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RESEARCH ARTICLE

Synthesis and Diagnosis of Nucleoside Analogues and Cytotoxic Effects against Normal Human Lymphocytes

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Abstract

Synthesis of nucleoside analogues and investigated that cytotoxic effects on the Normal Human Lymphocyte by MTT assay. Preparation of Mannich bases that derivative from thymidine fainally obtain crystals and purify by using silica gel filled with gasoline. In the current study is to evaluate the cytotoxic effects for the four compounds of nucleoside analogues at concentrations of (0.15mg/ml, 0.31mg/ml, 0.62mg/ml, 1.25mg/ml, 2.5mg/ml, 5mg/ml, 10mg/ml, and 20mg/ml) alongside against the freshly isolated and cultured normal human lymphocyte *in vitro*. The result refer to that cytotoxic effects of each type of nucleoside analogues used in the study and the growth inhibition rate (%) was associated to the concentration of nucleoside analogues, so that a 20mg/ml concentration of gave the highest growth inhibition rate, followed by the 10mg/ml, 5mg/ml and 2.5mg/ml, 1.25mg/ml concentrations, therefore the cytotoxic effects was concentration dependent. There was a significant difference between the concentrations when compared between the four kinds of nucleoside analogues, also statistical analysis related between the effects of different kind of nucleoside analogues showed great difference and there was a significant relationship between the inhibition rate and the concentrations of the materials tested ($P < 0.05$). The results visibly showed that nucleoside analogues have strong cytotoxic and cytostatic effects on studied the lymphocyte and the cytotoxic effects may be attributed to the role of these compounds in the DNA and RNA synthesis or play a role in the apoptosis. On the basis of these results we can conclude nucleoside analogues have potent cytotoxic effects against human lymphocytes *in vito* and the pathways for how the cells dies need further studies in the future studies in more details and more advanced tests and techniques.

Keywords: *Nucleoside analogues; Cytotoxicity; Normal human lymphocyte.*

Introduction

There are several of pyrimidines derivatives (cytosine, thiamine, and uracil) it have great biological significance due to DNA building units [1], studies have indicated that Manaik bases are of great important in medical fields are used as an anti-inflammatory, anti-malarial [2], and anti-cancer [3] and from prepared compounds through Manaik's reaction are compound 1 (analogue of thymidine), compound 2 (Osnervan) which is used in the treatment of Parkinson disease, and compound 3 which is used as analgesic of pain [5].

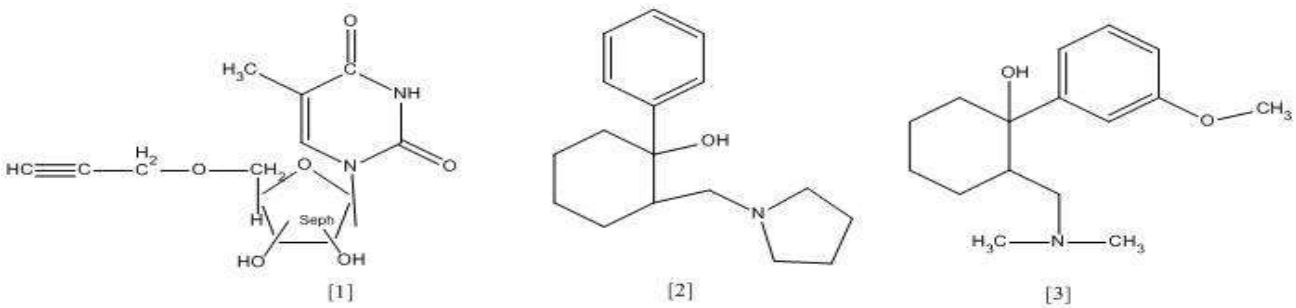

Figure 1: (Compound one, two and three)

Pyrimidines also used as immunosuppressant agent [6]. Cytotoxic nucleoside analogues and nucleobases were among the first chemotherapeutic agents to be introduced for the medical treatment of cancer. This family of Compounds has grown to include a variety of purine and Pyrimidine nucleoside derivatives with activity in both solid tumors and malignant disorders of the blood.

Nucleoside analogues and nucleobases are a pharmacologically diverse family, which includes cytotoxic compounds, antiviral agents, and immunosuppressive molecules. Cytotoxic nucleoside analogues are antimetabolites that interfere with the synthesis of nucleic acids.

These agents can exert cytotoxic activity by being incorporated into and altering the DNA and RNA macromolecules themselves, by interfering with various enzymes involved in synthesis of nucleic acids, or by modifying the metabolism of Physiological nucleosides [7].

