Molecular Targeting of CELL DEATH PATHWAYS IN CANCER THERAPY

Edited by
Prof. Dr. Ali Bilgili

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www.bidgeyayinlari.com.tr - bidgeyayinlari@gmail.com
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CHAPTER 1

Targeting Pyroptosis in Hepatocellular Carcinoma: From Molecular Mechanisms to Therapeutic Opportunities

Feray KÖÇKAR[1]
Nelin HACIOĞLU[2]
Esra TOKAY[3]

INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive malignancy with high mortality that develops against a background of chronic liver disease, cirrhosis, and persistent inflammation and is among the leading causes of cancer-related deaths worldwide. The molecular pathogenesis of HCC is determined not only by genetic alterations but also by complex inflammatory and immunoregulatory processes that occur in the tumor microenvironment (TME). For many years,

[1]Dr, Balıkesir University, Molecular Biology and Genetics, Orcid: 0000-0003-2572-8391
[2]Dr, Balıkesir University, Molecular Biology and Genetics, Orcid: 0000-0001-7884-7971
[3]Dr, Balıkesir University, Molecular Biology and Genetics, Orcid: 0000-0001-9993-2753

the basic approach in HCC treatment has been the induction of apoptosis in tumor cells; however, chemotherapy resistance and functional disorders in apoptosis pathways, frequently observed in clinical practice, have revealed the limited effectiveness of this strategy (Ferlay et al., 2014).

In this context, pyroptosis, which is directly related to innate immune responses, has become an increasingly important focus in hepatocellular carcinoma (HCC) biology. Pyroptosis is a highly inflammatory and immunogenic form of programmed cell death, characterized by inflammasome activation and gasdermin-mediated membrane pore formation (Wang et al.,2023). Despite the immunologically silent nature of apoptosis, the release of potent pro-inflammatory cytokines, such as IL-1β and IL-18, occurs during pyroptosis, which has the potential to shape immune cell infiltration and antitumor immune responses in the HCC tumor microenvironment (Vande Walle and Lamkanfi, 2016).

Recent studies have shown that inducing pyroptosis in HCC cells not only directly triggers tumor cell death but also contributes to the reprogramming of the immunosuppressive tumor microenvironment. In particular, the expression and activation levels of gasdermin family proteins are key drivers of pyroptosis in HCC, making these proteins potential therapeutic targets (Aglietti et al., 2017). Furthermore, it has been reported that targeted therapies and chemotherapeutic agents can simultaneously activate gasdermin-dependent pyroptosis along with apoptosis under certain conditions, opening the way for new combination strategies in treatment-resistant HCC cases (Liu et al.,2016).

This section aims to comprehensively address the molecular mechanisms of pyroptosis, its bidirectional (tumor-suppressive and tumor-promoting) roles in the HCC tumor microenvironment, and the potential of therapeutically targeting pyroptosis in terms of immunotherapy and combination therapy approaches.

2. Molecular mechanism of pyroptosis

2.1. Canonical inflammationosome-mediated pyroptosis in hepatocellular carcinoma

Canonical pyroptosis is a form of programmed cell death initiated by various inflammasome complexes and is characterized by the release of pro-inflammatory cytokines upon gasdermin-D (GSDMD) cleavage (Xia et al., 2019). Hepatocellular carcinoma (HCC) is characterized by chronic liver damage, persistent inflammation, and tissue regeneration, which offer a highly favorable biological environment for inflammasome activation. In this context, canonical pyroptosis plays a critical role in regulating immune responses and disease progression within the HCC tumor microenvironment.

The activation of canonical inflammasomes begins with the recognition of numerous intracellular and extracellular stimuli. Inflammasome sensors, also known as pattern recognition receptors (PAMPs), detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The detection of signals such as bacteria, viruses, cytosolic DNA, oxidative stress products, and cellular degradation components causes inflammasome sensors to activates downstream signaling pathways. This process results in the formation of multiprotein inflammasome complexes by inflammasome components, together with pro-caspase-1 and the adapter protein ASC (CARD-containing apoptosis-associated speck-like protein) (Hou et al., 2021).

In HCC, the NLRP3 inflammasome is particularly prominent and can be activated in hepatocytes and tumor-associated immune cells by numerous stimuli, such as bacterial products, viral infections, mitochondrial dysfunction, and reactive oxygen species. In contrast, the AIM2 inflammasome functions as a key sensor that responds to cytosolic double-stranded DNA in HCC cells, where genomic instability is significant. Following the formation of the

inflammasome complex, pro-caspase-1 is converted to active caspase-1, which is a central event in canonical pyroptosis. Activated caspase-1 simultaneously initiates two fundamental biological processes. First, it cleaves GSDMD, the driver of pyroptosis, exposing its N-terminus fragment. This N-terminal fragment disrupts cellular ion balance by creating pores in the plasma membrane of hepatocytes and HCC cells, leading to cell swelling and loss of membrane integrity. Second, caspase-1 converts inactive pro-IL-1β and pro-IL-18 into their biologically active forms. The release of these cytokines outside the cell through the resulting membrane pores leads to a strong inflammatory response in the HCC tumor microenvironment (Frank et al., 2019; Maltez et al.,2015).

High levels of IL-1β and IL-18 release resulting from canonical inflammasome-mediated pyroptosis can contribute to accelerated adaptive immune responses and increased immune cell infiltration. However, if this process becomes uncontrolled or chronic, the persistence of the inflammatory microenvironment can become a factor support HCC progression. Therefore, canonical pyroptosis should be considered a bidirectional biological process in HCC that can produce both tumor-suppressive and tumor-promoting outcomes depending on the context (Man et al., 2017; Strowig et al., 2012).

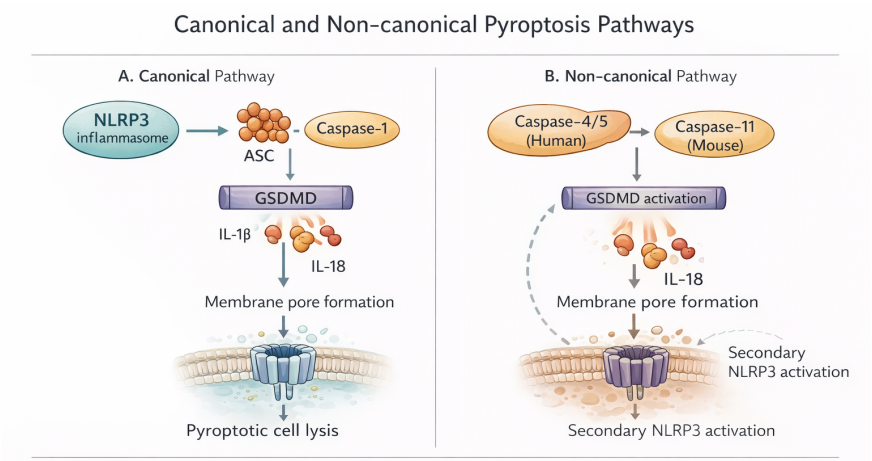

Figure 1: Schematic representation of canonical (caspase-1–dependent) and non-canonical (caspase-4/5/11–dependent) pyroptotic pathways leading to gasdermin D cleavage, membrane pore formation, inflammatory cytokine release (IL-1β and IL-18), and pyroptotic cell lysis.

2.2. Non-canonical inflammationosome-mediated pyroptosis in hepatocellular carcinoma

Unlike canonical inflammasome-mediated pyroptosis, the non-canonical pyroptosis pathway is not caspase-1 dependent and is mediated by caspase-4 and caspase-5 in humans (caspase-11 in mice). This pathway has unique biological significance in tumors associated with chronic inflammation, such as HCC, because it can be activated without the direct involvement of classical inflammasome sensors (NLRP3, AIM2, etc.) (Kayagaki et al., 2011; Ding and Shao, 2017).

The primary trigger for non-canonical pyroptosis is lipopolysaccharides (LPS) from gram-negative bacteria that reach the cell cytosol. Caspase-4/5/11 can directly recognize cytosolic LPS via the CARD domain at the N-terminus, leading to caspase cleavage and activation. This activation pattern does not require inflammasome sensors and directly initiates effector responses. Continuous exposure of the liver to microbial products via the gut-liver axis creates a suitable microenvironment for the activation of this pathway during HCC development. Caspase-4/5/11, activated in the cytosol, cleaves gasdermin-D (GSDMD), the pyroptotic executive protein, releasing the pore-forming N-terminus fragment. Oligomerized GSDMD-N fragments are transported to the cell membrane, leading to the formation of large transmembrane pores. This situation results in the disruption of ion balance, cell swelling, and pyroptotic cell death. In the context of HCC, this process can

directly lead to cell death in tumor cells and amplify inflammatory signals in the tumor microenvironment (Rühl and Broz, 2015).

The source of LPS in the cytosol is mostly gram-negative bacteria, and these molecules can be transported into the cell during infection or via bacterial outer membrane vesicles. Furthermore, it has been shown that the highly mobile group box-1 (HMGB1) protein, passively released from stressed or dying cells, triggers non-canonical pyroptosis by transporting extracellular LPS into the cytosol. This mechanism is particularly important in the HCC microenvironment, where chronic inflammation and tissue damage are prevalent (Kayagaki et al., 2015).

Notably, in the non-canonical pyroptotic pathway, caspase-4/5/11 does not directly induce maturation of IL-1β and IL-18. Instead, membrane pores and potassium ion ejection resulting from GSDMD cleavage stimulate secondary activation of the NLRP3 inflammasome. This activates caspase-1, leading to the maturation of IL-1β and IL-18. This demonstrates that the non-canonical pathway is not merely an independent cell death mechanism, but also operates in functional crosstalk with canonical inflammasome pathways (Kim et al., 2018; Rühl and Broz, 2015).

In conclusion, non-canonical inflammasome-mediated pyroptosis should be considered a regulatory mechanism in HCC that reinforces cellular responses to bacteria-derived inflammatory signals and canonical inflammasome activation. This bidirectional interaction is one of the key reasons why pyroptosis exerts context-dependent biological effects in the HCC tumor microenvironment.

3. Pyroptosis in Liver Physiology and Pathology

Owing to its inflammatory nature, pyroptosis is closely related to physiological and pathological processes in immunologically highly

active organs, such as the liver. The liver is central to the innate immune response, in addition to its metabolic functions, leading to pyroptosis playing a dual role in maintaining tissue homeostasis and pathological in chronic inflammation and disease development (Racanelli & Rehermann, 2006; Shi et al., 2017).

3.1 Pyroptosis in Normal Liver Homeostasis

Pyroptosis is an inflammatory, regulated cell death mechanism that plays a fundamental and indispensable role in the host development, survival, and defense against infections (Shi et al., 2017). In a healthy liver, pyroptosis functions as a protective mechanism that supports tissue repair and limits the spread of pathogens by ensuring the timely elimination of infected or damaged cells.

Hepatocytes, the most abundant cellular component of the liver, express inflammasome components at low basal levels under normal physiological conditions and maintain continuous molecular communication with nonparenchymal cells. This interaction is critical for maintaining hepatic homeostasis. Healthy hepatocytes actively contribute to the liver's immune surveillance process by expressing inflammasome sensors such as NLRP3, AIM2, and NLRC4 at baseline levels to detect cellular stress and potential danger signals (Wree et al., 2014; Zeng et al., 2020).

Kupffer cells, the resident macrophages of the liver, constitute the dominant macrophage population under healthy conditions and are the primary source of inflammasome activation and pro-inflammatory cytokines (IL-1β and IL-18). These cells regulate intercellular communication and coordinate the liver's innate immune response by continuously sensing both pathogen-derived molecular patterns (PAMPs) and damage-derived molecular patterns (DAMPs) through pattern recognition receptors (PRRs) (Gautier et al., 2012; Xu et al., 2021).

In this context, the balance of liver inflammation is based on the "double-edged sword" nature of pyroptosis. Moderate inflammasome activation and a controlled pyroptotic response within physiological limits preserve tissue integrity and promote wound healing. Disruption of this delicate balance and excessive or uncontrolled activation of pyroptosis leads to widespread cell death, severe tissue damage, fibrosis, and ultimately organ failure, causing physiological processes to evolve into pathological states (Zeng et al., 2020).

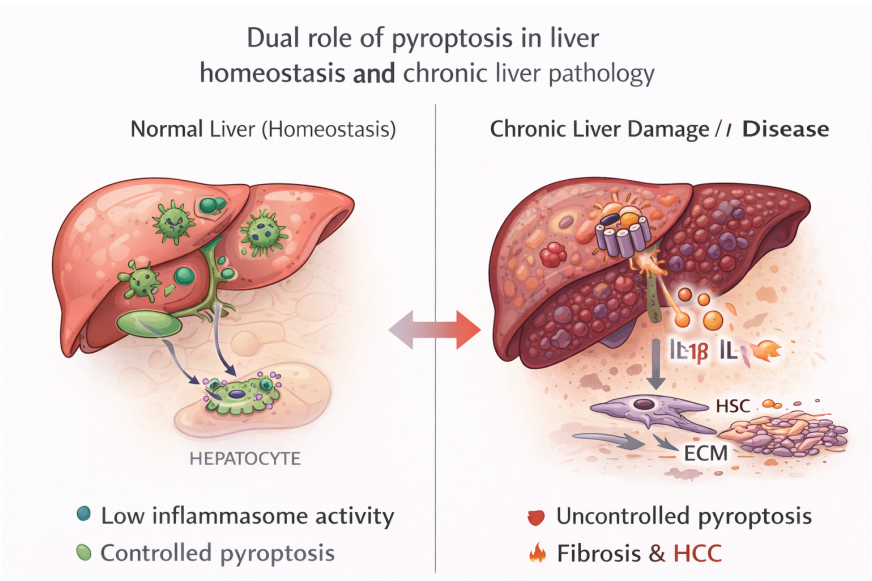

Figure 2: Dual role of pyroptosis in maintaining liver homeostasis and promoting fibrosis and hepatocellular carcinoma under chronic inflammatory conditions.

3.2 Pyroptosis in Chronic Liver Diseases

Chronic liver diseases are among the leading causes of morbidity and mortality worldwide, with inflammatory cell death mechanisms playing a central role in their pathogenesis. Pyroptosis, a regulated

form of cell death characterized by cell lysis and the release of potent pro-inflammatory cytokines (IL-1β and IL-18), is a critical regulator of the initiation and maintenance of the inflammatory cascade in response to chronic liver damage (Shi et al., 2017; Zeng et al., 2020). Excessive and prolonged pyroptotic activation in the liver contributes to the development of non-alcoholic fatty liver disease (NAFLD), alcoholic hepatitis, viral hepatitis, liver fibrosis, and ultimately hepatocellular carcinoma (HCC).

3.2.1 NAFLD and NASH

In non-alcoholic fatty liver disease (NAFLD), pyroptosis plays a central role in the progression from simple steatosis (NAFL) to non-alcoholic steatohepatitis (NASH), a more advanced and inflammatory phenotype. In NAFLD, increased free fatty acid levels and lipotoxicity lead to ceramide accumulation in hepatocytes, resulting in mitochondrial stress and increased reactive oxygen species (ROS) levels, which triggers the activation of the NLRP3 inflammasome. NLRP3-mediated caspase-1 activation leads to the production of IL-1β and IL-18, proteolytic cleavage of gasdermin D, and initiation of pyroptotic cell death (Wree et al., 2014).

In the NASH stage, pyroptosis is not limited to hepatocyte death but also exacerbates liver inflammation by increasing inflammatory cell infiltration and cytokine production. During this process, it has been shown that the expression of the GSDMD-N fragment, the executive form of pyroptosis, is significantly increased, and this increase is directly related to the degree of steatosis and the severity of lobular inflammation (Xu et al., 2021). Therefore, pyroptosis is considered a triggering and maintenance mechanism in the progression from NAFLD to NASH.

3.2.2 Alcoholic Hepatitis

In alcoholic hepatitis, pyroptosis is a key determinant of inflammation and hepatocyte damage resulting from excessive

alcohol consumption. Increased acetaldehyde production during ethanol metabolism results in mitochondrial dysfunction and accumulation of reactive oxygen species (ROS), triggering a significant oxidative stress response in hepatocytes. Thioredoxin-interacting protein (TXNIP), a major mediator of oxidative stress, is overexpressed under these conditions and directly stimulates the activation of the NLRP3 inflammasome. NLRP3-mediated caspase-1 activation leads to increased IL-1β and IL-18 production and the initiation of pyroptotic cell death through the cleavage of gasdermin D (GSDMD) protein. This process accelerates hepatocyte loss and exacerbates liver inflammation and tissue damage (Zhou et al. 2019).

The role of pyroptosis in the pathogenesis of alcoholic hepatitis is not limited to the canonical inflammatory pathways. Impairment of the intestinal barrier and increased circulating lipopolysaccharide (LPS) levels due to chronic alcohol consumption lead to the activation of the caspase-11 (caspase-4/5 in humans)-mediated non-canonical pyroptosis pathway. In this pathway, cytosolic LPS directly activates caspase-11/4, initiating GSDMD cleavage and membrane pore formation in the host cell. In experimental alcoholic hepatitis models, non-canonical pyroptosis plays a central role in maintaining hepatocyte death, cytokine release, and inflammatory cell infiltration (Yang et al., 2022).

