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DIFFERENTIAL EFFECT OF SELECTED METHYLXANTHINE DERIVATIVES ON RADIOSENSITIZATION OF LUNG CARCINOMA CELLS

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Aim: **Using caffeine as a reference derivative, this study was performed to investigate how other methylxanthine derivatives, theophylline, 3-isobutyl-methylxanthine and 1,3-dipropyl-7-methylxanthine, sensitize cells to radiation by modifying cell cycle checkpoints and inducing the apoptotic response. The effect of the methylxanthine derivatives was studied in response to gamma and ultraviolet radiation in a human large cell lung carcinoma cell line, null for p53, a normal lung epithelial cell line and the large cell lung carcinoma cell line stably transfected with p53.** ***Methods:*** **Effects of theophylline, 3-isobutyl-methylxanthine and 1,3-dipropyl 7-methylxanthine on cell-radiosensitization in comparison to caffeine tested by clonogenic survival assay, MTT assay, ELISA based apoptotic assay, flow cytometry, caspase-3 activity, TUNEL assay, and western blot analysis.** ***Results:*** **All the derivatives, except 3-isobutyl-methylxanthine, increased tumor cell sensitization to radiation by inducing apoptosis in the p53-null lung cancer cell line. The pattern of cell cycle progression revealed that these derivatives increased the number of cells in G1 phase by abrogating the G2/M checkpoint, directing the cells to apoptose through a p53-independent mechanism. In contrast, 3-isobutyl-methylxanthine was more potent than the other derivatives in radiosensitization of normal lung epithelial cells and the lung carcinoma cells stably transfected with wild-type p53. IBMX increased p53 protein level more than caffeine in lung carcinoma cells stably transfected with wild-type p53.** ***Conclusion:*** **Our results suggest that 3-isobutyl-methylxanthine might function through a p53-dependent mechanism.**
Key Words: **3-isobutyl-methylxanthine, caspase-3, p53, cell cycle, lung cancer.**

Lung cancer is the major cause of cancer death in the world and accounts for over 150,000 deaths annually in the United States [1]. Although extensive research is being done, lung cancer therapeutic outcomes have not significantly improved [2]. Non-small cell lung carcinoma (NSCLC) comprises about 75% of lung cancer cases and consists of adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. About 40% of patients with NSCLC undergo surgical resection of a localized tumor with curative intent. However, the majority of patients are diagnosed in advanced stages of the disease, which is usually less sensitive to chemotherapy or radiation than small cell lung carcinoma [3, 4]. Thus demands for efficient therapy are needed to control the growth and multiplication of lung carcinoma. The combination of radiotherapy and chemotherapy is an effective strategy to control the primary tumor, and diminish the chances of metastases [5, 6].

Radiation and many chemotherapeutic drugs damage DNA, resulting in a block in the cell cycle at G2/M, to repair the damaged DNA. Cell cycle checkpoints might contribute to the development of drug resistance, posing a formidable limitation in current cancer treatment [7]. Presumably, genotoxic agents that override cell cycle checkpoints could be used to sensitize cells towards more cell death [8, 9]. Caffeine, a methylxanthine, potentiates the lethal effects of toxic agents by overriding the G2/M checkpoint [10–13].

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Abbreviations used: ATM – ataxia telangiectasia mutated protein; ATR – ataxia telangiectasia related protein; DPMX – 1,3-dipropyl-7-methylxanthine; IBMX – 3-isobutyl-methylxanthine; NSCLC – non-small cell lung carcinoma.

It does so by inhibiting ataxia telangiectasia mutated protein (ATM) and ataxia telangiectasia related protein (ATR) [12] that are induced in response to gamma and ultraviolet (UV) radiation. p53, a downstream target of ATM and ATR, is a tumor suppressor protein that functions at G1/S and G2/M [14] and is an attractive target for cancer therapy. The override of the G2 block by caffeine is decreased in cells with wild-type p53 compared to those with mutant p53, making cancer cells with nonfunctional p53 more sensitive to radiation [15]. However, other groups showed that transfecting cells with wild-type p53 make cells more sensitive to irradiation [16–18].

The use of caffeine in sensitizing cells to radiation has been well documented in many cell lines [11, 19, 20]. However, the effect of other derivatives of methylxanthine on radiation-induced apoptosis of tumor and normal cells has not been characterized. Thus, we wanted to determine if other derivatives could also increase radiosensitivity, perhaps better than caffeine, and sensitize differentially between normal and tumor cells with different p53 genotypes. For the study, four different methylxanthine derivatives, caffeine, theophylline, 1,3-dipropyl-7-methylxanthine (DPMX), and 3-isobutyl-methylxanthine (IBMX), were tested for their potency at inducing apoptosis in a large cell lung carcinoma cell line, H1299 (null for p53), exposed to various doses of gamma and UV radiation. The response was compared to NL-20, a normal lung epithelial cell line, and p53-transfected H1299 stable cell line.

Theophylline, a nonspecific phosphodiesterase inhibitor that has bronchodilator effects, is used in the treatment of bronchial asthma and is a potential anti-inflammatory agent [21]. IBMX, also a phoshodiesterase inhibitor, affects many signal transduction pathways, such as cellular metabolism, including re-

lease of Ca^{2+} from intracellular stores [22], antagonism of adenosine receptor, and inhibition of phosphodiesterases [23]. DPMX is an antagonist of A2 adenosine receptors that are stimulated by adenylate cyclase in membranes of rat pheochromocytoma PC12 cells and human platelets [24]. The structures of the selected methylxanthine derivatives are illustrated in Fig. 1.

We investigated the effects of theophylline, DPMX, and IBMX on cell-radiosensitization in comparison to caffeine by clonogenic survival assay, MTT assay, ELISA based apoptotic assay, flow cytometry, TUNEL assay and caspase-3 activity and western blot analysis. We found that theophylline and DPMX increased the sensitivity of H1299 cells to radiation whereas IBMX did not have an effect. However, IBMX increased radiosensitization in normal lung epithelial and stably p53-transfected H1299 cells suggesting its function may be dependent on p53.

