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Cytotoxic Activity, Anti-migration and *in silico* Study of *o*-Coumaric acid on H1975 Non-small Cell Lung Cancer Cells

Dogukan Mutlu [1,a,*]

[1] *Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Üsküdar University, Istanbul, Türkiye*
**Corresponding author*

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ABSTRACT

Lung cancer represents the most common malignancy and remains the primary cause of cancer-related deaths worldwide. Phenolic acids, including o-coumaric acid (OCA), have attracted considerable attention due to their diverse biological activities, particularly their anticancer properties. This study evaluates the cytotoxic activity of OCA, along with its anti-migration effects and molecular docking analysis. The in vitro cytotoxicity of OCA on H1975 cells was assessed using the MTT assay and Acridine orange/ethidium bromide (AO/EB) staining, while its impact on cell migration was analyzed through an in vitro scratch assay. OCA demonstrated cytotoxic activity against H1975 cells, with an IC50 value of 8.107 mM, and inhibited cell migration by 38%. Additionally, in silico molecular docking was performed to investigate its interaction with the epidermal growth factor receptor (EGFR). Although OCA exhibited notable binding interactions with EGFR, including hydrogen bonding and pi-alkyl interactions, its binding affinity (−5.9 kcal/mol) was lower compared to Gefitinib (−8.5 kcal/mol), a known EGFR inhibitor. These findings suggest that while OCA holds potential as a therapeutic agent against non-small cell lung cancer, its efficacy may be enhanced through structural modifications, including the synthesis of derivatives, warranting further research into its industrial and clinical applications.

Keywords: *o*-Coumaric acid, Lung cancer, Cytotoxicity, Anti-migration, Molecular docking.

[a] *dogukan.mutlu@uskudar.edu.tr* *https://orcid.org/0000-0003-3259-5822*

Introduction

Lung cancer is a leading cause of cancer-related mortality globally, with non-small cell lung cancer (NSCLC) being the predominant subtype, accounting for over 80% of cases [1]. Most NSCLC patients are diagnosed at advanced metastatic stages, which renders them unsuitable for surgical intervention [2]. Chemotherapy remains a key treatment modality for cancer patients; however, chemotherapeutic agents often induce only temporary tumor regression. Over time, many cases develop drug resistance, leading to disease progression [3]. Consequently, numerous studies have been dedicated to elucidating the mechanisms underlying this resistance.

OCA, also known as trans-2-hydroxycinnamic acid, is a naturally occurring hydroxycinnamic acid widely found in various natural sources [4]. OCA is part of the human diet and contributes to the health benefits associated with consuming fruits, vegetables, whole grains, and mushrooms [5,6]. Its presence in these dietary sources is one reason why diets rich in fruits and vegetables are often recommended for their potential health-promoting properties.

OCA belongs to the group of phenolic compounds and has attracted the attention of researchers due to its various biological activities, particularly its potential anticancer effects [7-9]. The simple chemical structure of cinnamic acid, which includes a hydroxyl group (-OH) attached to the benzene ring, forms the basis for the antioxidant properties of extracts obtained from natural products containing OCA. These properties allow OCA to scavenge free radicals, protect cells from oxidative damage, and reduce the risk of diseases such as cancer [10-12].

However, studies on the biological activities of OCA are limited, and there are only a few reports on its anticarcinogenic activity. In this study, we evaluated the cytotoxic and anti-migration effects of OCA on H1975 cells, which are commonly used in NSCLC research due to their EGFR L858R/T790M double mutation. This mutation is known to cause resistance to first-generation tyrosine kinase inhibitors (TKIs), such as Gefitinib and Erlotinib, which target EGFR [13]. The presence of this mutation contributes to acquired resistance, making these drugs less effective in treating NSCLC patients with the L858R/T790M mutation [14,15]. Therefore, H1975 cells offer a useful model for investigating both the biology of EGFR-mutated NSCLC and the therapeutic potential of novel compounds like OCA. The findings suggest that OCA may have the potential to be developed as an anti-metastatic drug for human lung cancer.

