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Evaluation of anticancer effects of a novel proteasome inhibitor (Velcade), interferon (alpha-interferon) and antimyeloma antibodies on the growth of myeloma cells.

Thesis

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Biochemistry

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LIST OF ABBREVIATIONS

Ab : Antibody

ADCC : Antibody-dependent cellular cytotoxicity

AIF : Apoptosis inducing factor

Apaf-1 : Apoptotic protease activating factor 1

ATP : Adenosine triphosphate

BAX : BCL2-associated × protein

BID : BH3-interacting domain death agonist

BRM : Biological response modifier

Bz : Bortezomib

Ch-T : Chloramine –T

Ci : Curie

CR : A complete response

CRP : C-reactive protein

CTLs : Cytotoxic T lymphocytes

D : Dose

DC : Dendritic cells

DISC : Death-inducing signal complex

DMSO : Dimethyle sulfoxide

DTT : Dithiothreitol

FADD : Fas-associated death domain protein

FasL : Fas ligand

FBS : Foetal bovine serum

FCA : Freund's complete adjuvant

FIA : Freund's incomplete adjuvant

5-FU : 5-fluorouracil

G_0 : Gap 0

G_1 : Gap 1

G_2 : Gap 2

HDC : High-dose chemotherapy

HSCT : Hematopoietic stem cell transplant

IAP : Inhibitor of apoptosis

IFNs : Interferons

IFN-α : Alpha-Interferon

ISS : The International Staging System

KI : Potassium iodide

Mo : Monoclonal protein

M : Mitosis

MAbs : Monoclonal antibodies

MGUS : Monoclonal gammopathy of undetermined significance

MHC : Major histocompatibility complex

MIP-1α : Macrophage inflammatory protein-1α

MM : Multiple myeloma

6-MP : 6-mercaptopurine

Na ^{125}I : Sodium Iodide-125

Na OH : Sodium Hydroxide

$Na_2HPO_4.2H_2O$: Di-Sodium hydrogen orthophosphate

$Na_2S_2O_5$: Sodium metabisulphite

NaCl : Sodium chloride

$NaH_2PO_4.2H_2O$: Sodium dihydrogen orthophosphate:

$NaHCo_3$: Sodium bicarbonate

NK : Natural killer

OAFs : Osteoclast activating factors

p53 : protein 53 or tumor protein 53

PAbs : Polyclonal antibodies

PBS **:** Phosphate- buffered saline

PI **:** Propidium iodide

PR : Partial response

RANKL **:** RANK ligand

RIT : Radioimmunotherapy

S : Synthesis

T.C. : Total Counts of myeloma cells

T.C. /ml : Total Counts of myeloma cells/ml

TNF-α : Tumor necrosis factor-alpha

V(%) **:** Viability (%)

Introduction and Aim of the work

Multiple myeloma (MM), also known as myelomatosis or kahler's disease **(Raab et al., 2009)**, accounts for 1% of all cancers and the second commonest haemato-oncological diseases in the United Kingdom **(Devenney and Erickson, 2004)**. It is a neoplastic plasma cell disorder that is characterized by clonal proliferation of malignant plasma cells in the bone marrow microenvironment, monoclonal protein in the blood or urine, and associated organ dysfunction (**Marvelle and Tracey, 2012)**.

The proteasome is a large intracellular molecule with multicatalytic protease activities found both in the cytoplasm and the nucleus.It is an essential enzyme complex for the nonlysosomal, ATP-dependent proteolytic pathway , catalyzing the rapid degradation of intracellular proteins regulating cell cycle , apoptosis , cell adhesion, transcription , angiogenesis , and antigen presentation by MHC class I molecules. It is also essential for the rapid elimination of abnormal proteins, arising via mutation or by posttranslational damage such as oxidation. The catalytic core of this complex is found on the 20S proteasome subunit, a multicatalytic protease containing at least three peptidase activities: chymotryptic-like, tryptic-like, and post-glutamyl peptide hydrolyzing activities (**Alexei et al., 2012)**.

Bortezomib is a dipeptide boronic acid analog **(Wright, 2010)** that shows extreme selectivity of action towards cancer cells' proteasome, giving it a distinct advantage as a therapeutic agent. Its mode of inhibition is through reversible binding to the N-terminus threonine residue in the β-1 subunit of the catalytic core complex of the 26S proteasome **(Mohammad etal.,2011)**, leading to reversible inhibition of the chymotrypsin-like and proteolytic activity of the proteasome.This results in several

biological effects, including inhibition of the cell cycle, increased apoptosis, inhibition of NF-κB activity, induction of ER stress and sensitization of the tumor cells to drugs and CTL lysis **(Seki et al.,2010)**.

Immunotherapy, also called "biological therapy" is a promising treatment and an active area of cancer research for people with certain types of blood cancer. The development of immunotherapies is based on the concept that immune cells or their products (such as antibodies) that can recognize and kill cancer cells. They can be made in the laboratory and given to patients to treat cancer. Immunotherapies generally cause less severe short-term side effects than most chemotherapy or radiation therapy which not only destroys cancer cells but also affects rapidly dividing normal cells **(Glenn et al., 2010**).

The human alpha interferons (IFNs-α) are a family of structurally related proteins that exhibit antiviral, antiproliferative and immunoregulatory actions. Based on these properties, IFNs-α have been used as effective pharmacological agents in the treatment of certain malignant and viral diseases (**Michael and Christophe, 2010)**.

Antibodies protect the body against invading agents by one of following two ways: 1) by direct attack on the invader, and 2) by activation of the complement system, which has multiple means of destroying invading cells **(Charles et al., 1999)**. As therapy for cancer, antibodies can be injected into patients to seek out the cancer cells, potentially leading to disruption of cancer cell activities or to enhancement of the immune response against the cancer **(John and Christopher, 2007)**.

This study aims to evaluate the antitumor effect of novel anticancer drugs Bortezomib (Velcade). In addition, the effect of interferon (alpha-interferon) on the growth of myeloma cells was studied. Also the present study produced and evaluated the polyclonal antibodies against myeloma cells and studied the effect of the prepared antibodies (with and without labeling with radioactive isotopes) against the growth of myeloma cells (in vitro and in vivo). A correlation and combination among these drugs was performed to determine the most biologically active and its capability for application as cancer therapy. Also some biochemical parameters (Flow cytometry,Caspases, β2-microglobulin, liver functions and Kidney functions) were performed before and after treatment to evaluate the antitumor activity.

REVIEW OF LITERATURE

Multiple Myeloma

Multiple myeloma (MM) is a cancer of plasma cells (mature B lymphocytes) that usually arises in the bone marrow. Myeloma develops when plasma cells undergo a cancerous (or malignant) change and become myeloma cells. These myeloma cells multiply without any proper order, forming collections known as tumors that accumulate in different parts of the body, especially in the bone marrow and on the surfaces of different bones in the body. These tumors secrete chemicals that stimulate other bone marrow cells (osteoclasts) to remove calcium from the bone. As a result bones can become weaker, more brittle and break more easily **(Kyle and Rajkumar, 2008).**

The average patient age at diagnosis of multiple myeloma is about 68 years, and 99% of patients diagnosed are older than 40 years **(Palumbo et al., 2006)**. Although MM is incurable, it is treatable. Treatment is complex and may include chemotherapy, radiation therapy, and surgery **(Osman et al., 2001)**. Each treatment decision should be tailored to an individual patient's physical, emotional, financial, and medical needs. The goals of therapy include eradicating all evidence of disease, controlling the disease to prevent damage to target organs, preserving normal performance and quality of life, relieving pain associated with the disease, and managing the myeloma that is in remission **(Auwerda et al., 2007)**.

Pathophysiology

The uncontrolled growth of myeloma cells has many consequences, including skeletal destruction, bone marrow failure, increased plasma volume and viscosity, suppression of

normal immunoglobulin production, and renal insufficiency. The serum and/or urine M-protein is elevated and typically rising at the time of diagnosis **(Durie, 2008)**. The pathophysiology of myeloma is summarized in Table (1).

Bone Disease

The presence of abnormal protein has been linked with bone destruction. It has taken until quite recently to determine the mechanisms involved. The first clue was that both myeloma cells and increased numbers of osteoclasts are present at sites of bone destruction. Understanding of the mechanisms has evolved from the observation that myeloma cells produce osteoclast activating factors (OAFs) to the characterization of local cytokines such as IL-1β, IL-6, and TNF-α and -β; chemokines such as MIP-1α; and cell-cell adhesion processes involving av β3 integrin, all of which are involved in producing increased numbers and activity of osteoclasts. Most recently a substance called RANK ligand (RANKL) has been identified as a critical mediator of osteoclast activation. Besides activation of osteoclasts, the other characteristic feature of myeloma bone disease is inhibition of osteoblasts, which are responsible for new bone production and bone healing. "Coupling" between osteoclast and osteoblast function is responsible for normal bone remodeling and repair **(Terpos et al., 2007)**.

Anemia

Anemia is a characteristic feature of myeloma. Anemia may be caused by suppression of erythropoiesis by tumor-related cytokines, secondary to chemotherapy, renal insufficiency, and/or vitamin or iron deficiency **(Katzel et al., 2007)**.

Kidney Dysfunction

Impairment of kidney function is a common complication in myeloma patients. However, this does not mean that every patient will have this problem **(Dimopoulos et al., 2008)**. In some patients, myeloma proteins, especially Bence Jones light chains, cause renal injury by a variety of mechanisms ranging from tubular damage from large accumulations of precipitated light chains, to effects of myeloma proteins deposited as amyloid, or selective tubular damage resulting in the metabolic effects of an entity called Fanconi syndrome. Fanconi syndrome is a type of selective kidney tubular damage with leakage of amino acids and phosphates into the urine, which can cause metabolic bone disease. Other important factors related to kidney dysfunction in multiple myeloma patients are increased levels of calcium and/or uric acid, infection, and the effects of drugs **(Barosi et al., 2004)**.

Other Organs Dysfunctions

Myeloma cells can accumulate in bone marrow and/or in a variety of tissue sites and produce a broad range of potential complications.

• ***Neurologic Effects***: Nerve tissue is often affected in myeloma patients either by the direct antibody effects of myeloma proteins against nerves (e.g. myelin sheaths) or deposition of amyloid fibrils on nerves, thus impairing function. These effects result in peripheral neuropathies that must be distinguished from other causes of neuropathy such as diabetes mellitus. Because of the susceptibility to infection, viral infections of nerve tissue are quite common, most particularly varicella zoster (shingles) and Bell's palsy (partial facial paralysis).

• ***Plasmacytomas*** *:* Both in bone and soft tissue, plasmacytomas can result in compression or displacement of nerves, the spinal

cord, or even brain tissue. These pressure effects often represent a medical emergency and require immediate treatment with high doses of corticosteroids, radiation therapy, or neurosurgery.

• ***Infections :*** The predisposition to infections is perhaps the single most characteristic feature of myeloma patients besides the strong tendency for bone disease. The presence of active myeloma in the bone marrow results in impairment of normal immune functions, including normal antibody production (reflected by hypogammaglobulinemia), impaired T-lymphocyte function, and activated but aberrant monocyte/macrophage function. Some studies indicate that a factor issuing from the activated macro phages both enhances the activity of the myeloma, and inhibits normal immunoglobulin production and T-lymphocyte functions **(Katzel et al., 2007)**.

Staging and prognostic factors

The most widely used staging system for determining risk and prognosis since 1975 has been the Durie-Salmon Staging System **(Durie and Salmon, 1975)**. This staging system categorizes patients into 1 of 3 categories (stage I, II, or III) based on hemoglobin level, serum calcium level, the production rate of M protein, and the number of osteolytic lesions as shown in Table (2). Stages are further divided according to renal function and subclassified as A or B. Subclass A is relatively normal renal function (serum creatinine value less than 2.0 mg/dL) and subclass B is abnormal renal function (serum creatinine value greater than or equal to 2.0 mg /dL). The International Staging System (ISS) is a newer, more accurate system and has 3 stages based solely on results of serum albumin and beta2- microglobulin blood tests **(Greipp et al., 2005)** Table (3).

Table (1): Schema of Pathophysiology(Durie, 2008)

Skeletal Findings
• Solitary or multiple osteolytic lesions
• Diffuse osteoporosis (osteopenia)
Associated Effects Of Bone Destruction
• Elevated serum calcium
• Hypercalciuria (calcium increase in urine)
• Bone fractures
• Loss of height (vertebral collapse)
Extra Skeletal Myeloma (Rare)
• Soft tissue involvement, most commonly in head/neck area
(e.g., nasopharynx); also in liver, kidney and other soft tissue sites
Peripheral Blood
• Anemia
• Abnormal clotting
• Leukopenia
• Thrombocytopenia
• Plasma cell leukemia
• Circulating monoclonal B lymphocytes
(precursors of myeloma cells)
Plasma Protein Changes
• Hyperproteinemia (elevated protein)
• Hypervolemia (expanded volume)
• Monoclonal immunoglobulins
(IgG, IgD, IgA, IgM, IgE, or light chains only)
• Narrowed anion gap (low serum sodium)
• Elevated serum β2-microglobulin
• Decreased serum albumin
• Elevated serum IL-6 and C-reactive protein (CRP)
Kidney Abnormalities
• Proteinuria, casts without leukocytes or erythrocytes
• Tubular dysfunction with acidosis
• Uremia (kidney failure)
• Amyloidosis and renal dysfunction

Table (2): Durie and Salmon Staging System (Durie and Salmon, 1975).

Criteria	Measured myeloma cell mass (myeloma cells in billions/ m^2)*
STAGE I (low cell mass) All of the following: • Hemoglobin value <10 g/dL • Serum calcium value normal or <10.5 mg/dL • Bone X-ray, normal bone structure (scale 0) or solitary bone plasmacytoma only • Low M-component production rates IgG value <5.0 g/dL IgA value <3.0 g/dL Urine light chain M-component on electrophoresis <4 g/24h	600 billion myeloma cells*
STAGE II (intermediate cell mass) neither stage I nor stage III	600 to 1,200 billion Fitting myeloma cells*
STAGE III (high cell mass) One or more of the following: • Hemoglobin value <8.5 g/dL • Serum calcium value normal or >12 mg/dL • Advanced lytic bone lesions (scale 3) • High M-component production rates IgG value >7.0 g/dL IgA value >5.0 g/dL Urine light chain M-component on electrophoresis >12 g/24h	>1,200 billion myeloma cells*
SUBCLASSIFICATION (either A or B) • A: relatively normal renal function (serum creatinine value) <2.0 mg/dL • B: abnormal renal function (serum creatinine value) >2.0 mg/dL	
**myeloma cells in the whole body*	

Table (3): International Staging System (ISS) (Greipp etal., 2005).

STAGE	CRITERIA
I	Serum β2 microglobulin <3.5 mg/L Serum albumin ≥3.5 g/dL
II	Not I or III*
III	Serum β2 microglobulin >5.5 mg/L
*There are two possibilities for stage II: • Serum β2 microglobulin <3.5 mg/L, but serum albumin <3.5 g/dL or • Serum β2 microglobulin 3.5 – 5.5 mg/L irrespective of the serum albumin	

Diagnosis

Plasma cell dyscrasias can be divided into premalignant and malignant conditions. Monoclonal gammopathy of undetermined significance (MGUS) is a premalignant condition, whereas asymptomatic MM and active MM are malignant. Asymptomatic MM is differentiated from active myeloma by end-organ compromise designated by the acronym "CRAB" (hypercalcemia, renal insufficiency, anemia, or bone lesions) Table (4) **(Jed et al., 2007)**. MGUS is a premalignant condition that may progress to MM. MGUS has been found in 3.2% of persons aged 50 years or older and 5.3% of persons aged 70 years or older in the United States **(Kyle et al., 2006)**. MGUS is approximately 80 to 100 times more common than MM. Whereas the rate of progression of MGUS to MM is 1% per year, approximately three fourths of these individuals remain without conversion over 20 or more **(Bergsagel ,1995)**.

Table (4): Diagnosis in Multiple Myeloma **(Jed et al., 2007).**

Clinical features of end-organ damage: hypercalcemia, renal insufficiency, anemia, or bone lesions (CRAB)

Calcium levels increased: 0.5 mg/dL above the upper limit of normal or 10.5 mg/dL
Renal insufficiency: creatinine >2 mg/dL
Anemia: hemoglobin 2 g/dL below the lower limit of normal or hemoglobin <10 g/dL
Bone lesions: lytic lesions or osteoporosis with compression fractures (MRI or CT may clarify)
Other: symptomatic hyperviscosity, amyloidosis, recurrent bacterial infections (>2 episodes in 12 months)

Monoclonal gammopathy of undetermined significance (MGUS)

M protein in serum <3 g/dL
Bone marrow clonal plasma cells <10% and low level of plasma cell infiltration in a trephine biopsy (if done)
No evidence of other B-cell proliferative disorders
No CRAB
AL amyloid and the IgM paraprotien-related neurological syndromes would be instance of "MG associated with…"

Asymptomatic myeloma (smoldering myeloma)

M protein in serum >3 g/dL and/or bone marrow clonal plasma cells >10%
No CRAB

Symptomatic multiple myeloma

M protein in serum and/or urine
Bone marrow (clonal) plasma cells or plasmacytoma
+CRAB (Some patients may have no symptoms, but have CRAB.)

Treatment of Multiple Myeloma

The prognosis and treatment of an individual with multiple myeloma (MM) depends on many patient-specific factors, including age, overall state of health, and comorbidities. Currently, initiation of therapy is primarily determined by the stage of myeloma. One of the major treatment considerations for an individual with MM is the assessment of the patient's ability to receive high-dose chemotherapy (HDC) followed by hematopoietic stem cell transplant (HSCT). Disease-specific factors (e.g., bone lesions, anemia, and renal dysfunction) also may play a major role in treatment decisions **(Fonseca and San Miguel, 2007)**. Disease control, described in terms of molecular response, has been achieved with newer therapies in clinical trials. A complete response (CR) is defined as having no detectable monoclonal (M) protein in the serum and urine, normal percentage of plasma cells in bone marrow, no increase in size or number of osteolytic bone lesions, and the disappearance of soft tissue plasmacytomas **(Rajkumar and Durie ,2008)**. Partial response (PR) is defined as greater than or equal to a 50% reduction in serum M protein, maintained for a minimum of 6 weeks, greater than or equal to a 50% reduction in the size of soft-tissue plasmacytomas and no increase in the size or number of lytic bone lesions **(Richard et al.,2008)**. A summary of types of treatments is provided in Table (5).

Chemotherapy. There are numerous forms of oral and intravenous chemotherapy that are used as therapy for myeloma Table (6) **(San-Miguel et al., 2008)**.

Radiotherapy. Radiotherapy can be used to treat areas of bony pain caused by plasma cell destruction. Sometimes, plasma cells gather in solid tumors, called plasmacytomas, which may be treated with radiotherapy **(Kumar et al., 2003)**.

Supportive therapy. This therapy tries to get the body to fight cancer. It uses materials made by your own body or made in a

laboratory to boost, direct, or restore your body's natural defenses against disease. Biological therapy is sometimes called biological response modifier (BRM) therapy or immune-therapy.

Bone marrow transplantation. Bone marrow transplantation is commonly used to treat multiple myeloma. The procedure is done when the disease is considered to be in a remission (when the least amount of myeloma cells are present in the bone marrow). First, healthy stem cells are removed from the blood stream or from the marrow itself. These stem cells are frozen and stored in the laboratory. Next, high doses of chemotherapy are given to kill all the remaining myeloma cells in the bone marrow. Unfortunately, 100% of the myeloma cells cannot be killed with chemotherapy. Once the chemotherapy has been given, the healthy, frozen stem cells are re-administered to the patient. These cells reconstitute the bone marrow and immune system. This procedure is used to prolong remission, but unfortunately it is not curative. Occasionally, stem cells from a healthy donor are used rather than a patient's own stem cells. This procedure, called an allogeneic transplant, is much riskier than the procedure using one's own stem cells; however, it has the potential to cure the condition **(Bensinger, 2004)**.

Table (5): Myeloma Treatment Options (Durie, 2008).

1. Chemotherapy
2. High dose-therapy with transplant
3. Radiation
4. Maintenance therapy (e.g., alpha interferon, prednisone)
5. Supportive care:
   - Erythropoietin
   - Bisphosphonates
   - Antibiotics
   - Exercise
   - Emergency care (e.g., dialysis, plasmapheresis, surgery)
   - Pain medication
   - Growth factors
   - Brace/corset
6. Management of drug-resistant or refractory disease
7. New and emerging treatments:
   - Thalidomide and Revlimid® (IMiDs)
   - VELCADE® (proteasome inhibitor) and next-generation proteasome inhibitors in clinical trials
   - Doxil® (pegylated liposomal doxorubicin) to substitute for Adriamycin infusion
   - Trisenox® (arsenic trioxide) and ZIO-101 (organic arsenic) in clinical trials
   - Mini-allo (non-myeloablative) transplant
   - Heat shock protein -90 inhibitors in clinical trials
   - Agents targeting IL-6 and VEGF in clinical trials

Table (6): Some drugs used in treatment of myeloma

- Arsenic trioxide (Trisenox)
- Bortezomib (Velcade)
- Carmustine (BiCNU, BCNU)
- Cyclophosphamide (Cytoxan)
- Dexamethasone (Decadron)
- Doxorubicin (Adriamycin)
- Idarubicin (Idamycin)
- Interferon alfa (Roferon-A, Intron A)
- Lenalidomide (Revlimid)
- Melphalan (Alkeran)
- Pamidronate (Aredia)
- Prednisone
- Thalidomide (Thalomid)
- Vincristine (Oncovin)
- Zoledronic acid (Zometa)

Cell cycle

The cell cycle, or cell-division cycle, is the series of events that takes place in a cell leading to its division and duplication (replication). The cell cycle consists of four distinct phases: G_1 phase, S phase (synthesis), G_2 phase (collectively known as interphase) and M phase (mitosis) as declared in Fig (1) &Table (7). After cell division, each of the daughter cells begin the interphase of a new cycle **(Cooper, 2000)**.

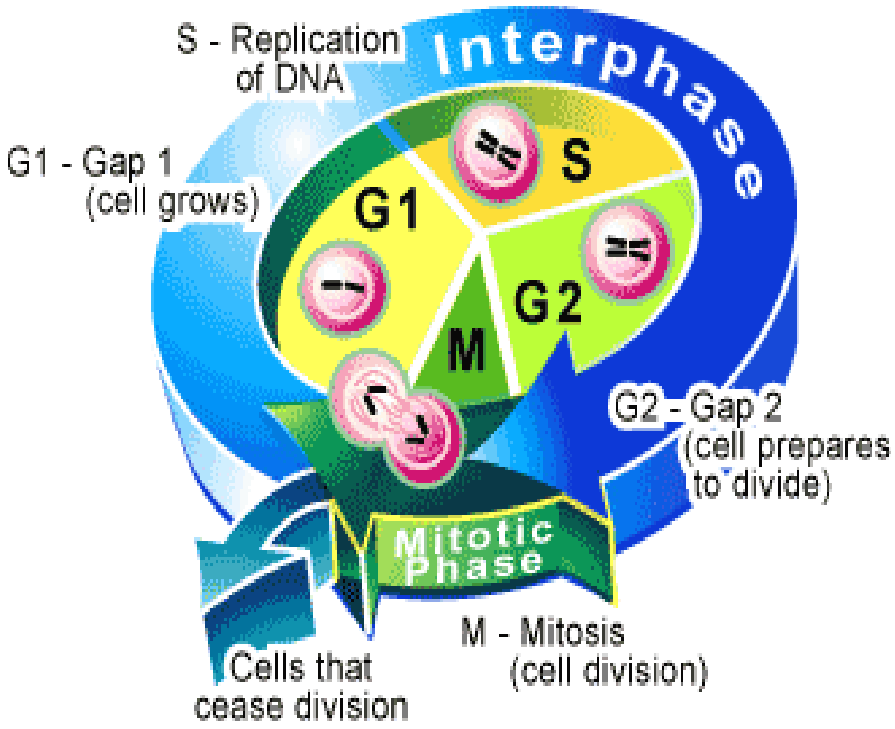

Fig. (1*):* Overview of the cell cycle (David, 2003)

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Table (7): Cell cycle phases

State	Phase	Abbreviation	Description
quiescent/ senescent	Gap 0	**G_0**	A resting phase where the cell has left the cycle and has stopped dividing.
Interphase	Gap 1	**G_1**	Cells increase in size in Gap 1. The *G_1 checkpoint* control mechanism ensures that everything is ready for DNA synthesis.
	Synthesis	**S**	DNA replication occurs during this phase.
	Gap 2	**G_2**	During the gap between DNA synthesis and mitosis, the cell will continue to grow. The *G_2 checkpoint* control mechanism ensures that everything is ready to enter the M (mitosis) phase and divide.
Cell division	Mitosis	**M**	Cell growth stops at this stage and cellular energy is focused on the orderly division into two daughter cells. A checkpoint in the middle of mitosis (*Metaphase Checkpoint*) ensures that the cell is ready to complete cell division.

Several proteins control the timing of the events in the cell cycle, which is tightly regulated to ensure that cells divide only when necessary. The loss of this regulation is the hallmark of cancer. Major control switches of the cell cycle are cyclin-dependent kinases. Each cyclin-dependent kinase forms a complex with a particular cyclin, a protein that binds and activates the cyclin-dependent kinase. The kinase part of the complex is an enzyme that adds a phosphate to various proteins required for progression of a cell through the cycle. These added phosphates alter the structure of the protein and can activate or inactivate the protein, depending on its function. There are specific cyclin-dependent kinase/cyclin complexes at the entry points into the G1, S, and M phases of the cell cycle, as well as additional factors that help prepare the cell to enter S phase and M phase. One important protein in the cell cycle is p53, a transcription factor that binds to DNA, activating transcription of a protein called p21. P21 blocks the activity of a cyclin- dependent kinase required for progression through G1. This block allows time for the cell to repair the DNA before it is replicated. If the DNA damage is so extensive that it cannot be repaired, p53 triggers the cell to commit suicide. The most common mutation leading to cancer is in the gene that makes p53 **(James and Donald, 2005)**.

Apoptosis

Apoptosis, or programmed cell death, is a highly regulated process that allows a cell to self-degrade in order for the body to eliminate unwanted or dysfunctional cells. During apoptosis, the genome of the cell will fracture, the cell will shrink and part of the cell will disintegrate into smaller apoptotic bodies. Unlike necrosis, where the cell dies by swelling and bursting its content in the area, which causes an inflammatory response, apoptosis is a very clean and controlled process where the content of the cell is kept strictly within the cell membrane as it is degraded as shown in Fig(2). The apoptotic cell will be phagocytosed by macrophages before the cell's contents have a chance to leak into the neighborhood **(Raff, 1998)**. Therefore, apoptosis can prevent unnecessary inflammatory response. Apoptosis is essential to embryonic development and the maintenance of homeostasis in multicellular organisms. In humans, for example, the rate of cell growth and cell death is balanced to maintain the weight of the body. During fetal development, cell death helps sculpt body shape, separating digits and making the right neuronal connections. In the immune system, cell death eliminates B cells and T cells that elicit autoimmune response and selects the most efficient lymphocytes to encounter an antigen in the process of affinity maturation **(Philip ,2009).**

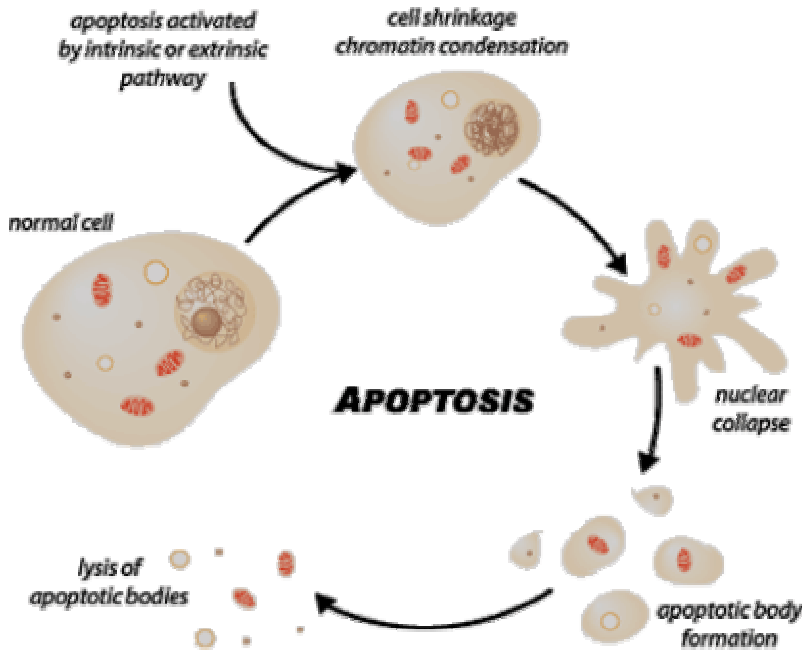

Fig. (2) Apoptosis- the programmed death of a cell (Philip ,2009).

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors, located on the cell membrane. In contrast, the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell **(Philip, 2009)**.

The Extrinsic Pathway: In the extrinsic pathway, signal molecules known as ligands, which are released by other cells, bind to transmembrane death receptors on the target cell to induce apoptosis. For example, the immune system's natural killer cells possess the Fas ligand (FasL) on their surface **(Csipo et al., 1998)**. The binding of the FasL to Fas receptors (a death

receptor) on the target cell will trigger multiple receptors to aggregate together on the surface of the target cell. The aggregation of these receptors recruits an adaptor protein known as Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors. FADD, in turn, recruits caspase-8, an initiator protein, to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 will be activated and it is now able to directly activate caspase-3, an effector protein, to initiate degradation of the cell. Active caspase-8 can also cleave BID protein to tBID, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome c in the intrinsic pathway **(Adrain et al., 2002)**.

The Intrinsic Pathway: The intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage and heat shock (Adrain et al., 2002). Upon receiving the stress signal, the proapoptotic proteins in the cytoplasm, BAX and BID, bind to the outer membrane of the mitochondria to signal the release of the internal content. However, the signal of BAX and BID is not enough to trigger a full release. BAK, another proapoptotic protein that resides within the mitochondria, is also needed to fully promote the release of cytochrome c and the intramembrane content from the mitochondria . Following the release, cytochrome c forms a complex in the cytoplasm with adenosine triphosphate (ATP), an energy molecule, and Apaf-1, an enzyme. Following its formation, the complex will activate caspase-9, an initiator protein. In return, the activated caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form an apoptosome, which in turn activates caspase-3, the effector protein that initiates degradation. Besides the release of cytochrome c from the intramembrane space, the intramembrane content released also contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and

Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP) **(Hague and Paraskeva, 2004)**.

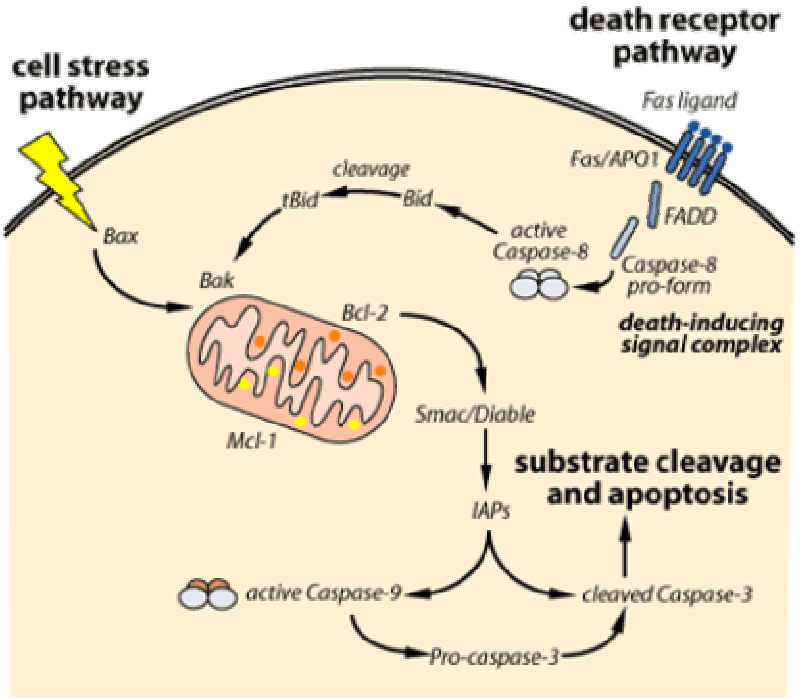

Fig. (3) The intrinsic and extrinsic pathways leading to apoptosis (Philip, 2009).

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Caspases

Caspases, or *c*ysteine-*asp*artic prote*ases* or **c**ysteine-dependent aspartate-directed proteases are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation **(Alnemri et al., 2011)**. Caspases are essential in cells for apoptosis, or programmed cell death, in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. Some caspases are also required in the immune system for the maturation of lymphocytes. Failure of apoptosis is one of the main contributions to tumor development and autoimmune diseases. Based on their proapoptotic functions, the caspases have been divided into two groups: initiators and effectors. First group of initiator (or apical) caspases (caspases-2, -8, -9, -10, and, probably, -11) activate the second-group of caspases (caspases-3, -6, and -7). The effector (or downstream) caspases are able to directly degrade multiple substrates including the structural and regulatory proteins in the cell nucleus, cytoplasm, and cytoskeleton **(Wang et al., 2005)**. Based on their homology in amino acid sequences, Caspases are divided into three subfamilies, as shown in Table (8) **(Ting et al., 2005)**.

Caspases are synthesised as a single chain of inactive zymogens, consisting of four domains: an N-terminal prodomain of variable length, a large subunit with a molecular weight of about 20 kDa, a small subunit (~10 kDa), and a linker region connecting these catalytic subunits (Salvesen and Riedl, 2008). The linker region is missing in some family members. Proteolytic cleavage of the caspase precursors results in the separation of large and small subunits with the production of a hetero-tetrameric complex (the active enzyme) consisting of two large and two small subunits **(Wolf and Green, 1999)**.

Caspases differ in the length and in the amino acid sequence of their N-terminal prodomain. The long prodomain (more than 90 amino acid residues) contains one of two modular regions essential for the interaction with adaptor proteins **(Ghavami et al., 2009).**

Table (8) Subfamily members of caspase family **(Ting et al., 2005)**.

Subfamily	Role	Members
I	Apoptosis activator	Caspase-2
		Caspase-8
		Caspase-9
		Caspase-10
II	Apoptosis executioner	Caspase-3
		Caspase-6
		Caspase-7
III	Inflammatory mediator	Caspase-1
		Caspase-4
		Caspase-5
		Caspase-11
		Caspase-12
		Caspase-13
		Caspase-14

Chemotherapy

Chemotherapy is a method of treating cancer by using one drug or a combination of drugs. Chemotherapy uses powerful drugs that work by slowing or stopping the cancer cells from growing, spreading or multiplying to other parts of the body. Chemotherapy can be used in combination with other treatments such as surgery or radiotherapy, to make sure all cancer cells have been eliminated. Chemotherapy drugs enter the bloodstream and therefore reach all parts of the body. Chemotherapy drugs destroy cancer cells by damaging them so they can't divide and grow. The drugs can also affect normal cells which are growing and dividing quickly and this can cause side effects. These are usually temporary because healthy cells quickly recover and permanent damage is rare with most chemotherapy treatments **(Hirsch, 2006)**.

Chemotherapy may be given in many ways:-

- **Injection**. The chemotherapy is given by a shot in a muscle in of the arm, thigh, or hip or right under the skin in the fatty part of the arm, leg, or belly.
- **Intra-arterial (IA)**. The chemotherapy goes directly into the artery that is feeding the cancer.
- **Intraperitoneal (IP)**. The chemotherapy goes directly into the peritoneal cavity (the area that contains organs such as intestines, stomach, liver, and ovaries).
- **Intravenous (IV)**. The chemotherapy goes directly into a vein.

- **Topically**. The chemotherapy comes in a cream that rubs onto the skin.
- **Orally**. The chemotherapy comes in pills, capsules, or liquids that you swallow.

Goals of chemotherapy

The major goals of chemotherapy are:

1. To destroy the cancer (combination chemotherapy). Chemotherapy can be used by itself or in combination with other treatments to cure many types of cancer.

2. To shrink a tumor before other treatments (neoadjuvant chemotherapy). Sometimes surgery or radiotherapy is more successful if chemotherapy is used first to reduce the size of the cancer tumor **(Chabner and Longo, 2006)**.

3. To destroy residual cancer cells after other treatments (adjuvant chemotherapy). Depending on the type of cancer, surgery and radiation therapy may not be able to remove all the cancer cells from the patient **(Pignon et al., 2000)**. Chemotherapy can be used as a follow-up to destroy any cancer cells that were missed.

4. To prepare the patient for a bone marrow or stem cell transplant (ablative chemotherapy). Some cancers can be treated with bone marrow or stem cell transplants from a healthy donor. Before the transplants take place, the cancer patient's original bone marrow is destroyed using high doses of chemotherapy drugs.

5. To relieve cancer symptoms (palliative chemotherapy). In some cases, chemotherapy can reduce the pain and other symptoms of cancer **(Skeel, 2003)**.

Types of Chemotherapy Drugs

Chemotherapy drugs can be divided into several groups based on factors such as how they work, their chemical structure, and their relationship to another drug. Some chemotherapy drugs are grouped together because they were derived from the same plant. Because some drugs act in more than one way, they may belong to more than one group.

Alkylating agents

Alkylating agents directly damage DNA to prevent the cancer cell from reproducing. As a class of drugs, these agents are not phase-specific; in other words, they work in all phases of the cell cycle. Alkylating agents are used to treat many different cancers, including acute and chronic leukemia, lymphoma, Hodgkin disease, multiple myeloma, sarcoma, as well as cancers of the lung, breast, and ovary. Because these drugs damage DNA, they can cause long-term damage to the bone marrow. In a few rare cases, this can eventually lead to acute leukemia. The risk of leukemia from alkylating agents is "dose-dependent," meaning that the risk is small with lower doses, but goes up as the total amount of drug used gets higher. The risk of leukemia after alkylating agents is highest 5 to 10 years after treatment **(Takimoto and Calvo, 2008)**.

There are many different alkylating agents, including:

- **nitrogen mustards:** such as mechlorethamine (nitrogen mustard), chlorambucil, cyclophosphamide (Cytoxan), ifosfamide, and melphalan
- **nitrosoureas:** which include streptozocin, carmustine (BCNU), and lomustine
- **alkyl sulfonates:** busulfan
- **triazines:** dacarbazine (DTIC), and temozolomide (Temodar)

- **ethylenimines:** thiotepa and altretamine (hexamethylmelamine)

The platinum drugs (cisplatin, carboplatin, and oxalaplatin) are sometimes grouped with alkylating agents because they kill cells in a similar way. These drugs are less likely than the alkylating agents to cause leukemia.

Antimetabolites

Antimetabolites are a class of drugs that interfere with DNA and RNA growth by substituting for the normal building blocks of RNA and DNA. These agents damage cells during the S phase. They are commonly used to treat leukemias, tumors of the breast, ovary, and the intestinal tract, as well as other cancers **(Joensuu , 2008)**. Examples of antimetabolites include 5-fluorouracil (5-FU), capecitabine (Xeloda), 6-mercaptopurine (6-MP), methotrexate, gemcitabine (Gemzar), cytarabine (Ara-C), fludarabine, and pemetrexed (Alimta).

Anti-tumor antibiotics

Anthracyclines

Anthracyclines are anti-tumor antibiotics that interfere with enzymes involved in DNA replication. These agents work in all phases of the cell cycle. Thus, they are widely used for a variety of cancers. Examples of anthracyclines include daunorubicin, doxorubicin (Adriamycin), epirubicin, and idarubicin. Other anti-tumor antibiotics include the drugs actinomycin-D, bleomycin, and mitomycin-C (**Minotti et al., 2004)**. Mitoxantrone is an anti-tumor antibiotic that is similar to doxorubicin in many ways, including the potential for damaging the heart. This drug also acts as a topoisomerase II inhibitor, and can lead to treatment-related leukemia. Mitoxantrone is used to treat prostate cancer, breast cancer, lymphoma, and leukemia **(Katzung, 2006)**.

Topoisomerase inhibitors

These drugs interfere with enzymes called topoisomerases, which help separate the strands of DNA so they can be copied. They are used to treat certain leukemias, as well as lung, ovarian, gastrointestinal, and other cancers. Examples of topoisomerase I inhibitors include topotecan and irinotecan (CPT-11). Examples of topoisomerase II inhibitors include etoposide (VP-16) and teniposide. Mitoxantrone also inhibits topoisomerase II **(Pommier et al., 2010)**.

Mitotic inhibitors

Mitotic inhibitors are often plant alkaloids and other compounds derived from natural products. They can stop mitosis or inhibit enzymes from making proteins needed for cell reproduction. These drugs work during the M phase of the cell cycle, but can damage cells in all phases. They are used to treat many different types of cancer including breast, lung, myelomas, lymphomas, and leukemias. These drugs are known for their potential to cause peripheral nerve damage, which can be a dose-limiting side effect **(Chun et al., 2007)**.

Examples of mitotic inhibitors include:

- **the taxanes:** paclitaxel (Taxol) and docetaxel (Taxotere)
- **epothilones:** ixabepilone (Ixempra)
- **the vinca alkaloids:** vinblastine (Velban), vincristine (Oncovin), and vinorelbine (Navelbine)
- **estramustine** (Emcyt)

Corticosteroids

Steroids are natural hormones and hormone-like drugs that are useful in treating some types of cancer (lymphoma, leukemias, and multiple myeloma), as well as other illnesses. When these drugs are used to kill cancer cells or slow their

growth, they are considered chemotherapy drugs. Corticosteroids are commonly used as *anti-emetics* to help prevent nausea and vomiting caused by chemotherapy, too. They are also used before chemotherapy to help prevent severe allergic reactions (hypersensitivity reactions). When a corticosteroid is used to prevent vomiting or allergic reactions, it is not considered chemotherapy. Examples include prednisone, methylprednisolone (Solumedrol) and dexamethasone (Decadron).

Miscellaneous chemotherapy drugs

Some chemotherapy drugs act in slightly different ways and do not fit well into any of the other categories. Examples include drugs such as L-asparaginase, which is an enzyme, and the proteosome inhibitor such as bortezomib (Velcade).

Other types of cancer drugs

Some other drugs and biological treatments are used to treat cancer, but are not usually considered "chemotherapy." While chemotherapy drugs take advantage of the fact that cancer cells divide rapidly, these other drugs target different properties that set cancer cells apart from normal cells. They often have less serious side effects than those commonly caused by chemotherapy drugs because they are targeted to work mainly on cancer cells, not normal, healthy cells. Many are used along with chemotherapy.

Targeted therapies

As researchers have come to learn more about the inner workings of cancer cells, they have begun to create new drugs that attack cancer cells more specifically than traditional chemotherapy drugs can. Most attack cells with mutant versions of certain genes, or cells that express too many copies of a particular gene. These drugs can be used as part of primary

treatment or after treatment to maintain remission or decrease the chance of recurrence. Only a handful of these drugs are available at this time. Examples include imatinib (Gleevec), gefitinib (Iressa), erlotinib (Tarceva), sunitinib (Sutent) and bortezomib (Velcade).

Differentiating agents

These drugs act on the cancer cells to make them mature into normal cells. Examples include the retinoids, tretinoin (ATRA or Atralin) and bexarotene (Targretin), as well as arsenic trioxide (Arsenox).

Hormone therapy

Drugs in this category are sex hormones, or hormone-like drugs, that alter the action or production of female or male hormones. They are used to slow the growth of breast, prostate, and endometrial (uterine) cancers, which normally grow in response to natural hormones in the body. These cancer treatment hormones do not work in the same ways as standard chemotherapy drugs, but rather by preventing the cancer cell from using the hormone it needs to grow, or by preventing the body from making the hormones **(Laurence et al., 2006)**.

Examples include:

- the anti-estrogens: fulvestrant (Faslodex), tamoxifen, and toremifene (Fareston)
- aromatase inhibitors : anastrozole (Arimidex), exemestane (Aromasin), and letrozole (Femara)
- progestins -- megestrol acetate (Megace)
- estrogens
- anti-androgens -- bicalutamide (Casodex), flutamide (Eulexin), and nilutamde (Nilandron)
- LHRH agonists -- leuprolide (Lupron) and goserelin (Zoladex)

Adverse effects

Chemotherapeutic techniques have a range of side effects that depend on the type of medications used. The most common medications mainly affect the fast-dividing cells of the body, such as blood cells and the cells lining the mouth, stomach, and intestines. Common side effect side effects include:

Nausea and vomiting/loss of appetite

Nausea and vomiting are common, but can usually be controlled by taking anti-nausea drugs, drinking enough fluids, and avoiding spicy foods. Loss of appetite (anorexia) may be due to nausea or the stress of undergoing cancer treatment. Drugs that have a high likelihood of causing nausea or vomiting include cisplatin, mechlorethamine, streptozocin, dacarbazine, carmustine, and dactinomycin. Those with moderate nausea-inducing potential include cyclophosphamide, doxorubicin, carboplatin, mitomycin, and L-asparaginase. Anticancer drugs with a low chance of causing nausea or vomiting include fluorouracil, methotrexate, etoposide, vincristine, and bleomycin **(Valerie, 2009).**

Hair loss

Some chemotherapy drugs cause hair loss (alopecia), but it is almost always temporary. Hair re-growth may not begin until several weeks have passed since the final treatment. This is the most common impact that chemotherapy has on the outer surfaces of the body **(Lynne, 2012).**

Anemia and fatigue

Low blood cell counts caused by the effect of chemotherapy on the bone marrow can lead to anemia, infections, and easy bleeding and bruising. Patients with anemia have too few red blood cells to deliver oxygen and nutrients to the body's tissues. Anemic patients feel tired and weak. If red blood cell levels fall too low, a blood transfusion may be given.

Infections

Patients receiving chemotherapy are more likely to get infections. This happens because their infection fighting white blood cells are reduced. The level of reduction can vary depending on the dose and schedule of treatments, and whether the drug is used alone or in combination with other anticancer agents. It is important for chemotherapy patients to avoid infection. When the white blood cell count drops too low, the doctor may prescribe medications called colony stimulating factors that help white blood cells grow. Neupogen and Leukine are two colony stimulants used as treatments to help fight infection **(Huang., 2000)**.

Easy bleeding and bruising

Platelets are blood cells that make the blood clot. When patients do not have enough platelets, they may bleed or bruise easily, even from small injuries. Patients with low blood platelets should take precautions to avoid injuries. Medicines such as aspirin and other pain relievers can affect platelets and slow down the clotting process.

Sores in the mouth

Chemotherapy can cause irritation and dryness in the mouth and throat. An inflammation in the mouth is called **stomatitis**. Painful sores may form that can bleed and become infected. Precautions to avoid this side effect include getting dental care before chemotherapy begins, brushing the teeth and gums regularly with a soft brush, and avoiding mouth washes that contain salt or alcohol. Good oral hygiene is important **(Lynne, 2012)**

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Neuropathy and other damage to the nervous system

Cancer patients may develop neurological problems due to the cancer or the anticancer drugs. A variety of problems can develop, including altered mental alertness, changes in taste and smell, seizures, and peripheral neuropathy (tingling and burning sensations and/or weakness or numbness in the hands and/or feet). Different drugs can lead to different types of neurological disorders **(Tannock et al., 2004)**.

Heart damage

Some anticancer drugs are damaging to the heart. In these cases, the dosage is closely monitored in an attempt to avoid heart damage. Specific drugs that may be toxic to the heart include doxorubicin, daunorubicin, high doses of cyclophosphamide, and in some cases 5-FU.

Kidney damage

A number of anticancer drugs can damage the kidney. Examples include high doses of methotrexate or 6- MP, as well as regular doses of L-asparaginase, cisplatin, mithramycin, streptozocin, and mitomycin C. Some kidney problems can be lessened by taking in adequate amounts of fluids. A secondary danger of kidney damage is that a less functional kidney can be more susceptible to further toxicity caused by other anticancer drugs that the patient is taking **(Chun et al., 2007)**.

