CELLULAR AND MOLECULAR PARAMETERS OF SANGUINARINE INDUCED BIMODAL CELL DEATH

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By

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ABSTRACT

Sanguinarine is a potential anti-cancer drug belonging to the benzophenanthridine alkaloids. K562 erythroleukemia cells treated with sanguinarine at concentrations of 1.5 μg/ml for 2 hours displayed the morphology of apoptosis or programmed cell death (PCD) and at 12.5 μg/ml, displayed the morphology of blister formation or blister cell death (BCD). This dual modality of cell death processes was coined "bimodal cell death" (BMCD). The present study is devoted to the cellular and molecular characterization of bimodal cell death.

Sanguinarine-induced PCD in the Bcl-2 (anti-apoptotic) low-expressing K562 erythroleukemia cells was found to have increased expression of the pro-apoptotic Bax protein as well as caspase-3 activation, whereas BCD was found to have neither. cDNA expression array analysis of sanguinarine induced PCD in K562 cells failed to reveal the presence of Bax at the gene transcript level, which suggests the relevance of post-translational modification of proteins as opposed to its *de novo* synthesis. On the other hand, high Bcl-2 protein expressing JM1 pre-B lymphoblastic cells, when treated with sanguinarine, failed to undergo the morphology of either PCD or BCD. Western blotting analysis of JM1 cells treated with sanguinarine showed an increase in Bcl-2 protein, over and above its high endogenous levels while there was no detectable Bax expression or caspase-3 activation. Bcl-2 appeared to have played the role of being anti-PCD as well as anti-BCD in JM1 cells during sanguinarine treatment. The role of several other members of the Bcl-2 family: Bad, Bak, Bcl-x_L, Bcl-x_S, Hrk, NBK, Bik, Bid as well as the tumour suppressor protein p53 was found to be negligible.

A comparative analysis of PCD and BCD in K562 and JM1 cells treated with sanguinarine was also done at the cellular level using the following methods: light and electron microscopy; terminal deoxynucleotidyl transferase (TdT) end labeling; ^{51}Cr release; trypan blue exclusion; propidium iodide exclusion and annexin-V-binding. These studies further characterized PCD and BCD at the cellular level in K562 cells, and at the same time highlighted the anti-PCD and anti-BCD roles played by Bcl-2 in sanguinarine-resistant JM1 cells.

Immunofluorescence-flow cytometry studies of protein expression in K562 cells treated with sanguinarine showed a high percentage of Bax positive cells with low levels of the nuclear transcription factor NF-κB in PCD, and a high percentage of Bax negative cells with high levels of NF-κB in BCD. JM1 cells showed elevated levels of Bcl-2 in response to sanguinarine treatment. This flow cytometry study also helped confirm the findings of Western blot analysis. Thus, the interaction between Bcl-2, Bax and NF-κB might be crucial to understand sanguinarine-induced PCD and BCD.

Sanguinarine was also found to overcome the P-glycoprotein-mediated drug efflux pump, widely believed to be associated with the classical multidrug resistant (MDR) phenomenon, in the vinblastin resistant CEM-VLB leukemia cells. Studies pertaining to the cellular and molecular parameters of sanguinarine induced PCD and BCD in the MDR CEM-VLB cells and in their wild type counterparts CEM-T4 (P-gp negative), showed that the bimodal cell death pattern in both cell lines was qualitatively similar. Moreover, flowcytometric studies in these cells showed an increased presence of sanguinarine in cells during BCD but not PCD. Thus, the phenomenon of BCD,

associated with elevated intracellular levels of sanguinarine, may have had a role to play in overcoming the P-gp mediated multidrug resistance.

Understanding different cell death mechanisms of individual anti-cancer agents may lead to their effective administration, alone or in combination with other established therapies. Accordingly, we have attempted to characterize the novel form of cell death-BCD, described in literature as the "blebbing phenomenon", and propose it as a form of cell death, distinct from apoptosis.

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Abbreviations

AC-DEVD	N-acetyl-Asp-Glu-Val-Asp
ATP	adenine triphosphate
BCD	blister cell death
BMCD	bimodal cell death
Bp	base pairs
BSA	bovine serum albumin
Ca^{++}	calcium
cDNA	complementary DNA
DNA	deoxyribonucleic acid
Ig	immunoglobulin
kD (kDa)	kilodalton
MDR	multidrug resistance
MDR	multidrug resistance
MHC	major histocompatibility complex
MRP	multidrug resistance – associated protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCD	programmed cell death
RNA	ribonucleic acid
RNase	ribonuclease
TCR	T cell receptor

TdT	terminal deoxynucleotidyl transferase
TNF	tumor necrosis factor
TUNEL	TdT mediated dUTP nick end labeling
UV	ultraviolet

Chapter 1

Background

1.1. Incidence of Cancer

In normal cell growth there exists a finely controlled balance between growth-promoting and growth-restraining signals such that proliferation occurs only when required. This process is disrupted in tumour cells with continued cell proliferation and loss of differentiation. In addition, the normal process of programmed cell death may no longer operate (Macdonald and Ford, 1997). Thus, in its simplest terms cancer represents an accelerating process of inappropriate, uncontrolled cell growth – a chaotic process within the order of biology. The number of cancer cases continues to rise, particularly among the elderly. This association is attributed to factors such as, duration of carcinogenesis, increased susceptibility of older tisuue to carcinogens and general systemic effects of aging of the body, for example, aging of the immune system (Balducci and Beghe, 2001). This rise in the incidence of cancer is accompanied by a corresponding increase in the interest in complementary therapies including the need for innovative anticancer substances (Morris *et al.*, 2000). It is our belief that a better understanding of the mechanisms of tumour cell killing by potential anti-cancer agents might contribute towards the discovery of novel therapies.

1.2. Overview of Cell Death: Apoptosis, Accidental Cell Death, The "Blebbing" Phenomenon and Necrosis

Increasing attention is now devoted to elucidating differences in cell death under pathological and physiological conditions. In fact, an *ad hoc* committee of the society of toxicologic pathologists are, at present, in the process of forging a consensus on the nomenclature of the various forms of cell death (Levin *et al.*, 1999). The pathways and identification of cell death is of key importance to the practice of diagnostic and research in toxicologic pathology (Trump *et al.*, 1997).

Studies of dying cells have showed that there are two major mechanisms involved in cell death, namely apoptosis (programmed cell death) and accidental cell death (Majno and Joris, 1995). Majno and Joris (1995) suggest that the most satisfactory term for cell death not by apoptosis is accidental cell death. According to these authors the term necrosis is inappropriate as it does not indicate a form of cell death, but merely refers to changes that take place after cells die. One well-known form of accidental cell death is ischemic cell death. The term "oncosis" has been proposed for ischemic cell death, as it is accompanied by swelling (Majno and Joris, 1995; Darzynkiewicz *et al.*, 1997).

Apoptosis is a process of programmed cell death in which a cell activates an intrinsic suicide mechanism that ultimately destroys the cell (Leach, 1998). This cell death process is necessary for development and homeostasis, for defence, and for removal of aging cells (Vaux and Strasser, 1996). The term apoptosis is a word derived from the Greek language: *apo,* for "apart" and *ptosis,* for "fallen" to describe the dropping off or falling off of petals from flowers or shedding of leaves from trees (Kerr *et al.*, 1972; Cohen, 1993; Ueda and Shah, 1994).

Apoptosis is also an active process, which requires energy in the form of ATP to maintain cellular integrity (Fesus *et al.*, 1991; Eastman, 1993). This energy is necessary along with RNA and protein synthesis to fabricate the molecules needed for the successful completion of apoptosis (Eastman, 1993). However, not all apoptotic cells are involved in *de novo* protein synthesis; it is well known that post translational modification (conformational changes) of already synthesized proteins can also lead to cell death by apoptosis (Reed, 2000; Liu *et al.*, 1994). In addition, this cell death process requires only single scattered cells (Ueda and Shah, 1994; Kerr *et al.*, 1994). The cell committed to undergo apoptosis first loses contact with its neighbours by receiving extracellular signals in the form of increased Ca^{++} (Fesus *et al.*, 1991; Ameisen, 1996). This causes rapid loss of water and ions resulting in condensation of cytoplasm and nuclear constituents (Ameisen, 1996). As the nucleus begins to condense it forms peripheral chromatin caps that line the nuclear membrane (Fesus *et al.*, 1991; Ueda and Shah, 1994). The nucleus continues to condense until it becomes dark and pyknotic, a characteristic that is a hallmark of an apoptotic nucleus (Cohen, 1993). Later, complex invaginations develop in the nuclear membrane resulting in multiple segmented nuclei (Ueda and Shah, 1994). As the cell loses cytoplasmic volume, the plasma membrane becomes ruffled and blebbed (Cohen, 1993). This wrinkling zeiotic plasma membrane leads to cellular fragmentation (Cohen, 1993). The fragments that are formed are called apoptotic bodies. These bodies contain the condensed cytoplasmic proteins and intact organelles along with nuclear fragments (Cohen, 1993). This process occurs very quickly within a time frame of about four hours without causing damage to surrounding cells or

tissues (Cohen, 1993). This rapid phagocytosis is critical in maintaining physiologic balance, thus avoiding an inflammatory response (Kerr *et al.*, 1994).

When the DNA of these apoptotic bodies is analyzed by gel electrophoresis, the pattern observed is one of distinct bands that give a "ladder" appearance. Each cleavage is at an interval of about 200 base pairs between the nucleosome linker regions (Cohen, 1993). These so called "ladder structures" of apoptotic nucleosomal DNA are widely used as biochemical markers of apoptosis (Zhitovsky *et al.*, 1994). It has been proposed that this event happens early in the process to serve as a protective measure to the nearby phagocytic cells by destroying the unwanted potentially damaged genetic information (Kerr *et al.*, 1994). However, some reports in the literature suggests that internucleosomal DNA fragmentation may not occur in some experimental systems (Liepins and Bustamante, 1994; Stewart, 1994), although considered to be a hallmark of apoptosis (Arends *et al.*, 1990; Compton, 1992).

Majno and Joris (1995) define "oncosis" as a form of cell death accompanied by cellular swelling, organelle swelling, blebbing, increased membrane permeability and usually accompanied by karyolysis. It is thought to evolve within 24 hours to typical necrosis and can be diagnosed by tests of permeability on whole cells, either in suspension (by dye exclusion tests) or by electron microscopy (using a colloidal marker). The mechanism of oncosis is supposed to be based on the failure of ionic pumps of the plasma membrane (Majno and Joris, 1995).

Interestingly, these authors also distinguish a third cell death modality, separate from oncosis and apoptosis; for which the term "blebbing" has been proposed. Blebbing, presumably a form of accidental cell death, is described as blister like fluid filled

structures, devoid of organelles. Blebs are thought to begin during the early stages of ischemic damage and are found to be initially reversible. Large blebs may burst, and it has been suggested that this may be the final blow to a dying cell (Majno and Joris, 1995).

The term necrosis derives from the Greek *nekros* (dead) and *osis* (condition), meaning "deadness". The term necrosis has been used for more than 2000 years to describe "dead changes of tissue visible to the naked eye" (Majno and Joris, 1995). It has more recently been defined as "death of tissue, usually as individual cells, groups of cells, or in small areas", "or a phase characterized by the break up of organized structure and function in the cell after it passes the point of no return during an acute cell injury" (Mergner *et al.*, 1990). All the above definitions indicate that necrosis is the status of death in cells or tissues, irrespective of the mechanism of death. Within the framework of classification of cell death proposed by Majno and Joris (1995), the necrotic step follows either oncosis or apoptosis and thus can be denoted either as oncotic or apoptotic "necrosis" respectively.

An identifying characteristic of necrosis is the morphological change that occurs when there is an increase in cell volume. This cellular swelling is caused by a change in the osmotic pressure gradient, which is due to loss of control of ion influx allowing the cell to take on more water (Samali *et al.*, 1996). As the cell volume increases, the chromatin inside the cell takes on a more flocculated pattern with a subsequent loss of plasma and organelle membrane integrity (Ueda and Shah, 1994). This eventually leads to cell rupture and the release of cellular components to the extracellular space. The consequence of cell lysis is the pathological effect these spillages have on the

neighbouring resident cells inducing catastrophic injury or even cell death (Ueda and Shah, 1994; Kerr *et al.*, 1994). This massive cellular damage is known as an "inflammatory response" (Ueda and Shah, 1994). DNA degradation of necrotic cells happens later by proteases destroying histone (DNA associated proteins) (Darzynkiewicz *et al.*, 1997).

Darzynkiewicz *et al.* (1997), proposes the term "cell necrobiology" to refer to the studies of events which occur during both apoptosis and oncosis, while another term "cell necrology" refers to the postmortem events, defined by Majno and Joris (1995) as necrosis. In contrast to apoptosis, which has been studied extensively, little is known about characteristic features of early necrotic cells, which may serve as markers for their identification (Darzynkiewicz *et al.*, 1997).

1.3. Regulation of Apoptosis

Apoptosis can be triggered by a wide variety of stimuli, including chemotherapeutic drugs, ultraviolet light irradiation, heat shock, cytokines, oxidative stress, growth factor deprivation, viral infections, genetic abnormalities, as well as normal differentiation and development (Reed, 1998b). Moreover, the initiation of apoptosis involves biochemical changes that might be unique to each apoptotic stimulus (Ucker, 1997).

At least two apoptotic pathways leading to the execution of apoptosis have been identified: a mitochondria-dependent pathway that is clearly governed by Bcl-2 family proteins and a parallel pathway involving activation of upstream caspase-8, such as those involved in Fas and TNF receptors signaling (Levy and Nelson, 2000).

Some cytokines, such as Fas ligand (FasL) and TNF-α, can bind to their receptors on the plasma membrane, causing trimerization of their receptors and thereby activation of an initiator caspase such as caspase-8 through interaction of the receptor with death adaptor proteins such as FADD (Fas-associated death domain) or TRADD (TNF receptor associated death domain) (Baker and Reddy, 1998; Nuñez *et al.*, 1998). In addition, other apoptotic stimuli, such as the anti-cancer therapeutic agent using cisdiamminedichloropplatinum (II) (cisplatin, CDDP), can cause mitochondria dysfunction. Mitochondrial dysfunction includes a reduction in the mitochondrial membrane potential, production of reactive oxygen species (ROS), opening of the permeability transition pore (PTP) and the release of the intermembrane space protein, cytochrome c (see review Gross *et al.*, 1999). In response to cytochrome c binding, the apoptotic proteinase activating factor-1 (Apaf-1) can form a complex with and then activate initiator caspase-9 (Li *et al.*, 1998; Zou *et al.*, 1997).

Interrelationships probably exist between these two pathways within the cell (Levy and Nelson, 2000). For example, Bid, a pro-apoptotic Bcl-2 family protein, is cleaved into two fragments by caspase-8 in response to signaling by Fas or TNF receptor. The C-terminal fragment of Bid then binds to mitochondria, thus initiates the mitochondria-dependent pathway to apoptosis (Li *et al.*, 1998; Luo *et al.*, 1998).

The mitochondria dependent pathway, in response to a variety of exogenous stimuli, communicates a death signal through modulation of genetically programmed proteins. These mainly comprise of the Bcl-2 family proteins, many of which reside in the inner and outer membrane of the mitochondria (Levy and Nelson, 2000).

1.3.1. The Bcl-2 Gene Family

The initial discovery of gene-directed cell death in *Caenorhabditis elegans* fuelled an intensive search for new genes exclusively involved in the regulation or execution of cell death pathways (Bakshi *et al.*, 1985; Cleary *et al.*, 1986). Many new cell death regulators have been identified, and several gene families largely comprised of cell death mediators have emerged. Much of our recent understanding concerning the molecular regulation of cell death originates with the bcl-2 family (Tsujimoto *et al.*, 1985). The B*cl-2* gene family includes both positive (Bax, Bcl-x_S, Bad, Bik) and negative regulators of apoptosis (Bcl-2, Bcl-x_L, Mcl-1) (Yang and Korsmeyer, 1996). Family members interact to form homodimers and heterodimers with other related and non-related proteins. Cell susceptibility to apoptosis is thought to be largely influenced by the relative ratios of pro-apoptotic and anti-apoptotic B*cl-2* family members.

The prototype of the family, Bcl-2 was originally cloned from the t(14:18) translocation break-point associated with follicular lymphoma and was subsequently identified as a negative regulator of apoptosis analogous to its homologue, ced-9 in *C. elegans* (Bakshi *et al.*, 1985; Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985; Cleary *et al.*, 1986; Tsujimoto and Croce, 1986). The Bcl-2 gene maps to 18q21 and is expressed at inappropriately high levels in those lymphomas possessing the t(14;18). Over-expression of Bcl-2 has been shown to protect a variety of cell lines from apoptosis induced by a broad range of signals, including growth factor deprivation, UV irradiation, oxidative stress, cytotoxic drugs and heat shock (Yang and Korsmeyer, 1996). The ability of Bcl-2 to protect against such a broad range of inducing signals suggests that the biochemical functions of this family form a late control point prior to the execution stage of apoptosis.

Bcl-2 does not protect against all death inducing signals, for example apoptosis induced by cytotoxic T lymphocytes (Vaux *et al.*, 1992), suggesting the presence of alternative pathways which circumvent this control point.

Members of the Bcl-2 family of proteins share two conserved regions which are referred to as BH1 and BH2 (Oltvai *et al.*, 1993; Yin *et al.*, 1994; Yin *et al.*, 1995) or domains B and C (Tanaka *et al.*, 1993; Hanada *et al.*, 1995), respectively. Both BH1 and BH2 domains appear to be important for mediating the physical interaction between Bcl-2 and other family members, most notably the dominantly acting death accelerator, Bax (Korsmeyer *et al.*, 1993; Yin *et al.*, 1994; Yang *et al.*, 1995; Hanada *et al.*, 1995; Sedlak *et al.*, 1995). The BH1 and BH2 domains do not appear to mediate Bcl-2 homodimerization. Rather, formation of Bcl-2 homodimers appears to be mediated by a conserved NH2 terminal region and referred to as domain A (Hanada *et al.*, 1995). It has been speculated that the ability of Bcl-2 to suppress cell death is a function of the relative proportion of Bcl-2 homodimers and Bcl-2/Bax heterodimers (Korsmeyer *et al.*, 1993; Yang *et al.*, 1995).

1.3.1.1. Bcl-2

The human Bcl-2 gene consists of three exons and spans approximately 230 kilo bases (kb) (Seto *et al.*, 1988). The Bcl-2 open reading frame spans exons 2 and 3 and encodes a 25 kilo-dalton (kDa) integral membrane protein (Seto *et al.*, 1988).

The human Bcl-2 consists of 239 amino acids and includes a 19-amino acid hydrophobic carboxy-terminal end which functions as a membrane anchor (Hockenbery *et al.*, 1990). The transmembrane domain may serve to optimize Bcl-2 function (Nguyen *et al.*, 1993; Nguyen *et al.*, 1994), but it has been shown in deletion mutants that cell

death suppression by Bcl-2 can be maintained in the absence of the membrane-spanning domain (Hockenbery *et al.*, 1993; Borner *et al.*, 1994;). Immunoelectron microscopic studies have demonstrated that the Bcl-2 protein preferentially localizes within the mitochondria at points where the inner and outer mitochondrial membranes are closely opposed to the endoplasmic reticulum and the nuclear envelope (Krajewski *et al.*, 1993; Akao *et al.*, 1994; Lithgow *et al.*, 1994).

Although Bcl-2 is subject to both tissue specific and developmental regulation, little is known about the molecular mechanisms of its functions. Recent evidence suggests that Bcl-2 may modulate intracellular signalling events associated with cell death induction. In this regard, a sustained modest increase in intracellular Ca^{++} is frequently implicated as an important cell death signalling mechanism (Nicotera *et al.*, 1994). This Ca^{++} signalling results initially from depletion of Ca^{++} from the endoplasmic reticulum followed by the influx of Ca^{++} across the cytoplasmic membrane. Bcl-2 has been shown to inhibit depletion of Ca^{++} from the endoplasmic reticulum and thereby prevent capacitative Ca^{++} entry into the cell (Lam *et al.*, 1994; Marin *et al.*, 1996). Additionally, Bcl-2 also appears to actively inhibit the intranuclear accumulation of Ca^{++} following cell death induction (Marin *et al.*, 1996).

Reactive oxygen species have also been implicated in the mediation of apoptotic cell death in some experimental systems. In this regard, Bcl-2 has been shown to inhibit the generation of reactive oxygen species or to significantly impede downstream oxidative damage resulting from the radicals once they have been generated (Korsmeyer *et al.*, 1993; Kane *et al.*, 1993; Akao *et al.*, 1994). However, it has also been demonstrated that Bcl-2 can inhibit cell death induction under conditions, which preclude

the formation of reactive oxygen species (Jacobson and Raff, 1995). Therefore, the ability of Bcl-2 to protect against the damaging effects of free radicals does not appear to account for Bcl-2 cell death suppression completely (Jacobson and Raff, 1995; Shimizu *et al.*, 1995).

1.3.1.2. Bax

The Bcl-2 associated protein, Bax, was first identified as a 21-kDa protein that coimmunoprecipitates with Bcl-2 (Oltvai *et al.*, 1993). Bax is a six exon, 4.5-kb gene. Bax shares 21% identity and 43% similarity with Bcl-2. The most conserved regions between the two molecules are the BH1 and BH2 domains encoded by exons 4 and 5, respectively. The junction of Bax exons 5 and 6 is identical to the exon 2 and 3 junction in the Bcl-2 gene (Oltvai *et al.*, 1993).

A complex pattern of alternative RNA splicing predicts three different splice variants of Bax (Apte *et al.*, 1995). Bax-α has a 1.0 kb RNA that encodes a 192-amino acid, 21 kDa protein with a transmembrane hydrophobic region. Bax-β has a 1.5 kb RNA that encodes a 218-amino acid, 24 kDa protein that lacks the transmembrane segment and is presumably a cytosolic form of Bax. Bax-γ lacks the 53 base pair (bp) exon 2, and consequently the reading frame in exon 3 is shifted. More recently, a Bax-δ cDNA clone was isolated which results from splicing of exon 2 to exon 4 and retains the carboxy-terminal transmembrane anchor as well as the BH1 and BH2 domains (Apte *et al.*, 1995). The functional significance of these individual Bax variants is, in large part, unknown. Bcl-2 family members function in part through protein-protein interactions and susceptibility of cells to apoptosis is determined by multiple competing dimerizations in which Bax is a common partner e.g. Bcl-2/Bax or Bcl-x_L/Bax (McDonnel *et al.*, 1996).

Enforced Bax expression may overcome Bcl-2 mediated cell death suppression and result in accelerated apoptotic cell death following a death inducing stimulus (Nguyen *et al.*, 1993). It has been suggested that the relative amounts of Bcl-2 and Bax may be considered as a "rheostat" which determines the susceptibility of a cell to undergo apoptosis (Korsmeyer *et al.*, 1993). According to this scheme, when Bcl-2 is in excess, Bcl-2 homodimers predominate and protect the cell from apoptosis. Conversely, when Bax is in excess, Bax homodimers predominate, and the cell becomes susceptible to apoptosis following exposure to an apoptotic signal (Korsmeyer *et al.*, 1993).

Immunohistochemical analysis of Bax protein tissue distribution showed that Bax is more expressed compared with Bcl-2 (Krajewski *et al.*, 1994). Although there is some overlap between Bax and Bcl-2 proteins their subcellular distribution is non-overlapping within several tissues. For example, Bax immuno staining was located in the base of the crypts of the small intestinal mucosa and surface epithelial cells in the colon. In contrast, Bcl-2 protein staining was mainly detected in the absorptive epithelium of the small intestine and the base of the colonic crypts. In the prostate, strong Bax immunostaining was detected in the androgen-dependent secretory epithelium, whereas Bcl-2 was only expressed in the androgen-independent basal cells (Krajewski *et al.*, 1994). Differential expression was also seen in the lymph nodes, where Bax is expressed in the germinal center lymphocytes known to exhibit high levels of apoptosis, while Bcl-2 expression was primarily by the interfollicular lymphocytes (Krajewski *et al.*, 1994).

The regulation of cell death displays lineage specific abberations in Bax knockout mice (Knudson *et al.*, 1995). The thymuses and spleens of Bax deficient mice were hyperplastic, secondary to an accumulation of T and B lymphocytes. However, the

ovaries contained unusual atretic follicles with excess granulosa cells. Additionally, the male Bax knockout mice were infertile secondary to disorders of the seminiferous tubules, accumulation of atypical premeiotic germ cells, and absence of mature haploid spermatocytes. These alterations were accompanied by multinucleated giant cells and dysplastic changes. Therefore, developmental absence of Bax protein can result in either hyperplasia or hypoplasia in a cell type-dependent manner (Knudson *et al.*, 1995).

The expression of Bax can be modulated at the transcriptional level by p53 (Miyashita and Reed, 1995). p53 is a sequence-specific DNA-binding protein, and known targets of p53 include genes associated with growth control, cell cycle checkpoints, DNA repair, and apoptosis (reviewed by Amundson *et al.*, 1998). The M1 myeloid leukemia cell line transfected with a temperature-sensitive p53 mutant expression vector exhibits rapid increase in Bax mRNA within 4 h of shifting cells to the permissive temperature (Zhan *et al.*, 1994). Bax mRNA was also upregulated following apoptosis induction by ionizing radiation but only in cell lines that possess wild-type p53 (Zhan *et al.*, 1994). The Bax gene promoter was shown to contain four p53-binding sites (Miyashita and Reed, 1995). These sites could be specifically transcriptionally transactivated by p53 in reporter plasmids driven by the Bax promoter. Together, these data suggest that Bax may function as a primary response gene in a p53-regulated apoptotic pathway (Miyashita and Reed, 1995). However, p53-independent apoptosis pathways were also found to exist (Strasser *et al.*, 1994).

Recent evidence suggests that Bax may play a role as a tumour suppressor. For example, Bax-α is expressed at high levels in normal breast tissue and is undetectable or expressed at low levels in breast cancers (Bargou *et al.*, 1995). Futhermore, reduced Bax

expression was associated with poor response to combination chemotherapy and shorter survival in women with metastic breast cancer. Direct experimental findings concerning the potential tumour suppressor activity of Bax has, however, not yet been reported (Bargou *et al.*, 1995).

It has been suggested that Bax, which contains a C-terminal transmembrane domain, displays regulated movement between cytosolic and mitochondrial compartments. In the cytosol of healthy cells the transmembrane domain of Bax is masked; assuming a latent conformation. Various apoptotic stimuli cause Bax to undergo a conformational change; the translocation to and insertion into mitochondria membranes. The nature of the signal that controls Bax activation remains largely unclear, though changes in cellular pH is thought to play a role (Reed, 2000).

1.4. Biochemical sequelae of apoptosis: Caspases

The regulation system of apoptosis in response to various stimuli involves the presence of at least two distinct checkpoints: one controlled by the Bcl-2/Bax family of proteins, another by the cysteine and possibly by the serine proteases (Darzynkiewicz *et al.*, 1997). Through several oncogenes and tumour suppressor genes, this system interacts with the machinery regulating cell proliferation and DNA repair proteases (Darzynkiewicz *et al.*, 1997). The programmed cell death pathway has been studied most successfully in the nematode *C. elegans* (Ellis and Horvitz, 1986), and it is from an appreciation of the components of this primitive pathway that the field derives much of the framework. The ced-3 gene was found to be a key regulator of apoptosis in these

nematodes. Humans contain at least ten homologs, complicating the assessment of their individual functions (Allen *et al.*, 1998).

Apoptosis triggered by all stimuli has in common the ablility to induce activation of a family of cysteine proteases called caspases, which cleave a variety of specific protein substrates (Cryns and Yuan, 1998; Nuñez *et al.*, 1998). Caspases implement cell death as the execution arm for apoptosis (Nuñez *et al.*, 1998). Caspases are important components of cell death pathways, normally present in the cell as zymogens that require proteolysis for activation of enzymatic activity. The mammalian caspases have been divided into upstream initiator caspases and downstream effector caspases, based on their sites of action in the proteolytic caspase cascade. Binding of initiator caspase precursors to activator molecules appears to promote procaspase oligomerization and autoactivation by enzymatic cleavage of the procaspase into fragments. Enzymatic activation of initiator caspases leads to proteolytic activation of downstream effector caspases and then cleavage of a number of vital proteins, including poly(ADP-ribose) polymerase (PARP), gelsolin, MEKK-1, and lamin (Cryns and Yuan, 1998). For example, expression of the gelsolin cleavage product in multiple cell types caused the cells to round up, detach from the plate, and undergo nuclear fragmentation (Kothakota *et al.*, 1997).

The caspases can be divided into three subfamilies based on sequence similarity and substrate specificity. The ICE subfamily comprises caspases-1, 4, 5 and 11, the CPP-32 (caspase-3) subfamily comprises caspases-3, 6, 7, 8, 9 and 10 and, to date, caspase-2 stands alone (Harvey and Kumar, 1998).