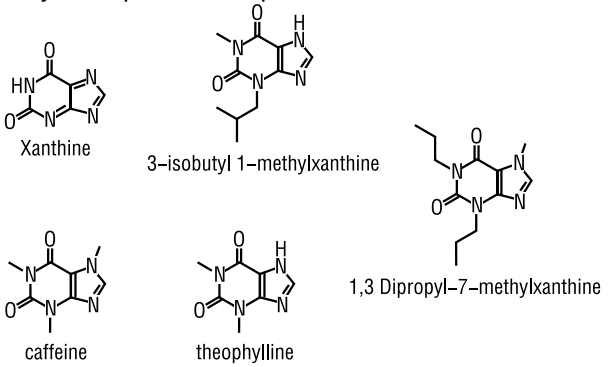

Fig. 1. The structure of selected methylxanthine derivatives

MATERIALS AND METHODS

Cell culture and drug treatment. H1299 cells that have a deletion of the p53 gene were derived from a human large cell lung carcinoma (provided by Jack Roth, M.D. Anderson Cancer Center). Cells were maintained in Dulbecco's modified essential media (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum, 100 Units/ml penicillin and 100 µg/ml streptomycin at 37 ˚C in a 5% CO_2 atmosphere (Gibco, USA). NL-20, a normal lung epithelial cell line (ATCC, USA) was maintained in DMEM/F-12 media (Gibco, USA) supplemented with 10% FBS, 100 Units/ml penicillin, 100 µg/ml streptomycin, insulin (1.2 g/l), transferrin (0.001 mg/ml), EGF (20 ng/µl) and hydrocortisone (500 ng/ml) at 37 ˚C in a 5% CO_2 atmosphere. H1299 cells were stably transfected with wild-type p53 and maintained in supplemented DMEM described above. Cells were transfected using Lipofectin (Invitrogen, USA) and stable transformants were selected with G418 (300 mg/L). Caffeine, theophylline, IBMX, and DPMX (Sigma, USA) were dissolved in cell culture media at concentrations of 1 mM and 4 mM.

Clonogenic survival assay. Cell survival following gamma and UV radiation was measured by clonogenic assays in monolayer in 10 cm plates. Cells were plated in triplicate and were grown for 2 days to approximately 70% confluency. The methylxanthine derivatives were dissolved in culture media at a final concentration of 4 mM and added to the plated cells 30 min prior to irradiation. Cells were irradiated with different doses of gamma radiation ranging from 2.5–10 Gy from a ^{137}Cs source (J. L. Shepherd and Associates, CA, USA) and UV radiation (10–30 J/m^2) using a 250 nm source (UVP Inc, CA, USA). After 24 h, the media containing the derivatives was removed and replaced with fresh media without the derivatives. The cells were grown for 14 days to monitor colony formation. Colonies of at least 50 cells were scored as clonogenic survivors. The surviving fraction was determined by the proportions of seeded cells that formed colonies after drug and radiation treatment relative to the control cells, which were irradiated either with gamma or UV radiation without prior drug treatment. Each data point was derived from the results of three independent experiments and expressed as mean ± standard deviation.

Cell viability was measured by the methyl tetrazolium (MTT) bromide mitochondrial activity assay as described previously (ATCC, Manassas, USA) [25]. Briefly, 4000–5000 cells/well in 100 µl of medium were seeded in a 96-well plate for 24 h prior to drug treatment. The media was then changed to media with derivative compounds and cells were treated with either gamma radiation (2.5 Gy) or UV radiation (10 J/m^2). After 24 h, 10 µL of 5 mg/ml MTT reagent was added to each well and incubated for 4 h. After incubation, 100 µl of detergent reagent was added to each well to dissolve the formazan crystals. The absorbance was determined at 570 nm. Cells treated only with irradiation were used as controls. Each assay was performed in triplicate and standard deviation determined.

Flow cytometric analysis. Cells were seeded at a density of 3–5 X 10^5/ 10 cm plate and incubated for 24 h before radiation. Media was changed to media containing the different derivatives 30 min before irradiation. Cells were exposed to 2.5 Gy gamma radiation or 10 J/m^2 UV radiation. 24 h after drug and radiation treatment, cells were harvested by trypsinization. The cells were washed with PBS and fixed with ice-cold 70% ethanol while vortexing. Finally, the cells were washed and resuspended in PBS containing 5 µg/ml RNase A (Sigma, USA) and 50 µg/ml propidium iodide (Sigma, USA) for analysis. Cell cycle analysis was performed using FACScan Flow Cytometer (Becton Dickinson) according to the manufacturer's protocol. Windows multiple document interfaces (WinMDI) software was used to calculate the cell-cycle phase distribution from the resultant DNA histogram, and expressed as a percentage of cells in the G0/G1, S and G2/M phases. The apoptotic cells were determined on the DNA histogram as a subdiploid peak.

Enzyme linked immunosorbent apoptosis assay. Cells were seeded at a density of 2 x 10^4/ well in a 96-well plate and incubated for 24 h. Media was changed to media containing the different derivatives 30 min before irradiation. Cells were exposed to 2.5–10 Gy gamma radiation or 5–30 J/m^2 UV radiation and then incubated for 24 h. An ELISA assay was performed using Cell Death Detection ELISA^{PLUS} kit (Roche-Applied Science, USA) that measures histone release

from fragmented DNA in apoptotic cells. Briefly, cells were lysed with 200-µl lysis buffer for 30 min at room temperature. The lysate was centrifuged at 200 g for 10 min. 150 µl of supernatant was collected, of which 20 µl was incubated with anti-histone biotin and anti-DNA peroxidase at room temperature for 2 h. After washing with incubation buffer three times, 100 µl of substrate solution (2,2`azino-di(3-ethylbenzthiazolin-sulphuric acid) was added to each well and incubated for 15–20 min at room temperature. The absorbance was measured using an ELISA reader (Spectra Max Plus) at 405 nm. The control group were cells treated with either UV or gamma radiation. Each assay was done in triplicate and standard deviation determined.