Respiratory problems

Cancer patients who have had radiation in the chest area are more susceptible to respiratory complications. Nitrosourea or bleomycin cause the most common type of respiratory toxicity, called pulmonary fibrosis.

Sexual function

Some drugs can lead to impaired sexual function. Alkylating agents and procarbazine may result in the absence of sperm in a man and the lack of menstruation in a woman. Patients of child-bearing age are usually told to refrain from conceiving while undergoing chemotherapy because of the defects it can cause in the fetus.

Vision problems

Some anticancer drugs can impact a person's vision. High doses of cyclophosphamide can cause blurred vision in children, while some alkylating agents can cause cataracts. Tamoxifen may be damaging to the retina, and cisplatin can damage the optic nerve. Conjunctivitis, commonly called pinkeye, is a treatable problem that occurs with many anticancer drugs.

Proteasomes

Proteasomes are protein degradative machines that are found in the nucleus and the cytoplasm. They are not only found in all eukaryotic organisms, but have also been found in archaebacteria. Proteasomes play an important role in the cell's life:

1. They remove abnormal and misfolded proteins from the cell.

2. They are involved in the cell's stress response, where they degrade Ub-conjugated regulatory proteins.

3. As part of the Ub system, they are involved in regulating the cell cycle.

4. They are involved in cellular differentiation (where they degrade transcription factors and metabolic enzymes).

5. They play an important role in the immune system by generating antigenic peptides that are presented by the major histocompatibility complex (MHC) class I molecules **(Lodish et al ., 2004).**

Protein degradation is as essential to the cell as protein synthesis. For example,

- to supply amino acids for fresh protein synthesis
- to remove excess enzymes
- to remove transcription factors that are no longer needed.

The 26S proteasome is a large multi-subunit complex critical for maintaining cell homeostasis through the destruction of cell signaling and regulatory proteins. It is composed of three major components: two 19S regulatory subunits and the 20S proteolytic core as given in (Fig. 4). The 20S core is made up of 2 α-

subunits and 2 β-subunits. The β-subunits contain the proteolytic activity including atryptic site, chymotryptic site, and a peptidylglutamyl site **(Almond and Cohen, 2002)**.

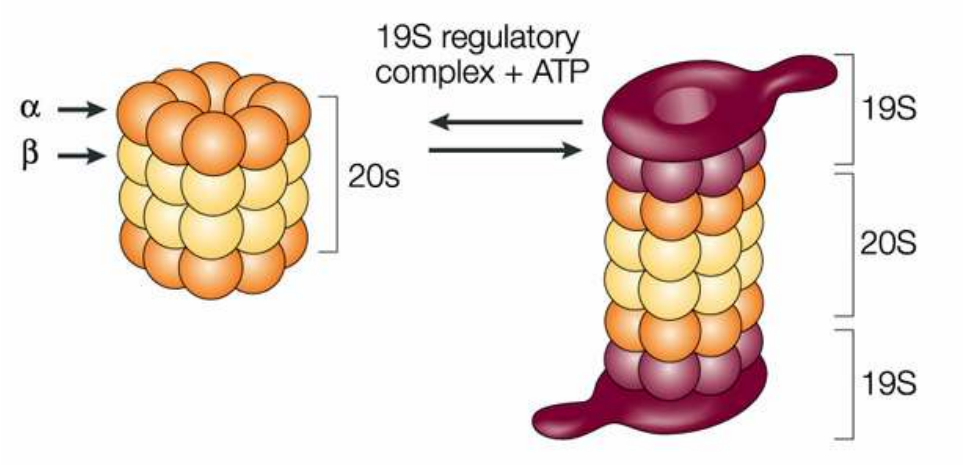

Fig. (4) :Proteasome structure (Julian A,2004).

Bortezomib inhibits the 26S proteasome by reversibly binding to the β5 subunit of the 20S core and inhibiting its chymotrypsin-like activity, thus disabling the ability of the proteasome to breakdown proteins and preventing further protein entry into the proteasome **(McConkey et al., 2008)**.

Bortezomib (*Velcade*)

Bortezomib (originally codenamed **PS-341**; marketed as **Velcade** by Millennium Pharmaceuticals) is the first therapeutic proteasome inhibitor to be tested in humans. It is approved in the U.S. for treating relapsed multiple myeloma and mantle cell lymphoma. In multiple myeloma, complete clinical responses have been obtained in patients with otherwise refractory or rapidly advancing disease **(Takimoto and Calvo, 2008)**.

Structure of Velcade

The chemical name for bortezomib, the monomeric boronic acid, is [(1R)-3-methyl-1-[[(2S)-1-oxo-3-phenyl-2-[(pyrazinyl-carbonyl) amino]propyl]amino]butyl] boronic acid.

Bortezomib has the following chemical structure:

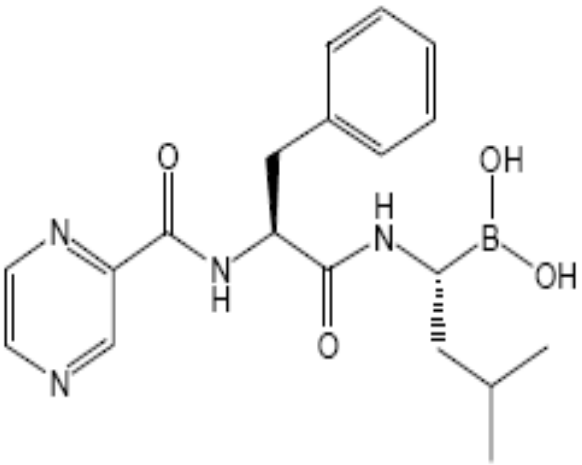

Fig. (5): Chemical Structure of Bortezomib (Andrey et al., 2011)

The molecular weight is 384.24Dalton. The molecular formula is $C_{19}H_{25}BN_4O_4$.The solubility of bortezomib, as the monomeric

boronic acid, in water is 3.3 to 3.8 mg/mL in a pH range of 2 to 6.5 **(Andrey et al., 2011)**.

Mechanism of action

Bortezomib works by inhibiting enzyme complex called proteasomes. Both normal cells and cancer cells contain proteasomes, which breakdown damaged and unwanted proteins into smaller components. Proteasomes also carry out the regulated breakdown of undamaged proteins in the cell, a process that is necessary for the control of many critical cellular functions. These smaller components are then used to create new proteins required by the cell. Proteasomes can be thought of as crucial to the cell's "recycling" of proteins. When Velcade inhibits proteasomes; the normal balance within a cell is disrupted. When proteasomes are inhibited in laboratory tests, cancer cells stop dividing. They also stop producing chemicals to stimulate other cancer cells. In addition, inhibition of proteasomes has caused cancer cells to die. Cancer cells appear to be more sensitive to these effects than normal cells, so that cancer cells die while normal cells can recover **(Irene et al., 2011).**

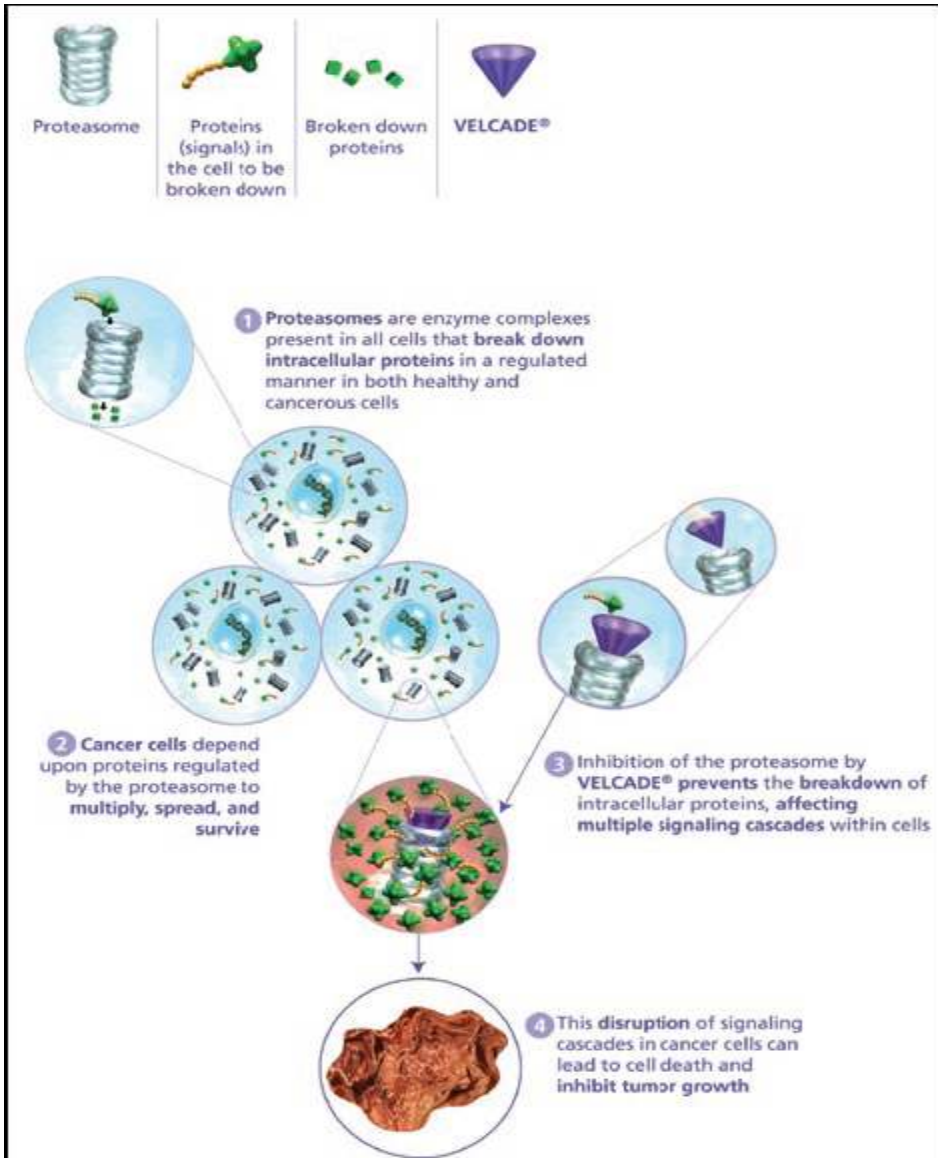

Fig. (6): Mechanism of action of Bortezomib (Irene et al., 2011).

By blocking the proteasome, Velcade can affect the growth and survival of myeloma cells through multiple mechanisms of action. **These include:**

1- Preventing the myeloma cells from sticking to bone marrow cells

2- Inhibiting the growth of new blood vessels which supply the myeloma cells with oxygen and nutrition

3- Altering the production of chemicals crucial for the growth and survival of myeloma cells

4- Velcade may also have a direct killing (cytotoxic) effect on the myeloma cells.

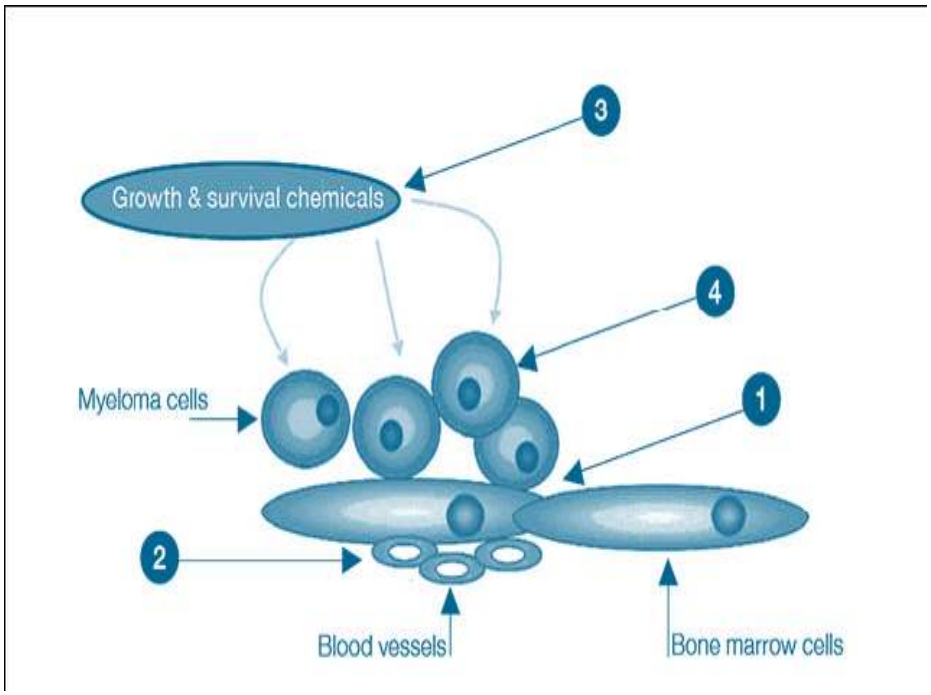

Fig. (7): The different ways that Bortezomib (Velcade) can affect multiple myeloma (Irene et al., 2011).

Side effects

Generally, most of the side effects associated with Velcade are manageable and predictable **(Kane et al., 2003)**.

Table (9): Possible Side Effects of Bortezomib

Common:	Less Common:
• Tiredness (fatigue) and weakness • Nausea and vomiting • Diarrhea and constipation • Decreased appetite • Decreased platelet count with increased risk of bleeding • Decreased red blood cell count with increased risk of anemia • Peripheral neuropathy (numbness, tingling, pain, or a burning feeling in the feet or hands) • Fever or fainting	• Headache • Decreased white blood cell count with increased risk of infection • Dehydration • Swelling • Problems with sleep • Difficulty breathing • Muscle cramps, muscle or joint pain • Dizziness, lightheadedness, • Blurred vision • Low blood pressure

Interferons

Interferons (IFNs) are proteins made and released by host cells in response to the presence of pathogens such as viruses, bacteria, or parasites or tumor cells. They allow for communication between cells to trigger the protective defenses of the immune system that eradicate pathogens or tumors. IFNs belong to the large class of glycoproteins known as cytokines.The IFNs are classified by their ability to bind to specific receptors termed Type I, Type II, and the recently-described Type III IFN receptors **(Hervas et al., 2011)**.

Type I Interferons

Type I IFNs, which include IFN-α and IFN- β have emerged as the most clinically useful IFNs for the treatment of cancer. They are secreted by nearly every cell in the body and are predominantly involved in cellular immune responses against viral infections **(Constantinescu et al., 1994)**. Type I IFNs induce expression of major histocompatibility complex (MHC) class I molecules on tumor cells and mediate the maturation of a subset of dendritic cells (DC)**(Trepiakas et al.,2009)**.They can also activate cytotoxic T lymphocytes)(CTLs), natural killer (NK) cells and macrophages . In addition to their immunologic effects ،the Type I IFNs can exert cytostatic and possibly apoptotic effects on tumor cells as well as anti-angiogenic effects on tumor neovasculature **(Sylvia and Kim, 2011)**.

Type II Interferons

The only member of this family is IFN-γ, which binds to a distinct receptor complex (IFN γ R1 and IFN γ R2) **(Pestka, 2007)**. The Type II IFN receptor is a subset of the type II cytokine receptors **(Mackall et al., 2011)**.

Type III Interferons

This recently-discovered family consists of IFN-λ1, IFN-λ2, and IFN- λ3 which activate an IL-10 receptor 2 subunit and IL-28 receptor α subunit complex **(Sylvia and Kim, 2011)**.

Possible Mechanisms For IFN'S Antitumour Action

The IFN may have direct effects on the tumour cells, for example it may be cytotoxic, affect the proliferation of the tumour cells, or induce cellular differentiation. Alternatively, or in addition, the IFN may have indirect effects for example on host immune functions, the tumour stromal cells, or the vascularization of the tumour. Several of these possibilities have been investigated in myeloma **(Michele et al., 2004)**.

Table(10)Possible mechanisms for IFNs antitumour action in myeloma

Direct effects
Antiproliferative
Cytotoxic:Pro-apoptotic effects on tumour cells
Sensitization to Fas-killing
Abrogation of growth:survival factor signalling
Synergism with chemotherapeutic agents
Indirect effects
Stimulation of immunological functions
Activation of cytotoxic T-lymphocytes
Activation of NK-cells
Activation of monocytes
Non-immunological host effects
Antiangiogenic effects
Interaction between stroma and malignant cells
Regulation of cytokine production

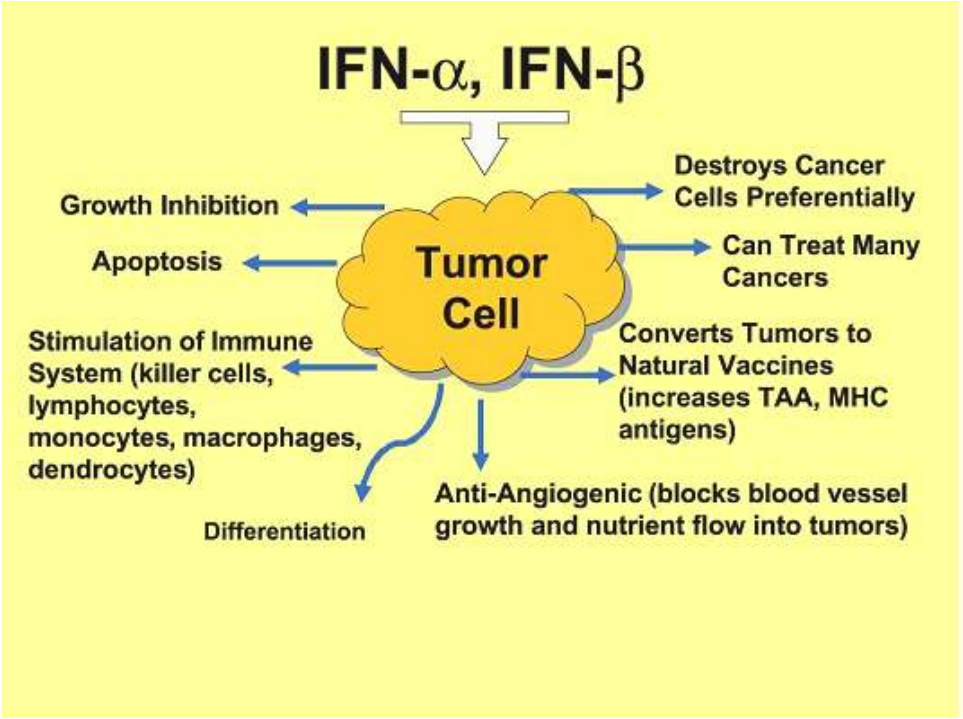

Fig.(8): Antitumor actions of interferons (Pestka, 2003).

Immunity and Immune system:

Immunity is defined as resistance to disease, specifically infections disease. The collection of cells, tissues, and molecules that mediate resistance to infections is called the immune system, and the coordinated reaction of these cells and molecules to infections microbes is the immune response.

Host defense mechanisms consist of innate immunity, which mediates the initial protection against infections, and adaptive immunity, which develops more slowly and mediates the later, even more effective, defense against infections .

Innate immunity

The term innate immunity (also called natural or native immunity) refers to the fact that this type of host defense is always present in healthy individuals, prepared to block the entry of microbes and to rapidly eliminate microbes that do succeed in entering host tissues.

The first line of defense in innate immunity is provided by epithelial barriers and by specialized cells and natural antibiotics present in epithelia, all of which function to block the entry of microbes. If microbes do breach epithelia and enter the tissues or circulation, they are attacked by phagocytes, specialized lymphocytes called natural killer (NK) cells, and several plasma proteins, including the proteins of the complement systems. All these mechanisms of innate immunity specifically recognize and react against microbes but do not react against noninfectious foreign substances. Different mechanisms of innate immunity may be specific for molecules produced by different classes of microbes. In addition to providing early defense against infections, innate immune

responses against infectious agents **(Abbas and Lichtman, 2004).**

Adaptive immunity

Adaptive immunity (also called specific or acquired immunity) is the type of host defense that is stimulated by microbes that invade tissues, that is, it adapts to the presence of microbial invaders.

The adaptive immune system consists of lymphocytes and their products, such as antibodies. Whereas the mechanism of innate immunity recognize structures shared by classes of microbes , the cells of adaptive immunity, namely , lymphocytes,express receptors that specifically recognize different substances produced by microbes as well as noninfectious molecules. These substances are called antigens. Adaptive immune responses are only triggered if microbes or their antigens pass through epithelial barriers and delivered to lymphoid organs where they can be recognized by lymphocytes. Adaptive immune responses generate mechanisms that are specialized to combat different types of infectious. For example, antibodies function to eliminate microbes in extracellular fluids, and activated T lymphocytes eliminate microbes living inside cells. For instance, antibodies (a component of adaptive immunity) bind to microbes, and these coated microbes avidly bind to and activate phagocytes (a component of innate immunity), which ingest and destroy the microbes.

There are two types of adaptive immunity, called humoral immunity and cell- mediated immunity that are mediated by different cells and molecules and are designed to provide defense against extracellular microbes and intracellular microbes, respectively as shown in (Fig.9). Humoral immunity is mediated by proteins called antibodies, which are produced by cells called B lymphocytes. Antibodies are secreted into the

circulation and mucosal fluids, and they neutralize and eliminate microbes and microbial toxins that are present in the blood and in the lumens of mucosal organs, such as the gastrointestinal and respiratory tracts. One of the most important functions of antibodies is to stop microbes that are present at mucosal surfaces and in the blood from gaining access to and colonizing host cells and connective tissues **(Charles et al., 2001)**.

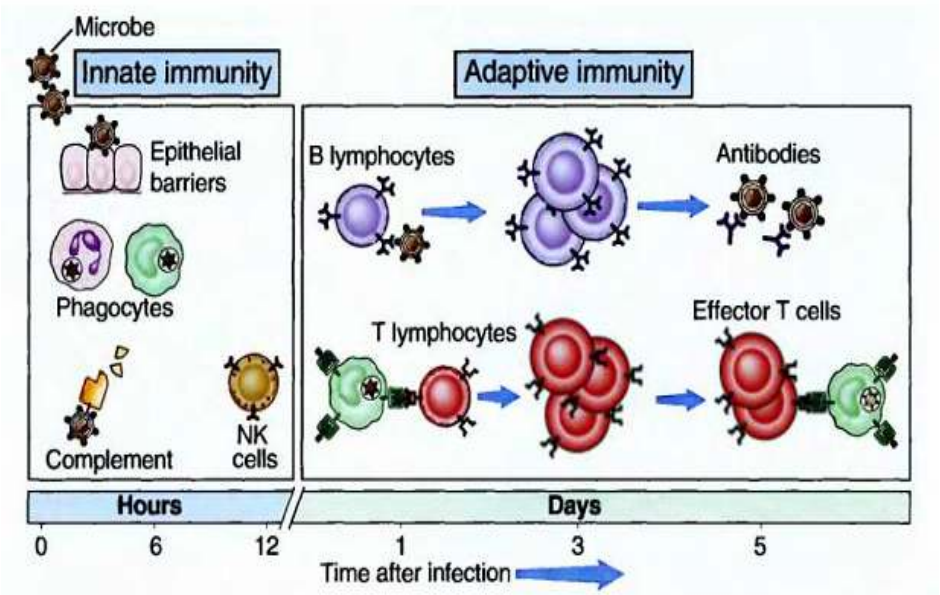

Fig. (9): The principal mechanism of innate and adaptive immunity (Abbas and Lichtman, 2004).

Defense against such intracellular microbes is called cell-mediated immunity because cells called T-lymphocytes mediate it. Some T-lymphocytes activate phagocytes to destroy microbes that have been ingested by the phagocytes into phagocyctic vesicles. The antibodies produced by B-lymphocytes are designed to specifically recognize antigens produced by intracellular microbes. Another important difference between B and T lymphocytes is that most T cells recognize only microbial protein antigens, whereas antibodies

are able to recognize many different types of microbial molecules, including proteins, carbohydrates, and lipids. Immunity may be induced in an individual by infection or vaccination (active immunity) or conferred on an individual by transfer of antibodies or lymphocytes from an actively immunized individual (passive immunity) **(Abbas and Lichtman, 2004).**

Antibodies

Antibodies (also known as immunoglobulins, abbreviated Ig) are heavy (~150 kDa) globular plasma proteins that are found in blood or other bodily fluids of vertebrates, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses **(Litman et al.,1993)**. They are typically made of basic structural units as presents in (Fig.10) each with two large heavy chains and two small light chains to form, for example, monomers with one unit, dimers with two units or pentamers with five units. Antibodies are produced by a kind of white blood cell called a plasma cell. There are several different types of antibody heavy chains, and several different kinds of antibodies, which are grouped into different isotypes based on which heavy chain they possess. Five different antibody isotypes are known in mammals, which perform different roles, and help direct the appropriate immune response for each different type of foreign object they encounter **(Eleonora and Nina, 2003).**

Though the general structure of all antibodies is very similar, a small region at the tip of the protein is extremely variable, allowing millions of antibodies with slightly different tip structures, or antigen binding sites, to exist. This region is known as the hypervariable region. Each of these variants can bind to a different target, known as an antigen **(Janeway, 2001)**. This huge diversity of antibodies allows the immune system to recognize an equally wide diversity of antigens. The

unique part of the antigen recognized by an antibody is called an epitope. These epitopes bind with their antibody in a highly specific interaction, called induced fit, that allows antibodies to identify and bind only their unique antigen in the midst of the millions of different molecules that make up an organism. Recognition of an antigen by an antibody tags it for attack by other parts of the immune system. Antibodies can also neutralize targets directly by, for example, binding to a part of a pathogen that it needs to cause an infection **(Rhoades and Pflanzer, 2002)**.

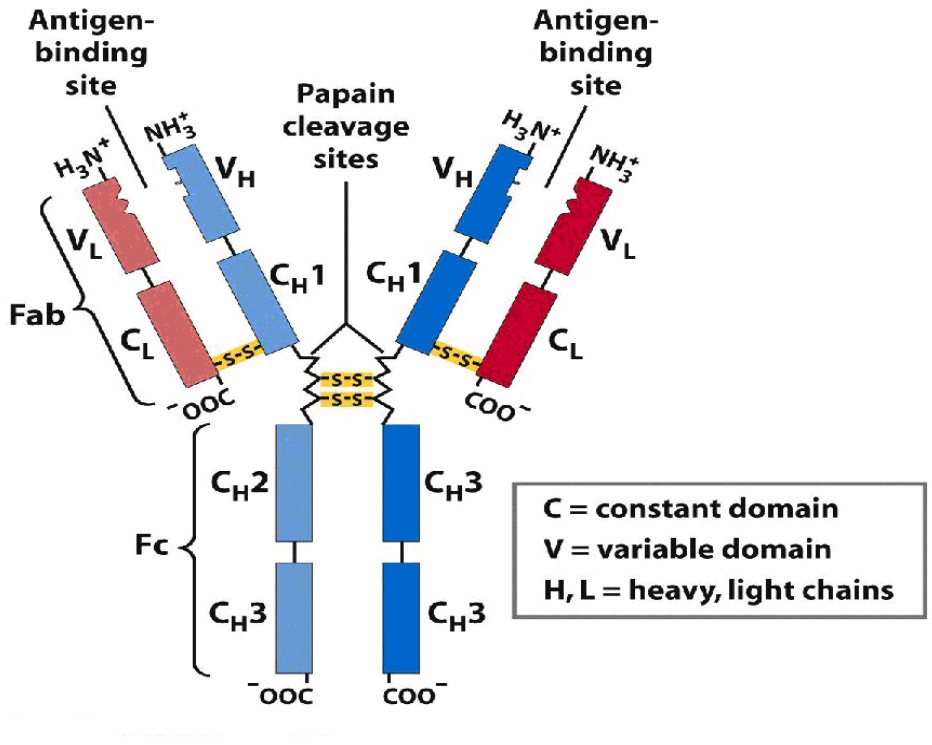

Fig (10): General structure of Antibodies (David and Michael, 2008).

Production of Antibodies:

Antibodies are serum immunoglobulins (Igs) that have binding specifically with particular antigens. Antibodies are therefore have an enormous utility in applications such as experimental biology, medicine, biomedical research, diagnostic testing, and therapy. Polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) can be used for these purposes, although the production of these antibodies requires the use of substantial numbers of animals with considerable animal welfare consequences. In the case of PAbs, animals are given injections of antigen or antigen/adjuvant mixtures for the induction of effective antibody responses, and it is usually necessary to collect blood to monitor antibody response during the experiment and to obtain the antibodies. In the case of MAbs, animals are given injections of antigen or antigen/adjuvant mixtures to induce specific B cells that are obtained from the spleen or lymph nodes to establish hybridomas. When in vitro production is not feasible, it is necessary to use animals for the production of antibodies by hybridomas in the abdominal cavity **(Marlies and Coenraad., 2005)**

Production of Polyclonal Antibodies:

Production of polyclonal antibodies include the following critical steps: (1) preparation of the antigen, (2) selection of the animal species, (3) selection and preparation of the adjuvant, (4) route of injection, (5) dosage and injection Schedule, and (6) blood collection which containing the polyclonal antibodies.

(1) Preparation of the Immunogen:-

It is important, when antibodies are produced, to consider immunogen features, which include the quality and quantity of the immunogen and the immunogen preparation. The specificity of the immune response obtained depends on the purity of the immunogen applied **(Leenaars et al., 1997)**.

(2)Selection of the Animal Species

It is important, when selecting the animal species for polyclonal antibodies production, to consider the following: (1) the amount of polyclonal antibodies needed, (2) the ease of obtaining blood samples, (3) the phylogenetic relationship between the antigen source and the animal species, and (4) the intended use of the polyclonal antibodies. The most frequently used animal species for polyclonal antibodies induction in the laboratory setting are the rabbit, mouse, rat, hamster, guinea pig, goat, sheep, and chicken **(Hanly et al.,1995)**. For the production of polyclonal antibodies, rabbits are used most often because of their convenient size, ease of handling and bleeding, relatively long life span, and adequate production of high-titer, high-affinity, precipitating antiserum **(Stills, 1994)**. When larger amounts of polyclonal antibodies are needed, farm animals such as sheep, goats, and horses are usually used. In some cases requiring large amounts of polyclonal antibodies, chickens may be used **(Erhard et al., 2000 and Schade et al., 1996)**.

The selection of an animal species is also influenced by the species from which the antigen is taken. The greater the phylogenetic distance between the animal species that is the source of the antigen and the species of the animal to be immunized, the better the immune response that will be evoked. The age of the animals is another important consideration because of that factor can influence the outcome of the immunization **(Hanly et al., 1995 and Hendriksen and Hau, 2003)**. In addition to the species, the sex of the animals must

also be decided. Traditionally, female animals are used for polyclonal antibodies production in as much as these animals can be group housed more successfully than males because females are more docile and less aggressive in social interaction **(Hendriksen and Hau, 2003).**

(3) Selection and Preparation of the Adjuvant:-

When the antigen to which antibodies are to be evoked is poorly immunogenic, the immune system requires a stimulus to induce an effective immune response. Adjuvants can be used for this purpose, and can direct an immune response against a more cellular or humoral response **(Cox and Coulter, 1997)**. There are various widely used adjuvants. One adjuvant is Freund's complete adjuvant (FCA), which consists of mineral oil mixed with detergent in aqueous solution and killed Mycobacterium Tuberculosis or Mycobacterium Butyrium to form water in oil emulsion. The antigen is contained in the water phase. Other microrganisms used as adjuvants are Bacilli Calmette-Guerin (BCG) is an attenuated strain Mycobacterium, Corynebacterium Parvum, and Bordetella Pertusis. Other adjuvants used are bacterial endotoxins and lipopolysaccharide (LPS) **(Benjamini and Leskowitz, 1991)**. Freund's incomplete adjuvant (FIA) lacks the added bacteria. Conventionally, primary immunization is made with FAC. The water in oil emulsion affects slow release of the antigen providing a semi-continues challenger to the immune system. The killed bacteria stimulate the T- helper lymphocytes to affect the immune response. Subsequent immunization can be in FAI as T-helper lymphocytes retain many of the initial challenger **(Edwards, 1990)**. In addition to the selection of the adjuvant, the preparation of the antigen/adjuvant mixtures may influence the outcome of the immunization experiment. With aseptic preparations, the first critical step is to minimize potential contamination, which may affect the quantity and quality of the antibodies as well as the welfare of the animal. The second step

in the case of an emulsion (e.g., FCA and FIA are water-in-oil emulsions) is to check the stability and quality of the emulsion. When FCA or FIA is used, a thick emulsion is the result of mixing the antigen and adjuvant **(Lindblad 2000).** The advantage of a thick emulsion is that it effectively protects the antigen against a quick degradation. The antigen is instead slowly released out of this emulsion and is therefore present a relatively long time for endocytosis and antigen presentation.

(4)Route of Injection

A variety of immunization procedures are available, however, no controlled study has been performed demonstrating the superiority of one technique over other **(Neischlag and Wickings, 1975)**. Intra-splenic, footpad, subcutaneaous and intra-muscular injections represent various routes for immunization. A variety evidence suggested that various routes of administration may be ranked in decreasing order of effectiveness as follows: lymph nodes > intra-articular > intra-muscular > intra-peritoneal > subcutaneous > intravenous **(Narra and Miller, 1975)**. The intra-dermal injection is widely used, intra-muscular, intra-peritoneal and intra-venous injection are rarely used, while footpad injection is extremely painful to the animal and should never be used **(Chard, 1982).**

(5) Dosage and Injection Schedule

Regardless of the mixture to be injected, it is imperative to use the smallest possible volume that induces a sufficient antibody response. The minimal volume depends on particular characteristics of the immunogen. Booster injections are always needed to obtain anti-sera of good titer and avidity. Practical experience suggested good results will be obtained using a booster dose given via the same routes (not necessary at the

same site) and using Freund's adjuvant incomplete on each occasion **(Hurn and Chantler, 1980)**.

(6) Blood Collection and Serum Separation

Various methods for blood collection from rabbits used, such as cardiac puncture, orbital sinus, nail insertion and auricular vein. These methods have danger to the life of the donor animal, poor volume yield, contamination and time consumed in obtaining large volume of blood **(Tiedong et al., 2004)**. A simple techniques for bleeding and withdrawing blood from rabbits ear vein has been describes by **Hope et al., 1969.**The procedure is efficient and not pernicious. Therefore, blood may be repeatedly collected from the same animal. It has been reported that blood collection is usually performed from the veins or can be collected from arteries of the rabbit's ear. For test bleeds, only a small quantity of blood is taken and the animal allowed to recovering. When the animal serum contains a high level of antibody, it may be advisable to sacrifice it and obtain a large volume of blood. Alternatively, weekly bleeding of rabbits and large animals will yield a lot of blood over one or two months. Antibodies are present in the serum fraction of blood so that the plasma must be separated from the blood as soon as possible after collection of the blood **(Johnstone and Thorpe, 1989).**

Preparation of Radiolabeled Antibodies

The choice of labeling method depends upon several factors, i.e preservation of the biological activity of the original molecules, sensitivity of the molecules to oxidation and the desired specific activity **(McFarlance, 1958)**. Iodination methods can be carried out using different methods as follows:

1. Insoluble oxidizing agents (Iodogen) method using water insoluble chloramide 1, 3, 4, 6- tetrachloro 3-α,6- α - diphenylglycoluril (Iodogen). The disadvantage of this method includes relatively long reaction time **(Fraker and Speck, 1978)**.

2- Enzymatic radioiodination method using enzyme lactoperoxidase to catalyze the oxidation of ^{125}I in the presence of very small amounts of hydrogen peroxide (H_2O_2) **(Marchalonis, 1969)**.

3- The chloramine -T (Ch-T) method is the most widely used method for radioiodination of small masses of protein, peptides and haptens or other molecules (containing a tyrosine ring) **(Hunter, 1971)**.

Ch-T is the sodium salt of the N-monochloro derivative of P-toluene sulfonamide. The Ch-T reaction is technically simple and rapid to perform **(Sherman et al., 1974)**. Na^{125} is oxidized by Ch-T in thepresence of the iodothyronines to be labeled, with the subsequent incorporation of [I^{125}] iodine into tyrosine-residues of iodothyronines in high yield **(Hunter, 1971 and Edwards, 1996)**. In aqueous solution, it breaks down slowly forming hypochlorous acid and is consequently a mild oxidizing agent. In the presence of Ch-T under slight alkaline conditions (pH 7.5), Na ^{125}I is oxidized forming cationic iodine +I. The iodine atoms substitute ortho to the hydroxyl group in the phenolic ring of tyrosine **(Bolton, 1985)**. Excess Ch-T is reduced by the addition of sodium metabisulphite as a reducing agent and free iodine is reduced to iodide .After the addition of an excess of unlabeled NaI or KI to act as acarrier for the high specific activity (I^{125}) iodide and a protein containing buffer to act as carrier for the labeled protein. The two are separated from each other by any suitable technique, often gel filtration **(John, 1996 and Lye and Hegesippe, 1984).**

Immunotherapy

Immunotherapy therapy (sometimes called biological, biotherapy, or biological response modifier therapy) is a relatively new addition to the family of cancer treatments that also includes surgery, chemotherapy, and radiation therapy. Biological therapies use the body's immune system, either directly or indirectly, to fight cancer or to lessen the side effects that may be caused by some cancer treatments.

Immunotherapy may be used to:

- Stop, control, or suppress processes that permit cancer growth.
- Make cancer cells more recognizable and, therefore, more susceptible to destruction by the immune system.
- Boost the killing power of immune system cells, such as T cells, NK cells, and macrophages.
- Alter the growth patterns of cancer cells to promote behavior like that of healthy cells.
- Block or reverse the process that changes a normal cell or a precancerous cell into a cancerous cell.
- Enhance the body's ability to repair or replace normal cells damaged or destroyed by other forms of cancer treatment, such as chemotherapy or radiation therapy.
- Prevent cancer cells from spreading to other parts of the body.

Immunotherapeutic approaches may be classified as shown in Table (11) as “tumor-specific” verus “tumor-

nonspecific" and active (stimulation of the host immune system) verus passive immunotherapies (transfer of effector molecules or cells, such as antibodies or CTLs, respectively, to the patient.Active specific immunotherapy aims to generate specific antitumor response through stimulation of the host immune system and is represented by cancer vaccines that usually have minimal toxicity profiles. Conversely, active nonspecific immunotherapies tend to have more significant side effects than the other immunotherapeutic modalities; they are probably the most toxic regimens but have also generated some of the most significant clinical responses to date.

Passive specific immunotherapy is another category of immunotherapy and involves the administration of reactive effector molecules, such as antibodies, or effector cells, such as CTLs.The recent clinical results obtained from antibody-mediated responses have rekindled much excitement for this modality.

Nonspecific passive immunotherapy is currently not frequently used. In the past, LAK cell therapy, representative of nonspecific passive immunotherapy, exhibited some successes, but it has now been replaced by the less operose and comparably effective single-agent IL-2 therapy **(Ronald et al., 2002).**

Table (11) Classification of Cancer immunotherapy **(Ronald et al., 2002).**

	Specific	*Nonspecific*
Active	Cancer vaccines Tumor cell vaccines Tumor lysates/ oncolysates Recombinate viral vaccines Peptide vaccines Naked DNA/RNA DC vaccines	Interferon alpha Interleukin 2 Tumor necrosis factor α Adjuvants Bacille Calmette - Guerin Dotex Coley,s toxins
Passive or Adoptive	Antibody therapy Lymphokine-activated NK cells Tumor- infiltrating lymophocyte Activated T cells	

Antibody Therapeutics

Antibody therapeutic mechanism through which antitumor antibody can mediate tumor cell killing directly or indirectly was presented in (Fig.11). Tumor specific antibodies will bind to tumors through recognition of tumor membranes antigens by the Fab portion may then be bound by the fc receptor on the surface of natural killer (NK) cells, resulting in lysis of the tumor cell. This process is termed antibody-dependent cellular cytotoxicity (ADCC) **(Santoni et al., 1979)**. Opsonization of tumor cells by antibodies can also recruit macrophages and, through phagocytosis, effect tumor cell killing by the macrophages. Additionally, bound antibodies on tumor cell membranes can also activate the complement cascade and effect

killing of the tumor cells through formation of membrane- lysis complexes. Recently, chemical modification of antibodies generated a family of conjugates that can affect tumor cell killing by specific targeting of a toxic molecular entity to the tumor cells mediated by the tumor-binding specificity of the antibodies **(Komen and John, 2002)**.

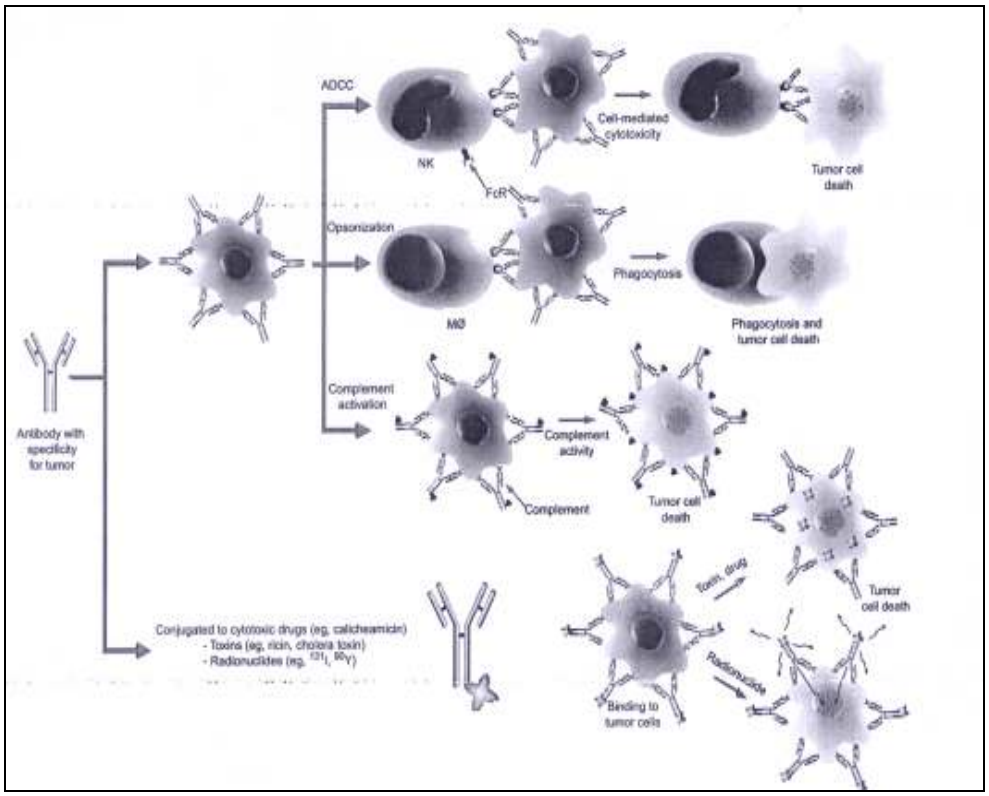

Fig. (11) Antibody therapeutics. Mechanisms through which antitumor antibody can mediate tumor cell killing directly (ADCC, Opsonization, complement activation) or indirectly through delivery of a cytotoxic radionuclide, drug, or other toxin to tumor) (Komen and John, 2002).

Radioimmunotherapy (RIT)

Radioimmunotherapy (RIT) uses an antibody labeled with a suitable radionuclide to deliver cytotoxic radiation to a target cell. In cancer therapy, an antibody with specificity for a tumor-associated antigen is used to deliver a lethal dose of radiation to the tumor cells. The ability for the antibody to specifically bind to a tumor-associated antigen increases the dose delivered to the tumor cells while decreasing the dose to normal tissues **(Milenic etal., 2004)**.

If the antibody labeled with γ- emitter radioisotope,it was used for radioimmunosintigraphy. If the antibody labeled with β- emitter radioisotope,it was used for radioimmunotherapy as shown in (Fig. 12) .

Possible mechanisms of radioimmunotherapy

When radiolabeled antibody binds to tumor cells it can cause tumor killing by

1. Self-killing (apoptosis) , programmed cell death triggered by the antibody
2. Complement-dependent cytotoxicity (CDC), where antibody fixes complement that kills the tumor cells
3. Antibody-dependent cellular cytotoxicity (ADCC), where effector cells (immune cells) kill the tumor cells

4. Ionizing radiation from the radioisotope damages the tumor cells, leading to cell death
5. Vaccine-like effect leading to adaptive immunity against cells that may survive initial treatment

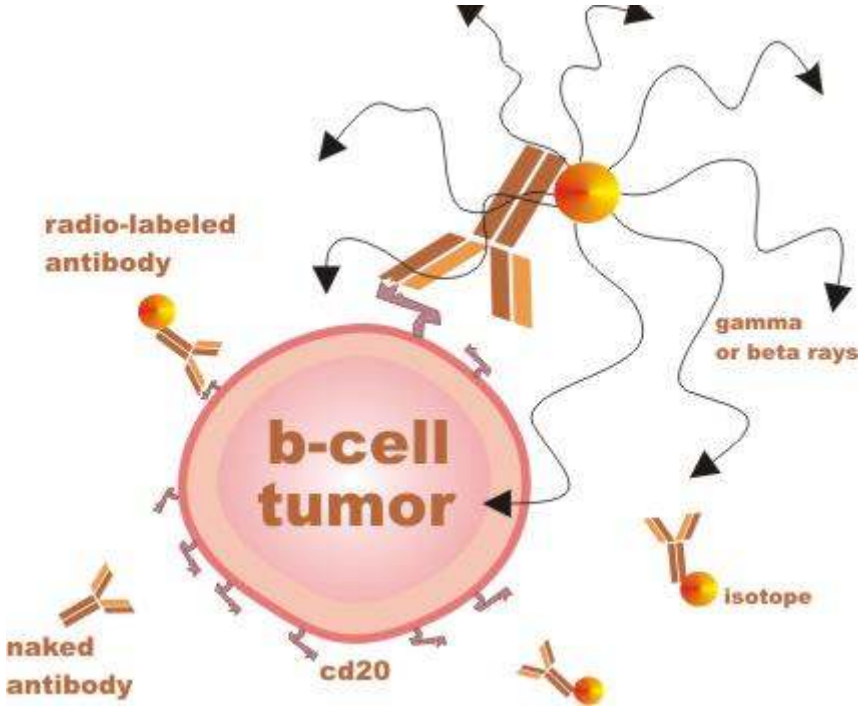

Fig. (12): Radioimmunotherapy (RIT) of Cancer

Gene therapy

Gene therapy essentially consists of introducing specific genetic material into target cells without producing toxic effects on surrounding healthy cells and tissue. It has been defined as the "genetic modification of cells of a patient in order to fight a

disease". Gene therapy includes both the transfer of new genetic material and the manipulation of existing genetic material.

At the present time, the most widely used gene therapy procedure follows these steps: (i) identification, isolation and amplification of the gene to be used in the treatment; (ii) extraction and in vitro culture of tissue cells from the patient to be treated; (iii) transfer of the therapeutic gene into these cells via a vector , using a gene that contains a promoting sequence to enable its expression and a marker to identify cells into which it is incorporated; and (iv) transfer into the patient of selected gene-containing cells. The theory is that when the gene exerts its normal physiological functions, the disease will be eliminated **(Sonia et al., 2008)**.

Types of gene therapy

Gene therapy may be classified into the two following types:

Somatic gene therapy

In somatic gene therapy, the therapeutic genes are transferred into the somatic cells, or body, of a patient. Any modifications and effects will be restricted to the individual patient only, and will not be inherited by the patient's offspring or lategenerations. Somatic gene therapy represents the mainstream line of current basic and clinical research, where the therapeutic DNA transgene (either integrated in the genome or as an external episome or plasmid) is used to treat a disease in an individual.

Germ line gene therapy

In germ line gene therapy, germ cells, i.e., sperm or eggs are modified by the introduction of functional genes, which are integrated into their genomes. This would allow the therapy to be heritable and passed on to later generations. Although this

should, in theory, be highly effective in counteracting genetic disorders and hereditary diseases, many jurisdictions prohibit this for application in human beings, at least for the present, for a variety of technical and ethical reasons **(Strachnan and Read, 2004)**.

Materials and Methods

Materials

Animals

●Rabbits: New Zealand White female Rabbits (2-3 kg body weight, three month old).

