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Cancer Letters 202 (2003) 161–171
CANCER Letters
www.elsevier.com/locate/canlet

N-Caffeoyltyramine arrests growth of U937 and Jurkat cells by inhibiting protein tyrosine phosphorylation and inducing caspase-3

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Received 15 April 2003; received in revised form 31 July 2003; accepted 1 August 2003

Abstract

N-Cinnamoyltyramine, *N*-caffeoyltyramine, *N*-feruloyltyramine, and *N*-sinapoyltyramine were synthesized and investigated to identify the most potent compound with anti-proliferation effect on HL-60, U937 and Jurkat cells. *N*-Caffeoyltyramine was the most potent with $GI_{50} = 10\ \mu M$. The treatment of the cells with *N*-caffeoyltyramine activated caspase-3 activity, and inhibited the growth of cells via decreasing in protein tyrosine kinase activity including epidermal growth factor receptor. These data indicate that *N*-caffeoyltyramine is most potent compound, inducing cell death of the cancer cells by inhibiting protein tyrosine kinases and activating caspase-3 activity.

Keywords: *N*-Caffeoyltyramine; Cell death; Capase-3; Tyrosine phosphorylation; Epidermal growth factor receptor

1. Introduction

Phytochemical research has been performed to explore their effects, particularly on cancers [1–7]. As part of the effort to find phytochemicals with anti-proliferation activity, numerous phytochemicals, including phenylpropenoic acid derivatives, have been screened and studied in our laboratory [8]. We reported previously that *N*-coumaroyltyramine is a potent phytochemical that arrests human tumor cells by inhibiting protein tyrosine kinases [8]. Because several *N*-coumaroyltyramine analogues have been reported in plants, four of these analogues were studied to determine whether they also have anti-proliferation activity and, if so, which of their structural moieties account for inhibition of cell growth of human cancer cells and consequent cell cycle arrest.

In this study *N*-cinnamoyltyramine, *N*-caffeoyltyramine, *N*-feruloyltyramine, and *N*-sinapoyltyramine were synthesized. These analogues were verified by LC–MS and used for characterization of their anti-proliferation activity as well as for determination of the structural moieties that were responsible for the activity. The data indicate that the hydroxyl groups of the phenylpropenyl group of *N*-caffeoyltyramine is the structural moiety responsible for the potent

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doi:10.1016/j.canlet.2003.08.010

anti-proliferation activity that leads to apoptosis by inhibiting protein tyrosine kinases and inducing caspase-3.

2. Materials and methods

2.1. Materials

Cinnamic acid, coumaric acid, caffeic acid, ferulic acid, sinapic acid, tyramine, DCM, 1,3-diisopropylcarbodiimide (DIC), *N*, *N*-dimethylformamide and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO). The FITC-conjugated anti-phosphotyrosine monoclonal antibody (clone PT66, mouse IgG1) and the isotype control, FITC-conjugated mouse IgG1 (MOPC-21) were also obtained from Sigma for use in analyses to determine the extent of phoshorylated tyrosine residues in intact cells. U937, HL-60, and Jurkat cells were purchased from ATCC (Manassas, VA).

3. Methods

3.1. Syntheses and Confirmation of *N*-coumaroyltyramine analogues

Syntheses were performed as described previously [8,9]. Briefly, the appropriate phenylpropenoic acids (cinnamic acid, coumaric acid, caffeic acid, ferulic acid, and sinapic acid) were dissolved in dichloromethane (DCM) and converted to the symmetrical anhydride with 1,3-diisopropylcarbodiimide. Tyramine dissolved in *N*, *N*-dimethylformamide (DMF) was added to the reaction mixture which was then stirred gently for 1 h. The synthesized products were recovered by precipitating with distilled water and washing with ethylacetate and 5% $NaHCO_3$ to remove the unreacted starting materials as described previously [8]. Synthesized N-coumaroyltyramine analogues were purified by HPLC (Waters, Milford, MA). For analysis by LC–MS, samples were purified as described previously [8]. LC was performed with an Agilent HP-1100 liquid chromatograph equipped with a Phenomenex LUNA C_{18} column (150 × 4.26 mm, 5 μm) operated at 25 °C at a flow rate of 0.2 ml/min using the elution buffer containing 0.1% formic acid. The run time was 25 min per sample.

3.2. Cell culture conditions

U937, HL-60, and Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cell viability was determined microscopically by trypan blue exclusion, and the number of cells was counted by hematocytometer [10].

