

Research Paper

Potential synergistic effect of phosphodiesterase inhibitors with chemotherapy in lung cancer

Kalliopi Domvri¹, Konstantinos Zarogoulidis¹, Nikolaos Zogas³, Paul Zarogoulidis¹✉, Savvas Petanidis⁴, Konstantinos Porpodis¹, Efrosini Kioseoglou³, Wolfgang Hohenforst-Schmidt²

1. Pulmonary Department-Oncology Unit, "G. Papanikolaou" General Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece
2. Sana Clinic Group Franken, Department of Cardiology / Pulmonology / Intensive Care / Nephrology, "Hof" Clinics, University of Erlangen, Hof, Germany
3. Gene and Cell Therapy Center, Hematology Department-Bone Marrow Transplantation Unit, "G. Papanikolaou" General Hospital, Thessaloniki, Greece
4. Department of Chemical Engineering, Aristotle University of Thessaloniki, Thessaloniki, Greece

✉ Corresponding author: Paul Zarogoulidis, M.D, Ph. D; Pulmonary Department-Oncology Unit, "G. Papanikolaou" General Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece. Fax: 00302310992424; Mobile: 00306977271974; E-mail: pzarog@hotmail.com

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Abstract

Purpose: Lung cancer remains the leading cause of cancer-related deaths worldwide and novel therapeutic approaches targeting crucial pathways are urgently needed to improve its treatment. Differentiation-based therapeutics (Methylxanthines) and phosphodiesterase inhibitors (type 4 and 5), have been implicated in cancer treatment. Our objectives were to capture any potential anti-tumor effect of these drug combinations with chemotherapeutic agents in vitro.

Methods: Theophylline as Methylxanthines, Roflumilast as phosphodiesterase type 4 (PDE4) inhibitor and Sildenafil as phosphodiesterase type 5 (PDE5) inhibitor are the drugs that we combined with the chemotherapeutic agents (Docetaxel, Cisplatin and Carboplatin) in vitro. Lung cancer cell lines (NCI-H1048-Small cell lung cancer-SCLC, A549- Non-small cell lung cancer-NSCLC) were purchased from ATCC LGC Standards. At indicated time-point, following 24h and 48h incubation, cell viability and apoptosis were measured with Annexin V staining by flow cytometry. Statistical analysis was performed by GraphPad Prism.

Results: In SCLC, following 48h incubation, platinum combinations of carboplatin with roflumilast and sildenafil ($p < 0.001$) and carboplatin with theophylline and sildenafil showed increased apoptosis when compared to carboplatin alone. Concerning the combinations of cisplatin, when combined with roflumilast, theophylline and sildenafil appeared with increased apoptosis of that alone ($p < 0.001$, 24h and 48h incubation). In NSCLC, the 24h incubation was not enough to induce satisfactory apoptosis, except for the combination of cisplatin with roflumilast and theophylline ($p < 0.05$) when compared to cisplatin alone. However, following 48h incubation, carboplatin plus sildenafil, carboplatin plus sildenafil, theophylline and roflumilast showed more cytotoxicity when compared to carboplatin alone ($p < 0.001$). Docetaxel combinations showed no statistically significant results.

Conclusion: The synergistic effect of PDE inhibitors with platinum-based agents has been demonstrated in lung cancer. Our suggestion is that these combinations could be used as additive and maintenance treatment in combination to antineoplastic agents in lung cancer patients.

Key words: lung cancer, theophylline, roflumilast, sildenafil, cisplatin, carboplatin, synergistic effect

Introduction

Lung cancer is still the most common type of cancer and the leading cause of cancer-related deaths

worldwide.[1-3]. Lung cancer is subdivided into two histological groups: Small Cell Lung Cancer (SCLC)

and Non-Small Cell lung Cancers (NSCLC) which accounts for almost 85% of all lung cancers.[4] Platinum-etoposide combination combined with thoracic and prophylactic cranial irradiation has been shown to improve outcome in limited-stage SCLC (TxNxM0) and in subgroups of extensive-stage SCLC (Tx,Nx,M1a/b), however, the clinical outcome for SCLC remains still discouraging.[5] Similarly, classical chemotherapy (platinum-doublet, taxanes, gemcitabine, pemetrexed) results in modest efficacy in NSCLC. At this point, the identification of molecular biomarkers such as EGFR inhibitors, have led to personalized therapy in NSCLC, but mechanisms of resistance remain to be elucidated.[6] Novel therapeutic approaches targeting crucial pathways are urgently needed to improve the treatment of lung cancer. Regarding neuroendocrine tumors with Ki-67 $\leq 20\%$ the formulation everolimus can be used.[7]

The aim of the multidrug therapy is a crucial treating option for lung cancer, though in several studies, multimodal therapeutic strategy resulted in superior efficacy, but at the expense of added toxicity.[8] [9] The success of multidrug therapy is based on acting simultaneously on different disease hallmarks.