●Mice: Female Balb/c mice (60 mice) with body weight of (range 20 - 25 gm) and age of 12 weeks old .The animals were kept at constant environmental and nutritional conditions throughout the experimental period and kept at room temperature (22±2) ºC with a 12 hr on/off light schedule. Standard food and water were allowed to mice all over the experiments.

Myeloma cell line

SP2 /OR myeloma cell line (Chapmen, 1998), Hammersmith hospital, London. UK.

Equipment:

- Laminar flow cabinet, TEISTAR BIO.11 A, Jose TAPIOLAS, 120, s 7245, TERRASSA-SPAIN.
- Incubator with water jacket (37 °C), CO2 supply and humidified environment is not necessary provided, (CO2 incubator).
- Inverted microscope for cell counting, Olympus, Japan.
- Deep freezer (-70 °C), Heraeus Instruments, Germany.
- Centrifuge: cell prep, dedicated cytology, Fisher scientific, LTD, UK.
- Liquid Nitrogen Refrigerator, LOCATOR, I.R.Thermolyme CRYO, Biological storage system, USA.
- Water bath, UK.
- PH meter, scientific instruments, UK.
- Multi-Crystal Gamma Counter, Berthold, LB 211, Germany.
- Magnetic stirrer
- Vacuum pump: Rotatory Van Vacuum Punp,D-97877,Werthein,Germany.
- Gravity oven, Asheville, N.C., U.S.A.
- Microanalytical Balance: Toplo, Model XE-100.Ading Denever Instrument Co,USA.
- Spectrophotometer
- Abbott AxSYM system.
- Epics flow cytometer.

Tools and Devices

●Haemocytometer, Boeckel-co, Scientific equipment Br and st wiefe 4.2000, Hamburg 11, western, Germany.

- Douple-hub-syringe:2x5 ml "Hamilton" luer lock syringes "syringe connector, Reno, Nivada, USA.
- Cryogenic work station, Bibby.
- Multipipettes: Eppindorf, ranged from 5-1000 µl,Germany.
- 0.22 um sterile membrane filters, Gelman Acrodisc DLL, Germany.

Plastic wares:

- 25 cm^2 tissue culture flasks.
- 75 cm^2tissue culture flasks.
- Sterile Pasteur, 1ml, 5 ml and 10 ml. Disposable plastic pipettes.
- Sterile 1 ml and 10 ml. Disposable plastic pipettes.
- Sterile centrifuge tubes, with cap, 15 ml.
- Sterile centrifuge tubes, with cap, 50 ml.
- Storage Ampoules, (2 ml). (ICN/Flow).
- Expanded polystyrene Box, 1 cm, wall thickness.
- Polystyrene Petri dishes (ICN/Flow).
- 12 well sterile tissue culture plate.
- 24 well sterile tissue culture plate.
- Sterile polystyrene containers. 60 ml and 100 ml (sterilin).
- 1 ml sterile syringes (Becton and Dickinson, Plastipak or equivalent)
- 70% ethanol in water bottle (EL Nasser pharmaceutical chemical co, Egypt).

- Sterile universal containers, 30 ml (sterile).
- Measuring cylinder 25 ml.

Chemicals, Reagents and Biological materials

●Bortezomib: (Velcade®, PS-341) was obtained from Millennium Pharmaceuticals Inc. (Cambridge, MA) and reconstituted with sterial normal saline (0.9%), to a stock concentration of 1 mg/ml prior to use in all assays.
The chemical name for bortezomib, the monomeric boronic acid, is [(1R)-3-methyl-1-[[(2S)-1-oxo-3-phenyl-2-[(pyrazinylcarbonyl) amino]propyl]amino]butyl] boronic acid. The molecular weight is 384.24. The molecular formula is $C_{19}H_{25}BN_4O_4$.

●IFN-Alpha: (specific activity = 3.3 x 10^6 U/mg) was purchased from Access Biochemical (San Diego, CA).

● Tissue culture medium , RPMI-1640, with 15mM HEPES buffer , sterile , powder , Sigma , USA (stored at 4°C).

- Foetal bovine serum (FBS): liquid, sterile, sigma, USA (stored at less -20ºC).
- Antibiotic antimycotic mixture (10.000u penicillin ,10mg streptomycin and 25 ug amphotericin B 1ml in 0.9 % Na Cl): liquid , sterile , sigma , USA (stored at less than 0°C) .
- L-glutamine (200mM solution), Hybri Max®, sterile filtered, Sigma, USA 1(stored at less than 0°C).
- Trypan blue dye (MOD), 0.5% w/v in normal saline ICN biological , CA , USA (stored at 15°C- 30 °C) .
- Freund's adjuvant complete (FAC sigma, USA (stored at 0°C -5°C).

- Freund's adjuvant Incomplete (FIA sigma, USA (stored at 0°C -5°C).
- Pristane (2, 6, 10, 14. Tetramethyl pentadecane) (Sigma, USA) .
- Dimethyle sulfoxide (DMSO, sterile. filtered, hybrid max®, Sigma, USA. (Stored at room temperature).
- Sodium Iodide-125 (Na ^{125}I), Radioactive concentration 3700 M Bq/ml. Half –life 59.9 days, Izotop.
- Di-Sodium hydrogen orthophosphate: $Na_2HPO_4.2H_2O$, Extra Pure, Merck, Germany.
- Sodium dihydrogen orthophosphate: $NaH_2PO_4.2H_2O$, Sigma Chemical Co, USA.
- Sodium chloride: NaCl, MW 58.44, Adwic Chemical Co., Egypt.
- Chloramine –T ($C_7H_7ClNO_{2S}Na$): N-Chloro-P-toluene sulfonamide sodium salts, Sigma Chemical Co, USA.
- Sodium metabisulphite ($Na_2S_2O_5$) : MW 190.1, S-1516 Sigma Chemical Co, USA
- Potassium iodide: KI, MW 166.01. Sigma Chemical Co.,P-2963, USA .
- Sodium bicarbonate: $NaHCo_3$, MW 84.01, LTD Dagenham, UK.
- β2-microglobulins kits (Sigma).
- Phosphate- buffered saline (PBS).
- Propidium iodide5 µg/mL (PI, sigma).

- RNAse10 µg/ml (Sigma).

Preparation of Reagents

1.Phosphate buffer (0.5 m,pH 7.4)(Stock solution):

It was prepared by dissolving 71.6 g $Na_2HPO_4.2H_2O$ and 15.3 g NaH2PO4.2H2O in 1 liter double distilled water (DDW) and stored at room temperature.

2. ***Phosphate buffer (0.05 m, pH 7.4)***:

It was freshly prepared by diluting the stock Phosphate buffer (0.5 M) 10- fold with DDW.

3. ***Phosphate buffer SALINE (PBS) (0.05 m,pH 7.4)***:

It was freshly prepared by diluting the stock Phosphate buffer (0.5 M) 10- fold with NORMAL SALINE (0.9%).

4. Sodium Chloride (Na Cl) (3 M)

175.5 g Na Cl were dissolved in 1 liter DDW.

5. **Sodium Hydroxide (Na OH) (0.01 N):**

0.4 g NaOH were dissolved in 1 literDDW.

6. Assay buffer:

The following reagents were added to each other and completed to 100 ml with DDW.

-10 ml Phosphate buffer (0.5 M), pH7.4.

-0.1 g sodium azide.

-0.1 g Bovine Serum Albumin.

-0.9 g sodium chloride.

Methods

1-Preparation of tissue culture medium

-Plain myeloma cell culture medium

Accurately 90% of final required volume of sterile distilled water was measured with temperature of 15-20 °C. While the water was gently stirring the powdered medium was added. The contents were stirred until dissolved. the original package was rinsed with a small amount of sterile distilled water to remove all traces of powder 2 g of sodium bicarbonate was added per liter of final volume of medium. The PH was adjusted into (7.0 ± 0.3).The additional water was added to bring the solution to final volume of one liter. The contents were sterilized immediately by filtration using 0.22 µm membrane filter. The medium was aseptically dispensed into a sterile container (Chapmen, 1998).

Myeloma cell culture medium

The myeloma cell culture medium was included the following constituents, 100 ml RPMI-1640, 10 ml Foetal bovine serum (FBS), 1ml Antibiotic Antimycotic and 1 ml L-Glutamine (Chapmen, 1998).

2-Maintenance of myeloma cells

Established myeloma cells were frozen as 1×10^7 cells / ml as 0.5 ml aliquots. One aliquot may be removed from liquid nitrogen. It is most convenient at this stage to handle only 10-15 ml medium in 25 cm^2 flasks until cell growth

enter log-phase and viability of > 95 % are achieved . Incubation was continued at 37 °C and the cells recounted with a viability check daily prior to adjustment of the cell count to 2.5-5×10^5 cells. This process was continued until a cell doubling time of 18-24 hrs was achieved with a viability of > 95%. Cell density should not be allowed to exceed 1-1.5×10^6 cells / ml. At this storage the total volume of cells can be expanded by transferring to 75 cm^2 flasks (max. vol. 50 ml) for freezing in liquid nitrogen to provide a secure stock of cells for future work (Chapmen, 1998).

3-Counting myeloma cells and viability checks

To determine total number of cells / ml and viability (%), 100 µl of cells suspension was mixed with 100 µl of vital dye (trypan blue). A drop of mixture was transferred into edge formed between the cover slip and the slide (a hemocytometer) and observed under the microscope where the living cells did not stain with the dye, while the dead cells stained with blue color. The myeloma cells (viable cells and dead cells were counted in the big squares (WBCs squares) of hemocytometer and the results were calculated as follow (Harlow and David, 1988).

$$\text{T.C of myeloma cells} = \frac{\text{Total number of cells}}{\text{Number of square counted}} \times 2 \times 104 = \text{number of cells/ml}$$

$$\text{Viable cells} = \frac{\text{Total number of viable cells}}{\text{Number of squares counted}} \times 2 \times 10^4 = \text{number of viable cells/ml}$$

$$\text{Viability (\%)} = \frac{\text{Number of viable cells /ml}}{} \times 100$$

Number of cells/ ml

4-Cryopreservation of SP2/OR myeloma cells

10 ml of freezing mixture {10% dimethyl sulfoxide (DMSO) in ice cold foetal bovine serum (FBS)} were prepared. The DMSO was add to the FBS drop wise with shaking and placed in ice in the cryogenic work station. Cells were counted and tested for viability, then centrifuged for 5 min at 900 rpm and the supernatant was discarded. The cells were resuspented at 1x 10^7 cells /ml in plain RPMI-1640 and washed (3) times with RPMI. The cells at 1x10^7 cells /ml (viability > 90%) were resuspended in the freezing mixture. The universal container was tapped to loosen the cell pellet, and then the freezing mixture was added drop wise with continuous shaking. Aliquots of 0.5 ml were prepared in tightly stoppered storage cryogenic vials , transferred to the expanded polystyrene cryobox , sealed with tape , placed at -70 °C overnight and finally stored in liquid nitrogen cryovessel (at – 185 °C) until required (Chapmen and Ratcliffe,1998) .

Production of Anti-myeloma Antibodies:-

Production of Anti-myeloma Antibodies was carried out by using seven healthy female mature White New-Zealand rabbits, 2-3 Kg. They were immunized with myeloma cells to raise anti-myeloma antibodies. Rabbits were kept under the same hygienic conditions, well balanced diet and water was supplied adlibitum. The production of Anti-myeloma Antibodies was carried out through the following steps:-

1.Preparation of myeloma-adjuvant mixture:

0.5 ml (1×10^7) of myeloma cells were emulsified with 1 ml of Freund's adjuvant (Complete or Incomplete). Freund's complete adjuvant (FCA) was used for primary injection while Freund's incomplete adjuvant (FIA) was used for booster doses (**R**). Emulsification was performed using Hamilton double-hub syringes connected to each other with narrow metallic tubing. The aqueous solution was placed in one syringe and the oily adjuvant in the other one. The aqueous solution was forced through the tubing into the oil in the other syringe. Then the mixture was forced back. This process was repeated many time until a stable water-in-oil emulsion is formed. Stability of the formed emulsion was tested by allowing a few drops to fall into beaker containing cold water. A stable emulsion remains white drop on the surface indicating the enclosure of the water phase within the oil phase (water-in-oil phase) according to **(R).**

2. Route of Injection

For primary immunization, each rabbit except control rabbit was received 1.5 ml of emulsion where 1.2 ml of emulsified mixture was injected subcutaneously over the shoulder at 6 sites (200 µl in each site). The remaining 0.3 ml of emulsified mixture was injected intramuscular at 2 sites (150 µl in each site).

-For the booster immunization, emulsified mixture was given in the same way except that Freund's complete adjuvant (FCA) was replaced by the Freund's incomplete adjuvant (FIA).

3. Immunization schedule:

Six immunization injection were administered, one primary and five booster injection, at one month intervals**(R).**

4. Blood sampling and harvesting the raised anti-sera:

The rabbits were bled for sampling through the marginal ear vein a week after the first booster injection and continued until the end of the immunization schedule at a week intervals, using a simplified device. A simple technique for withdrawing blood from rabbit's marginal ear vein has been described by Hope et al., (1969). Marginal ear was incised and a mild negative pressure was applied through the device to keep a continuous blood flow. Serum was separated from the blood without any additives and was kept under -20 C till testing **(R).**

Labeling of Anti-myeloma Antibodies

Chloramines –T method:

Into a vial containing 20 µl of serum 20 µl of 0.5 M phosphate buffer, pH 7.4 was added then mixed with 0.5 mCi 0f iodine-125. The reaction was started by the addition of 10 µl of phosphate buffer 0.05 M, pH 7.4

containing 20µg of chloramine -T. The reaction was allowed to proceed for 30 seconds and quenched by the addition of 10 µl of phosphate buffer 0.05 M, pH 7.4 containing 20µg of sodium metabisulfite. After that 20µg of KI in10 µl of phosphate buffer 0.05 M, pH 7.4 was added as a carrier.

The purification was carried out by transferring the iodination mixture into the top of a conditioned chromatographic column 35x1 cm loaded with PD-10. Elution was carried out using 0.05 M phosphate buffer containing 0.3% bovine serum albumin solution, and the flow rate was adjusted to drop per seconds. Elution pattern was monitored by counting radioactivity of fractions in a well type NaI (TI) scinitillation counter.

Effect of Velcade (Bortezomib) on myeloma cell line SP2OR in vitro

I-Growing of myeloma cells

Myeloma cells were cultured in the culture medium which prepared as mentioned above until cell growth enter log-phase and viabilities of > 95 % were achieved.

II-Preparation of variable doses of Velcade (Bortezomib)

The drug was diluted in culture media to prepare variable doses of (5, 10, 25, 50, 75 and 100 nM).

III-Addition of the prepared variable doses to myeloma cells

Velcade was added at variable doses (5, 10, 25, 50, 75 and 100 nM). Each dose was added into the myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily.

Effect of Alpha-interferon on myeloma cell line SP2OR in vitro

I-Growing of myeloma cells

The myeloma cells were grown as mentioned above

II-Preparation of variable doses of Alpha-interferon

The drug was diluted in culture media to prepare variable doses of (50, 100, 500, 1000, 5000 and 10000 IU/ml). The prepared variable doses of alpha interferon were added to myeloma cells.

III-Addition of prepared variable doses to myeloma cells

Alpha-interferon was added at variable doses of (50, 100, 500, 1000, 5000 and 10000 IU/ml) into myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily.

8-Induction of tumor in mice (Ascites)

A line of myeloma cell (SP2OR) was used in the induction of ascites. 0.5ml pristine (2, 6, 10, 14-tertramethyl-decanoicnacid) was injected intraperitoneal (i.p) then after 10-30 days the antigen incomplete Freund's adjuvant was injected. The injection was repeated on days 7 and 21 after first injection then myeloma cells were injected. The animals were maintained for 10-15 days till the tumor development was apparent as described by (Harlow and David, 1988). Treatment was started

after development of measurable tumor. Mice were used in this study and each mouse weighed daily.

Experimental design:

At the beginning of the experiment, mice were divided into six groups, The 1st group was control group, the 2nd group was ascites bearing mice, the3rd group was ascites bearing mice that treated with velcade, the 4th group was ascites bearing mice that treated with alpha- interferon, the 5th group was ascites bearing mice that treated with combined therapy between velcade and alpha- interferon and 6th group was ascites bearing mice that treated with myeloma- antibodies.

Groups are divided as followed:

1- **Control group (5 mice):** Animals served as untreated control group
2- **Ascites group (5 mice):** Mice bearing ascites

3- **Group [1]:** Mice bearing ascites and injected with velcade. This group was divided into four subgroup, each of 5 mice.

Subgroup (I): In this subgroup mice were injected with velcade at dose of **0.05 mg/kg** body weight twice a week for a total of 8 injections.

Subgroup (II): In this subgroup mice were injected with velcade at a dose of **0.1 mg/kg** body weight twice a week for a total of 8 injections.

Subgroup (III): In this subgroup mice were injected with velcade at a dose of **0.5 mg/kg** body weight twice a week for a total of 8 injections.

Subgroup (IV): In this subgroup mice were injected with velcade at a dose of **1.0 mg/kg** body weight twice a week for a total of 8 injections.

4- **Group [2]:** Mice bearing ascites and injected with alpha-interferon. This group was divided into three subgroup, each of (5) mice:

Subgroup (I): In this subgroup mice were injected with alpha-interferon at a dose of **0.05 mg/kg** body weight twice a week for a total of 8 injections.

Samples collection

Blood sampling: Directly, after animals were anaesthised, blood was collected from heart using insulin syringes, then the blood was transferred into sterile tubes and allow to stand for 15 minutes at room temperature, then centrifuged at 3000 r.p.m. for 15 minutes. Serum was separated and kept at -20 °C until used.

Determination of serum Creatinine:

Measurement of blood **Creatinine** was carried out according to the method described by Bartels and bohmer, 1972.

Pinciples

Creatinine in alkaline solution reacts with Picric acid to form a colored complex. The amount of the complex is directly proportional to the creatinine concentration.

Reagents:

- Standard Creatinine
- Reagent (R1a) Picric acid 35 mm/l
- Reagent (R1b) Sodium hydroxide 0.32 mmol/l

Working reagent: One volume of R1a (Picric acid) was added to one volume of R1b (NaOH). The mixture is stable for 3 days at +15 to +25°C.

Procedure

100 µl of sample and standard was added separately to 1 ml of working reagent placed at 25°C, mixed and after 30 seconds the absorbance A1 of the standard or sample was read. Exactly 2 minutes later, the absorbance A2 of the standard or sample was read.

Calculation

$A2 - A1 = \Delta A_{\text{sample or}} \Delta A_{\text{Standard}}$

Concentration of creatinine in serum=

$$\frac{\Delta A_{\text{sample}}}{\Delta A_{\text{Standard}}} \times \text{Standard conc. (mg/dl)} = \text{mg/dl}$$

Determination of serum Alkaline phosphatase :

The blood Alkaline phosphatase was measured colorimetric according to the recommendations of the Deutsche Gesellschaft fur Klinische Chemie, 1972.

Principles

p-nitrophenylphosphate $+H_2O \xrightarrow{ALK}$ phosphate + p-nitrophenol

Reagents

1. Reagent (R_1a) buffer

Diethanolamine buffer	1 mol/l, pH=9.8
Magnesium chloride	0.5 mmol/l

2. Reagent (R_1b) substrate

p-Nitrophenylphosphate (solution)	10 mmol/l

Procedure

In a clean dry tube, 20 µl of serum was added to 1ml of reagent, mixed and the initial absorbance was read at 405 nm and read again after 1, 2 and 3 minutes.

Calculation

$U/I = 2760 \times \Delta A$

Determination of serum Urea:

The blood urea was measured colorimetrically according to Fawcett and Scott, 1960.

Principle

Urea in serum is hydrolyzed to ammonia and carbon dioxide in the presence of urease. The ammonia is then measured photometrically by Berthelot´s reaction.

$Urea + H_2O \xrightarrow{Urease} 2NH_3 + CO_2$

NH_3 + hypochloride + phenol $\longrightarrow$ indophenol

(blue compound)

Reagents:

- Standard
- Reagent (R1): EDTA 116 mmol/l
  Sodium nitroprusside 6 mmol/l
  Urease 1g/l
- Reagent (R2): Phenol (diluted) 120 mmol/l
- Reagent (R3): Sodium hypochloride (diluted) 27 mmol/l
  Sodium hydroxide 0.14N

Procedure

10 µl of sample or standard was added separately to 100 µl of R1, mixed and incubated for 10 min at 37°C. Then 2.5 ml of R2 and 2.5 ml of R_3 were added to each tube, shacked and incubated for 15 min at 37°C. The color developed was measured using spectrophotometer, at 546nm against reagent blank. For blank tube 2.5 ml of R2 and 2.5 ml of R_3 were added to 100 µl of R1 and incubated for 15 min at 37°C

Calculation

$$\text{Serum Urea} = \frac{A_{\text{sample}}}{A_{\text{Standard}}} \times \text{Standard conc.} = \quad \text{mg/dl}$$

Determination of serum Uric Acid:

The blood uric acid was measured colorimetrically according to Fossati et al., 1980.

Principle

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoine} + CO_2 + H_2O_2$$

$2H_2O_2$+3, 5 -Dichloro -2- hydroxybenzenesulfonic acid+4-Aminophenazone $\xrightarrow{\text{Peroxidase}}$ N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzo-quinoneimine

Reagents:

- Standard
- Reagent (R1a).Buffer
  Hepes buffer 50 mmol/l, pH=7.0
  3, 5 -Dichloro -2- hydroxybenzenesulfonic acid 4 mmol/l

- Reagent (R1b) Enzyme Reagent

4- Aminophenazone	0.25 mmol/l
Peroxidase	≥ 1000U/l
Uricase	≥ 200 U/l

Calculation

$$\text{Serum Uric Acid} = \frac{A_{\text{sample}}}{A_{\text{Standard}}} \times \text{Standard conc.} = \text{mg/dl}$$

Determination of Serum Alanine Aminotransferase (ALT/GPT)

Serum Alanine Aminotransferase (ALT/GPT) was determined according to the method of Reitman and Frankel, 1957.

Principle

$$\alpha\text{- oxoglutarate} + \text{L-alanine} \xrightarrow{\text{GPT}} \text{L- glutamate} + \text{pyruvate}$$

Alanine Aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine

Reagents:

- Reagent (R_1).Buffer

Phosphate buffer	100 mmol/l, pH=7.0
L-alanine	200 mmol/l
α- oxoglutarate	2.0 mmol/l

- Reagent (R_2)

2,4-dinitrophenylhydrazine	2.0 mmol/l

Procedure

In clean dry test tube, 100 µl of serum was added to 0.5 ml of R_1 (buffer), mixed and incubated for exactly 30 min at 37°C. Then 0.5 ml of R_2 was added, mixed and incubated for exactly 20 min at 25°C. Then 5 ml Sodium hydroxide was added. At the same way blank was prepared replacing the sample with distilled water. The absorbance was measured spectrophotometrically at 546 nm against blank after 5 min.

Determination of Serum Aminotransferase (AST/GOT)

Serum Aspartate Aminotransferase (ALT/GPT) was determined according to the method of Reitman and Frankel, 1957.

Principle

α- oxoglutarate + L- aspartate $\xrightarrow{\text{GOT}}$ L- glutamate + Oxaloacetate

AST is measured by monitoring the concentration of Oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine

Reagents:

- Reagent (R_1).Buffer

Phosphate buffer	100 mmol/l, pH=7.0
L- aspartate	200 mmol/l
α- oxoglutarate	2.0 mmol/l

- Reagent (R_2)

2, 4-dinitrophenylhydrazine	2.0 mmol/l

Procedure

In clean dry test tube, 100 µl of serum was added to 0.5 ml of R_1 (buffer), mixed and incubated for exactly 30 min at 37°C. Then 0.5 ml of R_2 was added, mixed and incubated for exactly 20 min at 25°C.Then 5 ml Sodium hydroxide was added. AT same way blank was prepared replacing the sample with

distalled water. The absorbance was measured spectrophotometrically at 546 nm against blank after 5 min.

Determination of Hemoglobin concentration

Principle

Hemoglobin is first oxidized by potassium ferricyanide into methemoglobin which is converted into cyanmethaemoglobin by potassium cyanide. The absorbance of the cyanmethaemoglobin is monitored at 540 nm.

Reagents:

Potassium Ferricyanide	0.61 mmol/l
Potassium Cyanide	0.77 mmol/l
Potassium Phosphate	1.03mmol/l
Surfactant	0.1%v/v

Procedure

In a clean dry test tube, 20 µl of well mixed blood was added to 5ml of reagent, mixed and measured the absorbance at 540 nm against distilled water after 5 minutes.

Calculation

Hemoglobuin concentration = A_{sample} x 36.77 (g/dl)

11-Determination of β2-Microglobulins

By Abbott AxSYM system assay

Principle: AxSYM β2-microglobulins assay is based on the microparticle enzyme immunoassay (MEIA) technology. The AxSYM β2-microglobulins reagents and sample are added to the reaction cell in the following sequence: sample and all AxSYM β2-microglobulins required for one test are pipetted by the sampling probe into various wells of a reaction vessel (RV) in the sampling center. The RV is immediately transferred into the processing center. Further pipetting is done in the processing center by the processing probe. The reaction occur in the following sequence: the probe delivers the sample, specimen diluent and AxSYM solution 4 to the wells of the reaction vessel for diluting sample. An aliquot of the diluting sample.Anti- β2-M coated microparticles and AxSYM solution 4 are pipetted to one well of the reaction vessel. During the incubation of this reaction mixture the β2-microglobulins in the specimen binds to the Anti- β2-M coated microparticles forming an antibody-antigen complex. An aliquot of the reaction mixture is transferred to the matrix cell. The matrix cell is washed to remove unbound materials. Anti- β2-M: alkaline phosphatase conjugate is dispensed onto the matrix cell and binds to the antibody-antigen complex. The matrix cell is washed to remove unbounded materials. The substrate, Methylumbelliferyl phosphate, is added to the matrix cell and the rate of fluorescent product formation is measured by the MEIA optical assembly (Bataille and Durile, 1976)**.**

12-Cell cycle analysis

a-Preparation of samples

- Control sample: myeloma cells (1×10^6) cultured in media alone without treatment.
- Myeloma cells treated with melphalan: myeloma cells (1×10^6) treated with (0.1 mg/ml) melphalan for 24 hours.

- Myeloma cells treated with thalidomide: myeloma cells (1×10^6) treated with (100 µg/ml) for 24 hours & (150 µg/ml) thalidomide for 24 hours and 72 hours.
- Myeloma cells treated with thalidomide: myeloma cells (1×10^6) exposued to γ- radiation (6 gray) for 24 hours.

b-Procedure of flow cytometric analysis

The cells were washed twice with phosphate-buffered saline (PBS), fixed with 70% ethanol, and pretreated with 10 µg/ml of RNAse (Sigma). After a 30-min incubation at 37°C, Cells were stained with propidium iodide (PI; 5 µg/ml; Sigma), and cell cycle profile was determined by using the program M software on an Epics flow cytometer (Teru et al., 2000).

13-Colorimetric Analysis of Caspase-8 and -9 Activity

a-Preparation of samples

- Control sample: myeloma cells (1×10^6) cultured in media alone without treatment (5 samples).
- Myeloma cells treated with melphalan: myeloma cells (1×10^6) treated with (0.1 mg/ml) melphalan for 24 hours (5 samples).
- Myeloma cells treated with thalidomide: myeloma cells (1×10^6) treated with (100 µg/ml) for 24 hours & (150 µg/ml) thalidomide for 24 hours and 72 hours (5 samples).
- Myeloma cells treated with thalidomide: myeloma cells (1×10^6) exposued to γ- radiation (6 gray) for 24 hours (5 samples).

b-Caspase-8 and Caspas -9 assay

The cells were washed two times with 2 ml PSA and subsequently treated with lysis buffer for 10 min on ice bath to lyse cells (25µl of lysis buffer / $1x10^6$cells). The cell lysate was centrifuged at 10.000 xg for 1 min. The supernatant was transferred to a new tube and keep in ice bath. The enzymatic reaction for caspase activity was carried out in 20 ELISA wells. Each reaction requires 50µl of cell lysate, 50µlof 2x Reaction was added to each reaction (before using2x Reaction 10µl of fresh DTT stock (dithiothreitol) was added to 1ml of 2x Reaction). 5µl of caspase colorimetric substrate (LEHD-PNA) was added to each reaction, and then the plate was incubated at 37 °C for 1-2 hours. The absorbance of each well was detected using ELISA reader at wavelength 405 nm (Nicholas et al., 2002).

Results

In vitro study

Effects of Velcade, α-interferon , (Velcade+ α-interferon), Myeloma-Antibodies, Lablled Myeloma-Antibodies and (Velcade+ α-interferon+ Myeloma-Antibodies) on myeloma cell growth were

determined by treating SP2OR cells with variable doses of these drugs for six days.

Effects of bortezomib on growth of myeloma cells

Figure ( ) illustrates the effect of variable doses of Velcade (5, 10,20,30,50 and 100 nM) on the viability (%) of myeloma cells during lifespan of 6 days compared to control group. The viability of myeloma cells decreased at 5 nM from 95% to 87%, at 10 nM from 89% to 31%, at 20 nM from 73 |%to 11%, at 30 nM from 69% to 10% ,at 50 nM from 41% to 8%, and at 100 nM from 30% to 7%. These results indicated that cell growth was inhibited by bortezomib treatment in a dose- and time-dependent manner. Low doses of bortezomib (5nM) did not increase cell growth inhibition for 6 days.

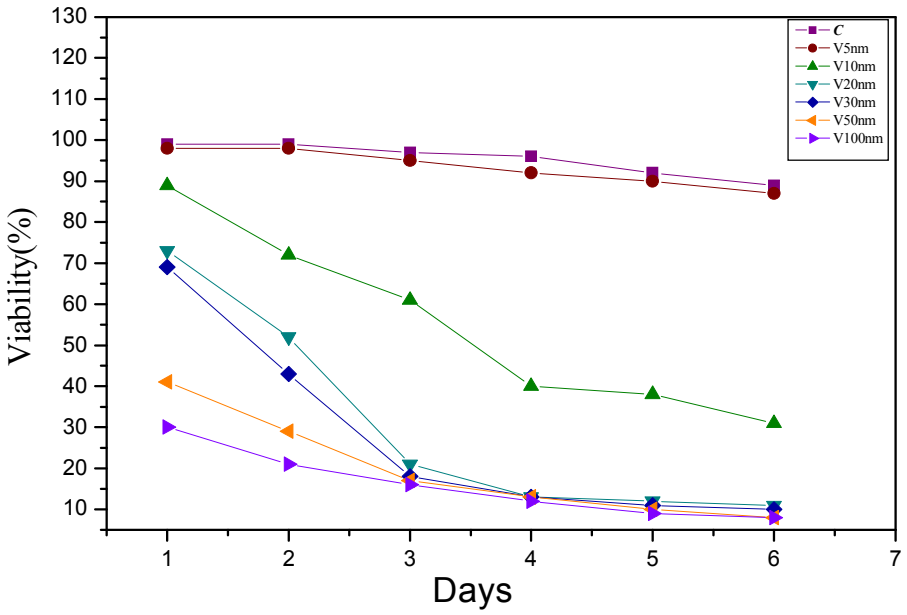

Fig.(1):Effect of variable doses of Velcade on viability % of myeloma cells.

Effects of IFN-α *on growth of myeloma cells*

Effect of IFN-α on the viability of myeloma cells was evaluated.IFN-α was applied at different doses (50, 100, 500, 1000, 5000 and 10000 IU/ml). As shown in (Fig. 2) the viability of myeloma cells decreased at 50 IU/ml from 98% to 88%, at 100 IU/ml from 91% to 70%, at 500IU/ml from 82 |%to 69%, at 1000IU/ml from 80% to 64% ,at 5000 IU/ml from 78% to 56%, and at 10000 IU/ml from 73% to 51%. These results indicated that the viability (%) decreased with increasing concentration of IFN-α.

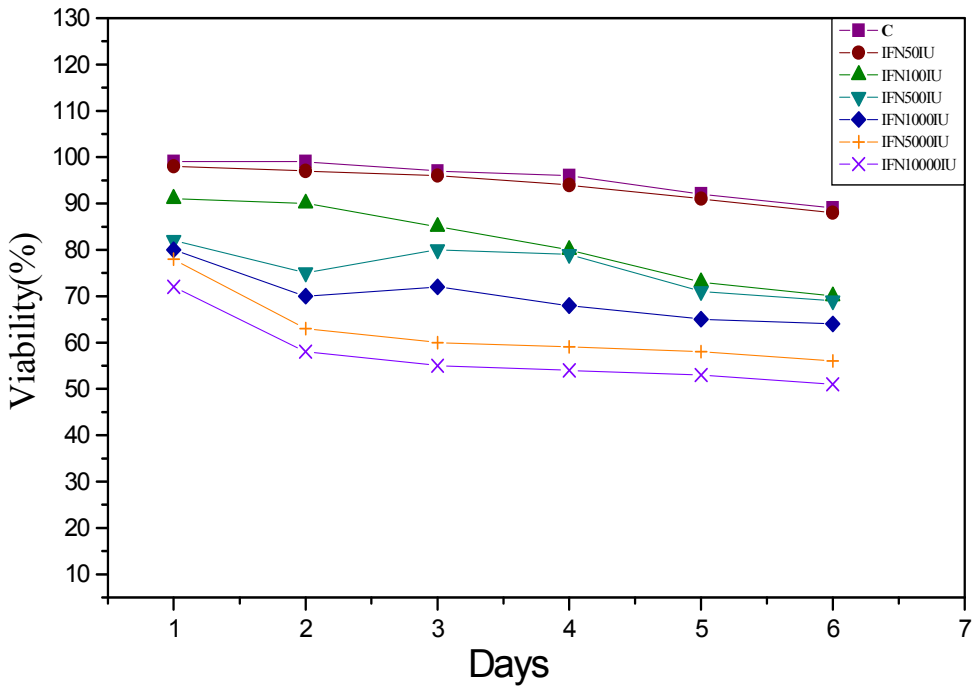

Fig. (2): Effect of variable doses of Alpha-Interferon on the viability % of myeloma cells.

Effects of bortezomib plus IFN-α *on growth of myeloma cells*

The effect of a combination of bortezomib and IFN-α on cell viability was investigated .It was observed from figure ( ) that viability of myeloma cells decreased at $D_1$from 96% to 75%, at $D_2$from 89% to 57%, at $D_3$from 56 |%to zero, at D_1 $D_4$from 15% to zero ,and reached to zero for D_5 & D_6. These results indicated that treatment with bortezomib plus IFN-α for 6 days resulted in more growth inhibition than either bortezomib or IFN-α alone in myeloma cells.

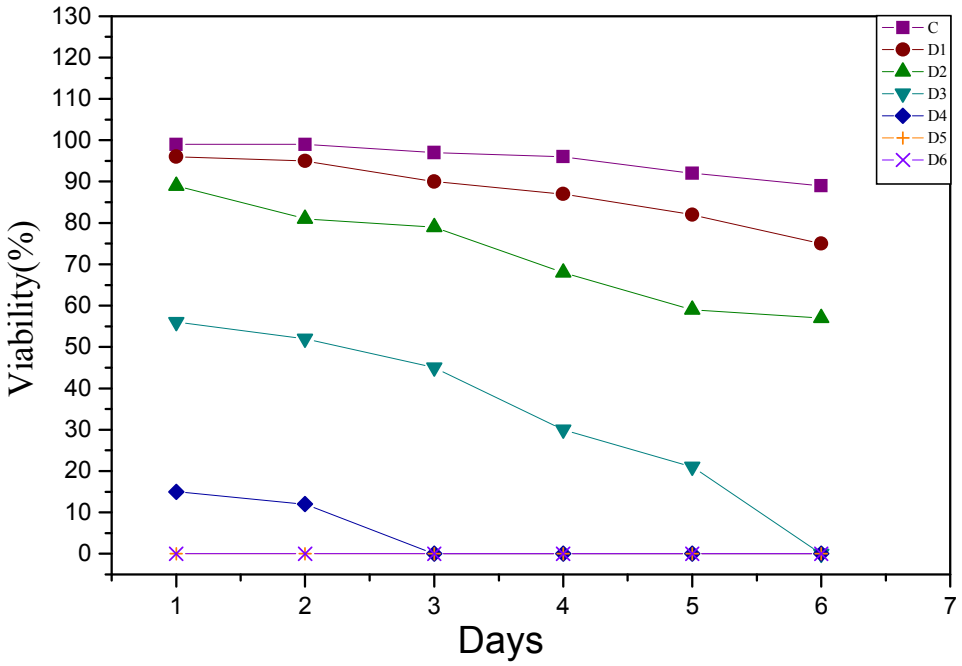

Fig.(3):Effect of variable doses of combined treatment with Velcade and Alpha-Interferon on the Viability % of myeloma cells.

Effects of Myeloma-Antibodies *on growth of myeloma cells*

As shown in (Fig.)_Myeloma-Antibodies reduced cell viability in adose-dependent and time-dependent manner.The viability of myeloma cells decreased at $D_1$from 98% to 79%, at $D_2$from 96% to 63%, at $D_3$from 90 |%to49%, at $D_4$from 86% to 42%, at from $D_5$79% to 35%, and at D_5 from62% to 29%.

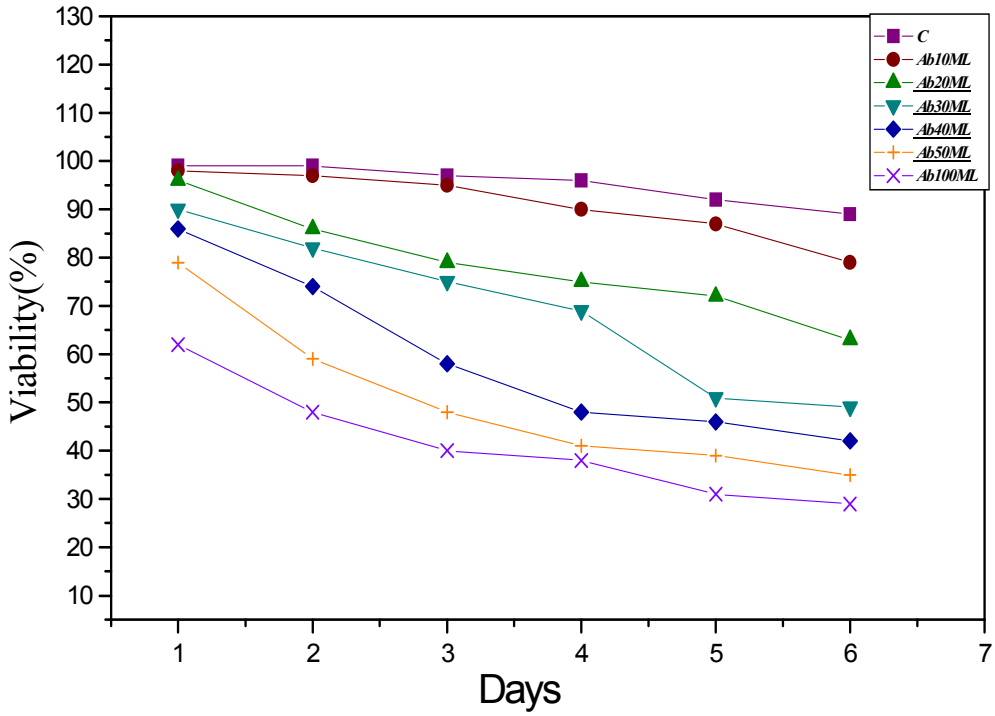

Fig.(4):Effect of variable doses of Myeloma-Antibodies on the viability % of myeloma cells.

Effects of Labeled Myeloma-Antibodies *on growth of myeloma cells*

Figure (15) illustrated that the viability of myeloma cells decreased at $D_1$from 89% to 62%, at $D_2$from 70% to 43%, at $D_3$from 14 |%to0, and reached to zero for D_5 & D_6.

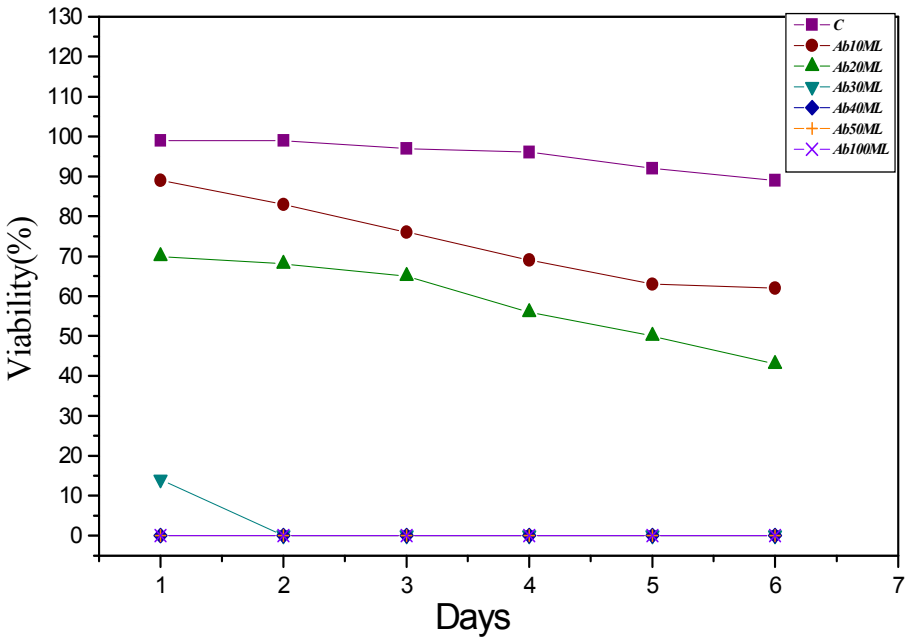

Fig. (5): Effect of variable doses of Labeled Myeloma-Antibodies on the viability % of myeloma cells.

Figure ( ) illustrates the effect of variable doses of Velcade+ IFN-α + Myeloma-Antibodies **on the viability (%) of myeloma cells during lifespan of 6 days compared to control group.**The viability of myeloma cells decreased at $D_1$from 79% to 19%, at $D_2$from 59% to zero, and reached to zero for D_5 & D_6.

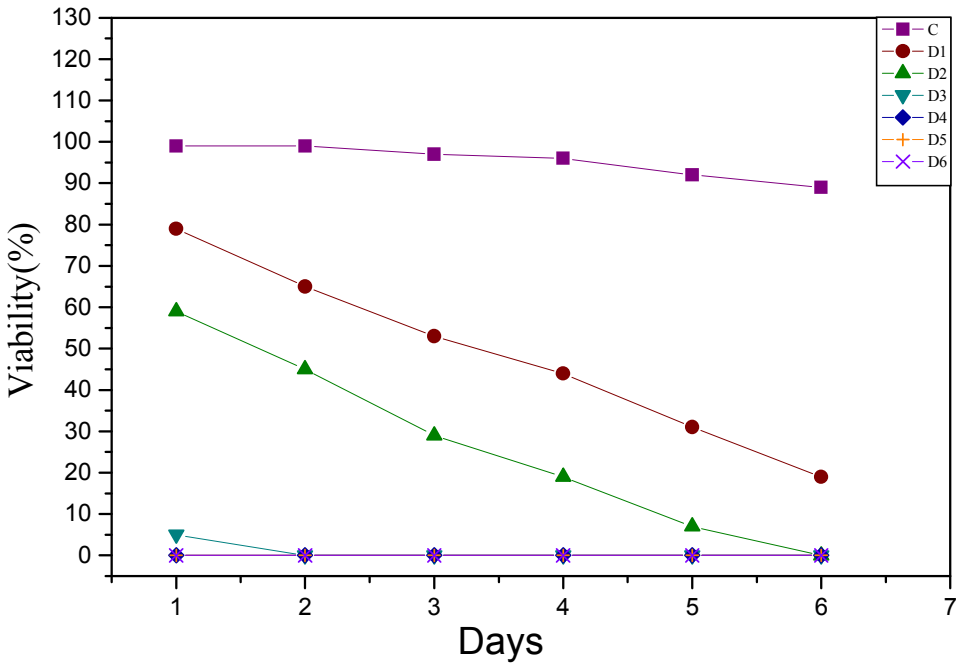

Fig.(6):Effect of variable doses of combined treatment with Velcade + Alpha-Interferon+ Myeloma-Antibodies on the viability % of myeloma cells.

In Vivo study

In the present study, the effect of different types of treatment (bortezomib, IFN-α, bortezomib+ IFN-α, myeloma-Antibodies and bortezomib+ IFN-α +myeloma-Antibodies) on tumor growth of ascites bearing mice were evaluated and compared with control group.

The results of ascites bearing mice untreated and treated with velcade are illustrated in table (19) and Fig().Before treatment the T.C /ml (mean ± SD) were ($15\times10^4\pm0.7\times10^4$ to $166.5\times10^4\pm3.6\times10$), while the viability % (mean± SD) were (82±2.0 to 97±1.5) for time intervals of 4 weeks. While after treatment with velcade (0.05, 0.1, 0.5 and 1.0 mg/kg) twice weekly for 4 weeks the T.C /ml (mean ± SD) decreased to($14\times10^4\pm1.4\times10^4$ to$111\times10^4\pm1.3\times10$),($13\times10^4\pm0.8\times10^4$ to $100\times10^4\pm0.7\times10^4$),($10\times10^4\pm0.7\times10^4$ to 0) and ($8\times10^4\pm0.7\times10^4$ to o) respectively. The viability % (mean ± SD) decreased to (75±2.9 to 91±1.4), (73±1.1 to 88±0.7), (61±1.0 to 0) and (42±0.8 to 0) respectively. From these results it can be concluded that animals treated at the two lowest PS-341 doses (0.05 and 0.1 mg/kg) showed inhibition of tumor growth compared with controls, but greatest inhibition of tumor growth was observed in mice treated with PS-341 at 0.5 and 1.0 mg/kg versus the control group.

