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Combination therapy enhanced the antitumor activity of artemisinin-iron in lung cancer Calu-6 cells

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Summary. *Aim of Study*: Combination therapy has become an important strategy for treatment of cancer and enhancing response rates. Artemisinin, a derivative from *Artemisia annua*, is a well-known potent antitumor component. Iron is considered as an essential component in the cytotoxicity of artemisinin. Butyric acid and miconazole both are shown to induce apoptosis in several cancer cell lines. The present study investigated the potential enhanced antitumor activity of artemisinin-iron on lung cancer adenocarcinoma cell line (Calu-6) when it is combined with butyric acid and/or miconazole. *Materials and Methods*: Cultured Calu-6 cells were divided into 12 flasks. The cells were treated with different doses of artemisinin (0.15, 0.3, 0.6 and 1.2 μg/ml) and iron (1 μmol/ml) with and without constant dose of butyric acid (55 μmol/ml) and/or miconazole (3 μmol/ml). The cells were monitored and photographed every 12 hours using an inverted microscope. After 60 hours, supernatants and cell extracts were examined for LDH (lactate dehydrogenase) concentration and total protein. Cell extract was examined for thiobarbituric acid-reactive substances (TBARS). Hematoxylin-eosin stained slides were prepared for microscopic examinations. *Results*: Biochemicalanalysis of artemisinin-iron treated cells revealed cell injury and reaction in a dose dependent manner. Concurrent combination of butyric acid and miconazole with artemisinin-iron caused significant increase in LDH concentration and TBARS when compared with the control group. Morphological changes include cell and nuclear swelling, vacuole formation, cellular detachment, pyknosis, karyolysis, necrosis and inhibition of new mitosis. *Conclusion*: Although artemisinin renders an excellent antitumor activity, its combination with butyric acid and miconazole provides a safe potent anticancer agent.

Key words: artemisinin, butyric acid, Calu-6, miconazoles

Introduction

Cancer in general and lung cancer in particular are considered as an important problem of major concern especially in low and middle-income countries. Lung cancer is responsible for 1.4 million deaths annually worldwide. Despite significant progress in the development of anticancer drugs, some adverse effects such as hair loss, damages to liver, kidney, bone marrow (1) and intrinsic or acquired resistance to these drugs 2), still remain unsolved. Recently, the use of naturally occurring components of traditional medicinal plants like artemisinin for treatment of cancer has increased significantly (3).

Artemisinin, a derivative from the traditional Chinese herb *Artemisia annua*, has a long history of use in traditional medicine for treatment of malaria fever due to its toxic effect on the malarial parasite, *Plasmodium falciparum* (4). It is a sesquiterpene lactone, containing an endoperoxide that is essential for its

cytotoxic activity (5). Artemisinin causes heme alkylation, protein alkylation, membrane damage, and loss of mitochondrial potential in plasmodium parasite (6).

In recent years, artemisinin and its analogues are shown to inhibit the growth of cancer cell lines (7-9). Carbon-based free radicals originating from reaction of endoperoxide with iron atoms remains one of the main causes of the artemisinin cytotoxicity. Compared to normal cells, cancer cells have more surface transferrin receptors which make them selectively vulnerable to cytotoxicity of artemisinin because of their high rate of iron uptake (10). Artemisinin exerts its toxicity in cancer cells mainly through induction of apoptosis; consequently leading to cell death (11) however , in some cases both apoptosis and necrosis are reported (6).

The antitumor activity of butyric acid and miconazole(12) have been proved. Short chain fatty acids like butyric acid are products of the bacterial fermentation of undigested carbohydrates in the colon (13). Butyric acid induces its toxicity through changing the chromosome structure , gene expression and differentiation in cancer cells (14). Due to some limitations such as rapid metabolism in the body and slow induction of apoptosis, alternative methods such as combination therapy 15) and using longer acting butyric acid (16, 17) have been employed. Miconazole terminates cell proliferation in tumor cells by G0/G1 cell cycle arrest and induction of apoptosis (12).

The aim of present study was to investigate the possible synergistic cytotoxic effect of artemisinin on lung cancer cell line Calu-6 in combination with butyric acid and miconazole.

