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Synthesis and Characterization of *N*-Coumaroyltyramine as a Potent Phytochemical Which Arrests Human Transformed Cells via Inhibiting Protein Tyrosine Kinases

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Numerous phytochemicals are believed to have beneficial effects on human health. *N*-Coumaroyltyramine accumulates in plants in response to wounding and pathogen attack. Due to the scarcity of *N*-coumaroyltyramine, its biological activities have not been studied in human cells. In this study, *N*-coumaroyltyramine was chemically synthesized and then purified by an HPLC with a UV-visible absorbance detector. Retention times of major peaks were 14.3 and 20.7 min, and the peak at 20.7 min was confirmed by LC-MS as *N*-coumaroyltyramine with a mass/charge (*m*/*z*) unit of 284.1. The synthesis procedure was relatively easy and had an acceptable yield (approximately 55%). The compound exhibited a new activity, suppression of growth of human tumor cells such as U937 and Jurkat cells. In addition, the suppressed growth of the cells was strongly associated with an increased percentage of cells in the S phase of the cell cycle progression. Furthermore, *N*-coumaroyltyramine was able to inhibit the protein tyrosine kinases including epidermal growth factor receptor (EGFR). This is the first report of the growth suppressing activity of *N*-coumaroyltyramine and its arrest of cells at the S phase of the cell cycle, possibly by inhibition of protein tyrosine kinases.

***Key Words:* *N*-coumaroyltyramine; phytochemicals; antiproliferation activity; tyrosine kinase inhibitor; cell cycle; epidermal growth factor receptor (EGFR).**

N-Coumaroyltyramine is a phytochemical accumulated by plants in response to wounding and pathogen attack (1–4). The accumulation of *N*-coumaroyltyramine seems to be followed by an increase in the tyramine *N*-coumaroyltransferase (THT) activity (5, 6). The phytochemical appears to be involved in fortifying the cell wall in response to pathogen attack by increasing rigidity as well as indigestibility of the cell wall (6–10). Although *N*-coumaroyltyramine has been identified and characterized in plants to some extent, its biological effects on human cells have not been investigated due to its low concentration in plants.

Currently, the importance of phytochemicals is rapidly being recognized, and much research is consequently focused on finding their beneficial effects and on evaluating their potential impact on human health (11–14). Numerous biological activities of phytochemicals have been discovered in the last decade (14, 15). However, studies of particular phytochemicals have not progressed, largely due to the scarcity of the pure phytochemicals. *N*-Coumaroyltyramine is one of the phytochemicals, the study of which has been seriously held up by the difficulty in obtaining a sufficient quantity of the phytochemical. To overcome this impasse and to elucidate biological activities of *N*-coumaroyltyramine in human cells, the compound was chemically synthesized, purified, and characterized. The synthesis was relatively easy, the yield was acceptable, and the synthesized compound was structurally identical to natural *N*-coumaroyltyramine. The potential biological activities of the synthesized compound were investigated, and during the investigation the synthesized compound was found to arrest tumor cells at a specific phase of the cell cycle. In this report, a convenient synthesis and the chemical characterization of *N*-coumaroyltyramine are described, and the antiproliferation effect of *N*-coumaroyltyramine is demonstrated for the first time, particularly its ability to arrest tumor cells such as U937 and Jurkat cells at the S-phase of the cell cycle, possibly via inhibiting protein tyrosine kinases.

Abbreviations used: EGFR, epidermal growth factor receptor; HPLC, high-performance liquid chromatography; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; MS, mass spectrometer.

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MATERIALS AND METHODS

Materials

Coumaric acid, tyramine, dichloromethane, 1,3-diisopropylcarbodiimide, and *N*,*N*-dimethylformamide were purchased from Sigma (St. Louis, MO). U937 and Jurkat cells were purchased from ATCC (Manassas, VA).

Methods

Synthesis of N-coumaroyltyramine. To synthesize *N*-coumaroyltyramine, coumaric acid and tyramine were used as starting materials. Coumaric acid was dissolved in dichloromethane (DCM), and then converted to the symmetrical anhydride with 1,3-diisopropylcarbodiimide (DIC) as described previously (16). Tyramine dissolved in *N*,*N*-dimethylformamide (DMF) was added to the reaction mixture which was then stirred gently for 1 h. Excess DCM was removed from the mixture by evaporation. The synthesized product was precipitated by the addition of water, and the precipitate was successively washed by ethyl acetate and 5% $NaHCO_3$ to remove the unreacted starting materials.

