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Review

Pancratistatin, an apoptic inducer: New horizon for targeted therapy in cancer

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Cancer is a major public health problem world wide and considered as the most dangerous disease and deaths are countable. Current cancer therapy does not target the specific target that causes the cancer and this leads to serious side effects which can be fatal. Targeted therapy is a new horizon for treating cancer at the specified sites with least side effects and more effective than the chemotherapy and other commercial methods used for cancer treatment. Different targets for cancer are mitochondria, Fas ligand, cell progress inhibition, cell membrane disruption, and induction of death receptor and enhance the apoptotic process by different pathways. Pancratistatin, a natural compound extracted from Hymenocallis littoralis, can selectively induce apoptosis by various pathways shows marked effectiveness on cancer cells. Apoptosis is one of the mechanisms, which remove the cells that are infected with pathogens or with abnormal cell cycle. Yield of pancratistatin and its derivatives are low and chemical synthesis required but not possible and hence the *E. coli* used for the transformation of organic compound for production of pancratistatin basic moiety. The present review article focuses on isolation of Pancrastatin and its possible mechanism of induction of apoptosis in jurkt cells and the different methods used to study an induction of apoptosis by them.

Key words: Pancrastatin, apoptosis, caspases, tumor necrosis factor (TNF).

INTRODUCTION

Cancer is a major public health problem and the burden of cancer is still increasing worldwide despite advances of diagnosis and treatment. It was estimated that in the year 2000, worldwide over 10 million new cases of cancer occurred (approximately 5.3 million men and 4.7 million women) and over 6 million people died from cancers (Parkin et al., 2000). Epidemiological studies have shown that many cancers may be avoidable. It is widely held that 80 - 90% of human cancers may be attributable to environmental and lifestyle factors such as tobacco, alcohol and dietary habits. Cancer prevention methods used today are still not promising as so many risk factors associated with them (Osborne et al., 1997). The most frequently affected organs are lung, breast, colon, rectum, stomach and liver. There is a need to find a new targeted therapy approach to cure different cancers with least side effects as it observed in current chemotherapy and radiation therapy. Today, a large number of drugs used to fight cancer are proapoptotic. The majority of proapoptotic cytotoxic drugs currently used to treat cancer patients take advantage of cell division itself in an attempt to achieve selective action, based on the more rapid division of cancer cells compared to their normal counterparts (Arkin, 2005) However, major problems with these molecules persist because they are not sufficiently selective for cancer cells, resulting in toxicity to normal cells and provoking widespread and serious consequences in patients (Arkin, 2005). Therefore, the need to identify potent anticancer agents that target tumor cells more selectively remains.

Natural products have played a highly significant role in the discovery and development of new drugs for the treatment of different cancers. More than 60% of drugs used in treatment of cancers are of natural origin. Plants of the Amaryllidaceae family have long been known for their toxicity and medicinal properties. Pancratistatin, a

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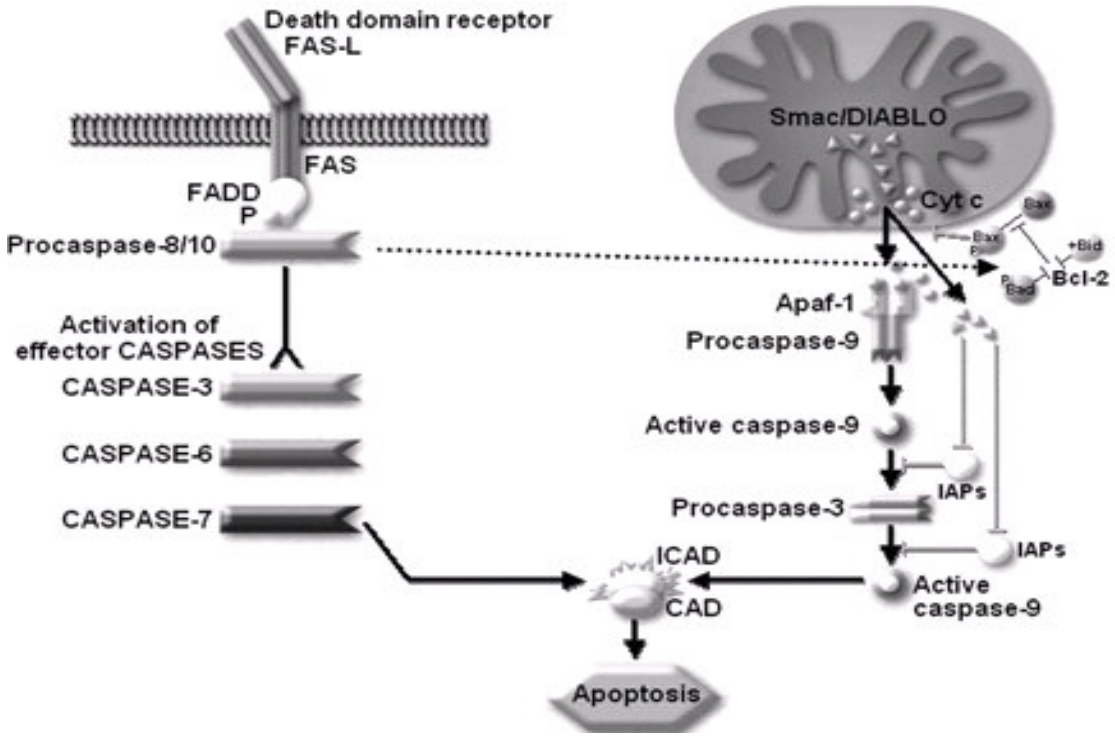

