

**T.C.
INONU UNIVERSITY
GRADUATE SCHOOL OF NATURE AND APPLIED SCIENCES**

**INVESTIGATION OF THE ANTICANCER EFFECT OF
SIMULTANEOUSLY APPLYING RNA-BASED P53 ACTIVATION
AND MDM2 INHIBITION IN DIFFERENT CANCER CELL LINES
CARRYING WILD-TYPE P53**

MASTER THESIS

İrem Nur MENEVŞE

Department of Molecular Biology and Genetics

Supervisor: Asst. Prof. Dr. Muhammed DÜNDAR

DECEMBER 2025

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**İNÖNÜ ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ MÜDÜRLÜĞÜ**

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**Tezi Hazırlayan: İrem Nur MENEVŞE
Yüksek Lisans Tezi**

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WORD OF HONOR

The master's thesis I submitted, titled "Investigation of The Anticancer Effect of Simultaneously Applying RNA-Based P53 Activation and MDM2 Inhibition in Different Cancer Cell Lines Carrying Wild-Type P53," was written by me without any assistance that would violate scientific ethics and traditions, and that all sources I have used are listed in the text and in the bibliography in accordance with the appropriate method. I affirm this with my honor.

İrem Nur MENEVŞE



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SYMBOLS AND ABBREVIATIONS

μL	: Microliter
ACTB	: Actin beta
AR	: Androgen receptor
ATM	: Ataxia-Telangiectasia Mutated
BCA	: Bicinchoninic acid
BAX	: BCL2 Associated X
BCL-2	: B-cell lymphoma 2
BID	: BH3 Interacting Domain Death Agonist
cDNA	: Complementary DNA
C_T	: Cycle threshold
CTR9	: Cln Three Requiring 9
DMEM	: Dulbecco's Modified Eagle Medium
DNA	: Deoxyribonucleic acid
E2F	: Early region 2 binding factor
ECL	: Enhanced chemiluminescence
EDTA	: Ethylenediaminetetraacetic acid
FBS	: Fetal bovine serum
FITC	: Fluorescein isothiocyanate
MDM2	: Murine double minute-2
mL	: Milliliter
mM	: Millimolar
mRNA	: Messenger RNA
nm	: Nanometer
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate-buffered saline
PI	: Propidium iodide
PUMA	: p53 upregulated modulator of apoptosis
PVDF	: Polyvinylidene Fluoride
RIPA	: Radioimmunoprecipitation assay buffer
rpm	: Revolutions per minute
SDS	: Sodium Dodecyl Sulfate

ÖZET

Yüksek Lisans Tezi

YABAN TİP P53 TAŞIYAN FARKLI KANSER HÜCRE HATLARINDA RNA TABANLI P53 AKTİVASYONU VE MDM2 İNHİBİSYONUNUN EŞ ZAMANLI UYGULANMASININ ANTİKANSER ETKİSİNİN ARAŞTIRILMASI

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p53–MDM2 eksenini, hücrelerel stres yanıtlarının merkezi bir düzenleyicisidir ve bozulması tümör gelişimi ile tedaviye direnç oluşumuna katkıda bulunur. Bu çalışma, yabancı tip p53 içeren A549 ve HCT116 hücre hatlarında p53–MDM2 eksenini modüle etmeye yönelik ikili bir RNA temelli stratejiyi araştırmıştır. Yaklaşım, p53 transkripsiyonunun küçük aktive edici RNA (sap53) ile artırılmasını ve p53'ün negatif düzenleyicisi olan MDM2'nin küçük engelleyici RNA (siMDM2) ile baskılanmasını içermiştir; her iki molekül de katyonik bir lipid taşıyıcı aracılığıyla hücrelere iletilmiştir. qPCR ve Western blot analizleri, sap53'ün p53 mRNA ve protein seviyelerini yükselttiğini, siMDM2'nin ise MDM2 ekspresyonunu azaltarak p53'ün degradasyonunu engellediğini ve endojen p53'ü stabilize ettiğini göstermiştir. Kombine uygulama, tekli uygulamalara kıyasla p53 birikimini daha fazla artırmış ve p21 ile PUMA gibi p53'ün aşağı akım hedeflerinin güçlü şekilde indüklenmesine yol açarak hücre döngüsü durması ve apoptozla ilişkili yolları aktive etmiştir. Fonksiyonel analizler, bu ikili yaklaşımın proliferasyonu azalttığını ve en belirgin canlılık düşüşünün kombinasyon grubunda gerçekleştiğini ortaya koymuştur. Ayrıca, migrasyon ve invazyon analizleri hücrelerel hareketliliğin baskılandığını ve en güçlü inhibisyonun kombine uygulamada olduğunu göstermiştir; bu durum, güçlendirilmiş p53 sinyalinin büyüme baskılanmasına ve metastatik potansiyelin azalmasına katkı sağladığını ortaya koymuştur. MDM2 ekspresyon paternleri, p53 aracılı transkripsiyonel artış ile siRNA kaynaklı baskılanma arasındaki dengeyi yansıtmıştır. Genel olarak bulgular, eşzamanlı p53 aktivasyonu ve MDM2 inhibisyonunun p53 sinyalini güçlendirdiğini, proliferasyon ve hareketliliği baskıladığını ve antitümör yanıtları artırdığını göstermiştir. Böylelikle, katyonik lipid içinde saRNA ve siRNA'nın eş zamanlı verilmesi, fonksiyonel p53'e sahip tümörler için umut verici bir RNA tabanlı tedavi stratejisi sunmuştur.

Anahtar Kelimeler: p53 Aktivasyonu, MDM2 İnhibisyonu, saRNA, siRNA, Lipid-bazlı RNA İletimi

ABSTRACT

Master Thesis

INVESTIGATION OF THE ANTICANCER EFFECT OF SIMULTANEOUSLY APPLYING RNA-BASED P53 ACTIVATION AND MDM2 INHIBITION IN DIFFERENT CANCER CELL LINES CARRYING WILD-TYPE P53

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The p53–MDM2 axis is a central regulator of cellular stress responses, and its dysregulation contributes to tumor development and therapeutic resistance. This study investigated a dual RNA-based strategy designed to modulate this axis in A549 and HCT116 cancer cell lines harboring wild-type p53. The approach involved activating p53 transcription through small activating RNA (sap53) while simultaneously suppressing its negative regulator MDM2 using small interfering RNA (siMDM2), both delivered within a cationic lipid carrier. qPCR and Western blot analyses showed that sap53 increased p53 mRNA and protein levels, whereas siMDM2 efficiently reduced MDM2 expression and stabilized endogenous p53 by preventing its degradation. Combined treatment enhanced p53 accumulation more than single applications and led to strong induction of downstream targets p21 and PUMA, indicating activation of pathways governing cell-cycle arrest and apoptosis. Functional assays demonstrated that this dual strategy reduced cellular proliferation, with the combination group showing the most pronounced decrease in viability. Moreover, migration and invasion assays revealed a marked suppression of cellular motility, with the combined treatment exerting the strongest inhibitory effect, exhibiting that reinforced p53 signaling contributed to growth inhibition and reduced metastatic potential. MDM2 expression patterns reflected the balance between p53-driven transcriptional induction and siRNA-mediated suppression. Overall, these findings indicated that simultaneous p53 activation and MDM2 inhibition amplified p53 signaling, suppressed proliferation and mobility, and strengthened antitumor responses. Therefore, co-delivery of saRNA and siRNA in cationic lipid offered a promising RNA-based treatment strategy for tumors that retain functional p53.

Keywords: p53 Activation, MDM2 Inhibitor, saRNA, siRNA, Lipid-based RNA Delivery

1. INTRODUCTION

Cells can constantly face a variety of stress factors, including DNA damage. Therefore, over time, stress factors can cause various genomic abnormalities, such as mutations and deletions, leading to genomic instability. These genomic irregularities can ultimately contribute to the development of cancer. An effective stress response mechanism is required to protect the cell's genome integrity from these adverse effects and to protect it from malignant transformation (Ozaki & Nakagawara, 2011). Among the molecules that coordinate this protective response, p53 is one of the most influential. It contributes to several regulatory networks that determine how a cell reacts to damage, including pathways controlling division, DNA repair capacity, programmed cell death, immune-related functions and metabolic balance (Zhang et al., 2022). Besides its transcription-dependent roles, p53 can localize to mitochondria, where it promotes apoptosis without requiring gene expression changes (Vaseva & Moll, 2009). Under normal physiological conditions, the amount of p53 inside the cell is kept very low. This is mainly due to the continuous tagging of p53 for proteasomal degradation by MDM2 (mouse double minute 2 homolog), which forms a feedback circuit with p53 to ensure tight (Haupt et al., 1997; Kubbutat et al., 1997). When DNA damage occurs, p53 begins to increase in the cell nucleus as a result of post-translational modifications such as phosphorylation and acetylation. These chemical modifications convert p53 from an inactive form to an active form, which may result from the dissociation of MDM2 from p53 (Prives & Hall, 1999; Vousden & Lu, 2002). Active p53 activates certain genes depending on the severity and type of DNA damage in the environment, thus contributing significantly to the initiation of apoptosis and arrest of the cell cycle (Ozaki & Nakagawara, 2011). Loss of function due to factors such as gene deletion or mutation in p53 disrupts the growth-death balance of cells, leading to immortality and ultimately cancer (Muller & Vousden, 2013). Indeed, alterations in p53 are detected in over half of all human cancer cases, illustrating its fundamental importance for tumor suppression (Rivlin et al., 2011). Because of this, therapeutic strategies designed to restore or enhance p53 activity have become a major focus of cancer research (Hu et al., 2021).

Approaches targeting p53 in cancer include preventing the degradation of wild-type p53 (Hu et al., 2021). An example of these approaches is the inhibition of MDM2-p53

interaction. MDM2 binds to the p53 protein with high affinity and negatively modulates its transcriptional activity and stability. Overexpression of MDM2, found in many human tumors, severely impairs p53 function (Vassilev et al., 2004). Interactions between p53 and MDM2 are known to tightly regulate p53 stability, and modifying this interaction can enhance p53 activity in cells that retain wild-type p53 (Xia et al., 2008). Several recent studies have shown that lowering MDM2 levels or blocking its association with p53 results in increased p53 activation and reduced tumor progression (Vassilev et al., 2004). For example, one study showed that reducing MDM2 expression with an antisense oligonucleotide targeting MDM2 in a cell line carrying wild-type p53 resulted in decreased MDM2-p53 interaction, increased p53 expression and activation, and triggered apoptosis (Chen et al., 1998). Another study showed that siRNA (small interfering RNA) mediated reduction of MDM2 expression caused an increase in p53 gene expression and protein levels and a decrease in tumorization in xenograft mice (Zhao et al., 2013).

Targeting wild-type p53 is not limited to reducing its interaction with MDM2 and preventing its proteasomal degradation. So far, p53 protein has been targeted in many ways to increase its activation and stabilization (Horn & Vousden, 2007). Among the mechanisms recently included in these studies is the direct targeting of wild-type p53 expression in an increasing direction. This RNA-based approach is based on increasing gene transcription. Small (or short) activating RNAs (saRNAs) specifically target gene promoter regions, leading to transcriptional gene activation. Therapeutic approaches using saRNA have been applied in recent years to target p53 and many other genes for different types of cancer (Wang et al., 2018). In a study using this approach, overexpression of p53 activated by saRNA was reported to suppress the growth of human prostate cancer cell (Ge et al., 2016). Another study demonstrated that increasing wild-type p53 expression via saRNA inhibited both tumor growth and metastasis in bladder cancer models (Wang et al., 2016). As understood from these studies conducted with different cell groups, increasing the gene transcription of non-mutant p53 via saRNA will have strategic importance as a therapeutic approach for cancers that contain wild-type p53.

Studies targeting MDM2 and wild-type p53 have not only been a unilateral approach, but have also shown effective results when targeted together. For example, one study investigated the simultaneous inhibition of MDM2 and overexpression of wild-type p53 in H1299 (p53 mutant) cells, which resulted in decreased cell proliferation and could lead to G1 cell cycle arrest (Liu et al., 2017). Considering all these, the simultaneous application of decreasing the expression of MDM2 and increasing the expression of wild-type p53 has the

hope to be a better therapeutic approach by providing a synergistic effect for many cancer types with wild-type p53. Thus, in our project, we aim to develop a novel RNA-based combined therapeutic strategy in different cancer cell lines (A549 and HCT116) harboring wild-type p53. For this purpose, we aim to simultaneously apply siRNA, which targets the messenger RNA of MDM2 and inhibits its expression (Chen et al., 2012), and saRNA, which targets the promoter region of p53 and increases its expression (Ge et al., 2016; Wang et al., 2016), and to see the enhancement of the anticancer effect obtained from their separate transfection. Accordingly, siRNA and saRNA will be incubated together in the same liposome with cancer cell lines in cell culture medium and cellular events such as cell viability, cell cycle arrest, induction of apoptosis, cell death, inhibition of migration and invasion will be examined. The obtained results will be compared with the control cell group (incubated with non-targeting siRNA and saRNA) and possible differences will be statistically analyzed. As a result of the analysis, the antitumor or anticancer properties of this new combined therapeutic approach will be evaluated. The fact that siRNA, which inhibits the expression of MDM2, and saRNA, which increases the expression of p53, will be applied to cancer cell lines in the same liposome for the first time in a combined manner makes the project unique. As a result of the results to be obtained, our study is expected to pioneer the development of new RNA-based combination therapeutic approaches.

1.1 Cancer

Cancer comprises a broad collection of disorders originating from various tissues, in which cells acquire the capacity to grow in an uncontrolled manner, invade nearby structures, and interfere with normal physiological processes (Brown et al., 2023). In addition, mutations in genes responsible for cell growth contribute greatly to cancer development (Golemis et al., 2018). One of the key characteristics of cancer cells is that they grow uncontrollably and abnormally. Thus, it becomes metastatic, invading surrounding tissues and can eventually become life-threatening (Hanahan & Weinberg, 2011). Many cancers consist of more than one risk factor. The use of tobacco products, alcohol abuse, physical inactivity, malnutrition and air pollution are the main causes of cancer. It is estimated that smoking alone, one of the strongest factors causing cancer, causes 21% of cancer-related deaths worldwide. Among other cancer-causing factors, alcohol consumption and low fruit and vegetable intake each accounted for 5% of cancer-related death (Danaei et al., 2005).

Cancer, a major social problem in the 21st century, continues to be a leading global health problem. Recent epidemiological data indicate that around 20 million new cancer diagnoses and nearly 10 million cancer-related deaths occurred worldwide in 2022. Research has shown that nearly one out of every five males and females develop cancer during their lifetime. It has also been reported that about one in nine males and one in twelve females pass away due to cancer (Bray et al., 2024). **Figure 1.1** (Bray et al., 2024) shows the most common types of cancer diagnosed in 185 countries by gender. Breast, stomach, colorectal, lung and prostate cancers are the cancers that cause the most deaths worldwide and have a higher incidence than other types of cancer (Pina-Sanchez et al., 2021).

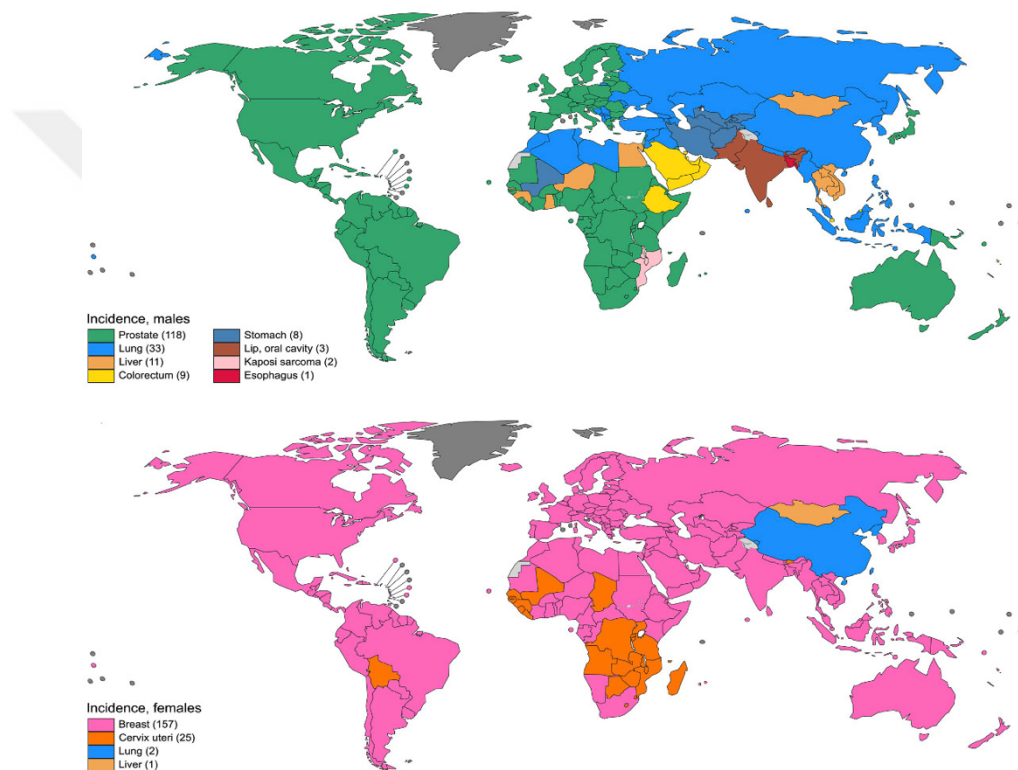


Figure 1.1 : Common types of cancer diagnosed in 185 countries by gender (Bray et al., 2024).

Cancer continues to attract intense scientific attention, largely because its global incidence is steadily rising and it remains one of the foremost contributors to early mortality worldwide (Bray et al., 2021). To date, many topics related to cancer have been studied, such as the origin, progression and development of cancer. Genomic, cellular and physical information has been obtained about cancer. This information has provided great clinical benefits for treatment improvements (Pina-Sanchez et al., 2021). However, the challenges and struggles in cancer prevention and cancer-related treatments remain (Sung et al., 2021).

1.1.1 Murine double minute 2 (MDM2) gene and its role in cancer

The MDM2 gene performs essential functions in the cell cycle, cellular stress responses, and maintaining their homeostasis. MDM2 is specifically responsible for the inhibitory regulation of the p53 tumor suppressor gene. The proto-oncogenic variant of MDM2, originally identified in a mouse tumor-derived cell line, was observed to be co-amplified with the related genes MDM1 and MDM3 (Haines, 1997). Structurally, the MDM2 locus spans approximately 25 kilobases and contains 12 exons organized under the control of two independent promoters. The resulting MDM2 protein is composed of 491 amino acids and features several well-defined functional regions (Freedman et al., 1999). The MDM2 protein, shown in **Figure 1.2**, consists of an N-terminal p53 binding domain, a nuclear localization signal (NLS), a nuclear export signal (NES), a zinc-finger domain, an acidic domain and a C-terminal RING-finger domain (Koo et al., 2022). The N-terminal p53-binding domain of MDM2 is the site where p53 interacts with this domain. The NLS domain is required for MDM2 transport from the cytoplasm to the nucleus and the NES domain directs MDM2 from the nucleus to the cytoplasm. Post-translational modifications occurring within the acidic domain, such as phosphorylation, can influence MDM2 stability and enhance its ability to promote p53 degradation. The C-terminal RING domain possesses E3 ubiquitin-ligase activity, enabling the ubiquitination of p53 and promoting its proteolysis (Inoue et al., 2016). Moreover, the zinc-finger region contributes to fine-tuning the regulatory control exerted by MDM2 on p53 (Koo et al., 2022).

