Vemurafenib (PLX4032, Zelboraf®), a BRAF Inhibitor, Modulates ABCB1-, ABCG2-, and ABCC10-Mediated Multidrug Resistance

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Abstract: In this study, we examined the in vitro effects of vemurafenib, a specific inhibitor of V600E mutated BRAF enzyme, on the response of cells overexpressing the ATP binding cassette (ABC) efflux transporters ABCG2, ABCB1, ABCC1 and ABCC10. Vemurafenib, at 5 μM and 20 μM, produced a significant concentration-dependent increase in the cytotoxicity of paclitaxel in cells overexpressing ABCB1 and ABCC10 and mitoxantrone in cells overexpressing ABCG2. Vemurafenib also significantly enhanced the accumulation of paclitaxel in cell lines overexpressing ABCB1 and ABCC10. Vemurafenib significantly increased the intracellular accumulation of mitoxantrone in cells overexpressing ABCG2. In contrast, vemurafenib did not significantly alter the sensitivity of ABCC1 overexpressing HEK/ABCC1 cells to vincristine. Finally, as determined by Western blotting, vemurafenib (20 μM) did not significantly alter the expression of ABCG2, ABCC10 or ABCB1. Thus, vemurafenib most likely reverses multidrug resistance by altering the transport function of these aforementioned ABC transporters, as opposed to affecting the expression of ABC proteins. The docking analysis of vemurafenib with the ABCB1 homology model also suggested that vemurafenib binds to the ABCB1 and ABCG2 drug binding site. These findings suggest that combination of specific inhibitors like vemurafenib with chemotherapeutic drugs may be used to overcome multidrug resistance in cells that overexpress ABCB1, ABCC10 and/or ABCG2 transporters.

Keywords: Vemurafenib, ABCC10, ABCG2, MDR.

INTRODUCTION

Metastatic melanoma has led to a steady increase in cancer-related deaths in the United States over the last decade [1]. It has been reported that more than 90% of melanomas express the BRAF V600E mutation (substitution of glutamic acid for valine at amino acid 600). The V600E mutation on the B-Raf gene and activation of the gene is instrumental in transducing downstream signaling in the mitogen-activated protein kinase (MAPK) pathway [2]. Thus, there have been extensive studies targeting the V600E mutated B-Raf to develop effective melanoma therapies [3, 4].

Recently, vemurafenib (PLX 4032, Zelboraf®), a highly selective B-Raf kinase inhibitor, has been approved by the US-Food and Drug Administration for treatment of metastatic and unresectable BRAF V600E+ melanomas [5, 6]. Vemurafenib improves the overall survival of patients by inhibiting the Raf-Mek-Erk signaling pathway which inhibits tumor growth [3, 7, 8]. Unfortunately, due to drug resistance initiated by various signaling molecules such as NRAS and PDGFRB, vemurafenib cannot be used to treat cancers that result from mutations in the B-Raf gene unrelated to V600E [9].

Over the last four decades, multidrug resistance (MDR) mediated by various mechanisms has posed several challenges in cancer treatment and resulted in chemotherapeutic failure [10]. Multidrug resistance occurs when cancer cells develop tolerance towards chemotherapeutic drugs via multiple mechanisms [11]. Numerous studies have shown that the ATP binding cassette (ABC) transporters, which belong to a super-family of transmembrane proteins, play a major role in mediating the active transport of chemotherapeutic drugs from cancer cells [12, 13]. The emergence of multidrug resistance in cancer is primarily mediated by overexpression of ABCB1, ABCG2 and ABCC1 members of the ABC super-family [14-16]. Recently, ABCC10, another ABC transporter belonging to sub-family C, has exhibited chemotherapeutic substrate
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Receptor tyrosine kinases (RTK) play a key role in cell signaling pathways and the resultant cross talk between different pathways makes them a target in the field of drug discovery and development. Recently, the FDA has approved several small molecule tyrosine kinase inhibitors (TKI) and a number of studies have elaborated on their potential as anti-cancer agents. Several research groups have reported that several pan-EGFR, Bcr-Abl, HER-2, VEGF and FGFR TKIs possess the ability to modulate ABC transporters and decrease MDR mediated resistance by these transporters [21]. TKIs, such as lapatinib, erlotinib, imatinib and nilotinib significantly reverse MDR in cells that overexpress ABCB1, ABCG2 and ABCC10 [22-27]. Therefore, TKIs are being extensively studied as potential modulators of MDR by inhibiting the energy-dependent efflux of the chemotherapeutic agents.