In these studies the cytotoxcity of nucleoside analogues on normal human peripheral lymphocytes, which are one of the immune system cells, which is an incorporated organization contain of specific cells and molecules. The most important are lymphocytes which are divided in to two types T- and B lymphocytes -, and natural killer (NK) cells.

Plasma cells which obtain from B cells excrete antibodies and T-lymphocytes are distinguishing in to many types of cells CD4 T-helper cells, and CD8 cytotoxic T-cells. Lymphocytes responsible for the immune response. Any kinds of toxic effect by lymphocyte lead to change in immune response [8, 9]. The assay that is used in this experiment is the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5diphenyltetrazolium bromide) tetrazolium reduction [10].

Material and Methods

Procedure of Preparation of Mannich Bases (1, 2, 3, 4) that Derivatives from Thymidine

Added (0.2 g, 0.1 mole) from thymidine to100 ml beaker contain 30 ml of ethanol and 0.1 mole of secondary amines (pyrvolidine, piprazin, piperidine, morpholin). Added 3-5 drops of glacial acetic acid then put the compounds in shaker water bath at (0-5 C) for 30 min.

Added (0.01 mole) from formaldehyde gradually with continuous stirring then the mixture was raised by a water bath for 3 hr. Keep the mixture in water bath at 5 C for 3 hr Separate the product by filtration and then get the precipitate, then wash with methanol and restores its crystals using absolute methanol and purify the material using silica gel filled with gasoline. Physical characteristics are shown in Table 1.

Table 1: Showed the Physical characteristics of what?

Comp. No.	Name of compounds	M.P C°	% yield	Colour
1	*β*- D- Ribofuranose -3- morpholino methyl thymine	Surup	61	White
2	*β*- D- Ribofuranose -3-piprazino methyl thymine	191 – 192	55.3	Crystal white
3	*β*- D- Ribofuranose -3- piperidino methyl thymine	176 – 177	72	Yellow
4	*β*- D- Ribofuranose -3- pyrrolidino methyl thymine	159 – 160	81	Off white

Isolation of Lymphocytes from Whole Blood

Five ml of blood were taken from normal healthy person and put in EDTA contain test tube. Five ml of Phosphate Buffered Saline (PBS) were added and mixed well by inverted the tube several time. Five ml of ficoll hypaque solution were taken and added to 15 ml tubes, carefully layered blood-PBS mixture on to the ficoll hypaque solution without mixing the blood with the ficoll hypaque, then the tubes centrifuged at 3000 rpm for 30 minutes. The buffy layer containing mononuclear cells was collected,

mixed with PBS, and centrifuge at 1500 rpm for 10 minutes for washing, and supernatant were removed from the tubes. The pellet of the normal lymphocytes resuspended in 5 ml of Roswell park Memorial institute medium (RPMI 1640) containing 20% fetal bovine serum. The cell counted and the lymphocytes were plated in 96-well cell culture plates, each must contain at least 10^4 cells/200µl, the plates were incubated in CO_2 for 24 hrs., to check the sterility and cell growth then used for cytotoxicity assay[11].

MTT Cytotoxicity Assay

Different concentrations from each of material of the four prepared compounds, eight concentrations used as follows in the concentrations (0.15 µg/ml, 0.31 µg/ml, 0.62 µg/ml, 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, and 20 µg/ml), after that, 200 µL from each concentration of the prepared compound was added in triplicate in to each of the wells containing human lymphocytes, complete medium was used as negative control.

After that, the 96-well plates were incubated in CO_2 incubator for 48 hours at 37ºC. After 48 hrs, 20 µl of MTT solution was added then incubated at 37ºC from 2-4 hours, during the life cells changed the MTT stain into formazan crystals. Then the plates centrifuged at 1000 rpm for 10 minutes and the medium removed from the plates using multichannel pipette and 50 µL of DMSO was added to each wells to solubilize the formazan dye, mixed for 10 minutes. Finally, the absorbance was read at 570 nm using ELISA instrument (Biotech, USA) and the percentage of inhibition rate was counted based on the formula below [12].

% Inhibition rate (IR) = absorption at 570nm for the negative control – absorption at 570 nm for test/absorption at 570nm for the negative control]*100

Statistical Analysis

The obtained results (The values of the examined parameters were assumed in terms of mean ± standard error) were statistically analyzed using Duncan's multiple range tests in SAS software (version 17; SAS Inc., Chicago, IL, USA). The grade of significance was $P > 0.015$ [13].