Purification of N-coumaroyltyramine. Synthesized N-coumaroyltyramine was further purified by HPLC (Waters, Milford, MA). The compound was first dissolved in pure methanol and then injected into an HPLC with a UV-visible absorbance detector (Waters, Milford, MA). A Nova-Pack C_{18} column and an isocratic buffer system (60:40; 50 mM Na-P:methanol) were used to purify *N*-coumaroyltyramine. Peaks were detected at $\lambda = 260$ by the UV-visible absorbance detector (Waters).

LC-Mass Spectrometer. HPLC samples were cleaned for analysis by LC-MS as follows: the sample was diluted with water to a concentration of 15% methanol, passed over a preconditioned 100 mg solid phase Bond-Elut Ph (SPE) column, washed with 1 ml 0.1% formic acid, eluted from the SPE column with 0.5 ml SPE column mobile phase containing 0.1% formic acid, water/methanol/acetonitrile/(26/60/14, v/v/v), and passed through a 0.2 micron filter. LC was performed with an Agilent HP-1100 liquid chromatograph equipped with a Phenmenex 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 SPE column mobile phase containing 0.1% formic acid. The LC was interfaced with a Thermo-Finnigan LCQ ion trap equipped with an ESI source. The ESI parameters were: spray voltage, 4.5 kV; capillary temperature, 200°C; capillary voltage, 32 V; tube lens offset, 25 V; sheath gas, 80%. Under these conditions two major chromatographic peaks, each with $[M + H]^+$ at m/z 300 (base peak), eluted at 14.6 and 20.7 min, respectively.

MS/MS spectrometer. MS/MS experiments were conducted on the protonated molecular ions (m/z 300) at 30% of end cap 5 V maximum.

Cell culture conditions. U937 and Jurkat cells were cultured in RPMI 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 (17).

Measurement of cell death. U937 and Jurkat cells were treated with several concentrations of *N*-coumaroyltyramine. Total cell death was measured by both the trypan blue exclusion method (17) and by a nonradioactive cell proliferation assay (Promega, Madison, WI). The nonradioactive assay is based on the cellular conversion of a tetrazolium salt into a blue formazan product that is easily detected at 570 nm (18).

Cell cycle analysis. Cell cycle analysis was performed by the method described previously (19, 20). To fix cells, U937 or Jurkat cells (2×10^6 cells/ml PBS) were added to 70% ice-cold ethanol. After 1 h, the fixed cells were centrifuged and the cell pellet was resuspended in 1 ml PBS containing 20 μg of RNase (1 mg/ml) and 18 μg of propidium iodide (1 mg/ml). DNA content of the cells was determined with a FACScalibur flow cytometer (Becton–Dickinson, San Jose, CA). Flow cytometric data files were collected for 10,000 events and analyzed by the CELLQuest program (Becton–Dickinson). Cell cycle distribution percentages were calculated using ModFit LT software 2.0 (Verity Software House, Inc., Topsham, ME). Calibration standards (LinearFlow Green and DNA QC Particle Kit) for the verification of instrument performance were purchased from Molecular Probes (Eugene, OR) and Becton–Dickinson, respectively.

