Ain shams Universirty
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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

Submitted for the Requirement of the Ph.D degree in

Biochemistry

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(M.Sc in Biochemistry)

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(2013)

جامعة عين شمس
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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
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Acknowledgment

*First and last thanks to **Allah** who gives me the power to go forward in away illuminated with his merciful guidance.*

*I am grateful and deeply obligated to **Prof.Dr. Ibrahim Hassan Borai**, Prof. of Biochemistry, Faculty Of Science,Ain Shams University, for valuable supervision, valuable revision and his precise flow up of this work.*

*I wish to express my sincere gratitude and deep appreciation to **Prof. Dr/ Mohammed Taha El-Kollaly**, Professer of Radiopharmaceutics , Atomic Energy Authority, for his valuable supervision, choosing the subject of research, overcoming the difficulties during this work, and continuous encouragement during the course of this study.*

*I also like to extend my sincere gratitude to **Dr.Kamel Abd El-Hameed Moustafa** , professor of Biochemistry, Labeled compounds department, Radioisotopes and production division, Atomic Energy Authority, for his hard work in this study, valuable advice to me and overcoming the difficulties.*

*I wish to express my deep appreciation to Dr. **Ahmed Abd El.aziz sayed** Assistant prof.of Biochemistry, Faculty Of Science , Ain Sham University, for his support and valuable advises to me.*

*I wish to express my deep appreciation to **Prof.Dr.Naser Mohammed El-Lahlob**y, Cancer Institute Center, Cairo University for his support and valuable advises to me.*

*I am very grateful to **Dr.Ibrahim Youssef Abd El-Ghany** Assistant professor of Biochemistry, Labeled compounds department, Radioisotopes and production division, Atomic Energy Authority, for his sustained encouragement, continuous support and great assistance throughout the work.*

I would like to express my deep thanks to my colleagues in the Labeled compounds department, Radioisotopes and production division, Atomic Energy Authority, for their help and support.

I would like to thank my colleagues in department of Biochemistry, Faculty Of Science, Ain Shams University, for their sincere advises and kind co-operation with me.

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.

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).**