Taken together, these findings suggest that pyroptosis is a bidirectional mechanism in alcoholic hepatitis, triggering the early inflammatory response and sustaining disease progression.

3.2.3 Viral Hepatitis

In Hepatitis B (HBV) and Hepatitis C (HCV) infections, pyroptosis plays a crucial role in both antiviral defense and disease progression as a vital component of the host immune response. Viral nucleic acids and structural proteins are detected in hepatocytes and resident immune cells of the liver via pattern recognition receptors (PRRs),

triggering the activation of inflammasomes, particularly through TLRs, RIG-I-like receptors, and cytosolic DNA/RNA sensors. This process activates various inflammatory complexes, primarily the NLRP3 and AIM2 inflammasomes, and initiates caspase-1-mediated pyroptosis.

During pyroptosis, the release of IL-1β and IL-18 is increased. While these cytokines enhance the antiviral immune response, uncontrolled activation can exacerbate liver inflammation. Another notable mechanism, particularly in chronic HBV and HCV infections, is that pyroptosis is not limited to infected hepatocytes but also occurs in neighboring "bystander" hepatocytes. This is associated with the spread of inflammatory signals and damage-related molecular patterns, leading to cell death even in cells that are not directly infected, thus expanding tissue damage (Guo et al., 2020).

In conclusion, while pyroptosis is an important part of the antiviral defense in viral hepatitis, it is considered a bidirectional mechanism that, in cases of chronic and excessive activation, perpetuates liver inflammation, leads to fibrosis, and, in the long term, predisposes to the development of hepatocellular carcinoma.

3.2.4 Liver Fibrosis

Pyroptosis is a critical process that supports the activation of hepatic stellate cells (HSCs), which are key cellular determinants of liver fibrosis, through direct and indirect mechanisms. Damage-related molecular patterns (DAMPs), inflammasome components, and pro-inflammatory cytokines (especially IL-1β) released from pyroptotic hepatocytes during liver damage are detected by HSCs, accelerating their differentiation from a quiescent phenotype to collagen-producing myofibroblasts. This indirect effect is considered a significant trigger for initiating the fibrotic response (Wree et al., 2014).

In addition, recent studies have shown that pyroptosis is not limited to hepatocyte-derived signals in fibrosis, but that inflammasome activation also occurs within HSCs themselves. Activation of the NLRP3 inflammasome in HSCs stimulates caspase-1-mediated signaling pathways, increasing TGF-β production and leading to excessive accumulation of extracellular matrix (ECM) components, particularly type I collagen (Zeng et al. 2020). Furthermore, the inflammatory microenvironment associated with pyroptosis strengthens the macrophage-HSC interaction, contributing to the maintenance of the fibrotic process.

Taken together, these findings indicate that pyroptosis is not merely a secondary consequence of liver fibrosis but an active mechanism that initiates and propagates the fibrotic response. Therefore, targeting pyroptosis and its inflammasome components offers a noteworthy approach for potential therapeutic strategies to limit fibrosis progression.

3.2.5 Hepatocellular Carcinoma

The role of pyroptosis in cancer is often described as a "double-edged sword," a dual nature that is particularly evident in hepatocellular carcinoma (HCC). On the one hand, pyroptosis can suppress tumor growth by inducing tumor cell death through inflammasome activation and gasdermin-mediated membrane pore formation. Conversely, chronic and uncontrolled pyroptosis can contribute to carcinogenesis by supporting the tumor microenvironment through prolonged inflammation, cytokine release, and immune cell reprogramming (Xu et al., 2021).

Analyses of HCC tissues revealed decreased caspase-1 expression and inflammasome activity, leading to the functional suppression of pyroptosis. This is considered an adaptation mechanism that allows HCC cells to evade inflammatory cell death and remain hidden from immune surveillance (Chu et al., 2016). Furthermore, it has been

reported that the expression levels of gasdermin family members (particularly GSDMD and GSDME) may be inversely related to HCC progression.

However, the reactivation of pyroptosis is a remarkable therapeutic approach for HCC treatment. Preclinical studies have shown that berberine, curcumin, and certain chemotherapeutic agents suppress the proliferation of HCC cells and enhance antitumor immunity by inducing pyroptosis (Chu et al., 2016; Xia et al., 2019). It has also been suggested that pyroptosis induction may increase sensitivity to immunotherapies by making the tumor microenvironment more immunogenic.

In light of these data, pyroptosis is considered not only a cell death mechanism in HCC but also a dynamic regulator shaping tumor biology and treatment response. A better understanding of the context-specific effects of pyroptosis will pave the way for the development of safe and effective pyroptosis-based treatment strategies for HCC.

4. Dysregulation of Pyroptosis Pathways in Hepatocellular Carcinoma

4.1 Changes in Expression Levels of Inflammasome Components in HCC

Inflammasomes are multiprotein complexes that are key regulators of the innate immune response and play a critical role in initiating pyroptosis. Transcriptomic and proteomic studies have revealed significant dysregulation in the expression of inflammasome sensors, particularly those belonging to the NLR family (NLRP3, NLRP6, and AIM2) in hepatocellular carcinoma (HCC) (Li et al., 2022; Gao et al., 2023).

The NLRP3 inflammasome exhibits bidirectional functions in HCC. Controlled NLRP3 activation promotes the maturation of IL-1β and IL-18 via caspase-1, supporting the antitumor immune response, while sustained NLRP3 activation under conditions of chronic liver inflammation can create an immunosuppressive tumor microenvironment, promoting tumor progression (Hu et al., 2021). In contrast, NLRP6 expression is frequently decreased in HCC tissues, and loss of NLRP6-mediated inflammasome activity is associated with increased tumor proliferation and poor prognosis (Gao et al., 2023).

Suppression of the AIM2 inflammasome also contributes to the disruption of pyroptotic signaling in HCC. Decreased AIM2 expression facilitates immune evasion by limiting caspase-1 activation and associated cytokine release, particularly supporting tumor cell survival in viral hepatitis-induced HCC (Li et al. 2022). These findings suggest that an imbalance between inflammasome components is a fundamental mechanism underlying the dysregulation of pyroptosis pathways in HCC.

4.1.1 Dysregulation of GSDMD and GSDME Expression in HCC

Gasdermin proteins are the executors of pyroptosis, with Gasdermin D (GSDMD) and Gasdermin E (GSDME) being the best-characterized members in cancer. In HCC, the expression and function of these two proteins differ significantly from those in other solid tumors (De Schutter et al., 2021).

GSDMD is cleaved by caspase-1 or non-canonical inflammatory caspases, triggering pore formation in the cell membrane. However, in HCC, despite the detection of inflammasome activation, decreased levels of active GSDMD-N fragments have been observed. This indicates a functional disconnect between

inflammasome sensing and the execution of pyroptosis, leading to the inhibition of effective inflammatory cell death (Hu et al. 2021).

In contrast, GSDME exhibits a remarkable paradox in HCC. GSDME, which is silenced in most solid tumors due to promoter hypermethylation, is overexpressed at both the mRNA and protein levels in HCC (Hu et al., 2021; Kahaer et al., 2025). High GSDME expression is associated with advanced tumor grade and poor overall survival, suggesting that GSDME may play a tumor-promoting role specific to HCC (De Schutter et al., 2021).

Mechanistic studies have revealed that GSDME frequently acts through pyroptosis-independent oncogenic pathways in HCC. Single-cell RNA sequencing analyses have shown that GSDME is highly expressed in tumor-associated macrophages and increases orientation towards the M2 phenotype by activating the PI3K–AKT signaling pathway. This situation contributes to the suppression of $CD8^+$ T cell function and the development of resistance to immunotherapy (Chen et al., 2024; Kahaer et al., 2025). However, it has been shown that the classical pyroptotic function of GSDME can be reactivated under appropriate pharmacological stimulation.

4.1.2 Caspase Dysregulation and the Execution of Pyroptosis in HCC

Caspases act as key molecular switches that determine whether a cell progresses towards apoptosis or pyroptosis. In HCC, the dysregulated expression of both inflammatory and apoptotic caspases significantly affects the efficiency of pyroptotic signaling (Shi et al., 2017). Caspase-1 activation has been reported to be suppressed in advanced HCC, which limits GSDMD cleavage and IL-1β/IL-18 release (Hu et al., 2021). Similarly, the heterogeneous expression of non-canonical inflammatory caspases contributes to insufficient pyroptosis induction (Li et al., 2022). Caspase-3 plays a critical role in HCC pyroptosis. Caspase-3, known as the main driver

of apoptosis, can reverse cell death during inflammatory pyroptosis by cleaving GSDME (Wang et al., 2017). However, in HCC, basal caspase-3 activity is often insufficient to initiate pyroptosis, despite high GSDME expression (Chen et al., 2023). In contrast, agents such as oxaliplatin, miltiron, and SIRT1 inhibitors can re-trigger GSDME-dependent pyroptosis by increasing caspase-3 activation and enhancing $CD8^+$ T cell infiltration (Zhang et al., 2020; Deng et al., 2025; Liu et al., 2024).

4.2. The Dual Role of Pyroptosis in Hepatocellular Carcinoma

Pyroptosis is a form of programmed cell death with strong inflammatory properties, characterized by inflammasome activation and gasdermin-mediated pore formation in the cell membrane. In the context of HCC, pyroptosis is a dual biological process that can exhibit both tumor-suppressive and tumor-progressive effects depending on the binding, cell type, and microenvironmental conditions (Wang et al., 2022; Tao & Yang, 2025).

4.2.1 Tumor Suppressive Roles of Pyroptosis

The tumor-suppressive effects of pyroptosis primarily occur through the direct induction of tumor cell death and enhancement of the antitumor immune response. The cleavage of GSDMD or GSDME by caspase-1 or caspase-3 as a result of inflammasome activation can lead to rapid and irreversible cell death in hepatocellular carcinoma cells. This process reduces the tumor cell burden, particularly in early-stage HCC (Wang et al., 2022). Pro-inflammatory cytokines, such as IL-1β and IL-18, released during pyroptosis, can generate an effective antitumor immune response by increasing dendritic cell activation and $CD8^+$ cytotoxic T lymphocyte infiltration in the tumor microenvironment. Some pyroptosis-associated molecular subtypes described by Wang et al. have been characterized by an “immune-warm” phenotype, and better immune activation and treatment

response potential have been observed in these subgroups (Wang et al., 2022).

Furthermore, gene signatures involving non-apoptotic cell death mechanisms have been shown to be associated with better overall survival in low-risk HCC subgroups. The maintenance of pyroptosis-associated gene expression has been linked to lower cancer stem cell characteristics and a more favorable immune microenvironment (Zhang et al., 2022). These findings support the notion that controlled pyroptosis activation may function as a tumor-suppressive mechanism in HCC.

4.2.2 The Tumor-Enhancing Roles of Pyroptosis

The aspects of pyroptosis that support tumor progression primarily arise through chronic inflammation, immune evasion, and formation of an immunosuppressive tumor microenvironment. In particular, prolonged and uncontrolled pyroptosis activation can lead to persistent inflammation, fibrosis, and ultimately, the creation of a fertile ground for tumor development in the liver tissue (Wang et al., 2022). Single-cell and spatial transcriptomic analyses have shown that pyroptotic activity is not homogeneous in the HCC tumor microenvironment but rather plays a tumor-promoting role in specific cell populations. Tao and Yang reported increased monocyte and macrophage infiltration in tumor regions with high pyroptosis scores, and these cells predominantly exhibited an immunosuppressive phenotype (Tao & Yang, 2025). This suggests that pyroptosis may serve to evade immunity rather than activating the immune system.

In particular, high expression of GSDME and other pyroptosis-related genes has been associated with poor prognosis in HCC. Wang et al. suggested that pyroptosis-associated “immune-warm” phenotypes paradoxically exhibit worse survival, which may be due to the suppression of effective antitumor responses despite high

immune cell infiltration (Wang et al., 2022). In this context, pyroptosis can contribute to immunotherapy resistance by increasing the expression of immune checkpoints, such as PD-L1 and CTLA-4 (Tao & Yang, 2025).

Additionally, pyroptosis-associated gene signatures are associated with increased TP53 mutation rates, high tumor stem cell counts, and poor TACE response in high-risk HCC patients. These data suggest that pyroptosis may play a role in promoting tumor aggressiveness and treatment resistance in advanced HCC (Zhang et al. 2022).

4.3. Interaction Between Pyroptosis and Tumor Microenvironment

Pyroptosis is a highly pro-inflammatory form of programmed cell death, characterized by the activation of inflammatory caspases (caspase-1, -4, -5, and caspase-3) and pore formation in the cell membrane via gasdermin proteins (especially GSDMD and GSDME) (Chen et al., 2016; Kovacs & Miao, 2017). Recent studies have shown that pyroptosis not only triggers tumor cell death but also directly shapes the immunological and inflammatory properties of the tumor microenvironment (TME) (Zheng et al., 2024).

4.3.1 Cytokine Release and Inflammatory Microenvironment

During piroptosis, potent pro-inflammatory cytokines, such as IL-1β and IL-18, are rapidly released owing to inflammasome activation. These cytokines increase inflammatory signaling in the TME, initiating both immune cell recruitment and generating proliferative signals in stromal and tumor cells (Wang et al., 2022). While acute and controlled piroptosis activation can enhance antitumor immunity by supporting dendritic cell maturation and $CD8^+$ T cell activation, chronic and sustained cytokine release contributes to the formation of a chronic inflammatory microenvironment that supports tumor development in HCC (Tao & Yang, 2025).

4.3.2 Immune Cell Infiltration

Pyroptosis-associated cytokines and danger signals (DAMPs) increase the infiltration of $CD8^+$ cytotoxic T cells, NK cells, monocytes, and macrophages into the tumor tissue. An "immune-warm" TME profile has been identified in HCC subtypes with high pyroptosis-associated gene signatures (Wang et al., 2022; Zhang et al., 2022). However, the functional nature of this infiltration remains unclear. Tao and Yang (2025) reported increased macrophage and monocyte infiltration in HCC samples with high pyroptosis scores, but these cells were mostly immunosuppressive. This suggests that pyroptosis can create a context-dependent tumor-suppressive or tumor-promoting microenvironment in the TME.

4.3.3 Relationship with Immune Checkpoint Molecules

One of the most critical effects of pyroptosis in the TME is the increased expression of immune checkpoint molecules. Inflammatory cytokines and interferon signaling can induce the expression of immunosuppressive molecules, such as PD-1, PD-L1, and CTLA-4, in tumor cells and tumor-associated immune cells (Zheng et al., 2024; Tao & Yang, 2025). Wang et al. (2022) showed that checkpoint genes were highly expressed in pyroptosis-associated immune-warm HCC subtypes, which may paradoxically be associated with a worse prognosis. These findings suggest that although pyroptosis initially increases immune activation, it may trigger adaptive immune evasion mechanisms.

4.4. Pyroptosis-Related lncRNAs in Hepatocellular Carcinoma

Long non-coding RNAs (lncRNAs) are RNA molecules that play a critical role in the regulation of gene expression at the transcriptional, post-transcriptional, and epigenetic levels of gene expression. In recent years, an increasing number of studies have shown that lncRNAs play important roles not only in tumor cell proliferation and metastasis but also in the regulation of programmed

cell death. In this context, lncRNAs associated with pyroptosis, a form of inflammatory cell death, have become a remarkable area of research in the molecular pathogenesis of hepatocellular carcinoma (Zhang et al., 2022; Tan and Yu, 2024).

Recent studies based on transcriptomic analyses have identified numerous lncRNAs that strongly correlate with the expression profiles of pyroptosis-related genes in HCC. Zhang et al. (2022) developed an lncRNA signature that can separate HCC patients into different prognostic groups using lncRNAs expressed in conjunction with pyroptosis-related genes. This signature was significantly associated with overall patient survival and immune characteristics of the tumor microenvironment. Similarly, Tan and Yu (2024) defined a prognostic index based on pyroptosis-related lncRNAs and demonstrated that it could be an independent predictor of clinical outcomes in HCC. These studies suggest that pyroptosis-related lncRNAs may not only be biomarkers of HCC but also functional molecules that reflect the biological behavior of the disease.

One of the most striking aspects of pyroptosis-related lncRNAs is their close relationship with the tumor microenvironment (TME). HCC samples with high pyroptosis-associated lncRNA scores generally exhibit a more “immune-warm” microenvironment profile. This profile is associated with increased infiltration of $CD8^+$ T-cells, NK cells, and macrophages into the tumor tissue (Zhang et al., 2022).

However, increased immune cell infiltration does not always result in an effective antitumor response. Tao and Yang (2025) demonstrated that pyroptosis-associated molecular subtypes simultaneously activate immunosuppressive signals despite the high presence of immune cells. In this context, pyroptosis-associated lncRNAs can increase inflammatory cytokine release and immune cell recruitment while paving the way for the development of immune escape mechanisms.

