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# X-ray Crystallographic Structure of Ibuprofen Bound to Human Adipocyte Fatty Acid Binding Protein at 1.4 Å Resolution

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**X-ray Crystallographic Structure of Ibuprofen Bound to Human  
Adipocyte Fatty Acid Binding Protein at 1.4 Å Resolution**

by

Anshu Sharma

A Thesis

Submitted to the Graduate Faculty of

St. Cloud State University

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science in

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## Abstract

The fatty acid-binding proteins (FABPs) are a family of nine structurally related proteins, which bind to long-chain fatty acids with high affinity. Fatty acid binding proteins were first discovered in 1972, while conducting studies on the factors that regulate the uptake of intestinal fatty acids in rats. These proteins are most abundantly found in the tissues engaged in active lipid metabolism. Adipocyte fatty acid binding protein or aP2 is a member of this family, which is found in adipocytes and macrophages and integrates metabolic and inflammatory responses (Furuhashi et al., 2007).

Studies on aP2 knockout mice have shown that aP2 null mice were more insulin sensitive compared to their wild type counterparts when kept on a high fat diet. This lipid chaperone plays an important role in metabolic syndromes, including type 2 diabetes and atherosclerosis. Targeting aP2 with small-molecule inhibitors can lead to a new class of therapeutic agents that may provide a model of study to prevent metabolic diseases such as type 2 diabetes.

This study focuses on the purification of aP2 by ion exchange chromatography in preparation for co-crystallization with ibuprofen. Previous attempts to do this have failed due to the tight binding of fatty acid at the binding site of the protein. To overcome this issue, delipidation was attempted by means of chromatography using the Lipidex 1000 sorbent. The purified protein was crystallized using the hanging drop method with seeding in the presence of ibuprofen. This study describes the crystal structure of human aP2 with bound ibuprofen at 1.4 Å resolution. Crystalline

aP2 was orthorhombic with cell dimensions of  $a = 32.20 \text{ \AA}$ ,  $b = 53.64 \text{ \AA}$ ,  $c = 74.44 \text{ \AA}$  and the space group was  $P2_12_12_1$ .

## **Acknowledgments**

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## Chapter 1: Introduction

### Fatty Acid-binding Proteins

The fatty acid-binding proteins (FABPs) are a family of nine structurally related proteins, which bind to long-chain fatty acids with high affinity. The intracellular lipid binding proteins provide cytosolic shuttles for the solubilization, transfer and storage of fatty acids in eukaryotic cells (Marr et al., 2006). They solublize hydrophobic ligands and transport them to appropriate metabolic systems for proper utilization (Baxa et al., 1989).

The members of the FABP family display a unique pattern of tissue expression (Ockner, 1990). As mentioned earlier, they are expressed in tissues involved in the metabolism of lipids. The members of the family include liver, intestinal, heart, adipocyte, epidermal, ileal, brain, myelin and testis fatty-acid binding proteins. As shown in table 1, the FABP's are not necessarily found in only one tissue. Different cell or tissue types have also been found to express many FABP isoforms (Furuhashi et al., 2007).

FABP's contribute to about 1-5% of the soluble intracellular proteins in adipocytes, cardiac myocytes and hepatocytes as they are required for the synthesis, storage and breakdown of lipids.

Table 1

*The family of fatty acid binding proteins and the expression of FABP gene.*

Common name	Alternative name	Tissue type
Liver FABP	L- FABP	Liver, intestine, pancreas, kidney, lung, stomach
Intestinal FABP	I- FABP	Intestine, liver
Heart FABP	H- FABP	Heart, skeletal muscle, brain, adrenal gland
Adipocyte FABP	A- FABP, aP2, ALBP,p15	Adipocyte, macrophage, dendritic cell
Epidermal FABP	E- FABP, mal1	Skin, tongue, adipose tissue, mammary gland
Ileal FABP	II- FABP	Ileum, ovary
Brain FABP	B- FABP	Brain
Myelin FABP	M- FABP	Peripheral nervous system
Testis FABP	T- FABP	Testis

An increase in fatty acid level results in increased levels of the FABP's. High levels of FABP's have been observed in skeletal muscle cells during diabetes and similar expression patterns have also been observed in adipocytes and hepatocytes. Though the regulation of expression of FABP's in different tissue types is not well understood, the pattern suggests that there is a built in mechanism, which responds to changing fatty acid levels in the cells.

### **Adipose Tissue and Adipocytes**

Adipose tissue stores energy as triglycerides and is also important in signaling energy balance status. Almost half of the adipose tissue cells consist of adipocytes.

There are two types of adipose tissue. White adipose tissue (WAT) stores energy as fat and Brown adipose tissue (BAT) converts energy into heat. Adipocyte fatty acid-binding protein (A-FABP) is nearly 1-3% of the total soluble adipocyte protein (Reese-Wagoner, 1999).

AP2 (FABP4) and mal1 (FABP5) are two isoforms of FABP, which are expressed in adipocytes and macrophages. Adipocyte fatty acid-binding protein is a dominant isoform in adipocytes. However, the expression of aP2 and mal1 is similar in activated macrophages. The regulation of aP2 and mal1 is through metabolic and inflammatory mediators. This information is an important link between obesity and inflammation because the inflammation that arises from adipocytes suppresses the insulin signaling pathway, thereby making way for type 2 diabetes (Wellen & Hotamisligil, 2005).

### **Trafficking of Fatty Acids in the Adipocyte**

Fatty acids, which are destined for adipose cells circulate in plasma as triglycerides attached to lipoprotein particles. Lipoprotein lipase hydrolyzes the triglyceride-lipoprotein complex liberating fatty acids, which are bound by serum albumin.

The mechanism by which albumin-bound fatty acids pass through the endothelial cell layer is not known. It has been postulated that it may interact with the adipocyte plasma membrane to stimulate fatty acid uptake. The effects of serum albumin on the uptake of long-chain fatty acids was studied by Trigatti and Gerber in 3T3-L1 adipocytes, a murine cell line which differentiated from preadipocytes to

adipocytes. The role of serum albumin in the uptake of long- chain fatty acids has been assumed to be the solubilization of fatty acid in the aqueous environment. Serum albumin provides a reservoir of bound fatty acid to replenish uncomplexed fatty acid, degraded by cellular uptake.

The experiment was conducted using 3T3-L1 adipocytes to test the cellular uptake of oleate in a saturable manner at all ratios of oleate to bovine serum albumin (BSA) was tested. It was found that oleate uptake was linearly proportional to the ratio of oleate to the BSA present. It was also found that an increase in the concentration of BSA had a stimulatory effect on oleate uptake. To test if the stimulatory effect was due to a direct interaction between BSA and the cell surface, BSA was labeled with a photo-reactive long-chain fatty acid. The results showed that 3T3-L1 adipocytes utilized BSA-complexed fatty acids as substrates for uptake, which also involved interaction between BSA and the cell surface (Trigatti & Gerber, 1995).

Trigatti and Gerber also identified that caveolin; a 3T3-L1 adipocyte plasma membrane protein has the capability of binding to photo-reactive fatty acid analogue as a possible transporter. Caveolin is a component of plasmalemmal vesicles, which form pits in the plasma membrane of adipocytes and other cell types.

The trafficking patterns for fatty acid have not been clearly established. It has been thought by some that the plasma membrane transporters carry fatty acids to acyl CoA synthetases, which are present on several internal membranes. Acyl CoAs are then utilized for the synthesis of membranes and triacylglycerol. The trafficking of

lipid binding protein affects only those acyl CoA synthetases which utilize long chain fatty acids (Bernlohr, Coea, & LiCatab, 1999).

### **Structure of FABP's**

Fatty acid-binding proteins are a family of small monomeric cytoplasmic proteins, with a molecular mass of 14-15 kDa. So far, nine members have been found. Their expression pattern depends on the type of tissue in which they are found. They are expressed most abundantly in tissues, which are involved in the metabolism of lipids (Furuhashi et al., 2007).

Expression of FABP's is determined by the metabolic demand of the cells. FABP's are lipid chaperones; they help in the transport of lipids to various compartments of the cells. The endoplasmic reticulum uses them for signaling, trafficking and synthesis of membranes. Mitochondria use them for oxidation, and the nucleus uses them for the regulation of transcription, mediated via lipids (Furuhashi et al., 2007).

The gene structure of FABP's is conserved. It consists of four exons and three introns. The members of this family share 15% to 70% sequence identity and their tertiary structure is superimposable and almost identical. A  $\beta$ -barrel is formed by 10  $\beta$ -strands, which run anti-parallel to each other around a ligand-binding cavity. The opening is enveloped on one side by the N-terminal helix-loop-helix cap domain (Erbay, Cao, & Hotamisligil, 2007).

The ligand is believed to enter the cavity through the region between the barrel and the lid. Inside the cavity, the carboxylate group interacts with a tyrosine and two

arginine residues by electrostatic forces of attraction. The helical N-terminus is thought by some to be crucial for electrostatic interaction between FABP's and membranes (Chmurzynska, 2006).

The fatty acid binding affinity directly relates with the hydrophobicity of the fatty acid. The binding of the ligand also inflicts a subtle change in the three dimensional structure of the protein, which may promote specific protein-protein or protein-membrane interactions thereby determining the function of each FABP (Storch, 2009).

