



Apoptin as a promising bio-weapon against transformed cells: A review

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Abstract

The menace of cancer is a growing problem world-wide affecting all categories of persons. For more than a decade, the development of a potent anticancer agent with the specificity of attacking transformed cells (malignant cells) without affecting normal cells (untransformed cells) have remained a holy grail clinically as it is proven to be fully successful in only a limited number of cancers. Targeting the transformed cells without affecting the normal cells is extremely difficult. The chameleon nature of transformed cells has been one of the hallmark limitation to the development of a clinically viable therapeutic agents for cancer patient as transformed cells (malignant cells) acquires biochemical properties such as been insensitive to anti-growth signals, self-sufficiency in growth signals, limitless replicative potentials, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis. Thus, this review focuses on a novel emerging gene product with a unique feature of selectively targeting transformed cells (cancer cells) while sparing normal (untransformed) cells. The discovery of a viral protein 3 derived from Chicken Anemia Virus called Apoptin has attracted attention as it possesses an inherent ability to specifically attack transformed cells without affecting the normal cells independent of p53 tumour suppressor gene status. The mechanistic insight for apoptin specificity against transformed cells has come from studies of cells in culture and genetic animal models. The selective apoptotic induction by Apoptin in transformed cells are mediated through the mitochondrial death pathway independent of the p53 tumour suppressor status. One of the principal difficulty in developing an efficient anti-cancer agent has always been in the therapeutic agent specificity to transformed cells (malignant cells) without affecting healthy bystander cells. In this review, we elucidate at the molecular level, the mechanistic event of apoptin specificity for attacking transformed cells without affecting normal cells. We also discuss the basic cellular processes and signaling pathways associated with apoptin's cancer selective toxicity.

Keywords: Apoptin, Apoptosis, Phosphorylation, Transformed cells, p53 tumour suppressor gene, Chicken Anaemia Virus.

1.0 Introduction

Advancement in current cancer therapeutic strategies have resulted in an increase in patient survival. Epidemiological studies and experimental evidences have shown cancer as a leading cause of death worldwide ^[1]. Cells are chronically faced with certain biochemical decisions to proliferate, differentiate and undergo programmed cell death (apoptosis) if life must continue with normalcy devoid of disorders or diseases, however, dysregulation in any of these biochemical processes results in pathological conditions such as cancer. Cancer is as a group of diseases characterized by the uncontrolled proliferation of cells and spread of abnormal cells ^[2,3]. Cancer cells are characterized by the fact that they keep replicating when they are actually supposed to be differentiated ^[4]. Nevertheless, cancer exerts a death toll of approximately 8.2 million people every year, amounting to 12% of total human mortality ^[5]. The global economic burden of cancer is 2-3% of global GDP, or approximately \$900 billion without the cost of treatment included, making it the most financially devastating disease ^[5]. Cancer causes 25% death in the United State, making cancer a major growing public health challenge in the US as well as in many other parts of the world affecting all categories of persons ^[6]. Lifestyle and environmental factors are the major causes of cancers (~80%) while genetic predisposition constitutes the remaining 20% ^[7]. Environmental contamination have become a major cause for concern, mainly because of their toxic, mutagenic and carcinogenic nature even at low concentration ^[8]. The occurrence of most common environmental contaminants that are perilous to life can be traced to anthropogenic sources ^[8,9].

A century of research on the origins of various cancers suggests mutations in cancer genes (tumor suppressors and oncogenes) ^[10]. Lack of equilibrium on the part of reactive oxygen species generation and the body battery defense system gives birth to oxidative stress ^[2]. Oxidative stress has been suggested in both experimental and clinical studies to play a major role in the pathogenesis of so many diseases such as cardiovascular disease and cancer ^[2]. The transformed cells (malignant cells) are genetically altered set of abnormal cells derived from normal cells and as such share numerous biological/genetic features, targeting one without affecting the other is extremely difficult.

Recently, a small protein called Apoptin has drawn a significant amount of attention due to its selective toxicity towards transformed cells. Apoptin is a viral protein 3 (VP3) derived from Chicken Anemia Virus (CAV) is known to possesses an inherent ability to specifically kill transformed cells independent of p53 tumor suppressor gene. The subcellular localization of apoptin appears to be crucial for this tumor selective activity ^[11]. In normal cells, apoptin resides in the cytoplasm, whereas in cancerous cells it translocates into the nucleus. The nuclear translocation of apoptin is largely controlled by its phosphorylation. In tumor cells, apoptin

causes the nuclear accumulation of survival kinases including Akt and is phosphorylated by CDK2. Thereby, Apoptin redirects survival signals into cell death responses. These features make Apoptin a potential candidate as a therapeutic and diagnostic tool in cancer treatment [11]. The two mechanistic properties of Apoptin are; the induction of apoptosis in human cancer/transformed cells without affecting the primary normal cells and also the induction of apoptosis in transformed cells occurring irrespective of the status of p53 tumour suppressor gene generated a particular interest to study cancer-specific processes [11].

The major challenge in the management and treatment of cancer patients over the years, whether by chemotherapy, radiotherapy or even excision surgery, has always been in administering the therapy specifically to transformed cells without affecting normal cells. Thus, an insight review on a promising anticancer agent and its mechanistic event with specificity of targeting transformed cells sparing healthy bystander cell will be an adage to the fight against cancer as the review on this perspective still remained an overlooked. Here, we review at the molecular level the mechanistic event and the signaling pathway by which Apoptin selectively attack transformed cells without affecting the normal cells, and also consider its clinical relevance in the fight against cancer.

2.0 Overview of Apoptin Protein

Apoptin is a small apoptosis inducing protein derived from chicken anemia virus (CAV), which belongs to Gyrovirus genus of Circoviridae family [11]. It is among the first tumour selective anti-cancer genes that have been isolated which induces p53-independent tumour cell-specific cell death [11]. Chicken anaemia virus (CAV) is a recently characterized single-stranded DNA virus of a novel type. It was first discovered in Japan in 1959. CAV particles have a diameter of 23-25 nm and contain a circular minus-strand DNA. The genome, a circular single-stranded DNA, codes for three viral proteins: VP1, the 52 kDa structural capsid protein; VP2, a 28 kDa non-structural protein with dual specificity phosphatase activity; and VP3, the smallest protein of about 13 kDa, known as Apoptin which induces apoptosis in erythrocyte precursors and thymocytes, resulting in immunodeficiency [12, 13, 14].