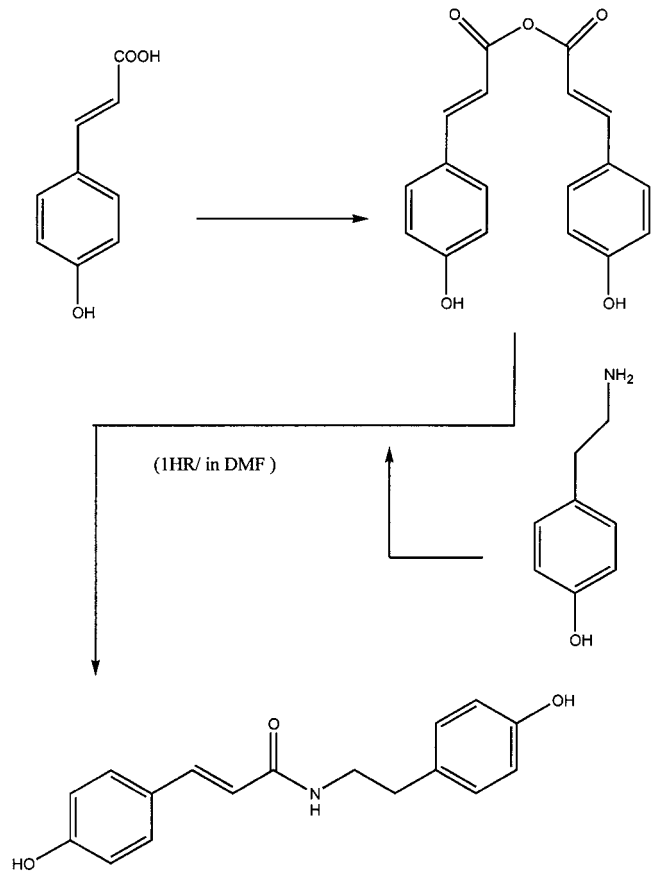

FIG. 1. Synthesis of *N*-coumaroyltyramine. (Top) 1,3-Diisopropylcarbodiimide (DIC) was a dehydrating reagent. (Bottom) Dimethylformamide was a solvent for tyramine. The yield was approximately 55%.

Determination of tyrosine kinase activity. Tyrosine kinase activity was determined by Western blot, using a specific anti-phosphotyrosine antibody (Sigma, St. Louis, MO). Cell extracts were prepared from U937 cells with or without the treatment of *N*-coumaroyltyramine (25 μM). EGFR tyrosine kinase activity (TK) was measured with a protein tyrosine kinase assay kit (Sigma). The activity was determined based on an ELISA with a TK-specific polymer substrate-coated microtiter plate. The assay procedure was performed according to the manufacturer's protocol.

RESULTS

Synthesis of N-Coumaroyltyramine

N-Coumaroyltyramine was synthesized with coumaric acid and tyramine as starting materials (see Materials and Methods). The symmetrical anhydride was generated (Fig. 1). Although coumaryl anhydride was poorly soluble in DCM and precipitated in the

reaction mixture, this was not a problem because the anhydride redissolved after the evaporation of DCM. Coumaric acid was completely dissolved by the addition of the DMF in which tyramine was dissolved. The synthesized product was easily precipitated by adding water, and the precipitate was washed and further purified by HPLC. Two major peaks were well separated and were detected at $\lambda = 260$ with retention times at 3, 7, and 10 min, respectively. The first and second peaks were unreacted tyramine and coumaric acid respectively because the retention times of these peaks were identical to respective retention times of tyramine and coumaric acid (data not shown).

LC-MS Analysis of N-Coumaroyltyramine

Structural analysis of the third peak was performed by an LC-MS as described in Methods. Prior to analysis by LC-MS, the second peak was subjected to a second HPLC procedure (see Materials and Methods). The analysis time was 25 min per sample, and two well-separated peaks were obtained with retention times of 14.3 and 20.7 min, respectively (Fig. 2A). The first peak was relatively smaller than the second one, and it seems contaminants leftover from previous HPLC purification. As expected, the first peak was verified by LC-MS as tyramine. The second peak with a retention time of 20.7 min was attributed to the newly synthesized compound because neither tyramine nor coumaric acid had a peak with that retention time. The major signals from the second peak were obtained at a mass/charge (*m/z*) unit of 284.1 (Fig. 2B), which is identical to the (*m/z*) unit of N-coumaroyltyramine. The second peak from the LC-MS was further analyzed with a MS/MS spectrometer. A major peak in the MS/MS chromatogram was derived from a daughter ion spectrum with base peak at *m/z* 163 attributable to the loss of the elements $C_8H_{11}NO$, which is consistent with the tyramine portion of the molecule (data not shown). These data indicated that the compound in the second peak in LC-MS was *N*-coumaroyltyramine.