Figure 1. Apoptosis pathways. The extrinsic or extrinsic pathway is triggered through the Fas death receptor. The second pathway is the intrinsic or extrinsic pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal. Both pathways converge to a final common pathway involving the activation of caspases that cleave regulatory and structural molecules and culminate in the death of the cell.

natural compound isolated from the plants of the Amaryllidaceae family has been proven to be most effective apotic inducer molecule in the treatment. The present review study investigates the synthesis of pancratistain, apoptic mechanism and how this compound is used in treatment of cancer through the induction of apoptic pathways.

APOPTOSIS AND ITS PATHWAYS

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide). In more specific way, Apoptosis is a form of programmed cell death in multicellular organisms involves a series of biochemical events leading to a characteristic cell morphology and death, in more specific terms, a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Kerr et al., 1972).

The apoptosis pathways

Apoptosis occurs through two main pathways, namely extrinsic or cytoplasmic pathway and intrinsic or mitochondrial pathway. Extrinsic or cytoplasmic pathway is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily (Irene et al., 2002). The intrinsic or mitochondrial pathway leads to the release of cytochrome-c from the mitochondria and activation of the death signal. At last converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell (Figure 1).

THE EXTRINSIC PATHWAY

Extrinsic pathway comprises several protein members including the death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death

domain, and caspases 8 and 10, which ultimately activate the rest of the downstream caspases leading to apoptosis (Figure1). Activation of the extrinsic pathway is initiated with the ligation of cell surface receptors called death receptors (DRs). Fas is a member of the tumor necrosis factor receptor superfamily and are also called Apo-1 or CD95. Other TNF receptors include TNFR1, DR3 (Apo 2), DR4 (tumor necrosis factor-related apoptosis-inducing ligand receptor 1 [TRAIL R1]), DR5 (TRAIL R2), and DR6. (Scaffidi et al., 1998).The membrane-bound FasL interacts with the inactive Fas complexes and forms the death-inducing signaling complex. The Fas death-inducing signaling complex contains the adaptor protein Fas-associated death domain protein and caspases 8 and 10 and leads to activation of caspase 8, which in turn can activate the rest of the downstream caspases. In some cells, the activation of caspase 8 may be the only requirement to execute death, while in other cell types; caspase 8 interacts with the intrinsic apoptotic pathway by cleaving Bid (a proapoptotic member of the Bcl-2 family), leading to the subsequent release of cytochrome-c. Regulators of the pathway include transcription factors such as NFκB and activating protein 1 that regulate the FasL gene; because it is a transcriptionally inactive gene other inhibitors of the pathway include FAP-1, Fas-associated-death-domain-protein like interleukin-1β-converting enzyme-like inhibitory protein, and the soluble decoy receptors such as DcR3, TRAIL R-3/DcR1, and TRAIL R-4/DcR2. These decoy receptors antagonize the stimulation of Fas by FasL though competition with the ligand (Irie et al., 2001).

The intrinsic pathway

BCL-2 is the key regulator of intrinsic pathway and overexpressed in malignancies. The Bcl-2 family includes proapoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, and antiapoptotic members such Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1. Antiapoptotic Bcl-2 members act as repressors of apoptosis by blocking the release of cytochrome-c, whereas proapoptotic members act as promoters. These effects are more dependent on the balance between Bcl-2 and Bax than on Bcl-2 quantity alone. Following a death signal, proapoptotic proteins undergo posttranslational modifications that include dephosphorylation and cleavage resulting in their activation and translocation to the mitochondria leading to apoptosis. All BH3-only molecules require multidomain BH3 proteins (Bax, Bak) to exert their intrinsic proapoptotic activity. In response to apoptotic stimuli, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome-c and second mitochondria-derived activator of caspase (also called direct IAP-binding protein with low pI). Cytochrome-c, once released in the cytosol, interacts with Apaf-1, leading to the activation of caspase-9 proenzymes. Active caspase-9 then activates caspase-3, which subsequently activates the rest of the caspase cascade and leads to apoptosis. Activated caspases lead to the cleavage of nuclear lamin and breakdown of the nucleus through caspase-3.

The final pathway

The final pathway executes the death signal, is the activation of a series of proteases termed caspases, but caspases-3, -6, -7, -8, and -9 are actively participating in apoptosis. The intrinsic and extrinsic apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribonuclease, and the caspase-activated deoxyribonuclease becomes active leading to nuclear apoptosis. The upstream caspases that converge to caspase-3 are caspases-9 and -8 in the intrinsic and extrinsic pathways, respectively. The downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, inhibitory subunits of endonucleaes (CIDE family), and finally, destruction of "housekeeping" cellular functions. Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing (Mancini et al., 1998).

