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BIOCHEMICAL MECHANISMS OF ETOPOSIDE; UPSHOT OF CELL DEATH

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ABSTRACT: Etoposide is an antineoplastic agent employed for healingvarious malignancies in human. It is a semi-synthetic derivative of podophyllotoxin and was introduced in 1971. The primary mode of action of etoposide is the inhibition of the function of the enzyme topoisomerase II. It stabilizes DNA-enzyme complex, and accumulation of this complex enhances the probability of double-stranded DNA breaks. However, etoposide can also causes DNA lesions in cancerous cells. This genotoxicity and antineoplastic activity of drug at the same time serve as the impetus for intensive research. Etoposide treatment causes permanent DNA breaks pertaining to its significant role in cell death and growth arrest in tumor cells. This review attempts to provide the deep knowledge of its possible biochemical pathways which are triggered in response to DNA breaks and leads to apoptosis and cell cycle arrest as well transcriptional activation of various death receptors. Molecular mechanism of drug efflux and activation of repairing proteins which can cause resistance in its action are also discussed. This current picture of known facts serve as a gateway to design more effective and efficient antitumor drugs, with new therapeutic strategies, combinations and modulation followed by the visualization of its biochemical mechanisms.

INTRODUCTION: Etoposide is an anti-cancer drug commonly known as VP-16 with its brand names Toposar VePesid, Lastet and NSC 141540. Etoposide has the chemical formula C29H32O13. Its scientific name is 4'-demethyl-epipodophyllotoxin 9-[4, 6-O-(*R*)-ethylidene-beta-D-glucopyranoside], 4' -(dihydrogen phosphate) and scientific number 33419-42-0.

Etoposide is classified as a "plant alkaloids" which make up a group of chemotherapy medications. These plant alkaloid drugs are segregated into three sub-divisionsbased on the nature of plant they derived.

Vinca alkaloids derive from the Periwinkle plant. Taxanes from the Pacific yew tree. Epipodophyllotoxins taken from the May apple plant.

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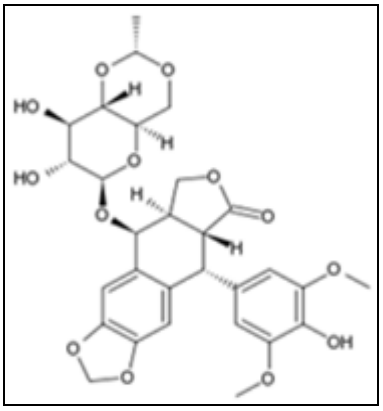

$C_{29}H_{32}O_{13}$

Synthesis:

Etoposide and teniposide make up the epipodophyllotoxin. It is a semi-synthetic derivative of podophyllotoxin and was first madein 1971 and approved by FDA in 1983. Podophyllotoxin (etoposide derivative) was first extracted from the rhizomes and dried roots of species of the genus *Podophyllinpeltatum* (May apple). It was first treated with the reagent hydrogen bromide to produce 1-bromo-1-deoxyepipodophyllotoxin then bromine was

replaced by a hydroxy group, resulting in 4-demethylepipodophyllotoxin. After that the 4-hydroxy group was coupled with 2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranose. The protecting group at the 4-hydroxy was removed by hydrogenolysis and the acyl groups by hydrolysis, and the cyclic *O*-4,6acetal was formed by reaction with acetaldehyde dimethyl acetal [1].

Target Action of Etoposide:

The principal cytotoxic objectfor etoposide is the enzyme topoisomerase II. Etoposide, an antineoplastic agent kills the cell by stabilizing enzyme-cleaved DNA complex. Topoisomerase is the enzyme which controls the topological structure of DNA. This enzyme involved in the temporary cutting, winding and unwinding of DNA. For this purpose it is critically important at several different steps in transcription, replication and chromosomal structure. Topoisomerases II cut both strands of a double-stranded DNA molecule, get ahead another duplex through the cut, and then reseal it again by the process that utilizes ATP. Etoposide interferes with the religation of DNA which causes stabilization of DNA–topoisomerase complex leads to the stabilization of cleavage complex. This accumulation generates permanent DNA strand breaks triggering recombination, mutagenesis, repair pathways and chromosomal translocations ultimately causingDNA strand break **(Fig. 1)**.

When these breaks are permanent in the cell, then initiate death pathways and eventually inhibit DNA synthesis. Etoposide is cell cycle dependent and phase specific drug andessentially affects S and G2 phases of cell cycle. This grounds forsignificant errors in DNA synthesis at the premitoticphase of cell division and can lead to death of cancerous cell. This cytotoxicity lies in its capability to form a stable complex between topoisomerase II and DNA ultimately inducing intensive DNA damage. This confirms the effect of etoposide on topoisomerase II by converting it into a cellular toxin and inducing crumbling of genome leading to cell cycle arrest and apoptosis [2].

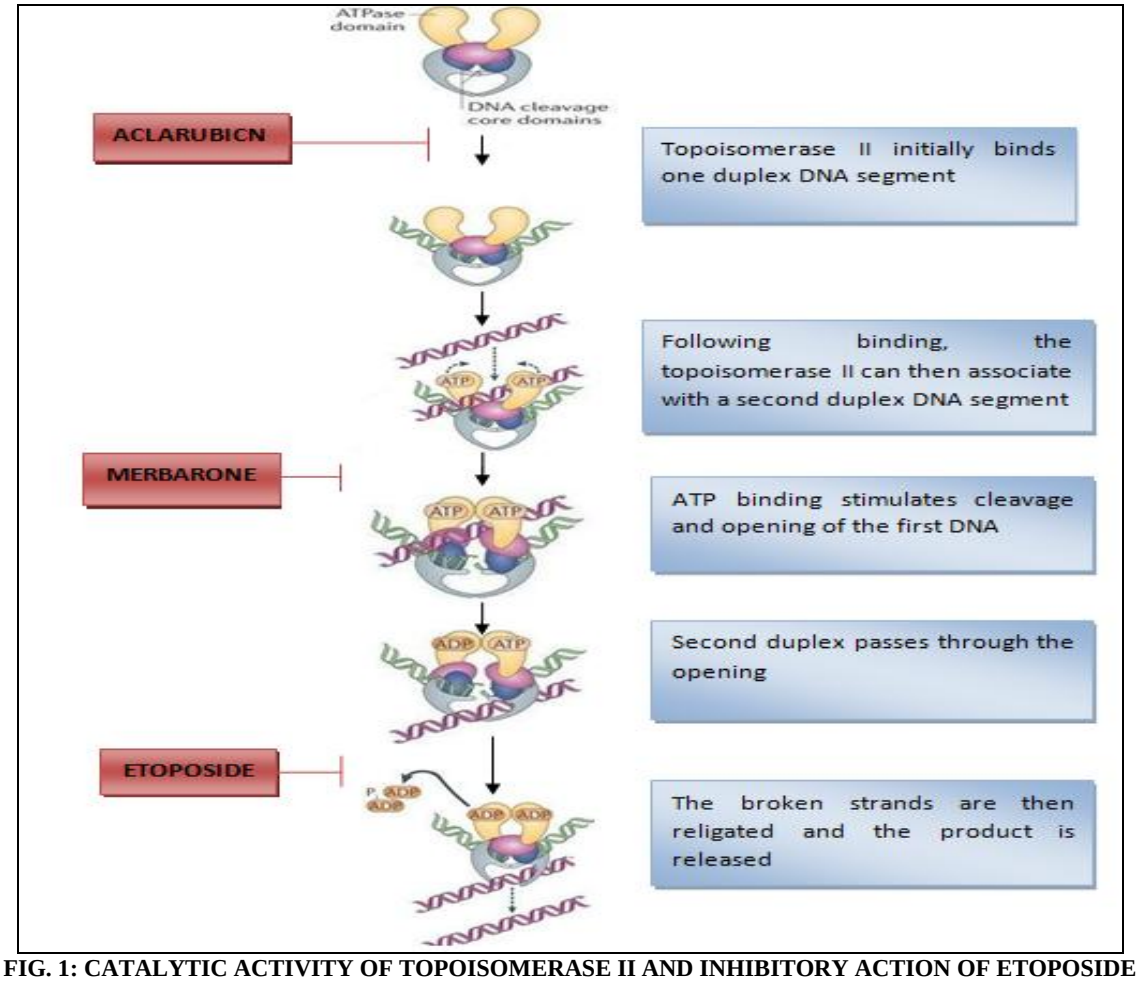

FIG. 1: CATALYTIC ACTIVITY OF TOPOISOMERASE II AND INHIBITORY ACTION OF ETOPOSIDE

Classification on the Basis of Functions:
Topoisomerase inhibitors belong to a class of drugs that blocks topoisomerases which may kill cancer cells. On the basis of their function they are classified in to three major groups.

- Topoisomerase II inhibitor compounds which interfere enzyme-DNA binding, the initial step of the cycle. These intercalating compounds include Aclarubicin and Suramin.

- These compounds form enzyme-cleaved DNA complex (cleavable complex) by stabilizing DNA topoisomerase II complexes and are termed as non intercalatorcomponuds. Etoposide, Teniposide and Saintopin are common drugs of this group.

- These are also non intercalator compounds that do not induce formation of cleavable complex by blocking ATP activity which results in the inhibition of topoisomerase II-catalyzed DNA cleavage. This group includes Merbarone and ICRF-187 [3, 4].

Indications:
Etoposide is used as FDA approved drug for treating testicular cancer and lung cancer however it is also used in prostate, bladder, stomach and uterine cancers, mycosis fungoides, Hodgkin's and non-Hodgkin's lymphoma, Wilm's tumor, Kaposi's sarcoma, Ewing's sarcoma, rhabdomyosarcoma and neuroblastoma. Its high-dose therapy may also be recommended in bone marrow transplant settings.

Therapeutic Combinations:
Oral etoposide has been reported as sole agent in different treatments. It is effective in combination therapies with other anti-cancer drugs to show some degree of success.