### **Structure of Adipocyte Fatty Acid-binding Protein**

The Adipocyte fatty acid-binding protein (A-FABP, ALBP, 422 protein, aP2 and p15) is a member of the intracellular lipid-binding protein family and it has a molecular mass of 14.5 kDa (Simpson, LiCata, Ribarik, & Bernlohr, 1999). The crystal structure of human aP2 is a  $\beta$ -barrel, which is formed by 10  $\beta$ -strands, which run anti-parallel to each other around a ligand-binding cavity. The opening is enveloped on one side by the N-terminal helix-loop-helix cap domain (Erbay et al., 2007).

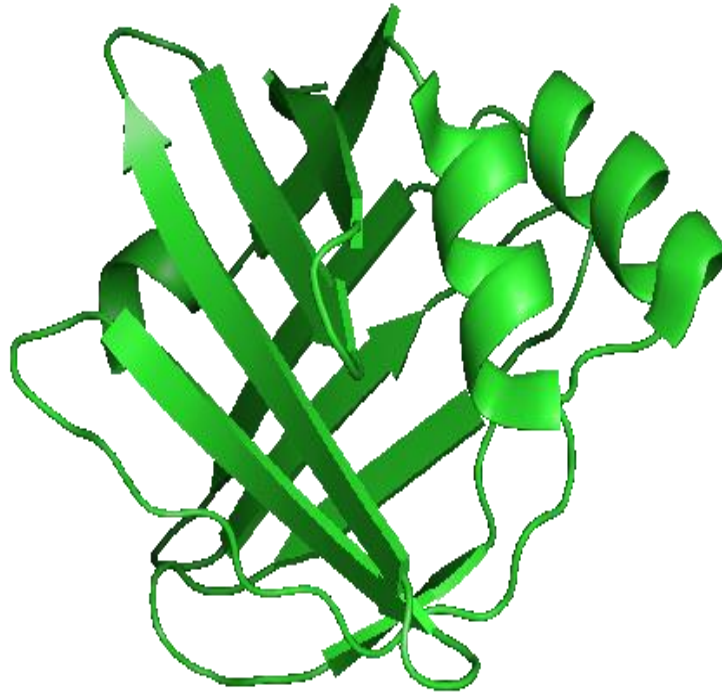


Figure 1. *The crystal structure of aP2 with the internal cavity for ligand binding.*

The inside of the barrel is a large water-filled cavity lined with polar and hydrophobic amino acids. The cavity is capable of receiving medium and long-chain fatty acids as well as other amphiphilic molecules. The ligand enters the cavity through the region between the barrel and the lid. The area near the lid along with the reverse turn loops is called 'portal' and is thought to be a region for the entry and exit for the ligand.

Inside the cavity, the carboxylate group of the fatty acid forms hydrogen bonds with the hydroxyl group of Tyr128 as well as the nitrogen atom of Arg126. The other oxygen atom of the fatty acid interacts with aP2 via water-mediated hydrogen bonds. The hydrophobic chain of the fatty acid binds in the pocket through hydrophobic interaction with various residues such as Phe16, Met20 and Phe57 (Marr et al., 2006).



## **aP2 and Type 2 Diabetes**

Obesity leads to dysfunction of adipose tissue involving metabolic, oxidative and endoplasmic reticulum stress. These factors activate stress-responsive signaling pathways, which result in metabolic, inflammatory and endocrine alterations, thereby contributing to the co-morbidities linked with obesity (Rudich, Kanety, & Bashan, 2007).

Obesity and the pathologies associated with it have become a global threat to human health. Previous studies have established that the body reacts to infection and obesity in a similar fashion. These conditions set off low-level chronic and local inflammation, which arises from the adipose tissue (Tuncman et al., 2006). The inflammatory responses are characterized by abnormal production of cytokines and acute phase reactants, which further turns on the inflammatory signaling pathways (Wellen & Hotamisligil, 2003).

The inflammatory responses normally work on metabolic support and energy redistribution, thus favoring catabolic pathways over the anabolic state, thereby curbing the insulin signaling pathway (Wellen et al., 2005). This inflammatory response paves the way for the progression of type 2 diabetes and other obesity related pathologies. Fatty acid binding proteins (FABP's) have been found to link metabolic and inflammatory responses in experimental models (Tuncman et al., 2006).

A study conducted by Makowski and Hotamisligil substantiates the claims mentioned above. Several models of FABP null mice have been created to study the

effects of altered fatty acid-binding capacity. Though all the models show alteration in lipid metabolism, only aP2-and mal1-deficient mice have been signaled for the development of metabolic syndrome. The phenotype of the aP2<sup>-/-</sup> mice did not show any defects in adipose tissue, reproduction, or growth.

The mice were kept on a diet high in fat to initiate stress on lipid pathways. When compared with aP2<sup>+/+</sup> controls, the aP2 null mice were protected from insulin resistance. Though the aP2 null mice had high levels of free fatty acid in the plasma, they had decreased cholesterol and triglyceride levels.

The aP2 deficient mice were then crossed with leptin deficient (ob/ob) mice to verify if the insulin sensitivity was due to the action of leptin. The results indicated that protection against insulin resistance was not due to leptin signaling. The two obesity models indicated that aP2 deficiency altered the metabolism of fatty acids and protected the animals from developing systemic insulin resistance (Makowski & Hotamisligil, 2004; Jequire, 2002).

A study was conducted on a genetic variation at the aP2 locus, which caused aP2 haploinsufficiency and was found to reduce the risk of type 2 diabetes. The alteration of the binding of the CAAT box/ enhancer-binding protein was found to reduce the transcription activity of the aP2 promoter, which resulted in a significant decrease in aP2 expression. The aP2 gene, mapped on 8q21 chromosome encodes a 131-aa protein. To study the genetic variation at the aP2 locus, 96 men and women were selected and the DNA sequence for the promoter and coding regions was determined (Tuncman et al., 2006).

A single nucleotide polymorphism (SNP) where Thymine (T) was replaced by Cytosine (C) at position -87 in the 5' promoter region was identified. The location of T-87C is at the binding site of the CAAT box/ enhancer-binding protein (C/EBP) (Tuncman et al., 2006). Due to the SNP, the recognition site gets altered. For the experiment, the binding of C/EBP $\alpha$ , which is a transcriptional factor for the differentiation of precursor cells into fat cells, was examined. The ability of the T-87C variant to interact with C/EBP $\alpha$  protein was tested and compared with the wild type. It was found that binding to the wild type CAAT box was much higher compared to the T-87C variant (Tuncman et al., 2006).

This finding was further pursued and studied by Wellen and Hotamisligil to find the association between metabolism and inflammation in reference to diabetes and inflammation. They argued that TNF- $\alpha$  is a link between inflammation and diabetes. Obese humans and mice, both show elevated levels of TNF- $\alpha$  in the adipose tissue. Mice lacking TNF- $\alpha$  show improved insulin sensitivity compared to the wild type. The study indicated that many mediators of the inflammatory response have similar pattern of expression as that of TNF- $\alpha$  during obesity.

Gokhan et al., have reported that mice with a null mutation in the aP2 gene, when placed on a high fat diet (40% of total calories in the form of fat), were significantly protected from hyperinsulinemia and insulin resistance compared to wild type. Also the obese aP2<sup>-/-</sup> animals failed to express TNF- $\alpha$ , a molecule implicated in obesity-related insulin resistance. They showed improved insulin sensitivity (Gokhan et al., 1996).

A study conducted by Uysal, Scheja, Wiesbrock, Bonner-Weir, and Hotamisligil indicated that genetically obese mice that lack aP2 have improved glucose and lipid metabolism. They found that aP2 deficiency in obese mice not only improved insulin sensitivity but also preserved the functioning of  $\beta$  cells.

Mice with aP2 deficiency were bred with mice having a heterozygous leptin gene. The cross between mice with the heterozygous leptin gene and aP2 deficiency generated OB/ob-aP2<sup>+/-</sup> mice. These mice were again intercrossed to generate OB/ob-aP2<sup>-/-</sup> and OB/ob-aP2<sup>+/+</sup> mice.

The cross amongst themselves produced either aP2<sup>+/+</sup> or aP2<sup>-/-</sup> animal. All ob/ob homozygous mice developed obesity. The results showed that ob/ob-aP2<sup>-/-</sup> mice had improved glucose metabolism compared to ob/ob-aP2<sup>+/+</sup> mice. Despite being extremely obese, aP2<sup>-/-</sup> mice showed similar results (Uysal et al., 2000).

### **The Inflammatory Pathways Leading to Insulin Resistance**

Almost two decades ago, the discovery of increased expression of the proinflammatory cytokine TNF- $\alpha$  in the adipose tissue of obese mice was the first indication that inflammatory mediators were associated with obesity. The changes in inflammatory signaling by adipocytes and the infiltration of immune cells into the adipose tissue results in obesity-induced insulin resistance and associated metabolic disease in animal models and humans. An important question, which arises when studying all the model systems, is why the ability to convert surplus energy into fat that has been preserved through the course of evolution becomes harmful to the body?

Nutritional abundance results in sequestering of the excess fuel in adipocytes, which is followed by continual demand on storage and processing of lipids. The adipocytes show signs of stress when they experience threshold. The stress is exhibited as hypertrophy (increase in the volume of adipose tissue due to lipid accumulation) within the cell, hypoxia (inadequate oxygen supply to the cells), disruption of mitochondrial function, production of reactive oxygen species, endoplasmic reticulum stress, apoptotic signaling, altered adipokine signaling, elevated production of TNF- $\alpha$  and increase in the release of fatty acid (Hummasti & Hotamisligil, 2010).

