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RESEARCH PAPER

# Pharmacological Modulation of Cytotoxicity and Cellular Uptake of Anti-cancer Drugs by PDE5 Inhibitors in Lung Cancer Cells

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## ABSTRACT

***Purpose*** Previous research has led to the recognition of a cGMP signaling pathway governing drug transport. This study is to investigate whether inhibitors of phosphodiesterase type 5 (PDE5), which increase intracellular cGMP levels, modulate the cytotoxicity and uptake of anti-cancer drugs in cancer cells.

***Methods*** The experiments were conducted with and without PDE5 inhibitors: dipyridamole, vardenafil, and/or sildenafil. The cytotoxicity of doxorubicin, cisplatin and oxaliplatin was determined in multiple cancer cell lines derived from different tissues. The cellular uptake of structurally diverse compounds was further examined in lung cancer cells with and without various endocytotic inhibitors. The tumor accumulation and the anti-tumor effect of trastuzumab were examined in a lung cancer xenograft mouse model.

***Results*** Dipyridamole could modulate the cytotoxicity of doxorubicin, cisplatin, and oxaliplatin in cancer cells. Particularly, PDE5 inhibitors increased cellular uptake of structurally diverse compounds into lung cancer cells both *in vitro* and *in vivo*. The effect of vardenafil on drug uptake could be blocked by endocytotic inhibitors. The growth of lung cancer xenograft in nude mice was significantly suppressed by addition of vardenafil to trastuzumab treatment.

***Conclusion*** PDE5 inhibitors may increase the efficacy of anti-cancer drugs by increasing endocytosis-mediated cellular drug uptake, and thus serve as adjuvant therapy for certain cancers such as lung cancer.

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## INTRODUCTION

Most of current chemotherapy is nonspecific and nonselective, which results in only a modest increase in survival and causes significant toxicity to the patient. The limitation in efficacy and safety underscores the urgent need to understand the biology of drug accumulation in cancer tissues and development of novel drug delivery strategies. It has been previously demonstrated that pharmacological modulation of a cyclic guanosine monophosphate (cGMP) pathway, which involves bradykinin (BK) (1–3), nitric oxide (NO) (4–6), cGMP (7,8), and potassium channel agonists (9–11), can selectively increase delivery of compounds, including chemotherapeutic drugs, to brain tumors.

The cGMP pathway has provided a map to develop therapeutic strategies for enhanced drug delivery to tumor tissues. In early studies, RMP-7, an analog of bradykinin, increased drug delivery into tumor tissues of both animals and humans and improve chemotherapeutic response in brain tumor patients (1,2,4,12). However, both BK and RMP-7 have a short disposition half-life in the body and cause the side-effect of hypotension (5,13), which make their clinical use difficult. NO donor compounds, such as L-arginine and hydroxyurea, and potassium channel ($K_{Ca}$ and $K_{ATP}$) agonists, such as NS1619, NS1851, minoxidil sulfate, and diazoxide, are also capable of increasing brain tumor permeability for drugs (6,9–11,14), although their ability to enhance chemotherapeutic efficacy remains to be determined. PDE5 inhibitors, which increase intracellular cGMP levels *via* their inhibition on cGMP-specific PDE5 (15,16), may be effective pharmacological modulators in the cGMP pathway. We have demonstrated that PDE5 inhibitors are very promising adjuvant therapy for the

treatment of brain tumors (7,8). There is also limited evidence showing that dipyridamole, a PDE5 inhibitor (17), could increase cellular permeability for anti-cancer drugs in a few cell lines derived from peripheral tumors (18–20). Nevertheless, it remains to determine how effective in non-brain tumors the delivery and efficacy of anti-cancer drugs can be enhanced by pharmacological modulators of the cGMP pathway, such as PDE5 inhibitors. This can be clinically very significant as those non-brain tumors such as lung cancer may have much higher prevalence while being life-threatening as well.

The present study is to investigate whether PDE5 inhibitors modulate the cytotoxicity and uptake of different anti-cancer drugs in different cancer cells that are derived from non-brain tumor tissues. At first, the effects of dipyridamole on the cytotoxicity of doxorubicin, cisplatin and oxaliplatin were determined in multiple cancer cell lines. We then focused on a metastatic lung cancer cell line, investigating if and how different PDE5 inhibitors including dipyridamole, vardenafil and sildenafil altered the cellular uptake of structurally diverse compounds. Lastly, potential effects of a PDE5 inhibitor on delivery and efficacy of an anticancer drug were examined *in vivo* in a lung cancer xenograft mouse model.

## MATERIALS AND METHODS

### Materials

Dipyridamole, cisplatin, and oxaliplatin were purchased from Sigma-Aldrich (St Louis, MO). Vardenafil (Levitra®) was obtained from the Bayer Pharmaceuticals Co. (West Haven, CT), sildenafil (Viagra®) from Pfizer, Inc (New York, NY), and doxorubicin hydrochloride (adriamycin) from Ben Venue Laboratories, Inc. (Bedford, OH). Trastuzumab (Herceptin®) was obtained from Genentech, Inc. (San Francisco, CA). $^{14}$C-carboplatin was customarily synthesized with PerkinElmer Inc. (Boston, Massachusetts). $^{14}$C-adriamycin was purchased from Moravek Biochemicals Inc. (Brea, California) and $^{14}$C-dextran from Sigma-Aldrich. Lipofectamine 2000 and Dulbecco's modified Eagle's medium (DMEM) medium were purchased from Invitrogen Inc. (Carlsbad, California). All other reagents except those specifically described below were commercially available.

