Sildenafil Enhances the Anticancer Activity of Paclitaxel in an ABCB1-Mediated Multidrug Resistance Xenograft Mouse Model

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Abstract: The overexpression of ATP-binding cassette (ABC) transporters can produce multidrug resistance (MDR) in cancer cells. Previous *in vitro* studies from our group reported that sildenafil significantly inhibits the efflux function of the ABCB1/P-glycoprotein transporter *in vitro*. This investigation examined the effect of sildenafil on the ABCB1 transporter-mediated MDR *in vivo*. A nude mouse ABCB1 overexpressing-xenograft model was used to examine the effect of sildenafil *in vivo*. The concentration of paclitaxel in tumors and plasma was analyzed using high performance liquid chromatography (HPLC). Sildenafil attenuated tumor growth synergistically, and this occurred without significant weight loss or other overt phenotypic changes. The action of sildenafil can be attributed to the inhibition of the ABCB1-mediated drug efflux, thereby increasing the concentration of paclitaxel in ABCB1-overexpressing tumors. The potentiation of the pharmacologic action of paclitaxel by sildenafil suggests that it may be useful in treating cancers that overexpress the ABCB1 transporter.

Keywords: ABCB1, multidrug resistance, paclitaxel, sildenafil.

INTRODUCTION

The development of drug resistance by cancer cells poses a major threat to the successful chemotherapy of cancer [1, 2]. One of the established mechanisms of multidrug resistance (MDR) is the increased expression of ATP binding cassette (ABC) transporters in tumor cells [3]. The ABC transporters are transmembrane proteins, comprised of 48 members, that are involved in transport of a wide variety of substrates, including ions, sugars, amino acids, vitamins, lipids and drugs [1, 4]. The overexpression of ABC transporters, such as ABCB1/P-glycoprotein (P-gp), ABCC/MRP subfamily (Multidrug Resistance Proteins) and ABCG2/BCRP/ MXR (Breast Cancer Resistance Protein, MitoXantrone Resistance protein,) have been implicated in the development of MDR [1, 3]. These ABC transporters can efflux a large number of structurally diverse anti-cancer drugs. The ABCB1 transporter is the most widely studied transporter and its overexpression is significantly correlated with MDR in tumor cells *in vitro* and *in vivo* [5, 6]. The ABCB1 transporter promotes the efflux of a wide spectrum of neutral and cationic hydrophobic drugs including vinca alkaloids, anthracyclines and taxanes. This aforementioned function of ABCB1 reduces the intracellular concentration of anticancer drugs below the cytotoxic level, thereby attenuating or even abolishing anticancer efficacy. Therefore, the blockade or inhibition of the ABCB1 transporter is a potential therapeutic strategy to surmount MDR in tumor cells. Indeed, three generation of ABCB1 inhibitors have been shown to potentiate the action of various anticancer drugs, sensitizing resistant cancer cells to specific anti-cancer drugs [6]. Recently, it was reported that the efflux function of ABCB1 transporters can be inhibited by protein tyrosine kinase inhibitors such as nilotinib and dasatinib [7]. Unfortunately, most of these compounds produced significant adverse or toxic effects in preclinical or clinical studies [6]. Thus, the development of safe and effective inhibitors of ABC transporters would be of importance in the clinical treatment of MDR cancer.

Sildenafil is a PDE5 inhibitor that is clinically approved for the treatment of erectile dysfunction and pulmonary arterial hypertension [8]. We have previously reported that sildenafil, an inhibitor of the enzyme cyclic guanosine 3', 5'-mono phosphate (cGMP)-specific phosphodiesterase type 5 (PDE5), reverses ABCB1- and ABCG2-mediated MDR *in vitro* [9]. Therefore, in the current study, we examined the effect of sildenafil on ABCB1-mediated resistance to

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paclitaxel using an ABCB1-overexpressing tumor-xenograft model in nude mice.

MATERIALS AND METHODS

Materials

Sildenafil was purchased from Toronto research chemicals (Toronto, Canada) and paclitaxel was obtained from LC labs (Woburn, MA). All other reagents and solvents were purchased from VWR (West Chester, PA).