Inhibition of the Growth of Transformed U937 and Jurkat Cells

Elucidation of the biological activities of new phytochemicals is key to understanding their effects on human cells. Because numerous phytochemicals have been reported to contain antiproliferation activity against human transformed cells (21), the investigation of the biological effects of *N*-coumaroyltyramine was focused initially on determining whether this compound contained anti-proliferation activity. U937 and Jurkat cells were chosen because they have been used extensively in similar experiments. The cells were treated for 18 h with various concentrations of *N*-coumaroyltyramine. As shown in Table 1, the number of dead cells increased with increasing concentrations of *N*-coumaroyltyramine. Dead cells were counted by trypan blue exclusion methods. The antiproliferation activity was confirmed by a nonradioactive cell proliferation assay (Promega), which measures the converted blue formazan product at 570 nm (18) (data not shown). Cell death proceeds in two independent ways: necrosis and apoptosis. Apoptotic processes such as the activation of ICE proteases, DNA fragmentation, morphological change, and plasma membrane leakage are commonly followed by characteristic cell arrest at specific phases of the cell cycle. The effect of *N*-coumaroyltyramine on the cell cycle was therefore studied.

Effect of N-Coumaroyltyramine on the Cell Cycle

As described above, the growth of both U937 and Jurkat cells was suppressed by micromolar concentrations of N-coumaroyltyramine. Cell arrest at specific stages of the cell cycle is often observed prior to the initiation of apoptotic processes. The effect of *N*-coumaroyltyramine on the cell cycle was therefore investigated with U937 cells. The cells were treated for 18 h with *N*-coumaroyltyramine (final concentration 30 μM). Since total cell number did not increase more than 10% of initial cell number at this concentration, *N*-coumaroyltyramine apparently suppressed the growth of U937 cells. The treatment also changed DNA contents at each phase from 46% (G0-G1), 6% (G2-M) and 47% (S) to 32%, 0%, and 67%, respectively (Fig. 3). The suppressed growth of U937 cells treated with *N*-coumaroyltyramine was associated with an increased percentage of cells in the S phase. This effect of *N*-coumaroyltyramine on the cell cycle was dependent on concentration and time; treatment with concentrations less than 50 μM and for times shorter than 18 h resulted in less dramatic changes in DNA content at each phase of the cell cycle. A similar result was obtained with the Jurkat cells treated with N-coumaroyltyramine (DNA contents at each phase: 30, 0, and 70%).

Inhibition of Tyrosine Kinase Activity by N-Coumaroyltyramine

Cell arrest often occurs due to reduced stimulation by essential growth factors. The stimulation was frequently initiated by phosphorylation process in cells, and the phosphorylation in tyrosine residues in proteins is assumed to be involved in abnormal growth of human tumor cells. Because *N*-coumaroyltyramine is capable of arresting U937 and Jurkat cells, the level of tyrosine phosphorylation was compared between U937 cells with and without treatment of *N*-coumaroyltyramine. As shown in Fig. 4, phosphotyrosine level was obviously reduced in U937 cells with the treatment. The epidermal growth factor receptor (EGFR) is a protein tyrosine kinase which is over-

