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RESEARCH ARTICLE

CHEMISTRY & BIODIVERSITY

Synthesis of New Cinnamoyl-Amino Acid Conjugates and *in Vitro* Cytotoxicity and Genotoxicity Studies

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Amino acid conjugates are described by the reaction of amino acids with bioactive organic groups such as vitamins, hormones, flavonoids, steroids, and sugars. In this study, 12 new conjugates were synthesized by reaction of cinnamic acid derivatives with various amino acids. Cytotoxic studies against four different human cancer cells (MCF7, PC-3, Caco-2, and A2780) were carried out by MTT assay method at five different concentrations. The structure-activity relationships based on the cell viability rates were evaluated. To compare the anticancer activities of the compounds using computational chemistry methods, they were docked against A2780 human ovarian cancer, Michigan Cancer Foundation-7 (MCF7), human prostate cancer (PC-3) and human colon epidermal adenocarcinoma (Caco-2) cell lines and compared with the standard 5-Fluorouracil. The results indicate that the efficacy of cinnamic acid derivatives increases with the presence of amino acids. Comet assay was conducted to understand whether the cell deaths occur through DNA damage mechanism and the results exhibit that the changes in the specified parameters were statistically significant ($p < 0.05$). Our study demonstrated that the compounds cause cell death through the formation of DNA damage mechanism.

Keywords: amino acid, cinnamic acid, cytotoxicity, DNA damage, synthesis.

Introduction

Amino acid conjugates are a general name given to biologically or physically active compounds, usually formed by a reaction between an amino acid and one or more nucleophilic/electrophilic active compounds such as flavonoids, vitamins, hormones, steroids, fatty acids, and several heterocyclic compounds.[1,2] The rationale behind the preparation of these conjugates is to enhance the activity of these compounds for pharmacological purposes. The increasing use of amino acid/peptide conjugates in drug designs in recent years has also made the synthesis of these compounds important.[3–9]

Cinnamic acid and its common derivatives are a class of organic compounds that are mostly isolated from plants and attract attention due to the double bond present in their structure that facilitates conjugation. Cinnamic acid and its natural and synthetic derivatives have a wide range of pharmacological activities such as anti-cancer,[10] anti-tuberculosis,[11] anti-inflammatory,[12] anti-parasitic,[13] and anti-microbial effects.[14] Cinnamic acid has long been used by humans as a component of plant-derived flavors.[15] Chemically, it is an aromatic fatty acid that is usually *trans*-geometry and consists of an acrylic acid group

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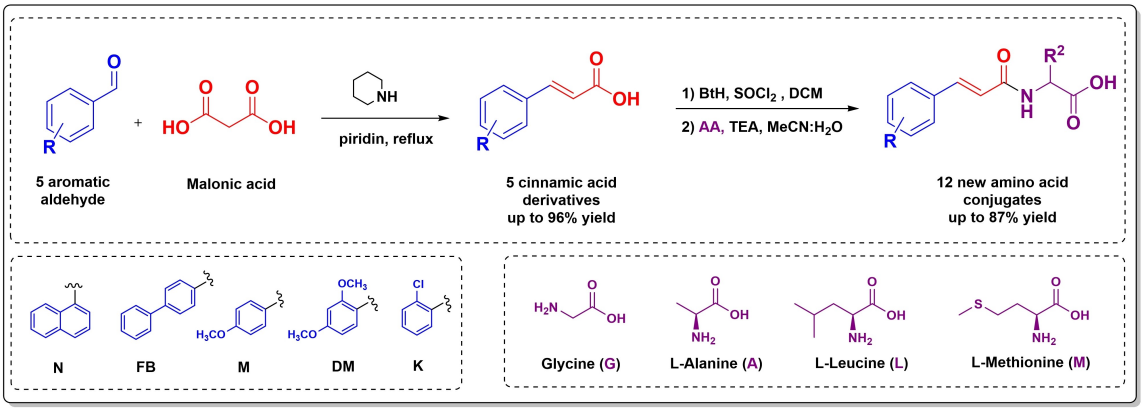
and a phenyl ring. These molecules have an unsaturated carbonyl fragment, which can be considered an active part, Michael acceptor, which is often used in the design of anticancer drugs.[16] In biological chemistry, cinnamic acid is an important intermediate in phenylpropanoid pathways, the precursor of lignin, the structural component of flavonoids and plants.[17–19] Additionally, cinnamic acid derivatives have been evaluated as pharmacologically active compounds due to their wide availability in plants and their low toxicity.[20,21] Besides having several biological activities,[22,23] they are often used as promising starting compounds for the development of new and highly effective drugs.