3.3. Cell death

Cells were treated with several concentrations of *N*-coumaroyltyramine analogues (for all experiments; all analogues were dissolved in 100 methanol, and the control and the samples were treated with the same volume of methanol). Total cell death was measured by both the trypan blue exclusion method [10] as well as by a non-radioactive cell proliferation assay (Promega, Madison, WI). The non-radioactive assay is based on the cellular conversion of a tetrazolium salt into a blue formazan product that is easily detected at 570 nm [11].

3.4. Caspase-3 activity

Caspase-3 activity was measured using ApoAlert Caspase-3 fluorescent assay kit (Clontech, Palo Alto, CA). Jurkat cells (1×10^6) were treated with *N*-caffeoyltyramine, and cell samples were harvested and prepared for assay at 0, 3, 6, 9 and 24 h. Caspase-3 activity was determined by measuring fluorescence from samples at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

3.5. DNA fragmentation assay

The assay was performed according to the instructions provided by the manufacturer (APO-DIRECT, BD Biosciences) with some modifications. Cells (2×10^6) were fixed in the sequential manner as described above. After overnight storage at −20 °C in 70% ethanol, cells were washed with PBS (2 ×) and then incubated for 2 h at 37 °C to label DNA breaks using the terminal deoxynucleotidyl transferase enzyme and fluorescein isothiocyanate deoxyuridine triphosphate (FITC-dUTP). Cells were stained for

total DNA using propidium iodide/RNase at room temperature for 30 min. The doubly stained cells were then immediately analyzed using the FACSCalibur flow cytometer as described above. The extent of DNA fragmentation in treated cells was expressed as the percentage increase in fluorescence caused by the binding of FITC-dUTP compared to the values for untreated controls. Staining of the positive and negative control cells supplied by the vendor served as additional controls for the assay.

3.6. Tyrosine phosphorylation by flow cytometry

Approximately 5×10^6 cells were sequentially fixed and permeabilized with antibodies by the method described by Imbert-Marcille et al. [12,13] but with some modification of the second step. Washed cells were first exposed to1% paraformaldehyde for 20 min at 4 °C. Cells were washed with PBS, suspended in 0.5 ml PBS, and rapidly treated with 5 ml chilled 70% ethanol. At this point, the cells could be stored at −20 °C for 1–7 days before flow cytometric analyses for phosphorylated tyrosine proteins. The fixed cells were recovered by centrifuging followed by washing with PBS before staining with detecting and control antibodies as described by Far et al. [13]. Extent of tyrosine phosphorylation in the cells was determined by measuring the increase in fluorescence produced by the FITC-labeled monoclonal antibody compared to the FITC-labeled isotype control antibody. Fluorescence events for 10,000 cells were collected and analyzed by flow cytometry (FACSCalibur cytometer with CellQuest software, Becton Dickinson, San Jose, CA).

3.7. Determination of EGFR tyrosine kinase activity

Epidermal growth factor receptor (EGFR) tyrosine kinase activity (TK) was measured with a protein tyrosine kinase assay kit (Sigma). Active epidermal growth factor (EGF) receptor purified from human A431 was used as a tyrosine kinase source (Upstate, Lake Placid, NY). Inhibitors were added 10 min before the peptide substrate was added to the assay mixtures. The activity was determined based on an ELISA assay with a TK-specific polymer substrate-coated micro-titer plate. The assay procedure was performed according to the manufacturer's protocol.

4. Results

4.1. Syntheses, and LC–MS and MS/MS analyses of N-cinnamoyltyramine, N-caffeoyltyramine, N-feruloyltyramine, N-sinapoyltyramine

N-Cinnamoyltyramine, *N*-caffeoyltyramine, *N*-feruloyltyramine, and *N*-sinapoyltyramine were synthesized using tyramine and cinnamic acid, caffeic acid, ferulic acid, and sinapic acid, respectively. Their chemical structures differ in the positions and numbers of hydroxyl groups on the benzene ring (Fig. 1). The synthesis was simple, and the yield of each analogue was greater than 55%. Synthesized products were purified by HPLC, and were analyzed by LC–MS as described in Section 2. The major signals from the peaks in LC–MS were obtained at estimated mass/charge (m/z) units of 267.1, 299.1, 313.1, 343.1, which are identical to the (m/z) units of *N*-cinnamoyltyramine, *N*-caffeoyltyramine, *N*-feruloyltyramine, and *N*-sinapoyltyramine (data not shown). Because all LC–MS procedures produced similar analyses of the four analogues, only the LC–MS chromatogram of *N*-caffeoyltyramine is presented in Fig. 2. *N*-Caffeoyltyramine exhibited two peaks in LC–MS (Fig. 2(A)). The signals of each peak were obtained at estimated mass/charge (m/z) units of 300, identical to the (m/z) units of protonated *N*-caffeoyltyramine. The two peaks in the LC–MS chromatogram appeared to be attributable to conformational isomers of *N*-caffeoyltyramine. This was proved by MS/MS. Each peak from the LC–MS was analyzed with a MS/MS spectrometer, and the peak in the MS/MS chromatograms was derived from daughter ion spectrums following the loss of the elements $C_8H_{11}NO$. This is consistent with the tyramine portion of the molecule (data not shown). The data indicated clearly that the compound in the peak of the LC–MS was *N*-caffeoyltyramine. The same LC–MS and MS/MS analyses for the other analogues were performed and confirmed as chemical structures of *N*-cinnamoyltyramine, *N*-feruloyltyramine, and *N*-sinapoyltyramine, respectively, (data not shown).

