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MASTER THESIS

INVESTIGATION OF ANTICANCER ACTIVITIES OF POTENTIALLY ACTIVE SMALL MOLECULE DRUG CANDIDATE MOLECULES

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Esranur AYDIN

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With much love and gratitude,

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LIST OF ABBREVIATIONS

ALK:	Anaplastic Lymphoma Kinase
AR:	Androgen Receptor
ATCC:	American Type Culture Collection
BRCA:	Breast Cancer Susceptibility
CAR-T Therapy:	Chimeric Antigen Receptor (CAR) T-cell Therapy
CT:	Computed Tomography
DCM:	Dichloromethane
DMEM:	Dulbecco's Modified Eagle Medium
DMF:	Dimethylformamide
DMSO:	Dimethyl sulfoxide
EGFR:	Epidermal Growth Factor Receptor
ER:	Estrogen Receptor
ERG:	ETS-related gene
FBS:	Fetal Bovine Serum
HBV:	Hepatitis B Virus
HCV:	Hepatitis C Virus
HER2:	Human Epidermal Growth Factor Receptor2
HPV:	Human Papillomavirus
IC-50:	Half-Maximal Inhibitory Concentration
NSCLC:	Non-Small Cell Lung Cancer
PET:	Positron Emission Tomography
PR :	Progesterone Receptor
PSA:	Prostate-Specific Antigen
PTEN:	Phosphatase and Tensin Homolog
RB:	Retinoblastoma
RPM:	Revolutions Per Minute
RPMI:	Roswell Park Memorial Institute Medium
SCLC:	Small Cell Lung Cancer
SF:	Serum-Free

TLC: Thin Layer Chromatography

TMPRSS2: Transmembrane Serine Protease 2

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1. ABSTRACT

INVESTIGATION OF ANTICANCER ACTIVITIES OF POTENTIALLY ACTIVE SMALL MOLECULES

Cancer is defined as the uncontrolled division and proliferation of cells in any part of the body. The most frequently seen cancers are lung, breast, and prostate cancer. Small molecules can be used for treatment of cancer. Hydrazones are related to aldehydes and ketones in structure. Oxadiazoles are heterocyclic, aromatic chemical compounds. These structures are present in already available small molecule anticancer drugs in the market. The aim of this project is to synthesize novel oxadiazole and hydrazone compounds and to determine the anticancer activities of them. In this study, fifteen new hydrazones and twelve new 1,3,4 oxadiazoles were synthesized. Hydrazones were used as starting molecules in order to obtain those 1,3,4 oxadiazoles. The anticancer activity of these compounds was investigated in vitro on A-549, MDA-MB-231, and PC-3 cell lines. MRC-5 was used as control. As a result of the study, the most promising molecules among the twenty-seven molecules were determined as AA-10, AA-9 and AA-26. When the activities of these molecules are examined, for the A-549 cell line, AA-10 coded hydrazone had the minimum IC-50 value of 13.39 μM. For the MDA-MB-231 cell line, AA-32 coded oxadiazole demonstrated the lowest IC-50 value with 22.73 μM. For PC-3, the hydrazone AA-9 showed activity with the lowest concentration of 9,389 μM. Most potent compounds were selected based on their minimum IC-50 values, not based on their specific activities to a single cell line. Toxicity levels on control group was also considered as a factor. A scratch assay was performed for A-549 and MDA-MB-231 cells, which have high migration capacity, for the 3 most potent molecules, and it was determined that these molecules did not show a significant antimetastatic effect.

Keywords: A-549, Anticancer Activity, Hydrazones, MDA-MB-231, Oxadiazoles, PC-3

2. ÖZET

POTANSİYEL AKTİF KÜÇÜK MOLEKÜLLERİN ANTİKANSER AKTİVİTELERİNİN İNCELENMESİ

Kanser, vücudun herhangi bir bölgesindeki hücrelerin kontrolsüz bölünmesi ve çoğalması olarak tanımlanmaktadır. En sık görülen kanser türleri akciğer, meme ve prostat kanseridir. Küöük moleküllü ilaçlar kanser tedavisinde aktif olarak kullanılmaktaır. Hidrazonlar, yapısal olarak aldehitler ve ketonlar ile benzerlik taşıyan kimyasal bileşiklerdir. Oksadiazoller ise hidrazonlar kullanılarak sentezlenebien heterosiklik, aromatik bir kimyasal bileşiklerdir. Bu yapılar piyasadaki küçük moleküllü kanser ilaçlarının yapısında hali hazırda yer almaktadırlar. Bu projenin amacı, çeşitli yeni hidrazon ve oksadiazol türevlerinin sentezlenmesi ve bu bileşiklerin antikanser aktivitelerinin belirlenmesidir. Bu çalışmada on beş yeni hidrazon ve on iki yeni 1,3,4 oksadiazol sentezlenmiştir. Bu bileşiklerin antikanser aktivitesi, A-549, MDA-MB-231 ve PC-3 hücre hatları üzerinde in vitro olarak araştırılmıştır. Kontrol grubu olarak MRC-5 kullanılmıştır. Çalışma sonucunda yirmi yedi molekül arasında en umut vaadeden moleküller AA-10, AA-9 ve AA-26 olarak belirlenmiştir. Aktiviteleri incelendiğinde A-549 hücre hattı için bulunan minimum IC50 değeri AA-10 kodlu hidrazona ait olup 13.39 μM olarak tespit edilmiştir. MDA-MB-231 hücre hattı için, AA-32 kodlu oksadiazol 22.73 μM ve PC-3 hücre hattı için AA-9 hidrazonu 9,389 uM konsantrasyonlarında en düşük aktiviteyi göstermiştir. En iyi aktiviteye sahip bileşikler seçilirken tek bir hücre hattı üzerinde gösterilen etkiden ziyada bütün kanser hatları üzerindeki genel etki ve control grubu üzerindeki toksisite düzeyi dikkate alınmıştır. En aktif 3 molekül için yüksek metastatik kapasiteye sahip olan A-549 ve MDA-MB-231 hücrelerine ayrıca yara iyileşme testi yapılmış ve bu moleküllerin hücrelerin metastatik kapasitesi üzerinde anlamlı bir etkisinin olmadığı tespit edilmiştir.

Anahtar Sözcükler: A-549, Antikanser Aktivite,Hidrazonlar, MDA-MB-231, Oksadiazoller, PC-3

3. INTRODUCTION AND PURPOSE

Cancer is the name given to a collection of diseases characterized by abnormal cells that develop and spread uncontrollably across the body. This disease might be fatal if not treated appropriately and on time. It is a complicated genetic, protein-related, and cellular condition, caused by a mix of genetic abnormalities and environmental factors (1).

Cancer is defined as unregulated cell division and proliferation in any part of the body. Many kinds of cancer develop tumors as a result of this growth. Depending on where these tumors begin in the body, there are numerous subtypes of cancer. While some traits are shared by all cancers, others are unique to specific types of cancer. Lung, breast, and prostate cancers are the most commonly seen ones (2).

According to the data given by the World Cancer Research Fund International, 18.094.716 million cancer cases were detected, and 9.884.402 individuals died from cancer worldwide in 2020 (3). In 2015, the estimated national cost of cancer care in the United States was $190.2 billion. Assuming constant prices, the anticipated cost of cancer in 2020 is $208.9 billion (4). This figure is predicted to rise to $246 billion by 2030, indicating a 34% increase (5). Those numbers reveal how important is the development of treatments against cancer.

Despite remarkable advances in cancer detection and therapy approaches in recent years, there is still an urgent need for new effective treatment options and pharmaceuticals to treat types of cancer. The discovery of more specific, and thus less dangerous, treatments for these malignancies will yield enormous benefits in the fight against cancer for millions of people all around the world. To that end, drug research and development studies against cancer are being conducted with great care both in our country and around the world.

Hydrazones are chemical compounds that are related to aldehydes and ketones in structure. Hydrazones are formed when the oxygen in aldehydes or ketones is replaced by the NNH_2 group. Anticancer (6,7), antiproliferative (8,9), antimicrobial (10,11), anti-inflammatory (12,13) and antiviral (14,15) characteristics have been demonstrated for hydrazone derivatives.

Oxadiazoles are a heterocyclic, aromatic chemical compound. They include two carbon atoms, two nitrogen atoms, and one oxygen atom. There are four isomers of oxadiazoles: 1,2,3 oxadiazoles, 1,2,4 oxadiazoles, 1,2,5 oxadiazoles, and 1,3,4 oxadiazoles. 1,3,4 of oxadiazoles have antibacterial (16,17), anti-inflammatory (18,19), antioxidant (20,21), anticancer (22,23), analgesic (19,23), and antiviral (24,25) properties.

Since these molecules show various pharmacological effects and are already in the core structures of a few pharmaceutical compounds on the market, it is possible for them to also show activity against cancer.

The aim of this project is to synthesize various new hydrazone compounds and to determine the anticancer activities of new 1,3,4-oxadiazole compounds based on these compounds.

In this project, dose, and time-dependent anticancer activity of specific 1,3,4 oxadiazole derivatives and hydrazones will be investigated in vitro in MDA-MB-231, A-549, and PC-3 lines. In this way, more detailed studies might be carried out on those candidate molecules that act well on the proliferation of cancer cells, and it will be possible to reveal which molecular pathways trigger this effect. It is also possible to synthesize different candidate molecules by taking these molecules as a starting point and modifying them according to obtained results.

It was also investigated whether these molecules have an effect on metastasis by performing a scratch test on the three molecules with the best activity.

4. LITERATURE REVIEW

4.1 Cancer

Cancer is the name given to a group of diseases caused by abnormal cells that grow and spread uncontrollably through the body. This condition can be fatal if not treated properly and on time. Cancer is a complex genetic, proteomic, and cellular disorder caused by a combination of genetic (hereditary or somatic) defects and external influences (1).

Even though all of the mechanisms that cause cancer and enable it to spread have yet to be fully understood, several risk factors that enhance cancer risk and cause bad prognosis, have been uncovered. Some of these risk factors are closely related to the individual's way of life. Risk factors such as excessive alcohol and tobacco consumption, obesity, lack of exercise, and dietary habits, and exposure to radiation and chemical carcinogens, for instance, are usually adjustable (26). With the exception of non-melanoma skin cancer, a minimum of 42% of recently diagnosed malignancies in the United States (2) or approximately 805.600 cases that will be diagnosed in 2022, are highly preventable.

However, under present circumstances, it is not possible to take precautions against inherited mutations that could cause cancer. Additionally, vaccinations against malignancies observed in the presence of infection, such as human papillomavirus (HPV), hepatitis B virus (HBV), and hepatitis C virus (HCV)-like virus-borne cancers, have been developed, and research in this field is progressing (27). Everyone is at risk of acquiring cancer, but the likelihood climbs rapidly with age; in the United States, 80 percent of cancer patients are 55 years old or older, and 57 percent are 65 years old or older (2).

Every year, the American Cancer Society publishes a report called “Cancer Statistics” that forecasts new cancer incidence and mortality rates in the United States for the upcoming year. Certain inferences are drawn in this article based on data collected by numerous partners working in various organizations around the country, and this data is analyzed by the National Center for Health Statistics.

Figure 4.1 (2) below shows the cancer diagnoses and cancer-related deaths predicted by the American Cancer Society for men and women in 2022.

	Estimated New Cases			Estimated Deaths		
	Both sexes	Male	Female	Both sexes	Male	Female
All sites	1,918,030	983,160	934,870	609,360	322,090	287,270
Oral cavity & pharynx	54,000	38,700	15,300	11,230	7,870	3,360
Tongue	17,860	12,880	4,980	2,790	1,830	960
Mouth	14,490	8,490	6,000	3,020	1,810	1,210
Pharynx	19,270	15,670	3,600	3,980	3,140	840
Other oral cavity	2,380	1,660	720	1,440	1,090	350
Digestive system	343,040	193,350	149,690	171,920	99,940	71,980
Esophagus	20,640	16,510	4,130	16,410	13,250	3,160
Stomach	26,380	15,900	10,480	11,090	6,690	4,400
Small intestine	11,790	6,290	5,500	1,960	1,110	850
Colon & rectum†	151,030	80,690	70,340	52,580	28,400	24,180
Colon	106,180	54,040	52,140			
Rectum	44,850	26,650	18,200			
Anus, anal canal, & anorectum	9,440	3,150	6,290	1,670	740	930
Liver & intrahepatic bile duct	41,260	28,600	12,660	30,520	20,420	10,100
Gallbladder & other biliary	12,130	5,710	6,420	4,400	1,830	2,570
Pancreas	62,210	32,970	29,240	49,830	25,970	23,860
Other digestive organs	8,160	3,530	4,630	3,460	1,530	1,930
Respiratory system	254,850	131,450	123,400	135,360	72,770	62,590
Larynx	12,470	9,820	2,650	3,820	3,070	750
Lung & bronchus	236,740	117,910	118,830	130,180	68,820	61,360
Other respiratory organs	5,640	3,720	1,920	1,360	880	480
Bones & joints	3,910	2,160	1,750	2,100	1,180	920
Soft tissue (including heart)	13,190	7,590	5,600	5,130	2,740	2,390
Skin (excluding basal & squamous)	108,480	62,820	45,660	11,990	8,060	3,930
Melanoma of the skin	99,780	57,180	42,600	7,650	5,080	2,570
Other nonepithelial skin	8,700	5,640	3,060	4,340	2,980	1,360
Breast	290,560	2,710	287,850	43,780	530	43,250
Genital system	395,600	280,470	115,130	68,260	35,430	32,830
Uterine cervix	14,100		14,100	4,280		4,280
Uterine corpus	65,950		65,950	12,550		12,550
Ovary	19,880		19,880	12,810		12,810
Vulva	6,330		6,330	1,560		1,560
Vagina & other genital, female	8,870		8,870	1,630		1,630
Prostate	268,490	268,490		34,500	34,500	
Testis	9,910	9,910		460	460	
Penis & other genital, male	2,070	2,070		470	470	
Urinary system	164,190	114,490	49,700	31,990	21,680	10,310
Urinary bladder	81,180	61,700	19,480	17,100	12,120	4,980
Kidney & renal pelvis	79,000	50,290	28,710	13,920	8,960	4,960
Ureter & other urinary organs	4,010	2,500	1,510	970	600	370
Eye & orbit	3,360	1,790	1,570	410	220	190
Brain & other nervous system	25,050	14,170	10,880	18,280	10,710	7,570
Endocrine system	47,050	13,620	33,430	3,330	1,650	1,680
Thyroid	43,800	11,860	31,940	2,230	1,070	1,160
Other endocrine	3,250	1,760	1,490	1,100	580	520
Lymphoma	89,010	48,690	40,320	21,170	12,250	8,920
Hodgkin lymphoma	8,540	4,570	3,970	920	550	370
Non-Hodgkin lymphoma	80,470	44,120	36,350	20,250	11,700	8,550
Myeloma	34,470	19,100	15,370	12,640	7,090	5,550
Leukemia	60,650	35,810	24,840	24,000	14,020	9,980
Acute lymphocytic leukemia	6,660	3,740	2,920	1,560	880	680
Chronic lymphocytic leukemia	20,160	12,630	7,530	4,410	2,730	1,680
Acute myeloid leukemia	20,050	11,140	8,910	11,540	6,730	4,810
Chronic myeloid leukemia	8,860	5,120	3,740	1,220	670	550
Other leukemia‡	4,920	3,180	1,740	5,270	3,010	2,260
Other & unspecified primary sites‡	30,620	16,240	14,380	47,770	25,950	21,820

Figure 4.1. Estimated Number of New Cancer Cases and Deaths By Gender In The United States in 2022 (2)

Even by looking at this table alone, the gravity of the situation becomes much more apparent. According to **Figure 4.1,** 1.918.030 new cancer cases and 609.360 cancer-related deaths are expected in the United States alone through 2022.

Lung cancer is expected to be the leading cause of death among these cancer types, with 350 fatalities every day for all genders. Furthermore, according to this study, breast cancer ranks highest with a thirty-one percent incidence rate in women and prostate cancer with twenty-one percent incidence in men among all other cancer types.

To quantify this rate according to the population of the United States in 2022, it is possible to talk about 287.850 cases of breast cancer cases that are anticipated to be newly diagnosed in women and 268.490 cases of prostate cancer that are predicted to be newly diagnosed in men. This statistic makes breast cancer the most frequently seen cancer in women at thirty percent. Moreover, when the reports issued every year are compared, it is found that the incidence of breast cancer is increasing at a rate of % 0.5 annually.

Similarly, according to the data published by the World Cancer Research Fund International, 18.094.716 million cancer cases were identified and 9.884.402 people died from cancer worldwide in 2020 (3). When these numbers are reflected in Turkey, **Figure 4.2.** below, which was developed by the Global Cancer Observatory using data from the International Agency for Research on Cancer, becomes noteworthy.

According to **Figure 4.2.** (3), lung cancer is the most common cancer with 41.264 cases in Turkey.

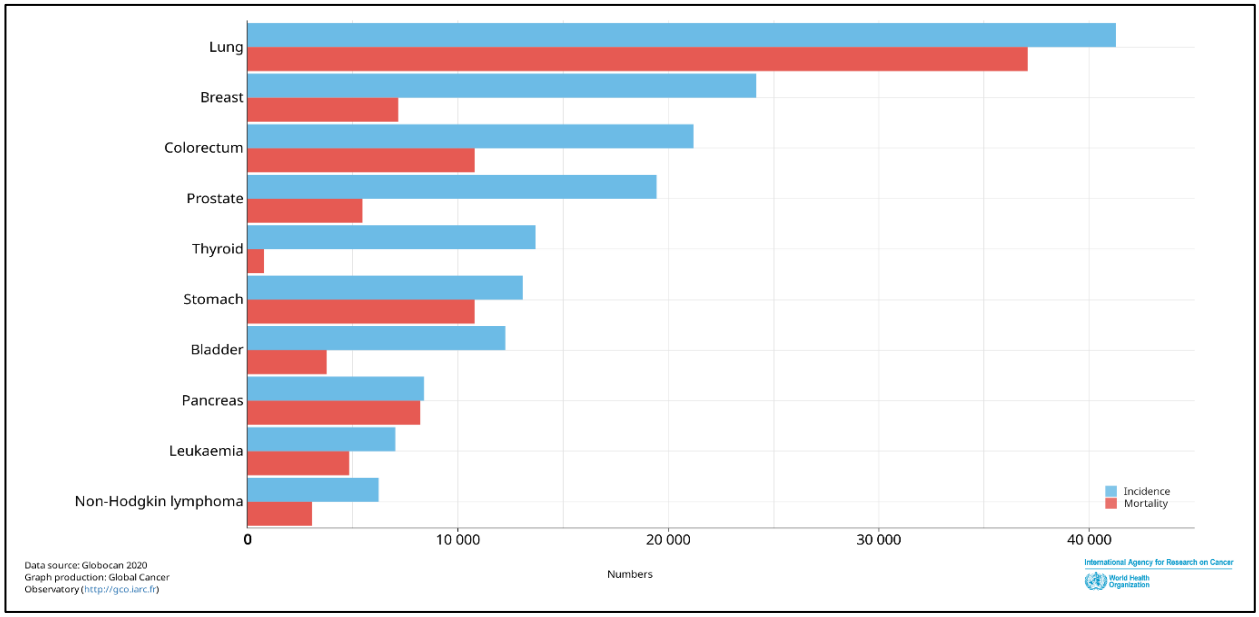


Figure 4.2. Estimated Number of Cancer Incidents and Deaths in Turkey (3)

Breast cancer comes in second with 24.147 new diagnoses, while colorectum cancer comes in third with 21.191 cases.

Lung cancer accounts for 37.070 deaths, followed by colorectum cancer and stomach cancer, each accounting for 10.798 deaths.

With these data, it can be clearly seen how much destruction cancer causes both in our country and in the world. Considering the burden of cancer on national economies, the importance of studies to develop a treatment against cancer grows even more.

