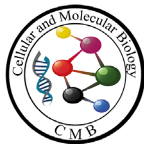

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Antiproliferative effects of EGFR inhibitor Cetuximab and parp inhibitor combination on non-small cell lung cancer cell line A549 and cervical cancer cell line HeLa

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ABSTRACT

In this study, the efficacy of Cetuximab and Parp inhibitor (Parp 1 inhibitor) used in targeted therapies, alone or in combination, on non-small cell lung cancer cell line A549 and cervical cancer cell line HeLa cells were evaluated. For this purpose different cell kinetic parameters were used. Cell viability, mitotic index, BrdU labelling index and apoptotic index were evaluated in experiments. In single applications Cetuximab at concentrations ranging from 1 mg/ml to 10 mg/ml and Parp inhibitor at concentrations 5 µM -7 µM - 10 µM were applied. IC_{50} concentration of Cetuximab for A549 was 1 mg/ml, the IC_{50} concentration of Cetuximab for HeLa was 2 mg/ml, the IC_{50} concentration of Parp inhibitor for A549 was 5 µM, and the IC_{50} concentration of Parp inhibitor for HeLa was 7 µM. In both single and combinations, there was a significant decrease in cell viability, mitotic index, BrdU labelling index and there was a significant increase in apoptotic index. A comparison of cetuximab, PARPi and combination applications showed the superiority of combined applications over single applications in all cell kinetic parameters used.

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Introduction

The combination of two or more chemotherapeutic agents targeting cancer pathways is an important part of cancer treatment (1, 2). Although the monotherapy application is widely used, it shows less effective than the combined application. Commonly used monotherapy applications cannot differentiate between actively proliferating healthy cells and cancer cells, resulting in toxic effects (3, 4). In contrast, combined therapy reduces the toxic effect as the agents are used to target different pathways and require lower doses (5, 6).

Among the cancer immunotherapy methods, the most transferred and approved to clinical applications for therapeutic purposes are monoclonal antibodies (7). Cetuximab is one of the main antibodies produced as a drug today. Cetuximab is a monoclonal antibody that targets EGFR and is clinically approved for use in cancer immunotherapy. Cetuximab is a chimeric antibody, meaning it contains both human and mouse protein sequences (8). The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein. It is a member of the subfamily of type I receptor tyrosine kinases, which includes HER1, HER2, HER3, and HER4. EGFR is constitutively expressed in most normal epithelial tissues (9). It has been determined that EGFR is overexpressed in many cancers. Overexpression of EGFR is associated with poor prognosis, shortened overall survival, and/or increased risk of metastasis. The activity of protein tyrosine kinases is tightly regulated, as they act as mediators responsible for cell growth, differentiation, and death (10). EGFR inhibitors are used in the treatment of different types of cancers in which the family of RTK has been found to be deregulated, which leads to overexpression and amplification of EGFR, which results in appropriate cellular stimulation (11).

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that is activated in response to DNA damage in eukaryotic cells. Activated PARP transfers ADP-ribose units from NAD+ to a nuclear protein such as histone, topoisomerase, DNA polymerase, DNA ligase, or itself. Excessive activation causes consumption of NAD^+ and ATP, resulting in cell dysfunction or necrosis. In addition, PARP is a caspase-independent apoptosis pathway through an apoptosis-inducing factor (12). Poly (ADP-ribose) polymerase-1 plays an important role in DNA repair, apoptosis, cell regulation, cell division, differentiation, transcriptional regulation and chromosome stabilization (13, 14). PARP-1 is a 113 kDa protein and consists of three main parts. The N-terminal end, the DNA binding region, is responsible for repairing damaged DNA. The central zone is responsible for the modification. The C-terminal catalytic domain transfers ADP-ribose structures from NAD^+ to the protein acceptor (13). The aim is to prevent cell damage. This ADP-ribose polymer structure is very important in repairing DNA damage (15). PARP inhibition causes the accumulation of single-stranded DNA breaks and their conversion into double-stranded DNA breaks (16). The use of PARP inhibitors in combination with chemotherapy, radiation, targeted drugs or immunotherapy is one of the strategies that can be used to improve patient outcomes (17).

In this current study, it was aimed to evaluate the expected anticancer effects on A549 cell line originating from human non-small cell lung cancer and the HeLa cell line originating from human cervical cancer as a result of the use of the EGFR inhibitor Cetuximab together with the

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PARP inhibitor.

