ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

SYNTHESIS OF NEUROPEPTIDE Y AND DEVELOPMENT OF AN ANTI-NPY MONOCLONAL ANTIBODY FOR PRECLINICAL TREATMENT OF PANCREATIC CANCER

NURDAN ÇAM

M.Sc. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

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Department:	Medical Biotechnology
Program:	Medical Biotechnology
Thesis Title:	Synthesis of Neuropeptide Y and Development of an Anti-NPY Monoclonal Antibody for Preclinical Treatment of Pancreatic Cancer
Student's name and Surname:	Nurdan Çam
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(Head of the Defense)

Jury Member
(Thesis Supervisor)

Jury Member

Jury Member

DECLARATION

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12.05.2023
Nurdan Çam

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LIST OF ABBREVIATIONS AND SYMBOLS

Boc	Tert-butyloxycarbonyl
°C	Celsius degree
DIC	N,N'-Disopropylcarbodiimide
DMF	N,N-Dimethylformamide
FCA	Freund's complete adjuvant
Fmoc	9-fluorenylmethyloxycarbonyl
g	Gram
HAT	Hypoxanthine-Aminopterin-Thymidine
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
IFA	Incomplete Freund's adjuvant
mAb	Monoclonal antibody
µl	Microliter
mg	Milligram
ml	Milliliter
MS	Mass spectrometry
NI	Neural invasion
NPY	Neuropeptide Y
OD	Optical density
Oxyma	Ethyl 2-cyano-2-(hydroxyimino)acetate
pAb	Polyclonal antibody
PDAC	Pancreatic ductal adenocarcinoma
PEG	Polyethylene glycol
PNI	Perineural invasion
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
UPLC	Ultra-performance liquid chromatography

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ÖZET

Nöropeptit Y'nin Sentezlenmesi ve Pankreas Kanserinin Preklinik Tedavisi için Anti-NPY Monoklonal Antikorunun Geliştirilmesi

Nöropeptitler, çeşitli katı tümörlerde anjiyogenez, uzak metastaz oluşumu ve tümör büyümesi gibi birçok süreçte yer alır. Bu alanda ortaya çıkan adaylardan biri nöropeptit Y (NPY) reseptörleridir. NPY'nin bilinen beş farklı reseptörü vardır ve bunların bazılarının ekspresyonu insan pankreas kanseri dokusunda da bulunmuştur. NPY ligandına doğrudan bağlanan ilaçlar, küçük moleküllü inhibitörler veya monoklonal antikorlar henüz ticari olarak mevcut değildir. Bu kapsamda, çalışmada pankreas kanserini önlemeye yönelik yeni tedavi yaklaşımları oluşturabilmek amacıyla NPY peptidinin sentezlenmesi ve hibridoma teknolojisi kullanılarak NPY moleküllerini spesifik olarak nötralize eden monoklonal antikorların üretilmesine yönelik araştırmalar yapılmıştır. Bu çalışmada, 36 aminoasitten oluşan NPY molekülünün peptit sekansından sentetik peptitlerin sentezi katı faz peptit sentezi metodu ile gerçekleştirilmiştir. Monoklonal antikor üretimi çalışmalarında kullanılmak üzere sentezlenen NPY peptidlerinin HPLC analizleri, saflaştırılmaları ve fizikokimyasal karakterizasyonu gerçekleştirildi. Monoklonal antikor üretiminin immünizasyon aşamasında, immünojen olarak kullanılan peptit, enjekte edilen peptit konsantrasyonu ve zaman aralıkları optimize edildi. Daha sonra, hibridoma teknolojisi uygulandı ve NPY peptidine yönelik monoklonal antikor üretiminin varlığı incelendi. Sonuç olarak, NPY peptidi başarıyla sentezlendi. Bağışıklama çalışmalarında, farelerde NPY peptidine karşılık yüksek bağışıklık tepkileri oluştu. Hibridoma teknolojisi ile monoklonal antikor üretimi çalışmaları sonunda, NPY antijenine karşı monoklonal antikor üretiminin varlığı tespit edilmiştir.

Anahtar Sözcükler: Nöropeptit Y, pankreas kanseri, kati faz peptit sentezi, monoklonal antikor, hibridoma teknolojisi

ABSTRACT

Synthesis of Neuropeptide Y and Development of an Anti-NPY Monoclonal Antibody for Preclinical Treatment of Pancreatic Cancer

Neuropeptides are involved in many processes such as angiogenesis, formation of distant metastasis and tumor growth in various solid tumors. One of the emerging candidates in this field is neuropeptide Y (NPY) receptors. NPY has five distinct known receptors, and their expression of some of them has also been found in human pancreatic cancer tissue. Drugs, small molecule inhibitors or monoclonal antibodies that bind directly to the NPY ligand are not yet commercially available. In this context, research has been carried out on the synthesis of NPY peptide and the production of monoclonal antibodies that specifically neutralize NPY molecules by using hybridoma technology in order to create new treatment approaches to prevent pancreatic cancer. In this study, the synthesis of synthetic peptides from the peptide sequence of the NPY molecule consisting of 36 amino acids was carried out by solid phase peptide synthesis method. HPLC analysis, purification and physicochemical characterization of NPY peptides synthesized for use in monoclonal antibody production studies were performed. In the immunization phase of monoclonal antibody production, the peptide used as immunogen, the injected peptide concentration and time intervals were optimized. Next, hybridoma technology was applied and the presence of monoclonal antibody production against the NPY peptide was examined. As a result, the NPY peptide was successfully synthesized. In immunization studies, high immune responses to the NPY peptide were elicited in mice. As a result of monoclonal antibody production studies with hybridoma technology, the presence of monoclonal antibody production against NPY antigen was detected.

Keywords: Neuropeptide Y, pancreatic cancer, solid phase peptide synthesis, monoclonal antibody, hybridoma technology

1 INTRODUCTION AND AIM

Based on the scientific basis of this thesis topic, this study aimed to synthesize NPY surface peptides and to determine the immune response that will occur as a result of immunization of this synthesized peptide in mice. In addition, one of the aims of this study is to produce monoclonal antibodies using this synthetic peptide based on the NPY peptide sequence. This study will be an important step towards the development of treatments for pancreatic cancer.

Therapies that directly target neural invasion have not yet been developed for pancreatic or other types of cancer. It has been determined that the amount of NPY is higher in cancer cells that invade into nerves in human pancreatic cancer patients. At the end of this study, preliminary studies will be carried out for the development of an antibody that directly inhibits NPY ligand. This will allow further studies on treatments that directly target neural invasion for pancreatic or other types of cancer.

2 BACKGROUND

2.1 Pancreas

The pancreas mainly regulates digestion and glucose metabolism. It has two functional components, the exocrine and endocrine pancreas (1). The exocrine component makes up a very large part of the pancreas. It consists mainly of acinar cells and ductal cells. Bicarbonate and digestive enzymes are released into the duodenum by this component. The endocrine component of the pancreas consists of the islets of Langerhans. Various hormones are secreted in this part, and these are insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide (2,3). Four special cell types found in these islets secrete these hormones (Figure 1) (4). Cells that regulate glucose utilization by producing glucagon and insulin are α- and β-cells, respectively. Pancreatic polypeptide and somatostatin, which modulate the secretory properties of other pancreatic cell types, are secreted by PP and δ-cells, respectively. The autonomic nervous system innervates both the exocrine and endocrine components, and their regulation is also provided in separate ways for normal activity (1).

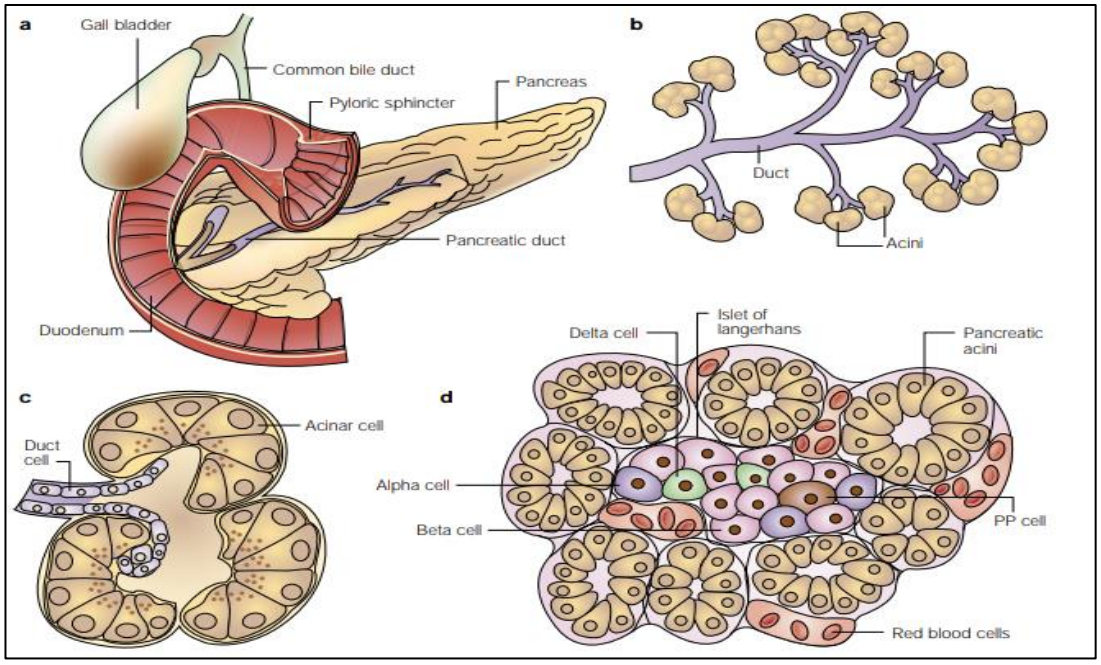

Figure 1. a. Anatomy of the pancreas. b. The exocrine pancreas. c. A single acinus. d. A pancreatic islet (endocrine pancreas) (4)

2.2 Pancreatic Cancer

Pancreatic cancer is the seventh leading cancer type worldwide in cancer-related deaths (5). The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), and 95% of patients cannot be cured. The five-year survival rate is the lowest compared to other cancer types and is characterized by a poor prognosis (6). In untreated patients, the average pancreatic cancer survival time is less than six months (7). It is predicted that by 2030, pancreatic cancer will take the second place as the leading cause of death in the United States (7). Pancreatic ductal adenocarcinoma, the most common type of pancreatic cancer arising from exocrine cells, accounts for approximately 95% of cases (8).

Neural invasion (NI) is one of the main factors causing the poor prognosis of pancreatic cancer (9). Tumor progression, local recurrence, and neuropathic pain are associated with neural invasion in pancreatic cancer (10). This NI term, used instead of the traditional term "perineural invasion (PNI)", is a relatively new and comprehensive term compared to PNI (11). In some studies, NI has been adopted mostly as the presence of pancreatic cancer cells on and along the perineurium (12,13). However, it has been demonstrated that PCa cells can also be found in the endoneural area, that is, between nerve fascicles (14,15).

The most important nerve-related problem for the treatment of clinical pancreatic cancer is PNI (1). PNI is the neoplastic invasion of tumor cells into or around the nerves (10,13,16–18). Compared with other gastrointestinal malignancies, the prevalence of PNI is much higher in PDAC (16,19–21). In addition, PNI is more severe in PDAC than in other gastrointestinal malignancies (19). Nevertheless, the reason why PDAC is associated with the highest rate of incidence of PNI is still unclear.

The potential to improve the prognosis of PDAC patients may be provided by PNI inhibition (1). Nonetheless, the mechanism behind PNI and crosstalk between cancer cells and nerves within the PNI are not clearly understood. In addition, the function of nerves in the initiation and progression of PDAC is still unclear. Elimination of these

uncertainties may provide new treatment strategies that target nerve cancer signaling, prevent PNI, and increase the survival rate of patients with PDAC.

2.3 Neuropeptide Y

Neuropeptide Y (NPY) is a highly conserved 36-amino acid peptide that was first isolated from porcine brain (22). This peptide is a part of a family of pancreatic polypeptides (PPs) and has high sequence similarity to peptides YY (PYY) and PP (23).

The amino acid sequence of NPY;

Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-TyrNH_2 (24).

NPY is particularly highly expressed in the cerebral cortex, hippocampus, hypothalamus, and amygdala (25). In addition, the expression of NPY is high in the spleen, liver, endothelial cells of blood vessels, heart and adrenal medulla (26). It has roles in many physiological functions such as cardiovascular and cognitive processes, food intake, reproduction, energy metabolism, and some neuroendocrine mechanisms (27–29). Receptor subtypes of NPY that mediate all these functions in humans are Y1, Y2, Y4, Y5, and Y6, and these receptors are all G-protein coupled (30). Activation of these G-protein coupled receptors (GPCR) modulates Ca^{2+} and K^{+} channels and also inhibits adenylyl cyclase and cyclic adenosine monophosphate accumulation (31,32).

Neuropeptides have been found to be involved in many processes such as angiogenesis, formation of distant metastasis and tumor growth in various solid tumors (33). There are many peptide hormone receptors that are overexpressed in human cancer, allowing an *in vivo* targeting in a diagnostic or therapeutic background. One of the emerging candidates in this field is NPY receptors. Effects of NPY-related peptides on tumor progression have been shown, particularly on cell proliferation,

angiogenesis, metastasis, and matrix invasion (34–36). In addition, increased expression of NPY and its G-protein coupled receptors has been demonstrated in PDAC (33). However, its association with neural invasion in pancreatic cancer is still has not been clarified yet.

As mentioned above, NPY has five distinct known receptors, and their expression of some of them has also been found in human pancreatic cancer tissue. Although it is not known exactly which of these receptors triggers nerve invasion, drugs, small molecule inhibitors or monoclonal antibodies that directly bind NPY are not yet commercially available. For these reasons, further studies are needed for new treatment approaches to prevent pancreatic cancer.

2.4 Solid Phase Peptide Synthesis

The development of solid phase peptide synthesis (SPPS) has eliminated the repetitive purification steps required at every stage, as in the classical solution-phase synthesis (37). This method is used as a standard method in peptide synthesis for up to 50 amino acid in the sequence. Bruce Merrifield introduced this method in 1963 and received the Nobel Prize in 1984 with this method. Peptides are attached to an insoluble solid polymer support (resin) and chemically synthesized with this method. This method is mainly based on the covalent attachment of the first amino acid (peptide C-terminus) to a solid polymer, and then subsequent coupling of amino acids step by step until the desired sequence is completed, and finally the separation of the synthesized peptide from the solid polymer. After the growing peptide chain is connected to the insoluble solid polymer and synthesis is completed, excess reagents and byproducts formed as a result of the reaction can be removed by filtering and washing.

2.4.1 Principles of solid phase peptide synthesis

The discovery of solid-phase synthesis (Merrified Method (37)) allowed the production of chemically modified peptides.

Solid phase peptide synthesis:

Assembling of a peptide bond from the C- to the N-terminus is provided by this method with using a solid resin bead (38). The addition of more than one amino acid at each step is prevented by protecting the N^{α}-amino group of the amino acid. Firstly, the carboxy group of the C-terminal amino acid is added to a linker group, hence adding it to the resin. After the first amino acid is attached to the resin, the temporary protecting group on the α-amino group is removed. Next, the second amino acid (with its protected N terminus) is connected via its C terminus to the N terminus of the first amino acid thus forming an amide bond. Filtration and washing processes are applied to remove excess reagents in the reaction. The N-terminal protecting group of the newly formed dipeptide added to the resin is removed subsequently. This cycle of peptide synthesis continues until the desired sequence is completed (Figure 2). In the final step of the synthesis, after deprotection of the N-terminal protecting group, the peptide is separated from the resin. Simultaneously, any side chain-protecting groups are removed.

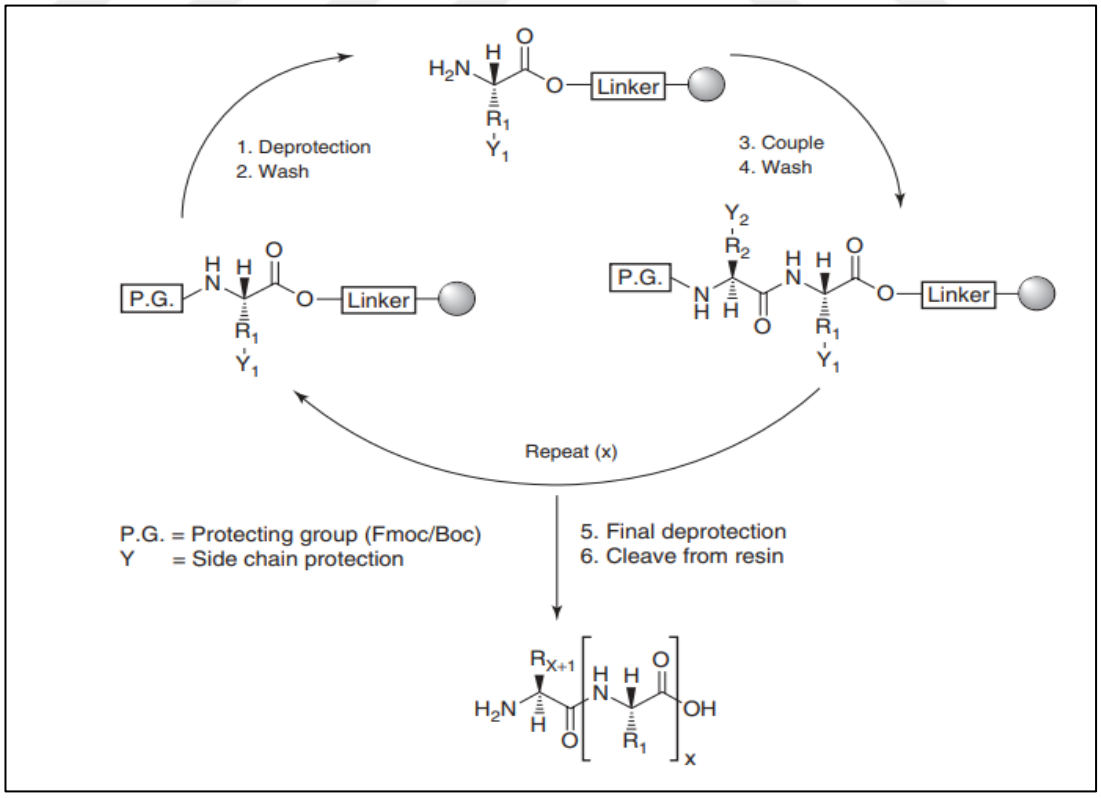

Figure 2. Solid-phase peptide synthesis (38)

2.4.2 Resins and linkers

Selection of suitable resins and linkers is crucial for successful peptide synthesis (39). It should be stable to all solvents to be used throughout the synthesis and to mechanical stability over a wide temperature range. It should have good swelling properties in the solvents used in the synthesis and all reagents should have easy access to the active sites. A number of resins and linkers with a wide range of applications have been developed to date. The choice of polymer support and linker in synthesis is directly related to the choice of coupling reagents, protecting groups, and cleavage conditions. Merrifield initially used polystyrene -based solid support (resin) which is still in use (37). Later, different resins have been developed. One of them is the resin based on polystyrene containing polyethylene glycol (PEG) chains (40) and the other is the resins consisting of PEG chains carrying specific crosslinkers (41).

As mentioned above, there are three types of resin depending on the polymer side group (39). These include polystyrene (PS)-based resins, PS-functionalized polyethylene glycol (PS-PEG) resins, and pure crosslinked PEG resins. PS resins are generally used in the synthesis of short to medium length peptides. PEG-based resins are mostly used for medium to long peptides or peptides with "difficult sequences". Successful performance can be achieved without any problems by choosing the right solid support in peptide synthesis (42). Today, the number of commercially available resins suitable for complex peptide synthesis is very high.

Linkers used in solid phase peptide synthesis have a very important role (39). They provide a linkage between the peptide and the solid support and prevent aggregation. Also, linkers have roles in protecting the C-terminal carboxy group. In addition, another role of the linker is its use in the C-terminal modification of the peptide. It is determinant in the proper selection of coupling reagents, protection groups and the cleavage conditions. The most widely used linkers for Fmoc -based solid phase peptide synthesis are shown in Table 1.

Table 1. The most widely used linkers for Fmoc -based solid phase peptide synthesis (Adapted from Ref (39))

Linker name	Final C-terminal functionality	Cleavage conditions
Rink amide linker	Peptide amides	95% TFA
Sieber amide linker	Protected peptide amides	1%-3% TFA
PAL linker	Peptide amides	95% TFA
Wang HMPA linker	Peptide acids	95% TFA
Wang HMBA linker	Protected peptide acids, amides, hydrazides, alcohols	Nu
SASRIN linker	Peptide acids	0.5%-1% TFA
2-Chlorotrityl chloride	Peptide acids	<0.5% TFA
Aryl hydrazide linker	Peptide amines or esters	Cu(II) cat, pyridine
BAL linker	Peptide acids, aldehydes, thioesters	95% TFA
Safety catch linker	Peptide thioesters	TMS-CHN2

2.4.3 Protection groups

2.4.3.1 Nα amino protecting groups

Solid phase peptide synthesis methods have been developed according to the groups used to protect the Nα-amino group (Figure 3) (39). In one of them, the tert-butyloxycarbonyl (Boc) group is used. This group can be deprotected with acids such as trifluoroacetic acid. Another group used in SPPS is 9-fluorenylmethyloxycarbonyl (Fmoc). This group can be deprotected with bases such as piperidine. Two basic methodologies, the Boc/Bzl methodology, also called Boc-SPPS, and the Fmoc/tBu methodology, also called Fmoc-SPPS, were developed based on the temporary protection and deprotection of amino acids. Due to the toxic and corrosive nature of hydrofluoric acid (HF), an apparatus is required for deprotection and cleavage in the Boc/Bzl methodology. Therefore, the Fmoc/tBu methodology is used more frequently.

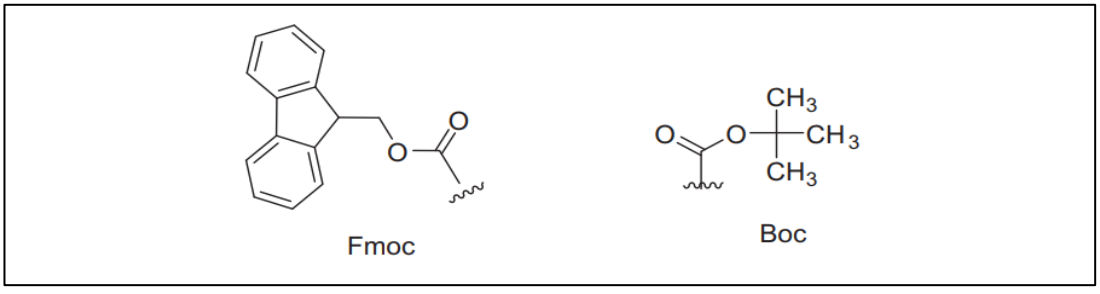

Figure 3. Nα amino protecting groups (39)

2.4.3.2 Side-chain protecting groups

In the past years, side-chain protecting groups in Fmoc-SPPS have been comprehensively developed and studied (43). The orthogonal strategy that allows deprotection of multiple protecting groups without affecting other protecting groups under well-defined reaction conditions, provides an advantage to the Fmoc/tBu methodology (44).

Amino acids with side-chains containing reactive functional groups are generally abundant in proteins (45). In solid phase synthesis, these potentially reactive groups are masked. The reasons for this are very harsh conditions in the synthesis and the need to reach the highest efficiency level in all chemical reactions. Common side chain protecting groups mainly used in Fmoc-SPPS are shown in Table 2 (39).

Table 2. Common side chain protecting groups in Fmoc-SPPS (Adapted from Ref (39))

TFA labile		**TFA stable**	
Protecting groups	**Amino acids**	**Protecting groups**	**Amino acids**
tBu	Asp, Glu: ester Ser, Thr, Tyr: ether	Alloc	Lys
Boc	Lys, Trp	Dde	Lys
Trt	Cys, His, Asn, Gln	Acm	Cys
Pbf	Arg		

2.4.4 Coupling reagents

In peptide synthesis, coupling is the joining of two amino acid residues to form a peptide bond (42). Coupling occurs when the amino group of one amino acid residue attacks the carbonyl group of the other amino acid containing the carboxyl activated by an electron withdrawing group. Activation of the carboxylic acid moiety of an amino acid with specific reagents is required to form a peptide bond (39). The most common coupling reagents used are shown in Figure 4.

