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Research Article

In vitro anticancer effect of theobromine in A549 non-small cell lung cancer cells

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Abstract

Objectives: Theobromine, a naturally occurring alkaloid, is now recognized as a viable option due to its potential therapeutic benefit in many types of cancers. This preliminary study was undertaken to clarify how theobromine affects the viability, apoptosis, and migration of human non-small cell lung cancer (NSCLC) A549 cells.

Methods: Water-soluble tetrazolium-1 (WST-1) assay was utilized to measure the cytotoxicity of theobromine (5-200 μM) in A549 cells for 24 and 48 hours. Flow cytometry analysis was used to detect apoptosis. A wound healing assay was carried out to evaluate the migration of A549 cells. Cells incubated with the IC50 concentration of cisplatin for 24 hours served as the reference group.

Results: A time- and dose-dependent effect of theobromine on the inhibition of A549 cell viability was dramatically observed. Theobromine treatment led to an increased apoptotic cell population and caspase 3/7 activity ($p<0.0001$). Furthermore, the migratory capacity of A549 cells was reduced in the cells treated with theobromine.

Conclusion: The results suggested that theobromine potently reduced the cell growth and migration capacity of NSCLC A549 cells by inducing caspase 3/7-mediated apoptosis. Additional studies are necessary to comprehend the antitumor mechanisms of theobromine against NSCLC and to provide helpful perspectives for more research.

Keywords: Apoptosis, A549 cells, caspase 3/7, cytotoxicity, migration, NSCLC, theobromine, viability

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Non-small cell lung cancer (NSCLC) is one of the most commonly encountered subtypes of lung cancer [1]. Prevailing pharmacological options for NSCLC, including chemotherapy, targeted therapy, and immunotherapy alone or in combination with surgery/radiation, are not completely effective and well-tolerated options due to their side effects, low specificity, intrinsic and/or acquired resistance to chemotherapeutic agents, and the lack of major advancements in the survival rates of patients [2, 3]. Among several anticancer drugs applied to lung cancer, cisplatin has been largely used as a first-line therapy in combination with other compounds [4]. However, limited usage of cisplatin in clinical settings has been reported due to its well-known side effects and consequential chemoresistance [5].

Due to limitations in early diagnostic techniques and the absence of clinical signs in the early stage of cancer, a large number of lung cancer patients are detected at advanced stages of the disease [6]. Thus, alternative therapeutic options which could offer better prognostication of the patients at early stages of cancer are thought to be quite advantageous. There has been a noticeable rise in the number of naturally obtained anticancer drugs that have been officially accepted for lung cancer therapy [7, 8]. Therefore, the identification and presentation of natural products with anticancer activity have gained interest for the management of lung cancer due to their low toxicity, accessible, and inexpensive properties [9].

Apoptosis represents a programmed cell death that is executed through the activation of the caspase family and followed

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by a set of morphological changes [10]. Natural compounds have demonstrated antitumor activity via the induction of apoptotic cell signaling in lung cancer cells [4]. Previous studies demonstrated that methylxanthines have anticancer and anti-inflammatory properties [11, 12]. Among natural products, theobromine is a natural methylxanthine found in Theobroma cacao, such as chocolate, and other foods including tea [13]. Theobromine is a caffeine derivative that can also be absorbed into the body via caffeine metabolized by the cytochrome P450 oxidase enzyme system [14].

It has been previously shown that theobromine has multiple biological properties along with its antitumor activity, anti-inflammatory, and antioxidant effects [15, 16]. The primary action mechanism of theobromine as a phosphodiesterase (PDE) inhibitor is mainly through increasing intracellular cyclic adenosine monophosphate (cAMP) [17]. High cAMP levels are linked to the induction of apoptosis, invasion, and spread of cancer cells [18]. Specifically, theobromine triggers apoptosis in HT-29 colorectal cancer cells [19], and decreases cell growth in human glioblastoma cells [13]. Theobromine is also capable of the inhibition of cell proliferation along with its antiangiogenic properties in human glioblastoma, ovarian, and lung cancers [13, 20, 21]. Furthermore, theobromine is able to improve the cytotoxic activity of doxorubicin in human VoLo colorectal cancer cells [22]. Moreover, theobromine has demonstrated promising anticancer activities indicating its ability to act as a potential therapeutic agent towards a number of malignancies [21, 23, 24].

Herein, the present study firstly assessed the cytotoxic, apoptotic, and antimigratory effects of theobromine treatment in A549 lung cancer cells.

