

# Molecular Targeting of Cell Death Signal Transduction Pathways in Cancer

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**Abstract:** Suppression of cell death (most often apoptosis) by survival signals, or by defects in cell death signal transduction pathways, is considered one of the obligate hallmarks of malignant transformation. However, molecular survival strategies to evade cell death only have relevance in the presence of pro-death signals. Discovery of the apoptotic properties of oncogenes responsible for increased tumor cell proliferation (e.g. c-Myc) provided the most important example for such signals and led to the concept of synthetic lethal targeting as a strategy of identifying cancer specific drug target molecules. Besides growth signal autonomy, other hallmarks of oncogenesis (insensitivity to anti-growth signals, limitless replicative potential, invasion and metastasis, angiogenesis and increased genomic instability) are also challenged by increased susceptibility to various forms of cell death. Therefore, cancer cells must acquire survival strategies to suppress these cell death/apoptosis mechanisms. Novel signal transduction therapies can target molecules involved in these strategies to trigger tumor specific cell death.

**Key Words:** Cell death, apoptosis, cancer.

## INTRODUCTION

The first challenge of this review is to define its topic. Individual cells can irreversibly lose cellular integrity and terminate metabolism in a multicellular organism by several biochemically and morphologically distinct mechanisms. The term "apoptosis" ("falling leaves", Greek) was introduced by two pathologists, Wyllie and Kerr in their landmark paper in 1972 [1]. They recognized that physiologic and several pathologic forms of cell death shares a number of common morphological features, such as cytoplasm shrinking with increased staining by eosin, nuclear condensation (kariopycnosis) and fragmentation (kariorexis), plasma membrane blebbing, formation of apoptotic bodies and detachment of the cells from the basement membrane. However, the first cell death research related Nobel Prize was awarded to three molecular genetists for their work in the field of programmed cell death in 2003. An explosion occurred in cell death research when the paradigms of cell death as a genetically determined ("programmed"), and biochemically and morphologically distinct cellular process were combined. The definition of apoptosis become clearer when caspases, the main executioner enzymes responsible for the above mentioned apoptosis hallmarks were discovered and the definition of apoptosis as a cell death process dependent on caspase activity, i.e. death by "thousand cuts" emerged [2, 3, 4].

However, the paradigm of cell death became less clear when it turned out that in the presence of caspase –inhibitors,

apoptosis inducers can still kill the cells by a necrosis-like mechanism which is still dependent on the activation of other enzymes, most often cathepsins [5]. Perhaps the most convincing examples for the existence of caspase-independent cell death signals came from experiments with death ligands. Death ligands trigger cell death *via* cell surface receptors, which leads to caspase activation. However, in peripheral T-cells, FasL can induce necrosis *via* the RIP molecule independently of caspase activation [6]. The existence of a caspase-8 independent cell death signaling pathway triggered by another death ligand, TRAIL, was also demonstrated in other cell types [7].

In our definition, active cell death is a cellular response to a non-lethal stimulus. In contrast, passive cell death occurs in the presence of unrepairable physical or chemical damage. It is easily acceptable that cell death induced by the activation of a cell surface receptor is an active form of cell death regardless of its biochemical and morphological characteristics. However, even "cytotoxic" agents, for example DNA damaging chemotherapeutic drugs can kill cells despite of the nearly complete repair of the initial damage [8]. Therefore, drug sensitivity depends not only on the extent of DNA damage but also on the active cellular response to the damage. This new view of cytotoxic drug action was revolutionary in our understanding of drug sensitivity and resistance of classic chemotherapeutic drugs. In addition, cell type specific regulation of apoptosis allowed the design of new selective drugs capable of modulating the cellular response.

Another way of defining signal transduction therapy targeting cell death pathways is to describe what is not meant by this. Cancer researchers working in the field of cell

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death/apoptosis, it is self-evident that “only a dead cancer cell is a good cancer cell”. However, in clinical oncology, control of tumor growth and spreading is often considered successful treatment without complete tumor response. Successful signal transduction therapies of cancer include targeting proliferation to slow down or permanently block tumor growth, inducing cellular senescence – a special form of irreversible cell cycle blocking –, inducing cellular differentiation of the cancer cell (“taming the cancer cell”), blocking invasion and metastasis and, most importantly, inhibiting tumor growth-related angiogenesis. In contrast to these approaches, the objective of cell death targeted signal transduction therapies is to directly induce tumor selective cell death.

This paper gives a short overview of basic cell death mechanisms and their analysis, cancer specific molecular changes that sensitize cancer cells to cell death, and cell death signaling pathways and their cancer related defects. In addition, the effects of cell death regulation on the sensitivity of tumors to cytotoxic drugs will be discussed. Finally, examples will be given on the use of these tumor specific molecular alterations in the development of signal transduction therapies.

### **BIOCHEMICAL CHARACTERISTICS AND MOLECULAR ORDERING OF APOPTOSIS**

Biochemical analysis of cell death revealed several common features during the apoptotic process. During the initiation phase, the signaling pathways induced by different trigger mechanisms converge to a common apoptosis execution pathway. One of the first biochemical signs of apoptosis is the disruption of the plasma membrane phospholipid asymmetry as phosphatidylserine “flips out” to the outer layer of the membrane [9].

These changes can be recognized by macrophages and neighboring cells, and the cells committed to apoptosis are quickly engulfed [9]. Rapid clearance of apoptotic cells is very important and can also explain why apoptosis was not recognized by researchers for a long time. The membrane changes can be detected using fluorescent-labeled Annexin-V protein or Merocyanin 540, which selectively binds phosphatidylserine on the cell surface [10].

In parallel, the pH in the cytoplasm becomes acidic and the mitochondria lose their membrane depolarization, which can be analyzed by mitochondrial fluorescent dyes, e.g., DiOC<sub>6</sub>, JC-1 and Rhodamine 123 [11]. Subsequently, cytochrome-C and dATP are released from the mitochondria leading to apoptosome formation and activation of effector caspases [12].

