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Supervisor Certification

We certify that this thesis was prepared under my supervision in the College of Science, Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

Signature:	Signature:
Supervisor:	Supervisor:
Dr.Majed H.AL-Gelawi	**Dr.Esmail K. Shubber**
Scientific Degree:	Scientific Degree:
Assist Professor	Professor
Date:	Date:

In review of the available recommendations, I forward this thesis for debate by the examining committee.

Signature:

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Date:

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:

Name:

Scientific Degree:

Date:

(Chairman)

Signature:

Name:

Scientific Degree:

Date:

(Member)

Signature:

Name:

Scientific Degree:

Date:

(Member)

I hereby certify upon the decision of the examining committee

Signature:

Name: **Dr. Laith A. Z. Al- Any**

Scientific Degree: Assistant Professor

Title: Dean of College of Science

Date:

أ لإ هـد ا ء

إلى من عجزت الكلمات عن وصفهم

وتلاشت المعاني و العبارات عن نعتهم

إلى رمز العطاء و الوفاء

إلى ينبوع حياتي

إلى من غمروني بحبهم

وساندوني وقت شدتي

إلى من هم اعز واقرب إلى نفسي

أهلي الأعزاء ، أبنتي الغالية

و زوجي الحبيب

اهدي جهدي المتواضع

شيماء

ألخلاصة

صممت الدراسة الحالية لتقييم بعض المعايير الخلوية الوراثية وكمية الحامض النووي ألرايبي المنقوص الأوكسجين DNA لخلايا الدم المحيطي (خلايا الدم اللمفاوية) ،لدى المرضى المصابين بسرطان ابيضاض الدم النقياني المزمن ، وتضمنت اختيار عشوائي لخمسة عشر مريضا، فضلا عن خمسة عشر شخصا سويا تم استعمال خلاياهم بوصفهم مجموعة سيطرة. تم تقدير ثلاثة معايير وراثية خلوي شملت المعامل ألارومي (BI)، المعامل الانقسامي (MI)، والتشوهات الكروموسومية (CAs) وخصوصا وجود كروموسوم فيلادلفيا (Ph+ve) ، في الخلايا اللمفاوية لمستزرع الدم، وتم توضيح حساسية الخلايا السرطانية لعقارين سامين خلويا هما الكليفك، و الميثوتركسيت، وقد استعملت أربعة جرع لعقار الكليفك (0.1, 1, 10, and 100µg/ml) وجرعتين لعقار الميثوتركسيت(MTX) (12.5,100µg/ml) واعتمادا على نتائج تقييم الحساسية فقد تم تقسيم المرضى إلى ثلاث مجاميع هي ذات حساسية (S)، متوسطي حساسية (MR) ، وغير مستجيبين (R)، وكانت مدياتها ، ٤٦,٧%، ١٣,٣%، ٤٠,٠%، على التوالي.

دل تقدير معايير الوراثية الخلوي (المعامل ألارومي، المعامل الانقسامي، والتشوهات الكروموسومية) على زيادتها في المستزرعات الخلوية غير المحتوية على العقارين لدى المرضى عند مقارنتهم مع مجموعة السيطرة السالبة ،وقد انخفضت قيم هذه المعايير عند معالجة المستزرعات الخلوية بالعقارين المضادين لخلايا ابيضاض الدم النقياني المزمن والخلايا اللمفاوية.

أظهرت نتائج فعالية العقارين أعلى تأثير لها في المعايير الوراثة الخلوية، من ناحية أخرى لوحظ بأن التركيز(100µg/ml) لكلا العقارين ،يستطيع إنهاء الانحراف في المعايير الوراثة الخلوية لدى المجموعتين الحساسة ، والمتوسطة الحساسية ، على النقيض من ذلك فقد أظهرت المجموعة غير المستجيبة مقاومة للعقارين التي تمت دراستهما بتركيزهما المحددة.

وتضمن البحث دراسة كمية الحامض النووي ألرايبي المنقوص الأوكسجين لمجموعتي المرضى (الحساسة ، وغير المستجيبة) بمقارنتها بمجموعة السيطرة ، عند دراستها خلال فترات محددة لحضن المستزرعات الخلوية (٢٤، ٤٨، ٧٢، و ٩٦ ساعة) عند معالجتها بالتركيز الفاصل (100µg/ml) لعقار الكليفك. أظهرت المجموعة غير المستجيبة للعقار أعلى زيادة ملحوظة بكمية الحامض النووي المنقوص الأوكسجين في مستزرعاتها الخلوية التي تحوي العقار والخالية منها، عند مقارنتها مع المجموعة الحساسة لمرضى ابيضاض الدم النقياني المزمن ، ومجموعة السيطرة.

الخلاصة

نستدل من هذه الدراسة، أن معايير الوراثة الخلوية (المعامل ألارومي (BI)، المعامل الانقسامي (MI)، والتشوهات الكروموسومية (CAs) وخصوصا وجود كروموسوم فيلادلفيا (Ph+ve)) وسيلة مناسبة لمعرفة مدى استجابة المرضى ومقاومتهم للعقار وطريقة علاجهم المثلى. فضلا عن ذلك تعد زيادة الحامض النووي الرايبي منقوص الأوكسجين في مستزرعات خلايا الدم اللمفاوية هي إحدى العلامات الواضحة لمقاومة العقار.

	List of Abbreviations
5-Brdu	5-Bromodeoxyuridine
abl	Abelson's mouse leukemia virus
AIDS	Acquired immunodeficiency
ALL	Acute lymphocyte leukemia
AML	Acute myelogenous leukemia
AP	Accelerated phase
ATP	Adenosine triphosphate
bcr	Breakpoint cluster region gene
BI	Blastogeneic index
BP	Blast phase
BUS	Busulfan
CAs	Chromosomal aberrations
CLL	Chronic lymphoid leukemia
CML	Chronic myeloid leukemia

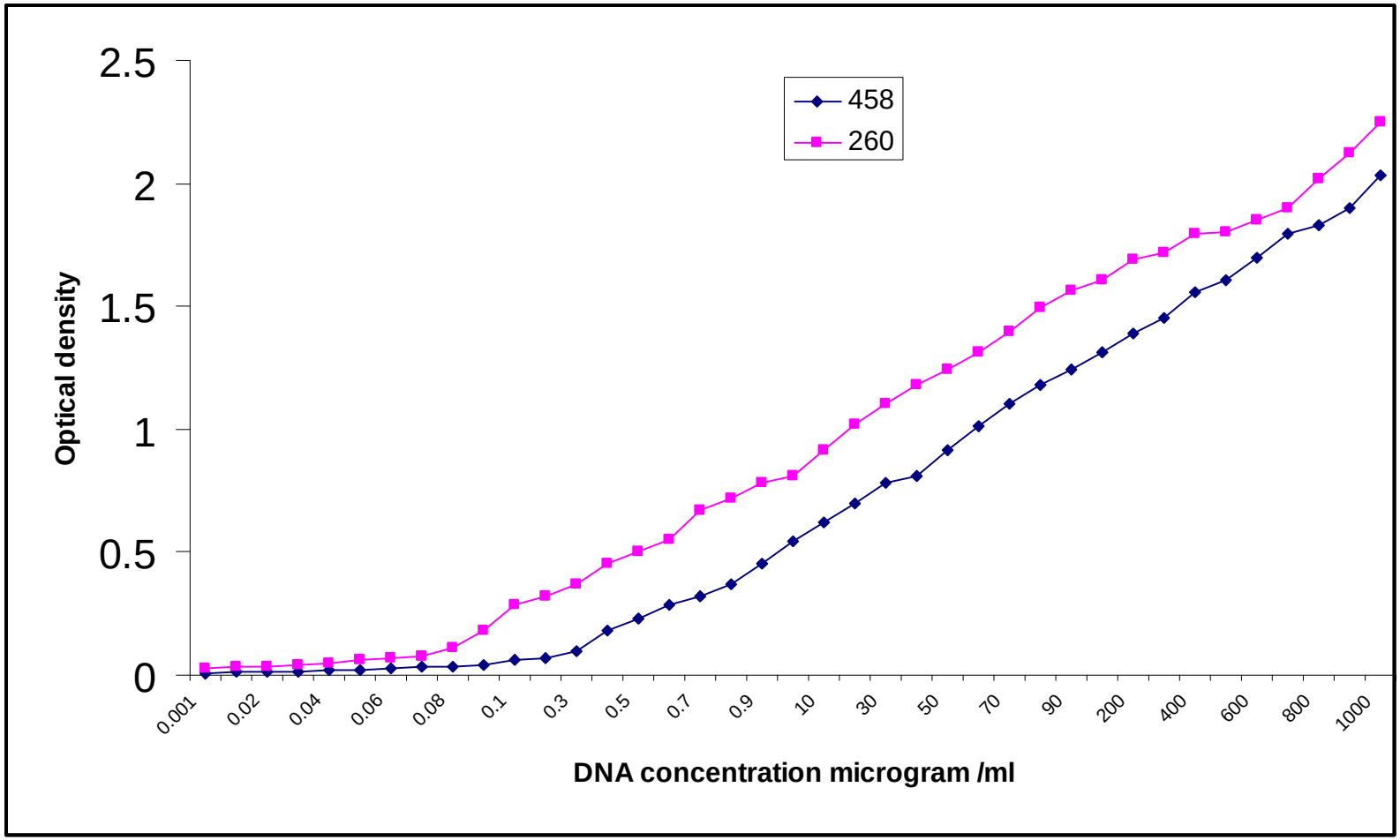
cmm	Cubic millimeter
CP	Chronic phase
DHFR	dihydrofolate reductase
DMs	amplified genes Double –minutes
DNA	Deoxyribonucleic acid
FDA	Food and Drug administration
FISH	In Situ Hybridization
G1	first gap phase
HIV	Human immunodeficiency virus
HSRs	Homogeneously staining regions
HTLV-I	Human T-cell leukemia virus I
HU	Hydroxyurea
INF-α	Interferon–alpha
MI	Mitotic index
MR	moderately resistance
MTX	Methoterxate