Caspase-3 activity. Caspase-3 activity was assayed according to manufacturer's protocol (Assay designs, USA). Briefly, $5x10^6$ cells were lysed in 100 µl lysis buffer containing 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM, 350 µg/ml PMSF and 5 mM DTT. Cell were homogenized by three cycles of freezing and thawing and then centrifuged to remove the cellular debris. Each sample was then incubated in buffer containing 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM EDTA supplemented with Ac-DEVD-AFC for 1 h at room temperature and then reaction was stopped with 1 N HCl. OD_{405} was measured using a spectrophotometer (Spectra Max Plus).

TUNEL and DAPI staining. For *in situ* detection of apoptotic cells, TUNEL assay was performed using DeadEnd™ fluorimetric tunnel system (Promega, USA). Cells were cultured on 4-chamber slides (VWR, USA) at a density of $2x10^4$ cells/chamber. After treatment with derivatives and 2.5 Gy of gamma irradiation, cells were washed with PBS and fixed by incubation in 4% paraformaldehyde (PFA) for 20 min at 4 °C. The fixed cells were then incubated with digoxigenin-conjugated dUTP in terminal deoxynucleotide transferase recombinant (rTdT)-catalyzed reaction and nucleotide mixture for 60 min at 37 °C in a humidified atmosphere and then immersed in stop/wash buffer for 15 min at room temperature. Cells were then washed with PBS to remove unincorporated fluorescein-12-dUTP. After washing with PBS, cells were incubated in 1 µg/ml DABI solution for 15 min in dark (data not shown). Cells were observed with fluorescence microscopy (RT slider Spot, Diagnostic Instruments, Inc) and photographed at 100X magnification.

Transfection of p53. H1299 cells were transfected with pCMV-p53 (human) plasmid using lipofeactamine (Gibco, BRL) as recommended according to manufacturer's protocol. Briefly, solution A and solution B (Lipofectin reagents, Invitrogen) were mixed and incubated for 20 min. The lipofectin-DNA solution was added to cells, plated at $1x10^5$/10 cM plates and incubated for 6 h at 37 °C in a CO_2 incubator. The media was aspirated and changed to normal media. G418 (300) mg/l (Gibco, BRL) was added to the culture to select from transfected cells. After 3 weeks, single independent cell clones were isolated and each clone was plated independently.

Western blot analysis. The expression of p53 was tested by Western blotting analysis using H1299 (p53-null cells) as control. The samples were normalized and the loading dye (50 mM Tris-Cl pH 6.8, 100 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added. The samples were heated at 95 °C for 5 min and the proteins separated on SDS-PAGE. Then, the proteins were transferred to Polyvinylidene Difluoride (PVDF) transfer membrane (Millipore Corporation, MA). The proteins were transferred onto the membrane using a semi-dry transfer apparatus (OWL). The membrane was blocked with 5% non-fat milk in PBS containing 0.25% Tween-20 (Sigma-Aldrich, MO) (PBS-T) at room temperature (RT) for 1 h and then incubated with the appropriate primary antibodies (Bp-53 monoclonal mouse antibody, Santa Cruz Biotechnology) at a 1 : 500 dilution in 5% milk PBS-T overnight at 4 °C. The membrane was washed quickly three times and then two 5 min wash and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (goat anti mouse antibody, Santa Cruz Biotechnology) at a 1 : 20,000 dilution in 5% milk PBS-T for 1 h at RT. The membrane was washed in PBS-T, three quick washes and three 15 min washes to remove unbound antibodies and proteins were detected by autoradiography using the ECL Advanced Western Blotting Detection Kit (Amersham Biosciences, NJ).

Statistical analysis. All data were analyzed by Student's *t*-test and expressed as mean ± standard deviation. A P-value < 0.05 was considered statistically significant. All of the experiments were done in triplicate.

RESULTS

Effect of methylxanthine derivatives on cell survival after radiation. Cell survival was measured by clonogenic assays after drug treatment and gamma or UV radiation. As expected, caffeine (the reference derivative) sensitized cells to gamma radiation (2.5–10 Gy) (Fig. 2, *a, b, c*) and UV (10–30 J/m^2) radiation (data not shown) in a dose-dependent manner. We tested whether the other methylxanthine derivatives could also radiosensitize cells and if the effect was dependent on p53 status. The effects of the derivatives were compared to the control group that consisted of cells treated only with irradiation.

Theophylline and DPMX induced sensitization of H1299 cells to gamma radiation and UV radiation (data not shown) as compared to the control ($P < 0.05$) but both were less potent than caffeine. IBMX failed to show any radiation-sensitizing effect on H1299 cells (see Fig. 2, *a*). To determine if the derivatives would have an effect on radiosensitivity dependent on p53, both NL-20 normal lung epithelial cells and H1299 transfected with p53 were analyzed. Interestingly, IBMX significantly decreased cell survival ($P < 0.05$) after 2.5 Gy gamma radiation and 10 J/m^2 UV radiation (data not shown) in NL-20 (see Fig. 2, *b*) and p53-transfected H1299 cells (see Fig. 2, *c*). DPMX

was the second most potent derivative at inducing radiosensitivity in these cells. Caffeine and theophylline also increased radiosensitivity but less effectively than IBMX and DPMX (see Fig.2, *b, c*).