Results and Discussion

Identified the prepared compounds (Nucleosides derivatives by Mannich reaction) by through physical properties as melting point, color, C-H-N analysis as shown below in table (2).Also identified the compounds by IR spectrophotometer, Necular Magnetic Resonance (H-NMR, C-NMR). IR spectrum showed disappear (H-N) binding band which was appeared at (3430-3380) that back to the base nitrogen thymine in all compounds, Other bands shown in Table (2) that confirm the prepared compounds.

Table 2: Shown the values of C.H.N and IR

Comp. No.	I.R						C.H.N			
	V(O-H) cm^{-1}	*V*(C=O) cm^{-1}	*V*(C-H) cm^{-1}	*V*(C-O-C) cm^{-1}	*V*(C-N) cm^{-1}	other	M. formula	%C Calc. Found.	%H Calc. Found.	%N Calc. Found.
1	3650	1716	2985	1250	1340	*V*(C-H) 2990 cm^{-1} of (CH_2) for morpholine				
2	3640	1720	2990	1290	1351	*V*(N-H) 3400 cm^{-1} of (CH_2) for piprazine				
3	3550	1730	2960	1300	1360	*V*(C-H) 2855cm^{-1} of (CH_2) for piperidine				
4	3560	1725	2895	1275	1330	*V*(C-H) 2860 cm^{-1} of (CH_2) for pyrrolidine				

For H-NMR spectrum shown in table (3) indicate the band (3.4-2.8ppm) of (H-N) for thymine is disappear, another band appeared in (1.7-2.2ppm) for (-CH_2-) of Mannich base that prepared addition to the other values which confirm the base substitution (pyrrolidine, piperidine, piprazin, morpholin) on nucleoside as shown below in Table 3.