Promising agents with antitumor action include cyclic nucleotide phosphodiesterase enzymes (PDEs) which are a large superfamily of enzymes that catalyze the hydrolytic breakdown of cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) that regulate various biological processes such as cell growth, energy homeostasis, muscle relaxation, and neuronal signaling.[10] Specifically, PDE-5, -6, and -9 are selective for cGMP and PDE-4, -7, and -8 are cAMP selective, whereas PDE-1, -2, -3, -10, and -11 are dual substrate-degrading isozymes.[11] According to several studies in various carcinomas such as breast cancers, colon adenocarcinoma, bladder squamous carcinoma, and lung cancers, it has been implied that PDEs may have antineoplastic effects by significantly lower cGMP levels than normal cells and may increase the specificity of a given chemotherapeutic agent.[1, 10, 12-15]

Roflumilast is the only PDE-4 inhibitor approved by the US Food and Drug Administration that targets inflammatory cells involved in triggering exacerbations of COPD.[16] It is catalyzed by cytochrome P450 1A2 and 3A4 to its active metabolite, roflumilast N-oxide, which is responsible for >90% of the total PDE-4 inhibitory activity of roflumilast.[17] Recently, it has been reported that roflumilast in the treatment of B-cell malignancies suppresses the activity of the oncogenic PI3K/AKT kinases, and

might have clinical activity in this setting.[18] In the same superfamily belongs Sildenafil, a PDE-5 inhibitor, which is used clinically for treating erectile dysfunction (ED) and pulmonary hypertension. Recently, sildenafil has shown to induce apoptosis in human tumors, such as colon carcinoma and chronic lymphocyte leukemia.[19, 20] Furthermore, PED5 expression has been shown to be increased on various cancers including breast, colon, bladder and lung cancer.[21-24] Besides sildenafil, other PED5 inhibitors are vardenafil and tadalafil.

Methylxanthines (MXs) are nonselective phosphodiesterase inhibitors, obtained from natural sources, contained in beverages (coffee, tea, etc.), whereas others have been used as therapeutic agents, and recently have been marked differentiative properties on tumor cells.[25, 26] The best known of these natural alkaloids are theophylline (TH, 1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), aminophylline (1,3-dimethyl-7H-purine-2,6-dione) and caffeine (1,3,7-trimethylxanthine). Theophylline, also known as 1,3-dimethylxanthine is being used as a bronchodilator drug in pulmonary diseases such as asthma or chronic pulmonary obstructive disease (COPD).[25, 27, 28] The main role of theophylline is acting as a non-specific adenosine antagonist, antagonizing A1, A2 and A3 receptors almost equally.[3] Investigated activities of theophylline derivatives, 7- and 8-positions include bronchospasmolytic[29, 30] anticancer, antimicrobial and circulatory blood system activity.[31] Concerning the antiproliferative and antimetastatic activities of TH, it has been reported to have beneficial effects alone or in combination with chlorambucil, in leukemia patients.[32, 33] Furthermore, TH was reported to negatively affect melanoma cell invasiveness in vitro and in vivo and to induce tumor cell differentiation through transglutaminase (TG, EC 2.3.2.13) activation.[34-36]

Taken together, these data have demonstrated a potential use of differentiation-based therapeutics such as Methylxanthines and phosphodiesterase inhibitors in cancer proliferation and apoptosis. In this study, we investigated the cytotoxicity of combined treatment of theophylline, roflumilast and sildenafil with platinum analogs or docetaxel on SCLC and NSCLC in vitro.