Materials and Methods

Cell Culture

A549 and HeLa cells used in the experiments were provided by American Type Culture Collection (ATTC Manassas, VA, USA). Both of cell lines regularly underwent two passages per week. A549 cell line was cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) and HeLa cell line was cultured in M199 (Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin (50 units/mL for both) and 10% bovine serum at 37 °C and 5% CO_2.

Cell Viability

The cytotoxicity of Cetuximab and Parp inhibitor (Parp 1 inhibitor) on the cells as a result of the application of scheduled concentrations was researched with the MTT test (18). Absorbance values of the experimental groups were measured by spectrophotometer at 570 nm by taking the 690 nm wavelength as reference.

Mitotic Index

For the determination of mitotic cells, cells were planted in 24-well plates containing $3x10^4$ cells for both cell lines. After cell seeding, cells were incubated 24 hrs. Cells treated with optimum concentrations were fixed with Carnoy's fixative at the end of the experimental periods. Then Feulgen method was applied and stained with Giemsa (19). For analysing MI, approximately 3000 cells were counted with a light microscope for each experimental group.

BrdU Labelling Index

BrdU (5-bromo-2'-deoxyuridine) was used to determine the DNA synthesis rate of A549 and HeLa cells after administration of IC_{50} concentration of Cetuximab and Parp inhibitor. This test is based on the determination of BrdU that binds to the genomic DNA of proliferating cells. BrdU was prepared according to the manufacturer's protocol and then detected via the spectrophotometric method (20).

Apoptotic Index

6-diamidino-2-phenylindole (DAPI) was used to determine the apoptotic cells. DAPI, a fluorescent dye, stains the nucleus of apoptotic cells. After culturing and inhibitor treatment, cells fixed with methanol: FTS mixture until staining was performed. For removing the dye PBS was used. A fluorescent microscope was used to identify apoptotic cells (21).

Statistics

All parameters (Cell viability, MI, BrdU % and AI) were evaluated according to the controls and each other. Therefore, in order to analyze the results one-way Anova test, Dunnett's test and Student's t-test were used. These statistical analyses were performed using SPSS statistics software (V22.0 IBM, Armonk, NY, USA). In the tests $p< 0.05$ level of significance was accepted.

Results

Cell Viability

In order to measure the effect of Cetuximab and Parp inhibitor (Parp 1 inhibitor) on the viability of A549 and HeLa cells and to determine the IC_{50} concentrations of these substances, Cetuximab at concentrations ranging from 1 mg/ml to 10 mg/ml and Parp inhibitor at concentrations 5 µM -7 µM - 10 µM were applied for 24 hours.

The absorbance values of A549 cell line for Cetuximab were $457,168x10^{-3}$; $239,254 x10^{-3}$; $113,321 x10^{-3}$; $110,235 x10^{-3}$; $97,145 x10^{-3}$ and $90,278 x10^{-3}$ respectively for control, 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml and 10 mg/ml (Figure 1A). The absorbance values of HeLa cell line for Cetuximab were $368,231x10^{-3}$; $224,768x10^{-3}$; $182,67x10^{-3}$; $154,239x10^{-3}$; $149,56x10^{-3}$ and $124,784x10^{-3}$ respectively for control, 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml and 10 mg/ml (Figure 1B).

The absorbance values of A549 cell line for Parp inhibitor were $457,168x10^{-3}$; $241,162x10^{-3}$; $203,573x10^{-3}$ and $178,64x10^{-3}$ respectively for control, 5 µM, 7 µM, 10 µM (Figure 2A). The absorbance values of HeLa cell line for Parp inhibitor were $368,231x10^{-3}$; $238,56x10^{-3}$; $185,854x10^{-3}$ and $116,528x10^{-3}$ respectively for control, 5 µM, 7 µM, 10 µM (Figure 2B).

