

INHIBITION OF SANGUINARINE INDUCED BIMODAL  
CELL DEATH BY AURIN TRICARBOXYLIC ACID BUT  
NOT BY CYCLOHEXIMIDE

CENTRE FOR NEWFOUNDLAND STUDIES

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**Inhibition Of Sanguinarine Induced Bimodal Cell Death By Aurin**  
**Tricarboxylic Acid But Not By Cycloheximide**

**By**

**© Sarathi C. Hallock**

A thesis submitted to the  
School of Graduate Studies  
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## **ABSTRACT**

The possible role of endonuclease and protein synthesis inhibition by aurintricarboxylic acid (ATA) and by cycloheximide (CHX) respectively was investigated in sanguinarine induced apoptosis (PCD) and blister cell death (BCD) in K562 erythroleukemia cells.

Studies in our laboratory have consistently shown that when K562 cells are treated with sanguinarine- a quaternary benzophenanthridine alkaloid which is reported to inhibit protein kinase C and nuclear factor NF- $\kappa$ B, at concentrations of 1.5  $\mu$ g/ml for 2hrs induced the morphology of PCD, and at concentrations of 12.5  $\mu$ g/ml for 2 hrs the morphology of BCD (blister formation). This phenomenon of dual cell death modality was termed “bimodal cell death” or BMCD (Liepins et al., 1996).

The role of endonuclease activity and protein synthesis in PCD and BCD was assessed by the capacity of ATA and CHX respectively, to inhibit these processes. This was investigated by pretreating cells with ATA and CHX prior to sanguinarine treatment and subsequently measuring their effects on bimodal cell death using a host of standard methods: light microscopy and quantitative morphology; electron microscopy; terminal dUTP mediated nick end labelling (TUNEL) assay; annexin V binding assay (fluorescence microscopic and flow cytometric analysis);  $^{51}\text{Cr}$  release assay; DNA content analysis by flowcytometry; oxygen consumption studies; trypan blue assay. Results show that, while ATA pretreatment of cells inhibited PCD almost completely and BCD by 30-40%, CHX pretreatment failed to inhibit PCD and BCD. This may indicate the importance of endonuclease in sanguinarine induced PCD and to some extent in

BCD. This may also signify the importance of post-translational modification of proteins rather than their *de novo* synthesis in both these forms of cell death.

Discovering new drugs and understanding their mechanisms of action may lead to more effective administration of these agents with other more established therapeutics in the treatment of cancer. To this end, better understanding the mechanism of PCD and identifying novel forms of cell death like BCD, would contribute in no small measure.

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## **Abbreviations**

ATA	Aurin tricarboxylic acid
CHX	Cycloheximide
PCD	Programmed cell death
BCD	Blister cell death
BMCD	bimodal cell death
ATP	adenine triphosphate
Ca <sup>++</sup>	calcium
TNF	tumor necrosis factor
TUNEL	TdT mediated dUTP nick end labeling
TdT	terminal deoxynucleotidyl transferase

## **1.0. Introduction**

### **1.1. Historical Perspective:**

Early evidence of the existence of two morphologically distinct types of cell death came from the Ph.D. studies of Australian pathologist John Kerr, working under the supervision of Sir Roy Cameron, at the University College Hospital Medical School in London (Allen, 1995; Kerr, 1965; Kerr, 1995). Kerr ligated portal vein branches to the left and median lobe of the rat liver (Kerr, 1965). These lobes shrank to one sixth of their original weight in 8 days, and the other lobes simultaneously underwent hyperplasia, while the total weight of the liver remained approximately constant. In the atrophying lobes, patches of confluent necrosis occurred around terminal hepatic venules, whereas periportal parenchyma, sustained by blood from the hepatic artery, survived. Individual hepatocytes outside the zone of necrosis, in the absence of inflammation gave rise to small round cytoplasmic masses, some containing pyknotic chromatin, a manifestation of cell death distinctly different from necrosis, which Kerr named *shrinkage necrosis*. Histochemistry for acid phosphatase activity showed that although lysosomes in periventricular necrotic cells ruptured with dispersal of enzyme product in the cytoplasm, lysosomes in the small round cytoplasmic masses stained discretely, indicating that the lysosomes were still intact. “Shrinkage Necrosis” of hepatocytes also occurred in the normal liver. This was a form of cell death morphologically distinct from necrosis. This cell death mechanism had features suggesting an intrinsically controlled active process that occurred under both physiological and pathological circumstances, was important in normal embryonic morphogenesis, and was also of great kinetic significance in tissues

and tumors (Kerr et al., 1972). Kerr, Wyllie and Currie in 1972, recognized that *necrosis* was an inappropriate term to describe active cell death occurring under physiological conditions, and proposed the term *apoptosis* for this process. This emphasized its role opposite to that of mitosis in regulating tissue size (Kerr et al., 1972). The word apoptosis was suggested by James Cormack, professor of classical Greek at the University of Aberdeen, and meant “falling off” or “dropping off” (Kerr et al., 1972). It had been used by Homer to describe the dropping off of leaves from trees in autumn (Wyllie, 1994).

### **1.2. The Concept Of Cell Death:**

Knowledge in the field of cell death has greatly increased in the past 20 years. In the course of this rapid advance, new concepts, such as apoptosis, appeared on the scene, and ancient terms such as necrosis came to be used in a new context, leading to much confusion. A recent review 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 of apoptosis versus necrosis” (Kerr et al. 1972). The complexity in defining the mode of cell death and in classification of different death mechanisms was recently reviewed by Majno and Joris (Majno and Joris, 1995). These authors convincingly argue that cell death and necrosis are two distinct events. Cell death according to these authors, is a process that leads to the point of no return; subsequent events are the postmortem changes, which these authors define as necrotic changes. In the case of induction of liver cell death by ischemia, for example, the irreversible point is at approximately 150 min after onset of oxygen deprivation (Majno and Joris, 1995). At that time, however, no

significant morphological changes can be seen, while the necrotic changes become visible only after 12 hours. Thus, the cells die, long before any morphological changes typical of necrosis can be detected. The term cell death “by necrosis”, which implies that necrotic changes accompany, appears to be contradictory and confusing. Additionally confusing is the traditional use of the term “necrosis” in pathology to describe gross tissue changes, visible by eye and occurring after cell death, i.e., attributed to postmortem cell changes (Majno and Joris, 1995).

The term “accidental cell death” to define the mode of cell death which is now generally denoted as cell necrosis, and “oncosis” to portray the early stages of accidental cell death was proposed by Majno and Joris. The term oncosis has been used by pathologists to describe cell death associated with cell swelling (oncos = swelling) which occurs during slow ischemia, e.g., as in the case of death of osteocytes entombed in bone tissue during osteogenesis. The features of oncosis are identical to those seen in the early phase of accidental cell death in a variety of cell systems. Within the framework of classification of cell death proposed by Majno and Joris, the necrotic step follows either oncosis or apoptosis and thus can be denoted either as “oncotic necrosis” or “apoptotic necrosis”, respectively (Majno and Joris, 1995). The common features of such late necrotic cells, regardless of whether they were dying by apoptosis or oncosis, are loss of plasma membrane transport (ability to exclude charged ions such as propidium iodide or trypan blue), autolytic processes, dissolution of the remnants of chromatin (karyolysis), etc (Majno and Joris, 1995).

### **1.3. Cell Necrobiology:**

The interest in cell death spans a wide range of diverse disciplines, including cell and molecular biology, oncology, immunology, embryology, endocrinology, hematology, neurology, plant biology, and biotechnology. Of particular interest are molecular mechanisms which predispose the cell to respond to an environmental or intrinsic signal by death and/or regulate the initial steps leading to the irreversible commitment to death. It is suggested that, since this subject is so wide and involves so many disciplines, a new field of biology and medicine is called for and the term “cell necrobiology” has been proposed to define this field (Darzynkiewicz et al., 1997). The term necrobiology refers to the life processes associated with morphological, biochemical and molecular changes which predispose, precede and accompany cell death, being derived from *necros* (death) and *bios* (life), reflecting their inevitably intertwined relationship. Cell necrobiology, thus, represents a wide area of research which includes different modes of cell death, the biological processes which predispose, precede and accompany cell death, as well as the consequences and tissue response to cell death. On the other hand the term “cell necrology” may be reserved to define specifically the field of studies dealing with the post-mortem changes, occurring in the cell which has passed the point of “no return” in its journey towards death (Darzynkiewicz et al., 1997).

### **1.4. Multidisciplinary Interest In Cell Necrobiology:**

A multistep complex mechanism regulates the cell's propensity to respond to various stimuli by apoptosis (Oltvai and Korsmeyer, 1994; Rowan and Fisher, 1997; Nagata,

1997; Goldstein, 1997; Kumar, 1997). The regulatory system involves the presence of at least two distinct stages, one controlled by the Bcl-2 family of proteins (Nagata, 1997; Goldstein, 1997; Kumar, 1997; Hockenberry, 1995; Lomo et al., 1996; Reed, 1994) and another by cysteine (Fernandez-Alnemri et al., 1995; Lazebnic et al., 1994; Nicholson, et al., 1995; Alnemri et al., 1996) and also possibly by serine (Bruno et al., 1992; Gorczyca et al., 1992, Hara et al., 1996; Weaver, et al., 1993) proteases. Among the proteins controlling the first stage, Bcl-2, Mcl-1, and Bcl-xl promote cell survival while Bax, Bcl-xs, Bak and, Bad enhance the cell's propensity to undergo apoptosis. All members of the Bcl-2 family contain well conserved domains that allow for the formation of homo- and hetero-dimers between members. The cysteine (ICE) proteases, recently renamed caspases (Alnemri et al., 1996), consist of at least 11 homologous enzymes with somewhat different substrate specificities. Caspases interact with the receptors transferring apoptotic signals through the plasma membrane such as Fas or TNF receptor 1 (TNFR1) via adaptor molecules such as FADD/MORT 1, through interactions between the well conserved death domains of these proteins (Nagata, 1997; Chinnaiyan, 1996; Kiston et al., 1996). There is a growing body of evidence that the Bcl-2 family of proteins act upstream of caspases (Nagata, 1997). Although not being direct modulators of caspases, the Bcl-2 proteins appear to activate these proteases perhaps by engaging their activator (Nagata, 1997; Krippner et al., 1996).

The wide interest in apoptosis stems from the realization that it is an active and, as mentioned above, highly regulated mode of cell death. In addition to the Bcl-2 family of proteins and caspases, a plethora of other molecules, which either promote or prevent

apoptosis, have been discovered to interact with the regulatory machinery of apoptosis. Some are the products of oncogenes or tumor suppressor genes, e.g., such as c-myc or wild type p53 (Evan et al., 1992; Clarke et al., 1993; Pavlovich et al., 1997). Others are viral proteins such as BHRF1, a product of the Epstein Barr virus, which is homologous to Bcl-2, or crmA, a product of cowpox virus, an inhibitor of caspases (Ray et al., 1992; Williams and Smith, 1993). These virally encoded proteins promote their intracellular propagation by postponing apoptosis. Given such a wide range of regulatory steps and molecules, there are many possibilities for interaction with the components involved in the regulation of apoptosis and thereby to modulate the cell's propensity to die. It is not surprising, therefore, that apoptotic mechanisms are the focus of interest of many researchers in the field of oncology.

Numerous strategies designed to utilize these mechanisms to modulate the sensitivity of the tumor and/or normal cells to anti-tumor agents, and, as a result, to increase the efficiency of treatment and/or to lower drug toxicity to the patient, have been considered (Darzynkiewicz, 1995; Dive and Hickmann, 1991; Fisher, 1994; Hannun, 1997; Hickmann, 1992; Kerr et al. 1994). Another finding promoting interest in apoptosis was the identification of the gene protecting cells from apoptosis (Bcl-2) as an oncogene (Tsujimoto et al., 1985). This finding revealed that not only a defect in the cell cycle results in uncontrolled cell proliferation but also the loss of the cell's ability to die on schedule may be a cause of cancer. More recently it has become apparent that tumor progression and increased malignancy are also associated with the change in the propensity of tumor cells to undergo spontaneous apoptosis (Hercbergs and leith, 1993;

Isaacs, 1993; Laidlaw and Potten 1992). The rate of spontaneous apoptosis in tumors as well as apoptosis induced by chemotherapy, therefore, have become areas of intense interest in oncology. In the latter case, it has been recently demonstrated that the ability of antitumor agents to induce apoptosis can be analyzed in the course of therapy, thereby providing the possibility of rapid assessment of their efficiency (Gorczyca et al., 1993; Li et al., 1994; Seiter et al., 1995; Halicka et al., 1997).

Another discipline where wide interest is focused on apoptosis, is immunology. Apoptosis plays a fundamental role in the clonal selection of T cells and is implicated in many other normal and pathological reactions (Cohen, 1993; Linette and Korsmeyer, 1994). Of particular interest is the mechanism of cell killing by NK lymphocytes, which is based in the use of the apoptotic effector machinery (Young et al., 1986). Furthermore, since progression of AIDS appears to be correlated with the rate at which T cells die by apoptosis, attempts are being made to monitor apoptosis of these cells and evaluate such measurements as markers of disease progression and prognosis (Echaniz et al., 1995; Meyaard et al., 1992).

Male fertility is a field where apoptosis appears to be of particular interest. It has been observed that DNA in chromatin of abnormal infertile sperm cells, in contrast to normal sperm cells, is excessively sensitive to heat or acid-induced denaturation (Evanson et al., 1980). The DNA in these cells also has numerous strand breaks (Gorczyca et al., 1993). Furthermore, the DNA in situ in sperm is more accessible to actinomycin D (Gledhill et al., 1971). These three features are also typical of DNA in the chromatin of apoptotic somatic cells (Hotz et al., 1992; Hotz et al., 1994). Therefore, it

was proposed that an apoptosis-like mechanism may be triggered to eliminate cells bearing various types of DNA damage, including mutations, even at late stages of spermatogenesis (Laidlaw and Potten, 1992). However, because such highly differentiated cells may have already lost many effectors of apoptosis, the process may be incomplete, resulting only in activation of an endonuclease which causes extensive DNA degradation, effectively eliminating such cells from the reproductive pool but not leading to their physical disintegration. Such spermatozoa may still have mitochondrial activity, normal motility, and, in some cases, even normal morphology (Evenson et al., 1980). Analysis of in situ DNA denaturability in sperm cells, assayed by flow cytometry, has become increasingly popular as a marker of infertility and in toxicology studies to assay the genotoxic effects of environmental agents (Evenson et al., 1993; Sailer et al., 1995).

#### **1.5. Apoptosis (cell death by suicide):**

There are three forms by which cells may commit suicide: by lysosomes, by free radicals, and by a genetic mechanism (apoptosis) (Majno and Joris, 1995). Thus, of these three, apoptosis stands out as a form of intentional suicide based on a genetic mechanism (Majno and Joris, 1995). Listed below are the salient features of apoptosis:

- (1). Apoptosis is a form of cell death characterized by morphological as well as biochemical criteria and can be considered as a counterpart of mitosis (Majno and Joris, 1995).
- (2). Morphologically the cell shrinks and becomes denser, as implied in the original name “shrinkage necrosis” (Kerr, 1971). The chromatin becomes pyknotic and packed into

smooth masses applied against the nuclear membrane (margination of chromatin), creating curved profiles that have inspired descriptive terms for over a century, such as half-moon-, horse-shoe-, sickle-, lancet and ship-like (navicular) (Schmaus and Albrecht, 1894; Rechsteiner, 1988). The nucleus may also break up (karyorhexis), and the cell emits processes (the budding phenomenon) that often contain pyknotic nuclear fragments. These processes tend to break off and become apoptotic bodies, which may be phagocytized by macrophages or neighbouring cells or remain free; however, the cell may also shrink into a dense, rounded mass, as a single apoptotic body (Kerr, 1971).

(3). There is little or no swelling of mitochondria or other organelles (Majno and Joris, 1995).

(4). Biochemically, the DNA is broken down into segments that are multiples of approximately 185 bp, due to specific cleavage between nucleosomes (Oberhammer et al., 1993).

(5). The process is under genetic control (Kerr et al., 1994; Wyllie, 1994) and can be initiated by an internal clock, or by extracellular agents such as hormones, cytokines, killer cells, and a variety of chemical, physical and viral agents.

(6). Apoptosis can run its course very quickly, even in minutes (Majno and Joris, 1995). For this reason apoptosis is remarkably unobtrusive in tissue sections (Weedon et al., 1979). In routine sections the best cytological marker of apoptosis is karyorhexis, especially in an isolated cell. Fortunately, a recent technical advance makes the identification of apoptosis a matter of simple histochemistry, a method that takes

advantage of the fact that the DNA breaking points (nicks) expose molecular endings that are chemically specific (Gavrieli et al., 1992).

(7). The rapidly developing tale of apoptosis warns us that generalizations are dangerous because, first, cell suicide does not always take the form of apoptosis; second, cell murder by cytotoxic lymphocytes leads to apoptosis; third, there seem to be several varieties of apoptosis (Martin, 1993) and fourth, different cell types may follow different rules (Virchow, 1971).

**Table 1:** Changes in cell morphology and molecular events during apoptosis.

### **(A) Morphological Changes**

- Cell Shrinkage
- Cell shape change
- Nuclear chromatin condensation
  - Loss of visually recognizable nuclear structure (framework)
  - DNA hyperchromicity
- Dissolution of nuclear envelope
- Nuclear fragmentation
- Condensation of cytoplasm
- Loss of cell surface structures (pseudopodia, microvilli)
- Formation of apoptotic bodies (“budding”, “blebbing”)
- Detachment of cells in cultures
- Phagocytosis of the apoptotic cell remains

### **(B) Biochemical and Molecular Events of Apoptosis**

- Increased ratio of apoptosis promoters vs inhibitors of the Bcl-2 family
- Mitochondrial changes
  - Decrease in the transmembrane potential
  - Leakage of cytochrome C
  - Oxidative stress (formation of ROI)
- Intracellular  $\text{Ca}^{++}$  rise
- Loss of asymmetry in plasma membrane phospholipids
- Activation of serine protease(s)
- Cascade-activation of caspases
  - Proteolysis of the “death substrates”
- Degradation of F actin and proteins other than “death substrates”
- Loss of DNA stability to denaturation
- Endonucleolytic DNA degradation
  - 50-300 kb fragments
  - Cleavage of internucleosomal DNA
- Activation of transglutaminase

### **1.6. Accidental Cell Death:**

For cell death not by apoptosis the most satisfactory term is accidental cell death. Necrosis is commonly used but it is not appropriate, because it does not indicate a form of cell death but refers to changes secondary to cell death by any mechanism, including apoptosis (Majno and Joris, 1995). Abundant data are available on one form of accidental cell death, namely ischemic cell death, which can be considered an entity of its own, caused by failure of the ionic pumps of the plasma membrane. Because ischemic cell death (in known models) is accompanied by swelling, the name oncosis was proposed for this condition (Majno and Joris, 1995). The term oncosis (derived from *oncos*, meaning swelling) was proposed in 1910 by von Recklinghausen precisely to mean cell death with swelling. Oncosis leads to necrosis with karyolysis and stands in contrast to apoptosis, which leads to necrosis with karyohexis and cell shrinkage (Majno and Joris, 1995).

Salient features of oncosis as defined by Majno and Joris are listed below:

- (1). Oncosis is a form of cell death accompanied by cellular swelling, blebbing, and increased membrane permeability.
- (2). It's mechanism is based on failure of ionic pumps on the plasma membrane.
- (3). It is caused, typically, by ischemia and possibly by toxic agents that interfere with ATP generation or increase the permeability of the plasma membrane.
- (4). It evolves within 24 hours to typical necrosis.
- (5). It is usually accompanied by karyolysis.

(6). It 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) (Hoffstein et al., 1975).

(7). The DNA breaks down in a nonspecific fashion (Wyllie et al., 1984).

(8). The cellular changes (increased permeability of the plasma membrane, cell swelling, organelle swelling and vacuolization, and simultaneous protein denaturation and hydrolysis) can only be hinted at by ordinary histological techniques.

Many experiments have shown that blebbing, described above, begins during the early stages of ischemic damage and is initially reversible (van Gorp et al., 1999). Large blebs may burst, and it has been suggested that this may be the final blow to a dying cell (Majno and Joris, 1995).

Thus, while apoptosis is characterized by the active participation of the affected cell in its own destruction, even to the point of triggering (in some cell systems) the *de novo* synthesis of cell death effector molecules, accidental cell death, generally results from the gross injury to the cell induced by an overdose of cytotoxic agents and is a passive, catabolic and degenerative process. An early event of accidental cell death is mitochondrial swelling, which is followed by the rupture of the plasma membrane and release of cytoplasmic constituents, including proteolytic enzymes, outside of the cell (Majno and Joris, 1995; Wyllie, 1992). Nuclear chromatin shows patchy areas of condensation and the nucleus undergoes slow dissolution (karyolysis). Necrosis triggers an inflammatory reaction in the tissue and often result in scar formation. DNA degradation is much less extensive during necrosis compared to apoptosis and the

products of degradation are heterogenous in size, forming a smear rather than discrete bands on electrophoretic gels (Gorczyca et al., 1992).

Cell death does not always manifest classical features of either apoptosis or necrosis. Many examples of cell death have been described in which the pattern of morphological and/or biochemical changes resemble neither typical apoptosis nor necrosis but often contain features of both (Cohen et al., 1992; Collins et al., 1992; Ormerod et al., 1994; Zakeri et al. 1993; Zamai et al., 1996; Akagi et al., 1995; Catchpoole and Stewart, 1993; Fukuda et al., 1993). In some cases, the integrity of the plasma membrane was preserved but DNA degradation was random, without evidence of internucleosomal cleavage. In other situations, DNA degradation was typical of apoptosis but nuclear fragmentation and other features of apoptosis were not apparent. Generally, while most hematopoietic lineage cell types are “primed” to undergo apoptosis and their death has typical features of apoptosis, the death of epithelial type cells or fibroblasts provide a more complex pattern that is sometimes difficult to classify. Furthermore, some drugs, which cause apoptosis, may additionally confuse the pattern of cell death due to drug-induced secondary effects on the cell. When apoptosis is triggered by drugs effecting cell structure and function, or by drugs effecting one or more pathways of the apoptotic cascade, particular features of apoptosis may not be apparent. Induction of apoptosis in the presence of inhibitors of proteases, for example, can lead to cell death without evidence of nuclear fragmentation or a decrease in the stability of DNA to denaturation (Hara et al., 1996). Likewise, prolonged cell arrest in the cell cycle induced by some drugs can result in growth imbalance that may dramatically alter cell

biochemistry and morphology. The apoptotic features of such cells are much different compared to untreated cells (Gong et al., 1995).

### **1.7. Early And Delayed Apoptosis. Relationship to the cell cycle:**

It has been observed in numerous studies that exposure of lymphocytes or hematological tumor cell lines to pharmacological concentrations of antitumor drugs or radiation induces their rapid apoptosis, which can be seen as early as 2-4 h after the onset of treatment (Gorczyca et al., 1992; Gorczyca et al., 1993). Likewise, apoptosis of lymphocytes induced by their activation, corticosteroids, or via engagement of the Fas receptor, by Fas ligand, or by TNF, also is rapid. In all these cases, the pattern of apoptosis is quite typical and the cells exhibit nearly all the characteristic apoptotic features, as listed in Table 1. During this early apoptosis the cells generally die in the same cell cycle, prior to mitosis. A term “homo-cycle” apoptosis has been proposed to define apoptosis which occurs in the same cycle in which the cells were initially exposed to the apoptosis triggering agent, prior to, or during mitosis. Often the cells die in the same phase of the cycle, without progressing to the next one. To define such death, the term “homo-phase apoptosis” applies (Halicka et al., 1997). An example of such apoptosis is rapid death of S phase cells during treatment of exponentially growing cell populations with DNA topoisomerase inhibitors (Gorczyca et al., 1993; Del Bino et al., 1991). In contrast to “homo-cycle apoptosis”, the term “postmitotic apoptosis” has been proposed to describe apoptosis which occurs in a cell cycle stage subsequent to the one in which the initial damage to the cell was induced (Halicka et al., 1997).

This subdivision is pertinent in relation to the possible differences in mechanisms triggering apoptosis. During homo-phase apoptosis, which for example, occurs in S phase, the cells do not traverse G1 or G2 check points. Therefore the product of the tumor suppressor gene p53, which controls these check points, may not play a significant sensitizing role, as it otherwise does in cells that traverse these check points. Anti-tumor strategies involving p53 and the checkpoints, therefore, may be different for drugs inducing homo-phase vs post mitotic apoptosis (Halicka et al., 1997).

While early apoptosis occurs between 30 minutes and 8 hours after induction of damage in the cell, apoptosis may also occur after a considerable delay. For example, exposure of cells to anti-tumor drugs at low concentrations or when transient (pulse) treatment with drugs or radiation is followed by growth in drug-free-media, the initial results may be perturbation of cell cycle progression (cytostatic effect). Subsequently, such cells may either resume cell cycle progression and even be capable of reproduction after removal of the drug from the medium, or they may die with a delay. Cell death in such a situation is often due not only to the primary damage but may also result from the accumulation of secondary changes that occurred during the cytostatic phase. One such change is growing imbalance. During inhibition of DNA replication, RNA and protein synthesis continue, but many genes which were initially damaged by the drug may become transcriptionally inactive. The cells die if products of these genes are essential for their survival. Less is known about whether altered ratios of overall RNA/DNA or protein/DNA, gross features characteristic of cell growth imbalance, may themselves contribute to cell death (Gorczyca et al., 1993; Del Bino et al., 1991).