Caspases are considered as initiators and central executioners of apoptosis [13]. 12 caspases are known in mammalian cells, but these proteins are phylogenetically conserved in the whole animal kingdom. These cysteine proteases cleave their substrates after aspartate residues. On the other hand, the residue before the aspartate determines the substrate affinity of different caspases. Caspases are synthesized as inactive proenzymes that are proteolytically processed to form an active tetrameric complex composed of two heterodimeric subunits of approx. 10 kD and 20 kD,

respectively. Conformational changes can induce autoproteolysis and activation of initiator caspases, i.e., caspase-8, -9 and -10. The subsequent proteolytic activation of effector caspases (caspase-3, -6 and -7) proceeds into a “caspase cascade” [14]. Caspases have several target proteins involved in genomic function, such as the repair enzymes PARP and DNA-PK; regulators of the cell cycle, e.g., RB, MDM-2, MEKK and PKC, and proteins of the nucleus and cytoskeleton, e.g., lamins, fodrins, gas2 and gelsolin [4]. The cleavage of structural proteins contributes to the morphological changes described above. The activity of caspases can be inhibited by synthetic peptide inhibitors. The proteolytic activation of caspases can be detected by western blot techniques or by fluorometric assays with synthetic substrates.

The caspase dependent cleavage of ICAD (inhibitor of the caspase-activated Dnase) allows the activation of CAD (caspase-activated Dnase) [15] and the cleavage of DNA initially into large (50-300 kb) and subsequently into small oligonucleosomal (~200 bp) fragments [16]. This DNA fragmentation is one of the major biochemical features of apoptosis that can be detected by gel electrophoresis (DNA “laddering”), TUNEL assays or sub-G1 reactions detected by flow cytometric methods extensively used in the present studies. However, in some cell lines, apoptosis can occur without DNA fragmentation. Furthermore, genomic fragmentation in non-apoptotic cells has been reported [17]. Apoptosis inducing factor (AIF) with endonuclease G can induce chromatin condensation and high molecular weight (50 kb) DNA fragmentation in the absence of CAD activity [18]. Apoptosis can be both dependent and independent on new protein synthesis [19,20].

The loss of cell membrane integrity is a relatively late event in the apoptotic process (also referred to as secondary necrosis) in contrast with necrosis, wherein the uptake of vital dyes (propidium iodide, trypan blue) precedes the random fragmentation of genomic DNA. It is common mistake to describe cell death as necrosis only based on the uptake of vital dyes at the end of a long *in vitro* experiment.

### **THE “INTRINSIC” MITOCHONDRIAL PATHWAY**

In most cases of cell death, the „point of no return” is permeabilization of the outer mitochondrial membrane leading to the release of toxic proteins [21] (Fig. 1). Membrane permeability is controlled by pro-apoptotic (e.g. BAX, BAK, BAD, BID, BIM, BMF, NOXA) and anti-apoptotic (e.g. BCL-2, BCL-XL) members of the BCL-2 family, inducing or preventing heterodimerization of pro-apoptotic members. Members of the BCL-2 family are located in the membranes of different cell organelles (i.e., mitochondria, endoplasmic reticulum, nucleus). Some of these proteins (e.g. BID, BAX, BAD) stay in the cytoplasm and a signal is required for translocation into a membrane, usually into the outer membrane of mitochondria. All family members contain at least one of the BCL-2-homologous domains (BH1-BH4). BH3 is responsible for the anti- or pro-apoptotic behavior, and certain pro-apoptotic members contain BH3 domains only. Functionally, VDAC (voltage dependent anionic channel) and ANT (adenine nucleotide translocator) are active participants of the control of permeability [22].

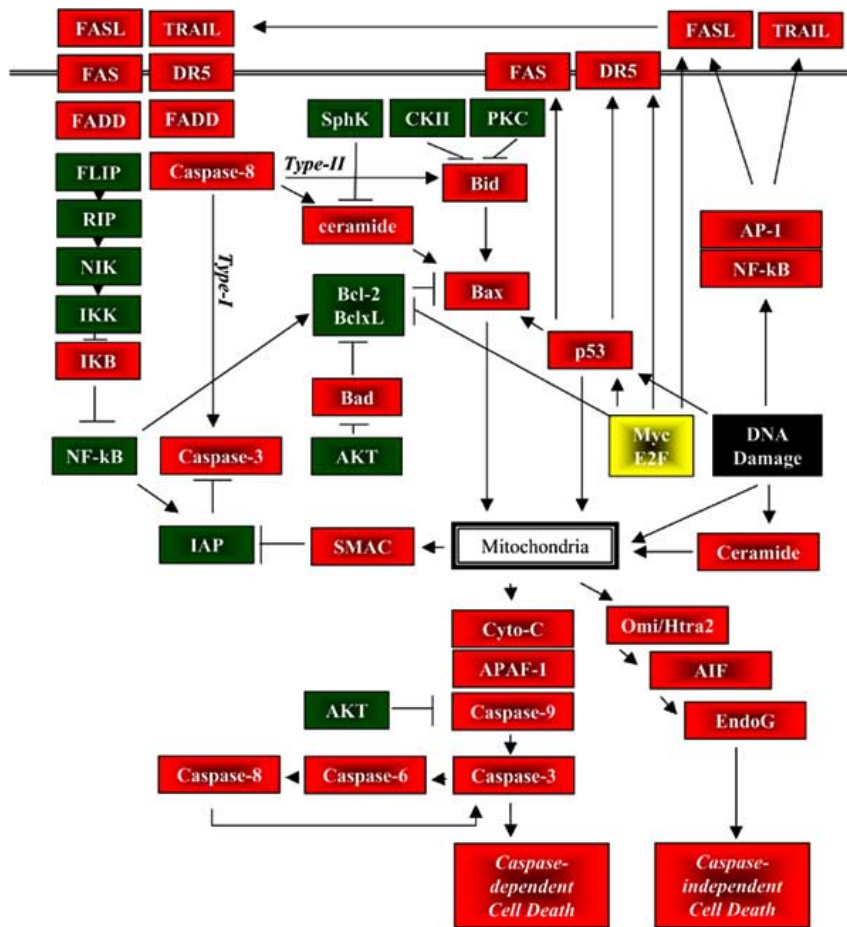


Fig. (1). The central regulation of cell death by multiple pathways.

