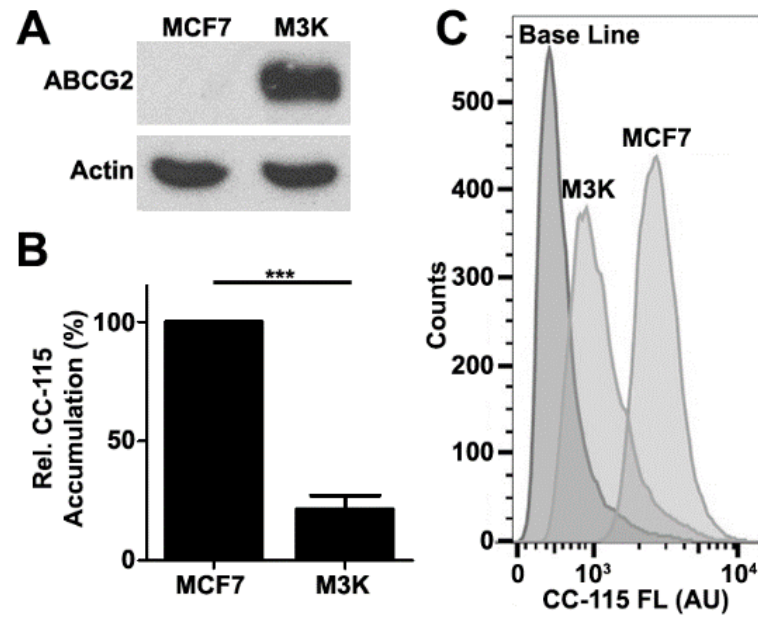

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Figure 3

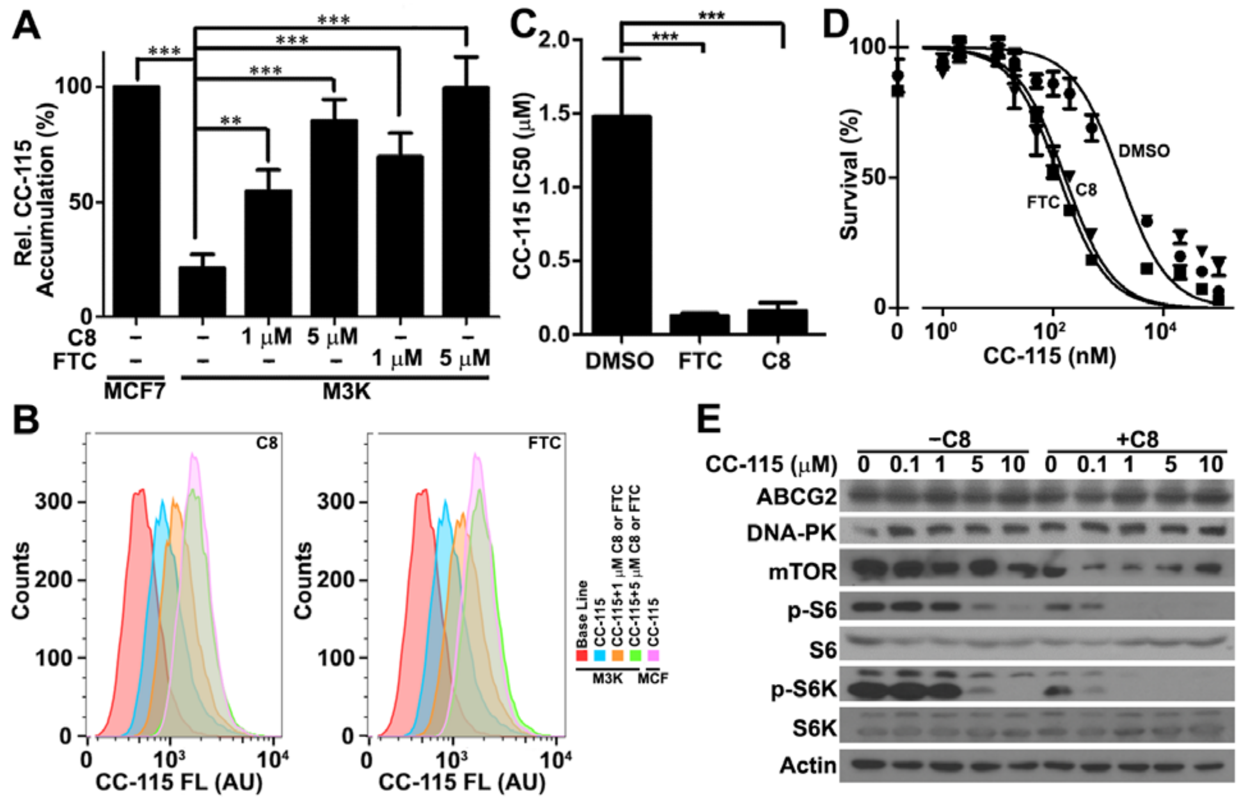

