The Efficient

and Specific Tumour

Cell Killing

by Modified Apoptin

By Chetan Kaher

Abstract

One in three people develops cancer. The vast majority of treatments are not successful due to their reliance on a functional p53 gene. Most cancers have mutations present in p53 or in other genes, which over-ride the effect of functional p53. Apoptin, a novel protein, has been found to induce apoptosis specifically in cancerous cells both *in vitro* and *in vivo* in a variety of tumour types, independent of the genetic aberration(s) causing the malignancy. To date Apoptin DNA has been introduced into cells by transfection or infection, with no studies using the Apoptin protein. A variety of proteins, when fused to the TAT-PTD protein from HIV have transduced various cells and still retained their function. Protein transduction has occurred in a receptor independent manor and been highly efficient. The 11 amino acids at the C terminus of Apoptin shares homology with TAT-PTD. This study modified the 3' end of Apoptin DNA into TAT-PTD, for the development of a protein based cancer therapy using the tumour specific properties of Apoptin and the transducing properties of TAT-PTD. Constructed DNA was checked by sequencing and cloned into a mammalian expression vector pcDNA3.1. Transfection into p53 deficient SAOS-2 cells revealed Apoptin-TAT was expressed in their nuclei, but with a slightly different pattern of expression compared to Apoptin. For further studies of the protein the constructed DNA was cloned into the bacterial expression vector pGEX-5X-3.

Contents

Introduction

1.1 Cancer

One in three people develop cancer in their lifetimes. Cancer is responsible for 24% of all mortalities and 250,000 new cases arise every year in the UK, with the incidence increasing [Key *et al*, 1997]. Cancer is a genetic disease, caused by uncontrolled cell proliferation and/or decreased programmed cell death due to defects in cell cycle regulating genes. The mammalian cell cycle is divided into five phases; G0 (quiescent state), G1 (gap 1), S (DNA synthesis), G2 (gap 2) and M (mitosis). Two broad categories of cell cycle regulating genes exist:

1. Proto-oncogenes, i.e. Jun, Ras, Bcl-2, whose normal activity is to promote cell proliferation. Mutations in these genes may create abnormal forms (oncogenes) which are excessively active, resulting in increased proliferation and/or decreased apoptosis.

2. Tumour suppressor genes (TSG), i.e. Rb, p53, BRCA 1& 2, whose normal function is to control the cell cycle, and induce cell cycle arrest or apoptosis in DNA damaged cells, or alternatively, repair the DNA damage and ensure accurate DNA replication.

Over a hundred tumour suppressor genes and proto-oncogenes, playing a role in cell-cycle have been identified, and a combination of mutations, deletions, translocations and duplications in several of these can cause cancer [Carson & Lois, 1995].

Cells have certain mechanisms in place to repair mutated or damaged DNA before they divide. Cells unable to repair this damage acquire mutations, which leads to genetic instability. If they accumulate enough mutations, believed to be about seven **[**Jones, 2000**]**, in genes controlling the cell cycle, there is a chance they may turn into cancerous (malignant) cells. Cells have developed mechanisms to prevent excessive mutations, including TSG mediated arrest in cell cycle (to prevent further proliferation) or the induction of apoptosis.

1.1.1 Apoptosis

Apoptosis is defined as programmed cell death, which is activated by specific genes (e.g. Bax) in a controlled manner. It is characterised by morphological changes, including the shrinkage of cells, fragmentation of their cytoplasm and segmentation of their nuclei [Kerr *et al*, 1994]. The apoptotic cells become fragmented into membrane enclosed apoptotic bodies, which are then phagocytosed by neighbouring cells [Schmitt & Lowe, 1999]. Apoptosis plays an important role in a variety of processes, including the formation of organs during embroygenesis, the regulation of the immune system and the elimination of DNA damaged cells, which threaten to become cancerous cells or are cancerous [Noteborn *et al*, 1998b]. Both apoptosis and cell proliferation is controlled by a variety of regulatory genes. Mutation in one or more of these genes may prevent the induction of apoptosis and increase growth, in DNA damaged cells, predisposing cells to cancer.

1.1.2 Tumour suppressor genes

p53

The TSG p53 is a DNA binding transcription factor and also has a regulatory function in the expression of large number of genes. Furthermore, it has a crucial role in the induction of apoptosis in DNA damaged cells. Most mutations in p53 result in its inactivation, however, occasionally certain types of mutations can convert it into a dominant oncogene [Levin *et al*, 1991]. Thus, mutations in p53 not only inhibit apoptosis, but can also stimulate cell division. More than 50% of cancers have mutations in the p53 gene [Lowe *et al*, 1994]. In normal cells p53 is kept in low levels by its interaction with MDM2, which signals the ubiquitin mediated degradation of p53 [Evan and Littlewood, 1998]. MDM2 has been found over-expressed in various tumour types, including those with wild-type p53 [Evan and Littlewood, 1998]. DNA damage causes the activation of many protein kinases, such as, ATM and DNA-PK, these in turn, phosphorylate p53 and/or MDM2 and prevent them from interacting, thus stabilising and activating p53. This causes the arrest of damaged cells in the G1 phase of the cell cycle, or causes the cell to undergo apoptosis [Campbell *et al*, 1994; Evan & Littlewood 1998]. p53 induces apoptosis, by an as yet undefined pathway (Fig. 1.1)**,** however, the induction of the oncogene Bcl-2 antagonist Bax, insulin like growth factor-1 (IGF) receptor and other p53 inducible genes (PIGs) and the simultaneous trans-repression of anti-apoptotic genes have all been demonstrated [Schmitt & Lowe, 1999; Evan & Littlewood, 1998]. It has also been noted that certain oncogenes, such as, Myc, also induce apoptosis in some circumstances [Evan & Littlewood, 1998]. Cells that do not express functional p53 protein are unable to arrest at the G1-S cell

cycle checkpoint or undergo apoptosis, and proceed unchecked through DNA synthesis [Ziegler *et al*, 1994]. This prevents the cells from repairing the incurred DNA damage prior to DNA replication and results in a higher rate of mutation.

pRb

The retinoblasoma tumour suppressor protein (pRb) also plays a critical role in cell cycle regulation. pRb acts to restrict cells in late G1 phase preventing the replication of cells. The D type cyclins and their associated kinases (cdk4 & cdk6) cause the phosphorylation and inactivation of pRb and the subsequent release of proteins, such as E2F, which are associated with pRb. This, further leads to the transcription of a number of genes, which initiate DNA synthesis (S phase) and cell proliferation [Alevizopoulos *et al*, 1997]. Thus, mutations of components in the Rb pathway, such as Rb, Cyclin D1 or the TSG $p16^{INK4a}$ (which inhibits cdk4 & cdk6 [Gius et al, 1999]) lead to a higher frequency of cancer [Bartek *et al*, 1997]. The Rb and p53 pathways are linked. $p21^{CIP1}$ is induced by a p53 dependent mechanism, which prevents the phosphorylation of pRb. This results in temporary cell arrest [Bartek *et al*, 1997]. Therefore mutations in components of the p53 regulated pathway effect the pRb regulated cell-cycle pathway.

1.1.3 Oncogenes

Over expression of anti-apoptotic genes, such as the proto-oncogenes Bcl-2 or the Bcl-2 related proteins e.g. Bcl-x and BAG-1, can render cells resistant to the induction of apoptosis and susceptible to oncogenic transformation, even in the presence of functional p53 [Danen Van Oorschot *et al*, 1999a]. Bcl-2, BAG-1 and Bcl-x inhibit p53-dependant apoptosis by acting down stream of p53 in the apoptotic pathway. They function by blocking the Bax mediated cytochrome c release from the mitochondria, which is known to be essential for downstream Apaf1 and caspase 9 activation (see section 1.6.1), which act to induce apoptosis (Fig. 1.1) [Desagher & Martinou, 2000; Liu *et al*, 1996; Rao and White, 1997; Schmitt & Lowe, 1999]. Over-expression of Bcl-2 by chromosomal translocations has been observed in several tumours, such as lymphoma, leukaemia and breast cancer. It is also over expressed in basal cell carcinomas and malignant melanomas [Noteborn *et al*, 1998b]. Other apoptosis inhibiting proteins include BCR-ABL oncogene, which is predominantly found in chronic myeloid leukaemia [Noteborn et al 1998b].

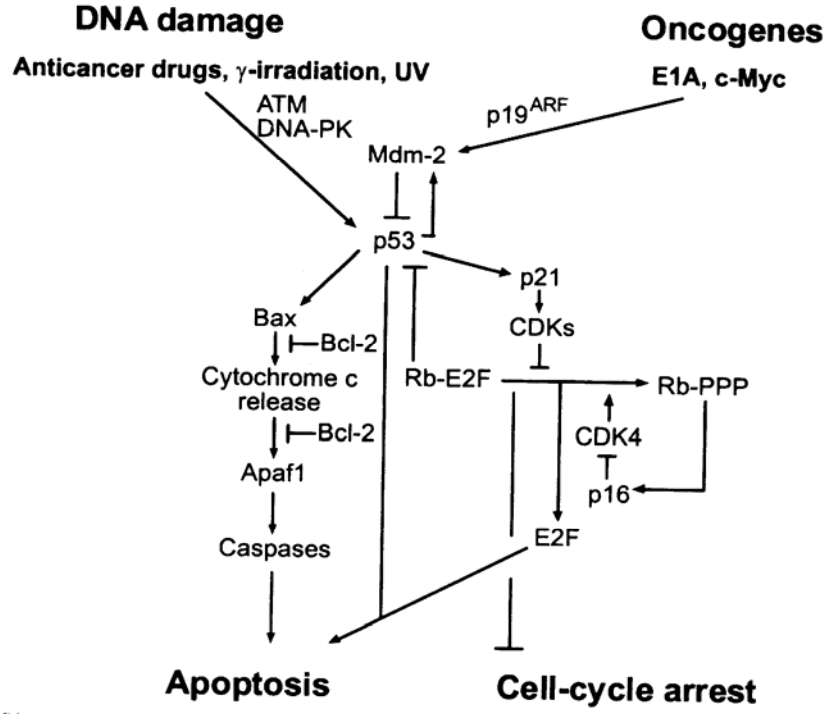


Fig. 1.1 The mechanisms by which DNA damage initiates pathways involved in apoptosis and cell cycle arrest [Schmitt & Lowe, 1999].

1.2 Head and Neck Cancer

Head and neck cancers encompase a wide range of malignant tumours involving multiple cell types. Squamous cell carcinoma of the head and neck is the sixth most common cancer world-wide [Jones 2000]. Smoking is the most important aetiological factor, with up to 90% of squamous cell carcinoma patients having a history of smoking [Jones 2000]. Molecular analysis of squamous cell carcinomas of the head and neck (SCCHN) has revealed a distinctive set of mutations, usually in the region of six to ten, which repeatedly occur. A high frequency of gene amplification and overexpression of oncogenes such as Myc, erbB-2, EGFR and cyclin D1, and mutations, deletions and hypermethylation of $p16^{INK4a}$ and p53 TSGs occur leading to their inactivation [Nagai 1999; Sartor *et al*, 1999]. Between 45-70% of head and neck cancers have mutations in p53 [Anderson, 2000], highlighting the importance of p53 in SCCHN. Despite advances in treatment over the last decades, approximately 50% of patients with SCCHN will die due to their cancer, a figure unchanged for the last 30 years [Gunnarskog *et al*, 1995]. This fact highlights the need for novel approaches.

1.3 Gene Therapy

Most cancer therapies (chemotherapy, radiotherapy and gene therapies) are dependent on a functional p53 to induce apoptosis in cancerous cells. However, the majority of cancers have a deficient or defective p53, or an activated anti-apoptotic oncogene (e.g. Bcl-2, BCR-ABL) in the presence of functional p53, which is believed to be the reason why most cancer therapies fail [Fan *et al*, 1994; Lowe *et al*, 1994]. Current cancer therapy routinely consists of a combination of surgery, chemo- and radiotherapy. Although they vary in their efficiency all have unpleasant side effects, highlighting the need for a highly specific and effective cancer therapy, with low toxicity.

The objective of gene therapy is the delivery and concomitant expression of gene products that either possess a therapeutic biological activity or induce an altered cellular phenotype. There are currently over 234 cancer gene therapy trials in progress across the world [Anderson, 2000]. Three distinct strategies have been developed: Mutation compensation, which involves gene(s) delivery to correct the molecular lesions causing the malignancy; Molecular chemotherapy, which involves gene delivery to kill the tumour cells or to increase their sensitivity to other therapies, and genetic immunotherapy, which attempts to increase the immunogenicity of tumour cells, to illicit an immune response from the host [Gomez-Navarro *et al*, 2000].

The initial euphoria that surrounded gene therapy has now subsided with the realisation that there are still many problems to overcome and no current gene therapy has proved completely successful.

Desired genes are incorporated into vectors. Vectors transduce cells and allow delivered gene(s) to be expressed intracellularly. As yet no gene delivery system (vector) has been able to efficiently transduce all the cells in a tumour, without being toxic to the host [Vile *et al*, 2000]. Vectors may be divided into viral (i.e. retro-, adeno-, lentiviral etc.) and non-viral (i.e. VP22, liposome, electroporation and naked DNA) [Kouraklis, 2000]. Poor efficiencies, induction of immune and inflammatory responses, possibility of mutagenesis, limited therapeutic gene length incorporation and variable expression times, are all disadvantages associated with viral vectors [Vile *et al*, 2000; Kouraklis, 2000]. The main draw-back non-viral vectors have is their poor *in vivo* efficiency (being unable to transduce a large number of cells) [Kouraklis, 2000]. Efficient gene delivery to cells in tumours is essential for cancer gene therapy to be successful. The current problems of gene delivery systems highlight the need for an efficient, non-toxic and specific mechanism of delivering gene(s) or their products into cells.