DEVELOPING APOPTOSIS-TRIGGERING THERAPEUTIC STRATEGIES

Apoptosis is a gene-controlled process and hence it is susceptible to genetic manipulation with therapeutic purposes. From a mechanistic point of view two types of approaches can be distinguished: 1. strategies that directly induce apoptosis, named here as pro-apoptotic approaches; and 2. strategies that modulate survival signaling pathways thereby facilitating the occurrence of apoptosis, called here permissive approaches. A summary of the stage of development of several strategies to induce tumor cell apoptosis is provided in Table 1.

Proapoptotic approaches

Apoptosis can be achieved through the exploitation of existing cellular players and pathways such as death receptors and caspases, or the introduction of exogenous proapoptotic molecules such as Apoptin. Proapoptotic strategies can involve: (a) direct introduction of proapoptotic players; (b) modulation of antiapoptotic molecules; or (c) restoration of tumor suppressor gene function.

Direct introduction of proapoptotic players

Activation of death receptor pathways: Receptors of the TNF-α3 super-family can be subdivided into two

Table 1. Strategy for induction of apoptosis.

Strategy	Target	Approach	Stage of development
Proapoptotic approaches			
Introduction of proapoptotic players	TRAIL	Recombinant therapy	Clinical trials palnned
	Apoptin	Gene therapy	Preclinical
	caspases	Gene therapy	Preclinical
Modulation of antiapoptotic genes or pathways	Mitochondria:		
	Proapoptotic molecules (Bax, BCL-Xs)	Gene therapy	Preclinical
	Down regulate antiapoptotic molecules (Bcl-2, Bcl-XL)		
	Direct effect on mitochondria	ODNs	Phase II/III
	Direct effect on pores		
		Lonidamine, arsenite	
		PK 11195	
Restoration or manipulation of tumor suppressor genes	p53	Gene therapy	Phase II/III
	Retinoblastoma	Gene therapy	Preclinical
	FHIT	Gene therapy	Clinical trials palnned
Permissive approaches	PI3k	LY294002	Preclinical
Oncogenes	Ras	Small molecules, ODNs	Phase II/III
	BCR-ABL	Small molecule (STI-571)	Phase III
	NFkB	ODNs	Phase I/II
	Proteasome inhibitors	PS-341	Phase II
	c-raf	ODNs	Phase II
	c-myb	ODNs	Preclinical
	Cell cycle modulators	UCN-01, flavopiridol	Phase III

groups, based on the presence or absence of a cytoplasmic DD Among the DD-containing members of the TNF super-family (death receptors) are TNFR-1, Fas (Apo-1 and CD95), DR3, DR4, DR5, and DR6. Binding of three ligand molecules to a homotrimeric death receptor molecule leads to clustering of the receptor DDs and aggregation of signaling molecules to form a functional DISC within the cell. Initiator procaspase-8, recruited to the DISC by virtue of its DEDs, becomes activated by autoproteolysis and dissociates from the DISC to initiate the activation of the caspase cascade.

a. Synthetic activation of caspases

Caspases are present in the cells as procaspases, and activation of procaspases requires cleavage at caspase consensus sites in their proenzyme structures, these enzymes can be activated either autocatalytically or by other hierarchical fashion. The caspase cascade includes “initiator” proteases, such as caspase-8, -9, and 10, which activate “machinery” proteases, such as caspase-3 and caspase-7 (Boldin, et al., 1996). Initiator caspases are activated by proapoptotic signals and contains prodomains that can physically link to adaptor molecules containing similar domains via homophylic interactions. The two major routes of activation of the caspase cascade, the death receptor and the mitochondria pathways, use DEDs and CARDs, respectively.

b. Apoptin

Apoptin (VP3), a Mr 14,000 protein derived from chicken anemia virus. In vitro results show that Apoptin is very active against cancer cells, without inducing toxicity to normal cells. This tumor-specific effect might be explained by the nuclear localization of the protein in tumor cells whereas in normal cells the protein localizes in the cytoplasm. Apoptin is equally active in genetically disrupted and potentially chemoresistant cells, such as p53-mutant, Bcl-2-overexpressing or BCR-ABL expressing tumor cells (Du et al., 2000).

Modulation of antiapoptotic players

Targeting mitochondria: Mitochondria contain apoptogenic proteins that are released into the cytoplasm during apoptosis which include cytochrome c, Smac/DIABLO, EndoG, heat shock protein-60, and apoptosis-inducing factor are released. Cytochrome C in the cytoplasm allows interaction with the CARD-containing adapter protein Apaf-1 (Costantini et al., 2000), ATP, and procaspase-9 via a CARD-CARD interaction forming a "apoptosome", ternary complex. This holoenzyme activates procaspase-9 and activated caspase-9 activates downstream caspase zymogens, starting the caspase cascade.