The viral protein 3 (VP3), a 13 kDa serine-threonine rich protein of 121 amino acids, has been shown to have the ability to induce programmed cell death (PCD) in chicken thymocytes both *in vitro* and *in vivo* in a fairly extensive body of scientific literature going back to the 1990s [15]. Because of its death-inducing abilities, the VP3 gene was renamed "APOPTIN". Interestingly, the apoptotic activity of Apoptin is not restricted to chicken thymocytes. Apoptin also causes PCD in various human tumor and transformed cells [16].

2.1 The Apoptin Sequence and Structure

Apoptin is composed of 121 amino acids, and does not show significant sequence homology with known cellular proteins (Figure 1). The C-terminal end of apoptin contains a bipartite nuclear localization sequence (NLS): NLS1 spans amino acids 82-88, and NLS2 residues 111-121 [16, 17]. In addition, apoptin contains a putative nuclear export sequence (NES) at residues 97–105. These recognition sequences drive the shuttling of apoptin in and out of the nucleus. Apoptin also harbors a short hydrophobic leucine-rich stretch (aa 33– 46) that is required for self-association as well as binding of promyelocytic leukemia protein and other interaction partners [18, 19]. It is interesting that the biologically active form of recombinant apoptin can form non-covalently attached globular multimers consisting of 30– 40 monomers [20] The formation of this multimeric complex appears to be facilitated through the interaction of the proline-rich hydrophobic regions in the N-terminus (aa1– 69) of each monomer. The C-terminal tip of each monomer, on the other hand, contains a NLS with an accessible phosphorylation site (Thr-108), which allows for its interaction with other proteins and for modification by kinases as shown in Figure 1 [11].

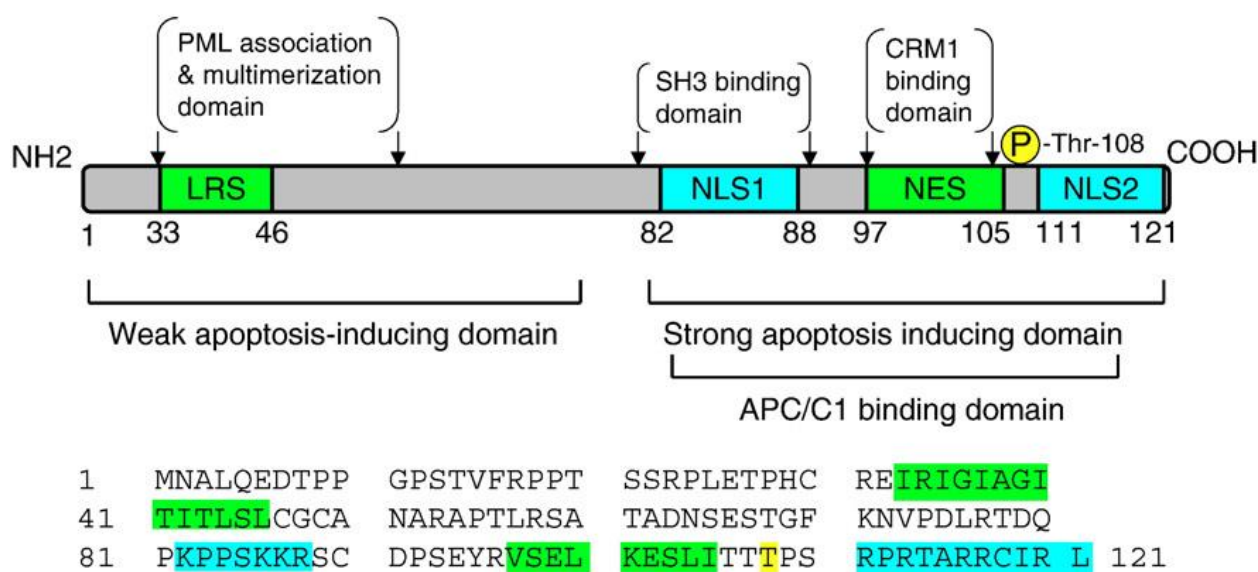


Figure 1: The primary structure of apoptin. The key domains and important sequences are indicated. Lower panel shows the sequence of apoptin [11].

2.3 Apoptin Interacting Molecules

The cellular localization of apoptin plays crucial role for its selective toxicity [21, 22]. However, for tumor specific toxicity, apoptin presumably requires additional interaction partners that activate specific signaling pathways in cancer cells (Figure 2) [11]. There are a number of molecules which interact with apoptin and appear to be important for the nuclear localization of apoptin or its tumor selective cytotoxicity (Table 1) [11].

Table 1: Apoptin-Interacting Molecules

S/N	Molecule	Biological Effect
1	DNA	Due to its basic structure, apoptin can directly interact with DNA, preferentially with double-stranded DNA and heterochromatin.
2	CyclinA-CDK2	CyclinA-CDK2 complex directly phosphorylates apoptin at Threonin 108 and contributes to the regulation of its subcellular localization.
3	Peptidyl-prolyl isomerase-like 3 (Ppil3)	Apoptin interact with Ppil3 may favor its cytoplasmic localization.
4	Hip-interacting protein (Hippi)	In non-cancerous cells, apoptin co-localize with Hippi in the cytoplasm, whereas in tumour cells Apoptin migrate to the nucleus while Hippi remains in the cytoplasm.
5	N-Myc interacting protein (Nmi)	The functional significance of Apoptin and Nmi interaction is not well understood.
6	APC1 (subunit of anaphase-promoting complex/cyclosome)	Apoptin interaction with APC1 may lead to mitotic cell cycle arrest and apoptosis.
7	Death effector domain-associated factor (DEDAF)	Coexpression of apoptin and DEDAF increases apoptosis as compared to the effects of either protein alone.
8	Promyelocytic leukemia protein (PML)	Sumoylated Apoptin interacts with PML in PML nuclear bodies. However, the disruption of the interaction of PML and apoptin does not affect apoptin's cytotoxicity.
9	Fas-associated death domain protein(FADD)	Upon over expression apoptin colocalizes with FADD, a component of the death receptor pathway, in so-called death effector filaments. The significance of this process is unknown
10	Bcl 10	Apoptin can colocalize with Bcl10, a regulator of apoptosis and NF-κB activation.
11	Importin-β1	Importin-β1 aids apoptin's nuclear translocation through the nuclear pore complex.