- Etoposide in combination with ifosfamide shows synergistic effect towards malignant tumors of childhood [5].

- In hormone- refractory prostate carcinoma combination of oral cyclophosphamide and oral etoposide is used [6].

- DICE (dexamethasone, ifosfamide, cisplatin, etoposide) combination is used as infusional chemotherapy for refractory non-Hodgkin's lymphoma [7].

Therapeutic Combinations in Lung Cancer:

- In combination with cisplatin it is also indicated for refractory small cell lung carcinoma [8, 9].

- The combination etoposide/ifosfamideor cisplatin/ifosfamideis effectual in treating non-small cell lung cancer. Combination of etoposide with ifosfamide is finer than combination with cisplatin owing to its better tolerability [10].

- Combinations of paclitaxel and etoposide have also been reported in treatment of lung cancer [11].

- Clinical trials of etoposide, cyclophosphamide, and vincristine for small-cell lung cancer appear to be an effective treatment [12].

Common Side Effects of Etoposide:
Thrombocytopenia:
Etoposide can reduce the platelet count by decreasing its production, which help in blood clotting. When platelets count is low it cause bleeding or bruising, such as nose bleeds, blood spots and rashes on the skin.

Leukopenia:
Low white blood count higher the risk of getting infections. White blood cells are important for inducing the immunity.

Alopecia:
This usually starts in 3–4 weeks after executing therapy, although it may happen earlier. Hair fall occurs completely.

Anorexia:
Etoposide can also cause metallic taste during chemotherapy and it lasts for several months. Normal taste usually comes back after treatment.

Fatigue:
Feeling exhausted is the most common associated side effect of chemotherapy, especially at the end of the therapy and remains for some weeks after treatment.

Less common side effect:
Anemia:
Etoposide can lessen the number of RBC's red blood cells ultimately causing anemia. This may make feel breathless and tired.

Nausea Vomiting Diarrhea:
Nausea and vomiting may begin after the treatment and last for up to five days. Etoposide can also cause diarrhea. This can easily controlled by medications.

Skin changes:
Itchy skin rashes may occur and skin may be darken butgenerally comes to normal in few months after the treatment.

Sleeplessness, headaches and confusion:
These effects occur only in high-dose treatment and are very rare.

Allergic reaction:
Allergic reaction includes high temperature, shivering, dizziness, a headache, reddening of the face, breathlessness, anxiety, and urticaria.

Adverse Reactions:
Acute leukemia:
Etoposide can cause second cancer usually acute leukemia that develops years later with etoposide treatment**.**

Influx of Etoposide:
Etoposide cellular uptake is diffusion dependent. Not any specialized transporter apparently takes part in etoposide transport while considerable saturatedsecretion occurs through intestinal tissues. Transport Once it enters in the cell, it has cytocidal effect. Etoposide phopshate, is not diffusable in the cell. Unless alkaline phosphatase cleaves its phosphate group and make it cytocidal agent, etoposide. Amphipathic nature of etoposide allows its association with hydrophobic domains of membrane proteins or memrane lipid [13].

Metabolism:
Etoposide metabolism is arbitrated by CYP3A4 and CPY3A5, both transcriptionally controlled by NR1I2. Further, etoposide can also be converted into the O-demethylated metabolites and, can be catalyzed by prostaglandin synthases or myeloperoxidase. These metabolites share same strength of hampering topoisomerase II and are more reactive than their parent drug. Inactivation of parent drug and metabolites is induced by glutathione and glucuronide conjugation. ABCC1, ABCC3 and ABCB1 are involved in efflux of conjugated or unconjugated forms of etoposide and represent the mechanisms of drug resistance. Etoposide glucuronide acts as a good substrate for both ABCC2 and ABCC3 and the elimination of this metabolite from the liver is almost completely dependent on these efflux pumps [14].

Efflux of Etoposide:
A family of transmembrane proteins, ATP-binding cassette (ABC) transporters, isinvolved in transportation of a wide array of substrates across biological membranes through an ATP dependent reaction. ATP-binding cassette (ABC) multidrug transporters, for instance P-glycoprotein (P-gp, ABCB1) and multi-drug resistance proteins (ABCC2 and ABCC3), may be have an important impact on cancer chemotherapy. From recent data it has been proved that ABCC1 is a good transporter for etoposide as a substrate. Furthermore, it has also seen that both ABCC2 and ABCC3 can moderately transport etoposide. ABCB1 transports various substrates across the cell membrane including chemotherapeutic agents such as etoposide and it is an important transporter for etoposide.

Transporters of Etoposide:
MDR1 (Multi-Drug Resistance1):
The (ABCB1) gene is located on chromosome 7q21.12 which encodes P-gp and used as a transporter in many hydrophobic substrates and anti-tumor drugs including etoposide, mainly expressed in organs such as intestine and liver involved in the excretion. In lung, P-gp express at the apical side of ciliated collecting ducts or ciliated epithelial cells, and also expressed on the apical and sideways surfaces of serous cells of

bronchial glands but not in mucus-secreting cells. The MDR (ABCB) has two distinct sites, H site and R site, for drug binding and transport that interacts in a positively supportive manner. The two sites have more or lessidentical affinity for etoposide. These transporters are present in the lung so are involved in treating small cell lung cancer which is mediated by etoposide.

Main transporters of etoposide are following:

1. Multidrug resistance protein 1 (ABCB1)
2. Multidrug resistance-associated protein 6 (ABCC6)
3. Canalicularmultispecific organic anion transporter 2 (ABCC3)
4. Multidrug resistance-associated protein 1 (ABCC1)
5. Multidrug resistance-associated protein 7 (ABCC10)
6. Canalicular multispecific organic anion transporter 1 (ABCC2)
7. ATP-binding cassette sub-family G member 2 (ABCG2)

ABC transporters can be expressed in tumor cells where they can actively efflux a broad spectrum of different anticancer drugs and also contribute to multidrug resistance. These efflux pumps have broad and substantially overlapping substrate specificities [15].

Drug Active Sites:

The drug is composed of a polycyclic ring system (rings A-B-C-D rings), a glycosidic moiety at C4 position, and a pendant ring (E–ring) at the C1 position. A, B, and E-rings of etoposide by substituents appear to be referred in the interaction between etoposide and human topoisomerase IIα in the binary enzyme–drug complex. The protons of the A–ring, the H5 and H8 protons of the B–ring, as well as the H2 and H6 protons and the 3′ and 5′methoxyl protons of the pendent E–ring interact with both enzymes in the binary protein-ligand complexes.

Substituents on E–ring of etoposide are necessary for drug action against topoisomerase II. E–ring is intimately associated with the protein in the binary complex. Since the E–ring has free rotation about the 1'–linkage to the C–ring, the H2' and H6' protons, as well as the 3′– and 5′–methoxyl groups. Although the 3′–methoxyl, 5′–methoxyl, and 4′–hydroxyl moieties of the E–ring all are important for etoposide task. The glycosidic moiety of etoposide, which is attached to the C–ring at the C4 position, does not interact with topoisomerase IIα in the binary enzyme-drug complex. D-ring of etoposide also does not appear to contact topoisomerase IIα in complex. Alterations in the D-ring may affect etoposide activity toward topoisomerase IIα in theternary enzyme drug–DNA complex [16, 17].

DNA enzyme complex, the most likely target of Etoposide:

Topoisomerase II use to generate transient double-stranded breaks in the backbone of nucleic acid. In order to sustain the integrity of the cleaved genetic material throughout drug activity, this enzyme forms proteineceous bridges that extend the nucleic acid break. This bridge is affixed by covalent phosphotyrosyl bonds, customary between the active site of the homodimeric enzyme and the newly created 5′ termini of DNA. Because of this cleavage complex (topoisomerase II-cleaved DNA complex) is normally short-lived intermediate in the catalytic cyclic activity of enzyme, which is endured by the cell. However, by the treatment of topoisomerase inhibitor drugs, when cleavage complexes present in high amount, become possibly toxic, endorsing illegitimate recombinations, frame shift mutations, permanent double-stranded DNA breaks and cell death. The cytotoxic prospective of topoisomerase II inhibitors has now been utilized clinically by the expansion of antitumor drugs. Rapidly proliferating cells are most susceptible to these agents because containing high concentrations of topoisomerase II in aggressive malignancies.

Etoposide induced scission reveals the ability to inhibit religation at specific sequences (rather than direct drug DNA) interactions that mediate cleavage complex formation. These conclusions are reliable with the recently proposed "positional

poison model" for the action of engendering to DNA lesions by topoisomerase II-targeted anticancer drug **(Fig. 2)**. Finally it is proposed that the primary pathway for the non-covalent enzyme-drug and DNA ternary complex formation is through etoposide-topoisomerase II interactions leads to DNA fragmentation and apoptosis [18].

