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Phosphodiesterase inhibitors as anti-cancer drugs

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Abstract

It is well known that high intracellular levels of cAMP can effectively kill cancer cells in vitro. Unfortunately substances elevating cAMP such as forskolin, 8-bromo-cAMP, 8-chloro-cAMP, monobutiryl or dibutiryl cAMP are not recommended to be used as anti-cancer drugs because of their high cytotoxicity. In contrast blockers of phosphodieterases such as theophylline and aminophylline, which could elevate intracellular cAMP, are commonly used as anti-asthma drugs reaching concentrations in the blood of 10–20 μg/ml. We tested the effectiveness of theophylline and aminophylline to induce cell death alone or in combination with common anti-cancer drugs such as cisplatin and gemcitabine (gemzar). We examined such drug combinations in the induction of cell death in a variety of carcinoma cell lines derived from human ovarian, prostate and lung cancer and in granulosa cell line transformed by SV40 and Ras oncogene. While theophylline could induce moderate cell death alone, at 20–25 μg/ml concentrations, aminophylline was ineffective at this concentration. Theophylline (at 15–25 ng/ml) was found in all four representative cell lines to synergize with gemcitabine or cisplatin to induce programmed cell death, which permits a reduction in the effective doses of cisplatin and gemcitabine by 2–3-fold. The effect of theophylline in induction of apoptosis involved reduction of intracellular levels of Bcl2. Such a reduction was proportional to the extent of apoptosis induced by theophylline as well as by the combined drug treatments. Therefore, we propose that theophylline should be considered as a potential anti-cancer drug in combination with other chemotherapeutic drugs. Screening of other phosphodiesterase blockers, which are not severely toxic, could open a possibility to improved chemotherapeutic cancer treatments with reduced undesired side-effects. A clinical trial, using theophylline as an anti-cancer drug, is currently being conducted in lung cancer patients.

Keywords: Apoptosis; Methylxantines; RAS; P53; MDM2 transformed granulosa cell line; Non-small cell lung cancer (NSCLC)

1. Introduction

Treatment of ovarian and other types of cancer with DNA-damaging agents is a common procedure [1–3]. Cisplatin exerts its cytotoxic effect via 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 [4–6] and the induction of apoptosis [7,8]. Unfortunately, drug concentrations used in chemotherapy are highly toxic also to normal cells and tissues [9]. Gemcitabine is a relatively novel anti-metabolite in clinical development that exhibits a broad spectrum of activity against leukemic cells and solid tumors, such as ovarian, lung and squamous carcinoma of head and neck, breast, pancreatic and colorectal cancers [10–13]. Its cytotoxicity is attributed to its ability to inhibit DNA synthesis and induce apoptosis [10,11]. Combination therapies of cisplatin and gemcitabine have proven effectiveness in cancer cells, demonstrating drug resistance to cisplatin alone, but unfortunately this drug combination is also toxic to normal cells and tissues, and therefore, could exert severe adverse side effects [14–17]. In an effort to reduce the dose and cytotoxicity of cisplatin, the drug was applied in experimental models with other drugs that elevate the intracellular levels of cyclic AMP, such as 8-bromo-cAMP or 8-chloro-cAMP [18] or the phosphodiesterase inhibitor theophylline, which is widely used to relieve asthma

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symptoms in humans [19–21]. Theophylline is rapidly, consistently, and completely absorbed when given in solution or as uncoated tablets that dissolve rapidly [22]. It is predominantly eliminated as metabolites formed by hepatic cytochrome P-450 isoenzymes, influenced by genetic factors, environmental agents (such as cigarette smoking) and various drugs [23]. To the best of our knowledge, there are no drug interactions between theophylline and cisplatin or gemcitabine. We have recently demonstrated that theophylline, when applied with cisplatin to ovarian carcinoma cell line or to granulosa cell line transformed with SV40 and Ras oncogene, can efficiently kill the cells [8]. By using pharmacological doses of theophylline, the effective concentrations of cisplatin and gemcitabine needed to kill the cells can be reduced by about 3-fold [8]. Unfortunately, a theophylline and cisplatin combination demonstrated severe nephrocytotoxicity in experimental animals [9]. On the other hand, aminophylline a more advanced anti-asthma drug did not elevate cisplatin cytotoxicity to kidney cells [9,24].