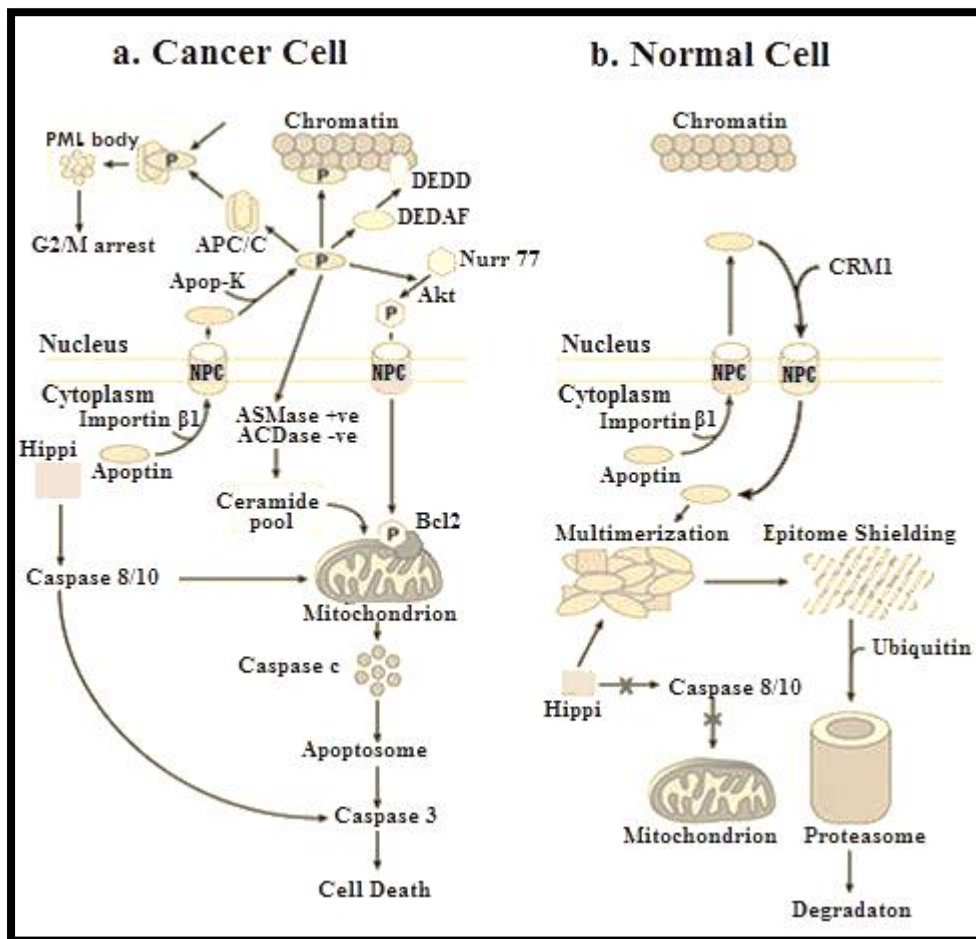


Figure 2: Apoptin interacting molecules in cancer cells and normal cells ^[11].

Apoptin interacts with peptidylprolylisomerase- like 3 (Ppil3) ^[23]. Ppil3 belongs to the cyclophilin family and shares 46% identity to cyclophilin A. Members of this family catalyze the cis–trans isomerization of peptidyl-prolylimidic bonds and play a role in mitochondrial maintenance, cell cycle progression and apoptosis. RNA mediated depletion of Pip13 leads to the migration of apoptin from cytoplasm to the nucleus. Interestingly, proline109 in apoptin was essential for the Ppil3 dependent cytoplasmic localization of apoptin ^[23].

Hippi, a protein interacting with Huntington-interacting protein 1 (Hip1), is also a binding partner for apoptin ^[24]. The Hippi–Hip1 complex can recruit caspase-8 and regulates apoptotic cell death in Huntington's disease. In noncancerous cells, apoptin co-localizes with Hippi in the cytoplasm, whereas in tumor cells apoptin migrates to the nucleus while Hippi remains in the cytoplasm ^[24]. The C-terminus region of apoptin is vital for the interaction with Hippi. As mentioned above, however, apoptin-mediated cell death is not compromised by a genetic deficiency of caspase8. Association of apoptin with cytoplasmic Hippi or Ppil3 may play a role in sequestering apoptin in cytoplasm and preventing apoptosis of normal primary cells ^[25].

N-Myc interacting protein (Nmi) associates with apoptin. It is an interferon inducible protein that can interact with transcription factors of Myc family. Nmi-like proteins enhance the

activity of Myc proteins that are involved in cell proliferation, differentiation as well as in apoptosis. The interaction of apoptin and Nmi account for the tumor specificity of apoptin's cytotoxicity, because Nmi is only expressed at low levels in normal tissues but highly upregulated in leukemia cell lines. Apoptin has been hypothesized to switch the Nmi/Myc-mediated transforming activity to an apoptotic activity. The restricted expression pattern of Nmi does not explain the broad antitumor activity of apoptin [26].

Apoptin associates with APC1, a subunit of anaphase-promoting complex/cyclosome (APC/C) and thereby inhibits the ubiquitin ligase function of APC/C. This interaction is present in cancer cells but is absent in noncancerous cells, which can be another possible mechanism for the tumor selective action of apoptin. Over expression of apoptin or RNA mediated depletion of APC1 subunit induces apoptosis in p53 null tumor cells, shows that apoptin may interfere with the APC/C pathway through binding to APC1. In future, APC/C may serve as a potential target for the development of novel anticancer therapies (Figure 2) [27].

Due to its basic structure apoptin binds to naked DNA, preferentially to heterochromatin. Bacterially expressed apoptin forms active protein multimers consisting of 30–40 monomers [20]. These apoptin multimers in turn can bind to DNA and cooperatively form distinct superstructures consisting of 20 apoptin multimers and approximately 3 kb DNA [28].

Apoptin can also interact with a pro-apoptotic protein, called death effector domain associated factor (DEDAF). DEDAF associates with death effector domain containing pro-apoptotic proteins and plays a role in transcriptional regulation [29]. Similar to apoptin, transient over expression of DEDAF can induce cell death in cancer cells, but not in normal cells. The cellular localization of DEDAF is similar to that of apoptin, as both co-localize into the nucleus of tumor cells. Co-expression of apoptin and DEDAF increases the apoptotic death rate as compared to the effects of expression of either of them alone (Figure 2) [29].

Apoptin also interacts with promyelocytic leukemia protein (PML) and localizes in PML nuclear bodies [30]. PML nuclear bodies are ordered protein complexes that associate with the nuclear matrix and play a role in apoptosis, DNA replication, repair, transcription, RNA transport and viral replication [31]. Many proteins residing in PML nuclear bodies, including apoptin, undergo a specific posttranslational modification, called sumoylation. A sumoylation deficient mutant of apoptin can still trigger apoptosis in wild type as well as in PML deficient cells suggest that neither sumoylation nor the interaction with PML are crucial for apoptin's proapoptotic activity. Although apoptin's interaction with PML appears dispensable for apoptosis, it involved in CAV replication (Figure 2) [30].