Sorafenib, a multikinase inhibitor targeting B-Raf, has been reported to modulate ABCB1- and ABCG2-mediated MDR [28, 29]. Another B-Raf inhibitor, vemurafenib, was recently approved for the treatment of metastatic melanoma in patients with BRAFV600E positive mutation [4]. Recently, it was reported that both Pgp and BCRP play an important role in the distribution of vemurafenib across the blood brain barrier [10]. In addition, the overexpression of ABCG2 has been identified a potential mechanism for resistance in BRAF (V600E) cancer by vemurafenib [30]. Therefore, in this study, using transfected cells, we conducted experiments to determine the interaction of vemurafenib with ABC transporters. We demonstrated that vemurafenib reverses MDR mediated by the overexpression of ABCB1-, ABCG2- and ABCC10- transporters.

2. MATERIALS AND METHODS

2.1. Materials

[³H]-paclitaxel and [³H]-MX were purchased from Moravek Biochemicals, Inc. (Brea, CA). Monoclonal antibodies BXP-21 (against ABCG2) and C-219 (against ABCB1) were acquired from Signet Laboratories, Inc. (Dedham, MA). Anti-actin monoclonal antibody (sc-8432) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Vemurafenib was provided by Selleck Chemicals (Houston, TX). Fumitremorgin C (FTC) was synthesized by Thomas McCloud Developmental Therapeutics Program, Natural Products Extraction Laboratory, NCI, NIH (Bethesda, MD) and it was a gift from Drs. Susan E Bates and Robert W. Robey. Mitoxantrone, doxorubicin, paclitaxel, docetaxel, verapamil, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell Lines and Cell Culture

HEK293/pDNA3.1, wild-type ABCG2-482-R2, mutants ABCG2-482-G2, and ABCG2-482-T7 cells were established by transfecting HEK293 with the empty pcDNA3.1 vector or pcDNA3.1 vector containing the full length ABCG2, coding either arginine (R), glycine (G), or threonine (T) at amino acid 482, respectively, and were cultured in medium with 2 mg/ml of G418 [34]. H460 and H460/MX20 cells were also kindly provided by Drs. Susan Bates and Robert Robey (NCI, NIH, Bethesda). Similarly, HEK293/pDNA3.1 cells were transfected with the ABCB1 gene to obtain HEK293/ABCB1 cells. ABCB1-transfected HEK/ABCB1 cell lines were kindly provided by Dr. Suresh Ambudkar (NCI, NIH, Bethesda). HEK293/ABCC10 cells were obtained by transfection of ABCC10 gene to HEK293/pDNA3.1 cells [35]. All of the cell lines were grown as adherent monolayers in flasks with DMEM culture medium (Hyclone Co., UT) supplemented with 10% bovine serum in a humidified incubator containing of 5% CO₂ at 37°C.

2.3. Preparation of Total Cell Lysates and Membrane Vesicles

Total cell lysates were prepared by harvesting the cells and rinsing twice with PBS. Cell extracts were prepared by incubating cells for 30 min with radioimmunoprecipitation assay (RIPA) buffer [1x PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 mg/ml leupeptin, 100 mg/ml p-aminophenylmethylsulfonyl fluoride(p-APMSF),and10 mg/ml aprotinin] with followed by centrifugation at 12,000xg at 4°C for 15 min. The supernatant containing total cell lysates was stored at -80°C until needed for experiments. The protein concentration was determined by bicinchoninic acid (BCA™)-based protein assay (Thermo Scientific, Rockford, IL).
2.4. Cell Cytotoxicity by MTT Assay

The MTT colorimetric assay was used to detect the sensitivity of cells to anticancer drugs. Cells were harvested with trypsin and resuspended in a final concentration of 5x10^3 cells/well for all cell lines. Cells were seeded evenly into 96-well multiplates (180 µl/well). For the reversal experiments, vemurafenib, verapamil, FTC, and cepharanthine (20 µl/well) were added, followed by different concentrations of the chemotherapeutic drugs (22 µl/well) into designated wells. After 96 h of incubation, 20 µl of MTT solution (4 mg/ml) was added to each well, and the plate was further incubated for 4 h, allowing viable cells to convert the yellow colored MTT into dark-blue formazan crystals. Subsequently, the medium was discarded, and 100 µl of dimethylsulfoxide (DMSO) was added into each well to dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPSYS Microplate Reader from DYNEX Technologies, Inc. (Chantilly, VA). The degree of resistance was calculated by dividing the IC50 (concentrations required to inhibit growth by 50%) for the MDR cells by that of the parental sensitive cells. The degree of the reversal of MDR was calculated by dividing the IC50 for cells with the anticancer drug in the absence of vemurafenib or other reversal compounds by that obtained in the presence of vemurafenib.