Materials and Methods

Cell cultures and reagents

The small cell lung cancer cell line [NCI-H1048 [H1048], ATCC® CRL-5853™) was purchased from ATCC LGC Standards. NCI-H1048 cells were cultured

in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM):F12 Medium culture medium, supplemented with 5% Fetal Bovine Serum (FBS) and with the following components, 0.005 mg/ml Insulin, 0.01 mg/ml Transferrin, 30nM Sodium selenite (final conc.), 10 nM Hydrocortisone (final conc.), 10 nM beta-estradiol (final conc.), extra 2mM L-glutamine (for final conc. of 4.5 mM), 5% fetal bovine serum (final conc.), all purchased from SIGMA. The non-small cell lung cancer cell line (A549, ATCC® CCL185™) was also purchased from ATCC LGC Standards. A549 cell line was cultured in DMEM, supplemented with 5% FBS, 1mM Penicillin-Streptomycin and 2mM L-glutamine, all purchased from SIGMA.

Lung cancer cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO₂ [37] and cultured in Coming's tissue culture flasks (25 and 75 cm²) according to the manufacturer's protocol. After cultures reached confluence, by microscope observation were then subcultured. Cells were detached with trypsin (1:250) 2.5 % and passaged. The indicated cell lines were seeded in 25 cm² flasks 0.7 × 10⁶ cells at a seeding density of 10⁶ cells for each cell. At confluence, at indicated time point, test compounds were added according to our protocol and after 24 h or 48h incubation apoptosis was measured (Table 1).

Table 1. Protocol of the experiment

Incubation of the drugs in lung cancer cell lines	
1.	Addition of chemotherapeutic agents alone for 2h
2.	Addition of PDE inhibitors alone for 2h
3.	Addition of combinations for 2h: chemotherapeutic agents (25µM) + PDE inhibitors (100µM) as follows: (sildenafil alone, roflumilast alone, theophylline alone, sildenafil + roflumilast, sildenafil + theophylline, roflumilast + theophylline, sildenafil + roflumilast + theophylline)
4.	After 24h and 48h incubation, apoptosis was measured

Test compounds

Drugs used in this study, include roflumilast (DAXAS® 500mg) as PED-4 inhibitor, sildenafil (Viagra® 50mg) as PED-5 inhibitor and theophylline as methylxines. These compounds were tested at concentration of 100µM. Docetaxel (10mg/ml, 140mg/5.5L), Carboplatin (10mg/ml, 350 mg/5.5L) and Cisplatin (1mg/ml, 90mg/5.5L) at concentration of 25µM are the chemotherapeutic agents that we combined with PDE inhibitors. All combinations were tested in triplicate (Table 1).

Analysis of the apoptotic cells with ANNEXIN V/ PI

Annexin V staining is used as a probe to detect cells that have expressed phosphatidylserine (PS) on

the cell surface, an event found in apoptosis as well as other forms of cell death. Propidium iodide (PI) is used as a DNA stain for both flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis [17], and microscopy to visualize the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells. The Annexin V kit used in this study was purchased from Immunostep (Salamanca, Spain). Data were acquired on a FACS Calibur (BD, Franklin Lakes, NJ, USA) instrument, and analyzed using the CellQuest Pro v6 software (BD) or FlowJo software vX.0.7 (Tree Star).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Multiple comparisons between experimental groups for one or more variables were performed using one- or two-way ANOVA, respectively, with Tukey's post-hoc test. Values of $p \leq 0.05$ were considered as statistically significant. All the analyses were undertaken using PRISM version 6.01 (GraphPad software, version 6, San Diego, CA, USA).

Results

In Small cell lung cancer cells, following 48h incubation, combinations of carboplatin with roflumilast and sildenafil ($p < 0.001$) and carboplatin with theophylline 3 and sildenafil showed increased apoptosis when compared to carboplatin alone (Table 3, Figure 1). Concerning the combinations of cisplatin, when combined with roflumilast, theophylline and sildenafil appeared with increased apoptosis of that alone ($p < 0.001$). In SCLC, the only combination that showed increased apoptosis following 24h incubation was cisplatin combined with roflumilast, theophylline and sildenafil that resulted in increased apoptosis when compared with cisplatin alone ($p < 0.001$) (Table 2, Figure 2). In Non-small cell lung cancer cells, the 24h incubation was not enough to induce satisfactory apoptosis, except for the combination of cisplatin with roflumilast and theophylline ($p < 0.05$) when compared to cisplatin alone (Table 4, Figure 4). However, following 48h incubation, carboplatin plus sildenafil, carboplatin plus sildenafil, theophylline and roflumilast showed more cytotoxicity when compared to carboplatin alone ($p < 0.001$) (Table 5, Figure 3). Docetaxel combinations with PDE inhibitors showed no statistically significant cytotoxicity when compared to them alone.

