Introduction

The removal of functionally dispensable, infected or potentially neoplastic cells is challenging and needs highly complicated and sensitive molecular mechanisms. Therefore, removal of unwanted cells is regulated by specialized and characteristically distinct programmed cell death (PCD) pathways, signifying their fundamental roles in homeostasis, host defense against pathogens, widely ranging pathologies and cancers.

Apoptosis is triggered through the activation of the apoptotic caspases and can operate either via an intrinsic or an extrinsic pathway. The intrinsic pathway is "switched on" by mitochondrial damage. Consequently, cytochrome c gets released from the mitochondrion into the cytoplasm, combines with APAF (apoptotic protease activating factor) and a pro-caspase-9 to form a signalosome known as "apoptosome" that activates caspase-9. Functionally active caspase-9 proteolytically processes and activates caspase-3, leading to cell death by cleavage of different cellular endogenous substrates.

Whereas, extrinsic pathway is activated by TRAIL, TNF, FasL which bind to death receptors (TRAIL-R, TNFR, Fas). Ligand-receptor interaction caused the oligomerization of these receptors leading to the recruitment and activation of caspase-8, which cleaved procaspase-3 to mediate apoptosis (1,2,3). Additionally, caspase-8 cleaved Bid and generated a truncated fragment, tBid. tBid translocated to the mitochondria and formed Bax/Bak pores on its surface, potentiated the release of cytochrome c and activated apoptosis (shown in figure 1). IAP (Inhibitor of apoptosis) proteins effectively blocked apoptotic death by inhibition of the activities of the caspases that executed programmed cell death. IAP protein family includes different members that have been extensively characterized. Structural studies have shown that one, two or three BIR (baculovirus IAP repeat) domains are critical for anti-apop-
totic activities. Mostly IAPs have a carboxy-terminal RING domain. This domain serves as a ubiquitin ligase. Counterbalancing of IAPs is very necessary and therefore, during apoptotic death, SMAC/DIABLO (Second mitochondria-derived activator of caspase), an IAP antagonist gets released from the mitochondria that enhances the activation of caspase within the apoptosome molecular machinery. Apoptosome is formed by cytochrome c, APAF1 (apoptotic protease activating factor 1) and caspase-9. SMAC/DIABLO counteracted IAPs via four-residue IAP-binding motif present in its amino terminal region. IAPs mediated inhibition of caspase activity depends on the IAP involved. XIAP (X-linked IAP) inhibited caspase 3, caspase 7 and caspase 9 and showcased characteristic uniquely ability to inhibit caspases by direct binding. Series of studies have shown that BIR2 and BIR3 domains of XIAP critically regulated caspase activation. Upon activation of procaspase 9, both SMAC and XIAP compete in a mutually exclusive manner for binding to the newly exposed four residues at the amino terminal of the smaller p12 subunit of caspase 9. This mutually exclusive binding actually determined the fate of the cell in context of execution or inhibition of apoptosis.

This four-residue peptide provided pharmaceutical basis for the design of SMAC mimetics (small peptide mimetics) that duplicate the binding activity of SMAC protein to XIAP, c-IAP1 and c-IAP2. Interaction of SMAC mimetics with c-IAP1 and c-IAP2 caused conformational changes in these proteins. These changes stimulated the endogenous E3 ubiquitin ligase function of c-IAP1 and c-IAP2, which resulted in the autoubiquitination and degradation. Furthermore, SMAC mimetics prevented the binding of XIAP to caspase-3, -7, or -9. Although endogenous SMAC effectively targeted XIAP, c-IAP1 and c-IAP2 for degradation, however SMAC mimetics can further be engineered to have more specificity and efficacy.

