

## **Eukaryotic Initiation Factor 4 A**

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### INTRODUCTION

The mechanisms governing the basic subsistence of eukaryotic cells are immensely complex; it is unsurprising therefore that regulation occurs at a number of stages of protein synthesis – the regulation of translation has become a well studied field (Gingras *et al* 1999). Human translational control is of increasing research interest as it has connotations in a range of diseases (Hollams *et al* 2002). Orthologues of many of the factors involved in human translation are shared by a range of eukaryotic organisms; some of which are used as model systems for the investigation of translation initiation, for example: sea urchin eggs and rabbit reticulocytes (Li *et al* 2001). Monod and Jacob were among the first to propose that “the synthesis of individual proteins may be provoked or suppressed within a cell, under the influence of specific external agents, and the relative rates at which different proteins may be profoundly

altered, depending upon external conditions” (Monod & Jacob 1961). Almost half a century after the flurry of postulations arising from the revelation of the central dogma of molecular biology, of which the preceding supposition by Monod and Jacob is an example; contemporary researchers still have much to learn about the modulation of genetic expression. Synthesis of protein from mature messenger RNA in eukaryotes is divided into translation initiation, elongation and termination, of these stages; the initiation of translation is the rate limiting step. Within the process of translation initiation; the bottleneck occurs shortly before the ribosome binds to the 5'

m<sup>7</sup>GTP facilitated by a number of proteins; it is at this stage that constrictions born of stress, amino acid starvation etc take effect. Eukaryotic initiation factor (eIF) complex 2 forms a ternary complex

with GTP and the initiator Met-tRNA – this process is regulated by guanine nucleotide exchange and phosphorylation and serves as the main regulatory element of the bottleneck of protein expression. Before translation can progress to the elongation stage; a number of initiation factors must facilitate the synergy of the ribosome and the mRNA and ensure that the 5' UTR of the mRNA is sufficiently devoid of secondary structure. Binding in this way is facilitated by group 4 eukaryotic initiation factors; eIF4 has implications in the normal regulation of translation as well as the transformation and progression of cancerous cells; as such it represents an interesting field of research.

5' UTR to render it more conducive to ribosomal binding and subsequent translation (Gorbalenya & Koonin 1993). Together these three proteins are referred to as eIF4F.

For maximal activity; eIF4A also requires eIF4B (80 kDa), which itself is enhanced by eIF4H (25 kDa) (Korneeva *et al* 2004). A study conducted by Bi *et al* into wheat germ seemed to indicate that eIF4A had a higher binding affinity for ADP than ATP except in the presence of eIF4B which increased the ATP binding affinity tenfold without affecting ADP affinity (Bi *et al* 2000). Once bound to the 5' cap of mRNA, this 48S complex then searches for the (usually) AUG start codon and translation begins.

#### ACTION

The repertoire of compounds involved in eukaryotic translation consists of initiation factor classes 1 – 6 (Hershey & Merrick 2000); eIF4 is responsible for the binding of capped mRNA to the 40S ribosomal subunit via eIF3. The mRNA cap is bound by eIF4E (25 kDa), eIF4G (185 kDa) acts as a scaffold for the complex whilst the ATP-dependent RNA helicase eIF4A (46 kDa) processes the secondary structure of the mRNA

#### GENE

In humans the gene encoding eIF4A isoform I has a transcript length of 1741bp, contains 11 exons and is located on chromosome 17 (Entrez Gene 2008)

#### PROTEIN

The 407 residue (Sudo *et al* 1992), 46 kDa (Belsham *et al* 2000) protein eIF4A is the prototypical member of the DEAD box helicase family, so called due to their conserved four-

residue D-E-A-D sequence. This family of helicases is found in a range of prokaryotic and eukaryotic organisms including humans, wherein they catalyse a variety of processes including embryogenesis and RNA splicing as well as translation initiation (Pause & Sonenberg 1993).