Adipocytes and macrophages have overlapping functions in obesity. When there is inflammation, macrophages co-localize in the adipose tissue. Macrophages express adipocyte gene products such as adipocyte/macrophage FABP aP2 (FABP4) and PPAR $\gamma$ , while adipocytes may express macrophage proteins such as TNF- $\alpha$  and IL-6.

The intracellular lipids can also have anti-inflammatory function. Peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) belong to the family of nuclear hormone receptor. LXRs regulate the homeostasis of cholesterol, fatty acid and glucose; oxysterols, the oxidative derivative of cholesterol are ligands of LXR. Macrophages undergo apoptosis in the absence of LXR. Thus LXR is crucial for the response of macrophages to infection (Wellen & Hotamisligil, 2005).

PPARs regulate the metabolism of carbohydrates, lipids and proteins. They also regulate the differentiation of preadipocytes into adipocytes. PPARs enhance

the transcription of genes, which are important for lipogenesis. Fatty acid is a ligand for PPAR. The activation of PPAR $\gamma$  inhibits inflammatory gene expression in macrophages and adipocytes through the suppression of NF- $\kappa$ B.

Preadipocytes under some conditions may differentiate into macrophages and show antimicrobial and phagocytic properties. Under normal conditions, insulin binds to the receptors on the insulin responsive cells thereby stimulating the insulin receptor. The insulin receptor is a tyrosine kinase; it autophosphorylates itself and many other substrates, including the members of the insulin receptor substrate family (IRS). Upon tyrosine phosphorylation the substrate interacts with a series of signaling proteins, thereby initiating different signaling pathways (Saltiel & Pessinb, 2002).

The exposure of cells to TNF- $\alpha$  or excess free fatty acids causes the inhibitory phosphorylation of the serine residue of IRS-1. This phosphorylation not only reduces the tyrosine phosphorylation of IRS-1 in response of insulin but also inhibits the association of IRS-1 with the insulin receptor. These events inhibit the downstream signaling and insulin action. Studies have shown that mice lacking the IRS-1 protein are insulin resistant but they do not develop diabetes (Saltiel & Pessinb, 2002).

High levels of TNF- $\alpha$  and free fatty acids are also potent activators of JNK (c-Jun N-terminal kinases). JNKs belong to the mitogen-activated protein kinase family that play an important role in cellular apoptosis and are responsive to stress stimuli such as cytokines.

JNK phosphorylates many cellular proteins, including IRS-1 and IRS-2.

Previous studies have indicated that the major JNK phosphorylation site in rat IRS-1 is located at Ser 307 and in humans is located at Ser 312. A study conducted by Aguirre, et al., on mammalian and yeast cells revealed that Ser 307 is near the PTB (phosphotyrosine binding domain) domain of IRS-1. Phosphorylation of this residue might disrupt the interaction between IRS-1 and the insulin receptor. These events might degrade IRS-1 or make it inaccessible to the activated insulin receptor, thereby impairing insulin action paving the way for type-2 diabetes (Aguirre et al., 2002).

The absence of JNK results in decreased adiposity, improved insulin sensitivity and enhanced insulin receptor signaling capacity. It has been shown that dietary and genetic (*ob/ob*) mice models have increased JNK activity in the tissues. Mice lacking either JNK1 (*Jnk1<sup>-/-</sup>*) or JNK2 (*Jnk2<sup>-/-</sup>*) and their controls (*Jnk1<sup>-/-</sup>*, *JNK<sup>+/-</sup>*) were kept on a high fat diet. On the high fat diet both control and *Jnk1<sup>-/-</sup>* mice developed obesity, but a section of adipose tissue from *Jnk1<sup>-/-</sup>* mice had decreased adipocyte size compared to the wild type. The absence of JNK-1 resulted in protection from obesity-induced insulin resistance (Hirosumi et al., 2002).

Due to infusion of lipids, there is an increase in the levels of intracellular fatty acid metabolites—e.g., diacylglycerol (DAG) and fatty acid COA's. DAG is an activator of PKC- $\theta$  and facilitates its translocation from the cytosol to the plasma membrane. These stimuli can also activate JNK1 and IKK $\beta$  along with PKC- $\theta$ . They associate with IRS-1 and phosphorylate IRS-1 at Ser 307, thereby impairing insulin action.

IKK $\beta$  is an enzyme and it is a protein subunit of I $\kappa$ B kinase. IKK $\beta$  inhibits insulin action by two pathways. In the first pathway, it phosphorylates IRS-1 at Ser 307. In the second pathway it phosphorylate the inhibitor of I $\kappa$ B. I $\kappa$ B plays a key role in regulating the immune response to infection. In the absence of the inhibitor, I $\kappa$ B gets activated and on sensing inflammation produces TNF- $\alpha$  and IL-6. Mice heterozygous for a null mutation in IKK $\beta$  are partially protected against insulin resistance (Wellen & Hotamisligil, 2005).

The inhibition of IKK $\beta$  by high dose aspirin treatment has shown to improve insulin signaling in humans (Hundal et al., 2002). All the above studies indicate that fatty acids are vital components of many biological processes and are crucial in the pathogenesis of many diseases. A-FABPs act at the intersection of metabolic and inflammatory pathways.

### **aP2 and Ibuprofen**

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have been implicated in improving insulin sensitivity. NSAIDs act as modulators of PPAR $\gamma$ . They activate PPAR $\gamma$  and induce adipogenesis by inhibiting NF- $\kappa$ B signaling pathway.

Indomethacin, a derivative of ibuprofen can induce the differentiation of the preadipocyte 3T3-L1 cell line and improve insulin sensitivity while functioning as PPAR $\gamma$  agonists (Zhang et al., 2009).

Ibuprofen has also been found to activate PPAR $\gamma$  and promote adipocyte differentiation. Rats, when treated with ibuprofen and Indomethacin, have shown reduced serum triglycerides and cholesterol levels Pharmacological agents that can



modify the function of A-FABP's are of interest for controlling lipid signaling pathways, metabolic regulation and inflammatory responses in a tissue specific or cell specific manner.

## Chapter 2: Materials and Methods

### Transformation Reaction

Bacterial cells, ECOS21, were transformed with the pET11b-aP2 plasmid vector, which has an insert for aP2 gene and ampicillin resistance selection marker. A microcentrifuge tube containing ECOS21 cells (50µl) was kept under tap water for 20 seconds. 1µl of plasmid DNA (2ng/µl) was added to it and was vortexed for 1 second.

The microcentrifuge tube was immediately placed on ice for 5 minutes. It was heat shocked at 42°C for 45 seconds and then immediately plated on an ampicillin containing LB agar plate. The plasmid used was antibiotic resistant, so only the cells that got transformed grew on the plate. The plates were kept overnight in a 37°C incubator.

### Expression of aP2

Single colonies of the E.coli strain harboring the plasmid containing the adipocyte lipid-binding protein insert were inoculated into 5 mL LB broth aliquots with 10 µl of ampicillin stock (100mg/ml) added. The test tubes were kept overnight in a shaking incubator, which was maintained at 37°C.

Next day, 500 µl of the media was inoculated in four flasks, each containing 500 ml LB broth. 500 µl of ampicillin stock were added to the flasks and they were kept in a shaking incubator. The culture was grown until it reached an OD of 0.6 @ 600nm. To induce the expression of aP2 protein, 1mM IPTG was added.

## **Harvesting aP2**

The culture was then allowed to grow 3 additional hrs and subsequently harvested by centrifugation using a JS-4.2 rotor @ 4,000rpm at 4°C for 20 min. 6.8 gm of cell paste was recovered from 1 liter of culture. The supernatant was discarded and the pellet was kept in a - 20°C freezer.

## **Cell Lysis**

The pellet was resuspended in 50 ml lysis buffer. The lysis buffer contained 50mM Tris and 20mM EDTA at a pH of 8.0. To disrupt the cells the resuspended pellet was passed through a M-110L pneumatic microfluidizer from Microfluidics. The lysate was centrifuged @ 14,000 rpm for 30 min, using a JA-20 rotor. This step removed the cell debris. The supernatant was collected and the pellet was discarded.

## **Purification of the Protein Using Q Sepharose Column**

Q Sepharose is a medium used for anion exchange chromatography. In anion exchange chromatography, a resin rich in positive charge is used and negatively charged proteins associate with the positive charge on the resin. The more negative the species, the tighter it binds to the column. Positively charged and neutral molecules do not bind and elute through the column. A study conducted by Singh and Bourdon found that the isoelectric point of aP2 is 7.6 (Singh, Rondeau, Hoareau, & Bourdon, 2007). Since, aP2 is relatively neutral at a pH of 7.4, it interacted very weakly with the column and eluted relatively unfettered.

As a column buffer 10mM HEPES and 1mM EDTA was used. The buffer was maintained at a pH of 7.4. The column was attached to an optical detector, which

detects absorbance @ 280nm. The absorbance is used as a proxy for protein concentration. 10ml of the supernatant were loaded onto the column. The fractions were collected until the detector showed a baseline of zero. The negatively charged proteins that were bound to the column were then eluted by washing the column with high salt (1M NaCl). The column was re-equilibrated by washing it with 10 column volumes of the buffer.