2.5. Western Blot Analysis

Equal amounts of total cell lysates (60 µg protein) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. After incubation in a blocking solution 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 membranes were immunoblotted overnight with primary monoclonal antibodies against either ABCB1 at 1:200 dilution or ABCG2 at 1:1000 dilution at 4 ºC, and were then incubated for 3 h at room temperature with horseradish peroxide (HRP)-conjugated secondary antibody (1:1000 dilution). The protein–antibody complex was detected by enhanced chemiluminescence detection system (Amersham, NJ). GAPDH was used as a housekeeping protein.

2.6. [3H]-Paclitaxel and [3H]-Mitoxantrone Accumulation

The effect of vemurafenib on the intracellular accumulation of paclitaxel in HEK293/pcDNA3.1 and HEK293/ABCB1 cells and HEK 293/pcDNA2.1 and HEK/ABCC10 was determined by measuring the intracellular accumulation of [3H]-paclitaxel in these cells. The intracellular accumulation of MX in ABCG2 was determined by measuring the intracellular accumulation of [3H]-MX in ABCG2 related cells. 1x10^5 cells were seeded in 6-well plates and pre-incubated with or without the inhibitor compounds for 96 h at 37°C. Intracellular drug accumulation was measured by incubating cells with 0.1 µM [3H]-paclitaxel or 0.1 µM [3H]-MX for 2 h in the presence or absence of the inhibitors at 37°C. The cells were washed three times with ice-cold PBS, typhsinized and lysed in 10 mM lysis buffer (pH 7.4, containing 1% Triton X-100 and 0.2% SDS). Each sample was placed in scintillation fluid and radioactivity was measured in a Packard TRI-CARB1 1900CA liquid scintillation analyzer from Packard Instrument Company, Inc. (Downers Grove, IL).

2.7. Molecular Modeling - ABCB1 and ABCG2

2.7.1. Ligand Structure Preparation

Vemurafenib structure was built using the fragment dictionary of Maestro v9.0 and energy minimized by Macromodel program v9.7 (Schrödinger, Inc., New York, NY, 2009) using the OPLSAA force field with the steepest descent followed by truncated Newton conjugate gradient protocol. The low-energy 3D structures of vemurafenib were generated by LigPrep v2.3 and the parameters were defined based on different protonation states at physiological pH±2, and all possible tautomers and ring conformations. The ligand structures obtained from the LigPrep v2.3 run were then used for generating 100 ligand conformations for each protonated structure using the default parameters of mixed torsional/low-mode sampling function. The conformations were filtered with a maximum relative energy difference of 5 kcal/mol to exclude redundant conformers. The output conformational search (Csearch) file containing at most 100 unique conformers of vemurafenib were used as input for docking simulations into each binding site of human ABCB1 and ABCG2.

2.7.2. Protein Structure Preparation

The X-ray crystal structure of mouse ABCB1 in apoprotein state (PDB ID: 3G5U), in complex with inhibitors QZ59-RRR (PDB ID: 3G6O), QZ59-SSS (PDB ID: 3G61) [31] and bacterial co-crystal structure of LmrA ATP-binding domain (PDB ID: 1MV5) as the template obtained from the RCSB Protein Data Bank were used to generate the homology model of human...
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ABCB1, based on our homology modeling protocol [32]. The refined human ABCB1 homology model was further used to generate different receptor grids for different sites (site 1-4) by selecting QZ59-RRR (site-1) and QZ59-SSS (site-2) bound ligands, all amino acid residues known to contribute to verapamil binding (site-3), two residues (Phe728 and Val982) known to be common to three previous sites (site-4) as previously reported by us [32]. Additionally to evaluate the possibility of vemurafenib interaction at the ATP-binding site, bound ATP ligand was selected for grid generation and ensuing docking simulation of vemurafenib at ATP-binding site. A homology model of ABCG2 was built based on the mouse apoprotein (PDB ID: 3G5U) [31] as the template and has been generated and a PDB file of a human homology model dimer form file was given to us by Rosenberg et al. [36]. A homology model of ABCG2 was energy minimized before initiating the preparation of the grid. To identify the drug binding sites on the ABCG2 homology model, we generated various grids based on the following residues as centroids: Arg482 (grid 1), Asn629 (grid 2), Arg383 (grid 3) and Leu241 along with Gly83 (grid 4). The choice of these residues was based on their involvement in ABCG2 function as determined through mutational experiments [37]. The grid 1 generated using Arg482 as the centroid was found to have the best docking score; hence, the docking discussion was based on binding mode of vemurafenib at this site. Glide v5.0 docking protocol was followed with the default functions (Schrödinger, Inc., New York, NY, 2009). The top scoring vemurafenib conformation at Arg482 site of ABCG2 and site 1 of ABCB1 was used for graphical analysis. All computations were carried out on a Dell Precision 470n dual processor with the Linux OS (Red Hat Enterprise WS 4.0).

