UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI
SCIENZE DELLA VITA E DELLA RIPRODUZIONE

DOTTORATO DI RICERCA IN BIOSCIENZE

CICLO XXII

TITOLO DELLA TESI DI DOTTORATO

STRUCTURAL FEATURES OF CATECHINS RESPONSIBLE FOR INHIBITORY ACTION ON SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1 (STAT1)

S.S.D. BIO/10

Coordinatore: Prof.ssa Marta Palmieri

Tutor: Prof.ssa Marta Vittoria Menegazzi

Dottoranda: Dott.ssa Martina Dal Bosco

A Camilla e Vincenzo

Index

Riassunto

Studi epidemiologici e ricerche sperimentali hanno rivelato che il tè verde ha molte proprietà sia come agente antitumorale che antiinfiammatorio. Tra tutti i polifenoli presenti nel tè verde, molti lavori mostrano che l'epigallocatechina gallato (EGCG) è il principale principio attivo in quanto esso è in grado di inibire l'angiogenesi, l'urochinasi, le metalloproteinasi e l'induzione dell'ossido nitrico sintasi inducibile (iNOS). Gli effetti benefici del tè verde si pensavano essere attribuiti alle spiccate proprietà antiossidanti dei polifenoli in esso contenuti, ma dati ottenuti negli anni passati nel nostro laboratorio hanno evidenziato una diversa azione più specifica.

Signal Transducers and Activators of Trascription (STATs) sono una famiglia di fattori trascrizionali che mediano l'azione di citochine coinvolte in numerose funzioni biologiche. Tra tutte la catechine presenti nel tè verde, solo EGCG era in grado di inibire l'attivazione del fattore trascrizionale Signal Transducer and Activator of Trascription 1 (STAT1), indotta dalla citochina interferone gamma (IFNγ). L'attivazione di STAT1, indotta da IFNγ, fa parte di alcune vie di trasduzione del segnale che innescano numerosi processi infiammatori, ed è necessaria per l'espressione di molteplici geni pro-infiammatori quali iNOS e il complesso maggiore di istompatibilità di calsse II DR alfa (MCH II DRA). La loro attivazione è stata riscontra in numerose malattie e EGCG, essendo in grado di inibirne l'espressione, risulta essere un buon coadiuvante nel trattamento di patologie infiammatorie STAT1 dipendenti.

15 nuove catechine gallato con struttura chimica analoga alla gallocatechina gallato (GCG), diastereoisomero *trans* dell'EGCG, sono state sintetizzate per il lavoro di ricerca qui proposto. Queste catechine di sintesi presentano un ridotto numero di ossidrili legati ai vari anelli della struttura rispetto alla GCG, composto di riferimento. I composti 2 e 3 presentano un minor numero di ossidrili sull'anello A, i composti 4, 5 e 6 mostrano una riduzione di ossidrili sull'anello B, i composti 7, 8, 9 e 10

presentano modifiche sull'anello D, mentre i composti rimanenti portano riduzioni di ossidrili su più anelli contemporaneamente.

Lo studio ha permesso di verificare:

1) In una linea cellulare di carcinoma mammario MDA MB 231, la capacità dei composti di sintesi studiati di inibire l'attivazione di STAT1 indotta da IFNγ, allo scopo di individuare la porzione di molecola minima necessaria e sufficiente per garantirne l'attività. I dati ottenuti indicano che la presenza di tre ossidrili in posizione 3′, 4′ e 5′ dell'anello B, e di almeno un ossidrile nell'anello D rendono le catechine capaci di inibire la fosforilazione di STAT1 in Tyr701 e Ser727, di bloccare il legame di STAT1 al DNA ed di impedire l'espressione di geni STAT1 dipendenti iNOS e MCH II DRA.

2) La diretta interazione tra la proteina STAT1 ricombinante e le catechine attive. L'ipotesi che, lungo la via IFNγ/STAT1, il target molecolare delle catechine studiate potesse essere la proteina STAT1 stessa è stata verificata mediante Surface Plasmon Resonance (SPR), Molecular Modelling, studi di mutagenesi sito specifica e saggi chinasici tra la proteina ricombinate STAT1 e l'enzima ricombinate Janus Kinase 2 (JAK2).

I nostri dati hanno permesso di suggerire un possibile, specifico ed efficiente meccanismo d'inibizione dell'attivazione di STAT1, da parte delle catechine. Ciò può rappresentare un punto d'inizio per lo sviluppo di nuovi composti capaci di modulare selettivamente l'attivazione di STAT1, utili nella prevenzione o terapia di patologie infiammatorie.

Abstract

In the last years, a great interest is emerging about green tea as a tool against human cancer development or inflammation, as pointed out by many reports describing the inhibitory action of epigallocatechin gallate (EGCG), the main polyphenol component of green tea, on angiogenesis, urokinase, metalloproteinases, and induction of inducible nitric oxide synthase (iNOS). The beneficial effect is believed to be due to their strong antioxidant activity. However, our laboratory evidence indicates the involvement of a more specific action.

Signal Transducers and Activators of Transcription (STATs) are nuclear factors mediating the action of cytokines involved in various biological functions. Among a number of catechins present in green tea extract, only epigallocatechin gallate (EGCG) exerts a strong inhibitory action on interferon gamma (IFNγ)-elicited activation of Signal Transducer and Activator of Transcription 1 (STAT1).

IFNγ-elicited STAT1 activation is important for iNOS and other STAT1 dependent genes expression, as major histocompatibility complex class II DR alpha (MCH II DRA).

15 new compounds, obtained by chemical modification of gallocatechin gallete (GCG), diastereomer of EGCG, were synthesized. They present fewer hydroxyl groups than GCG in ring A (compounds 1, 2 and 3) or in ring B (compounds 4, 5 and 6) or in ring D (compounds 7, 8, 9 and 10), or simultaneously in more than one ring (compounds 11, 12, 13, 14 and 15).

In this work we show:

1) The ability of some of this new synthetic compounds to inhibit IFNγ-elicited activation of STAT1, in human MDA MB 231 cell line, by showing the minimal chemical features necessary and sufficient for this activity. The synthetic catechins with three hydroxyl groups in 3′, 4′ and 5′ of ring B and at least one hydroxyl group in ring D are able to block STAT1 Tyr701 and Ser727 phosphorylation, STAT1 DNA-binding and STAT1 dependent iNOS and MCH II DRA gene expression.

2) The direct interaction between STAT1 recombinant protein and the anti-STAT1 catechins, identifying STAT1 itself as the real molecular target of catechins in the IFNγ/STAT1 pathway. This data was confirmed by Surface Plasmon Resonance (SPR) analysis, Molecular Modelling studies, Site-direct Mutagenesis, and STAT1- Janus Kinase 2 (JAK2) kinase assay.

In conclusion, we suggest a specific molecular mechanism of action of the catechins. These results may be considered the first step to the development of new anti-STAT1 molecules, that can be used as new drugs against inflammatory processes by modulation of STAT1 signaling.

1. Introduction

1.1   Inflammation

Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. However, inflammation and repair may be potentially harmful.

Inflammation is divided into acute and chronic patterns. Acute inflammation is relatively short duration and it is characterized by increased blood flow in the injured area. Leukocytes adhere to endothelium via adhesion molecules, transmigrate across the endothelium and migrate to the sites of injury under the influence of chemotactic agents. Phagocytosis of the offending agent follows, which may led to the death of the microorganism. During chemotaxis and phagocytosis, activated leukocytes may release toxic metabolites and proteases causing tissue damage.

Chronic inflammation is considered to be low-grade inflammation of prolonged duration. This includes some of the most common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and the chronic lung diseases. Both acute and chronic inflammation provoke the release of mediators, such as interleukins, tumor necrosis factor α, interferons and platelet-derived grow factor that can amplify the inflammatory response and influence its evolution.

There are two classes of anti-inflammatory agents: glucocorticoids and nonsteroidal anti-inflammatory drugs. Glucocorticoids inhibit phospholipase A2 , the enzyme that hydrolyzes the ester bond of membrane phospholipids with the release of arachidonate and prostaglandin production (Flower R. J., 1990).

Non-steroid anti-inflammatory drugs (NSAIDs) are the most widely prescribed for inflammatory diseases, these being the most critical worldwide pathologies, whereas extensive effort should be paid to the development of a new therapy. It is well known that NSAIDs exert their anti-inflammatory, analgesic and antipyretic action by inhibiting the activity of cyclooxygenase (COX), the prostaglandins synthesizing

enzyme (Vane J.R., 1971). Although the usefulness of NSAIDs has been widely documented, their prolonged administration often leads to undesirable side effect, especially in the gastrointestinal tract (Vane J. R. et al., 2003). Consequently, in the last decade many efforts have been paid to find new drugs, leading to the development of a new class of NSAIDs with improved gastrointestinal tolerability (Meyer-Kirchrath J., 2000).

1.2   STAT1 as a new molecular target of anti-inflammatory treatment

One of the hallmarks of the inflammatory response is the massive synthesis of nitric oxide (NO) by inducible NO synthase (iNOS), the expression of which is finely up-regulated by a number of pro-inflammatory cytokines such as interferon gamma (IFNγ), tumor necrosis factor alfa (TNFα) and interleukin 1beta (IL1β), together with gram-negative bacterial membrane-derived lipopolysaccharides (LPS) (Mariotto S. et al., 2004). The activation of iNOS provokes a massive production of NO which is deleterious for the cell.

Pro-inflammatory cytokines and LPS exert their action by activating specific nuclear factors regulating the expression of inflammatory genes. Signal Transducer and Activator of Transcription 1 (STAT1) principally mediate the action of IFNγ, while the nuclear factor-kappaB (NF-κB) mediates the signaling of TNFα, IL1β and LPS. The modulation of these nuclear factors has recently been considered a new strategy in the treatment of inflammatory diseases. Although NF-κB has so far received more attention (Lee J. I. et al.,1998; Zingarelli B. et al., 2003) we decided to study the critical role played by STAT1, which is activated in a number of related diseases (Carcereri de prati A. et al, 2005).

The inflammatory response proceeds without a minimum damage to the body if STAT1 activation is time-spatially well regulated. However, any deregulation of

STAT1 activation (i.e. excessive up- or down-regulation, prolonged activation, time-spatially erroneous activation, etc.) may cause a deleterious effect to tissue integrity (Lin T.S., 2000).

STAT1 hyperactivation is one of the most relevant phenomena in many pathologies correlated to both acute and chronic inflammation, such as ischemia/reperfusion injury, unstable angina, asthma, atherosclerosis, celiac disease and psoriasis. So the modulation of STAT1 activation may be a promising molecular target for a new class of anti-inflammatory drugs.

STAT1 protein belongs to a family of transcription factors that are inactive in the absence of specific receptor stimulation and are localized in the cytoplasm of unstimulated cells. Seven mammalian STAT proteins have been identified, STATs 1, 3, 4, 5A, 5B are between 750 and 795 amino acids long, whereas STATs 2 and 6 are nearly 850 amino acids long. These proteins are activated by a different group of cytokines and growth factors and all these proteins share structurally and functionally conserved five domains: a N-terminal domain (NH2), a coiled-coil domain (CCD), a DNA-binding domain (DBD), a linker domain (L), a Src homology 2 (SH2) domain (Fig.1). The transcriptional activation domain (TAD) at the carboxy (C) terminus is specific for the different STATs (Fig. 2).
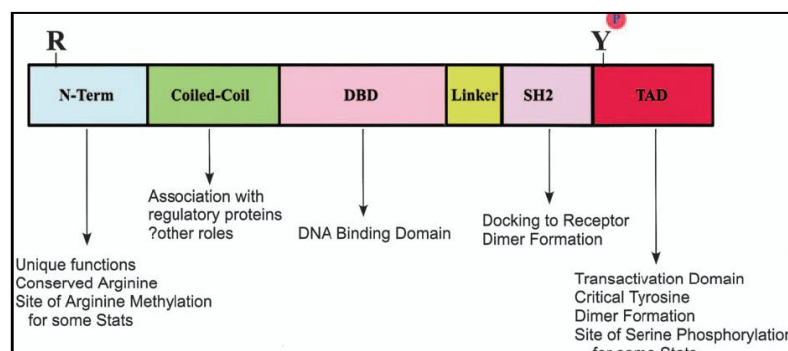

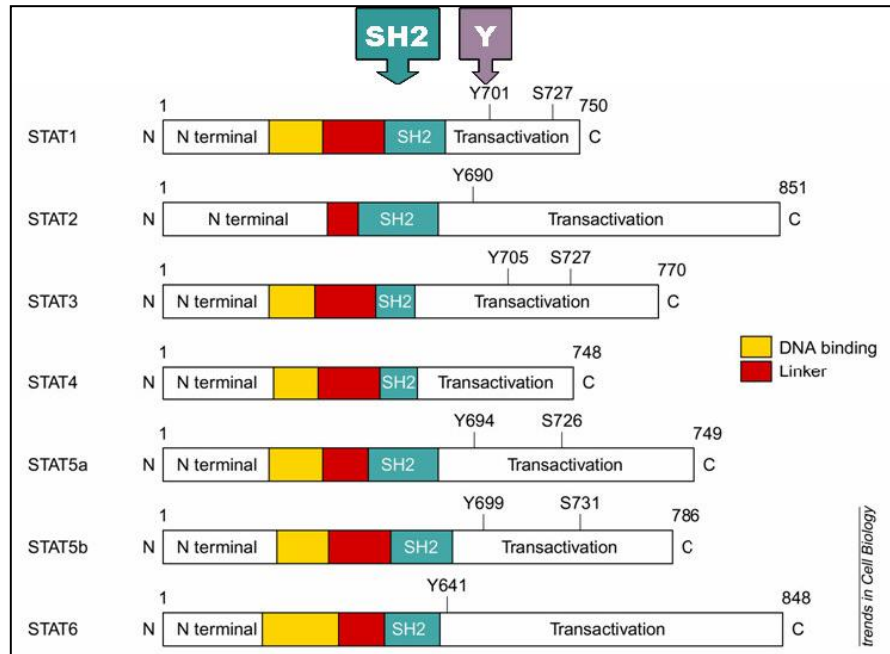**Figure 1: Conserve structural domains of STAT family proteins and their function** (Chen W., 2004)**.**
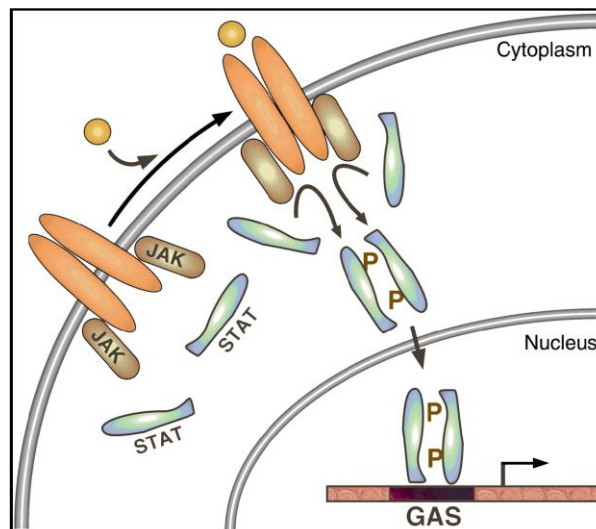
Figure 2: STAT proteins

Figure 3: JAK/STAT signaling pathway (Christian W. S., 2002).

1.3 The JAK/STAT pathway

The canonical Janus Kinase (JAK)/STAT signaling pathway is initiated by binding of a cytokine ligand to a cell-surface cytokine receptor (Fig. 3). The intracellular domains of many cytokine receptors are physically associated with tyrosine kinases of the JAK family. There are four mammalian JAKs (JAK1, 2, 3 and Tyk2) that are associated with specific cytokine receptors as can be seen in Table 1.

Binding of ligand triggers receptor dimerization/oligomerization and allows rapid transphosphorylation and activation of the receptor-associated JAKs. Activated JAKs then phosphorylate critical tyrosine residues on the receptor, creating STAT docking sites. In fact this leads to recruitment of specific STATs through their SH2 domains followed by single tyrosine phosphorylation of the bound STAT (Darnell J. E., 1997). Tyrosine phosphorylation of STATs can also be stimulated by binding of growth factors to receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor or platelet-derived growth factor receptor.

Table 1: Cytokines transduce their signals through specific sets of JAKs and STATs as indicated.

Receptors	Ligands	Jaks kinasi	STATs
IFN Family	IFNγ	TYK2, JAK1	STAT1, STAT2
	IFNγ	JAK 1, JAK 2	STAT1
	IL10	TYK 2, JAK 1	STAT3
Gp130 Family	IL6	JAK1, JAK2, TYK2	STAT3
	IL11	JAK 1	STAT3
	IL12	TYK2, JAK 2	STAT4
γC Family	IL2, IL7,IL15	JAK 1, JAK 3	STAT5
	IL4	JAK 1, JAK 3	STAT6
Receptor tyrosine kinases	EGF, CSF1		STAT1, STAT3, STAT5
	PDGF		STAT1, STAT3

After phosphorylation, STAT proteins form homodimers via reciprocal phosphotyrosine-SH2 interactions. Activated STAT dimers enter the nucleus via Importin-α dependent transport and bind to specific DNA targets. Nuclear import of different STAT proteins requires different transporters (e.g., STAT3 binds to importin-α3, STAT1 to importin-α5).

In order to drive gene expression, STAT proteins cooperate with numerous other transcription factors such as IRFs, Sp1, Jun, Fos, NF-κB and coactivators with functions in chromatin remodeling such as p300/CBP, PCAF, BRG1.

Maximal transcriptional activation requires serine (S) phosphorylation of a conserved motif (L)P(M)SP in the STAT C terminus. Dissociation from DNA allows dephosphorylation of STATs by nuclear protein tyrosine phosphatases (N-PTPs), such as SHP2, to complete the cycle of activation/inactivation. The unphosphorylated dimers associate with the nuclear export factor, chromosome region maintenance 1 (CRM1), for transport back to the cytoplasm where it can be reactivated (Meyer T. and Vinkemeier U., 2004) (Fig.4).
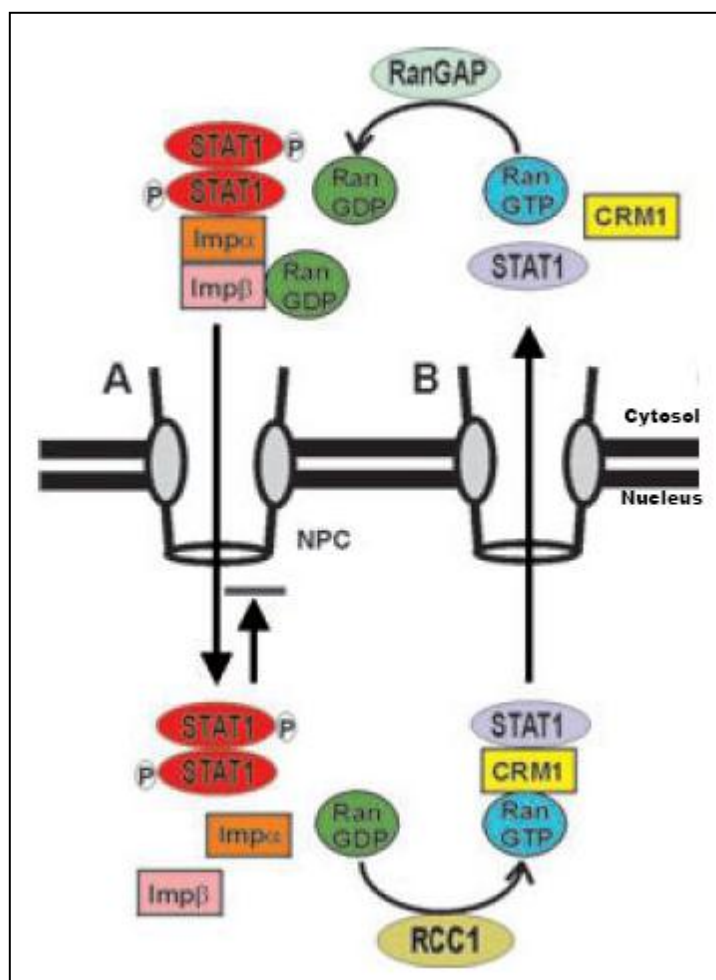
Figure 4: STATs at the nuclear envelope.
(A) Carrier-dependent import. Phosphorylated STAT dimers expose a dimer-specific nuclear localization signal and associate with importin α. Through importin β-mediated interactions with the interior of the nuclear pore (NPC) this complex migrates into the nucleus. The complex disassembles after the binding of RanGTP.
(B) Carrier-dependent export. Unphosphorylated STATs can bind to the exportin CRM1 via leucine-rich nuclear export signals and traverse the NPC. RanGTP enhances the interaction of CRM1 with cargo proteins. In the cytoplasm, the nucleotide hydrolysis of RanGTP leads to release of the cargo.

The negative regulators of the JAK/STAT pathway are well known; they include suppressors of cytokine signaling (SOCSs), protein tyrosine phosphatases (PTPs) and protein inhibitors of activated stats (PIAS) (Greenhalgh C. J. and Hilton D. J., 2001) (Fig.5).

SOCS proteins are a family of eight members, SOCSs 1 to 7 and CIS, they complete a simple negative feedback loop in the JAK/STAT circuit. Activated STATs

stimulate transcription of the SOCS genes and the resulting SOCS proteins bind directly phosphorylated JAKs to specifically inhibit JAK kinase activity (Starr K. et al., 1997). SOCS proteins can also bind phosphotyrosines on the receptors, so physically block the recruitment of STATs to the receptor (Endo T. A. et al., 1997; Shuai K. and Liu B., 2003; Chen X. P. et al., 2000).

PTPs, in the cytoplasm, reverse the activity of the JAKs. This class of enzymes includes SHP-1, SHP-2 and CD45. The best characterized of these is SHP-1, this contains two SH2 domains and can bind to either phosphorylated JAKs or phosphorylated receptors to facilitate dephosphorylation of these activated signaling molecules (Rane S. G. and Reddy E. P., 2000).