Discussion

Nowadays, novel combined chemotherapeutic treatments have led to a continuous improvement on

long-term survival and quality of life in patients in various cancers. However, new strategies are at need due to deleterious side effects and drug resistance obvious as poor efficacy for late stages of disease. More specifically, platinum containing drugs combined with other agents are used to treat a variety of cancer cell types, including lung cancer, as standard of care, showing significant effectiveness. Resistance to platinum-based drugs is a fact, affecting the efficacy and prognosis. The mechanisms of drug resistance are complicated, due to a variety of etiologies among others, such as abnormal expressions of membrane proteins, enhanced DNA repair functions, abnormal regulation mechanisms of apoptosis, and enhanced cellular detoxification function.[38]

The present study was designed to determine whether the addition of PDE inhibitors interacted with standard of care platinum-based chemotherapeutic agents to kill lung cancer cells in vitro. Our data show that in both lung cancer cell lines, platinum combinations with PDE inhibitors showed increased antiproliferative effect on lung cancer cell lines when compared to platinum monotherapy. In specific, the addition of sildenafil alone with cisplatin or carboplatin or with other PDE inhibitors (roflumilast and/or theophylline) combined, increased apoptosis in lung cancer cell lines. Cytotoxic effect of cisplatin exerts via the formation of mono-, inter-, and intra-strand cisplatin-DNA adducts, which can ultimately result in cell cycle arrest in G1, S, or G2-M phases [39-41] and induction of apoptosis.[42, 43] As for PDE-5 inhibitors, studies have reported that increase intracellular cGMP levels via their inhibition on cGMP-specific PDE5 [44, 45], suggesting that may be

effective pharmacological modulators in the cGMP pathway which has the map to enhance drug delivery to tumor tissues. Recently, Li et al demonstrated that PDE5 inhibition enhanced cytotoxicity likely due to the increase of drug uptake via endocytosis[46], suggesting it as adjuvant therapy for lung cancer. Besides, it has been reported that PDE5 inhibitors are overexpressed in many tumors, including lung, colon, breast, bladder, prostate and leukemia.[13, 47, 48] In the future we try and make a stratification with the expression of ki-67 in neuroendocrine tumors and the association with the efficacy of the drugs that we tested. Regarding the neuroendocrine tumors the ki-67 percentage plays a crucial role for treatment that should be followed. Nowadays if the percentage of the ki-67 is $\leq 20\%$ treatment with the pill everolimus can be provided, in the case of ≥ 21 then chemotherapy should be provided and when necessary along with radiotherapy.[7]

Furthermore, several studies have demonstrated that sildenafil showed antitumor activity in mouse models [49-52] and that PDE5 inhibition resulted in growth inhibition of tumor cells in vitro particularly by enhancing anticancer drugs.[20, 53-61] Also, sildenafil has been suggested as useful in mitigating the nephrotoxicity of cisplatin in rats.[2]

Other studies suggested that the role of sildenafil in enhancing the efficacy of anticancer drugs comes from the inhibition of ABCB1 (P-glycoprotein/P-gp) and ABCG2 (Breast Cancer Resistance Protein/BCRP).[62, 63] However, another study by Lin et al that performed an experiment on wild-type and ABCB1;BCG2 knockout mice to assess the potency and usefulness of sildenafil showed insufficient results for further clinical testing.[64]