Materials and methods

The cells

Human pulmonary adenocarcinoma (Calu-6) cell line was obtained from Pasteur Institute, Tehran, Iran. The cells were grown in 25 cm^2 cell culture plastic flasks containing RPMI 1640 medium (Roswell Park Memorial Institute medium, Buffalo, NY, USA) with 10% fetal bovine serum without antibiotics at 37°C in an incubator with humidified air and 5% CO_2. Media were refreshed every 72 hr until the cells filled the entire bottom of the culture flask (7–10 days).

Drugs

Artemisinin, 99% pure, was obtained from Sichuan Arts and Crafts Import and Export Corporation, Chengdu-China. Purification was tested with high performance liquid chromatography (HPLC). Artemisinin was dissolved in DMSO (dimethyl sulfoxide). A fresh innate source solution was produced for each treatment. Different doses of artemisinin (0.15, 0.3, 0.6 and 1.2 µg/ml) were used for treatments. Miconazole, butyric acid and iron were used with constant doses of 55 µmol, 3 µmol and 1 µgr/ml, respectively. (Sigma-Aldrich Corp., Milwaukee, USA). Cell cultures were divided into twelve groups including four variable artemisinin doses with constant dose of miconazole, butyric acid and iron treatments, four artemisinin and iron treatments (five to eight), one miconazole group , one miconazole, artemisinin (0.3 µg/ml) and iron group, one butyric acid, artemisinin (0.3 µg/ml) plus iron group and PBS as negative control group. Iron was added as ferrous sulfate heptahydrate.

Biochemical analysis

After 60 hours, the supernatant media from the individual cultures were recovered and centrifuged (5000 rpm for 5 minutes) in order to isolate floating cells. A total protein measurement and an enzyme analysis (LDH) were performed on the centrifuged supernatants and the cell fraction extracts. Total protein was measured by the Lowry method 18) with bovine serum albumin as standard. Lactate dehydrogenase activity of cells fraction and supernatant was measured by the Babson and Babson method (19). The total lipid peroxidation product, as indicated by malonyldialdehyde (MDA) formation in the cell homogenate, was assayed using the thiobarbituric acid reactive substances (TBARs) method (20). Cell fraction extracts were prepared by mixing centrifuged pellets and half of the cells of flask bottoms that were removed using a plastic stir rod. Cells were lysed using freeze-thaw cycles in a liquid nitrogen tank. Then all specimens were analyzed under light microscopy with a Neubauer slide. For the

last step, pellets were removed after centrifugation of cell lysates (21).

Light microscopy

Cellular changes were monitored and photographed under an inverted microscope every 12 hours until the end of the 60 hours exposure. After 60 hours, the remainder of the cell cultures were fixed with methanol and the plastic bottom of the flasks with fixed cells (cells that were not removed for extraction procedure) were stained with hematoxylin-eosin for microscopic analysis.

Statistical analysis

Data from culture supernatants and cell fraction extracts were analyzed using SPSS version 19.0.0 (SPSS, Inc., Chicago, USA) software and were presented as mean±S.D of three independent experiments. One-way ANOVA (analysis of variances) and Tukey (post hoc) were used. Levels of significance were represented in each result (P value ≤ 0.05 was considered significant).

Results

Biochemical analysis

Perceived LDH of supernatant solution in highest doses of artemisinin (1.2 and 0.6 ug/ml) plus miconazole, butyric acid and iron had led to a significant increase in comparison with the lowest doses of mentioned artemisinin (0.15 and 0.3 ug/ml) combinations (P<0.001). In addition, measured LDH in all groups except group four had noticeable increase with control group (P<0.001). In comparison of the first four groups with the second four groups without miconazole and butyric acid treatments, there was significant difference between the groups with equal amount of artemisinin (p<0.05). In the groups with different doses of artemisinin with iron (groups five to eight) significant elevation was observed in LDH of supernatants (P<0.01). Supernatant LDH level in the groups nine (miconazole), ten (artemisinin, miconazole and iron) and eleven (artemisinin, butyric acid and iron) was remarkably different with control group and increased. Results of measured LDH in cell fraction have shown significant difference between group one and two with control group (P<0.001), and amounts of LDH were increased in the group that received artemisinin as dose-dependent. Also, highest dose of artemisinin without miconazole and butyric acid (group five) showed noticeable LDH level enhancement in comparison with lowest dose of the same combination therapy (P<0.01). Regarding the LDH level of cell fraction, only groups one and five showed significant increases in contrast to control group (P<0.001). Total protein level of cell fraction was significantly different in the groups with highest and lowest dose of artemisinin (both with and without miconazole and butyric acid) and increased (P<0.05). Measured MDA in groups one, two, five and six (high doses of artemisinin) have shown significance difference with control group and enhanced remarkably (Table 1).