SCT	Allergenic stem cell transplantant
-ve	Negative
Ph	Philadelphia chromosome,
PBS	Phosphate buffer saline
PHA	-phytohaemoaglutinin
+ve	Positive
RQ-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
R	resistance
G0	Resting gap phase
RT-PCR	Reverse–Transcription Polymerase Chain Reaction
S	sensitive
G2	The second gap phase
S phase	The synthesis phase
WBCs	White blood cells
KCI	Potassium chloride

NaCl	Sodium chloride
HCI	Hydrochloric acid
$NaHCO_3$	Sodium Bicarbonate
Na_2HPO_4	Disodium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate

List of content

Figure (3-5):- Standard curve of DNA (thymus calf DNA) obtained at wave length 458nm, 260 nm



3-1 Drug Response

In order to evaluate the response of blood lymphocytes obtained from Chronic Myeloid Leukemia (CML) patients, the genotoxic effect of the two anti-cancer drugs (Gleevec, MTX) were tested, and the methods are presented in section (2-6-1). It is clearly showed that a significant reduction in BI, MI, CAs, were seen as a function of a drug concentrations (i.e. Concentration-dependent effect).

Accordingly the following concentrations (0.1, 1, 10, and 100 µg/ml of gleevec and 12.5,100 µg/ml of MTX) were selected as effective concentrations for further investigations in the study. To picture the type of response, a statistical was adopted, and this was previously described by Al-Murrani *et al,* 2001.

According to this method, the patients were divided in to three groups. They were sensitive (S), moderately resistance (MR), and resistant (R).The R group included patients whose lymphocytes showed continuous blastogenesis and mitosis with all concentrations of the drugs. The S and MR groups were defined according to the concept of confidence interval (upper and lower limits).

The lower limit was considered as a border line to separate the patients in to S and R groups. All patients who were below the border line were considered S, and those were above the border line were considered MR.

The results of such grouping are presented in table (3-1)

Out of the (15) CML patients, 7 (46.7%) were S, MR patients, were 2 (13.3%), while the R patients accounted for 6 (40.0%).

The patients sex distribution of these groups also presented in table (3-1).

Table (3-1) observed numbers and percentage frequentness of CML groups distributed by sex. (P>0.05)

Patient groups	Total no.	100%	Male no.	100%	Female no.	100%
Sensitive(S)	7	46.7	2	28.6	5	62.5
Moderate resistant(MR)	2	13.3	1	14.3	1	12.5
Resistant(R)	6	40.0	4	57.1	2	25.0
Total	15	100.0	7	100.0	8	100.0

3-2-Heamatology analysis

Total and differential account of leukocyte in the control (healthy untreated individual) and CML patients were presented in table (3-2) as a (mean ± SE).

The total account of white blood cells (WBCs) in table (3-2) showed a significant differences (P<0.05) between control (8647 ±294) and CML patients (15966±533). The overproduction of WBCs in CML patients that Result from a disorder in puriptential heamopoietic stem cells (Goldman *et al*, 1999) (Sattler *et al*, 1999)

Differential account of WBCs in both control and CML patients give significant result (P<0.05)

In neutrophils (8428±261 *vs.* 4786±66), lymphocytes (4748± 176 *vs.* 3384± 132) and basinophils, esoniophils as (10.71±0.5 *vs.* 12.67±0.2; 65.43±2.5 *vs.* 50.43±1.9) respectively. This result occurs under the normal range of differential WBCs counted (Greenberger *et al*, 1983; Verfaillie *et al*, 1997)

Table 3-2: Total and differential count of peripheral blood leukocyte in CML patients and control as a (mean ± SE) P<0.05

WBCs / Group	Total WBCs count	Neutrophils	Lymphocyte	Monocyte	Basophils	Eosinophils
Control	8647 ±294 a	4786±66 a	3384±132 a	379.5±54 a	12.67±0.2 a	65.43±2.5 a
CML patient	15966±533 b	8428± 26 b	4748±176 b	2695.2±184 b	10.71±0.5 a	50.43±1.9 a

(a, b) sample of significant differences between row

While in monocytes are presented in table (3-2) showed a significant increase (P<0.05) of CML patients and control from normal range about (18 – 25 % vs.2 – 8 %*)* (Seeley et *al*, 1998)

Increased monocytes in CML (2695.2 ± 184) instead of (379.5 ± 54) in control because the change alteration of genetic material in stem cells lead to change the proliferate and population of differentiated cells that a greatly expanded total myeloid mass (promyelocytes, myelocytes and metamyelocytes) (Mauritzson *et al*, 1999)

3-3-Cytogenetic parameter

Three cytogenetic parameters were used to evaluate the cytogenetic background of CML patients (S, MR, and R) and control before treating their lymphocytes with antileukemic drugs (gleevec and MTX). They were BI, MI, CAs and the most important CAs Ph+ve chromosome. The results are presented in table (3-3)

The control value of BI was (40.92±2.0), while in total CML patients BI was (35.15±2.5). This result showed a similar index in S group (32.01±2.0) and MR group as (34.00 ± 4.0). The R group also demonstrated a significant increased level of BI than S and MR groups (40.70 ±4.8) and near to BI of control. These BI value differences have non-significant level (P>0.05) for control and CML patients (total, groups).

The mitotic index (MI) between groups has non-significant level (P>0.05) for control and CML patients (total and groups), these result illustrated in table (3-3), when MI of control was observed (3.21 ±0.4), MI of total CML patients was (2.97 ± 0.26), while in S, MR, and R CML patients groups are (2.50 ±0.4; 2.00 ± 0.5 ; 3.77 ± 0.2) respectively.

Chromosomal aberrations (ring, gap, acentric, dicentric, chromatid break, chromosomal break, deletion and Ph+ve chromosome) of lymphocyte obtained from CML patients were higher than the corresponding value in control (1.634 ± 0.13 vs. 0.328 ± 0.03), such increased was more pronounced in the R groups (2.275 ± 0.04), and MR, S, CML patients (1.350 ±0.01; 1.188 ± 0.09) respectively. These differences were reached to significant level (P<0.05) corresponding to control.

Philadelphia chromosome, although it was observed in the total CML patients, while this defect not detectable in control (0.141 ± 0.03 vs. N.D.). These differences reached to a high significant level (P<0.05). However, the deviation maintained a significant level increased in the S group (0.04 ±0.00), and R group (0.300± 0.01), while non-significant level was observed in MR group (N.D.), when compared to control because not detectable Ph+ve chromosome.

The forthcoming analysis aimed to evaluate the cytogenetic backgrounds of control as well as CML patients before evaluating their response to (gleevec, MTX).

It was clear that the genetic make-up of CML has undergone some modification and such consequences may lead to establish the leukemic disease. It is always augmented that leukemia requires a genetic defects (mutation, translocation) before establishing a disease (Weisberg *et al,* 2000; Brandford *et al*, 2002).

The CAs results support the latter view, which was significantly increased in the patients. This parameter gives a good picture about disorder background of any genetic make-up.

A further evidence supporting such surplus may be observed in patients with breast cancer (Ahmed *et al*, 2006), and prostate cancer (Ghali *et al,* 2006), in which the rate of CAs were significantly increased in the patients.

The present results may clear also the importance of CAs in patient's resistance to gleevec and MTX. It was significantly progressed in MR and R patients. Therefore, the resistance may have a genetic background (i.e. mutation, gene amplification, and translocation) (Belloc *et al,* 1997; Kuo *et al*, 1998).

The Ph+ve chromosome may confirm such conclusion for its importance in patient's resistance to gleevec, and it was significantly observed in R patients (Ellen *et al,* 2000; Kasakayan *et al,* 2003).