Delayed apoptosis is also observed in the case of certain cell types, in particular cells of epithelial cell origin even following exposure to cytotoxic drug concentrations or radiation (Chang and Little, 1991; Shinohara and Nakano, 1993). It is possible that the cells, which do not undergo early apoptosis, lack the required effectors of apoptosis. The apoptosis-triggering event, in these cells, induces transcription of genes encoding the effector(s), which explains a delay in the execution of apoptosis. Inhibition of protein synthesis by cycloheximide, in such cases, generally protects the cells from apoptosis (however, as discussed later, cycloheximide may not inhibit apoptosis when post-translational modification of proteins rather than its de novo synthesis may be involved). For example T cell leukemic MOLT-4 cells do not undergo early apoptosis (Del Bino et al., 1990), most likely because they lack the action of the apoptosis-associated endonuclease, which is induced with a delay (Matsubara et al., 1994).

Interestingly, the phase of the cell cycle in which the cells undergo apoptosis in response to a particular drug is often different in the case of early vs delayed apoptosis. Transient exposure of cells to a drug which, for example, induces early apoptosis in S (e.g., teniposide) or in G2 M phase (taxol), followed by cell growth in drug-free medium, leads to cell death not only in S or G2 M but often in G1 phase (Halicka et al., 1997).

### **1.8. Incidence of apoptosis:**

Apoptosis has a significant role in physiological and developmental processes, but it also accounts for cell death occurring under a variety of pathological circumstances (Kerr and Harmon, 1991; Kerr et al., 1987; Kerr et al., 1994; Walker et al., 1988; Wyllie et al., 1980). An abbreviated report of its occurrence follows.

### **1.8.1. Normal Development:**

Many parallels can be drawn between developmental processes in lower species and mammals. Study of the finely regulated developmental cell death that occurs in the nematode *Caenorhabditis elegans* has provided insights into the genetic control of apoptosis in higher animals (Yuan and Horvitz, 1990). For example, ICE and bcl-2 protein are the homologues of the protein products of the *C. elegans* death modulating genes, ced-3 and ced-9.

During metamorphosis, regression of larval organs in lower vertebrates, for example, loss of tail as a tadpole matures into a frog, occurs by morphologically typical apoptosis (Kerr et al., 1974). In vertebrates apoptosis has been implicated in the development of most body systems (Gluckmann, 1951).

In 1966, Saunders showed that the interdigital tissue of amniota embryos underwent extensive but controlled cell death, allowing digits to emerge from the early foot plate (Saunders, 1996). Morphological studies of the regressing tissue showed apoptosis, correlating with the internucleosomal DNA fragmentation found on DNA gel electrophoresis (Garcia-Martinez et al., 1993; Kerr et al., 1987). During fusion of the palatine shelves, redundant epithelium undergoes apoptosis as fusion progresses (Farbman, 1968; Kerr et al., 1987).

Cell death during the development of the vertebrate nervous system has been studied extensively. It takes the form of apoptosis and involves a range of cell types, including astrocytes, oligodendrocytes (Barres et al., 1992), and neurons. Up to 50% of

neurones die soon after forming synaptic connections with their target cells: cell death is thought to reflect failure of neurones to receive specific survival factors from the target cells (Raff et al. 1993).

During intrauterine life, epithelial cells in the mammalian intestinal mucosa undergo apoptosis, allowing widening of the lumen and the formation of villi (Harmon et al., 1984). In the normal developing rat kidney, metanephric mesenchymal cells are programmed for apoptosis, dying if they fail to receive survival signals from ureteric bud cells (Koseki et al., 1982). Invasion of the mesenchyme by the ureteric bud, an outgrowth of the wolffian duct, blocks apoptosis, and the mesenchyme converts to epithelium and differentiates.

Apoptosis plays a key role in both the development and maintenance of the immune system (Williams, 1994). In the developing thymus, engagement of the T-cell receptor on immature T cells by self-antigen induces their death by apoptosis, self-reactive T-cells thus being eliminated. Induction of tolerance in B cells probably also involves apoptosis and a similar process deletes autoreactive B cells (Murphy et al. 1990; Smith et al., 1989).

### **1.8.2. Atypical Apoptosis During Terminal Cell Differentiation:**

In some tissues, cells undergoing terminal differentiation exhibit some but not all features of apoptosis. For example, normoblast nuclei undergo condensation in association with DNA cleavage to regularly sized fragments (Williamson, 1970). After displacement to one pole of the cell, the nucleated portion is recognized and

phagocytosed by macrophages, leaving behind an intact erythrocyte (Skutelsky and Danon, 1972). In the developing chicken lens, primary lens fiber cell nuclei undergo pyknosis and disappear after cleavage of DNA to form nucleosomal fragments: so by birth, the center of the lens comprises nonnucleated tissue (Appleby and Modak, 1977).

A specialized form of apoptosis also occurs in the skin as keratinocytes in the granular layer undergo terminal differentiation to form squames. Here, cells with classic apoptotic nuclear morphology are observed, and cleavage of their DNA to form nucleosomal-sized fragments can be demonstrated. Budding and cellular fragmentation associated with normal apoptosis do not occur. The cell forms a flattened anucleate keratinocyte on the skin surface (McCall and Cohen, 1991).

### **1.8.3. Normal Cell Turnover In Adult Tissues:**

Spontaneous apoptotic deletion of cells is a consistent feature of slowly and rapidly proliferating mammalian cell populations. In tissues that comprise predominantly postmitotic cells, such as the brain and the heart, apoptosis is not normally identified (Walker et al., 1988).

In slowly proliferating tissues, such as liver, prostate, pancreas and parotid gland, small numbers of apoptotic bodies are found in the normal tissue (Kerr, 1965; Kerr and Searle, 1973; Walker, 1987; Walker and Gobe, 1987). In the liver, these are almost always found in zone 3, next to the terminal hepatic venule (Benedetti et al., 1988). In the adult rat adrenal gland, where maintenance of the inner two thirds of the adrenal cortex is

finely regulated by serum ACTH levels, calculations suggest that over time the rate of apoptotic cell loss equals that of cell gain through mitosis (Wyllie et al., 1980).

In rapidly proliferating tissues, such as gastrointestinal epithelium, there are well-defined zones of both proliferation and apoptosis (Hall et al., 1994). In the small intestine, apoptotic cells are seen most frequently toward the tips of villi, although occasional apoptotic bodies are also identified close to the proliferative compartment within the intestinal crypts. Apoptotic bodies within the epithelium, and less frequently in the lamina propria, are seen also in the colon toward the top of crypts. Hall et al. have shown apoptosis with subsequent macrophage uptake of apoptotic bodies to be numerically more significant than luminal shedding, the latter traditionally regarded as the predominant mechanism of epithelial cell loss (Hall et al., 1994).

In lymph nodes, there is a high death rate among germinal center centrocytes, as they are selected for their ability to bind antigen (MacLennan, 1994). Those not selected, undergo apoptosis and become the tingible bodies seen in germinal center macrophages (Liu et al., 1989; Searle et al., 1982).

After releasing most of their cytoplasm as platelets, megakaryocyte remnants in the bone marrow die by apoptosis (Radley and Haller, 1983). Senescent neutrophils also undergo apoptosis, such cells being recognized and phagocytosed by macrophages, thus preventing release of their lysosomal enzymes as they die and augmentation of the inflammatory response (Savill, 1992). Varying erythropoietin levels regulate the extent of apoptosis of late erythroid progenitors, thereby controlling the size of the red blood cell pool (Koury, 1992; Koury and Bondurant, 1990).

#### **1.8.4. Cyclic Cell Loss In Adult Tissues:**

The size of many cell populations is under the control of growth factors or hormones, with cell deletion occurring when circulating levels of relevant factors fall. The cell loss invariably occurs by apoptosis (Ferguson and Anderson, 1981).

In the normal adult human breast, mitosis of duct and ductular epithelial cells is maximal at day 25 and apoptosis on day 28 (of a 28-day menstrual cycle) (Ferguson and Anderson, 1981). The apoptotic peak correlates with a sharp decrease in immuno-staining for bcl-2 protein in lobular epithelium (Sabourin et al., 1994). The apoptotic cells are phagocytosed by neighboring epithelial and myoepithelial cells and intraepithelial macrophages rather than being shed into glandular lumens (Ferguson and Anderson, 1981). Human endometrium shows increased loss of glandular epithelial cells by apoptosis in the late secretory, premenstrual and menstrual phases (Hopewood and Levison, 1976). Apoptotic cells and bodies are seen in epithelial cells and macrophages within and beneath the epithelium and rarely within gland lumens. Uterine epithelial cells surrounding the blastocyst at the implantation sites of mice and rats also undergo apoptosis, bringing the blastocyst into close association with endometrial stroma (Parr et al., 1987).

#### **1.8.5. Involution of organ tissue:**

Involution of the neonatal rat adrenal cortex occurs synchronously with known decreases in circulating ACTH in the neonatal period (Wyllie et al., 1973). In the first 3

to 5 days after birth, large numbers of apoptotic bodies are found in the adrenal cortex; subsequently the number falls to low levels. The lactating breast after weaning undergoes rapid involution, presumably related to falls in circulating prolactin levels (Walker et al., 1989). In the mouse, initial involution proceeds rapidly, with large numbers of apoptotic epithelial cells being shed into gland lumens. Subsequently, there is more gradual regression to the resting state, with apoptotic bodies derived from epithelial and myoepithelial cells mostly being phagocytosed by intraepithelial macrophages. Apoptosis of endothelial cells, resulting in regression of the capillary networks, accompanies the loss of glandular tissue. Internucleosomal DNA fragmentation characteristic of apoptosis accompanies involution (Strange et al., 1982).

Greater than 90% of ovarian follicles present at birth undergo atresia (Hsueh et al., 1994). Using a 3'- end labelling technique, DNA fragmentation characteristic of apoptosis is identified in atretic follicles of both chicken and porcine ovaries (Tilly et al., 1991). In postovulatory follicles, granulosa cells undergo apoptosis, whereas, thecal cells are relatively spared (Hurwitz and Adashi, 1993). In the regressing corpus luteum of sheep, endothelial cell apoptosis results in blood vessel depletion (Azmi and O'Shea, 1984). In other tissues, the stimulus to involution is unknown, but apoptosis remains the mechanism of cell loss. For example, spontaneous involution of hair follicles during catagen involves apoptosis of epithelial cells in the outer root sheath, with apoptotic bodies phagocytosed predominantly by adjacent epithelial cells (Weedon and Strutton, 1981). The resorption of tissues around erupting teeth also involves apoptosis (Schellens et al., 1982).

#### **1.8.6. Pathological Atrophy and Regression of Induced Hyperplasia:**

Pathological atrophy is also effected by apoptotic deletion of cells. Thus, falls in circulating levels of trophic hormones following castration induced atrophy of the rat prostate (Kerr and Searle, 1973). Exocrine gland atrophy after duct obstruction (Walker, 1987; Walker and Gobe, 1987), renal atrophy in experimental hydronephrosis (Gobe and Axelsen, 1987), and atrophy of the liver and kidney caused by mild ischemia (Gobe et al., 1990; Kerr, 1971) involve apoptosis. As in involution of the lactating breast and corpus luteum, apoptosis of endothelial cells may accompany the tissue atrophy (Walker, 1987; Walker and Gobe, 1987). Regression of induced hyperplasia is also generally effected by apoptosis. For example, deletion of proliferated bile ductules after the relief of common bile duct obstruction (Bhathal and Gall, 1985) and regression of hepatocyte hyperplasia after cessation of administration of cyproterone acetate occur by apoptosis (Borsch et al., 1985).

#### **1.8.7. Cell Mediated Immunity:**

Cell death induced by cytotoxic T-cells, K-cells and natural killer cells takes the form of apoptosis (Berke, 1994; Walker et al., 1988). Such death is part of immune surveillance: transformed and virally infected cells, which are potentially dangerous to the host, are recognized and removed. Induction of apoptosis in target cells by specifically sensitized T-lymphocytes may involve activation of cytoplasmic proteases in the target cell by engagement of the Fas receptor (also known as Apo-1 or CD95) (Enari

et al., 1995) or injection of granzyme B, a serine protease, through perforin pores in the target cell membrane (Shresta et al., 1995; Smyth and Trapani, 1995). T-cell mediated apoptotic cell death is also involved in transplant rejection (Krams et al., 1995), graft versus host disease (Ferrara and Deeg, 1991), acute and chronic hepatitis (Patel and Gores, 1995), primary biliary cirrhosis (Nakanuma et al., 1983) and lichen planus (Paus et al., 1993). Viral infection may also cause apoptosis through direct viral cytotoxicity, induction of tumor necrosis factor (TNF), or by producing conflicting growth signals (Shen and Shenk, 1995). Inappropriate induction of CD4<sup>+</sup> T-cell apoptosis by human immuno-deficiency virus (HIV) may be relevant to the pathogenesis of acquired immunodeficiency syndrome (AIDS) (Ameisen et al., 1994; Laurent-Crawford et al., 1991).

#### **1.8.8. Neoplasia, Cancer Therapeutic Agents, and Toxins:**

Although impaired induction of apoptosis as a result of abnormal *p53* or *bcl-2* gene expression undoubtedly contributes to the development and progression of many tumors, the execution phase must remain intact, as some apoptosis is present in virtually every malignant tumor (Kerr et al., 1994; Searle et al., 1973; Wyllie, 1985), often significantly retarding tumor growth (Kerr and Searle, 1972). Mild ischemia (Moor, 1987), infiltration by cytotoxic T-lymphocytes (Curson and Weedon, 1979), release of TNF- $\alpha$  by macrophages (Bellomo et al., 1992; Wright et al., 1992), and intrinsic activation of apoptosis, probably all contribute towards tumor regression. Radiotherapy, hyperthermia and chemotherapy promote tumor regression, at least partly through the

induction of apoptosis (Allan and Harmon, 1986; Hickman, 1992; Kerr et al., 1987; Kerr et al., 1994; Searle et al., 1982; Searle et al., 1975; Walker et al., 1988), but tumors lacking functional *p53* or expressing *bcl-2* are often resistant to these agents (Lowe et al., 1993). Toxins may induce apoptosis in normal tissues (Walker, 1988), often in a dose dependent manner, with low doses inducing apoptosis and high doses inducing necrosis (Kerr, 1972).

### **1.9. Medicinally Useful Herbs:**

Medicinally useful herbs have made a great contribution to medical practice today. Today about 75% of the worlds population still relies on plants, plant extracts and other tools of traditional medicine (Abelson, 1990). There are about 121 clinically useful prescription drugs worldwide that are derived from higher plants. About 74% of them came to the attention of the pharmaceutical houses because of their use in traditional medicine (Abelson, 1990). Among the drugs derived from plants are the anticancer agents vinblastine, vincristine and more recently the benzo-phenanthridine alkaloids. They provide some of our most valuable medications from plants (as listed below). The medical value of plants is due particularly to the chemicals which they contain but a few plants may help because of their physical properties. Herbs often contain several of these chemical groups, sometimes in the leaves, sometimes in the roots, or indeed in the whole plant. The main groups of chemicals involved in medical use are listed below (Jones, 1996):

- 1) Alkaloids: these complex nitrogenous compounds provide a number of our most potent drugs including morphine, quinine, caffeine and the novel class of benzo-phenanthridine alkaloids (sanguinarine, chelerythrine etc.).
- 2) Glycosides: these sugars are linked with a special complex chemical, e.g. the cardiac glycosides present in the foxglove. They include the saponins which can foam with water as in soapwort.
- 3) Essential oils: it is a misleading term. They are volatile fatty acids, often fragrant. Many have valuable antibacterial and antiparasitic actions. Some like peppermint have beneficial effects on the digestive organs.
- 4) Tannins: they have an astringent effect and are particularly present in those plants which have long had a tradition for stopping bleeding such as self-heal or the carpenter's herb (*prunella vulgaris*). Its use may have been helped by sphagnum moss which, with its great absorptive powers, has been used to stem serious bleeding since prehistoric times.
- 5) Mucilages: these are polysaccharides, chemically linked chains of sugars which tend to form a gel with water. They can be immensely helpful in certain respiratory diseases.
- 6) Bitter principles: these are non-poisonous bitter substances, particularly present in gentians, artemesias and hops. These act as digestive stimulants and also gives characteristic flavours.

- 7) Miscellaneous: there are many other groups including some which have not yet been synthesized by chemists and represent a great potential for future medicinal and domestic benefit.

#### **1.9.1. Alkaloids:**

An alkaloid is any of a class of nitrogen-containing natural products of plant origin that have an alkalilike, or basic, chemical nature. Some alkaloids are simple, monocyclic (one ring) amines, but many are very complex, polycyclic amines. Various family groupings are possible according to basic ring structure, such as pyridine or quinolone alkaloids. The term was coined in 1819 by the German pharmacist, K.F.W.Meissner (<http://www.entheogen.com/fatfreddie/chem/alkaloid.html>). Primitive tribes in various parts of the world have known for centuries that physiological effects can be obtained by eating or chewing the leaves, roots or bark of certain plants. By trial and error these tribes have found that some plant extracts cure diseases and others are deadly poisons.

Today thousands of different physiologically active compounds extracted from the leaves, barks, roots, flowers, and fruit of plants have been identified by chemists. Most of these are water insoluble, but they can be extracted from plant material by an acidic solution. Because they react with acid, these naturally occurring substances (most of which contain nitrogen-amides or amines) are called alkaloids, meaning “like a base” because bases react with acids.

An alkaloid is a physiologically active, nitrogen containing low molecular weight, base compound extracted from plant material (Pauli and Kutchan, 1998). A role in the chemical defence of the plant also emerges for alkaloids, causing this class of metabolites to be of both eco-chemical and pharmaceutical interest. Tetrahydrobenzylisoquinolone-derived alkaloids constitute a large number of structurally diverse molecules that vary widely in physiological activity. Examples of this class of alkaloids that have been employed in modern medicine are the muscle relaxant bisbenzylisoquinoline alkaloid (+)-tubacurarine used in abdominal surgery, the analgesic morphinan alkaloid morphine, the anti-tussive alkaloid codeine, the anti-microbial berberine alkaloid berberine and the anti-microbial and anti-tumor quaternary benzo[c]phenanthridine alkaloid sanguinarine (Pauli and Kutchan, 1998).

### **1.9.2. Sanguinarine:**

#### **1.9.2.1. Structure-Activity relationship:**

The quaternary benzo[c]phenanthridine alkaloid sanguinarine occurs in several genera of the families Papaveraceae (*e.g. Argemone, Bocconia, Chelidonium, Dicranostigma, Eschscholtzia, Glaucium, Hunnemannia, Hylomecon, Hypecoum, Macleaya, Meconopsis, Papaver, Platystemon, Romneya, Sanguinaria, Stylomecon and Stylophorum*), Berberidaceae (*Berberis* and *Mahonia*), Fumariaceae (*Corydalis, Dicentra* and *Fumaria*) and less abundantly in Menispermaceae (*Jateorhiza*), Ranunculaceae (*Thalictrum*), Rutaceae (*Zanthoxylum*), Sapindaceae (*Ptridophyllum*) and other taxa

(Sehmeller et al., 1997). The benzophenanthridine nucleus consists of four fused coplanar aromatic rings, with various functional groups on the terminal rings (Bajaj, 1990).

Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium) is a commercial drug (see table 3 for structure) derived from the root of *Sanguinaria canadensis* (blood root), *Chelidonium majus* L. (Greater Celandine) and other poppy-fumaria species. *Chelidonium majus* L., a perennial of Papaveraceae, is a common plant growing on the euroasiatic continent. The orange coloured sap of the stems and roots contains comparatively high concentrations of benzophenanthridine alkaloids, especially of the alkaloids chelidonine, chelerythrine, and sanguinarine and has a long history of being useful for the treatment of many diseases in european countries (Lenfeld et al., 1981). This plant is of great interest for its use also in chinese herbal medicine (Colombo and Bosisio, 1996). By standard methods more than twenty alkaloids have been detected in this plant. Three quaternary alkaloids from this plant, sanguinarine, chelerythrine and berberine, and a non quaternary one, chelidonine, have been separated by normal-phase chromatography (Han et al., 1991).

From the stucture-activity relationships point of view, two main remarks can be drawn: 1) For all the compounds so far synthesized and studied, 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). 2) The reactivity of the iminium toward nucleophilic attack (Rodds chemistry of carbon compounds, 2<sup>nd</sup> ed., 1978) has been put forward to explain the antileukemia activity of these alkaloids (Ulrichova et al., 1991). The iminium bond of any quaternary benzo[c]phenanthridine alkaloid is highly

susceptible to nucleophilic attack. The addition of nucleophiles such as water, thiols, amines, and alcohols is the most common reaction of sanguinarine (Simanek, In: The Alkaloids, Vol 26, Academic Press; Dostal and Potacek, 1990). However, such a transformation leads to unstable products, which could be responsible for the acute toxicity reported (Arisawa et al., 1984; Rodds chemistry of carbon compounds, 2<sup>nd</sup> ed., 1978).

#### **1.9.2.2. Pharmacological activities and applications:**

Many alkaloids of the benzo[c]phenanthridine family, including sanguinarine, display anti-tumor properties (Simeon et al., 1989). Apart from its anti-tumor properties, sanguinarine also shows antimicrobial activity against a wide variety of microorganisms including 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). The pseudobases of sanguinarine as well as their alkanolamines and pseudocyanides are more potent than the iminium ions (Mitscher et al., 1978). These findings are assumed to be the result of higher pseudobase hydrophobicity, which causes an increase in bioavailability to the microorganism. The pseudobase form can be reverted to the iminium ion form inside the microorganism. Sanguinarine possesses good antifungal activity against *Candida albicans* and the dermatophytes of trycophyton species *Epidermophyton floccosum*, *Microsporum canis*, and *Aspergillus fumigatus* (Hejmankova et al., 1984). Anti-inflammatory effects of sanguinarine have been demonstrated in carrageenan, kaolin, and formaldehyde induced

inflammation edema of the rat paw with activity similar to that of indomethacin when sanguinarine was administered orally at 5-10 mg/kg (Lenfeld et al., 1981). Following the oral administration of a 10 mg/kg dose a prolonged anti-inflammatory effect of sanguinarine was observed (Lenfeld et al., 1981). Sanguinarine demonstrates a local anesthetic action more pronounced than that of procaine (Kelenney, 1960). Sanguinarine also interferes with adrenaline and acetylcholine pharmacology models *in vitro* (Ulrichova et al., 1983; Hakim, 1954). The medicinal applications of sanguinarine are derived from hundreds of years of experience in traditional medicine. Native American Indians used extracts containing sanguinarine as expectorant and escharotic agents (Becci et al., 1987). A quaternary benzo[c]phenanthridine extract in combination with  $\text{ZnCl}_2$  was used in North America around 1910 as a necrotic agent for accessible neoplasms (Lewis and Elvin-Lewis, 1977). Sanguinarine is an ingredient in many over the counter products including toothpaste, mouthwash, cough and cold remedies and homeopathic preparations (Frankos et al., 1990).

The biochemical activities of sanguinarine includes the inhibition of protein kinase C (Janin et al., 1993; Gopalakrishna et al., 1995), NFkB (nuclear transcription factor) (Chaturvedi et al., 1997),  $\text{Na}^+/\text{K}^+$  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).

#### **1.9.2.3. Pharmacokinetics:**

The pharmacokinetics of sanguinarine have been widely studied. Following a 10mg/kg i.v. injection into rabbits, blood levels drop rapidly. The blood concentration of the alkaloid 15-20 min *post injectionem* was almost nondetectable, indicating fast elimination (Walterova et al., 1995). An *in vitro* dialysis of sanguinarine solutions against whole blood indicated that sanguinarine was not bound to the plasma, but preferentially adsorbed to red blood cells and this is in accord with *in vivo* evaluations (Dobbie and langham, 1961). Accumulated data also suggests that liver and GI tract are potential target tissues. In a study with humans 2.5-10% dispersions of sanguinarine in a degradable polymer gel were delivered subgingivally. Crevicular fluid showed detectable levels of sanguinarine for 12 days. However, no sanguinarine was detected in any blood sample during this period (Dunn et al., 1991).

#### **1.9.2.4. Toxicological evaluations and adverse effects:**

Acute toxicity of sanguinarine depends strongly on the mode of administration (Lenfeld et al., 1981; Kelenney, 1960,; Becci, et al., 1987; Frankos et al., 1990). A subcutaneous injection of sanguinarine results in an LD<sub>50</sub> for mice of 80 and 102 mg/kg, while an intravenous injection of sanguinarine results in an LD<sub>50</sub> of 15.9 mg/kg. The LD<sub>50</sub> in rats for i.v. administration was 29 mg/kg with sanguinarine. An oral LD<sub>50</sub> of sanguinarine in rats ranges from 1525 to 1624 mg/kg depending upon the additive. The acute dermal LD<sub>50</sub> in rabbits was over 200 mg/kg. Subchronic toxicity studies with sanguinarine in rats administered 1-15 mg/kg/day in the diet for a period of 14 days and

in monkeys given 10-60 mg/kg/day orally revealed no treatment related toxicity in any group apart from emesis and diarrhea, which was observed sporadically and only for limited time in monkeys at dosage levels > 30 mg/kg/day (Frankos et al., 1990). Intravenous administration of 5-15 mg/kg sanguinarine to cats caused an increase in blood pressure, tonicity, intestinal peristalsis, and contraction of the spleen (Kelenney, 1960). In reproductive and developmental toxicity, a fertility/reproduction study in rats has shown an increase in mortality at 100mg/kg/day. Reduced pup weight was observed at high doses only. Cardiovascular toxicity has been tested. Intravenous injections of sanguinarine (0.5-4.0 mg/kg) to guinea pigs produced a dose-related increase in the ventricular effective refractory periods (Whittle et al., 1980).