Materials and Methods

Cell culture and chemicals

The NSCLC A549 cells (ATCC) were cultured in DMEM (Capricorn Scientific) containing 10% FBS (Capricorn Scientific) and 1% Pen/Strep (Capricorn Scientific) at 37°C in 5% CO2 conditions. Theobromine was acquired from Sigma-Aldrich (catalog number: T4500) and dissolved in dimethyl sulfoxide (DMSO, Merck, Saint Louis, MO, USA) to have a 1 mM stock solution. Cisplatin (European Pharmacopoeia) was prepared with DMSO to obtain a 500 µM stock solution.

WST-1 cell viability assay

The WST-1 cytotoxicity test was used to determine the cytotoxicity against the A549 cell line. The principle of this assay is that cellular mitochondrial dehydrogenases convert the tetrazolium salt WST-1 into formazan crystals. A549 cells were proliferated and incubated (96-well). After incubation, theobromine and cisplatin were prepared at concentrations of 0, 5, 10, 25, 50, 100, and 200 µM and applied to the cells. The measurements were analyzed at the 24th and 48th hours. To eliminate the effects from DMSO, the concentrations were added to the negative control wells at a rate where theobromine and cisplatin were dissolved along with the medium. After adding 10 µL of the WST-1 solution (Cayman Chemical, cat. no. 10008883), the cells were incubated for 4 hours. Following incubation, the appropriate inhibitor concentration for the cells was determined via measuring absorbance values at 450 nm. Cisplatin was used as a reference drug. The percentage of cell viability in theobromine-treated groups was expressed as the ratio of the control group, which was assumed to be 100%. Furthermore, GraphPad Prism 9.1.0 programme was used to calculate the %50 inhibitory concentrations (IC_{50}) of theobromine and cisplatin via regression analysis.

Apoptosis detection by flow cytometry

The apoptosis induced by theobromine and cisplatin was examined with Annexin V-FITC and PI (Tonbo Biosciences, San Diego, CA). Adherent A549 cells (1×10^5/well) were incubated with IC_{50} values of theobromine (16.02 µM) or cisplatin (6.40 µM) for 24 hours at 37°C in 6-well plates. Then, the cells were stained with 5 µL of each of Annexin V-FITC and PI solution in binding buffer (15 minutes in the dark). Subsequently, the apoptosis rate was measured by ACEA NovoCyte flow cytometry (Agilent).

Caspase 3/7 activity assay by flow cytometry

To measure caspase 3/7 levels, A549 cells were treated with the IC_{50} concentration of theobromine (16.02 µM) or cisplatin (6.40 µM) and incubated overnight. After the 24-hour treatment period, the cells were trypsinized, collected, and analyzed for the detection of caspase 3/7 activity with Cell Meter™ Caspase 3/7 flow cytometry assay kit using ACEA NovoCyte flow cytometry instrument (Agilent).

Wound-healing assay

A549 cells were cultured in medium for full confluence. A sterile plastic pipette tip was used to artificially create a gap, and then cells were incubated with DMEM containing the IC_{50} values of theobromine and cisplatin for 24 hours. Subsequently, the wound area was photographed immediately at 0h with a light microscope (Olympus IX73), and the degree of wound healing was measured based on the area covered by cells at the indicated times (6, 12, and 24 hours) after the scratch.

Statistical analysis

Statistical analysis was analyzed with GraphPad Prism 9.1.0 program using Student's t-test and two-way ANOVA. Statistical significance level was accepted as $p<0.05$.

Results

Antiproliferative effect of theobromine on human A549 cells

A concentration- and time-dependent reduction in cell survival was detected in A549 cells after 24 and 48 hours of exposure to theobromine (5–200 µM) and cisplatin (1–50 µM) according to the WST-1 test (Fig. 1a, b). After treatment with theobromine for 24 hours, cell viability dropped to 72.5% at 5 µM and 66.5% at 10 µM (Fig. 1a). The viability of A549 cells was less than 36.5% at concentrations higher than 25 µM

