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Association of ABC Transporter With Resistance to FK866, a NAMPT Inhibitor, in Human Colorectal Cancer Cells

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Abstract. *Background/Aim: Nicotinamide phosphoribosyltransferase (NAMPT), a key enzyme in the NAD^+ biosynthetic pathway, is a drug target of potent anticancer candidates, including FK866 and other reported NAMPT inhibitors. However, it is known that NAMPT point-mutations render resistance to specific NAMPT inhibitors in several cancer cells. We investigated the resistance mechanisms of NAMPT inhibitor FK866 in human colorectal cancer (CRC) cells. Materials and Methods: We used CRC human cell line HCT116 to determine the expression profiles of FK866-sensitive parental HCT116 cells and FK866-resistant HCT116 ($HCT116R^{FK866}$) cells by DNA microarray analysis. The levels of multidrug resistance protein 1 (MDR1) were assessed via western blot. In addition, we analyzed the sensitivity of FK866 in parental HCT116 cells and $HCT116R^{FK866}$ cells by co-treatment with MDR1 inhibitor verapamil. Results: Our results revealed an association between ATP-binding cassette (ABC) transporter gene ABCB1 and resistance to NAMPT inhibitor FK866 in both $HCT116R^{FK866}$ cells and parental HCT116 cells. The expression of ABCB1, which encodes MDR1, was lower in $HCT116R^{FK866}$ cells than in parental HCT116 cells. Furthermore, the protein level of MDR1/ATP-binding cassette sub-family B member 1 (ABCB1) was 0.5-fold lower in $HCT116R^{FK866}$ cells than in parental HCT116 cells. Additionally, $HCT116R^{FK866}$ cells showed improved sensitivity to FK866 when co-treated with verapamil, an ABCB1 inhibitor. Interestingly, the efficacy of FK866 in parental HCT116 cells was the same for the treatment with FK866 alone or in combination with verapamil. Conclusion: The change in expression of ABCB1 plays a key role in CRC drug resistance to NAMPT inhibitor FK866. This suggests that the MDR1/ABCB1 mechanism may regulate the resistance of anticancer NAMPT inhibitor FK866.*

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Key Words: ABC transporter, drug resistance, FK866, NAMPT, NAMPT inhibitor, verapamil.

Nicotinamide phosphoribosyltransferase (NAMPT) is a rate-limiting enzyme in the salvage pathway of nicotinamide (NAM) in nicotinamide adenine dinucleotide (NAD^+) synthesis (1-4). NAMPT is a potential anticancer drug target, and many drug candidates have been developed that inhibit its enzymatic activity (1, 5), including FK866 (also known as APO866 and WK175) (6, 7), CHS-828 (also known as GMX1778) (8-11), GNE-617 (12), and STF-118804 (13). Acquired resistance to anticancer NAMPT inhibitors appears to be due to alterations in NAMPT itself (14-16). There have been previous reports that NAMPT point-mutations confer resistance to specific NAMPT inhibitors (14-16). Recently, we produced FK866-resistant $HCT116R^{FK866}$ cells from human colorectal cancer (CRC) HCT116 cells (17, 18). Whole-exome sequencing of the NAMPT gene revealed two single point-mutations, H191R and K342R, in NAMPT in $HCT116R^{FK866}$ cells, of which only K342R was present in the parental HCT116 cells (17, 18). Additionally, we used a NAMPT-immunoprecipitated proteomics approach to demonstrate that NAMPT in HCT116 cells, but not $HCT116R^{FK866}$ cells, interacted with the truncated POTE (expressed in prostate, ovary, testis, and placenta) ankyrin domain family member E (tPOTEE) and β-actin. Moreover, we reported that the NAMPT H191R variant in $HCT116R^{FK866}$ cells might prevent interaction with the two identified binding partners (tPOTEE and β-actin), resulting in cellular resistance to diverse NAMPT inhibitors (17). We also demonstrated that $HCT116R^{FK866}$