In 2015, the estimated national cost of cancer care was $190.2 billion in the United States. Assuming costs remain the same, estimated costs for cancer in 2020 were given as $208.9 billion (4). This number is expected to increase to $246 billion by 2030, representing a 34% increase (5).

4.1.2 Types of cancer and representing cell lines

Cancer is the uncontrollable division and proliferation of cells in any part of the body. This proliferation causes tumor formation in many types of cancer. As these tumors grow, they may interfere with the routine functioning of tissues and organs, depending on where they form in the body.

Tumor formation can occur in many different tissues. Therefore, there are many different subtypes of cancer. While some characteristics are shared by all malignancies, others are specific to certain forms of cancer. Tumors are generally examined under two main headings: solid and nonsolid tumors. Solid tumors include cancers such as prostate, breast, and lung. Non-solid tumors, on the other hand, spread and operate without forming a solid tumor such as lymphoma (28).

4.1.2.1 Breast cancer (29–31)

The most frequent type of cancer among women is breast cancer. It has a complicated and heterogenous structure when investigated at the molecular level. Generally, these differences at the molecular level are taken into account when choosing the treatment method.

HER2 (human epidermal growth factor receptor2), ER (estrogen receptor), PR (progesterone receptor), and BRCA gene family alterations are among the clinically relevant genetic mutations reported in breast cancer. Breast cancer is investigated in three main categories, taking these mutations and genetic differences into account.

Luminal A-like: There is no HER2 receptor. ER, PR or both receptors may be present. Its proliferation is low. %70 of the breast cancer patients fall into this category

Luminal B-like: HER2 receptor might be present. ER, PR or both receptors may be present. Unlike luminal A-like breast cancer, its proliferation is high. %15-20 of the breast cancer patients fall into this category

Triple-Negative: None of the ER, PR, and HER2 receptors are absent. %15 of the breast cancer patients fall into this category. Triple-negative breast cancer is more likely to reappear than other breast cancer types.

All types of breast cancer begin in the functional part of the breast known as the terminal duct lobular. Breast cancers diagnosed at an early stage have either remained in the breast or have migrated solely to the axillary lymph nodes. Breast cancer treatment is easier and more effective at this stage.

Endocrine therapy is applied after surgical operation in patients with ER. If the risk of recurrence of the disease is high, the treatment is supported by chemotherapy. In HER2-positive or triple-negative breast cancers, chemotherapy is applied before surgical operations in accordance with the molecular profile of the cancer. If cancer has spread to the lymph nodes, radiation therapy is also among the most frequently applied treatment methods.

Treatment is more challenging in aggressive and metastatic breast tumors. In luminal-like cancer types, endocrine therapy is also used accompanying chemotherapy. Patients in the triple-negative category receive chemotherapy and immunotherapy. Some drugs are available to block the HER2 receptor when it is present. If BRCA mutations are detected in aggressive and late-diagnosed breast cancers, it is recommended to conduct a risk assessment for other members of the family.

Breast cancer treatment strategies are getting more tumor- and patient-centered and individualized by the day. Only in this manner can the most effective and long-lasting treatment be achieved.

4.1.2.1.1 MDA-MB-231

The MDA-MB-231 cell line was isolated from a white, 51 years old woman with metastatic breast adenocarcinoma. Epithelial cancer cells were isolated from the fluid accumulated between her lungs and chest cavity. ATCC reference number of MDA-MB-231 is HTB-26 (32).

In **Figure 4.3** (33) below, the microscopic image of MDA-MB-231 in the cell culture can be seen.

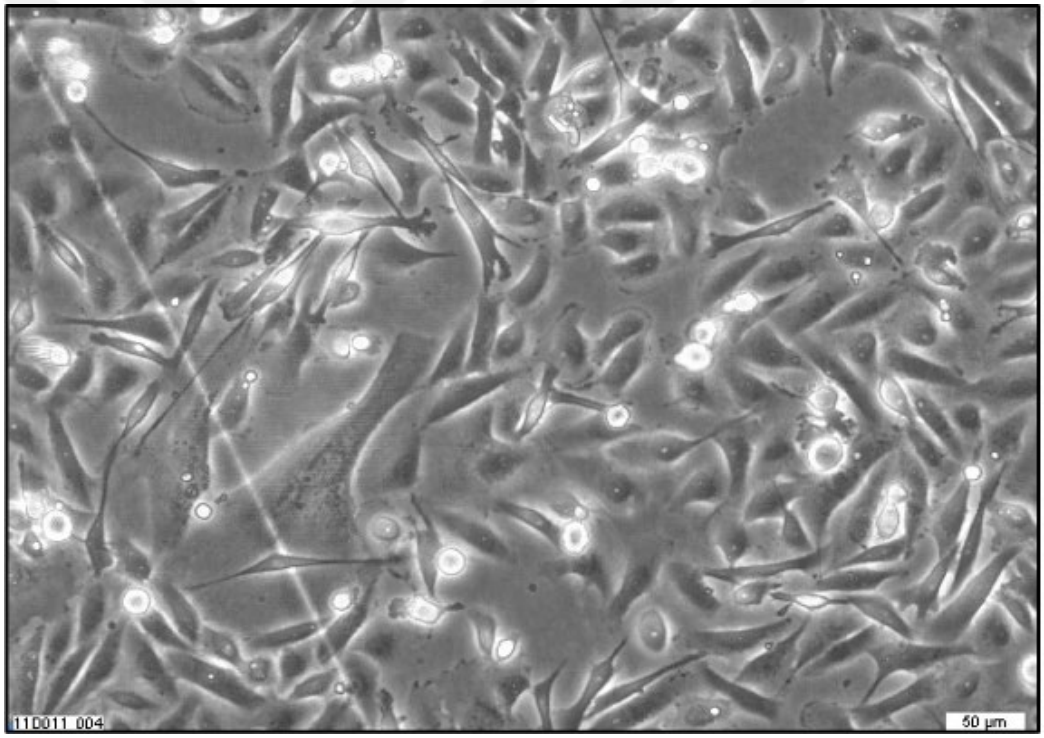


Figure 4.3. MDA-MB-231 Cells Under the Microscope (33)

This cell line is aggressive, prone to metastasis, and minimally differentiated. According to the molecular classifications of breast cancer, it belongs to the triple-negative breast cancer group (33). As a result, there is no expression of the estrogen receptor (ER), progesterone receptor (PR), or HER2 (human epidermal growth factor receptor 2) (29).

Triple-negative breast cancer is a particularly aggressive kind of breast cancer. It has few therapeutic alternatives. As a result, understanding the molecular pathways behind MDA-MB-231 and other triple-negative breast cancer cell lines is critical for developing new and improved therapeutic methods.

4.1.2.2 Prostate cancer (34)

The second most frequent type of cancer in men is prostate cancer. It is thought to be caused by mutations in epithelial cells in the prostate. The risk of prostate cancer increases with age. The great majority of cases are diagnosed in men over the age of sixty.

Mutations that cause prostate cancer occur in genes that control cell growth, proliferation, DNA damage control mechanisms, and apoptotic mechanisms. It has a rather heterogeneous structure. TMPRSS2-ERG fusion, deletions in PTEN and RB1 genes, P53 mutations, and MYC gene amplifications are among the indicators found commonly in prostate cancer. These mutations cause prostate cancer when they occur alone or in combination with other mutations. Prostate cancer typically spreads to local lymph nodes and bones.

4.1.2.2.1 PC-3

The PC-3 cell line was isolated from a white, 62 years old man with stage 4 prostatic adenocarcinoma. Epithelial cancer cells were isolated from his bone metastasis. ATCC reference number of PC-3 is CRL-1435 (35).

PC3 cells do not express androgen receptor (AR) or prostate-specific antigen (PSA) and grow in the absence of androgen. They are androgen-independent and do not express AR or PSA. They exhibit very aggressive behavior, which contrasts with the majority of clinical cases of prostate cancers (37).

In **Figure 4.4** (36) below, the microscopic image of PC-3 in the cell culture can be seen.

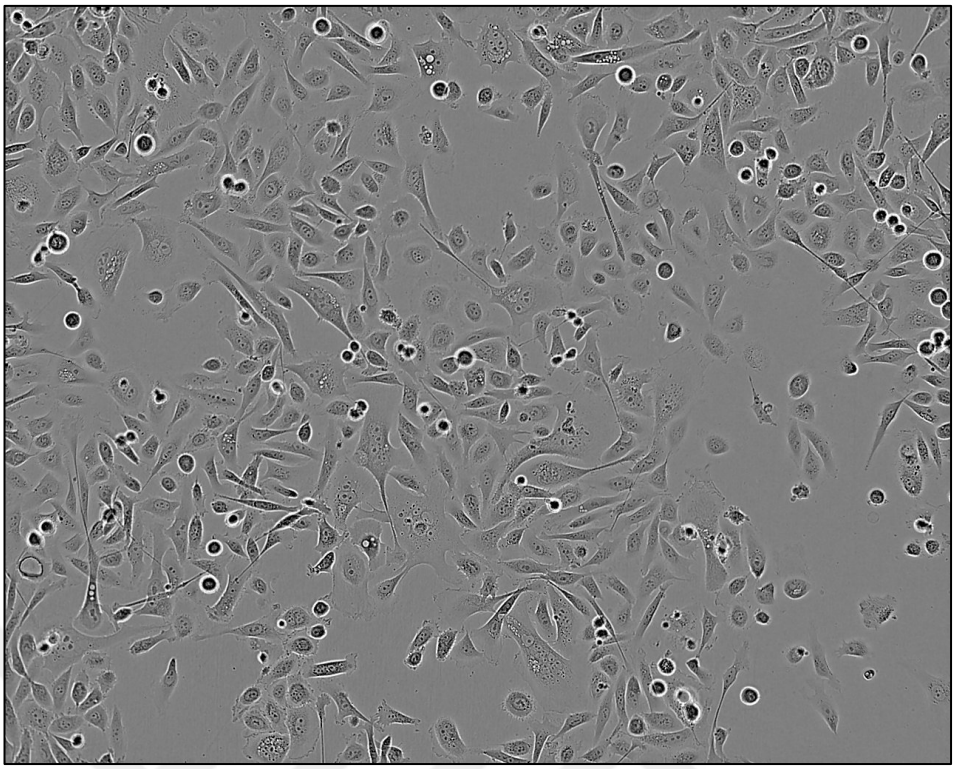


Figure 4.4 PC-3 Cells Under the Microscope (36)

4.1.2.3 Lung cancer

Lung cancer is classified into two categories. Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC).

4.1.2.3.1 Small cell lung cancer (38)

Small Cell Lung Cancer is generally more common in men than women. It can be brought on by smoking and being exposed to smoke. It progresses with symptoms such as difficulty in breathing, severe coughing, and coughing up blood.

Cancerous cells are small, and round in appearance. Cytoplasm levels are decreasing. In contrast to Non-Small Cell Lung Cancer, it is difficult to detect in the early stages of CT. If it can be diagnosed in the early stages, it is usually removed from the area by surgical intervention. Chemotherapy and radiotherapy may be used prior to surgery if considered necessary.

For metastatic versions, immunotherapy may be used in addition to chemotherapy. Inactivation of the p53 and RB tumor suppressor genes is the most common genetic alteration that causes SCLC. There is an increase in MYC gene activity, as well as modifications in autocrine and paracrine processes. Classification

is made at the molecular level by considering the expression levels of various transcription factors.

SCLC is a type of cancer with high metastatic ability. Because of its aggressive nature, the survival rate is low, and the period of survival following diagnosis is fairly short.

4.1.2.3.2 Non-small cell lung cancer (39)

Non-Small Cell Lung Cancer (NSCLC) is the most common kind of lung cancer, accounting for 85 percent of all cases. It is further classified as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.

CT and PET scans may usually be used to get a diagnosis. A biopsy is also used to confirm the diagnosis. Because it is a disease that is frequently discovered late and worsens throughout this time, the life expectancy is poor.

Although NSCLC has several genetic alterations, the most prevalent ones are mutations in EGFR (Epidermal growth factor receptor) and ALK (anaplastic lymphoma kinase).

The disease is treated with surgery, chemotherapy, immunotherapy, radiotherapy, and pharmaceuticals that target specific mutations.

4.1.2.3.3 A-549

The A-549 cell line was isolated from a white, 58 years old man with stage 4 prostatic adenocarcinoma. It is a human non-small cell lung cancer cell line. ATCC reference number of A-549 is CCL-185 (40).

In **Figure 4.5.** (41) below the microscopic image of A-549 in the cell culture can be seen.

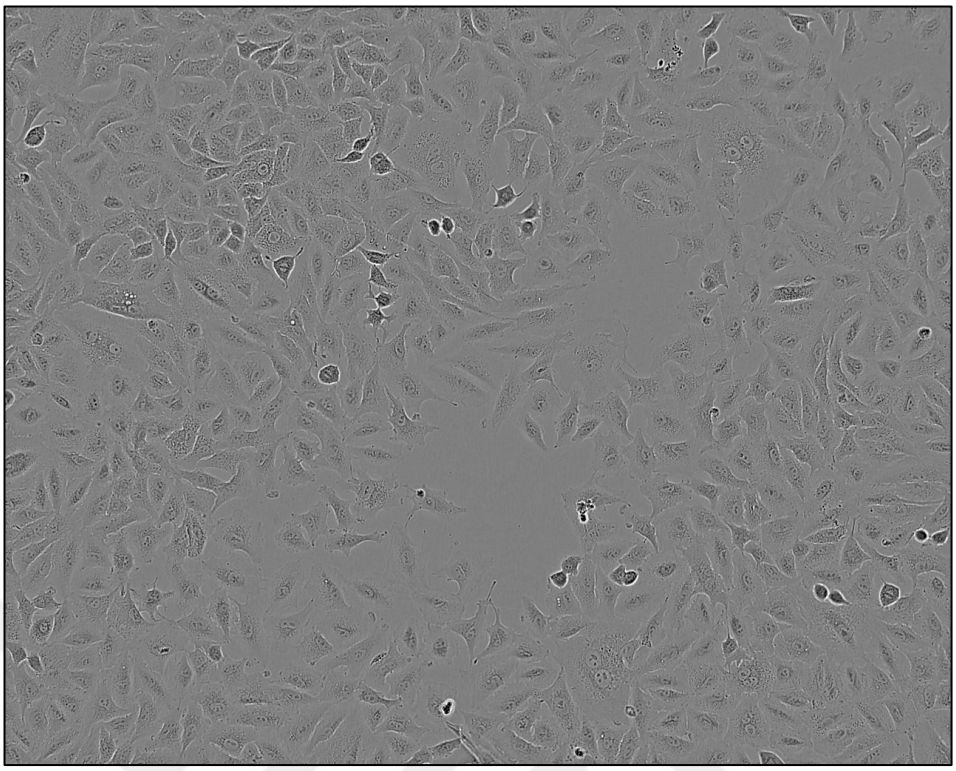


Figure 4.5. A-549 Cells Under the Microscope (41)

4.1.3 Treatment

Despite tremendous breakthroughs in cancer detection and therapeutic approaches in the previous years, there is still a compelling need for more efficient therapies and pharmaceuticals in order to treat various cancers. The development of more precise, and thus less harmful, medicines for these malignancies will give considerable benefits in the fight against cancer (42). In line with this goal, drug discovery and development studies against cancer continue with great care, both in our country and around the world.

There are many different cancer therapy options and procedures available today. The best treatment strategy is chosen from among these options based on the type of cancer addressed, how cancer progresses, and what is expected at the end of the treatment.

Some therapeutic approaches are only affecting a specific area. Surgical procedures and radiation therapy are two examples of those local therapies. Chemotherapy, immunotherapy, and certain pharmaceuticals are also commonly used

against cancer. However, these methods are affecting the entire body since they are not target based.

Surgical interventions in cancer are being used both in the diagnosis phase and in the treatment phase. Surgery, like any other type of treatment, can have complications and risks. Adverse reactions to the anesthetic drugs used during the operation, formation of clots and bleeding, infection, and damage to different organs due to the operation are among the main undesirable complications.

Chemotherapy is one of the most regularly utilized cancer treatments. The medicines employed in typical chemotherapies do not target a specific focus or area. They intend to kill tumor cells by causing cytotoxicity. Chemotherapy may be favored not just for treating cancer, but also for controlling it or suppressing specific symptoms. There are numerous chemotherapeutic drugs available. Many factors, such as cancer type, the extent of metastasis, and stage, are considered while determining the best chemotherapy (43).

Since chemotherapy affects other cells in the body, side effects are commonly seen. The most typical adverse effects are fatigue, nausea and vomiting, hair loss, weight loss, anemia, and becoming more prone to infection (44).

Radiotherapy is another common cancer treatment approach. It works by destroying cancer cells locally with high-energy particles or waves. Radiation damages the genetic material of cancer cells, which divide much faster than normal cells until they can no longer divide.

Although it is a targeted treatment approach, radiation can also penetrate the surrounding healthy tissue. However normal cells in the surrounding tissue divide considerably more slowly and have functional repair mechanisms, so they take less harm and can repair themselves (45).

Fatigue, some skin illnesses, headache, nausea, and hair loss are examples of radiation side effects. Supplemental medications can be used in some radiotherapy treatments to reduce radiation damage (46).

Immunotherapy is another type of treatment that is evolving and yielding promising outcomes. Parts of the patient's current immune system are utilized to fight

the disease in this therapeutic method. The body's previously existing defense mechanism can be engaged, or its effect can be amplified for this goal. As a result, the body fights cancer as part of its normal functioning. Another method is to provide patients with pharmaceuticals that resemble the immune system. Immunotherapy is used to treat cancer under various subtypes such as checkpoint inhibitors, cytokines, immunomodulators, and CAR-T cells (47).

Protein kinases control practically every element of cell life, and changes in their expression or gene mutations cause major abnormalities. One of the factors that have the most effective role in the formation and progression of cancer is kinases. These genes have mutations and remain active in cancer cells at all times. Cells can thus gain properties such as continual proliferation and division, which should normally be repressed, while also inhibiting apoptotic pathways (48). On the basis of these significant roles, cancer treatment strategies have also focused on kinases.

After imatinib, which was the first kinase inhibitor introduced in 2001, more than seventy kinase inhibitors have been introduced to the market. For instance, Tarceva also known as erlotinib is a small molecule protein kinase inhibitor that suppresses EGFR and has shown promising outcomes, particularly in non-small lung cancer (49).

Protein kinase inhibitors are not necessarily single-targeted. Nexavar or sorafenib, for example, is a FDA approved combined inhibitor that targets multiple types of kinases (50). Sutent and Sprycel are also protein kinase inhibitors that act on multiple targets. These drugs are used to treat kidney cancer, stomach cancer, colon cancers, and certain forms of leukemia (51,52).

Because of their small size, small molecule cancer drugs have been successfully used to reach the ligand-binding receptors located on the outer side of the cell membranes along with proteins located inside, including anti-apoptotic proteins, which is essential in transmitting downstream signaling for proliferation and metastasis (53).

No matter where the target is located within the cell, tiny molecule drugs can theoretically be created to target any part of a molecule. So, in order to pave the way for more extensive and effective use of small molecule drugs against cancer, it is important to prioritize small molecule drug design and development studies.

4.1.4 Challenges in drug design and discovery against cancer

Oncology drugs have typically had lower clinical development success rates than drugs in other fields, like cardiovascular disease (54).

Developing drugs against cancer is an extremely difficult and complex process. The amount spent on releasing a molecule as a drug is enormous, and at the end of the day, the number of molecules approved for use as a drug is extremely small. Despite this, new drug candidates continue to be developed and tested every day in the hope of being effective against cancer.

Although drug development studies against cancer are supported by many private companies, public institutions, and funds, the personalized nature of this disease, heterogeneity, subtypes, and staging differences make it very difficult to develop a one-size-fits-all drug against this disease (55)

Cancerous cells also gain resistance to drugs by modifying the receptors to which drugs bind, suppressing triggered signals, or creating other changes in the downstream pathway, and by developing some changes in the oncogenes. In **Figure 4.6.** (49) below the mechanisms cancer cells usually use to gain resistance against drugs are shown.