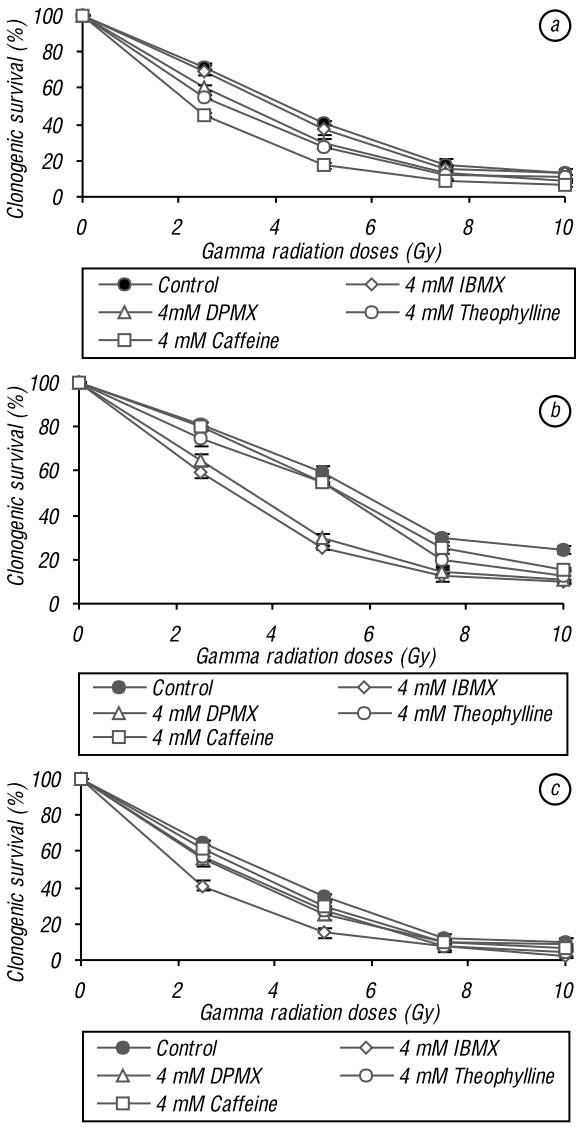

Fig. 2. Effect of different doses of gamma radiation on clonogenic survival in H1299 cells *(a)*, NL-20 cells *(b)* and wt-p53 H1299 transfected cells *(c)* with 4 mM of different methylxanthine derivatives. Each data point is an average of three independent experiments and expressed as M ± SD

To confirm the above results, the effect of the four different methylxanthine derivatives on radiosensitization was further investigated using the MTT assay. The results showed that transfecting cells with wild-type p53 increased radiosensitivity to 2.5 Gy gamma radiation (Fig. 3) and UV radiation (10 J/m^2) (data not shown). Moreover, it confirmed that IBMX caused increased sensitivity to radiation than the other derivatives, including caffeine. NL-20 cells were more resistant to radiation than H1299 and lung carcinoma cells stably transfected with wild-type p53. Caffeine was the most potent radiosensitizer in H1299 cells, followed by theophylline then DPMX. The decrease in cell viability by IBMX was not significantly different from control in H1299 cells but was a highly potent radiosensitizer in both normal lung epithelial and H1299 cells stably transfected with wild-type p53.

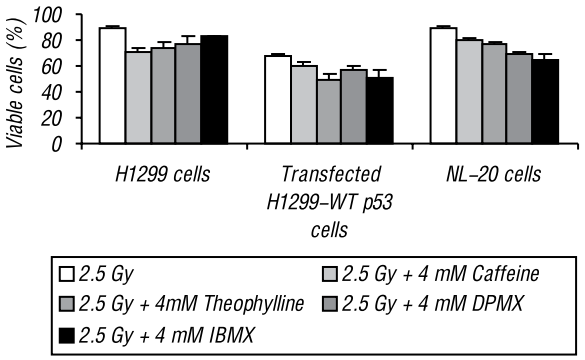

Fig. 3. Effect of gamma radiation (2.5 Gy) on cell viability of H1299 cells, NL-20 cells and wt-p53 H1299 transfected cells. All methylxanthine derivatives are in 4 mM concentration and compared to no treatment (100%). Each data point is an average of three independent experiments and expressed as M ± SD

Effect of methylxanthine derivatives on cell cycle checkpoints using flow cytometry. Flow cytometry was used to examine the effects of the derivatives on cell cycle checkpoints as well as cell proliferation or apoptosis. The percent of cells in G1, S, G2, and apoptosis were determined. There was an effect of the derivatives on G1 and G2 checkpoints as well as induction of apoptosis (Fig. 4, *a, b, c* and Tables 1, 2 , 3); however, no significant difference was found in the percent of cells in S phase. This analysis was done at 6, 12 and 24 h after UV (data not shown) or gamma radiation. H1299 cells that were irradiated and harvested 24 h post-irradiation showed an increase in the percent of G1 cells compared to G2, 47.4% *versus* 36.4%, respectively, which indicates that cells were arrested in G1 phase (see Table 1). However, both caffeine and theophylline significantly decreased the number of cells arrested in G2, 14.2% and 19.4%, respectively, compared to irradiated cells (36.4%), and increased the number of cells in G1 to 67.7 % and 62.1%, respectively, compared to the irradiated group (47.4%)(see Table 1). Also, they increased the percent of apoptosis after radiation in H1299 cells to 15% and 16%, respectively, compared to the irradiated group (10%). DPMX was less potent at causing a bypass of G2 but it did increase apoptosis to 18% *vs* 10% in irradiated cells. IBMX-treated H1299 cells were not significantly different from the control group in any of the phases of the cell cycle. (see Fig. 4, *a* and Table 1). Similar results with all the derivatives were seen after 10 J/m^2 UV radiation (data not shown).

Irradiation of NL-20 cells and p53-transfected H1299 cells with gamma radiation increased the number of cells in G2 compared to G1. When radiation was combined with any of the derivatives, the G2 checkpoint was bypassed causing more cells to enter G1 and more to apoptose (Fig. 4, *b, c* and Table 2, 3). IBMX induced the most apoptosis in the cells with wild-type p53, followed by DPMX and theophylline, then caffeine (see Fig. 4, *b, c* and Table 2, 3). Similar results were seen with UV radiation (data not shown).

