



# HHS Public Access

Author manuscript

*J Cell Biochem.* Author manuscript; available in PMC 2018 August 01.

Published in final edited form as:

*J Cell Biochem.* 2017 August ; 118(8): 2420–2429. doi:10.1002/jcb.25908.

## 2-Trifluoromethyl-2-Hydroxypropionamide Derivatives as Novel Reversal Agents of ABCG2 (BCRP)-Mediated Multidrug Resistance: Synthesis and Biological Evaluations

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### Abstract

It has been postulated that one of the biggest impediments to a successful chemotherapy is the phenomena of multidrug resistance (MDR) in cancer cells. One of the main mechanisms of MDR is overexpression of the ATP-binding cassette (ABC) transporters in cancer cells which alters absorption, distribution, metabolism and excretion of various chemotherapeutic drugs. Efforts have been made to find effective inhibitors of ABC transporters. However, none has been approved clinically. This study shows that a novel compound 3-chloro-N-(2-hydroxyphenyl)-4-(3,3,3-trifluoro-2-hydroxy-2-methylpropanamido) benzamide (compound 7d), one of the 2-trifluoromethyl-2-hydroxypropionamide derivatives could reverse ABCG2 (BCRP)-mediated MDR. Cytotoxicity studies show that compound 7d sensitizes the ABCG2-overexpressing cells to chemotherapeutic drugs mitoxantrone and SN-38, which are well-established substrates of the ABCG2 transporter. Western blotting results indicate that compound 7d does not significantly alter the protein level of the ABCG2 transporter. Accumulation and efflux studies demonstrate that compound 7d increases intracellular accumulation of mitoxantrone by inhibiting the function of ABCG2. Overall, these findings indicate a potential use for compound 7d as an adjuvant agent for chemotherapy to inhibit the function of the clinically relevant ABC transporter and sensitize tumor cells to chemotherapeutic drugs.

### Keywords

2-trifluoromethyl-2-hydroxypropionamide; ABCG2; multidrug resistance; ABC transporter

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**CONFLICTS OF INTEREST:** No potential conflicts of interest were disclosed.

## INTRODUCTION

Cancer chemotherapy becomes increasingly complicated because of the presence of multidrug resistance (MDR) [Gottesman, 2002]. MDR occurs due to the expression of ATP-binding cassette (ABC) transporters in cancer cells [Gottesman et al., 2002; Kathawala et al., 2015]. Current studies show that there are 48 human ABC transporter family members that are divided into 7 subfamilies from A to G according to the organization of their nucleotide-binding folds (NBFs) and 14 of them confers MDR [Dean et al., 2001; Tiwari et al., 2011]. Among those, ABC transporter subfamily B member 1 (ABCB1/Pgp), ABC transporter subfamily G member 2 (ABCG2/BCRP), and ABC transporter subfamily C member 1 (ABCC1/MRP1) have been reported as major factors in mediating resistance to certain anticancer drugs [Chen and Tiwari, 2011; Sodani et al., 2012]. ABCB1 was the first human ABC drug transporter to be discovered and it transported various chemotherapeutic drugs such as Vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes out of cells via an energy dependent process utilizing ATP [Gottesman et al., 2002; Juliano and Ling, 1976]. Similar to ABCB1, ABCC1 was discovered as another important transporter that conferred resistance to a range of chemotherapeutic substrates including anthracyclines, epipodophyllotoxins, Vinca alkaloids and taxanes [Cole et al., 1992; Sodani et al., 2012]. Subsequently, ABCG2 was identified that conferred resistance to diverse range of chemotherapeutics such as doxorubicin, mitoxantrone and topotecan [Doyle et al., 1998; Kathawala et al., 2015]. High ABCG2 expression has been found in a variety of solid tumors and in hematologic malignancies and has been correlated with poor clinical outcomes. Furthermore, ABCG2 is overexpressed in cancer stem cells that are able to self-renew and differentiate [Robey et al., 2007; Stacy et al., 2013]. Structurally, ABCG2 is a half-transporter composed of one nucleotide binding domain (NBD) and one transmembrane domain (TMD) that functions by homodimerization or heterodimerization [Mo and Zhang, 2012]. ABC transporters contribute to MDR by altering drug absorption, distribution, metabolism and excretion. For this reason, researchers have been looking for ways to inhibit ABC transporters and resensitize cancer cells to chemotherapeutic drugs [Goldberg et al., 1988; Polli et al., 2008; Shukla et al., 2011; Shukla et al., 2008; Tsuruo et al., 1981]. In recent years, tyrosine kinase inhibitors (TKI) have been in clinical use or used in clinical trials as targeted therapy for cancer treatment [Anreddy et al., 2014]. More recently, it has been reported that certain TKI may interact with ABC transporters by either being their substrates or inhibitors that compete with ATP on the catalytic site of the ATP-binding domain [Dai et al., 2008; Hegedus et al., 2002; Ozvegy-Laczka et al., 2005; Shi et al., 2009; Wang et al., 2014b]. Apart from the TKI, several studies have reported that ABC transporters can be inhibited by marine compounds, natural compounds and phosphodiesterase-5 (PDE-5) inhibitors that are mechanistically and structurally distinct [Kathawala et al., 2015]. One of the widely studied and an established ABCG2 reversal agent fumitremorgin C (FTC) is a fungal toxin of the diketopiperazines family of compounds. FTC is a specific, selective and potent inhibitor of ABCG2 [Rabindran et al., 2000]. However, no drug has been clinically approved as an inhibitor of ABC transporters due to their pharmacokinetic interactions and toxicity [Ahmed-Belkacem et al., 2006; Leonard et al., 2003; Wang et al., 2014a].

In this study, a variety of 2-trifluoromethyl-2-hydroxypropionamide derivatives were synthesized and their efficacy was evaluated. One compound, 3-chloro-*N*-(2-hydroxyphenyl)-4-(3,3,3-trifluoro-2-hydroxy-2-methylpropanamido)benzamide (compound 7d), was particularly active in ABCG2-overexpressing cells. Therefore, we conducted *in vitro* investigation of compound 7d such as, cytotoxicity, accumulation and alteration in protein expression in drug-selective and stably transfected ABCG2-overexpressing cells. Our results demonstrate that compound 7d could reverse ABCG2-mediated MDR by inhibiting the function of the transporter as opposed to alteration in protein levels.