Cancerous cells are extremely heterogeneous in the expression of relevant oncogenes. Thus therapeutic targeting of a single molecular abnormality i.e. p53, may have only an inconsequential impact on the whole tumour, due to mutations in other cell cycle control genes [Gomez-Navarro *et al*, 2000]. The delivery of molecular agents has proved toxic in hosts at the concentrations required for molecular chemotherapy to be effective and in addition to this, sub-populations of tumour cells exist which are resistant to the effects of these therapeutic molecules (drug resistance) [Kouraklis, 2000]. The main obstacle of genetic immunotherapy appears to be the tolerance the immune system exhibits in regard to therapeutic agents which are implemented to make

tumour cells more immunogenic [Gomez-Navarro *et al*, 2000]. The main gene therapy success has come when it is used in combination with chemotherapy, rather than alone [Khuri *et al*, 2000] and it is thus unlikely that gene therapy alone will play a curative role in cancer for a number of years [Vile *et al*, 2000].

One of the few successes has been the ONYX-15 virus, which replicates in and lyses cells lacking functional p53, in combination with chemotherapy (cisplatin and 5-fluorouracil). In a trial of 30 patients with head and neck cancer treated with this combination, 19 patients had objective responses, where their tumours regressed by more then 50% and 8 patients demonstrated complete responses with the total regression of their tumours. In a different trial the use of ONYX-15 alone only achieved partial regression in 15% of patients [Khuri *et al*, 2000].

In most cancer gene therapies, sustained and regulated expression of the transgene is not a prerequisite for effective treatment. The primary objective being either the direct induction of tumour cell death or the induction/enhancement of immune-mediated elimination of the tumour. The short term requirement for the presence of a therapeutic gene and the current limitations of gene therapy raises the possibility of achieving the same objective by direct delivery of the gene product itself, rather than the gene [Ford *et al*, 2001].

1.4 Protein Transduction

Protein transduction is described as the internalisation of proteins from the external environment, into the cell. This process relies on the inherent properties of a small number of proteins and peptides of being able to penetrate the cell membrane. The transducing property of these molecules can be conferred upon proteins which are expressed as fusions with them and thus offers an alternative to gene therapy, for the delivery of therapeutic proteins into target cells. The three most commonly used protein transduction vehicles are the antennapedia peptide, the VP22 protein and the HIV TAT (**t**ranscriptional **ac**t**i**vator) protein transduction domain [Reviewed by Ford *et al*, 2001], which was used in this study.

Green and Loewenstein and Frankel and Pabo (1988) independently showed that the HIV-1 TAT protein could enter cells in a receptor independent manner, when added to culture media. Nagahara *et al* (1998) later showed that an 11 amino acid sequence of TAT, between amino acids 47-57, was able to transduce cells rapidly and locate in the nucleus and cytoplasm. Indeed, Efthymiadas *et al* (1998) showed TAT traveled to, entered, and bound to components in the nucleus through an as yet unidentified pathway. The sequence of this domain of TAT is high in the basic amino acids (Fig. 1.2). It has been speculated that TAT binding to the cellular membrane is mediated through charge-interactions between the basic region of TAT- protein transduction domain (PTD) and negatively charged polysaccharides on the cellular membrane [Mann & Frankel 1991].

Tyr-[**Gly-Arg-Lys-Lys-Arg-Arg]**-Gln-Arg-Arg-Arg

Fig. 1.2. Sequence of TAT-PTD. The amino acids in brackets are believed to be the nuclear localisation signal [Nagahara *et al*, 1998; Schwarze *et al*, 2000].

TAT has been fused to over 50 proteins of various sizes, ranging from 15 - 120kDa, including β-galactosidase (over 1000 amino acids), which have subsequently transduced rapidly (within 20 mins) into a variety of cells [Schwarze *et al*, 1999]. TAT-PTD fused proteins transduce cells most efficiently when they are first denatured by urea (i.e. before being added to cells) [Nagahara *et al*, 1998]. Once they transduce the cell, they then correctly refold aided by molecular chaperones [Gottesman *et al*, 1997]. Nagahara *et al* (1998) showed that TAT-PTD fused with $p27^{Kip1}$, was able to transduce 100% of cells in less than 10 minutes, when added to their culture media. Encouragingly, $p27^{Kip1}$ still retained its biological activity when fused to TAT-PTD, of binding and inactivating cyclin E:Cdk2 complexes – to elicit cell migration in hepato-cellular carcinoma cells. Importantly, all eukaryotic cells tested to date are susceptible to TAT transduction [Schwarze *et al*, 2000]. TAT has also been able to transduce cells such as osteoclasts, which had previously been resistant to the expression of constructs by transfection or retroviral infection [Schwarze *et al*, 2000]. Most experiments have been conducted *in vitro*, but promisingly, *in vivo* experiments have also proved successful [Schwarze *et al*, 1999]. Lewin *et al* (2000) demonstrated that a 40-nm supraparamagnetic iron particle coated in thio-linked TAT-PTD, was also able to transduce cell membranes, showing TAT-PTD is able to transduce substances other then proteins. No toxic effects, have been noted in mice after multiple TAT-PTD injections [Schwarze *et al*, 1999].

The evidence above supports the possibilities for the development of new protein therapies for cancer and other diseases. However, it must be noted that transducing proteins readily enter all cells, thus, any therapeutic agent fused to TAT-PTD must be specific for only diseased cells or non-toxic to non-diseased cells. Therefore, fusing TAT-PTD to proteins displaying intrinsic cell specificity may be extremely promising for the development of novel therapies.

1.5 Chicken anaemia virus

Chicken anaemia virus (CAV) is a single-stranded circular DNA virus composed of 2319 nucleotides, that transiently causes severe anaemia by depletion of thymocytes and erythroblastoid cells in young chickens via apoptosis [Jeurissen *et al*, 1992; Noteborn *et al*, 1994]. The DNA encodes three proteins; VP1 (51.6kDa) being the capsid protein, VP2 (24kDa) of unknown function and VP3 (13.6kDa), which also has an undetermined function, but is known to be essential for viral replication [Noteborn et al, 1994]. In 1994, Noteborn *et al* found the expression of VP3 alone was enough to induce apoptosis in transformed chicken cells (T lymphoblastoid cells and Myeloid cells). This led to the renaming of VP3 to Apoptin, from **apop**tosis **in**duction.

1.6 Apoptin

Apoptin is a small protein of 121 amino acids, which does not resemble any other sequenced protein. It carries several known protein domains, including 2 nuclear-localisation signals and a nuclear-export signal [Danen Van Oorschot *et al*, 1997b]. It also has regions rich in proline or basic amino acids and overall it contains a high percentage of serine and theorine residues (see Appendix B) [Noteborn *et al*, 1991].

In 1995 Zhuang *et al* (a, b & c) found that Apoptin was also able to induce apoptosis in human malignant cells. It has since been shown to induce apoptosis in a variety of human transformed and/or tumourigenic cell lines, including; hepatomas, lymphomas, leukaemias, melanomas, breast and lung tumours, neuroblastomas, cholangio-, colon-, and squamous cell carcinomas [Noteborn 1998a; Zhuang *et al* 1995a; Danen-Van Oorschot *et al*, 1997b]. When these cell lines were transfected with an Apoptin encoding plasmid, 90-100% of them underwent apoptosis within 6 days. Danen-Van Oorschot *et al*, (1997b) showed when normal cell lines such as endothelial (HUVEC), epidermal (FSK-1), dermal (VH10) and human $CD3^+$ T cells, were transfected with Apoptin encoding plasmids, only 20% underwent apoptosis after 5 days. This was similar to the amount that underwent apoptosis when transfected with a control vector**.** In a more direct assessment, they compared the effect of transfecting Apoptin into VH10 and FSK-1 cells, versus their malignant counter parts, cell lines NW-18 and SCC-15 respectively. After 5 days 15% of the normal cells (VH10 and FSK-1) had undergone apoptosis, compared to around 75% of the cancerous NW-18 and SCC-15 cells. After six to seven days, 100% of the tumour cells expressing Apoptin underwent

apoptosis. All the studies so far have shown that Apoptin induces apoptosis specifically, in cancerous or transformed cells. To exclude the fact Apoptin may have delayed effects in normal cells, they transfected normal VH10 cells with either an Apoptin encoded plasmid or a control plasmid with neomycin resistance encoded. After 10 population doublings both sets of cells became senescent, showing that Apoptin was not toxic, and had no transforming activities in VH10 cells.

Apoptin induces apoptosis in a p53 independent manner, as cancerous cell lines, such as osteosarcoma (SAOS-2), with defective or absent p53 are as susceptible to Apoptin as those with functional p53 [Zhuang *et al*, 1995c; Noteborn *et al*, 1998a]. In addition Simian virus 40 (SV40) transformed tumourigenic fibroblasts NW-18 were also shown to undergo Apoptin-induced apoptosis [Danen Van Oorshchot *et al*, 1997b], further providing evidence that Apoptin induced apoptosis is p53 independent, as the SV40 transforming gene Large T antigen is known to bind to and inactivate p53.

Normal cells (VH10) transfected with the SV40 transforming gene Large T antigen are also susceptible to Apoptin-induced apoptosis [Noteborn 1998b], showing that the expression of a transforming protein, is enough to make normal cells susceptible to Apoptin-induced apoptosis. Apoptin has also been shown to be able to induce apoptosis in UV and X-ray irradiated cells from cancer prone individuals. For instance, irradiated cells from those with the inherited Li-Fraumeni syndrome, which is characterised by germ-line mutations in p53, are susceptible to Apoptin induced apoptosis, but normal cells from these individuals are not [Zhang *et al*, 1999; Noteborn, 1998b].

As explained, p53 dependant or independent apoptosis can be inhibited by over expression of oncogenes such as Bcl-2, BAG-1 and BCR-ABL. Apoptin has been shown to induce apoptosis in tumourigenic cell lines with high levels of Bcl-2, BCR-ABL and BAG-1 [Zhuang *et al*, 1995a; Danen Van Oorschot *et al*, 1997a, 1999b]. Interestingly, Zhuang *et al* (1995a) also found that Apoptin induced apoptosis faster in DoHH-2 cells, containing a high level of Bcl-2, than in K562 cells, with normal levels of Bcl-2. This was later confirmed by Danen Van Oorschot *et al* (1999b) who found that SAOS-2 cells transfected with both Apoptin and Bcl-2 underwent apoptosis to a significantly higher level than cells expressing Apoptin alone. This is surprising since Bcl-2 is known to inhibit p53 dependant or independent apoptosis. Immunoprecipitation assays show that Bcl-2 does not co-localise with Apoptin [Danen Van Oorschot *et al*, 1999a], thus it may be concluded that Bcl-2 plays an indirect role in the Apoptin mediated pathways in malignant cells. The Bcl-2 associating protein, BAG-1, can by itself, or in combination with Bcl-2, inhibit p53-induced apoptosis in human transformed cells [Danen Van Oorschot *et al*, 1997a]. Transfection studies with co-expression of Apoptin and BAG-1, revealed that Apoptin was still able to induce apoptosis, showing Apoptin acts via a BAG-1 independent pathway [Danen Van Oorschot *et al*, 1997a].

Recently, Pietersen *et al*, (1999) demonstrated that Apoptin, when cloned into a replication deficient adenovirus vector - AdMLP-VP3, was able to induce apoptosis in malignant cells *in vitro* and *in vivo* at a faster rate, than in transfection studies. Infection of normal rat hepatocytes with AdMLP-VP3 did not lead to an increase in apoptosis. In contrast, human hepatoma HepG2 and Hep3b cell lines, underwent a

rapid induction of apoptosis when infected with AdMLP-VP3, which was not observed when they were infected with controls (Fig. 1.3).

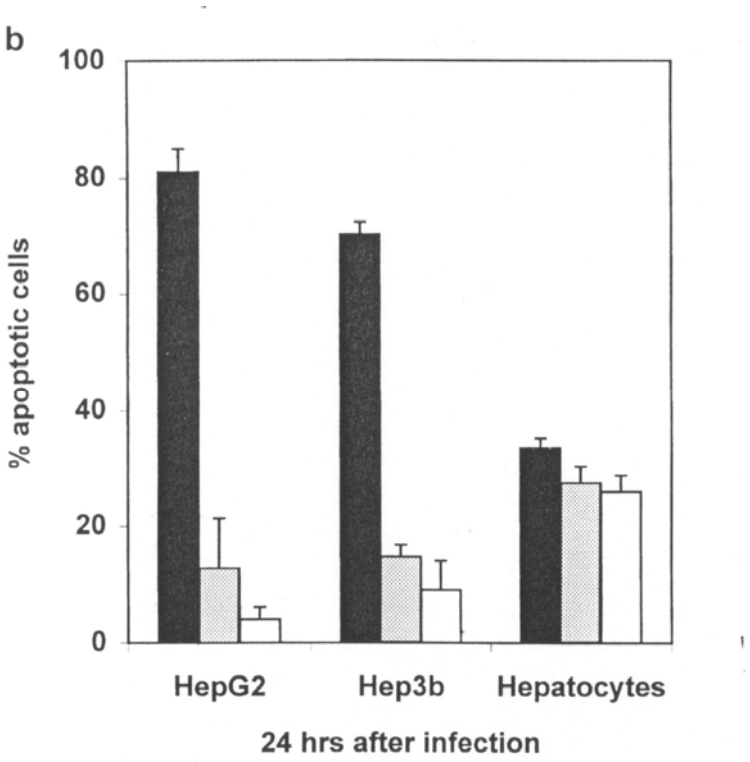


Figure 1.3 [Pietersen et al, 1999]. The effect of Apoptin on normal and malignant hepatocytes. HepG2, Hep3b and normal rat hepatocytes were fixed 24 h after infection. The percentage of cells that stained abnormally with DAPI was used as a relative indicator of apoptosis. The percentages are given for cells expressing Apoptin (black bars), β- galactosidase (grey bars) and non-infected cells. In each experiment at least 100 cells expressing Apoptin or β- galactosidase were examined.

In the first *in vivo* experiment, a single intratumoral injection of AdMLP-VP3 was administered into a xenogenic tumour of HepG2 cells (measuring 50 x 10^{-03} m^2) in nude mice. This resulted in a significant reduction of tumour growth when compared to tumours injected with control virus or saline [Pieterson *et al*, 1999] (Fig. 1.4).