Permeabilization can also result from the opening of a permeability transition pore in the inner mitochondrial membrane, which allows the accumulation of water and small molecules (up to 1.5 kDa), leading to the swelling of the intermembrane space and the rupture of the outer membrane. Among the released proteins, cytochrome-C, an important component of the respiratory chain, forms a complex (“apoptosome”) with ATP, APAF-1 (apoptotic protease activating factor 1) and caspase-9 [23, 24].

Other released proteins, Smac/DIABLO and OMI/Htra2 can inhibit the IAP proteins and thus facilitate the caspase cascade to direct activation. Through its protease activity, OMI/Htra2 can function also as an effector in the necrosis-like apoptosis [23, 24].

Endonuclease G can also contribute to both caspase-dependent and -independent cell death. Normally, apoptosis inducing factor (AIF) functions as an oxidoreductase in the intermembrane space, but becomes an active cell killer when released to the cytosol. It is translocated to the nucleus, and promotes – probably together with endonuclease G – chromatin condensation and high molecular weight (50 kb) DNA fragmentation [18].

**THE “EXTRINSIC” DEATH RECEPTOR PATHWAY**

Cells of higher metazoans activate their self-destructive mechanisms in response not only to environmental stress,

internal damage or lack of survival signals but also to the “instruction” of specific death signals [25, 26]. Death receptors are cell surface receptors that transmit apoptosis signals initiated by their specific “death ligands” (Fig. 1). Death receptors belong to the TNF receptor superfamily, which is defined by homologous cysteine-rich extracellular domains. There are six members of this family (TNFR1, FAS/CD95, DR4 (TRAIL-R1), DR5 (TRAIL-R2), DR3 (TRAMP), DR6). All of them contain an additional cytoplasmic “death domain”. These death domains enable the receptors to deliver apoptotic signals in sensitive cells upon binding to a specific ligand. Death ligands (TNF, FasL/CD95L, TRAIL/APO2L, DR3L) are type II transmembrane glycoproteins with the C-terminus displayed on the outside of the cell, a retained transmembrane region and cytoplasmic tails. Death ligands form oligomers, most often trimers, which are important for the ability to crosslink receptors. Proteolytic processing is a common posttranslational feature of death ligands. Death ligands bind and oligomerize their specific receptors. Several proteins have been identified that bind to the intracellular death domain of oligomerized death receptors to form the death inducing/signaling complex (DISC). The death domain signaling molecule, FADD [27, 28] recruit procaspase-8 molecules to the DISC [29, 30]. Inhibitory factors of the DISC that may be expressed include c-FLIP (FLICE-inhibitory protein) and FAP-1 (a Fas-associated phosphatase) [31].

In death receptor-mediated apoptosis, two main caspase-dependent signaling pathways have been demonstrated [32, 33]. In type I, the proposed ligation of death receptors leads to a strong caspase-8 activation at the DISC, which directly activates other caspases, including caspase-3, in the absence of mitochondrial involvement. In type II death receptor-mediated cell death, only small amounts of DISC are formed resulting in the activation of small amounts of caspase-8. Caspase-8 cleaves the cytosolic substrate Bid, and the proteolytically modified Bid induces conformational changes in Bax, which leads to pore formation in the mitochondrial membrane. This process can be blocked by an anti-apoptotic member of the Bcl-2 family [34]. Release of mitochondrial cytochrome-C and dATP triggers "apoptosome" formation from several caspase-9 molecules and the adapter molecule APAF-1. In the apoptosome, caspase-9 undergoes autoactivation and further activates the effectors caspases-3, -6 and -7 [34]. The active caspase-6 can activate further caspase-8 molecules and the apoptotic signal of death receptors can be further amplified [33, 34]. Release of Smac/DIABLO in turn suspend the inhibition of caspase-3 by IAP proteins, which opens a way to the direct type I signal pathway.

Death receptors can also activate caspase-independent signal transduction pathways. The sphingosine-ceramide pathway is one of the most ancient cell signal mechanisms. Sphingosine is the product of ceramide hydrolysis catalyzed by ceramidases, and has been shown to be rapidly produced during TNF- $\alpha$ -mediated apoptosis in human neutrophils [35] and rat cardiomyocytes [36]. Sphingosine, as well as C<sub>2</sub>-ceramide, induces cytochrome-C release from mitochondria in a caspase-independent mechanism, leading to the activation of caspase-9 and effector caspases, and to the processing of the substrate Bid, in a Bcl-xL-sensitive manner [37]. Treatment with exogenous C<sub>2</sub>-ceramide also results in increased sphingosine levels, and both sphingosine and C<sub>2</sub>-ceramide induces apoptosis in Jurkat cells – which may be inhibited by the overexpression of Bcl-2 –, where the characteristics of cell death resembles Fas-induced apoptosis [37]. Ceramide-induced apoptosis has also been determined to be Bax dependent [38]. Thus, the ceramide pathway can link death receptors to mitochondrial depolarization *via* the activation of BAX in a caspase-independent manner [39]. In addition, caspase-independent cell death induced by death receptor activation *via* RIP also has been described [6].