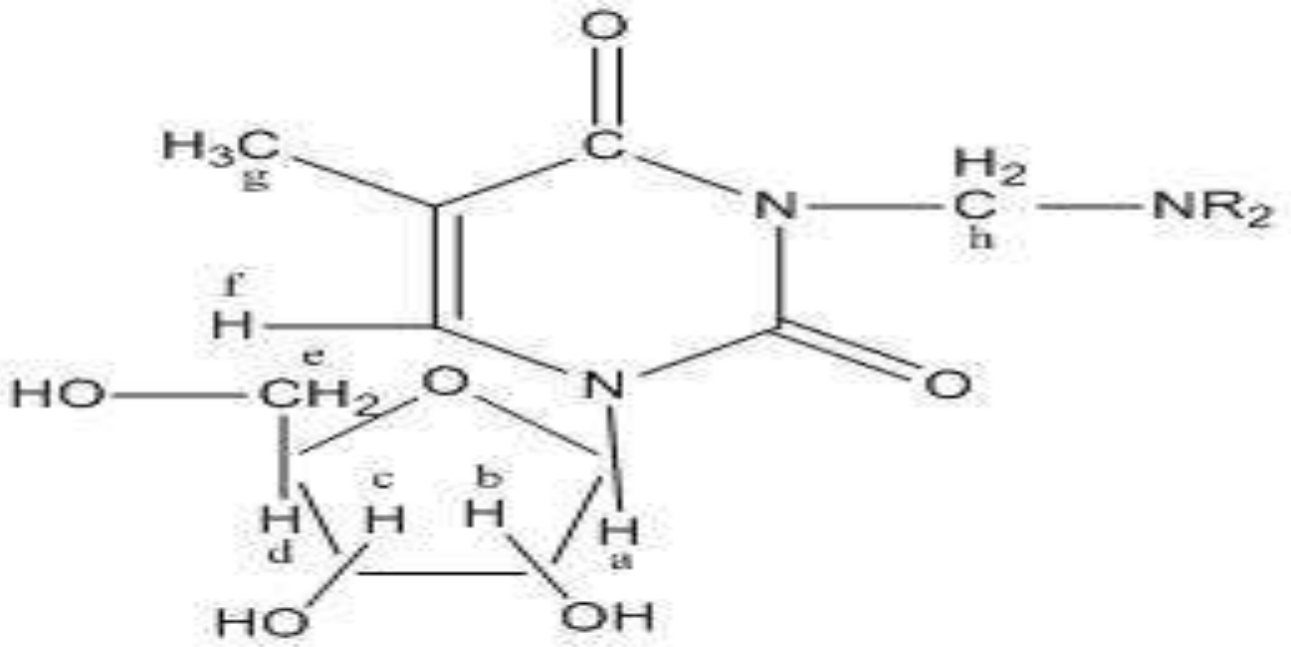

Fig. 2

Table 3: shown the values of C13.N.M.R For prepared compounds

Compd. No.	δ (ppm)									
	H_a	H_b	H_c	H_d	H_e	-OH	CH_3	-C=CH	-CH_2-	Other
1	2.8 , s	← 3.1 → , m		3.5 , q	4.5 ,d	9.5 , s	2.3 ,s	4.7 ,s	2.6 ,s	(2.9,t,2H for C near to N) and (3.3,t,2H for C near to O) for morpholine
2	2.6 , s	← 3.3 → , m		3.7 , q	4.0 , d	9.1 , s	2.1 , s	4.4 , s	2.4 , s	2.1,m,for H to piprazine
3	2.7 , s	← 3.2 → , m		3.5 , q	3.9 , d	9.0 , s	2.3 , s	4.2 , s	2.5 , s	2.3,m,for H piperidine
4	2.5 , s	← 3.1 → , m		3.4 , q	3.7 , d	9.3 , s	2.0 , s	4.1 , s	2.3 , s	2.4,m,for pyrrolidine

Also identified the prepared compounds by C.N.M.R which confirms the preparation of compounds as shown of their values in the Table (4).

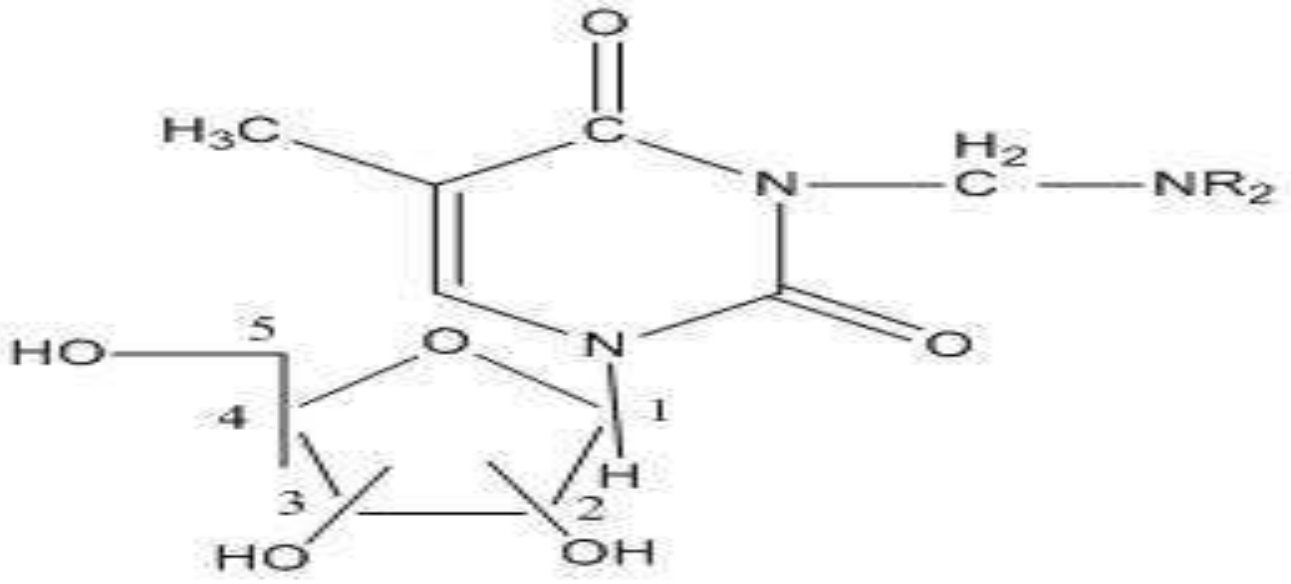

Fig. 3

Table 4: Shown the values of C13.N.M.R For prepared compounds

Compd. No.	δ (ppm)							
	C_1	C_2	C_3	C_4	C_5	CH_3	CH_2	Other
1	27	30 ↔ 34			37	39	41	(42 – 43)for thymine , (20 – 25) for morpholline
2	26	28 ↔ 35			38	40	42	(44 –51)for thymine , (21 – 24) for piprazine
3	24	26 ↔ 32			34	36	38	(40 – 44)for thymine , (22 – 26) for piperidine
5	24	27 ↔ 31			32	36	36	(39– 43)for thymine , (19 – 23) for pyrrolidine