Recombinant aP2 was identified by SDS-PAGE. The fractions containing aP2 were pooled and concentrated using a Centricon centrifugal filtration device. The protein was centrifuged using a JS 4.2 swing bucket rotor at 3,750 rpm for 10 min. The concentrator had a cut off of 10kDa and hence the buffer can flow through the membrane while retaining proteins above 10kDa.

### **Purification of the Protein Using SP Sepharose Column**

The concentrated protein was collected and loaded onto a sulfopropyl-Sepharose cation exchange column, which is used for cation exchange chromatography. The resin consists of long-chain dextran, a polymeric carbohydrate of many repeating units joined together by covalent bonds. Dextran is coupled to a cross linked agarose matrix. The stable agarose matrix and the flexible chains of dextran increases the exposure of SP charged groups and also allows the passage of charged protein molecules.

The column was equilibrated with 10mM HEPES and 1mM EDTA at a pH of 7.4. The fractions containing aP2 were identified by SDS-PAGE and pooled together.

They were concentrated using a Centricon centrifugal filtration device as described in the above step.

### **Removal of Fatty Acid by Lipidex 1000 Chromatography**

The concentrated protein (30 mg/ml) was warmed at 37 °C and applied to a pre-warmed Lipidex 1000 column equilibrated with buffer containing 10mM HEPES and 1mM EDTA at a pH of 6.0. This step is designed for temperature dependent removal of the sequestered fatty acids from protein-fatty acid complexes (Glatz and Veerkamp, 1983). The aP2 protein was left on the Lipidex column for 20 min at 37 °C.

The protein was then washed off the column using the column buffer. The fractions containing aP2 were pooled together and concentrated to 8.6 mg/ml using a Centricon centrifugal filtration device. A 10 fold molar excess of Ibuprofen was added to the concentrated protein and it was incubated at room temperature for 1 hour.

### **Protein Crystallization and Data Collection**

A 1M stock solution of Tris HCl at a pH of 8.0 and a 3.5M stock solution of Ammonium sulfate were prepared to serve as components of well solution. Table 2 shows the concentration range of ammonium sulfate (2.14 M to 2.42 M) and the concentration of Tris-HCl (0.1 M), used to determine conditions ideal for crystallization. The crystals were grown using the hanging drop method. A small droplet of the protein (1µl) was mixed with the crystallization reagent (1µl) and mounted on a siliconized cover slide; this slide was inverted over the reservoir. The initial concentration of reagent in the droplet was less than that of the reservoir.

The reservoir pulled water from the droplet until an equilibrium point was reached between them.

Small needle shaped crystals were formed within 4-5 days at room temperature. These crystals were isolated and used as seeds by transferring them into a 2  $\mu$ l drop of 8.6 mg/ml fresh aP2 and mother liquor. 1  $\mu$ l of fresh aP2 was added to it every day and big crystals were observed in 3-4 days.

The crystals were soaked in a cryoprotectant consisting of 1:4 glycerol and well solution and flash frozen in liquid nitrogen. For the purpose of data collection the crystal was mounted on a goniometer head, which was adjusted to center the crystal in the X-ray beam and also allowed rotation. The flash frozen crystal was held in a stream of cryo-cooled nitrogen gas.

The crystal diffracted the X-ray beam into discrete beams, which produced a reflection on the screen. The optical scanner measures the position and intensity of each reflection by measuring the optical absorbance of the spot on the screen. This information was sent to the computer in digital form.

The structure of human aP2 was solved by using the phases from the 2nnq model of the RCSB Protein Data Bank. The structure was initially refined using phenix refine without ibuprofen being modeled; clear electron density was seen for bound ibuprofen. Finally ibuprofen was manually modeled and refined to 1.4 Å resolution.

Table 2

*Crystallization conditions. Shows different conditions in a 24 well plate, which was used to grow crystals. The concentration range of Ammonium sulfate changes from 2.14 M- 2.42 M in the rows, whereas the concentration of Tris remains the same.*

<b>AS</b>						
<b>2.14M</b>	<b>2.196M</b>	<b>2.252M</b>	<b>2.308M</b>	<b>2.364M</b>	<b>2.42M</b>	
611	627	643	659	675	691	AS
100	100	100	100	100	100	Tris (0.1M)
289	273	257	241	225	209	Water
611	627	643	659	675	691	AS
100	100	100	100	100	100	Tris (0.1M)
289	273	257	241	225	209	Water
611	627	643	659	675	691	AS
100	100	100	100	100	100	Tris (0.1M)
289	273	257	241	225	209	Water
611	627	643	659	675	691	AS
100	100	100	100	100	100	Tris (0.1M)
289	273	257	241	225	209	Water

## Chapter 3: Results

### Protein Purification

The expression and purification protocol for recombinant aP2 was established by Chinander and Bernlohr. The protocol has been changed many times according to the need of the experiment. In this study aP2 was purified using anion and cation exchange chromatography and the purified protein was delipidated using Lipidex 1000 chromatography. The recombinant aP2 was identified by SDS-PAGE gel electrophoresis. The purified protein was then crystallized using the hanging drop method with seeding.

SDS-PAGE analysis in Figure 2 shows the crude bacterial lysate in lane 7. The bands across the 14.4 KDa protein marker corresponds to aP2 protein. Lane 7 and 8 both show a thick band for aP2, suggesting over-expression of the aP2 protein. Lane 1 shows the protein that was passed through the anion exchange column. It can be clearly seen that this step resulted in removing the interfering proteins by passing them through the column.



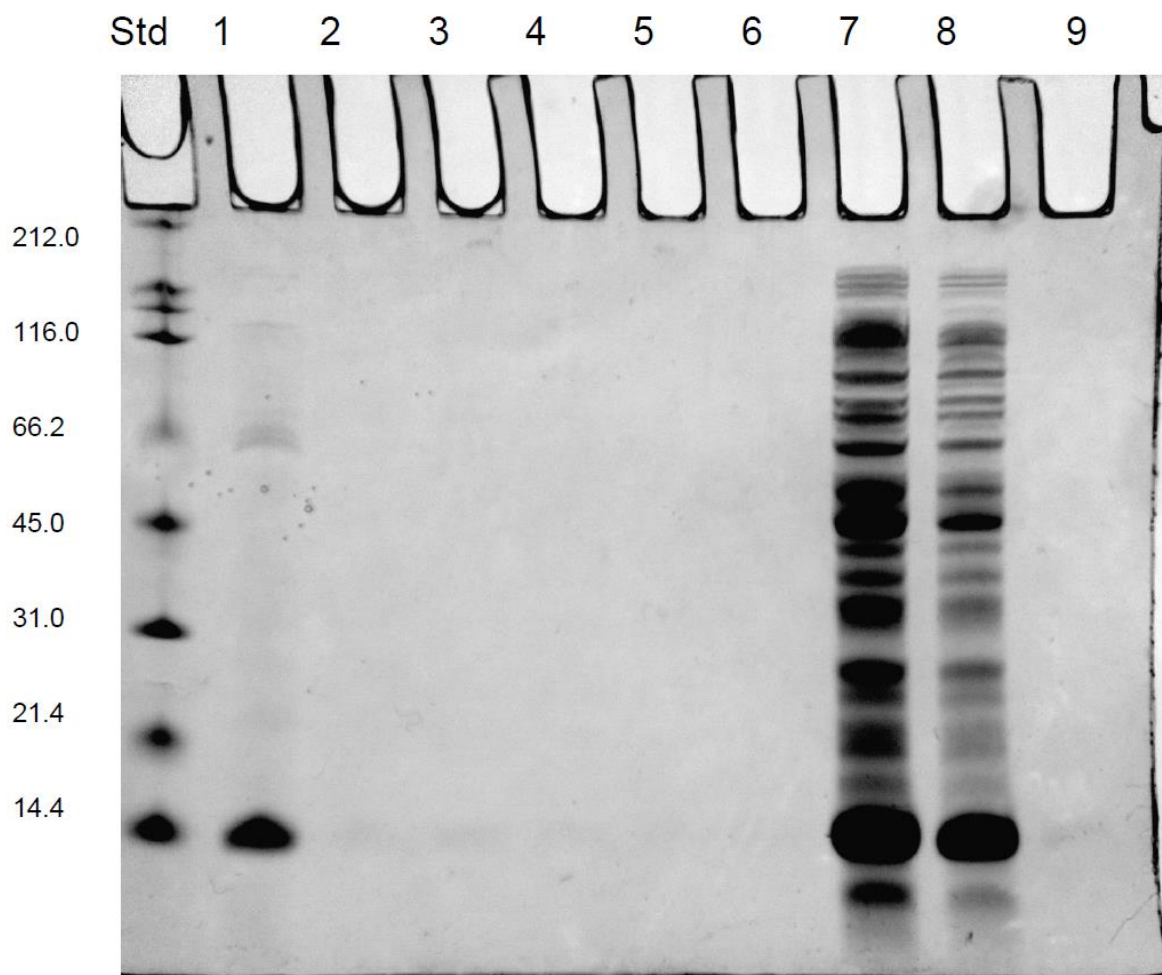


Figure 2

*SDS PAGE analysis. The numbers to the left of the gel show the molecular mass of the standards in kilodaltons. Lane 7 shows a sample of the bacterial lysate and in lane 8 the lysate was diluted. Lane 1 shows Q Sepharose purified protein.*

