Apoptosis and cell cycle arrest of human colorectal cancer cell line HT-29 induced by vanillin

KetLi Ho a, Latifah Saiful Yazana a,b, Norsharina Ismail a, Maznah Ismail a,b,*

a Nutraceutical and Nutrigenomics Program, Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
b Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

ABSTRACT

Background: Vanillin is responsible for the flavor and smell of vanilla, a widely used flavoring agent. Previous studies showed that vanillin could enhance the repair of mutations and thus function as an anti-mutagen. However, its role in cancer, a disease that is closely related to mutation has not yet been fully elucidated. Methods: Hence, this study investigated the cytolytic and cytostatic properties of vanillin against HT-29, a human colorectal cancer cell line. Methods used including cell viability assay, acridine orange (AO)–ethidium bromide (EB) double staining cell morphological analysis, Cell cycle analysis, annexin V–propidium iodide apoptosis test and 5-bromo-2-deoxyuridine (BrdU)-labeling cell proliferation assay. Results: Results showed that apoptosis was induced by vanillin and the IC50 for HT-29 and NIH/3T3 normal cell lines were 400 μg/ml and 1000 μg/ml, respectively. Different concentrations of vanillin arrest cell cycle at different checkpoints. 5-Bromo-2-deoxyuridine-labeling cell proliferation assay showed that G0/G1 arrest was achieved at lower concentration of vanillin (200 μg/ml) while cell cycle analysis by flow cytometer showed that G2/M arrest occurs at higher concentration of vanillin (1000 μg/ml). Conclusion: Cytolytic and cytostatic effects shown by vanillin showed that it could be a useful colorectal cancer preventive agent. Further in vivo study should be carried out to confirm that similar effects could happen in animals.

Background: Vanillin is responsible for the flavor and smell of vanilla, a widely used flavoring agent. Previous studies showed that vanillin could enhance the repair of mutations and thus function as an anti-mutagen. However, its role in cancer, a disease that is closely related to mutation has not yet been fully elucidated. Methods: Hence, this study investigated the cytolytic and cytostatic properties of vanillin against HT-29, a human colorectal cancer cell line. Methods used including cell viability assay, acridine orange (AO)–ethidium bromide (EB) double staining cell morphological analysis, Cell cycle analysis, annexin V–propidium iodide apoptosis test and 5-bromo-2-deoxyuridine (BrdU)-labeling cell proliferation assay. Results: Results showed that apoptosis was induced by vanillin and the IC50 for HT-29 and NIH/3T3 normal cell lines were 400 μg/ml and 1000 μg/ml, respectively. Different concentrations of vanillin arrest cell cycle at different checkpoints. 5-Bromo-2-deoxyuridine-labeling cell proliferation assay showed that G0/G1 arrest was achieved at lower concentration of vanillin (200 μg/ml) while cell cycle analysis by flow cytometer showed that G2/M arrest occurs at higher concentration of vanillin (1000 μg/ml). Conclusion: Cytolytic and cytostatic effects shown by vanillin showed that it could be a useful colorectal cancer preventive agent. Further in vivo study should be carried out to confirm that similar effects could happen in animals.

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most widely used flavoring agents. It has been used since year 1300s to make perfume, treat insect bite, heal wounds, treat coughing, stop blood spitting, increase muscular energy and stimulate sexual properties. In modern days, vanillin is proven as an anti-microbial agent and inhibitor of red blood cell sickling [1].