While Figures 3A and 3B showed the percent viability

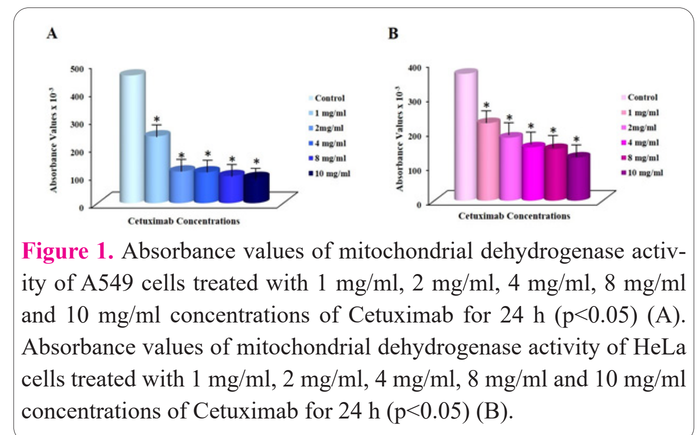

Figure 1. Absorbance values of mitochondrial dehydrogenase activity of A549 cells treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml and 10 mg/ml concentrations of Cetuximab for 24 h ($p<0.05$) (A). Absorbance values of mitochondrial dehydrogenase activity of HeLa cells treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml and 10 mg/ml concentrations of Cetuximab for 24 h ($p<0.05$) (B).

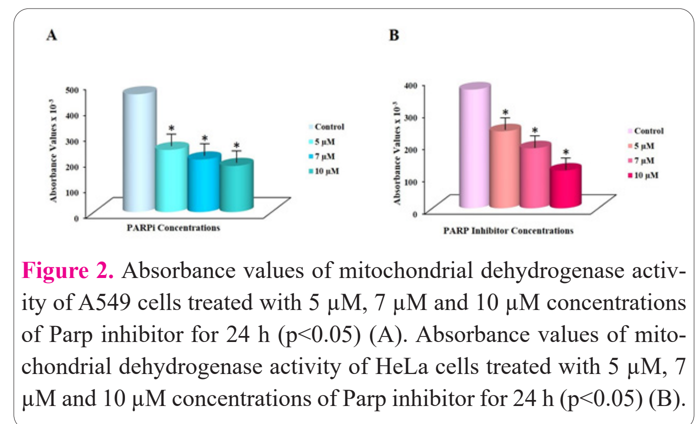

Figure 2. Absorbance values of mitochondrial dehydrogenase activity of A549 cells treated with 5 µM, 7 µM and 10 µM concentrations of Parp inhibitor for 24 h ($p<0.05$) (A). Absorbance values of mitochondrial dehydrogenase activity of HeLa cells treated with 5 µM, 7 µM and 10 µM concentrations of Parp inhibitor for 24 h ($p<0.05$) (B).

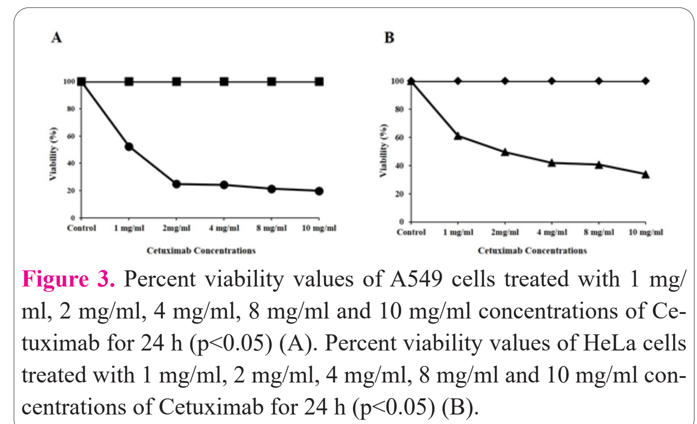

Figure 3. Percent viability values of A549 cells treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml and 10 mg/ml concentrations of Cetuximab for 24 h ($p<0.05$) (A). Percent viability values of HeLa cells treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml and 10 mg/ml concentrations of Cetuximab for 24 h ($p<0.05$) (B).

values of A549 and HeLa cells respectively, as a result of Cetuximab application, Figures 4A and 4B showed percent viability values of A549 and HeLa cells respectively as a result of Parp inhibitor application. When these values were examined, it was observed that the IC_{50} concentration of Cetuximab for A549 was 1 mg/ml, the IC_{50} concentration of Cetuximab for HeLa was 2 mg/ml, the IC_{50} concentration of Parp inhibitor for A549 was 5 µM, and the IC_{50} concentration of Parp inhibitor for HeLa was 7 µM.