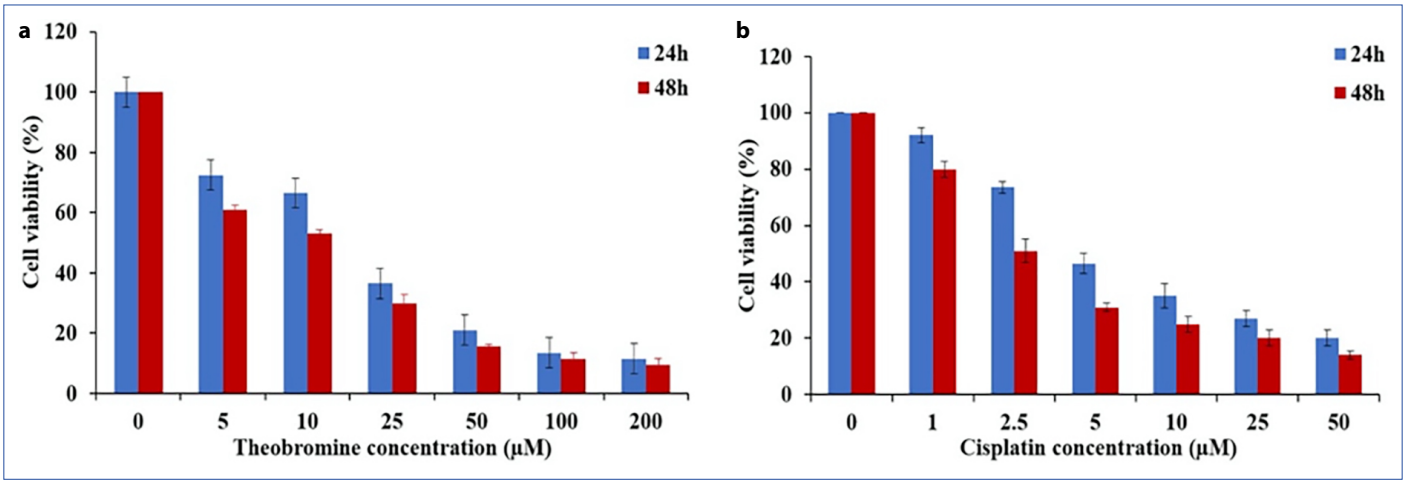

Figure 1. WST-1 assay showing cell viability of A549 after treatment with increasing doses of theobromine (a) and cisplatin (b) for 24 and 48 hours.
Data are displayed as the mean±SD. SD: Standard deviation.

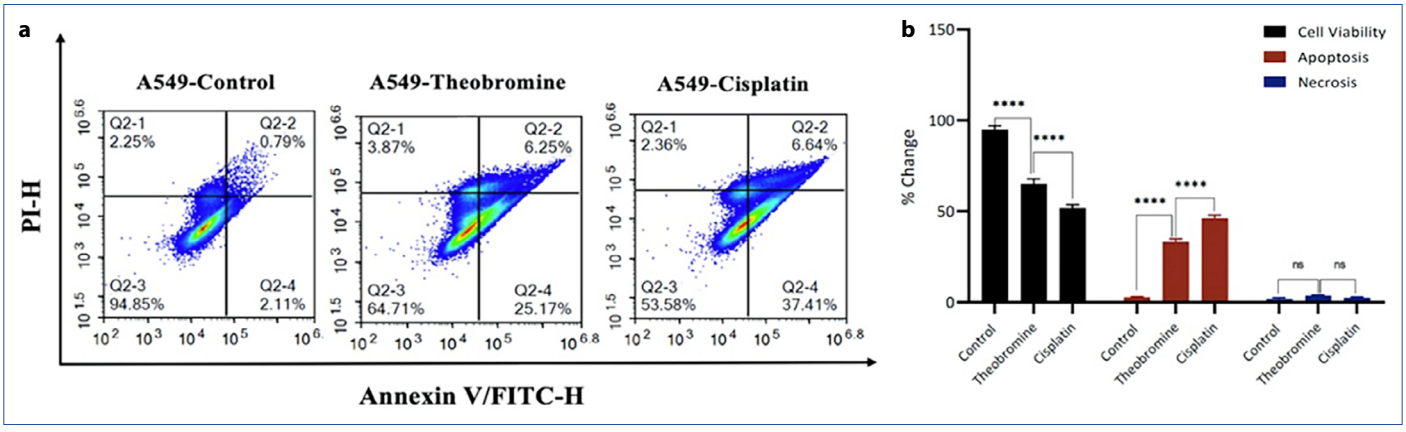

Figure 2. (a) Representative dot plots of A549 cells treated with theobromine and cisplatin at their IC_{50} concentrations for 24 hours for determination of apoptotic cell death by Annexin V/FITC-PI double staining. (b) The percentages of viable, apoptotic, and necrotic cells were statistically analyzed. The values are the representative of three independent experiments.

Data are displayed as the mean±SD. Significances are shown in comparison to control cells and theobromine-treated cells (****: p<0.0001). SD: Standard deviation; PI-H: Propidium iodide; FITC-H: Fluorescein isothiocyanate.

theobromine. For 48 hours of treatment, this cytotoxic effect of theobromine was observed more severely than for 24 hours of treatment (Fig. 1a). IC_{50} values of theobromine were calculated as 16.02 μM at 24 hours and 10.76 μM at 48 hours of treatment. Similar antiproliferative effects were also noted in cisplatin-treated A549 cells, as shown in Figure 1b, with IC_{50} values of 6.40 μM at 24 hours and 3.84 μM at 48 hours. Regarding the IC_{50} values of theobromine and cisplatin for 24 and 48 hours towards the A549 cell line, cisplatin, as the positive control, was more cytotoxic than theobromine.