2. Ovarian cancer

Ovarian cancer is the fifth leading cause of all female cancer-related deaths in North America, and it is the most lethal of all gynecologic cancers. Most ovarian cancers arise from the surface epithelium, a single layer of cells that cover the surface of the ovary. These epithelial cells are thought to play a role in ovulation and are responsive to hormones, growth factors, and cytokines, but the cellular and molecular events associated with ovarian carcinogenesis are poorly understood (reviewed in [25]). The current therapeutic regimens for ovarian cancer are largely ineffective in terms of long-term treatment. Although chemotherapy is the preferred treatment option, with cisplatin and paclitaxol (Taxol) as first-line chemotherapeutic agents, chemoresistance remains a major therapeutic hurdle. Moreover, the mechanisms that underline chemoresistance are not completely understood (reviewed in [26]). We have observed that theophylline synergizes with cisplatin killing the cells via induction of apoptosis at low concentrations equivalent to the recommended dose for anti-asthma activity, lowering considerably the EC50 for cisplatin (Figs. 1 and 2). Moreover, this synergism was associated with marked suppression of Bcl2 expression, which was shown to occur also by theophylline alone, suggesting a novel mechanism for the cytotoxicity exerted by this drug combination [8].

Since ovarian carcinoma cells may demonstrate resistance to cytotoxic drugs, the effect of theophylline alone in comparable concentrations used as an anti-asthma drug was used in rat ovarian follicular (granulosa) cells transformed by SV40 and RAS oncogene [27] and in human ovarian carcinoma cell line [9]. We found that in both cell lines theophylline induced a modest but significant elevation in the incidence of apoptosis, a combination of theophylline and cisplatine at concentration of 1 μM dramatically elevated the incidence of apoptosis [8]. This was evident both by flow cytometry analysis using propidium iodide labeling [8] (Fig. 1), and by the TUNEL technique [8] (Fig. 2). It should be noted that the concentration of theophylline used was in the pharmacological range for human asthma patients (5 μg/ml and 15 ng/ml, see Fig. 1B) while cisplatin concentrations were far below its ED50% lethal activity (7 μM), in the low range of concentration generally used for chemotherapy [16,28]. Also it was found that theophylline could synergize efficiently with gemcitabine (gemzar), which has been used recently in chemotherapy for the treatment of ovarian cancer especially in cells that were resistance to cisplatin [29–33]. Aminophylline, a derivative of theophylline, replacing theophylline as anti-asthma drug, did not show any synergistic effect on killing ovarian cancer cells below 80 μg/ml, which makes it an unfavorable candidate for use as an anti-cancer drug. Interestingly a combination of cisplatin and theophylline in pharmacological doses, used in our experiments did not enhance apoptosis in human derived non-transformed endothelial cell line (ECV304) [34,35]. This cell line is also used as a model for neovascularization, and therefore, it could be used as a good control for the modest effect of this drug combination on endothelial derived cells. In searching for the molecular mechanism underlying the apoptotic effect of theophylline alone or in combination with cisplatin, it was found that the survival gene Bcl2 was dramatically suppressed while expression of Bax and P53 remained unchanged [8] (Fig. 3). These studies delineate the potential of theophylline to be used as an anti-cancer drug in ovarian cancer, especially in these cancer cells that became resistance to some classical anti-cancer drugs such as cisplatin and taxol. This goal apparently could be achieved without raising the cytotoxicity in normal cells. Moreover, co-treatment of cancer patients with theophylline and chemotherapeutic drugs may eventually make it possible to lower the dose of anti-cancer drugs to cure ovarian cancer, and minimize negative side effects associated with chemotherapy.