Apoptin co-localizes with FADD (Fas associated protein with death domain) and Bcl10, a caspase recruitment domain containing protein, in cytoplasmic structures, called death effector

filaments [32]. Both FADD and Bcl10 are involved in apoptosis signaling by death receptors, though the significance of this co-localization for apoptin induced cell death is unknown. Through its proline rich sequence (aa81–86), apoptin interacts with the SH3 domain of the p53 regulatory subunit of phosphoinositide 3- kinase. Class I PI3 kinases play a central role in various regulatory processes, such as cell growth, survival and differentiation. When p85 is down regulated, apoptin is shuttled out of the nucleus, thereby imposing a negative regulation of apoptin induced cell death [35]. Apoptin derivatives devoid of the proline rich region do not interact with p85, are unable to activate PI3K and show impaired apoptosis induction. Thus, the interaction of apoptin with p85 subunit of PI3K appears to be essential for the cytotoxic activity of apoptin [33]. The role of PI3K/Akt pathway in cell survival is well established, there are examples in which PI3K and Akt also promote cell death [34-37].

Interestingly, as recently reported, the direct interaction of apoptin with Akt1 triggers nuclear trafficking of Akt and initiates apoptosis instead of a survival response [33, 38]. Agents such as etoposide, cisplatin, and interferon- β induce Akt1 phosphorylation that precedes the onset of caspase activation and apoptosis [39]. Transient activation of Akt1 supports cell survival, whereas its sustained activation leads to apoptosis [40].

3.0 Mechanisms of Apoptin Mediated Cell Death

One of the key properties of apoptin is its ability to induce tumor-specific cell apoptosis independently of p53 status without affecting the normal cells [28]. Thus, apoptin is similarly effective in killing tumor cells that are p53 deficient or either expresses wild-type or mutant p53. Apoptosis is typically mediated by intracellular cysteine proteases, called ‘caspases’ that function as initiators and executioners of the apoptotic process [41]. The activation of caspases is accomplished by two major signaling routes: the extrinsic death receptor and the intrinsic mitochondrial pathway.

The selective apoptotic induction by Apoptin in tumour cells (transformed cells) are mediated through the mitochondrial death (Intrinsic) pathway independent of the p53 status which involves; Activation of Apoptin (Phosphorylation of Apoptin), the release of Nurr77 transcription factor, Modulation of anti-apoptotic protein to pro-apoptotic protein, regulation of sphingolipid-ceramide pathway Involvement of Caspases. Apoptin mediated cell death is however independent of death receptors, as cells deficient in FADD or caspase8, the key regulators of the extrinsic apoptotic pathway remain sensitive to apoptin [25]. Moreover, blocking of death receptor CD95 with neutralizing antibodies does not affect apoptin induced cell death. In contrast to components of the death receptor pathway, apoptin mediated apoptosis

is strongly influenced by regulators of the mitochondrial death pathway, as for instance a deficiency of Apaf1 strongly protects tumor cells [25].

3.1 Nuclear Shuttling of Apoptin is Vital for its Apoptotic Action

The selective toxicity of apoptin to malignant cells is mainly attributed to its differential subcellular localization in tumor and normal cells. Apoptin predominantly localizes to the nucleus of cancer cells, whereas its accumulation in the nucleus is severely impaired in normal cells [23]. The shuttling of apoptin is regulated by both the bipartite nuclear localization sequence (NLS) and the N-terminal nuclear export sequence (NES) [21, 22, 42]. The NLS of apoptin is potentially active in both normal and cancer cells, as small amounts of apoptin can translocate to the nucleus in normal cells, have lower efficiency (Figure 3) [43]. Notably, a CRM1-regulated NES (aa 97–105) in apoptin that appears to be active in normal cells [42], but not in tumor cells, and thereby retains apoptin in the cytoplasm of normal cells (Figure 3).

Apoptin also contains a leucine-rich sequence (aa33–46) that is important for its interaction with PML and other proteins [18, 19]. Mutations that inactivate this sequence stretch not only reduce the ability of apoptin to associate with PML bodies, but also inhibit nuclear accumulation of apoptin in tumor cells [19].

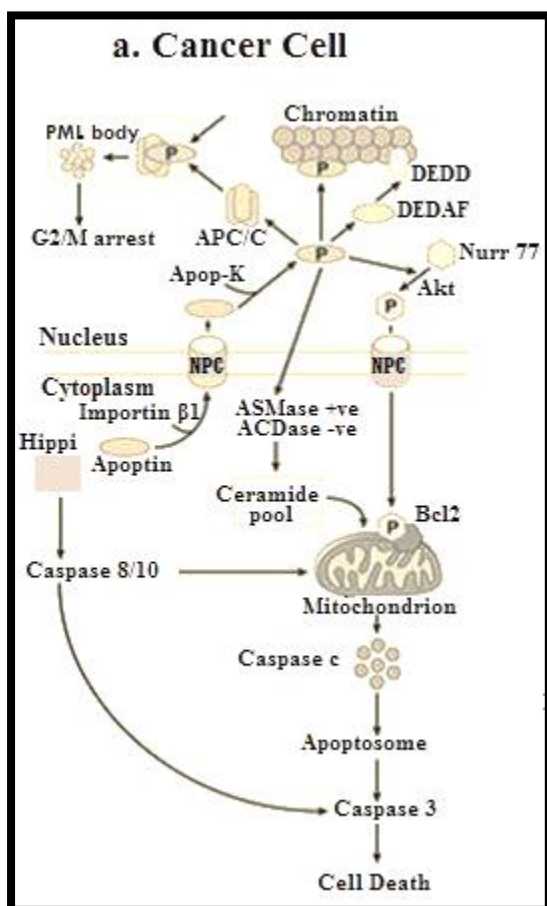


Figure 3: Schematic representation nuclear shuttling of apoptin [11].