3-4-Cytogenetic analysis of drug-treated lymphocytes cultured with PHA

3-4-1- Blastogeneic Index (BI)

The result of BI (mean±SE) of CML patients (S, MR, and R) and control in their response to four different concentrations of gleevec (0.1, 1, 10, and 100 µg/ml) and two concentrations (12.5, 100 µg/ml) of MTX drug are presented in table (3-4)

The two drugs reduced the blastogenic transformation of lymphocytes obtained from CML patients, and control. The degree of reduction was dependent on the drug concentrations and patients group (S, MR, and R)

A concentration (0.1µg/ml) of gleevec reduced the BI in the control to (37.98±1.7), and the reduction was observed in CML patients (32.02±2.5).This reduction has non-significant level ($P>0.05$). The S, MR, and R patients had BI value (31.14±1.5; 31.20 ± 0.04; and 36.60 ± 4.5) respectively.

Much more reduction was observed in the concentration (1µg/ml) of gleevec, especially in S patients (26.93 ± 1.7). This reduction was obtained in MR, R (30.00 ± 3.00; 32.96 ± 4.0 respectively), these differences not reached a significant level ($P>0.05$). The similar level was observed of BI in total CML patients (29.56± 2.2) compared to control (35.68 ± 1.78)

At a concentration (10µg/ml) gleevec showed a significant increased ($P< 0.05$) of BI in CML patients (25.42±2.1) compared to control (32.79 ±1.5). The higher reduction was observed in S patients (21.77± 1.8), while low reduction was obtained in MR patient (28.30± 3.0), and R (29.80 ± 3.0).

The MR patients (27.50±2.0) and R (29.60±4.0) were showed a low reduction in BI at a concentration (100 µg/ml) of gleevec, while the S patient and total patients were observed have a higher reduction in BI (20.75±1.8;

Table 3-4: Blastogenic index (BI) of lymphocyte obtained from CML patients and control after treatment with the drugs (gleevec, MTX), results showed as (mean± SE)

Drugs	Concentration (µg/µl)	Control mean± SE no.(15)	Patient no.(15) mean± SE			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	40.920±2.00 a	32.013±2.00 a	34.000±4.00 a	40.700±4.80 b	35.150±2.50 a
	0.1	37.980±1.70 a	31.140±1.50 a	31.200±0.04 a	36.600±4.50 a	32.027±2.50 a
	1	35.687±1.78 a	26.463±1.70 a	30.000±3.00 a	32.967±4.00 a	29.567±2.20 a
	10	32.793±1.50 a	21.775±1.80 B	38.300±3.00 ab	29.800±3.00 ab	25.420±2.10 b
	100	30.187±1.08 a	20.750±1.80 a	27.500±2.00 ab	29.600±4.00 ab	24.470±2.20 b
MTX	0.0	40.920±2.00 a	32.013±2.00 a	34.000±4.00 a	40.700±4.80 a	35.150±2.50 a
	12.5	33.580±1.90 a	27.912±1.50 a	28.500±2.00 a	35.333±4.00 a	30.920±2.00 a
	100	29.853±1.90 a	22.075±1.30 a	26.000±3.40 a	27.717±4.00 a	24.593±2.00 a

(a, b) sample of significant differences between columns

24.74± 2.2) respectively. These result has a significant level (P<0.05) when compared with control (30.18±1.08).

The blastogeneic response of blood lymphocytes grown in presence of MTX for (72 hr) was also reduced. Blastogeneic index value of control and patients at the two concentrations of MTX did not reach a significant level (P>0.05). A concentration (12.5 µg/ml) of MTX reduced the BI in CML patients S, MR, (27.91±1.5; 28.50±2.0 respectively), when compared to control (33.58±1.9). This reduction was also observed in R patients (35.33 ±4.0)

At the concentration (100 μg/ml) of MTX the higher reduction of BI in CML patients total (24.59±2.0) as in groups (S, MR, and R) when obtained after lymphocytes treated (22.07±1.3; 26.00 ±3.4; and 27.71±4.0) respectively.
The same reduction was obtained in control (29.85 ± 1.9).

As a general view, all groups of patients showed blastogenic resistance to drugs, and the resistance was clear in gleevec (10, and 100 μg/ml). The blastogenic transformation after stimulation with PHA is normally occurred, because PHA (mitogen) is able to cross-link the lymphocyte receptors, and induce their transformation (Roitt *et al,* 1998)

McCowage and Colleagues (1996) have demonstrated that the anticancer drugs may cause abnormalities in lymphocytes receptors involved in mitogen recognition; such effect may result in an inhibition of BI.
Resistant CML patients may develop some mutations that render their cells resistant to the drug; those patients have some gene associated with cell-cycle regulatory at several checkpoints, thereby affecting cellular proliferation (Iqbal *et al,* 2004).
Increased level of BI supports the latter view, which is in agreement with some recent investigations carried out in some malignancies (Darwesh M.F. *et al,* 2002).

3-4-2 Mitotic Index (MI)

The result of mitotic index (mean±SE) of CML patients (S, MR, and R), and control in their response to four different concentrations (0.1, 1, 10, and 100 μg/ml) of gleevec and two different concentrations (12.5, 100 μg/ml) of MTX, are presented in table (3-5)
The mitotic response of lymphocyte obtained from the patients and control exhibited a similar manner, in which, a reduced level of MI was observed

when the drug concentration was increased. However, some significant increased were noticed, and this was dependent on concentration of drugs, type, and patient groups (S, MR, and R).

At a concentration (0.1μg/ml) of gleevec was induced a significant reduction in MI but, not reached to significant level, in both control and patients (2.513±0.4; and 2.520± 0.15) respectively. In S patients was reached to (1.938±0.2), and similar significant level of reduction obtained for MR (1.400±0.2), and R patients (3.483±1.7)

For (1μg/ml) concentration of gleevec reduced the MI to (1.288 ±0.19) in S CML patients, and such concentration have the same effect on MR patients (1.100±0.05), and R group of CML patients (3.017±0.5). However, these differences were significant (P>0.05). MI was also reduced in control (2.073 ± 0.3) when compared with CML patients (1.967 ± 0.18).

A concentration (10 μg/ml) of gleevec reduced cell division at significant level (P<0.05) in R patient, it was had a low value of reduction (2.650±0.5) when compared to MR (0.5000 ±0.05), S patients (0.362 ± 0.18), and control (1.067±0.30).

Mitosis could not be detected at a drug response when treated with (100μg/ml) concentration of gleevec in each of control, and (S, MR) CML patients. The R group of CML patients reached a significant level (P<0.05) when it had a value (2.233±0.70).

For MTX at (12.5 μg/ml) concentration of drug, higher reduction of MI observed in control (0.560±0.09), and S patients (0.362±0.09). Moderate reduction was obtained at MR group (0.800±0.2) while low reduction of MI was observed in R group of CML patients, when it had a value (2.016±0.2). These differences had a high significant level (P<0.05).

At concentration (100 μg/ml) of MTX no detectable mitosis in control and (S, MR) groups of CML patients was observed. These results have a higher significant level ($P<0.05$) when compared with the MI reduction in R patients (0.588±0.07).

The forthcoming presentation clearly demonstrated that the patients groups have different response for drug effect. The resistance following sequence; R > MR > S, and each drug have specific effect to CML patients, this is interpreted by the fact each drug has a different mechanism of action, as well as, each drug may effect the cell-cycle at different stage (Vernol *et al*, 1988). For that the drug-related difference in mitotic response could be occurred by these factors.

Therefore, related cells did escape from the pro-apoptotic effect of gleevec and able to establish a mitosis of cells that can grow continuously in the presence of specific concentration. It is interesting to observed that, although these resistant cells are not killed by the tyrosine kinase inhibitors, and developed its resistance may be resulted by overexpression of multi-drug resistance protein, or by mutation (Fańcois *et al*, 2000)

On other hand, it was stated that one cause of MTX resistance in living cells result from DHFR gene mutation, as well as, in other genes such as ***p53,*** which are usually associated with DHFR gene amplification (Shubber et *al,* 2003).

Moreover, resistance is possible to ascribe for the transformation of proto-oncogenes to oncogenes in side the malignant cells (Weaver *et al*, 1989), such as reciprocal translocation in Ph+ve cells. Increased levels of Ph+ve cells in the CML patients, especially the R group, are in agreement with this concept.

3-4-3-Chromsomal aberrations (CAs)

The result of chromosomal aberrations (CAs) (mean ±SE) of CML patients (S, MR, and R) and control in their response to four concentrations of gleevec (0.1, 1, 10, and 100μg/ml) and two different concentrations (12.5 and 100μg/ml) of MTX are presented in table (3-6)

The type of chromosomal aberrations which have been observed are ring, gap, acentric, dicentric, chromatid break, chromosomal break, deletion and Ph+ve chromosome were showed in figure (3-1)(3-2)(3-3)(3-4). And the results of these chromosomal aberrations were presented in appendix C.