TRAIL-mediated pathway has gained overwhelming appreciation because of its characteristically distinct ability to induce killing of the cancer cells (4,5,6). These remarkable properties captivated the attention of researchers and multiple aspects related to TRAIL-driven pathway were uncovered. TRAIL-mediated apoptotic effects were explored in almost all cancers but gradually different research-groups started to note resistance against TRAIL-driven pathway. Resistance against TRAIL was puzzling and scientists started to deeply analyze the underlying mechanisms which abrogated TRAIL-driven pathway. There has been an exponential growth in the list of high-impact publication which comprehensively analyze multiple facets of TRAIL-driven pathway in different cancers. Excitingly, experts worked hard to resolve the outstanding questions related to TRAIL resistance mechanisms. Accordingly, scientists started to unlock the mystery of underlying mechanisms which caused TRAIL-resistance. It was suggested that downregulation of death receptors, overexpression of anti-apoptotic proteins and inactivation of pro-apoptotic proteins played critical role in regulation of TRAIL-mediated transduction cascade. Identification of the positive and negative regulators of TRAIL-mediated apoptotic pathway impelled researchers to analyze multiple target proteins in this network.

We have partitioned this multi-component review into sub-sections. We have summarized recently published cutting-edge researches related to TRAIL-driven pathway:

**Recent breakthroughs in TRAIL-driven signaling: fresh from the pipeline**

Ground-breaking discoveries in the past few years have revolutionized our understanding related to TRAIL-driven pathway.

It has recently been convincingly revealed that TRAIL-resistant sub-populations protect and enclose TRAIL-hypersensitive cells, thus causing an increase in TRAIL-resistance (7). TRAIL-resistant layers were formed at the interface of quiescent and proliferating cells and lacked both DR4 and DR5. Deprivation of nutrients and oxygen triggered an increase in the levels of DR5 in TRAIL-hypersensitive cells present within the inner spheroid layers. Cyclo-oxygenase (COX-II) inhibitors evoked DR5 expression in spheroids, most likely resulting from increased endoplasmic reticulum stress and thus re-sensitizing TRAIL-resistant cell layers to treatments (7).

Tristetraprolin, an ARE-binding protein has a critical role in the modulation of mRNA stability of death receptors (8). DR4 mRNA contained three AREs and DR5 mRNA contained four AREs in 3'-UTR. Tristetraprolin physically associated with AREs in DR4 and DR5 and enhanced the decay of DR4/5 mRNAs. Tristetraprolin overexpression in colon cancer cells enhanced TRAIL resistance but downregulation of tristetraprolin increased TRAIL sensitivity via DR4/5 expression (8).

It has previously been reported that TRAIL-resistant breast cancer cells demonstrated notably higher levels of autophagosomes (9). However, the use of autophagy inhibitors drastically reduced the number of autophagosomes. TRAIL-resistant breast cancer cells contain higher basal levels of autophagosomes. It is exciting to note that DR4 and DR5 co-localize with LC3-II within autophagosomes and upon disruption of autophagosome formation, shuttle back to the cell surface (9).

Internalization of death receptors is highly complicated. Internalized receptors can be transported from endosomes to the lysosomes where they can undergo degradation. RALB is essentially involved in trafficking of the vesicles and activation of the autophagosome assembly (10). Intriguingly, this function might be contributory to the accumulation of DR5 in RALB knockdown cancer cells. Chloroquine (lysosomal inhibitor) induced an increase in DR5 levels. Also, RALB inhibition evidently enhanced chloroquine-induced DR5 upregulation. However, chloroquine-driven increase in DR5 expression was abrogated in the cancer cells which transiently overexpressed RALB. Importantly, RALB overexpression resulted in an increased co-localization of endogenous DR5 with LAMP1. TRAIL treatment potentiated the binding of RALB with the DISC. However, RALB inhibition using pharmacologic strategies or RNAi approaches potently increased TRAIL-induced cell death in colorectal cancer cells (10).

TRAIL dose-dependently enhanced nuclear accumulation of DR4 and DR5 in pancreatic cancer cells (11). Moreover, DR4 and DR5 translocated into chromatin-
rich fractions. Study also provided clues about exit of death receptors. Exportin-1/chromosome region maintenance 1-homolog (CRM-1) is best-studied exporter of most nuclear proteins. CRM1 interacted with DR5 more efficiently as compared to DR4 (11).

It is interesting to note that recent publications have provided clues about nuclear accumulation of death receptors.

**TRAIL-resistance mechanisms**

Development of resistance against TRAIL is a major obstacle. Confluence of information pinpointed to an ever-increasing list of proteins which played central role in making cancer cells resistant to TRAIL.