## MATERIALS AND METHODS

### CHEMICALS

[<sup>3</sup>H]-mitoxantrone (4 Ci/mmol) was supplied by Moravek Biochemicals (Brea, CA). The monoclonal antibodies BXP-34 (used against ABCG2) were purchased from Signet Laboratories (Dedham, MA). The anti-actin monoclonal antibody (SC-8432) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The FTC was synthesized by the Thomas McCloud Developmental Therapeutics Program, Natural Products Extraction Laboratory, NCI, NIH (Bethesda, MD). Mitoxantrone, SN-38 and cisplatin were purchased from Tocris Bioscience (Ellisville, MO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and other chemicals were purchased from Sigma Chemical (St. Louis, MO). Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin/streptomycin and trypsin 0.25% were obtained from Hyclone Thermo Scientific (Logan, UT).

### CELL LINES AND CELL CULTURE

HEK293 cells were transfected with either the empty pcDNA3.1 vector or the pcDNA3.1 vector containing the full length ABCG2 with amino acid 482 coding either arginine (R), glycine (G), or threonine (T) and cultured in medium with G418 at 2 mg/ml [Robey et al., 2003; Robey et al., 2008]. Stably transfected HEK293/pcDNA3.1, wild-type ABCG2-482-R2, mutant ABCG2-482-G2 and mutant ABCG2-482-T7 cells were obtained from Drs. Susan. E. Bates and Robert W. Robey (NIH, MD). H460 is a human non-small cell lung cancer cell line and the resistant subline H460/MX20 were established with an additional 20 nM of mitoxantrone [Robey et al., 2001]. DMEM culture medium supplemented with 10% FBS and 1% Penicillin/Streptomycin was used to culture all of the cell lines under 5% CO<sub>2</sub> at 37°C as described previously [Yang et al., 2013; Zhang et al., 2014].

### THE SYNTHESIS OF 2-TRIFLUOROMETHYL-2-HYDROXYPROPIONAMIDE DERIVATIVES

The synthesis of 3-chloro-*N*-phenyl-4-(3,3,3-trifluoro-2-hydroxy-2-methylpropan amido) benzamide derivatives 7a-7o is outlined in Figure 1. 3,3,3-trifluoro-2-methyl-2-((trimethylsilyl)oxy) propanoyl chloride (compound 3) was prepared according to the reported procedures. However, when 1,3-Bis(trimethylsilyl)urea was not enough, a byproduct 3-chloro-1,1,1-trifluoro-2-methyl-3-oxopropan-2-yl,3,3-trifluoro-2-methyl-2-((trimethylsilyl)oxy) propanoate (compound 4) was synthesized. Another important intermediate 4-amino-3-chloro-*N*-(2-hydroxyphenyl)benzamide was prepared according to the reported procedures with slight modification [Zhichkin et al., 2008]. Briefly, 4-amino-3-

chlorobenzoic acid was simultaneously protected and activated with Vilsmeier reagent and then coupled with aniline to give 4-amino-3-chloro-*N*-(2-hydroxyphenyl)benzamide (compound 5) in good yield. Compound 5 reacted with compound 3 to give compound 7a-7i, while reacted with compound 4 to give compound 7j and 7k. 4-Amino-3-chlorobenzoic acid was easily transformed into 4-amino-3-chlorobenzoate (compound 6) in good yield. Then compounds 7l-7o were prepared according to the modified reported procedures using compound 6 and compound 3 [Bebernitz et al., 2000].

## CELL PROLIFERATION ASSAY

Cells were harvested with trypsin and resuspended in a concentration of  $6 \times 10^3$  cells/well. All cell lines were seeded evenly into 96 well plates with 180  $\mu$ l/well. Reversal agents (20  $\mu$ l/well) were added 1 h before adding different concentrations of chemotherapeutic drugs (20  $\mu$ l/well). After 72 h of incubation, each well received 20  $\mu$ l of MTT solution (4 mg/ml). The plates were then incubated for another 4 h to permit viable cells to convert the yellow colored MTT into dark-blue formazan crystals. Subsequently, the medium was discarded. In order to dissolve the formazan crystals, 100  $\mu$ l of DMSO was added into each well. An OPSYS Microplate reader from DYNEX Technologies, Inc. (Chantilly, VA) was used to measure absorbance at 570 nm. The degree of reversal of MDR was determined by dividing the IC<sub>50</sub> (concentrations required to inhibit growth by 50%) for the drug resistant cells by the IC<sub>50</sub> of the substrate in parental cells. The IC<sub>50</sub> values were calculated from survival curves using the Bliss method [Shi et al., 2006].

## PREPARATION OF CELL LYSATES AND WESTERN BLOT ANALYSIS

Cells were dissolved for 20 min on ice with a radioimmunoprecipitation assay (RIPA) buffer (PBS with 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 100 mg/ml p-aminophenylmethylsulfonyl fluoride) followed by centrifugation (12,000 rpm, 4°C for 20 min) to prepare the cell lysates. Equal amounts of total cell lysates (20  $\mu$ g protein) were resolved by sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. After incubation in 5% milk dissolved in TBST buffer (10 mM tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% tween 20) for 1 h at room temperature, the PVDF membranes were immunoblotted overnight with monoclonal antibody BXP-34 against ABCG2 at 1:500 dilution or Actin at 1:1000 dilution as an internal loading control. The PVDF membrane was then incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 dilution) for another 2 h. The protein-antibody complex was detected by an enhanced chemiluminescence detection system (Amersham, NJ).

## [<sup>3</sup>H]-MITOXANTRONE ACCUMULATION ASSAY

Equal amounts of HEK293/pcDNA3.1 or ABCG2-482-R2, ABCG2-482-G2 and, ABCG2-482-T7 cells ( $5 \times 10^6$ ) were preincubated at 37°C in DMEM (10% FBS) with and without compound 7d (5  $\mu$ M and 10  $\mu$ M) for 2 h. Subsequently, cells were incubated with 0.1  $\mu$ M [<sup>3</sup>H]-mitoxantrone for another 2 h with or without compound 7d. The medium was removed after 2 h and the cells were rinsed three times with cold PBS. A 200  $\mu$ l lysis buffer (pH 7.4, containing 1% Triton X-100 and 0.2% SDS) was added afterwards. A liquid scintillation analyzer from Packard Instrument Company, Inc. (Downers Grove, IL) was

used to measure the radioactivity. FTC (2.5  $\mu\text{M}$ ) was used as a positive control for ABCG2 reversal.