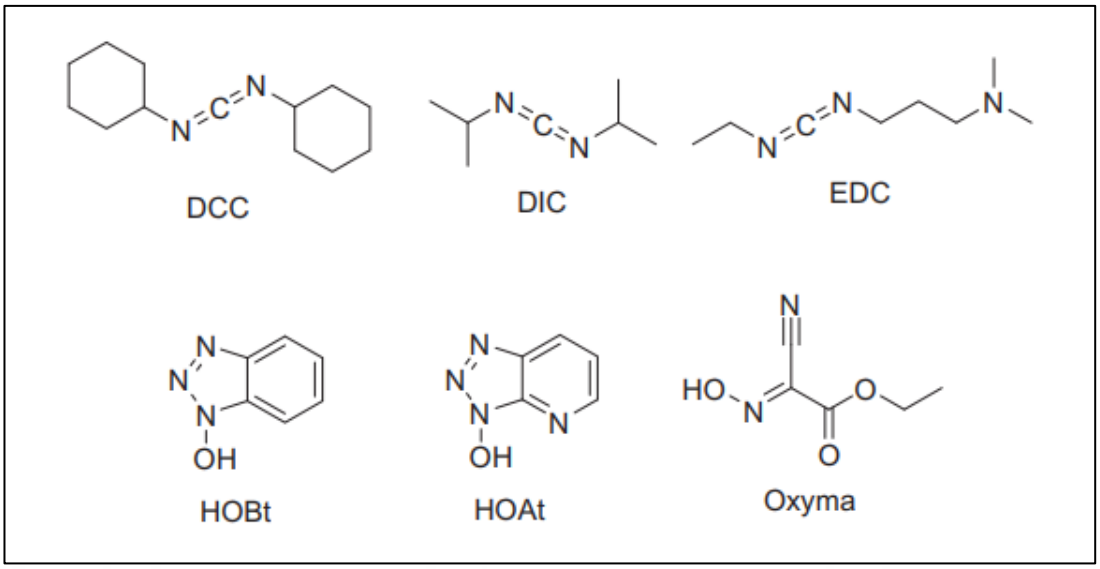

Figure 4. The most common coupling reagents (39)

Carbodiimide-based diisopropylcarbodiimide (DIC) and dicyclohexylcarbodiimide (DCC) are the most commonly used coupling agents (39). They contain two nitrogen atoms which are slightly basic. Thanks to this feature, the reaction between the acid and the carbodiimide can be triggered and O-acylisourea is formed as a result of this reaction. For automatic SPPS, it is preferred to use DIC, where the urea obtained is dissolved in solvents such as DCM or DMF. The addition of nucleophiles such as 1-hydroxy-7-azabenzotriazole (HOAt) or 1-hydroxybenzotriazole (HOBt) is necessary to avoid potential side reactions (epimerization, N-acylurea formation, etc.). In this way, less active benzotriazolyl ester formation is achieved (Figure 5) (46). The use of water-soluble carbodiimide-based N-ethyl-N0 -(3-dimethylaminopropyl) carbodiimide (EDC) is common in solution chemistry. The ethyl 2-cyano-2- (hydroxyimino) acetate (Oxyma), which significantly suppresses racemization in both automatic and manual synthesis, has been used recently (47).

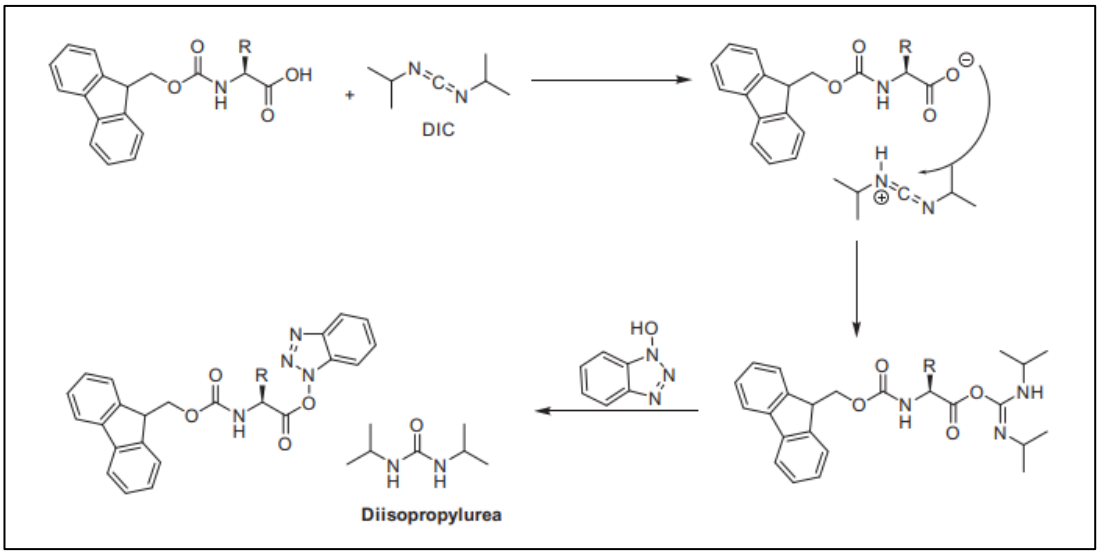

Figure 5. Coupling reaction by using DIC coupling agent (39)

2.4.5 Deprotection / cleavage

After the synthesis of the peptide with the desired sequence is complete, the peptide must be cleaved from the solid support (39). In addition, side-chain protecting groups must be removed. These processes are very important in order to obtain the desired peptide purely and efficiently. Trial experiments should be performed to treat the peptide with a cleavage cocktail and to determine optimum cleavage conditions. The reaction conditions, the completion time of the cleavage, and the scavengers used are the most important factors to consider to ensure a successful release. Any minor change in any of these will alter the chromatographic profile of the crude peptide.

The risk of side reactions that release carbocations during the cleavage process is quite high (39). Scavengers are used to trap these carbocations. While water is the most commonly used scavenger, organic reagents such as triisopropylsilane (TIS), triethylsilane (TES), 1,2 ethanedithiol (EDT), thioanisole, anisole, and thiophenol are also used as scavengers. In addition, 1,4-DL-dithiothreitol (DTT) is an alternative to EDT. The common scavenger reagents used are shown in Figure 6.

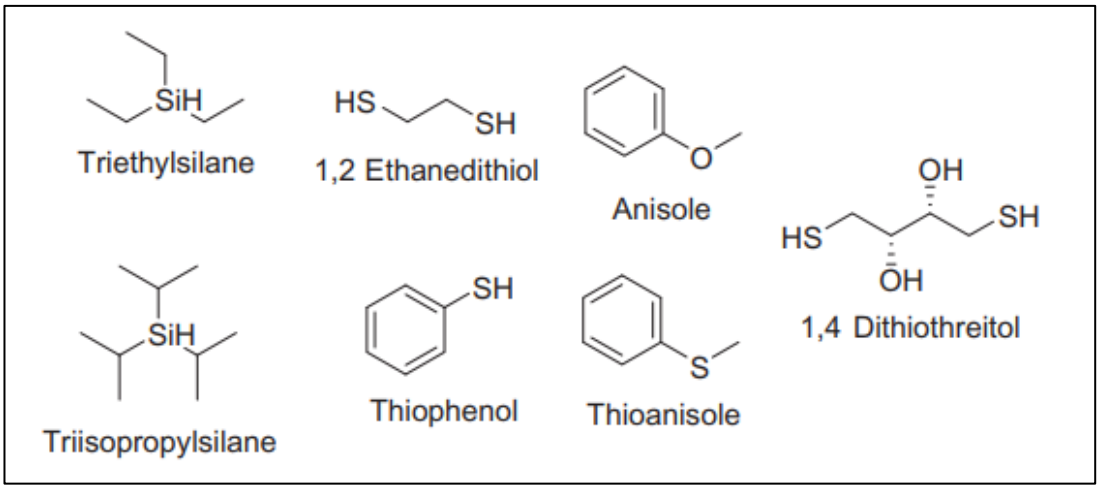

Figure 6. The common scavenger reagents used (39)

Preselection of the cleavage cocktail is hard because the side chain protecting groups, the type of peptide sequence, and the cleavable linker are important factors in determining this (43). The content and reaction time of some cleavage cocktails that have been used with modifications in recent years are given in Table 3 (39).

Table 3. Cleavage cocktails (Adapted from (Ref (39))

Mixture	**Reaction Time (h)**
TFA/phenol/water/TIS (88/5/5/2) (TES could be used instead of TIS)	1-4
TFA/phenol/water/thioanisole/EDT (85/5/5/2.5/2.5)	1-4
TFA/DTT/water/TIS (90/3/5/2)	1-4
TFA/thioanisole/EDT/anisole (90/5/3/2)	1-4
TFA/water/TIS (95/2.5/2.5)	1-4
TFA/EDT/water/TIS (92.5/2.5/3/2)	1-4

After the treatment time with the cleavage cocktail is completed, peptide precipitation is performed using cold diethyl ether or methyl tert butyl ether depending on the properties and size of the synthesized peptide (39). Chilled ether can be used to dissolve hydrophobic peptides, fully protected peptides, and short peptides (4-6 residues). Basically, peptide isolation can be achieved by precipitation, centrifugation, and freeze-drying processes. TFA/water/TIS (95: 2.5: 2.5 v/v/v) mixture can be applied in peptide syntheses using Rink Amid or Wang resins and Trt or tBu main side chain protecting groups. After this method, which is applied for 2-3 hours, peptides can be effectively isolated.

2.5 Monoclonal Antibody Production

Antibodies play an important role in the immune system and are glycoproteins known as immunoglobulins produced by B lymphocytes (Figure 7) (48,49). Antibodies, the main component of the humoral immune system, protect against invading pathogens such as bacteria and viruses (50). Antibodies contain two structural units consisting of light chains and heavy chains (51). Each light chain has one constant and one variable region, while each heavy chain has three constant and one variable regions. This variable region of antibodies important for invading pathogen and antigen recognition is responsible for these recognition interactions. In this antigen antibody recognition mechanism, which works with the lock and key principle, there is a specific paratope (lock) that binds to a specific antigen (key). Antibodies have crystallizable fragments (Fc) structures attached to antigen-binding fragments (48). There are five different types of heavy chains based on this (Fc) structure. Antibodies are grouped into five different isotypes as IgA, IgG, IgM, IgD and IgE, according to these differences in Fc regions (52).

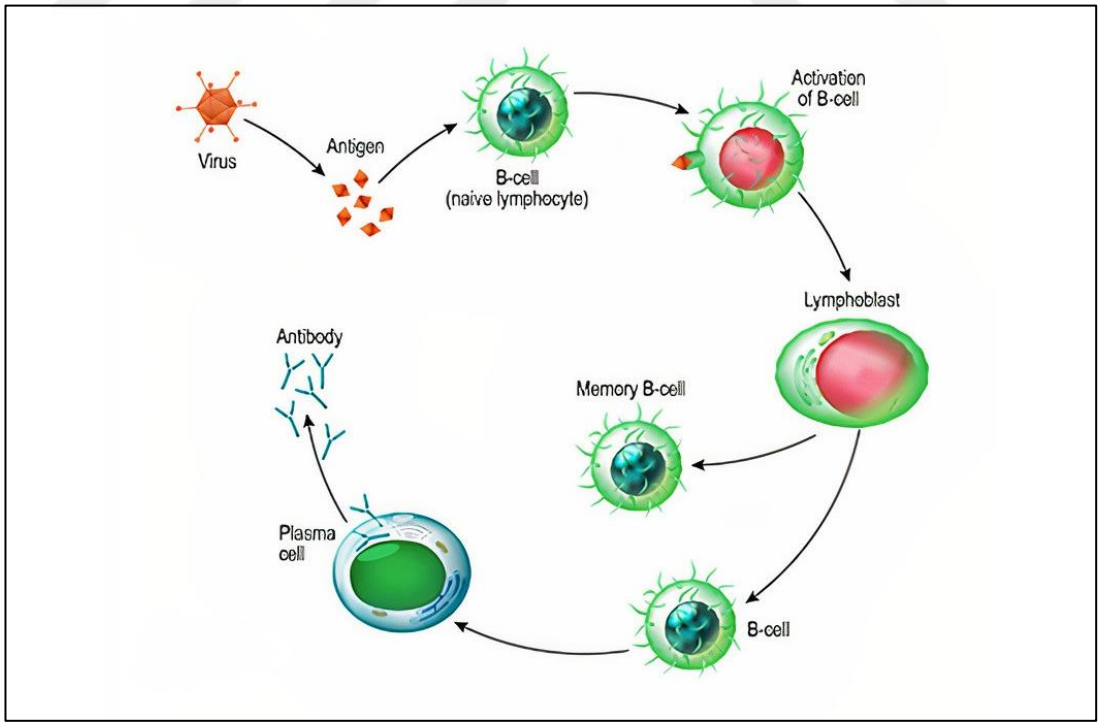

Figure 7. B cell activation and antibody production (49)

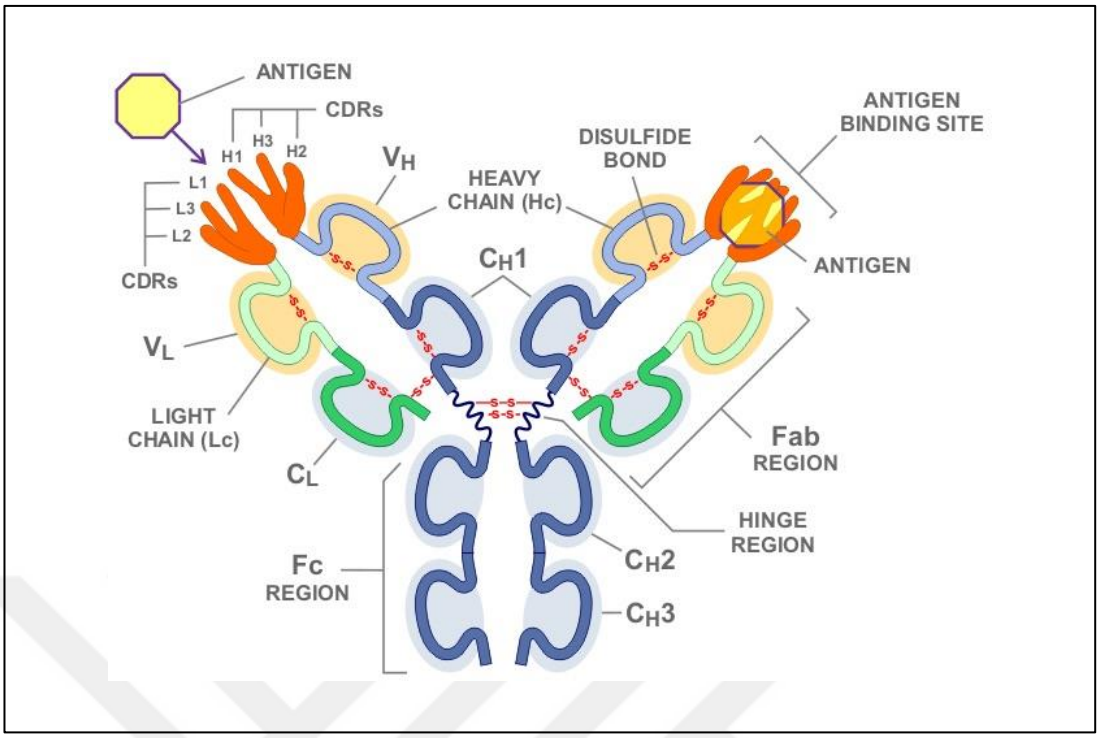

Figure 8. IgG antibody structure (53)

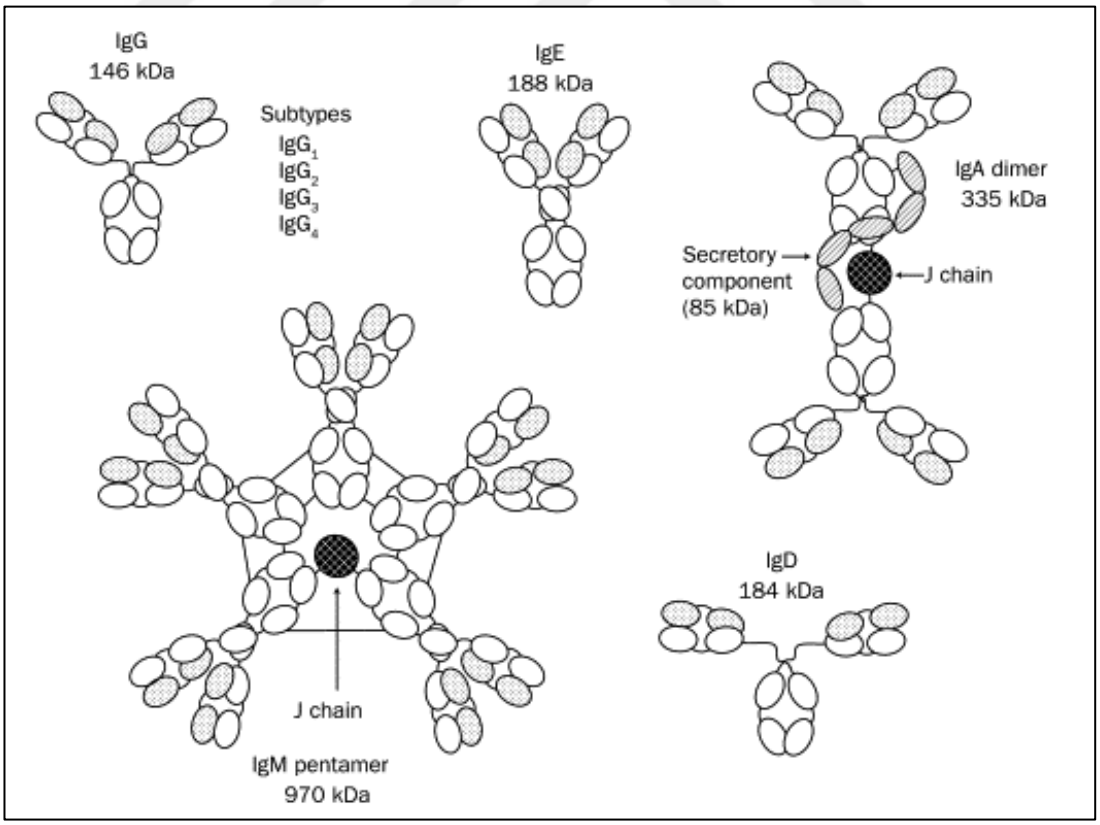

Figure 9. Human Ig classes (54)

Antibodies have long been used in a variety of applications in biomedical research (51). The properties of antibodies such as high specificity and selective binding have enabled them to be used in many applications such as magnetic cell sorting, therapeutic approaches, flow cytometry and immunoassays (55). There are two types of antibodies, polyclonal antibodies and monoclonal antibodies, according to their origin from the lymphocytes (56,57). These two types of antibodies both have advantages and disadvantages that make them equally suitable in a variety of applications (51).

2.5.1 Polyclonal antibodies

Polyclonal antibodies (pAbs), which are immunoglobulin molecules formed by different B cell lineages, react against multiple regions of a particular antigen, called epitopes (51). pAbs are a mixture of various antibodies as they are capable of recognizing multiple epitopes of the antigen (48). Production of pAbs aims to generate a high antibody titer against the specific antigen and is accomplished by injection of an immunogen into the animal with a prime-boost immunization strategy (51). Following immunization, pAbs can be used directly or purified by extraction of other serum protein components using affinity column chromatography. Purified or direct forms of polyclonal sera are attractive reagents for many purposes, such as their use as research or therapeutic reagents, due to their multi-epitope binding ability. One of the common uses of these polyclonal serums for many years is the treatment of toxin-producing bacterial and viral diseases (58).

The production of these antibodies can vary from batch to batch, and these blood-derived products carry the risk of blood-borne disease transmission (48). Another limitation of pAbs is that due to their low specificity, relatively high doses are required in studies to achieve the desired effect. In addition, its lack of use for the treatment of chronic diseases is another disadvantage. Because of all these disadvantages, the need for mAbs arises (51). mAbs are widely used today and are suitable for research due to their specificity, homogeneous nature, affinity, and high sensitivity (59).

2.5.2 Monoclonal antibodies

Monoclonal antibodies produced by a single clone of B lymphocytes are monospecific antibodies with high affinity and specificity that can bind to a single specific epitope of the antigen (51). Hybridoma-based technology for mAb production was used in 1975. By this method, indefinite amounts of mAb are produced continuously, with very minimal and acceptable variation from batch to batch. These antibodies can be produced against a particular epitope of a selected antigen or immunogen. With the development of these antibodies, the use of antibodies in various fields has expanded, thanks to their target specificity (60). mAbs have become an important tool in many fields as diverse as medicine, biochemistry and molecular biology (51).

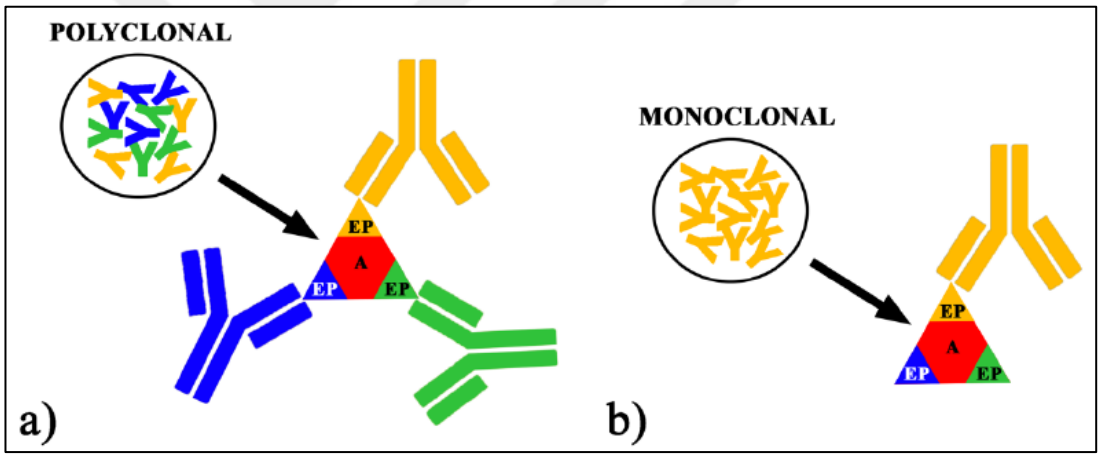

Figure 10. Polyclonal and monoclonal antibodies a) polyclonal antibody; b) monoclonal antibody. A: antigen; EP: epitope (61)

2.5.3 Hybridoma technology

In 1975, hybridoma technology was invented by Georges Kohler and Cesar Milstein, and in 1984 they received the Nobel Prize in medicine and physiology for this work (62). Production of hybridoma cells is accomplished by fusion of antibody-producing short-lived B cells with immortal myeloma cells (51). The hybridoma cell is a large amount of specific mAb. These hybridoma clones can be cryopreserved for an extended period of time to ensure continuous mAb production. A host animal has

the ability to produce highly specific, functional, and high-affinity mAbs, and this ability is used to generate hybridomas (63). Several mAbs developed over the years for use in the diagnosis, treatment and prevention of different diseases have been produced using this technology.

Early in the use of hybridoma technology, only murine antigens were used (51). The development of this field has made it a very important technology for producing mAbs against a wide variety of antigens and from different species. These species are humans (64,65), goats, sheep (66), cows (67), chickens (68), mice (69), rabbits (70), guinea pigs and rats (71). Mice are widely preferred as hosts for mAb production, followed by rabbits (51).

Hybridomas can be classified into two types, homo-hybridomas and hetero-hybridomas (51). Homo-hybridomas are formed when IgG-secreting B cells and their fusion partners are of the same species. Hetero-hybridomas are formed when antibody-secreting B cells and their fusion partners are of different species. Homo hybridomas are more genetically stable than hetero-hybridomas and secrete stable IgG. The reason for this is the gradual loss of chromosomal recombinants during the clonal selection stage due to genetic instabilities in hetero-hybridomas.