Protein O-GlcNAcylation is a hallmark post-translational modification that regulates different molecular mechanisms (12). O-GlcNAcylation enhanced TRAIL resistance, whereas O-GlcNAcylation inhibition effectively promoted TRA-8-induced apoptosis in TRAIL-resistant cancer cells. O-GlcNActransferase knockdown caused a notable increase in the efficacy of TRA-8 in mice xenografted with TRAIL-resistant cancer cells. Increased FADD binding to trimerized DR5 was noticed in the OGT silenced cancer cells. These findings clearly supported the concept that inhibition of O-GlcNAcylation promoted oligomerization of DR5 and FADD recruitment to form death inducing signaling complex (12).

CCR4-NOT transcription complex (CNOT), composed of 11 subunits has been shown to participate in multiple cellular processes (13). Moreover, CNOT2, one of the CCR4-NOT subunits played a central role in enhancing TRAIL-resistance. CNOT2 knockdown markedly increased TRAIL sensitivity in H1299 and A549 cells (13).

ROMO1 (Reactive oxygen species modulator-1) has also been shown to interfere with TRAIL-mediated apoptosis (14). TRAIL remarkably induced regression of tumors in mice xenografted with ROMO1-silenced HCT116 cancer cells. ROMO1 knockdown significantly lowered Bax ubiquitination by interfering with Parvin/Bax interaction (14).

EP300 gene encoded a histone acetyltransferase (p300) that regulated transcription via chromatin remodeling (15). P300 and CREBBP (CREB-binding protein) inhibition enhanced the expression levels of caspase-3, -7, -8 and 9 at the mRNA levels. Furthermore, downregulation of EP300 and CREBBP improved TRAIL-mediated apoptosis (15).

Protein phosphatase 2A (PP2A) played a critical role in TRAIL resistance (16). PP2A inhibitor LB-100 efficiently inhibited growth of the tumors in mice xenografted with MDA-468 breast cancer cells (16).

Methionine addiction is one of the hallmarks of cancer (17). Methionine restriction of methionine-addicted cancer cells increased DR5 expression. Methionine restriction increased tigatuzumab-induced activation of caspase and apoptotic death in BxPC-3 and MIA PaCa-2 cancer cells. Recombinant methioninase, a methionine-degrading enzyme is effective against different cancers. o-mETase and tigatuzumab markedly induced regression of tumors in mice xenografted with MIA PaCa-2 cancer cells (17).

Mixed lineage kinase domain-like (MLKL) also played a contributory role in the generation of extracellular and intraluminal vesicles (18). MLKL depletion heavily interfered with the intracellular trafficking of TRAIL/DR5 to degradative compartments. DR5 activation promoted rapid cleavage of substrates such as (AP2) adaptor protein 2. MLKL deficient cells revealed noteworthy increase in the cleavage of AP2 which clearly suggested that active TRAIL/DR complex promoted potent cleavage of proteins involved in clathrin-mediated endocytosis (18).

Tumor cells carrying truncated O-glycans are relatively resistant to TRAIL-induced apoptosis, while cells expressing extended O-glycans are more sensitive to TRAIL (19). DR4/5 carrying normal Sialyl-T antigen formed homo-oligomers that are essential for death signaling, while truncated O-glycans attenuated the formation of homo-oligmeric complexes in Jurkat and LOX cells and promoted the hetero-oligomeric complex formation between DR5 and DcRs in LOX cells (19). Collectively, the findings suggested that extended O-glycans promoted homo-oligomerization of DR4/DR5, which is essential for TRAIL-induced apoptosis. Truncated O-glycans prevented the homo-oligomerization between DR5 with DcR2 lacking the death domain, which attenuated the death signaling.

It has previously been reported that importin β1 transported DR5 to the nucleus (20). While, importin β1 knockdown upregulated cell surface expression of DR5 which resulted in an increased TRAIL sensitivity. Use of importin β1 inhibitors and agonistic anti-human DR5 (hDR5) antibody effectively reduced tumor growth in animal models (20).

JDP2 (Jun dimerization protein 2) is a bZip type transcriptional factor (21). Knockdown of JDP2 enhanced the expression of ATF4 target genes, including DR4 and DR5 in HeLa cells. Whereas, overexpression of JDP2 repressed ER stress-mediated transcriptional activation of DR5 which clearly suggested that JDP2 caused negative regulation of ATF4-mediated gene expression (21).