During the past 20 years, there has been an increasing interest in using vanillin as an anti-mutagenic agent. In 1986, Ohta et al. [2] first tested the anti-mutagenic effect of vanillin on bacteria and found that vanillin could reduce 4-nitroquinoline 1-oxide (4-NQO) and furylfuramide (AF-2) induced mutations. Subsequently, vanillin was reported to significantly reduce the mutations induced by ultraviolet light (UV), X-ray, ethynitrosourea (ENU) in V79 cell [3]. Mitomycin C (MMC) and methylmethane sulphonate (MMS) induced mutation in somatic cells of Drosophila melanogaster and mouse bone marrow cells also could be reduced by vanillin [4,5]. However, vanillin does not have anti-mutagenic effect on N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced mutations [6]. It is suggested that vanillin anti-mutagenic property is achieved by recA-dependent recombinational repair enhancement [7], error prone SOS repair inhibition [8], non-homologous DNA end-joining (NHEJ) inhibition [9], and reactive oxygen species scavenging [10].

As cancer is a disease initiated by genomic mutations, effective anti-mutagen like vanillin should possess anti-carcinogenic potential. Lirdprapamongkol et al. found that vanillin could suppress in vitro invasion and in vivo metastasis of mouse breast cancer cells [11]. King et al. [12] also reported that vanillin effectively repair mutation in colon cancer cell line. As vanillin's anti-mutagenic effect is confirmed in colon cancer cell line, this showed that colon cancer cell line would be a suitable cell line to investigate the relationship between vanillin anti-mutagenic effect and its cytotoxic effect. Furthermore, as vanillin is widely used as a flavoring agent, it is likely to interact with the colon.

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most widely used flavoring agents. It has been used since year 1300s to make perfume, treat insect bite, heal wounds, treat coughing, stop blood spitting, increase muscular energy and stimulate sexual properties. In modern days, vanillin is proven as an anti-microbial agent and inhibitor of red blood cell sickling [1].

During the past 20 years, there has been an increasing interest in using vanillin as an anti-mutagenic agent. In 1986, Ohta et al. [2] first tested the anti-mutagenic effect of vanillin on bacteria and found that vanillin could reduce 4-nitroquinoline 1-oxide (4-NQO) and furylfuramide (AF-2) induced mutations. Subsequently, vanillin was reported to significantly reduce the mutations induced by ultraviolet light (UV), X-ray, ethynitrosourea (ENU) in V79 cell [3]. Mitomycin C (MMC) and methylmethane sulphonate (MMS) induced mutation in somatic cells of Drosophila melanogaster and mouse bone marrow cells also could be reduced by vanillin [4,5]. However, vanillin does not have anti-mutagenic effect on N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced mutations [6]. It is suggested that vanillin anti-mutagenic property is achieved by recA-dependent recombinational repair enhancement [7], error prone SOS repair inhibition [8], non-homologous DNA end-joining (NHEJ) inhibition [9], and reactive oxygen species scavenging [10].

As cancer is a disease initiated by genomic mutations, effective anti-mutagen like vanillin should possess anti-carcinogenic potential. Lirdprapamongkol et al. found that vanillin could suppress in vitro invasion and in vivo metastasis of mouse breast cancer cells [11]. King et al. [12] also reported that vanillin effectively repair mutation in colon cancer cell line HCT116. As vanillin's anti-mutagenic effect is confirmed in colon cancer cell line, this showed that colon cancer cell line would be a suitable cell line to investigate the relationship between vanillin anti-mutagenic effect and its cytotoxic effect. Furthermore, as vanillin is widely used as a flavoring agent, it is likely to interact with the colon.

A lot of studies have been carried out to study vanillin anti-mutagenic effects. However, far too little attention has been paid to the impact of vanillin on cancer. Hence, the main purpose of this study was to determine the cytolytic and cytostatic effects of vanillin on colorectal cancer cell line HT-29.
2. Materials and methods

2.1. Materials

Vanillin, vanillic acid, sodium azide, acridine orange, ethidium bromide and Dulbecco's modified eagle's medium (DMEM) were purchased from Sigma Chemical (St.-Louis, USA). CellTiter 96® AQueous One Solution Cell Proliferation Assay was bought from Promega (Southampton, UK). Propidium iodide was purchased from Biovision (Mountain view, CA) while RNase A from Novagen (Madison, WI). APOPTEST™-FITC, mouse anti-BrdU was obtained from Dakocytomation (Kattendijke, Netherlands) and bovine serum albumin, 5-bromo-2-deoxyuridine (BrdU) were purchased from Calbiochem (Madison, WI). HT-29 and NIH/NIH/3T3 cell lines were purchased from the American Type Culture Collection (ATCC, USA). Other chemicals used were all of analytical grade.