4.2. Inhibition of the growth of human cancer cells

Because *N*-coumaroyltyramine has been reported to contain anti-proliferation activity against human

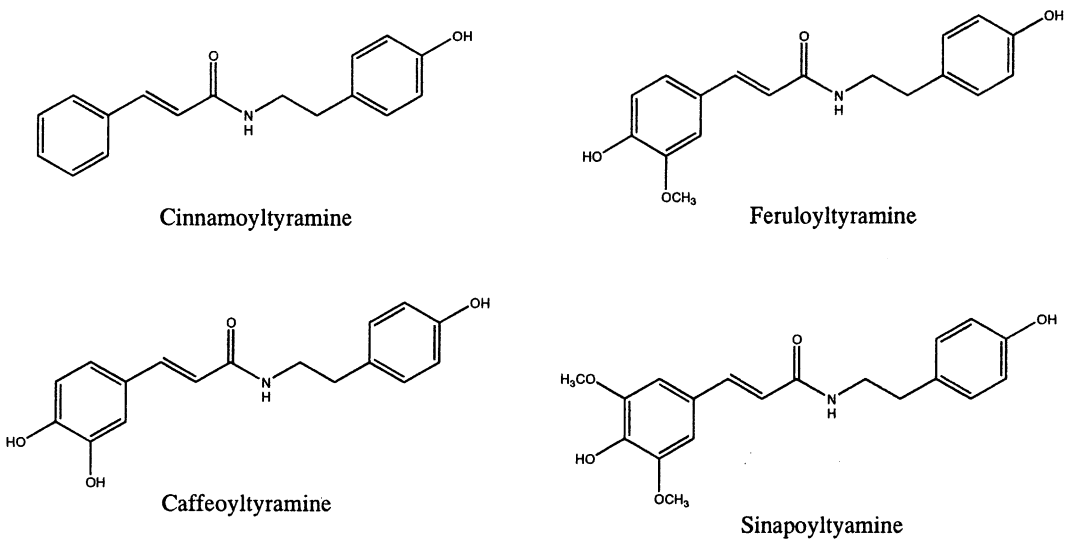


Fig. 1. *Chemical structures of N-cinnamoyltyramine, N-caffeoyltyramine, N-feruloyltyramine, and N-sinapoyltyramine*. Syntheses were performed as described in Ref. [8]. The yields were approximately 55%.

cancer cells [8], the activity of the four compounds (*N*-cinnamoyltyramine, *N*-caffeoyltyramine, *N*-feruloyltyramine, *N*-sinapoyltyramine) was investigated to determine whether these compounds are capable of inhibiting cell growth of human cancer cells. HL-60, U937, and Jurkat cells were used because these cells have been used extensively in cancer studies [14]. The cells were treated for 18 h with various concentrations of *N*-coumaroyltyramine and its analogues. As shown in Fig. 3, the number of living cells decreased with increasing concentrations of the analogues. The amount of cell death was determined by a trypan blue exclusion method and by a non-radioactive cell proliferation assay (Promega) [11]. In these experiments the anti-proliferation activities of the four analogues were compared to *N*-coumaroyltyramine. *N*-Caffeoyltyramine was the most potent. The potency in decreasing order was: *N*-Caffeoyltyramine $>$ *N*-coumaroyltyramine $\geq$ *N*-feruloyltyramine $>$ *N*-cinnamoyltyramine $\geq$ *N*-sinapoyltyramine. *N*-caffeoyltyramine began to suppress growth of cells 3 h after the treatment, but the rest of the compounds did not begin to suppress growth until 10–18 h after the treatment.