Cell Lines and Cell Culture

The human epidermoid carcinoma cell line KB-3-1 was selected in a stepwise manner to create cells resistant to colchicine. This was accomplished using increasing concentrations of colchicine to establish the ABCB1/P-gp-overexpressing drug-resistant cell line, KB-C2, with cells cultured in a medium with 2 µg/ml of colchicine as previously described [10]. Both cell lines were grown as adherent monolayers in flasks with DMEM (Hyclone Co., UT) supplemented with 10% fetal bovine serum in a humidified incubator containing of 5% CO_2 at 37°C.

Animals

Male athymic NCR (nu/nu) nude mice (18–25 g, age 5-6 wk) were purchased from Taconic Farms (NCRNU-M, Homozygous, Albino color) and were used for the ABCB1 overexpressing tumor-xenograft model. All mice were maintained on a 12 h light/12 h dark cycle, with ad libitum access to water and rodent chow. The mice were maintained at the St. John's University Animal Facility and were monitored closely for tumor growth by palpation and visual examination. This project was approved by the Institutional Animal Care & Use Committee (IACUC) at St. John's University and the research was conducted in compliance with the Animal Welfare Act and other federal statutes.

Nude Mice ABCB1-Xenograft Model

Briefly, KB-3-1 (1.2×10^6) and KB-C2 (1×10^7) cells were injected s.c. under the armpits. Tumors that fail to reach a volume of 20 mm^3 at the start of treatment were not used in this study. When the tumors reached a mean diameter of 0.5 cm (day 0), the mice were randomized into four groups (n=6) and treated with one of the following regimens: (a) vehicle (10% 0.1 N NaOH, 90% saline) (p.o., daily X 18), (b) paclitaxel (18 mg/kg, i.p., q3d X 6), (c) sildenafil dissolved in 10% 0.1 N NaOH, 90% saline (10 mg/kg, p.o., daily X 18), and (d) paclitaxel (18 mg/kg, i.p., q3d x 6) + sildenafil (10 mg/kg, p.o., daily x 18, given 1 h before giving paclitaxel). Tumor volumes were measured using caliper and body weights were recorded. The body weight of the animals was monitored daily to adjust the drug dosage and to observe treatment-related toxicities as well as disease progression. The two perpendicular diameters of tumors (termed A and B) were recorded every 3rd day and tumor volume (V) was estimated according to the following formula published previously [10].

$$V = \frac{\pi}{6}\frac{(A+B)^3}{2}$$

All animals were sacrificed by exposure to carbon dioxide and tumor tissues were excised and stored at -80°C. In separate experiments, mice bearing the KB-C2 tumor were given either vehicle or sildenafil (10 mg/kg) orally and 1 h later, they received paclitaxel (18 mg/kg) *via* the tail vein (n=3-6) and blood samples were collected periorbitally 15, 30, 60, 120 and 240 min after paclitaxel administration. The tumors were removed, weighed, and snap frozen in liquid nitrogen and stored at −80°C until analysis. Paclitaxel levels were quantified using HPLC analysis, as described previously [10].

HPLC Analysis of Paclitaxel in Plasma and Tissues - Chromatographic Conditions

The quantification of paclitaxel levels was conducted using an isocratic Shimadzu LC-20AB liquid chromatograph equipped with the Shimadzu SIL-20A HT auto sampler and LC-20AB pump connected to a Dgu-20A3 degasser (Columbia, MD). The column used was a reverse-phase, Phenomenex Luna C18 column (250 x 4.6 mm i.d., 5 µm; Phenomenex, Torrance, CA), with an ODS guard column (4 mm x 3 mm; Phenomenex). The injection volume was 20 µl, and the mobile phase used for the separation of paclitaxel in plasma and tissue homogenate samples consisted of acetonitrile and water (53:47, v/v), delivered at 1.0 ml/min flow rate. For paclitaxel detection, the Shimadzu UV SPD-20A detector was set at 227 nm. Data acquisition and analysis was achieved using LC Solution software version 1.22 SP1 (Shimadzu). All samples were analyzed in duplicate. Under these chromatographic conditions, the total run time was 15 min with a retention time of 12 min for paclitaxel. Standard curves for paclitaxel in plasma and tissue homogenates were prepared in the ranges of 25–5000 ng/ml.