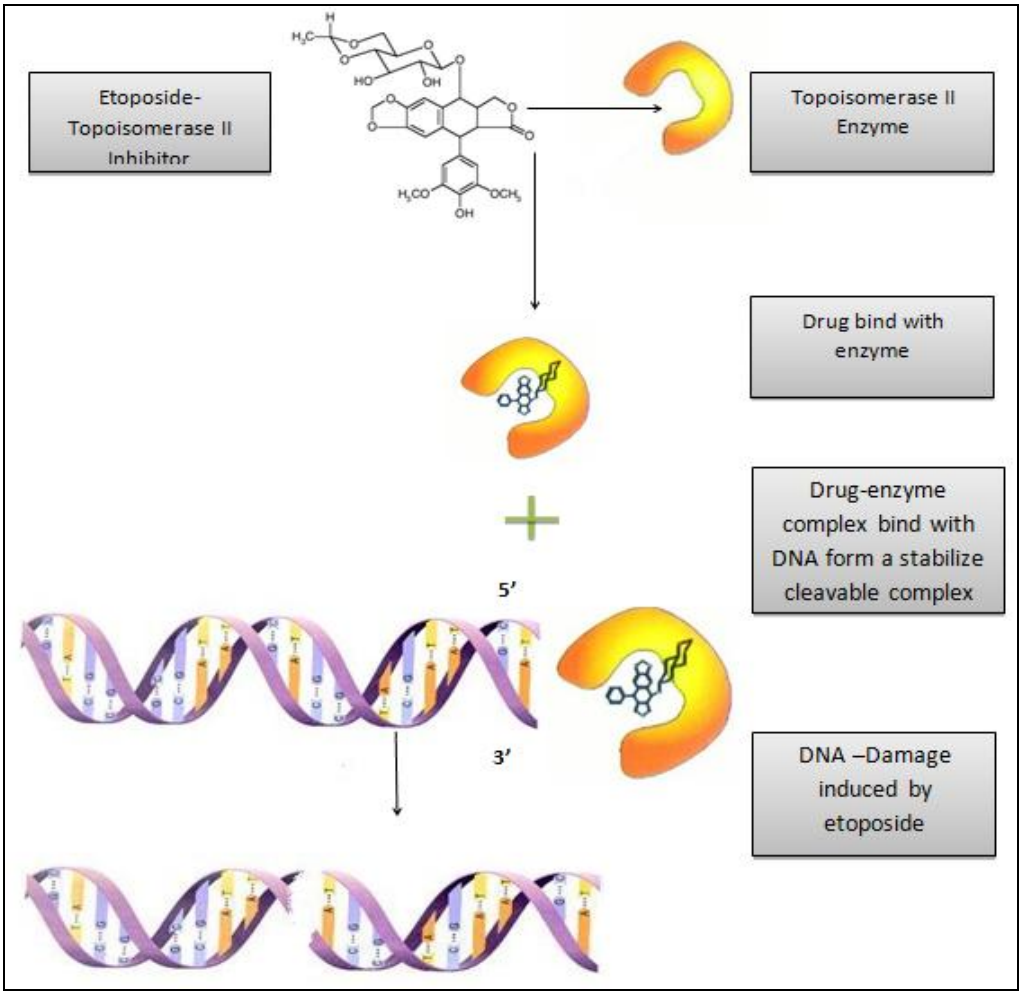

FIG. 2: DRUG BINDING AND STABILIZATION OF CLEAVABLE COMPLEX

Biochemical Mechanism of Action of Etoposide:

ATR mediated pathway in response to single strand break:

Etoposide acts after Topo II mediated DNA cleavage, in which Topo II enzyme covalently bind to DNA- 5′ termini, whereas DNA-3′ termini remains free cause the formation of lesions. These lesions develop in single-strand DNA gaps and double-strand DNA breaks. This cause the stimulation of ATR (Ataxia telangiectasia Rad3-related) pathway which is chiefly involved in the reaction to the halting of replication forks by etoposide, results in the development of extended single-stranded DNA regions [19]. Etoposide mediate ATR pathway entails the activity of the Ataxia telangiectasia Rad3-related (ATR) checkpoint kinase. Etoposide-induced DNA damage happens behind the replication fork and this damaged DNA is further transformed into extended single-stranded DNA region (ssDNA) which is coated with RPA (Replication protein A) [20]. ATR recognition of RPA-coated single strand DNA depends on ATR-interacting protein (ATRIP). ATR signaling is reliant on co-localization of the ATR-ATRIP complex with the Rad9-Rad1-Hus1 (9-1-1) complex, a heterotrimeric ring-shaped molecule. 9-1-1 complex carries a critical activator, TOPBP1 to ATR. TOPBP1 is obligatory for ATR activation that can arouse ATR activity [21].

Once ATR complex is amassed with DNA lesion prompts signals to synchronize cell cycle, and processes of repair and replication are commenced. ATR phosphorylate CHK1 on Ser317 and Ser345,

which is a trustworthymarker of CHK1 activation. Once CHK1 is phosphorylated, it release from chromatin material and further phosphorylates its substrates. Activated CHK1, phosphorylates Cdc25A protein phosphatase which uphold its ubiquitin-arbitrated proteolysis. Loss of Cdc25A leads to S-phase and G2-phases arrest in the of the cell division cycle. ATR signaling is critical for regulating replication either by the regulation of CHK1-CDC25 pathway [22, 23]. ATR is principally a replication stress response and it can also be activated by double strand DNA break **(Fig. 3)**. ATR is triggered more gradually and chiefly in S and G2 phases of cell cycle. This steady ATR activation at double-strand break site is a necessity for CDK-dependent DSB ends resection, which exposes a large single-stranded region. It can also be proposed that DSBs guides to ATM stimulation and the repair of DSBs can turn out RPA-coated ssDNA that elicits ATR pathway [22]. It may occur at TOPBP1 level by its phosphorylation through ATM. Phosphorylated TOPBP1 is more proficient activator of ATR [21].

ATR is one of the kinases which involves in the phosphorylation of Chk1 in reaction to genomic stress. P53 is also phosphorylated by Chk1 and this phosphorylation prompts downstream activation of p53 and its target genes i.e., Bax and PUMA (p53-upregulated modulator of apoptosis) ultimately leading to apoptosis, while transcriptional activation of p21 and 14-3-3 causes cell cycle arrest in G1 phase [24].

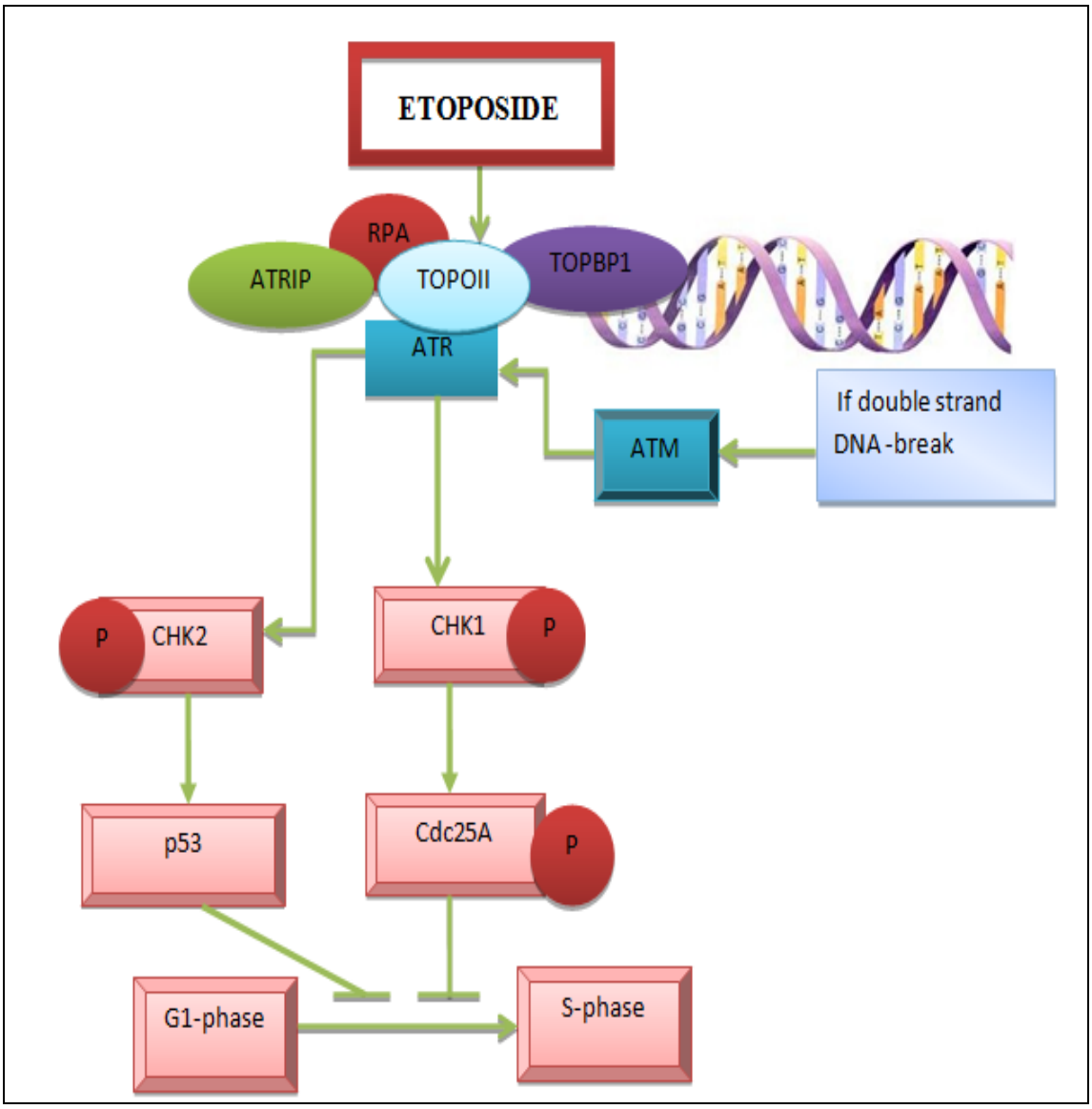

FIG. 3: ATR ACTIVATION BY ETOPOSIDE

ATM mediated pathway in response to double strand break:

Ataxia telangiectasia mutated (ATM) manganese dependent serine-threonine protein kinase is a tumor suppressor protein, signals the presence of DNA double strand break in mammalian cell. DNA damage results in ionizing radiations, replication error or chemicals. Chemotherapeutic drug etoposide also cause DNA damage. Etoposide, a topo II inhibitor drug stabilize cleavable complex (enzyme-DNA complex) cause DNA double strand break, leads to the activation of ATM, a key player in multiple signaling cascade in response to double strand DNA break and acts as a master switch and regulator in cell cycle progression. After etoposide treatment, ATM response to cell cycle checkpoints factors (chk factors) DNA repair or apoptosis **(Fig. 4)**. ATM is activated by MDNcomplex include three proteins the MRE11 (meiotic recombination-11), Rad50 and NBS1 (Nijmegen's breakage syndrome). ATM first phosphorylates NSB1 on several residues in response to DNA damage and MRE11/Rad50/NSB1 complex is needed for chk2 activation. The phosphorylation of p53 is controlled by CHK2 or ATM directly phosphorylates p53. When ATM phosphorylates serine 15 it activates p53 and N-terminal for chk2 activation [25].

