CC-115, a Dual Mammalian Target of Rapamycin/DNA-Dependent Protein Kinase Inhibitor in Clinical Trial, Is a Substrate of ATP-Binding Cassette G2, a Risk Factor for CC-115 Resistance

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ABSTRACT

CC-115, a triazole-containing compound, is a dual mammalian target of rapamycin (mTOR)/DNA-dependent protein kinase (DNA-PK) inhibitor currently in clinical trials. To develop this compound further, we investigated factors that may affect cellular response to CC-115. Previously, fatty acid synthase (FASN) was shown to upregulate DNA-PK activity and contribute to drug resistance; therefore, we hypothesized that FASN may affect cellular response to CC-115. Instead, however, we showed that CC-115 is a substrate of ATP-binding cassette G2 (ABCG2), a member of the ATP-binding cassette transporter superfamily, and that expression of ABCG2, not FASN, 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 that contributes 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 article, we report our findings on the potential mechanism of resistance to CC-115, a dual inhibitor of mTOR and DNA-PK currently in clinical trials. We show that CC-115 is a substrate of ABCG2 and can 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.

Introduction

The phosphoinositide 3-kinase (PI3) K-related kinase (PIKK) family of serine/threonine kinases includes ataxia-telangiectasia mutated, ataxia-telangiectasia mutated related, human suppressor of morphogenesis in genitalia-1, 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). In particular, DNA-PK plays an important role in nonhomologous end joining repair of DNA double-strand breaks (Hammel et al., 2010), whereas 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 (C_{16}H_{16}N_{8}O), 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 (Tsuij et al., 2017). Several ongoing clinical trials are testing CC-115 in an array of different cancer types, including phase 1 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 2 trial in glioblastoma. Early findings on CC-115 in CLL patients showed that seven of eight patients had a decrease in lymphadenopathy, with one partial response and three partial responses with lymphocytosis (Thijssen et al., 2016). Although these early results in CLL show promise for further development of CC-115, there is an observed variability in sensitivity. In fact, one of the eight 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.

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Materials and Methods

Materials. Antibodies against ABCG2 (no. MAB4146), DNA-PK (no. PC127), and actin (no. JLA-20) were from Millipore (Danvers, MA), Calbiochem (San Diego, CA), and Sigma (Saint Louis, MO), respectively. Antibodies against mTOR (no. 2983, 1:500), p-S6 (no. 2215, 1:1000), S6 (no. 2317, 1:200), p-S6K (no. 9234, 1:1000), S6K (no. 2708, 1:1000), p-AKT (no. 4051, 1:1000) were all from Cell Signaling (Danvers, MA). Antibody against DNA-PKcs (nos. PIMA513238, 1:1000) was from Invitrogen (Carlsbad, CA). S6K and p-S6K antibodies can also detect p85. Actin (no. JLA-20, 1:3000), anti-mouse secondary (no. A21281, 1:3000), and anti-rabbit secondary (no. A0545, 1:1000) antibodies were from Sigma. Cell Lines. MCF7 and M3K cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (no. 10-013-CV) (Corning, Manassas, VA) containing 10% fetal bovine serum (FBS) (no. A3160601) (Life Technologies, Grand Island, NY) with supplementation of 5 μg/ml G418, cloned, and propagated under the same conditions.

Cell Lysate Preparation and Western Blot. Cells were treated with the given concentration of CC-115 for 2 hours, and cell lysates were prepared as previously described (Liu et al., 2006) and separated by SDS-PAGE, followed by transfer onto polyvinylidene difluoride membranes. Western blot analysis was also performed exactly as we previously described (W. Zhang et al., submitted manuscript). 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). Human embryonic kidney cell line 293 (HEK293/ABCG2-venus and HEK293/Vec-venus were also established in a previous study and cultured in DMEM containing 10% FBS with 600 μg/ml G418 as previously described (W. Zhang et al., submitted manuscript). 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 μg/ml 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 conditions.