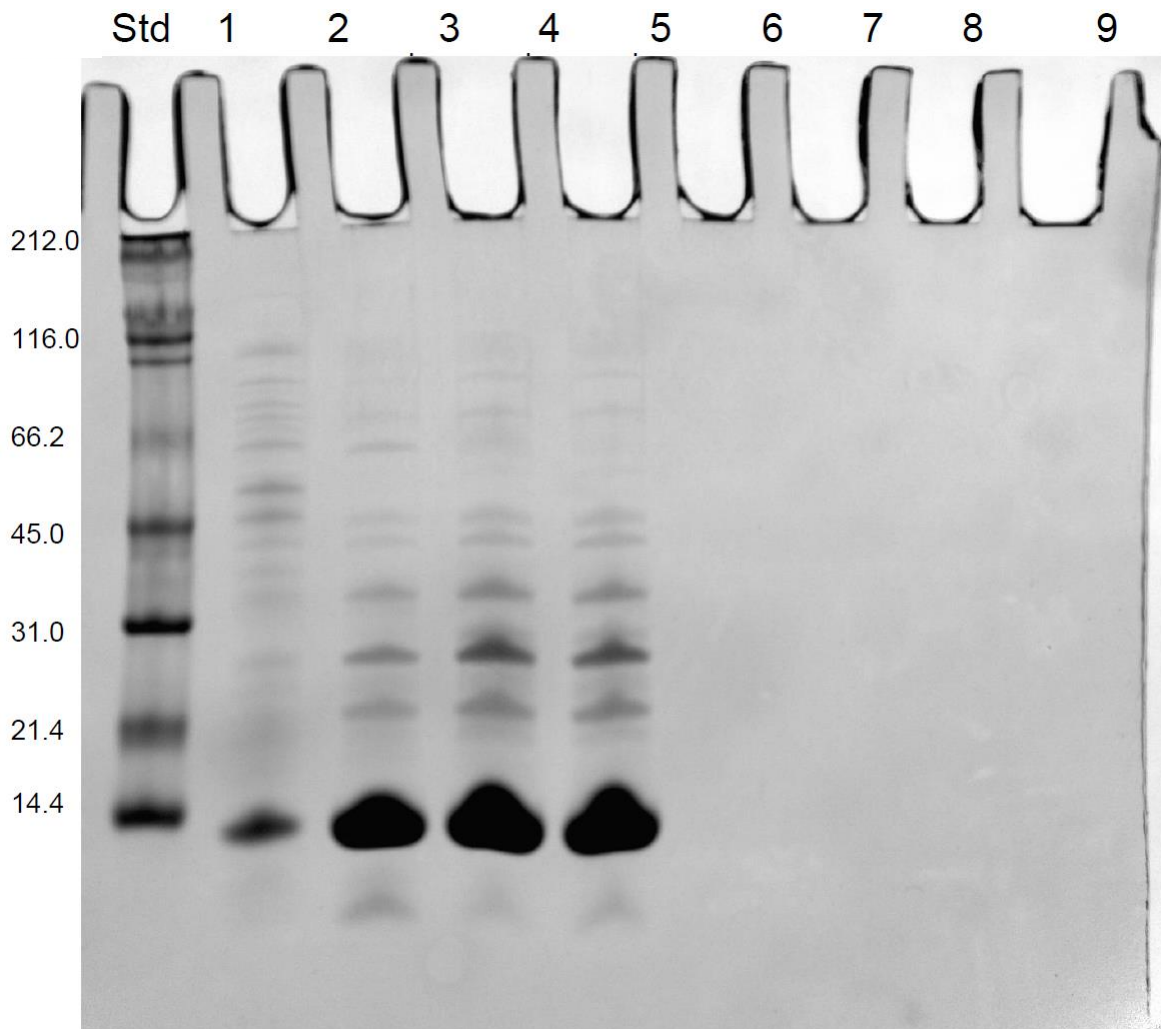


Figure 3

*The polyacrylamide gel electrophoresis analysis of the aP2 protein during the course of purification. The numbers to the left of the gel show the molecular mass of the standards in kilodaltons. Lane 1, E.coli extract; lane 2 pool from Q Sepharose column; lane 3 SP Sepharose purified protein and lane 4 shows the delipidated protein which was passed through the Lipidex 1000 column. A light smear on the gel that is migrating faster than aP2 is phospholipids. It sometimes co-purifies with aP2. The band across 14.4 KDa marker lane corresponds to aP2.*

During the course of purification starting from the bacterial lysate to SP Sepharose chromatography it was observed that aP2 was purified from the interfering proteins. A light smear on the gel that is migrating faster than aP2 are

phospholipids, which sometimes co-purifies with aP2 (Xu, Buelt, Banaszak, & Bernlohr, 1991).

The protein-bound fatty acid was removed from the aqueous solution in a temperature dependent manner using Lipidex 1000 chromatography at 37°C. Comparing lanes 3 and 4 from figure 3 it can be said that this was achieved without the loss of protein as the bands look equal in size.

### **Crystallization and Data Collection**

To examine whether the crystals were protein or salt crystals methylene blue was added to the drop harboring the crystals. Figure 4 clearly shows the presence of protein crystals as they turned blue in color. Figure 5 shows the seeding helped in growing bigger crystal. At 2 M ammonium sulfate and 10.4 mg/ml protein concentration small crystals were observed, as shown in panel A. These crystals were used as seeds for further crystallization experiments. Bigger crystals were observed at 2.42 M ammonium sulfate concentration and lower protein concentration between 8.6 to 8.0 mg/ml. These crystals gave diffraction data to 1.4 Å resolution. The entire set was collected to 1.4 Å resolution using the detector system. The statistical analysis of the combined x-ray data is presented in Table 3.

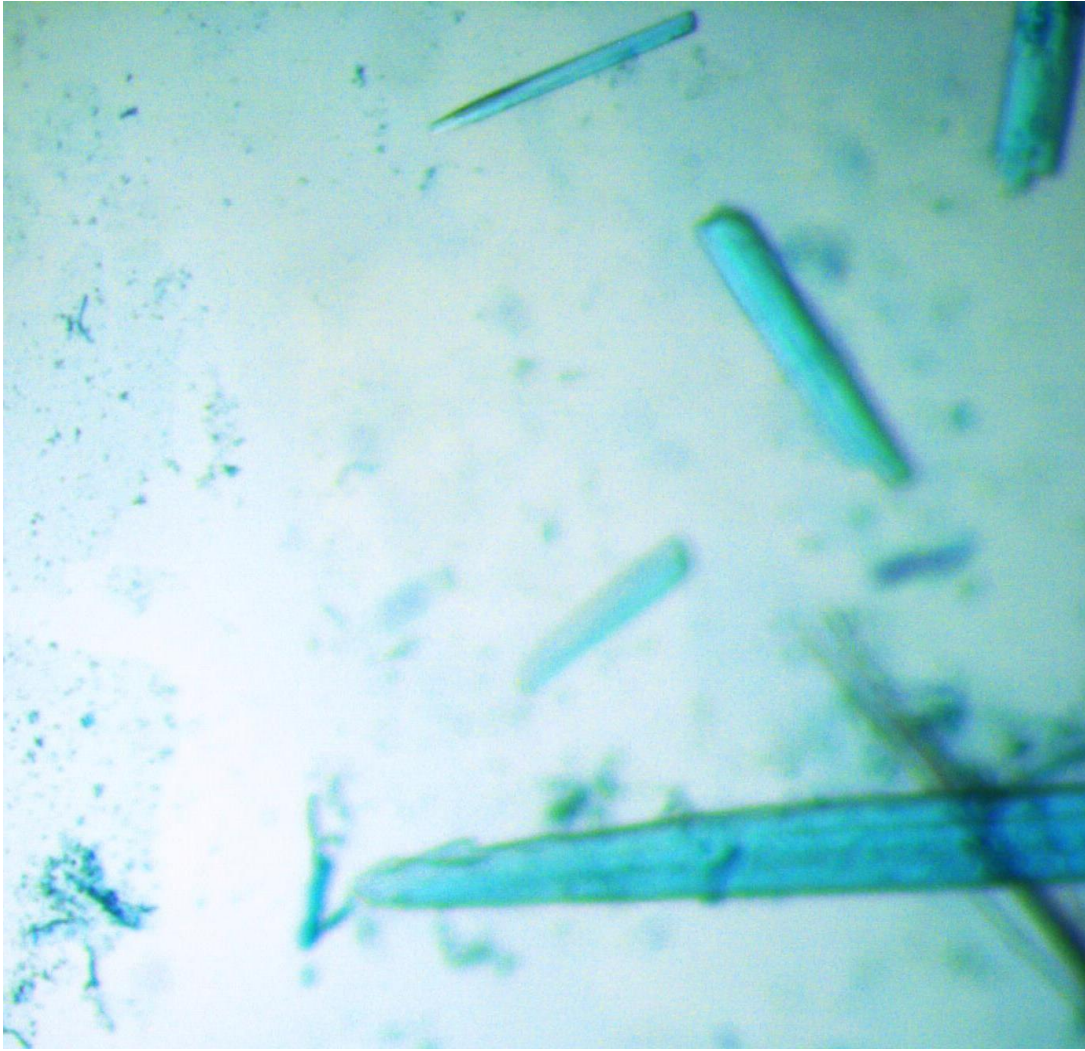


Figure 4

*Protein crystals stained with methylene blue. To test for protein crystals the crystals were stained with methylene blue. Crystals that appear blue corresponds to aP2 protein crystals.*