Theobromine promoted human A549 cell apoptosis

In agreement with the cytotoxicity results, the treatment of A549 cells with theobromine (16.02 μM) and cisplatin (6.40 μM) for 24 hours led to an increase in the rate of early and late apoptotic cells (31.42% and 44.05%, respectively) compared to control cells (2.9%) (Fig. 2a, b). The data indicated that theobromine has an antiproliferative effect on A549 lung cancer cells mediated by apoptosis induction. These results also indicated that the percentage of apoptotic cell death induced by cisplatin (44.05%) as a positive control was significantly higher than the theobromine-induced apoptosis rate (31.42%) ($p<0.0001$, Fig. 2b). In addition, A549 cells treated with theobromine and cisplatin exhibited a decreased population of viable cells by 64.71% and 53.58% in comparison to the non-treated group ($p<0.0001$, Fig. 2b).

Theobromine induced caspase 3/7 activity in A549 cells

To understand the mechanism of theobromine, we measured the caspase 3/7 levels as the markers of apoptosis induction via flow cytometry analysis [25]. The effects of theobromine on apoptosis coincide with the results obtained by the caspase 3/7 assay. The treatment of A549 cells with IC_{50} concentrations of theobromine for 24 hours resulted in augmented levels of caspase 3/7 activation when compared to the non-treated group ($p<0.0001$, Fig. 3a, b). As expect-

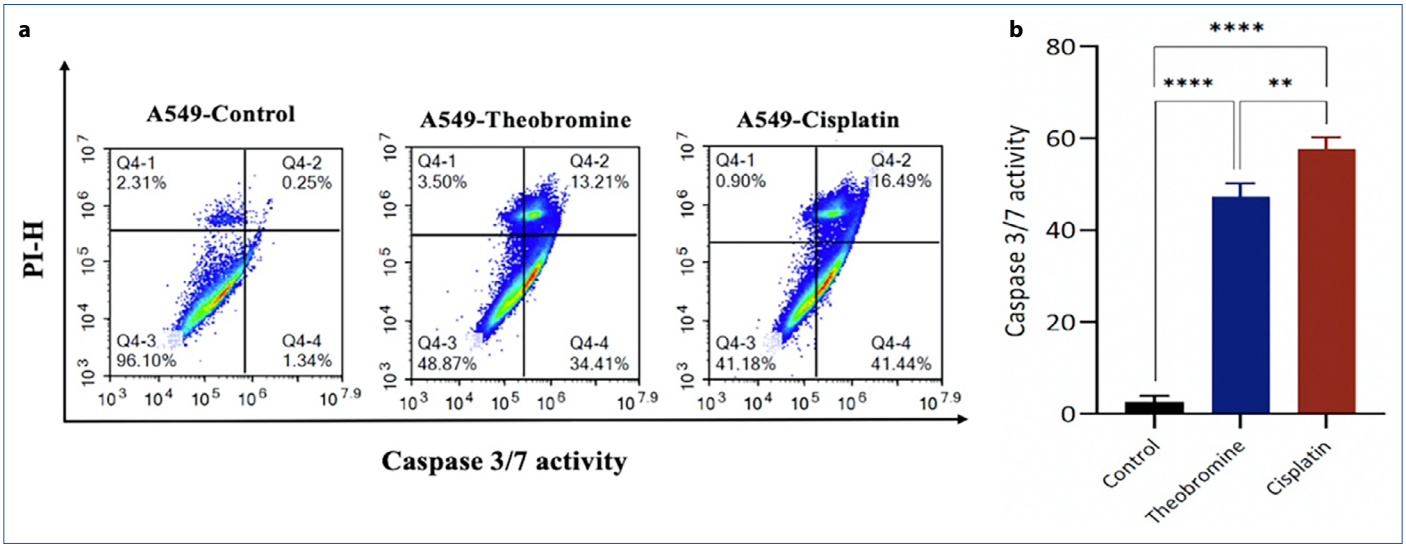

Figure 3. (a) Caspase 3/7 activity measured by flow cytometry in A549 cells treated with IC_{50} concentrations of theobromine or cisplatin for 24 hours. (b) Comparison of caspase 3/7 activity among the groups.

Data are displayed as the mean±SD. Significances are shown in comparison to control cells and theobromine-treated cells (**: p<0.01; ****: p<0.0001). SD: Standard deviation; PI-H: Propidium iodide; IC_{50}: Half-maximal inhibitory concentration.