Paradoxically, death receptors can activate survival signals, which attenuate their pro-death effect. Ligation of TNFR1, Fas and TRAIL receptors can induce the activation of NF- $\kappa$ B [40-46]. In the complex formed with Fas in CD3-activated human T lymphocytes, c-FLIP present in the DISC further recruits TRAF-1, TRAF-2, RIP and RAF-1 [44]. This leads to the activation of NF- $\kappa$ B and the Erk signaling pathways. RIP was also determined in the TNFR1 complex for downstream activation of NF- $\kappa$ B. Activation of NF- $\kappa$ B, as well as the protection of cells from TRAIL-induced apoptosis [32,33], can also upregulate the expression of anti-apoptotic genes including cIAP-1 and cIAP-2 [47], TRAF-1 and TRAF-2 [48], and c-FLIP [48, 49].

While type I caspase-dependent signaling pathway is inhibited by the IAP proteins and c-FLIP upregulated by NF-

kB, type II signaling is inhibited by the phosphorylation of Bid by PKC [50] and casein kinase II [51], which inhibits the proteolytic activation of Bid by caspase-8. The ceramide pathway is inhibited by the sphingosine kinase [52].

## TARGETING CELL DEATH SIGNALING IN CANCER USING THE SYNTHETIC LETHALITY CONCEPT

The fundamental change in our perception of the role of apoptosis in tumor biology came from a simple experiment [53]. Evan and co-workers cultured fibroblasts *in vitro* under serum deprived conditions. Under such conditions, cells were arrested in G1 cell cycle phase. However, this cell cycle block proved to be reversible, because cells started to grow again upon serum replacement. The expression of c-Myc decreased under low serum conditions and was induced in the presence of serum. In separate experiments, cells were transfected with a vector constitutively expressing the c-Myc oncogen. These cells grew faster in the presence but underwent *cell death* in the absence of serum. Thus, in the presence of unregulated, forced expression of the c-Myc oncogene, fibroblast survival is dependent on the presence of survival signals from serum components. Specifically, c-Myc over-expressing fibroblasts were rescued in serum free condition by the growth factors IGF-1 and PDGF [54].

In the normal tissue homeostasis cell number is mainly regulated by the availability of growth factors. Activation of growth factor receptors leads to the upregulation of proteins that initiate cell cycle progression, such as c-Myc, E2F-1 and AP-1. The intricate link between proliferation and apoptosis has been demonstrated by several groups since the original article of Evan and his co-workers [53, 54, 55, 56]. However, activation of anti-apoptotic pathways by the same growth factors ensures the survival of cells responding to physiological growth signals.

In their landmark review article "The Hallmarks of Cancer" [57], Hanahan and Weinberg described cancer as a combination of six hallmarks: 1) self-sufficiency in growth signals (for example, H-RAS mutation), 2) insensitivity to anti-growth signals (for example, loss of RB expression or TGF-beta insensitivity), 3) evasion of apoptosis (for example, production IGF survival factors), 4) limitless replicative potential (for example turning on telomerase), 5) sustained angiogenesis (for example, production of VEGF inducers), and 6) tissue invasion and metastasis (for example, inactivation of E-cadherin). In their model, these steps can occur in different order in different tumor types. We would like to offer a slightly different model in this review. We will argue that the evasion of apoptosis is an obligatory condition of the other five. In other words, all steps of tumor progression involve a molecular strategy for cell death inhibition. This also means that cancer cells are not more *resistant* to pro-apoptotic stimuli but more *dependent* on survival strategies than normal cells. Certain survival strategies can inhibit cell death caused by only one "hallmark", others can cover many oncogenesis related pro-apoptotic stress. Identification and targeting of these tumor type specific molecular survival strategies is an important objective in signal transduction therapies. Tumors pay the

price by risking apoptosis at each stage of tumor progression. Our goal is to exploit these potentially lethal mechanisms to induce tumor specific cell death.

The theoretical basis of using synthetic lethality based on the pro-apoptotic activity of c-Myc was formulated by Green and Evan in their review article “c-Myc: A Matter of Life and Death” [55]. It was suggested that cancers arise from the rare simultaneous acquisition of the two cooperating conditions that permit cell expansion: deregulated cell proliferation and suppressed apoptosis. Although cancer is more than just a matter of cell proliferation and apoptosis [57]. This new model of cancer biology implicates that unleashing apoptosis can activate cancer cell specific cell death, which justify the optimistic note of the authors: “Now, we want antibiotics for cancer.”

Based on this theoretical background, synthetic lethality as a concept in the molecular pharmacology of cancer research has been formulated and recently reviewed [58]. In brief, the concept comes from yeast genetics. Gene mutations are considered synthetic lethal if they induce cell death only in combination with another dysfunctional gene. Cancer is a result of gene mutations or false signal transduction pathways, which provide an opportunity to find lethal “partner” gene targets. These targets are only lethal in the presence of preexisting tumor-specific genetic and phenotypic alterations. In cancer, the basis for exploiting a gene as a cancer specific synthetic lethal partner may be both a loss and a gain of function.

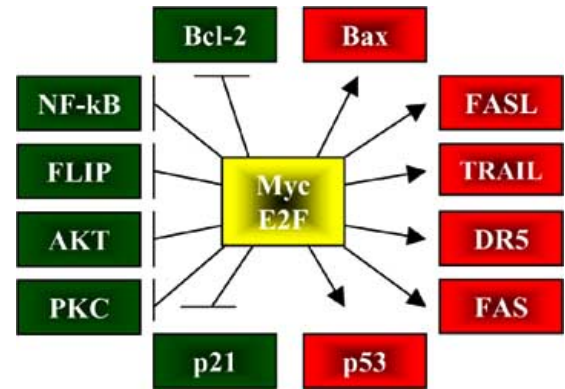
Often, these targets are the tumor specific survival strategies themselves. For example, upregulated or mutant anti-cell death signal proteins. Inhibition of these is the “specific targeting”. But, in theory, physiological survival proteins can become lethal targets in the background of tumor progression-related, pathological signaling. This drug development strategy is the “contextual targeting”.