Amide bond is a very common functional group found in small or complex natural and synthetic compounds. It has a very important place in metabolism due to being involved in biological processes such as enzymatic catalysis, transport/storage, and immune protection. Since it is a pharmacologically important functional group, 25% of the known drugs have a carboxamide group.[24]

In this study, the main step in the synthesis of amino acid conjugates is the formation of the amide bond that is obtained after activation of the carboxylic acid by coupling reagent benzotriazole. Using this methodology, prevent from possible racemization giving no change on configuration of amino acid. The benzotriazole compound is a heterocyclic aromatic compound with a benzene and triazole ring. Unlike the triazole ring, the benzene ring in benzotriazole provides extra conjugation to the structure with π-π interactions.[25,26] Due to its benefits, benzotriazole was used as a coupling reagent to obtain new cinnamoyl-amino acid conjugates. The study aims to synthesize cinnamoyl-amino acid conjugates and investigate the cytotoxic against numerous human cancer cell lines. In this context, 5 different aromatic aldehydes were selected, two of which carry an extra benzene ring and the other two containing mono and disubstituted methoxy groups, and their bioactivity was compared based on their structural differences. Amino acids have been preferred because of the various interactions they can make with alanine and leucine amino acids, which contain aliphatic side chains, and methionine with thioether side chain. Finally, research was focused on genotoxic activity and understanding the mechanism behind cell death if it occurs through DNA damage.

Results and Discussion

The general synthetic route of cinnamic acid derivatives and conjugates is shown in *Scheme 1* and all detailed spectroscopic and experimental data is given in *Supporting Information*. Cinnamic acids contain aromatic, olefinic, carboxylic acid proton and aliphatic proton depending on the type of substituent. When ^{1}H-NMR spectra of **MC** is examined (*Figure 1A*), carboxylic acid proton at 12.21 ppm, methoxy CH_3 protons as singlet at 3.80 ppm, olefinic proton (number 7) at 7.57–7.54 ppm, and olefin proton (number 8) at 6.41–6.37 ppm were observed. Aromatic protons 2–6 resonated in a low field due to the ortho-para directing effect of the methoxy group. The carbon NMR spectrum in *Figure 1B* is showed that the carboxylic acid carbon peak at 168.28 ppm, and the aromatic carbon (number 4) to which the methoxy group is bound is at 161.42 ppm, and the methoxy carbon peak at 55.78 ppm was clearly observed. In addition, aromatic =CH carbons resonate at 130.40



Scheme 1. Synthetic route for cinnamic acid derivatives and conjugates.

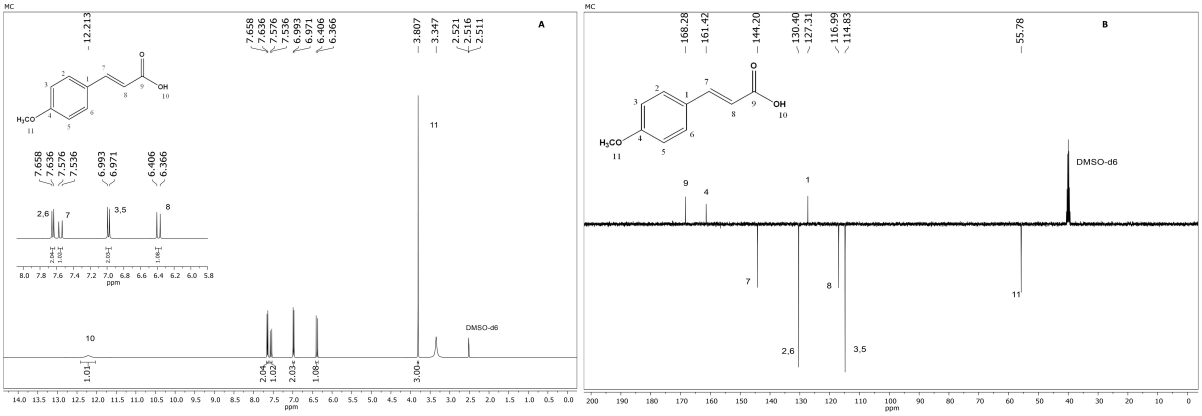


Figure 1. A) ^{1}H and (B) ^{13}C-APT NMR spectrum in (D_6)DMSO spectrum of compound **MC**.

and 114.83 ppm. Olefinic =CH carbons are seen at 144.20 ppm and 116.99 ppm. This shows that the cinnamic acid was purely obtained.