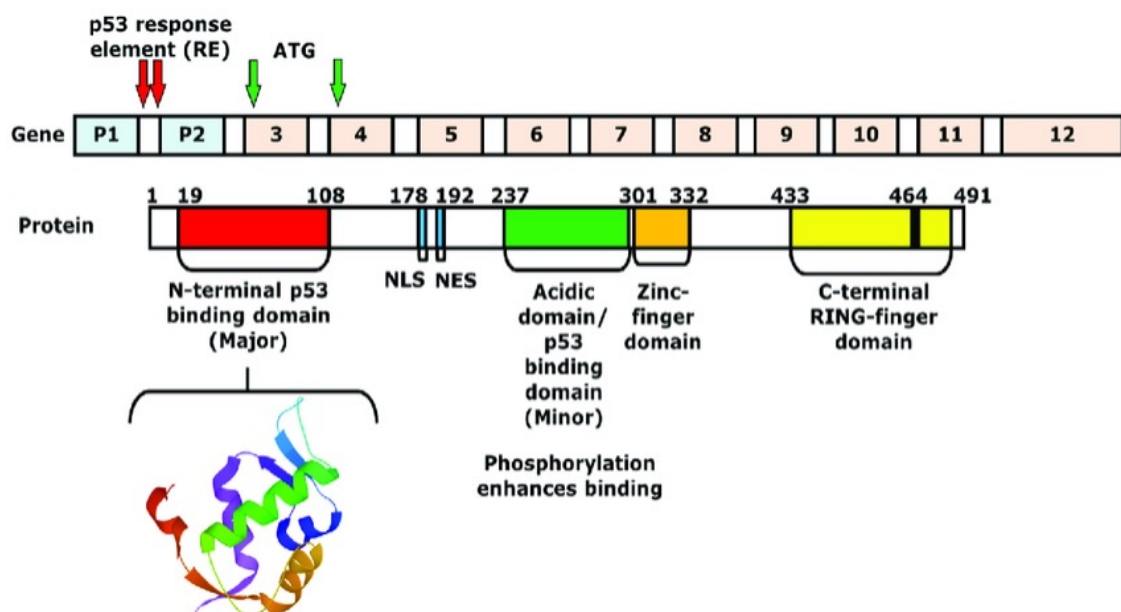


Figure 1.2 : Modeling the structure of the MDM2 gene and protein (Koo et al., 2022).

The MDM2 gene, which negatively regulates the p53 tumor suppressor, is classified as an oncogene due to its adverse effect on tumor development and is found in several types of human cancer. Overexpression levels of MDM2 with E3 ligase activity severely reduce the function of p53, leading to the rapid development of tumorigenesis (Zhao et al., 2014). MDM2 has a high expression in cancer cells, and MDM2 expression increases in cancer cells through p53-dependent and -independent mechanisms and gene amplification mechanism. (Levav-Cohen et al., 2005). The frequency of MDM2 gene amplification varies between 3.5% and 4.4% in human cancers in general (Sciot, 2021) and MDM2 amplification is more common in tumor types such as tissue sarcomas and osteosarcomas (Freedman et al., 1999).

MDM2 contributes to cell-cycle control by influencing the p53/pRb/E2F regulatory network. Alterations in this pathway—particularly those involving MDM2—can impair cell-cycle checkpoints and disturb the coordination of molecules that regulate cell-cycle progression (Levav-Cohen et al., 2005). In addition, the Rb protein, which normally regulates the E2F transcription factor, cannot bind to the E2F transcription factor due to mutations in MDM2 and the activity of E2F increases, causing cells to transition to S phase uncontrollably (Koo et al., 2022). Other factors involved in the cell cycle, such as these, are also degraded and consequently cause cells to aggressively grow and contribute to tumor formation (Haines, 1997).

Since MDM2 negatively regulates p53, p53-MDM2 interaction has become a remarkable and important target in cancer treatment (Konopleva et al., 2020). As a result of the disruption of this interaction, p53 begins to perform its function again, stopping cell-cycle progression and inducing programmed cell death in malignant cells. Considering these, small molecule inhibitors have been developed on this p53-MDM2 interaction (Koo et al., 2022). The most well-known of these inhibitors are Nutlins and RG7388 (Idasanutlin) (Konopleva et al., 2020). The basic logic of these inhibitors is that they bind MDM2 and phosphorylate p53. Thus, the p53 tumor suppressor starts to accumulate in the cell and stops the cell cycle and initiates apoptosis. Many more small molecule inhibitors such as these continue to be developed and clinical trials are ongoing (Koo et al., 2022).

1.1.2 TP53 tumor suppressor gene and its role in cancer

The p53 protein, which performs an essential function within the cell, is synthesized by the TP53 gene and has a vital role in the cell. One of the main functions of p53 is to prevent damage to the genome. (Borrero & El-Deiry, 2021). p53 protects genome integrity by arresting the cell cycle against any genotoxic stress in the environment, repairing damage to the DNA, or driving the cell to apoptosis in cases that cannot be corrected (Kato et al., 2003). The human p53 protein is composed of 393 amino acids and possesses several well-characterized functional domains that enable its regulatory activities. These include an N-terminal transactivation region responsible for initiating gene transcription, a proline-rich segment involved in apoptotic signaling, a central DNA-binding domain essential for its transcriptional role, an oligomerization/tetramerization region and a regulatory C-terminal domain (Bai & Zhu, 2006). **Figure 1.3** shows the domain and crystal structure of p53 (Koo et al., 2022).

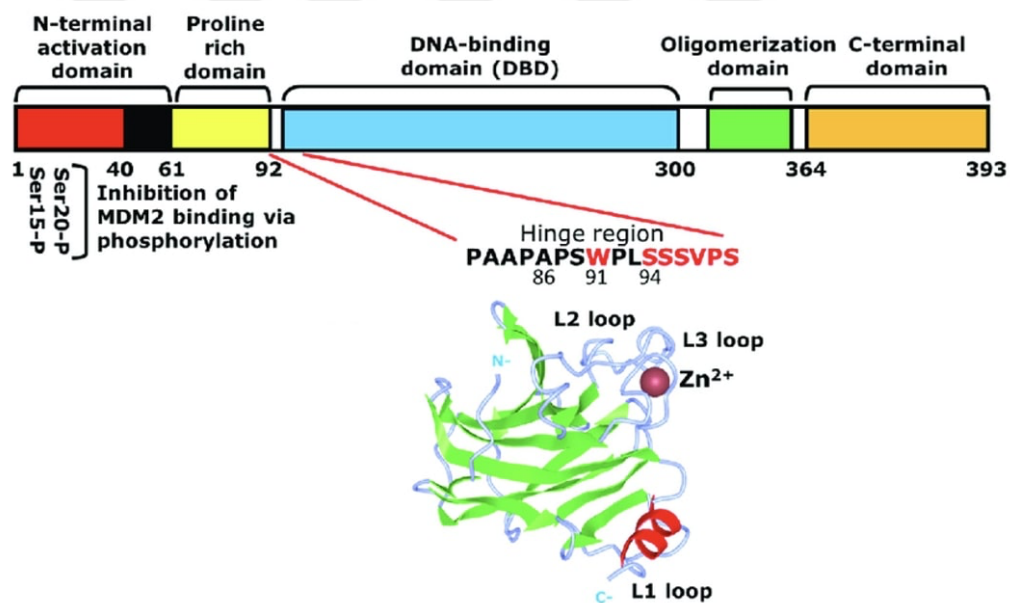


Figure 1.3 : Domain and crystal structure of p53 (Koo et al., 2022).

TP53, which normally prevents cells from becoming cancerous by controlling the cell cycle, is mutated in approximately half of human cancer types (Zawacka-Pankau, 2022). Mutation types such as missense, splice, frameshift, truncating are seen in TP53. Among these, missense mutation constitutes approximately 80% of TP53 mutations. As a result of mutations, p53 loses its function and mutant p53 can acquire new oncologic functions (Chen et al., 2022).

Among TP53 alterations, mutations that affect DNA contact residues or disrupt the structural integrity of the protein such as the well-known variants at codons R175, G245, R249, R282, R248 and R273 represent the most frequently observed mutation sites. These changes often endow the mutant protein with aberrant properties, enabling it to acquire functions that drive tumor progression, including enhanced proliferation, invasiveness and metastatic behavior. In addition, mutant p53 can influence numerous cellular processes, contributing to genome instability and altering the tumor microenvironment (Wang et al., 2023). The incidence of TP53 mutations varies widely across cancer types, but they are particularly common in malignancies of the lung, colon, ovary and stomach (Bai & Zhu, 2006).

To date, various vital roles of p53 in the cell have been revealed (Hu et al., 2021) and the negative consequences of the deficiency of this tumor suppressor have become a remarkable research topic, especially for the cancer field. Attempting to develop therapies by targeting p53, which has complex protein-protein interactions, has had many challenges (Hong et al., 2014). Despite these difficulties, therapeutic studies aimed at restoring p53 functions continue (Zawacka-Pankau & Selivanova, 2015).

1.2 The MDM2-P53 Pathway

Under conditions of cellular stress such as DNA damage p53 acts as a guardian of the genome by halting cell-cycle progression, initiating apoptosis and preventing the replication of damaged cells (Moll & Petrenko, 2003). However, it is important to keep the level of p53 in a normal cell low, as overexpression of p53 can disrupt the cell cycle and lead to untimely cell death (Bose & Ghosh, 2007). This regulation is primarily maintained by MDM2, an E3 ubiquitin ligase that directs p53 toward degradation, and thus restricts its activity under physiological conditions (Michael & Oren, 2002). MDM2 can achieve this suppression through several different mechanisms. MDM2 binds to the transactivation region of p53 and inhibits its transcription factor activity (Biderman et al., 2012). MDM2 also polyubiquitinates p53 for degradation by the 26S proteasome in the cytoplasm, rendering p53 inactive (Mendoza et al., 2014). MDM2 also contains a nuclear export signal that enables shuttling of p53 from the nucleus to the cytoplasm, effectively reducing its transcriptional activity (Biderman et al., 2012). While this is the case under normal conditions, when DNA damage occurs in the cell, a kinase called ATM (Ataxia-Telangiectasia Mutated) is activated and phosphorylates p53, preventing MDM2 from binding to p53. Thus, MDM2 cannot degrade p53 and p53 begins to accumulate in the cell

and performs its function (Bose & Ghosh, 2007). The p53-MDM2 feedback mechanism under stressful and non-stressful conditions is summarized in **Figure 1.4** (Anifowose et al., 2020).

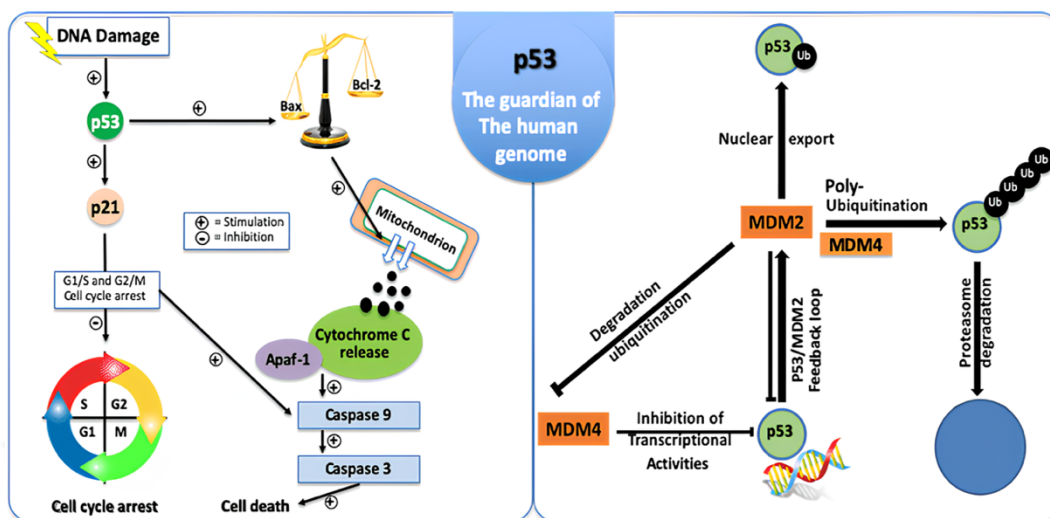


Figure 1.4 : Overview of the MDM2–p53 pathway (Anifowose et al., 2020).

When the MDM2 protein is overexpressed, it acts as an oncogene and starts to behave like a carcinogenic agent (Yao et al., 2024). Elevated MDM2 weakens the tumor-suppressive role of p53 by persistently targeting it for degradation, and amplification of the MDM2 gene has been documented in multiple human cancers (S. Wang et al., 2017). Upon overexpression of MDM2 in cancer cells, MDM2 continuously targets p53 for proteolytic degradation, as a result, p53 cannot fulfill its tumor suppressor function and the p53-MDM2 feedback mechanism loses its normal functioning and interaction. In such a case, p53-MDM2 interaction in cancer cells has become an important target for therapeutic intervention (Manfredi, 2010). Over the years, many strategies have been developed to target this interaction, especially MDM2, with agents such as antisense oligonucleotides and peptides (Wang et al., 2024). Among these efforts, nutlin derivatives and chalcone-based compounds have shown promising activity by either blocking MDM2’s p53-binding pocket or promoting modifications that enhance p53 stability. Preclinical studies indicate that inhibiting MDM2 can suppress cancer-cell proliferation and trigger apoptosis. Despite the variety of molecules designed to target this pathway, only a limited number—most notably Idasanutlin and the I3Ch series—have progressed to clinical evaluation. Many other compounds remain under investigation, with continued emphasis on therapeutics capable of effectively disrupting the MDM2–p53 axis in cancer cells (Koo et al., 2022).

1.3 Small Interfering RNA and Its Role in Cancer

Small interfering RNAs are double-stranded RNA molecules consisting of roughly 20–24 base pairs with sense and antisense chains (Ranasinghe et al., 2023). In 1998, Fire and colleagues (Fire et al., 1998) found that this duplex RNA was effective in gene silencing and that transferring the double-stranded RNA sequence into *Caenorhabditis elegans* caused suppression of gene expression (Saw & Song, 2020). In the RNA interference pathway, siRNA associates with the Argonaute-2 protein to assemble the RNA-induced silencing complex (RISC). In this step, Argonaute-2 cleaves the siRNA duplex into two strands—one referred to as the guide strand and the other as the passenger strand, which is subsequently broken down. The guide strand then directs the RISC complex toward a complementary mRNA target, enabling the complex to cleave and degrade that mRNA, ultimately reducing or eliminating its expression (Xiao et al., 2024). **Figure 1.5** illustrates the molecular mechanism underlying siRNA-mediated gene silencing (Ranasinghe et al., 2023).

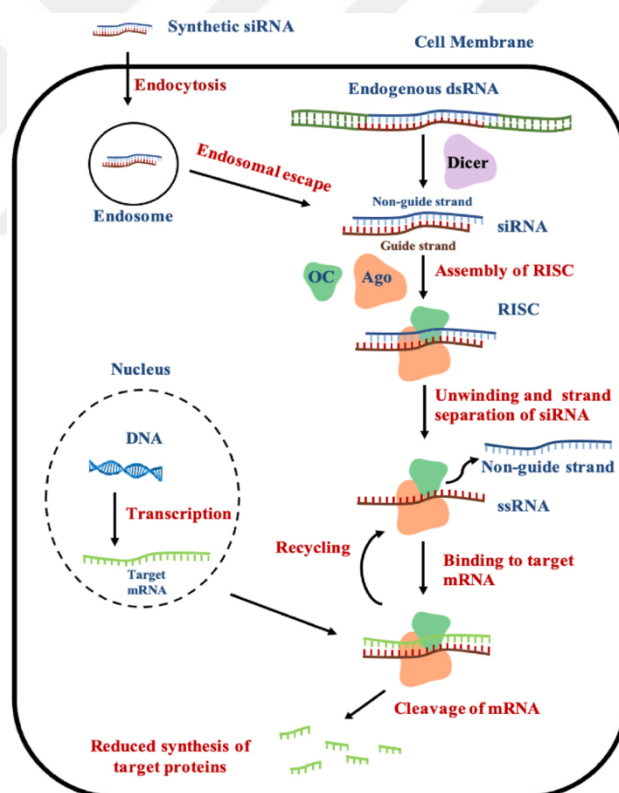


Figure 1.5 : Molecular mechanism of siRNA (Ranasinghe et al., 2023).

RNA interference, a powerful gene silencing mechanism, has been widely used in studies on hereditary diseases, infectious diseases, and especially cancer (Chen & Zhaori, 2011). Cancer has also been addressed with siRNA due to its diverse complex nature and

specific genes have been silenced with siRNA in cancer treatment (Isazadeh et al., 2023). To date, various synthetic siRNA therapeutics have inhibited invasion, metastasis, and reduced tumor growth within malignant cells (Hattab et al., 2021). This reduction has generally been achieved by targeting oncogenes in cancer cells and silencing their expression with siRNAs (Kamaruzman et al., 2019).

MDM2 is placed in the oncogene category considering its malignant properties, such as promoting cell growth and suppressing apoptosis and the p53 tumor suppressor gene (Zhao et al., 2014). Because MDM2 negatively regulates p53, it has become a therapeutic target and an area of interest when this regulation is disrupted in cancer cells (Vassilev, 2007). Multiple studies have investigated siRNA-mediated silencing of MDM2, demonstrating meaningful biological effects. For instance, silencing MDM2 in human lung cancer cells with two independent siRNA sequences resulted in markedly reduced cellular proliferation and enhanced apoptotic activity; the same intervention also improved the cells' sensitivity to radiotherapy (Guo et al., 2007). In another study, siRNA designed for the MDM2 gene was applied to MCF7 (breast cancer) cancer cell line. While levels of the antiapoptotic gene BCL2 declined, the expression of the proapoptotic genes BAX and BID increased significantly. As a result, cell growth stopped and apoptosis was induced (Kalantari et al., 2022). Another study conducted in H1299 p53-mutant cells assessed simultaneous MDM2 silencing and restoration of wild-type p53 activity. This combined approach produced pronounced suppression of proliferation and induced G1-phase arrest, highlighting the therapeutic potential of co-modulating this pathway (Liu et al., 2017).

siRNA is a remarkable approach to gene silencing with its powerful mechanism, and has also paved the way for studies on therapeutic intervention. Although there are many efforts to develop siRNA-based drugs, there are only a very few approved siRNA drugs today. This is because siRNA has challenges such as biostability, delivery efficiency and external factors. Therefore, there is a need to develop new methods or improve existing methods for the therapeutic intervention of siRNA (Ebenezer et al., 2025).