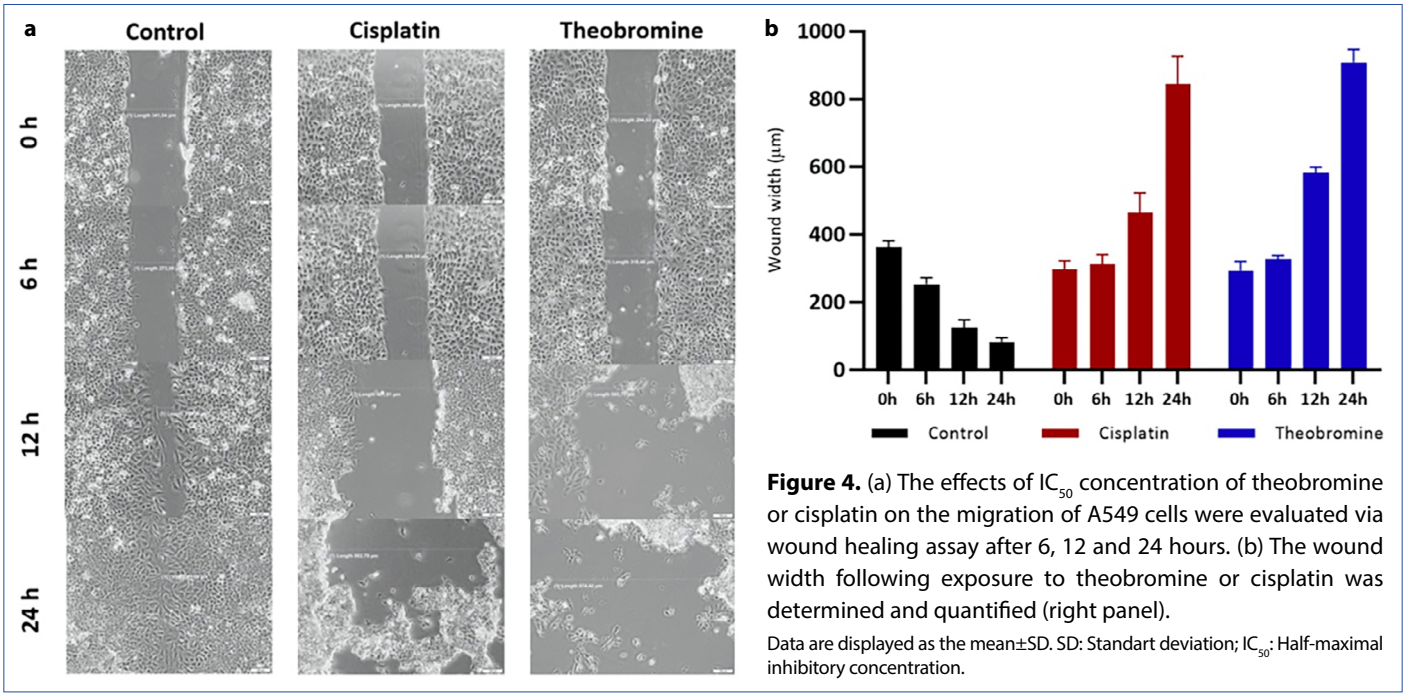

Figure 4. (a) The effects of IC_{50} concentration of theobromine or cisplatin on the migration of A549 cells were evaluated via wound healing assay after 6, 12 and 24 hours. (b) The wound width following exposure to theobromine or cisplatin was determined and quantified (right panel).

Data are displayed as the mean±SD. SD: Standart deviation; IC_{50}: Half-maximal inhibitory concentration.

ed, the data indicated that caspase 3/7 activity was higher in the cisplatin group (57.93%) than the theobromine group (47.62%) (p<0.01, Fig. 3a, b), which was also supported by the result of the apoptosis assay.

Theobromine reduced the migration ability of A549 cells

Theobromine prevented the migration of A549 cells. The migratory ability of A549 cells was significantly reduced with theobromine or cisplatin treatment for 24 hours as compared to control (p<0.0001, Fig. 4), suggesting that theobromine inhibited the migratory capacity of A549 cells. The relative wound width (area) following cisplatin treatment for 24 hours was smaller than that of theobromine-treated cells (p<0.01, Fig. 4).

Discussion

Theobromine is a product of caffeine metabolism and possesses a significant role in the health benefits of cocoa as a more stable and safe compound than caffeine [26, 27]. The existing literature has already established that theobromine has the ability to suppress the growth of several cancer types [13]. A previous study demonstrated the anticancer effects

of theobromine by the suppression of the angiogenic potential of human lung cancer cells [20, 28]. However, there are no specific studies showing the apoptotic and antimigratory effects of theobromine on A549 lung cancer.

Our experiments showed the dose-dependent inhibitory effects of theobromine on the viability of A549 cells. The percentage of live cells after 48 hours of theobromine treatment tended to be lower for all concentrations compared to those within 24 hours. In previous studies, theobromine decreased proliferation of HT-29 colon cancer cells [19] and the malignant glioma U87-MG cell line [13]. Theobromine cytotoxicity was observed at higher concentrations (100–500 µg/mL) in RAW 264.7 macrophages [29]. In contrast to our finding, Eguchi et al. [30] reported that theobromine (50–200 µM) did not show cytotoxicity in human A549 lung cancer cells.