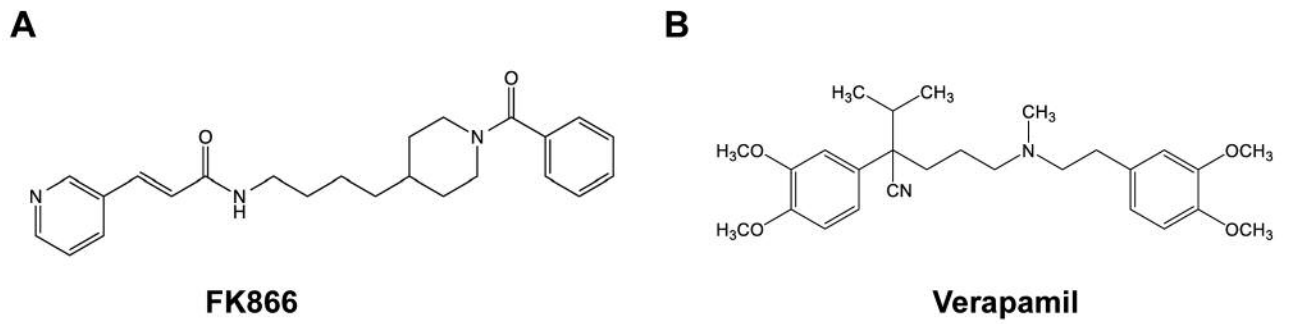

Figure 1. *Chemical structures of NAMPT inhibitor FK866 (A) and ABC transporter inhibitor verapamil (B).*

cells were more sensitive to the anticancer 5-fluorouracil and cisplatin and γ-ray irradiation compared with parental HCT116 cells (18).

Cancer drug resistance is a challenge that needs to be overcome to achieve effective anticancer chemotherapy. However, the underlying mechanisms can differ depending on the medicine, and common cancer resistance mechanisms include drug inactivation, drug efflux, drug-targeted alterations, bypass pathway activation, DNA damage repair, and cell death escape (19). Importantly, multidrug resistance (MDR) in cancer cells can significantly attenuate the response to chemotherapy (20-22). The major mechanism involved in conferring MDR is the overexpression of ATP-binding cassette (ABC) transporters, which can increase the efflux of drugs from cancer cells, thereby decreasing intracellular drug concentration (20-22).

The relationship between the mechanism of NAMPT resistance and ABC transporter is unelucidated. To this end, we investigated the association of ABC transporter with resistance to FK866, a NAMPT inhibitor, in human CRC HCT116 cells.

Materials and Methods

Reagents. The anticancer NAMPT inhibitor FK866 HCl was obtained from Focus Biomolecules (Plymouth Meeting, PA, USA) and was stored as a 10 mM stock in ultra-pure water at −20˚C. The ABC transporter inhibitor verapamil was obtained from FUJIFILM Wako Pure Chemical Corp. (Doshomachi, Osaka, Japan) and was stored as a 20 mM stock in DMSO at −20˚C.

Cell culture. The human CRC cell line HCT116 was obtained from the American Type Culture Collection (Manassas, VA, USA). FK866-resistant HCT116 (HCT116R^{FK866}) cells were developed according to the previously described method (17). Parental HCT116 and HCT116R^{FK866} cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an incubator a 37˚C in an atmosphere of 5% CO_2 at 100% relative humidity.

Clonogenicity assay. Clonogenicity assays were performed as previously described (17, 18). Briefly, parental HCT116 and HCT116R^{FK866} cells were dissociated with Accutase (NACALAI TESQUE INC., Nijo Karasuma, Kyoto, Japan), suspended in medium, inoculated into six-well plates (200 cells/well) in triplicate, and then incubated overnight. The cells were treated with various concentrations of drugs (FK866 only or co-treatment with verapamil), a concentration of DMSO, or ultra-pure water (control). After 10 days' incubation, the cells were fixed with a solution of 4% formaldehyde-phosphate-buffered saline and stained with 0.1% (w/v) crystal violet. The number of colonies per well were then counted.

