# Apoptozole Modulates ABCG2-Mediated Multidrug Resistance in Lung Cancer

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**Abstract:** Lung cancer is a widespread malignant tumor with a highmortality rate, often difficult to treat effectively due to chemoresistance. Given the close association between ABCG2 overexpression and cancer multidrug resistance, overcoming the issue of MDR caused by ABCG2 is still a challenge. In this study, we found that the small molecule apoptozole effectively suppresses the transport activity of ABCG2, thus mitigating multidrug resistance in lung cancer cells, overexpressing ABCG2, and improving the therapeutic effects of chemotherapy drugs mitoxantrone and topotecan. Additionally, apoptozole does not affect the expression level of ABCG2 protein. Molecular docking studies have demonstrated that apoptozole can firmly bind to the binding pocket of ABCG2. Our data present that apoptozole is an ABCG2 inhibitor and modulates ABCG2-mediated multidrug resistance in lung cancer.

**Keywords:** Lung cancer, Multidrug Resistance, ABCG2, Apoptozole, Chemotherapy Sensitivity, Molecular Docking.

## 1. INTRODUCTION

Generally, we refer to the phenomenon where cancer cells develop resistance to a variety of anticancer drugs with different structures and functions as multidrug resistance (MDR), thereby reducing the effectiveness of chemotherapy and adversely affecting the survival of cancer patients [1]. A significant factor contributing to cancer multidrug resistance (MDR) is the overexpression of ABCG2 in cancer cells, which enables them to expel anticancer drugs regardless of the concentration gradient [2]. ABCG2 is a transmembrane protein located on the cell membrane and is a member of the ATP-binding cassette (ABC) transporter family. By enhancing efflux activity, its overexpression lowers the intracellular concentration ofchemotherapy drugs, which in turn impacts the therapeutic outcomes [3, 4]. Chemotherapy drugs such as mitoxantrone, afatinib, osimertinib, the camptothecin derivative 9-aminocamptothecin, topotecan, irinotecan, and SN-38 are reported as the substratesof ABCG2 [5-8]. Therefore, overcoming MDR induced by the overexpression of ABCG2 has become a research hotspot.

Considering ABCG2's significant function in MDR, effective inhibitors of ABCG2 can assist in reversing MDR. A multitude of ABCG2 inhibitors have been discovered by numerous research teams, such as AZ32 [9], AZ-628 [10], febuxostat [11], GSK2606414 [12], KU55933 [13], NVP-TAE684 [14], OTS964 [15], VKIN-1 [16]. To date, the existing ABCG2 inhibitors still failed in clinical applications due to the limitations including efficiency and toxicities, etc, which further highlights the urgent need to develop novel efficient ABCG2 inhibitors. During the development of ABCG2 inhibitors, we found that the small molecule apoptozole (termed as Apoptosis Activator VII) might be a novel ABCG2 inhibitor. This study focused on the effects of apoptozole on ABCG2 activity and analyzed its role in ABCG2-mediated MDR in lung cancer.

## 2. MATERIALS AND METHODS

### 2.1. Reagents and Cell Culture

Topotecan HCl (#119413-54-6), lapatinib (#A8218), rhodamine 123 (#62669-70-9), mitoxantrone (#70476-

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82-3), and 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) (#298-93-1) were ordered from Jiangsu Aikang Biomedical R&D (Jiangsu, China), APExBIO (Texas, USA), D&B Biotech(Shanghai, China), Yuanye Biotech (Shanghai, China) , Sigma-Aldrich (Shanghai, China), respectively. Anti-vinculin (#sc-25336) and anti-ABCG2 antibody (#RLT0053) were purchased from Santa Cruz (Santa Cruz, California, USA) and Ruiying Biotech (Wuxi, China) respectively. The ABCG2-overexpressing human lung cancer MDR cell line H460/TPT10 was generated by stepwise selection of H460 in increasing concentrations of topotecanup to 10 μM [17]. Both H460 and H460/TPT10 cell lines were incubated in a culture chamber at 37°C with a $CO_2$ concentration of 5% in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum of CellMax (#SA301.02.V) from Lanzhou Minhai Bio-Engineering Co (Lanzhou, China).