1.4.1. The role of caspase-3 in apoptosis

The caspase-3- like subfamily is composed of caspase-3 and its closest homologues caspases-6, 7, 8, 9 and 10. Caspase-3 was first cloned by Fernandes-Alnemri *et al.* (Alnemri *et al.*, 1996), and shown to encode a 32 kDa cysteine protease homologous to ced-3, caspase-1 and caspase-2 that was capable of inducing apoptosis when overexpressed in insect cells. By sequence comparison with ICE, the active enzyme was thought to consist of sub units 20 kDa and 11 kDa in size, the only major difference between the sequences being the absence of a long amino-terminal pro-domain in caspase-3. Caspase-3 was subsequently identified as the protease responsible for cleaving poly(ADP-ribose) polymerase (PARP) (Allen *et al.*, 1998). PARP is a nuclear enzyme involved in DNA repair and was one of the first cellular substrates shown to be cleaved in apoptotic cells (Allen *et al.*, 1998). It has been recently demonstrated that caspase-3 activates the endonuclease called caspase-activated Dnase (CAD), which is responsible for the fragmentation of DNA, by specifically cleaving and inactivating the inhibitor of CAD (ICAD/DFF45) (Liu *et al.*, 1997; Enari *et al.*, 1998; Sakahira *et al.*, 1998).

1.5. Multidrug Resistance (MDR)

The challenge of clinical resistance to chemotherapeutics was recognized soon after introduction of the first anti-neoplastics. Drug resistance is now one of the major obstacles limiting the success of conventional anti-neoplastics. Biological mechanisms contributing to drug resistance may be present *de novo* and relate to inherent cellular features or may arise after exposure to anti-neoplastics (Szakacs *et al.*, 1998). Intrinsic drug resistance is believed to be responsible for the relatively poor response to

chemotherapy observed in patients with pancreatic, renal, and non-small cell lung cancer (Patel and Rothenberg, 1994). Cancers such as breast, ovarian, small cell lung cancer, the acute leukemias, and lymphomas, although associated with high initial response rates, have limited curative potential due to the acquisition of cellular mechanisms of drug resistance (Patel and Rothenberg, 1994). Pre-clinical investigations support a complex and redundant network of resistance mechanisms that include decreased intracellular drug accumulation, intracellular de-toxification, altered nuclear drug targets, impaired transduction of growth arrest signals and inherent cytokinetic resistance (Kellen, 1993). Many of these mechanisms represent physiological functions conserved in their malignant counterparts, and correlative studies indicate that some of these mechanisms may contribute to clinical chemotherapy resistance (Chaudhary and Robinson, 1991).

So far, three separate forms of MDR have been characterized in detail: classical MDR, non-Pgp MDR and atypical MDR. Although all three MDR phentypes have much in common with respect to cross-resistance patterns, the underlying mechanisms certainly differ (Nooter and Stoter, 1996).

1.5.1. Classical MDR

The classical MDR cell phenotype is characterized by a reduced ability to accumulate drugs, as compared to the parent cell lines, being most likely the main cause for the multidrug resistance. The reduced drug accumulation in classical MDR is due to activity of an energy-dependent unidirectional drug-efflux pump with broad substrate specificity. The MDR drug pump is composed of a transmembrane glycoprotein (P-gp) with a molecular weight of 170 kDa (Chen *et al.*, 1986), and is encoded by the so-called multidrug resistance gene. The glycoprotein is generally called P-glycoprotein, P

indicating permeability because it was originally thought that the glycoprotein regulated cellular permeability (Juliano and Ling, 1976). P-glycoproteins are about 1280 amino acids long, and are composed of two homologous halves; each half consists of six predicted transmembrane domains and intracellular loop with a consensus ATP-binding motif (Chen *et al.*, 1986). This architecture has been associated with both active transport and passive ion channel activities (Ames and Lecar, 1992). P-glycoproteins are post-translationally modified by glycosylation and phophorylation. Glycosylation of P-glycoprotein is not required for its multidrug transport function (Schinkel *et al.*, 1993), However, it has been suggested that N-glycosylation may contribute to correct folding, proper routing and stabilization of the molecule. P-glycoprotein uses energy in the form of ATP to transport drugs through a channel formed by the transmembrane segments (Horio *et al.*, 1988). It is assumed that the P-glycoprotein drug pump might act by extracting drug from the membrane at its external face, and its internal face by two related but functionally separable processes (Stein *et al.*, 1994). It has been a matter of debate as to whether the drug accumulation defect in classical MDR cells is due to decreased drug influx or an increased drug efflux (Stein *et al.*, 1994).

1.5.1.1. P-glycoprotein expression in normal tissues and tumours

P-glycoprotein is mainly found in specialized epithelial cells with secretory or excretory functions. In the liver, P-glycoprotein (P-gp) is present on the billiary surface of hepatocytes and small billiary ductules, in the pancreas on the luminal surface of the epithelial cells of small ductules, and in the kidney on the brush border of the proximal tubules (Thiebaut *et al.*, 1987). The colon and jejunum both show high levels of P-gp on the luminal surfaces of the mucosa. P-gp expression is also found in other specialized

epithelial cells such as sweat glands of the skin, cells lining in the trachea and major bronchi in the lung, glandular epithelial cells of the prostate, breast endometrium and thyroid, acinar cells of the pancreas, trophoblasts in the placenta (van der Valk *et al.*, 1990). P-gp expression is also detectable in hemopoietic cells in bone marrow and peripheral blood (Chaudhary and Robinson, 1991), and in capillary endothelial cells in human brain and testis (Cordon-Cardo *et al.*, 1990).

Expression of MDR1 gene has been detected in all tumour types, such as carcinomas, sarcomas, leukemias and lymphomas (Goldstein *et al.*, 1989; Cordon-Cardo *et al.*, 1990; Chan *et al.*, 1991; Center, 1993). Tumours that developed from tissues normally expressing high MDR1 levels, e.g. colon, liver, kidney, adrenal and pancreas, also have relatively high levels of MDR1 expression. Clinically, these tumours are all intrinsically drug resistant, i.e. have a very low response rate to chemotherapy. Other tumours occasionally have elevated MDR1 expression levels, but quite often show a lack of such expression. This group contains, among others, hematological malignancies, neuroblastomas, soft tissue sarcomas and cancer of the breast, ovary, lung and bladder. In general these tumours respond better to chemotherapy than the previous group, and even complete responses can be achieved. Unfortunately, a high percentage of patients relapse and become resistant to chemotherapy (Bosch and Croop, 1996).

1.5.1.2. Clinical relevance of the classical MDR phenotype

After the discovery that the P-glycoprotein molecule can be expressed in human tumors, clinical trials have been initiated with the aim to block the P-glycoprotein drug pump, and in that way make anti-cancer drugs more effective (Raderer and Scheithauer, 1993). Clinical modulation studies have been performed in cancers with an intrinsic

classical MDR phenotype, like colon and kidney cancer as well as in cancers that have acquired the classical MDR phenotype during the course of the therapy as is the case of some hematological malignancies (Jachez *et al.*, 1993; Keller *et al.*, 1992).

1.6. Sanguinarine

The Quaternary benzo[c]phenanthridine alkaloid sanguinarine occur in several genera of the families Papaveraceae, Berberidaceae, Fumariaceae and less abundantly in Menispermaceae, Ranunculaceae, Rutaceae and Sapindaceae (Schmeller *et al.*, 1997). An alkaloid is physiologically active, nitrogen containing low molecular weight, base compound extracted from plant material (Pauli and Kutchan, 1998). Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1.3-dioxolo[4,5-I]phenanthridinium) is a commercial drug derived from the root of *Sanguinaria canadensis* (bloodroot) and *Chelidonium Majus L.* (Greater Celandine) (Lenfeld *et al.*, 1981).

The iminium charge on the benzo[c]phenanthridine ring seem to be necessary for their biological action (Yves *et al.*, 1993; Arisava *et al.*, 1984; Monroe *et al.*, 1987). The iminium bond of any quaternary benzo[c]phenanthridine alkaloid is highly susceptible to nucleophilic attack (Yves *et al.*, 1993).

The pharmacological activities of sanguinarine are several. Many alkaloids of the benzo[c]phenanthridine family, including sanguinarine, display anti-tumour properties (Simeon *et al.*, 1989). Apart from its anti-tumour properties, Sanguinarine shows antimicrobial activity against a wide variety of microorganisms, which include bacteria, fungi and protozoa. Sanguinarine acts as both a bacteriostatic agent and a bactericide to major gram-positive and gram-negative bacteria (Odebiyi and Sofowora, 1979;

Vichkanova *et al.*, 1969; Dzin and Sacransky, 1985). Sanguinarine possess good antifungal activity against *Candida albicans* and the dermatophytes of trycophyton species *Epidermophyton floccosum, Microsporum canis,* and *Aspergillus fumigatus* (Hejmankova *et al.*, 1984). The anti-inflammatory effects of this drug have also been well documented (Lenfeld *et al.*, 1981). The biochemical activities of sanguinarine includes the inhibition of protein kinase C (Janin *et al.*, 1993; Gopalakrishna *et al.*, 1990), NFκB (nuclear transcription factor) (Chaturvedi *et al.*, 1997), Na^+/K^+ ATPase (Straub and Carver, 1975; Seifen *et al.*, 1979) and of tubulin assembly (Wolff and Knipling, 1993). By intercalation, sanguinarine forms a molecular complex with DNA, specifically binding to regions rich in guanosine-cytosine (Sen and Maiti, 1994); this intercalation, at least in part, may contribute towards tumour cell killing. Sanguinarine is at present an ingredient in many over-the-counter products including toothpaste, mouthwash, cough and cold remedies and homeopathic preparations, for its anti-bacterial and anti-fungal properties (Frankos *et al.*, 1990).

1.7. Objectives

From the foregoing review of the literature, it can be concluded that two major forms of cell death are identified: apoptosis and accidental cell death, and that following cell death, both types undergo postmortem changes collectively termed "necrosis". In addition, the regulation of apoptosis and the role of caspases were discussed. An introduction to the benzophenanthridine alkaloid sanguinarine, and to features of the classical MDR phenomenon was also provided.

As previously stated, the benzophenanthridine alkaloid sanguinarine has anticancer potential through induction of cell death. Studies in our laboratory have demonstrated that K562 cells, when exposed to sanguinarine for two hours at concentrations of 1.5 μg/ml and 12.5 μg/ml, displayed two different morphologies corresponding to two different modalities of cell death. At 1.5 μg/ml, cells displayed the formation of apoptotic bodies over the entire cell surface, consistent with the current criteria of apoptosis or programmed cell death (PCD). At concentrations of 12.5 μg/ml, cells displayed the morphology of large blister formation (commonly a single blister and usually not more than two) or "blister cell death" (BCD). Several reports in the literature have found K562 cells to be rather resistant to the induction of apoptosis (Kobayashi *et al.*, 1998); sanguinarine, on the contrary, was able to effectively overcome this resistance. Previous observations in our laboratory of apoptosis or programmed cell death (PCD) and blister cell death (BCD) induced by the semisynthetic benzophenanthridine alkaloid derivative Ukrain™ in K562 cells was termed "bimodal cell death" (BMCD) (Liepins *et al.*, 1996). My hypothesis is that apoptosis and BCD are two different cell death

processes, and the efficacy of anti-cancer drugs depend on the execution of both these processes; also, BCD, like apoptosis, may be genetically controlled.

The molecular mechanisms of cell death and its role in chemotherapy, and drug resistance are poorly understood. A thorough understanding of these mechanisms is indispensable for the search and development of novel chemotherapeutic agents (Reed *et al.*, 1994; Reed, 1995). Accordingly, the general objective of my study was to gain insight into the mechanisms of tumour cell killing by sanguinarine.

Recently, the semisynthetic benzophenanthridine alkaloid derivative Ukrain™ (NSC-631570), has shown to have efficacy towards human tumours (Susak *et al.*, 1996; Brzosko *et al.*, 1996), and is widely used as an unconventional mode of treatment (see entire issue of Drugs under Experimental and Clinical Research suppl. to vol. xxii, 1996). Ukrain was screened *in vitro* by the National Cancer Institute (NCI Development Therapeutics Program, USA), and found to have cytotoxic effects on their 60 human tumour cell lines (Liepins *et al.*, 1996). These facts on Ukrain™ prompted us to investigate the anti-cancer potential of another benzophenanthridine alkaloid-sanguinarine. Based on the model of sanguinarine induced bimodal cell death (i.e. PCD and BCD), my specific objectives were as follow:

1) Characterization of PCD (apoptosis) and BCD (blister cell death) induced by sanguinarine, in the form of a comparative study at the cellular level, using the low Bcl-2 expressing K562 erythroleukemia cells (Kobayashi *et al.*, 1998) and the high Bcl-2 expressing JM1 pre-B lymphoblastic cells (Samson *et al.*, 1996) (the low and high Bcl-2 expression in K562 and JM1 cells respectively, was also confirmed in our laboratory using western blotting techniques). K562 and JM1 cells were chosen based

on their respective Bcl-2 status. Thus, in addition to the characterization of sanguinarine-induced PCD and BCD, this comparative study also allowed to determine the importance of Bcl-2 in the susceptibility/resistance of tumour cells to sanguinarine. Methods used for this purpose included, light microscopy, electron microscopy, ^{51}Cr release, propidium iodide exclusion, trypan blue exclusion, annexin V binding and terminal deoxynucleotidyl transferase (TdT) end labelling.

2) Characterization of PCD and BCD induced by sanguinarine, in the form of a comparative study at the molecular level, using the low Bcl-2 expressing K562 cells and the high Bcl-2 expressing JM1 cells. The methodologies used for this purpose were western blotting technology and cDNA expression array studies. The role of the Bcl-2 family and caspases were analyzed.

3) Measuring Bcl-2, Bax and NF-κB (nuclear transcription factor) protein levels in K562 and JM1 cells using immunofluorescence-flow cytometric analysis in PCD and BCD induced by sanguinarine. This study also enables the confirmation of results obtained from western blotting.

4) Studying P-glycoprotein expression in the multidrug resistant CEM-VLB 1000 cell line and in its wild type counterpart CEM-T4, and characterization of PCD and BCD induced by sanguinarine at the cellular and molecular levels in both these cell lines. This study also enables, first, to observe the effects of sanguinarine in a cell system different to that of the above (i.e. K562 vs JM1 cells) and second, to evaluate the effects of sanguinarine in a Pgp positive multidrug-resistant (MDR) cell line.

It is hoped that this work may shed further light on the cellular and molecular parameters of PCD and BCD induced by sanguinarine, and to assess the roles played by

Bcl-2 and Pgp in sanguinarine-induced bimodal cell death. Hence, it is felt that this study would reveal, at least in part, the anti-cancer potential of sanguinarine, and discover the mechanisms by which it may induce tumour cell killing.

1.8. Co-authorship Statement

This thesis contains four research papers. The thesis author is the first author in all papers. The work done by each of the four authors is as follow:

(1) First author- the entire work concerning identification and design of the research proposal, practical aspects of the research, data analysis and manuscript preparation.

(2) Second author- assistance in the practical aspects in some of the experiments where radioactive substances were involved.

(3) Third author- advice on certain aspects of the research work, proofreading of manuscripts and the contribution of certain material.

(4) Fourth author- supervisor of thesis author.

Chapter 2

Sanguinarine Induces Bimodal Cell Death in K562 but not in High Bcl-2 Expressing JM1 Cells

Running Title: Sanguinarine Induces Cell Death in K562 but not in JM1 Cells

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Summary

Our previous studies with the low Bcl-2 expressing K562 cells have shown that, when treated with the putative anti-cancer drug sanguinarine, concentrations of 1.5 μg/ml induced the morphology of apoptosis or programmed cell death (PCD), while concentrations of 12.5 μg/ml induced a morphology of blister formation or blister cell death (BCD). To elucidate the possible role of Bcl-2 in this dual cell death modality induced by sanguinarine, K562 and the high Bcl-2 expressing JM1 cells were treated with sanguinarine concentrations of 1.5 μg/ml and 12.5 μg/ml respectively, and multiple parameters of their effects were studied using light and electron microscopy, terminal deoxynucleotidyl transferase (TdT) end-labeling, ^{51}Cr release, trypan blue exclusion, propidium iodide exclusion and annexin V binding. In general, it was found that, while K562 cells underwent PCD and BCD when treated with sanguinarine, JM1 cells failed to undergo either PCD or BCD under the same experimental conditions. Thus, the over-expression of anti-apoptotic Bcl-2 may have prevented sanguinarine from inducing PCD and BCD in JM1 cells. These results indicate that, resistance of JM1 cells to the alkaloid sanguinarine may have been due to an anti-BCD role played by Bcl-2, in addition to its widely reported anti-apoptotic role.

Introduction

Sanguinarine (SA) is a quaternary benzophenanthridine alkaloid derived from the root of the plant *Chelidonium Majus L.* or greater celandine (Lenfeld *et al.*, 1981). Among its many useful properties, the potential as a potent anti-cancer agent figure prominently (Ahmad *et al.*, 2000; Simeon *et al.*, 1989). Our previous studies have demonstrated that K562 human erythroleukemia cells, when exposed to sanguinarine at concentrations of 1.5 μg/ml and 12.5 μg/ml for two hours, displayed the morphologies of two different modalities of cell death: at 1.5 μg/ml the classical morphology of apoptosis or programmed cell death (PCD) and at concentrations of 12.5 μg/ml, the morphology of single blister formation or blister cell death (BCD) (Weerasinghe *et al.*, in press). Previous observations in our laboratory of apoptosis or programmed cell death (PCD) and blister cell death (BCD) induced by the semisynthetic benzophenanthridine alkaloid derivative Ukrain™ in K562 cells was termed "bimodal cell death" (BMCD) (Liepins *et al.*, 1996). Thus, this dual cell death modality induced by sanguinarine was also termed "bimodal cell death" (BMCD).

Programmed cell death (PCD), or apoptosis is a physiologically active cell death process, characterized by formation of apoptotic bodies, changes in membrane permeability, and nuclear DNA condensation and fragmentation (Liepins, 1989; Liepins and Bustamante, 1994). The latter event, i.e. internucleosomal DNA fragmentation, may not occur in some experimental systems (Liepins and Bustamante, 1994; Stewart, 1994), although considered to be a hallmark of apoptosis (Arends *et al.*, 1990; Compton, 1992). The deregulated expression of the anti-apoptotic Bcl-2 gene product was found to

contribute to multistep neoplasia through the suppression of cell death, or apoptosis, in transgenic mouse models (McDonnell *et al.*, 1996).

In order to assess the effects of the endogenous anti-apoptotic protein Bcl-2 in sanguinarine induced bimodal cell death (BMCD) i.e. PCD and BCD, we chose the JM1 human pre-B cell lymphoblastic cell line, which constitutively expresses elevated Bcl-2 gene product (Samson *et al.*, 1996), and the low Bcl-2 expressing K562 human erythroleukemia cell line (Kobayashi *et al.*, 1998). Our flow cytometric studies (as shown in the present study) and previous western blotting analysis have confirmed the high Bcl-2 expression in JM1 cells and its low expression in K562 cells, respectively. Both cell lines were studied in terms of their susceptibility to sanguinarine induced bimodal cell death (BMCD) in order to determine whether the elevated Bcl-2 gene product would protect JM1 cells from programmed cell death (PCD) and/or blister cell death (BCD). Results showed that sanguinarine effectively induced apoptosis or programmed cell death (PCD) and blister cell death (BCD) in K562 cells, whereas, JM1 cells showed resistance. This report also attempts to define a role (albeit a preliminary one) for BCD as a novel form of cell death and to discuss its relevance in Bcl-2 associated chemoresistance.

Materials and Methods

Cells and cell culture

The K562 erythroleukemia and JM1 pre-B cell lymphoblastic cell lines were purchased from ATCC (Rockville, MD, USA) and were routinely maintained as cell suspensions in RPMI-1640 medium supplemented with 10% fetal calf serum and L-glutamine in a humidified atmosphere containing 5% CO_2 at 37°C. All experiments were performed with K562 and JM1 cells during their exponential phase of growth.

Sanguinarine treatment

Sanguinarine HCl (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water to a concentration of 1.0 mg/ml. Serial dilutions were carried out (shortly before *in vitro* use), with tissue culture medium [RPMI-1640 + 10% fetal bovine serum (FBS)].

Sanguinarine was serially diluted in RPMI + 10% FBS to give a concentration range of 25 μg/ml to 0.19 μg/ml (8 dilutions) in flat bottom 24 well plates. Triplicate wells of each drug concentration was prepared and aliquots of 1×10^6 cells (K562 and JM1) per well were added. These tissue culture plates were subsequently incubated at 37^0 C/5% CO_2 for two hours.

Light microscopy

Cells treated with sanguinarine were studied and quantitated by light microscopy. The key morphological criterion for the detection of apoptosis was the formation of apoptotic bodies and the key criterion for blister cell death was the formation of cell surface blisters.

Electron microscopy

Cells were fixed with 2% glutaraldehyde, post fixed in 1% osmium tetroxide, dehydrated through a series of ethanols and embedded in TAAB 812 epoxy resin. Semi thin (0.5 μm) sections were cut axially, through all layers of each pellet to select areas for ultramicrotomy. Ultra thin sections were stained with lead citrate and 30% alchoholic uranyl acetate and then examined in a JEOL 100-Cx electron microscope.

Trypan blue assay

The vital dye, trypan blue, is excluded from putative live cells with intact plasma membranes (O'Brien *et al.*, 1997; Zhang *et al.*, 1990). The changes in membrane permeability in K562 and JM1 cells which were exposed to sanguinarine treatment and untreated controls, were evaluated in triplicate using flat bottom 96 well plates at 2×10^4 cells per well after 2 hours of incubation at 37°C. The exclusion of trypan blue (0.2%) was used as an indicator of the number of live cells. After 10 minutes of incubation with the dye, the number of trypan blue excluding cells was counted using a hemocytometer. The exclusion of trypan blue was used as an indicator of the number of live cells.

Propidium iodide assay

This cell viability assay is based on the exclusion of the fluorochrome, propidium iodide (Schmid *et al.*, 1994) by the cell membrane. K562 and JM1 cells treated with sanguinarine were evaluated for cell viability in triplicate in 96 flat bottom well plates at 2×10^4 cells per well and incubated for 2 hrs at 37°C. Thereafter, PI (5μg/ml in PBS) was added after cells were washed in PBS. After 10 minutes of incubation with the dye, the number of PI excluding cells was quantitated by hemocytometer using fluorescence microscopy.

^{51}Cr release assay

Cell injury was evaluated by $Na_2{}^{51}CrO_4$ release assay (Liepins, 1983; Liepins *et al.*, 1996). 2×10^6 cells from each cell line were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ and incubated for 90 minutes in a humidified incubator at 37°C/5% CO_2. Cells were washed three times with PBS + 2% FCS in order to remove unincorporated $Na_2{}^{51}CrO_4$.

Specific ^{51}Cr release of K562 and JM1 cells exposed to sanguinarine treatment and of untreated controls were evaluated using 96 V bottom well plates in duplicate at 2×10^4 cells per well, after two hours incubation at 37°C. The percentage of ^{51}Cr release was evaluated by the following formula.

$$\% \text{ Specific } {}^{51}Cr \text{ release: } \frac{(\text{Cells + Sanguinarine}) - \text{Spontaneous release}}{\text{Maximum } {}^{51}Cr \text{ release - spontaneous release}} \times 100$$

Spontaneous ^{51}Cr release is the amount of ^{51}Cr released by cells in the absence of the drug. Maximum ^{51}Cr release is the ^{51}Cr released by exposing the cells to 1N HCl.

Fluorescein-conjugated annexin V binding assay

After treatment with sanguinarine concentrations that induce PCD and BCD, K562 and JM1 cells (1×10^6 cells) were washed with PBS and incubated with annexin V fluorescein isothiocyanate (FITC) conjugate and propidium iodide (PI) utilizing an annexin V Fluos staining kit (Boehringer Mannheim Corp.). After labeling, cells were resuspended in binding buffer and analyzed using flow cytometry. FITC-fluorescence was measured at 530-545 nm and fluorescence of DNA-PI complexes at 575-606 nm. Cell debris was excluded from analysis by appropriate forward light scatter threshold setting (Leist *et al.*, 1997).

Terminal deoxynucleotidyl transferase (TdT) end-labeling assay

Specific 3'-hydroxyl ends of DNA fragments generated by endonuclease-mediated apoptosis are preferentially repaired by terminal deoxynucleotidyl transferase (TdT) (Gavrieli *et al.*, 1992). The TdT mediated nick end labeling assay has been developed to label these strand-breaks with streptovidin-horseradish peroxidase conjugated nucleotides followed by the addition of a substrate (TBL).

After sanguinarine treatment, cells were removed from individual wells, washed in PBS and fixed in 10% neutral buffered formaldehyde for 10 minutes. Cells were resuspended in 80% ethanol at 1×10^6 cells per ml. 50,000 cells were placed onto an electrostatically treated glass slide and air dried at room temperature. Cells were then permeabilized with proteinase K (Gavrieli *et al.*, 1992). Cell samples were then incubated for 60 minutes at 37°C in the presence and absence of exogenous TdT and streptavidin-horseradish peroxidase conjugated dNTP (deoxynucleotide triphosphate), followed by the substrate TBL according to the manufacturer's instructions (Sigma TACS ^{TM} In Situ Apoptosis Detection Kit, St. Louis, MO, USA). Cells were then examined and photographed under phase microscopy and counted to determine the percentage of cells with DNA nicking to total cells.

Detection of intracellular sanguinarine using flow cytometry

The auto-fluorescent nature of sanguinarine was used to measure its intracellular levels by quantitating the amount of fluorescence using a Becton Dickinson flow cytometer. Subsequent to drug treatment, cells were washed 3 times and subjected to flow cytometric analysis.

Flow cytometric analysis of Bcl-2 in K562 and JM1 cells

Flow cytometric analysis of Bcl-2 was carried out as described by Re *et al.* (1998). Briefly, after treatment with appropriate concentrations of sanguinarine, K562 and JM1 cells were fixed with 70% ethanol for 20 minutes at 4^0C. Cells were then washed twice with PBS and permeabilized with 0.5% Triton X 100 (Sigma, St. Louis, MO) in PBS for 10 minutes at 4^0C. After three washings with PBS, cells were resuspended in 10% goat serum for one hour at room temperature before adding anti-Bcl-2 monoclonal antibody (1:50, Santa Cruz Biotechnology, CA), and incubated overnight at 4^0C. The cells were then washed five times in PBS. Thereafter a polyclonal goat anti-mouse (1:100) antibody conjugated to fluorescein (FITC) was added to cells and incubated for 30 minutes at 37^0C. The cells were washed five times and analyzed by FACS (Becton-Dickinson, Palo Alto, CA).

Thereafter, the cells were gated for forward scatter (FSC) and side scatter (SSC) in order to rule out cell aggregates and debris. The negative controls consisted of identical secondary labeling without primary monoclonal antibody. The positive control was the expression of the HSP-70 housekeeping protein, which was detected by the anti HSP-70 monoclonal antibody (1:100 Santa Cruz).

Statistical Analysis

The results are presented as means ± SE. Statistical analyses were performed using the student's t test. Differences were considered significant when $p< 0.05$.

Results

K562 cells treated for 2 hrs with 1.5 μg/ml of sanguinarine at 37°C displayed formation of apoptotic bodies in over 96% of cells, whereas JM1 cells treated under similar conditions did not display the morphology of apoptosis (Figs. 2-1b and 2-1e respectively). K562 cells, treated with sanguinarine concentrations of 12.5 μg/ml displayed the morphology of single blister formation or blister cell death (BCD) in over 90% of cells (Fig. 2-1c), whereas JM1 cells subjected to the same experimental conditions failed to undergo this morphological phenomenon (Fig. 2-1f). Untreated control K562 cells and JM1 cells are shown in Figs. 2-1a and 2-1d respectively.

Electron microscopy of K562 cells exposed to concentrations of sanguinarine that induce apoptosis (1.5 μg/ml), showed the classic morphological changes, consisting of: formation of apoptotic bodies (containing organelles), chromatin condensation and nuclear fragmentation (Fig. 2-2b). When treated with sanguinarine concentrations that induce BCD (12.5 μg/ml), K562 cells displayed single blister formation (devoid of organelles), patchy chromatin condensation and an increase in cytoplasmic vacuolization (Fig. 2-2c). JM1 cells, on the other hand, failed to undergo either PCD or BCD as a result of sanguinarine treatment (Fig. 2-2e and Fig. 2-2f, respectively). However, JM1 cells treated with sanguinarine displayed an absence of microvilli, which was the only significant ultra-stuctural change noted (Figs. 2-2e & 2-2f).

The ^{51}Cr release assay, which is a universal assay of immuno-effector cell lysis of tumor cells, was used to measure cell injury (Liepins *et al.*, 1996). K562 cells showed 20±2.8% increase in specific ^{51}Cr release when treated with concentrations of

sanguinarine that induce PCD and 40±3.5% at concentrations that induce BCD. JM1 cells when treated with sanguinarine failed to show ^{51}Cr release (Fig. 2-3A).