Table (1): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with *Velcade.*

Days	Parameters measured	Mean±SD (n=5)				
		Control group	**Mice treated with Velcade (0.05mg/kg)**	**Mice treated with Velcade (0.1mg/kg)**	**Mice treated with Velcade (0.5mg/kg)**	**Mice treated with Velcade (1.0mg/kg)**
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$14\times10^4\pm1.4\times10^4$	$13\times10^4\pm0.8\times10^4$	$10\times10^4\pm0.7\times10^4$	$8\times10^4\pm0.7\times10^4$
	V. (%)	82±2.0	75±2.9	73±1.1	61±1.0	42±0.8
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$26\times10^4\pm1.8\times10^4$	$23\times10^4\pm0.8\times10^4$	$10\times10^4\pm0.7\times10^4$	$5\times10^4\pm0.7\times10^4$
	V. (%)	89±0.7	79±1.9	78±1.9	52±0.4	12±0.4
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$38\times10^4\pm0.7\times10^4$	$36\times10^4\pm1.1\times10^4$	$21\times10^4\pm0.7\times10^4$	$3\times10^4\pm0.5\times10^4$
	V. (%)	91±1.4	82±1.8	79±1.1	42±0.8	7±0.7
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$49\times10^4\pm0.7\times10^4$	$47\times10^4\pm0.7\times10^4$	$12\times10^4\pm0.4\times10^4$	$2\times10^4\pm0.4\times10^4$
	V. (%)	93±1.5	88±0.8	83±0.8	24±0.5	0
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$76\times10^4\pm1.8\times10^4$	$60\times10^4\pm1.1\times10^4$	$7\times10^4\pm0.7\times10^4$	0
	V. (%)	94±1.1	89±2.2	84±0.7	14±0.9	0
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$89\times10^4\pm0.8\times10^4$	$77.5\times10^4\pm1.1\times10^4$	$3\times10^4\pm0.7\times10^4$	0
	V. (%)	94±1.3	90±1.5	85±0.7	0	0
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$95\times10^4\pm2.5\times10^4$	$87\times10^4\pm0.7\times10^4$	0	0
	V. (%)	95±1.3	90±1.3	86±0.7	0	0
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$111\times10^4\pm1.3\times10^4$	$100\times10^4\pm0.7\times10^4$	0	0
	V. (%)	97±1.5	91±1.4	88±0.7	0	0

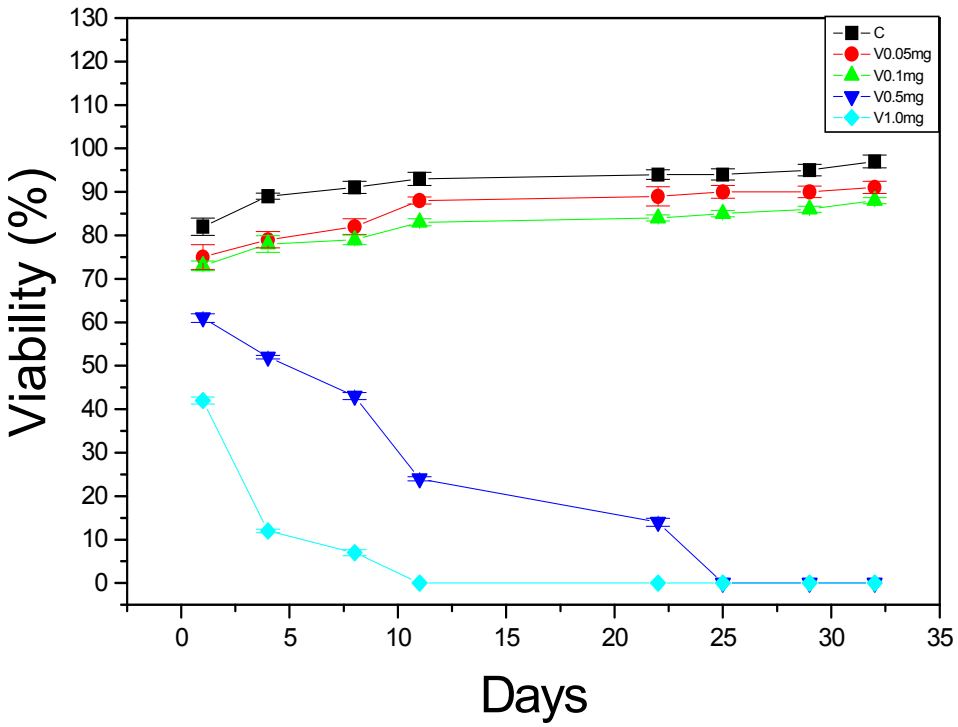

Fig.(7):Effect of variable doses of Velcade on viability % of myeloma cells of ascites bearing mice (mean ± SD)

Table (19) and Fig() represented the results of ascites bearing mice untreated and treated with IFN-α. Before treatment the T.C /ml (mean ± SD) were ($15\times10^4\pm0.7\times10^4$ to $166.5\times10^4\pm3.6\times10$), while the viability % (mean± SD) were (82±2.0 to 97±1.5) for time intervals of 4 weeks. While after treatment with IFN-α (10^3 IU, 10^4 IU and10^6) twice weekly for 4 weeks the T.C /ml (mean ± SD) decreased to ($14.5\times10^4\pm0.3\times10^4$ to $126\times10^4\pm0.3\times10^4$),($11\times10^4\pm0.4\times10^4$ to $72\times10^4\pm0.9\times10^4$) and ($9\times10^4\pm0.2\times10^4$ to $51\times10^4\pm0.4\times10^4$).The viability % (mean ± SD) decreased to (79±0.7 to 94±0.5), (71± 0.3 to17±0.7) and (68±0.7 to 70±0.7) respectively. From these results it can be concluded that animals treated with 10^4 IU and10^6 IFN-α showed inhibition of tumor growth compared with controls,

Table (2): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Alpha-interferon.

Days	Parameters measured	Mean±SD (n=5)			
			Mice treated with interferon (10^3 IU /kg)	**Mice treated with interferon (10^4 IU /kg)**	**Mice treated with interferon (10^6 IU /kg)**
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$14.5\times10^4\pm0.3\times10^4$	$11\times10^4\pm0.4\times10^4$	$9\times10^4\pm0.2\times10^4$
	V. (%)	82±2.0	79±0.7	71±0.3	68±0.7
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$29\times10^4\pm0.7\times10^4$	$13\times10^4\pm0.3\times10^4$	$11\times10^4\pm0.6\times10^4$
	V. (%)	89±0.7	85±1.9	69±0.7	58±0.9
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$37\times10^4\pm0.7\times10^4$	$21\times10^4\pm0.4\times10^4$	$17\times10^4\pm0.8\times10^4$
	V. (%)	91±1.4	89±0.7	53±0.7	47±0.4
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$5210^4\pm0.7\times10^4$	$39\times10^4\pm0.5\times10^4$	$23\times10^4\pm0.7\times10^4$
	V. (%)	93±1.5	91±0.8	48±0.7	31±0.7
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$68\times10^4\pm0.3\times10^4$	$44\times10^4\pm0.8\times10^4$	$37\times10^4\pm0.5\times10^4$
	V. (%)	94±1.1	92±0.4	29±0.7	19±0.7
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$89\times10^4\pm0.7\times10^4$	$62\times10^4\pm0.4\times10^4$	$44\times10^4\pm0.4\times10^4$
	V. (%)	94±1.3	93±0.7	23±0.7	13±0.7
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$108\times10^4\pm0.7\times10^4$	$68\times10^4\pm0.7\times10^4$	$48\times10^4\pm0.7\times10^4$
	V. (%)	95±1.3	94±0.7	19±0.7	9±0.8
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$126\times10^4\pm0.3\times10^4$	$72\times10^4\pm0.9\times10^4$	$51\times10^4\pm0.4\times10^4$
	V. (%)	97±1.5	94±0.5	17±0.7	7±0.7

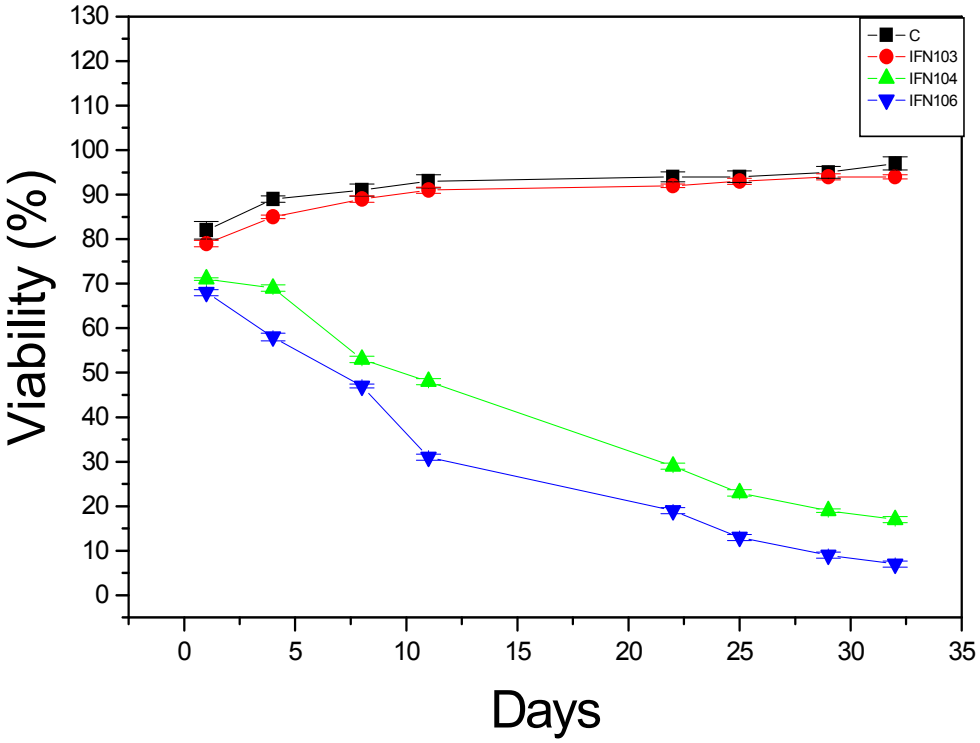

Fig.(8):Effect of variable doses of Alpha-Interferon on viability % of myeloma cells of ascites bearing mice (mean ± SD).

Table ( ) and Figure ( ) were demonstrated that, combination therapy with IFN-a plus bortezomib resulted in significant growth inhibition in **ascites bearing mice** compared with either agent alone.

Table (3): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with velcade plus α-interferon

Days	Parameters measured	Mean±SD (n=5)				
		Control group				
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$9\times10^4\pm0.5\times10^4$	$7\times10^4\pm0.4\times10^4$	$7.5\times10^4\pm0.8\times10^4$	$6\times10^4\pm0.6\times10^4$
	V. (%)	82±2.0	52±0.8	49±1.1	39±0.7	32±0.7
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$11\times10^4\pm0.7\times10^4$	$5\times10^4\pm0.4\times10^4$	$4\times10^4\pm0.4\times10^4$	$3\times10^4\pm0.7\times10^4$
	V. (%)	89±0.7	48±0.7	38±1.9	10±0.7	7±0.4
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$13\times10^4\pm0.7\times10^4$	$4\times10^4\pm0.7\times10^4$	$2\times10^4\pm0.3\times10^4$	$1.5\times10^4\pm0.3\times10^4$
	V. (%)	91±1.4	33±0.4	28±0.8	4±0.5	0
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$14\times10^4\pm0.4\times10^4$	$2.5\times10^4\pm0.3\times10^4$	$1.5\times10^4\pm0.7\times10^4$	0
	V. (%)	93±1.5	21±0.7	19±0.2	0	0
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$9\times10^4\pm0.8\times10^4$	$1.5\times10^4\pm0.3\times10^4$	0	0
	V. (%)	94±1.1	11±0.7	7±0.4	0	0
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$7\times10^4\pm0.5\times10^4$	0	0	0
	V. (%)	94±1.3	0	0	0	0
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$5.5\times10^4\pm0.3\times10^4$	0	0	0
	V. (%)	95±1.3	0	0	0	0
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$3\times10^4\pm0.7\times10^4$	0	0	0
	V. (%)	97±1.5	0	0	0	0

The results of ascites bearing mice untreated and treated with Myeloma-Antibodies are illustrated in table (19) and Fig().Before treatment the T.C /ml (mean ± SD) were ($15\times10^4\pm0.7\times10^4$ to $166.5\times10^4\pm3.6\times10$), while the viability % (mean± SD) were (82±2.0 to 97±1.5) for time intervals of 4 weeks. While after treatment with Myeloma-Antibodies (0.05, 0.1, 0.5 and 1.0 mg/kg) twice weekly for 4 weeks the T.C /ml (mean ± SD) decreased to ($14\times10^4\pm0.7\times10^4to117\times10^4\pm0.4\times10^4$),($12\times10^4\pm0.4\times10^4$ to $75\times10^4\pm0.7\times10^4$) and ($11\times10^4\pm0.2\times10^4$ to $58\times10^4\pm0.4\times10^4$) respectively. The viability % (mean ± SD) decreased to (81±0.7to90±1.5), (79±0.7 to 19±0.7) and (76±0.2 to12±0.4) respectively.

Table (4): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Myeloma-Antibodies .

Days	Parameters measured	Mean±SD (n=5)			
		Control group	**50µl**	100 µl	200 µl
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$14\times10^4\pm0.7\times10^4$	$12\times10^4\pm0.4\times10^4$	$11\times10^4\pm0.2\times10^4$
	V. (%)	82±1.0	81±0.7	79±0.7	76±0.2
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$30\times10^4\pm0.7\times10^4$	$24\times10^4\pm0.5\times10^4$	$16\times10^4\pm0.2\times10^4$
	V. (%)	89±0.7	86±0.9	74±0.7	61±0.7
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$38\times10^4\pm0.4\times10^4$	$31\times10^4\pm0.7\times10^4$	$19\times10^4\pm0.8\times10^4$
	V. (%)	91±1.4	89±0.9	69±0.4	58±0.7
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$49\times10^4\pm0.7\times10^4$	$41\times10^4\pm0.5\times10^4$	$27\times10^4\pm0.7\times10^4$
	V. (%)	93±1.5	90±0.8	53±0.7	48±0.7
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$66\times10^4\pm0.3\times10^4$	$42\times10^4\pm0.8\times10^4$	$36\times10^4\pm0.7\times10^4$
	V. (%)	94±1.1	90±0.7	39±0.4	25±0.7
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$83\times10^4\pm0.7\times10^4$	$59\times10^4\pm0.4\times10^4$	$42\times10^4\pm0.4\times10^4$
	V. (%)	94±1.3	91±0.7	29±0.7	18±0.7
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$98\times10^4\pm0.7\times10^4$	$63\times10^4\pm0.7\times10^4$	$50\times10^4\pm0.7\times10^4$
	V. (%)	95±1.3	93±0.7	22±0.3	13±0.9
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$117\times10^4\pm0.4\times10^4$	$75\times10^4\pm0.7\times10^4$	$58\times10^4\pm0.4\times10^4$
	V. (%)	97±1.5	90±1.5	19±0.7	12±0.4

Table (5): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with velcade+ α-interferon+ Myeloma-Antibodies

Days	Parameters measured	Mean±SD (n=5)				
		Control group				
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$8\times10^4\pm0.4\times10^4$	$6\times10^4\pm0.7\times10^4$	$4\times10^4\pm1.5\times10^4$	$2.5\times10^4\pm0.7\times10^4$
	V. (%)	82±2.0	49±0.7	32±0.8	21±0.4	6±1.3
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$9\times10^4\pm0.8\times10^4$	$4\times10^4\pm1.1\times10^4$	$3\times10^4\pm0.4\times10^4$	$1\times10^4\pm0.7\times10^4$
	V. (%)	89±0.7	36±0.7	28±0.8	10±0.7	0
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$11\times10^4\pm0.3\times10^4$	$3\times10^4\pm0.7\times10^4$	$2\times10^4\pm0.3\times10^4$	10
	V. (%)	91±1.4	29±0.4	13±0.4	0	0
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$12\times10^4\pm0.4\times10^4$	$1.5\times10^4\pm0.7\times10^4$	0	0
	V. (%)	93±1.5	18±1.1	9±0.2	0	0
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$7\times10^4\pm0.4\times10^4$	$1.5\times10^4\pm0.3\times10^4$	0	0
	V. (%)	94±1.1	9±0.7	0	0	0
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$7\times10^4\pm0.5\times10^4$	0	0	0
	V. (%)	94±1.3	0	0	0	0
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$5.5\times10^4\pm0.3\times10^4$	0	0	0
	V. (%)	95±1.3	0	0	0	0
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$3\times10^4\pm0.7\times10^4$	0	0	0
	V. (%)	97±1.5	0	0	0	0

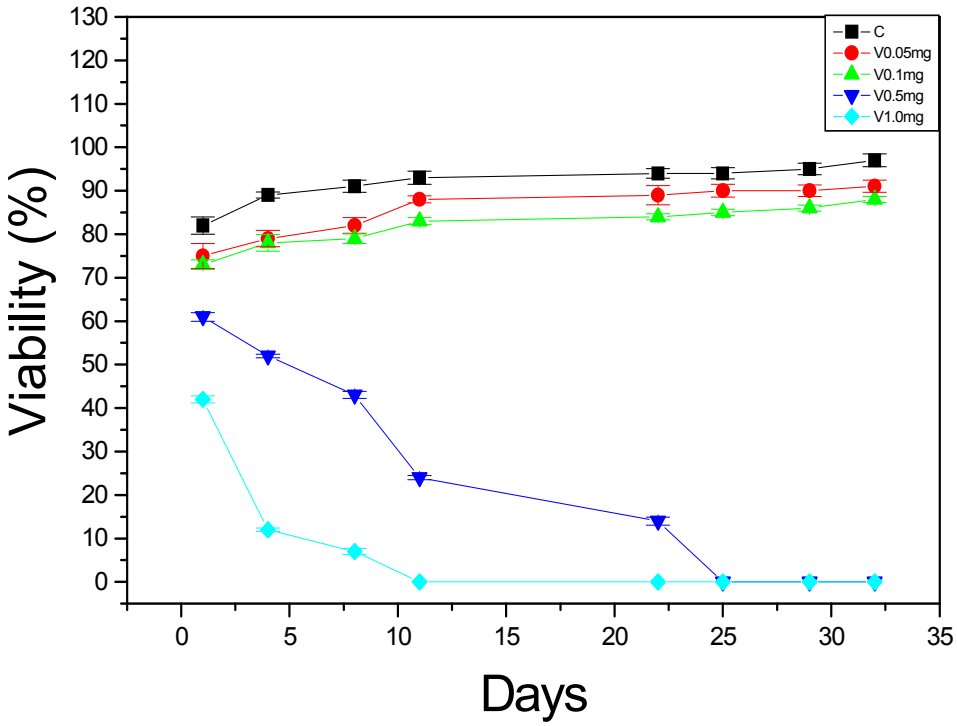

Fig.(7):Effect of variable doses of Velcade on viability % of myeloma cells of ascites bearing mice (mean ± SD).

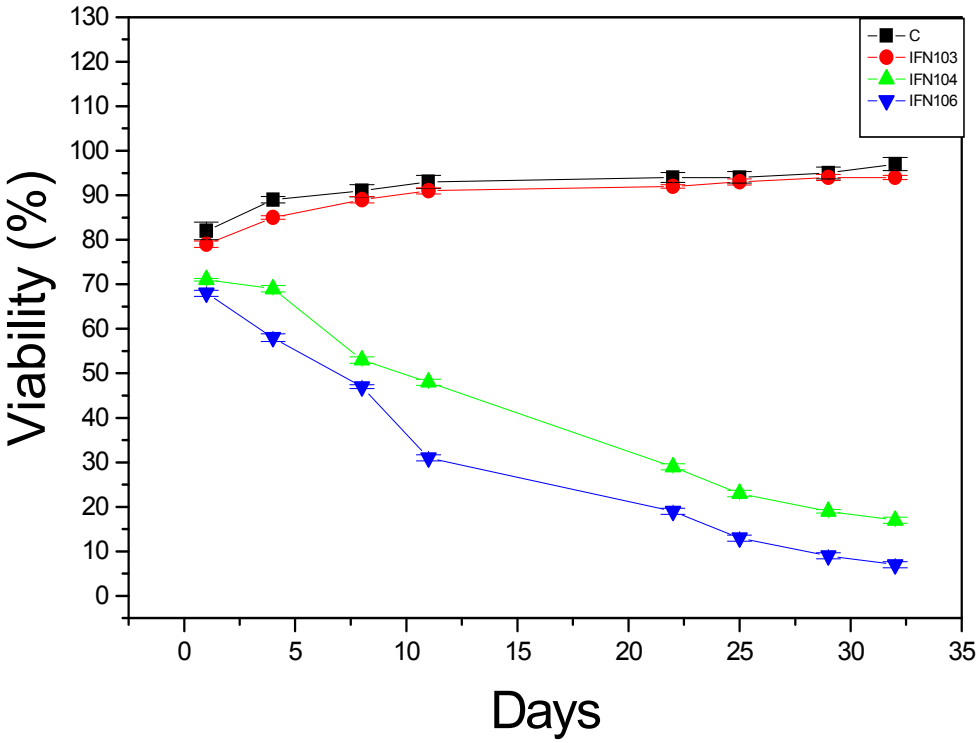

Fig.(8):Effect of variable doses of Alpha-Interferon on viability % of myeloma cells of ascites bearing mice (mean ± SD).

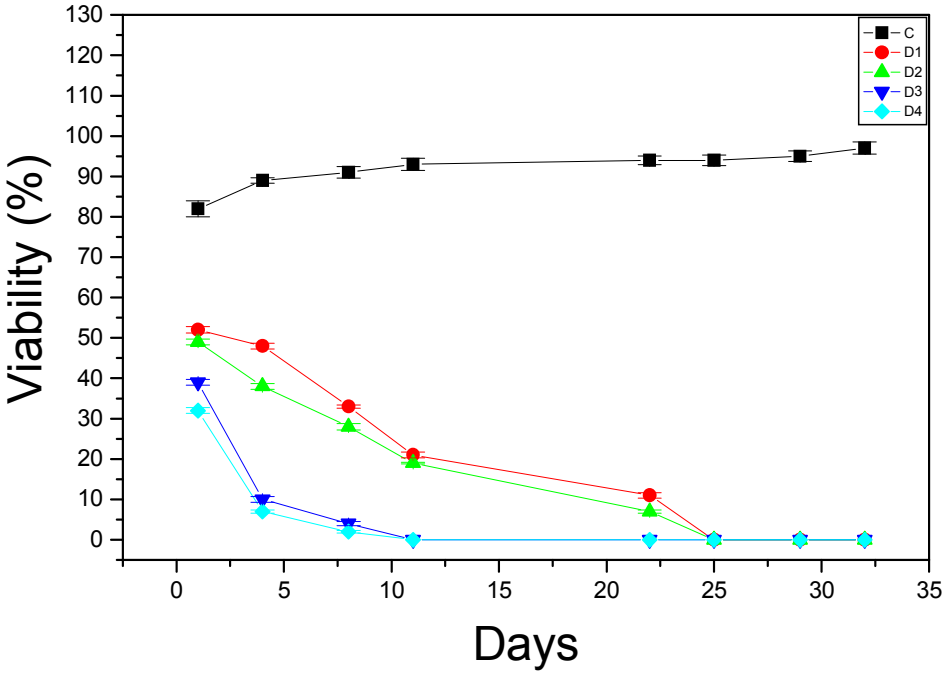

Fig. (9): Effect of variable doses of combined treatment with Velcade +Alpha-Interferon on the viability % of myeloma cells of ascites bearing mice (mean ± SD).

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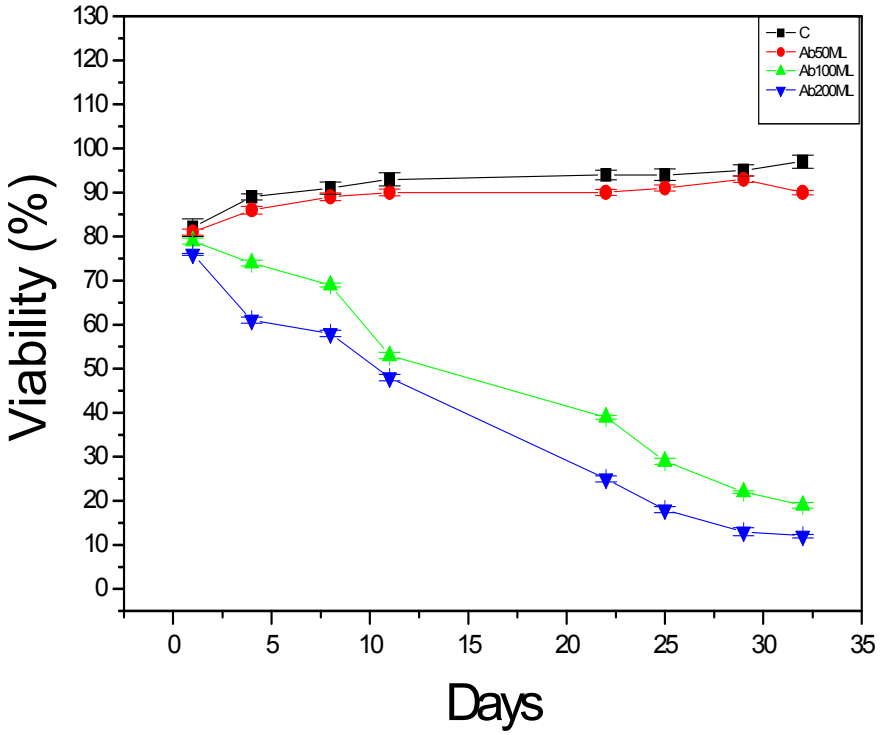


Fig.(10):Effect of variable doses of Myeloma-Antibodies on viability % of myeloma cells of ascites bearing mice (mean ± SD).

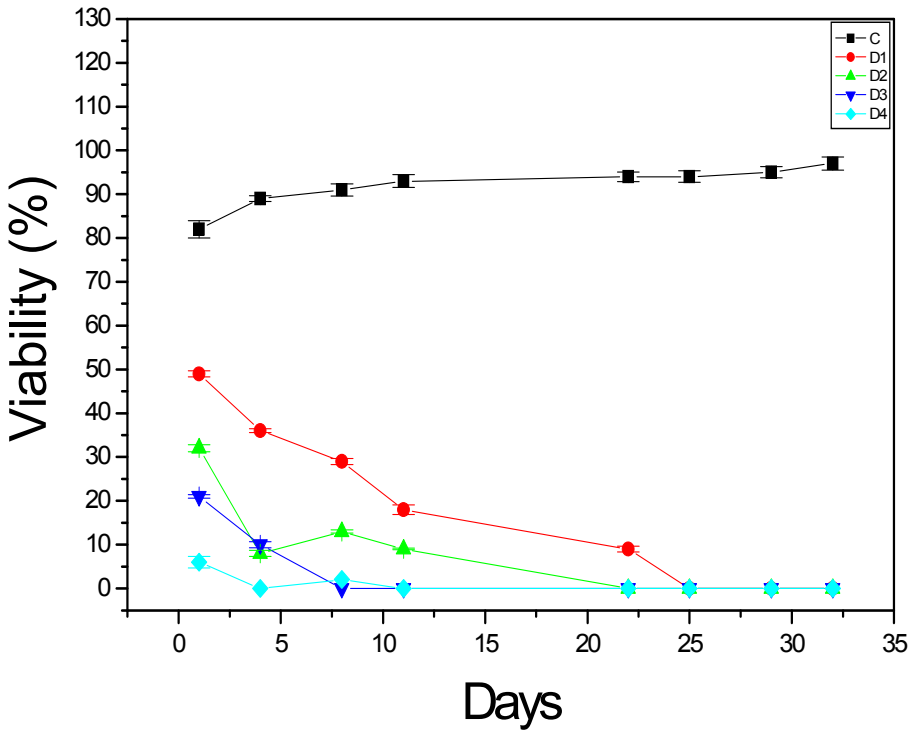


Fig. (11): Effect of variable doses of combined treatment with Velcade +Alpha-Interferon+ Myeloma-Antibodies on the viability % of myeloma cells of ascites bearing mice (mean ± SD).

Preparation and Purification of Radiolabeled Antibodies:

Radiolabeled ^{125}I-antibodies were produced applying Chloramine -T method and purified from radioiodination reaction mixture using PD-10 column chromatography. The Purification profiles of 125I-antibodies with Ch-T for rabbits **(**1, 2, 3, 4**)** are illustrated in **(**Figs **)**, respectively.

The results in Fig show that two identified peaks of radioactivity are obtained, one big and somewhat sharp for ^{125}I-antibodies tracer with good radiochemical yield and the other for free radioactive iodide with a relatively small peak. In addition it was clear that there was a good separation technique for the radioiodination reactants and resultant ^{125}I-antibodies.

Radiochemical yield was calculated from the purification chromatography to be **%.** Radiochemical purity was characterized by electropheretical analysis using elution buffer of 0.05 M phosphate buffer (pH 7.4) with time duration of three h. Radiochemical purity was calculated from the electrophoresis chromatogram to be **%** as illustrated in Fig.( ).

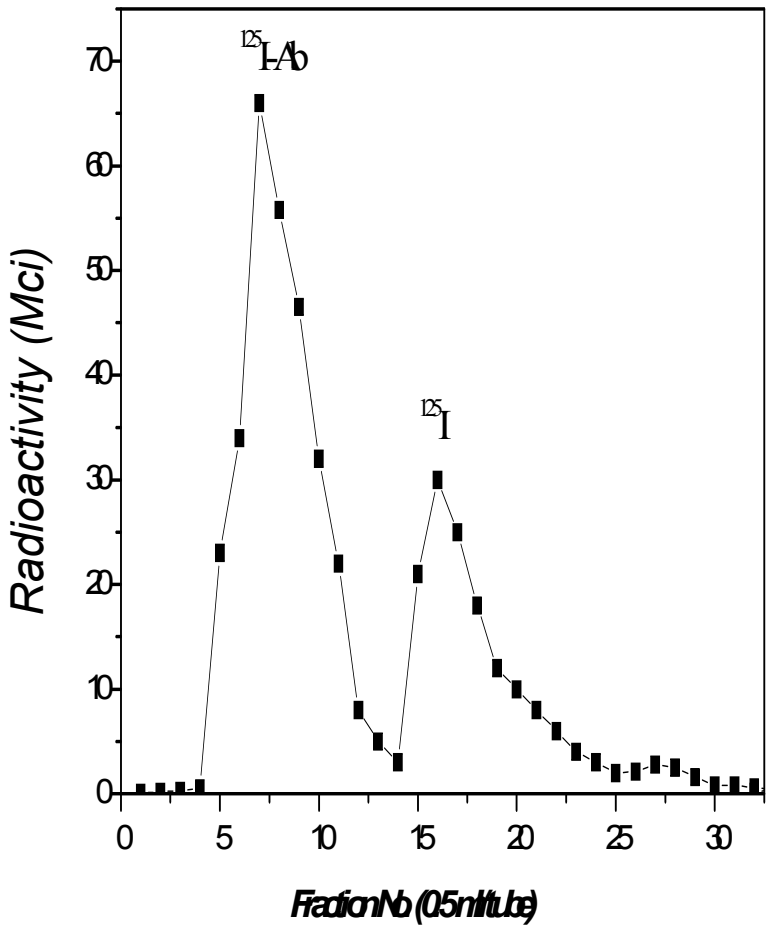


Fig. (12): Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (1))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method)

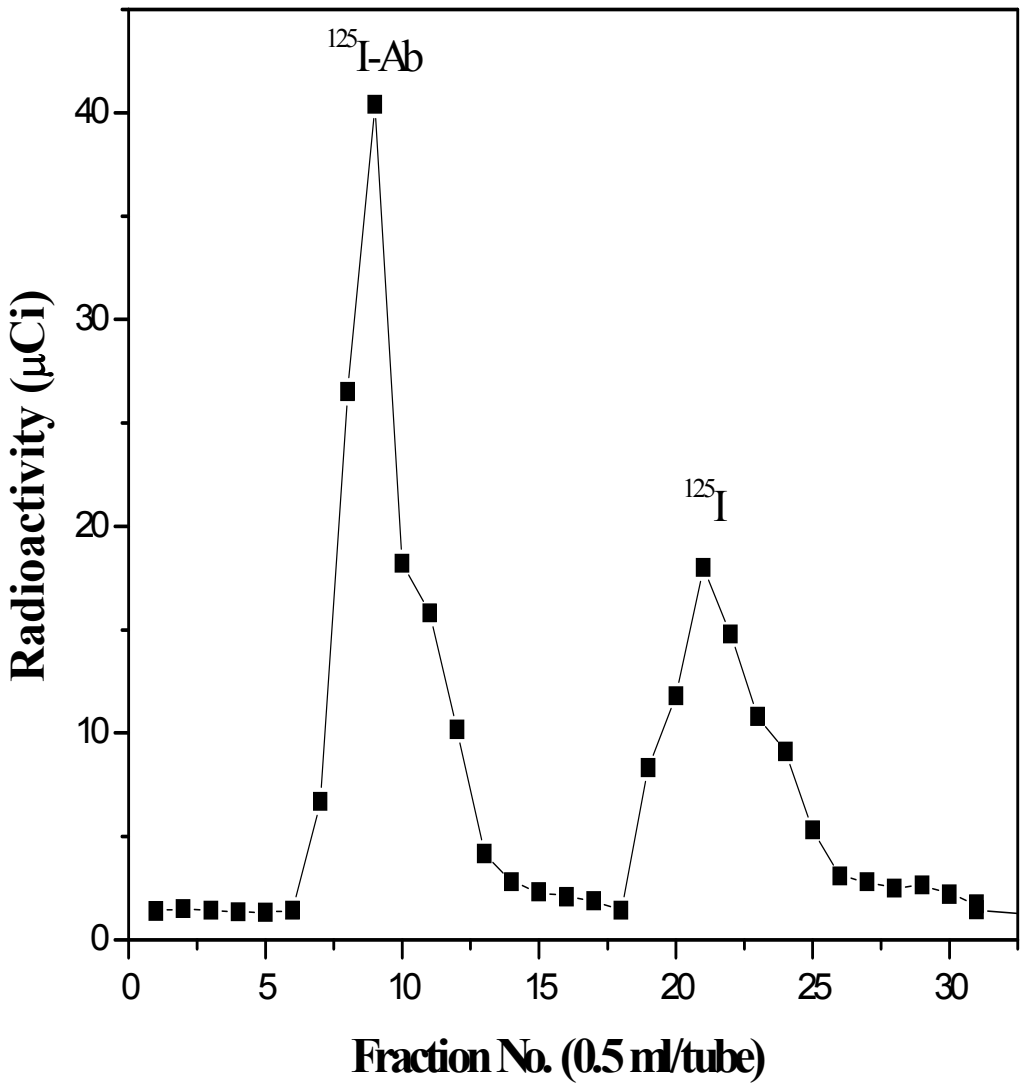


Fig. (13): Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (2))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method)

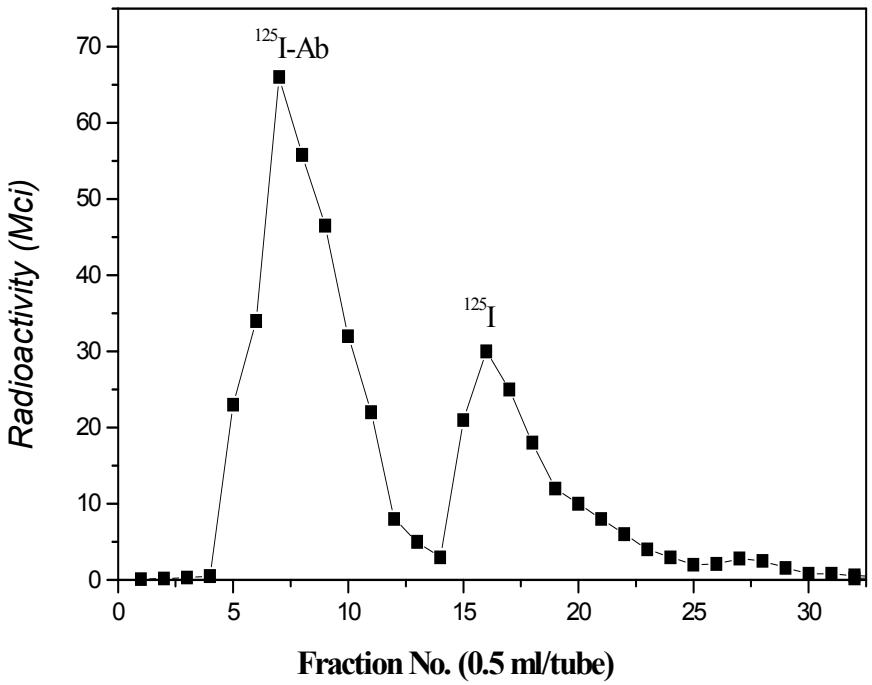


Fig. (14): Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (3))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method)

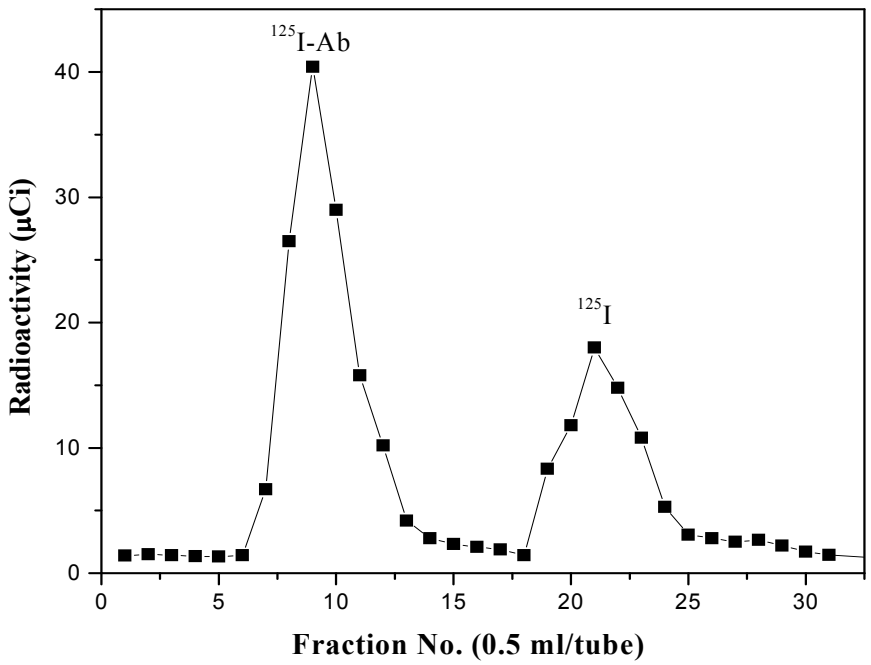

^{125}I-Ab
^{125}I
Radioactivity (µCi)
Fraction No. (0.5 ml/tube)
0
10
20
30
40
0
5
10
15
20
25
30

Fig. (15): Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (4))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method)

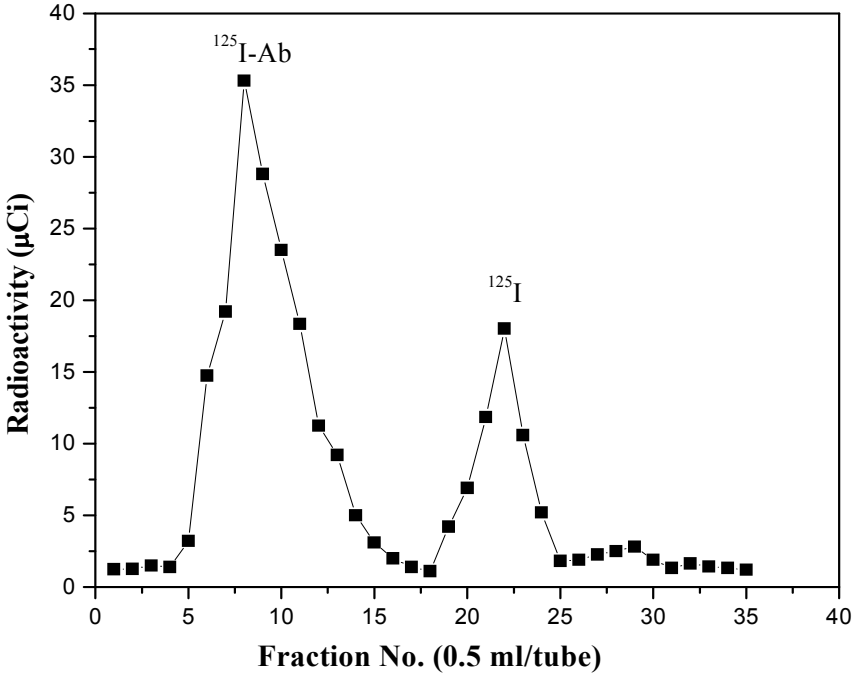


Fig. (16): Elution pattern of radioiodinated mixture of polying myeloma antibodies and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method)

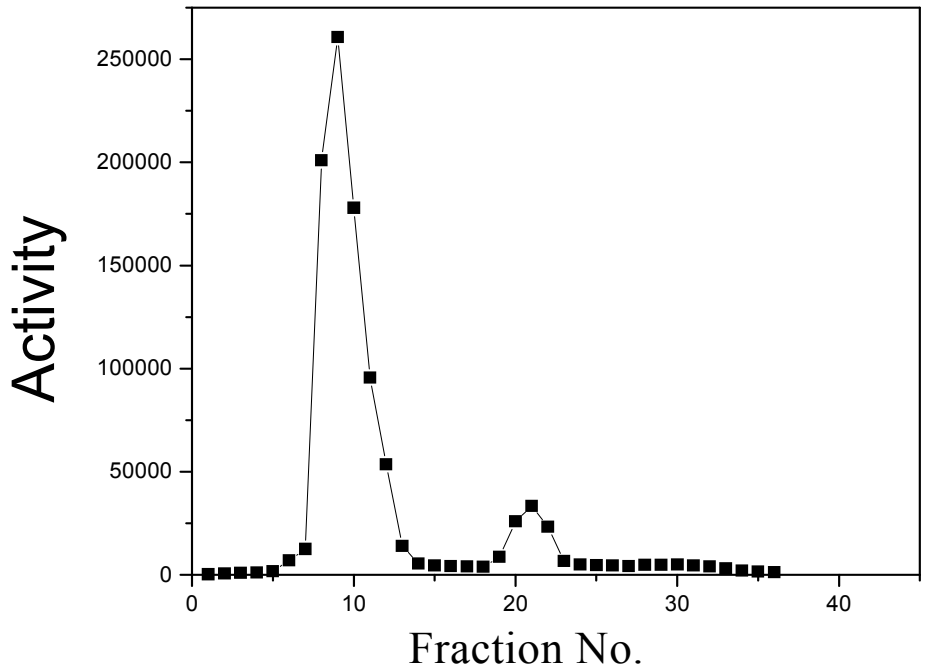

Activity
250000
200000
150000
100000
50000
0
0
10
20
30
40
Fraction No.

Fig. (17) Electrophoresis analysis of ^{125}I-antibodies

The total body weight of ascites bearing mice untreated and treated with IFN-α ,bortezomib , (IFN-α + bortezomib), Myeloma-Antibodies and (IFN-α + bortezomib+ Myeloma) Antibodies are illustrated in table (20). Before treatment the weight (mean ± SD) of the 5 mice was 35 ± 1.1 g. While after treatment with IFN-α,bortezomib , (IFN-α + bortezomib), Myeloma-Antibodies and (IFN-α + bortezomib+ Myeloma) the weight (mean ± SD) of the 5 mice decreased to **28±1.6**, **31±0.7** **25±1.5**, **27±1.3**and **24±1.8**g respectively after the 6th day of treatment and this due to the effect of melphalan, thalidomide and γ- radiation on the growth of myeloma cells.The results showed that treatment with ,,,,,,,,,,, decreased the total body weight as compared to the untreated bearing groups.

The results also showed that treatment with Cpds. 1, 7, 12, 15 and Cpd. 16 significantly eased the total body weight as compared to the untreated bearing mice, where the 16 showed the maximum decrease (5.3%) (Table 4). After the treatment with some of 1,3,4-

Groups	Weight(gm)		
	Number of mice	Mean weight±SD	range
Control	5	21±2.0	20—25
Untreated	5	39±1.5	38—42
Velcade	5	28±1.6	27—31
α-interferon	5	31±0.7	30—32
Velcade+ α-interferon	5	25±1.5	24—27
Myeloma-antibodies	5	27±1.3	26—28
Velcade+ α-interferon +Myeloma-antibodies	5	24±1.8	23—25

Table ( ): The weight of ascites bearing mice before and after treatment (mean ± SD).

Effects on Liver Marker Enzymes

Table shows the changes in liver Enzymes of treated and untreated mice. The serum **ALT, AST andALP activites were increased in the** Ascites bearing mice control group when compared to the normal group. Treatment with Bortezomib, IFN- **α**, Bortezomib+ IFN- **α**, **Myeloma-antibodies** and Bortezomib+ IFN- **α**+ **Myeloma-antibody** showed an increase in the level of serum **ALT, AST compared to untreated group.** Also, as compared to the native mice, the untreated group of mice showed an increase in the level of ALP**. The treatment increased the level of ALP** in the blood.

Table ( ) Change in liver marker enzymes ALT, AST and ALP in Ascites bearing group and treated groups compared to normal control (mean)

Parameter Groups	ALT(units/dL)	AST(units/dL)	ALP(units/dL)
Normal	**19.2±0.19**	**65.5±1.1**	**36±1.1**
Ascites bearing mice	**34.5±0.8**	**78.2±0.4**	**43.2±0.8**
Ascites bearing mice + Velcade	**45.4±0.3**	**85±0.7**	**51.3±0.7**
Ascites bearing mice+ α-interferon	**48.3±0.3**	**91.6±0.5**	**68±1.3**
Ascites bearing mice+ (Velcade+ α-interferon)	**57.6±0.4**	**96.1±0.2**	**73.2±0.8**
Ascites bearing mice+ Myeloma-antibodies	**39±0.4**	**81.4±0.7**	**49±0.7**
Ascites bearing mice+ (Velcade+ α-interferon +Myeloma-antibodies)	**59±0.5**	**98.2±0.7**	**77.1±0.5**

Effects on Kidney functions

The serum **Creatinine, Urea and Uric acid** were increased in the Ascites bearing mice control group compared to the normal group. The treated mice showed increase in the level of **Creatinine, Urea and Uric acid** in the serum of the blood as compared with the untreated groups**Table ( ) Change in Kidney functions Creatinine, Urea and Uric acid in Ascites bearing group and treated groups compared to normal control (mean)**

Parameter Groups	**Creatinine (mg/dL)**	**Urea (mg/dL)**	**Uric acid (mg/dL)**
Normal	**0.61±0.08**	**21±0.7**	**3.4±0.3**
Ascites bearing mice	**1.3±0.08**	**30±0.7**	**5.1±0.4**
Ascites bearing mice + Velcade	**1.6±0.1**	**46±0.7**	**6.2±0.2**
Ascites bearing mice+ α-interferon	**1.8±0.1**	**41±0.8**	**5.8±0.5**
Ascites bearing mice+ (Velcade+ α-interferon)	**1.9±0.08**	**530.8±**	**7.3±0.4**
Ascites bearing mice+ Myeloma-antibodies	**1.4±0.07**	**33±0.7**	**5.4±0.5**
Ascites bearing mice+ (Velcade+ α-interferon +Myeloma-antibodies)	**1.92±0.08**	**57±1.1**	**7.9±0.4**

Table ( ) Change in Hemoglobin (Hb) and Kidney functions in Ascites bearing group and treated groups compared to normal control (mean)

Parameter		Kidney functions

Groups	Hb	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)
Normal	**13.5**	**0.61**	**21**	**3.4**
Ascites bearing mice	**11.4**	**1.3**	**30**	**5.1**
Ascites bearing mice + Velcade	**10.2**	**1.6**	**46**	**6.2**
Ascites bearing mice+ α-interferon	**9.6**	**1.8**	**41**	**5.8**
Ascites bearing mice+ (Velcade+ α-interferon)	**9.2**	**1.9**	**53**	**7.3**
Ascites bearing mice+ Myeloma-antibodies	**11**	**1.4**	**33**	**5.4**
Ascites bearing mice+ (Velcade+ α-interferon +Myeloma-antibodies)	**8.9**	**1.92**	**57**	**7.9**

Parameter **Groups**	**Hb**		
Normal	**13.5**		

Ascites bearing mice	**11.4**		
Ascites bearing mice + Velcade	**10.2**		
Ascites bearing mice+ α-interferon	**9.6**		
Ascites bearing mice+ (Velcade+ α-interferon	**9.2**		
Ascites bearing mice+ Myeloma-antibodies	**11**		
Ascites bearing mice+ (Velcade+ α-interferon +Myeloma-antibodies)	**8.9**		

The data presented in table (21) showed that the β2-microglobulin values decreased after treatment with Bortezomib, IFN-α , Bortezomib+ IFN-α , Myeloma-antibodies and Bortezomib+ IFN-α +Myeloma-antibodies compared that of control

Groups	**β-2microglobulin**	
	Range	Mean ±SD
Control	2— 2.9	2.7±0.4
Bortezomib	1.1—1.6	1.4±0.14
IFN-α	1.5—2.1	1.9±0.2
Bortezomib+ IFN-α	0.9—1.4	1.2±0.18
Myeloma-antibodies	1.8—2.3	2.1±0.07
Bortezomib+ IFN-α+**Myeloma-antibodies**	0.7—1.2	0.9±0.1

Groups	**Caspase-8**		**Caspase-9**	
	Range	Mean ±SD	Range	Mean ±SD
Control		0.17		0.09
Bortezomib		0.28		0.2
IFN-α		0.22		0.17
Bortezomib+ IFN-α		0.25		0 23
Myeloma- antibodies		0.21		0.15
Bortezomib+ IFN-α**Myeloma-antibodies**		0.46		0.27

Flow cytometric analysis

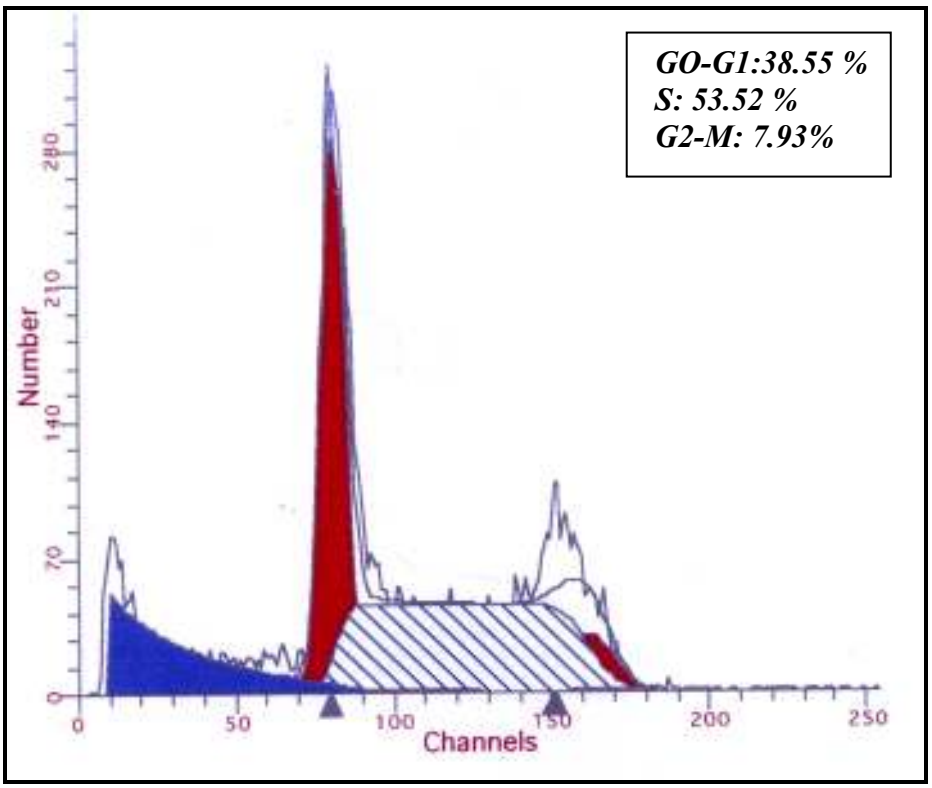


Fig. (28): Flow cytometric analysis of cell cycle profile of myeloma cell line (SP2OR) without treatment[control].