Material and Methods

Cell Culture

Human non-small lung cancer (H1975) cell line was purchased from American Type Culture Collection (ATCC). Cells were cultured with Dulbecco's Modified Eagle's

Medium (DMEM, Sigma-Aldrich, Germany) with 10% fetal bovine serum (FBS, Capricorn, Germany) and 100U/ml of penicillin, and 100 µg/ml of streptomycin (Capricorn) at 37 °C in a humidified 5% CO_2, as previously described [16].

Cytotoxicity Assay

Cytotoxicity was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, as described previously [17]. H1975 cells were plated into 96-well plates ($2×10^3$ cells/well). OCA was obtained from Sigma-Aldrich was dissolved in dimethyl sulfoxide (DMSO, Carlo Erba). Then different concentrations (0.625, 1.25, 2.5, 5, and 10 mM) of OCA were used to cytotoxicity for 24 hours. The final concentration of DMSO was kept below 1% (v/v), and the control group was treated with DMSO alone. After treatment, the culture medium was removed, 10 µl of MTT solution (5 mg/ml, Merck, USA) was added into each well and cells were kept at 37 °C. After 4 h incubation, the culture medium was aspirated and 100 µl of DMSO was added to each well, for solubilization of formazan crystals using an orbital shaker for 15 min at room temperature. Finally, cell viability was measured at 590 nm using an Epoch microplate spectrophotometer (BioTek, USA). The results are expressed as percentages of the treatment group compared to the control group. IC_{50} values was calculated using GraphPad Prism 9 (GraphPad Software, CA, USA).

AO/EB Double Staining Assay

H1975 cells were incubated, and treated with OCA at IC_{50} concentration as mentioned, after 95% alcohol fixation, cells were stained with acridine orange/ethidium bromide (AO/EB) mixture (1:1, 100 µg/ml), as described previously [18]. After washing for two times, the results were observed and photographed by fluorescent microscope (BX53F, Olympus, Japan).

In vitro Scratch Assay

The effect of OCA on the migration ability of H1975 cells was investigated by the scratch assay, as described previously [19]. Cells were seeded in 6-well plates ($3×10^4$ cells/well) and incubated for 24 hours. Then, the cell monolayers were scratched with a sterile 200 µl tip. After scratching, the wells were washed with phosphate buffered saline (PBS) twice to discard cell debris, and the DMEM was refreshed. Then, H1975 cells were treated with the IC_{50} dose of OCA for 24 hours. The wound areas were captured at 0, 24, 48, and 72 h with an inverted microscope (Oxion Inverso, Euromex, Netherlands) and the migration rate (%) was calculated with ImageJ software 1.53e (USA).

Molecular Docking Study

Molecular docking of OCA with EGFR was completed using the SeamDock web server [20]. Protein-ligand interactions were evaluated using AutoDock Vina with parameters set to a grid spacing of 1 Å, an energy range of 5 kcal/mol, and an exhaustiveness level of 8, while the docking box dimensions were configured as 33×27×18 Å. The docking models with high scores were selected. Figures were generated with SeamDock and BIOVIA Discovery Studio v.24.1.0. The interactions between OCA and EGFR were analyzed using Discovery Studio. Additionally, the inhibitor that was bound to *EGFR* was removed prior to the docking studies. The data for the three-dimensional (3D) structures of EGFR (PDB ID: 5D41) was used in this study.

Statistical Analysis

GraphPad Prism 9 (San Diego, CA, USA) was used for statistical analysis. The experiments were performed in triplicate, and the data are expressed as means ± SD. For comparisons between control and treatment groups, one-way ANOVA followed by Dunnett's test was performed.

Results and Discussion

MTT Assay

The MTT assay was conducted over a 24-hour incubation period to assess the cytotoxic activity of OCA against H1975 cells. As shown in Figure 1, within the concentration range of 0.625 to 2.5 mM, OCA did not exhibit statistically significant cytotoxicity. However, at higher concentrations, it inhibited cell viability in a dose-dependent manner, with an IC_{50} value of 8.107 ± 0.068 mM.

