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ÖZGÜN ARAŞTIRMA/ORIGINAL ARTICLE

Kolon Kanseri Hücrelerinde (DLD-1) Pirolidin Türevi Bileşiğin Antiproliferatif Aktivitesi

Antiproliferative Activity of Pyrrolidine Derivates Compound in Colon Cancer Cells (DLD-1)

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ÖZ

Amaç: Anti-kanser ilaç araştırmaları, çeşitli kanser türlerinde kemoterapötik tedavilerde önemli bir rol oynamaktadır. Pirolidin türevi bileşiklerin birçok araştırmacı tarafından güçlü bir anti-kanser bileşiği olduğu bildirilmiştir. Antiproliferatif aktiviteye sahip yeni ilaç adayları olduğu düşünülen pirolidin türevi bileşiklerin DLD-1 (insan kolon kanseri) ve CCD-18CO (normal kolon fibroblast) hücre hatları üzerindeki etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Pirolidin türevli bileşiklerin antiproliferatif aktivitesi DLD-1 ve CCD-18CO hücre hatlarında karşılaştırılarak farklı konsantrasyonlarda (25-100 uM) MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium bromide) ve RTCA (gerçek zamanlı hücre analizi) deneyleri ile 24 saat boyunca incelendi. MTT deneyinde veri setleri arasındaki farkların önemi, DLD-1 ve CCD-18CO hücre hatlarında SPSS 20.0 programıyla ANOVA ile istatistiksel olarak analiz edildi.

Bulgular: Pirolidin türevi bileşiklerin MTT yöntemi ile negatif kontrole göre DLD-1 kanser hücrelerinin sayısını azalttığı ve RTCA test sonuçlarına göre ise DLD-1 hücresinin baskılandığı belirlendi. Bu nedenle, bileşiklerin hücre proliferasyonunu inhibe ettiği ve antiproliferatif aktiviteye sahip olduğu gösterilmiştir.

Sonuç: Pirolidin türevi bileşiklerin DLD-1 kanser hücrelerinde antiproliferatif aktivite çalışmaları için ilk adım olabileceği ve gelecekteki çalışmalara rehberlik edeceği düşünülmektedir.

ABSTRACT

Objective: Anti-cancer drug research plays an important role for chemotherapeutic treatments in various types of cancer. Pyrrolidine derived compounds have been reported by many researchers to be a potent anti-cancer compound. It is aimed to investigate the effects of pyrrolidine-derived compounds that are thought to be new drug candidates with antiproliferative activity on DLD-1 (human colon cancer) and CCD-18CO (normal colon fibroblast) cell lines.

Material and Method: The antiproliferative activity of the pyrrolidine-derived compound was determined for 24 hours at different concentrations (25-100 µM) on DLD-1 and CCD-18CO cell lines by comparing MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium bromide) and RTCA (real-time cell analysis) assays. The significance of the differences between data sets in the MTT assay was analyzed statistically by ANOVA with SPSS 20.0 program for DLD-1 and CCD-18CO cell lines.

Results: It has been determined that pyrrolidine-derived compounds reduce the number of DLD-1 cancer cells according to negative control with the MTT method and suppress the DLD-1 cell according to the RTCA assay results. Thus, the compounds have been shown to inhibit cell proliferation and have antiproliferative activity.

Conclusion: Pyrrolidine-derived compounds will be the first step for antiproliferative activity studies in DLD-1 cancer cells and will guide the next studies.

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Introduction

About a million people are diagnosed with colon cancer every year in the world. Further studies related to the diseare are important because it is one of the cancer types that cause the most loss of life. Large bowel or colon cancer is among the most common cancer types in the world. It is a type of cancer that is more frequent in women and men, especially over the age of 50 (1,2).

We live in an era where world standards of living, the diagnosis and the treatment of diseases have been significantly improved and health care has ameliorated. With the recent increase in synthetic chemistry as a method of discovering and producing drugs, the potential of discovering new drug candidate compounds and providing new products for disease treatment and prevention is still important (2-4).