G_0 (quiescence state), G_1 (GAP1 phase), S (synthetic Phase), G2 (GAP2 phase), M (mitosis).

Figure (28) illustrated that cell cycle profile of myeloma cell without treatment. It was observed that the number of cells in G0/G1 phase was ***38.55*** %, S phase was ***53.52*** % and G2/M was ***7.93*** %.

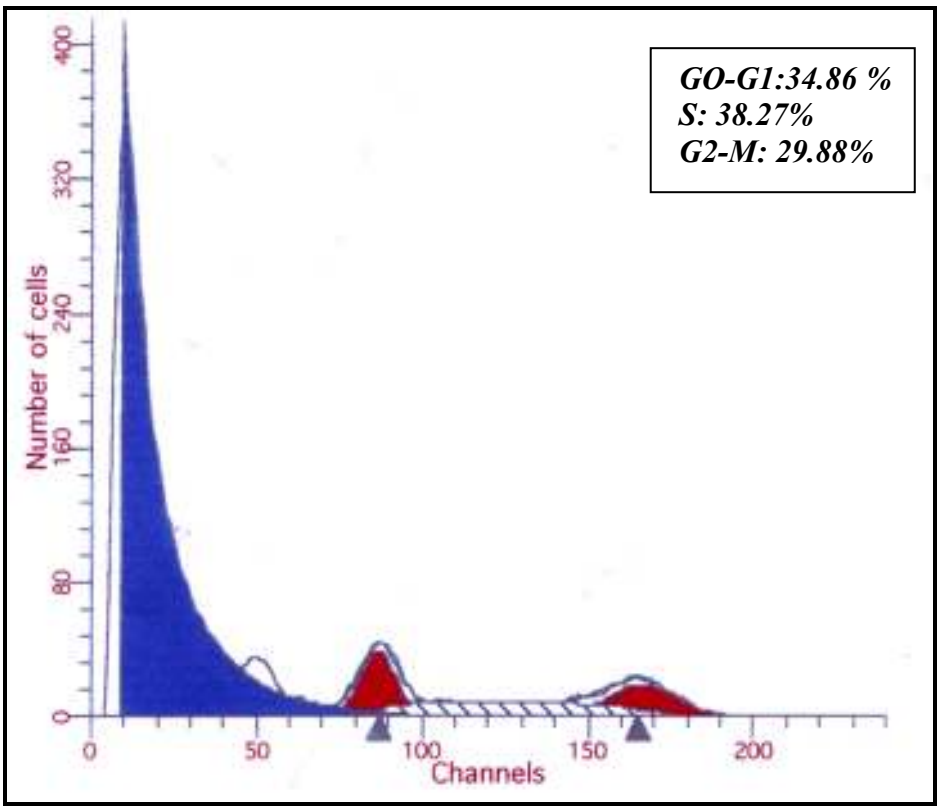


Fig.(31): Flow cytometric analysis for the effect of Bortezomib ( ) on cell cycle profile of myeloma cell line (SP2OR) .

The results of effect of **Bortezomib ( )**on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of **Bortezomib** treatment is illustrated in figure (29) where the number of cells in G0/G1 phase decreased from ***38.55*** to ***34.86*** %. It was observed that delay cell- cycle progression and cell cycle arrest at the S phase and G2/M phase.

The results of effect of **Bortezomib ( )** on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of **Bortezomib** treatment is illustrated in figure (29) where the percentage of cells in the G0/G1 phase decreased from ***38.55*** in the untreated control to ***34.86*** %, S phase decreased from ***53.52*** % in the untreated control to ***38.27*** %, while G2/M phase increased from 7.93***%*** to ***29.88%***. The above results suggested that bortezomib caused a G2/M arrest **myeloma cell** phase and an induction of apoptosis (Fig. 3).

of which was followed by the induction of apoptosis.

It was observed that delay cell- cycle progression and cell cycle arrest at the S phase and G2/M phase.

the percentage of cells in the
S + G2/M phase increased from 38.45% in the untreated control to

45%, 48.6% and 78.0%

%, while G0/G1 phase decreased from 58 to13.1%.

It was observed from figure (31) that the number of cells in the preG1 phase increased from (3.8% to 12.4%), decreased the number of cells in the G0/G1 phase (58% to 27.8%) and the number of cells in the S phase decrease from (16.5% to 8.6%) and increase the number of cells in the phase G2/M from (18.1% to 51.2%) after addition of (150µg/ml) for 24 hours.

Diss

treatment of myeloma cells with Bortezomib

resulted in cell cycle arrest at the G2/M phase and which was followed by the induction of apoptosis.

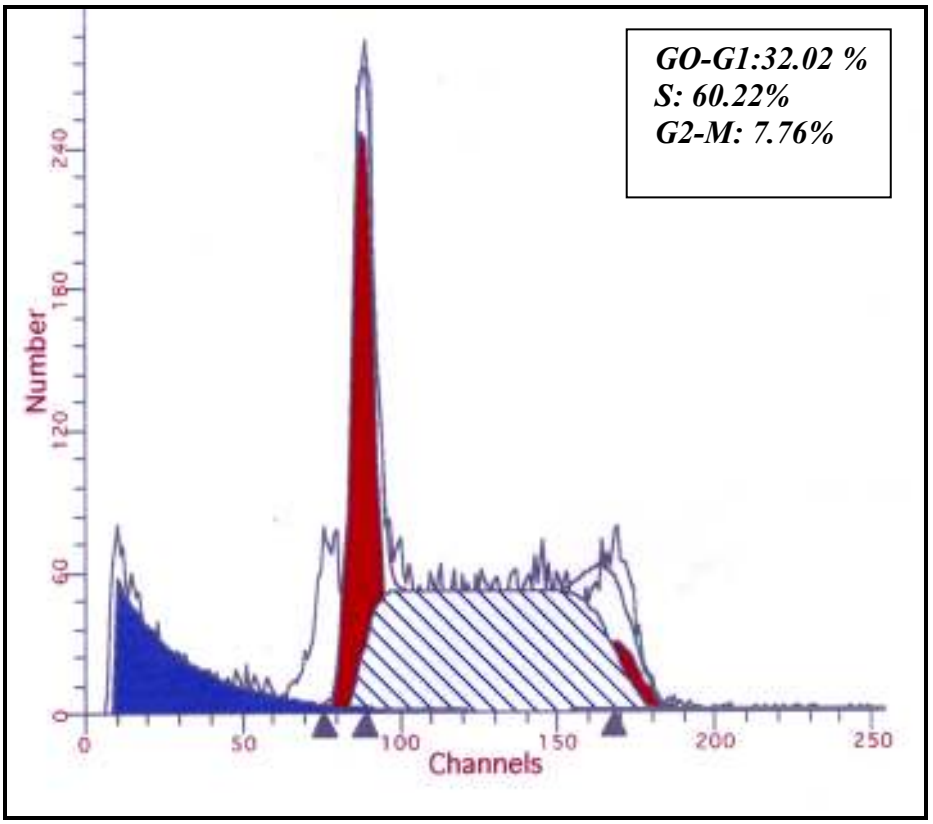


Fig.(31): Flow cytometric analysis for the effect of Myeloma-antibodies ( ) on cell cycle profile of myeloma cell line (SP2OR) .

It was observed from figure (31) that the number of cells in the G0/G1 phase decreased from (***38.55*** % to ***32.02*** %), increased the number of cells in the S phase (***53.52*** % to ***60.22%***) and the number of cells in the S phase decrease from (16.5% to 8.6%) and increase the number of cells in the phase G2/M from (18.1% to 51.2%) after addition of **Myeloma-**antibodies for 48 hours.

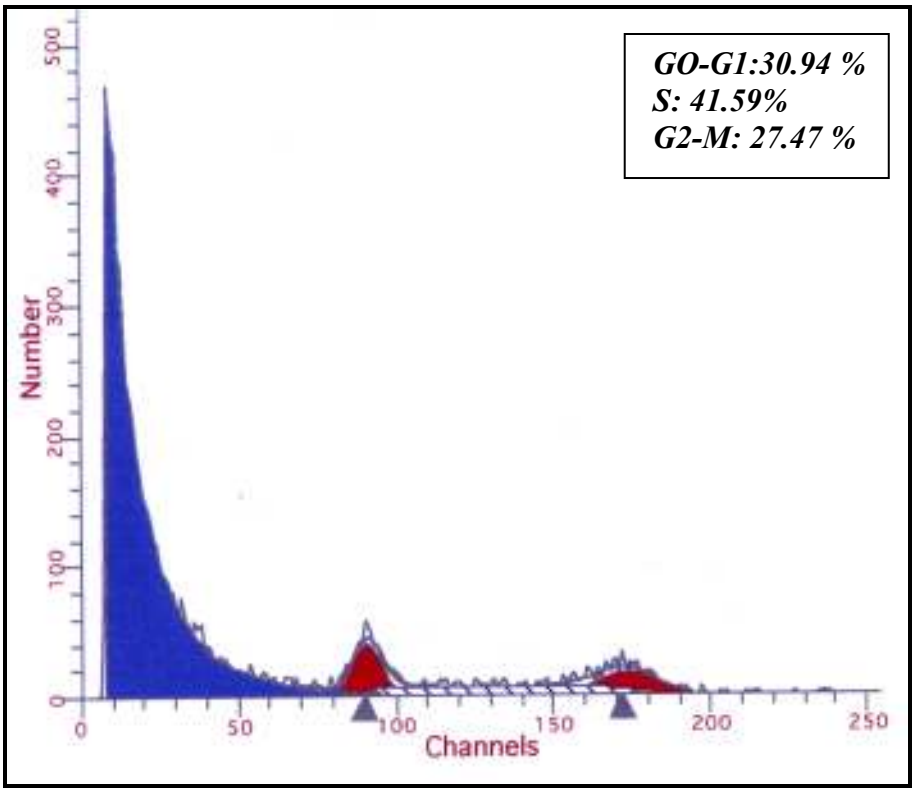
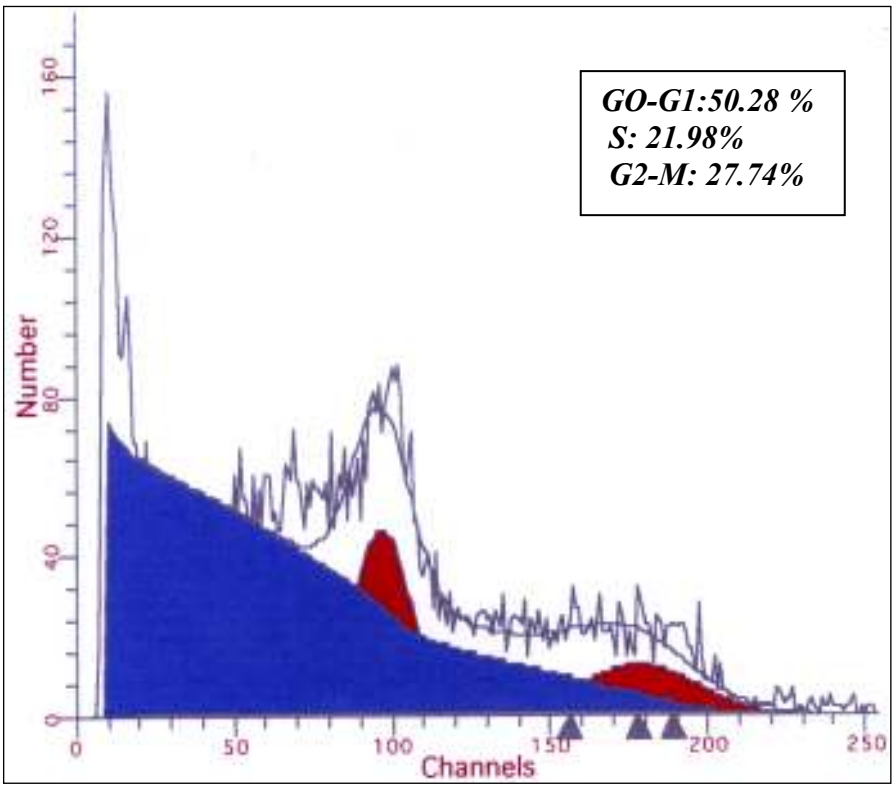


Fig.(31): Flow cytometric analysis for the effect of Bortezomib+ IFN-α **( ) on cell cycle profile of myeloma cell line (SP2OR) .**

The results of effect of **Bortezomib+ IFN-α ( )** on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of **Bortezomib** treatment is illustrated in figure (29) where the percentage of cells in the G0/G1 phase decreased from ***38.55*** in

the untreated control to ***30.94*** %, S phase decreased from ***53.52*** % in the untreated control to ***41.59*** %, while G2/M phase increased from 7.93***%*** to ***27.47 %.***The above results suggested that bortezomib caused a G2/M arrest **myeloma cell** phase and an induction of apoptosis (Fig. 3).



Diss

treatment of myeloma cells with Bortezomib
resulted in cell cycle arrest at the G2/M phase and which was followed by the induction of apoptosis.

The results of flow cytometric analysis of cell cycle profile of myeloma cell treated with IFN-α figure (33) revealed that the IFN-α induced growth inhibition was associated with cell accumulation in the G_1 phase, blockage of the cells entering from the G_1 to the subsequent S phase (a G_1 cell cycle arrest) leading to the growth cessation.

This cell accumulation in the G_1 phase is known as a G_1 cell cycle arrest (18). Thus, the IFN-α_{2b}/GSP combination may primarily target the G_1-S phase transition in the cell cycle, subsequently leading to the growth cessation.

Such study revealed that the IFN-α_{2b}/GSP-induced growth inhibition was associated with a 64% reduction in the S-phase cell popula-tion, due to a blockage of the cells entering from the G_1 to the subsequent S phase (i.e. a G_1 cell cycle arrest). This finding was also verified by analyzing G_1-specific cell cycle regulators: expressions of CDK2,

AB diss

Polyclonal antibodies are a possible alternative as they possess several inherent advantages. First, polyclonal antibodies raised against a selected target in hyper-immunized animals recognize the most immunogenic epitopes and so are 'naturally selected' for by the host [29]. This may also permit the development and manufacture of polyclonal antibodies that recognize multiple surface proteins and simultaneously activate multiple biochemical pathways leading to cell death. Second, polyclonal antibody therapeutics may be advantageous due to the high density of antibodies binding to the tumour cell surface. This would promote enhanced cross-linking of Fc receptors on effectors cells and efficient C1q binding in immunodeficient patients [31]. Finally, the PAb could preclude the development of tumour cell ''escape variants'', because the probability that tumour cells will simultaneously lose all target epitopes is extremely small Polyclonal antibodies are a possible alternative as they possess several inherent advantages. First, polyclonal antibodies raised against a selected target in hyper-immunized animals recognize the most immunogenic epitopes and so are 'naturally selected' for by the host [29]. This may also permit the development and manufacture of polyclonal antibodies that recognize multiple surface proteins and simultaneously activate multiple biochemical pathways leading to cell death. Second, polyclonal antibody therapeutics may be advantageous due to the high density of antibodies binding to the tumour cell surface. This would promote enhanced cross-linking of Fc receptors on effectors cells and efficient C1q binding in

immunodeficient patients [31]. Finally, the PAb could preclude the development of tumour cell ‘‘escape variants’’, because the probability that tumour cells will simultaneously lose all target epitopes is extremely small.

In this study, variable doses of myeloma antibodies were used for treatment of myeloma. The results obtained suggested that myeloma antibodies reduced cell viability in a dose-dependent and time-dependent manner by inhibiting proliferation or by inducing cytotoxicity in myeloma cell lines.

In ascites bearing mice treated with myeloma antibodies it was shown that the weight (mean±SD) of the animal and the viability (%) of myeloma cells were decreased. The results indicated that PAb reduced tumour growth by binding multiple surface antigens on myeloma cells and promoting apoptosis.

Treatment with PAb led to increased enzyme activities of caspases (8 and 9) at 48 h post-treatment and ultimately causing apoptosis by DNA fragmentation.

which suggested that the mechanism of apoptosis involved caspases activation.
which suggested that the mechanism of apoptosis involved caspases activation.

The results of flow cytometric analysis of cell cycle profile of myeloma cell treated with myeloma antibodies showed that myeloma antibodies increased the number of apoptotic cells and induced S phase arrest.

To confirm that PAb induced apoptosis of myeloma cells, we examined the activation of caspases-3, -8, and -9 by Western blotting (Fig. 3f). Indeed, treatment with PAb led to increased enzyme activities of these caspases at 48 h post-treatment and ultimately causing apoptosis by DNA fragmentation.

Polyclonal antibodies are a possible alternative as they possess several inherent advantages. First, polyclonal antibodies raised against a selected target in hyper-immunized animals recognize the most immunogenic epitopes and so are 'naturally selected' for by the host [29]. This may also permit the development and manufacture of polyclonal antibodies that recognize multiple surface proteins and simultaneously activate multiple biochemical pathways leading to cell death. Second, polyclonal antibody therapeutics may be advantageous due to the high density of antibodies binding to the tumour cell surface. This would promote enhanced cross-linking of Fc receptors on effectors cells and efficient C1q binding in immunodeficient patients [31]. Finally, the PAb could preclude the development of tumour cell ''escape variants'', because the probability that tumour cells will simultaneously lose all target epitopes is extremely small

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development of tumour cell ‘‘escape variants’’, because the probability that tumour cells will simultaneously lose all target epitopes is extremely small

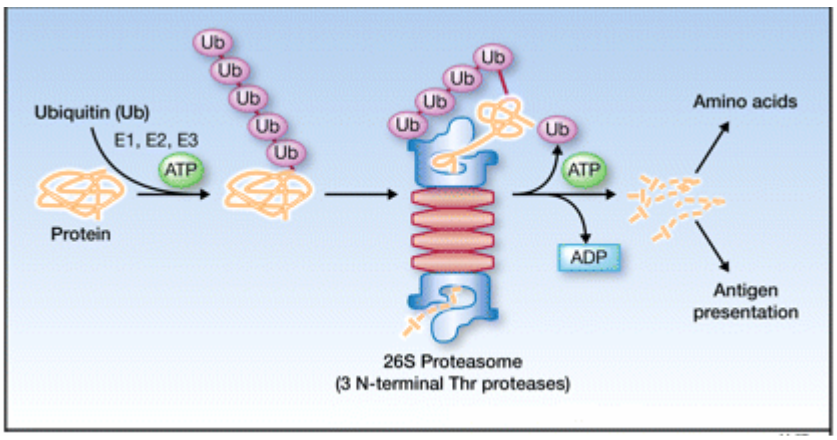

Figure 1. Protein degradation by the ubiquitin–proteasome pathway. A chain of 4 ormore ubiquitinmolecules are attached by the action of a series of ubiquitin ligases (E1, E2, E3) to 1 or more lysine residues on the target protein to be degraded. The ubiquitin–protein complex is transported to the proteasome, where the ubiquitin chain is removed, allowing the target protein to be unfolded by an ATP-dependent process and translocated to the interior of the proteasome, where it is degraded by 3 threonine (Thr) proteases to yield peptide fragments.

Materials

Animals

●Rabbits: New Zealand White Female Rabbits (five) (2-3 kg body weight, three months old).

●Mice: Female Balb/c mice (115 mice) with body weight of (range 20 - 25 gm) and age of 12 weeks old .The animals were kept at constant environmental and nutritional conditions throughout the experimental period and kept at room temperature (22±2) ºC with a 12 h on/off light schedule. Standard food and water were allowed to mice all over the experiments.

Myeloma cell line

SP2/OR myeloma cell line ,Hammersmith, London,UK.

Equipment:

- Biosafety Laminar flow cabinet, TEISTAR BIO.11 A, Jose TAPIOLAS, 120, s 7245, TERRASSA-SPAIN.
- CO_2 incubator with water jacket (37 °C), CO2 supply and humidified environment is not necessary provided, Heraeus Instruments, Germany.
- Inverted microscope for cell counting, Olympus, Japan.
- Deep freezer (-70 °C), Heraeus Instruments, Germany.
- Centrifuge: cell prep, dedicated cytology, Fisher scientific, LTD, UK.
- Liquid Nitrogen Refrigerator, LOCATOR,I.R.Thermolyme CRYO, Biological storage system, USA.
- Water bath, Cole Parmer instrument Co. Niles, Model 12500, USA.

- PH meter: Digital, Model 601, Orion Research Incorported, USA.
- Multi-Crystal Gamma Counter, Berthold, LB 211, Germany.
- Magnetic stirrer, Heidolph,Germany.
- Vacuum pump: Rotatory Van Vacuum Punp, D-97877, Werthein, Germany.
- Gravity oven, Asheville, N.C., U.S.A.
- Microanalytical Balance: Toplo, Model XE-100.Ading Denever Instrument Co,USA.
- Vortex Mixer: Model-231,Fisher Scientific Co, USA.
- Spectrophotometer UV-160A, Visible Recording Spectro-photometer, Shimadzu-Japan.
- Abbott AxSYM system.
- ELISA,Labsystems, RS 232,Finland.
- Flow cytometer Becton-Dickinson, San Jose, CA

Tools and Devices

●Haemocytometer, Boeckel-co, Scientific equipment Br and st wiefe 4.2000, Hamburg 11, western, Germany.

- Douple-hub-syringe: 2x5 ml "Hamilton" luer lock syringes "syringe connector, Reno, Nivada, USA.
- Cryogenic work station, Nalgene, USA.
- Multipipettes: Eppindorf, ranged from 5-1000 µl,Germany.
- 0.22 um sterile membrane filters, Gelman Acrodisc DLL, Germany.

Plastic wares:

- 25 cm^2 tissue culture flasks.
- 75 cm^2tissue culture flasks.
- Sterile Pasteur, 1ml, 5 ml and 10ml, disposable plastic pipettes.
- Sterile 1 ml and 10 ml, disposable plastic pipettes.
- Sterile centrifuge tubes, with cap, 15 ml.
- Sterile centrifuge tubes, with cap, 50 ml.
- Storage Ampoules, (2 ml). (ICN/Flow).
- Expanded polystyrene Box, 1 cm, wall thickness.
- Polystyrene Petri dishes (ICN/Flow).
- 12 well sterile tissue culture plate.
- 24 well sterile tissue culture plate.
- Sterile polystyrene containers. 60 ml and 100 ml (sterilin).
- 1 ml sterile syringes (Becton and Dickinson, Plastipak or equivalent)
- 70% ethanol in water bottle (EL Nasser pharmaceutical chemical co, Egypt).
- Sterile universal containers, 30 ml (sterile).
- Measuring cylinder 25 ml.

Chemicals, Reagents and Biological materials

- Bortezomib: (Velcade, PS-341) was obtained from Millennium Pharmaceuticals Inc. (Cambridge, MA) and reconstituted with sterial normal saline (0.9%), to a stock concentration of 1 mg/ml prior to use in all assays.The chemical name for bortezomib, the monomeric boronic acid, is [(1R)-3-methyl-1-[[(2S)-1-oxo-3-phenyl-2-[(pyrazinylcarbonyl) amino]propyl]amino]butyl] boronic

acid. The molecular weight is 384.24. The molecular formula is $C_{19}H_{25}BN_4O_4$.

- IFN-Alpha: (specific activity = 3.3 x 10^6 U/mg) was purchased from Access Biochemical (San Diego, CA).
- Tissue culture medium , RPMI-1640, with 15mM HEPES buffer , sterile , powder , Sigma , USA (stored at 4°C).
- Foetal bovine serum (FBS): liquid, sterile, sigma, USA (stored at less -20ºC).
- Antibiotic antimycotic mixture (10.000u penicillin ,10mg streptomycin and 25 µg amphotericin B 1ml in 0.9 % Na Cl): liquid , sterile , sigma , USA (stored at less than 0°C) .
- L-glutamine (200 mM solution), Hybri Max®, sterile filtered, Sigma, USA 1(stored at less than 0°C).
- Trypan blue dye (MOD), 0.5% w/v in normal saline ICN biological, CA, USA (stored at 15°C- 30 °C).
- Freund's adjuvant complete (FAC sigma, USA (stored at 0°C -5°C).
- Freund's adjuvant Incomplete (FIA sigma, USA (stored at 0°C -5°C).
- Pristane (2, 6, 10, 14. Tetramethyl pentadecane) (Sigma, USA) .
- Dimethyle sulfoxide (DMSO, sterile. filtered, hybrid max®, Sigma, USA. (Stored at room temperature).
- Sodium Iodide-125 (Na ^{125}I), Radioactive concentration 3700 M Bq/ml. Half –life 59.9 days, Izotop.

- Di-Sodium hydrogen orthophosphate: $Na_2HPO_4.2H_2O$, Extra Pure, Merck, Germany.
- Sodium dihydrogen orthophosphate: $NaH_2PO_4.2H_2O$, Sigma Chemical Co, USA.
- Sodium chloride: NaCl, MW 58.44, Adwic Chemical Co., Egypt.
- Chloramine–T ($C_7H_7ClNO_{2S}Na$): N-Chloro-P-toluene sulfonamide sodium salts, Sigma Chemical Co, USA.
- Sodium metabisulphite ($Na_2S_2O_5$) : MW 190.1, S-1516 Sigma Chemical Co, USA
- Potassium iodide: KI, MW 166.01. Sigma Chemical Co.,P-2963, USA .
- Sodium bicarbonate: $NaHCo_3$, MW 84.01, LTD Dagenham, UK.
- ELISA kits (Sigma)
- β2-microglobulins kits (Sigma).
- Phosphate- buffered saline (PBS).
- Stock Solution: 2 g trisodium citrate dihydrate, (3.4 mM), 2ml Igepal® (0.1% v/v) and 1044mg spermine tetrahyrdro-chloride, (1.5 mM) in 2 L of distilled water after adjusting to pH 7.6.
- Solution A: 15 mg Trypsin in 500 ml of Stock Solution (adjusted to pH 7.6)
- Propidium iodide 5 µg/mL (PI, sigma).
- Ribonuclease enzymes (RNase10 µg/m (Sigma).

Preparation of Reagents

1. Phosphate buffer (0.5 m, pH 7.4) (Stock solution):

It was prepared by dissolving 71.6 g $Na_2HPO_4.2H_2O$ and 15.3 g $NaH_2PO_4.2H_2O$ in 1 liter double distilled water (DDW) and stored at room temperature.

2. ***Phosphate buffer (0.05 m, pH 7.4)***:

It was freshly prepared by diluting the stock Phosphate buffer (0.5 M) 10- fold with DDW.

3. ***Phosphate buffer saline (PBS) (0.05 m, pH 7.4)***:

It was freshly prepared by diluting the stock Phosphate buffer (0.5 M) 10- fold with normal saline (0.9%).

4. Sodium Chloride (Na Cl) (3 M)

175.5 g Na Cl were dissolved in 1 liter DDW.

5. **Sodium Hydroxide (Na OH) (0.01 N):**

0.4 g NaOH were dissolved in 1 liter DDW.

6. Phosphate buffer: The following reagents were added to each other and completed to 100 ml with DDW.

-10 ml Phosphate buffer (0.5 M), pH7.4.

-0.1 g Sodium azide.

-0.1 g Bovine Serum Albumin.

-0.9 g Sodium chloride.

Methods

1-Preparation of tissue culture medium

- **Plain myeloma cell culture medium**

The RPMI-1640 powder was reconstituted by quantity sufficient sterile distilled water (900 ml). The contents were stirred until dissolved, and then 2 g of sodium bicarbonate was added, PH was adjusted into (7.0 ± 0.3). The additional water was added to bring the solution to final volume of one liter. The contents were sterilized immediately by filtration using 0.22 µm millipore membrane filter.The medium was aseptically dispensed into a sterile container **(Chapmen, 1998)**.

- **Myeloma cell culture medium**

The myeloma cell culture medium was included the following constituents, 100 ml RPMI-1640, 10 ml Foetal bovine serum (FBS), 1ml Antibiotic Antimycotic and 1 ml L-Glutamine **(Chapmen, 1998)**.

2-Maintenance of myeloma cells

Established myeloma cells were frozen as 1×10^7 cells / ml as 0.5 ml aliquots. One aliquot may be removed from liquid nitrogen. It is most convenient at this stage to handle only 10-15 ml medium in 25 cm^2 flasks until cell growth enter log-phase and viability of > 95 % are achieved . Incubation was continued at 37 °C and the cells recounted with a viability check daily prior to adjustment of the cell count to $2.5\text{-}5\times10^5$ cells. This process was continued until a cell doubling time of 18-24 hrs was achieved with a viability of > 95%. Cell density should not be allowed to exceed $1\text{-}1.5\times10^6$ cells / ml. **(Chapmen, 1998).**

3-Counting myeloma cells and viability checks

To determine total number of cells / ml and viability (%), 100 µl of cells suspension was mixed with 100 µl of trypan blue dye. A drop of mixture was transferred into edge formed between the cover slip and the slide (a hemocytometer) and observed under the microscope where the living cells did not stain with the dye, while the dead cells stained with blue color. The myeloma cells (viable cells and dead cells were counted in the big squares (WBCs squares) of hemocytometer and the results were calculated as follow **(Harlow and David, 1988)**.

$$\text{Total Counts of myeloma cells} = \frac{\text{Total number of cells}}{\text{Number of square counted}} \times 2 \times 10^4 = \text{number of cells/ml}$$

$$\text{Viable cells} = \frac{\text{Total number of viable cells}}{\text{Number of squares counted}} \times 2 \times 10^4 = \text{number of viable cells/ml}$$

$$\text{Viability (\%)} = \frac{\text{Number of viable cells /ml}}{\text{Number of cells/ ml}} \times 100$$

4-Cryopreservation of SP2/OR myeloma cells

10 ml of freezing mixture {10% dimethyl sulfoxide (DMSO) in ice cold foetal bovine serum (FBS)} were prepared. The DMSO was add to the FBS drop wise with shaking and placed in ice in the cryogenic work station. Cells were counted and tested for viability, then centrifuged for 5 min at 900 rpm and the supernatant was discarded. The cells were resuspented at 1x 10^7 cells /ml in plain RPMI-1640 and washed (3) times with RPMI. The cells at $1x10^7$ cells /ml (viability > 90%) were resuspended in the freezing mixture. The universal container was tapped to loosen the cell pellet, and then the freezing mixture was added drop wise with continuous shaking. Aliquots of 0.5 ml were prepared in tightly stoppered storage cryogenic vials , transferred to the expanded polystyrene cryobox , sealed

with tape , placed at -70 °C deep freezer overnight and finally stored in liquid nitrogen cryovessel (at –185 °C) until required **(Chapmen and Ratcliffe,1998)** .

Production of Antimyeloma Polyclonal Antibodies:-

Production of Antimyeloma polyclonal antibodies was carried out by using five healthy female mature White New-Zealand rabbits, 2-3 Kg. They were immunized with myeloma cells to raise anti-myeloma antibodies. Rabbits were kept under the same hygienic conditions, well balanced diet and water was supplied adlibitum. The production of Anti-myeloma Antibodies was carried out through the following steps:-

1. Preparation of myeloma-adjuvant mixture:

0.5 ml (1×10^7) of myeloma cells were emulsified with 1 ml of Freund's adjuvant (Complete or Incomplete). Freund's complete adjuvant (FCA) was used for primary injection while Freund's incomplete adjuvant (FIA) was used for booster doses**)**. Emulsification was performed using Hamilton double-hub syringes connected to each other with narrow metallic tubing. The aqueous solution was placed in one syringe and the oily adjuvant in the other one. The aqueous solution was forced through the tubing into the oil in the other syringe. Then the mixture was forced back. This process was repeated many times until a stable water-in-oil emulsion is formed. Stability of the formed emulsion was tested by allowing a few drops to fall into beaker containing cold water. A stable emulsion remains white drop on the surface indicating the enclosure of the water phase within the oil phase (water-in-oil phase) **(Marlies and Coenraad, 2005)**.

2. Route of Injection

For primary immunization, each rabbit except control rabbit was received 1.5 ml of emulsion where 1.2 ml of emulsified mixture was injected subcutaneously over the shoulder at 6 sites (200 µl in each site). The remaining 0.3 ml of emulsified mixture was injected intramuscular at 2 sites (150 µl in each site).

-For the booster immunization, emulsified mixture was given in the same way except that Freund's complete adjuvant (FCA) was replaced by the Freund's incomplete adjuvant (FIA) **(Leenars and Hendrickson, 1999)**.

3. Immunization schedule:

Six immunization injection were administered, one primary and five booster injection, at one month intervals **(Ostebold, 1982)**.

4. Blood sampling and harvesting the raised anti-sera:

The rabbits were bled for sampling through the marginal ear vein a week after the first booster injection and continued until the end of the immunization schedule at a week intervals, using a simplified device. A simple technique for withdrawing blood from rabbit's marginal ear vein has been described by Hope et al., (1969). Marginal ear was incised and a mild negative pressure was applied through the device to keep a continuous blood flow. Serum was separated after 6 months from the blood without any additives and was kept under -20 °C till testing **(Marlies and Coenraad, 2005)**.

ELISA Assay

Myeloma cells were grown overnight in the wells of poly-lysine-coated 96-well plates (1×10^6). The media was removed and the cells were washed three times in PBS. After washing, the cells were blocked with 5% skimed milk in blocking buffer (PBS containing 0.05% Tween-20 or PBST) for 1 h at room temperature. The blocking reagent was removed and the cells were washed three times in PBS before the Antimyeloma Antibodies was added. The Antimyeloma Antibodies was added and cells were incubated for 1 h at room temperature. The antibody was removed and the cells were washed three times in PBST. The secondary goat anti-rabbit antibody linked to alkaline phosphatase was added for 30 min. The cells were then washed three times in PBST. Subsequently, alkaline phosphatase substrate BCIP/NCP (Sigma) was added and absorbance was measured at 450 nm using a 96-well plate reader.

Labeling of Anti-myeloma Antibodies

Chloramines –T method:

Into a vial containing 20 µl of serum, 20 µl of 0.5 M phosphate buffer, pH 7.4 was added then mixed with 0.5 mCi 0f iodine-125. The reaction was started by the addition of 10 µl of phosphate buffer 0.05 M, pH 7.4 containing 20µg of chloramine -T. The reaction was allowed to proceed for 30 seconds and quenched by the addition of 10 µl of phosphate buffer 0.05 M, pH 7.4 containing 20µg of sodium metabisulfite. After that 20µg of KI in10 µl of phosphate buffer 0.05 M, pH 7.4 was added as carrier **(Hunter and Greenwood, 1962)**.The purification was carried out by transferring the iodination mixture into the top of a conditioned PD-10 chromatographic column 35x1cm. Elution was carried out using 0.05 M phosphate buffer containing 0.3% bovine serum albumin solution. Elution pattern was monitored by counting

radioactivity of fractions in a well type NaI (TI) scinitillation counter **(Graham, 1996).**

In Vitro Study:

◙ Effect of Bortezomib (Velcade) on myeloma cell line SP2OR

I-Growing of myeloma cells

Myeloma cells were cultured in the culture medium which prepared as mentioned above until cell growth enter log-phase and viabilities of > 95 % were achieved.

II-Preparation of variable doses of bortezomib

The drug was diluted in culture media to prepare variable doses of (5, 10, 20, 30, 50 and 100 nM).

III-Addition of the prepared variable doses to myeloma cells

Bortezomib was added at variable doses (5, 10, 20, 50, 75 and 100 nM). Each dose was added into the myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily **(Megan, 2007)**.

◙ Effect of Alpha-interferon on myeloma cell line SP2OR

I-Growing of myeloma cells

The myeloma cells were grown as mentioned above

II-Preparation of variable doses of Alpha-interferon

The drug was diluted in culture media to prepare variable doses of (50, 100, 500, 1000, 5000 and 10000 IU/ml).

III-Addition of prepared variable doses to myeloma cells

Alpha-interferon was added at variable doses of (50, 100, 500, 1000, 5000 and 10000 IU/ml) into myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily **(William et al., 2003)**.

◙ Effects of bortezomib plus IFN-α on myeloma cell line SP2OR

Variable doses of combined treatment with[bortezomib + Alpha-interferon] **D1** (5 nM Bz +50 IU/ml IFN-α), **D2**(10nM Bz +100 IU/ml IFN-α) , **D3**(20 nM Bz +500 IU/ml IFN-α), **D4**(30 nM Bz +1000 IU/ml IFN-α),**D5**(50 nM Bz +5000 IU/ml IFN-α), **D6**(100 nM Bz +10000 IU/ml IFN-α) were prepared and added into myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily **(Megan, 2007)**.

◙ Effects of Antimyeloma antibodies on myeloma cell line SP2OR

Antimyeloma antibodies was added at variable doses of (10, 20, 30, 50,100,200µl) into myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily.

◙ Effects of Labeled antimyeloma antibodies on myeloma cell line SP2OR

Labeled antimyeloma antibodies was added at variable doses of (10, 15, 20, 25, 30, 50 µCi/ml) into myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily.

◙ Effects of bortezomib +IFN-α + Myeloma-Antibodies on myeloma cell line SP2OR in vitro

Variable doses of combined treatment with [bortezomib +Alpha-interferon+ Antimyeloma Antibodies] **D1** (5 nM Bz +50 IU/ml IFN-α+10 µl Ab), **D2**(10nM Bz +100 IU/ml IFN-α+20 µl Ab) , **D3**(20 nM Bz +500 IU/ml IFN-α+30 µl Ab),**D4**(nM Bz +1000 IU/ml IFN-α+50 µl Ab), **D5**(50 nM Bz

+5000 IU/ml IFN-α+100 µl Ab), **D6**(100 nM Bz +10000 IU/ml IFN-α+200 µl Ab) were prepared and added into myeloma cells then the plate was incubated at 37ºc for 6 days. The total count and viability were calculated daily.

In Vivo Study:

Induction of tumor in mice (Ascites)

A line of myeloma cell (SP2OR) was used in the induction of ascites. 0.5ml pristine (2, 6, 10, 14-tertramethyl-decanoicnacid) was injected intraperitoneal (i.p) then after 10-30 days the antigen incomplete Freund's adjuvant was injected. The injection was repeated on days 7 and 21 after first injection then myeloma cells were injected. The animals were maintained for 10-15 days till the tumor development was apparent as described by **(Harlow and David, 1988)**. Treatment was started after development of tumor. Mice were used in this study and each mouse weighed daily.

Experimental design:

At the beginning of the experiment, mice were divided into eight groups, The 1st group was control group, the 2nd group was ascites bearing mice, the 3rd group was ascites bearing mice that treated with bortezomib, the 4th group was ascites bearing mice that treated with alpha- interferon, the 5th group was ascites bearing mice that treated with combined therapy between bortezomib and alpha-interferon , 6th group was ascites bearing mice that treated with myeloma- antibodies, 7th group was ascites bearing mice that treated with labeled antimyeloma antibodies and 8th group was ascites bearing mice that treated with combined therapy (bortezomib+ alpha-interferon+ myeloma- antibodies).

Groups are divided as followed:

1-Control group (5 mice): Animals served as untreated control group

2-Ascites group (5 mice): Mice bearing ascites

3-Group1: Mice bearing ascites and injected with bortezomib. This group was divided into four subgroup, each of 5 mice.

Subgroup (I): In this subgroup mice were injected intraperitoneal (i.p) with bortezomib at dose of 0.05 mg/kg body weight twice a week for a total of 8 injections.

Subgroup (II): In this subgroup mice were injected intraperitoneal with bortezomib at a dose of 0.1 mg/kg body weight twice a week for a total of 8 injections.

Subgroup (III): In this subgroup mice were injected intraperitoneal with bortezomib at a dose of 0.5 mg/kg body weight twice a week for a total of 8 injections.

Subgroup (IV): In this subgroup mice were injected intraperitoneal with bortezomib at a dose of 1.0 mg/kg body weight twice a week for a total of 8 injections **(Dharminder et al., 2005 and Richard et al., 2002).**

4-Group 2: Mice bearing ascites and injected with alpha-interferon. This group was divided into three subgroup, each of (5) mice:

Subgroup (I): In this subgroup mice were injected intraperitoneal with alpha-interferon at a dose of 10^3 IU /kg body weight twice a week for a total of 8 injections.

Subgroup (II): In this subgroup mice were injected intraperitoneal with alpha-interferon at a dose of 10^4 IU /kg body weight twice a week for a total of 8 injections.

Subgroup (III): In this subgroup mice were injected intraperitoneal with alpha-interferon at a dose of 10^6 IU /kg body weight twice a week for a total of 8 injections **(Angela et al., 2006)**.

5-Group 3: Mice bearing ascites and treated with bortezomib + alpha-interferon. This group was divided into four subgroup, each of (5) mice:

Subgroup (I): In this subgroup mice were injected intraperitoneal with a dose D1 (0.5 mg Bz +10^4 IU /kg IFN-α) twice a week for a total of 8 injections.

Subgroup (II): In this subgroup mice were injected intraperitoneal with a dose D2 (0.5 mg Bz +10^6 IU /kg IFN-α) twice a week for a total of 8 injections.

Subgroup (III): In this subgroup mice were injected intraperitoneal with a dose D3 (1.0 mg Bz +10^4 IU /kg IFN-α) twice a week for a total of 8 injections.

Subgroup (IV): In this subgroup mice were injected intraperitoneal with a dose D4 (1.0 mg Bz +10^6 IU /kg IFN-α**)** twice a week for a total of 8 injections **(Angela et al., 2006)**.

6-Group 4: Mice bearing ascites and injected with antimyeloma polyclonal antibodies. This group was divided into three subgroup, each of (5) mice:

Subgroup (I): In this subgroup mice were injected intraperitoneal with antimyeloma antibodies at dose of 50µl twice a week for a total of 8 injections.

Subgroup (II): In this subgroup mice were injected intraperitoneal with antimyeloma antibodies at dose of 100µl twice a week for a total of 8 injections.

Subgroup (III): In this subgroup mice were injected intraperitoneal with antimyeloma antibodies at dose of 200µl twice a week for a total of 8 injections.

7-Group 5: Mice bearing ascites and treated with labeled antimyeloma polyclonal antibodies. This group was divided into three subgroup, each of (5) mice:

Subgroup (I): In this subgroup mice were injected intraperitoneal with labeled antimyeloma antibodies 0.1mCi per mice.
Subgroup (II): In this subgroup mice were injected intraperitoneal with labeled antimyeloma antibodies 0.2mCi per mice.
Subgroup (III): In this subgroup mice were injected intraperitoneal with labeled antimyeloma antibodies 0.3mCi per mice (**Behr et al., 1998)**.

8-Group 6: Mice bearing ascites and treated with [bortezomib + alpha-interferon+ Antimyeloma Antibodies]. This group was divided into four subgroup, each of (5) mice:

Subgroup (I): In this subgroup mice were injected intraperitoneal with a dose D1 (0.5 mg Bz +10^4 IU /kg IFN-α+200µl Ab) twice a week for a total of 8 injections.
Subgroup (II): In this subgroup mice were injected intraperitoneal with a dose D2 (0.5 mg Bz +10^6 IU /kg IFN-α+200µl Ab) twice a week for a total of 8 injections.
Subgroup (III): In this subgroup mice were injected with a dose D3 (1.0 mg Bz +10^4 IU /kg IFN-α+200µl Ab) twice a week for a total of 8 injections.
Subgroup (IV): In this subgroup mice were injected intraperitoneal with a dose D4 (1.0 mg Bz +10^6 IU /kg IFN-α+200µl Ab**)** twice a week for a total of 8 injections.

Samples collection

Blood sampling: After 4 weeks directly animals were anaesthised, blood was collected from heart using insulin syringes, then the blood was transferred into sterile tubes and allow to stand for 15 minutes at room temperature, then centrifuged at 3000 r.p.m. for 15 minutes. Serum was separated and kept at -20 °C until used.