### **[<sup>3</sup>H]-MITOXANTRONE EFFLUX ASSAY**

Cells were exposed to the same procedure as stated in the drug accumulation experiment and then incubated in fresh medium at 37°C at various times (0, 30, 60, and 120 min) in the presence or absence of the inhibitor (compound 7d or FTC at 10 or 2.5  $\mu\text{M}$ , respectively). After washing three times with ice-cold PBS, the cells were lysed using 200  $\mu\text{l}$  lysis buffer and transferred to scintillation vials. Each sample was placed in scintillation fluid and radioactivity was measured in a liquid scintillation analyzer from Packard Instrument Company, Inc. (Downers Grove, IL).

### **STATISTICAL ANALYSIS**

All experiments were repeated at least three times and the differences were determined using the two-tailed student's t-test (Microsoft Excel 2010) and statistical significance was determined at  $p < 0.05$ .

## **RESULTS**

### **CYTOTOXICITY OF COMPOUNDS 7a-o IN ABCG2-OVEREXPRESSING CELL LINES**

Cytotoxicity assay was performed after treating different cell lines with increasing concentrations of compounds 7a-7o to determine the effect of compounds 7a-7o on cell viability. It was shown that the non-toxic concentration of 7a-7o was more than 30  $\mu\text{M}$  in HEK293/pcDNA3.1 and ABCG2 transfected cell lines, as well as in the lung cancer cell line H460 and the ABCG2 resistant subline H460/MX20. The cell viability of compound 7d in H460 and H460/MX20 cells, and HEK293/pcDNA3.1 and ABCG2 transfected cell lines are shown in Figure 2A and 2B, respectively.

### **SCREENING OF CHEMO-RESISTANCE REVERSAL ACTIVITIES OF COMPOUNDS 7a-7o ON ABCG2-OVEREXPRESSING CELL LINES**

A screening test was performed in H460 and its drug resistant subline H460/MX20, using mitoxantrone as the substrate for ABCG2 transporter. Compounds 7a-7o (10  $\mu\text{M}$ ) or positive control inhibitor of ABCG2 FTC (2.5  $\mu\text{M}$ ) was added. In the drug resistant subline H460/MX20, the resistance was 94.3 folds towards mitoxantrone compared to the parental H460 cell line. After adding 10  $\mu\text{M}$  of compound 7a-7o, the resistance folds decreased significantly, especially with compounds 7d, 7e and 7i. Compound 7d, 7e and 7i at 10  $\mu\text{M}$  significantly decreased the resistance to mitoxantrone to 6.5, 5.2 and 8.3 resistance folds, respectively (Table 1). Based on these results, we chose compound 7d in our following experiments.

### **THE EFFECT OF COMPOUND 7d ON REVERSAL OF ABCG2-MEDIATED MDR IN DRUG-SELECTIVE AND STABLY TRANSFECTED ABCG2-OVEREXPRESSING CELL LINES**

A concentration-dependent chemo-resistance reversal study was performed on the drug resistant subline H460/MX20 and its parental cell line H460 using different chemotherapeutic

drugs with various concentration of compound 7d (2.5  $\mu$ M, 5  $\mu$ M and 10  $\mu$ M) or FTC (2.5  $\mu$ M). The results are shown in Table 2. In parental cell line H460, the absence or presence of different concentrations of compound 7d did not alter the IC<sub>50</sub> values of chemotherapeutic drugs mitoxantrone and SN-38, probably because H460 did not express ABCG2 protein abundantly (Figure 5). In the drug resistant subline H460/MX20, the resistance folds were about 86.2 folds towards mitoxantrone and 185.2 folds towards SN-38 compared to the H460 cells [Kuang et al., 2012]. After adding compound 7d at 2.5  $\mu$ M, drug resistance decreased significantly to 22.0 folds towards mitoxantrone and 17.5 folds towards SN-38. Compound 7d at 5  $\mu$ M further decreased the drug resistance to 20.8 folds towards mitoxantrone and 8.1 folds towards SN-38. After pretreating cells with 10  $\mu$ M of compound 7d, the resistance to mitoxantrone significantly decreased to 6.3 folds towards mitoxantrone and 5.4 folds towards SN-38. FTC, the positive control inhibitor of ABCG2, at 2.5  $\mu$ M decreased the drug resistance folds to 4.8 folds towards mitoxantrone and 5.0 folds towards SN-38 which was comparable with compound 7d. H460/MX20 cells does not confer resistance to cisplatin, which is not a substrate of ABCG2 (Table 2). From Figure 3A and 3B, it can be observed that curves shifted to the left after co-incubation of anticancer drug with compound 7d at 2.5  $\mu$ M, 5  $\mu$ M and 10  $\mu$ M in H460/MX20 subline, while in Figure 3C compound 7d did not shift the cell viability curves for cisplatin.

To minimize various factors that may contribute to drug resistance in cancer cell lines, a chemo-resistance reversal study in HEK293/pcDNA3.1 and ABCG2 stably transfected cell lines ABCG2-482-R2, ABCG2-482-G2, ABCG2-482-T7 was carried out. Table 3 indicates that ABCG2 transfected cell lines are resistant to ABCG2 substrates mitoxantrone and SN-38. Compound 7d at 2.5  $\mu$ M, 5  $\mu$ M and 10  $\mu$ M enhanced the sensitivity of ABCG2 overexpressing cell lines to chemotherapeutic drugs mitoxantrone and SN-38 while it did not change the IC<sub>50</sub> values of empty vector transfected cell line HEK293/pcDNA3.1. These results are consistent with the drug resistant cancer cell line H460/MX20 and its parental cell line H460. The curves in Figure 4 showed that after co-incubating anticancer drug with compound 7d, the curves shifted to the left in the presence of the substrates mitoxantrone and SN-38 in ABCG2 transfected cell lines ABCG2-482-R2, ABCG2-482-G2, ABCG2-482-T7. Cisplatin was used as a negative control substrate of ABCG2 (Table 3 and Figure 4).

### THE EFFECT OF COMPOUND 7d ON THE EXPRESSION LEVELS OF ABCG2 PROTEIN

To determine whether down-regulating the protein levels of ABCG2 or inhibiting the function of ABCG2 caused the MDR reversal, Western blotting was performed. After incubating ABCG2-overexpressing subline H460/MX20 with compound 7d (10  $\mu$ M) at different time points (0, 24, 48, 72 h), the protein levels of ABCG2 were determined. Figure 5A clearly indicates that H460/MX20 expressed a much higher level of ABCG2 protein than that in the parental cell line H460, which did not change until 72 h. Similar results were obtained in the ABCG2-transfected cell lines as shown in Figure 5B.