### 2.2. Cytotoxicity Assay

Cells (3,000 cells per well) were seeded into 96-well plates and allowed to grow overnight. On the second day, various concentrations of agents were added. After 72 hours, MTT was added to each well at a final concentration of 0.5 mg/mL, and the plates were incubated in the cell culture incubator for 4 hours. The liquid in the 96-well plates was then carefully aspirated, and 50 μL dimethyl sulfoxide was added in each well to dissolve the formazan crystals formed by the cells. Absorbance was measured at 570 nm using a BioTek Synergy H1 microplate reader (Agilent Technologies, Santa Clara, CA, USA). The survival curves were calculated as previously described [18].

### 2.3. Drug Accumulationassay

Cells were seeded in 12-well plates at a density of 200,000 cells per well and 1 mL of complete culture medium was added. On the second day, cells were incubated in the specified concentrations of apoptozole or lapatinib for 60 minutes. Subsequently, cells were co-incubated with 10 μM mitoxantrone or10 μM rhodamine 123 for 2 hours. Afterward, cells were collected and analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) as previously reported [13].

### 2.4. Western Blot

Cells were suspended in the lysis buffer (1% nonidet P 40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate,0.03% aprotinin,10 ng/mL phenylmethanesulfonyl fluoride, and 1 μM sodium orthovanadate) for 30 minutes at 4°C. The protein supernatant was collected after centrifuged at the speed of 13,600 rpm for 10 minutes, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Then membranes were orderly incubated with the blocking buffer, primary antibodies, and secondary antibodies. Signals were detected with an Analytik Jena ChemiDoc XRS imaging system(Thuringia, Germany).

### 2.5. Docking Analysis

The structure of human ABCG2 protein was obtained from the Protein Data Bank (PDB ID: 6vxi). Molecular docking was performed using AutoDock to simulate the binding conformation of apoptozole with ABCG2. The binding conformations of apoptozole with ABCG2 were visualized using PyMOL.

### 2.6. Statistical Analysis

Statistical analysis of was conducted using GraphPad Prism 9. Student's t-test was employed to analyze data from different groups, with each dataset derived from at least three independent experimental repetitions. Significant differences between groups were indicated as $*p< 0.05$ and $**p< 0.01$.

## 3. RESULTS

### 3.1. Apoptozole Modulates ABCG2-Mediated Multidrug Resistance in Lung Cancer Cells

In order to investigate the effects of apoptozole (chemical structure shown in Figure **1A**) on ABCG2-mediated MDR in lung cancer, we first evaluate the cytotoxicity of apoptozole in H460 and H460/TPT10 cells. According to the MTT results, apoptozole showed no significant cytotoxicity to either cell line up to 100 μM (Figure **1B**). Based on this finding, we evaluated the sensitizing effects of apoptozole at the concentrations of 10 μM and 30 μM. We treated H460 and H460/TPT10 cells with 10 μM and 30 μM apoptozole in combination with ABCG2 substrates mitoxantrone and topotecan at various concentration gradients. The results were shown in Figure **1C-D**. Apoptozole dose-dependently increased the chemosensitivity of mitoxantrone and topotecan only in H460/TPT10 cells, not in H460 cells. The known ABCG2 inhibitor lapatinib showed a stronger effect than apoptozole. This finding indicates that apoptozole modulates ABCG2-mediated multidrug resistance in lung cancer.