Sanguinarine inhibits the hepatic drug-metabolizing enzyme system. Sanguinarine at 10 mg/kg i.p. prolonged pentobarbital sleep in rats (Dalvi and Peebles, 1981).

The presence of sanguinarine in low concentrations in the seed oil from *Argemone mexicana* and *Argemone ocroleuca* has been suggested as the sole etiologic agent responsible for epidemic dropsy, associated with glaucoma (Das and Khanna, 1997). The bioavailability data on sanguinarine and the animal toxicity and safety data suggest these benzo[c]phenanthridine alkaloids to have a wide margin of safety when administered orally or topically (Frankos et al., 1990; Lord et al., 1989).

#### **1.10. Sanguinarine Induced PCD (apoptosis) and BCD In K562 Cells *In Vitro*:**

Previous studies in our laboratory have demonstrated that K562 cells, when exposed to sanguinarine for 2 hrs 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  $\mu\text{g/ml}$ , cells displayed blebbing and shedding of membrane vesicles over the entire cell surface, consistent with the current criteria of apoptosis or programmed cell death (PCD). At concentrations of 12.5  $\mu\text{g/ml}$ , cells displayed the morphology of large blister formation (commonly a single blister and usually not more than two). This phenomenon was coined “blister cell death or BCD” (Liepins et al., 1996). Furthermore, these two morphologically distinct modalities of cell death were interphased by an apparently normal cell morphology at sanguinarine concentrations of 6.25  $\mu\text{g/ml}$ , which was dubbed as “silent period” based on the fact that these cells displayed a morphology similar to control cells not exposed to the drug (Liepins et al., 1996). The phenomena of PCD and BCD induced by sanguinarine was termed “bimodal cell death (BMCD)”.

K562 erythroleukemia cells express low levels of endogenous Bcl-2 protein and fairly high levels of endogenous Bax protein (Kobayashi et al., 1998). This expression is found to be intriguing as these cells are rather resistant to apoptosis, which is not consistent with the cells’ low levels of apoptosis-inhibiting Bcl-2 and high levels of apoptosis-facilitating Bax (Kobayashi et al., 1998). This cell line, originally established in the laboratory of Lozzio et al., are multipotential hematopoietic malignant cells that spontaneously differentiate into recognizable progenitors of the erythrocytic, granulocytic and monocytic series (Lozzio et al., 1981).

### **1.11. Aurin tricarboxylic acid (ATA):**

Aurin tricarboxylic acid is a heterogenous mixture of polymers (see table 3 for structure) that forms when salicylic acid is treated with formaldehyde, sulfuric acid and sodium nitrite (Gonzalez et al., 1979; Cushman et al., 1991a; Cushman et al., 1991b). This triphenylmethane dye or ATA, is an inhibitor of nucleases in vitro and has been shown to inhibit the following enzymes: DNase I, RNase A, S1 nuclease, exonuclease III, and restriction endonucleases Sal I, Bam HI, Pst I and Sma I (Hallick et al., 1977). ATA has also been reported to prevent apoptosis in nerve growth factor dependent sympathetic neurons and PC12 cells (Batistata and Greene, 1991). As ATA was initially shown to inhibit template binding to Q $\beta$  replicase (Blumenthal and Landers, 1973) and to prevent the binding of RNA to ribosomes (Webster and Zinder, 1969), it was postulated that this compound would inhibit the association of any nucleic acid binding protein with nucleic acid. The ATA molecule carries three carboxylic acid moieties that are negatively charged at physiological pH. It has been suggested that these groups facilitate extensive ionic binding of the molecule to positively charged groups on cellular proteins (Bina-Stein and Tritton, 1976). Nucleases, however, appear to be more sensitive to inhibition by ATA than many other enzymes (Bina-Stein and Tritton, 1976).

### **1.12. Objective Of Present Study:**

It is known that various factors maintain inhibition of apoptosis and when withdrawn result in induction of apoptosis. An example of this is interleukin-6 (IL6), which is a well known survival/growth factor for a murine B cell hybridoma, 7TD1 (Liu

et al., 1994). After IL6 deprivation, the cells became growth arrested and demonstrated signs considered to be the hallmark of apoptosis (Liu et al., 1994). It is also known that the expression of Bcl-2 inhibits apoptosis induced by various factors (Reed, 1994). An example of this is the work undertaken with sanguinarine in our laboratory (Weerasinghe et al., in preparation). It has been shown that the Bcl-2 gene product plays an anti apoptotic role in sanguinarine induced apoptosis. Chemicals that mimic the effects of these natural factors also inhibit the apoptotic process. This is exemplified by the inhibition of apoptosis by the endonuclease inhibitor ATA and by the protein synthesis inhibitor CHX (Liu, et al., 1994; Yanagihara and Tsumuraya, 1992)

The present study was designed to assess the underlying mechanisms and to characterize the PCD and BCD induced by sanguinarine in K562 cells. The initial objective was to discover chemical compounds with known biological effects that would inhibit the induction of PCD and/or BCD by sanguinarine. This was achieved by pretreatment of these cells with chemical compounds drawn from different groups, previously proven to be inhibitors of apoptosis. A list of such compounds, their respective drug actions and biological effects follows.

**Table 2:** A list of chemical compounds that regulate apoptosis, with their respective drug actions and biological effects (as described in “The Merck Index- An Encyclopedia of Chemicals, Drugs, and Biologicals”, 1999), used in the preliminary pretreatment screening test.

<b>AGENT</b>	<b>DRUG EFFECT</b>	<b>BIO. EFFECTS</b>
Aurintricarboxylic acid (Liu, et al., 1994)	metabolic inhibitor	endonuclease inhibitor
Cycloheximide (Liu et al., 1994, Yanagihara and Tsumuraya, 1992)	metabolic inhibitor	protein synthesis inhibitor
Actinomycin D (Atlante et al., 1998)	metabolic inhibitor	disruption of replication and transcription
Cyclosporin A (Crabtree and Clipstone, 1994)	metabolic inhibitor	Immunosuppressant.
Glutathione (Park et al., 2000; Yang et al., 2000)	endogenous antioxidant	major thiol compound of living plant or animal cell
Butylated hydroxy toluene (Anderson et al., 1999)	antioxidant	Antioxidant for food / antimicrobial activity
Dithiothreitol (Yu, et al., 2000; Liu et al., 1999)	antioxidant	Proteolytic agent
Mepacrine (Fabisiak, et al., 1998; Shin, et al., 1999; Jaattela, et al., 1995)	Phospholipase A2 (PLA2) inhibitor	Anti helminthic / parasite / protozoal
Verapamil (Ares, et al., 1997)	Ca <sup>2+</sup> channel blocker	Anti-arrythmic and vaso-dilating agent

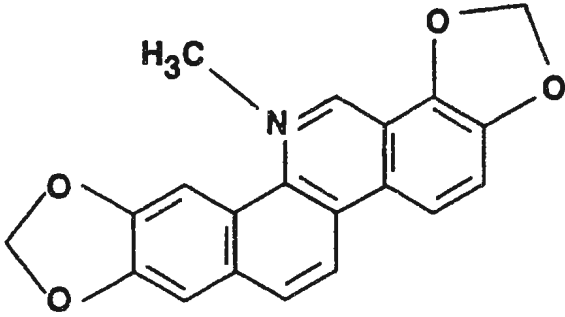
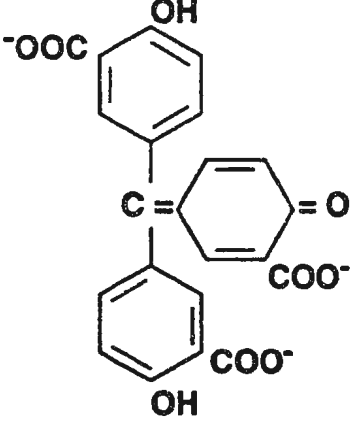
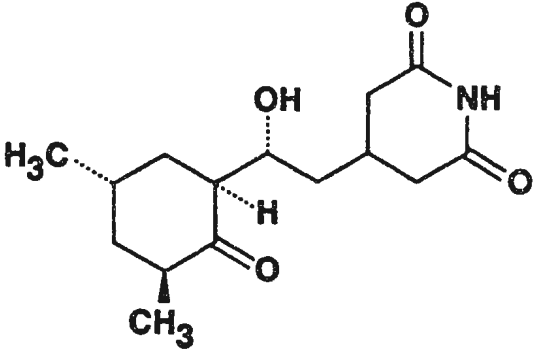
Nifedipine (Ares, et al., 1997)	Ca <sup>2+</sup> channel blocker	Anti-arrythmic and vaso-dilating agent
N-Acetyl-L-cysteine (Ferrari et al., 1995)	Mucolytic agent and thiol-based antioxidant	Inhibitor of HIV replication
Pargyline (Malorni et al., 1998; Tatton, et al., 1994)	MAO inhibitor	endonuclease inhibitor
3-Aminobenzamide (Malorni et al., 1995)	Inhibitor of poly-(ADP-ribose) polymerase (PARP)	Anti-inflammatory agent

Preliminary results of this screening test showed that only aurin tricarboxylic acid (ATA) was able to inhibit the morphology of PCD (considered a universal criterion for the identification of apoptosis) and BCD. Pretreatment of cells with ATA was able to completely inhibit the morphology of sanguinarine induced apoptosis, while the morphology of BCD was inhibited by about 30-40%. These preliminary data from light microscopic analysis were confirmed with more detailed study using a host of standard methods: quantitative light microscopy; electron microscopy for a more detailed study of certain organelles; “TUNEL” (TdT mediated dUTP nick end labelling) assay for DNA nicking; annexin V assay (by fluorescence microscopy and flowcytometric analysis) for the “phosphatidyl serine” (PS) flip;  $^{51}\text{Cr}$  release assay for membrane permeability; trypan blue exclusion assay for cell viability; flowcytometry studies for DNA content; oxygen consumption studies for cellular activity and mitochondrial function. As with ATA, these studies were also conducted with cycloheximide (see table 3 for structure) pretreatment. This was done regardless of the fact that CHX pretreatment was unable to inhibit sanguinarine induced PCD and BCD at the preliminary screening. Below-mentioned are the 4 primary reasons for undertaking experiments with CHX pretreatment parallel to that of ATA.

- 1). To confirm the results of the preliminary screening, that CHX, in fact does not inhibit the PCD nor BCD of sanguinarine.
- 2). To ascertain whether *de novo* protein synthesis was required for either modality of cell death as CHX was a proven inhibitor of apoptosis with compelling evidence of this in literature (Liu et al., 1994; Yanagihara and Tsumuraya, 1992).

- 3). To unveil even a subtle inhibition of sanguinarine induced PCD and/or BCD, despite not showing any inhibition in the screening preliminary test.
- 4). To have a protein synthesis inhibitor in CHX as a contrast to the endonuclease inhibitor ATA.

**Table 3:** Chemical structures of sanguinarine, aurin tricarboxylic acid and cycloheximide.

Name of Drug/ Chemical Compound	Structure
Sanguinarine	
Aurin tri-carboxylic acid	
Cycloheximide	

## **2.0. Materials and Methods**

### **2.1. Materials:**

The K562 erythroleukemia cell line was purchased from ATCC (Rockville, MD, USA). The drugs sanguinarine-HCl, ATA and CHX were purchased from Sigma Chemicals Co. (St. Louis, MO). Sanguinarine-HCl was 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. ATA and CHX were maintained as dry powder and were dissolved in RPMI just prior to use.

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. The “TUNEL” assay was purchased from Sigma Chemical Co. (St. Louis, MO). Both kits were stored and handled according to manufacturer’s instructions.

### **2.2. Cell culture:**

The K562 erythroleukemia cells were routinely maintained as a cell suspension in RPMI-1640 medium supplemented with 10% fetal calf serum and 2 ml L-glutamine in a humidified atmosphere containing 5% CO<sub>2</sub> at 37<sup>0</sup> C. All experiments were performed on K562 cells during their exponential phase of growth.

### **2.3. Chemical treatment:**

#### **2.3.1. Treatment of cells with sanguinarine:**

A working sanguinarine solution of 50 µg/ml was prepared from a stock solution of 1 mg/ml. 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 96 well plates. Triplicate wells for each drug concentration was prepared and aliquots of 10,000 cells (ATA pretreated, CHX pretreated or chemically untreated) per well were added. These tissue culture plates were subsequently incubated at 37<sup>0</sup> C + 5.0% CO<sub>2</sub> for two hours. Cells thus prepared were used in all subsequent experiments.

### **2.3.2. Pretreatment of cells with metabolic inhibitors:**

#### **2.3.2.1. Pretreatment of cells with ATA:**

A preliminary screening procedure was conducted in order to single out the optimum concentration and incubation period of ATA pretreatment that induced maximum inhibition of sanguinarine induced PCD and BCD. The concentrations of ATA used for this screening procedure were 50, 100 and 200 µM solutions, which were dissolved in RPMI + 10% FBS. The incubation periods for the pretreatment of cells with ATA were 2, 12, 24 and 48 hrs. These putative ATA concentrations were selected on the basis of the preliminary screening test and as per recent literature, eg. Liu et al., 1994. Since maximum inhibition of the morphology of sanguinarine induced apoptosis and BCD was achieved with pretreatment of ATA at concentrations of 100 µM for 2 hrs, all subsequent experiments were carried out under these ATA pretreatment conditions.

Thereafter, ATA pretreated cells were added to the serially diluted sanguinarine in 96 well plates, prior to a further incubation of 2 hrs. Triplicate wells of each drug concentration containing 10,000 cells per well were incubated in 37<sup>0</sup> C + 5% CO<sub>2</sub> for 2 hours. The pretreatment of cells with ATA and the subsequent treatment with sanguinarine as outlined above, were essential prerequisites for all later experiments.

#### **2.3.2.2. Pretreatment of cells with cycloheximide:**

The concentrations of CHX used were 0.1, 2.5 and 5.0 µg/ml. This was dissolved in RPMI + 10% FBS. The incubation period for the pretreatment of cells with CHX was done in four time periods; 2, 12, 24 and 48 hrs respectively. These concentrations and time periods were obtained by referring to literature (eg. Liu et al., 1994; Yanagihara and Tsumuraya, 1992) on this topic, in order to maximize the effect of CHX pretreatment. As CHX was not able to inhibit or change the PCD and BCD induced by sanguinarine in any significant way, a concentration of 2.5 µg/ml was used with a 2 hr incubation period for all subsequent experiments (the selection of a 2 hr incubation period was to be in line with a similar incubation period for ATA pretreatment).

#### **2.4. Quantitative Morphology by light microscopy:**

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 96 well plates. Cells were pretreated with ATA and CHX and exposed to sanguinarine as outlined in the “chemical treatment” section. The percentage of cell death (apoptosis or blister cell death) corresponding to each drug concentration was determined by quantitative light microscopy. Cells with the morphology of apoptosis and BCD were quantitated by hemocytometer after 2 hrs. The key morphological criterion of apoptosis was the formation of apoptotic bodies and the key criterion for blister cell death was the formation of a cell surface blister.

#### **2.5. Trypan blue assay:**

Charged cationic dye trypan blue is excluded from live cells with intact plasma membranes (Schmid et al., 1992; Schmid et al., 1994). The status of cell viability of

K562 cells exposed to ATA, CHX and sanguinarine (as described in the “chemical treatment” section) and of untreated controls were evaluated in triplicate, at 10,000 cells per well, in flat bottom 96 well plates. These experiments were conducted in triplicate for all 8 dilutions of sanguinarine. 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 were counted using a hemocytometer. The exclusion of 0.2% trypan blue was used as an indicator of the number of live cells.

## **2.6. <sup>51</sup>Cr release assay:**

Cell membrane permeability changes were evaluated by Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> release assay (Liepins et al., 1996). 2 x 10<sup>6</sup> of K562 cells were labelled with 200 µCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 90 minutes in a humidified incubator at 37°C, 5% CO<sub>2</sub>. Cells were washed three times with PBS + 2% FCS.

The changes in membrane permeability were evaluated in K562 cells pretreated with ATA and CHX, and subsequently treated with sanguinarine (as described in the “chemical treatment section), with untreated cells as controls. Experiments were conducted in triplicate at 2 x 10<sup>4</sup> cells per well and incubated (for two hours with sanguinarine) at 37°C using 96 V bottom well plates. The percentage of <sup>51</sup>Cr release was evaluated by the following formula.

% Specific <sup>51</sup>Cr release:

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

Maximum <sup>51</sup>Cr release - spontaneous release

Spontaneous <sup>51</sup>Cr release is the amount of <sup>51</sup>Cr released by cells in the absence of the drugs. Maximum Cr release is the <sup>51</sup>Cr released by exposing the cells to 1N HCl.

## **2.7. Electron Microscopy:**

Serial dilutions of sanguinarine 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. Upon exposure of cells to the appropriate concentrations of sanguinarine, ATA and CHX (as described in the “chemical treatment” section), these and untreated control K562 cells (250000 cells) were spun down in microcentrifuge tubes. Then the cells were fixed in situ, in 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% alcoholic uranyl acetate and then examined in a JEOL 100-Cx electron microscope (Liepins et al., 1976).

## **2.8. “TUNEL” assay:**

Specific 3'-hydroxyl ends of DNA fragments generated by endonuclease-mediated apoptosis are preferentially repaired by terminal deoxynucleotidyl transferase (TdT) (Raghuram et al., 1999; Gorczyca, 1993). The TdT mediated dUTP nick end labeling (TUNEL) assay has been developed to label these strand breaks by utilising streptavidin-horseradish peroxidase conjugated nucleotides followed by a substrate (TBL). This experiment was carried out using the “In Situ Apoptosis Detection Kit” (Sigma TACS TM, St. Louis, MO), according to the manufacturer’s instructions.

K562 cells (with and without pretreatment with ATA and CHX) were analyzed after two hours of incubation following sanguinarine treatment (as described in the “chemical treatment” section). Untreated K562 cells were used as negative controls.

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  $1 \times 10^6$  cells per ml., from which 50,000 cells were placed onto an electrostatically treated glass slide and air dried at room temperature. Cells were then permeabilized with proteinase K. Cell samples were then incubated for 60 minutes at 37°C in the presence and absence of exogenous TdT and incubated with streptavidin-horseradish peroxidase conjugated dNTP, 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 subsequently accounted for, to determine the percentage of TUNEL positive cells to total cells (the extent of apoptosis was determined from the appearance of labelled apoptotic bodies).

### **2.9. Fluorescein-conjugated annexin V binding assay:**

The annexin V binding status was measured by immunofluorescence microscopy and flowcytometry (Zhang et al., 1997; Shounan et al., 1998). After treatment with different drug concentrations (as described in the "chemical treatment" section) K562 cells ( $1 \times 10^6$  cells) were washed with PBS and incubated with annexin v-fluorescein isothiocyanate (FITC) conjugate and propidium iodide (PI) using an annexin v-Fluos staining kit (Boehringer Mannheim Co.) After labelling, cells were resuspended in binding buffer and analyzed using fluorescence microscopy and FACS analysis. FITC-fluorescence was measured at 530-545 nm and fluorescence of DNA-PI complexes at 575-606 nm. Cell debris were excluded from analysis by appropriate forward light scatter threshold setting. Four quadrants of the cytograms were set, using negative controls. Proportions of cells in each gradient were expressed as the percentage of the total

population. The relative cell numbers which showed early apoptotic changes in the form of annexin V positive and PI negative cells were shown in a graph.

#### **2.10. DNA content assay utilizing flow cytometry:**

The DNA content of K562 cells following drug exposure (ATA, CHX, and sanguinarine) was analysed using propidium iodide (PI) and flow cytometry (Liepins et al., 1996). For this purpose  $0.5 \times 10^6$  ATA and CHX pretreated K562 cells per well, were aliquoted in 12-well plates and exposed to eight serial dilutions of sanguinarine ranging from 25 µg/ml down to 0.19 µg/ml for 2 hrs at 37°C. After this time period each well received 2.0 ml PBS buffer containing 2.0% formaldehyde and cells were transferred to 12 × 75 mm tubes, pelleted by centrifugation and the medium removed. Cell pellets were treated with 200 µl of ice cold ethanol for 10 minutes and washed twice in PBS buffer and treated with RNAase (100 µg/ml) for 30 minutes at 37°C, followed by the addition of 200 µl of propidium iodide (50 µg/ml in 0.6% NP-40 detergent). The fluorescence intensity of cells was analysed in a Becton Dickinson flowcytometer. For each treatment, background fluorescence (endogenous fluorescence of sanguinarine) was subtracted from mean fluorescence to determine the mean DNA content of cells.

#### **2.11. Oxygen consumption measurements:**

Oxygen consumption by K562 erythroleukemia cells under experimental and control conditions was measured polarographically using Clark type YSI 5331 oxygen sensors in water jacketed, continuously stirred, chambers (model 5301) and digital amplifiers (model 16582, Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio) as described by Liepins and Hennen, 1977 (Liepins and Hennen, 1977; Liepins and Jayasinghe, 1985). The experimental cells were pretreated with ATA and CHX (as

outlined earlier) and treated with sanguinarine at 1.5 µg/ml and 12.5 µg/ml only inducing the morphologies of PCD and BCD, respectively. Temperature in the sample chambers was regulated by a continuous flow water bath Poly Temp System (Polyscience, model 80). The oxygen sensors were calibrated at various temperatures with oxygen saturated culture medium at the appropriate temperature. Oxygen measurements were carried out on 2 ml volumes of medium containing  $1 \times 10^6$  K562 cells/ml. Oxygen consumption was continually monitored and recorded using two-channel linear recorders. The relative oxygen consumption in experimental and control cells were calculated and expressed as arbitrary units.

### **3.0. Results**

As described in section 1.12. (objective of present study) a preliminary screen was conducted to identify the chemical compounds, that could inhibit PCD and BCD induced by sanguinarine in K562 cells. The rationale for selection of these chemical compounds was based on their capacity to inhibit apoptosis, as reported in literature. It was also described earlier that results of this preliminary screening showed that ATA inhibited PCD almost completely and BCD by 30-40%. Among the drugs that failed to inhibit PCD and BCD were the following: Cyclohexamide, Actinomycin D, Cyclosporine A, Glutathione, Butylated hydroxy toluene, Dithiothreitol, Mepacrine, Verapamil, Nifedipine, N-acetyl-L-cysteine, pargyline and 3 Aminobenzamide. The results documented below pertains to sanguinarine induced PCD and BCD as well as the effects of ATA and CHX pretreatment on both these forms of cell death.

#### **3.1. Light Microscopy and Quantitative Morphology:**

When K562 cells were treated with sanguinarine at concentrations of 1.5  $\mu\text{g/ml}$  for 2 hours, the characteristic morphology of apoptosis (formation of apoptotic bodies) was evident in >98% of cells [fig. 1A(b)]. Fig. 1A(c) shows the typical morphology of blister cell death (the formation of frequently one but rarely two blisters) in >95% of cells, when exposed to sanguinarine concentrations of 12.5  $\mu\text{g/ml}$  for 2 hours. The percentage of PCD and BCD was determined by quantitating apoptotic and blister cells using the following morphological criteria: formation of apoptotic bodies for the former and single blister formation for the latter.

Pretreatment of cells with ATA resulted in a complete (~100%) inhibition of the apoptosis [fig. 1B(b)] induced by sanguinarine and a reduction of BCD by about 30-40%

[fig. 1B(c)]. As shown in fig. 1B(a), ATA controls appear not to affect the morphology of K562 cells in any measurable way.

In contrast to ATA pretreatment, the pretreatment of CHX failed to inhibit the apoptosis and the blister cell death induced by sanguinarine [figs. 1C(b) and 1C(c)]. Cells treated with CHX only (CHX controls) did not show any changes, as shown in fig. 1C(a).

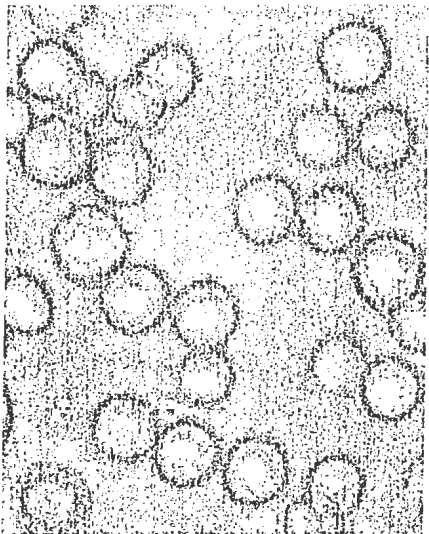
The graph of figure 1D between sanguinarine concentrations inducing PCD and BCD in K562 cells and the number of cells undergoing both these forms of cell death, summarizes the above findings.

**Figure 1 - Light Microscopy and Quantitative Morphology:**

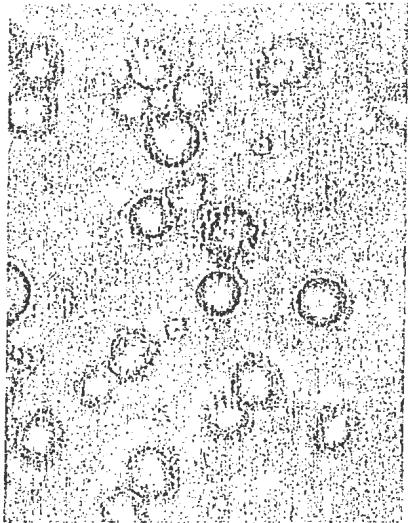
**Figure 1A:** Light microscopy pictures of cells treated with sanguinarine.