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Figure 4

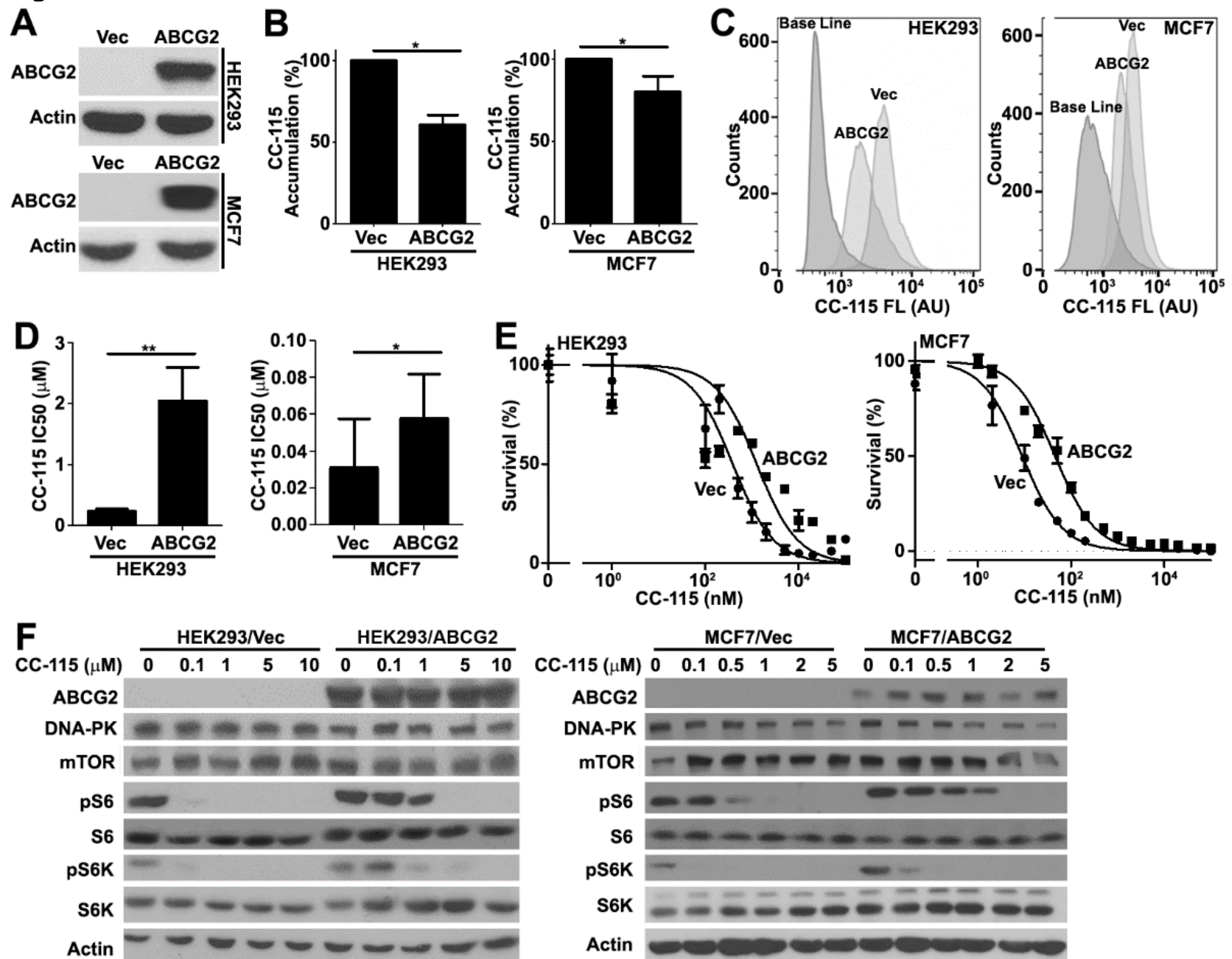

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Figure 5

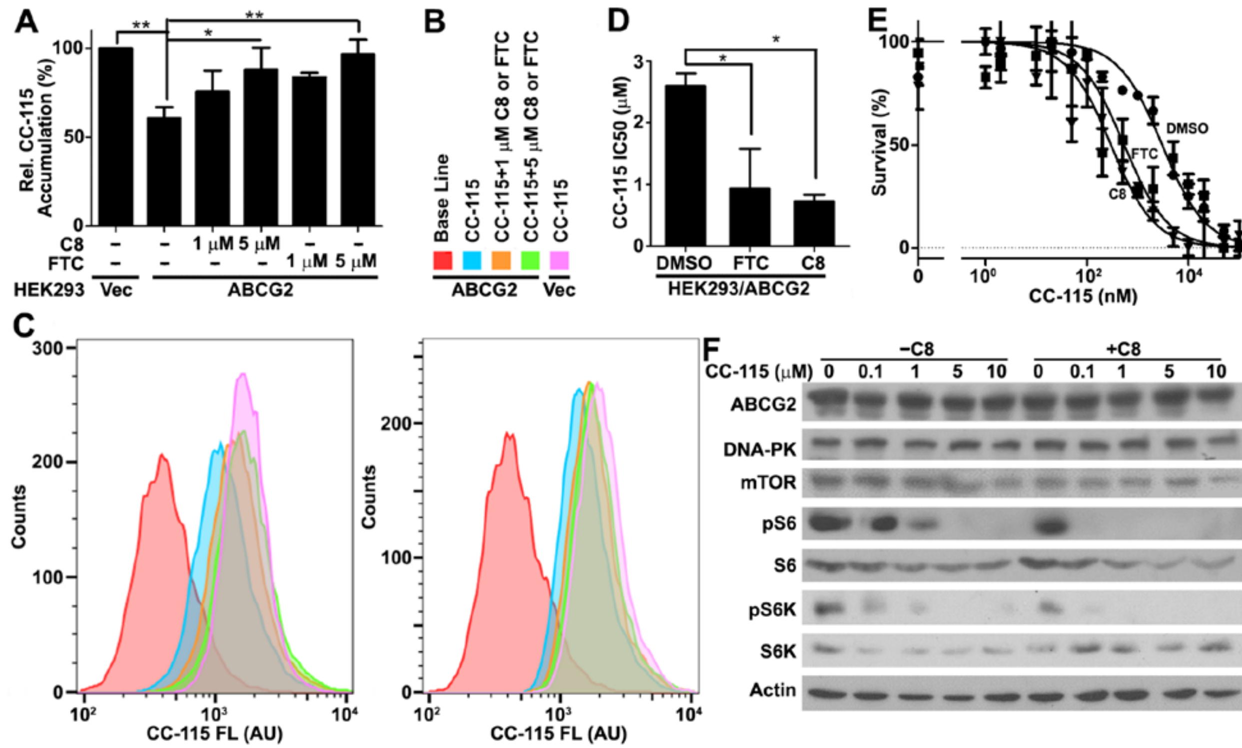