As shown for the parameters BI, MI, the CAs values were dependent on the type of drug, the concentration and the group of patients(S, MR, and R).

For gleevec (0.1μg/ml), the significant level ($P<0.05$) was observed in CAs value of CML patients when compared with control (1.435±0.16 *vs.* 0.206±0.03). These had similar manner obtained in the CAs value reduction in S patients (0.958±0.1), and MR patients (1.058±0.01), when compared with R patients (2.185±0.09).

Chromosomal aberrations were reached a higher significant level ($P<0.05$) at (1μg/ml) (0.098±.01) in control compared with R patients (2.065±0.1). Sensitive patient had a high CAs reduction when treated with this concentration (0.657±0.1); these results were observed in MR group (0.910±0.00) too. These two results get a high significant level ($P<0.05$)

At a concentration (10 μg/ml) of gleevec, low reduction of CAs observed in R patient (2.030±0.13), and MR patient (0.720±0.01). While a higher level of CAs reduction observed in cells from S patients and control (0.290± 0.01; 0.005±0.00) respectively, that showed a higher significant level ($P<0.05$)

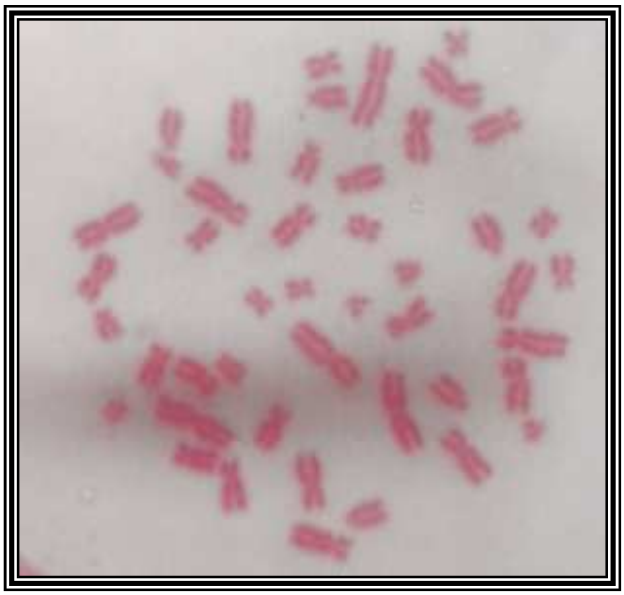


Figure (3-1) Normal chromosome in metaphase of human blood cells from human lymphocyte culture under (1000X).

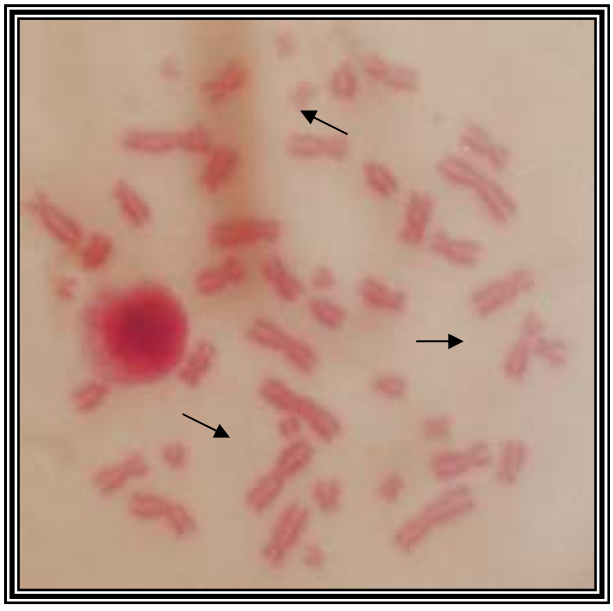


Figure (3-2) Chromosome in metaphase of blood cells from human lymphocyte culture containing (a-Gap, b-Ring, and c-acentric) under (1000X).

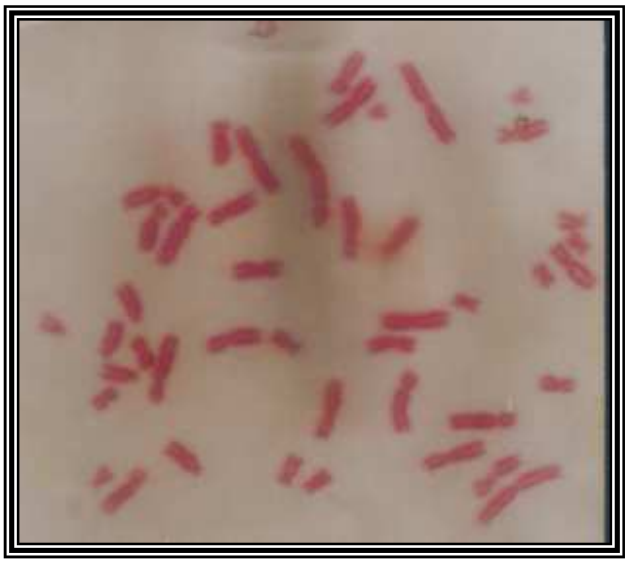


Figure (3-3) Chromosome in metaphase of blood cells from CML patient lymphocyte culture (Ph-ve chromosome) under (1000X).

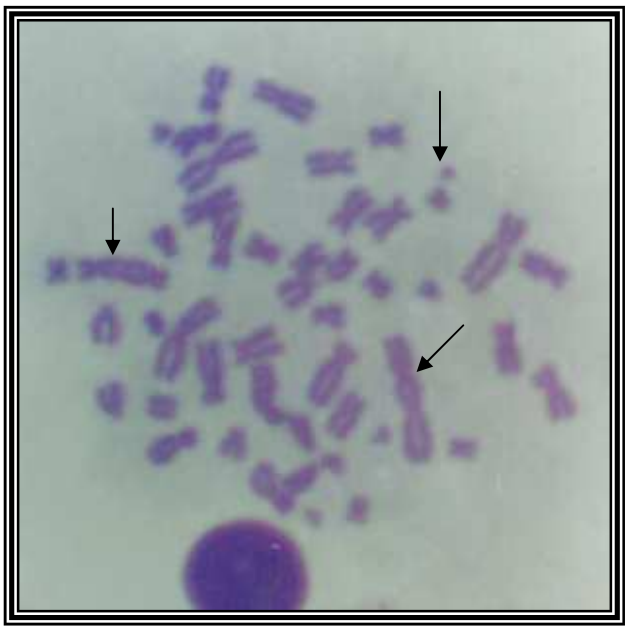


Figure (3-4) Chromosome in metaphase of blood cells from CML patient lymphocyte culture (Ph+ve chromosome) containing dicentric chromosome under (1000X).

Chromosomal aberrations were not detected in (S, MR groups) of CML patients and control at (100 μg/ml) concentration of gleevec because no metaphase found at this concentration. Chromosomal aberrations had a higher significant level ($P<0.05$) when compared R patient (2.063±0.18) with last results.

In MTX drug at (12.5μg/ml) concentration, high CAs reductions observed in each of S patients (0.016±0.01), MR patients (0.930±0.01), and control (0.300±0.05), while low reduction value of CAs in R patients group (2.126±0.17). All these results reached to the significant level ($P<0.05$). The same significant level observed in (100 μg/ml) of MTX treated lymphocytes. Chromosomal aberrations could not be determined in each of control and (S, MR) groups of CML patient, because no metaphase could be detected in this concentration, except in R patients which reached to significant level ($P<0.05$) when had a value (1.891±0.04).

Chromosomal aberrations were found in CML patients a result of genetic disorder in stem cells at (80%) of BP, AP of CML patients, but in few not all patients of CP CML patients (Watmore *et al,* 1985; Hagop *et al,* 2002 B)

Other reason the overproduction of WBCs in those patient lead to development of chromosomal abnormalities other than Ph+ve chromosome (Christina *et al,* 2002)

DNA damaged of cells and mistake in repaired system at any stages of cell cycle, and escaped the programmed cells death and apoptosis lead to trigger chromosomal abnormalities (Bonthron *et al,* 1998)

In sensitive response (S patients and control), and moderate resistance (MR) to drug for inhibition activities explained the reduction in cell proliferation as well as abnormalities in chromosomes, that reduced as a result of

increased programmed cell death (Paul *et al,* 2004), and DNA- repair system (Mohammad *et al,* 2004)

Resistance patients to gleevec and MTX are also depended on the presence of factors that inhibit apoptosis such as mutation in ***p53*** gene product, absence of retinoblastoma gene, increase the expression of ***bcl-2*** gene. These lead to inhibiting programmed cell death (Bertino *et al,* 1996)

In other hand, resistance may develop through several mechanisms lead to reactivation leukemic cells such as point mutation, gene amplification, or overexpression as well as multidrug resistance (Kuo *et al,* 1998)

Moreover patients with chromosomal aberration may be lead to develop resistance to anti-cancer drug such as deletions have a specific prognosis and drug resistance lead to disease relapse when compared to CML patients without this aberrations (Huntly *et al,* 2001)

3-4-4-Philadelphia chromosome (Ph+ve chromosome)

The results of Ph+ve chromosome (mean± SE) of CML patients (S, MR, and R) and control in their response to four different concentrations of gleevec (0.1, 1, 10, and 100 µg/ml) and two concentrations (12.5, 100 µg/ml) of MTX are presented in table (3-7) and as shown in figure (3-5)

When Ph+ve chromosome is the most important chromosomal aberrations in CML patients, and the chosen drug gleevec has a mechanism of action on the production of this abnormalities, while MTX used as indicator for mechanism of drug resistance. Explain it's effective by drug alone.