Cetuximab (1 mg/ml for A549 and 2 mg/ml for HeLa), PARPi (5 µM for A549 and 7 µM for HeLa) and combination (25% of IC_{50} concentration of Cetuximab (1 mg/ml) +25% of IC_{50} concentration of PARPi (5 µM) for A549 and 25% of IC_{50} concentration of Cetuximab (2 mg/ml) +25% of IC_{50} concentration of PARPi (7 µM) for HeLa) applications were carried out for both A549 and HeLa cells for 0-72 hours. The absorbance values of these applications are as follows. Absorbance values for A549 cell line decreased from 436,878 $x10^{-3}$ to 239,254 $x10^{-3}$ for Cetuximab, to 241,162$x10^{-3}$ for PAPRi and to 200,43 $x10^{-3}$ for combination at 24 h; decreased from 498,482$x10^{-3}$ to 227,734$x10^{-3}$ for Cetuximab, to 231,84$x10^{-3}$ for PARPi and to139,544 $x10^{-3}$ at 48 h; decreased from 534,65$x10^{-3}$ to 220,764 $x10^{-3}$ for Cetuximab, to 219,53$x10^{-3}$ for PARPi and to 97,54$x10^{-3}$ for combination at 72 h (Figure 5A). Absorbance values for HeLa cell line decreased from 368,231$x10^{-3}$ to 182,67$x10^{-3}$ for Cetuximab, to 185,854 $x10^{-3}$ for PAPRi and to 154,547$x10^{-3}$ for combination at 24 h; decreased from 389,643$x10^{-3}$ to 173,459$x10^{-3}$ for Cetuximab, to 176,374$x10^{-3}$ for PARPi and to 126,54$x10^{-3}$ at 48 h; decreased from 412,487$x10^{-3}$ to 150,43$x10^{-3}$ for Cetuximab, to 153,74$x10^{-3}$ for PARPi and to 101,43$x10^{-3}$ for combination at 72 h (Figure 5B).

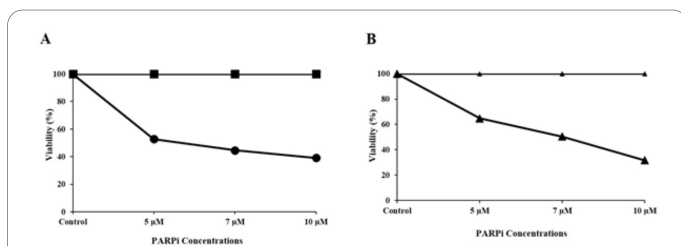

Figure 4. Percent viability values of A549 cells treated with 5 µM, 7 µM and 10 µM concentrations of Parp inhibitor for 24 h (p<0.05) (A). Percent viability values of HeLa cells treated with 5 µM, 7 µM and 10 µM concentrations of Parp inhibitor for 24 h (p<0.05) (B).

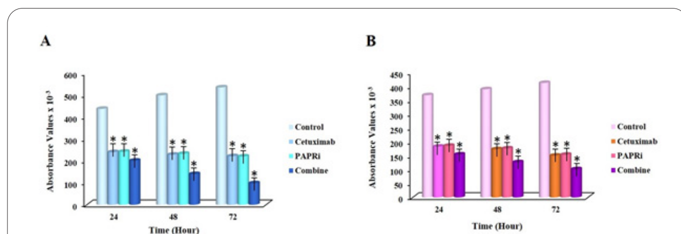

Figure 5. Absorbance values of mitochondrial dehydrogenase activity of A549 cells treated with Cetuximab (1 mg/ml), Parp inhibitor (5 µM) and combination (25% of IC_{50} concentration of Cetuximab (1 mg/ml) +25% of IC_{50} concentration of PARPi (5 µM)) for 0-72 h (p<0.05) (A). Absorbance values of mitochondrial dehydrogenase activity of HeLa cells treated with Cetuximab (2 mg/ml), Parp inhibitor (7 µM) and combination (25% of IC_{50} concentration of Cetuximab (2 mg/ml) +25% of IC_{50} concentration of PARPi (7 µM)) for 0-72 h (p<0.05) (B).