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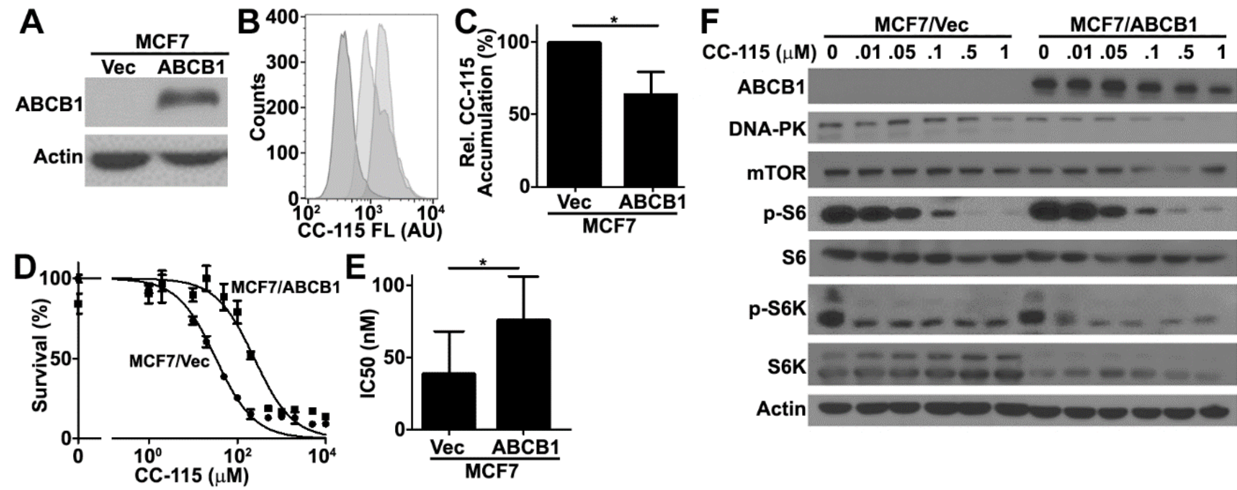
Figure 6



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