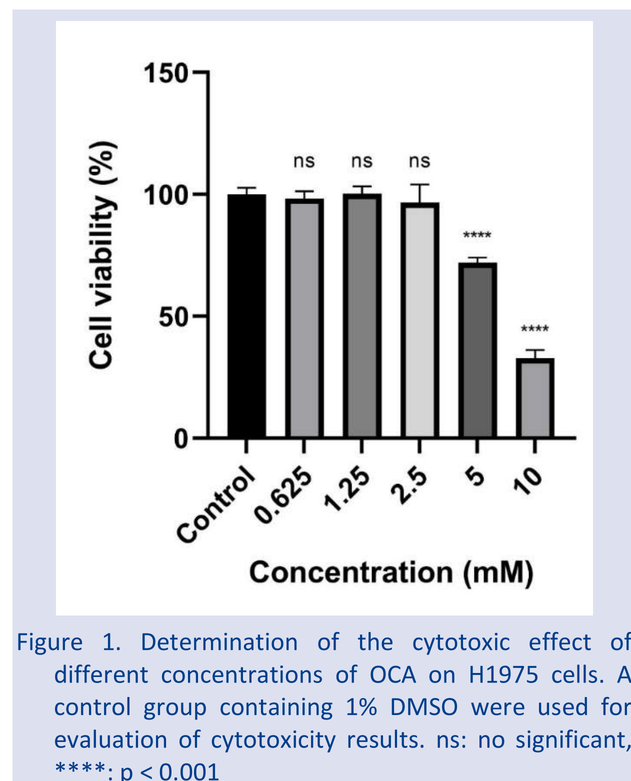

Figure 1. Determination of the cytotoxic effect of different concentrations of OCA on H1975 cells. A control group containing 1% DMSO were used for evaluation of cytotoxicity results. ns: no significant, ****: $p < 0.001$

Morphological Examination

After 24 hours of incubation, H1975 cells exposed to the IC50 concentration of OCA exhibited significant morphological changes compared to the control group (Fig. 2). These changes included nuclear condensation,

cellular degeneration, and a rounded morphology. In contrast, cells treated with 1% DMSO maintained a morphology similar to the control group after the incubation period.

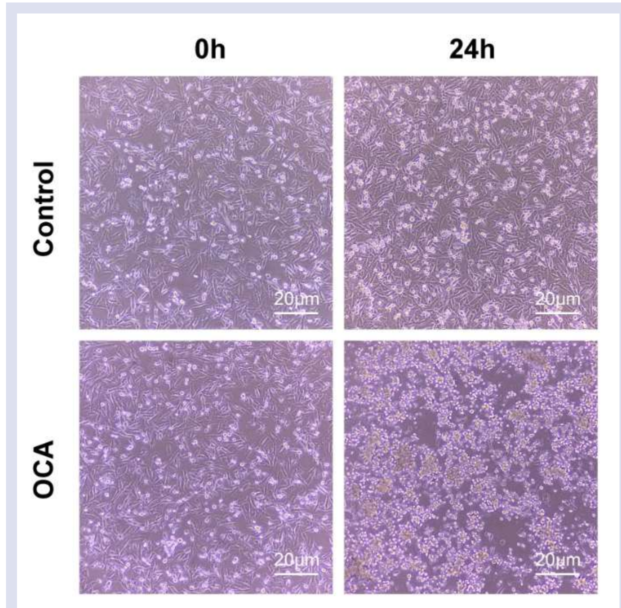

Figure 2. Morphological changes were observed in H1975 cells exposed to OCA at the end of 24 hours of incubation, compared to the control group at 10X magnification.