Mitotic Index

As a result of cetuximab, PARPi and combination applications, the proportions of cells in the mitotic phase of both cells were evaluated. Mitotic index values for A549 cell line decreased from 4,3% to 2,8% for Cetuximab, to 3,3% for PAPRi and to 2,03% for combination at 24 h; decreased from 4,9% to 2,2% for Cetuximab, to 2,1% for PARPi and to 1,7% at 48 h; decreased from 5,3% to 1,9% for Cetuximab, to 1,8% for PARPi and to 1,03% for combination at 72 h (Figure 6A). Mitotic index values for HeLa cell line decreased from 6,3% to 3,5% for Cetuximab, to 3,27% for PAPRi and to 2,19% for combination at 24 h; decreased from 6,9% to 2,9% for Cetuximab, to 2,8% for PARPi and to 1,37% at 48 h; decreased from 7,1% to 2,14% for Cetuximab, to 2,23% for PARPi and to 1,21% for combination at 72 h (Figure 6B).

BrdU Labelling Index

As a result of cetuximab, PARPi and combination applications, the proportions of cells in the synthesis phase of both cells were evaluated. BrdU labelling index values for A549 cell line decreased from 100% to 52% for Cetuximab, to 53% for PAPRi and to 48% for combination at 24 h; decreased from 100% to 47% for Cetuximab, to 49% for PARPi and to 41% at 48 h; decreased from 100% to 38% for Cetuximab, to 35% for PARPi and to 33% for combination at 72 h (Figure 7A). BrdU labelling index values for HeLa cell line decreased from 100% to 54% for Cetuximab, to 51% for PAPRi and to 46% for combination at 24 h; decreased from 100% to 49% for Cetuximab, to 45% for PARPi and to 34% at 48 h; decreased from 100% to 43% for Cetuximab, to 39% for PARPi and to 31% for

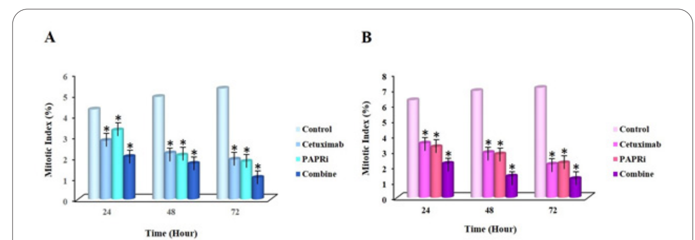

Figure 6. MI values of A549 cells treated with Cetuximab (1 mg/ml), Parp inhibitor (5 µM) and combination (25% of IC_{50} concentration of Cetuximab (1 mg/ml) +25% of IC_{50} concentration of PARPi (5 µM)) for 0-72 h (p<0.05) (A). MI values of HeLa cells treated with Cetuximab (2 mg/ml), Parp inhibitor (7 µM) and combination (25% of IC_{50} concentration of Cetuximab (2 mg/ml) +25% of IC_{50} concentration of PARPi (7 µM)) for 0-72 h (p<0.05) (B).

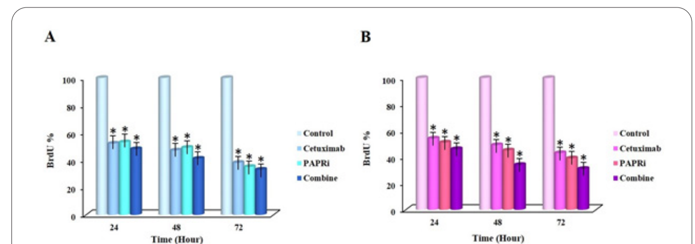

Figure 7. BrdU values of A549 cells treated with Cetuximab (1 mg/ml), Parp inhibitor (5 µM) and combination (25% of IC_{50} concentration of Cetuximab (1 mg/ml) +25% of IC_{50} concentration of PARPi (5 µM)) for 0-72 h (p<0.05) (A). BrdU values of HeLa cells treated with Cetuximab (2 mg/ml), Parp inhibitor (7 µM) and combination (25% of IC_{50} concentration of Cetuximab (2 mg/ml) +25% of IC_{50} concentration of PARPi (7 µM)) for 0-72 h (p<0.05) (B).

combination at 72 h (Figure 7B).

Apoptotic Index

As a result of cetuximab, PARPi and combination applications, the proportions of cells in apoptosis of both cells were evaluated. Apoptotic index values for A549 cell line increased from 3,3% to 4,1% for Cetuximab, to 3,6% for PAPRi and to 6,65% for combination at 24 h; increased from 3,11% to 4,32% for Cetuximab, to 4,04% for PARPi and to 7,26% at 48 h; increased from 3,48% to 5,34% for Cetuximab, to 4,52% for PARPi and to 7,85% for combination at 72 h (Figure 8A). Apoptotic index values for HeLa cell line increased from 3,39% to 3,88% for Cetuximab, to 5,23% for PAPRi and to 5,78% for combination at 24 h; increased from 3,63% to 3,94% for Cetuximab, to 6,04% for PARPi and to 6,33% at 48 h; increased from 3,74% to 4,31% for Cetuximab, to 6,41% for PARPi and to 8,01% for combination at 72 h (Figure 8B).