2.5.3.1 Monoclonal antibody production with hybridoma technology

Production of mAb with hybridoma technology is based on the immunization of a target antigen to laboratory animals and the fusion of B-cells formed in the animal at this stage with myeloma cells (Figure 11). Then, these hybrid cells are selected in HAT medium and finally, the cells that secrete the desired antibodies are screened.

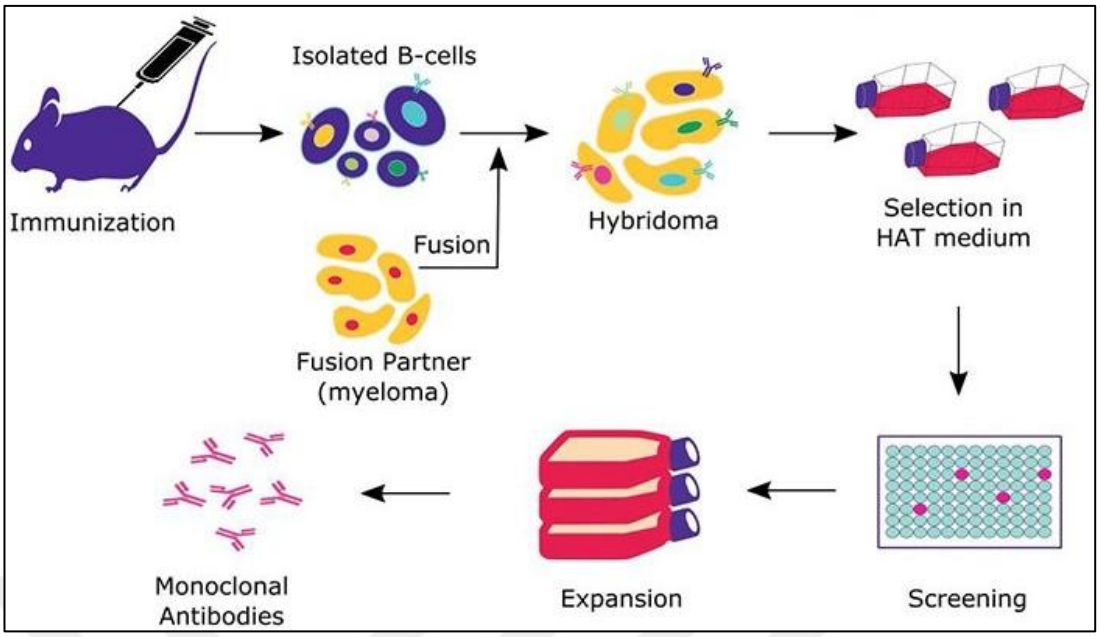

Figure 11. Hybridoma technology used for monoclonal antibody production (72)

The steps of mAb production with hybridoma technology are explained in detail below.

Immunization:

The first step in antibody production with hybridoma technology is immunization. The aim at this stage is to stimulate B cell differentiation into plasma B cells and memory B cells (48). Immunization administered to laboratory experimental animals such as rabbits or mice is injected with a selected antigen to which the antibodies are raised by a series of injections at intervals of several weeks. If, after several weeks of immunization by injection, sufficient antibodies are produced in the animal serum, the animal is sacrificed (73).

B lymphocyte isolation:

After immunization and sacrification of the animal showing the highest antibody titer, the animal's spleen is removed under aseptic conditions to isolate active B cells. Density gradient centrifugation is used to perform this step (48). Methods such as ELISA (73) or flow cytometry are used to determine the antibody presence in the

serum. Activated B lymphocytes that produce antibodies are found in the serum. These B lymphocytes are then fused with myeloma cells.

Preparation of myeloma cells:

A few weeks prior to fusion, myeloma cells are incubated in 8-azaguanine to obtain non-functional hypoxanthine-guanine phosphoribosyltransferase (HGPRT) genes (48). Inhibition of nucleotides assembly in the alternative salvage pathway is achieved by non-functional HGPRT, thereby sensitizing these cells to the HAT media (73). Therefore, it is the preferred method in hybridoma technology.

Fusion:

In the cell fusion process, activated B lymphocytes are fused with HAT-sensitive myeloma cells (48). Isolated activated B lymphocytes are centrifuged with HAT-sensitive myeloma cells in a fusion-promoting media. In this fusing process, polyethylene glycol (PEG) is used (73). In fusion with PEG, the plasma membranes of antibody-producing B lymphocytes and myeloma cells are fused, thus forming cells with more than one nucleus (48). Another method is used is electro-fusion using electric current. Electro-fusion, another method used alongside fusion with PEG, enables cells to fuse under the effect of an electric field.

Selection in HAT Medium:

In the presence of PEG, the cells are fused to form hybridoma cells (48). However, even if the fusion process is very efficient, only about 1 to 2% of hybridoma cells will be formed. In addition, only about 1 out of 100 cells will survive from these hybridoma cells. Ultimately, this process results in a number of unfused cells in the medium. These cells are cultured in HAT media for 10-14 days in order to select their fused cells from all these non-fused cells. HAT, the medium that provides selectivity, contains hypoxanthine-aminopterin-thymidine. Aminopterin contained in the HAT media blocks the nucleotide synthesis of cells via de nova synthesis pathway. Cells

treated with aminopterin survive in the presence of hypoxanthine and deoxythymidine using a secondary salvage pathway with functional hypoxanthine-guanine phosphoribosyltransferase (HGPRT) genes. Unfused B lymphocytes, which have a limited lifespan, naturally die within a few days. Unfused myeloma cells deficient in the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene also die. For these reasons, only Myeloma-B lymphocyte hybrid cells remain in the media. These cells are HGPRT positive with the functional HGPRT gene of B lymphocytes, and they can continue to grow indefinitely in the HAT media thanks to the immortality feature provided by myeloma cells (73).

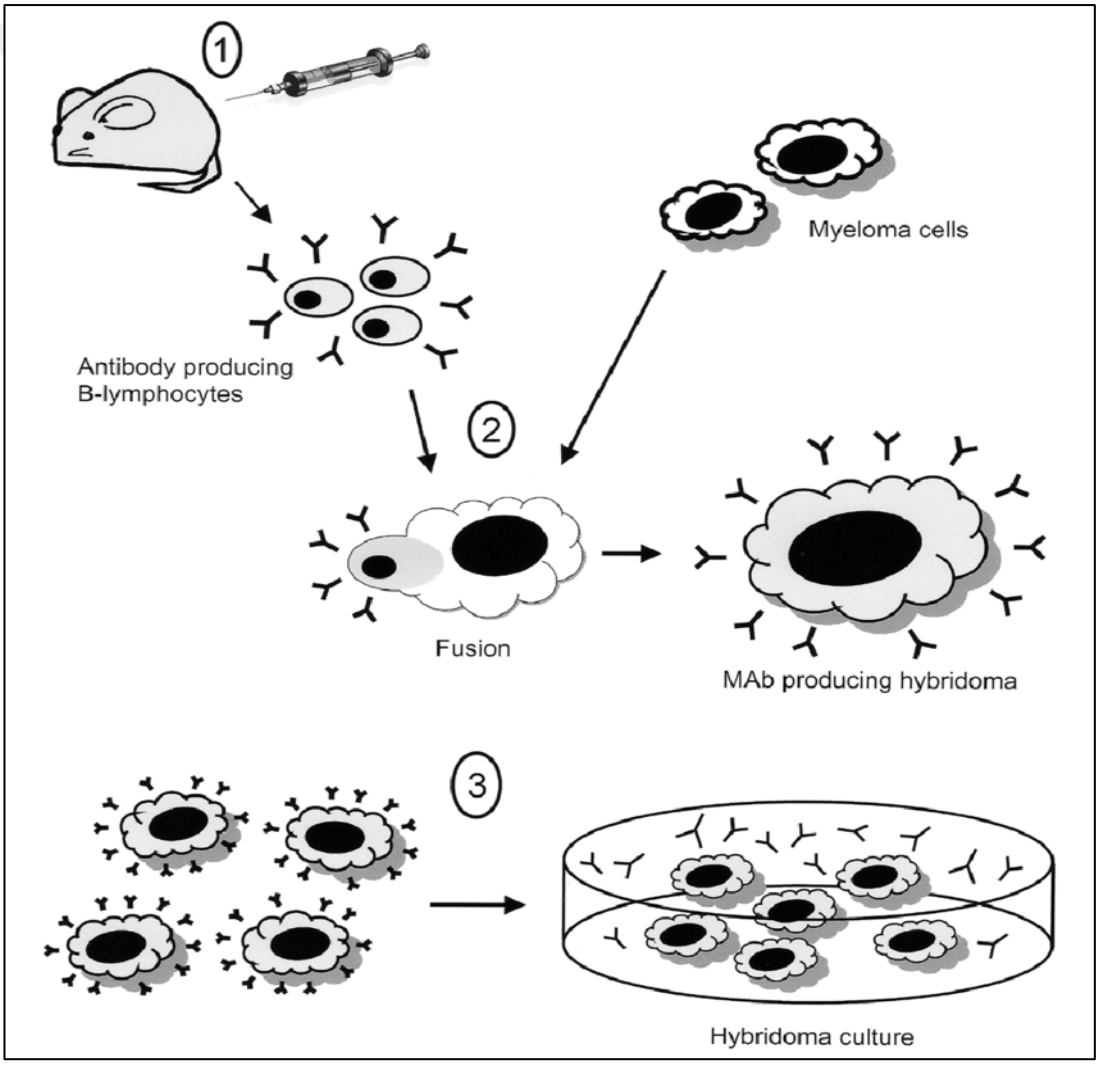

Figure 12. Hybridoma technology. 1. Immunization step and isolation of antibody-producing B lymphocytes from the spleen. 2. Fusion of B lymphocytes with myeloma cells and formation of hybridoma cells. 3. Propagation of hybridoma cells producing desired antibodies in culture (74)

Screening:

Hybridoma cells formed by HAT selection are transferred to ELISA plates with a single hybridoma cell in each well (48). In this process, the limiting dilution method (73) is used. The production of specific antibodies against a specific epitope occurs with the B cell line genes of the hybrid cells (48). This produced antibody is a monoclonal antibody. Hybridomas in other wells of the plate may produce antibodies directed against the same antigen but against another epitope. To separate and isolate these different hybridomas, screening is performed to select hybridomas that produce antibodies to specific epitopes (51).

Expansion:

To propagate hybridomas producing the desired antibodies, hybridomas are selected and transferred to large culture flasks or vessels (48). Two main methods are used for this process, in vitro and in vivo. Monoclonal antibody-producing hybridoma cells can be maintained and preserved with culture media (51).

In vivo:

Mice are used in the in vivo method to produce monoclonal antibodies. Injection of 105 to 110 viable hybridoma cells into mice are given intraperitoneally (48). A few weeks after the injection, the acidic fluid accumulated in the mouse is collected with a syringe.

In vitro:

Hybridoma cells are cultured under laboratory conditions in the in vitro method (48). Hybrid cells are grown in culture media and then monoclonal antibodies are isolated. This method reduces the risk of contamination from animals and is a more convenient method. Highly pure production of antibodies is achieved by in vitro antibody production (51,75).

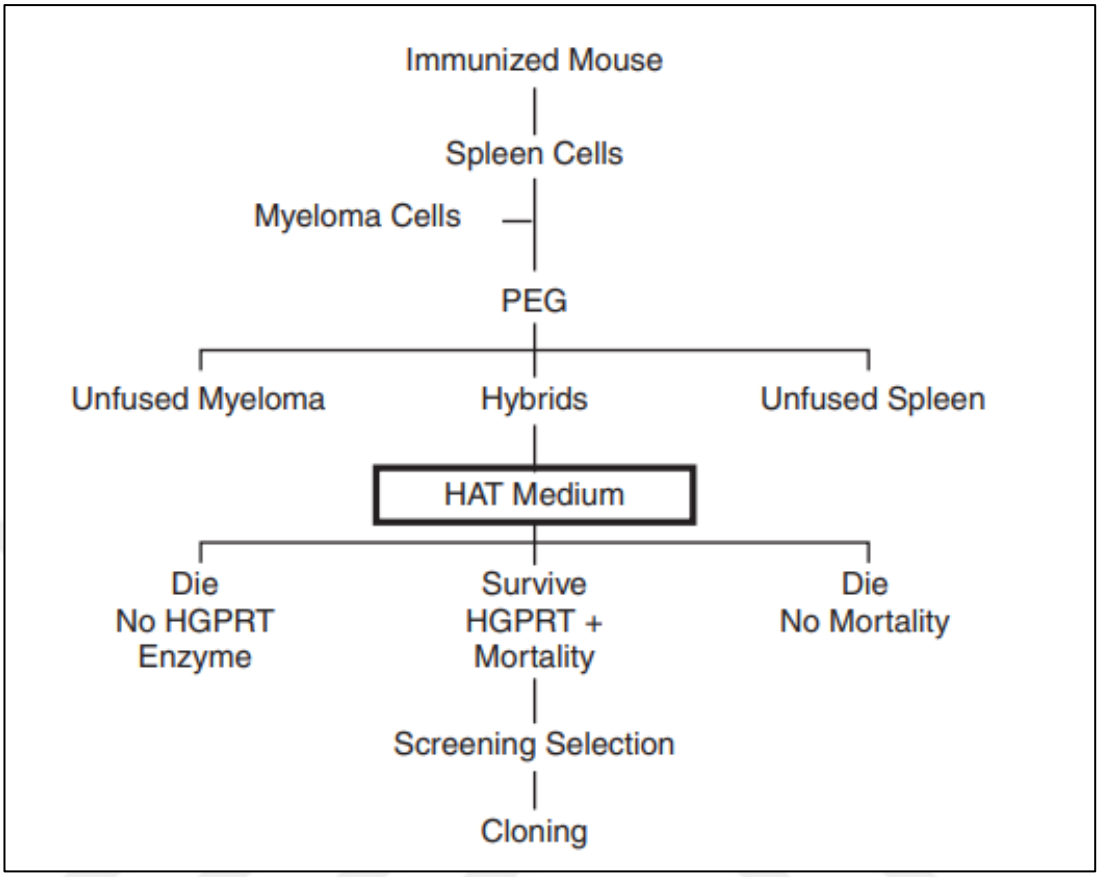

Figure 13. Process diagram of mAb production by hybridoma technology (76)

The use of hybridoma technology in the production of monoclonal antibodies provides many advantages (48). Production of highly pure and specific monoclonal antibodies can be achieved with hybridoma technology. An unlimited number of monoclonal antibodies can be produced. Also, the selection method used in this method is useful for arranging the right clones against a particular antigen. This technology is used in vaccine production research and in a variety of chemotherapeutic regimens to treat many cancers types. One of the advantages of antibodies obtained by using hybridoma technology is that they can be used directly (77). The fusion partner used in this methodology is myeloma cells with a remodeled transcriptional mechanism that consistently yield large amounts of antibodies and can be cryopreserved indefinitely for other purposes.

One of the difficulties encountered in hybridoma technology is the purification step of the antigen to be used so that it can generate a specific immune response (51). This step can be difficult in some cases and can limit the overall process and time in hybridoma production. Another challenge in this technology is the immunization phase of animals. Effective hybridoma production can be achieved with an effective immunization. Effective immunization with the selected antigen depends on important factors. These are dose of antigen, number of boosts, choice of adjuvant and the protocol used. In addition, multiple screening and cloning processes are required to identify antigen-specific hybridomas in hybridoma technology. Therefore, this methodology is a time-consuming and labor-intensive technology. With robotic screening methods, this difficulty has been alleviated somewhat (78).

Performing the screening process of antibody production methods on semi-solid selective medium greatly reduces the time required at this stage (51). This screening method is included in the sale of ready-to-use kits for the development of murine hybridoma. The use of murine hybridoma technology, which takes time and requires extra effort, is facilitated by this system. For the hybridoma technology selection stage, methylcellulose-based semi-solid selective medium is highly preferred (79). The use of this semi-solid medium reduces the possibility of loss of clones formed in the liquid medium due to rapid and excessive growth of cells. Clones selected in this semi-solid medium are then transferred to liquid growth medium and screening and expansion processes are applied.

Hybridoma technology has revolutionized fields such as immunology, toxicology, cell biology, biotechnology, medical and pharmaceutical research (48). The production of specific and sensitive mAbs in large quantities has expanded the use of mAbs in many fields. The mabs are widely used in the detection and diagnosis of cancers, as well as in the treatment of cancer.

The mAb market for the use of derived mAbs as a therapeutic and diagnostic reagent has grown tremendously over the past five years (51). In the early 1980s, therapeutic mAbs began to be developed commercially. The therapeutic mAb

developed to prevent kidney transplant rejection was the first mAb approved by the FDA in 1986. To date, mAbs have begun to take an important place in the treatment of a large number of diseases as therapeutic agents.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

Peptide Synthesizer (CEM), Peptide Razor (CEM), HPLC (Agilent), C18 Semi Preparative HPLC Column (Agilent), Xevo G2-XS QToF (MS) (Waters Coorp), Vortex (VWR), Pure Water Device (Advantage Milli Q), Freeze Dryer (Lyophilizer) (Labconco), Biosafety Cabinets (Thermo Scientific), Microplate Reader (Thermo Scientific Varioskan), NanoDrop One (Thermo Scientific). Centrifuge (Thermo Scientific), CO_2 Incubator (NUVE), Laminar flow hood (NUVE), Microplate Reader (BioTek), Hemacytometer, Inverted microscope (Zeiss), 96-well plates (High binding) (Greiner Bio-One), 96-well Black (Thermo Scientific), 75 cm^2 cell culture flasks (Sarstedt), 5-10-25 mL serological pipettes (Isolab), Serum gel blood tube (500 µL) (Sarstedt), Cell scraper (Corning), 15-50 mL falcon tubes (Greiner Bio-One), Cryotubes (Corning), 96-well cell culture plates (Greiner Bio-One), 40 µm cell strainer (Corning), Petri dishes (Greiner Bio-One), Injectors (B Braun Omnican), 21G needle (B Braun Omnican), Scissors (Geuder), Forceps (Geuder).

3.1.2 Chemicals

Oxyma (Sigma-Aldrich), Rink Amide Resin (Sigma-Aldrich), Acetonitrile HPLC Grade (Merk), N,N-Dimethylformamide (DMF) (aber), N,N-Diisopropylcarbodiimide (DCM) (Merck), Trifluoroacetic acid (TFA) (Sigma), Triisopropylsilane (TIS) (Across), Diethyl ether (Carlo Erba), Acetic acid (Sigma-Aldrich), Tween 20 (Sigma-Aldrich), Pierce™ Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific). Freund's complete adjuvant (FCA) (Sigma-Aldrich), Incomplete Freund's adjuvant (IFA) (Sigma-Aldrich), Dulbecco's phosphate-buffered saline (DPBS) (Gibco), Skim milk (BioFrox), Anti-Mouse Polyvalent Immunoglobulins (G,A,M)−Alkaline Phosphatase antibody produced in goat (Sigma-Aldrich), p-NPP alkaline phospatase substrate (Merck Millipore), Dulbeco’s Modified Eagle’s Medium

(DMEM) (Sigma-Aldrich), Fetal Bovine Serum (FBS) (Sigma-Aldrich), Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich), Gentamicin (Sigma-Aldrich), DMEM HG (Dulbecco's Modified Eagle's Medium with High Glucose) (Sigma-Aldrich), HEPES (Sigma-Aldrich), L-Glutamine (Sigma-Aldrich), Sodium Pyruvate (Gibco), Penicillin-Streptomycin (Gibco), Isoflurane (Dechra), Ethanol (Isolab), Polyethylene glycol (PEG) 4000 (Merck Millipore), Red Blood Lysis Buffer (ThermoFisher Scientific), Hypoxanthine-Aminopterin-Thymidine (HAT) supplement (50X) (Gibco), Hypoxanthine-Thymidine (HT) supplement (50X) (Gibco), Isostrip Mouse Monoclonal Antibody Isotyping Test Kit (Sigma-Aldrich), Fetal Calf Serum (FCS) (Sigma-Aldrich), 8-Azaguanine (Abcam), ClonaCell™-HY Hybridoma Kit (StemCell Technologies).

3.2 Methods

3.2.1 Solid-Phase synthesis and characterization

In this thesis, a synthetic peptide was synthesized from the peptide sequence of the Neuropeptide Y (NPY) molecule. Solid-phase peptide synthesis method, which was developed by Merrifield (37), was applied for the synthesis of NPY peptide. CEM, Liberty™ Blue and CEM Discover™ Microwave were used as the Automated Peptide Synthesizer (Figure 7). The Liberty™ Blue system has 20 positions for inserting natural amino acids, 5 positions for unnatural amino acids, and 12 positions for resin. The peptide sequence desired to be synthesized was defined to the device using the PepDriver software registered on the device.

NPY consists of 36 amino acids (22). The peptides synthesized in our thesis study are the 24 amino acid sequence (12-36) and 36 amino acid sequence (36) of NPY peptide and the amino acid composition of these sequences are given below:

NPY (12-36); PAEDMARYYSALRHYINLITRQRY
NPY (36); YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY

Figure 14. CEM, Liberty™ Blue Automated Microwave Peptide Synthesizer

Peptides are synthesized by assembling of amino acids from the C- to the N-terminus using Fmoc-based solid phase peptide synthesis strategy at different scales between 0.1 mmol and 0.05 mmol. These peptides were synthesized on a Rink Amide resin with a loading capacity of 0.70 mmol/g as a solid support. The amino acids used during synthesis contain protecting groups to prevent undesirable reactions.

The resin was incubated in N,N-Dimethylformamide (DMF) at least 30 minutes before synthesis and swelling was ensured. In the first step, the first amino acid is attached to the resin by the carboxy group at the C-terminus. When synthesis was initiated, the Fmoc protecting group at the N-terminus of the first amino acid attached to the resin was removed with piperidine (20% in DMF). Then, the second amino acid was added to the reaction with the activated carboxy group of the first amino acid to form an amide bond. N,N-disopropylcarbodiimide (DIC) and ethyl-2-cyano-2-(hydroxyimino) acetate (Oxyma) were used for activation of carboxyl groups. 0.2 M of the respective amino acid was used for each coupling step. Chemicals and excess

reagents were removed by washing. This cycle of peptide synthesis continued until the desired sequence was completed.

The amounts of reagents required at a synthesis scale of 0.1 mmol for the 24 amino acid sequence (12-36) of the NPY peptide are given in Table 4. In addition, the amounts of reagents required for the 36 amino acid sequence of the NPY peptide at a synthesis scale of 0.05 mmol are also given in Table 5.

When the peptide synthesis was finished, the resin was transferred to the CEM, Razor (Rapid Peptide Cleavage System) device and washed three times with DMF before cleavage process. Peptide cleavage from the resin and deprotection was performed with a mixture of trifluoroacetic acid (TFA) /triisopropylsilane (TIS)/H_2O (95: 2.5: 2.5 v/v/v) for 30-45 minutes at 38°C. The peptides were precipitated with cold diethyl ether (-20 °C) and centrifuged at 4500 rpm for 3 min. The precipitation process was repeated 3 times and the final precipitate was washed and dried. Information about synthesized peptides is given in Table 6.