### SELF-SUFFICIENCY IN GROWTH SIGNALS AND CELL DEATH

The classic example for the activation of autonomous growth signal is the direct activation of the c-Myc oncogene by chromosomal translocation, amplification or overexpression in many types of human cancer [59-61]. c-Myc is a “master” oncogene, which alone can contribute to many hallmarks of cancer. Besides the strong induction of proliferation (Hallmark #1), c-Myc induces the expression of VEGF (Hallmark #5) and downregulates the expression of E-cadherin (Hallmark #6). c-Myc is also a strong inducer of cell death *via* induction of several pro-apoptotic signal transduction mechanisms [55] (Fig. 1 and 2). Experimental proof for the ability of c-Myc to induce a complex tumor phenotype in the presence of powerful apoptosis inhibition came from experiments with transgenic mice with inducible c-Myc activity and Bcl-2 overexpression [60,61].

#### Activation of the “Intrinsic” Apoptotic Pathway by c-Myc

Deregulated activation of c-Myc requires the continuous stimulation of survival factors or pathologic disruptions of apoptotic signal transduction pathways induced by c-Myc. The most important of these pathways was demonstrated by



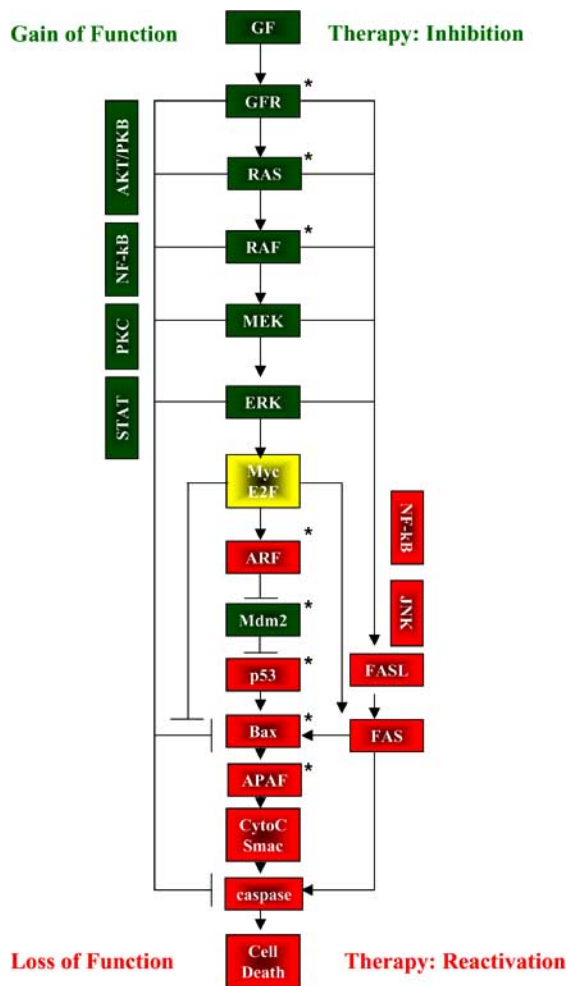
**Fig. (2).** c-Myc oncogene activates pro-apoptotic and inhibits anti-apoptotic proteins.

Cleveland and his group [62, 63] (Fig. 3). B cell lymphomas developed in c-Myc overexpressing transgenic mice were analyzed for secondary genetic alterations. It was found that ~25% of tumors harbored p53 mutations, another ~25% had overexpressed Mdm2, and a third ~25% showed a loss of ARF expression by hypermethylation. However, none of these alterations occurred simultaneously within the same tumor. For the remaining ~25% of tumors, other mechanisms were identified, such as the mutations of Bax [64]. Bax is a pro-apoptotic member of the Bcl-2 family. Upon activation by p53, Bax translocates into the mitochondria and triggers MOMP (mitochondrial outer membrane pore) opening and the release of cytochrome-C, AIF, smac/DIABLO, EndoG and omi/HTRA2. c-Myc directly represses the expression of the anti-apoptotic Bcl-2 and BclxL, which further shifts the balance toward Bax [65]. By putting together these experimental data, the c-Myc induced apoptotic pathway can be delineated. c-Myc activates p19/ARF and p19/ARF sequesters Mdm2 into the nucleolus. This leads to a reduced ubiquitination of p53 by Mdm2 and to a subsequent accumulation of p53. In turn, p53 upregulates Bax and downregulates Bcl-2, which directly activates the apoptotic machinery. Indeed, the disruption of this pathway at one point (p53 and Bax mutations, p19/ARF hypermethylation, Mdm2 amplifications) is the most common molecular feature of human cancers. Experiments with c-Myc transgenic mice also indicated that molecular strategies blocking individual pathways do not occur redundantly in one cancer cell. For example, in the presence of a p53 mutation, there is little selection pressure to induce the overexpression of Mdm2 or selection for Bax mutations. There is evidence for this rule also in human cancers. For example, Mdm2 amplification and p53 mutations are mutually exclusive in sarcomas [66, 67].

This main apoptotic signaling “highway” leads to the activation of the mitochondrial, “intrinsic” apoptotic signal transduction pathway. However, c-Myc can also activate the “extrinsic” death receptor induced cell death pathways (Figs. 1,2 and 3).