Results of trypan blue exclusion studies, to assess plasma membrane integrity, showed that during PCD and BCD >88% of K562 cells excluded trypan blue (Fig. 2-3B), as did JM1 cells treated with sanguinarine. Similarly, results of PI membrane permeability studies also found > 90% of PCD and BCD of K562 cells and > 90% of JM1 cells treated with sanguinarine excluding PI (Fig. 2-3C).

The annexin-V-assay was utilized for the detection of cell surface membrane phosphatidyl serine (PS) flip, which is known to be associated with apoptosis or PCD (Leist *et al.*, 1997). Sanguinarine induced apoptotic K562 cells showed 45±6.1% of annexin-V-positive cells, i.e. binding of annexin V to phosphatidyl serine on the outer surface of the cell membrane. K562 cells which underwent BCD did not show significant annexin-V-binding (16±2.4%). Also, JM1 cells treated with sanguinarine and untreated controls did not show the phosphatidyl serine (PS) flip (Fig.2-4).

DNA nicking in PCD and BCD of both cell lines was studied by the terminal deoxynucleotidyl transferase (TdT) end-labeling method. Results showed DNA nicking in >85% of K562 cells during PCD (Fig. 2-5b). Sanguinarine induced BCD in K562 cells failed to show DNA nicking (Fig. 2-5c). DNA nicking was also absent in JM1 cells treated with sanguinarine and in control untreated cells (Figs. 2-5e, 2-5f, 2-5d and 2-5a).

Measurements of intracellular sanguinarine based on its auto-fluorescence showed comparable levels in K562 and JM1 cells treated with concentrations of sanguinarine that induced apoptosis (Figs. 2-6b and 2-6e, respectively). However, BCD of K562 cells

showed higher levels of sanguinarine in comparison with JM1 cells treated with the same concentration of sanguinarine (Figs. 2-6c and 2-6f, respectively).

Both cell lines were examined for endogenous Bcl-2 levels in untreated controls and for possible changes in its expression after sanguinarine treatment utilizing immunofluorescence-flow cytometry. The percentage of untreated control K562 cells expressing Bcl-2 was 33.88% while the percentage of untreated control JM1 cells expressing Bcl-2 was 76.75% (Fig. 2-7).

Discussion

Apoptosis was originally recognized based on changes in cell morphology as revealed by light and electron microscopy (Kerr *et al.*, 1972; Wyllie *et al.*, 1992; Majno and Joris, 1995). Light and electron microscopic studies of K562 cells treated with sanguinarine showed the typical morphology of apoptosis: formation of apoptotic bodies and nuclear fragmentation. Other parameters of apoptosis in K562 cells treated with sanguinarine comprised of an increase in specific ^{51}Cr release denoting cell injury and a positive annexin-V-assay showing the phosphatidyl serine flip. The terminal deoxynucleotidyl transferase (TdT) end-lebeling assay show DNA nicking in >85% of apoptotic cells. However, exclusion of trypan blue and propidium iodide by apoptotic cells indicate plasma membrane integrity. The exclusion of these dyes by cells undergoing apoptosis is widely reported in literature (O'Brien *et al.*, 1997; Zhang *et al.*, 1990). In contrast, JM1 cells treated with the concentration of sanguinarine that induce apoptosis in K562 cells did not show the morphology or the other parameters characteristic of apoptosis. These observations coupled with the higher expression of Bcl-2 in JM1 cells (as opposed to K562 cells) strongly suggests the anti-apoptotic role of Bcl-2 in sanguinarine induced apoptosis.

Various reports in literature indicate that high expression of the anti-apoptotic Bcl-2 protein is associated with poor response to chemotherapy (Nakata *et al.*, 1998; Reed *et al.*, 1994). Results of the present study also indicate that Bcl-2 may be responsible for poor response to sanguinarine due to its anti-apoptotic nature.

Treatment of K562 cells with sanguinarine at concentrations of 12.5 μg/ml, induced the morphology of blister (usually one but rarely two) formation in >90% of

cells. Electron microscopical studies showed the blisters to be devoid of organelles. Other features of BCD revealed by electron microscopy were the absence of nuclear fragmentation and the presence of vacuolization and patchy chromatin condensation. The present study showed an elevation in ^{51}Cr release during BCD, higher than that of apoptosis, in K562 cells. BCD also showed the absence of both DNA nicking and the PS flip. However, it was shown that BCD excluded trypan blue and propidium iodide, denoting intact permeability of the cell membrane.

Necrosis does not indicate a form of cell death but refers to changes secondary to cell death by any mechanism (Levin *et al.*, 1999; Majno and Joris, 1995). It is known that early necrotic cells lose plasma membrane integrity, thus permitting the entry of trypan blue and propidium iodide into the cell (O'Brien *et al.*, 1997). The exclusion of trypan blue and PI by BCD indicate that it is not representative of early necrosis.

Blister formation is at best, only sporadically reported in literature (Liepins *et al.*, 1996; Phelps *et al.*, 1989). The phenomenon of BCD may resemble the "blebbing" described by Majno and Joris (Majno and Joris, 1995). Majno and Joris describes blebbing as blister like, fluid filled structures, typically devoid of organelles, that arise from the cell membrane and are apt to swell and burst. It is suggested that the mechanism of blebbing may appear to depend on a disconnection between the cell membrane and the underlying cytoskeleton (Majno and Joris, 1995; Phelps *et al.*, 1989). Trump *et al.* (1997) and Trump and Berezesky (1992) associates blebbing with increases in concentration of cytosolic Ca^{2+} $[Ca^{++}]_i$ and rearrangement of cytoskeletal proteins including filamentous actin, although Lemasters *et al.* (1987) saw no change in concentration of cytosolic Ca^{2+} $[Ca^{++}]_i$ during blebbing.

In contrast to K562 cells, JM1 cells treated with concentrations of sanguinarine inducing BCD in K562 cells, failed to undergo the morphological changes associated with BCD. Microscopy showed no significant changes in sanguinarine treated JM1 cells from untreated controls. Furthermore, sanguinarine treated JM1 cells failed to show the PS flip as well as DNA nicking, and showed no changes in ^{51}Cr release. On the basis of these results, the anti-apoptotic Bcl-2 may be associated with inhibition of the cell death processes of apoptotis and BCD. Thus, Bcl-2 may also be anti-BCD in addition to being anti-PCD. Evidence of Bcl-2 inhibiting the process of cytoplasmic blebbing has also been reported in recent literature (Ferri *et al.*, 2000). Hence, the anti-apoptotic Bcl-2 may further compound chemoresistance by additionally being anti-BCD in nature (at least, this may hold true in the case of sanguinarine treatment). Further characterization of BCD at the cellular and molecular levels might provide a new dimension to understanding the mechanisms of cell death and chemoresistance.

The role of BCD in tumour cell killing could be studied utilizing flow cytometric analysis by capitalizing on the fluorescent nature of sanguinarine itself. Sanguinarine exercises its anti-cancer properties (Simeon *et al.*, 1989), among other probable mechanisms, by forming a molecular complex with DNA by intercalation. More specifically, by binding to regions rich in guanosine-cytosine (Babich *et al.*, 1996; Sen and Maiti, 1994). Fig. 2-6 show a flow cytometric study of sanguinarine auto-fluorescence in K562 and JM1 cells treated with the drug. BCD, induced in the low Bcl-2 expressing K562 cells (Fig. 2-6c), showed high levels (48.5%) of red auto-fluorescence while high Bcl-2 expressing JM1 cells treated with the same concentration of sanguinarine (Fig. 2-6f) showed reduced levels (8.3%). Fluorescence microscopy found

the presence of sanguinarine predominantly in the nucleus of cells (data not shown). Literature suggests that the Bcl-2 protein is located on the nuclear membrane (and also on the mitochondrial and endoplasmic reticulum membranes) (Reed, 1998). Hence, an interesting assumption would be whether the nuclear membrane Bcl-2 is associated with preventing sanguinarine from entering the nucleus and thereby averting its intercalation with cellular DNA. On the other hand, it was shown that the level of auto-fluorescence in PCD of K562 cells was comparable to those of JM1 cells treated with the same concentration of sanguinarine. This points to the fact that, unlike in BCD, the intranuclear level of sanguinarine during PCD may not be Bcl-2 dependent.

It is reported that apoptosis is only rarely observed *in vivo* and may not be the sole cell death mechanism (Houghton, 1999). These findings emphasize the need to explore new forms of cell death mechanisms and corroborates our assertion that, at least in the case of sanguinarine, tumour cell death may also be induced by BCD- a hitherto less understood form of cell death. Sanguinarine induced bimodal cell death (BMCD) may serve as a unique model for the study of apoptosis and BCD (i.e. blebbing). Furthermore, this model facilitates better comparative analysis of both modalities of cell death, as they are induced in one and the same experimental system.

Figure 2-1: Light micrographs (original magnification- ×282) of K562 and JM1 cells treated with sanguinarine. Apoptosis (b) and blister cell death (c) in K562 cells treated with sanguinarine at 1.5 μg/ml and 12.5 μg/ml respectively for 2 hrs. JM1 cells, which express high endogenous levels of Bcl-2, are resistant to the induction of apoptosis (e) and blister cell death (f) at the corresponding sanguinarine concentrations. Control untreated K562 and JM1 cells are shown in figs. (a) and (d) respectively.

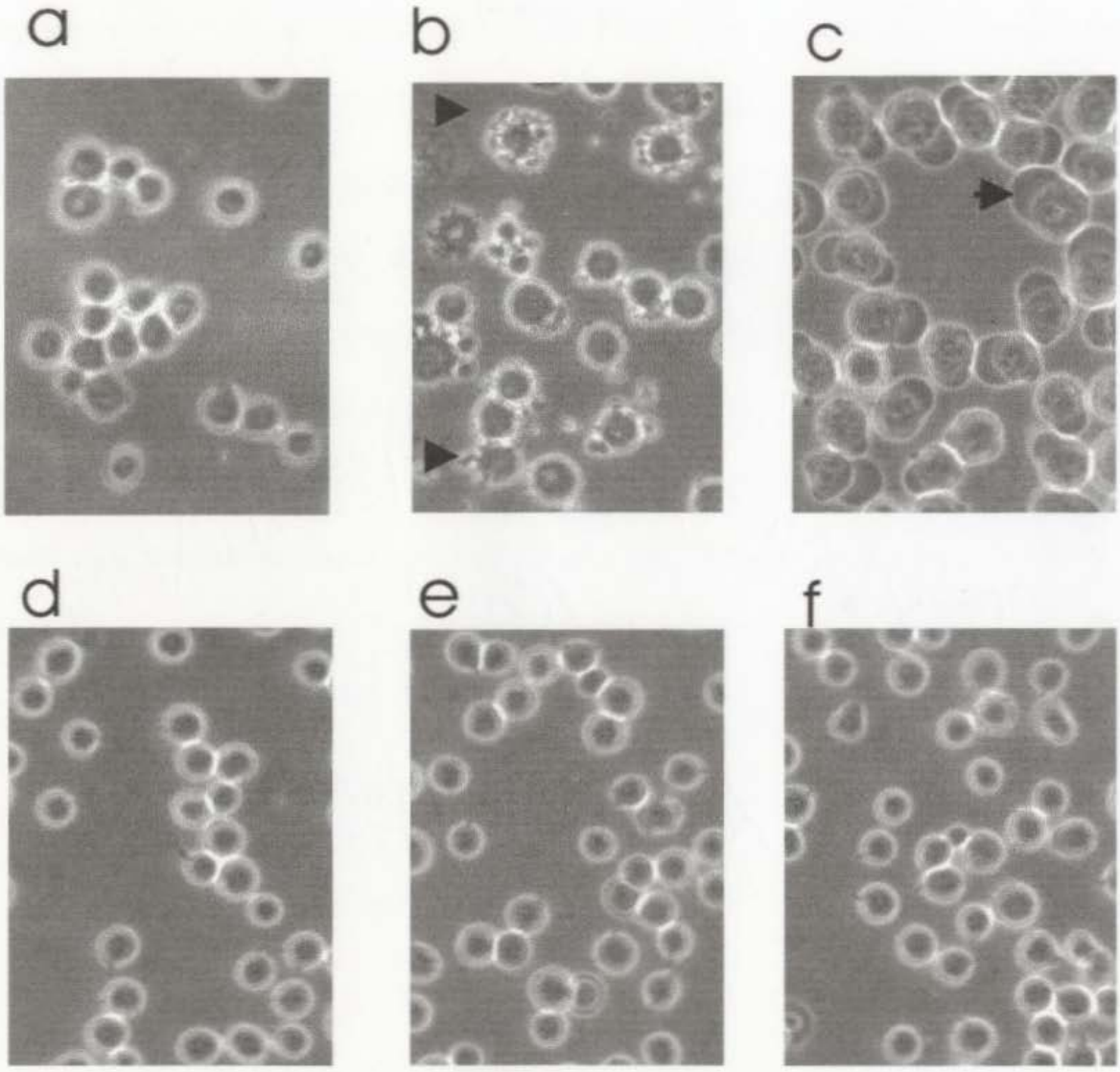

a
b
c
d
e
f

Figure 2-2: Electron micrographs (scale bar shown in respective figure) show control (a) apoptosis (b) and blister cell death (c) in K562 cells induced by treatment with sanguinarine 1.5 μg/ml and 12.5 μg/ml respectively for 2 hours. Also shown are, control JM1 cells (d), JM1 cells treated with sanguinarine 1.5 μg/ml (e) and JM1 cells treated with sanguinarine 12.5 μg/ml (f). Fig. (b) (PCD of K562 cells) show apoptotic bodies, nuclear fragmentation and chromatin condensation while fig. (c) (BCD of K562 cells) show blister formation, patchy chromatin condensation and an increase in vacuolization. As shown in figs. (e) and (f), the only ultra-structural change noted in JM1 cells when treated with sanguinarine (compared with control untreated cells) is the absence of microvilli.

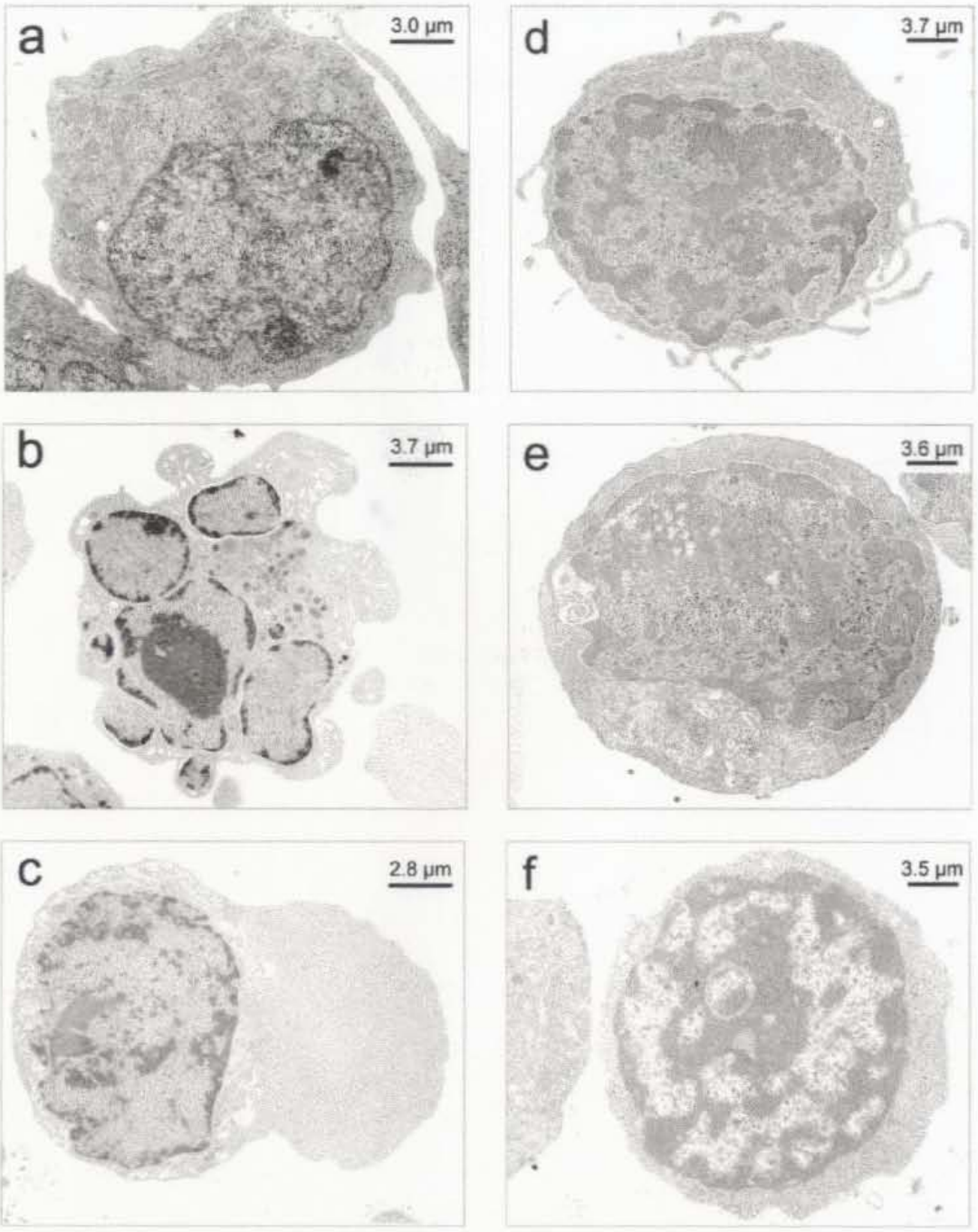

a
3.0 µm
d
3.7 µm
b
3.7 µm
e
3.6 µm
c
2.8 µm
f
3.5 µm

Figure 2-3A: Changes in membrane permeability to ^{51}Cr in K562 and JM1 cells when treated with sanguinarine concentrations of 1.5 μg/ml and 12.5 μg/ml. Each data point represents the mean ± SE for three experiments carried out in duplicate. The graph shows a 20 ±2.8% increase in ^{51}Cr in cells undergoing PCD and a 40 ± 3.5% (p value =0.004 vs control) increase in cells undergoing BCD in K562 cells but not in JM1 cells.

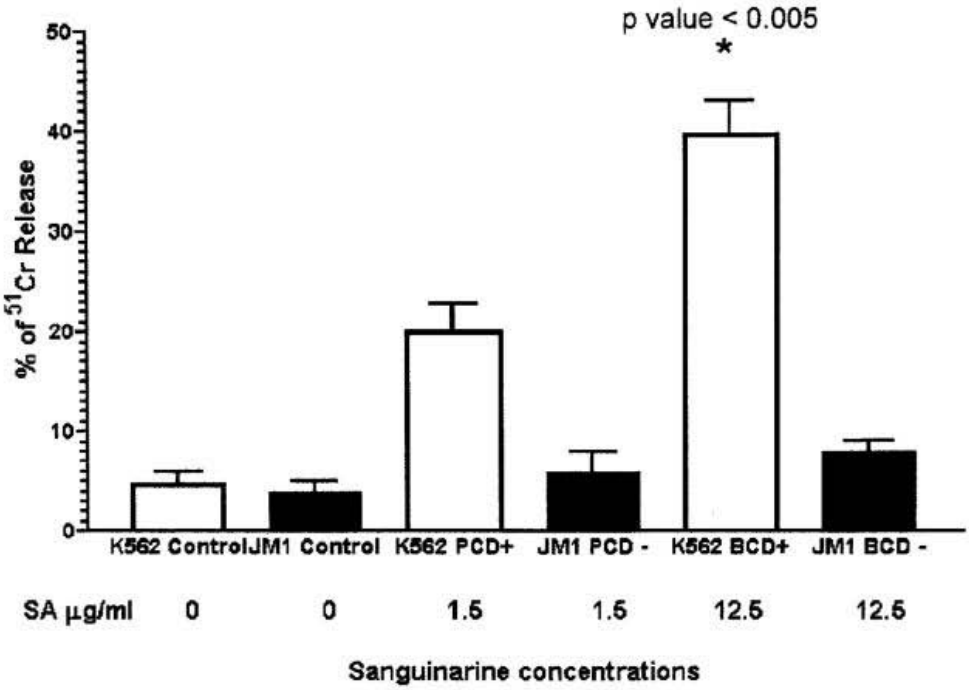

p value < 0.005
*
% of 51Cr Release
50
40
30
20
10
0
K562 Control
JM1 Control
K562 PCD+
JM1 PCD -
K562 BCD+
JM1 BCD -
SA µg/ml
0
0
1.5
1.5
12.5
12.5
Sanguinarine concentrations

Figure 2-3B: Quantitative trypan blue exclusion in sanguinarine induced apoptosis (at 1.5 μg/ml) and BCD (at 12.5 μg/ml) of K562 cells and of sanguinarine treated JM1 cells. The graph shows all cell samples of both cell lines (control, PCD and BCD) excluding trypan blue. Each data point represents the mean ± SE for three experiments carried out in triplicate.

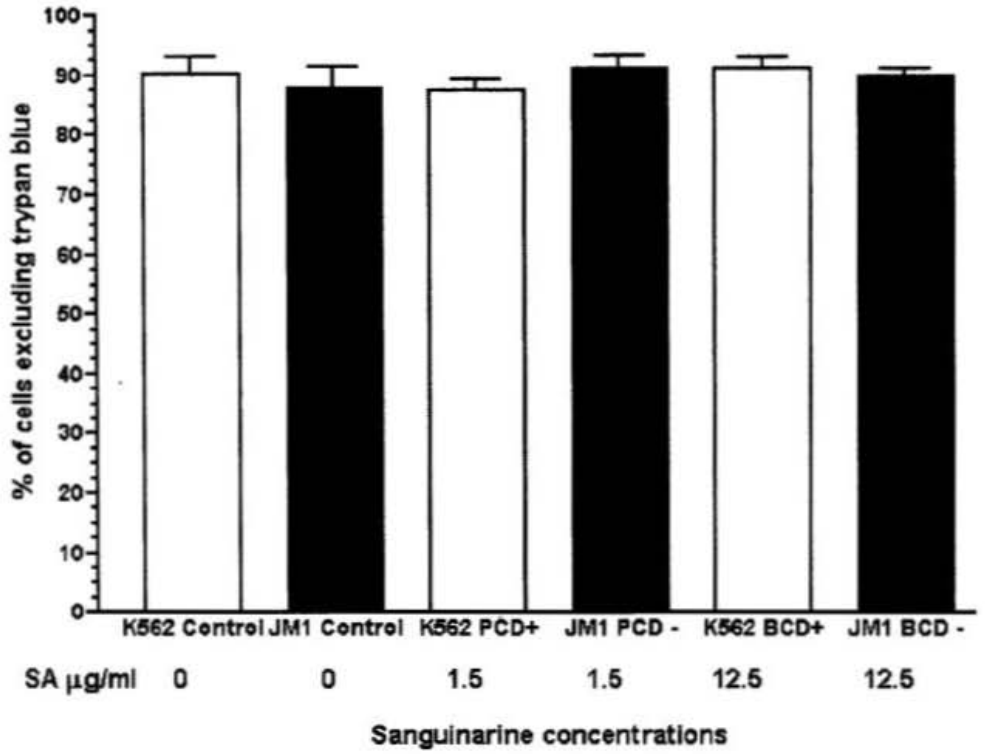

% of cells excluding trypan blue
100
90
80
70
60
50
40
30
20
10
0
K562 Control
JM1 Control
K562 PCD+
JM1 PCD -
K562 BCD+
JM1 BCD -
SA µg/ml
0
0
1.5
1.5
12.5
12.5
Sanguinarine concentrations

Figure 2-3C: Quantitative propidium iodide exclusion in sanguinarine induced apoptosis and BCD of K562 cells and of JM1 cells treated with sanguinarine. The graph shows all cell samples of both cell lines (control, PCD and BCD) excluding PI. Each data point represents the mean ± SE for three experiments carried out in triplicate.

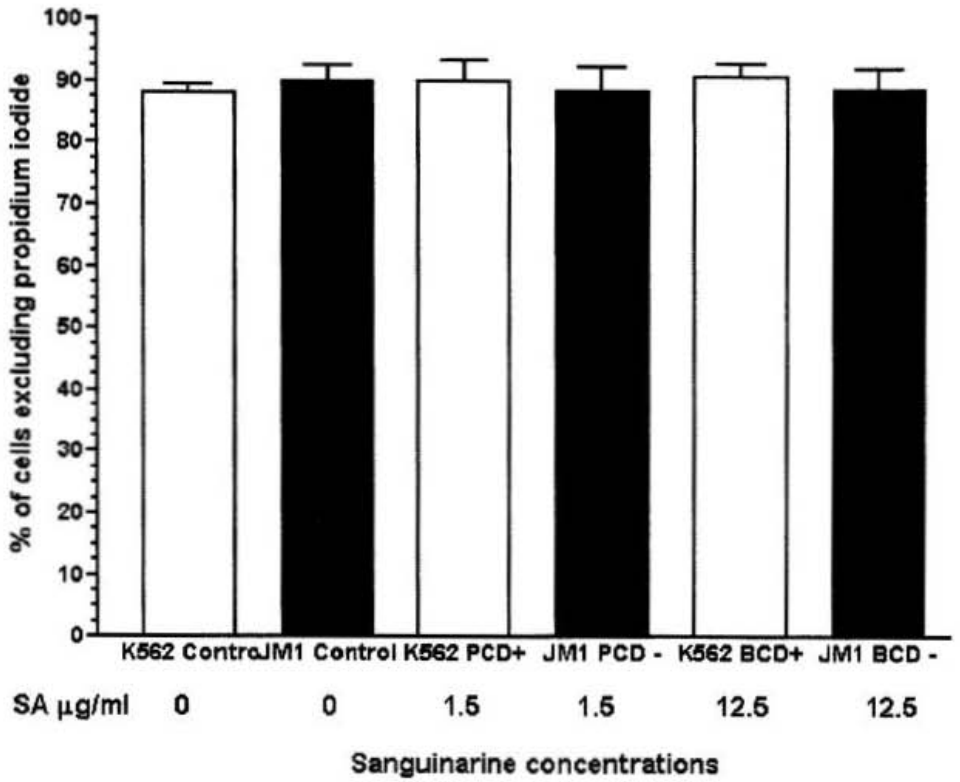

% of cells excluding propidium iodide
100
90
80
70
60
50
40
30
20
10
0
K562 Contro
JM1 Control
K562 PCD+
JM1 PCD -
K562 BCD+
JM1 BCD -
SA µg/ml
0
0
1.5
1.5
12.5
12.5
Sanguinarine concentrations

Figure 2-4: Cell surface membrane phosphatidyl serine (PS) flip as measured by the annexin V assay in sanguinarine induced apoptosis (1.5 μg/ml) and blister cell death (12.5 μg/ml) in K562 cells and in JMI cells treated with sanguinarine. After staining the cell samples with annexin V and FITC, the number of annexin V positive cells were quantitated by FACS analysis. Each data point represents the mean ± SE for three experiments carried out in triplicate. 45 ± 6.1% (p value = 0.03 vs control) of K562 cells undergoing PCD was positive for annexin V binding while all other cell samples (BCD of K562 cells, JM1 cells treated with sanguinarine and control untreated cells) did not show the PS flip.

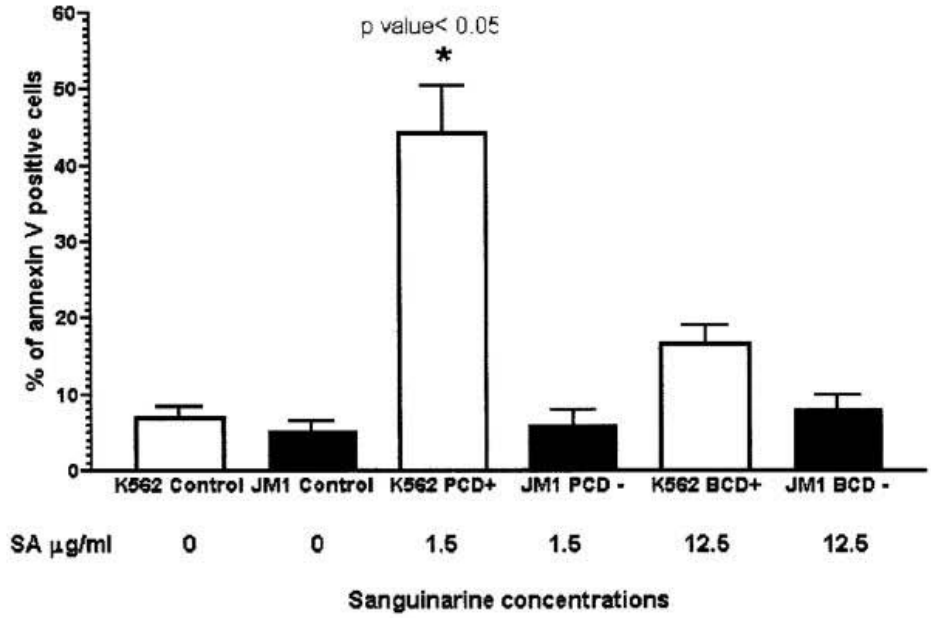

p value< 0.05
*
% of annexin V positive cells
60
50
40
30
20
10
0
K562 Control
JM1 Control
K562 PCD+
JM1 PCD -
K562 BCD+
JM1 BCD -
SA µg/ml
0
0
1.5
1.5
12.5
12.5
Sanguinarine concentrations

Figure 2-5: Terminal deoxynucleotidyl transferase (TdT) end labeling assay was utilized to detect damaged nuclear DNA in K562 and JM1 cells treated with sanguinarine (original magnification- ×200). Apoptotic K562 cells induced by sanguinarine showed DNA nicking (5b), whereas, BCD of K562 cells (5c), JM1 cells treated with either concentration of sanguinarine (5e and 5f), and untreated control cells (5a and 5d) failed to show nicking.

