ASSAYS FOR IDENTIFYING EFFECTORS OF THE INTERFERONS AND VIRAL INTERFERON-ANTAGONISTS

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ABSTRACT

Interferons (IFNs) are powerful cytokines synthesized and secreted by somatic cells of all mammalian species. They represent a large family of multifunctional proteins involved in antiviral defence, cell growth regulation and immune activation. However, aberrant IFN secretion has been implicated in a wide range of auto-immune diseases. Contrarily, in order to establish their infections in vivo most viruses must be able to circumvent the protective effect of the IFNs. Presented here, are approaches to high throughput screening aiming to the identification of molecular-inhibitors of both the IFN signalling response and the viral-antagonists of the same response. The strategies discussed in the following chapters are based on reporter assays quantifying IFN-induced expression of luciferase and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) genes. An HGPRT-reporter based assay was developed and tested against a library of compounds leading to the identification of para-nitrophenol, a suspected inhibitor of IFN-signalling. However the most attractive system proved to be a luciferase reporter- A549L cell- based approach to HTS. The latter was developed and extensively characterized presenting an attractive strategy to anti-IFN and antiviral compound identification. Results obtained through application of this system suggest another inhibitor of the IFN signalling cascade, curcumin. Curcumin was observed to interfere with IFN-induced activation of interferon signalling response elements and the related antiviral response as confirmed by virus-titre reduction (VTR) assays. In addition to the identification of compounds with anti-IFN properties a number of viral antagonists were delivered to reporter cells in order to establish the principles behind a HTS approach for ascertaining inhibitors of viral IFN-antagonism. Recovery of reporter activity in cells pretreated with IFN would suggest inhibition of the antagonist by the assayed compound.

Declarations

(i) I, Michael T. Valachas, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for higher degree.

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(ii) I was admitted as a research student in October 2002 as a candidate for the degree of Doctor of Philosophy in Molecular Virology; the higher study for which this is a record was carried out in the Faculty of Sciences at the University of St. Andrews between 2002 and 2006.

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(iii) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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ABBREVIATIONS

Units & Measurements

Da	Dalton
g	gram
hr	hour
l	litre
m	metre
min	minute
sec	second

Metric Prefixes

Kilo	10^{3}	K
Milli	10^{-3}	m
Micro	10^{-6}	μ
Nano	10^{-9}	n
Pico	10^{-12}	P

Proteins

ADAR	dsRNA-dependent adenosine diaminase
Bcl	B-cell leukemia
CaMK	Ca(2+)/calmodulin-dependent kinase
CPSF	Cleavage and Polyadenylation Specificity Factor
CREB	cAMP Response Element-Binding
CrkL	v-Crk avian sarcoma virus CT10 oncogene homolog-Like
cul4a	cullin 4a
DDB	DNA Damage Binding
eIF	eukaryotic Initiation Factor
FADD	Fas Associated protein withDeath Domain
FasL	Fas Ligand
GADD	Growth Arrest and DNA Damage
HGPRT	Hypoxanthine-Guanine PhosphoRibosylTransferase
HRP	HorseRadish Peroxidase
IFN	Interferon
IFNAR	IFN-Alpha Receptor
IFNGR	IFN-Gamma Receptor
IKK	Inhibitor of Kappa-b Kinase
IL	Interleukin
iNOS	inducible Nitric OxideSynthase
IPS	IFNβ-Promoter-Stimulator
IRAK	InteRleukin-Associated Kinase
IRF	Interferon Regulatory Factor
IRS	Insulin Receptor Substrate
ISGF	IFN Stimulated Gene Factor
JAK	Janus-Activaed Kinase
JNK	Jun N-terminal Kinase
MAP	Mitogen-Activated Protein
MAPK	MAP Kinase
MHC	Major Histocompatibility Complex
MKK	MAP Kinase Kinase
MyD88	Myeloid differentiation primary response gene 88
NAP	Nucleosome Assembly Protein
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor kB
NS	Non-Structural
OAS	OligoAdenylate Sunthetase
PABII	Poly(A)-Binding protein
peg.IFN	Pegylated IFN
PI	Phosphatidyl Inositol
PKR	Protein Kinase R
RACK	Receptor for Activated C Kinase
RIGI	Retinoic-acid-Inducible Gene I
RIP	Receptor-Interacting Protein
RNAP	RNA Polymerase
RTA	Replication and Transcription Activator
STAT	Signalling Transductors and Activators of Transcription
TAP	Transporter Associated with Antigen Processing
TBK	TANK-Binding Kinase
TBP	TATA Binding Protein
TLR	Toll-Like Receptor
TRAF	TNF Receptor-Associated Factor
TRIF	TIR domain-containing adapter inducing IFNβ
Tyk	Tyrosine kinase
UBCH	UBiquitin-Conjugating enzyme

Nucleic Acids

ds	Double stranded
ss	Single stranded
DNA	DeoxyRibonucleic Acid
RNA	RiboNucleic Acid
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
m-	Messenger
t-	Transport
Met-	Methylated

Viruses

Ade	Adenovirus
CMV	CytoMegaloVirus
EMCV	EncephaloMyocarditis Virus
HBV	Hepatitis B Virus
HHV	Human Herpes Virus
HIV	Human Immunodeficiency Virus
HPIV	Human ParaInfluenza Virus
HPV	Human Papilloma Virus
MeV	Measles Virus
MuV	Mumps Virus
PV	Polio Virus
RSV	Respiratory Syncytial Virus
SV	Simian Virus
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus
WNV	West Nile Virus

Syndromes & Conditions

AIDS	Acquired ImmunoDeficiency Syndrom
AITD	Autoimmune Thyroid Disease
BSE	Bovine Spongiform Encephalopathy
COPD	Cronic Obstructive Pulmonary Disorder
HAART	Highly Active Anti-Retroviral Treatment
IDDM	Insulin Dependent Diabetes Mellitus
RBM	RNA Binding Motif
SARS	Severe Acute Respiratory Syndrome
SLE	Systemic Lupus Erythematosus
Xe.P	Xeroderma Pigmentosum

Reagents

6TG	6- ThioGuanine
6TGMP	6-thioGuanilyic acid Mono-Phosphate
ATP	Adenosine Tri-Phosphate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	DimMethyl SulphOxide
FCF	Fetal Calf Serum
GDP	Guanosine Di-Phosphate
GTP	Guanosine Tri-Phosphate
MAPK	MAP Kinase
ODN	OligoDeoxyNucleotide
PMA	Phorbol Myristate Acetate
PNP	Para-NitroPhenol
PTB	PBS-Tween buffer

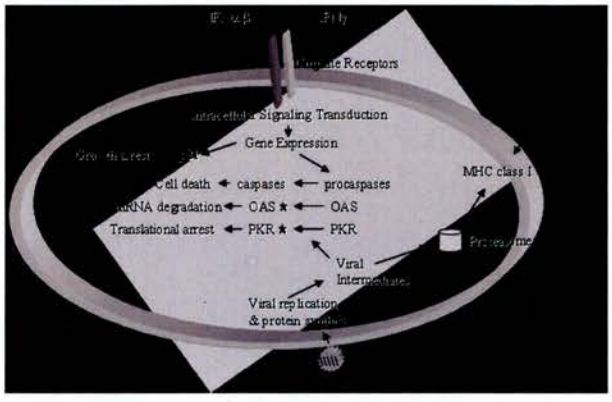
Miscellaneous

C.P.E.	CytoPathic Effect
CARD	CAspase Recruitment Domains
C-terminal	Carboxy- terminal
ELISA	Enzyme Linked ImmunoSorbent Assay
HAART	Highly Active Anti-Retroviral Treatment
HTS	High Throughput Screening
IRS	Insulin Receptor Substrate
ISC	Interferon Secreting Cells
ISG	Interferon Stimulated Gene
ISRE	Interferon Signalling Response Element
JH	Jak Homology
m.o.i	multiplicity of infection
MRSA	Methicillin Resistant Stapylococcus Aureus
NIH	National Institute of Health
NK	Natural Killer
N-terminal	Amino- terminal
ORF	Open Reading Frame
VTR	Virus Titre Reduction

CONTENTS

PART 1:

INTRODUCTION



History can be very unkind to bold statements. On December 1967 the U.S. Surgeon General Dr. William H. Stewart proudly proclaimed that we could close the book on infectious diseases (Stewart,1967). This statement, however uneducated it may sound, followed a trend of optimism, characteristic of an age and setting that had been successful in combating malaria, diphtheria and was within grasp of obliterating typhoid, tuberculosis, and bubonic plague. Since then the emergence of pathogens such as Ebola, HIV and countless others has not only "reopened" that book but has also edited a novel chapter. The excitement on the conquest over diseases such as smallpox and polio, to place the topic in a virological context, has now been overshadowed by the annual estimate of 2.77 million deaths due to HIV/AIDS (Morens, Folkers et al., 2004). Many factors have contributed to the rejuvenation of infectious diseases. Global transport, as the 2004 SARS outbreak very well illustrated, the increasing use of antimicrobials leading to the emergence of aptly named "superbugs" like MRSA, relaxation in

precautionary measures by the population and the growing industrialization of food production (the B.S.E. crisis) only offer a limited insight as to the causes of this rapid explosion of infectious diseases.

Along with rethinking measures insuring public health (too often sacrificed on the altar of short term profit) it is pivotal to ensure that the interdisciplinary research (involving but not limited to biomolecular, medical and biomedical sciences) continues to grow and suggest solutions for this evolutionary race.

Following on the foot steps of the early exceptional promises of using the body's own immune mechanisms to combat viral diseases, in this thesis I suggest, analyze and compare different approaches within the context of the Interferon Signaling Cascade and its related responses for the identification and development of novel antiviral and virological agents.

Chapter 1.1

The Interferons

1.1.1 Overview of the Interferons

1.1.1.1 Discovery of interferons

The phenomenon of viral interference, whereby infection by one virus impedes infection by another, had been known for many years prior to the 1957 experiments by Isaacs and Lindenmann that led to the identification of the cell-secreted substance responsible for this observation. Isaacs and Lindenmann, filtering out the virus, applied the medium of influenza infected chick cells to a fresh layer of cells and noted that the new layer was resistant to subsequent influenza superinfection (Lindenmann and Isaacs,1957). The pair deduced that a cell- secreted substance was responsible for this immunity and termed it as interferon (IFN). Over the following decades the importance of this novel substance would be comprehended and extensive studies for the characterization and elucidation of the mechanism of action of the interferons would be launched.

1.1.2.2 Nature of interferons

It is now understood that the material Isaacs and Lindenmann identified as IFN is a moiety within a wider family of cytokines collectively termed IFNs. These are categorized into two groups according to their structure, transcription patterns and receptor specificity (Stark et al., 1998). Type I interferons include IFNα, IFNβ, *IFNδ*, IFNε, IFNκ, *IFNτ*, IFNω, the four IFN-like cytokines IL28A, IL28B, IL-29 (conjointly termed as IFNλ)[1] and *limitin* (italics denote non-human IFNs). IFNγ is the only IFN belonging to the Type II family. The two types share no obvious structural homology yet they are linked by their related modes of action, induced genes and by, arguably, their most important attribute, the establishment of an

[1] A recent trend exists in classifying members of the IFNλ subfamily as Type III IFNs due to their cognate receptor binding apparatus being distinct from other members of IFNα family (Bartlett et al., 2005)

"anti-viral state" in the periphery of infection (Goodbourn et al., 2000). IFNs have additionally been implicated in oncogenesis and bone metabolism (Takaoka and Taninguchi, 2003). A summary of the biological properties of the IFNs appears in Figure 1.1.

IFNs α/β are produced within hours after infection by most cell types with plasmacytoid along with other dendritic cells (DC) being the predominant producer of the α subtypes[2] and fibroblasts for β. IFNγ is specific only to immune cells (T-lymphocytes being the main producer) with its main functional role being the immune modulation of T and Natural Killer (NK) cells. Interestingly, plasmacytoid dendritic cells (PDCs) can generate a billion IFNα molecules in a twelve hour period.

Upon viral infection, production of the IFN cytokines occurs en masse to limit the spread of virus and buy time for the adaptive immune system to mount an efficient response and clear the infection. The importance of the IFNs in innate immunity against viruses can be demonstrated in two ways. Primarily, knockout mice that do not express receptors for IFNα/β or IFNγ (although being capable of eliciting an adaptive response) are unable to effectively combat viral infections (reviewed in Goodbourn et al., 2000). Similarly a genetic defect in the IFN system (STAT1 deficiency) in humans is fatal at a young age on viral challenge (Dupuis et al., 2003). Secondarily most viruses through constant evolutionary pressure have found ways (although seldomly absolute) to circumvent the IFN response (reviewed in Weber et al., 2004) in order to establish the infection.

[2] historically these cells have been also addressed as Interferon Secreting Cells, (ISC)

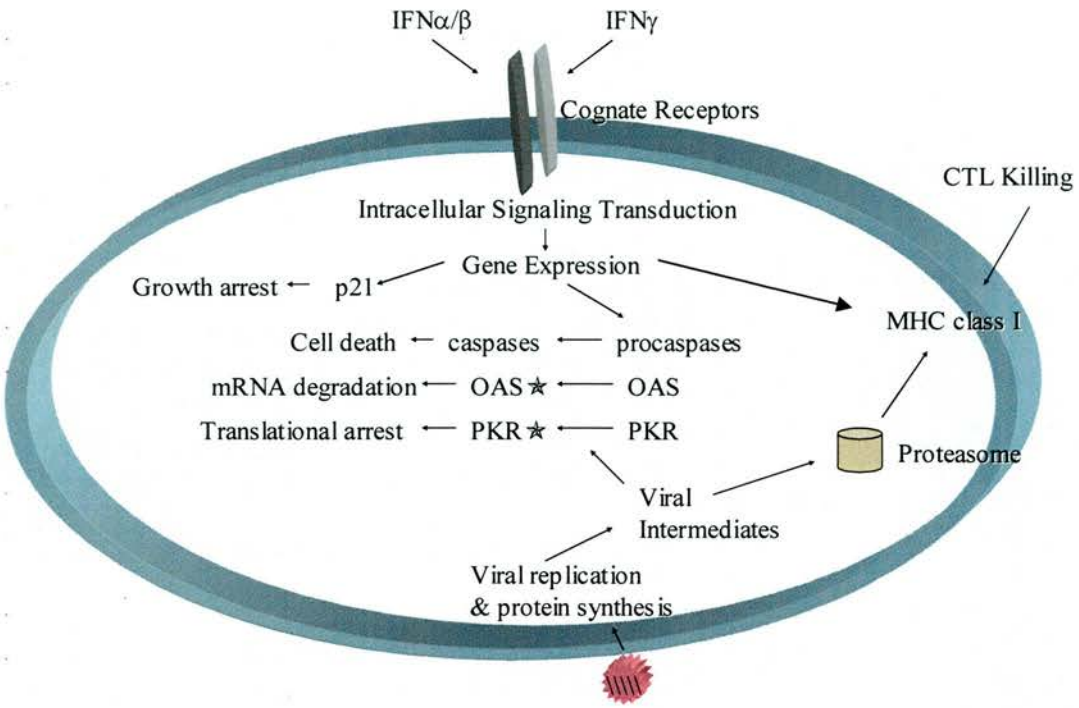


Figure 1.1: Biological Properties of the Interferons. IFNs regulate a number of cellular responses critically affecting the course of viral infection. Upon receptor binding and through signaling cascades the interferons stimulate transcriptional induction of genes involved in the antiviral response. Expression of inactive precursor molecules establishes an antiviral state within the cell preparing it for viral encounter. Activation of these precursor molecules often depends on the presence of a viral co-factor (often as in the case of the IFN inducible enzymes PKR and OAS, this co-factor is dsRNA, present as an intermediate of viral replication within the cell), ensuring inactivity within uninfected cells. IFNs can induce gene expression of proteins pivotal in both translational/transcriptional regulation and cell-cycle progression. Furthermore IFNs enhance expression of MHC class I molecules and profoundly affect presentation pathways. These measures are taken by cells in order to limit the infection and inform the periphery of their status. Details of the signaling pathways and the nature of the antiviral response are discussed in the text. Adapted from Goudbourn et al., 2000

1.1.2 Induction of the Interferons

In nature, interferon is produced upon viral infection by the detection of viral components within the cell or through physiological changes in the cellular environment. Similarly, in a laboratory environment IFN can be produced by incubating appropriate cells with a dsRNA inducer. The inducer varies in accordance to the interferon and cell type with species specificity.

Presented in this section are the mechanisms underlying IFN production. An overview of the extensively understood cascades behind IFNβ production (subsections 1.1.2.1/2/3) will be followed by discussing the mechanisms involved in IFNα induction (1.1.2.4). Finally, subsection 1.1.2.5 will address the production of IFNγ.

1.1.2.1 Induction of IFNβ

DsRNA, either being the viral genome itself or a by-product of viral nucleic acid metabolism (Jacobs, Langland, 1996), is generally accepted as a major stimulant of IFNβ production. DsRNA is an activator of protein kinase R (PKR), a serine threonine kinase belonging to a family of dsRNA binding kinases with a 65-68 amino acid binding sequence. Upon dsRNA binding PKR autophosphorylates (Galabru and Hovanessian, 1987) (Meurs et al., 1990) and through this event, activates the IKKβ subunit of the IκB kinase complex (Maran et al., 1994, Yang et al., 1995). In turn, the IκB kinase phosphorylates IκB. IκB functions as an inhibiting molecule associated and bound to the transcription factor NFκB in the cellular cytoplasm preventing the nuclear translocation of NFκB and thus expression of NFκB-stimulated genes. Phosphorylated IκB is targeted for ubiquitination and proteosomal degradation by an E3 ubiquitin ligase. Dissociation from IκB releases NFκB, which translocates to the nucleus. Meanwhile the Interferon Regulatory Factor (IRF) 3 is phosphorylated by virus-activated protein kinases namely IKKε and TBK1 (Sharma et al., 2003, Fitzgerald

et al., 2003) that have already been stimulated through[3] an RNA helicase: the retinoic-acid-inducible gene I (RIGI), or alternatively by Toll-like Receptors (TLRs) (Beutler, 2004). The phosphorylated IRF3 homodimerises and enters the nucleus where along with NFκB and other coactivators including p300 CREB-binding protein[4] and several copies of the HMG-I/Y accessory factor forms a polyfactorial transcription complex termed the "enhanceosome" (reviewed in Thanos, 1996) which binds the IFNβ promoter region and initiates the synthesis of mRNA.

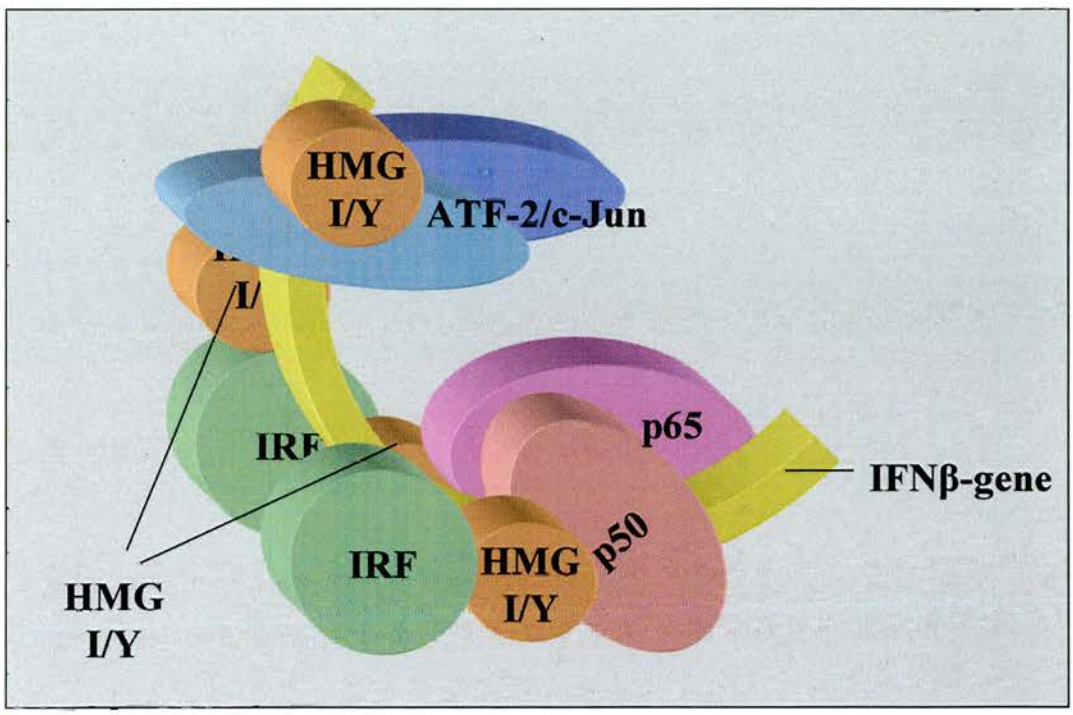


Figure 1.2 Enhanseosome formation model at the IFNβ promoter. The transcriptional activation of the IFNβ gene is dependent on the assembly of a higher order transcription complex, the enhanseosome. The high mobility group proteins (HMG) I/Y facilitate the binding of the ATF-2/c-jun factors, the p50/p65 subunits of NFκB and the IRF transcription factors. All proteins bind positive regulatory domains (PRD) on the IFNβ promoter facilitating transcriptional activation.

[3] a detailed overview exists in 1.1.2.2/1.1.2.3
[4] otherwise a protein of the "housekeeping" transcription complex.

1.1.2.2 ds-RNA signalling through Toll-like receptors

Toll-like receptors (TLRs) are type I transmembrane signalling molecules responding with high specificity to pathogen-associated ligands (reviewed in Medzitov, 2001). These can be lipids, proteins, carbohydrates and nucleic acids of invading microorganisms. The binding process triggers intracellular signalling cascades leading to inflammatory and immune responses against the invader. Although several molecules of the 11 member strong TLR family have been implicated in the antiviral response[5], TLR3, functioning as a cell surface dsRNA binding entity has been demonstrated to promote NFκB and IRF3 activation ultimately leading to IFNβ production (Alexopoulou, 2001). TLR3 through its critical adaptor protein TRIF can trigger at least four signalling pathways NFκB, IRF3 and the stress-activated pathways involving p38 and the c-Jun N-terminal kinases (JNK). Mice deficient in TRIF are totally defective in TLR3 signalling (Yamamoto et al., 2003). TRIF can stimulate IFN production in two distinct manners. In the first case TRIF recruits through association with NAP1[6], the protein kinase TBK1 and its relative IKKε that catalyze the phosphorylation and subsequent nuclear translocation of transcription factors IRF3 and IRF7. In the second model, TRIF assembles the adaptor molecule TRAF6 and receptor-interacting protein 1 (RIP1) leading to activation of the IKK complex and MAP kinases (Jiang et al., 2004). The activation of the IKK complex as seen earlier is able to promote IFNβ production; however TLR3 activation of IRFs 3/7 can lead to transcription of interferon signalling response element (ISRE) sequences[7] and thus the amplification of IFNβ production.

1.1.2.3 Role of RNA helicases in IFNβ production

Independently of TLR signalling, two cytoplasmic RNA helicases RIGI and the melanoma differentiation associated gene 5 (mda5) (belonging to the DExH/D protein family) have been implicated in interferonal production. Both molecules contain helicase domains responsible for dsRNA binding. Another shared feature

[5]The roles of two other TLRs, 7 and 9 will be addressed below in the context of IFNα-production.
[6] NAK-associated protein 1
[7] IFNα/β-inducible promoter elements of "antiviral" genes (discussed below in 1.1.3.2).

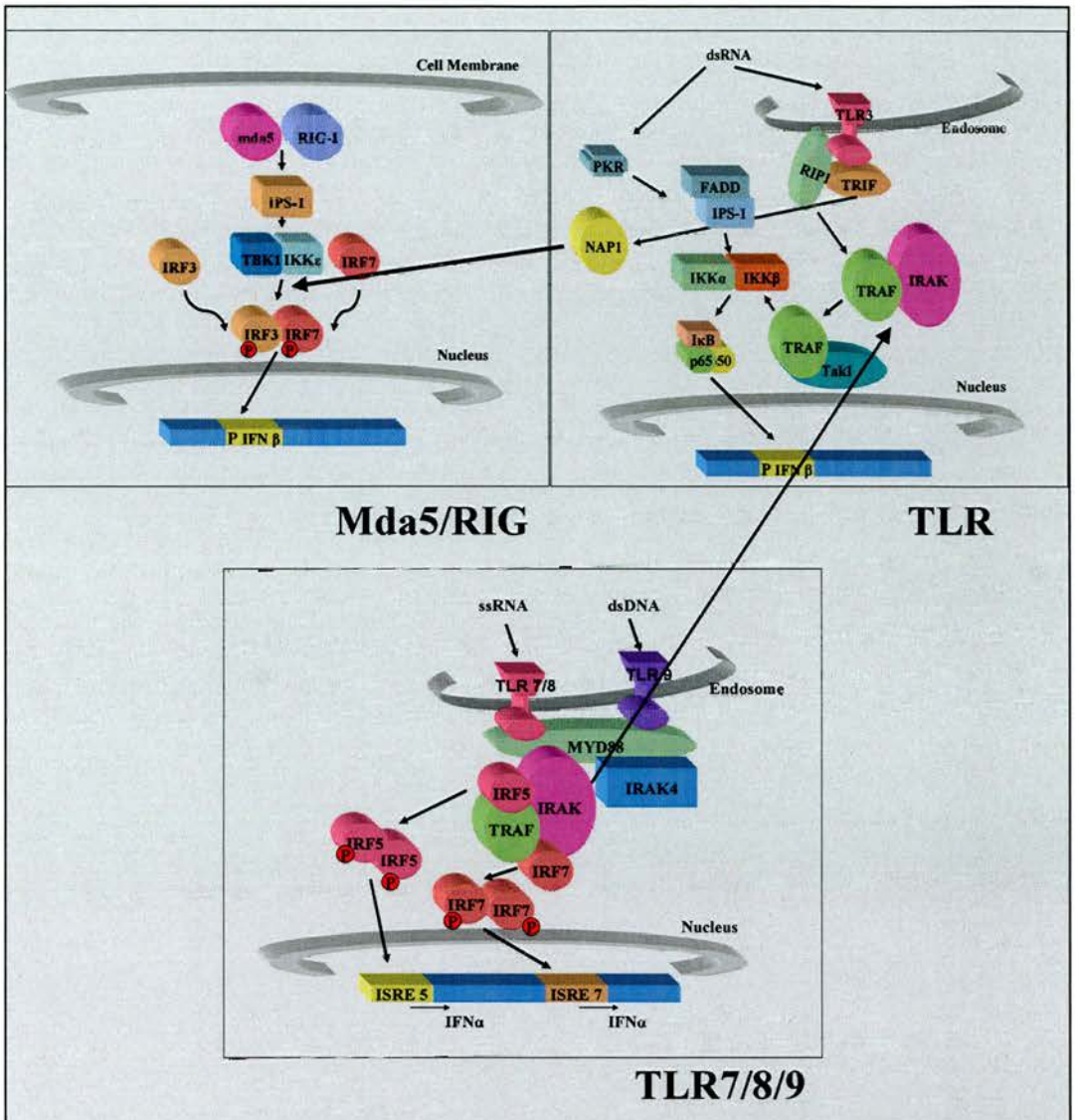


Figure 1.3: Schematic of IFN-induction pathways. The figure demonstrates the interactions leading to and crossregulation of type I IFNs discussed in the text. Presented clockwise: the mda5/RIGI pathway, the TLR3 dependent pathway and finally the TLR7/8/9 pathway for the induction of IFNα.

Transcriptional induction of the interferons relies on the presentation of ligands to cognate receptor molecules, like RIG I or PKR, or traditional receptors, like Toll Like Receptors. The process of viral replication proceeds with the production of viral intermediates or end products that can activate the appropriate receptors triggering their corresponding signaling cascades. and several copies of the HMG-I/Y accessory factor. For details to the upstream signaling cascades for transcription of the type I IFN genes refer to the text.

of these helicases is the existence of caspase-recruiting domains (CARD). CARDs are required for the initiation of the downstream signalling cascade leading to the activation of IRFs3/7 and NFκB. It is interesting to note that CARDs are highly unusual within the DExH/D family of proteins and RIGI and mda5 are the only identified members that contain such domains (Kang et al., 2002).

Both pathways (TLR3-dependent and TLR3-independent) converge at the level of TBK1/IKKε. However, RIGI/mda5 signalling requires the IFNβ-promoter-stimulator 1 (IPS1) (Kawai et al., 2005), an up to recently unidentified adapter protein. IPS1 contains an N-terminal CARD-like domain that can associate with RIG-I and mda5 and a C-terminal domain that has been reported to recruit RIP1 and Fas-associated death domain (FADD) protein leading besides the TBK1-IKKε-dependent dimerisation of IRFs to NFκB dependent gene transcription (Kawai et al., 2005).

1.1.2.4 Induction of IFNα genes

Production of IFNα from natural sources is induced by viral infection (for example, Sendai virus) of fibroblasts and leukocytes, each through distinct mechanisms. In contrast to IFNβ, which is encoded by a single gene, IFNα is derived from a diverse collection of related genes. The presence of IFNβ in the medium is required for IFNα production in fibroblast cells while this is not the case in leukocytes (Erlandsson, 1998). Although IFNβ dependent IRF7 synthesis can lead to the transcription of IFNα genes (Marie et al., 1998), the pathway for leukocyte IFNα production has not been clearly elucidated. TLR7 and TLR8 have been demonstrated to induce IFNα production by recognizing (in contrast to TLR3) GU-rich single stranded RNA (ssRNA) indicating that dsRNA is not the only physiological inducer of interferon production. Specifically TLR7 and TLR9 can induce IFNα production by respectively recognizing ssRNA and DNA. These TLRs mediate their signals through MyD88, an essential adaptor protein that upon ligand stimulation associates with the Interleukin associated kinases (IRAKs) 1/4, TRAF6 and the interferon regulatory factors 5 and 7 to form a signalling complex that catalyses IFN production by the homodimerisation of the corresponding IRFs.

1.1.2.5 Production of IFNγ

Presentation of mitogen[8] induces IFNγ in Th1 CD4+ helper T lymphocytes and virtually all CD8+ cells. Although being the subject of much research due to its versatility (as to the nature of pathogens it is employed against) and its implication in autoimmune disorders (Bach et al.,1997), the exact mechanism by which the signalling cascade for IFNγ production progresses remains elusive. The mitogen-activated protein kinases (MAPK) p38 and c-jun-N-terminal [kinase] (JNK) have been implicated in this process (Lu et al.,1999).The p38 MAPK family, consisting of four identified members (α,β,γ and δ) with direct homology, represents a very important class of Ser/Thr kinases. MAPKs are activated in response to cellular stress responses (including cytokine stimulation) and are involved in the phosphorylation of numerous transcription factors, apoptosis and, as the case is here, cytokine production. Pyridinyl imidazole compounds (specific inhibitors of the p38 MAPK) have been shown to inhibit induction of IFNγ genes (Rincon et al.,1998). Furthermore mice deficient for MAPK Kinase (MKK) 6, an upstream regulator of p38 MAPK, are unable to mount efficient cell mediated immune responses. The p53-responsive stress protein GADD45 has also been reported as an upstream regulator of the p38 MAPK. Over/under-expression of GADD45 proteins directly corresponds to IFNγ production status (Yang et al., 2001) (Lu et al., 2001).

In addition to MAPK, IL12 has also been shown to regulate IFNγ production. It acts so through STAT4 activation (Sinigaglia et al.,1999). IL12 initiated tyrosine phosphorylation of STAT4 by JAK/Tyk molecules leads to nuclear translocation of the molecule (Bacon et al., 1995). The interaction between STAT4 and Tyk2 is critical for IL12 dependent IFNγ expression (Sugimoto et al.,2003). However, for optimal induction the molecule has to be additionally phosphorylated on a serine residue, a task also involving the GADD45 proteins (Morinobu et al., 2002) in a MKK6/p38 MAPK-dependent manner (Visconti et al., 2000). In the nucleus of T-cells, binding sites for STAT4 and AP1 were discovered on the IFNγ promoter

[8] Mitogen: Proteins that stimulate cells to commence cell division, triggering mitosis.

region which may suggest mechanisms for the IL12 mediated IFNγ in these cells. (Barbulescu et al., 1998).

1.1.3 Interferon Signalling

After IFN has been induced and secreted in the cellular environment it can stimulate other cells driving them into the "antiviral state". In order to do so, cells must take up the cytokine by appropriate receptors on the cell membrane. IFN binding triggers intracellular signalling cascades that ultimately drive the transcription of genes related to the anti viral response. Section 1.1.3 addresses the signalling cascades activated by type I (1.1.3.1/2/3) and type II IFNs (1.1.3.4) binding to their specific cellular receptors.

1.1.3.1 Type I IFN signalling

Type I IFNs are thought to signal in numerous distinct pathways most of which have not been elucidated but all seem to require the presence of a common type I IFN receptor. These cognate receptors which demonstrate species specificity (reviewed in Pestca et al., 2004) are able to bind all subspecies of type I IFNs. Human cells and mainly fibroblasts, which produce the bulk of type I IFNs, are thought to (functionally) induce at least 13 subtypes of IFNα, one IFNβ and a single IFNω (Roberts et al., 1996) all of which bind the human version of the cell-membrane spanning IFNα/β receptor. The dimeric receptor consists of the 110kDa IFNAR1 and the (varying due to alternative splicing from 51kDa for the shortest form IFNAR2b to 91-100kDa for the longer gene products i.e. IFNAR2c) IFNAR2 subunits present two separate ligand binding sites[9] (Mogensen et al., 1999). Both receptors are encoded by genes at chromosome 21 and although there is only one type of IFNAR1, alternative processing of the IFNAR2 gene produces variants of the receptor subunit differing in their cytoplasmic domain length (Prejean and Colamonici, 2000) a fact that may provide insights to the diversity of the response via differential structural conformation of the apparatus (Platanias et al., 1996).

[9] although neither subunit on its own can bind IFNs in the high affinity manner, as such demonstrated by the two receptor apparatus (Cohen et al.,., 1995)

Type I Interferon signalling involves five stages of signalling initiation/continuation. IFN binding and receptor dimerization, initiation of a tyrosine phosphorylation intracellular cascade, STAT dimerization, nuclear translocation and binding to DNA sequences initiating transcription of Interferon stimulated genes (ISGs).

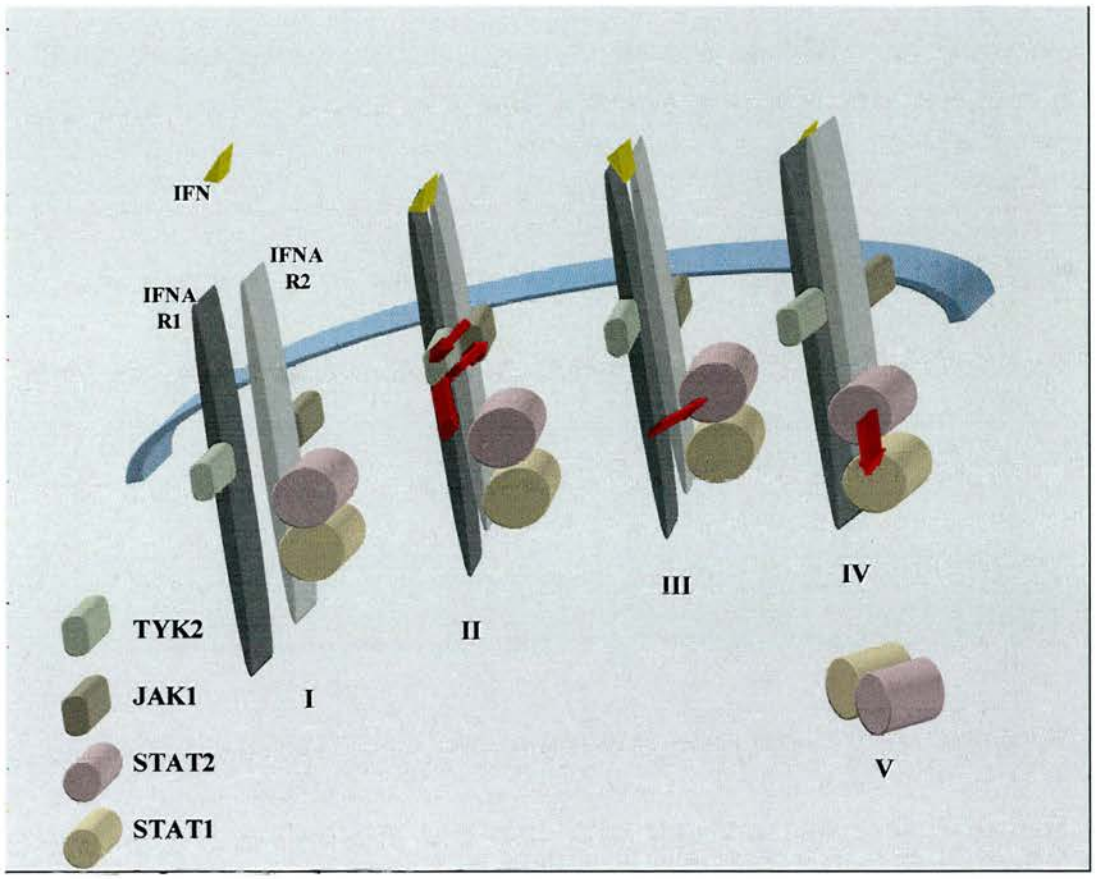


Figure 1.4: Binding of IFN to its receptor and initiation of the IFN signaling cascade. In the unstimulated cell the receptors exist dissociated on the cytoplasmic membrane. I: IFNAR1 is bound by tyk2 while IFNAR2 is complexed with jak1, STAT2 and in its presence, STAT1. II: Upon IFN stimulation the receptors dimerise facilitating the cross-phosphorylation of tyk2 and jak1. jak1 subsequently phosphorylates IFNAR1 on Tyr466. This event creates a docking site for STAT2 through the latter's SH2 domain. III: Through this interaction STAT2 is phosphorylated on Tyr690 in turn creating a docking site for the SH2 domain of STAT1. IV: the subsequent phosphorylation of STAT1 on Tyr701 dimerises the proteins which are subsequently IV: released in the cytoplasm.

1.1.3.2 Janus Kinases (Jaks) and Signalling Transductors and Activators of Transcription (STATs)

The main pathway, and the most understood, for the induction of IFN-stimulated genes (ISGs) requires, besides the two receptor subunits mentioned before, the activation of two members each of the JAK and STAT families and the transcription factor p48, also known as IFN regulatory factor 9 (IRF) (reviewed in Stark et al., 1998). The process possibly involves the presence of a receptor-associated tyrosine phosphatase SHP2 (David et al., 1996).

The JAKs are a family of tyrosine Janus kinases with its four known members being Jak1, Jak2, Jak3 and Tyk2. STAT4 discussed earlier in the context of IFNγ production is one of the seven members of the signal transducer and activator of transcription family collectively termed as STATs. Jak1 and Tyk2 are the Janus kinases that phosphorylate STAT1 and STAT2 during the IFNα/β signalling cascade while Jak1 and Jak2 are the activators of STAT1 for IFNγ signalling transduction which will be discussed below.