#### Activation of the “Extrinsic” Apoptotic Pathway by c-Myc

The strongest evidence for the connection between c-Myc and death receptor signaling has been indicated by the



**Fig. (3).** A linear signal transduction pathway leads from the proliferation signals toward cell death. Genetic or epigenetic alterations of genes involved in the signaling cascade are frequent in human tumors.

observation that c-Myc upregulates both Fas and FasL, and that neutralizing antibodies to the Fas ligand inhibit cell death when added to serum deprived cells overexpressing c-Myc [68]. Further, mouse embryonal fibroblasts from *lpr* or *gld* mice (defective in the FAS/CD95 or FASL/CD95L genes, respectively) are resistant to c-Myc-induced cell death [69]. FasL also appears to be a transcriptional target of c-Myc in some cell types [70]. Finally, overexpression of the dominant negative form of FADD also inhibits apoptosis induced by c-Myc [70]. In contrast, FADD knock out cells retain their sensitivity [71].

Connection between the two pathways is also supported by the dependence of Fas and TNF- $\alpha$ -induced apoptosis on the expression of c-Myc [70]. Sensitization by c-Myc is not attributable to increased receptor expression [70]. However, c-Myc may increase the level of cell surface Fas by activating p53-mediated expression and trafficking to the cell surface. At the level of the DISC, c-Myc can inhibit the expression of c-FLIP, an inhibitor of death receptor-induced apoptosis [72]. c-Myc inhibits the transcriptional activity of

NF $\kappa$ B induced by TNF- $\alpha$ . As a consequence, the anti-apoptotic protein A1 is not upregulated and cannot inhibit TNF- $\alpha$  induced cell death [73]. *In vivo*, the loss of FAS/CD95 expression in colon carcinoma compared to the abundant expression in the normal colon mucosa is a striking cancer related phenotype [74]. At least in a subset of colon carcinomas, hypermethylation of the promoter and the p53 enhancer region of FAS/CD95 gene seems to be responsible for this phenomenon [75]. Mutant RAS has been found to upregulate the expression of DNA methyl transferase [76]. The loss of APC leads to the deregulation of the WNT/Beta-catenine signal transduction pathway and to the subsequent upregulation of c-Myc in transformed colonic epithelium [77]. However, further progression would be self-limited by the increased apoptosis by c-Myc when RAS mutation occurs, which results in the downregulation of FAS/CD95 expression. This model also indicates that DNA methyl transferase is a synthetic lethal target in this type of colon carcinoma.

c-Myc upregulates another death receptor, DR5, as well as its specific ligand, TRAIL/APO2L [78]. However, it appears that there is no obligate selective pressure to develop survival mechanisms to block TRAIL-induced apoptosis during tumor progression. It is possible that TRAIL is released from tumor cells in an inactive form. Nevertheless, activation of DR5 is a proven synthetic lethal partner of the over-expressed c-Myc oncogen [78].

### GROWTH FACTOR RECEPTOR SIGNALING PATHWAY AND CELL DEATH

Under physiological conditions, cell proliferation is induced by growth factors. Growth factor receptor activation ultimately leads to the upregulation of c-Myc. However, the pro-apoptotic signals generated by c-Myc are suppressed by a parallel activation of survival signals by the same growth factor signaling pathway (Fig. 3). Oncogenic activation of growth receptors increase sensitivity to cell death. For example, oncogenic RAS sensitize cells to TRAIL-induced apoptosis *via* MEK [80, 81]. However, due to the activation of survival mechanisms, growth factor receptors and their downstream signal-transduction pathways can serve as “synthetic lethal” partners of autonom c-Myc activation.

The strict molecular order in growth factor receptor signaling is proven by the mutually exclusive presence of EGFR or RAS mutations in lung adenocarcinomas [82] or RAS and BRAF mutations in colon cancer [83] (Fig. 3).

The obvious cell-death suppressing molecular partners of c-Myc are the growth factor receptors themselves. The suppression of c-Myc-induced apoptosis by IGF-1 and PDGF has been demonstrated *in vitro* [54] and by EGF *in vivo* [60].

Downstream of the growth factor receptors, the common signal transduction protein is RAS. Genetic alterations in the RAS oncogene family are most frequently detected in cancer [84]. RAS genes were first identified as homologues to the viral oncogenes of transforming retroviruses [84, 85].

The cooperation between RAS and c-Myc during transformation is the classic model of multi-step oncogenesis

[84]. One aspect of this cooperation is the synergistic induction of cell proliferation. The other and probably even more important aspect is the inhibition of c-Myc-induced cell death by activated RAS. RAS activated by growth factor receptors or by activating mutations can also provide the necessary protection from c-Myc-induced apoptosis by activating survival signal transduction pathways. Kinases of these pathways are the obvious and popular targets of signal transduction therapies.

One of these kinase pathways activated by growth factor receptors and RAS is the RAF/MEK/ERK pathway [84]. MEK activates ERK kinases, which leads to an increased half life of phosphorylated c-Myc and to the induction of c-Jun and c-Fos, which results in the formation of the AP-1 transcription complex. AP-1-like sequences are present in the cyclin D1 promoter. Since the more well-known function of this pathway is the induction of cell cycle progression, researchers consider these proteins as targets to inhibit cell proliferation and not as a potential strategy to induce tumor specific cell death. In cells transformed by c-Myc and activated by RAS, inhibition of MEK induces apoptosis in a c-Myc activity-dependent manner, suggesting that increased MEK activity is at least one obligatory mechanism to maintain viability in c-Myc/RAS-transformed cells [86]. The ERK pathway plays a central role in regulating mammalian cell growth by relaying extracellular signals from ligand-bound cell surface tyrosine kinase growth factor receptors, such as the epidermal growth factor receptor (EGFR), HER-2, vascular EGFR (VEGFR), platelet-derived growth factor receptor (PDGFR), and MET, starting with the activation of RAS. The next critical step in this pathway involves activation of a family of serine threonine kinases known as RAF kinases. RAF kinases then phosphorylate and activate MEK1/2, which subsequently phosphorylates and activates ERK1/2 [87].