3.2 Apoptin Phosphorylation Plays a Crucial Role in Apoptotic Induction

The phosphorylation of apoptin is crucial in transducing apoptotic signals in cancer cells. Apoptin has a phosphorylation site at threonine-108 (Thr-108) adjacent to the nuclear export sequence (NES). Phosphorylation at Thr-108 appears to drive tumor-specific nuclear accumulation of apoptin via inactivation of the NES [44]. In contrast, Thr-108 phosphorylation only partially affects apoptin's pro-apoptotic activity, as an apoptin mutant devoid of this phosphorylation site retains the ability to induce apoptosis [33, 45]. Both N- and C-terminal regions of apoptin have the capacity to bind DNA and induce apoptosis independently, and that Thr-108 phosphorylation is required only for the C-terminus [45]. An apoptin mutant consisting of the first 69 amino acids is mainly located in the cytoplasm of human tumor cells, but harbors a distinct, albeit weaker, pro-apoptotic activity [21]. Furthermore, there is a cluster of three threonine residues (aa106–108) within the apoptin sequence. Mutation of Thr-108 still allows for the phosphorylation of apoptin at the adjacent threonine residues [11].

The mitogenic cyclin dependent kinase CDK2 is the principal kinase that directly phosphorylates apoptin at Thr-108 and is crucially required for apoptin-induced cell death (Figure 4) [38].

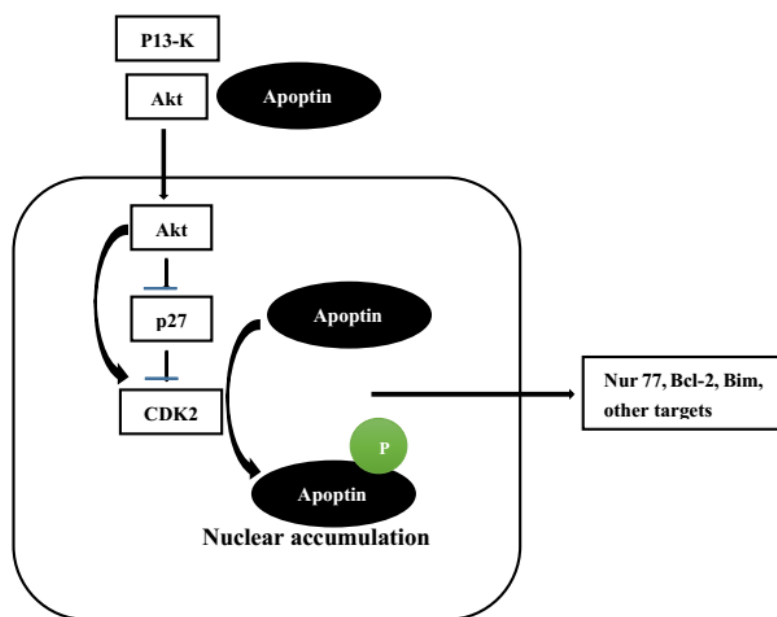


Figure 4: Apoptin Phosphorylation [11].

In tumor cells, Apoptin is phosphorylated by a cellular kinase and translocates to the nucleus where it accumulates. Subsequently, Apoptin activates the apoptotic machinery either through the mitochondria or other pro-apoptotic signalling pathways (e.g. Increase in ceramide pool) resulting in cell death (Figure 5). In contrast, in normal cells Apoptin shuttles back to the cytoplasm, becomes epitope shielded and eventually degraded in a proteasome-dependent manner (Figure 5) [46].

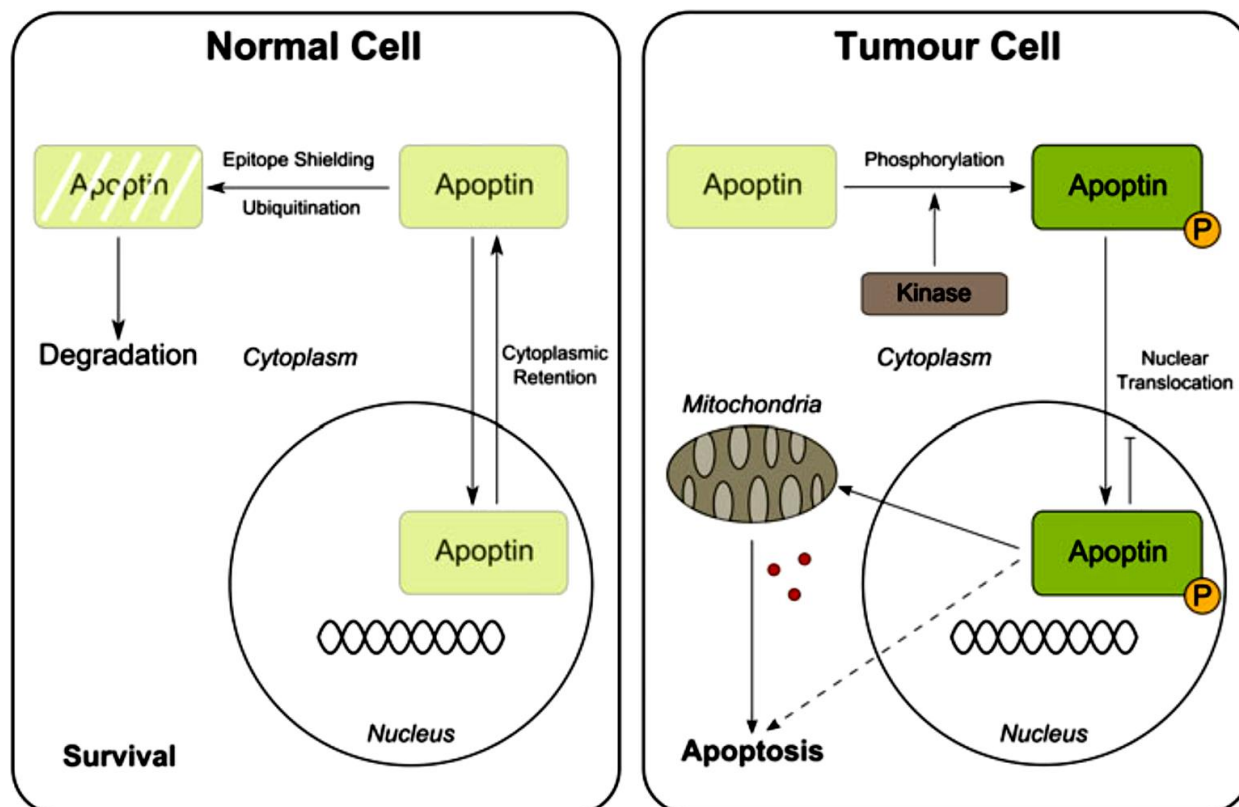


Figure 5: Model of Apoptin activation in tumor cells versus normal cells [47].

3.3 Phosphorylated Apoptin Induces the Nuclear Export of Nur77

Nur77 (also known as TR3, NGFI-B, TIS1 and NAK-1) is a transcriptional factor belonging to the superfamily of nuclear receptors [47, 48]. All members of the superfamily share a highly conserved structural organization with an amino-terminal region encoding activation function 1 (AF-1), followed by the DNA-binding domain (DBD), and the ligand-binding domain (LBD). The C-terminal region also encodes activation function 2 (AF-2) (Figure 6) [49].