2.2. Cell viability

HT-29 and NIH/3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were incubated in 5% CO₂ at 37 °C in 25 cm² flasks. Confluent cells were detached using 0.25% (w/v) trypsin–EDTA. Cell number and viability were determined using a haemocytometer after staining with trypan blue. Approximately 1 × 10⁵ cells were seeded into 96-well plates and incubated for 24 h. Vanillin and vanillic acid were then added into the wells. After 72 h, the cell viability was measured according to the manufacturer instruction. Briefly, 20 μl per well of The CellTiter 96® AQueous One Solution Reagent containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-2,5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES) was added and incubated for 1 h at 37 °C in a humidified, 5% CO₂ atmosphere. Viable cells colonies produce formazan which could be measured at 490 nm with a 96-well plate reader ( Opsys, USA).

2.3. Acridine orange (AO)–ethidium bromide (EB) double staining cell morphological analysis

Acridine orange is taken up by both viable and dead cells. It would fluoresce green when bound to double stranded DNA in living cells and fluoresce red when bound to single stranded DNA which dominates in dead cells. Ethidium bromide was excluded from living cells. However, late apoptotic or necrotic cells have ruptured membrane that allow the entrance of ethidium bromide to intercalate into DNA and fluoresce red. 5 × 10⁵ of HT-29 cells were seeded into each well of a 6-well plate and incubated for 24 h at 37 °C in a humidified, 5% CO₂ atmosphere. Vanillin was then added into the wells and incubated for 72 h. After incubation, cells were detached using 0.25% (w/v) trypsin–EDTA and washed once with phosphate buffer saline (PBS). Ten microlitres of monoclonal mouse anti-bromodeoxyuridine/FITC to bind to the double-strand of DNA. Thisstrand separation is essential for the monoclonal mouse anti-bromodeoxyuridine/FITC to bind to the BrdU. After strand separation, 3 ml of 0.1 M disodium borate anhydrous (pH 8.5) was added to neutralize the acid. The cells were then re-suspended in 100 μl of PBS–BSA–0.5% Tween 20. Ten microlitres of monoclonal mouse anti-bromodeoxyuridine/FITC was added into the suspension for 30 min while incubated at 4 °C. Cells were then washed with PBS-Tween20 again and finally PI was added with final concentration of 2 μg/ml. The samples were then read with a flow cytometer.

2.7. Statistical analysis

Data were expressed as mean ± S.D. and analyzed by Tukey’s test to determine the significance of differences between groups. A p-value lower than 0.05 or/and 0.01 was considered to be significant.

2.5. Annexin V–propidium iodide (AnnV–PI) staining apoptosis test

5 × 10⁵ of HT-29 cells were seeded into each well of a 6-well plate and after incubation for 24 h vanillin was added. The cells were then incubated for 24 h and 72 h. The subsequent procedures were carried out according to the instructions provided by the manufacturer of APOPTEST™-FITC kit. Briefly, cells were washed with PBS, suspended in binding buffer and then added with annexin-V FITC and propidium iodide (PI) for 10 min. The samples were then read by a flow cytometer.