Discussion

The accumulation of various genetic and epigenetic changes in many genes such as growth factors, growth factor receptors, angiogenic factors, cell cycle regulators or DNA repair genes are effective in all cancer types (22, 23).

Cytotoxic agents used in cancer chemotherapy reduce the number of neoplastic cells several times but cannot completely destroy them (24). For this reason, the use of targeted therapeutics, which have been developed with a much more rational approach, is increasing significantly in solid cancer patients. In recent years, as a result of a better understanding of the biochemical pathways in normal and cancer cells, target molecular structures that will slow down or stop the malignant process in cancer cells have begun to be defined in increasing numbers (25).

Excessive EGFR expression is an important marker for many cancer types, including small cell lung cancer and cervical cancer (23, 26, 27). Studies have shown that cetuximab inhibits the proliferation of EGFR-expressed cancer cells *in vitro* and inhibits tumor growth in xenograph models. It is also reported that its combined use with other chemotherapeutic agents increases its antitumor efficacy. Histological analyzes have also shown that cetuximab inhibits cell proliferation and initiates apoptosis. In addition, studies have shown that cetuximab triggers apoptosis of endothelial cells and antiangiogenic effects (28).

There are various studies that have tried combinations with parp inhibitors in chemotherapy, and the combination of PARP inhibitors with monoclonal antibodies is one of them (29). The fact that PARP inhibitors do not harm normal tissues due to their low side effects increases the interest in these inhibitors (30).

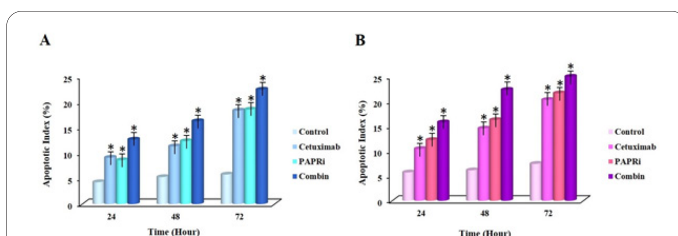

Figure 8. AI values of A549 cells treated with Cetuximab (1 mg/ml), Parp inhibitor (5 μM) and combination (25% of IC_{50} concentration of Cetuximab (1 mg/ml) +25% of IC_{50} concentration of PARPi (5 μM)) for 0-72 h ($p<0.05$) (A). AI values of HeLa cells treated with Cetuximab (2 mg/ml), Parp inhibitor (7 μM) and combination (25% of IC_{50} concentration of Cetuximab (2 mg/ml) +25% of IC_{50} concentration of PARPi (7 μM)) for 0-72 h ($p<0.05$) (B).

Various studies focus on the efficacy of the combination of monoclonal antibodies and Parp inhibitors (31). A study has shown that combined use of cetuximab and Parp inhibitor increases radiation sensitivity more than single use in head and neck squamous cell carcinoma (32). A study showed that Cetuximab increased the cytotoxic activity of Parp inhibitor in head and neck cancer cell lines. Increased susceptibility to this combination suggests that decreased double-strand break repair and increased DNA damage promote apoptosis (33).

In this study, the efficacy of Cetuximab and Parp inhibitor used in targeted therapies, alone or in combination, on non-small cell lung cancer cell line A549 and cervical cancer cell line HeLa cells, which are different cancer types, were evaluated. For this purpose, different concentrations of Cetuximab and Parp inhibitor were used. The values obtained as a result of cell viability measurements showed IC_{50} concentration for Cetuximab 1 mg/ml, 5 μM for Parp inhibitor in A549 cell line, IC_{50} concentration for Cetuximab 2 mg/ml and 7 μM for Parp inhibitor in HeLa cell line. Comparison of cetuximab, Parpi and combination applications showed superiority of combined application over single applications in all cell kinetic parameters used. The results obtained from this study showed that the combination of Cetuximab and Parpi shows promise in the treatment of different types of cancer independent of each other.

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Conflicts of interest

The author certifies that there is no conflict of interest.

Author's contribution

All authors are responsible for the manuscript equally.

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