Extraction of Paclitaxel from Plasma and Tissue Homogenate Samples

A simple one-step protein precipitation with acetonitrile was used for sample preparation. Tissues were homogenized in saline in the ratio of 1:2 (v/v). Paclitaxel was extracted from plasma and tissue homogenate samples by precipitation with acetonitrile in 1:1 and 1:2 ratios (v/v), respectively. Samples were vortexed for 1 min followed by centrifugation for 10 min at 10,000 rpm. The supernatant was transferred to insert vials from which 20 µl was injected onto the HPLC column. Samples with concentrations higher than the calibration range limit were appropriately diluted to fit within the working calibration curve.

Statistical Analysis

The data were analyzed using Student's *t*-test. The statistical significance was set at $P < 0.05$**.** All data were expressed as mean +/- standard deviation for at least n=3.

RESULTS

A dose of 18 mg/kg i.p. of paclitaxel was chosen based on our previous study [10] showing that this dose significantly reversed MDR in a mouse xenograft model. Sildenafil (10 mg/kg/day, p.o.) did not produce any visible toxicity or phenotypic changes in the male athymic NCR nude mice [11].

Paclitaxel (18 mg/kg, i.p.) significantly reduced the growth rate of ABCB1 overexpressing tumors in the NCR nude mouse (Figure **1C**). The treatment of mice with sildenafil and paclitaxel significantly potentiated the paclitaxel-induced inhibition of KB-C2 tumor volumes and growth rate compared to that of animals treated only with vehicle, sildenafil or paclitaxel ($P <$

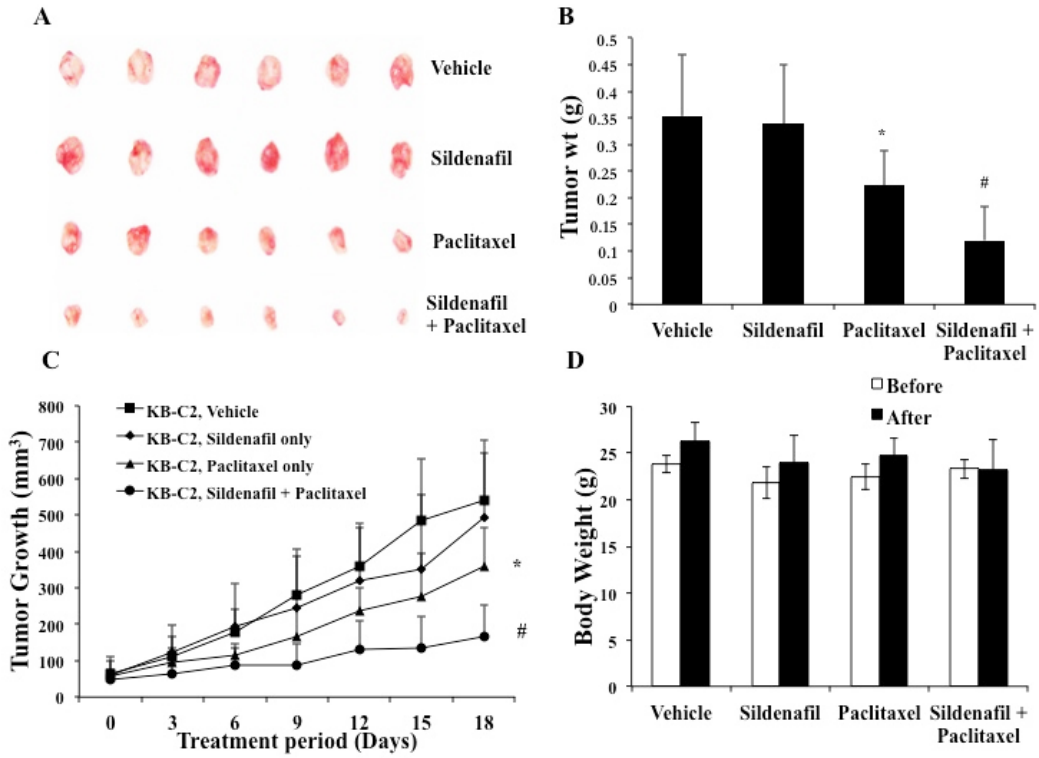

Figure 1: Sildenafil potentiates the efficacy of paclitaxel in an ABCB1 overexpressing tumor-xenograft model.