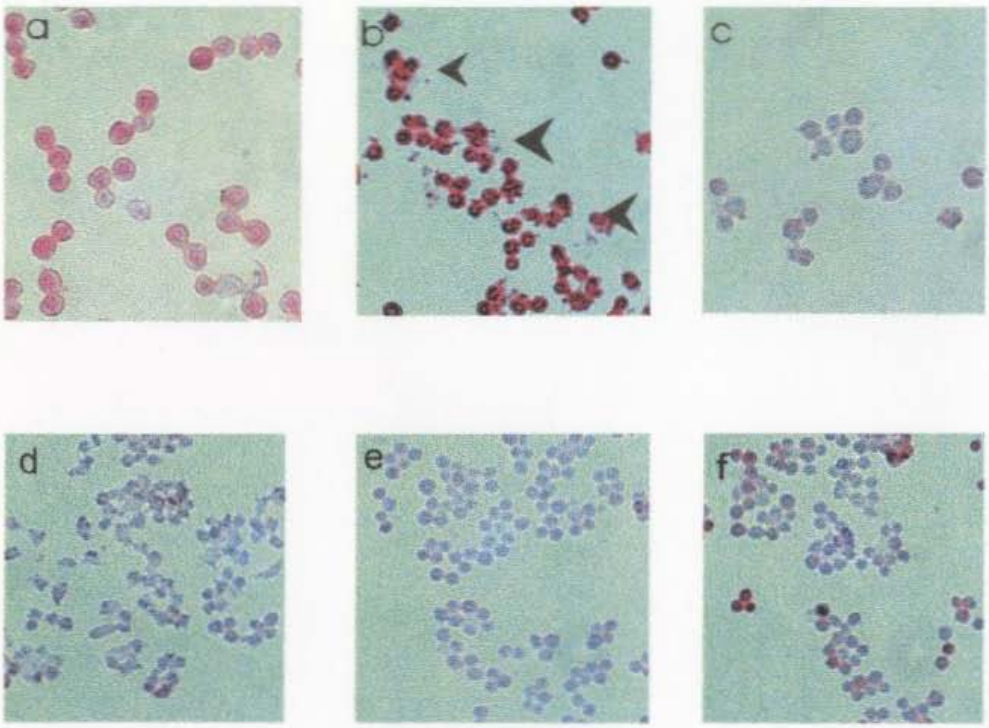

a
b
c
d
e
f

Figure 2-6: A flow cytometric dot-plot, showing sanguinarine auto-fluorescence in control (a), PCD (b) and BCD (c) of K562 cells as well as control (d) and sanguinarine treated JM1 cells (e and f). While BCD of K562 cells (c) showed a higher level of fluorescence than JM1 cells treated with a similar concentration of sanguinarine (f), the apoptosis of K562 cells (b) showed comparable levels of fluorescence to its JM1 counterparts (e).

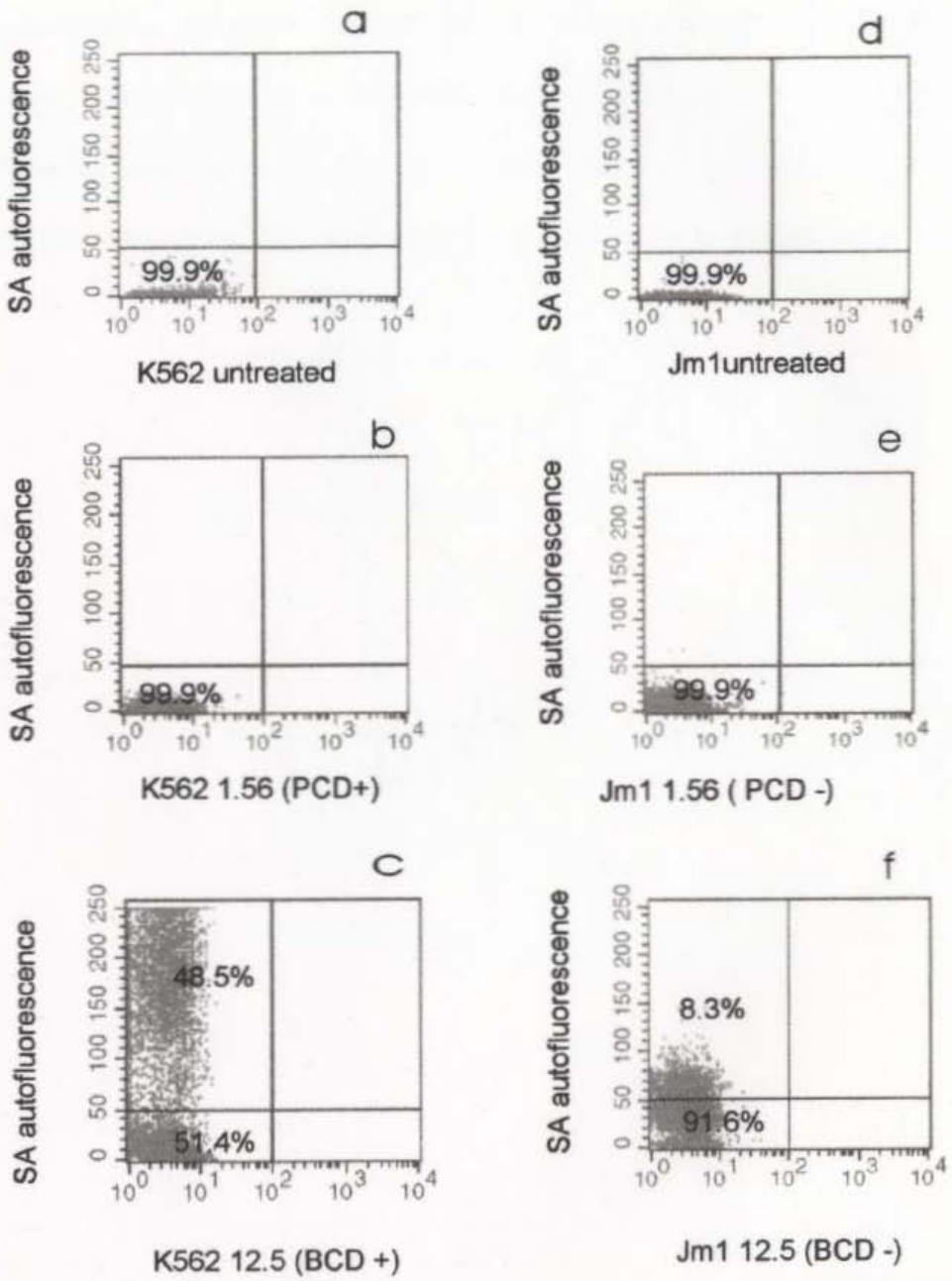
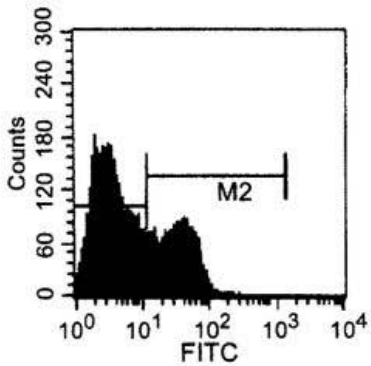

a
SA autofluorescence
99.9%
K562 untreated
d
SA autofluorescence
99.9%
Jm1untreated
b
SA autofluorescence
99.9%
K562 1.56 (PCD+)
e
SA autofluorescence
99.9%
Jm1 1.56 (PCD -)
c
SA autofluorescence
48.5%
51.4%
K562 12.5 (BCD +)
f
SA autofluorescence
8.3%
91.6%
Jm1 12.5 (BCD -)

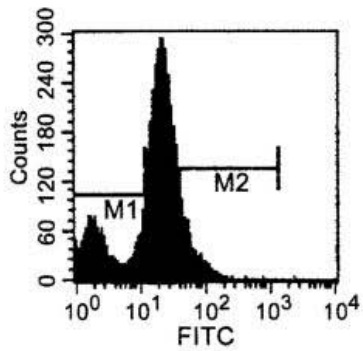
Figure 2-7: Percentages of cells in the histogram of flow cytometric analysis showing the expression of Bcl-2 in control (untreated) K562 (a) and JM1 (b) cells. (a) Histogram of Bcl-2 protein expression in untreated control K562 cells. The percentage of Bcl-2 positive cells is 33.88% and Bcl-2 negative cells is 66.77%. (b) Histogram of Bcl-2 protein expression in untreated control JM1 cells. The percentages of Bcl-2 positive cells and Bcl-2 negative cells are 76.75% and 24.02%, respectively. M1 denotes the percentage of cells negative for the protein while M2 denotes the percentage of positive cells.

a) K562 Control



M1=66.77% M2=33.88%

b) Jm1 Control



M1=24.02% M2=76.75%

Chapter 3

Role of Bcl-2 Family Proteins and Caspase-3 in Sanguinarine-Induced Bimodal Cell Death

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Abstract

Sanguinarine, a benzophenanthridine alkaloid, has anti-cancer potential through induction of cell death. Previously we have demonstrated that sanguinarine treatment at a low level induced apoptosis or programmed cell death (PCD) in the Bcl-2 low-expressing K562 human erythroleukemia cells, and a high level induced blister cell death (BCD); whereas Bcl-2 over-expressing sanguinarine-treated JM1 pre-B lymphoblastic cells displayed neither apoptosis nor BCD morphologies. Here, we report that sanguinarine-treated K562 cells, when analysed by western blot, showed significant increase in expression of the pro-apoptotic Bax protein in apoptosis, but not in BCD. cDNA expression array of PCD in K562 cells failed to reveal the presence of Bax at the gene transcript level, which suggests that this cell death process does not require *de novo* protein synthesis. Treated JM1 cells, on the other hand, showed an increase in the expression of Bcl-2 protein in both forms of cell death, but failed to show Bax expression. The role of other members of the Bcl-2 family remained negligible. Caspase-3 activation was observed in apoptosis of K562 cells but not in BCD or in sanguinarine-treated JM1 cells. These results suggest that sanguinarine in K562 cells induces apoptosis through increasing Bax and activating caspase-3, whereas sanguinarine-induced BCD involves neither. These results also suggest that in JM1 cells, Bcl-2 may be associated with susceptibility of cells to induction of apoptosis and BCD.

Introduction

Sanguinarine is a benzophenanthridine alkaloid derived from the plant, *Sanguinaria canadensis*, of the Papaveraceae family (Shamma and Guinaudeau, 1986). Its principle medicinal use to date is in dental products based on its antibacterial, antifungal, and anti-inflammatory activities, which reduce both gingival inflammation and supragingival plaque formation (Kuftinec *et al.*, 1990; Laster and Lobene, 1990; Godowski *et al.*, 1995). Sanguinarine has also been reported to have antiviral and, moreover, antitumor activity (Colombo and Bosisio, 1996; Faddeeva and Beliaeva, 1997; Ahmad *et al.*, 2000).

Biochemical studies indicate that sanguinarine has multiple cellular targets (for review, see Walterova *et al.*, 1995). For example, it can interact with and intercalate DNA (Nandi and Maiti, 1985), inhibit microtubule assembly (Wolff and Knipling, 1993), and inhibit a wide variety of enzymes, including Na^+/K^+ ATPase (Seifen *et al.*, 1979). Most interestingly, it is also a potent inhibitor of protein kinases (Wang *et al.*, 1997) and NF-κB (Chaturvedi *et al.*, 1997), which are involved in signal transduction pathways leading to cell proliferation and/or programmed cell death (PCD or apoptosis). Apoptosis is a process of active cell death characterized by cell surface blebbing, changes in membrane permeability and nuclear DNA condensation and internucleosomal fragmentation (Liepins, 1989; Liepins and Bustamante, 1994). The latter event is considered to be a hallmark of apoptosis (Arends *et al.*, 1990; Compton, 1992).

Our previous studies have demonstrated that K562 human erythroleukemia cells, when exposed to the alkaloid sanguinarine at concentrations of 1.5 μg/ml and 12.5 μg/ml for two hours, displayed the morphologies of two different modalities of cell death: at 1.5

μg/ml the classical morphology of apoptosis or programmed cell death (PCD) and at concentrations of 12.5 μg/ml, the morphology of single blister formation or blister cell death (BCD) (Weerasinghe *et al.*, in press). Previous observations in our laboratory of apoptosis or programmed cell death (PCD) and blister cell death (BCD) induced by the semisynthetic benzophenanthridine alkaloid derivative Ukrain™ in K562 cells was termed "bimodal cell death" (BMCD) (Liepins *et al.*, 1996). Thus, this dual cell death modality induced by sanguinarine was also termed "bimodal cell death" (BMCD).

Bcl-2 is known to be the prototype member of a family of genes that positively or negatively regulate apoptosis (Reed, 1998). Bcl-2 was also shown to be expressed in a variety of tissues and to inhibit the induction of apoptosis in many experimental systems (McDonnel *et al.*, 1996). The product of the Bax gene, another member of the Bcl-2 family that forms heterodimers, has been shown to induce the apoptotic cell death process (Yin *et al.*, 1994). Increased Bax expression may overcome Bcl-2-mediated cell death suppression following a death-inducing stimulus (Oltvai *et al.*, 1993). It has also been suggested that the relative amounts of Bcl-2 and Bax may determine the susceptibility of a cell to apoptosis (Korsmeyer *et al.*, 1993).

Apoptosis has been postulated to occur via execution by a family of cysteine proteases called caspases (Nuñez *et al.*, 1998). These death proteases are part of a proteolytic caspase cascade that is activated by diverse apoptotic stimuli from outside and inside of the cell. Enzymatic activation of initiator caspases, such as caspase-3, leads to proteolytic activation of downstream effector caspases and cleavage of a number of vital proteins, resulting in physiological, noninflammatory death and removal of apoptotic bodies by neighboring cells (Nuñez *et al.*, 1998; Salvesen, 1999).

This report indicates that sanguinarine-induced apoptosis in Bcl-2 low-expressing K562 erythroleukemia cells (Kobayashi *et al.*, 1998) involves Bax protein induction and caspase-3 activation, and that sanguinarine-induced BCD did not trigger caspase-3 activation and was Bax independent. In contrast, in Bcl-2 over-expressing JM1 pre-B lymphoblastic cells (Samson *et al.*, 1996), sanguinarine was unable to trigger caspase-3 activation or Bax induction. However, JM1 cells treated with sanguinarine showed an increase in the expresssion of Bcl-2.

Materials and Methods

Materials

The K562 human erythroleukemia cell line as well as the Bcl-2 over-expressing JM1 human B cell leukemia cell line were obtained from American Type Culture Collection (Rockville, MD, USA). These cells were cultured in RPMI 1640 medium supplemented with L. glutamine and 10% fetal calf serum. They were maintained in a 37°C/ 5% CO_2, fully humidified incubator and passaged 4-5 times weekly.

The drug sanguinarine was purchased from Sigma Chemicals (St. Louis, MO, USA) and maintained as a dry powder at room temperature. It was dissolved in deionized water at 1mg/ml, and used as a stock solution. Sanguinarine was subsequently diluted with RPMI 1640 in order to make a working solution of 50 μg/ml and serial dilutions thereof.

The ATLAS cDNA expression array was from Clontech Laboratories (Palo Alto, CA, USA) ^{32}P dATP (6.00 Ci/mmol) was purchased from DuPont New Research Products (Boston, MA, USA). Caspase 3 assay kit was from Becton Dickinson Co. (San Diego, CA, USA)

Cell culture

Cells were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD, USA) in 75 ml culture flasks to densities approximately 10^6 cells/ml.

Drug treatment

The following experimental format was used in order to prepare the cells for subsequent experiments. Logarithmically growing K562 and JM1 cells were placed in

60-mm dishes at approximately 3×10^6 cells/ml. Thereafter, serially diluted sanguinarine concentrations of 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5 and 25 μg/ml were added to the petri dishes and incubated for 2 hrs in a 37°C/5% CO_2 incubator. Thereafter, cells that underwent PCD and BCD (at concentrations of 1.5 μg/ml and 12.5 μg/ml respectively) were used for morphological analysis, caspase-3 assay and protein isolation from cells.

Quantitative morphology

The percentage of cell death, (i.e. apoptosis and blister cell death) corresponding to each drug concentration was determined by light microscopy. The key morphological criterion for apoptosis was the formation of apoptotic bodies and the key criterion for blister cell death was the formation of cell surface blisters. Number of apoptotic cells and blistering cells were counted using a hemocytometer.

Caspase-3 assay

Caspase-3 (CPP-32) activity was determined by measuring proteolytic cleavage of the specific fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC), based on the peptide sequence of caspase-3 cleavage site of poly (ADP-ribose) polymersase (Evdokiou, 1999). Specific caspase-3 inhibitor N-acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) was used as a negative control. Cells were cultured for 2 hrs in the presence of sanguinarine 1.5 μg/ml and 12.5 μg/ml with untreated cells as controls, washed in Phosphate Buffered Saline (PBS), and resuspended in NP-40 lysis buffer.

The cell lysate was added to each tube containing 8 μM of substrate in 1 ml of protease buffer {50 mM HEPES, 10% sucrose, 10 mM dithiothreitol and 0.1% 3 [(chloramido propyl) dimethyl amino] -1- propanesulphonate, pH 7.4} in the presence

and absence of the inhibitor Ac-DEVD-CHO, and the mixture was incubated at room temperature for 45 min. Caspase-3 activity was quantitated by measurement of yellow green fluorescence at 505 nm (excitation at 400 nm) due to release of AFC in ALS50 spectrofluorimeter (Perkin Elmer Co., Norwalk, CT).

PAGE-SDS and Western blotting

The effect of sanguinarine on expression of apoptosis-associated proteins including Bcl-2, Bax, Bak, Bad, Bik/Nbk, Bcl-X_S, Bid, Bcl-X_L, p53, and CPP-32, were determined by western blotting analysis using previously described methods (Pixton, 1990; Towbin *et al.*, 1979) with minor modifications. Briefly, 10^7 cells were pelleted, washed twice in Phosphate Buffered Saline (PBS), resuspended in 50 µl PBS and lysed in lysis buffer. The lysate protein was quantified using a DC protein assay kit (Amersham, Piscataway, NJ) and 20 µg protein/lane were boiled 5 min, resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose. The blots were blocked with PBS, 0.05% Tween 20 and 5% non-fat dried milk for 1 h at 22^0C, incubated in blocking solution containing primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), washed thrice for 5 min in TBS-T and then incubated with 1:2500 dilution of horseradish peroxidase conjugated secondary antibody (Santa Cruz) for 1 h at 22^0C. Blots were again washed thrice for 5 min in TBS-T and then developed by chemiluminescence (Amersham, Arlington Heights, IL).

RNA isolation

Total RNA was prepared according to Chomczynski and Sacchi and used for the ATLAS cDNA expression array (Chomczynski and Sacchi, 1987).

Briefly, cultured K562 cells (5×10^7 cells) and cells treated with sanguinarine 1.56 μg/ml for 2 hours were washed twice in PBS and immediately homogenized in 3 ml of ice cold Trizol (Life Technologies Inc., MD). After the addition of 600 μg of chloroform (Life Technologies Inc., MD) and a 15 minute centrifugation (10,000×g, 4^0C), RNA was ethanol-precipitated from the aqueous phase. Total RNA was resuspended in Rnase-free water and the concentration assessed by absorbency at 260 nm using an UV spectro-photometer.

cDNA array hybridization

Gene expression was analyzed using the ATLAS cDNA expression array (Vegh *et al.*, 1999) (a technique whose basic principle can be viewed as reverse Northern blotting), in untreated control and apoptotic cells of K562 cells. The apoptosis cDNA expression array consisted of 215 known apoptosis related genes belonging to eight functional groups: 1) cell cycle genes 2) P53 pathway genes 3) Bcl-2 family genes 4) Caspases 5) Caspase regulators 6) ligand and receptors 7) other regulators of apoptosis 8) house keeping genes. A mixture containing 1 μg of sample total RNA and 1 μl of 10× CDS primer (Clontech) was incubated for 2 min at 70°C. Afterwards, an 8 μl volume containing 1 μl of 10× dNTP, 0.5 μl of dithiotreitol (100 mM), 3.5μl of [^{32}P]αdATP (10mCi/ml), 1 μl of 25 mM $Mgcl_2$, 1 μl of 10× PCR buffer (Clontech) and 1μl of MMLV reverse transcriptase was added, and the solution was mixed and incubated for 25 min at 50°C. The reaction was terminated with the addition of 1 μl of 10× termination mix at room temperature. The reverse transcribed sample was applied to the center of the gel bed in a Chroma Spin-200 column (Clontech) and allowed to fully absorb into the resin. Successive column washes of 40 μl and 250 μl of diethylpyrocarbonate treated water

were followed by four successive fractionations using 100 μl of diethylpyrocarbonate treated water. The second and third fractions were pooled, mixed with 22 μl of 10× denaturing solution and incubated at 68°C for 20 min. Next, 5 μl of human Cot-1 DNA (1 μg/μl, Clontech) and 225 μl of 2× neutralizing solution were added and the labeled cDNA was then added to 5 ml of ExpressHyb hybridization solution (Clontech) with 150 μg/ml sheared salmon testis DNA (Sigma, St. Louis, MO, USA) to reach a final probe concentration of 1×10^6 c.p.m./ml and freshly applied to the ATLAS cDNA expression array membrane, which was prehybridized in 10 ml of ExpressHyb hybridization solution at 68°C in a roller bottle overnight. The membrane contains cDNA fragments representing 250 human apoptosis related genes. Each cDNA fragment is 200-500 bp long and is selected as a unique sequence without poly (A) tail, repetitive elements, or highly homologous sequences to minimize cross-hybridization and nonspecific binding of cDNA probes. The amount of each cDNA fragment on the membrane is 10 ng, and each cDNA fragment is immobilized in duplicate. Hybridization proceeded overnight at 68°C in a roller bottle. Membranes were stringently washed with agitation for 20 min in 200 ml of pre warmed (68°C) solution 1 (2× standard saline citrate, 1% sodium dodecyl sulfate) four times and solution 2 (0.1× standard saline citrate, 0.5% sodium dodecyl sulfate) twice. Afterwards, membranes were rinsed in 0.1× SSC and exposed to X-ray film for 3-7 days at –80°C.

Statistical analysis

The results are presented as means ± SE. Statistical analyses were performed using the student's t test. Differences were considered significant when $p< 0.05$.

Results

Sanguinarine-induced cell death

The effect of sanguinarine on cell death was examined in Bcl-2-overexpressing and low-expressing cells. Cell surface blebbing and shedding of apoptotic bodies (PCD) and blister formation (BCD) was examined by light microscopy and quantitated (Fig.3-1). Over 96% of 1.5 μg/ml sanguinarine-treated low Bcl-2 expressing K562 cells displayed apoptosis morphology (Fig.3-2a), whereas 12.5 μg/ml induced more than 90% BCD (Fig.3-2b). Using the same conditions, high Bcl-2 expressing JM1 cells displayed no apoptosis, and showed BCD in fewer than 10% of cells (Fig.3-1).

Effect of sanguinarine on expression of apoptosis-associated proteins

To study expression of apoptosis-regulating proteins of the Bcl-2 family and p53 during sanguinarine-induced apoptosis at 1.5 μg/ml and BCD at 12.5 μg/ml, Western blot analysis was used on K562 and JM1 cell extracts. Compared to untreated control K562 cells, the expression of pro-apoptotic Bax protein was found to be increased in sanguinarine-induced apoptosis (Fig.3-3). In contrast, sanguinarine-induced BCD was not accompanied by any significant increase in Bax protein expression. Treatment of JM1 cells with conditions that induce apoptosis and BCD in K562 cells failed to induce any significant change in Bax expression (Fig.3-3). In K562 cells, no change was observed in the low expression of the Bcl-2 protein in apoptosis and BCD. In contrast, JM1 cells showed an increase in Bcl-2 in low and high sanguinarine concentrations (Fig.3-4). However, the expression of pro-apoptotic Bak, Bad, Bik/Nbk, Bcl-X_S, Bid, and p53 and anti-apoptotic Bcl-X_L proteins was not significantly effected by sanguinarine in either cell line at low level or high level (individual data not shown).

K562 and JM1 cells contained high endogenous levels of the 32 kDa procaspase-3 (Fig.3-5). However, the 20 kDa activated caspase-3 product was only observed in apoptosis of K562 cells. Fluorimetric analysis results confirmed the activation of caspase-3 observed by Western blot analysis (Fig.3-6). Caspase-3 activation was seen in only K562 cells in the apoptosis-inducing concentration.

Results of cDNA expression array of PCD in K562 cells

This study enabled the simultaneous examination and analysis of changes at multiple gene transcript levels in sanguinarine induced apoptosis, as compared to untreated control K562 cells. The purpose of this study was to detect any changes in Bax expression or that of any other member of the Bcl-2 family of proteins at the gene transcript level. We observed no Bax gene (or any other member of the Bcl-2 family) expression in PCD at the mRNA level.

Discussion

K562 human erythroleukemia cells when treated with sanguinarine increase expression of the pro-apoptotic Bax protein during PCD while showing the morphological characteristics of apoptosis. In contrast, the Bcl-2-overexpressing JM1 cells when subjected to sanguinarine treatment showed an increase in the anti-apoptotic Bcl-2 expression with no changes in Bax expression and failed to show the morphological characteristics of apoptosis when treated with concentrations that induced apoptosis in K562 cells (1.5 μg/ml). These results suggest that the Bcl-2/Bax ratio may determine the susceptibility of cells to apoptosis during sanguinarine treatment. The proto-oncogenes Bcl-2 and Bax are known to inhibit and accelerate apoptosis, respectively (Reed *et al.*, 1994; Krajewski *et al.*, 1995). In addition, the ratio of the Bcl-2 and Bax proteins was found to correlate with the chemotherapeutic effect (Nakata *et al.*, 1998). It has also been observed that tumor cell apoptosis following chemotherapy is essential for chemotherapeutic efficacy (Barry *et al.*, 1990; Nakata *et al.*, 1998). Consistent with these studies, our findings indicate that the Bax/Bcl-2 ratio in cells could be a determining factor in the induction of apoptosis by sanguinarine. Another notable observation in the present study was that sanguinarine induced bimodal cell death (BMCD) in a p53 independent manner.

Considerable research has been devoted to study the activation of the protease cascade in drug-induced and other forms of apoptosis, as well as the ability of Bcl-2 and Bcl-2-related proteins to inhibit this process (Wang *et al.*, 1997; Irabo *et al.*, 1996). Sanguinarine treatment of Bcl-2 low-expressing K562 cells resulted in the activation/cleavage of caspase- 3 into 20 kDa breakdown products. The Bcl-2-

overexpressing JM1 cells, on the contrary, showed no breakdown products of caspase- 3. Therefore, these results show that, elevated Bcl-2 may be associated with failure to trigger caspase 3 activation, which is in agreement with several recent reports (Kojima *et al.*, 1996; Irabo *et al.*, 1996). These results also appear to be in agreement with recent literature, which suggests that in the case of some, but perhaps not all cytotoxic stimuli, Bcl-2 functions upstream of a critical cysteine protease involved in the degradation phase of apoptosis (Wang *et al.*, 1997). As opposed to the function of Bcl-2, increased levels of Bax promote caspase 3 activity in sanguinarine-induced apoptosis. In contrast, the absence of caspase 3 activation during BCD may indicate that this may not be a necessary element in the execution of BCD.

Compared to the abundance of literature pertaining to apoptosis, the phenomenon of BCD is, at best, only sporadically reported. This morphological phenomenon of blister formation may resemble "blebbing" as previously described by Majno and Joris (1995) and Phelps *et al.* (1989). Trump *et al.* (1997) and Trump and Berezesky (1992) associates blebbing with increases in concentration of cytosolic calcium and rearrangement of cytoskeletal proteins.