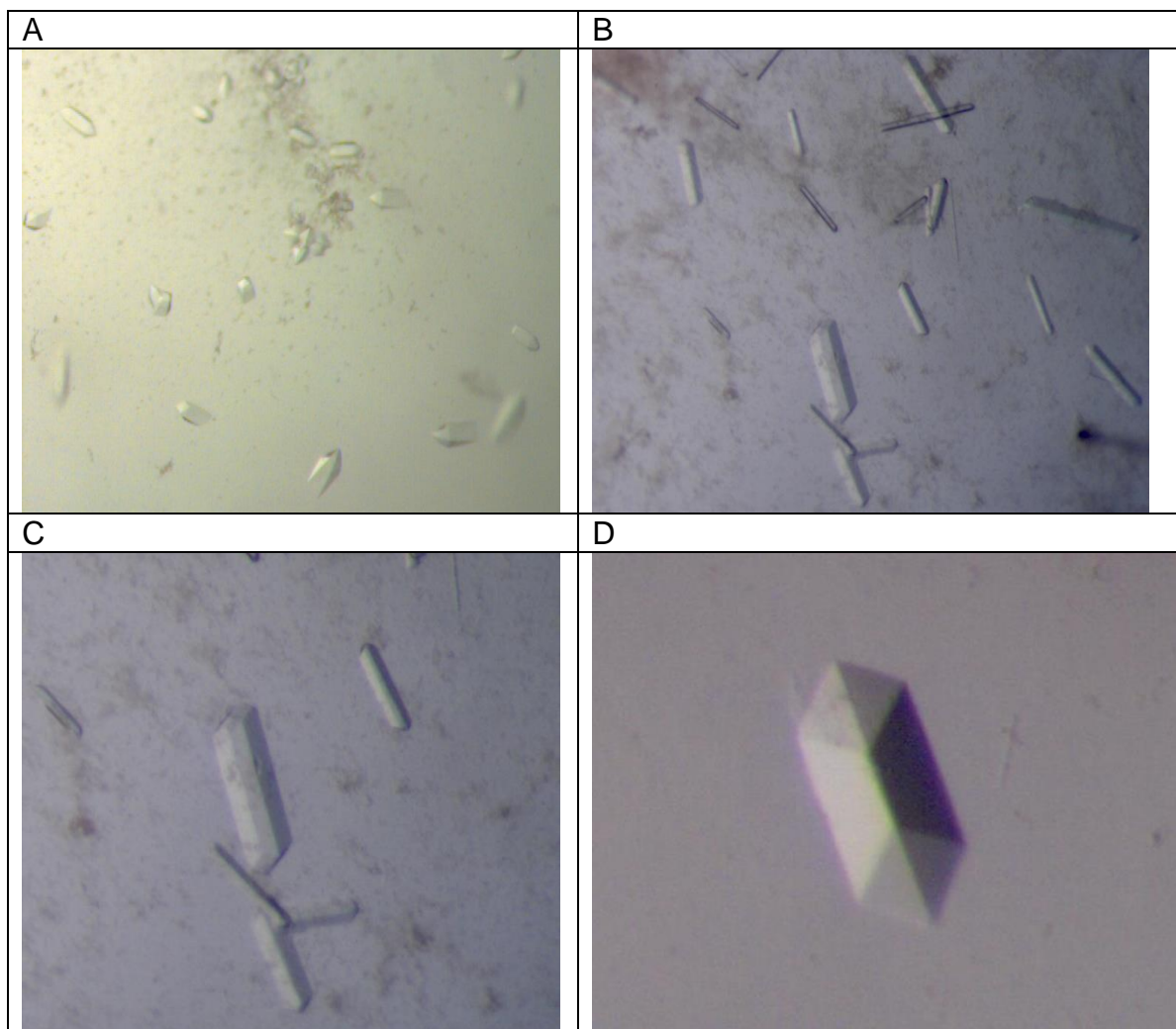


Figure 5

*Crystal images. The figure shows that changing the crystallization conditions produced bigger crystals. Panel A has shows many small crystals these crystals were used as seeds.*

Table 3

*X-ray data collection statistics of the native data set for crystalline aP2.*

Summary of data collection statistics			
Spacegroup	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>		
Unit cell dimensions	32.20	53.64	74.44
	90.00	90.00	90.00
Resolution range (Å)	29.56 - 1.40 (1.45 - 1.40)		
Total number of reflections	114166		
Number of unique reflections	24732		
Average redundancy	4.62	(2.27)	
% completeness	94.8	(67.6)	
Rmerge	0.039	(0.428)	
Reduced ChiSquared	0.97	(1.20)	
Output <I/sigI>	19.4	(1.4)	

The lattice type was orthorhombic with unit cell dimensions of  $a = 32.20 \text{ \AA}$ ,  $b = 53.64 \text{ \AA}$ ,  $c = 74.44 \text{ \AA}$ . The space group was P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> and there was one molecule of aP2 per asymmetric unit.

The Rmerge shows agreement between the averaged intensities for all observations of one reflection with the calculated intensities of individual observations of the same reflection. The individual intensities agree with their averages within 3.9%. The percentage completeness for the data set was 94.8% and each reflection was measured about 5 times.

### Refinement

For partly refined x-ray data clear electron density was seen for without ibuprofen being modeled. Finally ibuprofen was manually modeled as shown in Figure 6. The green density refers to positive electron density. Figure 7 shows the

difference map and the negative densities for the  $F_o - F_c$  are almost negligible. The backbone electron densities look continuous and it looks like a space filling model of the protein. The  $2F_o - F_c$  map shows agreement with the model and no empty electron densities have been observed.

The electron density for Ibuprofen was found to fit both stereoisomer of Ibuprofen as seen in Figure 8. Careful inspection of the model reveals that in the S form the oxygen at the O2 position of the carboxyl group of Ibuprofen formed hydrogen bonds with the hydroxyl group of Tyr 128 as well as with the NE nitrogen and NH2 nitrogen of Arg 126. The other oxygen at the O1 position also interacted with Tyr 128 via water mediated hydrogen bond. This bonding is not seen in the case of fatty acids.

In the R form, the oxygen at the O1 position of the carboxyl group of Ibuprofen formed hydrogen bonds with the hydroxyl group of Tyr 128 as well as the NE nitrogen of Arg 126. The oxygen at the O2 position formed a direct H bond with the NH2 nitrogen of Arg 126. These are the two key interactions for aP2 ligands.

The role of water in the binding site is to stabilize the bound ligand. Four new water molecules, which are shown in blue (Figure 9) were observed. They were found to interact with other water molecules.

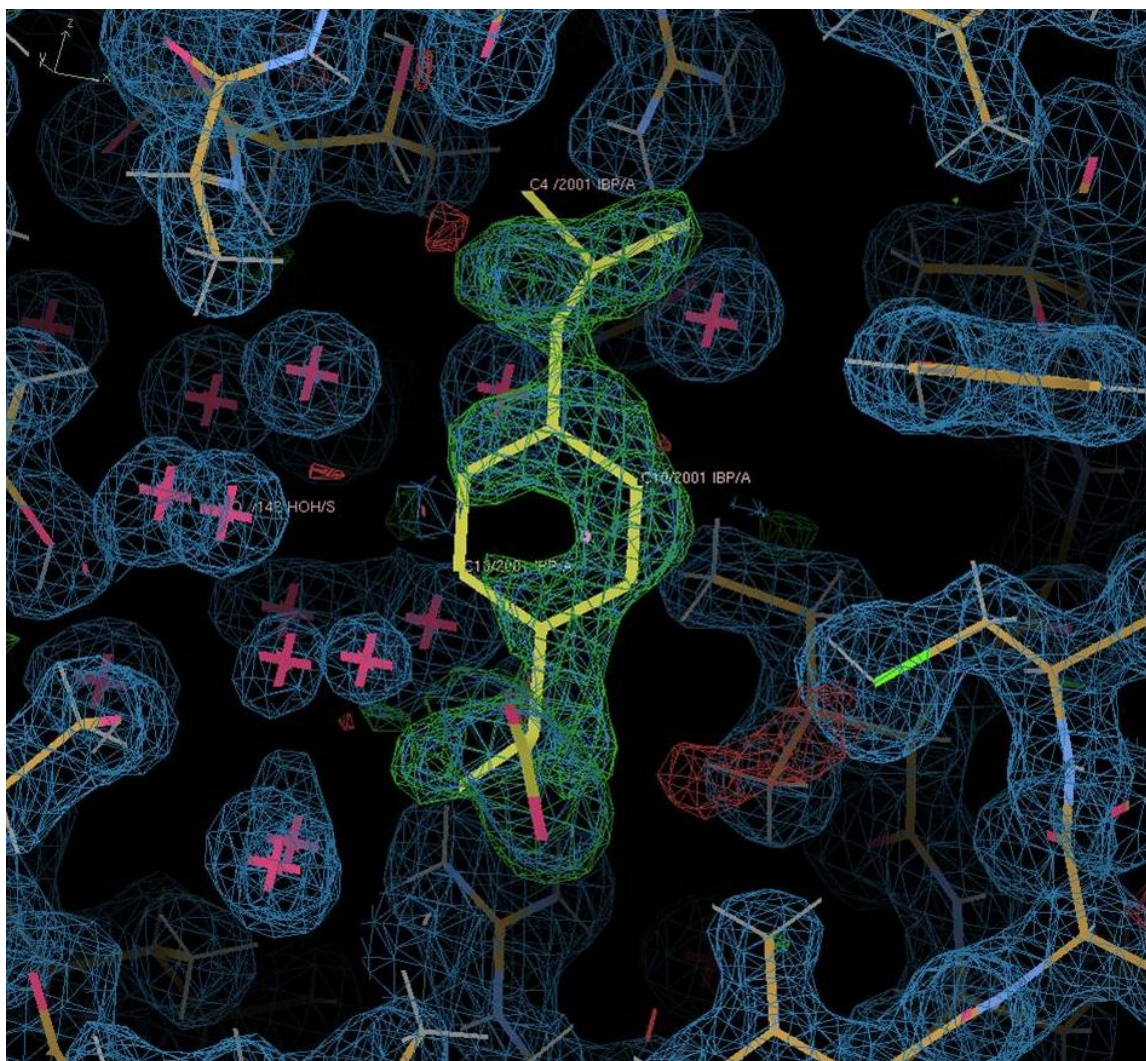


Figure 6

*Partly refined x-ray data. The figure shows the positive density, shown in green, was clearly seen for ibuprofen.*