DR4 is O-GlcNAcylated at 424th serine residue in the death domain to transduce the death signals intracellularly (22). Data indicated that DR4 was not modified by O-GlcNAc in majority of the TRAIL-resistant cancer cell lines. Interestingly, promoting DR4 O-GlcNAcylation restored sensitivity of cancer cells to TRAIL. O-GlcNAcylation-defective DR4 neither formed DISC nor translocated to aggregated platforms for clustering of receptors (22).

IITZ-01, a lysosomotropic autophagy inhibitor enhanced TRAIL-mediated apoptotic death (23). Cbl (Casitas B-lineage lymphoma) contributed to DR5 ubiquitination and degradation. IITZ-01 protected death receptors from degradation by decreasing the stability of Cbl. IITZ-01 also potently enhanced USP9X-dependent degradation of survivin in cancer cells (23).

CUL7 ubiquitinated caspase-8 with non-K48-linked polyubiquitin chains at K215 (24). These polyubiquitin chains showcased a unique feature as they did not target the substrates for degradation by proteasomes (shown in figure 1). CUL7-mediated ubiquitination of caspase-8 and notably reduced cleavage of caspase-8 and activa-
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TRAIL markedly reduced tumor growth in mice inoculated with CUL7-silenced MDA-MB-231 cancer cells (24).

Glucosamine and TRAIL combinatorially suppressed the expression of c-FLIP, BCL-XL, Mcl-1 and XIAP and promoted the translocation of BAK to the mitochondrial outer membrane thereby facilitating release of the cytochrome c and SMAC (25).

HuP10 is present in the nucleus. However, excitin-gly, TRAIL not only induced nuclear exit of HuP10 but also promoted its entry into mitochondria (26). HuP10 is shuttled from nucleus to the cytoplasm through CRM1/Exportin-1. Moreover, caspase-3 also played instrumental role in nuclear exit of HuP10. Findings indicated that HuP10 did not move out of the nucleus in caspase-3 knockdown cancer cells. Additionally, it was shown that HuP10 increased caspase-3 activity in cytoplasm (26).

Targeting of anti-apoptotic proteins

Bromodomain and extraterminal (BET) proteins (BRD2, BRD3, BRD4) work as epigenetic readers and master transcriptional coactivators and are now recognized cancer therapeutic targets (27). BET degraders such as ZBC260 and dBET represent a novel class of BET inhibitors that degrade BET proteins. BET degraders effectively induced apoptosis in sensitive NSCLC cells and were accompanied by reduction of Mcl-1 and c-FLIP levels. ZBC260 and TRAIL combinatorially induced apoptosis primarily through degradation of Mcl-1 and c-FLIP (27).

Trametinib, a MEK1/2 inhibitor potently degraded Mcl-1 through the proteosomal pathway (28). GSK-3β phosphorylated Mcl-1 at S159 and promoted Mcl-1 degradation. FBW7 (F-box and WD repeat domain-contai-
Natural products mediated restoration of TRAIL pathway

TRAIL-resistance has sparked an interest to search for wide-ranging bioactive chemicals from natural sources to restore apoptotic death in resistant cancers. Therefore, with the landmark achievements related to disentangling complicated web of proteins in TRAIL-pathway, medicinal chemists and natural product researchers contributed by identifying different natural products having premium medicinal properties and excellent ability to sensitize resistant cancer cells to TRAIL. Consequently, starting from transcriptional upregulation of death receptors, natural products have been shown to effectively stimulate extrinsic and intrinsic pathways and pharmacologically target anti-apoptotic proteins in different cancer cell lines and preclinical animal models.

Calcium/calmodulin dependent protein kinase kinase β (CaMKKβ) is an upstream kinase of AMPK (AMP-activated protein kinase) (31). CaMKKβ activated AMPK which consequently induced an increase in the levels of USP51 (ubiquitin specific peptidase 51). Hispidulin has been shown to trigger the activation of CaMKKβ/AMPK signaling axis to enhance the stability of Bim by USP51 in Caki cells. Hispidulin and TRAIL combinatorially induced regression of tumors in mice inoculated with Caki cells (31).