RAF-1 directly phosphorylates and inactivates BAD, phosphorylates and coimmunoprecipitates with Bcl-2, and also regulates the expression of BAG-1 and BAD in BCR-ABL-expressing cells, which may balance the pro-apoptotic signals generated as a consequence of the upregulated proliferative signals induced by Bcr-Abl [88, 89]. Mutations in Raf-1 have not been detected in human cancers. Somatic mutations of B-Raf are found in 60% of malignant melanomas and occur with moderate to high frequency in papillary thyroid carcinomas, colorectal, and ovarian cancers, strongly implicating the activation of B-Raf in tumorigenesis [90]. MEK-1 is a common mechanism for survival signaling by activated RAF. However, the role of the PI3K effector AKT is also demonstrated by the inhibitory effect of a dominant negative AKT mutant on RAF-1-induced cell survival. Moreover, a constitutively active form of AKT synergizes with RAF-1 in apoptosis suppression. These data strongly suggest a RAF effector pathway for cell survival that is mediated by MEK and AKT [91]. Caspase-9 is inhibited by phosphorylation at Thr 125, a conserved MAPK consensus site targeted by ERK2 *in vitro*, in a MEK-dependent manner in cells stimulated with epidermal growth factor (EGF) [92]. In keratinocytes, EGFR-dependent MEK activity contributes to both Bcl-x(L) expression and survival.

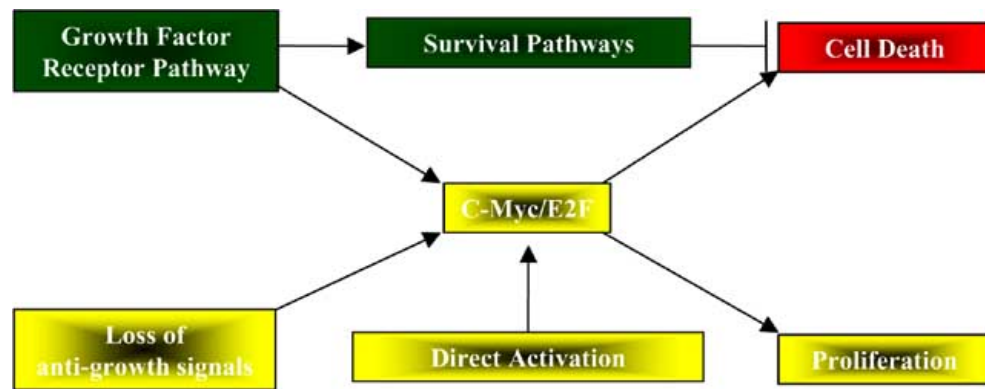
Other signaling pathways (i.e. phosphatidylinositol 3-kinase/AKT and phospholipase C gamma/protein kinase C alpha) are obligatory to keratinocyte survival but not to Bcl-x(L) expression [93]. Inhibitors of MEK/ERK signaling can lead to the upregulation of p53 and BAX and the release of the AIF from the mitochondria, which initiates caspase-independent cell death in breast cancer. Thus, MEK inhibitors might also be effective in the presence of a defective caspase cascade [94]. In melanoma cells, the 90-kDa ribosomal S6 kinase (RSK), a downstream effector in the MAPK signaling cascade, phosphorylates and inactivates Bad, a Bcl-2 homology 3-only pro-apoptotic protein. Constitutively activated RSK mutants inhibit apoptosis induced by the inhibition of MEK. Thus, the MEK/ERK/RSK pathway may present a potentially selective therapeutic target for the treatment of melanomas [95, 96].

### INSENSITIVITY TO ANTI-GROWTH SIGNALS AND CELL DEATH

The other hallmark of cancer is the loss of sensitivity to anti-growth signals. This leads to increased cell growth but also to increased susceptibility to cell death (Fig. 4). One classic example for the inability to respond to antigrowth signals is the loss of the Rb tumor suppressor gene [97]. After phosphorylation of Rb by Cyclin-D/CK4-6 complexes, E2F transcription factors (E2F1, E2F2, E2F3a) are released from Rb, which leads to the upregulation of genes essential for DNA replication and proliferation [98]. CDKs are inhibited by the CKIs p21, p27 and p16(INK4a). The loss of Rb, the overexpression of D-type cyclins and the loss of the CKI p16 are frequent events in oncogenesis [99]. However, there is plenty of evidence for the pro-apoptotic properties of E2F1 [99, 100]. E2F1 induces the expression of p19/Arf [101]. Activation of p19/ARF activates the ARF-MDM2-P53 pathway described above. However, E2F1 can activate cell death independently of p53 *via* upregulation of p73 [102] and Apaf1, an important adapter molecule of the apoptosome [103].

DNA damage activates the ATM/ATR kinases, and this leads to a direct activation of E2F1 (*via* phosphorylation) and a direct (or indirect *via* the Chk2 kinases) activation of p53 by the inhibition of Mdm2-mediated ubiquitination [104]. E2F1 and p53 cooperate in the induction of cell death in response to DNA damage. Thus inability to sequester E2F1 directs the signal transduction of p53 toward apoptosis instead of p21-mediated cell cycle arrest. p21/WAF1 (upregulated by p53) can also directly inhibit caspase-3 [105] and has been also shown to inhibit the initiator caspases (caspase-8 and caspase-10) in TRAIL induced apoptosis [106]. Thus, inability to upregulate p21 renders tumor cells more sensitive to both internal and death receptor-mediated pro-apoptotic signals.

The classic growth inhibitor cytokine TGF-beta induces cell cycle arrest in normal lymphocytes. In contrast, TGF-beta induces apoptosis in malignant lymphomas resistant to its growth inhibitory effect [107, 108]. In addition, TGF-beta has been shown to induce apoptosis in hepatoma cells, which is inhibited by the overexpression of Rb [109].