Recent studies have demonstrated that the AF-1 plays a major role in mediating Nur77 transactivation, while AF-2 is dispensable [50].

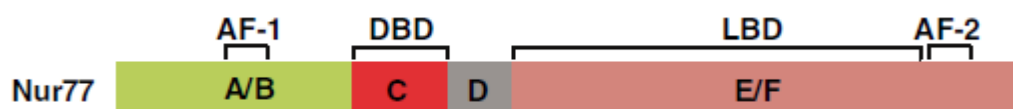


Figure 6: Schematic representation of Nur77. AF-1, activation function 1; DBD, DNA-binding domain; LBD, ligand-binding domain; AF-2, activation function-2 [51].

Nur77 is unique among nuclear receptors in that it is also an immediate-early gene induced by a diversity of extracellular stimulation, ranging from survival, differentiation and apoptosis. They work via distinct modes of action, and account for diverse biological activities of Nur77 [51].

Involvement of apoptin and Nur77 is essential in inducing programmed cell death. Phosphorylation of apoptin leading to its nuclear accumulation triggers the release of a transcription factor called Nur77, phosphorylated Nur77 transmit apoptotic signal from the nucleus to mitochondria, as it is shuttled from the nucleus to the cytoplasm upon transient expression of apoptin, Nur77 binds to Bcl 2 and change its properties from an anti-apoptotic to a pro-apoptotic molecule, leading to the activation of mitochondrial death pathway ^[52].

3.4 The Conversion of Bcl2 from an Anti-apoptotic to a Pro-apoptotic Molecule by Nur77

Nur77 interaction with Bcl2 represents the first example that a nuclear receptor interacts with a Bcl2 family member, providing the coupling of Nur77 nuclear receptor signaling to the Bcl2-mediated apoptotic machinery (Figure 7). In this regard, Bcl2 does not act as an anti-apoptotic protein in Nur77-mediated apoptosis. Instead, the interaction between Nur77 and Bcl2 triggers cytochrome c release and apoptosis ^[52]. The Nur77-Bcl2 interaction conformationally converts Bcl2 from an anti-apoptotic to a pro apoptotic molecule, providing a new approach to switch the Bcl2 phenotype (Figure 7). An important question is how Bcl2 is converted into a killer by Nur77 binding. All members of the Bcl2 family possess at least one of the four conserved BH domains ^[53]. Anti-apoptotic Bcl2 family members Bcl2 and Bcl-XL have hydrophobic pockets on their surfaces, which is essential for their anti-apoptotic effect, while their BH3 domains are buried. In contrast, pro-apoptotic Bcl2 family members have an exposed BH3 domain, which bind to the hydrophobic pockets of anti-apoptotic Bcl2 members (made up by their BH1, BH2 and BH3 domains) to inhibit their survival effect. The unstructured loop region of B50 amino acid length located between the BH4 (a1 helix) and BH3 (a2 helix) domains of Bcl2 plays an important, albeit highly complex role in regulating Bcl2 functions. Deletion of the loop region of Bcl2 blocks paclitaxel-induced apoptosis whereas caspase-mediated cleavage within the loop region of Bcl2 converts it into a pro-apoptotic molecule (Figure 7). ^[54, 55].

Modification of the loop region of Bcl2 or Bcl-XL by phosphorylation or deamidation is associated with downregulation of their anti-apoptotic activity ^[56]. Upon Nur77 binding to the loop of Bcl2, the hydrophobic binding groove of Bcl2 undergoes an extensive rearrangement, resulting in exposure of its BH3 domain. This conformational change is responsible for the conversion of Bcl2 from a cytoprotective to a cytotoxic molecule. It is likely that pro-apoptotic Bcl2 acts as a BH3-only protein to regulate the apoptotic potential of other Bcl2 family members. Activation of Bax or Bak requires their binding to BH3- only protein tBid ^[52].

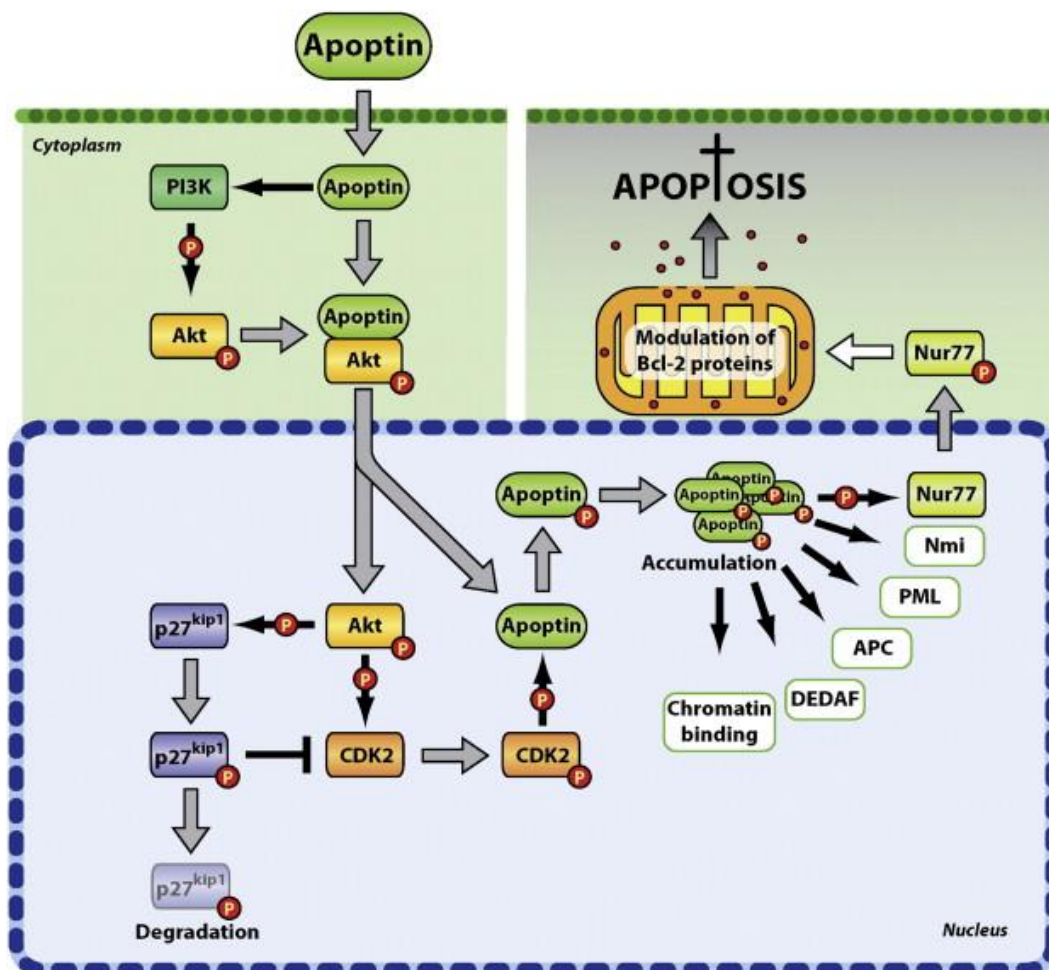


Figure 7: Schematic representation of Apoptin-mediated cell death [11].