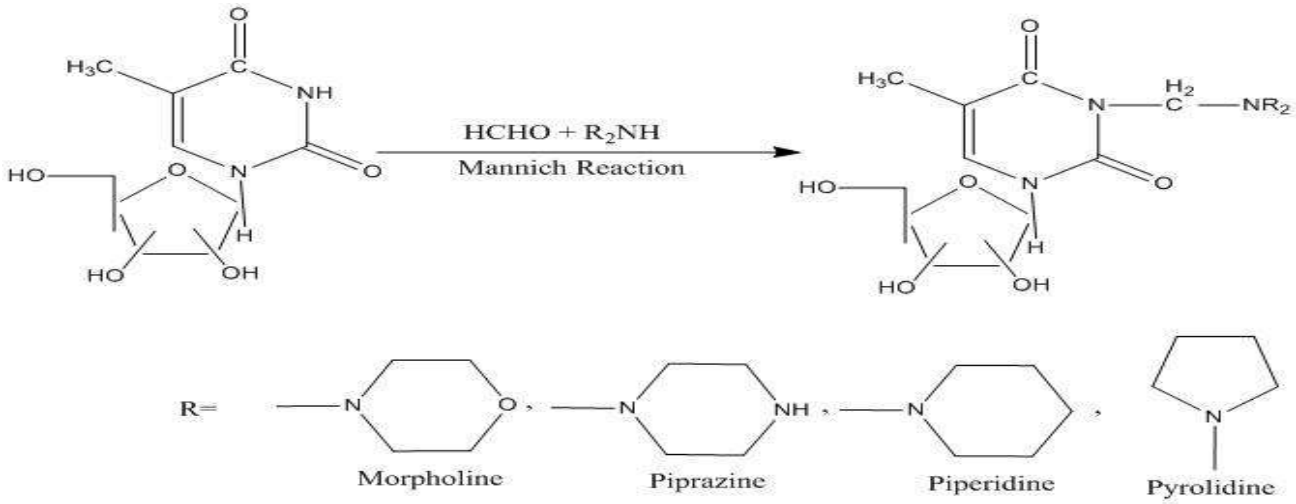

Fig 4: Synthesis of nucleoside

Cell culture can be a very sensitive and reproducible method for the preliminary screening of the inhibition rate of active ingredients on cells lines and isolated cells as several chemicals can be tested on human cells in controlled way[14][15], as the active agent can be well-tried on animal cells in highly controlled process. Cytotoxicity assays in general are able to observe many factors that possible inhibit the biochemical activity of many human cell lines [16].

A number of methods have been developed to evaluate the growth inhibition cell lines, like MTT assay [17]. In the introduce study the inhibitory effects of the nucleoside analogues at concentration (0.31mg/ml, 0.62mg/ml, 1.25 mg/ml, 2.5mg/ml, 5mg/ml, 10mg/ml, and 20mg/ml) with normal human lymphocytes in vitro were specified as distinguished already. The results showed in table (5) referred to the effects of each type of nucleoside analogues utilized in the study and the growth inhibition rate (%) was related to the nucleoside analogues concentration therefore a (20mg/ml) concentration the highest growth inhibition rate, then by (10%),(5%),(2.5%), and (1.25%), (0.62%) concentrations.

There was clear difference between the concentrations when compared between the four types of nucleoside analogues, statistical analysis showed a clear difference ($P <$ 0.0015).

Table 5: The cytotoxic effect expressed in inhibition rate percentage (%IR) for the four types of nucleoside analogues with different concentrations after 48 hours exposure on Normal Human Lymphocyte

Concentration %	Percentage of Growth inhibition (G.I %) in hep g2 cell line			
	B-D-Ribofuranose-3-morpholino methyl thymine	B-D-Ribofuranose-3-piprazino methyl thymine	B-D-Ribofuranose-3-piperidino methyl thymine	B-D-Ribofuranose-3-pyrrolidino methyl thymine
0.15 mg	37 ± 0.2	41.6 ± 0	34 ± 1.1	42.6 ± 0.087
0.31 mg	40± 0.1	44.3 ± 0.087	36.6 ± 1.1	45.3 ± 0.087
0.62 mg	41. ± 0.1	47.3 ± 1.1	38.3 ± 5.1	48.3 ± 0
1.25 mg	44 ± 0.087	50 ± 8.6	40.3 ± 0.2	50 ± 0
2.5 mg	46.6 ± 0.05	52.3 ± 8.6	42.3 ± 0	51.6 ± 0
5 mg	49.3 ± 0.1	55 ± 0.2	46.3 ± 0	53.3 ± 8.6
10 mg	51.6 ± 0.08	59 ± 0.2	50 ± 8.6	56 ± 0
20 mg	60 ± 0.2	63.3 ± 0.2	53.3 ± 8.6	58.6 ± 0.2