Due to their including the nitrogen, pyrrolidine compounds are utilized to construct effective antiproliferative activity (5). The pyrrolidine-derived compound has been reported to be a potent anti-breast cancer compound (6). It has detected cytotoxic effects of pyrrolidine dithiocarbamate in small cell lung cancer cells (7). 4T1, CT26, liver cancer (HepG2) and A549 were evaluated against cancer cells *in vitro* by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium bromide) analysis of the pyrrolidine-derived compound (8).

Most recent studies on cancer research are on antitumor, anti-proliferative, cell cycle suppressing, proapoptotic, anti-metastatic and anti-angiogenic activities in various cancer cell lines (9). Antitumor effects can be listed as: lowering ROS (reactive oxygen species) levels occurring in the body, showing antiproliferative effect, inducing apoptosis, showing anti-migration effect and inhibiting the angiogenesis (10).

Although the biological activities of pyrrolidine derivatives are known, its effect on DLD-1 (human colon cancer) has not been observed in the literature and remains uncertain.

In our study, we present a new drug candidate from pyrrolidine-derived compounds that will have antiproliferative activity in DLD-1 and CCD-18CO (normal colon fibroblast) cell lines. It was researched by comparing two different compounds I (start) and II (product). Pyrrolidine-derived compounds determined with MTT and RTCA (real-time cell analysis) assay by comparison in DLD-1 and CCD-18CO cell lines.

Material and Method

Chemistry

All used solvents were dried by standard methods and the reactions were usually carried out under an inert gas as a nitrogen atmosphere. 1H NMR spectra (400 MHz) and 13C NMR spectra (100 MHz) were recorded on Bruker spectrometers in CDCl3 as solvents. Mass spectrometric data (MS) were obtained by electrospray ionization (ESI). Melting points are uncorrected. Analytical thin-layer chromatography was performed on silica gel 60 plates. Column chromatography was performed on silica gel 60–200 mesh.

Synthesis of compound II

A mixture of methyl-2-((bis(propylthio)methylene) amino) acetate (1.0 mmol), N-phenyl maleimide (5.5 mmol) in dry toluene (10 mL) was refluxed at 185 °C under nitrogen atmosphere for 72 h to obtain a pale-white solid. The solid was purified by column chromatography using hexane-ethyl acetate (1.1) as the eluent.

White solid, yield 32%; mp 215 °C; Rf 0.065 (1:1; v:v, ethyl acetate:n-hexane); FTIR: 3159, 3063, 2963, 1780, 1730, 1709, 1596, 1574, 1498, 1424, 1377, 1204, 792, 759 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 1.022 (3H, t, *J*=7.3 Hz), 1.690-1.723 (4H, m), 3.008 (2H, fused isoindol ring proton, m), 3.64-3.711 (2H, fused isoindol ring proton, d, *J*=7.07 Hz), 3.948 (3H, $-OCH_3$, s), 7.280-7.49 (10H, m, Harom.), 7.675 (1H, NH, s) ppm, ^{13}C NMR (101 MHz, $CDCl_3$, ppm) δ 13.82, 13.717, 22.332, 32.030, 52.932, 53.842, 72.352, 126.129, 128.801, 129.010, 129.229, 131.326, 171.033, 172.288, 172.412 ppm; HRMS(EIC): Exact Mass 519.15; $[M+H]^+$ ion 520.1503, $[M-H]^+$ ion 518.1445 m/z.

Biological assays

Cell culture

In the study, DLD-1 (colon cancer) and CCD-18CO (normal colon), cell lines; In 25cm2 or 75cm2 flasks using 0.2 g/100 mL sodium bicarbonate, 10% fetal bovine serum (FBS) and RPMI-1640 (Roswell Park Memorial Institute) containing 1% penicillin/streptomycin and EMEM (Eagle's Minimum Essential Medium) media, 5% CO_2. It was produced by incubation for 24 hours at 37 °C temperature. After the cells were grown to 80% saturation, washing was applied with phosphate buffered saline (phosphate buffered saline; PBS). 1X Trypsin-EDTA was used for passaging the cells. In *in vitro* cell culture studies DLD-1 (colon cancer) (ATCC® CCL221™) and CCD-18CO (normal

colon epithelium) (ATCC® CRL-1459™) cell lines were used. Cancer cell lines indicated with ATCC numbers were obtained from the cell culture collection at Gebze Technical University. The study does not include human subjects. Therefore, ethics committee approval is not required for the study.