3. Lung cancer

There are some indications that high intracellular level of cAMP could arrest growth, induce apoptosis and attenuate cancer cell migration [35–39]. Cholera toxin, which could trigger elevation of intracellular cAMP levels, could also trigger apoptosis in human lung cancer cells [40]. Reduced DNA synthesis and cell viability has also been inspected in small carcinoma lung cell (SCLC) by treatment with the phosphodiesterase inhibitors such as IBMX, theophylline, caffeine of SCLC cells. However, relatively high concentrations of these drugs were used, which probably are toxic for in vivo experiments [41]. Also,

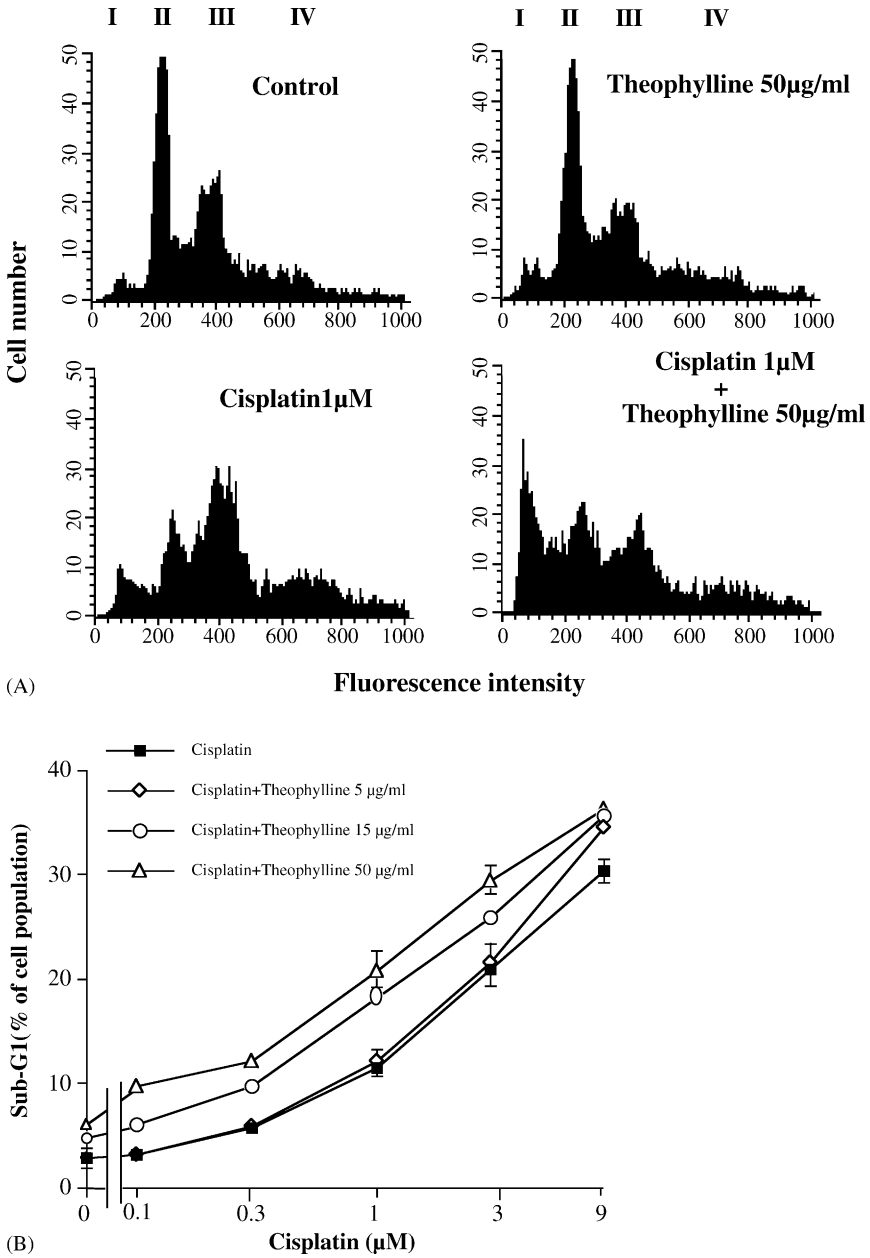
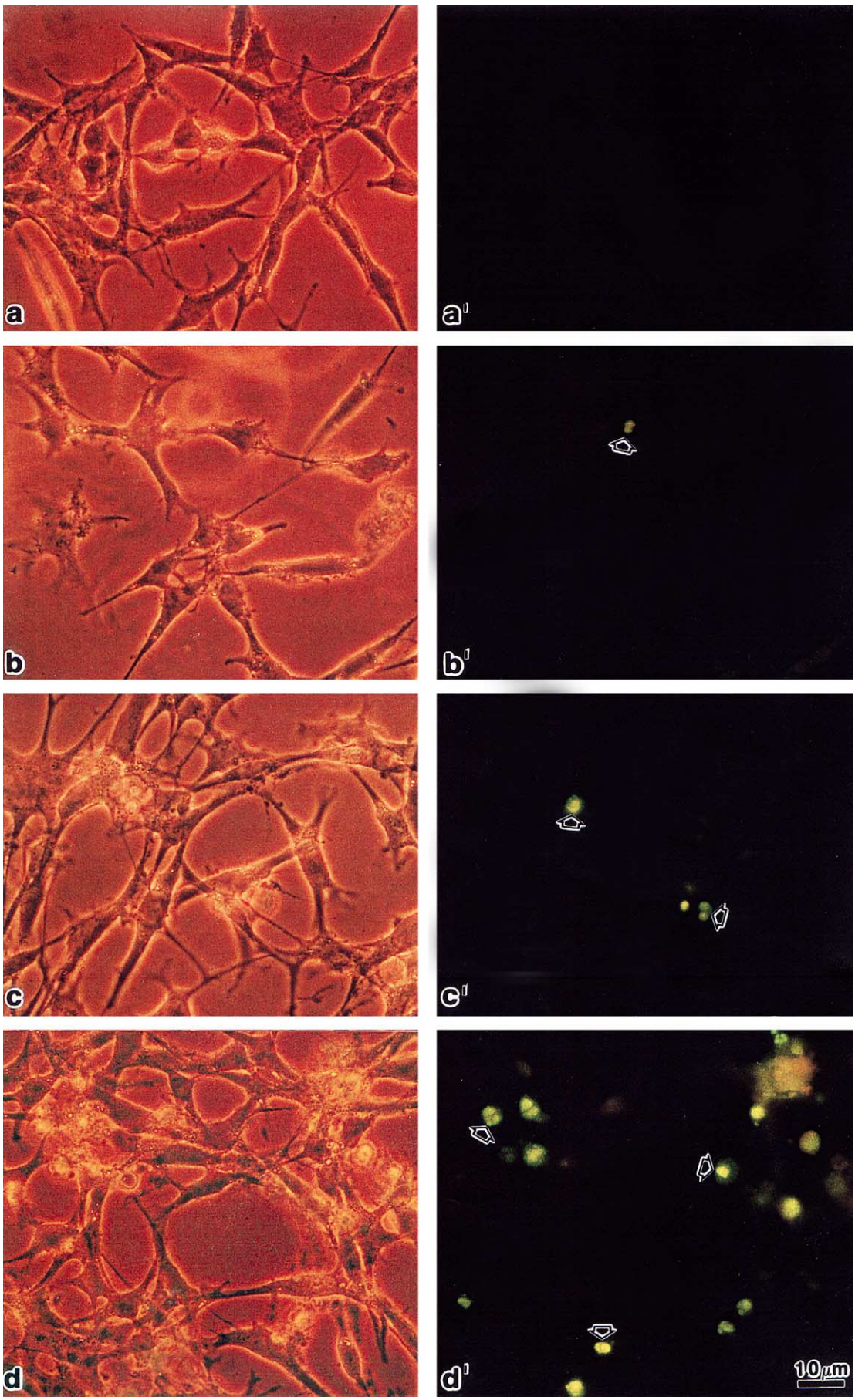


