Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

"BIOCHIMICA, BIOLOGIA MOLECOLARE E BIOTECNOLOGIE"

CICLO XXII

COORDINATORE Prof. BERNARDI FRANCESCO

STRATEGIES FOR ALTERATION OF PRO-INFLAMMATORY GENE EXPRESSION IN CYSTIC FIBROSIS

Settore Scientifico Disciplinare BIO/10

Dottorando
Dott. Mancini Irene

_____
(firma)

Tutore
Prof. Gambari Roberto

_____
(firma)

Anni 2007/2009

INDEX

INTRODUCTION

RESULTS

ABBREVIATIONS

ABC	ATP-binding cassette
ASL	Apical Surface Liquid
ATP	Adenosine triphosphate
BALF	Broncho-alveolar Lavage Fluid
cAMP	Cyclic Adenosine Monophosphat
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CFU	Colony-forming Units
COX	Cyclooxygenase
EMSA	Electrophoresis Mobility Shift Assay
ENaC	Epithelial Na^+ Channel
FBS	Fetal Bovine Serum
GC-FID	Gas Chromatography/Flame Ionization Detection
GC-MS	Gas Chromatography/Mass Spectrometry
GCs	Glucocorticoids
G-CSF	Granulocyte Colony-Stimulating Factor

HPLC	High-pressure Liquid Chromatography
ICAM-1	Intercellular Adhesion Molecule-1
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-6	Interleukin 6
IL-8	Interleukin 8
LPS	lipopolysaccharides
LTR	Long Terminal Repeat
MCP1	Monocyte Chemotactic Protein-1
MSDs	Membrane-Spanning Domains
NBDs	Nucleotide-Binding Domains
NF-kB	Nuclear factor k chain in B cells
NMR	Nuclear Magnetic Resonance
ODN	Oligodeoxynucleotide
PAO-1	*Pseudomonas aeruginosa* strain 1
PCR	Polymerase chain reaction
PE	Phycoerytrin
PGs	Prostaglandins

PKA	cAMP-dependent Protein Kinase
PMN	Polymorphonuclear Neutrophil
RANTES	Regulated on Activation Normal T Expressed and Secreted
RD	Regulatory Domain
RT-PCR	Retro-transcription Polymerase Chain Reaction
TdT	Terminal Deoxynucleotidyl Transferase
TF	Transcription Factor
TFD	Transcription Factor Decoy
TNF-α	Tumor Necrosis Factor-α

INTRODUCTION

1. CYSTIC FIBROSIS

Cystic Fibrosis (CF) is an autosomal recessive disorder that affects approximately one in 2500 births among most Caucasian populations, though its frequency may vary in specific groups [fig.1]. CF is caused by mutations in a single gene on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) which functions as a chloride channel in epithelial membranes.

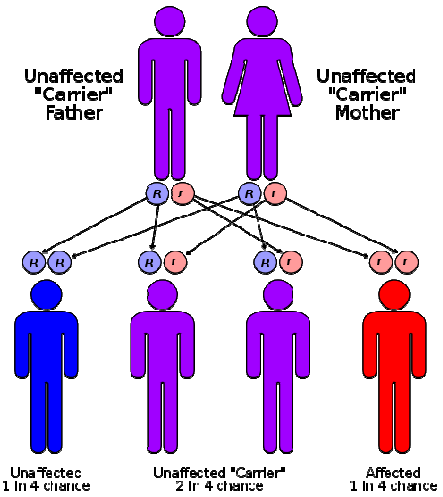


FIGURE 1 - Cystic fibrosis has an autosomal recessive pattern of inheritance.

1.1 HISTORY

Although the entire clinical spectrum of CF was not recognized until the 1930s, certain aspects of CF were identified much earlier. Indeed, literature from Germany and Switzerland in the 1700s warned ‘’Wehe dem Kind, das beim Kuß auf die Stirn salzig schmekt, er ist verhext und muss bald sterbe’’ or "Woe is the child who tastes salty from a kiss on the brow, for he is cursed, and soon must die," recognizing the association between the salt loss in CF and illness [1]. In the 19th century, Carl von Rokitansky described a case of fetal death

with meconium peritonitis, a complication of meconium ileus associated with cystic fibrosis. Meconium ileus was first described in 1905 by Karl Landsteiner [1]. In 1936, Guido Fanconi published a paper describing a connection between celiac disease, cystic fibrosis of the pancreas, and bronchiectasis [2]. In 1938 Dorothy Hansine Andersen published an article, "Cystic Fibrosis of the Pancreas and Its Relation to Celiac Disease: a Clinical and Pathological Study," in the *American Journal of Diseases of Children*. She was the first to describe the characteristic cystic fibrosis of the pancreas and to correlate it with the lung and intestinal disease prominent in CF [3]. She also first hypothesized that CF was a recessive disease and first used pancreatic enzyme replacement to treat affected children. In 1952 Paul di Sant' Agnese discovered abnormalities in sweat electrolytes; a sweat test was developed and improved over the next decade [4].

In 1988 the first mutation for CF, ΔF508 was discovered by Francis Collins, Lap-Chee Tsui and John R. Riordan on the seventh chromosome. Subsequent research has found over 1,000 different mutations that cause CF. Because mutations in the CFTR gene are typically small, classical genetics techniques had been unable to accurately pinpoint the mutated gene [5]. Using protein markers, gene-linkage studies were able to map the mutation to chromosome 7. Chromosome-walking and -jumping techniques were then used to identify and sequence the gene [6]. In 1989 Lap-Chee Tsui led a team of researchers at the Hospital for Sick Children in Toronto that discovered the gene responsible for CF in 1989. Cystic fibrosis represents the first genetic disorder elucidated strictly by the process of reverse genetics.

1.2 CLINICAL ASPECTS

Expression of the cystic fibrosis transmembrane conductance regulatory gene is found in areas that are lined with epithelial tissue. Some of these places include ciliated epithelium of high airways, the gastrointestinal tract, salivary and sweat glands, cervix, uterus, fallopian tubes, epididymis and the vas deferens. These

tissues all show some form of pathological involvement with cystic fibrosis [fig.2].

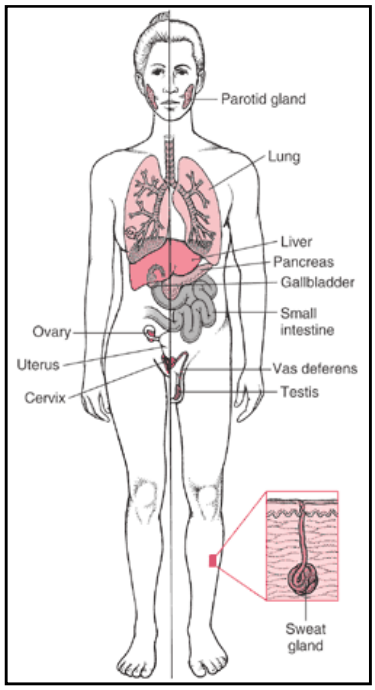


FIGURE 2 - Pathological involvement in cystic fibrosis.

Pulmonary disease: Lung disease is the major cause of morbidity and virtually all mortality: in patients with cystic fibrosis, progression of lung disease is insidious and patients may be relatively asymptomatic before irreversible changes and chronic bacterial colonization occur. The first detectable evidence of lung disease in patients with cystic fibrosis is infection and inflammation in bronchoalveolar lavage fluid (BLF), denoted by elevated counts of interleukin-8 and neutrophils and the presense of microorganisms [7, 8]. Overall, *Pseudomonas aeruginosa (PAO)* is the most common isolate, followed by *Haemophilus influenza* and *Staphylococcus aureus*: chronic colonization with *P. aeruginosa* is associated with a more rapid decline in lung function [9,10].

An exaggerated, sustained and axtended inflammatory response to bacterial and viral pathogens – characterized by neutrophil dominated airway inflammation – is the feature of lung disease in cystic fibrosis. Inflammation is present even in clinically stable patients with some lung disease and in young infants diagnosed by neonatal screening. Quantification of airway inflammation

is necessary to monitor its evolution over time and the effect of anti-inflammatory treatment. This monitoring remains a difficult task, since reliable non invasive markers of airway inflammation are not available.

Gastrointestinal, liver and pancreatic disease: Prior to prenatal and newborn screening, cystic fibrosis was often diagnosed when a newborn infant failed to pass feces (meconium). Meconium may completely block the intestines and cause serious illness. This condition, called meconium ileus, occurs in 10% of newborns with CF [11]. In addition, protrusion of internal rectal membranes (rectal prolapse) is more common in CF because of increased fecal volume, malnutrition, and increased intra–abdominal pressure due to coughing [12]. The thick mucus seen in the lungs has a counterpart in thickened secretions from the pancreas, an organ responsible for providing digestive juices that help break down food. These secretions block the movement of the digestive enzymes into the duodenum and result in irreversible damage to the pancreas, often with painful inflammation (pancreatitis) [13]. The lack of digestive enzymes leads to difficulty absorbing nutrients with their subsequent excretion in the feces, a disorder known as malabsorption. Malabsorption leads to malnutrition and poor growth and development because of caloric loss. Individuals with CF also have difficulties absorbing the fat-soluble vitamins A, D, E, and K. In addition to the pancreas problems, CF patients experience more heartburn, intestinal blockage by intussusception, and constipation [14]. Older CF patients may also develop distal intestinal obstruction syndrome when thickened feces cause intestinal blockage [15]. Thickened secretions also may cause liver problems in patients with CF. Bile secreted by the liver to aid in digestion may block the bile ducts, leading to liver damage. Over time, this can lead to cirrhosis, in which the liver fails to rid the blood of toxins and does not produce important proteins such as those responsible for blood clotting [16-17].

Endocrine disease: the pancreas contains the islets of Langerhans, which are responsible for making insulin, a hormone that helps regulate blood glucose. Damage of the pancreas can lead to loss of the isletcells, leading to a type of diabetes that is unique to those with the disease [18]. This Cystic Fibrosis

Related Diabetes (CFRD) shares characteristics that can be found in Type 1 and Type 2 diabetics and is one of the principal non-pulmonary complications of CF [19].

Osteoporosis: Vitamin D is involved in calcium and phosphorus regulation. Poor uptake of vitamin D from the diet because of malabsorption can lead to the bone disease osteoporosis in which weakened bones are more susceptible to fractures [20]. In addition, CF patients often develop clubbing of their fingers and toes due to the effects of chronic illness and low oxygen in their tissues.

Infertility: Infertility affects both men and women. At least 97 percent of men with cystic fibrosis are infertile but are not sterile and can have children with assisted reproductive techniques [21]. These men make normal sperm but are missing the tube (vas deferens), which connects the testes to the ejaculatory ducts of the penis [22]. Many men found to have congenital absence of the vas deferens during evaluation for infertility have a mild, previously undiagnosed form of CF [23]. Some women have fertility difficulties due to thickened cervical mucus or malnutrition. In severe cases, malnutrition disrupts ovulation and causes amenorrhea [24].

1.3 DIAGNOSIS AND MONITORING

Cystic fibrosis may be diagnosed by many different categories of testing including those such as, newborn screening, sweat testing, or genetic testing. The newborn screen initially measures for raised blood concentration of immunoreactive trypsinogen [25]. Infants with an abnormal newborn screen need a sweat test in order to confirm the CF diagnosis. Trypsinogen levels can be increased in individuals who have a single mutated copy of the CFTR gene (carriers) or, in rare instances, even in individuals with two normal copies of the CFTR gene. Due to these false positives, CF screening in newborns is somewhat controversial [26-27]. Therefore, most individuals are diagnosed after symptoms prompt an evaluation for cystic fibrosis. The most commonly used

form of testing is the sweat test. Sweat-testing involves application of a medication that stimulates sweating (pilocarpine) to one electrode of an apparatus and running electric current to a separate electrode on the skin. This process, called iontophoresis, causes sweating; the sweat is then collected on filter paper or in a capillary tube and analyzed for abnormal amounts of sodium and chloride. CF patients have increased amounts of sodium and chloride in their sweat [fig.3]. CF can also be diagnosed by identification of mutations in the CFTR gene [28].

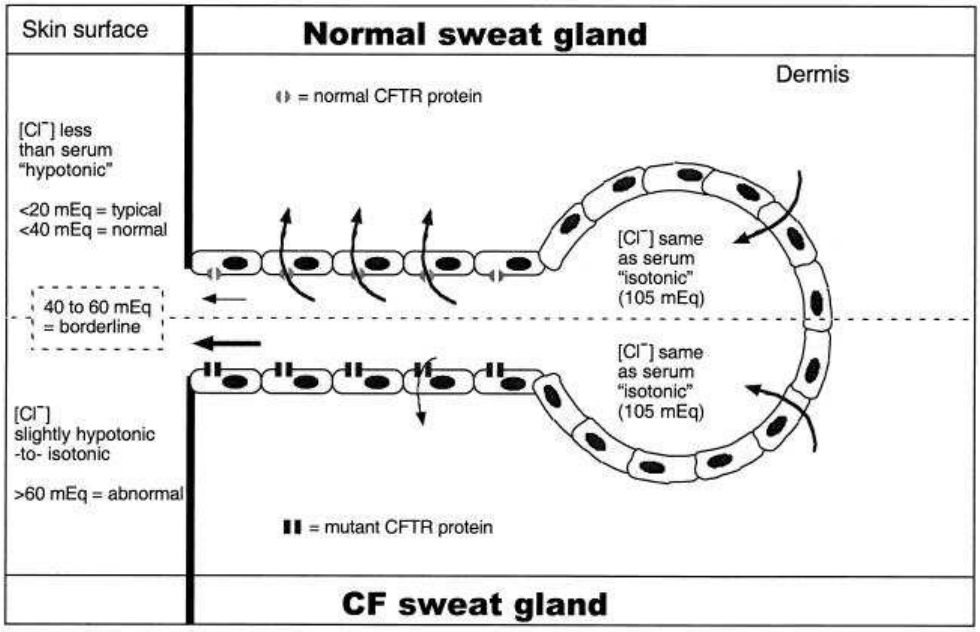


FIGURE 3 - Diagram of a sweat gland, showing paths taken by chloride ions (arrows) during secretion. In both normal and CF sweat glands in the dermis, chloride is present in secretions at a concentration of 105 mEq, equaling that in serum ("isotonic"). (Top) In the normal sweat gland, chloride is absorbed out of the sweat in a CFTR-dependent manner as the sweat travels from the gland to the skin surface. As a result, the chloride concentration in normal sweat is below that in serum ("hypotonic"), with <40 mEq considered normal and <20 mEq being typical. (Bottom) In the CF sweat gland, chloride absorption is hindered by defective CFTR function. As a result, sweat which reaches the skin surface has higher than normal chloride concentrations (>60 mEq).

A multitude of tests are used to identify complications of CF and to monitor disease progression. X-rays and CAT scans are used to examine the lungs for

signs of damage or infection. The examination of the sputum is required to isolate organisms which may be causing an infection or colonising the lower respiratory tract so that effective antimicrobial therapy can be provided. Culture for organisms such as *Burkholderia cepacia* (previously *Pseudomonas*) is required for candidates of lung transplantation as persisant bacterial colonisation reduces the chances of survival.

Pulmonary function tests measure how well the lungs are functioning, and are used to measure the need for and response to antibiotic therapy. Blood tests can identify liver abnormalities, vitamin deficiencies, and the onset of diabetes. DEXA scans can screen for osteoporosis and testing for fecal elastase can help diagnose insufficient digestive enzymes.

1.4 PRENATAL DIAGNOSIS

Couples who are pregnant or who are planning a pregnancy can themselves be tested for CFTR gene mutations to determine the degree of risk that their child will be born with cystic fibrosis. Testing is typically performed first on one or both parents and, if the risk of CF is found to be high, testing on the fetus can then be performed. Because development of CF in the fetus requires each parent to pass on a mutated copy of the CFTR gene and because CF testing is expensive, testing is often performed on just one parent initially. If that parent is found to be a carrier of a CFTR gene mutation, the other parent is then tested to calculate the risk that their children will have CF. CF can result from more than a thousand different mutations and it is not possible to test for each one. Testing analyzes the blood for the most common mutations such as ΔF508 - most commercially available tests look for 32 or fewer different mutations. If a family has a known uncommon mutation, specific screening for that mutation can be performed. Because not all known mutations are found on current tests, a negative screen does not guarantee that a child will not have CF [29]. In addition, because the mutations tested are necessarily those most common in the highest risk groups, testing in lower risk ethnicities is less successful because the mutations commonly seen in these groups are less common in the

general population. Couples who are at high risk for having a child with CF will often opt to perform further testing before or during pregnancy. In vitro fertilization with preimplantation genetic diagnosis offers the possibility to examine the embryo prior to its placement into the uterus. The test, performed three days after fertilization, looks for the presence of abnormal CF genes. If two mutated CFTR genes are identified, the embryo is not used for embryo transfer and an embryo with at least one normal gene is implanted. During pregnancy, testing can be performed on the placenta (chorionic villus sampling) or the fluid around the fetus (amniocentesis).

1.5 THERAPY

Although no definite cure has been discovered for cystic fibrosis as of yet, many treatments have been developed to improve the quality of life for those who suffer from this disorder. Since CF causes a build-up of mucus in the lung tissue, the respiratory diseases which result are often the actual cause of death, not CF itself.

1.5.1 ANTI-INFLAMMATORY THERAPY

Inflammation plays a major role in the pathophysiology of lung disease in CF. This respose is probably triggered primarily as a reaction to the inability of the affected lung to resist the invasion of the most common bacterial pathogens seen in this disease.

Anti-inflammatoty therapy can be provided in various forms: these include the use of oral corticosteroids which are potentially highly effective but which carry with them the risk of long-term systemic side effects.

Inhaled corticosteroids also have considerable potential because of their local action within the lung. Their potential therapeutic disadvantage is the difficulty of penetrating the viscid mucus which lines the airway in CF patients particularly as the disease progresses.

Other anti-inflammatory agents such as ibuprofen have considerable potential and have been the subject of various studies over the years.

More recently macrolides have come forward as potent anti-inflammatory agents and are beginning to have an established place in the therapeutic regimen for patients with long-term P*seudomonas aeruginosa* infection. Other agents which have been used include immunoglobulins and dornase alpha (DNase) that may have an anti-inflammatory role as well as being mucolytic.

1.5.2 CHEMOTERAPY

Many CF patients are on one or more antibiotic at all times, even when they are considered healthy, in order to prophylacticly suppress infection. Antibiotics are absolutely necessary whenever pneumonia is suspected or a lung function decline has been noticeable, and are usually chosen based on the results of a sputum analysis and the patient's past response. Many bacteria common in cystic fibrosis are resistant to multiple antibiotics and require weeks of treatment with Intravenous antibiotics such as vancomycin, tobramycin, meropenem, ciprofloxacin and piperacillin. This prolonged therapy often necessitates hospitalization and insertion of a more permanent IV such as a peripherally inserted central catheter (PICC) line or Port-a-Cath. Inhaled therapy with antibiotics such as tobramycin and colistin is often given for months at a time in order to improve lung function by impeding the growth of colonized bacteria [30, 31]. Inhaled antibiotic therapy with aztreonam is also being developed and clinical trials are underway [32]. Oral antibiotics such as ciprofloxacin or azithromycin are given to help prevent infection or to control ongoing infection [33]. Several of the antibiotics commonly used to treat CF patients, such as tobramycin and vancomycin, can cause hearing loss, damage to the balance system in the inner ear or kidney problems with long-term use. In order to prevent these side effects, the amount of antibiotics in the blood are routinely measured and adjusted accordingly.

1.5.3 OTHER METHODS TO TREAT LUNG DISEASE

Several mechanical techniques are used to dislodge sputum and encourage its expectoration. In the hospital setting, chest physiotherapy (CPT) is utilized; a respiratory therapist percusses an individual's chest with his or her hands

several times a day, to loosen up secretions. Devices that recreate this percussive therapy include the ThAIRapy Vest and the intra-pulmonary percussive ventilator (IPV).

Newer methods such as biphasic cuirass ventilation (BCV), and associated clearance mode available in such devices, integrate a cough assistance phase, as well as a vibration phase for dislodging secretions. Biphasic Cuirass Ventilation is also shown to provide a bridge to transplantation.These are portable and adapted for home use [34].

Physiotherapy is essential to help manage an individual's chest on a long term basis, and can also teach techniques for the older child and teenager to manage themselves at home. Aerobic exercise is of great benefit to people with cystic fibrosis. Not only does exercise increase sputum clearance but it also improves cardiovascular and overall health.Aerosolized medications that help loosen secretions include dornase alfa and hypertonic saline [35]. Dornase is a recombinant human deoxyribonuclease, which breaks down DNA in the sputum, thus decreasing its viscosity [36]. N-Acetylcysteine may also decrease sputum viscosity, but research and experience have shown its benefits to be minimal. Albuterol and ipratropium bromide are inhaled to increase the size of the small airways by relaxing the surrounding muscles.

As lung disease worsens, mechanical breathing support may become necessary. Individuals with CF may need to wear special masks at night that help push air into their lungs. These machines, known as bilevel positive airway pressure (BiPAP) ventilators, help prevent low blood oxygen levels during sleep. BiPAP may also be used during physical therapy to improve sputum clearance [37]. During severe illness, a tube may be placed in the throat (a procedure known as a tracheostomy) to enable breathing supported by a ventilator.

1.5.4 TREATMENT OF OTHER ASPECTS

Newborns with meconium ileus (bowel obstruction) typically require surgery, whereas adults with distal intestinal obstruction syndrome typically do not. Treatment of pancreatic insufficiency by replacement of missing digestive

enzymes allows the duodenum to properly absorb nutrients and vitamins that would otherwise be lost in the feces. Even so, most individuals with CF are advised take additional amounts of vitamins A, D, E, and K and eat high-calorie meals. So far, no large-scale research involving the incidence of atherosclerosis and coronary heart disease in adults with cystic fibrosis has been conducted. This is likely due to the fact that the vast majority of people with cystic fibrosis do not live long enough to develop clinically significant atherosclerosis or coronary heart disease.

Diabetes is the most common non-pulmonary complication of CF. It mixes features of type 1 and type 2 diabetes, and is recognized as a distinct entity, cystic fibrosis-related diabetes (CFRD) [38,39]. While oral anti-diabetic drugs are sometimes used, the only recommended treatment is the use of insulin injections or an insulin pump [40], and, unlike in type 1 and 2 diabetes, dietary restrictions are not recommended [38].

Development of osteoporosis can be prevented by increased intake of vitamin D and calcium, and can be treated by bisphosphonates, although adverse effects can be an issue [41]. Poor growth may be avoided by insertion of a feeding tube for increasing calories through supplemental feeds or by administration of injected growth hormone [42].

Sinus infections are treated by prolonged courses of antibiotics. The development of nasal polyps or other chronic changes within the nasal passages may severely limit airflow through the nose, and over time reduce the patient's sense of smell. Sinus surgery is often used to alleviate nasal obstruction and to limit further infections. Nasal steroids such as fluticasone are used to decrease nasal inflammation [43]. Female infertility may be overcome by assisted reproduction technology, particularly embryo transfer techniques. Male infertility may be overcome with intracytoplasmic sperm injection [44]. Third party reproduction is also a possibility for women with CF.

1.5.5 GENE THERAPY

Cystic fibrosis should be an ideal candidate for gene therapy, for four main reasons: (1) it is a single gene defect; (2) it is a recessive condition, with

heterozygotes being phenotypically normal (suggesting gene dosage effects are not critical); (3) the main pathology is in the lung, which is accessible for treatment; and (4) it is a progressive disease with a virtually normal phenotype at birth, offering a therapeutic window.

It has been suggested that only 5–10% of normal CFTR function is required to reverse the chloride channel defect [45], although it is not clear whether this has to be achieved in the majority of the airway epithelial cells, or whether a minority of cells expressing much higher levels would suffice. In clinical trials to date, two main vector systems have been harnessed to deliver the CFTR cDNA with appropriate promoter into host cells [46,47]. First, viral vectors with the CFTR cDNA incorporated into the viral genome exploit the efficiency of viruses to enter host cells and achieve relatively high levels of gene expression. Secondly, cationic liposomes mixed with plasmid DNA encoding CFTR enhance the transport of the DNA into host cells. Although cationic liposomes seem to generate a lower immune response than current viral vector systems, the levels of CFTR expression using this delivery system have been relatively poor.

The ideal vector system would have the following characteristics: (1) an adequate carrying capacity; (2) to be undetectable by the immune system; (3) to be non-inflammatory; (4) to be safe to the patients with pre-existing lung inflammation; (5) to have an efficiency sufficient to correct the cystic fibrosis phenotype; and (6) to have long duration of expression and/or the ability to be safely re-administered.

In-vivo gene therapy trials in patients with cystic fibrosis have been done with viral vectors and cationic lipids, [48, 49] however, long-term effects were not achieved. Repeat administration of adenovirus vectors reduces efficacy of transfection because of formation of specific antibodies, whereas lipids might not specifically target CFTR-expressing cells. Therefore, although much progress has been made in gene therapy, it is presently not a treatment option for patients with cystic fibrosis.

1.5.6 STEM CELLS

CF is a potential model disease for stem cell therapy because of the continuing lung inflammation and infection leading to damage, which could promote engraftment of stem cells. It is likely that infants and children with CF would be the best candidates for this sort of therapy, when they have faster cell turnover and before they have established lung damage: the ultimate therapy will be gene therapy to CF babies by utilizing their own stored umbilical cord blood stem cells [50].

Nevertheless, experiments to elucidate possible lung stem cell candidates are difficult because of the slow turnover of such cells, and therefore most animal studies use an injury model which promotes cell turnover. Moreover, basic questions remain unanswered. It is unknown which cell type should be targeted; current topical therapy targets the abundant surface epithelium, but it is the submucosal glands which expresses the highest CFTR in the lung.

1.5.7 LUNG TRANSPLANTATION

Double lung or heart-lung transplantation is a treatment option for patients with cystic fibrosis and end-stage lung disease. Overall survival of lung-transplant patients is poorer than for other organ transplantation, with 3-year survival of about 60% in patients with cystic fibrosis [51]. Generally, survival is better for adults than for children [52], but some centres have reported a survival benefit in children [53].

2. CFTR: CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

2.1 CFTR PROTEIN

Cystic fibrosis transmembrane conductance regulator (CFTR) is a phosphorylation-dependent epithelial Cl^- channel. It is located primarily in the apical membrane, where it provides a pathway for Cl^- movement across epithelia and regulates the rate of Cl^- flow. Thus CFTR is central in determining transepithelial salt transport, fluid flow, and ion concentrations. In the intestine, pancreas, and sweat gland secretory coil, CFTR plays a key role in fluid and electrolyte secretion, and in sweat gland duct and airway epithelia, it participates in fluid and electrolyte absorption.

CFTR can also regulate other membrane proteins [54]. The most thoroughly documented regulatory role for CFTR is its negative regulation of the amiloride-sensitive epithelial Na^+ channel (ENaC). CFTR decreases ENaC's open probability (Po) and reverses its usual increases to elevations of $[cAMP]_i$ [55]. When CFTR function is lost, the Na^+ conductance is markedly increased in CF human airways [56] and CF mouse nasal epithelia [57], but apparently not in human sweat ducts [58]. CFTR's channel and regulatory [59] functions require that it has to be phosphorylated by various kinases, especially cAMP-dependent protein kinase (PKA).

To summarize, CFTR is an anion channel and a channel regulator that plays multiple roles in epithelial transport. Although other functions have been proposed for CFTR, most present hypotheses of CF disease emphasize CFTR's roles in ion transport [fig.4].

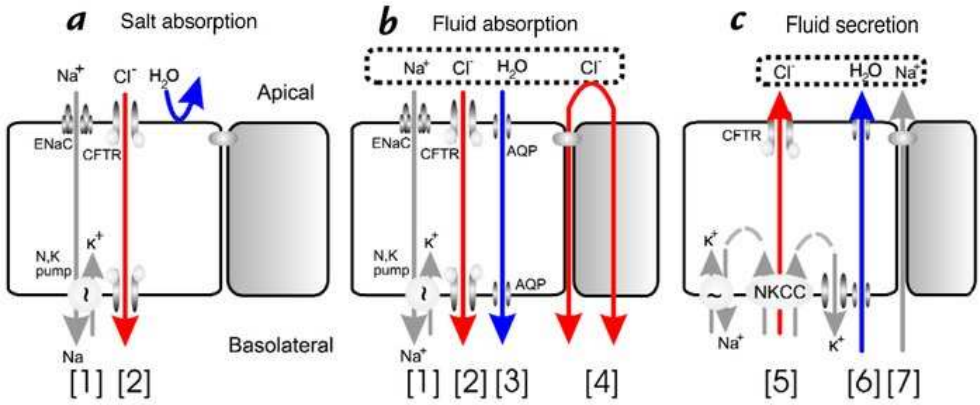
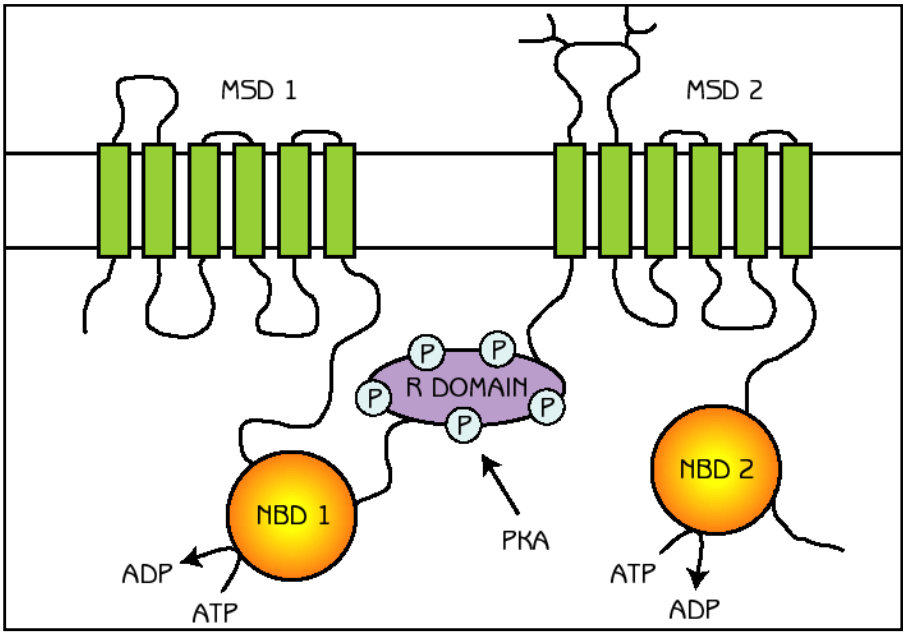


FIGURE 4 - CFTR's multiple roles in fluid and electrolyte transport. ***(a)*** Salt absorption. In the sweat duct, high apical conductance for Na^+ [1] and Cl^- [2] and relatively low water conductance allows salt to be reabsorbed in excess of water (hypertonic absorption) leaving a hypotonic luminal fluid. In the sweat duct CFTR is the only available anion conductance pathway, and when it is lost in CF the lumen quickly becomes highly electronegative and transport virtually ceases, resulting in high (similar to plasma) luminal salt. ***(b)*** Fluid absorption. In epithelia with high water permeability [3] relative to electrolyte permeability water will absorbed osmotically with salt to decrease the volume of luminal fluid. If no other osmolytes or forces are present, the salt concentration will remain unchanged. If water-retaining forces are present, permeant electrolytes can be reduced preferentially. The consequences of eliminating CFTR depend on the magnitude of such forces, the relative magnitude of alternate pathways for transepithelial anion flow [4], and how CFTR affects other ion channels. The high salt and low volume hypotheses differ on each of these points. ***(c)*** Anion-mediated fluid secretion. Secreting epithelia lack a significant apical Na^+ conductance. Basolateral transporters such as NKCC move Cl^- uphill into the cell; it then flows passively into the lumen via CFTR [5], K^+ exits basolaterally, Na^+ flows paracellularly [7] and water follows transcellularly [6]. Elimination of CFTR eliminates secretion [60].

CFTR is a member of the ATP-binding cassette (ABC) transporter family, and it contains the features characteristic of this family: two nucleotide binding domains (NBDs) and two six-membrane-spanning domains (MSDs) [59] [fig.5]. Moreover CFTR also contains the R domain (RD), a unique sequence not found in other ABC transporters or any other proteins. The R domain serves as the major physiologic regulator of the CFTR Cl^- channel. When cAMP level increase, cAMP-dependent protein kinase (PKA) phosphorylates the multiple serine residues within the R domain allowing ATP to open the channel.

PKA is the primary kinase that phosphorylates CFTR, although protein kinase C, cGMP-dependent protein kinase, and tyrosine phosphorylation can also stimulate channel activity [61].



FUGURE 5 - Model showing proposed domain structure of cystic fibrosis transmembrane conductance regulator (CFTR). MSD, membrane-spanning domain; NBD, nucleotide-binding domain; R, regulatory domain; PKA, cAMP-dependent protein kinase.

Once the R domain is phosphorylated, channel gating is regulated by a cycle of ATP hydrolysis at the NBDs. Finally, protein phosphatases dephosphorylate the R domain and return the channel to its quiescent state. At the moment, how phosphorylation activates the channel is not well understood. Some models propose that the R domain prevents the channel from opening and that phosphorylation relieves this inhibition. Other models suggest that phosphorylation of the R domain stimulates activity.