The role of mitochondria in apoptosis is complex and is considered as "point of no return" in the apoptotic process, and hence the manipulation of mitochondria activation as a potential therapeutic approach. Activation of mitochondria is accompanied by the translocation of cytochrome c from the mitochondrial intermembrane space into the cytoplasm and may involve a large mitochondrial conductance channel called the PTPC (Gross, 2002). Indirect mitochondria activation can be achieved by balance between proapoptotic and antiapoptotic members of the Bcl-2 family. This can be done either by downregulation of antiapoptotic molecules (e.g., antisense ODNs against Bcl-2) or by up-regulation of the proapoptoic counterparts (e.g., gene therapy with Bax) (Birnbaum, 1994).

Targeting IAPs: Caspase-9 is controlled by a regulatory system based on endogenous inhibitors. IAPs were originally identified in the genome of baculoviruses on the basis of their ability to suppress apoptosis in infected host cells. Members of the IAP family contain one to three modules of a common 70-amino acid zinc-binding motif called the baculoviral IAP repeat domain, which is critical for the antiapoptosis function. Several human cellular homologues of the baculovirus IAPs have been identified such as NAIP, c-IAP1, c- IAP2, XIAP, survivin, Apollon, Livin, and others. Another player in the balance between caspases and IAPs is the molecule Smac/DIABLO. This molecule is an apoptosis-promoting factor released by mitochondria that antagonizes the function of IAPs. In addition, another molecule called Omi/HtrA2 has been described recently as able to antagonize the antiapoptotic function of IAPs (Harris, 1996).

Restoration of function of tumor suppressor genes

Loss or mutation of p53 is a very common genetic abnormality in cancer. Initial Phase I p53-based gene therapy trials suggested that p53 replacement could lead to an increase in apoptosis in tumor cells and surrounding cells as a bystander effect. Reintroduction of other tumor suppressor genes has also resulted in an increase in tumor cell apoptosis. When both p16INK4 and wild-type p53 were transduced into cancer cells, a synergistic apoptotic effect was observed. Furthermore, re-expression of the FHIT tumor suppressor gene has also been associated with induction of apoptosis (Ji et al., 1999). Gene therapy strategies using FHIT are under development.

Apoptosis-permissive approaches

The ubiquitous distribution of the apoptotic machinery in cells requires that apoptosis be tightly controlled. Several intricate signaling pathways mediate survival messages and blockade of some of these pathways increase in apoptosis in cancer cells (Carlos et al., 2002). Inhibition of NFKB increases the apoptosis in cancer cell because normal cells NFKB is sequestered in the cytoplasm and inactive and hence its blockade by therapy would not harm normal cells. The 26S proteasome regulates protein turnover in eukaryotic cells. Because large repertoires of human proteins are regulated by the ubiquitin-mediated proteasome pathway, any alteration of this machinery could favor cell transformation through disturbances in cell cycle, tumor growth, and survival. Compounds PS-341 (Millennium Pharmaceuticals, Inc.) that inhibits the proteasome has been shown to be active in several animal models of inflammation and cancer. PI3k/Akt pathway is much more active in cancer cells than in normal cells may provide a potential increase of therapeutic index. Preclinical studies are ongoing and should address the potential effect that the blockade of the PI3k will have on the induction of apoptosis in cancer cells.

PANCRATISTATIN AND RELATED AMARYLLIDACEAE ALKALOIDS

Amaryllidaceae family and alkaloids

The Amaryllidaceae is the family of amaryllis and related plants. Members of the giant group Lilianae; the amaryllids are mostly bulbous plants, although some have a rhizome. Alkaloids derived from TYRAMINE combined with 3, 4-dihydroxybenzaldehyde via a norbelladine pathway, including galantamine, lycorine and crinine.

EXTRACTION OF PANCRATISTATIN FROM AMARYLLIDACEAE FAMILY

The bulb section of this native Hawaiian plant was extracted with dichloromethane-methanol-water and the extract was concentrated to obtain an aqueous phase.