Morphological changes

In the first four groups that received all the drugs with different doses of artemisinin, degenerative changes were observed after 12 hours. The first features were cellular and nuclear swelling and cellular detachment from the culture surface (Fig. 1). After 12 hours, more than 20% of the cells were detached from the surface in the highest dose of artemisinin. Severity of all changes was completely dose-dependent. Cellular vacuolization with numerous vacuoles (Fig. 2) and clumping of necrotic cells was observed in all groups at the end of the 60-hour exposure in a dose-dependent severity manner. The most observed nuclei change in higher doses was karyorrhexis (Fig. 2) but pyknosis and to a lesser extent karyolysis were observed, too. Also, some apoptotic figures were observable. With decrease in artemisinin dose, pyknosis was the most observed phenomenon. Degenerative changes acceleration decreased after 48 hours and cells showed more stability. At the end of exposure, only 30 % of the cells stayed attached in the group one with the highest dose of artemisinin.

In the second four groups that didn't receive miconazole and butyric acid, cell injury was observed af-

Table 1. Biochemical analysis of the cell cultures. Data are presented as Mean±Standard deviation.

Group	Drugs	LDH Medium	LDH Cell Fraction	Total Pro Cell Fraction	Total Pro Medium	MDA
1	Artemisinin (1.2 ug/mL), Miconazole, Butyric acid, Iron	2.12±0.04	14.6±0.41	5.2±0. 4	2.8±0.2	6.3±0.4
2	Artemisinin (0.6 ug/mL), Miconazole, Butyric acid, Iron	1.94±0.15	13.7±0.42	5.1±0. 3	2.4±0.2	6.2±0.2
3	Artemisinin (0.3 ug/mL), Miconazole, Butyric acid, Iron	1.92±0.05	12.5±0.38	5±0.3	2.3±0.1	5.8±0.2
4	Artemisinin (0.15 ug/mL), Miconazole, Butyric acid, Iron	1.41±0. 21	11.9±0.20	4.8±0.6	2.2±0.1	5.6±0.4
5	Artemisinin (1.2 ug/mL), Iron	1.93±0.05	13.7±0.35	4.7±0.6	2±0.3	5.6±0.2
6	Artemisinin (0.6ug/mL), Iron	1.87±0.06	13.4±0.18	4.5±0.7	1.8±0.1	5.6±0.1
7	Artemisinin (0.3 ug/mL), Iron	1.86±0.07	13.2±0.90	4.5±0.7	1.6±0.1	5.7±0.4
8	Artemisinin (0.15ug/mL), Iron	1.47±0.03	12.2±0.25	4.4±0.6	1.7±0.1	4.9±0.1
9	Miconazole	1.29±0.13	11.6±0.07	5±0.7	2.1±0.2	5.3±0.3
10	Artemisinin (0.3 ug/mL), Miconazole, Iron	1.79±0.9	13.5±0.36	5.7±0.1	2.3±0.2	5.7±0.7
11	Artemisinin (0.3 ug/mL) Butyric acid, Iron	1.87±0.08	13.5±0.26	4.8±0.6	2±0.4	6.3±1.2
12	Control	1.35±0.07	11.7±0.18	4.6±0.2	1.6±0.1	4.2±0.5