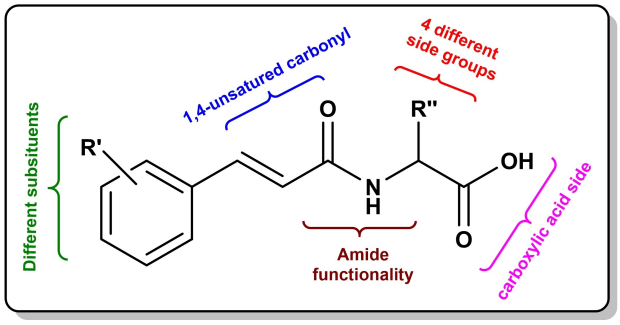
The most important peaks to be considered in the ^{1}H-NMR spectrum of analysis of amino acid conjugates containing carboxylic acid are the presence of the newly formed amide –NH proton and aliphatic protons in the side chain of the amino acid. In addition, the absence of aromatic protons of benzotriazole proves that the target compound is formed. Also, the presence of aromatic =CH protons and methoxy proton peak at 3.80 ppm from cinnamic acid can be shown as evidence. The aliphatic $-CH_2$ protons of glycine at 3.89–3.88 ppm as a doublet and carboxylic acid proton peak at 12.57 ppm proved that the desired product was formed. Characteristic groups expected to appear in the ^{13}C-NMR spectrum of amino acid conjugates in *Figure 2*; amide carbonyl and carboxylic acid carbonyl peaks of amino acid, aromatic =CH carbons, olefinic =CH carbons, and aliphatic carbon peaks. When the spectrum of **MCG** is examined, the presence of all the characteristic carbon peaks mentioned above is clearly visible. Carboxylic acid carbonyl carbon peak at 171.86 ppm, amide carbonyl carbon peak at 166.05 ppm, CH_2 proton peak of glycine at 41.28 ppm, and olefinic carbon peaks (numbers 7 and 8) at 139.36 ppm and 119.68 ppm, respectively, showed that the desired amino acid conjugate was synthesized.

In *Scheme 2*, the functional parts in the structure of the cinnamoyl-amino acid conjugate are given in general. The roles of these functional groups in cell interactions are also clearly seen in molecular docking studies.

In Vitro *Cytotoxic Activity*

Avanesyan et al. reported the antiradical activity of several cinnamic acid derivatives with different substitutions in the aromatic ring. They stated that the

Figure 2. A) ^{1}H and (B) ^{13}C-APT NMR spectrum in (D_6)DMSO spectrum of compound **MCG**.



Scheme 2. Active groups on cinnamoyl-amino acid conjugates that possibly interact with DNA.

type and location of the substituents on the aromatic ring increase or decrease the activity, and also the cinnamoyl part is the determining factor on the antiradical activity. It is well-known that cinnamic acid has an antimicrobial effect against pathogenic and spoilage bacteria although it restricts the use of low solubility in water.[27,28] In addition to natural sources, several synthetic cinnamates have shown strong inhibitory activity against HIV-1 integrase.[29,30]

When the efficacy rates of cinnamic acid derivatives against the A2780 cell lines at five different doses are compared among themselves (*Table 1*), it is obvious that all derivatives except naphthalene-containing derivatives are effective at all concentrations, especially at the 100 μM dose. Within this group of compounds, dimethoxy cinnamic acid (**DMC**) exhibits the highest effect at each dose. In this case, the effect of the presence of two electron-donating methoxy groups attached to the aromatic ring on cell death is more clearly seen compared to the derivative containing a single methoxy group (**MC**), and it gave more effective results compared to the derivative with an inductively electron-withdrawing chlorine group (**KC**), which makes the effectiveness of the methoxy group evident in this comparison.

Cell viability results of cinnamoyl conjugates containing glycine amino acid were examined on A2780 cell lines (*Table 2*), and the derivatives containing dimethoxy groups were most effective at 1, 5, and 25 μM doses. On the other hand, the compound containing phenylbenzyl group at 50 and 100 μM concentrations showed a significant effect. According to these results, the addition of glycine to phenylbenzyl-containing cinnamic acid seems to increase the effectiveness of this compound at higher doses.

According to overall results leucine-linked conjugate to the phenyl benzyl cinnamic acid derivative (**FBCL**), which is the most effective compound in all concentrations against four different cell lines among the target compounds is given in *Figure 3* and *Table 3*. The ineffectiveness of alanine and methionine amino acids in the phenyl benzyl group and the increase in the activities of leucine-containing compounds allow examining the effect of amino acid structures on cell death in terms of structural activity.