### Cell Culture

The cell lines in this study included a metastatic lung cancer cell line (NCI-H1915 or A549), cervix cancer cell lines (HeLa, KB-3-1, KB-CP20), breast cancer cell lines (MCF-7, BT-474,MDA-MB-231), liver cancer cell line (HepG2), ovary cancer cell lines (OA90, 2780 or A2780, 2780CP70 or A2780/CP70). KB-3-1 and KB-CP20 were provided by Dr. Michael Gottesman (NIH, Bethesda, MD). 2780 and 2780CP70 cells were from Dr. Michael J Birrer (Massachusetts General Hospital, Harvard Medical School, MA). All other cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown in DMEM, supplemented with 10% FBS, 4.5 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml), and were maintained in 75-cm$^2$ plastic flasks in 5% $CO_2$ at 37°C.

### Cytotoxicity Assessed by MTT Test

Cells were seeded in 96-well plates with a density of $4\times10^4$ cells/well. Twenty-four hours after seeding, a series of concentrations of the tested drugs were added into the plate wells. The medium was removed after 72-h incubation with the drugs. The MTT assay was conducted as described previously (21). In brief, each plate well was added the DMEM medium with 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4 h. Isopropanol with 0.1 N hydrochloric acid was then added to dissolve the MTT precipitate, and the absorbance of the colored solution quantified using a microplate reader (Bio-Rad. Hercules, CA) at a test wavelength of 570 nm and a reference wavelength of 690 nm.

### Drug Uptake in Cells

The same number of cells were seeded in 24-well plates and cultured until confluence. All uptake experiments were done as described previously with minor modifications (8). To verify the involvement of endocytotic pathways in drug uptake, the chemical inhibitors of endocytosis were used, including: filipin (8 μM) and methyl-β-cyclodextrin (5 mM) to inhibit caveolae-mediated endocytosis; amiloride (25 μM) to inhibit macropinocytosis; chlorpromazine (15 μM) and phenylarsine oxide (15 mM) to inhibit coated pit/clathrin endocytosis pathway (8,22,23). The cells were firstly incubated with and without an endocytotic inhibitor for 30 min at 37°C in the serum-free medium which was then replaced with that containing the inhibitor or not in addition to a PDE5 inhibitor (20 μM dipyridamole, 100 μM sildenafil, or 20 μM vardenafil) for another 30 min of incubation. Lastly, the medium was removed and replaced with that containing the uptake substrate, and cellular uptake was subsequently measured. To ensure conditions under which the uptake was not saturated, preliminary studies were conducted to determine compound uptake times and concentrations. The final uptake time of up to one hour was used for doxorubicin, cisplatin, dextran, and trastuzumab at concentrations of 50 nM, 10 μM, 10 μg/ml, and 25 μg/ml, respectively. The uptake was halted by removing the medium and washing the cells with ice-cold PBS buffer. After washing three times, the

cells were lysed in 300 μl of 1% (w/v) Triton X-100. Non-radiolabeled doxorubicin, cisplatin, and dextran, along with $^{14}C$ tracers were used in the uptake experiments. The radioactivity was counted by a multi-purpose scintillation counter (Beckman LS6500 Counter, Brea, CA). To detect the uptake of trastuzumab, the antibody compound was labeled with Alexa Fluor 680 (Invitrogen, Carlsbad, CA) using Xenofluor labeling kit (Caliper, Alameda, CA) as instructed by the vendor. Alexafluor-conjugated trastuzumab was quantitated using excitation at 680 nm and emission at 720 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). The protein concentrations were measured using a BCA protein assay kit (Bio-Rad. Hercules, CA) to normalize uptake values.

### Animal Studies

All procedures were carried out in accordance with NIH guidelines for animal experimentation, and the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the School of Pharmacy, University of Maryland Baltimore. Athymic nude mice (Charles River Laboratories International, Inc.) were housed under controlled conditions (21±2°C, humidity 60±10% and 12 h/12 h dark/light cycle) and had free access to food and water.

For the ectopic lung cancer mouse model, H1915 cells suspended in saline were mixed 1:1 with Matrigel (BD Biosciences) and subcutaneously inoculated into the right flank of each nude mouse. After inoculation, the skin incision was closed with a suture. Subcutaneous tumors were measured twice per week using calipers and their volumes were calculated using a standard formula ($width^2 \times length \times 0.5$).

For the studies of drug accumulation in the tumor, the tumor growth in the nude mice was allowed to reach a tumor diameter of 1 cm. The mice were divided into 6 groups ($n$=3–4 mice/group): saline (S) plus (+) doxorubicin (DOX); S + dextran (DEX); S + trastuzumab (TRA); vardenafil (V) + DOX; V + DEX; and V + TRA. The mice were firstly given either an oral dose of 10 mg/kg vardenafil or saline. One hour later, the mice were received by tail vein injection a $^{14}C$-radiolabel tracer of doxorubicin or dextran (0.1 μci/g), or the Alexafluor-conjugated trastuzumab (5-mg/kg). Three hours later, the mice were sacrificed. The xenograft tumors were isolated, gently washed, weighed and submerged in PBS, which were further homogenized completely. The homogenized tissues were centrifuged at 15,000 rpm for 10 min. The radioactivity (doxorubicin and dextran) in the supernatant was counted at multiple-purpose scintillation machine (Beckman LS6500 Counter, Brea, CA); while the fluorescence signal (Alexafluor-conjugated trastuzumab) was quantitated in the microplate reader.

To study the effect of vardenafil on the anti-tumor efficacy of trastuzumab, the nude mice inoculated with H1915 cells were divided into four groups ($n$=5/group): saline; vardenafil (the dose of 10 mg/kg, five times per week by oral, was based on our previous experience with a brain tumor model) (7); trastuzumab (the dose of 10 mg/kg, twice per week by tail vein was based on previous reports (8,24); and trastuzumab (10 mg/kg, intravenously, twice per week) plus vardenafil (10 mg/kg, orally, five times per week). The mice received their treatments beginning at day 7 after inoculation. All mice were sacrificed after 2 weeks of treatment. The tumor sizes before the treatment and after 2 weeks of treatment were compared among the four groups.