### COMPOUND 7d INCREASES THE INTRACELLULAR ACCUMULATION OF [<sup>3</sup>H]-MITOXANTRONE IN CELLS OVEREXPRESSING ABCG2

In order to understand whether compound 7d sensitizes cells to chemotherapeutic drugs by inhibiting the function of ABCG2, drug accumulation assay was performed. HEK293/pcDNA3.1 and ABCG2-transfected cells were incubated with [<sup>3</sup>H]-mitoxantrone with or without compound 7d or FTC at different concentrations for 2 h. Compound 7d (5 μM, 10 μM) significantly increased the intracellular concentrations of [<sup>3</sup>H]-mitoxantrone in ABCG2-transfected cells to the levels similar to those seen with FTC at 2.5 μM. However, compound 7d or FTC did not significantly influence the intracellular accumulation in HEK293/pcDNA3.1 cells (Fig. 6). These results suggest that the increased intracellular levels of [<sup>3</sup>H]-mitoxantrone in ABCG2 overexpressing cells might be due to the inhibitory effect of compound 7d on drug efflux function of ABCG2 transporter.

### COMPOUND 7d DECREASES THE EFFLUX OF [<sup>3</sup>H]-MITOXANTRONE FROM CELLS OVEREXPRESSING ABCG2

To determine whether compound 7d inhibits the efflux of mitoxantrone, thus increasing the intracellular accumulation of [<sup>3</sup>H]-mitoxantrone, an efflux assay was performed. Figure 7 shows that the ABCG2 overexpressing cell line ABCG2-482-R2 extrudes a greater amount of [<sup>3</sup>H]-mitoxantrone at various time points (0, 30 min, 60 min, 120 min) than the parental cell line HEK293/pcDNA3.1. After preincubating with compound 7d (10 μM), the efflux of [<sup>3</sup>H]-mitoxantrone is significantly inhibited. Similar results were found with the group treated with FTC at 2.5 μM. In the parental cell line HEK293/pcDNA3.1, compound 7d and FTC did not alter the rate of efflux of [<sup>3</sup>H]-mitoxantrone.

## DISCUSSION

The ABC transporters have been shown to cause efflux of the anti-cancer drugs from cancer cells, thus playing a major role in MDR [Breier et al., 2013; Sun et al., 2012]. Consequently, ABC transporter inhibitors can be used in combination with traditional chemotherapeutic drugs to increase the effectiveness of treatment by increasing the intracellular concentration of anticancer drugs [Kathawala et al., 2013; Kwak et al., 2010; Shi et al., 2011]. Several preclinical and clinical studies have been carried out to evaluate the feasibility of this approach [Kathawala et al., 2014b; Kuppens et al., 2007; Morschhauser et al., 2007].

Anilide derivatives of 2-trifluoromethyl-2-hydroxypropionamide were originally found to possess anti-androgen activities [Morris et al., 1991]. It was reported that some members of the derivatives act as potassium channel openers, possessing undesirable hypotensive activity [Grant et al., 1994; Ohnmacht et al., 1996]. Moreover, these compounds can also act as potent inhibitors of pyruvate dehydrogenase kinase *in vitro* and *in vivo* [Bebornitz et al., 2000]. To our knowledge, this is the first report of the effect of 2-trifluoromethyl-2-hydroxypropionamide derivatives on ABCG2-mediated MDR. The results showed that 2-trifluoromethyl-2-hydroxypropionamide derivatives can be used at an acceptably safe concentrations up to 30 μM. These results suggested that compound 7d can specifically target ABCG2 which could possibly lower the side effects of 2-trifluoromethyl-2-hydroxypropionamide derivatives.

The wild-type ABCG2-482-R2, mutant ABCG2-482-G2 and mutant ABCG2-282-T7 cells shows different responses to ABCG2 inhibitors as well as different resistant folds to ABCG2 substrates. According to previous reports, mutant type ABCG2 showed strong resistance to anthracyclines, doxorubicin, rhodamine but little resistance to methotrexate; whereas wild-type ABCG2 showed weak resistance to anthracycline and rhodamine but high resistance to methotrexate [Allen et al., 2002; Volk et al., 2002]. Three-dimensional structure analysis showed that ABCG2 substrate recognition sites are located in transmembrane domain and position 482 is located in the central lumen of transmembrane domain, which explained why the change of arginine at 482 can affect the substrate spectrum of ABCG2 [Cai et al., 2010]. It is known that FTC can block both wild type and mutant type ABCG2, therefore it was used as a positive control of ABCG2 reversal agent. Our results showed compound 7d could sensitize both wild-type ABCG2-482-R2 and mutant ABCG2 transfected cell lines to chemotherapeutic drugs in a concentration-independent manner, which was comparable to FTC. Thus, we conclude that compound 7d could possibly be developed as a more useful inhibitor in the clinics.

Compound 7d could reverse ABCG2-mediated MDR by either downregulating the protein expression of ABCG2 or inhibiting the function of ABCG2. In order to determine the effect of compound 7d on the protein level of ABCG2, Western blotting was performed after incubating the cells with compound 7d for different lengths of time. Western blotting results indicated that compound 7d did not alter the expression levels of ABCG2 protein up to 72 h in both drug selected ABCG2-overexpressing subline H460/MX20 and ABCG2 stably transfected HEK293 cell lines.

In order to look into whether compound 7d could inhibit the function of ABCG2, accumulation assay was performed on HEK293/pcDNA3.1 and ABCG2-overexpressing cell lines. The accumulation results indicated that in ABCG2 overexpressing cell lines, the intracellular concentration of [<sup>3</sup>H]-mitoxantrone increased significantly in presence of compound 7d with minimal increase in HEK293/pcDNA3.1 cells. Furthermore, the efflux assay showed that compound 7d significantly reduced the efflux of [<sup>3</sup>H]-mitoxantrone at different time points, thus indicating that increasing accumulation of [<sup>3</sup>H]-mitoxantrone inside the cells is due to reduced efflux activity. Overall, these results suggested that compound 7d can reverse ABCG2-mediated resistance to chemotherapeutic drugs by reducing the efflux and increasing the intracellular concentration of cancer chemotherapeutic drugs as opposed to decreasing the protein level of ABCG2.

Collectively, our study reported that compound 7d at safe concentrations could reverse ABCG2-mediated MDR to the ABCG2 chemotherapeutic substrate drugs in a concentration-dependent manner. These results warrant a preclinical investigation of compound 7d and its development as a potential ABCG2 inhibitor to surmount ABCG2-mediated MDR in clinics. Given that ABCG2 is an established cancer stem cell marker and clinically important transporter, we believe these results will provide a novel direction for the development of ABCG2 reversal agents.