These channels have several distinguishing characteristics:

- They have a small single-channel conductance (6–10 pS).
- The current-voltage (*I*-V) relationship is linear.
- They are selective for anions over cations.
- The anion permeability sequence is $Br^- \geq Cl^- > I^-$.
- They show time- and voltage-independent gating behavior.

- Their activity is regulated by cAMP-dependent phosphorylation and by intracellular nucleotides.

These features are conferred on CFTR Cl^- channels by the function of the MSDs, the NBDs, and the R domain.

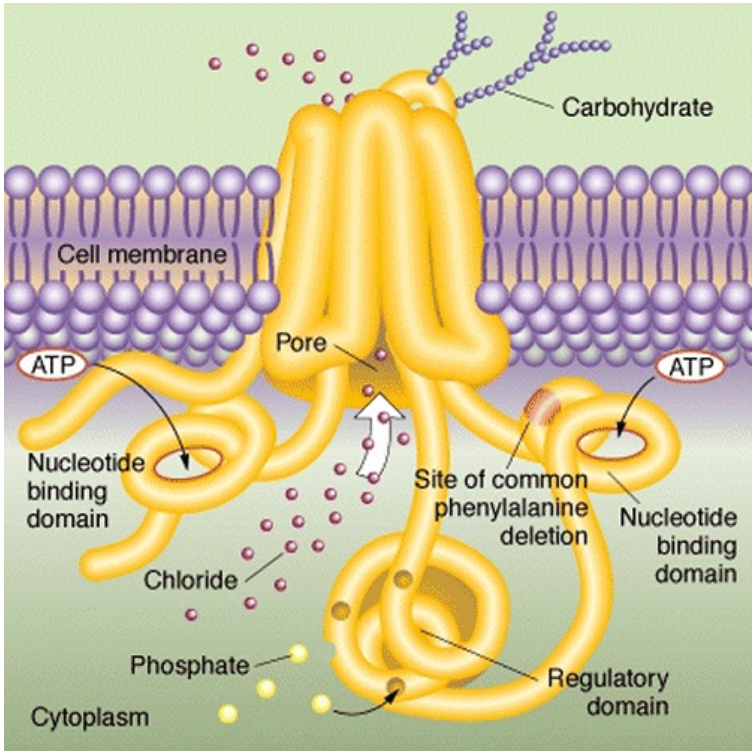


FIGURE 6 - CFTR structure.

In the proposed transmembrane topology [fig.6], which is supported by experimental evidence [62-64], 77% of the protein is in the cytoplasm, 19% in membrane spanning segments, and 4% in extracellular loops.

The minimum channel diameter was inferred to be 5.3 Å based on the size of the largest permeant anion [65]. Further studies using patches with larger numbers of channels showed that anions as large as lactobionate (10–13 Å in diameter) were slightly permeable. Thus, at least transiently, the diameter of the channel must be 10–13 Å [66]. In the presence of cytoplasmic ATP the large anions were only permeable from the cytoplasmic side [66]. The molecular basis for asymmetric permeation by large anions is unknown.

2.1.1 FROM CFTR TO LUNG DISEASE

The vexing problem of explaining CF lung disease using known properties of CFTR has recently been energized by two innovative but sharply different hypotheses [fig.7]. Interestingly, both hypotheses consider CFTR's role in salt absorption to be primary.

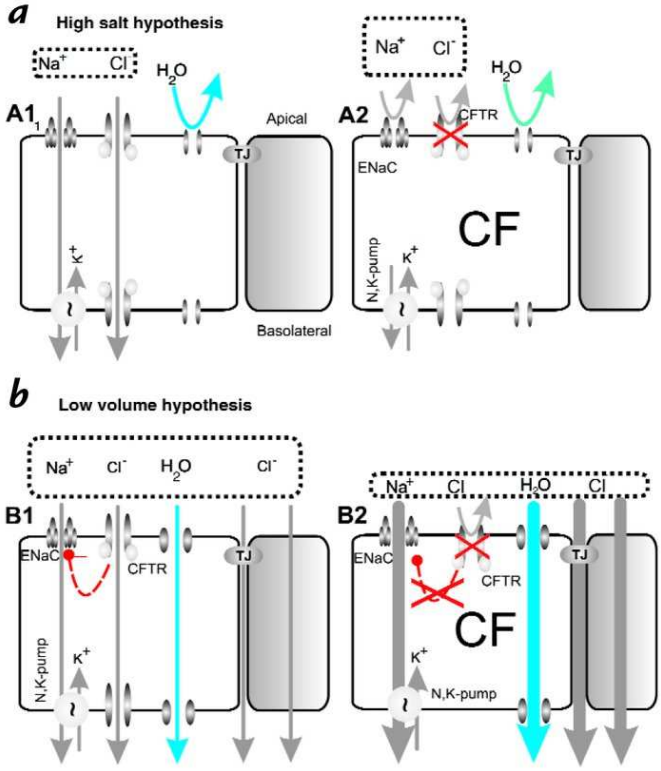


FIGURE 7 - Two hypotheses of how airway surface liquid (ASL) differs in healthy and CF lungs. ***(a)*** The high salt hypothesis [67,68] postulates that normal ASL has low levels of salt as a result of salt absorption in excess of water (*A1, left*). Even though the epithelium is water permeable, salt is retained in thin surface films by some combination of surface tension [69] and impermeant osmolytes [68]. In CF (*A2 right*), salt is poorly absorbed resulting in excessively salty ASL that disrupts natural mucosal antibiotics. Key features of the high salt model are: the lack of an appreciable shunt Cl^- conductance, central importance of CFTR's channel role, no specific role for inhibition of ENaC by CFTR, and a switch from isotonic volume absorption to hypertonic salt absorption as the surface layer thins and traps residual water. ***(b)*** The low volume hypothesis [73] postulates that normal ASL (*B1 left*) has salt levels approximately equal to plasma. In CF (*B2 right*), the removal of CFTR's inhibition of ENaC results in abnormally elevated isotonic fluid absorption which depletes the ASL and leads to reduced mucociliary clearance. Key features of the low volume model are the parallel pathway for Cl^-via shunt pathway(s) and inhibition of ENaC via CFTR.

The high salt hypothesis [67-68] emphasizes CFTR's function as an anion channel. According to this hypothesis, missing or defective CFTR causes reduced transepithelial Cl^- conductance, and by analogy with the sweat duct [70-71] this allows salt levels in the apical surface liquid (ASL) to remain at levels similar to those in plasma [fig.7]. The high salt in the ASL interferes with natural antibiotics such as defensins [72] and lysozyme.

In marked contrast, the low volume hypothesis [73] is based on CFTR's function as a regulator of other channels - in this case ENaC. According to this hypothesis, both normal and CF ASL have plasma-like levels of salt. CFTR mutations eliminate CFTR's inhibition of ENaC, and because there are significant shunt pathways for Cl^- in the airways, increased Na^+ transport drives increased absorption of Cl^- and water. Thus CF airways display accelerated isotonic fluid absorption that depletes ASL volume and dehydrates mucus, leading to obstruction and infection [74].

2.2 CFTR GENE

The gene responsible for CFTR was identified to reside on the long arm of chromosome 7 (7q.31.2) and subsequently isolated by positional cloning in 1989 [3-5,75] [fig.8].

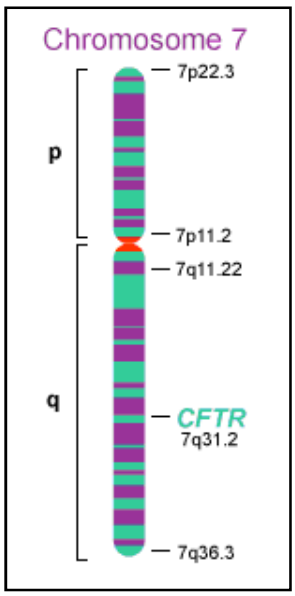


FIGURE 8 - The location of the CFTR gene on chromosome 7.

Availability of the gene sequence and direct mutation analysis were turning points in the history of CF and opened a new chapter of molecular and cellular studies in CF research.

The gene, named the CF transmembrane conductance regulator (CFTR), consists of a TATA-less promoter and 27 exons spanning about 215 kb of genomic sequence [fig.9-a] [76]. The CFTR gene encodes a 170 kDa (1480 amino acids) transmembrane protein with a symmetrical, multi-domain structure, consisting of two membrane-spanning domains (MSD1, MSD2), two nucleotide-binding domains (NBD1, NBD2) and a central, highly charged regulatory domain (RD) with multiple phosphorylation consensus sites [fig.9b-c] [5]. The principal function of CFTR is that of cAMP-regulated chloride transport at the apical membranes of epithelial cells but has also been implicated in many other processes such as regulation of other ion channels, membrane trafficking, pH regulation and apoptosis.

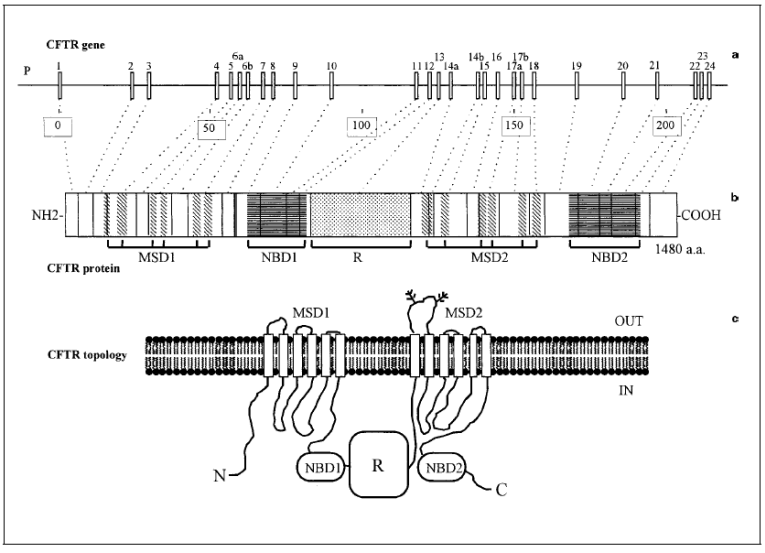


FIGURE 9 - Schematic diagram of the CFTR gene. **(a)** Structure of the CFTR gene consisting of promoter region (P) and 27 exons. The size of the introns is based on the sequences of genomic BAC clones 068P20 and 133K23 (submitted to GenBank by Washington University). **(b)** CFTR polypeptide with predicted domains (highlighted). **(c)** Topology of the CFTR protein relative to the cytoplasmic membrane and position of the most common mutation, ΔF508. MSD1 and MSD2 = Membrane spanning domains 1 and 2; NBD1 and NBD2 = nucleotide-binding domains 1 and 2; R = regulatory domain. Box: Deletion of 3 nucleotides, CTT (underlined), and subsequent loss of phenylalanine (underlined) at position 508 of the CFTR protein.

2.3 CFTR MUTATIONS

The first, and as it turned out, the most common CFTR defect identified among Caucasians was the ΔF508 mutation [fig.11], a 3-bp deletion in exon 10 causing a loss of phenylalanine at the amino acid position 508 of the protein product [fig. 9c][75]. Presently, 10 years after the discovery of the CFTR gene, more than 850 different alleles have been reported as proven or putative disease causing mutations (CFGAC website: http://www.genet.sickkids.on.ca/cftr/).

Feature	*How may it affect a phenotype?*
A - Type of mutation	Mutations that are predicted to prevent CFTR biosynthesis or produce grossly changed, unstable or nonfunctional proteins tend to have severe phenotypic consequences (type: nonsense, frameshift, splice, large in-frame deletion or insertion); effects of mutations that cause minor, local changes in the protein may range from mild to severe depending on other factors such as its molecular mechanism, amino acid change or location (type: missense, small inframe deletion or insertion)
B – Molecular mechanism	Immediate consequence of the mutation for CFTR biosynthesis, processing, trafficking to the membrane, stability and function (classes of mutations); the mechanisms of mutations are evaluated empirically on the molecular and cellular levels
C - Position in the gene (protein)	Applies particularly to minor, local changes (missense); mutations in structurally or functionally critical regions of the protein tend to correlate with more severe phenotypes when compared with mutations in less important regions; amino acid substitutions in highly conserved regions of CFTR protein may also have more severe phenotypic consequences than mutations from unconserved regions
D - Net molecular effect	The net amount of the functional CFTR present in the apical membrane of secretory epithelial cells, irrespective of type mutation or its mechanism; production of even a small amount of functional CFTR may be sufficient to prevent a severe disease (class IV and V mutations)
E - Intragenic modulators (complex alleles)	In some cases, a second change in the same allele may modulate the phenotypic effect of the primary mutation; for example, the missense mutation R117H associated with the 5T variant in the T-tract of the acceptor splice site of intron 8 is typically associated with the pancreatic sufficient form of CF but only with infertility (males) or asymptomatic presentation (females) when on the 7T background
F - Impact of second mutation	As a recessive disease, CF requires the presence of mutations in each allele; therefore, the type and molecular consequences of the second mutation in the genotype may be critical for the clinical outcome; for example, a severe allele is associated with PI only if the second mutation is severe; conversely, one mild mutation is sufficient to preserve pancreatic function, irrespective of the type of the second allele
G - Site of expression/organ pathophysiology/ secondary modulation	A phenotypic impact of a mutation also depends on where the mutation is expressed and organ-specific pathophysiology; in some organs like the pancreas, a CFTR genotype closely correlates with the severity of its phenotype (PI vs. PS); in lungs, with complex pathophysiology, the primary effect of a particular genotype may be considerably modulated by secondary genetic factors and environment

Table 1 - What determines a phenotypic effect of a mutation?

All types of mutations are represented (missense, frameshift, nonsense, splice, small and large in-frame deletions or insertions), and are distributed throughout the entire gene [fig.10]. The potential of a mutation to contribute to the severity of a CF phenotype depends on multiple factors [table 1, A-G].

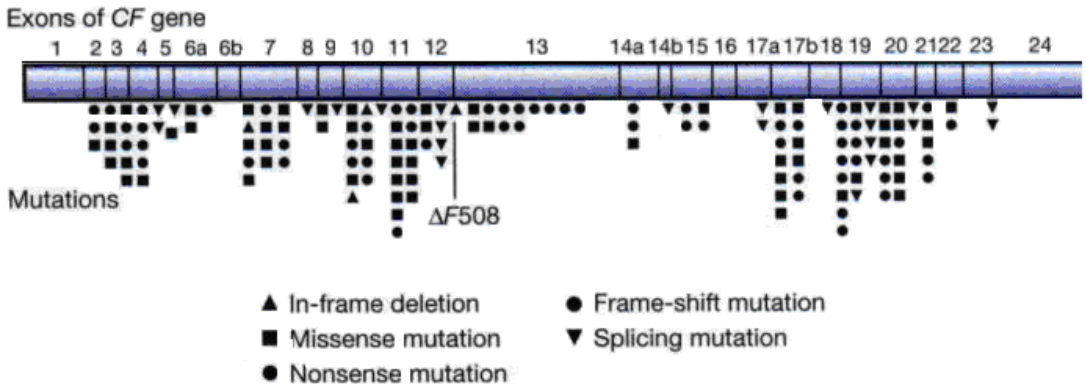


FIGURE 10 - Over 550 different mutations have been characterised in the CF gene CFTR.

Firstly, it is inherent to a characteristic mutation profile on the molecular and cellular levels as determined by its type (A), class (B) and position in the gene (C). Secondly, it depends on the other intragenic factors (E) such as a presence of other changes within the gene (complex allele), influence of a second mutation in the genotype (F) and secondary genetic and environmental factors associated with a pathogenesis of the affected organ (G). Due to a large number of disease-contributing mutations affecting the function of the CFTR gene to a different degree, a CFTR genotype has high potential as a primary source of phenotypic variability in CF.

2.3.1 MOLECULAR MECHANISMS OF CFTR MUTATIONS

Various mutations can be grouped into different classes [fig.11] based on their known or predicted molecular mechanisms of dysfunction and functional consequences for the CFTR protein.

Although schematic, such classification can often be a good indication of a mutation's severity and provides a rationale for their phenotypic consequences [table 1, B, D].

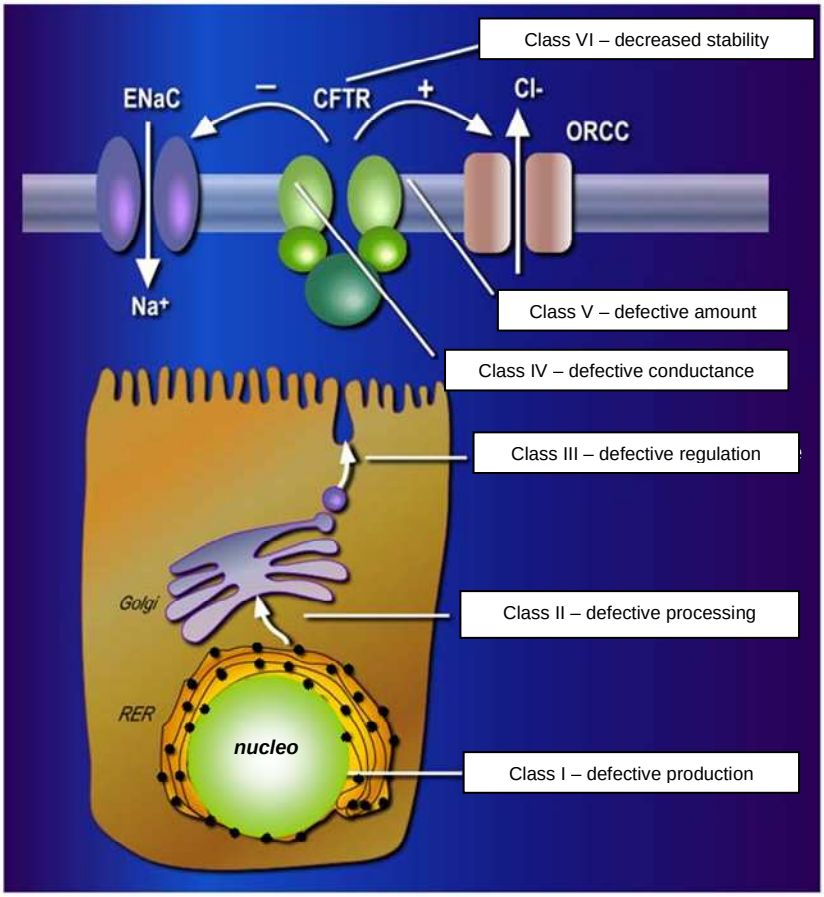


FIGURE 11 – Six classes of CFTR Mutations.

- **CLASS I: DEFECTIVE PROTEIN SYNTHESIS**

Net Effect: No CFTR Protein at the Apical Membrane. Mutations that belong to this category are associated with lack of biosynthesis or defective biosynthesis producing abnormal protein

variants (truncation, deletion, etc.). These are usually nonsense, frameshift, RNA splice-type mutations or missense mutations changing translation initiation codon (methionine at position 1). Due to their grossly altered structure, they tend to be unstable and efficiently cleared from the cell. In effect, virtually no functional CFTR is present at the apical membrane of the epithelial cells and as a consequence phenotypic effects of class I mutations tend to be severe.

- **CLASS II: ABNORMAL PROCESSING AND TRAFFICKING**

Net Effect: No CFTR Protein at the Apical Membrane. Many CFTR gene variants expressed in various heterologous systems fail to be properly

processed to a mature glycosylated form and transported to the apical membrane. Because of their absence in the membrane, these mutant CFTR variants are typically associated with severe CF phenotypes. Interestingly, some of the class II mutations – such as the most common ΔF508 mutation [fig.12] – if correctly processed, possess residual Cl channel activity and may lead to a sustained normal or only mildly affected phenotype. For this reason, mutations in this group – in particular the common ΔF508 deletion – are the targets of potential therapies, aimed at correcting the processing and delivery of a mutated CFTR protein to the apical membrane.

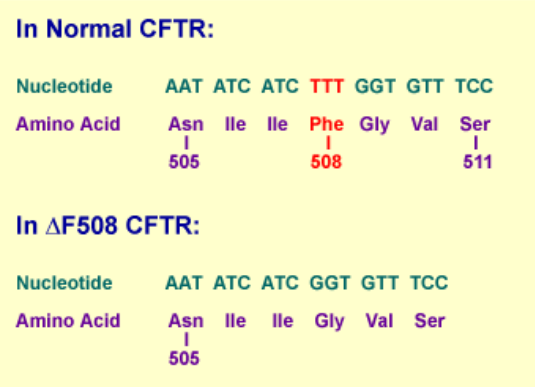


FIGURE 12 - The ΔF508 deletion is the most common cause of cystic fibrosis.

Recent studies of endogenous CFTR expression in CF-relevant epithelial tissues from patients homozygous for the ΔF508 mutation demonstrated however that immunolocalized distribution of CFTR in certain tissues (respiratory and intestinal) is indistinguishable from that observed in the corresponding wild-type cells [77]. The ΔF508 CFTR protein was absent only in sweat gland ducts of those patients. It is still possible that the ΔF508 CFTR is compartmentalized in vesicles in close proximity to the cell membrane and therefore gives the appearance of being embedded in the membrane. It should be noted, however, that the efficiency of processing and trafficking of the ΔF508 CFTR protein may vary conside ably between different epithelial cells. Therefore, different intracellular conditions may result in variable severity of tissue- or organ-specific pathophysiology.

- **CLASS III: DEFECTIVE REGULATION**

Net Effect: Normal Amount of Nonfunctional CFTR at the Apical Membrane. Mutations in this class affect the regulation of CFTR function by preventing ATP binding and hydrolysis at the nucleotide binding domains (NBD1; NBD2) required for the channel activation. Alterations within NBD1 (such as missense mutation G551D) may also affect CFTR regulation of other channels such as the outwardly rectifying chloride channel [78] or ROMK2 potassium channel [79]. This is an example of a mutation mechanism which affects some regulatory functions of CFTR in addition to its chloride transporting function.

- **CLASS IV: DECREASED CONDUCTANCE**

Net Effect: Normal Amount of CFTR with Some Residual Function at the Apical Membrane. Several mutations (R117H, R334W, R347P) were shown to affect the properties of CFTR single-channel conductance [80]. Interestingly, these mutations are located within MSD1 which is implicated in forming the pore of the channel [81] and the corresponding CFTR variants retain residual function. Alleles in this class are typically associated with a milder pancreatic phenotype: this includes patients with pancreatic sufficency (PS).

- **CLASS V: REDUCED SYNTHESIS/TRAFFICKING**

Net Effect: Reduced Amount of Functional CFTR at the Apical Membrane. Various mutations may be associated with reduced biosynthesis of fully active CFTR due to partially aberrant splicing (3849+10kbC→T) [82], promoter mutations or inefficient trafficking (A455E) [83, 84]. These mutations result in reduced expression of functional CFTR channels in the apical membrane. Class V mutations are associated with a milder CF phenotype.

- **CLASS VI: DECREASED STABILITY**

Net Effect: Functional but Unstable CFTR Present at the Apical Membrane. According to a new study, truncation of the C-terminus of CFTR leads to the marked instability of an otherwise fully processed and functional variant. These are usually nonsense or frameshift mutations (Q1412X, 4326delTC, 4279insA)

causing a 70- to 100-bp truncation of the Cterminusof the CFTR [85] and associated with severe CF presentation. The above classification categorizes CFTR mutations according to their molecular mechanisms and consequences for different aspects of CFTR biogenesis, metabolism and function but it does not exclude an association of a mutation with more than one mechanism (e.g. mislocalization and decreased function).

3. INFECTION AND INFLAMMATION IN CF

Although the cystic fibrosis transmembrane ion receptor (CFTR) gene and protein have been identified since seventeen years, it remains enigmatic how abnormalities in CFTR can cause chronic and persistent pulmonary infection and inflammation that lead to bronchiectasis and end-stage lung disease [86].

3.1 LUNG INFECTION

Healthy airways are sterile below the first bronchial division. Sterility is maintained, despite constant challenge from viruses and bacteria in the air that we breathe, by an elaborate hierarchy of defenses. Lung defenses are understood only in outline. The airway surfaces are covered with a thin film (~30 μm) of airway surface liquid (ASL) consisting of a periciliary sol and a mucus gel that are propelled toward the mouth by coordinated ciliary beating. Thus, mucociliary clearance, aided by cough, cleans the airways mechanically. The ASL is not simply saltwater, but is instead a rich broth of proteases/antiproteases, oxidants/antioxidants, antibiotics, and antibodies that work together to inactivate or destroy pathogens without undue collateral damage to the lungs. These mucosal mechanisms are backstopped by cellular immune mechanisms involving dendritic cells, neutrophils, and macrophages that are recruited and coordinated by signaling molecules in the ASL. Although the complex pulmonary defense system is understood in outline, details are lacking because of technical impediments that, together with the lack of appropriate animal models, help to explain why we are still struggling to understand CF lung disease. The pattern of CF lung disease is unlike that of any other lung disease.

In patients with cystic fibrosis, progression of lung disease is insidious and patients may be relatively asymptomatic before irreversible changes and chronic bacterial colonization occur.

Cough is the predominant symptom in the early stages of cystic fibrosis,

occurring in as many as 50% of patients by 10 month of age [87]; however, many patients do not have pulmonary symptoms. The first detectable evidence of lung disease in patients with cystic fibrosis is infection and/or inflammation in bronchoalveolar lavage fluid, denoted by elevated counts of interleukin-8 and neutrophils and the presence of microorganisms [88-91]. *Haemophilus influenza*, *Staphylococcus aureus* and *Pseudomonas aeruginosa (PAO)* are the most prevalent early pathogens, and most patients have colonization with at least 1 of these bacteria by 1 year of age [92, 93]. Early infections with *PAO* can be transient, and approximately half clear spontaneously [94-96]. Chronic colonization with *PAO* is associated with a more rapid decline in lung function [97, 98], especially if the isolate becomes mucoid [99-101]. Although most patients are initially infected with nonmucoid *P. aeruginosa*, it later transitions to a mucoid state. Mucoid *Pseudomonas* is much more difficult to treat and eradicate because it lives in a defensive mode of growth called biofilm [102, 103] [fig.13].

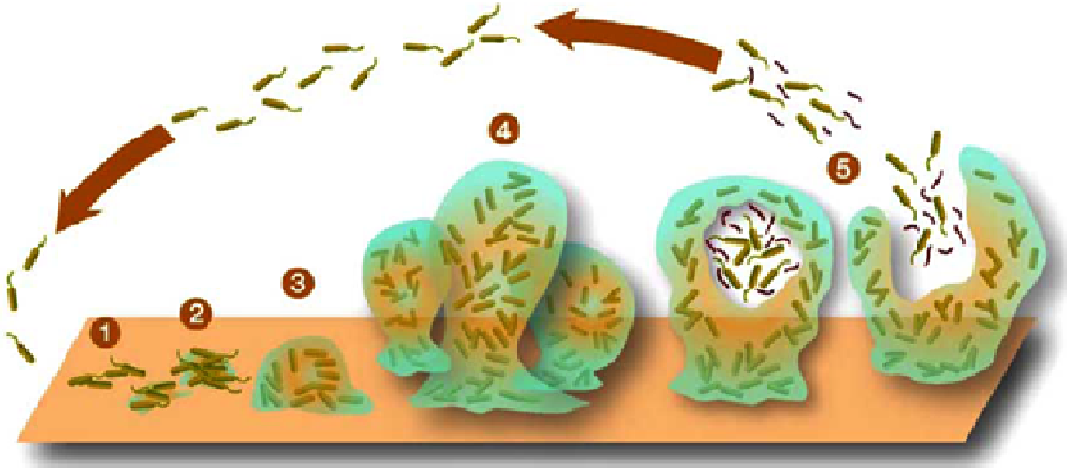


FIGURE 13: The biofilm life cycle of PAO. 1: individual cells populate the airway surface. 2: extracellular polymeric substances (EPS) are produced and attachment becomes irreversible. 3 and 4: biofilm architecture develops and matures. 5: single PAO cells are released from the biofilm.

Biofilms are communities of bacteria, enclosed in a self-produced matrix, attached to a surface [104]. Bacteria in a biofilm state exhibit increased

resistance to antibiotics [105] and host defense factors [106]. Therefore, clinically attainable antibiotic concentrations may not adequately clear biofilm infections, allowing the bacterial population to recover, persist and spread. Microscopic and physiologic evidence supports biofilm formation by *P. aeruginosa* in sputum from patients with cystic fibrosis [107]. Antibiotic-resistant, biofilm-forming mucoid *P. aeruginosa* are believed to play a dominant role in the progression of lung disease in patients with cystic fibrosis [99-101].

3.2 LUNG INFLAMMATION

The lungs, including mucus glands, are structurally normal at birth [108]. Soon after birth excessive endobronchial inflammation in the small airways has been demonstrated in both sputum culture positive and sputum culture negative patients. Broncho-alveolar lavage (BAL) fluid shows a predominantly neutrophil inflammation with elevated interleukin (IL)-8 and neutrophil elastase [109-112]. This persistent inflammation is the major cause of progressive lung injury and destruction leading to a decrease in lung function. This progressive loss of lung function is the main reason for the limited life expectancy of most patients with CF.

Virtually all patients with CF are chronically infected with one ore more bacterial species. The inflammatory response to infection appears to be more intense in patients with CF compared to non-CF patients. Additionally, it has been shown that the number of colony forming units in BAL fluid is directly related to the intensity of the inflammatory response with a significant increase in the number of inflammatory cells and an increase in IL-8 concentrations [113].

Acquisition and consequent chronic infection with mucoid strains of *P. aeruginosa* leads to an increase in the endobronchial inflammatory response to infection. It has been shown that CF cell lines produce more pro-inflammatory cytokines than normal cell lines in response to *P. aeruginosa* [114]. This “overproduction” of pro-inflammatory cytokines can be found in the ELF of CF airways [115]. Also significantly lower levels of the anti-inflammatory cytokine

IL-10 , which inhibits the production of pro-inflammatory cytokines as IL8 and IL6, are found. This imbalance of anti-inflammatory and pro-inflammatory cytokines results in an excessive and persistent inflammation in the CF airways. As a result lung function deteriorates more rapidly in *P. aeruginosa* positive patients compared with CF patients negative for *P. aeruginosa* [116, 117].

For all these reasons, understanding the pathophysiology of lung inflammation and thereby the pathogenesis of lung disease in CF is needed to improve current therapies and develop new therapeutic strategies.

3.3 CYTOKINES AND INFLAMMATORY MEDIATORS IN CYSTIC FIBROSIS

Airway disease in cystic fibrosis (CF) is characterised by chronic infection and an inflammatory response dominated by a neutrophilic infiltrate. There is incomplete understanding of the relationship between the abnormal CFTR gene product and the development of inflammation and progression of lung disease in CF [118].

Evidence suggests that airway inflammation in CF is associated with increased production of pro-inflammatory cytokines in the lung. Airway epithelial cells, macrophages, and neutrophils are all capable of producing cytokines.

Several studies have found elevated concentrations of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, and tumour necrosis factor-alpha (TNF-α) in the sputum and bronchoalveolar lavage fluid (BALF) of patients with CF [118]. Their synthesis is promoted by the transcription factor nuclear factor-kB (NF-kB), which plays an important role in intracellular signalling for the production of pro-inflammatory cytokines [119]. IL-10, IL-1 receptor antagonist protein (IRAP), and soluble TNF-α receptor (TNFsR) are anti-inflammatory cytokines that are relatively down-regulated in CF airway cells [120]. The principle action of IL-10 is to increase the synthesis of I-kB, the inhibitor of NF-kB. Downregulation of IL-10 leads to increased proinflammatory cytokines due to less inhibition of NF-kB actions [119]. Furthermore we

investigated on MCP1 (Monocyte Chemotactic Protein-1, a member of the small inducible gene -SIG- family, that plays a role in the recruitment of monocytes to sites of injury and infection), RANTES (Regulated on Activation Normal T Expressed and Secreted, a protein member of the interleukin-8 superfamily of cytokines, that is a selective attractant for memory T lymphocytes and monocytes), and G-CSF (Granulocyte Colony-Stimulating Factor, a colony-stimulating factor that stimulates the production of neutrophils). Table 2 lists the actions of both the proinflammatory and anti-inflammatory cytokines.

cytokines	Action
IL1	Primes neutrophils Increases adhesion of neutrophils to endothelium
IL8	Increases chemotaxis of neutrophils to site of inflammation Activates neutrophils Increases expression of adhesion molecules
TNFα	Increases chemotaxis of neutrophils to site of inflammation Increases adhesion of neutrophils to endothelium Induces synthesis of chemoattractant neutrophils Increases intermediary metabolism
IL6	Mediates acute-phase reaction Matures B-lymphocytes Activates T-lymphocytes
IL10	Inhibits secretion of TNFa and other cytokines Inhibits antigen presentation
IRAP	Inhibits IL-1 receptor binding Antagonises activities of IL-1
IL-1, interleukin-1; IL-8, interleukin-8; TNFa, tumour necrosis factoralpha; IL-6, interleukin-6; IL-10, interleukin-10; IRAP, interleukin-1 receptor antagonist protein.	

Table 2 – Actions of cytokines.