Fluorescence Microscopy

Fluorescence microscopy analysis was performed on AO/EB (100 µg/mL AO and 100 µg/mL EB) double-stained H1975 cells, both control and OCA-treated, following 24 hours of incubation. Viable cells have uniform green nuclei and the decrease in cell density is consistent with the MTT results (Fig. 3).

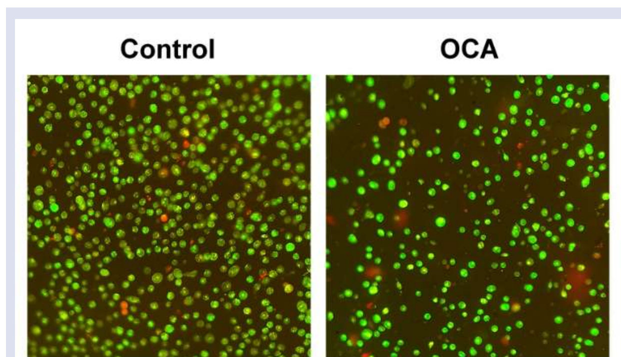

Figure 3. Fluorescence images of AO/EB staining. Viable cells have uniform green nuclei while dead and apoptotic cells exhibit a red and orange, respectively at 20X magnification.

Cell Migration

A scratch assay was performed to assess the effects of OCA on the migration of H1975 cells (Fig. 4). After a 24-hour treatment period, the medium containing the IC50 concentration of OCA was carefully removed from each well, the cells were washed twice with PBS, and fresh medium was added. The control group received culture medium supplemented with 1% DMSO as the final concentration.

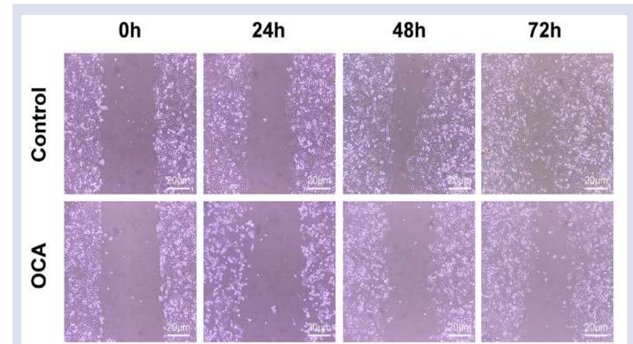

Figure 4. Representative photographs of cell migration using the *in vitro* scratch assay of OCA treated H1975 cells at 10X magnification. Photos were taken at 0, 24, 48, and 72 h.

In this study, the migration of control cells reached 90% at 72 h. However, the migration rate of cells treated with OCA was found to be 52% (Fig. 5). In other words, cell migration was restricted approximately two times (1.7-fold) compared to the control cells.

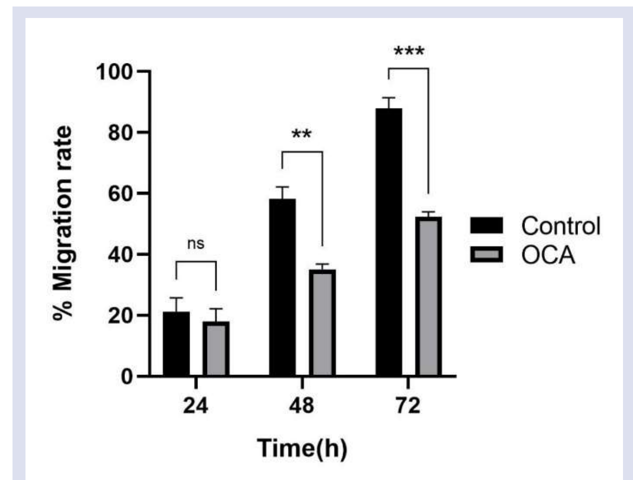

Figure 5. Percentage of migration rate for OCA treated cells compared to the control at 0, 24, 48, and 72 h. ns: no significant, **: $p<0.002$, ***: $p<0.0002$

Interaction Analysis

The 3D structure of EGFR was obtained from the PDB database as the receptor protein. The SMILES code of OCA (CID: 637540) and Gefitinib (CID: 123631) were retrieved from PubChem. OCA's binding interactions with the amino acids in the active site of EGFR were investigated through molecular docking studies. As shown in Figure 6, OCA revealed interactions with ALA1000, GLU1004, ASN771, VAL774, ARG776, GLN791, and LYS852, with a binding affinity of −5.9 kcal/mol in the best pose.