LDH level: unit/gram protein; Total protein: mg/mL; MDA: nmol/mg protein×10^{-3}

ter 36 hours in the highest dose. This cell injury was cellular and nuclear swelling as well as slight cellular detachment. The highest detachment rate was 10% in the highest dose after 60 hours. Some new mitosis was observed in two lower doses for the compensation of absent cells (Fig. 3). Necrosis was least change observed, and the most nuclear necrotic change was pyknosis; the most observed type of cell death was apoptosis (Fig. 4). The rate of apoptosis was artemisinin dose-dependent. In group 9 that received miconazole

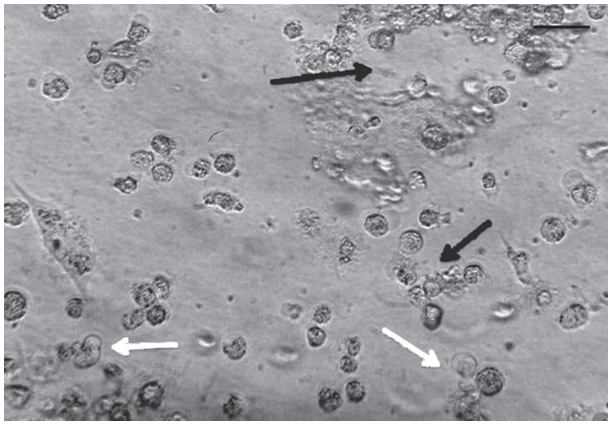

Figure 1. Cellular swelling (white arrows) and detachment (black arrows) in group 1 (artemisinin 1.2 ug/mL, miconazole, butyric acid, iron) in hour 36. Invert microscope (bar=30 μm).

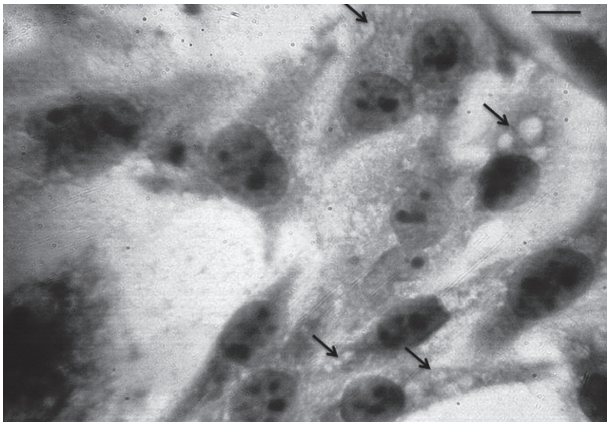

Figure 2. Cellular vacuolization with large and small vacuoles (arrows) in group 3 (artemisinin 0.3 ug /ml, miconazole, butyric acid, iron) in hour 60, karyorrhexis has started in nuclei. Invert microscope (bar=10 μm).

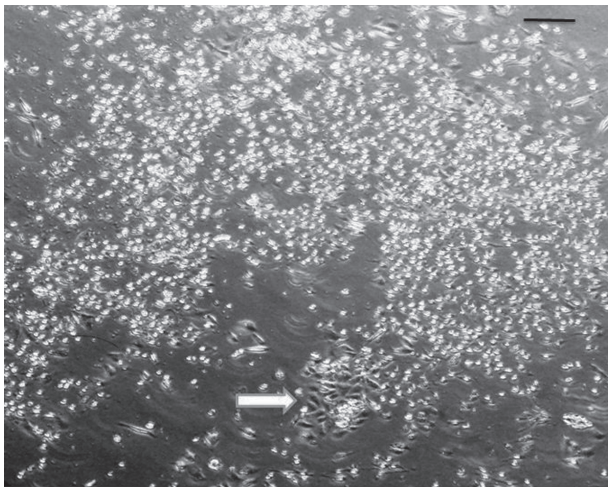

Figure 3. New mitoses in group 7 (artemisinin 0.3 ug/mL, iron) in hour 48 (arrow). Some rounded, detached and injured cells are observable. Invert microscope (bar=60 μm).