First Ph+ve chromosome could not be detected in control and MR group of CML patients in untreated lymphocytes culture cells and after these cell will be treated. That mean the normal healthy individual not have this type of chromosomal aberrations (reciprocal translocation) while in MR patient were when tested not be detected Ph+ve chromosome ascribed to either these patients had Ph-ve chromosome, but they had *bcr-abl* gene activity, or my them were treatment with gleevec after diagnosis acquired major, or complete cytogenetic response (Van *et al*, 1989; Richard *et al*, 2004), or may be the low number of patient in this group in our investigation. If we increased number patients may lead to observe this CAs.

Number of cells with Ph+ve chromosome was reduced as a function of drug types and concentrations. Significant reduction ($P<0.05$) of those cells was observed in groups after treatment with gleevec at (0.1 μg/ml) concentration in S patients which had a value (0.020±0.00) when compared with R group (0.270±0.04). The same significant reduction of those cells was observed at gleevec concentration (1 μg/ml) when S, and R groups of CML patients had a value (0.0012±0.00; 0.2850±0.05) respectively.

High significant level reach to ($P<0.05$) observed at (10μg/ml) concentration of gleevec in S group which had a value (0.00±0.00) when compared this result with R group was (0.255±0.04).

At (100 μg/ml) concentration of gleevec, Ph+ve chromosome cells could not be determined because metaphase could not be detected at this concentration. While R groups of CML patients reached to significant level ($P<0.05$) when had a value (0.286±0.05).

For MTX, low reduction of Ph+ve chromosome was observed in S group (0.026±0.01), and R group (0.243±0.05) of CML patients at (12.5 μg/ml), these result reached to significant level ($P<0.05$).

At (100μg/ml) concentration of MTX, Ph+ve chromosome could not be detected in S group because no mitosis was detected. R patients were had a significant level ($P<0.05$), R value was (0.215±0.05).

Reduction of Ph+ve chromosome in S patients when treated with gleevec that ascribed to the mechanisms action of drug lead to acquire complete cytogenetic response. Further evidence supporting such surplus may be observed in CML patients (Talapz *et al*, 2001; AL-Shummary *et al*, 2006)

Resistance patients (R patient) to gleevec and permit have a Ph+ve chromosome in their cells after treatment may develop through several mechanisms, mutation in ATP-binding site that prevent imitinib to recognize it (Shu *et al*, 1990; Shu *et al*, 1994). Or *bcr-abl* gene amplified or overexpression of protein leads to resistance (Gorre *et al*, 2001)

For MTX resistance in lymphocyte culture cells of CML patients that resulted by the mechanisms of MTX action be dependent on the origin of cell and on the enzyme activity (DHFR) (Mclvor *et al*, 2002; Colin *et al*, 2002)

The low reduction in Ph+ve chromosome may resulted by the action of MTX when stopped the cells at the S and G1 phase of cell-cycle and these cells under gone apoptosis(Nizami *et al*, 2001)

3-5-Cytogenetic analysis of Drug-treated lymphocyte cultured without PHA

3-5-1 Blastogeneic Index (BI)

The result of BI of control and CML patient cultured with out PHA was presented in table (3-8) and their response to different drug concentrations (0.1, 1, 10, and 100 μg/ml) of gleevec and (12.5, 100μg/ml) of MTX.

Blastogeneic Index of control not obtained when cultured lymphocyte cells from healthy individuals without PHA. MI not observed in all patients and control when cultured without PHA.

Blastogenic index (BI) of CML patients lymphocyte was compared when culture with and without PHA, the result shown in table (3-8) have a high significant level ($P<0.05$) in two type of drugs and at all concentrations, due to the high level of reduction was observed. Blastogeneic Index was obtained from lymphocytes cultured with mitogen give high value when compared it with culture without mitogen, these results were ascribed as the leukemic cells have decreased cell death as a primary mode of increased cell proliferation, due to genetic changes resulting in loss of programmed cell death and loss of the ability to regulate the cell cycle progression (Mosmann *et al,* 1983; Kastan *et al,* 1995). Therefore normal blood cells showed no growth when cultured without PHA while leukemic cells exhibited high growth rate.

3-7-DNA Estimation

3-7-1.Standard curve of DNA

Standard curve of DNA (μg/ml) (Thymus Calf DNA) is presented in figure (3-5). One of this standardization obtained at 260 nm of spectrophotometer, and other one obtained from 458 nm of spectrophotometer with UV light.

3-7-2.DNA measuring by fluorescence method

The DNA concentration (μg/ml) and cells accounted (1 x 10^5 cells/ml) result (mean±SE) of CML patients (S, R) and control in their response in the absence and presence cut-off concentration (100μg/ml) of gleevec, when cultured under different time, are presented in tables (3-9) (3-10).

As shown for the cytogenetic value, the DNA concentration is dependent upon the drug concentration.

In tables (3-9) (3-10) lymphocyte cell cultured with PHA in absence of gleevec in each of control, and CML patient(S, R), for different period of incubation (24, 48, 72, and 96 hr) that induced significant level of differences reached to ($P<0.05$).

DNA concentration (μg ml) increased with time when observed a result of DNA concentration at 24 hr period of incubation in control (0.426±0.01), S patients (0.532± 0.02), and R patients (1.003± 0.08), while this concentration increased approximately 3 time in S, R, groups of CML patient at 96 hr (6.000±0.00; 6.667± 0.05) respectively, but increased one time in control (0.960±0.03)

The results of lymphocyte cultured with PHA in presence of cut-off concentration (100μg/ml) of gleevec, showed a high reduction in DNA concentration in each of control and S group of CML patient while still

cells proliferate and DNA synthesis in R patients, At different period of incubation.

The DNA concentration of control and S patients (0.458±0.03; 0.582±0.03) respectively at (24 hr) period of culture incubation, these results were reduced when cultured reached (96 hr) of incubation to obtain a value (0.105 ±0.01) for control and (0.280± 0.01) of S patient. These gave a higher significant reduction value reached to ($P<0.05$) when compared with R group of CML patients, it was obtained value of DNA concentration at (24 hr) (0.573±0.05) ,while it was increased to reach a value of (3.260±0.20) at (96 hr) period of incubation with presence of gleevec (100µg/ml) .

As a general review sensitive behavior of control and S patient to gleevec, it was ascribed to the drug mechanism of action when inhibition growth rate (Carroll *et al*, 1997), DNA synthesis regulation in cells and induced apoptosis (Druker *et al*, 1996; Yuan *et al*, 1997; and Apperley *et al*, 2002)**.**

Resistance of R group of CML patients to gleevec, it was ascribed to several mechanisms of drug resistance (*p53* mutation, gene amplified, mutation, and multidrug resistance (Compos *et al,* 1992; Kipreos *et al,* 1992; and Bedi *et al,* 1999), or by the *bcr-abl* fusion gene properties for inhibition apoptosis of DNA-damaged cells or increased proliferate of cells by induction of growth factor genes (Bide *et al,* 1995; Slupianek *et al,* 2001)

3-7-3.Comparison of methods for DNA estimates

The result of DNA concentration measured spectrophotometricaly at 458 nm when direct estimation in fluorescence method and at 260 nm of DNA extracted from cells and then measured its absorbance, when cultured for (72 hr) with cells accounted are presented in table (3-11)

Table (3-11) Comparison method of DNA estimated, Cells accounted as (mean×10^5 cells/ml ± SE) and DNA concentration as (mean× µg / ml ± SE) at (72 hour) of incubation

Type of method		Gleevec concentration (µg / ml)	Control	Patients
DNA extraction method	DNA	0	0.730±0.03 a	2.680±0.90 a
		100	0.228±0.02 a	0.820±0.04 a
	Cells	0	7.800±2.50 a	30.100±4.30 a
		100	2.380±0.90 a	9.160±2.10 a
DNA plus fluorescence method	DNA	0	0.776±0.03 a	3.094±0.05 a
		100	0.185±0.02 a	0.994±0.05 a
	Cells	0	8.020±2.30 a	32.80±4.10 a
		100	1.900±0.80 a	11.52±4.30 a

This result obtained when lymphocytes cultured with PHA in presence and absence cut-off concentration of drug (100µg/ml) for 72 hr of incubation.