2.8. Statistical Analysis

All experiments were performed as triplicates and the differences were determined by using the Student’s t-test. The a priori level of statistical significance p < 0.05.

3. RESULTS

3.1. The Sensitivity of ABCB1-, ABCG2- and ABCC10-Overexpressing Cells to Anticancer Drugs is Increased in the Presence of Vemurafenib

The cell viability assay was performed to determine the effect of vemurafenib alone on MDR cells overexpressing ABCB1-, ABCG2- and ABCC10-transporters. Vemurafenib, at 5 μM, 10 μM and 20 μM, was studied as the optimum non-toxic concentration to modulate drug resistance in the reversal experiments. Vemurafenib, up to 20 μM, was relatively non-toxic as more than 90% cells surviving after incubation up to 72 h. (data not shown).

The effect of vemurafenib in combination with chemotherapeutic drugs was studied to determine if vemurafenib could significantly enhance the sensitivity of these cells to specific anticancer drugs. Vemurafenib (5 and 20 μM) produced a concentration-dependent decrease in the IC_{50} values of paclitaxel (Table 1), which is a substrate of ABCB1 in the HEK293/ABCB1 cells. Vemurafenib at 20 μM decreased the resistance from 114-fold to 34-fold in ABCB1 overexpressing HEK/ABCB1 cells. Verapamil, a known ABCB1

Table 1: The Effect of Vemurafenib on the ABCB1 Overexpressing Cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC_{50} ± SD (nM) (fold-resistance)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEK293/pcDNA3.1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>24.56 ± 3.10 (1.0)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>31.36 ± 2.94 (1.3)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>20.84 ± 3.98 (0.9)</td>
</tr>
<tr>
<td>+Verapamil 5 μM</td>
<td>27.89 ± 3.38 (1.1)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>978.97 ± 91.7 (1.0)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>947.18 ± 46.2 (1.0)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>936.21 ± 37.4 (0.9)</td>
</tr>
<tr>
<td>+Verapamil 5 μM</td>
<td>951.94 ± 72.2 (0.9)</td>
</tr>
</tbody>
</table>

*RResistance-fold was calculated from the IC_{50} value for either paclitaxel or cisplatin on HEK293/pcDNA3.1 cells in the presence of either vemurafenib or verapamil, or HEK293/ABCB1 cells with or without the vemurafenib or verapamil, divided by the IC_{50} values for paclitaxel and cisplatin of HEK293/pcDNA3.1 cells without vemurafenib or verapamil. Cell survival was determined by the MTT assay as described in "Materials and Methods". The (§) values in table represent the mean ± SD of at least three independent experiments performed in triplicate. Statistically, *, P < 0.05; **, P < 0.01, versus the control group (Student t-test).
inhibitor, at 5 μM, significantly sensitized the HEK293/ABCB1 cells to paclitaxel (Table 1). Vemurafenib (5 and 20 μM) concentration-dependently increased the sensitivity of ABC10 transfected HEK293/ABC10 cells to paclitaxel and docetaxel (Table 3). However, vemurafenib did not significantly alter the IC50 value of paclitaxel and docetaxel in the parental HEK293/pcDNA3.1 cells. Vemurafenib, at 20 μM, significantly reversed MDR in the ABC10 overexpressing cells that was comparable to cepharanthine (a known ABC10 inhibitor) at 2.5 μM (Table 2). Vemurafenib, at 5μM and 20μM, significantly decreased the IC50 values of ABCG2 substrates mitoxantrone from 134-fold to 64-fold and SN38 from 107-fold to 49-fold in ABCG2-overexpressing H460/MX20 cells (Table 3). However, vemurafenib did not alter the sensitivity of H460 lung cancer cells. Moreover, the reversal effect produced by vemurafenib in H460/MX20 cells was comparable to FTC, a known ABCG2 inhibitor. FTC, at 5 μM was able to completely reverse the resistance in ABCG2 overexpressing cells (Table 3). However, in H460 lung cancer cells, there were no significant changes in the IC50 values of either SN38 or MX in combination with vemurafenib (Table 3). In addition, vemurafenib did not significantly alter the IC50 values of cisplatin, which is not a substrate of ABCB1, ABCG2, or ABC10. Therefore, our results suggest that vemurafenib selectively sensitizes ABCB1-, ABC10 or ABCG2-overexpressing cells to their respective chemotherapeutic substrates.