2.6. 5-Bromo-2-deoxyuridine (BrdU)-labeling cell proliferation assay

5 × 10⁵ of cells were seeded into each well of a 6-well plate and incubated for 24 h before vanillin was added. Cells were then further incubated for 24 h, 48 h and 72 h. Prior to detachment, 5 μl of 10 μM BrdU was added into the media and incubated for half an hour. This would enable proliferating cells to take up the BrdU and incorporated them into the genome. Cells were then washed twice with 1 ml PBS–1% BSA and fixed with 5 ml 70% ethanol at −20 °C for 2 h. After fixation, 1 ml of 2 M hydrochloric acid–0.5% triton X-100 was added for 30 min at room temperature to separate the double-strand of DNA. This treatment with 10 μM BrdU was added into the media and incubated for half an hour. This would enable proliferating cells to take up the BrdU and incorporated them into the genome. Cells were then washed twice with 1 ml PBS–1% BSA and fixed with 5 ml 70% ethanol at −20 °C for 2 h. After fixation, 1 ml of 2 M hydrochloric acid–0.5% triton X-100 was added for 30 min at room temperature to separate the double-strand of DNA. This treatment is essential for the monoclonal mouse anti-bromodeoxyuridine/FITC to bind to the BrdU. After strand separation, 3 ml of 0.1 M disodium borate anhydrous (pH 8.5) was added to neutralize the acid. The cells were then re-suspended in 100 μl of PBS–BSA–0.5% Tween 20. Ten microlitres of monoclonal mouse anti-bromodeoxyuridine/FITC was added into the suspension for 30 min while incubated at 4 °C. Cells were then washed with PBS-Tween20 again and finally PI was added with final concentration of 2 μg/ml. The samples were then read with a flow cytometer.

Fig. 1. Treatment of (a) vanillin and (b) vanillic acid on HT-29 and 3T3 for 72 h. The cells survival were determined by MTS dye assay. **p < 0.01 from untreated cells.
3. Results

3.1. Cell viability

Both vanillin and vanillic acid were tested for cell viability. Vanillic acid was tested because it was the major metabolite of vanillin in human [11]. As shown in Fig. 1, vanillin showed cytolytic and cytostatic effects on HT-29 while vanillic acid did not show any cytolytic or cytostatic effect at concentration up to 500 μg/ml. The IC$_{50}$ of vanillin was 400 μg/ml while vanillic acid did not achieve IC$_{50}$ on HT-29. The IC$_{50}$ of vanillin against NIH/3T3 cells was 1000 μg/ml. It is apparent that vanillin is more cytolytic or/and cytostatic for the cancerous HT-29 cells than the normal NIH/3T3 cells. Since vanillic acid did not show cytotoxic effect on HT-29 cell line, its effect on normal cell line NIH/3T3 was not carried out.

The results obtained from the acridine orange–ethidium bromide double staining are shown in Fig. 2. Viable cells with intact DNA and nucleus give a round and green nuclei. Early apoptotic cells will have fragmented DNA which gives several green colored nuclei. Late apoptotic and necrotic cells DNA would be fragmented and stained orange and red. From the data it was clear that with increasing concentration of vanillin, the number of viable cells decreased tremendously. Besides, some cells exhibited typical characteristics of apoptotic cells like plasma membrane blebbing. However, the number of cells stained red did not increase. This indicates that most of the cells were not undergoing necrosis and cell death occurred primarily through apoptosis.

Fig. 2. Morphological study of HT-29 cells treated with various concentration of vanillin for 72 h. (a) 0 μg/ml; (b) 200 μg/ml; (c) 400 μg/ml; (d) 600 μg/ml; (e) 1000 μg/ml. The arrow is showing cells which were blebbing.
3.3. Cell cycle analysis by flow cytometer

Fig. 3 shows the effect of vanillin on the cell cycle of HT-29 cells. A slight increase ($p < 0.05$) of cell death was observed at 200 $\mu$g/ml and 400 $\mu$g/ml which was more pronounced at higher vanillin concentrations ($p < 0.01$). After 72 h, cell death was found to significantly ($p < 0.01$) increase to 20% compared to the control (5%) in cells treated with 600 $\mu$g/ml vanillin. When vanillin concentration was further increased to 1000 $\mu$g/ml, cell death was even increased to 30%. Besides, with high concentration of vanillin (1000 $\mu$g/ml), cell cycle arrest was observed at the G2/M checkpoint. After 24 h, only 24.5% of untreated cells were in G2/M stage but 42.99% of 1000 $\mu$g/ml vanillin treated cells were in G2/M stage.