Airway epithelial cells may be involved directly in the excess inflammation by several mechanisms. Pro-inflammatory cytokines arise from airway epithelial cells, as well as from macrophages and infiltrating neutrophils [Table 3].

	Neutrophils	Epithelial cells	Macrophages	Lymphocytes
IL-1 β	+	+	+++	
IL-6	+	+	+++	+
IL-8	+++	++	++	+
IL-10		++	+	++
TNFα	++	+	+++	+
IRAP	+		+++	
TNFsR	++	+	+	

IL-1β, interleukin-1beta; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; TNFα, tumour necrosis factor-alpha; IRAP, interleukin-1 receptor antagonist protein; TNFsR, tumour necrosis factor soluble receptor

Table 3 – Cellular source of cytokines produced in human airways.

Airway epithelial cells also express large numbers of the important pro-inflammatory adhesion molecule, ICAM-1. This adhesion molecule is a ligand for neutrophils, and adhesion is thought to result in increased IL-8 production, leading to persistence of neutrophils in the airway [121]. The fact that epithelial cells themselves are involved in cytokine release and are thought to play a major role in the local inflammation leads to further speculation that defective CFTR function (expressed most importantly in the epithelial cells) may be directly related to excessive inflammation.

The cytokines tumor necrosis factor-alpha (TNF-α), interleukin-8 (IL-8), interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1) have important roles in regulating neutrophil migration and the inflammatory response [122]:

- **ICAM-1**

Intercellular Adhesion Molecule-1 (ICAM-1, CD54) is a transmembrane glycoprotein molecule of the immunoglobulin superfamily. The protein is a type of intercellular adhesion molecule continuously present in low concentrations in the membranes of leukocytes and endothelial cells. Upon cytokine stimulation, the concentrations greatly increase. ICAM-1 can be induced by interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) and is expressed by the vascular endothelium, macrophages, and lymphocytes.

In cystic fibrosis, neutrophil migration from the bloodstream into the airway occurs through interactions between adhesion molecules on neutrophils and ligands on the vascular endothelium. One ligand for the CD11/CD18 leukocyte adhesion complex is the intercellular adhesion molecule-I which is expressed by endothelial cells [123] as well as by bronchial epithelial cells and alveolar type I cell [124-126]. A soluble form of ICAM- 1 (sICAM-1) circulates in the blood and is present in the bronchoalveolar lining fluid [127]. sICAM- 1 is often increased during inflammation; in particular, its concentration is elevated in tracheal aspirates of infants with bronchopulmonary dysplasia [128], as well as in the plasma or bronchoalveolar lavage (BAL) fluid of patients with idiopathic pulmonary fibrosis, asthma, and sarcoidosis [129-134].

- **IL8**

Interleukin-8 (IL-8) is a protein member of the α-chemokine family. This chemokine is one of the major mediators of the inflammatory response and it is produced by macrophages and other cell types such as epithelial cells.

There are more receptors of the surface membrane capable to bind IL-8. The most frequently studied types are the G protein coupled serpentine receptors CXCR1 and CXCR2. Expression and affinity to IL-8 is different in the two receptors (CXCR1 > CXCR2). Toll-like receptors are the receptors of the innate immune system. These receptors recognize antigen patterns (like lipopolysaccharides -LPS- in gram negative bacteria). Through a chain of biochemical reactions IL-8 is secreted and is an important mediator of the immune reaction in the innate immune system response.

Persistent infection with *Pseudomonas aeruginosa* increases interleukin-8 levels and causes dense neutrophil infiltrations in the airways of patients with chronic airway diseases, such as cystic fibrosis. Recently, it has been reported that nitrite reductase from *P. aeruginosa* enhances IL-8 gene promoter activity. Nitrite reductase enhanced nuclear localization of the NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) binding complex. Furthermore, nitrite reductase induced the degradation of IkBα, the major cytoplasmic inhibitor of NF-kB, and the expression of IkBα mRNA. These data support the critical role of the activation of NF-kB in nitrite reductase-induced IL- 8 gene expression in CF airway epithelium [135,136].

- **IL6**

IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-alpha and IL-1, and activation of IL-1ra and IL-10.
In CF patients IL-6 gene is up regulated following infection of CF bronchial epithelial cells with *P. aeruginosa*. It has been demonstrated the ability of the *P. aeruginosa* mitogen, exoenzyme S, to induce proinflammatory and immunoregulatory cytokines and chemokines, via NF-kB activation. Exoenzyme S strongly induced transcription of proinflammatory cytokines and chemokines,such as IL-6 and IL-8, and modest transcription of immunoregulatory cytokines. The response occurred early and subsided without evolving over time. These data suggest that cells responding to exoenzyme S would rapidly express proinflammatory cytokines and chemokines that may contribute to pulmonary inflammation in cystic fibrosis [137].

- **TNFα**

Tumor necrosis factor-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction.

The primary role of TNF-α is in the regulation of immune cells. TNF-α is also able to induce apoptotic cell death, to induce inflammation, and to inhibit tumorigenesis and viral replication, via NF-kB activation. Dysregulation of TNF-α production has been implicated in a variety of human diseases, such as cystic fibrosis and cancer [138]. Recombinant TNF is used as an immunostimulant.

Norman et al. have demonstrated that concentrations of TNF-α are higher in plasma from patients with cystic fibrosis than in normal controls and that its concentration is increased during episodes of acute respiratory infection. It has been found TNF-α in cystic fibrosis sputa, during times of clinical stability, at concentrations that have been shown to produce neutrophil migration, respiratory burst, and degranulation in vitro [139]. This provides evidence in favour of the hypothesis that lung damage, due to chronic infection, is occurring during periods of apparent wellbeing.

3.4 NF-kB IN DEFENSE AND DISEASES

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA.

3.4.1 NF-kB: A KEY ROLE IN INFLAMMATION

NF-κB regulates host inflammatory and immune responses and cellular growth properties [140] by increasing the expression of specific cellular genes. These include genes encoding at least 27 different cytokines and chemokines, receptors involved in immune recognition such as members of the MHC, proteins involved in antigen presentation, and receptors required for neutrophil adhesion and migration [141]. Cytokines that are stimulated by NF-κB, such as IL-1β and TNF-α, can also directly activate the NF-κB pathway, thus establishing a positive autoregulatory loop that can amplify the inflammatory response and increase the duration of chronic inflammation. NF-κB also stimulates the expression of enzymes whose products contribute to the

pathogenesis of the inflammatory process, including the inducible form of nitric oxide synthase (iNOS), which generates nitric oxide (NO), and the inducible cyclooxygenase (COX-2), which generates prostanoids [142]. The NF-κB pathway is likewise important in the control of the immune response. It modulates B-lymphocyte survival, mitogen-dependent cell proliferation, and isotype switching, which lead to the differentiation of B lymphocytes into plasma cells [143]. In addition, NF-κB regulates IL-2 production, which increases the proliferation and differentiation of T lymphocytes [144]. Thus, activation of NF-κB leads to the induction of multiple genes that regulate the immune and the inflammatory response.

In addition to activating the expression of genes involved in the control of the immune and inflammatory response, the NF-κB pathway is also a key mediator of genes involved in the control of the cellular proliferation and apoptosis. Antiapoptotic genes that are directly activated by NF-κB include the cellular inhibitors of apoptosis (c-IAP1, c-IAP2, and IXAP), the TNF receptor–associated factors (TRAF1 and TRAF2), the Bcl-2 homologue A1/Bfl-1, and IEX-IL [145].

3.4.2 NF-kB FUNCTION AND REGULATION

NF-κB is composed of homo- and hetero-dimeric complexes of Rel family polypeptides, which are characterized by an N-terminal RHD (Rel homology domain), which mediates DNA binding, nuclear localization and subunit dimerization [140]. Mammals express five NF-κB proteins, namely RelA (p65), RelB, c-Rel, NF-κB1 p50 and NF-κB2 p52 [fig.14A]. NF-κB1 and NF-κB2 are synthesized as large precursors of 105 (p105) and 100 kDa (p100) respectively. These are partially proteolysed by the proteasome (termed processing), which removes their C terminal halves, to produce the active NF-κB1 p50 and NF-κB2 p52 subunits. RelA and c-Rel both have C-terminal transactivation domains in their RHDs and can strongly activate transcription from NF-*κ*B binding sites in target genes. RelB also contains a transactivation domain in its RHD and can function as an NF-kB activator when complexed with p50 or p52. However, RelB/RelA heterodimeric complexes are inhibitory, since they cannot bind DNA

[146]. NF-κB1 p50 and NF-κB2 p52 lack a transactivation domain, and can only promote transcription when heterodimerized with a transactivating Rel subunit [147]. Homodimers of p50 and p52 function as transcriptional repressors, but can stimulate transcription when bound to the IκB-like nuclear protein BCL-3 [148-150].

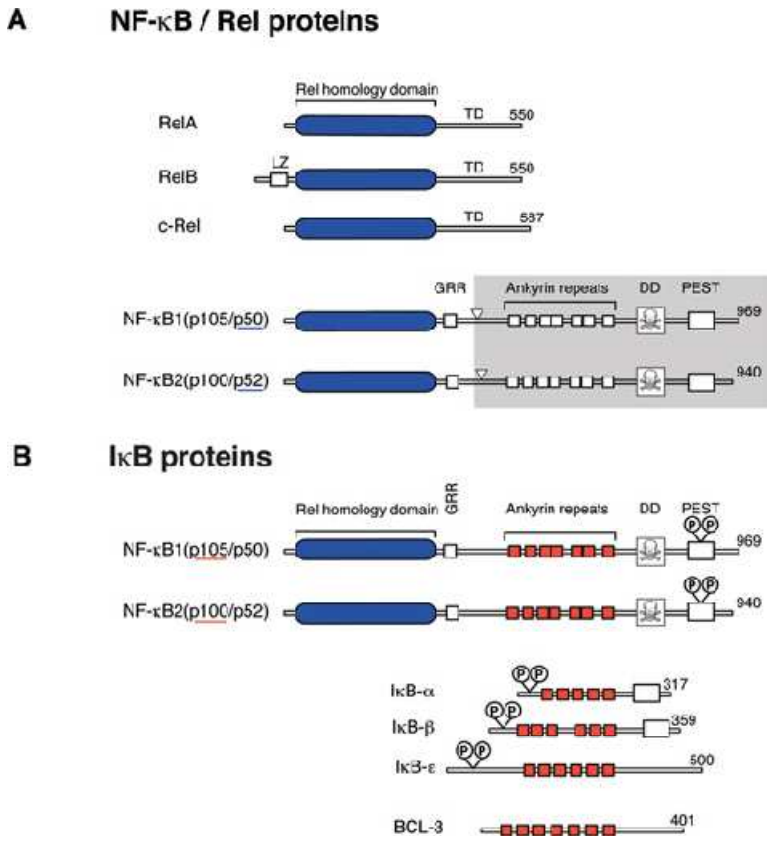


FIGURE 14 - Schematic representation of members of the Rel/NF-**κ**B **(A)** and I*κ*B **(B)** families of proteins Rel/NF-*κ*B proteins are characterized by their conserved N-terminal RHDs (blue) which mediate DNA binding, nuclear localization and subunit dimerization. I*κ*B proteins each contain ankyrin repeat regions (red), which interact with RHDs of NF-*κ*B subunits to prevent nuclear translocation. The numbers of amino acids in each protein are shown on the right. In **(A)** arrowheads point to the C-terminal residues of p50 and p52, which are produced by proteolytic processing of the C-terminal halves p105 and p100 respectively (grey shading). In **(B)** the positions of the IKK phosphorylation sites, which regulate the inducible proteolysis of I*κ*B proteins, are shown. LZ, leucine zipper; TD, transactivation domain.

In unstimulated cells, NF-κB dimers are inactive, since they are sequestered in the cytoplasm by interaction with inhibitory proteins termed IκBs (inhibitors of

NF-κB) [140]. In response to stimulation with agonists, IκBs are proteolytically degraded by the proteasome, releasing associated NF-κB dimers to translocate into the nucleus and modulate gene expression [fig.15]. The transcriptional activity of certain NF-κB dimers is additionally regulated by phosphorylation, providing an extra level of control of NF-κB activation [151].

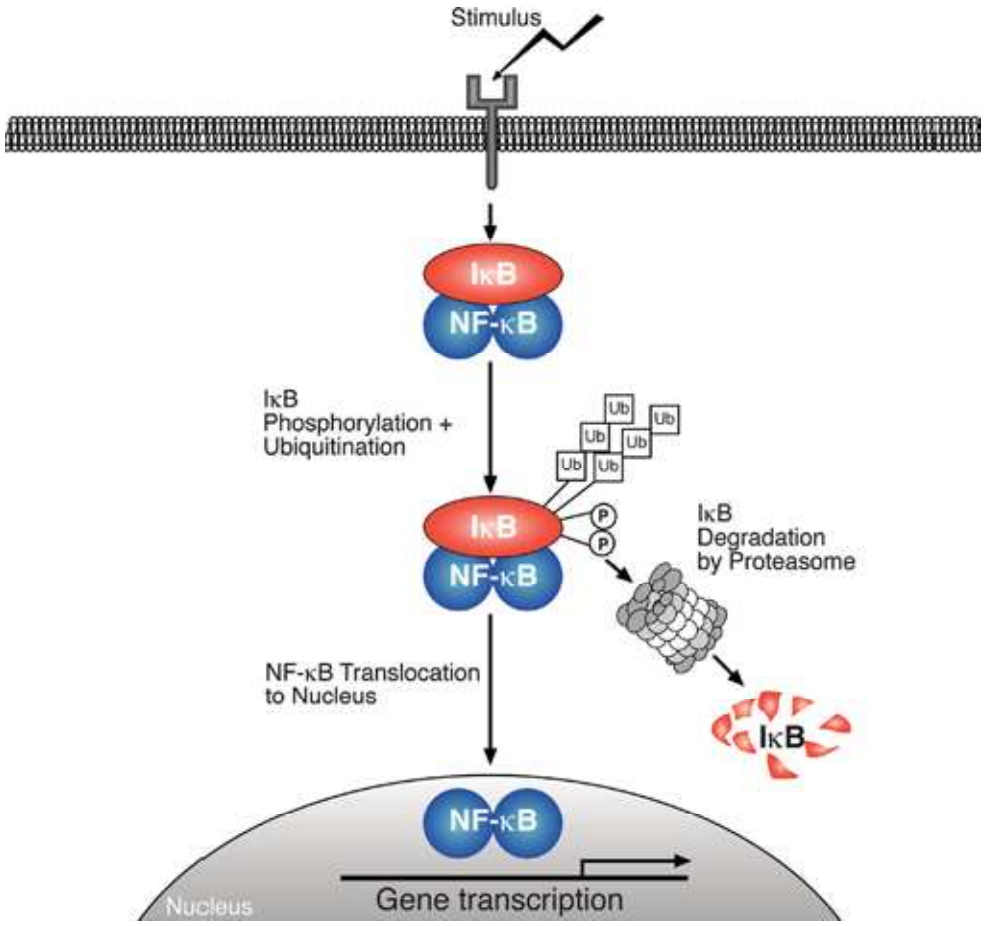


FIGURE 15 - Model of the generic NF-*κ*B activation pathway

Various stimuli induce the phosphorylation (-□) and subsequent polyubiquitination (-Ub) of I*κ*Bs, which are then targeted for degradation by the 26S proteasome. Associated NF-*κ*B dimers are thereby released to translocate into the nucleus, where they bind to the promoter regions of NF-*κ*B-responsive genes to modulate their expression. The transactivating capacity of nuclear NF-*κ*B dimers can also be regulated by phosphorylation.

I*κ*Bs comprise a family of structurally related proteins, each of which contain multiple ankyrin repeats that interact with the nuclear localizing signals of RHDs to prevent nuclear translocation of Rel subunits [fig.14B]. This family includes

IκBα, IκBβ and IκBε, which are composed of a central ankyrin repeat region and an N-terminal regulatory domain, which controls their inducible degradation. The precursor NF-κB proteins, NF-κB1 p105 and NF-κB2 p100, also function as IκBs as a result of ankyrin repeat regions in their C-terminal halves. Alternate promoter usage of the NF-κB1 gene can generate an mRNA encoding only the C-terminal IκB-like portion of p105, termed IκBγ, but this has so far only been reported in murine cells [152, 153].

3.4.3 INHIBITION OF NF-kB: A STRATEGY IN ANTI-INFLAMMATORY THERAPIES

The induction of proinflammatory genes usually results from the increased activity of a subset of transcription factors that have thus become attractive targets in the development of novel anti-inflammatory drugs. Among the inducible transcription factors that control inflammatory gene expression, nuclear factor NF-kB plays a central and evolutionarily conserved role in coordinating the expression of various soluble proinflammatory mediators (as cytokines and chemokines) and leukocyte adhesion molecules [140].
A wide range of genes involved in inflammation contain functional kB-sites within their promoters and are induced by NF-kB [154]: thus, NF-kB could be proposed as one target to down-modulate the immune processes involved in different pulmonary diseases, including CF [155], by using of drugs, synthetic molecules, natural products and normal or recombinant proteins that could act at several steps to interfere with NF-kB activation [fig.16].
Various classes of NF-kB inhibitors are discussed below:

- **NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)**

Acetylsalicylic acid (ASA) and sodium salycilate function as an anti-inflammatory agents by inhibiting cyclooxygenase (COX), an enzyme activity essential to the production of prostaglandins (PGs) [156]. More recent studies demonstrate that the mechanism of inhibition by both ASA and sodium

salycilate occurs by means of binding and blocking the ATP binding site of IKKβ [157]. Intriguingly, expression of the COX-2 encoding gene, believed to be responsible for the massive production of PGs at inflammatory sites, is transcriptionally regulated by NF-kB [158, 159]. The ability of ASA and sodium salicylate to inhibit NF-kB activity and kB-dependent gene expression has been tested experimentally in a wide range of cell types and conditions. For example, pretreatment of primary synovial fibroblasts or lung epithelial cells with ASA blocks IL-1β and TNF-α – induced NF-kB activation so that the subsequent expression of IL-6 and IL-8 is inhibited [160, 161].

Furthermore Ibuprofen inhibits NF-kB activation in T cells [162] as well as COX-2 expression and PGE2 production in murine macrophages. Among the other NSAIDs that have been investigated, acetaminophen inhibits the binding of NF-kB to DNA in LPS- and interferon-γ – treated macrophages and thus precludes expression of inducible nitric oxide synthase [163]. Moreover, sulindac, an NSAID that is both structurally and pharmacologically related to indomethacin, decreases IKKβ kinase activity and consequently inhibits NF-kB activation [164]. Lastly, tepoxalin, which inhibits both COX and 5-lipoxygenase, blocks NF-kB activation in Jurkat T-cells and HeLa cells.

Thus, the findings that salicylates and glucocorticoids can inhibit NF-kB activity are among a growing body of data indicating that drugs specifically designed to target NF-kB activation might be clinically useful for the treatment of diseases that involve inflammation, as cystic fibrosis [165-169].

- **GLUCOCORTICOIDS**

Glucocorticoids (GCs) and other corticosteroids are the most widely used anti-inflammatory, immunosuppressive drugs. GCs function to downregulate the expression of genes involved in inflammation [170]. Previous studies demonstrated that GCs increase the expression of an IkB molecule, named IkBα, so that NF-kB is retained in the cytoplasm and thus rendered inactive [165, 167].

The notion that NF-kB inhibition underlies the anti-inflammatory capacity of GCs is also supported by animal studies. In a mouse model of acute inflammation (carrageenin - induced air pouch inflammation), treatment with the synthetic steroid dexamethasone reduces the number of cells that stain positively for activated NF-kB [171]. In rats, dexamethasone not only inhibits TNF-α production and hepatic NF-kB activation associated with peritoneal sepsis [172], but also inhibits LPS-induced activation of NF-kB, restores myocardial contractility, and increases myocardial IkBα protein levels [173]. Finally, a recent study of normal human subjects demonstrates that intravenous injection of hydrocortisone increases IkBα levels and reduces the translocation of NF-kB into the nuclei of peripheral mononuclear cells [174], which supports the experimental model originally proposed for the molecular action of GCs [165, 167].

- **ANTIOXIDANTS**

The generation of reactive oxygen species (ROS) by phagocytic leukocytes (neutrophils, monocytes, macrophages, and eosinophils) is one of the most important hallmarks of the inflammatory process. By oxidizing essential cellular components of invading pathogens, reactive radicals and oxidants also represent the first line of defense against microorganisms [175]. In addition to promoting general cytotoxicity, ROS may also act to upregulate proinflammatory gene expression by activating NF-kB, a process that is itself sensitive to the cellular redox state [176].

Diverse agents that cause oxidative stress can activate NF-kB [177], and numerous stimuli that activate NF-kB, including cytokines, phorbol esters, LPS, and CD3 engagement, increase the levels of intracellular ROS [178]. This generation of ROS, however, is cell- and stimulus-specific. Thus, whereas stimulation with the proinflammatory cytokines IL-1 and TNF-α leads to substantial intracellular ROS in lymphoid and monocytic cell lines, no such increases have been observed in epithelial cell lines derived from ovaries, colon, breast, or cervix [178].

- **ANTISENSE OLIGODEOXYNUCLEOTIDES**

Antisense oligodeoxynucleotides constitute a potentially important family of therapeutic compounds for the treatment of a range of diseases [179]. The use of antisense technology to inhibit expression of NF-kB proteins has been evaluated both in vitro and in vivo in a variety of experimental systems.

Antisense targeting of the NF-kB p50 subunit can reduce IgM and IgG synthesis in B cells [180], and can significantly reduce maturation and proliferation of CD25/IL-2R T-cells [181]. Antisense strategies to reduce expression of the p65 subunit greatly inhibits the expression of cell adhesion molecules in endothelial and smooth muscle cells in vitro [182]. In vivo, antisense inhibition of p65 blocks tumor growth in nude mice, prolongs allo- and xenograft survival [183, 184], and alleviates septic shock in LPS-treated animals [185, 186]. Clearly, the therapeutic potential of antisense molecules directed against NF-kB and the components required for its expression and activation warrants further investigation.

- **PROTEASOME INHIBITORS**

Signal-induced phosphorylation and ubiquitination of IκB and its degradation by the 26S proteasome precede NF-κB nuclear translocation. Inhibitors of proteasome function reduce the degradation of IκB to prevent activation of the NF-κB pathway. A variety of peptide aldehydes, including MG101, MG132, and MG115, make up one class of agents that inhibit the protease activity of the proteasome [187, 188].

Proteasome inhibitors of another class, including lactacystin, block protein degradation activity by acylating a threonine residue in one of the key proteasome subunits. Finally, a group of boronic acid peptides, including PS-341, are extremely potent inhibitors of proteasome function [189, 190]. Recently, PS-341 has shown promise as an adjunct to cancer chemotherapy by inhibiting activation of the NF-κB pathway. It is also possible that inhibitors of the ubiquitin ligase that mediates IκB ubiquitination may be a useful target in

preventing proteasome degradation of IκB. Thus, a variety of potential inhibitors of proteasome function may have a role interrupting the NF-κB pathway.

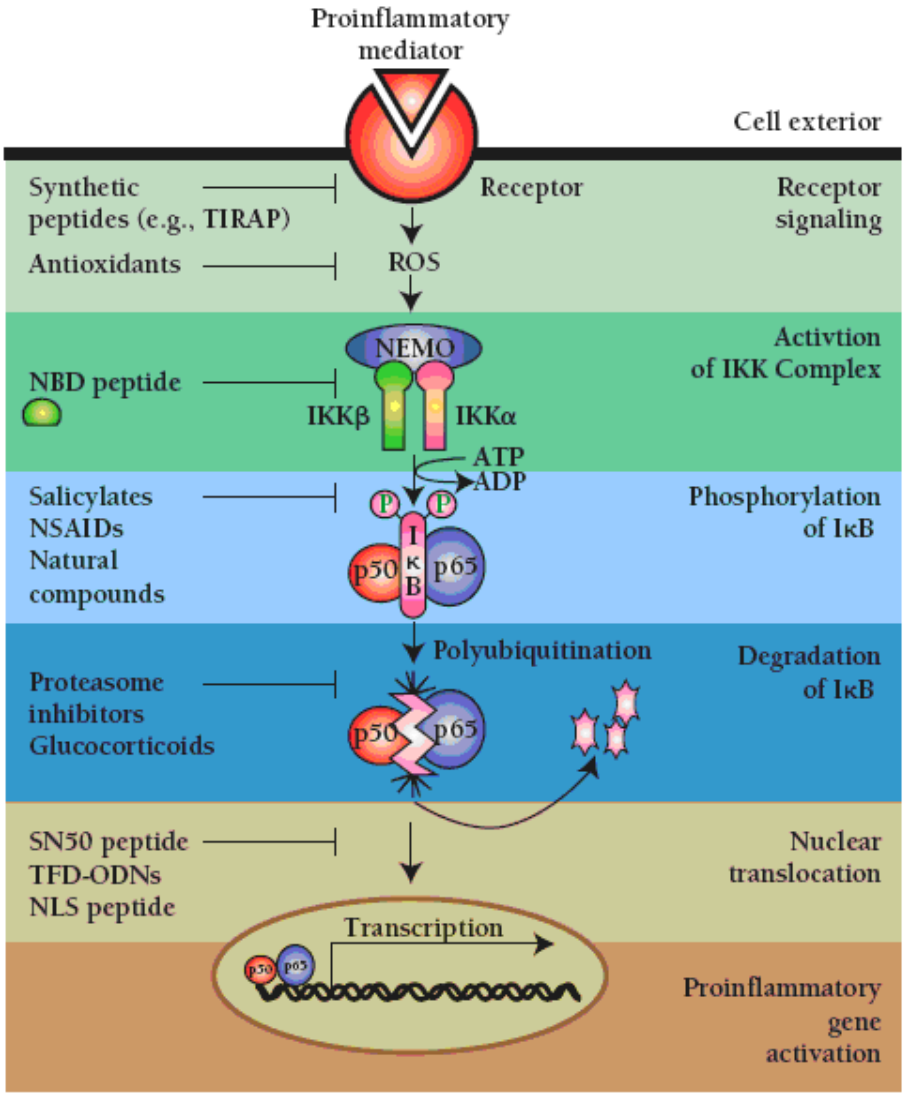


FIGURE 16 - Inhibition of the NF-κB pathway. A schematic illustrating the steps involved in the activation of the NF-κB pathway. Numerous drugs, natural products, and normal or recombinant proteins can act at several of these steps to interfere with NF-κB activation.

In our lab, we focused our attention on two different strategies with the aim of down-regulate the pro-inflammatory gene expression:

- transcription factor decoy (TFD)
- natural products as source of new anti-inflammatory componuds

3.5 OLIGODEOXINUCLEOTIDES AS THERAPEUTIC DECOYS

Synthetic oligonucleotides have recently been the object of many investigations, aimed to develop sequence-selective molecules able to modulate, either positively or negatively, transcription of eukaryotic genes. Alteration of transcription could be obtained by using synthetic oligonucleotides mimicking target sites of transcription factors (the transcription factor decoy -TFD- approach). This could lead to either inhibition or activation of gene expression, depending on the biological functions of the target transcription factors.

Because transcription factors can recognize their relatively short binding sequences even in the absence of surrounding genomic DNA, short double-stranded oligodeoxynucleotides (ODNs) bearing the consensus binding sequence of a specific transcription factor have been explored as tools for manipulating gene expression in living cells [fig.17].

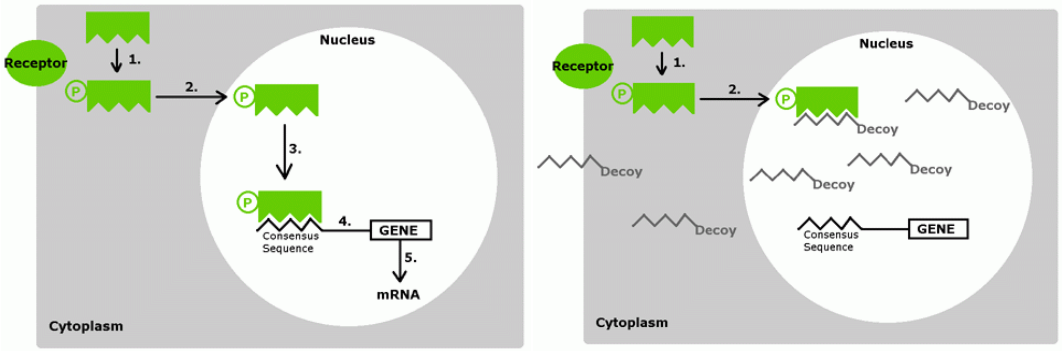


FIGURE 17 - Under a stimulus, there is a receptor mediated activation of the IKK complex, that induces a phosphorylation of IkBα (1), that leaves NF-kB and allow it to enter the nucleus(2) and binding specific sequences (3), starting the transcription of specific genes and mRNA (4-5). If specific sequences, complementary to the consensus region on gene target promoter to which the transcription factors bind, are injected into the cells, there is a block in the specific gene transcription.

These synthetic decoys "compete" for binding of the transcription factor with consensus sequences in target genes. If delivered into the cell in sufficient concentrations these "decoys" thus have the potential to attenuate the binding of the transcription factor to promoter regions of target genes and thus

attenuate the function of the transcription factor to regulate the expression of its target gene(s). Transfected at high concentrations these decoys have been reported in the literature to completely block transcription factor function. Clearly they represent powerful research tools for studying gene regulation both in vitro and also more recently in vivo [191, 192].

Yoshimura et al. reported the successful *in vivo* transfer of NF-kB decoy ODN to reduce neointimal thickening in rat carotid artery model, providing a new therapeutic strategy for restenosis after angioplasty. Since a rapid and transient activation of NF-kB after balloon injury plays a crucial role in the formation of neointima, they demonstrated that inhibition of transient NF-kB activation should be a new therapeutic tool. To block NF-kB activation, they utilized a 'decoy' *cis* element, since decoy ODN can bind NF-kB and thereby block its activation. Using this technology, they already reported the successful inhibition of NF-kB activation in a rat myocardial infarction model [193]. Therefore, they examined the inhibition of NF-kB activation after balloon injury by *in vivo* transfection of NFkB decoy ODN into balloon-injured blood vessels. As expected, it was observed that activation of NF-kB in the injured vessels was successfully inhibited by NF-kB, but not scrambled, decoy ODN *in vivo*. These studies reveal the importance of NFkB activation in neointimal formation after vascular injury, since transfection of NFkB decoy ODN inhibited neointimal formation accompanied by a decrease in NFkB activity.

Furthermore, Penolazzi et al. reported the in vivo effects of a decoy oligonucleotide targeting the nuclear factor κB (NF-κB) on osteoclasts during forced orthodontic tooth movement in rats [194]. Wistar rats were subjected to orthodontic forces, in the absence or presence of treatment with a decoy molecule mimicking a nonsymmetric NF-κB binding site (5'-CGC TGG GGA CTT TCC ACG G-3'). TUNEL staining of fragmented DNA revealed that treatment with NF-κB decoy but not with scramble double-stranded oligodeoxynucleotides (ODN) induced a high level of osteoclast apoptosis in vivo. Immunohystochemical analysis for death receptor Fas revealed strong positivity only in samples treated with NF-κB decoys, demonstrating that osteoclasts are sensitive to death induction via Fas signaling. Induction of

apoptosis in osteoclasts could be a strategy for treatment of excessive osteoclast activity in pathologic conditions such as osteoporosis, peri-articular osteolysis, inflammatory arthritis, Paget's syndrome and tumour-associated osteolytic metastases.

Moreover, the lung Infection with *P. aeruginosa* is known to induce expression of genes of the innate immunity by activating different TFs, such as NF-kB, AP-1, Elk-1, and NF–IL-6 [195-197]. Since NF-kB plays a well known pivotal role in inflammatory processes, we choose it as the first molecular target for the TF decoy approach.

3.6 NATURAL PRODUCTS

Classic therapies based on corticosteroids [170] and ibuprofen [162], as well as chemiotherapy, have been employed in CF patients, but they have demonstrated limited efficacy or occurrence of undesired effects, suggesting the importance of investigations aimed at identifying alternative therapeutic targets and novel anti-inflammatory molecules [198, 199, 200-203].

In this respect, we are studying natural products as sources for the discovery of new drugs on the basis of their widespread application in ethnic and traditional medicine to cure several human diseases, such as muscular and neuronal diseases, mycosis, heart and respiratory diseases, atherosclerosis [204-206].

As an activator of many pro-inflammatory cytokines and inflammatory processes the modulation of the NF-kB transduction pathway is a principal target to alleviate the symptoms of such diseases as arthritis, inflammatory bowel disease and asthma. Natural products and some extracts are reviewed and assessed for their activity and potency as NF-kB inhibitors. A large number of compounds are currently known as NF-kB modulators and include the isoprenoids, most notably kaurene diterpenoids and members of the sesquiterpene lactones class, several phenolics including curcumin and flavonoids such as silybin. Additional data on cellular toxicity are also highlighted as an exclusion principle for pursuing such compounds in clinical

development. In addition, where enough data exists some conclusions on structure-activity relationship are provided.

The treatment of inflammatory conditions with plants is widely reported [207-209]. Natural products are already providing lead compounds in the search for inhibitory small molecules but only a few are beginning to be commercially used. One example is a veterinary product under development for the treatment of canine osteoarthritis (Phytopharm 2001a) and inflammatory bowel disease (Phytopharm 2001b).