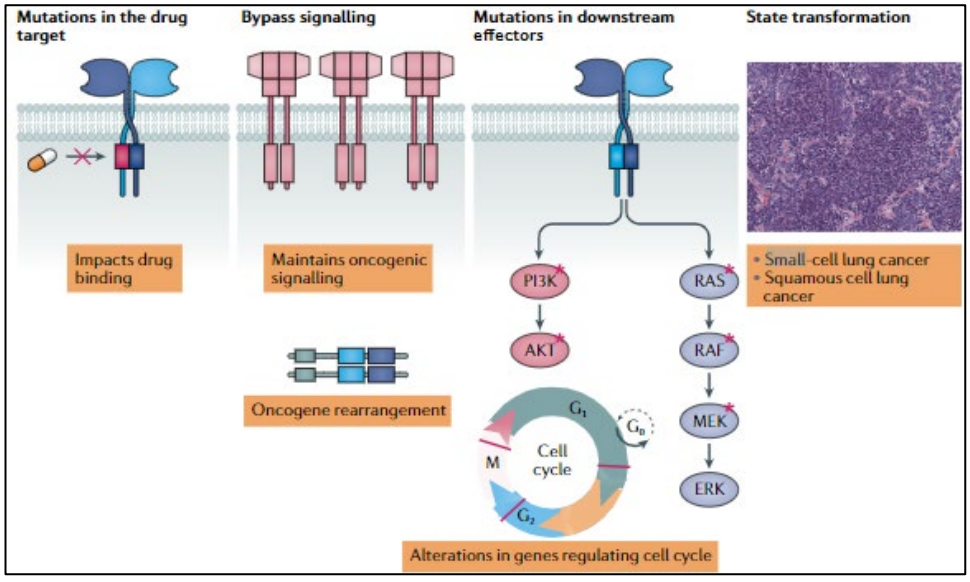


Figure 4.6. Drug Resistance Mechanisms of Cancer Cells (49)

As shown in **Figure 4.6.**, it is critical to produce a molecule that is powerful against cancer while also ensuring that this molecule is not destroyed by cancerous cells' defense and survival processes.

4.2 Hydrazones

Hydrazones are organic molecules that are similar to aldehydes and ketones. When the oxygen in aldehydes or ketones is replaced with the NNH_2 group, hydrazones are generated. The structure of hydrazones can be seen in **Figure 4.7.** below.

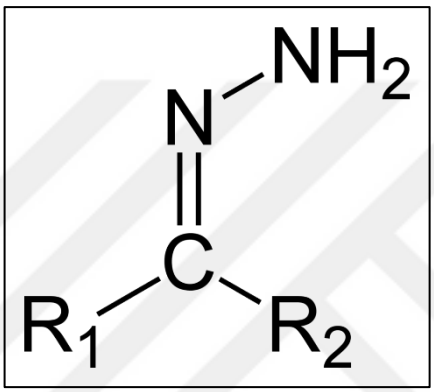


Figure 4.7. Chemical Structure of Hydrazones

Because of hydrazones' diverse applications ranging from therapeutic agents to herbicides and pesticides this structure is recognized as a favored structure. This scaffold, which is represented by the fusion of the amide and imine subunits, offers pharmacophoric sites for hydrogen-bond acceptor and donor sites, as well as interaction with a diverse spectrum of amino-acid residues (6)

Hydrazone derivatives have been shown to have anticancer (6,7), antiproliferative (8,9), antimicrobial(10,11), anti-inflammatory (12,13), and antiviral (14,15) properties.

Various molecular structures and drug categories often associated with hydrazone scaffolds can be seen in **Figure 4.8.** (6)

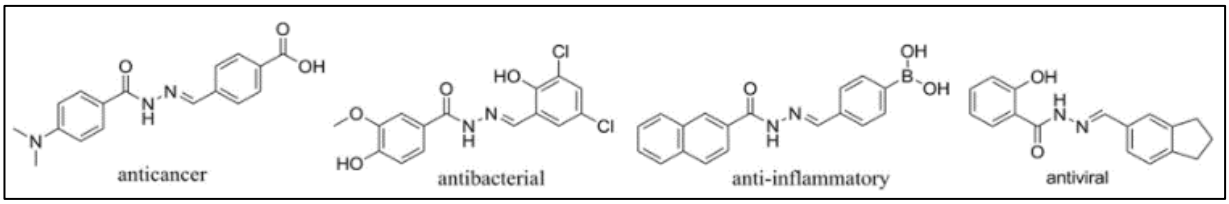


Figure 4.8. Derivatives of Hydrazones with Various Pharmacological Activity Categories (6)

4.3 Oxadiazoles

Oxadiazoles are a type of heterocyclic, aromatic, chemical substance. They have two carbon, two nitrogen, and one oxygen atom. Oxadiazoles come in four isomers: 1,2,3 oxadiazoles, 1,2,4 oxadiazoles, 1,2,5 oxadiazoles, and 1,3,4 oxadiazoles. In **Figure 4.9.** below, different oxadiazole isomers can be seen.

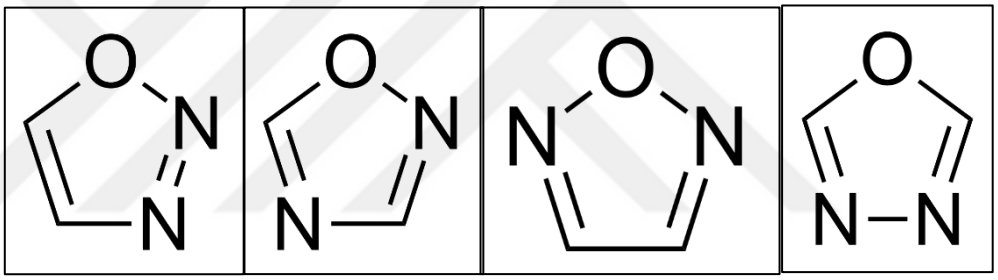


Figure 4.9. 1,2,3 oxadiazole; 1,2,4 oxadiazole; 1,2,5 oxadiazole; 1,3,4 oxadiazole's chemical structures

Oxadiazoles are common motifs in druglike compounds, and they are commonly employed as bioisosteric substitutes for ester and amide properties (56).

Many drugs in the market have already had oxadiazoles in their core structures. Isentress (raltegravir) is a drug that has an antiretroviral effect and is used to treat HIV (57), Butalamine which is used to dilate the vessels (58), Fasiplon which is used to prevent or treat the symptoms of anxiety (59) and Picovir (pleconaril) which is used as an antiviral agent (60), and oxolamine which is used as a cough suppressant (61); have oxadiazoles in their chemical structures as it can be seen in **Figure 4.10.** (59).

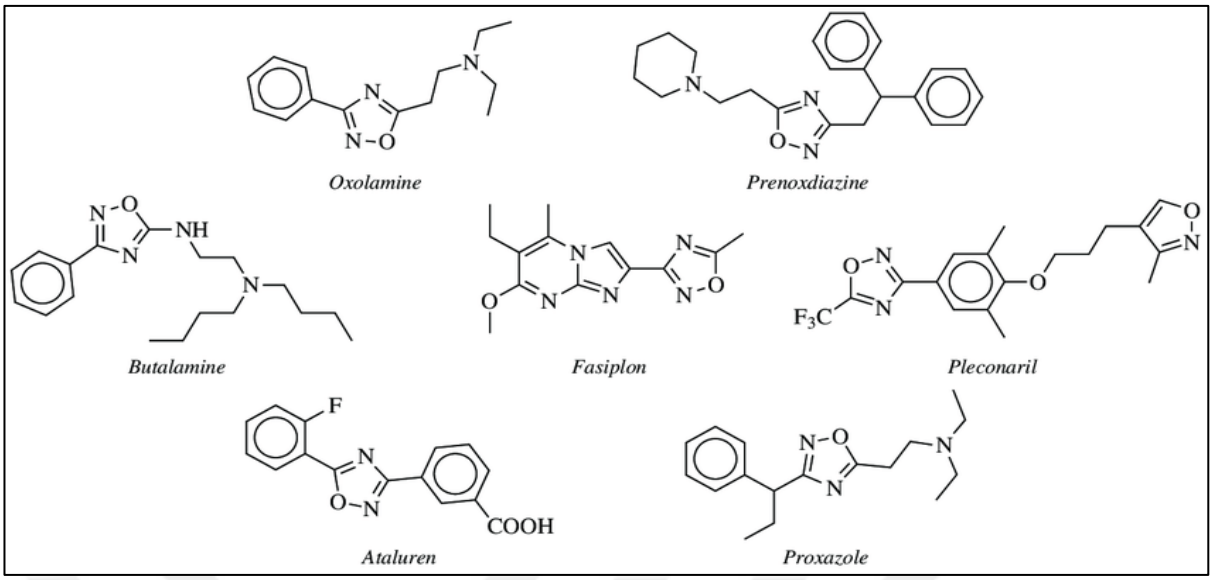


Figure 4.10. Chemical Structures of Commercial Drugs that Includes Oxadiazoles (59)

1,3,4 of oxadiazoles have antibacterial (16,17), anti-inflammatory (18,19), antioxidant (20,21), anticancer (22,62), analgesic (19,23), and antiviral (24,25) properties. Due to various unique advantages, 1,3,4 oxadiazoles are also found in the structures of various pharmaceuticals already available on the market.

Fenadiazole which is a sedative (63) and Thiodazosin which is an antihypertensive (64) for example, comprises 1,3,4 oxadiazole in their composition, as do Zibotentan which is a candidate from Astra Zeneca in cancer treatment as an Endothelin-A Antagonist(65).

5. MATERIALS and METHODS

5.1 Materials

5.1.1 Disposables

The plastic materials and disposable consumables used during this study and their brands are listed below.

Pipette tips (1000 µl, 200 µl, and 10 µl) were from Gilson. 5 ml serological tips were from Falcon, and 10 ml serological tips were from Capp. 15 ml and 50 ml centrifuge tubes were also from Capp. PVDF Membrane syringe filters at 0.22 µm and 0.45 µm were from GVS. 2 ml microcentrifuge tubes were from BioPointe and 1.5 ml microcentrifuge tubes were from Axygen. T25 flasks, 25 cm^2 Rectangular Canted Neck Cell Culture Flask with Vent Cap (catalog number 430639) and T75 flasks, 75cm^2 U-Shaped Canted Neck Cell Culture Flask with Vent Cap (catalog number 430641U) were both acquired from Corning.

Cryovials were from Thermo Fisher Scientific. 96 well black plates with clear bottom, were obtained from Corning Costar (catalog number 3904). 6-well clear plates (catalog number 3516) were from Corning Costar as well.

5.1.2 Chemicals

The chemicals used during the study and other products required for cell culture and the brand and catalog numbers of these products are listed below.

DMEM with 4.5 g/l Glucose, with L-Glutamine, Sodium Pyruvate, with 3.7 g/l $NaHCO_3$ (catalog number P04-03590), and RPMI 1640 with 2 mM L-Glutamine, with 1 mM Pyruvate, with 4.5 g/l Glucose, with 10 mM Hepes, with 1.5 g/l $NaHCO_3$ (catalog number P04-18047) liquid media were both from Pan Biotech. Cell Culture Dissociation Reagent was Trypsin-EDTA (0.25%), with phenol red (catalog number 25200056); Fetal Bovine Serum, qualified, heat-inactivated, Brazil (catalog number 10500064); Penicillin-Streptomycin (10,000 U/ml) (catalog number 15140122) and L-Glutamine (200 mM) (catalog number 25030081) were all from Thermo Fisher Scientific. Dimethyl sulfoxide for cell biology (catalog number 1264ML100) was from Neofroxx.

5.1.3 Laboratory equipment

The devices used during the work, and the brand and model information of these devices are given below.

The biosafety cabinet was from the Safe Fast brand and model Classic Class II. The incubator was from the Thermo Scientific brand, model Forma 310 Direct Heating CO_2. The centrifuge was from Thermo Scientific, SL8 model. SpectraMax i3x Multi-Mode Detection Platform device was used as a spectrophotometer with a plate reader. The microscope was from the ZEISS brand, Axio Vert.A1 Invert The water. The water bath was from the DAIHAN brand, Digital Precise Water Bath, model WB-22. Hemacytometer was from Marienfield. Pure water and ultrapure water were obtained from distilled water appliances by Elga. Vortex was from Vert Scientifica brand, Advanced Vortex Mixer with Variable Speed, model ZX3. Shaker was from the Heidolph brand, Polymax 1040 platform shaker model. -20 ºC freezer was from Siemens and -80 ºC was from Aucma.

5.1.4 Cell lines

Four types of cell lines were included in this study: Human breast carcinoma (MDA-MB-231/ATCC code: HTB-26), human prostatic carcinoma (PC-3/ATCC code: CRL-1435), human lung cancer epithelial cells (A549/ATCC code: CCL-185), and human fetal lung (MRC-5/ATCC code: CCL-171). All of those cells were already available in the laboratory.

5.2 Methods

5.2.1 Preparation of used chemicals in cell culture

Complete media were prepared 50 ml at a time, separately for each cell line, to preserve the freshness of the prepared media more easily and minimize the risk of contamination. For this reason, the percentages were calculated over 50 ml and the media was prepared as described below.

5.2.2 Complete Dulbecco's Modified Eagle Medium (DMEM)

A serological pipette was used to transfer 44 mL of DMEM to a 50 ml tube. It was supplemented with 5 mL of FBS (10% of 50 ml), 500 µl of L-Glutamine (%1 of

50 ml), and 500 µl of Penicillin/Streptomycin (%1 of 50 ml) with the help of a micropipette.

The tube was capped and gently inverted to mix the contents. Complete DMEM was maintained at 4 ºC degrees throughout its use degrees throughout its use

5.2.3 Complete Roswell Park Memorial Institute (RPMI) 1640

A serological pipette was used to transfer 44 ml of RPMI to a 50 ml tube. It was supplemented with 5 ml of FBS (10% of 50 ml), 500 µl of L-Glutamine (%1 of 50 ml), and 500 µl of Penicillin/Streptomycin (%1 of 50 ml) with the help of a micropipette.

The tube was capped and gently inverted to mix the contents. Complete RPMI was maintained at 4 ºC degrees throughout its use.

5.2.4 Fetal Bovine Serum (FBS)

In order to heat inactivate the FBS, the bottle that contains FBS is kept in a water bath at 56 ºC for 30 minutes. During this time, the bottle was rotated every 5-10 minutes to ensure an even heat distribution.

FBS was filtered twice, once with 0.45 µm filters and subsequently with 0.22 µm filters. Then, the FBS was divided into 50 mL tubes under the biosafety cabinet and stored at -20 ºC until used.

5.2.5 Cell Titer-Glo Reagent

To produce the reagent, the Cell Titer-Glo Buffer, and Cell Titer-Glo Substrate, which were already included in the purchased kit, were defrosted and brought to room temperature before use, as directed in the instructions.

When they were balanced at room temperature, Cell Titer-Glo Buffer was added into the amber container that holds Cell Titer-Glo Substrate to restructure the lyophilized enzyme-substrate blend. To achieve a homogenous mixture, the bottle was lightly inverted until the contents were mixed thoroughly. Cell Titer-Glo Reagent consists of this mixture.

Then, the prepared reagent was aliquoted into 2 mL microcentrifuge tubes and put up to -20 ºC in order to be stored until use.

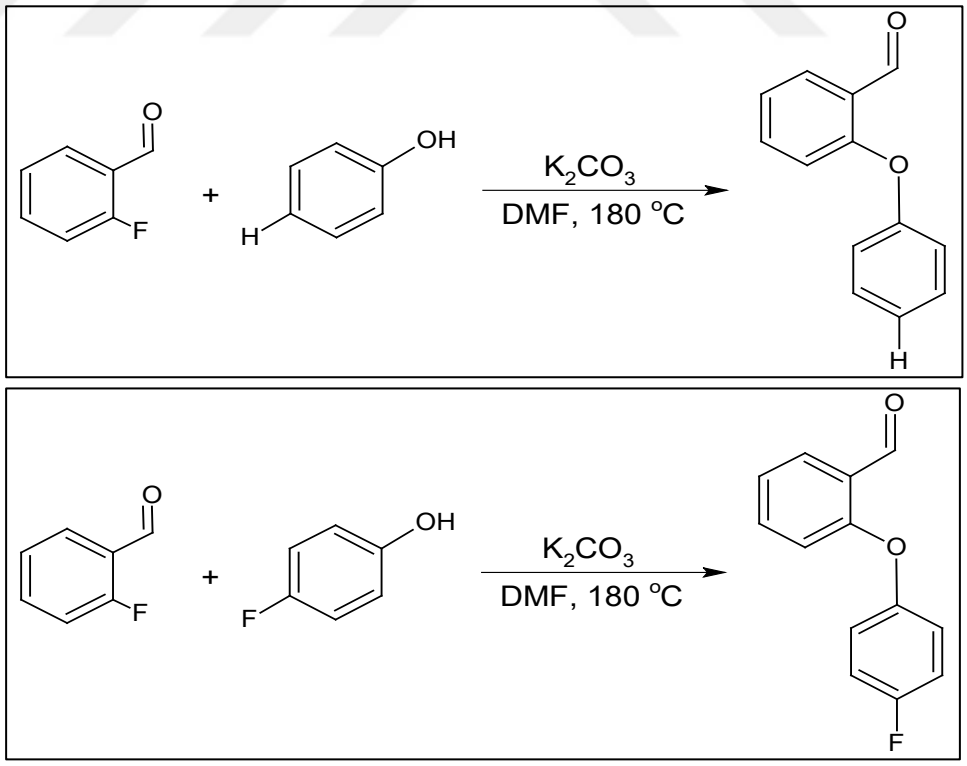
5.2.6 Preparation of drug candidate molecules

Hydrazones and oxadiazoles to be used in this study were synthesized by Assoc. Prof. Dr. Hatice Başpınar Küçük and her team, who are currently working in the Department of Organic Chemistry at Istanbul Cerrahpaşa University. Molecules were delivered in microcentrifuge tubes, sealed with parafilm, and labeled respectively.

5.2.6.1 Synthesis of starting compounds

To begin the synthesis of hydrazones, which would eventually be utilized to make oxadiazoles, three benzaldehyde derivative aldehydes were synthesized by using Ullman Kapling Reaction. 5 mmol of 2-fluorobenzaldehyde and phenol were dissolved in 10 ml of DMF for this reaction. The resulting mixture was then treated with 5 mmol K_2CO_3 and refluxed for 2 hours at 180 °C under a nitrogen atmosphere. After cooling to room temperature, the organic phase was separated using DCM. Column chromatography was used to purify the resulting product.

The reaction used for the synthesis and the benzaldehyde derivatives obtained is given below in **Figure 5.1.**



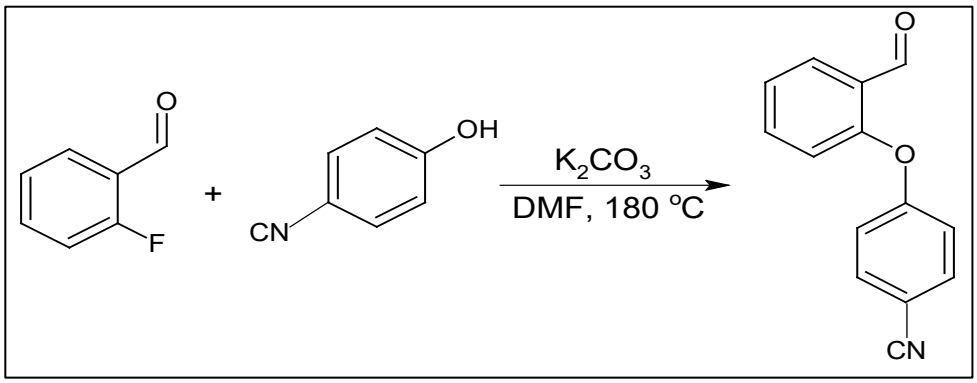


Figure 5.1. Synthesis of Benzaldehyde Derivatives

5.2.6.2 Synthesis of hydrazones

The hydrazones employed for this study were synthesized through the condensation reaction of 2-arenoxybenzaldehydes and several hydrazones.