Tyk2 and Jak1 are associated with the cytoplasmic domains of IFNAR1 and IFNAR2 respectively (Li et al., 1997). The corresponding STATs in cells untreated with IFN appear to pre-associate with IFNAR2 recruited from their cytosolic latent monomerities with STAT2 appearing to be necessary for the interaction (Li et al., 1997). Upon IFNα/β binding the two subunits of the receptor are assembled (Novick et al., 1994). This event brings Tyk2 and Jak1 into proximity allowing their cross-phosphorylation which in turn induces tyrosine phosphorylation of IFNAR1 at Tyr466 (Colamonici et al.,1994). This phosphorylation leads to STAT2 binding to the receptor via its src homology-2 (SH2) domain (Yan et al.,1996). A further phosphorylation event at Tyr690 of STAT2 recruits and phosporylates, at Tyr701, the SH2 region of STAT1. The phosphoactivated STAT1 and STAT2 moieties then form a heterodimer that dissociates from the receptor complex and is released in the cytoplasm[10].The exact

[10] Heterodimers of these STATs with unknown implications have also been reported in the absence of an IFN stimulus (Stancato et al., 1996).

mechanism for the nuclear translocation of the heterodimer remains elusive. Inside the nucleus STAT1/STAT2 interact with IRF9[11] (Veals et al., 1992) to form the ISGF3 complex and drive transcription of ISGs. These ISGs contain a consensus [GAAAN(N)GAAA][12] sequence promoter element (Goodbourn et al., 2000) termed the IFN signalling response element (ISRE). The p38 MAP kinase (mentioned before in the context of IFN production), although not required for DNA binding by the ISGF3 complex, appears to be essential for IFNα-stimulated transcription of ISREs (Li et al., 2004).

It was recently reported that the human herpes virus 8 (HHV8), the etiological agent for Kaposi's Sarcoma encodes an ISRE binding protein, the replication and transcription activator (RTA) selectively inducing cellular ISGs and regulating the viral K14 and ORF74 open reading frames containing ISRE-like sequences (Zhang et al., 2005). ISGs are induced during the viral lytic phase of HHV8 replication and progression from a latent to a lytic phase is characterized by K14-ORF74 gene expression (Jeong et al., 2001), so interactions between viral proteins and ISREs/ISRE-like sequences may play an important role in cytopathogenic progression. An elaborate account on the antagonism of the IFN related responses by a variety of viruses is presented in Chapter 1.2)

Alternative pathways have also been suggested for IFN type I signalling. Several STAT-complexes have been identified after IFN stimulation of the receptors, resulting from the association of diverse STAT combinations such as STAT1 homodimers, STAT1/STAT3 dimers, STAT3 homodimers and others including STAT5. Interestingly CrkL [13] is tyrosine phosphorylated in an interferon-dependent manner and associates with STAT5 (phosphorylated at Serines 725/730) to drive transcription of ISGs (Uddin et al., 2003). Compared to the familiar ISGF3 complex targeting ISRE sequences, all the "exotic" combinations of STATs, namely STAT 1/1, 1/3, 3/3, 5/5, 1/2 (without IRF9) and CrkL/STAT5 are aimed at ISGs holding GAS elements (GAS will be discussed below as part of the IFN type II signalling transduction cascade).

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[11] IRF9 is otherwise known as p48.
[12] N represents any nucleotide
[13] CrkL is a v-crk sarcoma virus oncogene homologue functioning as an activatator of the RAS and JUN kinase signalling pathways containing one SH2 and two SH3 domains.

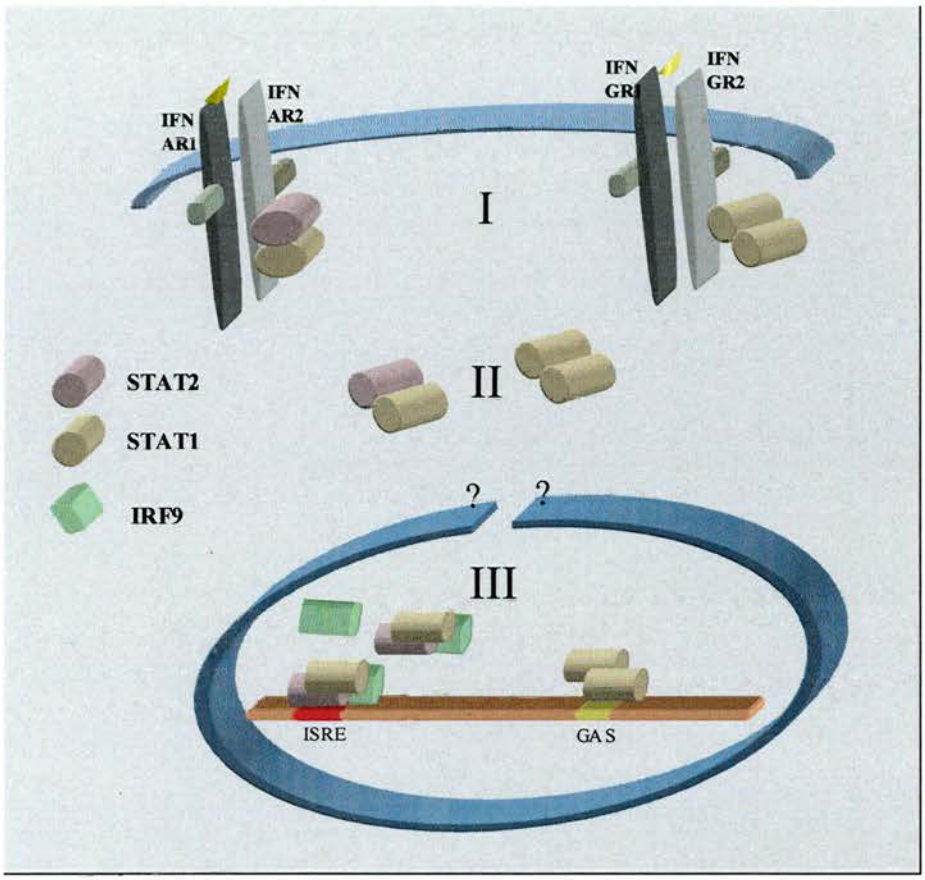


Figure 1.5: The Signalling pathway activated by IFNα/β and IFNγ. I: After IFN is bound by the appropriate receptor II: STAT homodimers are activated and released in the cytoplasm. III: upon nuclear translocation the phosphorylated STAT1/STAT2 molecules associate with IFR9(p48) to bind ISRE consensus sequences, promoting antiviral gene expression. Phosphorylated STAT1 homodimers resulting from IFNγ signaling directly bind GAS elements regulating type II-dependent transcription.

1.1.3.3 Alternate signalling pathways

While the Jak/STAT- IRF9 cascade is the major mechanism of IFN signalling it is not unique. Murine cells lacking insulin receptor substrate (IRS) 1 and 2 are unresponsive to the antiproliferative effects of IFNα/β (Uddin et al.,1997) suggesting the importance of the IRS/PI-3' kinase pathway although very little is known on this. Very little is also known for two more, namely, the CBP/crk and

the vav-oncogene proposed pathways, activated upon IFNα/β stimulation of the IFNAR/JAK complexes, with implications on proliferation and malignancy (reviewed in Platanias and Fish.,1999). Compelling evidence though has implicated the p38 MAP kinase in this process as well.

The observation that type I IFNs activate the p38 MAPK pathway (Uddin et al., 1999) besides directly linking IFNγ production through IFN type I signalling illustrates a novel mechanism for ISG transcription. Although the exact progression of this cascade remains largely unknown, its importance within the IFN type I signalling cascade is increasingly being realized (Li et al.,2005). Following IFN binding to IFNAR, the mediators of the signal (thought to be the Janus kinases associated with the receptor) activate the small G-protein Rac1. The Rac1/p38 system, either in correlation with STAT molecules or even independently, acts to regulate ISGs interferon-dependent transcription. Notably the p38 MAP kinase subtype α appears essential for type I IFN-dependent gene expression (Lee et al., 2003).

1.1.3.4 Type II IFN signalling

Type II IFN signalling is initiated by IFNγ binding to its cognate receptor IFNGR also composed of two subunits the 60kDa IFNGR1 and the 62kDa IFNGR2 (Bach et al., 1997) and requires Jak1, Jak2 and STAT1 to transduce the signal and activate transcription of IFNγ-inducible genes. Jak 1 and Jak 2 are proximally associated with IFNGR1 and IFNGR2 respectively in unstimulated cells without any strong interactions keeping the receptor subunits together (Bach et al.,1997). Dimeric IFNγ binds two IFNGR1 units creating sites for the binding of two IFNGR2 molecules thereby bringing the associated inactive Jak1 and Jak2 kinases in proximity for their subsequent auto- and trans- phosphorylation activation (reviewed in Stark et al., 1998) with Jak2 being activated first (Briscoe et al., 1996). Jak1 phosphorylates IFNGR1 at Tyr440 introducing a docking site of five amino-acids near the C-terminal end of the receptor for the recruitment of STAT1 molecules via their SH2 regions (reviewed in Bach et al., 1997). A phosphorylation event at Tyr701 of STAT1 (the same residue required for

activation by type I IFNs, as discussed previously) results in the release and dimerisation of two STAT1 molecules and subsequent translocation to the nucleus. This translocation is independent of the actin cytoskeleton/microtubule network (Lillemeier et al., 2001) and through an elusive mechanism can be largely achieved by free diffusion within the cytoplasmic and nuclear cellular compartments (Kerr et al., 2003). Inside the nucleus the upstream regulatory domains of IFNγ-inducible genes contain GAS (TTNCNNNAA) consensus sequences coupled to antimicrobial genes and genes regulating antiproliferative functions.

IFNγ can indirectly influence the transcription of ISRE elements by up-regulating transcription of IRFs. Previously IRF9 was discussed as an essential component of ISRE stimulation by IFNα/β with similar properties held by IRFs 1 and 2. While IRF1 can induce stimulation of ISRE containing elements IRF2 can inhibit it (Harada et al., 1989) allowing the modulation of the IFN response.

1.1.3.5 STAT1 phosphorylation in Type I and II signalling

Although not required for nuclear translocation and not demonstrating any regulatory effect on the process, both in the context of IFN type I and II signalling, the efficacy of STAT1-mediated transcription of corresponding sequences is heavily dependent on a second, Tyr701-phosphorylation-dependent phosphorylation (Kovarik et al., 2001) on Ser727. Markedly, an 80% loss of its activation potential is observed when the residue is mutated to an alanine (Wen et al.,1995). There are questions as to the nature of the mediator, yet a MAP-like kinase seems to be responsible (Goodbourn et al., 2000). The aforementioned p38 MAPK is the enzyme considered responsible for this activity in mouse fibroblasts (Goh et al., 1999) and fetal brain astrocytes (Lee et al., 2003) but not in macrophages (Kovarik et al.,1999) and other cell types where the Ca(2+)/calmodulin-dependent kinase (CaMK) II has been attributed that role (Nair et al.,2002). To further complicate things, p38 MAPK appears to be able to phosphorylate Ser727 of STAT1 independent of IFN stimulation (Ramsauer et al., 2002).

1.1.4 Interferon Stimulated Genes and the Innate Immune Response

The upstream interferon activated pathways are capable of providing a number of pre-formed solutions to the problems arising from pathogen infection. This response is non-specific (when contrasted to the antigenic specificity of the adaptive leg of the immune system) but decisive in the progress of the initial stages of disease and/or pathogenesis. Cessation of protein synthesis (as to limit virion processing and amplification), controlled (apoptotic) death of infected cells, the upregulation of demonstration pathways and recruitment of adaptive elements are the hard wired, delaying tactics employed by interferonal immunity. Although different subtypes of type I IFNs share similar mechanisms for the propagation of their signals, specific responses are more effectively induced by different subtypes (Kontsek, 1994). IFNβ seems to be a more potent inhibitor of cellular proliferation than IFNα (Morrison et al., 2001) and different cells demonstrate via their IFNAR different specificities to the plethora of IFNα subtypes (Pfeffer, 1997). In addition to these observations, the selective activation of different sets of ISGs proposes the versatility that is required for efficient, effective and non-wasteful use of cellular resources[14]. A variety of processes mirror the diversity in which the aforementioned themes are applied and these include: up-regulation of Protein Kinase R (PKR), the 2',5'-Oligoadelylate synthetase (OAS), the Mx GTPases, the RNA-specific adenosine deaminase (ADAR), the induction of a nitric oxidesynthase (iNOS2) and the upregulation of elements in classes I and II of MHC presentation pathways.

1.1.4.1 Protein Kinase R

Human PKR is an IFN inducible 62-68kDa protein comprised of 551 amino acids (Kuhen et al., 1996). It consists of a regulatory N-terminal domain holding two dsRNA binding motifs (dsRBM I and dsRBM II) and a catalytic C-terminus with

[14] although overlapping of functions is apparent for reasons that will be discussed below when viral countermeasures against the IFN response are considered.

conserved, catalytic sub-domains responsible for its protein kinase activities (Meurs, 1990). In IFN pre-treated cells PKR predominately resides in the cytosol (Pestka et al.,1987) in an inactive form. Following dsRNA binding, PKR is phosphorylated (either intra- or extra- molecularly) resulting in a conformational change revealing the C-terminus and thus rendering the enzyme active (Meurs, 1990, Katze et al.,1991,Clemens and Elia, 1997). PKR is thought to function as a homodimer with two molecules binding a single dsRNA moiety and cross phosphorylating each other. The binding of PKR to dsRNA seems to be irrespective of the nucleic acid sequence (although certain RNAs are more potent activators than others) (Robertson and Mathews, 1996) but questions arise as to the size requirements of the molecule. In most cases the minimal requirements for interaction with PKR is dsRNA of 30-50bp. However, there have been reports of 11bp of dsRNA interacting with the protein. In contrast, the optimal activators are duplex RNA molecules with a length of approximately 80bp (Manche et al., 1992). At least six proteins have been identified as substrates for PKR phosphorylation: the enzyme itself, the transcription factor IκB, the α subunit of the eukaryotic translation initiation factor 2a (eIF-2a), the M-phase specific dsRNA binding phosphoprotein MPP4, the NFAT 90kDa protein as well as the human immunodeficiency virus (HIV) Tat protein(reviewed in Samuel,2001).

The best characterized function of PKR is the phosphorylation of eukaryotic initiation factor (eIF)-2α. eIF-2 is a hetero trimeric GTPase that binds Met-tRNA and delivers it to the 40S subunit of the ribosome in a GTP-dependent matter to trigger initiation of translation. When the initiation phase is completed eIF-2α now complexed with GDP (hence inactive) is released from the ribosome. The exchange of GDP for GTP on eIF-2α is catalyzed by the exchange factor eIF-2B which re-activates and recycles eIF-2. Phosphorylated eIF-2α interacts strongly with eIF-2B so PKR-mediated phosphorylation of eIF-2α at Ser51 leads to translational arrest by impairing the eIF-2B-catalysed guanine nucleotide exchange reaction, ultimately preventing eIF-2α recycling (Robertson and Mathews, 1997).

PKR was considered the context of IFN production, and was shown in response to dsRNA stimuli to activate NF-κB, a component of the signalling cascade that

results in the transcription of IFNβ, thereby enhancing the production and secretion of the cytokine. This fact along with reports of PKR affecting the activity of STAT1 (Wong,1997) and IRF1 (Kumar et al.,1997) demonstrate PKR's ability in enhancement of the signalling cascades and hence acceleration of the responses towards viral clearance.

Another strategy for reducing viral load and dissemination of infectious particles by the cellular mechanism is the apoptotic death of the host. PKR has been identified as a major effector contributing to the processes underlying apoptosis. Besides the antiviral activities of the enzyme, these observations link PKR with antiproliferative mechanisms demonstrating the versatility of the IFN response. Studies of human malignancies and tumour cell lines suggest that, in general, patients bearing tumours with a higher PKR content have a more favourable prognosis (reviewed in Jagus et al.,1999). Mice lacking the N-terminal domain/dsRNA binding domain of PKR are still able to mount an apoptotic response against viral intrusion, an ability that is lost when the catalytic domain is missing (Abraham et al., 1999), thereby suggesting that the C-terminus of the protein is responsible for PKR's apoptotic properties. The exact mechanisms linking PKR to apoptosis have not been elucidated yet. Additionally, in a PKR dependent manner, presentation of dsRNA to cells leads to increased synthesis of Fas ligand (FasL) and its cognate receptor (Balachandran et al.,1998). FasL is a type II transmembrane protein belonging to the tumour necrosis factor (TNF) family and is predominantly found on activated T cells. The trimeric form of FasL binds and activates its receptor triggering several apoptotic pathways including the initiation of a cascade of caspase stimulation through activation of caspase 8. Although PKR is a component of innate immunity, with pivotal roles in antiviral-antiproliferative functions, homozygous disruptions of its native genes still do not render mice defenceless against viral attack (Abraham et al., 1999) demonstrating the adaptability, complexity and multi-factorial nature of the interferon response that does not rely solely on the presence of a single antiviral enzyme.

1.1.4.2 2'5' Oligoadenylate synthetase (OAS) / RNAseL

One of the first groups of interferon-inducible enzymes to be characterized was the 2',5' OAS. As their name indicates this family of synthetases holds a 2',5'-phosphodiester bond and was the first biologically synthesized molecule to be identified with such a linkage. The OAS catalyze the synthesis of 2-5A oligoadenylates[15] from ATP bearing the general structure ppp(A2'p)nA (Kerr, and Brown, 1978). GTP may also be utilized making the structure pppG2'p5'G (Rebouillat and Hovanessian, 1999). The 2-5A oligomers bind with high affinity to RNase L, a latent endoribonuclease that upon activation by the adenylates dimerises and initiates degradation of cellular and viral mRNAs (Dong and Silverman, 1995).

Three forms of 2'5'OAS differing in size have been identified: a small with one OAS unit (OAS1), a medium with two (OAS2) and a large form bearing three (OAS3). In humans all forms stem from distinct genes clustered over 130kb on the 2'5'-OAS locus on chromosome 12 with all of the forms containing an ISRE element in their 5' flanking region. Alternative splicing yields isoforms of the three types of OAS that are able to oligomerize with each other producing associations that vary in their localization, concentration of dsRNA required for activation and the optimal conditions required for maximum enzymatic activity (reviewed in Samuel 2000). For their activation, 2'5'OASs, not unlike PKR, require dsRNA binding in corresponding nucleotide binding regions that are separate from their catalytic domains. However, the RNA binding regions of 2'5' OAS and PKR share no obvious homology, and speculation lies as to the ability of other cofactors, besides dsRNA, to activate certain forms of the enzyme. For example fructose 1-6 bisphosphate has been reported to stimulate 2'5'OAS in the absence of dsRNA (Suhadolnic et al.,1990).

The binding of functional oligoadenylates (synthesized in response to dsRNA activation of OAS), to the inactive/latent RNAse L moieties permits their dimerisation which in turn bestows the endoribonuclease catalytic activity needed for the subsequent processing and degradation of RNAs. Although all mRNAs are

[15] Normally dimers to pentamers.

liable to cleavage by RNAse L, the enzyme shows more specificity, due to stereometric effects, to viral nucleotides. The reason behind that comes from the instability of its activators the 2-5As. These oligoadenylates are very labile molecules, so tend to activate RNAse L molecules in their proximity, that is the area of their synthesis by 2'5'OAS, which in turn is the area of dsRNA presence and hence viral genomic processing.

mRNAs are not the sole targets of RNAse L, translational arrest in a greater scale can be mediated by binding and disrupting cellular 18S and 28S ribosomal RNA leading to ribosomal inactivation (Iordanov et al., 2000). Experiments in mice with homozygous deletions of the RNAse L genes (RNAse $L^{-/-}$) demonstrate impaired antiviral responses when challenged with different viruses, but they additionally show defects in apoptosis in a variety of tissues (Zhou et al., 1997), implicating the enzyme in this process as well. Although degradation of ribosomes can induce a ribotoxic stress response contributing to apoptosis (Iordanov et al., 1997), RNAse L may also need to degrade additional ribonucleotides eventually leading to activation of JNK. Continued phosphorylation of JNK in this fashion may trigger the mitochondrial pathway of apoptosis. Studies reveal that 2-5A-activation of RNase L leads to cytochrome c release from the mitochondria into the cytoplasm and thus, to caspase activation and apoptosis (Li et al., 2004, Rusch et al., 2000). RNAse L also appears to be one of the links between innate and adaptive immunity. In skin allograft rejection experiments in RNAse $L^{-/-}$ mice and subsequent histologic examinations, a dramatic reduction in inflammatory infiltrates was observed suggesting a delay in T-cell priming or a deficiency in immune cell trafficking (Silverman et al., 2002).

1.1.4.3 The Myxoma (Mx) proteins

The Mx proteins are dynamin-like GTPases that have been implicated at conferring various degrees of resistance to a wide range of RNA viruses including DNA viruses with genomic RNA intermediates (reviewed in Haller and Kochs, 2002). They were first identified in mice demonstrating increased resistance when challenged with influenza A virus (Horisberger et al., 1983). The Mx proteins in

humans are encoded by loci on chromosome 21 and exist in two forms MxA and MxB. While MxA is an interferon-induced molecule and a major contributor to the interferon-actuated antiviral state there is speculation as to the functions of MxB. The latter do not demonstrate antiviral qualities, appear to be independent of interferon stimulation like most other members of the dynamin superfamily but, most interestingly, cells lacking expression of MxB show impairment in the nuclear import process and defects in cell cycle progression (King et al., 2004). MxA proteins on the other hand appear to inhibit trafficking of another kind, more relative to this thesis: the intracellular transport of viral components (Stranden et al., 1993).

MxA, a 76kDa protein, binds GTP with low affinity and demonstrates a high GDP turnover (hydrolysis) rate (Richter et al., 1995). As in other dynamin-like GTPases the GTP tripartite binding domain (and catalytic site) of MxA exists in its N-terminus followed by a central interactive domain and a leucine zipper as its GTPase effector domain in the C-terminal region. The cellular localization of the protein is in the cytoplasm, forming two kinds of assemblies. In uninfected IFN-primed cells MxA monomers self-assemble to form oligomers by intermolecular interactions of the N-terminal leucine zipper region with the central association domain, while in infected populations monomers of MxA bind viral nucleocapsids and nucleocapsid-like targets and aggregate on them.

These observations, according to Otto Hallers group in Freiburg suggest a plausible and elegant mode of action. The induction of the interferon signalling cascade leads to the transcription of the MxA genes only temporarily. In uninfected cells the proteins form the oligomeric complexes mentioned above, perhaps, as a mechanism to ensure a more stable (compared to the MxA monomer) and continual presence in the cytoplasm. In this form the MxA oligomer transiently releases monomeric MxA that act as tracking probes for viral targets. Once the cell is infected the MxA monomers bind the viral nucleocapsid /nucleocapsid-like structures and addition of further monomers produces a massive complex that is targeted away from the virion-assembly locations within the cell thus disrupting the generation of viable virus particles (Haller and Kochs 2002).

1.1.4.4 The dsRNA-dependent adenosine diaminase (ADAR1)

The systems discussed above offer only a limited insight into the interferon antiviral response. Notably mice deficient in all of the pathways mentioned above i.e. PKR, RNAseL and Mx1 (the murine homologue of human MxA) are still able to mount, however limited, antiviral responses (Zhou et al., 1999). The dsRNA-dependent adenosine deaminase (ADAR1) is an enzyme that interferes with the posttranscriptional modification of dsRNAs. ADAR1 is a 136kDa interferon-inducible protein that catalyses the conversion of adenosine into inosine (reviewed in Goudbourn et al.,2000). More specifically, ADAR1 catalyses the hydrolytic C-6 deamination of adenosines converting them to inosine on RNAs thus destabilizing dsRNA associations that may occur as viral genome or intermediates of viral nucleic acid metabolism. The inosine-uracil bonding is considerably weaker than the normally occurring adenosine-uracil pairing resulting in weak interactions (base-pairing) of the dsRNA helices. S. Goodbourn's otherwise excellent review on the interferons (Goodbourn et al., 2000) mentions that "since many viral RNAs go through a dsRNA-based replicative intermediate, this [the relative instability of the processed dsRNA] has the effect of being mutagenic" referencing the reports of genomic substitution consistent with ADAR activity. This statement could be misleading since ADAR supports mutagenesis in a more direct matter. Conversion of adenosine to inosine by deamination changes the purine base from adenine to hypoxanthine. This base, being a precursor of the purines adenine and guanine, is recognized by the cellular nucleotide processing machinery as guanine thus linking ADAR-dependent deamination directly to mutagenesis.

1.1.4.5 Immunomodulatory actions of IFNs

The common theme of the strategies we have seen so far is the inhibition of virus growth at a cellular level through apoptosis or inhibition of viral replication. The result of these actions is the reduction of viral load. However, additionally, the IFNs hold immunomodulatory properties. Both types of IFNs, through distinct mechanisms, are able to act as regulators of several arms of the immune response;

either by controlling the maturation and actions of certain immune cell types or by moderating the secretion of cytokines from infected or effector cells. The major histocompatibility complex (MHC) molecules type I and II are complexes of cell surface proteins that bind processed peptides of internal or external origin respectively and present them to T-lymphocytes to elicit an immune response if the antigens are recognized as foreign to the organism. Both types of IFNs upregulate expression of MHC I proteins while only IFNγ can promote the expression of MHC II, thereby on one hand promoting CD8+ T cell and on the other, CD4+ T cell responses (reviewed in Goodbourn et al.,2000). STAT1-/- mice demonstrate severe impairments to even the basal expression of MHC I molecules and animals lacking IFN receptors show similar deficiencies (Lee et al., 1999). Furthermore the action of IFNs through differential expression of the enzymatic proteasomal subunits diversifies quantitatively and qualitatively the presentation of peptides to CD8+ T cells. The proteasome normally consists of three subunits x, y and z but IFNγ activation results in reduced expression of these molecules and promotes the expression of the LMP2, LMP7 and MECL1 enzymatic subunits. Differential assembly, thus, of the organelle results in proteasomes with different specificities against the peptides to be presented thereby increasing the diversity of molecules displayed. Quantitative changes to the MHC systems can occur directly by upregulation of the MHC I proteins or by enhancing the expression of TAP1 and TAP2 transporters that mediate the translocation of the antigens from the proteasome to bind the MHC class I apparatus (Goodbourn et al., 2000, Boehm et al., 1997, Epperson et al., 1992, York and Rock 1996).

IFNs can also exert their immunomodulatory effects by enhancing the activity or promoting maturation of certain immune cell types. One unarguable role of IFNs is their ability to enhance the cytotoxicity of NK cells. NK cells are critical mediators of innate immunity prior to the maturation of the adaptive immune response (reviewed in French and Yokoyama, 2003). The primary effector mechanisms of these cells are cytokine secretion (including IFNγ) and cell mediated cytotoxicity manifested by the secretion of pore forming proteins (perforins) and other serine proteinases that result in the exocytotic killing of target cells (Loh et al., 2005). IFNα activation of NK cells leads to increased

perforin mRNA expression levels, in turn leading to increased cytotoxicity in NK cells (Kaser et al., 1999). Bridging innate and adaptive immune responses IFNs can stimulate monocyte differentiation into short lived dendritic cells (Buelens et al., 2002), the antigen-presenting cells that, among other functions, act to prime naïve T-Cells. Memory T-cell division and their accelerated turnover come as results from IFN type I induction of different cytokines including IL15. Furthermore type I IFNs, independently of IL15, are able to promote survival of activated T cells (Tough et al., 1999, Marrack et al., 1999).

1.1.5 Interferons and Autoimmunity

The fragile balance between immunity and autoimmune disorder is illustrated in many conditions characterized by an overzealous immune system. Upregulation of interferons has been detected in a range of autoimmune diseases like arthritis and autoimmune encephalomyelitis, while aberrant activation of IFNγ-producing Th1 cells that recognize self-peptides of the myelin sheath, such as myelin basic protein and proteolipid protein, is the prominent autoimmune etiology of Multiple Sclerosis (Mouzaki et al., 2004). However, the most studied relationship between elevated expression of IFNs and disease comes from research on Systemic Lupus Erythematosus (SLE).

1.1.5.1 Role of IFNs in Systemic Lupus Erythematosus

SLE is regarded as the prototypic systemic autoimmune disease with a prevalence of 0.06% in white populations, more common in females with a strong hereditary component (although environmental factors such as viruses and bacteria cannot be excluded). SLE is characterized by the production of mononuclear antibodies that lead to subsequent end organ damage (reviewed in Henry and Mohan, 2005). Type I IFNs have been proposed as playing a pivotal etiopathogenic role in the development and maintenance of the disease process. Raised serum levels of IFNα have been recorded in SLE and these levels have been correlated with disease progression and severity (Bengtsson et al., 2000). Interestingly several signs and symptoms in SLE mimic those in influenza or those encountered during IFNα therapy, namely: fever, fatigue, myalgia, arthritic pain and leucopenia (Ronnblom and Alm, 2003). Furthermore patients treated with IFNα for cancer or infectious conditions like hepatitis can spontaneously develop SLE providing a causative role for the cytokine in the initiation of the disease process (Ronnblom et al., 1990 and Ioannou et al., 2000). IFNα in SLE is secreted by activated plasmocytoid dentritic cells (pDCs) sensitized by complexes consisting of autoantibodies and autoantigens containing RNA and DNA released by cells undergoing apoptosis as a result of the disease (Lovgren et al., 2004). A recent genomic study on families with a history of SLE identified polymorphisms in the IRF5 and Tyk2 genes (as

seen above the corresponding gene products play a pivotal role in the production and signalling propagation of IFNs) that displayed strong signals of joint linkage and association with SLE (Sigurdsson et al., 2005).

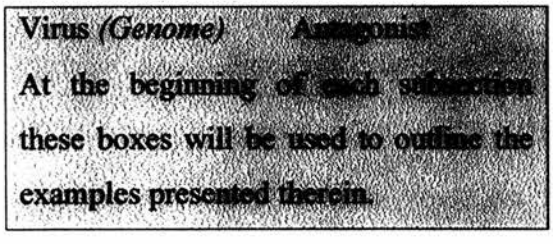
1.1.5.2 Autoimmunity through IFN treatment

In similarity with SLE, patients treated with IFNα for Hepatitis C are prone to autoimmune thyroid disease (AITD). Furthermore high endogenous levels of the cytokine are associated with naturally occurring AITD and it is concluded that IFNα is one of the environmental factors capable of triggering onset of the disease in genetically susceptible individuals (Prummel and Laurberg, 2003). Furthermore the products of IFN stimulation e.g. PKR, Mx proteins and RNAseL have been implicated in the onset of insulin dependent diabetes mellitus (IDDM) and poly (I):(C), the IFN inducer discussed above, can induce diabetes even in diabetes resistant mouse strains (Sobel et al., 1992). Besides the examples mentioned above IFNα therapy has provided insights to the autoimmunogenicity of the cytokine and has been associated with the development of psoriasis, thyroiditis, autoimmune gastritis, interstitial pneumonitis, myasthenia gravis, autoimmune hemolytic anemia, Raynaud's syndrome, Vogt–Koyanagi–Harada disease, and rheumatoid arthritis (reviewed in Devendra et al., 2004).

Chapter 1.2

Viruses Strike Back

The previous section provided an overview of the interferon response and how an intact pathway can confer immunity to an infected organism through the vast arsenal of antiviral mechanisms activated upon successful propagation of the signalling/activation cascades. The key words in the above statement, for reasons that will become clear below are: intact and successful. In the face of such a powerful defense system inducing apoptosis of infected cells, disruption of viral replication and recruitment of an equally, if not more, potent adaptive immune response, viruses have evolved in ways allowing them to circumvent these problems. In fact all viruses that have survived the ongoing evolutionary war and still trouble us today seem to be able to antagonize IFN, in varying degrees. The efficacy of these countermeasures often dictates such important features of virus "life" cycle like pathogenicity and host range. Furthermore, the lengths that some viruses go into in order to inhibit the IFNs and the resulting responses illustrates the importance of the system. Suppression of the response can occur at virtually any level of the IFN system through mechanisms intriguing either in their simplicity or complexity. However these measures are seldom absolute and in most cases the adaptive leg of the immune system kicks in to clear the infection.



The format of this section (1.2) is based on that of 1.1, presenting examples of viral counter-interferon strategies in accordance to their occurrence; starting with inhibition of IFN production (1.2.1), continuing with disruption of IFN signalling (1.2.2) and briefly highlighting the suppression of the IFN-actuated gene products involved in the innate immune process (1.2.3). Considering their multi-functionality there are many cases where individual viral proteins can disrupt more than one part of the IFN system. These among certain other antagonists of the system will be discussed in detail as they present either theoretical or model targets for

chemotherapy. This section concludes with a focus point on the special case, due to its extensive use in the experimental part of this project, of the SV5 V protein.

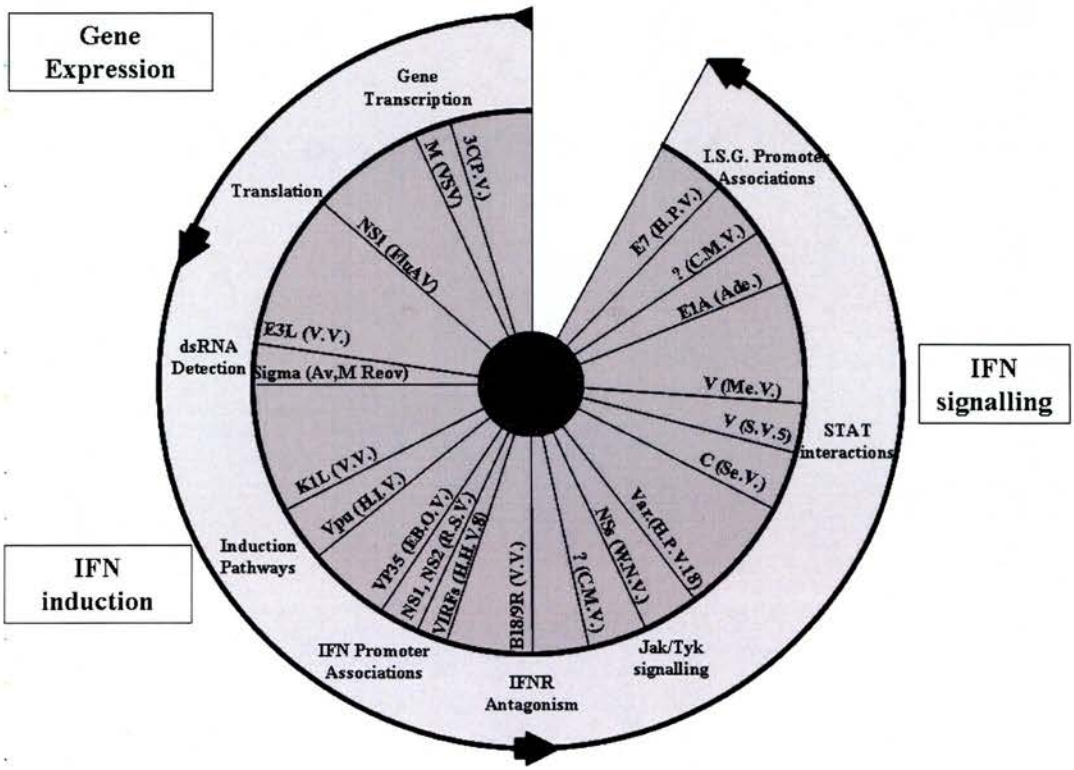


Figure 1.6: Schematic representation of IFN viral antagonism. The figure demonstrates the viral examples that will be discussed in the text. Description of anti-IFN viral antagonism will, counter-clockwise, start with the antagonism of host gene expression, proceed with examples of IFN-production inhibition and will conclude with attenuation of the IFN signalling pathway. Key steps of the progression also presented. Adapted from Haller et al., 2006

1.2.1. Inhibition of Interferon Production

A strategy employed by a wide range of viruses in order to establish their infections is the downregulation of host gene expression. This can be achieved by inhibition at any level of expression: transcription, RNA processing, translation or any combination thereof. The most common advantage associated with this viral strategy is resultant abundance of cellular resources that the virus can manipulate for its own replication (Knipe, 1996). However there are several examples (reviewed in Lyles, 2000) of viruses defective through mutations in their ability to downregulate host protein synthesis that can replicate as efficiently as their wild-type counterparts in most cells, lagging behind only in cell types capable of mediating powerful innate immune responses. This observation diverts the advantage of disrupting gene expression, from access to plentiful resources, to repression of host antiviral responses, including the IFN system.

1.2.1.1 *Viral inhibitors of host gene expression*

Polio Virus (+ve ssRNA)	**3C**
Vesicular Stomatitis Virus (dsDNA)	**M**

A classic example for downregulation of host gene expression is the 3C protease of poliovirus (PV, a picornavirus). The protein normally catalyzes the secondary cleavage[16] of the viral polyprotein, (Porter, 1993) but it also catalyzes the inhibition of host transcription by targeting all three host RNA polymerases (Clark et al., 1991,1993). In the best studied example, 3C targets the TATA box-binding protein (TBP) and cleaves one or more of three Gln-Gly motifs contained in that protein (Das and Dasgupta, 1993). Since TBP is responsible for recruiting various transcription factors to the promoter initiation site including RNA polymerase II (RNAPII), this action directly terminates RNAPII-dependent transcription. Additionally and through an unidentified mechanism the 3C protease has been shown to inhibit RNAPI-dependent transcription as well as RNAPIII by targeting

[16] Two cleavages are required for activity. The initial cleavage is mediated by the viral 2A protease

the transcription factor TFIIIC (Clark and Dasgupta, 1990). The inhibition of the three host polymerases is a strategy evolved by members of distinct viral families. Vesicular stomatitis virus (VSV), a rhabdovirus (as opposed to poliovirus being the prototype picornavirus) uses its Matrix (M) protein to obtain similar results. In its role within viral replication and assembly the M protein attaches the viral nucleocapsid to the plasma membrane and induces the budding process that generates the viral envelope (Lenard, 1996). However, in a genetically separate role and by unclear mechanistics the M protein has also been shown to inhibit transcription by RNAP I, II and III (Ahmed and Lyles 1998).

1.2.1.2 *Viral inhibitors of RNA modification.*

The subsequent logical step for viral inhibition would be host RNA processing. The dominant example for this strategy comes from influenza A virus, executed through its Non-structural (NS) 1 protein. Influenza NS1 is a 230 amino acid protein, consisting of two functional domains: an RNA binding/dimerisation domain on the N-terminus and an effector domain on the C-terminal region (Quian et al., 1994). In contrast to VSV and PV, influenza virus replicates inside the nucleus of host cells and NS1 functions in that context to inhibit post-transcriptional modification of the host RNA, including: mRNA transport control, splicing, translation and polyadenylation (other anti-IFN properties of NS1 will be discussed separately). NS1 specifically inhibits nuclear export of mRNAs containing 3'poly(A) ends (Quian et al., 1994) by the interaction of its C-terminal effector domain with the cleavage and polyadenylation specificity factor (CPSF), an essential protein component of the cellular 3'-end processing machinery that modifies precursor mRNAs (Nemeroff et al., 1998). Additionally, NS1 targets the subsequent poly-adenylation step of mRNA modification by binding the nuclear poly(A)-binding protein (PABII) that is required for elongation by poly(A) polymerase (Chen et al., 1999). Since most viral gene expression (although not all) proceeds via unspliced mRNA intermediates another obvious target for inhibition would be the spliceosome. *In vitro* experiments have demonstrated the ability of NS1 to bind to the U6 snRNA,

preventing the molecule's interaction with other snRNAs thereby inactivating the spliceosome and disabling nuclear export of mRNAs (Qiu et al., 1995).

1.2.1.3 *Viral Targeting of dsRNA detection*

Vaccinia Virus (dsDNA)	E3L
M. Reovirus (dsRNA)	σ3
Av. Reovirus (dsRNA)	σA

The most common, but not the unique, inducer of IFN production is dsRNA. As expected, viruses based on RNA genomes are more likely to proceed with replication or metabolism of their genetic information through dsRNA. In fact DNA viruses are less potent activators of IFN precisely because they generally produce less dsRNA (reviewed in Jacobs and Langland, 1996). Many viruses encode proteins that bind dsRNA, through dsRNA binding regions similar to host proteins like PKR, making it unavailable for the intracellular surveillance proteins that initiate IFN production procedures. Such a tactic besides interfering with IFN production may make the virus immune to the antiviral actions of proteins that use dsRNA as an activator, like PKR and 2'-5'OAS, illustrating the popularity of dsRNA sequestering proteins among different viruses.