3.5 Apoptin Regulation of Sphingolipid-Ceramide Pathway Leading to Apoptosis

Apoptin increases ceramide by upregulation of acid sphingomyelinase, resulting in increased hydrolysis of sphingomyelin to ceramide and downregulation of acid ceramidase resulting in decreased deacetylation of ceramide to sphingosine leading to increase ceramide pool (Figure 8) [57]. Using a gene therapy approach, Liu et al., (2006) showed that treatment of human prostate cancer cells with an adenoviral vector containing green fluorescent protein–tagged apoptin (AdGFP-Apoptin) *in vitro* resulted in inhibition of cell proliferation and induction of apoptosis [58]. Furthermore, they demonstrated that AdGFP-Apoptin-mediated apoptosis was independent of various apoptotic regulators, including p53, caspase-3, Bcl2, Bax, survivin, FLIPs, XIAP, and CIAP [58].

Ceramide is a second messenger signaling molecule that is increased by cellular stress induced by diverse therapeutic agents, such as tumor necrosis factor- α , Fas ligand, radiation, and chemotherapeutic drugs [59]. However, apoptin increases ceramide pool which will act to alter the mitochondrial permeability transition pore leading to the releases of pro-apoptotic proteins

such as cytochrome c and subsequent activation of caspases resulting in programmed cell death (Apoptosis) (Figure 8).

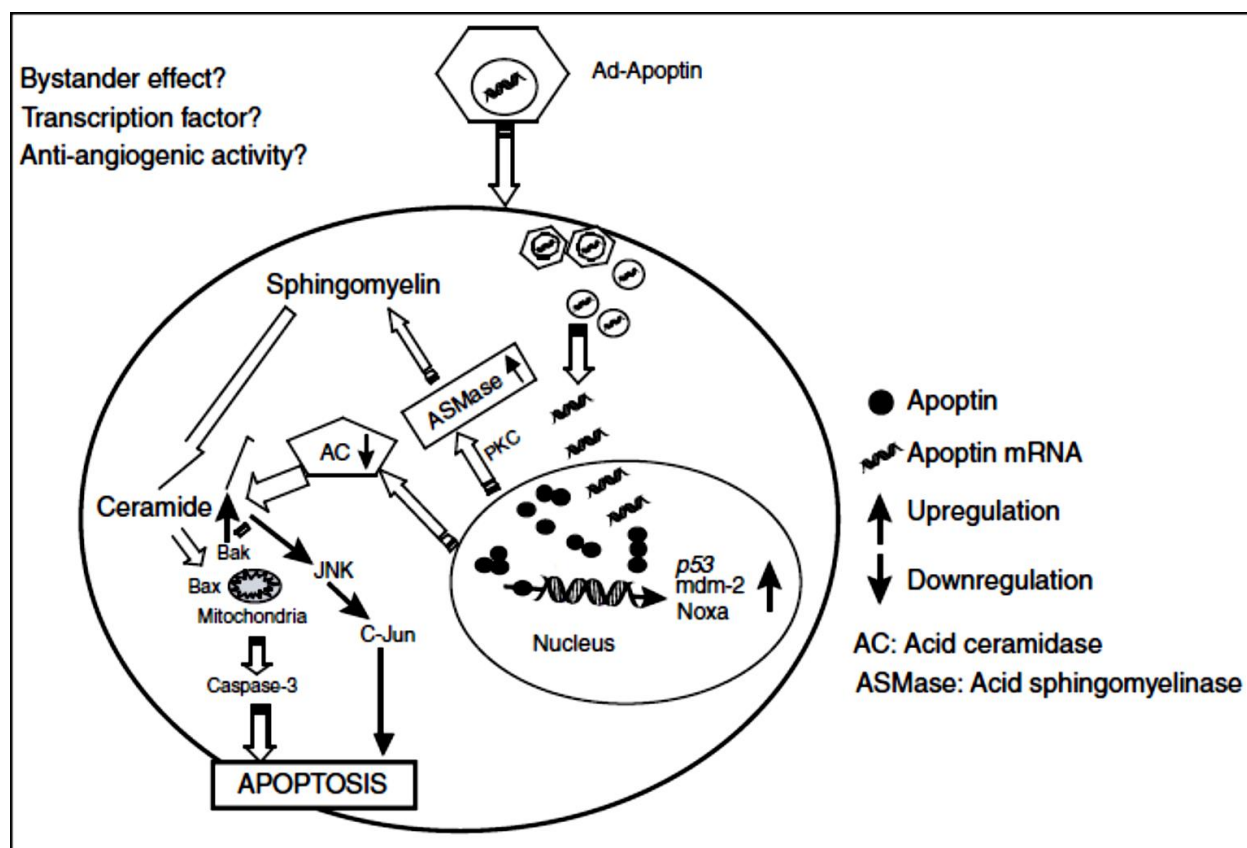


Figure 8: AdGFP-Apoptin-mediated ceramide signaling and tumor cell killing [57].

4.0 Cellular Activities of Apoptin

In normal cells (Untransformed cells), apoptin are localized in the cytoplasm in which they undergoes multimerization and upon transient expression to the nucleus by Exportin- β 1, they are rapidly shuttled out by chromosomal maintenance 1 protein (CRM 1) back to the cytoplasm which they undergoes epitope shielding and subsequently ubiquitination involving three enzymatic processes which are Ubiquitin activation enzyme, Ubiquitin conjugating enzyme and ubiquitin ligation [11]. The cascade of these enzymatic processes were initially referred to as “The Molecular Kiss of Death”, this is further degraded following proteosomal degradation. Thus, apoptin are degraded in the cytoplasm of an untransformed cell (Figure 9) [11]. Conversely, in transformed cells (Tumour cells), Apoptin are activated by phosphorylation with cellular kinases and are shuttled out of the cytoplasm to the nucleus where they accumulates to generate biochemical signaling involving activation, release and translocation of a transcription factor as well as upregulation and downregulation of biological processes leading to programmed cell death (Apoptosis) through mitochondrial death (Intrinsic) pathway [11].