2 mmol aldehyde was put into 10 to 20 ml of anhydrous ethanol for this. The mixture was then treated with 2 mmol of hydrazine and refluxed for 2-3 hours. At this point, TLC was used to control the reaction. After the reaction was completed, the mixture was allowed to cool to room temperature. The precipitated crystals were rinsed with aqueous ethanol and filtered. Recrystallizing the crude crystals obtained from ethanol gave pure hydrazones.

The general reaction scheme utilized for this purpose is shown in **Figure 5.2.** below.

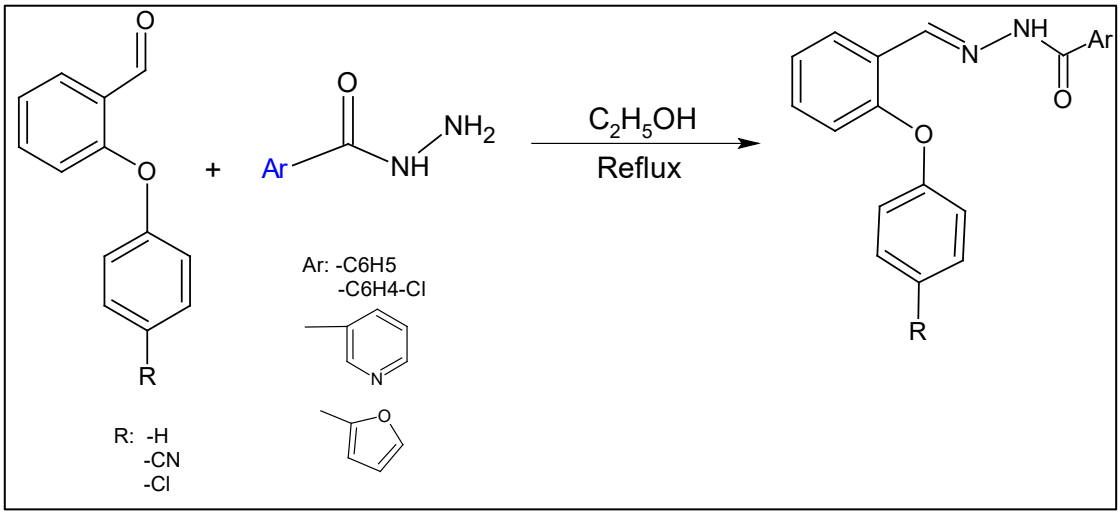


Figure 5.2. Synthesis of Hydrazones out of Benzaldehyde Derivatives

In order to obtain several hydrazones, H, CN, Cl, C_6H_5, and C_6H_4-Cl, were substituted in the positions specified by R1 and Ar in the overall reaction mechanism

shown in **Figure 2.** As a result of these combinations reacting with each other, fifteen hydrazones were obtained.

5.2.6.3 Synthesis of oxadiazoles

Utilizing hydrazones as a starting component, I_2 catalyzed oxidative cyclization was used to synthesize 1,3,4 oxadiazoles.

1 mmol of hydrazone was dissolved in 5 ml of DMSO for this. The reaction was given 3 mmol K_2CO_3 and 1.2 mmol iodine. It was mixed for 1 to 4 hours at 100 °C. TLC was used to check the reaction.

After the reaction had finished, the mixture was cooled to room temperature and treated with 5% $Na_2S_2O_3$. It was extracted with ethyl acetate and a separating funnel. The mixture was washed with saturated NaCl, and the organic phase was dried with Na_2SO_4. After filtration, it was taken to the evaporator. Column chromatography was used to purify the residue. Due to purification challenges at this point, three oxadiazoles were removed from the study, leaving a total of twelve 1,3,4 oxadiazoles for this study.

5.2.6.4 Preparing stock solutions

Stock solutions were prepared at 50 mM for all molecules in the study. In this way, the same amounts could be used for all molecules in calculations, regardless of molecular weight or any concentration differences. **Table 5.1.** and **Table 5.2.** below give the molecular weights of the small molecules and their respective amounts that were weighed for the preparation of 50 mM stock solutions.

Table 5.1. 50 mM Stock Solution Preparations for Hydrazones

Hydrazones	Molecular Weights	50 mM Stock Solution
AA-5	316 g/mol	7.9 mg in 500 µl DMSO
AA-7	350 g/mol	8.75 mg in 500 µl DMSO
AA-8	317 g/mol	7.925 mg in 500 µl DMSO
AA-9	306 g/mol	7.65 mg in 500 µl DMSO
AA-10	338 g/mol	8.45 mg in 500 µl DMSO
AA-11	341 g/mol	8.525 mg in 500 µl DMSO

AA-12	375 g/mol	9.375 mg in 500 µl DMSO
AA-13	342 g/mol	8.55 mg in 500 µl DMSO
AA-14	331 g/mol	8.275 mg in 500 µl DMSO
AA-15	363 g/mol	9.075 mg in 500 µl DMSO
AA-17	350 g/mol	8.75 mg in 500 µl DMSO
AA-18	384 g/mol	9.6 mg in 500 µl DMSO
AA-19	351 g/mol	8.775 mg in 500 µl DMSO
AA-21	340 g/mol	8.5 mg in 500 µl DMSO
AA-20	372 g/mol	9.3 mg in 500 µl DMSO

Table 5.2. 50 mM Stock Solution Preparations for Oxadiazoles

Oxadiazoles	Molecular Weights	50 mM Stock Solution
AA-26	314 g/mol	7.85 mg in 500 µl DMSO
AA-16	348 g/mol	8.7 mg in 500 µl DMSO
AA-27	315 g/mol	7.875 mg in 500 µl DMSO
AA-28	304 g/mol	7.6 mg in 500 µl DMSO
AA-29	339 g/mol	8.475 mg in 500 µl DMSO
AA-23	373 g/mol	9.325 mg in 500 µl DMSO
AA-30	340 g/mol	8.5 mg in 500 µl DMSO
AA-24	329 g/mol	8.225 mg in 500 µl DMSO
AA-31	348 g/mol	8.7 mg in 500 µl DMSO
AA-32	382 g/mol	9.55 mg in 500 µl DMSO
AA-33	349 g/mol	8.725 mg in 500 µl DMSO
AA-34	338 g/mol	8.45 mg in 500 µl DMSO

Precision scales were used to weigh the quantities listed for each molecule in **Table 5.1.** and **Table 5.2.** above, which were then transferred to 2 ml microcentrifuge tubes. Precision scales were used to weigh the quantities listed for each molecule in the tables, which were then all transferred to 2 ml microcentrifuge tubes separately. After making sure that they were completely dissolved and formed a homogeneous

mixture, the prepared stock solutions were transferred to separate microcentrifuge tubes by passing them through 0.22 µm filters. Throughout their use, each tube was individually labeled and stored at 4 ºC.

5.2.6.5 Preparing different concentrations out of stock solutions

Dose ranges for use in the study were determined as 300 µM, 200 µM, 100 µM, 50 µM, and 25 µM. Since 50 mM stock solutions were prepared for all molecules beforehand, the following dilutions could be applied for all molecules.

Figure 5.3. below illustrates the steps for preparing solutions with the concentrations of 300 µM, 200 µM, 100 µM, 50 µM, and 25 µM.

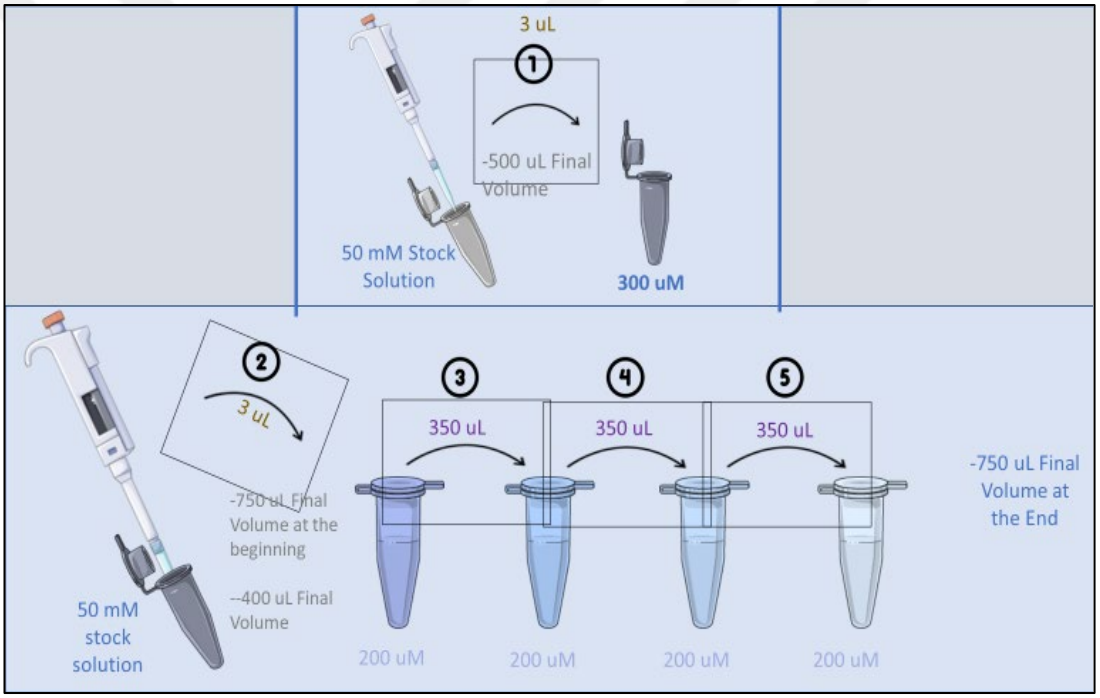


Figure 5.3. Preparation of Different Concentrations out of Stock Solutions

As **Figure 5.3.** shows clearly, to prepare a 300 µM solution, 3 µl was taken from the previously prepared 50 mM stock solution and diluted with proper complete media inside of a 2 ml microcentrifuge tube until the final volume was up to 500 µl.

For 200 µM, once again 3 µl was taken from the previously prepared 50 mM stock solution and diluted with proper complete media. But this time the final volume

was set up to 750 µl since from this point on serial dilution was performed to achieve the rest of the concentrations.

So, in order to prepare 100 µM, 50 µM, and 25 µM concentrations, 2 ml microcentrifuge tubes were all filled with 350 µl complete media and put in order respectively. Then by starting from the 200 µM concentration's microcentrifuge tube, 350 µl from each the previous concentration were transferred to the next one after a throughout mix.

The medium used in these preparations was chosen to be the same medium used when culturing the cell lines. That is, complete DMEM was used for MDA-MB-231, MRC-5, and PC-3, while complete RPMI was preferred for A549.

These steps were repeated over each molecule, and various concentrations were prepared in this way to be used on cells. These solutions were not prepared beforehand; instead, they were prepared right before the application.

5.2.7 Cell culturing

In this study, a total of four cell lines, three from different cancer types and one regular fibroblast, were used. Human breast carcinoma (MDA-MB-231), human prostatic carcinoma (PC-3), and human lung cancer epithelial cells (A549) were the cancerous cells, while the fibroblasts in the control group were human fetal lung (MRC-5) cells.

Since the cells come from various sources and have different characteristics, some of them required different types of products to be handled. The same protocol and requirements were used for MRC-5, PC-3, and MDA-MB-231 cells. They were all cultured in high glucose DMEM with L-glutamine and sodium pyruvate. However, the A549 cells required a different form of media than the others, so relevant changes have been made by changing the cell medium from high glucose DMEM to RPMI 1640.

5.2.7.1 Opening cell lines

The steps for opening cells mentioned below apply to all cell types in this study. Cell media was chosen in accordance with the cells, and all lines were opened in the same manner.

The cryovials that contain the cells were taken from -80 ºC. and placed in a hot water bath which heated to 37 ºC, keeping the neck of the vial dry by not submerging the vial completely. Cryovials were kept in the bath until they thaw while gently twirling them in water. As soon as the contents were melted, cryovials were taken out of the water bath and pat dry with a paper towel. After that, they were thoroughly sprayed with 70% ethanol solution before being placed in the biosafety cabinet.

In a 15 ml tube, 9 ml of serum-free (SF) media which was appropriate for the cell type was added. The contents of the cryovial were mixed with SF medium with a dripping motion with the help of a micropipette. This mixture was then centrifuged at 1500 rpm for 5 minutes at 4 ºC. The tube was removed from the centrifuge and sprayed with 70% ethanol solution before getting placed in the biosafety cabinet.

The supernatant was removed without disturbing the pellet sitting at the bottom of the tube. At this stage, a minimal amount of supernatant was allowed to remain on top of the pellet in order to prevent cell loss. The pellet was dissolved by pipetting with 1 ml of the complete medium that suits the cell type. A serological pipette was used to transfer 4 mL of complete medium to a T25 cell culture flask. The cell solution in the 15ml tube was added to this flask in drops without touching the pipette to the edges.

The flask was gently shaken so that the cells were evenly dispersed on the surface of the flask. The cells were placed in an incubator at 37 °C and 5% CO_2. The cells were checked after 24 hours, and their medium was changed if necessary.

5.2.7.2 Passaging

Cells were evaluated every other 24 hours. When the cell density reached 80%, the cells were passaged to ensure the culture's long-term viability.

For this, the flask containing the cells was taken from the incubator and the waiting medium on top of the cells was discarded. Cells were washed with serum-free (SF) medium. Meanwhile, to avoid detaching the cells, the medium was directed to the flask's ceiling rather than directly to the cells adhering to the flask's bottom.

According to the size of the flasks, T25 flasks received 1 mL of 0.25 percent trypsin-EDTA, while T75 flasks received 2 mL of 0.25 percent trypsin-EDTA. For the enzyme to show activity, the flask was placed in the incubator for 4 minutes. When

the time was up, it was checked with the help of a microscope whether the cells adhered to the surface were detached or not. If necessary, an extra minute of waiting in the incubator was offered. The cells were then collected after washing the flask surface with the complete medium that was at least three times the amount of enzyme used. Depending on the next step of the experiment, cells were collected in a single 15 ml tube or multiple 15 ml tubes at this stage.

Tubes containing the collected liquids were centrifuged at 1500 rpm for 5 minutes at 4 °C. Before being placed in the biosafety cabinet, the tube was taken out of the centrifuge and sprayed with %70 ethanol solution.

The pellet at the bottom of the tube was not disturbed while the supernatant was removed. To limit cell loss at this step, only a small amount of supernatant was permitted to stay on top of the pellet. Pipetting 1 ml of complete media that is appropriate for the cell type was used to dissolve the pellet. 4 ml of complete media was transferred into a T25 cell culture flask using a serological pipette. If the cells were being transferred into a T75 flask this amount was set as 6 ml. The cell solution in the 15ml tube was dropped into the flask without touching the pipette to the flask's edges. The flask was gently shaken to ensure that they were evenly distributed on the surface.

5.2.7.3 Freezing

In case of an event of an experimental difficulty, it is important to have some return points. Therefore, in various passage numbers during the study, the cells were frozen and stored in order to provide an opportunity to go back and repeat the experiment if necessary while also keeping stock.

This was accomplished by removing the flask containing the cells from the incubator and discarding the waiting media on top of the cells. Serum-free (SF) media was used to wash the cells. Similarly, the medium was directed to the flask's ceiling rather than straight to the cells clinging to the flask's bottom to avoid disconnecting them.

The flask was placed in the incubator at 37 °C for 4 minutes to allow the enzyme to become effective. When the timer ran out, the cells attached to the surface were

examined under a microscope to see if they were detached. After washing the flask surface with the complete medium, which was at least three times the amount of enzyme used, the cells were collected. The recovered liquids were centrifuged in 15 mL tubes at 4 °C for 5 minutes at 1500 rpm.

After this stage, the cells were dissolved in the freezing medium instead of the complete medium. To prepare the freezing medium, 90% FBS and 10% DMSO were mixed together.

Cells that were thoroughly dissolved by pipetting were distributed to cryovials. 1-2 ml were put into each one. Cryovials, which are tightly closed and labeled appropriately, were first placed in the freezer at -20°C. After being held here for less than an hour, they were then transferred to -80 °C. In this way, gradual freezing was provided.

5.2.8 Effects of candidate molecules on cell proliferation

5.2.8.1 Culturing the cells in 96 well plate

Cells were seeded into 96 well plates to see the effect of different doses on cells. For this, an equal number of cells had to be seeded per well. For this reason, the cells were removed from the flasks by using 1 ml of 0.25% trypsin/EDTA and incubated in the incubator at 37 °C for 4 minutes, then 4 ml of complete medium was added to the cells and the mixture was transferred to a 15 ml tube. After centrifugation at 1500 rpm and 4 °C for 5 minutes, the supernatant in the 15 ml tube was removed.

The cell pellet was suspended with 1 ml complete medium. If the cells to be counted at this stage, covered the surface of the flask and were too dense, the cells could also be dissolved in 2 ml of complete medium in order to provide convenience during counting. 5 µl were taken from the cell suspension and diluted with 45 µl complete medium. 10 µl were taken from this mixture for counting.

Cells were counted with the help of a hemocytometer and cultured into 96 well black plates at eight thousand cells in 100 µl per well. Plates were kept in the incubator for 24 hours. When the states of the cells were examined under the microscope, it was determined that they had reached 80% density, and the treatment of candidate molecules took place.

5.2.8.2 Giving candidate molecules to cells

After 24 hours, the complete medium on top of the cells was withdrawn without touching the tip of the pipette on the bottom surface of the wells. Then, candidate molecules, the preparation of which was explained in detail above, were applied at concentrations of 300 µM, 200 µM, 100 µM, 50 µM, and 25 µM in triplicate wells for each dose of each molecule. 100 µl were given each well.

Plates were put into the incubator for 48 hours. The state of cells was checked in the first 24 hours to see the effect of candidate molecules under the microscope.

5.2.8.3 Cell Titer Glo

The Cell Titer-Glo Luminescent Cell Viability Assay is a standardized way to evaluate the viable cell count in cultures depending on ATP. The presence of ATP indicates the existence of living cells with active metabolisms. The Cell Titer-Glo Assay is also optimized for multi-well plate layouts, so it is advantageous in this type of study where there are multiple molecules and several cell lines.

Cell titer assay allows for measurement with a single reagent applied directly to cultured cells. There is no need for further processes before application, such as washing the cells and removing the media from them. The introduced reagent promotes cell lysis and generates a luminescent signal proportional to the amount of ATP present in the lysate. The intensity of this signal is proportional to the number of alive cells in the culture (66). The luciferase enzyme is used to calculate the Cell Titer Glo measurement. The working principle of the Cell Titer Glo Assay is given in **Figure 5.4.** (67) below.