In Vitro *Genotoxicity Studies*

Cancer is one of the most important diseases that cause death in the world, and therefore, several research and development are carried out in order to develop new drug candidates with minimal side effects and high efficiency in the diagnosis and treatment of cancer. In this context, especially cinnamic acid and its derivatives have received great attention due to their potential as anti-cancer agents.[31–35] It is one of the possible hypotheses that most of the anticancer drugs used in the clinic exert their effects through the inhibition of DNA-bound proteins/enzymes or by interacting directly with DNA.[36,37] As a result of compound-DNA interaction studies, it has been suggested that cinnamic acid derivatives have DNA binding abilities.[33] Comet analysis results from DNA damage studies (*Table S5* and *S6*) of cinnamic acid and conjugates in human prostate cancer cell lines (LNCaP, PC-3), human colon cell lines (Caco-2), and human ovarian cancer (A2780) cell lines at the 100 μM dose TL, TI, OTM, HI, and HD parameters exhibited changes were observed and these changes were statistically significant. As a result, we came to the conclusion that cell deaths

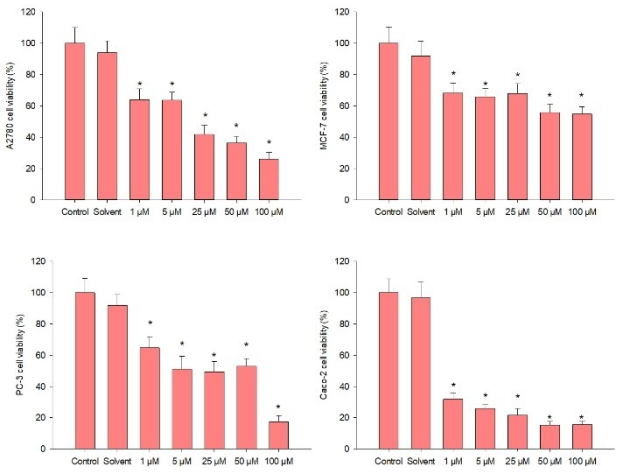


Figure 3. Cell viability graphs for FBCL against four human cancer cell lines.

Table 1. Cell viability results of cinnamic acid compounds against A2780 cell lines.

Entry	Control	Solvent	1 μM	5 μM	25 μM	50 μM	100 μM	$LogIC_{50}/IC_{50}$
MC	100 ± 10.12	93.92 ± 7.57	**70.11 ± 5.32***	**58.73 ± 6.20***	**61.43 ± 6.92***	**50.17 ± 8.05***	**38.67 ± 3.26***	1.595/39.39
DMC	100 ± 10.12	93.92 ± 7.57	**62.07 ± 7.42***	**54.09 ± 9.28***	**49.15 ± 6.79***	**39.51 ± 5.45***	**28.37 ± 4.23***	1.268/18.53
FBC	100 ± 10.12	93.92 ± 7.57	**64.41 ± 5.89***	**63.18 ± 8.86***	**46.37 ± 6.29***	**35.12 ± 4.81***	**31.88 ± 3.29***	1.293/19.62
KC	100 ± 10.12	93.92 ± 7.57	**70.91 ± 6.96***	**61.32 ± 8.87***	**62.31 ± 7.13***	**51.14 ± 5.92***	**41.31 ± 5.51***	1.637/43.4
NC	100 ± 10.12	93.92 ± 7.57	98.56 ± 9.25	96.24 ± 10.29	93.01 ± 12.78	93.24 ± 10.47	97.04 ± 9.91	3.16/14.47

proceeded through the mechanism of DNA damage. Images obtained from Comet Assay trials for FBCL are given in Figure 4. Obtained results are given as tables in the supplementary file.