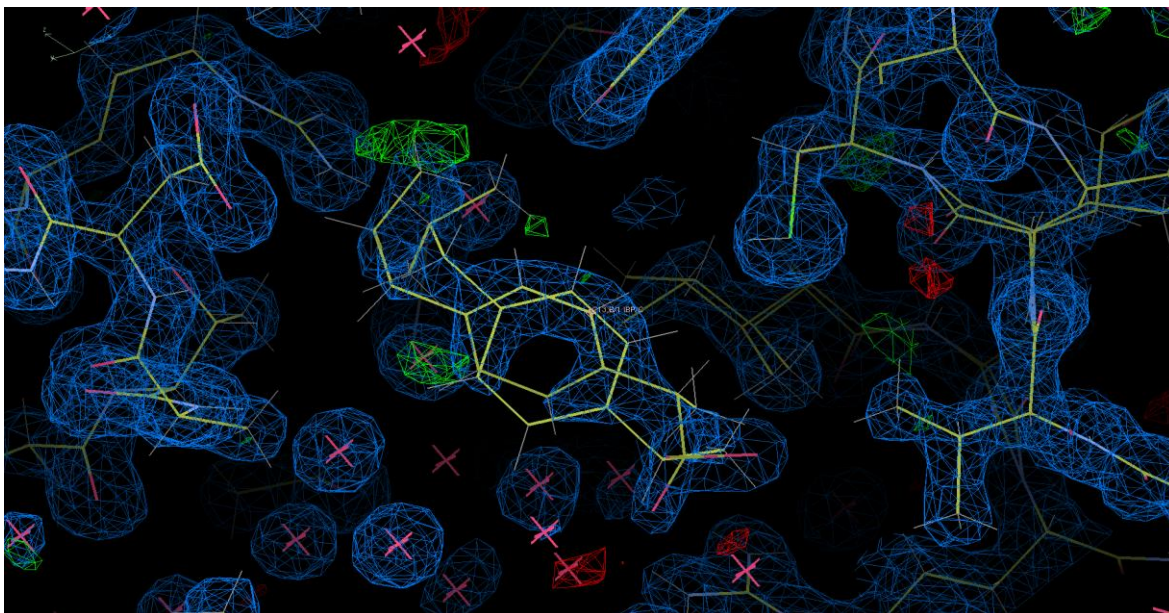
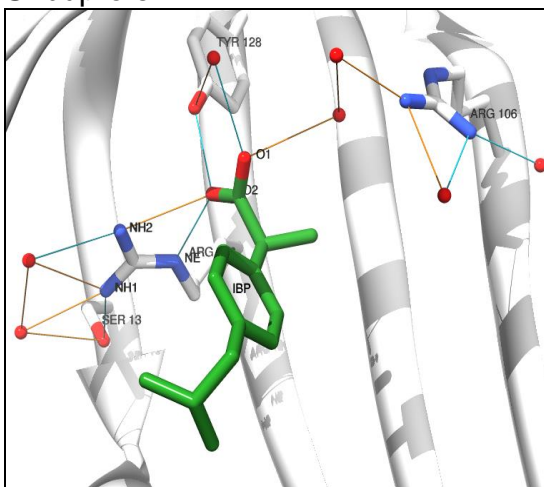


Figure 7

*Electron density map using the final phases showing both stereoisomer of Ibuprofen.*

S Ibuprofen



R Ibuprofen

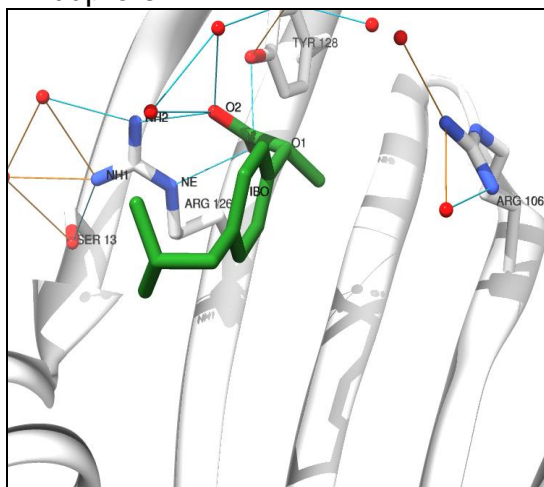


Figure 8

*Interactions between ibuprofen carboxylate group and the protein. A portion of the refined model of aP2 with bound ibuprofen showing hydrogen bonds with the neighboring amino acids indicated by red and blue lines.*

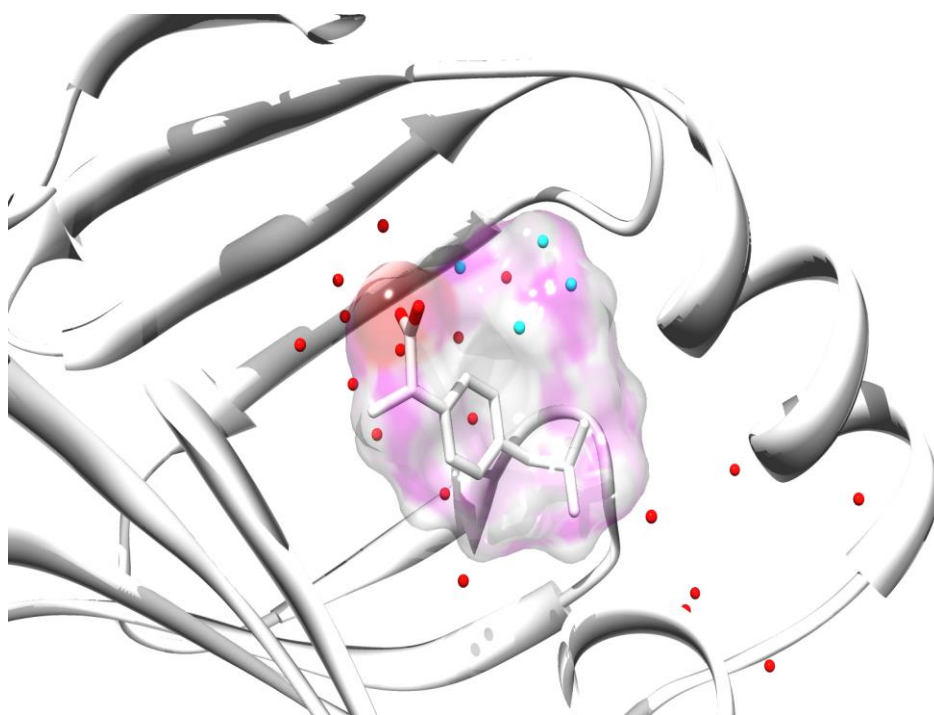


Figure 9

*Ibuprofen with additional water molecules. A space filling model of fatty acid in the background showing ibuprofen displacing the fatty acid. The waters shown by blue color are the water molecules that are added during this process.*

## Chapter 4: Discussion

Fatty acid bound to aP2 protein cannot be compared to an enzyme-substrate complex, where the enzyme-binding site resembles the shape of the substrate. The binding cavity of aP2 is different from the shape of the fatty acid and permits other ligands to bind. Compounds having a lipophilic backbone linked to an acid moiety, usually a carboxylate binds at the active site. Ibuprofen and other NSAIDs, such as indomethacin, share this structural similarity. Ibuprofen has been shown to increase peroxisomal  $\beta$ -oxidation with indomethacin being less active (Foxworthy, Perry, & Eacho, 2003).

The focus of this study was the purification of the aP2 protein and its co-crystallization with Ibuprofen to study its interaction with the amino acids that play a key role in the binding of a ligand. The aP2 protein was expressed in *E. coli* and it was purified using ion exchange chromatography. The overexpression of aP2 protein can be judged by looking at Figure 2. The purification was monitored by SDS-polyacrylamide gel electrophoresis. The aP2 protein was separated from the contaminant proteins and the results are summarized in Figure 3, which shows homogenous aP2 across the 14.4 KDa marker.

Crystallization experiments were successful using macroseeding. The crystals gave diffraction data to 1.4 Å resolution. The statistical details of the combined x-ray data set are recorded in Table 3. The lattice type was orthorhombic and the unit cell dimensions were,  $a = 32.20 \text{ \AA}$ ,  $b = 53.64 \text{ \AA}$ ,  $c = 74.44 \text{ \AA}$ .