These result was gave by matching the number of CML patients and control, the period of incubation, and the drug concentration for get the optimal method (easy, rapid, simple) in our study for DNA estimated.

At each kind of methods cells were accounted and matching with DNA concentration.

Direct method, DNA concentration had a value (0.185±0.02) for control, when cells account had a value (1.900±0.80) and the DNA concentration for patients (0.994±0.05) when cells account had a value (11.52±4.30) in presence of drug. These result not reached a significant level ($P<0.05$), when compared with DNA concentration measured after extraction from cells (DNA extraction method) when a value (0.228±0.02) in control, when cells accounted (2.380±0.90) and (0.820±0.04) of patients when cells accounted was (9.160±2.10).

DNA concentration of control and CML patients in absence of drug had a value (0.776±0.03; 3.094±0.05) respectively when cells accounted for control and CML patients had a values (8.020±2.30; 32.80±4.10) respectively, in fluorescence method no reached significant level when compared with extraction method when DNA concentration of control and CML patients had a value (0.730±0.03; 2.680±0.90) respectively. The cells account for each of control was (7.800±2.50) and for patients were (30.100±4.30). The DNA concentration in each method not reaches significant level, these result was supported with other investigation such as (Sutcliffe *et al*, 1970)

Fluorescence yield from Hochest-DNA complex is strongly dependent on the composition and structure of DNA. Hochest fluorescence is higher in AT-rich DNA than in CG-rich DNA (Mordy *etal,* 1991).

Extraction of DNA from cells and then measured its concentration has many difficult, such as excessive shearing the DNA molecules during handling, or companied with denatured, damaged and hydrolyzed DNA (Dell`anno *et al,* 1998).

In this study, suggested to use simple, rapid, easy method for DNA measuring (Hochest-DNA complex) for crude homogenized lymphocytes culture when compared with DNA extraction.

These results support other investigation such as (Labarca *et al*, 1980) (Leff *et al*, 1995).

2-1- Equipments and apparatus:

The following equipments and apparatus were used in this study.

Equipments and apparatus	Company
Autoclave	Gallenkamp (England)
Balance	Ohans (France)
Cold incubator	Gallenkamp (England)
Compound light microscope	Olympus (Japan)
Distillator	Gallenkamp (England)
Electric oven	Gallenkamp (England)
Freezer	Eshtar (Iraq)
Hemocytometer	Witeg (Germany)
Hotplate with magnetic stirrer	Gallenkamp (England)
Laminar air flow	Hermal labortechnik(Germany)
Micropipette	Witeg (Germany)
Ph-meter	Orien research(USA)
Portable centrifuge	Hermal labortechnik(Germany)
Refrigerated centrifuge	Sanyo(UK)
Refrigerator	Eshtar (Iraq)
Sensitive electric balance	Metler (Switzerland)
Shaker Incubator	GFL (Germany)
Spectrophotometer	VWRcompany-Branson(USA)
Spectrophotometer with uv light	Hermal labortechnik(Germany)
Ultrasonic corporation	VWRcompany-Branson(USA)

Vortex	Buchi (Switzerland)
Water path	Gallenkamp (England)

2-2- Chemical Material

The following chemical materials were used in this study and its company

MATERIAL	ORIGIN COMPANY
5-Bromodeoxyuridine(5-Brdu)	BDH (England)
Absolute Methanol	Fluka (Switzerland)
Antibiotic(Streptomycin, Penicillin)	Samara drug factory(Iraq)
Calf thymus DNA	Fluka (Switzerland)
Chloroform	BDH (England)
Colchicine	Houde (France)
EDTA	BDH (England)
Ethanol	Fluka (Switzerland)
Fetal calf serum	Sigma (USA)
Giemsa stain	Sigma (USA)
Glacial acetic acid	Fluka (Switzerland)
Gleevec	Novaritis (Switzerland)
Glycerol	BDH (England)
Heparin	Denemarca-arcDenem
Hepes	Sigma (USA)
Hochest 33258	Sigma (USA)
Hydrochloric acid (HCI)	BDH (England)
Isoamylalcohol	Fluka (Switzerland)
KCI	Fluka (Switzerland)

KH_2PO_4	Sigma (USA)
Leishmen stain	Instatements of Sera and Vaccine(Iraq)
MTX	Fluka (Switzerland)
Na_2HPO_4	Sigma (USA)
NaCl	Sigma (USA)
NaH_2PO_4	Sigma (USA)
$NaHCO_2$	Sigma (USA)
Phenol	Fluka (Switzerland)
Phytohaemoaglutinin (PHA)	Radiobiology center of the Ministry of Science and Technology(Iraq)
Proteinase-K	Sigma (USA)
RPMI-1640	Flow laboratories(UK)
SDS	Fluka (Switzerland)
Tris-base	Fluka (Switzerland)
Tris-HCl	Fluka (Switzerland)

2-3- Solution

2-3-1 Antibiotic solution

Two antibiotics were used in the cultures.

Streptomycin, it was prepared by dissolving (1gm) from it with (100ml) of sterile DDW (deionized distil water)

Penicillin, it was prepared by dissolving (100000 unit) in (100 ml) of sterile DDW. Both antibiotic sterilized by (0.22µm) filter unite (filtration) under an aseptic condition, and store at (-20°C) until used.

(As prepared in biotechnology department of al-Nahrain university)

2-3-2- 5-Bromodeoxyuridine (5-Brdu)

One tablet (50mg) of 5-Brdu dissolved by (35ml) of PBS and the solution was sterilized by filtration throughout (0.22µm) sterile filter, final concentration (1.43mg /1ml). Store in dark bottle at (-20°C) until used (Shubber *et al*, 1987).

2-3-3- Colchicine

The stock solution was prepared by dissolving one tablet (1mg) in (10ml) of DDW to make it's concentration (100µg/ml), then from this stock solution (1ml) was taken and mixed with (9ml) of sterile DDW to reach the final concentration (10µg/ml). Sterilized and store at (-20°C) (Marlise *et al*, 1997).

2-3-4-Phytohaemoaglutinin (PHA)

It was obtained as freezed solution from Radiobiology center of the Ministry of Science and Technology (Iraq) from this solution mixed (1ml) with (19ml) of normal saline (0.85%). Sterilized by filtration throughout (0.22µm) sterile filter and store at (-20°C) until used.

2-3-5- Sodium Bicarbonate ($NaHCO_3$)

- $NaHCO_3$ (4.4%) for media prepared. It's prepared by dissolving (4.4gm) of NaHCO3 in (100ml) of DDW sterilized and store at (-20°C).

- $NaHCO_3$ (0.5%) for Giemsa stain working solution preparation.

It was prepared by dissolving (0.5 gm) of it in (100 ml) of DDW and kept at (4°C) (Allen *et al*, 1977).

2-3-6- Potassium chloride (KCl)

Hypotonic solution (0.075M) (It was prepared immediately)

The solution was prepared by dissolving (5.85gm) from KCl in (1 liter) of DDW, adjust pH at (7.2), and put in an incubator at (37°C) before using (Passarge *et al*, 2001).

2-3-7- Heparin solution (5000IU/ml)

Heparin was prepared by mixing (1ml) of heparin with (24ml) of sterile DDW and distribution in sterile tubes until used. Store in refrigerator at (4°C)(AL-Amiry *et al*, 1999).

2-3-8-Normal saline (0.85%)

It was prepared by dissolving (8.5gm) of NaCl with one liter of DDW, sterilized and store at (-20°C) until used.

2-3-9-Phosphate buffer saline (PBS)

It was prepared by dissolving the following materials in DDW, and the final volume brought-up (1 liter). The pH of the buffer was adjusted to (7.2), these chemicals are:

Material	Chemical name	Ingredient in gm
Sodium Chloride	NaCl	8.00
Potassium Chloride	KCl	0.20
Disodium hydrogen phosphate	Na_2HPO_4	1.15
Potassium dihydrogen phosphate	KH_2PO_4	0.20

This solution was sterilized by autoclaving and stored at (4°C) (Verma and Babu *et al*, 1989).

2-3-10-Fixative solution:

This solution was freshly prepared by mixing 3:1 v/v absolute methanol to glacial acetic acid, then kept at (4°C) at least (15 min) before use (Shubber *et al,* 2003)

2-3-11- Giemsa stain:

- **Stock solution**

This stain was prepared by dissolving (1gm) of Giemsa stain powder in (33 ml) of glycerol and put in water bath at (37°C) for (2hr), with shacking from time to time ,

Then this solution was cool down with room temperature for (60 min), then added (60 ml) of absolute methanol with shacking it continually.