### Statistics and Data Analysis

All data were expressed as the mean±standard deviation (SD). The $IC_{50}$ values were obtained by fitting F, the percentage of the maximal cell growth at different drug concentrations, to the equation $F=100\times[1-C^{\gamma}/(IC_{50}{}^{\gamma}+C^{\gamma})]$ using WinNonlin (Pharsight, Sunnyvale, CA). The maximal cell growth was the cell growth in the medium without any anti-cancer compounds; C is the concentration of the anti-cancer compound and γ is the slope factor. Resistant factor (RF), which is the ratio of the $IC_{50}$ for an anti-cancer drug alone to that of its combination with dipyridamole, was calculated in the analysis of cytotoxicity data. The *in vitro* data were from a representative experiment performed in triplicate or quadruplicate and all experiments were repeated at least twice. Data were analyzed statistically using the unpaired *Student's t* test and the analysis of variance (ANOVA) followed by *Dunnett's* test, when appropriate. A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

### Effects of PDE5 Inhibitor Dipyridamole on Cytotoxicity of Doxorubicin in Different Cancer Cells

Dipyridamole, a PDE5 inhibitor (17), has been showed an anti-cancer activity in a few cancer cell lines either alone or in combination with a chemotherapeutic agent such as doxorubicin and cisplatin (18–20). To explore the potential of PDE5 inhibition as an adjunctive therapy for different cancers, we evaluated the cytotoxicity of dipyridamole alone and its effect on the cytotoxicity of doxorubicin in a variety of cancer cell lines derived from different tissues, including lung, cervix, breast, liver, and ovary. Dipyridamole alone showed cytotoxicity in all of the cancer cell lines studied, with the IC50s ranged from 29.4 to 215 μM (Fig. 1). The ovary 2780CP70 (IC50: 29.4 μM) was most sensitive to

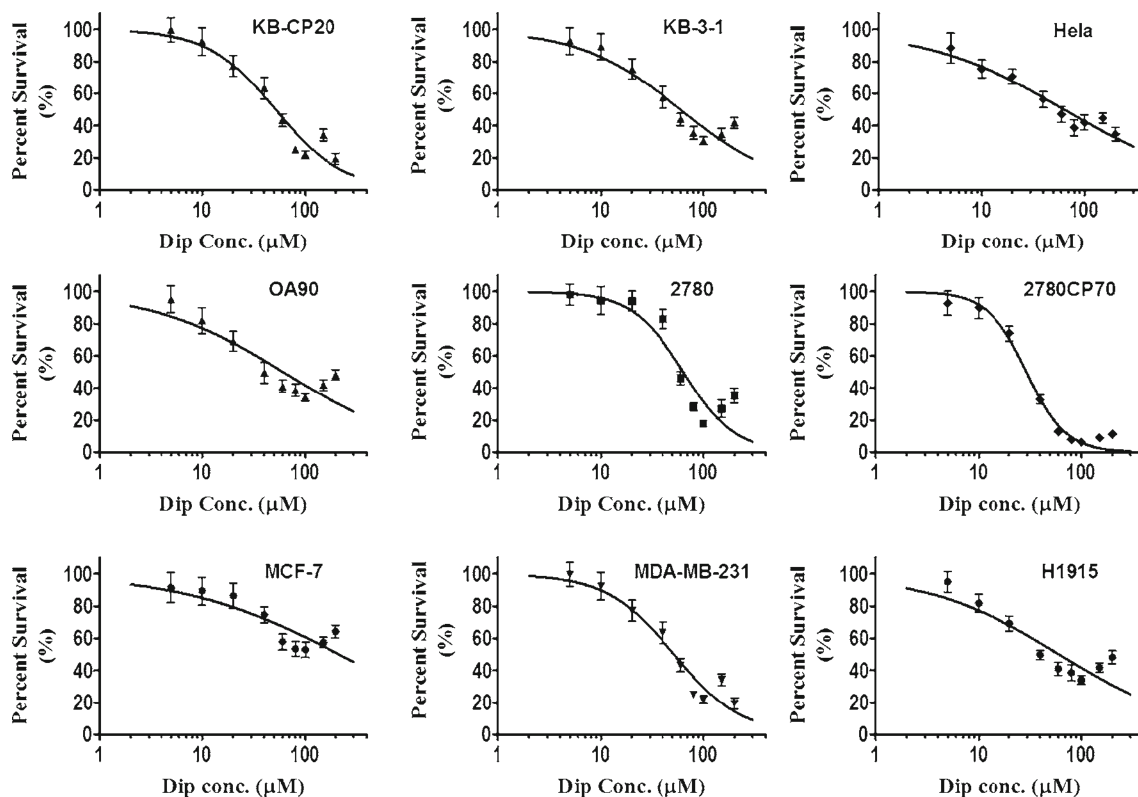


**Fig. 1** Cytotoxicity of dipyridamole in different cancer cell lines. The cancer cells ($3 \times 10^3 - 6 \times 10^3$/well) of different tissue derivation were incubated in 96-well plates with dipyridamole at a range of concentrations (0–200 μM) for 48 h. The cell viability was determined by MTT assay as described in the Methods. The cell viability without dipyridamole treatment is defined as 100%. All data points are mean±standard deviation (SD) from three replicates.

dipyridamole treatment, while the MCF-7 was somewhat resistant to dipyridamole treatment (IC50: 215 μM). The IC50s were higher than the clinical plasma concentrations of dipyridamole. We then examined the combinational cytotoxicity of dipyridamole and an anticancer agent, doxorubicin. Resistant factor (RF, the ratio of the $IC_{50}$ for doxorubicin alone to that of its combination with dipyridamole) was used to determine the effect of dipyridamole on the cytotoxicity of doxorubicin. Dipyridamole (20 μM) enhanced the sensitivity of most cell lines to doxorubicin cytotoxicity (Tables I). Of note, the sensitivity of the metastatic lung cancer H1915 cells was enhanced by 15 folds by dipyridamole (Fig. 2a). However, dipyridamole reduced the sensitivity in breast BT-474 (RF: 3.00), cervix KB-3-1 (RF: 2.97) and KB-CP20 (RF: 2.63) cells. The data suggested that dipyridamole might be an effective adjunctive therapy for doxorubicin to kill certain cancer cells, in particular lung cancer cells.