## Acknowledgments

**CONTRACT GRANT SPONSOR:** 1) National Institutes of Health; 2) St. John's University Research Seed Grant.

**CONTRACT GRANT NUMBER:** 1) 1R15CA143701; 2) 579-1110-7002.

We thank Drs. Susan E. Bates and Robert W. Robey (NIH, MD) for providing FTC, H460, H460/MX20, HEK293/pDNA3.1 and ABCG2-transfected cell lines.

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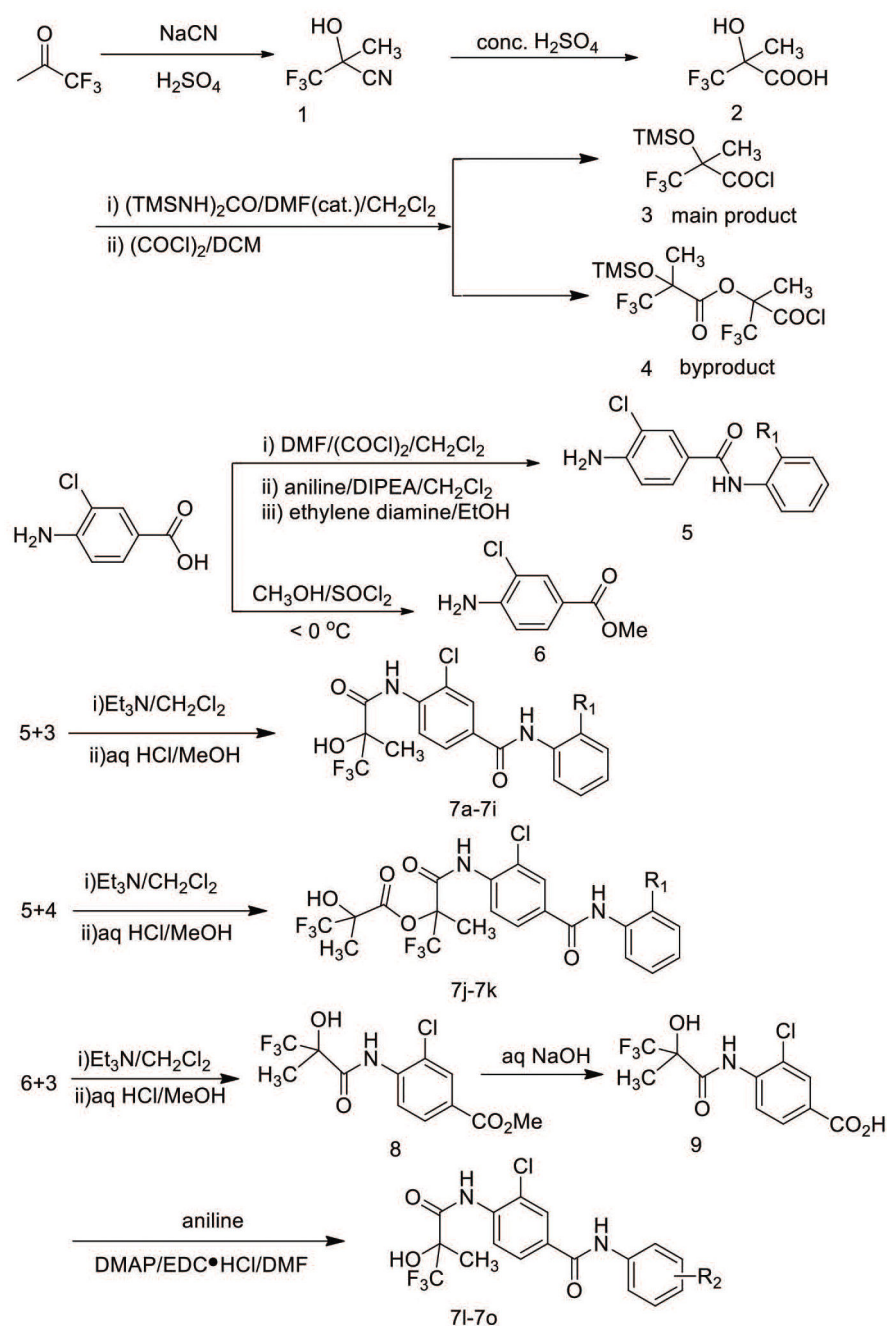
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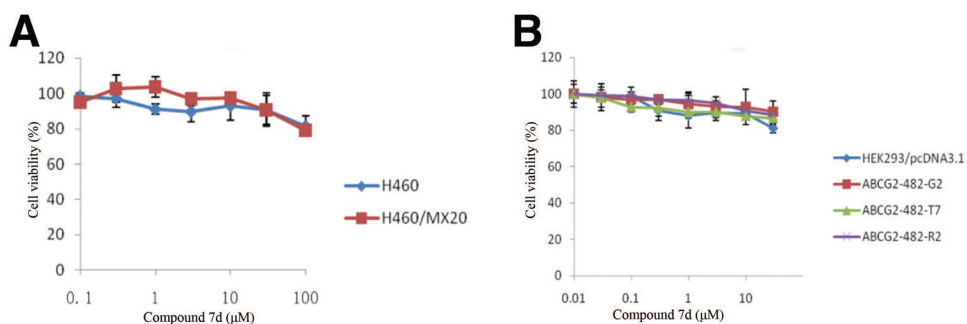
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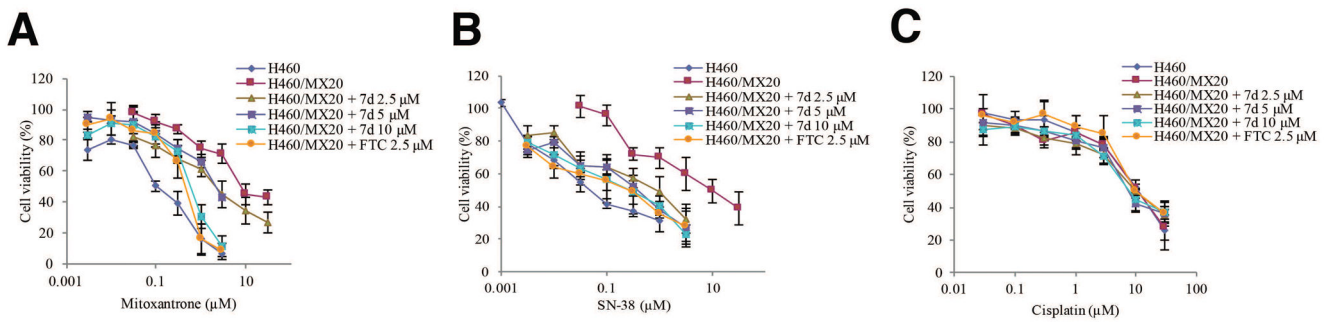
**Figure 1. Synthesis of 3-chloro-N-phenyl-4-(3,3,3-trifluoro-2-hydroxy-2-methylpropanamido)benzamide derivatives 7a-7o**

Outline of scheme of 2-trifluoromethyl-2-hydroxypropanamide derivatives. Synthesis was carried out according to reported procedures as described in the “Materials and methods” section.