The cytotoxic effects of the different concentrations of the (ß- D- Ribofuranose -3- morpholino methyl thymine) after 48 hours incubation with normal human lymphocytes data shown in figure(). The statistical analysis showed from results that ß-D-Ribofuranose-3-morpholinomethylthymine concentration dependent of inhibition rate when the concentration increased the inhibition rate increased also against normal human lymphocytes, this cell killing may be attributed to many factors one of them could be the materials integrated in the DNA and RNA synthesis pathways that led the cells to die or may be increased the apoptosis which lead to increase the cell death and the inhibition rate increased, the cells may dead by one of the explained or supposed possibilities, it needs for more and detailed study about the pathway of the cell death.

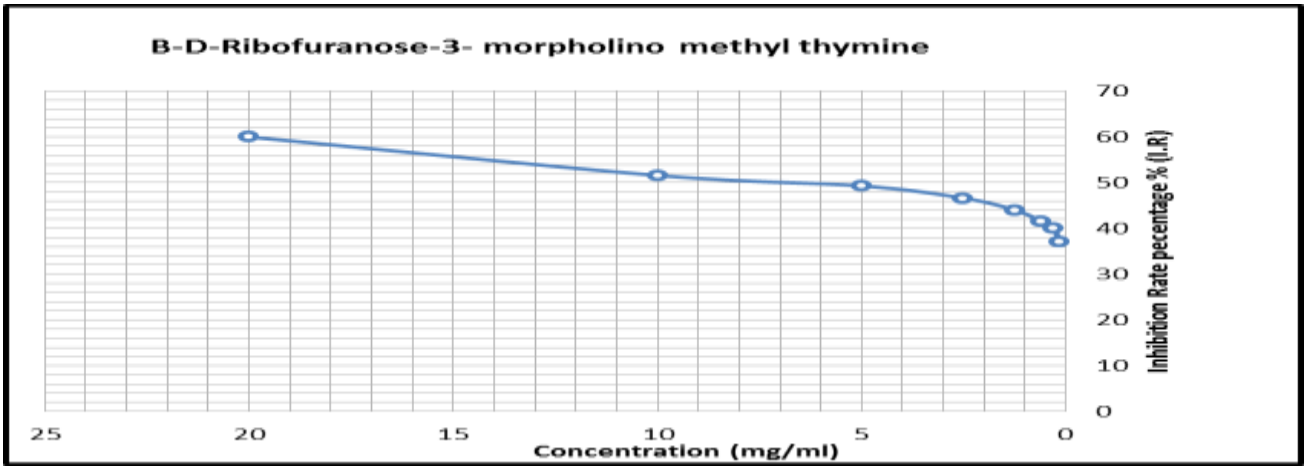

Fig 5: The cytotoxic effect expressed as the inhibition rate percentage (%IR) for different Concentrations of *β*- D-Ribofuranose -3- morpholino methyl thymine after 48 hours incubated on normal human lymphocytes

In Figure (5) also showed that concentrations used (0.31, 0.62, 1.25, 2.5, 5, 10, and 20 mg/ml) respectively, that toxic effect was strong, against the normal human lymphocytes, also showed increased inhibition rate with the increasing concentrations the inhibition rate were (37%, 40%, 41.6%, 44%, 46.6%, 49.3%, 51.6%, and 60%) respectively. The cytotoxic effect of different concentrations of the (- D-Ribofuranose-3-piprazinomethylthymine) in same way as used with first nucleoside analogue incubate with normal human lymphocytes for 48 hours is shown in figure().