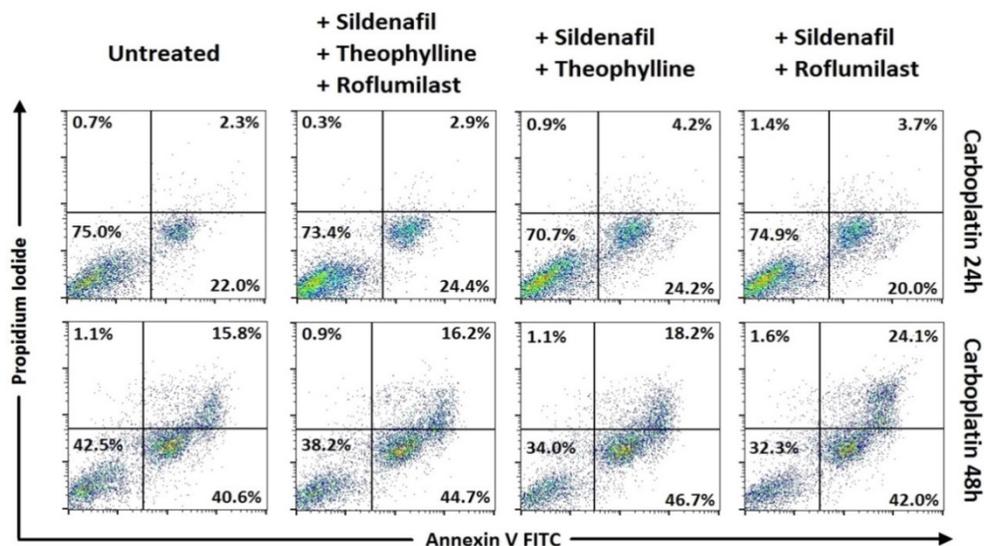


Figure 1. Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for SCLC. Combinations of phosphodiesterase inhibitors with carboplatin, with statistically significant differences.

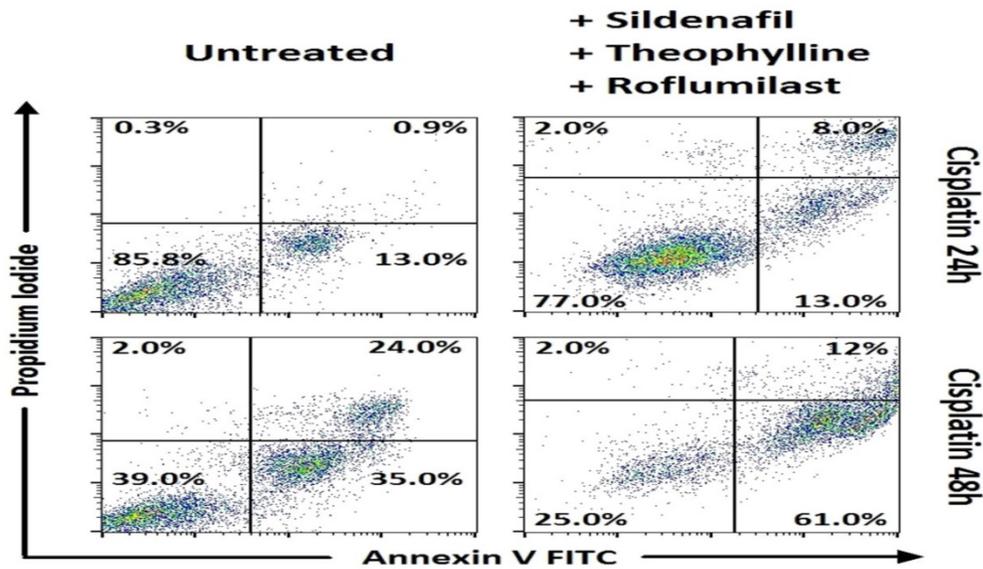


Figure 2. Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for SCLC. Combinations of phosphodiesterase inhibitors with cisplatin, with statistically significant differences.

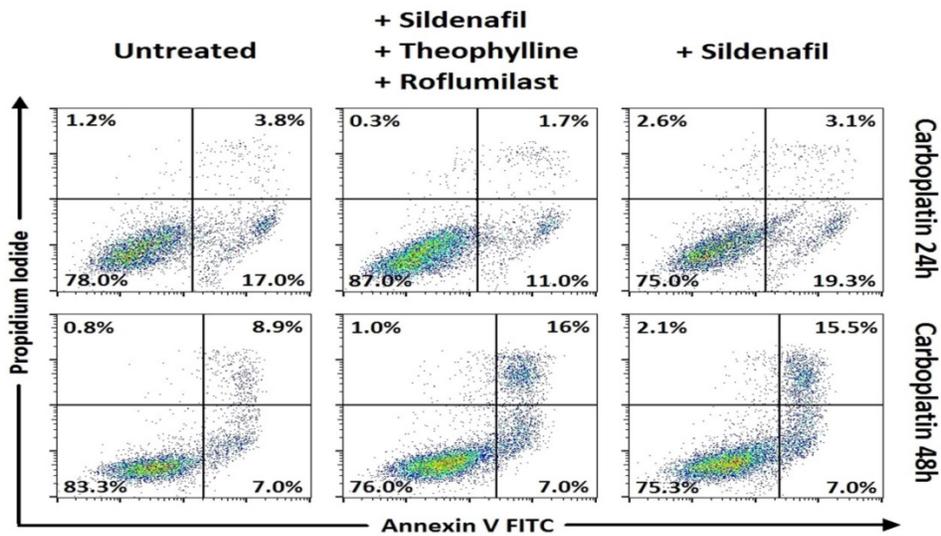


Figure 3. Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for NSCLC. Combinations of phosphodiesterase inhibitors with carboplatin, with statistically significant differences.