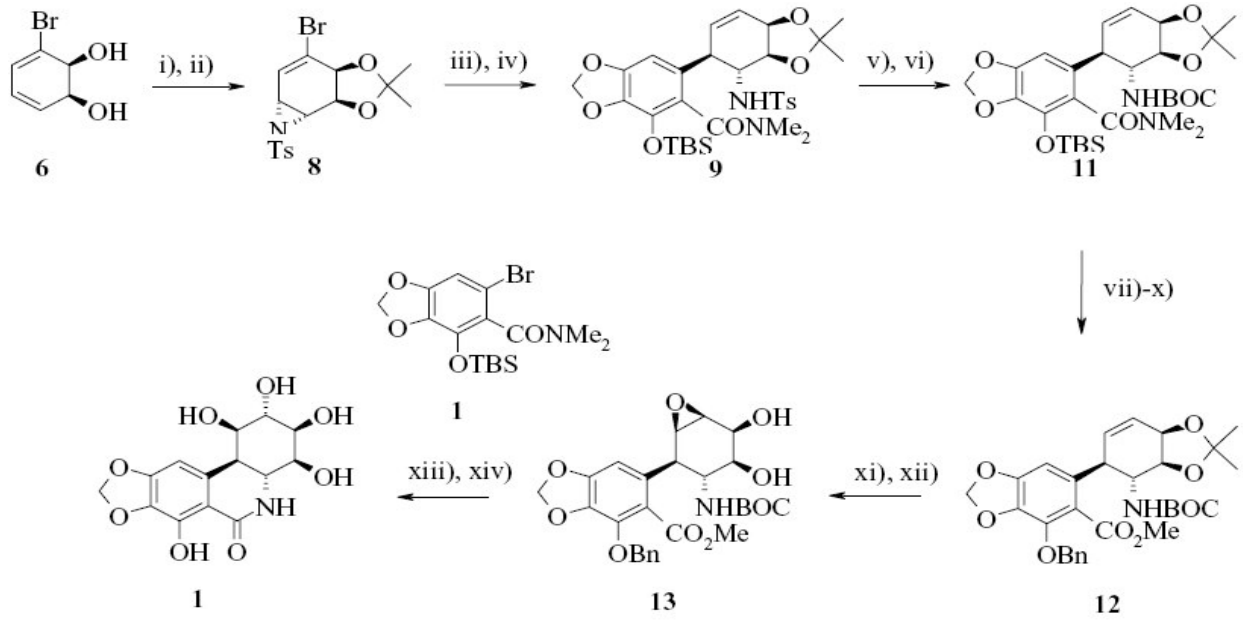

Figure 2. First enantioselective total synthesis of pancratistatin.

Organic compounds were extracted with n-butanol and purification of the resulting crude product was undertaken using gel permeation chromatography (Sephadex LH-20) and precipitation/crystallization to give the pure natural product. This experimental procedure is very effective for laboratory-scale isolation of pancratistatin, but cost is too high for large scale production (Pettit et al., 1986). The rare tropical bulbs of *H. littoralis* seem to be a good source of pancratistatin as it can be isolated from these in the order of 100 to 150 mg/kg when bulbs are obtained from the wild in Hawaii. However, the compound has to be commercially extracted from field- and greenhouse-grown bulbs or from tissue cultures cultivated, for example, in Arizona, which generate lower levels of pancratistatin (a maximum of 22 mg/kg) even in the peak month of October. After October, when the bulb becomes dormant, levels of pancratistatin drop to only 4 mg/kg by May. Field-grown bulbs, which show monthly changes in pancratistatin content, generate somewhat smaller amounts (2-5 mg/kg) compared to those grown in greenhouse cultivated over the same period. Consequently and to date, the best sources of pancratistatin are rare, tropical, and subtropical bulbs of *Z. flava*, *H. kalbreyeri*, and *H. littoralis* when these plants are cultivated in their native region. (Laurent et al., 2008)

SYNTHESIS OF PANCRATISTATIN AND ITS DERIVATIVES

Bioavailability of pancratistatin is poor and solubility too and hence chemical synthesis and its modification to lead moiety can approached by using chemoenzymatic synthesis. This can be achieved by recombinant strain, Toluene dioxygenase (TDO) over expressed *E. coli* mJM109 (pDTG601).

This converts bromobenzene to bromochrial diol 5 and dihydrodiodehydrogenase (DHDH) enzyme from similar organism, *E coli* JM109 (pDTG602), which leads to functionalized catechols 6. The diol derived from bromobenzene was converted to aziridine 8 and combined with the functionalized benzamide 10 to yield, in four steps, the core of the target with four of its six centers correctly installed, and Figure 2.

Reagents and conditions

i) DMP, p-TSA, (cat.), acetone, r.t.; ii) PhI=NTs, $Cu(acac)_2$, CH_2CN, r.t., 45% (over 2 steps); iii) Bu_3SnH, AIBN, THF, reflux, 78%; iv) 1, s-BuLi, TMEDA, THF, CuCN, r.t., -78°C; v) s-BuLi, THF, $(BOC)_2O$; vi) Na/anthracene, DME, -78°C; vii) SMEAH/morpholine, THF, -45°C; viii) BnBr, K_2CO_3, DMF; ix) $NaClO_2$, KH_2PO_4; x) CH_2N_2; xi) HOAc,THF, H_2O, 60°C; xii) t-BuOOH, $VO(acac)_2$, C_6H_6, 60°C; xiii) H_2O, BzONa (cat.), 100°C; xiv)H_2, Pd(OH)2/C, EtOAC.

MECHANISM OF PANCRATISTATIN AND ITS DERIVATIVES

Pancratistatin (PST) has been shown to possess anti-cancer potential but still specificity and mechanism of PST to cancer cells action remain unknown. But study shows that PST induced apoptosis selectively in cancer cells and that the mitochondria may be the site of action of PST in cancer cells.