In the nucleus, STAT activity is restrained by PIAS proteins. The PIAS family proteins are a group of 5 STAT interacting proteins that bind to activated STAT dimers and prevent them from binding DNA.
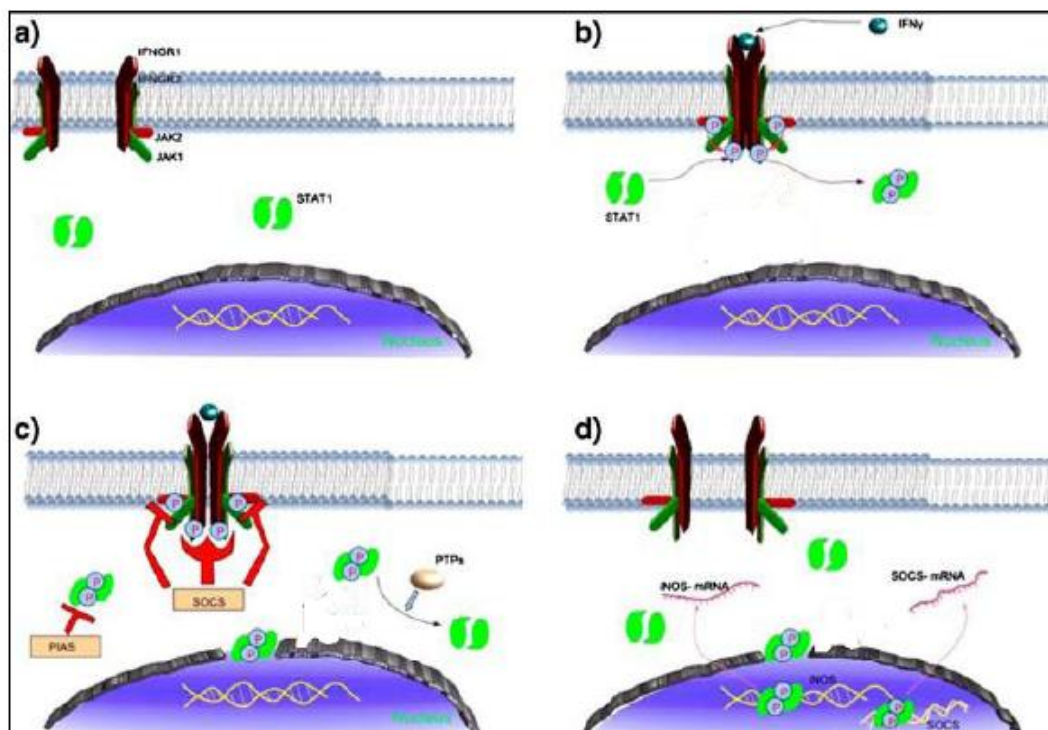
Figure 5: STAT1 cascade. a) Normal condition, all the player of STAT1 cascade are present in the resting state. b) Activation of IFNγ-elicited STAT1 and its up-regulation. Cellular membrane-bound IFNγ receptor (IFNGR 1/2) recognizes, among different cytokines, specifically IFNγ. Upon recognition, IFNγ activates IFNGR1/2 leading to activation of neighbouring JAK 1/2. Activated JAK 1/2 phosphorylate tyrosine residue(s) of themselves, IFNGR 1/2 and STAT1. Tyrosine-phosphorylated STAT1 forms dimers, thus achieving the capacity to enter the nucleus. c) Down-regulation of JAK 1/2 may be tuned by other proteins such as PIAS, PTPs including SHP-1 and SOCS. d) Induction of STAT1-dependent genes. Activated STAT1 recognizes the promoter regions of specific genes encoding proteins deeply involved in the inflammatory response such as iNOS and SOCS.

1.3.1 The principal players of IFNγ-elicited STAT1 pathway

IFNγ receptor belongs to the class II cytokine receptor family and has two subunits (Fig. 5): IFNGR1, the ligand-binding chain, also known as the α chain, and IFNGR2, the signal-transducing chain, also known as the β chain. IFNGR chains lack intrinsic kinase/phosphatase activity and so must associate with signaling machinery for signal transduction. The intracellular domain of IFNGR1 contains two functionally importance sequences: JAK1 binding motif LPKS (Leu-Pro-Lys-Ser), a membrane-

proximal sequence, and the STAT1 binding sites YDKPH (Tyr-Asp-Lys-Pro-His) . This last motif contains an essential Y440 phosphorylation site that is phosphorylated during signal transduction to allow STAT1 recruitment to receptor. The intracellular domain of IFNGR2 contains a functionally important box1/box2 sequence, required for Jak2 association.

Upon interaction with IFNγ, IFNGR1 dimerize and become associated with two IFNGR2. Receptor assembly leads to activation of JAK1 and JAK2 and phosphorylation of the tyrosine 440 residue on the intracellular domain of IFNGR1. This leads to the recruitment and phosphorylation of STAT1, which forms homodimers and translocates to the nucleus to activate a wide range of target genes. After signaling, the ligand-binding chains of receptor are internalized and dissociate; the chains are then recycled to the cell surface.

JAKs are a family of tyrosine kinases costitutively associated with a proline-rich membrane proximal domain of the receptor (Ihle I. N., 2001). Upon ligand stimulation, receptors undergo the conformational changes that bring JAKs into proximity of each other, enabling activation by trans-phosphorylation (Remy I. et al., 1999) (Fig. 6).
There are four members of the JAK family in mammals, Jak1, Jak2, Jak3 and Tyk2. Jak1, Jak2 and Tyk2 are expressed ubiquitously, whereas the expression of Jak3 is restricted to cells of the myeloid and lymphoid lineages (Leonard W. and O'Shea J. J.,1998, Witthun B. A. et al., 1994).
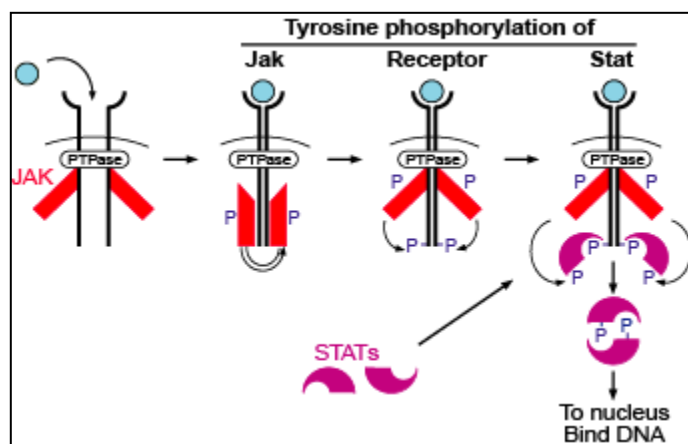
Figure 6: Mechanism of STAT1 tyrosine phosphorylation by JAK.

STAT1 protein, similarly to other STATs proteins, shares structurally and functionally conserved five domains:

N-terminal domain, comprising approximately 130 amino acids. Several studies suggest that N-terminal domain promotes cooperativity of binding to tandem GAS elements (Xu X. et al., 1996). Other studies have suggested that the N-terminal STAT domain promotes interaction with the transcriptional coactivator CBP/p300 (Horvath C. M., 2000), the PIAS family (Shuai K., 2000), receptor domains (Leung S. et al., 1996), and that it regulates nuclear translocation (Strehlow I. and Schindler C., 1998).

Coiled-coil domain consists of four α-helices (approximately amino acid 135–315). The crystal structures of STAT1 reveals that this domain protrudes about 80A° laterally from the core structure (Chen X. et al., 1998). This forms a large predominantly hydrophilic surface that is available for specific interactions with other helical proteins such as p48/IRF9, c-Jun and N-myc (Zhang J. G. et al., 1999a; Zhu M. H. et al., 1999). More recently, studies have also implicated the coiled-coil domain in receptor binding, tyrosine phosphorylation and nuclear export (Zhang T. et al., 2000).

DNA-binding domain, approximately amino acid 320–480, recognizes interferon gamma activated site (GAS) element and activates transcriptional activity. Since the DNA binding domain is likely to exhibit a different conformation prior to activation,

it is appealing to consider the possibility that it may have additional functions (McBride K. M. et al., 2000).

Linker domain, amino acids 488–576 connects the DNA-binding domain with the SH2 domain. The STAT1 crystal structure reveals that helix α10 of the linker domain interacts with the SH2 domain, and that helix α6 interacts with strand β11 of the DNA binding domain (Chen X. et al., 1998). These observations suggest that DNA binding capacity can be regulated by structural changes in the SH2 domain.

SH2 domain plays an important role in signaling through its capacity to bind to specific phosphotyrosine motifs. Consistent with this, its structure (amino acids 580–680) is well conserved. It consists of an anti-parallel β-sheet flanked by two α-helices, which form a pocket. An absolutely conserved STAT1 arginine (Arg602), which mediates the interaction with phosphate, lies at the base of this pocket. The ability of this SH2 domain to recognize specific phosphotyrosine motifs plays an essential role in three signaling events: (1) recruitment to the cytokine receptor through recognition of specific receptor phosphotyrosine motifs, (2) association with the activated JAK and (3) STAT homodimerization (Shuai K. et al., 1994). As highlighted in the crystal structures of STAT1, the dimerization depends on the interaction between the SH2 domain of one STAT monomer and the tyrosine phosphorylated tail segment of the other monomer. The crystal structure of STAT1 bound to DNA shows that the dimeric interaction between two SH2 domains are crucial for the formation of a DNA binding clamp wraps almost entirely around the duplex (Chen X. et al., 1998) (Fig. 7).

The *Transcriptional Activation Domain*, at carboxy-terminal domain, is poorly conserved among the STATs. This domain contains a critical tyrosine residue (Y~700) which is phosphorylated when the molecule is activated. In particular activated STAT1 is phosphorylated in tyrosine 701.

TAD regulates unique transcriptional responses, but how the STAT carboxy-terminus regulates these responses remains to be determined. However important progress has been made, for example it has been determined that the transcriptional activity of

several STATs can be modulated through serine phosphorylation (Decker T. and Kovarik P., 2000; Schroeder K. et al., 2004).

The IFNγ/JAK/STAT1 cascade appears to cross-talk with other signal transduction pathways in addition to STAT1, in fact IFNγ activates PI3K/Akt, MKK/ERK1/2 and p38 MAP kinase pathways (Nguyen H., 2001; Blanchette J., 2003) that are responsible for the STAT1 serine phosphorylation enhancing STAT1 transcriptional activity, as mentioned before.

Since STAT1 cascade requires multiple phosphorylation steps, it is likely to be potentially achieved at various steps (fig. 5). As described in literature, diverse agents may act at multiple levels to inhibit the STAT1 signal transduction pathway such as JAK tyrosine phosphorylation, STAT1 serine and tyrosine phosphorylation or STAT1 DNA binding.
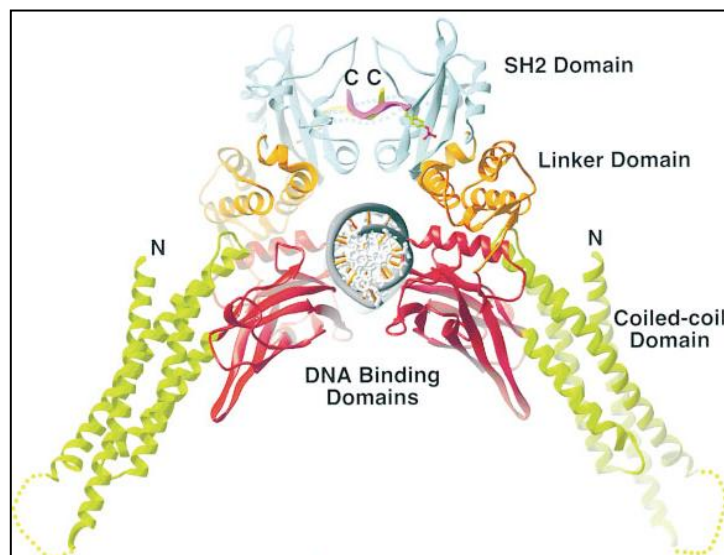

Figure 7: The crystal structure of the STAT1 DNA complex (Chen X., 1998)**.**

1.4 Inducible Nitric Oxide Synthase

Nitric oxide (NO) synthase is a widely distributed enzyme catalyzing the synthesis of NO from L-arginine. There are three isoforms of NO synthase encoded by distinct genes, including the constitutively expressed neuronal and endothelial NO synthase (nNOS, eNOS) and iNOS.

iNOS is normally absent in resting cells (Kleinert H. et al., 2000). After activation of cells by different inducers, iNOS is expressed and is active for hours to days as a "high-output" enzyme (MacMicking J. et al., 1997). The high amounts of NO produced by iNOS may be beneficial for their microbicidal, antiviral, antiparasitic, and antitumoral action (MacMicking J.et al., 1997; Bogdan C., 2001). However, an aberrant iNOS induction (high concentrations of NO, up to millimolar concentration, around the site of inflammation) is likely to have detrimental consequences and seems to be involved in the pathology of several human diseases (Kroncke K. D. et al., 1998). The iNOS enzyme is expressed in various organs, including the lungs and intestine, where an overproduction of NO contributes significantly to vascular failure and end-organ damage during endotoxemia and to diseases such as asthma, short- and long-term lung disease, and septic shock (Barnes P. J. and Liew F. W., 1995; Kro¨ncke K. D. et al., 1998; Kleinert H. et al., 2000; Bogdan C., 2001). Enhanced iNOS expression is observed in patients with inflammatory bowel disease (Cavicchi M. and Whittle B. J., 1999), celiac disease (van Straaten E. A. et al., 1999), ulcerative colitis, and Crohn's disease (Guslandi M., 1998). Therefore, pharmacological suppression of iNOS-dependent NO production may be helpful in the treatment of these diseases. The regulation of iNOS expression is cell- and species specific, and a variety of signal transduction pathways are involved (Kleinert H. et al., 2000). Regulation of the transcription of the iNOS gene is believed to be the most important control mechanism for iNOS expression. JAK/STAT pathway has been shown to be essential for human and murine iNOS expression (Gao J. et al., 1997; Kleinert H. et al., 1998; Ganster R. W. et al., 2001; Menegazzi M. et al., 2001; Yao Y. et al., 2003). iNOS induction in human cells seems to be partially dependent on activation of the

NF-κB signal pathway (Kleinert H. et al., 1998). Studies using the human iNOS promoter showed important regulation of the iNOS promoter activity by the transcription factors NF-κB, activator protein (AP) 1, and STAT1 (de Vera M. E. et al., 1996; Kleinert H. et al., 1998; Kleinert H. et al., 2000; Ganster R. W. et al., 2001). Ganster et al. (2001) described the dependence of human iNOS promoter activity on a composite GAS/NF-κB element located at position -5.8 kb in the human iNOS promoter (Ganster R. W. et al., 2001). Therefore, the IFNγ/JAK/STAT pathway seems to be a good target for the inhibition of human iNOS promoter activation and hence iNOS expression in pathologic situations.

1.5 Major Histocompatibility Complex Class II

The major histocompatibility complex (MCH) class II plays an important role in cellular immunity, recognition, and differentiation. The MHC class II (MHC II) genes are composed of human leukocyte antigen (HLA) -DQ, -DP, -DM and –DR, the latter is the most abundant form. These encode α and β chains of heterodimeric cell surface molecules that present processed antigens to $CD4^+$ T-lymphocytes. Non-immune cells lack constitutive expression of MCH class II, however, in most of these cells MCH class II expression can be induced by IFNγ (Benoist C. et al., 1990; Glimcher L. H. et al., 1992).

The regulation of MHC II genes occurs primarily at the transcriptional level, and a non DNA-binding protein, class II transactivator (CIITA), has been shown to be the master control factor for MHC II transcription (Rohn W. M. et al., 1996; Van den Else P. J. et al., 1998). The induction of MHC II genes transcription by IFNγ occurs relatively slowly and depends on the presence of the transcription factors STAT1 (Meraz M. A. et al., 1996). STAT1 induces transcription of CIITA by direct binding to CIITA promoter. CIITA is both essential and sufficient for MHC II expression (Steimle V. et al., 1994).

High expression of MHC class II molecules has been postulated to be involved in the progression of various inflammatory diseases, such as rheumatoid arthritis, insulin-dependent diabetes mellitus, inflammatory bowel disease and multiple sclerosis (Grusby M. J. et al., 1995). Indeed MHC II expression was found on the endothelium in atherosclerotic lesions obtained from patients who died of cardiovascular or neurologic diseases (Van der Wal A. C. et al., 1992). An increased expression of MHC II molecules in arterial tissue from transplanted hearts was predictive of arteriosclerosis and graft failure (Labarrere C.A. et al., 1997).

Hence MCH II expression in non-immune cells may indicate an inflammatory state, which can compromise the integrity of the tissue. So the inhibition of this gene expression can improve organ condition.

1.6 The Catechins of Green Tea

Green tea is tea made solely with the leaves of *Camellia sinensis* that have undergone minimal oxidation during processing. There are two major varieties that characterize this species: *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica*, that are members of the Theaceae family.

Green tea originates from China and has become associated with many cultures in Asia from Japan and South Korea to the Middle East. Many varieties of green tea have been created in countries where it is grown. These varieties can differ substantially due to variable growing conditions, processing, and harvesting time. Tea is manufactured in three basic forms: black tea (78%), green tea (20%) and oolong tea (2%). Unlike black and oolong tea, green tea production does not involve oxidation of young tea leaves. Green tea is produced from steaming fresh leaves at high temperatures, thereby inactivating the oxidizing enzymes and leaving the polyphenoles content intact.

Thus, green tea extract (GTE) is rich in powerful anti-oxidant polyphenoles. The polyphenoles found in tea are more commonly known as catechins and comprise 30–40% of the extractable solids of dried green tea leaves.

The main catechines in green tea are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) (Fig. 8). EGCG, found in the highest concentration in green tea, is the most active and best researched of all green tea ingredients.

Of all the teas consumed in the world, green tea is well studied for its health benefits (Mukhtar H. and Ahmad N., 1999). Green tea is generally considered a safe, non-toxic beverage and its consumption is usually without side effects. Several epidemiological investigations in various countries reveal beneficial effects of green tea (Hirvonen T. et al. 2000; Klatsky A. L. et al. 1993).

Green tea polyphenols have demonstrated significant anti-oxidant, anti-carcinogenic, anti-inflammatory, thermogenic, probiotic and anti-microbial properties in numerous human, animals and *"in vitro"* studies (Choi Y. B. et al. 2004; Crespy V. and Williamson G., 2004; Kakuda T., 2002; Sung H. et al. 2000). It has been demonstrated that ingestion of green tea significantly increased human plasma anti-oxidant capacity *"in vivo"* (Serafini M. et al. 1996). Tea polyphenols have also been noted to induce apoptosis and cell cycle arrest in a wide array of cell lines (Levites Y. et al. 2002) thus retarding the growth and development of neoplasms. In addition, the protective effects of tea catechines were evaluated by the attenuation of nitric oxide formation from inducible NO synthase (Chan M. M. et al. 1997).

The molecular basis of the action of this catechins has for long time been elusive. Recently some hints on the possible molecular mechanism of the antitumor or anti-inflammatory effects of green tea have emerged: EGCG inhibits urokinase (Jankun J. et al., 1997), an enzyme indispensable for tumor invasion and metastasis, and angiogenesis (Cao Y., 1999), which is crucial for all types of tumor. In addition, EGCG efficiently suppresses the activity of matrix metalloproteinase-2 and -9, essential for tumor cells in cutting through basement membrane barriers (Liotta L. A. et al., 1980; Lin Y. L. and Lin J. K., 1997), thus leading to an efficient inhibition of

angiogenesis. Unlike to our cellular system, in the macrophage-derived cell line EGCG was found to exert a strong inhibitory action on NF-κB activation (Gupta S. et al., 2004), this seem to indicate that EGCG exerts a distinct action on the nuclear factor activation in different cells lines. EGCG also blocks the induction of expression of iNOS in lipopolysaccharide (LPS) activated mouse macrophages (Moncada S., 1992).

Studies with [³H]EGCG, labeled with ³H, shows the distribution of [³H]EGCG in mouse organs by oral administration. Radioactivity was found in various organs as digestive tract, lung, breast, liver, pancreas, skin, kidney, brain, uterus, ovary and testes. This indicated that [³H]EGCG was incorporated into cells of organs, in particular these studies showed that radioactivity was incorporated into cytosol and nuclei of cells (Okabe S. et al., 1997).
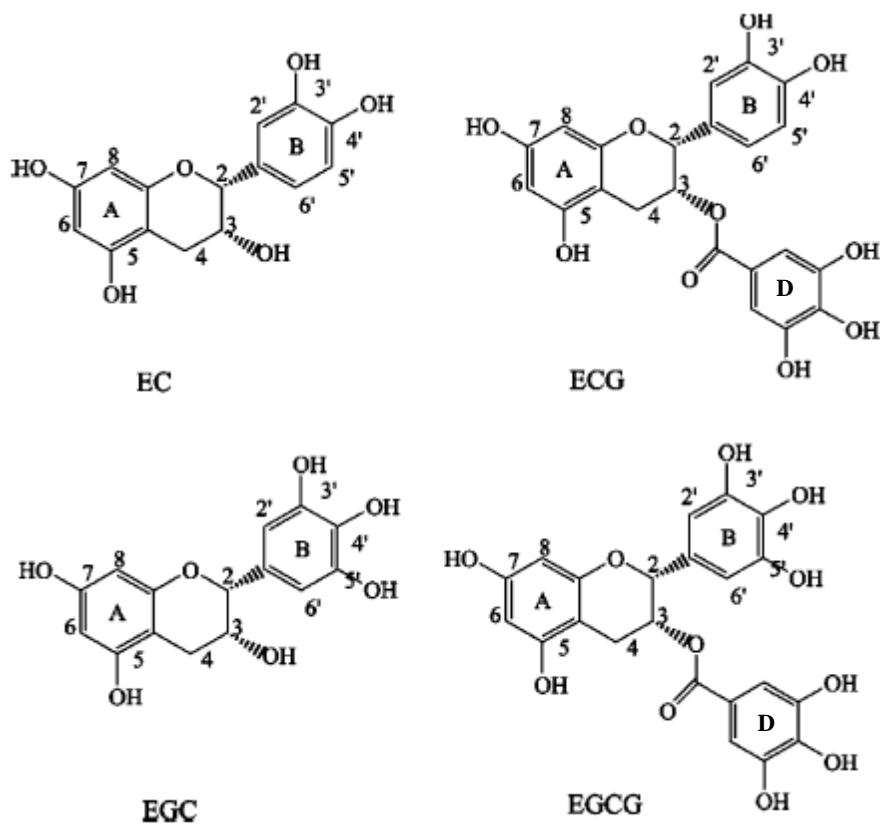
Figure 8 Structure of catechins present in Green Tea leaves.