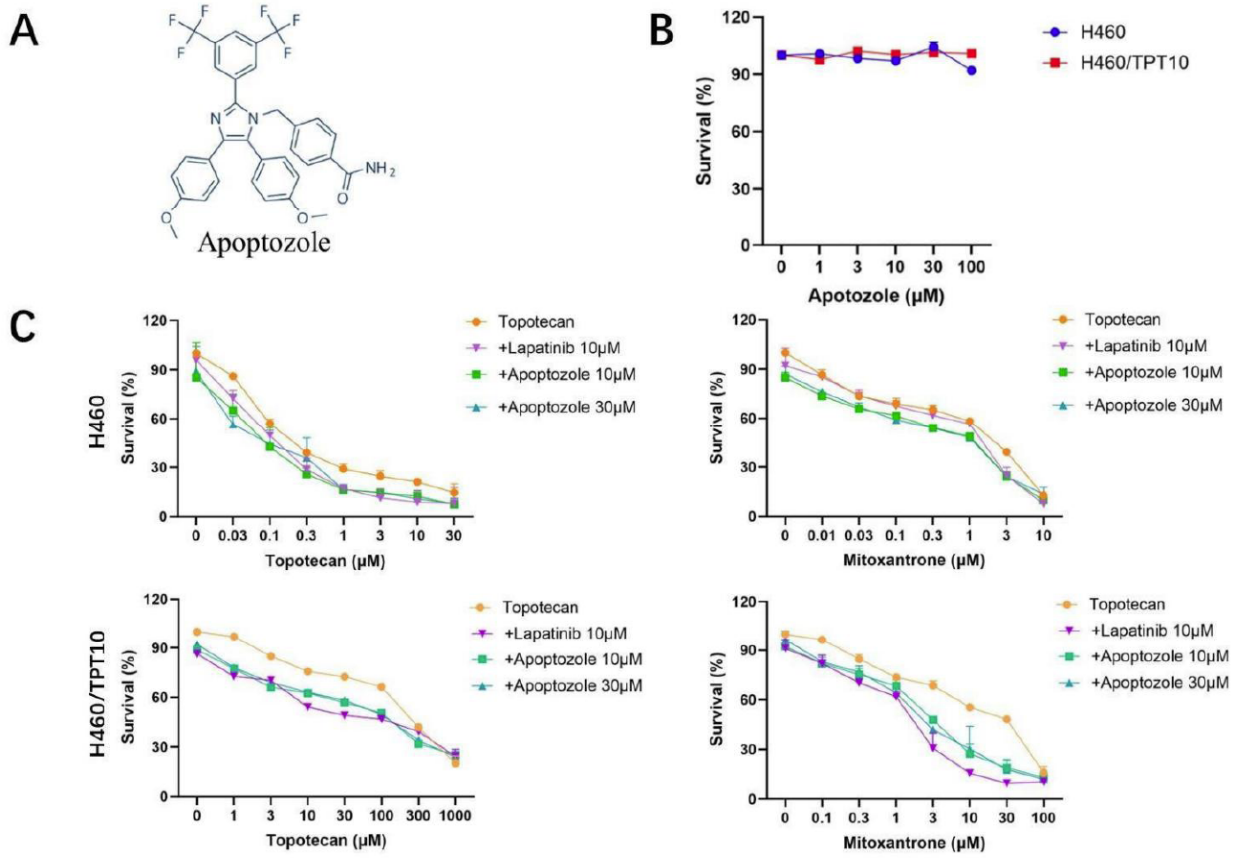


**Figure 1:** Apoptozole modulates ABCG2-mediated multidrug resistance in lung cancer cells. (**A**) Chemical structure of apoptozole. The cells were treated with the indicated agents for 72 hours and examined via MTT assay. The representative cell survival curves are shown in (**B–D**).

### 3.2. Apoptozole Augments Mitoxantrone and Rhodamine 123 Accumulation in ABCG2-Overexpressing Lung Cancer Cells

Given that ABCG2 functions as an efflux pump on the cell membrane, we hypothesized that the reversal MDR effect of Apoptozole might be achieved by inhibiting the efflux pump activity of ABCG2. We conducted drug accumulation experiments of ABCG2 substrates mitoxantrone and rhodamine 123 in H460 and H460/TPT10cells. According to the results (Figures **2A-B**), the intracellular levels of mitoxantrone and rhodamine 123 in H460/TPT10 cells were lower than those in H460 cells. Apoptozole dose-dependently increased the intracellular levels of mitoxantrone and rhodamine 123 only in H460/TPT10 cells, not in H460 cells. These results suggest that apoptozole augments mitoxantrone and rhodamine 123 accumulation in ABCG2-overexpressing lung cancer cells.