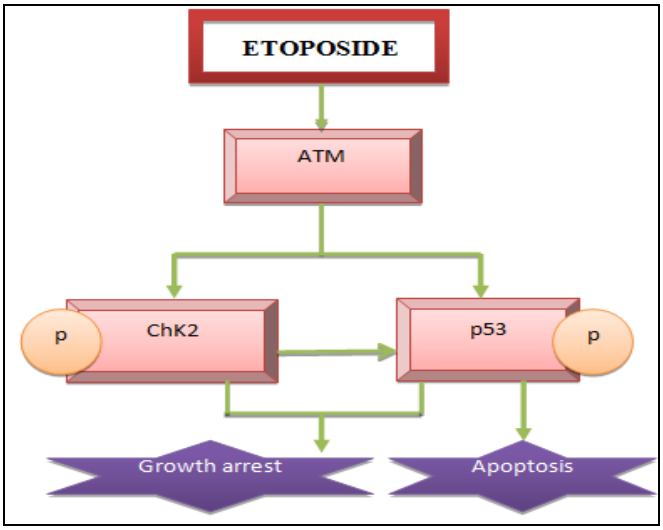

FIG. 4: MECHANISM OF ATM IN GROWTH ARREST AND APOPTOSIS

P53, dependent functions:

P53 is tumor suppresser and have key function in the cellular response towards genomic toxicity [26]. Etoposide cause the phosphorylation of p53 within 6 hrs. A stress signal is conveyed to the p53 protein by post-translational modifications and answers the activation of the p53 protein as a transcriptional factor that instigates a program of cell cycle arrest andcellular senescence. Further p53-responsive and interactive proteins are produced by transcriptional network of p53 that interrelate with anoutsized number of other signal transduction pathways and account for a numerous positive and negative auto-regulatory feedback loops. Many factors regulate p53 activation and its downstream response depends on cellular environment. Etoposide treatment is liable to a speedy and widespreadinitiation of cell death and additional increase in p53 and PUMA expressions as well as Bax processing. Transcriptional activation of gene encoding p21 and 14-3-3 leads to G1 cycle arrest [27, 28].

P53 and Growth Arrest:

Transcriptional activation of p53 can inhibit both G1 to S and G2 to M phase of cell cycle. P53 mediated transcriptional induction of p21 has a foremost role in mediating G1 arrest. In cell cycle progression, p21 can inhibit Cdk2 which requires for S phase, resulting in the buildup of hypophosphorylated Rb (retinoblastoma) directing to G1 to S phase arrest **(Fig. 5)**. The M phase progression entails Cdc2 which can be hindered by several proteins like p21, GADD45 or 14-3.3. A direct transcriptional repression of cdc2/cyclin B by p53 also results in G2 to M phase arrest. P53 controls the expression of these inhibitory proteins to provoke growth arrest [29].

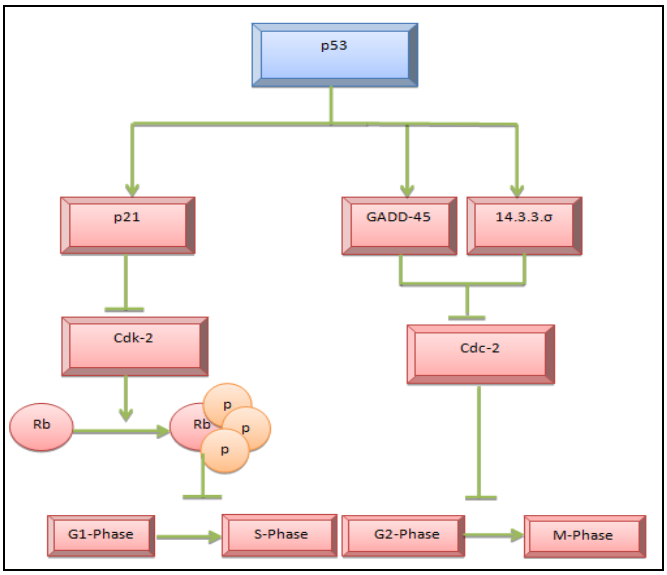

FIG. 5: P53 ROLE IN GROWTH ARREST

P53 and apoptosis:

Etoposide induce apoptosis in p53 dependent-pathway, activated in response to diverse stress stimuli under the action of ATM [26]. p53, a transcription factor mediates its diverse functions by two routes to apoptosis either by "intrinsic or mitochondrial" cell death pathway or "extrinsic pathway".

P53 Induced Intrinsic pathway:

Intrinsic apoptotic pathway is triggered, through application of the DNA-damaging topoisomerase inhibitors etoposide [30]. Phosphorylation of p53 results in the up-regulation of proteins in the nucleus induce expression or transcriptional activation of proapoptotic Bcl-2 family proteins (BAX, NOXA, PUMA, BID, CD95, APAF-1, DR5, p53AIP1) implicated in apoptosis [31, 32, 33]. The intrinsic apoptotic pathway is dictated by the expression of Bcl-2 family proteins, directs the release of cytochrome c from the mitochondria leads to apoptosis through caspase cascade **(Fig. 6)**. NOXA and PUMA genes are direct transcriptional targets of p53 and have critical role for p53-induced apoptosis. Increased expression of PUMA promotes mitochondrial translocation and mulitmerization of Bax [34].

Bcl-2 family comprises anti-apoptotic (pro-survival) and pro-apoptotic proteins. Bcl-2 family includes: anti apototic proteins, whose members are most structurally similar to Bcl-2, such as Bcl-X_L, and pro-apoptotic proteins Bax, Bak and BH3-only proteins [35, 36]. Inhibition of proapoptotic proteins through antiapoptotic Bcl-2 members is prevented by activated BH3-only proteins. Further a conformational change in Bax and Bak proteins occur which direst their insertion into mitochondrial membrane through oligomerization and pores formation. This whole leads to release of proapoptotic factors from mitochondria into cytochrome c and a series of events is initiated in which binding of cytochrome c with Apaf1 occurs and apoptosome is formed that consequently triggers caspase cascade causing cell death [37].

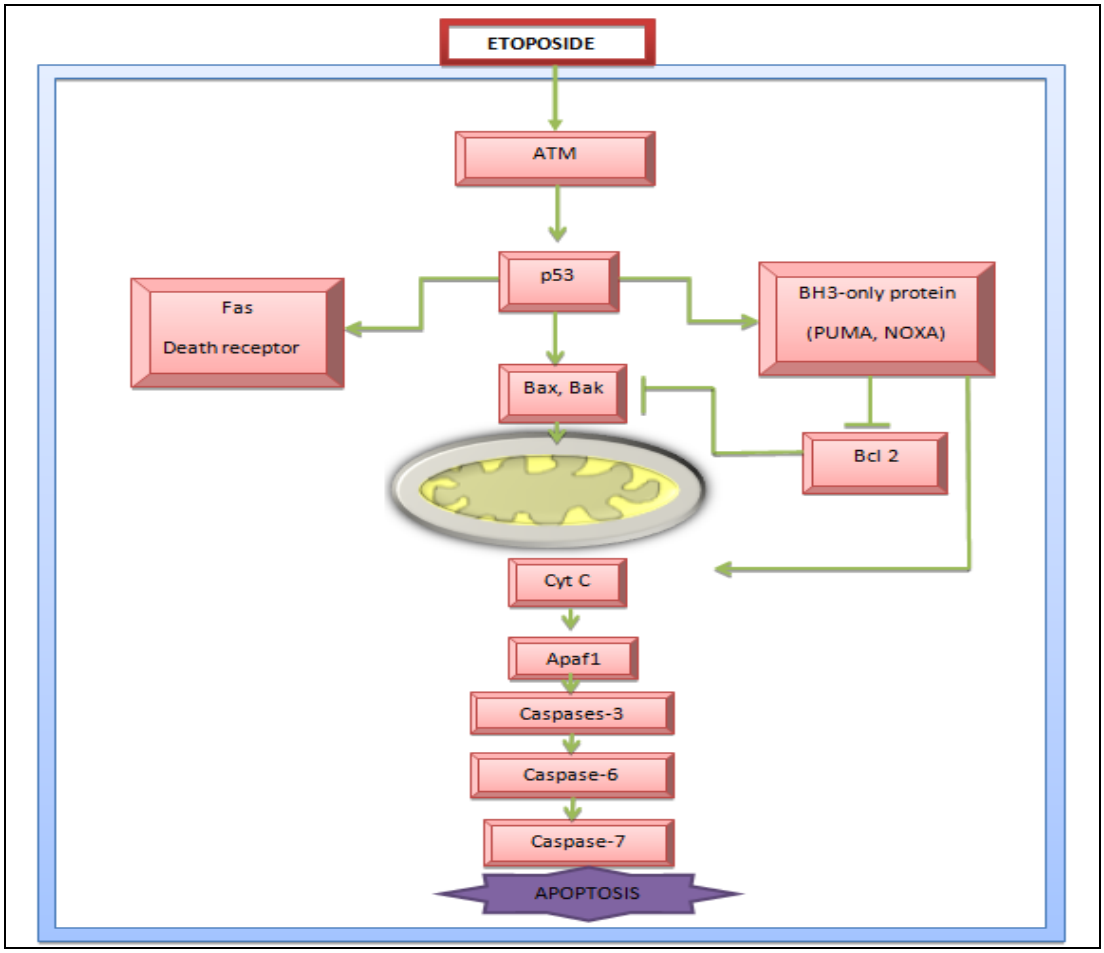

FIG. 6: INTRINSIC APOPTOTIC PATHWAY BY P53

P53 Induced Extrinsic pathway:

Etoposide through its genotoxic function can up-regulate p53 and turn on death receptor pathway. P53 activates various genes by transcription, involved in cell death as Fas receptor [38]. Etoposide may rapidly up-regulate death receptor Fas or Fas ligand by this means directly triggering the apoptotic pathway [39]. Upon an apoptotic stimulus, stem helices are allowed by Fas death domains to interact and stabilize the open form. FADD (Fasassociated death domain) can then bind to the Fas molecules; this consequence is procedural interlinked DISC (death-inducible signaling complex) formation. This allows activation of caspase 8, via its oligomerisation causes the induction of apoptosis [40]. DISC places the molecules of procaspase-8 in a spatial orientation favorable for their activation [41, 42]. c-FLIP has a high affinity to bind to the DISC and is structurally similar to caspase-8 so it is an apoptotic inhibitor [43].