Pyroptosis-associated lncRNAs have been shown to be closely related to the expression of immune checkpoint molecules in HCC. Zhang et al. (2022) reported a significantly increased expression of immunosuppressive molecules, such as PD-1, PD-L1, and CTLA-4, in patients with a high-risk pyroptosis-associated lncRNA signature. This suggests that pyroptosis-associated lncRNAs may be determinants of T cell depletion and immunotherapy responses. In contrast, "immune-hot" tumors with a pyroptosis-associated lncRNA profile may be more susceptible to immune checkpoint inhibitors. The pyroptosis-associated molecular subtypes identified by Wang et al. (2022) allowed for the identification of potential patient subgroups in terms of immunotherapeutic approaches. This indicates that pyroptosis-associated lncRNAs can be an important tool for patient stratification and the development of personalized treatment strategies.

5. Pyroptosis and Immunotherapy in Hepatocellular Carcinoma

5.1 Immunological Landscape of Hepatocellular Carcinoma: Barriers to Effective Immunotherapy

Hepatocellular carcinoma (HCC) is a tumor that develops multilayered immune evasion mechanisms during its development and therefore has a limited response to immunotherapy. Although clinically validated immune checkpoint inhibitors (especially PD-1/PD-L1 blockade) aim to reactivate the T cell-mediated antitumor response, a sustained clinical response is achieved in only a limited subgroup of HCC patients (Llovet et al., 2021).

This limited efficacy is primarily due to the immunologically "cold" or "immune-excluded" phenotype of the HCC tumor microenvironment (TME). HCC lesions typically show low $CD8^+$ cytotoxic T cell infiltration, in contrast to a predominance of regulatory T cells (Tregs), myeloid-derived suppressor cells

(MDSC), and tumor-associated macrophages (TAM). This cellular composition severely restricts T cell function through cytokines (TGF-β and IL-10) and metabolic barriers that suppress the antitumor immune response (Ringelhan et al., 2018).

Furthermore, because HCC develops against a background of chronic inflammation and fibrosis, physical barriers such as hypoxia, a rigid extracellular matrix, and impaired vascular structure prevent immune cells from effectively penetrating the tumor. This situation limits treatment success because even when T cell "brakes" are removed with PD-1/PD-L1 inhibitors, a sufficient number of functional effector cells cannot reach the tumor site. Therefore, the fundamental problem in HCC is not only T cell inhibition but also the inability to initiate effective immune responses.

In this context, blocking immune checkpoints alone is insufficient to increase the effectiveness of immunotherapy in HCC; additional strategies that reshape the tumor microenvironment and strongly activate the immune system are needed.

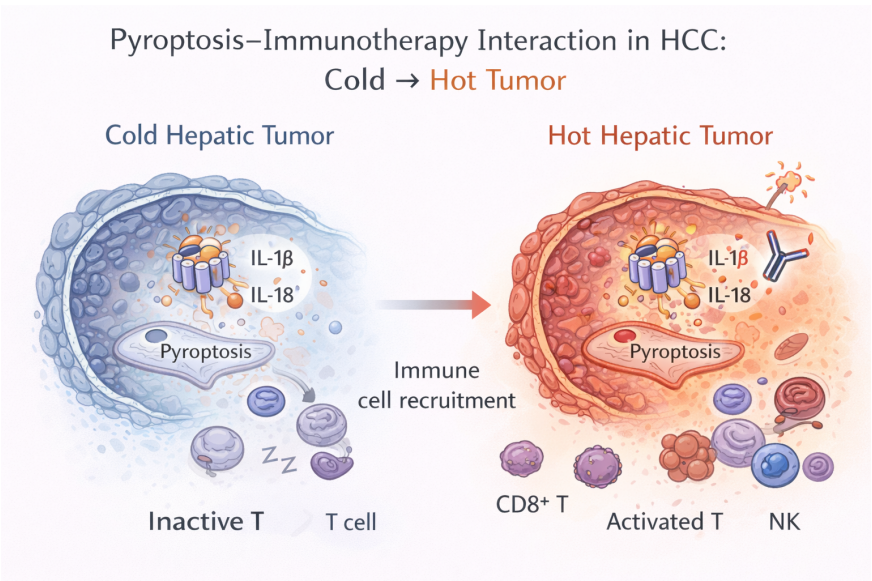

Figure 3: Pyroptosis converts immunologically cold HCC tumors into hot tumors, enhancing immune activation and immunotherapy responses.

5.2 Pyroptosis as an Immunogenic Cell Death Modality in Hepatocellular Carcinoma

Unlike classical apoptosis, pyroptosis is a regulated form of cell death that occurs in response to strong inflammatory signals and exhibits characteristics of immunogenic cell death (ICD). In this process, the activation of gasdermin family proteins (especially GSDMD and GSDME) leads to the formation of pores in the cell membrane, swelling of the cell, and lysis, ultimately resulting in the dispersion of cellular contents into the tumor microenvironment (Shi et al., 2017).

The importance of pyroptosis in HCC is not limited to the elimination of tumor cells. Damage-related molecular patterns (DAMPs), pro-inflammatory cytokines such as IL-1β and IL-18, and tumor antigens released during pyroptotic cell death transmit a strong “danger” signal to the immune system, initiating an antitumor response. In this respect, pyroptosis stands out as a mechanism that can trigger the first step of immune activation, which is lacking in HCC (Xu et al., 2021). While apoptosis is generally a "silent" form of cell death and is often tolerated by the immune system, pyroptosis, due to its inflammatory nature, enables the maturation of dendritic cells, increases antigen presentation, and strengthens T cell priming. These features make pyroptosis a critical form of ICD that can reprogram the immune response in HCC (Tang et al. 2020).

In particular, in tumors developing against a background of chronic inflammation, such as HCC, the controlled and targeted induction of pyroptosis can make tumor cells "visible" to the immune system, enabling the transformation of immunologically cold tumors into warm tumors. Thus, pyroptosis functions not only as a cell death

mechanism but also as a trigger that reshapes the microenvironment for immunotherapeutic intervention. Owing to these characteristics, pyroptosis has the potential to overcome insufficient immune activation, one of the main obstacles to immunotherapy in HCC, and forms the basis for rational combinations with immune checkpoint inhibitors.

5.3 Pyroptosis-Mediated Remodeling of the Tumor Microenvironment in Hepatocellular Carcinoma

The tumor microenvironment (TME) is a key regulatory system that determines the biological behavior, immune response, and treatment sensitivity of hepatocellular carcinoma (HCC). Pyroptosis has emerged as a powerful transformative mechanism that, owing to its inflammatory and immunogenic properties, enables not only the elimination of tumor cells but also the reprogramming of the cellular and molecular components of the HCC microenvironment.

5.4 Pyroptosis-Driven Immune Cell Recruitment and Activation

During pyroptotic cell death, gasdermin-mediated pore formation in the cell membrane and cellular lysis, along with the release of damage-associated molecular patterns (DAMPs) such as IL-1β, IL-18, ATP, and HMGB1, create a powerful chemotactic and activator signaling network in the tumor microenvironment (Shi et al., 2017). These signals contribute to the strengthening of antitumor immunity, particularly by increasing the migration of $CD8^+$ cytotoxic T cells and natural killer (NK) cells into the tumor bed.

In the context of HCC, this process is critically important because most HCC lesions exhibit an “immune-desert” or “immune-excluded” phenotype that is poor in immune cells. The inflammatory microenvironment triggered by pyroptosis breaks this immune silence, enabling effector cells to enter the tumor tissue. Experimental studies have shown that inducing pyroptosis increases

the expression of T cell attractant chemokines, such as CXCL9 and CXCL10, which are directly related to T cell infiltration (Wang et al., 2020).

5.5 Effects on Antigen-Presenting Cells and T-Cell Priming

Pyroptosis plays a central role in initiating adaptive immune responses by facilitating the efficient presentation of tumor antigens to the immune system. DAMPs released from pyroptotic cells stimulate the maturation and migration of dendritic cells (DCs), thereby increasing their antigen-presenting capacity. This allows for enhanced T cell priming and expansion of tumor-specific T cell clones (Tang et al., 2020).

Given the antigen presentation defects and inadequate T cell activation frequently observed in HCC, pyroptosis is considered a critical step in reshaping the microenvironment. Specifically, unlike apoptosis, GSDME-mediated pyroptosis has the potential to compromise immune tolerance by presenting an antigenic load in an immunogenic context (Rogers et al., 2017).

5.6 Modulation of Immunosuppressive Cell Populations

Pyroptosis-mediated microenvironment reprogramming is not limited to the activation of effector immune cells; it also affects the immunosuppressive cell populations that dominate the HCC TME. Increased inflammatory signals can promote a shift of TAMs tumor-associated macrophages TAMs from an M2-like immunosuppressive phenotype to an M1-like pro-inflammatory phenotype. This phenotypic change supports the immunotherapeutic response by reducing the suppression of T cell function (Ringelhan et al., 2018). However, excessive or chronic pyroptotic activation can have the opposite effect through the sustained release of IL-1β and IL-18, leading to the re-establishment of an immunosuppressive microenvironment by promoting Treg or MDSC expansion. This highlights the double-edged nature of pyroptosis in HCC.

5.7 Synergistic Interplay Between Pyroptosis and Immune Checkpoint Blockade in Hepatocellular Carcinoma

Immune checkpoint inhibitors (ICIs) aim to reactivate the T cell-mediated antitumor response, particularly by targeting the PD-1/PD-L1 axis. However, the clinical efficacy of these agents in HCC is limited in most patients because of inadequate antigen presentation, low T cell infiltration, and an immunosuppressive tumor microenvironment (TME). In this context, pyroptosis offers strong potential for functional synergy by providing the necessary immunological preconditions for ICI efficacy.

5.7.1 Pyroptosis-Driven Antigen Release and Enhancement of Tumor Immunogenicity

The effectiveness of immunotherapy depends on the immune system's ability to recognize antigens from tumor cells, the processing of these antigens by professional antigen-presenting cells, and the initiation of a T cell response. However, in hepatocellular carcinoma (HCC), since a large proportion of tumor cells are eliminated through non-immunogenic (“silent”) cell death pathways, such as apoptosis, tumor antigens are not adequately recognized by the immune system, and an effective immune priming process does not occur (Ringelhan et al., 2018).

In this context, pyroptosis stands out as a form of cell death that radically alters antigen presentation. During pyroptosis, inflammasome activation (mostly NLRP3 or AIM2) leads to caspase-1 or caspase-4/5/11 activation in non-canonical pathways. These caspases proteolytically cleave gasdermin proteins (specifically GSDMD or GSDME), leading to the formation of large pores in the cell membranes. Consequently, the cell swells, undergoes lysis, and its contents are released uncontrollably into the extracellular space (Shi, Gao & Shao, 2017; Xu et al., 2021).

This release includes not only tumor-associated antigens but also damage-associated molecular patterns (DAMPs), such as ATP and HMGB1, and potent pro-inflammatory cytokines such as IL-1β and IL-18. These molecules trigger signal transduction via TLRs and purinergic receptors in dendritic cells, promoting cell maturation, MHC class I and II expression, and increased expression of costimulatory molecules. This activates the cross-presentation of tumor antigens and enhances $CD8^+$ T cell priming (Tang et al., 2020).

Preclinical HCC models have shown that inducing pyroptosis increases antigen presentation capacity, accelerates T cell clonal expansion, and significantly enhances antitumor immune response (Wang et al., 2020). These data suggest that pyroptosis plays a central role in overcoming inadequate tumor immunogenicity, which is one of the earliest and most critical barriers to immunotherapy in HCC.

5.7.2 Pyroptosis-Mediated T-Cell Infiltration Creates a Permissive Context for Checkpoint Blockade

The tumor microenvironment of hepatocellular carcinoma (HCC) is characterized by dense fibrotic stroma, impaired vascular structure, and marked hypoxia. These structural and metabolic features often render HCC an immunologically "cold" tumor phenotype by preventing the effective infiltration of immune cells into tumor tissue (Ringelhan et al., 2018). In this context, the access of $CD8^+$ T cells and natural killer (NK) cells, which are necessary for the effectiveness of immune checkpoint inhibitors (ICIs), to the tumor bed is severely restricted.

Pyroptosis plays a critical role in overcoming infiltration barriers. Pro-inflammatory cytokines, such as IL-1β and IL-18, along with damage-associated molecular patterns (DAMPs) released during pyroptotic cell death, generate a potent inflammatory response in the tumor microenvironment. This inflammatory environment creates a

chemotactic gradient for $CXCR3^+$ $CD8^+$ T cells and NK cells, particularly by increasing the expression of interferon-induced chemokines, such as CXCL9 and CXCL10 (Wang et al., 2020). Consequently, HCC lesions that were previously devoid of immune cells become enriched with effector immune cells.

This cellular redistribution is not limited to a numerical increase in immune cells; it also contributes to the functional reprogramming of the tumor microenvironment (TME). Increased T cell infiltration enables more efficient recognition of tumor antigens and strengthens the local immune response of the body. Thus, pyroptosis significantly enhances the biological efficacy of PD-1/PD-L1 blockade by creating a "permissive immune context" necessary for ICIs.

Consequently, pyroptosis is considered a central regulatory mechanism in HCC that facilitates the transformation of "cold" tumors into "hot" and immunotherapy-responsive phenotypes by overcoming the physical and biochemical barriers that limit immune cell entry.

5.7.3 Relief of T-Cell Exhaustion Through Combined Pyroptosis Induction and PD-1/PD-L1 Blockade

In hepatocellular carcinoma (HCC), chronic antigen exposure from tumor cells leads to T-cell exhaustion over time, characterized by increased PD-1 expression, decreased cytotoxic capacity, and reduced cytokine production (Ringelhan et al., 2018). This exhausted phenotype is considered one of the main biological barriers limiting the clinical efficacy of immune checkpoint inhibitors.

Pyroptosis is an important complementary mechanism for reversing the suppressed state of the immune response. Tumor antigens, damage-associated molecular patterns (DAMPs), and pro-inflammatory cytokines, such as IL-1β and IL-18, released during

pyroptotic cell death, strongly stimulate dendritic cell activation, antigen presentation, and the recruitment of $CD8^+$ cytotoxic T cells into the tumor microenvironment. This process promotes a numerical increase and functional reactivation of T cells.

However, T cells activated by pyroptosis can be depleted in a chronic inflammatory environment. PD-1/PD-L1 blockade plays a critical role in ensuring the continuity of the pyroptosis-induced T cell response. In preclinical HCC models, PD-1 blockade applied in conjunction with pyroptosis induction has been shown to significantly increase IFN-γ production, granzyme B and perforin expression, and cytotoxic activity against tumor cells. This combination strategy has been reported to provide more pronounced tumor shrinkage and prolonged antitumor response than pyroptosis or PD-1 blockade alone (Tang et al., 2020; Wang et al., 2020).

In conclusion, the combined use of pyroptosis and PD-1/PD-L1 blockade offers a mechanistically grounded and rational strategy for overcoming T cell depletion in HCC. This approach has the potential to enhance the clinical efficacy of immunotherapy by simultaneously targeting the initiation and maintenance of immune responses.

5.8 Preclinical Evidence Supporting Combination Strategies in HCC

Preclinical studies in hepatocellular carcinoma (HCC) models have shown that inducing pyroptosis through pharmacological or genetic methods, when combined with immune checkpoint inhibitors (ICIs), provides significant and consistent antitumor effects. These studies demonstrate that the response to PD-1/PD-L1 blockade is significantly increased, and the tumor microenvironment shifts to a more immunogenic profile, particularly when gasdermin-mediated pyroptosis is activated (Zhang et al., 2020).

Inducing pyroptosis in experimental HCC models enhances dendritic cell activation and $CD8^+$ cytotoxic T cell infiltration by

increasing antigen release and inflammatory cytokine production in tumor tissue. In this context, pyroptosis supports early immune response steps, such as antigen presentation and T cell priming, which are necessary for the efficacy of ICIs. When administered in combination with ICIs, the functional continuity of these activated T cells is maintained, and the cytotoxic activity against tumor cells becomes more sustained.

In particular, in models where GSDME-mediated pyroptosis is activated, it has been reported that pro-inflammatory cytokines are increased in the tumor microenvironment, immunosuppressive cell populations (e.g., Tregs and MDSCs) are relatively reduced, and antitumor immunity becomes dominant (Zhang et al., 2020). These findings suggest that pyroptosis not only enhances tumor cell death but also creates a favorable immunological context for ICIs therapy.

These preclinical data demonstrate that the combination of pyroptosis and ICIs has the potential to become a clinically significant treatment strategy for HCC. It is also suggested that biomarkers associated with pyroptosis, particularly GSDME expression levels, may be helpful in predicting which patients might benefit from these combination therapies. This approach could contribute to the development of more rational and personalized immunotherapy strategies for HCC.

5.9 Enhancing Immunotherapy with Pyroptosis Using Pharmacological and Natural Compounds

The immunogenic properties of pyroptosis make pharmacological targeting of this form of cell death and its combination with immunotherapeutic approaches an attractive strategy for cancer treatment. In immunosuppressed tumors, such as hepatocellular carcinoma (HCC), agents that trigger pyroptosis can reshape the tumor microenvironment, enhancing the effectiveness of immune checkpoint inhibitors.