### 3.3. Apoptozole has No Effect on ABCG2 Protein Expression and its Binding Pattern with ABCG2

To explore whether apoptozole affects ABCG2 protein expression, H460/TPT10cells were treated with 30 μM apoptozole for 3 and 72 hours. Then, ABCG2 protein expression was detected by Western blot. Apoptozole has no effect on ABCG2 protein expression (Figure **3A**). We further conducted molecular docking to understand the interaction between apoptozole and ABCG2. As shown in Figures **3B-C**, apoptozole is located within the binding pocket of ABCG2 and engaged in hydrophobic interactions with hydrophobic amino acid residues on ABCG2, including Val-401, Thr-435, Phe-439, Val-442, Thr-538, Leu-539, Thr-542, Ile-543, and Val-546. Apoptozole forms the intermolecular hydrogen bonds with Phe-439 and Thr-542 and an F-O bond with Thr-435, thereby obtaining a stable binding conformation.

## 4. DISCUSSION

Apoptozole is originally developed as an apoptosis activator in multiple cancer cells by binding to heat shock protein 70 (HSP70) and heat shock cognate protein (HSC70) with dissociation constants of 0.14 and 0.21μM [19]. Further study has showed that apoptozole suppresses HSC70 ATPase activity through binding to its ATPase domain to enhance

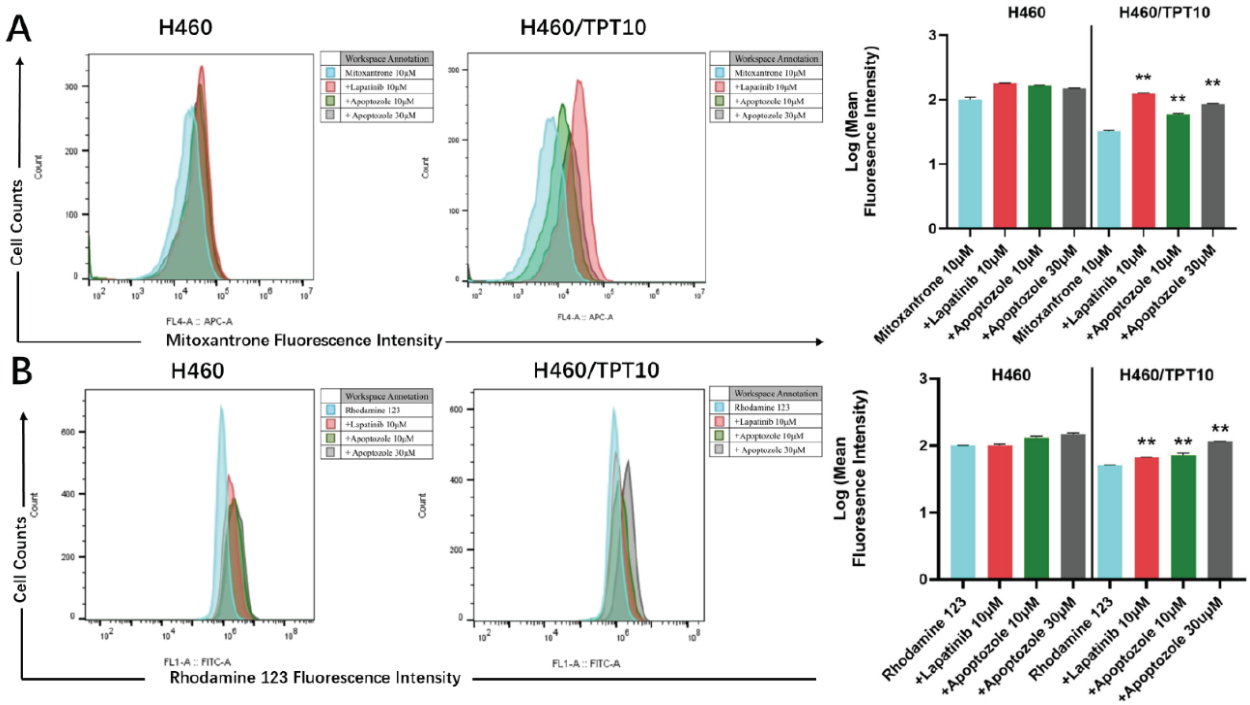


**Figure 2:** Apoptozole augments mitoxantrone and rhodamine 123 accumulation in ABCG2-overexpressing lung cancer cells. Cells were pre-incubated with apoptozole or lapatinib for 1 hour, then incubated with 10 μM (**A**) mitoxantrone, (**B**) rhodamine 123 for 2 hours, followed by flow cytometry detection. ** $p$< 0.01 compared to the corresponding control groups (n = 3).