To determine the space groups the computer program examined the x-ray data for symmetry and systematic absences, which described the internal symmetry in the unit cell. It observed the reflections with  $h, k$  or  $l = 2n$  along the reciprocal axes. This indicated that the space group was  $P2_12_12_1$ . The data set was 98.4 % complete. Each reflection was shown to be measured 5 times. Redundancy improved accuracy because when the reflection was measured more than once its average was more accurate than the single observation.

The R-work was 0.1557 and R-free value was 0.1984. The R-free was computed with a set of intensities that were not included during refinement. R-work evaluated the whole data set but R-free was a quality control process, which gave the information on how accurate the current model was when it measured the intensities that were not included in the refinement. During refinement the R-free value was higher compared to R-work. R-free value decreased with the R-work when refinement was in process showing agreement between the calculated and the observed data.

Figure 8 shows that the electron density for Ibuprofen was found to fit both stereoisomers of Ibuprofen and in both the forms. The carboxyl group of Ibuprofen formed hydrogen bonds with the hydroxyl group of Tyr 128 as well as the NE nitrogen of Arg 126. The binding of fatty acid within the cavity appears due to the network of hydrogen bond between four species: 1) carboxylate group of the fatty acid, 2) the two guanidine group from Arg 106 and Arg 126, 3) hydroxyl group of Tyr 128, and

4) the intervening water molecule located between the carboxylate group and Arg 106.

Figure 8 clearly shows that the fourth condition was not met in both the S and the R forms. In the S form, the interaction between the carboxylate group and Arg 106 was mediated via two water molecules and in the R form, no interaction was found. This can be the reason that points towards the possibility of ibuprofen being a weak inhibitor of  $\alpha P2$  protein. It was also observed that Ibuprofen did not encounter hydrophobic interaction with Phe 57. The four additional water molecules shown in Figure 9 were found and they may assist in stabilizing ibuprofen at the binding site. Ibuprofen is a non-steroidal anti-inflammatory drug and a cyclooxygenase inhibitor. It has been implicated as a potent PPAR $\gamma$  activator. Cyclooxygenases (COX) regulates the production of eicosanoids and exists in two isoforms COX-1 and COX-2. COX-1 is expressed constitutively in most tissues. It regulates the basal levels of prostaglandins (PGs), because their increased levels trigger inflammation. The expression of COX-2 is induced by inflammatory cytokines and is responsible for high levels of prostaglandins (Linton & Frazio, 2003). Ibuprofen and indomethacin both are nonspecific to COX and higher doses can be harmful to the body with a variety of detrimental side effects like gastrointestinal ulceration.

The information on the influence of NSAIDs on PPARs may be used to design drugs that can minimize the potential for unwanted side effects. The  $\alpha P2$  inhibitor should also be selective versus muscle FABP and epidermal FABP. Epidermal FABP is expressed in various tissues including adipose, macrophage, stomach, heart,

brain, liver, muscle, lung and endothelial cells. The muscle FABP is expressed in heart and skeletal muscle. It has been related to the oxidation of fatty acid in these tissues, which is important for their proper function. A non-selective inhibitor can cause side effects, thus selectivity for mFABP over eFABP is crucial for an aP2 inhibitor (Sulsky et al., 2007).

BMS309403 is a potent aP2 inhibitor that has been designed logically to have selectivity over mFABP and eFABP. It competitively inhibits the interaction of fatty acid with the binding cavity. A fluorescent 1,8-anilino - 8 naphthalene sulfonate (ANS) binding displacement assay was used to study the binding of the inhibitor to aP2, muscle FABP and mal1. The inhibitor constant value of BMS309403 towards aP2 was found to be 2nM. This was much lower compared with 250nM for muscle FABP and 350 nM for mal1. The high binding affinity of BMS309403 and selectivity for aP2 can be attributed to the interaction of the ligand with the key residues i.e. Tyr128, Arg 126 and Arg106.

AP2 inhibition by BMS309403 simulates the effects of the aP2 null mutation and reduces the development of atherosclerotic lesions. These changes may be brought about by effects on macrophage mediated inflammatory pathways given that treatment with the inhibitor decreases the production of various pro-inflammatory cytokines by macrophages as well increases APOA1 mediated cholesterol efflux (decreasing the concentration of cholesterol esters in macrophages).

The inhibitor also produces effects in adipose tissue and elsewhere downregulating adipocyte pro-inflammatory cell signaling pathways and reducing

expression of pro-inflammatory cytokines. Physiological effects include increased blood free fatty acids, reduced triglycerides, reduced adiponectin and increased adipocyte and whole body insulin sensitivity. Unlike the effect of the aP2 null mutation, the inhibitor did not produce a compensatory upregulation of Mal1 expression and reduced fatty infiltration of the liver as compared to the aP2 null mutant. Models like BMS309403 can provide a class of therapeutic agents for diseases such as type-2 diabetes (Furuhashi et al., 2007).

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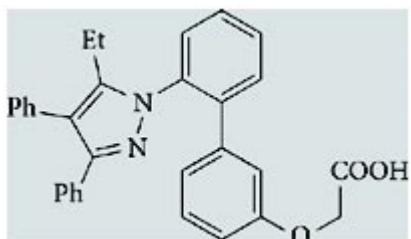
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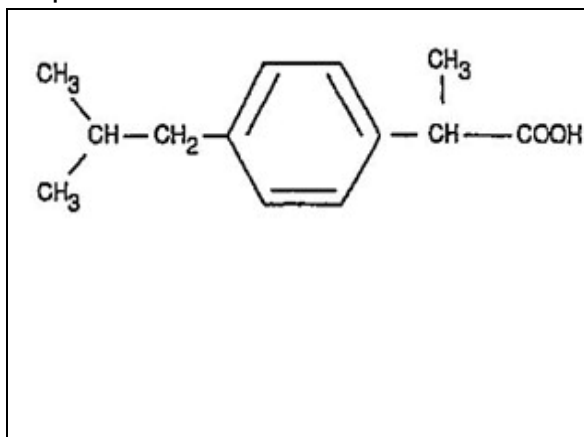
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## Appendix

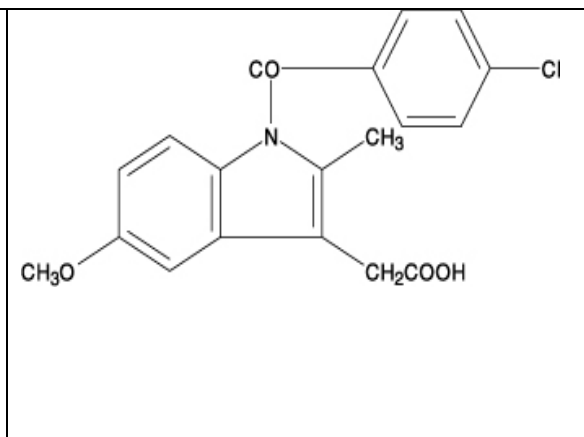


BMS309403

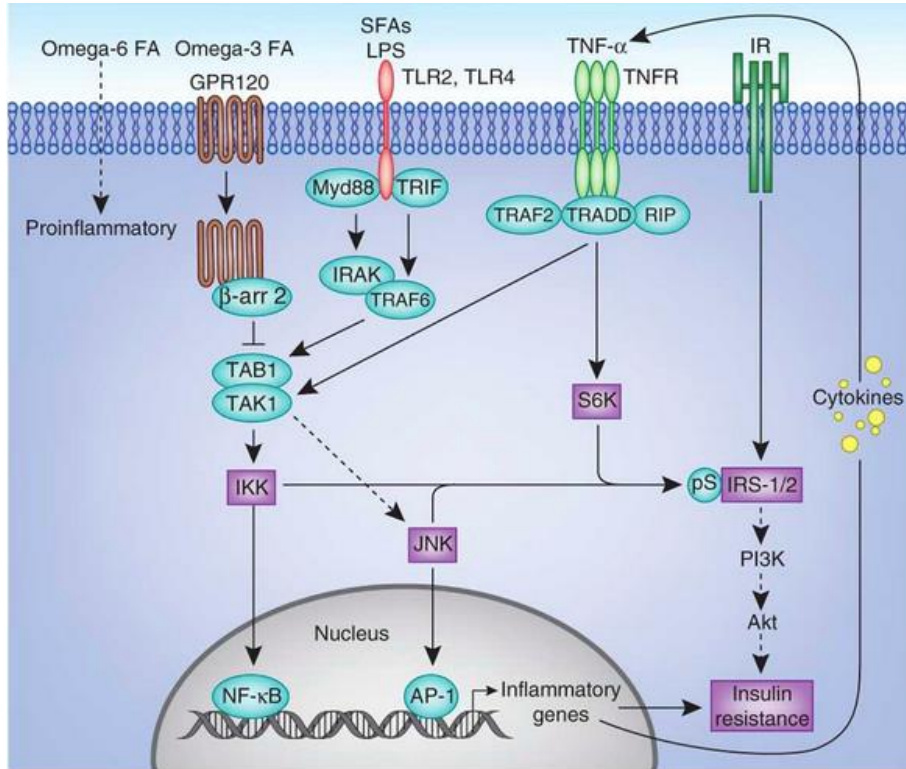
Ibuprofen



Indomethacin



The chemical structure of Ibuprofen, Indomethacin and BMS309403



Olivia Osborn and Jerrold M Olefsky. (2012). The cellular and signaling networks linking the immune system and metabolism in disease. *Nature Medicine* 18, 363-374

*The figure shows inflammatory signaling pathways, which are involved in the development of insulin resistance.*