Table 4. Required reagent amounts for 24 amino acid sequence (12-36) of NPY peptide

Reagent	**Volume (mL)**	**Mass (g)**
Alanine (Ala)	9	0.57
Arginine (Arg)	24	3.12
Asparagine (Asn)	3	0.36
Aspartate (Asp)	3	0.25
Glutamine (Gln)	3	0.37
Glutamate (Glu)	3	0.26
Histidine (His)	3	0.38
Isoleucine (IIe)	6	0.43
Leucine (Leu)	6	0.43
Methionine (Met)	3	0.23
Proline (Pro)	3	0.21
Serine (Ser)	3	0.24
Threonine (Thr)	3	0.24
Tyrosine (Tyr)	12	1.11
Main Wash (DMF)	614	
Deprotection (Piperidine)	125	
Activator (DIC)	34	
Activator Base (Oxyma)	17	
Resin (Rink Amide) Mass Required: 0.143 g		

Table 5. Required reagent amounts for 36 amino acid sequence of NPY peptide

Reagent	Volume (mL)	Mass (g)
Alanine (Ala)	6	0.38
Arginine (Arg)	14	1.82
Asparagine (Asn)	3	0.36
Aspartate (Asp)	5	0.42
Glutamine (Gln)	2	0.25
Glutamate (Glu)	3	0.26
Glycine (Gly)	2	0.12
Histidine (His)	2	0.25
Isoleucine (IIe)	3	0.22
Leucine (Leu)	3	0.22
Lysine (Lys)	2	0.19
Methionine (Met)	2	0.15
Proline (Pro)	6	0.41
Serine (Ser)	3	0.24
Threonine (Thr)	2	0.16
Tyrosine (Tyr)	8	0.74
Main Wash (DMF)	878	
Deprotection (Piperidine)	145	
Activator (DIC)	47	
Activator Base (Oxyma)	24	
Resin (Rink Amide) Mass Required: 0.071 g		

Table 6. Properties of synthesized peptides (80)

Peptide	NPY (12-36)	NPY (36)
Molecular Weight (g/mol)	3000.44	4271.74
Net Charge at pH 7.0	3.09	1.09
Isoelectric Point	10.22	9.16
% Hydrophilic Residues	38 %	42 %

3.2.1.1 Purification

HPLC (Agilent Technologies 1260 Infinity) analyzes of the synthesized peptides were performed. Purification was carried out at 214 nm and 280 nm wavelengths with the help of UV detector using Agilent Technologies VariTide RPC 250x10mm ID column, which is suitable for semi-prep system and peptide purification. Semi preparative HPLC was performed at flow rate of 2 ml/min. Two different mobile phases were used; mobile phase A: 0.05% TFA in H_2O and mobile phase B: 0.025% TFA in acetonitrile. Analyzes were performed with a linear gradient of 5-80% B in 45

minutes. The gradient method used was determined according to the retention time and % B range determined in the peptide analysis. Peptides were separated by peak based on HPLC. The purified peptides were frozen at -80°C and then lyophilized to be ready for use in experiments.

3.2.1.2 LC-MS / MS analysis

Physicochemical characterization of synthetic peptides was performed with analytical devices. Characterization of the synthesized peptides was performed by high-resolution mass spectrometry using Electrospray (ESI) mass spectrometry (MS) coupled to Waters H-Class Bio ultra-performance liquid chromatography (UPLC). The UPLC and mass spectrometry parameters used for the characterization of the peptides are shown in Table 7 and Table 8, respectively.

Table 7. UPLC Experimental Parameters

UPLC Parameters					
Mobile Phase A	%100 Ultra Pure Water				
Mobile Phase B	%100 Acetonitrile				
Mobile Phase C	%1 Trifluoroacetic acid				
Column Feature	ACQUITY UPLC Peptide CSH C18, 130 Å, 1.7 µm, 2.1 × 100 mm, (Part No:186006937)				
Injection Volume	10 µL				
Column Temperature	40 °C				
Sampler Temperature	10 °C				
UV Wavelength	214 nm				
Analysis Time	25 min				
Gradient Table and Flow Rates	Time (min)	%A	%B	%C	Flow Rate (mL/min)
	0	85	5	10	0.200
	2	85	5	10	0.200
	9	70	20	10	0.200
	12	50	40	10	0.200
	16	20	70	10	0.200
	18	0	90	10	0.200
	20	0	90	10	0.200
	21	85	5	10	0.200
	25	85	5	10	0.200

Table 8. Mass Spectrometry Experimental Parameters

Mass Spectrometry Parameters	
Mass Spectrometry	Xevo G2-XS QToF
Software	UNIFI v1.9.4
ESI Polarity	Positive
Analysis Mode	Sensitivity
Source Parameters	Capillary Voltage (kV): 2.0 Kon Voltage (V): 50 Source Temperature (°C): 100 Desolvation Temperature (°C): 300 Con Gas Flow Rate (L/h): 50.0 Desolvation Gas Flow Rate (L/h): 600.0
Scan Time	0.500 sec
Low Collision Energy	4 eV
Collision Energy Range	25 eV – 30 eV
m/z Mass Range	50 – 2000 Da
Data Collection Tİme	0 – 25 min

3.2.1.3 Fluorometric peptide concentration measurement

Pierce™ Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific) was used for measuring the concentration of peptides. Lyophilized peptide was dissolved in dH_2O and the assay protocol was followed according to the manufacturer's instructions. 10 µL of each standard or sample replicate was added to one well of a black 96-well plate (Thermo-Fisher Scientific). Next, 70 µL of Fluorometric Peptide Assay Buffer was added followed by 20 µL of Fluorometric Peptide Assay Reagent. After 5 minutes of incubation at room temperature, the fluorescence was measured using an excitation of 390nm/emission of 475nm using a Varioskan Microplate Reader.

3.2.1.4 NanoDrop to quantify peptide preparations at 205 nm

The lyophilized peptide was dissolved in DPBS containing 0.5% Acetic acid and 0.1% Tween 20 with different concentrations. Each peptide concentration (5 mg/mL, 3 mg/mL, 2 mg/mL, 1, 0.85 mg/mL and 0.7 mg/mL) was measured by NanoDrop One (Thermo Scientific). There are three different options in the NanoDrop One Protein A205 application (Figure 8). The Scopes method option was selected and extinction

coefficient can be determined automatically by this method. The measurements were taken at 205 nm. Especially in the concentration measurements of proteins containing significant amounts of tryptophan (Trp) and tyrosine (Tyr) residues, a more accurate ε_{205} is obtained by using this method.

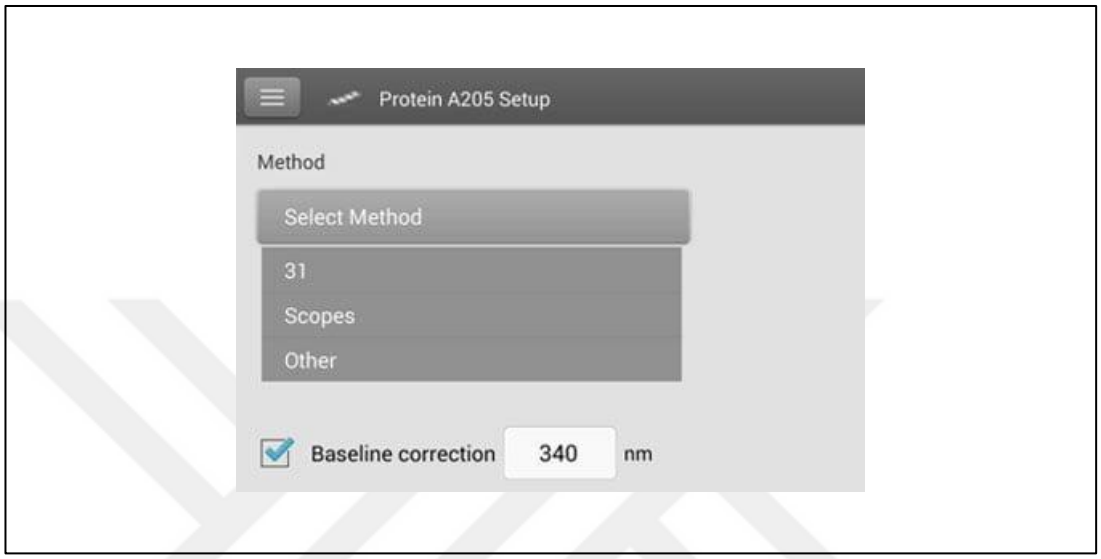

Figure 15. NanoDrop One Protein A205 methods

3.2.2 Monoclonal antibody production

3.2.2.1 Peptide injection/immunization

After successfully obtaining the NPY molecule, this peptide was used in the monoclonal antibody production part of the study. The first synthesized 24 amino acid sequence of NPY peptide (12-36) was used for immunization of mice numbered N1-N15, while 36 amino acid sequence of NPY peptide was used for mice numbered N16-N25.

Six- to ten-week-old BALB/c mice were immunized intraperitoneally with different concentrations of NPY. Intraperitoneal immunization of BALB/c female mice with NPY antigen is shown in Figure 16. Freund's complete adjuvant (FCA) was used in the first immunization, and Incomplete Freund's adjuvant (IFA) was used in subsequent immunizations. Mice used for immunization in this thesis study, peptide

used as immunogen, injected peptide concentration and time intervals are shown in Table 9.

Figure 16. Intraperitoneal immunization of BALB/c female mice with NPY antigen

N1-N5 mice were immunized once intraperitoneally with a total of 300 µl of peptide-containing solution at 10-day intervals. For immunization of each mouse, 50 µg of NPY (12-36) peptide was dissolved in 150 µl of PBS and mixed with 150 µl of adjuvant. N6-N10 mice were immunized once intraperitoneally with a total of 300 µl of peptide-containing solution, one week apart. For immunization of each mouse, the NPY peptide concentration applied to the N1-N5 mice was applied. In the later stages of the study, the peptide concentration used to immunize N11-N15 mice was increased to 100 µg NPY for each mouse. Mice were immunized once intraperitoneally with a total of 300 µl of peptide-containing solution, one week apart. Finally, N16-N20 mice were immunized once intraperitoneally, one week apart. For immunization of each mouse, 125 µg of NPY (36) peptide was dissolved in 150 µl of PBS containing 0.5% Acetic acid and 0.1% Tween 20 and mixed 150 µl of adjuvant. In the immunization of N21-N25 mice, the immunization method (peptide type, concentration and dose) applied to the N16-N20 mouse group was re-applied.

Table 9. Immunization schedule of mice with NPY antigen

Mice	Peptides used as a immunogen	Peptide Concentration (µg)	Time interval for immunization
N1-N5	NPY peptide (12-36)	50	At 10-day intervals
N6-N10	NPY peptide (12-36)	50	At 7-day intervals
N11-N15	NPY peptide (12-36)	100	At 7-day intervals
N16-N20	NPY peptide (36)	125	At 7-day intervals
N21-N25	NPY peptide (36)	125	At 7-day intervals

3.2.2.2 Plasma collection

For detection of antibody response in mice, blood samples were obtained and blood plasma was collected from these samples. Following the first week of immunizations, blood samples (about 45 µl) were collected from the cheek of each mouse once a week after vaccinations using IFA adjuvant into commercially available serum separator tubes. Collection of blood sample from BALB/c female mice is shown in Figure 17. Obtained blood samples were centrifuged at 16.000 x g for 5 minutes at 4°C. Plasma samples obtained after erythrocyte separation with gel were stored at -20 °C for further studies.

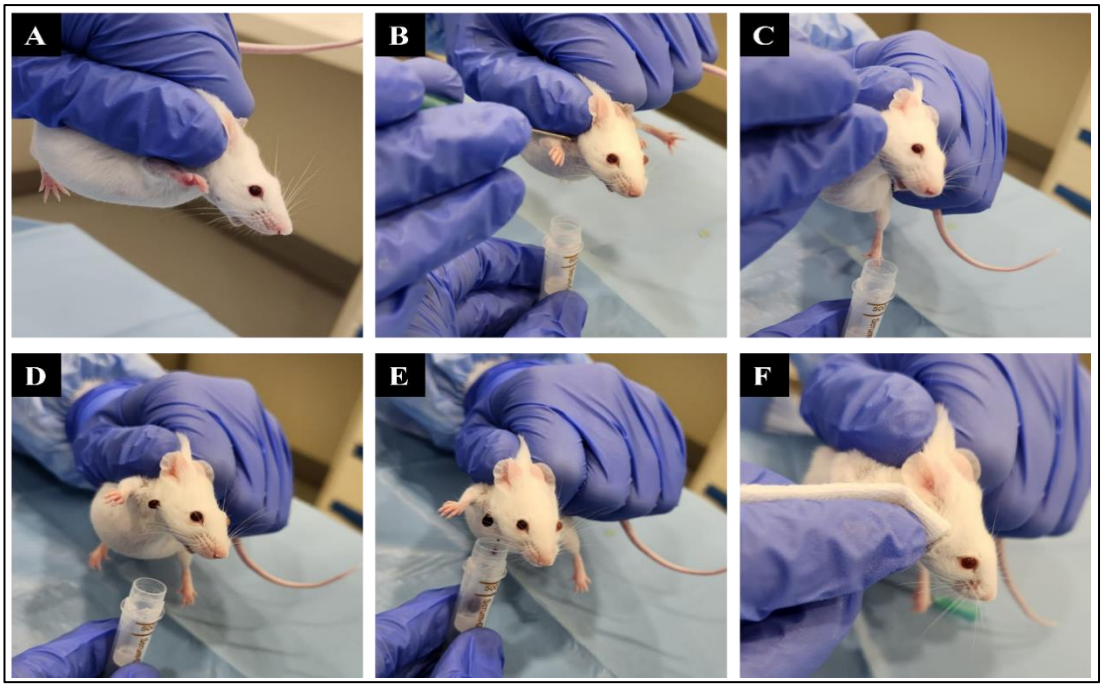

Figure 17. Collection of blood sample from BALB/c female mice (shown in order of all stages from A to F)

3.2.2.3 Detection of antibody response by ELISA method

The immune response to NPY antigen in mice was determined by ELISA method. In this method, plasma samples collected regularly once a week were used, and the efficiency of antibody response against NPY antigen was regularly checked in each mouse. Plasma samples from animals previously showing a high antibody response were used as positive controls in the ELISA test. The yellow color appearance of the samples confirms mAb production against the NPY antigen (Figure 18). The mouse showing the highest antibody titer was selected for the fusion experiment.

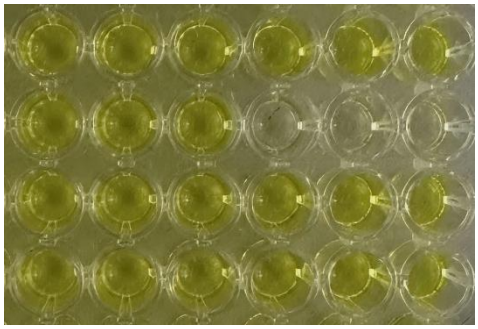

Figure 18. The appearance of the plate as a result of the ELISA test applied to the plasma samples collected from mice

3.2.2.3.1 ELISA

First, wells of 96-well plate were coated with 100 µl of 40 ng of NPY antigen and incubated overnight at 4°C after the plate was sealed. Then, the plate was washed 3 times with washing buffer (0.05% Tween 20 in PBS) to remove unbound antigens. Coated wells were incubated for 1 hour at 37°C with 100 µl of blocking buffer (1% skim milk in PBS) to block non-specific sites. After blocking, the plate was washed 3 times with washing buffer and the plate was incubated for 1 hour at 37°C with 100 µl of plasma samples diluted 1:100 with PBS, positive controls (plasma samples showing high antibody response to NPY antigen) and negative controls (only PBS). After incubation, the plate was washed again 3 times with washing buffer and 100 µl of alkaline phosphatase conjugated goat anti-mouse polyvalent (IgG, IgM, IgA) antibody

diluted 1:1000 in PBS was added to the wells and incubated at 37°C for 1 hour. The plate was washed 3 times for the last time with washing buffer and finally 100 µl of p-NPP alkaline phosphatase substrate was added to each well and incubated at 37°C for 1 hour. Antibody response to NPY antigen in mice was measured by reading optical density (OD) values at 450 nm using a microplate reader.

3.2.2.4 In vitro studies

Culture medium and cells were prepared in a sterile laminar flow. To prevent any contamination by microorganisms, 70% ethanol and UV light of the laminar flow cabinet were used. All liquid materials used for cell culture were heated in a 37°C water bath before use. The cell types used in this study are shown in Table 10.

Table 10. Cell types used in this study

Source	Name of Cell
F0, CRL-1646	Myeloma Cells
BALB/c mouse peritoneal cells (fibroblasts)	Feeder Cells
Cells from spleen of BALB/c mouse immunized with NPY	B Lymphocytes

3.2.2.4.1 Cell thawing

Cells stored in the freezer (-80°C) were thawed in 37°C water bath and then transferred to a sterile 15 mL tube and diluted with 10 mL of pre-warmed growth medium. In order to remove DMSO from the cells, they were first centrifuged at 300 x g for 5 minutes. Next, the supernatant was discarded and the cell pellet was dissolved with (1 mL) fresh medium. Resuspended cells were transferred to the T75 culture flasks and an additional 11 mL medium was added. Culture flasks were incubated in a 5% CO_2 incubator at 37 °C.

3.2.2.4.2 Cell counting

Cell counting was carried out by using a hemacytometer. 10 µl of cell suspension was taken and added between the hemocytometer and cover glass. The cells in all four

outer squares were counted by using inverted microscope. The average cell count from the squares was multiplied by 10^4 to find the number of cells per milliliter. Thus, the approximate number in the cell suspension was determined.

3.2.2.4.3 Subculture of myeloma cells

F0 myeloma cells were grown in DMEM HG medium containing 10% FBS, 1% HEPES (1 M), 1% L-Glutamine, 1% Sodium Pyruvate and 1% Penicillin/Streptomycin. Cells adhered to the culture flask surface were removed with a cell scraper. Next, cells were transferred to a 50 mL centrifuge tube and centrifuged at 300 x g for 5 min. The obtained cell pellet was resuspended with 1 mL of medium. After cell counting, cells were splitted at the desired rate and seeded into new T75 culture flasks. Then, an additional 11 mL medium was added and the culture flasks were incubated in a 5% CO_2 incubator at 37 °C.

3.2.2.4.4 Myeloma cell freezing

Myeloma cells adhered to the culture flask surface were removed with a cell scraper. Next, cells were transferred to a 50 mL centrifuge tube and centrifuged at 300 x g for 5 min. The resulting cell pellet was resuspended with freezing medium (90% FCS, %10 DMSO) and transferred to cryotubes. Finally, cryotubes were stored at -80°C for further studies.

3.2.2.5 Hybridoma technique with f0 myeloma cells fusion

3.2.2.5.1 Feeder cell isolation

For better fusion, feeder cells were prepared and seeded into 96-well plates one day before fusion of spleen cells and myeloma cells. Feeder cells provide required growth factor releases for hybridomas. Feeder cells can be obtained from healthy and untreated mouse. The BALB/c mouse is preferred in this study. For feeder cell isolation process, 5 to 10 mL of DMEM HG medium containing 50 µg/mL Gentamicin

antibiotic solution was prepared in a 50 mL falcon tube, sealed with paraffin and placed in an ice-filled foam container.

Isoflurane was used to anesthetize the mouse. The mouse was then euthanized using the cervical dislocation technique and sterilized with 70% ethanol. All processes were carried out under laminar flow to prevent contamination. The euthanized mouse was placed on the dissecting board in the dorsal position. The skin of the mouse was carefully peeled off without damaging the peritoneum. 5 mL of DMEM HG medium containing Gentamicin was injected into the peritoneal cavity of the mouse using a 21G needle. The injection was applied carefully so as not to puncture the internal organs with the needle. The injected medium was left for about 1-2 minutes and was taken back from the inner peritoneal membrane with feeder cell mixture. The medium containing the feeder cells was collected up to 3 to 5 mL and transferred to a new 50 mL falcon tube and stored in an ice-filled foam container.

Feeder cells harvested for the fusion process were added to 10 mL of DMEM HG medium containing 20% FBS, 1% HEPES (1 M), 1% L-Glutamine, 1% Sodium Pyruvate and 50 µg/mL Gentamicin or 1% Penicillin/Streptomycin. Cells were resuspended with this medium to approximately 100 mL. Next, 100 µl of feeder cells per well were seeded into 96-well plates. Finally, 96-well plates were incubated in a 5% CO_2 incubator at 37 °C.

3.2.2.5.2 Spleen cell isolation

The mouse showing the highest antibody titer was selected. Three to four days before the fusion, the selected mouse was injected with 150 µl of NPY peptide antigen (without adjuvant) as a booster. Before the fusion process, the necessary materials were prepared. 1 g of PEG 4000 was dissolved in 1 mL of PBS and 1 mL of dH2O and autoclaved. Autoclaved PEG 4000 was incubated at 37 °C overnight. Feeder cells were checked to avoid any contamination. Culture mediums used in the fusion process were prepared.

Isoflurane was used to anesthetize the mouse. The mouse was then euthanized using the cervical dislocation technique and sterilized with 70% ethanol. All processes were carried out under laminar flow to prevent contamination. The euthanized mouse was placed on the dissecting board. The skin of the mouse was carefully peeled off without damaging the peritoneum. The peritoneal wall was cut using sterile scissors and forceps. Next, the spleen and lymph nodes were removed from the body. The removed spleen and lymph nodes were cleaned of fatty tissues in a petri dish containing DPBS. Then, they are transferred to a new 50 mL falcon tube containing DMEM HG medium containing 10% FBS, 1% HEPES (1 M), 1% L-Glutamine, 1% Sodium Pyruvate and 1% Penicillin/Streptomycin. In this study, the same medium without serum was used in some of the spleen isolation experiments. Isolation of spleen cells is shown in Figure 19.

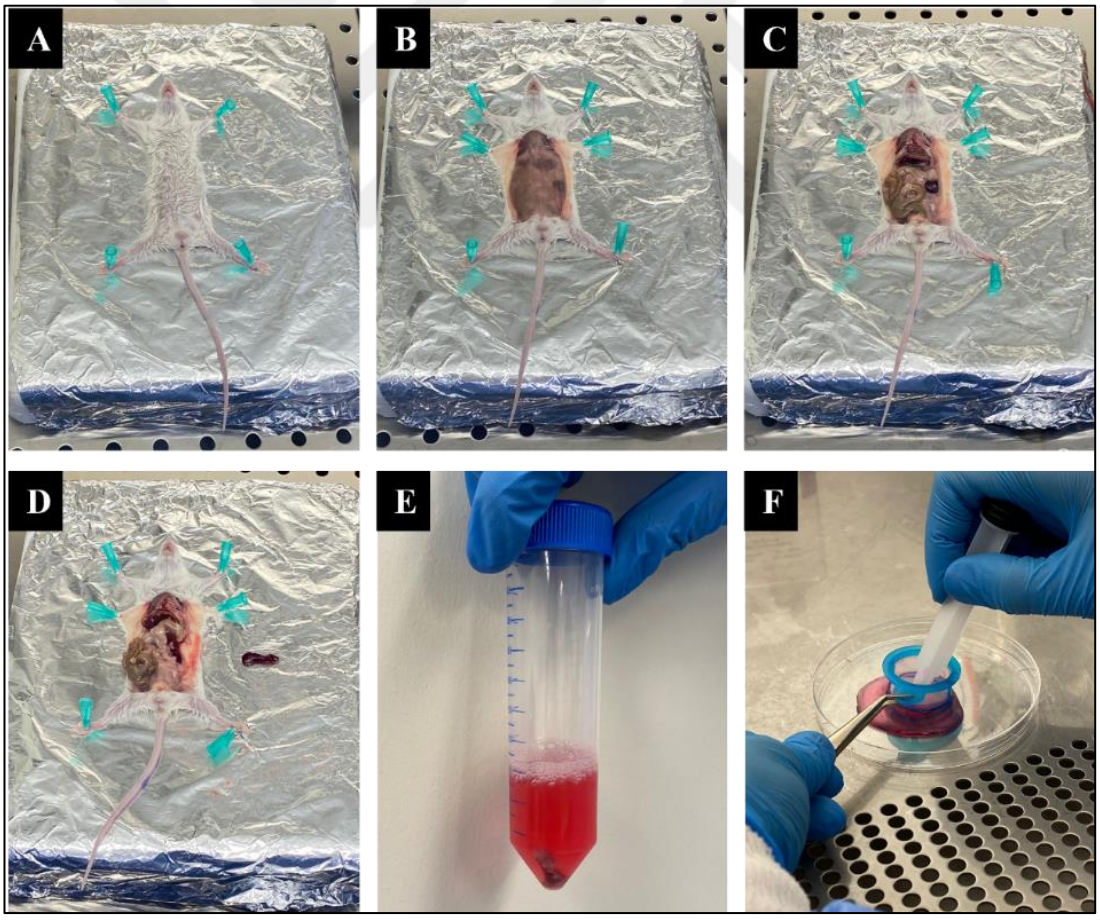

Figure 19. Isolation of spleen cells (shown in order of all stages from A to F)

Tissues were transferred to a sterile petri dish with the help of a forceps. Then, it was placed in a 40 µm cell strainer and crushed with the help of a sterile syringe pump. Cells were filtered and washed using DMEM HG complete medium (1% HEPES, 1% L-Glutamine, 1% Sodium Pyruvate and 1% Penicillin/Streptomycin) containing 10% FBS. The filtered cells were collected in a 50 mL falcon tube and centrifuged at 300 x g for 5 minutes at 4°C. This step was repeated three times. Next, the supernatant was discarded and the cell pellet was resuspended with 2.5 mL of ice-cold Red Blood Lysis Buffer. After 2.5 minutes of incubation at room temperature, cells were resuspended by making up 35 mL with DMEM HG complete medium containing 10% FBS. It was then centrifuged at 300 x g for 5 minutes at 4°C. This step was repeated until clear supernatant was observed. Finally, the resuspended cells were counted.