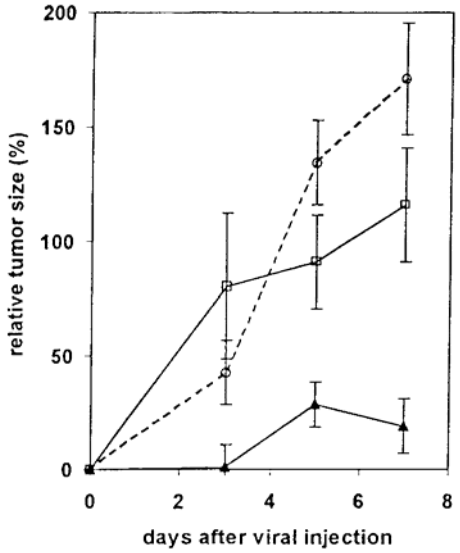


Figure 1.4 [Pietersen et al, 1999]. Anti-tumour effect of Apoptin. The rate of tumour growth is depicted relative to the initial size of tumours treated with ADMLP-VP3 (black triangles), AdMLPas - VP3 anti-sense (open squares) or saline (open circles).

Experiments in rats, where repeated intravenous doses of AdMLP-VP3 were administered, were well tolerated, indicating that the Apoptin expressing virus can be safely administered without serious side effects in rats. The animals showed a normal increase in weight and looked healthy, and upon autopsy no tissues appeared abnormal [Pieterson *et al*, 1999].

1.6.1 Mechanism of Action

The mechanism of Apoptins' action is yet to be elucidated. In all the Apoptin studies mentioned above it was found that normal cells, not susceptible to Apoptin induced apoptosis, have Apoptin located in the cytoplasm, whereas in malignant or transformed cells, Apoptin always localises in the nucleus. Zhuang *et al*, (1995a; 1995c) and Danen-Van Oorschot *et al*, (1997b), demonstrated that a truncated Apoptin – with 11 amino acids deleted at its C terminus, displayed less efficient nuclear localisation, in combination with delayed and reduced apoptotic activity in malignant cells. This suggests that the last 11 amino acids may serve as a nuclear localisation signal. Zhuang *et al*, (1995b) showed that the adenovirus 5 protein, E1B-21kDa, prevented the nuclear localisation of Apoptin and the induction of apoptosis in osteosarcoma cell lines. However, in the human hepatoma cell line Hep3b, it was unable to prevent the nuclear localisation of Apoptin, and apoptosis was induced. Electron microscope studies of chicken mononuclear cells showed that Apoptin co-localises with cellular chromatin [Noteborn *et al*, 1994], suggesting that it interacts with nuclear chromatin, perhaps to unwind its structure. This evidence implies the nuclear localisation of Apoptin is important for its function.

The mechanism by which Apoptin translocates to the nucleus in tumour cells is, as yet, unknown. It is possible that in normal cells it interacts with components in the cytoplasm, which keep it there. These components may be lost when the cell is transformed. Alternatively it may be that malignant and transformed cells may express an unidentified protein, which somehow causes Apoptin to translocate to the nucleus.

The fact that Apoptin acts independently of p53, Bcl-2 and BCR-ABL but in a Bcl-2 stimulated manner, suggests it triggers apoptosis by a yet uncharacterised pathway.

Caspases play a central role in most apoptotic pathways. They are known to cleave and regulate many proteins involved with apoptosis [Danen-Van Oorschot *et al*, 2000; Green, 1998; Cheng *et al*, 1997]. Caspases may be divided into; upstream initiator caspases and downstream effector caspases. Different apoptotic signals activate different initiator caspases, in turn activating downstream effector caspases, resulting in a cascade of caspase activation, leading to apoptosis. Danen-Van Oorschot (2000) demonstrated that Apoptin induced apoptosis requires the activation of downstream caspase 3 and perhaps other downstream caspases, but not the activation of the upstream caspases 1 and 8, showing that Apoptin acts downstream in the apoptotic pathway.

In light of the above evidence, the unique ability of Apoptin to induce apoptosis efficiently and specifically in cancer cells, independent of p53, Bcl-2 or BCR-ABL status, but in a Bcl-2 stimulated manner, suggest Apoptin may be an attractive candidate for cancer therapy. The activity of Apoptin in transformed cells may serve as a diagnostic tool for detection of individuals at an increased risk for hereditary cancer and pre-maligant lesions.

1.7 Project Aim

The potential of Apoptin as an effective agent in cancer therapy and the remarkable cell transducing properties of TAT-PTD, preludes to an exciting cancer therapy, in which a recombinant protein could be used to specifically target and kill a large percentage of malignant cells.

Comparison of the amino acid sequence of TAT-PTD and the 11 amino acid (aa) C terminal nuclear localisation signal of Apoptin, have revealed homology between the two (Fig 1.5).

Tyr-Gly-**Arg-Lys-Lys-Arg-Arg**-Gln-**Arg-Arg-Arg** *TAT-PTD*

...Arg-Pro-**Arg**-Thr-Ala-**Lys-Arg-Arg**-Ile-**Arg**-Leu *11aa from the C terminus of Apoptin*

Figure 1.5. The amino acid sequence of TAT-PTD and the C terminus of Apoptin [Schwarze *et al*, 2000; Noteborn *et al*, 1991].

Both sequences convey nuclear localisation signals to their respective proteins [Schwarze *et al*, 2000; Zhuang *et al*, 1995a; 1995c; Danen-Van Oorschot *et al*, 1997b] and both have a high percentage of the basic amino acids Arginine and Lysine (in bold). It was thus thought that modifying the end 11aa nuclear localisation signal of Apoptin with TAT-PTD, would convey cell transducing properties to Apoptin, which would enable it to be used alone as an effective cancer therapy.

The aim of this project is to modify the 11 codons at the 3' end of Apoptin DNA into TAT-PTD and to 1) clone this into a mammalian expression vector to check if the protein is expressed in a tumour cell line and compare its pattern of expression with that of Apoptin, and, 2) clone it into bacterial expression vectors to enable the expression of protein. Further studies by the Oral Oncology group will then elucidate the effectiveness of the recombinant protein in SCCHN cell lines, followed by other tumour cell lines.

In this project a VP3 gene obtained from a different CAV isolate, with two mismatched base pairs compared to Apoptin DNA (Appendix B) which results in two changes in the aa sequence, will be studied in tandem to determine if this gene possesses the same properties as Apoptin. One of the mismatches is in the 11 aa at the C terminus of Apoptin (Fig. 1.6).

Arg- Pro-Arg-Thr-Ala-**Arg**-Arg-Arg-Ile-Arg-Leu *11aa from the C terminus of VP3*

Arg-Pro-Arg-Thr-Ala-**Lys**-Arg-Arg-Ile-Arg-Leu *11aa from the C terminus of Apoptin*

Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg *TAT-PTD*

Figure 1.6 Comparison of the amino acid sequence of the C terminus of VP3, Apoptin and TAT-PTD. The mismatch between Apoptin and VP3 occurs at the sixth aa from the C terminus, where Lysine is replaced by Arginine (in bold) [Lars Guelen (unpublished), Noteborn *et al*, 1991 Schwarze et al, 2000].

2.0 Materials & Methods

All reagents used were of analytical grade or equivalent, and obtained from standard suppliers, except where mentioned. Apoptin, VP3 and TAT DNA were kindly supplied by Lars Guelen MSc, Oral Oncology Group, GKT Dental Institute, London, UK. For composition of buffers, solutions and culture media see Appendix A.

For convenience Apoptin-TAT-PTD and VP3-TAT-PTD constructed DNA will be called Ap-TAT and VP3-TAT respectively.

2.1 Production of Ap-TAT and VP3-TAT DNA

2.1.1 Primer design

Primers were designed so the 11aa 3' end of Apoptin and VP3 DNA could be modified into TAT (Fig. 2.1), with an overlap of 11 bases (see Appendix B and section 1.7 for DNA and amino acid sequence of Apoptin, VP3 and TAT-PTD). The primers were designed in accordance to recommendations set out by Dieffenbach *et al* (1995).

VP3/Apoptin Forward Primer 5' ATG-AAC-GCT-CTC-CAA-GAA-G 3'

Reverse Primer 5' TTA-CCG-TCT-TCT-TTC-CCT-TCT-TTT-CTT-TCG-GCC-ATA- ***GCT-GGG-AGT-AGT-GG*** *3'*

Figure 2.1 Forward and reverse primer design. The reverse primer consists of a TAT-PTD region and an Apoptin binding region, the latter being shown in bold type.

The codons of the reverse primer were designed to obtain the highest possible homology with the 3' Apoptin/VP3 DNA sequence (Appendix B), while still retaining the TAT amino acid sequence when translated. The primers were made by MWG Biotech, Germany.

2.1.2 Production and Amplification of Ap-TAT and VP3-TAT DNA

Polymerase chain reaction (PCR) was carried out in a HYBAID Omnigene® PCR machine with 20ng DNA (1.25µl DNA) in a total volume of 100µl solution, containing 0.5µl (50pmol) of each primer; 1µl 25mM deoxyribonucleotidetriphosphates (dNTPs); 10µl 10X Pfu buffer (Stratagene™, USA Promega™, USA) solution; 1µl 2.5U/µl of Pfu DNA polymerase (Promega) or Pfu Turbo® DNA polymerase (Stratagene) and 85.75µl of autoclaved water. Using Pfu polymerase a very low mutation frequency (1 X 10^{-6} per base pair duplication) was assured [Cline *et al*, 1996], due to inherent 3'-5' proof reading (exonuclease) mechanisms**.** The reaction was performed as follows:

- 94°C for 2 min - to denature the DNA

- 10 cycles - 94°C for 30 sec - to denature DNA
  46°C for 30 sec - to anneal reverse primer
  72°C for 30 sec - polymerase extension step

- 35 cycles - 94°C for 30 sec - to denature DNA
  56°C for 30 sec - to anneal both primers
  72°C for 30 sec - polymerase extension step

- 72°C for 5 min - polymerase extension

The annealing temperatures (T_m) were estimated using the following formula: $T_m \approx 2(N_A + N_T) + 4(N_G + N_C)$, where N equals the number of adenine (A), thymine (T), guanine (G), or cytosine (C) bases in the primer [Dieffenbach *et al*, 1995]. As there was not 100% homology between the reverse primer and the 3' Apoptin/VP3 sequence, the first 10 cycles were completed at 46°C to enable the annealing of the non-specific reverse primer and amplify the Ap-TAT/VP3-TAT sequences. Subsequently, the annealing temperature was raised to 56°C to anneal the forward primer and obtain maximum amplification of the sequence.

One unit (0.25µl) of SuperTaq DNA Polymerase (HT Biotechnology, UK) was added to 25µl of the PCR reaction mixture and incubated at 72°C for 30 min, in order to add deoxyadenosinetriphosphate (dATP) to the 3' end of the DNA. This is required for ligation into pCR2.1® TOPO vector (Invitrogen Life Technologies, UK) which has single thymidine overhangs (Appendix C). The samples were stored at -20°C.

2.1.3 Agarose gel electrophoresis

DNA from each reaction was separated and analysed by electrophoresis through a 1% agarose gel. Gels were prepared by mixing 1g of agarose powder (Kramel Biotech, UK) in 100ml 1X TAE buffer and dissolved by boiling in a microwave on medium heat for 3 min. The solution was taken out and allowed to cool to 50°C before 5µl of 10mg/ml ethidium bromide was added. Ethidium bromide intercalates between the bases of DNA and fluoresces on exposure to UV light, allowing the visualisation and quantification of DNA. The solution was then mixed and poured into a sealed off casting plate, air bubbles were removed and combs were added to make DNA wells. The gels were allowed to set for about 30 min, after which the combs and seals were removed. The gels were subsequently placed in an electrophoresis tank, which was filled with 900ml of 1X TAE. A mixture of 25µl DNA solution in the presence of 1X loading buffer was then loaded into the wells. A 100bp DNA ladder - 10µl (50ng/µl - New England BioLabs (NEB), USA) was used to measure the size of DNA fragments and estimate concentrations. Electrophoresis was carried out at 100V for approximately 45 min, the gels were subsequently visualised under UV light.

2.2 Cloning of Ap-TAT and VP3-TAT for sequencing

2.2.1 Purification of PCR product

The inserts were cut out of the gel using a sterile scalpel. The DNA was then extracted from gel using the QIAEX II® Gel Extraction Kit protocol, and dissolved in 40µl 0.1X TE (QUIAX II handbook, February 1999, QIAGEN, Germany).

2.2.2 Ligation of Ap-TAT and VP3-TAT into pCR2.1® TOPO vector

Constructed DNA was cloned into pCR2.1® TOPO vector for sequencing. The reaction was carried out using the TA cloning kit (Invitrogen). The following components were mixed, in order:

- 1µl sterile water
- 1µl pCR2.1® TOPO vector (linearised)
- 3µl DNA (Stratagene - Ap-TAT, VP3-TAT and Promega -Ap-TAT, VP3-TAT)

The components were gently mixed with a pipette and left to stand at room temperature for 5 min.

2.2.3 Transformation and growing of competent cells with recombinant plasmid

To 50µl of ice thawed OneShot® TOP10F′ competent *E. Coli* cells (Invitrogen) 2µl of the ligation mixture was added (on ice) and mixed. The heat shock process was then used to transform the cells [Sambrook et al, 1989a]; the mixture was incubated for on ice for 30 min and then at 42°C for 45 sec (which increases uptake of plasmid DNA) and finally on ice for 2 min. Next, 400µl of LB medium (Appendix A) was added and the cells were incubated for 40 min at 37°C, being shaken every 10 min to promote growth. Subsequently 150µl of the bacterial suspension along with 50µl 4% X-Gal was plated onto LB-agar plates (Appendix A) supplemented with 50µg/ml ampicillin, and incubated overnight at 37°C. Colonies that contained plasmid DNA with inserted PCR product could be identified as white colonies because of the insertional inactivation of the *LacZ* gene in the pCR2.1® TOPO vector. White colonies were picked from each plate and incubated shaking overnight at 37°C in 2ml LB medium containing 50µg/ml ampicillin.