Icarin stimulated the expression of DR4 and DR5 (32). Additionally, it was shown that Icarin triggered C/EBP homologous protein (CHOP) mediated upregulation of DR5. ERK activation was found to be necessary for the upregulation of death receptors. ERK inhibition markedly suppressed Icarin-induced expression of DR4 and DR5 (32).

Pseudolaric Acid B is a diterpenoid obtained from the root barks of Pseudolarix kaempferi Gordon tree. It has been noted to increase TRAIL-sensitivity (33). Intraperitoneal injections of ethanolic extracts of Pseudolarix kaempferi markedly reduced tumor growth in mice subcutaneously injected with HN22 cells (33).

NEO214 was chemically synthesized by covalent linkage of perillyl alcohol with rolipram using a carbamate bond (34). NEO214 was tested for efficacy against intracranial athymic tumor bearing mice and intracranial immunocompetent syngeneic mice. NEO214 effectively inhibited tumor growth in mice intracranially implanted with glioblastoma cells. To analyze the pharmacological properties of NEO214 in syngeneic immunocompetent models, GL261 mouse glioma cells were intracranially implanted into C57bl/6. To confirm whether or not NEO214 stimulated CHOP and DR5 in immunocompetent brain tumor models, GL261 mouse glioma cells were implanted into the frontal cortex of immunocompetent syngeneic mice. Results clearly suggested that NEO214 stimulated CHOP and DR5 (34).

Honokiol enhanced TRAIL-sensitivity by lowering the levels of c-FLIP and survivin (35). Detailed studies revealed that honokiol interfered with STAMBPL1-mediated stabilization of c-FLIP and survivin. Ectopic expression of STAMBPL1 interfered with the degradation of c-FLIP and survivin by honokiol (shown in figure 1). Whereas, levels of survivin and c-FLIP were noted to be reduced in STAMBPL1-silenced in Caki cells. STAM-

BPL1 directly interacted with c-FLIP and survivin and enhanced the stability of these proteins (35).

GALNT14 belongs to a large subfamily of glycosyltransferases. GALNT14-induced DR5 O-glycosylation and promoted TRAIL sensitivity of cancer cells (36). Oridonin dose- and time dependently induced an increase in the levels of GALNT-14 in Caki and A549 cells. Combinatorial treatment induced glycosylated-high molecular weight DR5 in A549 and Caki cells. Collectively, these findings supported the concept that DR5 glycosylation played a vital role in enhancing the TRAIL sensitivity by oridonin (36).

There is a rapid increase in the identification of natural products having remarkable ability to restore apoptosis in TRAIL-resistant cancers (37-47).

Biochemical modifications

Threonine 166 is an important phosphorylation site and studies have shown that Thr-166 phosphorylation is essential for ROS-mediated Lys-167 ubiquitination of c-FLIP protein (48). Therefore, it seems clear that generation of ROS acts as a trigger for post-translational modifications of c-FLIP. It will be paramount to analyze additional anti-apoptotic proteins tagged for degradation in oxidative stress-induced cancer cells.

HECTD3, an E3 ubiquitin ligase interacted with DED (death effector domains) of caspase-8 and ubiquitinated caspase-8 with K63-linked polyubiquitin chains (49). K63-linked polyubiquitin did not sort caspase-8 for degradation but considerably reduced activation of caspase-8 (49).

Membrane-associated RING-CH (MARCH) ubiquitin ligase family played critical role in ubiquitination of DR4. Ligase-dead MARCH-8 variant was unable to ubiquitinate DR4. Moreover, Lysine 273 was important for MARCH-8 mediated ubiquitination of DR4 (50).

KDM4A small-molecule inhibitor compound-4 (C-4) has been shown to stimulate expression of TRAIL and DR5 (51). Nuclear receptor co-repressor (NCoR)-HDAC complexes transcriptionally downregulated TRAIL and DR5. C-4 induced dissociation of KDM4A and NCoR-HDAC complexes and promoted attachment of CBP (histone acetyltransferase) to upregulate TRAIL and DR5 (51).

Xenografted mice based studies

Vemurafenib is a selective inhibitor of BRAF kinase and highly effective against anaplastic thyroid cancer cells (52). Vemurafenib potently enhanced TRAIL-induced apoptosis and induced regression of tumors in mice xenografted with 8505C cancer cells (52).