3.2.2.5.3 Fusion

For fusion, spleen cells and myeloma cells were combined in the same 50 mL falcon tube at a ratio of 1:2 or 1:4. (1X spleen cells; 2X or 4X myeloma cells). It was then centrifuged at 300 x g for 5 minutes at 4°C and the supernatant was discarded. 1 mL of the previously prepared PEG 4000 solution was slowly added to the cells within 1 minute and then pipetted for homogenization. 4 mL of DMEM HG (without FBS) medium was added very slowly within 2 minutes. Then, 20 mL of DMEM HG (without FBS) medium was added very slowly within 2 minutes. Finally, 25 mL of DMEM HG medium containing 20% FBS was added very slowly within 1 minute and then pipetted for homogenization. Cell mixture were incubated at 37°C for 1 hour and then centrifuged at 300 x g for 5 minutes at 4°C. The supernatant was discarded and the resulting cell pellet was resuspended with DMEM HG complete medium containing 20% FBS and 2% HAT supplement. Finally, 100 µl of hybrid cells per well were seeded into 96-well plates containing pre-seeded feeder cells. The 96-well plates were incubated in a 5% CO_2 incubator at 37 °C for 10 days.

After 10 days of incubation, medium exchange was made with DMEM HG complete medium containing 20% FBS and 1% HT supplement. The medium was exchanged again 10 days later with DMEM HG complete medium containing only

20% FBS. Regularly, each well of the 96-well plate was checked for healthy cells and colony formation.

Following the immunization of the N1-N5, N6-N10, N11-N15 and N16-N20 mouse groups, the mouse showing the highest antibody titer was selected, and the above-described method was applied for the fusion process.

After the immunization of the N21-N25 mouse group, the fusion experiments were continued by making some changes in the hybridoma technique applied for the other groups. After selecting the mouse with the highest antibody titer in this group, myeloma cells were incubated with 8-azaguanine at different concentrations (20 µg/mL; 200 µg/mL) 3 days before the fusion experiment. At the same time, myeloma cell cultivation was continued in medium without 8-azaguanine. The fusion process applied for the other groups was applied with these myeloma cells.

For the fusion process of one of the mice with high antibody titer in N21-N25 mice group, another method for hybridoma selection and cloning, a methylcellulose-based semi-solid medium, was used. ClonaCell™-HY Hybridoma kit was applied according to the manufacturer's instructions after spleen isolation from the selected mouse.

Reagents and media used in monoclonal antibody production with the ClonaCell™-HY Hybridoma kit is shown in Table 11.

Table 11. Reagents and media used in monoclonal antibody production with the ClonaCell™-HY Hybridoma kit

Hybridoma Kit Products	**Description and Purpose**
Medium A	Culture medium for hybridoma and myeloma cells
Medium B	Medium used to wash cells before and during the fusion process
Medium C	Medium used to promote the viability of hybridoma cells before selection with HAT
Medium D	Semi-solid methylcellulose-based medium used for hybridoma selection and cloning (includes HAT supplement and serum)
Medium E	Media used for growth and expansion of hybridoma cells after selection by HAT
PEG	Reagent for the fusion process

Necessary preparations were completed before the fusion process with the hybridoma kit. One week before fusion, the cultivation of myeloma cells was performed using Medium A. Myeloma cells in Media A adhered to the culture flask surface were removed with a cell scraper. Next, cells were transferred to a 50 mL centrifuge tube and centrifuged at 300 x g for 10 minutes. The obtained cell pellet was resuspended with 30 mL of medium B and centrifuged at 300 x g for 10 minutes. This process was repeated two more times for a total of three washes. Finally, the resulting cell pellet was resuspended with 25 mL of Medium B. The volume of the cell suspension containing 2 x 10^7 viable myeloma cells was calculated and kept at room temperature until fusion process.

For the isolation of spleen cells after spleen isolation from mice, spleen was transferred to a sterile petri dish with the help of a forceps. Then, it was placed in a 40 µm cell strainer and crushed with the help of a sterile syringe pump. Cells were filtered and washed using 30 mL of Medium B. The filtered cells were collected in a 50 mL falcon tube and centrifuged at 300 x g for 10 minutes. This process was repeated two more times for a total of three washes. Finally, the resulting cell pellet was resuspended with 25 mL of Medium B. The volume of the cell suspension containing 1 x 10^8 viable spleen cells was calculated and kept at room temperature until fusion process.

For fusion, 1 x 10^8 spleen cells and 2 x 10^7 myeloma cells were combined in the same 50 mL falcon tube. It was then centrifuged at 400 x g for 10 minutes and the supernatant was discarded. The obtained cell pellet was disrupted by slowly tapping the bottom of the falcon tube. 1 mL of PEG was slowly added to the cells within 1 minute without stirring. Immediately afterward, cells were stirred with pipette tip within 1 minute. 4 mL of Medium B was added very slowly within 4 minutes. An additional 10 mL of Medium B was then added slowly to the cell mixture. Then, this cell mixture was incubated at 37°C water bath for 15 minutes. After incubation, 30 mL of Medium A was slowly added to the cells and centrifuged at 400 x g for 7 minutes, and then the supernatant was discarded. Finally, the cell pellet was resuspended with 40 mL of Medium A and centrifuged again at 400 x g for 7 minutes. The resulting cell pellet was resuspended with 10 mL of Medium C. Cells resuspended in Media C were

transferred to a T75 culture flask containing 20 mL of Medium C. The flask was incubated for 16 to 24 hours in a 5% CO_2 incubator at 37 °C.

After 16 to 24 hours of incubation, cells were transferred to a 50 mL centrifuge tube and centrifuged at 400 x g for 10 minutes. Then, the supernatant was discarded. The cell pellet was resuspended with 10 mL of Medium C. Then, cells in Media C were transferred to the bottle containing 90 mL of Medium D. After mixing the bottle, it was waited for 15-20 minutes for the bubbles formed in the bottle to rise. Finally, 9.5 mL of cell suspension was added very slowly, evenly distributing in the bottom, to each of the 10 x 100 mm culture dishes using a 12 mL syringe. The culture dishes were incubated for 10 to 14 days in a 5% CO_2 incubator at 37 °C.

After 14 days, the presence of colonies in the culture dishes was observed with the naked eye and under the microscope. Colonies formed were isolated using a pipettor set to 10 µl and pipette tips. Colonies previously collected with these sterile pipette tips were pipetted into a 96-well plate, each well containing 200 µl of Medium E. Each well was then pipetted to resuspend the colonies. The plate was then incubated for 3 to 4 days in a 5% CO_2 incubator at 37 °C. Supernatant samples collected from cells were used for identification of monoclonal antibodies with ELISA and isostrip tests.

3.2.2.6 Identification of monoclonal antibodies with ELISA and isostrip tests

In order to determine the specific antibody level of the hybrid clones obtained after fusion, the ELISA method, which was previously applied to mouse plasma samples, was applied. The only difference was that instead of 100 µl of plasma sample diluted 1:100 with PBS, 100 µl of supernatant from wells containing clones was used. Based on positive ELISA results, the subtype of produced antibodies was confirmed with Mouse Monoclonal Antibody Isotype test kits. The caps of the development tubes of the isotyping strips were removed. Supernatant samples were diluted 1:10 with 1% BSA in PBS. 150 mL of sample was added to the development tube.

4 RESULTS

4.1 Solid-Phase Peptide Synthesis

In this thesis, solid phase peptide synthesis method was used for the synthesis of NPY peptides, as described in the materials and method chapter. CEM, Liberty™ Blue and CEM Discover™ Microwave were used as the Automated Peptide Synthesizer and peptides were synthesized by assembling of amino acids from the C- to the N-terminus using Fmoc-based solid phase peptide synthesis strategy. The characterization of the synthesized peptides was carried out by various chromatographic methods (such as HPLC, LC-MS). Finally, the concentration of synthesized peptides was measured.

First, the 24 amino acid sequence of NPY peptide (12-36) was synthesized.

Synthesized NPY (12-36) sequence; PAEDMARYYSALRHYINLITRQRY

Information on the NPY sequence consisting of 24 amino acids is given in Figure 20.

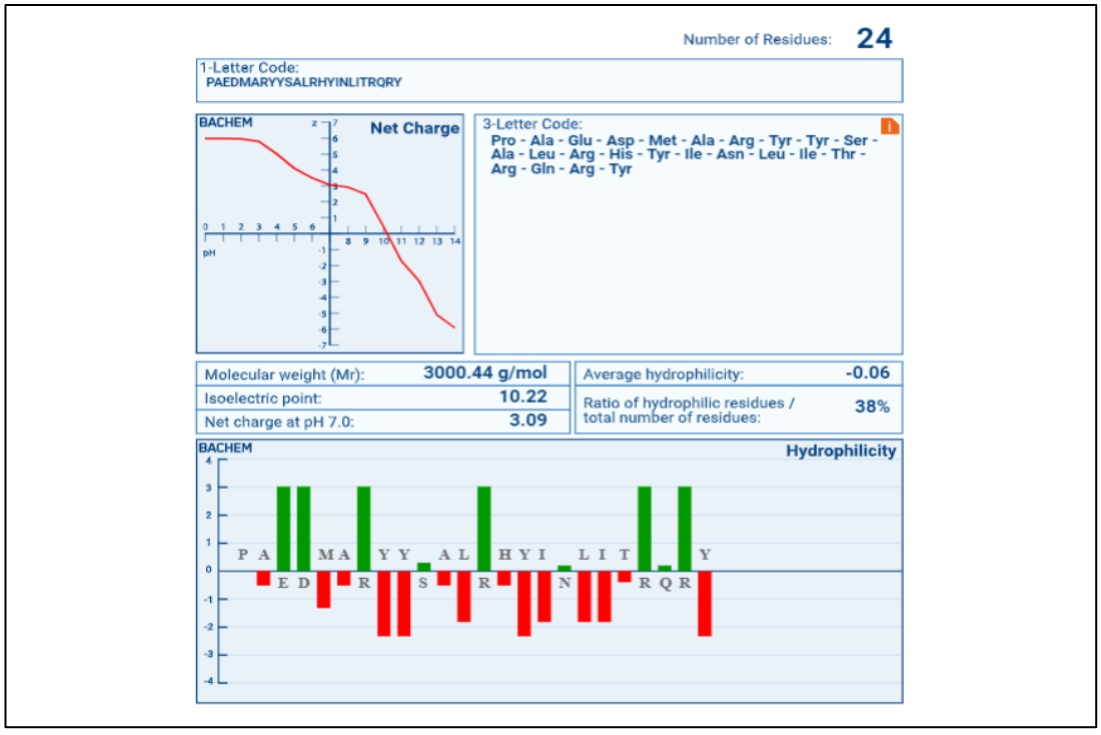

Figure 20. Information on the NPY sequence consisting of 24 amino acids (80)

In the later stages of the study, the peptide from which the entire 36 amino acid sequence was synthesized was used.

NPY (36) sequence; YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY

Information on the NPY sequence consisting of 36 amino acids is given in Figure 21.

Figure 21. Information on the NPY sequence consisting of 36 amino acids (80)

The PEPFOLD program was used to estimate the three-dimensional (3D) structures of the synthesized NPY peptides (Figure 22). NMR solution structure of human NPY (36) is given in Figure 23.

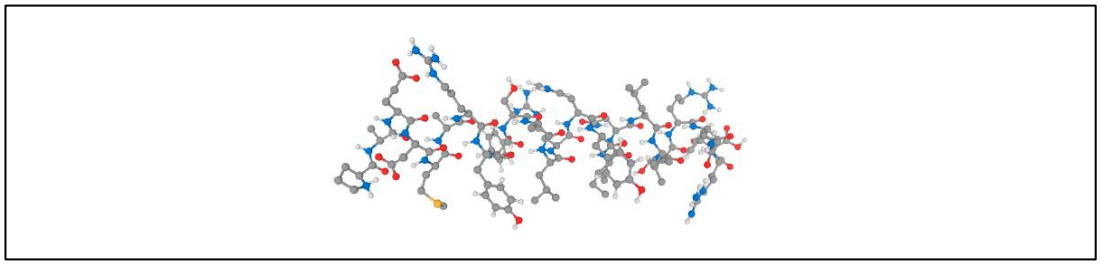

Figure 22. Estimated 3D structure of NPY (12-36)

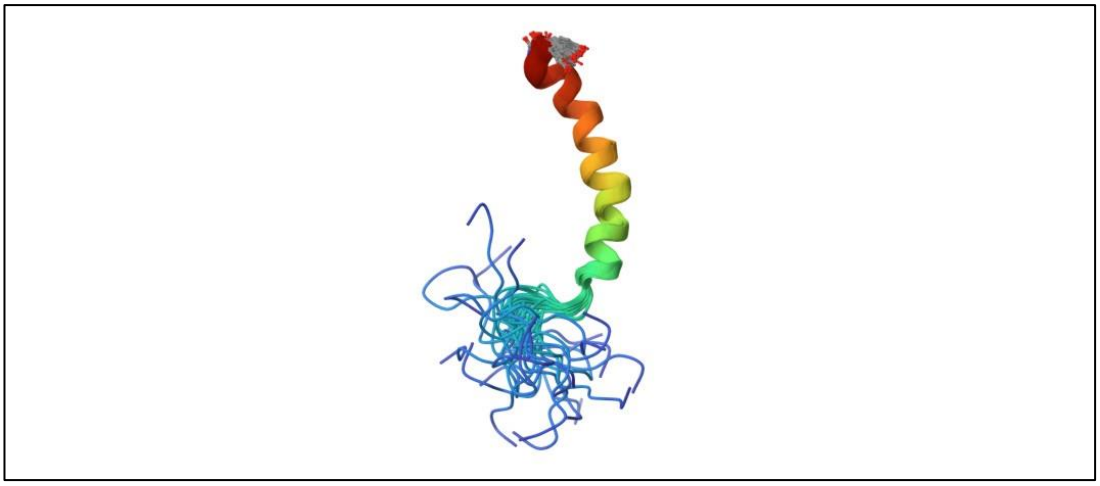

Figure 23. NMR solution structure of human NPY (36, 81)

4.2 HPLC Analysis of Peptides

HPLC analysis of the synthesized peptides was performed. The gradient method used in this analysis was determined according to the retention time of the synthesized peptides and the percentage of B solution.

The retention time of NPY (12-36) was 24.34 min. According to the appearance of the main peak in this HPLC chromatogram, purification was carried out by collecting only the molecules belonging to the main peaks. HPLC chromatogram of NPY (12-36) peptide is shown in Figure 24.

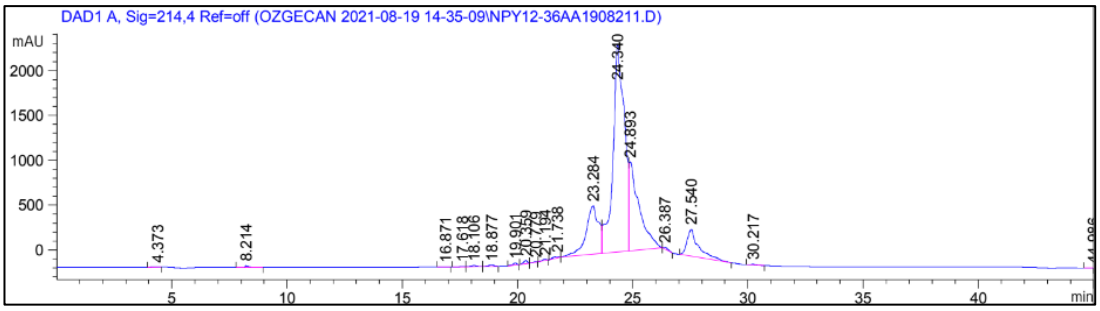

Figure 24. HPLC chromatogram of NPY (12-36) peptide

The retention time of NPY (36) was 25.089 min. According to the appearance of the main peak in this HPLC chromatogram, purification was carried out by collecting

only the molecules belonging to the main peaks. HPLC chromatogram of NPY (36) peptide is shown in Figure 25.

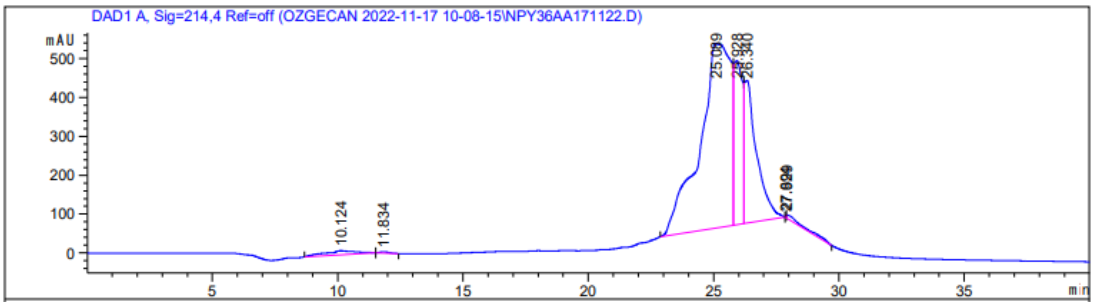

Figure 25. HPLC chromatogram of NPY (36) peptide

4.3 LC-MS / MS Analysis Results

For the synthesized NPY (12-36) peptide, peak was detected in UPLC system with UV detector in 13.75 minutes (Figure 26). Confirmation that the desired peptide has been synthesized is shown by the MS/MS spectrum of the 13.75 min peak (Figure 27). The sequences of the synthesized peptide were confirmed by peptide mapping analysis using LC-MS / MS analysis.

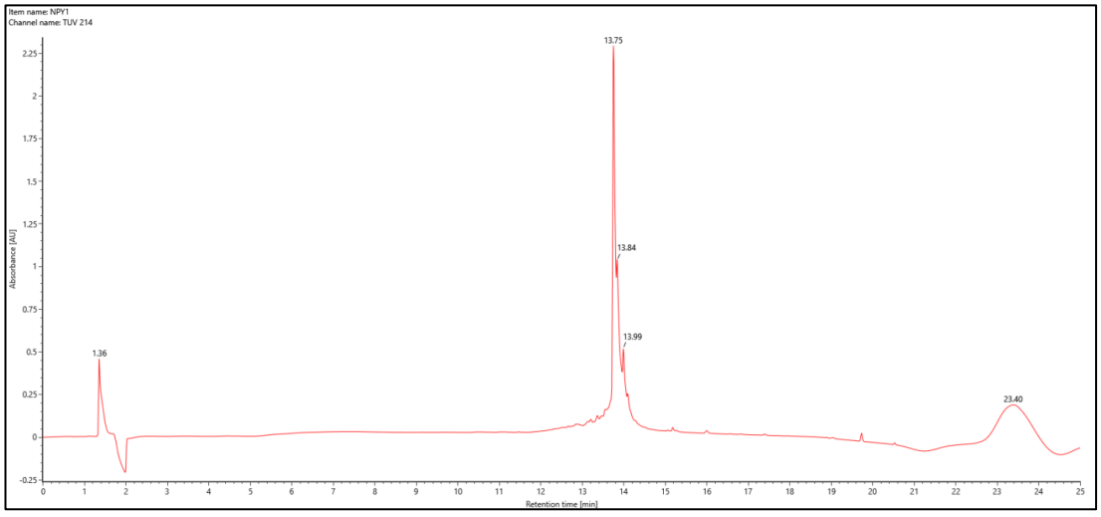

Figure 26. LC-UV Chromatogram of NPY (12-36) peptide

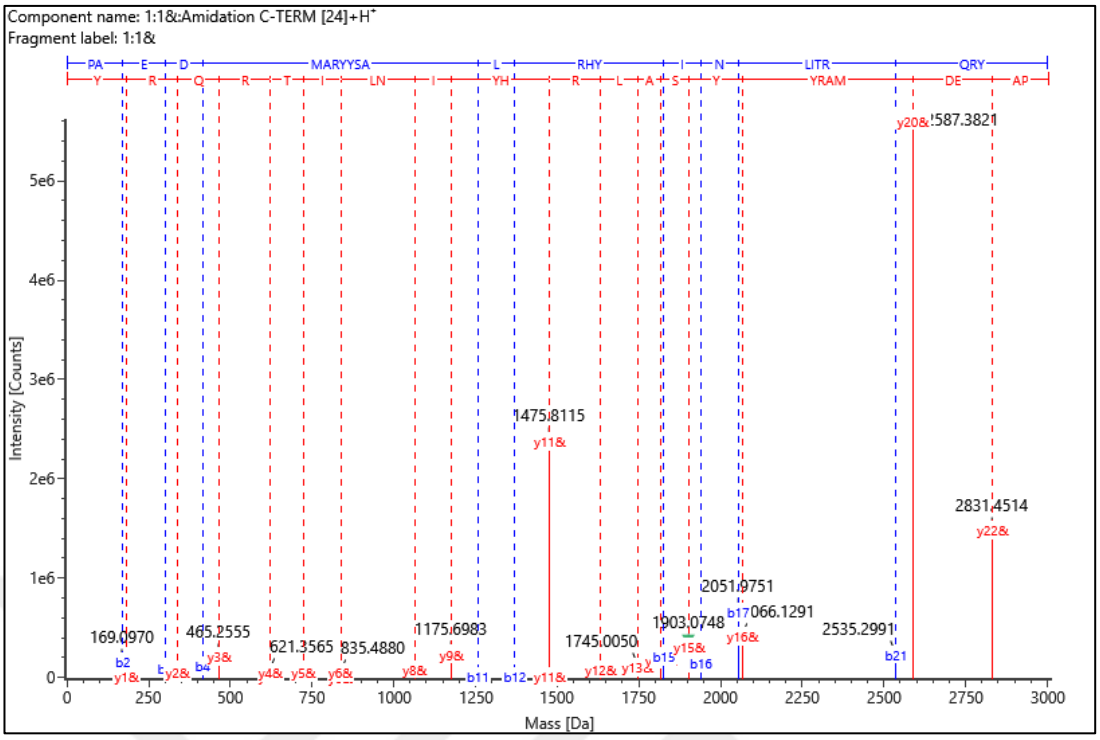

Figure 27. MS / MS Mass Spectrum of NPY (12-36) peptide

For the synthesized NPY (36) peptide, peak was detected in UPLC system with UV detector in 13.96 minutes (Figure 28). Confirmation that the desired peptide has been synthesized is shown by the MS/MS spectrum of the 13.96 min peak (Figure 30). The sequences of the synthesized peptide were confirmed by peptide mapping analysis using LC-MS / MS analysis.