Sorafenib, a long-used treatment for HCC, is known primarily as a multikinase inhibitor targeting kinases such as RAF/MEK/ERK and VEGFR. However, recent studies have shown that sorafenib's effects are not limited to direct antiproliferative effects on tumor cells; it can also modulate the immune response within the tumor microenvironment. Preclinical studies have reported that sorafenib induces caspase-1 and gasdermin-mediated pyroptosis by increasing the activation of inflammasomes in tumor-associated macrophages. This process enhances the activation of natural killer (NK) cells and their cytotoxic response against tumor cells by increasing IL-1β and IL-18 release (Zhang et al. 2020). In this context, sorafenib is considered an indirect immunomodulator that can make immunologically “silent” HCC lesions more immunogenic via pyroptosis.

Additionally, natural compounds offer significant potential for combined therapy strategies targeting pyroptosis. Berberine, a natural isoquinoline alkaloid, induces pyroptosis in HCC cells by triggering inflammasome activation and gasdermin-mediated membrane pore formation. Berberine-induced pyroptosis suppresses tumor cell proliferation and promotes immune cell recruitment into the tumor microenvironment by increasing inflammatory cytokine production (Chu et al., 2016).

Similarly, curcumin can stimulate inflammasome activation and caspase-1-dependent pyroptosis while suppressing pro-tumor signaling pathways, such as NF-κB and STAT3. This dual effect of curcumin is important because it reduces tumor cell survival and enhances the immune response through immunogenic cell death. Other naturally occurring diterpenoids, such as miltirone, have been reported to suppress tumor growth and support antitumor immune responses by triggering pyroptosis in HCC cells (Wang et al., 2019).

The common feature of these pharmacological and natural agents is their potential to utilize pyroptosis not only as a mechanism of cell

death but also as a microenvironment regulator that enhances the sensitivity to immunotherapy. However, given the potent inflammatory nature of pyroptosis, the clinical use of these agents requires careful dosing and timing. It should be noted that in HCC developing against a background of chronic liver disease, uncontrolled inflammation can exacerbate liver damage.

In conclusion, pharmacological induction of pyroptosis via sorafenib and various natural compounds offers a promising approach to enhance immunotherapy in HCC. In the future, rational combinations of these agents with immune checkpoint inhibitors and the identification of pyroptosis-based biomarkers to guide patient selection will play critical roles in improving clinical success.

6.Conclusion

Hepatocellular carcinoma develops against a background of chronic liver disease and persistent inflammation, and the interactions between cell death mechanisms and the tumor microenvironment significantly shape the disease biology. As discussed in this book chapter, pyroptosis is a highly inflammatory and immunogenic form of programmed cell death mediated by inflammasome activation, inflammatory caspases, and gasdermin-mediated pore formation. The frequent suppression of apoptosis in HCC and its contribution to treatment resistance make pyroptosis an alternative and complementary therapeutic target.

However, the role of pyroptosis in HCC is context-dependent and bidirectional in nature. Controlled activation can promote dendritic cell maturation, T cell priming, and $CD8^+$ T/NK cell infiltration by increasing tumor antigen release, whereas chronic and uncontrolled inflammation can facilitate tumor progression and immune evasion. Therefore, understanding dysregulation of the inflammasome–caspase–gasdermin axis and evaluating pyroptosis-associated

signatures (e.g., gasdermin expression and pyroptosis-associated lncRNAs) as biomarkers is critically important.

In conclusion, the controlled and targeted modulation of pyroptosis, particularly in rational combinations with immune checkpoint blockade, offers novel therapeutic opportunities for HCC.

Kaynakça

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CHAPTER 2

MOLECULAR MECHANISMS OF NEWLY SYNTHESIZED MOLECULES IN CANCER TREATMENT

1. Ayşe Nur COŞKUN DEMİRKALP[1]

Introduction

Cancer is a heterogeneous group of diseases resulting from the interaction of genetic and environmental factors, and is among the leading causes of morbidity and mortality worldwide (Sung et al., 2021). Despite advances in diagnostic methods and treatment protocols, chemotherapy resistance, metastatic spread tendency, and low survival rates in many cancer types limit the effectiveness of current treatment approaches (Vasan et al., 2019). Furthermore,

[1] Asst. Prof. Dr., Kırşehir Ahi Evran University, Medical Services and Techniques
Orcid: 0000-0002-8125-7670

many classical anticancer agents can cause serious side effects on healthy cells, and long-term use increases toxicity. This situation increasingly highlights the need for new, more selective, low-side-effect profile, and molecularly targeted therapeutic strategies (Arruebo et al., 2011).

Newly synthesized molecules offer significant potential in modern cancer treatment thanks to their unique chemical structures, functional groups, and selective interactions with specific biological targets. Advances in chemical synthesis technologies and the widespread use of structure-activity relationship (SAR) analyses have enabled researchers to develop more rational drug designs targeting specific biomolecular targets (Ertl, 2020). Schiff base derivatives, metal complexes, heterocyclic compounds, semi-synthetic agents derived from natural products, redox-active molecules, and lipophilic next-generation compounds are among the most frequently studied structures in this context (Patel et al., 2021). These molecules, thanks to their unique chemical properties, can induce apoptosis in cancer cells, suppress proliferation, or target metabolic vulnerabilities observed in tumors.

One of the most important advantages of newly synthesized molecules in cancer treatment is their capacity to target multiple biological pathways simultaneously. Frequent cell cycle control disorders in cancer cells, overexpression of anti-apoptotic proteins, enhanced tolerance to reactive oxygen species (ROS), overactivation of survival pathways such as PI3K/Akt and MAPK/ERK, and epigenetic reprogramming are key processes intervened by these compounds (Hanahan, 2022). In addition, the immunosuppressive nature of the tumor microenvironment, inflammatory signaling, angiogenesis, and molecular changes related to metastatic processes are also among the current targets of next-generation molecular agents (Quail & Joyce, 2017).

This book chapter aims to comprehensively address the fundamental biochemical and molecular mechanisms of action of newly synthesized molecules in cancer treatment. In particular, cell cycle regulation, intrinsic and extrinsic apoptosis pathways, oxidative stress and redox imbalance, modulation of signal transduction networks, and epigenetic mechanisms will be discussed in detail. Furthermore, cell culture models used in the preclinical evaluation of newly synthesized molecules, toxicity analyses, real-time cell monitoring systems, and in vivo experimental approaches will also be addressed. The information presented here aims to provide a scientific basis for new molecule development studies and to offer researchers a holistic perspective on the process from synthesis to clinical application.

Classification of Newly Synthesized Molecules

Newly synthesized products developed for use in cancer treatment are examined under a very broad classification based on their chemical structures, pharmacophore groups, biological targets, and cellular mechanisms of action. The classification of new generation molecules is not based solely on chemical bonds; factors such as the molecule's protein binding affinity, intracellular distribution, bioavailability, metabolic stability, redox properties, and interaction with the tumor microenvironment are also among the key determining criteria (Patel et al., 2021; Ertl, 2020). This section comprehensively addresses the types of newly synthesized molecules; the chemical properties, biological effects, potential for use in cancer treatment, and literature findings of each class are discussed in detail.

Schiff bases are compounds formed through the condensation of carbonyl compounds with primary amines, characterized by the presence of an imine (–C=N–) functional group. The anticancer potential of this chemical class has been recognized

for decades, with evidence indicating that their biological activity is markedly enhanced when coordinated with metal ions to form metal complexes (Da Silva et al., 2011). The imine bond exhibits a strong affinity for metal coordination, while the presence of aromatic rings increases lipophilicity, thereby facilitating cellular membrane permeability. Additionally, conjugated structures enhance the ability of Schiff bases to intercalate with DNA. The anticancer effects of Schiff base derivatives are mediated through multiple mechanisms, including DNA intercalation and inhibition of topoisomerase activity (Patel et al., 2021), activation of apoptosis via mitochondrial membrane potential collapse (Gürbüz et al., 2019), induction of oxidative stress through increased reactive oxygen species (ROS) production, and inhibition of key regulatory proteins such as cyclin-dependent kinases (CDKs), epidermal growth factor receptor (EGFR), and Bcl-2.In particular, copper-Schiff base complexes are among the strongest candidates showing selective toxicity in cancer cells (Tardito & Marchiò, 2009).

Metal Complexes, The clinical success of cisplatin paved the way for the development of anticancer compounds based on metal ions, and this area remains one of the most important topics in drug development research (Barry & Sadler, 2013).

Platinum Complexes, Cisplatin, carboplatin, oxaliplatin, and new generation Pt(IV) pro-drugs. They halt replication by forming intrastrand and interstrand cross-links in DNA. New Pt(IV) complexes are more stable and become active in cancer cells by being reduced to Pt(II) (Johnstone et al., 2014).

Copper Complexes, Copper-based complexes have attracted considerable attention as anticancer agents due to their intrinsic redox activity and ability to participate in biologically relevant electron transfer reactions. When coordinated with Schiff base, salicylic acid, or triazole ligands, copper complexes exhibit pronounced anticancer effects, which are largely attributed to

enhanced stability, improved cellular uptake, and selective accumulation in tumor cells. A key mechanism underlying their cytotoxic activity involves the induction of oxidative stress through Fenton-like reactions, leading to excessive production of reactive oxygen species (ROS). Elevated ROS levels cause oxidative damage to DNA, proteins, and lipids, ultimately triggering mitochondrial dysfunction and apoptosis. Moreover, the redox cycling between Cu(I) and Cu(II) states enables sustained ROS generation, contributing to the potent anticancer activity of these complexes (Denoyer et al., 2015).

Ruthenium-based compounds, such as NAMI-A and KP1019, have emerged as promising alternatives to platinum-based chemotherapeutic agents due to their lower systemic toxicity and distinct biological profiles. Unlike classical platinum drugs that primarily exert cytotoxic effects, ruthenium complexes have demonstrated notable antimetastatic activity, inhibiting tumor invasion and dissemination rather than directly targeting rapidly dividing cells. Their ability to mimic iron in biological systems facilitates selective uptake by tumor tissues, while their versatile coordination chemistry allows for fine-tuning of ligand structures to optimize pharmacokinetic and pharmacodynamic properties. These features, combined with reduced nephrotoxicity and improved tolerability, position ruthenium complexes as attractive candidates for next-generation anticancer therapies (Kia et al., 2019).

Iron- and manganese-based complexes are distinguished by their strong redox-active properties, which enable them to disrupt redox homeostasis within cancer cells. Through redox cycling, these metal complexes can induce excessive oxidative stress, leading to non-apoptotic forms of regulated cell death such as necroptosis and ferroptosis. In particular, Fe(III) complexes have attracted significant interest due to their ability to promote lipid peroxidation and iron-dependent reactive oxygen species generation, key

hallmarks of ferroptosis. The induction of ferroptotic cell death is especially promising for the treatment of aggressive and therapy-resistant tumors, where conventional apoptosis-based strategies often fail. Manganese complexes further contribute to oxidative stress by modulating mitochondrial function and redox signaling pathways, enhancing their anticancer potential. Collectively, these properties highlight iron and manganese complexes as emerging candidates in redox-based anticancer strategies (Stockwell et al., 2017).

Heterocyclic Structures: More than 60% of modern anticancer drugs contain heterocyclic scaffolds, underscoring their central role in contemporary drug design (Chen et al., 2020). Heterocyclic structures possess unique electronic and steric properties that enable highly specific interactions with protein targets involved in cancer progression. Among the most prominent classes, quinazoline derivatives are well known for their efficacy as epidermal growth factor receptor (EGFR) inhibitors, exemplified by clinically approved agents such as erlotinib and gefitinib. Pyrimidine-based compounds have been extensively developed as inhibitors of cyclin-dependent kinases (CDKs) and phosphoinositide 3-kinase (PI3K), thereby interfering with key signaling pathways that regulate cell cycle progression and survival. Benzimidazole derivatives exert anticancer effects primarily through microtubule destabilization, leading to mitotic arrest and subsequent cell death. In addition, indole and indazole derivatives play critical roles in the modulation of apoptotic signaling pathways by targeting multiple molecular mediators. Collectively, many heterocyclic anticancer agents not only suppress survival and proliferation signaling but also inhibit matrix metalloproteinases (MMPs), which are closely associated with tumor invasion and metastasis, highlighting their multifunctional therapeutic potential.

Natural products represent one of the richest and most productive sources for anticancer drug discovery, with approximately 48% of FDA-approved anticancer agents being either natural products or their derivatives (Newman & Cragg, 2020). These compounds exhibit remarkable structural diversity and biological specificity, enabling them to modulate multiple cancer-related pathways. Major classes include flavonoids such as quercetin and apigenin, which exert anticancer effects primarily through suppression of the PI3K/Akt signaling pathway; terpenoids, including paclitaxel (taxol) and artemisinin, which induce cytotoxicity via microtubule stabilization and reactive oxygen species (ROS) generation; alkaloids such as vinblastine and camptothecin, well known for their ability to inhibit topoisomerase activity; and polyphenols such as resveratrol, which have been shown to modulate epigenetic mechanisms involved in tumor progression. To overcome limitations associated with poor bioavailability or instability of native natural products, semi-synthetic derivatives—including docetaxel and topotecan—have been developed, offering improved pharmacokinetic properties, enhanced potency, and increased clinical applicability.

Redox-Active Molecules: The dysregulated redox balance and elevated basal levels of reactive oxygen species (ROS) in cancer cells render redox-active molecules particularly attractive as anticancer agents (Trachootham et al., 2009). Compounds within this class exploit the vulnerability of tumor cells to oxidative stress by further increasing ROS levels beyond the cellular tolerance threshold. Quinone derivatives induce cytotoxicity primarily through free radical–mediated DNA damage and redox cycling processes, while N-oxide–containing molecules can undergo selective bioreductive activation in hypoxic tumor regions, thereby enhancing tumor selectivity and minimizing damage to normal tissues. Isothiocyanates exert anticancer effects by modulating phase

II detoxification enzymes and disrupting cellular redox homeostasis. Notably, redox-active molecules demonstrate heightened efficacy in tumors with elevated glutathione levels, where adaptive antioxidant mechanisms can be overwhelmed, leading to oxidative damage, mitochondrial dysfunction, and subsequent cell death.

Lipophilic compounds readily traverse cellular membranes and exert selective cytotoxic effects on cancer cells by preferentially targeting mitochondria, which play a central role in tumor cell metabolism and survival. These molecules accumulate within the mitochondrial matrix driven by the elevated mitochondrial membrane potential characteristic of cancer cells, leading to mitochondrial membrane depolarization, suppression of ATP production, and release of cytochrome c into the cytosol. Subsequent activation of caspase cascades ultimately results in apoptotic cell death. Among this class, molecules conjugated with the lipophilic cation triphenylphosphonium (TPP^+) have gained considerable attention in recent years due to their efficient mitochondrial targeting and enhanced anticancer efficacy. By selectively disrupting mitochondrial bioenergetics and redox homeostasis, TPP^+-conjugated compounds represent a promising strategy for the development of next-generation mitochondria-directed anticancer therapeutics (Modica-Napolitano & Aprille, 2017).

Novel Molecules Designed as Kinase Inhibitors: Newly developed molecules targeting aberrantly activated kinases in cancer cells constitute the core of modern targeted cancer therapy. Dysregulated kinase signaling plays a pivotal role in tumor initiation, progression, angiogenesis, and therapy resistance, making kinases highly attractive therapeutic targets. Key kinases frequently targeted in anticancer drug development include epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), anaplastic lymphoma kinase (ALK), BCR–ABL fusion kinase, phosphoinositide 3-kinase (PI3K), and the mammalian target

of rapamycin (mTOR). Many newly designed heterocyclic molecules exhibit high affinity for the ATP-binding pockets of these kinases, enabling selective inhibition of kinase activity and downstream oncogenic signaling pathways. Through rational drug design and structure–activity relationship optimization, these inhibitors achieve improved selectivity and potency, contributing to enhanced therapeutic efficacy and reduced off-target toxicity.

Molecular Mechanisms of Action

The therapeutic efficacy of newly synthesized anticancer molecules is largely determined by their ability to target fundamental biological processes that are dysregulated in cancer cells. Malignant cells exhibit a wide spectrum of molecular abnormalities, including uncontrolled proliferation, evasion of apoptosis, genomic instability, metabolic reprogramming, hyperactivation of intracellular signaling pathways, and epigenetic dysregulation. Consequently, contemporary drug development strategies have shifted from single-target approaches toward the design of multifunctional molecules capable of modulating multiple cellular pathways simultaneously (Hanahan, 2022). In this context, newly developed anticancer compounds exert their effects through diverse molecular mechanisms, which are discussed in this section under the headings of cell cycle regulation, apoptotic signaling pathways, oxidative stress and redox homeostasis, modulation of intracellular signaling cascades, epigenetic regulation, and interactions with the tumor microenvironment.