## Effects of PDE5 Inhibitor Dipyridamole on Cytotoxicity of Platinum Compounds in Different Cancer Cells

Platinum compounds including cisplatin, carboplatin and oxaliplatin are most active anticancer agents that are in treatment regimens for 80% of cancer patients (25–27). To further determine the potential of dipyridamole as an adjunctive therapy, we also assessed its effects on the cytotoxicity of cisplatin and oxaliplatin in a set of cancer cell lines. Dipyridamole (20 μM) had cytotoxicity additive to that of cisplatin in the metastatic lung cancer H1915 cells (RF: 0.68, Fig. 2b) and the breast MCF-7 cells (RF: 0.77). However, dipyridamole decreased the sensitivity to cisplatin in KB-3-1 (RF: 2.41) and KB-CP20 (RF: 1.49) cells, with no effect in other cell lines (Table II). The cisplatin-resistant cervix KB-CP20 cells were derived from the cisplatin-sensitive cervix

**Table I** Cytotoxicity, Expressed as IC50, of Doxorubicin (Dox), and its Combination with Dipyridamole (Dip) in Different Cancer Cell Lines

| Cell lines | Dox only $IC_{50}$ (nM) | Dox + Dip $IC_{50}$ (nM) | RF |
|---|---|---|---|
| KB-CP20 | 1190 ± 384 | 3140 ± 1080 | 2.64 |
| KB-3-1 | 36.8 ± 16.8 | 109 ± 12.7 | 2.97 |
| Hela | 290 ± 94.7 | 124 ± 29.7 | 0.43 |
| OA90 | 369 ± 48.4 | 312 ± 83.7 | 0.85 |
| BT474 | 1940 ± 492 | 5830 ± 2540 | 3.01 |
| HepG2 | 253 ± 13.4 | 194 ± 23.9 | 0.77 |
| MCF-7 | 530 ± 72.0 | 349 ± 59.1 | 0.66 |
| MDA-MB-231 | 1740 ± 100 | 715 ± 50.3 | 0.41 |
| NCI-H1915 | 1450 ± 541 | 94.6 ± 19.1 | 0.07 |

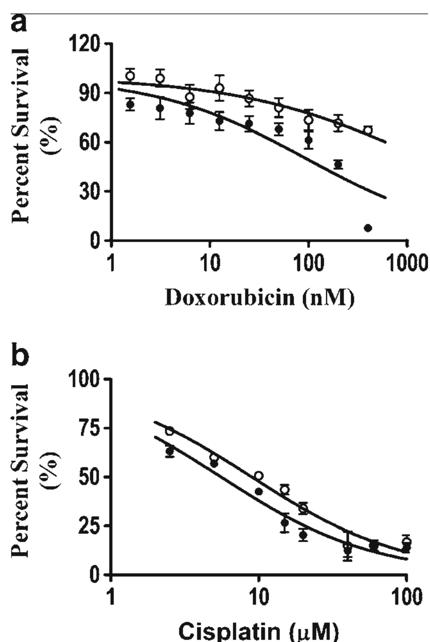


**Fig. 2** Effect of dipyridamole on the cytotoxicity of doxorubicin and cisplatin in H1915 lung cancer cells. NCI-H1915 cells were incubated with doxorubicin (0–400 nM) (**a**) or cisplatin (0–100 $\mu$M) (**b**) at a range of concentrations, with (●) and without (○) 20 $\mu$M of dipyridamole, for 48 h. The cell viability was determined by MTT assay as described in the Methods. The cell viability without any drug treatment is defined as 100%. All data points are mean ±standard deviation (SD) from three replicates.

KB-3-1 cells (28). Our data suggested that dipyridamole might not have any benefits in overcoming cisplatin resistance in these cancers. To our surprise, dipyridamole significantly decreased the sensitivity to the cytotoxicity of oxaliplatin in all of the cancer cell lines studied (Table II). In particular, the sensitivity in KB-CP20 cells (RF>1,000) was remarkably reduced by the co-treatment of dipyridamole. Overall, our cytotoxicity experiments with different chemotherapeutic agents indicated that the effects of the PDE5 inhibitor dipyridamole on cellular chemoresponse were dependent on cell lines and drugs. Both cisplatin and doxorubicin are among active agents for lung cancers. The data suggested that dipyridamole most likely served as an adjunctive therapy to kill certain lung cancer cells.

### Effect of PDE5 Inhibitors on Cellular Uptake of Structurally Diverse Compounds in H1915 Cancer Cells

To understand the mechanism why dipyridamole could enhance chemoresponse in lung cancer cells, we performed *in vitro* cellular uptake studies to determine whether PDE5 inhibitors enhance the transport of drugs into the metastatic lung cancer H1915 cells. Moreover, to ascertain a mechanistic role by PDE5 inhibition, we used two specific PDE5 inhibitors, sildenafil and vardenafil, in addition to dipyridamole. As shown in Fig. 3, all three PDE5 inhibitors significantly enhanced the uptake of doxorubicin and carboplatin into the H1915 cells, with the most effects observed with vardenafil. Our subsequent studies were thus conducted with vardenafil. To further confirm whether PDE5 inhibitor-mediated increase of drug transport was translated to anti-cancer sensitization, we also examined the effect of vardenafil (20 μM) on the cytotoxicity of doxorubicin and carboplatin in H1915 cells. As dipyridamole did above, vardenafil significantly increased the cytotoxicity of doxorubicin and carboplatin in the H1915 cells (Fig. 4). We asked whether vardenafil could have a general effect on the uptake of different compounds in the lung cancer cells. Two additional compounds, dextran and trastuzumab, which represented hydrophilic polymers and macromolecules respectively, were studied. Vardenafil (20 μM) significantly increased the uptake of these two compounds into H1915 cells as well (folds *vs.* control, $P<0.05$, Fig. 5). Interestingly, the effects by vardenafil seemed to be reversely correlated with the molecular weights of the compounds, with the most increase of uptake shown for