3.4. Annexin V–propidium iodide (AnnV–PI) staining apoptosis test

Fig. 4 shows that at higher concentrations of vanillin (400 $\mu$g/ml, 600 $\mu$g/ml and 1000 $\mu$g/ml) some changes could be observed over time. After 24 h, only 3.86% of untreated cells entered early apoptosis stage but 6.87% (200 $\mu$g/ml), 6.11% (400 $\mu$g/ml), 7.04% (600 $\mu$g/ml) and 7.16% (1000 $\mu$g/ml) of treated cells have entered early apoptosis stage. After 72 h, only 5.73% of untreated cells entering late apoptosis but 19.06% (400 $\mu$g/ml), 24.32% (600 $\mu$g/ml) and 24.86% (1000 $\mu$g/ml) of treated cells entering late apoptosis. Besides, compared with 15.61% of untreated cells, 15.61% and 18.40% of 600 $\mu$g/ml and 1000 $\mu$g/ml treated cells entering into secondary necrosis respectively. The differences between untreated cells and treated cells were all significant ($p < 0.05$).

3.5. 5-Bromo-2-deoxyuridine (BrdU)-labeling cell proliferation assay

This assay is a more sensitive way to investigate vanillin ability to arrest HT-29 cells in G0/G1 stage compared to the cell cycle analysis by flow cytometer. For cells that are just starting to enter into S phase, the changes in DNA content may not be significant enough in cell cycle analysis but would showed a difference in 5-bromo-2-deoxyuridine-labeling cell proliferation assay. Anti-proliferative effect of vanillin on HT-29 cells is displayed in Fig. 5. After 24 h, all vanillin-treated cells growing ability dropped to approximately 60% for all concentrations. Furthermore, after
48 h, the cells ability to proliferate had been tremendously reduced to 20% at 600 μg/ml. These showed that vanillin could arrest cells in G0/G1 stage. However, after 72 h, proliferation of vanillin treated cells increased while untreated cells decreased. This might be due to the limiting factors in the culture medium of untreated cells. As the nutrient and space in the well of untreated cells decreased, the condition was less favorable for the growth of cells. However, the nutrients in wells containing vanillin treated cells were still abundant, thus allowing the growth of cells.

4. Discussion

Much study has been carried out to investigate the anti-mutagenic property of vanillin for the last two decades. Vanillin shows promising results especially in aiding recombinational repair to fix single strand break, double strand break and interstrand crosslink. As cancer is a disease closely related to genomic mutation, it is logical to postulate that vanillin does have anti-cancer property. Besides, the low toxicity of vanillin also prompted the use of vanillin as anti-cancer agent to reduce the side effect targeting some common features available in many kinds of cancer cells. A potential candidate for this common feature could be the inhibition of AP-1 transcription factor complex. AP-1 activity has been reported to be essential for many cancers including classical Hodgkin lymphoma, anaplastic large cell lymphoma, breast cancer, skin tumors, lung cancer and hepatocellular carcinoma [14].