The potentiation of antitumor efficacy of paclitaxel by sildenafil in ABCB1 overexpressing (KB-C2) epidermoid carcinoma xenograft model is shown. Three independent experiments were carried out in athymic NCR nude mouse implanted (s.c.) with KB-C2 cells. **A**) A representative picture of the excised KB-C2 tumor sizes from different mice is shown at the end of the treatment period. **B)** The bar graph represents the mean tumor weight (n=6) of the excised KB-C2 tumor from different mice. The treatment groups used were: (a) vehicle (daily, p.o.), (b) sildenafil (10 mg/kg, p.o., daily) (c) paclitaxel (18 mg/kg, i.p., q3d X 6) (d) paclitaxel (18 mg/kg, i.p., q3d x 6) + sildenafil (10 mg/kg, p.o., daily, given 1 h before giving paclitaxel). Each column represent the mean determinations and the bars represent the SD. *, $P < 0.05$ versus vehicle group; #, $P < 0.05$ versus the paclitaxel group. **C)** Changes in tumor volume with time in an ABCB1-xenograft model are shown. The points represent the mean tumor volume for each treatment group (n=6). Each point on the graph represents the mean tumor volume (mm^3) on a specific treatment day and the bars represent the SD. *, $P < 0.05$ versus the vehicle group; #, $P < 0.05$ versus paclitaxel alone group. **D)** Changes in mean body weight before and after treatment are shown in the bar graph.

0.05; Figure **1A**, **1B** and **1C**). In addition, there was no significant difference in body weight or phenotypic changes between the treatment groups (Figure **1D**). These results suggest that sildenafil potentiates the efficacy of paclitaxel by inhibiting the efflux function of the ABCB1 transporter. There was no significant difference in the KB-3-1 (parental cell line) tumor size between paclitaxel alone or combination treatment group (data not shown).

Separate experiment was designed to analyze the effect of sildenafil on the concentration of paclitaxel in plasma and ABCB1 overexpressing tumors. Paclitaxel (18 mg/kg) was administered intravenously (to get clear idea about the pharmacokinetic of paclitaxel) with or without oral sildenafil (1 h before paclitaxel administration). The concentrations of paclitaxel were increased in the ABCB1 overexpressing tumors after combination treatment compared to mice treated only with paclitaxel (Figure **2B**). In addition, sildenafil (10 mg/kg) also significantly increased the paclitaxel concentration in the plasma (Figure **2A**).

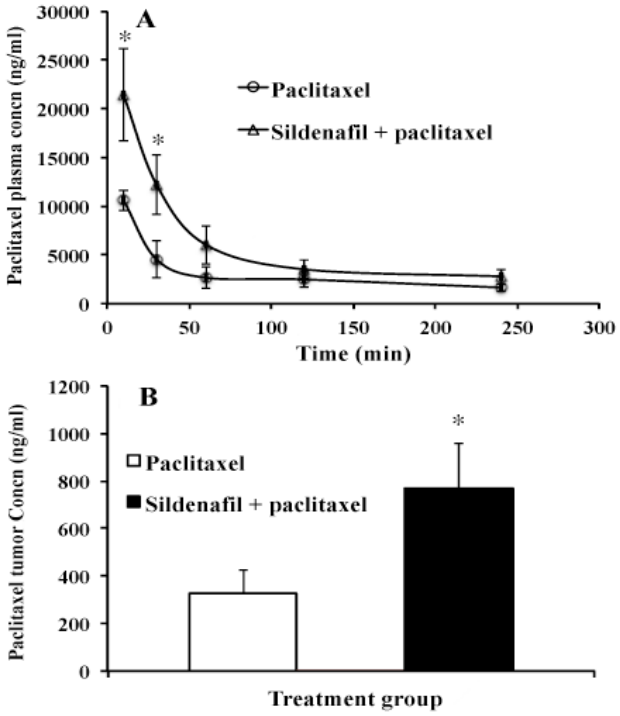

Figure 2: Sildenafil increases the concentration of paclitaxel in plasma and tumors in an ABCB1 overexpressing tumor-xenograft model.