Our results show that low Bcl-2 expressing K562 cells treated with sanguinarine 12.5 μg/ml, underwent BCD without significant changes in the expression of Bcl-2 and Bax. Furthermore, the elevated Bcl-2 expressing JM1 cells when exposed to a similar concentration of the drug failed to undergo the morphology of BCD and at the same time showed an increase in Bcl-2 with no change in Bax expression. Thus, it appears that cell susceptibility to BCD may be associated with Bcl-2 levels, as opposed to the Bax/Bcl-2 ratio in PCD. The increase of Bcl-2 during sanguinarine treatment of JM1 cells (which

may have prevented these cells from undergoing PCD and BCD) may have been brought about by a mechanism downstream of the initiation of the toxicity by the drug. Recent literature has also shown evidence of inhibition of cyplasmic blebbing by Bcl-2 (Ferri *et al.*, 2000). This increased expression of Bcl-2 may change the expression of drug targets or drug transport across the plasma membrane or it may change the capacity of the cell to repair damage induced by the drug. More importantly, this may indicate that the overexpression of the Bcl-2 gene is another factor contributing to drug resistance by assuming a dual role of anti-PCD and anti-BCD.

Studies of sanguinarine-induced PCD in K562 cells using cDNA expression analysis showed, contrary to expectations, the lack of Bax at the gene transcript level. This indicates that the increase in cellular protein expression in sanguinarine-induced bimodal cell death may have been the result of post-translational modification of proteins rather than its synthesis *de novo*. This notion was further strengthened by our studies, which showed the failure of the protein synthesis inhibitor cycloheximide to inhibit PCD, and BCD in K562 cells (data not shown). This illustrates the importance of the endogenous Bcl-2 levels in JM1 cells, in determining the resistance of these cells to sanguinarine induced PCD and BCD. In contrast, as aforementioned, the low Bcl-2 expressing K562 cells, despite being considered a drug resistant cell line (Kobayashi *et al.*, 1998) underwent PCD and BCD with relative ease, when induced by sanguinarine. The resistance of JM1 cells to PCD and BCD mediated by endogenous Bcl-2 during sanguinarine treatment, albeit via different pathways, may help understand the mechanism of chemoresistance, at least for sanguinarine.

Apoptosis is not the sole cell death mechanism (Houghton, 1999). Thus, the need to explore new forms of cell death mechanisms remains. Our findings demonstrate that, at least in the case of sanguinarine, tumor cell death may also be induced by BCD, a hitherto less understood form of cell death. We believe that the discovery of novel anti-cancer agents and their individual patterns and mechanisms of cell death may have an important bearing in overcoming chemoresistance.

Figure 3-1: Quantitative morphological analysis of K562 and JM1 cells. K562 cells treated with sanguinarine 1.5 μg/ml show the morphology of apoptosis in about 96% of cells (* p value = 0.0003 versus control), and when treated with concentrations of 12.5 μg/ml, the morphology of BCD (single blister formation) in over 90% of cells (* p value = 0.0004 versus control). However, JM1 cells treated with the same concentrations of sanguinarine failed to show apoptosis, and BCD was shown in about 10% of cells.

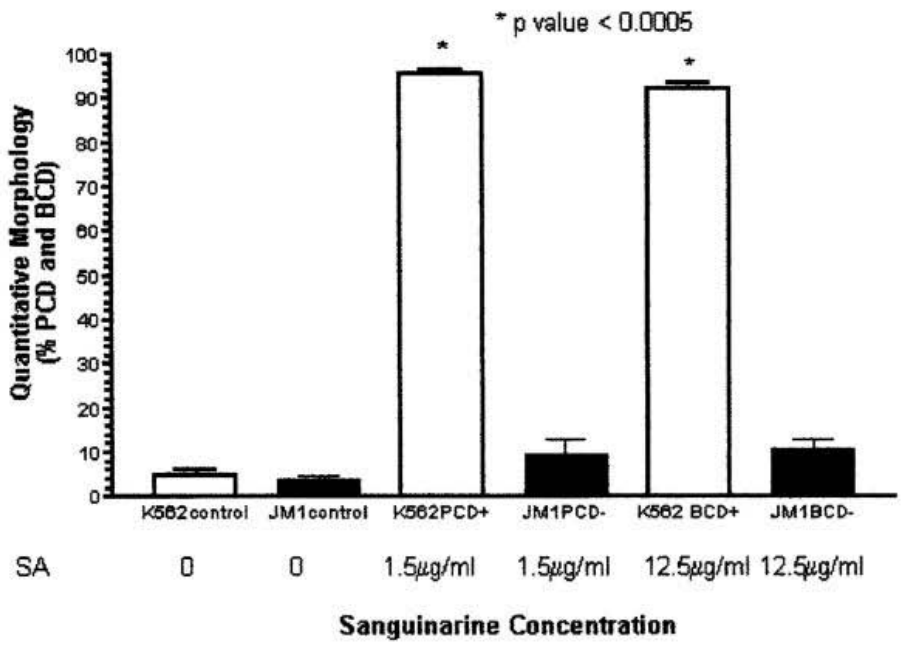

* p value < 0.0005
Quantitative Morphology (% PCD and BCD)
100
90
80
70
60
50
40
30
20
10
0
K562control
JM1control
K562PCD+
JM1PCD-
K562 BCD+
JM1BCD-
SA
0
0
1.5µg/ml
1.5µg/ml
12.5µg/ml
12.5µg/ml
Sanguinarine Concentration

Figure 3-2: Electron micrographs (original magnification- x15000) of sanguinarine induced PCD and BCD of K562 cells. The figure shows (a) apoptosis and (b) blister cell death in K562 cells induced by treatment with sanguinarine 1.5 μg/ml and 12.5 μg/ml respectively for 2 hours. Fig. (a) show apoptotic bodies, nuclear fragmentation and chromatin condensation, while fig. (b) show blister formation, patchy chromatin condensation and an increase in vacuolization.

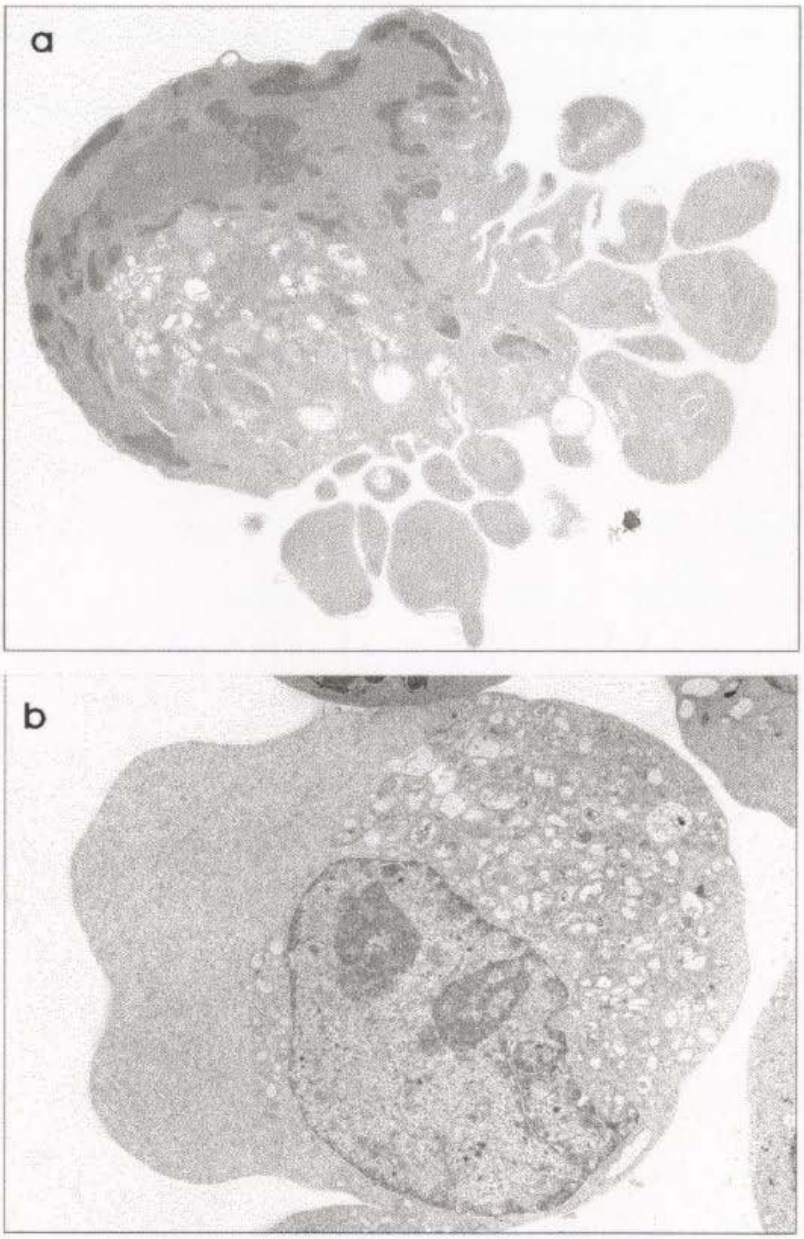
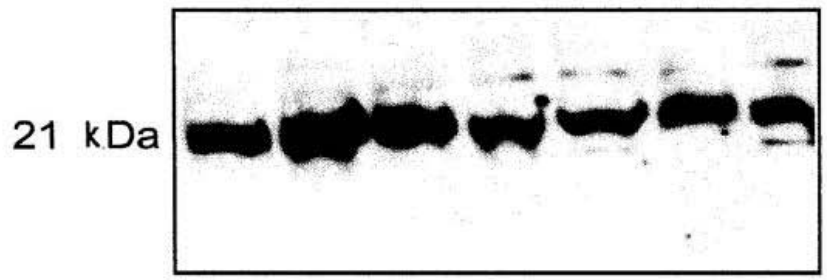

a
b

Figure 3-3: Western blot for Bax protein expression in K562 cells (lanes 1, 2, 3) and JM1 cells (lanes 4, 5, 6)[untreated (lanes 1 & 4) or treated with sanguinarine 1.5 μg/ml (lanes 2 & 5) or 12.5 μg/ml (lanes 3 & 6)], using a total cell extract of 50 μg per lane. Lane 7 shows positive control for Bax antibody. Results show an increased thickness in the Bax band in PCD of K562 cells.



	1	2	3	4	5	6	7
SA μg/ml	0	1.5	12.5	0	1.5	12.5	-
	K562	**K562**	**K562**	**JM1**	**JM1**	**JM1**	**+cont**
		PCD+	**BCD+**		**PCD-**	**BCD-**	

Figure 3-4: Western blot for Bcl-2 protein expression in K562 cells (lanes 1, 2, 3) and JM1 cells (lanes 4, 5, 6) [untreated (lanes 1 & 4) or treated with sanguinarine 1.5 μg/ml (lanes 2 & 5) or 12.5 μg/ml (lanes 3 & 6)], using a total cell extract of 20 μg per lane. Lane 7 shows positive control for Bcl-2 antibody. JM1 cells treated with sanguinarine showed an increase in Bcl-2 expression. The levels of Bcl-2 in treated and untreated K562 cells were relatively insignificant.

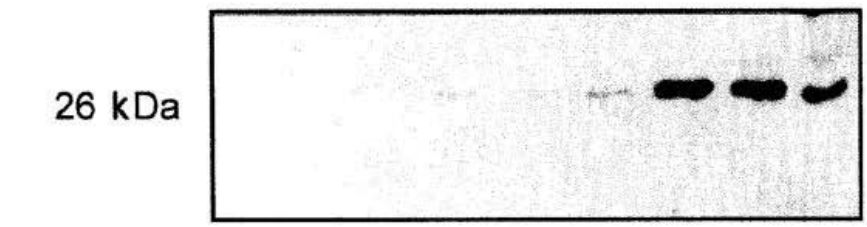
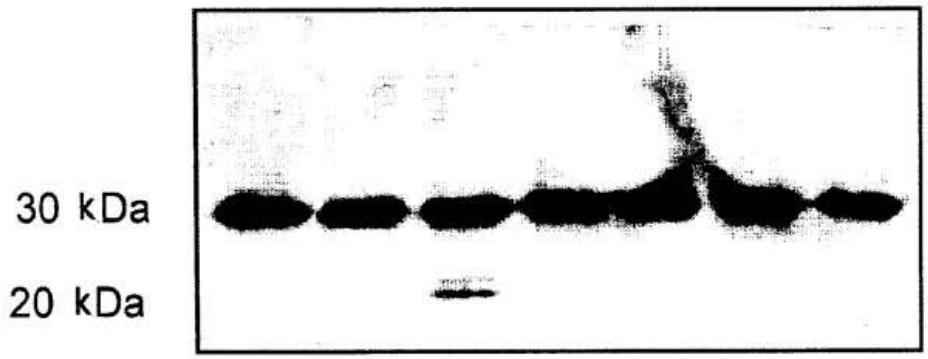
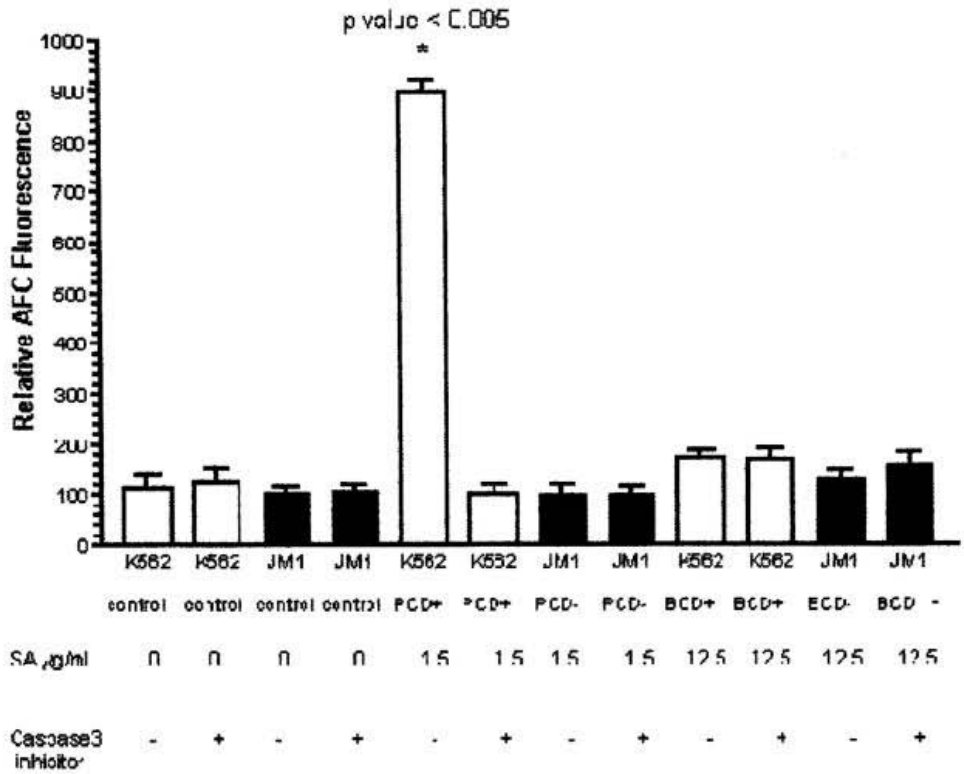

26 kDa
1 2 3 4 5 6 7
SA μg/ml
0 1.5 12.5 0 1.5 12.5 -
K562 K562 K562 JM1 JM1 JM1 +cont
PCD+ BCD+ PCD- BCD-

Figure 3-5: Western blot for Caspase-3 in K562 cells (lanes 2, 3, 4) and JM1 cells (lanes 5, 6, 7) [untreated (lanes 2 & 5) or treated with sanguinarine 1.5 μg/ml (lanes 3 & 6) or 12.5 μg/ml (lanes 4 & 7)], using a total cell extract of 20 μg per lane. Lane 1 shows positive control for Caspase-3 antibody. Results show only PCD of K562 cells with 20 kDa breakdown products of caspase-3 and hence its activation.



	1	2	3	4	5	6	7
SA µg/ml	-	0	1.5	12.5	0	1.5	12.5
	+cont	**K562**	**K562**	**K562**	**JM1**	**JM1**	**JM1**
			PCD+	**BCD+**		**PCD-**	**BCD-**

Figure 3-6: Caspase-3 activation assay in K562 and JM1 cells treated with sanguinarine. Fluorimetric analysis of caspase-3 activation was positive during apoptosis of K562 cells (* p value = 0.003 versus control) but negative during BCD. JM1 cells treated with 1.5 μg/ml and 12.5 μg/ml of sanguinarine did not show caspase-3 activation. Caspase-3 activation in sanguinarine induced apoptosis in K562 cells was diminished upon treatment with the caspase-3 inhibitor (DEVD-CHO). Results represent the mean ± SE from 3 independent experiments.


p value < 0.005
*
Relative AFC Fluorescence
1000
900
800
700
600
500
400
300
200
100
0
K562 K562 JM1 JM1 K562 K562 JM1 JM1 K562 K562 JM1 JM1
control control control control PCD+ PCD+ PCD- PCD- BCD+ BCD+ BCD- BCD -
SA µg/ml 0 0 0 0 1.5 1.5 1.5 1.5 12.5 12.5 12.5 12.5
Caspase3 inhibitor - + - + - + - + - + - +

Chapter 4

Bax, Bcl-2 and NF-κB Expression in Sanguinarine Induced Bimodal Cell Death

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Abstract

The apoptosis related proteins: Bax, Bcl-2 and NF-κB were analyzed in sanguinarine induced apoptosis and blister cell death (BCD) of K562 erythroleukemia cells, and in sanguinarine treated high Bcl-2 expressing JM1 pre-B lymphoblastic cells, utilizing immunofluorescence–flow cytometry. Sanguinarine induced apoptosis of K562 cells was found to have increased Bax expression and decreased NF-κB, whereas BCD showed a decrease in Bax expression and an increase in NF-κB. In contrast, high Bcl-2 expressing JM1 cells when exposed to the same concentrations (and duration) of sanguinarine that induced PCD and BCD in K562 cells, failed to show the respective morphologies while showing a concomitant increase in Bcl-2. Results from studies with K562 cells suggest that Bax is pro-apoptotic, and also that an increase in NF-κB may be associated with BCD. Results from studies with JM1 cells suggest that Bcl-2 is anti-apoptotic and anti-BCD. Results from JM1 cells strengthen the assumption in literature of the central role Bcl-2 plays in chemoresistance by assuming an anti-PCD role. These results also suggest that in JM1 cells, Bcl-2 may further complicate chemoresistance by being anti-BCD in nature, in addition to its anti-PCD role.

Introduction

Our previous studies have demonstrated that K562 human erythroleukemia cells, when exposed to the alkaloid sanguinarine at concentrations of 1.5 μg/ml and 12.5 μg/ml for two hours, displayed the morphologies of two different modalities of cell death: at 1.5 μg/ml the classical morphology of apoptosis or programmed cell death (PCD) and at concentrations of 12.5 μg/ml, the morphology of single blister formation or blister cell death (BCD) (Weerasinghe *et al.*, in press). Previous observations in our laboratory of apoptosis or programmed cell death (PCD) and blister cell death (BCD) induced by the semisynthetic benzophenanthridine alkaloid derivative Ukrain™ in K562 cells was termed "bimodal cell death" (BMCD) (Liepins *et al.*, 1996). Thus, this dual cell death modality induced by sanguinarine was also termed "bimodal cell death" (BMCD). Furthermore, these two morphologically distinct modalities of cell death were interphased by a period of apparently normal cell morphology (silent period) based on light microscopy and a relatively lower degree of ^{51}Cr release than that during blister cell death (BCD) (Liepins *et al.*, 1996).

Sanguinarine (13-methyl [1,3]benzodioxolo[5,6-C]-1,3-dioxolo[4,5-i]phenanthridinium) is derived from the root of *Sanguinaria canadensis* and other poppy-fumaria species (Chaturvedi *et al.*, 1997; Bajaj *et al.*, 1990). It belongs to the benzophenanthridine family of alkaloids and has a wide range of potentially useful medicinal properties, such as antifungal, antiprotozoal, antibacterial and antiviral activities (Walterova *et al.*, 1995). Sanguinarine is also a known PKC (protein kinase C) inhibitor (Gopalakrishna *et al.*, 1990), a DNA intercalator (binding to guanosine-cytosine rich regions) (Babich *et al.*, 1996; Sen and Maiti, 1994), a putative topoisomerase I and II

inhibitor (Hsiang *et al.*, 1989; Zhang *et al.*, 1990), an inhibitor of tubulin assembly (Wolff and Knipling, 1993) and an inhibitor of Na^+/K^+ ATPase (Seifen *et al.*, 1979; Straub and Carver, 1975).

Programmed cell death (PCD), or apoptosis, is a physiological cell death process characterized by the formation of apoptotic bodies, changes in membrane permeability, elevated oxygen consumption rates and nuclear DNA condensation with or without fragmentation (Liepins, 1989; Liepins *et al.*, 1996). The latter event, i.e., internucleosomal DNA fragmentation, may not occur in some experimental systems (Stewart, 1994; Liepins and Bustamante, 1994), although considered to be the hallmark of apoptosis (Arends *et al.*, 1990; Compton, 1992). Sanguinarine induced apoptosis did not show DNA laddering (internucleosomal DNA fragmentation) in gel electrophoresis (Liepins *et al.*, 1996). However, DNA nicking was demonstrated in these cells by the terminal deoxynucleotidyl transferase (TdT) end labeling method (Weerasinghe *et al.*, in preparation).

Membrane permeability studies with K562 cells have shown that both PCD and BCD are associated with increases in ^{51}Cr release. However, the cytoplasmic membrane of both PCD and BCD was found to exclude trypan blue, denoting viability (Weerasinghe *et al.*, in preparation). The phosphatidyl serine (PS) flip associated with apoptosis, assessed by the annexin V assay, was positive in PCD but negative in BCD (Weerasinghe *et al.*, in preparation).

Bcl-2 was found to be activated in the majority of follicular non-Hodgkins's lymphomas (Reed *et al.*, 1994). Initially, the function of Bcl-2 was viewed, like many other oncogenes, as promoting proliferation. Recently, it has been shown to function as

an inhibitor of apoptosis (Korsmeyer, 1999). The product of the Bax gene, another member of the Bcl-2 family that forms heterodimers, was also found to be involved in the apoptotic cell death process (Yin *et al.*, 1994). Increased Bax expression may overcome Bcl-2 mediated cell death suppression and result in accelerated apoptotic cell death following a death-inducing stimulus (Oltvai *et al.*, 1993). It has also been proposed that the relative amounts of Bcl-2 and Bax may be considered as a "rheostat" which determines the susceptibility of a cell to undergo apoptosis (Korsmeyer *et al.*, 1993).

The transcription factor NF-κB has been recognized as an anti-apoptotic regulator (Kajino *et al.*, 2000). However, the mechanism by which NF-κB blocks apoptosis is still controversial (Kajino *et al.*, 2000). NF-κB encompasses a family of inducible transcription factors that play a critical role in the regulation of gene expression in response to various environmental challenges, including inflammatory stimuli, infectious agents and oxidative stress (Baeuerle, 1998). It is also suggested that *de novo* protein synthesis is dispensable for anti-apoptotic effects of NF-κB and support the possibility that NF-κB could exert its anti-apoptotic action through protein-protein interaction (Kajino *et al.*, 2000).

A multitude of methods has been described to identify apoptotic cells by flow cytometric analysis (Darzynkiewicz *et al.*, 1997). The present study is aimed at understanding the inter-relationship between Bax, Bcl-2 and NF-κB in the twin cell death modalities of programmed cell death (PCD) and blister cell death (BCD) induced by sanguinarine in the low Bcl-2 expressing K562 cells, and comparing these results vis-à-vis those obtained in similar experiments in the high Bcl-2 expressing JM1 cells, treated with the same concentrations (and duration) of sanguinarine that induce PCD and BCD in

K562 cells, utilizing immunofluorescence-flow cytometry. The JM1 human pre-B cell lymphoblastic cell line constitutively expresses elevated endogenous levels of the Bcl-2 gene product (Samson *et al.*, 1996), while the K562 human erythroleukemia cells express low levels (Kobayashi *et al.*, 1998) (these findings are also confirmed in the present study).

Materials and Methods

Materials

The K562 erythroleukemia cell line as well as the JM1 human B cell leukemia cell line were obtained from ATCC (Rockville, MD). RPMI 1640, L-glutamine and fetal calf serum were from GIBCO BRL (Gaithersburg, MD). Sanguinarine was purchased from Sigma Chemicals (St. Louis, MO) and maintained as a dry powder at RT. Sanguinarine was dissolved in deionized water and used as a stock solution of 1 mg/ml. Primary antibodies for Bcl-2 and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NF-κB antibody was kindly provided by Dr. Karen Mearow (Memorial University of Newfoundland). Secondary antibodies conjugated to FITC were also purchased from Santa Cruz Biotechnology.

Cell culture

Bcl-2 over expressing JM1 B cell lymphoma and K562 erythroleukemia cell lines were cultured in RPMI 1640 medium supplemented with L-glutamine and 10% fetal calf serum (GIBCO, BRL), at 37^0C/8% CO_2 in a humidified incubator.

Sanguinarine treatment

Sanguinarine HCl (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water at a concentration of 1.0 mg/ml. Serial dilutions were carried out (shortly before *in vitro* use) with tissue culture medium [RPMI-1640 + 10% fetal Bovine Serum (FBS)]. The serial dilution of sanguinarine with RPMI-1640 + 10% FBS in 24 flat bottom well plates yielded a concentration range of 25 μg/ml to 0.19 μg/ml (8 dilutions). Triplicate wells of each drug concentration were prepared and aliquots of 1 x 10^6 cells

(K562 or JM1) per well were added. These tissue culture plates were subsequently incubated at 37^0 C + 5.0% CO_2 for two hours.

Light microscopy

Cells treated with sanguinarine were studied and quantitated by light microscopy. The key morphological criterion for the detection of apoptosis was the formation of apoptotic bodies and the key criterion for blister cell death was the formation of cell surface blisters.

Electron microscopy

Cells were fixed with 2% glutaraldehyde, post fixed in 1% osmium tetroxide, dehydrated through a series of ethanols and embedded in TAAB 812 epoxy resin. Semi thin (0.5 μm) sections were cut axially, through all layers of each pellet to select areas for ultramicrotomy. Ultra thin sections were stained with lead citrate and 30% alchoholic uranyl acetate and then examined in a JEOL 100-Cx electron microscope.

Flowcytometric analysis

Flow cytometric analysis was carried out as described by Re *et al.* (1998). Briefly, after treatment with appropriate concentrations of sanguinarine, K562 and JM1 cells were fixed with 70% ethanol for 20 minutes at 4^0C. Cells were then washed twice with PBS and permeabilized with 0.5% Triton X 100 (Sigma, St. Louis, MO) in PBS for 10 minutes at 4^0C. After three washings with PBS, cells were resuspended in 10% goat serum for one hour at room temperature before adding anti-Bcl-2, Bax and NF-κB monoclonal antibodies. (1:50, 1:90, 1:100, respectively) and incubated overnight at 4^0C. The cells were then washed five times in PBS. Thereafter, a polyclonal goat anti-mouse (1:100) or polyclonal goat anti-rabbit (NF-κB 1:75) antibody conjugated to fluorescein

(FITC) was added to cells and incubated for 30 minutes at 37^0C. The cells were washed five times and analyzed by FACS (Becton-Dickinson, Palo Alto, CA).

Thereafter, the cells were gated for forward scatter (FSC) and side scatter (SSC) in order to rule out cell aggregates and debris. The negative controls consisted of identical secondary labeling without primary monoclonal antibody. The positive control was the expression of the HSP-70 housekeeping protein, which was detected by the anti HSP-70 monoclonal antibody (1:100).

Results

K562 cells treated for 2 hrs with 1.5 μg/ml of sanguinarine at 37°C displayed formation of apoptotic bodies in over 96% of cells, whereas JM1 cells treated with a similar concentration of sanguinarine did not display the morphology of apoptosis (Figs. 4-1b and 4-1e respectively). K562 cells, treated with sanguinarine concentrations of 12.5 μg/ml displayed the morphology of single blister formation or blister cell death (BCD) (Fig. 4-1c), whereas JM1 cells failed to undergo this morphological phenomenon, when treated with the same sanguinarine concentration (Fig. 4-1f). Untreated control K562 cells and JM1 cells are shown in Figs. 4-1a and 4-1d respectively. Electron micrographs of apoptotic K562 cells showed the classic morphological changes, consisting of: formation of apoptotic bodies (containing organelles), chromatin condensation and nuclear fragmentation (fig. 4-2a). Electron micrographs of sanguinarine induced BCD in K562 cells showed single blister formation (devoid of organelles), patchy chromatin condensation and an increase in cytoplasmic vacuolization (fig. 4-2b).

Both cell lines were examined for changes in the pro-apoptotic Bax, anti-apoptotic Bcl-2 and in the anti-apoptotic nuclear transcription factor NF-κB proteins, subsequent to sanguinarine treatment utilizing immunofluorescence-flow cytometry. The percentages of cells expressing the respective proteins were studied in PCD and BCD of K562 cells, and also in sanguinarine treated JM1 cells, as compared to untreated controls.