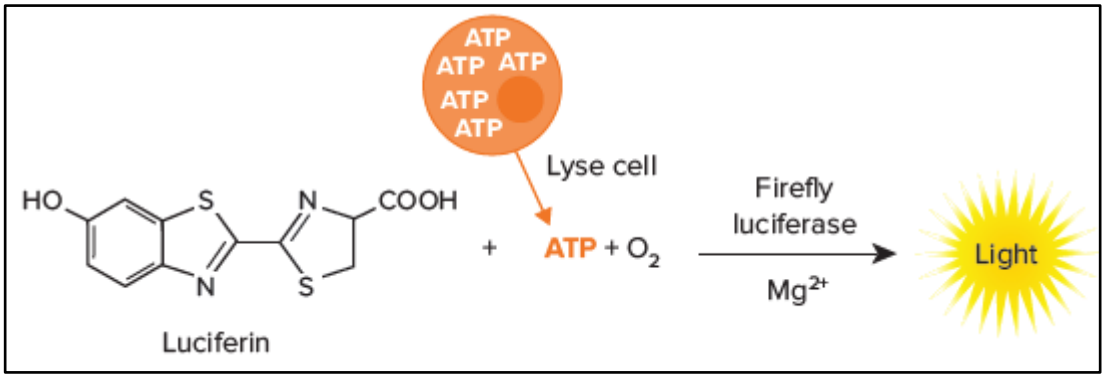


Figure 5.4. Reaction Mechanism of Luciferase in Cell Titer Glo (67)

Compared to other measurement methods, Cell Titer Glo has many advantageous features such as fast results, high sensitivity, and providing a stable and long-lasting signal. Due to these advantages, Cell Titer Glo was chosen as the cell viability assay in this study. The reagent, which had been prepared as previously described, aliquoted, and stored at -20°C, was brought to room temperature. Then, 10 µl of reagent was dropped onto the cells' medium. Meanwhile, since the reagent is light sensitive, care was taken to maintain the surroundings as darkened as possible. After adding the reagent to all of the wells, the plate was covered with aluminum foil, so the light was cut off. The plate was shaken for 5-10 minutes on top of a shaker to mix the components. The signal was then stabilized by leaving it on the bench for 1-2 minutes without shaking. Spectramax instrument was used to take measurements.

In some wells, the absorbance value of the background was measured by keeping only the medium in those wells without any cells present. The mean of these measured values was subtracted from the other measurements. In this way, the deviation caused by the medium was eliminated. In the GraphPad Prism program, the average of the three repetitions for each dose was calculated. This software was used to normalize data and calculate IC50 values out of the normalized versions.

5.2.9 Scratch assay

For the scratch assay, MDA-MB-231 and A-549 cells were seeded. 100.000 cells in 1.5 ml complete medium per well on a 6-well plate were cultured as it is described in detail above. Then 6 well plates were incubated at 37 °C for 24 hours. After 24 hours, cells that had accumulated were scratched out using the tip of a 200 µl pipette. The media on top of the cells were then discarded, and the cells were treated for 48 hours with three molecules AA-9, AA-10, and AA-26.

For MDA-MB-231 the dosages were 40 µM for AA-9, 120 µM for AA- 10 and 30 µM for AA-26. For A-549 the dosages were 55 µM for AA-9, 15 µM for AA- 10 and 15 µM for AA-26.

The cell states were evaluated under the microscope, and during the follow-up, markings were made to get photos from the same place. Under the microscope, images

of the determined reference points were recorded right after treatment, 24 hours later, and 48 hours later.

6. RESULTS

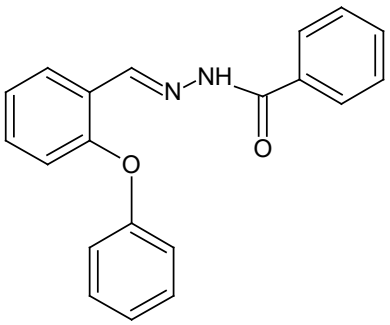
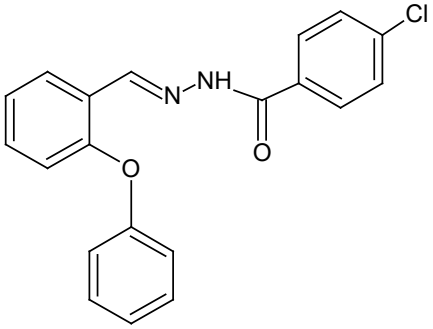
6.1 Novel Small Molecules

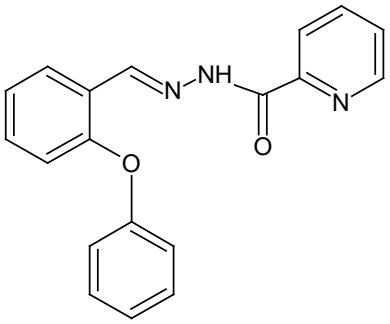
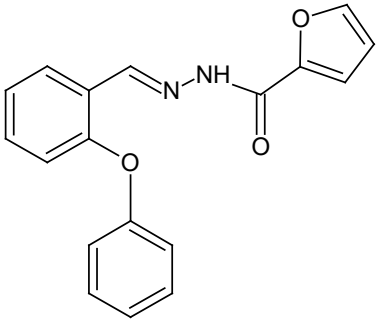
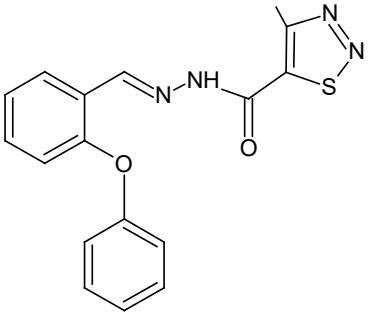
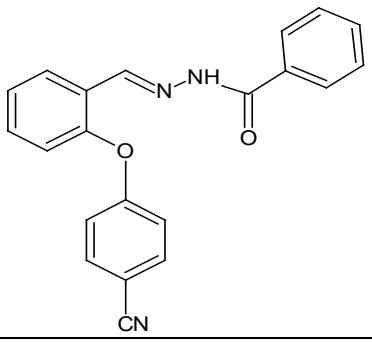
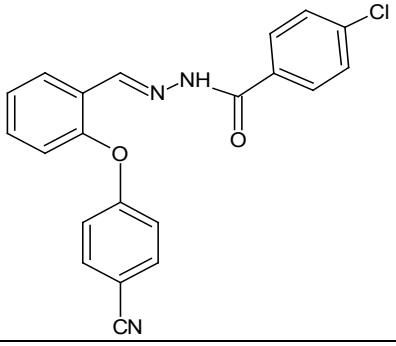
Assoc. Prof. Dr. Hatice Başpınar and her team members from Istanbul Cerrahpaşa University Organic Chemistry Department synthesized fifteen novel hydrazones and twelve novel 1,3,4 oxadiazoles. Hydrazones were used as starting points in order to obtain those 1,3,4 oxadiazoles. Three out of fifteen of those 1,3,4 oxadiazoles were hard to purify so only twelve were used in this study.

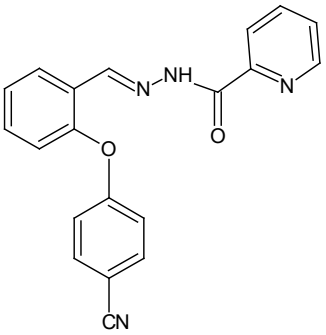
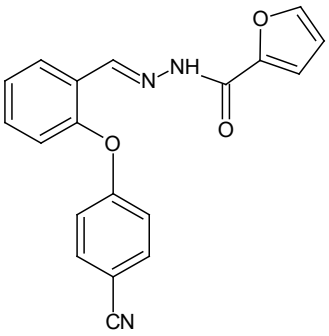
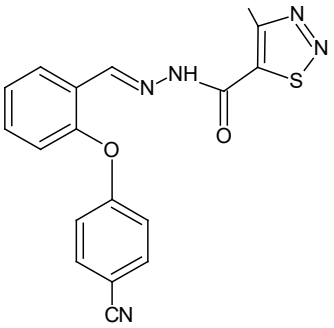
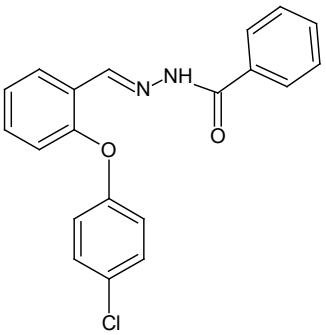
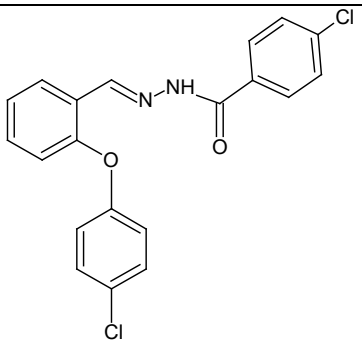
6.1.1 Hydrazones

Twelve novel hydrazones have already been synthesized. Their molecular weights and chemical structures are given in **Table 6.1.** below.

Table 6.1. Chemical Structure and Molecular Weight of Each Novel Hydrazone

Hydrazones	Chemical Structures	Molecular Weight
AA-5		316 g/mol
AA-7		350 g/mol

AA-8		317 g/mol
AA-9		306 g/mol
AA-10		338 g/mol
AA-11		341 g/mol
AA-12		375 g/mol

AA-13		342 g/mol
AA-14		331 g/mol
AA-15		363 g/mol
AA-17		350 g/mol
AA-18		384 g/mol

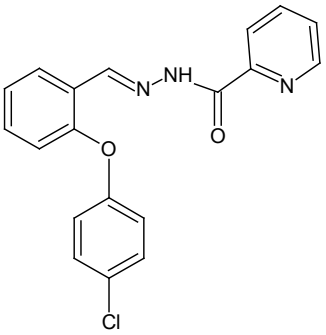
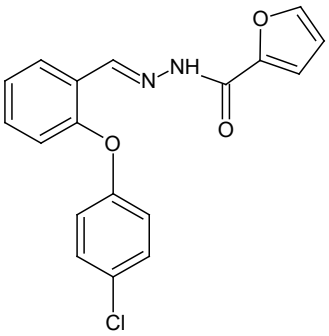
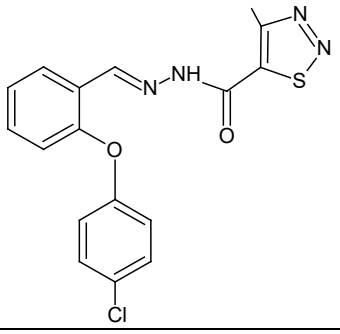
AA-19		351 g/mol
AA-21		340 g/mol
AA-20		372 g/mol

Table 6.1. shows that novel hydrazones have different molecular weights ranging from 306 g/mol to 384 g/mol. It is aimed to provide an understanding of the effect of different additions and deletions of different groups with various modifications on anticancer activity.

6.1.2 Oxadiazoles

Twelve novel 1,3,4 oxadiazoles have already been synthesized. Their molecular weights and chemical structures are given in figures below.