- a) Control untreated cells.
- b) Apoptosis induced by sanguinarine.
- c) Blister cell death induced by sanguinarine.

a



b



c

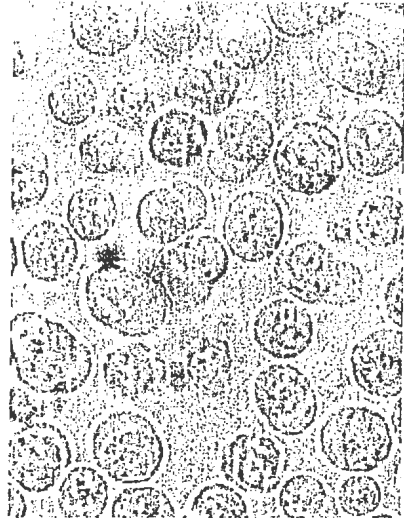
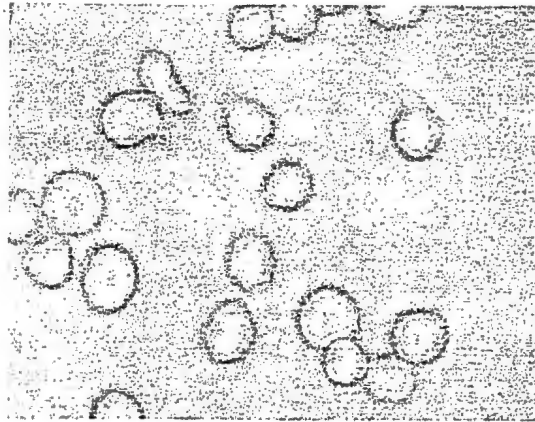
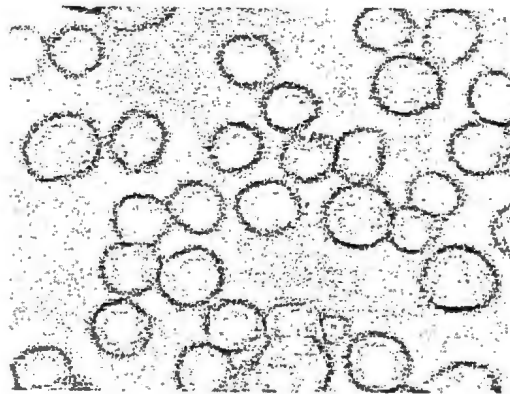


Figure 1B: Treatment of cells with sanguinarine and ATA.

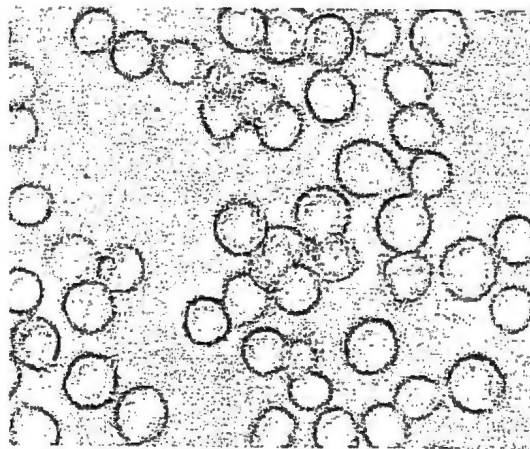
- a) Cells treated with ATA only (ATA controls)
- b) Cells treated with ATA and sanguinarine (at concentrations that induced apoptosis in K562 cells). ATA pretreatment completely inhibited the apoptotic process.
- c) Cells treated with ATA and sanguinarine (at BCD inducing concentrations in K562 cells). ATA pretreatment inhibited sanguinarine induced BCD by 30-40%.



c



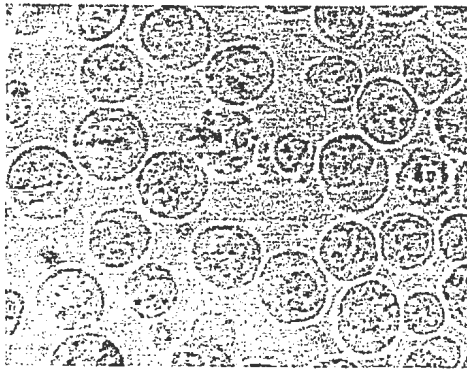
b



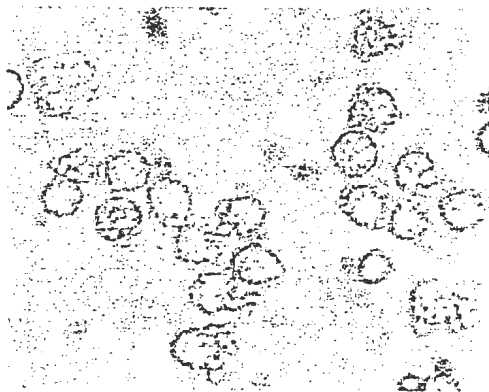
a

Figure 1C: Treatment of cells with sanguinarine and CHX.

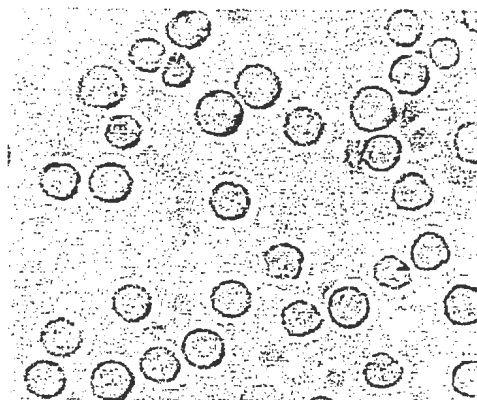
- a) Cells treated with CHX only (CHX controls).
- b) Apoptosis induced by sanguinarine but with CHX pretreatment. Pretreatment with CHX failed to inhibit sanguinarine induced apoptosis.
- c) Blister cell death induced by sanguinarine with CHX pretreatment. Pretreatment with CHX failed to inhibit BCD.



c

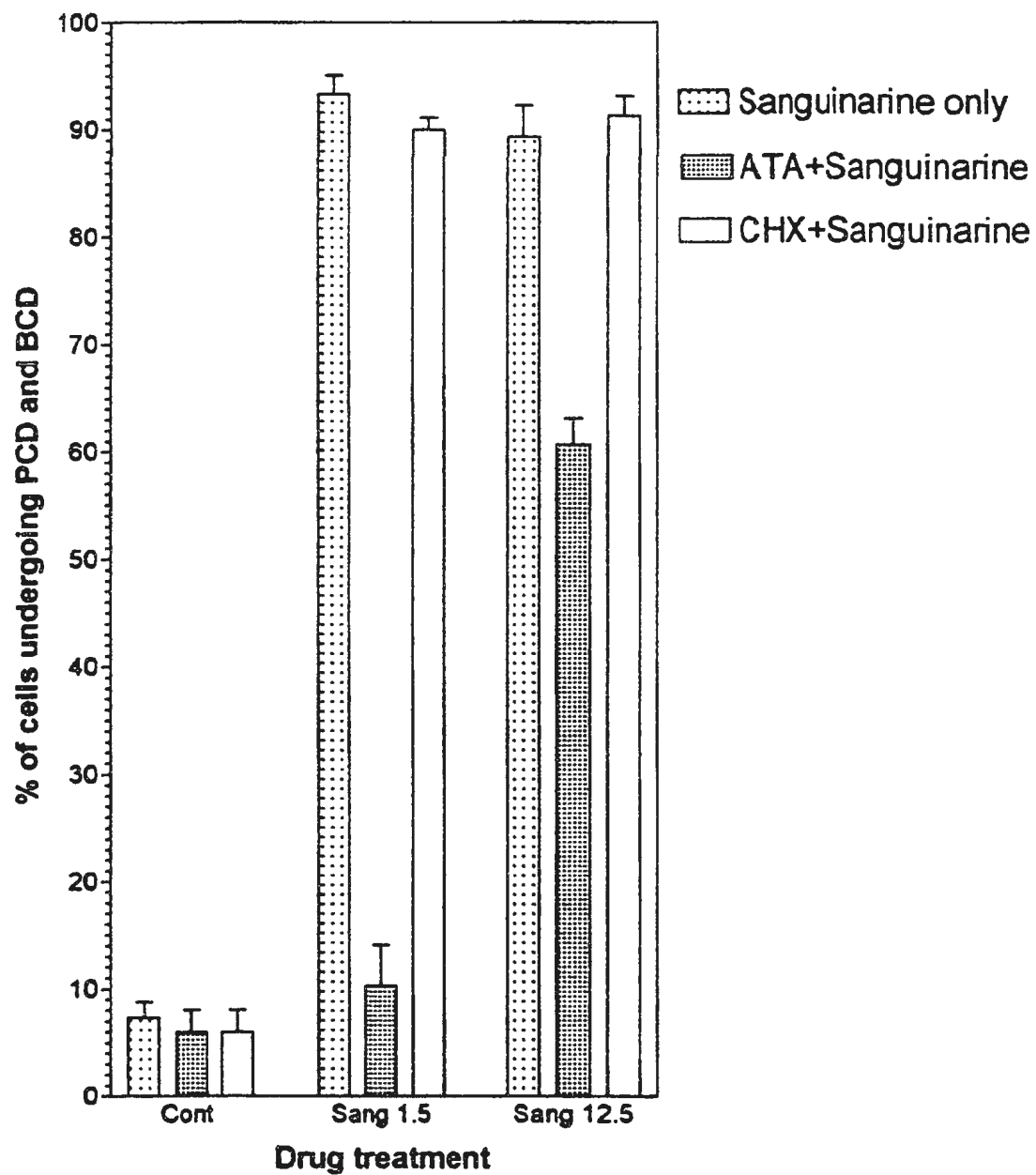


b



a

Figure 1D: The graph of figure 1D between sanguinarine concentrations that induce PCD and BCD in K562 cells and the number of cells undergoing both these forms of cell death, summarizes the above findings in quantitative morphology. Results show that while ATA pretreatment inhibited the morphological characteristics of PCD and BCD (albeit, to different degrees), CHX pretreatment failed to do so.

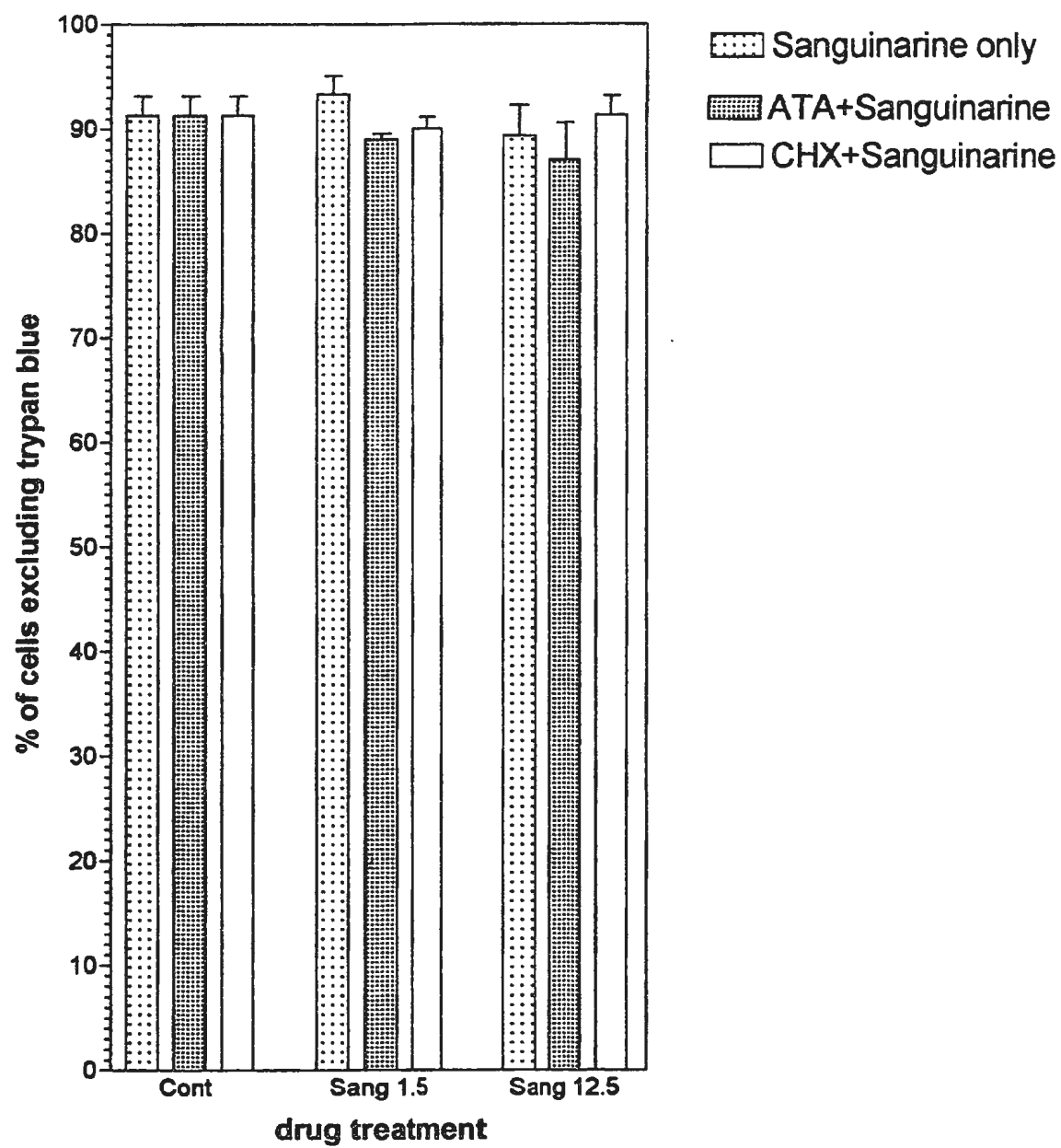


### **3.2. Trypan Blue Exclusion Assay:**

The trypan blue exclusion assay was performed to study cell viability. As shown in the graph of fig. 2, between the concentrations of sanguinarine and the percentages of cells excluding trypan blue, reveals that all cell samples (controls, sanguinarine induced PCD and BCD, and cells pretreated with ATA and CHX) exclude trypan blue, hence deemed as viable when assessed by this in-vitro criterion.

**Figure 2 – Trypan blue exclusion assay:**

The graph of figure 2, between the concentrations of sanguinarine treatment and the percentage of cells excluding trypan blue show that all cell samples, whether treated with sanguinarine only or pretreated with ATA or CHX excluded trypan blue.



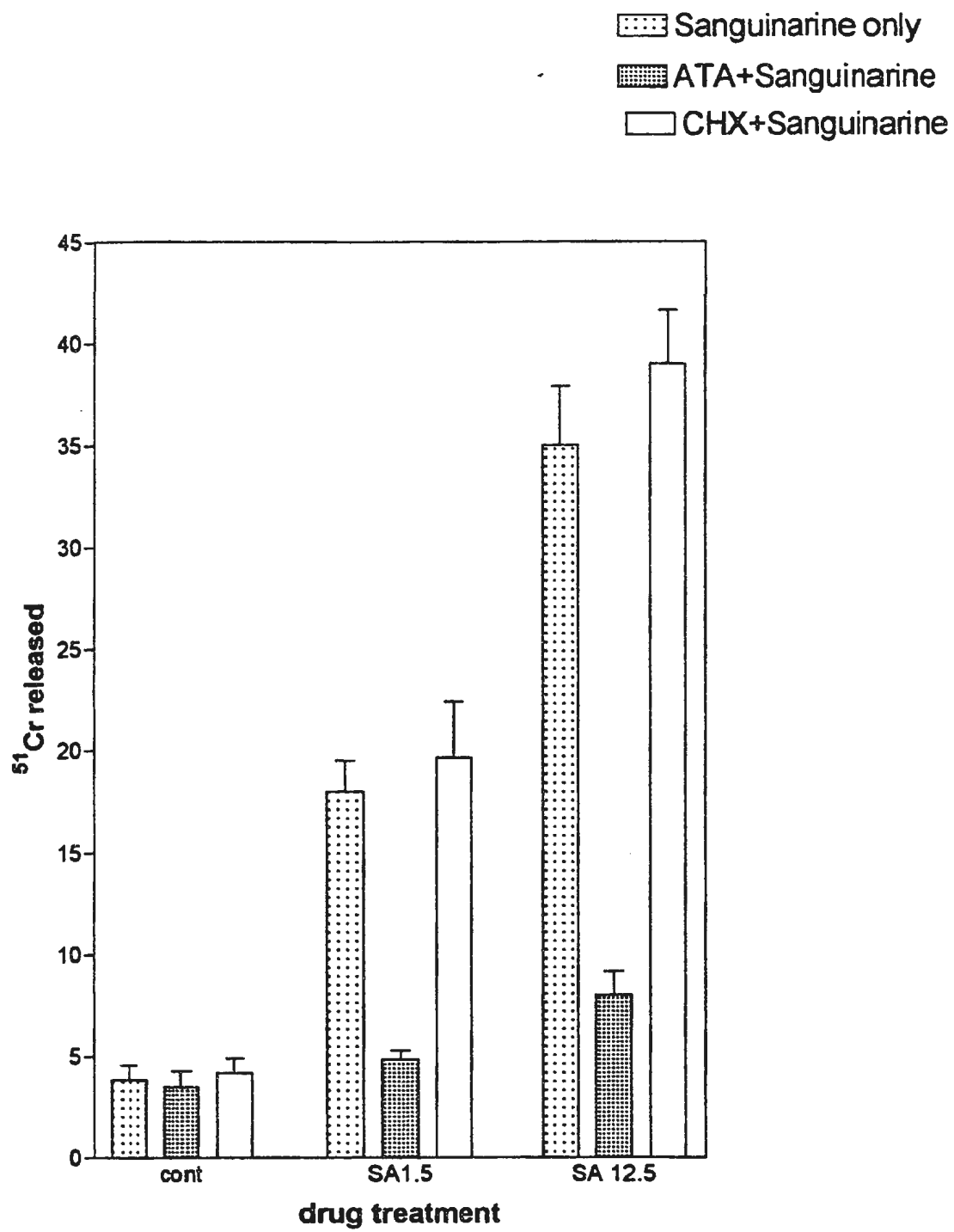
### **3.3. $^{51}\text{Cr}$ Release Assay:**

Changes in cell membrane permeability were assessed utilizing the  $^{51}\text{Cr}$  release assay.

$^{51}\text{Cr}$  release in sanguinarine induced PCD and BCD in sanguinarine treated as well as ATA and CHX pretreated cells were studied and presented in a bar graph in fig. 3. As demonstrated by the graph, the apoptotic cells showed an increase in  $^{51}\text{Cr}$  release relative to untreated control cells while cells that underwent BCD showed the highest increase. Results of cells treated with ATA and sanguinarine show a demonstrable decrease in the levels of  $^{51}\text{Cr}$  released by cells at concentrations of sanguinarine that induce apoptosis. The  $^{51}\text{Cr}$  released by treatment of cells with ATA and sanguinarine at concentrations inducing BCD too were lower as compared to those of BCD, without ATA pretreatment. ATA controls showed a level of  $^{51}\text{Cr}$  release that was comparable to that of untreated control cells. CHX pretreatment appears not to have changed the basic levels of  $^{51}\text{Cr}$  release of sanguinarine induced apoptosis and BCD.

**Figure 3 –  $^{51}\text{Cr}$  release assay:**

The graphs in figure 3 show the percentage of  $^{51}\text{Cr}$  released vs the cell samples (control, PCD and BCD) during sanguinarine treatment only and with ATA and CHX pretreatment. Results show a significant rise in  $^{51}\text{Cr}$  release during apoptosis and an even higher (and more rapid) increase during BCD in cells treated with sanguinarine only when compared with untreated control cells. ATA pretreatment appears to inhibit the  $^{51}\text{Cr}$  released in PCD as well as BCD. ATA controls (ATA pretreatment only) appear to have the same levels of  $^{51}\text{Cr}$  release as untreated controls. CHX pretreatment appears to have had no effect on sanguinarine induced PCD and BCD.



### **3.4. Transmission Electron Microscopy (TEM):**

The phenomena of apoptosis and BCD induced by sanguinarine were studied using transmission electron microscopy. Figs. 4A(a), 4A(b) and 4A(c) show electron micrographs of an untreated cell, apoptotic cell and BCD induced by sanguinarine, respectively. As shown in fig. 4A(b), morphological changes of apoptosis are evident in the apoptotic cell as compared to the normal untreated cell [fig. 4A(a)]. Condensation of the cytoplasm with a change in cell shape and size, absence of microvilli, nuclear chromatin condensation, cell shrinkage are some of the changes in apoptotic cells. Chromatin condensation is evident at the periphery of the nucleus with the areas of condensed chromatin acquiring a concave shape, with a uniform and smooth appearance. Nuclear fragmentation or karyorrhexis and the presence of apoptotic bodies are also evident in sanguinarine induced apoptosis. These apoptotic bodies contain nuclear fragments and cellular organelles. The plasma membrane also show blebbing (resembling budding in yeast) on the surface of cells. Fig. 4A(c) shows a cell that has undergone BCD. Cellular swelling and blister formation are evident in BCD, along with patchy chromatin condensation and vacuolization of the cytoplasm. The prominent single blister (rarely two) is devoid of organelles.

Figs. 4B(a), 4B(b) and 4B(c) display cells pretreated with ATA. ATA pretreatment causes a complete inhibition of the blebbing and shedding of membrane vesicles in sanguinarine induced apoptosis and a partial inhibition of single blister formation of BCD. Fig. 4B(a) shows an electron micrograph of a control cell treated with ATA only, while Fig. 4B(b) and 4B(c) show cells treated with ATA plus sanguinarine at concentrations that induce apoptosis and at concentrations inducing BCD, respectively. As evident from fig. 4B(a), ATA pretreatment alone (ATA controls) appeared to have no significant effect, as compared to untreated control cells [Fig. 4A(a)]. Fig. 4B(b) show

the inhibition of apoptosis with the absence of both apoptotic bodies and nuclear fragmentation. However, a slight increase in chromatin condensation and cytoplasmic vacuoles were observed, as compared to sanguinarine induced apoptosis. The presence of microvilli was also noted in ATA pretreated cells. Fig. 4B(c), on the other hand, show the absence of blister formation (although it should be noted that ATA pretreatment inhibited BCD only by about 30-40%). Patchy chromatin condensation and an increase in the cytoplasmic vacuoles are also seen along with an absence of microvilli.

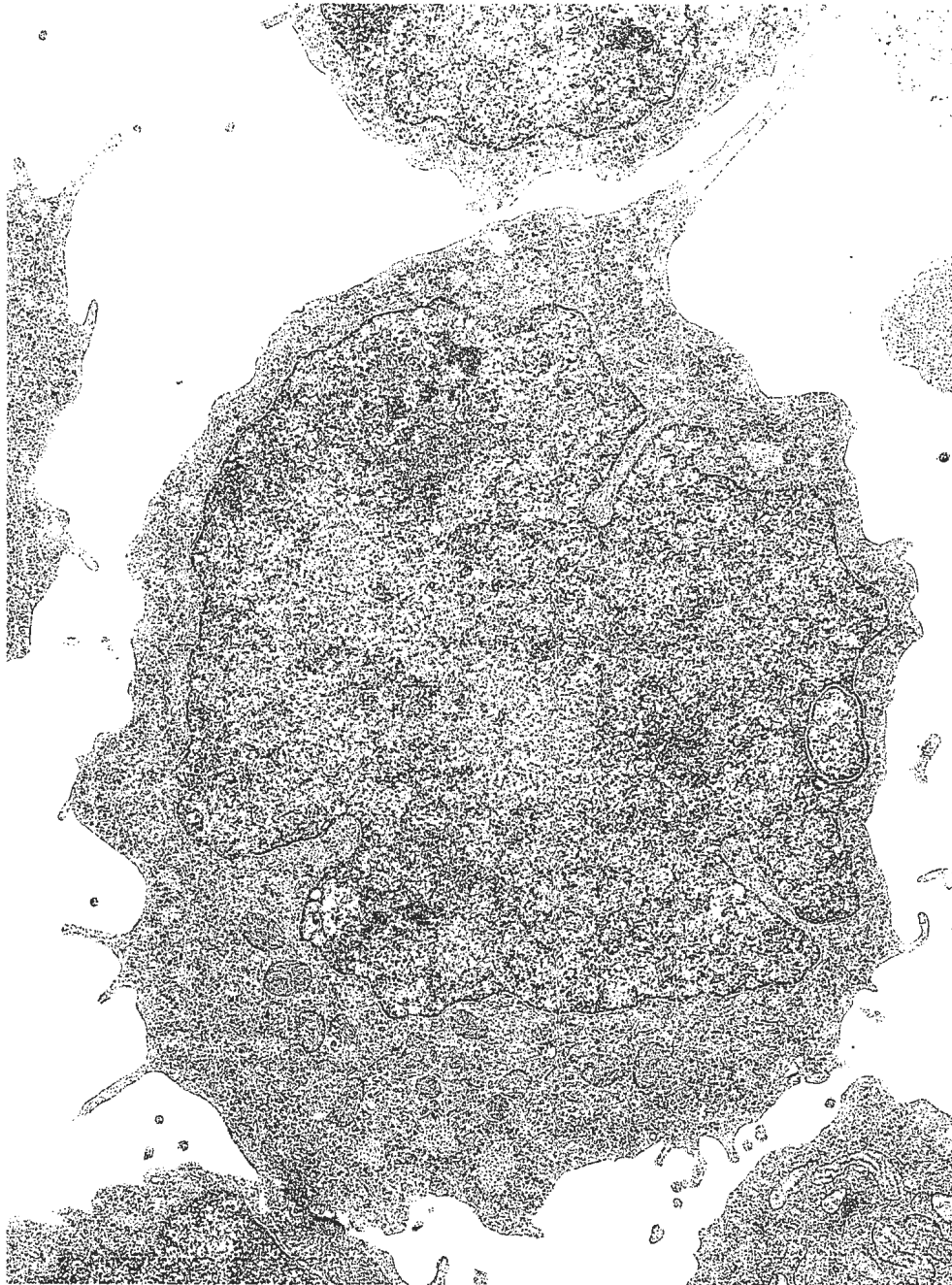
As displayed in Figs. 4C(b) and 4C(c), the pretreatment of CHX had no effect on sanguinarine induced apoptosis and BCD respectively, while fig. 4C(a) demonstrates that CHX exposure alone (CHX controls) does not lead to any significant change in cell morphology.