However, vanillic acid that is the major metabolite of vanillin in human and rats did not show any cytolytic and cytostatic effects. This supports the previous findings reported by Durant [9] that the aldehyde group of vanillin is important for its function. In the acidic orange–ethidium bromide dual staining assay, reduction in cancer cell number and also feature of apoptosis like cells blebbing can be seen. This provides morphological proof to qualitatively show that vanillin could induce apoptosis and inhibit cell growth. In the cell cycle analysis, vanillin induced cell death was shown by an increase in the sub-G1 area for treated cells. Besides, with high concentration of vanillin, a G2/M arrest has been observed. This arrest would allow additional time for the repair of breakage type chromosomal aberrations [15]. It should be noted that in cell cycle analysis only 12% of cell death occurred when HT-29 cells were treated with 400 μg/ml of vanillin. However, in cell viability assay, 50% of cell viability reduction occurred for 400 μg/ml of vanillin treated HT-29 cells. This is due to different parameters were measured in each assay. Cell viability assay detects the number of viable cells but could not tell the percentage of the cells undergoing cell death and the percentage of cells undergoing cycle arrest separately. In contrast, cell cycle assay can differentiate cell death and arrested cells separately. Thus, the 50% cell viability shown in cell viability assay is actually the total of cell death, G0/ G1 arrest and G2/M arrest that were measured in cell cycle analysis and 5-bromo-2-deoxyuridine-labeling cell proliferation assay.

As cell death could be divided into necrosis or apoptosis, it is essential to find out the mode of cell death induced by vanillin. Apoptosis is more favorable than necrosis because it is a programmed cell death and does not trigger inflammatory responses. In this study, cells were finally going into secondary necrosis. It should be noted that secondary necrosis is different from normal necrosis. When apoptosis occurs in vivo, the apoptotic body will be engulfed by macrophage or adjacent cells and thus not going through lysis. However, when apoptosis occurs in vitro the apoptotic body will ultimately swell and lyse due to the absence of phagocytes. As vanillin induced cell death follow the sequence of early apoptosis, late apoptosis and secondary necrosis it is concluded that vanillin actually induce cell death via apoptotic rather than necrotic pathway.

By using the BrdU incorporation assay developed by Dolbeare et al. [16], the ability of vanillin to arrest cells in G0/G1 was determined. From the results, it seems that vanillin is a better cytostatic agent than a cytolytic agent. With lower concentration (200 μg/ml) of vanillin, G0/G1 arrest could still happen while cytolytic effect will only show when vanillin concentration is higher than 400 μg/ml according to the cell cycle analysis.

The findings obtained in this study seem to be consistent with DNA microarray results reported by Cheng et al. [14] that vanillin could change the expression of genes responsible for cellular process, cell cycle, cell death, cancer progression and induce G2 arrest with high concentration of vanillin. Genes involved in DNA damage and oxidative damages also found regulated by vanillin in colon cancer cell line HCT16 cells [12].

In conclusion, this study has shown that vanillin possesses cytolytic and cytostatic properties. It could induce apoptosis and inhibit cancerous cell growth. These findings add to a growing body of literature on the functional use of vanillin. However, the concentration of vanillin needed to carry out all those functions is high. This might be due to vanillin is a better cytostatic agent than a

Fig. 4. Annexin V–PI flow cytometry analysis of HT-29 cells treated with μg/ml, 200 μg/ml, 400 μg/ml, 600 μg/ml and 1000 μg/ml of vanillin. The cells could be divided into four groups: viable, early apoptosis, late apoptosis and secondary necrosis.

Fig. 5. Anti-proliferative effects of vanillin on HT-29 cells treated with various concentrations of vanillin for 24 h and 48 h. *p < 0.05, **p < 0.01 compared to the control cells.
cytolytic agent. As cytostatic agent usually needs longer treatment time \[11\], lower concentration of vanillin might still be able to show effects if a longer treatment time is allowed. Besides, even though the concentration of vanillin needed is high, its effectiveness might be increased when used together with other chemotherapeutic drugs due to synergistic effect.

As current studies are focused on in vitro investigation of vanillin effect, further research might explore vanillin effects in vivo. Since vanillin is consumed widely in foods, we could anticipate that vanillin might be beneficial for the prevention and treatment of colorectal cancer.

**Conflicts of interest**

This research project is carried out by the financial support from Research University Grant Scheme (vote 91087), Universiti Putra Malaysia.

**Acknowledgement**

The authors wish to merit Research University Grant Scheme (vote 91087), Universiti Putra Malaysia for providing financial support to carry out the research project.

**References**