Molecular Docking Studies

The activities of chemical species against biological receptors can be studied at the molecular level by computational methods. The molecular docking process can determine the interactions between the minimalized protein structure of cell lines and drug candidate ligands.[38] This way, binding energies, interaction types and interaction modes of ligands with target proteins are predicted at the molecular level. DockingServer was used for docking calculations of compounds.[39] The geometry optimization of the ligands was performed with the MMFF94 method on the server. Gasteiger method for charge calculation, pH = 7.0 and grid maps (x, y and z) 90 × 90 × 90 Å were used.[40] The structure of the ovarian tumor (OTU) domain in complex with ubiquitin (PDB ID: 3 C0R) was selected for the human ovarian cancer cell lines (A2780).[41] The construct of 10-formyltetrahydrofolate and 5,10-Metenyltetrahydrofolate synthetase (MTHFS) (PDB ID: 3HY3) was preferred for the Michigan cancer foundation-7 (MCF7) cell lines.[42] Inhibition of MTHFS in human MCF-7 breast cancer cells are determined to arrest the growth of cells. The crystal structure (PDB ID: 3QUM) of prostate antigen (PSA) with high affinity and PCa-selective antibody was determined for the human prostate cancer (PC-3) cell lines.[43] The serologically defined colon cancer antigen structure from Homo sapiens (PDB ID: 2HQ6) was determined for the human colon epidermal adenocarcinoma (Caco-2) cell lines.[44] To predict the biological activities of the investigated compounds on the determined target proteins, the binding

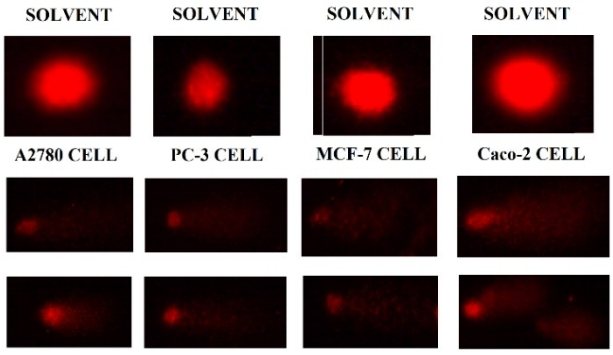


Figure 4. Images obtained from Comet Assay trials for **FBCL**.

Table 2. Cell viability results of cinnamoyl-glycine conjugates against A2780 cell lines.

Entry	Control	Solvent	1 μM	5 μM	25 μM	50 μM	100 μM	$LogIC_{50}/IC_{50}$
MCG	100 ± 10.12	93.92 ± 7.57	**54.44 ± 5.56***	**47.437 ± 5.76***	**49.23 ± 5.49***	**46.81 ± 3.39***	**44.81 ± 5.85***	1.41/25.72
DMCG	100 ± 10.12	93.92 ± 7.57	**46.13 ± 4.29***	**41.49 ± 5.22***	**43.43 ± 3.57***	**40.02 ± 4.46***	**36.92 ± 4.24***	0.8/6.31
FBCG	100 ± 10.12	93.92 ± 7.57	**57.74 ± 5.48***	**60.86 ± 7.029***	**49.59 ± 6.77***	**37.82 ± 5.80***	**34.70 ± 5.26***	1.342/21.96
KCG	100 ± 10.12	93.92 ± 7.57	**65.37 ± 5.12***	**64.40 ± 6.01***	**61.58 ± 5.45***	**59.59 ± 5.16***	**53.24 ± 4.66***	1.797/62.64
NCG	100 ± 10.12	93.92 ± 7.57	93.17 ± 8.29	93.95 ± 8.88	93.39 ± 9.64	91.20 ± 9.56	89.10 ± 10.89	2.817/655.8

energy between ligand and reference 5-FU with the target protein is given in *Table 4*.

As seen in *Table 4*, experimentally the most active substances against the A2780 cell lines are DMCG and KCA. In the docking results, the binding energies between DMCG and KCA and the 3C0R target protein were −5.62 and −5.74 kcal/mol, respectively. Compounds with high experimental anticancer activity against the MCF-7 cell lines are FBCG, MCA and FBC, respectively. According to the docking results, the most advantageous molecules in terms of activity are FBCG, MCA and FBC, and their binding energies are −8.79, −7.71 and −7.14 kcal/mol, respectively. Besides, FBCL compound also has high anticancer activity in docking results. According to the data obtained from the docking results, the examined compounds have the highest binding energies with the 3HY3 target protein. In general, anti-cancer activities against the PC-3 cell lines are lower against other cancer types in both experimental and computational results. Among the molecules studied, FBC and FBCL have high anticancer activity against the 3QUM target protein. According to the experimental and docking results for the Caco-2 cell lines, FBCL is the most active molecule. The binding energy of FBCL and FBCG compounds with the 2HQ6 target protein is −6.24 and −5.99 kcal/mol, respectively.

In general, the anticancer activity of the investigated chemical species is higher than the reference 5-FU. In addition, when the molecular structures of the studied compounds are examined in general, it can be said that the alkyl groups of the compounds containing the phenyl group, which are substituted at the amine α position, increase the chemical reactivity. Another noteworthy issue is that the biphenyl structure is an important moiety of its inhibitory activity against biological receptors. The docking poses of the compounds exhibiting high biological activity against each cell lines are given in *Figure 5*.