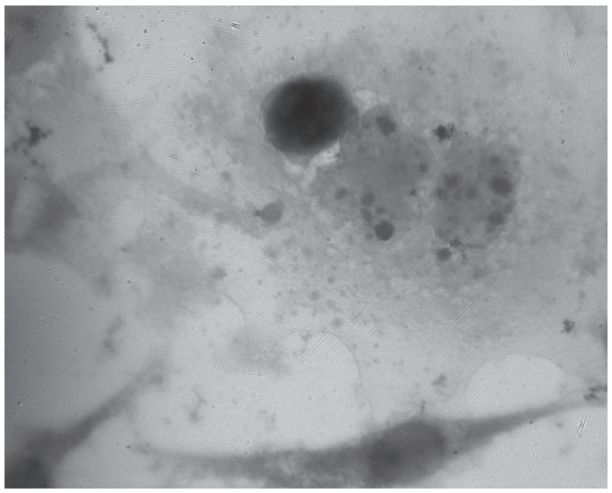

Figure 4. Three apoptotic cells in group 5 (artemisinin 1.2 ug/mL, iron) in hour 60 (arrows). H&E staining (bar=60 μm).

alone, the changes were similar to the first four groups but they were slighter, with the pyknotic as the most apparent cellular change. At the end of 60 hour, exposure, 40% of cells were detached from the surface. Some apoptotic figures were obvious, too. Cells in the group 10 that were exposed to artemisinin, miconazole and iron didn't show any important change before 24 hours but after that the changes were compared to the first 4 groups with lower intensity. In group 11 that received artemisinin, butyric acid and iron, the most prominent feature was apoptosis. Although necrosis

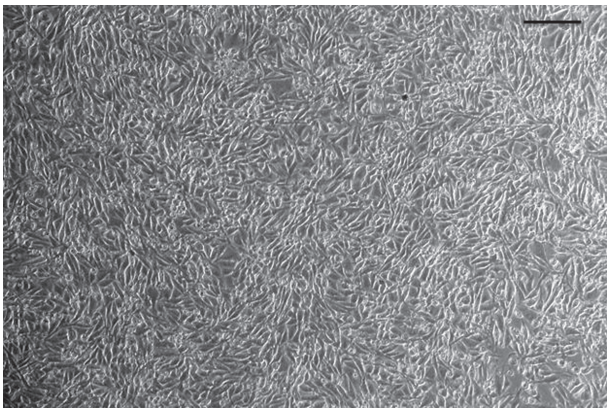

Figure 5. Control group of Calu-6 cells in hour 60. Invert microscope (bar=60 μm)

with pyknotic nuclei was present, a few karyorrhexis were also noted. Control group did not display any significant changes (Fig. 5).

Discussion

Although some studies have shown the effectiveness of combination therapy for artemisinin and its analogs (13, 22), our reported synergist cytotoxic effect on the Calu-6 cell line was not reported. *Artemisia* antitumor property was first reported on Ehrlich ascites tumor cells 23). Iron triggers and markedly enhances the cytotoxicity of artemisinin when it is preloaded with cancer cells. It is believed that presence of iron is essential for potent activity of artemisinin in cancer cells (6). Iron-mediated release of reactive oxygen species (ROS) and/or carbon-centered radicals causes DNA damage, mitochondrial depolarization and apoptosis in tumor cells (24). In the present study, the addition of iron is intended to enhance the effect of artemisinin. The anticancer activities of artemisinin have been postulated to involve cell cycle arrest, apoptosis and/or angiogenesis (25).

The inability of the cells to defend generated free radicals efficiently usually culminates in a destructive consequence known as oxidative stress. Oxidative lipid injuries, namely lipid peroxidation (LPO) by hydrogen peroxide, superoxide anion, and hydroxyl radicals, produce a progressive loss of membrane fluidity, thus reducing membrane potential and increasing its perme-

ability. Malonyldialdehyde (MDA) is the primary end product of lipid peroxidation and its increase indicates oxidative stress induced by ROS (26).

In the present study, high doses of artemisinin (1.2 and 0.6 ug/mL) significantly increased the MDA-level. Recent study revealed that generation of ROS as a consequence of electron transport chain activity is an initiating event in artemisinin cytotoxicity and seems to be essential for apoptosis induction. Tumor cells are more susceptible to ROS damage due to their lower expression of antioxidant enzymes compared to that of normal cells (27).