Poxviruses, including vaccinia virus, encode for E3L, a 190 amino acid protein containing a C-terminal dsRNA binding domain. (Watson et al.,1991). Compared to the wild type virus, E3L-deleted vaccinia virus (E3L$^-$) is sensitive to IFN treatment (Beattie et al., 1995). Both these observations suggest an apparent role for E3L, and indeed the protein binds dsRNA, making it unavailable for detection and preventing the activation of proteins that require the molecule as a substrate. Beyond that, E3L directly binds to the dsRNA domain of PKR (Sharp et al., 1998) inhibiting both the PKR-dependent pathway for induction of IFN and of the antiviral response mediated by the kinase. IFN induction, as seen previously, is dependent on the phospho-activation of IRF3 and IRF7. Inhibition of PKR, however, does not prevent phosphorylation of these transcription factors and IFN induction can still occur. E3L has been observed to inhibit phosphorylation of

these factors; and interestingly, an E3L mutant incapable of binding dsRNA retains this ability, suggesting both a dsRNA-independent phosphorylation of IRF3/7 (and subsequent induction of IFNs) and the possibility of E3L inhibition of the unidentified kinase (Smith et al., 2001).

Most mammalian reovirus strains are also capable of inducing host protein shut-off and encode proteins targeting the innate immune system. Considering the dsRNA nature of the viral genome it would only be natural to hypothesize a dsRNA sequestering protein defending the genetic information against attack by IFNs and their actions. Indeed this comes in the form of the reovirus major outer capsid protein, σ3, a 40 kDa cytoplasmic protein with a dsRNA binding region at the C-terminus (reviewed in Jacob and Langlands,1998). σ3 belongs to a diverse family of proteins found across species serving distinct functions sharing only dsRNA binding properties through a highly structured ~85 amino acid domain (Miller and Samuel, 1992). Similarly to E3L, σ3 is also an inhibitor of PKR suggesting a role in translational control (Lloyd and Shatkin 1992). Furthermore, σ3 expression aids replication of the otherwise IFN-sensitive E3L$^-$ vaccinia virus (Beattie et al., 1995). Mutations of σ3 altering the affinity of the protein towards dsRNA also alters the sensitivity of the virus towards IFN (Bergeron et al., 1998). Avian reovirus S1133 encodes the σA protein, homologous to σ3, also capable of dsRNA binding and with higher affinity when compared to its mammalian counterpart. No dsRNA binding motifs have been recognized within the amino acid sequence of the protein but the affinity for the nucleic acid can be compared to that of molecules like PKR and E3L. It still remains under investigation whether σA protein is the main reason behind the avian reovirus' insensitivity to IFN treatment.

1.2.1.4 *Viral targeting of the NFκB cascade*

Vaccinia Virus (dsDNA)	K1L
HIV (+ve ssRNA)	Vpu

One key step in the induction of the IFNs is the dissociation of NFκB from its inhibitor IκB. Furthermore, NFκB has been reported to be a mediator of IFN-independent innate antiviral responses against RNA viruses like human parainfluenza virus (HPIV) 3 and respiratory syncytial virus (RSV) (Bose et al., 2003). It would thus be logical to assume viral inhibitors of the transcription factor, however NFκB inhibition would make the host cell liable to apoptosis (antiapoptotic properties of NFκB reviewed in Van Antwerp et al., 1998). Furthermore, certain viruses rely on NFκB activation for their gene expression and even activate it (reviewed in Hiscot et al., 2001). Evolution though, has granted viruses with an increased perception of dialectics and viruses will circumvent the antiapoptotic/antiviral response by other mechanisms (reviewed in Cuff and Ruby, 1996). A variety of viral proteins have been identified as specific inhibitors of NFκB (indirectly NFκB inhibition can occur, for example, by the dsRNA sequestering proteins).

The vaccinia virus (VV) K1L gene product provides one such example. By preventing phosphorylation and subsequent degradation of IκBα, possibly through interactions with IKK or with the kinases that act upstream of IKK, VV can inhibit NFκB-dependent gene expression (Shisler and Jin, 2004). Similarly, the HIV1 Vpu protein [otherwise implicated in CD4 degradation (Willey et al.,1992)] affects IκBα by preventing its degradation (Akari et al.,2001). Vpu is an integral membrane protein that demonstrates a high affinity for TrCP, a key component of the E3 ubiquitin ligase complex that regulates ubiquitin-related proteasomal degradation (Margotin et al., 1998) of proteins including IκBα. The interaction between Vpu and TrCP was found to severely impair TNFα-induced NFκB activation and subsequent gene expression (Bour et al.,2001).

As mentioned above, there is a delicate balance between viral suppression and viral activation of NFκB, and this exact balance affects critical aspects of virus biology as pathogenicity and viral persistence. Measles virus (MeV) demonstrates this principle perfectly. The epithelial cells of the respiratory tract are the primary targets of measles infection and in these cells MeV directly induces NFκB activation, promoting increased transcription of the viral genes. The infection, though the upregulation effects of NFκB activation, is thereby characterized by increased cytokine (amongst them IFNα/β) secretion (Helin et al., 2001) contributing to pathogenicity. On the other hand, the virus suppresses or fails to mediate phosphorylation of IκBα and the subsequent activation of NFκB in neurons (Dhib-Jalbut et al., 1999). This action results in limited cytokine production and hence, restricted antiviral response; with the ultimate consequence being the establishment of a state of permanent infection in these cells.

1.2.1.5 *Viral targeting of promoter associations*

Ebola Virus (-ve ssRNA)	VP35
bRSV (-ve ssRNA)	NS1,NS2
HHV8 (dsDNA)	Various

Other viruses trying to limit IFN production "drop the hassle" (together with the resultant advantages, as seen with MeV) of inhibiting NFκB activation altogether for another strategy: the inhibition of transcription factor interactions with the IFN promoters. In the case of IFNβ this could be achieved by preventing interactions of the IRF transcription factors with the four PRD regions of the IFNβ promoter through: direct binding, phosphorylation inhibition, secretion of viral homologues with a dominant negative effect on IFNβ transcription, prevention of IRF dimerisation or IRF cytoplasmic retention.

Ebolavirus, one of the most lethal human viruses with fatality rates up to 90%, uses extremely potent methods to circumvent the IFN response; a major contributor if not the actual cause, for its high virulence. One such tactic is the inhibition of IFNβ production. Through an unclear mechanism the Ebolavirus

VP35 protein targets the phosphorylation of IRF3. Inactive IRF3 shuttles between the nucleus and cytoplasm unable to contribute to the formation of the transcriptional complex required to drive IFNβ-mRNA production (Basler et al., 2003). Similar to Ebolavirus, bovine respiratory syncytial virus (bRSV), through the cooperation of its two non-structural proteins NS1 and NS2, also blocks enhanceosome formation by inhibiting the C-terminal IRF3 phosphorylation (Bossert et al., 2003).

Molecular "piracy", or the encoding of homologues to host proteins is a common feature of DNA viruses especially in members of the poxvirus and herpesvirus families. Human herpes virus 8 (HHV8), the viral etiologic agent of Kaposi's sarcoma produces a variety of such homologues mimicking (and ultimately inhibiting) a multitude of cytokines and cytokine response pathways including Bcl2, IL6 and IRF3 (Moore et al.,1996). The competitive inhibition through a conserved tryptophan-rich DNA binding domain at the N-terminal end of the viral homologue to IRF3 (vIRF3) is enough to cause a 50% reduction in IFN synthesis (Lubyova and Pitha, 2000). This reduction, though, is relatively inefficient, suggesting that other cellular factors are substituting for IRF3 to induce enhanceosome formation in its absence. One potential candidate is IRF1. But expectedly enough, besides encoding for vIRF3, HHV8 produces a viral homologue of IRF1 as well (vIRF1) (Li et al., 1998) ensuring an efficient blockade on IFNβ synthesis. Although vIRFs possess only 13% amino acid homology to the human proteins in total they share a 70% residue identity in their DNA binding motifs (Zimrig et al., 1998) suggesting that competition occurs inside the nucleus for DNA binding at the appropriate positions.

An alternative way to inhibit IRFs is by direct binding. The E6 oncoprotein (initially implicated in p53 ubiquitination) of the "high risk" (for cervical cancer) human papilloma virus 16 (HPV16) has been demonstrated to directly interact with IRF3 (Ronco et al., 1998). The specific interaction of E6 with IRF3 results in a substantial reduction of IFN mRNA production. Binding of IRF3 seems to be distinct from that of p53 and it does not cause the degradation of the transcription factor (Ronco et al., 1998). However, inhibition of IFN production by HPV16 possibly provides an insight to the nature of the infection, both in terms of viral

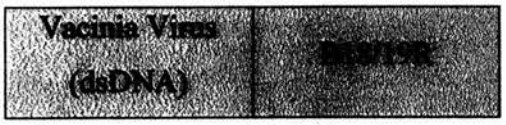
persistence and possibly as a contributor to oncogenesis. Recent studies have also implicated the severe acute respiratory syndrome (SARS) coronavirus in a molecular blockade of the interferon induction pathway. SARS virus in contrast to other members of the coronavirus family (Bardoux et al., 1998) is an inefficient IFNβ inducer and the syndrome is treated with exogenous IFN. SARS virus appears to inhibit IFN production by directly interfering with the functions of IRF3 by inhibiting efficient phosphorylation, dimerisation and subsequent nuclear translocation of the transcription factor (Spiegel et al., 2005)

1.2.1.6 *Alternative antagonism of IFN production*

The examples mentioned above are not unique and there are several documentations of other viral proteins interfering with the induction of type I IFNs e.g. the Thogoto virus M protein (Hagmeier et al., 2003) , Sendai C and V proteins targeting of signalling pathways leading to IRF3 activation (Komatsu et al., 2004), the V protein of SV5 (Andrejeva et al., 2001). What is clear though, is that the inhibition of IFN induction is a very efficient tactic, within the context of viral replication, and very popular among different viruses ranging from large DNA viruses like Vaccinia virus down to compact RNA viruses like Influenza virus. What demonstrates the efficiency of the IFN mediated antiviral response even more is that disruption of IFN production is not enough, as many of the viruses discussed above also target secreted IFN and the signalling cascade initiated upon IFN binding to its receptors.

1.2.2 Inhibition of Interferon signalling

1.2.2.1 *Antagonism of IFN binding to its receptors*



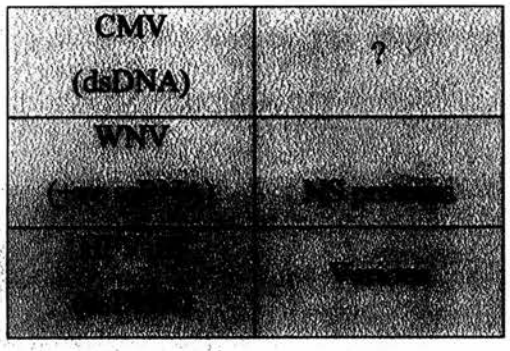
Antagonism of IFN signalling, similarly to IFN production, can occur at any stage within the cascade. Disruption of interferonal stimulation at the receptor level is the first phase affected by viral antagonists of signalling. The concept of viral homologues is not restricted to the level of IFN induction and in this case, viruses can produce homologues of the IFN receptor.

Vaccinia virus (VV) and other members of the orthopoxviridae family (including camelpox and mousepox) encode proteins that antagonize both types of IFN binding to their cognate receptors (reviewed in Smith et al., 1998). VV inhibits IFNα/β signalling through an IFN binding protein, the product of the viral B18R or B19R (depending on the strain) open reading frame (Colamonici et al., 1995, Symons et al., 1995). In contrast to the cellular fibroconnectin type II IFNARs, B18R uses three immunoglobulin (Ig)-like domains to bind IFNs with a high affinity (Smith and Chan, 1991). In addition, this homologue binds IFNs without demonstrating any species specificity for the cytokine (specificity to an organism's "own" IFNs being a major characteristic of the IFNARs) (Symons et al., 1995).

B18R protein comes in two forms: a 52kDa version that attaches to the host membrane "scavenging" IFN binding in competition with the IFNARs, and a larger 60-65kDa form that is secreted in the extracellular environment. While the advantage of having a soluble receptor floating around the medium binding secreted IFNs with high affinity is obvious, the larger B18R has been observed on the surface of uninfected cells. This intriguing observation led to the realization by Alkami et al.,. (2000) of a novel mechanism of viral immune evasion: the secretion of viral proteins that bind to cellular surfaces of uninfected cells. In this

novel strategy the infected cell produces B18R which can attach to the cell surface of infected cells competing for IFNα/β, be released in the medium scavenging IFN secreted by other cells or even bind the cell surface of uninfected cells in the periphery of the infection desensitizing them of the viral presence and disrupting them from going into the antiviral state.

1.2.2.2 *Antagonism of signalling molecules*



Since both type I and II IFNs signal through related mechanisms (the Jak/STAT pathways) and many of the signalling components are common to both, there are clear advantages to the inhibition of the transducers. This can be achieved at all levels of the cascade: association of the Janus kinases (Jaks) with the receptor complex, disruption of STAT phosphorylation and subsequent dimerisation, interference with nuclear translocation of STAT dimers or other associated transcription factors, ending with the inhibition of the formation of an active transcription complex to drive the antiviral response.

Human cytomegalovirus (CMV), a major cause of pathogenesis in immunocompromised carriers, within its arsenal of powerful immune evasion methods ensuring viral persistence (reviewed in Miller et al., 2002) efficiently targets the signalling cascade as a way of limiting the direct or indirect antiviral actions of IFNs. Infection with CMV downregulates Jak1, through an unidentified mechanism involving the proteasome. Jak 1, as seen earlier, is an integral part of the signalling cascade, common for both types of IFNs. This decrease in Jak1 limits the subsequent tyrosine phosphorylation of the corresponding STATs leading to inadequate downstream transcriptional activity (Miller et al., 1998). In addition to Jak1 CMV also negatively regulates the levels of p48(IRF9) (the STAT associated cofactor required for transcriptional activation) preventing the

formation of ISGF3 (Miller et al., 1999), thus blocking IFNα/β-dependent gene expression in two efficient steps.

Although CMV infections are widespread and asymptomatic to most, another blocker of the IFN signalling cascade West Nile virus (WNV) is a major cause of viral encephalitis. Indeed WNV replication was found to be unaffected by IFNα treatment suggesting that the virus is able to attenuate the cascade (Diamond et al.,2000). The non-structural proteins (NS) of WNV have been implicated in preventing phosphoactivation of both Jak1 and Tyk2 (Guo et al.,2005). Guo et al.,. (2005) have proposed, among other possible mechanisms, NS1 binding to the IFN receptors prohibiting Jak association, or, binding and inhibition of the two associated Jaks: Jak1 and Tyk2 with the latter suffering increased suppression of its tyrosine kinase abilities. However the exact nature of this inhibition remains unclear.

The HPV-18 E6 oncoprotein (distinct from the example of HPV-16 in 1.2.1.5) has been implicated as an inhibitor of IFN type I signalling also by preventing Jak function. Specifically E6 interacts with the Jak-homology (JH) domains of Tyk2 that are required for binding to the intracellular domain of the receptor (Li et al.,1999). This specific inhibition of Tyk2 explains the unresponsiveness to IFNα but not to IFNγ (in contrast to the above example of CMV-mediated interference with Jak1) illustrating HPV18 E6 as a specific inhibitor of type I signalling.

1.2.2.3 Focus Point: Antagonism of STAT interactions by Paramyxoviruses

Sendai Virus (-ve ssRNA)	**C**
Simian Virus 5 (-ve ssRNA)	**V**
Measles Virus (-ve ssRNA)	**V**

The subsequent level for viral attenuation of the IFN signalling cascade is the inhibition of STAT complex formation. This can be achieved in two levels either by preventing the phosphorylation of the transducers, or by preventing their association. Since the V protein of SV5

was extensively used as a model protein for viral inhibition of IFN signalling throughout this work and because much of the research on this step of has been done on paramyxoviruses, the focus of this section will be on the accessory proteins of these viruses and their interactions with the STATs.

The inhibitory effects of certain paramyxoviruses have been known for over forty years. The initial observations that cells infected with human parainfluenza virus (HPIV) 3 (Hermodsson, 1963) and Sendai virus (SeV) (Cantrell and Valle,1965) enabled the growth of IFN sensitive viruses were characterized at the molecular level only recently. In the meantime, other paramyxoviruses belonging to the *Paramyxovirinae* subfamily were also identified to interrupt IFN signalling, a property otherwise limited to DNA viruses (Goodbourn et al.,2000).

The examples of antagonists offered below will be drawn exclusively from the ***paramyxoviridae*** family after a short introduction to the organization of the viral genome that allows for the expression of inhibitory molecules against the STATs.

Genome organization of Paramyxovirus IFN antagonists

Paramyxoviruses are nonsegmented negative (–ve) sense, single stranded RNA viruses and classified under the order of *Mononegavirales*. Across three genenera belonging to the *Paramyxovirinae* (*Respirovirus*, *Rubulavirus* and *Morbillivirus*) the members of the family contain a multifunctional P gene encoding for multiple proteins by RNA editing or by overlapping reading frames. In the case of *Rubulavirus* the P gene encodes for two proteins, P and V, with a common N-terminus while the P genes of *Respirovirus* and *Morbillivirus* also contain information for the generation of a third group of proteins labeled C.

SeV (belonging to *Respirovirus* genus) encodes up to eight proteins through the single P gene. Although the P mRNA of SeV is a faithful transcript of the P gene, during de novo viral mRNA synthesis two G residues are added to the editing site to produce alternative proteins via alteration of the reading frame, including V. This process is reversed within the Rubulaviruses (as SV5 and hPIV2) where V is

the faithful copy of the P gene and the addition alters the transcript to encode for P. The C proteins are produced by an open reading frame (ORF) located upstream of the editing site of the P ORF in the +1 reading frame. While the P protein is a component of the viral polymerase and thus an essential component for viral replication, the accessory proteins C and V have now being shown to act as IFN antagonists, and indeed recombinant SeV with C and V knockouts in vivo is non-pathogenic (Delenda et al., 1998).

Sendai Antagonism

SeV, a model Paramyxovirus uses its C protein to antagonize IFN signalling. It does so both by directly binding to STAT1 preventing its activation (Takeuchi et al., 2001) and by targeting STAT1 for degradation (Strahle et al., 2003). SeV C interacts with STAT1 in two ways: a) by preventing IFN-induced Tyr701-STAT1 phosphorylation and dimerisation while in other cases[17] b) by inducing Tyr701 phosphorylation but decreasing STAT1 levels in an IFN signalling-independent manner (Garcin et al., 2003). While there are obvious advantages in the decrease of STAT1 levels or inhibition of its phosphorylation the reasons behind the induction of phosphorylation are unclear. It has been suggested by Garcin et al.,. (2003) that the C-phosphorylated STAT1 in contrast with its native Jak-mediated phosphorylation is unable to dimerise with the Tyr690-phosphorylated STAT2 as the heterodimer is formed in a concerted fashion at the cytosolic face of the IFNAR.

Simian Virus 5 Antagonism

The V gene product of the SV5 P gene has been identified as a potent inhibitor of the IFN response by obstructing besides IFN production the signalling pathway that leads to ISG transcription (Didcock et al., 1999a). A significant decrease in STAT1 levels is observed as early as 4hrs after infection with the virus. This effect is reversed by incubating cells with proteasome inhibitors including MG132 implying a role for the proteasome in the observed decrease in STAT1 levels

[17] where alternative variants of the C protein are concerned

(Didcock et al., 1999b). Further proof implicating SV5 V protein in this process comes from transfections of the protein into a variety of cell lines all responding by decreased STAT1 levels and reduced ISG transcription. SV5 V interacts with STAT2 (in vivo and in vitro) and the presence of both STAT1 and STAT2 is required for the proteasomal degradation of STAT1 (Parisien et al., 2002). Furthermore, V was also observed to interact with the p127 (DDB1) subunit of the UV damage DNA binding protein (Lin et al., 1998) an interaction that, besides slowing down the host cell cycle (Lin et al., 2000), is essential for V-induced-STAT1 proteasomal degradation (Andrejeva et al., 2002). The model suggested in the aforementioned paper for the STAT1 degradation is that the V protein acts as a bridge between STAT1/STAT2 and DDB1, which then binds to cullin-4A (cul-4A) (a member of the cullin family of ubiquitin-protein ligases) in a ubiquitin E3 ligase complex, resulting in the ubiquitination and degradation of STAT1. A more detailed presentation of V antagonism exists in 1.2.4 Focus Point..

Human Parainfluenza Virus 2 antagonism

Another Rubulavirus, the human parainfluenza virus 2 (hPIV2) uses its V protein to disrupt IFN signalling by targeting STAT2 for degradation. After infection with the virus or transfection of V into cell cultures , STAT2 levels rapidly decline within the cell, in a manner that can partially be alleviated by proteasomal inhibition (Young et al., 2000 and Parisien et al., 2001). This degradation of STAT2 instead of STAT1 may render the cells unresponsive to IFNα/β but, in contrast to SV5 V induced inhibition of STAT1 which protects the virus from the actions of both type I and type II IFNs, hPIV2 V expressing cells are to an extent susceptible to IFNγ. Similarly to SV5 V, hPIV2 V also mediates its actions through the assembly of the DDB1/cul4a apparatus and the polyubiquitination, and subsequent degradation of the corresponding transducers can be achieved *in vitro* by using rabbit reticulocyte lysates (Precious et al., 2005). Interestingly the *in vitro* attempts to elucidate the molecular mechanisms behind STAT degradation for both viruses, followed up by cell culture experiments, demonstrated that in contrast to SV5 V, specifically targeting STAT1, hPIV2 V is responsible for the polyubiquitination of both STAT1 and STAT2 (Precious et al.,2005).

Antagonism by other Rubulaviruses

The V proteins of the Rubulaviruses SV41 and mumps virus (MuV) have also been implicated in the disruption of the IFN signalling cascade by the degradation of STAT1 via a mechanism partially involving the proteasome (Gotoh et al.,2001). Mumps virus uses the V protein's C-terminal, cysteine rich domain to evade the actions of IFNs by the degradation of STAT1 (Kubota et al., 2001). Additionally to STAT1 binding, MuV V has been reported to associate strongly with RACK1 (Kubota et al., 2002), the adaptor molecule between unphosphorylated STAT1 and the IFN receptor (Usacheva et al., 2001). The interaction between MuV V and RACK1 prevents the adaptors association with the receptor preventing thus the phosphoactivation of STAT1. MuV V also targets STAT3 for degradation, adding not only to the disruption of IFN signalling but most interestingly, STAT3 being a mediator of anti-apoptotic signals (Darnell, 2002), implying a mechanism behind the oncolytic properties of MuV (Ulane et al., 2003).

Measles Antagonism

By using a mechanism of inhibition distinct to the *Rubulavirus*, measles virus (MeV, a *Morbilivirus*) via its V protein antagonizes IFNα/β and IFNγ signalling. Affinity purification of V results in associations with STAT1/2/3 but not with cul4A or DDB1 demonstrating that MeV V in contrast to the examples mentioned above does not act by preventing transducer phosphorylation/dimerisation, instead it acts by preventing the nuclear translocation of STATs (Palosaari et al., 2003).

In addition, conveniently linking this section with the following, the purification experiments of MeV V also showed interaction with IRF9 perhaps as a method insuring complete inactivation of the ISGF3 complex. This property of MeV is unique among paramyxoviruses but inhibition of ISGF3 formation and the subsequent transcription of ISGs, as will be described below, is a common strategy employed by a range of viruses.

1.2.2.4 *Viral antagonism of p48(IRF9)*

Adenovirus dsDNA	E1A
Cytomegalovirus (dsDNA)	?
HPV-16 (dsDNA)	E7

Having discussed the effect of paramyxoviral antagonists on STAT associations, the subsequent level of IFN signalling disruption that will be addressed is the inhibition of p48. p48 is the third partner (along with STAT dimers) in the formation of the ISGF3 complex and a common target of viral inhibition. Presented below are two modes of IFN signalling inhibition achieved through degradation and sequestering of p48.

Adenovirus is a potent inhibitor of IFN with adenovirus infected cells being more susceptible to viral infection and allowing rescuing of IFN-sensitive viruses (Anderson and Fennie, 1987). IFN antagonism in the signalling level is mediated by the viral early region 1A (E1A) proteins. By alternative splicing two major products are transcribed: a 243kDa variant made from 12S E1A mRNA and a larger, 289kDa, protein from the 13S E1A mRNA. Both proteins have important implications in regulating cell growth and the transcription of host/viral genes (Nevins, 1995) and most importantly in our context, modulating the IFN cascade (Ackrill et al., 1991). Expression of E1A in HeLa cells results in a decrease in both STAT1 and p48 levels resulting in the transcriptional inhibition of both ISRE and GAS elements, thereby affecting the expression of ISRE/GAS- driven genes (Leonard and Sen, 1996). Expression of E1A, though, in cell lines derived from HT1080 cells is only marked by a reduction in p48 levels leaving STAT1 unaffected (Leonard and Sen, 1997). Loss of p48 accounts for the loss of IFNα/β function while retaining transcription through GAS elements, most frequently associated with type II IFN. Overexpression of p48 in these cells restored ISRE transcriptional activity (Leonard and Sen, 1997) illustrating that E1A acts by disrupting the IFN-induced transcription of ISRE elements through prevention of ISGF3 formation by direct downregulation of p48 expression. Another virus

infection also characterized by loss of p48 is that caused by hCMV. As seen previously the virus is capable of disrupting several steps of the signalling cascade leading to ISG transcription and one of those is the formation of ISGF3 resulting from hCMV mediated downregulation of p48 through an unknown mechanism (Miller et al., 1999)

p48 is also the target of HPV16 through its E7 protein (previously the E6 protein of HPV16 was discussed in the context of IFN production inhibition) affecting type I IF-stimulated transcription of ISRE sequences. Protein-protein interaction studies revealed that E7 binds through a 20aa sequence[18] directly to p48 and expression of E7 results in prevention of ISGF3 formation. (Barnard and McMillan, 1999). The importance of both E6 and E7 mediated-loss of IFN function is jointly emphasized firstly by the practice of administering exogenous IFN to infected individuals and perhaps more importantly by the latest attempts to create vaccines targeting these antigens (reviewed in Mandic and Vujcov, 2004).

[18] otherwise characterized as a sequestering sequence for pRB, a tumour suppressor protein (reviewed in Motoyama et al.,., 2004).

1.2.3. Antagonism of IFN-actuated antiviral products

As discussed in 1.1.4. successful IFN signalling leads to the expression of very potent antiviral enzymes. As expected a vast array of individual viruses target these products in order to sustain their replication and to prevent host cells from attaining the antiviral state.

1.2.3.1 *Inhibition of PKR and 2'-5' OAS/RNAseL*

In 1.2.1.3 the concept of PKR inhibition by dsRNA binding proteins was discussed in the context of IFN production. However there are examples of viruses like poliovirus that mediate the degradation of the enzyme. Poliovirus replication is heavily dependent upon dsRNA metabolism, so naturally due to PKR affinity for dsRNA the virus has to evade the enzyme. It accomplishes this by inducing degradation of the kinase. The model argued by Black et al. follows a very clever mechanism: poliovirus introduced dsRNA (or RNA with extensive double stranded features) is bound by PKR along with bivalent cationic trace elements (like magnesium or manganese) that induce though electrostatic interactions a conformational change in the protein. The altered enzyme is detected by uncharacterized host or viral proteases and is subsequently completely proteolysed (Black et al.,1993).

2'-5' OAS/RNAseL is also a common target for viral suppression by viral antagonists. While dsRNA sequestering proteins mentioned above have the double function of antagonizing both PKR and 2'-5' OAS/RNAseL, there are cases of specific inhibition of the enzyme. HSV, for example, synthesizes 2'5'A derivatives that act as antagonists preventing the activation of RNAseL (Cayley et al., 1984). Furthermore, HIV1 and EMCV induce specific RNAse L inhibitors (RLIs) down-regulating the molecule's enzymatic activity by antagonizing 2'5'A binding to RNAseL (Martinand et al., 1998).

Sections 1.2.1 to 1.2.4 provided typical examples of viral antagonists of the IFN response in respect to their level of occurrence within the appropriate pathways

incorporating an in-depth view on the inhibition of STAT interactions by paramyxoviruses. The SV5 V protein there, was presented in similar detail with other examples. In addition to section 1.2.3 (for reasons explained below), SV5 V will be discussed in 1.2.5 in greater detail through its interactions and molecular mechanisms of action.

1.2.4 Focus Point: SV5 and the V protein

1.2.4.1 Simian Virus 5

Simian Virus 5 is one of the etiological agents behind canine "kennel cough". SV5, although its name indicates a simian host[19], naturally infects dogs. However there is additional evidence for human infection in children (Hsiung, 1972, Goswami *et al.*, 1984).

As mentioned previously the SV5 V protein is a potent IFN signalling antagonist. However, the protein has also been identified as an inhibitor of IFN production. Due to this versatility and to the fact that a large portion of this treatise is based on the properties of SV5 V, the mechanisms of inhibition employed by the antagonist will be viewed in considerable detail

1.2.4.2 Genomic Organisation of the V protein

SV5 V is the faithful mRNA copy of the V/P gene of the viral genome. The 222aa gene product is a multifunctional protein, originally, demonstrated to interact with the viral nucleoprotein (NP) (Randall and Bermingham, 1996), a property that may enable V to act as a chaperone keeping the NP soluble. Besides this initial attribute, SV5 V has been assigned other functions like ssRNA binding properties, and its zinc binding C-Terminal domain has been implicated in IFN antagonism. Wild type SV5(wtSV5) is a poor inducer of IFNs (Didcock et al., 1999). Studies with recombinant SV5 V with a deleted C-Terminus (rSV5VΔC) and wtSV5 demonstrated elevated levels of IFNβ-mRNA and severe impairment in IFNβ-production-inhibition by the recombinant virus in comparison to wtSV5 (Poole et al., 2002). The observation was confirmed by studies showing N-terminal deletions up to 126aa being tolerated[20]. In comparison, deletions at the C-terminus

[19] SV5 was first isolated from rhesus monkey kidney cells and named in the assumption of the animal as the natural host of the virus.
[20] i.e. Not having an effect on V functionality in the context of IFN production attenuation.

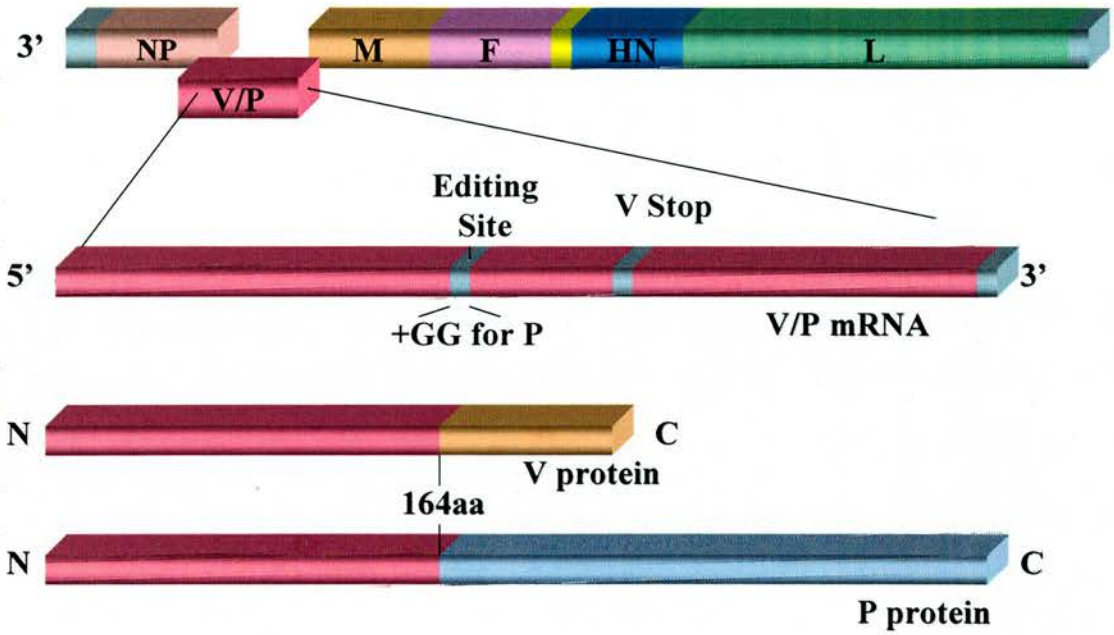


Figure 1.7: Representation of the SV5 genome organisation and its V/P gene expression strategy. The SV5, –ve sense, RNA genome consists of seven genes encoding for eight proteins (NP,V/P, M, F, SH,HN and L). The V/P gene encodes for two distinct proteins through mRNA editing. Whilst V mRNA is a faithful gene transcript, insertion of two non-templated guanine residues (GG) alters the ORF to produce P mRNA.

or point mutations of the C-terminal conserved cysteine residues, disabled the antagonistic effect of SV5 V on IFNβ production.

1.2.4.3 SV5 V blocks IFN production

While SV5 V will antagonize IFN induction through transfected (i.e. intracellular) dsRNA, the disruption is not absolute. Additionally, V protein expression has no observed effect on extracellular dsRNA stimulation or signalling mediated

through TLR3 (Andrejeva et al., 2004). However, the V protein carboxy-terminal domain, of not only SV5 but of most paramyxoviruses (including representatives across all three genera of the Paramyxovirinae), binds specifically to mda5, the IFN-inducible helicase mentioned in the previous chapter, that along with RIG I is an integral part of IFNβ production. This association between paramyxovirus V and mda5 results in the significant inhibition of IFNβ transcription in response to intracellular dsRNA (Andrejeva et al., 2004).

1.2.4.4 SV5 V blocks IFN signalling

It was illustrated before that SV5 V causes disruption of IFN signalling through STAT1 proteasomal degradation. However the underlying mechanics of this inhibition are jointly both points of controversy and also paradigms of ingenious modelling. It has been verified that V binds DDB1 through its C-terminal end, and that this interaction directly correlates with the ability of V to induce STAT1 degradation as demonstrated in studies where siRNA was raised against DDB1 in HeLa cells expressing V. In the face of limited DDB1 expression, STAT1 levels recovered within the cells and IFN signalling was restored, indicating that insufficient levels of DDB1 deprive the associations required by SV5 V for its ability to target STAT1 (Andrejeva et al., 2002). The cellular UV damage-specific DNA binding protein (UVDDB) consists of the 127kDa DDB1 and the 48kDa DDB2 subunits. Under physiological conditions DDB1 is complexed with DDB2[21]. Expression of V in cells derived from patients with the disease disrupts IFN signalling indicating that association with DDB2 is not required for STAT1 proteasomal targeting. Additionally, in non-Xe.P cell lines the association of DDB1 with V inhibits DDB1 interactions with DDB2 (Leupin et al., 2003).

In normal uninfected cells the DDB1 subunit of UVDDB has been shown to interact with Cullin 4a (Cul4a). Cullins are highly conserved proteins required for the assembly of large multiprotein complexes like E3 ubiquitin ligase complexes responsible for polyubiquitination and subsequent proteasomal degradation of substrate molecules. They play a pivotal role in cell cycle control, development

[21] This interaction is impaired in patients suffering from one subtype of the condition Xeroderma pigmentosum

and genomic stability. Cul4a and DDB1 have been reported to form part of an E3 ligase complex regulating c-Jun activity (Hu et al., 2004). Coimmunoprecipitation studies of V and DDB1 also "pull-down" Cul4a. Additionally siRNA raised against Cul4a results in a 20% reduction of V-induced STAT1 degradation (Ulane and Horvath 2002) providing evidence for the direct role of Cul4a in the process of inhibition of IFN signalling.

Before Cul4a was implicated in the degradation apparatus, experiments with STAT2-deficient cells revealed that STAT1 degradation is also dependent on the presence of STAT2 and additionally besides STAT1 and STAT2 no other component of the signalling cascade is required. These findings led to the observation that V targeted STAT1 degradation is irrespective of IFN signalling (Andrejeva et al., 2002). Further experiments on the nature of the interaction revealed that V was not able to induce the degradation of exogenously delivered STAT1 unless the low levels of STAT2 in the cell were boosted by additional transfection of STAT2 genes thus indicating that the degradation of STAT1 not only requires the presence of STAT2 but also that the two transducers need to be expressed in a similar stoichiometry.

These observations based on protein-protein interactions indicate that the V protein of SV5 can induce the formation of a novel ubiquitin ligase complex termed the V-dependent degradation complex (VDC) including STAT1, STAT2 DDB1 and possibly Cul4a. Controversy arises though as to the details of VDC action and assembly. In the core of the controversy is the observation that V can oligomerize to form large spherical structures. In one proposed model of the VDC, V moieties assemble to large complexes, while one molecule of V binds STAT1 and STAT2. The remaining elements (DDB1, Cul4a and possibly Roc1) of the ubiquitination complex are bound to adjacent V molecules juxtaposing the substrate sub-complex with the enzymatic one, allowing more efficient ubiquitin transfer from the E2 ubiquitin-conjugating enzyme to STAT1 (Ulane et al., 2005).

The second working model for the VDC complex, based on in-vitro attempts to reproduce STAT1 poly-ubiquitination, proposes SV5 V as an adaptor in linking DDB1 to STAT2 to facilitate STAT1 targeting. In uninfected cells STATs do not

associate with the enzymatic machinery. Upon infection with SV5, the V protein acts as a linker bringing the enzymatic portion of the assembly in proximity to the STAT1/STAT2 complex. The formation of the E3 ligase complex then requires the recruitment of other cellular proteins (including E1 and UBCH5a) and subsequently catalyzes the addition of ubiquitin to STAT1, thus labelling STAT1 for degradation. Upon dissociation of STAT1 from the complex, STAT2 is either released into the cytoplasm, to recruit further STAT1 molecules, or remains complexed to the rest of the apparatus awaiting novel substrate (Precious et al., 2005).

Contrary to the illustrated importance of the C-terminus of V in the process of IFN antagonism, studies of two canine isolates of SV5 (CPI- and CPI+) differing in three amino-acid positions demonstrated that sequence alterations within the N-terminus can affect the ability of V to disrupt IFN signalling[22] (Chatziandreou et al., 2002). Infection with CPI+ leads to STAT1 degradation and hence a blockade of the IFN signalling cascade in both canine and human cells while CPI- infection does not have any effect on STAT1 degradation, thus making CPI- an interferon sensitive virus. To restore functionality to the V protein of CPI-, sequential mutation of all three residues distinguishing the two strains is required. IFN sensitivity however of CPI- in canine cells resulted in the clearance of the virus from most cells while in the remaining ones CPI- established persistent infections. Interestingly, both CPI+ and CPI- are able to inhibit IFNβ promoter activation, which demonstrates that residues in the N-terminal region of V are not important for IFNβ production attenuation. These observations demonstrate a further property of V, that the abilities of SV5 V to block IFN production and IFN signalling can be clearly distinguished.

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[22] CPI- being the virus with the amino-acid substitutions.

Chapter 1.3

Therapeutics and Innate Immunity

1.3.1 Immune pathways as targets for pharmaceutical intervention

1.3.1.1 *Background*

Historically, medical practice has played a pivotal role in combating a range of infectious diseases, and with the complements of pharmacology the arsenal of diagnostic and therapeutic tools has continuously expanded, offering a range of anaesthetics, antiseptics, antibiotics and vaccines. However, one field, in which chemotherapeutical approaches have traditionally been weak and largely ineffective, extends in the development of agents that deal with viral infections. Vaccinations have provided immunity, comfort and survival in the face of potent killers. Furthermore, through vaccination, forms of global pestilence such as smallpox and largely polio have been eradicated. In contrast, there are but limited approaches in treating patients that have already contracted viral diseases besides suggesting analgesics, the intake of plenty of fluids and rest while the disease is left to run its course.