4.3. Activation of caspase-3 by N-caffeoyltyramine

Cell death is related to cellular and molecular events in the cells [15–17] and occurs via two independent cell death processes: necrosis or apoptosis. Necrotic cell death is an accidental cell death that does not require any cellular or molecular mechanism [18–21]. Apoptosis, however, does require programmed cellular and molecular events, such as caspase enzymes activation. In this study, CPP32/Caspase-3 activation (an ICE-family protease) was assessed with an ApoAlert-Caspase assay kit. As shown in Fig. 4, both *N*-caffeoyltyramine and *N*-coumaroyltyramine induced caspase-3; but the course of caspase-3 activation by the two phytochemicals was quite different. *N*-Caffeoyltyramine began to activate caspase-3 as early as 3 h after the treatment, and its activity reached a maximum at 6 h; but *N*-coumaroyltyramine had no effect on the activity until 6 h after treatment, and the maximal activity was achieved around 9 h. If a low concentration (5 μM) of *N*-caffeoyltyramine was used, the activation of caspase-3 was reduced four-fold. These data indicate that *N*-caffeoyltyramine can creates a cellular environment conductive to caspase-3 activation earlier than *N*-coumaroyltyramine.

4.4. DNA fragmentation by N-caffeoyltyramine

Activation of ICE-family proteases (CPP32/Caspase-3) eventually leads to the degradation of chromosomal DNA by activating deoxyribonuclease.