The cell cycle is a tightly regulated process governed by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. In cancer cells, these regulatory mechanisms are frequently disrupted, and the inactivation of critical checkpoints at the G1/S or G2/M transitions leads to uncontrolled cell proliferation (Malumbres & Barbacid, 2009). Newly synthesized anticancer molecules are

capable of inducing cell cycle arrest through multiple mechanisms, including inhibition of CDK2, CDK4/6, and CDK1, suppression of cyclin D1 and cyclin E expression, and activation of CDK inhibitors such as p21 and p27. In particular, small heterocyclic molecules have been shown to exhibit high affinity for the ATP-binding pockets of CDKs, resulting in effective suppression of kinase activity (Asghar et al., 2015). Moreover, numerous Schiff base derivatives and kinase inhibitor candidates have been demonstrated to induce cell cycle arrest at the G0/G1 or G2/M phases in cancer cells, an effect that is often followed by the activation of apoptotic pathways (Otto & Sicinski, 2017).

Apoptosis is a vital form of programmed cell death that plays a central role in maintaining organismal homeostasis. In cancer cells, however, the overexpression of anti-apoptotic proteins and suppression of pro-apoptotic factors enable malignant cells to evade apoptosis and survive under conditions that would normally trigger cell death (Pfeffer & Singh, 2018).

A substantial proportion of newly synthesized anticancer molecules exert their cytotoxic effects by targeting the mitochondria-mediated intrinsic apoptotic pathway. This mechanism involves activation of the pro-apoptotic proteins Bax and Bak, suppression of anti-apoptotic members such as Bcl-2 and Bcl-xL, loss of mitochondrial membrane potential, and subsequent release of cytochrome c into the cytosol. These events lead to the sequential activation of initiator caspase-9 and executioner caspase-3, culminating in apoptotic cell death (Youle & Strasser, 2008). Lipophilic and mitochondria-targeted molecules are particularly effective in this context, as they can directly accumulate within the mitochondrial membrane and induce selective toxicity. This effect is especially pronounced in tumor cells with high metabolic activity and elevated mitochondrial dependence (Modica-Napolitano & Aprille, 2017).

Certain newly synthesized anticancer agents are capable of initiating apoptosis through activation of death receptors located on the cell surface, including Fas (CD95) and tumor necrosis factor–related apoptosis-inducing ligand receptors (TRAIL-R1/R2). Engagement of these receptors leads to the formation of the death-inducing signaling complex (DISC) and subsequent activation of initiator caspase-8. Activated caspase-8 not only directly triggers downstream executioner caspases but also induces truncation of the BH3-only protein Bid, thereby establishing critical crosstalk between the extrinsic and intrinsic mitochondrial apoptotic pathways. This amplification loop enhances apoptotic signaling and contributes to the effective elimination of cancer cells, highlighting the therapeutic relevance of death receptor–mediated apoptosis in anticancer drug development (Ashkenazi, 2015).

Cancer cells characteristically generate higher basal levels of reactive oxygen species (ROS) compared to normal cells; however, they concurrently develop robust antioxidant defense mechanisms that enhance their tolerance to oxidative stress (Trachootham et al., 2009). Newly synthesized redox-active molecules exploit this vulnerability by further perturbing redox balance, thereby pushing intracellular ROS levels beyond the threshold compatible with cell survival. These compounds induce cytotoxicity through multiple mechanisms, including enhancement of ROS production, depletion of intracellular glutathione (GSH) levels, and suppression of antioxidant enzymes. Metal complexes and quinone derivatives are particularly effective in mediating oxidative stress–induced cytotoxicity through these pathways (Liou & Storz, 2010). Excessive ROS accumulation results in widespread macromolecular damage, including DNA strand breaks, lipid peroxidation, and protein oxidation, ultimately triggering programmed and non-programmed cell death pathways such as apoptosis, necroptosis, or ferroptosis (Stockwell et al., 2017).

Key Signaling Pathways in Cancer Cell Survival and Proliferation: Among the principal signaling pathways involved in cancer cell survival, proliferation, and therapy resistance are the PI3K/Akt/mTOR, MAPK/ERK, JAK/STAT, and NF-κB pathways, all of which are frequently dysregulated in malignant cells.

PI3K/Akt/mTOR Pathway: The PI3K/Akt/mTOR signaling cascade plays a critical role in regulating cellular growth, metabolism, and evasion of apoptosis. Aberrant activation of this pathway is a hallmark of many cancer types and is closely associated with tumor progression and therapeutic resistance. Numerous newly synthesized small molecules have been shown to exert anticancer effects by attenuating Akt phosphorylation, suppressing mTOR activity, and inducing autophagy, thereby disrupting pro-survival signaling and sensitizing cancer cells to cell death (Hua et al., 2019).

MAPK/ERK Pathway: Aberrant activation of the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) pathway is closely associated with enhanced cell proliferation, invasion, and metastatic potential in cancer. Persistent stimulation of this signaling cascade promotes uncontrolled growth and resistance to apoptosis. Heterocyclic molecules and kinase inhibitors have demonstrated significant efficacy in suppressing MAPK/ERK signaling by inhibiting upstream kinases or directly targeting ERK activation, thereby reducing tumor cell proliferation and metastatic behavior (Dhillon et al., 2007).

NF-κB and STAT3 Signaling: The transcription factors nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) play pivotal roles in inflammation, cell survival, immune evasion, and angiogenesis in cancer. Constitutive activation of these pathways contributes to tumor progression and resistance to therapy. Recent studies have shown that newly synthesized anticancer agents can inhibit NF-κB nuclear translocation and suppress STAT3 activation, leading to

downregulation of pro-survival and pro-inflammatory gene expression and enhanced susceptibility of cancer cells to apoptosis (Yu et al., 2014).

Regulation of Epigenetic Mechanisms: Epigenetic alterations play a fundamental role in cancer development and progression by inducing heritable yet reversible changes in gene expression without altering the underlying DNA sequence. Key epigenetic mechanisms include DNA methylation, histone modifications, and chromatin remodeling, all of which contribute to the stable silencing of tumor suppressor genes and aberrant activation of oncogenic pathways (Jones & Baylin, 2007). Newly synthesized epigenetic modulator molecules exert anticancer effects through diverse mechanisms, including inhibition of DNA methyltransferases (DNMTs), leading to reactivation of tumor suppressor genes; inhibition of histone deacetylases (HDACs), resulting in chromatin relaxation and enhanced transcriptional activity; and inhibition of bromodomain and extraterminal (BET) proteins, which suppress oncogene expression. Notably, epigenetic agents offer significant therapeutic advantages in combination treatment strategies, particularly for chemotherapy-resistant tumors, by restoring drug sensitivity and modulating multiple cancer-associated pathways simultaneously (Dawson & Kouzarides, 2012).

Effects on the Tumor Microenvironment and Metastasis: Next-generation anticancer molecules are designed not only to target cancer cells directly but also to modulate the tumor microenvironment, which plays a critical role in tumor growth, immune evasion, and metastatic dissemination. Key processes within the tumor microenvironment include angiogenesis, extracellular matrix remodeling, and interactions with immune cells. Newly synthesized compounds have been shown to exert anti-metastatic effects through multiple mechanisms, including suppression of vascular endothelial growth factor (VEGF) signaling,

inhibition of matrix metalloproteinases such as MMP-2 and MMP-9, and blockade of epithelial–mesenchymal transition (EMT). By interfering with these processes, these agents limit tumor invasion, migration, and colonization of distant organs, highlighting their potential as effective strategies for controlling cancer progression and metastasis (Quail & Joyce, 2013).

Future Perspectives

The development of newly synthesized molecules in cancer treatment is not limited to obtaining effective compounds; it also encompasses multi-dimensional approaches such as target-specific delivery of these molecules, increasing their bioavailability, reducing their toxicity, and improving their clinical success. In this context, nanoparticle-based delivery systems, combination therapies, and artificial intelligence-assisted drug design are among the most important strategies shaping the future of anticancer drug development processes.

Nanoparticle-Based Drug Delivery Systems: Nanotechnology has attracted increasing interest in cancer therapy due to its strong potential to optimize the pharmacokinetic and pharmacodynamic properties of newly synthesized anticancer molecules. Nanoparticle-based delivery systems encompass a wide range of platforms, including liposomes, polymeric nanoparticles, dendrimers, metal nanoparticles, and solid lipid nanoparticles. These systems can prolong the retention of therapeutic agents within tumor tissues while significantly reducing off-target toxicity in healthy tissues. Encapsulation of newly developed molecules into nanoparticulate carriers effectively addresses classical challenges in drug development, such as poor aqueous solubility, rapid metabolic degradation, and nonspecific biodistribution. Moreover, passive targeting can be achieved by exploiting the enhanced permeability and retention (EPR) effect characteristic of tumor vasculature. In

addition to passive targeting, active targeting strategies further enhance tumor selectivity through surface functionalization of nanoparticles with antibodies, peptides, or ligands such as folate and transferrin. Recent advances in the development of smart nanoparticle systems—responsive to redox conditions, pH changes, or enzymatic activity—enable controlled and stimuli-triggered drug release by leveraging the unique biochemical features of the tumor microenvironment. Collectively, these approaches significantly improve the therapeutic efficacy of newly synthesized molecules while minimizing systemic side effects, highlighting the growing importance of nanotechnology in modern anticancer drug delivery (Peer et al., 2007; Torchilin, 2014).

Combination Therapies and Synergistic Approaches: The heterogeneous nature of cancer cells and their capacity to develop adaptive resistance mechanisms significantly limit the long-term efficacy of single-agent therapies. Consequently, the use of newly synthesized molecules in combination with existing chemotherapeutic agents, targeted therapies, or immunotherapies is increasingly regarded as one of the most promising strategies in modern cancer treatment. Combination therapies aim to suppress multiple biological pathways within tumor cells through the simultaneous or sequential administration of agents with distinct mechanisms of action. For instance, combining a newly synthesized compound that induces apoptosis with a DNA-damaging chemotherapeutic agent can result in synergistic cytotoxic effects. Similarly, novel molecules with epigenetic regulatory properties have been shown to restore sensitivity to conventional chemotherapy. In addition to enhancing therapeutic efficacy, combination strategies allow for dose reduction of individual agents, thereby minimizing toxicity and improving patient compliance. Preclinical combination studies conducted in cell culture systems and in vivo models provide a robust scientific foundation for clinical

translation. Therefore, early evaluation of the combination potential of newly synthesized molecules has emerged as a critical component of anticancer drug development pipelines (Al-Lazikani et al., 2012; Bayat Mokhtari et al., 2017).

AI-Assisted Drug Design: Artificial intelligence (AI) and machine learning approaches are transforming the landscape of drug discovery and development by introducing unprecedented levels of efficiency and precision. In particular, AI-based methodologies applied during the design phase of newly synthesized molecules offer substantial advantages in terms of time and cost reduction. These approaches enable highly accurate predictions across multiple stages of drug development, including virtual screening, molecular docking, structure–activity relationship (SAR) modeling, and toxicity prediction. Deep learning algorithms trained on large chemical and biological datasets facilitate the identification and design of molecules with high affinity for specific biological targets, thereby reducing the need for extensive experimental screening of large compound libraries. As a result, the most promising candidates can be prioritized early, significantly improving the efficiency of laboratory-based studies. Moreover, AI-assisted models can predict key pharmacokinetic parameters such as lipophilicity, bioavailability, metabolic stability, and potential toxicity, contributing to more informed decision-making in lead optimization. Looking ahead, the integration of AI-driven drug design with nanoparticle-based delivery systems and combination therapy strategies is expected to play a pivotal role in the advancement of personalized cancer treatments. This holistic and multidisciplinary paradigm aims to enhance the clinical success rates of newly synthesized anticancer molecules (Zhavoronkov et al., 2019; Vamathevan et al., 2019).

Conclusion

Cancer is a highly heterogeneous disease shaped by complex genetic, epigenetic, and environmental interactions, which limits the effectiveness of conventional single-target therapies. This complexity necessitates the development of more selective, multitargeted, and personalized treatment strategies. In this context, newly synthesized molecules have emerged as promising candidates in modern oncology due to their diverse chemical structures and multifaceted mechanisms of action.

This chapter has summarized the anticancer potential of newly synthesized molecules within the framework of their chemical classifications, molecular mechanisms, preclinical evaluation strategies, and structure–activity relationships. Key mechanisms underlying their therapeutic effects include cell cycle regulation, reactivation of apoptotic pathways, disruption of redox homeostasis, modulation of critical intracellular signaling cascades, and epigenetic regulation. Together, these multidimensional activities highlight the potential of novel compounds to overcome major clinical challenges such as chemoresistance and tumor adaptation.

Rational drug design approaches, supported by SAR analyses and molecular modeling, play a central role in optimizing the efficacy and selectivity of these molecules. Looking forward, the integration of nanoparticle-based delivery systems, combination therapy strategies, and artificial intelligence–assisted drug design is expected to significantly enhance the clinical translation and success of newly synthesized anticancer agents.

In conclusion, newly synthesized molecules represent not only alternative therapeutic options but also key components of next-generation targeted and personalized cancer treatments. Continued interdisciplinary collaboration will be essential to translate these advances into effective and sustainable clinical solutions.

Kaynakça

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CHAPTER 3

PROGRAMMING CELL DEATH AND CANCER TREATMENT WITH GENE TRANSFECTION

CANDAN ALTUNTAŞ[1]
GÖKHAN DURUKSU[2]
YUSUFHAN YAZIR[3]

Introduction

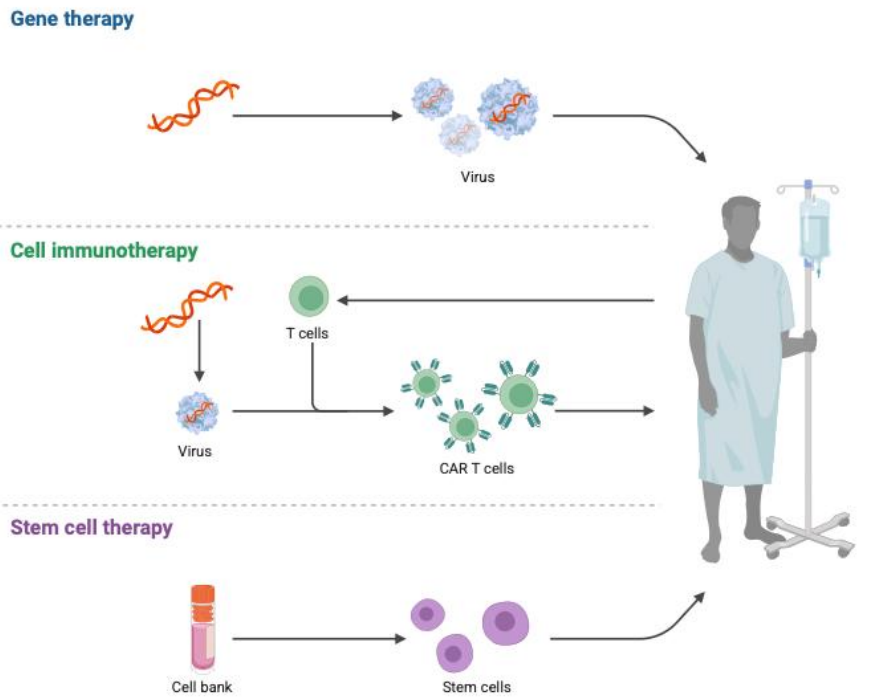
In light of the uncontrolled proliferation of cancer cells, the induction of cell death is regarded as a treatment modality against these cells. Therapeutic methods, such as chemotherapy and radiotherapy, have been developed to eradicate malignant cells and have been associated with cancer treatment for a considerable period. However, these treatments also result in the death of numerous normal cells and the occurrence of undesirable side effects. The present study hypothesizes that focusing on cancer cells, as opposed to the induction of regional damage and the consequent

[1]Dr. Kocaeli University, Stem Cell and Gene Therapies Research and Practice, Orcid: 0000-0002-2551-1849
[2] Assoc. Prof. Dr., Kocaeli University, Department of Stem Cell, Institute of Health Sciences, Orcid: 0000-0002-3830-2384
[3] Prof. Dr., Kocaeli University, Department of Histology and Embriyology, School of Medicine, Orcid: 0000-0002-8472-0261

reduction in cell viability, will prevent the occurrence of these deleterious side effects. When cells become non-viable, they are exposed to cell death mechanisms that have evolved for this purpose and are programmed to activate across species, a process termed "apoptosis" (Dl, 1994: 777; Moyer, Tanaka & Cheng, 2025: 303). This type of cell death differs from necrosis and autophagy and occurs as a result of a physiological suicide process rather than a hostile environment.

In a global context, gene therapy is defined as a treatment approach that involves the correction of a genetic defect by the addition of a normal/healthy sequence of a defective or missing gene, thereby correcting the underlying disorder (Friedmann, 1992: 93). The early focus of gene therapy on rare diseases caused by harmful monogenic (single-gene) defects is well documented (Vrellaku et al., 2024: 3220). Nevertheless, advancements in this domain have demonstrated that gene therapy techniques may possess a broad spectrum of potential applications (Wirth, Parker & Ylä-Herttuala, 2013: 162). Although the traditional way of treating cancer involves the use of certain chemotherapeutic agents, the development of chemoresistance during the therapeutic process is one of the main factors causing many forms of chemotherapy to fail (Gottesman, 2002: 615). Despite the remarkably accomplishments in recent years in the field of target identification strategies, cancer gene therapy remains in its infancy (Das et al., 2015: 259). Notwithstanding the local administration of these recombinant viruses, the potential exists for their dissemination to other tissues, a phenomenon attributable to the advanced vascular structure characteristic of tumor tissue. This, in turn, can lead to undesirable outcomes.