Since the emergence of AIDS and appreciation of both its devastating and profit-building potential we are witnessing a surge in the development of novel antiviral agents and treatments. These agents, having their roots in the advancements made in the characterization of structure and function of viruses, are products of the greatly expanding insights into the genetic, immunological and molecular function of organisms. Advances in techniques, especially in the fields of robotics, synthetic and biological chemistry, as well as the availability of compounds isolated from natural sources now permit the high throughput screening (HTS) of vast libraries of chemical entities in our search for effective antiviral treatments. With the compounds and the screening methods, currently or potentially, available

the pressure lies on identifying the targets and building viable models to test for antiviral (or anti-inflammatory) activity.

Modulation of immune responses has always been the main goal in the development of medicinal drugs. Traditionally, the targets identified as candidates for chemotherapy are often considered as belonging to the adaptive leg of the immune system. However advances in characterization and increasing information concerning innate immunity present novel grounds for pharmaceutical research especially at the points where both arms of immunity meet. By comprehending the nature of ligand binding to cognate cellular receptors and by elucidating the molecular pathways activated upon initiation of the corresponding signalling cascades we are presented with numerous targets for medicinal intervention.

Innate immunity lies in the forefront of host defense and mediates such diverse responses as: the recognition of infection through recognition of foreign ligands or host signalling molecules (e.g. the IFNs), inflammation, microbial clearance and cell death. All these aspects of innate immunity provide a diverse range of targets for therapeutic agents affecting approaches to chemotherapy on infection, autoimmunity and cancer. The increasingly realized importance of the innate immune system and the opportunities it offers for pharmaceutical research is best illustrated by the U.S. National Institute of Health (USNIH) attempt through a five-year plan to "develop an encyclopaedia of innate immune system activity" (Oplinger, 2003). There are multiple advantages in targeting adaptive immune pathways besides the diverse and pivotal functions within microbial clearance. Microbial resistance is a major player in this approach since modulating the innate immune system and boosting its effectiveness is largely unaffected by this increasingly alarming issue.

1.3.1.2 *TLR and NFκB*

Research on innate immunity pathways has recently been focused on the Toll like receptor (TLR)-gene family and the NFκB signalling cascade. Since analogous research within the IFN field has so far been poor and since both cascades are involved upstream of IFN activation an overview of the approaches utilized for therapeutic targeting of the TLR innate immune system cascades will illustrate how similar approaches can be manipulated for modulating the interferonal response to combat viral infection and autoimmunity. As indicated earlier, signalling from TLRs 3, 4, 9 proceeds through activation of both the NFκB and IRF 3, 5 and 7 pathways for the transcription of genes crucial to the antiviral response including those of the IFNs. Although neither pathway alone critically determines the induction of the response, mutations affecting them all result in substantially decreased antiviral responses (reviewed in Ulevitch, 2004). Stimulation, therefore, by small-molecule or other agonists has the potential of augmenting resistance to viral infections. Research on the TLRs has catalyzed the design of novel candidate immunotherapeutics and vaccine adjuvants, including unmethylated CpG oligodeoxynucleotides (ODNs), either as bacterial DNA or synthetic molecules. ODNs were observed to activate plasmacytoid dendritic cells (PDCs) through TLR9 and hence stimulate IFNα/β production (Krieg 2002). Furthermore, CpG ODNs were found to enhance antigen uptake and stimulation of antigen-presenting cells. These reagents have since been used in clinical trials for a range of viral diseases including HIV. CpG ODNs are regarded most effective as an immune adjuvant in HIV disease in the setting of highly active antiretroviral therapy (HAART)-induced control of HIV replication (Jiang et al., 2005). Interestingly, not only to highlight the relevance of TLR-mediated induction of the IFNs but also to illustrate the importance of research on reagents targeting innate immune pathways, bacterial CpG ONDs were also found to enhance or even trigger pathogenicity through TLR9 in systemic autoimmunity, within the IFNα/β pertinent topic, of lupus (Lenert et al., 2005).

Numerous studies have also taken place on the NFκB signalling pathway. It is possible to use inhibitors of the IKK complex to treat chronic inflammatory

diseases like asthma and chronic obstructive pulmonary disorder (COPD), where sterile inflammation resulting from TLR activation is important (Caramori et al., 2004). While both subunits IKKα and IKKβ of the kinase have been selected as potential targets for therapeutical intervention, most progress has arisen from the development of selective small-molecule inhibitors of IKKβ. Furthermore, a small molecule selective inhibitor of IKKβ was recently identified as an agent preventing oedema formation and pulmonary inflammation in mice (Ziegelbauer et al., 2005). And whilst the list of intracellular inhibitors for IKKβ keeps expanding [including the recent addition of Linus Pauling's favourite drug, Vitamin C (Carcamo et al., 2002)] so does the list of novel intracellular signalling targets both within the NFκB pathway and its downstream elements, especially the IFNs.

As we are now favoured with detailed knowledge on ligands, receptors and signal transducers concerning the IFN pathway, most components of the cascade could be faced as targets for treatment. Since there have been little advances in pharmaceutically targeting either the pathway itself (stimulating it for anti-viral purposes or suppressing it for anti-inflammatory applications) or the viral agonists of the cascade, most of the medicinal applications of the cytokine are focused on supplementing loss of function with exogenous administration of IFN. While IFN treatment was scarce until the 1980s, recombinant DNA technology allowed expression within bacteria, permitting massive increase in production and IFN application. In combination with chemotherapy and radiation therapy, IFN is delivered to chronic sufferers of various types of systemic cancer. However the first example of IFN supplementation in combating a viral disease was for the treatment of Hepatitis C for which the substance was approved in the USA in 1991. Modern IFN treatment takes advantage of the longer half life of pegylated - IFN [(IFN in addition with polyethylene glycol (peg.IFNα)].

The textbook IFN treatment for chronic hepatitis B today is the intramuscular or subcutaneous administration of 5 million units daily or 10 million units thrice a week of peg.IFNα for a total of 16 weeks including treatment with nucleoside analogues like lamivudine and adefovir. The current focus of treatment is to clear

HBV infection by suppressing viral replication. Patient/treatment status is established at any one time by monitoring serum HBV DNA levels, serum alanine aminotransferase, seroconversion and loss of HBV e and surface antigens. Patient total recovery is mirrored by complete loss of the HBV surface antigen in the blood (8% for IFN treated patients or 1% of untreated patients in a year). Due to the nature of the treatment the patient has to be monitored for severe side effects including autoimmune reactions. Hepatitis C is harder to treat and monotherapy with each of the two current treatment options, IFN and ribavirin is disappointing. Prognosis though is markedly improved upon combination of both agents with up to 70% responding[23] to the combined 3 million units of IFN three times a week with ribavirin for less than two years) treatment (Lagget and Rizzetto, 2003).

[23] Note: responding does not mean cured

1.3.2 High Throughput Screening

1.3.2.1 Screening of compound libraries

High throughput screening (HTS) can be defined as the process of identifying molecular entities with biological activity by assaying large collections of chemical compounds. These collections, usually ranging from 10^2 to 10^6, of virtual or existing compounds can be either in-house synthesized or externally obtained. Once the molecular libraries are assembled they can be stored in a 96-well plate (or higher) format in order to be screened against the desired targets. These libraries can range from natural products to semi-synthetic natural product analogues to purely synthetic compounds; assayed as crude extracts involving 10-100s of compounds (this usually applies to natural compound libraries) or as purified single chemical structures. While identification of single entity bioactive compounds in the second case is self evident, involving analytical skills only if the compound has not been characterized, identifying bioactive ingredients within mixtures of molecules obtained from natural sources may involve multiple rounds of chemical purification and bio-assaying. These added levels of complexity encountered by assaying a mixture of compounds have contributed to the decline in pharmaceutical research into natural products.

The development of combinatorial chemistry and the generation of large molecular compound collections has allowed rapid assaying of 'screen-friendly' synthetic chemical libraries. Furthermore the increasing availability of biological (structural and/or functional) information enables increasing target identification prompting for shorter dug discovery timelines. As the range of synthetic possibilities and the number of molecular structures available are continuously increasing the process of compound selection has become overwhelmingly crucial (Oprea, 2000).

1.3.2.2 Characteristics of Natural vs Synthetic compound screening

Between 1981 and 2002 approximately half (49%) of the 877 small molecule New Chemical Entities (NCE) were based on natural products and their analogues (Newman et al., 2003). Despite this success (following the 1970s-1980s trend towards phytochemical approaches to pharmaceutical discovery) the last decades have seen a decline in natural product research. However, unrealized expectations from research and development on fully synthetic molecules for treatment of infectious diseases (a traditionally strong field of natural products), as well as emerging trends on the field, have reversed the balance and we are currently faced with a renewed interest in natural products for lead-compound generation. Compounds for lead generation within the context of HTS should demonstrate high molecular diversity within the boundaries of reasonable drug-like properties. Natural product libraries have been long established as sources of high chemical diversity, biochemical specificity as well as incorporating a broader distribution of molecular properties (molecular mass, octanol-water partition coefficient and diversity of ring systems) (Lee and Schneider, 2001) readily satisfying Lipinskis 'rule of five'[24]. Comparative studies across libraries (reviewed in Koehn and Carter, 2005) have revealed that natural products hold a higher number of chiral centres and increased steric complexity than their combinatorial or synthetic counterparts. Statistical analysis of natural and combinatorial compound libraries has also highlighted some of the differences encountered in their applications on HTS. While synthetic molecules have a higher ratio of aromatic ring structures to total heavy atoms, naturally obtained products exhibit a higher number of solvated H-bond acceptors and donors. Additionally, the two also differ in that synthetic drugs tend to posses a higher number of nitrogen-, suphur-, and halogen-containing groups in contrast to the higher number of oxygen atoms displayed by their natural counterparts.

Current drug discovery focuses on the interactions of small molecules with appropriate macromolecular targets. And while a virtually infinite number of

[24] Analysis of the World Drug Index led to the development of the 'rule of five' identifying several properties generally applicable to oral administering of small molecules. These properties are molecular mass < 500Da, no. of H-bond donors < 5, no. of H-bond acceptors < 10 octanol-water partition coefficient < 5 (Lipinski et al.,2001)

small molecules can readily be synthesized to ensure diversity in the library collection and thereby increase the probabilities for favourable associations, in practice biological systems tend to respond better to structures possessing certain properties collectively termed as 'biological friendliness' (Martin, 2001). And indeed the first large synthetic libraries some of them containing well over a million compounds failed to deliver the expected high hit rates. An insight in to the reasons behind the absence of a satisfactory number of leads even within these diverse libraries comes from structural and genomic data indicating that the number of unique protein folds is much smaller compared to the number of protein families sequence similarity predicts. Instead proteins seem to favour conserved architectures established by molecular evolution (Koehn and Carter, 2005). Naturally obtained compounds or synthetic compounds offering high similarity to them, can therefore be regarded as a favoured population of structures selected by environmental or evolutionary pressure to interact with the natural architectures of biological macromolecules.

AIMS AND OBJECTIVES

As seen in he preceding chapter the IFN response is a pivotal modulator of immunity in the face of viral infection. Furthermore disruption of the delicate balance between IFN-functionality and autoimmunity was seen to play a critical role in the development and progression of diseases like lupus. It was therefore suggested that attenuation of the aberrant IFN production and signalling transduction characteristic of a plethora of autoimmune diseases, could prove beneficial to sufferers. In addition, innate resistance to viral infection and replication as demonstrated by cell cultures proves an impediment to vaccine design and virus isolation from clinical samples.

In this study, disruption of the IFN-signalling pathway through small molecules was seen as an attractive approach in circumventing the problems encountered by undesired/ uncontrolled IFN-cascade activation. In order to identify effectors of the signalling pathways leading to expression of ISGs the first part of this project aimed at the development of assays suitable for HTS of compound libraries. The objectives to be met were the development of the methodology, the theoretical evaluation and the initial assay runs confirming functionality of such HTS system.

IFN- related responses were also described as targets of viral antagonism aiming at the unhindered replication and the establishment of the infection. In accordance to that, viruses unable to circumvent the IFN-responses are effectively disabled in vivo. It was thus suggested that inhibition of the viral antagonists involved in the attenuation of the IFN signalling response would effectively destabilize progression of the infection leading to improved chances of virus-clearance by the immune system. Towards that goal, a series of approaches aiming to the identification of inhibitors of viral antagonists of the IFN response were considered and applied. Again, the objectives of these approaches were the development and theoretical evaluation of system functionality.

PART 2:

MATERIALS AND METHODS

	6 well Plate	*12 well plate*	*24 well plate*	*96 well plate*
Lysis Buffer	300µl	150µl	75µl	25µl
Luciferase Buf.	300µl	150µl	75µl	25µl
Luciferin Buf.	100µl	50µl	25µl	7.3µl
Total Volume	700µl	350µl	175µl	57.3µl

This section outlines the primary methodology that was applied in the experimental part of this treatise.

2.1 Cells and Viruses

2.1.1 Mammalian cell culture

2.1.1.1 Basic cell lines

In this work, cells of human and simian origin were used:

Human cells

- *293*: Human embryo kidney cells, transformed with sheared human Ad5 DNA. Cell line provided by Prof. R.T.Hay, University of Dundee, UK

-*2fTGH*: (Pellegrini et al, 1989; McKendry et al, 1991); diploid fibroblasts. Cell line provided by Dr. S. Goodbourn (St. George's Hospital Medical School, London, UK).

-*Hep2*: Human negroid cervical carcinoma cells. Cell line obtained from the European Collection of Cell cultures (ECACC)

Simian cells

-*Vero*: Simian fibroblast-like cells, originally from kidney cells of *Cercopithecus aethiops* (African Green Monkey). Obtained from ICN Pharmaceuticals Ltd., UK

2.1.1.2 Cell lines with altered properties

In addition to the basic cell lines listed above, cell lines encompassing altered properties were also used:

Externally obtained cell lines

-*Hela57A*: human transformed cell line, derived from cervical carcinoma cells; containing an integrated copy of the 3 × κB ConA luciferase reporter plasmid. Luciferase is expressed upon activation of the NFκB cascade through the appropriate stimulus (e.g. TNF). Cell line provided by Prof. R.T.Hay, University of Dundee, UK

-*A549L*: human epithelial lung carcinoma cell line, containing an integrated copy of ISRE-IFN responsive promoter elements coupled to luciferase. Luciferase is expressed upon activation of IFN signalling. Cell line provided by Günther R. Adolf (NBE Discovery Boehringer Ingelheim Austria GmbH)

Cell lines engineered for the purposes of this project

-*293C4*: As above, but containing an integrated copy of the p(9-27)4tkΔ(-39)lucter-IRESpuro plasmid.

- *A549L/SV5V*: As above; lentivirus-produced containing the integrated V-expressing gene of the Simian Virus 5.

- *A549L/SeV5V*: As above; lentivirus-produced containing the integrated V-expressing gene of Sendai Virus.

-*A549L/NS1Vic*: As above; lentivirus-produced containing the integrated NS1-expressing gene of the influenza A/Victoria/3/75(H3N2L) strain

-*A549L/NS1pr8*: As above; lentivirus-produced containing the integrated NS1-expressing gene of the influenza A/PR/8/34 (H1N1) strain

-*A549L/vIRF1*: As above; lentivirus-produced containing the integrated vIRF1-expressing gene of the Human Herpes Virus 8 (Kaposi's Sarcoma Virus).

The preparation of these cell lines is discussed below.

2.1.1.3 Cell Culture

Maintenance of cell cultures

Cell cultures were maintained in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen Ltd., UK) supplemented (except were noted otherwise) with 10% foetal calf serum. This solution, in the text will be addressed as "normal medium". Cells were routinely passaged, trypsinised (Trypsin, EDTA; Becton Dickinson UK Ltd.) and diluted according to the growth rate of the cell line.

Storage of cultured cells

Adherent cells in 75cm^2 were trypsinised, resuspended in 1ml of normal growth medium, pelleted at 400x*g* for 5mins and resuspended in 10ml of freezing medium (10%DMSO, 30%FCS in DMEM). The resulting cell suspension was aliquoted into the required number of pre-labelled, sterile cryovials (1ml/vial) ensuring firm application of the lid. Vials were placed in polysterene boxes and left at -70°C for 16hrs before being transferred into liquid nitrogen containment for long-term storage.

Resuscitation of stored cells

Cryovials were removed from liquid nitrogen and the cells rapidly thawed in 30second cycles between a 37°C water bath and room temperature. The thawed cells were

centrifuged at 250xg for 5 minutes. The resulting cell pellet was removed from its freezing medium (through aspiration of the supernatant) and was resuspended in 1ml of normal growth medium. The suspension was tranfered into appropriate flasks containing normal growth medium and allowed to recover at37°C for 24hrs. After recovery the medium was replaced to remove DMSO traces of the freezing medium.

2.1.1.4 Transfection of mammalian cells

Adherent mammalian cells were tranfected with DNA 24hrs after trypsinisation using the Fugene™ 6 reagent (Roche Diagnostics, UK) according to the manufacturers instructions

2.1.2 Viral vectors and infection of mammalian cell lines

2.1.2.1 EMC virus

For the virus-titre reduction (VTR) assays cells were infected with 5pfu/μl of Encephalomyocarditis virus in the presence of varying concentrations of IFN.

2.1.2.2 Preparation of adenoviral delivery vectors

Production of Adenovirus

The ISRE-luciferase region of the p(9-27)4tkΔ(-39)lucter plasmid was inserted into the pV60 adenoviral plasmid encoding for a minority of adenoviral gene. The resulting plasmid pV60-luc, in order to produce infectious adenovirus needed to recombine with the p17 adenoviral plasmid containing the majority of the adenovirus genome plasmid.

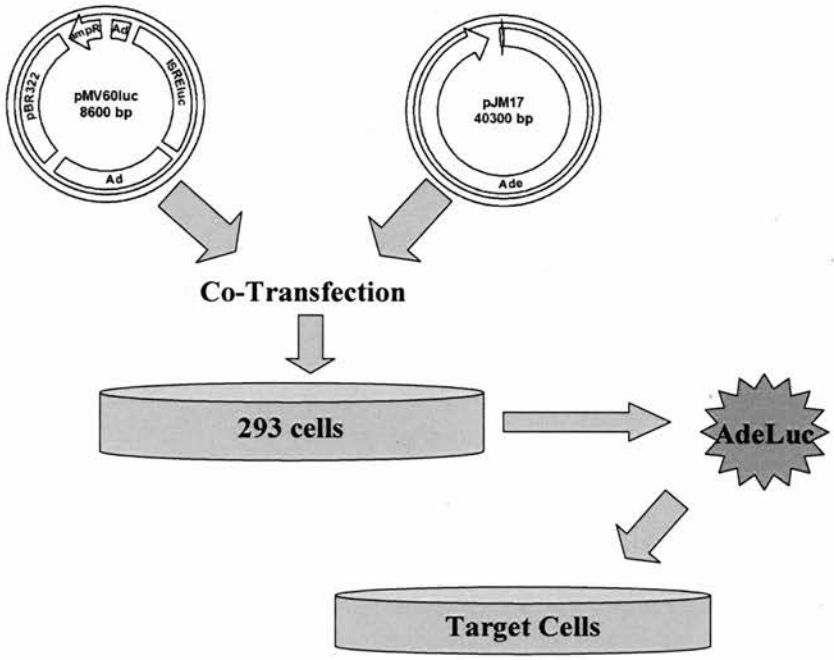


Figure 2.1: Schematic Representation of recombinant adenovirus production. 293 cells are co-transfected with pMV60 plasmid containing the desired genes and pJM17 containing the majority of Ade5 genome. The resulting transduction produces infectious adenovirus which can be used for gene delivery to target cells.

293 cells were grown to 60% confluence in 75cm2 flasks. The cells were subsequently co- transfected with the two adenoviral plasmids [pV60 (either containing the ISRE-luciferase encoding sequences or the V protein of SV5) and pJM17] using the Fugene™ 6 transfection protocol. The cells were incubated with normal growth medium in their native flasks until the observance of an adenovirus-induced cytopathic effect (C.P.E.). The C.P.E. was observed 10 days post infection. Medium from these flasks was removed and applied onto fresh monolayers of 293 cells, grown in 6 well plates, to screen for reporter expression. On confirmation of luciferase activity through adenoviral delivery the original infected 293-containing stock was removed from their flasks by gentle tapping and decanting into 15ml falcon tubes. The suspension was centrifuged at 400rpm for 10mins after which the supernatant was removed. The pellet formed by the infected 293 cells was washed once with PBS. The pellet was then resuspended in 1ml of PBS and

1 ml of Arcton and the suspension was vigorously shaken for 30mins. After subsequent centrifugation at 2000r.p.m. three layers were formed with the aqueous containing the adenovirus.

Adenovirus purification through plaque assays

293 cells were seeded in 6 well plates and gently rocked to ensure equal distribution of cells. The cells were grown in normal growth medium until 80% confluence was attained. The media was subsequently replaced with 2%FCS containing medium and the cells were infected with serial dilutions of the original adenoviral stock (see section on the production of adenovirus) and incubated with CO_2 within a gas box at 37°C for 2hrs on a rocker. At 2hrs post infection the supernatant was replaced with overlay medium (DMEM;2%FCS containing 3% agararose and 8mM $MgCl_2$). The medium solidified at room temperature after 30mins and the plates were transferred to 37°C/5%CO_2 incubators. The cells were allowed to incubate for 6-8 days until plaques (resulting from the infection of individual adenoviral clones) formed onto the monolayers. Material from the plaques containing the purified virus was removed and applied onto fresh 293 monolayers for amplification. The amplified virus was extracted with Arcton (as previously described) and aliquots were applied onto Vero monolayers to screen for expression of the genes of interest.

2.1.2.3 Preparation of Lentiviral delivery vectors.

Recombinant Lentivirus encoding desired sequences was produced by the co-transfection of 4.5μg of a transfer vector pDlNotIpuro (carrying the genes of interest) with 3μg of the packaging plasmid pCMVR8.91 (expressing the HIV-1 gag/pol, tat and rev genes) and 3μg of the envelop plasmid pMD-G (expressing VSV-G genes). The plasmid "cocktail" was tranfected using the Fugene™ 6 protocol into 293T; 80% confluent monolayers grown in normal growth medium in 75cm2 flasks. At 24hrs post transfection the medium was replaced with 10ml of fresh medium. The supernatant containing the produced

lentivirus was collected after a further 48hr and a 72hr period. The supernatant was centrifuged at 2000xg for 5mins and was subsequently passed through 0.45μm filters (Tuffrin Membrane, Life Science, UK). In order to concentrate the virus in solution the filtrate was transferred into VIVASPIN-20 tubes (100,000 MWCO PES; VIVASCIENCE, UK) and centrifuged at 3000xg. The virus was aliquoted in 1ml vials and stored in -80 °C.

For determining viral titres, Hep2 cells (30% confluent in 6-well plates) were infected with serial dilutions of the viral stock in the presence of 8μg/ml Polybrene for 48hrs and subsequently selected with 2μg/ml puromycin. After a 10 day selection period, plates were fixed (Fixation solution: 10% v/v Formaldehyde) for 2hrs and subsequently stained with crystal violet dye (1% w/v crystal violet, 20% v/v ethanol, 1% v/v methanol)for 10mins. After washing the surviving colonies were counted and the viral titre was calculated as colony forming units (c.f.u.)/ml

2.1.3 Preparation of stable cell lines

2.1.3.1 Preparation of stable mammalian cell lines by transfection

To produce a 293 mammalian cell line permanently expressing the genes of interest, monolayers of the target cell line were grown in a $25cm^2$ flask to 50-75% confluence and transfected with 3μg of the DNA encoding for the protein of interest using the lipid-based Fugene™ 6 transfection protocol. The plasmid DNA constructs were based on the pIRESpuro2 and p(9-27)4tkΔ(-39)lucter plasmids, facilitating the selection of transfected cells in the presence of IFN as described below. At 24hrs post transfection, the cells were diluted and plated out into 60cm Petri dishes at varying dilutions. The culture media was subsequently supplemented with 10^4 i.u./ml of IFN (rHuIFN-a_/D) to stimulate the

inducible expression of puromycin resistance sequences. The medium was supplemented with varying concentrations of puromycin after 24hrs . The cells were incubated under these conditions for 4 days after which the medium was replaced with normal growth medium for 3 days to allow cell growth (incubation with IFN prevents cell replication) regular changes of IFN- , IFN/puromucin- containing and normal growth media for 56 days until he formation of resistant colonies was apparent. The individual colonies were picked, amplified and screened for expression of the gene of interest. The colony providing satisfactory reporter activation was re-selected in a further 56-day selection cycle.

2.1.3.2 Preparation of stable mammalian cell lines by lentiviral delivery of genes of interest.

A549 target cells were grown in a $25cm^2$ flask to 50-75% confluence. The cells were transduced with the recombinant lentivirus of interest (m.o.i of 0.1) in the presence of 8μg/ml Polybrene in normal growth medium. At 48hrs post infection (p.i.) the infected cells expressing the genes of interest and a selection marker (puromycin resistance) were selected with varying concentrations of puromycin. The cells were incubated in these conditions for 14 days until the formation of resistant colonies was apparent. These colonies were subsequently trypsinised together so as to create a "mixed" population of resistant cells. The mixed cell line, without colony isolation procedures, can result in the homogenous expression of the gene of interest by 95% of the cells.

2.2 Protein Analysis

2.2.1 Immunofluorescence

For immunofluorescence analysis, cells were grown on 10mm-diameter coverslips (General Scientific Co. Ltd., UK). A detailed account of the staining procedure can be found in Randall and Dinwoodie, 1986. Cells were primarily treated with fixing solution (5% v/v formaldehyde, 2% w/v sucrose in PBS) for 10 min, then permeabilised (0,5% Nonidet-p40, 10% w/v sucrose in PBS) for 5 min and washed repeatedly in 1% v/v FCS;PBS. To detect the proteins of interest, cell monolayers were incubated with 10-15 μl of appropriately diluted SV5-P-k (Randall et al., 1987) antibody for 1hr (primary antibody). Cells were subsequently washed (1% v/v FCS in PBS) several times and incubated for 1hr with a secondary goat anti-mouse Ig Texas Red-conjugated antibody (Seralab, UK) and DAPI (0.5μg/ml; Sigma-Aldrich Co Ltd., UK) for nuclear staining. All reactions were performed at room temperature and antibody treatments were allowed to progress in a humidified chamber in order to prevent desiccation of the monolayers. Following staining, samples were washed with PBS, mounted with coverslips using Citifluor AF-1 mounting solution (Citifluor Ltd., UK) and examined under a Nikon Microphot-FXA immunofluorescence microscope.

2.2.2 SDS-PAGE and Immunoblotting

2.2.2.1 SDS-PAGE

Protein samples were prepared in gel electrophoresis sample buffer (50mM Tris-HCl pH 7.0, 0.2% SDS, 5% 2-mercaptoethanol and 5% glycerol) and heated at 100°C for 5mins prior to electrophoresis analysis. Polypeptides were separated through SDS-PAGE (7-12% bis-acrylamide) in 0.75mm mini-slab gels of the Bio-Rad mini-protean II

electrophoresis system, by electrophoresis at 150 to 180V until maximum resolution of polypeptide bands.

2.2.2.2 Immunoblotting

Cells were washed twice in PBS prior to harvesting and subsequently lysed in gel electrophoresis sample buffer. Cell lysates were then sonicated and heated at 100°C for 5mins. Samples were analysed by SDS-PAGE, and the proteins were transferred to nitrocellulose membranes using a Trans-Blot Cell (Bio-Rad, UK), assembled according to the manufacturers instructions. The apparatus was submerged in transfer buffer (0.025M Tris, 0.19M glycine, 20% v/v methanol) in a tank and run at 200mA for 2hrs. Following electroblotting, the membrane was rinsed with de-ionised water and incubated in blocking buffer (10% w/v skimmed milk powder, 0.2% v/v Tween-20; PBS) for an hour to block non-specific binding sites. Proteins of interest were detected with the appropriate antibodies by incubating the membrane in antibody suspension (1/1000) dilution in blocking buffer for 1hr. The membranes were subsequently washed thrice in 0.2% v/v Tween-20; PBS, incubated in blocking buffer and prior to secondary antibody reaction (1/200 in blocking buffer) the membrane was rinsed another three times. The membrane was left to react for 1hr on a rocker. The membrane was subsequently washed and protein bands were visualized through enhanced chemiluminescence (ECL) (Amersham Biosciences Ltd., UK) according to the manufacturers instructions.

2.2.3 Protein expression

2.2.3.1 Expression of GST-fusion proteins

E. coli cells were transformed with a GST-V construct (bacterial transformation described in 2.3.2.2) and a single colony was inoculated with 10ml liquid Luria Bertani (LB) medium (10g/L bactotryptone, 5g/L yeast extract, 10mM NaCl, ph7.0) with ampicillin and incubated at 37°C for 16hrs. Cells were subcultured at 1/100 in 2L and

incubated at 37°C with shaking until the density of growth reached an OD600=0.5. Cells were induced with 1mM IPTG and incubated at 30°C with shaking for 3hrs. Cells were harvested by centrifugation at 4,500 x g for 10mins at 4°C. The resulting cell pellet was re-suspended in cell re-suspension buffer (50mM Tris-HCl, pH 8.0, 200mM NaCl, 1mMDDT) containing protease inhibitors (Comp. Mini EDT-free Protease Inhibitor tablets, Roche Diagnostics Ltd., UK) 500µl was used for a 10ml test culture, 7ml for 1L and 14ml for 2L culture. Cells were broken using sonication (10 amplicons, in 3 cycles of 30sec on ice) Lysates were ultracentrifuged at 15,000 rpm for 3hrs. The protein containing supernatant was removed, filtered (0.45µm) and stored on ice prior to further purification.

2.2.3.2 Glutathione-Agarose Preparation

Glutathione-agarose beads were prepared by the addition of 83mg glutathione-agarose (Sigma-Aldrich Co.,UK) to 16ml of sterile distilled water and allowed to swell at room temperature for 30mins. The beads were washed in 10ml distilled water, subsequently pelleted and resuspended in 2ml of distilled water, resulting in a 50% suspension. Glutathione-agarose columns were prepared by loading a suitable amount of 50% bead suspension into a 2ml column (Pierce Biotech. Inc.). The beads were allowed to settle and were subsequently washed.

2.2.3.3 Purification of GST-V

The soluble protein extract obtained at 2.2.3.1 was applied to the glutathione-agarose columns described above. The flow through was collected and re-applied to the column so to ensure maximum binding of GST-V onto the beads. The column was washed with 10 column volumes of the appropriate wash buffer (50mM Tris-HCl, pH 8.0, 200nM NaCl) until no protein could be detected in 5µl of flow-through applied onto nitrocellulose strips and stained by Methylene Black. GST-V was eluted from the column with 10mM Glutathione in 1ml fractions. Staining by Methylene Black onto

nitrocellulose determined the peak fractions. The resulting protein was quantified by SDS-PAGE against standard dilutions of BSA.

2.2.4 Luciferase reporter assays and determination of relative protein expression levels.

Cell monolayers to be assayed for luciferase reporter activity in 6-, 12-, 24-, and 96 well plates were carefully washed once with PBS. The cells were subsequently lysed with corresponding volumes (Table 2.1 lists the buffer volumes used across the range of assay formats) of Lysis buffer (25mM Tris phosphate pH7.8, 8mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1% v/v Triton X-100) for 2mins on a rocking platform. After lysis, ATP containing Luciferase Buffer (30% glycerol, 0.8mM ATP, 2% BSA, 12.5mM Tris phosphate pH7.8, 4mM $MgCl_2$, 0.5mM DTT, 0.5mM EDTA, 0.5% v/v Triton X-100) was added to the samples. The extract was transferred to a luciferase curvette and luciferin pigment was supplemented (1.5mM in TE buffer) by the appropriate injector. Pigment injection and subsequent (10sec delayed) luminescence measurements were performed by a luminometer (VICTOR^2 1420; Perkin Elmer Lifesciences)

Table 2.1 Reagent/Buffer volume against corresponding assay format

	6 well Plate	*12 well plate*	*24 well plate*	*96 well plate*
Lysis Buffer	300µl	150µl	75µl	25µl
Luciferase Buf.	300µl	150µl	75µl	25µl
Luciferin Buf.	100µl	50µl	25µl	7.3µl
Total Volume	700µl	350µl	175µl	57.3µl

2.3 Nucleic acid analysis and processing

2.3.1 Plasmid DNA

2.3.1.1 Plasmid vectors and constructs

Mammalian cells were tranfected with the *Luciferase reporter plasmid*, in order to perform luciferase activity measurements in accordance to the requirement of the experiment. Furthermore the reporter and its promoter were extensively used in the engineering of constructs in the creation of adenovirus and the creation of stable cell lines. ***Backbone plasmids*** were the basic plasmids used for the construction of the various vectors. ***Plasmids encoding for viral proteins*** were used for the construction of lentivirus capable of delivery of these genes into target cell lines. Finally virus production plasmids were the plasmids encoding for essential genes in the production of lentivirus and adenovirus. This section will list the starter plasmids used for cloning while individual cloning strategies appear below as a separate section.

Inducible luciferase reporter plasmid

- IFN-a/b-responsive plasmid; *p(9-27)4tkΔ(-39)lucter* (King and Goodbourn, 1998): the plasmid contains four tandem repeat sequences of the ISRE from the IFN-inducible gene, 9-27 fused to the firefly luciferase gene. Provided by Dr. S. Goodbourn (St. George's Hospital Medical School, UK)

Backbone Plasmids

- *pIRESpuro2*; The plasmid vector used for the production of stable cell lines (see above) The plasmid enables the expression of the puromycin resistance gene from a single

transcript (the puromycin resistance gene is translated from an internal ribosome entry site (IRES) derived from the encephalomyocarditis (EMC) virus) under the control of the human cytomegalovirus (hCMV) major intermediate early promoter (Clonetech UK)

-pMV60 adenoviral plasmid: Adenovirus type 5 vector containing part of adenovirus genome including a promoter-less HindIII cloning site (Wilkinson and Akrigg, 1992)..

Plasmids encoding for viral proteins

-*pEF.SV5V*; plasmid encoding for the V protein of the SV5 W3 wild-type strain. Its construction has been reported by Didcock and colleagues (1999)

-pCA14/V Adenoviral SV5-V transfer plasmid containing the left inverted terminal repeat (LTR), packing signal and sequence overlapping the pJM17 adenoviral plasmid (described below) Obtained from Yun Hsiang Chen, University of St. Andrews, UK.

-pLenti V, SeV, Vic75NS1, pr8NS1, HHV8vIRF1. Lentivirus vectors based on the pDlNotIpuro vector, constructed by and obtained from Yun Hsiang Chen, University of St. Andrews, UK

Virus production plasmids

-pJM17;Adenoviral plasmid containing most of the viral genome of Ad5 dl309. The vector lacks the DNA packaging signal of the E1 region. Plasmid was obtained from Yun Hsiang Chen, University of St. Andrews, UK

- pCMVR8.91; Lentiviral derived vector expressing the HIV-1 gag/pol, tat and rev genes

- pMD-G: envelope vector required for the production of infectious lentivirus (expressing VSV-G genes)

2.3.1.2 Preparation of plasmid DNA

For small scale preparations, bacterial cultures of 5ml were grown for 16hrs at 37°C on a shaker. DNA was extracted from cells using the Qiagen DNA mini-prep (Thermo Hybaid Limited, UK) or the FastPlasmid Mini (Eppendorf, UK) kits according to the manufacturers instructions.

For large scale preparations, bacterial cultures of 500ml were grown overnight at 37°C on a shaker. Similarly to small scale preparations, DNA was extracted from cells and purified on silica gel membrane columns, using the Qiagen DNA maxi-prep kit (Thermo Hybaid Limited, UK according to the manufacturers instructions.

The prepared DNA was quantified by measuring sample OD260nm (where 50ng/µl for OD260nm=1) in a UV spectrophotometer (UVICON 923, Bio-Tek Kontron Instruments Ltd., UK). The purity (in terms of protein carry-over) of the preparation was assayed by calculatingOD260nm/OD280nm ratio (preparations OD260nm / OD280nm $\leq$ 1.8 were deemed unacceptable). Quantifications were confirmed by agarose gel electrophoresis (described below).

2.3.2 DNA processing and cloning

2.3.2.1 Polymerase chain reaction (PCR)

DNA sequences were amplified using: Taq polymerase (Promega UK Ltd.) Vent polymerase (New England Biolabs UK Ltd.) or the in-house produced PfuI (Obtained from Dr P. Coote, University of St. Andrews, UK) according to the manufacturers recommendations on a GeneAmp PCR System 2400 (Applied Biossystems).

Oligonucleotide primers used in PCR reactions (obtained from Oswel Research Products Ltd., UK), were designed taking into consideration a number of parameters: GC content/frequency at the starting and terminal region of the oligonucleotide, melting temperature T_M , sequence variation and finally restriction enzyme site usage.

Information about primers used in the present study are included in the Cloning Strategies sub-chapter in the end of the Materials and Methods section.

2.3.2.2 Restriction enzyme digestion of DNA

Analytical restriction enzyme digestion

In analytical restriction enzyme digestions 1-3μg, of DNA were incubated with 2 units of the appropriate restriction enzyme/s (Promega UK Ltd. Or New England Biolabs UK Ltd.) with the corresponding reaction buffer in a total volume of 10μl made up by the addition of de-ionised water. The reactions were held in the appropriate temperature for 2-4hrs.

Preparative restriction enzyme digestion

For Preparative restriction enzyme digests, 1-10µg of DNA was digested with similar conditions as for the analytical reaction to a total reaction volume of 50µl.

2.3.2.3 Vector Dephosphorylation

Calf Intestinal Alkaline phosphatase reaction

Following digestions of the vector and insert DNA, the vector was de-phosphorylated with 0.01u/pmol-of-ends of Calf intestinal alkaline phosphatase CIAP (Promega UK Ltd.). The dephosphorylation reaction included CIAP buffer (Promega UK Ltd.) made up to the desired volume with deionised water. The reaction was carried out for 15mins at 37°C, 15mins at 56°C and after a further addition of 0.01u/pmol-of-ends of CIAP for 15 mins at 37°C followed by a further, 15min incubation at 56°C.

Shrimp Alkaline Phosphatase reaction

Alternatively, dephosphorylation was carried out by 1u/ml of Shrimp Alkaline Phosphatase (SEP). The dephosphorylation reaction included SAP buffer (Promega UK Ltd.) made up to the desired volume with deionised water. The reaction was carried out for 15mins at 37°C followed by incubation at 65°C for a further 15mins.

Prior to DNA construct ligation reactions, digested and dephosphorylated DNA fragments were purified by using Qiagen silica membrane gel columns (Thermo Hybaid Limited, UK) according to the manufacturers instructions.

2.3.2.4 Agarose gel electrophoresis

DNA was analysed by gel electrophoresis in horizontal mini-gels of 0.7-1% w/v agarose (Sigma-Aldrich Co Ltd., UK) in TBE buffer (0.045M Tris-borate, 0.001M EDTA). Prior to electrophoresis, DNA samples were mixed with the appropriate volume of DNA loading buffer (Promega UK Ltd.). Samples were run along with DNA MW markers (1kb ladder; Promega UK Ltd.) at 70-100 V in TBE buffer supplemented with 1μg/ml ethidium bromide. After the desired band resolution was attained electrophoresed DNA was visualized through UV irradiation (Gel Doc 2000 UV transilluminator/photography system; Bio-Rad, UK). If the samples were to be subsequently used for cloning UV exposure of the gel was kept to a minimum to prevent the formation of thymidine dimers.