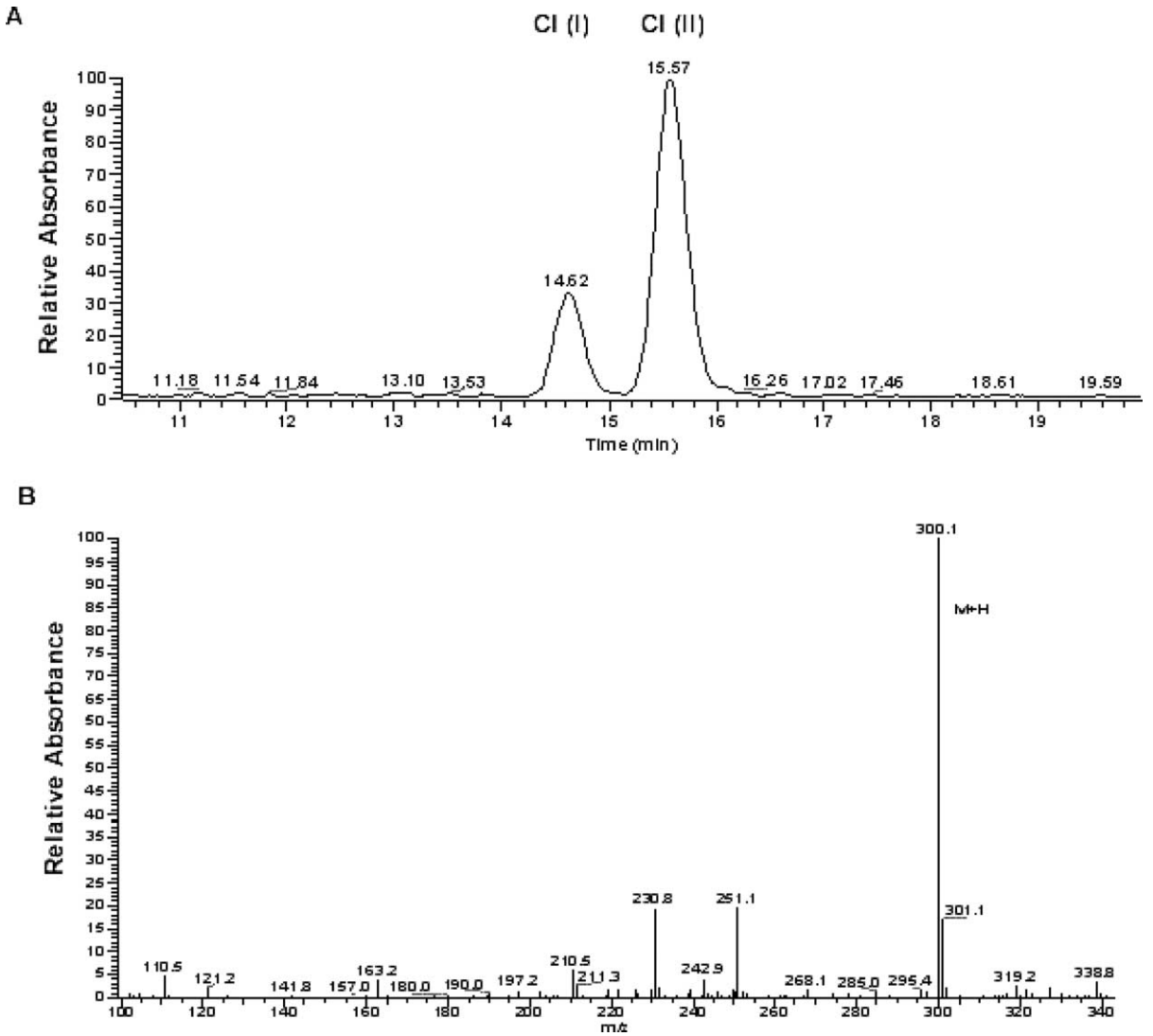
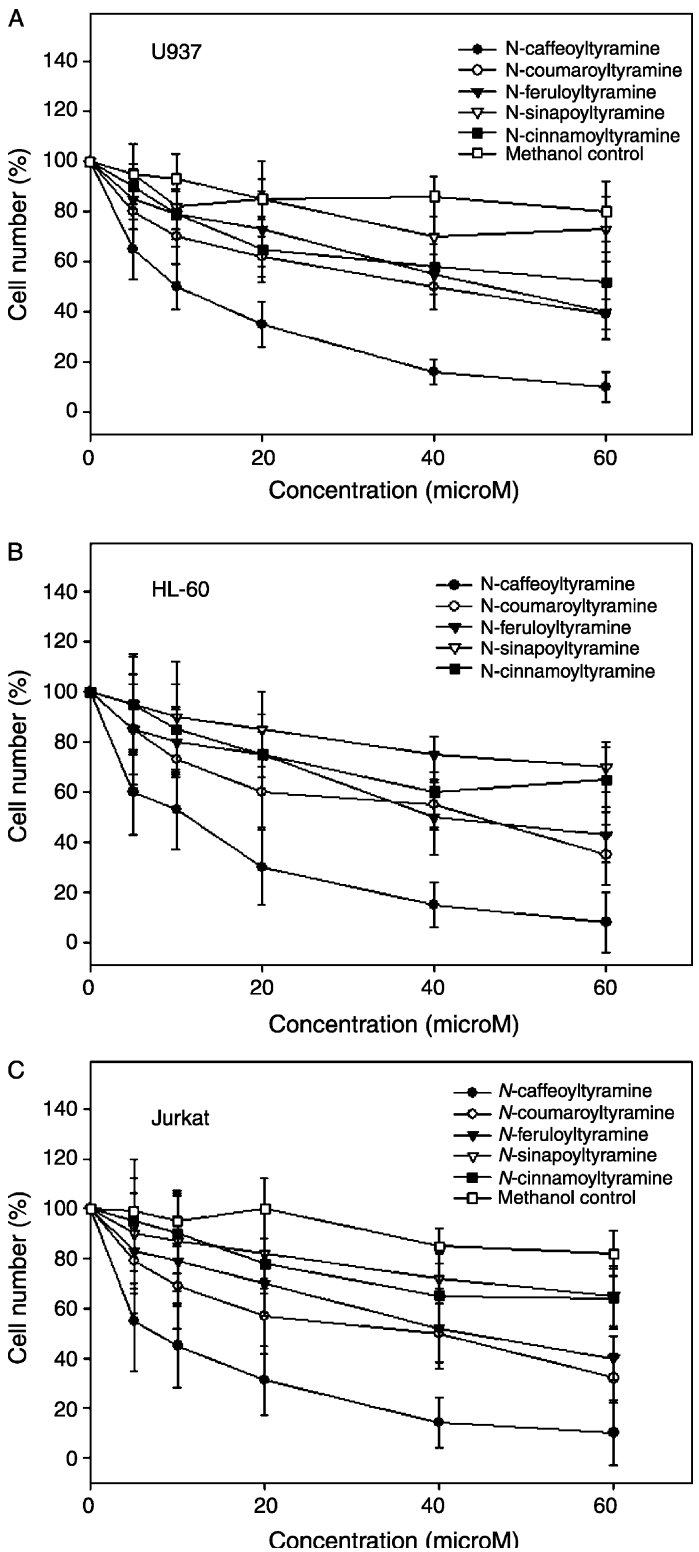


Fig. 2. *LC–MS chromatograms*. (A) Separation of synthesized *N*-caffeoyltyramine by LC–MS. The analysis time was 25 min per sample. Two peaks were well separated with retention times of 14.6 and 15.5. These two peaks correspond to each conformational isomers (CI (I) and CI (II)) of *N*-caffeoyltyramine (see Section 4). (B) Mass spectra of the *N*-caffeoyltyramine detected at *m*/*z* (299). The mass spectrum is the full scan (*m*/*z* 100–500) with the background ions subtracted. Both peaks have a molecular mass of 299 as indicated by their protonated ion at *m*/*z* 300 with full scans that are almost identical.