3.2. Vemurafenib does not Alter the Expression of ABCB1, ABCG2 and ABC10

The reversal of ABC transporter mediated MDR can result from a decrease in the expression of ABCB1, ABCG2 or ABC10 protein levels. To determine the effect of vemurafenib on protein expression, ABCB1, ABCG2 and ABC10 overexpressing cells were incubated with 20 μM of vemurafenib for 24, 48, 72 and 96 h. The results indicated that vemurafenib did not significantly alter the protein expression levels in ABCB1, ABCG2 and ABC10 (data not shown). Therefore, vemurafenib’s reversal of MDR is not due to an alteration of ABCB1, ABCG2 or ABC10 protein expression.

3.3. Effect of Vemurafenib on the Intracellular Accumulation of [3H]-Paclitaxel in ABCB1 and ABC10 Overexpressing Cells and [3H]-MX in ABCG2 Overexpressing Cells

In order to elucidate the effect of vemurafenib on the function of ABCB1, ABC10 and ABCG2, the change in the accumulation of known chemotherapeutic substrates for ABCB1, ABC10 and ABCG2 transporters in cells overexpressing ABCB1, ABC10 and ABCG2 was determined. The change in intracellular levels of [3H]-paclitaxel, a known substrate of ABCB1, was measured in the presence or absence of vemurafenib in both HEK293/pcDNA3.1 and

Table 2: The Effect of Vemurafenib on the ABC10 Overexpressing Cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 ± SD3 (nM) (fold-resistance)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEK293/pcDNA3.1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>10.30 ± 0.30 (1.0)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>9.02 ± 0.43 (0.87)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>9.74 ± 0.54 (0.95)</td>
</tr>
<tr>
<td>+Cepharanthine 2.5 μM</td>
<td>9.61 ± 0.61 (0.93)</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>21.19± 1.31 (1.0)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>26.19 ± 4.69 (1.23)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>22.18 ± 3.81 (1.04)</td>
</tr>
<tr>
<td>+Cepharanthine 2.5 μM</td>
<td>19.12± 0.34 (0.9)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>947.71 ± 61.2 (1.0)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>899.58 ± 86.7 (0.9)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>886.43 ± 17.4 (0.9)</td>
</tr>
<tr>
<td>+Cepharanthine 2.5 μM</td>
<td>835.34 ± 75.2 (0.9)</td>
</tr>
</tbody>
</table>