2.3.2.5 Purification of DNA fragments from agarose gels

DNA fragments of interest, analysed by gel electrophoresis, was obtained by excision of the resolved bands from the gel and subsequent purification on Qiaquick gel extraction columns (Thermo Hybaid Limited, UK) according to the manufacturers instructions.

2.3.2.6 DNA ligation reactions

DNA fragments containing cohesive ends obtained after gel purification were pooled together (molar ratios of insert/vector 2-5 / 1), heated for 5minsat 45°C to disrupt potential annealing of cohesive ends and incubated in ice to prevent reannealling. Following this procedure, 1 unit of T4 DNA ligase and the appropriate buffer (Promega UK Ltd.) was added to the mixture. The reaction was optionally supplemented with water to a final volume of 10μl and incubated at 16°C for 16hrs.

2.3.2.7 Nucleotide sequence analysis

Nucleotide sequence analysis was performed on purified DNA samples by the fluorescent dideoxynucleotide method, using an ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer Life Sciences Ltd., UK). Analysis was carried out by Mr A. Houston at the (now defunct) DNA Sequencing Unit of the University of St. Andrews. Nucleotide sequence data were processed using a variety of computer algorithms including Gene Jockey II Sequence Processor (BioSoft, UK), DNA™ Strider 1.2 (C. Marck, France)

2.3.3 Bacterial transformations

2.3.3.1 Bacterial cell culture

The bacterial strain used as hosts for gene expression, cloning and transformations was the *Escherichia Coli* (*E. Coli*) strain DH5α [*supE*44, Δ*lac*U169(ϕ*80d lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *rel*A1]. Bacteria were grown in liquid Luria-Bertani (LB) medium containing 10g/l bacto-tryptone (Becton Dickinson UK limited), 5 g/l yeast extract (Becton Dickinson UK limited) and 10mM NaCl pH7.5, or plated on solid LB medium supplemented with 1.5% w/v agar (Becton Dickinson UK limited) and 1M $MgSO_4$ in 90 mm-diameter Petri dishes (Scientific Laboratory Supplies Ltd., UK). When required media were supplemented with ampicillin (100μg/ml)

2.3.3.2 Preparation of competent bacterial cells

Fresh bacterial 10ml cultures were diluted a 100-fold in fresh LB medium and incubated at 37°C in a shaking incubator. When the cell density reached an OD600nm of 0.5-0.7 units the cells were removed from their incubator and were placed on ice for 20mins. The

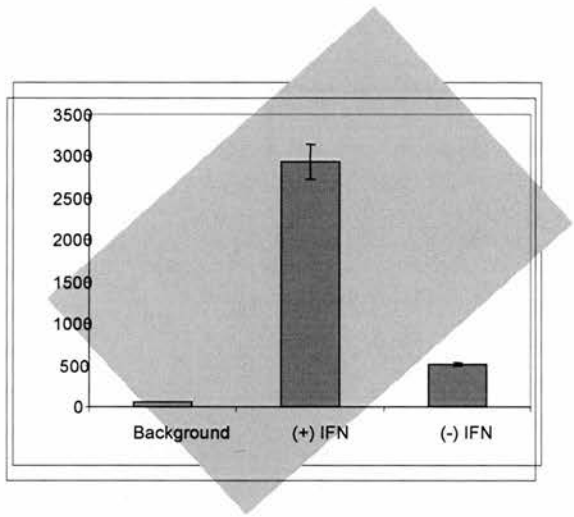
cultures were subsequently centrifuged at 3500rpm for 15mins and the cell pellet was resuspended in 15ml of filter-sterilised TFB1 buffer (30mM KOAc, 10mM $CaCl_2$, 50mM MnCl, 100mM RbCl, 15% v/v glycerol, the pH was adjusted to 5.8 with acetic acid). The suspension was incubated on ice for 30 min before centrifugation 3500rpm for 15mins. The pellet was resuspended in 4ml filter-sterilised TFB2 buffer (10mM MOPS pH 6.5, 75mM $CaCl_2$, 10mM RbCl, 15% v/v glycerol, the pH was adjusted to 6.5 with KOH). The cell suspension was aliquoted, quick-frozen in liquid nitrogen and stored at -70°C.

2.3.3.3 Transformation of competent bacterial cells

DNA (1μg) was applied directly to heat-shock competent ice-thawed bacteria and the cells were left to incubate on ice for 30mins. Cells were subsequently immersed in a 42°C water bath for a heat-shock treatment of 90 sec. The suspension was re-incubated on ice for 5mins. The cells were subsequently introduced, in order to allow expression of their newly acquired ampicillin resistance genes, to 500μL of LB medium and were incubated with shaking at 37°C for 1hr. The culture was centrifuged for 1 min at 13000 x g and the resulting pellet was subsequently resuspended in 100μl LB medium. The cells were then plated onto solid LB-agar medium containing ampicillin in 90mm diameter Petri dishes. The dishes were incubated at 37°C for 16 hrs before ampicillin-resistant colonies could be visualized and manipulated

PART 3:

RESULTS



Having overviewed the mechanisms, the potency and the significance of the IFN response in disease (both viral and autoimmune) we are presented with a system that regulates a number of important immune actions. Manipulation of such a system through small molecule effectors could potentially yield diverse benefits. For example, inhibition of the response in cell lines involved in vaccine production/development could in fact permit increased viral yields of vaccine-strain viruses. Furthermore, antagonism of the response in patients with SLE could alleviate symptoms developed in the progression of the disease. Alternatively, inhibition of viral-interferers of the IFNs would render the replication of virus in infected cells defective and permit the institution of the "antiviral state" in uninfected cells.

Identifying small molecules that tamper with the IFN responses can be achieved by high throughput screening of combinatorial or natural product libraries. An optimal assay for ascertaining effectors of the response would encompass three properties: it should permit accurate large scale screening of numerous compounds, the time scale for each screening cycle should be rapid and finally each cycle should proceed through limited and preferentially automated steps. As for the technical requirements of such a system, these properties translate into: the assay has to permit screening of commercially and in-house obtained libraries usually appearing in 96- or 384- well plates (with the ensuing restrictions such formats impose on reagent volume, handling etc.), within a limited time scale one must be enabled to screen the maximal number of compounds and finally the automation of the system and the near-synchronous introduction of compounds/stimuli to the assay (for example: while it is possible to manually pipette 384 compounds into 384 well plates it is not desirable).

Presented in this part (3) are the approaches utilized and the results obtained in the development of assays accommodating these principles both as means for identification of compounds with anti-IFN properties (3.1) and compounds with anti-viral activity (3.2, 3.3). The nature of this work (as a technical evaluation of functionality across a range of approaches to HTS) demands a certain amount of overlapping between the preceding chapter on materials and methods and the results. Therefore, methods (or protocols) essential to assay development that are novel or are regarded as refinements on the basic approaches outlined in Chapter 2 are included in their appropriate sections below.

Chapter 3.1

Development of Cell Based Systems for High Throughput Screening of Compounds with anti-IFN properties

A key step in the development of assays permitting the HTS of compound libraries is the ability to quantify cellular processes in response to compound introduction. The approach taken in this part of the project utilizes the properties of two reporter systems: luciferase (3.1.1) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (3.1.2). Each will be discussed separately below, while the final leg of this section (3.1.3) contains results obtained from taking advantage of a combined approach to the screening of compounds and the identification of inhibitors of the IFN response.

3.1.1 Luciferase reporter-based assays

In order to distinguish between a functional and a disrupted IFN signalling cascade in compound-treated cells, bioluminescence through firefly luciferase was utilized. The common principle behind the assays described below is the coupling of interferon signalling response elements (ISRE) to *Photinus pyralis* luciferase encoding sequences. When cells are stimulated with IFN and the signal is effectuated at the level of ISRE activation, the luciferase gene is expressed. Luciferase, catalyses the oxidation of luciferin pigments. The energy input of this reaction (in the form of ATP) is transformed into light, quantifiable through equipment measuring luminescence (luminometer) (Schematic 3.1). Respectively, in the absence of IFN stimulation or through disruption of the signalling cascade leading to ISRE activation, the cells fail to demonstrate inducible-reporter expression.

Schematic 3.1.1 Luciferase bioluminescence: IFN signaling activates its corresponding ISRE sequences which in turn promote the expression of luciferase genes. Luciferase catalyses the oxidation of the supplemented luciferin pigment in a two step reaction to yield oxyluciferin. The energy supplied to the reaction in the form of ATP is converted to light. The reaction proceeds as follows:

Luciferin combines with adenosine triphosphate (ATP) to form luciferyl adenylate and pyrophosphate (PPi) on the surface of the luciferase enzyme. The luciferyl adenylate remains bound to the enzyme

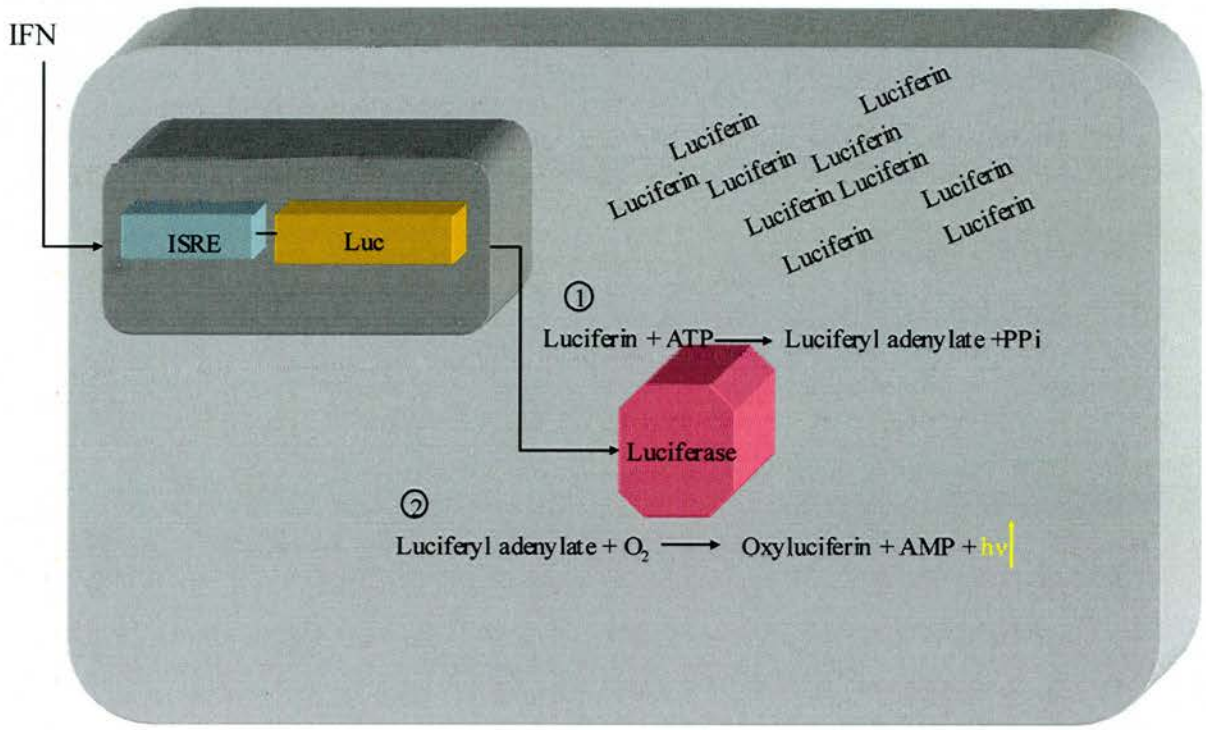
1. luciferin + ATP → luciferyl adenylate + PPi

The luciferyl adenylate combines with oxygen to form oxyluciferin and adenosine monophosphate (AMP). Light is given off and the oxyluciferin and AMP are released from the enzyme's surface:

2. luciferyl adenylate + O_2 → oxyluciferin + AMP + light

Light emitted by the process can then be quantified by luminescence measuring equipment.

Sch. 3.1


IFN
ISRE
Luc
Luciferin
Luciferin
Luciferin
Luciferin
Luciferin
Luciferin
Luciferin
Luciferin
Luciferin
Luciferin
①
Luciferin + ATP → Luciferyl adenylate +PPi
Luciferase
②
Luciferyl adenylate + O_2 → Oxyluciferin + AMP + hν

3.1.1.1 Transient transfections of luciferase reporter plasmids

The first strategy for delivering ISRE-luciferase sequences in cells in order to quantify their responses to IFN treatment was the transient transfection of an IFNα/β-responsive reporter plasmid p(9-27)4tkΔ(-39)lucter (King and Goodbourn,1998) into 293 cells. As mentioned above, one of the properties of an ideal screening system is the ability to screen candidate compounds in a format suitable for automation. Furthermore, for an economically viable screening model, the format of the screening system must be such that allows maximum compound screens against the average total cost (ATC) [25] of the assay. With these requirements in mind, the assay was conducted in two formats differing in their surface areas (a 6-well and a 96-well format). The 6-well plate (large surface area) format would be used as a positive control demonstrating general system functionality. Similarly, quantifiable differences in inducible-reporter expression, in the presence or absence of IFN stimulation, in the smaller format (96-well plate), would demonstrate the systems' suitability for economically meaningful automation.

293 human cells in 75cm^2 flasks were transfected with 6μg of p(9-27)4tkΔ(-39)lucter plasmid. Cells were subsequently trypsinised and seeded in 6-, and 96-well plates. After a 24 hour recovery period cells were induced with IFN for 4 hours. The resulting expression of luciferase was measured by inducing luminescence after cell lysis as described in the materials and methods section.

The responses (Figures 3.1.1, 3.1.2) indicate the functionality of the assays within these specific assay formats. While the difference between the presence and the absence of an IFN stimulus is apparent and quantifiable in the 6-well plates (the largest format) (Figure 3.1.1), as the format is decreased so is the sensitivity of the

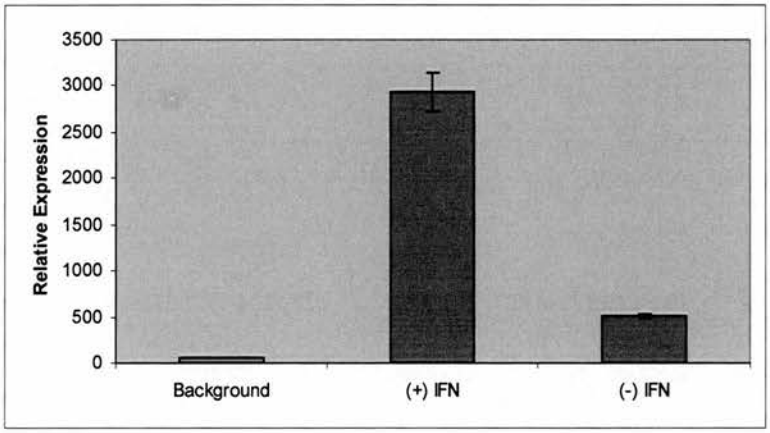
[25] Average total cost (in this context) is the sum (Σ) of all the screening costs divided by the number of units (or compounds) screened (Q, level of output). $ATC = \Sigma cost / Q$

Figure 3.1.1[1] (left)**: Transient transfections of p(9-27)4tkΔ(-39)lucter (pISRE-luc) reporter into 293 cells grown in 6 well plates**. 293 cells grown in 75cm^2 flasks were transfected with 6 μg of the pISRE-luc. reporter plasmid. Cells were trypsinised 24 h post-transfection and seeded in 6-well plates. After a 24 h recovery period the cells were stimulated with 2x10^4 i.u. of IFN for 4hrs before lysis and luciferase activity measurements. The figure demonstrates the luciferase activity measurements obtained when cells were incubated with or without IFN.

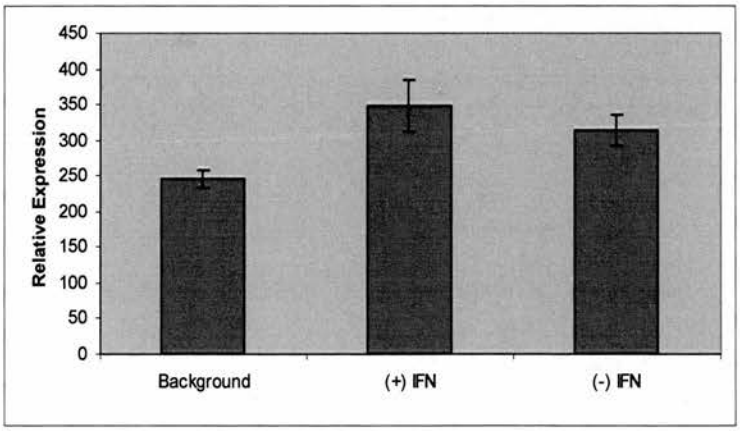
Figure 3.1.2[1] (right)**: Transient tranfections of p(9-27)4tkΔ(-39)lucter reporter into 293 cells grown in 96 well plates**. 293 cells grown in75cm^2 flasks were transfected transfected with 6μg of the pISRE-luc. reporter plasmid. Cells were trypsinised 24hrs post-transfection and seeded in 96-well plates. After a 24hr recovery period the cells were stimulated with 2x10^3 i.u. of IFN for 4hrs before lysis and luciferase activity measurements. This figure demonstrates the measurements obtained in the presence or absence of an IFN stimulus.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

Fig.3.1.1/2



3.1.1 6-well plate format



3.1.2 96-well plate format

measuring equipment to the luminescence emitted by transfected cells. In the smallest format (96-well plates) differentiation between stimulated and unstimulated cells is not only unquantifiable but both conditions of treatment are indistinguishable from the background measurement (Figure 3.1.2). It would be safe to assume that this marked decrease in sensitivity to luminescence between the two formats is dependent jointly on the transfection efficiency of the target cell line as well as the insensitivity of the response to IFN stimulation. While the number of reporter expressing (transfected) cells in the 6-well plate format is sufficient to signal reporter activation, the reduction of the surface area (and hence the cell number) in the 96-well plate is not accommodated by the sensitivity of the luminometer's sensors.

The failure to distinguish between stimulated and unstimulated groups in the 96-well plate format renders this approach impracticable since large scale compound screening would minimally require a perceptible difference between responsive and unresponsive groups in this format.

3.1.1.2 *Adenoviral delivery of ISRE-luciferase to selected cell lines*

Since transient transfections proved inefficient in terms of reporter expression in a 96-well plate format, delivery of the reporter genes through an adenoviral vector was seen as an alternative strategy. Adenoviral delivery is a popular concept (reviewed in Zhang, 1997) utilizing the transfer of genes into target cell lines through infection with the vector. Adenoviruses have distinct advantages over other delivery methods, including high titres, the infection of dividing and nondividing cells, and a broad natural tropism that can be altered by capsid modifications (Sandig et al., 2000).

Engineering of recombinant adenovirus requires the coupling of the desired genes into the genome encoding for infectious adenovirus. The resulting vector can be used to infect target cells delivering desired sequences and ultimately allowing their

expression by the infected population. Bypassing problems like transfection efficiency, adenovirus encoding for an IFN-responsive reporter (AdeLuc) was seen as a more favourable option to the one presented in 3.1.1.1. Furthermore, delivery of the desired genes can be controlled by adjustment of the multiplicity of the infection (m.o.i), allowing the expression of multiple copies of the reporter per cell, thereby increasing the sensitivity of the assay.

In order to produce virus expressing the inducible reporter[26] the ISRE-luciferase region of the p(9-27)4tkΔ(-39)lucter vector was cloned (Cloning Strategy no1) in the pV60 adenoviral plasmid producing the "positive" luciferase inducible clone, ***pV60luc*** (Figure 3.1.3). This vector proved a potent reporter of the IFN signalling cascade activation with expression patterns demonstrating a 7-fold difference between the presence and the absence of stimulus in 6-well plates. Compared to transient transfections with the p(9-27)4tkΔ(-39)lucter reporter plasmid, transfection with the pV60luc vector allowed more efficient induction of the reporter in response to IFN stimulation

pV60luc was co-transfected with an adenoviral helper plasmid accommodating the greater part of adenovirus genes to produce infectious adenovirus[27]. Production of virus was confirmed by observing the resulting cytopathic effect (C.P.E.) in the replication-permissive 293 cell line[28]. The adenovirus was amplified and Arcton-extracted. A fraction of the extract was applied on Vero cells seeded in 6-well plates. 24 hrs post infection the monolayers were stimulated by IFN addition and 4hrs later luciferase measurements were taken. Figure 3.1.4 demonstrates the transduction by recombinant adenovirus of the Vero monolayers. Encouragingly, reporter activation in response to IFN stimulation of infected cells implied the

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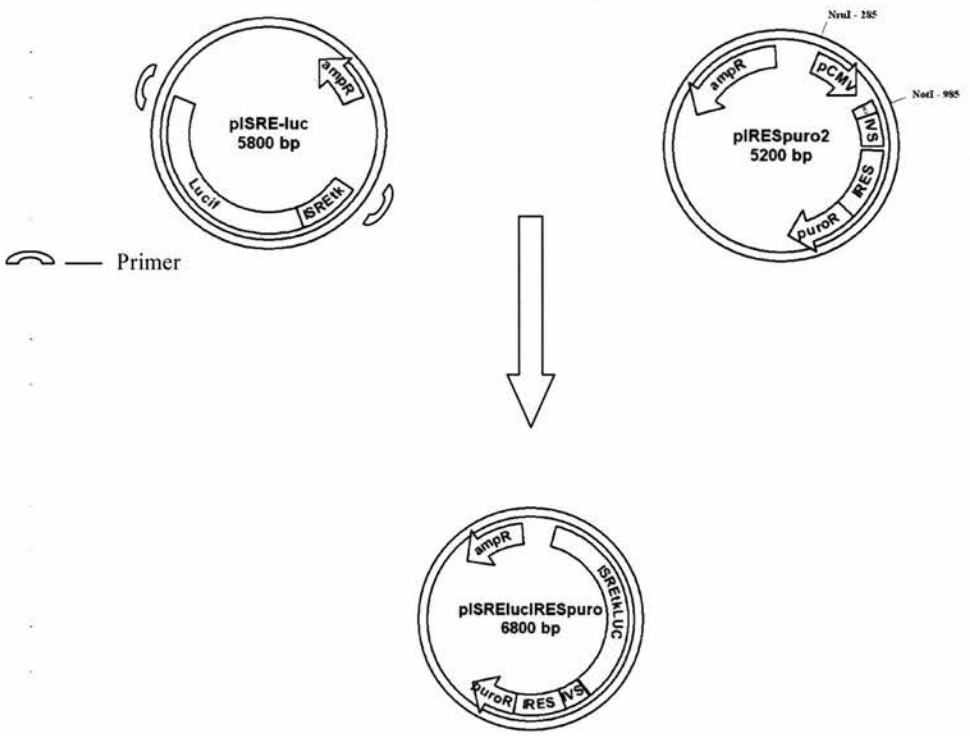
[26] The methodology of production of infectious adenovirus encoding genes of interest appears on 2.1.2.2.

[27] However, the recombination process leads to the production of a "mixed population" of adenovirus, only a minority of which may are "positive clones" (i.e. adenovirus encoding the genes of interest)

[28] As a safety precaution the E1A region of the recombinant adenovirus (essential for viral replication) has been removed making the vector uninfectious. The 293 cell line allows the replication of the virus by constitutively expressing the E1A gene (Graham et al., 1977)

Cloning Strategy 1



Cloning Strategy 1: Construction of pISRElucIRESpuro: The ISRE-luciferase region of the p(9-27)4tkΔ(-39)lucter (pISRE-luc) plasmid was amplified through PCR. The sequence obtained was subsequently cleaved with *Not I* and *Nru I* restriction enzymes (RE). The pIRESpuro2 vector was cleaved with the same REs in order to remove the pCMV promoter and ligate in its place the ISRE-luciferase cassette. The construct obtained was assayed for inducible-luciferase activity and after confirmation was used for the creation of the 293C4 reporter cell line.

Primers used

$IRES_{FOR}$: CGCGCG**TCGCGA**GAAACATAAAATGAATGC

$IRES_{REV}$: ATAAGAAT**CGGCCCG**CGCTGAATACATTTTA

Fig. 3.1.3

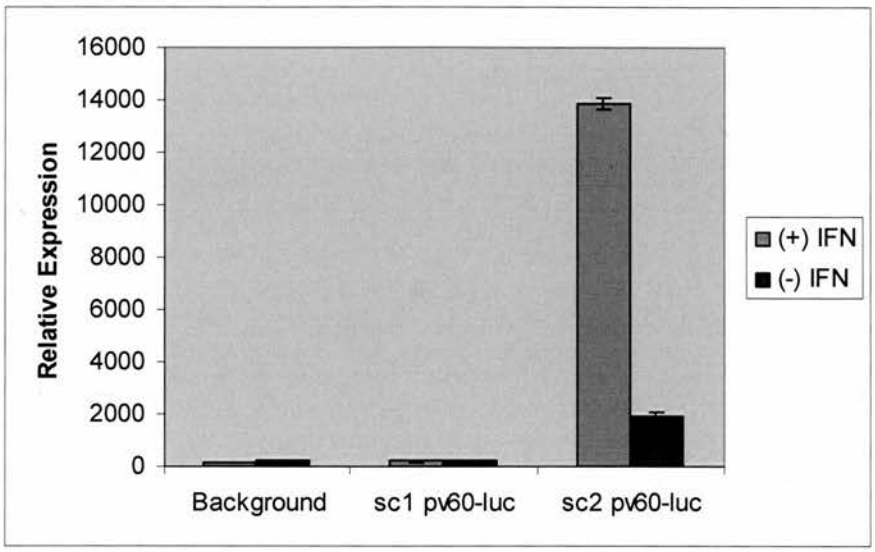


Figure 3.1.3[1]: Luciferase measurements of pv60-luc IFN-inducible reporter plasmid. Several clones obtained through cloning of the ISRE-region into the pv60 adenoviral construct were tested for luciferase activity. DNA was transfected into 293 cells in 6-well plates. After a 24 hr period the cells were stimulated with 10^4 i.u./ml of IFN for 4 hrs before lysis and luciferase activity measurements. sc1 presents a typical negative and sc2 the optimal positive clone.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

Fig. 3.1.4

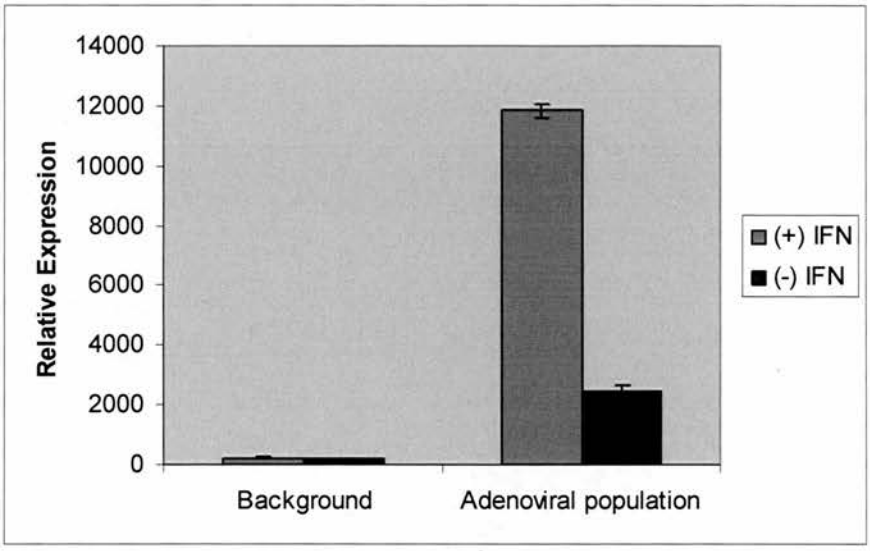


Figure 3.1.4[1]**: Luciferase activity of Vero cells infected with Adenovirus ("mixed population").** The adenovirus generated (see text for details) was applied onto Vero monolayers in 6 well plates. 24 hrs post infection the monolayers were stimulated by IFN addition and 4hrs later luciferase measurements were taken.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained

presence of recombinant, reporter-carrying adenovirus within the "mixed population". The term "mixed population" is used to describe the Arcton extract, containing reporter virus and un-recombined "wild type" adenovirus

Attempts, however, to isolate a single positive clone from the unselected ("mixed") viral population were unsuccessful. The extracted virus was applied onto monolayers of 293 cells which were grown in 6-well plates. Over 200 (of the resulting) plaques were picked and incubated with fresh 293 cells in 25cm^2 flasks, all of which contained infectious adenovirus as confirmed by the resulting C.P.E. The medium from these flasks was subsequently passed through 0.4μm filters, to remove cellular debris, and was applied onto Vero cells. All of the infections failed to demonstrate luciferase activity. Considering that the mixed adenovirus was able to transduce luciferase into cells and none of the clones obtained through plaque purification demonstrated reporter activity, these results imply that the recombination process was inefficient in producing the desired virus (with an undetermined ratio of less than 0.5%).

In a further effort to obtain a positive clone, the pV60luc construct was sent to G. Wilkinson at the Cytomegalovirus and Adenovirus Group of Cardiff University, specializing in replication-deficient adenoviral vectors. The material sent back, however, demonstrated lower luciferase activity levels to the in-house produced virus (data not shown). Furthermore, additional assays to isolate a positive clone also failed to deliver and out of the 20 isolates tested no clone was found to demonstrate reporter activity.

3.1.1.3 *Creation of an IFN-responsive luciferace reporter cell line*

Meanwhile, due to the unsatisfactory development of AdeLuc as a means of controlled reporter expression, the problem of inducible-reporter delivery was re-approached, this time with the goal of engineering a stable cell line expressing luciferase under the control of ISRE. This stable cell line would be produced

through puromycin selection of cells expressing luciferase coupled to puromycin resistance- conferring genes jointly under the control of ISRE.

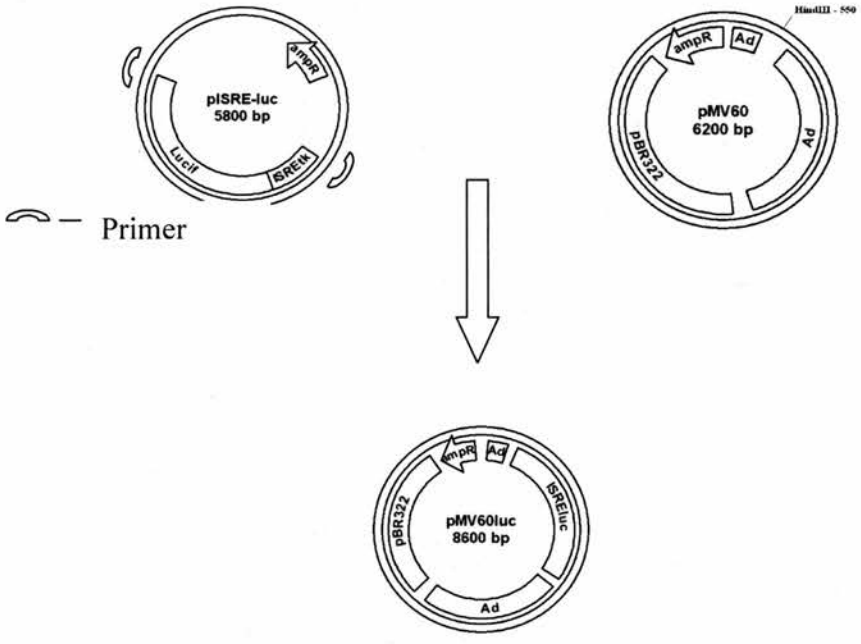
In order to create a vector for transfection with selection capability the ISRE-luc region of the p(9-27)4tkΔ(-39)lucter plasmid was inserted in a puromycin resistance conferring vector (pIRESpuro2) (Cloning Strategy 2). This new vector would then drive joint (through IRES) luciferase and puromycin-resistance protein expression under the control of the ISRE. After obtaining a functional inducible clone the plasmid was transfected into 293 cells. Following selection under IFN/puromycin, 10 surviving colonies were grown individually and subsequently assayed for inducible luciferase activity (data not shown). A single colony demonstrating the highest sensitivity to IFN was grown and reselected in puromycin, after which a single positive clone, C4, was chosen. The resulting 293C4 cell line was then assayed for luciferase activity across a range of assay formats to determine the sensitivity of this approach.

Cells were grown in 6-, 12-, 24-, 96- well plates and stimulated with IFN. Cells assayed in the 6-well plate format demonstrated a satisfactory response to IFN stimulation (Figure 3.1.5). However, similarly to the case of the transient transfections (3.1.1.1), IFN-dependent light emission in samples within 96-well plates was indistinguishable between stimulated and unstimulated groups (Figure 3.1.6). Results reporting IFN activity across the formats assayed demonstrate decrease in sensitivity (i.e. decrease in fold-difference activation of the reporter between stimulated and unstimulated cells) when screening within a smaller format (Figure 3.1.7).

3.1.1.4 *Introducing a means for quantifying assay functionality*

As mentioned above a desirable screening assay would have to be adaptable to, at least, a 96-well plate format. Furthermore, within the confines of such a format, luciferase activation measurements between stimulated/responsive and

Cloning Strategy 2



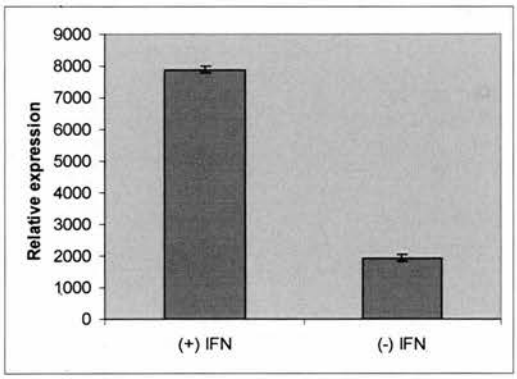
Cloning Strategy 2: Construction of pMV60luc: The ISRE-luciferase region of the p(9-27)4tkΔ(-39)lucter (pISRE-luc) plasmid was amplified through PCR. The sequence obtained was subsequently cleaved with *HindIII* restriction enzyme. The pMV60 vector was cleaved with the same RE in order to insert the ISRE-luciferase cassette. The construct obtained was assayed for inducible-luciferase activity and after confirmation was used for the engineering of recombinant adenovirus.

Primers used

$HINDIII_{FOR}$: GGCGCGC**AAGCTT**GCTGAATACAGTTACATTTTA

$HINDIII_{REV}$: GGCGCGC**AAGCTT**CAACATAAAATGAATGC

Fig. 3.1.5/6



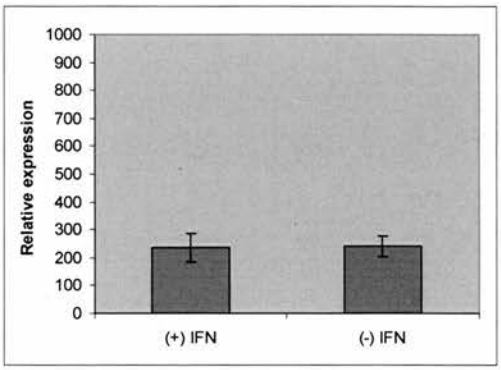


Figure 3.1.5[1]: 293C4 luciferase activity measurements in a 6 well plate format. 293C4 cells were grown in 6 well plates (DMEM/10%F.C.S.). The cells were either stimulated or left untreated with 10^4 i.u. of IFN for 4hrs before lysis and luciferase activity measurements. The figure represents these measurements.

Figure 3.1.6[1]: 293C4 luciferase activity measurements in a 96 well plate format. 293C4 cells were grown in 96 well plates (DMEM/10%F.C.S.). The cells were either stimulated or left untreated with $2x10^3$ i.u. of IFN for 4hrs before lysis and luciferase activity measurements. The figure represents these measurements.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

Fig. 3.1.7

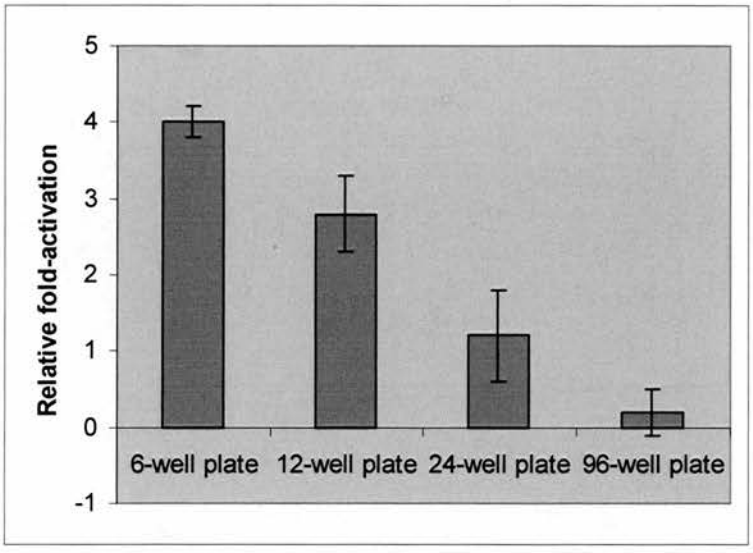


Figure 3.1.7[1]**: Analysis of 293C4 reporter cell line activation across assay formats.** The reporter cell line was grown in 6w-, 12w-,24w- and 96w- plates. The cells were subsequently stimulated with $2x10^4$, 10^4, $5x10^3$, $2x10^3$ i.u. of IFN correspondingly or left untreated. The resulting luciferase measurements in the presence of IFN stimulus were divided to the ones obtained in the absence of one producing the ratio of reporter expression.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

unstimulated/ unresponsive groups needs to be apparent and quantifiable. Optimally the difference in fold-activation between the groups must be sufficient so as to enable the observer to clearly assess possible attenuation of the response in the presence of a compound candidate. In order to determine system functionality, based on these requirements the relative-reporter activations obtained across the formats tested can be graphically analyzed to reveal efficacy and to predict practicability in untested formats.

IFN-specific light emission can be linearly plotted against an increasing assay format to yield an, algebraic, linear equation in the form of $y = ax + b$ where for an x experimental format you are presented with a corresponding y fold activation difference between stimulated and unstimulated groups signifying the sensitivity of the assay. The value a represents the slope of the line equating to the technical accuracy obtained between formats (with the ideal value being 1) and b representing the y intercept or the fold-difference activation obtained at the minimal format. A zero or negative b value indicates a non-functional assay format (Schematic 3.2).

This analysis was applied on the engineered 293C4 cell line. The resulting line clearly demonstrates the aforementioned observation for the inefficiency of a screening system based on 293C4 cells (Figure 3.1.8)

After the presentation of the aforementioned data on the following section (3.1.2) an alternate assaying system based on the HGPRT reporter and its implementation within a HTS approach will be discussed.

Schematic 3.2: Qualitative linear adaptation of two hypothetical assays in terms of fold-difference activation against increasing plate formats. Keeping the format of the assay constant, reporter activation in the presence of an IFN stimulus is divided by the measurements obtained in the absence of IFN. The resulting product represents the fold difference reporter activation (FDRA) for that specific assay format. The calculation is repeated at least once for an alternate assay format. The FDRA values can then be plotted against their corresponding formats to yield a functionality curve. This linear adaptation algebraically follows a $y = ax + b$ equation with the gradient (slope) of the line a representing by definition the change in the y coordinate divided by the corresponding change in the x coordinate, between two distinct points on the line. a can thus represent the technical accuracy of the measuring equipment and is relevancy lies on demonstrating the range of formats that can be assayed by using the corresponding system. Of more importance is the b value of the equation representing the y intercept of the line or the FDRA value obtained by the minimal assay format [by definition b is the point at which the line intersects the line x=0 (the y-axis)]. The analysis is required primarily to predict the functionality of the assay system in decreasing formats when all external conditions are kept constant (same equipment, same treatment etc.).