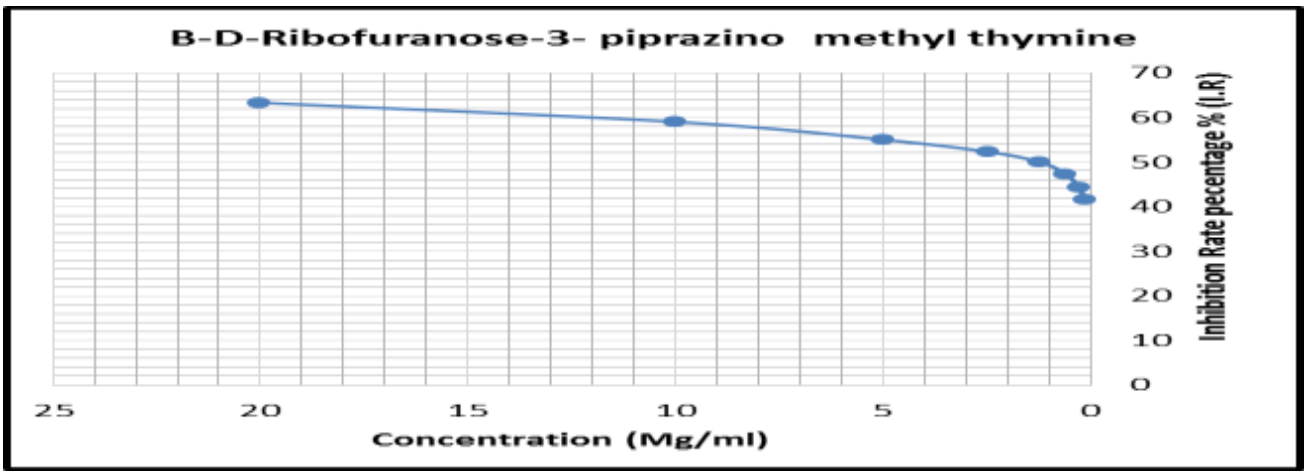

Fig 6: The cytotoxic effect expressed as the inhibition rate percentage (%IR) for different Concentrations of *β*- D-Ribofuranose -3- piprazino methyl thymine after 48 hours incubated on normal human lymphocytes

In Figure (6) also shows that concentrations used (0.31, 0.62, 1.25, 2.5, 5, 10, and 20 mg/ml) respectively that toxic effect was strong, with Normal human lymphocytes showing inhibition increased with increasing concentration that shown inhibition (41.6%, 44.3%, 47.3%, 50%, 52.3%, 55%, 59%, 63.3%) respectively. The cytotoxic effect of the(- D-Ribofuranose -3- piperidino methyl thymine), after used different concentrations in the same way as used with first and second nucleoside analogue incubate with normal human lymphocytes for 48 hours is shown in figure().

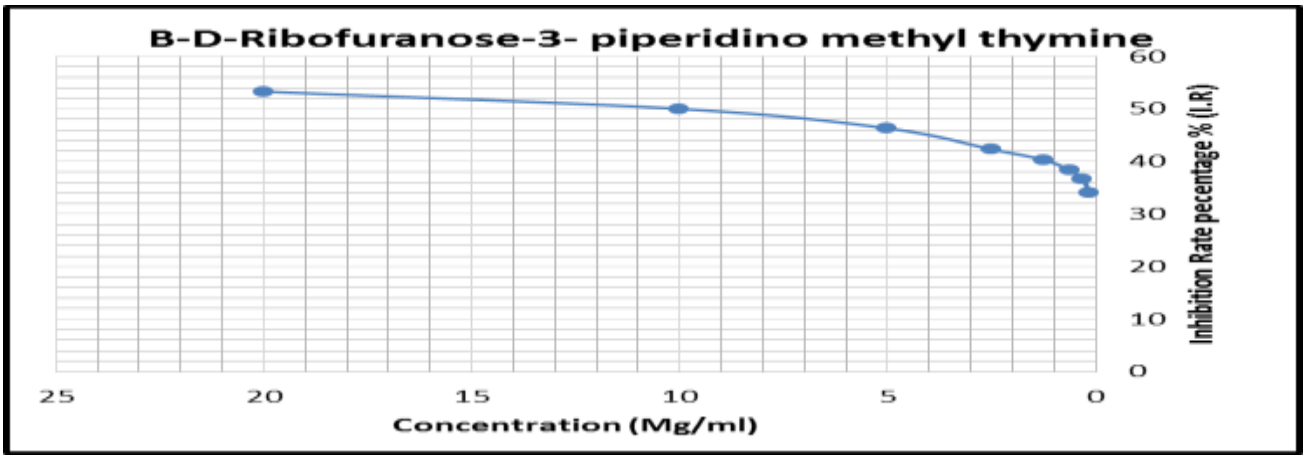

Fig 7: The cytotoxic effect expressed as the inhibition rate percentage (%IR) for different Concentrations of *β*- D-Ribofuranose -3- piprazino methyl thymine after 48 hours incubated on normal human lymphocytes

In Figure (7) also shows that concentrations used (0.31, 0.62, 1.25, 2.5, 5, 10, and 20 mg/ml) respectively that toxic effect was strong, with Normal human lymphocytes showing inhibition increased with increasing concentration that shown inhibition (34 %, 36.6 %,38.3 %, 40.3 %, 42.3%,46.3 %, 50 %, and 53.3 %)respectively Finally the cytotoxic effect of different concentrations of the(*β*- D- Ribofuranose -3- pyrrolidino methyl thymine) in same way as used with the previous nucleoside analogues incubate with normal human lymphocytes for 48 hours is shown in figure().