1.7 EGCG inhibits JAK/STAT1 pathway

Our previous study examined the inhibitory action of catechins of Green Tea on IFNγ-elicited STAT1 activation (Menegazzi M. et al., 2001). Among all green tea catechins, only EGCG, added to MDA MB 231 cells one hour before the induction with IFNγ, inhibited the STAT1 activation dose-dependently; the estimated value of the concentration giving 50% inhibition (IC_{50}) was about 10 µM. On the contrary EGCG, in this cell system, failed to inhibit NF-κB, a transcriptional factor activated by a mix of cytokines: TNFα, IL1β, LPS and IFNγ.

Western blot analyses showed that EGCG, dose dependently, reduced the amount of tyrosine phosphorylation.

We believed that EGCG may be a promising candidate as a drug modulating STAT1 signaling devoid of undesirable side effects.

1.8 The synthetic catechins

Some catechins were synthesized by the group of prof. Sergio Romeo, Faculty of Pharmacy, University of Milan. These new synthetic compounds were analogous to Gallocatechin gallate (GCG; 2S, 3R), which is the *trans* diastereomer of Epigallocatechin gallate (EGCG; 2R, 3R). GCG was easier to synthesize and it is more stable than *cis* diastereomer (EGCG), in fact high temperature converts EGCG into GCG.

EGCG and GCG are characterized by a benzyl ring (ring A) with two hydroxyl groups in position 5 and 7, a pyranose ring (ring C), fused with the previous ring,

which binds in position 2 a second benzyl ring (ring B), with three hydroxyl groups in 3′, 4′ and 5′, and in position 3 a gallate group. In this work the benzyl ring of gallate group is called ring D. In EGCG the ring B and gallate group are in *cis* conformation, while in GCG are in *trans* conformation (Fig. 9).

The synthetic catechins are racemic mixture (2S, 3R; 2S, 3S) and have fewer hydroxyls than GCG in ring A, or ring B, or D or simultaneously in multiple rings; this aspect will be described in results section.
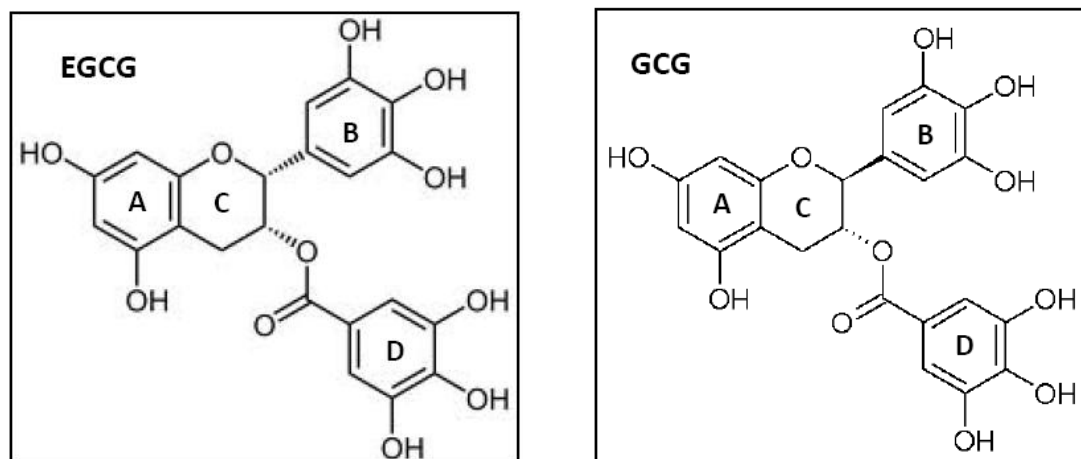

Figure 9: Structure of EGCG and GCG.

2. The aim of the work

Previous data in our laboratory, as briefly mentioned in introduction section, have demonstrated that, among all catechins present in green tea leaves extract, only EGCG is able to inhibit IFNγ-elicited STAT1 activation, a transcriptional factor that plays a pivotal role at the early phase of the inflammation in regulating the expression of a number of inflammation related genes (Carcereri de Prati A. et al., 2005). The inflammatory response is closely related and modulated by the action of cytokine and pro-and anti-inflammatory mediators. The loss of this homeostasis is crucial for the onset of chronic inflammatory diseases. In literature it is reported that the beneficial action of green tea dinking can be extended to inflammatory disease.

In this work 15 synthetic catechins, obtained by chemical modification of GCG, diastereomer of EGCG, will be studied to identify the minimal structural feature that preserve the anti-STAT1 activity. The reduced number of hydroxyl groups in rings A, B, D and simultaneously in more rings will be considered, and the activity of these modifications in IFNγ-elicited STAT1 pathway and in STAT1 dependent genes induction will be evaluated.

Since STAT1 cascade is up and down regulated in a complex manner, STAT1 modulation can be potentially achieved at various steps. In this work a possible moleculer target of these compounds in the IFNγ/JAK/STAT1 pathway will be investigated. Several considerations were assumed and a possible direct interaction between some catechins and STAT1 protein will be verified by Surface Plasmon Resonance and molecular modelling analysis.

In the future, the possibility to know the chemical features for anti-STAT1 activity and the putative interaction site of the molecular target may be useful to synthesize new anti-inflammatory drugs with specific effect on modulation of STAT1 signaling, without undesirable secondary effects of actually used anti-inflammatory drugs.

3. Materials and Methods

3.1 Materials

Chemical reagents were purchased from Sigma-Aldrich Company, Milan, Italy, unless otherwise specified.

3.2 The catechins

Epigallocatechin gallate (EGCG) and Gallocatechin gallate (GCG) were purchased from Sigma-Aldrich, while the synthetic catechins (compounds 1-15) were prepared by the group of prof. Sergio Romeo, Faculty of Pharmacia, University of Milan, modifying two synthetic methods reported in literature (Li L. And Chan T. H., 2001; Zaveri N. T., 2001).

3.3 Cells culture

Human mammary carcinoma cell line (MDA MB 231, American Tissue Culture Collection) were cultured in Dulbecco Modified Eagle's Medium (DMEM, BioWhittaker, Cambrex Bio Science, Belgium) supplemented with 10% fetal bovine serum (FBS, Biowitthaker, Cambrex Bio Science, Belgium), gentamycin 40 µg/ml, penicillin 100 UI/ml and streptomycin 100 µg/ml in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Cells were plated onto 6 cm diameter dishes for Electrophoretic Mobility Shift Assay (EMSA) and for RNA extraction, and 10 cm diameter dishes for Western Blot. At 70% confluence cells were cultured in serum free DMEM for 3-4 h prior to exposure to different compounds. After 1h of pre-treatment in the presence of different concentration of catechins, the activation of STAT1 transcription factor was elicited by addition of IFNγ (30 ng/ml). The cells were harvested after 1 hour for EMSA and Western Blot analysis or 30 hours for Real-Time PCR experiments.

For protein extraction the cells were washed in cold phosphate saline buffer (PBS, BioWhittaker, Cambrex Bio Science, Belgium) to stop the reaction, harvested by scraping, centrifuged for 5 minutes at 1500 x g and the pellets were stored at -80°C until use.

3.4 Nuclear extracts

Nuclear extracts were prepared from cultured cells according to Osborn et al. (Osborn et al., 1989). Cellular pellets were solubilized for 10 minutes at 4°C in 20-30 µl of a strongly hypotonic solution containing a detergent, to break down the membrane phospholipids, (HEPES pH 7.9 10 mM, Nonidet-P40 0.1%, KCl 10 mM, $MgCl_2$ 1.5 mM, DTT 0.5 mM). The solution was added of protease inhibitors: complete EDTA-free Protease Inhibitor (Roche) 1X, 10 µg/ml leupeptin, 5 µg/ml antipain and pepstatin, 1 mM PMSF. This hypotonic solution allowed water to go into the cells, by osmosis, so that the cells broke out. Lysed cells were centrifuged at 4°C for 10 minutes at 2300 x g and supernatant, containing membranes and cytosolic fraction, was discarded. Pellets of cell nuclei were suspended for 15 minutes in 10-15 µl of hypertonic solution (HEPES pH 7.9   20 mM, NaCl 420 mM, $MgCl_2$ 1.5 mM, Glycerol 25%, EDTA 0.2 mM, DTT 0.5 mM) containing a mixture of proteases inhibitors: complete EDTA-free protease inhibitors (Roche) 1X, leupeptin 10µg/ml, antipain and pepstatin 5µg/ml, PMSF 1 mM. This last solution brought, by osmosis, water out of nuclei so they were squeezed and allowed to release nuclear proteins. The samples were centrifuged at 25000 x g for 30 minutes at 4°C, the supernatants containing nuclear proteins were stored at -80°C until use.

3.5 Quantification of protein concentration

Protein concentration in the nuclear extracts was determined using the Bradford method (Bradford, 1976).

The Bradford assay involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. The dye exists in three forms: cationic (red), neutral (green), and anionic (blue); when the dye binds to proteins, it is converted to a stable blue form (λ_{max} at 595 nm). The quantity of proteins can be estimated by determining the amount of dye in the blue form, this is usually achieved by measuring with spectrophotometer (Beckman DU650) the absorbance of the solution at 595 nm and comparing this to a

standard curve generated by the reaction of known amounts of a standard protein Bovine Serum Albumin (BSA).

3.6 Electrophoretic mobility shift assay (EMSA)

Using non-denaturing polyacrylamide gel electrophoresis, ^{32}P end-labeled DNA fragment, when binds specifically to a protein, retards its electrophoretic mobility showing discrete bands corresponding to the individual protein-DNA complexes.

The double stranded oligonucleotide, containing the STAT1 binding site (sis-inducible factor-binding recognition element, SIE/M67) from the c-Fos promoter (5′-GTCGACATTTCCCGTAAATCG-3′) (Wagner B. J. Et al., 1990) was purchased from MWG Biotech. The lyophilized oligonucleotide was solubilized in Tris-EDTA (100 pmol/μl) and 5 pmol of forward and reverse oligonucleotides were annealed at 95°C in Tris HCl 1M pH 8, EDTA 50 mM and NaCl 5M. Then it was radiolabeled with [γ-^{32}P]ATP (PerkinElmer) and T4 Polynucleotide Kinase (PNK, USB Corporation, Ohio, USA). The T4 polynucleotide kinase catalyzes the transfer of the terminal pohosphate of ATP to 5′ hydroxyl termini of DNA. The oligonucleotide (10 pmoles) was combined with T4 PNK buffer (10 X) 5 μl, T4 PNK 3 U/μl, [γ-^{32}P]ATP 20 pmoles and water up to 50 μl. The mixture was incubated at 37°C for 30 minutes and the reaction was terminated by heating at 65°C for 5 minutes. The ^{32}P end labelled oligonucleotide was purified from precursor ^{32}P ATP by Sephadex G-50 column (GE Healthcare).

10 μg of nuclear extract were incubated at room temperature for 20 min with 2-5 x 10^{4}cpm of the ^{32}P-labeled double stranded oligonucleotide, in a 15 μl of reaction mixture containing HEPES 20 mM pH 7.9, KCl 50 mM, glycerol 10%, DTT 0.5 mM, EDTA 0.1 mM, poly(dI-dC) 1 μg, salmon sperm DNA 1 μg. Poly(dI-dC) and salmon sperm DNA were added to reduce proteins' non-specific bonds to the ^{32}P-labeled DNA. Products were fractioned by electrophoresis on a non denaturing 5% polyacrilamide gel in TBE (Tris base 50 mM, Boric acid 50 mM, EDTA 0.5 mM).

The gel was previously balanced for 30 minutes at 100 Volt and it was carried out in half past two hours at 140 Volt. At the end of the electrophoresis the gel was dried by gel dryer (BioRad) for 40 minutes and exposed to a photographic plate Kodak X-AR. The intensity of the retarded bands was measured with a Phosphoimager (Molecular Dynamic, Milan, Italy).

3.7 Western Blot

The Sodium Dodecyl Sulphate – Polyacrilamide Gel Electrophoresis (SDS-PAGE) followed Laemmli's method (Laemmli, 1970).

Cells were lysed on ice with HEPES 20 mM pH 7.4, NaCl 420 mM, Nonidet P40 1%, EGTA 1 mM, EDTA 1 mM and complete EDTA-free protease inhibitors (Roche) 1X for 20 minutes, and centrifuged at 4°C for 30 minutes at 25000 x g. Pellets were discarded and supernatants were store at –80°C until use. Total cellular proteins (40-50 µg/lane) were added of reducing buffer (Sample Buffer: Tris HCl 1 M pH 6.8, SDS 20%, glycerol 20%, bromophenol blu 0.25% and β-mercaptoethanol 5%), denatured by boiling for 5 minutes and immediately cooled on ice for 5 minutes. The samples were fractionated by SDS-PAGE in a 7.5% gel. A marker of known molecular weight (Rainbow Molecular Weight Marker, Amersham Biosciences) was also fractionated in the same gel. The electrophoresis was performed in running buffer (Tris HCl 25 mM pH 8.3, Glycin 192 mM, SDS 0.1%) at 15 mA until the stacking gel was passed, and at 25 mA in the running gel. Proteins were electroblotted onto PVDF (polyvinyldene fluoride) membrane (Immobilion P, Millipore, Bedford, MA, USA), previously activated 1 minute in methanol, by BioRad Mini Protean II TM system for 1 hour at 300 mA on ice in the transfer solution (Tris HCl 25 mM pH 8.3, Glycine 192 mM, Methanol 10%). The membranes were incubated with a blocking solution (Albumin Bovine Serum 5%, Tris HCl 10 mM pH 7.5, NaCl 100 mM, Tween 20 0.1%) for 1 hour at room temperature under agitation. Then they were incubated over night at 4°C under agitation with a primary polyclonal antibody anti-phosphoSTAT1 Tyr701 (Cell

Signaling Technology, MA, USA) diluted 1:700 in blocking solution or with a polyclonal antibody anti-phosphoSTAT1 Ser727 (UPSTATE Biotechnology) diluted 1:300 in blocking solution. The membranes were washed three times for 10 minutes with washing solution (Tris HCl 10 mM pH 7.5, NaCl 100 mM, Tween 20 0.1%) and incubated with a secondary antibody anti-rabbit IgG-peroxidase conjugate (Cell Signaling Technology, MA, USA) diluted 1:2000 in blocking solution for 1 hour at room temperature under agitation. Membranes were washed again three times for 10 minutes to dry out excess of the antibody.

The immunoreactive proteins on the blot were detected using "Immun-Star™ WesternC™ Chemiluminescent Kit" (BIO RAD), an enhanced chemiluminescence detection system, by Bio-Rad ChemiDoc XRS.

Following the analysis of phosphorylated STAT1, the blots were stripped and reprobed with anti-Total STAT1 antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:1000.

3.8 Real-Time PCR

MDA MB 231 cells cultured in 6 cm diameter dishes (2.5×10^6 cells) were processed to purify total RNA using RNeasy® Mini Kit (QIAGEN). The biological samples were lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivated RNase to ensure purification of intact RNA. Etanol was added to provide appropriate binding conditions, and the sample was applied to an RNeasy Mini spin column, where the total RNA bound to the membrane while contaminant were efficiently washed away. High quality RNA was then eluted in 60 µl RNase-free water.

Purified RNA was diluted 1:30 in RNase-free water in quartz cuvette, the concentration of RNA was determined measuring the absorbance at 260 nm (absorption spectrum of each sample $\lambda_{210-310}$; peak-point at λ_{260}) in Beckman DU650 spectrophotometer.

To perform Real-Time PCR using RNA as a starting template, RNA had first to be reverse transcribed into cDNA. For generating first-strand cDNA to use in Real-Time

quantitative PCR, SuperScript® VILO cDNA Synthesis Kit (Invitrogen) was used. As indicated in guidelines, 1 µg of RNA was combined in a tube on ice with 2 µl of SuperScript Enzyme Mix, which included SuperScript RT enzyme and recombinant ribonuclease inhibitor, and with VILO reaction mix, which included random primers, $MgCl_2$, and dNTPs in a buffer formulation that had been optimized for RT-PCR . Finally was added free water up to 20 µl. The reverse transcription was performed with Rotor Gene 6000, Corbett machine, using the following program: 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 85°C to block reaction.

Real-Time PCR was performed using QuantiTect® SYBR® Green PCR kit (QIAGEN). This kit was composed by a PCR Master Mix containing fluorescent dye SYBR green I, which binds all double stranded DNA molecules, emitting a fluorescent signal on binding; HotStarTaq DNA Polymerase which is a Taq DNA Polymerase modified form of enzyme, it is provided in an inactive state and has no enzymatic activity at ambient temperature but is activated by incubation of 15 minutes at 95°; a buffer which contains a balanced combination of KCl and $(NH_4)_2SO_4$ to promoting a high ratio of specific to non specific primer binding during the annealing step of each PCR cycle. Finally the master mix contains ROX passive reference dye. The fluorescence from ROX dye does not change during the course of Real-Time PCR, but provides a stable baseline to which PCR-related fluorescent signals are normalized.

In a PCR tube 12,5 µl of QuantiTect SYBR Green PCR Master Mix were mixed with 2,5 µl of QuantiTect Primer Assays of genes iNOS or MCH II DRA, that were ready to use and they were guaranteed to provide specific and sensitive quantification; finally, 9 µl of free water were added to the reaction mix . This mix was added to 1 µl of cDNA, which corresponds to 50 ng of RNA.

The program and instrument used to occur and analyze reaction was RotorGene 6000, Corbett; the Real-Time PCR program was the following:

1. 15 minutes at 95° activation HotStarTaq DNA Polymerase
2. 15 seconds at 94° denaturation
3. 30 seconds at 55° annealing

4.   30 seconds at 72° extension

The steps from 2 to 4 were repeated 45 times.

3.9 STAT1 expression in *E. coli*

- **Strains and media**

The *E. coli* strains used were:

XL1Blue (genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*q*Z∆M15* Tn*10* (Tetr)]) for the amplification of the pET-22b plasmid containing the STAT1 sequence.

BL21 (DE3) (genotype: F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3)) for protein expression.

Bacterial cultures were grown in *Luria Bertani Broth* (LB) medium. To prepare the LB medium, NaCl 0.5%, Tryptone 1% and Yeast extract 0.5% were suspended in water. The obtained solution was autoclaved at 121°C and the appropriate antibiotic, in this case ampicillin, was added at final concentration of 100 μg/ml. Solid cultures were grown in LB medium plus agar 1.5%.

- **pET-22b plasmid**

The vector used to clone and express STAT1 wild-type protein was pET-22b (QIAGEN).

The plasmid size is 5493 bp. It contains a closely arranged series of synthetic restriction sites called "polylinker", that are unique to the polylinker and hence provide a variety of targets that can be used to clone foreign DNA fragments; a *bla* sequence, coding for β-lactamase, which gives ampicillin resistance; a *lacI* sequence. This last sequence encodes the lac repressor that controls protein expression from a T7 promoter, which carry the lac operator. It maintains target gene transcriptionally silent in the uninduced state, while expression is induced by the addition of Isopropil β-D-tiogalattopiranoside (IPTG) to the bacterial culture.

pET-22b enables to add a tag of six histidine residues at the C-terminus of the cloned protein to facilitates purification, which is performed in a Nichel Sepharose column by affinity chromatography.
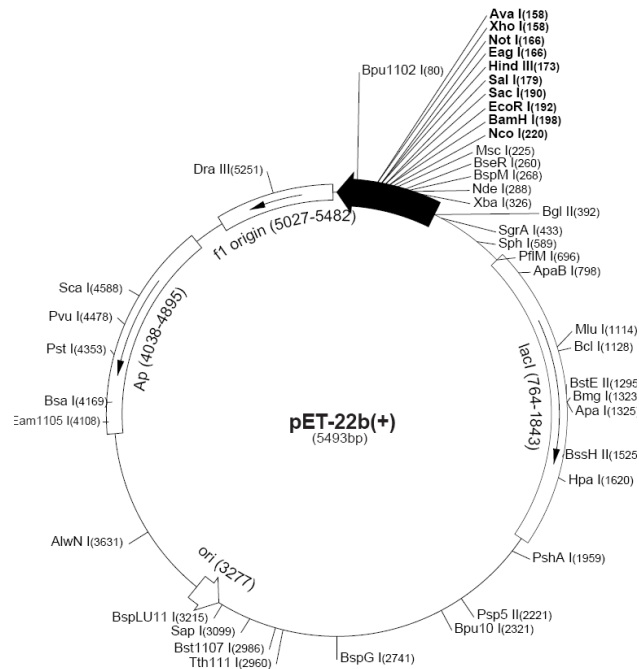
Figure 10: Map of pET-22b plasmid

- **Amplification of STAT1 insert**

The STAT1 clone of *E. coli* was purchased from Invitrogen (IMAGE clone ID 2452685, GenBank accession no. AI923499). The cDNA encoding for STAT1 protein was amplified by PCR. Appropriate primers were designed that include restriction sites for two different endonuclese, at 5′ and 3′ ends of inserted sequence, in order to have complementary sticky ends and to allow the subsequent cloning into the expression plasmid pET-22b.

The primers for STAT1 cloning in pET-22b were:

STAT1_ET-for: GGTGGTCATATGAGCACAGTGATGTTAGAC

STAT1_ET-rev:

AATGTCGACGGAACCACGCGGAACCAGCACTTCAGACACAGAAAT

The CATATG and GTCGAC sequences are the restriction sites for Nde I and Sal I, respectively.

Moreover, the STAT1_ET-reverse primer allowed to include at the C-terminal end of the STAT1 sequence, just before the His-Tag, a sequence (GGAACCACGCGGAACCAG) that encodes a cleavage site for thrombin. This will allow to cleave the His-Tag after the protein purification.

The PCR reaction was performed by a Mastercycler personal Thermocycler (Eppendorf®); the reaction mix was composed of: plasmid DNA (50 ng) ; primer forward (1 µM); primer reverse (1 µM); dNTPs (200 µM); Buffer 1X (stock 10X); *Pfu* DNA polymerase (Jena Bioscience) (1 unity); demineralized water DNasi free (up to 50 µl).