Figure 1. *Cell and gene therapies (created with BioRender.com).*



1.1 Solving Problems in Viral Gene Transfer

A significant benefit of viral vector-based gene therapies is its capacity to target specific cells, a process contingent on the substantial interaction between viral proteins and host receptors that are serotype-specific. However, the efficacy of viral delivery can vary for several reasons. In addition to immune system responses, the treatment approach may be a contributing factor. The absence or

reduction of receptor expression in the target cell or tissue has been demonstrated to result in a concomitant reduction in gene transfer efficiency. It is important to note that cancer cells do not constitute a homogeneous population; consequently, targeting a single cellular surface receptor may not be the most effective strategy. From this standpoint, the following approaches may be considered:

- Eliminating the adverse effects of the innate immune response to viral vectors is an important approach. As tissue-specific macrophages and Kupffer cells are critical in reducing this effect, the choice of molecules injected with viral vectors, such as polyethylene glycol (PEG) and similar synthetic polypeptides, can be modified. It has been demonstrated that PEGylation, a process which involves the coating of viral particles with poly(lactic-co-glycolic acid) (PLGA) or poly-[N-(2-hydroxypropyl) methacrylamide], has a detrimental effect on viral cell targeting and reduces host-vector interactions (Abel, Angel, Kiguchi & Di, Giovanni, 2009: 1350).
- Complement activation, a component of the immune system, has been identified as a significant impediment to the efficacy of gene transfer via viral mechanisms. PEG has been shown to masks important proteins involved in virus recognition, thereby protecting viral particles from the immune system. An alternative approach suggests that the incorporation of host cell proteins into the structure of the viral capsid may serve as a means of preventing complement activation (Vanderplasschen, Mathew, Hollinshead, Sim & Smith, 1998: 7544).
- An alternative approach involves the recombinant expression of the soluble form of CD59 in viruses, which

prevents the accumulation of the membrane attack complex and lysis of these viruses by the complement (Gandhi, Cashman & Kumar-Singh, 2011: e21621).

- Another approach to enhance gene transfer efficacy is the use of pharmacological agents that suppress the immune system. Compared to alternative approaches, this method is distinguished by its simplicity and ease of application, as it does not involve pre-design or recombinant modifications. For prophylactic purposes, IL-10 and rapamycin (an immunosuppressive drug) have been used in combination in virus-mediated gene transfer, with immune responses to exogenously administered genetic products blocked in *in vivo* animal models (Nayak et al., 2009: 1523).
- The use of a range of viruses for gene transfers has led to enhanced efficacy, attributable to the potent stimulation of the immune response by adenoviruses. The utilization of alternative viral vector systems, such as lentiviruses and adeno-associated viruses, has been demonstrated to elicit diminished immune responses (Batty & Lillicrap, 2024: 2945).

A significant challenge that undermines the efficacy of treatment is that viruses administered intravenously for therapeutic purposes primarily interact with cells found in the liver and spleen (Shashkova, Doronin, Senac & Barry, 2008: 5896). Furthermore, factors such as vector neutralization, non-cell-specific binding of viruses, retention of viral particles by cells or tissues, and the inability of viruses to detect tumor cells behind the endothelial matrix barrier limit treatment outcomes (Das et al., 2015: 259). Adenovirus serotype 5 (Ad5) vectors have been employed to enhance the therapeutic efficacy of certain types of cancer by

infecting target cancer cells through expressing of coxsackie and adenovirus receptors (CARs). These receptors are poorly expressed in human cancer cells (Coyne & Bergelson, 2006: 119; Philipson & Pettersson, 2004: 87).

The objective of this study was to enhance binding to integrin-binding viral protein subunits (HI-loops) on the capsid surface by structurally incorporating an RGD (Arg-Gly-Asp) motif into adeno-associated viruses (AAVs) for the development of viral therapy, even without targeting CAR expression (Nicol, 2005: 291). One of the earliest documented studies involved a virion design comprising both an RGD motif and a poly-lysine ligand (heparin sulfate proteoglycan) (Wu et al., 2002: 1647). In a separate study, a CAR-non-targeted chimeric adenovirus vector containing Ad3 fiber proteins incorporated into the Ad5 capsid was developed (Krasnykh, Mikheeva, Douglas & Curiel, 1996: 6839). These virions (Ad.5/3) have demonstrated antiviral efficacy in cancer cells with low CAR expression, including melanoma, ovarian cancer, renal cancer, squamous cell carcinoma, glioma, prostate cancer, pancreatic cancer and colorectal cancer (B. Azab et al., 2012: 2145; B. M. Azab et al., 2014: 34; Rupesh Dash et al., 2011: 8785; R Dash et al., 2010: 447; Eulitt et al., 2010: 1290; Hamed et al., 2013: 171; Haviv et al., 2002: 4273; Kanerva et al., 2002: 275; Kawakami et al., 2003: 1262; Park et al., 2011: 368; Rein et al., 2005: 1327; Rivera et al., 2004: 1694; Sarkar et al., 2014: 125; Volk et al., 2003: 511).

Natural tropism imposes limitations on the capacity of adenoviruses to target specific tissue/cell types, and transduction of non-target tissues due to natural tropism can result in undesirable side effects of viral applications. Consequently, both CAR and αv integrin-binding interactions have been modified to prevent CAR-associated natural tropism (Einfeld et al., 2001: 11284). In other similar designs, Ad vectors consisting of both fibrin and wild-type fibers have been developed that present a 6-His motif, demonstrating

gene transduction specific to both CAR and the artificial 6-His receptor. The incorporation of a peptide sequence (SY-GYLPLP, a vascular endothelial cell-targeting peptide) or an antibody mimetic termed "affibody" into the HI loop to facilitate cellular-specific targeting has led to enhanced gene transfer in various cancer cell lines (Das et al., 2015: 259). Adenoviruses expressing an anti-EpCAM antibody conjugated with a neutralizing fiber-inhibiting antibody have been developed to target the adhesion molecule (EpCAM) found on tumor cells (Haisma et al., 1999: 1469).

1.2 Regulation of Gene Transfer Specific Expression in Cells

The most significant consideration in the utilization of viral vectors for the transfer of diverse genetic sequences into cells for cancer treatment is the assurance of tumor-specific expression. The utilization of selective promoters, which are tailored to tumors or cancer cells, resulting in the limitation of expression in specific cellular subpopulations, represents an approach that has been extensively investigated through both *in vitro* studies and live animal experiments. The identification of an appropriate tumor-selective/specific promoter is a critical step in this approach. Rodriguez et al. (1997) designed a tumor-selective promoter by inserting a prostate-specific antigen (PSA) promoter/enhancer sequence upstream of the E1A gene, resulting in a prostate-specific oncolytic virus. However, it should be noted that the designs of viruses are not limited to the PSA promoter/enhancer. Furthermore, a surviving promoter for glioma (Van Houdt et al., 2006: 583), the β-catenin-responsive promoter for colorectal and liver cancer (Fuerer & Iggo, 2002: 270) and the tyrosinase promoter for melanoma (Nettelbeck, Rivera, Balagué, Alemany & Curiel, 2002: 4663) are a few examples of promoters utilized to direct viral replication genes in specific cancers. Similarly, oncolytic viruses have been developed using a cyclooxygenase-2 (Cox2) promoter to target various types of cancer, including cervical, ovarian, and

pancreatic cancers (Hoffmann & Wildner, 2006: 374). MUC1/DF3 (Fukazawa et al., 2010: 3), alpha-fetoprotein (AFP) (Hallenbeck et al., 1999: 1721), and squamous cell carcinoma antigen-2, which targets squamous cell carcinoma (Hamada et al., 2010: 545), as well as the glial fibrillary acidic protein promoter for GBM, have been validated at the preclinical level and have been demonstrated to be effective in various animal models.

Numerous adenovirus types have been engineered for this purpose, either as monotherapy or in combination with chemotherapy. As outlined in the following section, some of these include viruses containing the p53 gene, which is expressed under the CMV promoter (Ad5CMV-p53) (clinicaltrials.gov, Clinical Trial No. The following references are relevant to the present study: NCT00004038, NCT00003450, NCT00004225, NCT00044993, NCT00003649, NCT00004041 and NCT00003167. The following references are also relevant to the present study, albeit to a lesser extent: Ad5-yCD/mutTKSR39rep-hIL12 (NCT03281382, NCT00406939 (IL-12 only)), an oncolytic adenovirus engineered for IL-12 therapy that expresses two suicide genes and human IL-12. The Ad5 virus was modified to contain the human CD40L gene with an RSV promoter (AdCD40L) (NCT00891748). In addition, an adenoviral vector that expresses the herpes thymidine kinase gene (AdV-tk) has been developed (NCT00589875, NCT00300521, and NCT00005057). Concurrent with viral studies, non-viral clinical trials are being conducted in parallel, though in smaller numbers: cationic liposomes conjugated with an anti-transferrin receptor single-chain antibody fragment (TfRscFv), containing the tumor suppressor gene RB94 (NCT01517464) or liposomes containing epidermal growth factor receptor (EGFR) antisense DNA (NCT00009841).

Despite the extensive range of designs and methodologies that have been developed for *in vitro* studies in the literature during

clinical research, there is a limited number of studies that have reached the clinical trial stage and have been successfully completed. This situation can be attributed to preconceived notions and biases against gene therapy and virus-based studies. Moreover, owing to the expeditious and unambiguous responses engendered by immunotherapy, the chimeric antigen receptor (CAR) T-cell therapy approach, an *ex vivo* T-cell-based treatment modality, has undergone a marked increase in utilization.

1.3 Alternative Approaches in Cancer Treatment

CAR T-cell therapy is an immunotherapeutic approach that involves the genetic modification of a patient's T cells to express a synthetic receptor that can recognize and target a specific antigen on tumor cells (Brudno, Maus & Hinrichs, 2024: 1924; Marofi et al., 2021: 81). The distinctive and precise impact of CAR T-cells is typically attributable to the integration of the extracellular antigen-binding domains of antibodies (single-chain variable fragments (scFv) and heavy chain variable region (VH)-based ligands) with the intracellular signaling mechanism of the T cell receptor (TCR) CD3 chain (Rahbarizadeh, Ahmadvand & Moghimi, 2019: 41). Despite the encouraging developments witnessed in recent years, the reasons for the failure of CAR T-cell therapy are multifactorial and may not be resolved by synthetic biological advances alone (Dimitri, Herbst & Fraietta, 2022: 78).

Gene therapy has the potential to enhance the efficacy of T cells with targeted modifications and reduce treatment risks by redirecting the specificity of the immune response, in conjunction with genome editing. The CRISPR system, when employed in conjunction with CRISPR and related Cas endonucleases, which have recently been frequently used as gene regulators, constitutes a powerful gene-editing technology that potentially allows targeting multiple genes in T cells to improve cancer immunotherapy

(Schmidt et al., 2022: eabj4008; Stadtmauer et al., 2020). Gene therapy and genome editing approaches have the potential to modify T cell dysfunction and enhance immune targeting. The interaction between programmed cell death protein 1 (PD-1) on activated T cells and PD-1 ligand (PD-1L) on cancer cells limits the lethal effects of chimeric antigen receptor (CAR) T cells on solid tumors (Hu et al., 2019: 365). It has been demonstrated that the function and persistence of T cells can be enhanced by the removal of both the endogenous T cell receptor (TCR) and the immune checkpoint molecule programmed cell death protein 1 (PD-1) from the genome sequence using CRISPR-Cas9 technology. Furthermore, the PD-1 deletion process has the capacity to enhance safety and mitigate the toxicity that may be induced by autoimmunity (NCT03399448). As demonstrated in the relevant literature, the presence of tumor cells expressing programmed death ligand 1 (PD-L1) reduces the efficacy of chimeric antigen receptor (CAR) T cells (anti-CD19 4-1BB), thereby decreasing treatment effectiveness. To reverse this suppressed antitumor response, PD-1 negative anti-CD19 CAR T cells were generated by targeting the Pdcd1 gene in T lymphocytes using CRISPR-Cas9-mediated gene editing and lentiviral transduction.

Figure 2. *CART therapy in cancer (created with BioRender.com).*

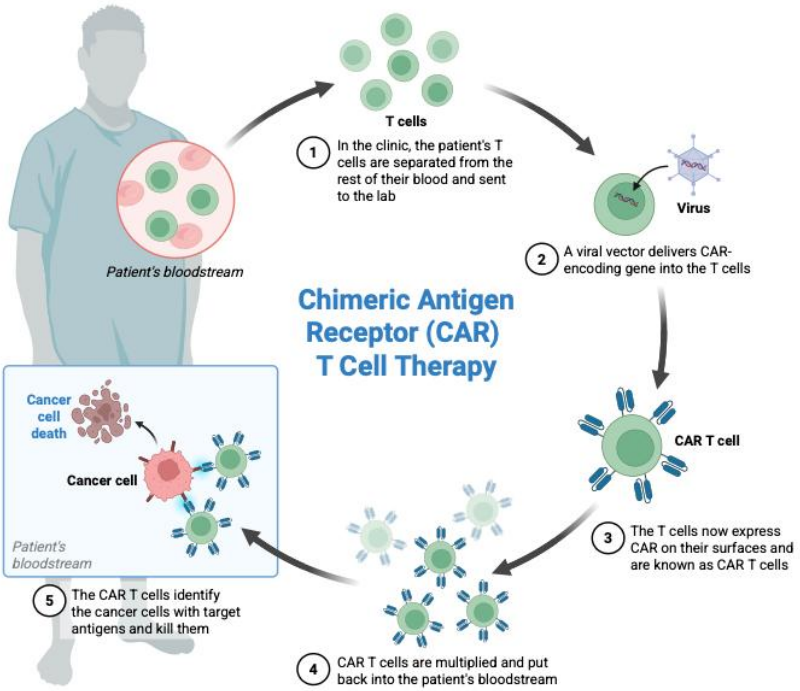
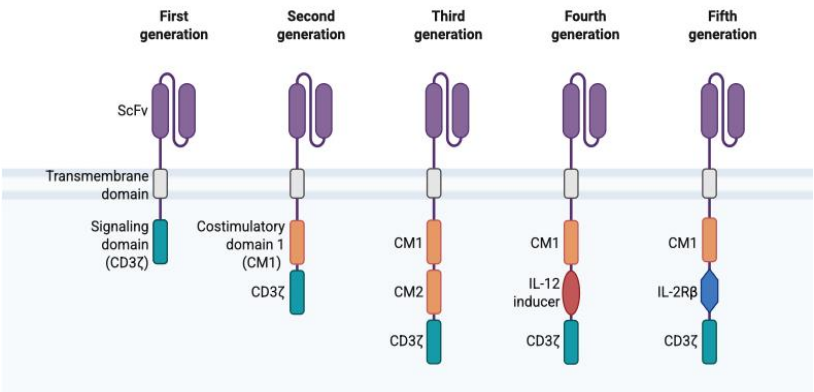


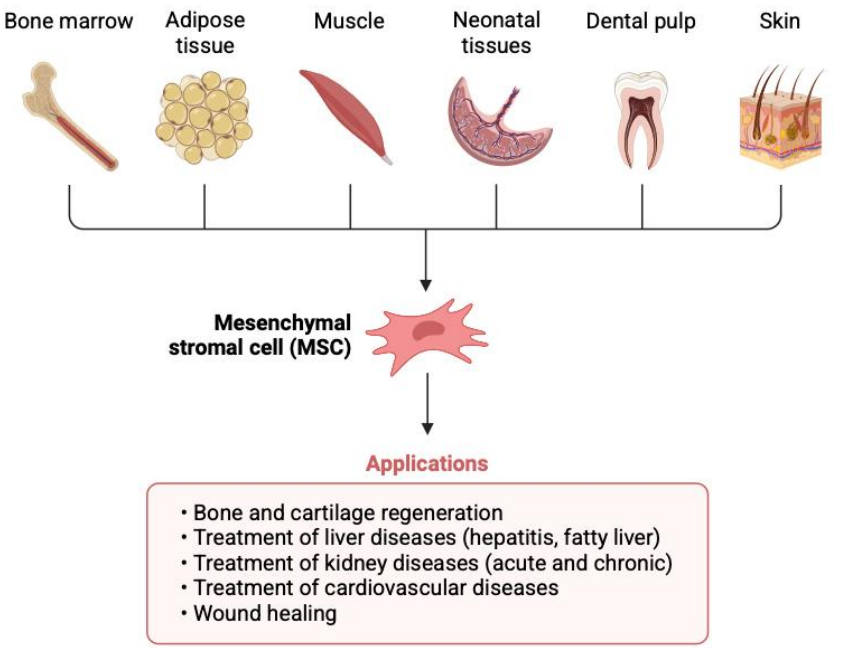
Figure 3. *Structure of chimeric antigen receptors (CAR) (created with*



1.4 A Different Tool for Gene Transfer: Adult Stem Cells

Considering the self-renewal and differentiation capabilities of stem cells, their role in tissue regeneration following damage is well understood. Furthermore, under normal conditions, where no damage has occurred, these cells perform regulatory functions that ensure the continuity of tissue and organ functions through the substances they release into the environment. However, these stem cells, particularly mesenchymal stem cells (MSCs), exhibit tumor tropism (tendency to migrate toward tumor sites) and can fuse with cancer cells under certain conditions, exerting effects on them. Despite the absence of a comprehensive understanding of the underlying mechanisms, MSC effects vary across different tumor types (De Becker & Riet, 2016: 73; Feng & Chen, 2009: 717; Marofi,

Figure 4. *Sources and clinical use of mesenchymal stem cells (created with BioRender.com).*



Vahedi, Biglari, Esmaeilzadeh & Athari, 2017: 1770; Shah, 2012: 739).