**Figure 4 - Electron Micrographs (magnification 3000 x 3):**

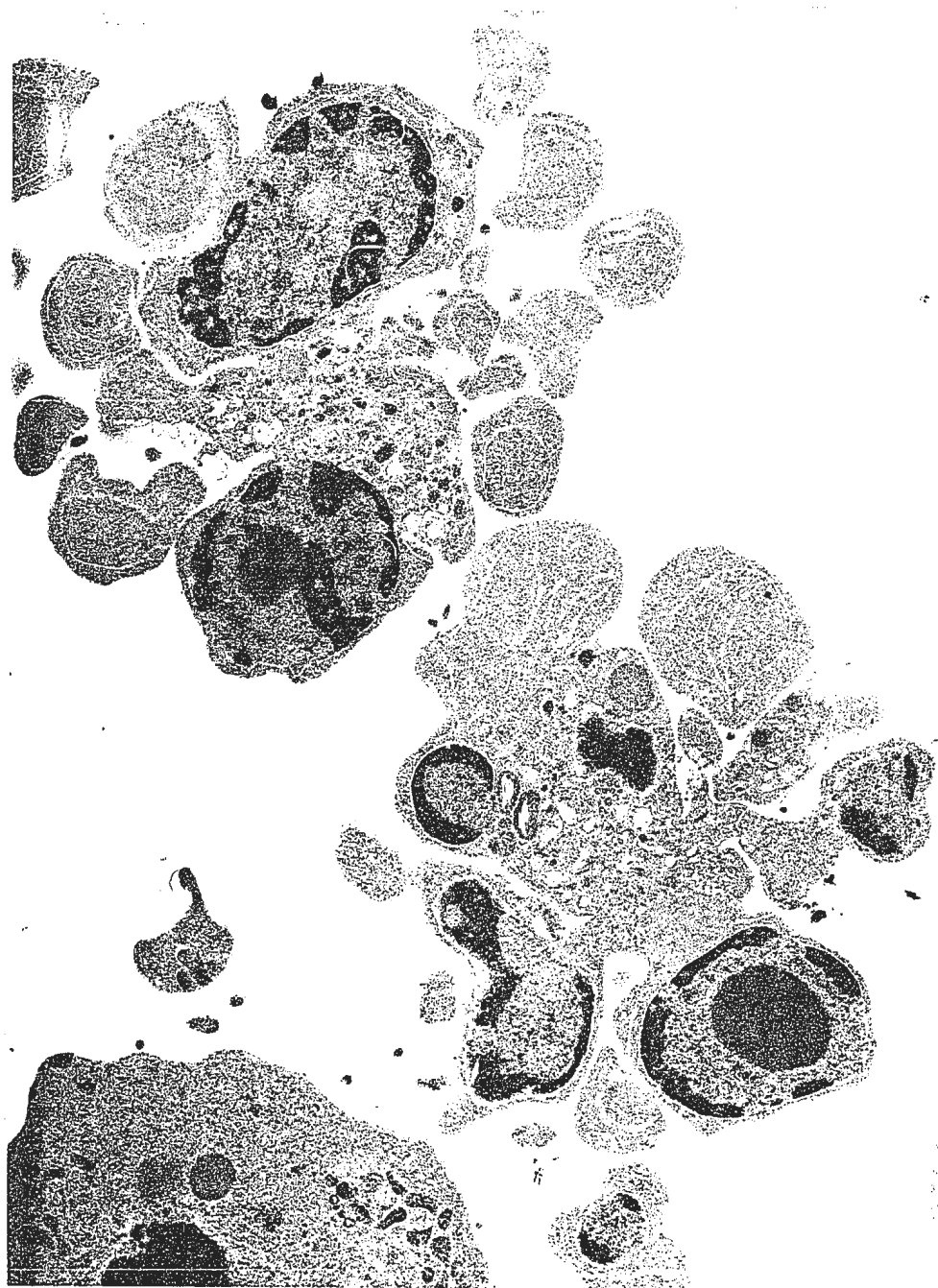
**Figure 4A:** Transmission electron microscopy pictures of cells treated with sanguinarine only.

- a) Control untreated cell
- b) Apoptotic cell induced by sanguinarine. Apoptotic bodies contain nuclear fragments, cellular organelles and concave shaped chromatin condensation. Microvilli are not present in apoptosis.
- c) Blister cell death in a cell induced by sanguinarine. The large blister is devoid of organelles. The nucleus show swelling (unlike the fragmentation of apoptosis) along with nuclear condensation. An increase in the number of vacuoles is also prominent.

(a)



(b)



(c)

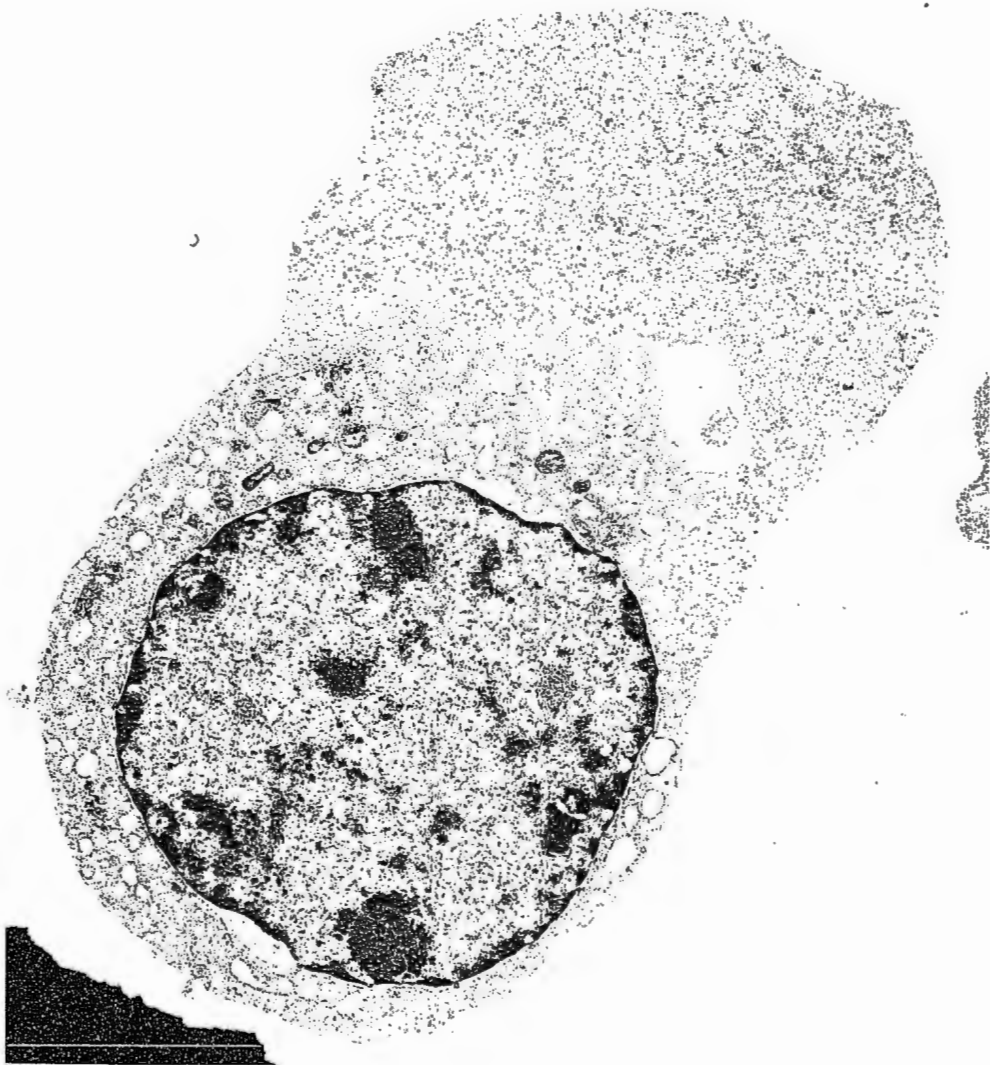
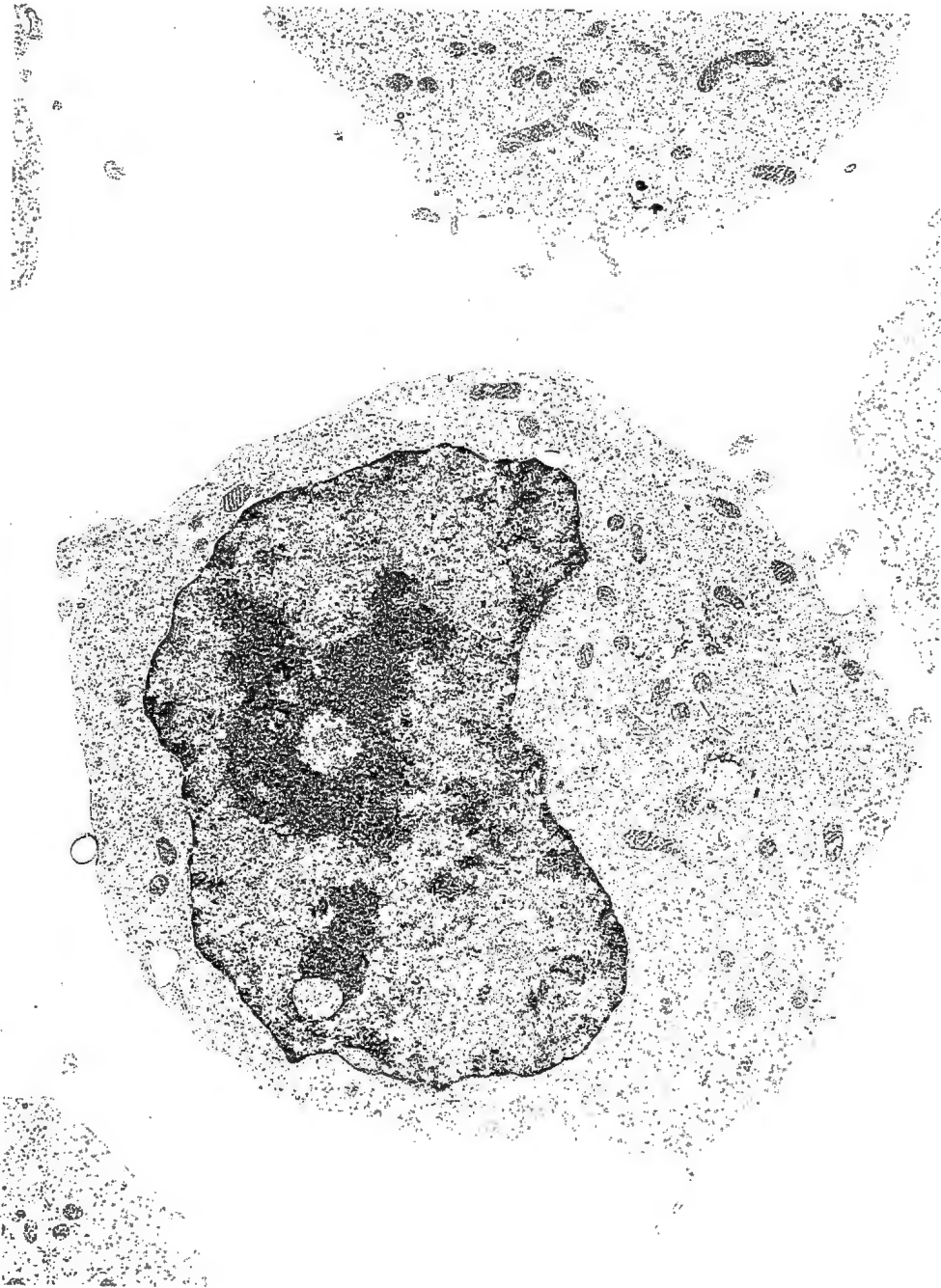


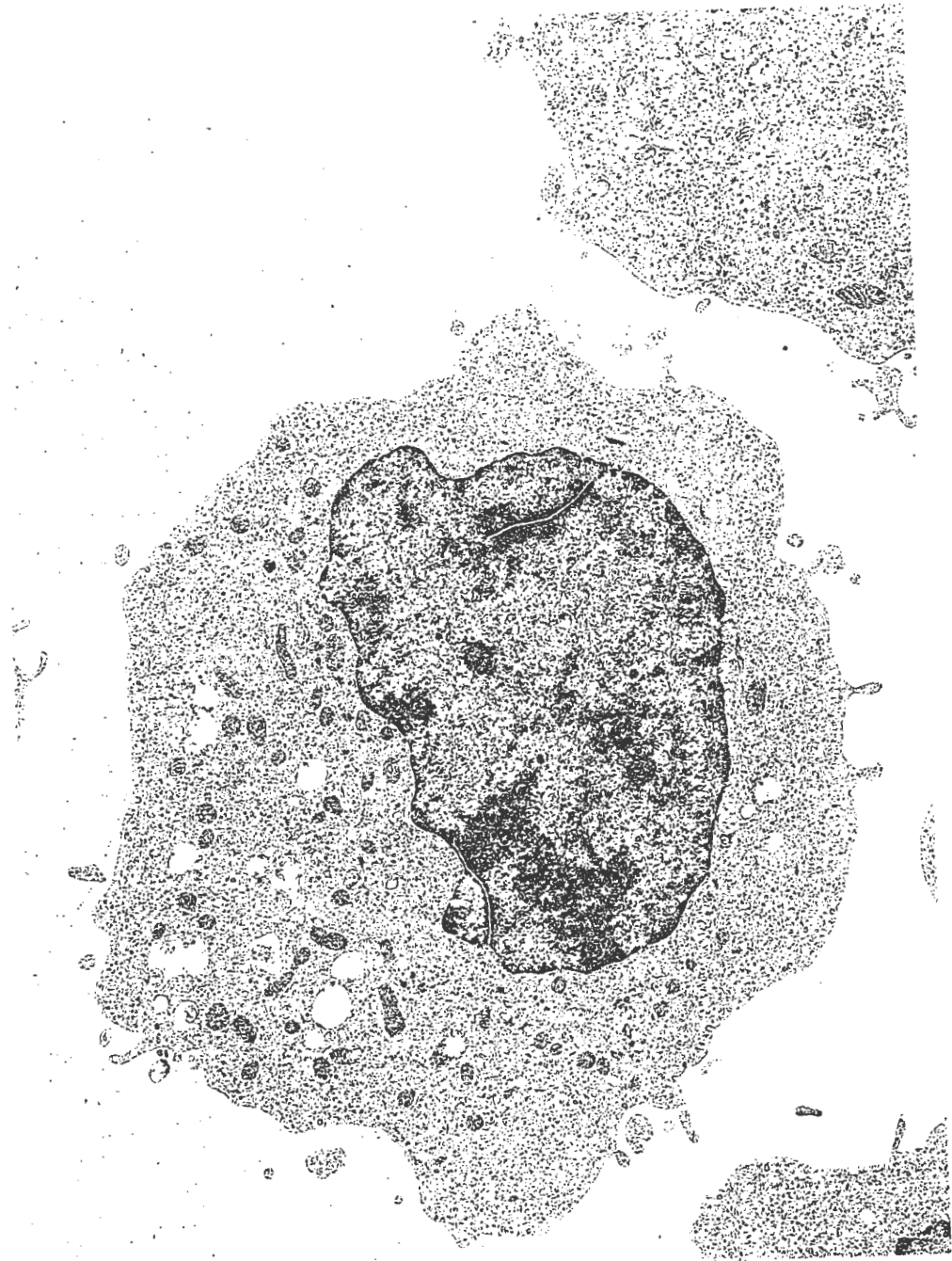
Figure 4B: Transmission electron microscopy graphs of cells treated with ATA and sanguinarine.

- a) Cell treated with ATA only. ATA pretreatment does not appear to have any significant effect on cell morphology.
- b) Cell treated with ATA and sanguinarine (at concentrations that induce apoptosis in K562 cells). Cells show an inhibition in the formation of apoptotic bodies and nuclear fragmentation.
- c) Cell treated with ATA and sanguinarine (at concentrations that induce BCD in K562 cells). The cell shows no blister formation although only 30-40% of cells displayed this inhibition. An increase in vacuoles is noted while microvilli were conspicuous by their absence.

(a)



(b)



(c)

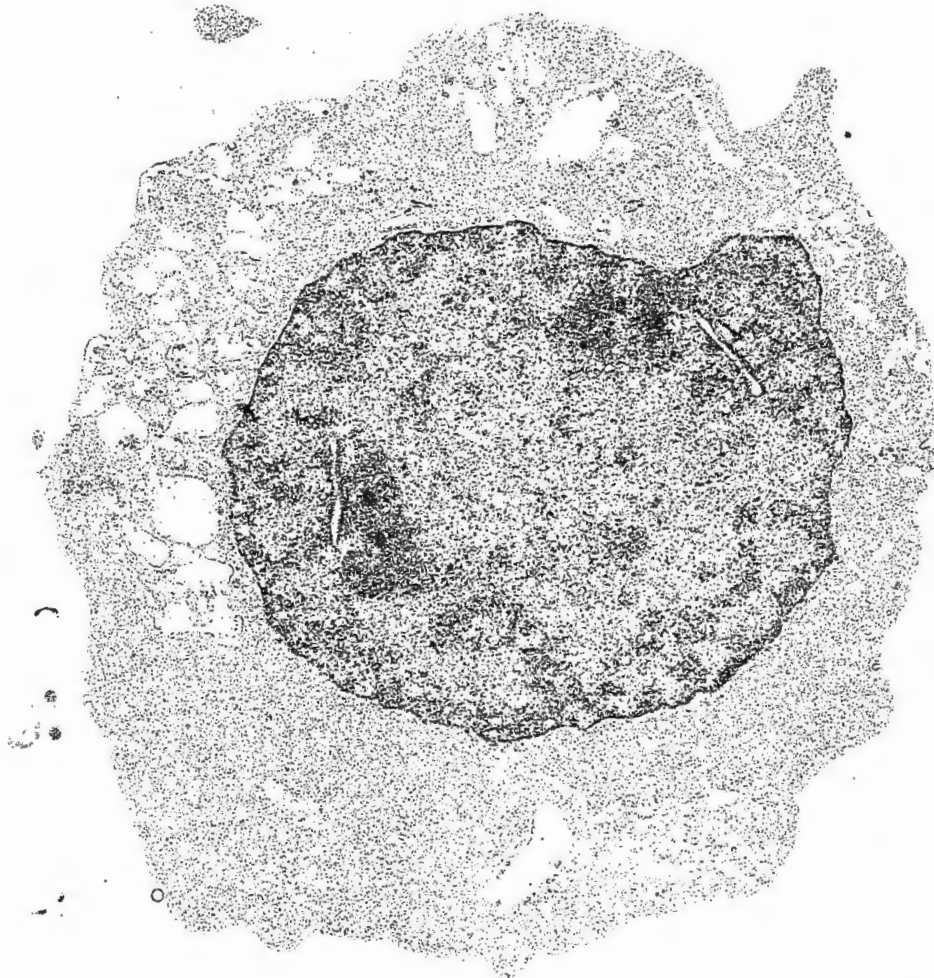
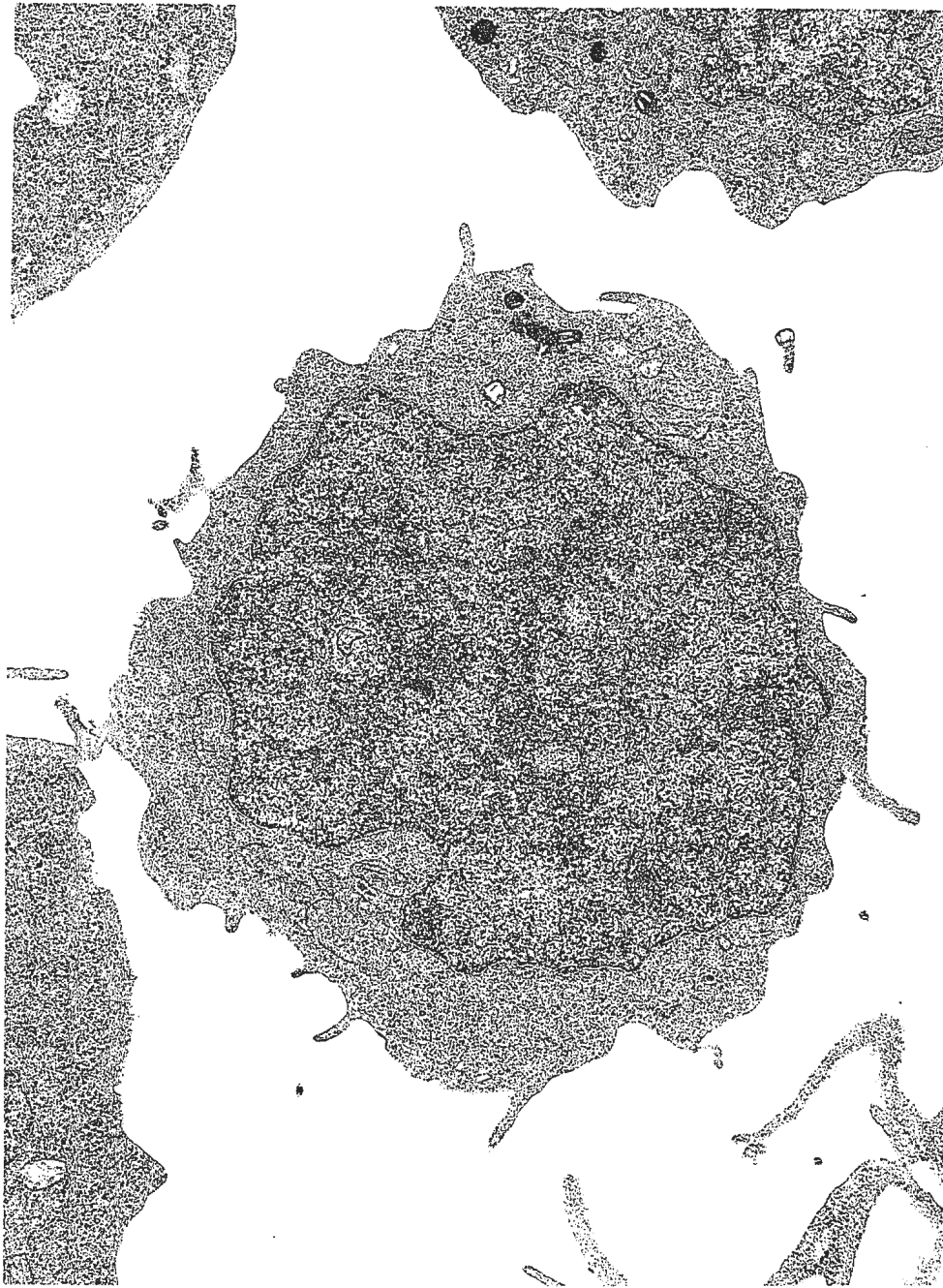


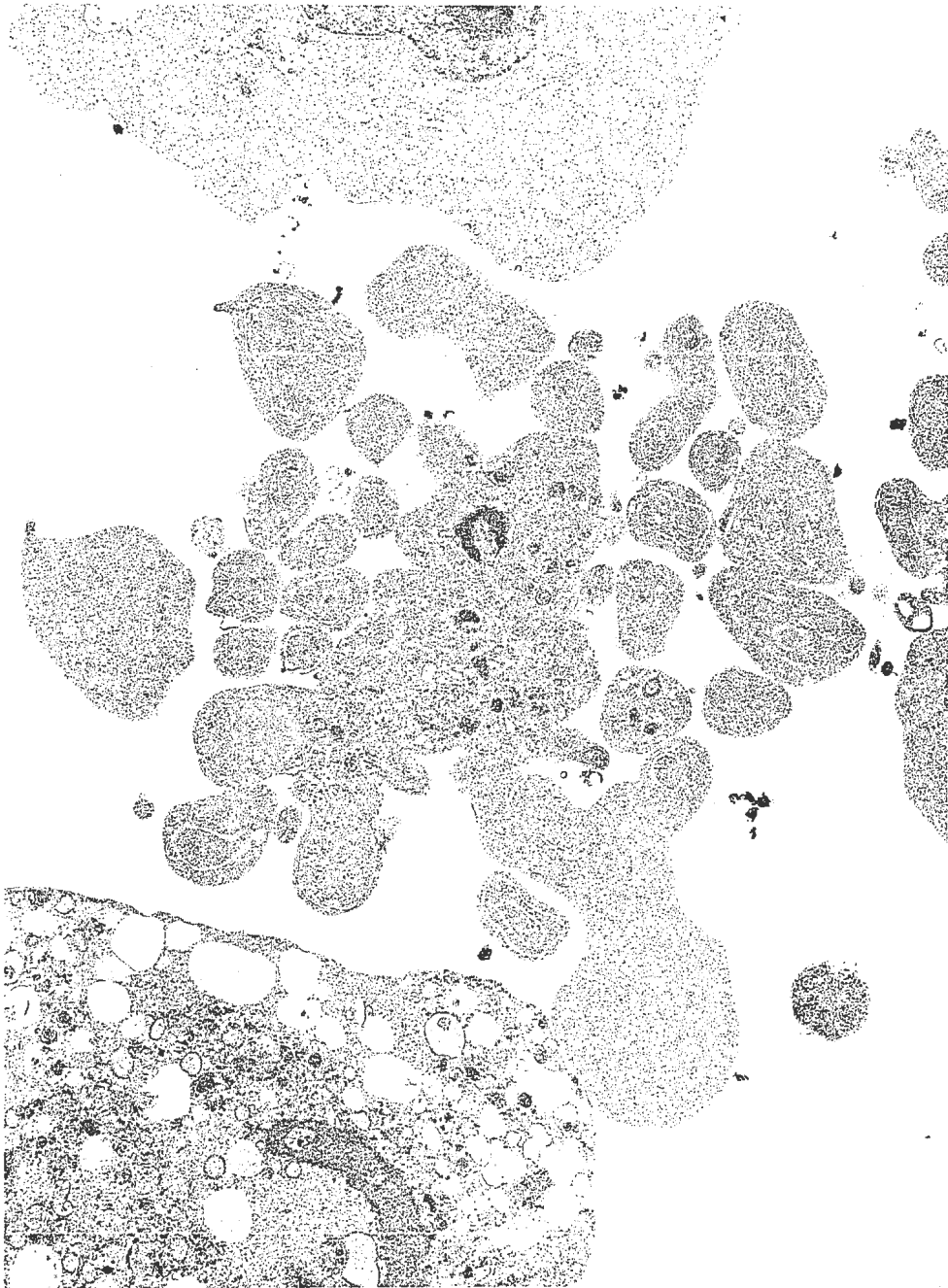
Figure 4C: Transmission electron microscopy graphs of cells treated with CHX and sanguinarine

- a) Cell treated with CHX only. CHX pretreatment appears to have no effect on cell morphology.
- b) Apoptotic cell induced by sanguinarine, pretreated with CHX. CHX pretreatment appears to have no effect on the morphology of the apoptosis of sanguinarine.
- c) BCD induced by sanguinarine, pretreated with CHX. CHX pretreatment seems to have no effect on BCD inhibition either.