The *Pfu* polymerase is an high fidelity DNA polymerase which exhibits the lowest error rate of any thermostable DNA polymerase. The enzyme replicates DNA at 72°C, catalyzing the polymerization of nucleotides into duplex DNA in the 5´-3´ direction. *Pfu* Polymerase also possesses a 3´-5´ exonuclease (proofreading) activity. Base misinsertions that may occur during polymerization are rapidly excised by the proofreading activity of the polymerase. It extends at approximately 2 kb/min.

The PCR program was the following:

1. 5 minutes at 95° starting denaturation
2. 60 seconds at 95°C denaturation
3. 60 seconds at 58°C annealing
4. 70 seconds at 72°C extension
5. 10 minutes at 72°C final extension

The steps from 2 to 4 were repeated 35 times.

The STAT1 insert is composed of 581 amino acid (1743 bp), molecular weight 67139.2 Dalton.

After the amplification reaction, the PCR sample was run on a 0.8% agarose gel in TAE buffer (Tris-Acetate 40 mM pH 8, EDTA 1 mM). Ethidium bromide (0.5 µg/ml

final) was added to the gel. The latter is a fluorescent dye used to stain DNA, that intercalates between the bases of DNA.

The samples containing DNA were mixed with a loading buffer, which contains glycerol to allow the sample to "fall" into the gel wells, and a tracking dye, which migrates in the gel and allows the visual monitoring or how far the electrophoresis had proceeded. Along with the PCR reaction, a sample of a DNA size marker (1kb ladder, Sigma-Aldrich®) was pipetted into one of the wells to verify the correct length of the PCR products.

When gel was solidified, the samples were pipetted into the wells and a current of 80 volt was applied. After about 40 minutes of running, the gel was observed by an ultraviolet transilluminator.

The desired PCR product was purified from the gel using QIAEX®II Gel Extraction kit (QIAGEN). The purified DNA was resupended in Tris-HCl 10 mM pH8.5.

- **Amplification and extraction of pET22b plasmid DNA from *E. coli* cultures**

A colony of *E. coli* tansformed with the plasmid pET22b was incubated overnight in 3 ml of LB medium. The cells were centrifuged at 13000 x g for 1 minute and the plasmid DNA was extracted by the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). The kit employs a modified alkaline-SDS lysis procedure followed by adsorption of the DNA onto a silica matrix in the presence of high salt concentration. Contaminant are then removed by a spin-wash step. Finally the bound plasmid DNA is eluted in 50 µl of Tris-HCl 10 mM pH 7.5.

- **Digestion of the STAT1 insert and of the pET-22b plasmid**

Amplified cDNA encoding STAT1 and the plasmid pET-22b were cut with the same restriction enzymes Nde I and Sal I (Jena Bioscience) so that to create complementary sticky ends which facilitate the subsequent ligation. The reactions were incubated at 37°C for 3 hours. Then the enzymes were inactivated by incubation at 65°C for 20 minutes.

A Calf Intestinal Alkaline Phosphatase (CIAP, Sigma-Aldrich®, 1U/µl) was added to the pET-22b plasmid, after digestion; the reaction took place for 1 hour at 37°C. CIAP treatment prevents self-ligation of the vector because removes the 5′ phosphate from both termini of the linear vector.

The digested plasmid and insert were isolated by agarose electrophoresis and purified by the QUIAEX®II Gel Extraction Kit (QIAGEN).

- **Ligation of the STAT1 insert and of the pET-22b plasmid**

In order to improve the efficiency of the ligation, a proper insert/vector ratio is required. To this aim, we determined insert and vector concentration by estimating DNA amount by agarose gel electrophoresis.

The ratio of STAT1 insert to destination plasmid was 5:1, it was calculated by using the equation:

Ng insert= 5*(kb insert)*(ng plasmid)/(kb plasmid);

T4 DNA ligase (Promega) was used for the ligase reaction, and the mixture was incubated overnight at 4°C.

- **Preparation of competent *E. coli* cells using CaCl$_2$**

The product of the ligase reaction was then introduced into the *E. coli* XL1-blue strain by a reaction of transformation.

One colony of *E.coli* cells, grown on an LB agar plate at 37°C overnight, was placed in 5 ml LB media and was allowed to grow overnight at 37°C under agitation. The 5 ml of overnight culture were transferred into 200 ml of LB media in a flask and the cells were allowed to grow under agitation at 37°C until the OD$_{600}$ of 0.4-0.6. Cells were transferred into 4 polypropylene falcon tubes (50 ml) and centrifuged at 4°C for 10 minutes at 3000 x g. Media was poured off and cells were resuspended in 25 ml of cold 50 mM CaCl$_2$, previously filtered and sterilized through a 0.22 µm filter, and were incubated on ice for 30 minutes. Cells were centrifugated at 4°C for 10 minutes at 3000 x g. Supernatant was poured and cells were resuspended (by pipetting) in 5 ml cold 50 mM CaCl$_2$ containing 15% glycerol. 300 µl were transferred into sterilized Eppendorf tubes (1.5 ml) placed on ice. The cells were frozen in liquid nitrogen and stored at -80°C. These cells could be used for transformation .

- **Transformation of competent *E. coli* cells by heat shock to amplify the product plasmid**

XL1-blue strain of *E. coli* was used to amplify the plasmid. The competent cells were removed from -80°C storage and placed on ice. As the cells were thawed, 150 µl of these cells were transferred into Eppendorf tube (1.5 ml) and 30 ng of DNA obtained by ligation with the plasmid was added; tubes were kept on ice for 30 minutes. Then, tubes with DNA and bacteria were placed into a water bath at 42°C for 45 seconds and subsequently on ice for 5 minutes. 200 µl of LB was added and tubes were incubate for 45 minutes in agitation at 37°C. The resulting cultures were spread on LB agar with ampicillin (100 µg/ml) and were allowed to grow overnight at 37°C.

- **PCR Screening of Transformant Colonies**

After obtaining colonies from a transformation reaction, some colonies were resuspended in 5 µl of water, a PCR reaction was carried out using Taq polymerase and the same reaction mix and program used for amplification of insert. The PCR samples and 1kb molecular weight marker were run on 0.8% agarose gel and visualized at UV lamp.

Since the PCR colonies might give "false-positives", some single transformant colonies of XL1-Blue were inoculated with 3 ml of LB, containing ampicillin, and incubated overnight at 37°C. The plasmid DNA was extract by GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) and checked by sequencing (BMR genomics).

- **Expression of the STAT1 protein in the BL21 strain of *E.coli***

Cells of the BL21 strain of *E.coli* were transformed by heat shock, as described before, with 50 ng of the pET22b vector containg the insert encoding from STAT1.

A colony of the transformed cells was placed in 3 ml LB media with ampicillin and allowed to grow for 6 hours at 37°C under agitation. Then, the 3 ml culture was put in 20 ml of LB with ampicillin and allowed to grow overnight at 37°C in agitation. The overnight culture was transferred into 1 L LB media with antibiotic in a flask, the cells were allowed to grow at 37°C under agitation until the OD_{600} reached a value of 0.6-0.8, which represent the exponential growth phase. At this point IPTG (0.5 mM)

44

was added to the culture medium and cells were allowed to grow overnight at 20°C under agitation.

Before the induction with IPTG and after nearly 16 hours of induction, 1 ml of culture was centrifuged at 12000 x g and suspended in Sample-Buffer. The induction was verified by SDS-PAGE and by Western Blot using a specific antibody against STAT1 (Santa Cruz Biotechnology).

The pellet of 1 L culture was resuspended in Tris 20 mM pH 7.5, NaCl 0.5 M, imidazole 10 mM, β-Mercaptoethanol 5 mM and PMSF (phenylmethylsulfonyl fluoride) 0.2 M. Cells were sonicated, frozen in liquid nitrogen and stored at -80°C until use.

- **STAT1 protein purification**

STAT1 protein was purified by affinity chromatography, that is based on the specific and reversible interaction between a particular protein to be purified and a ligand previously immobilized on a stationary phase. The process can be thought as an entrapment, because the target molecule with a particular property is trapped on a solid or stationary phase, while others molecules in solution are not retained.

Recombinant proteins may be easily purified by immobilized metal ion chromatography (IMAC). In this work HisTrap FF crude (GE Healthcare) column was used. It is prepacked with NiSepharose™ resin, which consists of highly cross-linked agarose beds with an immobilized chelating group. The column is precharged with Ni^+ ions that are able to form a complex with this residues, thus Ni^+ ions selectively bind those proteins that have this residues exposed on their surface.

The *E. coli* lysate was thawed at 37°C in a water bath, added of Leupeptin and Pepstatin 100 µg/l and centrifuged 12000 x g at 4°C for 30 minutes. The supernatant was filtered with a gauze and loaded on the column by FPLC AKTA (GE Healthcare) synstem.

The protein was eluted by adding a competitive molecule, i.e. imidazole. A linear gradient of imidazole (10 mM – 500 mM) was applied in Tris-HCl 20 mM pH7.5, NaCl 0.5 M.

The different eluted fractions and the unbound fraction were analyzed by SDS-PAGE.

The gel was then stained by a staining solution (25% v/v ethanol; 10% v/v glacial acetic acid; 0.15% p/v comassie brilliant blue) for 30 minutes and then was bleached a by destaining solution (25% v/v ethanol; 10% v/v glacial acetic acid).

3.10 Site-directed mutagenesis of STAT1 protein

Site-directed mutagenesis was performed using QuikChange®II Site-Directed Mutagenesis Kit (Stratagene) which allows to generate mutants by a rapid three-steps procedure (Fig. 11).

Amplification is a PCR reaction which utilizes the supercoiled double-stranded DNA (pET-22b vector) containing the cDNA encoding for STAT1 as template, and two synthetic oligonucleotide primers, each complementary to opposite strands of the vector and containing the desired mutation.

The mutagenic oligonucleotide primers used to consent the Q518A and the H568A variants were the following:

Q518A-FOR:CTCAATGTGGAC**GCG**CTGAACATGTTG

Q518A-REV:CAACATGTTCAG**CGC**GTCCACATTGAG

H568A-FOR:CTCATTAAAAAA**GCG**CTGCTCCCTCTC

H568A-REV:GAGAGGGAGCAG**CGC**TTTTTTAATGAG

The site-directed mutagenesis was performed using *PfuUltra* ™ high-fidelity DNA polymerase.

Cycling parameters for site-directed mutagenesis were:

1. 5 minutes at 95°C for initial denaturation
2. 30 seconds at 95°C for denaturation
3. 60 seconds at 55°C for annealing
4. 10 minutes at 68°C for extension
5. 10 minutes at 68°C for final extension

The steps from 2 to 4 were repeated 16 times in an Eppendorf® termocycler.

The amplification products were digested with *Dpn* I enzyme for 3 hours at 37°C. *Dpn* I is a specific endonuclease which digests the parental (non-mutated), methylated dsDNA. A part of the products was pipetted in a 0.8% agarose gel to confirm the amplicon presence.

XL1-Blue competent cells were then transformed with 5 µl of the digested DNA, as previously described, and the STAT1 sequence was sequenced to check the presence of desired mutation.
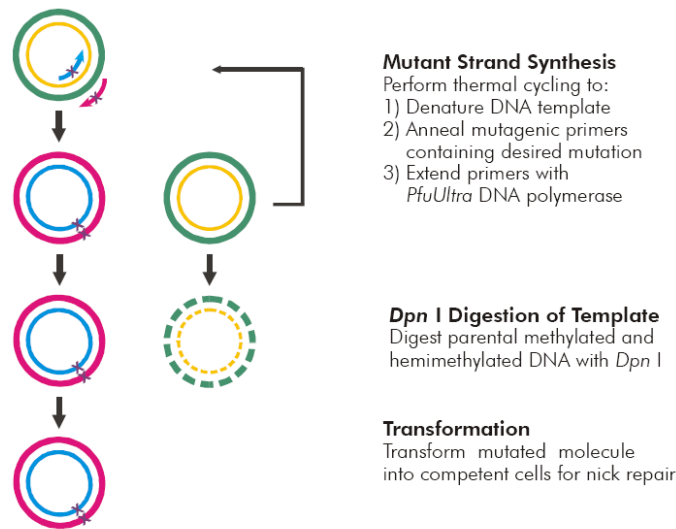
Figure 11: Overview of QuikChange II site-directed mutagenesis method

3.11 Surface Plasmon Resonance (SPR) analysis

Study of the interaction between STAT1 protein and catechins were carried out by Surface Plasmon Resonance (SPR) analysis using "BIAcore 2000 biosensor system" (BIAcore inc., Piscataway, NJ, USA).

SPR is an *"in vitro"* technique based on an optical phenomenon, and can simultaneously detect interactions not only between proteins but also between

proteins and small molecules. BIAcore instruments consist of sensing optics, an automated sample delivery system, and a computer for instrument control, data collection and data processing. Furthermore, careful data analysis lead to evaluate kinetics parameters of the interaction.

Experiments were performed on disposable sensor chip CM5 (carboxylated dextran chip). The chip has two flow channel, in one flow channel STAT1 protein (the ligand), 0.05 mg/ml in sodium acetate buffer 10 mM pH 4.0, was immobilized by an amine coupling reaction kit (BIAcore Inc., Uppsala, Sweden) according to manufacturer's instructions. First a EDC/NHS solution (freshly mixed 0.2 M *N*-ethyl-*N*9-dimethylaminopropylcarbodiimide and 50 mM *N*-hydroxysuccinimide) was injected for 7 minutes to activate the surface, and next STAT1 was injected, followed by 1 M ethanolamine at pH 8.5 for 7 minutes to stop further binding. The other flow cell without STAT1 was used as control.

After activation and immobilization of STAT1, each catechin (the analyte) in running buffer (HBS-EP buffer, BIAcore) was injected into the flow cell containing immobilized STAT1, appropriate concentration ranges of compounds were determined from anticipated preliminary experiments. Analysis were performed with EGCG, GCG, compounds 1, 2, 3, 7, 8 and 9 at concentrations of 0, 0.2 - 50 µM, and with compounds 4, 5, 6, 10 to 15 at concentrations of 5 to 200 µM in the final volume 50 µL. Elution rate was 30 µLmin^{-1} for each experiment. At operative temperature (25°C) one analytical cycle consisted of 6 minutes of buffer flow, followed by 6 minutes of test compound at reported concentration in HBS-EP buffer and 8 minutes of buffer flow to follow the dissociation phase. Before the successive cycle the surfaces were regenerated with 30 seconds injections of 5 mM NaOH followed by HBS-EP buffer for 6 minutes. Sensograms expressed in resonance unit (RU) for each compound were analyzed with BIAevaluation software and BIAsimulation software (BIAcore Inc.).

3.12 Computer modelling

The molecular modelling was performed in collaboration with prof. Perahia group, "Laboratoire de Modelization et Ingenierie des Proteines" (University of Paris-sud, Orsay France).

The search of putative interaction sites of catechins with STAT1 was carried out using Q-sitefinder (http://www.bioinformatics.leeds.ac.uk/qsitefinder/) on the X-ray structure of STAT1 (pdb entry 1yvl.pdb Laurie, A.T. and Jackson, R.M. 2005). Docking simulations were carried out using Autodock v.4 (Morris G.M. 1998) which was recently reported as part of the most accurate softwares for that matter (Bursulaya B.D et al., 2003; Jenwitheesuk E. et al. 2003). This docking software was used with full flexibility of the ligands but with limited protein flexibility. The initial geometry of the ligands was obtained by minimization with the semi empirical method AM1 by using Gaussian03 (Frisch M. J. et al. 1998; Pettersen E. F. et al. 2004; Pedretti A. 2004) program in vacuum. ADTOOLS, the graphical interface of Autodock was used for adding the polar hydrogens for docking calculations and for assigning the atomic charges of the protein.

Calculations with Autodock were performed using a particularly accurate procedure using the Lamarckian algorithm with a population size of 100, a number of energy evaluations of 2.0×10^6, a generation number of 27,000, a mutation and crossover rates of 0.02 and 0.8 respectively. The energies were evaluated by using atomic affinity potentials for each atom type of the ligand calculated by Autogrid v3.0.5. Furthermore, the number of runs was set to 100 to explore a large number of poses of the highest affinity and the Solis and Wets algorithm was used to relax the best 10 % of the obtained conformations.

3.13 STAT1/JAK2 Kinase assay

JAK2 kinase causes STAT1 phosphorylation in Tyr701 and its activation. A kinase semiquantitative assay was improved to assess the change in wild-type and mutated STAT1 phosphorylation following catechin treatment. It was performed under

agitation at 37°C in 30 µl volume composed of reaction buffer (Tris-HCl 20 mM pH 7.5; ATP 100 µM; $MgCl_2$ 50 mM), STAT1 (recombinant purified protein) and JAK2 (recombinant enzyme, Millipore) at different concentrations (STAT1 500 ng - 5 µg; JAK2 3 ng – 300 ng). The reaction was stopped by adding 10 µl of Sample Buffer 4X, proteins were denatured at 95°C for 5 minutes, were fractionated by electrophoresis on 10% SDS-PAGE and analyzed by Western Blot using antibodies anti-phosphoSTAT1 Tyr701 (Cell Signaling Technology, MA, USA) and anti-phosphoJAK2 Tyr1008 (Millipore, Bedford, MA, USA) diluted 1:700 and 1:500, respectively, in blocking solution. To set up the kinase assay, different reaction times (from 2 to 20 minutes) were performed.

Once the better condition for the kinase activity was detected, catechins were pre-incubated at different times (10 and 30 minutes) with STAT1 recombinant protein to identify the optimal interaction time. The best condition for JAK2 Kinase assay was: 30 minutes of pre-incubation of the catechins at 37°C under agitation with wild-type and mutated STAT1 (0,16µg/µl); 5 minutes of kinase reaction with JAK2 (0,16ng/µl) at 37°C under agitation.

3.14 Statistic

When necessary data were expressed as means ± S.D. of at least three independent experiments and statistical analysis for single comparison was performed using Student's t-test. The criterion for statistical significance was $P < 0.01$.

4. Results

4.1 Synthetic Catechins

A number of catechins were synthesized by the group of prof. Sergio Romeo, Faculty of Pharmacy, University of Milan.

As already mentioned, these compounds were analogous to GCG, diastereomer of EGCG, because GCG was more stable and easier to synthesize than EGCG. To study the importance of rings A, B, D and their hydroxyl groups on the IFNγ-elicited STAT1 activation, new catechins were synthesized with fewer hydroxyl substituents than GCG in rings A, B and D (Fig. 12 and Table 2), in order to analyze the minimal necessary and sufficient structure for anti-STAT1 activity.

As shown in table 2, compound 1 is the racemic mixture in 3 of GCG (2S, 3R; 2S, 3S) and was considered as the reference compound in the different experiments. Compounds 2 and 3 present less hydroxyl groups in ring A, while compounds 4, 5 and 6 have reduction of hydroxyls in ring B, changes in ring D can be noticed in compounds 7, 8, 9 and 10. Finally compound 11 to 15 have less hydroxyl groups in more than one ring simultaneously.
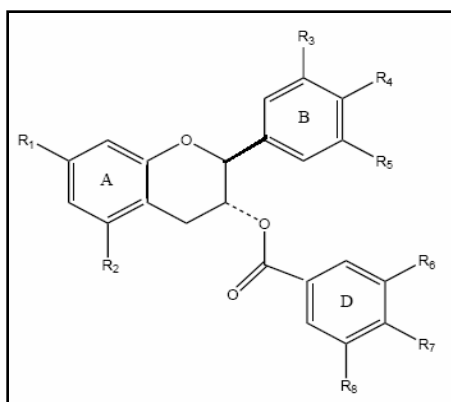
COMPOSTO	R1	R2	R3	R4	R5	R6	R7	R8
1	OH	OH	OH	OH	OH	OH	OH	OH
2	OH	H	OH	OH	OH	OH	OH	OH
3	H	H	OH	OH	OH	OH	OH	OH
4	OH	OH	H	OH	OH	OH	OH	OH
5	OH	OH	H	OH	H	OH	OH	OH
6	OH	OH	H	H	H	OH	OH	OH
7	OH	OH	OH	OH	OH	H	OH	OH
8	OH	OH	OH	OH	OH	OH	H	OH
9	OH	OH	OH	OH	OH	H	OH	H
10	OH	OH	OH	OH	OH	H	H	H
11	OH	H	OH	H	OH	OH	OH	OH
12	OH	OH	H	OH	OH	OH	H	OH
13	OH	H	OH	H	H	OH	OH	OH
14	H	H	H	H	H	OH	OH	OH
15	H	H	H	H	H	H	H	H

Figure 12: General structure of catechin gallate   **Table 2: Synthetic Catechins**

4.2 Structural features of anti-STAT1 synthetic catechins

4.2.1 DNA binding activity

Previously study reported that 20 μM EGCG reduced completely STAT1 DNA-binding activity in MDA MB 231 cells treated with IFNγ (Menegazzi M. et al., 2001). To examine the importance of stereochemistry in this action, GCG was further tested for its capacity to reduce STAT1 DNA-binding activity. EMSA showed that GCG exerted an inhibitory action in the same content to EGCG (Fig. 13), indicating that the two stereoisomers cannot be distinguished regarding their capacity to inhibit STAT1 DNA-binding activity. In order to envisage the structural features involved in this activity, a structure activity relationship study was performed using MDA MB 231 cell line. The synthetic catechins at different concentrations were administered to the cells one hour before the cell stimulation with IFNγ (30 ng/ml) for 1 hour. As shown in figure 14, at 20 μM compounds 1 and 2 showed a strongest inhibitory action, reducing STAT1 DNA-binding activity to less than 30 % of that of induced cells. The estimated IC_{50} was around 12 μM for compound 1 and 17 μM for

compound 2. At the same concentration (20 µM) compound 3 reduced STAT1 DNA-binding activity to 70 % of that of induced cells with estimated IC_{50} to be around 28 µM. Compounds 7, 8 and 9 reduced STAT1 DNA-binding activity, with estimated IC_{50} to be around 33, 37, 31 µM respectively. All other synthetic catechins were inactive in reducing STAT1 DNA-binding activity (Fig. 14 and data not shown).