Survival Assays. We plated 500 to 4000 cells/well in a 96-well plate and cultured overnight, followed by treatment with increasing concentrations of CC-115 in the absence or presence of doxorubicin at its IC$_{50}$ concentration for 3 days, and cell survival was measured using methylene-blue staining (Oliver et al., 1989) or 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For methylene blue assay, cells were first fixed with methanol for 30 minutes and stained with 1% (w/v) methylene-blue in 10 mM borate buffer, pH 8.5, for 30 minutes. Excess dye was removed by aspiration, and plates were washed three times with ddH$_2$O. The stains were then released using a 1:1 mixture of 100% ethanol and 0.1 M HCl, followed by determination of O.D.$_{570}$nm. For MTT assay, cells were treated with 5 mg/ml thiazolyl blue tetrazolium bromide in phosphate-buffered saline (PBS) for 4 hours at 37°C, and cells were collected by centrifugation. Finally, dimethylsulfoxide 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 hours, and cell lysates were prepared as previously described (Liu et al., 2006) and separated by SDS-PAGE, followed by transfer onto polyvinylidene difluoride membranes. Western blot analysis was also performed exactly as we previously described (Xu et al., 2004, 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 hours 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 minutes at 37°C. Then, 10 μM CC-115 or dimethylsulfoxide control was added to the cells, followed by incubation at 37°C for 30 minutes with shaking every 10 minutes. Cells were then washed with PBS twice and subjected to analysis using flow cytometry with an excitation at 405 nm and emission at 421 nm.

Fig. 1. FASN does not contribute to DNA-PK inhibitor 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 (A and E), followed by Tukey post hoc test ($n = 3$, ***$P < 0.005$, **$P < 0.01$, ***$P < 0.001$). DNA-PK, DNA-PKcs.
Statistical Analysis for all experiments was run using Prism Graphpad (San Diego, CA). Results are presented as mean ± standard deviation. IC₅₀ values were calculated from three biologic replicates, each run in triplicate. Western blots were run on four 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 the MCF7/AdVp3000 (M3K) 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 Fig. 1, A and B, M3K cells are much more resistant than MCF7 cells to CC-115 (IC₅₀ of ~2.2 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 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 Fig. 1C) and tested their response to CC-115 compared with their respective control cells (MCF7/Vec and M3K/Scr). As shown in Fig. 1A and Supplemental Fig. 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 DNA-PK.

To ensure that FASN expression does not contribute to resistance to DNA-PK inhibition, we tested a DNA-PK selective inhibitor, NU7441. As shown in Fig. 1D and Supplemental Fig. S2, alteration of FASN expression did not change cellular response to NU7441, although M3K cells are slightly more resistant to NU7441 than the parental MCF7 cells. DNA-PK activation occurs in response to DNA damage,
therefore, we also tested whether FASN causes resistance to DNA-PK inhibition in the presence of DNA damage. As shown in Supplemental Fig. S3, no change occurred in the cellular response to NU7441 in the presence of doxorubicin at its IC_{20} concentration. No significant difference in cellular response to CC-115 was seen in the presence of doxorubicin at its IC_{20} concentration, although there may be a trend toward resistance (Supplemental Fig. S3).

Because CC-115 is a dual mTOR/DNA-PK inhibitor, we next tested whether its inhibition of the mTOR pathway was impaired in the drug-resistant M3K cells. As shown in Fig. 1E, CC-115 inhibition of constitutive activation of S6K and phosphorylation of S6, downstream targets of mTOR, were significantly impaired in M3K cells 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 Drug-Resistant M3K Cells.** To investigate further 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 Fig. 2A), by first determining the accumulation of CC-115 in M3K versus MCF7 cells. As shown in Supplemental Fig. S4, CC-115 is autofluorescent with an emission wavelength at 421 nm, which was used to monitor its intracellular accumulation. As shown in Fig. 2, B and C, CC-115 accumulation was much lower in M3K cells, with an ∼80% reduction compared with MCF7 cells.