By molecular docking, the binding modes and types of secondary chemical interactions between the target protein and the studied molecule can be determined.[45] The docking poses of the compounds with high activity against the selected target proteins, as well as the interaction types between the compounds and the target proteins are given in *Figure 1*. Secondary chemical interactions are very important in docking studies. The interaction strengths of the chemical species with biologically active molecules depend on the interaction types.[46] It may be a pioneer to examine the interaction types for drug

Table 3. Cell viability results of FBCL against all cancer cell lines.

Cell type	Control	Solvent	1 μM	5 μM	25 μM	50 μM	100 μM	$LogIC_{50}/IC_{50}$
A2780	100 ± 10.12	93.92 ± 7.57	**61.05 ± 6.87***	**63.87 ± 5.04***	**41.76 ± 5.81***	**36.51 ± 4.02***	**26.19 ± 4.09***	1.218/16.5
PC-3	100 ± 9.29	91.94 ± 7.02	**64.79 ± 7.02***	**51.04 ± 8.31***	**49.44 ± 6.69***	**52.92 ± 4.81***	**17.44 ± 3.81***	1.314/20.6
MCF-7	100 ± 10.41	91.99 ± 9.34	**68.29 ± 6.36***	**65.86 ± 5.39***	**68.06 ± 6.20***	**55.93 ± 5.29***	**54.84 ± 4.75***	1.828/67.31
Caco2	100 ± 8.92	96.8 ± 9.93	31.88 ± 3.97	26.00 ± 2.33*	21.62 ± 4.26*	15.33 ± 2.27*	15.62 ± 2.11*	−0.095/0.801

Table 4. Binding energies (kcal mol^{-1}) with investigated compounds and selected target proteins.

Entry	3C0R	3HY3	3QUM	2HQ6
DMC	−4.04	−5.80	−4.18	−4.58
DMCG	−5.62	−6.19	−4.37	−4.40
DMCM	−4.41	−6.57	−3.99	−4.81
MC	−3.88	−5.51	−4.85	−4.76
MCG	−4.20	−6.18	−4.37	−4.70
MCA	−4.57	−7.71	−4.44	−5.10
FBC	−4.60	−7.14	−5.62	−5.08
FBCG	−4.65	−8.79	−4.17	−5.99
FBCL	−5.09	−8.06	−5.84	−6.24
KC	−4.47	−6.27	−5.50	−4.46
KCG	−5.18	−7.25	−4.97	−5.44
KCA	−5.74	−7.17	−4.85	−5.50
5-FU	−3.58	−4.80	−3.54	−3.92

candidates that are considered to be developed. The studied molecules and target proteins representing the A2780 and Caco-2 cell lines generally form hydrogen bonds. It also includes hydrophobic interactions between molecules and target proteins. Polar and hydrophobic interactions exist between the MCF-7 cell lines and the investigated compounds. Hydrogen bonding, polar and hydrophobic interactions occur between the target protein corresponding to the PC-3 cell lines and the compounds.

Conclusions

Cinnamic acids and their amino acid conjugates were successfully synthesized and characterized. Cell viability tests were performed against 4 different cancer lines at five different doses for new conjugates and initial cinnamic acids. Most of the conjugates showed cytotoxic effects at all doses. Cytotoxic compounds were studied using the comet assay method to determine whether cell death was caused by DNA damage. DNA damage analysis at the dose where the compounds are most effective was carried out by the comet assay method and was applied to the cell lines at a dose of 100 μM. The results indicate that compounds were effective on TL, TI, and OTM values of the cells and this effect was statistically significant ($p < 0.05$). Our study demonstrated that the compounds cause cell death through the formation of DNA damage mechanism. According to the calculated docking parameters, the investigated compounds were found to have higher activity than the anticancer standard substance. In addition, it was estimated that there was a general trend between