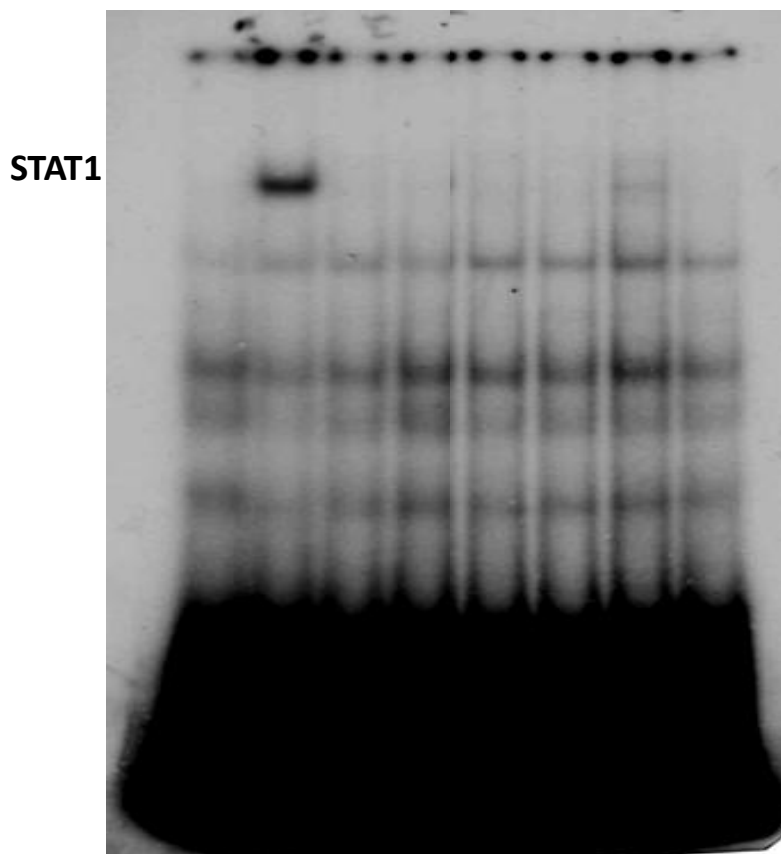| Compounds	–	–	EGCG		GCG		1	
[µM]	–	–	20	40	20	40	20	40
IFNγ	–	+	+	+	+	+	+	+

Figure 13: Effects of EGCG, GCG and compound 1 on STAT1 activation.

Figure 13 shows DNA binding activity of STAT1 in MDA MB 231 cell line untreated or induced by IFNγ in the presence or absence of different concentrations µM of EGCG, GCG and compound 1. Cell nuclear extracts (10 µg) were incubated with a ^{32}P-labeled double-stranded oligonucleotide containing binding sequence for STAT1 and separated by nondenaturing PAGE. The specificity of the retarded bands was demonstrated by competition with 100-fold excess of specific unlabeled oligonucleotide (not shown). The gel is representative of three separate experiments.
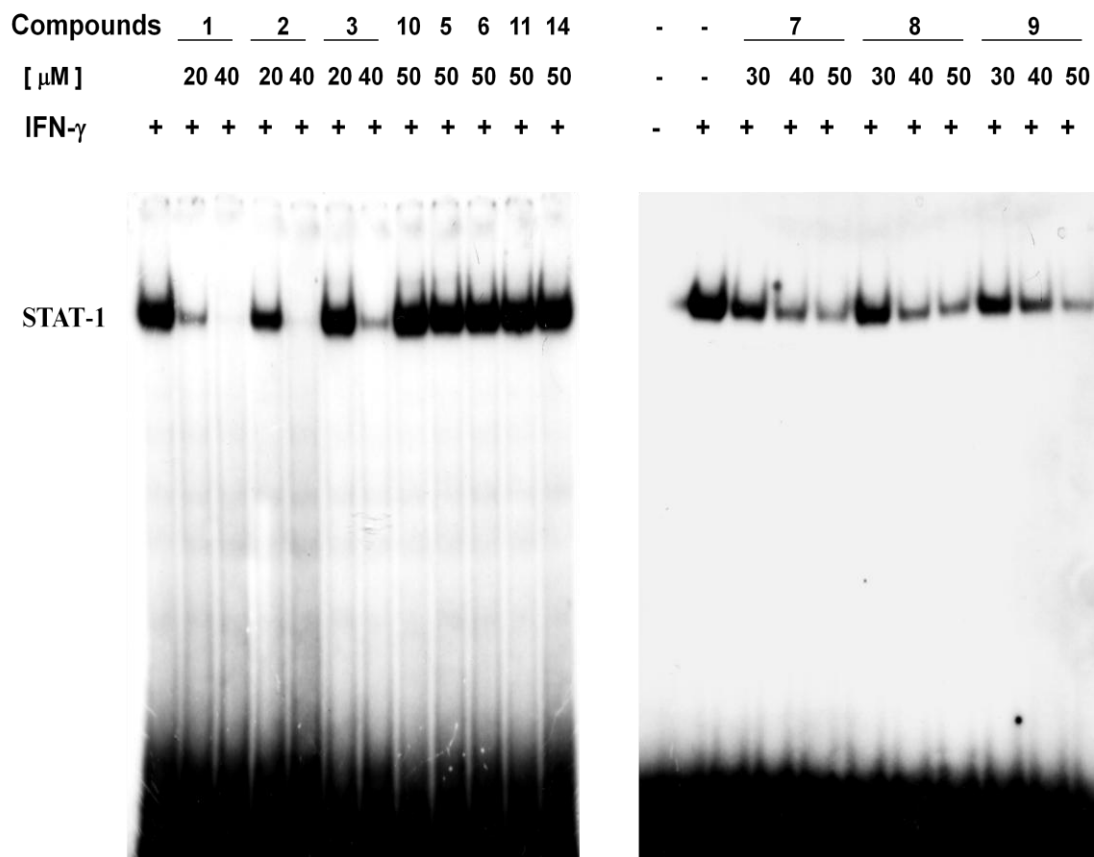
Figure 14:   Effect of synthetic catechins on STAT1 activation.

Figure 14 shows DNA binding activity of STAT1 in MDA MB 231 cell line untreated or induced by IFNγ in the presence or absence of different concentrations μM of synthetic catechins (each compound is identified by a number as shown in table 3). Cell nuclear extracts (10 μg) were incubated with a ^{32}P-labeled double-stranded oligonucleotide containing binding sequence for STAT1 and separated by nondenaturing PAGE. The specificity of the retarded bands was demonstrated by competition with 100-fold excess of specific unlabeled oligonucleotide (not shown). Each gel is representative of five separate experiments.

4.2.2 STAT1 phosphorylation

In the IFNγ/JAK/STAT1 pathway, STAT1 activation occurs after the phosphorylation of STAT1 proteins by JAKs. Phosphorylation in Tyr701 of the two STAT1 monomers is the necessary and sufficient condition for dimerization (Schindler C. et al., 1992; Shuai K. et al., 1992), nuclear translocation and DNA binding. Although phosphorylation in Ser727 of STAT1 is not necessary to DNA binding of this transcription factor, however, it is very important for a maximal transcriptional activity. So, to clarify the anti-STAT1 activity of these compounds, western blot analysis were performed in total cellular extracts, using two rabbit polyclonal antibodies that recognized STAT1 phosphorylated in Tyr701 and Ser 727, respectively.

The western blot showed high level of phosphorylated Tyr701 STAT1 1 hours after the induction with IFNγ (30 ng/ml) (Fig. 15). The pre-treatment of active synthetic catechins (compounds 1, 2, 3, 7, 8 and 9) reduced Tyr701 phosphorylation, in particular compounds 1, 2 and 7 acting stronger than compounds 3, 8 and 9 (Fig. 15). GCG and compound 1, which is the racemic form of GCG, had similar activity (Fig.15).

To test the ability of these molecules to inhibits the phosphorylation in Ser727 of STAT1, a second blot was hybridized with anti-posphoSTAT1(Ser727) antibody, as shown in figure 15. The control cells show slight phosphorylation, compounds 1, 2 and 9, and to lesser content compounds 3, 7 and 8 compared to the induced sample, inhibited this phosphorylation.

No significant changes in the amount of total STAT1 protein was observed (Fig. 15).
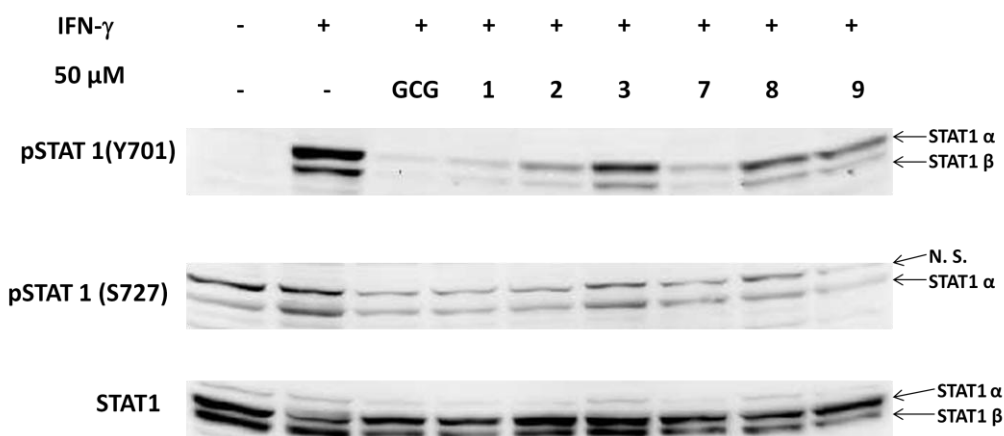
Figure 15: Effect of synthetic catechins on Tyr701 and Ser727 phosphorylation of STAT1 in MDA MB 231 cells treated with IFNγ.

50 µg of total extracts of cells treated as in figure 14 were separated by denaturing SDS-PAGE, electroblotted onto PVDF membrane and probed with an antibody recognized phosphorylated STAT1 in Tyr701, and Ser727. Top panel shows immunoblot of STAT1 phosphotylated in Tyr701, the antiphosphoSTAT1 Tyr701 antibody was diluted 1:700 in BSA 5%; in the central panel the same samples are probed with antiphosphoSTAT1 Ser727 (lowest band), the antibody was diluted 1:300 in BSA 5%. Lower panel shows the same samples and blot stripped and reprobed with an antibody recognizing STAT1 total protein, diluted 1:1000 in milk 5%. Data shown are representative of three independent experiments. Note that there are two forms of STAT1: STAT1 α and STAT1 β. The first contains two phosphorylation sites at Tyr701 and, in its carboxyl-terminal domain, Ser727. STAT1 β is an alternatively spliced version of STAT1 that lacks 38 carboxyl-terminal residues, including Ser727. The blots for STAT1 or phosphoSTAT1 Tyr701 detect the doublet of STAT1 α (upper band) and β (lower band), whereas antiphosphoSer727 detects the single STAT1 α band; the upper band of this blot is a non specific band (N.S.).

4.2.3 Modulation of gene expression

Since IFNγ/JAK/STAT1 pathway elicits the expression of different STAT1 dependent genes, the ability of the anti-STAT1 synthetic catechins to inhibit the expression of two genes was studied. The genes considered in this work encode for inducible Nitric Oxide Synthase (iNOS) and for Major Histocompatibility Complex Class II DR alpha (MHC II DRA). While the expression of MCH II genes is induced by IFNγ alone, in human cells, other different cytokines are also required to have a full iNOS induction. In this work MDA MB 231 cells were pre-treated with synthetic catechins for one hour, then they were induced only with IFNγ (30 ng/ml) for 30 hours, to exclude the involvement of NF-κB transcription factor. Total RNA were purified from cells and the amount of specific mRNAs was quantified with Real-Time PCR as described in Materials and Methods and shown in the histograms (Fig. 16 and 17).

Although IFNγ alone elicited low level of iNOS gene induction in MDA MB 231 cell line (about two fold), figure 16 shows that only compounds 1, 2, 3, 7, 8 and 9 were able to inhibit iNOS expression at different manner. The amount of MCH II DRA mRNA levels are shown in figure 17. This histogram shows that: 1. treatment with IFNγ alone gives a very good gene induction (10 fold) in comparison to the control sample; 2. compounds 1, 2, 3, 7, 8 and 9 are able to completely inhibit the MCH II DRA gene expression, in fact they all have almost the same value of mRNA level of control sample. All other synthetic catechins are inactive (data not shown). Only a trend of reduction of MCH II DRA gene expression is shown after pre-treatments with compounds 5 and 10; this in not statistically significant for $P < 0,01$.

Figure 16: Inhibition of iNOS gene expression after IFNγ induction.

Cells MDA MB 231 were pre-treated with different catechins for 1 hour and induced with IFNγ (30ng/μl) for 30 hours; total RNA was extracted, purified and quantified as described in materials and methods. The histogram shows iNOS mRNA levels quantified by Real-Time PCR assay. Compounds 1, 2, 3, 7, 8 and 9 are able to inhibit iNOS gene expression, while compounds 5 and 10 are not. Values are averages of three independent experiments ± S. D. *P<0.01 vs. IFNγ treated cells.
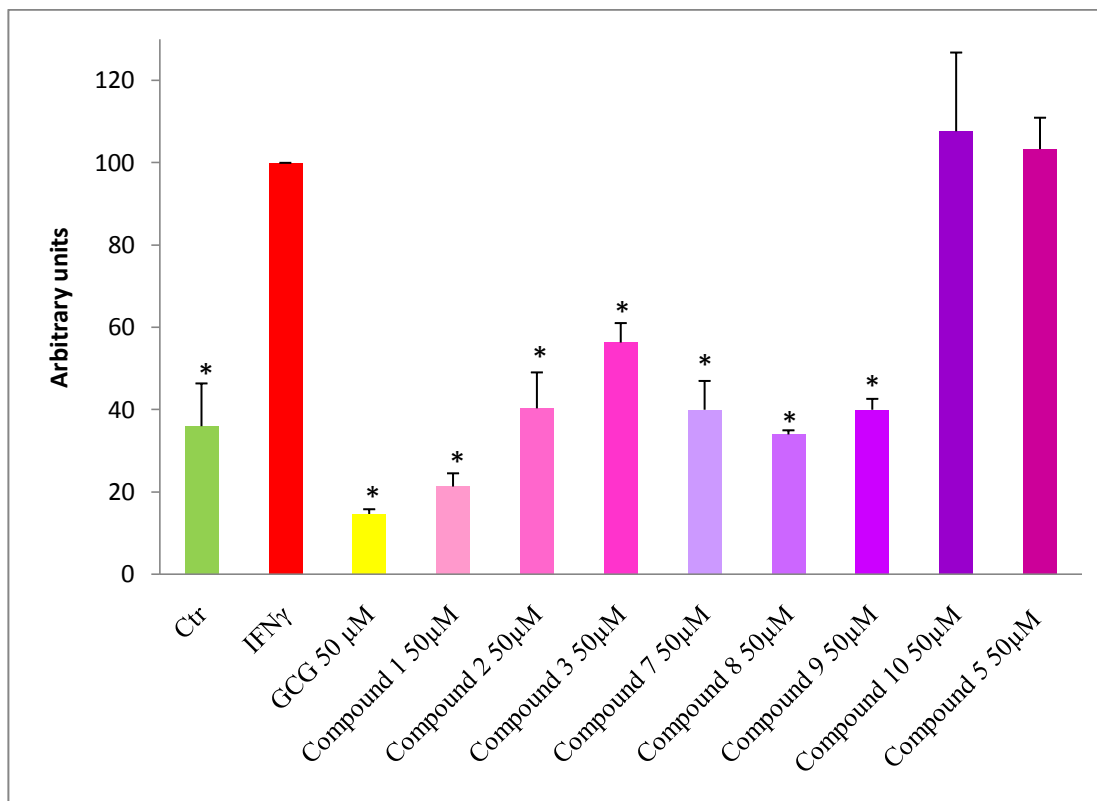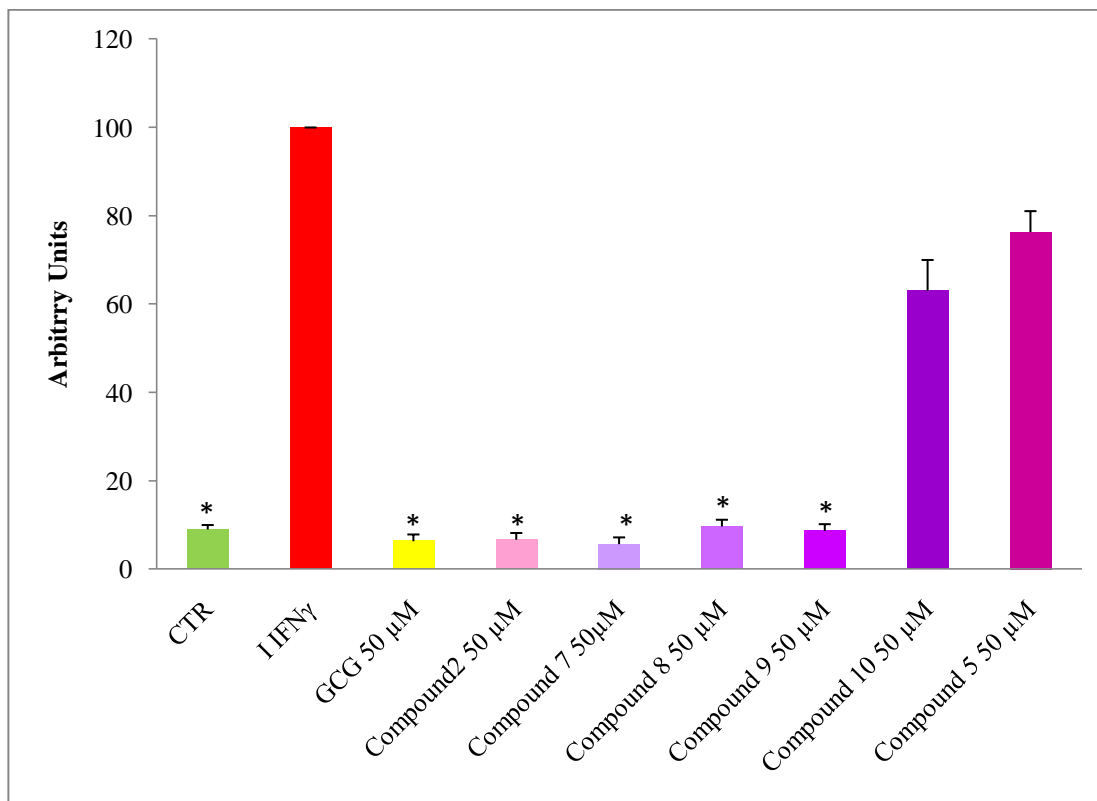
Figure 17: Inhibition of MCH II DRA gene expression after IFNγ induction.

Cells MDA MB 231 were pre-treated with different catechins for 1 hour and induced with IFNγ (30ng/µl) for 30 hours; total RNA was extracted, purified and quantified as described in materials and methods. The histogram shows MCH II DRA mRNA levels quantified by Real-Time PCR assay. Compounds 1, 2, 3, 7, 8 and 9 are able to inhibit MCH II DRA gene expression, while compounds 5 and 10 are not. Values are averages of three independent experiments ± S. D. *P<0.01 vs. IFNγ treated cells.

4.3 Direct interaction between anti-STAT1 catechins and STAT1 protein

4.3.1 Surface Plasmon Resonance analysis

Okabe et al. study, using radiolabeled catechins, showed that natural catechins are able to cross plasmatic membrane and enter into the cells (Okabe S. et al., 1997). So the possible target of the anti-STAT1 molecular mechanism should be an intracellular target and we verified in this work whether catechins interact directly with STAT1 protein itself.

In order to evaluate this hypothesis Surface Plasmon Resonance (SPR) analysis were performed. First STAT1 protein was cloned in a vector suitable for expression in bacteria (pET22b), expressed in BL21 strain of *E.coli* and purified by affinity chromatography, as described in Materials and Methods. Then STAT1 was immobilized on sensor chip and the interaction with catechins was analyzed by BIAcore instrument.

Analysis of sensograms by "BIAevaluation software" and "BIAsimulation software" provided k_a and k_d values and dissociation constant (K_D) was calculated and showed in Table 3. Figure 18 and 19 show some examples of interaction or no interaction between STAT1 protein and some compounds. First part of response curves (rising graph) represents the association phase between STAT1 and the considered compounds, and allows to measure the association rate constant (k_a); the second part of responsive curves (descending graph) represents the dissociation phase and gives the dissociation rate constant (k_d). In the better sensograms there is also an equilibrium phase between association and dissociation, which represents the steady state and allows to calculate the dissociation constant ($K_D = k_d / k_a$).

As shown in figures 18 and 19, sensograms clearly indicated that not only EGCG and GCG but also all catechins that reduced STAT1 activity (compounds 1, 2, 3, 7, 8 and 9) were able, in different ways, to interact directly with STAT1 protein.