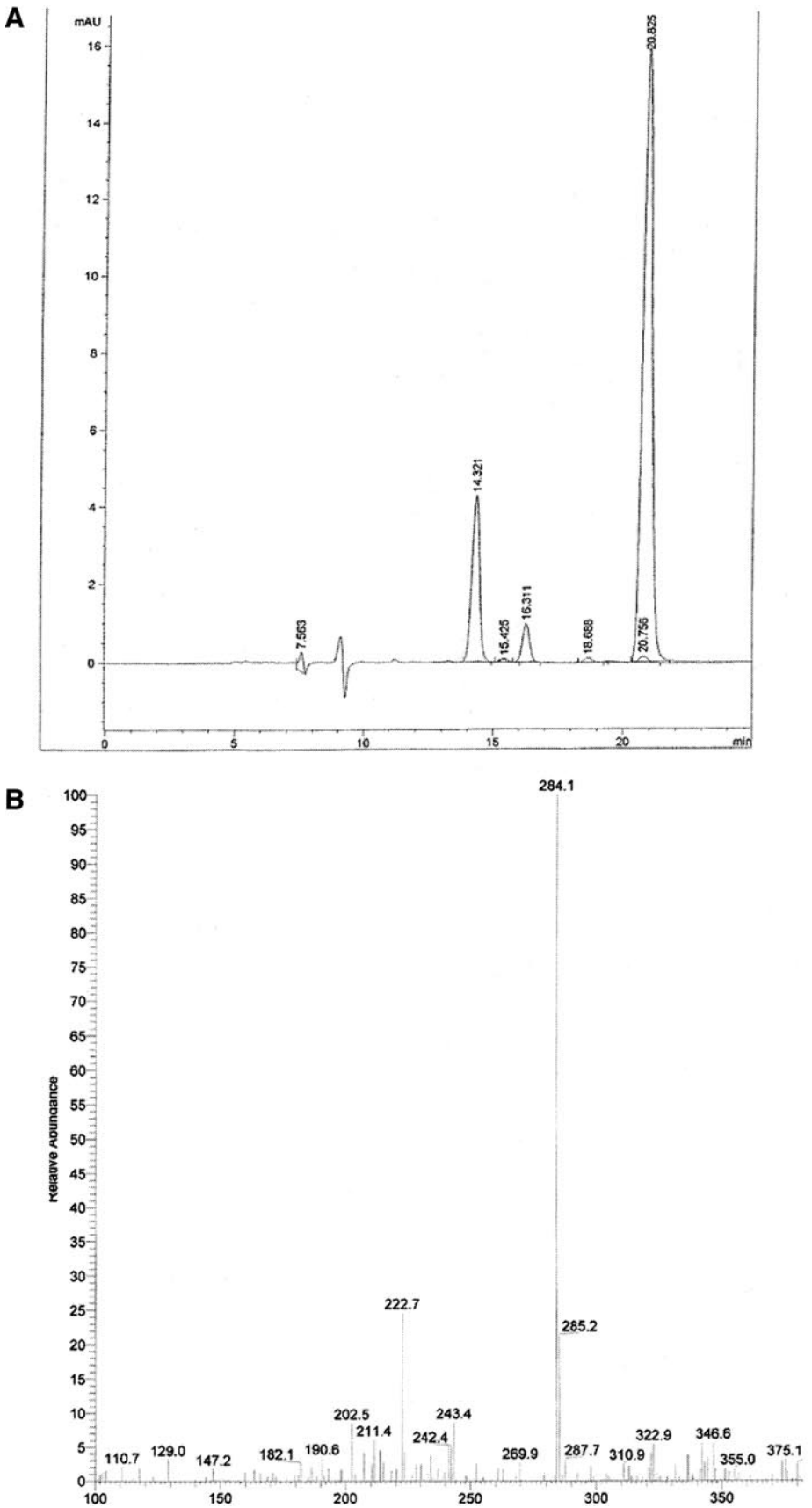

FIG. 2. LC-MS chromatograms. (A) Separation of synthesized *N*-coumaroyltyramine by LC-MS. The analysis time was 25 min per sample, and two peaks were well separated with retention times of 14.3 and 20.7. (B) Mass spectra of the *N*-coumaroyltyramine detected at *m/z* 284.1.

TABLE 1

Effect of *N*-Coumaroyltyramine on U937 and Jurkat Cells

N-Coumaroyltyramine (μM)	U937 cells	Jurkat cells
0	10×10^5	10×10^5
30	$(6.0 \pm 0.5) \times 10^5$	$(6.8 \pm 0.7) \times 10^5$
60	$(5.0 \pm 0.4) \times 10^5$	$(4.5 \pm 0.5) \times 10^5$
90	$(4.2 \pm 0.5) \times 10^5$	$(4.0 \pm 0.6) \times 10^5$

Note. The cells (1×10^6) were treated for 18 h with various concentrations of *N*-coumaroyltyramine. Dead cells were counted by both a trypan blue exclusion method and by a non-radioactive cell proliferation assay (Promega) which measures the converted blue formazan product at 570 nm.