Sch 3.2 represents two ideal (a = 1) systems: one functional and the other non functional. What distinguishes the systems is their b value. Assaying by the first results in "adequate", quantifiable FDRA values in the minimal format and an expectance of quantifiable FDRA values even if the assay format is decreased (transposing the y axis correspondingly). Assaying by the second system results in "low" or unquantifiable FDRA vales signifying that this system is not functional (if $b \leq 1$) or in any case worse than the first in quantifying differences of reporter activation in (since a=1 in both) any of the relevant assay formats.

Sch 3.2

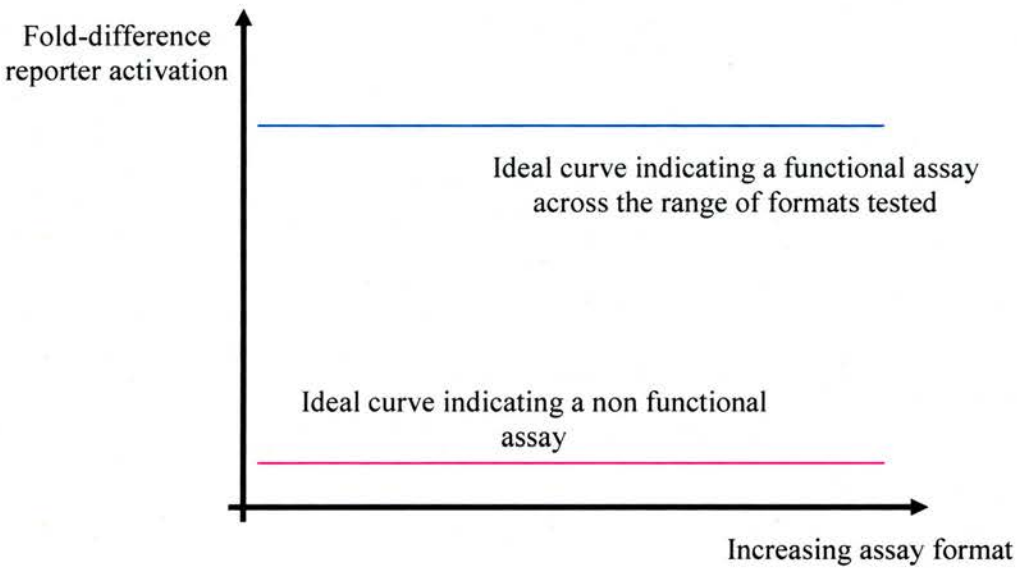

Fold-difference
reporter activation
Ideal curve indicating a functional assay
across the range of formats tested
Ideal curve indicating a non functional
assay
Increasing assay format

Fig. 3.1.8

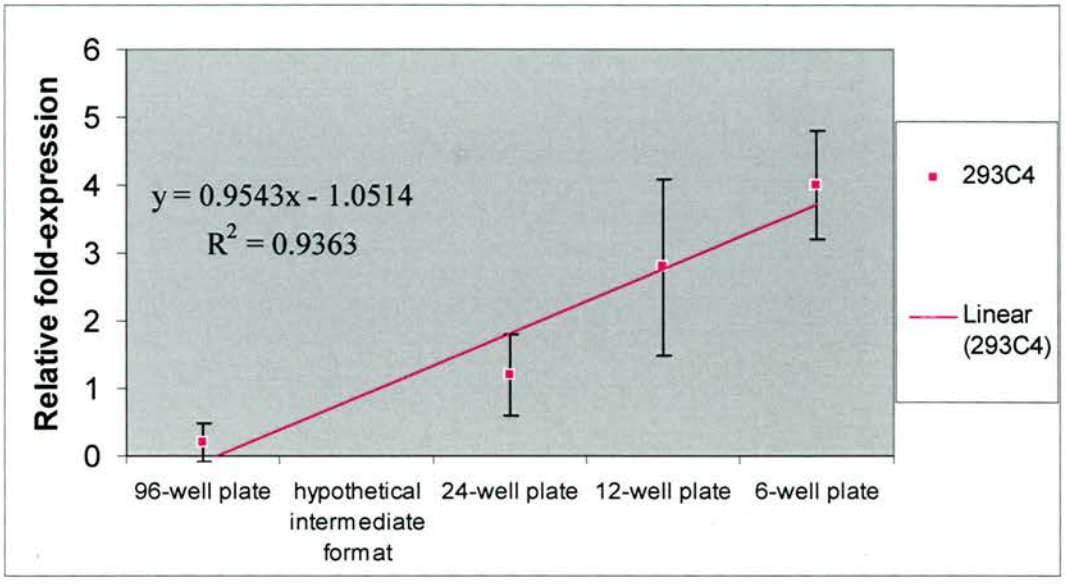


Figure 3.1.8[1]: Linear representation of the relative luciferase activations demonstrated by the 293C4 reporter cell line. The data presented in 3.1.1.3 was used to graphically represent the differential responses to IFN stimulation of the reporter cell line covering a range of plate formats. The curve obtained can predict the functionality or otherwise of these assays when the format of the assay is decreased (i.e. smaller cell counts, smaller plate formats) when keeping the relative in-well volume concentration of IFN constant (1/100 dilutions of 10^6i.u./ml stock solutions or equivalent)

[1] The data presented in this graph is the raw data obtained from (Fig. 3.1.6) so the verification principles are applied here as well. R^2 represents the correlation coefficient of the curves. The closer the coefficient is to 1, the stronger the correlation between the variables (or roughly: the accuracy of the best fit line in relation to the data points) .

3.1.2 Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) reporter-based assay

The unsatisfactory demonstration of a sufficient and quantifiable fold-activation difference between stimulated and unstimulated groups in 96-well plates through delivery of inducible luciferase reporters required re-evaluation of the reporter system. A new approach was tried, one that utilised the properties of another reporter cell line, 2fTGH.

2fTGH cells contain the 6-16 IFN-inducible-promoters coupled to HGPRT (Hypoxanthine-guanine phosphoribosyltransferase) encoding sequences (Pellegrini et al., 1989). HGPRT catalyses the conversion of hypoxanthine and guanine to their respective 5'-mononucleotides and plays a pivotal role in the metabolic salvage of purines in mammalian cells[29] (Rincon-Limas et al., 1991). In the presence of 6-Thioguanine (6TG), in the cell medium, HGPRT catalyses 6TG conversion into 6-thioguanilyic acid (6TGMP)[30]. Intracellular activation by 6TG results in incorporation into DNA as a false purine base which is toxic to the growing cell.

Observing HGPRT-mediated cytotoxicity permits assaying the IFN status of cells incubated with the drug. A functional IFN signalling cascade allows expression of the HGPRT upon IFN stimulation which then catalyses cell killing by 6TG added to the medium. Contrarily, a disruption of ISRE activity and its dependent HGPRT expression leaves the cells unaffected by 6TG in the surrounding medium. By discerning cell survival or death the IFN-signalling status of the group can be evaluated.

---

[29] Complete deficiency in the enzyme in humans results to Lesch-Nyhan syndrome, a severe neurological disorder.

[30] 6TGMP interferes with the synthesis of guanine nucleotides by its inhibition of purine biosynthesis by pseudo-feedback inhibition of glutamine-5-phosphoribosylpyrophosphate amidotransferase, a unique enzyme to the de novo pathway of purine ribonucleotide synthesis. TGMP also inhibits the conversion of inosinic acid (IMP) to xanthylic acid (XMP) by competition for the enzyme IMP dehydrogenase. Thioguanine nucleotides are incorporated into both the DNA and the RNA by phosphodiester linkages with a cytotoxic effect.

3.1.2.1 Testing the functionality of a 2fTGH-based assay

2fTGH cells were seeded in the wells of a 96-well plate with a density of ~18000 cells/well. The cells were left to recover for 24 hours after which the medium was replaced with DMEM, 10%F.C.S. containing 10^2 or 10^3 i.u./ml of IFN. After 24hrs the medium was supplemented with 6-Thioguanine (6TG) (selection medium containing 300μM or 30μM 6TG). The cells were monitored daily over the course of a week to observe signs of cell death due to IFN-induced expression of HGPRT. The cells were subsequently fixed and stained with crystal violet dye (Figure 3.1.9).

The assay yielded encouraging results with observed toxicity of 6TG in IFN-treated cells in a 96-well plate format. Since cell death was apparent in the groups treated with 10^2 i.u./ml of IFN and 6TG (30μM) at 7 days post 6TG addition these conditions were established for the subsequent experiments. However the presence of a limited number of stained cells (cells having ultimately survived 6TG treatment) called for further refinement of the assay.

3.1.2.2 Refinement and further examination of the assay

As incorporation of 6TG to the genomic material of the cell takes place at the level of DNA synthesis, toxicity is dependent on cell cycle progression. Since replication occurs at the S-phase of the cell cycle, actively dividing populations are more susceptible to 6TG-mediated killing. Prolonging the S-phase in replicating cells could lead into the reduction of background survival.

In order to test possible interference with 6TG killing of cells with in-well confluence[31], varying densities of cells (*a*: 18000-, *b*: 9000-, *c*: 5400- cells/well) were seeded in a 96-well plate. After 24 hours of post-trypsinisation recovery, HGPRT expression was induced with IFN, and 6TG was added after a further 24

[31] In-well confluence results in contact inhibition, slowing down the cell cycle and restricting S-phase progression.

Fig. 3.1.9

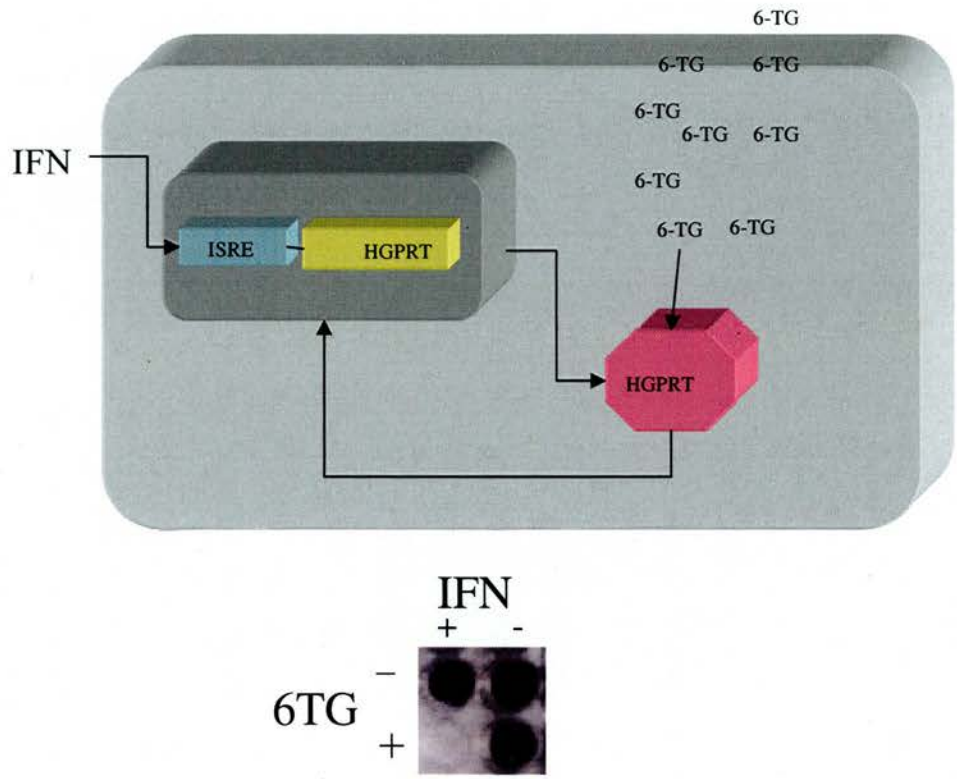


Figure 3.1.9: HGPRT-mediated cell selection. 2fTGH cells were seeded in 96 well plates in DMEM, 10%FCS. The cells were left untreated or stimulated with 10^3 i.u. of IFN for 24hrs before addition of 6-TG (300μM or 30μM). Cells were incubated under these conditions for 7 days (with the medium/IFN/6-TG being renewed every two days). Cells were subsequently fixed in a 10% Formalin/PBS solution and stained with a 1% Crystal Violet dye. Cells that were pretreated with IFN in the presence of 6-TG were killed, due to HGPRT expression, while the unstimulated group survived the treatment.

hours. The cells reached in-well confluence in varying times according to their original densities with group *a* wells appearing confluent at the time of IFN addition. Seven days post- 6TG addition, cells were fixed and stained. Figure 3.1.10 demonstrates that the cells attaining confluency faster (group *a*) were marginally more resistant to the toxic effects of 6TG.

In another attempt to determine the effect of cell-cycle progression on the sensitivity of the assay, ~18000 2fTGH cells were grown in a 96 well format with varying amounts of foetal calf serum. Three concentrations were tested *a*: 10%, *b*: 4% and *c*: 2% of foetal calf serum. The cells were expected to have a more rapid cell cycle progression in the presence of excess nutrients. Again the conditions described above were observed and following staining on the seventh day (Figure 3.1.11) cell killing did not seem to be largely influenced by the speed of cell cycle progression. Even though the 10% FCS- grown group exhibited a minority of stained cells after 6TG treatment (possibly as a result of rapid population and confluence in the well), reduction of the cell number seeded on the plate was seen as a preferable way to counteract background survival.

These findings led to the refinement of the already functional assay by decreasing the number of seeded cells from ~18000 to ~9000 cells per well while retaining the additional conditions originally observed (DMEM, 10% v/v FCS, 10^2 i.u./ml IFN, 30μM 6TG).Having established the principles and the methodology the assay was tested against a compound library.

3.1.2.3 Screening of a compound library using the 2fTGH-based reporter assay

A collection of synthetic compounds and their starting materials were obtained from N. Westwood (School of Chemistry, University of St. Andrews). The compounds were dissolved in DMSO and came in a 96 well plate format. 2fTGH cells were

Fig. 3.1.10/11

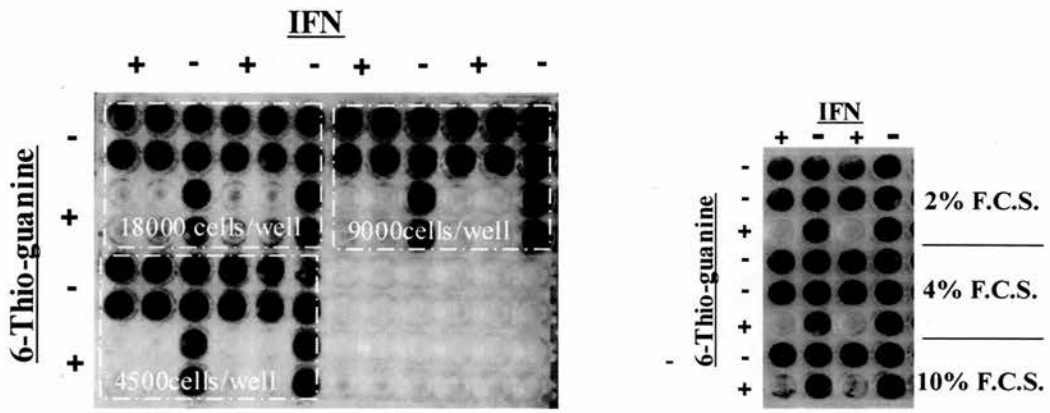


Figure 3.1.10 (left)**: Effect of cell density on the 2fTGH cell assay.** 2fTGH cells were seeded in 96-well plates with varying densities and they were treated with the same conditions as those in the previous experiments. After a six day incubation period in the presence and absence of IFN stimulus and 6TG the cells were fixed and stained. The figure shows the differences in cell killing between the 4500; 9000 cells/well groups and the, more dense, 18000cell/well group.

Figure 3.1.11 (right)**: Effect of cell cycle progression speed on the 2fTGH assay**. 9000 2fTGH cells were seeded in individual wells of 96-well plates. The cells were grown in DMEM supplemented with varying concentrations of fetal calf serum and treated in the same conditions with the previous assays. After a six day incubation period the cells were fixed and stained, revealing again an effect on the 6TG mediated cell killing of cells. The group that was grown in the presence of 10% F.C.S. demonstrated a limited resistance to the toxic effects of 6TG incorporation in the growing DNA chains of cells.

seeded in 96 well plates and grown for 24hrs in DMEM, 10%FCS. The compounds were delivered to the cells through pin transfer 24 hrs prior to IFN stimulation and 48hrs before 6TG addition. The plates were monitored over the week-long course of treatment and the cells were subsequently fixed and stained. Figure 3.1.12 displays one of the stained plates.

Unexpectedly the number of positive hits (i.e. wells with stained cells of varying densities), close to 50% of the wells tested, exceeded the expectations behind such an assay. A possible account for the high cell survival (besides an expected anti-IFN mechanism) could come from screened products disrupting cell growth. 6TG-mediated cytotoxicity was partial in other wells suggesting a probable interference of compounds in the cell cycle progression. These effects could also be attributed to stress-related responses triggered by activation of stress pathways in response to compound incubation [32](see Chapter 4: Discussion).

[32] For example, compounds interfering with the pH of the growth medium could result in triggering stress related pathways with various effects on the cell-cycle. Alternatively, because of the "irrationality" of HTS some of the compounds tested could act as fixatives, fixing the cells on incubation.

Fig. 3.1.12

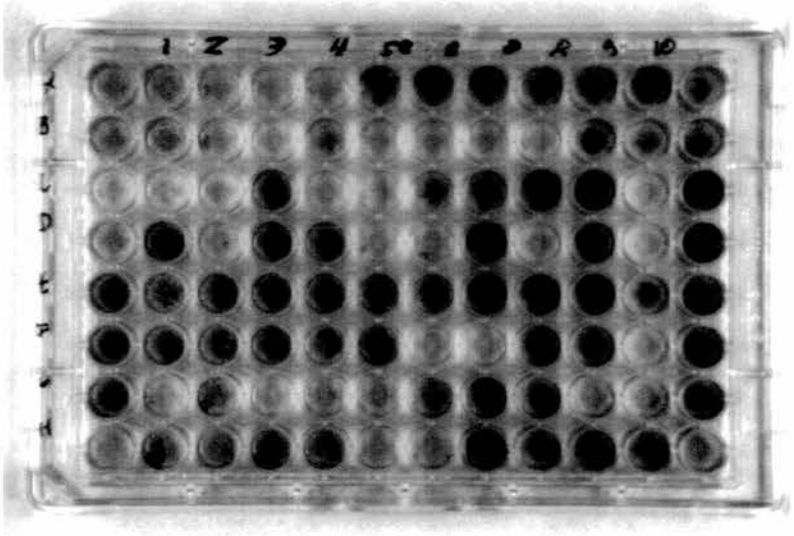


Figure 3.1.12: Compound collection screening. 2fTGH cells were seeded in 96 well plates and grown in DMEM/10%FCS. Compounds were delivered to the cells through 96wp pin arrays. After 24hrs cells were stimulated with 103 i.u. of IFN and the next day with 6TG (30μM). Cells were incubated in these conditions for 7 days (medium/IFN/6TG was renewed every three days and compounds were re-introduced on the first 3-day period via pin transfer). After fixation cells were stained with crystal violet.

The 96-well plate presented above demonstrates typical results obtained from the assay. Note the large number of "positive hits" appearing as wells containing stained cells and also the difference in color intensity among the wells that accommodated live cells prior to fixation.

3.1.3 Characterization of a novel A549L reporter cell line and its applications on a combined 2fTGH/A549L screening assay

The unexpected results generated through the HGPRT-reporter screen (in the face of both refinement-steps and test-runs) suggested the unreliability of such a system on its own. At this point a luciferase reporter cell line based on A549 cells (A549L) became available[33]. Similarly to the 293C4 (discussed in 3.1.1.3) the A549L cell line expresses luciferase under the control of inducible ISREs.

Before proceeding with investigations on the properties of a combined 2fTGH/A549L screening assay (discussed below in 3.1.3.1) the characterization of the cell line will be addressed.

3.1.3.1 Analysis and characterization of the A549L cell line

The externally obtained, IFN-responsive luciferase reporter A549 cell line (A549L) was tested for functionality in the confines of a luciferase reporter assaying system. A549L cells were highly responsive to IFN stimulation in the 6-well plate format (Figure 3.1.13) demonstrating a 14-fold +/-IFN reporter activation and statistically substantial responsiveness in the 96 well plate format (Figure 3.1.14). In fact, when tested across the spectrum of plate formats (Figure 3.1.15) the A549L cell line demonstrated high sensitivities to IFN-controlled reporter expression. These encouraging results, especially the responsiveness of the cell line within a 96-well plate format, suggest that the system on its own can stand as a favourable approach

[33] externally obtained from Gunther Adolf (NBE Discovery Boehringer Ingelheim Austria GmbH)

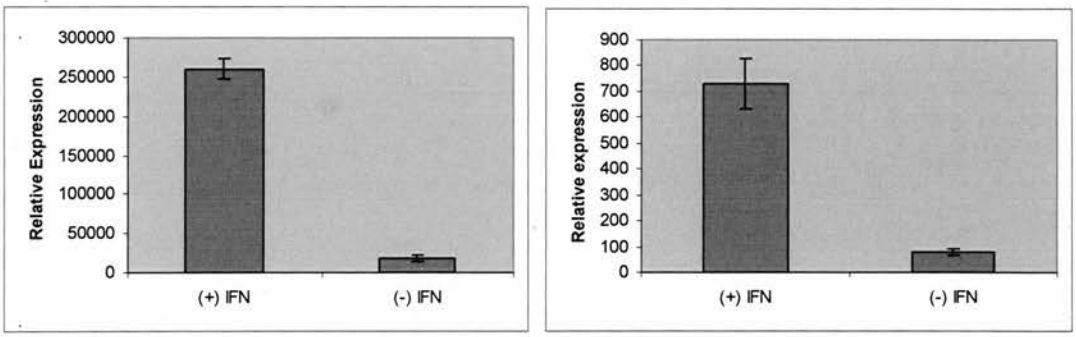


Figure 3.1.13[1]: A549L luciferase activity measurements in a 6 well plate format. A549L cells were grown in 6 well plates (DMEM/10%F.C.S.). The cells were either stimulated or left untreated with $2x10^4$ i.u. of IFN for 4hrs before lysis and luciferase activity measurements. The figure represents these measurements.

Figure 3.1.14[1]: A549L luciferase activity measurements in a 96 well plate format. A549L cells were grown in 96 well plates (DMEM/10%F.C.S.). The cells were either stimulated or left untreated with $2x10^3$ i.u. of IFN for 4hrs before lysis and luciferase activity measurements. The figure represents these measurements.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

Fig. 3.1.15

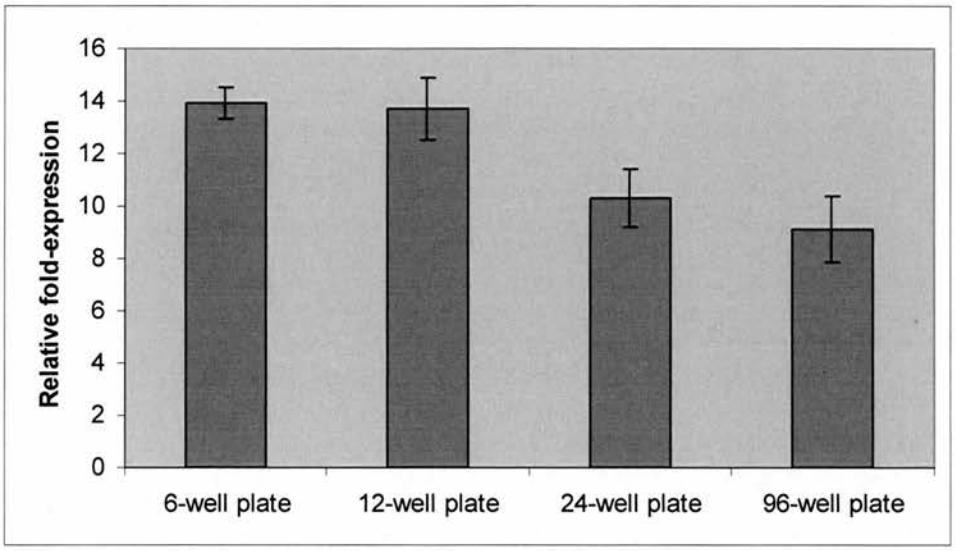


Figure 3.1.15[1] **: Analysis of A549L reporter cell line activation across assay formats.** The reporter cell line was grown in 6-, 12-, 24- and 96- well plates. The cells were subsequently stimulated with $2x10^4$, 10^4, $5x10^3$,$2x10^3$ i.u. of IFN correspondingly or left untreated. The resulting luciferase measurements in the presence of IFN stimulus were divided to the ones obtained in the absence of one producing the ratio of reporter expression.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

to HTS. Alternatively the properties of the A549L cells-line could be applied to test/verify results obtained through the 2fTGH cell-based approach.

Figure 3.1.16 presents the qualitative linear quantification of both the 293C4 and A549L cell lines, where the 293C4 cells obey a $y= 0.95x -1.05$ equation and the A549L line follows an equation of the $y=1.32x + 7.46$ form. This analysis when applied to the 293C4 and A549L cell lines directly demonstrates the differences in sensitivity of these cells to IFN stimulation across a range of assay formats (from 6- to 96-well plates).

3.1.3.2 A549L- based approach allows screening for effectors of IFN induction in 6-well plates

In the introductory section, it was discussed that inhibition of the IFN response *in vivo* can be achieved by inhibition of IFN production. It would, therefore, also be of interest to establish a screening system aiming to identify molecular inhibitors of IFN production. Since ISRE activation lies downstream of IFN production it was expected that stimulation of the IFN promoters would lead to expression, translation and subsequent release of IFN to the medium that would then stimulate expression of ISRE sequences.

Since intracellular and extracellular dsRNA detection by the cell proceeds through distinct pathways[34], Poly(I):poly(C) (PolyI:C) was transfected (so as to stimulate intracellular dsRNA detection pathways) in the first group while the inducer was supplemented (so as to stimulate extracellular pathways) to the medium of the second group. Luciferase activity measurements were taken after 24 hours. Figure 3.1.17a demonstrates that while there was a statistically significant difference of a 3-fold magnitude between stimulated and unstimulated cells on the PolyI:C transfected group, the group that was incubated with the effector-supplemented medium did not respond to the stimulus. The unresponsiveness to extracellular

[34] For example, the RIGI/mda5 and TLR3 pathways reviewed in 1.1.2

Fig. 3.1.16

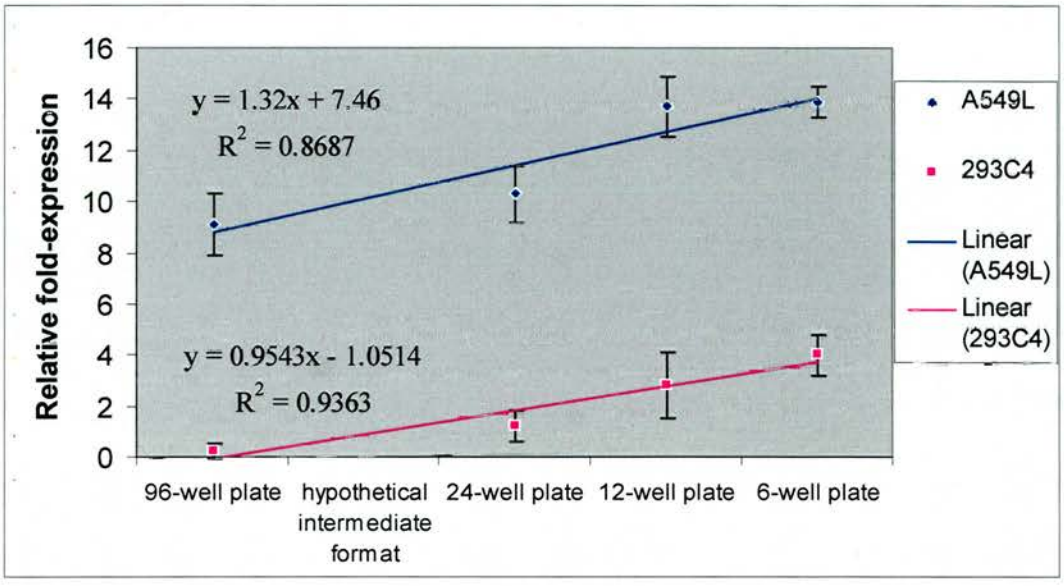


Figure 3.1.16[1]: Linear representation and analytical comparison of the relative luciferase activations demonstrated by the A549L and 293C4 reporter cell lines. The data presented in 3.1.6 and 3.1.14 was used to graphically represent the differential responses to IFN stimulation of the two reporter cell lines covering a range of plate formats. The curves obtained can predict the functionality, or otherwise, of these assays when the format of the assay is decreased (i.e. smaller cell counts, smaller plate formats) when keeping the relative in-well volume concentration of IFN constant (1/100 dilutions of 10^6i.u./ml stock solutions or equivalent).

[1] The data presented in this graph is the raw data obtained from (3.1.6 and 3.1.14) so the verification principles are applied here as well. R^2 represents the correlation coefficient of the curves. The closer the coefficient is to either −1 or 1, the stronger the correlation between the variables (or roughly: the accuracy of the best fit line in relation to the data points) .

Fig. 3.1.17a/b

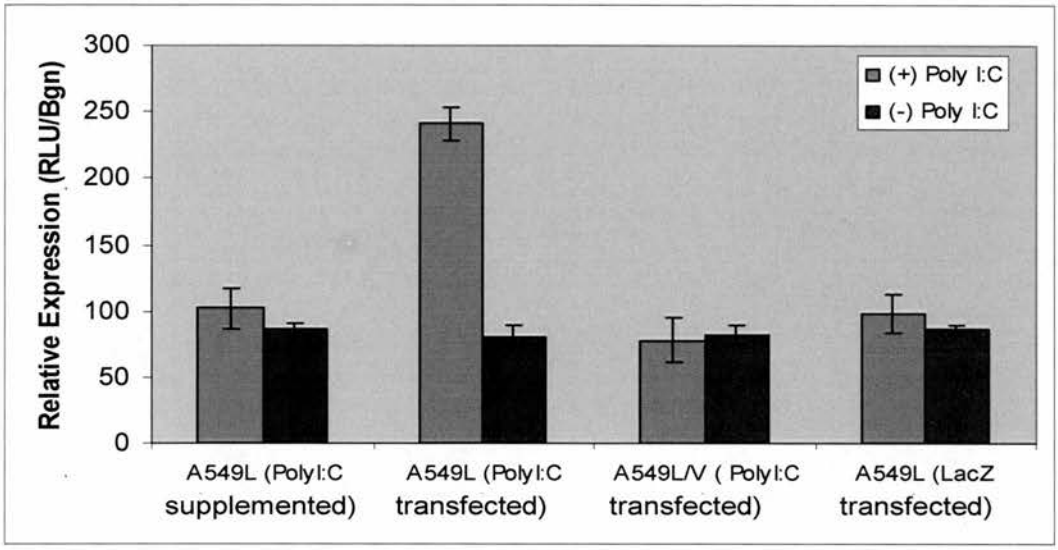


Figure 3.1.17a/b[1]**: Poly(I):poly(C) stimulation of A549L reporter cell line. 17a:** A549 cells were seeded in 6 well plates. A549L were transfected or had their medium supplemented with 1μg of poly(I):poly(C) with a group remaining unstimulated (transfected with pLacZ) as a control. The cells were incubated with or without the inducer for 24hrs prior to lysis and luciferase activation measurements. **-17b:** A further group of cells (A549L/V) were cotransfected with both 1μg of poly(I):poly(C) and 1μg of pV plasmid encoding for SV5 V. The cells were incubated with or without the inducer for 24hrs prior to lysis and luciferase activation measurements

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

dsRNA by the A549L cells can be explained due to the lack of TLR3 receptors by A549 cells. Furthermore transfection of the inducer in smaller formats (12-, 24-, 96-well plates) failed to demonstrate a substantial activation that would permit large scale screening of compounds outside the six well plate format.

The qualitative linear adaptation (Figure 3.1.18) of the assay in terms of fold-difference activation against increasing plate formats produced a functionality equation of $y = 0.63x - 0.71$, marking this approach ineffective for mass screening of candidate compounds interfering with production of IFNs.

Within the restrictions of this large 6-well plate format the assay proved functional when the DNA of a known antagonist of both IFN production and the IFN-stimulated signalling cascade was transfected in the cells. 1μg of the V gene of SV5 was co-transfected with 1μg PolyI:C in the wells of an A549L-seeded six-well plate and luciferase measurements were taken 48 hours later for both the V-expressing A549L cell and the naïve polyI:C stimulated A549L control group. Figure 3.1.17b [labelled as 3.1.17a/b] demonstrates that the inhibition of ISRE expression caused by the viral antagonist can be observed in a 6-well plate format suggesting the functionality of the assay as a system for detecting inhibition of ISRE expression in the presence of an inhibitor. Furthermore, A549L-poly(I)-poly(C) stimulation could be used through viral-gene delivery (in this case transfection) for identifying protein antagonists of both legs of the IFN response. Their characterization of the A549L cell line's differential properties in the context of IFN production and signalling appears as Figure 3.1.19.

Having analyzed, characterized and established the functionality of the A549L cell line the following section will address an A549L-based approach attacking the problems encountered in 3.1.2.3 with the initial 2fTGH-based compound library screen.

Fig. 3.1.18

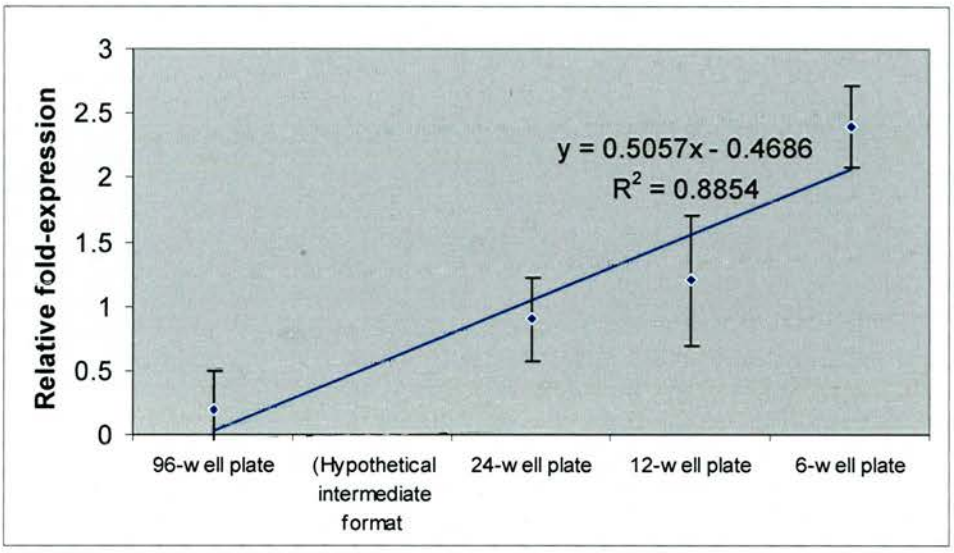


Figure 3.1.18[1]**: Linear representation of relative reporter activation through poly(I):(C) stimulation of A549L cells.** A549L cells were transfected with 6μg of poly(I):(C) or pLacZ (acting as a control) in their native 75cm^2 flasks . The cells were subsequently split and seeded in 6- , 12-, 24-, and 96 well plates. Following a 24hr recovery period luciferase measurements were obtained. Stimulated luciferase activity was divided to the measurements obtained by the corresponding controls. These values were used to construct the functionality curve for the assay.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained. R^2 represents the correlation coefficient of the curves.

Fig 3.1.19

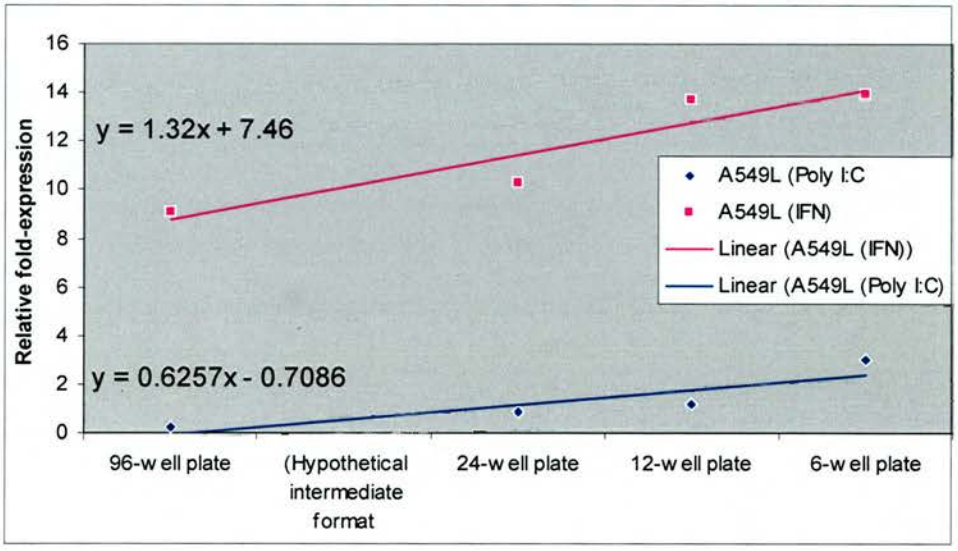


Figure 3.1.19: Linear representation and analytical comparison of the relative luciferase activations demonstrated by the A549L cell line under differential stimulation. The data presented in 3.1.13 and 3.1.17 was used to graphically represent the differential responses to IFN and poly(I):poly(C) stimulation of the reporter cell line covering a range of plate formats. The curves obtained can predict the functionality of these assays when the format of the assay is decreased (i.e. smaller cell counts, smaller plate formats).

3.1.3.4 *Determining the compounds to be re-evaluated*

The samples that (in 3.1.2.3) demonstrated the highest 2fTGH survival were selected for re-evaluation against the A549L reporter system. With ~50% (47/96) of the wells (post compound/IFN/6TG treatment) containing fixed and stained cells of varying density an arbitrary number of samples was chosen to be re assayed.

The 6 wells of the 96-well plate that demonstrated highest cell survival as indicated by the colour density upon staining, were chosen through their ability to absorb light in the visible spectrum when irradiated with saturating amounts of light. The plate was inserted in a chamber where lighting could be adjusted from saturating to obsolete levels through a rheostat. When the plate was illuminated to saturation the contrast was increased gradually to the point where the six wells with the highest absorbance were identified.

3.1.3.5 Luciferase evaluation of the selected samples

After the six compound samples that conferred the highest resistance to IFN/6TG treatment were identified, they were delivered to individual wells in a 6- well plate format seeded with A549L cells. The cells were incubated for 24hrs with the corresponding compounds and were subsequently stimulated with $2x10^4$ IFN for 4hrs. Luciferase activity measurements were then obtained (Figure 3.1.20). The resulting data confirmed the presence of false hits even amongst the most seemingly positive samples. However, the luciferase data for the compound 1.C8 suggested the compound as an inhibitor of the IFN signalling mechanism. The compound referred as 1.C8 was identified as 4-nitrophenol or para-nitrophenol (PNP) and it was shown by both assays (2fTGH- and A549L- based) to affect IFN signalling.