This solution was filtered throughout filter–paper (0.45µm) and stored in dark bottle under room temperature.

- **Working solution of Giemsa stain** (Allen *et al,* 1977)

This solution was prepared by mixing the following

1 ml	Stock giemsa stain
40ml	DDW
0.5ml	$NaHCO_2$ (0.5%)
1.25ml	Absolute methanol

2-3-12 Buffer of DNA Estimation

2-3-12-1- Buffer A

This buffer was (0.05 M) NaH_2PO_4, (2.0M) NaCl, ($2x10^{-3}$ M) EDTA, with pH obtained at (7.4). This was prepared by dissolving (0.59gm) NaH_2PO_4, (11.6gm) of NaCl and (0.0744gm) of EDTA in

(100ml) of sterile DDW and obtained pH at (7.4), filtered with (0.45µm) filter paper, and store at (4°C) until used (Labarca *et al*, 1980).

2-3-12-2- Hochest stain solution

*Stock was prepared by dissolving (1.5 mg) of Hochest stain powder in (30ml) of sterile DDW to get concentration (50µg/ml).

*(1µg/ml) Hochest stain prepared by mixing (1ml) of stock solution with (49 ml) of sterile DDW, and kept in dark bottle at (4ºC) until used .

*(0.1µg/ml) Hochest stain prepared by mixing (1ml) of stock solution with (9 ml) of sterile DDW, and kept in dark bottle at (4ºC) until used. (Labarca *et al*, 1980)

2-3-12-3- Buffer B (lyses buffer)

It was containing (0.01mM) Tris–HCl pH8, (1mM) EDTA, (0.1M) NaCl, (1%) SDS, and (0.2 mg/ml) proteinase K.

*Proteinase K (0.2 mg/ml) was prepared by dissolving (2mg) of enzyme with (10ml) of sterile DDW, store at (4ºC) until used.

*Buffer B prepared by dissolving (0.2422gm) Tris–HCl, (0.074gm) EDTA, (0.58gm) NaCl and (1gm) SDS with (100ml) of sterile DDW, sterilized by filtration throughout (0.22µm) filter-paper. Stored at (4ºC) until used (Sambork *et al*, 1989; Mahavash *et al*, 2000)

2-3-12-4- 5M NaCl

This was prepared by dissolving (29 gm) of NaCl in (100ml) of sterile DDW with shacking, after that filtered with (0.45µm) filter–paper. This solution was store at (4ºC) until used (Iqbal *et al*, 2004).

2-3-12-5- Phenol /Chloroform/Isoamy1 alcohol

This was prepared by mixing 25:24:1v/v/v of phenal, chloroform and isoamyl alcohol. This solution kept at (4ºC) before use (Iqbal *et al*, 2004).

2-3-12-6- TE-Buffer (10Mm Tris-base-1Mm EDTA)

This was prepared by dissolving (0.121gm) Tris–base and (0.0292gm) EDTA in (100ml) of DDW, sterilized with (0.22µm) filter –paper, and store at (4ºC) until use (Iqbal *et al*, 2004)

2-3-13- Leukocyte diluents's solution

It's was prepared by mixing (2ml) of glacial acetic acid with (98ml) of DDW. Few drops (3-5) of ethylene blue were added as a color detector, kept at (4ºC) until used (Catalano *et al*, 2002).

2-3-14 Leishmen stain

- Lieshmen kit was supplied from institute of Sera and Vaccine, Baghdad-Iraq.
- Lieshmen buffer was prepared by dissolving of (1.36 gm) KH_2PO_4, and (3.25gm) Na_2HPO_4 in (100ml) of sterile DDW.

(Catalano *et al*, 2002)

2-3-15- Culture media preparation (RPMI-1640)

It was prepared by the following components:

RPMI-1640 media base	10gm
Fetal Calf Serum(FCS)	100ml
Sodium bicarbonate (4.4%)	15ml
Antibiotic solution	10ml
5-Brdu solution	10ml
Hepes	3gm

Media volume was completed to (1000ml) by adding sterile DDW approximately (865ml), adjusted pH to (7.2).

Culture media preparation was done under an aseptic condition. Culture media was sterilized by filtration throughout (0.22µm) filter–paper, and then distributed in sterile (10ml) vacutainer test tube about (2ml) media then store at (-20°C) until used .

Tow tubes were incubated overnight at (37°C), no contamination could occur during preparation and distribution in autoclaved tubes (Shubber *et al*, 1991).

2-3-16- Anti-cancer drug preparation

2-3-16-1- Preparation of Gleevec solution

Gleevec (Imitinib myestalte)(Novartis), kindly provided by Dr. Ali Muslim (The National Center of Heamatology, Baghdad, Iraq) at a concentration (100mg/capsul),was prepared by dissolving it in (100ml) of sterile PBS, and kept at (-20°C) as stock until use.

From this stock (1000µg/ml) prepared other drug concentrations (100, 10, 1 µg/ml) by serial dilution with sterile PBS, and store in vial at (-20°C). From this concentrations (1000, 100, 10, and 1 µg/ml) of gleevec take (0.25ml) were added to blood culture to get gleevec concentrations (100, 10, 1, and 0.1 µg/ml) per culture media respectively (Paul *et al,* 2004).

2-3-16-2- Methotrexate preparation (MTX)

Methotrexate (Fluka), kindly provided by Dr E. K. Shubber (Radiobiology center of the Ministry of Science and Technology, Baghdad, Iraq) at concentration (25 mg/ml in vial), was prepared at two concentration.

*(0.5µg/0.1ml) MTX, was prepared by mixing (0.04ml) of MTX with (200ml) of sterile DDW, prepared under an aseptic condition. It was store in sterilized vial at (-20°C) until used .From this concentration (0.1 ml)

was added to blood culture media to get (12.5µg/ml) MTX concentration per culture media.

*(4µg/0.1ml) MTX, was prepared by mixing (0.16ml) of MTX with (100ml) of sterile DDW, these prepared under an aseptic condition. It was store in sterilized vial at (-20°C) until used. From this concentration (0.1ml), was added to blood culture media to get (100µg/ml) MTX concentration per culture media (Shubber *et al*, 2003).

2-4- Subjects

A samples of (30) subject were participated in this study.

The subjects were divided into two groups.

*The first included (15) healthy normal individuals (6 males, 9 females) as a control group.

*The second included (15) patients (7 male, 8 females) with a clinical diagnosis of chronic myeloid leukemia (CML). They were clinically detected as CML patients in the National Center of Hematology, AL-Yarmok, Baghdad, Iraq.

The diagnosis was made by the consultant medical staff in the center, and it was confirmed by a laboratory blood and bone marrow examination. Both, patients and controls, were matched for age and sex. The age had arranged of (14-70) years old.

2-5- Blood collection

The blood samples were collected during the period from November 2004 to July 2005. From each subject (3-4 ml) of peripheral blood was obtained by a vein –puncture using disposable syringe, after that blood put in sterile test tube recoated with heparin.

The blood was placed in a cool–box and transferred to the laboratory. These samples were used for cytogenetic analysis and DNA quantity.

2-6-Cytogenetic analysis

2-6-1- Cytogenetic analysis

Cytogenetic analysis included assessments of blastogenic index (BI), mitotic index (MI), chromosomal aberrations (CAs). Such assessments were carried out after blood cell culturing: (As shown in figure (2-1))

2-6-1-1- Blood cells culturing

Blood cell culturing for cytogenetic analysis was performed following the procedure of Shubber *et al* 2000 with some modification:

a- Culture was set-up in a (10ml) sterilized test–tube containing (2ml) culture medium (RPMI-1640).

b- Add (0.25ml) of PHA solution. This step was skip when test as with out PHA.

c- Add (0.25ml) of prepared drug (anti–cancer drug, each tube had different concentration of it's for testing culture). This step was skip when test as control (with out drug) and mixing well.

d- After that this mixture cultured with (0.25ml) of peripheral blood sample.

e- The culture was incubated for (72) hr, at (37°C), with mixing twice a day.

All these steps were made under an aseptic condition.

2-6-1-2- Cell harvesting (Vermu and Babu *et al*, 1989) with simple modification,

After (70 hr) of incubation, colchicine (0.2ml) was added to each tube with shaking, and then transferred back to the incubator. After that the cells were harvested as the following:

1- The tube was centrifuged at (2250 rpm) for (15 min),
2- The supernatant was discarded and a little media was left over cell pellet.