MSCs have become an attractive gene therapy target due to their natural propensity to migrate towards inflammatory and tumor microenvironments, their relatively low immunogenicity profiles, and their suitability for *ex vivo* genetic programming. These cells can exert their effects either by directly expressing therapeutic transgenes (e.g. cytokines, pro-apoptotic ligands and enzymes), or by delivering oncolytic viruses and nucleic acids (e.g. mRNA, siRNA and miRNA) to the target tissue. In brain tumors in particular, their ability to indirectly cross the blood–brain barrier and target the surgical cavity/residual tumor makes MSCs ideal for localized gene delivery in glioma. However, the context-dependent nature of the interaction between MSCs and tumors, and the potential for pro-tumor effects, necessitates careful selection of indications and engineering strategies (Antoon, Overdevest, Saleh & Keating, 2024: 3545; Lan, Luo & Wei, 2021: 195).

In the perspective of oncology, glial tumors are one of the area's most intensively studied for MSC-based gene therapies. A phase I study was conducted to evaluate the intertumoral administration of patient-derived adipose MSCs carrying the adenoviral thymidine kinase (HSV-TK) gene in recurrent glioblastoma. The study demonstrated the technical feasibility and acceptable safety of the approach, and also showed prolonged progression-free survival in some patients (ADSC-HSV-TK + ganciclovir) (Al-kharboosh et al., 2020: 443). A clinical trial is currently underway in glioblastoma, investigating the delivery of oncolytic adenovirus DNX-2401 loaded into mesenchymal stem cells (MSCs). In this study, the MSCs' objective is to deliver the virus, thereby enhancing its spread into tumor tissue (Chen, Ene, Lang & Kan, 2022: 533). MSC-derived EVs (exosomes/vesicles) have also emerged as a cell-free, potentially safer option for gene

delivery. For example, studies have demonstrated target-suppressive effects in glioma models with miR-124-loaded MSC-EVs (Katakowski et al., 2013: 201).

CONCLUSION

The potential of gene therapy can only be realized through the translation of that potential into tangible clinical benefits. In order to achieve such benefits, it is necessary to establish a safe, effective and sustainable treatment platform, as well as to achieve permanent and long-term therapeutic effects. To achieve this objective, it is imperative to comprehensively understand the biological limitations, immune response risks, vector-derived toxicities, off-target genetic alterations, and technical challenges related to the production and application processes that hinder gene therapy approaches. In addition to these obstacles, the development of innovative strategies to prevent or minimize potential side effects is an indispensable step in improving treatment safety. However, the safety and efficacy parameters, which are decisive factors in the clinical success of any treatment approach, require even more careful evaluation in the context of gene therapy. It is imperative to precisely identify the therapeutic genes that have the potential to halt or reverse the progression of the disease. In addition, it is crucial to ensure the delivery of these genes to target cells and tissues with high specificity to achieve long-term expression. These factors are instrumental in determining the success of clinical interventions over an extended period. Consequently, future advances in gene therapy

will depend not only on technological innovations, but also on safety, efficacy, and clinical applicability.

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CHAPTER 4

LÖSEMİ HÜCRE HATLARINDA KAFEİK ASİT FENETİL ESTER (CAPE)'İN İLAÇ DİRENCİ ÜZERİNE ETKİSİ

MAHMUT SAMİ İNCE[1]

Giriş

Akut myeloid lösemi (AML), hematopoetik kök hücrelerin farklılaşmasındaki bozukluk ve kontrolsüz çoğalma sonucunda ortaya çıkan, blastik hücrelerin baskın olduğu bir hastalıktır (Shipley & Butera, 2009: 649). Hastalığın tedavisinde kullanılan standart kemoterapötik yaklaşımlar başlangıçta etkili olsa da, birçok olguda tedaviye yanıtsızlık veya erken nüks gözlenmektedir. Bu olumsuz tablonun en önemli nedenlerinden biri çoklu ilaç direncidir (multidrug resistance, MDR). MDR; hücre içine giren ilaçların etkinliğini azaltan çeşitli biyolojik mekanizmaları içerir. MDR1 geni tarafından kodlanan P-glikoprotein başta olmak üzere MDR ilişkili protein, akciğer direnç proteini ve meme kanseri direnç proteini gibi efluks pompaları ilaçların hücre dışına atılmasını sağlar. Ayrıca topoizomeraz ve glutatyon sentetaz enzim aktivitelerindeki artış ve anti-apoptotik bcl-2 geninin aşırı ekspresyonu da sitotoksik etkilere

[1] Uzman Doktor, Konya Şehir Hastanesi, İç Hastalıkları ve Yoğun Bakım, Orcid: 0000-0003-4832-1404

karşı direncin artmasına katkıda bulunmaktadır (Wood & ark., 1994: 504).

Doğal ürünlerin antikanser potansiyeli üzerine yapılan araştırmalar son yıllarda giderek artmıştır. Bu ürünlerden biri olan propolis, bal arıları tarafından toplanan reçineli bir maddedir ve bünyesinde pek çok biyolojik açıdan aktif bileşen barındırır. Propolisin en dikkat çekici bileşenlerinden biri kafeik asit fenetil ester (CAPE)'dir. CAPE'in antienflamatuar, antiviral ve antikanser özellikleri pek çok hücre kültürü modelinde gösterilmiştir. HeLa, HL60 ve MCF7 hücre hatlarında yapılan deneysel çalışmalar, CAPE'in oksidatif stres mekanizmaları üzerinden antikanser etki gösterebildiğini ortaya koymuştur. Bunun yanında, CAPE'in HL60 hücrelerinde apoptozu uyardığı, bu etkiyi de bcl-2 ekspresyonunu azaltarak, bax ekspresyonunu artırarak ve kaspaz-3 aktivasyonunu tetikleyerek gerçekleştirdiği rapor edilmiştir (Son & Lewis, 2002: 468; Chen & ark., 2001: 5615; Borrelli & ark., 2002: 38; Bhimani & ark., 1993: 4528)

Bu bağlamda, çalışmamızda CAPE'in HL60, K562 ve NB4 lösemik hücre hatları üzerindeki etkilerinin incelenmesi ve özellikle ilaç direncinin aşılmasındaki rolünün değerlendirilmesi amaçlanmıştır.

Materyal ve Yöntem

Bu çalışmada üç farklı insan lösemik hücre hattı kullanıldı. HL60 hücre hattı akut promiyelositik lösemi tanılı bir hastadan elde edilmiş olup in vitro koşullarda farklılaşabilme özelliği taşımaktadır (Gallagher & ark., 1979: 713). NB4 hücre serisi, t(15;17) translokasyonu taşıyan insan akut promiyelositik lösemi olgusundan türetilmiş olup retinoik asit ile granülositik maturasyon göstermektedir (Lanotte & ark., 1991: 1080). K562 hücreleri kronik myeloid löseminin blastik kriz fazından türetilmiş, philadelphia

kromozomu pozitif olup bcr/abl füzyon geni ekspresyonu nedeniyle apoptoza dirençlidir (Lozzio & Lozzio, 1975: 321).

Hücreler %10 fetal bovin serum, %1 L-glutamat ve %1 penisilin/streptomisin içeren RPMI-1640 besiyerinde, %5 CO_2 içeren 37 °C'deki inkübatörde kültüre edildi.

Hücre sayımı için 5 mL hücre süspansiyonu 1200 rpm'de 5 dakika santrifüj edildi, süpernatant uzaklaştırıldı ve pellet 3 mL ortam içinde yeniden süspanse edildi. Daha sonra 50 µL hücre süspansiyonu ile 50 µL tripan mavisi karıştırıldı. Bu karışımdan 10 µL Thoma lamına alınarak hücre sayımı yapıldı. Mililitre başına hücre sayısı şu formül ile hesaplandı: Hücre/mL = Hücre sayısı × 10^4 × 2 (dilüsyon katsayısı).

Hücre canlılığının değerlendirilmesi için 25000 hücre/mL yoğunluğunda süspansiyon hazırlandı ve opak duvarlı 96 kuyucuklu plakalara ekildi. Azasitidin, desitabin, sitarabin (ara-C) ve CAPE farklı dozlarda eklendi ve hücreler 37 °C'de 4 gün inkübe edildi. Sonrasında her kuyucuğa 50 µL CellTiter-Glo® çözeltisi eklendi ve oda sıcaklığında 5 dakika inkübe edildi. Hücre canlılığı, üretici firmanın önerileri doğrultusunda CellTiter-Glo® lüminesans testi ile değerlendirildi.

İstatistiksel analizler GraphPad Prism v6 yazılımı ile yapıldı. Karşılaştırmalarda Student t-testi kullanıldı ve $p<0.05$ anlamlı kabul edildi.

Bulgular

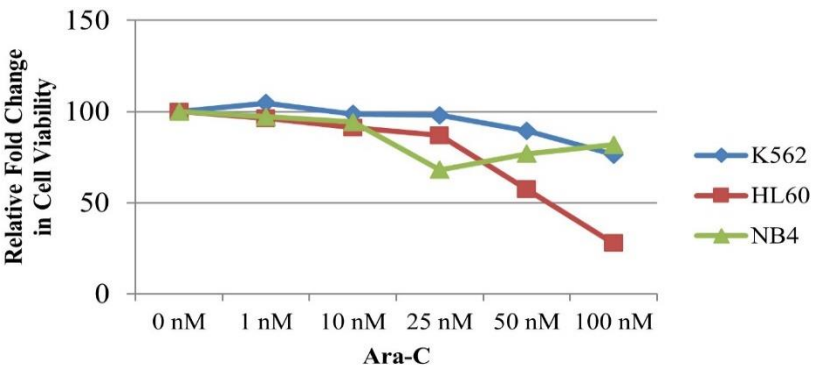
Çalışmada ilk olarak ara-C uygulamasının hücre canlılığı üzerine etkisi incelendi (Tablo 1). K562 hücrelerinde 100 nM ara-C uygulamasından sonra canlılık %76,40, NB4 hücrelerinde %81,6 olarak belirlendi. HL60 hücrelerinde ise 50 nM konsantrasyonda canlılık %57,39 düzeyine indi. Bu bulgular, özellikle K562 hattında

olmak üzere tüm hücre serilerinin Ara-C'ye karşı belirgin direnç geliştirdiğini ortaya koydu (Şekil 1).

Tablo 1. Farklı Ara-C Dozlarında Hücre Canlılığı

Ara-C Konsantrasyonu	Hücre Hatları % Canlılık ± SD (Standart Sapma)		
	K562	HL60	NB4
0 nM	100±3.57	100.00±12.09	100±21.86
1 nM	104.64±10.08	96.27±38.24	97.16±2.69
10 nM	98.68±14.39	91.14±30.88	94.33±3.97
25 nM	97.99±0.71	86.94±6.66	68.14±12.42
50 nM	89.45±5.30	57.39±6.09	76.89±10.47
100 nM	76.40±4.03	27.81±3.06	81.86±20.14

Şekil 1. Ara-C Dozlarına Bağlı Hücre Canlılığı Değişimi (Rölatif Kat Artışı)

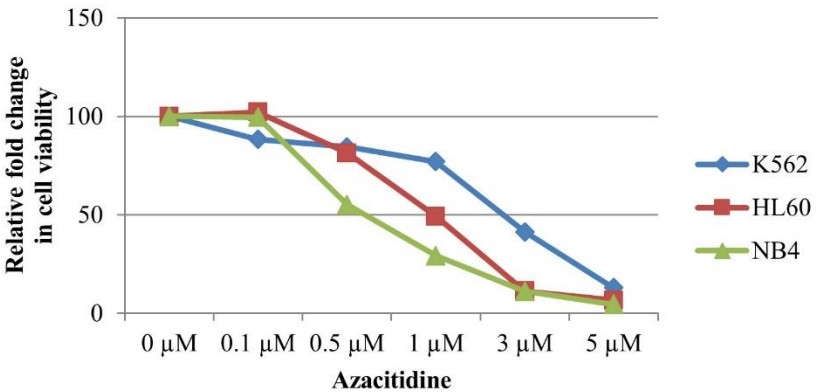


Azasitidin uygulamasında 1 µM konsantrasyon, HL60 ve NB4 hücrelerinde canlılığı sırasıyla %49,29 ve %29,2'ye düşürürken, K562 hücrelerinde %77,01 düzeyinde korundu (Tablo 2). Böylece Ara-C sonuçlarına benzer biçimde K562 hücrelerinin azasitidine karşı da daha dirençli olduğu saptandı (Şekil 2).

Tablo 2. Farklı Azasitidin Dozlarında Hücre Canlılığı

Azasitidin Konsantrasyonu	Hücre Hatları % Canlılık ± SD		
	K562	HL60	NB4
0 µM	100±8.97	100±1.27	100±47.34
0.1 µM	88.34±1.56	102.14±13.07	99.63±60.86
0.5 µM	84.56±8.67	81.32±21.59	55.20±26.06
1 µM	77.01±7.73	49.29±6.10	29.20±8.39
3 µM	41.37±12.45	11.25±1.21	11.09±2.45
5 µM	12.98±3.71	6.60±0.48	4.54±0.57

Şekil 2. Azasitidin Dozlarına Bağlı Hücre Canlılığı Değişimi (Rölatif Kat Artışı)

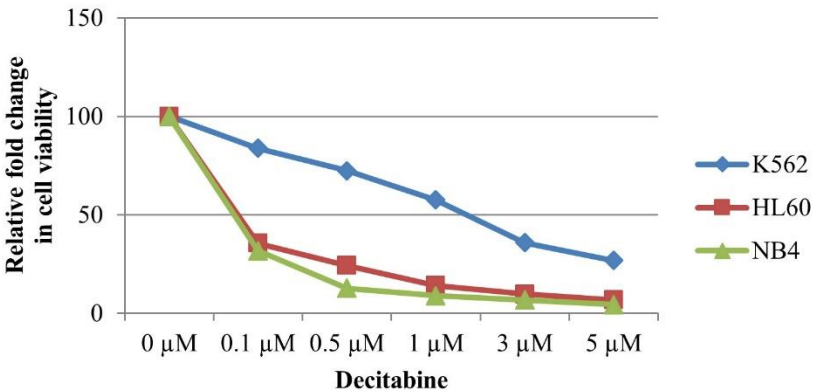


Desitabin uygulamalarında da benzer bir eğilim gözlendi. 0,5 µM dozunda HL60 ve NB4 hücrelerindeki canlılık sırasıyla %24,26 ve %12,71'e kadar azalırken, K562 hücrelerinde %72,30 düzeyinde kaldı (Tablo 3). Daha düşük doz olan 0,1 µM desitabin, HL60 ve NB4'te %50'nin üzerinde canlılık azalmasına yol açarken, K562 hücre hattı bu koşullarda da direnç gösterdi (Şekil 3).