Fig. 3.1.20

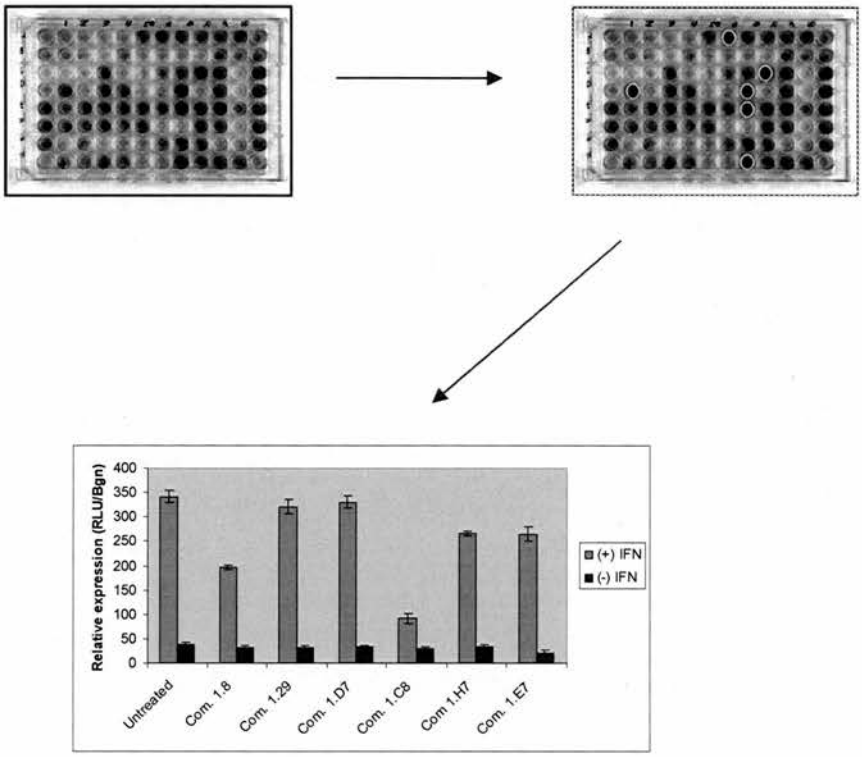
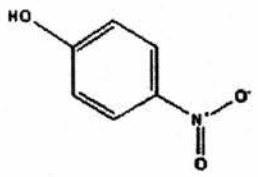


Figure 3.1.20[1]: Luciferase analysis of compounds identified as "hits" from the 2FT-gH based screening assay. The compounds that demonstrated highest survival rates were re-screened for inhibitory activity in A549L cells. The cells were incubated for 24hrs with the corresponding compounds and were subsequently stimulated with $2x10^4$ IFN for 4hrs. Luciferase activity measurements were subsequently obtained. The only compound that scored as a valid "hit" was 1.C8.

[1] The results shown here are the average measurements obtained from 2 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained

3.1.3.6 Further analysis of PNP inhibition of IFN mechanisms



4-nitrophenol (PNP)

Once a suspected effector was identified, further assays were conducted in order to assess its properties as a small-molecule inhibitor of the IFN signalling response. To determine the specificity of the observation the compound was re-evaluated in A549L and HeLa57A IFN-responsive luciferase expressing cell lines. HeLa57A cells (addressed in 2.1.1.2) are reporters of NFκB signaling, containing an integrated copy of the 3 × κB ConA luciferase reporter plasmid. Activation of the NFκB signaling cascade by inducers like tumor necrosis factor (TNF) or phorbol myristate acetate (PMA) results in the expression of luciferase genes, allowing for the quantification of the response.

A control group of 293 cells transfected with a constitutively expressing luciferase vector was also assayed to determine any interference of the compound with the technical aspects of the assay (whether chemical interactions with assay components interferes with reporter activity measurements [35]). Two concentrations were tested: an 800x and a 2000x final dilutions of the original stock of unknown molecular concentration. Figure 3.1.21 shows the luciferase measurements obtained after a 24 hr incubation of the three cell lines with PNP and a 4 hr IFN stimulation.

Incubation of A549L cells with PNP, resulted in a 4-fold inhibition of luciferase expression in IFN-treated cells compared to the untreated group. Additionally, both the control 293 and HeLa57A cells remained unaffected by PNP treatment. 293 cells failed to demonstrate any effect of PNP on their reporter activation levels indicating that the drug did not affect either their expression pattern nor interfere with the luciferase assay. Similarly no significant responsiveness was shown by PMA-stimulated HeLa57A cells in the presence and the absence of PNP. These

[35] For example a chromophoric compound could diffract light in a way interfering with the luciferase measuring equipment.

Fig. 3.1.21

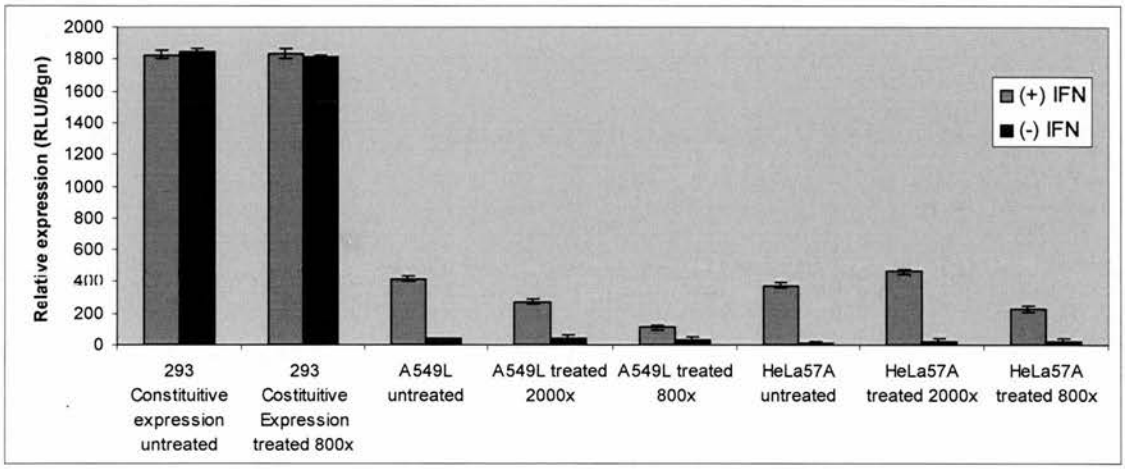


Figure 3.1.21[1]**: The effect of para-nitrophenol on reporter expression proceeding through IFN and NFκB signaling**. A549L, HeLa57A and 293 cells (transfected with pLuc) were seeded in 6 well plates for 24 hrs prior to PNP introduction. The cells were incubated with two stock dilutions of the compound (1/2000 and 1/800 of the final volume) for a further 24hrs. A549L cells were activated with $2x10^4$ i.u. of IFN, HeLA57A with phorbol myristate acetate (PMA) (10 ng/ml) for 4hrs before lysis and luciferase activity measurements.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

results implicate PNP as a specific inhibitor of IFN-signalling in contrast to a general inhibitor of gene expression.

3.1.3.7 *Identifying the working concentration of PNP*

Since the concentration of PNP in the compound plate was undetermined, a working concentration of the compound had to be established prior to large scale experimentation. A dose-response assay was constructed both to detect appropriate dose levels for IFN inhibition and to reveal additional information on the cytotoxicity of the compound.

A549L cells were seeded in the wells of a 96-well plate. The medium of the individual wells was supplemented with medium containing doubling dilutions of PNP ranging from 0.5mM to $1.53x10^{-6}$mM. The cells were incubated for 48hrs with the compound and were subsequently stimulated with IFN for 4hrs prior to luciferase activity measurements. The resulting dose-response curve appears as Figure 3.1.22. The highest concentrations proved lethal to the A549L cells and cell death was apparent both after microscopical evaluation of the plate status and by the complete lack of luciferase expression upon measurement. The subsequent dilutions of the compound did not appear to harm the cells and, curiously, cells responded to PNP treatment with no marked effect on their luciferase expression levels as compared to the untreated group. Possibly this effect could be ascribed to stress responses related to toxicity. However, this response gradually declined as PNP concentration in the medium was decreased reaching a plateau from 10^{-3}mM up to $2x10^{-4}$mM. Finally, the recessing lower concentrations were, expectedly, marked with a steep recovery of luciferase expression levels.

3.1.3.8 *Virus-titre reduction (VTR) assay on cells treated with PNP*

The compound proved to interfere with IFN signalling as reported both by the HGPRT- and the luciferase-based experiments. In order to verify these observations

Fig. 3.1.22

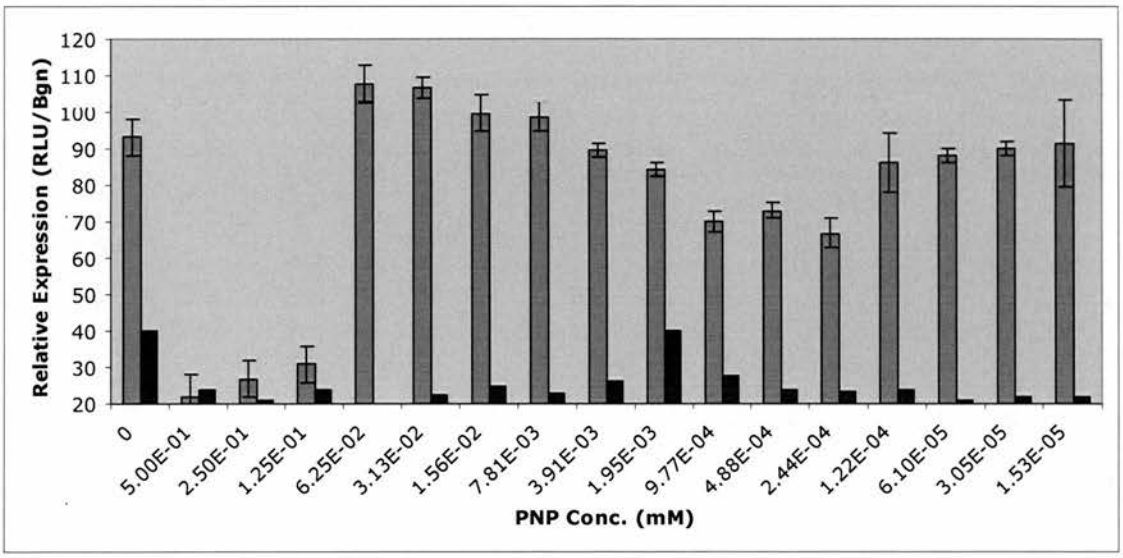


Figure 3.1.22[1]: Dose-response curve to identify working concentration of PNP inhibition of reporter activation. A549L cells were seeded in 96 well plates. The cells were pretreated with doubling dilutions of PNP for 48 hrs prior to stimulation with $2x10^3$ i.u. of IFN. The cells were monitored daily for physiological changes revealing extensive cell death for the highest concentrations. Following a 4hr activation period with IFN the cells were lysed and luciferase activity measurements ensued.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained.

and evaluate their significance, compound treated cells underwent a virus titre reduction (VTR) assay. Detecting the IFN status of the cell (treated in the presence or absence of PNP) by the infection with an IFN sensitive virus would confirm disruption of the IFN signalling responses.

To estimate IFN signalling inhibition within a viral infection setting cells must not be able to produce IFN as viral infection will induce IFN thereby protecting the cells. For this reason, Vero cells, lacking the simian IFNβ promoter (Mosca and Pitha, 1986) effectively defective in IFN synthesis, were seeded in the wells of a 96-well plate. The cells were subsequently treated with doubling dilutions of PNP ranging from 10^{-3} up to $3x10^{-5}$ for 24hrs. The medium was supplemented with doubling dilutions of IFN for a further 24hrs. After that, the cells were infected with encephalomyocarditis (EMC) virus with an m.o.i. of 0.1. After 7 days the medium was removed and the cells were fixed and stained with crystal violet dye. Figure 3.1.23 shows the extent of the resulting infection and survival/death of the Vero monolayers. Cells incubated with the higher concentrations of PNP demonstrated impaired resistance to viral infection compared to PNP-untreated cells containing the same amount of protective IFN. In fact these results suggest that cells are 10-times more receptive to EMCV infection when incubated with $10.^{-3}$mM PNP, an observation that concurs with the initial observations of IFN signalling inhibition.

The data from PNP treatment of the reporter cells and from the EMC-VTR assay suggested that the compound does indeed have an effect on IFN signalling. To summarise these points, PNP was found to promote cell survival in 2fTGH cells treated with IFN/6TG, PNP-incubated A549L cells failed to demonstrate reporter activity levels comparable to untreated cells and finally (perhaps most importantly) cells treated with PNP, in the presence of IFN, were more susceptible to viral infection by EMCV.

Fig. 3.1.23

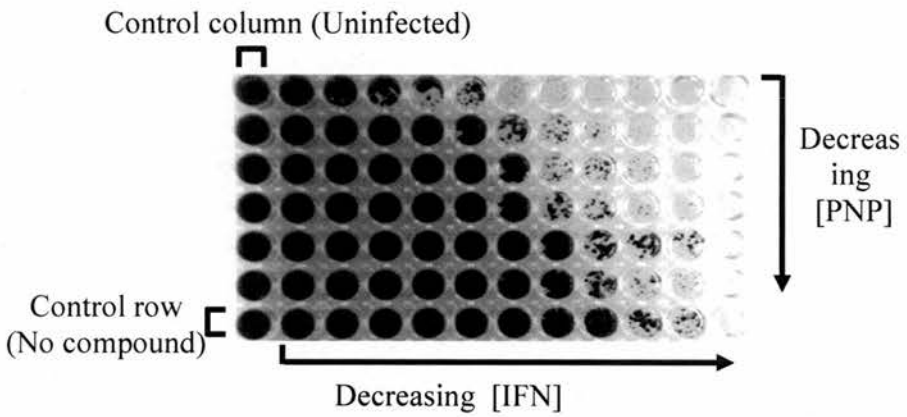
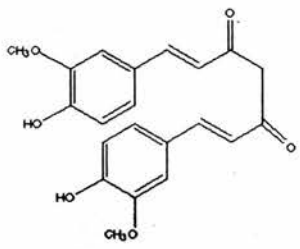


Figure 3.1.23[1]**: EMCV- IFN sensitivity assay for PNP treated Vero cells**. Vero cells were seeded in 96 well plates. Compound was introduced to the medium 24hrs and IFN at 48hrs post seeding. The cells across the plate were treated with increasing doubling dilutions of IFN (the maximal IFN concentration being $2x10^2$ i.u. in a final volume of 200µl). Vertically the cells were treated with increasing doubling dilutions of PNP (maximal compound concentration being 10^{-3} mM). The plate was infected (excluding the control group which was also unstimulated by IFN) with an m.o.i. of 0.1 with EMC virus. The control row was left untreated with PNP. The infection was monitored throughout a 7 day period after which the cells were fixed and stained with Crystal Violet dye.

[1] Appearing on the figure is a typical 96 well plate derived from the first of 2 equivalent assays.

3.1.4 Curcumin inhibits IFN signalling in human A549L cells

The effects of PNP on the IFN signalling cascade have not been published. Therefore, to prove the functionality of the assaying system an established effector of cellular responses was needed to function as a positive control.



Curcumin

The anti-inflammatory properties of (1E, 6E)-1,7 – bis (4-hydroxy - 3 - methoxyphenyl) - 1,6-heptadiene - 3, 5 - dione or commonly known as curcumin have long been established especially in the field of cancer biology. Beside its preventive and/or curative effects in experimental animal models of cancer and several neurodegenerative diseases, curcumin has been characterized as an inhibitor of important anti-inflammatory pathways including the inducible nitric oxide synthase (iNOS) and lipooxygenase (LOS) (reviewed in Bengmark, 2006). Alongside iNOS and LOS, curcumin in the earliest report on its antioxidant properties was found to inhibit IκBα degradation, induced through stimulation of the NFκB pathway (Brennan and O'Neil, 1998).

In addition to NFκB, previous reports have implicated curcumin in the inhibition of Jak/STAT interactions in brain microglial cells (Kim et al., 2003) with implications on the progression of Alzheimer's disease. Since Jak/STAT signalling is the pathway most commonly discussed in the context of IFN signalling the compound was assayed for inhibition using the lung derived A549L cell line (3.1.3.2).

3.1.4.1 *Effect of Curcumin on HeLa57A-luciferase reporter cells*

To investigate the effect of curcumin and in fact establish the functionality of a luciferase screening assay when faced with natural product molecular candidates, the compound was tested on the NFκB reporter HeLa57A cell line.

The compound was delivered to the medium of HeLa57A monolayers within a 12-well plate format. The cells were incubated in the presence of the compound for 48hrs with varying concentrations (9 doubling dilutions of a 20 μM final concentration in the most concentrated sample). The cells were subsequently stimulated with the known NFκB-inducer, PMA for 4 hrs and luciferase measurements produced a working concentration dose response curve (Figure 3.1.24).

The results of this assay directly correlate with previous reports (Brennan and O'Neil, 1998) of curcumin acting as an inhibitor of the NFκB signalling cascade with an 80% inhibition of luciferase activity when cells were incubated with a 20μM concentration of the compound.

3.1.4.2 *Effect of Curcumin on A549L lung cells*

A549L luciferase reporter cells were seeded in a 12-well plate format and were incubated with varying concentrations of the compound (identical to the concentrations used in 3.1.3.1 on the HeLa57A cell line). After a 48hr incubation period the cells were stimulated with IFN for 4hrs and luciferase activity measurements were taken. Besides blocking NFκB signalling, curcumin was also found to inhibit IFN signalling (Figure 3.1.25). The maximal concentration of curcumin used was 20μM and the 48hr incubation period affected IFN-induced luciferase expression with a 45-65% inhibition of reporter activity.

Having shown that curcumin affects IFN-induced luciferase expression it would be of interest to determine whether this effect could be reproduced in a restricted format. Reporter-activity inhibition by curcumin in a 96-well plate format would confirm the validity of the screening approach in the context of large scale HTS. Encouragingly, the observation of luciferase downregulation was confirmed. A549L cells were seeded in a 96-well plate and incubated through pin transfer with

Fig. 3.1.24

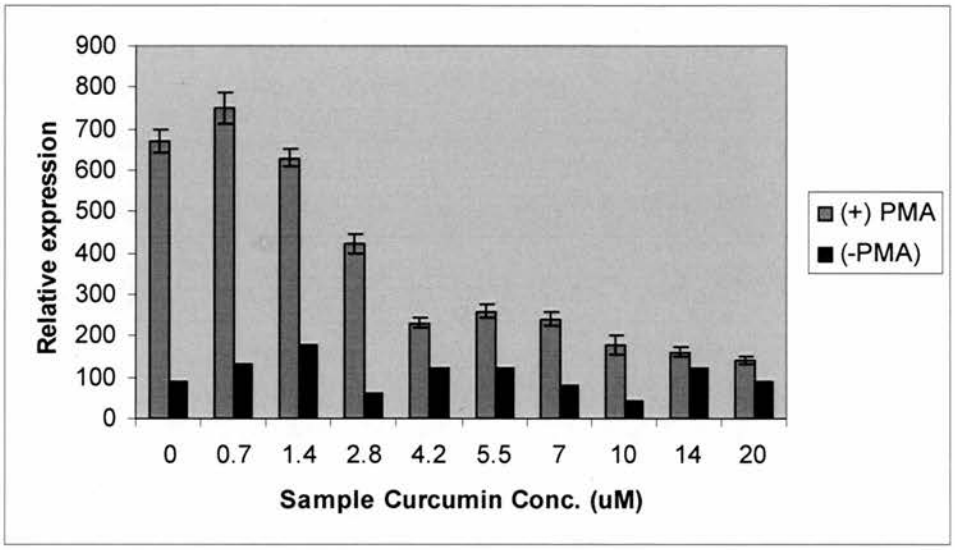


Figure 3.1.24[1]: Establishment of NFκB inhibition (and its resulting quantification through the reporter assay) by curcumin on HeLa57A cells. HeLA 57A cells were grown in 12 well plates in medium supplemented with varying concentrations of curcumin for 48 hrs. The cells were stimulated with PMA (10ng/ml) for four hours before lysis and luciferase reporter measurements.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained

Fig. 3.1.25

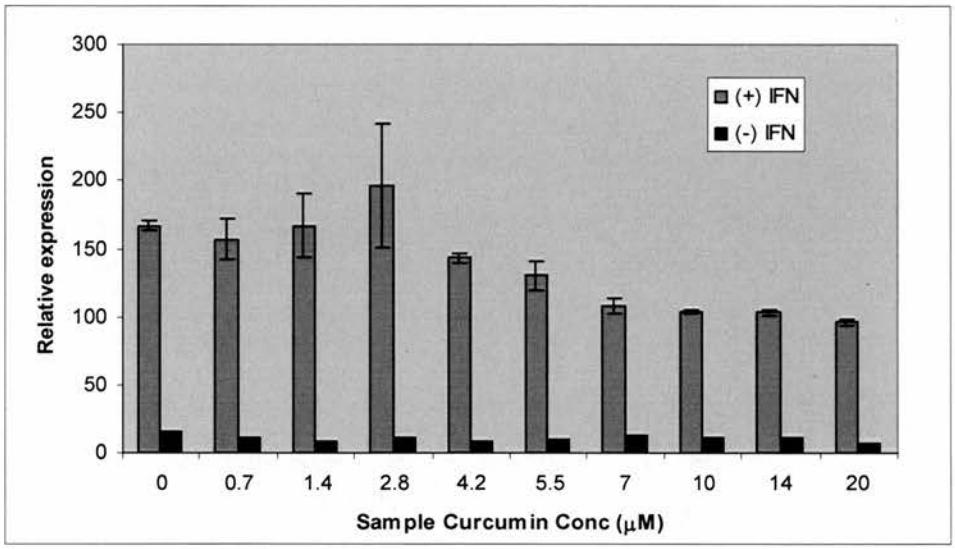


Figure 3.1.25[1]**: Curcumin inhibits IFN-induced reporter activation in human A549L cells.** A549L cells were seeded in 12 well plates in medium supplemented with varying concentrations of curcumin for 48 hrs. The cells were stimulated with 10^4 i.u. of IFN for 4 hrs before lysis and luciferase reporter measurements.

[1]The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained

curcumin, for 48hrs prior to IFN stimulation. Inhibition of luciferase activity was both statistically significant and apparent. However, the restricted format demonstrated inhibition levels lower than those observed in the 12-well plate format ~25% (Figure 3.1.26)

3.1.4.3 *Comparative analysis of curcumin inhibition levels between A549L and HeLa57A cell lines.*

The dose response curves obtained from incubation of human cell lines with curcumin in 3.1.3.1 and 3.1.3.2 were normalized to the maximum luciferase activity (the levels of reporter activation obtained from untreated cells after stimulation with the corresponding effector). Figure 3.1.27 shows the comparative analysis of the dose-inhibition levels attained by using the same concentrations of curcumin on both the A549L and HeLa57A cell lines. Although inhibition was observed in both cases, the levels of antagonism on the NFκB signalling pathway are markedly higher than those observed for the IFN-responsive cell line. It was also noted that both groups demonstrated a similar pattern in their inhibition progression as the concentration of curcumin was increased reaching a plateau of optimal inhibition levels with concentrations >7μM of the polyphenol.

3.1.4.4 *VTR-assay on cells treated with varying concentrations of curcumin*

To estimate IFN signalling inhibition in the context of cell survival upon viral challenge, Vero cells, defective in IFN production, were seeded in the wells of a 96-well plate. The cells were subsequently treated with 4 doubling dilutions of curcumin starting with 10μM final concentration of the compound for 24hrs. The medium was then supplemented with doubling dilutions of IFN for 24hrs. The next day the cells were infected with encephalomyocarditis virus with an m.o.i. of 0.1.

Fig. 3.1.26

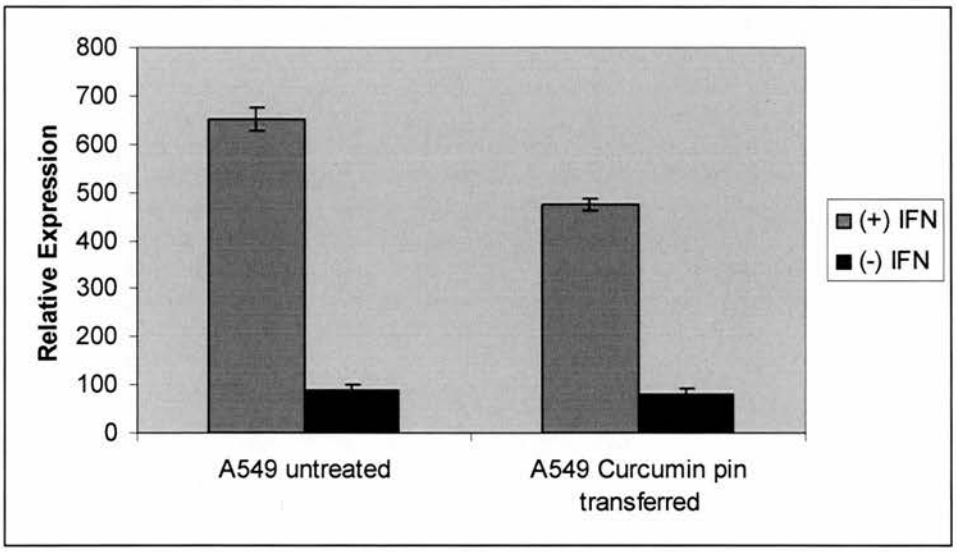


Figure 3.1.26[1]: Pin transfer of curcumin to A549L cells in a 96-well plate format. A549L cells were seeded in a 96-well plate. Compound was delivered to the wells through pin transfer of curcumin from a 10mM stock solution. Cells were incubated with the compound for 48 hrs prior to a 4hr stimulation period with $2x10^3$ i.u. of IFN. Cells were subsequently lysed and luciferase activity measurements were taken.

[1]The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained

Fig 3.1.27

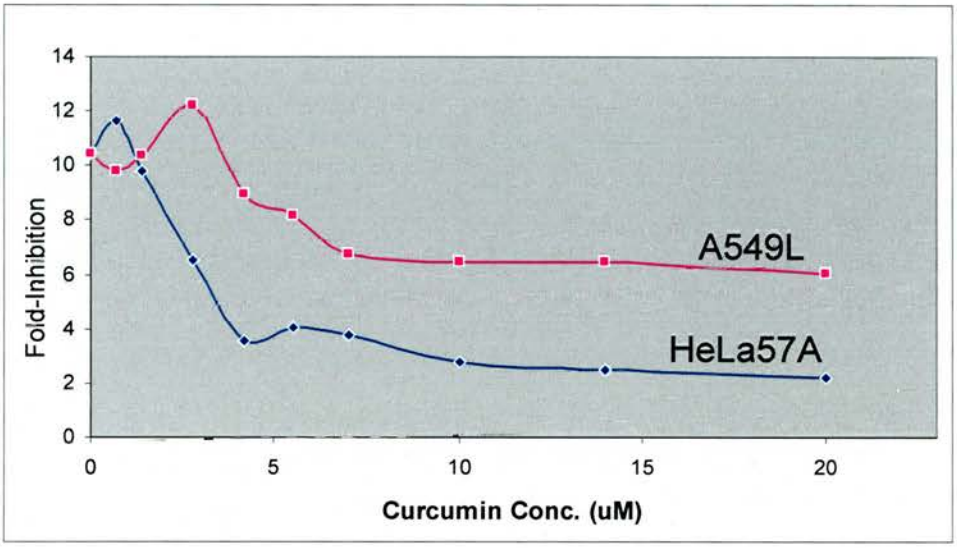


Figure 3.1.27[1]**: Comparative analysis of curcumin inhibition levels between A549L and HeLa57A cell lines.** The data obtained from the dose response curves in 3.1.24 and 3.1.25 were nomalised to uninhibited luciferase activity. Activity in the presence of IFN was divided to activity in the absence of stimulus. The value obtained for each compound concentration represents the relative fold-activation and is graphically presented.

[1] The data presented in this graph is the raw data obtained from (3.1.24 and 3.1.25) so the verification principles are applied here as well

Fig. 3.1.28

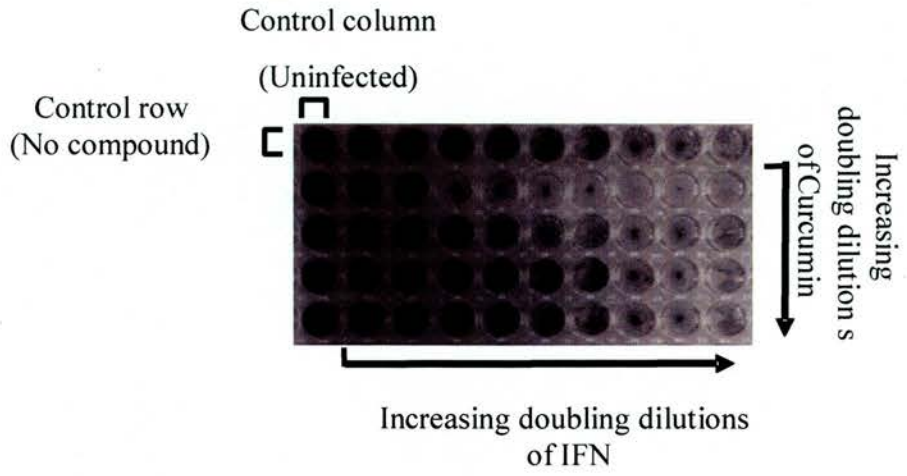


Figure 3.1.28[1]**: EMCV- IFN sensitivity assay for curcumin treated Vero cells**. Vero cells were seeded in 96 well plates. Compound was introduced to the medium 24hrs and IFN at 48hrs post seeding. The cells across the plate were treated with decreasing concentrations of IFN (the maximal IFN concentration being $2x10^2$ i.u. in a final volume of 200µl). Vertically the cells were treated with decreasing concentrations of curcumin (maximal final compound concentration being 20 µM). The plate was infected (excluding the control group which was also unstimulated by IFN) with an m.o.i. of 0.1 with EMC virus. The control row was left untreated with curcumin. The infection was monitored throughout a 7 day period after which the cells were fixed and stained with Crystal Violet dye.

[1] Appearing on the figure is a typical 96 well plate derived from the first of 2 equivalent assays.

After 7 days the cells were fixed and stained with crystal violet dye. Figure 3.1.28 shows the extent of the resulting infection and survival/death of the cell monolayers. Cells that were incubated with the higher concentrations of curcumin demonstrated impaired resistance to viral infection compared to untreated cells treated with the same amount of IFN. These results suggest that cells become 6-8 times more receptive to EMCV infection when incubated with 10mM curcumin.

These results indicate the functionality of a luciferase assaying system (as confirmed by the inhibition of reporter activity in HeLa57A cells). Furthermore, the inhibition of reporter activation by IFN in a 96 well plate format in A549L cells suggested the relevance of this strategy as a system for high-throughput screening of compounds antagonizing IFN signalling responses. In addition, the results obtained suggest curcumin as an inhibitor of IFN-induced ISRE activation.

Chapter 3.2

Development of cell based assays for High Throughput screening of compounds with antiviral properties

Having established reporter systems for screening of small molecular inhibitors of the IFN signalling response, the same technical principles were revised and applied within the context of antiviral compound identification. The common aim shared by the strategies described below is the inhibition of viral antagonists of the IFN response and the restoration of IFN signalling. Genes of viral origin encoding suspected or identified antagonists of the IFN signalling cascade were delivered into target cell lines with IFN-inducible reporter potential. If the viral protein expressed by the reporter cells was indeed an antagonist of the IFN signalling cascade, reporter activity after IFN stimulation would be restricted. Ultimately these reporter/viral-protein expressing- cell lines could be tested against compound libraries to identify inhibitors of the antagonists. Antagonist inhibition by candidate compounds could then be determined by the restoration of reporter activity in cells treated with that compound.

Delivery of viral genes into target cell lines was approached in two distinct ways. Section 3.2.1 focuses on the engineering of a recombinant adenovirus coding for the SV5 V protein, an established antagonist of IFN processes. Section 3.2.2 describes a lentiviral system for the delivery of a variety of viral proteins to reporter cell lines.

3.2.1 Adenoviral delivery of the SV5 V protein antagonist to reporter cell lines

One of the best studied antagonists of the IFN response is the SV5 V protein. An adenoviral construct coding for the V protein of SV5 V (pAdeV) was obtained from Y.H. Chen (University of St. Andrews) and was co-transfected with the corresponding helper plasmid into 293 cells, a permissive as to adenoviral growth and replication cell line[36]. Infectious adenovirus encoding for V (AdeV) was obtained, purified and re-grown in a larger scale.

3.2.1.1 *Adenoviral delivery of SV5 V to 2fTGH cells*

2fTGH cells were introduced earlier as a reporter cell line. Although these cells did not prove to be suitable as a screening system for IFN-antagonistic compounds (3.1.2.3), the 2fTGH-based approach is clearly an effective system for distinguishing between IFN responsive and unresponsive cells. In this system, survival of cells expressing SV5 V, in the presence of IFN and 6TG, would provide proof of principle, for the technical aspects, of the effectiveness of adenoviral delivery as means of establishing the foundations of a screening system.

2fTGH cells were seeded and grown in $75cm^2$ flasks for 24hrs. The medium was replaced with DMEM containing AdeV for the test group and adenovirus encoding for β-galactosidase (AdeLacZ) for the control group. The cells were subsequently infected with an m.o.i. of 10 for 2hrs at 37°C. The infected cells were trypsinised and the cells were seeded in 96-well plates. The cells were allowed to recover for 24hrs. Following this recovery period, cells were either treated with 10^2 i.u./ml of IFN or left untreated. After a further 24hrs the medium was supplemented with 30μM concentration of 6TG and left to incubate for 7 days with daily monitoring. When cell death was observed the cells were fixed and stained with crystal violet

[36] The process is described in 2.1.2.2

dye. The subsequent observation (Figure 3.2.1) verified the hypothesis and cells that had a functioning IFN response were killed by the presence of 6TG and IFN while those that were either untreated with IFN or expressed the V protein survived.

These results suggested that the 2fTGH/AdeV system was functional in the context of compound screening for inhibitors of the viral antagonist.

3.2.1.2 *Adenoviral delivery of SV5 V protein to A549L reporter cell line.*

The encouraging results obtained through the 2fTGH-based system provided the background as the same principles were later applied on the A549L cell line. These cells were discussed extensively in 3.1 where they were established as a favourable approach to anti-IFN compound screening. In this section these cells are alternatively viewed as the basis of a system for identification of inhibitors of viral antagonists of the IFN signalling.

A549L cells were seeded and grown in $75cm^2$ flasks for 24hrs. Their medium was subsequently replaced with medium containing AdeLacZ for the control group and AdeV for the test group. The cells were infected with an m.o.i of 10 with the corresponding viruses for 2hrs at 37°C. The flasks were trypsinised and cells were seeded in 96-well plates. After a recovery period of 24hrs the cells were stimulated or left untreated with IFN and luciferase measurements were taken.

Figure 3.2.2 shows that both groups of cells demonstrated an increase in their reporter activity compared to the uninfected control group. While this was expected for the AdeLacZ group, these results were unexpected for the AdeV infected cells. While this observation could suggest that the levels of V expression were not sufficient to block the response further experimentation disproved this.

The experiment was repeated but the cells were allowed a further 24 hr recovery period post infection/trypsinisation meaning that the cells were stimulated with IFN

Fig. 3.2.1

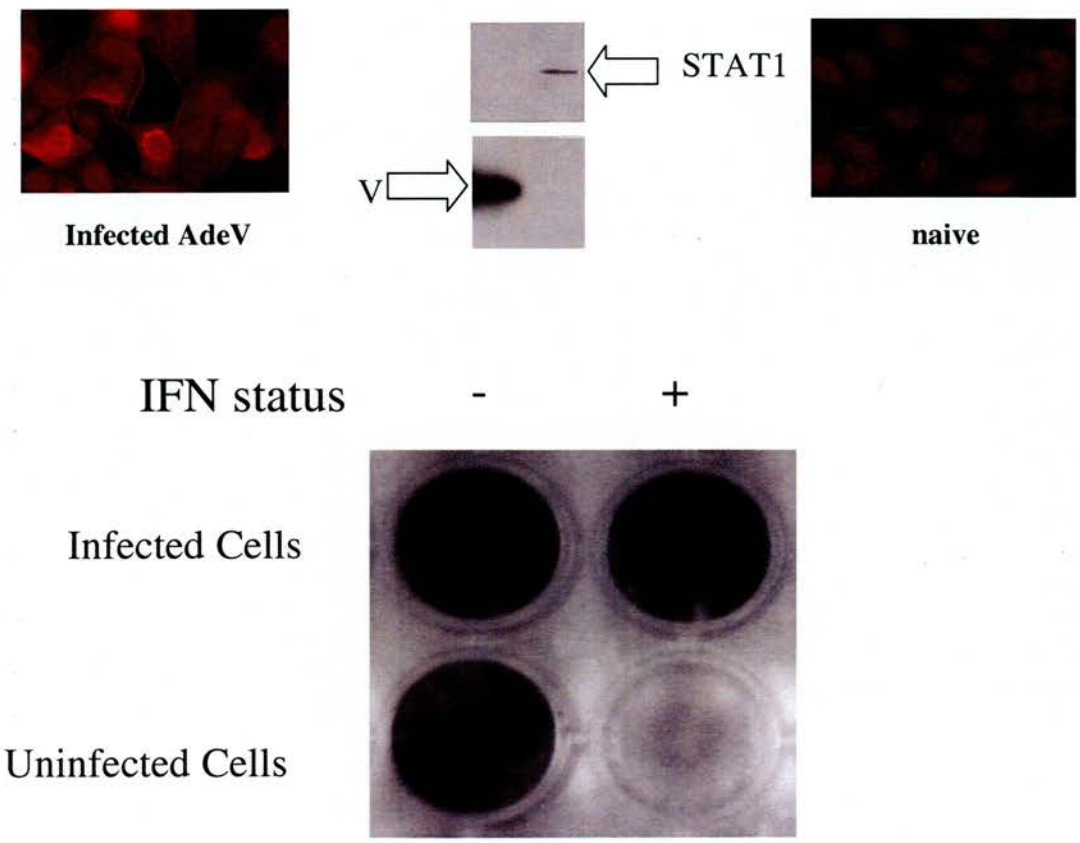


Figure 3.2.1[1]: Adenoviral delivery of SV5 V protein to 2fTGH cells. 2fTGH cells were infected with AdeV (m.o.i. 10) and subsequently seeded in 96 well plates along with a control group of uninfected cells. Following a 24 hr recovery period the medium was either supplemented with 10^2 i.u. of IFN or not and at 48hrs post infection 6-TG was added. The plate was monitored throughout a week with the medium (IFN and 6-TG, where applicable) being renewed every three days. The cells were subsequently fixed and stained with crystal violet.

[1] The figure appearing is derived from a typical 96 well plate acquired from at least 3 equivalent assays. The pictures for the expression of SV5 V were performed by Dr. Yun-Hsiang Chen (University of St. Andrews)

at 48hrs post-infection for 4hrs. The AdeV-infected group displayed a ~5 fold decrease in luciferase activity while the AdeLacZ continued to show elevated levels of reporter activity associated with IFN production upon viral infection. The difference between the data obtained from the two experiments could be attributed to IFN being expressed as a result of adenovirus infection thereby activating ISREs prior to reporter activity measurements. The additional 24 hr recovery period allowed the virus-induced ISRE activity to subside (see 4.2.1)

The combined results from both experiments appear on Figure 3.2.2. This significant knockout of IFN production/signalling in AdeV-infected reporter cells marks this platform as a strong candidate system for HTS for molecular inhibitors of SV5 V.