**Fig. (4).** A model to predict response to signal transduction therapies. Growth factor receptor pathway means the Receptor-RAS-RAF-MEK pathway. Survival signal are (AKT, PKC, NFkB etc.).

### TUMOR PROGRESSION BEYOND INCREASED CELL PROLIFERATION AND CELL DEATH

After the acquirement the first two hallmarks of cancer, cancer cells are challenged by new pro-death circumstances. All the remaining hallmarks (limitless replicative potential, sustained angiogenesis, tissue invasion/metastasis and genomic instability) increase cancer cell sensitivity to cell death and provide an opportunity to identify synthetic lethal targets for the induction of tumor cell specific cell death.

During the first phase of oncogenesis, increased proliferation in the absence of telomerase expression leads to the erosion of telomeres [110, 111]. Shortened telomeres activate the ATM-ARF-P53-P21 pathway to induce a permanent cell cycle block (replicative senescence) or apoptosis. Suppression or dysfunction of this pathway is required to surpass this phase. Further cell cycles lead to a marked chromosome instability, which results in the death of the majority of tumor cells (crisis). New clones expressing the telomerase enzyme emerge as survivors. However, the survival of cancer cells will depend on telomerase activity, which is therefore an obvious target of cancer therapy [110, 111].

As tumors grow, the available nutrients and oxygen decreases. Hypoxia induces p53 *via* the ATR/ATM-ARF-MDM2 pathway [112]. Physiologically, p53 upregulates p21 and decreases cell proliferation to adapt to tissue oxygenation. In the presence of excess E2F-1, p53 induces apoptosis. The complete dysfunctionality of this pathway leads to extreme anoxia and necrosis. Paradoxically, evasion of apoptosis by high activity of the important survival signal Akt/PKB can be disadvantageous for the survival of cancer cells under these conditions. Akt/PKB activates mTOR, which suppresses autophagy. The inability to activate autophagy and consume non-vital macromolecules and cell organelles to maintain viability renders tumor cells dependent on uninterrupted supply of nutrients [113]. HIF-1-mediated upregulation of VEGF and the subsequent *de novo* angiogenesis improve the situation, but neovascularisation is often slower than tumor growth, which maintains hypoxia in the tumor [112].

One of the most elegant experimental demonstration of the apoptotic barrier inhibiting tissue invasion was provided by Pelengaris *et al.* [60]. Activation of c-Myc in the epidermal keratinocytes in mice induced tissue growth,

which resembled a tumor by microscopic pathomorphological evaluation. However, cells at an attempt to invade underwent apoptosis at a certain distance from the basal membrane. Thus, in the absence of cell-cell contacts, matrix anchor and overexpression of local growth factors, c-Myc induced cell death. Next, epithelial carcinoma cells entering the blood stream are challenged by a special form of cell death referred to as “anoikis” or “anoicosis” [114]. Anchorage-independent growth is a hallmark of oncogenic transformation. Epithelial cancer cells require special survival mechanisms to maintain viability, therefore provide an opportunity for selective cancer therapy. For example, the inhibition of MEK or the simultaneous inhibition of ERK and mTOR-p70 (S6K) induces anoicosis in K-RAS-transformed rat fibroblasts and breast cancer cell lines [115].

Genome instability is considered the enabling feature of cancer cells because increased mutation rate accelerates tumor progression. However, accumulation of mutant proteins increases the dependence on HSP chaperons to maintain proper folding. Non-functional mutants need to be quickly degraded by the proteasome. HSP inhibitors and proteasome inhibitors are being tested in clinical trials or are already applied in cancer treatment [116].

Increased genomic instability is accompanied by decreased cell cycle check point regulation. The consequence is the inability of the cell to arrest in the cell cycle for sufficient time for repair and proceeds to mitosis with major DNA damage. The result can be a special form of cell death, the mitotic catastrophe (see later in more detail).

Hanahan and Weinberg did not mention evasion of the immune response as a cancer hallmark. However, certain tumor types can be “attacked” by FasL and granzyme-B expressing cytotoxic T cells or TRAIL-expressing NK cells [117, 118]. Evasion of death receptor induced apoptosis or inhibition of caspase-activation by granzyme can be a “matter of life or death”.

### TUMOR-SPECIFIC CELL DEATH WITH SIGNAL TRANSDUCTION THERAPY

Typically, cell death (apoptosis) signal transduction therapies are considered to include three major approaches: 1) inhibition of survival signals; 2) restoration of cell death signaling; and 3) direct activation of cell death pathways activated by death ligands or pro-apoptotic proteins.











NIMA	=	Never in mitosis gene A9
NNK	=	Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NRAS	=	Neuroblastoma rat sarcoma viral oncogene homolog
NSCLC	=	Non-small-cell lung cancer
PARP	=	Poly-ADP-ribose-polymerase
PCD	=	Programmed cell death
PCR	=	Polymerase chain reaction
PDGFR	=	Platelet-derived growth factor receptor
PI3K	=	Phosphoinositide 3-kinase
PKC	=	Protein kinase C
PLK	=	Polo-like kinase
RAF	=	v-raf murine sarcoma viral oncogene homolog
RAS	=	Rat sarcoma viral oncogene homolog
RIP	=	Receptor interacting protein
SMAC	=	Second mitochondria derived activator of caspases
SSCP	=	Single strand conformational polymorphism
SphK	=	Sphingosine kinase
STAT	=	Signal transducer and activator of transcription
TGFR	=	Transforming growth factor receptor
TGF- $\alpha$	=	Transforming growth factor – alfa
TK	=	Tyrosine kinase
TKR	=	Tyrosine kinase receptor
TNF	=	Tumor necrosis factor
TRAIL	=	TNF-alfa related apoptosis inducing ligand
VEGF	=	vascular endothelial growth factor

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Received: 06 October, 2005

Accepted: 10 October, 2005