MTT assay

MTT analysis is a method in which the amount of cell proliferation is determined based on the colorimetric measurement of the color change occurring in the cells incubated for enzymatic activity due to the reduction of formazon dyes or MTT. Cytotoxic or proliferative effects of any therapeutic agent on the cell can be determined with this method.

The possible cytotoxic effect of the pyrrolidine compound on DLD-1 and CCD-18CO cell lines was applied with the MTT kit according to the manufacturer's instructions for use.

One day before the application of the MTT method, 100 µl RPMI and EMEM medium were prepared with the cell counted into a 96-well plate ($1x10^4$/ well) and cultivated in the wells. The microplate was placed in an incubator set at 37 °C and 5% CO_2 for 24 hours, allowing the cells to adhere to the surface. After 24 hours of incubation, pyrrolidine compounds prepared in serial dilutions (100 µM - 25µM) were added to the wells. After incubation, 100 µl of MTT (5 mg/ mL) solution was added to the cells and it was left for 2 hours and then the reaction was terminated by adding 100 µl of DMSO (dimethylsulphoxide) to the wells. Incubated cells were measured with microplate reader spectrophotometer at 570 nm absorbance value in three replications (11).

Statistical analysis

The significance of differences between data sets was analyzed statistically by ANOVA with SPSS 20.0 program for DLD-1 and CCD-18CO. The conclusions were indicated as ID50 ± SE (standard error of the mean) for cell lines.

Real time cell analysis (RTCA) assay

Cells would be incubated in RPMI medium and EMEM medium containing 10% FBS at 5% CO and 37 °C. During the experiment, cells were loaded into the E-plates of the iCELLingence RTCA device at $1x10^4$ cells / 400 µL in order to determine cell proliferation and possible anticancer activities of the compounds. After the cells were expected to adhere to the E-plates for 24 hours, (100 µM - 25µM) the appropriate concentrations of the synthesized compounds were determined and the effects of the compounds on cell proliferation in real time were monitored for up to 48 hours. The increase in cell index values was considered to be an increase in cell proliferation, and a decrease in these values was an inhibition of cell proliferation and / or antiproliferative activity as an indicator of cell death (12).

Results

The methyl 2-((bis(propylthio)methylene) amino) acetate and pyrrolidine derivative were characterized by ESI-MS, 1 H - 13C NMR analyses. The pyrrolidine derivative, Methyl-1,3,5,7-tetraoxo-2,6-diphenyl-8-(propylthio) decahydro-4,8-epiminopyrrolo[3,4-f] isoindole-4(1H) carboxylate, were efficiently synthesized from a mixture of methyl 2-((bis(propylthio)methylene)amino) acetate, N-phenylmaleimide in dry toluene under refluxing reaction conditions. The syntheses of compounds I have been previously reported (13-15) and compound II was synthesized according to previously published procedures (Figure 1).

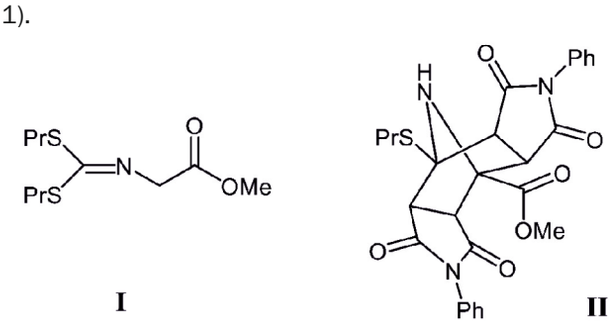

Figure 1. Chemical structures of the imine of glycine methyl ester and pyrrolidine-derived compound.