Because CPP32/Caspase-3 was clearly activated by *N*-caffeoyltyramine, DNA fragmentation experiment was performed to determine whether the activation of CPP32/Caspase-3 can cause DNA fragmentation in the Jurkat cells treated by *N*-caffeoyltyramine. As shown in Fig. 5, more than 70% DNA fragmentation occurred in 30 μM of *N*-caffeoyltyramine. Less DNA fragmentation (24%) was observed when the cells were treated with *N*-caffeoyltyramine (15 μM). The DNA fragmentation data are very consistent with CPP32/Caspase-3 data. Magnitude of the Caspase-3 activation is positively correlated to the degree of


A
U937
N-caffeoyltyramine
N-coumaroyltyramine
N-feruloyltyramine
N-sinapoyltyramine
N-cinnamoyltyramine
Methanol control
Cell number (%)
Concentration (microM)
B
HL-60
N-caffeoyltyramine
N-coumaroyltyramine
N-feruloyltyramine
N-sinapoyltyramine
N-cinnamoyltyramine
Cell number (%)
Concentration (microM)
C
Jurkat
N-caffeoyltyramine
N-coumaroyltyramine
N-feruloyltyramine
N-sinapoyltyramine
N-cinnamoyltyramine
Methanol control
Cell number (%)
Concentration (microM)

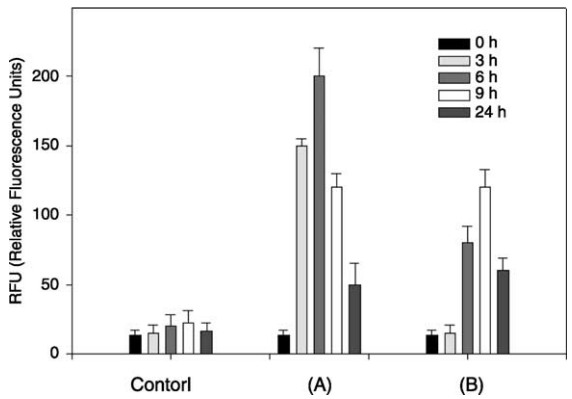


Fig. 4. *Induction of caspase-3 by N-caffeoyltyramine*. Jurkat cells (approximately 0.8×10^6 cells) were treated with *N*-caffeoyltyramine (A) and *N*-coumaroyltyramine (B) (final concentration 30 μM). The activation of capase-3 enzyme was measured by ApoAlert Caspase-3 fluorescent assay kit (Clontech, Palo Alto, CA). Data points represent the mean ± SD of more than three samples. Both treatments produced significant activation of caspase-3 compared to the control values. Caspase-3 activation was significant in the cells treated with *N*-caffeoyltyramine (A) ($p < 0.05$, ANOVA, Student–Newman–Keuls Method).

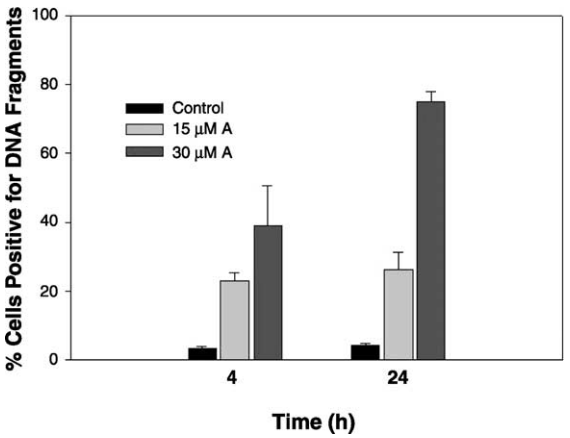


Fig. 5. *DNA fragmentation by N-caffeoyltyramine*. Approximately 2×10^6 Jurkat cells were treated with 15 and 30 μM *N*-caffeoyltyramine (A) or not (Control) for the times indicated. The extent of DNA fragmentation in treated cells was expressed as the percentage increase in fluorescence caused by the binding of FITC-dUTP compared to the values for untreated controls. Initial values for control cells did not differ from those determined at 4 and 24 h and are not included. Values represent the mean ± for three samples. Both treatments produced significant increases in DNA fragmentation compared to the control values. At the same time points, cells treated with 30 μM A demonstrated significantly higher amounts of DNA fragmentation compared cells treated with 15 μM A ($p < 0.05$, ANOVA, Student–Newman–Keuls Method).

DNA fragmentation. These two data suggest strongly that *N*-caffeoyltyramine induces cell death via apoptotic processes.