2.2.4 Mini-preparation of plasmid DNA

Plasmid DNA was isolated by a method described by Sambrook, *et al* (1989a), as follows; 1.5ml of the bacterial cultures were placed in eppendorf tubes and spun at 13k revolutions per minute (rpm) for 5 min. The supernatant was carefully removed and the pellet resuspended in 100µl of cold mini-prep (MP) I solution (Appendix A), which initiates cell lysis. Next, 200µl of freshly prepared MP II (Appendix A) was added, mixed (by inverting) and subsequently incubated at 25°C for 5 min to complete cell

lysis and denature the cell's DNA and protein. The mixture was incubated on ice for 5 min after the addition of 150µl of cold MP III (Appendix A), to selectively renature plasmid DNA and precipitate chromosomal DNA, followed by centrifugation (13krpm/5min). The plasmid containing supernatant was then carefully removed and transferred into a fresh eppendorf tube. Next, 400µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the supernatant to remove any proteins present, and subsequently mixed by inverting the tubes five times. The mixture was centrifuged (13krpm/5min) and the top aqueous phase containing the plasmid removed, with care not to disturb the protein interface layer or the bottom organic phase. The aqueous layer was incubated for 45 min at -70°C after the addition of 800µl cold 100% ethanol, which served to precipitate and concentrate the plasmid DNA and remove any salts present from the MP solutions. The mixture was then spun (13krpm/15min) and the ethanol removed with due attention not to disturb the DNA pellet. The mixture was subsequently washed twice to remove residual salt as follows; 1ml of 70% ethanol was added to the tube, which was subsequently mixed, centrifuged (13krpm/5min) and the supernatant carefully removed. The DNA pellet was then air dried for 20 min and resuspended in 30µl 0.1 TE (Appendix A) with 10ng/ml RNAse A (to destroy residual RNA) and stored at -20°C.

2.2.5 Identification of plasmids containing inserts

*Eco*RI 10U/µl (MBI Fermentas, Lithuania) was used to cut the inserts out of pCR2.1® TOPO vector and check their length on agarose gel. A digestion pre-mix was made on ice for 24 reactions. Aliquots of 17µl (14µl water, 2µl buffer and 1µl *Eco*RI) were placed into fresh eppendorfs and 3µl of the plasmid DNA was added. The digestions were incubated for one hour at 37°C. The mixtures, in the presence of 1X loading buffer, were run on a 1% agarose gel (see section 2.1.3) along with 10µl (50ng/µl) 100 bp DNA ladder (NEB), as a molecular weight marker to check the size of inserts and estimate the concentration of plasmid DNA.

2.2.6 Sequencing

The reactions yielding the highest amounts of plasmid with insert were selected for sequencing. Approximately 1µg (10µl) of each sample of plasmid DNA was sent to Cambridge Bioscience Ltd, UK for sequencing.

2.3 Cloning of Ap-TAT and VP3-TAT into a Mammalian expression vector

Results from sequencing showed that Stratagene VP3-TAT and Promega Ap-TAT DNA sequences were correct, and were therefore used for subsequent experimentation.

Ap-TAT and VP3-TAT were cloned into the mammalian expression vector pcDNA3.1®+ (Invitrogen – Appendix C). pcDNA3.1+ was selected as it is known to express proteins in a wide range of mammalian cells, using a cytomegalovirus promoter. This would enable it to be used to test the protein in a variety of cell types in the future.

2.3.1 Transformation and amplification of pCR2.1® TOPO vector containing Ap-TAT and VP3-TAT

To amplify the DNA of Ap-TAT and VP3-TAT, approximately 200ng (1µl) plasmid DNA was removed from the mini-prep DNA (section 2.2.4) and diluted to a concentration of approximately 10ng/µl in 0.1TE. Next, 1µl of this dilution was transformed into 50µl of ice thawed OneShot® TOP10F′ competent *E. Coli* cells and grown following the protocol outlined in section 2.2.3, with the omission of X-GAL plating**.** Colonies were picked from each plate, added to 2ml LB medium containing 50µg/ml ampicillin and incubated shaking overnight.

2.3.2 Mini-preparation of plasmid DNA

Plasmid DNA was extracted using the TENS method [Zhou *et al*, 1990]. The cultures (1.5ml) were placed in eppendorf tubes and centrifuged (13krpm/5 min). Supernatant was removed from the cells leaving approximately 50µl, and the cells were resuspended by vortexing them briefly. Next, 300µl of TENS solution (Appendix A) was added to the suspension and vortexed, followed by the addition of 150µl of sodium acetate (pH5.2) and subsequent vortexing for 5 sec. This served to lyse the cells, denature their DNA and precipitate chromosomal DNA and other cellular components. The mixture was centrifuged (10krpm/5min) and the plasmid DNA containing supernatant removed and transferred to a new eppendorf. Next, 900µl of cold ethanol was added and the mixture spun (10krpm/10 min), to precipitate plasmid DNA. The supernatant was removed carefully and the pellet air dried for 20 min. The pellet was then resuspended in 50µl 0.1 TE containing 10ng/ml RNAse A. To determine the yield of the mini-prep method, one tenth volume plasmid DNA and 1X loading buffer (diluted in distilled water) was run on 1% agarose gel (section 2.1.3) along with 10µl (50ng/µl) 1kb DNA ladder.

2.3.3 Restriction enzyme digestion, gel electrophoresis and isolation of inserts

The inserts (Ap-TAT and VP3-TAT) were cut out of pCR2.1® TOPO vector using *Eco*RI as follows; 16µl of plasmid DNA (approx. 500ng VP3-TAT and 1µg Ap-TAT, section 2.3.2) was added to a mixture of 2µl (20U) *Eco*RI and 2µl (10X) *Eco*RI buffer, and gently mixed by pipetting before incubation at 37°C for 2 h. The mixture was subsequently run on 1% agarose gel with the addition of 1X loading buffer as described in section 2.2.5. The inserts were purified using the QIAGEN gel extraction kit (section 2.2.1).

2.3.4 Transformation and amplification of pcDNA3.1

pcDNA3.1+ (1µl - 10ng/µl) was transformed using the same protocol described in section 2.3.1. One colony was picked from the plate, added to 100ml LB medium containing 50µg/ml ampicillin and incubated shaking overnight at 37°C.

2.3.5 Maxi-preparation of pcDNA3.1

The Maxi-preparation of pcDNA3.1+ DNA was carried out following the QIAGEN® protocol (Qiagen Plasmid purification handbook July 1999 p12-15) with the DNA pellet resuspended in 250µl 0.1 TE. A 0.5µl sample in the presence of 1X loading buffer was run on 1% agarose gel along with 10µl (50ng/µl) 1kb DNA ladder, to estimate the yield of the maxi-prep procedure.

2.3.6 Restriction enzyme digestion, gel electrophoresis and isolation of pcDNA3.1+

pcDNA3.1+ was digested with *Eco*RI. Approximately 1µg of maxi-prepared pcDNA3.1+ (section 2.3.5) was added to a mixture of 2µl (20U) *Eco*RI and 2µl (10X) *Eco*RI buffer, and made up to a final volume of 20µl with distilled water. This was then mixed by pipetting and incubated at 37°C for 2 hours. The mixture was subsequently run on 1% agarose gel after the addition of 1X loading buffer with 10µl (50ng/µl) 1kb DNA ladder (see section 2.1.3). The insert was isolated following the QIAGEN gel extraction protocol (see section 2.2.1).

2.3.7 Calf Intestinal Phosphate Treatment of pcDNA3.1

Calf Intestinal Phosphate (CIP) treatment was carried out according to Sambrook *et al* (1989a) to prevent the linearised vector self-ligating during the ligation reaction. Initially, 4.5µl of 10X buffer 2 (NEB) and 0.5µl of CIP (10K U/ml NEB) was added to 40µl *Eco*RI digested and isolated pcDNA3.1+ (section 2.3.6). This was incubated for 60 min at 37°C to dephosphorylate the end termini of pcDNA3.1+. The reaction was stopped by incubating the mixture for 15 min at 75°C, followed by the addition of 10µl 10% sodium dodecyl sulphate (SDS), 2µl Proteinase K (20mg/ml) and 43µl distilled water and subsequent incubation for 2h at 56°C. The dephosphorylated linearised plasmid was purified by the addition of an equal volume (100µl) phenol: chloroform: isoamylalcohol (25:24:1), which was subsequently mixed by inverting, and spun (13krpm/1min). Next, 80µl of the aqueous top phase, containing DNA, was transferred into a new eppendorf and 120µl 0.1 TE was added to the remaining subtracted mixture,

which was subsequently mixed and spun (13krpm/1 min). The top 120µl phase of this was then added to the 80µl removed phase in the new eppendorf. To further purify the plasmid DNA an equal volume of chloroform (200µl) was added, mixed (by inverting), and spun (13krpm/1min). In a new eppendorf, 20µl 3M sodium acetate was added to 180µl of the top phase of the mixture and mixed. Monovalent cations (i.e. sodium acetate) are needed in order to precipitate DNA in ethanol [Sambrook *et al*, 1989a]. To precipitate the DNA, 600µl of ethanol was added and mixed by inverting. The mixture was incubated at -70°C for 15 min and subsequently centrifuged (13krpm/15min). The supernatant was removed and the DNA pellet air dried for 20 min. The pellet was then resuspended in 20µl 0.1 TE. To verify the presence of CIP treated vector and determine its yield one tenth volume was run on gel as described in sections 2.2.1 and 2.3.2.

2.3.8 Ligation of Ap-TAT and VP3-TAT into pcDNA3.1

Reaction mixtures were set up on ice, using a molar insert:vector ratio of approximately 3**:**1, estimated from gel pictures.

In order,

VP3-TAT		*Ap-TAT*
3.5µl distilled water		2µl distilled water
1.5µl 10X T4 ligase buffer	*(NEB)*	1µl 10X T4 ligase buffer
1µl T4 DNA ligase	*(NEB, 400U/µl)*	1µl T4 DNA ligase
8µl VP3-TAT	*Eco*RI *dig. (aprox. 100ng)*	5µl Ap-TAT
1µl pcDNA3.1+	*Eco*RI *digested CIP treated*	1µl pcDNA3.1+

A control where the VP3-TAT insert was replaced by distilled water was also prepared to check for vector self-ligation. The mixtures were mixed by pipetting and incubated for 2h at 25°C.

2.3.9 Transformation, amplification and purification of constructed plasmids

From the ligation mixture (section 2.3.8) 1µl was transformed into 50µl of ice thawed OneShot® TOP10F′ competent *E. Coli* cells and grown following the protocol outlined in section 2.2.3, with the omission of plating X-Gal. Colonies of Ap-TAT were picked and grown shaking overnight at 37°C in 2ml LB medium containing 50µg/ml ampicillin. Plasmid DNA was isolated using the mini-prep procedure outlined in section 2.2.4.

2.3.10 Orientation of inserts

The orientation of the insert was checked by *Pst*I (MBI fermentas) digestion, in which 3µl of the plasmid DNA (section 2.3.9) was added to a mixture of 14µl distilled water, 2µl 10X buffer R (MBI Fermentas) and 1µl (10U) *Pst*I. This was mixed by pipetting and subsequently incubated for 1 h at 37°C. The mixtures along with 10µl (50ng/µl) 100bp DNA ladder were then run on 1% agarose gel (section 2.1.3) in the presence of 1X loading buffer.

2.4 The expression of Ap-TAT

The expression of Ap-TAT was checked in a p53 deficient osteosarcoma (SAOS-2) cell line. The cells were transfected with either pcDNA3.1+ containing Ap-TAT; pcDNA3.1+ containing Apoptin (to compare effects); pcDNA3.1+ or mock-transfected by a method modified from Sambrook *et al* (1989b). After two days, the cells were stained with 4', 6-diamino-2-phenylindole (DAPI) and a mouse anti-VP3 monoclonal antibody (available in the lab.), to check for protein expression.

2.4.1 Amplification of pcDNA3.1 containing Ap-TAT

The *Pst*I digestion revealed which mini-preparation mixtures contained sense orientation Ap-TAT. Approximately 50ng (1µl) of sense Ap-TAT mini-prepared plasmid, estimated in comparison to 500ng 100bp DNA ladder was transformed into 50µl of ice thawed OneShot® TOP10F′ competent *E. Coli* cells and grown following the protocol outlined in section 2.2.3, with the omission of plating X-Gal. One colony was picked from the plate and grown in 100ml LB medium containing 50µg/ml ampicillin and incubated shaking overnight at 37°C. Maxi-prep was carried out following the QIAGEN® protocol, with the exception of step 8 in which the recentrifugation step was replaced by filteration using Whatman filter paper (Whatman Ltd, UK). The DNA pellet resuspended in 250µl 0.1 TE. A 1µl sample in the presence of 1X loading buffer was run on 1% agarose gel along with 10µl (50ng/µl) 1kb DNA ladder, to estimate the yield of the maxi-prep procedure.

2.4.2 Seeding of SAOS-2 cells for transfection

SAOS-2 cells (supplied by Lars Guelen) were seeded a day before transfection in 4 X 6 cm dishes, at a density of 3 X 10^5 cells per 6 cm dish (estimated by counting the number of cells in a 0.1µl chamber of a haemocytometer, Weber Scientific International , UK) in 3ml Dulbecco's Modified Eagle Medium (DMEM – Appendix A), supplemented with 10% Foetal Calf Serum (FCS), 1X penicillin/streptomycin (Appendix A) and 1mM sodium pyruvate.

2.4.3 Preparation and transfection of DNA into cells

Approximately 5µg of plasmid DNA (estimated from gel electrophoresis pictures, in comparison to 500ng DNA ladders) was used to transfect SAOS-2 cells. pcDNA3.1+ containing Apoptin; pcDNA3.1+ containing Ap-TAT (see section 2.4.1); pcDNA3.1+ (see section 2.3.5) was precipitated in 3X volume 100% ethanol and 0.1X volume 3M sodium acetate and then incubated at -70°C for 20 min. The mixture was subsequently centrifuged (13krpm/3min) and the supernatant removed. The DNA pellet was then washed with 70% ethanol to remove residual salts. The eppendorfs were filled to the top with 70% ethanol, which served to sterilise the tube, centrifuged (13krpm/1 min) and the supernatant removed under sterile conditions. The DNA was dissolved in 180µl distilled water, followed by the addition of 15µl 2.5M calcium chloride and 150µl 2X N,N-bis [2-hydroxyethyl]-2-aminoethane sulphonic acid buffered saline (2X BES), while vortexing. The mixture was subsequently incubated for 15 min at 25°C. This last step served to form a calcium phosphate-DNA coprecipitate, which markedly increases the uptake of DNA by cells, by an unknown mechanism [Sambrook *et al*,

1989b]. Next, 300µl co-precipitated DNA along with distilled water (control) was added dropwise to cells, one day post-seeding. The 3ml culture medium (section 2.4.2) was replaced with 3ml of new medium one day post-transfection to remove any harmful components from the transfection procedure.