MEDI3039 is an effective multivalent DR5 agonist. It has recently been tested for efficacy against breast cancer in xenografted mice (53). MEDI3039 induced regression of the tumors in mice injected with MDA-MB-231T cancer cells. MEDI3039 was also preclinically assessed for efficacy and any possible hazardous effects at multiple doses and more injections to evaluate dose-dependent effects and to assess if more doses might prevent disease relapses. MEDI3039 not only significantly inhibited lung metastases but also inhibited growth of established experimental metastasis in xeno-
It has been scientifically established that c-Myc played dualistic role in carcinogenesis. Elevated c-Myc activity has been reported in breast cancer cells having high metastasizing potential (54). Increased c-Myc functions are crucial for invasive outgrowths in the brain microenvironment. However, despite highly oncogenic activities, MDA-MB-231 (brain metastasizing cells) demonstrated considerably enhanced susceptibility to TRAIL-induced apoptotic death. c-Myc–deleted-MDA-MB-231 (brain metastasizing cells) exhibited reduced TRAIL sensitivity but restoration of c-MYC re-sensitized them to TRAIL-induced apoptotic death. MDA-MB-231 (brain metastasizing cells) were injected into the arterial circulation of immune-deficient mice and later mice were treated with TRAIL. TRAIL caused significant reduction in the growth of brain metastases in xenografted mice (54).

Bone seeking MDA-MB-231-cancer cells were injected into the left ventricle of anesthetized mice (55). Intra-peritonally administered dasatinib significantly reduced number of metastases. Moreover, dasatinib promoted significantly higher relative gain and relative lower loss in the bone volume of xenografted mice. Collectively, these findings supported the fact that dasatinib prevented metastases of osteotropic MDA-MB-231 cells to the bone (55).

Co-encapsulation of the negatively charged siHSP70 and positively-charged TRAIL worked with remarkable synergy (56). Complementary activity of TRAIL and siHSP70 synergistically inhibited tumor growth in mice inoculated with A549. Furthermore, TRAIL and siHSP70 co-treatment also potently inhibited metastatic spread of cancer cells to the lungs as evidenced by noteworthy reduction in the metastatic nodules on the surface of lungs (56).

Concluding remarks

There has always been a quest to search for the anti-cancer agents having valuable efficacy and minimum off-target effects. Specialized killing of cancer cells is yet another stumbling block that confounds standardization of therapies. Discovery of TRAIL is regarded as a landmark in the field of molecular oncology. Branching trajectories of TRAIL-pathway have enabled researchers to not only unravel cancer killing pathways but also the strategies to therapeutically target different anti-apoptotic proteins which abrogate TRAIL-mediated apoptotic death in variety of cancers. Excitingly, anti-apoptotic proteins have been comprehensively analyzed and emerging evidence has highlighted how these proteins escaped from ubiquitination and degradation. Identification of natural and synthetic agents having minimum off-target effects and maximum efficacy has remained an overarching goal in molecular oncology. Moreover, regulation of TRAIL-induced cascade by non-coding RNAs is also a very exciting area of research and there is a gradual carving of this facet of TRAIL-pathway. More importantly, noteworthy increase in the progression of TRAIL-based therapies in various phases of clinical trials is indeed encouraging and promising. Therefore, keeping in view the wealth of information sequentially gathered in the past two decades will certainly help the researchers in overcoming TRAIL-resistance pathways and inclusion of TRAIL-based therapies in the list of clinically approved drugs.

Restoration of apoptotic pathway is an essential aspect of molecular oncology which is evidenced by the exponentially growing number of TRAIL-pathway-related publications. More importantly, such a framework holds the potential of far-reaching breakthroughs in the treatment of cancer, for cancer biologists and of bridging the knowledge gaps in understanding how TRAIL-pathway operates in different cancers and their subtypes. In-depth research related to TRAIL-based therapies has started to gain momentum and this remarkable strength of interest will enable cross-disciplinary collaborative works and push this exciting field of research forward to get a step closer to individualized medicine.

References

27. Zong D, Gu J, Cavalcante GC, Yao W, Zhang G, Wang S, Owo-

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Cell Mol Biol (Noisy le Grand) 2020 | Volume 66 | Issue 8

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