Results of flow cytometric studies of K562 cells in relation to the pro-apoptotic Bax show an increase in the percentage of cells expressing high levels during PCD (≈81.79%) (fig. 4-3b) and a decrease during BCD (≈26.71%) (fig. 4-3c), compared with untreated controls (≈43.50%) (fig. 4-3a). On the other hand, JM1 cells treated with the

same concentrations (and duration) of sanguinarine that induced PCD and BCD in K562 cells, did not show any significant change in Bax protein expression (figs. 4-3e and 4-3f), when compared with untreated controls (fig. 4-3d).

When treated with sanguinarine, the percentage of K562 cells expressing Bcl-2 failed to show significant change during PCD (40.41%) (fig. 4-4b) and BCD (34.80%) (fig. 4-4c) compared to control untreated cells (33.88%) (fig. 4-4a). In contrast, JM1 cells treated with sanguinarine (figs. 4-4e and 4-4f) showed an increase in the Bcl-2 content, as compared to untreated controls (fig. 4-4d). Although failing to show any significant change in the percentage of cells expressing Bcl-2, the intensity of FITC fluorescence was in fact significantly higher during sanguinarine treatment (given the logarithmic scale by which FITC fluorescence is presented in the graph) (figs, 4-4e and 4-4f) as compared to untreated controls (fig. 4-4d). This shift to the right is indicative of an increased expression of Bcl-2.

Results of NF-κB expression in PCD and BCD of K562 cells showed an interesting pattern. The percentage of K562 cells expressing NF-κB during PCD showed a significant decrease (17.38%) (4-5b), compared to untreated controls (42.23%). However, during BCD of K562 cells induced by sanguinarine, 60.15% of cells contained high levels of NF-κB (4-5c). In contrast, results of JM1 cells treated with concentrations of sanguinarine inducing PCD and BCD in K562 cells, show no significant change in the percentage of cells expressing NF-κB (81.13% and 80.85%, respectively) (figs. 4-5e and 4-5f), when compared with untreated controls (83.65%) (4-5d).

Discussion

K562 cells exposed to sanguinarine at concentrations of 1.5 μg/ml for two hours were found to increase the expression of Bax and decrease the expression of NF-κB, while demonstrating characteristics of the classical morphology of apoptosis. In contrast, high Bcl-2 expressing JM1 cells, when exposed to a similar concentration and duration (1.5 μg/ml for two hours) of sanguinarine, failed to show the morphology of apoptosis. Furthermore, these cells when treated with 1.5 μg/ml sanguinarine showed an increase in Bcl-2 expression, but no significant change was detected in the levels of Bax and NF-κB. These results suggest that sanguinarine induced apoptosis increases the expression of Bax while decreasing NF-κB in K562 cells. On the other hand, high levels of endogenous Bcl-2 in JM1 cells and its increased expression during sanguinarine treatment may have been associated with preventing these cells from undergoing apoptosis.

It has been reported that the proto-oncogene Bcl-2 inhibits apoptosis and confers drug resistance to malignant cells while Bax accelerates apoptosis (Korsmeyer, 1999; Reed *et al.*, 1994; Krajewski *et al.*, 1995). The ratio of the Bcl-2 and Bax proteins was found to correlate with the chemotherapeutic effect (Yang and Korsmeyer, 1996; Krajewski *et al.*, 1995). These findings held true in PCD induced by sanguinarine. Our results were also in agreement with the widely held notion of NF-κB being anti-PCD (Kajino *et al.*, 2000; Wang *et al.*, 1996); the high endogenous levels of NF-κB in JM1 cells might be associated with the resistance of these cells to PCD. It has also been suggested that NF-κB can induce the expression of genes that contribute to proliferation such as Bcl-2 (Mercurio and Manning, 1999). When treated with sanguinarine, JM1 cells expressing high levels of endogenous NF-κB, increased Bcl-2 expression. Thus, it

appears that the increase in the Bax/Bcl-2 ratio on the one hand and reduction of NF-κB on the other, would represent a reasonable approach to cancer drug development, if the ultimate goal was to create the necessary conditions for the facilitation of PCD in tumor cells.

When K562 cells were exposed to sanguinarine concentrations of 12.5 μg/ml for two hours, they displayed the morphology of blister formation, which was previously found to release ^{51}Cr but exclude trypan blue (data not shown) (Liepins *et al.*, 1996). The exclusion of trypan blue and PI by BCD indicate that it is not representative of early necrosis, as early necrotic cells lose plasma membrane integrity permitting the entry of trypan blue into the cell (O'Brien *et al.*, 1997).

Blister formation is at best, only sporadically reported in literature (Liepins *et al.*, 1996; Phelps *et al.*, 1989). The phenomenon of BCD may resemble the "blebbing" described by Majno and Joris (1995). Majno and Joris (1995) describe blebbing as blister like, fluid filled structures, typically devoid of organelles, that arise from the cell membrane and are apt to swell and burst. It is suggested that the mechanism of blebbing may appear to depend on a disconnection between the cell membrane and the underlying cytoskeleton (Majno and Joris, 1995; Phelps *et al.*, 1989). Trump *et al.* (1997) and Trump and Berezesky (1992) associate blebbing with increases in concentration of cytosolic Ca^{2+} $[Ca^{++}]i$ and rearrangement of cytoskeletal proteins including filamentous actin, although Lemasters *et al.* (1987) saw no change in concentration of cytosolic Ca^{2+} $[Ca^{++}]i$ during blebbing. The rupture of these large cell surface blebs is thought to represent the abrupt transition from reversible to irreversible cell injury (Lemasters *et al.*, 1987).

When protein expression of BCD in response to sanguinarine treatment was analyzed by immunofluorescence-flow cytometry we observed a decrease in Bax expression and an increase in NF-κB levels. The decrease in Bax and increase in NF-κB during BCD in K562 cells were diametrically opposite to that which was observed during apoptosis. In contrast, the high Bcl-2 expressing JM1 cells exposed to the concentration and duration (12.5 μg/ml for two hours) of sanguinarine that induced BCD in K562 cells, failed to undergo the morphological changes of BCD. Protein expression analysis by flow cytometry showed that changes in the expression of Bax and NF-κB were minimal. However, the anti- apoptotic Bcl-2 protein showed an increase in expression during sanguinarine treatment. These results suggests that while the decrease in Bax and increase in NF-κB may be associated with BCD induced by sanguinarine (as found in K562 cells), on the other hand, high levels of Bcl-2 may be associated with protection against sanguinarine induced BCD (as found in JM1 cells).

Thus, from a pharmacological standpoint, the correlation between Bcl-2 and chemoresistance was found to be convincing in the treatment of tumor cells with sanguinarine. The role of high Bcl-2 expression and poor response to chemotherapy are corroborated by recent reports in literature (Yang and Korsmeyer, 1996). The findings of the present study suggest that the anti-apoptotic Bcl-2, apart from inhibiting the apoptotic process might have also inhibited the process of BCD. Thus, Bcl-2 may also be anti-BCD in addition to being anti-PCD. Evidence of Bcl-2 inhibiting the process of cytoplasmic blebbing has also been reported in recent literature (Ferri *et al.*, 2000). Hence, the anti-apoptotic Bcl-2 (as discussed earlier) may further compound chemoresistance by

additionally being anti-BCD in nature (at least, this holds true in the case of sanguinarine treatment).

It is stated that apoptosis is only rarely observed *in vivo* and may not be the sole cell death mechanism (Houghton, 1999). Results of the present study show that sanguinarine, apart from PCD, may induce tumour cell death by BCD-a hitherto less understood form of cell death. The discovery of novel forms of cell death induced by anti-cancer agents and identifying their mechanisms may have an important bearing in overcoming chemoresistance.

Figure 4-1: Light micrographs (original magnification- x282) of K562 and JM1 cells treated with sanguinarine. Apoptosis (b) and blister cell death (c) in K562 cells treated with sanguinarine at 1.5 μg/ml and 12.5 μg/ml respectively for 2 hrs. JM1 cells, which express high endogenous levels of Bcl-2, are resistant to the induction of apoptosis (e) and blister cell death (f) at the corresponding sanguinarine concentrations. Control untreated K562 and JM1 cells are shown in figs. (a) and (d) respectively.

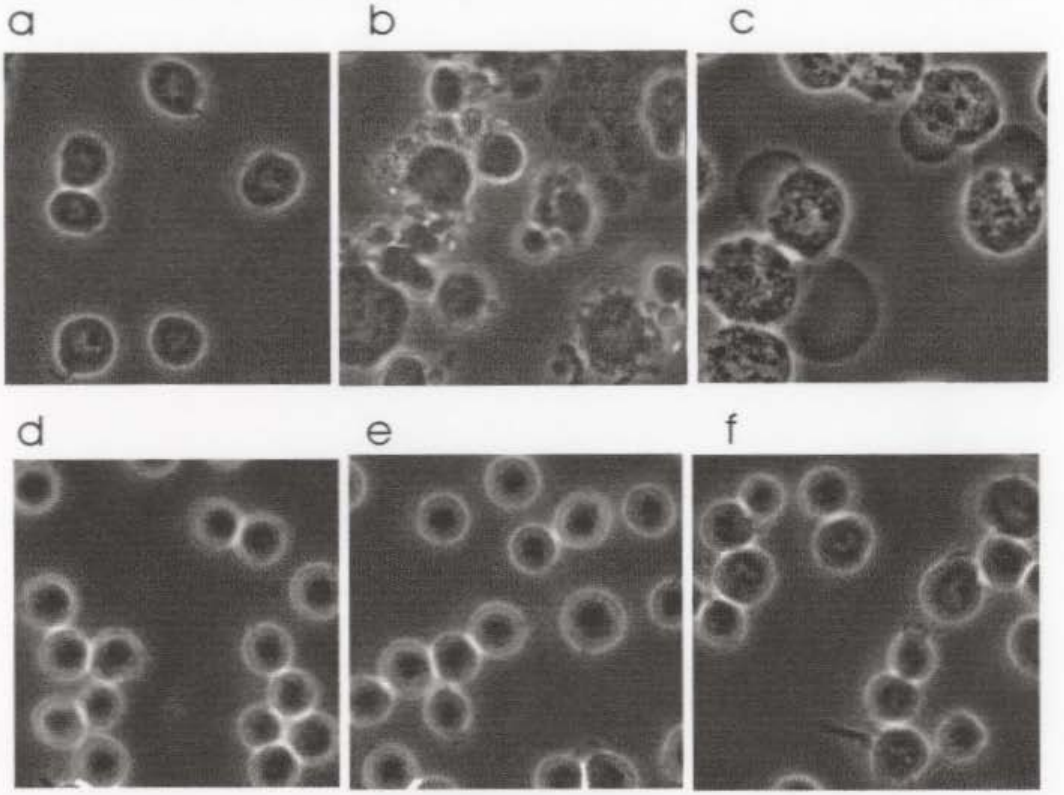
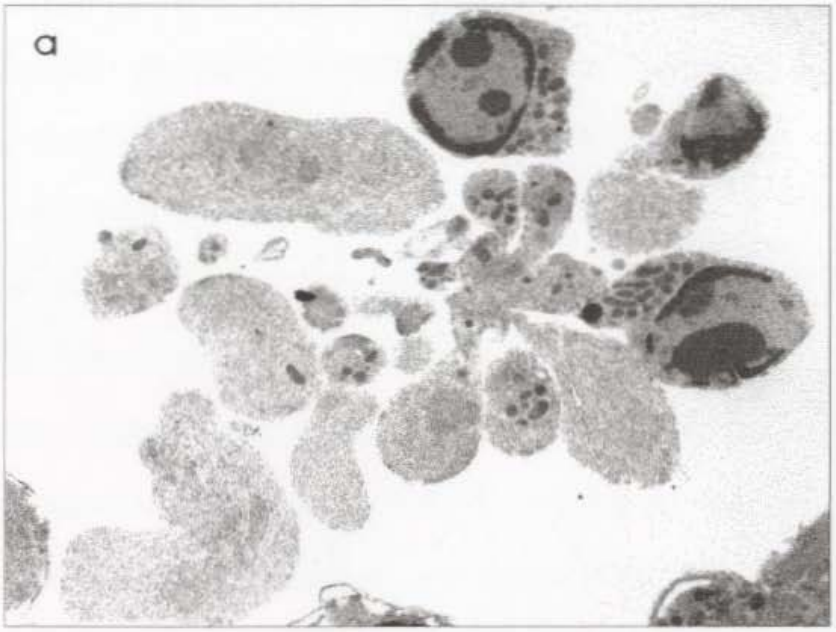

a
b
c
d
e
f

Figure 4-2: Electron micrographs (original magnification- x15000) of sanguinarine induced PCD and BCD of K562 cells. The figure shows (a) apoptosis and (b) blister cell death in K562 cells induced by treatment with sanguinarine 1.5 μg/ml and 12.5 μg/ml respectively for 2 hours. Fig. (a) show apoptotic bodies, nuclear fragmentation and chromatin condensation, while fig. (b) show blister formation, patchy chromatin condensation and an increase in vacuolization.


a

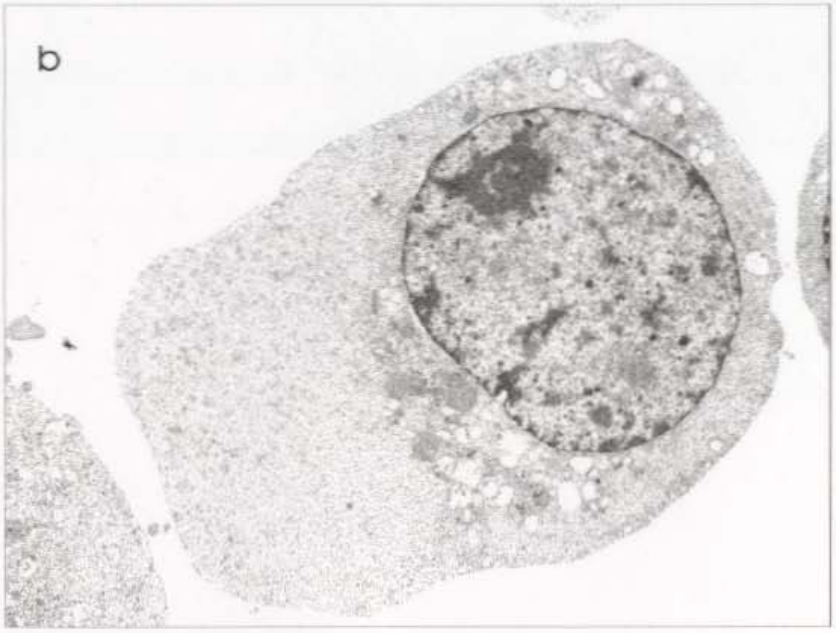
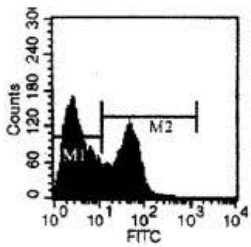

b

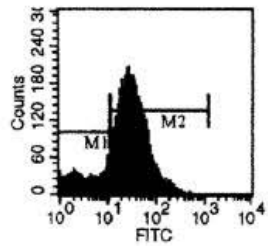
Figure 4-3: Flow cytometric analysis of Bax in K562 and JM1 cells by histogram. (a) Bax protein expression in untreated control K562 cells. The percentage of Bax positive cells is 43.5% and Bax negative cells are 56.99%. (b) Bax protein expression in sanguinarine induced apoptotic K562 cells. The percentages of Bax positive cells are 81.79% and Bax negative cells is 18.79%. (c) Bax protein expression in K562 cells that underwent sanguinarine induced BCD. The percentage of Bax positive cells is 26.71% and the percentage of Bax negative cells is 73.85%. (d) Bax protein expression in untreated control JM1 cells. The percentages of Bax positive cells and Bax negative cells are 26.64% and 74.02%, respectively. (e) Bax protein expression in JM1 cells exposed to the concentration and duration of sanguinarine that induce apoptosis in K562 cells (1.5 μg/ml). The percentage of Bax positive cells and Bax negative cells are 39.11% and 61.63%, respectively. (f) Bax protein expression in JM1 cells exposed to the concentration and duration of sanguinarine that induced BCD in K562 cells (12.5 μg/ml). The percentages of Bax positive cells and Bax negative cells are 37.48% and 63.89%, respectively.

a



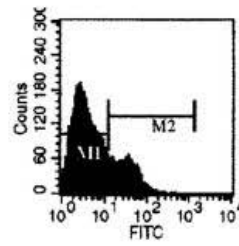
M1=56.99% M2=43.5%

b



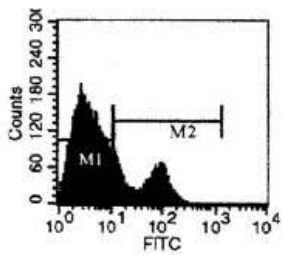
M1=18.79% M2=81.79%

c



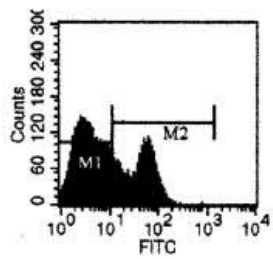
M1=73.85% M2=26.71%

d



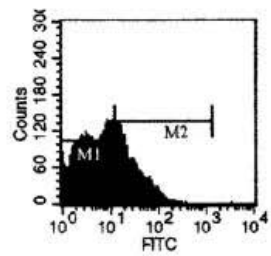
M1= 74.02% M2=26.64%

e



M1=61.63% M2=39.11%

f



M1=63.89% M2= 37.48%

Figure 4-4: Flow cytometric analysis of Bcl-2 in K562 and JM1 cells by histogram. (a) Bcl-2 protein expression in untreated control K562 cells. The percentage of Bcl-2 positive cells is 33.88% and Bcl-2 negative cells is 66.77%. (b) Bcl-2 protein expression in sanguinarine induced apoptotic K562 cells. The percentage of Bcl-2 positive cells is 40.41% and Bcl-2 negative cells is 60.05%. (c) Bcl-2 protein expression in K562 cells that underwent sanguinarine induced BCD. The percentage of Bcl-2 positive cells is 33.80% and the percentage of Bcl-2 negative cells is 66.33%. (d) Bcl-2 protein expression in untreated control JM1 cells. The percentages of Bcl-2 positive cells and Bcl-2 negative cells are 77.46% and 22.95%, respectively. (e) Bcl-2 protein expression in JM1 cells exposed to the concentration and duration of sanguinarine that induce apoptosis in K562 cells (1.5 μg/ml). The percentages of Bcl-2 positive cells and Bcl-2 negative cells are 82.06% and 18.03%, respectively. (f) Bcl-2 protein expression in JM1 cells exposed to the concentration and duration of sanguinarine that induce BCD in K562 cells (12.5 μg/ml). The percentages of Bcl-2 positive cells and Bcl-2 negative cells are 76.75% and 24.02%, respectively.

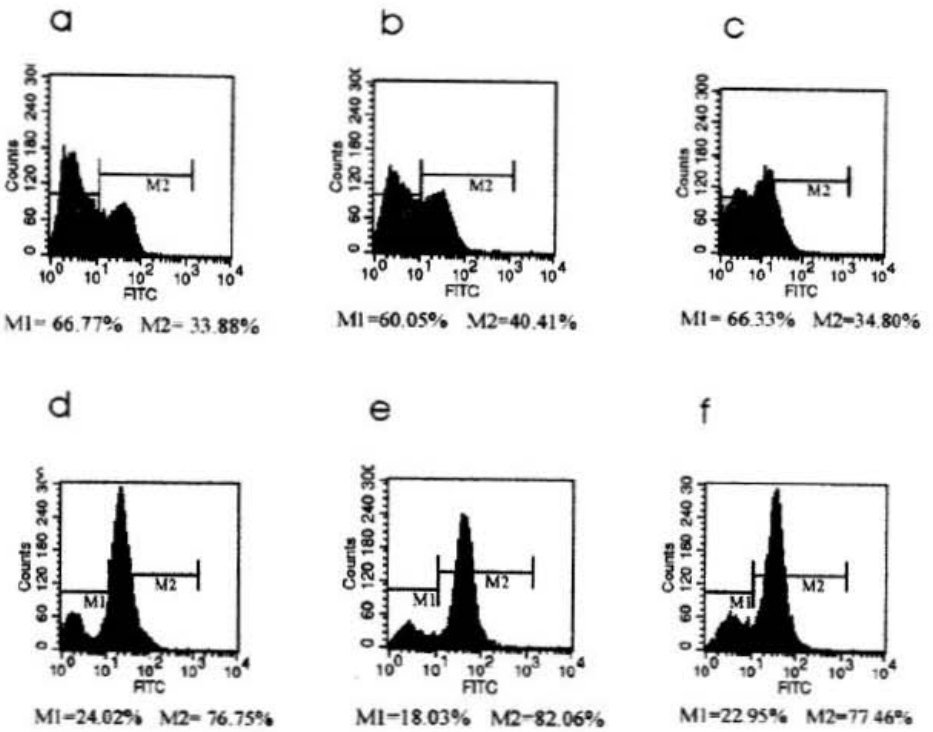
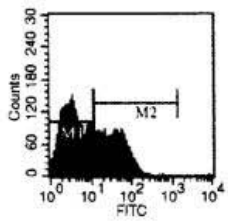

a
b
c
d
e
f
Counts
FITC
M1
M2
M1= 66.77% M2= 33.88%
M1=60.05% M2=40.41%
M1= 66.33% M2=34.80%
M1=24.02% M2= 76.75%
M1=18.03% M2=82.06%
M1=22.95% M2=77.46%

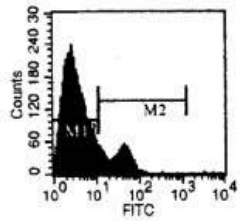
Figure 4-5: Flow cytometric analysis of NF-κB in K562 cells by histogram. (a) NF-κB protein expression in untreated control K562 cells. The percentage of NF-κB positive cells is 42.23% and NF-κB negative cells is 58.39%. (b) NF-κB protein expression in sanguinarine induced apoptotic K562 cells. The percentage of NF-κB positive cells is 17.38% and NF-κB negative cells is 83.17%. (c) NF-κB protein expression in K562 cells that underwent sanguinarine induced BCD. The percentage of NF-κB positive cells is 60.14% and the percentage of NF-κB negative cells is 41.28%. (d) NF-κB protein expression in untreated control JM1 cells. The percentages of NF-κB positive cells and NF-κB negative cells are 83.65% and 16.74%, respectively. (e) NF-κB protein expression in JM1 cells exposed to the concentration (and duration) of sanguinarine that induce apoptosis in K562 cells (1.5 μg/ml). The percentages of NF-κB positive cells and NF-κB negative cells are 81.13% and 19.33%, respectively. (f) NF-κB protein expression in JM1 cells exposed to the concentration and duration of sanguinarine that induced BCD in K562 cells (12.5 μg/ml). The percentages of NF-κB positive cells and NF-κB negative cells are 80.85% and 19.63%, respectively.

a



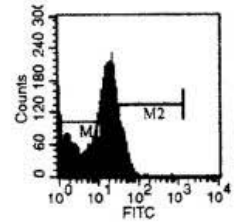
M1=58.39% M2= 42.23%

b



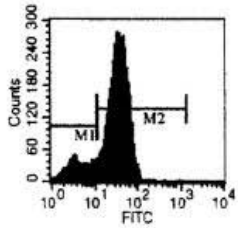
M1=83.17% M2=17.38%

c



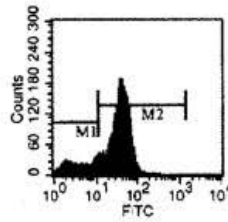
M1=41.28% M2=60.15%

d



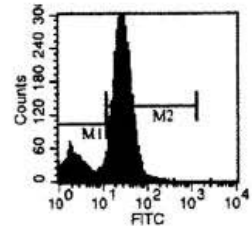
M1=16.74% M2=83.65%

e



M1=19.33% M2=81.13%

f



M=19.63% M2=80.85%

Chapter 5

Induction of Bimodal Cell Death by Sanguinarine in Multidrug-Resistant CEM-VLB 1000 Cells

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Abstract

The multidrug-resistant (MDR), permeability-glycoprotein (Pgp) positive CEM-VLB 1000 human leukemia cells and its wild type CEM-T4, when treated with the alkaloid sanguinarine, were found to undergo apoptosis or programmed cell death (PCD) at concentrations of 1.5 μg/ml and blister cell death (BCD) at concentrations of 12.5 μg/ml. Pgp positive cells are known to be encoded by the multidrug-resistance gene (MDR1), and characterized by a reduced ability to accumulate drugs. However, when intracellular levels of sanguinarine were measured by flow cytometric analysis in CEM-VLB 1000 cells treated with sanguinarine, we observed low levels during PCD, but high levels during BCD. Furthermore, the cell death processes of PCD and BCD of both cell lines were found to be qualitatively similar when assessed by light microscopy, terminal deoxynucleotidyl transferase (TdT) end-labeling, annexin V binding, trypan blue exclusion and clonogenic assay. Western blotting analysis revealed an increase in the Bax/Bcl-2 ratio and activation of caspase 3 in PCD but not BCD in both cell lines. Therefore, we conclude that sanguinarine may overcome the phenomenon of MDR in CEM-VLB 1000 cells by inducing PCD through increasing the Bax/Bcl-2 ratio and activating caspase 3, and BCD, which involved neither.

Introduction

Sanguinarine (or bloodroot) belongs to the benzophenanthridine alkaloid group, which are biosynthesized from phenylalanine in plants of the *Papaveraceae* family (Dostal and Potacek, 1990; Imanek, 1985). This alkaloid has been reported to display a wide spectrum of biological effects, such as antifungal, antimicrobial, analgesic and moreover, anti-cancer properties (Dostal and Potacek, 1990; Imanek, 1985; Vavrecková and Ulrichová, 1994; Ahmad *et al.*, 2000). Its principal use today is in over-the-counter products including toothpaste, mouthwash, cough and cold remedies, and homeopathic preparations (Babich *et al.*, 1996). Sanguinarine has also been reported to be a protein kinase C (PKC) and NF-κB (nuclear transcription factor κB) inhibitor (Gopalakrishna *et al.*, 1990; Chaturvedi *et al.*, 1997), a DNA intercalator (binding to guanosine-cytosine rich regions) (Babich *et al.*, 1996; Sen and Maiti, 1994), a topoisomerase I and II inhibitor (Hsiang *et al.*, 1989; Zhang *et al.*, 1990), an inhibitor of tubulin assembly (Wolff and Knipling, 1993) and an inhibitor of Na^+/K^+ ATPase (Seifen *et al.*, 1979; Straub and Carver, 1975).

Resistance of malignant tumors to chemotherapeutic agents remains a major cause of failure in cancer therapy (Ling, 1997). A multigene family designated MDR (Endicot and Ling, 1989, Kang *et al.*, 2000) has been identified, the members of which encode a family of membrane glycoproteins termed P- glycoproteins (Nooter and Herweijer, 1991; Borst, 1991; Juranka *et al.*, 1989; Endicot and Ling, 1989). P-glycoprotein appears to function as an energy-dependent transport pump capable of effluxing cytotoxic agents and thereby decreasing their intracellular concentration (Hamada and Tsuruo, 1988). The

MDR gene, overexpressed in multidrug-resistant cell lines, is associated with poor prognosis for therapy (Ling, 1997; Bosch and Croop, 1996).

Apoptosis or PCD is a highly regulated process that results physiologically in the selective death of cells. The cell death pathway may be conceived as a series of intracellular decision points where death regulatory genes are activated in response to physiologic or pathologic signals. The Bcl-2 family of genes is a major component of this pathway, serving as both positive and negative regulators of cell death. The intracellular level and the ratio of Bcl-2 and Bcl-2-related proteins dictate if a cell should engage in the apoptotic program (Reed, 1997a; Reed, 1997b). It has also been suggested that the relative amounts of Bcl-2 and Bax may be considered as a "rheostat" which determines the susceptibility of a cell to undergo apoptosis (Korsmeyer *et al.*, 1993).

Apoptosis has been known to be executed by a family of cysteine proteases called caspases (Nuñez *et al.*, 1998). The activation of caspase-3 leads to proteolytic activation of downstream effector caspases and the cleavage of a number of vital proteins, resulting in the orderly demise of cells (Nuñez *et al.*, 1998; Salvesen, 1999). In contrast to apoptosis, little is known about cell death by blister formation.

Our previous studies have demonstrated that K562 human erythroleukemia cells, when exposed to the alkaloid sanguinarine at concentrations of 1.5 μg/ml and 12.5 μg/ml for two hours, displayed the morphologies of two different modalities of cell death: at 1.5 μg/ml the classical morphology of apoptosis or programmed cell death (PCD) and at 12.5 μg/ml, the morphology of single blister formation or blister cell death (BCD) (Weerasinghe *et al.*, in press). Previous observations in our laboratory of apoptosis or programmed cell death (PCD) and blister cell death (BCD) induced by the semisynthetic

benzophenanthridine alkaloid derivative Ukrain™ in K562 cells was termed "bimodal cell death" (BMCD) (Liepins *et al.*, 1996). Thus, this dual cell death modality induced by sanguinarine, a potent anti-cancer agent (Ahmad *et al.*, 2000), was also termed "bimodal cell death" (BMCD).