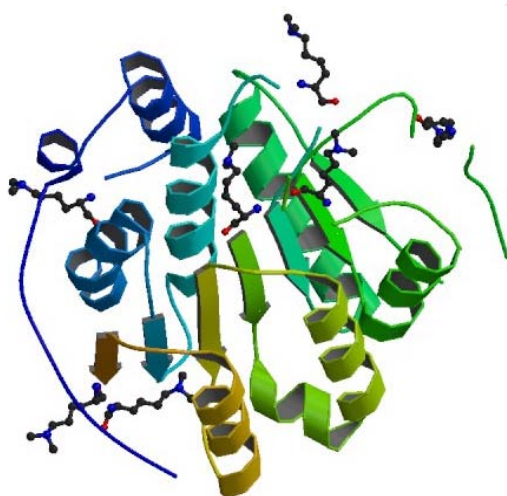


Figure 1 Ribbon diagram of eIF4A

Crystallographic analysis of yeast eIF4A carried out by Carruthers *et al* (2000) revealed that the molecule is approximately 80 Å in length and has a “dumbbell” shape where the proximal section represents an 11 residue (18 Å) linker postulated to confer a degree of flexibility and distension to the molecule in solution (Figure 1). eIF4A is an abundant cytoplasmic protein (Lin *et al* 2008); at over three copies per ribosome it is the most abundant translation

initiation factor (Bordeleau *et al* 2006).

### ISOFORMS

Three isoforms of eIF4A exist; I and II share 95% amino acid similarity and have been found simultaneously in rabbit reticulocyte eIF4F in a ratio of 4:1 respectively (Yoder-Hill *et al* 1993). The third isoform; eIF4A III, which shares only 65% similarity to the other isoforms is believed to be a core component of the exon junction complex involved in pre-mRNA splicing (Bordeleau *et al* 2005).

### RESEARCH INTEREST

As previously mentioned, the cell cycle is a complex process relying upon the precise execution of a large number of tightly-regulated sequential stages, the importance of efficacious regulation is highlighted by the potentially disastrous consequences of abnormal cellular proliferation. The effective diagnosis and treatment of cancer are among the highest medical priorities of the Western world, approximately 7.6 million deaths in 2007 (American Cancer Society 2007) were attributed to cancers of various types. The intense scientific, humanitarian and financial interest in oncology has lead

to the investigation of any avenue of research that may have a chance of yielding an effective treatment, one such avenue is the reliance of cancerous (neoplastic) cells upon higher levels of translation initiation factors in certain circumstances.

In order for a cell to become neoplastic, a mutation must occur in one or more of a set of genes, the primary function of which is to govern proliferation, these are termed proto-oncogenes, the wild type forms of oncogenes. The mutation that eventually causes abnormal proliferation may also occur in a tumour-suppressor gene which would normally act to negate or prevent DNA damage, an example of a tumour suppressor is the transcription factor p53 which can suspend the cell cycle to allow for DNA repair and even initiate apoptosis (programmed cell death) if the damage proves irreparable (Harms & Chen 2005).

Studies by Kozak (1991) indicated that many proteins involved in proliferation are regulated at the translational level. The mRNAs of such genes tend to possess more complex secondary structures in their 5' untranslated regions which inhibit the ribosome upon its scanning of the

mRNA to find the start codon (Sonenberg *et al* 1996); the ribosome must rely upon the helicase activity of eIF4A to unwind or 'melt' these structures before translation can begin. It has been postulated that a number of oncogenes require far greater levels of every member of the eIF4F complex (eIF4G, E, A and associated other factors) for successful expression compared to normal genes. This is believed to be attributable to the large and highly structured 5' untranslated regions (UTRs) of oncogenes, exemplified by those possessed c-myc and Bcl-xL (Darveau *et al* 1985). The rapid sequestration of eIF4A by oncogenes in malignant neoplasia reduces the available cytoplasmic levels of the helicase, along with the proteins necessary for its processive activity, as a result; the proliferating cancer cells must overexpress these translation initiation factors to address the deficit.