Blocks the progression of cell cycle at least at G0/G1 and S phases

Pancratistatin derivatives, 1-O-(3-hydroxybutyryl) pancratistatin (HBP) and 1-O-(3-O-b -D-glucopyranosylbutyryl) pancratistatin (GBP), showed strong cytostatic activity against rat embryo fibroblast 3Y1 at concentrations less than 1 mM. When the effect on cell cycle progression was examined in 3Y1 fibroblasts HBP, GBP, and pancratistatin inhibited the progression of 3Y1 fibroblasts from G0/G1 to S phase. While effect of HBP and GBP on the progression was evaluated in promyelocytic leukemia HL-60RG cells synchronized at G0/G1 phase, the cells did not progress into S phase and accumulated in sub G0/G1 phase, which indicated apoptotic cells. These findings suggest that of Amaryllidaceae alkaloids, HBP blocks the progression of cell cycle at least at G0/G1 and S phases and GBP does at least at G0/G1 phase, resulting in apoptosis induction in tumor cells. (Mutsuga et al., 2002)

Triggering the activation of death receptor and release of cytochrome C

Amaryllidaceae isocarbostyril narciclasine induces marked apoptosis mediated cytotoxic effects in human cancer cells but not in normal fibroblasts by triggering the activation of the initiator caspases of the death receptor pathway (caspase-8 and caspase-10) in human MCF-7 breast and PC-3 prostrate carcinoma cells. The formation of the Fas and death receptor 4 (DR4) death-inducing signaling complex was clearly evidenced in MCF-7 and PC-3 cancer cells. Caspase-8 was found to interact with Fas and DR4 receptors on narciclasine treatment. Narciclasine-induced downstream apoptotic pathways in MCF-7 cells diverged from those in PC-3 cells, where caspase-8 directly activated effector caspases such as caspase-3 in the absence of any further release of mitochondrial proapoptotic effectors. In MCF-7 cells, the apoptotic process was found to require an amplification step that is mitochondria-dependent, with Bid processing, release of cytochrome C, and caspase-9 activation. High selectivity of narciclasine to cancer cells might be linked to activation of the death receptor pathway. Normal human fibroblasts appear approximately 250-fold less sensitive to narciclasine, which does not induce apoptosis in these cells probably due to the absence of death receptor pathway.

Early activation of caspase-3 and the flipping of phosphatidyl serine

Pancratistatin seemed to show more specificity than VP-16 or paclitaxel as an efficient inducer of apoptosis in human lymphoma (Jurkat) cells, with minimal effect on normal nucleated blood cells. Caspase-3 activation and exposure of phosphatidyl serine on the outer leaflet of the plasma membrane were earlier events than the generation of ROS and DNA fragmentation observed following pancratistatin treatment. This indicates a possible involvement of caspase-3 and plasma membrane proteins in the induction phase of apoptosis. Pancratistatin does not cause DNA double-strand breaks or DNA damage prior to the execution phase of apoptosis in cancer cells (Kekre, 2005).

METHODS OF APOPTOSIS INDUCTION IN JURKT CELLS BY AMARYLLIDACEAE ALKALOIDS

Induction of apoptosis in Jurkt cells by Amaryllidaceae alkaloids

A systematic synthetic approach has been used to determine the minimum cytotoxic pharmacophore from the pancratistatin ring system and find the trans-fused b/c-ring system containing the 2, 3, 4-triol unit in the C-ring. It has also been noted that the 2, 3-diol derivatives have significant activity, indicating that the C3-hydroxyl is a moderating influence. The C7 phenolic and C1 hydroxyl functions are not essential. Here evaluation of the structure- activity relationships for two AMD alkaloids, AMD4 and AMD5 (Figure 3). Both these compounds lack the multiple hydroxyl groups but instead have a methoxy group that varies in orientation between AMD4 (α) and AMD5 (β). Our results indicated that like PST, AMD5 has the capability of selectively inducing apoptosis in cancer cells while sparing normal cells, albeit at a concentration 10-fold higher.

Hoechst staining method

This method was used to study apoptosis induction by pancrastatin alkaloids. In this, Jurkat cells were incubated with different concentrations of AMD4 and AMD5 for up to 72 h. The degree of apoptosis was observed by Hoechst staining, where condensed, brightly stained nuclei indicated apoptotic cell death. The number of apoptotic nuclei was expressed as a percentage of the total number of cells in a dose dependent manner; alkaloid AMD5 at a concentration of 10 μM incited apoptosis in over 40% of Jurkat cells after 48 h (Figure 4 and 5a). In contrast to this finding, alkaloid AMD4 had a minimal effect on cancer cell viability under similar treatment conditions.

Annexin-V assay method

Annexin-V assay was carried out at several time-points in order to monitor phosphatidyl serine flipping to the outer leaflet of the plasma membrane, which is a characteristic apoptotic event. Annexin-V staining is specific to

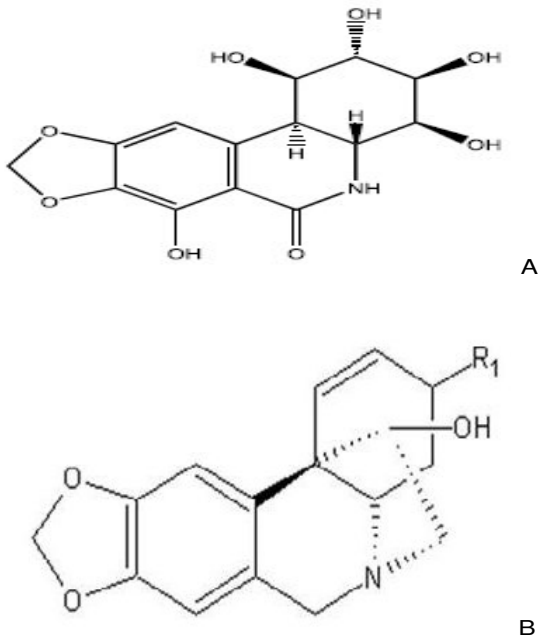

Figure 3. Chemical structure of native Pancratistatin (A) and of Amaryllidaceae alkaloids AMD4 and AMD5 (B). The Amaryllidaceae compounds have a methoxy group that varies in orientation between AMD4 (α) and AMD5 (β).