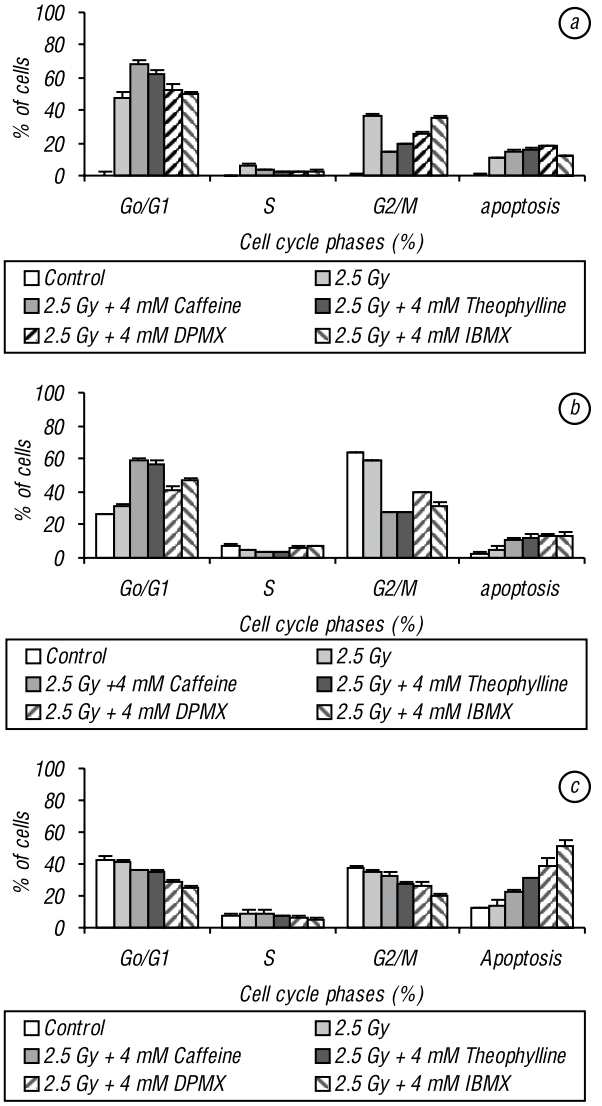

Fig. 4. Impact of methylxanthine derivatives on cell cycle phases in H1299 (a) cells, NL-20 (b) cells and wt-p53 H1299 (c) transfected cells at 2.5 Gy of gamma radiation and 24 h duration interval. Each data point is the mean of three independent experiments and expressed as M ± SD. Statistical difference was shown in Tables 1, 2 and 3

Table 1. Effect of different methlxanthine derivatives on cell cycle of H1299 cells

	Go/G1 %	S %	G2/M %	Apoptosis %
Control	60.5 ± 2.0	4.3 ± 0.5	14.1 ± 1.8	4.7 ± 1.0
IR	47.4 ± 4.0	5.6 ± 2.1	36.4 ± 2	10.7 ± 0.5
IR + Caffeine	67.7 ± 3.0*	3.1 ± 0.4*	14.2 ± 0.2*	15.0 ± 0.5*
IR+ Theophylline	62.1 ± 2.1*	2.5 ± 0.3*	19.4 ± 0.5*	16.0 ± 1.0*
IR + DPMX	53.0 ± 3.0*	2.9 ± 0.1*	26.0 ± 1.0*	18.1 ± 0.4*
IR + IBMX	50.0 ± 1.0	3.0 ± 0.2*	35.0 ± 1.0	12.0 ± 0.4

Control is non treated cells. All others were treated with 2.5 Gy IR. Results are expressed as M ± SD and the difference is significant (indicated by *) only if $P < 0.05$ compared to radiation alone

Table 2. Effect of different methlxanthine derivatives on cell cycle in NL-20 cells

	Go/G1 %	S %	G2/M %	Apoptosis %
Control	26.4 ± 1.0	7.7 ± 1.0	63.3 ± 1.2	2.7 ± 0.2
IR	30.8 ± 1.5	4.7 ± 0.3	59.1 ± 0.2	5.4 ± 1.4
IR + Caffeine	58.8 ± 1.2*	3.3 ± 0.4	27.2 ± 0.5*	10.7 ± 1.3*
IR + Theophylline	57.0 ± 2.3*	3.6 ± 0.3	27.5 ± 0.5*	11.9 ± 2.0*
IR + DPMX	41.4 ± 2.0*	6.5 ± 0.8*	39.2 ± 0.3*	12.9 ± 1.0*
IR + IBMX	47.3 ± 1.0*	7.2 ± 0.4*	31.9 ± 1.3*	13.6 ± 2.5*

Control is nontreated cells. All others were treated with 2.5 Gy IR. Results are expressed as M±SD and the difference is significant (indicated by *) only if $P < 0.05$ compared to radiation alone.

Table 3. Effect of different methlxanthine derivatives on cell cycle in p53 stably transfected H1299 cells

	Go/G1 %	S %	G2/M %	Apoptosis %
Control	42.9 ± 1.9	7.7 ± 1.0	37.5 ± 0.8	11.9 ± 0.6
IR	41.7 ± 1.1	9 ± 2.0	35.2 ± 1.0	14.1 ± 2.9
IR + Caffeine	35.7 ± 1.0	9 ± 2.6	32.5 ± 2.1	22.8 ± 0.4
IR + Theophylline	34.9 ± 1.0	7 ± 1.0	27.1 ± 1.4*	31 0.4*
IR + DPMX	28.4 ± 1.2*	6 ± 1.0	26.4 ± 2.0*	39.2 ± 5.0*
IR + IBMX	24.9 ± 1.0*	4.8 ± 1.7*	19.5 ± 1.9*	50.8 ± 4.2*

Control is nontreated cells. All others were treated with 2.5 Gy IR. Results are expressed as M ± SD and the difference is significant (indicated by *) only if $P < 0.05$ compared to radiation alone.