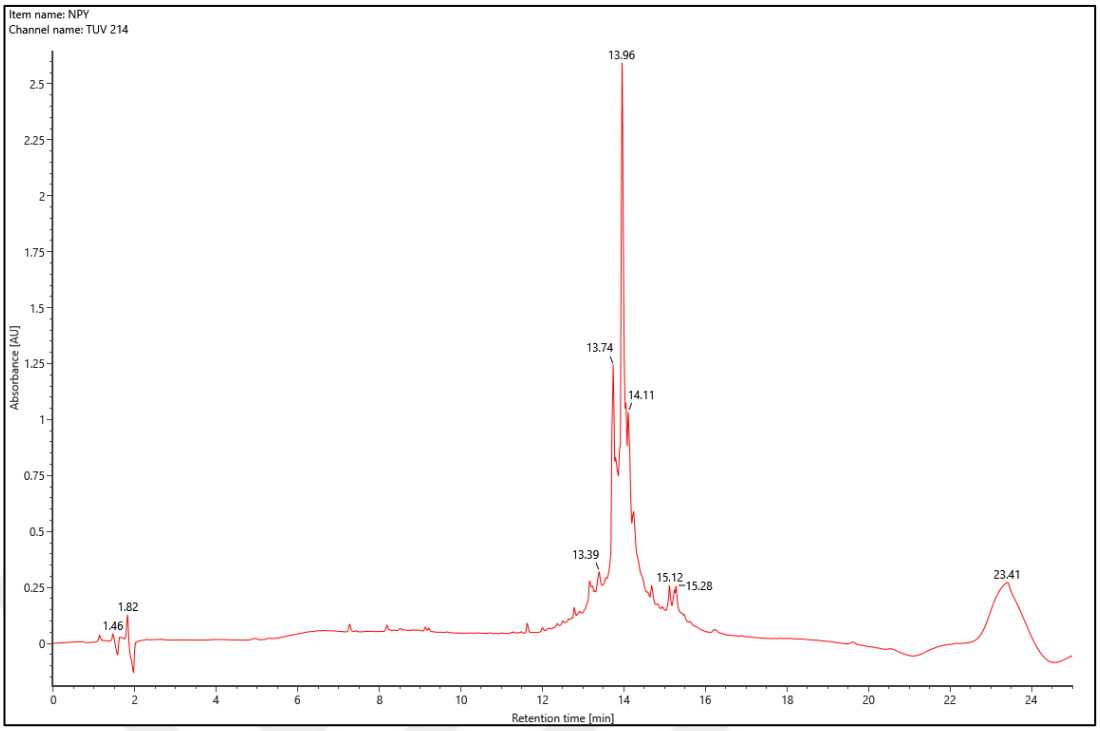

Figure 28. LC-UV Chromatogram of NPY (36) peptide

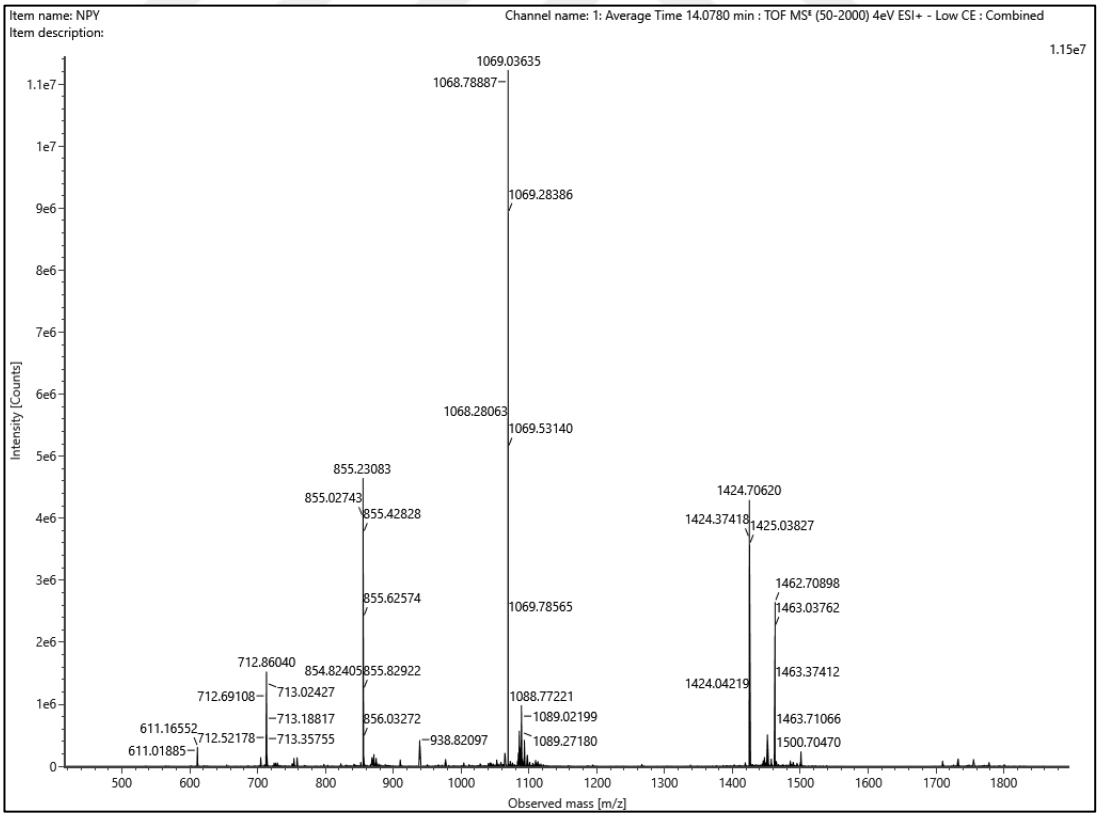

Figure 29. Mass Spectrum of NPY (36) peptide

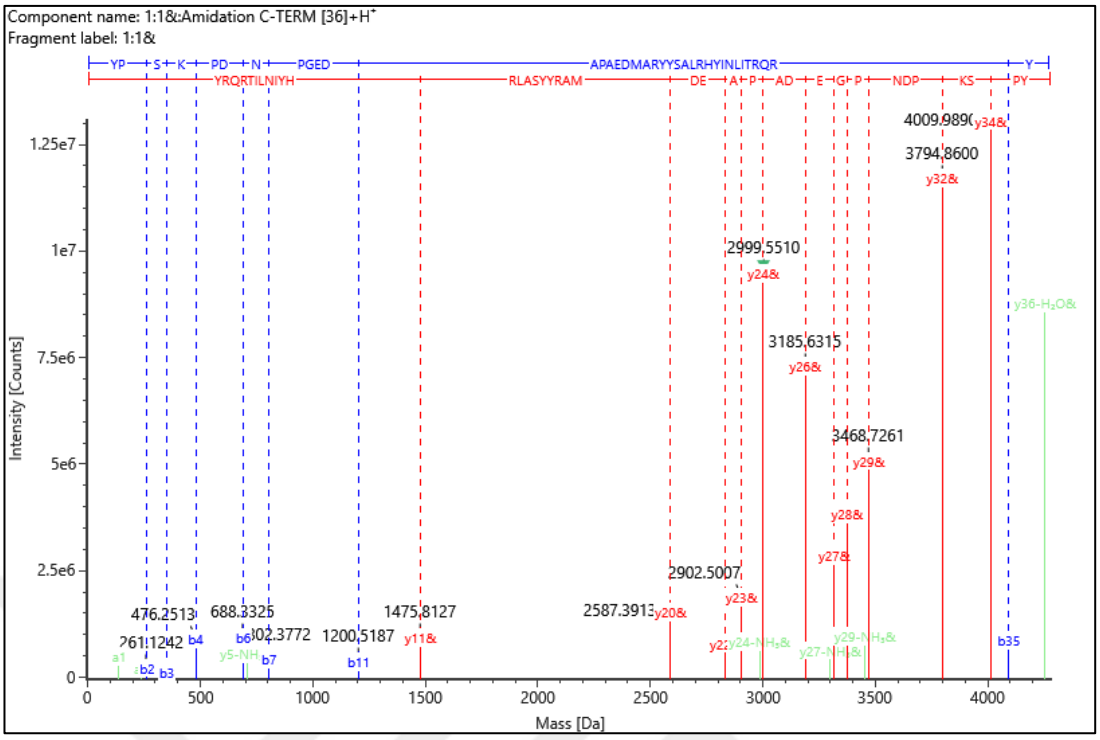

Figure 30. MS / MS Mass Spectrum of NPY (36) peptide

4.4 Fluorometric Peptide Concentration Measurement

The concentration of synthesized and purified peptides (NPY 12-36) was measured with the Pierce™ Quantitative Fluorometric Peptide Assay. Peptide samples were completely solubilized prior to performing this test. Thermo Scientific Peptide Digest Assay Standard was used as a reference standard. Serial twofold dilution was applied to this standard. A standard curve was constructed by plotting the fluorescence values of the standards against their respective concentrations. In this assay, the sample concentration is outside the molecular weight of the peptide to be measured, 900-1500Da, and therefore the samples were measured in µM. For this, the standard curve graph was plotted according to the range of 841-6.6 µM (Figure 31). It was then converted from µM to µg/mL. For this, the following formula is used:

Concentration (µg/mL) = Peptide molecular weight (Da) × Concentration (µM)/1000

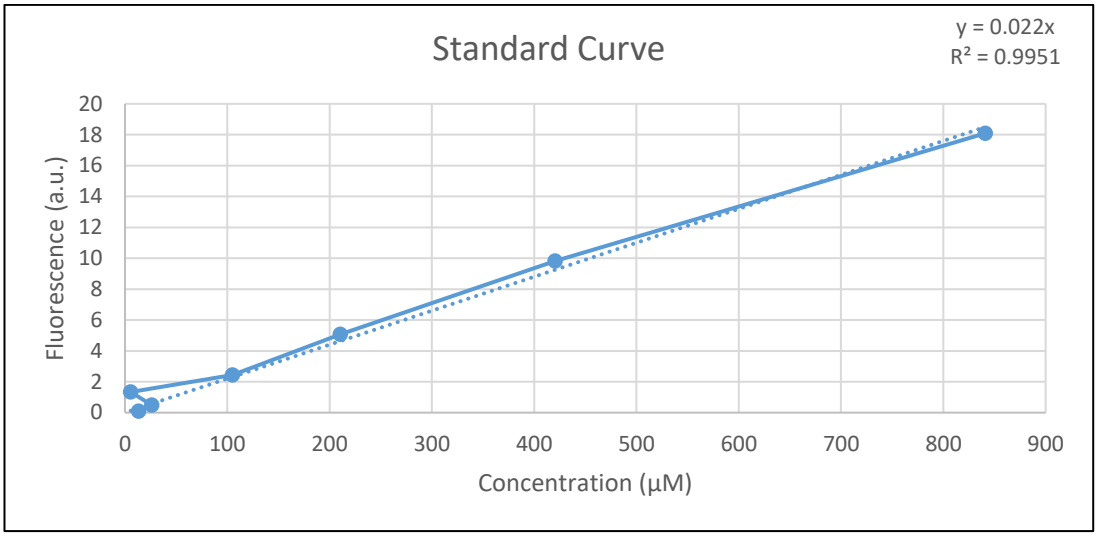

Figure 31. Standard curve for NPY peptide

The assay protocol was followed according to the manufacturer's instructions. The absorbance values of the peptide samples were measured using an excitation of 390nm/emission of 475nm using a Varioskan Microplate Reader. Finally, the mass of the synthesized peptides was determined using the generated standard curve. The mass of the peptides was calculated using the following formula:

Mass (g) = Concentration (mol/L) x Volume (L) x Molecular Weight (g/mol)

The molecular weight of NPY (12-36) is 3000.43 g/mol, while the molecular weight of NPY (36) is 4271.74 g/mol. 10 mg of NPY (12-36) peptide was used in immunization studies.

4.5 NanoDrop to Quantify Peptide Preparations at 205 nm

NanoDrop One was used for the quantification of the synthesized NPY (36) peptide. Firstly, the synthesized and lyophilized NPY (36) peptide was dissolved in DPBS containing 0.5% Acetic acid and 0.1% Tween 20 at concentrations of 5 mg/mL, 3 mg/mL, 2 mg/mL, 1 mg/mL, 0.85 mg/mL and 0.7 mg/mL. The Scopes method option was selected, and extinction coefficient can be determined automatically by this method. The measurements were taken at 205 nm.

The result of measurements for each peptide concentration at 205 nm using NanoDrop One (Scopes Method) is shown in Table 12. According to these results, the peptide was finally dissolved at a concentration of 1 mg/mL and again measured in NanoDrop One.

Table 12. Measurements for each peptide concentration (5 mg/mL, 3 mg/mL, 2 mg/mL, 1 mg/mL, 0.85 mg/mL and 0.7 mg/mL) using Scopes Method (n=5)

Different concentrations of NPY (36) peptide (mg/mL)	**Concentration (Scopes Method) (mg/mL)**	**A205**	**A280**
5	3.31 ± 0.02	113.01 ± 0.69	6.78 ± 0.21
3	2.30 ± 0.02	76.83 ± 0.69	4.10 ± 0.12
2	1.55 ± 0.01	53.52 ± 1.31	3.31 ± 0.33
1	0.96 ± 0.06	32.93 ± 2.82	2.01 ± 0.41
0.85	0.83 ± 0.03	28.05 ± 1.55	1.60 ± 0.26
0.7	0.68 ± 0.03	22.71 ± 1.21	1.24 ± 0.23

Table 13. Measurements for stock peptide concentration (1 mg/mL) using Scopes Method (n=5)

Concentration of stock NPY (36) peptide (mg/mL)	**Concentration (Scopes Method) (mg/mL)**	**A205**	**A280**
1	0.84 ± 0.03	27.49 ± 0.99	1.35 ± 0.05

According to NanoDrop One results, a stock NPY (36) peptide solution at a concentration of 0.84 ± 0.03 mg/mL was prepared to be used in the immunization processes of monoclonal antibody production experiments and stored at -20 °C (Table 13).

4.6 Detection of Antibody Response

Six- to ten-week-old BALB/c mice were used for the immunization process. Mice used for immunization in this thesis study, peptide used as immunogen, injected peptide concentration and time intervals are shown in Table 9 in the Materials and Methods section.

Following the first week of immunizations, weekly blood samples were collected to detect antibody response in mice and blood plasma was obtained from these samples. The immune response to NPY antigen in mice was determined by ELISA using these obtained plasma samples. Plasma samples from animals previously showing a high antibody response were used as positive controls in the ELISA test.

For immunization of N1-N5 mice, 50 µg NPY (12-36) peptide was dissolved in 150 µl of PBS and mixed with 150 µl of adjuvant for intraperitoneal injection. In order to observe the effect of NPY peptide on the immunization process in this group, N4 and N5 were determined as control animals. These mice were injected with only PBS with adjuvant solution instead of the peptide. The results of the immune response to the NPY antigen for N1-N5 mice were determined by ELISA test, and the graph in Figure 32 shows the change of immune responses in N1-N5 mice up to the 6th injection.

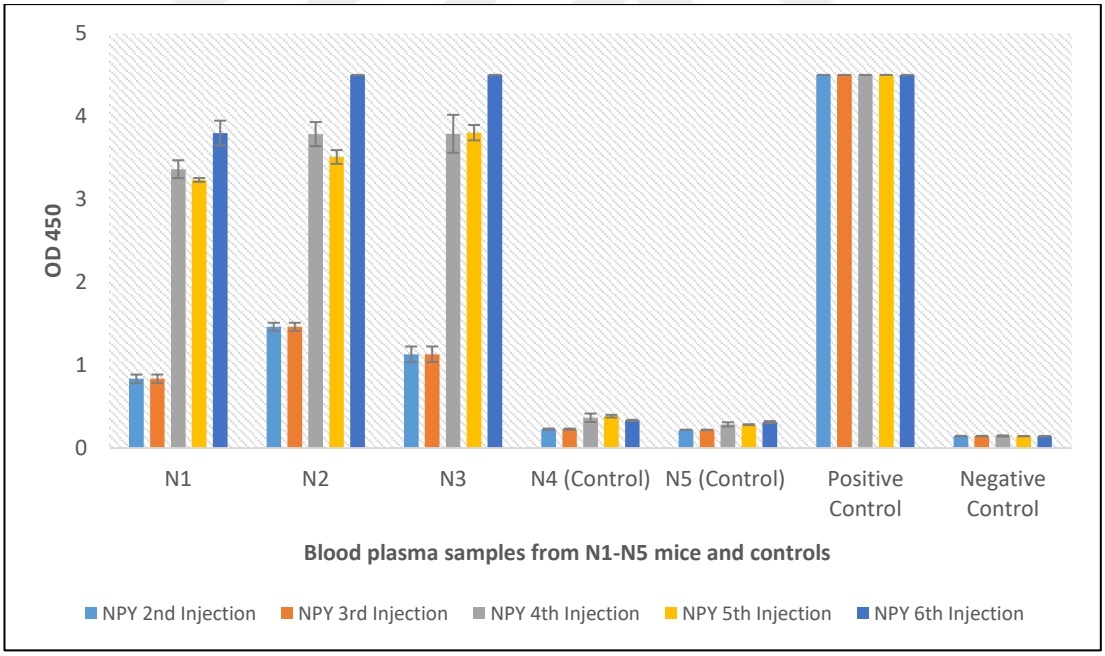

Figure 32. Change in immune responses of N1-N5 mice according to the number of injections

According to the ELISA test results, N2, N3 and N1 mice showed the highest antibody titer with OD 450 values of 4.50, 4.50 and 3.80 at the end of the 6th injection,

respectively. Mice N2, N3, and N1, respectively, were selected for the fusion experiment.

Following fusions of N1-N5 mice, N6-N10 mice were immunized with the NPY (12-36) peptide once intraperitoneally, one week apart. In immunization, the NPY peptide concentration applied to N1-N5 mice was applied. The results of the immune response to the NPY antigen for N6-N10 mice were determined by ELISA test, and the graph in Figure 33 shows the change of immune responses in N6-N10 mice up to the 9th injection.

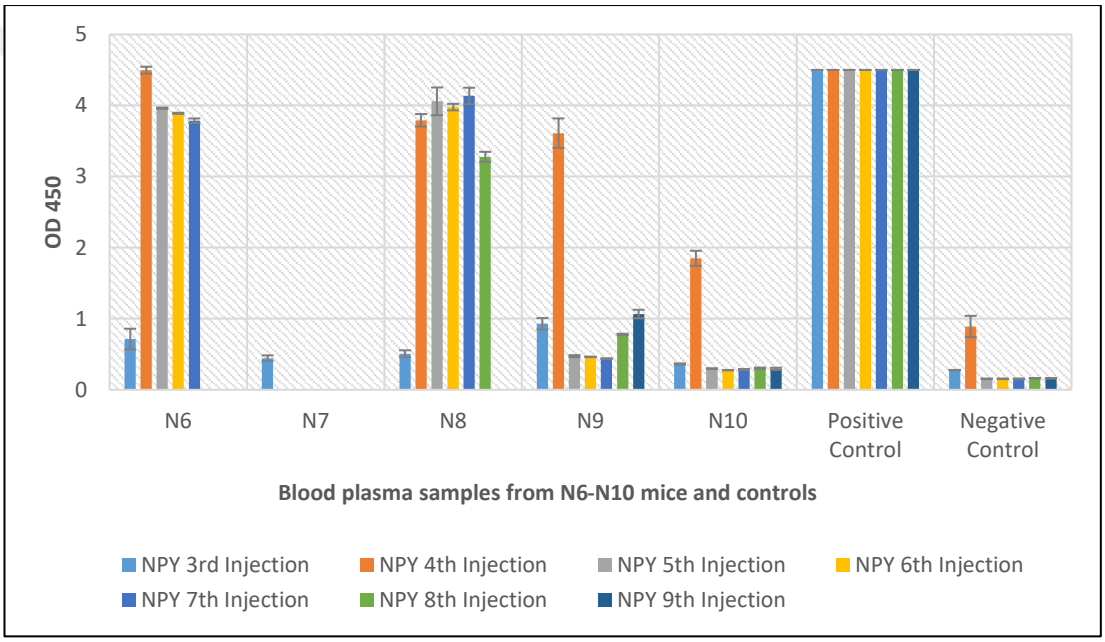

Figure 33. Change in immune responses of N6-N10 mice according to the number of injections

Isolation of spleen cells (B lymphocytes) from mice and fusion with myeloma cells was continued using the hybridoma technique, depending on the changes in the weekly immune responses of the N6-N10 mice (Figure 33). The N7 mouse died after the 3rd injection for unknown reason.

According to the ELISA test results, N6 and N8 mice showed higher antibody titer in contrast to other mice. Since the OD 450 values of the N6 mouse decreased as the number of injections increased, this mouse was first selected for the fusion experiment.

At the end of the 7th injection, the OD 450 value of the N6 mouse was 3.79. Immediately afterwards, the N8 mouse showed a lower value at the end of the 8th injection than at the 7th injection, although normally it showed higher values as the number of injections increased. For this reason, N8 mouse were also selected for the fusion experiment at the end of the 8th injection. At the end of the 8th injection, the OD 450 value of the N8 mouse is 3.28. Then, at the end of the 9th injection, N9 and N10 mice with OD 450 values of 1.07 and 0.30, respectively, were included in the fusion experiment.

In the later stages of the study, the concentration of NPY (12-36) peptide used as an immunogen was increased from 50 µg to 100 µg for N11-N15 mice, since the immune response was decreased in the group of mice N6-N10 (Figure 33). Following fusions of N6-N10 mice, N11-N15 mice were immunized once intraperitoneally with a total of 300 µl of peptide-containing solution, one week apart. The results of the immune response to the NPY antigen for N11-N15 mice were determined by ELISA test, and the graph in Figure 34 shows the change of immune responses in N11-N15 mice up to the 5th injection.

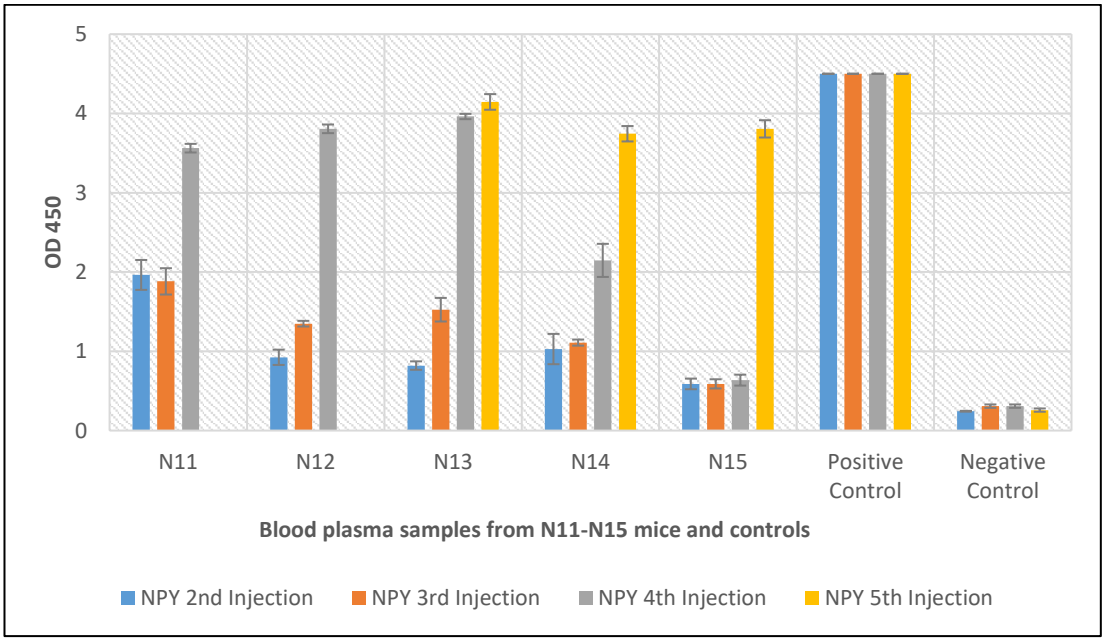

Figure 34. Change in immune responses of N11-N15 mice according to the number of injections

According to the ELISA test results, N12 and N11 mice with OD 450 values of 3.81 and 3.56, respectively, at the end of the 4th injection were selected for the fusion experiment. Next, N13, N15 and N14 mice with OD 450 values of 4.25, 3.81, 3.74, respectively, at the end of the 5th injection were selected for the fusion experiment.

For immunization of N16-N20 mice, 125 µg of NPY (36) peptide consisting of 36 amino acids was dissolved in 150 µl of PBS containing 0.5% Acetic acid and 0.1% Tween 20 and mixed 150 µl of adjuvant for intraperitoneal injection. The results of the immune response to the NPY antigen for N16-N20 mice were determined by ELISA test, and the graph in Figure 35 shows the change of immune responses in N16-N20 mice up to the 3rd injection.