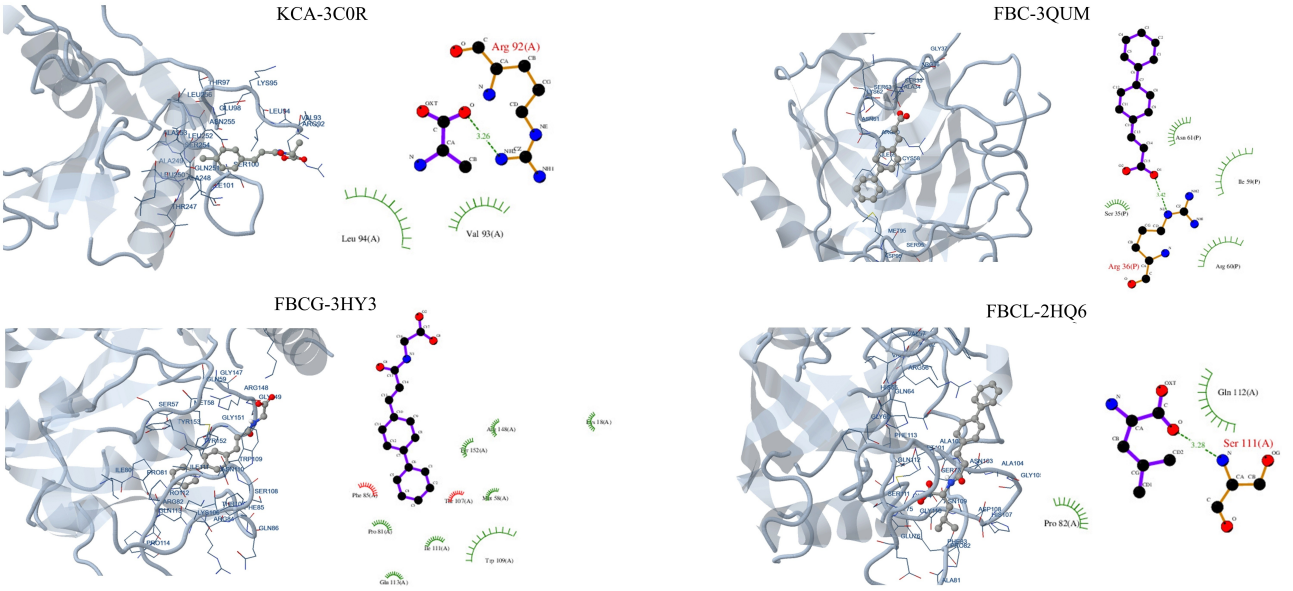


Figure 5. Docking poses of certain compounds and target proteins.

the experimental and the docking results and that the biphenyl group increased the anticancer activity. The results suggest that amino acid conjugates show promising cytotoxic and genotoxic activity and that new studies will be designed on drug development research.

Experimental Section

Materials and Measurements

All the chemicals were bought from Sigma–Aldrich and Merck. All amino acids used in this work were chosen in L-stereoisomer form. ^{1}H, ^{13}C-APT NMR and FT-IR spectra of compounds were recorded by using Bruker DPX-400 MHz and Perkin Elmer FT-IR spectrometer, respectively. LECO 932 CHNS-O apparatus and Bruker Microflex LT MALDI-TOF MS spectrometer were used for microanalysis and mass spectra, respectively. Human prostate (PC-3), breast (MCF-7), and ovarian (A2780) cancer cell cultures were provided by ATCC (American Type Culture Collection). Penicillin, trypsin, streptomycin, newborn calf serum, and Dulbecco's modified Eagle's medium (DMEM) were provided from Hyclone (Waltham, MA, USA). CO2 incubator from Panasonic, biological safety cabinet from Nuve MN-120, microplate reader from BioTEK spectrophotometer, cell maintenance and control from Reverse Microscope SOIF-XDS, and for sterilization Nuve was preferred.

General Synthetic Procedures for Cinnamic Acid Derivatives

The malonic acid (2.2 equiv.), aromatic aldehyde (1.0 equiv.), piperidine (0.2 equiv.), and pyridine (5.7 equiv.) as solvent was added to a 250 ml reaction flask which was then arranged for reflux overnight. The reaction was monitored by thin layer chromatography and after completion of the reaction, the mixture was added to a 600 ml baker which contained 400 ml distilled water. Initially, a precipitate occurred. Then pH of the mixture was brought up to 5 and let the mixture was stirred for a couple of hours. The precipitated was filtered and the obtained solid was dried. Yields were between 85 and 95 %.[47,48]

General Synthesis Procedures of Cinnamoyl-Amino Acid Conjugates

Amino acid (1.0 equiv.) was dissolved in MeCN/H_2O (7 : 3) and then triethylamine (1.2 equiv.) was added to the solution at room temperature. After 15 min, cinnamoyl-benzotriazole (1.0 equiv.) compound was added to the mixture and stirred overnight. The reaction was monitored via thin layer chromatography. After the completion of the reaction, all reaction solvent was removed. The obtained crude product was precipitated in distilled water (200 ml). The

obtained precipitate was filtered and dried. Yields were up to 87%.