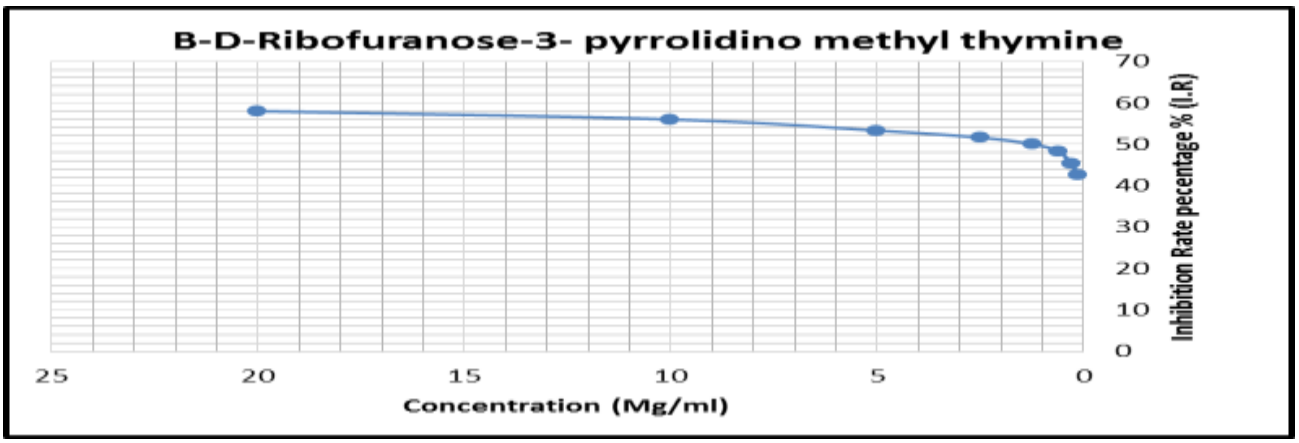

Fig 8: The cytotoxic effect expressed as the inhibition rate percentage (%IR) for different Concentrations of *β*- D-Ribofuranose -3- pyrrolidino methyl thymine after 48 hours incubated on normal human lymphocytes

In figure (8) also shows that concentrations used (0.31, 0.62, 1.25, 2.5, 5, 10, and 20 mg/ml) respectively that toxic effect was strong, with Normal human lymphocytes showing inhibition increased with increasing concentration that shown inhibition (42.6 %, 45.3 %, 48.3 %, 50 %, 51.6 %, 53.3 %, 56 %, 58.6 %) respectively. In the last four figures shown the cytotoxic effect of nucleoside analogues on the normal human lymphocyte the study detected when increase concentration of nucleoside analogues increase the inhibition rate which were used MMT assay.

The present data are in accordance with many other studies worldwide, in which different types of nucleoside analogues have been tested on many types of cell lines.

That studies shown the action of the nucleoside analogue go through an initial rate-limiting phosphorylation after that a nucleoside kinase, which leads to the presentation of a monophosphate metabolite. A next phosphorylation step is then carry out by nucleoside monophosphate kinase, and the next phosphorylation step is carry out by nucleoside diphosphate kinase.

Triphosphates can be integrated in nucleic acids, in contender with their normal coordinate, or they can prevent nucleic acid synthesis by inhibiting basics enzymes such as polymerases. Ribonucleotide reductase M1 (RRM1), a fundamental enzyme interested in nucleotide metabolism, can be suppressed both by diphosphorylated and triphosphorylated analogues.

Catabolic enzymes may decrease the amount of dynamic metabolites, including deaminases and 5′-nucleotidases. The cellular effects stimulated by nucleoside and nucleotide analogues are described [18]. When lymphocytes death ability to inhibition of immune system that is lead to raise the happening of infectious disease and tumors [19].

Conclusion

This study investigate that nucleoside analodus give rise to cell death in human lymphocytes but it want to more studies to learning main effect on the molecular mechanism to be used a therapeutic agent to treatment of immune system disease.

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