The controlled biological process of apoptosis controls the ratio of cell death to survival, and evading apoptosis is a characteristic of most forms of cancer [10]. The activation of the caspase family members is a key regulatory mechanism of apoptosis [25]. Annexin V-FITC and PI staining and the caspase 3/7 activity assay were employed to check whether this inhibitory impact of theobromine on cell viability was related to the induction of apoptosis. The results demonstrated a vital induction in caspase 3/7 activity and a marked increase in the percentage of apoptotic cells in the theobromine-treated group compared to the control cells. According to the findings, the apoptotic mechanism of theobromine in A549 cells is likely through the induction of caspase 3/7 activity. Similarly, Cadoná et al. [19] demonstrated the induction of apoptosis and enhanced sensitivity to chemotherapeutic drugs after theobromine treatment in HT-29 colon cancer cells. In addition, theobromine demonstrated anticancer activity in the malignant glioma cell line and an animal model of colon cancer through downregulation of the Akt/mTOR signaling pathway, which is a mechanism involved in the inhibition of apoptosis [13, 31]. In addition, theobromine-based derivatives induced apoptosis with its antiangiogenic properties in different cancer cell lines [32, 33]. Theobromine *in vitro* showed significant inhibitory activity on PARP-1, as a substrate of activated caspase 3/7, which is an important target for anticancer therapy [34]. In the 3T3-L1 preadipocytes, theobromine has been reported to induce G0/G1 arrest [35]. According to *in vivo* research, oral treatment with theobromine dramatically diminished the tumor weight in the Balb/c mouse with sarcoma cells [24], which could support the antiapoptotic effects of theobromine.

As well, the effects of caffeine are partly mediated by the products of its metabolism, such as theobromine, and the precise effects of theobromine vary greatly based on the type of cell and the amount of caffeine [26]. Numerous other investigations revealed that caffeine causes apoptosis, cell cycle arrest, and inhibition of viability in different cancer cells [36–38]. Caffeine also induces caspase 3/7 activity in human osteosarcoma cells [39]. Furthermore, caffeine has led to apoptosis in a skin epidermal cell line [36]. In human lung carcinoma cells, Qi et al. [38] showed that radiation-induced apoptosis was enhanced by 5 mM caffeine, but also caffeine alone caused apoptosis and a tumor suppressor protein p53-independent G1 phase arrest.

We further used a wound-healing assay to demonstrate the effect of theobromine on migration. Here, we observed that theobromine also suppressed the migration of the A549 lung cancer cell line in a time-dependent manner. Moreover, theobromine seemed to suppress migration more potently than cell viability at its IC_{50} value, indicating that the antimetastatic action of theobromine might not depend only on cytotoxicity. In a study by Eissa et al. [32], a new theobromine derivative dramatically reduced the migratory capabilities of HepG2 cancer cells. Caffeine treatment (10 µg/mL) did not affect the migration ability of A549 and HeLa cancer cells [40].

Conclusion

Our research revealed that theobromine induced apoptosis and enhanced caspase 3/7 activation, which in turn prevented A549 cells from proliferating and migrating. However, this article offers preliminary data that will be the basis for future studies. Thus, more fundamental researches are needed to verify favorable *in vivo* antitumor activity of theobromine to elucidate its exact mechanisms as an anticancer agent.

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References

1. Molina JR, Yang P, Cassivi SD, Schild SE, Adjei AA. Non-small cell lung cancer: Epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 2008;83(5):584–94. [CrossRef]
2. Guo Q, Liu L, Chen Z, Fan Y, Zhou Y, Yuan Z, et al. Current treatments for non-small cell lung cancer. Front Oncol 2022;12:945102. [CrossRef]
3. Liu J, Xiang Y, Fang T, Zeng L, Sun A, Lin Y, et al. Advances in the diagnosis and treatment of advanced non-small-cell lung cancer with EGFR exon 20 insertion mutation. Clin Lung Cancer 2024;25(2):100–8. [CrossRef]
4. Huang CY, Ju DT, Chang CF, Muralidhar Reddy P, Velmurugan BK. A review on the effects of current chemotherapy drugs and natural agents in treating non-small cell lung cancer. Biomedicine (Taipei) 2017;7(4):23. [CrossRef]