With a detailed analysis of sensograms obtained with catechins that exert a stronger inhibitory action on STAT1 activity (GCG, EGCG, compounds 1, 2 and 3), two dissociation constants could be calculate: K_{D1} (nM) which indicated high affinity of STAT1 binding and K_{D2} (µM) at low affinity. To explain these data, we suppose the presence of two putative interacting sites for the catechins on STAT1 protein with different affinity constants. The K_{D1} values estimated for GCG (16.9 nM), EGCG (23 nM), compound 1 (37.3 nM) and compound 2 (27,6 nM) indicated that the interaction between STAT1 and these compounds might be very tight at one of putative interaction site (Table 3). Interaction between STAT1 and compound 3 (534 nM) also appeared strong. Compounds 7, 8 and 9, with less anti-STAT1 activity, showed mild STAT1 interaction. Indeed, analysis of their sensograms (Fig. 18) determined only one K_D value (11.8 µM, 2.42 µM, 7.36 µM respectively) (Table 3). Other catechins such as compounds 5, 6, 11, 12 and 14 with high K_D value, varying from 50 to 2 x 10^2 µM, showed very weak interaction (Table 3). Compounds 4, 10, 13 and 15 did not produce analysable sensograms suggesting that there is not at all STAT1 interaction with these catechins (Fig. 19).
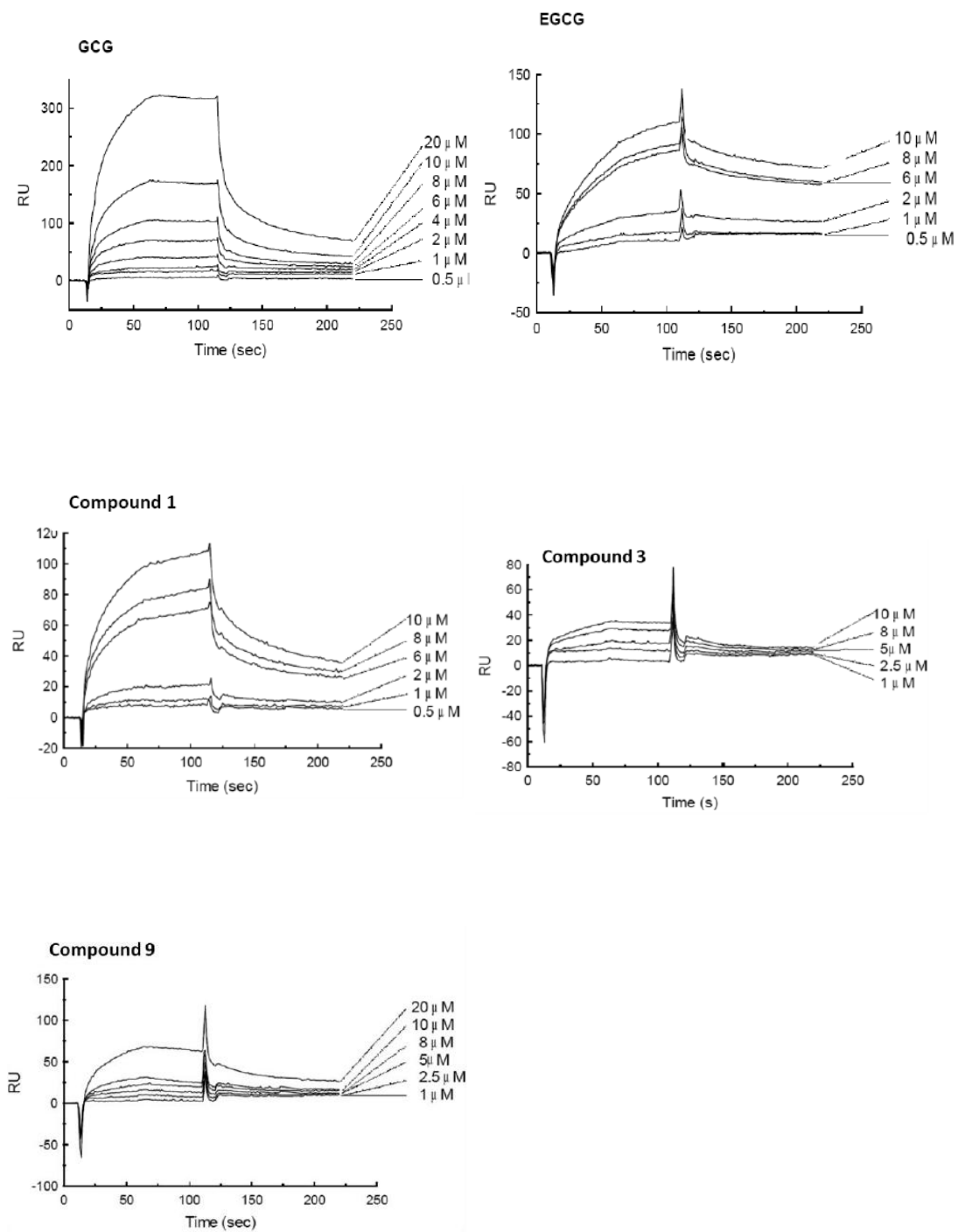
Figure 18:

Examples of sensograms of anti-STAT1 catechins.
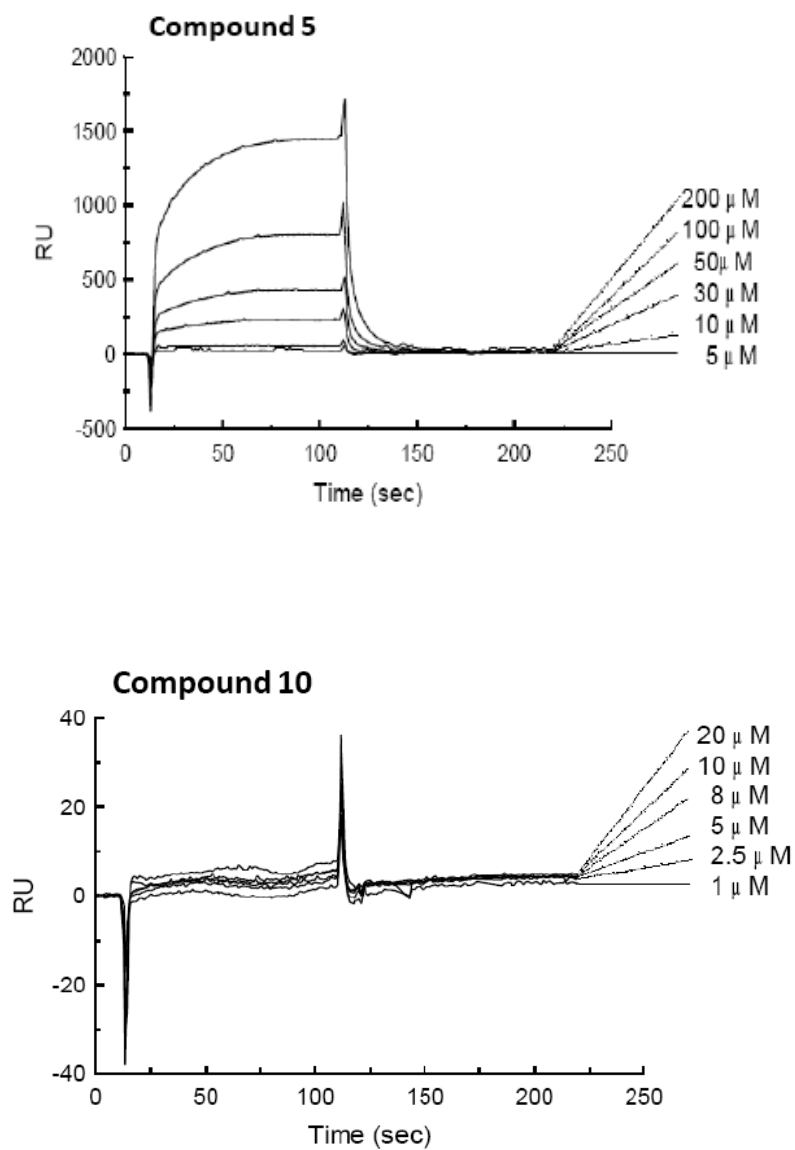
Figure 19:

Examples of sensograms indicating very weak or no interaction between STAT1 and compounds 5 and 10.

Compounds	K_{D1} (nM)	K_{D2} (µM)
EGCG	23	2.55
GCG	16.9	2.19
1	37.3	3.11
2	27.6	15.2
3	534	7.85
K_D (µM)		
4	-	
5	7.30×10^2	
6	7.11×10^2	
7	11.8	
8	2.42	
9	7.36	
10	-	
11	4.83×10^2	
12	52.3×10^2	
13	-	
14	2.54×10^2	
15	-	

Table 3:

Estimated values of the dissociation constants ($K_D = k_d / k_a$) of the catechins tested.

4.3.2 Computer Modelling Studies

The possible interaction between catechins and STAT1 was analyzed by molecular modelling studies, in order to determine possible catechin binding sites on the STAT1 structure. This analysis was performed in collaboration with Prof. Perahia of the "Laboratoire de modelization et ingenierie des proteins" of the University Paris-sud.

Starting from the X-ray structure of STAT1 (pdb entry 1yvl) specific different software were applied which allowed the identification of five major putative binding sites for GCG (Fig. 20). Two of them were located at the interface between the linker and SH2 domains (sites 1 and 2). The three remaining sites have a location closest to the region of STAT1 that interacts with DNA: one is located in the DNA-binding domain (site 3) and the two others are located at the interface between the DNA-binding domain and the linker domain (sites 4 and 5). The putative sites 3, 4 and 5 were ignored in this work since the free energy of the most stable docked orientations of the catechin in these sites was significantly higher than that of sites 1 and 2 (site 1: -10.90; site 2: -10.84; site 3: -8.96; site 4: -7.72; site 5: -7.65 kcal/mol). Furthermore, our data have shown that EGCG and GCG were not able to block the STAT1-DNA-binding in EMSA assay when they were added directly to the nuclear extracts containing STAT1 already activated proteins in the incubation mixture (data not shown). This could indicate either that catechins do not interact with the STAT1 in its DNA-binding domain or that the two catechins are not able to bind to already phosphorylated STAT1 proteins.

In order to better understand the nature of catechins-STAT1 interaction at the molecular level, docking calculations were performed for all the tested catechins, as reported in the Materials and Methods section. These simulations predict good binding affinities for anti-STAT1 compounds both at site 1 and 2. The detailed analysis of the best predicted orientations of the ligands in the different cavities shows that the hydroxyl groups of ring B seem to be very important for stabilizing catechins-STAT1 complexes. These groups were oriented towards the interior of the

cavity in both sites (Fig. 21) and seem to interact with residues Arg512, Gly513 and Gln518 on site 1 (Fig. 22 a), and with His568, Lys566 and Leu570 on site 2 (Fig. 22 c). However non-anti-STAT1 ligands, with fewer hydroxyls in ring B than GCG (compound 5) or without hydroxyls in ring D (compound 10), were oriented with the ring B towards the exterior of the cavity, interacting with other residues as shown in figures 22 b and d that represents the best pose of compound 5 in the two different sites. Thus on the basis of these analyses, the tested ligands could be divided into two subgroups, one of compounds that show good affinities of STAT1 binding (EGCG, GCG, compounds 1, 2, 3, 7, 8 and 9) and the other that show less affinities (compound 4, 5, 6 and 10 to 15).
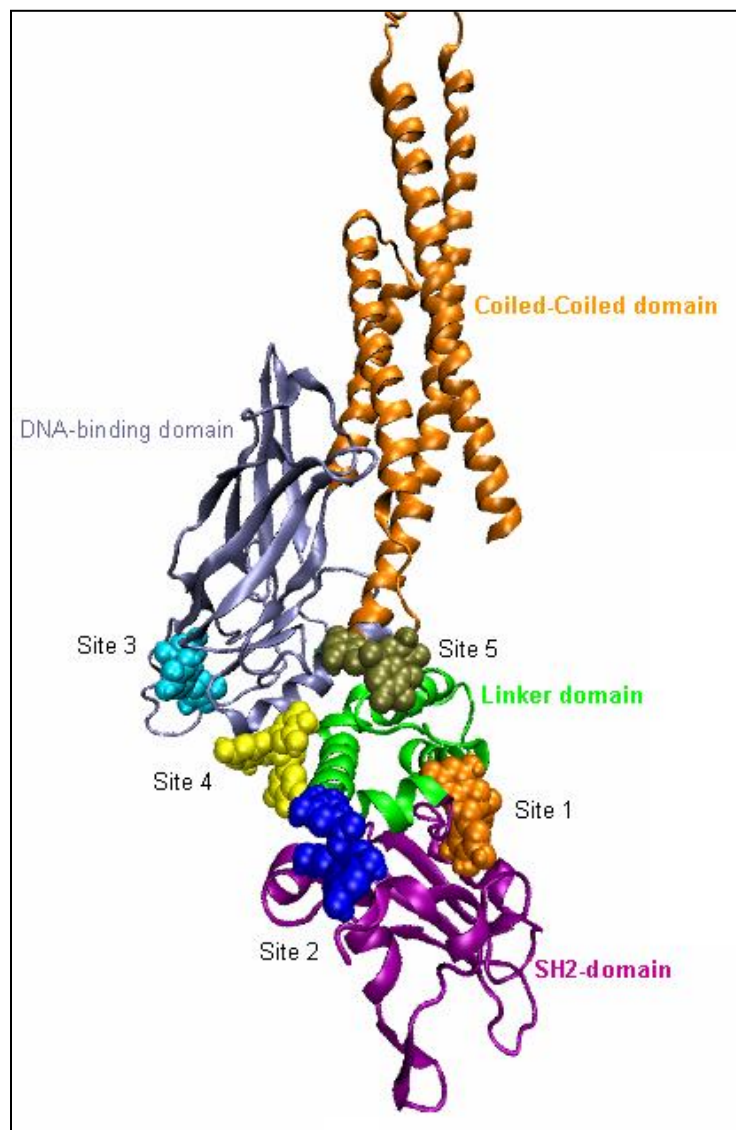
Figure 20: Location of STAT1 – GCG potential binding sites. Schematic view of STAT1 represented in cartoon without the N-domain (structure pdb reference 1YVL); the different domains as well as the five binding sites are indicated.
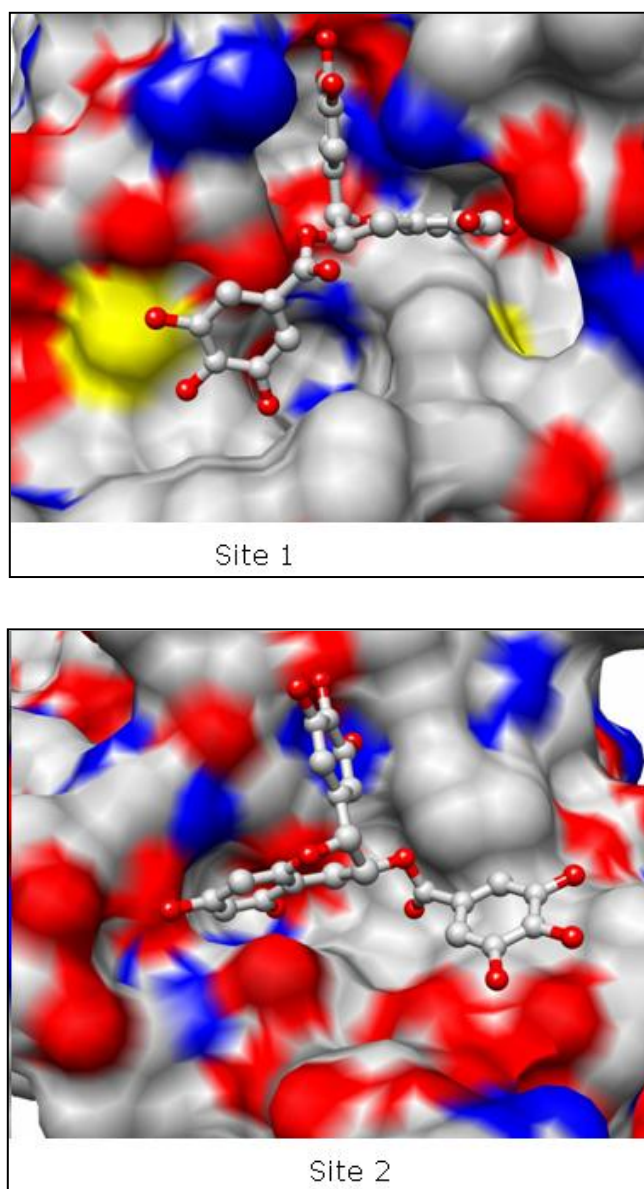
Figure 21: Surface representation of the two binding sites colored by the element type; oxygen (red), nitrogen (blue), carbon (white) and sulphur (yellow). GCG is represented in stick and ball.
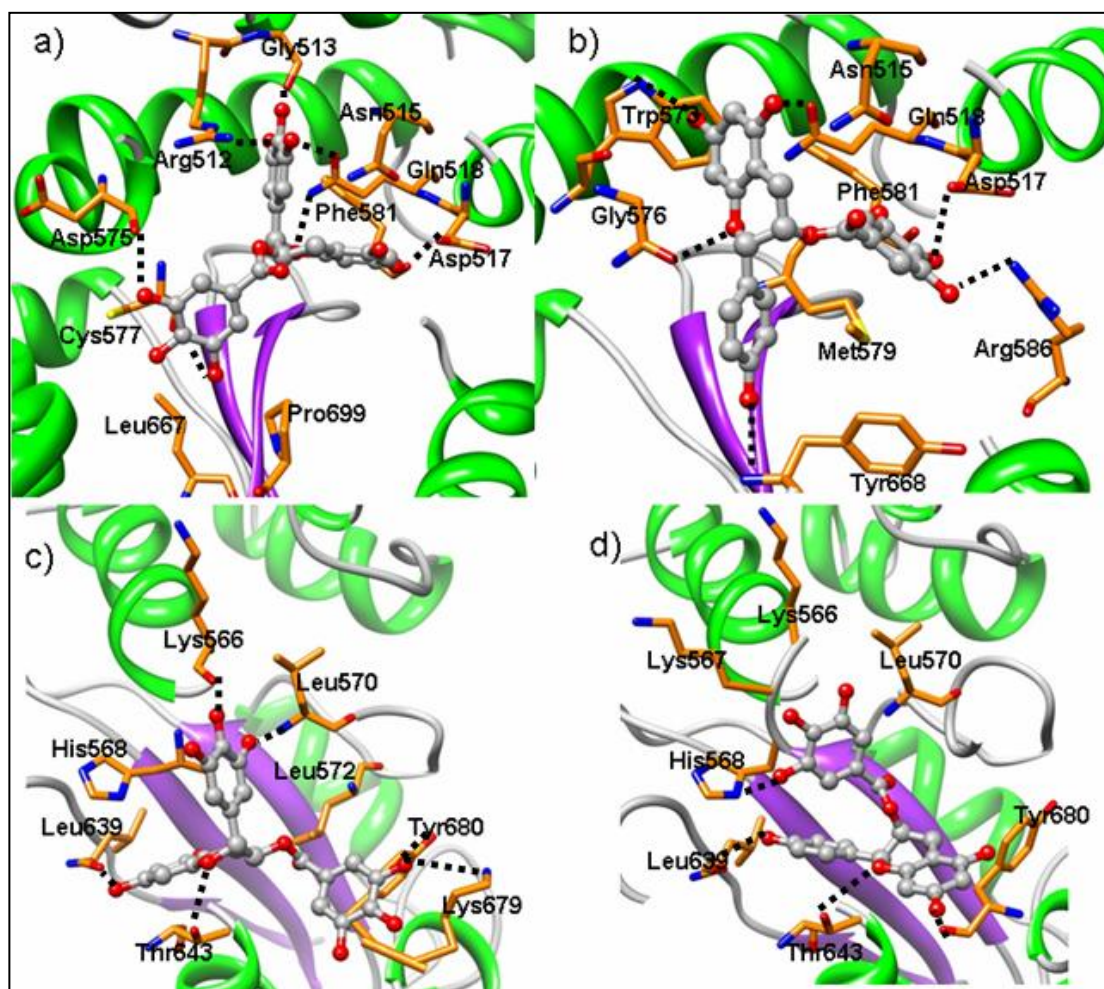
Figure 22: Representations of the best Autodock pose of GCG and compound 5 in the two binding sites. The ligand is in balls and sticks, interacting residues are in sticks and the secondary structure is in cartoon. a) GCG in site 1, b) compound 5 in site1, c) GCG in site 2 and d) compound 5 in site 2.

4.3.3 Site-directed mutagenesis

Molecular modelling studies have shown that most of the ligands considered could interact with STAT1 protein by their hydroxyls groups on the B and D rings. In site 1 anti-STAT1 catechins seem to bind to the side chains of Arg512, Gly513, Asp517, Gln518, Asp575 and Cys577; in site 2 catechins seem to bind to the main peptide chain of Lys566, Leu570, Lys679 and Tyr680, as well as to the side chain of His568. Moreover the latter amino acid seems to bind to another α chain domain, probably by interacting with Leu639. In this way the two chain domains could form a pocket for ligand insertion.

To test the possibility that Gln518 and His568 could be important for the catechin-STAT1 interaction on site 1 and 2 respectively, site-directed mutagenesis experiments were performed. Both Gln518 located in site 1 and His568 in site 2 were replaced with Ala (QA and HA, respectively) and the catechins affinities for the mutant form of STAT1 were analyzed by Surface Plasmon Resonance.

The obtained dissociation constants (K_D) are reported in Table 4 and indicate that the replacement of Gln518 of STAT1 with Ala (QA) produce two dissociation constants. For each catechin the K_{D1} (nM) value is not modified, while K_{D2} (µM) value is higher than that of STAT1 wild-type. This data strongly suggest that Gln518 could take part of site 1 and that this site is identified as the less affinity site for the catechins binding.

On the other hand, when His568 is replaced by Ala (HA), K_{D1} (nM) disappears while K_{D2} (µM) values increase with respect to the K_{D2} values of STAT1 wild type protein. On the basis of these results it is possible to conclude that His568 take part of site 2, which is characterized by an higher affinity with respect to site 1 for the ligands considered. Moreover our data demonstrate that His568 is essential for anti-STAT1 catechins binding.

Compounds	STAT 1 WT		STAT 1 QA		STAT 1 HA	
	K_{D1}(nM)	K_{D2}(µM)	K_{D1}(nM)	K_{D2}(µM)	K_{D1}(nM)	K_{D2}(µM)
GCG	16.9	2.19	28.6	240	-	22.4
2	27.6	15.2	14.2	68.4	-	99
3	534	7.85	607	137	-	69.4

Table 4 : Estimated values of the dissociation costants of the catechins tested with mutant STAT1 in Gln518 (QA) and in His568 (HA), in relation to STAT1 protein wild type (WT).

4.3.4 JAK2 kinase assay

The first step of the activation of STAT1 protein is the phosphorylation at Tyr701; our data demonstrated that some catechins provoke a reduction in Tyr701 phosphorylation. To demonstrate that the direct interaction between active catechins and STAT1 protein could prevent Tyr701 phosphotylation, a kinase assay was performed using JAK2 recombinant enzyme since JAK2 is the protein kinase responsible to phosphorylation of STAT1 in Tyr701.

As reported in Materials and Methods section, in the reaction buffer STAT1 recombinant protein (0,16 µg/µl) was incubated with Jak2 recombinant protein (0,16 ng/µl) for 5 minutes after 30 minutes of the pre-incubation time with the catechins.

Figure 23 shows the ability of anti-STAT1 catechins, as GCG and compound 2, to reduce the STAT1 phosphorylation in a dose-dependent manner. While, the inability of inactive compounds to prevent STAT1 phosphorylation was showed in figure 24 (compounds 5 and 10). In addition all catechins were ineffective to inhibit the phosphorylation status of activated JAK2.