1.4 Small Activating RNA and Its Role in Cancer

Small activating RNAs (saRNAs) are short RNA duplexes, approximately 21 nucleotides long, that boost gene expression by recognizing promoter regions of specific genes and stimulating transcriptional activity (Ghanbarian et al., 2021). Small activating RNAs, like RNA interference, require the Argonaute-2 protein, but unlike RNAi, saRNAs

carry out their target gene-inducing intervention in the nucleus of the cell (Voutila et al., 2017). Once transported into the nucleus, saRNA associates with Argonaute-2 and recruits additional regulatory proteins involved in transcriptional activation. The saRNA–AGO2 complex localizes to promoter sequences of the selected gene, where it initiates transcriptional enhancement. This process involves several components, including RNA helicase A and CTR9 (Cln three requiring 9), which together assemble the RNA-induced transcriptional activation (RITA) complex. The RITA complex then engages with RNA polymerase II, facilitating increased transcriptional output from the targeted promoter region. Beyond its transcriptional effects, saRNA activity can also influence chromatin structure. Through interactions with chromatin-remodeling factors and histone-modifying enzymes, saRNA may contribute to epigenetic changes that further reinforce gene activation (Qian et al., 2025). The functional mechanism of saRNA is presented in **Figure 1.6** (Qian et al., 2025).

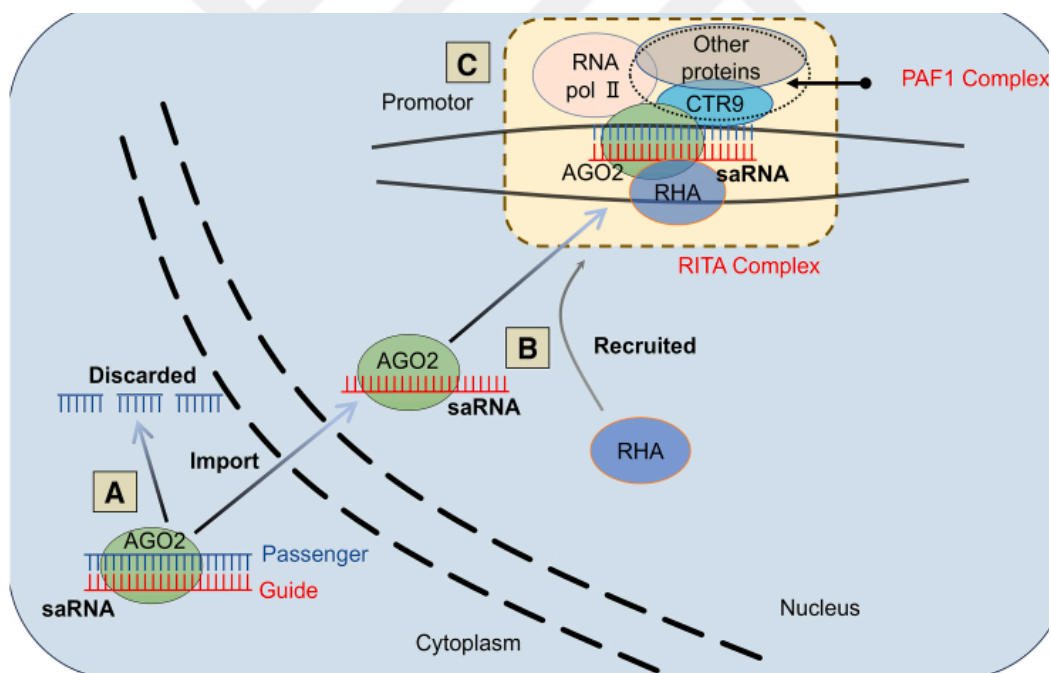


Figure 1.6 : Mechanism of saRNA (Qian et al., 2025).

To date, saRNA has been involved in research aimed at addressing various diseases such as metabolic conditions, neurological degenerative diseases, and cancer (Qian et al., 2025). In particular, saRNA-mediated therapies for cancer have been shown to have great potential. saRNA can regulate the reversal of cancer cell problems caused by cancer-associated genes, such as cancer cell escape from apoptosis, uncontrolled cell invasion and

migration, cell cycle disruption and resistance to chemotherapy (Zhou et al., 2018). In order to regulate these processes, saRNA intervention in cancer suppressor genes can provide a powerful fight against cancer. For example, in one study, the expression of the p53 tumor-inhibiting gene was increased in bladder malignant cells via saRNA, and as a result, cells were paused in the G0/G1 phase, colony formation and cellular growth were significantly reduced, and both migration and invasion were suppressed (Wang et al., 2016). In another study, a study was conducted with three human colorectal cancer cells to learn the activities of p21-saRNA-322 targeted to the p21 gene in human colorectal cancer. In addition, a xenograft colorectal tumor model was created in mice to learn the in vivo tumor suppression ability of p21-saRNA-322. As a result of all these, cell proliferation and colony formation were reduced, cellular senescence was observed, and tumor growth was significantly reduced in tumor models created in mice (L.-L. Wang, H.-H. Guo, et al., 2017). Studies have also been conducted on prostate cancer with saRNA. The effects of Notch1-saRNA-1480 on the human prostate cancer cell line PC3 were examined. Notch1 has an essential function shaping the future behavior of cells, and Notch1 messenger RNA and protein levels were significantly increased after Notch1-saRNA-1480 transfection. Moreover, levels of androgen receptor (AR) and vascular endothelial growth factor (VEGF) gene expression were markedly reduced. Consequently, the proportion of apoptotic cells rose considerably, and the invasive and migratory abilities of cells were strongly suppressed. Tumor volume and growth were also significantly decreased in in vivo experiments (Ma et al., 2020). In an additional investigation, the expression of the HIC-1 (Hypermethylated in Cancer 1) tumor-suppressor gene, which is epigenetically silenced in breast cancer, was targeted for reactivation using saRNA. dsHIC1-2998 saRNA was delivered to MCF7 and MDA-MB-231 breast cancer cell lines. As a result, colony inhibition was significantly induced, mRNA levels of MDA-MB-231 cells increased 3.37-fold compared to the control group, while mRNA levels in MCF7 cells increased 6.52-fold, showing a significant increase. In general, the cell cycle was arrested in G1/G0 phases in both cell lines (Zhao et al., 2015).

Since their discovery, saRNAs have been used in various studies, especially on human cancers. Their therapeutic potential on tumor suppressor genes has been investigated. These studies have yielded promising results. However, studies are ongoing to improve problems such as target specificity, off-target effects and efficiency (Yoon & Rossi, 2018).

1.5 Materials Used in Transfection of Small RNAs

Small RNAs are of great importance in therapeutic applications and research with their ability to regulate protein function, support transcriptional activation of genes or silence protein expression of certain genes (Yan et al., 2022). Because RNAs are very sensitive molecules, they can encounter systematic and cellular barriers to access genes within the cell (Yan et al., 2022). For small RNAs to act on the target inside the cell, the delivery of small RNAs into the cell is crucial. However, there are several challenges to efficient delivery such as poor stability, endosomal escape, off-target effects (Fus-Kujawa et al., 2021; Pandey et al., 2024).

Chemical modifications as well as various means of transportation are used to ensure efficient transmission and overcome obstacles and challenge. By chemically modifying the ribose, terminal and backbone portions of RNAs, significant improvements in serum stability, incorporation into RISC and immune system evasion can be achieved (Zhang et al., 2023). Among chemical modifications, 2'-O-methyl (2'-OMe) and 2'-deoxy-2'-fluoro (2'-F) modifications in the 2' region of the RNA backbone are commonly used and enhance nuclease stability as well as in vitro stability (Allerson et al., 2005). Along with these, stability and efficient transmission can also be improved by remodeling the ribose ring, base modifications, modifications in the 5' end region of the chain, or regulation of the poly(A) tail length in the 3' region (Shi et al., 2024).

In addition to chemical modifications, different delivery systems have been developed to protect RNA from degradation by external factors such as RNase and other nucleases due to its negatively charged and unstable nature (Yan et al., 2022). Nanocarriers must have basic properties such as compatible immunogenicity, non-toxicity, biocompatibility, biodegradability, and stability in order to efficiently transport RNA. Numerous nanocarriers are available for RNA delivery, including polymeric, lipid-polymeric, exosome, inorganic, and cell-based (Golubovic et al., 2023). Especially in clinical settings, lipid-based nanocarriers are generally preferred for RNA delivery due to their advantages such as good biocompatibility and proven track record of success in biomedical applications (Yi Xue et al., 2015). Commonly used types of nanocarriers are shown in **Figure 1.7** (Giri et al., 2023).

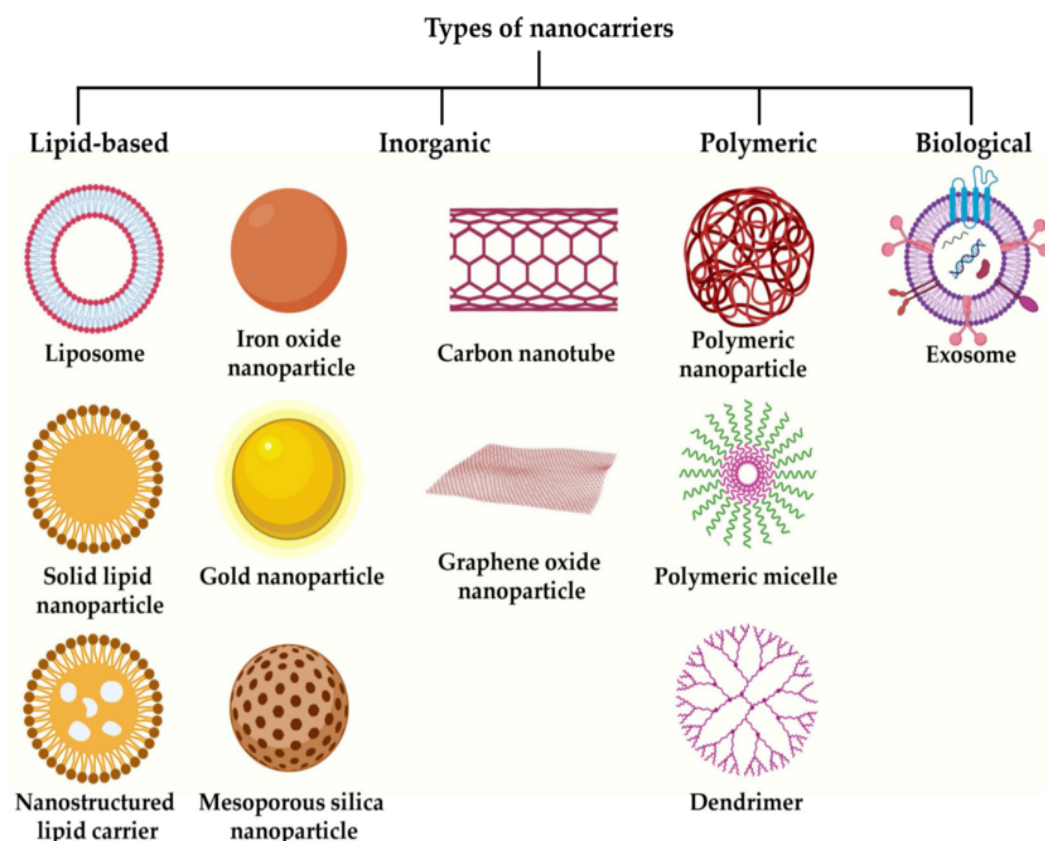


Figure 1.7 : Commonly used types of nanocarriers (Giri et al., 2023).

Liposomes, which are valuable for RNA transport, are spherical vesicles formed by the arrangement of phospholipids in a double layer, containing an aqueous phase inside (Haque et al., 2024). Liposomes are considered safe nanocarriers due to their unique structures, which are biodegradable and highly biocompatible, enabling the delivery of various drugs (Giri et al., 2023). Liposomes are also of great importance in RNA therapies for the delivery of RNA. Since RNA molecules are structurally unstable, they are quickly broken down by nucleases within cellular environments when delivered naked, but this degradation is prevented by liposomes. (Gao et al., 2023). Additionally, lipid nanoparticles and liposomes composed of ionizable and cationic lipids for RNA therapies can increase loading efficiency more effectively by facilitating the endosomal escape of RNA (Jung et al., 2022). In recent years, there has been a great deal of research on vehicles for lipid-based drug delivery. Although lipid-based formulations such as liposomes show therapeutic advantages in biomedical applications, there are still challenges to overcome. More translational research is needed in areas such as production, targeting, and toxicology (Sercombe et al., 2015).

2. MATERIALS AND METHODS

In the study, small RNAs were complexed with cationic lipid, and cells were transfected with these complexes. Tests and analyses were performed after transfection. The overall workflow of the study is presented in **Figure 2.1**.

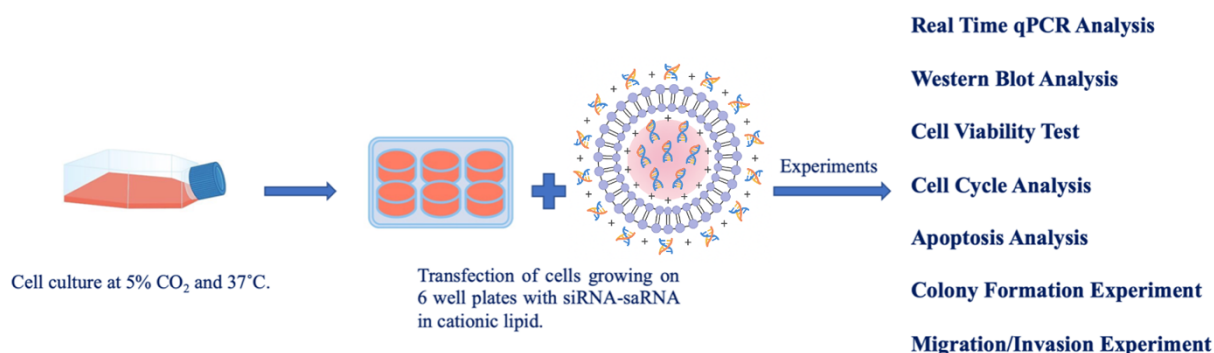


Figure 2.1: General steps of the study.

2.1 Cell Lines

In the study, human lung A549 cell line (ATCC CCL-185) and human colorectal carcinoma HCT116 cell line (ATCC CCL-247) were used. All experiments were performed under identical conditions for both cell lines.

2.2 Small RNA Molecules

Small RNA molecules such as siMDM2 (siRNA-MDM2), which targets and silences the mRNA of the MDM2 gene (Chen et al., 2012), sap53 (dsP53-285), which targets the p53 promoter sequence that initiates transcription and induces transcriptional gene activation and human nonhomology RNA sequence, referred to as control RNA (dsControl), were synthesized using sequence information obtained from existing publications (Ge et al., 2016; Wang et al., 2016). The synthesis and procurement of small RNAs were carried out through biotechnology company (Qiagen). The target and sequence information for siMDM2, sap53, and control RNA molecules is provided in **Table 2.1**.

Table 2.1 : Target and sequence information for small RNAs.

Small RNAs	Target Gene	Target Area	Antisense Strands (5'→3')	Sense Strands (5'→3')
siMDM2	MDM2	mRNA	AGAUGACUGUAGGCCCAA GCGTT[dT][dT]	GCUUGGCCUACAGUCU ACUUTT[dT][dT]
sap53	p53	Promoter	UUAUGGAAGGCUUCCGU AA[dT][dT]	UUAUCGGAAAGGCUU CCUCAA[dT][dT]
Control RNA	–	–	UCUACUGUCACUCAGUAG U[dT][dT]	ACUACUGAGUGACAG UAGA[dT][dT]

2.3 Verification of Small RNA Molecule Loading onto Cationic Lipids Using Gel Electrophoresis

The efficiency of cationic lipids in forming siRNA (or saRNA) complexes was assessed using gel electrophoresis prior to the transfection step in cell culture (Khatri et al., 2014). For each sample, the small RNA–cationic lipid mixtures were combined with a 6X nucleic acid loading buffer (Fermentas Life Sciences). The preparations were then loaded onto a 0.5% agarose gel prepared in TBE buffer, containing 10.80 g/L Tris base, 5.5 g/L boric acid, and 0.58 g/L EDTA (PanReac AppliChem). Ethidium bromide was incorporated into the gel at a final concentration of 0.5 µg/mL to enable visualization. The samples were subjected to electrophoretic separation at a constant voltage of 90 V, which was maintained for 20 minutes. Afterward, the gel was visualized under UV light (G: BOX Chemi XRQ, Syngene). GeneTools software (Syngene) was used to determine the amount of siRNA (or saRNA) that was not loaded onto cationic lipids. The cationic lipid-siRNA (or saRNA) complex formation efficiency was determined using the following equation:

$$\% \text{ siRNA (or saRNA) - Cationic lipid complex} = [(Total \text{ Small RNA} - Unloaded \text{ Small RNA}) / (Total \text{ Small RNA})] \times 100$$

In this formula, RNA_{total} and RNA_{unloaded} represent the total siRNA (or saRNA) and free siRNA (or saRNA) amounts obtained during preparation, respectively.

2.3 Cell Culture

A549 and HCT116 cell lines preserved in cryovials at -80°C were revived by gently adding pre-warmed Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Capricorn) and 1% penicillin–streptomycin (Gibco). They were then placed into sterile Falcon tubes, followed by centrifugation at 1000 rpm for approximately 7 minutes (Nüve, NF800R). Following centrifugation, the supernatant was removed, and the remaining cell pellet was dissolved by mixing with fresh culture medium and cultured in 25 cm^2 (Greiner CELLSTAR®) culture flasks. After the cells in the flask were checked with the live cell movie analyzer (JuLI™ Br, NanoEntek), Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 . Passaging was initiated when confluency reached 70–80%. The spent medium was aspirated, and cells were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS). Subsequently, 1.5 ml of 0.25% Trypsin-EDTA (Gibco) was used to detach the cells. The flasks were then returned to the 5% CO_2 incubator for about 5 minutes to allow detachment. The cells were separated from the flask surface, and 3 ml of fresh medium was added to inhibit trypsin activity. The contents were transferred to a sterile tube and then centrifuged again at 1000 rpm for 7 minutes. After discarding the supernatant the pellet was gently mixed into an appropriate amount of medium to obtain a homogeneous cell suspension. These cells were prepared for subsequent experimental procedures.

2.4 Transfection

A mixture of a certain amount of cells and a certain amount of trypan blue dye was transferred to a Thoma slide for counting the cells prepared for culture. Counting was performed using the JuLI™Br (NanoEntek) device. Following quantification, 1×10^5 viable cells were plated into each well of a 6-well plate containing 3 ml of complete medium (10% FBS and 1% penicillin–streptomycin). A549 and HCT116 cultures were allowed to attach and stabilize for a period of 24 hours. Before the transfection procedure began, the culture medium was replaced with fresh medium. Transfections were carried out using the cationic-lipid reagent LipoFectMax™ (ABP Biosciences), following the recommended workflow provided by the manufacturer. The cationic-lipid–RNA complexes were prepared in 1 ml of serum-free medium so that the final small RNA concentration reached 50 nM, based on prior complex-formation efficiency calculations. The cells were left to incubate for 6–8 hours. After incubation, 1 ml of medium containing 20% FBS was added to each well containing

cells. The cells were incubated for 24 hours in an incubator at 5% CO₂ and 37 °C. The medium used by the cells was removed. To complete the transcription process, 3 ml of fresh medium was added to the cells. Six application groups were applied for both cell lines in the transfection procedures. The treatment groups for the cells were named as follows: Control (untransfected cells), mock (cells exposed only to the cationic lipid-based transfection reagent without any RNA molecules), Control RNA (cells transfected with cationic lipid-Control RNA complex), sap53/Control RNA (cells transfected with cationic lipid-sap53-Control RNA complex), siMDM2/Control RNA (cells transfected with cationic lipid-siMDM2-Control RNA complex), and sap53-siMDM2 (cells transfected with cationic lipid-sap53-siMDM2 complex). Following the transfection process, the cells were used in other stages of the experiment.