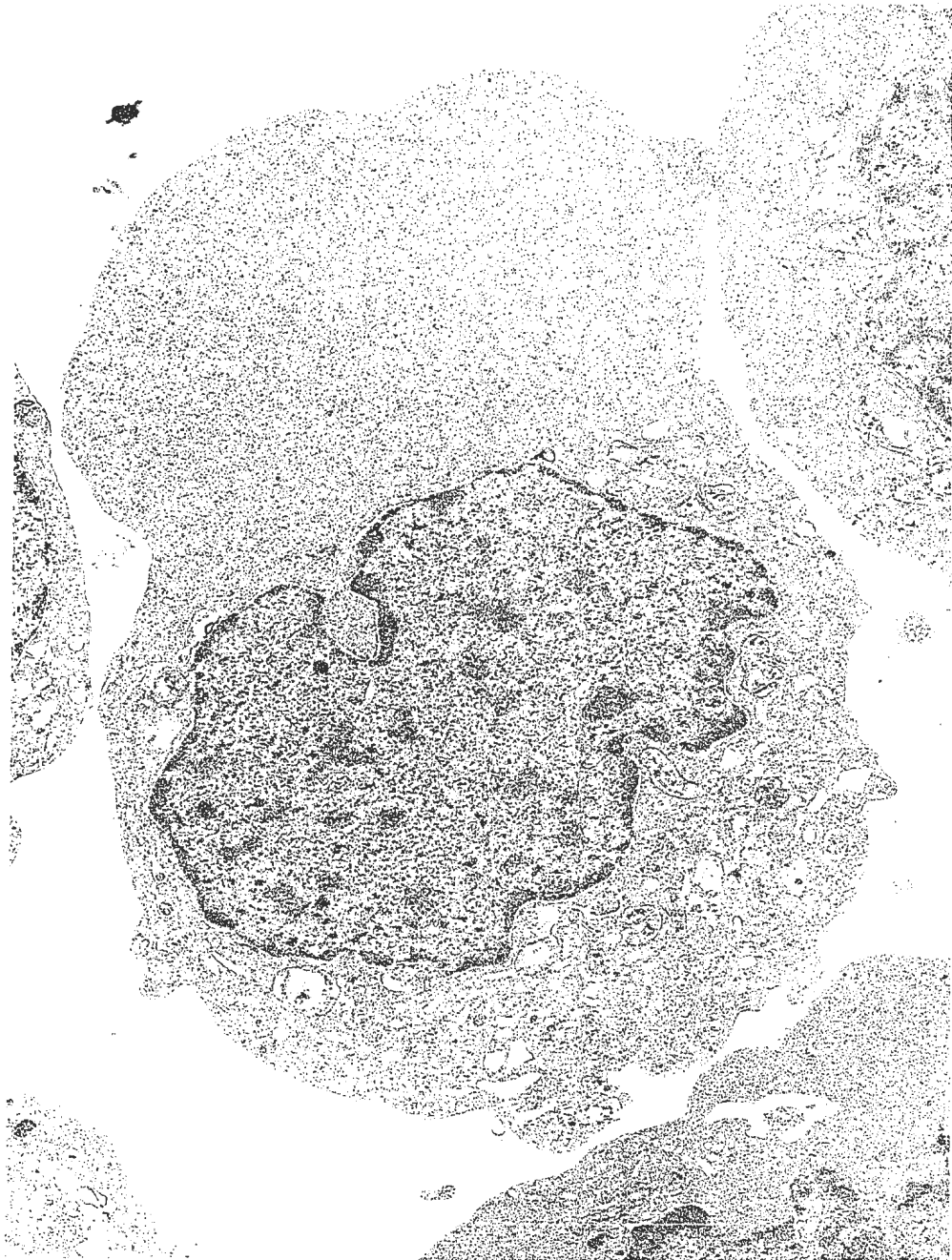
(a)



(b)



(c)



### **3.5. “TUNEL” Assay:**

The Terminal Deoxyribonucleotidyl Transferase Mediated dUTP Nick End Labelling Assay for the detection of DNA fragmentation (nicking) was done in control and sanguinarine treated cells. Figs. 5A, 5B and 5C show the results of the “TUNEL” (TdT mediated dUTP nick end labelling) assay in light micrographs. While Fig. 5A shows the results in sanguinarine induced PCD and BCD, fig. 5B shows the effects of ATA pretreatment and fig. 5C the effects of CHX pretreatment on these two forms of cell death.

Sanguinarine induced apoptosis displayed a positive “TUNEL” assay [Fig. 5A(b)] denoting DNA nicking, while, control untreated cells displayed a negative “TUNEL” assay [Fig. 5A(a)]. Fig. 5A(c) show a negative “TUNEL” displayed by sanguinarine induced BCD.

Fig. 5B(b) showed a negative “TUNEL” in cells treated with ATA and sanguinarine at concentrations inducing apoptosis, denoting an inhibition in DNA nicking of this form of apoptosis. Figs. 5B(a) and 5B(c) shows negative “TUNEL” for ATA only (ATA controls) and ATA and sanguinarine (at concentrations inducing BCD) treated cells respectively.

Figs. 5C(a) and 5C(c) display negative “TUNEL”, while the result of 5C(b) is positive. Fig. 5C(a) show CHX only (CHX controls) treated cells, while fig. 5C(b) show cells treated with CHX and sanguinarine at apoptosis inducing concentrations and fig. 5C(c) show CHX and sanguinarine at BCD inducing concentrations.

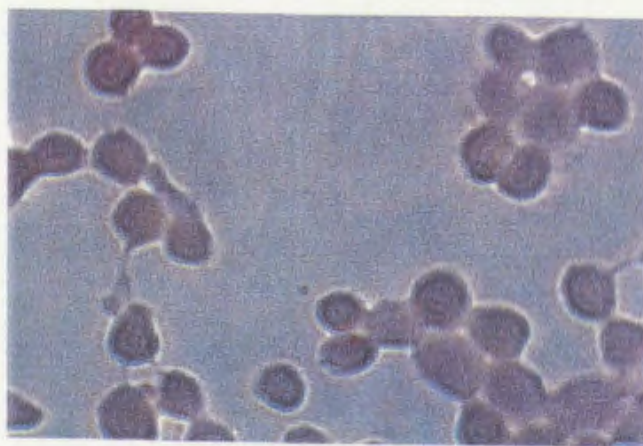
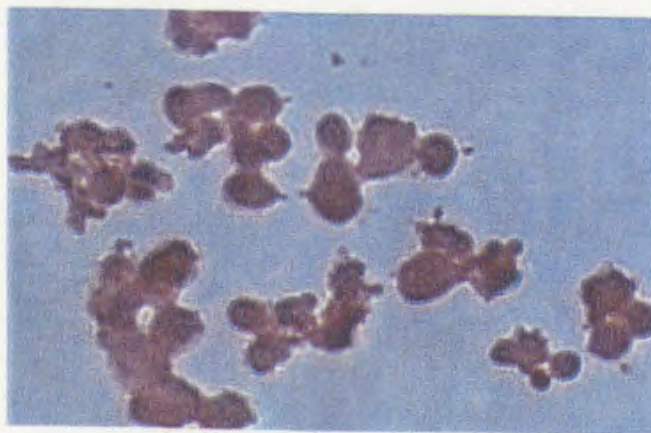
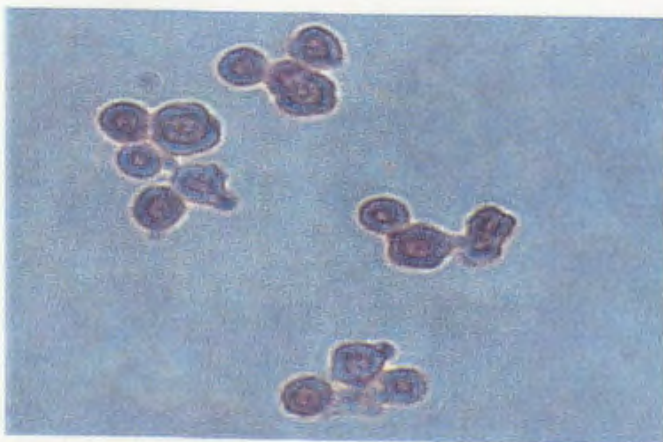
The graph of figure 5D between sanguinarine concentrations inducing PCD and BCD and the percentage of TUNEL positive cells in both these forms of cell death, summarizes the above findings.

**Figure 5 – “TUNEL” Assay:**

Light micrographs of the results of the “TUNEL” (TdT mediated dUTP nick end labelling) assay to detect DNA strand breaks. The dark spots within the cells denote a positive “TUNEL”.

Figure 5A: Pictures of cells treated with sanguinarine.

- a) Untreated control cells show negative “TUNEL”.
- b) Apoptotic cells treated with sanguinarine show positive “TUNEL”.
- c) BCD induced by sanguinarine (at 12.5  $\mu\text{g/ml}$ ) show negative “TUNEL”.



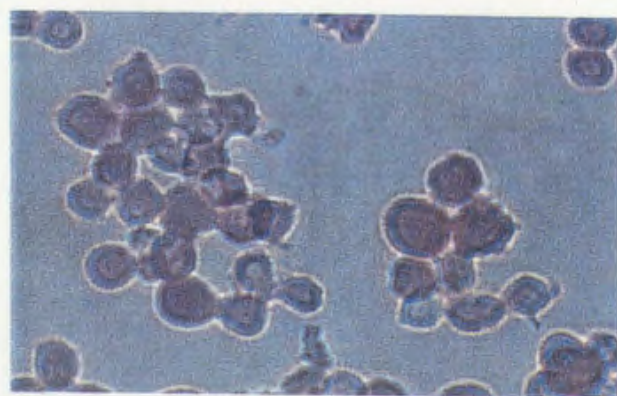
c

b

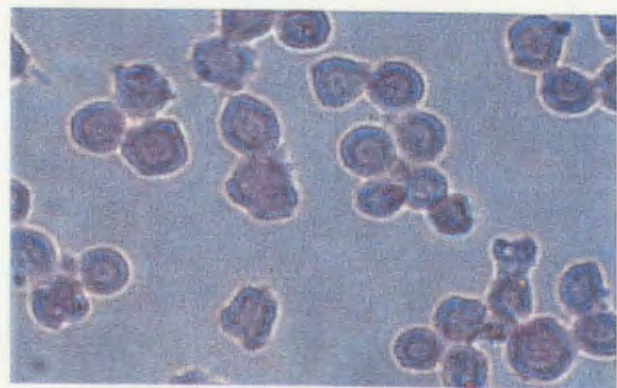
a

Figure 5B: Pictures of cells pretreated with ATA and then exposed to sanguinarine.

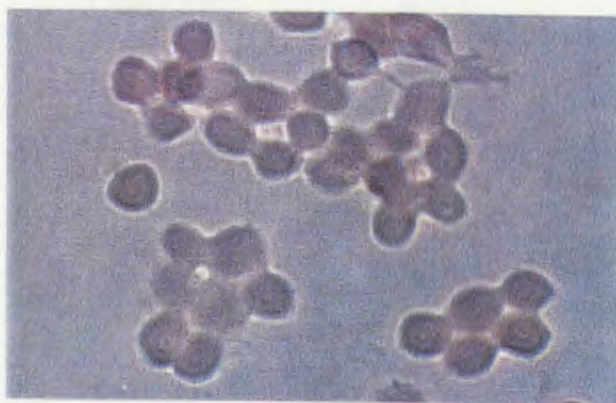
- a) Cells treated with ATA only show negative “TUNEL”.
- b) Cells treated with ATA and sanguinarine (at concentrations inducing apoptosis in K562 cells) show negative “TUNEL”.
- c) Cells treated with ATA and sanguinarine at concentrations inducing BCD show negative “TUNEL”.



c



b



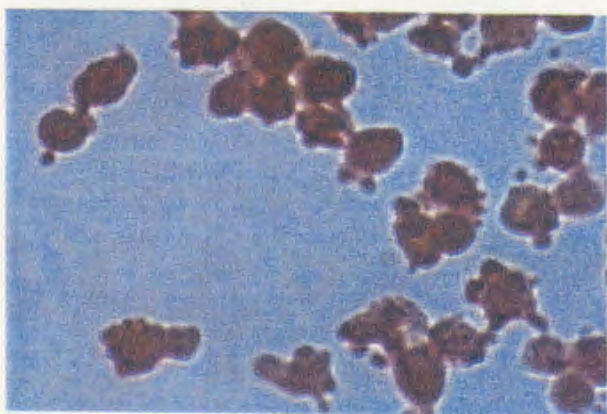
a

Figure 5C: Pictures of cells pretreated with CHX and then exposed to sanguinarine.

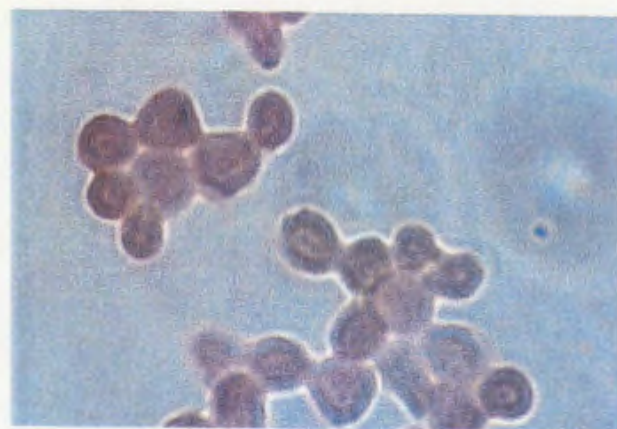
- a) Cells treated with CHX only. “TUNEL” negative.
- b) Apoptotic cells treated with CHX and sanguinarine. “TUNEL” positive.
- c) BCD cells treated with CHX and sanguinarine. “TUNEL” negative.



c

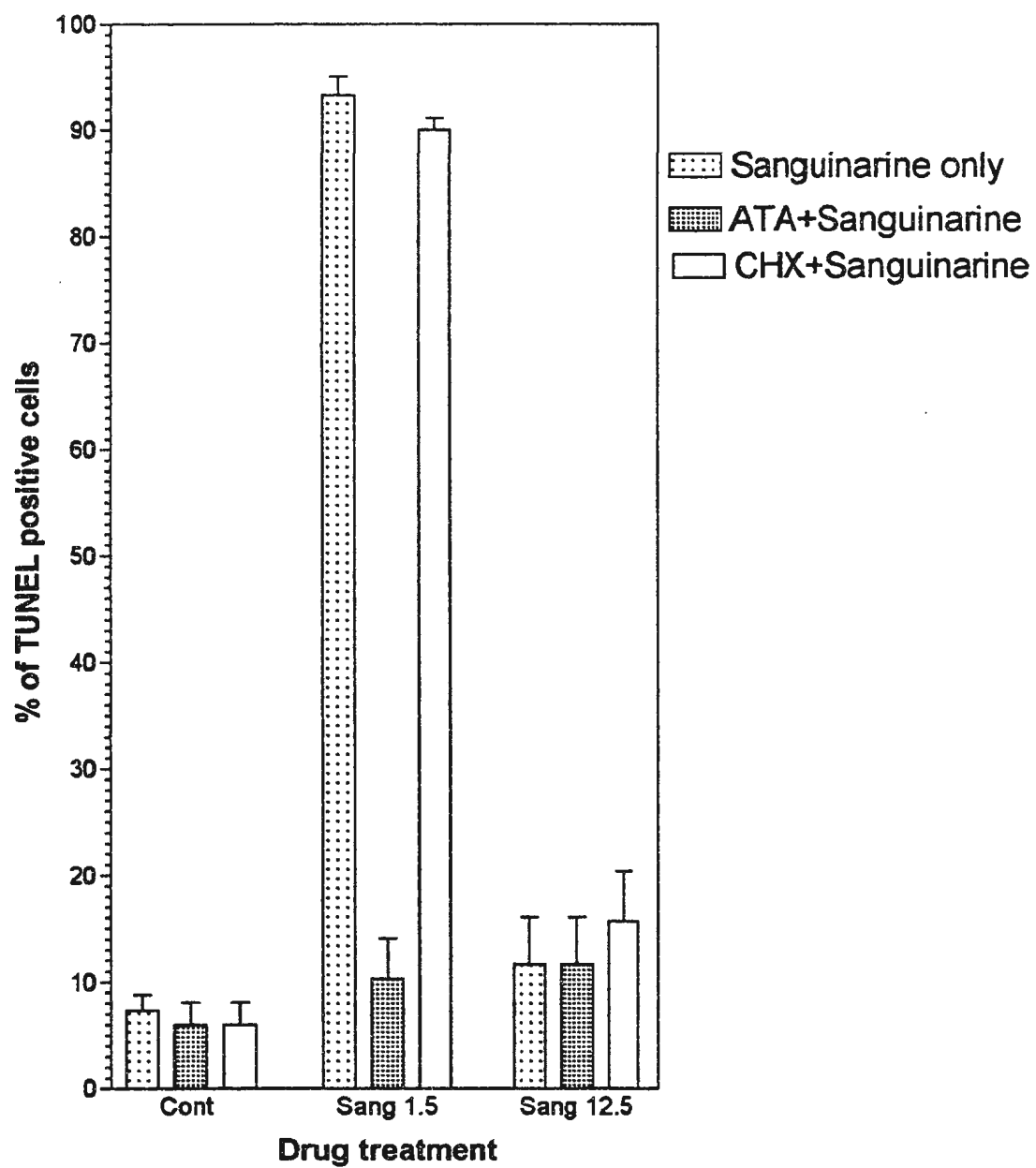


b



a

Figure 5D: The graph of figure 5D between sanguinarine concentrations that induce PCD and BCD and the percentage of TUNEL positive cells in both these forms of cell death, summarizes the above findings. Results show that while ATA pretreatment appears not to have any significant effect on the TUNEL results of BCD, it certainly shows an inhibition of DNA nicking observed during sanguinarine induced PCD. The outcome of TUNEL during sanguinarine treatment remained unchanged despite CHX pretreatment.



### **3.6. Annexin V – Fluorescence Microscopy:**

The light micrographs of Fig. 6 show the results of the annexin V assay, utilized to assess the phosphatidyl serine flip. Fig. 6A shows the results of cells that underwent sanguinarine induced cell death (PCD and BCD), Fig. 6B shows the results of cells treated with ATA and sanguinarine while Fig. 6C shows the results of cells treated with CHX and sanguinarine (the green fluorescence denotes the occurrence of the phosphatidyl serine (PS) flip, while the yellow nuclear fluorescence is due to sanguinarine).

As shown, while untreated control cells had a negative result [Fig. 6A(a)] the apoptotic cells induced by sanguinarine were positive for the phosphatidyl serine flip [Fig. 6A(b)]. Sanguinarine induced BCD showed a negative result [Fig. 6A(c)].

Fig. 6B(b) representing ATA and sanguinarine treated cells at doses of 1.5  $\mu\text{g/ml}$  (concentrations inducing apoptosis in K562 cells) did not show the PS flip. ATA controls and cells treated with ATA and sanguinarine at doses of 12.5  $\mu\text{g/ml}$  (concentrations inducing BCD in cells), shown in figs. 6B(a) and 6B(c) respectively, did not demonstrate a positive phosphatidyl serine flip.

Figs. 6C(a), 6C(b) and 6C(c) represents CHX pretreated cells. While apoptosis [fig. 6C(b)] showed a positive annexin V, BCD [fig. 6C(c)] and CHX controls [fig. 6C(a)] showed negative results.

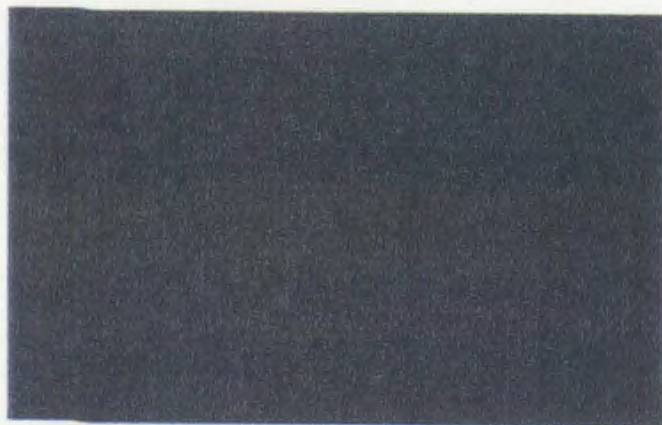
**Figure 6 - Annexin V- Fluorescence Microscopy:**

Light micrographs of the results of the annexin v assay utilized to study the phosphatidyl serine flip. The green fluorescence identifies cells that were positive for the phosphatidyl serine flip and is located on the cell surface. The bright yellow observed in some cells are the result of the fluorescence of sanguinarine in the nuclei.

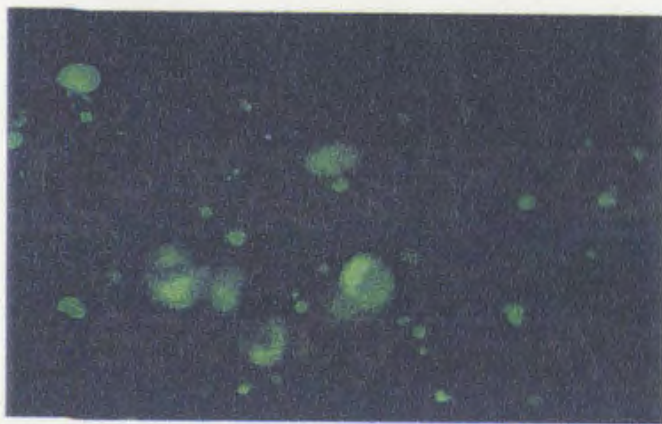
Figure 6A: Light micrographs of cells treated with sanguinarine.