The molecular docking study of OCA with EGFR revealed the following bonding interactions between the ligand and protein residues: 2.10 Å and 2.58 Å long hydrogen bonds with GLN791 and VAL774, respectively;

2.97 Å and 1.40 Å long unfavorable donor-donor interactions between ASN771 and LYS852 of the target protein and OCA, respectively; 4.78 Å and 5.26 Å long pi-alkyl interactions with ARG776 and ALA1000, respectively; and a 4.75 Å long pi-anion interaction with GLU1004.

Gefitinib, a known EGFR inhibitor, demonstrated a binding affinity of −8.5 kcal/mol in the best pose, outperforming OCA in its interaction strength with the EGFR active site (Fig. 6). Gefitinib formed hydrogen bonds with key residues, including ASP770 (2.58 Å), ARG776 (2.03 Å), ARG999 (2.35 Å), GLU1005 (2.75 Å), and ASP1006 (1.97 Å). Additionally, it exhibited a pi-cation interaction with LYS852 (4.58 Å) and a Pi-anion interaction with GLU1005 (4.26 Å). Gefitinib also established alkyl interactions with ARG999 (4.98 Å) and ALA1000 (3.91 Å), as well as a Pi-alkyl interaction with ARG776 (4.56 Å). These interactions highlight Gefitinib’s strong and versatile binding capability with the EGFR active site.

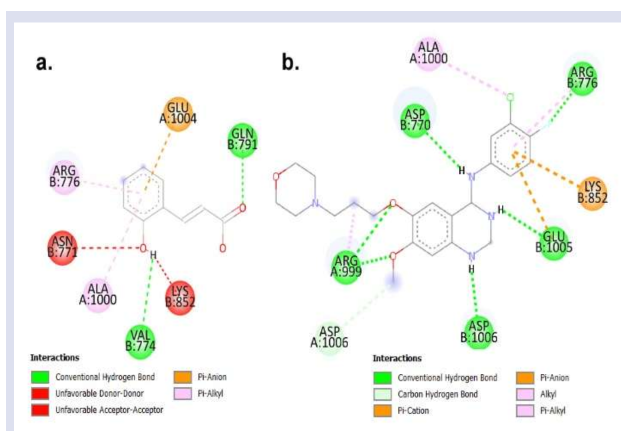

Figure 6. Representative images of molecular docking. Dotted lines show the interactions of a. OCA with EGFR (binding affinity: −5.9 kcal/mol) and b. Gefitinib with EGFR (binding affinity: −8.5 kcal/mol)

This higher affinity can be attributed to the formation of a greater number of hydrogen bonds, as well as diverse secondary interactions (pi-cation and pi-anion interactions) with residues critical for EGFR binding. While OCA demonstrated notable interactions with residues such as GLU1004, GLN791, and VAL774, it showed unfavorable donor-donor interactions with ASN771 and LYS852, potentially reducing its binding efficiency. In contrast, Gefitinib formed favorable interactions with key residues, including ASP770, ARG776, and GLU1005, which are essential for robust binding. Furthermore, Gefitinib's interaction with LYS852 via a pi-cation bond and its engagement with both GLU1005 and ARG776 through pi-anion and pi-alkyl interactions, respectively, underscore its versatility in binding modes.