2.5 RNA Isolation and Real-Time qPCR Analysis

Seventy-two hours after transfecting A549 and HCT116 cells with cationic lipid-small RNA complexes, the medium used by the cells in each well of the 6-well plate was removed. The cells were then washed twice with PBS. Subsequently, 500 µL of 0.25% Trypsin-EDTA was dispensed into each well, and the plates were kept in a 37 °C incubator for roughly five minutes to facilitate detachment. Cells separated from the plate base were neutralized with 1 mL of fresh medium and transferred to sterile Eppendorf tubes. The culture medium-cell solution was centrifuged at 1000 rpm for five minutes (Sigma 1-14 microcentrifuge). After discarding the supernatants, total RNA was isolated from the resulting cell pellets using the EcoPure Total RNA Kit, following the procedure recommended by the manufacturer. RNA quantity and purity were examined using a Denovix DS-11 FX+ spectrophotometer/fluorometer. Complementary DNA (cDNA) synthesis was carried out using the OneScript® Plus cDNA Synthesis Kit. For each RT-qPCR reaction, 100 ng of cDNA, 10 µL of 2× Master Mix (GoTaq® qPCR Master Mix, Promega) and 200 nM of both forward and reverse primers (Sentebiolab) were combined. DNase- and RNase-free water was added until the final reaction volume was 20 µL. Prior to experimental runs, optimal annealing temperatures were determined using gradient PCR within a ±5 °C range specified in the primer documentation. **Table 2.2** shows the primer sequence information for the targeted genes. RT-qPCR was performed on the StepOnePlus™ Real Time PCR System (Applied Biosystems) using StepOne™ Plus v2.3 software. Briefly, the PCR steps were performed on the device as follows: In the PCR reaction, cDNAs were treated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10

seconds and 60°C for 30 seconds. In the post-processing phase, cycle threshold (C_T) values generated by StepOne™Plus v2.3 software were used to analyze the expression levels of the target genes used in the study. The relative expression levels of the relevant genes were compared between different cell groups using the livak method ($2^{-\Delta\Delta C_T}$) (Livak & Schmittgen, 2001) with ACTB used as the reference (housekeeping) gene.

Table 2.2 : RT-qPCR primer sequences.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
p53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
MDM2	TGTTTGGCGTGCCAAGCTTCTC	CACAGATGTACCTGAGTCCGATG
p21	GCCCA GCTTGACACGAGCAG	GCCGGGTTTGTGAGTGGTGAGA
PUMA	ACGACCTCAACGCACAGTACGA	CCTAATTGGGCTCCATCTCGGG
ACTB	CACCATTGGCAATGAGCGG TTC	AGGTCTTTGCGGATGTCCACGT

2.6 Protein Isolation and Western Blot Analysis

A549 and HCT116 cell lines transfected with cationic lipid–small RNA complexes were collected 72 hours following transfection. Culture medium from the 6-well plate was first removed, and the adherent cells were rinsed twice with PBS. To separate the cells from the plate base, 500 µL of 0.25% trypsin-EDTA was added to each well, and the cells were incubated at 37 °C for approximately 5 minutes. Cells separated from the plate base were neutralized with 1 mL of fresh medium and transferred to microcentrifuge tubes. The tubes were centrifuged at 1000 rpm for 5 minutes (Sigma 1-14). After discarding the supernatant, the pellet was washed once more with PBS and centrifuged again. For protein extraction, the pellets were resuspended in RIPA lysis buffer (1X; 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1.0 mM EDTA, 0.5% sodium deoxycholate and 0.1% SDS; PanReac AppliChem, pH 7.4) supplemented with protease and phosphatase inhibitors (Thermo Fisher). For the SDS-PAGE step of the Western blot, 25 µg of protein from six groups of samples belonging to each cell line was mixed with 2X Laemmli sample buffer (0.125 M Tris base, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol) and heated at 95 °C for 5 minutes. The protein lysates were then separated on SDS-PAGE gels at 100 V for approximately 100 minutes using an SDS-PAGE running system (Mini PROTEAN Tetra Cell). After SDS-PAGE, transfer paper pre-soaked in transfer buffer (1X semi-dry transfer buffer; 47.6 mM Tris-base,

39.3 mM glycine, and 20% methanol) was placed in the Western Blot transfer system (Thermo Scientific™ Pierce™ Power Blotter), and PVDF (GVS) membranes were placed on top of this paper. The run gel was then placed on top of these, and finally covered again with transfer paper. Proteins were blotted using the Western Blot transfer system using 25 V and 1.3 A for 8 minutes to transfer them to the membrane. The membranes were subsequently placed in a blocking solution containing 5% non-fat milk in 1× TBST (25 mM Tris-base, 150 mM NaCl, 0.1% Tween-20; pH 7.6) and gently agitated at room temperature (44 rpm) for 1 hour. After incubation, the primary antibody was added at a 1/1000 dilution to a 1× TBST solution containing 5% milk powder, and incubation with the primary antibody solution was carried out at 4 °C overnight. The membranes were then incubated for 1 hour at room temperature on a shaker set to 44 rpm in blocking solution containing 5% non-fat milk prepared in 1× TBST (25 mM Tris-base, 150 mM NaCl, 0.1% Tween-20; pH 7.6). After the washing steps were completed, immunochemical detection was achieved using an ECL (Enhanced Chemiluminescence) detection kit, and the protein band intensities were analyzed using GeneTools software (Syngene). During the analysis, protein band intensities provided by the device's software were compared to relatively evaluate the expression levels of each protein between different cell groups, with β -actin served as the internal loading control. Details of the antibodies employed for western blot analysis are summarized in **Table 2.3**.

Table 2.3 : Antibody information used in western blot analysis.

Antibody	Company	Catalog Number
Anti-MDM2	Affinity Biosciences	AF0208
Anti-p53	Abcam	ab131442
Anti-p21	Elabscience	E-AB-66357
Anti-PUMA	Elabscience	E-AB-67059
Anti- β -actin	Elabscience	E-AB-40338

2.7 Cell Viability Assay

After 72 hours of incubation following the transfection procedure, the culture medium was aspirated from each well, followed by two washes with PBS. Subsequently, 500 μ L of 0.25% trypsin solution was added to each well and the plates were placed in a 37

°C incubator for approximately 5 minutes to allow detachment. Once the cells were released, 1 mL of fresh growth medium was added to stop the enzymatic activity, and the suspension was transferred into tubes and centrifuged at 1000 rpm for 5 minutes. The pellets were gently resuspended in complete medium, and 1000 cells were seeded into each well of 96-well plates. Cell viability was evaluated over five consecutive days using the CellTiter Blue® Cell Viability Assay (Promega). At each measurement point, the culture medium was aspirated and replaced with 100 µL of fresh medium containing 10 µL of CellTiter-Blue® reagent. The plates were kept at 37 °C for 2 hours. Following incubation, absorbance values at 570 nm and 600 nm were recorded using a microplate spectrophotometer (Agilent Biotek Epoch). The recorded absorbance values were used to compare viability among the experimental groups.

2.8 Apoptosis Analysis

At seventy-two hours post-transfection, cells were cleared of culture medium, followed by two consecutive PBS washes applied to each experimental group. Cell detachment was achieved through exposure to 500 µL of 0.25% Trypsin-EDTA, with incubation carried out briefly at 37 °C. Fresh medium (1 mL) was introduced to recover detached cells, which were subsequently pelleted by centrifugation at 1000 rpm for 5 minutes. Cell pellets were then brought into suspension using Annexin V binding buffer. Quantification of apoptotic populations was performed via the Annexin V-FITC/PI Apoptosis Kit (E-CK-A211, Elabscience). For each sample, 100 µL of cell suspension was transferred to assay tubes. Subsequently, 5 µL Annexin V-FITC (90 µg/mL) and 10 µL propidium iodide (0.5 mg/mL) were added, and samples were gently mixed and the staining reaction was allowed to proceed for 15 minutes at room temperature under light-protected conditions. After staining, 400 µL of Annexin V binding buffer was added to each tube. Data acquisition was carried out using a BD Accuri™ C6 Plus flow cytometer. Fluorescence signals were detected in the FITC FL1 channel at 533/30 nm and the PI signal in the FL2 channel at 585/40 nm. Data acquisition and apoptosis quantification were performed using BD Accuri™ C6 Plus software.

2.9 Cell Cycle Analysis

The culture medium from small RNA-transfected cells was aspirated, and each well was rinsed twice with PBS to remove residual medium. Subsequently, enzymatic detachment was initiated by adding trypsin, followed by a 5-minute incubation period at 37 °C, after

which the cells were collected for further processing. After detachment, 1 mL of fresh medium was added to neutralize the trypsin, and the cell suspension was transferred into tubes. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes. After centrifugation, the supernatant was carefully removed, and 500 μ L of 70% ethanol was added dropwise while gently vortexing the tubes. The ethanol-fixed cells were maintained at 4 °C for 1 hour, after which centrifugation was repeated at 1000 rpm for 5 minutes, followed by a single PBS wash. The pellets were resuspended in 400 μ L PBS and exposed to 0,1 mg/mL RNase A at 37 °C for 30 minutes. Following RNase treatment, propidium iodide (0,05 mg/mL) was introduced, and samples were gently mixed and allowed to incubate for 15 minutes under light-protected conditions. Flow-cytometric acquisition was performed using a BD Accuri™ C6 Plus system (BD Life Sciences) equipped with a 585/40 nm bandpass filter for PI detection. Data for each experimental group were collected and analyzed using BD Accuri™ C6 Plus software.

2.10 Colony Formation Assay

A549 and HCT116 cells transfected with cationic lipid-small RNA complexes were harvested 72 hours after transfection, the used culture media were removed from a 6-well culture plate, and each cell group was washed twice with PBS. Enzymatic detachment was initiated by adding 500 μ L of 0.25% trypsin to each well, with incubation carried out for 5 minutes at 37 °C. After trypsinization, 1 mL of fresh medium was supplied to neutralize the enzyme, and the resulting cell suspension was transferred into tubes and centrifuged at 1000 rpm for 5 minutes. The obtained pellet was gently resuspended in fresh medium, and each well of a 6-well plate received 1000 cells. Cell growth was monitored for 10 days, and the medium was refreshed every two days. On the 10th day, a staining solution containing 0.5% crystal violet, 25% methanol, and distilled water was prepared to stain the cells. Before staining, the cells were rinsed twice with PBS, followed by fixation with ice-cold 100% methanol for 30 minutes. After fixation, the methanol was discarded, and approximately 2 mL of the staining solution was added to each well. Plates were kept at room temperature for 30 minutes to allow colony staining. After the incubation period was over, the dyes were removed and excess dye residues in the colonies were washed away with distilled water. Colony formation efficiency was calculated according to the following formula "Colony formation rate (%) = (number of colonies/number of seeded cells) $\times$ 100".

2.11 Migration and Invasion Assay

The old medium of cells transfected with small RNAs was removed, and each well was washed twice with PBS. Then, 500 μ L of 0.25% trypsin solution was added to each well and incubated for 5 minutes in a 37°C incubator. 1 mL of fresh medium was added to the incubated cells, and the cells were centrifuged at 1000 rpm for 5 minutes. Cells were dissolved in a serum-free culture medium. The Boyden chamber assay was performed using a polycarbonate membrane (Transwell® Permeable Supports, Polycarbonate (PC) Membrane, 3428) to culture cells and perform the experiments. For invasion experiments, 1 mL of extracellular matrix solution (Corning® Matrigel® Basement Membrane Matrix, 354234) was spread across the polycarbonate membrane surface and allowed to polymerize at 37 °C for 30 minutes. Following gel solidification, serum-containing medium was introduced into the lower compartment of each insert. Previously transfected cells, maintained in serum-free conditions, were placed into the upper chamber such that each insert received 1×10^5 cells. The plates were maintained under standard culture conditions (37 °C, 5% CO₂) for 24 hours to permit cell migration or invasion. Cells that traversed to the underside of the membrane were fixed using 100% methanol for 1 hour at 4 °C. After fixation, methanol was discarded and membranes were stained with 0.5% crystal violet for 1 hour at room temperature. The membranes were then washed with distilled water until excess dye was removed. Once the membranes had dried, cells present beneath the membrane were enumerated using light microscopy. The migration and invasion capacities between the groups were compared using the cell numbers obtained for each group in the formula "Migration-invasion (%) = [(number of cells crossing the membrane in each application group)/(number of cells crossing the membrane in the control group)] x 100".

2.12 Statistical Analysis

For each cell line used in the study, experimental groups were created as cells transfected only with cationic lipid (Mock), cells transfected with cationic lipid-small RNA complexes (Control RNA), untransfected cells (Control), as well as single siMDM2/Control RNA and sap53/Control RNA transfection groups and sap53/siMDM2 combination groups. Statistical comparisons between experimental groups within each cell line were carried out using Student's t-test. The symbol '*' was used to indicate a significant difference in the comparison of sap53/Control RNA, siMDM2/Control RNA and sap53/siMDM2 groups with the Control RNA/Control RNA group, the symbol '#' was used to indicate a significant

difference in the comparison of sap53/siMDM2 combined transfection with single sap53/Control RNA and siMDM2/Control RNA groups, and non-significant (ns, $p \geq 0.05$) was used if no significant difference was observed between them. All analyses were performed on three biological replicates for each cell line. The $p < 0.05$ values obtained as a result of the analyses indicate that the difference between the experimental groups is statistically significant, while $p < 0.001$ or $p < 0.01$ indicate a greater statistical difference between the values of the groups.



3. RESULTS

3.1 Morphology of Cell Lines

Morphological images of A549 and HCT116 cells obtained using the JuLI™ Br cell imaging device are shown in **Figure 3.1**.

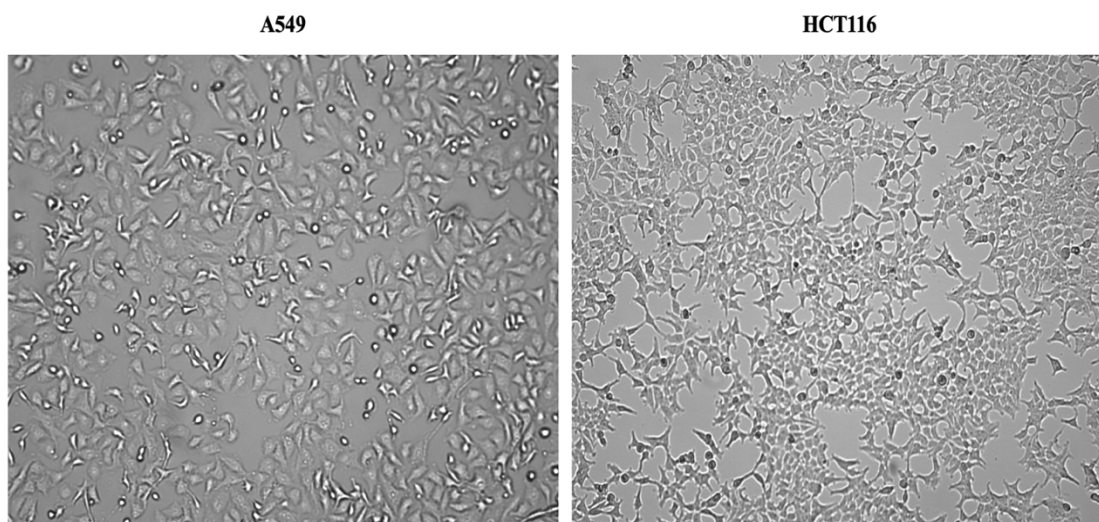


Figure 3.1: Morphological images of A549 and HCT116 cell lines.

3.2 Verification of Small RNA Molecule Loading onto Cationic Lipids Using Gel Electrophoresis

Before the transfection step for each cell line, the formation of complexes between small RNAs and cationic lipids was determined by gel electrophoresis. Gel electrophoresis was performed with the cationic lipid-small RNA complexes and free RNA molecules. The gel was then visualized under UV light (G:BOX Chemi XRQ, Syngene, Cambridge, England). GeneTools software (Syngene, Cambridge, England) was used to determine the amount of siRNA or saRNA not loaded onto cationic lipids. Based on cationic lipid-siRNA or saRNA complexation efficiency calculations, the complex formation efficiency was determined to be approximately 91%. **Figure 3.2** shows gel images of free RNAs and cationic lipids complexed with small RNAs.

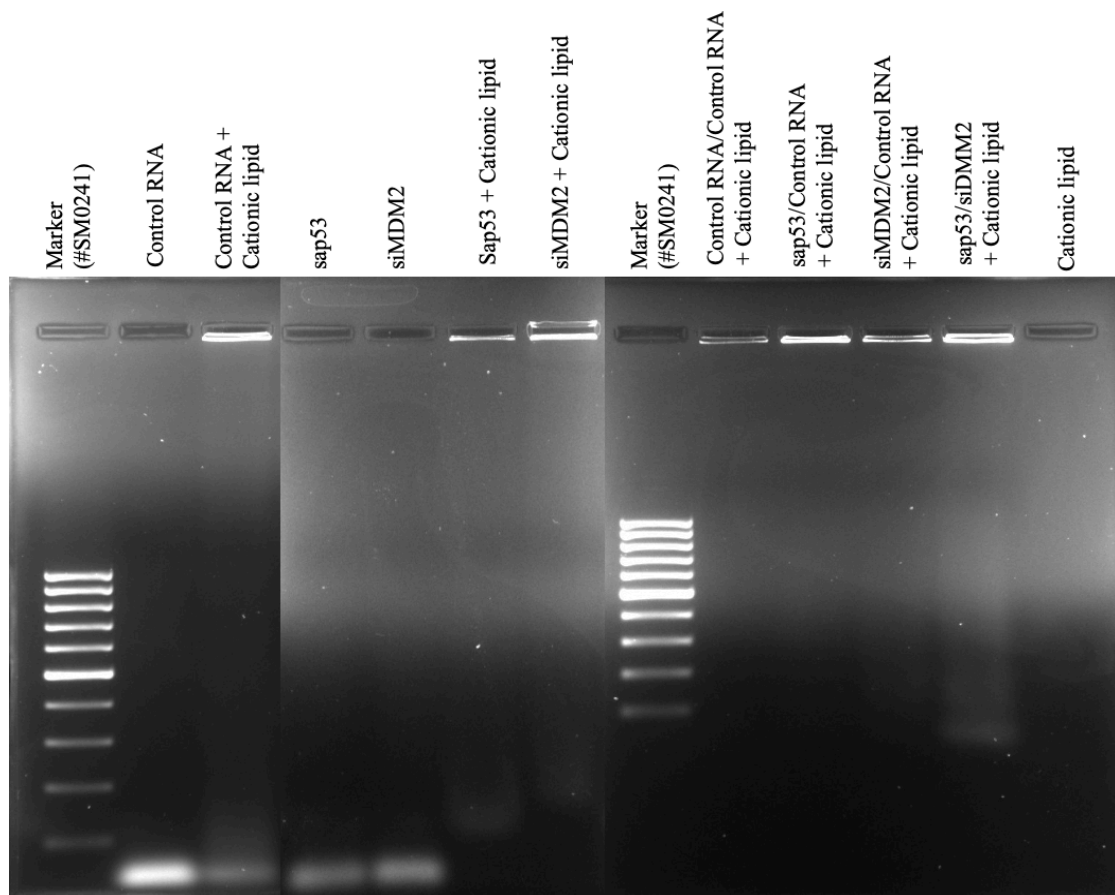


Figure 3.2: Gel images of complexation analysis of small RNAs with cationic lipid.