**Table II** Cytotoxicity, Expressed as IC50, of Cisplatin (Cis), Oxaliplatin (Oxa), and Their Combination with Dipyridamole (Dip) in Different Cancer Cell Lines

| Cell lines | Cis only $IC_{50}$ ($\mu$M) | Cis+Dip $IC_{50}$ ($\mu$M) | RF | Oxa only $IC_{50}$ ($\mu$M) | Oxa+Dip $IC_{50}$ ($\mu$M) | RF |
|---|---|---|---|---|---|---|
| KB-CP20 | 26.1 ± 1.66 | 39 ± 4.56 | 1.49 | 155 ± 29.9 | > 155,000[a] | >1,000 |
| KB-3-1 | 3.23 ± 0.55 | 7.8 ± 0.75 | 2.42 | 5.36 ± 0.72 | 23.18 ± 3.89 | 4.33 |
| Hela | 10.7 ± 1.75 | 11.2 ± 1.80 | 1.06 | 14.9 ± 3.18 | 35.42 ± 9.08 | 2.38 |
| OA90 | 14 ± 0.848 | 13.1 ± 0.88 | 0.94 | 61.4 ± 10.1 | 210.2 ± 19.6 | 3.43 |
| BT474 | 86.4 ± 11.9 | 96.8 ± 5.56 | 1.12 | 130 ± 56.4 | 375 ± 49.6 | 2.88 |
| HepG2 | 8.47 ± 0.88 | 8.81 ± 1.35 | 1.04 | 7.0 ± 1.46 | 19.2 ± 3.09 | 2.75 |
| MCF-7 | 34.8 ± 3.99 | 26.7 ± 3.08 | 0.77 | 14.1 ± 3.08 | 36.14 ± 8.44 | 2.56 |
| MDA-MB-231 | 121 ± 5.49 | 116 ± 8.25 | 0.96 | 346 ± 81.5 | 2,003 ± 884 | 5.79 |
| H1915 | 9.02 ± 0.56 | 5.58 ± 0.41 | 0.62 | 49.9 ± 19.3 | 1,300 ± 863 | 26 |

[a] The concentrations of oxaliplatin used could not achieve more than 50% of cell death, and the IC50 was estimated to be more than 155, 000 $\mu$M by the software

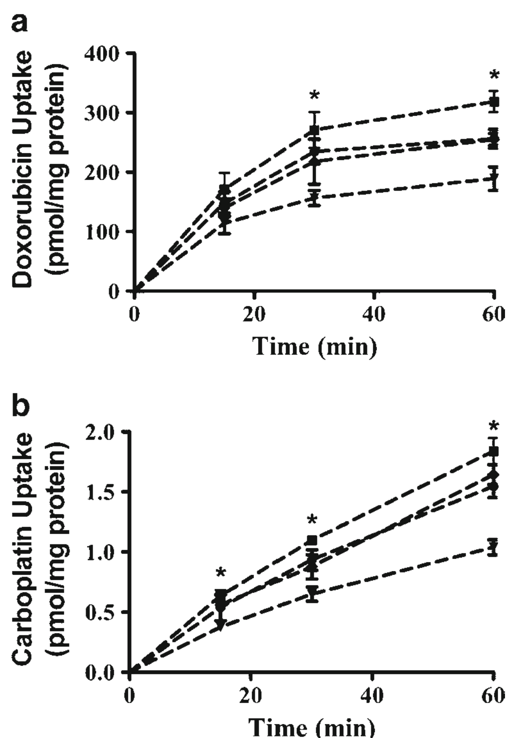


**Fig. 3** Effect of PDE5 inhibitors on the cellular uptake of doxorubicin and carboplatin in H1915 cells. The cells were incubated with doxorubicin (50 nM) (**a**) or carboplatin (10 μM) (**b**) in H1915 cells without (▼, control) and with a PDE5 inhibitor (■, 20 μM vardenafil; ●, 100 μM sildenafil; or ♦, 20 μM dipyridamole) for the indicated period of times. The cellular uptake of doxorubicin or carboplatin was determined as described in the Methods. *$p<0.05$; the uptake in the presence of a PDE5 inhibitor was statistically more than that in the absence of any inhibitor.

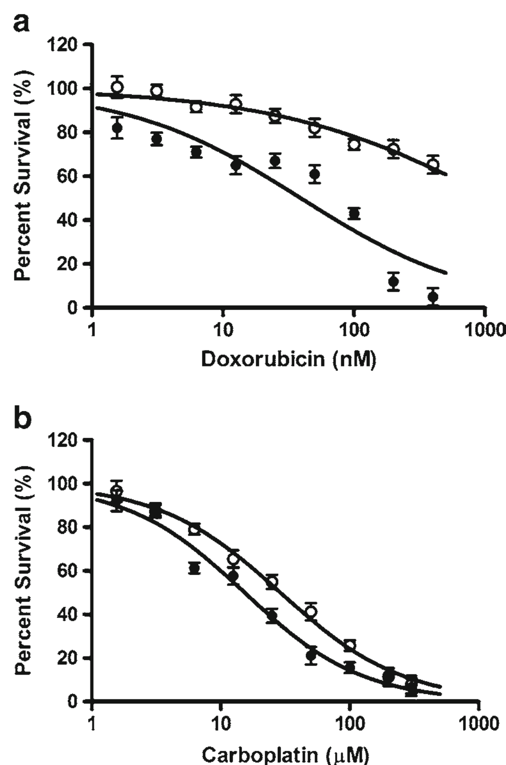


**Fig. 4** Effect of vardenafil on the cytotoxicity of doxorubicin and carboplatin in H1915 cells. The cells were incubated with doxorubicin (0–400 nM) (**a**) or carboplatin (0–300 μM) (**b**) at a range of concentrations, with (●) and without (○) 20 μM of vardenafil, for 48 h. The cell viability was determined by MTT assay as described in the Methods. The cell viability without any drug treatment is defined as 100%. All data points are mean±standard deviation (SD) from three replicates.

trastuzumab. Our data suggested that PDE5 inhibitors might enhance the chemosensitivity of anti-cancer drugs by increasing their cellular uptake in lung cancer cells, *via* a mechanism that was underlying the transport of structurally diverse compounds across the membrane.