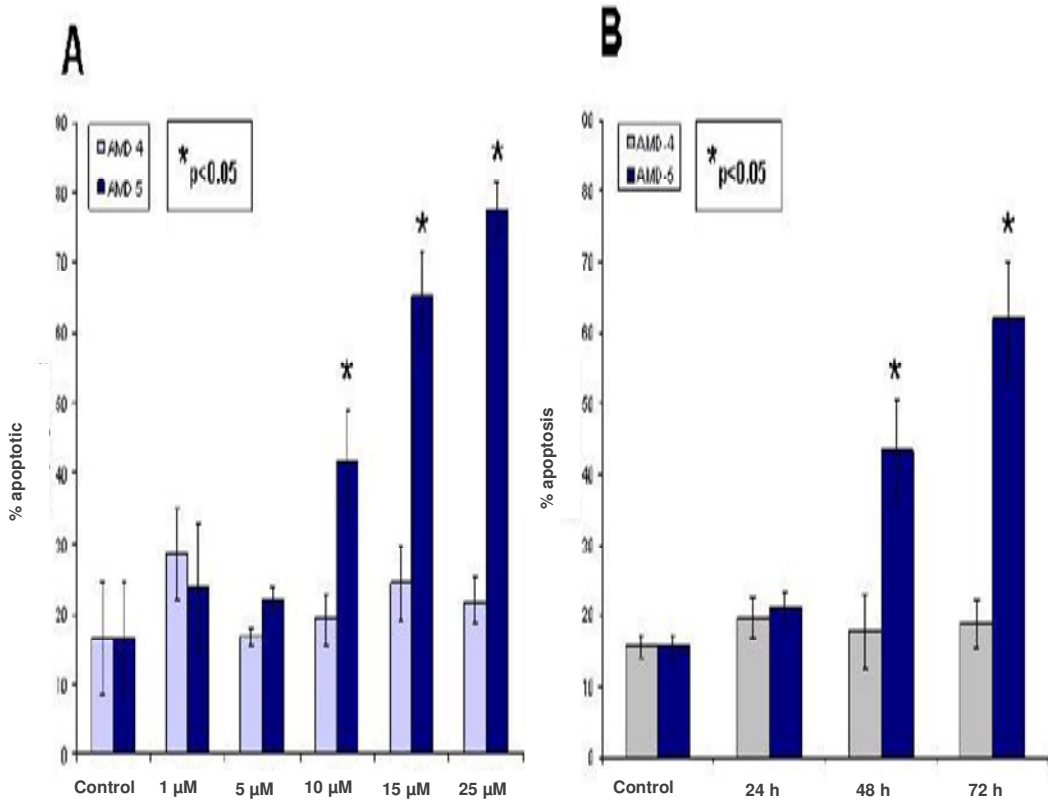

Figure 4. A Dose-response curve for Jurkat cells treated with various concentrations of either AMD4 or AMD5 for 48 h. B. Time Course: A measurement of the degree of apoptosis induced bin Jurkat cells treated with 10 µM of either AMD4 or AMD5 over 72 h.