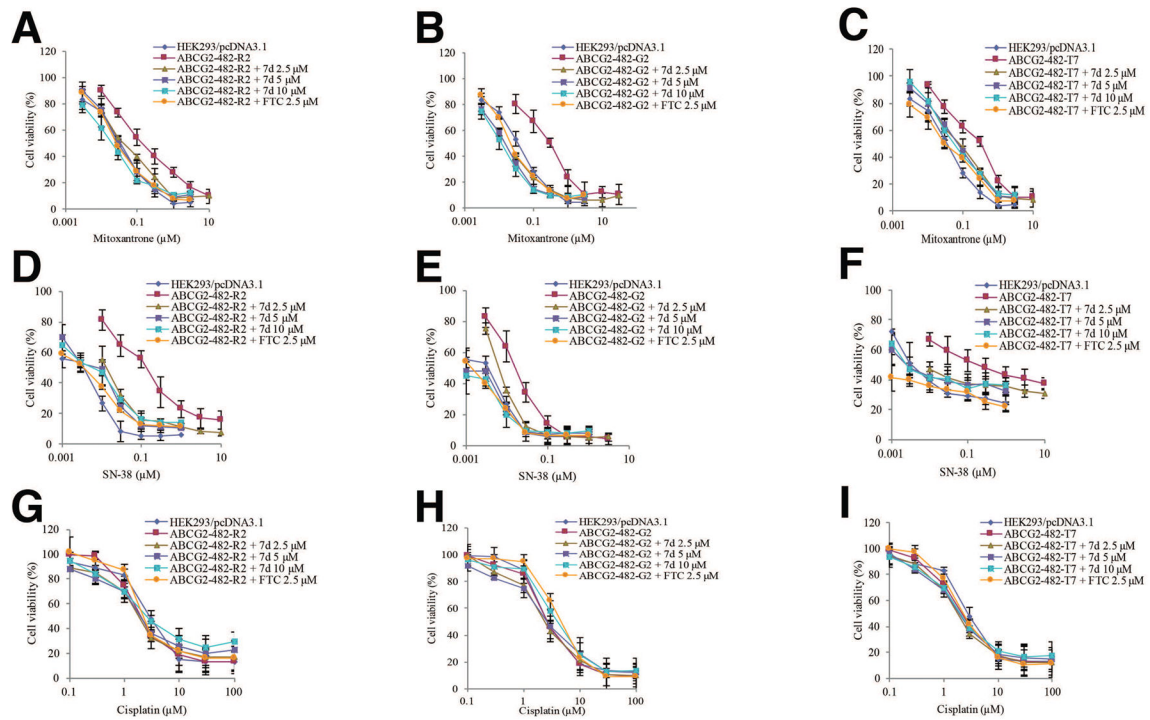
**Figure 2. The cytotoxicity curves of compound 7d in different cell lines**

The cell viability curve of compound 7d in H460 and drug resistant subline H460/MX20 (A), the cell viability curve of compound 7d in HEK293/pcDNA3.1, ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 cells (B). Cytotoxicity was determined by MTT assay as described in the “Material and methods” section. Points with error bars represent the mean  $\pm$  standard error. The above figures are representative of three independent experiments, each done in triplicates.



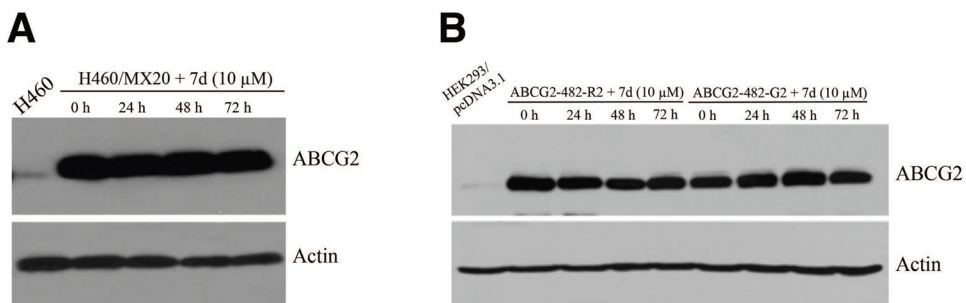
**Figure 3. The effect of compound 7d on the sensitivity of drug resistant subline H460/MX20 to anticancer drugs**

H460 cells and H460/MX20 cells were seeded in 96-well plates 24 h before compound 7d was added. After 1 h preincubation of compound 7d, mitoxantrone (A), SN-38 (B), or cisplatin (C) was added and cells were further incubated for 72 h. Cell viability was measured by the MTT assay as described in the “Material and methods” section. Points with error bars represent the mean  $\pm$  standard error. The above figures are representative of three independent experiments, each done in triplicates.