## Role of Endocytosis in Drug Uptake Modulation by PDE5 Inhibitors in H1915 Cancer Cells

Previous studies suggest that endocytotic vesicular transport is a mechanism for the increase of chemotherapeutic uptake into brain tumors by pharmacological modulation (9). Here we examined the role of three major endocytotic pathways clathrin- and caveolae-mediated transport, as well as macropinocytosis, in the increased uptake induced by vardenafil in H1915 cells (Fig. 6). Caveolae-mediated endocytosis can be inhibited by filipin and methyl-β-cyclodextrin (22,23). The vardenafil-enhanced uptake of doxorubicin was abolished by the pretreatment of the cells with filipin or methyl-β-cyclodextrin ($P<0.001$ *vs.* vardenafil only). We then examined the role of macropinocytosis by using its inhibitor, amiloride (22,29). Amiloride had a similar effect to those of filipin and methyl-β-cyclodextrin. However, the clathrin pathway inhibitors, chlorpromazine and phenylarsine oxide, only slightly decreased the effect by vardenafil ($P>0.05$ *vs.* vardenafil only).

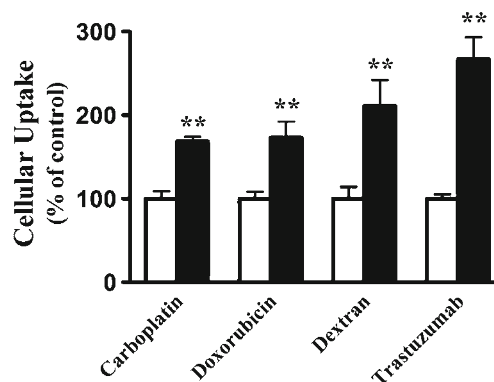


**Fig. 5** Effect of vardenafil on the cellular uptake of different compounds in H1915 cells. The cells were incubated with doxorubicin (50 nM), carboplatin (10 μM), dextran (10 μg/ml), or trastuzumab (25 μg/ml) in H1915 cells with (solid bars) and without (blank bars) 20 μM of vardenafil for 30 min. The cellular uptake of doxorubicin, carboplatin, dextran, or trastuzumab was determined as described in the Methods. To scale different compounds in the same figure, the uptake of different compounds was normalized to the percentage of their respective control. **$p<0.01$; the uptake in the presence of vardenafil was statistically more than that in the absence of vardenafil (control).

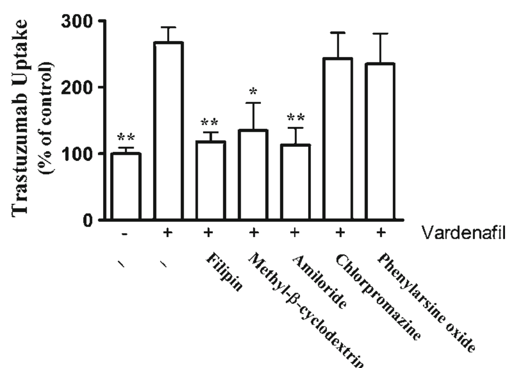


**Fig. 6** Effects of endocytic pathway inhibitors and vardenafil on the cellular uptake of trastuzumab in H1915 cells. The cells were incubated with and without various endocytotic inhibitors for 30 min before addition of vardenafil or control medium for another 30 min. The cells were then continuously incubated with trastuzumab (25 $\mu$g/ml) in the presence or absence of the endocytotic inhibitors and vardenafil for 30 min. The cellular uptake of trastuzumab was determined as described in the Methods. The endocytotic inhibitors are: filipin (8 $\mu$M) and methyl-β-cyclodextrin (5 mM) as the inhibitors of caveolae endocytic pathway, amiloride (25 $\mu$M) as the inhibitor of macropinocytosis, chlorpromazine (15 $\mu$M) and phenylarsine oxide (15 mM) as the inhibitors of the coated pit/clathrin pathway (8,22,23). The uptake without any treatment by endocytotic inhibitors and vardenafil (control) is defined 100%. *$p < 0.05$; **$p < 0.01$ *vs.* vardenafil treatment alone.

The data suggested that stimulation of caveolae and macropinocytosis pathways might play an important role in the effects of PDE5 inhibitors on drug uptake in lung cancer cells.

### Effect of PDE5 Inhibitor Vardenafil on Drug Accumulation and Anti-tumor Efficacy of Trastuzumab in a Mouse Tumor Model

Lastly, we tested whether PDE5 inhibition could also have any effects on drug accumulation and anti-cancer efficacy of a drug *in vivo*. After oral doses of vardenafil (10 mg/kg) or saline,

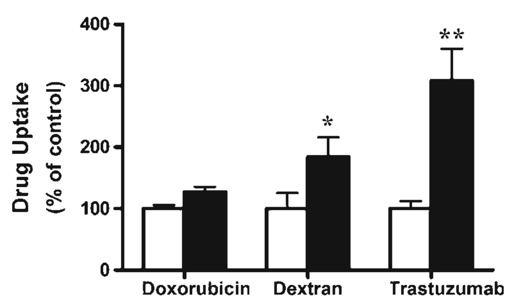


**Fig. 7** Effect of oral vardenafil on the accumulation of different compounds in flank lung cancer tissues of mouse model. The nude mice were implanted with H1915 human non-small lung cancer cells. The tumor growth in the nude mice was allowed to reach a tumor diameter of at least 1 cm. The mice were given either an oral dose of 10 mg/kg vardenafil (solid bars) or saline (blank bars). One hour later, the mice were received by tail vein injection of $^{14}$C-doxorubicin (0.1 $\mu$ci/g), $^{14}$C-dextran (0.1 $\mu$ci/g) or trastuzumab (5 mg/kg). The tumor levels of doxorubicin, dextran, or trastuzumab were determined as described in the Methods. Three to five mice were used for each group. To scale different compounds in the same figure, the uptake of different compounds was normalized to the percentage of the control saline groups, respectively. *$p < 0.01$ *vs.* control.