2.4.4 Preparation of cells for immunocytochemistry

Two days post-transfection, the medium of the transfected cells was removed. The cells were washed with 3ml versene (Appendix A), followed by the addition of 300µl 1X trypsin and subsequent incubation for 2 min at 37°C. This served to detach the cells from the base of the plate. To inhibit further trypsinisation, the cells were resuspended in 1ml of culture medium (section 2.4.2). The suspension was then placed in eppendorf tubes and centrifuged (1krpm/5min), the supernatant removed and the cells subsequently washed with 1ml phosphate buffered solution (PBS - Appendix A).

2.4.5 Staining of SAOS-2 cells

The cells were placed on slides using a Shandon Cytospin (Shandon, UK)**,** where 100µl of the cell suspension (section 2.4.5) was spun for 5 min at 800 rpm onto slides. The cells were fixed by incubation in acetone for 20 min at -20°C, which also serves to remove some of the cell's lipid, so that all cellular proteins and nucleic acids become accessible to added antibodies [Johnston and Thorpe, 1996]. The acetone was removed by washing with 1ml PBS three times. To prevent unspecific binding of monoclonal antibodies, slides were blocked by incubation with 3% bovine serum albumin (BSA) in PBS for 30 min at 25°C. The cells were stained with anti-VP3 monoclonal antibody,

diluted 1:100 in PBS containing 3% BSA [personal communication Lars Guelen] by incubation at 4°C, overnight. Unbound antibody was washed off by washing three times with PBS. Next, the slides were incubated in anti-mouse immunoglobulin G (IgG) antibody (Sigma, USA) conjugated to fluorescein isothiocyanate (FITC) diluted 1:200 in PBS, for 1h. The slides were again washed three times with PBS and mounted in 4',6-diamino-2-phenylindole (DAPI) contained in Vectashield® mounting medium, diluted 1:10 in Vectashield® mounting medium (both supplied by Vector, Canada). DAPI is known to stain intact nuclei strongly but apoptotic ones irregularly and weakly [Telford *et al*, 1992]. It is excited by light with a wavelength (λ) of 360nm and emits a blue light at λ = 460nm [Vector - Vectashield® mounting medium with DAPI datasheet]. FITC conjugated anti-mouse IgG antibody binds to anti-VP3 monoclonal antibody. FITC emits green light at a λ = 525nm when excited by light at λ=495nm [Sigma anti-mouse IgG antibody datasheet].

2.5 Cloning Ap-TAT and VP3-TAT into pGEX-5X-3 bacterial expression vector

Ap-TAT was cloned into the bacterial expression vector pGEX-5X-3 (Amersham Pharmacia Biotech, UK - Appendix C). The pGEX system is designed for the cloning of DNA and expression of recombinant proteins in *E. coli.* Targeted genes are expressed under the control of a *tac* promoter. pGEX-5X-3 expresses the desired protein in *E.Coli* fused to a Glutathione S-transferase (GST) tag protein. The tag aids in the purification of the protein from *E.Coli* after it has been expressed. The fused protein incorporates a cleavage site allowing the tag to be removed after purification with Factor Xa. The restriction enzymes used to clone inserts into expression vectors were selected to ensure Ap-TAT and VP3-TAT would be read "in-frame," and that the plasmid or insert would not be cut adversely.

2.5.1 Transformation, Amplification and Maxi-preparation of pGEX-5X-3

The amplification, maxi-preparation and checking of pGEX-5X-3 (50ng/ml) was carried out in exactly the same way as pcDNA3.1 as described in section 2.3.4 and 2.3.5.

2.5.2 Restriction enzyme digestion, gel electrophoresis and isolation of pGEX-5X-3

Approximately 1µg (5µl) pGEX-5X-3 maxi-prep DNA was added to a mixture of 2µl (10X) buffer 4 (NEB) and 2µl (20U) *Sma*I (NEB) made up to a final volume of 20µl with distilled water and subsequently incubated for 2 h at 25°C. This created an open vector with "blunt-ends." The mixture was then run on 1% agarose gel (as described in

section 2.1.3) in the presence of 1X loading buffer along with 10µl (50ng/µl) 1kb DNA ladder. The linearised plasmid was isolated following the QIAGEN gel extraction protocol and dissolved in 40µl of 0.1 TE. The vector was then CIP treated to prevent vector self-ligation according to the procedure outlined in section 2.3.7.

2.5.3 Restriction enzyme digestion, gel electrophoresis and isolation of inserts

The inserts (Ap-TAT and VP3-TAT) were cut out of pCR2.1® TOPO vector with EcoRI and subsequently treated with Klenow DNA Polymerase (NEB), at a concentration of 1 unit per µg insert DNA, to fill in single-stranded DNA achieving "blunt-ends." To 16µl mini-prep DNA (1µg Ap-TAT and 500ng VP3-TAT section 2.3.2), a mixture of 2µl (10X) buffer 2 and 2µl (20U) EcoRI was added and incubated at 37°C for 2 h. The samples were then incubated for 20 min at 65°C to inactivate EcoRI and the following reactions were set up:

1µl Klenow (1U/µl)	0.5µl Klenow (1U/µl)
1µl 2mM dNTPs	1µl 2mM dNTPs
20µl Ap-TAT EcoRI dig. mix	20µl VP3-TAT EcoRI dig. mix

The mixtures were incubated for 15 min at 25°C. To inactivate the Klenow, the mixtures were incubated for 15 min at 75°C after the addition of 4µl 50mM EDTA.

The inserts were run on 1% agarose gel along with 10µl (50ng/µl) 1kb DNA ladder in the presence of 1X loading buffer and subsequently isolated as described in section

2.2.1).

2.5.4 Ligation of Ap-TAT and VP3-TAT into pGEX-5X-3

Reaction mixtures using Ap-TAT and VP3-TAT were set up on ice, using a molar insert:vector ratio of 3:1, (estimated in comparison to 500ng DNA ladders), as follows:

In order,

1µl distilled water

1µl ligase buffer

1µl T4 DNA ligase

2µl (approx. 100ng) *Sma*I restricted pGEX-5X-3

5µl (approx. 25ng) Klenow treated, *Eco*RI restricted Ap-TAT or VP3-TAT inserts

A control where the Ap-TAT/VP3-TAT insert was replaced by distilled water was also prepared to check for vector self-ligation. The mixtures were mixed by pipetting and incubated for 2h at room temperature.

2.5.5 Transformation, amplification and purification of constructed plasmids

The preparation of constructed plasmid DNA was completed according to the protocol outlined in section 2.3.9. Colonies of Ap-TAT were picked and grown shaking overnight at 37°C in 2ml LB medium containing 50µg/ml ampicillin. Mini-preparation of grown colonies was completed according to the procedure outlined in section 2.2.4.

2.5.6 Orientation of Inserts in pGEX-5X-3

The orientation of the insert was checked by *Pst*I digestion, in which one tenth volume of constructed plasmid DNA was added to a mixture of, in order, 14µl distilled water, 2µl 10X buffer R and 1µl (10U) *Pst*I. This was mixed by pipetting and subsequently incubated for 1 h at 37°C. Next, loading buffer was added to a final concentration of 1X and the mixtures run on 1% agarose gel along with 10µl (50ng/µl) 1kb DNA ladder (section 2.1.3).

3.0 Results

3.1 Generation of Ap-TAT and VP3-TAT DNA

The Ap-TAT and VP3-TAT DNA was synthesised using PCR (section 2.1.2). DNA fragments were present at the expected length (Fig. 3.1) of Ap/VP3-TAT (366 bp including a 3 bp stop codon - Appendix B). Two different Pfu enzymes from Promega and Stratagene were used.

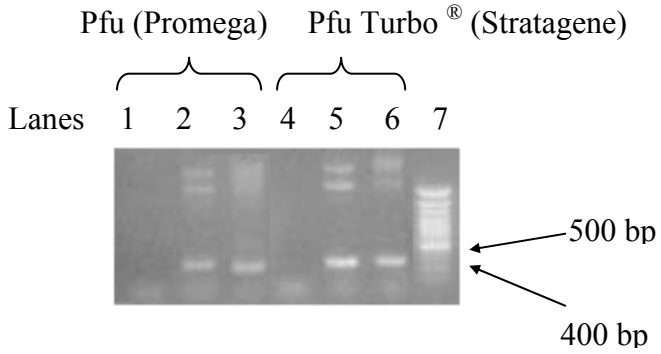


Figure 3.1. Ap-TAT and VP3-TAT products from PCR. Constructs (Stratagene and Promega) run on 1% agarose gel stained with ethidium bromide. Lanes: 1 and 4 show negative controls samples with complete PCR mixtures except for template DNA; 2 and 5 show Ap-TAT product; 3 and 6 show VP-3 TAT product; lane 7 shows 500ng molecular weight marker - 1kb DNA ladder.

3.1.2 Cloning of inserts into pCR2.1® TOPO vector

Inserts were cloned into pCR2.1® TOPO vector and transformed into *E.Coli* cells, six colonies were grown and mini-prepared. The plasmids were restricted with *Eco*RI (section 2.2.4) and run on 1% agarose gel (Fig. 3.2). DNA samples with the highest yield of correctly sized insert were selected for sequencing (Fig. 3.2): Stratagene VP3-TAT (lane 1) and Ap-TAT (lane 3); Promega VP3-TAT (lane 2) and Ap-TAT (lane 6).

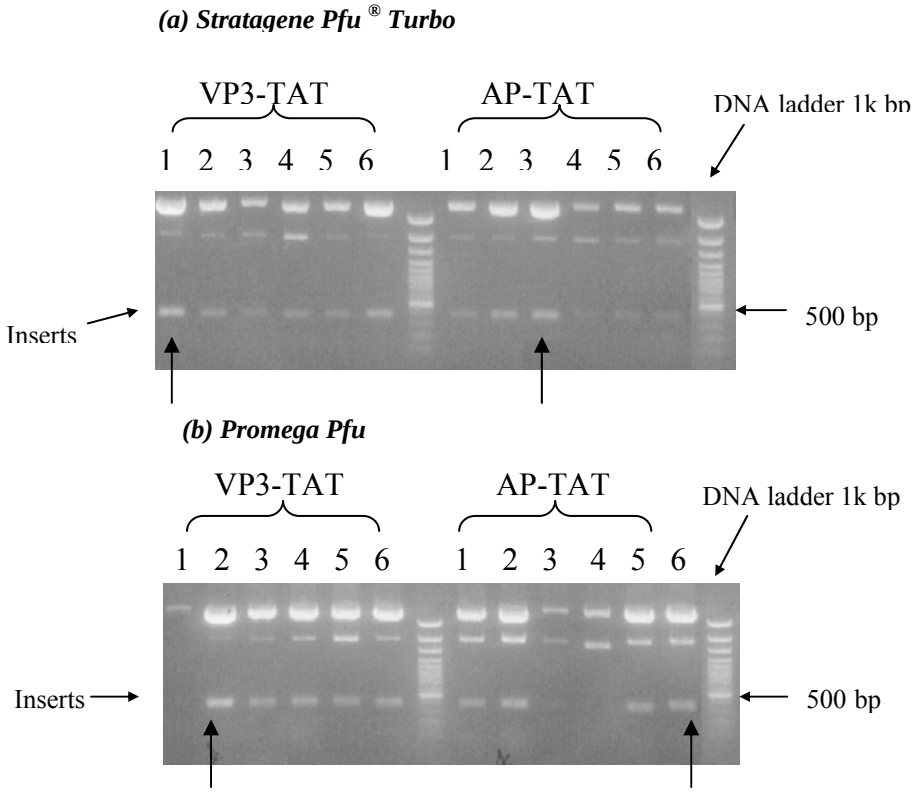


***Figure 3.2. pCR2.1® TOPO vector containing Ap-TAT and VP3-TAT PCR DNA digested with Eco*RI.** (a) Ap-TAT and VP3-TAT produced by PCR using Stratagene Pfu® Turbo; (b) Ap-TAT and VP3-TAT produced using Promega Pfu. Digestions run on 1% agarose gel stained with ethidium bromide. The 1k bp DNA ladder (500ng) is shown in the middle and final lanes.

It should be noted that there is a strange bond of approximately 1200bp in all the samples in Figure 3.2. As there is the same bond in all the samples, DNA contamination was most likely be in either the 0.1 TE, distilled water, *Eco*RI enzyme or the 10X buffer for *Eco*RI. Subsequent testing with all four revealed the contamination was actually in the Eco*RI* enzyme (results not shown), which was discarded.

3.1.3 Sequencing of Ap-TAT and VP3-TAT inserts contained in pCR2.1® TOPO vector

Sequencing results from Cambridge Bioscience Ltd, showed that the Ap-TAT and VP3-TAT DNA sequences were made as specified (Fig. 3.3 and Appendix B). Cambridge Bioscience Ltd were unable to determine the sequence of Stratagene Ap-TAT for an unspecified reason. Stratagene VP3-TAT (Fig. 3.3a) and Promega Ap-TAT (Fif. 3.3c) DNA sequences were selected for further experimentation.

3.2 Cloning of Ap-TAT and VP3-TAT into pcDNA3.1

The inserts were cut out of pCR2.1® TOPO vector with *Eco*RI, purified, and cloned into *Eco*RI digested, CIP treated, pcDNA3.1. The amounts of insert and vector for a molar cloning ratio of Approximately 3:1 was estimated by comparing the amounts of insert/vector to 500ng DNA ladder from gel electrophoresis pictures (Fig. 3.4).