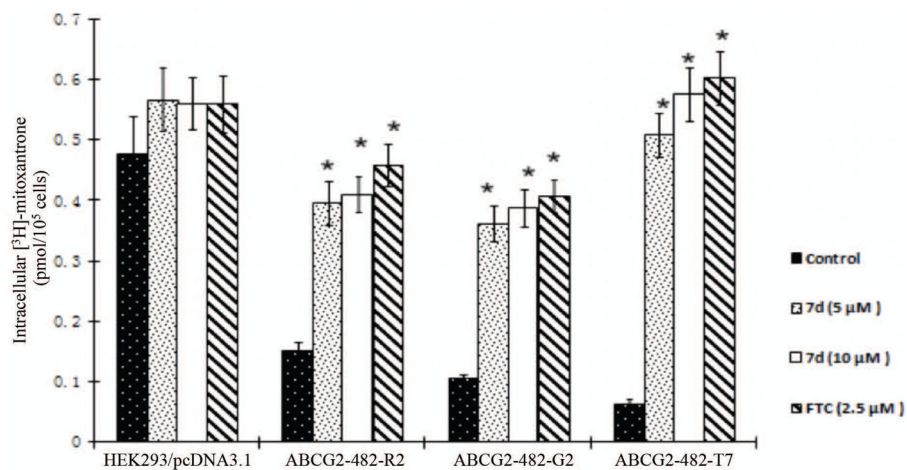


**Figure 4. The effect of compound 7d on the sensitivity of ABCG2 transfected cell lines to anticancer drugs**  
 HEK293/pCDNA3.1 cells and ABCG2 transfected cell lines ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 cells were seeded in 96-well plates for 24 h before compound 7d was added. After 1 h preincubation of 7d, mitoxantrone was added in ABCG2-482-R2 group (A), ABCG2-482-G2 group (B), ABCG2-482-T7 group (C); SN-38 was added in ABCG2-482-R2 group (D), ABCG2-482-G2 group (E), ABCG2-482-T7 group (F); cisplatin was added in ABCG2-482-R2 group (G), ABCG2-482-G2 group (H), ABCG2-482-T7 group (I) and cells were incubated for 72 h. Cell viability was measured by the MTT assay as described in the “Material and methods” section. Points with error bars represent the mean  $\pm$  standard error. The above figures are representative of three independent experiments, each done in triplicates.

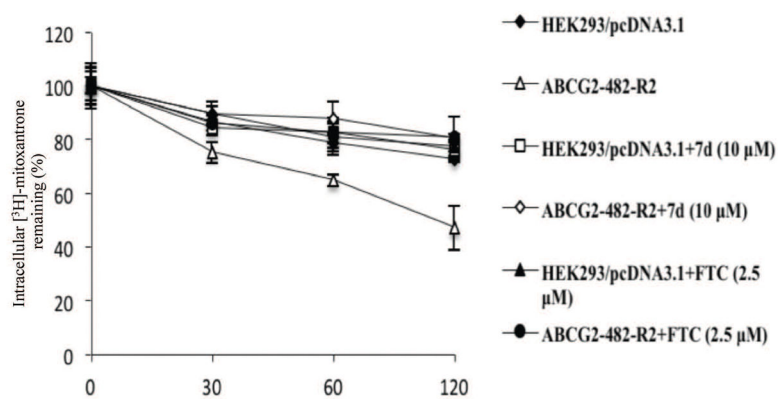




**Figure 5. The effect of compound 7d on the expression of ABCG2 protein**  
 Cells were treated with compound 7d (10 μM) for 72 h. Western blot experiments for ABCG2 were performed on H460 and H460/MX20 cell lysates (A), and HEK293/pCDNA3.1, ABCG2-482-R2 and ABCG2-482-G2 transfected cell lysates (B) as described in “Materials and methods” section.



**Figure 6. The effect of compound 7d on the accumulation of [<sup>3</sup>H]-mitoxantrone**  
 The accumulation [<sup>3</sup>H]-mitoxantrone in empty vector transfected HEK293/pcDNA3.1, ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 cells with or without compound 7d treatment. Columns are the mean of triplicate determinations. \*,  $p < 0.05$  versus the control group.



**Figure 7. The effect of compound 7d on the efflux of [<sup>3</sup>H]-mitoxantrone**  
The effect of compound 7d at 10 μM on retention of [<sup>3</sup>H]-mitoxantrone over a period of time in HEK293/pcDNA3.1 and ABCG2-482-R2. Data points represent the means ± SD.

**Table 1**

The effect of compounds 7a-7o at 10  $\mu$ M on ABCG2-mediated drug resistance in H460 and H460/MX20 cell lines.

Drugs	IC <sub>50</sub> $\pm$ SD <sup>a</sup> (nM) (Resistance fold)	
	H460	H460/MX20
<b>Mitoxantrone</b>	108.4 $\pm$ 27.0 (1.0) <sup>b</sup>	10234.6 $\pm$ 391.2 (94.3)
+compound 7a 10 $\mu$ M	267.9 $\pm$ 65.2 (2.5)	1683.1 $\pm$ 142.2 (15.5) *
+compound 7b 10 $\mu$ M	40.4 $\pm$ 12.8 (0.4)	3998.6 $\pm$ 687.5 (36.9) *
+compound 7c 10 $\mu$ M	95.5 $\pm$ 10.4 (0.9)	1032.6 $\pm$ 554.1 (9.5) *
+compound 7d 10 $\mu$ M	84.1 $\pm$ 15.0 (0.8)	706.1 $\pm$ 183.0 (6.5) *
+compound 7e 10 $\mu$ M	172.8 $\pm$ 14.0 (1.6)	562.8 $\pm$ 45.1 (5.2) *
+compound 7f 10 $\mu$ M	66.4 $\pm$ 10.9 (0.6)	1461.8 $\pm$ 148.2 (13.5) *
+compound 7g 10 $\mu$ M	62.9 $\pm$ 4.2 (0.6)	1179.2 $\pm$ 329.0 (10.9) *
+compound 7h 10 $\mu$ M	53.3 $\pm$ 2.1 (0.5)	1224.3 $\pm$ 498.1 (11.3) *
+compound 7i 10 $\mu$ M	80.0 $\pm$ 16.3 (0.7)	894.8 $\pm$ 55.4 (8.3) *
+compound 7j 10 $\mu$ M	165.2 $\pm$ 18.2 (1.5)	1363.4 $\pm$ 268.3 (12.6) *
+compound 7k 10 $\mu$ M	98.1 $\pm$ 7.9 (0.9)	1880.6 $\pm$ 107.0 (17.3) *
+compound 7l 10 $\mu$ M	93.1 $\pm$ 7.7 (0.9)	3265.6 $\pm$ 423.1 (30.1) *
+compound 7m 10 $\mu$ M	110.6 $\pm$ 11.7 (1.0)	5152.8 $\pm$ 645.8 (47.4) *
+compound 7n 10 $\mu$ M	101.6 $\pm$ 14.5 (0.9)	5452.9 $\pm$ 630.7 (50.3) *
+compound 7o 10 $\mu$ M	120.1 $\pm$ 23.7(1.1)	4261.4 $\pm$ 543.7 (39.3) *
+FTC 2.5 $\mu$ M	83.2 $\pm$ 12.6 (0.8)	583.6 $\pm$ 89.4 (5.4) *

<sup>a</sup>Values represent mean  $\pm$  SD of at least three independent experiments, each performed in triplicate.

<sup>b</sup>Resistance fold was calculated by dividing the IC<sub>50</sub> values of mitoxantrone in H460/MX20 cells in the absence or presence of reversal agents by the IC<sub>50</sub> values of substrates of H460 cell line.