the nude mice implanted with H1915 cells in the flank were intravenously injected with doxorubicin, dextran, or trastuzumab. Vardenafil significantly increased tumor accumulation of dextran and trastuzumab, while only a trend was seen for doxorubicin in the mice (Fig. 7). The *in vivo* effect by vardenafil was also more significant for the larger molecules. The *in vivo* data, together with those *in vitro* (Figs. 3, 4, and 5) supported that PDE5 inhibitors might enhance non-specific endocytotic activities instead of the activity of a specific drug transporter. The finding of vardenafil-induced increase of trastuzumab accumulation in tumor tissues led us to examine the anti-tumor efficacy of trastuzumab *in vivo*. The lung cancer- bearing mice were treated with trastuzumab (10 mg/kg, twice per week, intravenously) beginning at day 7 after tumor implantation, with oral administration of vardenafil (10 mg/kg, 5 times per week) or saline. We observed that the tumor growth in the nude mice was significantly slower in the combination (vardenafil *plus* trastuzumab) treatment group, when compared to those groups of single treatment (vardenafil alone, trastuzumab alone, or no treatment control) (Fig. 8). Notably, either trastuzumab alone or vardenafil alone did not suppress tumor growth in the present study.

## DISCUSSION

In the present study, we made exciting observations on the effects of PDE5 inhibitors on the cytotoxicity and cellular uptake of anti-cancer drugs. Specifically, we found that PDE5 inhibition enhanced cytotoxicity of doxorubicin and cisplatin in different cancer cells; particularly in lung cancer

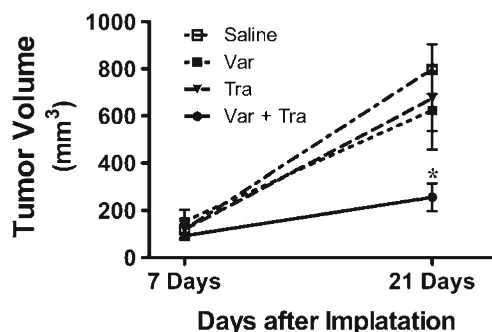


**Fig. 8** Effect of oral vardenafil on tumor growth in the lung cancer xenograft mouse model receiving trastuzumab treatment. The nude mice were implanted with H1915 human non-small lung cancer cells. Seven days after tumor cell inoculation, the mice were divided into four groups ($n = 5$ mice/group): saline; vardenafil (Var); trastuzumab (Tra); and trastuzumab (10 mg/kg, intravenously, twice per week) plus vardenafil (10 mg/kg, orally, five times per week) (Var+Tra). Vardenafil was orally administrated to the mice five times per week at the dose of 10 mg/kg. Trastuzumab was injected to the mice twice per week by tail vein at the dose of 10 mg/kg. The tumor size was measured as described in the Methods. All mice were sacrificed after 2 weeks of treatment. The tumor sizes before the treatment and after 2 weeks of treatment were compared among the four groups. *$p < 0.05$ *vs.* other three groups.

cells in which we further demonstrated that the enhanced cytotoxicity was likely due to the increase of drug uptake *via* endocytosis. Consistent findings were obtained *in vivo* that the PDE5 inhibitor vardenafil increased drug concentrations in a cancer xenograft in mice; and that the tumor growth was significantly suppressed by addition of vardenafil to the treatment by the anticancer trastuzumab. As the primary cellular consequence of PDE5 inhibition is an elevated level of cGMP (15,16), the results from the present study in non-brain tumor cells are in line with those from previous reports that pharmacological modulation of a cGMP pathway may selectively increase delivery of chemotherapeutic drugs to brain tumors (1–7,9–11).

Low intracellular drug concentrations are among major obstacles toward a successful cancer therapy. In the past, focus has been particularly on understanding drug efflux as a major resistance mechanism, which only leads to mild success in improving chemotherapy for most cancer types including lung cancers. Whereas great effort would be still put on this direction, it is clear that alternative mechanisms and approaches are needed. Understanding the biology of drug uptake across cellular membrane is moving higher priority. We have previously reported that the inhibition of PDE5 by vardenafil and sildenafil increases the concentrations of chemotherapeutics in brain tumors (7,8). Moreover, others have reported that dipyridamole increases cisplatin concentrations and toxicity in breast carcinoma cells (18), larynx cancer cells (19), and ovarian carcinoma cells (20). In this study, we have extended those previous findings by demonstrating the desirable effects of PDE inhibitors on chemoresponse in a variety of cancer cells. However, the effect of dipyridamole on cytotoxicity of platinum compounds appeared to be rather mild in H1915 cells. It may be due to the increased platinum uptake as illustrated in our transport assays. Alternatively, PDE5 inhibitors may also offer benefits as an apoptosis modulator. *In vivo* apoptotic rates increase in an orthotopic lung cancer model treated with the PDE5 inhibitor exisulind (30,31). Our data showed that dipyridamole alone had cytotoxic effects on multiple cancer cell lines. Overall, the findings of the present study have provided rationales to use PDE5 inhibitors as novel adjuvant therapy for certain malignant tumors such as lung cancers.