Tablo 3. Farklı Desitabin Dozlarında Hücre Canlılığı

Desitabin Konsantrasyonu	Hücre Hatları % Canlılık ± SD		
	K562	HL60	NB4
0 µM	100.00±16.77	100.00±14.96	100.00±11.68
0.1 µM	83.73±20.15	35.49±10.41	31.70±6.98
0.5 µM	72.30±10.98	24.26±1.97	12.71±4.10
1 µM	57.49±17.79	14.06±4.11	8.89±2.56
3 µM	35.81±12.47	9.75±1.88	6.84±1.34
5 µM	26.82±6.91	6.84±0.55	4.34±0.47

Şekil 3. Desitabin Dozlarına Bağlı Hücre Canlılığı Değişimi (Rölatif Kat Artışı)

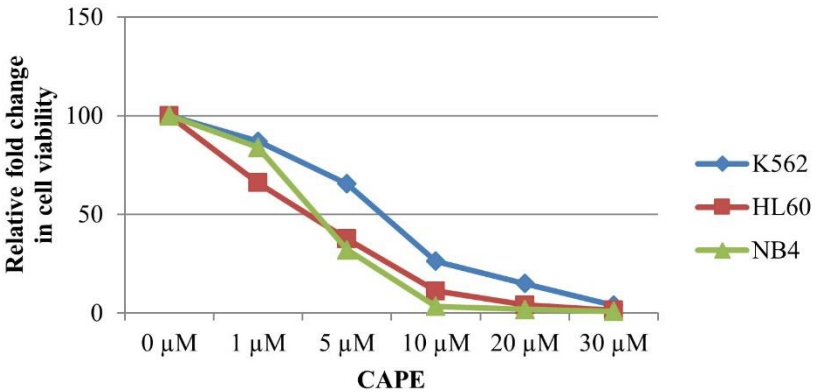


CAPE tedavisi tüm hücre serilerinde canlılığı anlamlı ölçüde azalttı. 5 µM konsantrasyonda K562'de canlılık %65,41 iken, HL60 ve NB4 hücrelerinde sırasıyla %37,54 ve %32,51 olarak saptandı (Tablo 4). Bu konsantrasyon, HL60 ve NB4 hücrelerinde %50'nin üzerinde azalma sağlarken, K562'de benzer bir etkinin ortaya çıkması için yaklaşık 7–8 µM düzeylerine ihtiyaç duyuldu (Şekil 4). Bu nedenle 5 µM CAPE, kombine ilaç deneyleri için uygun bir konsantrasyon olarak kabul edildi.

Tablo 4. CAPE Dozlarına Bağlı Hücre Canlılığı

CAPE Konsantrasyonu	Hücre Hatları % Canlılık ± SD		
	K562	HL60	NB4
0 µM	100±23.75	100±7.86	100±45.71
1 µM	87.03±13.25	65.98±16.82	83.86±19.01
5 µM	65.41±10.45	37.54±4.87	32.01±0.79
10 µM	26.21±5.68	11.14±1.62	3.41±0.14
20 µM	14.97±0.96	4.13±0.07	1.89±0.68
30 µM	3.90±0.19	1.49±0.23	0.82±0.08

Şekil 4. Farklı Dozlarda CAPE'in Hücre Canlılığı Üzerine Etkisi



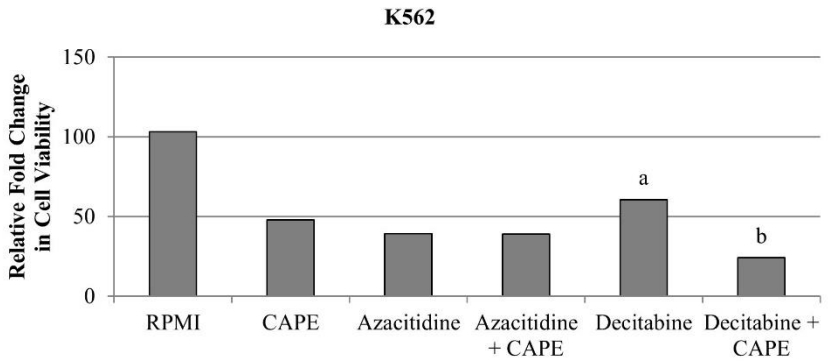
Dirençli olduğu bulunan K562 hücre hattında desitabin ile CAPE birlikte uygulandığında canlılık %60,65'ten %23,99'a düştü ve bu azalma istatistiksel olarak anlamlı bulundu ($p<0.05$). Buna karşın, azasitidin ile CAPE kombinasyonu benzer bir etki göstermedi (Tablo 5). Desitabin ile CAPE'nin birlikte kullanımı, desitabin tek başına uygulandığında elde edilenden daha belirgin bir canlılık azalması oluşturdu (Şekil 5).

Tablo 5. Sitozin Nükleozid Analogları ile CAPE'in Birlikte Kullanımının Hücre Canlılığı Üzerine Etkisi

	K562 Hücre Hattı % Canlılık ± SD
RPMI	103.07±5.74
CAPE (5 µM)	47.88±3.29
Azasitidin (1 µM)	39.23±14.46
Azasitidin + CAPE	38.99±7.69
Desitabin (1 µM)	60.65±1.20[a]
Desitabin + CAPE	23.99±3.44[b]

a-b: $p<0.05$

Şekil 5. K562 Hücre Serisinde CAPE'nin Azasitidin ve Desitabine Direnç Üzerine Etkisi



Ara-C'ye dirençli hücrelerde CAPE eşliğinde tedavi canlılık üzerinde dramatik bir azalma yarattı. K562 hücrelerinde canlılık %66,30'dan %16,88'e, HL60 hücrelerinde ise %83,24'ten %10,05'e geriledi (Şekil 6–7). NB4 hücrelerinde Ara-C tek başına anlamlı bir etki oluşturmazken, CAPE ile birlikte uygulandığında canlılık %42,72'ye düştü (Tablo 6, Şekil 8). Bu azalmalar Ara-C tekli tedavi

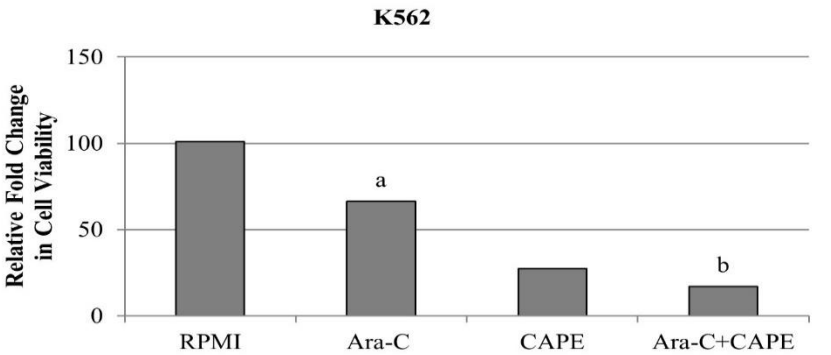
ile karşılaştırıldığında istatistiksel olarak anlamlı bulundu (sırasıyla $p<0.01$; $p<0.001$ ve $p<0.05$).

Tablo 6. CAPE ve Ara-C Kombinasyonunun Hücre Canlılığına Etkisi

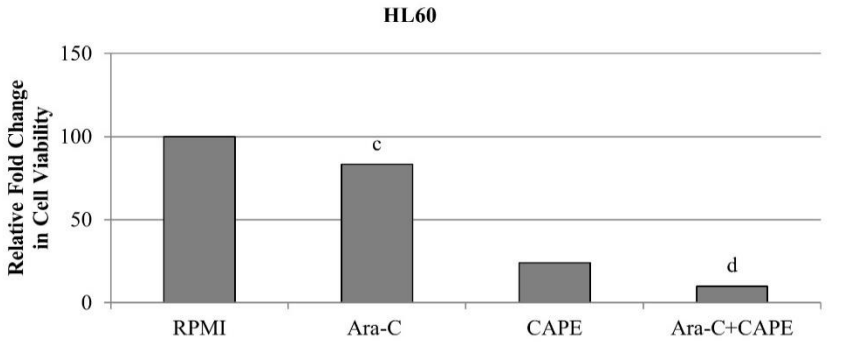
	Hücre Hatları % Canlılık ± SD		
	K562	HL60	NB4
RPMI	101.15±4.51	100.00±10.16	100.04±18.73
Ara-C (25 nM)	66.30±1.27[a]	83.24±13.09[c]	122.25±31.7[e]
CAPE (5 µM)	27.44±2.54	23.91±1.52	76.28±5.18
Ara-C+CAPE	16.88±3.62[b]	10.05±1.55[d]	42.72±5.05[f]

a-b, c-d: $p<0.001$; e-f: $p<0.05$

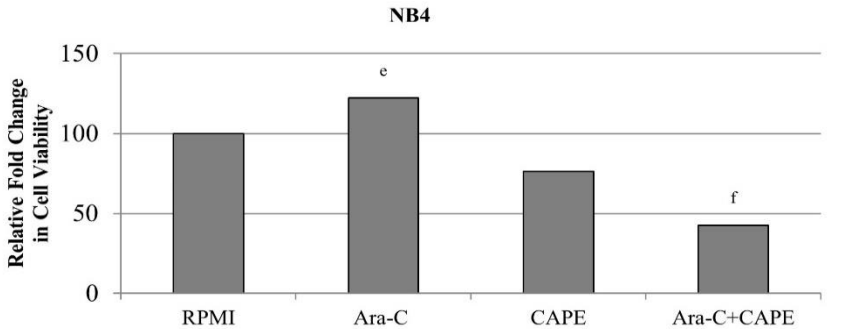
Şekil 6. K562 Hücre Serisinde Ara-C ve CAPE'in Kombine Kullanımının Hücre Canlılığı Üzerine Etkisi



Şekil 7. HL60 Hücre Serisinde Ara-C ve CAPE'in Kombine Kullanımının Hücre Canlılığı Üzerine Etkisi



Şekil 8. NB4 Hücre Serisinde Ara-C ve CAPE'in Kombine Kullanımının Hücre Canlılığı Üzerine Etkisi



Tartışma

Akut myeloid lösemi (AML), genetik ve epigenetik değişiklikler sonucu kemik iliği, kan ve diğer dokularda blast hücrelerinin birikimi ile karakterize ciddi bir hematolojik malignitedir. Hastaların yaklaşık %30'u genç erişkinlerde, %50'si

ise ileri yaş grubunda uygulanan tedavilere yanıt verememektedir (Shipley & Butera, 2009: 649). Goldie ve Coldman dirençli hücrelerin tanı anında mevcut olduğunu, tedaviye duyarlı hücreler elimine edildikçe bu klonların çoğaldığını ve hastalık seyrini belirlediğini göstermiştir (Goldie & Coldman, 1983: 923). Tedaviye yanıtsızlığın birçok hasta ve hastalık ilişkili faktöre bağlı olduğu bilinmekle birlikte, ilaç direnci başarısızlığın en önemli nedenlerinden biri olarak öne çıkmaktadır (Mesa & Tibes, 2012: 1496).

Ara-C, AML tedavisinde yaygın olarak kullanılan nükleozid analoglarından biridir. DNA polimeraz inhibisyonu veya DNA zincirine bağlanarak uzamayı durdurması aracılığıyla etki gösterir (Johnson, 2001: 929). Direnç mekanizmalarının tam olarak açıklanamamış olmasına karşın, ilacın aktif metaboliti olan ara-CTP düzeyini azaltan mekanizmaların temel rol oynadığı bildirilmektedir. HL60 ve K562'nin de yer aldığı beş farklı hücre hattında yapılan bir çalışmada, ara-C'ye dirençli alt klonların varlığı saptanmış; membran taşıyıcı kapasitelerinde azalma, fosforilasyonda hız kısıtlayıcı enzim olan deoksisitidin kinaz (DCK) aktivitesinde düşüş ve defosforilasyondan sorumlu sitozolik nükleotidaz-II (CN-II) aktivitesinde artış belirlenmiştir. Özellikle ara-C'nin hücre içine girişini sağlayan hENT1 taşıyıcısındaki azalma, DCK aktivitesindeki düşüş ve CN-II aktivitesindeki artış direnç gelişiminde kritik faktörler olarak tanımlanmıştır (Negoro & ark., 2011: 911).

Son yıllarda AML tedavisinde azasitidin ve desitabin gibi sitozin nükleozid analogları da kullanılmaya başlanmıştır. Bu ajanlar düşük dozlarda DNA'ya entegre olarak DNA metiltransferazları geri dönüşsüz şekilde bağlar, böylece hipometilasyon oluşturarak tümör baskılayıcı genlerin yeniden aktivasyonuna yol açar (Jones & Baylin, 2002: 415; Lübbert & Minden, 2005: 38; Yoo & Jones, 2006:

37). Bu mekanizma sayesinde yoğun tedavi alamayan hastalarda mortalite ve morbiditede azalma sağlanabilmektedir.

Bizim çalışmamızda da literatür ile uyumlu şekilde HL60, NB4 ve özellikle K562 hücrelerinin Ara-C'ye dirençli olduğu görüldü. Bcr/abl füzyon geninin eksprese edildiği K562 hattındaki direnç, Philadelphia kromozomu pozitif akut lösemilerde tedaviye yanıtsızlığı açıklayan mekanizma ile tutarlıdır. Nitekim U937 ve HL60 hücrelerinde Ara-C sonrası apoptoza özgü morfolojik değişiklikler rapor edilirken, K562'de benzer bulgulara rastlanmamıştır (Akiyama & ark., 1999: 774).

Azasitidin ve desitabin tedavilerine karşı HL60 ve NB4 hücrelerinde belirgin canlılık azalması gözlenirken, K562 hattında direnç devam etmiştir. Özellikle desitabinin düşük dozlarda daha güçlü etkinlik göstermesi literatürle uyumludur. Hollenbach ve ark., HL60 ve THP-1 hücrelerinde yaptıkları karşılaştırmada her iki ajanın da DNMT-1 yıkımına, DNA hipometilasyonuna ve DNA hasarına yol açtığını, ancak bu etkinin desitabinle 2–10 kat daha düşük konsantrasyonlarda elde edildiğini göstermiştir. Bununla birlikte azasitidinin 1 μM üzerindeki dozlarda daha etkili olduğu, total protein sentezini azalttığı, RNA'ya inkorporasyonu nedeniyle hücre döngüsü üzerinde farklı etkiler gösterdiği bildirilmiştir (Hollenbach & ark., 2010: e9001).

Çalışmamızda doğal bir bileşik olan propolisin etkin maddesi CAPE'nin, HL60 ve NB4 hücrelerinde 5 μM gibi düşük konsantrasyonlarda, K562'de ise 7–8 μM düzeylerinde canlılığı azalttığı belirlendi. Ara-C, azasitidin ve desitabine dirençli K562 hattında dahi CAPE'nin etkili olması, farklı mekanizmalar aracılığıyla dirençli hücrelerde de etkinlik gösterebileceğini düşündürdü. Literatürde CAPE'nin glioma, meme kanseri, melanom ve T hücreli ALL dahil farklı hücre hatlarında düşük mikromolar konsantrasyonlarda antiproliferatif etki gösterdiği rapor edilmiştir (Avci & ark., 2011: 41; Brudzynski & Carlone, 2004: 389; Lee &

ark., 2003: 2281; Rossi & ark., 2002: 530). Bu veriler CAPE'nin farklı yolaklar üzerinden antikanser etki gösterebileceğini desteklemektedir.

Bizim bulgularımızda da CAPE'nin Ara-C, azasitidin ve desitabin ile birlikte kullanılması, özellikle K562'de desitabin kombinasyonu dışında tüm dirençli hücre hatlarında canlılık azalmasını belirgin şekilde artırdı. Literatürde CAPE'nin bu ilaçlarla kombinasyonuna ilişkin çalışmaya rastlanmamıştır. Elde edilen sinerjik etkinin apoptoz yolakları, antioksidan mekanizmalar ve çoklu ilaç direnç genleri üzerindeki etkilerinden kaynaklanabileceği düşünülmektedir. Nitekim CAPE'nin HL60 hücrelerinde DNA fragmantasyonu, bcl-2 baskılanması, bax artışı ve kaspaz-3 aktivasyonu yoluyla apoptoz indüklediği; glutatyon düzeylerini azaltarak proliferasyonu engellediği ve NF-κB yolaklarını inhibe ettiği gösterilmiştir (Chen, Shiao & Wang, 2001: 143; Son & Lewis, 2002: 468; Lee & ark., 2008: 987). Ayrıca CAPE'nin MDR1 ekspresyonunu baskıladığı ve bu yolla kemoterapiye duyarlılığı artırabileceği de rapor edilmiştir (Omene, Wu & Frenkel, 2012: 1279).

Desitabin ile CAPE kombinasyonu dirençli hücrelerde belirgin bir duyarlılık sağlarken, azasitidin ile aynı etkinin görülmemesi bu iki ajanın mekanizmalarının farklı olduğuna işaret etmektedir. Desitabin ve Ara-C'nin hücre içinde benzer yollarla aktif metabolitlerine dönüştüğü, DCK aktivitesine bağımlı oldukları ve özellikle bcr/abl füzyonu ile tirozin kinaz aktivitesi artmış K562 hattında direnç gelişiminin daha belirgin olduğu bilinmektedir (Qin & ark., 2007: 4225). Buna karşın azasitidine direnç gelişiminde G2-M kontrol noktasının yeniden yapılanması ve UCK-2 mutasyonunun rol oynadığı gösterilmiştir (Sripayap & ark., 2014: 294). Çalışmamızda CAPE'nin bu farklı mekanizmalara etki ederek direnç gelişimini engelleyebileceği ve özellikle tirozin kinaz aktivitesi artmış hücrelerde ilaca duyarlılığı artırabileceği öne sürülmektedir.

Sonuç olarak, CAPE’nin HL60, NB4 ve K562 hücre serilerinde proliferasyonu baskıladığı, tek başına veya özellikle desitabin ve Ara-C ile birlikte kullanıldığında direnç mekanizmalarını kırarak tedavi etkinliğini artırdığı saptandı. Bu veriler, CAPE’nin gelecekte dirençli lösemilerde alternatif tedavi stratejilerinde değerlendirilebilecek güçlü bir aday olabileceğini göstermektedir.

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