Figure 6.1. Synthesis of AA-26 Using AA-5 As Starting Component

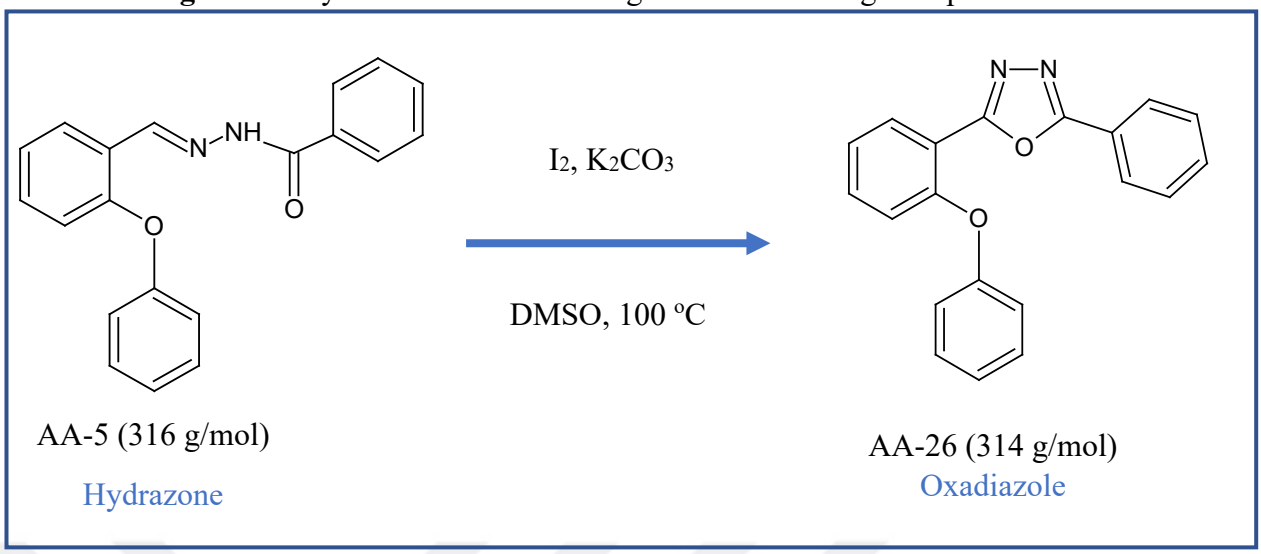


Figure 6.2. Synthesis of AA-16 Using AA-7 As Starting Component

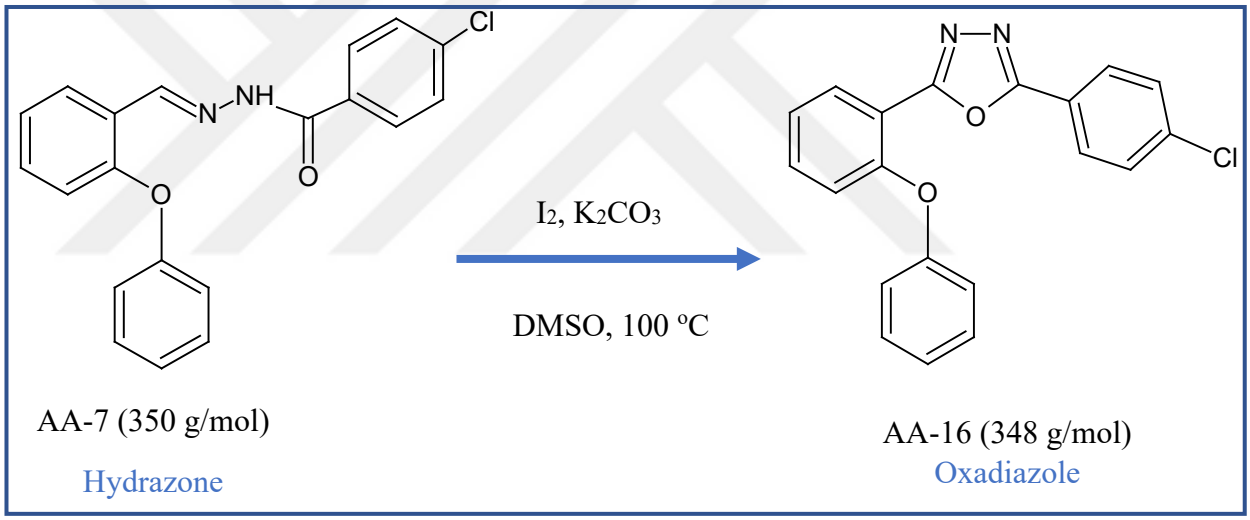


Figure 6.3. Synthesis of AA-27 Using AA-8 As Starting Component

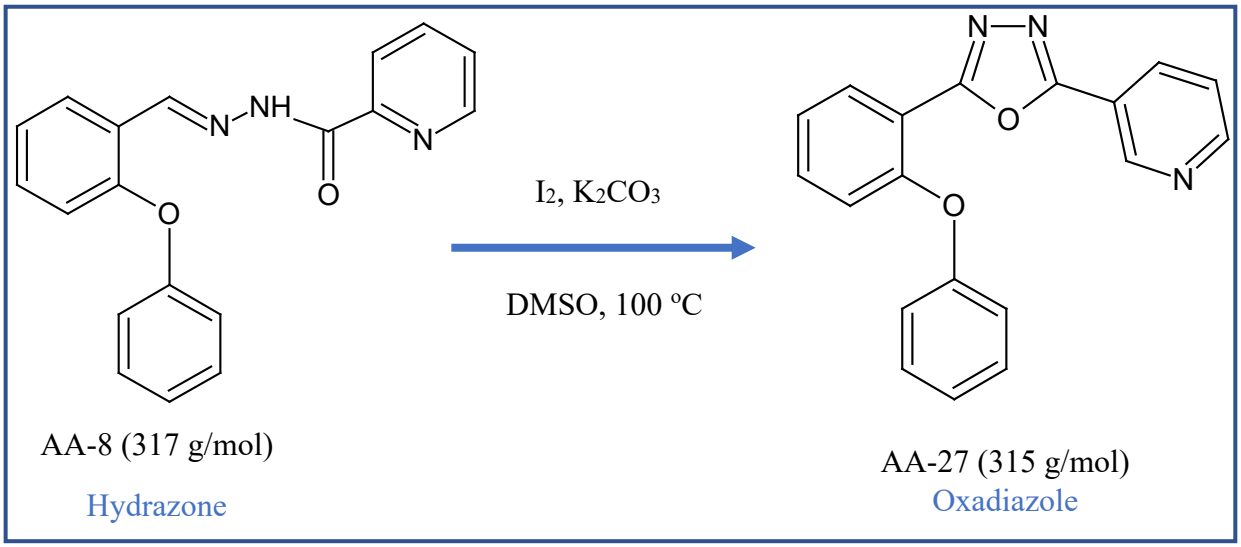


Figure 6.4. Synthesis of AA-28 Using AA-9 As Starting Component

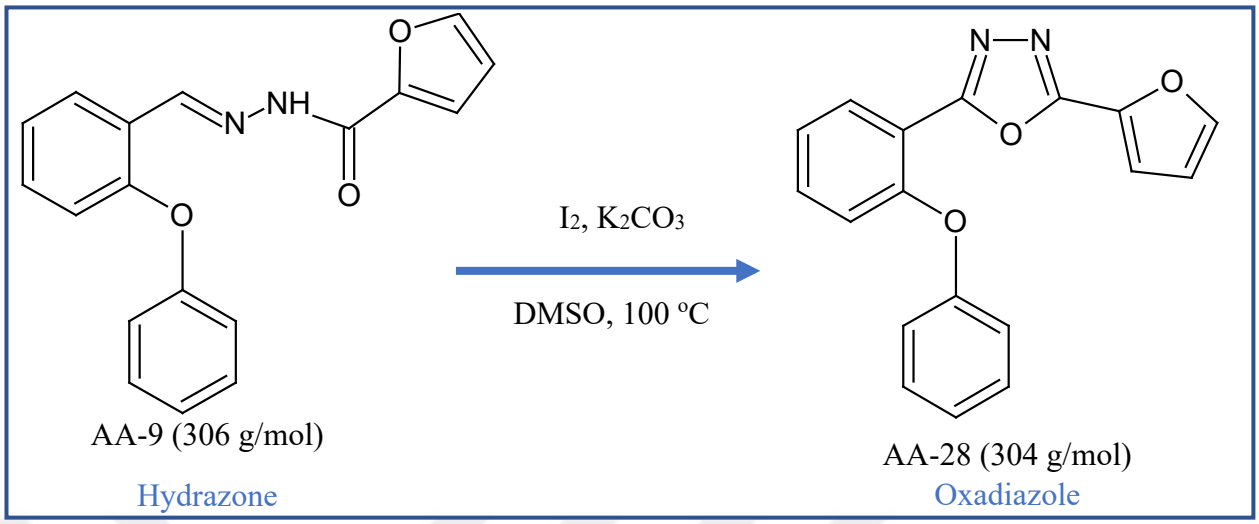


Figure 6.5. Synthesis of AA-29 Using AA-11 As Starting Component

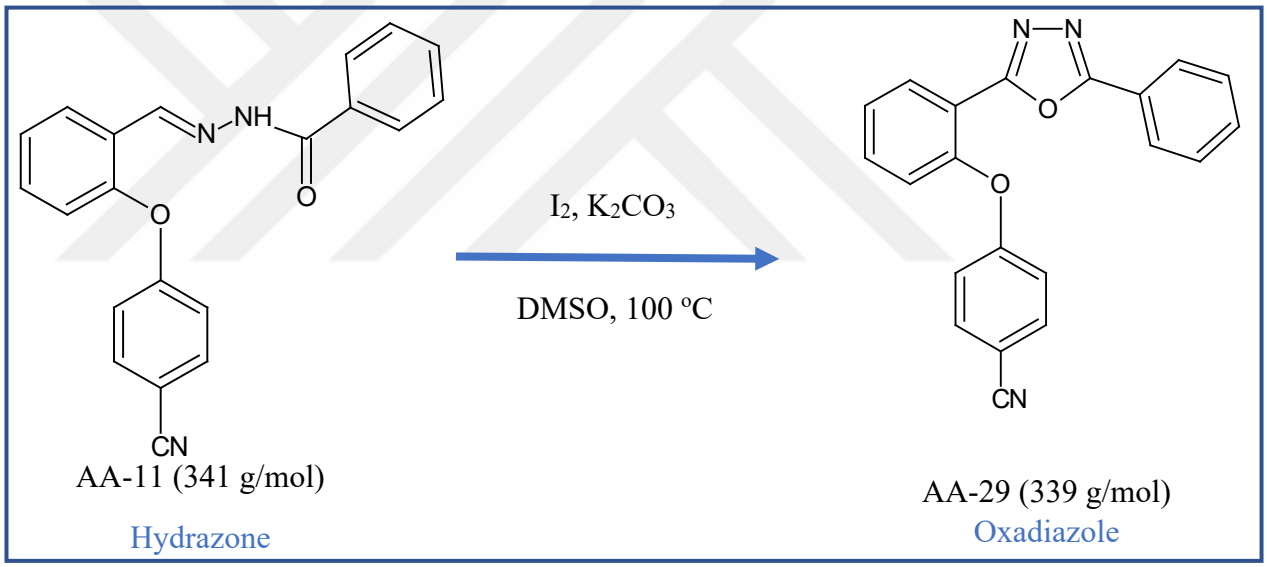


Figure 6.6. Synthesis of AA-23 Using AA-12 As Starting Component

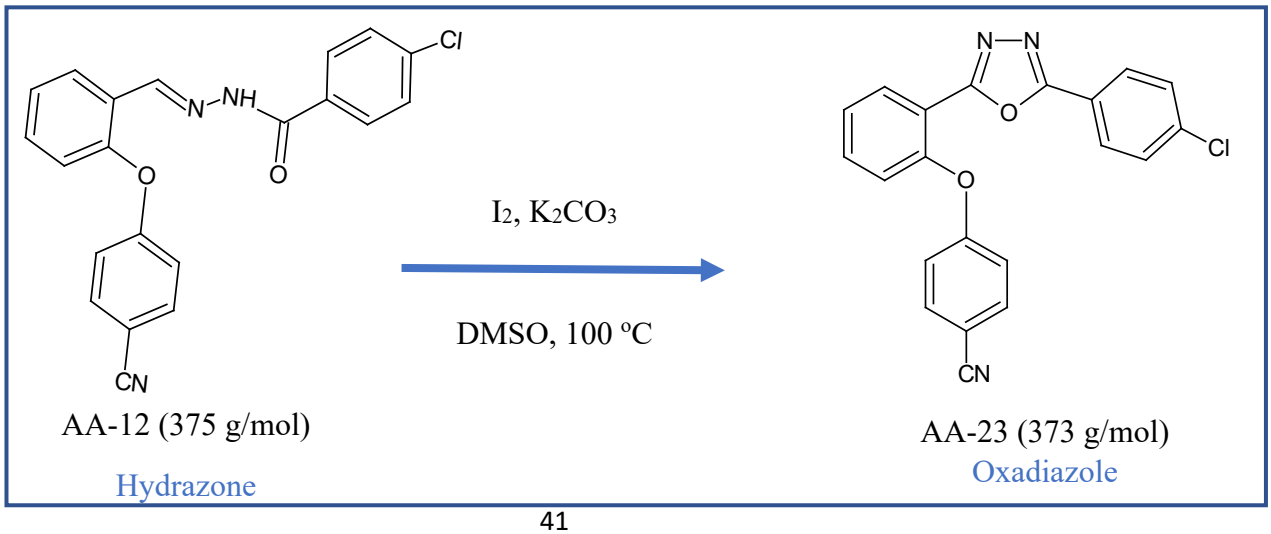


Figure 6.7. Synthesis of AA-30 Using AA-13 As Starting Component

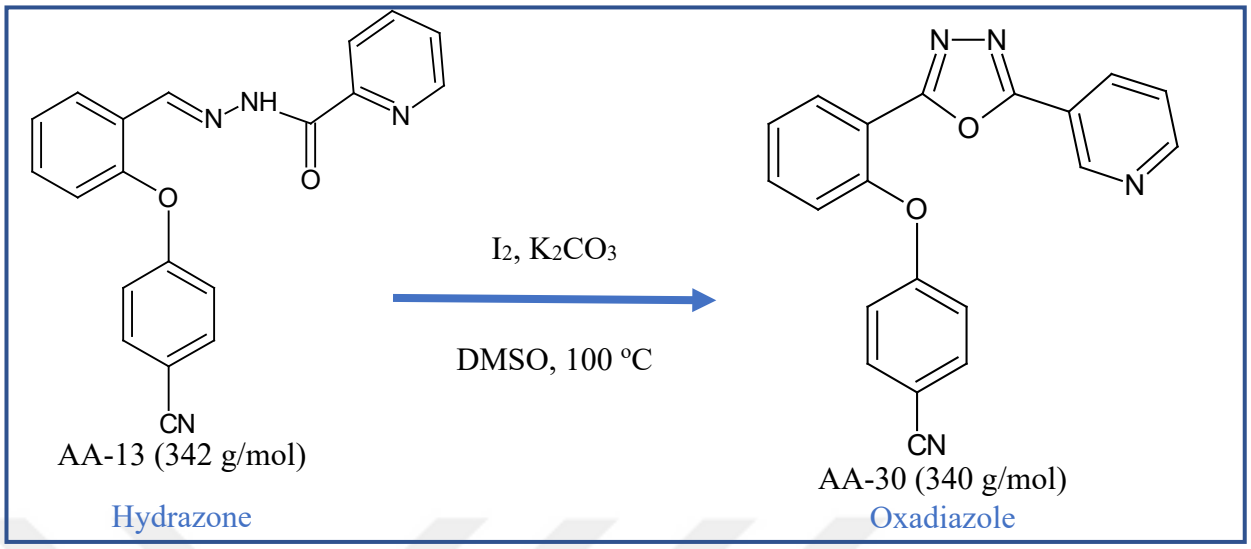


Figure 6.8. Synthesis of AA-24 Using AA-14 As Starting Component

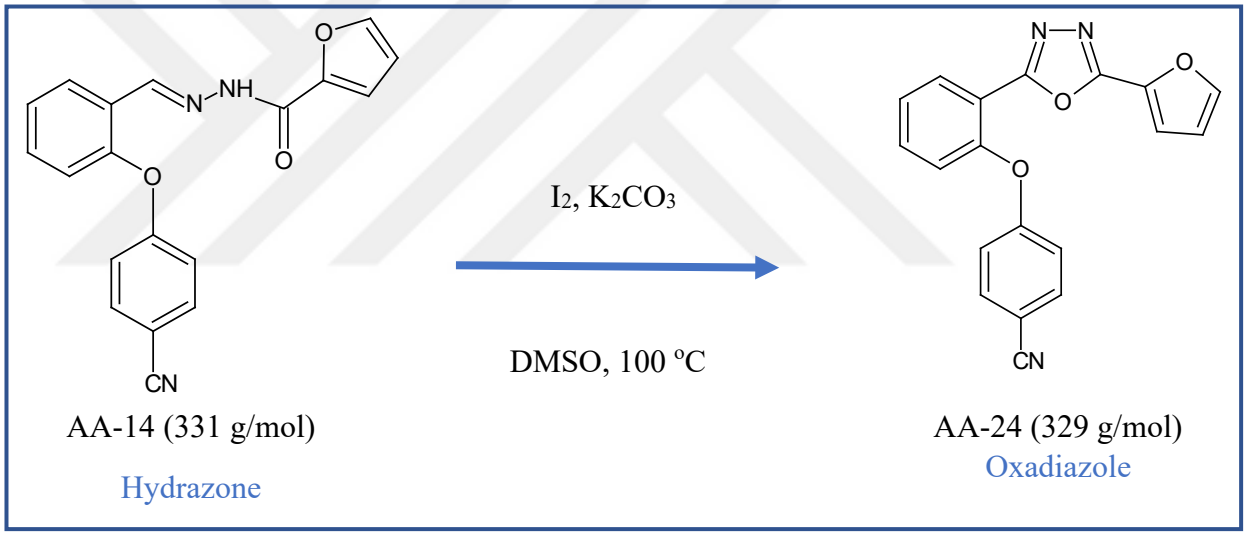


Figure 6.9. Synthesis of AA-31 Using AA-17 As Starting Component

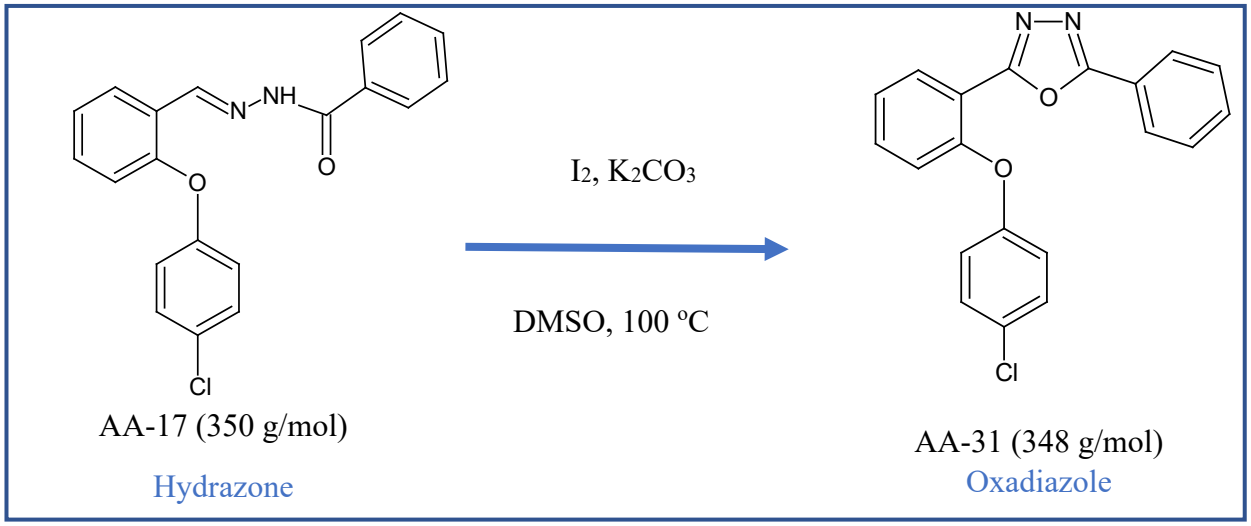
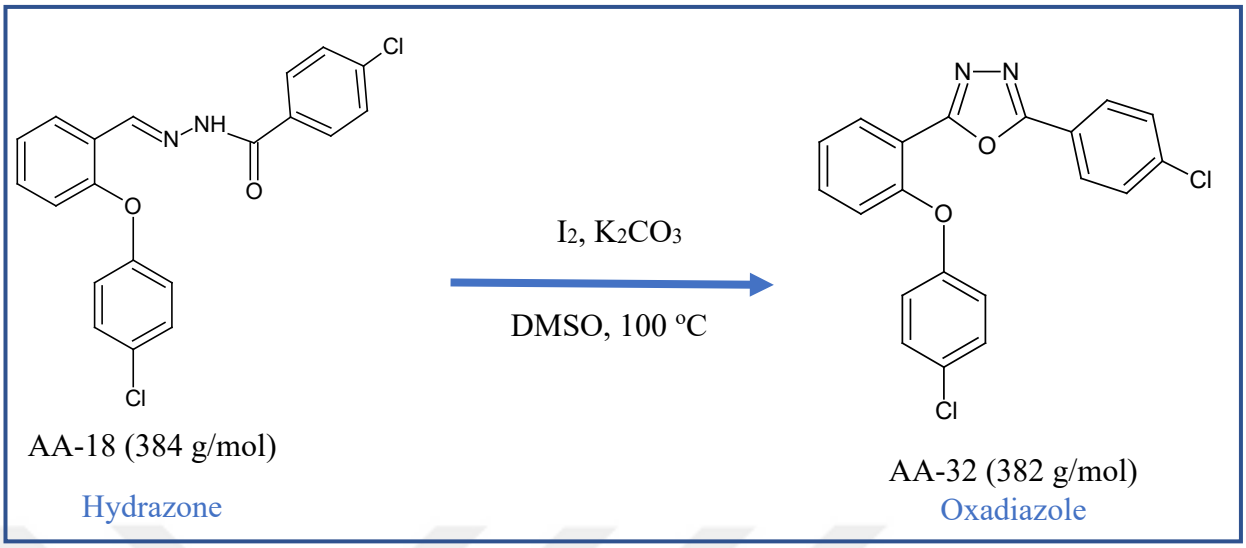


Figure 6.10. Synthesis of AA-32 Using AA-18 As Starting Component



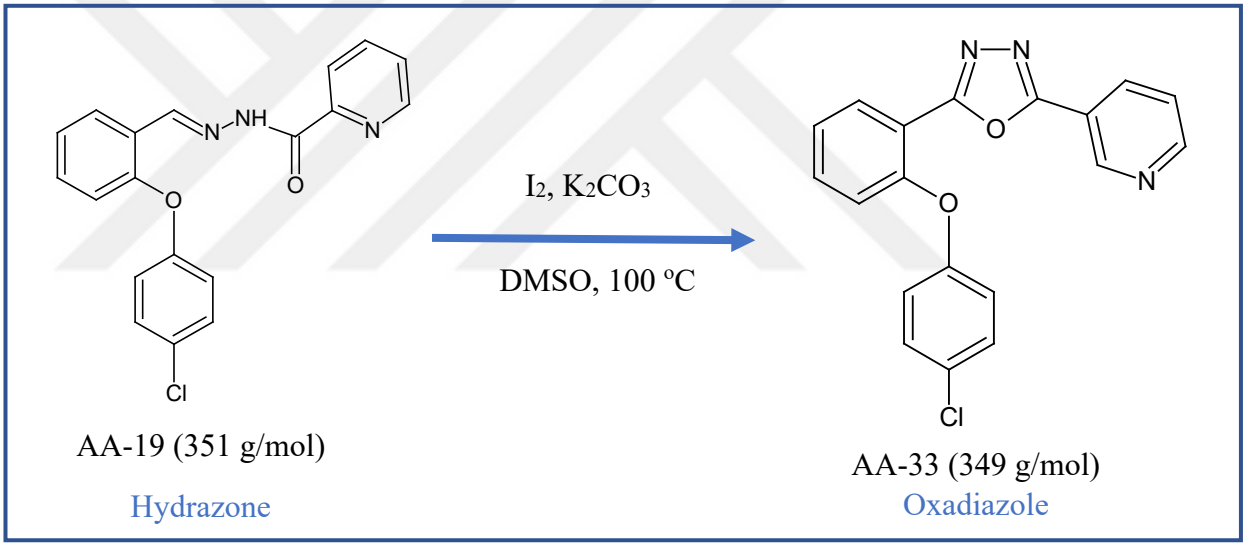
AA-18 (384 g/mol)

Hydrazone

AA-32 (382 g/mol)

Oxadiazole

Figure 6.11. Synthesis of AA-33 Using AA-19 As Starting Component



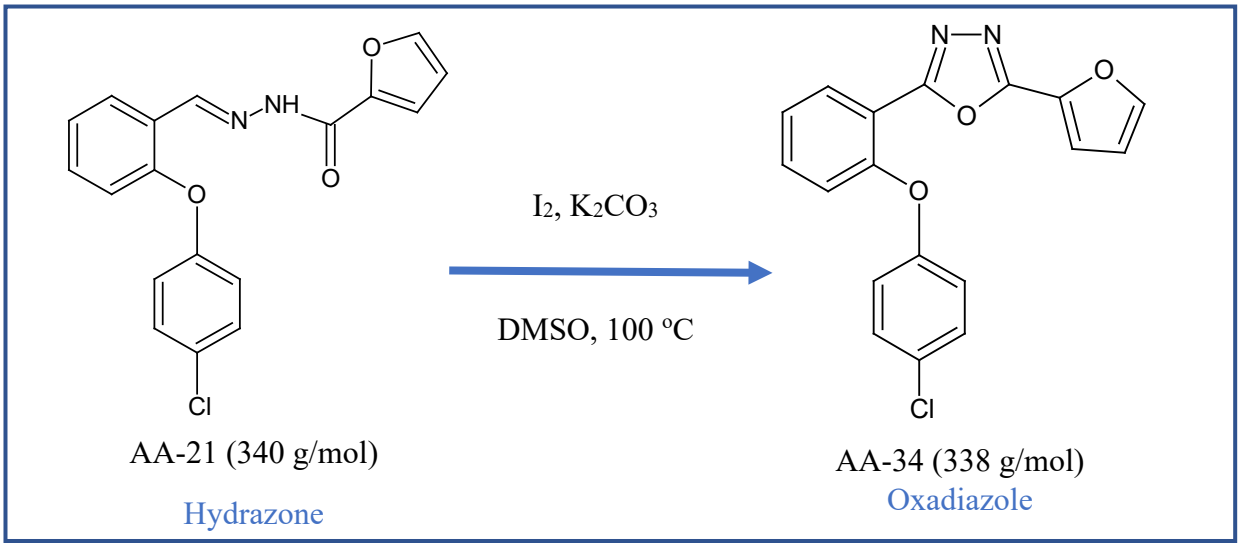
AA-19 (351 g/mol)

Hydrazone

AA-33 (349 g/mol)

Oxadiazole

Figure 6.12. Synthesis of AA-34 Using AA-21 As Starting Component



AA-21 (340 g/mol)

Hydrazone

AA-34 (338 g/mol)

Oxadiazole

When the foregoing reactions and the 1,3,4 oxadiazoles are inspected, it can clearly be seen that they all have different molecular weights ranging from 306 g/mol to 384 g/mol. Since the 1,3,4 oxadiazoles are obtained by cyclization of the respective hydrazones, each oxadiazole is 2 g/mol lighter than the corresponding hydrazone.

6.2 Anticancer Activity

6.2.1 Effect of molecules on different cell lines

Cell viability was investigated on A-549, MDA-MB-231, PC-3 cancerous cell lines, and MRC-5 fibroblast lines. The effect of molecules on different cell lines is given in **Table 6.2.** below.

Table 6.2. IC-50 Values of All Hydrazones and Oxadiazoles Based on Each Cell Line

Molecules	A-549	MDA-MB-231	PC-3	MRC-5
AA-5 (H1)	28,65 µM	53,02 µM	47,75 µM	16,89 µM
AA-26 (O1)	29,28 µM	24,02 µM	65,96 µM	74,45 µM
AA-7 (H2)	72,81 µM	137,5 µM	25,27 µM	24,44 µM
AA-16 (O2)	152,2 µM	46,92 µM	82,23 µM	36,02 µM
AA-8 (H3)	207,5 µM	50,84 µM	86,02 µM	43,46 µM
AA-27 (O3)	109,4 µM	42,62 µM	120,9 µM	12,82 µM
AA-9 (H4)	49,79 µM	31,46 µM	9,389 µM	44,66 µM
AA-28 (O4)	102,1 µM	78,6 µM	54,31 µM	40,07 µM
AA-10 (H5)	13,39 µM	108,3 µM	18,09 µM	86,96 µM
AA-11 (H6)	36,9 µM	77,2 µM	65,91 µM	56,82 µM
AA-29 (O6)	45,79 µM	32,66 µM	34,16 µM	141,1 µM
AA-12 (H7)	31,84 µM	45,08 µM	66,53 µM	62,75 µM
AA-23 (O7)	64,78 µM	55,74 µM	126,8 µM	16 µM
AA-13 (H8)	73,04 µM	65,8 µM	78,34 µM	66,87 µM
AA-30 (O8)	78,58 µM	38,2 µM	78,22 µM	23,07 µM
AA-14 (H9)	208,5 µM	205,7 µM	84,56 µM	31,66 µM
AA-24 (O9)	72,85 µM	23,07 µM	41,37 µM	23,07 µM
AA-15 (H10)	26,56 µM	25,9 µM	18,39 µM	37,06 µM
AA-17 (H11)	121,9 µM	32,69 µM	46,35 µM	66,87 µM
AA-31 (O11)	89,98 µM	79,2 µM	65,83 µM	58,34 µM
AA-18 (H12)	39,43 µM	77,7 µM	30,2 µM	14,55 µM
AA-32 (O12)	36,26 µM	22,73 µM	38,42 µM	51,87 µM
AA-19 (H13)	71,77 µM	55,51 µM	38,12 µM	37,93 µM
AA-33 (O13)	137,47 µM	108,5 µM	124,1 µM	124,1 µM

AA-21 (H14)	39,36 µM	37,85 µM	30,39 µM	50,55 µM
AA-34 (O14)	28,84 µM	30,37 µM	25,6 µM	35,59 µM
AA-20 (H15)	89,65 µM	100,4 µM	27,03 µM	7,784 µM

As noted in **Table 6.2.**, different compounds exhibit the lowest IC-50 values in different cell lines. For the A-549 cell line, AA-10 coded hydrazone has the minimum IC-50 value of 13.39 µM. For the MDA-MB-231 cell line, however, AA-32 coded oxadiazole demonstrated the lowest IC-50 value with 22.73 µM. For PC-3, which is the last cancerous cell line in this study, the hydrazone AA-9 showed activity with the lowest concentration of 9,389 µM.