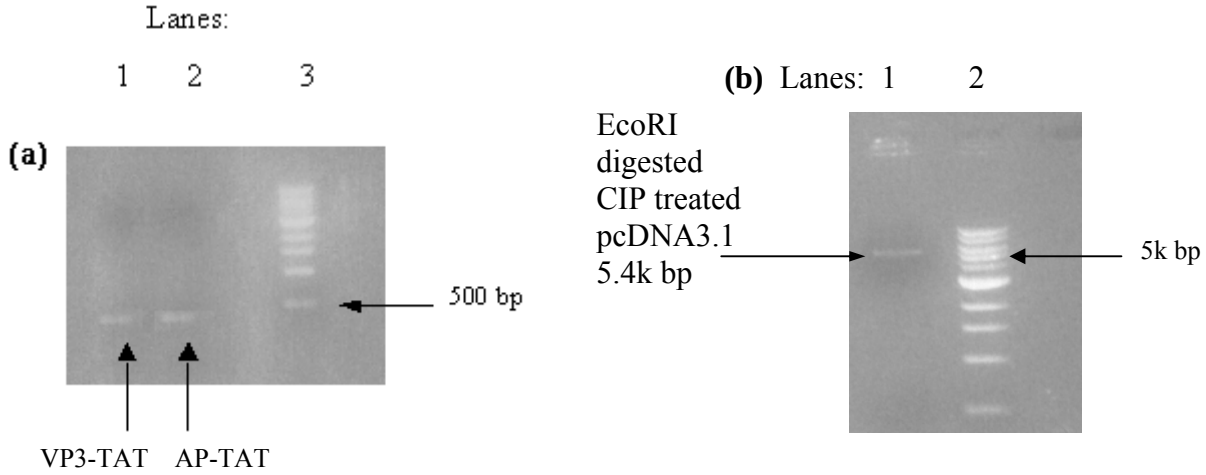
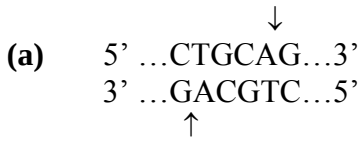


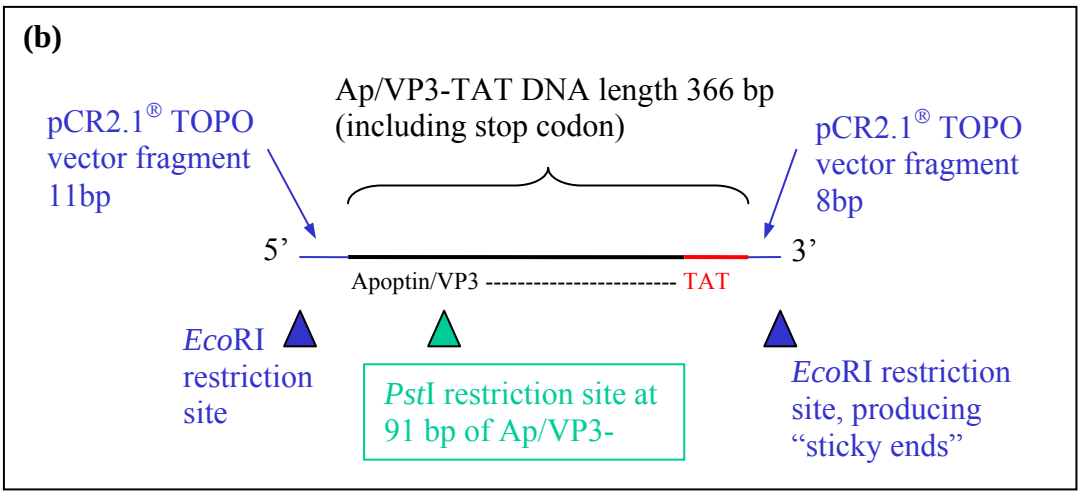
Fig 3.4 EcoRI restricted insert and vector (CIP treated) (a) A 2µl sample of purified *Eco*RI digested VP3-TAT (lane 1) and Ap-TAT (lane 2) and 500ng 1kb marker DNA ladder (lane 3), (b) A 2µl sample of purified CIP treated *Eco*RI digested pcDNA3.1 (lane 1), 500ng 1kb DNA ladder (lane 2) run on 1% agarose gel stained with ethidium bromide.

A similar number of colonies were formed in VP3-TAT transformed *E.Coli* cells compared to the empty vector control transformation (section 2.3.8). This indicated that the transformation was unlikely to have been efficient. It was thus decided to continue with Ap-TAT contained plasmid, colonies of which were far in excess of the empty vector control transformation. After culturing the orientation of inserts was determined by digesting the isolated mini-prep plasmid with *Pst*I (Fig. 3.5). Three of the mini-preparations had fragments present at the expected lengths (292 bp, Fig. 3.5d) for pcDNA3.1+ containing Ap-TAT sense orientation (Fig. 3.5e).

(a)



(b)

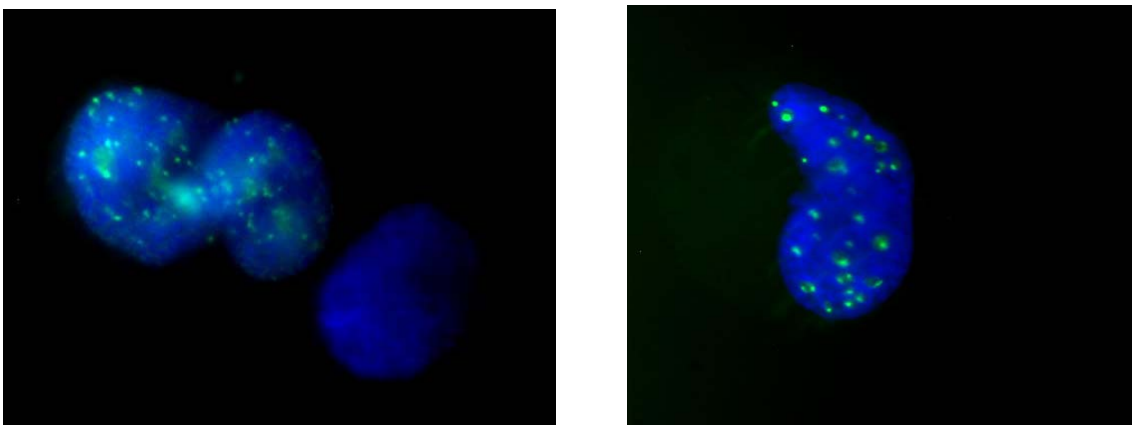


3.3 The expression of Ap-TAT

Plasmids containing sense orientation Ap-TAT (Fig. 3.5e – lane 3) were amplified in *E.Coli* and maxi-prepared (section 2.4.1). Subsequently p53 deficient SAOS-2 cells were transfected using the calcium phosphate co-precipitation method with 5μg of the following plasmids; pcDNA3.1 containing Ap-TAT; pcDNA3.1 containing Apoptin; pcDNA3.1; or a mock transfection with water in place of DNA. Two days post-transfection expression of the transgene was analysed. Cells were fixed, blocked and stained with DAPI and anti-VP3 monoclonal antibody (section 2.4.6, Fig. 3.6). Using florescence microscopy it was observed that Apoptin and Ap-TAT SAOS-2 transfected cells contained a small number of cells which stained positively with anti-VP3 antibody in their nuclei. No staining was observed in cells transfected with empty pcDNA3.1 or the mock transfection. The Apoptin transfected cells had a more diffuse pattern of expression in their nuclei (3.6a) compared to the Ap-TAT transfected cells, which had a more granular pattern, particularly occupying the nucleoli (Fig. 3.6b).

(a) Apoptin stained nuclei **(b) Ap-TAT stained nuclei**

Apoptin and DAPI staining



Apoptin staining

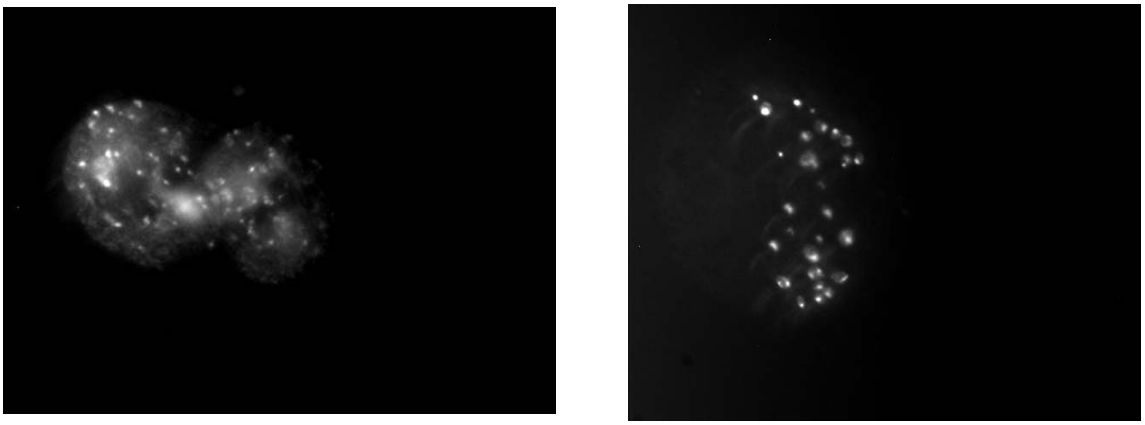


Figure 3.6 Expression of Apoptin and Ap-TAT in SAOS-2 cells (X1000). DAPI stains DNA blue, FITC conjugated mouse IgG antibody stains anti-VP3 antibody green. (a) Apoptin expression in SAOS-2 nuclei, (b) Ap-TAT expression in SAOS-2 nuclei.

3.4 Cloning of Ap-TAT and VP3-TAT into pGEX-5X-3

Ap-TAT and VP3-TAT were cloned into the bacterial expression vector pGEX-5X-3 so the proteins could be expressed, purified and tested in further studies. pGEX-5X-3 was amplified, digested with *Sma*I (creating "blunt ends" Fig. 3.7a) and subsequently CIP treated to prevent self ligation (Fig. 3.7b). Ap-TAT and VP3-TAT inserts were cut out of pCR2.1® TOPO vector with *Eco*RI, purified (Fig. 3.5a), followed by treatment with Klenow to create "blunt-ends" (Fig. 3.7c). The yield of VP3-TAT was very low. It was thus decided to continue with Ap-TAT (Fig. 3.7c). Ap-TAT was then cloned into pGEX-5X-3, transformed into *E.Coli* cells and plated. After mini-preparation, one tenth volume of plasmid DNA was digested with *Pst*I to determine the orientation of the inserts (Fig. 3.5b, 3.8a/b). One of the mini-preparations had fragments present at the expected length (1256 bp Fig. 3.8b) for pGEX-5X-3 containing Ap-TAT sense orientation (Fig. 3.8c).

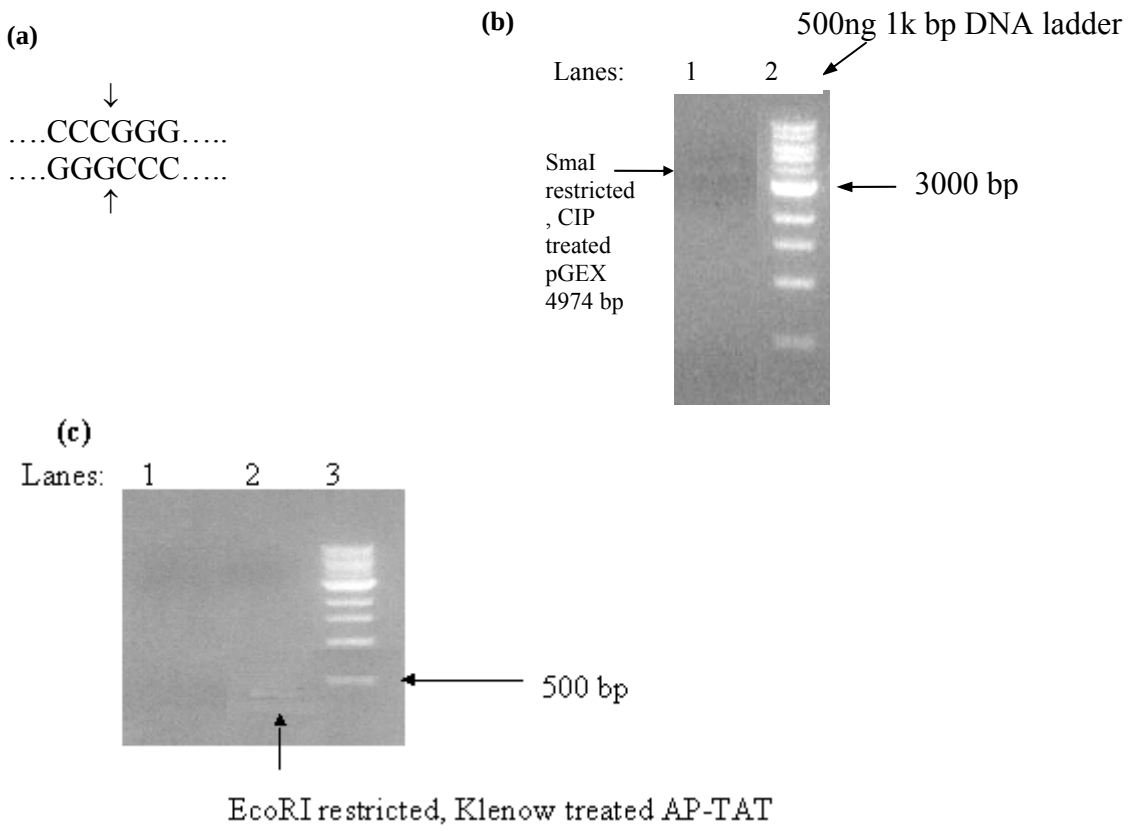


Fig. 3.7 Treated inserts and vector. (a) *Sma*I DNA sequence recognition site. (b) *Sma*I restricted, CIP treated pGEX-5X-3**,** lane 1; 500ng 1kb DNA ladder lane 2 (c) *Eco*RI restricted, Klenow treated VP-TAT lane 1; *Eco*RI restricted, Klenow treated Ap-TAT lane 2; 500ng 1kb DNA ladder lane 3. Fragments run on 1% agarose gel stained with ethidium bromide.

4.0 Discussion

The aim of this project was to modify the 11 codons at the 3' end of Apoptin DNA into TAT-PTD in the hope of combining the tumour specific killing properties of Apoptin with the cell transduction ability of TAT for the development of a protein based cancer therapy. Ap-TAT was cloned into the mammalian expression vector pcDNA3.1 and transfected into p53 deficient SAOS-2 cells to test for expression. Ap-TAT was further cloned into a bacterial expression vector for future expression and testing of the protein.