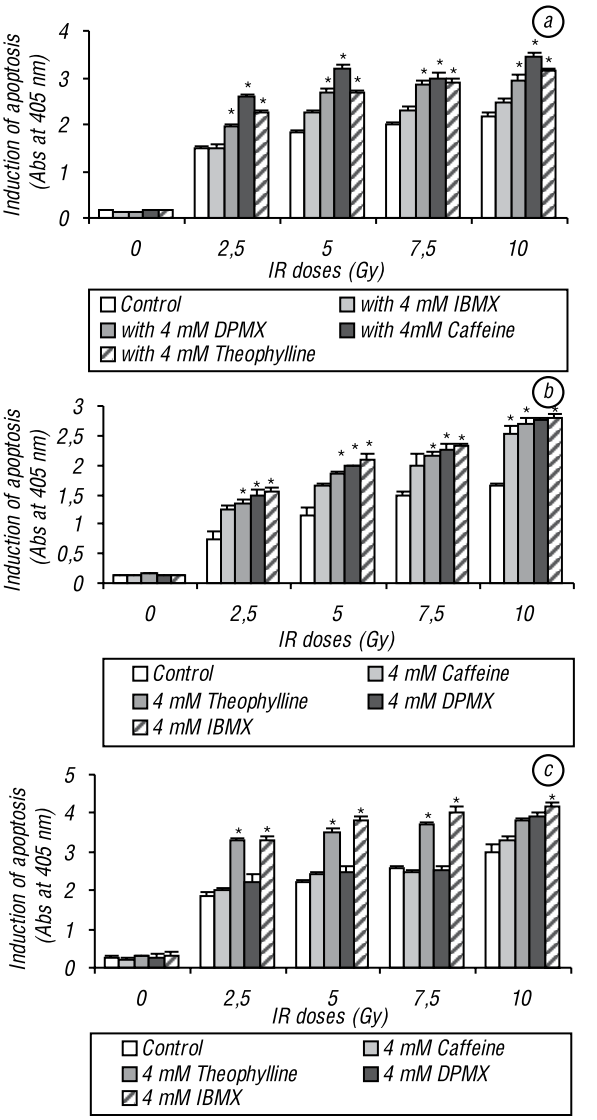

Fig. 5. Induction of apoptosis in H1299 cells *(a),* NL-20 cells *(b)* and wt-p53 H1299 transfected cells *(c)* with methylxanthine derivatives after different doses of gamma radiations ranging from (2.5–10 Gy) and 24 h duration interval. Each data point is the mean of three independent experiments and expressed as M±SD. Induction of apoptosis represents absorbance at 405 nm

Apoptotic response by methylxanthine derivatives after radiation. Apoptosis was examined in H1299, NL-20 and p53-transfected H1299 cells (Fig. *5, a, b, c*) after 2.5-10 Gy gamma radiation or 10-30 J/m^2 UV radiation (data not shown) by ELISA to detect histone release. In reference to the control group, caffeine induced significant increase in apoptosis compared to other derivatives at all doses in H1299 cells (see Fig. 5, *a*). NL-20 cells were more resistant to

gamma radiation (see Fig. 5, *b*) and UV radiation (data not shown) than p53-null cells. In contrast to the results in H1299 cells, IBMX was the most potent derivative in NL-20 cells followed by DPMX, theophylline then caffeine. Interestingly, in H1299-p53 transfected cells, IBMX and theophylline were similar in inducing apoptosis but more potent and significant from caffeine, DPMX and control group (see Fig. 5, *c*).

Caspase-3 activity. Caspase-3 activity was measured using DEVD peptide-nitroanilide (pNA) after 24 h of exposure to 4 mM of caffeine and IBMX in conjunction with 2.5 Gy of gamma radiation. The results showed that IBMX increased caspase-3 activity in p53 stably transfected cells while caffeine has non-significant effect. In contrast, in H1299 cells, caffeine increased caspase-3 activity compared to IBMX (Fig. 6).

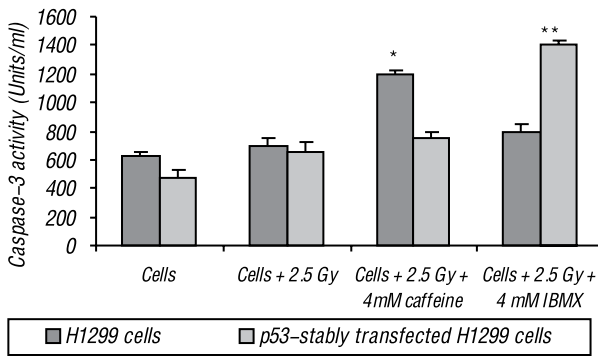

Fig. 6. Caspase-3 activity was determined in both H1299 and p53-stably transfected cells. IBMX increases the activity of caspase-3 compared to irradiated group and irradiated group in combination with caffeine. Results are expressed as M ± SD. *represents $p < 0.05$compared to irradiated group in H1299 cells and ** represents $p < 0.05$compared to irradiated group in p53 stably transfected H1299 cells.

TUNEL assay. TUNEL assays were performed in order to ascertain induction of apoptosis by 4 mM caffeine or IBMX in H1299 and p53-stably transfected cells in conjunction with 4 Gy of irradiation (Fig. 7). In H1299 cells, the assay revealed the presence of nuclear condensation and TUNEL-positive cells after treating cells with 2.5 Gy and 4 mM caffeine compared to irradiation alone (see Fig. 7, *a*). Moreover, similar results were obtained after treating p53 stably transfected H1299 cells with 2.5 Gy and 4mM IBMX. Whereas no induction of apoptosis was found in control non treated group (see Fig. 7, *b*).

p53 protein expression. To further investigate the effect of IBMX on p53, H1299 cells were stably transfected with p53 and expression of p53 was determined (Fig. 8, *a, b*). p53 protein increased in the presence of caffeine slightly more than gamma radiation alone. Interestingly, IBMX greatly increased the stabilization or induction of p53 protein levels in conjunction with gamma radiation. This is consistent with the increased apoptosis seen in the p53 transfected cells (see Fig. 5, *c*, Fig. 6).