4.5. Suppression of tyrosine phosphorylation by *N*-caffeoyltyramine

Stimulation of cells by external growth factors frequently leads to phosphorylation of proteins in cancer cells. The phosphorylation of tyrosine residues of proteins is assumed to be involved in abnormal growth of human tumor cells [22,23]. Because *N*-coumaroyltyramine has already been reported to induce apoptotic cell death and inhibit protein tyrosine phosphorylation in cancer cells [8], the effect of *N*-caffeoyltyramine on the inhibition of protein tyrosine phosphorylation was investigated. Total tyrosine phosphorylation in Jurkat cells was determined by flow cytometry with FITC-labeled monoclonal antibody against phosphotyrosine. As shown in Fig. 6, the phosphotyrosine level was reduced time-dependently in the cells treated with *N*-caffeoyltyramine (A) compared to those treated with *N*-coumaroyltyramine (B). The inhibition of phosphorylation of tyrosine residues was also dose-dependent (data not shown). The data suggest that *N*-caffeoyltyramine may induce the arrest of cell growth and cause cell death by inhibiting tyrosine kinases and/or triggering programmed cell death processes.

4.6. Inhibition of EGFR tyrosine kinase activity by *N*-caffeoyltyramine

The EGFR is a protein tyrosine kinase which is over-expressed in various types of cancers, and EGFR

Fig. 3. *Effect of N-coumaroyltyramine analogues on growth of U937, HL-60, and Jurkat cells*. The cells (approximately 0.8×10^6 cells) were treated for 18 h with the analogues. Dead cells were measured by the trypan blue exclusion method [10] as well as by a non-radioactive cell proliferation assay (Promega, Madison, WI). The two method produced a similar result, so the data produced using the trypan blue exclusion method are presented herein. Data points in all figures represent the mean ± SD of more than 3 samples.

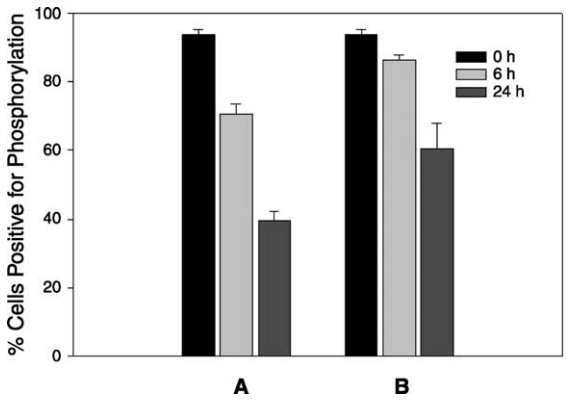


Fig. 6. *Reduction of tyrosine phosphotyrosine by N-caffeoyltyramine*. Approximately 1×10^6 Jurkat cells were treated with *N*-caffeoyltyramine (A) and *N*-coumaroyltyramine (B) (final concentration 30 μM). Data points represent the mean ∀ SD of more than three samples. Cells were fixed and permeabilized as described in Section 2, and extent of tyrosine phosphorylation in the cells was determined by measuring the increase in fluorescence produced by the FITC-labeled monoclonal antibody compared to the FITC-labeled isotype control antibody. Both treatments produced significant reduction of tyrosine phosphorylation compared to the control values. Reduction of tyrosine phosphotyrosine was significant in the cells treated with *N*-caffeoyltyramine (A) ($p < 0.05$, ANOVA, Student–Newman–Keuls Method).

tyrosine kinase inhibitor can block signal transduction pathways implicated in proliferation and survival of cancer cells [24,25]. Because *N*-caffeoyltyramine suppressed the growth of the cancer cells as well as the level of phosphotyrosine, the inhibitory effect of *N*-caffeoyltyramine on EGFR tyrosine kinase was determined with an EGFR tyrosine kinase assay kit. As shown in Table 1, *N*-caffeoyltyramine inhibited a protein tyrosine kinase EGFR in a dose-dependent manner. The data indicate that *N*-caffeoyltyramine is a potent inhibitor of tyrosine kinases including EGFR. Thus it is possible that *N*-caffeoyltyramine may induce the arrest of cell growth and even cause cell death by inhibiting tyrosine kinases and/or EGRF tyrosine kinase.

Table 1
Inhibition of EGFR tyrosine kinase by *N*-caffeoyltyramine

N-caffeoyltyramine (μM)	EGFR tyrosine kinase (%)
0	100
20	50 ± 14
40	36 ± 6
60	23 ± 3
80	15 ± 2

EGFR tyrosine kinase (TK) activity was measured with a protein tyrosine kinase assay kit (Sigma, St Louis, MO). Active EGF receptor purified from human A431 was used as a tyrosine kinase source (Upstate, Lake Placid, NY). The assay procedure was performed according to the manufacturer's protocol. The residual activities were presented as % of the original activity.