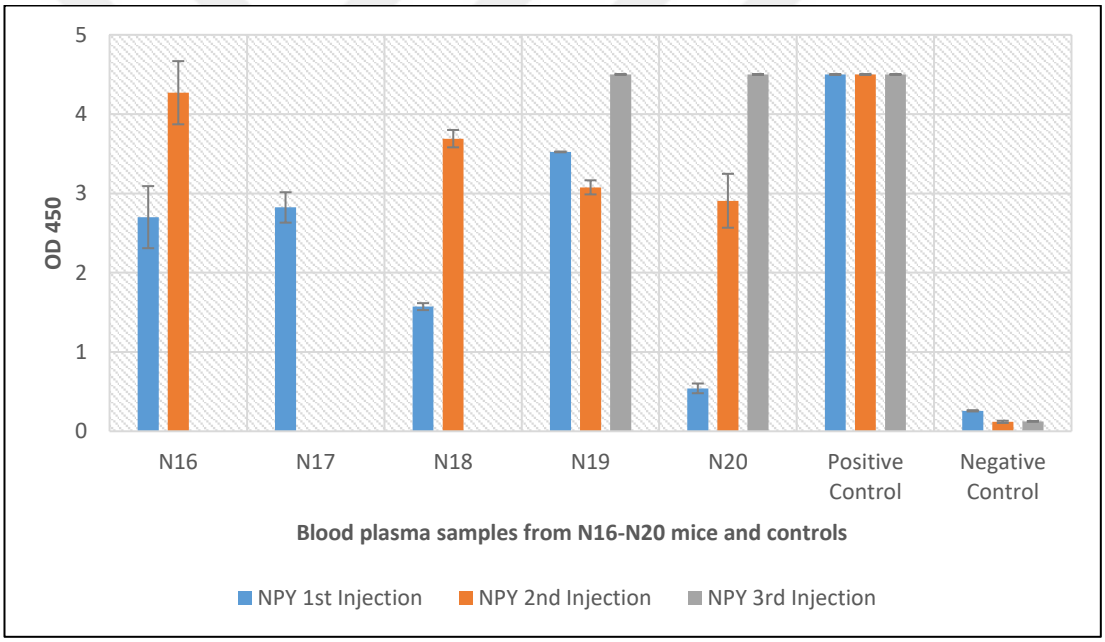

Figure 35. Change in immune responses of N16-N20 mice according to the number of injections

Fusion experiments were continued using the hybridoma technique according to the changes in weekly immune responses of N16-N20 mice immunized using the newly synthesized NPY (36) peptide. The N17 mouse died after the 1st injection for unknown reason.

According to the ELISA test results, N16 and N18 mice showed the highest antibody titer at the end of the 2nd injection with OD 450 values of 4.27 and 3.69, respectively. Both were selected for the fusion experiment. Then, at the end of the 3rd injection, the OD 450 values of N19 and N20 mice were measured as overflow (4.5). Therefore, both were selected for the fusion experiment.

In the immunization of N21-N25 mice, the immunization method (peptide type, concentration and dose) applied to the N16-N20 mouse group was re-applied. The results of the immune response to the NPY antigen for N21-N25 mice were determined by ELISA test, and the graph in Figure 36 shows the change of immune responses in N21-N25 mice up to the 3rd injection.

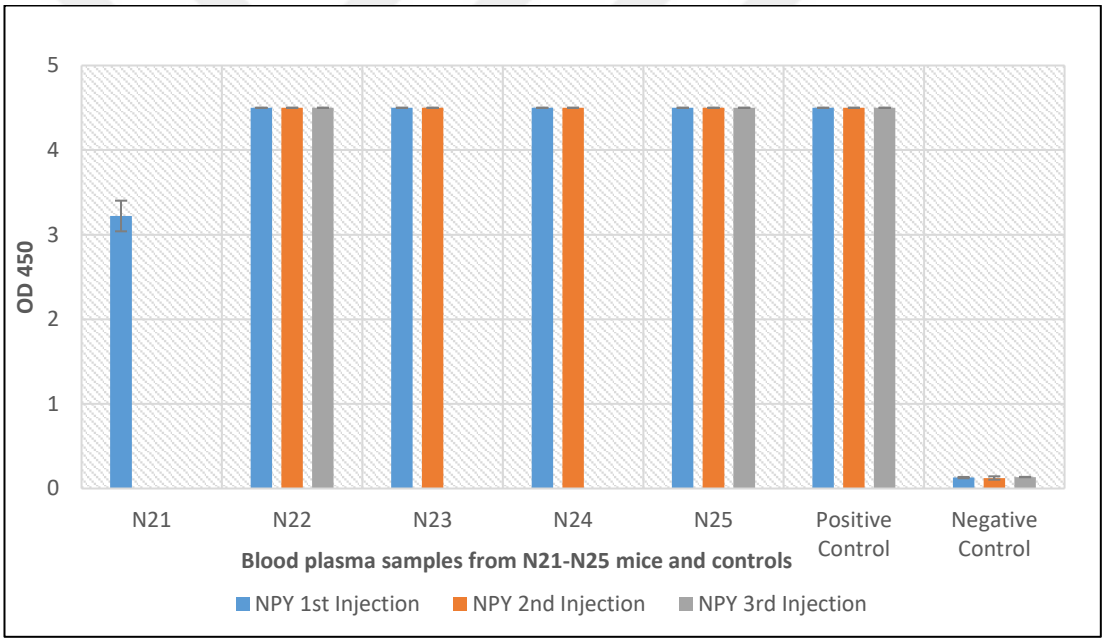

Figure 36. Change in immune responses of N21-N25 mice according to the number of injections

According to the changes in weekly immune responses of N21-N25 mice, fusion experiments were continued by making some changes in the hybridoma technique previously applied for other immunization groups. In this group, The N21 mouse died after the 1st injection for unknown reason.

In addition, since the OD 450 values were measured as overflow in the ELISA test results, the ELISA test was applied by diluting the plasma samples collected from the mice at the ratio of 1:500 in order to determine which mouse had the higher antibody titer (Figure 37).

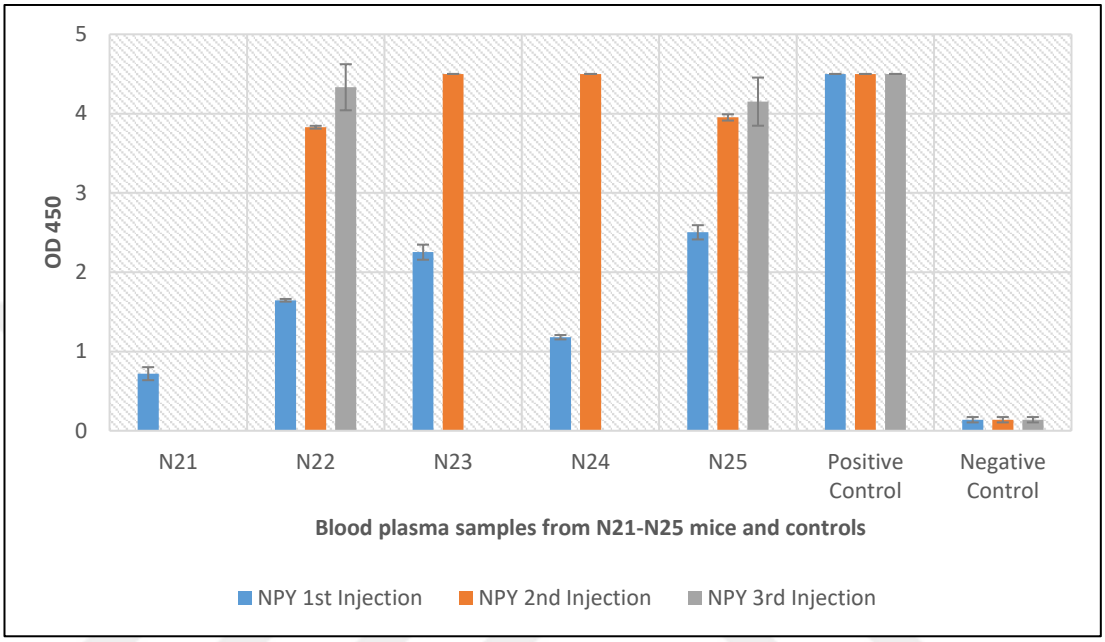

Figure 37. ELISA test results for plasma samples (diluted at the ratio of 1:500) collected from the N21-N25 mice according to the number of injections

According to the ELISA test results applied by diluting the plasma samples collected from N21-N25 mice at a ratio of 1:500, N23 and N24 mice showed the highest antibody titer at the end of the 2nd injection with an OD 450 value of 4.50. For this reason, N23 and N24 mice were selected for the fusion experiment at the end of the 2nd injection. Then, at the end of the 3rd injection, the OD 450 values of N22 and N25 mice were 4.33 and 4.15, respectively. Finally, both were selected for the fusion experiment.

4.7 Fusion with Hybridoma Technique

Feeder cells were isolated from mouse peritoneum 2-3 days before fusion and seeded into 10 96 well plates. Spleen was isolated from mouse for fusion procedure. B cells were isolated from the spleen and transferred to a 50 ml falcon. For the fusion

process, counted myeloma cells and spleen cells were combined at a ratio of 1:4. These cells were then fused in the presence of PEG 4000. The cells obtained after the fusion process were seeded into 10 96 well plates on which the feeder cells were seeded before. Cells were incubated for 10 days in DMEM HG complete medium containing 20% FBS and 2% HAT supplement. 10 days after fusion, cells were transferred from HAT-containing medium to HT-containing medium. Clone formation in hybrid cells was regularly examined under the microscope.

No colony formation was observed in the wells of the 96-well plates in the microscopic observations of the cells formed as a result of the fusion experiment performed following the immunization of N1-N5, N6-N10, N16-N20 mice groups. The appearance of hybrid cells during HAT selection according to regular examination under the microscope is shown in Figure 38.

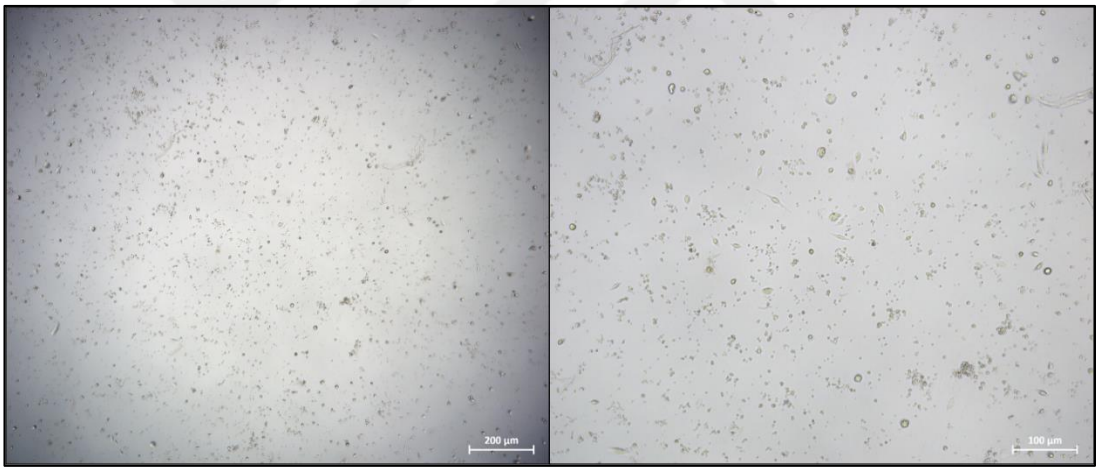

Figure 38. Hybrid cells during HAT selection (HAT selection in liquid medium). Scale bars are 200 µm (5X) and 100 µm (10X) respectively

After the hybridoma technique applied for the N13 mouse, the wells in which clone formation was observed were determined according to regular examinations under the microscope in the 96-well plate (Figure 39).

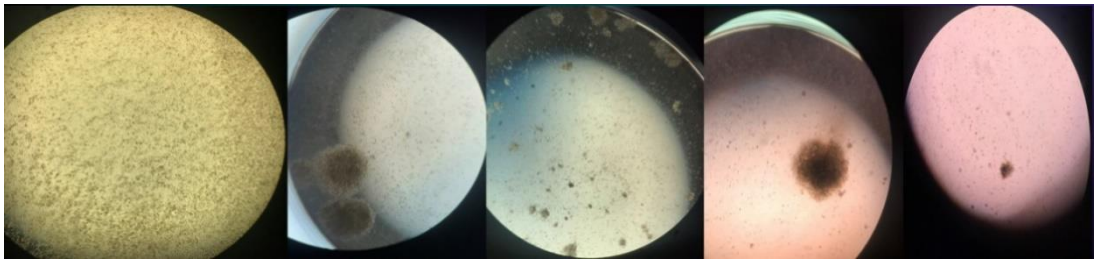

Figure 39. Microscopical views of the clones formed in hybrid cells after hybridoma technique applied to N13 mouse

For hybridoma selection and cloning, cells fused in a methylcellulose-based semi-solid medium were seeded in 100 mm culture dishes. Cells were incubated for 10 to 14 days. Clone formation in hybrid cells was examined under a microscope. The appearance of clone formed after 14 days in hybrid cells incubated in methylcellulose-based semi-solid medium is shown in Figure 40. In addition, microscopic views of clones of different sizes grown on the same plate are shown in Figure 41.

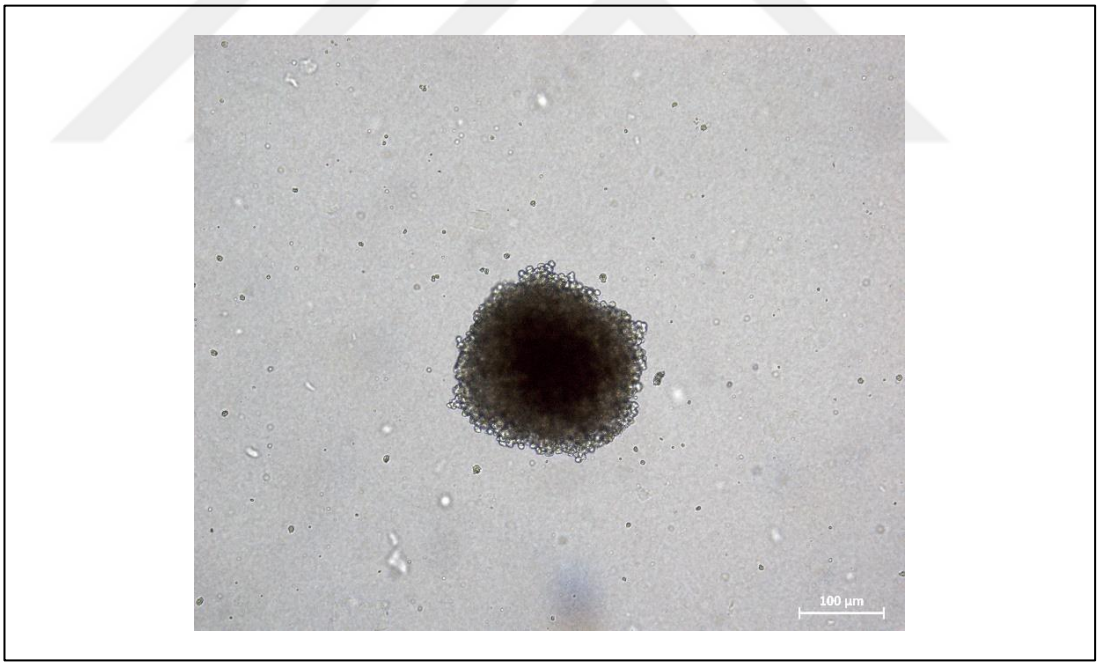

Figure 40. The appearance of clones formed after 14 days in hybrid cells incubated in methylcellulose-based semi-solid medium. Scale bar is 100 µm (10X)

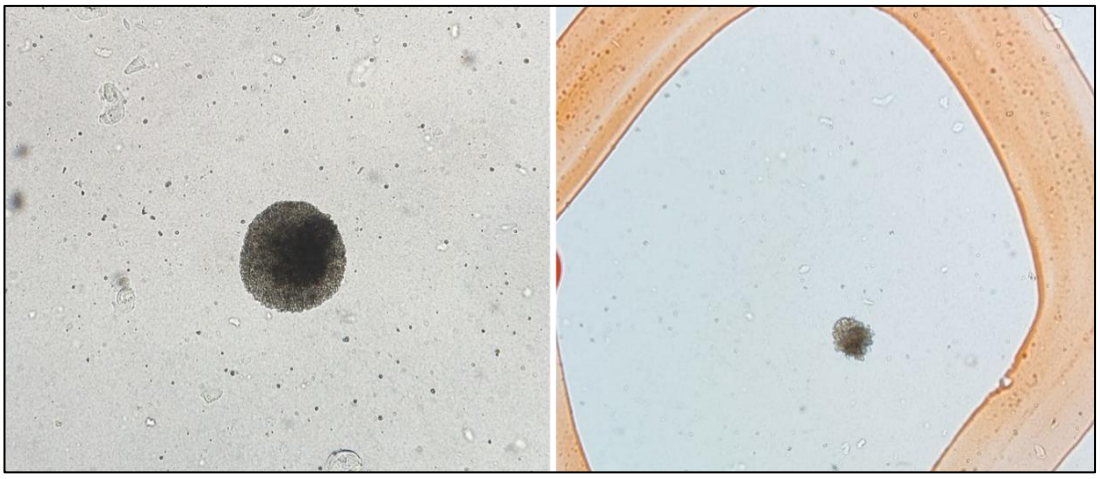

Figure 41. Microscopic views of clones of different sizes in hybrid cells incubated in methylcellulose-based semi-solid medium

The formed colonies were isolated, and the collected colonies were transferred to the medium used for growth and expansion of hybridoma cells after selection with HAT. The cells were then incubated for 3 to 4 days. Microscopic views of harvested and resuspended colonies are shown in Figure 42.

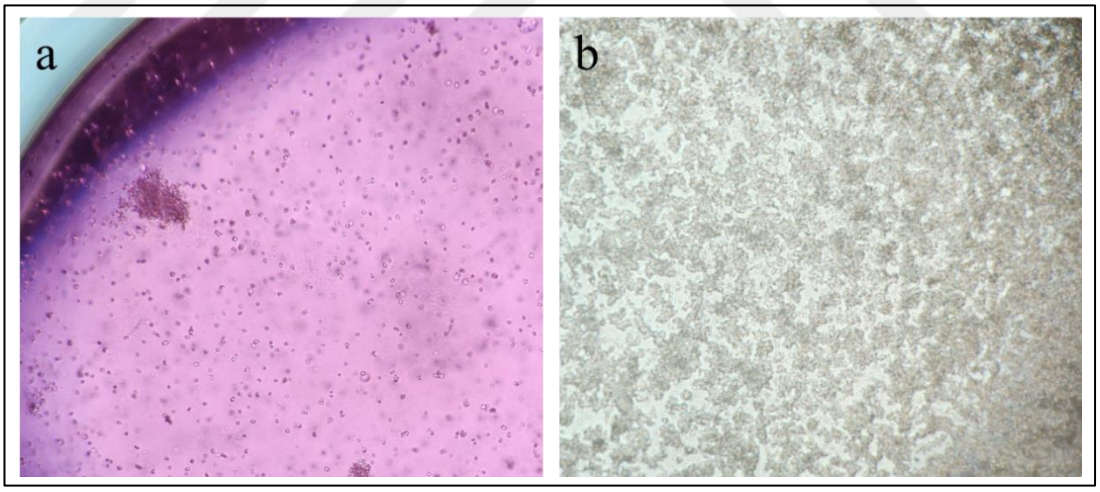

Figure 42. Microscopic views of harvested and resuspended colonies. a: 1st day, b: 3rd day

4.8 Identification of Monoclonal Antibodies with ELISA and Isostrip Tests

ELISA screening was performed regularly to detect the presence of mAb production against NPY antigen in hybrid cells formed after all fusion experiments and HAT selection.

The OD 450 values of the supernatant samples from hybrid cells of N16 and N18 mice, together with the positive and negative control samples, in the ELISA test are shown in the graph in Figure 43. According to ELISA results, mAb production against NPY antigen could not be detected in hybrid cells of N16 and N18 mice. In addition, positive results were not obtained in the ELISA screenings regularly applied for the N1-N5, N6-N10, N16-N20 mice groups.

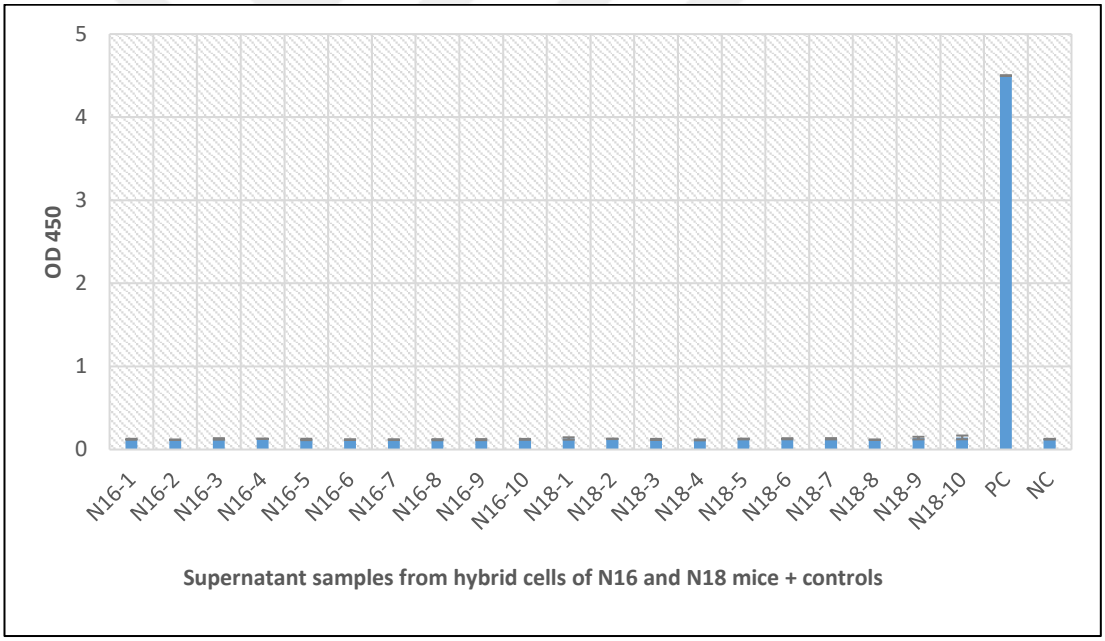

Figure 43. ELISA screening results applied to hybrid cells of N16 and N18 mice to detect the presence of mAb production against NPY antigen

A commercial Mouse Monoclonal Antibody Isotype assay kit was used to determine the presence of mAb in cells with colony formation observed. Figure 44 shows the results of the isotyping performed. IgG3, κ was found in selected cells.

Figure 44. Results of strip isotyping for hybrid cells of N13 mouse

The OD 450 values of the supernatant samples from hybrid cells that harvested from methylcellulose-based semi-solid medium, together with the positive and negative control samples, in the ELISA test are shown in the graph in Figure 45. According to the ELISA test results, the OD 450 value of one of the hybrid cells collected from methylcellulose-based semi-solid medium was determined as 1.53. Thus, the presence of mAb production against NPY antigen was detected in the ELISA test.