**Sensitization of M3K Cells to CC-115 by Inhibiting ABCG2.** To determine whether ABCG2 is responsible for the reduced CC-115 accumulation in M3K cells, we tested the effect of ABCG2-specific inhibitors C8 and FTC (Rabindran et al., 2000; Peng et al., 2009) on CC-115 accumulation in M3K cells. As shown in Fig. 3, A and 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 (Fig. 3, C and D). Furthermore, inhibiting ABCG2 with C8-sensitized M3K cells to CC-115 inhibition of mTOR activation of S6K (Fig. 3E).

**ABCG2 Overexpression Reduces CC-115 Accumulation in Both HEK293 and MCF7 Cells.** To determine further the role of ABCG2 in CC-115 efflux and resistance, we took advantage of human embryonic kidney cell line 293 and MCF7 cells stably transfected with ABCG2 (HEK293/ABCG2-venus, which contains a venus tag and MCF7/ABCG2, which

Fig. 4. Effect of ABCG2 overexpression on CC-115 accumulation and resistance. (A) Western blot analysis of ABCG2 expression in HEK293/Vec versus HEK293/ABCG2-venus and MCF7/Vec versus MCF7/FASN cells. (B and C) CC-115 accumulation in HEK293/Vec versus HEK293/ABCG2-venus and MCF7/Vec versus MCF7/ABCG2 cells as determined using FACS. (D and E) CC-115 IC_{50} and representative dose-dependent survival curves of HEK293/Vec versus HEK293/ABCG2-venus and MCF7/Vec versus 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 versus HEK293/ABCG2-venus and MCF7/Vec versus 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. The t test was used to compare CC-115 accumulation (B) and IC_{50} (D) (n = 3 to 4; *P < 0.05, **P < 0.01, ***P < 0.001). DNA-PK, DNA-PKcs.
does not), and their respective control cells transfected with vector (HEK293/Vec, venus tagged, and MCF7/Vec) (see Fig. 4A). Using these cells, we first determined CC-115 accumulation as described already. As shown in Fig. 4, B and 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 Fig. 4, D and 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 (Fig. 4F). We also tested the effect of CC-115 on mTORC2 by determining its effect on AKT phosphorylation. As shown in Supplemental Fig. 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 whether inhibiting the function of ABCG2 could reverse the ectopic ABCG2-induced resistance and accumulation reduction of CC-115. As shown in Fig. 5, A–C, both C8 and FTC significantly reversed ABCG2-induced CC-115 accumulation reduction. Moreover, both C8 and FTC significantly reduced CC-115 IC₅₀ in HEK293/ABCG2-venus cells (Fig. 5, D and 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 (Fig. 5F), and mTORC2, as indicated by AKT phosphorylation (Supplemental Fig. S5B).

**Role of ABCB1 in CC-115 Resistance.** It is known that ABC transporters involved in drug resistance have overlapping substrates; thus, we next tested whether other ABC transporters, such as ABCB1, may also contribute to CC-115 resistance. For this purpose, we took advantage of MCF7 cells that overexpress ectopic ABCB1 (BC19 cells) and tested CC-115 accumulation and resistance compared with vector-transfected MCF7 cells (MCF7/Vec) (Fig. 6A). As shown in Fig. 6, B and C, CC-115 accumulation is significantly reduced in BC19 compared with the control MCF7/Vec cells. The IC₅₀ of CC-115 was also significantly increased in BC19 cells compared with the MCF7/Vec cells (Fig. 6, D and E). When cells were treated with increasing concentrations of CC-115, there was a slight change in S6K and S6 phosphorylation compared with MCF7/Vec cells (Fig. 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 used as markers to stratify patients into treatment groups for future clinical trials. The observed variability in CLL patient response in a past trial (Loscher and Potschka, 2005), which may affect the outcome of these patients, who were subjected to 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 a higher affinity to ABCG2 than to ABCB1. It is also possible that the expression level of ABCB1 is less than that of ABCG2 as discussed already for ABCG2 in different expressing host cells. These possibilities remain to be tested in future studies.

Although FASN upregulates 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 (submitted manuscript), a mediator of FASN–DNA damage-activated up-regulation of DNA-PK activity (Wu et al., 2016a). Although it remains to be determined, the failure of FASN overexpression in causing resistance to DNA-PK inhibition may be due to the fact that FASN overexpression leads only 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.

**References**


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