Plasma paclitaxel concentration at different time points **A)** Paclitaxel concentrations in KB-C2 tumors **B)** with or without sildenafil treatment is shown. In the combination treatment group, 10 mg/kg sildenafil was given orally, 1 h before giving 18 mg/kg paclitaxel *via* the tail vein. Blood was collected periorbitally from mice at different time point, centrifuged at 4ºC and the supernatant was collected and stored at -80ºC until analysis. Mice were euthanized and tumor tissue was collected and stored at -80º C until analysis (n=3-6). *, $P < 0.05$ versus the paclitaxel group.

DISCUSSION

Previously, we reported that *in vitro*, sildenafil significantly sensitized ABCB1-overexpressing MDR cells to their substrate anticancer drugs [8]. Based on these findings, we conducted the current study to ascertain if sildenafil would potentiate the anticancer efficacy of the ABCB1 substrate paclitaxel *in vivo,* using the ABCB1-overexpressing murine tumor-xenograft model. In the current study, sildenafil significantly potentiated the anti-cancer efficacy of paclitaxel, as evidenced by the reduction in KB-C2 tumor size and growth rate in tumors xenografted in athymic nude mice after 18 days of treatment. In addition, sildenafil alone or in combination with paclitaxel did not produce significant changes in body weight or phenotype compared to the vehicle group or paclitaxel alone. This suggests that the combination of paclitaxel and sildenafil is efficacious in treating ABCB1-overexpressing tumors in a nude mouse xenograft model without producing significant visible adverse effects or toxicity. Our pharmacokinetic results indicated that sildenafil significantly increased the plasma and tumor levels of paclitaxel, a substrate for the ABCB1 transporter (Figure **2**). Sildenafil's potentiation of the anti-cancer efficacy of paclitaxel most likely due to its inhibition of the efflux activity of the ABCB1 transporter [9], thereby increasing the intracellular levels, and subsequently, the cytotoxicity of paclitaxel. The increase in paclitaxel plasma levels by sildenafil may result from the fact that both drugs in mice are metabolized by hepatic CYP450 [12, 13]. In addition, inhibition of liver ABCB1 by sildenafil can also affect the biliary clearance and may result in increased plasma levels of paclitaxel. Tentatively, these results, provided they can be extrapolated to humans, suggest that sildenafil may increase the likelihood of successfully combating MDR in cancer due to the overexpression of ABCB1 transporter. However, it is possible that the sildenafil-induced increase of paclitaxel levels may increase the risk of neurotoxicity and myelotoxicity, although this remains to be determined.

Our results are in contrast to those of Lin *et al*., who reported that sildenafil (10 and 50 mg/kg; p.o.) did not significantly potentiate the efficacy of doxorubicin against murine ABCB1-mediated MDR *in vivo* [14]. This discrepancy may be due to differences in the animal model and dosing regimens. For example, Lin

et al. used the syngeneic mouse tumor model, whereas we used the athymic nude mouse xenograpft model. In addition, we treated animals for over an 18-day period vs. 7 days. However, in our study as well as that of Lin *et al.*, the tumor volume in the combination treatment group was not significantly different from the anticancer drug alone treatment group on day 7 [14]. However, our pharmacokinetic results are congruent with those of Lin *et al.* [14].

In conclusion, the current *in vivo* study indicates that p.o. sildenafil potentiates the anticancer activity of paclitaxel in a nude mouse xenograft model at clinically relevant plasma drug concentration. The combination of sildenafil with ABCB1 substrate anticancer drugs may increase the probability of the successful treatment of patients with ABCB1-mediated MDR cancer.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

ABBREVIATIONS

ABC = ATP-binding cassette

ABCB1/P-gp = (P-glycoprotein)

MDR = multidrug resistance

PDE-5 = phosphodiesterase type 5

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