Microarray analysis. RNA extraction was performed as described previously (23, 24). Briefly, total RNA was extracted using QIAshredder spin columns and an RNeasy Mini Kit (both QIAGEN, Hilden, Germany) as per the manufacturer's instructions. DNA microarray analysis of the parental HCT116 and HCT116R^{FK866} cells was performed by TAKARA Bio Inc. (Shiga, Japan) using a SurePrint G3 Human Gene Expression 8×60K v3 Microarray (Agilent Technologies, Santa Clara, CA, USA) to determine the expression profiles of the cells.

Western blot analysis. Western blot analysis was performed as described previously (17, 25). The following antibodies were used: rabbit anti-MDR1 (1:1,000; Abcam, Cambridge, UK), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:20,000; Trevigen, Gaithersburg, MD, USA), and horseradish peroxidase-labelled anti-rabbit immunoglobulin (Ig)G (1:20,000; GE Healthcare, Pittsburgh, PA, USA).

Statistical analysis. The data were presented as the means±standard deviation (SD). The significance of differences among groups was evaluated using Student's *t*-test; $p<0.05$ was considered statistically significant.

Results

To investigate the resistance mechanisms of NAMPT inhibitor FK866 (Figure 1A) in human CRC, we analyzed the comprehensive gene expression profiles of FK866-resistant HCT116R^{FK866} cells and FK866-sensitive parental HCT116

Table I. *Gene expression of ABCB1 in parental HCT116 and HCT116R^{FK866} cells as determined by microarray analysis.*

Gene symbol	Gene description	S (signal)	R (signal)	FC (R/S)	*p*-Value
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	153.0	73.5	0.48	0.004

S, FK866-sensitive parental HCT116 cells; R, FK866-resistant HCT116R^{FK866} cells; FC, fold change (HCT116R^{FK866} cells *vs*. HCT116 cells). The signal values represent the average signal intensity for the microarray probe (probe ID: A_23_P82523) from three independent experiments.

Table II. *Gene mutation status of multidrug resistance gene ABCB1 in HCT116 and HCT116R^{FK866} cells.*

Gene symbol	Gene mutations	
	HCT116	HCT116R^{FK866}
ABCB1	*mt*(Ile1145Ile)*het*	*mt*(Ile1145Ile)*het*
	mt(Ser893Ala)*het*	*mt*(Ser893Ala)*het*
	mt(Asp603His)*het*	*mt*(Asp603His)*het*
	mt(Gly412Gly)*het*	*mt*(Gly412Gly)*het*
	mt(Asn21Asp)*het*	*mt*(Asn21Asp)*het*

mt, Mutation-type; *het*, heterozygous.

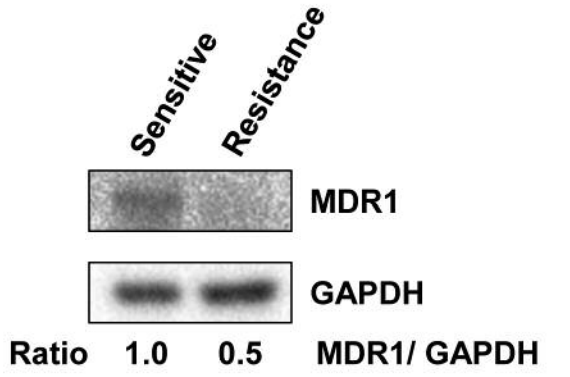

Figure 2. *Protein levels of MDR1 in FK866-resistant HCT116R^{FK866} and parental HCT116 human CRC cells. Whole-cell lysates were prepared from parental HCT116 and HCT116R^{FK866} cells. Protein levels of MDR1 and GAPDH were examined by western blot analysis. The expression of GAPDH was used as an internal control. Data are representative of at least three independent experiments. Levels of MDR1 protein are represented by the ratio of MDR1 density to GAPDH density relative to the value for parental HCT116 cells. Results represent the averages of three independent experiments.*