The objective of the present study was to characterize the effects of sanguinarine treatment in the multidrug-resistant CEM-VLB 1000 human leukemia cells and in their wild type CEM-T4 cells. We found that sanguinarine induced PCD and BCD in Pgp-positive CEM-VLB 1000 cells and in their wild type Pgp-negative CEM-T4 cells, at the same concentrations and under the same experimental conditions. This report is also an attempt to better understand the possible mechanisms by which sanguinarine overcomes the drug efflux actions of Pgp in MDR cells.

Materials and Methods

Materials

The CEM-VLB 1000 and CEM-T_4 cell lines were kindly provided by Dr. Victor Ling (University of British Columbia, Canada). These cells were cultured in RPMI 1640 medium supplemented with L. glutamate and 10% fetal calf serum. They were maintained in a 37°C/5% CO_2 fully humidified incubator and passaged 4-5 times weekly.

Annexin-V-fluos staining kit for the detection and quantification of apoptosis and differentiation from necrosis at the single cell level was purchased from Boehringer Mannheim Corp (Indianapolis, IN). The kit was stored and handled according to manufacturer's instructions.

The drug sanguinarine ([1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-I]phenanthridinium, 13-methyl-, chloride[9CI]) was purchased from Sigma Chemicals (St. Louis, MO) and maintained as a dry powder at room temperature. It was dissolved in deionized water at 1 mg/ml and used as a stock solution. Sanguinarine was subsequently diluted with RPMI 1640 in order to make a working solution of 25 μg/ml.

Drug treatment

The following experimental format was used in order to prepare the cells for subsequent experiments. Logarithmically growing CEM-VLB 1000 and CEM-T4 cells were placed in 60 mm dishes at approximately 3×10^6 cells/ml. Thereafter, serially diluted sanguinarine concentrations of 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5 and 25 μg/ml were added to the petri dishes and incubated for 2 hrs in a 37°C/5% CO_2 incubator. Cells that underwent PCD and BCD (at concentrations of 1.5 μg/ml and 12.5 μg/ml respectively) were used for morphological analysis, caspase-3 assay and protein isolation from cells.

Light microscopy

Cells treated with sanguinarine were studied and quantitated by light microscopy. The key morphological criterion for the detection of apoptosis was the formation of apoptotic bodies and the key criterion for blister cell death was the formation of cell surface blisters.

Trypan blue assay

Charged cationic dye trypan blue is excluded from putative live cells with intact plasma membranes. The changes in membrane permeability in CEM-VLB 1000 and CEM-T4 cells which were exposed to sanguinarine treatment and in untreated controls, were evaluated in triplicate wells at 2×10^4 cells per well after 2 hours of incubation at 37°C using flat bottom 96 well plates. After 10 minutes of incubation with the dye trypan blue (0.2%), the number of trypan blue excluding cells was counted using a hemocytometer. The exclusion of 0.2% trypan blue was used as an indicator of the number of live cells.

Clonogenic assay

This method for assessing cloning efficiency of cells was done as previously described by Grant *et al.* (1991), with minor modifications. Briefly, cells treated with sanguinarine and untreated controls were plated, washed twice and resuspended in RPMI 1640. After determining cell densities by coulter counter, 400 cells were seeded in 35-mm tissue culture plates containing 1 ml of RPMI, 20% fetal calf serum and 0.3% bacto agar (Difco, Detroit, MI). The plates were placed in a 37^0C, 5% CO_2, fully humidified incubator for 10-11 days. Thereafter, colonies were stained with 2% crystal violet in methanol and counted in an inverted microscope.

Terminal deoxynucleotidyl transferase (TdT) end-labeling assay

Specific 3'-hydroxyl ends of DNA fragments generated by endonuclease-mediated apoptosis are preferentially repaired by terminal deoxynucleotidyl transferase (TdT) (Gavrieli *et al.*, 1992). The TdT mediated nick end labeling assay has been developed to label these strand-breaks with streptovidin-horseradish peroxidase conjugated nucleotides followed by the addition of a substrate (TACS Blue Label™ or TBL).

After sanguinarine treatment, cells were removed from individual wells, washed in phosphate buffered saline (PBS) and fixed in 10% neutral buffered formaldehyde for 10 minutes. Thereafter, cells were resuspended in 80% ethanol at 1×10^6 cells per ml. 50,000 cells were placed onto an electrostatically treated glass slide and air-dried at room temperature. Cells were then permeabilized with proteinase K (Gavrieli *et al.*, 1992) and incubated for 60 minutes at 37°C in the presence and absence of exogenous TdT and streptavidin-horseradish peroxidase conjugated dNTP (deoxynucleotide triphosphate), followed by the substrate TBL according to the manufacturer's instructions (Sigma TACS™ in situ Apoptosis Detection Kit, St. Louis, MO). Cells were then examined and photographed under phase microscopy and counted to determine the percentage of cells with DNA nicking to total cells.

Fluorescein-conjugated annexin-V-binding assay

After treatment with sanguinarine concentrations that induce PCD and BCD, CEM-VLB 1000 and CEM-T4 cells (1×10^6 cells) were washed with PBS and incubated with annexin-V fluorescein isothiocyanate (FITC) conjugate and propidium iodide (PI) utilizing an annexin-V-Fluos staining kit (Boehringer Mannheim Corp, Indianapolis, IN).

After labeling, cells were resuspended in binding buffer and analyzed using flow cytometry. FITC-fluorescence was measured at 530-545 nm and fluorescence of DNA-PI complexes at 575-606 nm. Cell debris was excluded from analysis by appropriate forward light scatter threshold setting (Leist *et al.*, 1997).

Expression of Pgp in cell surface

CEM-VLB 1000 and CEM-T4 Cells were incubated for 30 mins with MDR1 antibody (0.5 μg for 10^6 cells) or non-specific murine IgG2a. After washing with PBS twice, cells were incubated with FITC conjugated F(ab')2 fragment of sheep anti-mouse immunoglobulin (50 μl of 1:200 dilution). After 30 mins, cells were washed with PBS twice and held at 4^0C in the dark until analysis by flowcytometry (Kunikane *et al.*, 1997).

Detection of sanguinarine auto-fluorescence

Subsequent to drug treatment, cells were washed 3 times and subjected to flow cytometric analysis. Measuring of intracellular sanguinarine fluorescence was done using a Becton Dickinson flow cytometer.

SDS-PAGE and Western blotting analysis

The following experimental format was used in order to prepare the cells for subsequent western blotting analysis. Logarithmically growing CEM-VLB 1000 and CEM-T4 cells were placed in 15 mm petri dishes at around 3×10^6 cell/ml. Thereafter, serially diluted sanguinarine concentrations of 0.19, 0.39, 0.78, 1.56, 3.12, 6.125, 12.5 μg/ml were added to the petri dishes and incubated for 2 hrs in a 37°C/5% CO_2 incubator.

Expression of Bcl-2, Bax, Bak, Bad, Bik/Nbk, Bcl-X_S, Bid, Bcl-X_L, p53, and CPP-32 proteins were determined by western blotting analysis using previously described methods with minor modifications (Pixton, 1990; Towbin *et al.*, 1979). Briefly, after

treatment with sanguinarine, whole cell pellets (1×10^7 cell/dish) were washed twice in PBS, resuspended in 50 µl PBS and lysed by adding lysis buffer solution. The homogenates were quantified using DC protein assay. Equal amounts of proteins (20 µg/well) were boiled for 5 minutes, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% stacking gel and 12% resolving gel) and electroblotted to nitrocellulose. After blocking with PBS tween 20 (0.05%) and with 5% non-fat dried milk for 1 hr at 22°C, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For the study of CPP-32, primary antibody was used at 1:250. Blots were washed 3 times for 5 min in TBS-T and then incubated with 1:2500 dilution of horseradish peroxidase conjugated secondary antibody (Santa Cruz) for 1 hr at 22°C. Blots were again washed 3 times for 5 minutes in TBS-T and then developed by chemiluminescence (Amersham, Arlington Heights, IL).

Statistical analysis

The results are presented as means ± SE. Statistical analyses were performed using the student's t test. Differences were considered significant when $p< 0.05$.

Results

Morphological studies by light microscopy showed that CEM-VLB 1000 and CEM-T4 cells, when treated with sanguinarine at concentrations of 1.5 μg/ml displayed the morphology of apoptosis in over 90% of cells, and at concentrations of 12.5 μg/ml, blister formation in over 85% (Fig.5-1). Results of trypan blue studies for cell viability showed over 90% of PCD and BCD of both cell lines excluding trypan blue (fig.5-2). The clonogenic assay to measure cell viability showed no colonies in PCD and BCD of both cell lines as compared with control untreated cells (fig.5-3).

DNA fragmentation in PCD and BCD of both cell lines was assessed by the terminal deoxynucleotidyl transferase (TdT) end-labeling method. Results showed DNA nicking in over 88% in PCD of both cell lines but not in BCD (fig.5-4).

The annexin-V-assay was utilized for the detection of cell surface membrane phosphatidyl serine (PS) flip, which is known to be associated with apoptosis or PCD (Leist *et al.*, 1997). Sanguinarine-induced apoptotic CEM-VLB 1000 and CEM-T4 cells showed over 60% of annexin-V positive cells, i.e. binding of annexin-V to phosphatidyl serine on the surface of the cell membrane as compared to about 15% in untreated control cells. However, cells of both cell lines that underwent BCD did not show the PS flip (fig.5-5).

Detection of Pgp expression in cell surface was done using immunofluorescence-flow cytometry with FITC fluorescence. Over 99% of CEM-VLB 1000 cells displayed FITC fluorescence when treated with the MDR1 antibody. In contrast, MDR1 antibody-treated CEM-T4 cells showed no FITC fluorescence. CEM-T4 and CEM-VLB 1000 cells treated with the isotype IgG (negative controls) did not show fluorescence (fig.5-6).

Flow cytometric detection of sanguinarine uptake by measuring its auto-fluorescence showed relatively low levels in PCD of both cell lines but considerably higher levels in BCD (fig.5-7). Intracellular sanguinarine levels in PCD and BCD of CEM-T4 cells were comparable to those of PCD and BCD of CEM-VLB 1000 cells, respectively.

Western blotting analysis was done to study the protein expression during sanguinarine-induced PCD and BCD in both cell lines. These studies were limited to analyzing the expression of the Bcl-2 family gene products. The expression of the 21 kDa pro-apoptotic Bax protein was found to be moderately increased in sanguinarine-induced PCD in both cell lines as compared to untreated control cells. In contrast, sanguinarine-induced BCD did not show any significant change in Bax protein expression (fig.5-8A). No change was observed in the expression of the 26 kDa anti-apoptotic Bcl-2 protein in neither PCD nor in BCD in either of the cell lines (fig.5-8B). The 32 kDa endogenous caspase 3 was inherent in both cell lines. However, it's 20 kDa breakdown products, denoting activation, was only observed in PCD of both cell lines but not in BCD (5-8C). The expression of pro-apoptotic Bak, Bad, Bik/Nbk, Bcl-X_S, Bid, p53 and anti-apoptotic Bcl-X_L proteins was not significantly affected by sanguinarine in either cell line at low level or high level (individual data not shown).

Discussion

Our results show that, Pgp-positive CEM-VLB 1000 and Pgp-negative CEM-T4 cell lines underwent similar patterns of PCD and BCD, both qualitatively and quantitatively during sanguinarine treatment. When exposed to sanguinarine at concentrations of 1.5 μg/ml and 12.5 μg/ml for 2 hours, both cell lines showed the morphology of PCD in over 90% of cells and BCD in over 85% of cells, respectively. In contrast to BCD, the PCD induced by sanguinarine showed DNA nicking and the phosphatidyl serine (PS) flip, commonly associated with PCD or apoptosis. Both PCD and BCD were found to have membrane viability by the exclusion of trypan blue but failed to have long term survival in the clonogenic assay.

Western blotting analysis revealed a moderate up-regulation of the Bax protein in sanguinarine-induced PCD in both cell lines, while Bcl-2 levels remained unchanged. Our previous studies have shown that high levels of endogenous Bcl-2 protect JM1 pre-B lymphoblastic cells from undergoing bimodal cell death, when treated with sanguinarine (Weerasinghe *et al.*, in press). These findings illustrate the importance of the Bax/Bcl-2 ratio in the induction of PCD (Korsemeyer, 1999). Furthermore, this ratio has been shown to correlate with the chemotherapeutic effect (Nakata *et al.*, 1998; Yin *et al.*, 1994). On the other hand, protein expression analysis of BCD revealed no significant changes in Bcl-2 family protein expression as compared to untreated control cells and did not appear to show the increased expression of Bax seen in PCD. Other members of the Bcl-2 family, including Bak, Bad, Bik, Bcl-X_S, Bcl-X_L and Bid, as well as the tumor suppressor p53 appear not to play any significant role in either PCD or BCD.

Immunofluorescence and flow cytometry studies showed the presence of the transmembrane glycoprotein- Pgp in the MDR CEM-VLB 1000 cells but not in CEM-T4 cells. We have also shown the elevated presence of sanguinarine-fluorescence in cells by flow cytometry in BCD of both cell lines relative to PCD. Thus sanguinarine may have overcome the Pgp-mediated drug efflux pump by inducing a less understood form of cell death- BCD, and not by PCD which was also induced by the drug. Sanguinarine is known to exert it's anti-cancer properties, among other mechanisms, by forming a molecular complex with DNA by binding to regions rich in cytosine-guanosine (Sen and Maiti, 1994; Babich *et al.*, 1996). The influx of drug into the cell in BCD may aid or even potentiate sanguinarine intercalation, and contribute towards its potency as an anti-tumor agent.

Necrosis represents not a form of cell death but changes secondary to cell death by any mechanism (Majno and Joris, 1995). It is known that early necrotic cells lose plasma membrane integrity, thus permitting the entry of trypan blue and propidium iodide into the cell (O'Brien *et al.*, 1997). The exclusion of trypan blue and propidium iodide by BCD in cells indicate that BCD is not representative of early necrosis. Blister formation is at best, only sporadically reported in literature (Liepins *et al.*, 1996; Phelps *et al.*, 1989). The phenomenon of BCD may resemble the "blebbing" described by Majno and Joris (Majno and Joris, 1995). Majno and Joris describe blebbing as blister-like, fluid-filled structures, typically devoid of organelles that arise from the cell membrane and are apt to swell and burst. Trump *et al.* (1997) and Trump and Berezesky (1992) associates blebbing with increases in concentration of cytosolic Ca^{2+} [Ca^{++}]i and rearrangement of cytoskeletal proteins including filamentous actin. The rupture of these

large cell surface blebs is thought to represent the abrupt transition from reversible to irreversible cell injury (Lemasters *et al.*, 1987).

Considerable attention has been devoted to studying the activation of the protease cascade in drug-induced and other forms of apoptosis, as well as on the ability of Bcl-2 and related proteins to inhibit this process (Wang *et al.*, 1997). Our results showed that sanguinarine-induced PCD activated caspase 3, while BCD failed to do so. Our previous studies have shown that sanguinarine treatment of Bcl-2 low-expressing K562 cells resulted in the activation/cleavage of caspase-3 into 20 kd CPP-32 breakdown products, whereas the Bcl-2 over-expressing JM1 cells showed no breakdown products of CPP-32 (results not shown). Therefore, in the presence of elevated Bcl-2, sanguinarine may have been unable to trigger caspase 3 activation, which is in agreement with several recent reports (Kojima *et al.*, 1996; Irabo *et al.*, 1996). These results also appear to be consistent with recent literature, which suggests that in the case of some, but perhaps not all cytotoxic stimuli, Bcl-2 functions upstream of a critical cysteine protease involved in the degradation phase of apoptosis (Wang *et al.*, 1997). As opposed to Bcl-2, increased levels of Bax promotes caspase 3 activity in sanguinarine-induced apoptosis, which is corroborated in the present study. In contrast, the absence of caspase 3 activation during BCD may indicate that this may not be a necessary element in the execution of BCD.

Apoptosis is not the sole cell death mechanism (Houghton, 1999). Our findings show that, at least in the case of sanguinarine, tumor cell death may also be induced by BCD-a hitherto less understood form of cell death. Furthermore, sanguinarine may overcome the phenomenon of MDR in the CEM-VLB 1000 cell line by inducing BCD as

well as PCD. Sanguinarine may therefore be explored as a potential anti-cancer drug in reversing drug-resistance in the chemotherapy of common malignancies.

Figure 5-1: Light micrographs (original magnification- ×282) of CEM-VLB 1000 and CEM-T4 cells treated with sanguinarine. Control, apoptosis and blister cell death (BCD) of CEM-VLB 1000 cells are shown in figs. (a), (b) and (c), while control, apoptosis and BCD of CEM-T4 cells are represented by figs. (d), (e) and (f), respectively. Both cell lines treated with sanguinarine at 1.5 μg/ml and 12.5 μg/ml respectively for 2 hrs showed the morphology of apoptosis (arrows) and BCD (arrows).

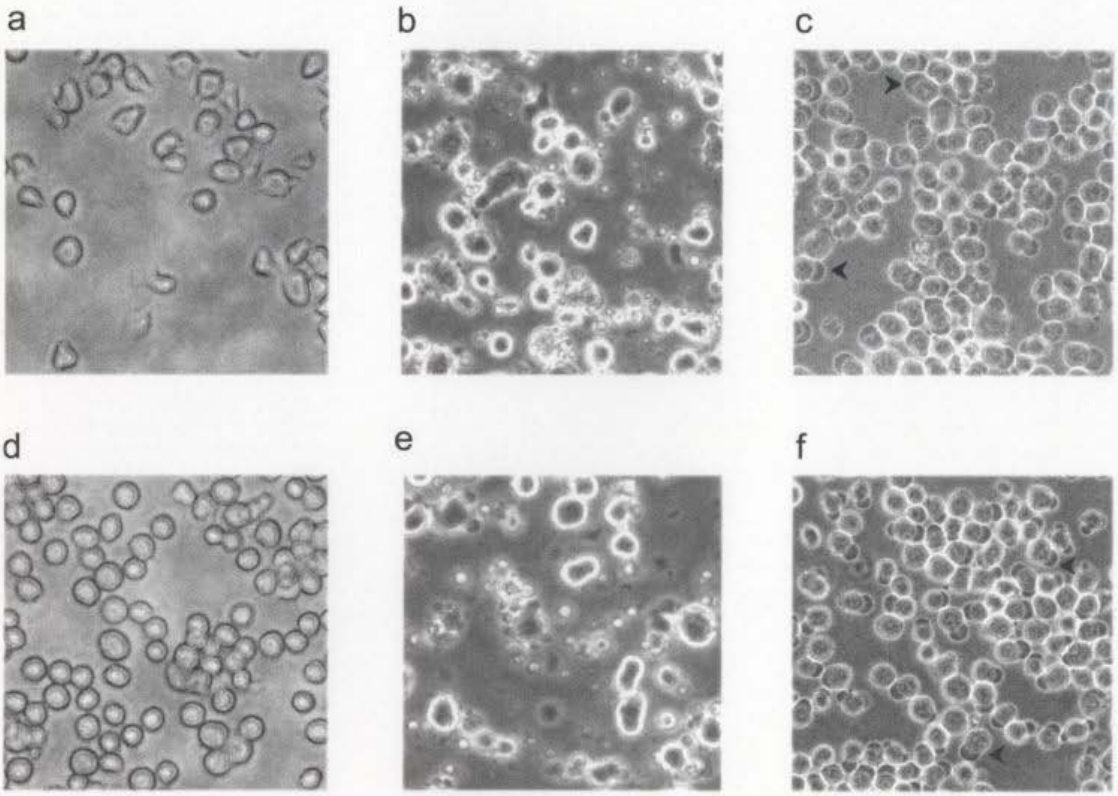

a
b
c
d
e
f

Figure 5-2: Trypan blue exclusion assay in sanguinarine treated CEM-VLB 1000 and CEM-T4 cells. As shown, quantitative trypan blue exclusion showed over 90% of cells of both cell lines that underwent PCD or BCD excluding the dye. Results represent the mean ± SE from 3 independent experiments.

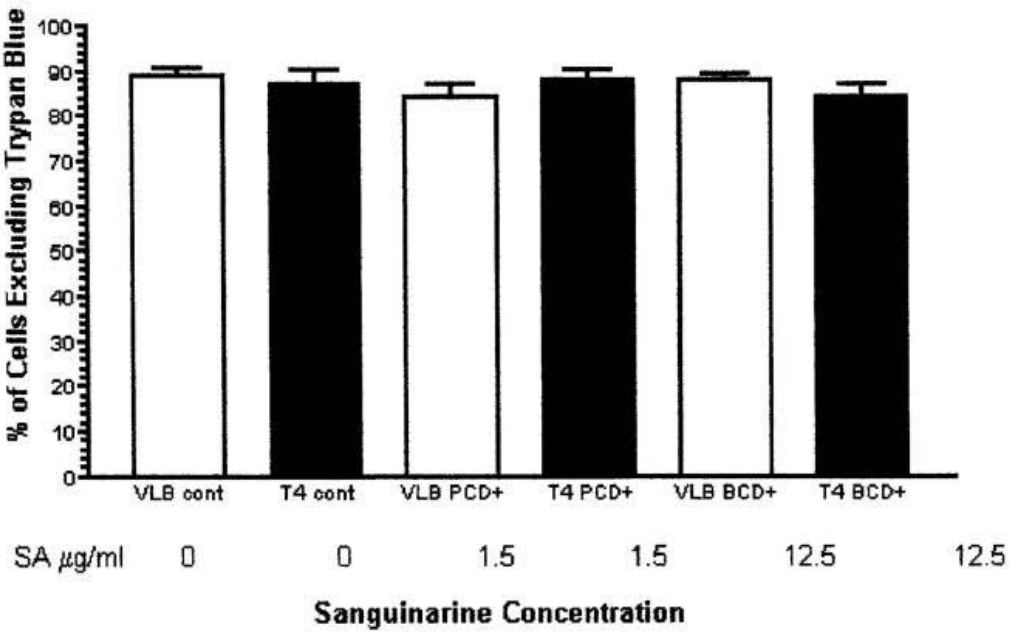

% of Cells Excluding Trypan Blue
100
90
80
70
60
50
40
30
20
10
0
VLB cont
T4 cont
VLB PCD+
T4 PCD+
VLB BCD+
T4 BCD+
SA µg/ml 0 0 1.5 1.5 12.5 12.5
Sanguinarine Concentration

Figure 5-3: Clonogenic assay for CEM-VLB 1000 and CEM-T4 cells treated with sanguinarine. The graph shows survival cell fraction, expressed as the ratio of the colony forming cells of drug treated and untreated cells. PCD and BCD of both cell lines induced by sanguinarine showed no colony formation. Experiments were repeated three times.

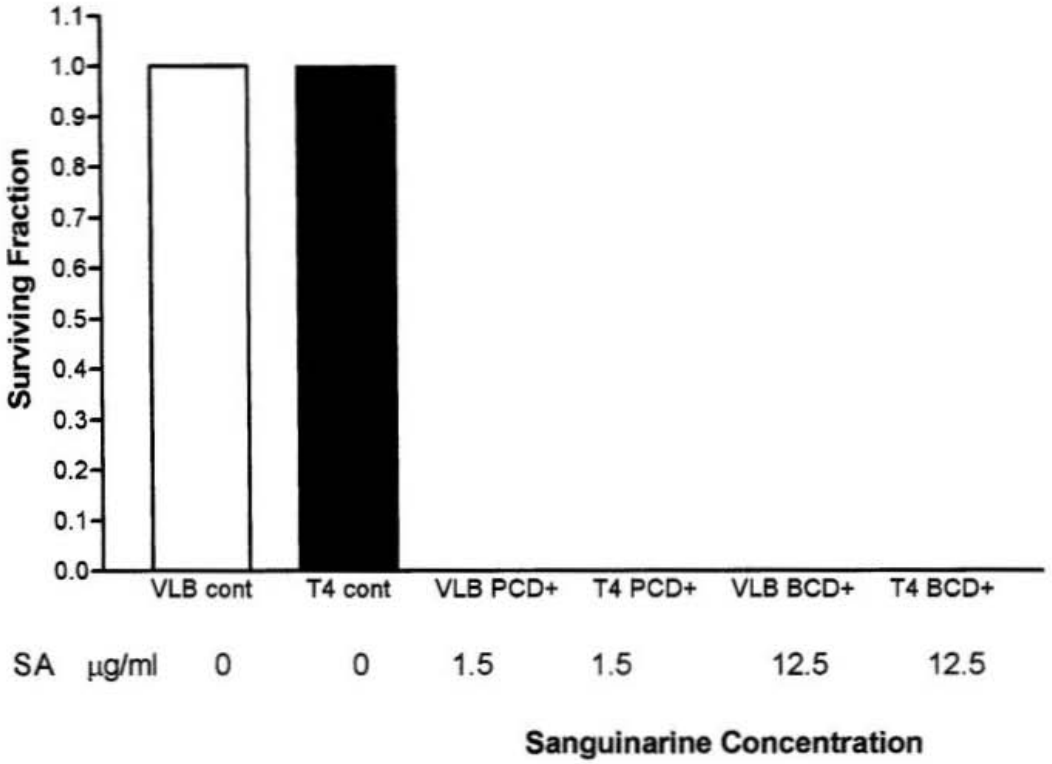

Surviving Fraction
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
VLB cont
T4 cont
VLB PCD+
T4 PCD+
VLB BCD+
T4 BCD+
SA µg/ml
0
0
1.5
1.5
12.5
12.5
Sanguinarine Concentration

Figure 5-4: Terminal deoxynucleotidyl transferase (TdT) end labeling assay in CEM-VLB 1000 and CEM-T4 cells treated with sanguinarine was used to detect DNA damage. Sanguinarine-induced apoptotic cells of both cell lines showed DNA nicking (b and e), whereas, BCD of both cell lines (c and f) as well as untreated controls (a and d) failed to show DNA nicking.

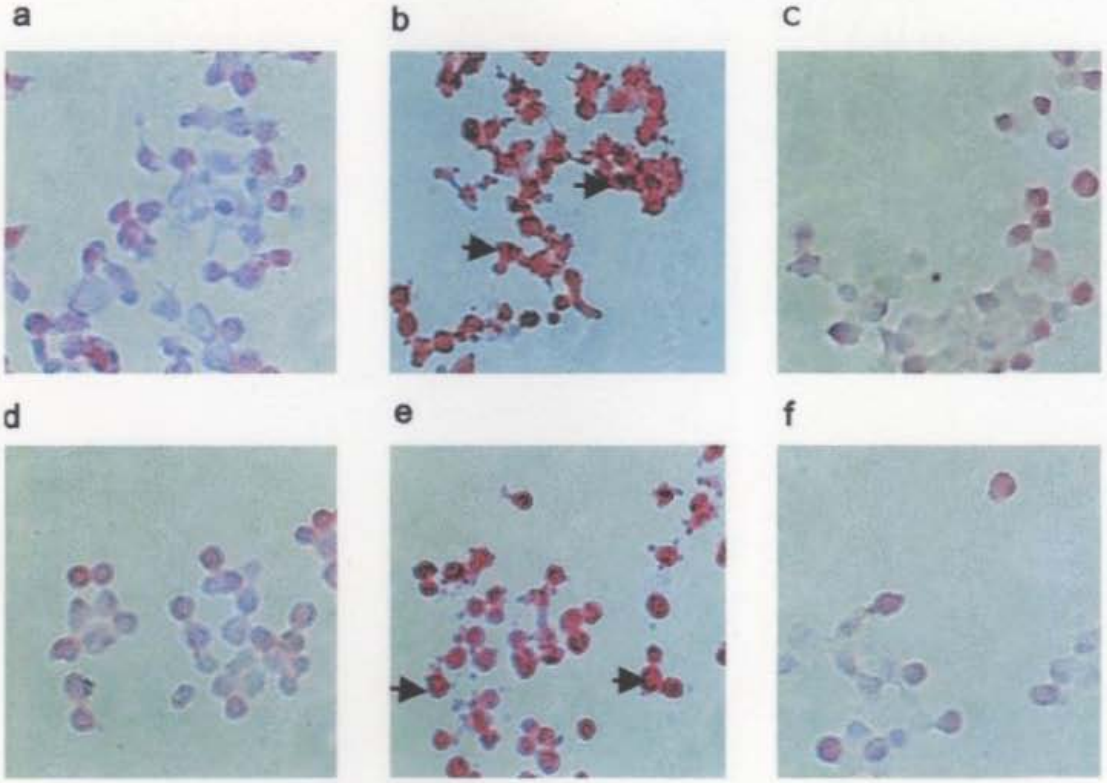

a
b
c
d
e
f

Figure 5-5: Annexin-V-binding assay in CEM-VLB 1000 and CEM-T4 cells treated with sanguinarine. The cell surface membrane phosphatidyl serine flip in sanguinarine induced apoptosis (1.5 μg/ml) and blister cell death (12.5 μg/ml) in both cell lines were measured using annexin-V and FITC staining with subsequent quantitation of annexin-V positive cells by FACS analysis. As shown, apoptosis of both cell lines had annexin-V-binding while all other cell samples (control and BCD) failed to show the PS flip. Each data point represents the mean ± SE from three independent experiments. *, $p<0.05$ is the statistical significance of the difference in annexin-V-binding between sanguinarine-treated CEM-VLB 1000/CEM-T4 cells at apoptosis-inducing concentrations and controls of these respective cell lines.