Fig. 1. Synergistic induction of apoptosis by theophylline and cisplatin in HRP-53-10 cells. DNA breakdown in HRP-53-10 cells during apoptosis. Cells were incubated with each concentration (5, 15 and 50 µg/ml) of theophylline singly and in the presence of increasing concentration of cisplatin (from 0.1 to 9 µM) for 24 h in DMEM/F12 medium containing 5% FCS. Cells were fixed with methanol, stained with propidium iodide and examined by FACS. The data in B are expressed as the sub-G1 population, which serve as an index for the incidence of apoptosis. Data in panel (A) were taken from representative points in panel (B). Values of cisplatin-theophylline (15 and 50 µg/ml) treated cells are significantly different from cells treated with cisplatin alone at 0.1–9 µM ($P < 0.001$) (from [8] by permission).

theophylline administration to mice reduced hepatic and pulmonary colonization of B16 melanoma cell line. In another study cAMP was found to decrease chemotaxis, invasiveness and lung colonization of H-ras transformed mouse fibroblasts which exhibit a highly malignant phenotype with the ability to produce large tumors and to colonize the lung after tail vain injection [42]. The drugs in the above study used relatively high, and toxic doses. Therefore, this study examined if theophylline and aminophylline could arrest growth and induced apoptosis in typical non-small lung carcinoma cells (NSCLC) (H1299), using low pharmacological doses of theophylline and aminophylline [43].

Moreover, since cancer cells and in particular NSCLC can become refractory to widely used chemotherapeutic drugs such as cisplatin [44–46], we tested the drug combination of cisplatin and gemcitabine (Gemzar) with theophylline or aminophylline. It was found that theophylline induced apoptosis in the cultured H1299 cell line at concentrations as low as 30 µg/ml, reaching an ED50% at 100 µg/ml. In contrast, aminophylline induced apoptosis at concentrations of 300 µg/ml, and 17% apoptosis was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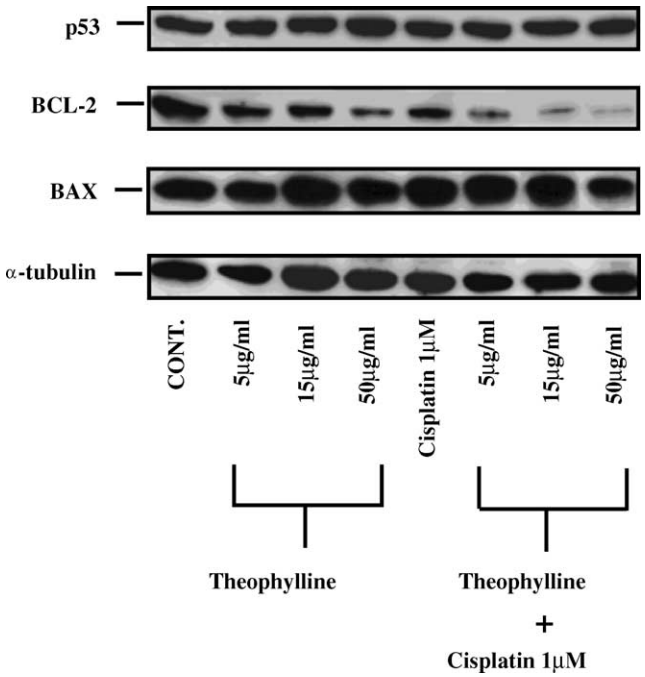

Fig. 3. Expression of p53, Bcl-2 and Bax in controls, theophylline and/or cisplatin-treated HRP-53-10 cells. Cells were incubated with each concentration (5, 15 and 50 μg/ml) of theophylline singly and in the presence of 1 μM concentration of cisplatin for 24 h in DMEM/F12 medium containing 5% FCS. Cell lysates were prepared, and Western blot analysis was performed using specific antibodies to p53 (PAb421 protein expression directed against human and mouse), Bax and Bcl2.