3.3 Real-Time qPCR Analysis

The RNA obtained from the experimental groups in the A549 and HCT116 cell lines was converted to cDNA, and a qPCR assay targeting p21, PUMA, p53, MDM2, and ACTB was performed. The mRNA expression levels of the analyzed genes were assessed based on the obtained Ct values.

In both cell lines, all groups were first compared to the Control group, then the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups were compared to the Control RNA/Control RNA group, and finally the co-transfection group sap53/siMDM2 was compared to the single transfection groups sap53/Control RNA and siMDM2/Control. In the A549 cell line, p53 transcript levels remained essentially unchanged in the Mock and Control RNA/Control RNA groups when assessed against the Control group, and their levels were similar to those of the Control group. The sap53/Control RNA group showed approximately 6-fold higher expression compared to the Control group, while the siMDM2/Control RNA group showed an approximately 5.5-fold increase. The sap53/siMDM2 group showed an approximately 6-fold increase. In comparisons using the

Control RNA/Control RNA condition as the reference point, the sap53/Control RNA and siMDM2/Control RNA groups showed an approximately 4-fold increase ($p<0.01$), while sap53/siMDM2 showed an approximately 4.1-fold increase ($p<0.01$). The sap53/siMDM2 co-transfection group displayed a 1.08-fold elevation relative to the sap53/Control RNA group (ns) and 1.11-fold increase when assessed against the siMDM2/Control RNA group (ns). When evaluated against the baseline Control condition, p21 transcript abundance in the A549 cell line showed similar values in the Mock and Control RNA/Control RNA groups. The p21 levels in the sap53/Control RNA and siMDM2/Control RNA groups demonstrated an approximately 6-fold elevation relative to the Control group. Co-transfection of sap53/siMDM2 resulted in an approximately 6.1-fold increase. When p21 measurements were interpreted using the Control RNA/Control RNA group as the comparator, the sap53/Control RNA group showed a 3.02-fold rise, the siMDM2/Control RNA group exhibited a 2.1-fold increase, and the sap53/siMDM2 group displayed a 3.21-fold enhancement ($p<0.01$). p21 expression in the sap53/siMDM2 group showed a 1.06-fold increase relative to the sap53/Control RNA group, and a 1.52-fold increase when assessed against the siMDM2/Control RNA group ($p<0.05$). In the A549 cell line, PUMA transcript levels increased 1.72-fold in the Mock group and 1.33-fold in the Control RNA/Control RNA group relative to the Control condition. The sap53/Control RNA group displayed a 3.96-fold increase, the siMDM2/Control RNA group showed a 3.46-fold increase, and the sap53/siMDM2 group showed a 4.63-fold rise relative to the Control group. PUMA transcript abundance exhibited a 2.3-fold increase in the sap53/Control RNA group with reference to the Control RNA/Control RNA group, whereas the siMDM2/Control RNA group demonstrated a 2.01-fold increase. The sap53/siMDM2 group displayed a 2.69-fold elevation relative to the Control RNA/Control RNA group ($p<0.01$). The co-transfection group sap53/siMDM2 showed a 1.17-fold increase relative to the sap53/Control RNA group (ns) and a 1.34-fold increase relative to the siMDM2/Control RNA group ($p<0.05$). The A549 cell line showed an approximately 1.69-fold increase in MDM2 expression levels in the Mock group and the Control RNA/Control RNA group using the Control group as the reference. The sap53/Control RNA group showed a 2.45-fold increase, while the siMDM2/Control RNA group showed a 0.034-fold decrease. The sap53/siMDM2 co-transfection group showed a 1.34-fold increase. Compared to the Control RNA/Control RNA group, the sap53/Control RNA group showed a 1.45-fold increase. In contrast, the siMDM2/Control RNA group exhibited a 0.015-fold decrease, and the sap53/siMDM2 group showed a 0.71-fold decrease. Compared to the sap53/siMDM2 group, the

sap53/Control RNA group showed an increase relative to the sap53/siMDM2 group, while the siMDM2/Control RNA group showed a significant decreases.

The HCT116 cell line exhibited approximately 1.7-fold and 1.89-fold increases in p53 transcript levels in the Mock and Control RNA/Control RNA groups, respectively, when evaluated against the Control condition. The sap53/Control RNA group, siMDM2/Control RNA group, and sap53/siMDM2 co-transfection group displayed increases of 5.56-, 5.22-, and 6.16-fold, respectively. When benchmarked against the Control RNA/Control RNA reference, the sap53/Control RNA group exhibited a 2.94-fold rise, the siMDM2/Control RNA group showed a 2.76-fold increase, and the sap53/siMDM2 group demonstrated a 3.25-fold increase ($p < 0.01$). The sap53/siMDM2 co-transfection group showed 1.11-fold and 1.18-fold elevations relative to the sap53/Control RNA and siMDM2/Control RNA groups, respectively (ns). In the HCT116 cell line, p21 transcript abundance increased by approximately 2-fold using the Control group as the reference, whereas the Mock condition did not differ from baseline. The sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups demonstrated increases of 4.16-, 4.78-, and 4.52-fold, respectively, when evaluated against the Control condition. Using the Control RNA/Control RNA group as reference, the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups exhibited increases of 4.19-, 4.81-, and 4.56-fold, respectively ($p < 0.01$). The sap53/siMDM2 co-transfection group did not display a statistically significant difference relative to the individual sap53/Control RNA and siMDM2/Control RNA groups (ns). For PUMA expression in the HCT116 cell line, transcript levels rose by 1.65-fold and 1.57-fold in the Mock and Control RNA/Control RNA groups, respectively, compared with the Control group. The sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups demonstrated increases of 4.97-, 5.3-, and 5.72-fold, respectively. PUMA transcript levels increased by 3.16-, 3.38-, and 3.64-fold in the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups relative to the Control RNA/Control RNA condition ($p < 0.01$). No meaningful change was detected in the sap53/siMDM2 co-transfection group when compared with the sap53/Control RNA and siMDM2/Control RNA single-transfection groups (ns). For MDM2 expression in the HCT116 cell line, the Mock and Control RNA/Control RNA groups showed a 1.95- and 1.83-fold increase, respectively, compared to the Control group. The sap53/Control RNA group showed a 2.07-fold increase, while the siMDM2/Control RNA group showed significant 0.031-fold decrease and the sap53/siMDM2 group showed a 1.74-fold increase. Compared to the Control RNA/Control RNA group, no significant change was observed in the sap53/Control RNA and

sap53/siMDM2 groups (ns) while siMDM2/Control RNA showed a significant decreases. The sap53/siMDM2 co-transfection group did not show a significant increase when evaluated against the sap53/Control RNA group, but it did show an increase when evaluated against the siMDM2/Control RNA group. The relative mRNA expression levels of target genes in A549 and HCT116 cell lines are shown in **Figure 3.3** and **Figure 3.4**.

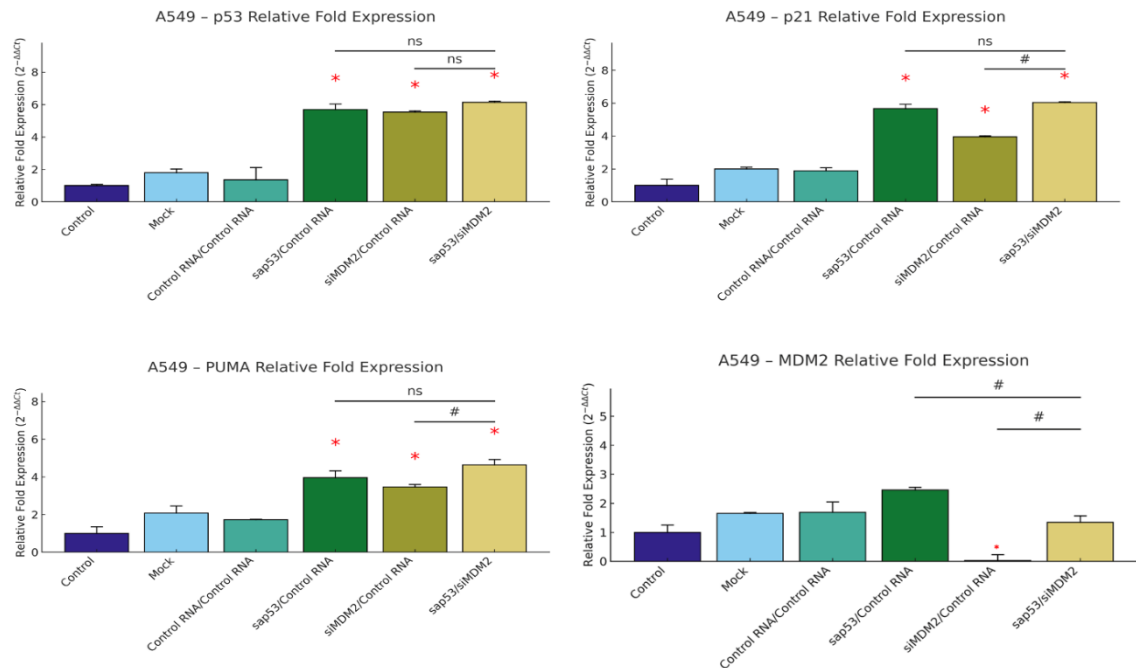


Figure 3.3 : mRNA expression levels of targeted genes in A549 cell line.

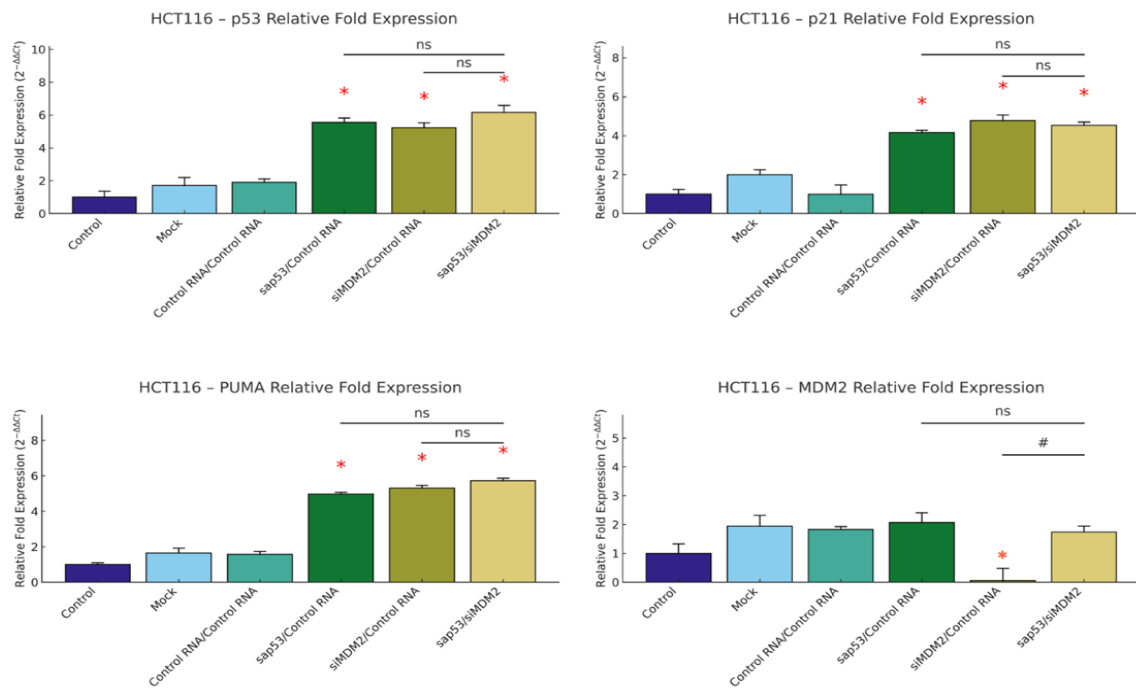


Figure 3.4 : mRNA expression levels of targeted genes in HCT116 cell line.

3.4 Protein Expression Analysis by Western Blot

Protein isolation was performed from each group of A549 and HCT116 cell lines. The effects on p53, MDM2, p21, and PUMA protein levels were evaluated. Protein band intensities were analyzed using GeneTools software (Syngene).

In the p53 western blot analysis performed on the A549 cell line, the Mock group showed a similar profile to the Control group, while the Control RNA/Control RNA group showed a slightly greater increase when evaluated against the Control group. The sap53/Control RNA group, siMDM2/Control RNA group, and sap53/siMDM2 group showed approximately 2.5-, 2-, and 3-fold increases, respectively. When contrasted with the Control RNA/Control RNA group, the p53 level in the sap53/Control RNA group increased by 2-fold ($p<0.05$), while the p53 level in the siMDM2/Control RNA group increased by approximately 1.5-fold ($p<0.05$). The sap53/siMDM2 group showed an approximately 2.3-fold increase in p53 levels ($p<0.01$). The sap53/siMDM2 co-transfection group showed a 1.2-fold ($p<0.05$) and 1.5-fold ($p<0.01$) increase in p53 levels when evaluated against the sap53/Control RNA and siMDM2/Control RNA single transfection groups, respectively. Thus, as expected, p53 levels increased with sap53 application, while p53 levels increased with siMDM2 application. The sap53/siMDM2 co-transfection group showed the strongest effect on p53 levels.

In the analysis of MDM2 protein levels in the A549 cell line, the Mock group exhibited a slight elevation relative to the Control condition, while the Control RNA/Control RNA group showed a similar mild increase. The sap53/Control RNA group demonstrated an approximately 1.9-fold rise in MDM2 protein levels when contrasted with the Control group, whereas the siMDM2/Control RNA group displayed a significant reduction ($p<0.05$). In the sap53/siMDM2 co-transfection group, MDM2 expression increased by approximately 1.2-fold. The sap53/Control RNA group exhibited a statistically significant 2.7-fold increase in MDM2 expression ($p<0.05$), while the siMDM2/Control RNA group showed a marked decrease ($p<0.05$). The sap53/siMDM2 group presented an approximately 1.6-fold increase. When the co-transfection group was evaluated relative to the sap53/Control RNA and siMDM2/Control RNA single-transfection groups, a significant decrease and increase in MDM2 expression, respectively, were observed ($p<0.05$).

Regarding p21 protein levels in the A549 cell line, the Mock group exhibited a slight reduction relative to the Control condition, whereas the Control RNA/Control RNA group showed an increase. The sap53/Control RNA group demonstrated an approximately 2.4-fold elevation in p21 protein levels. In the siMDM2/Control RNA group, p21 protein levels

increased by approximately 1.6-fold. In the sap53/siMDM2 co-transfection group, a strong rise of about 3-fold was detected. When assessed relative to the Control RNA/Control RNA condition, the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups exhibited approximately 2-fold ($p<0.05$), 1.3-fold ($p<0.05$), and 2.5-fold ($p<0.01$) increases, respectively. Comparisons between the co-transfection group and the single-transfection groups revealed a 1.25-fold (ns) and 1.9-fold ($p<0.05$) increase relative to the sap53/Control RNA and siMDM2/Control RNA groups, respectively, indicating that the combined treatment resulted in a more pronounced increase in p21 expression than the individual treatments.

In the analysis of PUMA protein levels in the A549 cell line, a slight decrease was observed in the Mock group when contrasted with the Control group, while the Control RNA/Control RNA group showed an approximately 1.3-fold increase. An approximately 2-fold elevation in PUMA levels was observed in the sap53/Control RNA group, while the siMDM2/Control RNA group demonstrated an approximately 1.5-fold increase. The sap53/siMDM2 group showed an approximately 2.05-fold increase. When evaluated against the Control RNA/Control RNA group, the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups showed an approximate increase in PUMA levels of 1.48-fold ($p<0.05$), 1.07-fold (ns), and 1.52-fold ($p<0.05$), respectively. The sap53/siMDM2 co-transfection group showed a small increase in protein levels compared to the sap53/Control RNA group (ns). When evaluated against the siMDM2/Control RNA group, it showed an approximately 1.41-fold increase ($p<0.05$).

In the western blot evaluation of p53 protein levels in the HCT116 cell line, the Mock group exhibited a 1.2-fold elevation relative to the Control condition, while the Control RNA/Control RNA group displayed a comparable increase. The sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups showed increases of approximately 2.16-, 2.08-, and 2.42-fold, respectively. When interpreted against the Control RNA/Control RNA reference, p53 levels in the sap53/Control RNA group rose by 2.06-fold ($p<0.05$), whereas the siMDM2/Control RNA group exhibited an increase of about 1.9-fold ($p<0.05$). The sap53/siMDM2 co-transfection group demonstrated an approximately 2.31-fold elevation ($p<0.05$). Relative to the sap53/Control RNA and siMDM2/Control RNA single-transfection groups, the co-transfection group showed 1.12-fold (ns) and 1.16-fold (ns) increases, respectively.

In the analysis of MDM2 protein levels in the HCT116 cell line, the Mock group and the Control RNA/Control RNA group showed increases of approximately 1.25- and 1.1-fold,

respectively, when assessed relative to the Control condition. The sap53/Control RNA group exhibited an approximately 2.43-fold elevation in MDM2 levels, whereas the siMDM2/Control RNA group showed a significant reduction. In the sap53/siMDM2 group, MDM2 expression increased by about 1.25-fold. The sap53/Control RNA group demonstrated a statistically significant 1.94-fold increase relative to the Control RNA/Control RNA reference group ($p<0.05$). The siMDM2/Control RNA group presented a significant decrease in MDM2 levels ($p<0.05$). The sap53/siMDM2 co-transfection group exhibited an increase of roughly 1-fold. Within the co-transfection group, MDM2 levels decreased by half and increased by half, respectively, in comparison with the sap53/Control RNA and siMDM2/Control RNA single-transfection groups ($p<0.05$).

In the analysis of p21 protein levels in the HCT116 cell line, a 1.19-fold increase was detected in the Mock group, while the Control RNA/Control RNA condition exhibited a 1.21-fold elevation relative to the baseline Control. The sap53/Control RNA group showed an approximately 2.47-fold rise in p21 expression. In the siMDM2/Control RNA group, p21 levels increased by about 1.88-fold. A pronounced elevation of approximately 2.78-fold was observed in the sap53/siMDM2 co-transfection group. When evaluated against the Control RNA/Control RNA reference, the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups showed increases of 2.04-fold ($p<0.05$), 1.55-fold ($p<0.05$), and 2.3-fold ($p<0.01$), respectively. The co-transfection group demonstrated 1.13-fold (ns) and 1.48-fold ($p<0.05$) higher p21 expression in comparison with the sap53/Control RNA and siMDM2/Control RNA single-transfection groups, indicating a stronger induction of p21 protein levels with the combined treatment.