\* :  $p < 0.05$

**Table 2**

Compound 7d effectively sensitizes drug selective ABCG2-overexpressing cancer cells to the substrate anticancer drugs

Drugs	IC <sub>50</sub> ± SD <sup>a</sup> (nM) (Resistance fold)	
	H460	H460/MX20
<b>Mitoxantrone</b>	108.5 ± 18.9 (1.0) <sup>b</sup>	9353.2 ± 876.8 (86.2)
+compound 7d 2.5 μM	98.5 ± 9.5 (0.9)	2390.0 ± 517.3 (22.0) *
+compound 7d 5 μM	74.9 ± 16.0 (0.7)	2253.3 ± 432.1 (20.8) *
+compound 7d 10 μM	87.5 ± 6.6 (0.8)	680.9 ± 256.1 (6.3) *
+FTC 2.5 μM	86.8 ± 8.7 (0.8)	525.4 ± 120.4 (4.8) *
<b>SN-38</b>	52.4 ± 5.6 (1.0) <sup>b</sup>	9702.8 ± 1008.4 (185.2)
+compound 7d 2.5 μM	55.4 ± 7.3 (1.1)	914.6 ± 83.9 (17.5) *
+compound 7d 5 μM	41.2 ± 13.2 (0.8)	426.5 ± 52.6 (8.1) *
+compound 7d 10 μM	46.2 ± 8.7(0.9)	281.7 ± 49.3 (5.4) *
+FTC 2.5 μM	40.6 ± 9.4(0.8)	264.1 ± 28.0 (5.0) *
<b>Cisplatin</b>	9608.9 ± 733.1 (1.0) <sup>b</sup>	10356.6 ± 1065.2 (1.1)
+compound 7d 2.5 μM	8917.6 ± 681.7 (0.9)	9339.4 ± 729.4 (1.0)
+compound 7d 5 μM	10323.4 ± 1087.8 (1.1)	9162.6 ± 821.6 (1.0)
+compound 7d 10 μM	8172.9 ± 765.5 (0.9)	8251.4 ± 743.5 (0.9)
+FTC 2.5 μM	11568.4 ± 980.1 (1.2)	10767.2 ± 850.4 (1.1)

<sup>a</sup>Values represent mean ± SD of at least three independent experiments, each performed in triplicate.

<sup>b</sup>Resistance fold was calculated by dividing the IC<sub>50</sub> values of mitoxantrone, SN-38, or cisplatin of H460/MX20 cells in the absence or presence of reversal agents by the IC<sub>50</sub> values of substrates of H460 cell line.

\* :  $p < 0.05$

**Table 3**

Compound 7d effectively sensitizes stably transfected ABCG2-overexpressing cells to the substrate anticancer drugs

Drugs	IC <sub>50</sub> ±SD <sup>a</sup> (nM) (Resistance fold)			
	HEK293/pcDNA3.1	ABCG2-482-R2	ABCG2-482-G2	ABCG2-482-T7
<b>Mitoxantrone</b>	37.8 ± 2.3 (1.0) <sup>b</sup>	158.9 ± 37.2 (4.2)	309.3 ± 12.0 (8.2)	299.8 ± 62.6 (7.9)
+compound 7d 2.5 μM	37.6 ± 6.1 (1.0)	39.4 ± 5.9 (1.0) *	23.6 ± 2.0 (0.6) *	60.1 ± 8.2 (1.6) *
+compound 7d 5 μM	38.4 ± 4.7 (1.0)	34.3 ± 6.2 (0.9) *	18.9 ± 2.5 (0.5) *	81.2 ± 13.8 (2.1) *
+compound 7d 10 μM	38.0 ± 9.5 (1.0)	20.8 ± 3.0 (0.6) *	17.4 ± 3.9 (0.5) *	73.7 ± 6.5 (1.9) *
+FTC 2.5 μM	30.4 ± 2.2 (0.8)	22.0 ± 8.9 (0.6) *	21.5 ± 3.0 (0.6) *	40.5 ± 4.9 (1.1) *
<b>SN-38</b>	3.3 ± 0.7 (1.0) <sup>b</sup>	41.6 ± 4.3 (12.6)	179.7 ± 17.2 (54.5)	269.3 ± 51.8 (81.7)
+compound 7d 2.5 μM	2.2 ± 0.5 (1.0)	7.3 ± 1.5 (2.2) *	21.4 ± 2.5 (6.5) *	12.0 ± 1.1 (3.6) *
+compound 7d 5 μM	2.0 ± 0.6 (0.6)	0.8 ± 0.2 (0.25) *	8.2 ± 1.3 (2.5) *	6.5 ± 1.2 (2.0) *
+compound 7d 10 μM	1.7 ± 0.6 (0.5)	0.8 ± 0.3 (0.26) *	11.0 ± 1.6 (3.3) *	4.8 ± 0.8 (1.5) *
+FTC 2.5 μM	2.8 ± 0.5 (0.8)	1.0 ± 0.5 (0.3) *	5.9 ± 0.7 (1.8) *	5.4 ± 0.3 (1.7) *
<b>Cisplatin</b>	3068.2 ± 253.4 (1.0) <sup>b</sup>	2359.0 ± 247.5 (0.8)	2931.2 ± 308.6 (1.0)	2650.3 ± 324.1 (0.9)
+compound 7d 2.5 μM	2614.7 ± 543.6 (0.9)	2512.8 ± 485.1 (0.8)	3051.6 ± 577.7 (1.0)	2117.9 ± 643.7 (0.7)
+compound 7d 5 μM	2823.6 ± 991.7 (0.9)	2340.9 ± 342.9 (0.8)	3107.4 ± 266.1 (1.0)	2342.9 ± 203.5 (0.8)
+compound 7d 10 μM	2635.6 ± 250.7 (0.9)	2778.6 ± 159.3 (0.9)	2606.6 ± 344.7 (0.8)	2602.4 ± 250.0 (0.8)
+FTC 2.5 μM	2211.3 ± 543.9 (0.7)	2516.8 ± 163.9 (0.8)	2911.2 ± 259.1 (0.9)	2566.7 ± 253.0 (0.8)

<sup>a</sup>Values represent mean ± SD of at least three independent experiments, each performed in triplicate.

<sup>b</sup>Resistance fold was calculated by dividing the IC<sub>50</sub> values of mitoxantrone, SN-38, or cisplatin of ABCG2 overexpressing cell lines in the absence or presence of reversal agents by the IC<sub>50</sub> values of substrates of HEK293/pcDNA3.1 cell line.

\*:  $P < 0.05$