aResistance-fold was calculated from the IC50 value for either vinblastine, paclitaxel, cisplatin or cepharanthis on HEK293/pcDNA3.1 cells in the presence of either vemurafenib or cepharanthis, or HEK293/ABC10 cells with or without the vemurafenib or cepharanthis, divided by the IC50 values for vinblastine, paclitaxel and cisplatin of HEK293/pcDNA3.1 cells without vemurafenib/cepharanthish. Cell survival was determined by the MTT assay as described in “Materials and Methods”. The § values in table represent the mean ± SD of at least three independent experiments performed in triplicate. Statistically, *, P < 0.05; **, P < 0.01, versus the control group (Student t-test).
HEK293/ABCB1 cells. The intracellular accumulation of [3H]-paclitaxel in ABCB1-overexpressing HEK293/ABCB1 cells was significantly lower compared to HEK293/pcDNA3.1 cells after 96 h of incubation (Figure 2). Vemurafenib, at 20 μM, produced a significant increase in the intracellular levels of [3H]-paclitaxel HEK293/ABCB1. The increase in intracellular paclitaxel accumulation in ABCB1 overexpressing cells was comparable to verapamil at 10 μM. However, the intracellular level of [3H]-paclitaxel in HEK293/pcDNA3.1 cells was not altered by either vemurafenib or verapamil (Figure 2). Additionally, the intracellular level of [3H]-MX was measured in cells overexpressing ABCG2 in the presence or absence of vemurafenib after 96 h of incubation (Figure 3). Vemurafenib produced a significant concentration-dependent increase in the intracellular levels of [3H]-MX in ABCG2 overexpressing H460/MX20 cells (Figure 3). However, there was no significant change in the intracellular levels of [3H]-MX in parental H460 cells incubated with vemurafenib or FTC (Figure 3). Further, similar results were obtained when ABCC10 overexpressing HEK293/ABCC10 cells showed a decrease in the accumulation of [3H]-paclitaxel after 96 h incubation compared to the HEK293/pcDNA3.1 cells. In the presence of vemurafenib at 20 μM, there was a significant increase in the intracellular levels of [3H]-paclitaxel which is a ABCC10 substrate. The results obtained in HEK293/ABCC10 cells incubated with 20 μM vemurafenib was comparable to cephapirin at 2.5 μM and the parental HEK293/pcDNA3.1 cells were unaffected by presence or absence of the inhibitors (Figure 4). The increase in intracellular accumulation of the specific substrates [3H]-paclitaxel in ABCB1 and ABCC10 overexpressing cells and [3H]-MX in the ABCG2 overexpressing cells in the presence of 20 μM vemurafenib suggests that vemurafenib inhibits the efflux function of ABCB1, ABCC10 and ABCG2 and thus plays a role in mediating MDR reversal.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 ± SD (nM) (fold-resistance)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantrone</td>
<td>11.94 ± 0.71 (1.0) 1598.37 ± 70.20 (133.8)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>10.59 ± 0.31 (0.9) 1433.69 ± 73.4 (120.0)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>8.13 ± 0.17 (0.7) 762.03 ± 96.62 (63.8)*</td>
</tr>
<tr>
<td>+FTC 5 μM</td>
<td>7.6 ± 0.21 (0.6) 54.85 ± 0.55 (4.6)*</td>
</tr>
<tr>
<td>SN38</td>
<td>74.02 ± 0.63 (1.0) 7888.14 ± 198.6 (106.56)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>66.49 ± 0.32 (0.9) 6759.22 ± 145.4 (91.34)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>55.57 ± 0.83 (0.8) 3656.45 ± 91.23 (49.4)**</td>
</tr>
<tr>
<td>+FTC 5 μM</td>
<td>46.74 ± 0.22 (0.6) 283.54 ± 1.49 (3.8)*</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1142.45 ± 145.3 (1.0) 1294.67 ± 158.42 (1.1)</td>
</tr>
<tr>
<td>+Vemurafenib 5 μM</td>
<td>1056.42 ± 94.21 (0.9) 1130.54 ± 109.27 (1.0)</td>
</tr>
<tr>
<td>+Vemurafenib 20 μM</td>
<td>1021.27 ± 102.65 (0.9) 1092.58 ± 148.93 (1.0)</td>
</tr>
<tr>
<td>+FTC 5 μM</td>
<td>1083.79 ± 110.22 (0.9) 1105.45 ± 183.67 (1.0)</td>
</tr>
</tbody>
</table>

*Resistance-fold was calculated from the IC50 value of either mitoxantrone, SN-38 or cisplatin on H460 cells in the presence of either vemurafenib or FTC, or H460/MX-20 cells with or without the vemurafenib or FTC, divided by the IC50 values for mitoxantrone, SN-38 and cisplatin of H460 cells without the vemurafenib or FTC. Cell survival was determined by the MTT assay as described in “Materials and Methods”. The (§) values in table represent the mean ± SD of at least three independent experiments performed in triplicate. Statistically **, P < 0.01, versus the control group (Student t-test).
Figure 2: The effect of vemurafenib on the accumulation of [3H]-paclitaxel in HEK293-pcDNA3.1 and HEK/ABCB1 cells. The intracellular accumulation of [3H]-paclitaxel was measured after incubation with or without vemurafenib and verapamil for 96 h at 37°C and then incubated with 0.1 μM [3H]-paclitaxel for another 2 h at 37°C.