There are a multitude of approaches for identifying new pharmaceuticals. In the field of natural product biology, ethnopharmacological as well as bioprospecting approaches have received renewed attention in recent years. The concept of ethnopharmacology specifically aims to develop plant-based drugs for more widespread local use either as pure compounds or plant extracts (phytotherapy) [210]. Concurrent with this analysis is the requirement for the documented use of useful plants by indigenous peoples (ethnobotany).

Thus, screening libraries of plant extracts for their anti-inflammatory activity, in suitable respiratory epithelial cells in vitro and pre-clinical animal models in vivo, could select candidate molecules of potential therapeutic effect. These anti-inflammatory molecules for respiratory diseases could be administered both systemically (e.g. orally) or locally (e.g. by aerosol).

In our lab, we investigated the anti-inflammatory effect of a series of medicinal plant extracts, including *Bergamot* from Lebanon, *Emblica officinalis* and *Saraca asoka* from Bangladesh

3.6.1 BERGAMOT

Bergamot is a small and roughly pear-shaped citrus fruit, [fig.18] growing on small trees known as bergamots (*Citrus bergamia Risso, Rutaceae*).

Bergamot plants, despite their uncertain species definition and phytogeographical origin, are mainly cultivated in Ionian coastal regions of southern Italy, Argentina, and Brazil for their fruits. Bergamot fruits are traditionally employed as raw material in cosmetics and cosmeceutics because

of their essential oil flavor and in dietary and herbal products as crude drugs because of the eupeptic properties and therapeutic activities against digestive disorders [211].

FIGURE 18 – *Citrus Bergamia.*

In the past, Bergamot essential oil has been used in the folk medicine as antiseptic, to facilitate wound healing and as antihelminthic. Actually, likewise other essential oils, Bergamot essential oil is widely used in aromatherapy, a complementary medicine highly diffused in the industrialized countries to improve mood and mild symptoms of stress-induced disorders such as anxiety [212], depression [213], behavioural disturbances in dementia [214] and chronic pain [215, 216]. Individual components of essential oils reach the blood, cross the blood-brain barrier and enter the central nervous system (CNS) following inhalation, dermal application [217], intraperitoneal (i.p.) or subcutaneous (s.c.) injection and oral administration [218]. Physiological and pharmacological studies, combined with phytochemical analyses, indicate that a variety of essential oils exert specific effects on the CNS [219].

Recently, Hiroyasu Inoue et al. have released a study confirming the inflammation reducing power of many aromatherapy essential oils. The oils shown to reduce inflammation through suppression of the COX-2 pro-

inflammatory enzyme included herbs, like thyme, clove and fennel; rose; eucalyptus; and even the citrus bergamot. They suppress the inflammation COX-2 mediated in a manner similar to resveratrol, the chemical linked with the health benefits of red wine. The oils considered to have the strongest anti-inflammation activity used in aromatherapy were not even examined, which can mean that there are naturally a great many compounds found in essential oils that have an anti-inflammatory effect [220].

3.6.2 *EMBLICA OFFICINALIS*

Emblica officinalis is commonly known as Amla or the Indian gooseberry [fig.19], which is a member of a small genus *Emblica* (Family *Euphorbiaceae*) and is extensively found all over India, as well as Sri Lanka, Malaysia, China, Pakistan and Bangladesh.

FIGURE 19 – *Emblica Officinalis.*

The tree is small to medium sized, reaching 8 to 18 m in height, with a crooked trunk and spreading branches. The branchlets are glabrous or finely pubescent, 10–20 cm long, usually deciduous; the leaves simple, subsessile and closely

set along branchlets, light green, resembling pinnate leaves. The flowers are greenish-yellow. The fruit is nearly spherical, light greenish yellow, quite smooth and hard on appearance, with 6 vertical stripes or furrows.

The fruits of *Emblica officinalis* have been reported to contain constituents with variable biological activity: they have been used for thousands of years in the traditional Indian medicine for the treatment of several diseases. For many years the therapeutic potential of the fruits was attributed to their high content of ascorbic acid: about 1 g of vitamin C per 100 ml of fresh juice.

In 1996 professor Shibhnath Ghosal of Banaras Hindu University discovered that *Emblica* fruits do not contain ascorbic acid neither in free nor in conjugated form, but it contains two new hydrolysable tannins with low molecular weight (B1000), called emblicanin A (2,3-di-*o*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-d-lactone) and emblicanin B (2,3,4,6-bis-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-d-lactone) and other tannins like punigluconino (2,3-di-O-galloyl-4,6-(*S*)-hexahydroxydiphenoylgluconicacid) and pedunclagin (2,3,4,6-*bis*-(*S*)-hexahydroxydiphenoyl-D-glucose) already isolated in other species, *Punica granatum* in particular. These two new tannins have a very strong antioxidant action. The two emblicanins A and B have been found to preserve erythrocytes against oxidative stress induced by asbestos, generator of superoxide radical. Emblicanin A oxidates when put in contact with asbestos becoming emblicanin B and together they have a stronger protective action to erythrocytes than vitamin C. Moreover they improve the efficacy of vitamin C in reducing dihydroascorbic acid to ascorbic acid.

The same recycling process has been observed in the rutin-vitamin C combination. Fruit extract has been shown to have an antimutagenic activity in Ames test. Recently fruits have been tested for their antiviral activity, particularly for inhibiting reverse transcriptase in the replication of retroviruses like HIV-1. Some active ingredients inhibit the replication of the virus through a non competitive action with respect to the dTTP but competitive action with respect to the template primer.

Other studies have shown that the fruit enhances the immunodefence and has a hypolipidemic effect. *Emblica* fruit is also effective in acute pancreatitis therapy and like hepatoprotective [221-229].

Recent studies by Sachdanandam et al. illustrated the beneficial outcome of the Siddha drug Kalpaamruthaa (KA) in reducing the pathological lesions caused by the proinflammatory cytokines in adjuvant induced arthritis (AIA) in rats. KA, consisting of *Semecarpus anacardium* nut milk extract (SA), dried powder of *Emblica officinalis* fruit and honey, significantly regulated the inflammation in arthritic joints by reducing extracellular matrix degradation and cartilage and bone destruction via down regulating the levels of TNF-a and IL-1β, as well the levels of acute phase proteins with appreciable increase in the levels of immunoglobulins in arthritic rats [230].

3.6.3 *SARACA ASOKA*

The Ashoka tree (*Jonesia Ashok* or *Sarac Indica*) is a plant belonging to the Caesalpiniaceae subfamily of the legume family [fig.20]. It is an important tree in the cultural traditions of the Indian Subcontinent and adjacent areas.

FIGURE 20 – *Saraca asoka* plant and fruits.

As a wild tree, the asoka is a vulnerable species. It is becoming rarer in its natural habitat, but isolated wild asoka trees are still to be found in the foothills

of central and eastern Himalayas, in scattered locations of the northern plains of India as well as on the west coast of the Subcontinent near Mumbai.

The asoka tree is considered sacred throughout the Indian subcontinent, especially in India and Sri Lanka. This tree has many folklorical, religious and literary associations in the region. Highly valued as well for its handsome appearance and the color and abundance of its flowers, the asoka tree is often found in royal palace compounds and gardens as well as close to temples throughout India. The asoka tree has a symbolic importance in Buddhism. Queen Māyā of Sakya is said to have given birth to the Buddha under an asoka tree in a garden in Lumbini.

The earliest chronicled mention is in the Ayurvedic treatise, the Charka Samhita (100 A.D.), in which Asoka is recommended in formulations for the management pain with relation to uterus (gynecological) as anodynes. The Bhavprakasha Nighantu, commonly known as the Indian Materia Medica (1500 A.D.), cites the plant as a uterine tonic: actually *Saraca asoka* is very famous for its use in treating gynecological disorders, it is known as an astringent to treat excessive uterine bleeding from various causes (including hormone disorders and fibroids), but also for regulating the menstrual cycle and, in various complex formulas, as a tonic for women. The bark, rich in tannins and cyanidins (red colored compounds), is the primary medicinal part [231-233].

Chloroform, methanol, aqueous and ethanolic extracts of the stem bark of Saraca indica were investigated for their antibacterial and antifungal activity against standard strains of Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Klebsiella pneumoniae, Proteus mirabilis, Salmonella typhimurium and Streptococcus pneumoniae and the fungi: Candida albicans and Cryptococcus albidus. Methanolic and aqueous extract exhibited antimicrobial activity with MIC ranging from 0.5-2% and 1-3% respectively. Methanolic extract exhibited the strongest activity against both bacteria and fungi [234].

Different forms of uses of Asoka herb are described:

- *Asoka fresh or dried bark*: it is bitter, astringent and sweet in taste. It has stimulating effect on endometrial and the ovarian tissue. It is useful in internal bleeding, hemorrhoids, ulcers, uterine affections, menorrhagia especially due to uterine fibroids, meno-metrorrhagia, leucorrhoea and pimples.
- *Asoka fresh flower*: it is an excellent uterine tonic and is used in cervical adenitis, biliousness, syphilis, hyperdipsia, burning sensation, hemorrhagic dysentery, piles, scabies in children and inflammation.
- *Asoka dried flowers*: they are used in diabetes.
- *Asoka seeds*: they are used in treating bone fractures, strangury and vesicle calculi.

We suggest that *Saraca asoka*, with its lengthy history of human use in traditional medicine, deserves consideration as a candidate drug for suppressing IL-8-dependent lung inflammation in CF.

AIM OF THESIS

Cystic fibrosis is an autosomal recessive genetic disorder common in Caucasian population caused by mutations in the CFTR gene and characterized by extensive lung inflammation. One of the hallmarks in CF is a chronic infection sustained by strikingly few pathogens including *Staphylococcus aureus* and *Haemophilus influenza*e, whereas in the course of their lifetime CF patients become progressively colonized by *Pseudomonas aeruginosa*. Elevated concentrations of pro-inflammatory cytokines and chemokines have been found in the bronchoalveolar fluid of patients with CF [118, 119]. Consistent with these findings, experimental models support the concept that interaction of *P.aeruginosa* with CF respiratory epithelial cells promotes PMN recruitment, mainly by releasing the chemokine interleukin 8 (IL-8) [116, 117]. Within the lumen of CF airways, nucleic acids derived from PMNs further reduce mucociliary clearance, and proteases released from PMNs mediate epithelium injury, progressive bronchial wall damage, bronchiectasis, and peribronchiolar fibrosis. Therefore, the exaggerated, PMN-dominated inflammatory process is thought to critically contribute to the gradual decline in CF lung function [113, 114].

Novel pharmacological correctors and potentiators of mutated CFTR protein leave many unanswered questions concerning their application in CF, including the degree of correction of the defect that can be obtained, the safety in long term administration and the efficacy in reverting the extensive lung inflammatory pathology [86]. Although anti-bacterial therapy has been shown to be more promising since early eradication therapy has been established in some centers, it remains questionable whether all patients will profit from this strategy [30-33]. In addition, besides the fact that anti-inflammatory molecules have demonstrated potential benefits in CF patients, enthusiasm has been tempered either by the limited efficacy or the occurrence of undesired effects. Therefore considering that at present no fully satisfactory anti-inflammatory treatment is available for clinical use in CF patients, investigation on novel anti-

inflammatory molecules for the treatment of lung inflammation is considered a major priority in the CF community (www.cff.org).

With respect to pro-inflammatory mechanisms, *P. aeruginosa* is known to stimulate surface respiratory epithelial cells to express and release IL-8 through activation of the transcription factor NF-kB [114, 115]. Besides IL-8, a large number of other genes contain kB sites in their 5'-upstream untranslated regions, including genes encoding cytokines, chemokines, and adhesion molecules that orchestrate PMN transbronchial migration, such as TNF-α, IL-1, GRO-γ, and intercellular adhesion molecule (ICAM)-1, together with different other genes regulating innate immunity. Thus, NF-kB has been proposed as one target to down-modulate the immune processes involved in different pulmonary diseases, including CF.

The transcription factor (TF) decoy approach has been proposed to modulate gene expression *in vitro*. This approach is based on the intracellular delivery of double-stranded oligodeoxynucleotides causing inhibition of the binding of TF related proteins to the different consensus sequences in the promoter of specific genes. Several decoy oligodeoxynucleotides have been studied to pursue the modulation of expression of genes relevant to human diseases. Due to its central role in immune response and cell cycle regulation, TF decoy oligodeoxynucleotides targeted to NF-kB have been studied in other and our laboratories [118-121].

To date, the inhibition of IL-8 expression by NF-kB decoy molecules has been limited both conceptually, by the potential lack of specificity related to the wide role of NF-kB in regulating expression of several genes, and practically, by the limited efficiency obtained in the preliminary experiments [235-237]. Thus, we addressed the issues of efficiency and specificity by testing the effect of novel NF-kB decoy sequences on the expression of different genes induced by *P. aeruginosa* in human bronchial epithelial cells carrying either the wild type or the mutant CFTR gene.

An other approach has been studied in our lab to modulate the pro-inflammatory gene expression *in vitro*. This second approach is based on extracts from plants used in ethnic medicine that are of great interest, as many

of them are known to retain anti inflammatory properties. This was reviewed by Darshan and Doreswamy [238], who reported about the anti inflammatory activity of drugs derived from 38 medicinal plants. In this review a clear role of botanical plants (including polysaccharides, terpenes, curcuminoids and alkaloids) was reported as alleviating inflammatory diseases, such as arthritis, rheumatism, acne skin allergy and ulcers. The most potent in curing inflammatory diseases were found to be plants containing polysaccharides [238]. In the present study, we analyzed the effects of *Bergamot*, *Emblica officinalis* and *Saraca asoka* extracts on the production of cytokines and chemokines in cystic fibrosis IB3-1 cells [239-241] induced to hyper secretion of inflammatory cytokines and chemokines by TNF-α [242-244].

In our studies we investigated the ability of the plant extracts and their main constituent drugs derived, to interfere with specific bindings between target DNA sequences and Rel/NF-kB proteins by EMSA assays (Electrophoretic Mobility Shift Assay). To use this compound for *in vitro* experiments, anti-proliferative assays have been made in cystic fibrosis IB3-1 cells model.

After determining the IC50 values (concentrations of extracts leading to 50% inhibition of IB3-1 cell growth), we analyzed the inhibitory effect of these compounds on gene expression of interleukin 8 (IL8) that is directly involved in the inflammation pathway via NF-kB activation. In particular, IB3-1 cells have been treated with different concentration of *Bergamot*, *Emblica officinalis, Saraca asoka* plant extracts and with increasing concentration of their main identified constituents *bergamottin, bergapten, citropten, pyrogallol*, *5-OH-isoquinoline, digitoxin*; the isolated RNA has been retro-transcribed (*Retro-Transcription PCR*) and amplified for the gene of interest IL-8 and the housekeeping GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

The plant extracts resulted more interesting have been used on IB3-1 cells induced with TNF-α (Tumor Necrosis Factor-α) to evaluate the inhibitory activity in this inflamed cellular model, since previous studies demonstrated that TNF-α cytokine, present in high concentration in CF patients, induces inflammatory mediators expression. Finally, Bio-Plex technology has been used to analyze the potential inhibitory effect of plant extracts on the expression of cytokines in

IB3-1 cells induced by TNF-α. The Bio-Plex suspension array system helps to reveal a more complete view of the effects of candidate drugs on the biology of cells.

MATERIALS AND METHODS

1. CELL CULTURES

IB3-1 cells (LGC Promochem), derived from a CF patient with a DF508/W1282X mutant genotype, were immortalized with adenovirus 12-SV40 [fig.21]. The cells were grown in LHC-8 basal medium (Biofluids), supplemented with 5% FBS in the absence of gentamycinin in a humidified atmosphere of 5% CO2 in air and 37℃ [239]. Treatment with TNF-α (80 ng/ml) was performed on 70% confluent cells for 24 hours to induce the inflammation on IB3-1 cells.

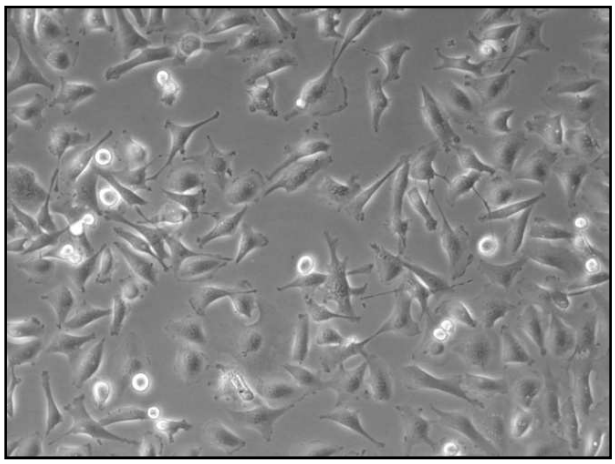


FIGURE 21 – IB3-1 cells

2. CELL INFECTION WITH *PSEUDOMONAS AERUGINOSA*

PAO1, a well-characterized, nonmucoid motile laboratory strain of *P. aeruginosa*, kindly donated by Alice Prince (Columbia University, New York, New York), was grown in trypticase soy broth (TSB) or agar (TSA) (Difco, Detroit, MI) [239]. Bacteria colonies from overnight cultures on TSA plates were grown with shaking in 20 ml TSB broth at 37℃ until an optical density (at 660 nm) corresponding to 1 x 10^9 colony-forming units (CFU)/ml was reached, as determined by dilution plating. Bacteria were washed twice in PBS and finally

diluted in each specific serum-free culture medium before infection. All cell lines were infected with ranging doses of PAO1 at 37℃ for 4 hours.

3. DECOY OLIGODEOXYNUCLEOTIDES

Decoy oligodeoxynucleotides (ODNs) mimicking the NF-kB consensus sequences identified in the HIV-1 long terminal repeat (LTR) of the promoter region of HIV-1 gene (sense: 5'-CGC TGG GGA CTT TCC ACG G-3') were used. A ''scrambled'' ODN sequence (sense: 5'-CAC AAA GTG TAA CAG TCT-3') was used in each experiment. Synthesis and high-performance liquid chromatography–grade purification was obtained from Sigma. Sense and anti-sense freeze-dried ODNs were resuspended in DNAse-free sterile water at the stock concentration 5 mg/ml. Annealing to obtain double-stranded ODNs was performed by mixing equimolar concentrations of both forward and reverse strands (1 mg/ml in 150 mM NaCl, final concentration), incubating the solution at 100℃ for 5 minutes, then leaving it overnight at room temperature.

4. TRANSFECTION OF CELLS WITH DECOY ODNS

IB3-1 cells have been transfected with ODNs by mixing the double-stranded ODNs with the cationic liposome Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Lipofectamine 2000 (4ml) was diluted in 1ml LHC-8 serum-free cell culture medium (Biofluids), according to the manufacturer's instructions. Double-stranded decoy or scrambled ODNs (2 mg) were added and incubated for 10 minutes. Liposome:DNA complexes in LHC-8 serum-free medium were added to IB3-1cells and incubated at 37℃ and 5% CO_2 for 6 hours. The cells were washed twice with culture serum-free medium and left at 37℃ and 5% CO_2 for a further 18 hours before infection with *P. aeruginosa*.

5. PLANT EXTRACTS AND CHEMICAL

Bergamot oil was extracted by cold pressing of peels, and it is made up of 93-96% volatile compounds high quantities of oxygenated compounds.
The dried fruits of *Emblica officinalis* were extracted with absolute ethanol and the yield was 9.33%.
The stem barks of *Saraca asoka* were also extracted with absolute ethanol and the obtained yields were 14.16%.
Bergamottin, bergapten and *citropten* were purchased from Extrasynthese (Lyon, France) and resuspended in ethanol and 3% dimethil-sulfoxide (DMSO). *Pyrogallol*, *5-OH-hydroquinoline*, *Digoxin* were furnished from Sigma-Aldrich (Milwaukee, WI, USA) and resuspended in water (*pyrogallol*) or methanol (*5-OH-hydroquinoline* and d*igoxin*). All the resuspended compounds were conserved at -20℃ protected from light.

6. ASSAYS OF *IN VITRO* ANTIPROLIFERATIVE ACTIVITY

In vitro antiproliferative activity was assayed as follows. Cells were seeded at the initial concentration of 30.000 cells/cm^2 and then cultured in the absence or in the presence of increasing concentration of plants extracts or purchased chemical drugs. The medium was not changed during the induction period. For the IC50 (concentrations of extracts leading to 50% inhibition of IB3-1 cell growth), we determined the values of cell number/ml after 3 days of culture, when untreated cells are in the log phase of cell growth.

7. PREPARATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared from IB3-1 cells as described [246]. Cell were washed twice with PBS and detached by trypsinization. Nuclear proteins were obtained by hypotonic lysis, followed by high-salt extraction treatment of nuclei

Protein concentration was determined using Bio-Rad protein assay. Nuclear extracts were brought to a concentration of 0.5 mg/ml for electrophoretic mobility shift assay (EMSA) experiments.

8. ELECTROPHORESIS MOBILITY SHIFT ASSAY (EMSA)

Electrophoresis mobility shift assay (EMSA) was performed by using double-stranded ^{32}P-labelled oligonucleotides as target DNA [247, 248]. Oligodeoxynucleotides were labeled with γ^{32}-P-ATP using 10 Units of T4-polinucleotidekinase (MBI Fermentas, St. Leon-Rot, Germany) in 500 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA in the presence of 50 mCi γ^{32}-P-ATP) in a volume of 20 µl for 45 minutes at 37℃. Reaction was brought to 150 mM NaCl, and 150 ng complementary oligodeoxynucleotide was added. Reaction temperature was increased to 100℃ for 5 minutes and left diminishing to room temperature overnight. Binding reactions were set up as described elsewhere in binding buffer (10% glycerol, 0.05% NP-40, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10 mM MgCl2), in the presence of poly(dI:dC).poly(dI:dC) (Pharmacia, Uppsala, Sweden), 2-5 µg of crude nuclear extracts [246] and 0.25 ng of labeled oligonucleotide, in a total volume of 20 µl [247, 248]. After 30 minutes of binding at room temperature samples were electrophoresed at constant voltage (200 V for 1 hr) through a low ionic strength (0.25x TBE buffer) (1x TBE = 0.089 M Tris-borate, 0.002 M EDTA) on 6% polyacrylamide gels until tracking dye (bromophenol blue) reached the end of a 16 cm slab. Gels were dried and exposed for autoradiography with intensifying screens at -80℃. In these experiments, DNA/protein complexes migrate through the gel with slower efficiency.

To study inhibition of protein/DNA interactions, addition of the reagents was as follows: (a) poly(dI:dC).poly(dI:dC); (b) labelled oligonucleotides mimicking the binding sites for transcription factors to be modulated; (c) *Saraca asoka*, *Emblica officinalis* or *Bergamot* extracts; (d) binding buffer; (e) nuclear factors.

The nucleotide sequences of double-stranded target DNA utilized in these experiments were 5'-CGC FGG GGA CTT TCC ACG G-3' (sense strand, NF-kB) (Sigma Genosys, Cambs, UK).

To study the activation of NF-kB in the inflammatory cascade, we exposed our model system of IB3-1 cells to the *P. aeruginosa* laboratory strain PAO1 infection: EMSA was perfomed as previously described with double-stranded synthetic oligodeoxynucleotides mimicking the NF-kB–binding site present in the promoter of the IL-8 gene (IL-8 NF-kB, sense: 5'-AAT CGT GGA ATT TCC TCT-3') incubated with nuclear extracts from IB3-1 cells or purified NF-kB p50 dimer protein (Promega, Madison, WI) were used at the specified concentrations and poly(dI:dC) (1 mg per reaction) was also added to abolish nonspecific binding.

For quantitative determinations, the bands corresponding to protein-DNA complexes were analyzed using the BIO-RAD Gel Doc 2000 (Bio-Rad Laboratories). The values obtained in the reactions performed in the presence of plant extracts or purified NF-kB p50 dimer protein were compared to control untreated reactions.

9. QUANTIFICATION OF IL-8 TRANSCRIPTS AND OTHER PRO-INFLAMMATORY GENES

Total RNA was isolated (Trizol reagent, Invitrogen), retro transcribed (Reverse Transcription System, Promega Corporation, Medison, USA) and the resulting cDNA was quantified by relative quantitative real-time PCR [245]. The sequences of the oligonucleotides used for amplification of IL-8 mRNA were: 5'-GTG CAG TTT TGC CAA GGA GT-3' (forward) and 5'-TTA TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC-3' (reverse); for β-actin mRNA: 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' (forward); 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3' (reverse). PCR was performed in a final volume of 50 μl containing 50 mM KCl, 10 mM TRIS-HCl pH 8.8, 1.5 mM $MgCl_2$ by using 1U/reaction of Taq DNA polymerase, 100 μM dNTPs, 0.5 μM

PCR primers. The 30 PCR cycles used were as follows: denaturation, 30s, 94 ℃; annealing, 60s, 68 ℃; elongation, 60s, 72℃. T he length of the IL-8 PCR product was 236 bp [240, 241]. The marker pUC mix 8 (Fermentas, Milan, Italy) at 0.5 μg/lane was used.

For quantitative real-time PCR reaction, 0.5/20 μl aliquots of cDNA were used for each Sybr Green real-time PCR reaction to quantify the relative tissue expression of IL-8 transcripts and other pro-inflammatory genes. Primer sequences and concentration are shown in Table 4. Primer sets were purchased from Sigma-Genosys (The Woodlands, TX).

Primer	Sequence (5' to 3')	Accession n°	nM
IL8-F	GACCACACTGCGCCAACA	AF385628.2	15
IL8-R	GCTCTCTTCCATCAGAAAGTTACATAATTT		15
GROγ-F	CCGGACCCCACTGCG	M36821	2.5
GROγ-R	TTCCCATTCTTGAGTGTGGCTA		15
ICAM1-F	TATGGCAACGACTCCTTCTCG	NM_000201	15
ICAM1-R	CTCTGCGGTCACACTGACTGA		15
IP10-F	AGTGGCATTCAAGGAGTACCTCTC	NM_001565.1	15
IP10-R	GATGCAGGTACAGCGTACGGT		2.5
RANTES-F	CTACACCAGTGGCAAGTGCTC	NM_002985.2	15
RANTES-R	TTTCGGGTGACAAAGACGACT		2.5
MIP1α-F	CTACTTTGAGACGAGCAGCCAG	NM_002983.1	45
MIP1α-R	GGTTAGGAAGATGACACCGGG		15
TNFα-F	GGACCTCT CTCTAATCAGCCCTC	NM_000594	2.5
TNFα-R	TCGAGAAGATGATCTGACTGCC		2.5
IL1β-F	CTCCACCTCCAGGGACAGGA	BT007213.1	45
IL1β-R	GGACATGGAGAACACCACTTGTT		15
IL6-F	CGGTACATCCTCGACGGC	NM_000600	2.5
IL6-R	CTTGTTACATGTCTCCTTTCTCAGG		45

TABLE 4 – Primers for quantification of gene expression

Each 25 µl of total reaction volume contained 0.5 µl of cDNA, 10 pmol of primers, 1x iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA). Real-time PCR reactions were performed for a total of 40 cycles using an iCycler IQ® (Bio-Rad Laboratories, Hercules, CA). The relative proportions of each template amplified were determined based on the threshold cycle (*Tc*) value for each PCR reaction. The ΔΔCt method was used to compare gene expression data. Each sample was quantified in duplicate from at least two independent experiments. Mean ± S.D. values were determined for each fold difference. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA served as internal standards (housekeeping gene). Duplicate negative controls (no template cDNA) were also run with every experimental plate to assess specificity and indicate potential contamination.

10. CYTOKINE PROFILES

Cytokines in tissue culture supernatants released from the cells under analysis, were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA) [249, 250] as described by the manufacturer.

The Bio-Plex system is a flexible, easy-to-use multiplex analysis system that permits the simultaneous analysis of up to 100 different biomolecules (proteins, peptides, or nucleic acids) in a single microplate well. By multiplexing with the Bio-Plex system, researchers can dramatically increase the amount of useful information from rare or volume-limited samples such as cell colture supernatants, and decipher complex interrelationships among proteins involved in signal transduction pathways. The microplate platform allows the automated analysis of 96-well plates with a throughput of more than 1,800 assay points (for a 23-plex assay) in 30 minutes.

The Bio-Plex suspension array is built around three core technologies. The first is the family of fluorescently dyed microspheres, or beads, from Luminex Corp. The second is a flow cytometer with two lasers and associated optics to measure biochemical reactions that occur on the surface of the microspheres,

and the third is a high-speed digital signal processor to efficiently manage the fluorescent output. These technologies are brought together in the Bio-Plex workstation [fig.22].

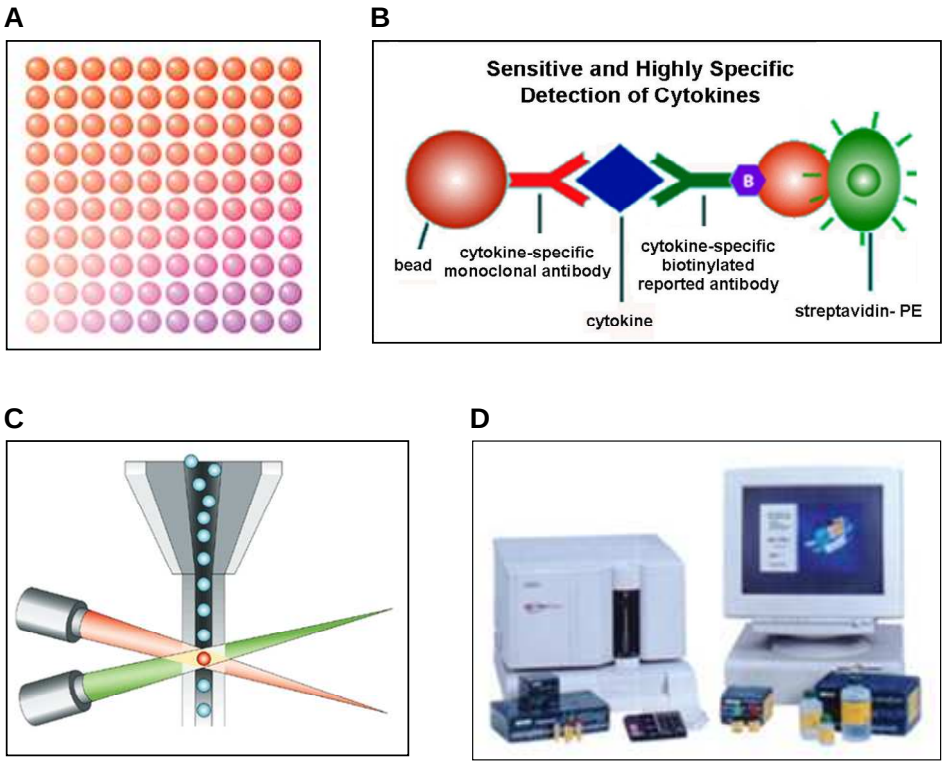


Figura 22 - **(A)** 100-Plex Luminex^{TM}; **(B)** schematic sandwich immunoassay; **(C)** Red classify laser e Green reporter laser; **(D)** Bio-Plex workstation (BIORAD).

The Bio-Plex suspension array system uses the multiplexing technology of Luminex Corp. to enable the simultaneous quantitation of up to 100 analytes. This technology uses polystyrene beads internally dyed with differing ratios of two spectrally distinct fluorophores. Each fluorophore can have any of 10 possible levels of fluorescent intensity, thereby creating a family of 100 spectrally addressed bead sets [fig.22 (A)].

Bio-Plex assays contain dyed beads conjugated with monoclonal antibodies specific for a target protein or peptide such as a cytokine or a phosphoprotein. Each of the 100 spectrally addressed bead sets can contain a capture antibody specific for a unique target protein. The antibody-conjugated beads are allowed to react with sample and a secondary, or detection, antibody in a microplate well to form a capture sandwich immunoassay. Multiplex assays can be created

by mixing bead sets with different conjugated antibodies to simultaneously test for many analytes in one sample [fig.23].