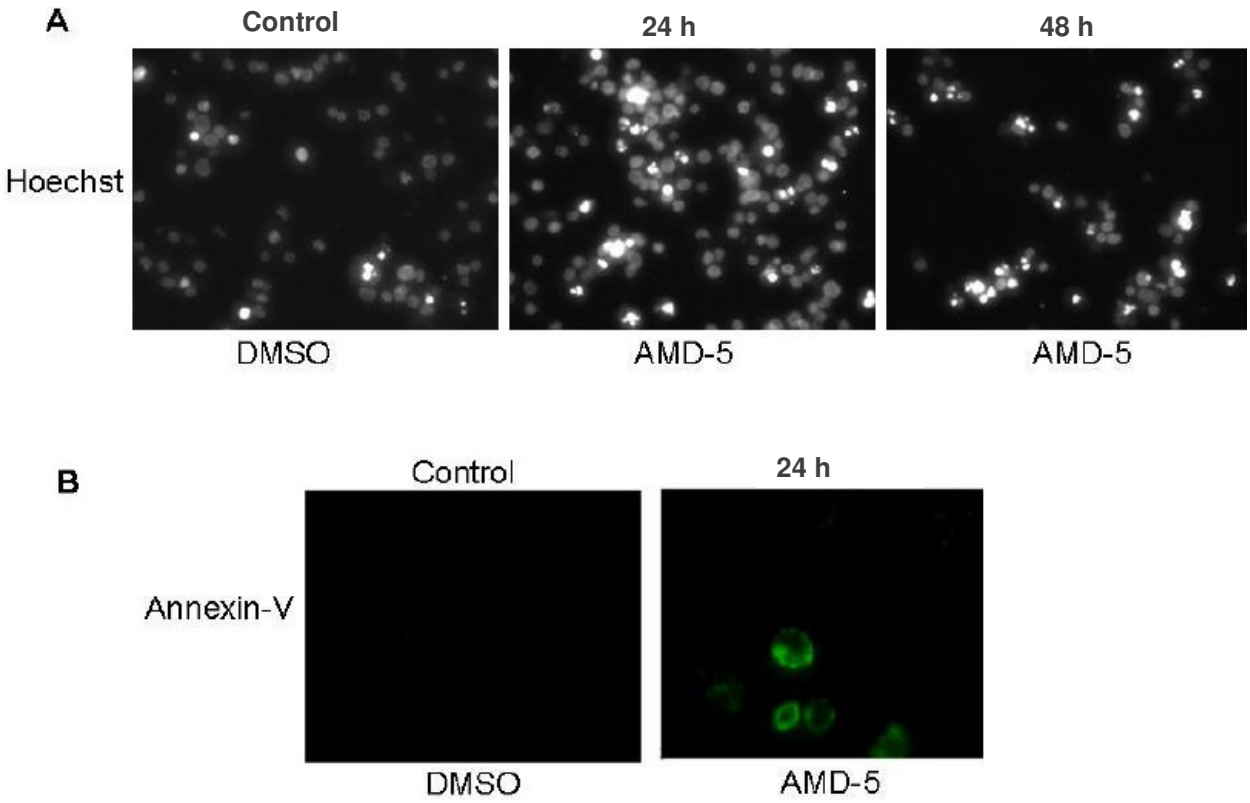

Figure 5. Morphology of Jurkat cells treated with AMD5 at 10 µM for either 24 h or 48 h: A. Nuclear Morphology of Jurkat cells treated with AMD5 at 10 µM for either 24 or 48 h. Jurkat cells were stained with cell-permeable Hoechst 33342 dye to observe apoptotic nuclear morphology. Apoptotic nuclei are brightly stained and condensed when compared to healthy nuclei. The control was treated with DMSO solvent. Magnification: 200×. B. Annexin-V Binding Assay: Jurkat cells were treated with 10 µM AMD5 for 24 h. Jurkat cells were incubated with Annexin-V 488 Alexa-Fluor conjugate to observe phosphatidyl-serine flipping from the inner to the outer leaf of the plasma membrane. Cells undergoing apoptosis will have more Annexin-V substrate bound, and thus appear brighter than healthy cells. Magnification: 400×

apoptotic cells, and background staining is low in unaffected cells (Kekre et al., 2005). Jurkat cells incubated with 10 µM AMD5 resulted in a high incidence of phosphatidylserine flip; approximately 45% of Jurkat cells were observed to be labeled with Annexin-V-FITC (Figure 5 b).

TUNEL assay method for apoptosis induction in jurkt cells

TUNEL (terminal transferase dUTP nick end labeling) assay show that DNA fragmentation occur after the alkaloid AMD5 treatment at 10 µM. activation of caspase-3,-7 and -9 are also increased 3 fold as compare with the control by AMD5 rather than where only slightly increase of caspase-3 activation. Effect of amaryllidaceae alkaloid AMD5 on the normal mononuclear cells was observed by the Hoechst dye for apoptotic morphology and results show that minimal effect on viability of normal nucleated blood cells in comparison to untreated cells. Effect of AMD4 was not tested. Functionalized phenanthridone skeleton present in natural Amaryllidaceae alkaloids may be a significant common element for selectivity against cancer cells, and the configuration of the methoxy-side groups is responsible for higher binding affinity to the target protein/s thus making for a more efficient anti-cancer agent (Carly et al., 2007).

CONCLUSION

Apoptosis is strictly regulated by cell machinery using Fas, Caspases, Death receptor and TNF receptors. Pancratistatin, alkaloids from the amaryllidaceae family induce the apoptosis in cancer cell by different mechanisms like up-regulation of Fas, increase in caspase-3, destabilization of mitochondrial membrane potential and flipping of phosphatidyl serine. Pancratistatin is an inducer of the apoptosis in the cancer cells and produce lesser cytotoxic effect on the normal cells. AMD5 exhibit potent effect on cancer cell viability that the AMD4. This cytotoxicity effect of Pancrastatin

(Amaryllidaceae alkaloids) is attributed by the presence of functionalized phenanthridone skeletonin in natural Amaryllidaceae alkaloids. This natural product can reveal and unveil the horizon for the new-targeted therapy in cancer due to its least toxicity on normal healthy cells and proved to be an effective and promising drug candidate for cancer therapy. Due to lesser yield of pancratistatin from the plant source, chemical synthesis by chemoenzymatic process is a significant pathway for making library for the pancratistatin and its derivatives.

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