The kinase assay with mutant STAT1 in His568Ala (Fig. 25) demonstrates that active compounds are unable to reduce the STAT1 Tyr701 phosphorylation, suggesting that the STAT1-catechins interaction on the site 2 is important to preserve the inhibitory action on STAT1 Tyrosine phosphorylation.

| GCG | – | 5 | 25 | 50 | – | – | – |
| Compound 2 | – | – | – | – | 25 | 50 | – |

pSTAT1

STAT1

pJAK2

JAK2

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
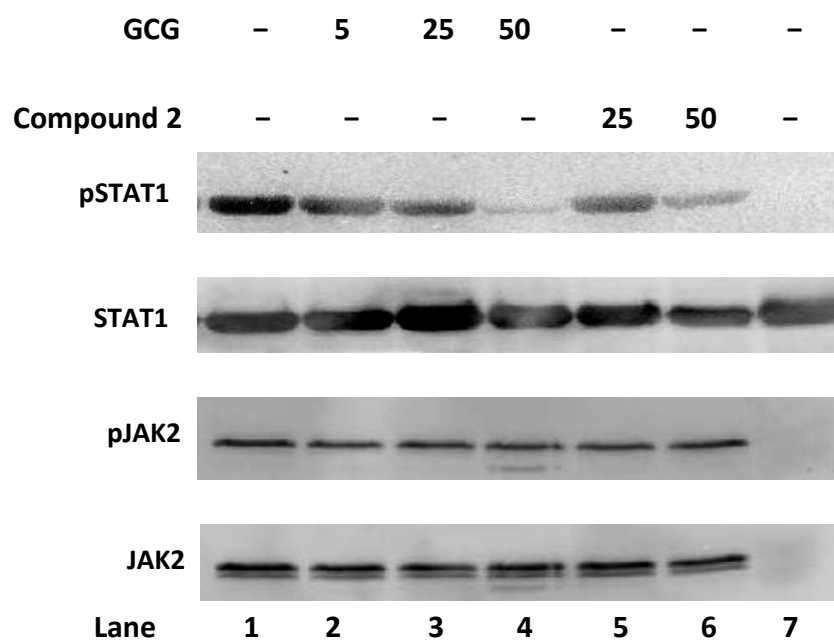
Figure 23: Kinase assay of active compounds. GCG and compound 2 were pre-incubated for 30 minutes at different doses with STAT1 recombinant protein. JAK2 recombinant protein was added for 5 minutes to the reaction mixture thus the samples were analyzed by western blot with anti-phosphoSTAT1 (Tyr701) and anti-phosphoJAK2 (Tyr1007) antibodies. Blots were stripped and reprobed with antibodies recognizing STAT1 or JAK2 proteins. Lane 1 shows reaction between STAT1 and JAK2 in reaction buffer; lane 7 shows STAT1 protein alone. Data shown are representative of three independent experiments.

Compound 10 (µM)	–	5	25	50	–	–	–	–
Compound 5 (µM)	–	–	–	–	–	5	25	50

pSTAT1(Tyr701)

STAT1

pJAK2(Tyr1007)

JAK2

Lane	1	2	3	4	5	6	7	8

Figure 24: **Kinase assay of non active compounds**. The compounds 5 and 10 were pre-incubated at different concentration for 30 minutes with STAT1 recombinant protein. JAK2 recombinant protein was added for 5 minutes to the reaction mixture, thus the samples were analyzed by western blot with anti-phosphoSTAT1 (Tyr701) and anti-phosphoJAK2 (Tyr1007) antibodies. Blots were stripped and reprobed with antibodies recognizing STAT1 or JAK2 proteins. Lane 1 shows STAT1 protein alone; lane 5 shows reaction between STAT1 with JAK2 in reaction buffer. Data shown are representative of three different experiments.

GCG	–	–	25	50	–	–
Compound 2	–	–	–	–	25	50

pSTAT1 (Tyr701)

Lane	1	2	3	4	5	6

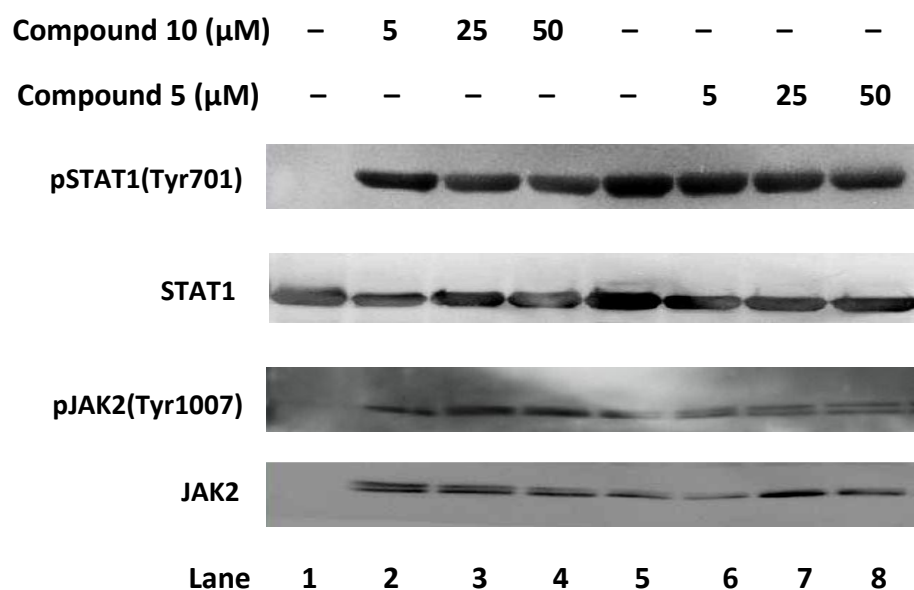**Figure 25**: **Kinase assay with mutated STAT1 recombinant protein (H568A).** GCG and compound 2 were pre-incubated for 30 minutes at different dose with STAT1 recombinant protein mutated in His568 with Ala. JAK2 recombinant protein was added for 5 minutes to reaction mixture, thus samples were analyzed by western blot with anti-phosphoSTAT1 (Tyr701) antibody. Lane 1 shows mutated STAT1 protein alone; lane 2 shows reaction between mutated STAT1 and JAK2 in reaction buffer. Data shown are representative of three independent experiments.
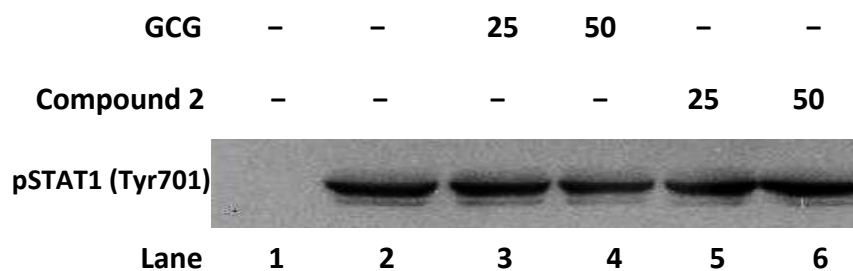
5. Discussion

An increasing body of evidence indicates beneficial actions of green tea drinking that might be extended to a number of pathologies correlated to inflammation (Choi Y. B. et al., 2004; Crespy V. and Williamson G., 2004; Dona M. et al., 2003; Kakuda T., 2002). Previous work in our laboratory demonstrated that EGCG efficiently inhibits interferonγ (IFNγ)-elicited phosphorylation of tyrosine residue 701 and DNA-binding activity of STAT1 in different human carcinoma-derived cell lines (Menegazzi M. et al., 2001). STAT1 almost exclusively mediates the action of IFNγ, playing a pivotal role at the early phase of inflammation in regulating the expression of a number of inflammation related genes (Carcereri De Prati A. et al., 2005).

Despite some reports indicate the critical involvement of the change in redox state in modulating STAT1 activation (Grimm M. et al., 2002; Kim H. et al., 2001; Pawate S. et al.,2004; Pointer M. E. et al., 1999), our results show that all catechins present in green tea extract, excluding EGCG, are unable to down-regulate STAT1 activation, suggesting that EGCG may not exert its activity using exclusively its antioxidant capacity.

In this work we have tried to answer two questions on:
1. the structural features of the catechins for their anti-STAT1 activity;
2. the molecular target and mechanism of action of the catechins in inhibiting STAT1 activation.

New synthetic catechins, obtained by GCG chemical modification, was synthesized to answer these questions; as first, a study to understand a structure activity relationship has been done.

The stereochemistry of the catechin gallate compounds does not influence the anti-STAT1 activity. As a matter of fact, GCG, trans diastereomer of EGCG, is able to inhibit STAT1 activation and presents DNA-binding inhibitory level in EMSA assays (IC$_{50}$ 10-15 μM) similar to that of EGCG. Compound 2, lacking in one hydroxyl group in 5 of ring A, have similar anti-STAT1 activity (IC$_{50}$ 17 μM) in comparison to compound 1, racemate of GCG, (IC$_{50}$ 12 μM).

Compound 3, lacking in both the hydroxyl groups in 5 and 7 of ring A, shows a lower anti-STAT1 activity (IC_{50} 28 μM). Compounds 7, 8 and 9, that present two hydroxyl groups in R_7 and R_8, or two hydroxyl groups in R_6 and R_8 or only one hydroxyl group in R_7 of ring D, retain a reduced anti-STAT1 DNA-binding activity (IC_{50} 33, 37, 31 μM, respectively). Since compound 10, lacking in all hydroxyl groups in ring D, is completely inactive, at least one hydroxyl group in ring D must be essential for the anti-STAT1 activity.

The synthetic catechins lacking in hydroxyls in ring B (compounds 4, 5 and 6) are fully inactive, this indicates that the natural structure of ring B must be preserved to retain the inhibitory action on STAT1 activation.

More than one hydroxyl group was removed simultaneously in rings A, B and D in compounds 11, 12, 13, 14 and 15. Compounds 11, 12 and 13 are characterized by removal of hydroxyl groups in rings A and B, while in compound 14 has been modified only rings B and D and in compound 15 has been modified rings A, B and D. All these compounds resulted inactive, thus confirming the importance of the three substituents of ring B for the STAT1 inhibition.

Western blot studies demonstrate the ability of the synthetic compounds, lacking in some hydroxyl substituents, to inhibit the IFNγ-elicited STAT1 phosphorylations in Tyr701 and Ser727, that are necessary for STAT1 activation and for a maximal transcriptional activity. These analysis confirm the results obtained in EMSA assays and show that the chemical features for anti-STAT1 activity are: the presence of three hydroxyl groups in ring B and at least one hydroxyl group in ring D.

IFNγ-elicited activation of STAT1 is correlated to acute inflammatory diseases, such as heart ischemia/reperfusion injury (Takagi Y. et al., 2002; Stephanou A. et al., 2003; West D.A. et. Al., 2004), and chronic inflammatory diseases such as asthma (Sampath D. et al, 1999; Quarcoo D. et al., 2004), AIDS (Magnani M. et al., 2003; Miller E. D. et al., 2003), atherosclerosis (Wang X. Q. et al., 2002; Woods M. et al., 2003), celiac disease (Mazzarella G. et al., 2003; Monteleone I. et al., 2004), chronic inflammatory bowel disease (Schreiber S. et al., 2002), diabetes (Cottet S. et al.,

2001; Suk K. et al., 2001), psoriasis (Giustizieri M. L. et al.,2002) and rheumatoid arthritis (Yokota A. et al., 2001; Kasperkovitz P. V. et al.; 2004).

The inhibitory effects of catechins against IFNγ-elicited STAT1 phosphorylation is important to block the expression of some STAT1 dependent genes, deeply involved in mediating inflammatory processes. iNOS, normally silent, seems to be involved in the pathology of several human diseases (Kroncke K. D. et al., 1998). Massive amount of NO produced by iNOS may lead to tissue damage (Miwa M. et al., 1987).

Preliminary experiments showed that in human mammary carcinoma cell line MDA MB 231, iNOS induction strictly depends on IFNγ signaling and STAT1 activation, nevertheless LPS and cytokines such as TNFα and IL1β have synergistic effects for full iNOS expression by NF-κB activation. Although the activation of STAT1 is not exclusively elicited by IFNγ but by other cytokines (IFN α/β and IL10; Seidel H. M. et al., 2000), the biological effects of IFNγ are mainly mediated by STAT1 homodimers (Meraz M. A. et al,1996). In this work MDA MB 231 cells were induced for 30 hours only with IFNγ to exclude the involvement of other transcription factors. The IFNγ-induction of MDA MB 231 cells was not the best condition for iNOS expression since it leads to an induction of only two fold. Nevertheless, we showed that compounds 1, 2, 3, 7, 8 and 9 were able to inhibit iNOS induction with different degree. All other compounds, as compound 5, lacking in one hydroxyl substituent in ring B, or compound 10, lacking in all hydroxyl groups in ring D, are unable to induce iNOS expression.

Despite non-immune cells normally lack constitutive expression of MCH class II DRA gene, this gene expression in MDA MB 231 cells, can be induced by IFNγ. The regulation of MHC class II genes family occurs primarily at the transcriptional level; the master control factor for MHC class II transcription is class II transactivator (CIITA) (Rohn, W. M., 1996). IFNγ induces the expression of CIITA gene (Chang C. H. et al., 1994, Steimle V. C. A. et al., 1994). Thus, MCH II DRA expression in non-immune cells may indicate an inflammatory state, which can compromise the

integrity of the tissue. The inhibition of these gene expression can improve organ condition and inhibit graft failure.

Although MCH II DRA induction is under the control of different transcriptional factors (Meraz M. A. et al, 1996; Hobart M. et al., 1997), a single treatment of MDA MB 231 cells with IFNγ gives a full induction of MCH II DRA gene expression. In this experimental condition, compounds 1, 2, 3, 7, 8 and 9 completely inhibit MCH II DRA expression to the levels of non-induced cells. The others catechins, lacking of the previously mentioned chemical features, present an irrelevant capability to inhibit MCH II DRA expression.

These data reinforce the previous evidences obtained by EMSA and Western Blot showing that the minimal structure of catechins for the inhibition of STAT1 dependent genes expression is the presence of three hydroxyl groups in ring B and one hydroxyl group in ring D.

The importance of modulation of iNOS, MCH II DRA and other STAT1 dependent genes expression involved in inflammatory and immune response is well known (Bach E.A. et al, 1999; Chatterjee-Kishore M. et al,2000). Our catechins may play a significant role to control these inflammatory processes.

The second aim was to identify the molecular target of inhibitory action of catechins in the IFNγ/JAK/STAT1 pathway. Our results demonstrate that the earlier event in this pathway concern the inhibition of STAT1 phosphorylation. Thus, the possible targets of this inhibition could be upstream: IFNγ binding to its specific receptor (IFNGR1/2), and JAK Tyrosine phosphorylation, or STAT1 Tyrosine 701 and Serine 727 phosphorylation itself.

The possible interaction of the catechins with IFNGR was not evaluated. Previous studies with radiolabelled catechins showed the ability of these compounds to enter into the cell (Okabe S. et al., 1997). Moreover, our preliminary results showed action of GCG also after washout catechins by changing cell medium, suggesting that the molecular target might be an intracellular protein.

Activation of JAK 1 and 2 by tyrosine phosphorylation is reported to cause STAT1 and STAT3 activation (Leonard W. et. Al, 1998). Since EGCG and GCG fail to inhibit interleukin6-elicited activation of STAT3 in the same cell line (Menegazzi M. et al., 2001), the possibility that catechins reduce JAK1 or 2 activity to inhibit STAT1 phosphorylation is unlikely. Involvement of tyrosine phosphatases in STAT1 inhibitory action of catechins also seems to be quite improbable, since they may regulate the tyrosine-phosphorylated state of a wide spectrum of proteins including STAT3. SOCS1/3, that down-regulate STATs activation, could be a target of catechins. Since the notion that SOCS gene expression is induced mainly by STAT1 activation itself renders this possibility unlikely.

We took into account the idea that the target of STAT1-inhibiting action of catechins might be STAT1 protein itself. To evaluate this hypothesis first we analyzed, by Surface Plasmon Resonance method, the interaction between immobilized STAT1 and all different catechins. SPR analysis indicate that EGCG, GCG and the synthetic catechins with the presence of three hydroxyl groups in ring B and, at least, one hydroxyl group in ring D, directly interact with STAT1 protein with high affinity. Moreover, EGCG, GCG and compounds 1, 2 and 3 present two K_D values (nM and µM) which lead to suppose the presence of two binding sites with different affinities on STAT1 protein. These data are in line with computer modelling analysis indicating that GCG may strongly interact with STAT1 at two sites, site 1 and site 2, close to SH2 domain. This binding could affect the tyrosine 701 phosphorylation.

Site-directed mutagenesis shows that site 2 has higher affinity for catechins than site 1, as replacing His568 of site 2 with Ala, K_{D1} (nM) disappears. Probably this amino acid is important to save the binding pocket for catechins, which seemed to be stabilized by His568 bound to opposite α chain domain. On the other hand without His568 there is no interaction with hydroxyl groups of ring B of catechins in site 2. Moreover, this mutation probably provokes a structural change in all STAT1 protein so that to decrease the binding affinity of the catechins for STAT1 protein also at site 1, as shown by the nearly 10 fold increase of K_{D2} values.

Since synthetic catechins 4, 5, 6, 10, 11, 12, 13, 14, 15 have not inhibitory action on IFNγ-elicited STAT1 activation, and they are unable to directly interact with STAT1, we suggest that the three hydroxyl groups in ring B are critical for their specific binding to STAT1. The docking analysis data seem to support this hypothesis showing different orientations of active and inactive catechins in STAT1 binding sites. The compounds able to inhibit IFNγ-elicited STAT1 activation seem to direct the three hydroxyls of ring B into the cavity, stabilizing catechin-STAT1 complexes. The compounds unable to inhibit STAT1 activation, seem to orient the ring B toward the outside of the cavities of STAT1 pocket.

This data may suggest that inhibitory action of considered catechins on STAT1 DNA-binding activity is due to its capacity to strongly and directly interact with STAT1 protein. The ability of anti-STAT1 catechins to directly interact with STAT1 with high affinity at critical sites close to SH2 domain underlines a novel mechanism leading to an efficient and specific inhibition of STAT1 activation. In particular, the very high affinity of the compounds 1, 2 and 3 towards putative binding site 2 is in line with the specificity of their action towards the STAT1 pathway shown in this study. Since, STAT3, having a slightly different SH2 domain with respect to that of STAT1, was not inhibited by anti-STAT1 catechins, adding further support to the above-mentioned mechanism. This may also explain how anti-STAT1 catechins disturb IFNγ-elicited phosphorylation of both Tyr701 and Ser727, both of which are situated near putative anti-STAT1 catechins binding sites.

Our results obtained by Western Blot analysis showed that MDA MB 231 cells, pre-treated with active catechins, had lower level of phosphorylated STAT1. This was confirmed by STAT1-JAK2 kinase assay data showing that the *"in vitro"* STAT1 phosphorylation in Tyr701 is inhibited, in a dose dependent manner, by catechins' pre-treatment. On the contrary, JAK2 auto-phosphorylation does not change after pre-treatment with active catechins in presence of STAT1, suggesting that STAT1 is the real target of inhibitory action of these compounds. Therefore, this assay supports the

idea that direct binding of the anti-STAT1 catechins with STAT1 protein is an important event to affect STAT1 phosphorylation in Tyr701 by JAK2 enzyme.

In conclusion our results indicate that the inhibitory action of anti-STAT1 catechins should not be attributed only to their antioxidant activity. This work indicates that among a number of catechins examined, the compounds with a common structural features including the presence of three hydroxyl groups in ring B and at least of one hydroxyl group in the ring D are able to inhibit STAT1 activation. Among all the possible targets in IFNγ/JAK/STAT1 pathway, this chemical features allow catechins to directly interact with STAT1 protein. The catechins-STAT1 interaction is very tight and seems to prevent STAT1 phosphprylation in Tyr701 by JAK2, and represent a novel, specific and efficient molecular mechanism for inhibition of IFNγ-elicited STAT1 activation.

This study, furthermore, may be considered the first step to the development of new anti-STAT1 molecules, able to prevent or to switch off inflammatory diseases.

6. Bibliography

- Bach, E.A., Kaplan, D.H., and Schreiber, R.D. (1999) Biochemistry, mechanism of action, and biology of the interferons. In *Inflammation, basic principles and clinical correlates* (Gallin, J.I., and Snyderman, R. Eds.) pp. 487-503, Lippincott Williams & Wilkins, Philadelphia; Ramana, C.V.

- Barnes PJ and Liew FY (1995) Nitric oxide and asthmatic inflammation. *Immunol Today* **16:**128–130.

- Benoist, C. and D. Mathis (1990). "Regulation of major histocompatibility complex class-II genes: X, Y and other letters of the alphabet." *Annu Rev Immunol* **8**: 681-715.

- Blanchette, J., M. Jaramillo, et al. (2003). Signalling events involved in interferon-gamma-inducible macrophage nitric oxide generation. *Immunology* **108**(4): 513-522.

- Bogdan C (2001) Nitric oxide and the immune response. *Nat Immunol* **2:**907–916.

- *Bradford MM (1976)Anal Biochem 72: 248-254.*

- Bursulaya BD, Totrov M, Abagyan R and Brooks CL, 3rd (2003) Comparative study of several algorithms for flexible ligand docking. *J Comput Aided Mol Des* **17**: 755-63.

- Carcereri de Prati A, Ciampa AR, Cavalieri E, Zaffini R, Darra E, Menegazzi M, Suzuki H and Mariotto S (2005) STAT1 as a new molecular target of anti-inflammatory treatment. *Curr Med Chem* **12**: 1623-29.

- Cao, Y. and R. Cao (1999). Angiogenesis inhibited by drinking tea. *Nature* **398**(6726): 381.

- Cavicchi M and Whittle BJ (1999) Regulation of induction of nitric oxide synthase and the inhibitory actions of dexamethasone in the human intestinal epithelial cell line, Caco-2: influence of cell differentiation. *Br J Pharmacol* **128:**705–715.

- Chang, C. H., J. D. Fontes, M. Peterlin, and R. A. Flavell. 1994. Class II transactivator (CIITA) is sufficient for the inducible expression of major histocompatibility complex class II genes. *J. Exp. Med. 180:1367*.