cells using DNA microarray technology (data not shown). In the microarray analysis, the expression of multidrug resistance-related *ABCB1* was decreased in HCT116R^{FK866} cells compared with parental HCT116 cells (Table I). Previously, we used whole-exome sequencing analysis to demonstrate that the gene mutation status of *ABCB1* was similar in HCT116R^{FK866} and parental HCT116 cells (Table II) (18). However, the relationship between the mechanism of NAMPT inhibitor FK866 resistance and ABC transporter remained unelucidated. Therefore, we investigated the association of ABC transporter and FK866 resistance in our established human CRC FK866-resistant HCT116R^{FK866} (17) and FK866-sensitive parental HCT116 cells. ABCB1/MDR1 is a glycosylated 170-kDa transmembrane protein that is encoded by the *ABCB1* gene, and the drug efflux pump of the family of ABC transporters is well known (20-22). Microarray analysis revealed that the gene expression of *ABCB1* was 0.5-fold lower in HCT116R^{FK866} cells than in parental HCT116 cells (Table I). For western blot validation, the protein level of *ABCB1* was 0.5-fold lower in HCT116R^{FK866} cells than in parental HCT116 cells (Figure 2). These findings suggested that MDR1/ABCB1 was expressed at lower levels in FK866-resistant HCT116R^{FK866} cells compared to parental HCT116 cells.

To explore how the multidrug-resistant *ABCB1* function conferred resistance to NAMPT inhibitor FK866, we analyzed the sensitivity of FK866 in HCT116R^{FK866} cells and parental HCT116 cells by co-treatment with verapamil (Figure 1B), an ABC transporter ABCB1 inhibitor (20, 21). Interestingly, HCT116R^{FK866} cells showed improved sensitivity to FK866 when co-treated with verapamil. In contrast, the sensitivity of FK866 in parental HCT116 cells was unaffected by the verapamil co-treatment (Figure 3). The 50% effective concentration (EC_{50}) of FK866 in parental HCT116 and HCT116R^{FK866} cells with or without verapamil co-treatment was determined by colony formation assay. The EC_{50} of FK866 in HCT116R^{FK866} cells was higher with FK866 alone (EC_{50}=6,650 nM in FK866) compared with co-treatment with verapamil (EC_{50}=2,700 nM in FK866). In contrast, the EC_{50} of FK866 in parental HCT116 cells was lower with FK866 alone (EC_{50}=11 nM in FK866) compared to co-treatment with verapamil (EC_{50}=15 nM in FK866) (Figure 3A and Table III). Regarding the sensitivity index of FK866, HCT116R^{FK866} cells were 2.5-fold more sensitive to FK866 when co-treated with verapamil. In contrast, HCT116 cells were almost similar in sensitivity to FK866 with verapamil co-treatment. These findings suggested that *ABCB1* machinery regulates NAMPT inhibitor FK866 resistance in HCT116R^{FK866} cells.