4.1 Cloning into pcDNA3.1+

Cambridge Bioscience Ltd confirmed that the Ap-TAT and VP3-TAT sequences were made as specified (Fig. 3.3).

The 3' end of the VP3 gene (from a different CAV isolate – Appendix B) was modified into TAT-PTD, to compare it with Ap-TAT. Unfortunately even though we had the correct sequence (Fig. 3.3a/b) we were unable to amplify the plasmid containing VP3-TAT DNA. After cloning VP3-TAT into pcDNA3.1+, *E.Coli* cells were transformed and plated, however colony numbers were similar to the cells transformed with just pcDNA3.1+. This indicated the ligation reaction may not have been successful. Time constraints and the primary objective of cloning Ap-TAT dictated the postponement of cloning VP3-TAT into pcDNA3.1+. Ap-TAT was successfully cloned into pcDNA3.1+ using the same concentrations of ingredients as the VP3-TAT reaction (section 2.3.8). The concentration of VP3-TAT DNA was less than Ap-TAT DNA

(Fig. 3.4a), thus future experiments should increase the amount of VP3-TAT for a successful ligation reaction.

4.2 The Expression of Ap-TAT

Transfection of p53 deficient SAOS-2 cells with Ap-TAT was inefficient, with approximately 0.3% of cells transfected. Transfection of cells is pH sensitive [Sambrook et al, 1989b]. The poor transfection efficiency may have been due to an unwanted pH change in 2X BES. Putatively, trypsinisation of cells may have obliterated already weak apoptotic cells, preventing them from being stained.

The binding ability of the anti-VP3 monoclonal antibody to Ap-TAT was unknown as we do not know which epitope the antibody binds to. Staining revealed the antibody used was able bind to Ap-TAT, with a similar affinity to which it binds Apoptin (Fig. 3.6). This indicates the antibody most likely binds to a region in the first 111 residues of Apoptin.

4.2.1 Localisation of Ap-TAT

Ap-TAT stained positively in the nucleoli of transfected cells, as was the case for Apoptin (Fig. 3.6). This confirmed results previously reported for Apoptin [Danen-Van Oorschot *et al,* 1997b]. This first preliminary experiment suggests the nuclear expression of Ap-TAT is much more granular and less diffuse than Apoptin. Ap-TAT, like Apoptin, locates in the nucleoli of SAOS-2 cells. However, it is not yet known if it interacts with the same components that Apoptin interacts with, and further if it is

capable of inducing apoptosis. Conceivably, Ap-TAT may be binding to different components in the nuclei, dictated by TAT, giving it a different function.

The intracellular localisation of TAT-PTD fused proteins seems to depend on the properties of the protein TAT-PTD is fused to. Rho is a protein that causes the development of actin stress fibres in osteoblasts. When fused to Rho, TAT-PTD remains in the cytoplasm to cause the formation of actin fibres [Chellaiah *et al*, 2000]. The expression of Ap-TAT (Fig. 3.6) has confirmed previously reported results [Schwarze *et al*, 2000] that some TAT-PTD fused proteins locate in the nucleus of cells. TAT-PTD is known to travel to and bind to components in the nucleus of cells, care of its inherent NLS [Fig. 1.2 and Schwarze *et al*, 1999]. Considering this, it is perhaps surprising that different TAT-PTD fused proteins locate in different intracellular compartments. It would appear that the location signals of proteins (pre-fusion to TAT-PTD) over ride any effect of the TAT-PTD NLS and dictate the cellular localisation of the fused protein.

Apoptin is located in the cytoplasm of normal cells. In malignant/transformed cells Apoptin localises in the nucleus to induce apoptosis [Zhuang *et* al, 1995c]. Proteins fused to TAT-PTD have been shown to retain their biological function [Schwarze *et al*, 2000]. This would support the possibility that Ap-TAT retains the tumour specificity of Apoptin. Zhuang *et al* (1995c) showed that truncated Apoptin without its C-terminal NLS was still able to induce apoptosis in cancerous cells but at a much slower rate compared to wild-type Apoptin. This indicates the C-terminus of Apoptin is important for its action but not essential. Thus, modifying the C-terminal NLS of Apoptin, as has occurred here, should still induce tumour specific apoptosis, but perhaps at an altered

rate. Further, TAT-PTD and the C-terminal NLS of Apoptin share homology (Fig. 1.5). Thus, it is also possible that modifying the C-terminal NLS of Apoptin into TAT-PTD will not alter its function. Putatively, if the modified protein's location is dictated by TAT-PTD or if the altered amino acid sequence of Apoptin is essential for inducing tumour specific apoptosis, Ap-TAT may not induce apoptosis at all, or alternatively, localise in the nuclei of normal cells, inducing apoptosis. As yet the importance of the 2nd NLS of Apoptin [Danen Van OorSchot *et al*, 1997b] is unknown as no experiments have investigated its importance.

4.2.2 Possible mechanisms of action of Apoptin and Ap-TAT

In this study Ap-TAT and Apoptin were found to localise in the nucleoli (Fig. 3.6). If Ap-TAT does induce tumour specific apoptosis, then its mechanism of action is likely to be similar to that of Apoptin. The nucleolus serves to package newly synthesised ribosomal RNA with proteins to form ribosomes, which further translocate to the cytoplasm to construct cellular proteins [Alberts *et al*, 1994]. One of the events in apoptosis is the suppression of protein synthesis [Alberts *et al*, 1994]. It may be that Apoptin acts in the nucleoli to prevent ribosomal generation and subsequent protein formation. Apoptin induced apoptosis has been shown to require caspase-3 activation (section 1.6.1). It has also been shown to co-localise with nuclear chromatin [Noteborn *et al*, 1994]. Putatively, Apoptin induces apoptosis by more than one pathway.

Further knowledge on Apoptins' mechanism of action may come from studying proteins with similar characteristics. Death effector domain (**DED**) containing **D**NA-binding protein (DEDD) is a ubiquitously expressed 318 aa protein [Stegh *et al*, 1998].

It is normally found in the cytoplasm of cells. Upon activation of CD95, it translocates to the nucleolus to inhibit cellular biosynthetic activity and induce apoptosis [Stegh *et al*, 1998]. Studying the mechanism by which it translocates to the nucleolus may help in understanding how Apoptin translocates. Stegh *et al* (1998) hypothesised that an inhibitor exists in the cytoplasm which masks one of the NLSs of DEDD and retains DEDD in the cytoplasm. On activation of CD95, the inhibitor is cleaved which results in its degradation and/or its release from DEDD, resulting in the uncovering of the NLS and the nuclear translocation of DEDD. The endonuclease CAD, which translocates to the nucleus during apoptosis, has been shown along with its cytoplasmic inhibitor – ICAD, to operate by this mechanism [Sakahira *et al*, 1998]. Similarly, there may be an inhibitor present in the cytoplasm of normal cells which prevents the nuclear localisation of Apoptin. The expression of this inhibitor may be lost in transformed/malignant cells. Although, because DEDD also induces apoptosis in normal cells, studies using it will not elucidate the reason why Apoptin is tumour specific. The melanoma differentiation associated (MDA) protein MDA-7, like Apoptin, has also been found to induce apoptosis specifically in various tumour cell lines [Su *et al*, 1998, Jiang *et al*, 1996]. Su *et al* (1998) showed MDA-7 induced apoptosis correlated with an increase in BAX: Bcl-2 ratio. MDA-7 is also known to translocate from the cytoplasm to the nucleus in tumour cells. However, unlike Apoptin, it does not have a NLS, and the way in which it translocates to the nucleus is believed to be mediated by cytosolic chaperones, such as HMC [Su *et al*, 1998]. Thus, it is also possible that malignant cells express a cytoplasmic chaperone which mediates the nuclear localisation of Apoptin. While the mechanism of action of Apoptin and MDA-7 may not be the same, it is conceivable that they use the same mechanism to

differentiate between malignant and non-malignant cells. Thus, future studies with MDA-7 may give indications to the way in which Apoptin operates.

4.3 Cloning into pGEX-5X-3

The cloning of Ap-TAT into pGEX-5X-3 was successful (Fig. 3.8). This will allow the expression, purification and urea denaturing of Ap-TAT for further testing. The cloning of VP-TAT into pGEX-5X-3 was unsuccessful. After *Eco*RI and Klenow treatment, a very low yield of VP3-TAT remained (Fig. 3.7c). Our primary objective was to clone Ap-TAT into pGEX-5X-3, thus it was decided to postpone the cloning of VP3-TAT.

4.4 Potential problems

TAT is the transcriptional activator gene product of HIV-1. The full length TAT protein has been shown to stimulate the growth of Kaposi's sarcoma-derived cells, by activating transcription of specific genes [Ford *et al*, 2001]. Transgenic mice with full length TAT, preferentially develop Kaposi's sarcoma [Ford *et al*, 2001]. It is yet unknown if TAT-PTD is similarly able to activate the transcription of genes in cells. This is something which must be monitored closely. If the TAT-PTD is shown to be devoid of toxic effects *in vivo*, it will be of great use in a variety of applications.

TAT is a protein and as such will act as an antigen to the immune system, thus any long term treatments using it may be hindered by an immune response. Thus far in limited *in vivo* experiments both Apoptin and TAT have been demonstrated, independently, to

be safe in animals [Pietersen *et al*, 1999, Schwarze *et al*, 1999]. More research is required to determine whether Ap-TAT will be non-toxic and effective *in vivo*.

4.5 Future work

Many questions remain about both Apoptin and TAT due to their relatively recent discoveries. I have gained a studentship to further research Ap-TAT this summer. The very first study must determine whether Ap-TAT is capable of inducing apoptosis in tumour cells. Apoptin is known to induce apoptosis in between 80-90% of SAOS-2 cells six days after transfection [Zhuang *et al*, 1995c]. To determine if Ap-TAT is also able to induce apoptosis in SAOS-2 cells, after transfecting cells using the same methods and controls described in section 2.4, the cells should be left for six days before fixing, blocking and then staining with anti-VP3 monoclonal antibody and DAPI. Cells expressing Ap-TAT will display irregularly stained nuclei, if they are undergoing apoptosis [Telford *et al*, 1992]. If Ap-TAT does induce apoptosis in SAOS-2 cells, the next experiment is the transfection of a panel of normal and tumour cells with pcDNA3.1+ containing Ap-TAT. This will determine whether the tumour specificity of Apoptin is retained with Ap-TAT. If the tumour specific activity of Apoptin is retained, it would be a potentially promising development in cancer therapy. However, even if the tumour specific ability is not retained and Ap-TAT is found to induce apoptosis in normal cells, it could still be a promising development with a variety of potential clinical applications. Ap-TAT could be a protein which induces a natural cell death in all cells efficiently. Thus, it may be able to be used to reduce/remove benign/malignant tissue growths.

After the determination of specificity, the Ap-TAT protein will be expressed in *E.Coli* cells, isolated and then denatured in urea. Urea denaturation is necessary for efficient transduction of TAT-PTD fused proteins [Nagahara *et* al, 1998]. However, the concentration of urea for the most efficient transduction varies with the type of fused protein [Schwarze *et al*, 2000]. Thus the concentration of urea leading to the most efficient transduction of Ap-TAT must be determined. The purified protein will then be tested by adding it to the medium of a number of cell lines, beginning with SCCHN cell lines, to determine its efficiency. The results of these experiments will dictate further research.

To discover the mechanism by which Apoptin and Ap-TAT act, it is very important to find out what they interact with. Future studies should see if they interact with other proteins involved in apoptosis such as DEDD or CAD. The DNA damage induced apoptosis pathway proteins, caspase 9 and Apaf 1 [Schmitt and Lowe 1999], have not been studied to see if they interact with Apoptin. Also, the cytosolic chaperone HMC, which may be responsible for the nuclear translocation of MDA-7 may have some function in Apoptin induced apoptosis. These possible interactions could be studied using immunoprecipitation assays, with the relevant antibodies.

Apoptin has a similar C terminus to TAT-PTD (Fig. 1.6). It would be interesting to see if the Apoptin protein is capable of transducing cells, while still retaining its function, which has not been shown before.

4.6 Conclusion

Here, in a preliminary study, we have successfully constructed and expressed a novel gene which is hypothesised to encompass the tumour specific killing ability of Apoptin and the cell transducing properties of TAT-PTD. Only after much experimentation will we discover if this constructed protein has the exciting potential envisaged. The next important step will be to discover if the protein induces tumour specific apoptosis, followed by elucidating its efficiency and toxicity *in vitro* and *in vivo*. In theory, the potential of this protein is great. However, like many other exciting proteins in cancer research it should be viewed with caution, bearing in mind it has not been shown to have an effect in any cells as yet.

The cloning of VP3-TAT into pcDNA3.1+ and pGEX-5X-3 proved unsuccessful. However following the recommendations outlined above (section 4.1 and 4.3) future experiments should be more fruitful. It will be particularly interesting to see if the mismatches in VP3 (Appendix B), cause VP3-TAT to have a different effect compared to Ap-TAT, inferring the mismatched base(s) are essential for function.

Bibliography

1. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D., (1994). The Nucleus. In: *Molecular Biology of the Cell*, 3rd Edition (by same authors). Garland Publishing, New York, pp379-383.

2. Alevizopoulos, K., Vlach, J., Hennecke, S., Amati, B., (1997). Cyclin E and c-myc promote cell proliferation in the presence of $p16^{INK4}$ and of hypophosphorylated retinoblastoma family proteins. *The EMBO Journal* 16(17): 5322-5333.

3. Anderson, W.F., (2000). Gene therapy scores against cancer. *Nature Medicine* 6(8): 862.

4. Bartek, J., Bartkova, J., Lukas, J., (1997). The retinoblastoma protein pathway in cell cycle control and cancer. *Experimental Cell Research* 237: 5322-5333.

5. Birnboim, H.C., & Doly, J., (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* 7(6): 1513-23.

6. Campbell, C., Quinn, A.G., Angus, B., Farr, P.M., Rees, J.L., (1994). Wavelength specific patterns of p53 induction in human skin following exposure to UV radiation. *Cancer Research* 53: 2697-2699.