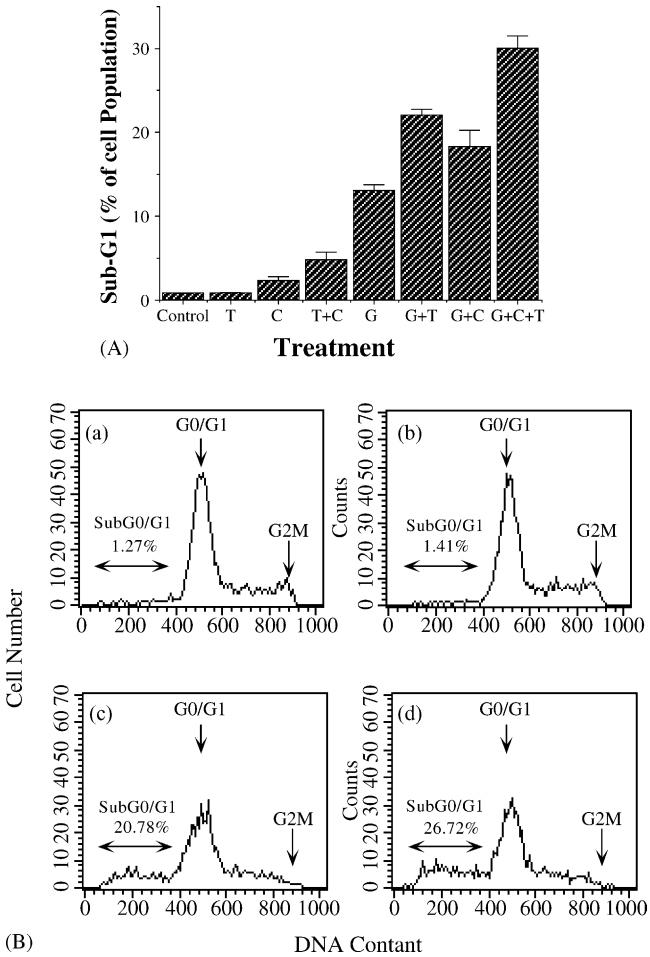

Fig. 4. The effect of theophylline with different drug combination on induction of apoptosis in H1299 non-small lung carcinoma. Concentration: theophylline (T), 20 μg/ml; cisplatin (C), 0.3 μg/ml; gemcitabine (G), 10 ng/ml. (A) Data were obtained from triplicate plates for each treatment (mean ± S.D.). Incidence of apoptosis was determined by FACS analysis following labeling of DNA with propidium iodide. (B) DNA profile of selected treatments following FACS analysis, which was repeated three times with essentially similar results. a: control, b: T, c: G + C, d: G + C + T.

evident at concentrations as high as 900 μg/ml, which is a lethal dose for in vivo treatment. Cisplatin induced apoptosis with ED50% of 0.8 μg/ml, while gemcitabine induced apoptosis with ED50% of 20 ng/ml [43]. Using a combination of 20 μg/ml of theophylline (calculated as an effective but not toxic anti-asthma drug) with 10 ng/ml gemcitabine or with 0.3 μg/ml cisplatin significantly elevated incidence of apoptosis compared to gemcitabine or cisplatin alone at similar concentrations (Figs. 4 and 5). In contrast, an observed synergistic effect between aminophylline and gemcitabine was evident only at concentrations of 80 μg/ml and 10 ng/ml, respectively. However, no effect was apparent in combination doses of aminophylline (80 μg/ml) with cisplatin (0.3 μg/ml) [43]. The combined treatments involved reduction in the intracellular level of the anti-apoptotic Bcl2 gene product. This corresponded with the extent of apoptosis induced by the various drug combinations. Thus, theophylline was found significantly more effective than aminophylline in increasing the sensitivity of the H1299 lung cancer cells to the induction of cell death by gemcitabine and cisplatin. Combination of theophylline with these drugs may permit a reduction in the effective dose needed in chemotherapy treatment of lung cancer patients [43]. Interestingly, it was recently found that theophylline can arrest growth of prostate cancer cells and induce apoptosis in combination with anti-cancer drugs.