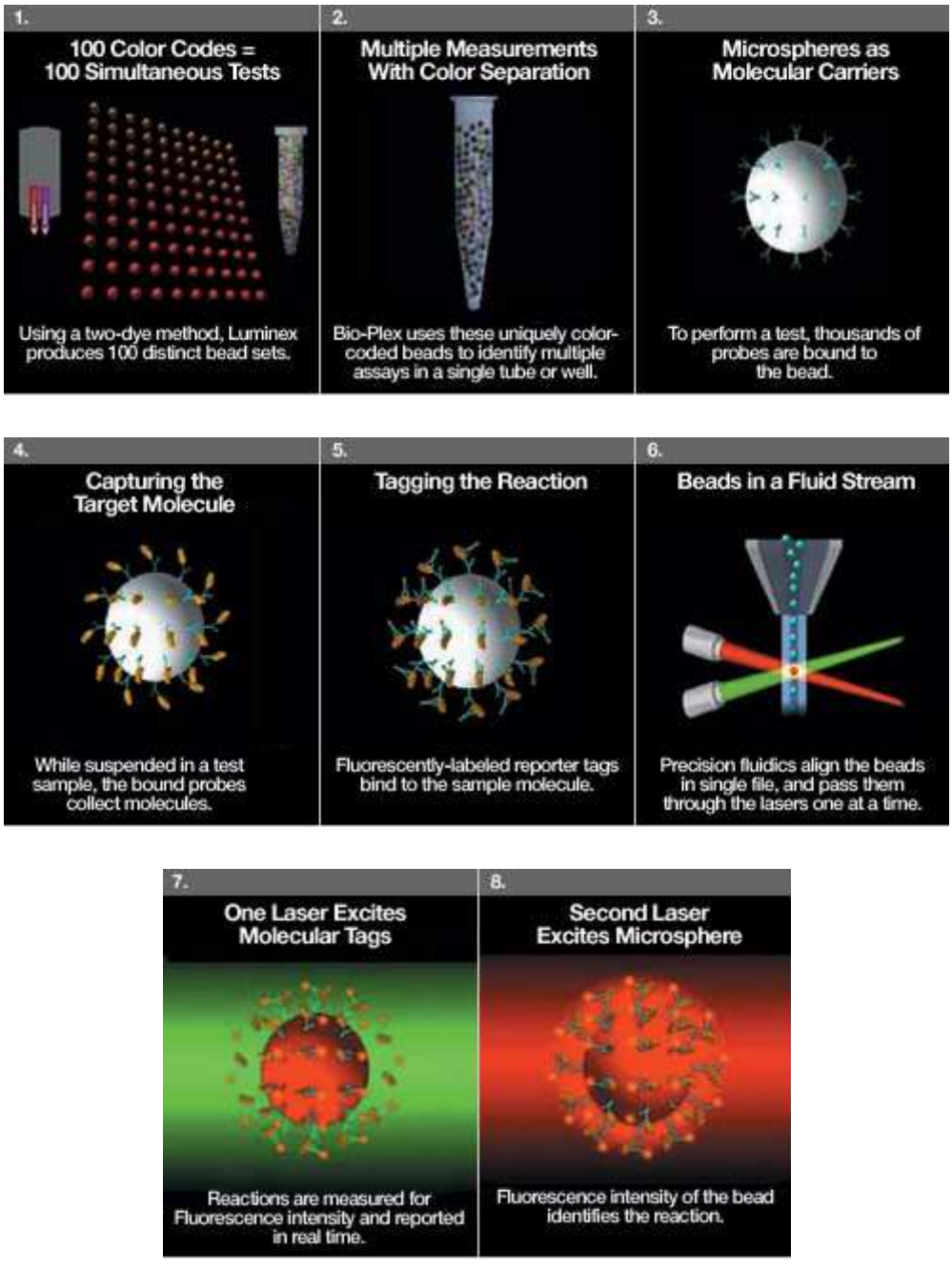


FIGURE 23 - Bio-Plex technology

The assay solution is drawn into the Bio-Plex array reader, which illuminates and reads the sample. When a red diode "classification" laser (635 nm) in the

Bio-Plex array reader illuminates a dyed bead, the bead's fluorescent signature identifies it as a member of one of the 100 possible sets. Bio-Plex Manager software correlates each bead set to the assay reagent that has been coupled to it (for example, an IL-2 capture antibody coupled to bead #36). In this way the Bio-Plex system can distinguish between the different assays combined within a single microplate well. A green "reporter" laser (532 nm) in the array reader simultaneously excites a fluorescent reporter tag (phycoerythrin, or PE) bound to the detection antibody in the assay. The amount of green fluorescence is proportional to the amount of analyte captured in the immunoassay. Extrapolating to a standard curve allows quantitation of each analyte in the sample. Patented digital signal-processing algorithms provide simultaneous real-time acquisition of classification and reporter signal output from thousands of beads per second, supporting up to 100 x 96 = 9,600 analyte measurements from each 96-well plate.

In our experiments, the custom-made Bio-Plex human cytokine Human 7-Plex Panel, which included seven cytokines [IL-1rα, IL-6, IL-8, G-CSF, MCP-1 (MCAF), RANTES, VEGF] were used. 50 µl of cytokine standards or samples (supernatants from treated cells) were incubated with 50 µl of anti-cytokine conjugated beads in 96-well filter plates for 30 min at room temperature with shaking. Plates were then washed by vacuum filtration three times with 100 µl of Bio-Plex wash buffer, 25 µl of diluted detection antibody were added, and plates were incubated for 30 min at room temperature with shaking. After three filter washes, 50 µl of streptavidin-phycoerythrin was added, and the plates were incubated for 10 min at room temperature with shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer, and samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad Laboratories, Hercules, CA).

11. GC-FID AND GC-MS ANALYSES

The sample extracts were analyzed and the relative peak areas for different components weighted by gas chromatography-flame ionization detection (GC-FID). The relative percentages were determined using GC TRACE Thermoquest, equipped with autosampler Triplus Thermo Electron Corporation. The column was VF-5ms, 30 m x 0.25 mm. Flow rate was 1.0 mL/min He, and split 1:50. Injector temperature, 300 ℃; detector temperature, 350 ℃. Oven temperature was initially 55 ℃, raised to 100 ℃ a t a rate of 1 ℃/min, and then raised to 250 ℃ at a rate of 5 ℃/min and finally held at that temperature for 15 min. One µl of each sample (15 mg/mL CH_2Cl_2) was injected. The percentage composition of different components was computed by the normalization method from the GC peak areas (data integration software: Jasco-Borwin version 1.5, JMBS Developments, Fontaine, France), without correction factors. Identification was performed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer with electron impact and hooked to a NIST (National Institute of Standards and Technology) library. A Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm) was used. Operating conditions were as follows: injector temperature 300 ℃; FID temperature 300 ℃, Carrier (Helium) flow rate 1.0 mL/min and split ratio 1:50. The MS (mass spectrometry) conditions were as follows: ionization voltage, 70 eV; emission current, 10 µAmp; scan rate, 1 scan/s; mass range, 29-400 Da; trap temperature, 150 ℃, transfer line temperature , 300 ℃. The main compounds were identified by comparing their relative retention time, KI (Kovats Index) and the MS fragmentation pattern with pure compounds and by matching the MS fragmentation patterns with the above mentioned mass spectra libraries and with those in the literature [251]. To determine the Kovats index of the components, an alkenes mixture (C_8–C_{24}) was added to the sample extracts before injecting in the GC–MS equipment and analyzed under the same conditions as above.

12. HPLC ANALYSIS

High-pressure liquid chromatography (HPLC) analysis was performed to quantify the main constituents. Therefore, pure commercial standards were used as external standards to set up and calculate appropriate calibration curves. The experimental conditions were performed using a Jasco modular HPLC (Tokyo, Japan, Model PU 2089) coupled to a Diode Array apparatus (MD 2010 Plus) linked to an injection valve with a 20 µL sampler loop. The column used was a Tracer extrasil ODS2 25x0.46 cm, with a flow rate of 1.0 mL/min. The mobile phase employed consisted of the solvent solution B (methanol) and A (water/ formic acid = 95/5). The gradient system adopted was characterized by 4 steps: 1. isocratic, with solvent solution B/A=40/60 (%), for 2 min; 2. the solvent solution B raised progressively from 40 to 60% in 18 min (from min 2 to min 20) until reaching the ratio B/A=60/40 (%); 3. the solvent solution B then raised 100% in 4 min (from min 20 to min 24); 4. the solvent solution ratio reached B/A=40/60 in 4 min (from min 24 to min 28). Injection volume, 40.0 µl. Chromatograms were recorded and peaks from sample extracts were identified by comparing their spectra with spectra obtained with pure standards. Peak area was determined by integration using dedicated Borwin software (Borwin ver. 1.22, JMBS Developments, Grenoble, France). The qualitative and quantitative analysis of each extract was performed 3 times.

13. ^{1}H NMR FINGERPRINTING ANALYSIS

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini-400 spectrometer operating at 399.97 MHz and at temperature of 303 K. Sample extracts (10 mg) were dissolved in deuterated chloroform (0.8 mL) in a 5mm NMR tube; solvent signal was used for spectra calibration (^{1}H 7.26 ppm). ^{1}H spectra were run using a standard pulse sequence “s2pul”. ^{1}H NMR spectra in $CDCl_3$ of the main constituents of plant extracts were compared with spectra obtained at the Spectral Database for Organic Compounds (SDBS free site

organized by National Institute of Advanced Industrial Science and Technology -AIST- Japan).

14. STATISTICAL ANALYSIS

Data are presented as the mean ± SEM from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student's t-test. A P value < 0.005 was considered statistically significant.

RESULTS

1. INDUCTION OF PRO-INFLAMMATORY GENES BY *P. AERUGINOSA* IN IB3-1 CELLS

Respiratory epithelial cells paving the conductive airways are known to play a key role as early sensors of the presence of pathogens by expressing a series of soluble molecules aimed at recruiting and activating immune cells. *P. aeruginosa*, a critical pathogen in CF chronic lung pathology, is known to induce a proinflammatory response in respiratory epithelial cells. Therefore, we exposed the bronchial epithelial cell line IB3-1, which expresses the DF508/W1282X-mutated CFTR protein, to the *P. aeruginosa* laboratory strain PAO1 for 4 hours, and total RNA was extracted to quantify transcripts. As shown in figure 24, PAO1 induced a very strong up-modulation of (1) IL-8 and GRO-γ, which recruit polymorphonuclear neutrophils (PMNs); (2) of ICAM-1, which cooperates as an adhesion molecule in driving the migration of leukocytes through both endothelial and epithelial monolayers; and (3) of the pro-inflammatory cytokines IL-1β and IL-6 in IB3-1 cells. These data confirm the role of bronchial epithelial cells in the early induction of pro-inflammatory stimuli upon exposure to *P. aeruginosa*.

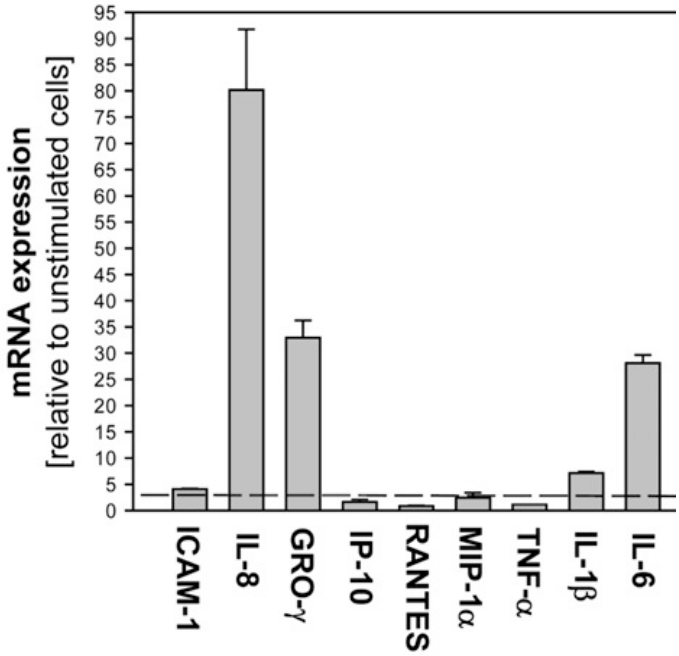


FIGURE 24 - Effects of *P. aeruginosa*-dependent cytokine transcription on IB3-1 cells.

2. SEARCH FOR TRANSCRIPTION FACTOR BINDING SITES WITHIN THE PROMOTERS OF PRO- INFLAMMATORY GENES

The *P. aeruginosa* infection of bronchial epithelial cells is known to induce expression of genes of the innate immunity by activating different TFs, such as NF-kB, AP-1, Elk-1, and NF–IL-6 [195-197]. To propose a TF decoy approach to down-modulate gene expression, the analysis of the promoter sequences of the target genes is very important. To this end, we evaluated the presence of TF signals within the genes modulated by PAO1 treatment of IB3 cells. The analysis reported in figure 25 describes the presence of several sequences that are putative targets of transcription factors. Among these sequences, NF-kB binding sites are present in all the promoters analyzed. Since NF-kB plays a well known pivotal role in inflammatory processes, we choose it as the first molecular target for the TF decoy approach.

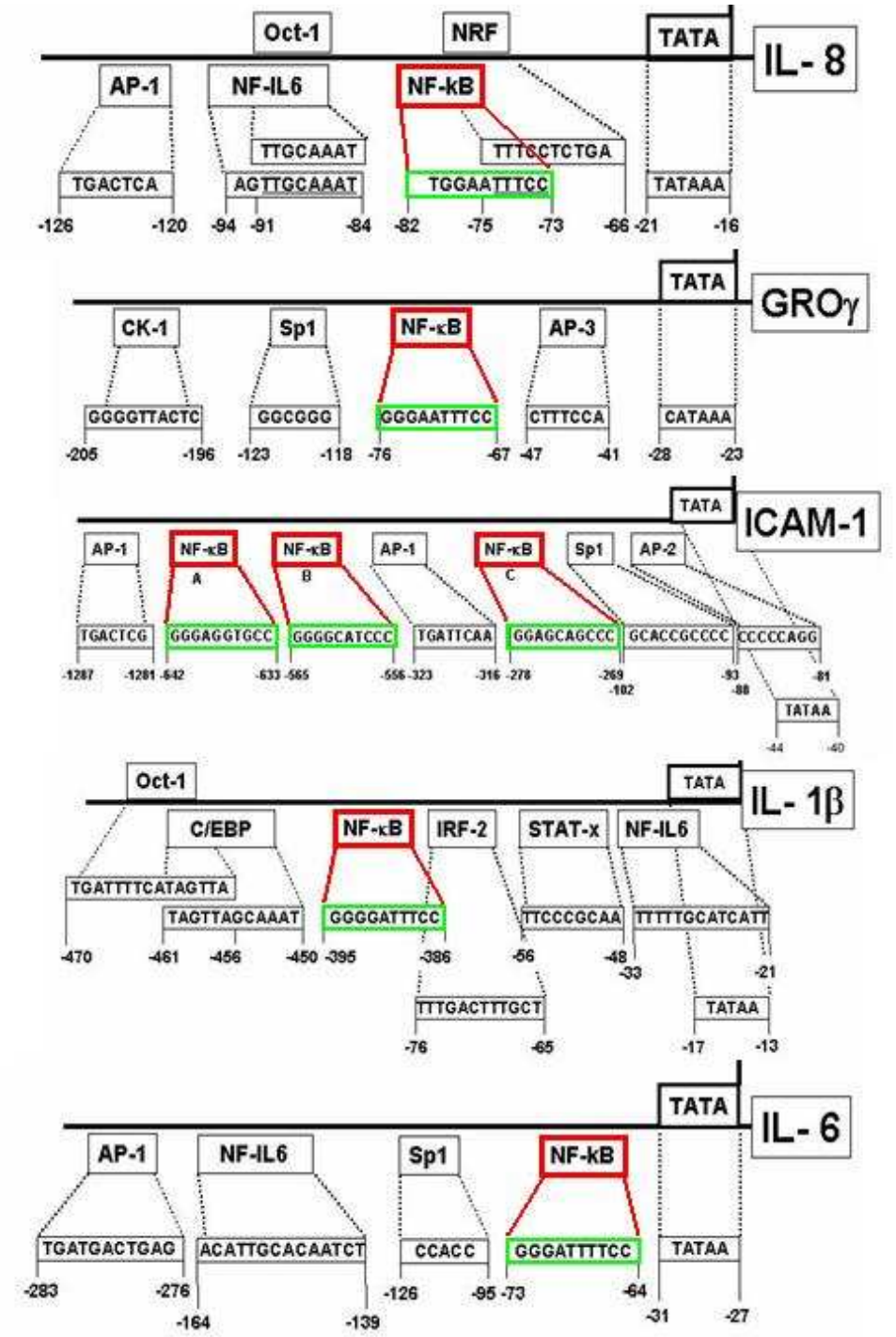


FIGURE 25 - Schematic representation of the position of the consensus sequences of the major transcription factors identified in the 59-untranslated regions of IL-8, GROγ, ICAM-1, IL-1β and IL-6 genes (35, 44–47). Transcription factor consensus sequences have been completed by the free-access TF-search software (http://www.cbrc.jp/research/db/TFSEARCH.html).
The positions of the TATA box and of the consensus sequences identified have been reported relatively to the translation start sites of each gene.

3. TRANSCRIPTION FACTOR DECOY

3.1 EFFECT OF NF-kB DECOY ODNs ON NUCLEAR EXTRACTS

Due to its role in recruiting PMNs in the bronchial wall and lumen of the patients, down-modulation of IL-8 is considered a key target to reduce the damage of the CF airway tract. Since consensus sequence for the transcription factor NF-kB is contained in the promoter of IL-8 gene, the CF bronchial IB3-1 cells were transfected with TF decoy ODNs that have been previously designed to mimic the DNA binding site of NF-kB and tested in their capacity to interfere with the NF-kB dependent transcription [235]. A double-stranded ODN homologous to the NF-kB binding site present in the LTR of the immunodeficiency type I virus (HIV-1 LTR NF-kB decoy ODN) has been previously demonstrated by our research group to efficiently interact with NF-kB p52 transcription factor [252]. Moreover, a new decoy ODN homologous to the NF-kB consensus sequence identified in the promoter of the IL-8 gene (IL-8 NF-kB decoy ODN) was designed and prepared for this purpose. We exposed our model system of cells to the *P. aeruginosa* laboratory strain PAO1 and studied the activation of NF-kB by EMSA. As shown in figure 26-A, *P. aeruginosa* increases NF-kB TF, as expected from previously reported data.

To test the ability of the decoy ODNs to compete for the binding of NF-kB TF with the sequence contained in the promoter of IL-8, all the decoy ODNs were incubated with nuclear extracts from IB3-1 cells in the presence of a radiolabeled probe 100% homologous with the IL-8 NF-kB sequence, and EMSA was performed. Complete inhibition of interaction of the ^{32}P labeled IL-8 NF-kB probe with NF-kB proteins present in the nuclear extracts (NF-kB:DNA complex) was obtained with very low concentrations of the IL-8 NF-kB cold probe and both HIV-1 LTR 5 and 10 ng, as shown in figure 26-B. To further validate the specificity of the competition, the same ODNs were used in the binding experiments of the ^{32}P-labeled IL-8 NF-kB probe with purified TF NF-kB

subunits. Pre-incubation of the HIV-1 LTR ODN with purified NF-kB p50 dimer completely abolish the binding to the ^{32}P-labeled IL-8 NF-kB probe, as reported

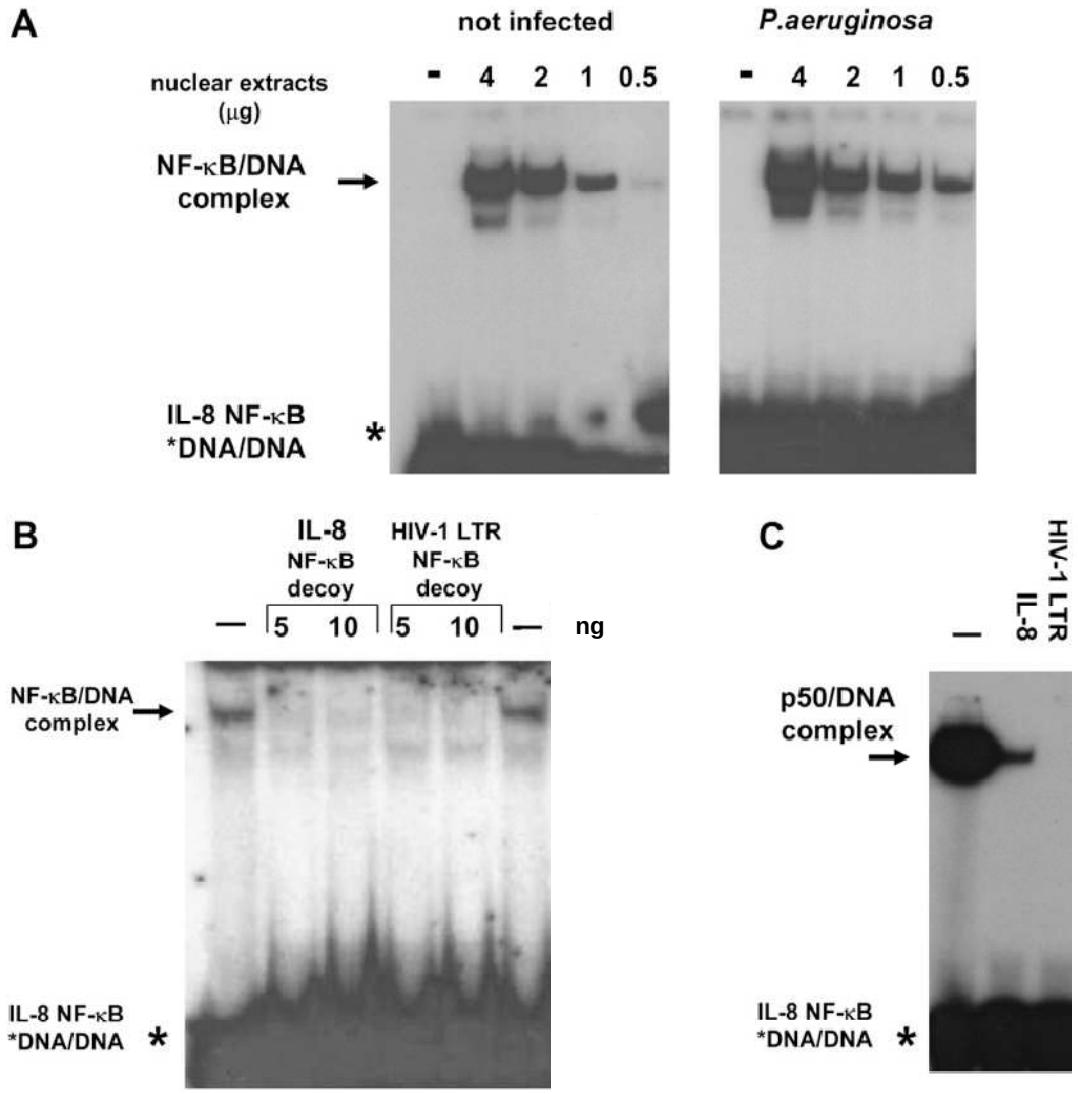


FIGURE 26 - Effect of decoy ODNs on molecular interactions between nuclear proteins and the ^{32}P-labeled IL-8 NF-Kb DNA target molecule. **(A)** IB3-1 cells ($2x10^6$ cells/Petri dish) were infected with *P. aeruginosa* laboratory strain PAO1 (20 cfu/cell) or PAO1 suspension medium (not infected) for 2 hours. Nuclear extracts (from 0.5 to 4 mg) were incubated for 20 minutes in the presence of ^{32}P-labeled IL-8 NF-kB 18-mer probe. Protein:DNA complexes were separated by polyacrilamide gel electrophoresis. The arrow indicates the protein:DNA complexes. The asterisk indicates the free ^{32}P-labeled 18-mer probe; (-) indicates ^{32}P-labeled 18-mer probe without nuclear extracts. **(B)** Nuclear extracts from IB3-1 cells (3 mg) were incubated for 20 minutes in the absence (-) or presence of the indicated amounts (5 or 10 ng) of the HIV-1 LTR NF-kB or IL-8 NF-kB cold probe DNA/DNA molecules. After this incubation period, a further 20-minute incubation step was performed in the presence of ^{32}P-labeled IL-8 NF-kB 18-mer probe. **(C)** Purified NF-kB p50 dimer protein (10 ng) was incubated for 20 minutes in the absence (-) or presence of 100 ng of the HIV-1 LTR NFkB or IL8 NF-kB cold probe DNA/DNA molecules.

in figure 26-C. Interestingly, the competition of the HIV-1 LTR ODN was even more effective than that obtained with the IL-8 NF-kB decoy ODN, with a core sequence 100% homologous to the consensus sequence contained in the promoter of IL-8, that shows the presence of a residual, albeit very small, p50:DNA complex [fig.26-C]. Altogether, these EMSAs provide proof of principle of the competition of these NF-kB decoy ODNs for the DNA consensus sequence contained in the promoter of the IL-8 gene.

3.2 EFFECT OF NF-kB DECOY ODNs IN IB3-1 CELLS

To test the effect of ODNs on gene transcription, complexes of cationic liposomes with NF-kB or scrambled ODNs were pre-incubated with IB3-1 cells 24 hours before exposure to the PAO1 laboratory strain of *P. aeruginosa* for a further 4 hours. HIV-1 LTR NF-kB decoy ODN strongly inhibited transcription of IL-8 mRNA, whereas, as reported in the dose-response experiment shown in figure 27-A, the IL-8 NF-kB decoy ODN resulted in a less potent effect with respect to bacteria-treated cells, either when compared with untreated or scrambled ODN-treated IB3-1 cells. The dose-response experiments reported in figures 27-B suggest that the HIV-1 LTR NF-kB exhibits its maximal inhibitory effect already at a low concentration.

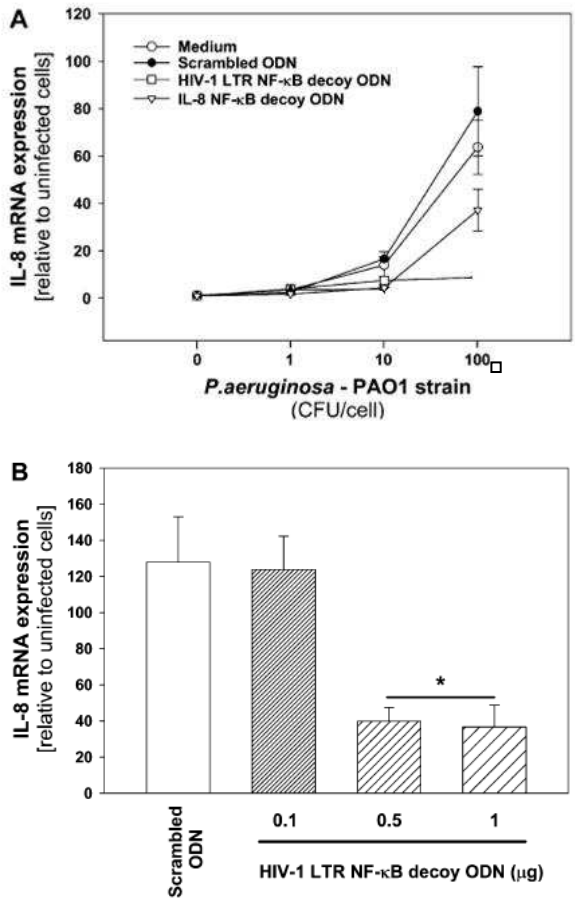


FIGURE 27 - Effect of NF-kB decoy ODNs on *P. aeruginosa* dependent induction of IL-8 mRNA in IB3-1 cells. **(A)** Cells were pre-incubated for 24 hours with HIV-1 LTR, IL-8 NF-kB decoys, scrambled ODNs, or medium alone before infection with *P. aeruginosa*, PAO1 strain. Total RNA was extracted 4 hours after infection and IL-8 mRNA was quantified as described in MATERIALS AND METHODS. **(B)** Effect of HIV-1 LTR NF-kB decoy ODN on IL-8 transcription after infection with PAO1 (100 CFU/cell) for 4 hours. Data are mean 6 SEM and are representative of three separate experiments.

Since DNA-binding sequences for the TF NF-kB have been reported also in the promoters of the other four genes that we found induced by *P. aeruginosa* in IB3-1 cells, as described in figure 24, the question arose if whether, in addition to IL-8, the NF-kB decoy ODNs inhibit the transcription of other pro-inflammatory genes induced by PAO1. HIV-1 LTR NF-kB decoy ODN did not show inhibitory effects on the *P. aeruginosa*-dependent transcription of GRO-γ, ICAM-1, and IL-1β and only minor and not significant effects were observed of the expression of IL-6 gene, as shown in figure 28.

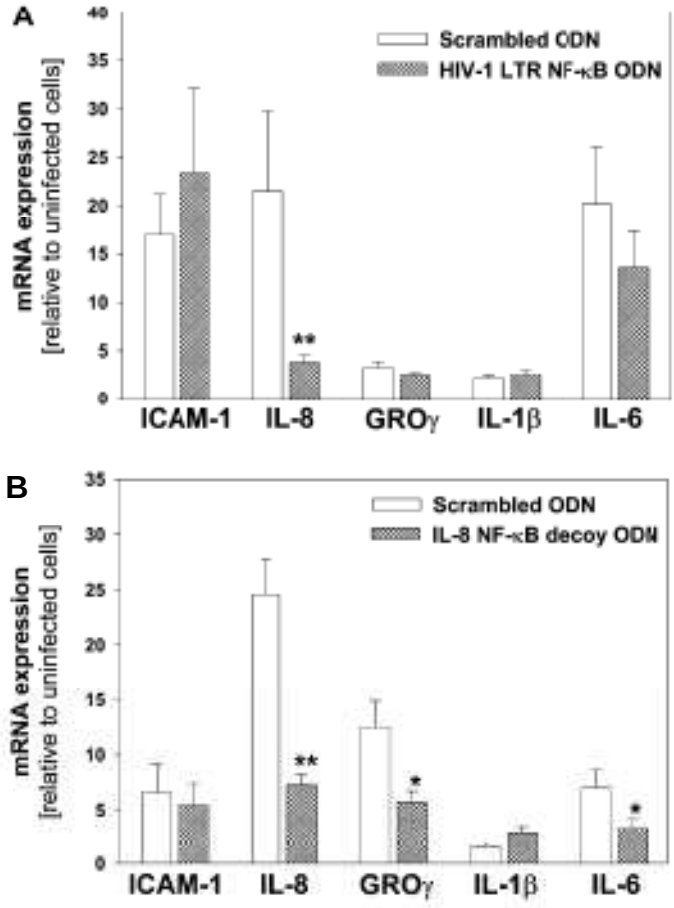


FIGURE 28 - Effect of NF-kB decoy ODNs on induction of different cytokines. HIV-1 LTR NF-kB decoy ODN was tested on transcription of ICAM-1, IL-8, GROγ, IL-1β, and IL-6 genes in **(A)** IB3-1 bronchial cells derived from patients with CF after infection with *P. aeruginosa*. **(B)** Effect of IL-8 NF-kB ODN decoy on cytokine transcripts induced by infection with *P. aeruginosa* in IB3-1 cells. Decoy or scrambled ODNs were pre-incubated with IB3-1 cells for 24 hours before infection with PAO1 (150 cfu/cell) for 4 hours. Total RNA was extracted and processed for quantification of transcripts as described in MATERIALS AND METHODS. Values are mean 6 SEM of four separate experiments. Significance in Student's t test between each scrambled and decoy ODNs: *P<0.05 or **P<0.01.

The newly designed IL-8 NF-kB decoy ODN was also tested on the transcription of the different genes induced by *P. aeruginosa*. As shown in figure 28-B, IL-8 NF-kB decoy ODN inhibits IL-8 transcription induced by *P. aeruginosa*, although with lower efficiency than that observed with HIV-1 LTR NF-kB, together with a partial inhibition of *P. aeruginosa* dependent transcription of GRO-γ and IL-6. So far, the main message from these results indicates that newly designed decoy ODNs directed against the transcription factor NF-kB can inhibit *P. aeruginosa* dependent transcription of IL-8. Since different NF-kB decoy ODNs

have been previously tested in respiratory cells stimulated exposed to the pro-inflammatory cytokine TNF-α, obtaining a much lower degree of inhibition [236], we performed further experiments, exposing IB3-1 cells to either TNF-α and IL-1β, with the aim to gain preliminary information on whether the difference was more likely related to the ODN sequences or the pro-inflammatory challenge used. Both pro-inflammatory stimuli induced transcription of the same pattern of genes induced by *P. aeruginosa*, as shown in figures 29-A and 29-B. However, when cells were challenged with both stimuli and *P. aeruginosa*, the most effective HIV-1 LTR NF-kB decoy ODN inhibited significantly IL-8 mRNA induced by PAO1 and IL- 1β but had a much more limited effect on TNF-α dependent transcription of this chemokines, as reported in figure 29-C.