5. Dasari S, Njiki S, Mbemi A, Yedjou CG, Tchounwou PB. Pharmacological effects of cisplatin combination with natural products in cancer chemotherapy. Int J Mol Sci 2022;23(3):1532. [CrossRef]
6. Ruiz-Ceja KA, Chirino YI. Current FDA-approved treatments for non-small cell lung cancer and potential biomarkers for its detection. Biomed Pharmacother 2017;90:24–37. [CrossRef]
7. Naeem A, Hu P, Yang M, Zhang J, Liu Y, Zhu W, et al. Natural products as anticancer agents: Current status and future perspectives. Molecules 2022;27(23):8367. [CrossRef]
8. Spira A, Ettinger DS. Multidisciplinary management of lung cancer. N Engl J Med 2004;350(4):379–92. [CrossRef]
9. Talib WH, Alsayed AR, Barakat M, Abu-Taha MI, Mahmod AI. Targeting drug chemo-resistance in cancer using natural products. Biomedicines 2021;9(10):1353. [CrossRef]
10. Carneiro BA, El-Deiry WS. Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol 2020;17(7):395–417. [CrossRef]
11. Miwa S, Sugimoto N, Shirai T, Hayashi K, Nishida H, Ohnari I, et al. Caffeine activates tumor suppressor PTEN in sarcoma cells. Int J Oncol 2011;39(2):465–72.
12. Saito T, Sugimoto N, Ohta K, Shimizu T, Ohtani K, Nakayama Y, et al. Phosphodiesterase inhibitors suppress Lactobacillus casei cell-wall-induced NF-κB and MAPK activations and cell proliferation through protein kinase A- Or exchange protein activated by cAMP-dependent signal pathway. ScientificWorldJournal 2012;2012:748572. [CrossRef]
13. Sugimoto N, Miwa S, Hitomi Y, Nakamura H, Tsuchiya H, Yachie A. Theobromine, the primary methylxanthine found in Theobroma cacao, prevents malignant glioblastoma proliferation by negatively regulating phosphodiesterase-4, extracellular signal-regulated kinase, Akt/mammalian target of rapamycin kinase, and nuclear factor-kappa B. Nutr Cancer 2014;66(3):419–23. [CrossRef]
14. Monteiro J, Alves MG, Oliveira PF, Silva BM. Pharmacological potential of methylxanthines: Retrospective analysis and future expectations. Crit Rev Food Sci Nutr 2019;59(16):2597–625. [CrossRef]
15. Azam S, Hadi N, Khan NU, Hadi SM. Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. Med Sci Monit 2003;9(9):BR325–30.
16. Fuggetta MP, Zonfrillo M, Villivà C, Bonmassar E, Ravagnan G. Inflammatory microenvironment and adipogenic differentiation in obesity: The inhibitory effect of theobromine in a model of human obesity *in vitro*. Mediators Inflamm 2019;2019:1515621. [CrossRef]
17. Yoneda M, Sugimoto N, Katakura M, Matsuzaki K, Tanigami H, Yachie A, et al. Theobromine up-regulates cerebral brain-derived neurotrophic factor and facilitates motor learning in mice. J Nutr Biochem 2017;39:110–16. [CrossRef]
18. Ahmed MB, Alghamdi AAA, Islam SU, Lee JS, Lee YS. cAMP signaling in cancer: A PKA-CREB and EPAC-centric approach. Cells 2022;11(13):2020. [CrossRef]
19. Cadoná FC, Machado AK, Azzolin VF, Barbisan F, Dornelles EB, Glanzner W, et al. Guaraná a caffeine-rich food increases oxaliplatin sensitivity of colorectal HT-29 cells by apoptosis pathway modulation. Anticancer Agents Med Chem 2016;16(8):1055–65. [CrossRef]
20. Skopińska-Różewska E, Sommer E, Demkow U, Chorostowska-Wynimko J, Bałan B, Rózycka B, et al. Screening of angiogenesis inhibitors by modified tumor-induced angiogenesis (TIA) test in lung cancer. Rocz Akad Med Bialymst 1997;42(Suppl 1):287–96.
21. Barcz E, Sommer E, Sokolnicka I, Gawrychowski K, Roszkowska-Purska K, Janik P, et al. The influence of theobromine on angiogenic activity and proangiogenic cytokines production of human ovarian cancer cells. Oncol Rep 1998;5(2):517–20. [CrossRef]
22. Środa-Pomianek K, Michalak K, Palko-Łabuz A, Poła A, Dzięgiel P, Puła B, et al. Cytotoxic and multidrug resistance reversal activity of phenothiazine derivative is strongly enhanced by theobromine, a phytochemical from cocoa. Eur J Pharmacol 2019;849:124–34. [CrossRef]
23. Lentini A, Kleinman HK, Mattioli P, Autuori-Pezzoli V, Nicolini L, Pietrini A, et al. Inhibition of melanoma pulmonary metastasis by methylxanthines due to decreased invasion and proliferation. Melanoma Res 1998;8(2):131–7. [CrossRef]
24. Bałan BJ, Siwicki AK, Pastewka K, Demkow U, Skopiński P, Skopińska-Różewska E, et al. Synergistic activity for natural and synthetic inhibitors of angiogenesis induced by murine sarcoma L-1 and human kidney cancer cells. Adv Exp Med Biol 2017;1020:91–104. [CrossRef]
25. Shim MK, Yoon HY, Lee S, Jo MK, Park J, Kim JH, et al. Caspase-3/-7-specific metabolic precursor for bioorthogonal tracking of tumor apoptosis. Sci Rep 2017;7(1):16635. [CrossRef]
26. Martínez-Pinilla E, Oñatibia-Astibia A, Franco R. The relevance of theobromine for the beneficial effects of cocoa consumption. Front Pharmacol 2015;6:30. [CrossRef]
27. Baggott MJ, Childs E, Hart AB, de Bruin E, Palmer AA, Wilkinson JE, et al. Psychopharmacology of theobromine in healthy volunteers. Psychopharmacology (Berl) 2013;228(1):109–18. [CrossRef]
28. Gil M, Skopińska-Różewska E, Radomska D, Demkow U, Skurzak H, Rochowska M, et al. Effect of purinergic receptor antagonists suramin and theobromine on tumor-induced angiogenesis in BALB/c mice. Folia Biol (Praha) 1993;39(2):63–8.
29. Lee HW, Choi IW, Ha SK. Immunostimulatory activities of theobromine on macrophages via the activation of MAPK and NF-κB signaling pathways. Curr Issues Mol Biol 2022;44(9):4216–28. [CrossRef]
30. Eguchi H, Kimura R, Onuma S, Ito A, Yu Y, Yoshino Y, et al. Elevation of Anticancer drug toxicity by caffeine in spheroid model of human lung adenocarcinoma A549 cells mediated by reduction in Claudin-2 and Nrf2 expression. Int J Mol Sci 2022;23(24):15447. [CrossRef]
31. Shojaei-Zarghani S, Yari Khosroushahi A, Rafraf M. Oncopreventive effects of theanine and theobromine on dimethylhydrazine-induced colon cancer model. Biomed Pharmacother 2021;134:111140. [CrossRef]
32. Eissa IH, Yousef RG, Elkady H, Elkaeed EB, Alsfouk AA, Husein DZ, et al. Identification of new theobromine-based derivatives as potent VEGFR-2 inhibitors: Design, semi-synthesis, biological evaluation, and in silico studies. RSC Adv 2023;13(33):23285–307. [CrossRef]