However, whether the effect of PDE5 inhibitors on chemotherapy is beneficial or not may be dependent on cancer types and chemotherapeutics. In the present study, dipyridamole actually caused moderate resistance to the cytotoxicity of doxorubicin and cisplatin in a few cancer cell lines. In particular, the cytotoxicity of oxaliplatin was reduced by dipyridamole in all of the studied cell lines. The mechanisms underlying the dipyridamole-induced chemoresistance are currently unclear. Since cancer patients always receive polypharmacy, the present study also raises concerns on possible compromised chemosensitivity due to the use of PDE5 inhibitors in patients.

Three major cellular mechanisms have been suggested to account for the transfer of molecules across the membranes: passive diffusion, transporter protein-mediated process, and endocytosis. Passive diffusion is thought to be important for drugs to cross hydrophobic membranes (32). The process is mainly related to physiochemical characteristics of an individual drug. Drug transporters have become a focus in the fields of pharmaceutical research since last decade. In general, transporter proteins mediate the transfer of specific small molecules across the membrane. Several families of proteins have been extensively characterized as drug transporters. These include the ATP-binding-cassette (ABC) family (33), $H^+$-oligopeptide cotransporters of solute carrier family 15 (SLC15A) (34), the solute carrier organic anion transporting polypeptide family (SLCO or SLC21A) (35), and the organic cation-anion-zwitterion transporter family (SLC22A) (36). Because of its specificity, the transporter protein-mediated process does not explain the transport across membranes for a majority of drugs. Endocytosis is defined as the transport of molecules from the extracellular milieu through the formation of a vesicle at the plasma membrane. This transport event circumscribes both soluble and membrane-bound cargos. Endocytosis has been a subject of intense research in cell biology. It is regarded as an important mechanism for macromolecule therapeutics including antibodies, viral vectors, and nanoparticles to cross the membrane (37,38). However, emerging evidence suggests that endocytosis also plays an important role as a general transport mechanism for drugs including small molecules to cross the membrane (8,9,39–43). In the present study, we have further provided such evidence that PDE5 inhibitor-induced increase in drug uptake involves endocytosis, specifically caveolae-dependent transport and macropinocytosis.

It should be noted that the drug uptake mediated *via* endocytosis is non-specific. In the present study, the uptake of not only the anti-cancer drugs but also the hydrophilic polymer dextran was enhanced by PDE5 inhibition. However, the endocytosis induced by PDE5 inhibitors could be specific to anticancer drugs in the treatment of cancers as PDE5 has been reported to be overexpressed in many carcinomas and as a predominant isoform of PDEs in carcinoma cell lines in culture, including lung cancer, colonic adenocarcinoma, breast cancer, bladder cancer, prostate cancer, and leukemia PDE5 has been also detected (30,44,45). As expected, the endocytosis-mediated drug transport was most efficient for the macromolecule trastuzumab in this study. Since macromolecule therapeutics have been increasingly used in clinic, future studies are highly necessary to determine the interaction between these macromolecules and PDE5 inhibitors in pursuit of synergistic therapy.

The molecular mechanism underlying PDE5 inhibitors-induced endocytosis remains to be determined. PDE5 inhibitors, *via* their inhibition on cGMP-specific PDE5, increase the intracellular levels of cGMP that is an important second messenger molecule. We speculate that increased levels of cGMP would cause the activation of certain molecules that play a critical role in endocytosis in the lung cancer cells. One of such molecules might be cGMP-dependent protein kinase (PKG) which has been shown to induce activation of potassium channels such as $K_{Ca}$ channels in a variety of cells. Activation of potassium channels causes cellular membrane hyperpolarization which lead to increased vesicular transport across cellular membrane in brain tumor cells (9,10). Very recently, activation of PKG has also been reported to upregulate phosphatidylinositol-4,5-bisphosphate, thereby accelerating endocytosis (46). Notably, endocytosis has also been reported to be involved in the maturation, trafficking, and membrane turnover of transporter proteins for small compounds (39–43). Consistently, we showed the effects by PDE5 inhibitors and endocytosis inhibitors on the uptake of doxorubicin and platinum compounds. However, those chemical inhibitors of endocytosis may not be specific to endocytotic process only. Future studies using specific genetic approaches such as shRNAs toward the proteins required for endocytosis are necessary to characterize the mechanism underlying PDE5 inhibitors-induced endocytosis.

We have observed a potential synergistic therapy from the combination of vardenafil and trastuzumab. Trastuzumab is a humanized monoclonal antibody for the treatment of patients with breast, lung, or prostate cancers that overexpress HER-2 (Human Epidermal Growth Factor Receptor 2). We showed that oral administration of vardenafil increased the accumulation of trastuzumab in the cancer xenograft at the flank of nude mice. Consistently, the co-administration of vardenafil also led to an enhanced anti-tumor effect of trastuzumab in the mice. The synergistic effect is interesting because trastuzumab targets cell-surface HER-2 receptors which are highly expressed in H1915 cells (47), and does not have to be transported into cancer cells for initiation of its therapeutic effects. One explanation is that vardenafil may enhance the receptor-mediated endocytosis of trastuzumab and its associated HER-2. This might cause the increased detection of trastuzumab in the tumors and reduced HER-2 in cell surface that is available for further ligand signaling. Alternatively, PDE5 inhibitors such as vardenafil may enhance vesicular permeability *via* increased endocytosis in vessel endothelial cells (8) and bring more trastuzumb molecules to cancer cells. Future effort is needed to understand the molecular mechanism underlying the PDE5 inhibition-induced increase in anti-tumor efficacy of trastuzumab.

In conclusion, the present study has demonstrated that PDE5 inhibitors can modulate the cytotoxicity and uptake of anti-cancer drugs in certain cancer cells. The results from lung cancer cells suggest that PDE5 inhibitors may exert their effects through modulation of endocytosis. Oral administration of the PDE5 inhibitor vardenafil significantly increases the accumulation and enhances the anti-tumor effect of trastuzumb in a xenograft mouse model of lung cancer. Giving the fact that all the PDE5 inhibitors used in the present study are already FDA approved, we expect fewer barriers in translation of our findings to future clinical studies.

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