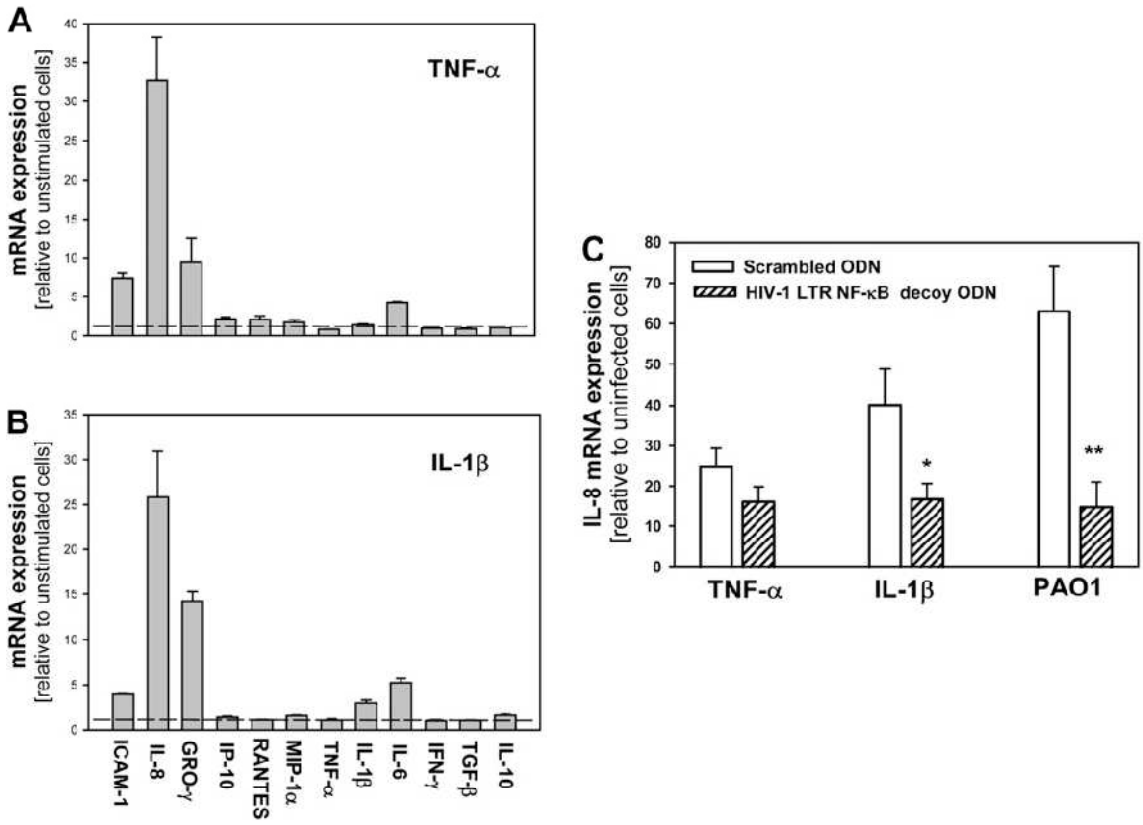


FIGURE 29 - Effect of different pro-inflammatory stimuli on transcription of IL-8 mRNA. Induction of cytokine transcription by **(A)** TNF-α (50 ng/ml) and **(B)** IL-1β (10 ng/ml) in IB3-1 cells. Cells were seeded, serum starved for 18 hours, and incubated with the different stimuli for 4 hours. **(C)** Effect of HIV-1 LTR NF-kB decoy ODN on IL-8 transcription in IB3-1 cells treated with different stimuli. Decoy or scrambled ODNs were pre-incubated with IB3-1 cells for 24 hours before stimulation for 4 hours by IL-1β (50 ng/ml), TNF-α (10 ng/ml), or infection with PAO1 (100 CFU/cell) for 4 hours. Total RNA was extracted and processed for quantitation of transcripts as described in MATERIALS AND METHODS. Values are mean 6 SEM of three separate experiments. Significance in Student's t test between each scrambled and decoy ODNs: *P<0.05 or **P<0.01.

4. NATURAL PRODUCTS

4.1 BERGAMOT EXTRACTS

In the laboratory of professor Sacchetti, commercial mature *C. bergamia* fruits belonging to three different stocks from organic farming in southern Italy were purchased and manually processed to completely remove the epicarp. The raw plant material obtained (150 g for each sample stock) was immediately suspended in 600 mL of chloroform and processed for the extractions. The suspension was homogenized for 5 min and submitted to sonication in an ultrasound bath (Ultrasonik model 104X, Ney Dental Inc.) in the dark at a constant temperature of 25 °C. Subsequently, the samples were filtered and centrifuged for 20 min at 3000 rpm. The residue was re-extracted with 400 mL of chloroform following the same procedure previously described. The collected chloroform extracts, named Extract 1, were partially reduced in volume with a rotavapor and then completely dried under nitrogen flow. The total extraction yield was 3.6 ± 0.2%. Aliquots (5 g) of these sample extracts (Extract 1) were subjected to fractional distillation at 350-400 Pa and 40°C, as described elsewhere [253], to completely remove volatile terpene compounds and partly oxygenated chemicals. The sediment obtained at the end of the distillation corresponded to the Extract 2 samples with an extraction yield of 40.3 ± 0.8%. One gram of samples of Extract 2 was then resuspended in 20 mL of diethyl ether, vigorously shaken for 30 min, and centrifuged. The supernatant was removed, and the residue, corresponding to Extract 3 samples, was collected, dried under nitrogen flow, and weighed (yield = 65.1 ± 1.5%). For all laboratory processing care was taken to protect the operations from light and oxidizing conditions.

4.1.1. EFFECT OF BERGAMOT EXTRACTS ON THE EXPRESSION OF IL-8 mRNA IN IB3-1 CELLS FOLLOWING TNF-α TREATMENT

In order to determine effects of bergamot extracts on mRNA accumulation, the expression of IL-8 gene was studied by RT-PCR analysis of RNA extracted from TNF-α treated IB3-1 cells cultured in the presence of increasing concentrations of bergamot extracts for 24 hours. These concentrations have been chosen according to IC_{50} values obtained in IB3-1 cell antiproliferative experiments reported in figure 30. As target gene, we have decided to study the accumulation of IL-8 mRNA because IL-8 gene (a) is one of the most expressed interleukin in IB3-1 cells [241], (b) it is strongly induced following TNF-α treatment [241], (c) it is clearly involved in inflammatory processes associated with CF [15,16].

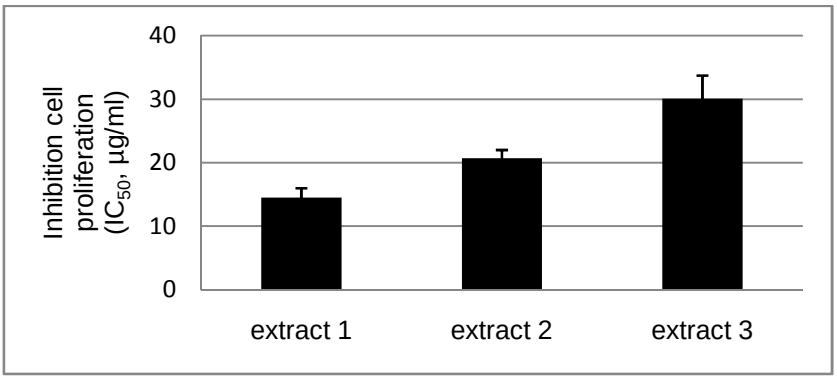


FIGURE 30 - Inhibition of cell proliferation (IC_{50}, µg/ml) of bergamot extracts on cell growth of IB3-1 cells. The IC_{50} values were obtained from three independent experiments ± SD.

Panel A of figure 31 shows representative results obtained by amplifying RNA from untreated IB3-1 cells (triangles), TNF-α treated IB3-1 cells (squares) or IB3-1 cells treated with IC_{50} extract 3 (crosses) using primer specific for IL-8 and GAPDH RNA sequences. The GAPDH gene was used as reference gene in order to normalized the IL-8 mRNA expression. Clear induction of IL-8 transcripts following TNF-α treatment is evident. Significant inhibitory effects were detected following exposure of TNF-α treated IB3-1 cells to IC_{50} concentration of extract 3. Panel B of figure 31 presents the complete set of the

results obtained by the real-time quantitative RT-PCR data on the effects of bergamot extracts on IL-8 mRNA accumulation with respect to GAPDH transcripts. The first set of results consistently obtained is the strong increase of IL-8 mRNA transcript in TNF-α treated IB3-1 cells (average, 34 fold, $p < 0.01$). In addition, the results obtained indicate that IL-8 mRNA expression is much lower in TNF-α treated IB3-1 cells cultured in the presence of bergamot extracts. More in detail, extract 1 and extract 3, even when used at IC_{25} concentrations, display high level of inhibition of TNF-α induced IL-8 mRNA accumulation. IC_{50} concentrations are necessary to obtain similar inhibitory effects using extract 2.

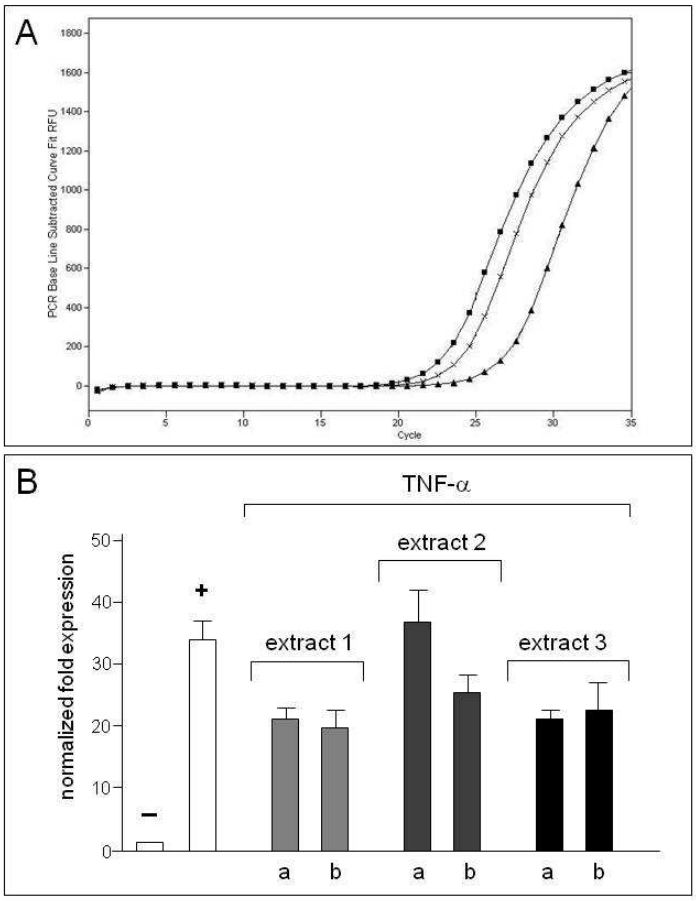


FIGURE 31 – Effects of bergamot extracts on IL-8 mRNA accumulation. IB3-1 were treated with 80 ng/ml TNF-α in the presence of increasing concentrations of bergamot extracts for 24 hours, RNA was extracted and RT-PCR performed with primers specific for IL-8 and β-actin RNA sequences. **(A)** Representative quantitative RT-PCR analysis. Triangles: RNA from untreated IB3-1 cells; squares: TNF-α treated IB3-1 cells; crosses: IB3-1 cells treated with IC_{50} extract 3. **(B)** Effects of bergamot extracts on IL-8 mRNA accumulation with respect to β-actin transcripts. (a): IC_{25} of extract; (b): IC_{50} of extract.

4.1.2. EFFECT OF BERGAMOT EXTRACTS ON THE EXPRESSION OF IL-8 GENES INDUCED IN IB3-1 CELLS FOLLOWING TNF-α TREATMENT: A BIO-PLEX ANALYSIS

In consideration of the inhibitory activity on IL-8 mRNA accumulation, Bio-plex experiments were performed to verify the possible effects of bergamot extracts on the release of this protein. IB3-1 cells were treated with 80 ng/ml of TNF-α and increasing amounts (IC_{25}-IC_{50}-IC_{75}) of bergamot extracts. After 24 hours, recovered supernatants were analyzed using the Bio-Plex human cytokine IL-8 single-plex (Bio-Rad). When IB3-1 cells were induced with TNF-α and cultured in the presence of bergamot extracts, some important differences in the release of IL-8 occurred, as reported in figure 32. As it is evident the TNF-α induced release of IL-8 (pg/ml) is strongly reduced using extract 1 and 3 at IC_{50} concentrations. The effect was found to be concentration-dependent. The results obtained indicate a strong inhibition of release of IL-8 using extracts 3, even when it was used at IC_{25} concentration, while extract 2 showed inhibitory activity active only at IC_{75} concentration. These data suggest that bergamot extracts might specifically inhibit the expression of the IL-8 pro-inflammatory gene. These data are in agreement with the results obtained using the RT-PCR approach [fig.31], and sustain the concept that bergamot extracts are potent inhibitors of the TNF-α induced expression of the IL-8 gene in IB3-1 cells.

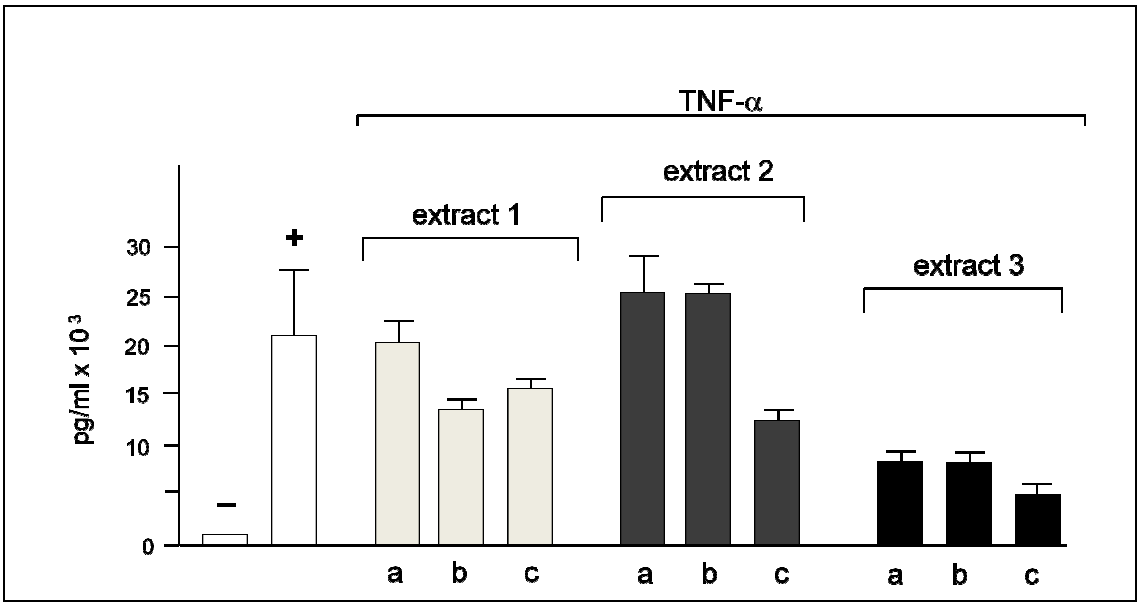


FIGURE 32 – Effects of bergamot extracts IL-8 release. IB3-1 cells were treated with 80 ng/ml of TNF-α and increasing amounts (a: IC_{25}; b:IC_{50}; c:IC_{75}) of bergamot extracts. After 24 hours, recovered supernatants were analyzed using the Bio-Plex human cytokine IL-8 single-plex (Bio-Rad). (-): untreated IB3-1 cells; (+):IB3-1 cells treated with TNF-α.

4.1.3. PHYTOCHEMICAL INVESTIGATION

In consideration of the biological activity shown in figures 31 and 32, the phytochemical investigation of the three bergamot extracts was performed in order to obtain a qualitative and quantitative determination of the chemical constituents of the extracts, with particular reference to non-volatile fraction chemicals, i.e. coumarins and psoralens. We are grateful to professor Gianni Sacchetti who performed the GC-FID and GC-MS analyses.

Normally bergamot oil, extracted by cold pressing of peels is made up of 93-96% volatile compounds, such as monoterpenes, in particular 25-53% limonene, and high quantities of oxygenated compounds, such as linalool (2-20%) and linalyl acetate (15-40%) [254]. A variable percentage of the essential oil (4-7%) consists of nonvolatile compounds as pigments, waxes, and above all coumarins (e.g., citropten) and psoralens (e.g., bergapten and bergamottin) [fig.33].

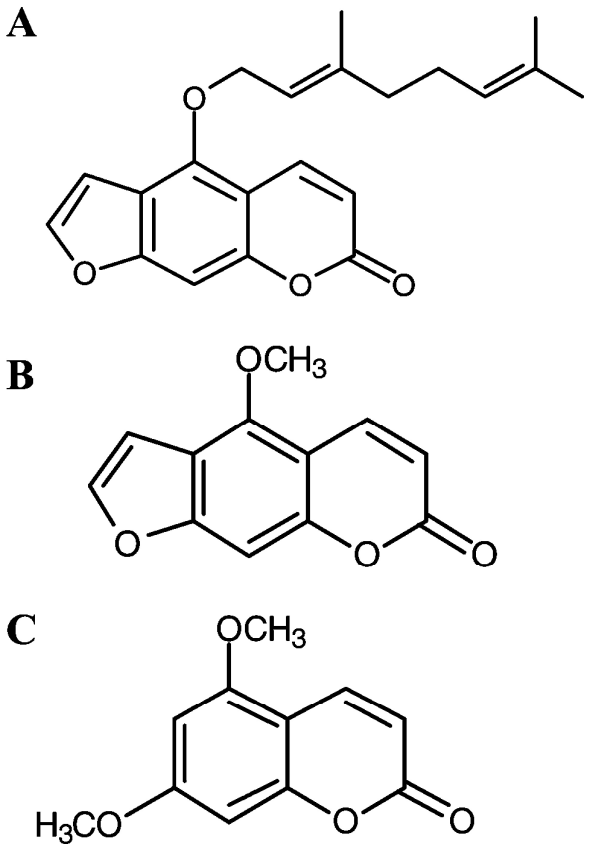


FIGURE 33 - Molecular structures of bergamottin (5-geranyloxypsoralen, **A**); bergapten (5-methoxypsoralen, **B**); and citropten (5,7-dimethoxycoumarin, **C**).

The link between these phytochemical aims and the bioactivity assays targets was to determine the role, if any, of the non-volatile fraction, or of their constituents individually, with or without the synergic interaction of volatile chemicals, in the modulation of IL-8 gene expression in *in vitro* cell models. For the chloroform extract (extract 1 samples), composition was similar to that in bergamot essential oil with two significant fractions: (a) monoterpenes and their correlated compounds and (b) coumarins and psoralens [255]. The extraction procedures followed led to products (extract 2 and extract 3 samples) with a progressive decrease of volatile compounds and an increase of the nonvolatile fraction (coumarins and psoralens) as determined by semiquantitative combined GC-FID and GC-MS analyses [Table 5]. Extract 1 samples showed linalyl acetate (36.12%), linalool (27.35%), and limonene (18.78%) as the main constituents, as for bergamot essential oil, whereas

coumarins and psoralens made up 4.64% [Table 1]. Extract 2 samples showed an important reduction of linalyl acetate (27.45%) and a dramatic decrease of other terpene compounds. Many minority chemicals were not detectable, whereas others, such as limonene, showed a reduction of 93.2%. Extract 3 samples gave an even more noticeable reduction of terpene compounds, with linalyl acetate (0.43%), γ-terpinene (0.17%), and β-myrcene (0.53%) as the only volatile fraction chemicals, against an increase of coumarins and psoralens of 45.5 and 95.2% with respect to extract 2 and 3 samples, respectively [table 5].

		area %[b]		
identified compound	KI[a]	Extract 1	Extract 2	Extract 3
α-pinene	939	0.56 ± 0.04	nd	nd
sabinene	976	0.60 ± 0.05	nd	nd
β-pinene	979	0.73 ± 0.05	nd	nd
β-myrcene	991	2.53 ± 0.21	2.51 ± 0.18	0.53 ± 0.06
α-terpinene	1017	0.10 ± 0.01	nd	nd
p-cymene	1025	0.13 ± 0.02	nd	nd
limonene	1027	18.78 ± 1.17	1.27 ± 0.11	nd
trans-*E*-ocimene	1050	0.15 ± 0.02	nd	nd
γ-terpinene	1060	6.37 ± 0.35	1.06 ± 0.12	0.17 ± 0.02
linalool	1097	27.35 ± 1.89	3.62 ± 0.23	nd
α-terpineol	1189	0.13 ± 0.01	nd	nd
nerol	1230	0.22 ± 0.02	0.56 ± 0.07	nd
linalyl acetate	1257	36.12 ± 2.48	27.45 ± 1.88	0.43 ± 0.04
geranial	1267	0.28 ± 0.02	0.89 ± 0.06	nd
γ-terpinyl acetate	1349	0.11 ± 0.03	1.75 ± 0.11	nd
neryl acetate	1362	0.31 ± 0.03	1.57 ± 0.11	nd.
geranyl acetate	1381	0.19 ± 0.02	1.42 ± 0.13	nd
caryophyllene	1409	0.37 ± 0.04	0.70 ± 0.08	nd
trans-α-bergamotene	1435	0.38 ± 0.03	1.03 ± 0.09	nd
β-bisabolene	1506	0.58 ± 0.07	1.23 ± 0.11	nd
coumarins, psoralens[c]		4.64 ± 0.44	52.60 ± 2.56	96.54 ± 2.34

[a]KI, Kovats indices calculated with Varian Factor Four VF-5 ms. [b]Calculated with GC-FID; nd, not detectable. [c]Only citropten and bergapten were detectable on GC-MS.

TABLE 5 - Composition of Bergamot Extracts Determined by GC-MS and GC-FID

HPLC analyses to detect and quantify coumarins and psoralens reputed to be representative of the nonvolatile fraction and of the bioactivity of bergamot

crude drug and derived products [212-218, 255] were performed by doctor Ilaria Lampronti, who is acknowledged for her work. This showed a homogeneous qualitative profile with respect to the occurrence of citropten, bergamottin, bergapten, bergaptol, and 5-geranyloxy-7-methoxycoumarin in all bergamot extract samples. A total amount corresponding to 5.83% was found in extract 1 samples, 37.84% in extract 2 samples, and 89.36% in extract 3 samples [table 6]. Bergaptol and 5-geranyloxy-7-methoxycoumarin were not detectable in the three extracts, whereas the other compounds (citropten, bergamottin, bergapten) were always identified. The ratio among the detected chemicals changed greatly in the different kinds of samples. In samples of extract 1 bergamottin and bergapten represented 2.57 and 2.89% respectively, whereas citropten was detected in lower concentrations (0.37%). In samples of extract 2 concentrations of bergamottin, bergapten, and citropten were 9.44, 27.26, and 1.14%, whereas in samples of extract 3 concentrations were 3.10, 85.75, and 0.51%, respectively [table 6].

EXTRACT	CITROPTEN	BERGAMOTTIN	BERGAPTEN	BERGAPTOL	5-GERANYLOXY-7-METHOXYCOUMARIN
1	0.37 ± 0.03	2.57±0.08	2.89±0.07	*nd	Nd
2	1.14±0.07	9.44±0.17	27.26±0.59	nd	Nd
3	0.51±0.03	3.10±0.06	85.75±1.18	nd	Nd

*Nd: not detectable

Table 6 - Coumarins and Psoralens Percentage (w/w ± SD) in the Bergamot Extracts Determined by HPLC Analyses.

^{1}H NMR [fig.34] was performed in the laboratory of professor Saccheti, who is acknowledged, on all of the samples belonging to the three different extracts in order to have a fingerprint of the bergamot phytocomplexes. NMR can potentially perform in a single-step analysis the assessment of all organic compounds that constitute a phytocomplex [256 and references cited therein]. For the three extracts examined it was possible to detect, through a single NMR analysis, the presence of psoralens, coumarins, and monoterpenes. This NMR approach allows with good approximation the relative amount of each detectable component by comparison of the integrals of typical not overlapped

signals assigned to these chemicals to be obtained [256, 257]. In this specific case we considered two allyl protons on limonene (4.72 ppm), double doublet of proton on C2 on linalool (5.89 ppm) and linalyl acetate (5.97 ppm), doublet of proton onC3 on citropten (7.95 ppm), and doublet of proton near the oxygen on the furanic ring of bergapten (7.01 ppm) and bergamottin (6.93 ppm).

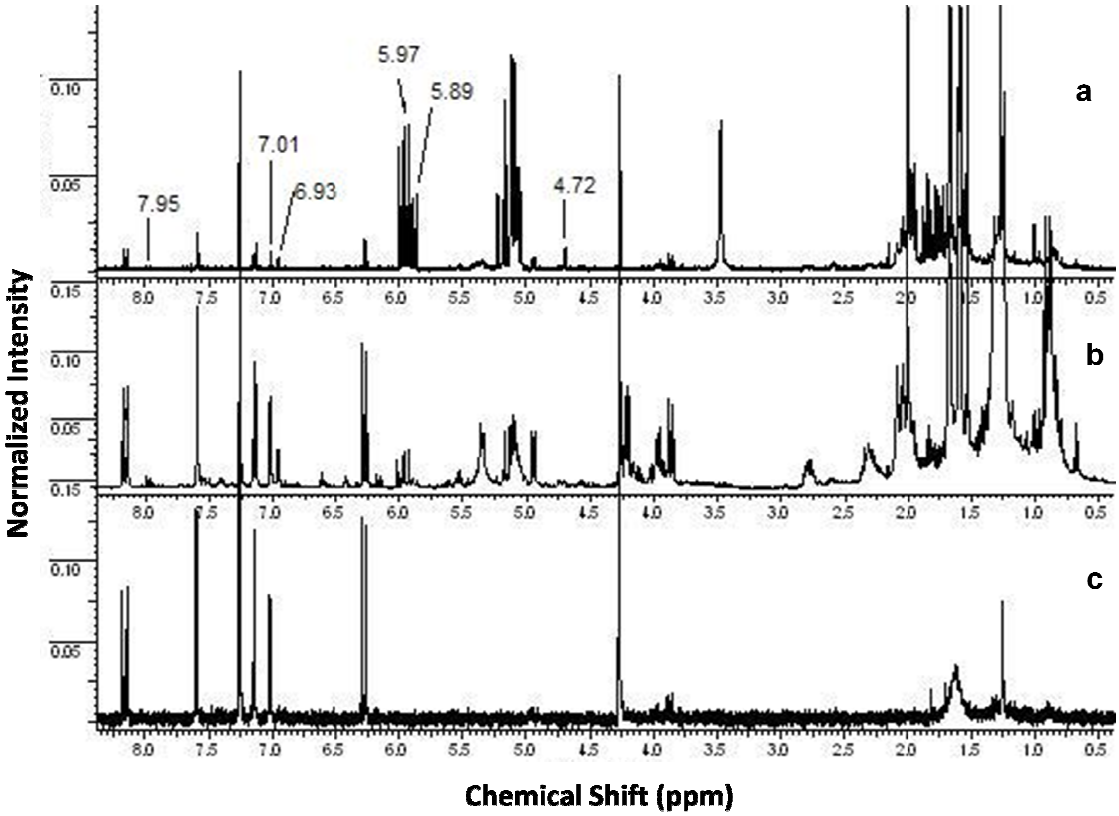


FIGURE 34 - ^{1}H NMR spectra of samples belonging to Extract 1 (a), Extract 2 (b), and Extract 3 (c).

In consideration of the fact that extract 3 was the most active on inhibition of TNF-α induced IL-8 mRNA accumulation and IL-8 secretion by treated IB3-1 cells, we hypothesized that the coumarin/psoralen fractions of the extracts were among those responsible for the biological activity. Therefore, the activity of citropten, bergapen and bergamottin was further analyzed.

4.1.4. EFFECT OF IDENTIFIED COMPOUNDS IN BERGAMOT EXTRACTS ON EXPRESSION OF IL-8 GENES IN TNF-α TREATED IB3-1 CELLS

In order to determine effects of three principal identified compounds on IL-8 mRNA accumulation, we analyzed by semi-quantitative RT-PCR the level of IL-8 mRNA in TNF-α treated IB3-1 cells cultured in the presence of increasing concentrations of bergamottin, bergapten, citropten for 24 hours [fig.35]. These concentrations were chosen according to IC_{50} values obtained in IB3-1 cell antiproliferative experiments (citropten 235µM ± 38,2 SD, bergapten 155,8 µM ± 43,6 SD, bergamottin 43,25 µM ± 6,2 SD)

The preliminary results obtained indicate that IL-8 mRNA expression is lower in TNF-α treated IB3-1 cells cultured in the presence of bergapten or citropten at IC_{50} concentrations, while bergamottin does not show inhibitory effects at the same concentrations [fig.35-A]. The β-actin mRNA (used as housekeeping gene) accumulation does not change following bergamot compunds treatment [fig.35-B].

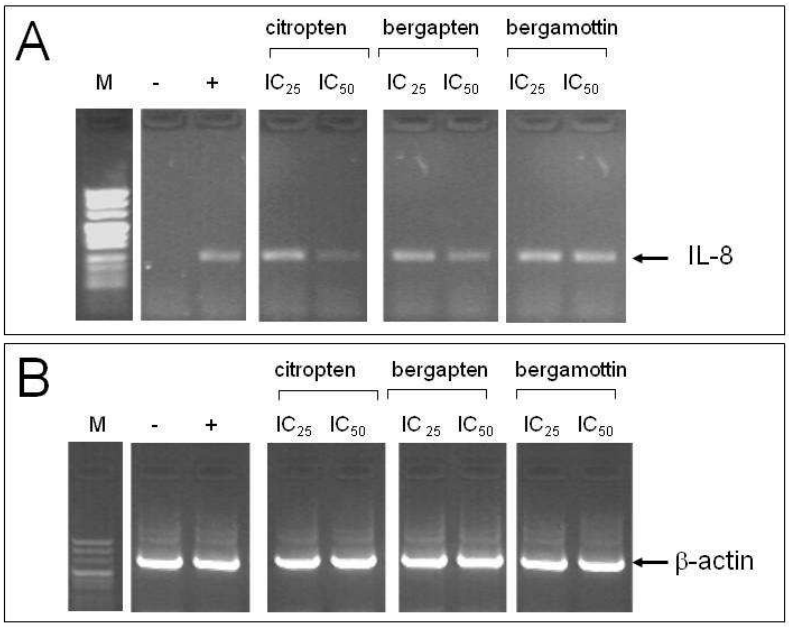


FIGURE 35 - Effects of citropten, bergamottin and bergapten on IL-8 mRNA accumulation: semi-quantitative RT-PCR analysis. IB3-1 were treated with TNF-α in the presence of IC_{25} or IC_{50} concentrations of citropten, bergapten and bergamottin as indicated for 24 hours, RNA was extracted and RT-PCR performed with primers specific for IL-8 and β-actin RNA sequences. (-): untreated IB3-1 cells; (+):IB3-1 cells treated with TNF-α.

On the basis of this first set of results, showing that bergapten and citropten exhibit an interesting inhibitory activity on mRNA accumulation, to better quantify inhibitory effects of these two identified compounds, we performed real-time quantitative RT-PCR analysis on RNA extracted from IB3-1 cells induced with TNF-α and treated with IC_{25} and IC_{50} concentrations of citropten or bergapten, comparing IL-8 mRNA accumulation with that of the internal controls β-actin mRNA. Panel A of figure 36 shows the representative results obtained by amplifying RNA from untreated (triangles), TNF-α (squares), citropten (diamonds, IC_{50}) and bergapten (crosses, IC_{50}) treated IB3-1 cells using primer specific for IL-8 RNA. Clear induction of IL-8 transcripts, following TNF-α treatment is evident, but this increase is reduced after treatment with citropten or bergapten. Particularly citropten is more efficient than bergapten to decrease IL-8 mRNA accumulation. This result is more evident in panel B of figure 36, showing a summary of the data obtained by real-time quantitative RT-PCR were performed to investigate effects of bergapten and citropten at two different concentrations (IC_{25} and IC_{50}) on IL-8 mRNA accumulation. Significant inhibitory effects on IL-8 mRNA expression normalized to β-actin transcripts were detected following exposure of TNF-α induced IB3-1 cells to citropten and bergapten at IC_{25} concentrations yet. Moreover, citropten was found to be more active than bergapten at the same concentrations. These data are in agreement with data obtained by semi-quantitative RT-PCR [fig.35], and confirm the potent inhibitory activity of citropten and bergapten on expression of the IL-8 gene in IB3-1 cells.

The results shown in figure 36-B have been also confirmed using Bioplex technology to analyze the release of IL-8 protein (pg/ml) in supernatants recovered from TNF-α induced IB3-1 cells treated with citropten and bergapten at IC_{50} and IC_{25} concentrations. These supernatants were collected before to perform the RNA extraction for real time PCR analysis. The results, shown in figure 36-C, confirmed the inhibitory activity of citropten and bergapten (citropten>bergapten) on expression of IL-8 protein in IB3-1 cell model.