Non-small cell lung cancer (NSCLC) remains one of the most common and fatal cancers in humans. The cure rate is low while the mortality rate is high. Most of the patients

Fig. 2. Apoptosis in HRP-53-10 cells incubated with theophylline and/or cisplatin. Cells were incubated at 37 °C with 5% FCS for 24 h (a, a′), in the presence of 50 μg/ml of theophylline (b, b′), or in the presence of 1 μM cisplatin (c, c′), or with a combination of theophylline and cisplatin at the above concentrations (d, d′). Cells were fixed with 3% paraformaldehyde and stained for apoptotic nuclei according to the TUNEL method. (a–d) Phase contrast microscopy; (a′–d′) fluorescent microscopy of identical fields. Note low incidents of apoptosis nuclei (green fluorescence, *wide arrows*) subsequent treatment with theophylline or cisplatin alone and high incidence of apoptotic nuclei in cell cultures co-stimulated with theophylline and cisplatin (from [8] by permission).

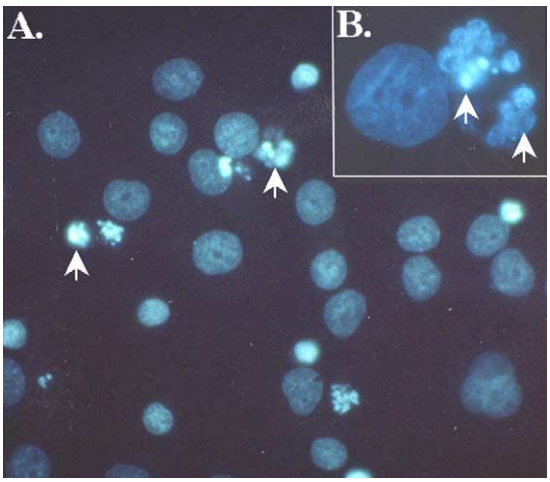

Fig. 5. Synergistic effect of gemcitabine (10 ng/ml) and theophylline (20 μg/ml) on apoptosis in H1299 cell line. Cells were fixed with 4% paraformaldehyde, treated with DAPI and photographed with a Zeiss microscope. Arrows indicate apoptotic nuclei. Magnification in (A) 800× and in (B) 1800×.

diagnosed are in advanced stages, i.e. IIIB or IV, in which the disease is no longer curable, although it is amenable to chemotherapy with or without radiation therapy [47]. Chemotherapy has been advocated in non-small cell lung cancer since it provides superior care in terms of survival and cost. However, the absolute survival rate is not pronounced, namely an additional 1.5 months, and a 10% increase in one year survival [48]. New hope for successful treatment of NSCLC has been generated by the introduction of new drugs such as gemcitabine. Monotherapy or cisplatin-based combined chemotherapy with these agents has shown improved care in terms of survival and quality of life [49]. Cisplatin and gemcitabine combination have been recently accepted as a treatment option for NSCLC. The reported response rates range between 40–50% and 1-year survival rates range between 35–60% [49]. The side effects of the cisplatin and gemcitabine combination include nausea, vomiting, fever, malaise, nephrotoxicity, neurotoxicity, bone marrow depression, alopecia, and others. Improving the response rate to chemotherapy and decreasing the clinically significant side effects, are the aims of medical oncology in the 21st century. Theophylline may improve the symptoms of dysponea by its action as a bronchodilator as well as an anti-cancer drug. In review of our observation concerning the synergistic apoptotic effect between gemcitabine and theophylline in the H1299 lung cancer cell line [43], the theophylline plus gemcitabine combination may be as effective as cisplatin plus gemcitabine, and this may have important clinical implications. In preliminary experiments we tested the effect of theophylline alone or in combination with relatively low concentrations of cisplatin and/ or gemcitabine on survival of prostate cancer cells. Theophylline synergized significantly with the other drugs to induce apoptosis in these cell (Hirsh et al., unpublished observations).