In addition, there are studies that have reported a concern about using PDE inhibitors. Based on evidence linking the use of PDE with increased risk of melanoma supported by recent studies [65-69], Pottegard et al conducted two parallel case-control studies, using the Danish Nationwide Health Registries (DNHR) and the Kaiser Permanente Northern California (KPNC) electronic health records, concluding to little evidence for a causal association between PDE inhibitors use and risk of melanoma.[70]

To our knowledge, this is the first report to combine various PDE inhibitors with chemotherapy in lung cancer cell lines. In our study, the addition of sildenafil plus theophylline with or without roflumilast combined with carboplatin or cisplatin

showed increased apoptosis when compared with platinum-based drugs alone. Despite the high pharmacological doses [71] and questionable protective effect against cisplatin nephrotoxicity [72], theophylline has been proposed as a potential anti-cancer drug combined with chemotherapeutic regimens for more than a decade.[36, 43, 73, 74] Specifically, the synergistic effect of theophylline with chemotherapeutic drugs comes from the induction of apoptosis and the interference with the cell cycle.[73] As for roflumilast, a number of publications have suggested the antitumor activity of this PDE4 inhibitor.[18, 75-77] According to researchers its anticancer action is related to its ability to influence glucocorticoid (GC) sensitivity in the malignant lymphocyte.[78]

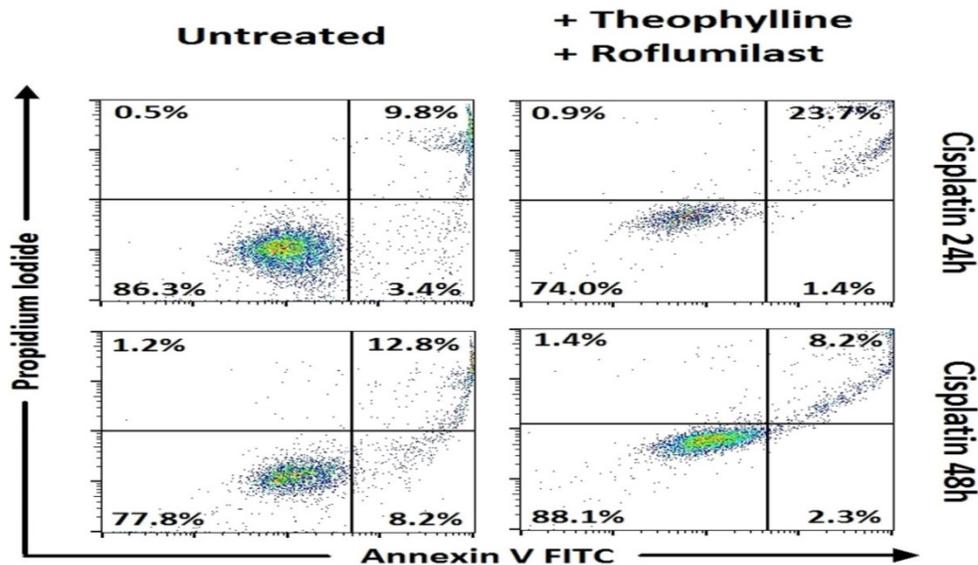


Figure 4. Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for NSCLC. Combinations of phosphodiesterase inhibitors with cisplatin, with statistically significant differences.

Table 2. Means ± standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry in SCLC cancer cell lines after 24h incubation with drugs (representative data).