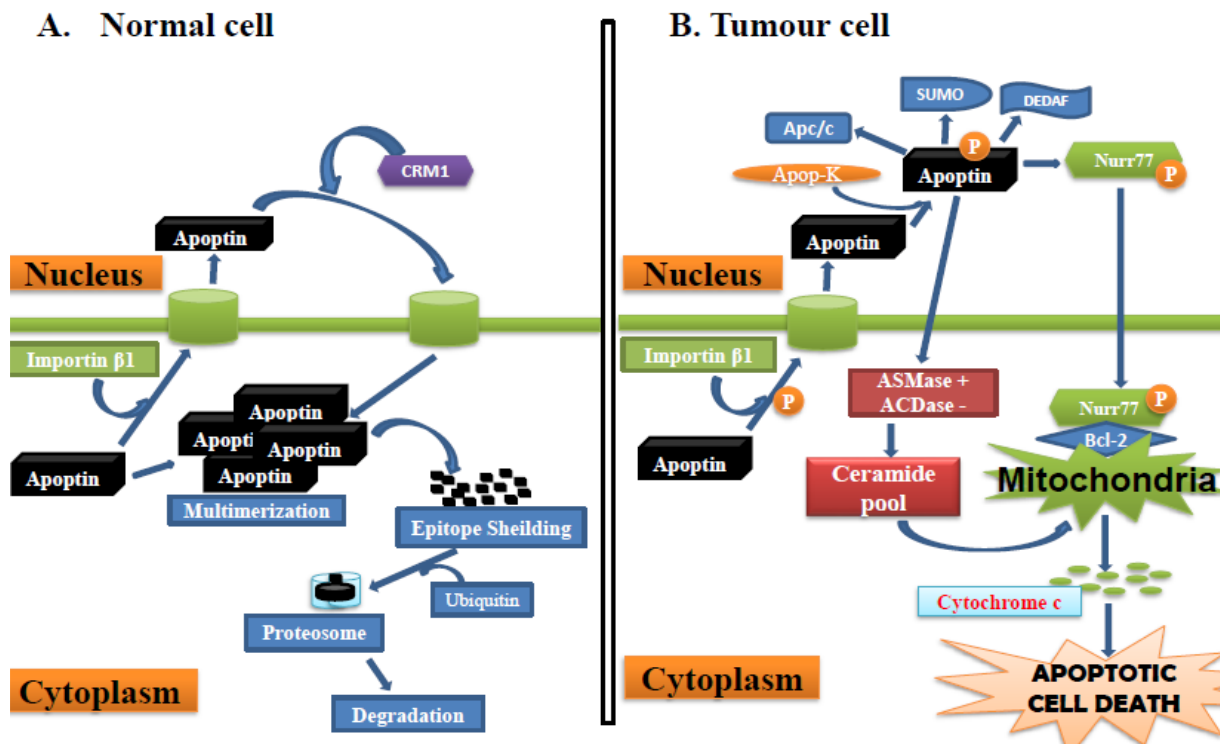


Figure 9: Modified schematic representation of how Apoptin works in cells ^[11].

5.0 Overview of Apoptin Clinical Perspectives

The ability of apoptin to induce apoptosis independently of p53 and to switch survival pathways into pro-apoptotic signaling carries an enormous pharmacological potential ^[11]. These remarkable features might be directly employed for the use of apoptin in cancer therapy. In addition, targeting of apoptin's interaction partners might be an approach to achieve tumor specificity and to design new anti-tumour therapies. Several preclinical studies have shown that apoptin improves also the efficacy of traditional therapy, while apoptin itself shows no adverse side effects ^[11].

Recent studies demonstrate that down regulation of surviving together with the expression of apoptin significantly increases the percentage of apoptotic cells compared to the effects seen upon surviving loss or apoptin expression alone ^[60, 61]. Surviving belongs to the family of inhibitors of apoptosis proteins (IAP) and is highly expressed in several tumors. These findings suggest that a combined strategy using apoptin and a down regulation of surviving could be effective in controlling the growth of tumors that are otherwise resistant to traditional chemotherapy. Another study has shown that the combined treatment of tumors with recombinant apoptin and IL-18 induced a strong Th1 response against Lewis lung carcinomas that was associated with a significant inhibition of tumor growth ^[62]. Similarly, a combined therapeutic approach with recombinant adenovirus expressing apoptin and etoposide showed an additive cytotoxicity on human osteosarcoma U2OS cells ^[63]. Also, when paclitaxel

treatment was combined with apoptin, the survival of p53- positive, p53-negative and p53-mutant cancer cells was compromised at a significantly lower dose of cytotoxic chemotherapy. These observations indicate that apoptin in combination with conventional chemotherapeutic agents offers an effective strategy for cancer treatment [63].

The tumor selective cell death by apoptin has now been demonstrated in a great number of tumor cells using various techniques including adenoviral transfer, transient over expression or introduction of cell permeable apoptin constructs. While all these studies confirmed a tumor specific effect, the most challenging issue is still the development of efficient delivery forms of apoptin at the tumor lesion.

Conclusion

In conclusion, myraids of current cancer treatments lack specificity in killing transformed cells without causing severe negative effects to normal cells in the body. Apoptin being a novel gene product derived from Chicken Anemia Virus (CAV), has the ability to selectively kill transformed cells without affecting normal cells. This specificity and efficiency of killing transformed cells has made apoptin a subject of much interest and speculation. The molecular event by which apoptin selectively induced apoptosis in transformed cells is mediated through mitochondrial death pathway independent of p53 tumor suppressor gene status. Understanding the killing efficiency and specificity of apoptin against transformed cells, sparing the normal cells as well as the mechanisms utilized by apoptin in attacking transformed cells will lay a significant bedrock towards developing a potent anticancer agent against all types of cancer.

Significance Statement

This review unravels the molecular mechanisms and signaling pathways of apoptin specificity against transformed cells (cancer cells) that can be beneficial for cancer treatment, diagnosis and management. In addition, this review will help the researchers to uncover the critical areas of developing a potent anticancer agent that can selectively attack cancer cells without affecting normal cells of which many researchers were not able to explore. Thus a new theory on the mechanisms of chameleon nature of transformed cells (cancer cells) that offers resistance to anticancer agent may be arrived at.

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