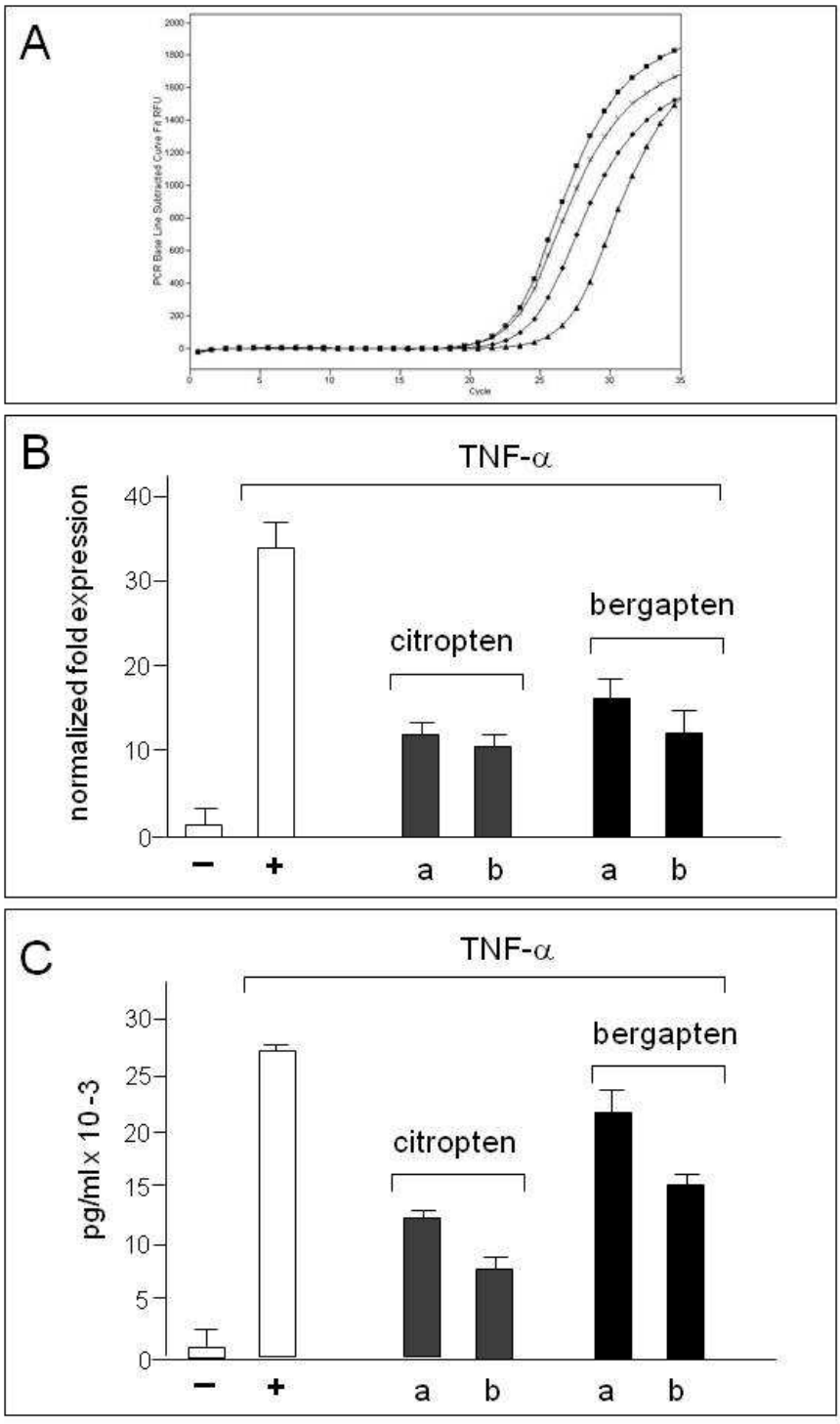


FIGURE 36 – Effects of citropten and bergapten on IL-8 gene expression. **(A-B)**: mRNA accumulation studied by quantitative RT-PCR analysis; **(C)** Bio-plex analysis of IL-8 protein present in supernatants. **(A)** representative RT-PCR analysis. triangles: untreated IB3-1 cells; squares: TNF-α treated IB3-1 cells; diamonds: IB3-1 cells treated with citropten; crosses: IB3-1 cells treated with bergapten. **(B)** Summary of the effects of citropten and bergapten on IL-8 mRNA accumulation with respect to β-actin transcripts. **(C)** Bio-plex analysis, using the Bio-Plex human cytokine IL-8 single-plex (Bio-Rad). (-): untreated IB3-1 cells; (+):IB3-1 cells treated with TNF-α.

4.2 *EMBLICA OFFICINALIS*

The dried fruits of *Emblica officinalis* were extracted with absolute ethanol and the yield was 9.33%. This ethanolic extract of *Emblica officinalis* was defatted with petroleum ether and the defatted extract was successively fractionated with different solvent systems on the polarity basis. The solvents were 100% dichloromethane, 25% ethylacetate-dichloromethane, 50% ethylacetatedichloromethane, 75% ethylacetate-dichloromethane, 100% ethylacetate, butanol and acetone and the remaining aqueous portions were separated.

4.2.1 GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF THE FRACTIONS OF *EMBLICA OFFICINALIS* EXTRACTS

In order to identify the putative antiproliferative compound present within unfractionated extracts and n-butanol fraction from *Emblica officinalis*, gas chromatography-mass spectrometry (GC-MS) analysis was performed by doctor Ilaria Lampronti, who is acknowledged. Gas chromatography coupled to mass spectrometry allows purification and identification of single components from biological extracts. *Emblica officinalis* extracts and the respective n-butanol fraction samples were diluted to a final volume of 1 ml with methanol (approximately 1 mg/ml). One µl of each solution was injected into the gas chromatograph with an appropriate microsyringe, chromatographed using a fused-silica capillary column and analyzed with a quadrupole mass spectrometric detector. The resulting chromatograms are shown in figure 37-A and C, and demonstrate that in *Emblica officinalis* extracts 3 peaks with different retention times (r.t., 13.4, 20.9, 22.3 min) are present. The mass spectrometric analysis [fig.37-B, 37-D, and data not shown] demonstrated that these 3 peaks correspond to 3 derivatives: a) pyrogallol (or 1,2,3-benzenetriol); b) tetradecahydro-1,4-dimethyl-7- (1- methylethyl)-1-phenantrene

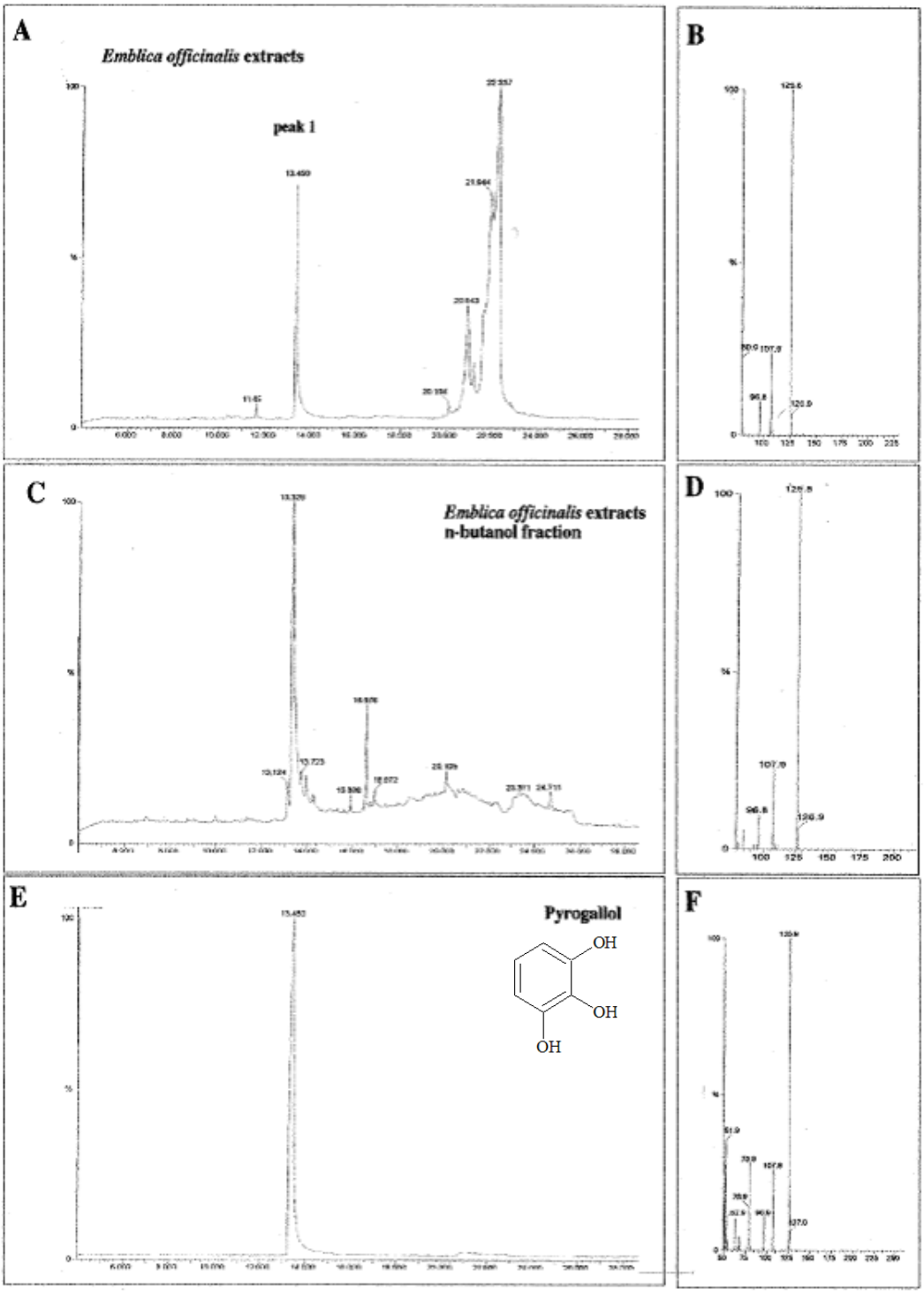


FIGURE 37 - **(A**, **C**, **E)**, Ion chromatograms of unfractionated *Emblica officinalis* extracts **(A)**, n-butanol fraction of *Emblica officinalis* extracts **(C)**, pyrogallol **(E)**.
(B, **D**, **F)**, Mass spectrum of peak 1 of unfractionated *Emblica officinalis* extracts **(B)**, peak 1 of n-butanol fraction of *Emblica officinalis* extracts **(D)** and pyrogallol **(F)**.

methyl ester; and c) decahydro-4H-cyclopentacycloocten-4-one respectively, identified by using the NIST library (Mass Spectrometry Data Handling System, version 1.12, Fisons, Thermo Finnigan, San Jose, CA). In the chromatogram of the n-butanol fraction of *Emblica officinalis* extracts [fig.37-C] we can observe only one major peak at 13.3 min (r.t.) corresponding to pyrogallol [fig.37-D], also present in *Emblica officinalis*. The retention time (r.t.) and the fragmentation pathways are the same as shown in figure 37-A and B. Moreover, we analyzed commercial pyrogallol for comparison with the two peaks found in *Emblica officinalis* extracts and the respective n-butanol fraction samples. The r.t. and the fragmentation pathways corresponded perfectly [fig. 37-E and 37-F]. Accordingly, we hypothesized that the antiproliferative activity of *Emblica officinalis* extracts was due to pyrogallol. M/z values: a) pyrogallol (*Emblica officinalis extracts*): 80.0, 96.8, 107.9, 125.8, 126.9; pyrogallol (*Emblica officinalis*, n-butanol fraction): 80.0, 96.8, 107.9, 125.8, 126.9; pyrogallol (Aldrich): 79.9, 96.9, 107.9, 125.9, 127.0; b) 81.0, 99.0, 121.0, 123.0, 150.0, 164.0, 186.9, 207.0, 279.2, 306.1, 324.2; c) 95.0, 96.0, 98.0, 123.0, 124.0, 130.8, 166.0, 195.0, 212.1, 241.1, 279.1.

4.2.2 EFFECTS OF *EMBLICA OFFICINALIS* EXTRACTS ON *IN VITRO* PROLIFERATION OF IB3-1 CELLS

In order to obtain preliminary information on the biological properties of the employed extracts, their effects on cell growth were examined. The extracts isolated from *Emblica officinalis* were first tested on IB3-1 cells. Figure 38 summarizes the data derived from three independent experiments. IB3-3 cells were seeded at the initial concentration of 30.000 cells/cm^2 and then cultured in the absence or in the presence of 0.05-500 µg/ml of *Emblica officinalis* extracts or purchased pyrogallol. For the IC_{50} (concentrations of extracts leading to 50% inhibition of IB3-1 cell growth), we determined the values of cell number/ml after 3 days of culture, when untreated cells are in the log phase of cell growth.

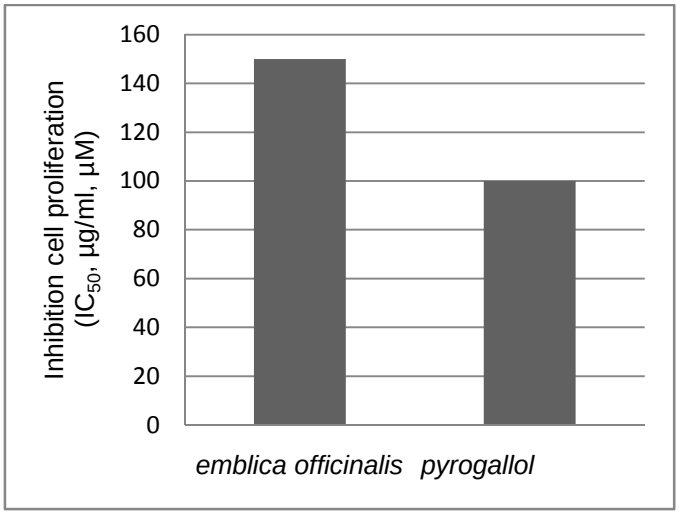


FIGURE 38 - Inhibition of cell proliferation (IC50, µg/mL or µM) of *Emblica officinalis* extracts and identified compound pyrogallol on cell growth of IB3-1 cells. The IC_{50} values were obtained from three independent experiments ± SD.

4.2.3 EFFECT OF *EMBLICA OFFICINALIS* EXTRACTS ON THE EXPRESSION OF PRO-INFLAMMATORY GENES INDUCED IN IB3-1 CELLS BY TNF-α TREATMENT

To verify the potential anti-inflammatory activity of *Emblica officinalis* extracts and its main constituent pyrogallol, Bio-plex experiments were performed evaluating the release of pro-inflammatory cytokines and chemokines on supernatant of IB3-1 cell model. Accordingly with the results of proliferation activity assays [fig.38], IB3-1 cells were treated with increasing amounts ($IC_{12,5}$-IC_{50}) of *Emblica officinalis* extracts or (IC_{25}-IC_{50}) pyrogallol and with or without 80 ng/ml of TNF-α. After 24 hours, recovered supernatants were analyzed using the Bio-Plex human cytokine 7-plex (Bio-Rad). The results shown in table 7 demonstrated that since the amount of 50 µg/ml *Emblica officinalis* is able to reduce the release of VEGF (*Vascular Endothelial Growth Factor*) and IL-8 proteins, by 20 and 37% respectively. Less relevant is the activity of *Emblica officinalis* on down regulation of IL-12, G-CSF, MCP-1, RANTES e IL-10 gene expression. Moreover, pyrogallol seems to down regulate gene transcription of G-CSF, RANTES, VEGF, by 44, 73 and 63% respectively, and especially of IL8 by 51%.

SAMPLE	G-CSF	MCP-1	RANTES	VEGF	IL-6	IL-8
Control	14,99	5,59	210,35	12.449,02	505,06	568,54
Emblica 150 µg/ml	12,84	4,82	240,13	9.747,39	477,29	357,07
Emblica 50 µg/ml	15,04	6,32	293,95	14.051,81	635,65	410,66
Pyrogallol 150 µM	8,25	3,74	56,19	4.577,81	1.908,5	279,32
Pyrogallol 100 µM	11,23	5,27	140,25	6.694,74	1.852,45	285,17

TABLE 7 - Effect of *Emblica officinalis* extracts on release of cytokines and chemokines. IB3-1 cells were cultured in the presence of increasing amounts (150 and 50 µg/ml), as indicated, of *Saraca asoka* extracts or pyrogallol (150 and 100 µM) for 24 hours. The release of the indicated cytokines and chemokines is reported as pg/ml.

When IB3-1 cells were induced with 80 ng/ml of TNF-α and cultured in the presence of increasing amounts *Emblica officinalis* extracts or pyrogallol (as described above) for 24 hours, some important differences in the release of cytokines and chemokines occured, which are reported in the table 8.

SAMPLES	G-CSF	MCP-1	RANTES	VEGF	IL-6	IL-8
Control	*0.55	OOR <	38,63	649,72	18,13	165,44
C + (TNF-α)	6,36	79,5	137,1	292,95	220,69	1.713,57
Emblica 150 µg/ml	3,28	13,09	17,78	170,71	541,76	1.458,25
Emblica 50 µg/ml	5,81	19,10	42,73	220,47	560,80	2.520,49
Pyrogallol 150 µM	4,45	70,76	22,58	171,25	248,75	1.158,23
Pyrogallol 100 µM	2,45	78,90	73,52	24,49	201,81	1.197,79

OOR=out of range
*= extrapolated value

TABLE 8 - Effect of Emblica officinalis extracts on TNF-α dependent release of cytokines and chemokines. IB3-1 cells were induced with 80 ng/ml of TNF-α and cultured in the presence of increasing amounts (150 and 50 µg/ml), as indicated, of Saraca asoka extracts or pyrogallol (150 and 100 µM) for 24 hours.The release of the indicated cytokines and chemokines is reported as pg/ml.

The positive control of the experiment is represented by IB3-1 cells induced with TNF-α without *Emblica officinalis* extracts or components treatment. As it is evident the TNF-α induced release of the cytokines and chemokines of the panel. These data suggest that *Emblica officinalis* extracts inhibit especially the expression of MCP-1 (-83%), RANTES (-87%), VEGF (-41%), IL-8 (-14%), and the pyrogallol the expression of RANTES genes.

In consideration of the inhibitory activity on IL-8 release, RT-PCR experiments were performed to verify the possible effects of *Emblica officinalis* extracts on the expression of this gene.

4.2.4 EFFECT OF *EMBLICA OFFICINALIS* EXTRACTS AND IDENTIFIED COMPOUND PYROGALLOL ON EXPRESSION OF IL-8 IN TNF-α TREATED IB3-1 CELLS

In order to determine the effects of *Emblica officnalis* extracts and pyrogallol on mRNA accumulation, the expression of IL-8 gene was analyzed by RT-PCR analysis of RNA extracted from TNF-α treated IB3-1 cells cultured in the presence of increasing concentration of *Emblica officinalis* extracts for 24 hours [fig.39]. These concentrations were chosen according to IC_{50} values obtained in IB3-1 cell antiproliferative experiments reported in figure 38.

The preliminary results obtained indicate that IL-8 mRNA expression is lower in TNF-α treated IB3-1 cells cultured in the presence of *Emblica offcinalis* at IC_{25} (100μg/ml) concentrations, while pyrogallol does not show inhibitory effects at the higher concentrations IC_{75} (150μM) [fig.39-A]. The β-actin mRNA (used as housekeeping gene) accumulation does not change following *Emblica officinalis* and pyrogallol treatment [fig.39-B].

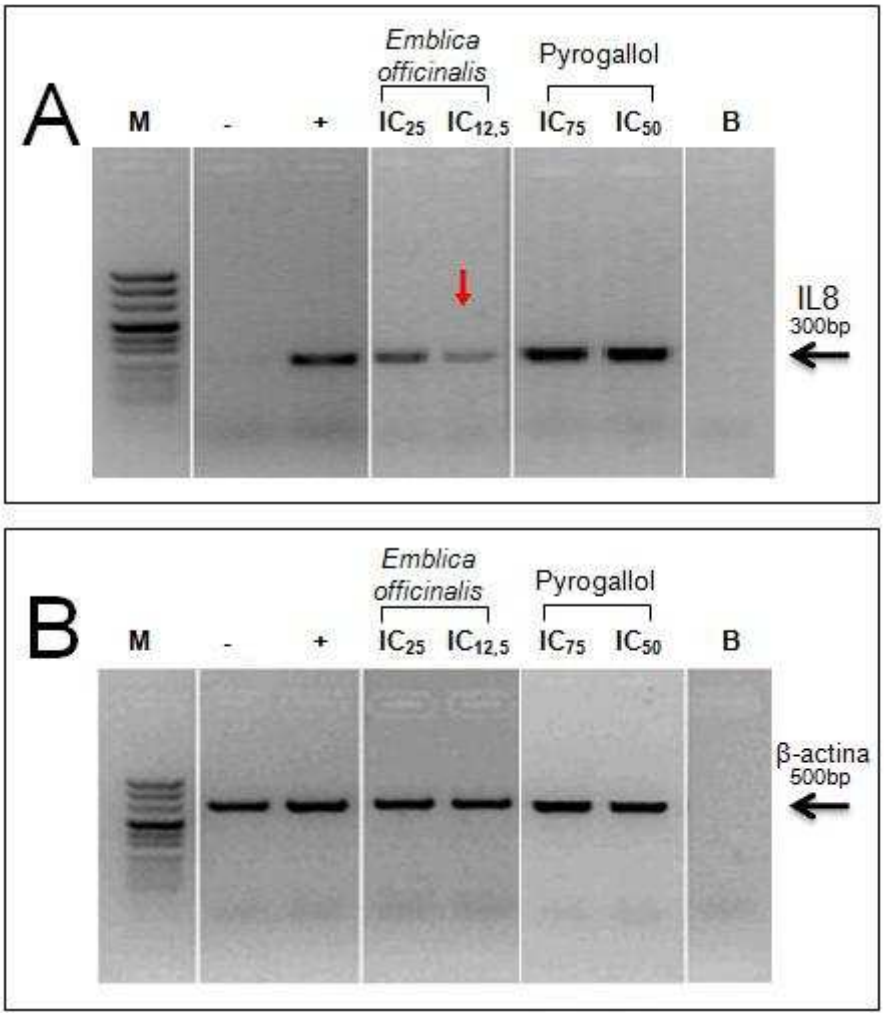


FIGURE 39 - Effects of *Emblica officinalis* and pyrogallol on IL-8 mRNA accumulation: semi-quantitative RT-PCR analysis. IB3-1 were treated with TNF-α in the presence of IC_{25} or IC_{50} concentrations of *Emblica officinalis* and pyrogallol as indicated for 24 hours, RNA was extracted and RT-PCR performed with primers specific for IL-8 and β-actin RNA sequences. (-): untreated IB3-1 cells; (+):IB3-1 cells treated with TNF-α.

4.2.5 *IN VITRO* EFFECT OF *EMBLICA OFFICINALIS* EXTRACTS ON NF-kB TRANSCRIPTION FACTOR ACTIVITY

The ability of *Emblica officinalis* extracts to interfere with NF-kB binding to DNA was investigated, given that the transcription factor NF-kB plays a critical role in regulation of a large number of specific pro-inflammatory genes expression. As reported in figure 40, a dose dependent effect was observed, indicating the ability of *Emblica officinalis* extracts to completely inhibit NF-kB interaction with its cis element, when used at 100, 50, and 25 µg/reaction. The sensitivity of NF-kB/DNA interactions to *Emblica officinalis* extracts was demonstrated to be related to the type of plant extracts and not to the extracting buffers, since no inhibitory effects were observed (a) with the extracting buffer and (b) other extracts from medicinal plants, such as *Oroxylum indicum, Cuscuta reflexa, Paederia foetida, Hygrophilla auriculata, Ocimum sanctum* [data not shown and 258].

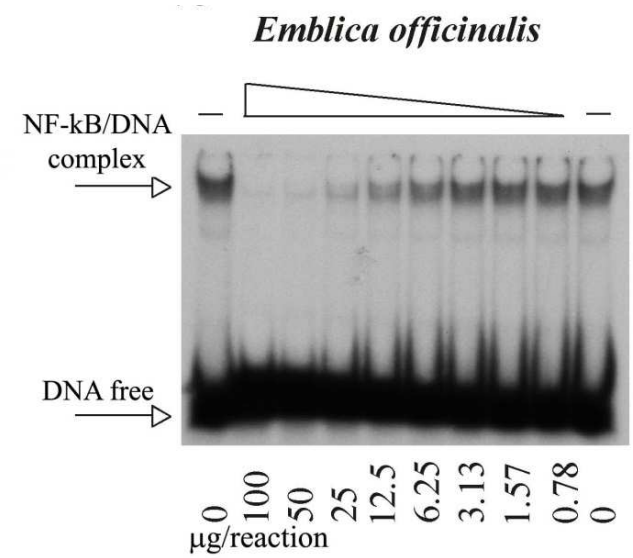


FIGURE 40 - Analysis by electrophoretic mobility shift assay of the effects of *Emblica officinalis* extracts on NF-kB DNA binding activity. Nuclear extracts from the IB3-1 cell line were incubated with ^{32}P-labelled oligonucleotides (*) NF-kB
in the presence of different amounts (100, 50, 25, 12.5, 6, 3, 1.5, 1 µg) of *Emblica officinalis* extracts. Protein/DNA complexes and free probe are indicated by arrows.

4.3 *SARACA ASOKA*

The stem bark of *Saraca asoka* was extracted with absolute ethanol in a cold extraction process [245, 259 and 260]. The yield was 14.16%. The chemical composition has been determined by GC-MS [fig.41] as reported elsewhere [258] by doctor Ilaria Lampronti, who is acknowledged.

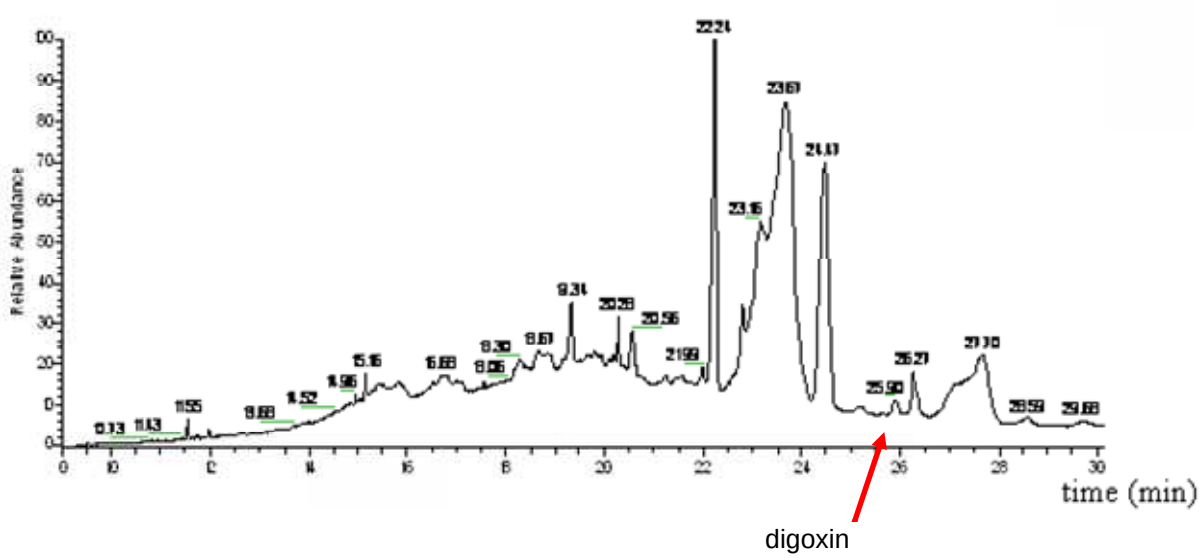


FIGURE 41 - Gas chromatography-mass spectrometry analysis of the etanol fraction of *Saraca asoka* stem bark extracts.

The ion chromatogram revealed in the etanol fraction of the *Saraca asoka* stem bark extracts a major peak corresponding to Digoxin [fig.42].

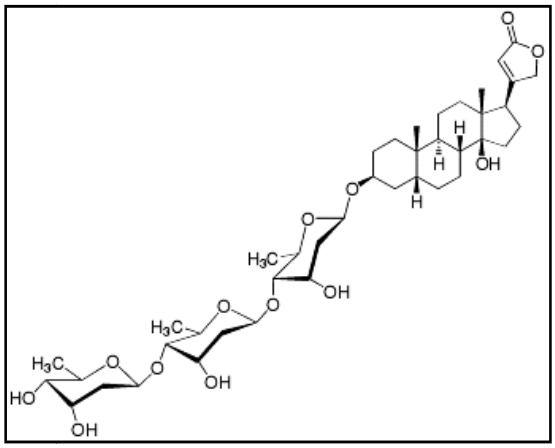


FIGURE 42 – Digoxin structure.

4.3.1. INDUCTION OF CYTOKINE AND CHEMOKINE RELEASE IN IB3-1 CELLS TREATED WITH TNF-α

In order to determine the effects of TNF-α treatment on the release of chemokines and cytokines, cystic fibrosis IB3-1 cells were treated with 80 ng/ml of TNF-α for 24 hours and the supernatants derived from a same number of TNF-α treated IB3-1 cells was analyzed using the Bio-Plex human cytokine Human 7-Plex Panel (Bio-Rad). The results obtained are shown in figure 43.

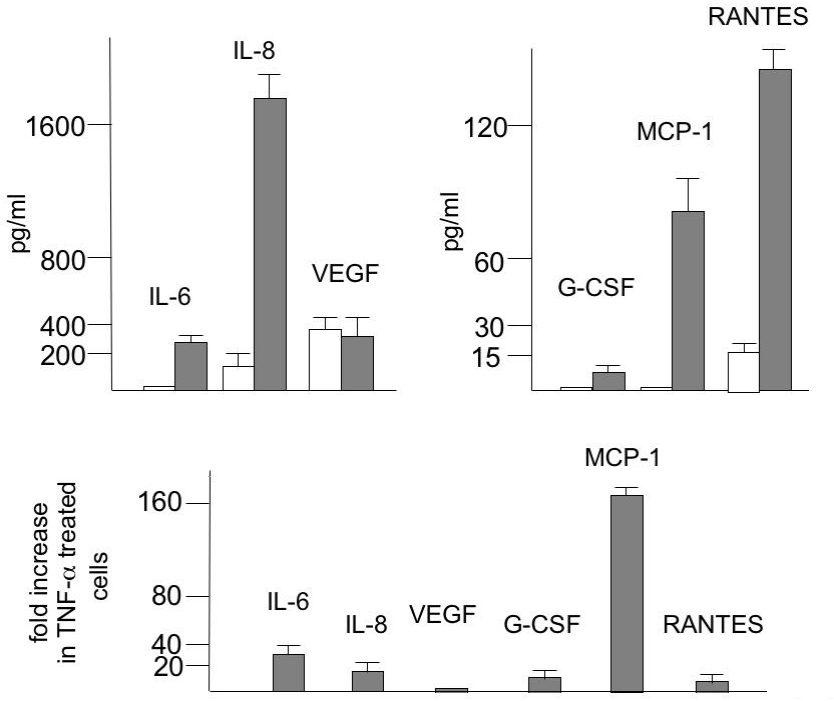


FIGURE 43 - Effect of TNF-α on cytokine and chemokine release in IB3-1 cells. IB3-1 cells (50,000/cm^2) were seeded and incubated in the absence (white boxes) or in the presence (grey boxes) of 80 ng/ml of TNF-α for 24 hours. Supernatants of same numbers of TNF-α treated IB3-1 cells were analyzed using the Bio-Plex Human 7-Plex Panel (Bio-Rad). The results are presented as pg/ml (upper panel). In the lower panel the data are presented as fold increase of cytokine and chemokine release in TNF-α treated cells in respect to untreated IB3-1 cells.

An increase of IL-6, IL-8, G-CSF, MCP-1 and RANTES was appreciable. VEGF is secreted at high levels in IB3-1 cells, but its release was not further induced by TNF-α. The highest fold induction was observed for MCP-1; the highest absolute levels of release (pg/ml) were observed for IL-8. These data are in agreement with results showing a close relationship between TNF-α stimulation

and IL-8 production by CF epithelial cells. In addition, our data are of interest, because they sustain the concept that TNF-α treated IB3-1 cells represent a system reproducing a high level of expression of proteins relevant for CF. In fact, IL-6, G-CSF, MCP-1 and RANTES are all associated with inflammation, as reported in several research papers and reviews [113, 114].

4.3.2. EFFECT OF *SARACA ASOKA* EXTRACTS ON *IN VITRO* PROLIFERATION OF IB3-1 CELLS

When IB3-1 cells are treated with increasing concentrations of *Saraca asoka* extracts the results shown in figure 44-A were obtained. Cell number/ml was assessed after 3 days of cell growth. No inhibitory effects were found with up to 50 μg/ml *Saraca asoka* extracts. IC_{50} was found to be 86.2 $\pm$ 4.7 μg/ml.