Caspase8 mediated apoptotic pathway:

Outer mitochondrial membrane localized caspase 8 slices cytosolic procaspase 3 directly and turns onit. Once caspase-3 is activated during etoposide-induced apoptosis, MST1 will be cleaved and activated.MST1 (mammalian STE20-like kinase 1) is a serine/threonine kinase, over-expression of which brings about apoptotic morphological changes such as chromatin condensation. The cleaved N-terminal kinase domain of MST1 translocates into the nucleus and mediates the phosphorylation of histone H2AX, and the phosphorylated H2AX then recruits caspase-activated DNase to mediate DNA fragmentation. During the procedure of etoposide-induced apoptosis, the breakage of MST1 robustly comes with strong H2AX phosphorylation at Ser-139 recruits caspase-activated DNase to mediate DNA fragmentation [44]. The caspase 3 cleaves DFF45 (DNA fragmentation factor 45), dissociates from DFF40, inducing oligomerization of DFF40 (a heterodimeric factor of DFF40) that has DNase activity.

Caspase 8 mediated intrinsic pathway:

Etoposide induced Caspase 8 pathway is activated through FADD/TRADD-dependent mechanisms not only trigger extrinsic apoptotic pathway via caspase 3, also recruit mitochondrial apoptotic signaling [45, 46].

Activated caspase 8, by the DISC cleavage, directly activates caspase 3 as well as breaks the pro-apoptotic Bcl-2 family member Bid, forming the truncated form of Bid, t-Bid. t-Bid then triggers apoptotic pathway by translocation in to outer mitochondrial membrane (OMM) and promotes oligomerization of Bax or Bak, results the release of cytochrome *c* and other apoptogenic proteins from the intermembrane space (IMS) of the mitochondria into the cytosol [47]. This step can be antagonized by the anti-apoptotic Bcl-2 family members. The released cytochrome c, interacts with Apaf-1 (Apoptotic protease-activating factor 1) and, in the presence of (d)ATP, gets attached to adapter protein Apaf-1 and activates it, as a consequence caspase 9 are recruited, causing the formation of apoptosome, stimulation of caspase 9, and subsequent execution of caspases, caspase 3, 6 or 7 which propagate further caspase processing events, leads to cell death [48].

Caspase8 induced mitochondrial fragmentation:

Caspase 8 target Bid, a direct activator of integral polytopicER membrane protein Bap31, the cytosolic tail of which is cleaved by caspase-8 to produce proapoptoticBap20 [45, 46]. It can lead to severe ER stress by provoking Ca^{2+} release and enhanced mitochondrial fission. In addition to this, p20 also interferes with Bap31-mediated protein trafficking and causes a speedy conduction of ER calcium signals. These Ca^{2+} signals stimulate DRP1 (dynamin-related protein) dependent organelle fission and cause the release of cytochrome c. Anti-apoptotic members of Bcl-2 family fundamentally targets ER calcium homeostasis and Bap31/procaspase-8 complexes [49].

These evidences suggest that signaling pathway via etoposide treatment activate p53 which stimulate caspase 8 causes the cleavage of BAP31 to BAP20 on the ER. ER causes the Ca^{2+} release uptake by mitochondria **(Fig. 7)**. When Ca^{2+} level exceed mitochondrial fission occurs, eventually contribution to apoptosis by interrupting mitochondrial integrity and function [50, 51].

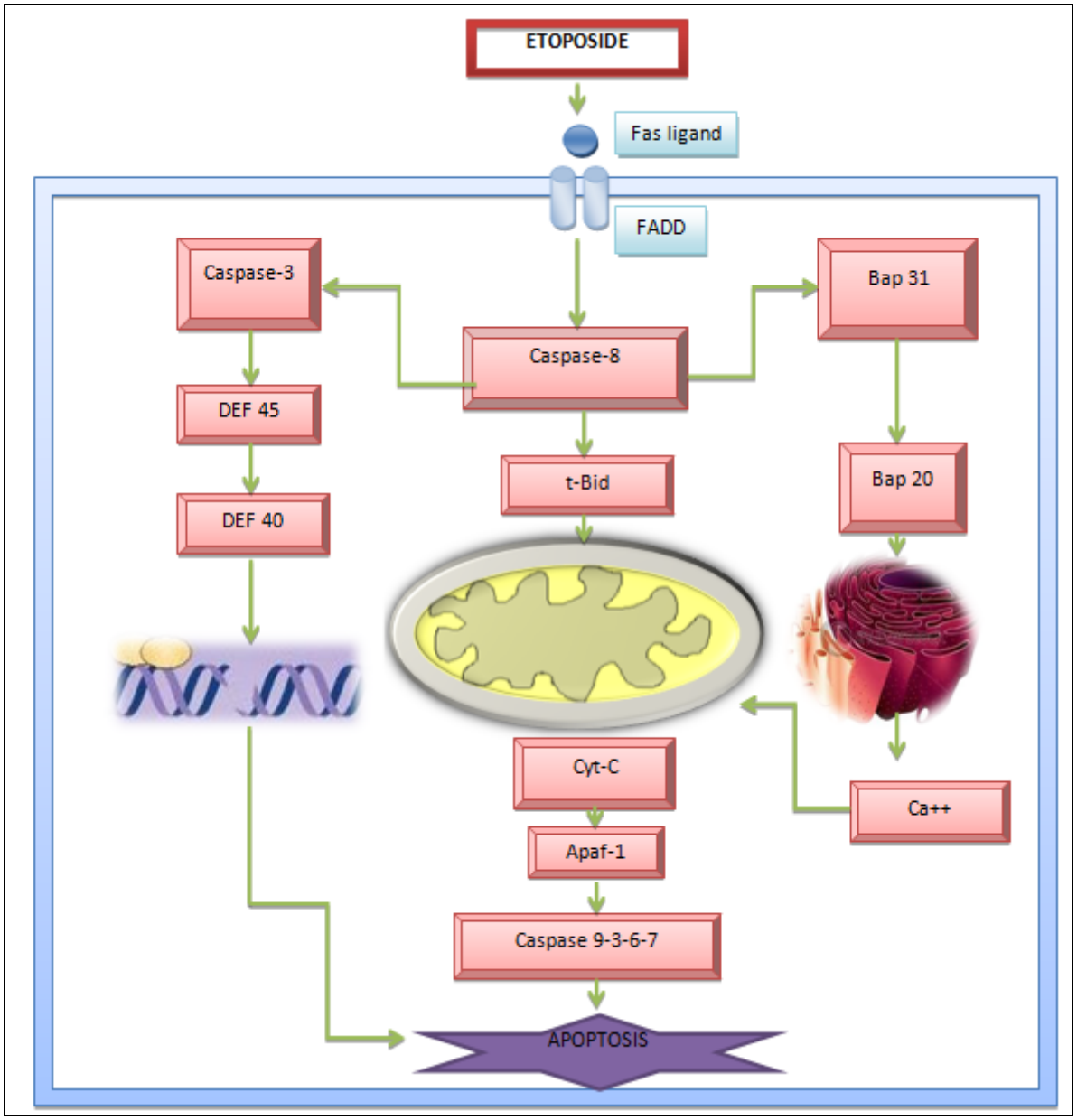

FIG. 7: ROLE OF CASPASE 8 IN APOPTOSIS

Role of NF-kB:

Etoposide causes DNA damage, treated by the main DNA damage sensor p53 which is trans-activated by p53 provoked protein with death domain (PIDD) and activates NF-kB. Upon DNA damage NF-kBshifts to the nucleus and activate transcription of DR4 receptor gene. However, it is observed that a dynamic induction of the p53-dependent phosphorylation of FADD at Ser194 brings a crucial change in sensitization to DNA damage by augmenting MEKK1 pathway. The phosphorylation of p65 is carried out through p53 dependent death receptor expression. Other receptors like DR3, DR4 and DR5 are also stimulated by etoposide. Cells can express a copiously functional relation among p53 and p65 by Fas receptor assembly which are highly receptive to etoposide. Fas receptor has ability to activate p65 in FasL-independent manner and caspase-dependent apoptosis as well p65 induce apoptotic death by caspase-independent manner [38, 52]. Tumor necrosis related apoptosis inducing ligand (TRAIL/Apo-2L) that attaches to death receptor-4 (DR4, TRAIL-R1) and death receptor-5 (DR5, TRAIL-R2) is a protein thatpersuades cell death in a multiple type of cancersrather than normal non-transformed cells. Etoposide augmented DR4 protein levels escorts to apoptosis with the TRAIL induced cell death pathway.Etoposide express DR5 gene through the

collaboration of transcription factors NF-kB and p53 by binding with DR5. The DR4 protein resides on the plasma membrane; upon attachment with TRAIL it activates caspase-8 and forms the DISC (death inducing signaling complex) to executedeath signaling program ensuing apoptosis.