FIG. 4. Western blot of proteins with phosphotyrosine. Approximate 1×10^6 U937 cells were treated for 18 h with *N*-coumaroyltyramine. Proteins with phosphotyrosine were visualized using biotin-conjugated monoclonal ant-phosphotyrosine. The first and second lanes are control (1), third and fourth (2) 10 μM, fifth and sixth (3) 30 μM, seventh and eighth (4) 60 μM of *N*-coumaroyltyramine. At the bottom, Western blot for glyceraldehydes-3-phosphate dehydrogenase is shown to demonstrate protein samples loaded in the gel are similar.

expressed in various types of cancers, and EGFR tyrosine kinase inhibitor can block signal transduction pathways implicated in proliferation and survival of cancer cells (22–24). Because *N*-coumaroyltyramine suppressed the growth of the cancer cells as well as the level of phosphotyrosine, the inhibitory effect of N-coumaroyltyramine on EGFR tyrosine kinase was determined with an EGFR tyrosine kinase assay kit. As shown in Table 2, *N*-coumaroyltyramine inhibited a protein tyrosine kinase (EGFR) in a dose-dependent manner. The data indicate that *N*-coumaroyltyramine is a potent inhibitor of tyrosine kinases including EGFR. Thus it is possible that *N*-coumaroyltyramine may induce the arrest of cell growth and even cause cell death by inhibiting tyrosine kinases and/or EGRF tyrosine kinase.

DISCUSSION

Many diverse phytochemicals exist in nature, and a great number of them have been traditionally used for treating specific diseases (25–27). Massive screening as well as chemical modification of phytochemicals would lead to the discovery of some modern drugs. Our recent work has focused on determining the biological activities of phytochemicals in human cells, a key step in understanding their effects on human health (28–30). This work, however, was often hampered by the difficulty of obtaining a sufficient amount of the phytochemicals. *N*-Coumaroyltyramine is one of the phytochemicals that would occur in very small quantities. It was therefore chemically synthesized, purified, and characterized for investigating its biological effects. The synthesis was easy and had a good yield, and the synthesized compound was structurally identical to N-coumaroyltyramine in plants. While we were exper-

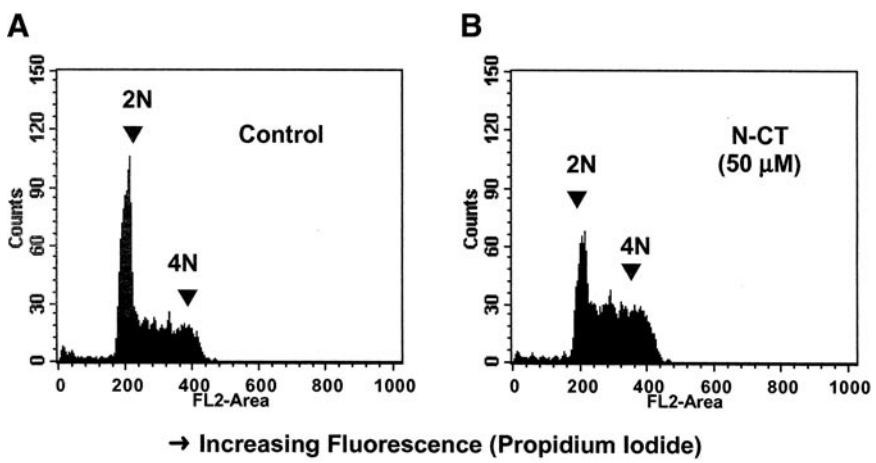

FIG. 3. Effect of *N*-coumaroyltyramine on the cell cycle progression of U937 cells. U937 cells (approximately 0.8×10^6 cells) were treated for 18 h with *N*-coumaroyltyramine (final concentration 30 μM). DNA content of the cells was determined by a FACScalibur flow cytometer (Becton–Dickinson, San Jose, CA). (A) control cells (47.7% at G0–G1, 8.5% at G2–M, 43.7% at S). (B) The cells treated with *N*-coumaroyltyramine (21.1% at G0–G1, 0.85% at G2–M, 78.0% at S).

TABLE 2

Inhibition of Tyrosine Kinase by *N*-Coumaroyltramine

N-Coumaroyltyramine (μM)	U937 cells (%)	Jurkat cells (%)
0	100	100
30	80 ± 11	85 ± 9
60	50 ± 6	55 ± 8
90	40 ± 3	43 ± 5
120	25 ± 2	30 ± 4

Note. EGFR tyrosine kinase activity (TK) was measured with a protein tyrosine kinase assay kit (Sigma, St. Louis, MO). The assay procedure was performed according to the manufacturer's protocol.

imenting with the synthesized n-coumaroyltyramine, we found that it arrested the cell cycle at S-phase and induced cell death in both U937 and Jurkat cells, possibly via inhibition of tyrosine kinase activity.