The exposure of Calu-6 cells to artemisinin caused a marked increase in LDH level. LDH is a stable cytoplasmic enzyme. The extracellular appearance of LDH is a marker of cell damage. This increase was more noticeable in high doses of artemisinin compared with low doses or untreated cells. These findings suggest that artemisinin induces a concentration-dependent decrease in cell viability, which is accompanied by elevated lipid peroxidation and irreversible cell membrane damage as indicated by LDH release.

Morphological changes also proved dose and time-dependent antitumor activity of artemisinin against Calu-6 cells. The test was performed in triplicate to ensure the accuracy of the results. We add miconazole and butyric to our mixture to give the blend more anti-cancer power. According to the previous researches miconazole dose dependently arrested various cancer cells at the G0/G1 phase of the cell cycle. p53-signaling pathway was suspected to be involved in mediating miconazole-induced apoptosis and growth arrest in human cancer cells 12). Although we found some apoptosis in miconazole-treated groups, in our research miconazole caused a marked necrosis in the Calu-6 cell line and necrotic figures were more obvious than apoptotic ones. Furthermore, these necrotic features became more frequent as the dose of artemisinin was decreased in the first four groups. However, in the present study, the combination of miconazole (group ten) did not result in a significant increase in artemisinin cytotoxicity. Similarly, the combination of butyric acid and artemisinin did not show significant difference when compared with artemisinin alone (group eight). Previously this synergistic cytotoxicity between sodium butyrate and dihydroartemisinin was described on the human lymphoblastoid leukemia cell line (13). Butyric acid is believed to induce apoptosis through histone deacetylation enzyme inhibition and further decondensation of chromatin, which finally leads to endonuclease-mediated apoptosis (28). Interestingly, this synergistic effect was significant when both butyric acid and miconazole combined with artemisinin. This difference was obvious regarding the high LDH and MDA level of first four groups compared to control group and short onset (12 hours) of observed morphological changes. Butyric acid was assumed to facilitate the accessibility of anticancer drugs to DNA by loosening the chromatin (17, 13); however, other possible involving mechanisms need to be elucidated. Taken together, our data did not show significance different between butyric acid and miconazole (groups ten and eleven), as there was no significance different between groups ten and eleven. It is recommended that increased doses of these agents or their practical analogs be used in order to observe or improve this synergist effect. Some derivatives of butyrate have shown more powerful activity than butyrate itself (29), and in some cases this activity was specific to its analogs.

References

1. Jaracz S, Chen J, Kuznetsova LV, *et al.* Recent advances in tumor-targeting anticancer drug conjugates. Bioorg Med Chem 2005; 13: 5043-54.
2. Han YH, Moon HJ, You BR, *et al.* Effects of carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone on the growth inhibition in human pulmonary adenocarcinoma Calu-6 cells. Toxicology 2009; 265: 101-7.
3. Nam W, Tak J, Ryu JK, *et al.* Effects of artemisinin and its derivatives on growth inhibition and apoptosis of oral cancer cells. Head Neck 2007; 29: 335-40.
4. Sadava D, Phillips T, Lin C, *et al.* Transferrin overcomes drug resistance to artemisinin in human small-cell lung carcinoma cells. Cancer Lett 2002; 179: 151-6.
5. Van Agtmael MA, Eggelte TA, van Boxtel CJ. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. Trends Pharmacol Sci 1999; 20: 199-205.
6. Crespo-Ortiz MP, Wei MQ. Antitumor activity of artemisinin and its derivatives: from a well-known antimalarial agent to a potential anticancer drug. J Biomed Biotechnol 2011; 2012: 247-597.
7. Paik IH, Xie S, Shapiro TA, *et al.* Second generation, orally active, antimalarial, artemisininderived trioxane dimers with