- a) Untreated control cells. Negative annexin V.
- b) Cells treated with sanguinarine at 1.5  $\mu\text{g/ml}$ . Positive annexin V.
- c) Cells treated with sanguinarine at 12.5  $\mu\text{g/ml}$  for 2 hrs. Negative annexin V.

a



b



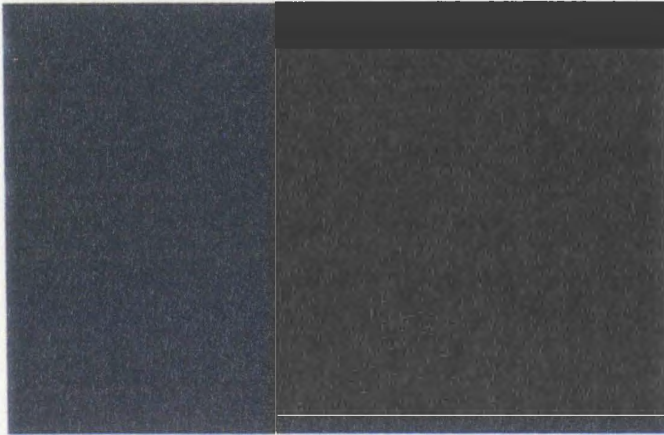
c



Figure 6B: Light micrographs of cells pretreated with ATA and then exposed to sanguinarine

- a) Cells treated with ATA only. Negative annexin V.
- b) Cells treated with ATA and sanguinarine at concentrations inducing apoptosis. Negative annexin V.
- c) Cells treated with ATA and sanguinarine at concentrations that induce BCD. Negative annexin V.

a



b



c

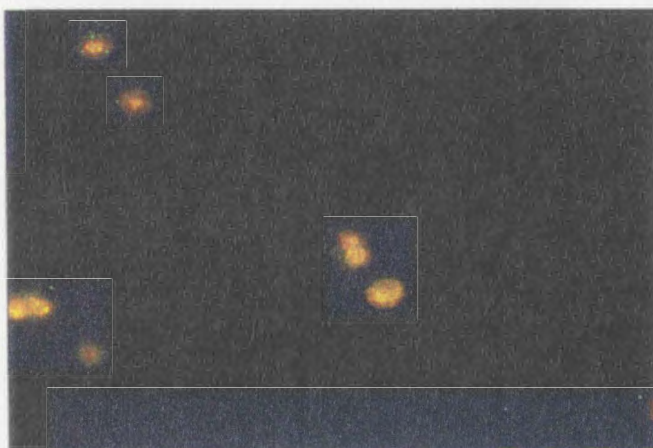
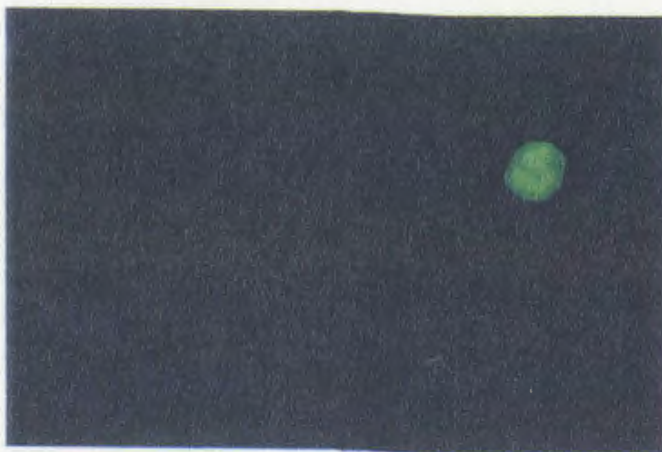


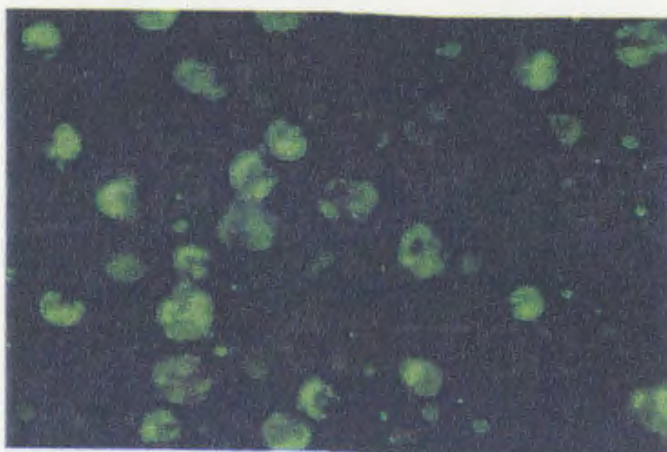
Figure 6C: Light micrographs of cells pretreated with CHX and then exposed to sanguinarine.

- a) Cells treated with CHX only. Negative annexin V.
- b) Cells treated with CHX and sanguinarine at concentrations inducing apoptosis.  
Positive annexin V.
- c) Cells treated with CHX and sanguinarine at concentrations inducing BCD.  
Negative annexin V.

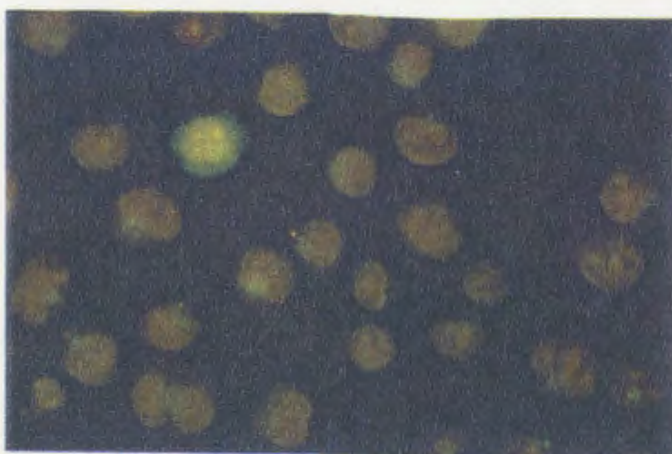
a



b



c

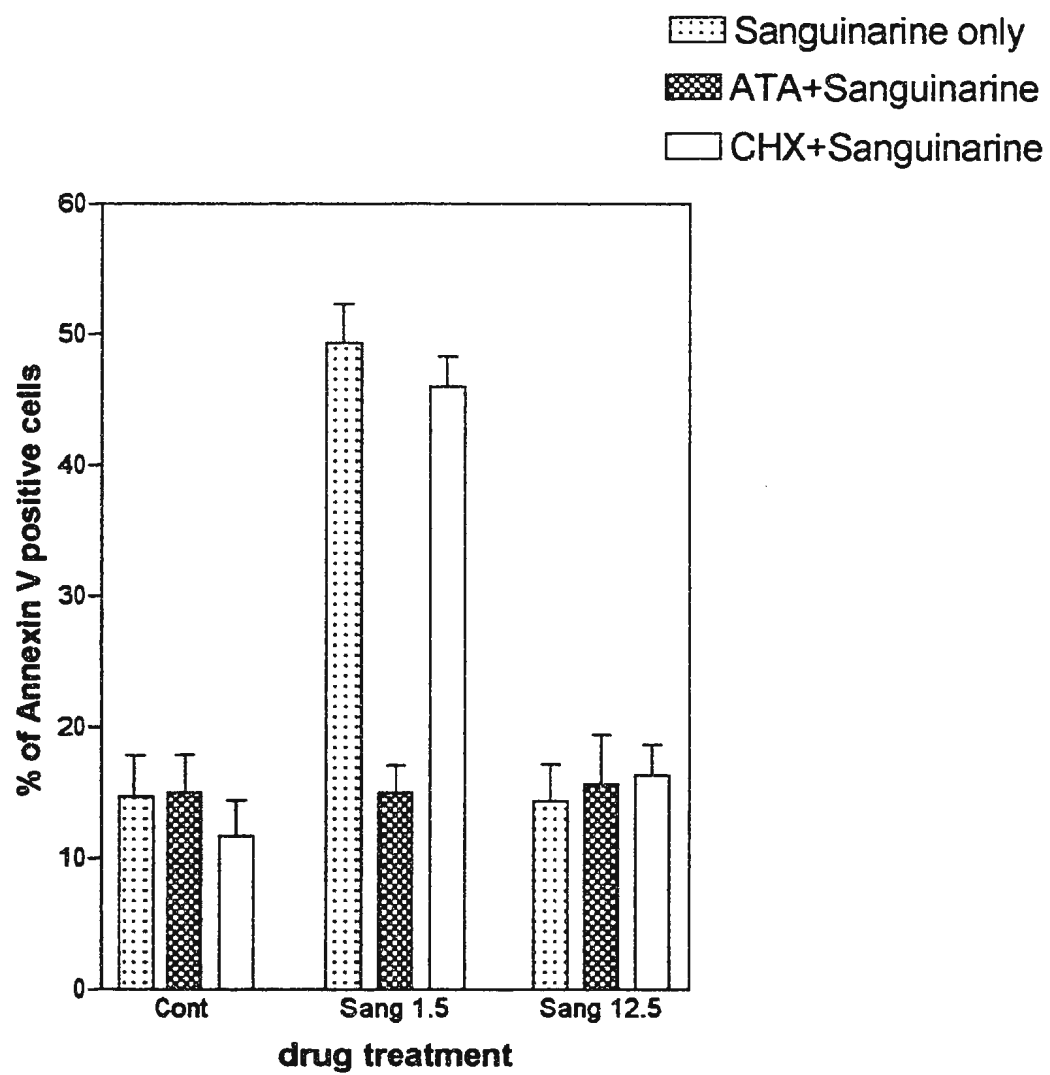


### **3.7. Annexin V Assay – Flow cytometry:**

The results of the annexin V assay were also assessed by flowcytometry. The bar graph as shown in Fig. 7 displays the percentage of annexin v binding in cells. An increase in annexin V binding was observed in the apoptosis induced by sanguinarine and also in the cells pretreated with CHX that underwent apoptosis. As shown, all other cell samples (untreated controls, sanguinarine induced BCD, ATA controls, ATA and sanguinarine treated, CHX controls and CHX and sanguinarine treated at concentrations inducing BCD) did not show any significant annexin v binding.

**Figure 7 – Annexin V-Flow cytometry:**

The graph between the percentage of annexin V positive cells and the various cell samples (control, PCD and BCD), show the quantitation of the phosphatidyl serine flip by FACS analysis using annexin V. These results confirm the above fluorescence microscopy results. The phosphatidyl serine flip was positive in apoptotic cells of sanguinarine and in apoptotic cells induced by sanguinarine but with CHX pretreatment. All other cell samples showed negative annexin V.



### **3.8. Cellular DNA Content-Flow cytometry:**

The DNA content in cells was assessed using flow cytometric analysis. The DNA histogram of Fig. 8 shows the DNA content versus the number of cells.

The DNA content of untreated control cells, apoptotic cells induced by sanguinarine and cells that underwent sanguinarine induced BCD was assessed (Fig. 8A). While apoptotic cells show no change in DNA content (comparable with control untreated cells), the cells that underwent BCD display a shift to the right denoting an apparent increase in DNA content.

A histogram reflecting the DNA content of cells pretreated with ATA and subsequently exposed to sanguinarine is shown in Fig. 8B. Results show that while ATA pretreatment had no effect on the DNA content in cells treated with apoptosis-inducing concentrations of sanguinarine, ATA pretreatment reduced the shift to the right, which was observed during BCD. The DNA content of ATA controls was comparable to that of untreated control cells.

A DNA histogram of the flow cytometric analysis of DNA content in cells pretreated with CHX and subsequently exposed to sanguinarine is shown in Fig. 8C. Results showed that CHX pretreatment had no effect on the DNA content induced by sanguinarine, i.e., while the DNA content level shifted to the right in BCD, there was no change in PCD as compared to CHX controls. CHX controls showed a DNA content level comparable to that of untreated control cells.

**Figure 8 – Cellular DNA Content – Flow cytometry:**

Flow cytometry analysis of DNA content by histogram using propidium iodide.

Figure 8A: Histogram of untreated cells and cells treated with sanguinarine. Results show increased DNA content in BCD but not in apoptosis.

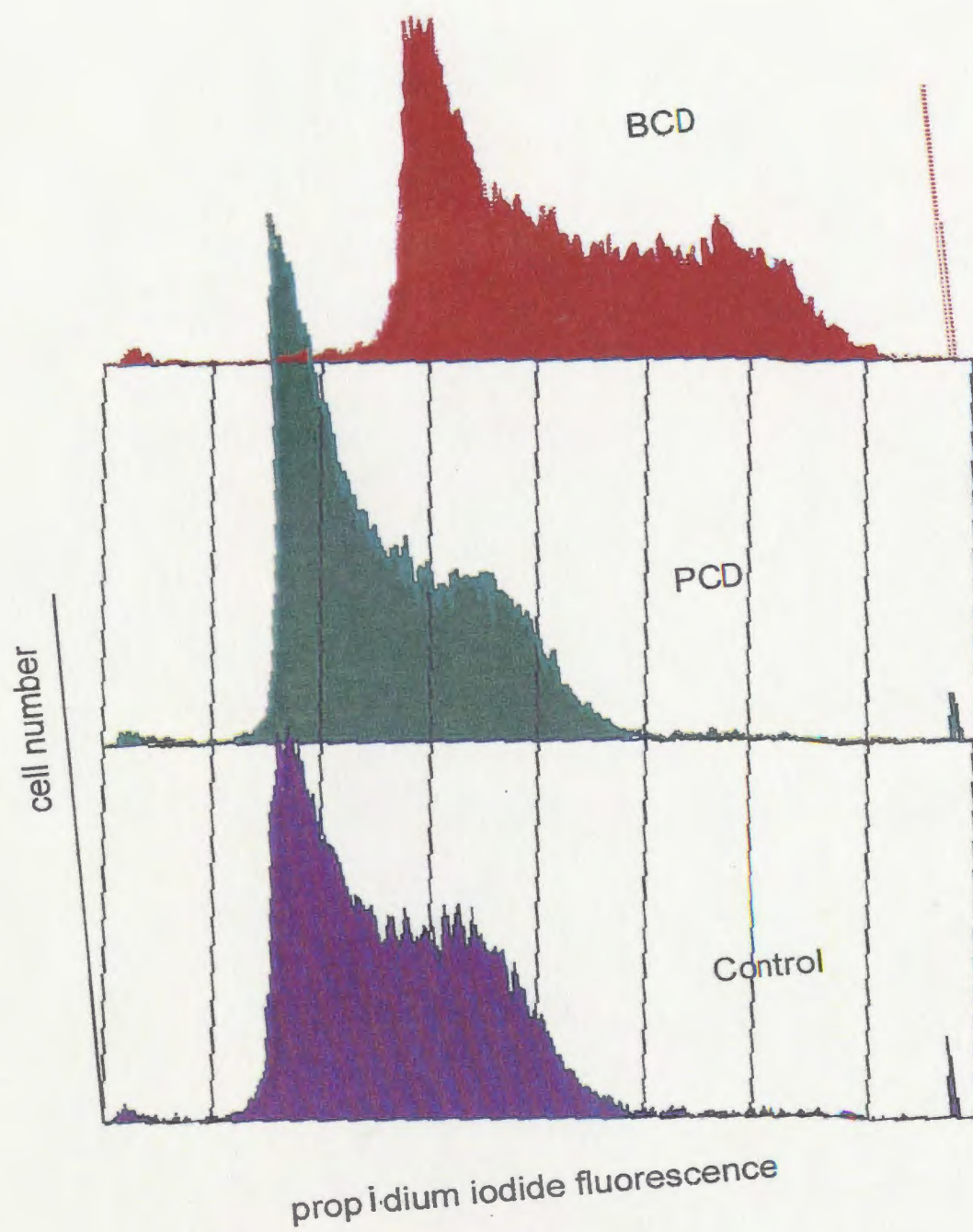


Figure 8B: Histogram of cells pretreated with ATA and then exposed to sanguinarine. While ATA pretreatment reduced the characteristic shift to the right of BCD, it had no effect on the DNA content of PCD.

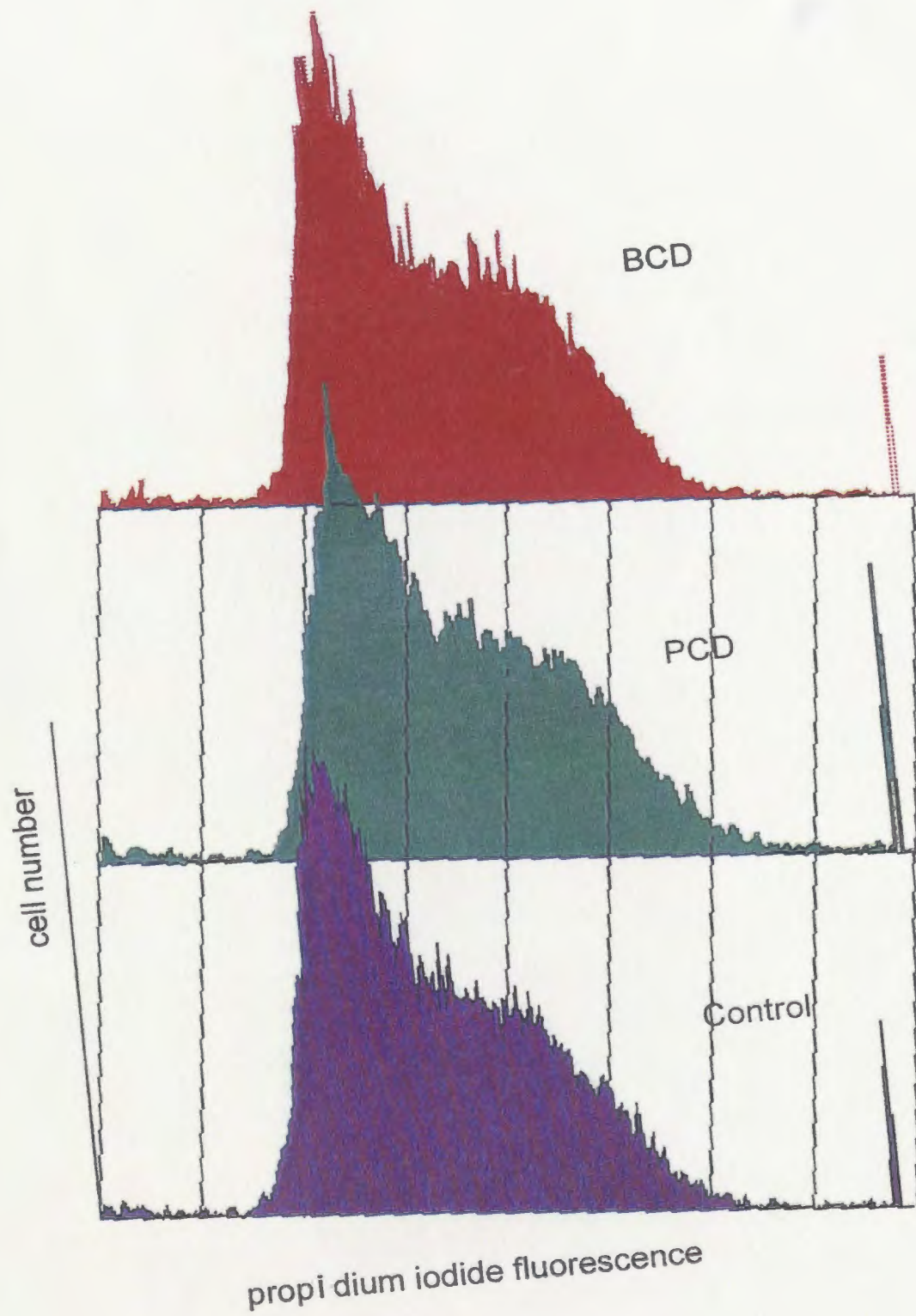
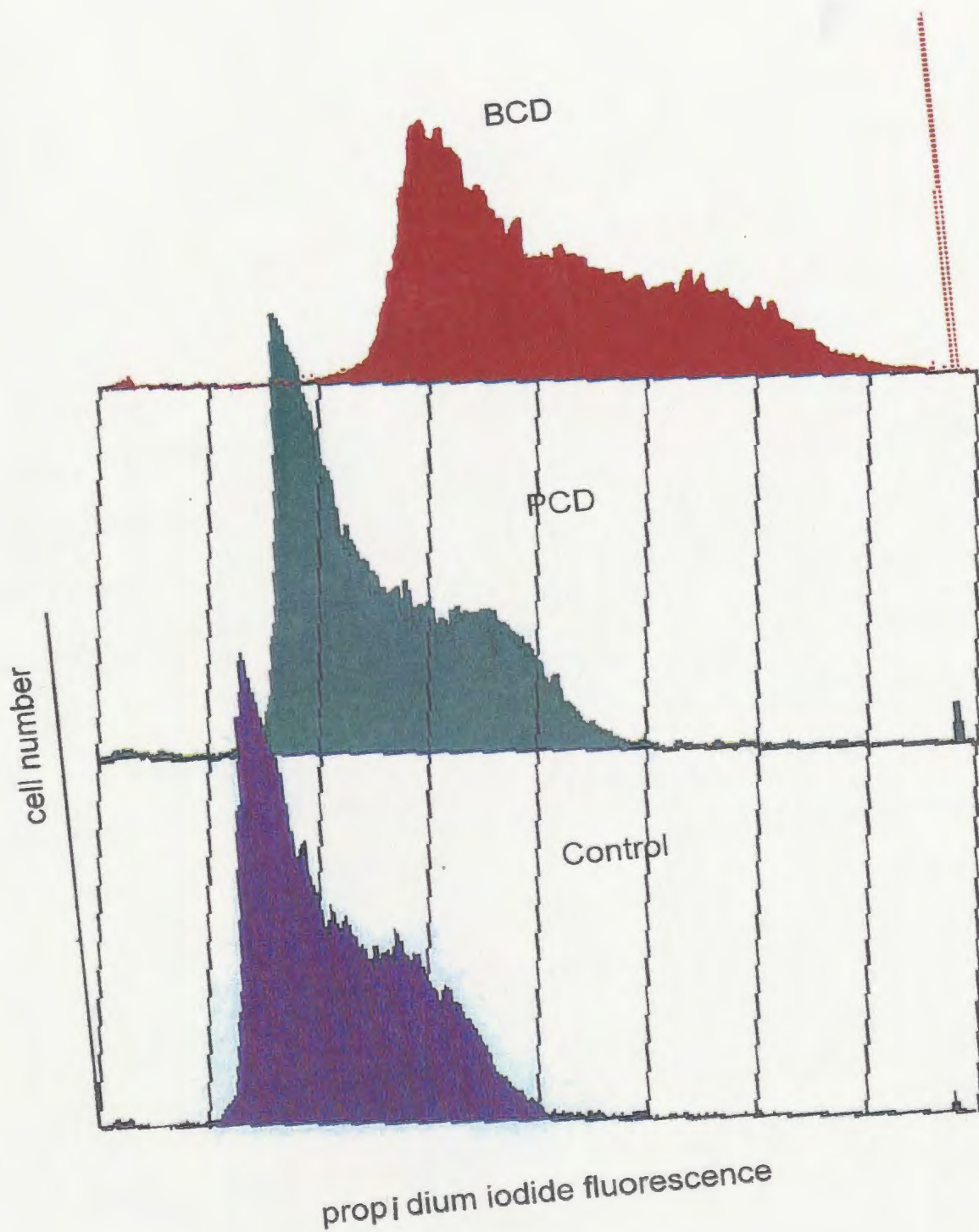


Figure 8C: Histogram of cells pretreated with CHX and then exposed to sanguinarine. CHX pretreatment appears to have no effect on DNA content neither of PCD nor of BCD.



### **3.9. O<sub>2</sub> Consumption Studies:**

The results of this study, designed to investigate the O<sub>2</sub> consumption rates of cells, are depicted in Fig. 9. These O<sub>2</sub> consumption graphs show O<sub>2</sub> consumption rates by cells versus time.

Fig. 9A shows the results of O<sub>2</sub> consumption studies of untreated control cells, apoptotic cells and in cells that underwent BCD induced by sanguinarine. Untreated control cells showed a linear O<sub>2</sub> consumption rate during the first two hours. The cells that underwent apoptosis showed an accelerated rate of O<sub>2</sub> consumption with time, followed by a rapid decline. The first 30 minutes of exposure to sanguinarine showed a relatively sharp increase in the O<sub>2</sub> consumption rate over that of controls, followed by a rate comparable to that of controls between 30-60 minutes and a rate lower than that of controls between 60-120 minutes. In sharp contrast, cells that underwent BCD displayed comparatively lower rates of O<sub>2</sub> consumption after the first 30 minutes.

Fig. 9B shows the results of O<sub>2</sub> consumption studies of cells pretreated with ATA. ATA pretreatment increased the O<sub>2</sub> consumption rates overall, in both sanguinarine-induced apoptosis and BCD. However, as opposed to sanguinarine induced apoptosis, ATA pretreatment prevented the accelerated oxygen consumption during the initial half-hour of sanguinarine exposure. The O<sub>2</sub> consumption rate of ATA controls (cells treated with ATA only) showed an O<sub>2</sub> consumption rate similar to untreated controls.

Fig. 9C showed the results of O<sub>2</sub> consumption studies of cells pretreated with CHX. CHX pretreatment appears to have no significant effect on O<sub>2</sub> consumption rates of sanguinarine induced PCD and BCD. The O<sub>2</sub> consumption rates of CHX controls (cells treated with CHX only) were similar to that of untreated controls.

**Figure 9 - Graphs depicting O<sub>2</sub> consumption levels with time:**

Figure 9A: Graph showing O<sub>2</sub> consumption levels in arbitrary units with time in untreated controls, apoptotic cells induced by sanguinarine and in cells that underwent sanguinarine induced BCD. The apoptotic cells showed an initial rapid phase of accelerated oxygen consumption (especially in the first half-hour of sanguinarine exposure), and later a slowing down and gradually tapering off at 2 hours. In contrast, the control untreated cells adopted a steady rate of oxygen consumption and continued beyond the second hour. BCD tends to have a fairly even rate for the first half-hour, after which the rate declines rapidly.