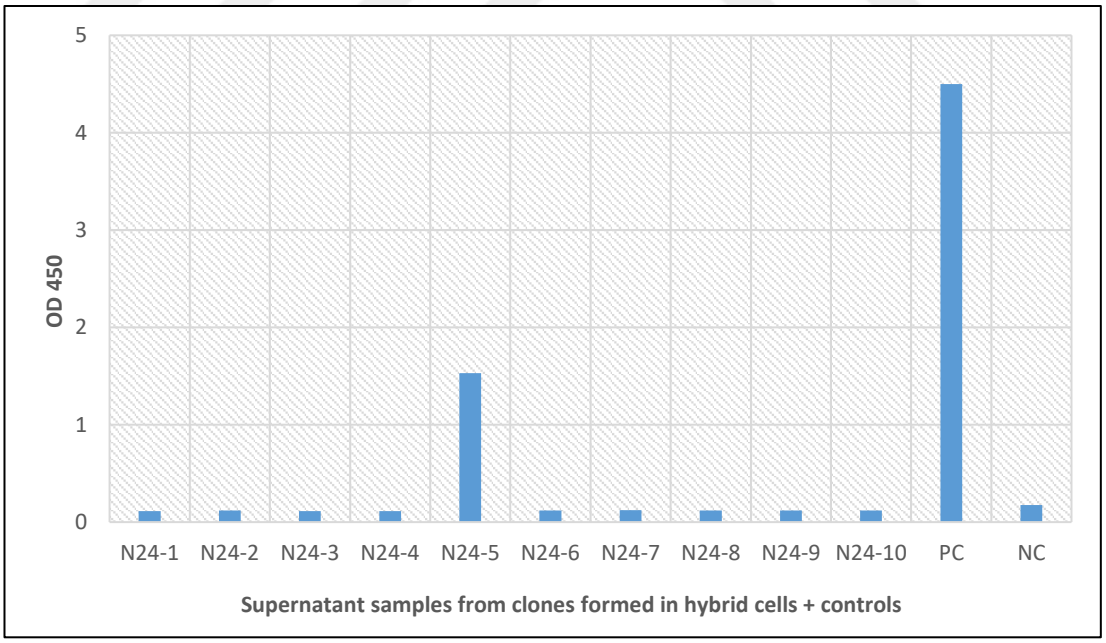

Figure 45. ELISA screening results applied to hybrid cells harvested from methylcellulose-based semi-solid medium to detect the presence of mAb production against NPY antigen

The results of strip isotyping for hybrid cells harvested from methylcellulose-based semi-solid media are shown in Figure 46. IgM, κ was found in selected cells.

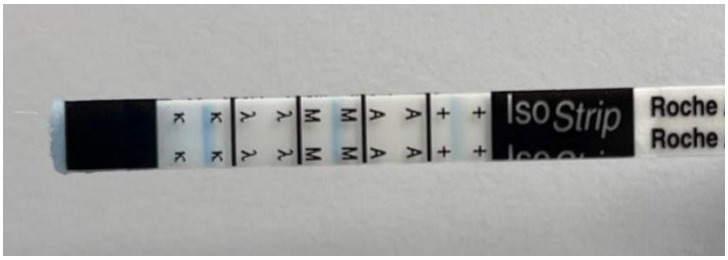

Figure 46. Results of strip isotyping for hybrid cells harvested from methylcellulose-based semi-solid medium

5 DISCUSSION

The substance that can cause a specific immune response if it enters the organism and can specifically react with the product formed as a result of this response is called complete antigen (82). There are also molecules known as partial or incomplete antigens, also called haptens. Although these molecules have the ability to form immune complexes by being recognized by an antibody, they cannot induce an immune response in the organism. By coupling another (usually larger) reactive substance with these molecules, an immune response can be induced. Therefore, not all antigen molecules are immunogens. However, all immunogenic molecules are also antigens. In this context, we can say that antigenicity and immunogenicity are different terms from each other. The ability to induce an immune response is called immunogenicity. On the other hand, the ability to combine specifically with the products of the immune response is called antigenicity. The effectiveness of molecules defined as antigens is determined by their features such as foreignness, molecular size and structural relationships, degradability and structural stability.

Molecular size is a crucial characteristic for a molecule's ability to elicit a high immune response (82). In general, large molecules elicit a higher immune response than small molecules. Some macromolecules have better recognition by the immune system. The most effective immunogens are polysaccharides and proteins. Nucleic acids and lipids generally show poor immunogenicity if they are not coupled to polysaccharides or proteins.

According to the situation called tolerance, the cells in the immune system do not respond to the organism's own antigens (82). Cells involved in the immunogenic interaction respond to foreign molecules that are even slightly different from the organism's own antigens, which is the basis of immune function. These foreign molecules, which differ in their immunogenicity and degree of inducing the immune response, the more different from the organism's own antigen, the higher the immune response will be.

For a molecule to be recognized as an immunogen, it must be structurally stable (82). Highly flexible and structurally unstable molecules such as gelatin have poor immunogenicity. Likewise, proteins show higher immunogenicity compared to carbohydrates, lipids, and nucleic acids, which are composed of large repeating polymers.

If antigens do not dissolve or degrade when they enter the organism, they cannot display their immunogenic properties because immune system cells can only recognize small molecular components of the immunogenic molecule (82). Stainless steel pins and plastic joints used in implants that can be placed in the tissue are highly preferred in medicine because these substances do not degrade and do not create an immune response in the organism.

Peptides began to be used as immunogens with the development of peptide-based vaccines and the discovery of their potential applicability. There are important considerations when using peptides as immunogens (82). We can count these as the length of the peptide, the sequence of the peptide, whether the peptide to be used will bind to a carrier protein, and if so, the type of carrier, the method to be used in the evaluation and analysis of the immune response that will occur when it is given to the organism, and the protocol to be used in immunization. Increasing the length of the peptide to be used as an immunogen to 12 amino acids increases its ability to induce an immune response, however short peptides of consisting of 6 amino acids have also been demonstrated to produce antiserum (83). A secondary structure can be produced by even longer peptides mimicking that in complete proteins, making them more likely to elicit an immune response similar to that produced by native protein (84).

Solid phase peptide synthesis method has become a frequently used method in research since it overcomes the purification steps required at each step during peptide synthesis in solution. Therefore, the peptides used in this study were synthesized by this method.

At the beginning of the study, there was a dissolution problem with the NPY peptide consisting of 36 amino acids. If the antigens do not dissolve or degrade when they enter the organism, since they cannot show their immunogenic properties, first of all, the 24 amino acid sequence of the NPY (12-36) peptide was synthesized and studies on the production of monoclonal antibodies with this synthesized peptide were initiated. At the same time, studies on solving this dissolution problem continued in parallel with other experiments.

In the later stages of the study, the peptide from which the entire 36 amino acid sequence was synthesized was used. The solubility problem of the NPY peptide, which consists of 36 amino acids, has been overcome by finding a suitable solvent that can dissolve this peptide. In general, if the peptides used as immunogens are longer, depending on the number of amino acids they contain, and also larger in molecular size, the immune response to this molecule will be higher. In this context, the results of immunizations with the NPY peptide consisting of 36 amino acids in the monoclonal antibody production section of this study showed that the mice gave high immune responses to this peptide even after the first injections.

In hybridoma production, immunization and also screening for specificity can be performed using crude antigen (76). However, the high purity of the antigen to be used in the immunization stage will provide a significant advantage by increasing the chance of success in hybridoma production and reducing the time for procedures. In this context, HPLC analysis was performed for the purity of the peptides synthesized and used in this study. Confirmation of the synthesis of the desired peptide as well as confirmation of the synthesized peptide sequences were performed by LC-MS / MS analyzes.

In the production of monoclonal antibodies, adjuvants are used in the immunization process of antigens. These immunological adjuvants, which non-specifically increase or modify the immune response to the antigen used for immunization, increase the potency of the antigen in this process (85). By administering the antigen together with the adjuvant, an immunogen depot is formed

because the introduced antigen is slowly released into the immune system over a long period of time. In addition, immune stimulation can be optimized by delivering a general immunostimulant and antigen to the same microenvironment where they can interact with lymphocytes and antigen presenting cells at the same time.

The most widely well-known and used adjuvants developed by Freund are water-in-oil emulsions (86,87). These emulsions, which are a mixture of an equal volume of oil with an aqueous solution of soluble antigen, are produced by the emulsification process (85). In this process, the antigen-containing water droplets in a viscous emulsion form particulates and are retained in the oil. After the mixing process, the components in the mixture do not separate and these thick emulsions remain stable are important points for the adjuvant activity. The addition of immunostimulants to water-in-oil emulsions can increase the potency of the emulsion. Complete Freund's adjuvant (CFA) consists of mineral oil, aqueous antigen solution, an emulsifier, and finally heat-killed Mycobacterium tuberculosis. This M. tuberculosis is a highly strong immunomodulator. The adjuvant consisting of the same components except for M. tuberculosis is Incomplete Freund's adjuvant (IFA).

These Freund's adjuvants, which are commonly used with antigens, act as stimulators in the production of good quality antibodies at high concentrations (85). There are some important factors in the use of these adjuvants. These emulsions, which are difficult to produce, can cause the degradation of protein antigens (88). In addition, it can cause symptoms such as sterile abscesses, granulomas and ulcerations after administration of Freund's adjuvants and is inherently toxic (89,90). For these reasons, it is recommended to use CFA only for the first immunization in order to prevent these complications during the immunization phase in antibody production studies. It is recommended to use IFA for subsequent subsequent immunizations. In this study, Freund's complete adjuvant (FCA) was used in the first immunization and Incomplete Freund's adjuvant (IFA) was used in the subsequent immunizations to increase the potency of the NPY antigen.

The life span of the mice selected for use in the study and possible fibroblast development with age should be considered (76). The ideal age range for immunization studies is 6-8 weeks. It is recommended that at least three mice be immunized with the same method, since premature deaths may occur due to various causes in immunization studies. In this study, six- to ten-week-old BALB/c mice were immunized in groups of five, each for different immunization approaches in terms of the peptide used as the immunogen, the injected peptide concentration, and the time intervals. A final injection of boost (antigen solution without adjuvant) to mice selected for monoclonal antibody production will increase the likelihood that only high-affinity B cells will be activated, thereby yielding high-affinity monoclonal (76). Accordingly, a final injection of boost with antigen solution without adjuvant was administered after adequate antibody titer was achieved in mice.

The schedule to be implemented in immunization, which is the first step in monoclonal antibody production with hybridoma technology, is important in order to achieve successful results. Sufficient time is required for affinity maturation and also for isotype class switching (76). After the host animal's immune system is first exposed to a specific immunogen, IgM antibody will be produced first. After this response, immunoglobulin isotype class switch can occur by individual B cells. A somatic gene recombination is thought to occur at the heavy chain locus during B-cell development (91). Meanwhile, the variable epitope binding region of the immunoglobulin remains the same and the heavy chains change from IgM to IgG. The specific isotype class switching is thought to be directed by the cytokines IL-4 and IL-5. This class switching and affinity maturation begin in 4 to 5 days (92). The immune system will have antibodies of the IgA and IgG subclasses if booster immunization is administered after a rest time interval of at least 3 weeks.

After the immunization step, the B lymphocytes of the animal used in this process are activated (76). Antibodies are then produced that recognize hundreds or thousands of epitopes of the antigen used in immunization. The extent of the immune response is determined by the immunogenicity of the antigen administered to the host animal. These antibodies produced by a variety of different B cells are polyclonal by the B cell

clones from which they originate. At the same time, we can say that the polyclonal serum consists of monoclonal antibodies. However, when antibody-synthesizing B cells are obtained from an animal, they naturally die rapidly upon cultivation in vitro. Therefore, these cells need to be immortalized. When a single B cell is isolated from the polyclonal mixture and given the property of immortality, it is monoclonal. Antibodies produced by a single B cell clonal line are called monoclonal antibodies. These B cells, which can produce an antibody that usually recognizes a single epitope of 6 to 12 amino acids, are all the same at the epitope binding site, even if they produce hundreds of antibodies. As a result of the fusion of this single B cell with a myeloma cell, an immortal hybridoma (B cell line) is formed that can produce monoclonal antibody against a single epitope.

Animal selection is limited to those with compatible mutant cell lines for the fusion process (76). The common use of rats and mice as donor animals is because most mutant cell lines are derived from the BALB/c mouse. As long as it does not elicit a weak immune response to a specific immunogen, the BALB/c strain is chosen because it does not cause histocompatibility problems when combined with the BALB/c myeloma line at the fusion stage of the study. SP2/0-Ag 14, S194/5.XXO, P3-X63-Ag8.653, and FO are the most commonly used mutant cell lines in studies. These myeloma cell lines, commonly used in fusion processes, do not secrete immunoglobulin and are all derived from BALB/c (93). Accordingly, FO myeloma cell was used in this study.

During the hybridization phase of two different cells, chemical and/or physical manipulation is used, in which the cells adhere and fuse to each other (76). Hybrid cells formed as a result of this fusion process contain the DNA of both different cells, so when all DNA is considered, they are tetraploid. The chemical to be used in this process is very important because this chemical can cause very harmful effects for cells and cause cells to be exposed to high stress and die. The most used chemical and the most important element in fusion is polyethylene glycol (PEG). The use of PEG ensures that the cells adhere to each other. With the PEG effect, the two cells are fused to form one membrane and contain most of the DNA from both. In this study, PEG

was used for the fusion of myeloma cells and B lymphocytes isolated from the spleen of mice.

Immediately after the fusion process, different cell types appear in the culture medium (76). However, in the selection stage, other cells die, except for unfused mutant cells and hybridomas. The most widely used method for specifically selecting only hybridomas is the use of mutant myeloma cell lines lacking the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) for fusion and growing the cells in a medium that provides Hypoxanthine-Aminopterin-Thymidine (HAT) selectivity (94). Aminopterin in HAT-supplemented media blocks the primary biosynthetic pathway for nucleic acids (76). Normal cells produce HGPRT in the presence of hypoxanthine and thymidine and can use a secondary salvage pathway, but mutant cell lines cannot use this salvage pathway because they cannot synthesize this enzyme. Hybridomas will be the only surviving cells, taking the ability to produce HGPRT from spleen cells and immortality from mutant cell line, when continued cultivation in HAT medium for selection purposes.

Maintenance of hybrid cells in culture after the fusion process is performed is very important as they are in a fragile state (76). The most important time for these cells to survive is the first 10 days after fusion. Hybrid cells can contain about 80 chromosomes because a mouse diploid cell has 40 chromosomes. The majority of chromosomes in hybrid cells are lost in the first few days, as the fusion process of cell membranes can cause unstable and traumatic events. This causes cell growth to stop, cell death, and the ability to synthesize immunoglobulins to disappear. During this critical first few days, some hybrid cells may divide, while other hybrids may not divide for a longer period of time, and many cell deaths may occur. For these reasons, a lot of cell death will occur in the environment where these cells are present during the cultivation and an unhealthy culture environment will be formed. Maintaining hybrid cells in separate wells is critical.

During HAT selection in fusion, all non-fused myeloma cells must die (76). Then, the cells should be taken to HT medium where aminopterin is absent in the medium.

As the cells divide, the pH of the culture medium will change and therefore its color will change from red to orange. The determination of the cells that need to be feed can be realized by this color change. Some of the cultured hybrid cells may not grow and may also divide at different rates in growing cells. Hybrid cells receive genetic information from both cells, but some may be insufficient in their ability to produce antibodies. Therefore, not all hybrid cells in the medium produce antibodies. These hybrids that cannot produce antibody may overtake hybrid cells capable of producing antibodies in the same well.

Regular screening should be performed for all cells, as hybrid cells failing to produce antibodies can outgrow potential hybrid cells that can produce antibodies (76). The care of cells that show a negative result in antibody production can be dispensed with. It may take time for some hybrid cells to produce antibodies, so the maintenance of these cells may be terminated after several screening tests. In addition, cells that initially show a positive result on the first scan may later show a negative result. This may be due to the possibility that post-fusion unfused B lymphocytes produce immunoglobulin before they die. For this reason, it is very important to apply the repeated screening process to the cells and in this study, the ELISA screening test was frequently applied for the cells obtained after the fusion process.

Isolating and multiplying a hybridoma cell is called cloning (76). Cells in the medium that produce antibodies and grow from only one well can appear as a single cell line. However, other cells that may or may not produce antibodies can be found in the media of this cell line. Cloning is done to separate the colonies from hybrid cells that do not produce antibodies and also to ensure that the hybrid cells are monoclonal. At the same time, chromosome loss can occur at any time of cultivation. Hybridomas are less stable and very sensitive. Therefore, it is very important to perform the cloning process as soon as possible after selection. Limited dilution in the liquid phase is the most widely used cloning method. Dilution of cells is performed by taking only one cell alone into separate plate wells. However, it is thought that the colonies appearing in the liquid in this process may be caused by the shifting of the cells. Therefore, semi-solid media can also be used for the cloning process.

The use of semi-solid selective medium for hybridoma selection and cloning greatly reduces the time required at this step (51). For the hybridoma technology selection step, methylcellulose-based semi-solid selective medium is highly preferred (79). The use of this semi-solid medium reduces the possibility of loss of clones formed in the liquid medium due to rapid and excessive growth of cells. Clones selected in this semi-solid medium are then transferred to liquid growth medium and screening and expansion processes are applied. In this study, following the immunization studies of the last mouse group, the semi-solid method of selection and cloning was applied to one of the mice with high antibody titer using the ready-to-use ClonaCell™-HY hybridoma kit.

As mentioned earlier, different cell types such as unfused myeloma cells, spleen cells, fused spleen-spleen cells, myeloma-myeloma cells, and myeloma-spleen cells (hybrid) can arise during the fusion process. Therefore, selection of cells other than hybrid cells is a critical step in monoclonal antibody production. For this purpose, 'Hypoxanthine-Aminopterin-Thymidine' (HAT) selection medium is generally used. In our study, we also used HAT selection method. The aminopterin contained in the HAT medium blocks the primary purine synthesis pathway (95). However, cells treated with aminopterin synthesize purine nucleotides using an alternative salvage pathway catalyzed by the enzymes hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and thymidine kinase (TK) in the presence of hypoxanthine or thymidine.

In the fusion process, myeloma cells compatible with this selection are used. Alternative myeloma cell lines can be treated, for example, with medium containing 8-azaguanine (96). Myeloma cells that cannot synthesize the HGPRT enzyme correctly cannot use the alternative pathway and thus die in the HAT environment. B lymphocytes, which have a limited lifespan in vitro, die naturally in the cultivation carried out for the purpose of selection. Hybrid cells can continue to grow in the HAT environment with the necessary enzymes provided by B lymphocytes and the immortality feature provided by myeloma. In this study, myeloma cells compatible with HAT selection were used for the fusion process. Despite the high antibody titers obtained in mice at the immunization stage, most hybrid cells did not form colonies

after fusion. For this reason, fusion was also performed with myeloma cells treated with different concentrations of 8-azaguanine to be completely sure that the myeloma cell line we used was sensitive to the HAT medium. As a result, the same results were obtained in experiments with myeloma cells untreated with 8-azaguanine.

In this study, the NPY (12-36) peptide was used for the group of N1-N5 mice, which was the first group to be immunized, and it was understood that this peptide did not cause any problems for N1-N3 mice according to the first trials. To observe the effect of NPY (12-36) peptide on the immunization process in this group, N4 and N5 were designated as control animals and these mice were injected with PBS containing only adjuvant solution instead of the peptide. According to the ELISA test results, the immune responses to the NPY peptide showed a higher OD 450 value for N1-N3 mice as expected compared to control mice. This group of mice was immunized at 10-day intervals. As the number of injections increased, the N1-N3 mice began to show higher antibody titers. However, colony formation was not observed according to the fusion results, and the presence of mAb production against NPY antigen could not be detected in regular ELISA screening tests. It was thought that the level of antigen in the immune system decreased due to the aging of the mice and therefore the immunization time interval. Therefore, N6-N10 mice were immunized one week apart with the same peptide concentration.

In immunization of N6-N10 mice, problems in the formation of immune response were observed in N9 and N10 mice, although the same peptide concentration was administered in a more recent period. At the same time, another problem was the slowing of the immune system observed in mice as the number of continued injections increased, and the spleen cells isolated for the fusion process had passed the appropriate time interval for their functions. In the later stages of the study, the concentration of NPY (12-36) peptide used as an immunogen was increased from 50 µg to 100 µg for N11-N15 mice, since the immune response was decreased in the group of mice N6-N10.

After immunization of N11-N15 mice with a higher concentration of NPY (12-36) peptide, no problems in the formation of immune response were observed in these mice. This group of mice was selected earlier for fusion experiments, before the number of injections increased too much, in order to prevent the reduced immune response due to aging. Colony formation was observed according to the fusion results. Isotyping was performed to determine the presence of mAb in cells with colony formation and IgG3, κ was found in selected cells. However, the presence of mAb production against NPY antigen could not be detected in ELISA test. In the fusion results for this group, it was shown that the obtained hybrid cells were able to form colonies capable of producing antibodies. However, it is not fully understood whether antibodies produced by hybrid cells are produced specifically against the NPY peptide. If the peptides used as immunogens are longer, depending on the number of amino acids they contain, the immune response to this molecule will be higher. In this context, it was thought that the ability of B lymphocytes to produce antibodies with higher specificity against the NPY peptide could be enhanced by the use of NPY (36) peptide, which consists of 36 amino acids as immunogen. Therefore, NPY (36) peptide was used for immunization of N16-N20 mice.

Immunization for the N16-N20 mouse group were performed at a dose of 125 µg with the NPY (36) peptide consisting of 36 amino acids, which was synthesized later in the study. Immunization results of N16-N20 mouse groups with the NPY (36) peptide showed higher immune responses to this peptide than other immunization mouse groups, even after the first injections. However, colony formation was not observed according to the fusion results, and the presence of mAb production against NPY antigen could not be detected in regular ELISA screening tests. Due to the very small amount of antibodies produced by clones in the medium, possible healthy clones may not be selected in regular ELISA screening results. Against this possibility, frequent ELISA screenings for hybrid cells were performed and regularly observed under the microscope. As a result of ELISA screening tests and observations performed under the microscope after the fusion experiments, the cultivation of hybridoma cells without active colonies was terminated. This problem can be seen from time to time in hybrid clones produced using hybridoma technology. One of the

reasons for this problem is that there are twice as many chromosomes in the hybrid cell formed by the fusion of two cells, and these chromosomes can move away from the cell over time during the fusion process. In this case, if the discarded chromosomes have genes related to the synthesis of the antibody, the produced clone stops the production of antibodies. Since it was previously shown that hybrid cells can form colonies capable of producing antibodies, it was thought that one of the reasons why colony formation was not observed in this group may be due to the loss of rare clones that may occur during hybridoma selection and cloning.

NPY consists of 36 amino acids (22). Since the peptide in which all 36 amino acid sequences of the NPY peptide was synthesized was used in the immunization of the N16-N20 mouse group and high immune responses were formed against this peptide, the immunization approach was not changed for the N21-N25 mouse group. As a result of the immunization of the N21-N25 mouse groups, high immune responses were formed against the NPY peptide as in the previous group. Different approaches were applied for hybridoma selection and cloning in the fusion process. For the fusion process of the N21-N25 mouse group, a methylcellulose-based semi-solid medium was used to prevent the loss of rare clones that may occur during hybridoma selection and cloning. Colony formation was observed according to the fusion results. Isotyping was performed to determine the presence of mAb in cells with colony formation and IgM, κ was found in selected cells. In addition, the presence of mAb production against NPY antigen was detected in the ELISA tests of this group.

In this study, it has been shown that hybrid cells can form colonies capable of producing antibodies at the end of all immunization studies and hybridoma production studies. In addition, the presence of mAb in cells with colony formation was determined by isotyping. Finally, the presence of mAb production against NPY antigen was detected in regularly performed ELISA tests.

6 CONCLUSION

Pancreatic cancer has an extremely poor prognosis and causes an important health problem worldwide. Neural invasion is one of the main factors causing the poor prognosis of pancreatic cancer. There are many peptide hormone receptors that are overexpressed in human cancer, allowing an in vivo targeting in a diagnostic or therapeutic background. One of the emerging candidates in this field is NPY receptors. NPY has five distinct known receptors, and their expression has also been found in human pancreatic cancer tissue. Although it is not known exactly which of these receptors triggers nerve invasion, drugs, small molecule inhibitors or monoclonal antibodies that directly bind NPY are not yet commercially available. For these reasons, further studies are needed for new treatment approaches to prevent pancreatic cancer.

In this study, NPY peptide was successfully synthesized, and high immune responses were obtained as a result of immunization of this synthesized peptide in mice. In addition, cells capable of producing monoclonal antibodies were developed using this synthetic peptide based on the NPY peptide sequence. Overall, this study includes preliminary studies for the development of antibodies that directly inhibit NPY ligand. It is thought that the findings of this study will allow further research on treatments that directly target neural invasion for pancreatic or other types of cancer. This study is of great importance for research into the development of therapies that directly target neural invasion in pancreatic cancer.

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8 APPENDIX

APPENDIX 1. Ethical Approval of This Study

APPENDIX 1. Ethical approval of this study (continued)

9 CURRICULUM VITAE