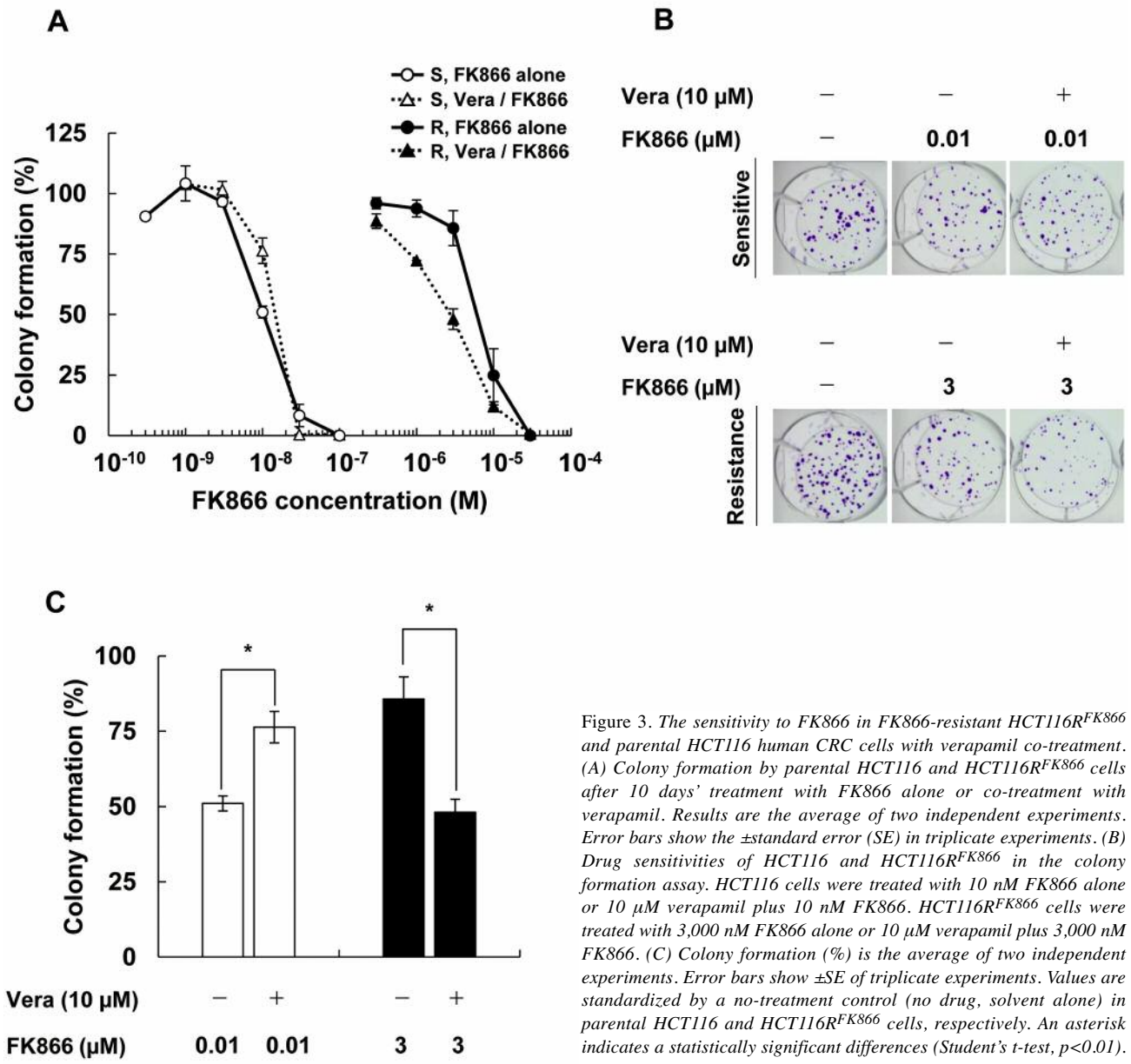

Figure 3. *The sensitivity to FK866 in FK866-resistant HCT116R^{FK866} and parental HCT116 human CRC cells with verapamil co-treatment. (A) Colony formation by parental HCT116 and HCT116R^{FK866} cells after 10 days' treatment with FK866 alone or co-treatment with verapamil. Results are the average of two independent experiments. Error bars show the ±standard error (SE) in triplicate experiments. (B) Drug sensitivities of HCT116 and HCT116R^{FK866} in the colony formation assay. HCT116 cells were treated with 10 nM FK866 alone or 10 μM verapamil plus 10 nM FK866. HCT116R^{FK866} cells were treated with 3,000 nM FK866 alone or 10 μM verapamil plus 3,000 nM FK866. (C) Colony formation (%) is the average of two independent experiments. Error bars show ±SE of triplicate experiments. Values are standardized by a no-treatment control (no drug, solvent alone) in parental HCT116 and HCT116R^{FK866} cells, respectively. An asterisk indicates a statistically significant differences (Student's t-test, p<0.01).*