In the analysis of PUMA protein levels in the HCT116 cell line, both the Mock group and the Control RNA/Control RNA group exhibited slight elevations relative to the Control condition. The sap53/Control RNA group displayed an approximately 2.2-fold increase in PUMA expression, whereas the siMDM2/Control RNA group showed an approximately 1.5-fold rise. The sap53/siMDM2 co-transfection group demonstrated an approximately 3.8-fold increase. When normalized to the Control RNA/Control RNA reference group, the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups exhibited increases of approximately 1.83-fold ($p<0.05$), 1.25-fold (ns), and 3.17-fold ($p<0.05$), respectively. In the comparison of single-transfection groups, the sap53/siMDM2 group showed about a 1.73-fold ($p<0.05$) increase in PUMA protein relative to the sap53/Control RNA group, and a 2.53-fold increase relative to the siMDM2/Control RNA group ($p<0.05$). Overall, the co-transfection approach produced a more substantial rise in PUMA expression

than either single application. **Figure 3.5** and **Figure 3.6** show protein band images and graphs of the data obtained from the groups in the A549 and HCT116 cell lines.

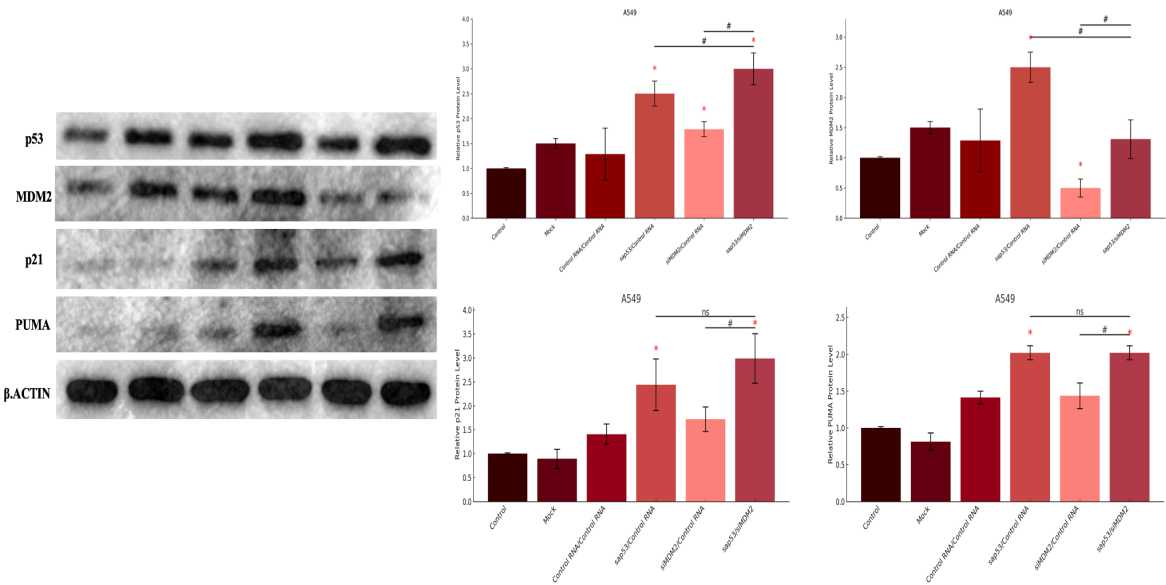


Figure 3.5 : Relative expression levels of targeted proteins in different treatment groups in the A549 cell line.

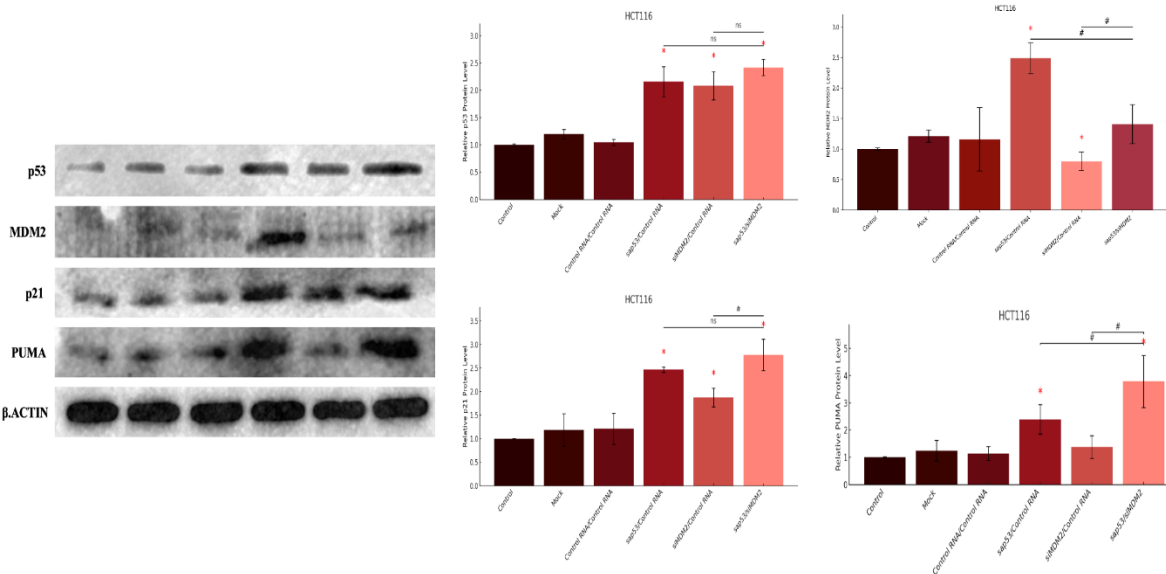


Figure 3.6 : Relative expression levels of targeted proteins in different treatment groups in the HCT116 cell line.

3.5 Cell Viability Test

Cell viability measurements were performed over five days using the resazurin assay to observe the effects of each cell group on cell proliferation in A549 and HCT116 cell lines. Absorbance values were recorded in the 570-600 nm wavelength range, and analyses were performed on three biological replicates.

In the A549 cell line, the Mock and Control RNA/Control RNA groups displayed comparable proliferation patterns, showing steady increases throughout the experiment. The sap53/Control RNA group exhibited reduced viability on days 3, 4, and 5, with values of 28%, 19.2%, and 33.2%, respectively. The siMDM2/Control RNA group likewise demonstrated lower viability, measured at 32.3%, 25.8%, and 31.9% on the corresponding days. The sap53/siMDM2 co-transfection group showed even greater reductions in cell proliferation, exhibiting decreases of 49.6%, 44.1%, and 50.3% on days 3, 4, and 5, respectively. Relative to the Control RNA/Control RNA group, both the sap53/Control RNA and siMDM2/Control RNA single-transfection groups demonstrated ~25% reductions in viability ($p < 0.05$). A pronounced decline of 47.5% ($p < 0.01$) was observed in the sap53/siMDM2 group when compared with the same reference group. The co-transfection condition also displayed significantly lower viability than either single-transfection group, showing an additional 28% reduction ($p < 0.01$). This indicates that the combined sap53 and siMDM2 treatment exerted a stronger inhibitory effect on cell growth than either component alone.

In the cell viability analysis performed on the HCT116 cell line, the Mock and Control RNA/Control RNA groups showed slight fluctuations compared to the Control group, but for the most part, they exhibited an increase in cell proliferation at a similar rate. sap53/Control RNA showed a decrease in cell viability of 33.96%, 35.73%, and 39.88% on days 3, 4, and 5, respectively, when evaluated against the Control group. The siMDM2/Control RNA group showed decreases in cell viability of 38.79%, 36.57%, and 44.93% on days 3, 4, and 5, respectively. The sap53/siMDM2 co-transfection group strongly reduced cell proliferation by 53.48%, 53%, and 53.83% on days 3, 4, and 5, respectively, compared to the Control group. When evaluated against the Control RNA group, the sap53/Control RNA group showed a 30.5% decrease ($p < 0.01$) from day 2 to day 5, while the siMDM2/Control RNA group showed a 35% decrease ($p < 0.05$) in cell proliferation. The sap53/siMDM2 co-transfection group showed the strongest suppression of cell proliferation at a rate of 48.5% ($p < 0.01$). The sap53/siMDM2 co-transfection group showed a 26.5% decrease in cell proliferation compared to the sap53/Control RNA group ($p < 0.01$), while the

siMDM2/Control RNA group showed a 20% decrease ($p<0.01$). The data graph showing the 5-day cell viability in different treatment groups of A549 and HCT116 cell lines is shown in **Figure 3.7** and **Figure 3.8**.

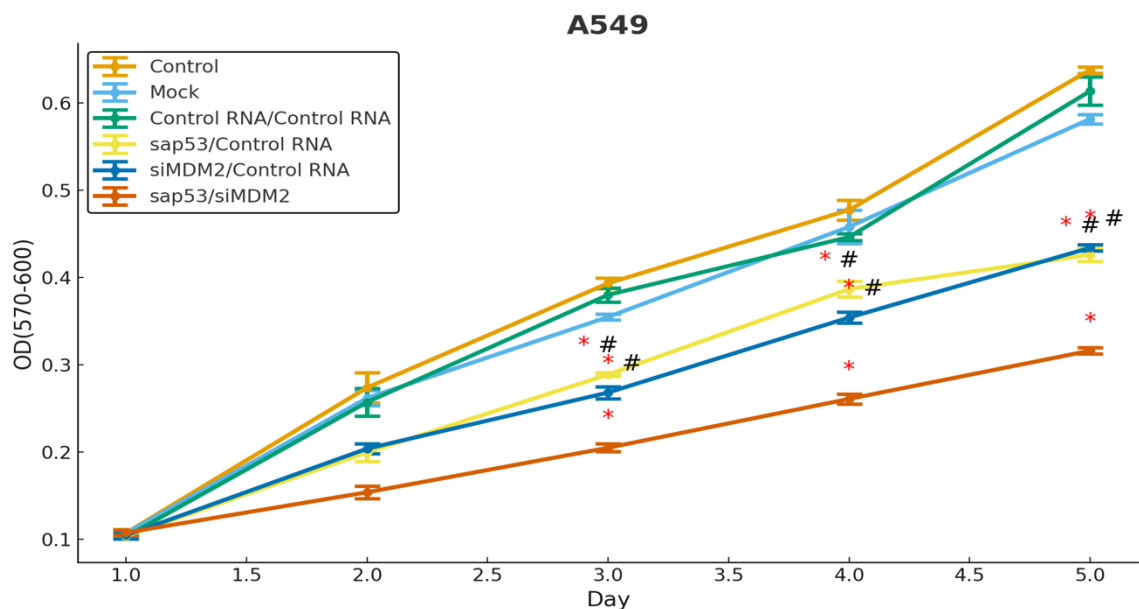


Figure 3.7 : Analysis of 5-day cell viability in different treatment groups of A549 cell line.

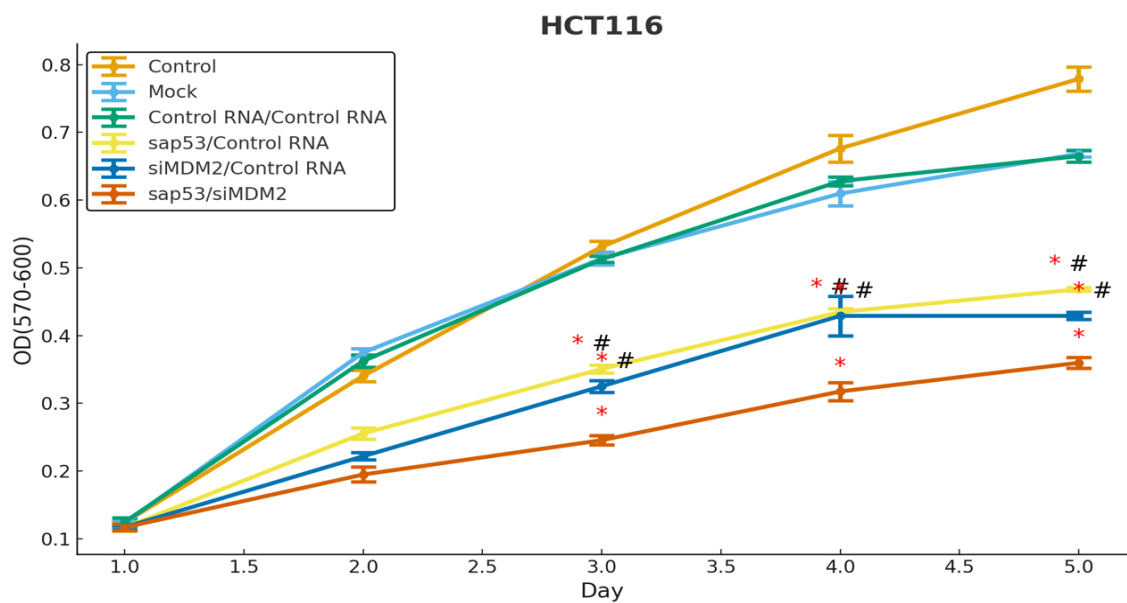


Figure 3.8 : Analysis of 5-day cell viability in different treatment groups of HCT116 cell line.

3.6 Cell Cycle Analysis

Cell cycle analysis was conducted to assess how cationic lipid–small RNA complexes influenced the distribution of A549 and HCT116 cells within the G0/G1, S, and G2/M phases. The proportion of cells in each phase was quantified for all experimental groups, and variations between the groups were evaluated.

In the A549 cell line, the G0/G1 phase ratios were determined to be 4.3%, 8.3%, 18.3%, 16.5%, and 23.9% for the Mock, Control RNA/Control RNA, sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups, respectively, relative to the Control group. Within the G0/G1 phase comparisons, the sap53/Control RNA group exhibited a 10% elevation, the siMDM2/Control RNA group an 8.2% increase, and the sap53/siMDM2 co-transfection group a 15.6% rise when contrasted with the Control RNA/Control RNA reference group ($p < 0.01$). The sap53/siMDM2 co-transfection group was also compared with the single sap53/Control RNA and siMDM2/Control RNA groups. For the G0/G1 phase, the sap53/siMDM2 group showed a 5.6% and 7.4% increase, respectively, compared to the sap53/Control RNA and siMDM2/Control RNA groups ($p < 0.01$). For the S phase, relative to the Control group, the Mock group experienced a 3.3% reduction, while the Control RNA/Control RNA group demonstrated a 3% decrease. The sap53/Control RNA and siMDM2/Control RNA groups showed decreases of 2.8% and 1.7%, respectively. The sap53/siMDM2 co-transfection group exhibited the most prominent suppression with an approximately 14.9% decline. When contrasted with the Control RNA/Control RNA group, the sap53/Control RNA group showed no notable variation, the siMDM2/Control RNA group displayed a 1.3% increase, and the sap53/siMDM2 group showed an 11.8% reduction ($p < 0.01$). The co-transfection group showed a 12–13% decrease relative to both single-transfection groups ($p < 0.01$). In the G2/M phase, no significant alteration was observed in the Mock group relative to the Control condition. The Control RNA/Control RNA, sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups showed reductions of 5.4%, 16%, 14.5%, and 8.8%, respectively. When compared to the Control RNA/Control RNA group, the sap53/Control RNA group displayed a 10% decline, and the siMDM2/Control RNA group showed a 9.9% reduction ($p < 0.01$). The sap53/siMDM2 co-transfection group showed a 3.5% decrease ($p < 0.05$). Relative to the single-transfection groups, the sap53/siMDM2 group exhibited increases of 7.3% and 5%, respectively. The analyses revealed that the sap53/Control RNA group and the sap53/siMDM2 co-transfection

group arrested the cell cycle in the G0/G1 phase for the A549 cell line and also caused significant decreases in the S and G2/M phases.

In HCT116 cells, the proportions in the G0/G1 phase were 4.1%, 10.3%, 16.3%, 15.0%, and 24.8% for the Mock, Control RNA/Control RNA, sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups, respectively, when contrasted with the Control group. Among these, the sap53/Control RNA group showed a 6.4% increase, the siMDM2/Control RNA group showed a 5% increase, and the sap53/siMDM2 co-transfection group showed a 13.7% increase compared to the Control RNA/Control RNA group ($p < 0.01$). The sap53/siMDM2 co-transfection group also demonstrated a notably stronger enrichment in G0/G1 relative to both single-transfection groups, at 8.3% and 9.8%, respectively ($p < 0.01$). In the S phase analysis, Mock, Control RNA/Control RNA, sap53/Control RNA, and siMDM2/Control RNA groups showed decreases when contrasted with the Control group, but for the S phase, the sap53/siMDM2 co-transfection group achieved the most significant decrease at a rate of 14.6% when evaluated against the Control group. In the S phase analysis, no significant change was observed in the sap53/Control RNA and siMDM2/Control RNA groups compared to the Control RNA/Control RNA group. However, the sap53/siMDM2 co-transfection group for the S phase showed a significant decrease of 12.1% when contrasted with the Control RNA/Control RNA group ($p < 0.01$). In the S phase analysis, the sap53/siMDM2 co-transfection group showed a significant decrease of 12.8% and 13.8%, respectively, compared to the sap53/Control RNA and siMDM2/Control RNA single transfection groups ($p < 0.01$). Thus, it was observed that the co-transfection group significantly suppressed the S phase, during which DNA is synthesized. In the G2/M phase, the Mock group showed a 3.3% decrease compared to the Control group, while the Control RNA/Control RNA group showed an 8.7% decrease. The sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups showed decreases of 14.8%, 14.3%, and 10.6%, respectively. In the G2/M phase analysis, comparisons with the Control RNA/Control RNA group showed decreases of 6.7% and 5.7% in the sap53/Control RNA and siMDM2/Control RNA groups, respectively ($p < 0.01$). The sap53/siMDM2 co-transfection group showed no significant change. The sap53/siMDM2 co-transfection group showed increases of 4.7% and 3%, respectively, compared to the sap53/Control RNA group and the siMDM2/Control RNA group. In this case, the co-transfection group partially alleviated the suppression in the G2/M phase. Cell cycle analysis results for the A549 and HCT116 cell lines are shown in **Figure 3.9** and **Figure 3.10**, respectively.