#### eIF4A AS A DRUG TARGET

The potential efficacy of selecting eIF4A isoform one as a specific drug target has been corroborated by a number of studies, in 1997, Eberle *et*

al from the University of Berlin demonstrated that *in vitro* human melanoma cell cultures consistently overexpressed eIF4A, recording average mRNA levels to be 5.6 times those of parallel non-neoplastic human melanocytes. Eberle also found that eIF4A isoform 2, eIF4B (the stimulating cofactor of eIF4A (Ochs *et al* 1999)), eIF4E and eIF4G do not exhibit the same abnormal expression levels and, as such are precluded from becoming drug targets with the same potential effect as eIF4A isoform one. As a result of this differential, combined with the relative complexity of its function; eIF4A is the most common target of all known inhibitors of the eIF4F complex (Moerke *et al* 2007).

#### CURRENT INHIBITORS OF EIF4A

The logical progression, upon the identification of eIF4A as a molecule more heavily relied on by cancerous cells, is the screening of compounds with inhibitory effects with a view to developing these into therapeutic agents. Several candidate compounds exist currently but none of these have formed the basis of anti-cancer drugs yet.

#### PATEAMINE A

The thiazole-containing polyene bis-lactone pateamine A (PatA) (Figure 2) was identified as a candidate by Bordeleau *et al* in 2005 as part of a

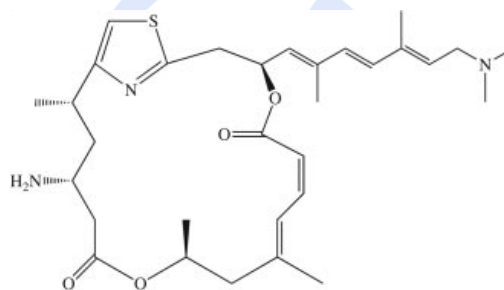


Figure 2 Pateamine A

high throughput screening strategy to identify inhibitors of eukaryotic protein synthesis. High throughput screening of small molecule libraries remains a popular approach to drug discovery despite the lower than expected numbers of drug candidates generated. During the 14 years between 1988 and 2002, the discovery of only 70 drug candidates is attributable to high throughput screening (Mullin 2004). In this period also, however, 60% of the drugs introduced were derived from natural compounds. Bordeleau combined these two avenues of discovery by including natural products into the library and, out of the 90,000+ compounds screened, identified a single eukaryotic translation initiation inhibitor. Pateamine A was isolated from the marine sponge *Mycale*

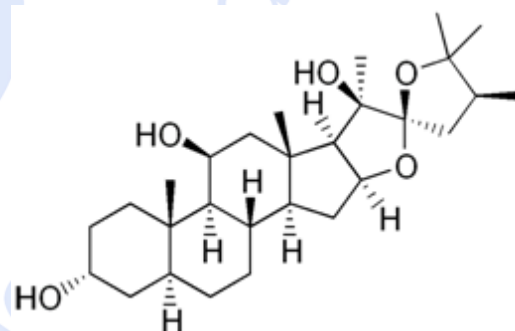
(Figure 3) found off the coast of New Zealand (Romo *et al* 1998).



**Figure 3** The marine sponge  
*Mycale*

Pateamine A differs greatly from hippuristanol in its inhibitory action; it enhances the ability of eIF4A to bind to ATP and mRNA, stimulates RNA-dependent ATP hydrolysis and also acts as an agonist of the helicase activity of the molecule (Bordeleau *et al* 2005). Pateamine does not affect the complexed eIF4A (eIF4A<sub>c</sub>) but overstimulation of the free cytosolic eIF4A (eIF4A<sub>f</sub>) causes impromptu, non-specific unwinding of mRNA templates resulting in interference of translation initiation and elongation. Alongside this activity, pateamine also causes eIF4A<sub>f</sub> to become tightly bound to the RNA template so that the helicase is eventually sequestered away from its cytosolic reservoir and therefore unavailable to form the eIF4F complex necessary

for effective translation initiation (Bordeleau *et al* 2005). Despite the high specificity of pateamine A for eIF4A even among the DEAD box helicase family (Bordeleau found that it had no affinity for the related Ded1p helicase) and the fact that it shows greater efficacy than hippuristanol in similar conditions (Mazroui 2006); there are a number of problems with the development of pateamine into an anti-cancer drug. Unlike hippuristanol, pateamine binds irreversibly to eIF4A potentially resulting in its degradation and the permanent inactivation of the cellular