MTT and RTCA analyses were performed to investigate whether the pyrrolidine-derived compounds are antiproliferative in cell lines. Similar effects were observed in both MTT and RTCA assays. The two compounds were evaluated against two cell lines. For the antiproliferative effects of the compounds, DLD-1 cancer cell line and CCD-18CO normal cell line were used *in vitro*. For this purpose, cells were incubated with compounds (I-II) in multiple concentrations (25, 50, 100 µM) for 24 hours at 37 °C (Figure 2 and 3). Cell antiproliferative effect optical density (OD) values were calculated at 570 nm. The pyrrolidine-derived compounds used in the study stands out as an anti-cancer agent. Based on the optical density (OD) values, the effects of the compounds against *in vitro* proliferation are as follows: II>I.

In addition, it was found that compounds I and II had antiproliferative effects against normal cell lines.

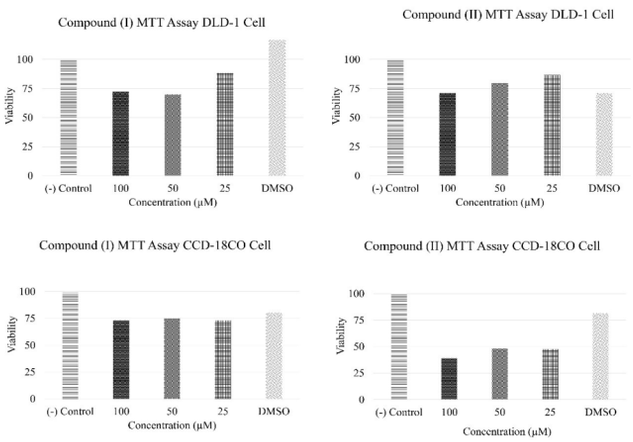

Figure 2. MTT assay of increasing concentrations of compounds on DLD-1 and CCD-18CO cells.

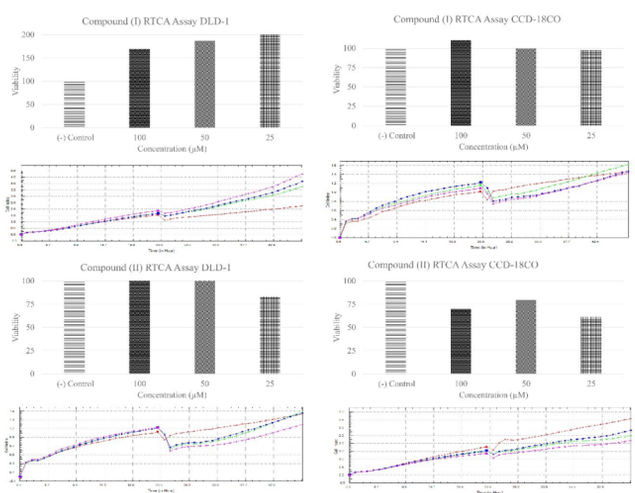

Figure 3. Real-time cell analysis of increasing concentrations of compounds on DLD-1 and CCD-18CO cells.
(Red; Negative control, Green; 100 µM, Blue; 50 µM, Pink; 25 µM)

Discussion

Spiro [pyrrolidine-thiazolo-oxindoles] derivatives was tested antiproliferative assay and exhibited broad biological activity on HepG2, MCF-7 (breast cancer), and HCT-116 (colon cancer) cell lines *in vitro* (16). 4-(pyrrolidine-2,5-dione-1-yl) phenol compound exhibited significant anticancer activity for HT-29 (colon) cancer cell line (17). Pyrrolidine/piperidine substituted 3-amido-9-ethylcarbazole derivatives were assessed antiproliferative effect by the MTT assay on HT-29 (colon cancer) and SH-SY5Y (neuroblastoma cancer) cells. The compounds demonstrated an acetylcholinesterase inhibition activity, antioxidant activity, and antiproliferative effect on cancer cells (18).