Cell cycle analysis

a-Preparation of samples

- Control sample: myeloma cells (1×10^6) cultured in media alone without treatment.
- Myeloma cells treated with bortezomib: myeloma cells (1×10^6) treated with (20nM) bortezomib for 48 hours.
- Myeloma cells treated with IFN-α: myeloma cells (1×10^6) treated with (10^3IU/ml) IFN-α for 48 hours
- Myeloma cells treated with bortezomib Plus IFN-α: myeloma cells(1×10^6) treated with (20nM Bz+10^3IU/ml IFN-α) for 48 hours
- Myeloma cells treated antimyeloma antibodies **:** myeloma cells (1×10^6) treated with (200 µl Ab) for 48 hours
- Myeloma cells treated labeled antimyeloma antibodies **:** myeloma cells (1×10^6) treated with (15µCi LAb) for 48 hours
- Myeloma cells treated with bortezomib + IFN-α + Antimyeloma Antibodies: myeloma cells (1×10^6) treated with (20nM Bz +10^3IU/ml IFN-α +200µl Ab)

b-Procedure of flow cytometric analysis

Cells were washed twice with phosphate-buffered saline (PBS) ,250 mL of Solution A (trypsin buffer pH 7.6) was added and the cells were incubated for 10 min at room temperature, followed by the addition of 200 mL of Solution B (trypsin inhibitor and RNase buffer) and incubation for a further period of 10 min at room temperature. Finally, 200 mL of cold Solution C (propidium iodide stain solution) was added and the cells were incubated on ice for 10 min in the dark. The samples were analyzed by flow cytometer (Becton-Dickinson, CA, USA) **(Teresa and Robert ,2004).**

Determination of β2-Microglobulins

By Abbott AxSYM system assay

-Preparation of samples

- Control sample: myeloma cells (1×10^6) cultured in media alone without treatment {5 samples}.
- Myeloma cells (1×10^6) treated with (20nM) bortezomib for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (10^3IU/ml) IFN-α for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (20nM BZ+10^3IU/ml IFN-α) for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (200 µl Ab) for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated **(**15µCi LAb) for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (20nM Bz +10^3IU/ml IFN-α +200µl Ab) {5 samples}

Principle&Method: AxSYM β2-microglobulins assay is based on the microparticle enzyme immunoassay (MEIA) technology. The AxSYM β2-microglobulins reagents and sample are added to the reaction cell in the following sequence: sample and all AxSYM β2-microglobulins required for one test are pipetted by the sampling probe into various wells of a reaction vessel (RV) in the sampling center. The RV is immediately transferred into the processing center. Further pipetting is done in the processing center by the processing probe. The reaction occur in the following sequence: the probe delivers the sample, specimen diluent and AxSYM solution to the wells of the reaction vessel for diluting sample. An aliquot of the diluting sample.Anti- β2-M coated microparticles and AxSYM solution 4 are pipetted to one well of the reaction vessel. During the

incubation of this reaction mixture the β2-microglobulins in the specimen binds to the Anti- β2-M coated microparticles forming an antibody-antigen complex. An aliquot of the reaction mixture is transferred to the matrix cell. The matrix cell is washed to remove unbound materials. Anti- β2-M: alkaline phosphatase conjugate is dispensed onto the matrix cell and binds to the antibody-antigen complex. The substrate, Methylumbelliferyl phosphate, is added to the matrix cell and the rate of fluorescent product formation is measured by the MEIA optical assembly **(Bataille and Durile, 1976).**

Colorimetric Analysis of Caspase-8 and -9 Activity

a-Preparation of samples

- Control sample: myeloma cells (1×10^6) cultured in media alone without treatment {5 samples}.
- Myeloma cells (1×10^6) treated with (20nM) bortezomib for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (10^3IU/ml) IFN-α for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (20nM BZ+10^3IU/ml IFN-α) for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (200 µl Ab) for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated **(**15µCi LAb) for 48 hours {5 samples}.
- Myeloma cells (1×10^6) treated with (20nM Bz +10^3IU/ml IFN-α +200µl Ab) {5 samples}

b-Caspase-8 and Caspase -9 assay

The cells were washed two times with 2 ml PSA and subsequently treated with lysis buffer for 10 min on ice bath to lyse cells (25µl of lysis buffer / 1x10 ^{6}cells). The cell lysate was centrifuged at 10.000 xg for 1 min. The

supernatant was transferred to a new tube and kept in ice bath. The enzymatic reaction for caspase activity was carried out in 20 ELISA wells. Each reaction requires 50µl of cell lysate, 50µlof 2x Reaction was added to each reaction (before using2x Reaction 10µl of fresh DTT stock (dithiothreitol) was added to 1ml of 2x Reaction). 5µl of caspase colorimetric substrate (LEHD-PNA) was added to each reaction, and then the plate was incubated at 37 °C for 1-2 hours. The absorbance of each well was detected using ELISA reader at wavelength 405 nm **(Cristina et al., 2008)**.

♦ Determination of serum Creatinine level:

Measurement of serum **Creatinine** was carried out according to the method described by **Bartels et al., 1972**.

Principle

Creatinine in alkaline solution reacts with Picric acid to form a colored complex. The amount of the complex is directly proportional to the creatinine concentration.

Reagents:

- Standard Creatinine
- Reagent (R1a) Picric acid 35 mm/l
- Reagent (R1b) Sodium hydroxide 0.32 mmol/l

Working reagent: One volume of R1a (Picric acid) was added to one volume of R1b (NaOH). The mixture is stable for 3 days at +15 to +25°C.

Procedure

100 µl of sample and standard was added separately to 1 ml of working reagent placed at 25°C, mixed and after 30 seconds the absorbance A1 of the standard or sample was read. Exactly 2 minutes later, the absorbance A2 of the standard or sample was read.

Calculation

$A2 - A1 = \Delta A_{\text{sample or}} \Delta A_{\text{Standard}}$

Concentration of creatinine in serum=

$$\frac{\Delta A_{\text{sample}}}{\Delta A_{\text{Standard}}} \quad X \quad \text{Standard conc. (mg/dl)} = \quad \text{mg/dl}$$

Normal values are: 0.2-0.9 (mg/dl)

♦ Determination of serum Urea:

The blood urea was measured colorimetrically according to **Fawcett and Scott, 1960**.

Principle

Urea in serum is hydrolyzed to ammonia and carbon dioxide in the presence of urease enzyme. The ammonia is then measured photometrically by Berthelot´s reaction.

$$\text{Urea} + H_2O \xrightarrow[\text{enzyme}]{\text{Urease}} 2NH_3 + CO_2$$

NH_3 + Sodium hypochloride + phenol ⟶ indophenol (blue compound)

Reagents:

- Standard
- Reagent (R1): EDTA 116 mmol/l
  Sodium nitroprusside 6 mmol/l
  Urease 1g/l
- Reagent (R2): Phenol (diluted) 120 mmol/l
- Reagent (R3): Sodium hypochloride (diluted) 27mmol/l
  Sodium hydroxide (2.5 ml) 0.14N

Procedure

10 µl of sample or standard was added separately to 100 µl of R1, mixed and incubated for 10 min at 37°C. Then 2.5 ml of R2 and 2.5 ml of R_3 were added to each tube, shacked and incubated for 15 min at 37°C. The color developed was measured using spectrophotometer, at 546nm against reagent blank. For blank tube 2.5 ml of R2 and 2.5 ml of R_3 were added to 100 µl of R1 and incubated for 15 min at 37°C

Calculation

$$\text{Serum Urea} = \frac{A_{\text{sample}}}{A_{\text{Standard}}} \times \text{Standard conc.} = \quad \text{mg/dl}$$

Normal values are: 8-33 (mg/dl)

♦ Determination of serum Uric Acid:

The blood uric acid was measured colorimetrically according to **Fossati et al., 1980**.

Principle

Uric acid+ O_2+ $2H_2O$ $\xrightarrow{\text{Uricase}}$ Allantoine + CO_2 + H_2O_2

$2H_2O_2$ + 3,5-Dichloro-2-hydroxybenzenesulfonic acid + 4-Aminophenazone $\xrightarrow{\text{Peroxidase}}$ N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzo-quinoneimine

Reagents:

- Standard
- Reagent (R1a).Buffer
  Hepes buffer 50 mmol/l, pH=7.0
  3, 5 -Dichloro -2- hydroxybenzenesulfonic acid 4 mmol/l
- Reagent (R1b) Enzyme Reagent

4- Aminophenazone	0.25 mmol/l
Peroxidase	≥ 1000U/l
Uricase	≥ 200 U/l

Working reagent: Dissolve one vial of R1b in appropriate amount of R1a

Procedure

100 µl of sample and standard was added separately to 1 ml of working reagent placed at 37°C, mixed and after 5 min the absorbance of the standard or sample was read.

Calculation

$$\text{Serum Uric Acid} = \frac{A_{\text{sample}}}{A_{\text{Standard}}} \times \text{Standard conc.} = \text{mg/dl}$$

Normal values are: 3-7 mg/dl

♦ Determination of serum Alkaline phosphatase activity:

The blood Alkaline phosphatase was measured colorimetric according to the recommendations of the **Deutsche Gesellschaft fur Klinische Chemie, 1972.**

Principle

$$\text{p-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALK}} \text{phosphate} + \text{p-nitrophenol}$$

Reagents

1. Reagent (R_1a) buffer

Diethanolamine buffer	1 mol/l, pH=9.8
Magnesium chloride	0.5 mmol/l

2. Reagent (R_1b) substrate

p-Nitrophenylphosphate (solution)	10 mmol/l

Procedure

In a clean dry tube, 20 µl of serum was added to 1ml of reagent, mixed and the initial absorbance was read at 405 nm and read again after 1, 2 and 3 minutes.

Calculation

$U/I = 2760 \times \Delta A$

Normal values are: 35-96(U/I)

♦ Determination of Serum Alanine Aminotransferase activity (ALT)

Serum Alanine Aminotransferase (ALT) was determined according to the method of **Reitman and Frankel, 1957**.

Principle

$$\alpha\text{- oxoglutarate} + \text{L-alanine} \xrightarrow{\text{GPT}} \text{L- glutamate} + \text{pyruvate}$$

Alanine Aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine

Reagents:

- Reagent (R_1).Buffer

Phosphate buffer	100 mmol/l, pH=7.0
L-alanine	200 mmol/l
α- oxoglutarate	2.0 mmol/l

- Reagent (R_2)

2,4-dinitrophenylhydrazine	2.0 mmol/l

Procedure

In clean dry test tube, 100 µl of serum was added to 0.5 ml of R_1 (buffer), mixed and incubated for exactly 30 min at 37°C. Then 0.5 ml of R_2 was added, mixed and incubated for exactly 20 min at 25°C. Then 5 ml of 0.4 N Sodium hydroxide was added. At the same way blank was prepared replacing the sample with distilled water. The absorbance was measured spectrophotometrically at 546 nm against blank after 5 min.

Normal values are: 17-77(U/I)

♦ Determination of Serum Aminotransferase activity (AST)

Serum Aspartate Aminotransferase (AST) was determined according to the method of **Reitman and Frankel, 1957**.

Principle

$$\alpha\text{- oxoglutarate + L- aspartate} \xrightarrow{\text{GOT}} \text{L- glutamate + Oxaloacetate}$$

AST is measured by monitoring the concentration of Oxaloacetate hydrazone formed with 2, 4 dinitrophenylhydrazine

Reagents:

- Reagent (R_1).Buffer

Phosphate buffer	100 mmol/l, pH=7.0
L- aspartate	200 mmol/l
α- oxoglutarate	2.0 mmol/l

- Reagent (R_2)

2, 4-dinitrophenylhydrazine	2.0 mmol/l

Procedure

In clean dry test tube, 100 µl of serum was added to 0.5 ml of R_1 (buffer), mixed and incubated for exactly 30 min at 37°C. Then 0.5 ml of R_2 was added, mixed and incubated for exactly 20 min at 25°C. Then 5 ml of 0.4 N Sodium hydroxide was added. At same way blank was prepared replacing the sample with distalled water. The absorbance was measured spectrophotometrically at 546 nm against blank after 5 min.

Normal values are: 54-298 (U/I)

♦ Determination of Haemoglobin concentration

Principle

Haemoglobin is first oxidized by potassium ferricyanide into methemoglobin which is converted into cyanmethaemoglobin by potassium cyanide. The absorbance of the cyanmetcyanmet-haemoglobin is monitored at 540 nm.

Reagents:

Potassium Ferricyanide	0.61 mmol/l
Potassium Cyanide	0.77 mmol/l
Potassium Phosphate	1.03mmol/l
Surfactant	0.1%v/v

Procedure

In a clean dry test tube, 20 µl of well mixed blood was added to 5ml of reagent, mixed and measured the absorbance at 540 nm against distilled water after 5 minutes.

Calculation

Haemoglobuin concentration = A_{sample} x 36.77 (g/dl)
Normal values are: 10.2-13.6 g/dl

Results

In vitro study

Effects of Bortezomib, α-interferon, (Bortezomib + α-interferon), Myeloma-Antibodies, Lablled Myeloma-Antibodies and (Bortezomib + α-interferon+ Myeloma-Antibodies) on myeloma cell growth were determined by treating SP2OR cells with variable doses of these drugs for six days.

Effect of Bortezomib (Velcade) on growth of myeloma cells

Fig. (13) illustrates the effect of variable doses of Bortezomib (5, 10,20,30,50 and 100 nM/ml) on the viability (%) of myeloma cells during lifespan of 6 days compared to control group. The viability of myeloma cells decreased at 5 nM from 95% to 87%, at 10 nM from 89% to 31%, at 20 nM from 73 % to 11%, at 30 nM from 69% to 10%, at 50 nM from 41% to 8%, and at 100 nM from 30% to 7%. These results indicated that cell growth was inhibited by bortezomib treatment in a dose-and time-dependent manner. Low doses of bortezomib (5nM) did not increase cell growth inhibition for 6 days.

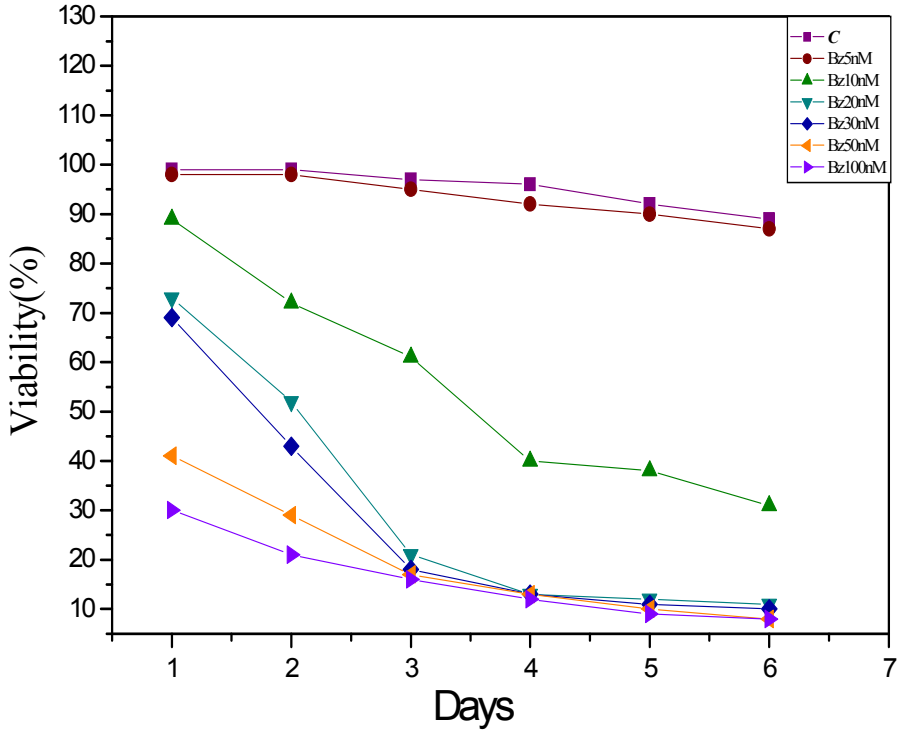

Fig. (13): Effect of variable doses of Bortezomib on the viability % of myeloma cells.

Effect of IFN-α on growth of myeloma cells

Effect of IFN-α on the viability of myeloma cells was evaluated. IFN-α was applied at different doses (50, 100, 500, 1000, 5000 and 10000 IU/ml). As shown in Fig.(14) the viability of myeloma cells decreased at 50 IU/ml from 98% to 88%, at 100 IU/ml from 91% to 70%, at 500IU/ml from 82 % to 69%, at 1000IU/ml from 80% to 64% , at 5000 IU/ml from 78% to 56%, and at 10000 IU/ml from 73% to 51%. These results indicated that the viability (%) decreased with increasing concentration of IFN-α.

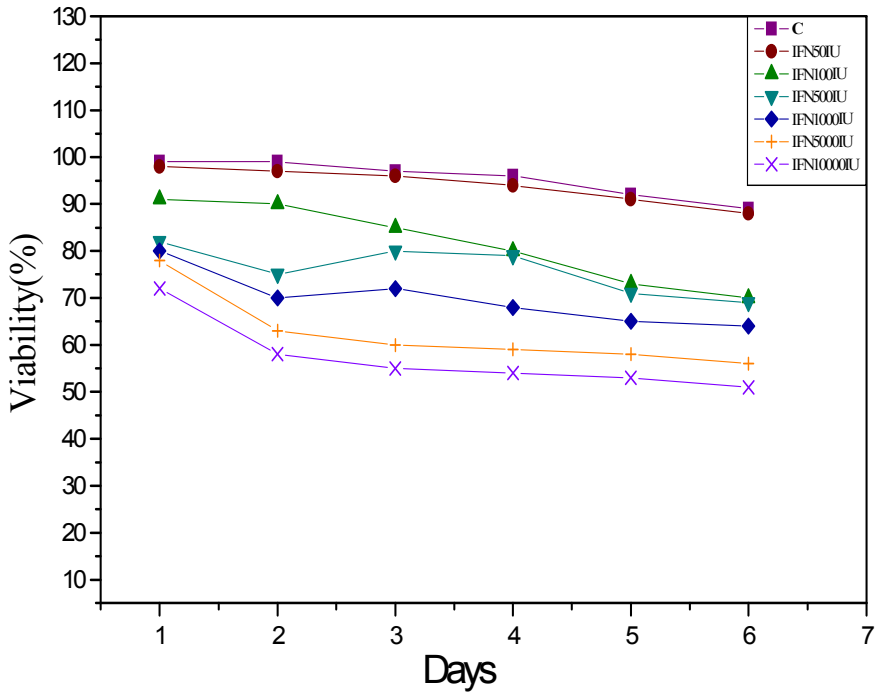

Fig. (14): Effect of variable doses of IFN-α on the viability % of myeloma cells.

Effects of bortezomib plus IFN-α on growth of myeloma cells

The effect of a combination of bortezomib and IFN-α on cell viability was investigated. It was observed from Fig. (15) that viability of myeloma cells decreased at D_1 from 96% to 75%, at D_2 from 89% to 57%, at D_3 from 56 % to zero, at D4 from 15% to zero, and reached to zero for D_5 & D_6. These results indicated that treatment with bortezomib plus IFN-α for 6 days resulted in more growth inhibition than either bortezomib or IFN-α alone in myeloma cells.

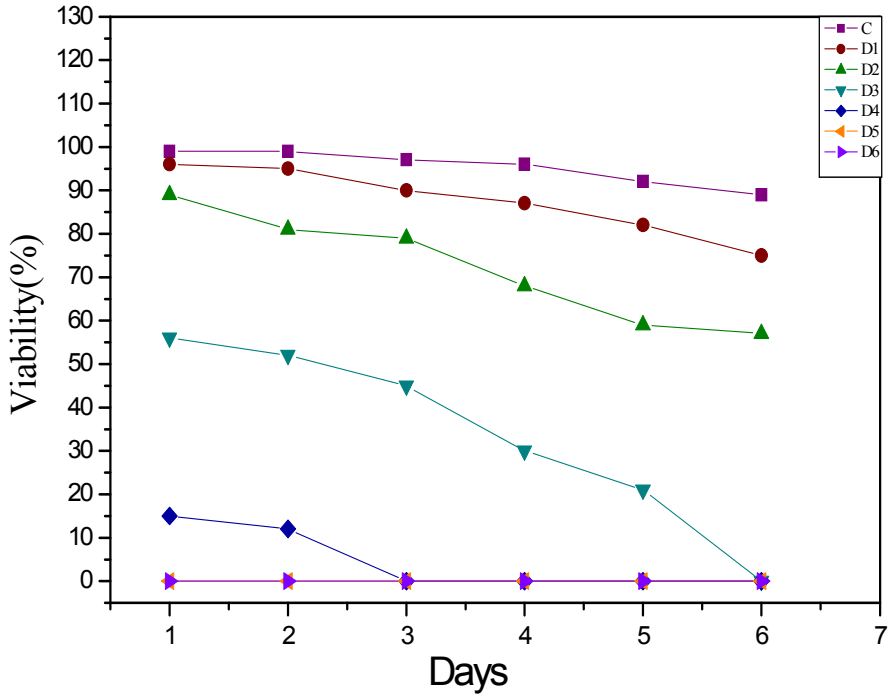

Fig. (15): Effect of variable doses of combined treatment with Bortezomib and IFN-α on the viability % of myeloma cells.

Effect of Antimyeloma polyclonal antibodies on growth of myeloma cells

As shown in Fig. (16) Myeloma-Antibodies reduced cell viability in a dose-dependent and time-dependent manner. The viability of myeloma cells decreased at 10 µl from 98% to 79%, at 20 µl from 96% to 63%, at 30 µl from 90 % to 49%, at 50 µl from 86% to 42%, at from 100 µl 79% to 35%, and at 200 µl from 62% to 21%. These results indicated that antimyeloma-Antibodies induced cell growth inhibition.

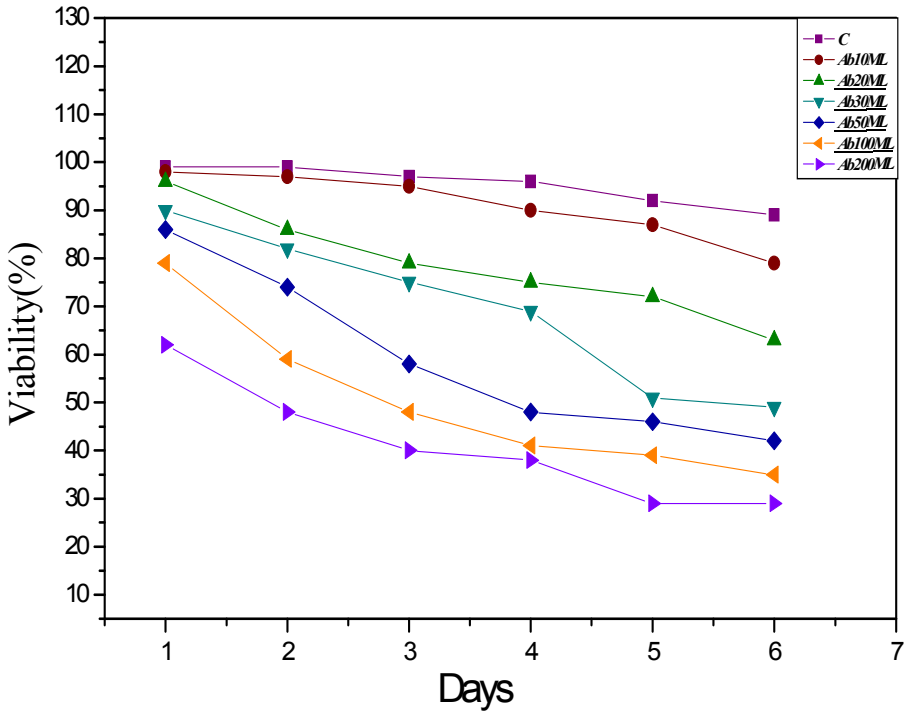

Fig. (16): Effect of variable doses of Myeloma polyclonal antibodies on the viability % of myeloma cells.

Effect of Labeled Antimyeloma polyclonal antibodies on growth of myeloma cells

The results of effect of labeled antimyeloma Antibodies on cell viability are illustrated in Fig. (17). The viability of myeloma cells decreased at 10 µCi/ml from 89% to 62%, at 15 µCi/ml from 70% to 43%, at 20 µCi/ml from 14 % to 0, and reached to zero for 25 ,30and50µCi/ml.

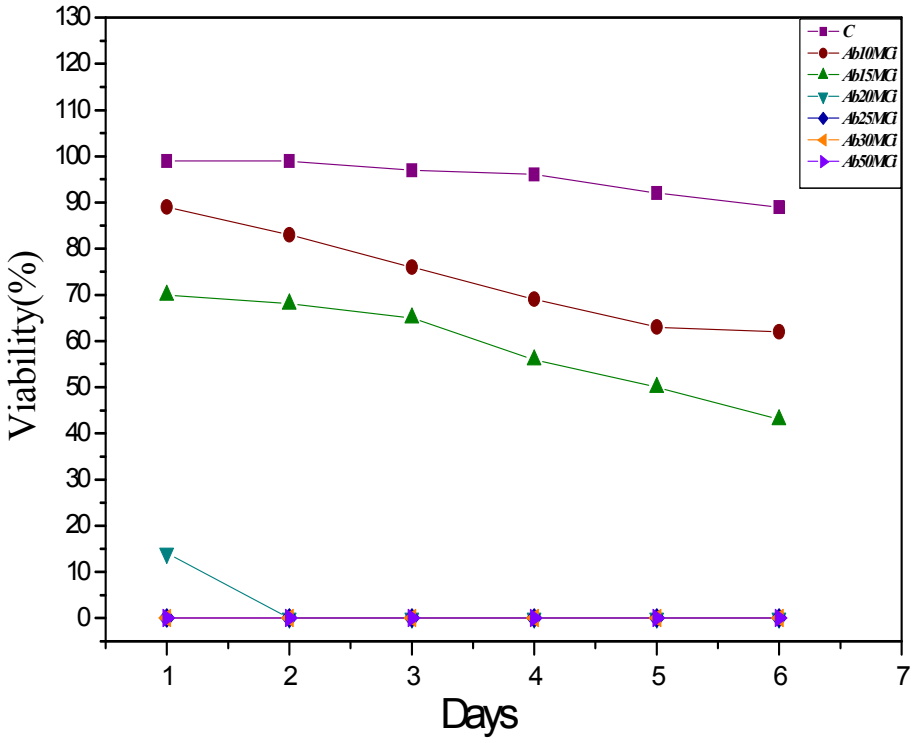

Fig.(17): Effect of variable doses of Labeled Myeloma polyclonal antibodies on the viability % of myeloma cells.

Effects of bortezomib +IFN-α + Myeloma-Antibodies on growth of myeloma cells

Fig. (18) illustrates the effect of variable doses of bortezomib + IFN-α + Myeloma-Antibodies on the viability (%) of myeloma cells during lifespan of 6 days compared to control group. The viability of myeloma cells decreased at D_1 from 79% to 19%, at D_2 from 59% to zero, and reached to zero for D_3, D_4, D_5 and D6.

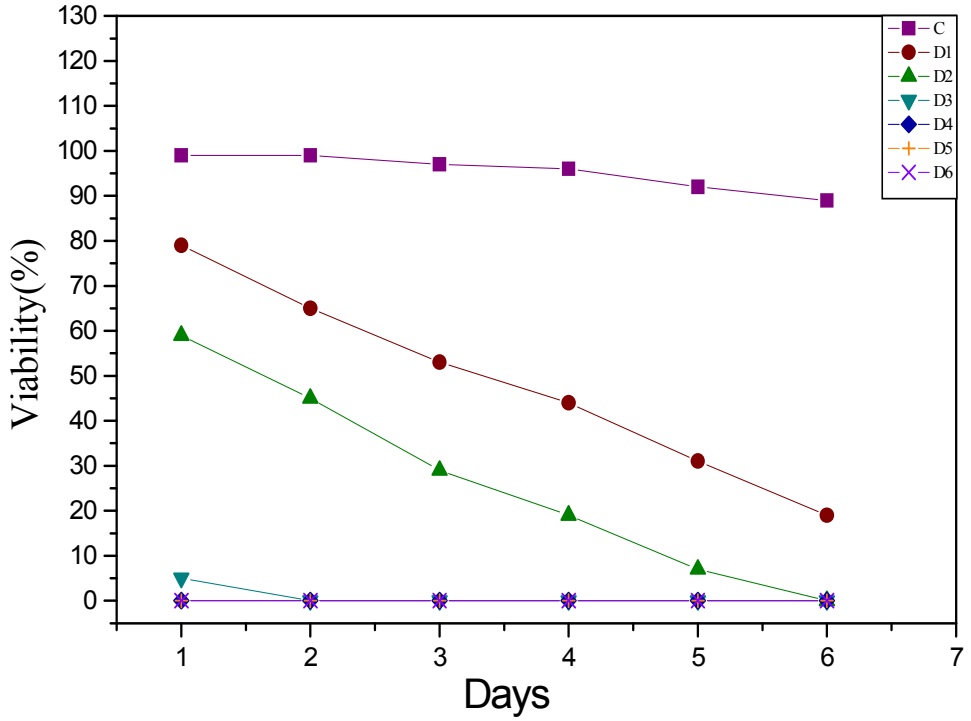

Fig. (18): Effect of variable doses of combined treatment with bortezomib + IFN-α + Myeloma-Antibodies on the viability % of myeloma cells.

In Vivo study

In the present study, the effect of different types of treatment [Bortezomib, IFN-α, (Bortezomib+ IFN-α), Antimyeloma antibodies, Labeled antimyeloma antibodies and (Bortezomib+ IFN-α +Antimyeloma antibodies)] on tumor growth of ascites bearing mice were evaluated and compared with control group.

The results of ascites bearing mice untreated and treated with bortezomib were illustrated in table (12) and (Fig.19). Before treatment the T.C /ml (mean ± SD) was ($166.5\times10^4\pm3.6\times10^4$), while the viability % (mean± SD) was (97±1.5) for time intervals of 4 weeks. While after treatment with bortezomib (0.05, 0.1, 0.5 and 1.0 mg/kg) twice weekly for 4 weeks the T.C /ml (mean ± SD) decreased to ($111\times10^4\pm1.3\times10^4$), ($100\times10^4\pm0.7\times10^4$), (0) and (0) respectively. The viability % (mean ± SD) decreased to (91±1.4), (88±0.7), (0) and (0) respectively. From these results it can be concluded that animals treated at the two lowest doses Bortezomib (0.05 and 0.1 mg/kg) showed inhibition of tumor growth compared with controls, but greatest inhibition of tumor growth was observed in mice treated with bortezomib at 0.5 and 1.0 mg/kg versus the control group.

Table (12): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Bortezomib.

Days	*Parameters measured*	Mean±SD (n=5)				
		Control group	Mice treated with bortezomib (0.05mg/kg)	Mice treated with bortezomib (0.1mg/kg)	Mice treated with bortezomib (0.5mg/kg)	Mice treated with bortezomib (1.0mg/kg)
1	*T.C / ml*	$15\times10^4\pm0.7\times10^4$	$14\times10^4\pm1.4\times10^4$	$13\times10^4\pm0.8\times10^4$	$10\times10^4\pm0.7\times10^4$	$8\times10^4\pm0.7\times10^4$
	V. (%)	82±2.0	7 5±2.9	73±1.1	61±1.0	42±0.8
4	*T.C / ml*	$31\times10^4\pm2.4\times10^4$	$26\times10^4\pm1.8\times10^4$	$23\times10^4\pm0.8\times10^4$	$10\times10^4\pm0.7\times10^4$	$5\times10^4\pm0.7\times10^4$
	V. (%)	89±0.7	79±1.9	78±1.9	52±0.4	12±0.4
8	*T.C / ml*	$40\times10^4\pm1.8\times10^4$	$38\times10^4\pm0.7\times10^4$	$36\times10^4\pm1.1\times10^4$	$21\times10^4\pm0.7\times10^4$	$3\times10^4\pm0.5\times10^4$
	V. (%)	91±1.4	82±1.8	79±1.1	42±0.8	7±0.7
11	*T.C / ml*	$56\times10^4\pm6.8\times10^4$	$49\times10^4\pm0.7\times10^4$	$47\times10^4\pm0.7\times10^4$	$12\times10^4\pm0.4\times10^4$	$2\times10^4\pm0.4\times10^4$
	V. (%)	93±1.5	88±0.8	83±0.8	24±0.5	0
22	*T.C / ml*	$79\times10^4\pm4.2\times10^4$	$76\times10^4\pm1.8\times10^4$	$60\times10^4\pm1.1\times10^4$	$7\times10^4\pm0.7\times10^4$	0
	V. (%)	94±1.1	89±2.2	84±0.7	14±0.9	0
25	*T.C / ml*	$93.5\times10^4\pm6.5\times10^4$	$89\times10^4\pm0.8\times10^4$	$77.5\times10^4\pm1.1\times10^4$	$3\times10^4\pm0.7\times10^4$	0
	V. (%)	94±1.3	90±1.5	85±0.7	0	0
29	*T.C / ml*	$112.5\times10^4\pm8.8\times10^4$	$95\times10^4\pm2.5\times10^4$	$87\times10^4\pm0.7\times10^4$	0	0
	V. (%)	95±1.3	90±1.3	86±0.7	0	0
32	*T.C / ml*	$166.5\times10^4\pm3.6\times10^4$	$111\times10^4\pm1.3\times10^4$	$100\times10^4\pm0.7\times10^4$	0	0
	V. (%)	97±1.5	91±1.4	88±0.7	0	0

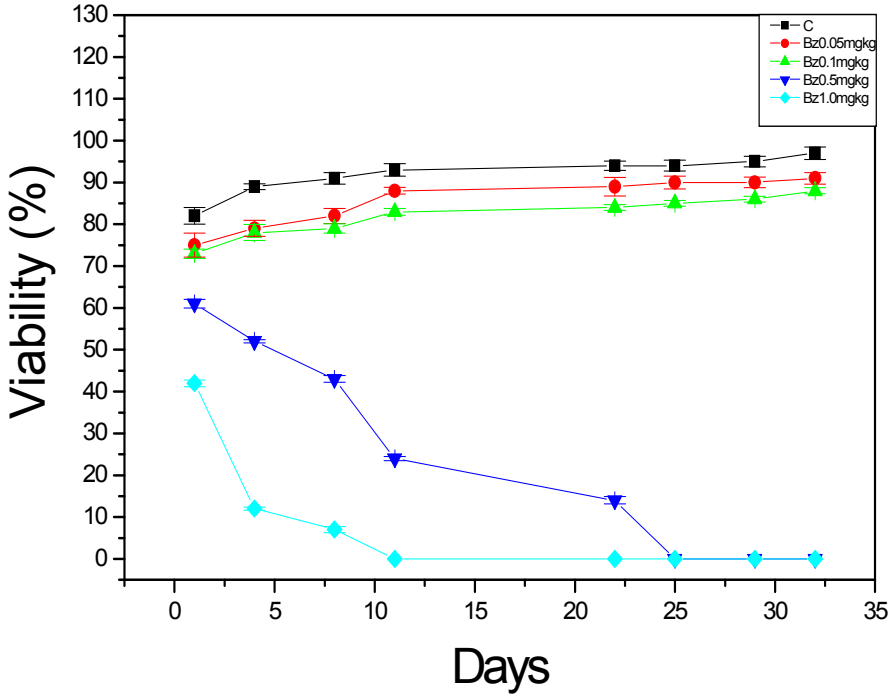

Fig. (19): Effect of variable doses of Bortezomib on viability % of myeloma cells of ascites bearing mice (mean ± SD)

Table (13): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Alpha-interferon

Days	*Parameters measured*	Mean±SD (n=5)			
		Control group	**Mice treated with interferon (10^3 IU /kg)**	**Mice treated with interferon (10^4 IU /kg)**	**Mice treated with interferon (10^6 IU /kg)**
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$14.5\times10^4\pm0.3\times10^4$	$11\times10^4\pm0.4\times10^4$	$9\times10^4\pm0.2\times10^4$
	V. (%)	82±2.0	79±0.7	71±0.3	68±0.7
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$29\times10^4\pm0.7\times10^4$	$13\times10^4\pm0.3\times10^4$	$11\times10^4\pm0.6\times10^4$
	V. (%)	89±0.7	85±1.9	69±0.7	58±0.9
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$37\times10^4\pm0.7\times10^4$	$21\times10^4\pm0.4\times10^4$	$17\times10^4\pm0.8\times10^4$
	V. (%)	91±1.4	89±0.7	53±0.7	47±0.4
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$52\times10^4\pm0.7\times10^4$	$39\times10^4\pm0.5\times10^4$	$23\times10^4\pm0.7\times10^4$
	V. (%)	93±1.5	91±0.8	48±0.7	±0.7
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$68\times10^4\pm0.3\times10^4$	$44\times10^4\pm0.8\times10^4$	$37\times10^4\pm0.5\times10^4$
	V. (%)	94±1.1	92±0.4	29±0.7	19±0.7
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$89\times10^4\pm0.7\times10^4$	$62\times10^4\pm0.4\times10^4$	$44\times10^4\pm0.4\times10^4$
	V. (%)	94±1.3	93±0.7	23±0.7	13±0.7
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$108\times10^4\pm0.7\times10^4$	$68\times10^4\pm0.7\times10^4$	$48\times10^4\pm0.7\times10^4$
	V. (%)	95±1.3	94±0.7	19±0.7	9±0.8
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$126\times10^4\pm0.3\times10^4$	$72\times10^4\pm0.9\times10^4$	$51\times10^4\pm0.4\times10^4$
	V. (%)	97±1.5	94±0.5	17±0.7	7±0.7

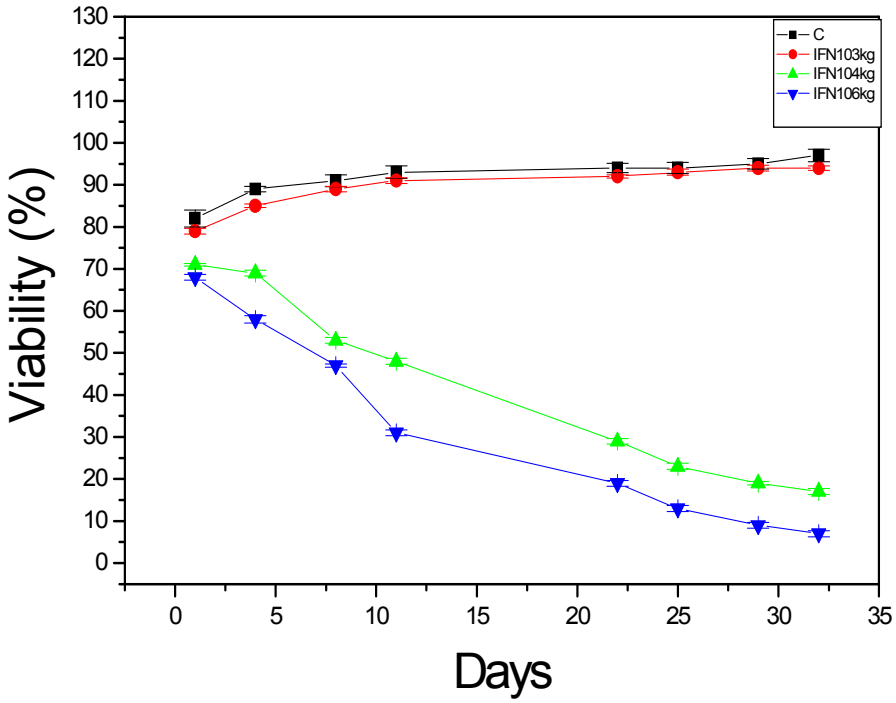

Fig.(20):Effect of variable doses of IFN-α on viability % of myeloma cells of ascites bearing mice (mean ± SD).

Table (13) and (Fig.20) represented the results of ascites bearing mice untreated and treated with IFN-α. Before treatment the T.C /ml (mean ± SD) was ($166.5\times10^4\pm3.6\times10$), while the viability % (mean± SD) was (97±1.5) for time intervals of 4 weeks. While after treatment with IFN-α (10^3, 10^4and10^6 IU/kg) twice weekly for 4 weeks the T.C /ml (mean ± SD) decreased to ($126\times10^4\pm0.3\times10^4$), ($72\times10^4\pm0.9\times10^{4)}$ and ($51\times10^4\pm0.4\times10^4$).The viability % (mean ± SD) decreased to (94±0.5), (17±0.7) and (7±0.7) respectively. From these results it can be concluded that animals treated with 10^4 and10^6 IU/kg IFN-α showed inhibition of tumor growth compared with controls**.**

Table (14): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Bortezomib plus α-interferon

Days	Parameters measured	*Mean±SD (n=5)*				
		Control group	**D1**	**D2**	**D3**	**D4**
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$9\times10^4\pm0.5\times10^4$	$7\times10^4\pm0.4\times10^4$	$7.5\times10^4\pm0.8\times10^4$	$6\times10^4\pm0.6\times10^4$
	V. (%)	82±2.0	52±0.8	49±1.1	39±0.7	32±0.7
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$11\times10^4\pm0.7\times10^4$	$5\times10^4\pm0.4\times10^4$	$4\times10^4\pm0.4\times10^4$	$3\times10^4\pm0.7\times10^4$
	V. (%)	89±0.7	48±0.7	38±1.9	10±0.7	7±0.4
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$13\times10^4\pm0.7\times10^4$	$4\times10^4\pm0.7\times10^4$	$2\times10^4\pm0.3\times10^4$	$1.5\times10^4\pm0.3\times10^4$
	V. (%)	91±1.4	33±0.4	28±0.8	4±0.5	0
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$14\times10^4\pm0.4\times10^4$	$2.5\times10^4\pm0.3\times10^4$	$1.5\times10^4\pm0.7\times10^4$	0
	V. (%)	93±1.5	21±0.7	19±0.2	0	0
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$9\times10^4\pm0.8\times10^4$	$1.5\times10^4\pm0.3\times10^4$	0	0
	V. (%)	94±1.1	11±0.7	7±0.4	0	0
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$7\times10^4\pm0.5\times10^4$	0	0	0
	V. (%)	94±1.3	0	0	0	0
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$5.5\times10^4\pm0.3\times10^4$	0	0	0
	V. (%)	95±1.3	0	0	0	0
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$3\times10^4\pm0.7\times10^4$	0	0	0
	V. (%)	97±1.5	0	0	0	0

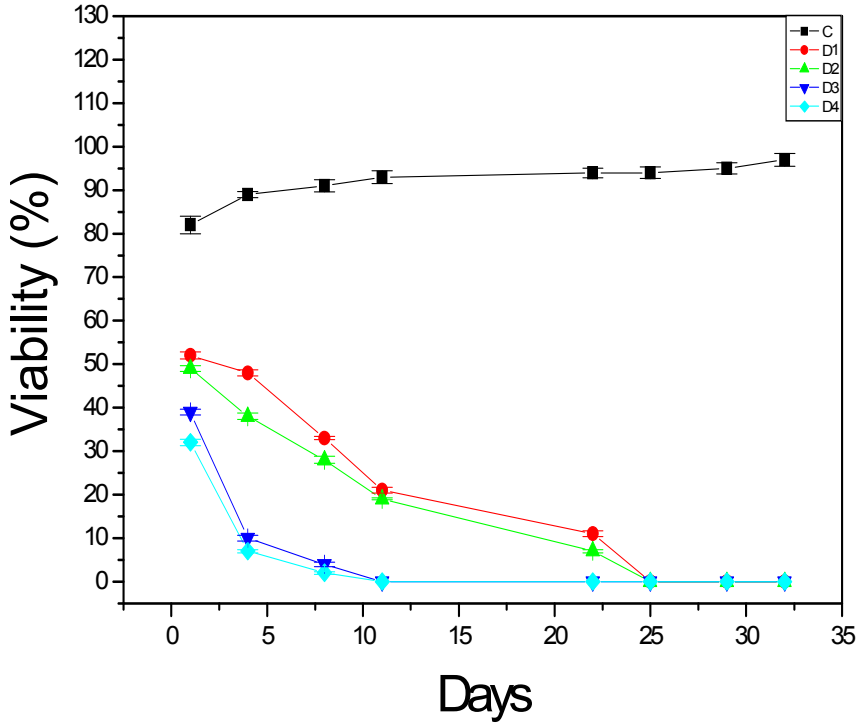

Fig. (21): Effect of variable doses of combined treatment with Bortezomib + IFN-α on the viability % of myeloma cells of ascites bearing mice (mean ± SD).

The results in table (14) and (Fig.21) were demonstrated that combination therapy with IFN-α plus bortezomib resulted in significant growth inhibition in ascites bearing mice compared with either agent alone. Combined treatment with Bortezomib and IFN-α may induce synergistic effect and promote apoptosis in myeloma cell lines.

Table (15): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Antimyeloma-antibodies

Days	Parameters measured	Mean±SD (n=5)			
		Control group	**50µl**	**100 µl**	**200 µl**
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$14\times10^4\pm0.7\times10^4$	$12\times10^4\pm0.4\times10^4$	$11\times10^4\pm0.2\times10^4$
	V. (%)	82±1.0	81±0.7	79±0.7	76±0.2
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$30\times10^4\pm0.7\times10^4$	$24\times10^4\pm0.5\times10^4$	$\times10^4\pm0.2\times10^4$
	V. (%)	89±0.7	86±0.9	74±0.7	61±0.7
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$38\times10^4\pm0.4\times10^4$	$31\times10^4\pm0.7\times10^4$	$19\times10^4\pm0.8\times10^4$
	V. (%)	91±1.4	89±0.9	69±0.4	58±0.7
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$49\times10^4\pm0.7\times10^4$	$41\times10^4\pm0.5\times10^4$	$27\times10^4\pm0.7\times10^4$
	V. (%)	93±1.5	90±0.8	53±0.7	48±0.7
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$66\times10^4\pm0.3\times10^4$	$42\times10^4\pm0.8\times10^4$	$36\times10^4\pm0.7\times10^4$
	V. (%)	94±1.1	90±0.7	39±0.4	25±0.7
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$83\times10^4\pm0.7\times10^4$	$59\times10^4\pm0.4\times10^4$	$42\times10^4\pm0.4\times10^4$
	V. (%)	94±1.3	91±0.7	29±0.7	18±0.7
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$98\times10^4\pm0.7\times10^4$	$63\times10^4\pm0.7\times10^4$	$50\times10^4\pm0.7\times10^4$
	V. (%)	95±1.3	93±0.7	22±0.3	13±0.9
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$117\times10^4\pm0.4\times10^4$	$75\times10^4\pm0.7\times10^4$	$58\times10^4\pm0.4\times10^4$
	V. (%)	97±1.5	90±1.5	19±0.7	12±0.4

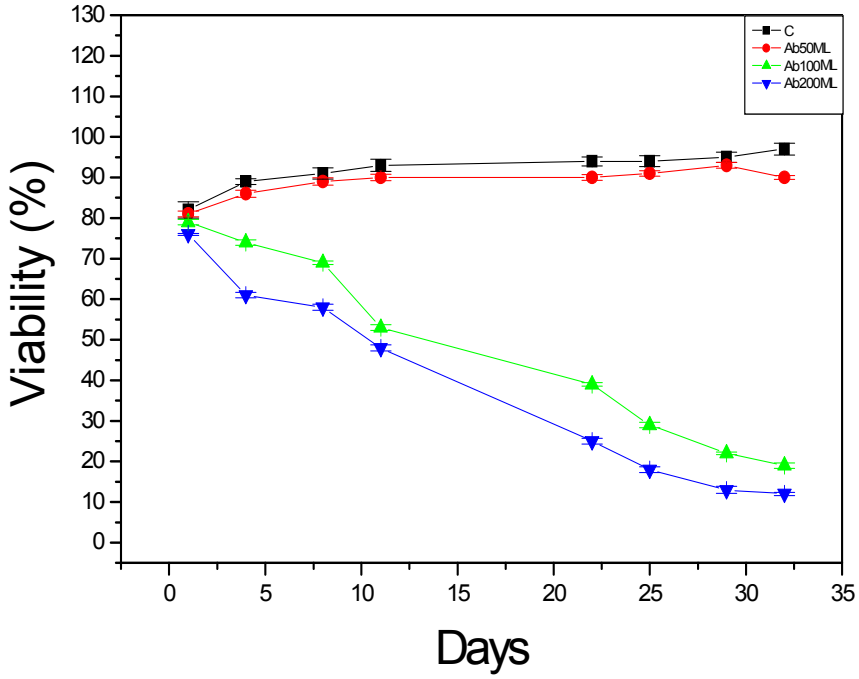

Fig.(22):Effect of variable doses of Myeloma-Antibodies on viability % of myeloma cells of ascites bearing mice (mean ± SD).

The results of ascites bearing mice untreated and treated with Myeloma antibodies are illustrated in table (15) and (Fig.22). Before treatment the T.C /ml (mean ± SD) was ($166.5\times10^4\pm3.6\times10$), while the viability % (mean± SD) was (82±2.0) for time intervals of 4 weeks. While after treatment with Myeloma-Antibodies (50,100,200 µl) twice weekly for 4 weeks the T.C./ml (mean ± SD) decreased to ($117\times10^4\pm0.4\times10^4$), ($75\times10^4\pm0.7\times10^4$) and ($58\times10^4\pm0.4\times10^4$) respectively. The viability % (mean ± SD) decreased to (90±1.5), (19±0.7) and (12±0.4) respectively.