- Chan MM, Fong D, Ho CT, Huang HI (1997) Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. *Biochem Pharmacol* 54(12):1281–1286

- Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell Jr, J.E., Kuriyan, J., (1998). Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. *Cell* 93, 827–839.

- Chen XP, Losman JA, Rothman P (2000). SOCS proteins, regulators of intracellular signaling. *Immunity*;13:287-90.

- Choi YB, Kim YI, Lee KS, Kim BS, Kim DJ (2004) Protective effect of epigallocatechin gallate on brain damage after transient middle cerebral artery occlusion in rats. *Brain Res* 1019(1–2):47–54

- Cottet, S.; Dupraz, P.; Hamburger, F.; Dolci, W.; Jaquet, M.; Thorens, B. (2001). SOCS-1 protein prevents Janus Kinase/STAT-dependent inhibition of beta cell insulin gene transcription and secretion in response to interferon-gamma. *J Biol Chem* **276**(28): 25862-25870.

- Crespy V, Williamson G (2004) A review of the health effects of green tea catechins in vivo animal models. *J Nutr* 134(12 Suppl):3431S–3440S.

- Darnell JE Jr (1997) STATs and Gene Regulation. *Science* **277**: 1630-1635.

- Decker, T., Kovarik, P., (2000). Serine phosphorylation of STATs. *Oncogene* 19, 2628–2637.

- de Vera ME, Shapiro RA, Nu¨ssler AK, Mudgett JS, Simmons RL, Morris SM, Billiar TR, and Geller DA (1996) Transcriptional regulation of human inducible nitricoxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc Natl Acad Sci USA* **93:**1054–1059.

- Dona M, Dell'Aica I, Calabrese F, Benelli R, Morini M, Albini A, Garbisa S (2003) Neutrophil restraint by green tea: inhibition of inflammation,associated angiogenesis, and pulmonary fibrosis. *J Immunol* 170(8):4335–4341.

- Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, et al (1997). A new protein containing an SH2 domain that inhibits JAK kinases. N*ature*;387:921-4.

- Flower R. J., (1990). Lipocotin. *Prog. Clin. Biol. Res*. 349:11-25

- Frisch MJ, TG, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Zakrzewski VG, Montgomery JA Jr, Stratmann RE, Burant JC Dapprich S, Millam JM, Daniels AD, Kudin KN, Strain MC, Farkas O, Tomasi J, Barone V, Cossi M, Cammi R, Mennucci B, Pomelli C, Adamo C, Clifford S, Ochterski J, Petersson GA, Ayala PY, Cui Q, Morokuma K, Malick, DK, Rabuck AD, Raghavachari K, Foresman JB, Cioslowski J, Ortiz JV, Baboul AG, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Gomperts R, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Gonzalez C, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Andres JL Gonzalez C, Head-Gordon M, Replogle ES, Pople JA (1998) Gaussian, Inc., Pittsburgh, PA.

- Ganster RW, Taylor BS, Shao L, and Geller DA (2001) Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B. *Proc Natl Acad Sci USA* **98:**8638–8643.

- Gao J, Morrison DC, Parmely TJ, Russell SW, and Murphy WJ (1997) An interferon-γ-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-γ and lipopolysaccharide. *J Biol Chem* **272:**1226–1230.

- Giustizieri, M.L.; Albanesi, C.; Scarponi, C.; De Pita, O.; Girolomoni, G., (2002). Nitric oxide donors suppress chemokine production by keratinocytes in vitro and in vivo. *Am J Pathol* **161**(4): 1409-1418.

- Glimcher, L. H. and C. J. Kara (1992). Sequences and factors: a guide to MHC class-II transcription. *Annu Rev Immunol* **10**: 13-49.

- Greenhalgh, C. J. and Hilton, D. J. (2001). Negative regulation of cytokine signaling. *Journal of Leukocyte Biology* **70,** 348-356.

- Grimm M, Spieker M, De Caterina R, Shin WS, Liao JK (2002) Inhibition of major histocompatibility complex class II gene transcription by nitric oxide and antioxidants. *J Biol Chem* 19:26460–26467.

- Grusby, M. J., and L. H. Glimcher. (1995). Immune responses in MHC class II-deficient mice. *Annu. Rev. Immunol. 13:417.*

- Gupta S, Hastak K, Afaq F, Ahmad N, Mukhtar H (2004). Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor kappa B and induction of apoptosis. *Oncogene*. 23(14):2507-22.

- Guslandi M(1998) Nitric oxide and inflammatory bowel diseases. *Eur J Clin Investig* **28:**904–907.

- Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P (2000) Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers. *Stroke* 31(10):2301–2306.

- Hobart, M., Ramassar, V., Goes, N., Urmson, J., and Halloran, P. F. (1997) IFN regulatory factor-1 plays a central role in the regulation of the expression of class I and II MHC genes in vivo*J. Immunol.* **158,** 4260–4269

- Horvath, C. M. (2000). STAT proteins and transcriptional responses to extracellular signals. *Trends Biochem Sci* 25(10): 496-502.

- Ihle, J.N. 2001. The Stat family in cytokine signaling. *Curr. Opin. Cell Biol.* 13, 211–217.

- Jankun, J., R. W. Keck, et al. (1997). "Inhibitors of urokinase reduce size of prostate cancer xenografts in severe combined immunodeficient mice." *Cancer Res* **57**(4): 559-563.

- Jenwitheesuk E and Samudrala R (2003) Improved prediction of HIV-1 protease-inhibitor binding energies by molecular dynamics simulations. *BMC Struct Biol* **3**, 2.

- Kakuda T (2002) Neuroprotective effects of the green tea components theanine and catechins. *Biol Pharm Bull* 25(12):1513–1518

- Kasperkovitz, P.V.; Verbeet, N.L.; Smeets, T.J.; van Rietschoten, J.G.; Kraan, M.C.; van der Pouw Kraan, T.C; Tak, P.P.; Verweij, C.L. (2004). Activation of the STAT1 pathway in rheumatoid arthritis. *Ann Rheum Dis* **63**(3): 233-239.

- Kim H, Lee TH, Hwang YS, Bang MA, Kim KH, Suh JM, Chung HK, Yu DY, Lee KK, Kwon OY, Ro HK, Shong M (2001) Methimatazole as an antioxidant and immunomodulator in thyroid cells: mechanism involving interferon gamma signaling and H2O2 scavenging. *Mol Pharmacol* 60:972–980

- Klatsky AL, Armstrong MA, Friedman GD (1993) Coffee, tea, and mortality. *Ann Epidemiol* 3(4):375–381

- Kleinert H, Boissel JP, Schwarz PM, and Fo¨rstermann U (2000) Regulation of the expression of nitric oxide synthase isoforms, in *Nitric Oxide: Biology and Pathobiology* (Ignarro LJ ed) pp 105–128, Academic Press, New York.

- Kleinert H, Wallerath T, Fritz G, Ihrig-Biedert I, Rodriguez-Pascual F, Geller DA, and Fo¨rstermann U (1998) Cytokine induction of NO Synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-kB-signaling pathways. *Br J Pharmacol* **125:**193–201.

- Kro¨ncke KD, Fehsel K, and Kolb-Bachofen V (1998) Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol* **113:**147–156.

- Laurie AT and Jackson RM (2005) Q-SiteFinder: an energy-based method for the prediction of protein-ligand binding sites. *Bioinformatics* **21**, 1908-16.

- Labarrere, C. A., Nelson, D. R., and Faulk, W. P. (1997) Endothelial activation and development of coronary artery disease in transplanted human hearts. *J. Am. Med. Assoc.* **278,** 1169–1175

- Laemmli UK (1970) *Nature* **227**: 680-685.

- Lee, J.I.; Burckart, G.J. *J. (*1998) Nuclear factor kappa B: important transcription factor and therapeutic target." *J Clin Pharmacol* **38**(11): 981-993.

- Leonard, W., O'Shea, J.J., 1998. JAKS and STATS: Biological implications. *Annu. Rev. Immunol.* 16, 293–322.

- Leung, S., Li, X., Stark, G.R., 1996. STATs find that hanging together can be stimulating. *Science* 273, 750–751.

- Levy, D.E., and Darnell, J.E., Jr. (2002). Stats: Transcriptional control and biological impact. *Nat. Rev. Mol. Cell Biol.* 3, 651–662.

- Levites Y, Amit T, Youdim MB, Mandel S (2002) Involvement of protein kinase C activation and cell survival/ cell cycle genes in green tea polyphenol (-)-epigallocatechin 3-gallate neuroprotective action. *J Biol Chem* 277(34):30574–30580.

- Li L, Chan TH. Enantioselective synthesis of epigallocatechin-3-gallate (EGCG), the active polyphenol component from green tea. *Org Lett.* 2001 3(5):739-41.
- Lin, T.S.; Mahajan, S.; Frank, D.A. (2000) STAT signaling in the pathogenesis and treatment of leukemias. *Oncogene* **19**(21): 2496-2504.

- Lin YL, Lin JK (19997). (-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. *Mol Pharmacol.*52(3):465-72.

- Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature.* Mar 6;284(5751):67-8.

- MacMicking J, Xie QW, and Nathan C (1997) Nitric oxide and macrophage function. *Annu Rev Immunol* **15:**323–350.

- Magnani, M.; Balestra, E.; Fraternale, A.; Aquaro, S.; Paiardini, M.; Cervasi, B.; Casabianca, A.; Garaci, E.; Perno, C.F. (2003). Drug-loaded red blood cell-mediated clearance of HIV-1 macrophage reservoir by selective inhibition of STAT1 expression. *J Leukoc Biol* **74**(5): 764-771

- Mao, X., Ren, Z., Parker, G.N., Sondermann, H., Pastorello, M.A., Wang, W., McMurray, J.S., Demeler, B., Darnell, J.E., Jr., and Chen, X. (2005). Structural bases of unphosphorylated STAT1 association and receptor binding. *Mol. Cell 17*, 761–771.

- Mariotto, S.; Menegazzi, M.; Suzuki, H. (2004) Biochemical aspects of nitric oxide. *Curr Pharm Des* **10**(14): 1627-1645.

- Mazzarella, G.; MacDonald, T.T.; Salvati, V.M.; Mulligan, P.; Pasquale, L.; Stefanile, R.; Lionetti, P.; Auricchio, S.; Pallone, F.; Troncone, R.; Monteleone, G. (2003). Constitutive activation of the signal transducer and activator of transcription pathway in celiac disease lesions. *Am J Pathol* **162**(6): 1845-1855.

- McBride, K.M., McDonald, C., Reich, N.C., (2000). Nuclear export signal located within theDNA-binding domain of the STAT1transcription factor. *EMBO J*. 19, 6196–6206.

- Menegazzi M, Tedeschi E, Dussin D, De Prati AC, Cavalieri E, Mariotto S, and Suzuki H (2001) Anti-interferon gamma action of epigallocatechin-3-gallate mediated by specific inhibition of STAT1 activation. *FASEB J* **15:**1309–1311.

- Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., et al. (1996)

Targeted disruption of the STAT1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84**, 431–442

- Meyer, T., and Vinkemeier, U. (2004). Nucleocytoplasmic shuttling of STAT transcription factors. *Eur. J. Biochem. 271*, 4606–4612.

- Meyer-Kirchrath, J. and K. Schror (2000). Cyclooxygenase-2 inhibition and side-effects of non-steroidal anti-inflammatory drugs in the gastrointestinal tract. *Curr Med Chem* **7**(11): 1121-1129.

- Miller, E.D.; Smith, J.A.; Lichtinger, M.; Wang, L.; Su, L. (2003). Activation of the signal transducer and activator of transcription 1 signaling pathway in thymocytes from HIV-1-infected human thymus. *AIDS* **17**(9): 1269-1277.

- Miwa, M., Stuehr, D.J., Marletta, M.A., Wishnok, J.S., and Tannenbaum, S.R. (1987) Nitrosation of amines by stimulated macrophages. *Carcinogenesis* **8**, 955–958

- Moncada, S. (1992). Nitric oxide gas: mediator, modulator, and pathophysiologic entity. *J Lab Clin Med* **120**(2): 187-191.

- Monteleone, I.; Monteleone, G.; Del Vecchio Blanco, G., Valvassori, P.; Cucchiara, S.; MacDonald, T.T.; Pallone, F. (2004). Regulation of the T helper cell type 1 transcription factor T-bet in coeliac disease mucosa. *Gut* **53**(8): 1090-1095.

- Morris GM, Goodcell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ (1998) Automated Docking Using a Lamarckian Genetic Algorithm and Empirical Binding Free Energy Function. *J Comput Chem* **19:** 1639-1622.

- Mukhtar, H.; Ahmad, N. Cancer Chemoprevention: Future Holds in Multiple Agents. *Toxicol. Appl. Pharmacol. (*1999), *158*, 207-210.

- Nguyen H, Ramana CV, Bayes J, Stark GR. (2001).Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression *J Biol Chem* 276(36):33361-8.

- Okabe S., Suganuma M., Hayashi M., Sueoka E., Komori A. and Fujiki H. (1997) Mechanism of growth inhibition by tea polyphenols on human luna cancer cell line, PC-9. Jpn. J. *Cancer Res*., 88, 639-643.

- Osborn L, Kunkel S and Nabel G J (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc Natl Acad Sci U.S.A*. **86** : 2336-2340.

- Pawate S, Shen Q, Fan F, Bhat NR (2004) Redox regulation of glial inflammatory responses to lipopolysaccharide and interferon gamma. *J Neurosci Res* 15:540–551.

- Pedretti A, Villa L and Vistoli G (2004) VEGA--an open platform to develop chemo-bio-informatics applications, using plug-in architecture and script programming. *J Comput Aided Mol Des* **18**: 167-73.

- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC and Ferrin TE (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**: 1605-12.

- Pointer ME, Daynes RA (1999) Age-associated alterations in splenic iNOS regulation: influence of constutively expressed IFN-gamma and correction following Oupplementation with PPARalpha activators or vitamin E. *Cell Immunol* 1:127–136.

- Quarcoo, D.; Weixler, S.; Groneberg, D.; Joachim, R.; Ahrens, B.; Wagner, A.H.; Hecker, M.; Hamelmann, E. (2004) Inhibition of signal transducer and activator of transcription 1 attenuates allergen-induced airway inflammation and hyperreactivity. *J Allergy Clin Immunol* **114**(2): 288-295.

- Rane SG and Reddy EP (2000) Janus kinases: components of multiple signaling pathways. *Oncogene* **19**: 5662-5679.

- Remy, I., Wilson, I.A., Michnick, S.W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. *Science* 283, 990–993.

- Rohn, W. M., Y.J. Lee, and E. N. Benveniste. 1996. Regulation of class II MHC expression. *Crit. Rev. Immunol. 16:311*

- Sampath, D., Castro, M., Look, D.C., and Holtzman, M.J. (1999) Constitutive activation of an epithelial signal transducer and activation of transcription (STAT) pathway in asthma. *J. Clin. Invest.* **103**, 1353–1361.

- Schindler, C., K. Shuai, et al. (1992). Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* **257**(5071): 809-813.

- Schreiber, S.; Rosenstiel, P.; Hampe, J.; Nikolaus, S.; Groessner, B.; Schottelius, A.; Kuhbacher, T.; Hamling, J.; Folsch, U.R.; Seegert, D. (2002). Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. *Gut* **51**(3): 379-385.

- Schroder K, Hertzog PJ, Ravasi T and Hume DA (2004) Interferon-γ: an overview of signals, mechanisms and functions. *J Leukocyte Biology* **75**: 163-180.

- Serafini M, Ghiselli A, Ferro-Luzzi A (1996) In vivo antioxidant effect of green and black tea in man. *Eur J Clin Nutr* 50(1):28–32.

- Seidel, H.M., Lamb, P., and Rosen, J. (2000) Pharmaceutical intervention in the JAK/STAT signaling pathway. *Oncogene* **19**, 2645–2656.

- Shuai, K. (2000). Modulation of STAT signaling by STAT-interacting proteins. *Oncogene* 19, 2638–2644.

- Shuai, K., Horvath, C.M., Huang, L.H., Qureshi, S.A., Cowburn, D., Darnell Jr, J.E., (1994). Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* **76**(5): 821-828.

- Shuai, K., and Liu, B. (2003). Regulation of JAK-STAT signalling in the immune system. *Nat. Rev. Immunol. 3*, 900–911.

- Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, et al (1997). A family of cytokine-inducible inhibitors of signalling. *Nature*;387:917-21.

- Stephanou, A.; Latchman, D.S. (2003). STAT-1: a novel regulator of apoptosis. *Int J Exp Pathol* **84**(6): 239-244

- Steimle, V., C. A. Siegrist, A. Mottet, B. Lisowska-Grospierre, and B. Mach. (1994). Regulation of MHC class II expression by interferon-γmediated by the transactivator gene CIITA. *Science 265:106*.

- Strehlow, I., Schindler, C. (1998). Amino terminal signal transducer and activator of transcription (STAT) domains regulate nuclear translocation and STAT deactivation. *J. Biol. Chem*. 273, 28049–28056.

- Suk, K.; Kim, S.; Kim, Y.H.; Kim, K.A.; Chang, I.; Yagita, H.; Shong, M.; Lee, M.S. (2001). IFN-gamma/TNF-alpha synergism as the final effector in

autoimmune diabetes: a key role for STAT1/IFN regulatory factor-1 pathway in pancreatic beta cell death. *J Immunol* **166**(7): 4481-4489.

- Sung H, Nah J, Chun S, Park H, Yang SE, Min WK (2000) In vivo antioxidant effect of green tea. *Eur J Clin Nutr* 54(7):527–529.

- Takagi, Y.; Harada, J.; Chiarugi, A.; Moskowitz, M.A. (2002). STAT1 is activated in neurons after ischemia and contributes to ischemic brain injury. *J Cereb Blood Flow Metab* **22**(11): 1311-1318.

- Van den Elsen, P. J., S. J. P. Gobin, M. C. J. A. van Eggermond, and A. Peijnenburg. 1998. Regulation of MHC class I and II gene transcription: differences and similarities. *Immunogenetics 48:208.*

- Van der Wal, A. C., Das, P. K., Tigges, A. J., and Becker, A. E. (1992) Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions *Am. J. Pathol.* **141,** 1427–1433

- Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs." *Nat New Biol* **231**(25): 232-235.

- Vane, J.R.; Botting, R.M. (**2003)** The mechanism of action of aspirin. *Thromb Res* **110**(5-6): 255-258.

- Van Straaten EA, Koster-Kamphuis L, Bovee-Oudenhoven IM, van der Meer R, and Forget PP (1999) Increased urinary nitric oxide oxidation products in children with active coeliac disease. *Acta Paediatr* **88:**528–531.

- Wagner, B.J., Hayes, T.E., Hoban, C.J., and Cochran, B.H. (1990) The SIF binding elementconfers sis/PDGF inducibility onto the c-fos promoter. *EMBO J*. **9**, 4477–4484.

- Wang, X.Q.; Panousis, C.G.; Evans, G.F.; Zuckerman, S.H. (2002). Interferon-gamma-mediated downregulation of cholesterol efflux and ABC1 expression is by the Stat1 pathway. *Arterioscler Thromb Vasc Biol* 22(5): e5-9.

- West, D.A.; Valentim, L.M.; Lythgoe, M.F.; Stephanou, A.; Proctor, E.; van der Weerd, L.; Ordidge, R.J.; Latchman, D.S.; Gadian, D.G. (2004). MR image-guided investigation of regional signal transducers and activators of transcription-1 activation in a rat model of focal cerebral ischemia. *Neuroscience* **127**(2): 333-339.

- Witthun BA, Silvenoinrn O, Miura O, Lai KS, Cwik C, Liu ETIhle JN (1994) Involvment of Jak3 Janus kinase in signaling by interleukins 2 and 4 inlymphoid and myeloid cells. *Nature* **370**: 153-57.

- Woods, M.; Wood, E.G.; Bardswell, S.C.; Bishop-Bailey, D., Barker, S.; Wort, S.J.; Mitchell, J.A.; Warner, T.D. (2003). Role for nuclear factor-kappaB and signal transducer and activator of transcription 1/interferon regulatory factor-1 in cytokine-induced endothelin-1 release in human vascular smooth muscle cells. *Mol Pharmacol* **64**(4): 923-931

- Xu, X., Sun, Y.L., Hoey, T., Cooperative, D.N.A. (1996). Binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science* 273, 794–797.

- Yao Y, Hausding M, Erkel G, Anke T, Forstermann U, and Kleinert H (2003) Sporogen, S14–95 and S-curvularin, three inhibitors of human inducible nitricoxide synthase expression isolated from fungi. *Mol Pharmacol* **63:**383–391.

- Yokota, A.; Narazaki, M.; Shima, Y.; Murata, N.; Tanaka, T.; Suemura, M.; Yoshizaki, K.; Fujiwara, H.; Tsuyuguchi, I.; Kishimoto, T. (2001). Preferential and persistent activation of the STAT1 pathway in rheumatoid synovial fluid cells. *J Rheumatol* **28**(9): 1952-1959.

- Zaveri NT. Synthesis of a 3,4,5-trimethoxybenzoyl ester analogue of epigallocatechin-3-gallate (EGCG): a potential route to the natural product green tea catechin, EGCG. *Org Lett.*;3(6):843-6.

- Zhang, T., Kee, W.H., Seow, K.T., Fung, W., Cao, X. (2000). The coiledcoil domain of Stat3 is essential for its SH2 domain-mediated receptor binding and subsequent activation induced by epidermal growth factor and interleukin-6. Mol. *Cell. Biol*. 20, 7132–7139.

- Zhang, J.G., Farley, A., Nicholson, S.E., Willson, T.A., Zugaro, L.M., Simpson, R.J., Moritz, R.L., Cary, D., Richardson, R., Hausmann, G., Kile, B.J., Kent, S.B., Alexander, W.S., Metcalf, D., Hilton, D.J., Nicola, N.A., Baca, M. (1999a). The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc. Natl. Acad. Sci*. USA 96, 2071–2076.

- Zhu, M.-H., John, S., Berg, M., Leonard, W.J., (1999). Functional association of Nmi with Stat5 and Stat1 in IL-2- and IFNgamma-mediated signaling. *Cell* **96**(1): 121-130.

- Zingarelli, B.; Sheehan, M.; Wong, H.R. (2003) Nuclear factor-kappaB as a therapeutic target in critical care medicine. *Crit Care Med* 31(1 Suppl): S105-111.