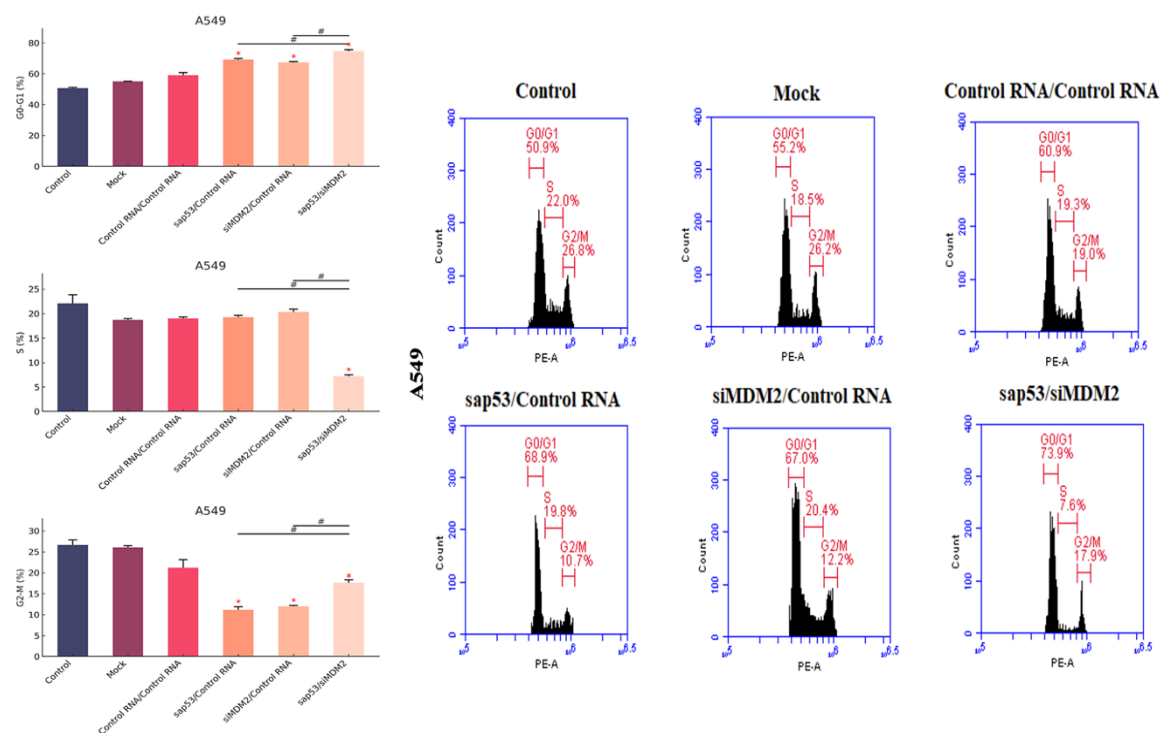


Figure 3.9 : Results of cell cycle analysis in the A549 cell line.

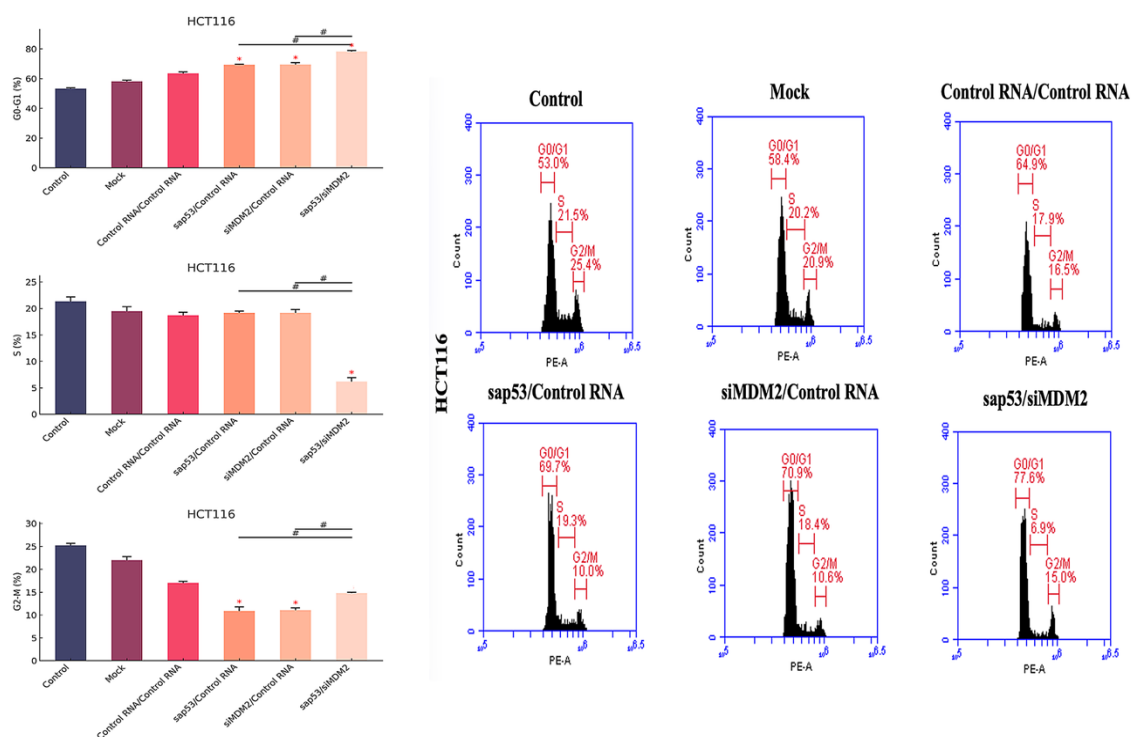


Figure 3.10 : Results of cell cycle analysis in the HCT116 cell line.

3.7 Apoptosis Analysis

Annexin V-FITC/PI flow cytometry analyses were conducted to evaluate the effects of cationic lipid–small RNA complexes on apoptotic cell death in A549 and HCT116 cell lines. Data acquisition and analysis were carried out using the BD Accuri™ C6 Plus flow cytometer.

For the A549 cell line, the proportion of apoptotic cells was 3.57% in the Control group and 6.20% in the Mock group. In the Control RNA/Control RNA group, apoptosis was measured at 5.93%. The percentages of apoptotic cells in the sap53/Control RNA, siMDM2/Control RNA and sap53/siMDM2 groups were 26.27%, 31.80% and 45.97%, respectively. Taking the Control RNA/Control RNA group as the reference, apoptosis levels were 20.34% higher in the sap53/Control RNA group and 25.87% higher in the siMDM2/Control RNA group ($p < 0.01$). In the sap53/siMDM2 co-transfection group, the apoptotic rate exceeded that of the Control RNA/Control RNA group by 40.04% ($p < 0.001$). When the co-transfection group was evaluated relative to the single-transfection conditions, apoptosis increased by 19.7% ($p < 0.05$) compared with the sap53/Control RNA group and by 14.17% ($p < 0.01$) when contrasted with the siMDM2/Control RNA group. These findings indicate that the combined sap53 and siMDM2 treatment markedly potentiated the apoptotic response in A549 cells.

In apoptosis analyses performed on the HCT116 cell line, a 3.2% apoptosis rate was detected in the Control group. In the Mock and Control RNA/Control RNA groups, apoptosis rates of 5.33% and 4.67%, respectively, were detected. The apoptosis rate was 27.93% in the sap53/Control RNA group, while it was 34.4% in the siMDM2/Control RNA group. The apoptosis rate was 47.9% in the sap53/siMDM2 group. The apoptosis rate in the sap53/Control RNA group was found to be 23.26% higher than in the Control RNA/Control RNA group ($p < 0.01$). In the siMDM2/Control RNA group, the increase in apoptosis rate was found to be 29.73% compared to the Control RNA/Control RNA group ($p < 0.01$). In the sap53/siMDM2 co-transfection group, the apoptosis rate was found to be 43.23% higher ($p < 0.001$). The increase in apoptosis rate in the sap53/siMDM2 co-transfection group was 19.97% ($p < 0.05$) and 13.50% ($p < 0.01$) compared to the sap53/Control RNA and siMDM2/Control RNA single transfection groups, respectively. Thus, it was determined that the single groups also increased apoptosis, but the co-transfection group made this increase even more pronounced. The results of apoptosis analyses performed on different groups of A549 and HCT116 cell lines are shown in **Figure 3.11** and **Figure 3.12**.

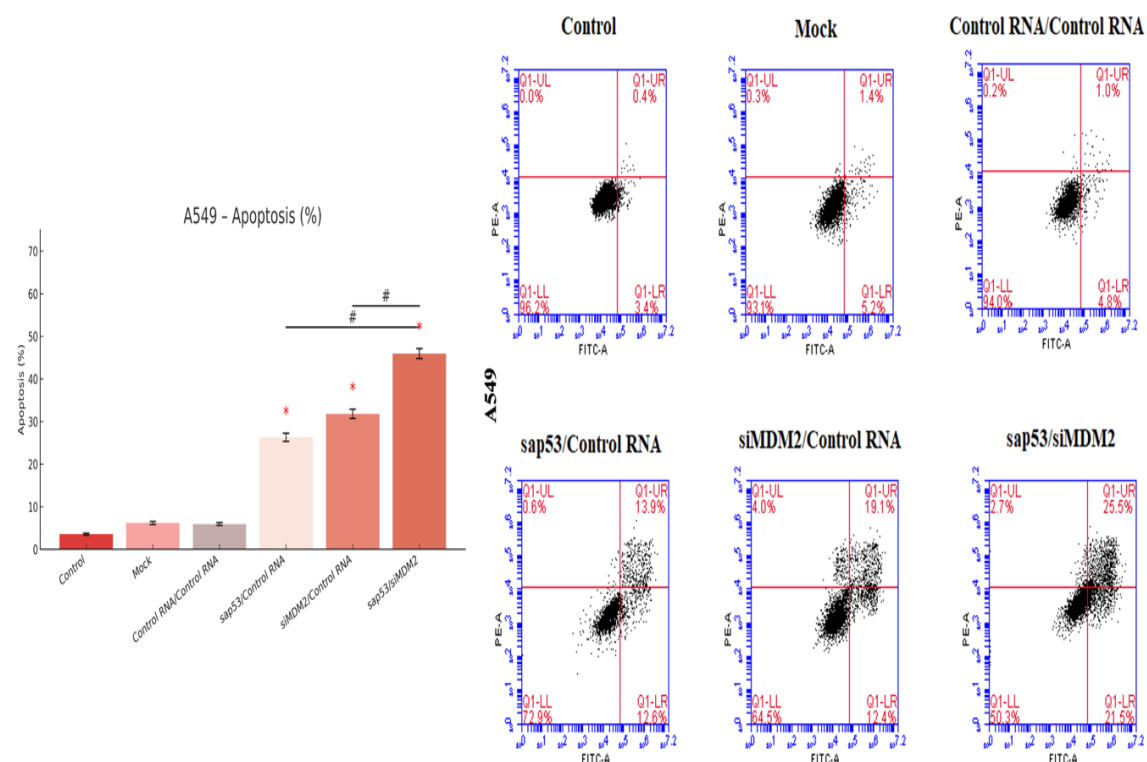


Figure 3.11 : Results of apoptosis analysis in the A549 cell line.

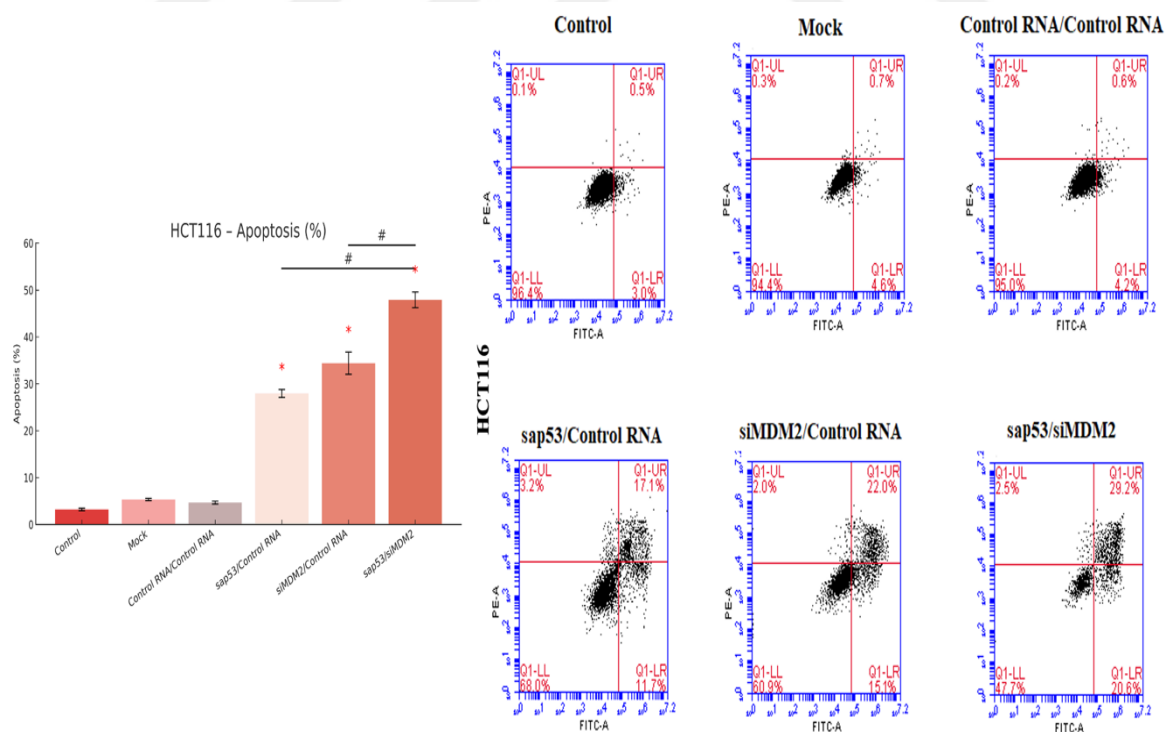


Figure 3.12 : Results of apoptosis analysis in the HCT116 cell line.

3.8 Colony Formation Assay

A colony formation assay was performed on A549 and HCT116 cell lines, and the effects of cationic lipid-small RNA complexes on the colony-forming capacity of the cells were evaluated.

All groups for the A549 cell line were when evaluated against the Control group. While the colony formation rate in the Mock group decreased by 21.7% compared to the Control group, the colony formation rate in the Control RNA/ Control RNA group increased by 1.5%. The colony formation rate in the sap53/Control RNA group decreased by 45% compared to the Control group, while colony formation in the siMDM2/Control RNA group decreased by 43%. In the sap53/siMDM2 group, colony formation decreased by 68%. Cationic lipid–small RNA groups were also compared with the cationic lipid–human nonhomology RNA sequence group, the Control RNA/Control RNA group. The colony formation rate of the sap53/Control RNA group showed a 45.8% decrease compared to the Control RNA/Control RNA group ($p<0.05$). The colony formation rate of the siMDM2/Control RNA group showed a 43.7% decrease compared to the Control RNA/Control RNA group ($p<0.01$). The colony formation rate of the sap53/siMDM2 group was found to be 68.6% lower than that of the Control RNA/Control RNA group ($p<0.001$). The sap53/siMDM2 group, co-transfected with sap53 and siMDM2, was also compared with the sap53/Control RNA and siMDM2/Control RNA single transfection groups. The sap53/siMDM2 group showed a 42% decrease in colony formation compared to the sap53/Control RNA group and a 44% decrease compared to the siMDM2/Control RNA group ($p<0.05$).

All groups for the HCT116 cell line were when contrasted with the Control group. Compared to the Control group, the colony formation rate decreased by 28.6% in the Mock group, while the colony formation rate decreased by 15.6% in the Control RNA/Control RNA group. While this colony formation rate decreased by 55.2% in the sap53/Control RNA group, it decreased by 42.8% in the siMDM2/Control RNA group. In the sap53/siMDM2 group, this colony formation rate decreased by 55.8%. Cationic lipid–small RNA groups were also compared with the cationic lipid–human nonhomology RNA sequence group, the Control RNA/Control RNA group. The colony formation rate of the sap53/Control RNA group showed a 46.9% decrease compared to the Control RNA/Control RNA group ($p<0.001$). The colony formation rate of the siMDM2/Control RNA group showed a 32.3% decrease compared to the Control RNA/Control RNA group ($p<0.01$). The colony formation rate of the sap53/siMDM2 group was found to be 47.7% lower than that of the Control

RNA/Control RNA group ($p<0.001$). The sap53/siMDM2 group, co-transfected with sap53 and siMDM2, was also compared with the sap53/Control RNA and siMDM2/Control RNA single transfection groups. The sap53/siMDM2 group showed a 1.5% decrease in colony formation compared to the sap53/Control RNA group. When contrasted with the siMDM2/Control RNA group, it showed a 22.7% decrease ($p<0.05$). In both A549 and HCT116 cells, MDM2 silencing and p53 activation significantly reduced colony formation in single groups.

The sap53/siMDM2 co-transfection group increased the decrease in colony formation rate to an even higher level. Colony formation assay results for the A549 and HCT116 cell lines are shown in Figure 3.13 and Figure 3.14, respectively.

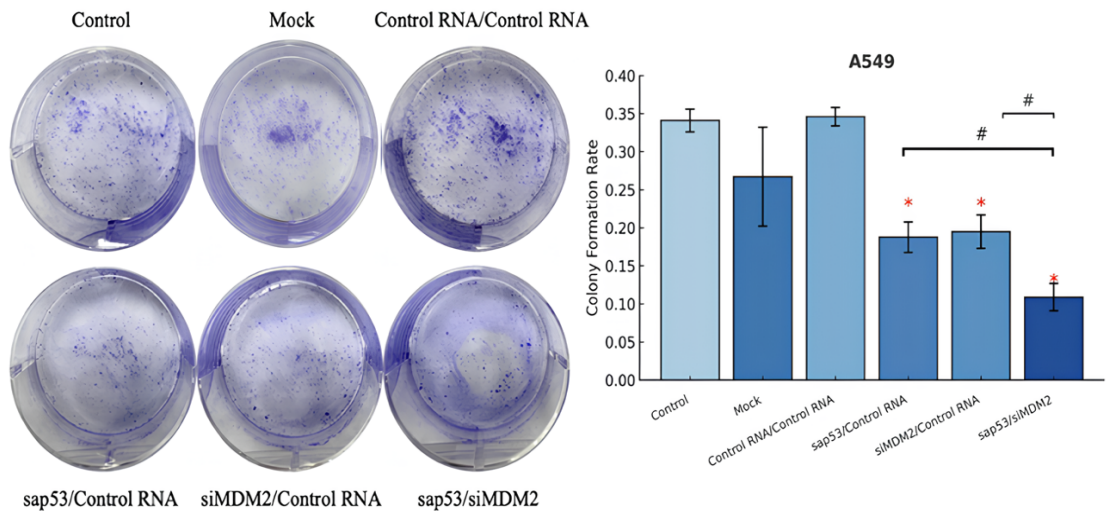


Figure 3.13 : Images and analysis of colony formation assay for the A549 cell line.

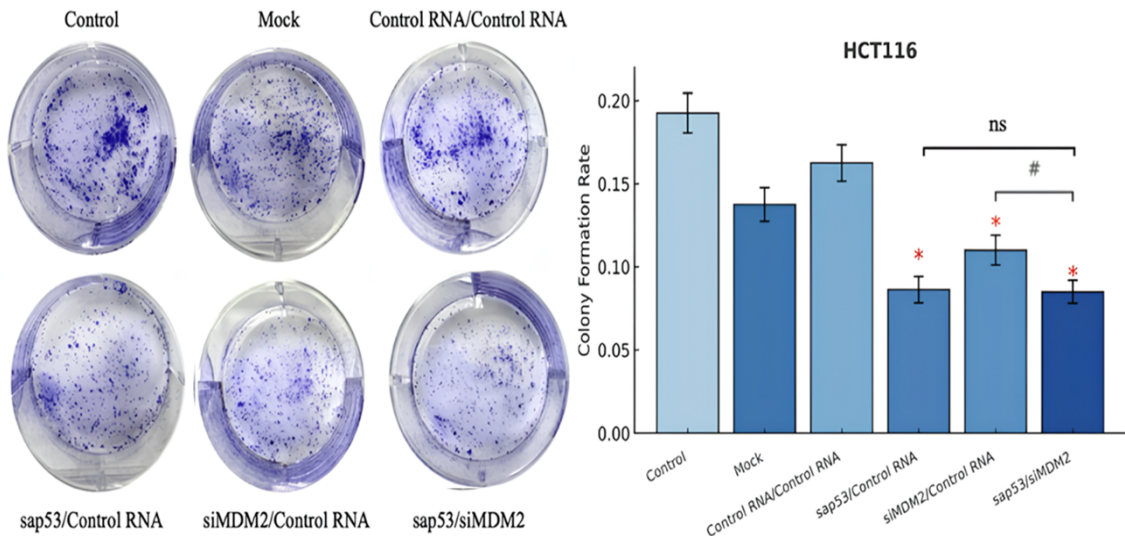


Figure 3.14 : Images and analysis of colony formation assay for the HCT116 cell line.