We compared the pyrrolidine-derived with two different cell viability tests to evaluate their antiproliferative activities. The results of two different methods showed similar characteristics. The antitumor effect against DLD-1 cancer cell line gave the most effective result for compound II (100 µM). It should be noted that the pyrrolidine-derived compound has an antiproliferative effect in the CCD-18CO normal cell. We compared the concentrations of compounds I and II in two cell lines. As a result of ANOVA analyses (SPSS 20.0), there was a significant difference between them. To figure out the relationships among groups, we also performed post hoc analyses. For this reason, firstly, the homogeneity of the variance test result was observed and depending on these results, different tests were applied. Games-Howell test were used for multiple comparisons of compound I and compound II in DLD-1 and CCD-18CO cell lines ($p < 0.005$) (Table 1) (Figure 4 and 5).

Table 1. *In vitro* antiproliferative effect of the cell lines were investigated by MTT assay after treating compound I and II with varying concentrations of DLD-1 and CCD-18CO cell lines for 24 h. The acquired data were evaluated using SPSS 20.0 analysis and defined as IC_{50} values.

Compounds	Concentration	Cell lines ID50 [µm] ± SE	
	(µM)	DLD - 1	CCD - 18CO
I	25	0.9420	0.8320
	50	0.7470	0.8540
	100	0.7730	0.8320
II	25	0.7420	0.5340
	50	0.6850	0.5370
	100	0.6120	0.4360

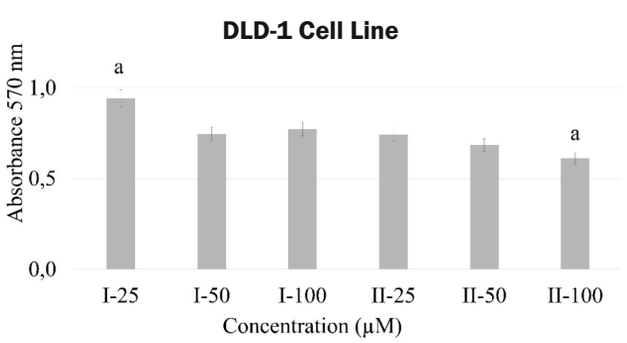

Figure 4. Comparison between compounds I and II in terms of antiproliferative activity. a indicates significant difference for DLD-1 cell line.

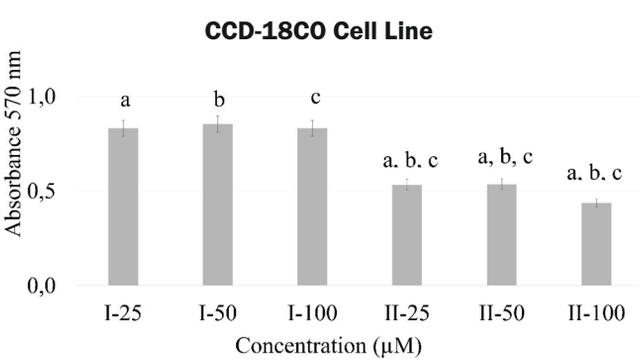

Figure 5. Comparison between compounds I and II in terms of antiproliferative activity. a, b, c indicates significant difference for CCD-18CO cell line.

Conclusion

In a nutshell, the antiproliferative activity of the pyrrolidine-derived compound was determined on DLD-1 and CCD-18CO cell lines by comparing MTT and RTCA assays. The significance of differences between data sets in MTT assay was analyzed statistically by ANOVA with SPSS 20.0 program for DLD-1 and CCD-18CO cell lines.

In the literature studies with pyrrolidine-derived compound, antiproliferative activity studies were not found to be against DLD-1 and CCD-18CO cell lines. This study has the first and only feature in DLD-1 cell lines for these compounds. We think that the pyrrolidine-derived compound has the potential to prevent proliferation in pharmaceutical research and will be useful in further studies to develop new derivatives.

Yazarlık Katkısı: Fikir/Hipotez: SM, MG, TY Tasarım: SM, MG, TY Veri toplama/Veri işleme: SM Veri analizi: SM, MG, TY Makalenin hazırlanması: SM, MG, TY

Etik Kurul Onayı: Gerek yoktur.

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