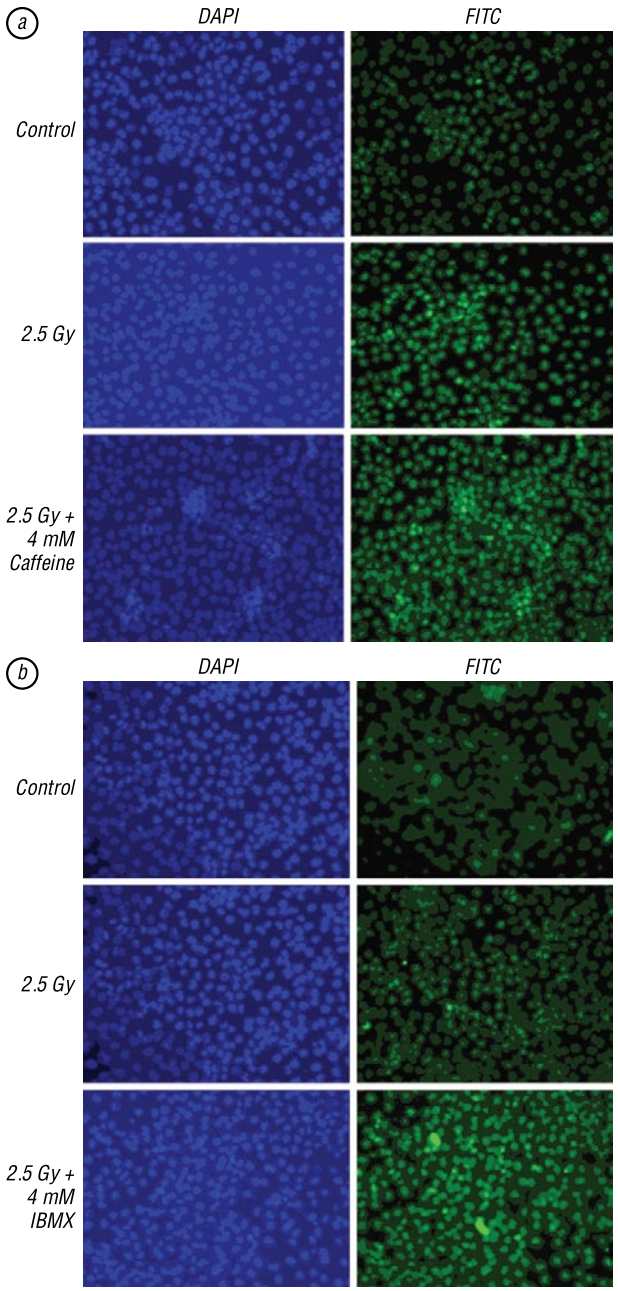

Fig. 7. Characterization of induced-cell death in H1299 cells *(a)* and p53 stably transfected cells *(b)*. Cells were treated with DAPI and stained via TUNEL assay. For each group, cells treated with 2.5 Gy of gamma radiations and 2.5 Gy of gamma radiation with 4 mM of caffeine and IBMX. DAPI was used to stain the nuclei and FITC represent, fluorescein-12 dUTP labeled DNA

DISCUSSION

Cell cycle checkpoints in G1 and G2 are activated in response to UV or gamma radiation allowing time for DNA repair to take place. After complete repair, the cells resume cycling. However, when the damage in cells is severe and cannot be repaired, the cells undergo apoptosis and die. Many researchers are challenged to discover novel drugs or new DNA damaging agents to preferentially kill tumor cells. Our results show that derivatives of methylxanthine differentially affect the cell cycle and apoptosis and their mechanism may depend on the cell´s p53 status.

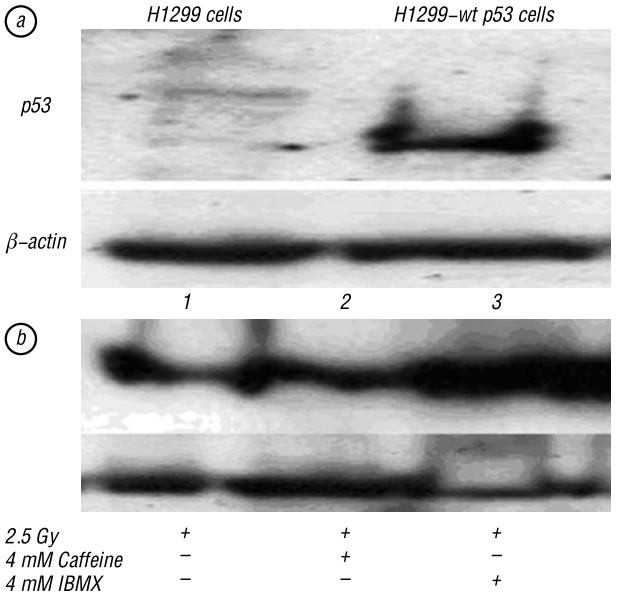

Fig. 8. Western blot analysis. *a*. Expression of p53 was determined in H1299 and in p53 stably transfected cells. *b*. p53 expression was compared, in stably transfected cell treated with 2.5 Gy, 2.5 Gy + 4 mM caffeine, 2.5 Gy + 4 mM IBMX (lanes *1, 2* and *3*). ß-Actin was used as an internal control to monitor equal protein sample loading

After radiation-induced DNA damage, ATM and ATR become activated and initiate signal transduction pathways that regulate G2 arrest [26, 27]. Downstream substrates of these kinases such as Chk1, Chk2 and Cdc25C are required for the initiation or maintenance of G2-phase arrest after genome damage [28]. The G2/M DNA damage checkpoint blocks mitosis thereby preventing the transmission of DNA lesions to daughter cells. The majority of cancer cells often have a corrupted G1 cell cycle checkpoint, which might contribute to carcinogenic processes and to the limited responses of tumors to radiotherapy [7]. However, this may contribute to making them more vulnerable to anticancer agents that interfere with G2 arrest, because the G2 checkpoint is their last defense after DNA damage [29]. The pharmacological bypassing of G2 checkpoints is powerful strategy for increasing the response to radiotherapy [8, 9, 30]. Hence, combined treatment with cytotoxic agents is designed to circumvent the radiation-induced G2 checkpoint while at the same time maintaining the benefits of an intact G1 checkpoint in normal cells [7, 31, 32].