Considering the activities of these compounds in other cell lines, hydrazone AA-10 demonstrated activity at 18.09 µM for PC-3 and 108.3 µM for MDA-MB-231. For oxadiazole AA-32, activity was detected at 36.26 µM in A-549 and 38.42 µM in PC-3. Finally, IC-50 values of 49.79 µM for A-549 and 31.46 µM for MDA-MB-231 were computed for hydrazone AA-9. The values of these compounds on the fibroblast cells MRC-5 which were utilized as a control group in this study were determined to be 44.66 µM for AA-9, 86.96 µM for AA-10, and 51.87 µM for AA-32.

The letters H and O are given next to the molecules in the table to indicate whether they are hydrazone or oxadiazole, as well as numbers to understand which oxadiazole is produced from which hydrazone. As a result, it is apparent from the table that for instance AA-26 oxadiazole, encoded as O1, was synthesized utilizing AA-5 hydrazone, which is encoded as H1.

When the activities of the oxadiazoles and the corresponding hydrazones on the A-549 cell line were compared, it was reported that the oxadiazoles of O3, O9, O11, O12, and O14 showed higher activity in lower dosages than their hydrazones. The hydrazones H1, H2, H4, H6, H7, H8, and H13 were shown to be more active than their oxadiazoles.

When the same comparisons were performed on the MDA-MB-231 line, it was discovered that, unlike A-549, oxadiazoles generally outperformed hydrazones. O1, O2, O4, O6, O8, O9, O12, and O14 are more active than their hydrazones, while H3, H7, H11, and H13 are more active than their oxadiazoles.

When these results were analyzed for PC-3, the final cancerous line in this study, only O6, O8, O9, and O14 oxadiazoles outperformed their hydrazones. There is little difference between the activity of O8 oxadiazole and H8 hydrazone. remaining hydrazones are more active than their oxadiazole competitors.

It was discovered that MRC-5, which acted as the control group, was inhibited by O3, O4, O7, O8, O9, O11, and O14 oxadiazoles at lower concentrations than their hydrazones.

When all lines and molecules are considered, it is seen that O9 and O14 oxadiazoles have higher activity than hydrazones in all cancer lines and also in the control group. For the remaining molecules, it is not possible to generalize the results that are valid for all of the cell lines. Activities differ depending on the lines.

Because of the high number of molecules and the execution of research with several cell lines, it is critical to depict the data in **Table 6.2.** over graphs. The following **Figure 6.13.** depicts the effects of all molecules on all cell lines.

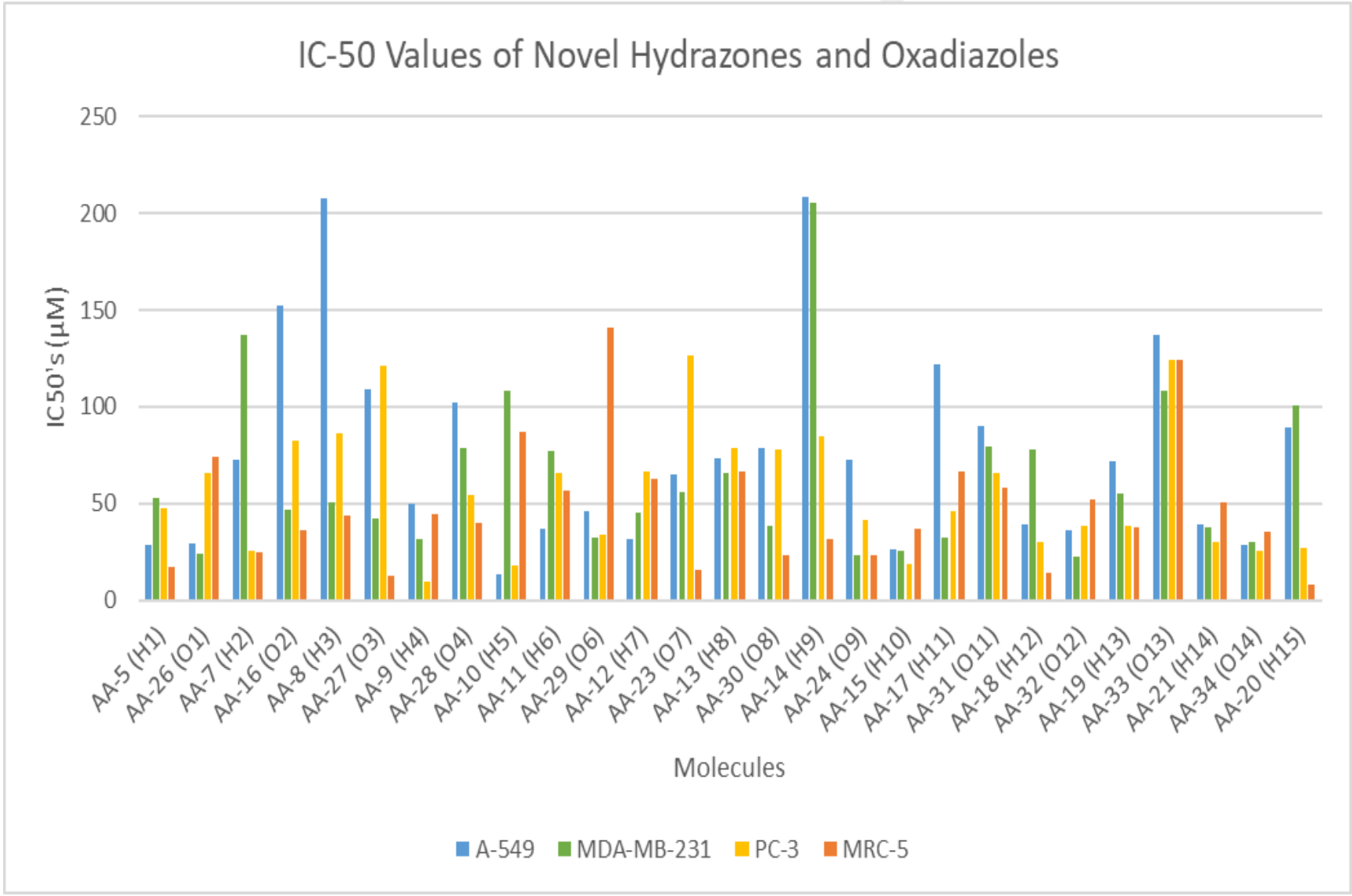
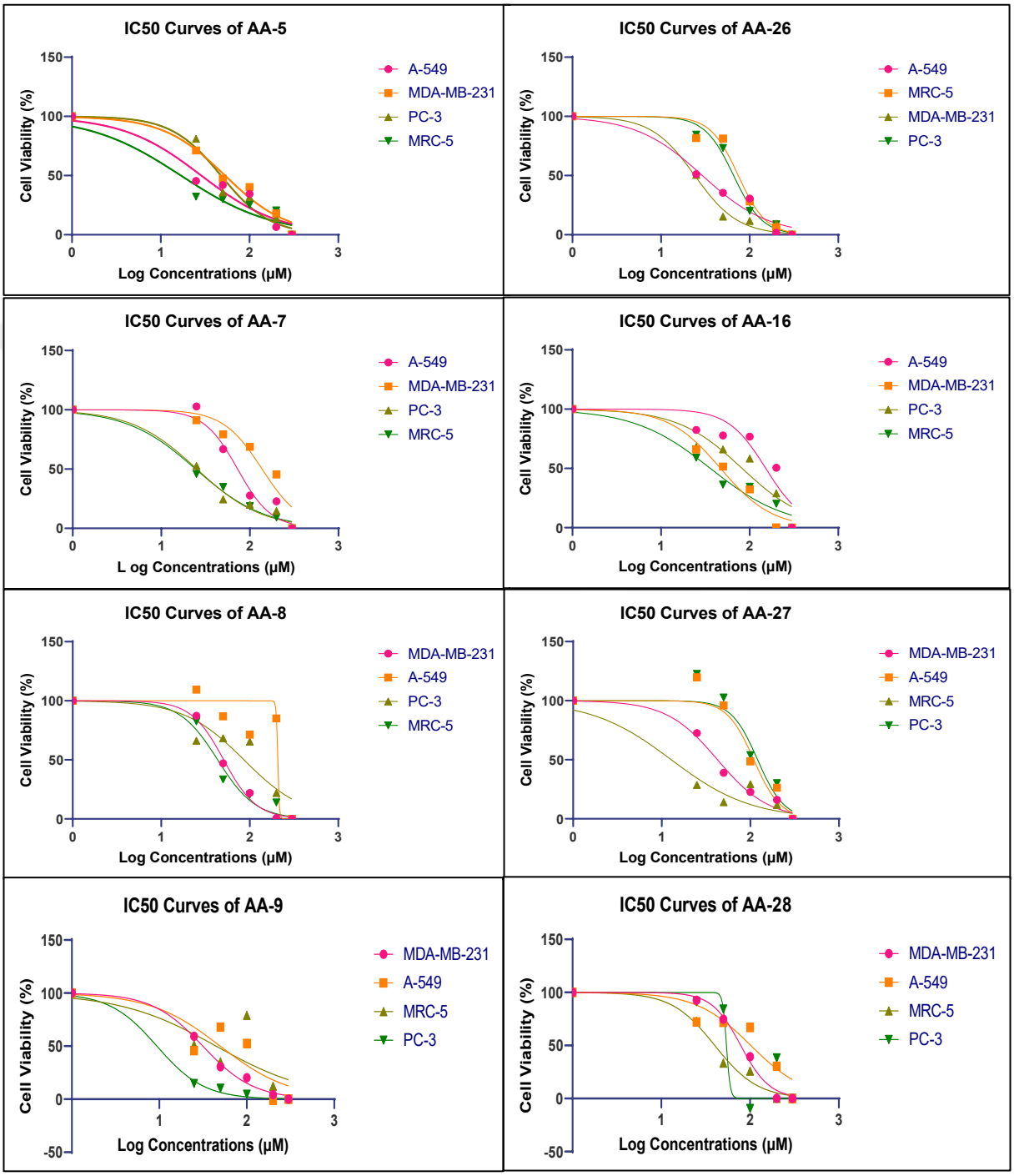
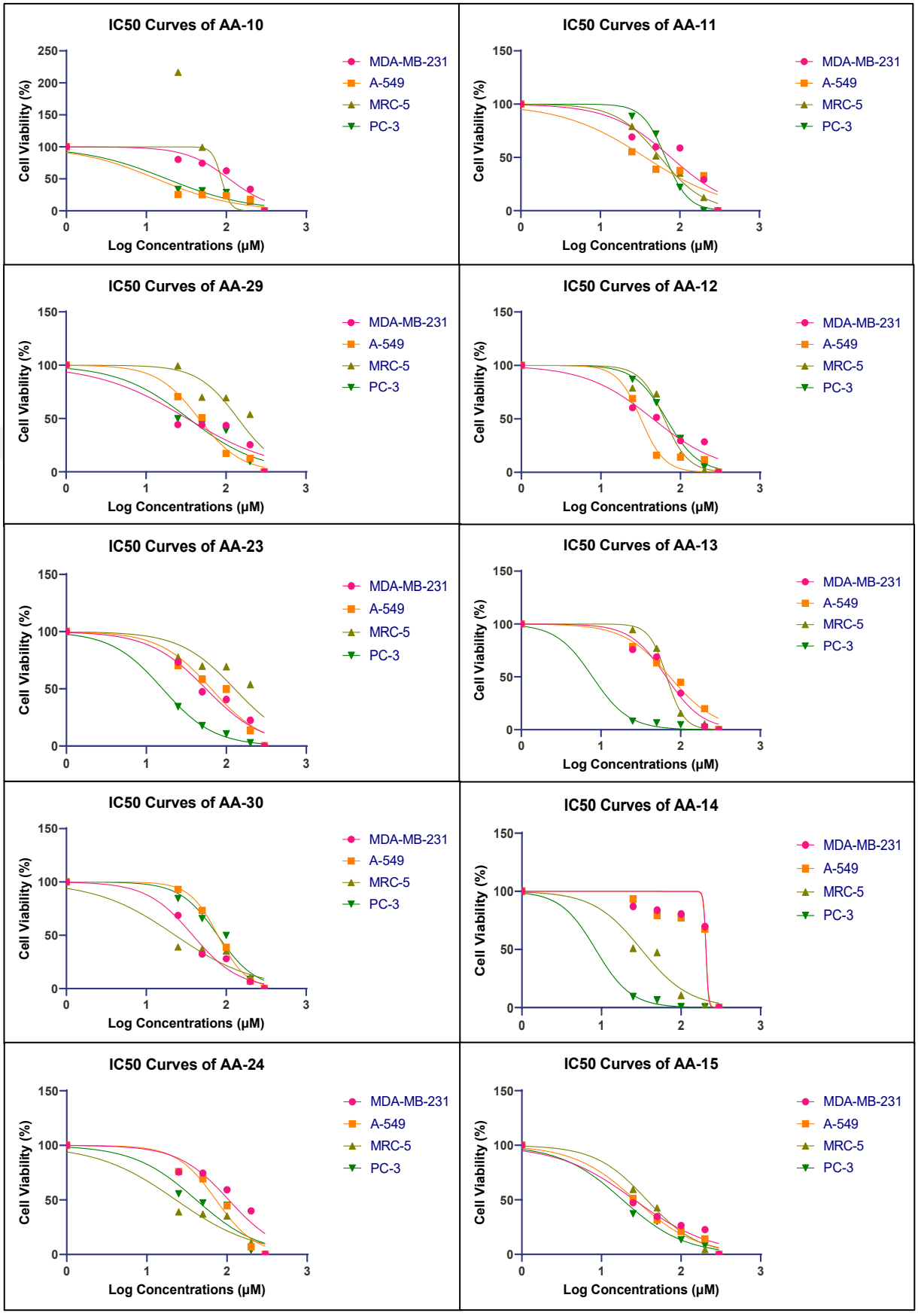


Figure 6.13 IC50 Values of Novel Hydrazones and Novel 1,3,4 Oxadiazoles on All of The Cell Lines

The data in **Table 6.2.** was used to construct molecule-based IC50 curves for all cell lines.




IC50 Curves of AA-10
Cell Viability (%)
Log Concentrations (μM)
MDA-MB-231
A-549
MRC-5
PC-3
IC50 Curves of AA-11
IC50 Curves of AA-29
IC50 Curves of AA-12
IC50 Curves of AA-23
IC50 Curves of AA-13
IC50 Curves of AA-30
IC50 Curves of AA-14
IC50 Curves of AA-24
IC50 Curves of AA-15

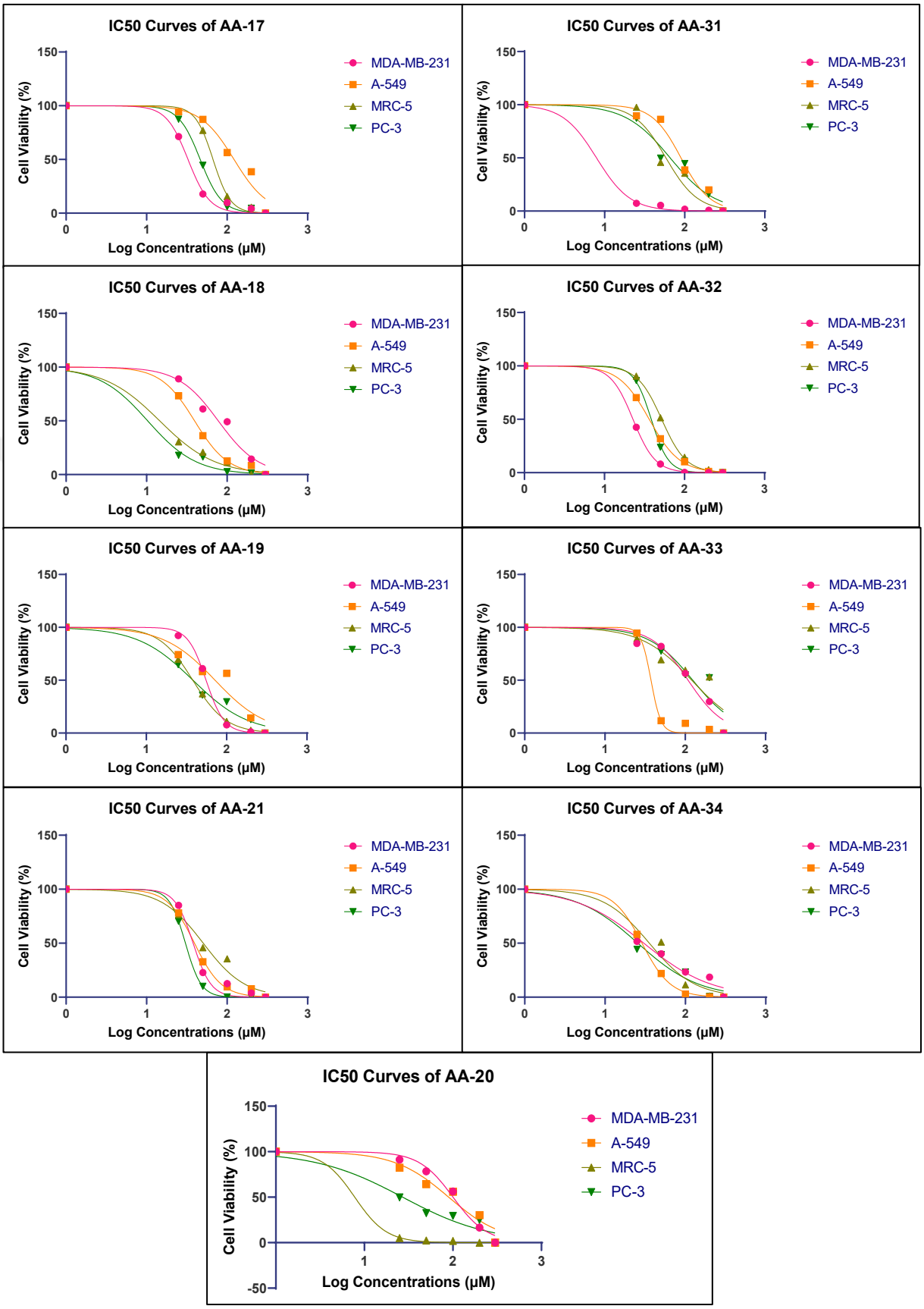


Figure 6.14. IC50 Curves of All Molecules in All Cell Lines

6.2.2 Scratch assay

Since A-549 and MDA-MB-231 are two of the most metastatic cell lines investigated in this study, a scratch assay was performed to see if the three compounds with the greatest activity had any antimetastatic influence on these lines.