Figure 3: The effect of vemurafenib on the accumulation of [3H]-mitoxantrone in H460 and H460/MX20 cells. The intracellular accumulation of [3H]-mitoxantrone was measured after incubation with or without vemurafenib or FTC for 96 h at 37°C and then incubated with 0.1 μM [3H]-mitoxantrone for another 2 h at 37°C.

[31]. To understand the binding mechanism of vemurafenib to the homology model of human ABCB1 at a molecular level, docking studies were performed on all of the possible binding sites [32]. The best docking score for the docked conformation of vemurafenib was found at site-1, indicating this site as most likely to bind vemurafenib. Therefore, the binding interaction of vemurafenib within the site 1 of ABCB1 is discussed here and shown in Figure 5.

One of the sulfonamide oxygen atoms of vemurafenib is involved in a hydrogen bond interaction with the Tyr307 (-NHSO2---HO-Tyr307, 1.7 Å) and
another oxygen atom is involved in an electrostatic interaction with Gln725 (-NHSO2--HNH-Gln725, 2.7 Å). The nitrogen atom of the pyridine ring interacts electrostatically with the side chain of Tyr953 (-N---HO-Tyr953, 3.4Å). Moreover, the chlorophenyl and pyrrolopyridine rings of vemurafenib are located within a hydrophobic pocket lined by the side chains of Met69, Phe72, Phe336, Phe732, Leu975, Phe978 and Val982. The difluorophenyl ring is stabilized by hydrophobic contacts with the side chains of Phe336, Val982 and Ala985. The propyl group of vemurafenib forms hydrophobic contacts with the side chain of Phe728, Ala729, Phe732 and Val982. Few residues like Phe336 and Val982 form hydrophobic contacts with all hydrophobic parts of vemurafenib. The culmination of these numerous interactions suggests that vemurafenib binds to the ABCB1 drug-binding site (site-1) with high affinity.

The Glide predicted docked model of vemurafenib at the Arg482 centroid-based grid of ABCG2 illustrated in Figure 5B. The difluorophenyl-propane-sulfonamide substituents of vemurafenib are stabilized through hydrophobic contacts with the side chains of F507, F511, L626, W627 and H630. The NH of the same sulfonamide function of vemurafenib is involved in two electrostatic interactions with the nitrogen atom present in the side chains of imidazole group two of H630, present in the dimer form of ABCG2 (NH---N-His630, 4.4 Å) and (NH---N-His630, 4.3 Å) may be responsible for the conformational lock of the dimer form of the ABCG2, which may lead to weaken the ABCG2 activity of vemurafenib. One of the fluorne atoms in the trifluoromethyl substituent may participate in an electrostatic interaction with the side chain of Asn629 (-CF3---H2NOC-Asn629, 2.8 Å), whereas the other fluorine atom may interact with the side chain of His630 (-CF3---HN-His630, 3.9 Å). As shown in Figure 5B, the 4-chlorophenyl ring of vemurafenib is stabilized through the hydrophobic side chains of M483, Y518, Y570 and I573. The NH of pyrrole group and Nitrogen atom of the pyridine ring are involved in two hydrogen bond formation with the side chains of S486 (-NH---OH-Ser486, 1.8 Å) and Y570 (-N---HO-Tyr570, 2.1 Å) respectively (Figure 5B). The favorable binding affinity of vemurafenib towards ABCG2, suggests that it may significantly modulate the ABCG2 transporter and its substrates.