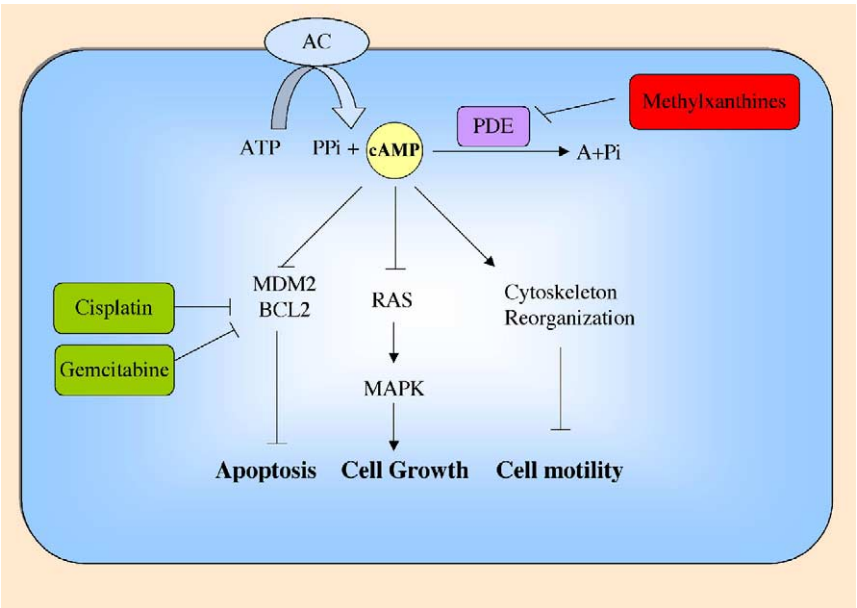

Fig. 6. Possible mechanism of methylxanthines, such as theophylline and aminophylline as anti-cancer drug. Methylxanthines block phosphodiesterase (PDE), which may down regulate the formation of cyclic AMP (cAMP) catalyzed by adenylate cyclase (AC). Elevated levels of cAMP as a consequence of PDE inhibition may down regulate MDM2, which is known to block the apoptotic stimuli generated by p53 [51,52]. Theophylline, cisplatin and/or gemcitabine may synergize in down regulation of the survival protein Bcl-2 [43,54], thus stimulate apoptosis in the cancer cells. High intracellular levels of cAMP induced by methylxanthines can down regulate RAS/MAPK signaling leading to arrest cell growth. High levels of cAMP, induced by methylxanthines inhibition of PDE, may lead to cytoskeleton reorganization and block of cell motility [55]. Thus methylxanthines may affect apoptosis, growth and spreading metastasis of the cancer cells.

4. Mechanism of methylxantine arresting of cancer cell growth and its relevance to treatment of human cancer

The exact molecular mechanism by which methylxantines could arrest cancer cell growth, and attenuate metastatic spread is still obscure. This group of compounds, which are potent inhibitors of phosphodiesterase activity, could elevate intracellular levels of cAMP (Fig. 6). Elevation of cAMP in a variety of cancer cells may suppress RAS activity [50] and as a consequence it could reduce the constitutive activity of MAPK, which could be relatively high in cancer cells. On the other hand, cAMP could attenuate Bcl2 [8] and MDM2 [51,52] intracellular levels. Reduction in Bcl2 expression, which is considered as a survival factor, and MDM2, which blocks the proapoptotic activity of P53, could lead to enhanced apoptosis (Fig. 6). Attenuation of cell migration by theophylline may also be exerted by cAMP, which can lead to cytoskeleton reorganization via specific phosphorylation of specific components of the microtubular network [53–55]. It is hoped that treatment of patients suffering from ovarian cancer, lung cancer and prostate cancer with combination of theophylline therapies will enable dosage lowering of cisplatin and gemcitabine, thereby reducing side effects and increasing anti-cancer activity. This treatment would apply up to 20 μg/ml of theophylline in the blood and body fluid [19,21,56], but significantly reduce the necessary amount of the anti-cancer drugs and their undesirable side effects. Further controlled randomized clinical studies are necessary to validate the in-vitro assays reported.

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