Ceramide Production by Etoposide:

Ceramide, a tumor-suppressor lipid is generated, when cells come across stimuli of apoptotic signaling, for instance radiation (UV and γ-irradiation), death receptors (CD95 and TNF receptor), or subjected to genotoxic chemotherapy. Etoposide causes accumulation of ceramide derived from SM hydrolysis under the action of SMase, functions as an important mediator of the apoptosis of glial cell induced by etoposide [53].

Ceramide up-regulate thioredoxin-interacting protein (Txnip) which is a tumor suppressor gene, so, etoposide, by this way is involved in provoking apoptosis. Txnip; also termed as vitamin D3 up-regulated protein 1, endogenously hampers thioredoxin, which is a small redoxprotein.Thioredoxin associates with ASK1 and encourages ASK1 degradation and ubiquitination to restrain ASK1-arbitrated apoptosis by inhibiting ASK1 kinase activity. Elevated Txniplevels results in ASK1 instigation.

MAPK related apoptotic pathway:

ASK1 activation:

ASK1 after being phosphorylated and oligomerization with threonine residues in its activation loop is dissociated from thioredoxin. Txnip induction by ceramide, cause an increase dephosphorylation at the inhibitory residue Ser83 and phosphorylation of ASK1 at activation residue Thr845. Thioredoxin blocks activity of apoptosis signal-regulating kinase 1 (ASK1) and also decrease the consequent ASK1-dependent apoptosis. Txnip eradicates this effect, therefore, Txnip possesses proapoptotic role and is a stress-responsive protein. Txnip signaling regulateASK1, p38 MAPK- and JNK-mediated pathways, as well as endoplasmic reticulum (ER) stress death signaling pathways. ASK1 is the member of MAPKKK family. Ceramide induces apoptosis through phosphorylation of p38 MAPK and JNK [54].

Activation of MEKK1:

Etoposide causes DNA damage and activates MEKK1 (Mitogen-activated protein kinase) and NF-kB a protein. MEK kinase 1 (MEKK1) induces apoptosis through both caspase amplification and transcriptional activation or regulation. MEKK1 activates through apoptotic pathway leads to the cleavage by caspase 3-like protease into the 91kDa kinase domain in reaction to DNA damaging or genotoxicmediators such as etoposide [55].

Fas-induced activation of MEKK1 via caspase 3 or related proteases slices MEKK1. FADD phosphorylation contributes to the activation of MEKK1 pathway at Ser194, can up-regulate MEKK1 pathway. In apoptosis, caspase 3 and related proteases break MEKK1 at the peptide bond after D68 and sets it free from a compartment, triton-insoluble cytoplasmic complex. MEKK1 involved in pro-survival and pro-apoptotic pathways is an activator of JNKc-Jun N-terminal kinase.

MEKK1 activates the (JNK); as well as it turns on IKK causing the degradation of IkB and the stimulation of NF-kB.MEKK1 phosphorylates the inhibitor IkB leads to its breakdown proceeding with activation of NF-Kb.

JNK Activation:

The regulation of JNK is done by multipleMAPKKK' proteins, involving MEKK1,MEKK4 and ASK1, that is regulated by Rac1 and GTPases.The MAPKK, MKK4/7, dually phosphorylates JNK within the Thr^{138}-Pro-Tyr^{185} motif (pTPpY) in the catalytic core of active JNK.
JNK is also induced by the PKCδ activation is needed for the unrelenting activation of JNK and the inactivation of ERK. PKCδ is activated through caspase dependent manner eventually leading the stimulation of JNK and inhibition of ERK. ERK1 and ERK2 has critical role in mitogenic signaling and oncogenesis. There is also evidence that activation of ERKs stimulates protection against apoptosis mediated factors **(Fig. 8)**. On the other hand JNK activation is significant and sometimes ample to provoke apoptosis [56].

JNK prompts extrinsic apoptotic signaling through up regulation of death receptors and their ligands.

JNK persuades apoptosis by diverse mechanisms, including cytochrome release and needs the pro-apoptotic (Bcl-2) family proteins, Bax (Bcl-2 associated x protein) and Bak1 (BCL-2 antagonist killer1). Active JNK robustlyconnected to transcription-dependent apoptotic signaling, chiefly through the commencement of c-Jun and other AP-1 proteins such asJunD, JunB and ATF-2 and may influence apoptosis by up regulating apoptosis-related genes [57].

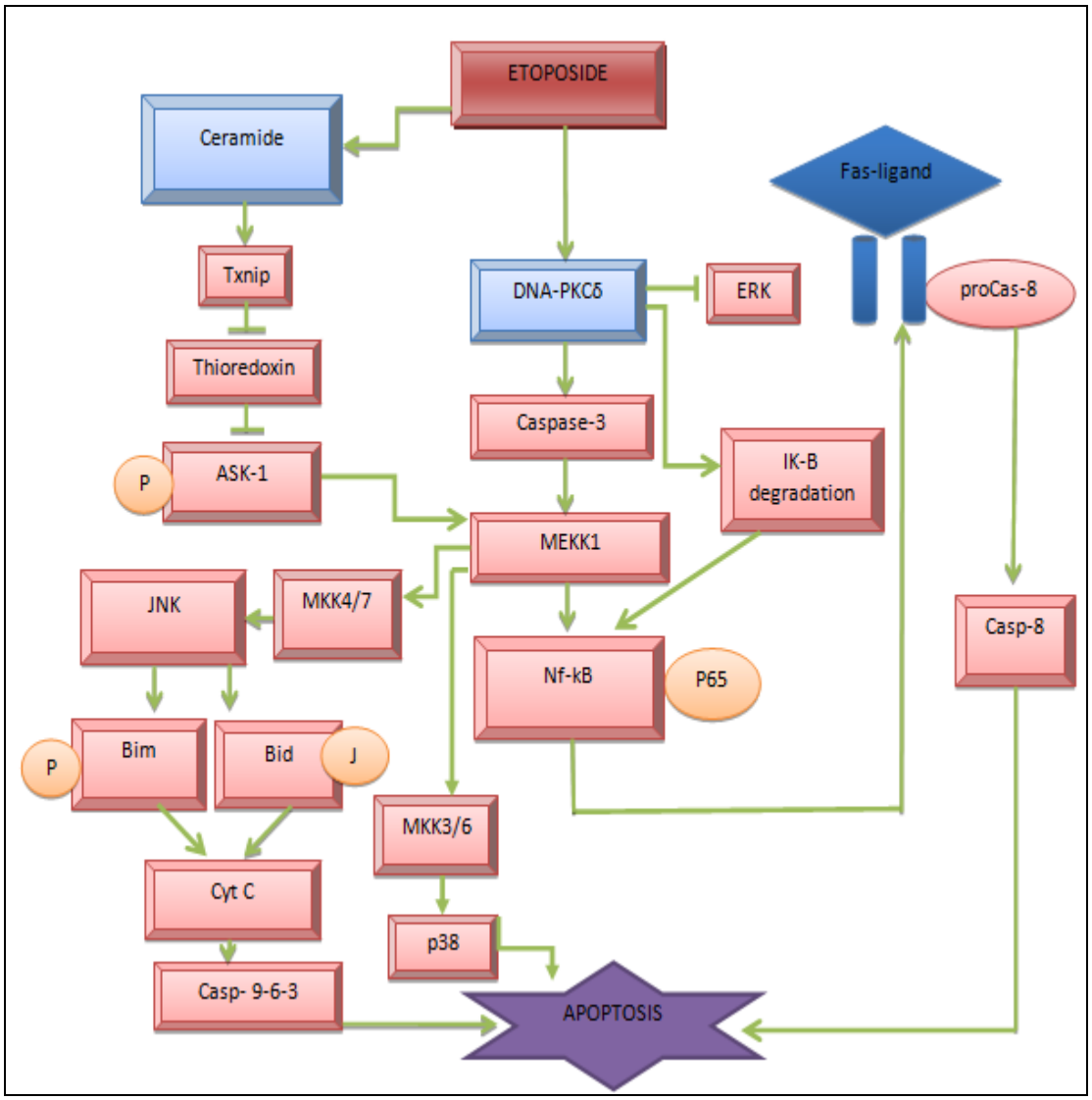

FIG. 8: ACTIVATION OF MAPK FAMILY PROTEINS VIA DNA-PK'S AND CERAMIDE

c-Abl pathway:

Cellular responses to genotoxic stresses after etoposide treatment including the activation of c-Abl, JNK and p53 expression. Apoptosis resulted in the degradation and activation of c-Abl after etoposide treatment, activates c-Abl kinases. The activated c-Abl plays a role in amplification of apoptosis induced by etoposide [58, 59, 60].

The proto-oncoprotein c-Abl is a member of the Src family of non-receptor tyrosine kinases. c-Ablis ubiquitously expressed in the cells and concentrates both in the cytoplasm and nucleus where it isengaged in definite roles. c-Abl protein includes diverse domains which shows high affinity for phosphorylated tyrosine like Src homology 2 (SH2) domain. SH3 (Src78homology 3) domain binds to proline-rich domains containing PxxP motif as well as c-Abl SH3 binding proteins proved fruitful in regard to characterize its immediate downstream targets proteins, such as DNA-PK, ATM, the Abl family proteins and transcriptional factor p73. The exact mechanism of c-Abl activation is not known however on the basis of data it was seen that its

activity relies on ataxia-telangiectasia-mutated (ATM) gene. Atm is a nuclear member of a family of phosphatidylinositiol-3-kinase like enzymes which phosphorylates c-Abl by binding at serine 465 residue. It is also reported that with regard to DNA-PK it used to activate a similar sequence of events [61].