33. Eissa IH, Yousef RG, Elkady H, Elkaeed EB, Alsfouk AA, Husein DZ, et al. Design, semi-synthesis, anti-cancer assessment, docking, MD simulation, and DFT studies of novel theobromine-based derivatives as VEGFR-2 inhibitors and apoptosis inducers. Comput Biol Chem 2023;107:107953. [CrossRef]
34. Geraets L, Moonen HJ, Wouters EF, Bast A, Hageman GJ. Caffeine metabolites are inhibitors of the nuclear enzyme poly(ADP-ribose)polymerase-1 at physiological concentrations. Biochem Pharmacol 2006;72(7):902–10. [CrossRef]
35. Jang YJ, Koo HJ, Sohn EH, Kang SC, Rhee DK, Pyo S. Theobromine inhibits differentiation of 3T3-L1 cells during the early stage of adipogenesis via AMPK and MAPK signaling pathways. Food Funct 2015;6(7):2365–74. [CrossRef]
36. He Z, Ma WY, Hashimoto T, Bode AM, Yang CS, Dong Z. Induction of apoptosis by caffeine is mediated by the p53, Bax, and caspase 3 pathways. Cancer Res 2003;63(15):4396–401.
37. Ito K, Nakazato T, Miyakawa Y, Yamato K, Ikeda Y, Kizaki M. Caffeine induces G2/M arrest and apoptosis via a novel p53-dependent pathway in NB4 promyelocytic leukemia cells. J Cell Physiol 2003;196(2):276–83. [CrossRef]
38. Qi W, Qiao D, Martinez JD. Caffeine induces TP53-independent G(1)-phase arrest and apoptosis in human lung tumor cells in a dose-dependent manner. Radiat Res 2002;157(2):166–74. [CrossRef]
39. Igarashi K, Yamamoto N, Hayashi K, Takeuchi A, Kimura H, Miwa S, et al. Non-toxic efficacy of the combination of caffeine and valproic acid on human osteosarcoma cells *in vitro* and in orthotopic nude-mouse models. Anticancer Res 2016;36(9):4477–82. [CrossRef]
40. Xu H, Wang L, Shi B, Hu L, Gan C, Wang Y, et al. Caffeine inhibits the anticancer activity of paclitaxel via down-regulation of α-tubulin acetylation. Biomed Pharmacother 2020;129:110441. [CrossRef]