EGFR is a membrane bound receptor with tyrosine kinase activity. Protein tyrosine phosphorylation is a central event that mediates signal transduction events involved in a wide range of cellular processes such as cell-cycle progress, transcriptional regulation, cell transformation, proliferation, differentiation, and apoptosis (22). Mammalian cell cycle control and signal transduction pathways are well orchestrated to maintain steady-state homeostasis of the cells (31, 32). An imbalance of homeostasis can result in the progression of cancer or in apoptosis, causing the cells to be locked into either an active proliferation or a doomed death state (32, 33). For the last decade, increased understanding of these processes has led to the development of anti-cancer drugs that intervene at specific molecular steps involved in carcinogenesis. These drugs include inhibitors of cell cycle progression, inhibitors of tyrosine kinase receptors, and inhibitors of other molecules involved in intracellular signal transductions (34, 35). The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that is overexpressed in various tumor cells (22, 23). Due to the involvement of its overexpression in carcinogenesis, selective EGFR tyrosine kinase inhibitors have been relentlessly sought in order to block signal transduction pathways implicated in proliferation and survival of cancer cells. As a result of basic cancer research, several drugs have been developed. One of them is STI-571 (CGP or 57148B) that is used to treat chronic myeloid leukemia (CML). STI-571 is a tyrosine kinase inhibitor that works by interrupting a specific enzyme found only in the cells of certain cancers but is absent in healthy, non-cancerous cells (36, 37).

Because *N*-coumaroyltyramine can inhibit tyrosine kinases including EGRF tyrosine kinase, and because it can arrest the growth of transformed U937 and Jurkat cells at S-phase, understanding its mechanism of action may be important. Cell death can occur via two separate pathways (necrosis and apoptosis). Necrotic cell death is an accidental cell death that does not require any cellular or molecular mechanism. Apoptosis, however, does require programmed cellular and molecular events; it displays several biological and morphological hallmarks that can be used to determine whether apoptosis is occurring. Four criteria that can be used to determine this are: the activation of the family of interleukin-1β converting enzyme (ICE) proteases, phosphatidylserine externalization, DNA fragmentation, morphological change, and plasma membrane leakage (38, 39). Because treatment of U937 and Jurkat cells with *N*-coumaroyltyramine induced cell death, an experiment was performed to determine whether cell death was due to necrosis or apoptosis. For the experiment, activation of ICE-family proteases (CPP32/Caspase-3) was assessed with an ApoAlert-Caspase Assay Kit (Clontech) according to the manufacturer's protocol. The activity of CPP32 protease was measured by fluorometry. This experiment indicated that in U937 and Jurkat cells treated with *N*-coumaroyltyramine cell death might be occurring by an apoptotic process (data not shown). The effect of *N*-coumaroyltyramine on apoptosis is being investigated with other apoptotic assays to confirm this result.

As demonstrated above, *N*-coumaroyltyramine seems a potent phytochemical to inhibit EGFR tyrosine kinase, to arrest the growth of the transformed cells at S phase, to induce an apoptotic process, and eventually to cause cell death. Understanding of the molecular mechanisms of drugs is an essential step in developing drugs with greater efficacy and specificity than conventional chemotherapy drugs. In general, several synthetic inhibitors of protein tyrosine kinases (tyrphostins B44, B46, B50, and B56) are believed to inhibit protein tyrosine kinase activity by competing with the binding of ATP. The mechanism by which *N*-coumaroyltyramine inhibits EGFR tyrosine kinase, however, remains unknown. Studies should therefore be performed to elucidate the mechanism involved in the EGFR tyrosine kinase inhibition in the future. In nature, there are several analogues of *N*-coumaroyltyramine, including *N-trans*-caffeoyltyramine, *N-trans*-feruloyltyramine, and *N*-sinapoyltyramine (5, 6). Since these analogues share considerable structural resemblance to *N*-coumaroyltyramine, the effects of the analogues on EGFR tyrosine kinase should also be investigated. The outcomes of current and future studies will contribute to developing new agents for inhibiting the growth of cancer cells as well as epidermal growth factor receptor tyrosine kinase.

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