Table (16): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Labeled antimyeloma antibodies

Days	Parameters measured	Mean±SD (n=5)			
		Control group	**0.1mCi**	**0.2mCi**	**0.3mCi**
1	***T.C / ml***	$15\times10^4\pm0.7\times10^4$	$13\times10^4\pm0.4\times10^4$	$11\times10^4\pm0.7\times10^4$	$8\times10^4\pm0.2\times10^4$
	V. (%)	82±1.0	79±0.9	72±0.4	51±0.
4	***T.C / ml***	$31\times10^4\pm2.4\times10^4$	$23\times10^4\pm0.7\times10^4$	$14\times10^4\pm0.4\times10^4$	$6.5\times10^4\pm0.4\times10^4$
	V. (%)	89±0.7	68±0.7	57±0.7	43±0.2
8	***T.C / ml***	$40\times10^4\pm1.8\times10^4$	$28\times10^4\pm0.7\times10^4$	$21\times10^4\pm0.7\times10^4$	$6\times10^4\pm0.7\times10^4$
	V. (%)	91±1.4	45±0.5	36±0.5	19±0.7
11	***T.C / ml***	$56\times10^4\pm6.8\times10^4$	$32\times10^4\pm0.3\times10^4$	$19\times10^4\pm0.8\times10^4$	$5\times10^4\pm0.7\times10^4$
	V. (%)	93±1.5	34±0.8	28±0.7	12±0.4
22	***T.C / ml***	$79\times10^4\pm4.2\times10^4$	$41\times10^4\pm0.7\times10^4$	$12\times10^4\pm0.3\times10^4$	$4\times10^4\pm0.7\times10^4$
	V. (%)	94±1.1	29±0.7	22±0.4	9±0.7
25	***T.C / ml***	$93.5\times10^4\pm6.5\times10^4$	$20\times10^4\pm0.7\times10^4$	$10\times10^4\pm0.7\times10^4$	$3\times10^4\pm0.4\times10^4$
	V. (%)	94±1.3	23±0.7	19±0.5	3±0.7
29	***T.C / ml***	$112.5\times10^4\pm8.8\times10^4$	$15\times10^4\pm0.4\times10^4$	$7\times10^4\pm0.4\times10^4$	0
	V. (%)	95±1.3	21±0.3	16±0.3	0
32	***T.C / ml***	$166.5\times10^4\pm3.6\times10^4$	$12\times10^4\pm0.4\times10^4$	$5\times10^4\pm0.7\times10^4$	0
	V. (%)	97±1.5	19±0.5	13±0.7	0

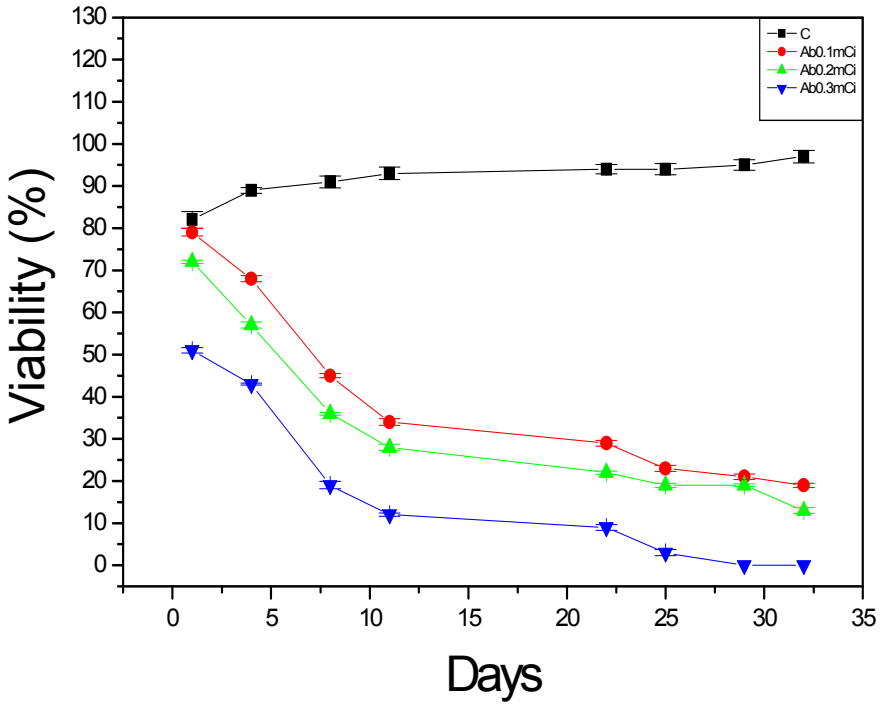

Fig.(23):Effect of variable doses of Labeled myeloma-antibodies on viability % of myeloma cells of ascites bearing mice(mean ± SD).

Table (16) and (Fig.23) represented the results of ascites bearing mice untreated and treated with Labeled myeloma antibodies. Before treatment the T.C /ml (mean ± SD) was ($166.5\times10^4\pm3.6\times10$), while the viability % (mean± SD) was (97±1.5) for time intervals of 4 weeks. While after treatment with Labeled myeloma antibodies (0.1, 0.1 and 0.1mCi), the T.C /ml (mean ± SD) decreased to ($12\times10^4\pm0.4\times10^4$), ($5\times10^4\pm0.7\times10^{4)}$ and (0).The viability % (mean ± SD) decreased to (19±0.5), (13±0.7) and (0) respectively. From these results it can be concluded that the effect of labeled antimyeloma antibodies on myeloma cells growth inhibition was more effective than that of antimyeloma antibodies without labeling**.** This is due to the cytotoxic effect of ionizing radiation.

Table (17): Total count and viability % of myeloma cells of untreated and treated ascites bearing mice with Bortezomib + α-interferon + Myeloma-Antibodies

Days	Parameters measured	Mean±SD (n=5)				
		Control group	D1	D2	D3	D4
1	*T.C / ml*	$15\times10^4\pm0.7\times10^4$	$8\times10^4\pm0.4\times10^4$	$6\times10^4\pm0.7\times10^4$	$4\times10^4\pm1.5\times10^4$	$2.5\times10^4\pm0.7\times10^4$
	V. (%)	82±2.0	49±0.7	32±0.8	21±0.4	6±1.3
4	*T.C / ml*	$31\times10^4\pm2.4\times10^4$	$9\times10^4\pm0.8\times10^4$	$4\times10^4\pm1.1\times10^4$	$3\times10^4\pm0.4\times10^4$	$1\times10^4\pm0.7\times10^4$
	V. (%)	89±0.7	36±0.7	28±0.8	10±0.7	0
8	*T.C / ml*	$40\times10^4\pm1.8\times10^4$	$11\times10^4\pm0.3\times10^4$	$3\times10^4\pm0.7\times10^4$	$2\times10^4\pm0.3\times10^4$	0
	V. (%)	91±1.4	29±0.4	13±0.4	0	0
11	*T.C / ml*	$56\times10^4\pm6.8\times10^4$	$12\times10^4\pm0.4\times10^4$	$1.5\times10^4\pm0.7\times10^4$	0	0
	V. (%)	93±1.5	18±1.1	9±0.2	0	0
22	*T.C / ml*	$79\times10^4\pm4.2\times10^4$	$7\times10^4\pm0.4\times10^4$	$1.5\times10^4\pm0.3\times10^4$	0	0
	V. (%)	94±1.1	9±0.7	0	0	0
25	*T.C / ml*	$93.5\times10^4\pm6.5\times10^4$	$7\times10^4\pm0.5\times10^4$	0	0	0
	V. (%)	94±1.3	0	0	0	0
29	*T.C / ml*	$112.5\times10^4\pm8.8\times10^4$	$5.5\times10^4\pm0.3\times10^4$	0	0	0
	V. (%)	95±1.3	0	0	0	0
32	*T.C / ml*	$166.5\times10^4\pm3.6\times10^4$	$3\times10^4\pm0.7\times10^4$	0	0	0
	V. (%)	97±1.5	0	0	0	0

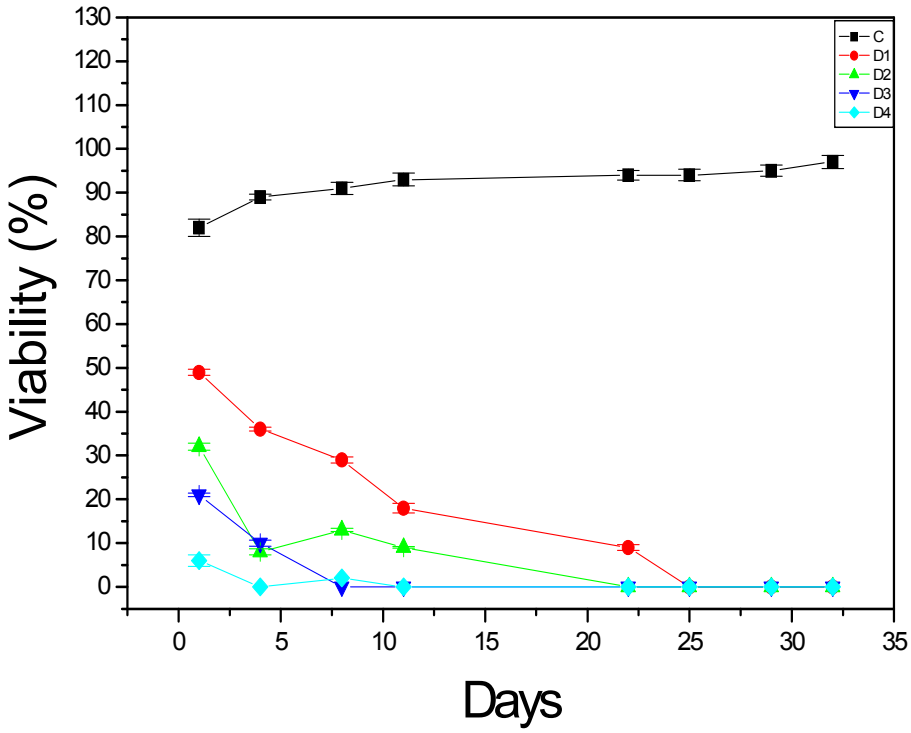

Fig. (24): Effect of variable doses of combined treatment with Bortezomib + IFN-α +Myeloma-Antibodies on the viability % of myeloma cells of ascites bearing mice (mean ± SD).

The results obtained in table (17) and (Fig. 24) indicated that triple combinations of (Bortezomib + IFN-α +Myeloma-Antibodies) are more effective than double combinations or each agent alone.

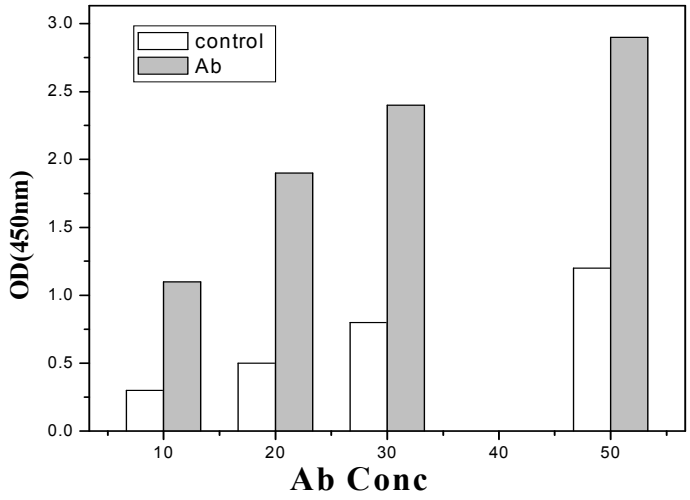

Fig. (25): ELISA results of binding of antimyeloma polyclonal antibodies to myeloma cells.

Preparation and Purification of Radiolabeled Antibodies:

Radiolabeled ^{125}I-antibodies were produced applying Chloramine -T method and purified from radioiodination reaction mixture using PD-10 column chromatography. The Purification profiles of ^{125}I-antibodies with Ch-T for rabbits **(**1, 2, 3, and 4**)** are illustrated in **(**Figs.26, 27, 28, 29**)**, respectively. The results in Fig.(30) show that two identified peaks of radioactivity are obtained, one big and somewhat sharp for ^{125}I-antibodies tracer with good radiochemical yield and the other for free radioactive iodide with a relatively small peak. In addition it was clear that there was a good separation technique for the radioiodination reactants and resultant^{125}I-antibodies. Radiochemical yield was calculated from the purification chromatography to be 70%**.** Radiochemical purity was characterized by electropheretical analysis using elution buffer of 0.05 M phosphate buffer (pH 7.4) with time duration of three h. Radiochemical purity was calculated from the electrophoresis chromatogram to be 84 % as illustrated in Fig. (31).

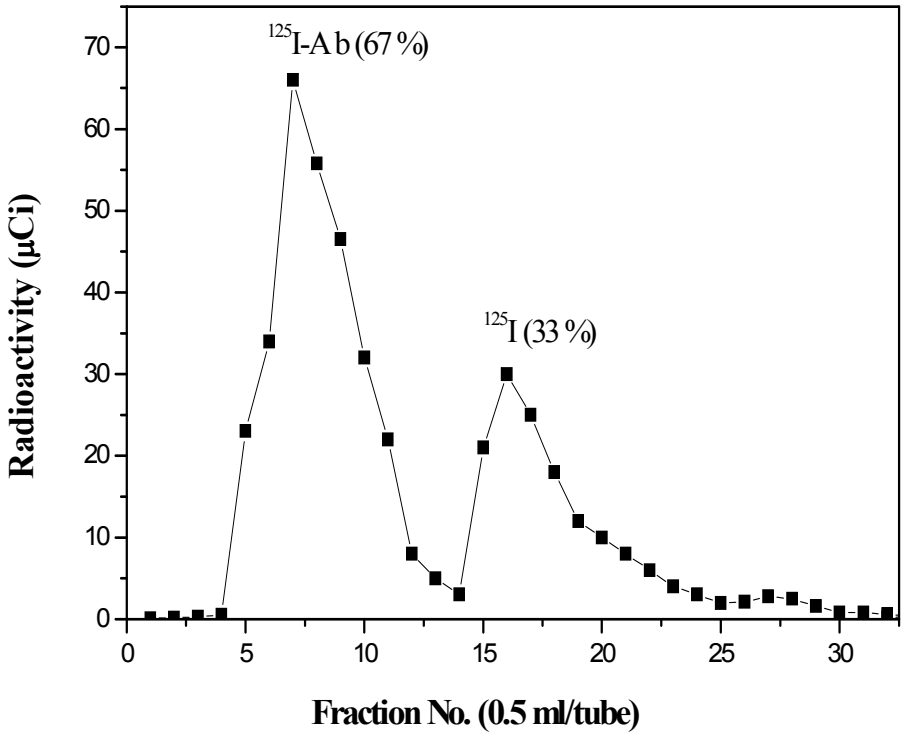

Fig. (26): Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (1))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method).

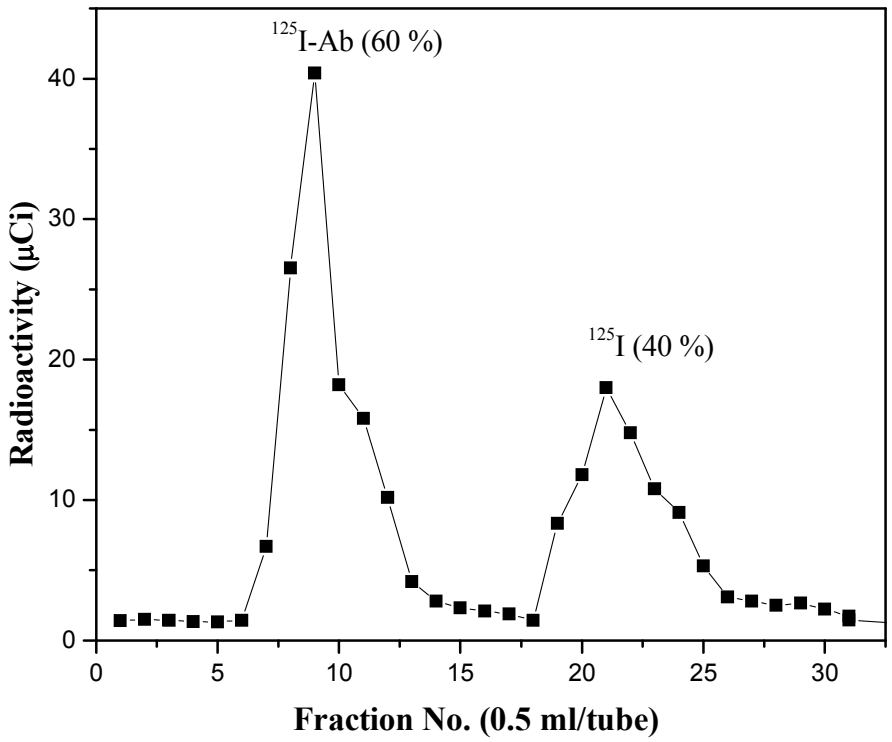

Fig. (27): Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (2))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method).

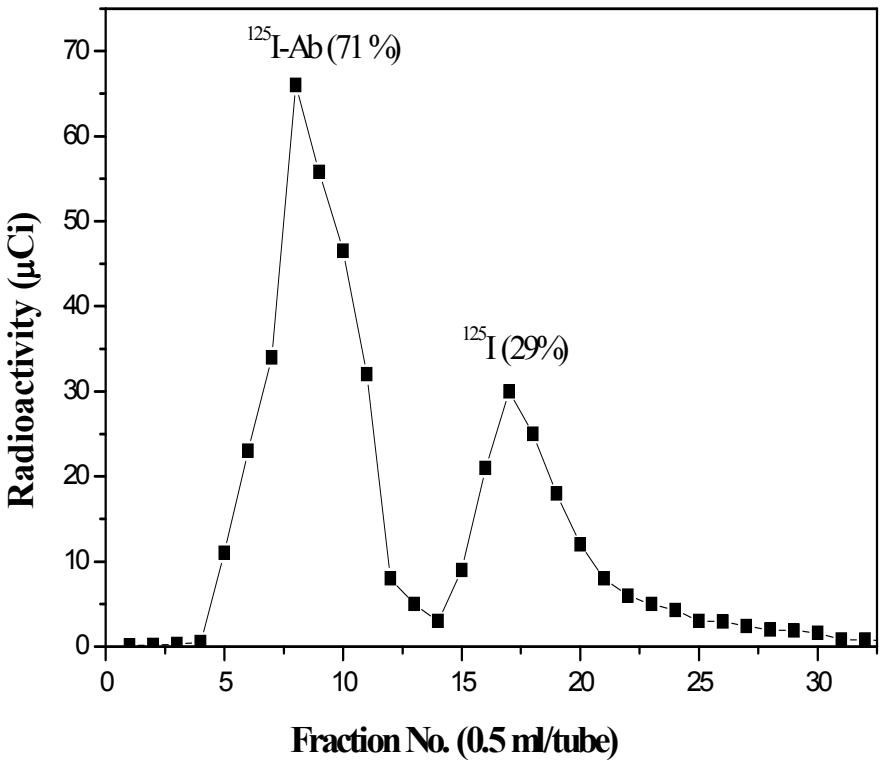

Fig.(28):Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (3))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method).

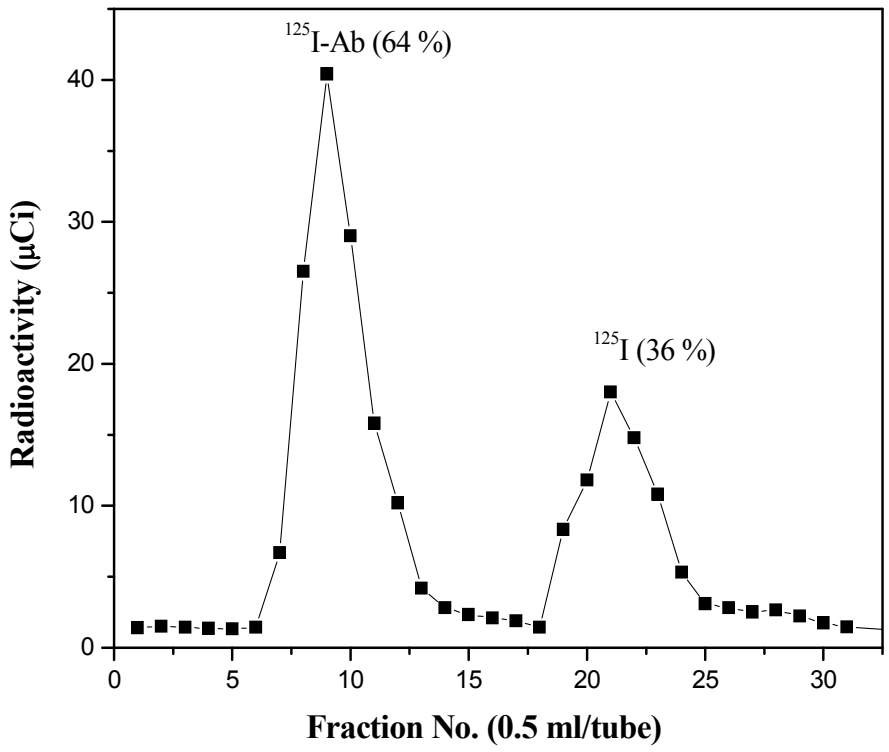

Fig. (29): Elution pattern of radioiodinated mixture of myeloma antibodies *(Rabbit (4))* and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method).

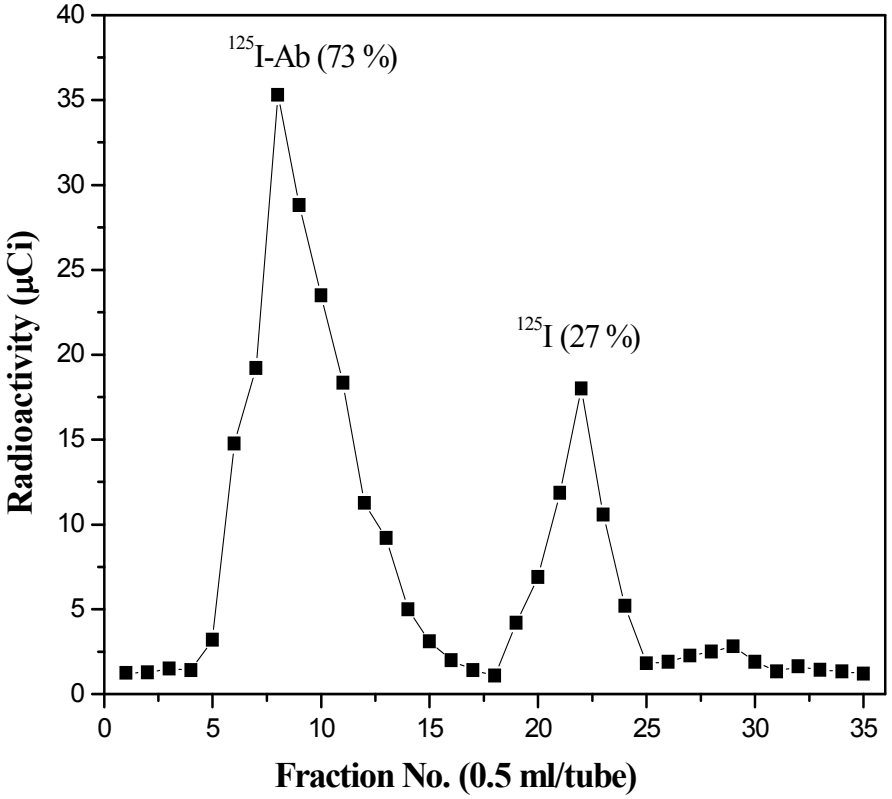

Fig. (30): Elution pattern of radioiodinated mixture of polying myeloma antibodies and purification of ^{125}I-antibodies tracer using PD-10 column (Ch-T method).

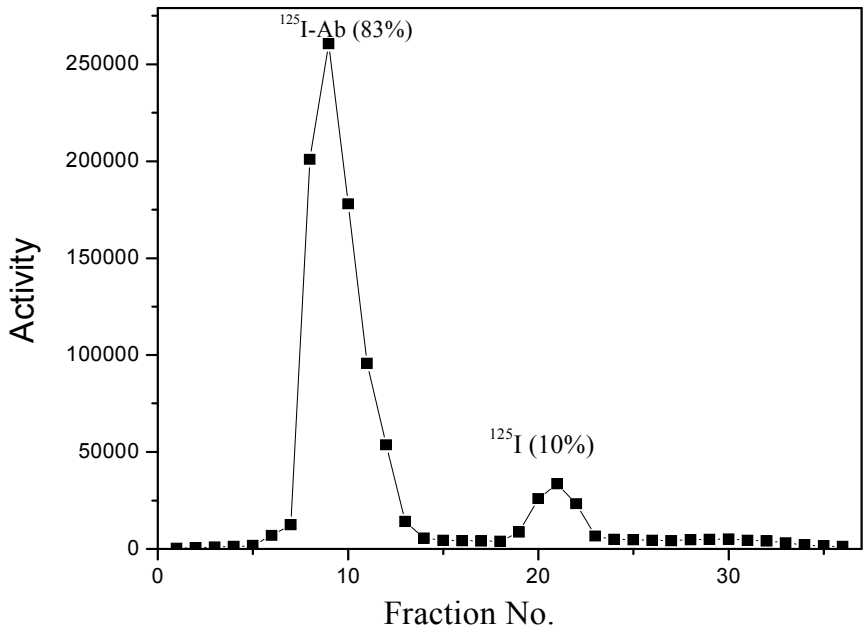

Fig. (31) Electrophoresis analysis of ^{125}I-antibodies.

Flow cytometric analysis

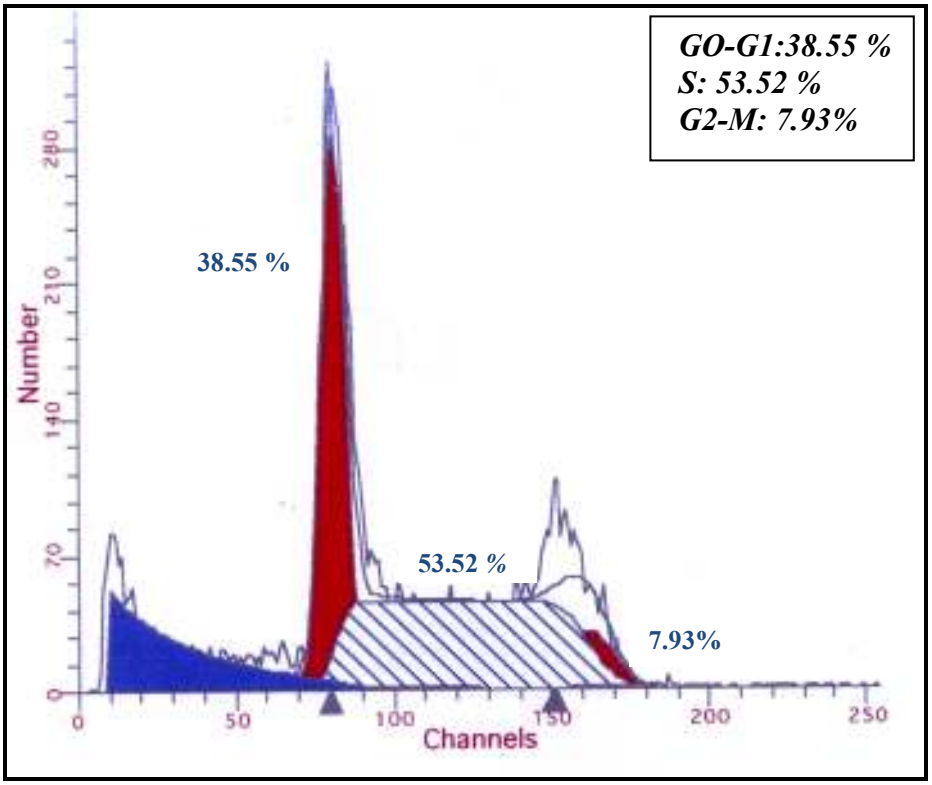

Fig. (32): Flow cytometric analysis of cell cycle profile of myeloma cell line (SP2OR) without treatment[control].

G_0 (quiescence state), G_1 (GAP1 phase), S (synthetic Phase), G2 (GAP2 phase), M (mitosis).

Figure (32) illustrated that cell cycle profile of myeloma cell without treatment. It was observed that the number of cells in G0/G1 phase was 38.55 %, S phase was 53.52 % and G2/M was 7.93 %.

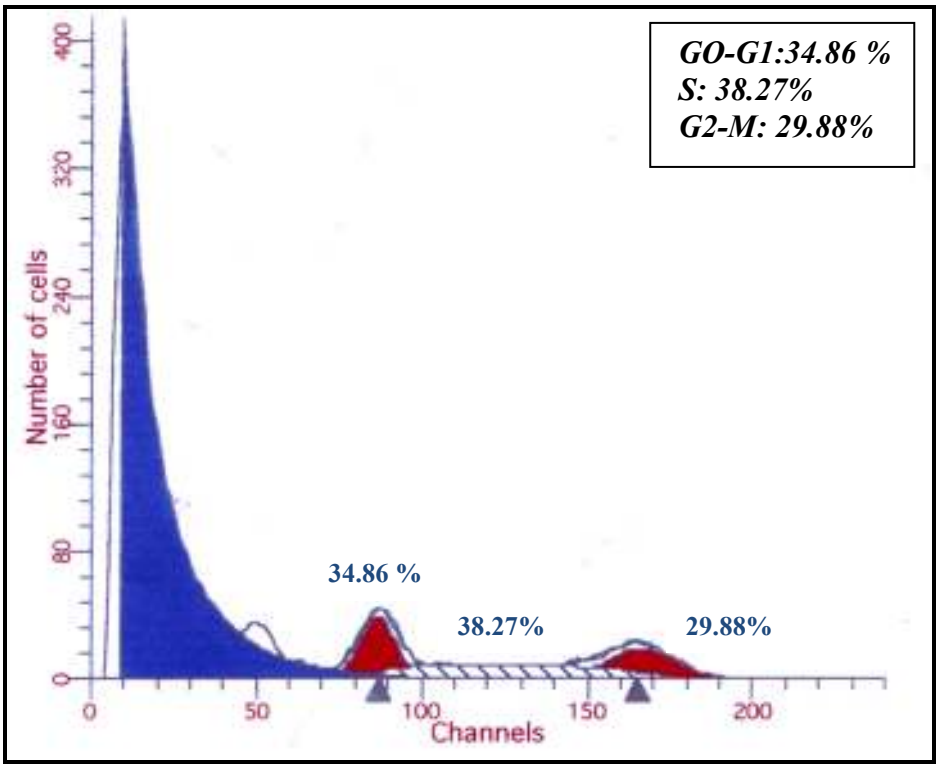

Fig.(33): Flow cytometric analysis for the effect of Bortezomib (20nM/ml) on cell cycle profile of myeloma cell line (SP2OR) .

The results of effect of Bortezomib (20nM/ml) on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of Bortezomib treatment is illustrated in figure (33) where the percentage of cells in the G0/G1 phase decreased from 38.55 in the untreated control to 34.86 %, S phase decreased from 53.52 % in the untreated control to 38.27 %, while G2/M phase increased from 7.93% to 29.88%. The above results suggested that Bortezomib caused a G2/M arrest myeloma cell phase and an induction of apoptosis.

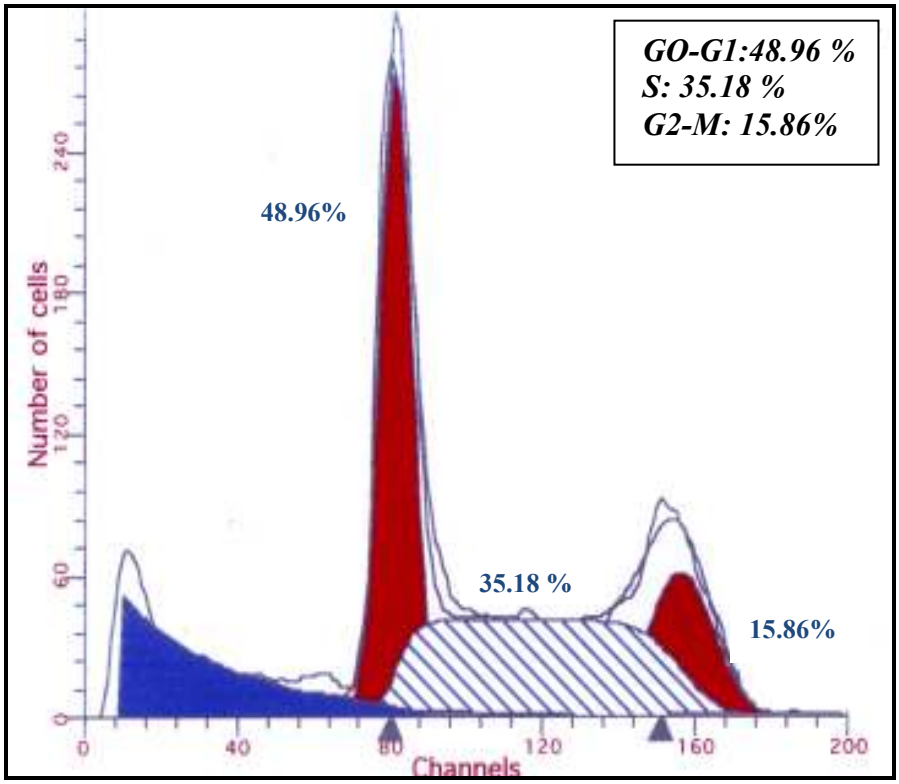

Fig. (34): Flow cytometric analysis for the effect of Alpha-interferon (10^3 IU/ml) on cell cycle profile of myeloma cell line (SP2OR) .

The results of effect of Alpha-interferon (10^3 IU/ml) on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of IFN-α treatment is illustrated in figure (34) where the percentage of cells in the G0/G1 phase increased from 38.55 in the untreated control to 48.96 %, S phase decreased from 53.52 % in the untreated control to 35.18 %, while G2/M phase increased from 7.93% to 15.86%. The above results revealed that IFN-α caused a G1 cell cycle arrest leading to the growth cessation.

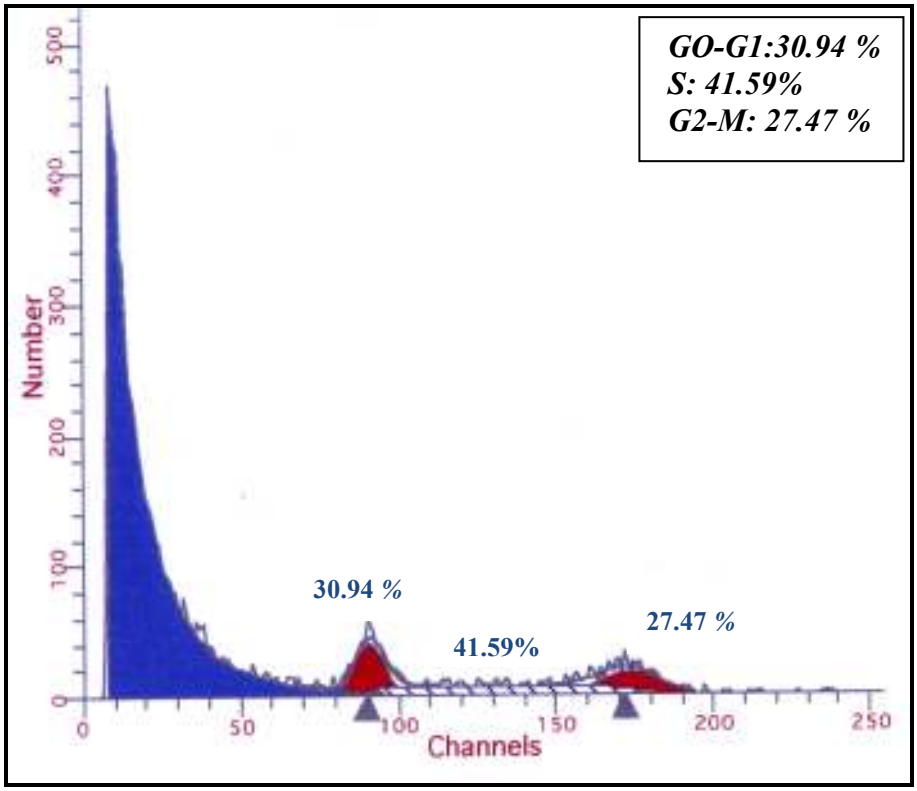

Fig.(35): Flow cytometric analysis for the effect of Bortezomib+ IFN-α (20nM/ml + 10^3 IU/ml) on cell cycle profile of myeloma cell line (SP2OR) .

The results of effect of Bortezomib+ IFN-α (20nM/ml + 10^3 IU/ml) on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of Bortezomib+ IFN-α treatment is illustrated in figure (35) where the percentage of cells in the G0/G1 phase decreased from 38.55 in the untreated control to 30.94 %, S phase decreased from 53.52 % in the untreated control to 41.59 %, while G2/M phase increased from 7.93% to 27.47 %. The above results suggested that bortezomib plus IFN-α induced a greater level of apoptosis compared with either agent alone.

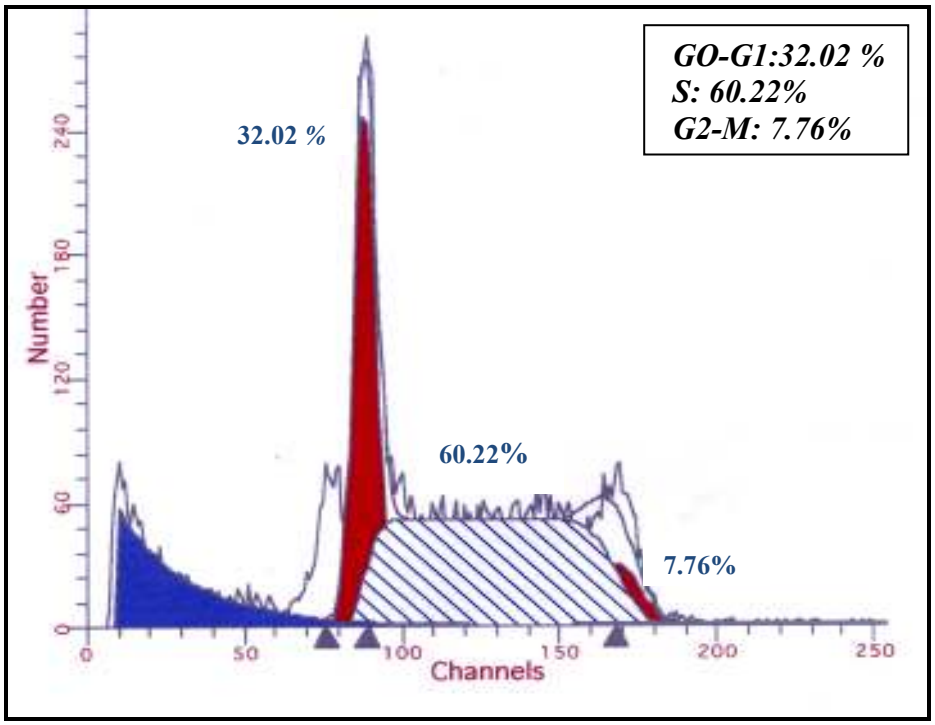

Fig. (36): Flow cytometric analysis for the effect of Myeloma-antibodies (200µl) on cell cycle profile of myeloma cell line (SP2OR).

It was observed from figure (36) that the number of cells in the G0/G1 phase decreased from 38.55 % to 32.02 % increased the number of cells in the S phase 53.52 % to 60.22% and the number of cells in the phase G2/M changed from 7.93 % to 7.76 % after addition of Myeloma-antibodies for 48 hours.

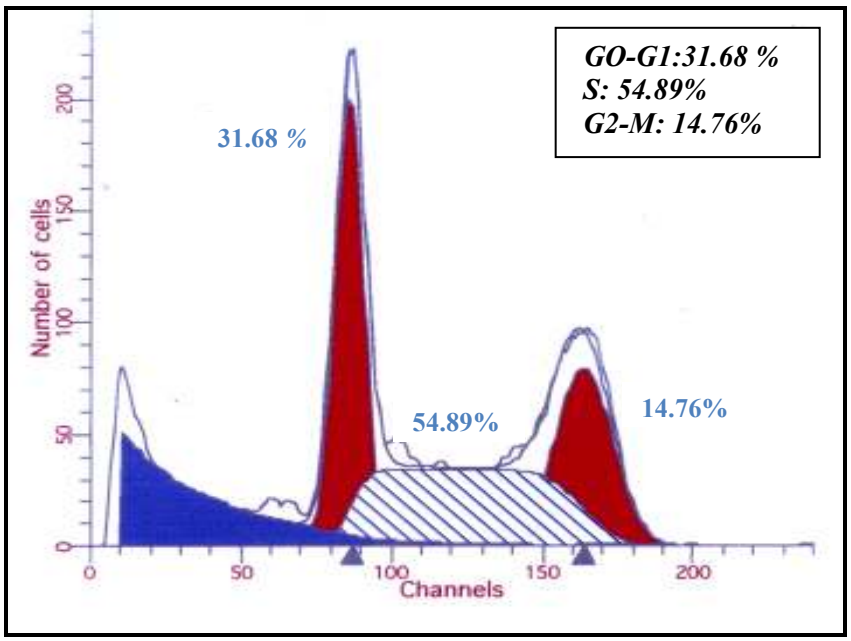

Fig. (37): Flow cytometric analysis for the effect of Labeled myeloma-antibodies (15µCi) on cell cycle profile of myeloma cell line (SP2OR).

The results of effect of Labeled myeloma-antibodies on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of treatment is illustrated in figure (37) where the percentage of cells in the G0/G1 phase decreased from 38.55 in the untreated control to 31.68 %, S phase increased from 53.52 % in the untreated control to 54.89%, while G2/M phase increased from 7.93% to 14.76%. The above results suggested that Labeled myeloma-antibodies induced S phase and G2/M phase arrest and an induction of apoptosis.

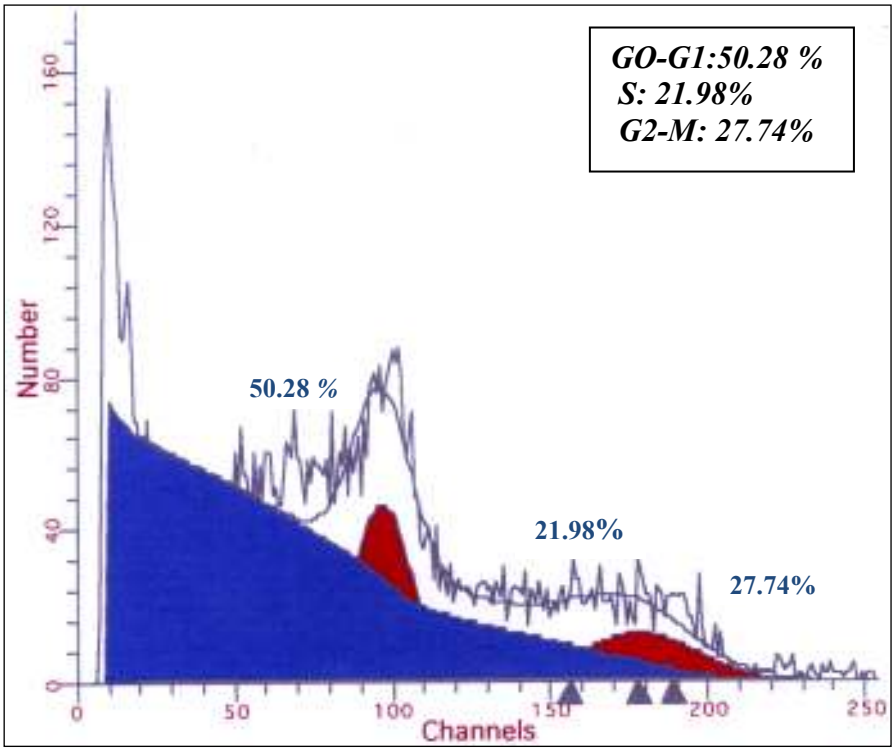

Fig. (38): Flow cytometric analysis for the effect of Bortezomib+ IFN-α + Myeloma antibodies on cell cycle profile of myeloma cell line (SP2OR).

The results of effect of (Bortezomib+ IFN-α+ Myeloma-antibodies on cell cycle profile of myeloma cell line (SP2OR) after 48 hours of treatment is illustrated in figure (38). The results indicated that triple combinations increased number of apoptotic cells and induced a greater level of apoptosis compared to double combinations or either agent alone in myeloma cell line.

Table (18) Effects of Bortezomib, α-interferon, (Bortezomib + α-interferon),Labeled myeloma antibodies,Myeloma antibodies, and (Bortezomib + α-interferon+ Myeloma-Antibodies) on Cell Cycle Phase Distribution.

Conditions	% of Cells in Cell Cycle Phases		
	G0/G1	S	G2/M
Control	38.55	53.52	7.93
Bortezomib	34.86	38.27	29.88
α-interferon	48.96	35.18	15.86
Bortezomib + α- interferon	30.94	41.59	27.47
Myeloma-antibodies	32.02	60.22	7.76
Labeled myeloma antibodies	31.68	54.89	14.76
Bortezomib+α-interferon + Myeloma antibodies)	50.28	21.98	27.74

The total body weight of ascites bearing mice untreated and treated with Bortezomib, IFN-α, (Bortezomib + IFN-α), Myeloma antibodies, Labeled myeloma antibodies and (IFN-α + Bortezomib+ Myeloma Antibodies) are illustrated in table (19). Before treatment the weight (mean ± SD) of the 5 mice was 35 ± 1.1 g. While after treatment with IFN-α,bortezomib , (IFN-α + Bortezomib), Myeloma antibodies, Labeled myeloma antibodies and (IFN-α+Bortezomib+Myeloma Antibodies) the weight (mean ± SD) of the 5 mice decreased to 28±1.6, 31±0.7, 25±1.5, 27±1.3, 26±1.4 and 24±1.8 g respectively after the four weeks of treatment and this due to the effect of drugs on the growth of myeloma cells. The results showed that treatment with these drugs decreased the total body weight as compared to the untreated bearing groups.

Table (19): The weight of ascites bearing mice before and after treatment (mean ± SD).

Groups	Weight(g)		
	Number of mice	**Mean weight±SD**	**Range**
Control	**5**	**21±2.0**	**20-25**
Untreated	**5**	**39±1.5**	**38-42**
Bortezomib	**5**	**28±1.6**	**27-31**
α-interferon	**5**	**31±0.7**	**30-32**
Bortezomib + α-	**5**	**25±1.5**	**24-27**
Myeloma-antibodies	**5**	**27±1.3**	**26-28**
Labeled myeloma antibodies	**5**	**26±1.4**	**25-29**
Bortezomib + α-interferon+Myeloma antibodies	**5**	**24±1.8**	**23—25**

The data presented in table (20) showed that the β2-microglobulin values decreased after treatment with Bortezomib, IFN-α , Bortezomib+ IFN-α , myeloma antibodies, Labeled myeloma antibodies and Bortezomib+ IFN-α +Myeloma-antibodies compared that of control. These results indicated that these drugs inhibit the growth of myeloma cells lead to decrease levels of β2-microglobulin.

Table (20):The level of β2-microglobulin (mg/L) in Myeloma cells treated with Bortezomib, IFN-α , Bortezomib+ IFN-α , Labeled myeloma antibodies ,Myeloma-antibodies and Bortezomib+ IFN-α +Myeloma-antibodies compared that of control (Mean±SD).

Groups	β-2microglobulin(mg/L)	
	Range	Mean ±SD
Control	2 - 2.9	2.7±0.4
Bortezomib	1.1-1.6	1.4±0.14
IFN-α	1.5-2.1	1.9±0.2
Bortezomib+ IFN-α	0.9-1.4	1.2±0.18
Myeloma-antibodies	1.8-2.3	2.1±0.07
Labeled myeloma antibodies	1.2-1.9	1.7±0.2
Bortezomib+ IFN- α + Myeloma-antibodies	0.7—1.2	0.9±0.1

The results obtained in table (21) demonstrated that treatment with Bortezomib, IFN-α , Bortezomib+ IFN-α , Myeloma-antibodies, Labeled myeloma antibodies and Bortezomib+ IFN-α +Myeloma-antibodies increased activation of caspases 8 and 9 in myeloma cells compared that of control leading to induction of extrinsic and intrinsic apoptotic pathways.

Table (21): The activities of Caspase-8 and Caspase-9 in Myeloma cells treated Bortezomib, IFN-α , Bortezomib+ IFN-α , Labeled myeloma antibodies, Myeloma-antibodies and Bortezomib+ IFN-α +Myeloma-antibodies compared that of control (Mean±SD).