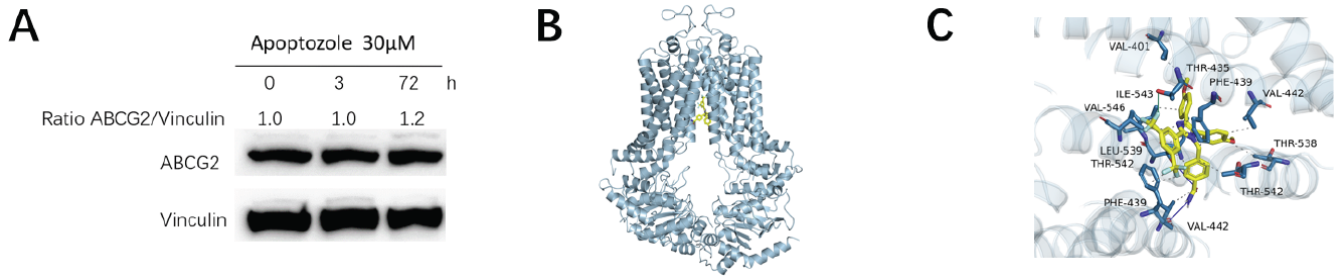


**Figure 3:** Apoptozole has no effect on ABCG2 protein expression and its binding pattern with ABCG2. (**A**) Western blot detection of ABCG2 expression levels in TOP10 cells treated with 30 μM apoptozole at the indicated time points. (**B**) The optimal docking position of apoptozole within the human ABCG2 binding pocket generated by AutoDock Vina (shown in yellow). (**C**) An enlarged view of the highlighted area showing the interaction of apoptozole with ABCG2 residues Val-401,Thr-435, Phe-439, Val-442, Thr-538,Leu-539, Thr-542, Ile-543, and Val-546.

membrane trafficking of mutant cystic fibrosis transmembrane conductance regulator and its chloride channel activity in cystic fibrosis cells [20]. Apoptozole does not bind to other types of HSPs, including HSP40, HSP60, and HSP90, and induces cancer cell death through caspase-dependent apoptosis by blocking the binding of APAF-1 with HSP70 [21]. Apoptozole interrupts the interaction of clathrin with Hsc70, thereby resulting in the accumulation of transferrin in clathrin-coated vesicles and inhibition of release of free $Fe^{3+}$ from clathrin-coated vesicles during transferrin receptor-mediated endocytosis [22]. Apoptozole strengthens immune responses to protein antigens by increasing the release of Th1 and Th2-type cytokines and inducing the generation of antibodies with anelevated IgG2c/IgG1 ratio [23]. Furthermore, apoptozole can prevent HSP70 from binding to NLRP3, decrease NLRP3 degradation, and modulate NLRP3-induced microglial pyroptosis, thus facilitating the development of postoperative cognitive dysfunction [24].

In this study, we observed that apoptozole could inhibit thetransportactivity of ABCG2, enhance the chemosensitivity of mitoxantrone and topotecan in ABCG2-overexpressing lung cancer cells, thereby antagonizing ABCG2-mediated MDR in lung cancer. Additionally, apoptozole does not alter the expression levels of ABCG2protein, but firmly binds to the binding pocket of ABCG2. However, the effects of apoptozole

on ABCG2-mediated MDR in lung cancer *in vivo* and the indirectly influence ABCG2 or cell survival by apoptozole's known interactions with HSP70/HSC70 need to be further investigated. Interestingly, a glutathione-responsive nanoplatform containing cisplatin prodrug, apoptozole and D-peptide was designed to suppress the energy metabolism and lysosomal activity of cancer cells, thus keeping cancer cells vulnerable to cisplatin and decreasing the risk of cancer metastasis [25]. Another nanodiamond-based nanoplatform containing doxorubicin，hyaluronic acid，protamine sulphate, the photosensitizer indocyanine green and apoptozole was constructed to enhance mild-temperature photothermal/chemo combination therapy against triple negative breast cancer [26].

## 5. CONCLUSION

In summary, our data present that apoptozole is an ABCG2 inhibitor and modulates ABCG2-mediated multidrug resistance in lung cancer.

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## CONFLICT OF INTEREST

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