Drug combinations	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Untreated cells SCLC	2.6±1.5	7.7 ±1.5	4.3±1.4	86.9±3.5
Carboplatin	1.7±3	2±2.5	21±1.5	74.5±3
Carboplatin + sildenafil + theophylline + roflumilast	1.3±1	3±3	23±2	71.4±3.2
Carboplatin + theophylline + sildenafil	1.6±1	4±2.3	23±1	71.1±3
Carboplatin + roflumilast + sildenafil	1±0.5	4±2	21±2.5	75.4±2.1
Cisplatin	1.3±2	1±1.5	12±3	85±2
Cisplatin + sildenafil theophylline + roflumilast	1.5±1	7.7±1.2	14±2.1	76±2.8

Besides platinum-based regimens, in our study, we combined docetaxel with the same PDE inhibitors (sildenafil, roflumilast, theophylline). However, we found no significant differences when compared to docetaxel monotherapy. This chemotherapeutic drugs stop mitosis in the M phase of the cell cycle, whereas platinum analogs are cell cycle non-specific agents.[40]

In conclusion, although the synergistic effect is documented, the benefit of PDE inhibitors on chemotherapy may be dependent on cancer types and

chemotherapeutics. Further laboratory research and clinical studies are required to fully understand the mechanisms of drug interaction and clinical utility of this therapeutic approach. We argue that the next step for our research could be to propose a phase I trial of these combinations to determine safe doses of the PDE inhibitors combinations to be followed by phase II trials in conjunction with platinum, in patients who have displayed platinum resistance. This perhaps would show the ability of these combinations to re-sensitize tumors to platinum and at the same time suggesting lower doses of chemotherapeutic drugs.

Table 3. Means ± standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry in SCLC cancer cell line after 48h incubation with drugs (representative data).

Drug combinations	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Untreated cells SCLC	1.5±1	3.9 ±1.3	4.2±1	90±3.2
Carboplatin	1±1.2	16±1.5	41±2	43±3.2
Carboplatin + sildenafil + theophylline + roflumilast	1±1	16±2.8	45±3.5	40±3
Carboplatin + theophylline + sildenafil	1.4±1.1	18±2	47±1.6	35±2.2
Carboplatin + roflumilast + sildenafil	2±0.9	25±4	41±1.1	33±2.8
Cisplatin	2.4±1.1	23.5±2.5	36±3	38±2.1
Cisplatin + sildenafil theophylline + roflumilast	1.8±1.1	10.5±2	62±2.2	25.4±2.9

Table 4. Means \pm standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/ Propidium iodide by flow cytometry in NSCLC cancer cell line after 24h incubation with drugs (representative data).

Drug combinations	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Untreated cells NSCLC	0.5 \pm 0.6	5.5 \pm 1.4	1.8 \pm 1	92 \pm 3.3
Carboplatin	2 \pm 1.1	3 \pm 1	16 \pm 2.2	79 \pm 3
Carboplatin + sildenafil + theophylline + roflumilast	1 \pm 0.8	1.5 \pm 1	12 \pm 2	86 \pm 3.1
Carboplatin + sildenafil	2.6 \pm 1.2	3 \pm 1.5	19 \pm 1.7	75 \pm 2.8
Cisplatin	0.5 \pm 0.8	9 \pm 1.5	3 \pm 1.4	88 \pm 3
Cisplatin + theophylline + roflumilast	0.5 \pm 1.1	24 \pm 4	1 \pm 2	75 \pm 3.5

Table 5. Means \pm standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/ Propidium iodide by flow cytometry in NSCLC cancer cell line after 48h incubation with drugs (representative data).

Drug combinations	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Untreated cells NSCLC	0.5 \pm 0.6	5.5 \pm 1.4	1.8 \pm 1	92 \pm 3.3
Carboplatin	0.9 \pm 1	8.9 \pm 2	7 \pm 1.9	83 \pm 3.1
Carboplatin + sildenafil + theophylline + roflumilast	1.1 \pm 0.5	17 \pm 1.3	6.5 \pm 2	75 \pm 3.3
Carboplatin + sildenafil	2 \pm 1	15 \pm 2.5	7.3 \pm 2.3	75 \pm 2.6
Cisplatin	0.5 \pm 0.9	11 \pm 2.5	10 \pm 2.3	79 \pm 2.5
Cisplatin + theophylline + roflumilast	0.8 \pm 0.9	7 \pm 1.9	2 \pm 0.8	91 \pm 3

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The abstract of this paper was presented at the 23rd Panhellenic Congress that took place in Thessaloniki, Greece. It was presented as poster with the following authorship/information: AB063. Potential synergistic effect by combination of PED-4 and PED-5 inhibitors plus theophylline in lung cancer? Ann Transl Med. 2016 Nov; 4(22): AB063. doi: 10.21037/atm.2016.AB063 <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5159351/>

Competing Interests

The authors have declared that no competing interest exists.

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