Groups	Caspase-8		Caspase-9	
	Range	Mean ±SD	Range	Mean ±SD
Control	0.14-0.2	0.17±0.02	0.04-0.12	0.09±0.03
Bortezomib	0.19-0.31	0.28±0.04	0.13-0.22	0.2±0.02
IFN-α	0.15-0.25	0.22±0.03	0.09-0.21	0.17±0.04
Bortezomib+ IFN-α	0.18-0.29	0.25±0.04	0.16-0.25	0.23±0.03
Myeloma antibodies	0.13-0.23	0.2±0.04	0.08-0.19	0.15±0.04
Labeled myeloma antibodies	0.17-0.24	0.23±0.03	0.1-0.23	0.18±0.02
Bortezomib+ IFN- α +Myeloma-antibodies	0.41-0.48	0.46±0.02	0.19-0.29	0.26±0.05

Effects on Liver Marker Enzymes

Table (22) shows the changes in liver Enzymes of treated and untreated mice. The serum ALT, AST and ALP activites were increased in the Ascites bearing mice control group when compared to the normal group. Treatment with Bortezomib, IFN- α, Bortezomib+ IFN- α, Labeled myeloma antibodies ,Myeloma antibodies and Bortezomib + IFN- α+ Myeloma antibodies showed an increase in the level of serum ALT, AST compared to untreated group. Also, as compared to the native mice, the untreated group of mice showed an increase in the level of ALP. The treatment increased the level of ALP in the blood.

Table (22): Change in liver marker enzymes ALT, AST and ALP in Ascites bearing group and treated groups compared to normal control (mean± SD).

Groups	Parameter		
	ALT(units/dl)	AST(units/dl)	ALP(units/dl)
Normal	19.2±0.19	65.5±1.1	36±1.1
Ascites bearing mice	34.5±0.8[a]	78.2±0.4 [a]	43.2±0.8 [a]
Ascites bearing mice + Bortezomib	45.4±0.3 [b]	85±0.7 [b]	51.3±0.7 [b]
Ascites bearing mice+ α-interferon	48.3±0.3 [b]	91.6±0.5 [b]	68±1.3 [b]
Ascites bearing mice+ (Bortezomib + α-interferon)	44.6±0.4 [b]	83.1±0.2 [b]	50.2±0.8 [b]
Ascites bearing mice+ Myeloma antibodies	38±0.4 [b]	81.4±0.7 [b]	48±0.7 [b]
Ascites bearing mice+ Labeled myeloma antibodies	41±0.3 [b]	85.3±0.3 [b]	49.5±0.7 [b]
Ascites bearing mice+ (Bortezomib + α-interferon +Myeloma antibodies)	42±0.5 [b]	80.2±0.7 [b]	77.1±0.5 [b]

[a] $p < 0.05$ Ascites bearing group vs normal group; [b] $p < 0.05$ treated vs. Ascites bearing mice group.

Effects on Haemoglobin (Hb) and Kidney functions

As shown in Table (23) haemoglobin was decreased in Ascites bearing mice control group when compared to the native mice group. Treatment with Bortezomib, IFN- α, Bortezomib+ IFN- α, Myeloma-antibodies,Labeled myeloma antibodies and Bortezomib+ IFN- α+ Myeloma-antibody decreased the haemoglobin level as compared with the Ascites bearing mice control group,while treatment with Myeloma antibodies and Bortezomib+ IFN- α+ Myeloma antibodies increased the Hb content and RBC counts to more or less normal levels. Also the serum Creatinine, Urea and Uric acid were increased in the Ascites bearing mice control group compared to the normal group. The treated mice showed increase in the level of Creatinine, Urea and Uric acid in the serum of the blood as compared with the untreated groups.

Table (23): Change in Haemoglobin (Hb) and Kidney functions in Ascites bearing group and treated groups compared to normal control (mean± SD).

Groups	Parameter			
	Hb (g/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)
Normal	12.9±0.3	0.61±0.08	21±0.7	3.4±0.3
Ascites bearing mice	11.4±0.4 [a]	1.3±0.08 [a]	30±0.7 [a]	5.1±0.4 [a]
Ascites bearing mice + Bortezomib	10.2±0.3 [b]	1.5±0.1 [b]	39±0.7 [b]	5.4±0.2 [b]
Ascites bearing mice+ α-interferon	9.6±0.5 [b]	1.7±0.1 [b]	41±0.8 [b]	5.8±0.5 [b]
Ascites bearing mice+ (Bortezomib + α-interferon)	10.8±0.4 [b]	1.6±0.08 [b]	43±0.8 [b]	5.3±0.4 [b]
Ascites bearing mice+ Myeloma-antibodies	11±0.2 [b]	1.4±0.07 [b]	33±0.7 [b]	5.4±0.5 [b]
Ascites bearing mice+ Labeled myeloma antibodies	9.9±0.3 [b]	1.5±0.07 [b]	35±0.7 [b]	5.9±0.5 [b]
Ascites bearing mice+ (Bortezomib + α-interferon +Myeloma-antibodies)	10.9±0.4 [b]	1.6±0.08 [b]	42±1.1 [b]	5.2±0.4 [b]

[a] $p < 0.05$ Ascites bearing group vs normal group; [b] $p < 0.05$ treated vs. Ascites bearing mice group.

Parameter / Groups	Hb	Kidney functions		
		Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)
Normal	**13.5±0.3**	**0.61±0.08**	**21±0.7**	**3.4±0.3**
Ascites bearing mice	**11.4±0.4**	**1.3±0.08**	**30±0.7**	**5.1±0.4**
Ascites bearing mice+Bortezomib	**10.2±0.3**	**1.6±0.1**	**46±0.7**	**6.2±0.2**
Ascites bearing mice+ α-interferon	**9.6±0.5**	**1.8±0.1**	**41±0.8**	**5.8±0.5**
Ascites bearing mice+(Bortezomib + α-interferon)	**9.2±0.4**	**1.9±0.08**	**530.8±**	**7.3±0.4**
Ascites bearing mice+ Myeloma-antibodies	**11±0.2**	**1.4±0.07**	**33±0.7**	**5.4±0.5**
Ascites bearing mice+(Bortezomib + α-interferon +Myeloma antibodies)	**8.9±0.4**	**1.92±0.08**	**57±1.1**	**7.9±0.4**

The total body weight of ascites bearing mice untreated and treated with Bortezomib, IFN-α, (Bortezomib + IFN-α), Myeloma-Antibodies and (IFN-α + Bortezomib+ Myeloma Antibodies) are illustrated in table (17). Before treatment the weight (mean ± SD) of the 5 mice was 35 ± 1.1 g. While after treatment with IFN-α,bortezomib , (IFN-α + Bortezomib), Myeloma-Antibodies and (IFN-α + Bortezomib+ Myeloma Antibodies) the weight (mean ± SD) of the 5 mice decreased to 28±1.6, 31±0.7, 25±1.5, 27±1.3 and 24±1.8 g respectively after the four weeks of treatment and this due to the effect of drugs on the growth of myeloma cells. The results showed that treatment with bortezomib , IFN-α , bortezomib , (IFN-α + bortezomib), Myeloma-Antibodies and (IFN-α + bortezomib+ Myeloma Antibodies) decreased the total body weight as compared to the untreated bearing groups.

Table (22): The weight of ascites bearing mice before and after treatment (mean ± SD).

Groups	Weight(gm)		
	Number of mice	**Mean weight±SD**	**range**
Control	**5**	**21±2.0**	**20—25**
Untreated	**5**	**39±1.5**	**38—42**
Bortezomib	**5**	**28±1.6**	**27—31**
α-interferon	**5**	**31±0.7**	**30—32**
Bortezomib + α-interferon	**5**	**25±1.5**	**24—27**
Myeloma-antibodies	**5**	**27±1.3**	**26—28**
Bortezomib + α-interferon +Myeloma-	**5**	**24±1.8**	**23—25**

Parameter Groups	ALT(units/dL)	AST(units/dL)	ALP(units/dL)
Normal	**19.2±0.19**	**65.5±1.1**	**36±1.1**
Ascites bearing mice	**34.5±0.8[a]**	**78.2±0.4 [a]**	**43.2±0.8 [a]**
Ascites bearing mice + Bortezomib	**45.4±0.3 [b]**	**85±0.7 [b]**	**51.3±0.7 [b]**
Ascites bearing mice+ α-interferon	**48.3±0.3 [b]**	**91.6±0.5 [b]**	**68±1.3 [b]**
Ascites bearing mice+ (Bortezomib + α-interferon)	**57.6±0.4 [b]**	**96.1±0.2 [b]**	**73.2±0.8 [b]**
Ascites bearing mice+ Myeloma-antibodies	**39±0.4 [b]**	**81.4±0.7 [b]**	**49±0.7 [b]**
Ascites bearing mice+ (Bortezomib + α-interferon +Myeloma-antibodies)	**59±0.5 [b]**	**98.2±0.7 [b]**	**77.1±0.5 [b]**

The data presented in table (21) showed that the β2-microglobulin values decreased after treatment with Bortezomib, IFN-α , Bortezomib+ IFN-α , Myeloma-antibodies and Bortezomib+ IFN-α +Myeloma-antibodies compared that of control.

Table (21): The level of β2-microglobulin in Myeloma cells treated with Bortezomib, IFN-α , Bortezomib+ IFN-α , Myeloma-antibodies and Bortezomib+ IFN-α +Myeloma-antibodies compared that of control (Mean±SD).

Groups	β-2microglobulin	
	Range	Mean ±SD
Control	**2— 2.9**	**2.7±0.4**
Bortezomib	**1.1—1.6**	**1.4±0.14**
IFN-α	**1.5—2.1**	**1.9±0.2**
Bortezomib+ IFN-α	**0.9—1.4**	**1.2±0.18**
Myeloma-antibodies	**1.8—2.3**	**2.1±0.07**
Bortezomib+ IFN- α + Myeloma-antibodies	**0.7—1.2**	**0.9±0.1**

The results obtained in table (21) **demonstrated** that treatment with Bortezomib, IFN-α , Bortezomib+ IFN-α , Myeloma-antibodies and Bortezomib+ IFN-α +Myeloma-antibodies increased activation of caspases 8 and 9 compared that of control.

Table (21): the Activities of caspase-8 and caspase- 9 in Myeloma cells treated Bortezomib, IFN-α , Bortezomib+ IFN-α , Myeloma-antibodies and Bortezomib+ IFN-α +Myeloma-antibodies compared that of control (Mean±SD).

Groups	**Caspase-8**		**Caspase-9**	
	Range	Mean ±SD	Range	Mean ±SD
Control	0.14-0.2	0.17±0.02	0.04-0.12	0.09±0.03
Bortezomib	0.19-0.31	0.28±0.04	0.13-0.22	0.2±0.02
IFN-α	0.15-0.25	0.22±0.03	0.09-0.21	0.17±0.04
Bortezomib+ IFN-α	0.18-0.29	0.25±0.04	0.16-0.25	0.23±0.03
Myeloma- antibodies	0.13-0.23	0.2±0.04	0.08-0.19	0.15±0.04
Bortezomib+ IFN-α**Myeloma-antibodies**	0.41-0.48	0.46±0.02	0.19-0.29	0.26±0.05

	Hb		
Normal	**13.5**		
Ascites bearing mice	**11.4**		
Ascites bearing mice + Velcade	**10.2**		
Ascites bearing mice+ α-interferon	**9.6**		
Ascites bearing mice+ (Velcade+ α-interferon	**9.2**		
Ascites bearing mice+ Myeloma-antibodies	**11**		
Ascites bearing mice+ (Velcade+ α-interferon +Myeloma-antibodies)	**8.9**		

Parameter Groups	**Creatinine (mg/dL)**	**Urea (mg/dL)**	**Uric acid (mg/dL)**
Normal	**0.61±0.08**	**21±0.7**	**3.4±0.3**
Ascites bearing mice	**1.3±0.08**	**30±0.7**	**5.1±0.4**
Ascites bearing mice + Velcade	**1.6±0.1**	**46±0.7**	**6.2±0.2**
Ascites bearing mice+ α-interferon	**1.8±0.1**	**41±0.8**	**5.8±0.5**
Ascites bearing mice+ (Velcade+ α-interferon)	**1.9±0.08**	**530.8±**	**7.3±0.4**
Ascites bearing mice+ Myeloma-antibodies	**1.4±0.07**	**33±0.7**	**5.4±0.5**
Ascites bearing mice+ (Velcade+ α-interferon +Myeloma-antibodies)	**1.92±0.08**	**57±1.1**	**7.9±0.4**

The total body weight of ascites bearing mice untreated and treated with Bortezomib, IFN-α, (Bortezomib + IFN-α), Myeloma-Antibodies and (IFN-α + Bortezomib+ Myeloma Antibodies) are illustrated in table (17). Before treatment the weight (mean ± SD) of the 5 mice was 35 ± 1.1 g. While after treatment with IFN-α,bortezomib , (IFN-α + Bortezomib), Myeloma-Antibodies and (IFN-α + Bortezomib+ Myeloma Antibodies) the weight (mean ± SD) of the 5 mice decreased to 28±1.6, 31±0.7, 25±1.5, 27±1.3 and 24±1.8 g respectively after the four weeks of treatment and this due to the effect of drugs on the growth of myeloma cells. The results showed that treatment with bortezomib , IFN-α , bortezomib , (IFN-α + bortezomib), Myeloma-Antibodies and (IFN-α + bortezomib+ Myeloma Antibodies) decreased the total body weight as compared to the untreated bearing groups.

Table (17): The weight of ascites bearing mice before and after treatment (mean ± SD).

Groups	Weight(gm)		
	Number of mice	**Mean weight±SD**	**range**
Control	**5**	**21±2.0**	**20—25**
Untreated	**5**	**39±1.5**	**38—42**
Bortezomib	**5**	**28±1.6**	**27—31**
α-interferon	**5**	**31±0.7**	**30—32**
Bortezomib + α-interferon	**5**	**25±1.5**	**24—27**
Myeloma-antibodies	**5**	**27±1.3**	**26—28**
Bortezomib + α-interferon +Myeloma-	**5**	**24±1.8**	**23—25**

Discussion

Cancer is a group of diseases characterized by unregulated cell growth and the invasion and spread of cells from the site of origin, or primary site, to other sites in the body. Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. Other cancer promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, and thus present in all cells from birth. Complex interactions between carcinogens and the host genome may explain why only some develop cancer after exposure to a known carcinogen. Genetic abnormalities found in cancer typically affect two general classes of genes. Cancer-promoting oncogenes are often activated in cancer cells, giving those cells new properties, such as hyperactive growth and division, protection against programmed cell death, loss of respect for normal tissue boundaries, and the ability to become established in diverse tissue environments. Tumor suppressor genes are often inactivated in cancer cells, resulting in loss of normal functions in those cells, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system **(Lauren, 2008)**.

Multiple myeloma (MM) is aclonal B cell malignant disease that is characterized by the proliferation of plasma cells in the bone marrow(BM) in association with monoclonal protein in the serum and/or urine, immuneparesis, skeletal destruction ,renal dysfunction, anemia, hypercalcemia and lytic bone diseases **(Thanh et al.,2012)**.

This study aims to evaluate the antitumor effect of novel anticancer drugs Bortezomib (Velcade). In addition, the effect of interferon (alpha-interferon) on the growth of myeloma

cells was studied. Also the present study produced and evaluated the polyclonal antibodies against myeloma cells and studied the effect of the prepared antibodies (with and without labeling with radioactive isotopes) against the growth of myeloma cells (in vitro and in vivo). A correlation among these drugs was performed to determine the most biologically active and its capability for application as cancer therapy. Also some biochemical parameters were performed before and after treatment to evaluate the antitumor activity.

Bortezomib, formerly known as PS-341, is a boron containing molecule that specifically and reversibly inhibits the threonine residue of the 26S proteasome, an enzyme complex that plays a key role in the cell by regulating protein degradation in a controlled fashion. Proteins that are no longer required, including those involved in cell cycle control, apoptosis and cell signaling, are tagged with ubiquitin which directs them to the proteasome which subsequently degrades them **(Adams, 2004)**. This process maintains the balance of inhibitory and stimulatory proteins involved in cell cycle, thus inhibition of the proteasome results in a loss of the tight control of the process with a build up of cell cycle and regulatory proteins leading to cell death **(Antonia et al., 2006)**.

Bortezomib is a novel proteasome inhibitor showing antitumor activity against many tumors, primarily multiple myeloma.In this study, variable doses of Bortezomib were used for treatment of myeloma cells, and the effective dose was determined. The results showed that cell growth was inhibited by Bortezomib treatment in a dose-dependent and time-dependent manner **(Adams, 2002)**. These studies indicated that Bortezomib possesses potent growth inhibitory effects on myeloma cells in vitro and suggested that Bortezomib down-regulates growth and survival kinases, induces apoptotic cascades, inhibits the proteasome-ubiquitin pathway, and

induces stress responses **(Chen et al., 2011 and Jagannath 2005)**.

Bortezomib was also shown to inhibit the growth of myeloma cells in mice **(Leblanc et al., 2002).**When the mice become ascetic by injection of myeloma cells into the peritoneal cavity, the abdominal cavity of the animals enlarged due to the increase of myeloma cells count inside the abdominal cavity leading to increasing the weight of the animal. After intraperitoneal injection of the bortezomib with effective dose according to the weight of the animal (0.5 and 1.0 mg/kg) into the peritoneal cavity, the weight (mean±SD) of the animal was decreased and the mean viability of myeloma cells reached zero **(Richard et al., 2002)**. This is due to the effect of injected Bortezomib on the growth of myeloma cells inside the peritoneal cavity of the animal. The present study suggested that bortezomib induces tumor cell apoptosis, suppression of neoangiogenesis, and dose-dependent proteasome inhibition in vivo **(Lisa and Alexandra, 2012)**.

Interferons are soluble proteins produced naturally by cells in response to viruses. It has both anti-proliferative and immunomodulating properties and is one of the first examples of a biological response modifier used to treat the haematological malignancy multiple myeloma. Its role in the treatment of multiple myeloma has been as a single induction agent, a co-induction agent with other chemotherapy regimens, and as maintenance therapy after conventional chemotherapy or complete remission after autologous or allogeneic transplantation **(Khoo et al., 2011)**.

In this study, variable doses of Interferon-α were used for treatment of myeloma cells, and the effective dose was determined. The data showed a dose and time dependent antiproliferative effect of IFN-α on myeloma cells. IFN-α act on

the tumor cell to decrease cell proliferation and promote apoptosis. This strongly indicated direct effects of IFN- α on myeloma cells **(Joseph et al., 2010)**.

In ascites bearing mice treated with Interferon-α it was shown that the weight (mean±SD) of the animal and the viability(%) of myeloma cells were decreased after intraperitoneal injection of doses 10^4 and 10^6 IU/kg compared to that of control mice. These studies indicated that Interferon-α enhanced inhibition of cell growth and promote apoptosis and its indirect activity on the immune cells which activates them to kill the tumor cells **(Sylvia and Kim, 2011)**.

So these results indicated that the effect of Bortezomib on myeloma cell division was more effective than that of Interferon- α, where Interferon- α only decreased the viability (%) but couldn't reach it to zero.

Because some drugs work better together than alone, two or more drugs are often given at the same time (combination therapy). In vitro and in vivo studies showed that combined treatment with bortezomib and IFN-α resulted in marked increase levels of growth inhibition, apoptosis and tumor regression when compared with either agent alone, suggesting a significant potential of bortezomib in combination with other chemotherapeutics to enhance antitumor activity, reduce toxicity, and overcome drug resistance **(Megan,2007 and Angela et al.,2005)**.

Polyclonal antibodies immunotherapy possess several inherent advantages. First, polyclonal antibodies raised against a selected target in hyperimmunized animals recognize the most immunogenic epitopes and so are ‘naturally selected’ for by the host **(Newcombe** and **Newcombe, 2007)**. This may also permit the development and manufacture of polyclonal antibodies that

recognize multiple surface proteins and simultaneously activate multiple biochemical pathways leading to cell death. Second, polyclonal antibody therapeutics may be advantageous due to the high density of antibodies binding to the tumour cell surface. This would promote enhanced cross-linking of Fc receptors on effectors cells and efficient C1q binding in immunodeficient patients. Finally, the PAb could preclude the development of tumour cell ‘‘escape variants’’, because the probability that tumour cells will simultaneously lose all target epitopes is extremely small (**Sharon et al., 2005)**.

In this study, variable doses of produced antimyeloma antibodies were used for treatment of myeloma. The results obtained suggested that myeloma antibodies reduced cell viability in a dose-dependent and time-dependent manner by inhibiting proliferation or by inducing cytotoxicity in myeloma cell lines via two immune mechanisms include antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity **(Ben-Kasusa et al., 2007)**.

In ascites bearing mice treated with antimyeloma antibodies it was shown that the weight (mean±SD) of the animal and the viability (%) of myeloma cells were decreased. The results indicated that antimyeloma antibodies reduced tumour growth by binding multiple surface antigens on myeloma cells and promoting apoptosis. The results suggested that myeloma antibodies may have utility as a component of a clinical regimen for myeloma malignancies **(Borghaei et al., 2009)**.

The present study suggested that the effect of labeled antimyeloma antibodies on myeloma cells growth inhibition was more effective than that of antimyeloma antibodies without labeling **.**This is due to the cytotoxic effect of ionizing radiation **(David, 2002)**.

In vitro and in vivo studies suggested that triple combinations of (Bortezomib + IFN-α +Myeloma-Antibodies) induced a greater level of growth inhibition and apoptosis compared to double combinations (Bortezomib + IFN-α) or either agent alone in myeloma cell line. Triple combinations of are more Efficacious than double combinations or each agent alone. The present study suggested that triple combinations represent a novel treatment strategy for inducing a direct, apoptotic effect on tumor cells.

Effect on cell cycle

The cell cycle has four sequential phases. Arguably the most important phases are S phase, when DNA replication occurs, and M phase, when the cell divides into two daughter cells. Separating S and M phase are two gap phases referred to as G1 and G2. G1 follows on from mitosis and is a time when the cell is sensitive to positive and negative cues from growth signaling networks. G2 is the gap after S Phase when the cell prepares for entry into mitosis **(Murray and Hunt, 1993)**. G0 represents a state when cells have reversibly withdrawn from the cell division cycle in response to high cell density or mitogen deprivation **(Zetterberg and Larsson, 1985)**. Deregulation of the cell cycle underlies the aberrant cell proliferation that characterizes cancer and loss of cell cycle checkpoint control promotes genetic instability **(Gareth and Kai, 2012)**.

By performing the cell cycle for untreated and treated cells with Bortezomib the results obtained indicated treatment of myeloma cells with Bortezomib (20nM/ml) resulted in cell cycle arrest at the G2/M phase and which was followed by the induction of apoptosis **(Lisa and Alexandra, 2012**).

The results of flow cytometric analysis of cell cycle profile of myeloma cell treated with IFN-α (10^3 IU/ml) revealed that the IFN-α induced growth inhibition was associated with cell accumulation in the G1 phase, blockage of the cells entering from the G1 to the subsequent S phase (a G1 cell cycle arrest) leading to the growth cessation **(Andrew et al., 2012 and Matsui et al., 2003).**

Flow cytometric analysis of cell cycle profile of combined treatment suggested that (bortezomib plus IFN-α) induced a greater level of growth inhibition, arrest at the G1 phase and G2/M phase apoptosis compared with either agent alone.

The results of flow cytometric analysis of cell cycle profile of myeloma cell treated with myeloma antibodies showed that myeloma antibodies increased the number of apoptotic cells and induced S phase arrest lead to apoptosis.

The results of flow cytometric analysis of cell cycle profile of myeloma cell treated with labeled myeloma antibodies revealed that labeled myeloma antibodies inhibited myeloma cell proliferation by inducing cell cycle arrest at G2/M phase and S phase lead to apoptosis.

Flow cytometric analysis of triple combinations suggested that (Bortezomib + IFN-α +Myeloma-Antibodies) increased number of apoptotic cells and induced a greater level of apoptosis.

Effect on β2-microglobulin

Human β2M, an 11.6-kDa nonglycosylated polypeptide, interacts with and stabilizes the tertiary structure of the MHC class I α-chain .There is a close association of free β2M with many haematological malignancies, such as multiple myeloma,

leukemias and lymphomas. Elevated levels of β2M in serum are present and correlate with a poor patient outcome in MM. In fact, the serum levels of β2M are one of the most important prognostic factors for patients with MM, giving a reliable survival prediction **(Jing and Qing, 2011)**.

β2-microglobulin concentration was measured for treated and untreated cells. The results obtained demonstrated the β2-microglobulin values decreased after treatment with Bortezomib, α-interferon, (Bortezomib + α-interferon), Labeled myeloma-antibodies, Myeloma antibodies, and (Bortezomib+α-interferon+ Myeloma antibodies) compared to that of control and this indicated that these drugs inhibit the growth of myeloma cells **(Klaus et al.,2009 and Alexanian et al.,1985)**.

Effect on Caspases

Caspases, a family of aspartate-specific cysteine proteases, play major role in apoptosis and a variety of physiological and pathological processes. Fourteen mammalian caspases have been identified and can be divided into two groups: inflammatory caspases and apoptotic caspases. Based on the structure and function, the apoptotic caspases are further grouped into initiator/apical caspases (caspase-2, -8, -9, and -10) and effector/executioner caspases (caspase-3, -6, and -7) **(Ying and Guo-Qiang, 2011)**. Caspase-8 activation is triggered by binding of death ligands to their cognate receptors, whereas caspase-9 is activated as a result of mitochondrial cytochrome crelease and the subsequent formation of an apoptosome complex **(Ghavami et al., 2009)**.

In order to gain a better understanding of the apoptotic pathways activated by Bortezomib, α-interferon, (Bortezomib + α-interferon), Labeled myeloma antibodies, Myeloma antibodies, and (Bortezomib + α-interferon+ Myeloma

antibodies) in myeloma cells, and to delineate the mediators responsible for this process, we cultured the cells in the presence or absence of these drugs and subsequently investigated the activation of caspases 8 and 9.

The obtained results demonstrated that Bortezomib induced activation of caspase-8 and caspase-9 in myeloma cells leading to induction of extrinsic and intrinsic apoptotic pathways. The recorded data are in keeping with **(Lisa and Alexandra, 2012 and Mitsiades et al., 2002)**.

Our data demonstrated that IFN- α induced activation of caspases 8 and 9, this caspases activation seems to be of major importance for the execution of IFN-α induced apoptosis **(Lena et al., 2002)**.

Combined treatment with bortezomib and IFN-α increased activation of caspases 8 and 9 led to increased levels of growth inhibition, apoptosis and tumor regression when compared with either agent alone **(Gregory et al., 2008).**

Treatment with Myeloma antibodies led to increased enzyme activities of caspases (8 and 9) at 48 h post-treatment and ultimately causing apoptosis by DNA fragmentation. Treatment with Labeled myeloma antibodies induced activation of caspase 9 more than caspase 8 and causing apoptosis.

The obtained results revealed that triple combinations of (Bortezomib + IFN-α +Myeloma antibodies) induced a greater level of caspases activation and enhanced growth inhibition and apoptosis compared to double combinations (Bortezomib + IFN-α) or either agent alone in myeloma cell line.

Effect on Biochemical parameters

The presence of tumor in human body or in experimental animals is known to affect many functions of the vital organs in the body even when the site of the tumor does not interfere directly with the function of these organs **(DeWys, 1982)**.

In cancer chemotherapy, other major problems are myelosuppression and anemia **(Price and Greenfeild, 1954)**. The anemia encountered in tumor-bearing mice is mainly due to reduction in RBC or hemoglobin percentage or both, which occur either due to iron deficiency or due to hemolytic or myelopathic conditions **(Sinclair et al., 1990)**. Treatment with Bortezomib, IFN- α, [Bortezomib+ IFN-α],Myeloma-antibodies and Labeled myeloma-antibodies decreased the hemoglobin level as compared with the Ascites bearing mice control group **(Teresa et al.,2008 & Peter et al.,2012)** , while treatment with Myeloma-antibody and [Bortezomib+ IFN- α+ Myeloma-antibodies] increased the Hb content to more or less normal levels.

The serum Creatinine, Urea and Uric acid were increased in theAscites bearing mice control group compared to the normal group. The treated mice showed increased in the level of Creatinine, Urea and Uric acid in the serum of the blood as compared with the untreated groups.

Since liver is considered to be the main organ of drug activation, detoxification, and other metabolic reactions, activity of some liver marker enzymes were measured in the plasma. The serum ALT, AST and ALP activates were increased in the ascites bearing mice control group when compared to the normal group. Treatment with these drugs showed an increase in the level of serum ALT, AST compared to untreated group

(**Youngwoon et al., 2012 & Paul and Michael, 2001)**. Also, as compared to the native mice, the untreated group of mice showed an increase in the level of ALP. The treatment increased the level of ALP in the blood (**Evangelos et al., 2007)**. The results suggested that the consumption of free amino acids in building the proteins of rapidly dividing tumor cells might result in the disturbances of the enzyme activity in the liver **(Abu-Sinna et al., 2003)**.

Summary

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to deregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity. Multiple myeloma (MM) is characterized by the accumulation of malignant plasma cells in the bone marrow leading to impaired hematopoiesis and bone diseases, which includes mainly lytic lesions, pathological fractures, hypercalcemia and osteoporosis.

Chemotherapy is the systemic treatment of cancer with anticancer drugs. Chemotherapy uses powerful drugs that work by slowing or stopping the cancer cells from growing, spreading or multiplying to other parts of the body.

Extensive studies were run all over the world during the last years to discovery some new drugs which possess anticancer effect with less toxicity and have the ability to increase the survival time.

In the current study Bortezomib was employed as chemotherapeutic drug for the treatment of myeloma cells where a variable dose of Bortezomib (5, 10,20,30,50 and 100 nM/ml) were used for treatment of myeloma cells in vitro. The results obtained indicated that the T.C/ml was decreased by increasing the drug conc. compared to that of control group. These results illustrated the effect of Bortezomib on the growth of myeloma cells, where the myeloma cell division was decreased while the older cells were deteriorated so that the T.C/ml were decreased. Also the viability of myeloma cells were significantly decreased after 72 hours of addition at drug concentration 20, 30, 50 and 100 nM/ml). The present results

indicated that the effective dose of Bortezomib used for treatment of myeloma cells was 20 nM/ml, where at this concentration the viability and T.C/ml of the cells significantly decreased after 72 h of addition of the drug while the drug concentration was at low concentration, thus the side effect of the drug to normal cells decreased. The effective dose of Bortezomib were used for treatment of ascites bearing mice (in vivo) and the results indicted that the average weight of mice and viability of myeloma cells were 39±1.5gm and 82±2.0% before treatment and 28±1.6 gm and zero after treatment by a dose 0.5 and 1.0 mg/kg after 4 weeks of intraperitoneal injection of the drug.

Also Interferon-α was used as chemotherapeutic drug for the treatment of myeloma cells where variable doses of Interferon-α (50, 100, 500, 1000, 5000 and 10000 IU/ml) were used for treatment of myeloma cells in vitro. The T.C/ml and viability were decreased by increasing the drug conc. and reached to minimum value after 3rd day at drug concentration 10^4 IU/ml compared to that of control group. The results obtained indicated that the effective dose of IFN-α was 10^4 IU/ml, where the viability was significantly decreased after 3rd day of addition of IFN-α and the effective dose was at minimum concentration thus the side effect of the drug to normal cells will decreased.

The results of ascites bearing mice (in vivo) treated with IFN-α indicated that the average weight and viability % of myeloma cells of ascetic fluid of the mice decreased from 39±1.5 gm to 31±0.7gm for body weight of mice and (from 89±0.7 % to 7±0.7%) for viability of myeloma cells at IFN-α concentration10^4 IU and 10^6 IU /kg after 4 weeks of intraperitoneal (i.p.) injection of the drug. The results indicated that the effect of Bortezomib on myeloma cell division was

more effective than that of Interferon- α, where Interferon- α only decreased the viability (%) but couldn't reach it to zero.

In vitro and in vivo studies showed that combined treatment with bortezomib and IFN-α resulted in marked increase levels of growth inhibition, apoptosis and tumor regression when compared with either agent alone. Bortezomib and IFN-α induced synergistic apoptosis in myeloma cell lines.

Immunotherapy uses the body's immune system, either directly or indirectly, to fight cancer or to lessen the side effects that may be caused by some cancer treatments. In this study, we produced antimyeloma polyclonal antibodies by immunizing five New-Zealand white rabbits with SP2/OR myeloma cells.

As shown in the present study, antimyeloma polyclonal antibodies inhibited proliferation and induced apoptosis of myeloma cell lines in vitro and induced apoptosis after serial intraperitoneal injection of PAb in ascites bearing mice in vivo. The results obtained revealed that PAb is an effective agent for in vitro and in vivo induction of apoptosis in multiple myeloma.

Our results indicated that PAb decreased tumor growth by binding multiple surface antigens on myeloma cells and promoting apoptosis. The results suggested that PAb may have utility as a component of a clinical regimen for myeloma malignancies.

The present study confirmed that the effect of labeled antimyeloma antibodies on myeloma cells growth inhibition was more effective than that of antimyeloma Antibodies without labeling. This is due to the cytotoxic effect of ionizing radiation.

The data recorded that triple combinations of (Bortezomib + IFN-α +Myeloma-Antibodies) were superior in its anticancer potential than double combinations (Bortezomib + IFN-α) or either agent alone in myeloma cell line. The present study suggested that triple combinations represent a novel treatment strategy for inducing a direct, apoptotic effect on tumor cells

Also some confirmatory tests were performed to confirm the effect of the previously used treatment on the growth of myeloma cells, these tests include β2-microglobulin, caspases 8 and 9 and flow cytometric analysis. β2-microglobulin was measured for myeloma cells before and after treatment. Where β2-microglobulin values before treatment was 2.7±0.4 mg/dl while after treatment with Bortezomib, α-interferon, (Bortezomib+α-interferon), myeloma-Antibodies, Labeled Myeloma-Antibodies and (Bortezomib + α-interferon+ Myeloma-Antibodies) were (1.4±0.14, 1.9±0.2, 1.2±0.18, 2.1±0.07 and 0.9±0.1 mg/dl) respectively and this indicated that the conc. of β2-microglobulin decreased by treatment of myeloma cells. This suggested that these drugs inhibit the growth of myeloma cells.

The cell cycle profile was performed for myeloma cells before and after treatment and the results obtained indicated that these drugs were effective against the growth of myeloma cells.

Also caspases enzymes 8 and 9 were measured for myeloma cells before and after treatment. These enzymes play essential roles in apoptosis , necrosis, and inflammation. The results suggested that these drugs induced activation of caspase-8 and caspase-9 in myeloma cells leading to induction of extrinsic and intrinsic apoptotic pathways. Also some biochemical parameters were performed before and after treatment to evaluated side effects of these drugs.

Also Some biochemical parameters were performed before and after treatment

Conclusion

In vitro and in vivo studies showed that combined treatment with bortezomib and IFN-α resulted in marked increase levels of growth inhibition, apoptosis and tumor regression when compared with either agent alone. Bortezomib and IFN-α induced synergistic apoptosis in myeloma cell lines.

The data recorded that triple combinations of (Bortezomib + IFN-α +Myeloma-Antibodies) were superior in its anticancer potential than double combinations (Bortezomib + IFN-α) or either agent alone in myeloma cell line. The present study suggested that triple combinations represent a novel treatment strategy for inducing a direct, apoptotic effect on tumor cells

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IFN-A and Bortezomib Overcome Bcl-2 and Mcl-1 Overexpression in
Melanoma Cells by Stimulating the Extrinsic Pathway of Apoptosis
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Additively Enhanced Antiproliferative Effect of Interferon Combined with Proanthocyanidin on Bladder Cancer Cells
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Anti-tumour activity of interferon-alpha in multiple myeloma: role of interleukin 6 and tumor cell differentiation.

Title	Anti-tumour activity of interferon-alpha in multiple myeloma: role of interleukin 6 and tumor cell differentiation.
Publication Type	Journal Article
Year of Publication	2003
Authors	Matsui W, Huff CA, Vala M, Barber J, Smith DB, Jones RJ
Journal	British journal of haematology
Volume	121
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Pagination	251-8
Date Published	2003 Apr
Abstract	Interferon-alpha (IFN-alpha) is a pleotropic cytokine that has clinical activity against a wide variety of malignancies, including multiple myeloma (MM). In vitro, IFN-alpha has diverse effects on both normal and malignant cells, however, the exact mechanisms responsible for its clinical anti-tumour activity remain unclear. We found that IFN-alpha inhibited MM cell proliferation in association with cell cycle arrest at G1 and limited the clonogenic growth of both MM cell lines and primary patient specimens. At the doses tested, IFN-alpha was not cytotoxic, but induced terminal plasma cell differentiation resulting in the loss of clonogenicity. These activities were markedly enhanced by the major MM growth factor interleukin 6 (IL-6). Moreover, IL-6 was required for this process, as neutralizing antibodies against IL-6 inhibited the effects of IFN-alpha. IL-6 also induced MM cell terminal differentiation when combined with a second, unrelated, antiproliferative agent bryostatin-1, suggesting that its differentiating activities are

preferentially enhanced in the presence of agents that inhibit cell cycling. These results suggest that the differentiating activities of IFN-alpha may play a role in its clinical antimyeloma activity and provide the rationale for clinical differentiation therapy in MM.

Liver function

Detrimental Effect of the Proteasome Inhibitor, Bortezomib in Bacterial Superantigen- and Lipopolysaccharide-induced Systemic Inflammation
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Molecular Therapy vol. 18 no. 6, 1143–1154 june 2010

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INF (*AB

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IF HB

Interferons in the treatment of multiple myeloma

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Ascites bearing mice+ (Bortezomib + α-interferon)	**57.6±0.4 [b]**	**96.1±0.2 [b]**	**73.2±0.8 [b]**
Ascites bearing mice+ Myeloma-antibodies	**39±0.4 [b]**	**81.4±0.7 [b]**	**49±0.7 [b]**
Normal	**59±0.5 [b]**	**98.5±0.7 [b]**	**76.1±0.5 [b]**
Ascites bearing mice+ (Bortezomib + α-interferon +Myeloma- antibodies)	**34.5±0.8[a]**	**78.2±0.4 [a]**	**43.2±0.8 [a]**
Ascites bearing mice	**45.4±0.3 [b]**	**85±0.7 [b]**	**51.3±0.7 [b]**
Ascites bearing mice +	**48.3±0.3 [b]**	**91.6±0.5 [b]**	**68±1.3 [b]**
Ascites bearing mice+ α-			

Parameter

Groups **ALP(units/dL)** **AST(units/dL)** **ALP(units/dL)**

Groups	Parameter			
	Hb	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)
Normal	13.5±0.3	0.61±0.08	21±0.7	3.4±0.3
Ascites bearing mice	11.4±0.4	1.3±0.08	30±0.7	5.1±0.4
Ascites bearing mice + Bortezomib	10.2±0.3	1.6±0.1	46±0.7	6.2±0.2

Ascites bearing mice+ α-interferon	**9.6±0.5**	**1.8±0.1**	**41±0.8**	**5.8±0.5**
Ascites bearing mice+ (Bortezomib + α-interferon)	**9.2±0.4**	**1.9±0.08**	**530.8±**	**7.3±0.4**
Ascites bearing mice+ Myeloma-antibodies	**11±0.2**	**1.4±0.07**	**33±0.7**	**5.4±0.5**
Ascites bearing mice+ (Bortezomib + α-interferon +Myeloma-antibodies)	**8.9±0.4**	**1.92±0.08**	**57±1.1**	**7.9±0.4**

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http://onlinelibrary.wiley.com/advanced/search/results?searchRowCriteria[0].queryString="Thomas Moritz"&searchRowCriteria[0].fieldName=author&start=1&resultsPerPage=20

الملخص العربي

يعتبرالسرطان من مئات الأمراض الشائعة في هذا العصر و هو عبارة عن نمو غير طبيعي للخلايا التي تسببها العديد من التغييرات في التعبير الجيني مما يؤدي إلى اختلال التوازن بين تكاثر الخلايا وموت الخلايا و الانتقال و النمو من مكان منشاها لامكان اخرى فى الجسم. و لو لم يتم التحكم فى انتشار هذ ه الخلايا فأن السرطان سوف يؤدى إلى موت الكائن الحى. تتميز المايلوما السرطانية (MM) بتراكم خلايا البلازما الخبيثة في نخاع العظم مما يؤدي إلى ضعف تكون الدم وأمراض العظام.

اتجهت الابحاث فى السنوات الاخيرة إلى تكثيف الدراسات لاكتشاف بعض الأدوية الجديدة المضادة للأورام بحيث تكون لها القدرة على القضاء على الورم مع احتفاظها بسمية منخفضة على بقية أعضاء الجسم مما يؤدى إلى تقليل نسبة الوفيات فى مرضى السرطان. تهدف هذه الدراسة إلى تقييم الطرق المختلفة المستخدمة لعلاج مثل هذه الأورام السرطانية و تمت الدراسة على خلايا المايلوما السرطانية وتم فيها استخدام طرق مختلفة من العلاج اولهما العلاج الكيماوي باستخدام عقاري (بورتيزوميب و الإنترفيرون ألفا) و ثانيهما العلاج المناعى بانتاج و تقييم النشاط المضاد للأورام للأجسام المضادة عديدة النسيلة على نمو خلايا المايلوما السرطانية و ثالثهما العلاج المناعى الاشعاعى و ذلك بترقيم الأجسام المضادة و استخدامها في العلاج. و قد تمت هذه الدراسة علي مرحلتين فالمرحلة الاولي تمت بالمعمل بأطباق المزارع والمرحلة الثانية تم دراستها في حيوانات التجارب (فئران من نوع بالب سي).

العلاج الكيماوي هو علاج باستخدام أدوية كيماوية تُعرف بالعقاقير المضادة للسرطان، تقوم بالقضاء على الخلايا السرطانية ومنع انتشارها لأجزاء أخرى من الجسم. قد تم دراسة تأثير التركيزات المختلفة لعقاربورتيزوميب(الفالكيد) (نانومول / مللي) على نمو خلايا المايلوما السرطانية و حساب العد الكلى و درجة الحيوية لها قبل و بعد العلاج لمدة ٦ أيام لتحديد التركيز الفعال و المؤثر على نمو خلايا الميلوما السرطانية و قد أظهرات النتائج ان العد الكلى و درجة الحيوية لخلايا المايلوما السرطانية تقل بزيادة الجرعات المستخدمة و قد وجد أن التركيز الامثل للعلاج بهذا العقار هو ٢٠ نانومول / مللي حيث أن هذا التركيز يؤثر على نمو الخلايا و حيويتها بعد ٧٢ ساعة و في الوقت نفسه فهذا التركيز غير مرتفع بالدرجة التي تؤثر على الخلايا الطبيعة . و تم احداث استسقاء داخل الغشاء البريتوني لفئران التجارب بحقن خلايا المايلوما السرطانية بداخلها و دراسة تأثير التركيز الفعال على نمو خلايا المايلوما بها بعد حساب التركيز المستخدم حسب وزن الحيوان و أظهرت النتائج التأثير الفعال لهذا العقار حيث أنه قبل العلاج كان وزن الفأر

٤٠ جرام و درجة الحيوية لخلايا المايلوما داخل الغشاء البريتوني ٨٦% بعد ٤ أسابيع من حقن العقار مرتين اسبوعا أصبح الوزن ٢٧ جرام و درجة الحيوية صفر.

كما تم دراسة تأثير التركيزات المختلفة لعقار الإنترفيرون ألفا(وحدة دولية / مل) و تم حساب العد الكلى و درجة حيوية لخلايا الميلوما السرطانية قبل و بعد العلاج لمدة ٦ أيام و قد أظهرت النتائج أن العد الكلى و درجة الحيوية لخلايا الميلوما السرطانية تقل بزيادة الجرعات المستخدمة من العقار و قد وجد أن التركيز الامثل للعلاج بهذا العقار هو ١٠٠ ميكروجرام حيث أن هذا التركيز يؤثر على نمو الخلايا و حيوتها و في نفس الوقت فهذا التركيز غير مرتفع بالدرجة التي تؤثر على الخلايا الطبيعة. و تم احداث استسقاء داخل الغشاء البريتوني لفئران التجارب بحقن خلايا ميلوما سرطانية بداخلها و دراسة تأثير التركيز الفعال لعقار الإنترفيرون ألفا على نمو خلايا الميلوما بها بعد حساب التركيز المستخدم حسب وزن الحيوان و أظهرت النتائج التأثير الفعال لهذا العقار حيث انه قبل العلاج كان وزن الفار ٤٠ جرام و درجة الحيوية لخلايا الميلوما داخل الغشاء البريتوني٩٢% و بعد ٤ أسابيع من حقن العقار اصبح الوزن ٣٣ جرام و درجة الحيوية ١٧%.

وقد أظهرت الدراسة أن العلاج المشترك بين (بورتيزوميب +الإنترفيرون ألفا) أدى إلى زيادة ملحوظة في تثبيط نمو وموت خلايا المايلوما و تضائل الورم مقارنة باستخدام احد العقارين منفردا.

العلاج المناعى (العلاج البيولوچى) و هو عبارة عن استخدام الجهاز المناعى للجسم نفسه بطرقة مباشرة او غير مباشرة لعلاج الخلايا السرطانية و ايضا يستخدم العلاج المناعى فى تقليل الثار الجانبية التى تحدث فى الطرق التقليدية الاخرى من العلاج مثل العلاج الكيماوى و العلاج الإشعاعى وقد تم إنتاج الأجسام المضادة بتطعيم خمسة من الأرانب النيوزيلاندية البيضاء بخلايا المايلوما . و قد تم الحقن الابتدائى باستخدام محفز فروند الكامل متبوعا بخمس حقنات تنشيطة متتالية باستخدام محفز فروند غير الكامل و تم ايضا ترقيم الأجسام المضادة باستخدام اليود-١٢٥ المشع بطريقة الكلورامين- تى وتمت تنقيته باستخدام طرق الفصل الكروماتوغرافي. وقد أظهرت النتائج التأثير القوي والفعال للأجسام المضادة على نمو الخلايا و حيويتها فى أطباق المزراع و فى الفئران. وقد اكدت الدراسة ان الأجسام المضادة المرقمية أكثر فعالية من الأجسام المضادة الغير مرقمية ويرجع ذلك إلى تأثير الإشعاع المؤين على نمو الخلايا.

وقد أوضحت النتائج أن الجمع بين (بورتيزوميب +الإنترفيرون ألفا+ الأجسام المضادة) أكثر فعالية على تثبيط نمو الخلايا المايلوما بمقارنة بالعلاج المزدوج أواستخدام احد العقارين منفردا و قد اقترحت هذه الدراسة أن العلاج الثلاثى يمثل استراتيجية جديدة لعلاج مثل هذه الأورام السرطانية .

وقد تم أيضا إجراء بعض التجارب الحيوية لتأكيد مدى تأثير الطرق العلاجية السابقة فى علاج خلايا المايلوما السرطانية و هذه التجارب تشمل تحديد المراحل المختلفة لنمو الخلايا باستخدام تحليل الفلو سيتوميتري والذي يوضح المراحل المختلفة لعملية الانقسام الخلوي وكذلك تم تقدير تركيز مادة البيتا-٢ ميكروجلوبين والتي يبين تركيزها تحديد معدل نمو الخلايا وأيضا قياس أنزيمات الكاسبس ٨&٩ والتي يؤثر وجودها على معدل موت الخلايا وقد أظهرت النتائج التي تم الحصول عليها التأثير القوي والفعال للطرق العلاجية التي تم استخدامها من علاج كيماوي ومناعى و مناعى اشعاعى والتي تشير إلى تأثيرها على الحد من نمو خلايا المايلوما السرطانية.

جامعة عين شمس
كلية العلوم

تقييم النشاط المضاد للأورام لاحد المثبطات البروتوسومية والانترفيرون والأجسام المضادة لخلايا الميلوما على نمو خلايا الميلوما السرطانية

رسالة مقدمة للحصول على درجة الدكتواره فى الكيمياء الحيوية
مقدم من

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