**Figure 4** Hippuristanol

machinery of translation initiation (Bordeleau *et al* 2006) making unmodified pateamine an extremely toxic compound (Bordeleau *et al* 2008). Pateamine A has also previously been shown to possess immunosuppressive properties (Romo *et al* 1998).

## HIPPURISTANOL

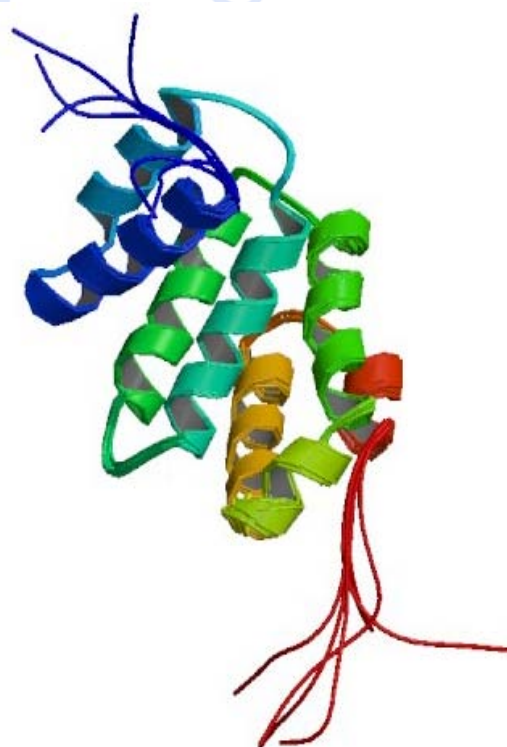
Hippuristanol was also discovered by Bordeleau *et al* in 2006 as a highly specific inhibitor of cap-dependent translation; they found that a dose of 1  $\mu\text{M}$  was sufficient to reduce translation by 60% in Krebs-2 extracts (Bordeleau *et al* 2006). The molecule itself is a 462.66  $\text{g mol}^{-1}$  cytotoxic polyoxygenated steroid (Figure 4) which was originally isolated from the gorgonian coral *Isis hippuris* (Figure 5) found off the coast of Okinawa.

Bordeleau showed that hippuristanol acts by weakening the ability of eIF4A to bind to RNA; further postulating that hippuristanol may bind in a reversible manner to a number of conserved motifs (IV – VI) within the C terminus of eIF4A. ATP hydrolysis and mRNA binding are believed to be the responsibility of motif IV while V and VI form the active closed conformation of eIF4A (Bordeleau *et al* 2006). The application of hippuristanol as an

anti-cancer agent has consistently played second string to its potential as a treatment for viral pathogenesis as a large number of viruses also rely comparatively heavily upon eIF4A for the pre-translation 5' processing of their mRNA.

#### PDCD4

In 2003 Yang *et al* identified that pcd4 (programmed cell death 4), a human gene believed to be involved in the inhibition of promoter-induced neoplastic transformation and the activation of AP-1 dependent transcription required for transformation also acted as a direct



inhibitor of eIF4A. The protein itself consists of 469 amino acid residues and is 51.7 kDa (Figure 6). Recent x-



Figure 5 The gorgonian coral *Isis Hippuris*

ray crystallographic analysis of the pdcd4 has determined that it possesses two MA3 domains which bind in tandem to the N terminal domain of eIF4A, competing with both RNA and eIF4G for the site and in so doing, inhibiting the action of eIF4A (Suzuki *et al* 2008).