4. DISCUSSION

Multidrug resistance has been a major challenge in the success of cancer chemotherapy and the overexpression of ABC transporters is an one of the major mechanisms for development of multidrug resistance. In this study, we examined the effect of vemurafenib on cells overexpressing ABC transporters. Our findings indicate that in vitro, vemurafenib inhibits the efflux of chemotherapeutic drugs from the cancer cells.
One of the major findings of this study was that vemurafenib significantly increased the sensitivity of established ABCB1 substrate in HEK/ABCB1 cells (Table 1) and ABCC10 substrates in HEK/ABCC10 cells (Table 2). However, vemurafenib (20 \( \mu \)M) did not sensitize the parental HEK293/pcDNA3.1 to the anticancer drugs used in this study (Tables 1 and 2). In addition to ABCB1 and ABCC10, vemurafenib (20 \( \mu \)M) increased the IC\(_{50}\) of ABCG2 substrates mitoxantrone and SN38 in ABCG2 overexpressing H460/MX20 cells (Table 3). We observed that vemurafenib-induced sensitization was specific to the ABC transporter overexpressing cells and the parental HEK293/pcDNA 3.1 and H460 cells were not significantly affected by 5 or 20 \( \mu \)M of vemurafenib. These findings suggest that based on the cell viability assay, vemurafenib significantly reverses ABCB1-, ABCG2- and ABCC10-mediated drug resistance and increases the sensitivity of these cells to their specific substrates.
Vemurafenib significantly enhanced the intracellular accumulation of $[^3H]$-MX in ABCG2 overexpressing H460/MX20 cells (Figure 3). Similarly, vemurafenib, in a concentration-dependent manner, significantly enhanced the intracellular accumulation of $[^3H]$-paclitaxel in ABCB1 overexpressing HEK293/ABCB1 cells (Figure 2). The results were consistent with the cell survival assay, suggesting that vemurafenib interacts synergistically with both ABCB1 and ABCG2 substrates and sensitizes the ABCB1- and ABCG2-mediated MDR cells to specific anticancer drugs. Similarly, in ABCC10 overexpressing HEK/ABCC10 cells, vemurafenib significantly increased the sensitivity of cells to anticancer drugs. The incubation of HEK/ABCC10 cells with vemurafenib (20 μM) for 96h, significantly increased the paclitaxel accumulation in HEK/ABCC10 cells. The increased accumulation of ABC transporter substrates post vemurafenib exposure confirms the ability of vemurafenib to reverse multidrug resistance and improve the cytotoxicity effect of current chemotherapeutic drugs. Our findings confirm the recent report highlighting the importance of ABCB1 and ABCG2 in preventing the penetration of vemurafenib across the blood brain barrier and their roles in limiting distribution of vemurafenib.

There are different mechanisms that can resensitize ABC transporter-overexpressing cells to anti-cancer drugs. Consequently, we determined the effect of vemurafenib on the regulation of the protein expression of ABC transporters. Vemurafenib did not significantly alter the protein expression of cells that overexpress ABCB1, ABCC10 and ABCG2 transporters (data not shown). These findings further confirm that vemurafenib is a functional modulator of ABC transporters and that this has no significant attenuation of the protein expression. Thus, the reversal effect of vemurafenib on ABCB1, ABCG2 and ABCC10 in MDR cells is most likely due to its inhibition of the efflux of chemotherapeutics.

Until now, attempts to obtain the co-crystal structures of human ABCB1 and ABCG2 have been unsuccessful due to their membrane bound nature and ensuing difficulties in isolation and purification. Hence, we used human ABCB1 and ABCG2 homology models. To identify the most appropriate binding site for vemurafenib, docking studies were performed on all possible binding sites of ABCB1 as described by Aller et al. [31] and referenced critical residues shown by site-directed mutagenesis experiments for ABCG2. After exploring the docking simulations of vemurafenib at all of these binding sites, the most favorable binding site was identified and the binding energy scores for vemurafenib against human ABCB1 and ABCG2 indicate high-affinity binding. Moreover, vemurafenib appears to exhibit all of the pharmacophoric features, such as hydrophobic groups and/or aromatic ring centers, hydrogen-bond acceptors and hydrogen-bond donor groups that have been described critical for binding at ABCB1 and ABCG2 transporters [33]. By understanding the nature of the molecular interaction gained through molecular modeling studies structural clues to obtain potent inhibitors of this targets may be revealed.

Our data clearly suggests an important role of vemurafenib in inhibiting the function of ABC transporters. Furthermore, it is highly likely that vemurafenib binds to ABCB1 and ABCG2 transporters. However, the MDR reversal potential of vemurafenib needs to be further established by studying the transport kinetics of most likely due to its vemurafenib and its effect on drug efflux mechanisms. In conclusion, vemurafenib, a clinically approved drug for treatment of BRAF$^{V600E}$ metastatic melanoma, reverses ABCB1-, ABCG2- and ABCC10-mediated MDR. Vemurafenib significantly increases the intracellular drug accumulation of chemotherapeutic substrates in ABCB1-, ABCG2- and ABCC10-overexpressing cells, but does not affect the expression of the ABC transporter proteins. Further studies are required to ascertain the interaction of vemurafenib with the ABC transporters. In addition, the determination of effect of vemurafenib in in vivo models (using tumor xenografts) would provide information on its potential use with other drugs for the treatment of drug resistance in cancer.

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CONFLICT OF INTEREST

None declared.

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