5. Discussion

A primary focus of our research is to determine the biological activities of new potent phytochemicals and to elucidate their molecular mechanisms [8,26–29]. The phytochemicals and their analogues can then hopefully be utilized as preventive and/or therapeutic compounds for specific diseases such as diabetes, heart diseases, and cancers [8,26–37]. This objective can be more easily accomplished by investigating a potent phytochemical and its analogues together to determine which structural moieties influence biological activities [38,39]. In this study, *N*-caffeoyltyramine and its analogues were chemically synthesized in order to investigate their anti-proliferation effects. Examination of the relation of the structures to anti-proliferation activity suggests that the hydroxyl group in the benzene ring is important because 4-hydroxylation is essential for activity and additional 3-hydroxylation enhances it. This deduction is also supported by the lesser potency of ferulic acid (4-hydroxy and 3-methoxylcinnamic acid) than caffeic acid. Methoxylation in the benzene ring is apparently not as potent as hydroxylation for anti-proliferation activity. 3 and 4-Hydroxylations of phenylpropenyl group of *N*-caffeoyltyramine may have potent antioxidant properties that can modulate redox-sensitive proteins, and the modulation seem to be associated with the inhibition of EGFR. This study suggest that the hydroxylation, and its number and position in benzene rings are very important for modulating EGFR inhibitory activities as well as anti-proliferation activities of the analogues.

Understanding cellular and molecular events is a key step in evaluating biological effects of new compounds on human cells. Cell growth goes through four different phases of the cell cycle (G0/G1, S, G2, M). Because *N*-caffeoyltyramine inhibited growth of human tumor cells, cell cycle analyses were

performed to determine whether cells treated by the compound were arrested in a specific phase of the cell cycle. The analyses were performed with Jurkat cells treated with *N*-caffeoyltyramine. The treatment changed DNA contents at each phase from 46% (G0/G1), 47% (S) and 6% (G2/M) to 32, 67 and 0%, respectively (unpublished data). The suppressed growth of Jurkat cells treated with *N*-caffeoyltyramine was associated with an increased percentage of cells in the S phase, which is consistent with the data from the cells treated with *N*-coumaroyltyramine [8]. This effect of *N*-caffeoyltyramine on the cell cycle was dependent on concentration and time. However, changes in DNA content at each phase of the cell cycle were not observed, if the cells were treated with higher concentrations of *N*-caffeoyltyramine (higher than 20 μM; unpublished data). This may be due to early entry of the cells into apoptosis processes that precluded arrest at a specific phase of the cell cycle.

Because treatment of Jurkat cells with *N*-caffeoyltyramine induced cell death, an experiment was performed to determine whether cell death was due to necrosis or apoptosis. There are several hallmark events in apoptotic processes such as the activation of ICE proteases, DNA fragmentation, morphological change, and plasma membrane leakage [22,23,40,41]. To determine the cell death process, activation of ICE-family proteases (CPP32/Caspase-3), DNA fragmentation, and morphological change were assessed. Activation of caspase-3 and DNA fragmentation were clearly demonstrated in this paper. Also, morphological change (cell size shrinkage) was also observed (data not shown here). The results indicated that cell death in Jurkat cells treated with *N*-caffeoyltyramine was induced via an apoptotic process (activation of ICE-family proteases (CPP32/Caspase-3), DNA fragmentation, and morphological change).

Protein tyrosine phosphorylation is a central signal pathway involved in mediating various cellular processes such as cell-cycle progress, transcriptional regulation, cell transformation, proliferation, differentiation, and apoptosis [18–21,25]. The critical role of tyrosine phosphorylation in cell proliferation processes is demonstrated by the overexpression of EGFR in various tumor cells [24,25]. Due to the involvement of tyrosine phosphorylation in carcinogenesis, tyrosine kinase inhibitors have been relentlessly sought in order to inhibit signal pathways implicated in proliferation and survival of cancer cells. Caffeic acid and its derivatives were previously reported to have potential to inhibit TK [42–45]. The data indicated that *N*-caffeoyltyramine, a caffeic acid derivative, could inhibit the tyrosine kinase in a time and dose-dependent manner. As described above, *N*-caffeoyltyramine is the most potent phytochemical of the analogues tested for arresting growth of the transformed cells at S phase, for inhibiting EGFR tyrosine kinase, for inducing an apoptotic process, and eventually for causing cell death. Future studies will therefore focus on developing *N*-caffeoyltyramine derivatives inducing cell death via inhibiting tyrosine kinases including EGFR specifically and effectively. The outcome of such studies will contribute to developing new agents for controlling the growth of cancer cells.

Acknowledgements

We thank Vincent Flanagan for analyzing LC–MS samples.

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