3.9 Migration and Invasion Assay

Migration and invasion analyses were performed using the Boyden chamber assay to evaluate the migration and barrier crossing abilities of cells in different groups of A549 and HCT116 cells. Cells transfected with cationic lipid-small RNA complexes were incubated for 24 hours, after which migratory and invasive cells in each group were counted, and analyses were performed using the data obtained.

Migration and invasion analyses were carried out in the A549 cell line. In the migration assay, both the Mock group and the Control RNA/Control RNA group displayed higher migratory activity relative to the Control condition. In contrast, cells transfected with sap53/Control RNA exhibited a 17.6% reduction in migration. A more pronounced decrease of 37.2% was observed in the siMDM2/Control RNA group. The dual-transfection group (sap53/siMDM2) demonstrated the strongest inhibition, with migration declining by 83.1%. When the results were evaluated using the Control RNA/Control RNA group as the reference, the sap53/Control RNA group showed a 27.1% decrease in migration ($p<0.05$), whereas siMDM2/Control RNA led to a 44.4% reduction ($p<0.01$). The combined sap53/siMDM2 treatment further enhanced this suppressive effect, reducing migration by 85% ($p<0.001$). Moreover, the co-transfection group migrated 79.4% ($p<0.01$) and 73% ($p<0.01$) less than the sap53/Control RNA and siMDM2/Control RNA single-transfection groups, respectively, indicating that the dual strategy exerted a stronger inhibitory influence on cell motility than either single application. Thus, while the single transfection groups also suppressed migration, the co-transfection group showed a stronger migration-suppressing effect. In invasion analyses performed using the A549 cell line, the Mock group again showed higher invasive capacity compared with the Control group. Cells compared with Control RNA/Control RNA showed an invasion pattern similar to the control condition. The sap53/control RNA group showed an invasion rate of 55.5%, while the siMDM2/control RNA group showed an invasion rate of 49.9%. In the sap53/siMDM2 co-transfection group, invasion decreased to 29.6%. Relative to the sap53/Control RNA and siMDM2/Control RNA groups, the co-transfection condition showed 46.6% ($p<0.05$) and 40.6% ($p<0.05$) lower invasion, respectively. Although each single-transfection treatment suppressed invasion to some extent, the combined sap53/siMDM2 approach exhibited a more substantial inhibitory effect.

Migration and invasion analyses were performed on the HCT116 cell line. In the migration analysis, the Mock and Control RNA/Control RNA groups showed similar migration levels compared to the Control group. A 38.4% decrease in migration was

observed in the sap53/Control RNA group. A similar decrease of 34.4% was observed in the siMDM2/Control RNA group. The co-transfection of sap53 and siMDM2 caused a more substantial reduction, lowering migration by 53.2%. When the sap53/Control RNA, siMDM2/Control RNA, and sap53/siMDM2 groups were compared with the Control RNA/Control RNA reference, migration declined by 35.9%, 31.8% ($p<0.05$), and 51.3% ($p<0.01$), respectively. The combined treatment was additionally compared with each single-transfection condition. The sap53/siMDM2 group showed 24.8% lower migration than the sap53/Control RNA group ($p<0.05$) and 28.4% lower migration than the siMDM2/Control RNA group ($p<0.01$). Although both single-transfection strategies effectively inhibited migration, the dual application further amplified this suppressive effect. Thus, although single groups showed a significant suppression of migration, the sap53/siMDM2 co-transfection group was found to inhibit cell migration more strongly. In the invasion assay performed in HCT116 cells, the Mock group exhibited lower invasive capacity than the Control group. Cells treated with Control RNA/Control RNA displayed an invasion pattern that closely resembled the Control condition. The sap53/Control RNA treatment reduced invasion by 61.3% compared with the Control group, while siMDM2/Control RNA decreased invasion by 83.8%. The sap53/siMDM2 co-transfection resulted in the strongest inhibitory outcome, reducing invasion by 89.1%. In comparisons with the Control RNA/Control RNA group, the sap53/Control RNA group inhibited invasion by 60.2% ($p<0.05$), while the siMDM2/Control RNA group suppressed invasion by 81% ($p<0.01$). The sap53/siMDM2 co-transfection group showed an 88.8% reduction in invasion ($p<0.01$). The invasion inhibition rates of the sap53/siMDM2 combination group compared to the sap53/Control RNA and siMDM2/Control RNA single transfection groups were 27.8% ($p<0.05$) and 32.7% ($p<0.01$), respectively. The visualizations and analyses of the migration and invasion experiments performed on A549 and HCT116 cell lines are shown in **Figure 3.15** and **Figure 3.16**.

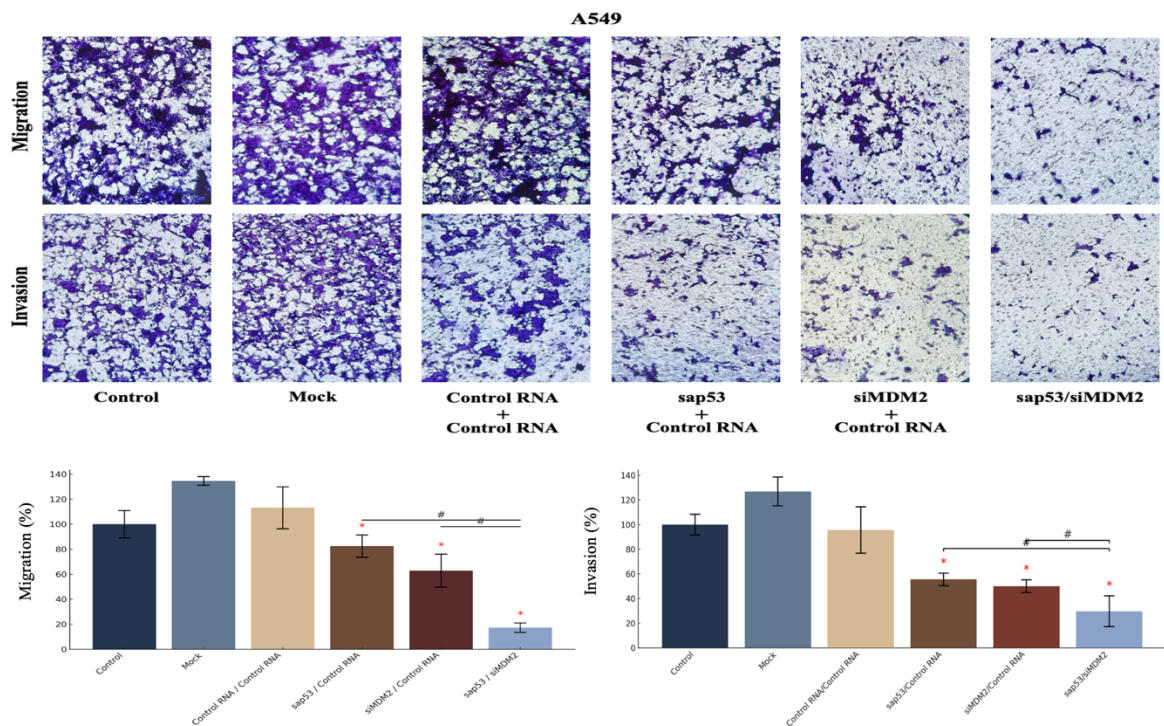


Figure 3.15 : Migration and invasion results of A549 cell line groups.

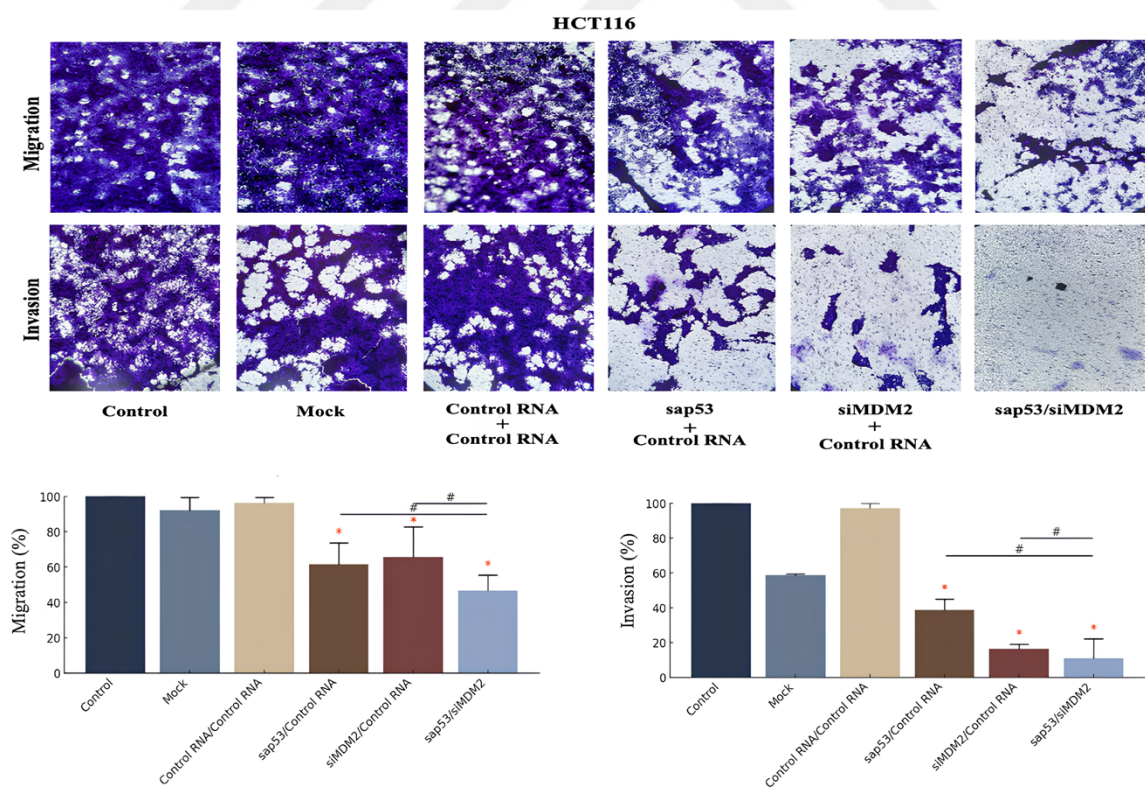


Figure 3.16 : Migration and invasion results of HCT116 cell line groups.

4. DISCUSSION

The tumor suppressor gene p53 plays a central role in maintaining cellular integrity and preserving genomic stability and is therefore widely recognized as the “guardian of the genome.” Through its involvement in diverse molecular pathways, p53 regulates numerous cellular processes and contributes to the pathogenesis of a broad range of diseases, including autoimmune, metabolic, cardiovascular disorders, and particularly cancer (Kung & Weber, 2022). Notably, p53 is among the most frequently altered genes in human malignancies, underscoring its critical importance in tumor suppression (Dolgin, 2017). Upon activation, p53 induces a variety of antitumor responses, including apoptosis, cell cycle arrest, and cellular senescence, thereby limiting malignant progression (Kasthuber & Lowe, 2017).

Under physiological conditions, intracellular p53 levels are tightly controlled by the E3 ubiquitin ligase activity of MDM2, which promotes p53 degradation through a well-established negative feedback loop (Lindström et al., 2022). In response to cellular stress, this balance is transiently disrupted, allowing p53 accumulation and activation. However, in many cancer cells, dysregulation of the p53–MDM2 axis—most commonly through MDM2 overexpression or amplification—leads to persistent suppression of p53 activity, enabling tumor cells to evade growth control and apoptotic signaling (Clegg et al., 2008).

In this study, a dual-target RNA-based strategy was employed in A549 and HCT116 cancer cell lines harboring wild-type p53 to investigate how simultaneous transcriptional activation of p53 and suppression of its negative regulator MDM2 influence cellular responses. The results demonstrate that both sap53-saRNA-mediated activation of p53 transcription and siMDM2-mediated inhibition of MDM2 independently enhance p53 signaling and modulate downstream gene expression. These findings are consistent with previous studies emphasizing the pivotal role of the p53–MDM2 axis in coordinating cellular stress responses and tumor suppressive mechanisms (Ozaki & Nakagawara, 2011; Vousden & Lu, 2002).

Activation of p53 through sap53-saRNA resulted in increased p53 expression at both the mRNA and protein levels, in agreement with reports showing that small activating RNAs targeting promoter regions can efficiently induce gene transcription (Ge et al., 2016; Wang et al., 2016). In parallel, siMDM2 treatment reduced MDM2 expression, thereby stabilizing p53 by limiting its ubiquitin-mediated degradation, a mechanism well documented in the

literature (Haupt et al., 1997; Kubbutat et al., 1997; Michael & Oren, 2002). When both RNA molecules were delivered together, p53 levels generally exceeded those observed following single treatments. However, the magnitude of p53 accumulation was not always proportionally greater, suggesting the presence of intrinsic regulatory constraints within the p53 signaling network. In particular, p53-driven transcriptional induction of MDM2 likely acts as a buffering mechanism that limits excessive p53 accumulation during combined treatment (Prives & Hall, 1999).

The functional consequences of p53 pathway modulation were reflected in the expression patterns of key downstream targets, including p21 and PUMA. Upregulation of p21 indicates effective engagement of p53-dependent cell cycle regulatory mechanisms, whereas increased PUMA expression supports activation of intrinsic mitochondrial apoptotic pathways (Vousden & Lu, 2002; Vaseva & Moll, 2009). These molecular changes suggest that concurrent modulation of p53 and MDM2 influences not only p53 stabilization but also the transcriptional programs governing cell cycle control and apoptosis.

To place these findings in context, previous studies have largely relied on single-target strategies to modulate the p53–MDM2 axis. Investigations using p53-targeting saRNAs have demonstrated increased p53 and p21 expression accompanied by G0/G1 cell cycle arrest and reduced cellular proliferation (Ge et al., 2016; Wang et al., 2016). Moreover, Wang et al. reported that p53 activation suppresses cancer cell migration and invasion, highlighting the role of p53 in regulating metastatic potential (Wang et al., 2016). In contrast, a study targeting MDM2 with siRNA reported approximately 44.53% inhibition of cell proliferation and an increase in apoptotic cell populations to approximately 30.45%, although the analysis was limited to phenotypic outcomes without direct molecular validation of p53 pathway components (Chen et al., 2012).

In the present study, sap53 and siMDM2 were evaluated both individually and in combination using molecular and functional assays in A549 and HCT116 cell lines. qPCR analyses revealed robust induction of p53 and p21 expression in both models, while western blot analyses confirmed corresponding increases at the protein level, with the most pronounced elevations observed in the combined treatment groups. Functionally, the sap53–siMDM2 combination produced the strongest antitumor phenotype, characterized by enhanced apoptotic responses, accumulation of cells in the G0/G1 phase, and marked suppression of cellular migration and invasion. These effects consistently exceeded those observed with single-agent treatments, indicating that simultaneous transcriptional

activation of p53 and inhibition of its negative regulator amplifies p53-dependent cellular outcomes.

Although both A549 and HCT116 cell lines exhibited similar overall response patterns, differences in the magnitude of molecular and functional changes were observed. These variations likely reflect cell-type-specific regulatory contexts, baseline stress response capacities, and intrinsic proliferation characteristics of the two cancer models. Such differences underscore the importance of cellular context in shaping p53 signaling outcomes and highlight the necessity of evaluating p53-based therapeutic strategies across multiple tumor types.

A key strength of this study lies in the co-delivery of sap53 and siMDM2 within the same cationic lipid carrier, representing a coordinated and innovative approach for modulating the p53–MDM2 pathway. Lipid-based nanocarriers are widely recognized for enhancing RNA stability, cellular uptake, and intracellular bioavailability (Giri et al., 2023; Jung et al., 2022). By enabling synchronized intracellular delivery of both RNA molecules, this system allows transcriptional activation of p53 and suppression of its negative regulator to occur concurrently rather than sequentially, thereby maximizing pathway activation and downstream biological effects.

Taken together, the findings of this study demonstrate that increasing p53 expression while concurrently limiting its degradation results in a more robust activation of p53-dependent pathways. The dual-target RNA-based strategy effectively enhances apoptotic responses, induces cell cycle arrest, and suppresses cellular migration and invasion in cancer cells retaining wild-type p53. These results provide strong foundational evidence that combined RNA-based modulation of transcriptional and post-translational regulatory layers may overcome limitations associated with single-target approaches. Although further *in vivo* studies are required to evaluate therapeutic efficacy, safety, and delivery performance, the present findings highlight the conceptual and mechanistic promise of dual RNA-based strategies targeting the p53–MDM2 axis.

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PROJECTS:

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CONFERENCE PAPERS:

- **Dündar, M., Menevşe, İ. N., Çam Derin, D., Gültekin, E., & Koç, A.** (2024). *Combined delivery of p53 activating and KRAS inhibitory small RNA significantly inhibited cell proliferation and motility in human lung cancer cells.* 9th International Congress of Molecular Biology Association of Turkey, İzmir, Türkiye, September 12-14, p. 83.
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PUBLICATIONS:

- **Menevşe, İ. N., Taskın, I. I., Sancar, P. Y., & Kürşat, M. (2024).** Investigation the Cytotoxic and Antimicrobial Effect of Ranunculus Poluninii. *Bitlis Eren Üniversitesi Fen Bilimleri Dergisi*, 13(2), 393-399.
- **Gürünlüoğlu, K., Satılmış, B., Gül, M., Dünder, M., Göktürk, N., Akbulut, S., ... & Demircan, M. (2024).** The impact of subdermal adipose derived stem cell injections and early excision on systemic oxidative stress and wound healing in rats with severe scald burns. *Burns*, 50(8), 2056-2069.
- **Uğur, Y., Menevşe, İ. N., Dünder, M., Karci, H., Zengin, R., & Güzel, A. (2025).** Comparative chemical and biological evaluation of Urtica dioica extracts obtained by methanol and hexane: antioxidant, cytotoxic, apoptotic, and antimicrobial potentials. *BMC Complementary Medicine and Therapies*, 10.1186/s12906-025-05211-3. Advance online publication.
- **Dünder, M., Çam Derin, D., Menevşe, İ. N., Gültekin, E., & Koç, A. (2025).** Targeted TP53 and KRAS modulation via AuNP-mediated RNA delivery suppresses cancer progression. *Current Gene Therapy*, DOI: 10.2174/0115665232425141251028050552 (Accepted).