For MDA-MB-231 the dosages were 40 μM for AA-9, 120 μM for AA- 10 and 30 μM for AA-26. For A-549 the dosages were 55 μM for AA-9, 15 μM for AA- 10 and 15 μM for AA-26. After the application, three photos were taken; immediately, 24 hours later, and 48 hours afterward. In **Figure 6.15.** below, the pictures of each time stamp for all three molecules can be seen.

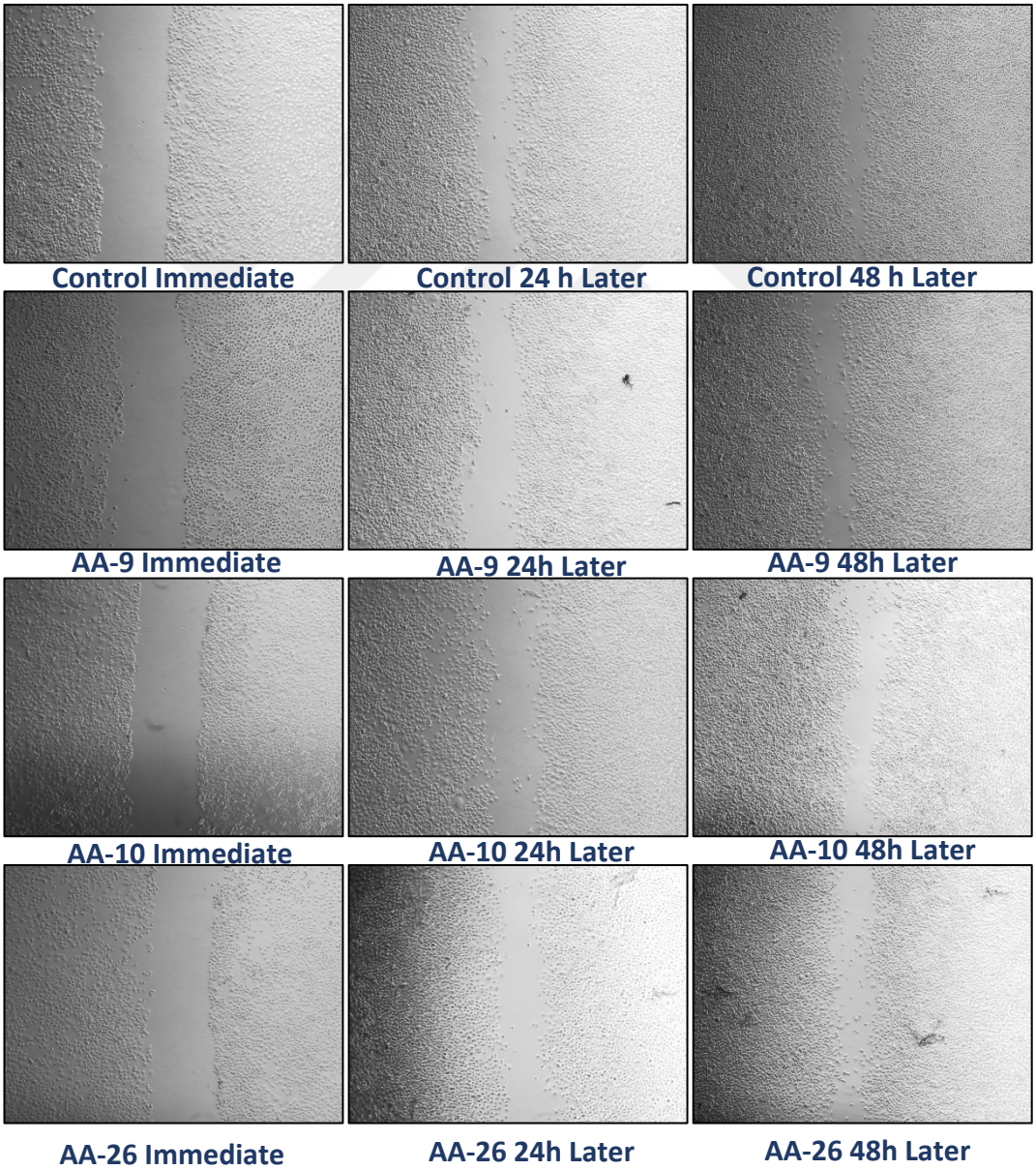


Figure 6.15. Scratch Assay Images of AA-9, AA-10 and AA-26 on A-549 cell line

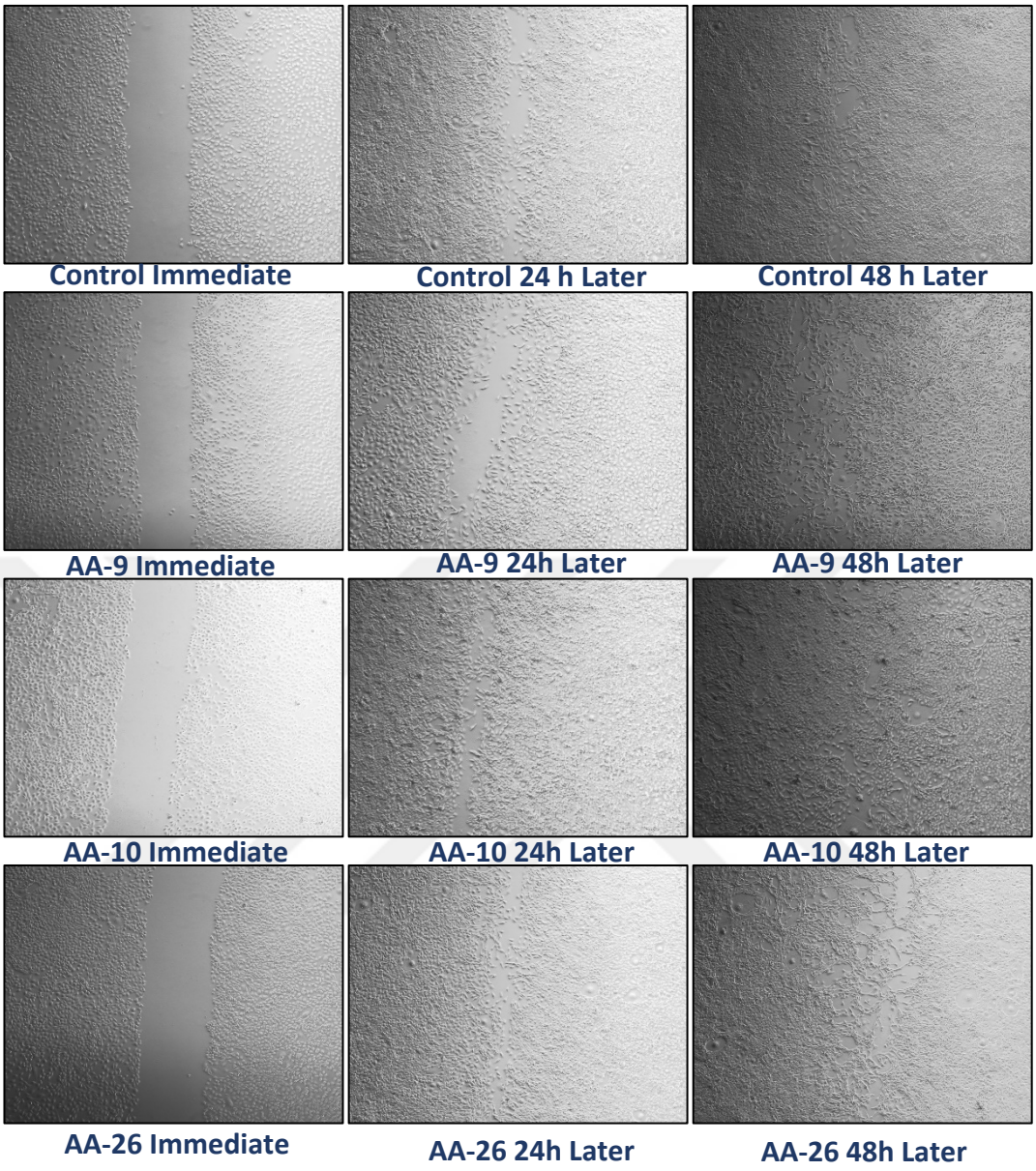


Figure 6.16. Scratch Assay Images of AA-9, AA-10 and AA-26 on MDA- MB-231 Cell Line

When the healing rates of the wounds were compared, it was determined that the added molecules did not cause a significant change in the wound healing rate.

In order to make comparisons, each application was made in triplicate for all three molecules, the averages and standard deviations were taken, and the p values were calculated by considering the sample size.

7. DISCUSSION AND CONCLUSION

In this study, the anticancer activities of fifteen novel hydrazone and twelve novel oxadiazole molecules were investigated by calculating IC-50 values on A-549 (human lung cancer epithelial cells), MDA-MB-231 (human breast carcinoma), and PC-3 (human prostatic carcinoma) cell lines.

The cytotoxic activities of the synthesized compounds were investigated at 300 µM, 200 µM, 100 µM, 50 µM, and 25 µM. According to the data obtained as a result of these scans, a scratch assay was performed for the three most promising compounds, AA-9 hydrazone, AA-10 hydrazone, and AA-26 oxadiazole. In this way, it was desired to examine whether these molecules show an antimetastatic effect.

When the cytotoxicity levels in the A-549 cell line are investigated, the best IC-50 value was found to belong to the AA-10 hydrazone (IC-50:13,39 µM). This was followed by the hydrazones AA-15 (IC-50:26,26 µM) and AA-5 (IC-50:28,65 µM) in terms of activity.

The inhibitory effect seen on the A-549 cell line was compared to the control group, the MRC-5 cell line. AA-10 hydrazone (IC-50:13,39 µM), AA-29 oxadiazole (IC-50:45,79 µM), and AA-26 oxadiazole (IC-50:29,28 µM) were found to have a high effect on A-549 while being less hazardous to MRC-5. Their IC-50 values were 86.89 µM, 141,1 µM, and 74,45 µM respectively for the MRC-5 cell line. Although AA-5 hydrazone (IC-50:28,65 µM) and AA-15 hydrazone (IC-50:26,56 µM) have a greater effect than these compounds at lower concentrations, they should be investigated more closely because they also show similar toxic effects on MRC-5 in relatively low concentrations as well.

When the cytotoxicity values of all hydrazones and their counterpart oxadiazoles were tested in the A-549 cell line, no significant and general connection was identified between them.

The first pair, AA-5 hydrazone (IC-50:28,65 µM) and AA-26 oxadiazole (IC-50:29,28 µM), labeled as H1 and O1, demonstrated activity in similar concentrations. AA-11 hydrazone (IC-50:36,9 µM), and AA-29 oxadiazole (IC-50:45,79 µM), labelled as H6 and O6; AA-13 hydrazone (IC-50:73,04 µM) and AA-30 oxadiazole (IC-50:78,58 µM)- labelled as H8 and O8; AA-18 hydrazone (IC-50:39,43 µM) and

AA-32 oxadiazole (IC-50:36,26 μM), labelled as H12 and O12, and finally, AA-21 hydrazone (IC-50:39,36 μM) and AA-34 oxadiazole (IC-50:28,84 μM), labelled as H14 and O14, had also similar activities with each other.

However, it is not possible to make this inference for the other seven pairs used in the study. An almost twofold difference in activity was seen between the AA-7 hydrazone (IC-50:72,81 μM) and the AA-16 oxadiazole (IC-50:152,2 μM), labeled as H2 and O2. Similarly, between the AA-8 hydrazone (IC-50:207,5 μM) labeled as H3 and the AA-27 oxadiazole (IC-50:109,4 μM) labeled as O3; between AA-9 hydrazone (IC-50:49,79 μM) and AA-28 oxadiazole (IC-50:102,1 μM) labeled as H4 and O4; between AA-12 hydrazone (IC-50:31,84 μM) and AA-23 oxadiazole (IC-50:64,78 μM) labeled as H7 and O7; between AA-19 hydrazone (IC-50:71,77 μM) and AA-33 oxadiazole (IC-50:137,47 μM) labeled as H13 and O13, a nearly twofold difference was also observed. This difference favors hydrazones in the A-549 cell line for all of the listed pairings.

There was only one case where oxadiazoles performed better than hydrazones. A nearly three-fold difference in favor of oxadiazole between AA-14 hydrazone (IC-50:208,5 μM) and AA-24 oxadiazole (IC-50:72,81 μM), labeled as H9 and O9 was observed.

Seven of the top ten molecules with the highest effect on A-549 in the ranking based on the lowest IC-50 values are hydrazones, and three are oxadiazoles. Three of the top five most active compounds are hydrazones, with two being oxadiazoles. Based on these findings, it was determined that hydrazones were more effective than oxadiazoles on the A-549 cell line.

When a similar analysis was performed on the MDA-MB-231 cell line, it was discovered that the best IC-50 value was found to belong to AA-32 oxadiazole (IC-50:22,73 μM). This was followed by the oxadiazoles AA-24 (IC-50:23,07 μM) and AA-26 (IC-50:24,02 μM).

The inhibitory effect seen on the MDA-MB-231 cell line was compared to the control group, the MRC-5 cell line for this study. Three molecules that provide high activity while causing minimal damage to normal tissue were determined as AA-29 oxadiazole (IC-50:32,66 μM), and AA-26 oxadiazole (IC-50:24,02 μM), and AA-32

oxadiazole (IC-50:22,73 μM). AA-26 and AA-32 are molecules that both show the highest activity for MDA-MB-231 and are less destructive on normal tissue.

When the cytotoxicity levels of hydrazones and their counterpart oxadiazoles were tested in the MDA-MB-231 cell line, no significant and general connection was identified between the pairs.

Listed pairs of AA-9 hydrazone (IC-50:31,46 μM) and AA-28 oxadiazole (IC-50:78,6 μM) labeled as H4 and O4, AA-12 hydrazone (IC-50:45,08 μM) and AA-23 oxadiazole (IC-50:55,74 μM) labeled as H7 and O7, AA-17 hydrazone (IC-50:32,69 μM) and AA-31 oxadiazole (IC-50:78,2 μM) labeled asH11 and O11, finally, AA-19 hydrazone (IC-50:55,51 μM) and AA-33 oxadiazole (IC-50:108,5 μM) labeled H13 and O13 were found to have more active hydrazones than oxadiazoles in their matchings.

While these activity differences are more than double in pairs of AA-9 hydrazone (IC-50:49,79 μM) and AA-28 oxadiazol (IC-50:102,1 μM) labeled as H4 and O4, AA-19 hydrazone (IC-50:71,77 μM) and AA-33 oxadiazol (IC-50:137,47 μM) labeled as H13 and O13, and AA-17 hydrazone (IC-50:121,9 μM) and AA-31 oxadiazol (IC-50:89,98 μM) labeled as H11 andO11. The difference is very small when compared to each other in AA-12 (IC-50:31,84 μM) hydrazone and AA-23 oxadiazol (IC-50:64,28 μM) labeled as H7 and O7.

On the MDA-MB-231 cell line, the oxadiazoles demonstrated greater activity at lower doses in all remaining pairings in this study.

According to the ranking based on the lowest IC-50 values, four of the first ten molecules that demonstrated the greatest effect on MDA-MB-231 were hydrazones, and six were oxadiazoles. Four of the first five molecules are oxadiazoles, with only one being hydrazone. Based on these findings, it was determined that oxadiazoles were more effective than hydrazones on the MDA-MB-231 cell line.

Finally, when the PC-3 cell line was evaluated, it was revealed that the AA-9 (IC-50:9,389 μM) A-10 (IC-50:18,09 μM), and AA-15 (IC-50:18,39 μM) hydrazones had the highest IC-50 values on this cell line.

When compared to the inhibitory impact on MRC-5, the three molecules that provides activity while causing the least amount of damage to normal tissue were

identified as AA-10 and AA-9 hydrazones, as well as AA-29 oxadiazole. Their IC-50 values on MRC-5 was 86,96 μM, 46,66 μM, 141,1 μM respectively.

When the cytotoxicity levels of hydrazones and their counterpart oxadiazoles were tested in the PC-3 cell line, no significant and general connection was identified between the pairs. Listed pairs of AA-11 hydrazone (IC-50:65,91 μM) and AA-29 oxadiazole (IC-50:34,16 μM) labeled as H6 and O6, AA-14 hydrazone (IC-50:84,56 μM) and AA-24 oxadiazole (IC-50:41,37 μM) labeled as H9 and O9, and finally, AA-21 hydrazone (IC-50:30,39 μM) and AA-34 oxadiazole (IC-50:25,6 μM) labeled as H14 and O14 were found to have more active oxadiazoles than hydrazones in their respective pairs.

AA-13 hydrazone (IC-50:78,34 μM) labeled as H8 and AA-30 oxadiazole (IC-50:78,22 μM) labeled O8 have very close activity ranges in the PC-3 cell line. The hydrazones demonstrated higher activity at lower doses in all remaining couples that were used in this study.

According to the ranking based on the lowest IC-50 values, eight of the first ten molecules that showed the best effect for PC-3 were hydrazones, and two were oxadiazoles. Only one of the first five molecules is oxadiazole, while the other four are hydrazones.

As in the A-549 cell line, when a general evaluation is made based on all of the compounds, hydrazones are found to be more effective than oxadiazoles in the PC-3 cell line.

Additional scratch assay on MDA-MB-231 and A-549 cell lines showed that these three compounds did not provide significant antimetastatic effects on those cell lines.

Throughout the study, it was discovered that the hydrazones employed in the production of oxadiazoles did not generate a consistent difference in biological activity when compared with their counterparts in any of the cell lines. According to research, molecules have distinct actions in different forms of cancer. Hence, it is not possible to construct a solid conclusion that applies to all cell lines.

It may be possible to re-evaluate selected candidate compounds as an anticancer agent once again if some changes in the chemical structures of potent compounds are

made to reduce their toxicity and boost activity against cancer. Most promising candidate molecules can be used as starting molecules for this purpose.

In conclusion, when all of the data obtained during the study was reviewed together, AA-9 and AA-10 hydrazones, and AA-26 oxadiazoles were judged to be the most potent compounds among the twenty-seven molecules tested. While making this evaluation, the effects of molecules on all of the cancerous cell lines and toxicity on normal cells were taken into account. AA-10 hydrazone, which is considered to be the most potent molecule, showed a particularly selective effect in PC-3 and A-549 cell lines. The toxicity level of this molecule on the MRC-5 cell line is quite low. AA-10 hydrazone has a thiadiazol heterocyclic aromatic ring in its structure. Thiadiazols are commonly investigated structures by medicinal chemists. This heterocyclic ring has a mesoionic character which lets candidate molecules that carry this ring in their structures to be able to effectively permeate the cell membrane and has more powerful connection with biological targets. AA-10 has a lower impact on MDA-MB-231 cells than A-549 and PC-3 cells and MDA-MB-231 cells are triple-negative cells that do not express receptors on their surfaces. So, it is thought that the heterocyclic structure of thiadiazol in the structure of the molecule has an effect on the binding capacity of the receptors. (68)

Synthesizing the derivatives of this compound and analyzing their activities will enable more active molecules to be synthesized in the future. AA-10 hydrazone, which is regarded to be the most effective chemical, had a very selective effect in PC-3 and A-549 cell lines. The toxicity level of this chemical on the MRC-5 cell line is quite low. It is hypothesized that the heterocyclic structure of thiadiazol in the structure of the molecule has an effect on the binding ability to the receptors. Synthesizing the derivatives of this compound and analyzing the activity will enable more active molecules to be generated.

Although the oxadiazole of this molecule is expected to be more active on MDA-MB-231, the oxadiazole of AA-10 hydrazone could not be synthesized and purified due to chemical challenges. Therefore, in this investigation, the biological activity data of the oxadiazole of AA-10 hydrazone could not be included. By adding different functional groups, AA-10 derivatives and oxadiazoles belonging to these derivatives can be investigated further in terms of their activities against different types of cancer.

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