Caspases cleave c-Abl into at least three fragments. Different caspases like Caspase 8, caspase 10 and the effector caspase 3 cleave c-Abl, and sequential cleavage depends on the action of different caspases.c-Abl may be involved in the G1-S checkpoint with an intact kinase domain. G1 arrest mechanism by c-Abl is unclear, however several observations shown that c-Abl binds with p53. c-Abl-p53 complex up regulates p21 in the DNA damage signaling pathway that causes G1 arrest. c-Abl encourages the functions of p53 by enhancing the expression of the target gene p21 and by enhancing its transcriptional functions. Indirect job of c-Abl in G1 arrest now obvious from that c-Abl can control the p53 level by inhibiting Mdm2-mediated degradation of p53 and supporting p53 accumulation [62].

p73 Mediated Growth Arrest:

p53 and its homologue p73 have highly significant sequences and functional similarities. p73 is an important developmental gene and also expected as a tumour suppressor. However, p73 activation after DNA damage induces by a way that is distinct from that of p53. p73, a member of the p53 gene family, which is phosphorylated by c-Abl. P73, a high sequence homologuos of p53 induces both cell cycle arrest G1 and apoptosis**(Fig. 9)**. Unlike p53, it can directly interact with c-Abl. The induction of G1-arrest by c-Abl remains unclear. The verity that p73 is able to encourage p21 expression, leads to G1 growth arrest [63, 64].

p73 Mediated Apoptosis:

Molecular mechanisms through which p73 induces apoptosis are remain unclear. p73, confined in the nucleus and remains in nucleus during apoptosis, indicates that p73 has indirect effect on Bax translocation. p73 can also directly transactivate PUMA and this direct effect of PUMA on Bax conformation and mitochondrial relocalization propose that there is a molecular association between mitochondrial apoptotic pathway and p73 [65, 66]. p73 mediated direct transactivation of PUMA occur rapidly and stronger than direct transactivation of Bax. PUMA used to translocates Bax into mitochondria and provides the fast conformational alterations of Bax from an inactive to an active state which can causes Bax mitochondrial translocation and release of cytochrome c induced by p73 mediated apoptosis [66]. Evidence provides several possible downstream effectors activation which are the transcription factors p73 and p53 and also the stress-activated kinases, p38 and JNK [67].

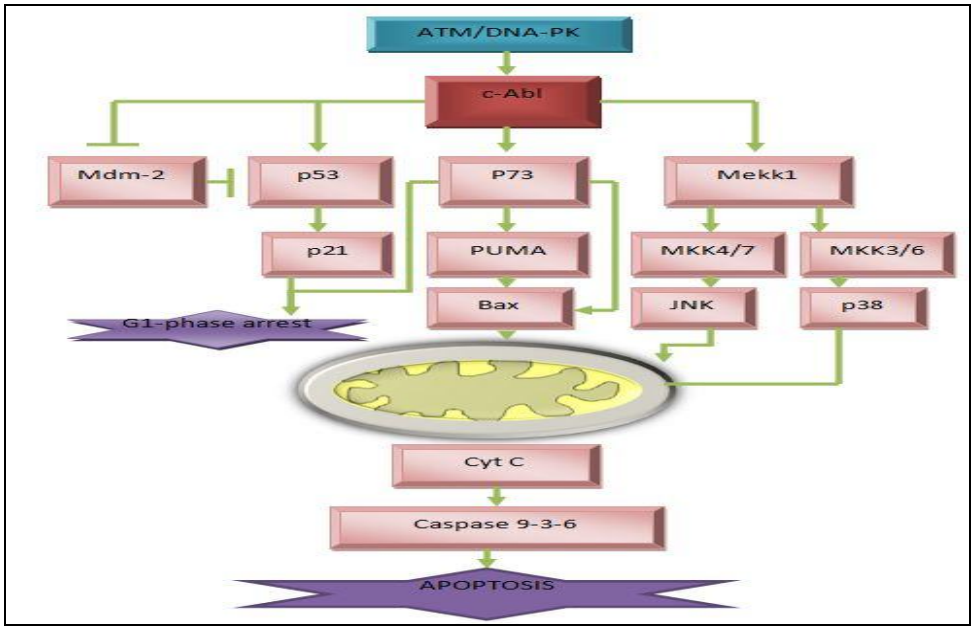

FIG. 9: cABL ACTIVATION AND ROLE OF p73 ISOFORM

Molecular Basis of Etoposide Resistance:

Ras/Raf/MEK/ERK pathway and cell survival:

After growth factor stimulation of the appropriate receptor, results in the packing of membrane-bound Ras with GTP. Ras can be activated by growth factor receptor tyrosine kinases [GFRTK]. Ras/GTP then engages Raf to membrane where it is activated.Both Raf and Ras are proto-oncogenes, and brings about the deregulation of the ERK signaling that entails anti-apoptotic pathway. In more than 30% of common cancers the deregulation of ERK pathways occur, therefore, Raf kinases are a striking target for novel treatments with aim to reverse the deregulation process**(Fig. 10)**. Raf-1 acts as deactivator of apoptosis via its target MEK/ERK that are capable to inhibit activated caspases; through stimulation of the anti-apoptotic factor NF-kB. NF-kBcontrols the expression of IAPs, phosphorylates Bad and cause itto relocate into cytosol from mitochondria, in this mannershielding the mitochondria [68]. Activated ERK can be translocated to the nucleus and phosphorylate additional transcription factors like CREB, Elk-1, Fos and globin transcription factor 1 (Gata-1) and others which can bind with the promoters of many genes, including growth factor and cytokine genes. These genes are vital in promoting growth and averting apoptosis of multiple cell types [69].

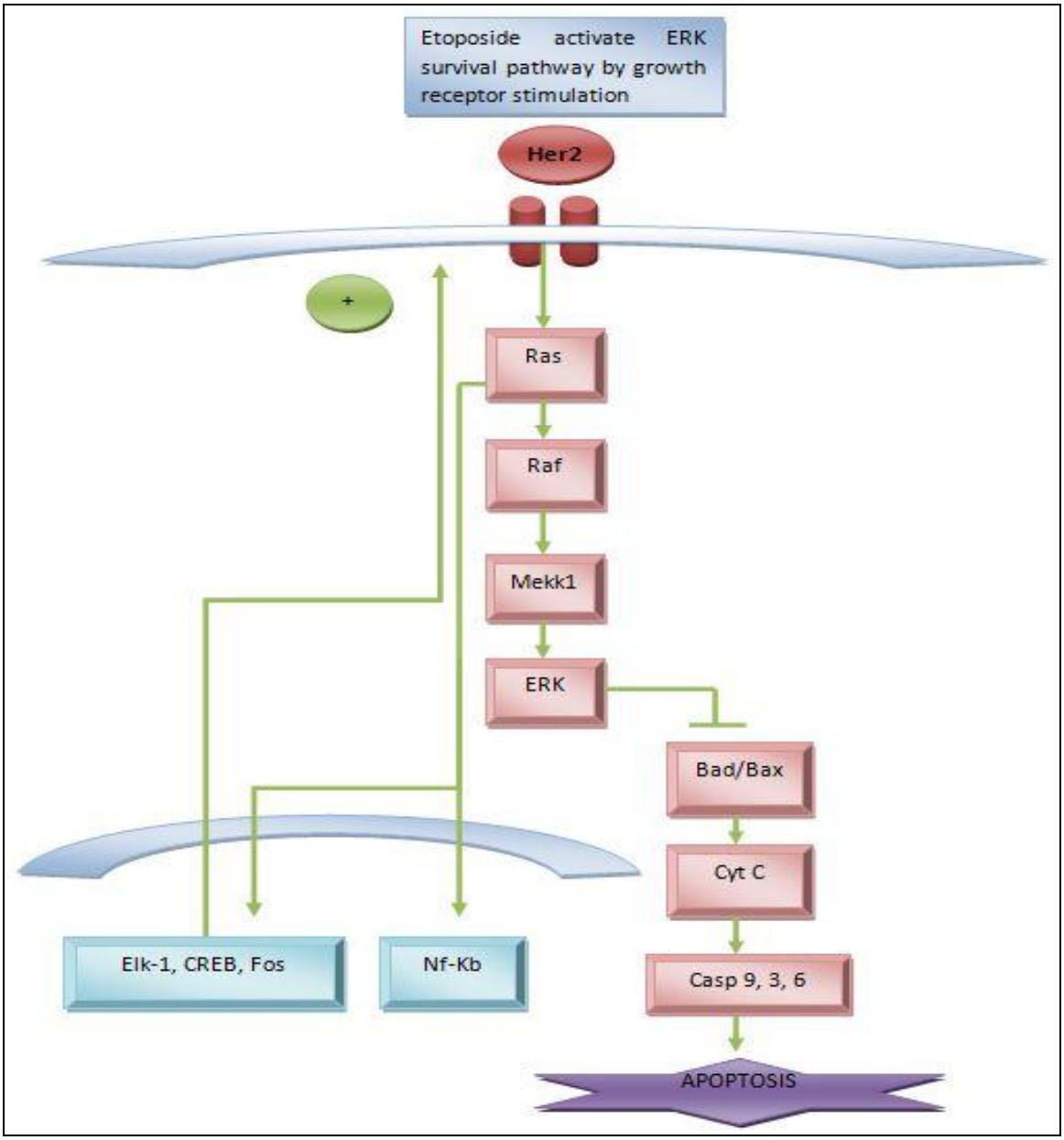

FIG. 10: ERK SURVIVAL PATHWAY

Role of AKT in cell survival:

The mainhindrance to booming treatment of cancer is the resistance in chemotherapy. Etoposide can stimulates PI3K (phosphoinoside-3 kinase) and Akt, activities in concentration and time-dependent manner [70]. Akt is a Serine/threonine kinase protein that is engaged as a mediator in many physiological responses such as stimulation of cell proliferation and inhibition of apoptosis. Three mammalian isoforms of Akt are currently known that are Akt1/PKB-Alpha, Akt2/PKB-Beta and Akt3/PKB-Gamma.

Growth factor receptor mediates signal transduction pathway that has been involved in granting resistance to chemotherapy on cancer cells via Akt apoptotic resistant pathway. HER2 (Human Epidermal Growth Factor Receptor 2) emerged to has a higher phosphorylation level of Akt. HER2 cause Akt activation through PI3K which associates with an augmented resistance of cells to several chemotherapeutic agents like etoposide [71, 72]. PI-3K is recruited to the phosphotyrosine residues and converts PIP2 into PIP3. PI3K can also be activated by Ras. The actions of Akt in the cell are diverse and numerous, but all eventually result in cell-proliferation and cell survival.