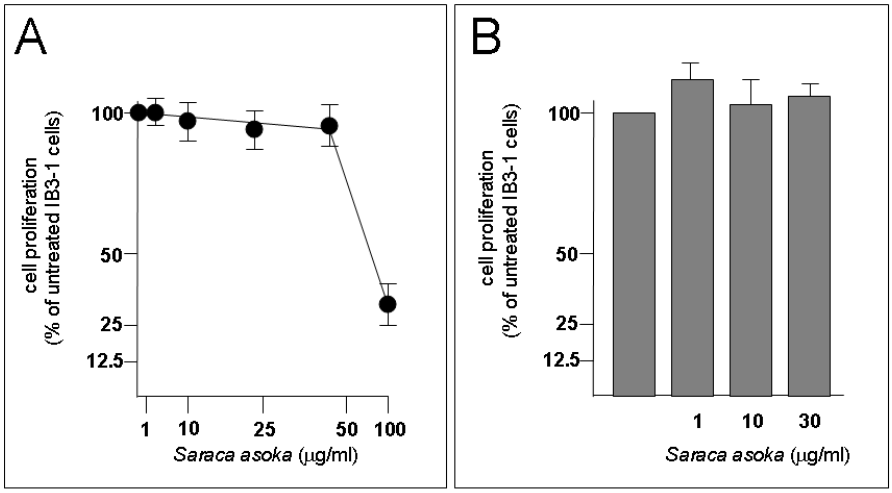


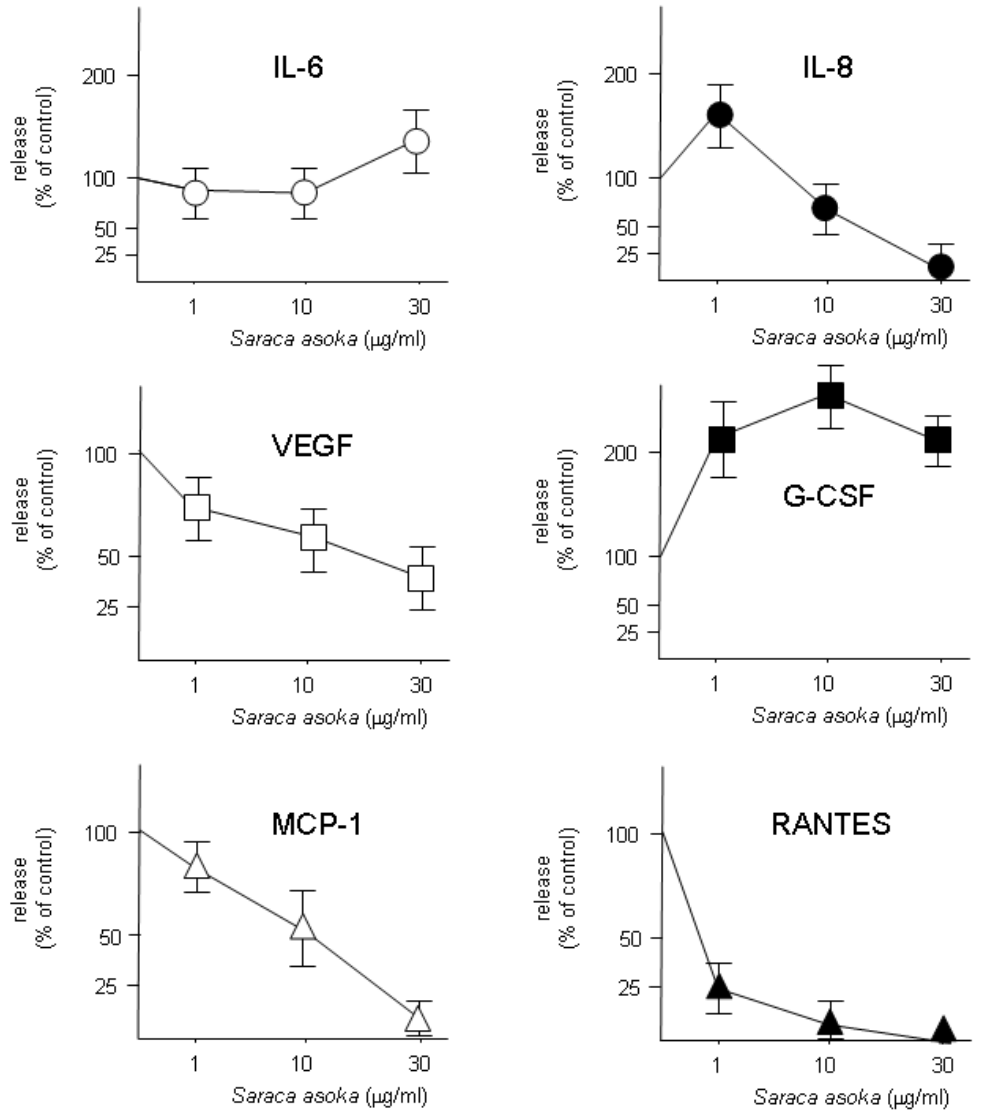
FIGURE 44 - Effect of *Saraca asoka* extracts and TNF-α on *in vitro* proliferation of IB3-1 cells. **(A)**. IB3-1 cells (50,000/cm^2) were seeded and incubated in the absence or in the presence of the indicated concentrations of *S. asoka* extracts. Cell number/ml was assayed after 3 days of cell growth and compared to the values obtained in untreated control cells. **(B)**. IB3-1 cultured in the presence of 80 ng/ml of TNF-α were treated for 3 days with *S. asoka* extracts (1, 10 and 30 μg/ml) and cell number compared with untreated cells, cultured in the absence of both TNF-α and *S. asoka* extracts. The results are the average from three independent experiments + SD.

Figure 44-B shows that treatment of IB3-1 cells with 80 ng/ml of TNF-α in the presence of increasing amounts (1, 10 and 30 μg/ml) of *Saraca asoka* extracts

does not cause significant alteration in the cell growth rate. Accordingly, no significant increase in the proportion of trypan-blue excluded cells was observed following treatment with either TNF-α (80 ng/ml), *Saraca asoka* extracts (1, 10 and 30 μg/ml), or both (not shown). These data demonstrate that TNF-α and *Saraca asoka* extracts are not cytotoxic on IB3-1 cells under the experimental condition employed.

4.3.3. EFFECT OF *SARACA ASOKA* EXTRACTS ON THE EXPRESSION OF PRO-INFLAMMATORY GENES INDUCED IN IB3-1 CELLS BY TNF-α TREATMENT

When IB3-1 cells were induced with 80 ng/ml of TNF-α and cultured in the presence of increasing amounts (1, 10 and 30 μg/ml) of *Saraca asoka* extracts for 24 hours, some important differences in the release of cytokines and chemokines occured, which are reported in the representative experiment shown in figure 45. As it is evident [see also the summary Table included in figure 45] the release of some TNF-α induced cytokines/chemokines is strongly reduced. The effect was found to be concentration-dependent. The results obtained indicate a strong inhibition of release of IL-8, MCP-1 and RANTES. Low level of inhibition was on the contrary detected analyzing the release of VEGF. No inhibitory effects on IL-6 and G-CSF release were found. These data suggest that *Saraca asoka* extracts might inhibit the expression of pro-inflammatory genes, the most important of which is IL-8. Considering the cell number in each well, in a representative experiment untreated IB3-1 secreted an average of 0.16 ± 0.02 pg of IL-8/10^6 cells/24 hours. This value increased to 2.8 ± 0.6 when IB3-1 cells were treated with TNF-α, and was found to be 3.0 ± 0.6, 1.8 ± 0.4 and 0.3 ± 0.15 if the cells were cultured with TNF-α in the presence of 1, 10 and 30 μg/ml extracts from *Saraca asoka*, respectively [the summary of the results obtained is included in figure 44]. In consideration of the inhibitory activity on IL-8 release, RT-PCR experiments were performed to verify the possible effects of *Saraca asoka* extracts on the expression of this gene.



	IC_{50}
IL-6	no inhibition
IL-8	12.5 ± 4.5
VEGF	18.1 ± 4.1
G-CSF	no inhibition
MCP-1	10.2 ± 3.5
RANTES	0.53 ± 0.18

FIGURE 45 - Effect of *Saraca asoka* extracts on TNF-α dependent release of cytokines and chemokines. IB3-1 cells were induced with 80 ng/ml of TNF-α and cultured in the presence of increasing amounts (1, 10 and 30 µg/ml), as indicated, of *Saraca asoka* extracts for 24 hours. The release of the indicated cytokines and chemokines is reported as % of control (TNF-α treated cells). In the Table shown in the lower-right part of the panel summary results from three experiments are included.

4.3.4. *SARACA ASOKA* EXTRACTS INHIBIT IL-8 mRNA EXPRESSION

In order to determine the effects of *Saraca asoka* extracts on mRNA accumulation, the expression of IL-8 gene was analyzed by RT-PCR analysis of RNA extracted from TNF-α treated IB3-1 cells cultured in the presence of 1 and 10 μg/ml of *Saraca asoka* extracts. The expression of IL-8 was chosen for the following reasons: (a) IL-8 is one of the most expressed interleukin in IB3-1 cells [fig.43]; (b) it is strongly induced following TNF-α treatment [fig.43]; (c) it is clearly involved in inflammatory processes associated with CF [261,262]. Interestingly, IL-8 exhibit the highest release also in CF cells infected with *Pseudomonas aeruginosa* [240].

The results obtained clearly indicate that IL-8 mRNA expression is much lower in TNF-α treated IB3-1 cells cultured in the presence of 10 μg/ml *Saraca asoka* extracts. No inhibitory effects were found on IL-8 mRNA accumulation in TNF-α treated IB3-1 cells cultured with 1 μg/ml *Saraca asoka* extracts.

In figure 46-A the preliminary results are shown obtained by semi-quantitative RT-PCR, indicating that (a) IL-8 mRNA expression is induced by TNF-α treatment and (b) much lower accumulation of IL-8 mRNA is detectable in RNA from TNF-α treated IB3-1 cells cultured in the presence of 10 μg/ml *Saraca asoka* extracts. In order to better quantify the effects of *Saraca asoka* extracts real-time quantitative RT-PCR reactions were also performed, comparing IL-8 mRNA accumulation with that of the internal controls β-actin and GAPDH mRNA sequences. Panel B of figure 46 shows the representative results obtained by amplifying RNA from untreated (open symbols) or TNF-α treated (closed symbols) IB3-1 cells using primer specific for IL-8 and GAPDH RNA sequences as indicated. Clear induction of IL-8 transcripts, and no changes of GAPDH mRNA accumulation following TNF-α treatment is evident. Significant inhibitory effects were detected following exposure of TNF-α treated IB3-1 cells to 10 μg/ml *Saraca asoka* extracts, while low effects were found with 1 μg/ml *Saraca asoka* extracts. These data are in agreement with data obtained using the Bioplex approach [fig.45], and sustain the concept that extracts from

Saraca asoka are potent inhibitors of the TNF-α induced expression of the IL-8 gene in IB3-1 cells.

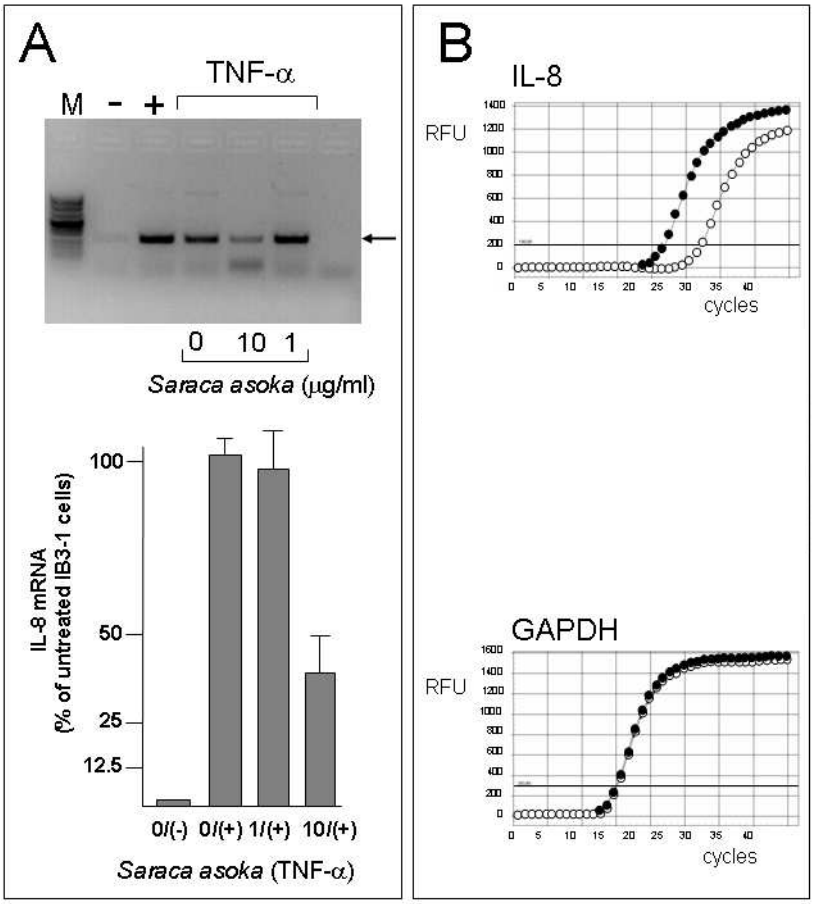


FIGURE 46 - Effect of *Saraca asoka* extracts on TNF-α dependent induction of IL-8 mRNA accumulation. IB3-1 cells were induced with 80 ng/ml of TNF-α and cultured in the presence of 1 and 10 μg/ml of *Saraca asoka* extracts for 24 hours. IL-8 mRNA was quantified by semi quantitative RT-PCR **(A)** or real-time quantitative RT-PCR **(B)**. **(A)** In the upper panel a representative semi quantitative RT-PCR experiment is shown; arrow indicates the IL-8 specific PCR generated fragments. In the lower panel the quantitative analyses of two independent experiments are shown. The data are indicated as % of control TNF-α untreated cells. **(B)** Representative real-time quantitative RT-PCR experiments representing amplifications of RNA from untreated (open symbols) or TNF-α treated (closed symbols) IB3-1 cells using primers specific for IL-8, and GAPDH RNA sequences, as indicated.

4.3.5. *SARACA ASOKA* EXTRACTS INHIBIT INTERACTIONS BETWEEN NF-kB TRANSCRIPTION FACTOR AND TARGET DNA SEQUENCES

In order to determine the effects of *Saraca asoka* extracts on possible molecular targets important for the onset of inflammatory state in CF cells, the possible effects on the interactions between the nuclear transcription factor kB (NF-kB) and target DNA sequences were analyzed by EMSA. NF-kB is indeed involved in the inflammatory state of CF [263-265]. Moreover NF-kB is induced, together with IL-8, in *Pseudomonas aeruginosa* infected CF cells. The results, shown in figure 47, clearly demonstrate that increasing amounts of *Saraca asoka* extracts fully suppress the interactions between NF-kB and target DNA elements.

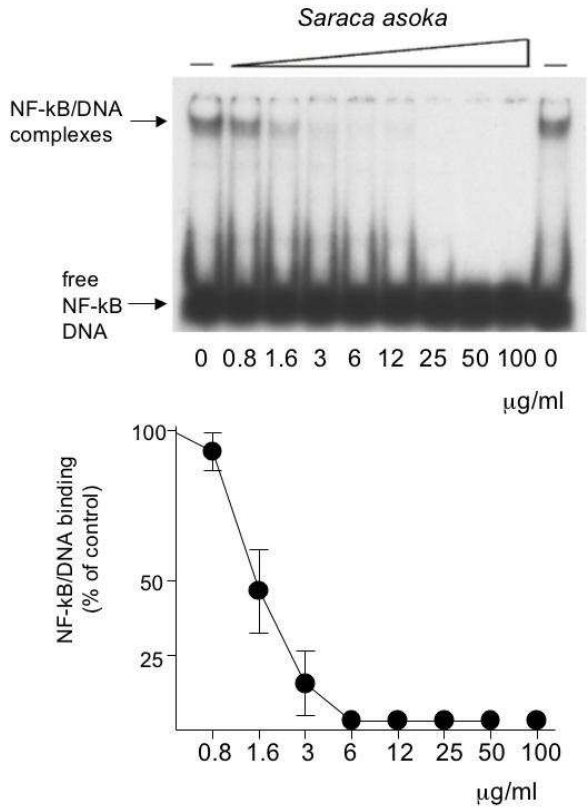


FIGURE 47 - Effect of Saraca asoka extracts on molecular interactions between nuclear proteins and 32P-labeled NF-kB DNA target molecules. Protein/DNA complexes were separated by polyacrylamide gel electrophoresis. The arrow indicates the protein/DNA complexes. * indicates the free 32P-labeled probe. A. Nuclear extracts from IB3-1 cells (4 µg) were incubated for 20 min in the absence (-) or presence of the indicated amounts of Saraca asoka extracts. After this incubation period, a further 20-min incubation step was performed in the presence of 32P-labeled NF-kB HIV-1 DNA sequences.

DISCUSSION AND FUTURE PERSPECTIVES

One of the clinical feature of cystic fibrosis (CF) is a deep inflammatory process, which is characterized by production and release of cytokines and chemokines, among which interleukin 8 (IL-8) represent one of the most important [109-112]. In fact, high levels of IL-8 are exhibited in CF cells infected with *Pseudomonas aeruginosa* or induced with TNF-α [118]. Accordingly, there is a growing interest in developing therapies against CF in order to reduce the excessive inflammatory response in the airways [99, 100] of CF patients. In this respect, among the inducible transcription factors that control inflammatory gene expression, nuclear factor NF-kB plays a central role.

In this thesis, we investigated on two different strategies with the aim of down-regulate the pro-inflammatory gene expression interfering with NF-kB activation pathway: (a) the transcription factor decoy approach; (b) screening of new therapeutic molecules identified in extracts from medicinal plants, such as Bergamot, *Saraca asoka* [259, 260] and *Emblica officinalis*, since they have been reported to exhibit anti-inflammatory activities in several studies [221-229].

1. TRANSCRIPTION FACTOR DECOY

In the past few years, the transcription factor decoy approach has been applied to modulate gene expression both *in vitro* [191, 192] and *in vivo* [193, 194], suggesting that this approach has potential value in non viral gene therapy. In this thesis, we applied a TF decoy strategy aimed at down-modulating the expression of pro-inflammatory genes in wild-type and CF bronchial epithelial cells infected with *P. aeruginosa*. Transfection of bronchial cells with decoy ODNs mimicking the NFkB sequences contained in the HIV-1 LTR or IL-8 genes clearly inhibited *P.aeruginosa*–dependent expression of IL-8.

A TF decoy strategy to down-modulate IL-8 expression was first proposed by

Cooper and colleagues in human monocytes stimulated with IL-1β [235]. In that experimental model, transfection of a 10-nucleotide decoy ODN 100% homologous to the core sequence of NF-kB identified in IL-8 promoter produced an average 60% inhibition of the IL-1β - dependent expression of IL-8 gene. Application of TF decoy ODNs has been tried by Griesenbach and coworkers with a different 20-nucleotide NFkB decoy ODN in CF tracheal epithelial cells stimulated with TNF-α, as they obtained only an average 40% inhibition of IL-8 secretion [236]. The same decoy ODN was unable to inhibit IL-6 secretion after bleomycin-induced pneumonia in murine lungs [237]. How can we reconcile these previous results with the highly efficient inhibition of IL-8 expression we report in the present thesis? Different decoy ODN sequences have been used in our experiments with respect to all the previous studies, which could, by itself, explain the discrepancies. In addition, Cooper and colleagues [235] used decoy ODNs without flanking regions, which could be more easily degraded by nucleases cleaving at the 5' or 3' ends of the ODNs, thus reducing their effectiveness in competing for active NF-kB. Also, the application of different pro-inflammatory stimuli could have impact on the efficiency of the NF-kB decoy ODNs, a stimulus that we chose considering this pathogen particularly relevant to study anti-inflammatory approaches for CF lung pathology. From this point of view, we report here that the HIV-1 LTR NF-kB decoy ODN has a lower potency of inhibition when IB3-1 cells have been stimulated with TNF-α instead of IL-1β and *P. aeruginosa* [fig.29-C]. This is in agreement with the weak inhibition that Griesenbach and coworkers [236] observed after using the same stimulus. This can be partly explained considering that TNF-α activates a panel of receptors, adapters, and kinases [266], which is quite different than those induced by *P. aeruginosa*, thus suggesting that transcription factors other than NF-kB could have a predominant role. Thus differences in the pro-inflammatory stimuli used could have an important role besides that played by the choice of the sequences of the decoy ODNs.

NF-kB proteins comprise a family of transcription factors involved in the control of a large number of genes related to cellular growth, developmental processes, apoptosis, and immune and inflammatory responses (for synopsis see http://

www.NF-kB.org). As far as our bronchial epithelial model system is concerned, we found that *P. aeruginosa* induces transcription of IL-8, GROγ, ICAM-1 IL-1β, and IL-6 genes, all of them containing NF-kB consensus sequences in their promoters, as summarized also in figure 25. How can we possibly explain that the HIV-1 LTR NF-kB decoy ODN strongly inhibits the *P. aeruginosa* - dependent transcription of IL-8, but not that of the other four genes induced in parallel in IB3-1 cells? We initially considered that the consensus sequences for NF-kB are not 100% homologous in the genes regulated by this transcription factor. Many nucleotide variations could be present, as summarized in the general NF-kB consensus usually reported (5'-GGG[A/G]NN[C/T][C/T]CC- 3'), which in principle accounts for the possibility of 2^7 different variants. In our specific case, none of the seven NF-kB consensus sequences identified in the promoters of the five genes that we found induced by *P. aeruginosa* are perfectly homologous with at least one of the others. Consequently, different degrees of homology between the HIV-1 LTR NF-kB decoy ODN and the consensus sequences in the promoters can be observed. Thus we hypothesized that an important parameter modulating the efficacy the NF-kB decoy ODNs in inhibiting transcription of IL-8, GRO-γ, ICAM-1, IL- 1β, and IL-6 should be the degree of homology between decoy ODN and consensus sequences.

For instance, the three consensus sequences identified within the promoter of ICAM-1 gene present the highest number of mismatches with the HIV-1 LTR NF-kB decoy ODN that prompted us to test the effect of new decoy ODNs designed with 100% homology. Disappointingly, none of the three ICAM-1 NF-kB decoy ODNs inhibited *P. aeruginosa* - dependent ICAM-1 transcription, even after transfecting the three decoy ODNs together [data not shown 267]. Although this unsuccessful attempt could be related to different parameters (e.g., length of the ODN, choice of the 5'- and 3'-flanking nucleotides, secondary structure, etc.), overall these negative results seem to basically disprove the hypothesis. Second, it should be noted that the HIV-1 LTR NF-kB decoy ODN is even more homologous to the consensus sequences reported in the GRO-γ and IL-6 than in the IL-8 promoter, whereas we proved the decoy

ODN being effective in inhibiting transcription of the latter but not of the former genes [fig.28]. Third, the IL-8 NF-kB decoy ODN, which is much more homologous than the HIV-1 LTR NF-kB decoy ODN with the consensus sequence in the promoter of IL-8, is less efficient in inhibiting IL-8 expression than the HIV-1 LTR NF-kB decoy ODN and inhibits partially also the transcription of GRO-γ and IL-6. Therefore it becomes apparent that the extent of homology between decoy ODN and consensus sequences is important but not sufficient to explain all the differences in effectiveness of each NF-kB decoy ODN in inhibiting the transcription of the five genes that we observed induced by *P. aeruginosa* in bronchial epithelial cells. Extensive analyses of a large series of ODNs mutated in their core consensus and/or flanking regions need future investigation to thoroughly answer these questions. A further explanation of the different efficiency of NF-kB decoy ODNs could be related to the complex interplay of transcription factors in the expression of the different genes of interest in the present study. Surface structures of *P. aeruginosa*, like flagellum and pili, interact with different receptors expressed on respiratory epithelial cells, leading eventually to activation of transcription factors such as NF-kB, AP-1, Elk-1, and NF–IL- 6, as already reported [195-197]. Interestingly, all the five genes we found induced by *P. aeruginosa* contain in their promoters one or more consensus sequences for NF-kB and some of them also for the transcription factors AP-1, NF-IL-6, and Sp-1 [fig.25]. Therefore, we hypothesize that the competition of the decoy ODN for the binding of NF-kB to its consensus sequence is not sufficient to inhibit drastically GRO-γ, ICAM-1, IL-1β, and IL-6 transcription also because of the hierarchical roles of AP-1, NF-IL-6, and Sp-1 in respect to NFkB for the transcription of some of those genes.

As far as the mechanism of action of the decoy ODNs is concerned, we can infer various suggestions. The degree of homology between specific decoy ODNs and the consensus sequences for the corresponding TF is important but not sufficient to predict the efficiency in inhibiting gene transcription. Efficiency of each decoy ODN could depend on the secondary structure and the kind of flanking regions of the ODNs, and on the hierarchy of the different TFs in regulating expression of specific genes. Thus, from a theoretical point of view,

our results strengthen the utility of the TF decoy strategy to provide useful insights on the issue of regulation of gene transcription. Conversely, as a practical consequence useful to applications in human diseases, decoy ODNs appear to present the advantage of a much higher gene specificity than previously expected. For instance, one may have anticipated a broad inhibitory effect of our NF-kB decoy ODNs on all the five genes containing NF-kB consensus sequences, whereas experimental results showed that only IL-8 is strongly inhibited with selected decoy ODNs. Considering the therapeutic applications of TF decoy ODNs to lung disease of patients with CF and/or to other human lung diseases in which the innate immunity is involved, we propose here a hint for inhibiting master genes in CF pulmonary pathology. This means that novel anti-inflammatory approaches focused on specific target genes relevant to specific diseases, without having too broad and undesired effects on the innate immune response, could be seriously taken into consideration. On the opposite side, knowing the redundancy of this response, a disease-oriented intervention should be tailored, focusing on the inhibition of the relevant pro-inflammatory genes with complementary TF decoy molecules. Different molecular approaches can be investigated to obtain a novel anti-inflammatory intervention. Strictly single-gene-related inhibitory approaches could be devised considering the application of anti-sense and Silencing inhibitory RNA (SiRNA) nucleotides. However, due to the redundancy of the inflammatory response to *P. aeruginosa* observed in CF lungs, inhibition of one chemokine (e.g., IL-8) could be bypassed by other potent chemoattractants (e.g., Gro-γ). A much broader inhibitory approach such as the modulation of the receptors for pathogens [268] would present the disadvantage of reducing the wide array of genes induced by pathogen-associated molecular patterns that are critical to maintain the proper anti-infective defenses against pathogens. In terms of receptor blockade, those for chemokines could also be a theoretically interesting target [269]. However, since chemokine receptors are expressed on circulating immune cells, the systemic delivery of antagonist or competing molecules could raise safety concerns. All this considered, TF decoy ODNs directed toward NF-kB regulating pro-inflammatory genes transcribed in CF

respiratory epithelial cells exposed to *P. aeruginosa* could present the advantage of extending or reducing the range of target genes by changing the ODN sequence, as observed also in the results presented here. In the perspective of application in the respiratory tract, NF-kB decoy ODNs conjugated with novel lipoplexes have been successfully utilized in murine lungs, obtaining effective inhibition of endotoxic shock (39). However, we are perfectly aware that the delivery of decoy ODNs in the conductive airways still requires extensive research and development, in particular to ensure sufficient stability in respect to degradation in the biological fluids. Recent progress obtained in other and our laboratories with PNA-chimeric TF decoy molecules that are significantly more permeable to the plasma membrane and resistant to the nucleases contained in serum and other biological fluids [247, 248, 252, 271-273] are rendering closer the possibility of extending the TF decoy strategy to in vivo pre-clinical experiments.

2. NATURAL PRODUCTS

- ***EMBLICA OFFICINALIS* AND *SARACA ASOKA***

Since extracts from medicinal plants have been reported in literature [275-278] to exhibit anti-inflammatory activities, it could be interesting to analyze the basis of this activity and the possible applications in biomedicine.

In this respect *Emblica offcinalis* and *Saraca asoka* [231, 233, 279] deserves great attention. These medicinal plants have been reported to be useful in traditional indian medicine for the treatment of several diseases. Fruits of *Emblica offcinalis* are used for the treatment of a number of diseases, such as dyslipidemia and atherosclerosis, as hepatoprotective, antibacterial and anti-inflammatory agent [221-229]. In many cases, *Emblica officinalis* has been shown to be a potent free radical scavenging agent thereby preventing carcinogenesis and mutagenesis [227]. Whereas, *Saraca asoka* is especially relied upon as an astringent to treat excessive uterine bleeding of different

origin, but also for regulating the menstrual cycle and, in various complex formulas, as a tonic for women.

In spite of these promising features, the reports on the biological effects of extracts from *Emblica officinalis* and *Saraca asoka* are few.

In this thesis we studied the effects of *Emblica officinalis* and *Saraca asoka* extracts on TNF-α mediated induction of interleukin and chemokines in cystic fibrosis IB3-1 cells, considering that: (a) in cystic fibrosis epithelium high levels of interleukin (for instance IL-6) [280, 281] and chemokines (for instance IL-8) [261, 262] are found; (b) this effect is thought to be mediated, at least in part, by TNF-α [243, 282] and (c) targeting IL-8 is considered of great interest in the anti-inflammatory treatment of CF patients [198, 201, 241, 245].

The first conclusion of the results presented in this thesis is that both *Emblica offcinalis* and *Saraca asoka* extracts are strong inhibitors of IL-8 expression, measured at the mRNA (using semi-quantitative as well as real-time quantitative RT-PCR assays) and protein level. This was determined in cystic fibrosis IB3-1 cells, either TNF-α-untreated (data not shown), or stimulated with TNF-α.

To validate these results, cytokines and chemokines in culture supernatants released from cystic fibrosis IB3-1 cells treated with TNF-α were measured by Bio-Plex cytokine assay. When IB3-1 cells were induced with TNF-α and cultured in the presence of *Emblica officinalis* extracts, some important differences in the release of MCP-1 (-83%), RANTES (-87%), VEGF (-41%), IL-8 (-14%) occurred compare to the control (IB3-1 cells induced with TNF-α without plant extracts treatment). Similar effects were found after the treatment of induced cells with increasing amount of *Saraca asoka* extracts: the results show a down-regulation in MCP-1, RANTES, VEGF and IL-8 expression, by 92%, 99%, 54% and 96% respectively. These data suggest that *Emblica offcinalis* and *Saraca asoka* extracts might inhibit the expression of the IL-8 pro-inflammatory gene. These data are in agreement with the results obtained using the RT-PCR experiments, and sustain the concept that these extracts could be inhibitors of the TNF-α induced expression of the IL-8 gene in IB3-1 cells. All these factors are important in the onset of the inflammatory state in CF

patients.

With respect to the anti-inflammatory activity of *Emblica officinalis* extracts, the identification of bioactive compounds is of interest to restrict biopharmaceutical drug design to single or few compounds. Gas chromatography-mass spectrometry (GC-MS) analyses showed pyrogallol as the common compound present both in unfractionated and n-butanol fraction of *Emblica officinalis* extracts. The Bio-plex analysis on supernatant from TNF-α induced IB3-1 cells suggest that pyrogallol inhibits especially the expression of RANTES (-83%) gene, but no significant data were obtained on regulation of IL-8 expression. These results were confirmed by RT-PCR analysis of RNA extracted from TNF-α treated IB3-1 cells cultured in the presence of increasing concentration of pyrogallol: IL-8 mRNA expression does not show inhibitory effects at the higher pyrogallol concentrations.

The data obtained indicate that *Emblica offcinalis* and *Saraca asoka* extracts inhibit NF-kB/DNA interactions. Nevertheless it is not clear the mechanism of action of *Emblica officinalis* and *Saraca asoka* extracts although an attempt was made to determine whether the plant extracts retain an inhibitory activity on the transcription factor NF-kB, which is critical for IL-8 gene expression and involved in CF inflammation. Phytochemical analysis will be performed to understand the main contituents of the extracts in order to evaluate which molecules are responsible for the downregulation on IL-8 expression. Further experiments to determine other biochemical targets of *Saraca asoka* and *Emblica offcinalis* extracts and the inhibitory effects on IL-8 gene expression in other cellular pathological systems and murine models are needed.

- **BERGAMOT**

The major result of this thesis is the finding that extracts obtained from bergamot (*Citrus bergamia* Risso) epicarps, contain components displaying an inhibitory activity on IL-8. This effect has been confirmed both at the mRNA

levels and the protein release in the CF cellular model IB3-1, induced with TNF-α and treated with low concentrations of bergamot extracts. The qualitative and quantitative determination of the chemical constituents of the extracts, with particular reference to non-volatile fraction chemicals, has demonstrated the presence of citropten, bergamottin and bergapten in all bergamot extract samples and with different amounts (%w/w) [table 5 and 6]. In particular the coumarin/psoralen portion was the major constituent of the most active among the extracts (extracts 3). Accordingly, we hypothesized that the coumarin/psoralen portion of these extracts were among the biological active components. On the other hand, the data reported do not clarify why extract 2 exhibited the lower biological activity. It should be noted that the composition of extract 1 and extracts 2 is complex and, therefore, the presence of compounds exhibiting opposite activity or interfering with the coumarin/psoralen compounds cannot be excluded. A second achievement of the results here presented is the demonstration that, among the preminent coumarin/psoralen compounds, bergapten and citropten were the most active molecules to reduce mRNA level of IL-8 in TNF-α treated IB3-1 cells at IC_{50} concentrations, while bergamottin did not show inhibitory effects at the same [fig.35] and at higher doses (data not shown). These results have been confirmed by real-time quantitative RT-PCR and Bio-plex analyses. In particular citropten is more efficient than bergapten in decreasing IL-8 mRNA/protein accumulation in IB3-1 cells.

These results clearly indicate that bergapten and citropten are strong inhibitors of IL-8 expression and could be proposed as potential anti-inflammatory molecules to reduce lung inflammation in CF patients. Further experiments are

required to determine: (a) other biochemical targets of citropten and bergapten as well as whether the inhibitory effects on IL-8 gene expression are reproducible in other cellular pathological systems in which this chemokine plays a role; (b) safety, potential toxicity, *in vivo* bioavailability, stability and whether suitable concentrations can be reached following in vivo administration. Moreover, it should be considered that coumarin compounds, as reported in literature, display anti-proteinase activity [274]. For example, serin proteinase is capable to degradate the matrix proteins and is involved in several inflammatory respiratory diseases as CF. It could interesting to investigate and correlate the anti-inflammatory effect of bergapten and citropten with their possible anti-proteinase activity. This double role of these comppunds could be useful for the treatment of lung inflammation in CF patients.

Last but not least, it would be of interest to see whether the effect of bergamot and its extracts could play the same role with different inflammation stimuli, such as IL-1β or PAO-1.