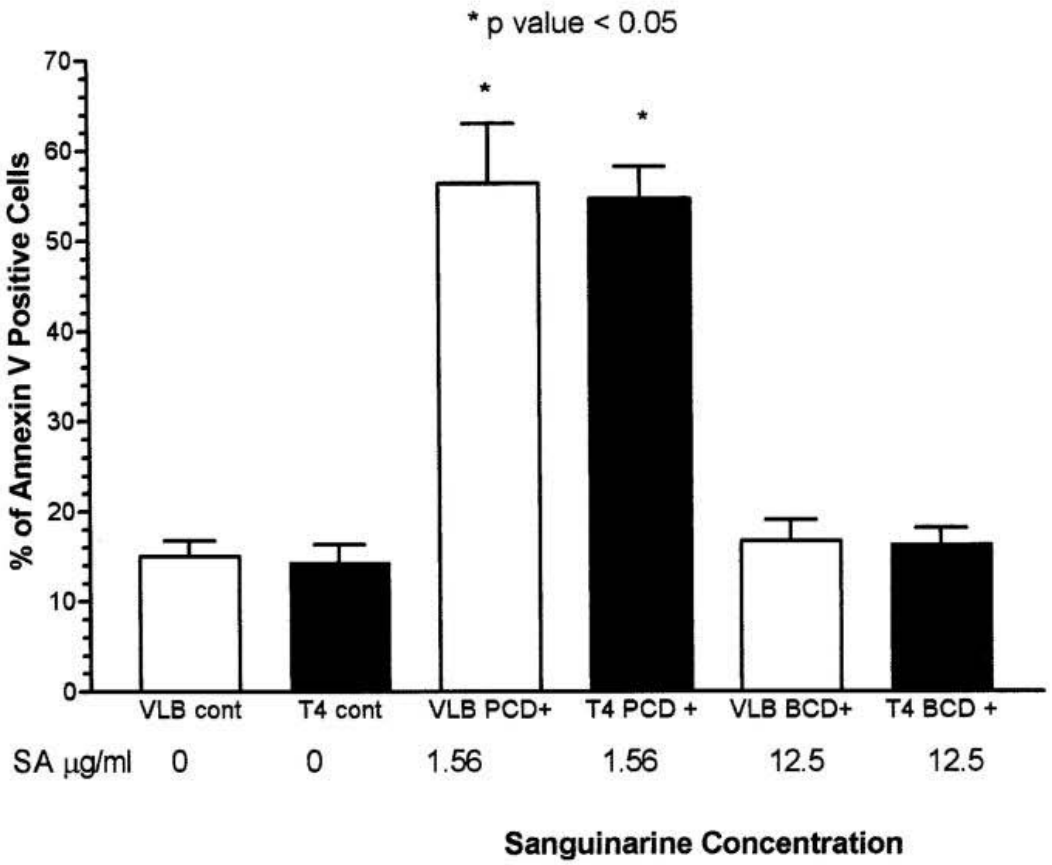

* p value < 0.05
% of Annexin V Positive Cells
70
60
50
40
30
20
10
0
*
*
VLB cont
T4 cont
VLB PCD+
T4 PCD +
VLB BCD+
T4 BCD +
SA µg/ml 0 0 1.56 1.56 12.5 12.5
Sanguinarine Concentration

Figure 5-6: Cell surface Pgp expression in CEM-VLB 1000 and CEM-T4 cells. Peaks "b" and "d" show Pgp-positive CEM-VLB 1000 cells and Pgp-negative CEM-T4 cells, respectively. As shown in the histogram over 95% of CEM-VLB 1000 cells were Pgp positive. Both "a" and "c" peaks show Pgp-negative controls. The X-axis shows the intensity of FITC fluorescence and Y-axis shows cell counts.

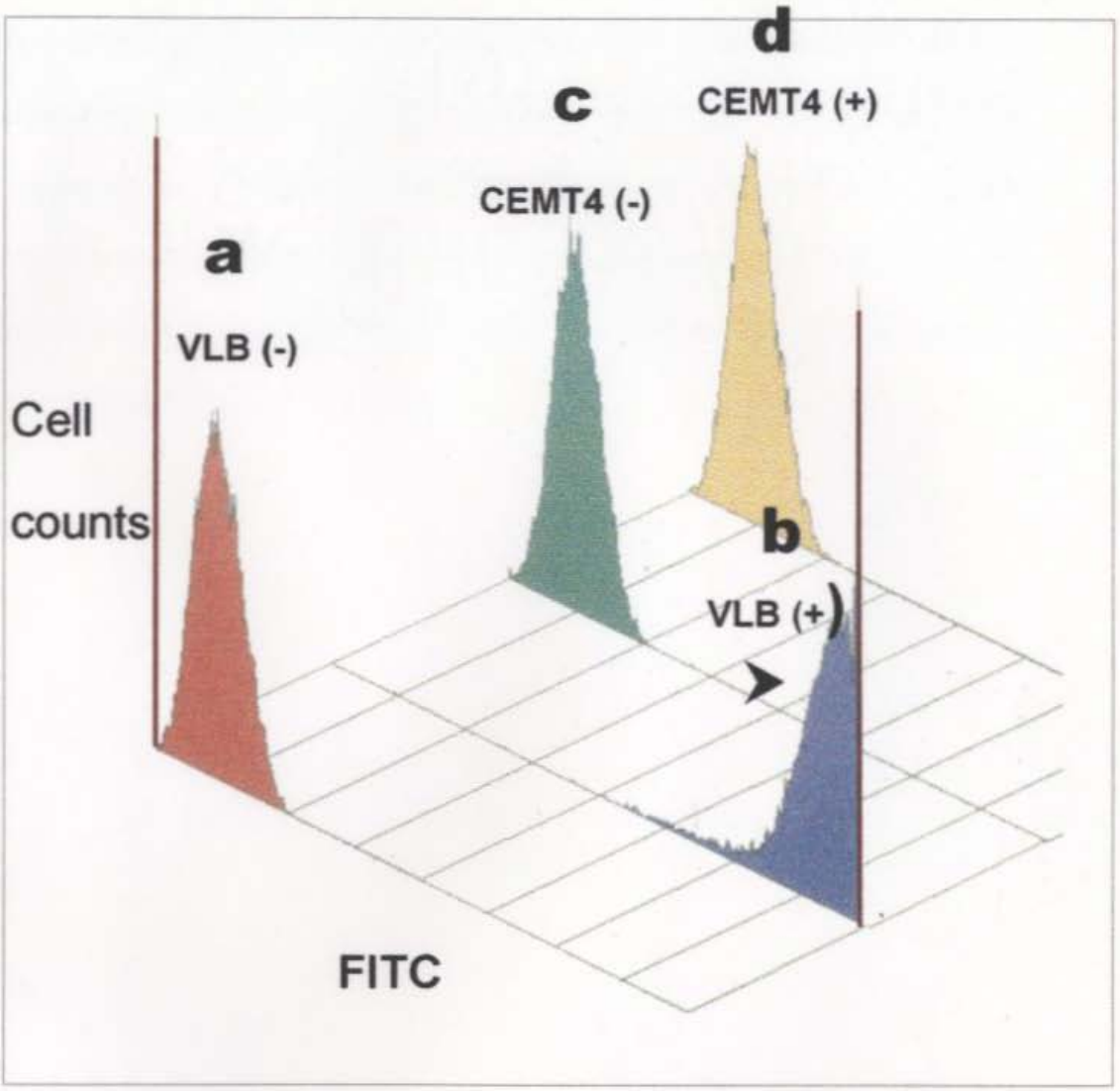
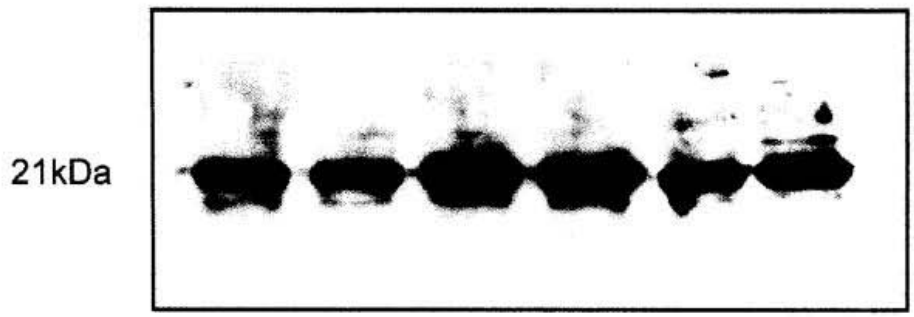

d
c
CEMT4 (+)
CEMT4 (-)
a
VLB (-)
Cell
counts
b
VLB (+)
FITC

Figure 5-7: Flow cytometric dot plots for the detection of sanguinarine auto-fluorescence in CEM-T4 and CEM-VLB 1000 cells. Propidium iodide (PI) fluorescence levels in PCD of CEM-VLB 1000 and CEM-T4 cells are represented by (b) and (e), and that of BCD are represented by (c) and (f), respectively. Control untreated CEM-VLB 1000 and CEM-T4 cells are shown in (a) and (d), respectively. The X-axis show FITC fluorescence intensity and Y-axis show intensity of PI fluorescence. Sanguinarine fluorescence levels in PCD and BCD of CEM-VLB 1000 cells were comparable to that in PCD and BCD respectively, of CEM-T4 cells.

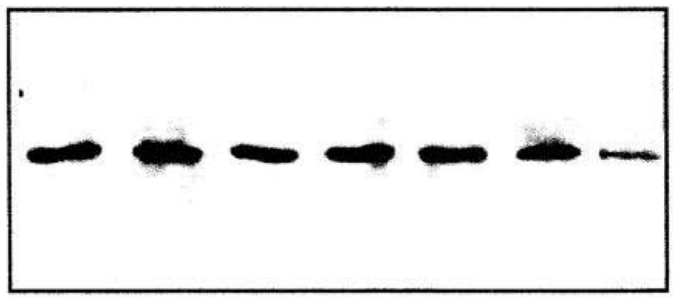
a
b
c
d
e
f
Auto fluorescence of SA
Auto fluorescence of SA
Auto fluorescence of SA
Auto fluorescence of SA
Auto fluorescence of SA
Auto fluorescence of SA
0 50 100 150 200 250
10^0 10^1 10^2 10^3 10^4

Figure 5-8A: Western blot of Bax protein expression in PCD and BCD of CEM-VLB 1000 and CEM-T4 cells treated with sanguinarine. Lanes 1, 3 and 5 show control, PCD and BCD of CEM-T4 cells, while lanes 2, 4 and 6 show control, PCD and BCD of CEM-VLB 1000 cells. As shown, the 21 kDa Bax band is moderately elevated in PCD of both cell lines. The elevation of the Bax band in BCD was not significant.



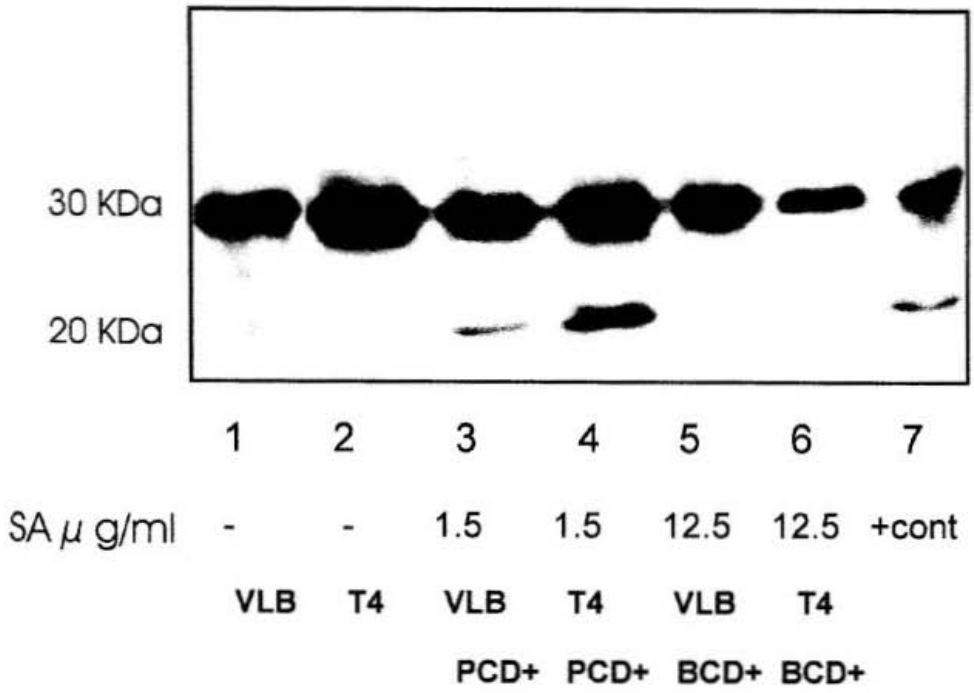
	1	2	3	4	5	6
SA μ/ ml	-	-	1.5	1.5	12.5	12.5
	VLB	T4	VLB PCD+	T4 PCD+	VLB BCD+	T4 BCD+

Figure 5-8B: Western blot of Bcl-2 protein expression in PCD and BCD of CEM-VLB 1000 and CEM-T4 cells treated with sanguinarine. Lanes 1, 3 and 5 show control, PCD and BCD of CEM-T4 cells, while lanes 2, 4 and 6 show control, PCD and BCD of CEM-VLB 1000 cells. Lane 7 is the positive control. Results show no change in the 26 kDa Bcl-2 levels in sanguinarine treated cells.



	1	2	3	4	5	6	7
SA μg/ml	-	-	1.5	1.5	12.5	12.5	+cont
	T4	**VLB**	**T4**	**VLB**	**T4**	**VLB**	
			PCD+	**PCD+**	**BCD+**	**BCD+**	

Figure 5-8C: Western blot of CPP-32 protein expression and of its 20 Kd breakdown product in PCD and BCD of CEM-VLB 1000 and CEM-T4 cells. Lanes 1, 3 and 5 show control, PCD and BCD of CEM-T4 cells, while lanes 2, 4 and 6 show control, PCD and BCD of CEM-VLB 1000 cells. Lane 7 is the positive control. Results show the 20 kDa breakdown product only in PCD of both cell lines.


30 KDa
20 KDa
1 2 3 4 5 6 7
SA μ g/ml - - 1.5 1.5 12.5 12.5 +cont
VLB T4 VLB T4 VLB T4
PCD+ PCD+ BCD+ BCD+

Chapter 6

Summary and Future Directions

Summary and Future Directions

Sanguinarine-induced bimodal cell death in our view is a unique phenomenon, about which very little is reported in literature. Its components PCD (or apoptosis) and BCD (as we have chosen to name it) are two different forms of cell death. The information reported on PCD on an almost daily basis is truly extraordinary. This stands in sharp contrast to the paucity of literature available on BCD. During the course of my present study period, we have found that bimodal cell death (BMCD), i.e. apoptosis and BCD induced by sanguinarine a convincing model for the study of both these cell death phenomena. As pointed out in the preceding chapters, the percentage of apoptosis and BCD induced by sanguinarine in K562 erythroleukemia cells, the MDR CEM-VLB cells or in their wild type counterparts CEM-T4 cells are > 90% and >85% respectively. This feature alone would make sanguinarine-induced bimodal cell death very attractive for the study of apoptosis and BCD.

In essence, the present study leading to this thesis revolved around several key issues, which we believe, are inter-related. These broadly comprise of, characterizing sanguinarine-induced PCD and BCD at the cellular and molecular levels, investigating the effects of Bcl-2, Bax/Bcl-2 ratio and the multidrug-resistant protein P-gp in the susceptibility of cells to sanguinarine, and also determining the role of BCD as a novel form of cell death. These issues were addressed within the context of four chapters (papers).

We found that the Bcl-2 low-expressing K562 cells (Kobayashi *et al.*, 1998), when treated with sanguinarine concentrations of 1.5 μg/ml, underwent the morphology of apoptosis, and when treated with concentrations of 12.5 μg/ml, underwent the

morphology of BCD. Characterization of the cellular parameters of sanguinarine-induced PCD and BCD in the Bcl-2 low-expressing K562 cells was done by measuring multiple parameters, using light and electron microscopy, ^{51}Cr release, TdT end labelling, annexin-V-binding and trypan blue and propidium iodide exclusion assays (see table 1).

Table 1: Characteristics of apoptosis or programmed cell death (PCD) vs blister cell death (BCD)

Criteria	Apoptosis or programmed cell death (PCD) in K562 cells	Blister Cell Death (BCD) in K562 cells
morphology as studied by light and electron microscopy	displayed formation of apoptotic bodies containing organelles, chromatin condensation, and nuclear fragmentation	displayed formation of blisters (often one, but rarely two) devoid of organelles, patchy chromatin condensation, and increase in vacuolization
cell injury as measured by ^{51}Cr release assay	showed a relatively lower degree of injury; approximately 20% of ^{51}Cr released	showed a relatively higher degree of injury; approximately 40% of ^{51}Cr released
cell viability and plasma membrane integrity as measured by trypan blue and propidium iodide dye exclusion assays	demonstrated viability by excluding both dyes	demonstrated viability by excluding both dyes
membrane PS flip as detected by the annexin-V-assay	showed PS flipping	showed no PS flipping
DNA nicking as measured by the TdT end labelling method	showed DNA nicking	showed no DNA nicking
Bax protein expression as measured by western blotting and flow cytometry	increase in Bax expression	no change in Bax expression
Bcl-2 protein expression as measured by western blotting and flow cytometry	no significant change in Bcl-2 expression	no significant change in Bcl-2 expression
NF-κB protein expression as measured by flow cytometry	decrease in NF-κB expression	increase in NF-κB expression

Caspase-3 involvement/activation as measured by western blotting and fluorimetric assay	Caspase-3 activation involved	Caspase-3 activation not involved

The differences between PCD and BCD, in light of these cellular assays were discussed. In general, all the above experiments showed significant differences between both forms of cell death, with the exception of trypan blue and propidium iodide dye exclusion assays. This leads us to the topic of BCD versus Necrosis.

Often BCD is associated with the necrotic process; thus leading to confusion. As we have noted elsewhere, the nomenclature of cell death is constantly being debated by the society of toxicologic pathologists and other regulatory bodies, and thus is an evolving process (Levin *et al.*, 1999). We subscribe to the theory that necrosis does not represent a form of cell death, but merely constitutes changes secondary to cell death. BCD by virtue of excluding propidium iodide and trypan blue may not represent necrosis. We have also observed morphologic similarities between BCD and the "blebbing phenomenon" found in the literature (Majno and Joris, 1995; Trump *et al.*, 1997). This was discussed at length in the preceding chapters.

Bcl-2 high-expressing JM1 cells, when treated with concentrations of sanguinarine that induce PCD and BCD in K562 cells, failed to undergo the corresponding morphologies which were observed in K562 cells treated with sanguinarine. This failure by JM1 cells to undergo the relevant morphologies was accompanied by an increase in the levels of Bcl-2 at both concentrations. Sanguinarine treatment of K562 cells, on the contrary, showed an increase in Bax at the lower concentration with no significant change in Bcl-2 levels (see table 1). These results clearly show the importance of the Bax/Bcl-2 ratio and its association with susceptibility of cells to sanguinarine-induced apoptosis; the increase in this ratio is also widely reported in literature to be associated with the

apoptotic process (Korsemeyer, 1993). The detection of this ratio may prove to be a valuable prognostic marker in oncology.

Interestingly, as aforementioned, treatment of the Bcl-2 high-expressing JM1 cells with sanguinarine at low and high concentrations resulted in an increase in the levels of Bcl-2. Our experiments show that the increase in Bcl-2 is a better indication of the susceptibility of cells to sanguinarine-induced apoptosis and BCD, than the endogenous Bcl-2 levels in cells. Further evidence of this was seen in experiments with CEM-VLB 1000/CEM-T4 cells. Both cell lines, despite showing moderate levels of endogenous Bcl-2, underwent bimodal cell death (BMCD) with relative ease when treated with the high and low concentration of sanguinarine; there was no increase in levels of Bcl-2 in either cell line, compared to untreated controls. In JM1 cells, the concomitant increase of Bcl-2 when treated with sanguinarine may be associated with the resistance to apoptosis and BCD that these cells demonstrate. The Bcl-2 gene product is widely associated with chemoresistance by virtue of its anti-apoptotic nature (Reed, 1998; Korsemeyer, 1999). Recent evidence indicates that Bcl-2 may also inhibit the "blebbing" phenomenon (Ferri *et al.*, 2000). We propose that the chemoresistance caused by the increase in Bcl-2 might be associated with its dual role of anti-PCD and anti-BCD, and thus may have potential clinical implications.

The cell death processes of PCD and BCD were also characterized at the molecular level. The expression of Bcl-2 family proteins, p53 and caspase-3 activation was assessed in both forms of cell death using western blotting technology. Results indicate that sanguinarine induced apoptosis involves caspase-3 and Bax by post-translational modification, whereas sanguinarine induced BCD involves neither caspase 3 nor Bax in

K562 cells. Evidence of *de novo* protein synthesis-independent apoptosis is well demonstrated in the literature (Reed, 2000). These results of western blotting studies were confirmed using immunofluorescence-flow cytometry; that of increase in pro-apoptotic Bax in sanguinarine-induced apoptotic K562 cells and the increase of Bcl-2 in JM1 cells, when treated with the drug.

We also discussed the involvement of the nuclear transcription factor NF-κB in sanguinarine-induced PCD and BCD (chapter 4). Immunofluorescence–flow cytometry studies showed that in response to sanguinarine treatment, K562 cells decreased NF-κB during PCD; this was in sharp contrast to the increase in NF-κB during BCD. The anti-apoptotic nature of NF-κB is well known (Kajino *et al.*, 2000), and corroborates our findings of reduction in NF-κB in apoptosis. In contrast, the increase in NF-κB in BCD may be associated with a pro-BCD function.

Sanguinarine was also found to overcome the P-glycoprotein mediated drug efflux pump in multidrug-resistant (MDR) CEM-VLB 1000 cells. This we attributed, as discussed earlier, to the influx of sanguinarine into the cell during BCD, thereby facilitating its intercalation with DNA. It was shown that PCD and BCD induced by sanguinarine in CEM-VLB 1000 cells and in its wild type counterparts CEM-T4 (P-gp negative) were qualitatively similar. This was demonstrated when sanguinarine induced PCD and BCD in both cell lines was assessed by standard methods, at both cellular and molecular levels. Western blotting analysis revealed an increase in the Bax/Bcl-2 ratio and activation of caspase 3 in PCD but not BCD in both cell lines. Therefore, we concluded that sanguinarine may overcome the phenomenon of MDR in CEM-VLB 1000 cells by inducing PCD through increasing the Bax/Bcl-2 ratio and activating caspase 3,

and BCD, which involved neither. Sanguinarine may therefore be explored as a potential anti-cancer drug in reversing drug-resistance in the chemotherapy of common malignancies.

We have dwelled on describing the paucity of information on non-apoptotic forms of cell death, despite having found that apoptosis may not be the sole cell death mechanism (Houghton, 1999; Trump *et al.*, 1997). We believe that the identification and characterization of the different modalities of cell death induced by potential anti-cancer drugs may be important in the war against cancer. Understanding different cell death mechanisms of individual anti-cancer agents may lead to their effective administration, alone or in combination with other established therapies. Thus, the need to identify and characterize novel cell death modalities of potential anti-cancer agents remains.

Based on our results, we may be able to hypothesize on a probable genetic regulation of BCD, as was also suggested by Trump *et al.* (1997) and Shirai (1999). Many would find this statement rather presumptuous. It is for this precise reason that we propose this line of thought as an avenue for essential future investigations. Further analysis of BCD at the cellular and molecular levels (especially at the early stages and at different time periods) might shed more light on this novel cell death modality. It is known that many factors such as, cell death inducing agent, its dose and the type of cell line contribute to the molecular changes that are triggered during cell death (Yanagihara and Tsumuraya, 1992). As such, in general, it is desirable that protein expression of BCD be studied in different cell lines, with several death inducing agents at different doses and time periods of exposure. As previously stated, our cell lines (with the exception of JM1) showed BCD when exposed to sanguinarine concentrations of 12.5 μg/ml, for 2 hrs. We are

particularly interested in studying the protein expression patterns at earlier time periods, i.e. 15, 30, 60 and 90 mins, when K562 cells are exposed to sanguinarine at the BCD inducing concentration of 12.5 μg/ml. It will also be interesting to know the changes at the gene transcript level at these time periods, when analyzed using cDNA array technology. These studies may help to reveal any possible genetic regulation of BCD.

Similarly, sanguinarine-induced apoptosis may also be studied along the lines described above, i.e at 15, 30, 60 and 90 mins of exposure of K562 cells, at the apoptosis-inducing drug concentration of 1.5 μg/ml. This may help reveal the protein expression patterns in apoptosis, particularly of the Bcl-2 family and the Bax/Bcl-2 ratio, at the early stages. This ratio, as also reported by other investigators, could be a valuable prognostic tool for common malignancies (Korsemeyer *et al.*, 1993; Korsemeyer, 1999). Thus, in a clinical setting, early detection of this ratio is of value. We are also keen on further investigating the lesser-known role of the increase in the anti-apoptotic Bcl-2, as a factor contributing towards chemoresistance. As aforementioned, K562 cells, CEM-VLB 1000 and its wild type underwent apoptosis and BCD when induced by sanguinarine while showing no increase in Bcl-2 levels; sanguinarine-treated JM1 cells, on the contrary, showed an increase in Bcl-2 levels while resisting apoptosis and BCD. Therefore, it would be interesting whether this phenomenon could be observed during sanguinarine treatment in other cell lines with varying levels of endogenous Bcl-2.

As mentioned before, we found no *de novo* protein synthesis in apoptosis as was also reported in the literature (Reed, 2000). Reed (2000) has suggested that, in apoptosis, post-translational modification of Bcl-2 proteins might take place by their phosphorylation/dephosphorylation and that conformational changes of Bax is associated

with its translocation to and insertion into mitochondria membranes. Based on this, it would be interesting to study these possible changes of Bcl-2 and Bax in relation to our experiments, using elctrophoretic mobility shift assay and immuno-electron microscopy, respectively.

In order to further strengthen our results, we would also like to transfect our cell lines with Bax and Bcl-2 and repeat the experiments with sanguinarine. K562 cells transfected with Bcl-2 as well as JM1 cells transfected with Bax could be used for this purpose. These experiments may further confirm the value of the Bax/Bcl-2 ratio in sanguinarine-induced apoptosis and the consistency of the association of chemoresistance with the increase in Bcl-2 levels in apoptosis and BCD, as observed in our studies. As described by Kobayashi et al. (Kobayashi *et al.*, 1998), these transfections may also give us the opportunity of studying spontaneous apoptosis due to Bax transfection and compare that with the apoptosis induced by sanguinarine.

Finally, this thesis may not be complete without reference to the potential anti-cancer effects of sanguinarine, about which many reports are found in the literature. As previously described in detail, our results showed that sanguinarine induces tumor cell death by PCD and BCD in K562 cells, found to be rather resistant to apoptosis and also to overcome the P-gp mediated MDR phenomenon. It would be interesting to document the effects of sanguinarine *in vivo*. Dose-response studies with sanguinarine may be done in tumor-bearing mice; multiple parameters can be measured including tumour size and immunological indices such as immune effector cell cytolytic activity.

The past few years have seen a rapid advance in the understanding of cell death. As new concepts such as apoptosis appeared and the discovery of new and additionally

forms of cell death are pursued, some conceptual and semantic strains developed; a recent reviewer saw fit to conclude that "there is no field of basic cell biology and cell pathology that is more confusing and more unintelligible than is the area between apoptosis versus necrosis" (Majno and Joris, 1995).

In closing, I would like to quote from a review by Majno and Joris-"We would like to point out two facts. First, oncosis and apoptosis are merely two forms of cell death, among many others that remain to be described. Consider, for example, that form of cell death that makes histopathology possible, namely death by fixation. Histological fixatives are designed to produce the perfect crime, death without visible traces. What shall we call it? Second, in these days of molecular pathology, it is well to remember that the marvelous story of apoptosis was initiated by a very simple, morphological observation, accessible to the microscopes of our great-grandfathers."

Would BCD attract the same attention as apoptosis?

We believe, yes.

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