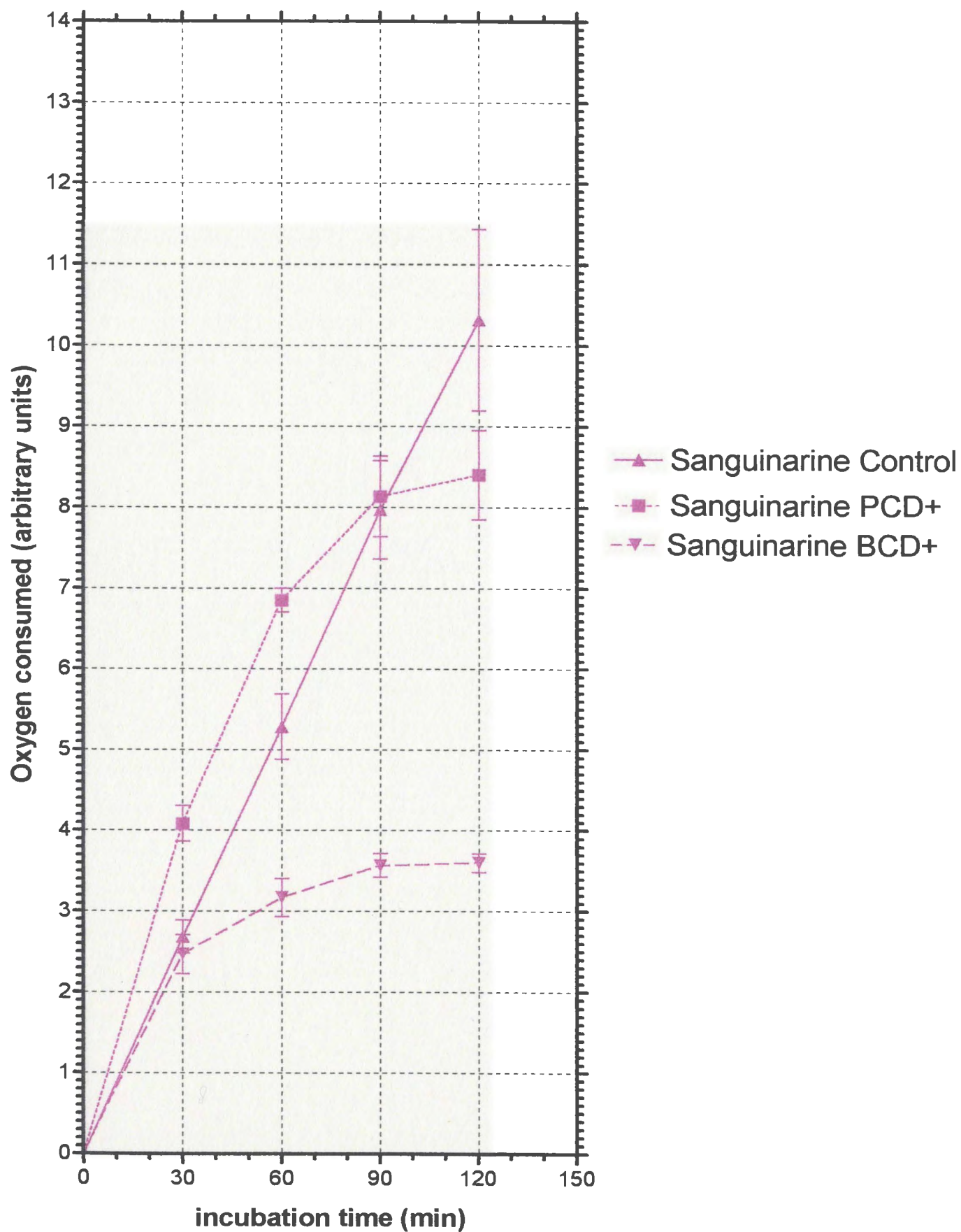


Figure 9B: Graph showing O<sub>2</sub> consumption rates in cells treated with ATA only (ATA controls) and cells pretreated with ATA and later exposed to concentrations of sanguinarine inducing apoptosis and BCD. As opposed to sanguinarine induced apoptosis, ATA pretreatment did not show the accelerated oxygen consumption rate during the initial half hour of sanguinarine exposure nor did it show the slowing down of the oxygen consumption rate which was seen in cells treated with sanguinarine only. The oxygen consumption rate was almost similar to that of untreated controls.

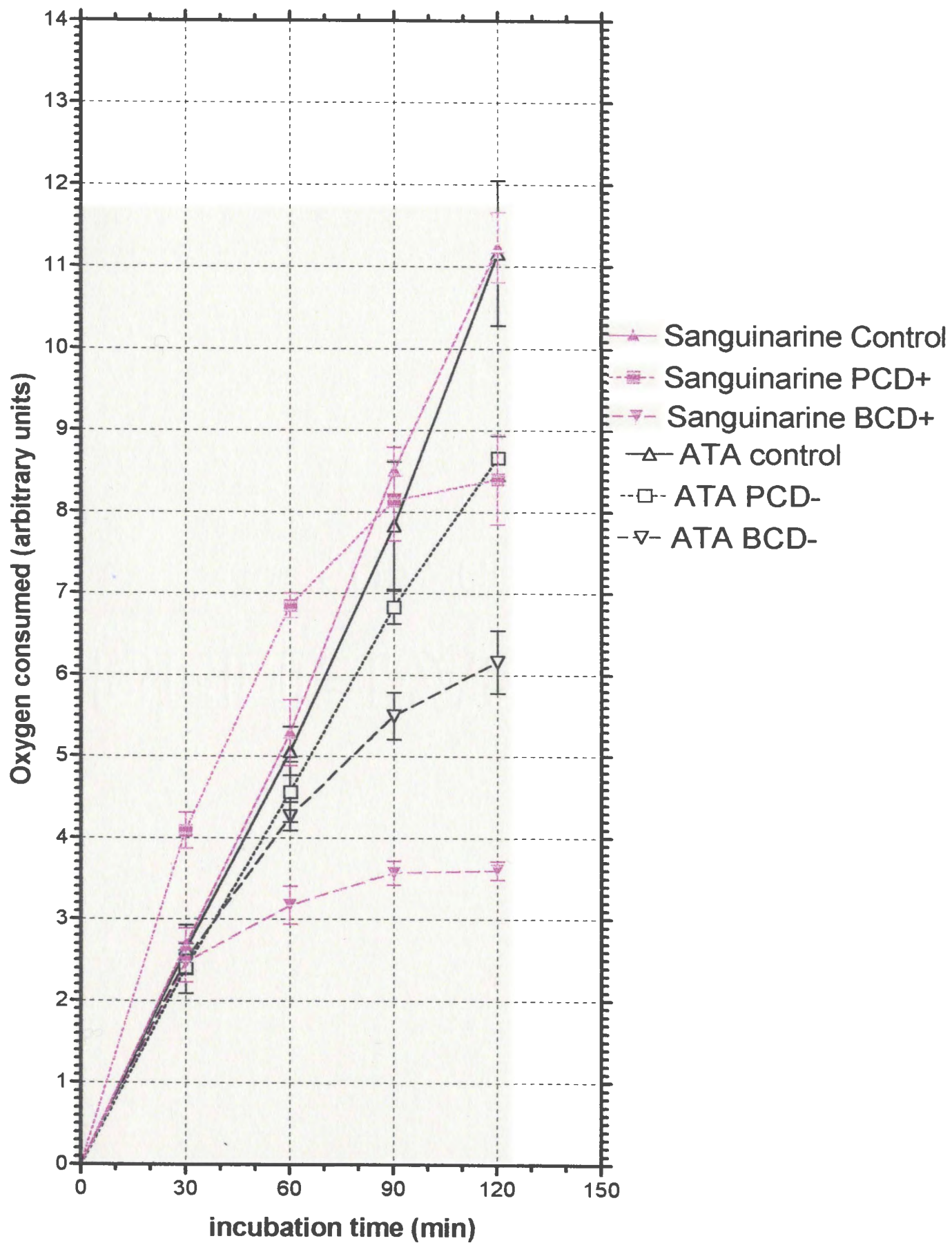
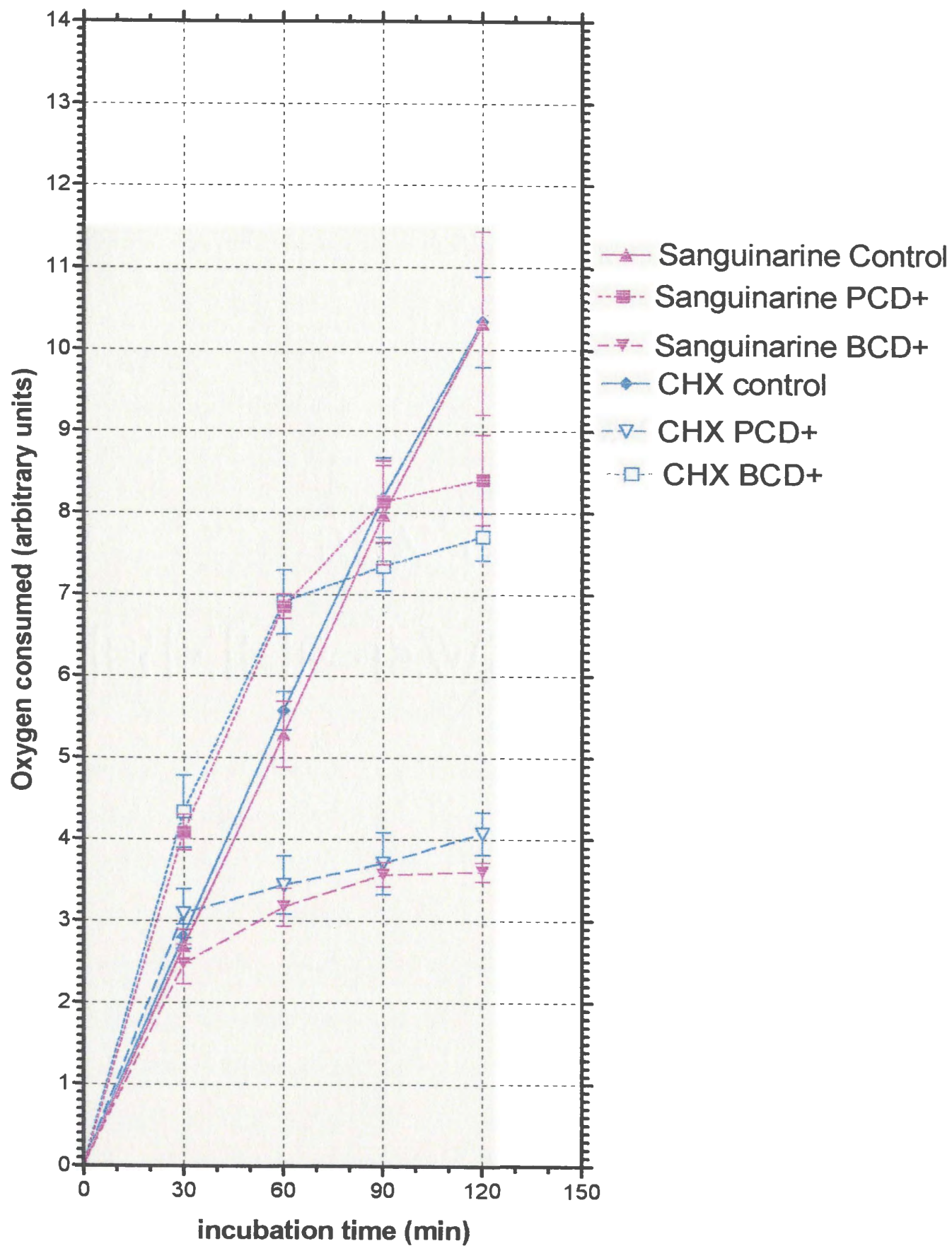


Figure 9C: As shown in this graph CHX pretreatment appear to have no effect on the O<sub>2</sub> consumption rates of sanguinarine induced PCD and BCD.



#### **4.0. DISCUSSION**

Previous studies in our laboratory demonstrated that K562 cells when exposed to sanguinarine, display the classical morphology of apoptosis at 1.5 µg/ml and the morphology of BCD at 12.5 µg/ml, when observed by light microscopy. When pretreated with different concentrations of ATA for various time periods (as outlined in “methods”) it was found that ATA pretreatment at 100 µM for 2 hrs. completely inhibited the morphology of sanguinarine induced apoptosis, while the number of cells demonstrating the morphology of BCD was reduced by 30-40%.

Apoptosis is generally been found to correlate with DNA fragmentation ( Liepins and Younghusband, 1987 ) and is considered to be the hallmark of apoptosis ( Arends et al., 1990; Compton, 1992 ). This DNA fragmentation is thought to occur at the internucleosomal regions due to the activation of a specific endonuclease (Barry and Eastman, 1993, Majno and Joris, 1995, Arends et al., 1990, Commpton, 1992, Wyllie et al., 1992). Not all cells, however, manifest a strict correlation between the morphology of apoptosis and nuclear DNA fragmentation (Ucker et al., 1992). Initially, DNA is cleaved at the sites of attachment of chromatin loops to the nuclear matrix, which results in the appearance of discrete 300-50 kb size fragments (Oberhammer et al., 1993). Subsequently, DNA is preferentially cleaved between nucleosomes. The products are discontinuous DNA fragments representing nucleosomal and oligonucleosomal sized DNA fragments. They generate a characteristic “ladder” pattern during agarose gel electrophoresis. However, in many cell types, DNA cleavage during apoptosis does not proceed to inter-nucleosomal sized sections but rather proceeds only to 300-50 kb size DNA fragments (Oberhammer et al., 1993; Cohen et al., 1992; Collins et al. 1992; Ormerod et al., 1994; Zakeri et al., 1993; Zamai et al., 1996). Thus, it should be stressed that, because in many cell types DNA degradation during apoptosis does not progress to

internucleosomal segments but stops at 300-50kb fragments, the absence of the former segments (no "DNA laddering") is not evidence of the lack of apoptosis. The presence of a large number of DNA strand breaks is a very specific marker of apoptosis (Gorczyca et al., 1992). Their detection, by the assay employing exogenous TdT, has therefore, become one of the main criteria in studies of cell death. The reaction catalyzed by exogenous TdT ("end labeling", "tailing", "TUNEL") or DNA polymerase (nick translation) is aimed at detecting the 3' OH ends in DNA breaks by attaching to them biotin or digoxigenin conjugated nucleotides ( Gorczyca et al., 1992; Gorczyca et al., 1993; Li et al. 1996 ). Previous studies in our laboratory showed a lack of DNA fragmentation in sanguinarine induced apoptosis, analyzed by standard agarose gel electrophoresis (Liepins et al., 1996). The "TUNEL" assay to reveal the presence of DNA fragmentation in the apoptosis induced by sanguinarine showed nicking of DNA strands. When pretreated with ATA, apart from an inhibition of the morphology of apoptosis, a negative "TUNEL" denoting lack of DNA strand fragmentation was also observed. Inhibition of DNA strand nicking may be in conformity with the endonuclease inhibiting function of ATA (Hallick et al., 1977 ).

The remaining results of the present study pertaining to the parameters of apoptosis, confirms the above notion of the inhibition of apoptosis induced by sanguinarine, by ATA. Results of light and electron microscopy studies show the lack of formation of apoptotic bodies and nuclear fragmentation when pretreated with ATA, despite having a marginal increase in vacuoles and a degree of patchy chromatin condensation. ATA pretreatment resulted in a decrease in membrane permeability as well, denoted by a decrease in the  $^{51}\text{Cr}$  released, as opposed to the concomitant release of  $^{51}\text{Cr}$  in apoptosis. The absence of the phosphatidyl serine flip demonstrated by a negative annexin V assay in ATA pretreated cells, was in sharp contrast to that of apoptosis

induced by sanguinarine. As opposed to the oxygen consumption rates of sanguinarine induced apoptosis, ATA pretreatment did not show the accelerated oxygen consumption during the initial half hour of sanguinarine exposure nor did it show the tapering off of it at 2 hrs of sanguinarine exposure. The high oxygen consumption rates (during the first half hour) associated with the sanguinarine induced apoptotic process in the present study may represent elevated levels of cellular respiration providing the energy required for the apoptotic process. These results were in keeping with earlier observations in our laboratory of significantly higher oxygen requirements of apoptotic mastocytoma P815 cells than nonapoptotic cells in cold shock induced apoptosis (Liepins et al., 1985). Alternatively, the formation of reactive oxygen intermediates, reported to play a role in some cytolytic processes, may account for some or all of the elevated oxygen consumption rates (Nathan et al., 1982; Keisari et al., 1984; Guthrie et al., 1984). Whereas, the tapering off, of the oxygen consumption rates (denoted by the plateauing of the curve in fig. 8) at the end of the 2 hr period of sanguinarine exposure, may indicate the dying process. The initial reduction coupled with the subsequent stabilization of oxygen consumption rates when pretreated with ATA is in consonance with the inhibition of the sanguinarine induced apoptotic process.

Conversely, pretreatment of cells with the protein synthesis inhibitor cyclohexamide (CHX), failed to inhibit the apoptosis induced by sanguinarine. This is confirmed by the results of light and electron microscopy, "TUNEL",  $^{51}\text{Cr}$  release, annexin V and oxygen consumption assays. Failure to inhibit sanguinarine induced apoptosis by cycloheximide may have been due to, either protein synthesis not being a factor in the apoptosis induced by this drug or that the 2 hour exposure to the drug not being adequate to exercise the capacity of cycloheximide to inhibit protein synthesis and consequently, to inhibit the apoptotic process. This notion is corroborated by recent

findings in our laboratory utilizing western blotting techniques and cDNA expression arrays. Apoptotic cells analyzed by western blotting techniques showed an increase in the pro-apoptotic protein Bax and a decrease in the anti apoptotic gene products of Bcl-2 and NFkB (Weeresinghe et al., in preparation), while cDNA expression studies showed no changes in mRNA levels pertaining to these gene products.

These results suggest that, while ATA successfully counteracted the apoptosis induced by sanguinarine, CHX failed to do so. Thus, even though protein synthesis inhibition has been reported to be effective in the inhibition of apoptosis in certain experimental conditions (Williams et al., 1990; Yanagihara et al., 1992; Wyllie et al., 1987), this appears not to be the case in sanguinarine induced apoptosis of K562 cells. On the other hand, the inhibition of endonuclease by ATA (Hallick et al. 1977) was successful in inhibiting the apoptotic process induced by sanguinarine. Thus, it could be assumed that, rather than de novo protein synthesis, what is more important for the sanguinarine induced apoptotic process is the post-translational modification of the already synthesized proteins.

The effects of CHX on apoptosis may depend on many factors including the cell death inducing agent, its dose and the type of cell line (Yanagihara et al. 1992). Yanagihara et al., reports inhibition of apoptosis induced by TGF- $\beta_1$  (transforming growth factor  $\beta_1$ ) in human gastric scirrhous carcinoma cell lines HSC-39 and HSC-43 by CHX. This might suggest the requirement for new protein synthesis in TGF- $\beta_1$  induced cell death. At the same time, the authors assert that, the pretreatment of CHX had no effect upon TGF- $\beta_1$  mediated hepatoma cell death. The authors also claim that, at lower TGF- $\beta_1$  concentrations, CHX partially prevented TGF- $\beta_1$  induced cell death in a dose dependent fashion (Yanagihara et al., 1992). Evidence of CHX acting as a stimulant of apoptosis is also found in literature (Thomas and Hersey, 1998).

The phenomenon of single blister formation or BCD is at best, only poorly reported in literature. As previously mentioned, this phenomenon of BCD is induced when K562 cells were treated with sanguinarine concentrations of 12.5 $\mu$ g/ml for 2 hrs. Cell surface blister formation in hepatocytes induced by hypoxia has been reported to produce blebbing and/or blister formation in more than 70% of cells (Lemasters et al. 1987). This morphology was found to be independent of intracellular calcium fluxes, and the rupture of these large cell surface blebs was considered by the authors to represent the abrupt transition from reversible to irreversible cell injury.

As opposed to apoptosis induced by sanguinarine, BCD was characterized by a negative "TUNEL" assay denoting the absence of DNA nicking, a negative annexin V assay showing the absence of the phosphatidyl serine flip and a DNA content analysis by flowcytometry with a shift to the right denoting increased ploidy. Roberts et al., reports that taxol induces a nominal degree of polyploidy in K562 cells when treated with a concentration of 10  $\mu$ M for 24 hrs, although there had been no indication of any degree of cell ploidy at short term intervals, i.e., four hours (Roberts et al., 1990). Membrane permeability assessment of BCD in the present study by  $^{51}$ Cr release assay showed a much higher release than that of apoptosis while oxygen consumption studies showed a consumption rate much lower and distinctly different to that of apoptosis. These results also denote that BCD is a cell death process with reduced levels of energy. This is in sharp contrast to sanguinarine induced apoptosis, which presumably, is a higher energy requiring process as shown by a higher oxygen consumption rate at least during its initial stages. The earlier plateauing of the oxygen consumption curve in BCD (fig. 9) also suggests that mitochondrial dysfunction occurs much earlier than that of apoptosis. Electron microscopic studies show cytoplasmic swelling by way of blister formation, nuclear swelling sans the nuclear fragmentation of apoptosis and patchy chromatin

condensation. However, it was also shown that cells, which underwent BCD, excluded trypan blue indicating cell viability and a functional cell membrane. The exclusion of trypan blue by the cell membrane shows that BCD is not representative of early necrosis (Wyllie et al., 1984). It is generally accepted that necrosis does not indicate a form of cell death but refers to changes secondary to cell death by any mechanism (Majno and Joris, 1995). These characteristics of BCD may point to it being representative of an early stage of oncosis. Oncosis is deemed to be a part of accidental cell death accompanied by swelling (Majno and Joris, 1995). Thus, BCD could be hypothetically construed as an early stage of oncosis. Accordingly, this may also serve as a future “model” for the study of oncosis.

When pretreated with ATA, sanguinarine induced BCD was reduced by 30-40% as assessed by light microscope while CHX pretreatment of cells had no effect on its reduction. Thus, it could be asserted that, while the inhibition of protein synthesis plays no role in BCD, the inhibition of endonuclease appears to retard the BCD process. ATA pretreatment in BCD also show a relative increase in oxygen consumption, decrease in <sup>51</sup>Cr release and a decrease in DNA content or ploidy as compared to BCD without ATA pretreatment. These results point to an inhibition of sanguinarine induced BCD during ATA pretreatment of cells.

Further studies are warranted to determine the molecular changes of ATA pretreatment in BCD, with a view to demystifying the partial inhibition of it during ATA pretreatment. ATA pretreatment of cells appeared to have no effect in transmission electron microscopic, TUNEL, as well as annexin V studies when compared to BCD without ATA pretreatment. Contrary to ATA, CHX pretreatment appears not to effect any of the parameters of sanguinarine induced BCD. Thus, similar to the assumptions made regarding the failure of CHX to inhibit apoptosis, it could be argued that, either this

process of sanguinarine induced BCD does not necessitate protein synthesis or that the 2 hr period of sanguinarine exposure does not provide sufficient time for the protein synthesis inhibiting process of CHX to take effect.

Another noteworthy feature observed during the induction of BCD, was the intensity of the sanguinarine auto-fluorescence itself. This is demonstrated in the fluorescence light microscopic pictures of fig. 6, designed to detect the phosphatidyl serine flip in the annexin V assay. Fig. 6A shows the yellow fluorescence of sanguinarine (which incidentally, also happens to fall within the green fluorescence range) during BCD [fig. 6A(c)] but absent during apoptosis [fig. 6A(b)](which shows only the green fluorescence of FITC conjugated annexin V). The spectre of an increased presence of sanguinarine in BCD may be at least partly responsible for the tumor cell killing ability of this anti-cancer drug. This assumption is fortified by the fact that sanguinarine exercises it's anti-cancer properties (Sen and Maiti, 1994; Babich et al., 1996), among other probable mechanisms of actions, by forming a molecular complex with DNA through intercalation; more specifically, by binding to regions rich in guanosine-cytosine (Sen and Maiti, 1994; Babich et al., 1996). Previous studies in our laboratory have also confirmed that sanguinarine overcomes the multi-drug resistant (MDR) phenomenon, in the Pgp positive MDR CEM-VLB human leukemia cell line (Liepins et al., 1996 (abstract); Weerasinghe et al., in preparation). Therefore, the overcoming of the MDR phenomenon by sanguinarine could hypothetically be associated with the induction of BCD. Thus, the efficacy of sanguinarine as an effective anti-cancer agent could well be the result of the induction of BCD, in addition to the usual PCD, which is widely reported in literature. It has been stated that apoptosis is only rarely observed in vivo and may not be the sole cell death mechanism and that this observation emphasizes the need to explore

new forms of cell death mechanisms (Houghton, 1999). In view of this grave necessity to identify new forms of cell death, the phenomenon of BCD looms large.

The present study attempted to shed more light on some of the biochemical mechanisms of sanguinarine induced PCD and BCD, while revealing multiple parameters of both these forms of cell death in a tumor cell line. It has been reported that, the efficacy of anti-cancer drugs may hinge on their ability to alter the apoptotic regulatory machinery of cancer cells (Earnshaw et al. 1999). In keeping with this statement by Earnshaw et al., it might be equally compelling to assume that unravelling the molecular make-up of BCD might bear fruit in terms of better comprehending the role of BCD in tumor cell killing. This might even lead to a better understanding of chemo-resistance and the ability to overcome this monumental obstacle to finding a cure for cancer.

The discovery of new and effective anti-cancer agents and unraveling their mechanisms of action is an important step in the war against cancer. Knowledge of mechanisms of action of drugs could help device a formula for the more efficient and judicious use of these agents as combination therapy with other more established therapeutics for the effective treatment of drug resistant forms of cancer.

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