Caffeine has been widely used as an inhibitor of the G2 checkpoint. Addition of caffeine to cells will abrogate G2/M checkpoint responses to many genotoxic stresses including UV, gamma radiation and other drugs [33, 34]. Caffeine's method of action is most probably through the metabolism of both purines and pyrimidines, transferring methyl groups to thymidine, adenine, and guanine in de novo synthesis [35]. DNA synthesis can proceed through two distinct pathways depending on the available intracellular pools: a *de novo* pathway synthesizing nucleic acids from precursors and a salvage pathway utilizing preformed purine rings such as xanthine [36]. The demethylation of caffeine results in a consequent accumulation of xanthine, altering the balance between purines and pyrimidines, which could account for the inhibition of cell proliferation by caffeine.

Our data showed a significant enhancement of radiation-induced toxicity of lung cancer cells without functional p53 compared to normal lung epithelial cells and p53-transfected H1299 cell when irradiated in a proliferative state in the presence of caffeine, theophylline, or DPMX. The data from clonogenic survival and MTT viability assays showed that caffeine is more potent than other derivatives in sensitizing H1299 cells to radiation. However, caffeine was less potent than all the other derivatives in NL-20 cells and p53-transfected H1299 cells, which is consistent with previous data that caffeine preferentially sensitizes p53-deficient cells to DNA damage by disruption of the G2/M checkpoint [37, 38]. Theophylline and DPMX were less potent than caffeine in H1299 cells. IBMX failed to elicit any response in these cells. However, in normal lung epithelial cells and p53-transfected H1299 cells, IBMX was a highly potent radiosensitizer. This suggests that IBMX may function through a different mechanism than the other methylxanhine derivatives, possibly in p53-dpendent.

Many drugs have been used for the treatment of lung cancer cells by inducing apoptosis through pathways that are p53-dependent and independent [39, 40]. Our data clearly showed that all the methylxanthine derivatives induced apoptosis in H1299 in a p53-independent mechanism after UV radiation and gamma radiation except IBMX. Theophylline and DPMX also abrogated the G2 checkpoint and directed more cells to apoptosis in normal and tumor cells. However, in H1299 cells, IBMX increased the percent of cells in G1 phase but it did not direct any cells to apoptose. In contrast, in normal lung epithelial cells and p53-transfected H1299 cells, IBMX was the most potent derivative at inducing apoptosis. The Western blot analysis demonstrated that p53 was overexpressed in p53-stably transfected cells in the presence of IBMX than caffeine combined with gamma radiations. These results were further confirmed by increase caspase-3 activity in the presence of IBMX in p53 stably transfected cells. The results were also supported by TUNEL assay. These results indicate that IBMX might function through a different mechanism than the other methylxanthine derivatives. Because IBMX may kill surrounding normal cells as or more efficiently than tumor cells, it might not serve as a therapeutic for lung cancers that possess wild-type p53. However, studies utilizing more cell lines with variations of p53 status should be done to address this issue.

The possible conclusion of the differential effect between caffeine and IBMX is the substitution of the methyl group at N3 in caffeine by a bulkier isobutyl group in IBMX and/or absence of a methyl group at N7 in caffeine. Though DPMX is bulkier than IBMX, due to the presence of two propyl groups and one methyl group, its action was less potent than IBMX. This might conclude that the isobutyl group at N3 makes

the xanthine ring more reactive and functional in cells with functional p53 than the other derivatives. Further analysis should be done to investigate the molecular mechanism of IBMX.

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ДИФФЕРЕНЦИАЛЬНЫЙ ЭФФЕКТ ОТДЕЛЬНЫХ ПРОИЗВОДНЫХ МЕТИЛКСАНТИНА НА РАДИОЧУВСТВИТЕЛЬНОСТЬ КЛЕТОК КАРЦИНОМЫ ЛЕГКОГО

Цель: **провести сравнительный анализ влияния кофеина и других производных метилксантина (тиофилин (ТФ), 3-изобутил-метилксантин (3-ИБМК), 1-3-дипропил-7-метилксантин (1-3-ДП-7МК)) на радиосенсибилизацию клеток крупноклеточной карциномы легкого человека, не содержащих гена p53, нормальных эпителиальных клеток легкого и крупноклеточной карциномы легкого, стабильно трансфецированной геном p53. Изучали влияние производных метилксантина на реакцию клеток на гамма- и ультрафиолетовое облучение.** ***Методы:*** **радиосенсибилизацию клеток под действием кофеина, ТФ, 3-ИБМК и 1-3-ДП-7МК исследовали при помощи клоногенного анализа, МТТ-анализа, иммуноферментного метода, адаптированного для выявления апоптотических клеток, проточной цитометрии, спектрофотометрического метода определения каспазы-3, TUNEL-метода, а также иммуноблоттинга.** ***Результаты*****: все производные метилксантина, за исключением 3-ИБМК, повышали чувствительность опухолевых клеток к радиации путем индукции апоптоза клеток рака легкого, лишенных гена p53. Эти производные повышали число клеток в G1 фазе путем блока перехода G2/M клеточного цикла, вызывали апоптоз по p53-независимому механизму. В то же время 3-ИБМК проявил себя как более мощный радиосенсибилизатор нормальных эпителиальных клеток легкого, стабильно трансфецированных диким типом гена p53. 3-ИБМК повышал уровень p53 белка в большей степени, чем кофеин в клетках карциномы легкого, стабильно трансфецированных диким типом гена p53.** ***Выводы:*** **результаты позволяют предположить, что действие 3-ИБМК осуществляется по p53-зависимому пути.**

Ключевые слова: **3-изобутил-метилксантин, каспаза-3, p53, клеточный цикл, рак легкого.**