7. Carson, D.A., & Lois, A., (1995). Cancer progression and p53. *Lancet* 346: 1009-1011.

8. Chellaiah, M.A., Soga, N., Swanson, S., McAllister, S., Alvarez, U., Wang, D., Dowdy, S.F., Hruska, K.A., (2000). Rho-A is critical for osteoclast podosome organisation, motility and bone resorption. *Journal of Biological Chemistry* 275: 11993-12002.

9. Cheng, E.H., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K., Hardwick, J.M., (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* 278: 1966-1968.

10. Cline, J., Braman, J.C., Hoegrefe, H.H., (1996). PCR fidelity of Pfu polymerase and other thermostable DNA polymerases. *Nucleic Acids Research* 24: 3546-3551.

11. Danen-Van Oorschot, A.A., Den Hollander, A., Takayama, S., Reed, J., Van der Eb, A.J., Noteborn, M.H., (1997a). BAG-1 inhibits p53 induced but not apoptin induced apoptosis. *Apoptosis* 2: 395-402.

12. Danen-Van Oorschot, A.A., Fischer, D.F., Grimbergen, J.M., Klein, B., Zhuang, S., Falkenburg, J.H., Backendorf, C., Quax, P.H., Van der Eb, A.J., Noteborn, M.H., (1997b). Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proceedings of the National Academy of Sciences of the United States of America* 94(11): 5843-7.

13. Danen-Van Oorschot, A.A., Van der Eb, A.J., Noteborn, M.H., (1999a). BCL-2 stimulates Apoptin-induced apoptosis. *Advances in Experimental Medicine & Biology* 457: 245-9.

14. Danen-Van Oorschot, A.A., Van der Eb, A.J., Noteborn, M.H., (2000). The chicken anemia virus-derived protein apoptin requires activation of caspases for induction of apoptosis in human tumor cells. *Journal of Virology* 74(15): 7072-8.

15. Danen-Van Oorschot, A.A., Zhang, Y., Erkeland, S.J., Fischer, D.F., Van der Eb, A.J., Noteborn, M.H., (1999b). The effect of Bcl-2 on
Apoptin in 'normal' vs transformed human cells. *Leukemia* 13 Suppl 1:S75-7.

16. Desagher, S., Martinou, J,C., (2000). Mitochondria as the central control point of apoptosis. *Trends in Cell Biology* 10(9): 369-77.

17. Dieffenbach, C.W., Lowe, T.M.J., Dveksler, G.S., (1995). General concepts for PCR Primer Design. In: *PCR Primer, A Laboratory Manual* (by same authors). Cold Spring Harbor Laboratory Press, USA, pp 135-155.

18. Efthymiadis, A., Briggs, L.J., Jans, D.A., (1998). The HIV-1 Tat Nuclear localisation sequence confers novel nuclear import properties. *The Journal of Biological Chemistry* 273(3): 1623-1628.

19. Evan, G., & Littlewood, T., (1998). A matter of life and cell death. *Science* 281: 1317-1322.

20. Fan, S., El-Diery, W.S., Bae, I., Freeman, J., Jondle, D., Bhatia, K., Fornace, A.J. Jr, Mcgrath, I., Kohn, K.W., O'Conner, P.M., (1994). p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Research* 54: 5824-5830.

21. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L.L., Pepinsky, B., Barsoum, J., (1994). Tat-mediated delivery of heterologous proteins into cells. *Proceedings of the National Academy of Sciences USA* 91(2): 664-8

22. Ford, K.G., Souberbielle, B.E., Darling, D., Farzaneh, F., (2001). Protein transduction: an alternative to genetic intervention? *Gene Therapy* 8: 1-4.

23. Frankel, A.D., and Pabo, C.O., (1988). Cellular uptake of the Tat protein from the human immunodeficiency virus. Cell 55: 1189-1193.

24. Ganly, I., Soutar, D.S., Kaye, S.B., (2000). Current role of gene therapy in head and neck cancer. *European Journal of Surgical Oncology* 26: 338-343.

25. Gius, D.R., Ezhevsky, S.A., Becker-Hapak, M., Nagahara, H., Wei, M.C., Dowdy, S.F., (1999). Transduced p16INK4a peptides inhibit hypophosphorylation of the retinoblastoma protein and cell cycle progression prior to activation of Cdk2 complexes in late G1. *Cancer Research* 59(11): 2577-80.

26. Gomez-Navarro, J., Curiel, D.T., Douglas, J.T., (2000). Gene therapy for cancer. *European Journal of Cancer* 35(14): 2039-2057.

27. Gottesman, S., Wickner, S., Maurizi, M.R., (1997). Protein quality control: triage by chaperones and proteases. *Genes & Development* 11(7): 815-23.

28. Green, D.R., (1998). Apoptotic pathways: the road to ruin. *Cell* 94: 695-698.

29. Green, M., and Loewenstein, P.M., (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus TAT trans-activator protein. *Cell* 55: 1179-1188.

30. Gunnarskog, J., Stenbeck, M., Holm, M.E., (1995). Cancer survical in Sweden in 1961-1991: Lip, oral cavity and mesopharynx. *Acta Oncologica* 34 (supplement 4): 5-10

31. Jeurissen, S.H., Wagenaar, F., Pol, J.M., Van der Eb, A.J., (1992). Chicken anaemia virus causes apoptosis of thermocytes after in vivo infection and of cell lines after in vitro infection. *Journal of Virology* 66: 7383-7388.

32. Jiang, H., Su, Z-z., Lin, J.J., Goldstein, N.I., Young, C.S.H., Fisher, P.B., (1996). The melanoma differentiated associated gene mda-7 suppresses cancer cell growth. *Proceedings of the National Academy of Science USA* 93, 9160-9165.

33. Johnston, A., and Thorpe, R., (1996). Immunocytochemistry, Immunohistochemistry and Flowcytofluorimetry. In: *Immunochemistry in Practice* 3rd Ed. Blackwell Science, Oxford, pp 322-323.

34. Jones, A.S., (2000). Tumour suppressor genes and head and neck cancer. *Advances in Oto-Rhino-Laryngology* 56: 249-260.

35. Kerr, J.F., Winterford, C.M., Harmon, B.V., (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 73: 2013-2026.

36. Key, F., Forman, D., Pike, M.C., (1997). Epidemiology of cancer. In: Franks, L.M., and Teichs, N., (eds.) *Introduction to the cellular and molecular biology of Cancer*. 3rd Edition, Oxford University Press Inc, New York.

37. Khuri, F.R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I.F., Romel, L., Gore, M., Ironside, J., MacDougall, R.H., Heise, C., Randlev, B., Gillenwater, A.M., Bruso, P., Kaye, S.B., Waun Ki Hong., Kirn, D.H., (2000). A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nature Medicine* 6: 879-885.

38. Kouraklis, G., (2000). Gene therapy for Cancer: From the laboratory to the patient. *Digestive Diseases and Science* 45(6): 1045-1052.

39. Levin, A.J., Momand, J., Finlay, C.A., (1991). The p53 tumour suppressor gene. *Nature* 351: 453-455.

40. Lewin, M., Carlesso, N., Tung, C-H., Tang, X-W., Cory, D., Scadden, D.T., Weissleder, R., (2000). Tat peptide derivatised magnetic nanoparticles allow in vivo tracking tracking and recovery of progenitor cells. Nature Biotechnology 18: 410-414.

41. Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147-157.

42. Lowe, S.W., Bodis, S., McClatchy, A., Remington, L., Ruley, H., Fisher, D.E., Housman, D.E., Jacks, T., (1994). p53 status and the efficiency of cancer therapy in vivo. *Nature* 266: 807-810.

43. Mann, D.A. & Frankel, A.D., (1991). Endocytosis and targeting of exogenous HIV-1 TAT. EMBO 10: 1733-1739.

44. Nagai, M.A., (1999). Genetic alterations in head and neck squamous cell carcinomas. *Brazilian Journal of Medical and Biological Research* 32(7): 887-904.

45. Nagahara, H., Vocero-Akbani, A.M., Snyder, E.L., Ho, A., Latham, D.G., Lissy, N.A., Becker-Hapak, M., Ezhevsky, S.A., Dowdy, S.F., (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27^{Kip1} induces cell migration. *Nature Medicine* 4(12): 1449-1452.

46. Noteborn, M.H., Danen-Van Oorschot, A.A., Van der Eb, A.J., (1998a). The Apoptin gene of chicken anaemia virus in the induction of apoptosis in human tumourigenic cells and in gene therapy of cancer. *Gene Therapy and Molecular Biology* 1: 399-406.

47. Noteborn, M.H., De Boer, G.F., Van Roozelaar, D., Karreman, C., Kranenburg, O., Vos, J.G., Jeurissen, S.H.M., Hoeben, R.C., Zantema, A., Koch, G., Van Ormondt, H., Van der Eb, A.J., (1991). Charcterization of cloned chicken anaemia virus DNA that contains all elements for the infectious replication cycle. *Journal of Virology* 65: 3131-3139.

48. Noteborn, M.H., Zhang, Y.H., Van der Eb, A.J., (1998b). Apoptin specifically causes apoptosis in tumor cells and after UV-treatment in untransformed cells from cancer-prone individuals: a review. *Mutation Research* 400(1-2): 447-55.

49. Noteborn MH. Todd D. Verschueren CA. de Gauw HW. Curran WL. Veldkamp S. Douglas AJ. McNulty MS. Van der EB AJ. Koch G., (1994). A single chicken anaemia virus protein induces apoptosis. *Journal of Virology* 68(1): 346-51.

50. Pietersen, A.M., Van der Eb, M.M., Rademaker, H.J., Van den Wollenberg, D.J., Rabelink, M.J., Kuppen, P.J., Van Dierendonck, J.H., Van Ormondt, H., Masman, D., Van de Velde, C.J., Van der Eb, A.J., Hoeben, R.C., Noteborn, M.H., (1999). Specific tumor-cell killing with adenovirus vectors containing the apoptin gene. *Gene Therapy* 6(5): 882-92.

51. Rao, L., & White, E., (1997). Bcl-2 and the ICE family of apoptotic regulators: making a connection. *Current Opinion in Genetics and Development* 7: 52-58.

52. Sakahira, H., Enari, M., Nagata, S., (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391: 96-99.

53. Sambrook, J., Fritsch, E.F., Maniatis, T., (1989a). Plasmid Vectors. In *Molecular Cloning: A Laboratory Manual*, Book 1, 2nd Ed (by same authors), Cold Harbor Laboratory Press, USA, pp 1.21-1.78, E.10.

54. Sambrook, J., Fritsch, E.F., Maniatis, T., (1989b). Expression of Cloned Genes in Cultered Mammalian Cells. In *Molecular Cloning: A Laboratory Manual*, Book 3, 2nd Ed (by same authors), Cold Harbor Laboratory Press, USA, pp 16.30-16.39.

55. Sartor, M., Steingrimsdottir, H., Elamin, F., Gaken, J., Warnakulasuriya, S., Patridge, M., Thakker, N., Johnson, N.W., Tavassoli, M., (1999). Role of p16/MTS1, cyclin D1 and Rb in primary oral cancer and oral cancer cell lines. *British Journal of Cancer* 80: 79-86.

56. Schmitt, C.A., & Lowe, S.W., (1999). Apoptosis and Therapy. *Journal of Pathology* 187: 127-137.

57. Schwarze, S.R., Ho, A., Vocero-Akbani, A., Dowdy, S.F., (1999). In vivo protein transduction: Delivery of a biologically active protein into the mouse. *Science* 285: 1569-1572.

58. Schwarze, S.R., Hruska, K.A., Dowdy, S.F., (2000). Protein transduction: unrestricted delivery into cells? *Trends in Cell Biology* 10: 290-295.

59. Stegh, A.H., Schickling, O., Ehret, A., Scaffidi, C., Peterhansel, C., Hofman, T.G., Grummt, I., Krammer, P.H., Peter, M.E., (1998). DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. *The EMBO Journal* 17(20): 5974-5986.

60. Su, Z-Z., Madireddi, M.T., Lin, J.J., Young, C.S.H., Kitada, S., Reed, J.C., Goldstein, N.I. and Fisher, P.B. (1998). The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in

nude mice. *Proceedings of the National Academy of Science USA* 95: 14400-14405.

61. Telford, W.G., King, L.E., Fraker, P.J., (1992). Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. *Cytometry* 13: 137-143.

62. Vile, R.G., Russell, S.J., Lemoine, N.R., (2000). Cancer gene therapy: hard lessons and new courses. *Gene Therapy* 7: 2-8.

63. Ziegler, A., Jonason, A.S., Leffell, D.J., Simon, J.A., Sharma, H.W., Kimmelman, J., Remington, L., Jacks, T., Brash, D.E., (1994). Sunburn and p53 in the onset of skin cancer. *Nature* 372: 773-776.

64. Zhang, Y.H., Abrahams, P.J., Van der Eb, A.J., Noteborn, M.H., (1999). The viral protein Apoptin induces apoptosis in UV-C-irradiated cells from individuals with various hereditary cancer-prone syndromes. *Cancer Research* 59(12): 3010-5.

65. Zhuang, S.M., Landegent, J.E., Verschueren, C.A., Falkenburg, J.H., Van Ormondt, H., Van der Eb, A.J., Noteborn, M.H., (1995a). Apoptin, a protein encoded by chicken anemia virus, induces cell death in various human hematologic malignant cells in vitro. *Leukemia* 9 S1: S118-20.

66. Zhuang, S.M., Shvarts, A., Jochemsen, A.G., Van Oorschot, A.A., Van der Eb, A.J., Noteborn, M.H., (1995b). Differential sensitivity to Ad5 E1B-21kD and Bcl-2 proteins of apoptin-induced versus p53-induced apoptosis. *Carcinogenesis* 16(12): 2939-44.

67. Zhuang, S.M., Shvarts, A., Van Ormondt, H., Jochemsen, A.G., Van der Eb, A.J., Noteborn, M.H., (1995c). Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis in human osteosarcoma cells. *Cancer Research* 55(3): 486-9.

68. Zhou, C., Yang, Y., Jong, A.Y., (1990). Mini-prep in ten minutes. *BioTechniques* 8: 172-173.