#### Figure 6 Pcd4

Despite the recognised potential of pdcd4 as a chemotherapeutic inhibitor of eIF4A in neoplastic cells; interest is largely concentrated on its role in cancerous transformation.

Along with their significant roles in cancerous cells, eIF4 members, which preside over the recruitment phase of translation initiation, are implicated in the processes which may lead to a cell undergoing transformation and becoming cancerous in the first place. The availability of eIF4E and eIF4A for eIF4F formation is regulated by mTOR (mammalian target of rapamycin) which determines the state of phosphorylation of the 3 eIF4E binding proteins (4E-BP) and modulates the action of the kinase (S6K1) responsible for phosphorylation of pdcd4 in response to mitogens. Upon phosphorylation, 4E-BPs are released from their usual

bond with eIF4E; releasing it for use in translation initiation, once pdcd4 is phosphorylated, it is degraded, thereby ceasing to inhibit eIF4A activity and allowing it to also enter the eIF4F complex (Bordeleau *et al* 2008). It has been shown that there exist elevated levels of eIF4 components in already cancerous cells but a number of studies have also demonstrated that introducing ectopically expressed eIF4E and eIF4G can transform cells in culture (Lazaris-Karatzas *et al* 1990). This is consistent with the fact that dysregulation of the mTOR pathway (which regulates 4A and E activity) can lead to transformation (Beevers *et al* 2006).

#### SILVESTROL

Bordeleau recently (2008) identified a further inhibitor of eIF4A derived from a natural source. The cyclopenta benzofuran flavagline (Figure 8) silvestrol was originally isolated from the fruits and twigs of *Aglaia silvestris* (Figure 7), a species of plant belonging to the mahogany family (Meliaceae) (Hwang *et al* 2004).



the discovery of further eIF4A inhibitors. The purpose of this effort is twofold; to learn more about the, still relatively enigmatic, process of 4A mediated translation and also to test successful compounds as candidates for cancer therapy as the current inhibitors have certain limitations in this application. The high-throughput screens will be divided into *in vitro* and *in vivo* components.

#### IN VITRO SCREEN

In 1998, Rodgers *et al* performed an *in vitro* assay of eIF4A helicase activity which involved the synthesis of oligonucleotides consisting of short radiolabelled and long (cold) RNA strands, they demonstrated the eIF4A could function *in vitro* without the need for any coactive molecules such as eIF4B and successfully unwind and separate the annealed short and long duplex. This approach cannot be adapted to work as a high throughput screen due to the northern analysis required following each iteration. The actual mechanism by which the *in vitro* assay will operate relies upon the fact that ethidium bromide is twenty times more fluorescent when associated with double rather than single stranded RNA; if the helicase

operates as normal then it will unwind and eventually dissociate the duplex leading to a reduction in fluorescence. The duplex to be used is a palindromic RNA hairpin (RHP1) developed by Yang *et al* and shown to inhibit translation without eIF4A. To enhance the precision of the screen, a further differential between double and single stranded RNA will be introduced, in the form of RNase T1 which cleaves only single stranded RNA. The above components will be combined in 96 well plates each containing a different small molecule with effective inhibitory molecules showing an increased level of ethidium bromide fluorescence.

#### IN VIVO SCREEN

The parallel live-cell screen, for which HeLa cultures will be used, is important for a number of reasons; it may be used to select against cytotoxicity as such compounds would be fatal to the cell and also select against compounds that do not pass readily into the cell. Such a system would also be conducive to RNA interference as an effective positive control, an impossibility in the *in vitro* system. The palindromic

RNA hairpin developed by Yang *et al* used in the *in vitro* screen will be cloned into the vector pcmvDsRedExpressDR between destabilised *Renilla* luciferase and firefly luciferase genes under the control of a cmv promoter. A control plasmid (pcmvZsGreenExpressDR) will be cotransfected which will not contain the hairpin to serve to differentiate between overall protein and eIF4A mediated protein expression.

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