Fig. 3.2.2

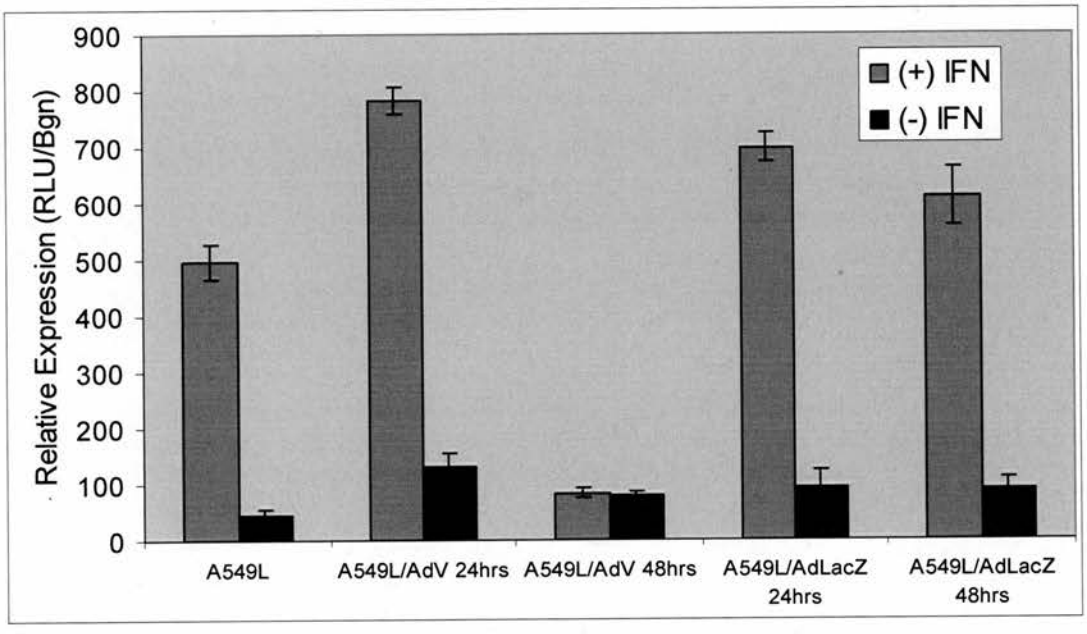


Figure 3.2.2[1]: Adenoviral delivery of SV5 V protein in A549L cells. A549L cells were infected with AdeV (m.o.i. 10) or AdeLacZ (m.o.i. 10) for 2hrs at 37°C and were subsequently seeded in 96 well plates along with a control group of uninfected cells. A 24- or 48-hour recovery period was observed. Subsequently cells were treated +/-10^3 i.u./ml IFN. After 4 hrs of stimulation the cells were lysed and luciferase activity measurements were taken.

[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained

3.2.2 Lentiviral delivery of viral protein antagonists to IFN responsive reporter cell line

Although adenoviral delivery of desired genes to IFN-responsive cell lines was satisfactory, in order to circumvent various technical obstacles (for an elaborate account of the choice see Discussion), the creation of stable cell lines expressing viral genes was seen as a more favourable approach to HTS methodology.

The proteins to be delivered were two known antagonists (of paramyxoviral origin) of IFN signalling, SV5 V protein and Sendai Virus V, as well as three proteins with undetermined effects on the IFN signalling cascade: the Influenza A (pr8 strain) virus NS1, the Influenza A (Victoria strain) virus NS1 and the HHV8 vIRF1 proteins[37].

3.2.2.1 Creation of stable cell lines expressing viral proteins through lentivirus infection.

Lentiviral constructs of the desired genes coupled to puromycin resistance conferring sequences were obtained and co-transfected with the corresponding helper plasmids in permissive 293FT cells (the full procedure is described in detail in section 2.1.2.3. Lentivirus produced by 293FT cells and released in the medium of infection was used to infect monolayers of A549L cells grown in 25cm^2 flasks. The cells were left to incubate with the virus for 48hrs. After trypsinisation cells were transferred into 75cm^2 flasks and after a further 24hr recovery period, cells having acquired antibiotic resistance were selected in the presence of puromycin. Cells were then assayed for viral protein expression through immunofluoresence (Figure 3.2.3).

[37] The mode of action of these proteins is discussed in the introductory section 1.2

Figure 3.2.3

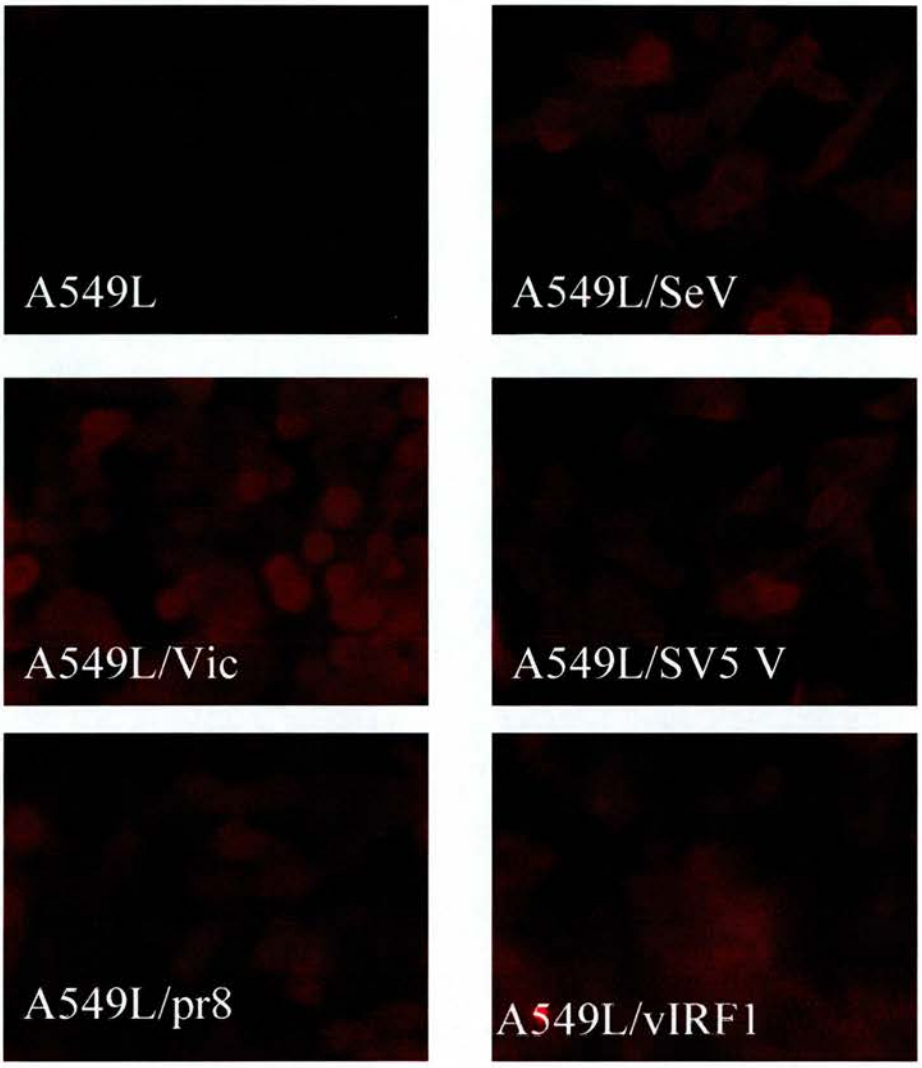


Figure 3.2.3: Immunofluorescence analysis of A549L cells expressing viral antagonists. A549L cells were transduced with lentivirus encoding for viral proteins suspected to antagonize IFN responses. Cells subsequently underwent immunofluorescence analysis to confirm viral protein expression.

3.2.2.2 *Luciferase activity measurements of the viral protein expressing cell lines.*

The A549L cells stably expressing the suspected viral antagonists of the IFN signalling response were seeded in six well plates and were subsequently stimulated with IFN for 4hrs. The luciferase activity measurements obtained after the stimulation period appear on Figure 3.2.4. As expected the SV5 and SeV V proteins inhibited the induction of luciferase in response to IFN but the experiment also produced novel data for the case of the influenza NS1 proteins.

Stimulation with IFN of the A549L/NS1pr8 cell line resulted in a massive ~5 fold activation of the ISRE-luciferase promoter when compared to the A549L cells. This cell line also demonstrated increased reporter activity in the absence of an IFN stimulus. The implications of these results are currently unknown but this background activation of ISRE promoters may come as a result of NS1 interference with other cellular proteins. These observations were reversed in the case of A549L/NS1vic IFN stimulation where the luciferase signal was reduced in levels comparable to those attained with the inhibition of the signalling cascade by the two V protein antagonists.

Antagonism of the response in a somewhat lesser extent to A549L/SV5V and A549L/SeV cell line (yet still apparent and statistically significant) was also exhibited by the A549L/vIRF1 cell line. The creation of these cell lines and specifically the ones that displayed inhibition of the response provided a delicate alternative approach to HTS for molecular inhibitors of these proteins.

Fig. 3.2.4

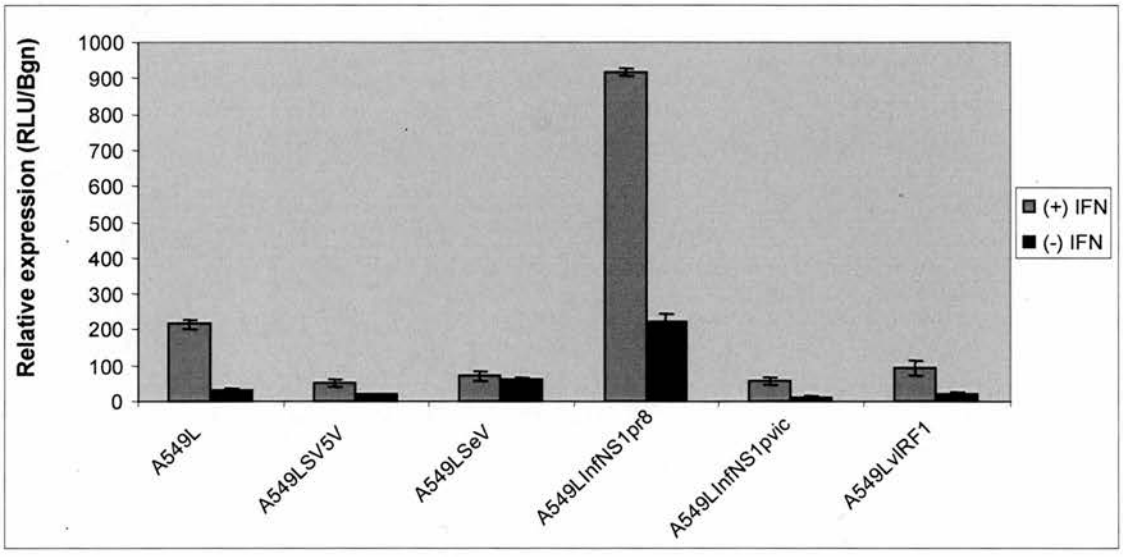


Figure 3.2.4[1]: Lentiviral delivery of viral proteins effecting the IFN response to A549L cells. A549L cells and A549L cells expressing viral proteins were seeded in 6-well plates. After a 24 hr recovery period the cells were stimulated with $2x10^4$ i.u. of IFN for 4 hrs prior to lysis and luciferase assay measurements.

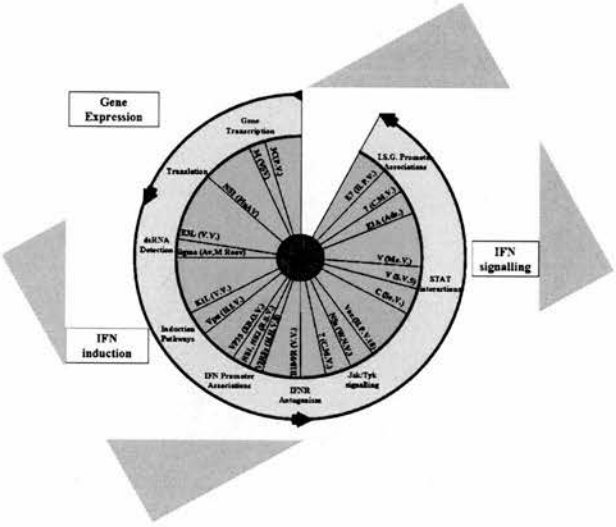
[1] The results shown here are the average measurements obtained from at least 3 similar assays. The error bars appearing on the graphical representations represent the root mean square (or standard) deviation from the average values obtained

Summary

Presented in this part were approaches to high throughput screening aiming to the identification of molecular-inhibitors of both the IFN signalling response and the viral-antagonists of the same response. The systems developed and described were based on reporter assays quantifying IFN-induced expression of luciferase and Hypoxanthine-guanine phosphoribosyltransferase. Delivery of the reporters and the viral-suspected antagonists was achieved through transfection, and transduction by adenoviral and lentiviral vectors. These strategies overall proved successful and led to the identification of two compounds (PNP and Curcumin) as inhibitors of IFN-signalling. Additionally A549L cell lines carrying integrated copies of viral genes were created and now form the backbone of a HTS strategy aiming at the identification of compounds with anti-viral properties.

PART 4:

DISCUSSION



Chapter 4.1

Insights from the development of screening systems for ascertaining negative regulators of IFN signalling

The functional importance of the IFN signalling cascade was highlighted in the previous chapters. Attenuation of the response (a common feature of viral infection) was seen as a measure ensuring the establishment of the infection (1.2). In addition, autoimmune pathogenesis caused either directly or as a consequence of uncontrolled IFN secretion was discussed in 1.5. The first part of this study concentrated on the development of cell-based approaches aimed at the identification of small molecule inhibitors of IFN signalling.

Previous reports have suggested the use of engineered viruses defective in their capacity to circumvent the IFN response as an approach to vaccine production (Didcock et al., 1999, Garcia-Sastre et al., 1998). Such a virus is effectively attenuated in vivo, while retaining its replication potential. Development of live-attenuated vaccines proceeds through growth and amplification of the desired viral strain in cell culture. Engineered virus, lacking the means to evade the IFNs is thus susceptible to their action in culture resulting in restricted growth and viral yield. This problem has been approached by the use of cell lines unable to produce or respond to IFN with the most notable example being Vero cells (Mosca and Pitha, 1986). However, not all viruses grow well in Vero cell culture and not all cells are appropriate for vaccine development. An approach tackling this problem has been the engineering of cells to express IFN antagonists like the SV5 V protein (Young et al., 2003), allowing the growth and increased yield of IFN sensitive viruses. While this approach presents an attractive model for improving virus yield, it faces alternative

shortcomings. The time scale for the insertion of viral antagonists into cells approved for vaccine development and the subsequent period for proper regulatory certification of the novel cell line are not ideal. A possible approach circumventing these problems would be the introduction of a reagent into the culture medium that inhibited IFN signalling, thereby facilitating increased viral yield of IFN sensitive viruses irrespective of the cell line used.

Additionally to vaccine development, IFN-induced autoimmunity and the severe side-effects of unregulated IFN production or treatment can potentially be addressed through inhibition of the signalling cascade between IFN stimulation and ISG expression. In this context, an inhibitor of IFN signalling would act similarly to the anti-inflammatory drugs, widely available, for the treatment of "overzealous" cytokine action.

Identification of such an inhibitor ideally proceeds through HTS of compound libraries. An appropriate platform for this purpose should have the following attributes: 1.The system must be sensitive enough to enable screening in the restrictive format of 96- or 384-well plates, 2.The assay cycle (the time required for each assay run) should be expeditious, allowing maximum screens per time period, 3. The screening procedure should proceed through limited and preferably sequentially automated steps, 4. The disbursement for each "run" should not be restrictive, 5. An alternate screening method should be used for verifying the results obtained through the first screen-run. This second screening system would not require the sensitivity of its predecessor, however it must be conclusive as to the effect of the suspected inhibitor.

This section (4.1) discusses the approaches taken in developing an effective and functional system for HTS and provides the conclusions reached by their practical application.

4.1.1 Transient transfection of luciferase reporter

Transient transfection of the inducible reporter plasmid into 293 cells for the purpose of HTS proved to be an inefficient strategy. Contrary to the high transfection rates characteristic to that specific cell line (easily achieving 80-90% efficiency), reporter activity in 96 well plates seeded with reporter-transfected 293 cells was minimal, challenging the sensitivity of the measuring equipment. The failure to assess differences between groups of IFN stimulated and unstimulated cells in the restrictive format can be attributed to the relatively weak promoter elements of the ISRE-luciferase plasmid. When an alternate construct, using a stronger (Mx) IFN-inducible promoter element instead of ISRE was transfected into 293 cells, the response was readily quantifiable (data not shown). However, even if the reporter was substituted with the new, more sensitive plasmid, the approach of transient transfections as means for HTS (for reasons explained below) would still be sub-optimal.

Transient transfection of reporter places an extra step into each assay cycle: the transfection of the reporter. Each $75cm^2$ flask culture contains enough cells to seed two 96-well plates, therefore for every two assay runs a transfection step is required. Furthermore, many compound libraries do not allow spaces for control wells in individual plates, so in order to standardize the transfection step-related technical inconsistencies cell count measurements are needed, as are specific calculations of the relative amounts of reporter DNA/transfection reagent. While these actions are not in themselves wholly restrictive, on the downstream applications of such a system, they do not reflect an ideal screening assay.

The problems surrounding this method are aggravated when alternate cell lines are used. In fact, 293 cells make a poor cell line for extensive handling. The cells attach weakly to the seeded surfaces, and monolayers can be easily disturbed by compound introduction through pin transfer. However, alternative cell lines for this purpose, like Hep2 or Vero cells, are characterized by very low transfection efficiency rates making them also unsuitable for this approach.

Nevertheless, for small-scale screening of compounds readily available in sufficiently large quantities (so as to perform the screening in 6-well plates) this method is ideal. The difference in reporter activity between stimulated and unstimulated cells is readily quantifiable in 6 well plate formats. Furthermore, this approach does not require extensive preparation (as is the case for adenoviral or lentiviral delivery of the reporter), nor does it go through the time-consuming steps required for the generation of a stably expressing cell line.

4.1.2 Adenoviral delivery of inducible reporter

In the above section, three major obstacles in assay development were presented, a weak promoter, transfection efficiency and cell sensitivity. As means of bypassing the problems arising from the application of transient transfections, alternative approaches were considered. Adenoviral delivery of genes is a common and effective strategy. By controlling the number of particles (and thus the number of reporter copies) infecting a single cell (i.e. the multiplicity of the infection), the original weakness of the promoter could be counteracted. An m.o.i of 10 would therefore, statistically at least, ensure 10 copies of the inducible reporter gene for every cell in culture. Additionally, this strategy directly counteracts the problem of transfection efficiency since virtually every cell is potentially infected by the vector. Furthermore, this system offers a wide range of choice for the target cell line.

With these advantages considered, attempts to engineer a recombinant adenovirus expressing the luciferase gene under the control of an inducible promoter were launched. The adenovirus plasmid constructed with the inserted promoter and reporter sequences was functional, as confirmed by reporter assays, gel electrophoresis and DNA sequencing. Recombination with the helper plasmid in 293 cells produced infectious adenovirus that was used to successfully transduce (as inducible-luciferase activity was recorded) target Vero cells. However, encouraging as these initial results were, the assay system was affected by an overwhelmingly common problem in recombinant adenovirus production: wild-type (or empty-vector)

adenovirus contamination (Sandig et al., 2000; Anderson et al., 2000; Cheshenco et al., 2001). All attempts to obtain a reporter-expressing virus from adenovirus engineered both in-house and externally from adenovirus vector specialists were disturbingly futile. While expression of the desired vector was apparent in the unselected preparation (as confirmed by luciferase-activity assays) no single reporter-encoding clone could be obtained by plaque assay purification. A possible reason accounting for the high prevalence of contaminant virus may be the size and the nature of the insert. The pISRE-luc region of the p(9-27)4tkΔ(-39)lucter plasmid that was inserted into the adenoviral genome is approximately 2.5 Kb in length. During the recombination process the replication machinery may selectively ignore this large insert. Furthermore, the four tandem repeats of the synthetic ISRE promoter element could again be selectively eliminated in the packaging phase of viral replication.

While the attempts in isolating recombinant adenovirus were unsuccessful, this strategy is potentially useful. Given the length of time for the preparation of an adenovirus encoding for desired sequences (reaching approximately 3 months, including two selection cycles) adenovirus generation is a slow process. However, after obtaining a responsive reporter virus, further implementation of the strategy is relatively easy due to the high titres characteristic of adenovirus. Additionally, generation and purification of the virus needs to be done only once. Subsequent amplification solely requires adenovirus infection of 293 cells and Arcton extraction.

4.1.3 Creation of an IFN-responsive luciferase reporter cell line 293C4

Yet another approach in developing a reporter system was the creation of a stable cell line expressing luciferase under the control of an IFN-inducible promoter. The 293C4 line, generated by puromycin selection of pISRElucIRESpuro transfected 293 cells also proved to be a weak reporter of IFN activity in 96 well plate formats. Additionally, a number of the problems that applied to the transient transfections of the vector were also encountered in this approach. Again the weakness of the

promoter was apparent in the optimal (for HTS) formats. Similarly, transfection, IFN treatment, selection and growth in the course of at least 6 months did not produce a cell line that was less sensitive to handling. Transfection efficiency however, was negated by 2 cycles of puromycin selection ensuring that all cells were expressing the reporter. Overall, while the principle behind a stable reporter cell line is the most favourable approach to HTS (as will be discussed below), an assay based on 293C4 cells is not. The qualitative linear adaptation (QLA) of the cell line ($y = 0.95\ x - 1.05$) perfectly describes the potential of this approach. While pitiful for large scale compound screening, these cells do provide a viable alternative to transient transfections of IFN inducible reporters as laboratory diagnostic means in large formats (6- and 12- well plates).

4.1.4 HGPRT reporter-based assay

Departing from the luciferase theme of the above sections temporarily, the HGPRT reporter system will be addressed. Taking advantage of the properties of the 2fTGH cell line, this approach was expected to provide the means for clear distinction between IFN responsive and unresponsive cells. Accordingly, the initial observations of cell survival in untreated cells (compared to cell death in IFN treated cells) in the presence of 6TG, were encouraging. Refinement steps were taken until a suitable protocol for HTS was developed. Prior to actual HTS the system appeared to work perfectly. However, when tested against a small compound library, the number of "hits" returned was unexpectedly high (~50%). Unless the comound library was a "gold mine", cell survival in the presence of half the compounds screened suggested the unreliability of this assay.

As mentioned previously, one of the prerequisites of 6TGMP incorporation into the genome of the cell is dependent upon cell cycle progression. Thioguanine nucleotides are solely integrated in the cellular DNA and RNAs during the S-phase of the cell cycle. Therefore, cells not actively dividing are "immune" to the effects of the drug. Furthermore, densely populated monolayers are characterized by torpid cell cycle progression resulting from contact inhibition. In addition to

these general effects possibly accounting for the observed ambiguity of the assay, are impedimenta more specific to the nature of HTS.

HTS is an "irrational" (compared to the "rational" approaches utilizing structural and synthetic methods) approach to drug development. This entails that the compounds screened are random, as are their effects on the screened cell culture. These compounds could therefore interfere not with the IFN signalling cascade, as such, but with the technical aspects of the assay itself.

For example Brønsted-Lowry interactions between the compound tested and the cellular medium can alter the cellular environment triggering pH-related stress responses, including the release of reactive oxygen species that can cause growth arrest.

Similarly, the formation of other free radicals can have a similar effect. Furthermore as mentioned in 3.1.2.3, some of the tested compounds can act as fixatives, fixing the cells during treatment. These cells when stained later will appear as cells (ultimately) having survived 6TG treatment.

All of the above factors can potentially interfere with the functionality of a screening system. The unsatisfactory development of the 2fTGH cell line-based assay for the purposes of HTS highlights an additional requirement of the ideal screening system: *6.* The reporter assay should not be affected by cell growth/growth arrest.

4.1.5 Characterisation and application of a novel IFN-responsive reporter A549L cell line

Returning to the application of luciferase vectors for assaying the status of IFN signalling in response to compound screening the suitability of the A549L cell line will now be addressed. A549L cells proved to be ideal for the purposes of this study.

While the principle behind A549 functionality was the same as for the 293C4 cell line (i.e. luciferase genes under the control of ISRE promoter elements), A549 cells demonstrated augmented sensitivity to IFN treatment, as reported by luciferase activity measurements. The cells responded to IFN treatment in 6 well plates with a 14-fold activation of the reporter. Furthermore, and more substantially, these cells demonstrated relative expression between unstimulated and IFN-treated cells in 96 well plates of a 9-fold order of magnitude. This latter observation confirmed the functionality of an assaying system based on A549L cells.

The QLA of the A549L cells gave an equation of $y = 1.32\ x + 7.46$, providing the theoretical background for its application. In addition to describing the functionality of the approach in 96 well plates, the QLA predicts functionality even in smaller formats. These results in correlation with the other properties characteristic of the cell line (robustness of the cells in response to handling, human origin) suggest a powerful and efficient system for the identification of negative regulators of the IFN signalling cascade.

The validation of the A549L line as means of assaying IFN signalling functionality as well as the increased sensitivity in response to IFN treatment, suggested a further application for the cells. Logically, since IFN signalling lies downstream of IFN production it was hypothesised that stimulation of the cells to produce IFN would lead to quantifiable activation of ISREs via the binding of the cytokine to cell receptors in culture. This would enable further application of the cell line, beyond its already established basis as an assaying system for interferers of IFN signalling, as part of a screening system for effectors of IFN production. Cells that were transfected with dsRNA in 6 well plates responded to the inducer with a relative, two fold activation of luciferase expression. While this value is low, permitting (as can be seen by the corresponding QLA) screening only in 6 well plate formats, it provides proof of principle for a similar approach. The reasons behind the inefficiency of the cell line as a reporter of IFN production status partially can be attributed to the poor transfection efficiency of the cell line (30-40%). In an attempt to bypass this problem,

dsRNA was supplemented in the medium in order to activate extacellular-dsRNA detection pathways. However, in hindsight, this approach was flawed due to the lack of TLR3 receptors by cells of the A549L family, and that was mirrored by the luciferase activity measurements. All these aspects considered, a system jointly reporting luciferase activity in response to IFN production and IFN signalling is possible.

Circumventing these problems, a cell line can be designed bearing strong promoter elements, and expressing the proteins involved in extracellular/intracellular dsRNA signalling. Delivery of intracellular dsRNA could either be achieved by transfection of the inducer in high-transfection-efficiency cells or by their transduction with an appropriate vector. As for extracellular dsRNA a prerequisite for the engineered reporter cell line would be the expression of TLR3. Furthermore an alternative reporter system could be developed utilising luciferase under the control of the IFNβ-promoter. While this approach would be ideal for the purposes of screening for IFN-induction inhibitors it could not be used for modulators of the signalling leg of the response.

Establishment of the functionality of the cell line led to the identification of two negative regulators of the IFN signalling cascade in A549L and Vero cells: 4-nitrophenol (or para-nitrophenol, PNP) and (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (or curcumin).

4.1.6 Identification of the negative regulators of the IFN signalling response PNP and Curcumin.

The test-run of the HGPRT reporter assay against a combinatorial compound library identified numerous "hits". A portion of these was re-examined using the newly-established A549L-based approach. A single compound, PNP was recognized as a "hit" from both assays. In order to establish the significance of these observations *in vivo* a virus-titre reduction (VTR) assay was conducted.

VTR assays provide an accurate account of the effects of IFN-antagonists on the system. Infecting cells treated with varying dilutions of IFN with an IFN-sensitive virus (in this case EMCV) provides clues as to IFN signalling status of the tested culture. These assays have been adapted for identifying viral antagonists of IFN signalling. Naïve cells and cells expressing suspected viral antagonists are pre-treated with identical concentrations of IFN. If the naïve cells are protected from virus infection and cells expressing the viral protein allow EMCV replication, as identified by the resulting CPE, this demonstrates that the viral protein is negating the protective effects of IFN. The same principle can be effectively applied for the identification of reagents either promoting or abrogating the effects of IFN. While this system is the most accurate screening method for IFN antagonism, its uses within HTS are limited due to the excessive demands of each assay run (in its current form, each 96-well-plate-run can, practically, assay less than 12 compounds or compound concentrations including controls with unrealistic costs in consumables)[38].

PNP treatment of Vero cells "scored" positive in the VTR assays that were conducted, in that it reduced the protective effects of IFN. In fact the cells were found to be 10-times more receptive to EMCV infection when treated with PNP, confirming the attenuation of the IFN signalling response in Vero cells within the confines of this assay.

Previous studies have implicated Curcumin in the inhibition of anti-inflammatory pathways (Brennan and O'Neil, 1998; reviewed in Bengmark, 2006). A HTS screening system originally developed within R.T. Hay's lab (whilst at St. Andrews) based on the NFκB responsive luciferase reporter HeLa57A cells was used to confirm these observations and establish the functionality of such an approach. Indeed, curcumin was seen to inhibit the activation of NFκB in HeLa cells as reported by the 80% reduction of luciferase activity in these cells.

[38] However, proper refinements can be made to the system allowing the economical screening of 94 compounds or compound dilutions , including two controls (data not presented)

Besides the effects of curcumin on the NFκB pathway, a report of Jak-STAT signalling inhibition in brain microglia (in the context of IFNγ signalling) (Kim et al., 2004) by the compound was published. Because of the common components between IFNs α/β and IFNγ, it seemed appropriate to test it against the A549L reporter system to investigate whether curcumin affects type I signalling as well. The compound was seen to inhibit luciferase expression after IFN treatment by 45-60%. The observation was confirmed by a VTR assay in Vero cells demonstrating that the cells were 6-8 times more receptive to EMCV infection in cells pre-treated with curcumin. Furthemore, HTS aims at the identification of lead compounds that can be subsequently altered (synthetically) to increase their potency. Therefore it can be assumed that chemical alteration of curcumin may lead to derivatives amplifying its inhibitory potential.

In addition, during the writing up stages of this treatise a publication appeared presenting data in agreement with what is presented here (Lee et al., 2005). The aforementioned paper reports inhibition of NFκB and the NF-κB-regulated gene Cyclo-oxygenase 2 (COX2) by curcumin in IFN treated A549 cells. This study is of interest not only because of the related cell line used, but mainly because of their observation that both of these factors are expressed in response to IFNα treatment of A549 cells. Furthermore, the authors present data showing the downregulation of IFNα-induced NFκB and COX2 by treatment with curcumin. These effects when viewed in the light of the data presented here seem to be a direct consequence of IFN-signalling inhibition by curcumin. While further investigations are required to elucidate the exact mechanism of inhibition in the context of IFNα/β signalling the most attractive model, proposed by Kim et al., (2004) is the inhibition of Jak phosphorylation.

Chapter 4.2

Insights from the development of screening systems for the identification of inhibitors of viral IFN-antagonism

In addition to the development of screening systems for the identification of compounds interfering with the IFN signalling cascade, a series of approaches aiming at ascertaining inhibitors of viral antagonists of the same response were investigated. Applying the same principles as before (reporter based approach to HTS), engineered or transduced cell lines encoding viral IFN-antagonists of the response were adapted for use in the context of antiviral compound identification. The reporter systems described in the first part are sensitive enough to demonstrate interference with the IFN cascades. Reporter systems expressing viral antagonists of IFN demonstrated substantial reduction in their IFN-inducible reporter gene expression patterns. Recovery of reporter activity after compound treatment would therefore signify the identification of an inhibitor of the viral IFN antagonist.

Engineering the cells to express viral proteins was achieved through infection with delivery vectors of viral origin. The first approach described below focused on the uses of adenoviral vectors carrying the genes of interest as means of transducing target cell lines. The second approach aimed at the creation of stable cell lines through lentivirus delivery. Departing from the cell-based approaches, a further concept was examined, in the form of an *in vitro* enzyme immunoassay.

Overall, all the systems proved functional for the purposes of HTS. However, the absence of established inhibitors of any of the antagonists jointly signifies both the relevance of this assay as well as the lack of proof-of –principle controls. Each will

be separately addressed below in the light of their individual relative merits and disadvantages.

4.2.1 Adenoviral delivery of SV5 V protein to 2fTGH and A549L reporter cell lines

The first approach towards the establishment of a valid reporter system for HTS of antiviral compounds was the transduction of reporter cell lines by adenovirus expressing the V protein of SV5. While antiviral drug development against SV5 infection is not the "holy grail" of pharmaceutical research, assays aiming towards that goal provide a working model for the application of similar methodology against other targets. The reasons behind the choice of the V protein as an IFN-antagonist stem from the fact that its mechanisms of action against IFN-signalling have been relatively well established (e.g. Andrejeva et al., 2002). (as compared with other viral proteins mediating the same effect). Furthermore, work within a paramyxovirus-oriented group results in an abundance of related material becoming available.

After the successful engineering of adenovirus encoding for SV5 V, 2fTGH cells were transduced. Cells expressing the protein antagonist survived IFN and 6TG treatment. While this result as a report of system functionality was encouraging, what property of V contributes to this effect has been debated. Besides its well documented function in antagonism of IFN production and signalling, SV5 V has been reported to slow down cell cycle progression (Lin and Lamb, 2000). As seen above attenuation of the cell cycle can lead to "immunity" against 6TG treatment. However, that does not seem to be the case in this example. Slow cell cycle progression can in fact be beneficial for the 6TG mediated killing. While this may sound contradictory to what was discussed earlier, in truth it is not. Slow cell cycle progression can mean that certain phases of the sequence can be prolonged. In fact the V protein appears to do exactly that. It slows down the cell cycle by prolonging the S phase (ibid.). Prolonged S phase, correlates with an extended period in which 6TG can be incorporated to the

genomic material. Therefore it is logical to assume that the observed survival of the cells is not an artefact but indeed an effect of IFN signalling attenuation.

However effective the principle behind this system, a drawback exists. The assay identifies "hits" as cell death within the corresponding wells, therefore compounds that are toxic to the cells will "score" in the assay. While this does not have an effect within the context of assay methodology for identifying inhibitors of IFN signalling (as "hits" are defined as cell survival in response to IFN/6TG treatment), it does present problems in assay application for identifying inhibitors of viral antagonists. It is expected that these problems are exaggerated when screening synthetic compound libraries, since these collections are more likely to contain toxic molecules in comparison to natural product libraries.

In contrast, another assay was developed bypassing this problem. A549L cells proved ideal in quantifying IFN responsiveness. Their transduction with adenovirus expressing SV5 V was successful and in response, the cell line demonstrated reduced IFN-dependent luciferase activity. A minor setback was observed when luciferase activities were measured 24hrs post infection and their values were found elevated compared to the control A549L uninfected control group. This observation can be explained as a consequence of viral infection by the vector leading to IFN production. However, the solution was straightforward, and currently the assay protocol suggests measurement of reporter activity at 48hrs post infection (in order to allow the response to infection to subside). Otherwise, this system does present an attractive model for identifying inhibitors of viral antagonists of the IFN signalling cascade.

4.2.2 Creation of stable lines expressing viral proteins based on A549L cells by lentivirus.

Recombinant lentivirus transduction is a highly efficient and straightforward way of creating stable cell lines expressing foreign genes. Two known antagonists of IFN signalling and three uncharacterized (as IFN signalling is concerned) proteins of viral

origin were permanently transduced into A549L cells. The purpose of this experiment was dual, to establish a screening system for inhibitors of the viral antagonists and to determine the effect of the other proteins on the IFN cascade. The V proteins of Sendai and SV5 paramyxoviruses have long been established as antagonists of IFN signalling. The cell lines engineered to express these proteins did respond predictably to IFN treatment. Both the A549L/SeV and the A549L/SV5V lines demonstrated minute reporter activities in response to IFN compared to the uninfected cell lines. Whilst adenoviral delivery of the SV5 V protein did, as a basis for a screening system, prove effective, the A549L/SV5V cell line surpasses it in adaptability and functionality for one simple reason: it expresses the antagonist permanently. The consequences of that may seem trivial but they do come into play when large scale HTS is concerned. Stable expression of SV5 V entails higher reproducibility as well as the the avoidance of three time determining steps within the AdeV strategy. Compared to adenovirus, the lentivirus vector can be more easily produced and does not require extensive purification steps. In addition, infection of cells prior to each assay run is now obsolete saving at least 24 hrs from the assay duration. Furthermore, a further 24 hrs can be spared as there is no requirement for a recovery period as the one allowed after AdeV infection.

In addition to the creation of the SV5 and SeV V protein cell lines, the NS1 proteins of the vic75 and pr8 strains of the Influenza A virus as well as the vIRF1 of HHV8 were also delivered by lentivirus to A549L cells. While the observations after IFN treatment are not conclusive, they can provide insights as to the effect of these proteins on the IFN signalling cascade. A549L/vIRF1 cells were seen to inhibit luciferase activity in response to IFN treatment. The NS1 proteins of the different influenza virus strains had a differential effect on IFN-dependent ISRE activation in their A549L based cell lines. A549L/pr8 seemed to induce reporter activity in response to IFN treatment. In contrast, A549L/vic75 appeared to inhibit the same response. While it could be assumed that these effects were representative of viral protein action, specifically on IFN signalling, a recent publication (Hayman et al., 2005) with additional basis rejects this. While confirming the observations on ISRE

induction and inhibition by the pr8NS1 and vic75NS1, respectively, their data do not indicate this as a specific effect, instead as a by-product of their expression. For example inhibition of PKR by a viral antagonist, as is the case for pr8NS1 can enhance general translation (Salvatore et al., 2002) and therefore lead to elevated luciferase levels.

These observations may seem to place questions about the specificity of the A549L-based system as a reporter of the antagonistic properties of viral proteins on the IFN signalling cascade. However and most importantly, these findings suggest an additional use for this approach. The A549L based system can not only be used to identify inhibitors of IFN-antagonists but molecules that inhibit/interfere with the properties on ANY protein, as long as it affects (directly or indirectly and in a quantifiable manner) ISRE dependent luciferase activity. For example, even though NS1 has not been implicated as a specific effector of IFN signalling, it was observed to induce reporter expression. Therefore the A549L/pr8/NS1 cell line could be assayed against compound libraries in order to identify inhibitors of the NS1-aggrevated luciferase expression. Theoretically, restoration of reporter activity to normal levels in compound treated-pr8NS1 expressing cells would signify inhibition of the viral protein.

In addition to the cell based approaches discussed in the context of anti-viral compound identification the foundations of an in vitro assay were laid aiming at the screening for inhibitors of specific interactions of viral antagonists and their targeted proteins (these data were not included in the Results part). This novel approach took form in the development of an enzyme linked immunobinding assay quantifying the interaction between viral protein antagonists of the IFN signalling pathway and their cellular targets. The model used here was again SV5 V. As described earlier the interaction between the V protein and DDB1 is of pivotal importance to the establishment of SV5V infection as it is a prerequisite of STAT1 degradation. Failure of V to bind DDB1 results in failure of the virus to antagonize IFN signalling. In the preliminary experiments, GST purified V was bound to ELISA 96 well plates and

reacted with baculovirus-produced DDB1. The complex was then treated with an antibody against DDB1 conjugated to a reporter like horseradish peroxidase. Antibody-bound DDB1, if in association with V, would remain on the plate and would subsequently be detected by peroxidase activity. If plate-bound V was treated with compounds inhibiting the interactions with DDB1 the corresponding wells would not demonstrate any reporter activity, thereby identifying a "hit".

This approach is still in development, however initial runs have proved extremely successful (data not shown). While this approach could prove a powerful tool within the arsenal of HTS systems it has its own inherent weakness. For example, while the cell-based approaches discussed previously offer insights as to the toxicity of a candidate compound, this assay cannot. Hits identified by this assay could therefore include strong denaturing agents (a common side effect of compounds encountered in combinatorial/synthetic libraries). Thus, the requirement of an additional cell culture assay, in order to confirm IFN signalling attenuation, is self-evident. Contrarily, one of the advantages behind such an assay is that the mechanism of inhibition by actual hits would be easier to elucidate, as this assay takes into to account the protein-protein interactions of only two proteins.

Conclusions (and the award goes to....)

Overall the approaches tested proved satisfactory. However, the A549L-based screening methods were by far the most accurate, efficient and versatile approaches. Jointly in the context of anti-IFN and anti-viral compound screening, stable cell lines expressing on one hand the reporter and on the other the reporter and the antagonist could provide the backbone of an effective HTS system. However, on its own, no approach can be conclusive; therefore an additional system examining the hits obtained must be applied. The ideal verification system for that purpose would be the EMCV VTR assays. These systems working together are effective (as the case study of curcumin confirmed) and present an appealing methodology for HTS aimed at identifying inhibitors both of IFN signalling and viral-IFN antagonists.

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