Discussion

NAMPT, a key enzyme in the NAD^+ biosynthetic pathway, is a molecular target of potent anticancer candidates, including FK866 and other reported NAMPT inhibitors (1-4). However, many studies have previously reported that NAMPT point-mutations render resistance to specific NAMPT inhibitors in several cancer cell types (14-16). We investigated the resistance mechanisms of NAMPT inhibitor FK866 in human CRC HCT116 cells (17, 18). Our findings revealed that multidrug resistant-related *ABCB1* (the gene encoding MDR1) is expressed at a lower level in FK866-resistant HCT116R^{FK866} cells compared to parental HCT116 cells (Figure 2 and Table I). We previously demonstrated that FK866-resistant HCT116R^{FK866} cells, which are resistant to other classes of NAMPT inhibitors (*e.g.*, CHS-828, GNE-617, and STF118804), were more sensitive to the anticancer medicines 5-fluorouracil and cisplatin compared to parental HCT116 cells (18). Interestingly, HCT116R^{FK866} cells showed a 2.5-fold higher sensitivity (*i.e.*, less resistance) to FK866 with verapamil co-treatment. This suggests that ABCB1 may be involved in the efflux of NAMPT inhibitor FK866 in HCT116R^{FK866} cells. We consider that FK866-resistant HCT116R^{FK866} cells become resistant to NAMPT inhibitor FK866 by down-regulating

Table III. *Sensitivity to FK866 in parental HCT116 and HCT116R^{FK866} cells with co-treatment of verapamil.*

Cell line	FK866 (EC_{50}, nM)		
	Verapamil (–)	Verapamil (+)	Sensitivity index
HCT116	11	15	0.7
HCT116R^{FK866}	6,650	2,700	2.5

The cells were treated as described in the legend to Figure 3. The EC_{50} values are the average of triplicate determinations obtained from two independent experiments. The sensitivity index was determined as the EC_{50} values of FK866 alone divided by the EC_{50} values of FK866 in combination with verapamil and FK866 in HCT116 and HCT116R^{FK866} cells, respectively.

ABCB1 expression. Importantly, the sensitivity of FK866 in parental HCT116 cells was unaffected by verapamil co-treatment. Interestingly, many previous reports demonstrated that the overexpression of *ABCB1* is responsible for the resistance against anticancer drugs, *e.g.*, cisplatin, etoposide, paclitaxel, and doxorubicin, in several cancer cells (20, 26-28). Conversely, our finding suggests that the lower expression of *ABCB1* is associate with anticancer FK866 resistance in the FK866-resistant HCT116R^{FK866} cells. These results indicated the difference in the ABCB1 function between HCT116R^{FK866} cells and parental HCT116 cells. We propose that this difference was regulated by post-transcriptional modification (*e.g.*, protein modification by phosphorylation, the interaction of long non-coding RNAs, or other factors). We are currently investigating the association of ABC transporter ABCB1 and other family members with NAMPT inhibitor FK866 resistance mechanisms in human CRC HCT116 cells. Our data indicate that FK866-resistance in human CRC cells is responsible for the diverse mechanisms; NAMPT mutation, differences in the components of NAMPT complex, and different drug efflux machinery. Finally, these novel findings provide a better understanding of the resistance mechanisms of the anticancer NAMPT inhibitors.

Conflicts of Interest

None.

Authors' Contributions

AS: Conceived and designed the experiments. YO and AS: Performed the experiments. YO, AS, YK, TA, FU and ST: Analyzed the data. YO and AS: Wrote the paper.

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