All the Spectroscopic data can be found in supporting material.

Determination of in Vitro *Cytotoxic Activity*

Human breast (MCF-7) and human ovarian cancer cell lines (A2780) in Dulbecco's modified Eagle's medium (DMEM) culture in 4 mM L-glutamine, with 4500 mg/L glucose with the addition of 10 mM non-essential amino acids for the culture of breast cancer cells were maintained. At 37 °C in 5% CO_2 humidified incubator was used to maintain the cells.

In Vitro *Cytotoxic Assay (MTT Assay)*

MTT assay methods were used to screen synthesized compounds against cancer cells.[49,50] Active mitochondria were used to cleave 3-(4,5-dimethylthiazol)-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide to form completely soluble dark blue formazan in acidified isopropanol that is detected by a microtiter plate reader. To detect living and growing cells the assay provides no radioactive usage. In different concentrations (1, 5, 25, 50, and 100 μM) of agents, breast cancer cell was plated in 96-well flat bottom tissue culture plates and treated with agents The incubation of culture cells was maintained at 37 °C at 5% CO2 humidified incubator for 24 h. The formazan crystals were dissolved in 0.04 N (100 mL) in isopropanol and readings were recorded by using a 570 nm filter (Biotek Synergy HTX). Each data represents an average of 10 measurements.[51] All cell viability results were determined considering control cells.

Statistical Analysis

Quantitative data were verified as mean ± standard deviation (SD), and normal distribution was confirmed using the Kolmogorov-Smirnov test. Quantitative data were analyzed using the Mann–Whitney U test with Bonferroni correction, known as the post-hoc test, followed by the Kruskal–Wallis H test. All p values < 0.05 were considered statistically significant. All analyses were done by IBM SPSS Statistics 22.0 for Windows. The $LogIC_{50}$ values of the compounds were determined using the% cell viability values with the GraphPad Prism 6 program.[52]

Genotoxicity Studies

Comet assay is used for determining DNA damage in several mammalian cell types such as lymphocytes,[37] sperm cells,[53] epithelial cells,[35] and cancer cells[54] by single-cell gel electrophoresis. In this study, to determine DNA damage the alkaline comet assay technique with minor modifications was preferred which is described in our previous study.[34] 100 ml of 0.7% normal melting-point agarose in PBS was used to coat the microscope slides at room temperature and in dark dried for 24 h. Before the comet assay experiments, cell viability was first assessed by the trypan blue exclusion test, with analyzes initiated as soon as the percentage of viable cells was greater than 90%. Cultured PC-3, A,2780, Caco-2, and MCF-7 cells were incubated for one day at the highest concentration (100 μM) at which the compounds were effective. Slides placed after lysis in a horizontal electrophoresis tank (Bio-Rad, USA) filled with cold neutral electrophoresis buffer were electrophoresed for 20 min at 25 V (0.83 V cm^{-1}) and 300 mA. The buffer (0.4 M Tris, pH 7.5) was used to neutralize the slides at 4 °C for 5 min and then 20 μg/ml ethidium bromide for spotting. At the end, the slides were covered with a coverslip. To prevent form additional DNA damage entire process was performed in the dark.

DNA damage analysis was counted using a fluorescence microscope (Leica, Germany) equipped with appropriate filters at 200x magnification, twenty-five cells per slide and two slides per sample, and images were saved for offline analysis. The images obtained based on the TI, TL, and OTM parameters of DNA damage for each substance were analyzed in Argenit Kameram software (Ankara, Turkey). Comet test data were analyzed using one-way ANOVA, post-position Tukey HSD test was performed.[55] All results were expressed as mean ± SEM or SD, and $p < 0.05$ was considered to be statistically significant.

Supporting Information

FT-IR, MALDI-TOF MS, ^{1}H, and ^{13}C-APT NMR spectra of all synthesized compounds were given in the *Supporting Information*.

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Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Author Contribution Statement

E. Çalışkan completed syntheses of all compounds. S. Sandal, S. Tekin, D. A. Öztürk and K. Koran carried out cytotoxicity and genotoxicity studies. E. Çalışkan, A. O. Görgülü, and A. Çetin were contributed to writing of original manuscript. E. Çalışkan and K. Koran carried out the structural characterization of compounds. S. Erkan and D. A. Öztürk carried out molecular docking experiments. E. Çalışkan completed the final version of the revised manuscript.

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