Numerous in vitro studies have demonstrated the ability of OCA to induce apoptosis in various cancer cell lines. For instance, OCA has been shown to trigger apoptosis in human breast adenocarcinoma MCF-7 cells by activating caspase enzymes, which play a pivotal role in the apoptotic process [21]. Cai et al. (2021) highlighted that OCA inhibits prostate cancer cell growth by targeting the androgen receptor (AR)/β-catenin axis and initiating apoptosis [22]. Similarly, Jung et al. (2012) observed that OCA induced apoptosis in glioblastoma cells through reactive oxygen species (ROS) production and activation of caspase-dependent pathways [23]. In colorectal cancer cells, OCA suppressed proliferation and induced apoptosis by interfering with the TGF-β signaling pathway [24].

Additionally, OCA has been reported to inhibit cell proliferation in both malignant (HSG1) and non-malignant (S-G; GN61) cells. The NR50 value was determined as 6.4 mM for HSG1 cells, while the values for S-G and GN61 cells were 6.7 mM and >10 mM, respectively [7]. Sen et al. (2013) further demonstrated the anti-proliferative effects of OCA, with EC50 values of 4.95 mM in MCF-7 cells and 7.39 mM in HepG2 cells. Their findings also revealed OCA's ability to induce apoptosis in breast cancer cells, alongside its impact on xenobiotic metabolism and potential drug interactions [8,9].

While prior studies have reported OCA's anticancer effects in various cancer types, this study is the first to explore and confirm OCA's cytotoxic and anti-migration effects on EGFR-mutated H1975 NSCLC cells, a clinically relevant model of drug-resistant lung cancer. After 24 hours of treatment, OCA exhibited significant cytotoxicity at concentrations above 2.5 mM, reducing H1975 cell viability to approximately 30% at a 10 mM concentration. Consistent results were observed in the AO/EB assay. Moreover, OCA markedly inhibited migration in H1975 cells compared to the control group. Unlike previous studies that primarily focused on apoptosis induction or proliferation suppression, our findings also highlight OCA's strong anti-migration properties in lung cancer cells, which are critical for metastasis prevention.

However, the IC_{50} value of OCA in H1975 cells (8.107 mM) is relatively high, suggesting that achieving effective cytotoxicity in clinical settings may be challenging due to physiological limitations. For instance, a recent study reported cytotoxic effects within a dose range of 100–500 µM in U-138 MG glioblastoma cells treated with OCA derivatives [25]. These findings underscore the necessity of developing strategies to enhance OCA's efficacy at lower concentrations. Potential approaches include structural modifications to produce more potent derivatives, combination therapies to exploit synergistic effects with other chemotherapeutic agents, and advanced drug delivery systems to optimize its pharmacokinetics and bioavailability. Such advancements are critical for bridging the gap between OCA's promising in vitro effects and its potential clinical applications.

It is well known that many cases of NSCLC involve overexpression of EGFR. As such, EGFR is a targeted receptor in the treatment of lung cancer [26]. The use of currently available EGFR inhibitors for NSCLC treatment still faces various shortcomings, particularly the high failure rate due to resistance [27]. For this reason, OCA was docked with the EGFR macromolecule, and the results showed that OCA has the potential to inhibit receptors that are overexpressed in NSCLC. The docking results suggest that while OCA shows some potential as an EGFR

ligand, its lower binding affinity and the presence of unfavorable interactions make it less effective compared to Gefitinib. Future studies could explore structural modifications of OCA, including the synthesis of derivatives, to enhance its binding properties and interaction profile, potentially improving its inhibitory activity against EGFR.

This study contributes to the understanding of OCA's anticancer properties by demonstrating its specific activity against H1975 cells. While further research is necessary to validate these findings, the results highlight OCA's potential role as a supplementary or alternative approach in lung cancer treatment, particularly in addressing the challenges posed by drug resistance.

Conclusion

This study suggests that OCA may serve as a promising alternative bioactive agent for NSCLC. This is the first examination of the antiproliferative effects of OCA on H1975 cells. Further research is needed to elucidate the molecular mechanisms underlying the potential anti-cancer activities of OCA.

Conflict of interest

There are no conflicts of interest in this work.

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