high stability, efficacy, and anticancer activity. J Med Chem 2006; 49: 2731-4.
8. Posner GH, McRiner AJ, Paik IH, *et al*. Anticancer and antimalarial efficacy and safety of artemisinin-derived trioxane dimers in rodents. J Med Chem 2004; 47: 1299-301.
9. Jiao Y, Ge CM, Meng QH, *et al*. Dihydroartemisinin is an inhibitor of ovarian cancer cell growth1. Acta Pharmacol Sin 2007; 28: 1045-56.
10. Lai H, Sasaki T, Singh NP, Messay A. Effects of artemisinin-tagged holotransferrin on cancer cells. Life Sci 2005; 76: 1267-79.
11. Singh NP, Lai HC. Artemisinin induces apoptosis in human cancer cells. Anticancer Res 2004; 24: 2277-80.
12. Wu CH, Jeng JH, Wang YJ, *et al*. Antitumor effects of miconazole on human colon carcinoma xenografts in nude mice through induction of apoptosis and G0/G1 cell cycle arrest. Toxicol Appl Pharmacol 2002; 180: 22-35.
13. Singh NP, Lai HC. Synergistic cytotoxicity of artemisinin and sodium butyrate on human cancer cells. Anticancer Res 2005; 25: 4325-31.
14. Gleave ME, Sato N, Sadar M, *et al*. Butyrate analogue, isobutyramide, inhibits tumor growth and time to androgen-independent progression in the human prostate LNCaP tumor model. J Cell Biochem 1998; 69: 271-81.
15. Rabizadeh E, Bairey O, Aviram A, *et al*. Doxorubicin and a butyric acid derivative effectively reduce levels of BCL-2 protein in the cells of chronic lymphocytic leukemia patient. Eur J Haematol 2001; 66: 263-71.
16. Rephaeli A, Blank-Porat D, Tarasenko N, *et al*. In vivo and in vitro antitumor activity of butyroyloxymethyldiethyl phosphate (AN-7), a histone deacetylase inhibitor, in human prostate cancer. Int J Cancer 2005; 116: 226-35.
17. Niitsu N, Kasukabe T, Yokoyama A, *et al*. Anticancer derivative of butyric acid (pivalyloxymethyl butyrate) specifically potentiates the cytotoxicity of doxorubicin and daunorubicin through the suppression of microsomal glycosidic activity. Mol Pharmacol 2000; 58: 27-36.
18. Lowry OH, Rosebrough NJ, Farr AL, *et al*. Protein measurement with the Folin phenol reagent. J biol Chem 1951; 193: 265-75.
19. Babson AL, Babson SR. Kinetic colorimetric measurement of serum lactate dehydrogenase activity. Clin Chem 1973; 19: 766-9.
20. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzym 1978; 52: 302-10.
21. Hackett RL, Shevock PN, Khan SR. Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals. Urol Res 1994; 22: 197-203.
22. Wu GS, Lu JJ, Guo JJ, *et al*. Synergistic anti-cancer activity of the combination of dihydroartemisinin and doxorubicin in breast cancer cells. Pharmacol Reports 2013; 65: 453-9.
23. Woerdenbag HJ, Moskal TA, Pras N, *et al*. Cytotoxicity of artemisinin-related endoperoxides to Ehrlich ascites tumor cells. J Nat Prod 1993; 56: 849-56.
24. O'Neill PM, Barton VE, Ward SA. The molecular mechanism of action of artemisinin-the debate continues. Molecules 2010; 15: 1705-21.
25. He R, Mott BT, Rosenthal AS, *et al*. An artemisinin-derived dimer has highly potent anti-cytomegalovirus (CMV) and anti-cancer activities. PLoS One 2011; 6: e24334.
26. Shahbazfar AA, Zare P, Mohammadpour H, *et al*. Effects of different concentrations of artemisinin and artemisinin-iron combination treatment on Madin Darby Canine Kidney (MDCK) cells. Interdiscip Toxicol 2012; 5: 30-7.
27. Bostwick DG, Alexander EE, Singh R, *et al*. Antioxidant enzyme expression and reactive oxygen species damage in prostatic intraepithelial neoplasia and cancer. Cancer 2000; 89: 123-34.
28. Hague A, Manning AM, Hanlon KA, *et al*. Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. Int J Cancer 1993; 55: 498-505.
29. Nudelman A, Ruse M, Aviram A, *et al*. Novel anticancer prodrugs of butyric acid. 2. J Med Chem 1992; 35: 687-94.

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