Akt hampers apoptosis by inhibiting the formation of the BAD/BclXLcomplex and phosphorylate the BAD component of this complex. In its antiapoptotic role Akt triggers IKK, which finally activates NF-kB and cell survival**(Fig. 11)**. Another interaction of Akt can be seen with death protease caspase 9, activity of which is decreased by direct inhibition through Akt. YAP, the target of Akt is another transcriptional factor that combines with 14-3-3 proteins and causes cytoplasmic localization and nuclear export. Akt is a major survival protein, and phosphorylates p21; a member of CDK inhibitors family, involved in cell cycle arrest and curtails cell proliferation. Akt also phosphorylates Ser 166 on MDM which then enters into nucleus to target p53 for degradation. Akt also activates mTOR which promotes cell proliferation by the synthesis of protein [73, 74].

Phosphorylation of XIAP (X linked inhibitor of apoptosis) through Akt hinders its autoubiquitination thus condensing apoptosis. The activity of Akt is impeded negatively by PTEN (Phosphatase and Tensin Homolog), SHIP (SH2-Containing Inositol Phosphatase) and CTMP (Carboxyl-Terminal Modulator Protein).

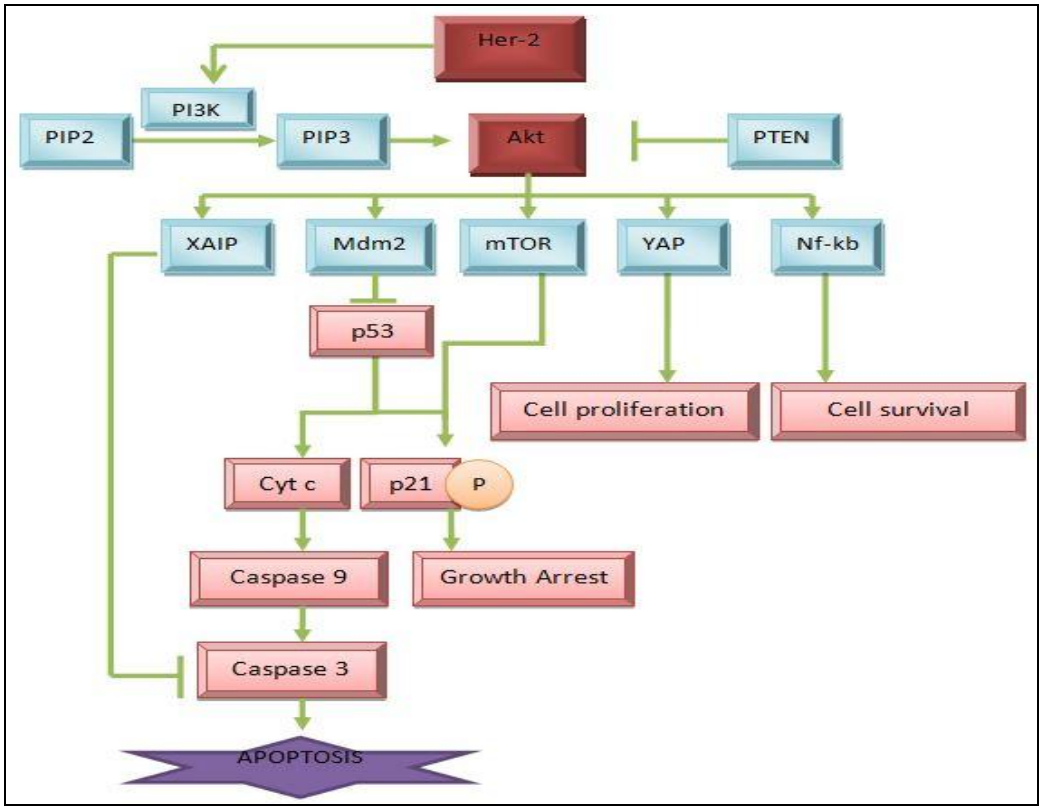

FIG. 11: AKT SURVIVAL PATHWAY

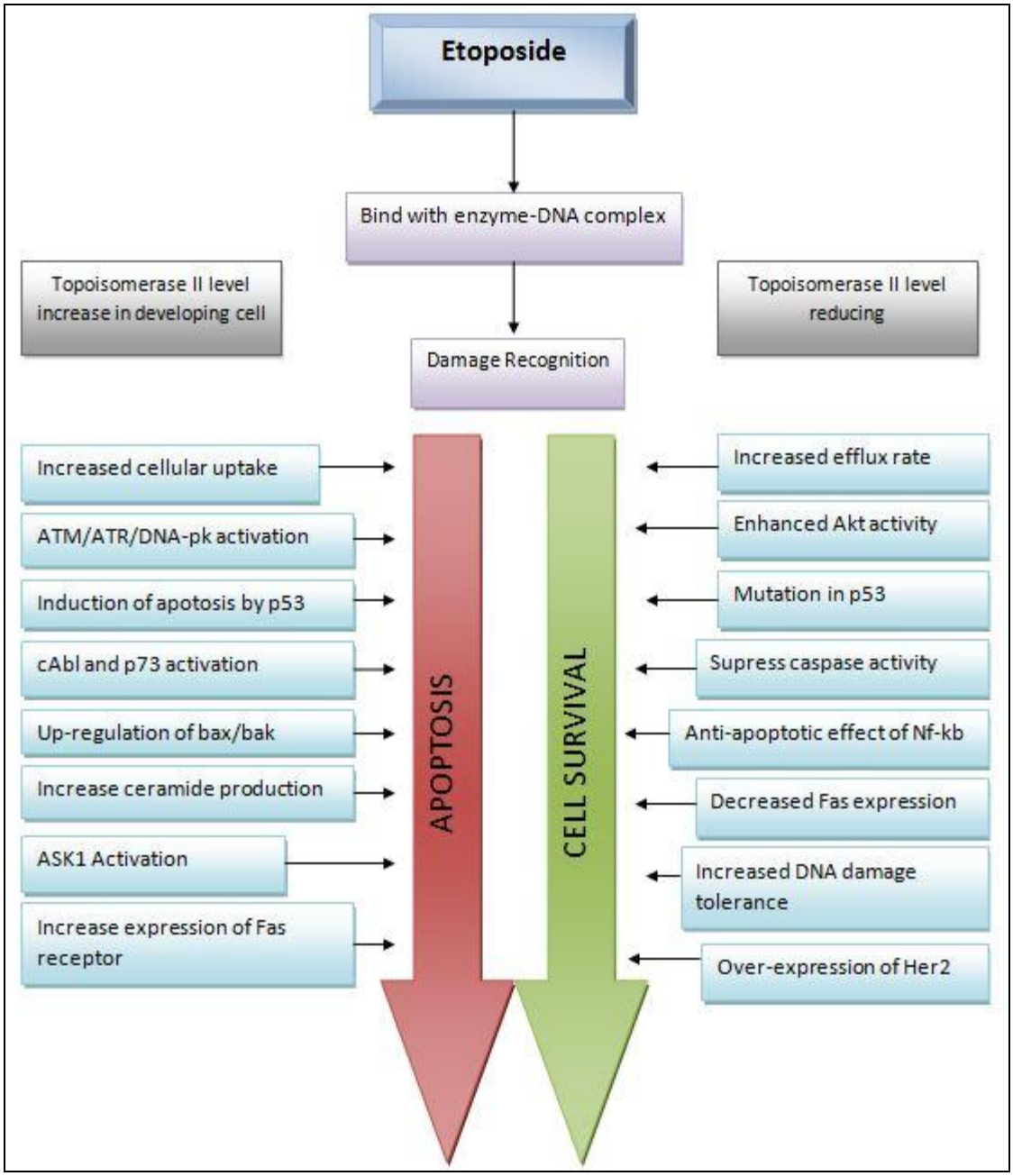

FIG. 12: CROSSTALK BETWEEN APOPTOTIC AND RESISTANT FACTORS

CONCLUSION: This review summarizes the molecular mechanism of etoposide, displaying its cytotoxic activity against a wide variety of tumors. Etoposide is a plant alkaloid derived from podophyllotoxin, and its mechanism of action purposed for its antineoplastic activity based on its interaction with topoisomerase II enzyme, causing DNA damage ultimately leading to cell death. In response to etoposide, DNA damage sensor proteins ATM, ATR, DNA-PKs activate signal transduction pathways. P53 dependent pathway causes growth arrest, apoptosis and induction of (Fas/TRAIL) death receptors. Increased production of ceramide contributes in activation of ASK1/MEKK1/JNK/p38 which have diverse role in apoptosis.

Etoposide mode of action not only havemeticulous role in apoptosis, but also encumber the skill of drug to bringdown apoptosis. Down-regulation of apoptotic signaling pathway is fundamentallyan entireattribute of resistance. Mechanisms that haltpropagation of DNA damage signal to apoptoticapparatus include activation of PI3-K/Akt and Ras/Raf via Her2 pathway.

Future Perspective:

In order to ameliorate the potency and selectivity of anti-cancer drugs, novel strategies need to be developed. Despite the significant role of etoposide in tumors, acquired resistance mediated by DNA repair enzymes or proteins, may impede apoptosis in preclinical settings. Fortunately, the understanding of major biochemical pathways of etoposide has opened new direction of pharmacological manipulations in chemotherapy. Advanced modulation of key cellular pathways directed to circumvent etoposide resistance may be complementary way to develop alternative strategies. In future, cancer therapy by etoposide must be directed to look for adjuvant drugs that effect biochemical mechanism that can bypass drug resistance, increase its influx rate and transportation in cell, rather to exclusively search for a specific drug with targeting particular cellular constituents.

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