3- The pellet was re-suspended in (5ml) of warmed hypotonic solution with a continuous gentle mixing.
4- The tubes placed in incubator for (20-25min) at (37ºC). Each tube shaking time to time.
5- The tubes centrifuged at (2250 rpm) for (15 min).
6- The supernatant was discarded and the pellet was gently mixed, and few drops of cold fixative solution were added as drop-wise with mixing to give a total volume (5ml).
7- The tubes were kept in the refrigerator overnight.
8- The tubes centrifuged at (2250 rpm) for (15 min).
9- Step 6 and 8 were repeated until the supernatant become clear.
10-After final washing, the cells were suspended in a small volume of fixative (pellet plus fixative) approximately (1ml). This suspension may be used immediately to make slides, or may be stored for several days before the slides are made, storage should be at (4ºC).

All these steps made under a septic condition.

2-6-1-3- Slide preparation

The slides should be clean, wet, child, grease-free before use, so wiped them with absolute alcohol. This was done as following:

1- The slides were cleaned by hot DDW and detergent.
2- Deluge in 2% HCl until used.
3- Then before one day of used washed with sterile DDW and deluge with DDW overnight in refrigerator.
4- These slides then put in cold fixative before used.
5- The suspended cells were mixed well with pasture pipette, then (5-7) drops of cells suspension were dropped from a high distance approximately (90cm) on to wet, chilled, grease –free slides.

Slides was put slant to permit spreading the drops on it, then slides were left to air-dry. (Passarge *et al*, 2001)

2-6-1-4- Staining (Shubber *et al*, 1987)

After slides were dried labeling by marker to know them. Staining slides with working Giemsa prepared as pre-description in (2-3-11) for (30 min) by putting slides in jar containing working–Giemsa.

After that by forceps take slides and washing with DDW.

Staining with Giemsa used to study MI, BI of cells under (40X) of compound light microscope. And CAs of cells using oil immersion lenses (100X) of compound light microscope.

2-6-1-5- Blastogeneic Index analysis (BI)

Blastogenic index was determined as a percentage of blast cell (mitogen stimulated cells) to a total number of growing cells (1000 cells), using The following formula:

$$BI\% = \frac{\text{Number of blast cells}}{\text{Total number of cells (1000)}} \times 100$$

(Al-Shawk *et al*, 1999)

2-6-1-6- Mitotic index (MI)

Mitotic index was determined as a percentage of mitotic cells to interphase nuclei in (1000) cells, using the following formula:

$$MI\% = \frac{\text{Number of dividing cells}}{\text{1000 cells}} \times 100$$

(Shubber *et al*, 1999)

2-6-1-7- Chromosomal aberrations (CAs)

The prepared slides were examined under the oil immersion lenses (100X) for (25-100) divided cells per each blood lymphocytes cultures

and the cells should be at the metaphase stage of the mitotic division where the CAs are clear and the percentage of these aberrations was estimated (Passarge *et al*, 2001).

2-6-2. Experimental design

3-6-2-1- Standardization

- **Standardization of colchicine**

In order to select the colchicine dose that can give well metaphase with good number and shape of chromosome. These concentrations of colchicines used (0.1,0.2,0.3) add through three different time before culture harvested (2hr,1hr,1/2hr) to take an optimal concentration with optimal time .

They were tested against the cells of healthy individuals. It was found that two concentrations at two different times resulted in good values [(0.2ml) for (2hr), and (0.1ml) for (1hr)]. Second experiment did by comparison between these two concentrations and time by measuring BI, MI with good chromosome.

This found (0.2ml) of colchicine at (2hr) added before harvesting its more effect than other with good result.

- **Standardization of drug Doses**

In order to select the drugs concentrations that can inhibit blastogeneic, mitosis, or both, four concentrations (0.1, 1, 10, and 100µg/ml) of gleevec and two concentrations (12.5, 100µg/ml) of MTX employed. They were tested against the cells of (15) healthy individuals. It was found that a dose of (100µg/ml)of gleevec and MTX can inhibit BI and MI in all individuals, when these result were repeated against cell, some patients showed resistance to a concentration of (100µg/ml) of gleevec and MTX . Therefore, for test the effects of the drug on healthy and CML individuals, all concentrations of each drug were employed.

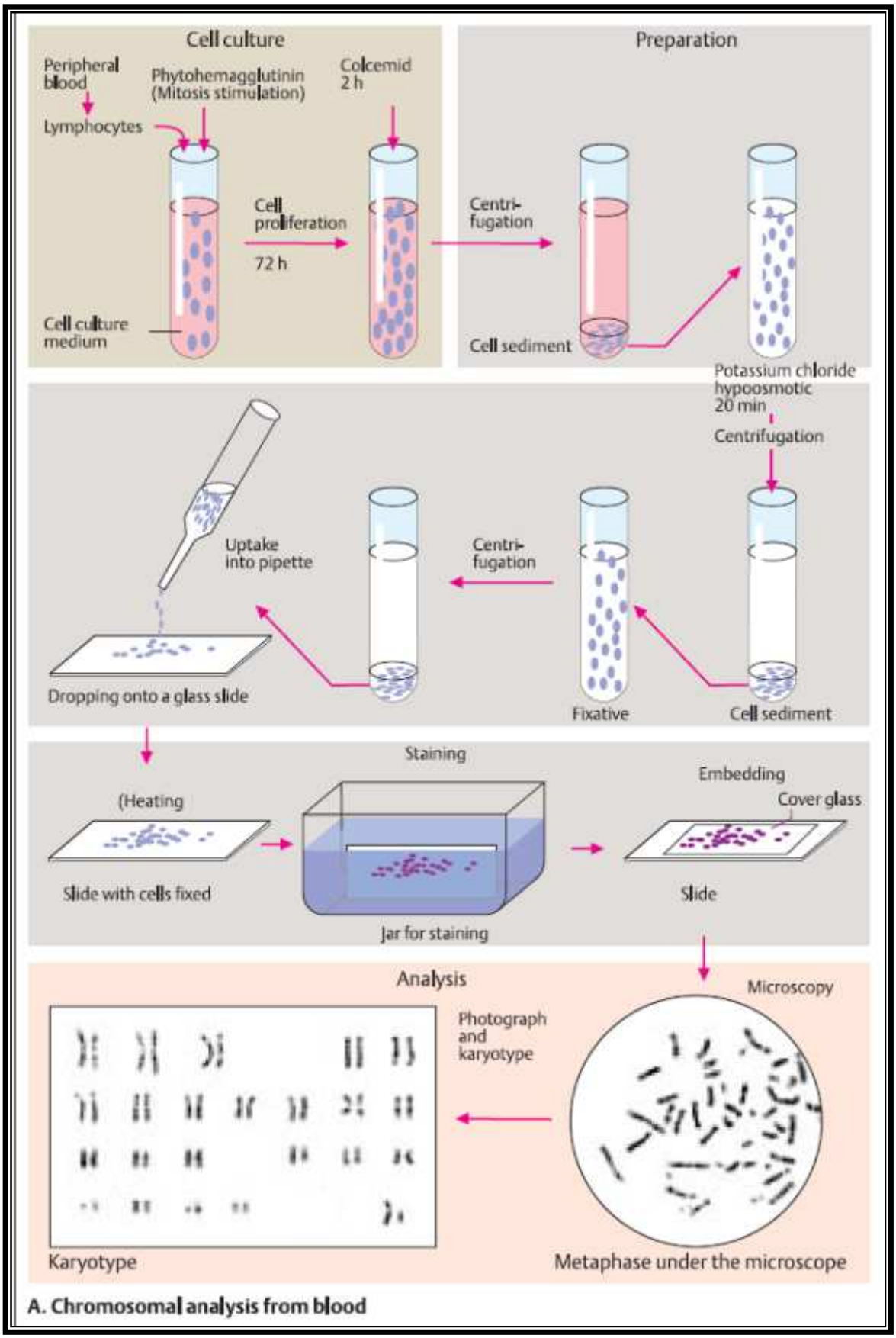


Figure (2-1) Chromosomal analysis from blood (Passarge *et al*, 2001)

2-6-2-2- Assessment of drug effects

For each subject (patients or controls), 14 cultures were set–up. The first culture was a drug–free, while the rest were treated with the concentration of each drugs as outlined in (2-6-1-1), (7) cultures with PHA and other (7) cultures with out PHA.

To assess the drug effect, the BI, MI and CAs were evaluated. The scheme of experimental design is show in figure (2-2)

2-7- Hematology analysis

Total and differential count of human blood leukocytes, all subjects (control and patients) which were used for DNA quantity and cytogenetic experiment when vein-punctured before cultured, study on its total and differential counts.

2-7-1- Total count of Leukocytes (Catalano *et al*، 2002)

1-The blood was taken by vein-puncture and put to heparinized tube.

2-A diluting solution (0.4 ml) was pipette in to test tube.

3- The heparinzed blood (0.1ml) was pipette and mixed well with diluting fluid for at least 2 minutes.

4-The hemocytometer was sited up with its cover glass in position, by a pasture pipette both sides of the hemocytometer were filled with the diluted blood.

5-The cells were allowed 2 minutes to be slatted.

6-The cells were count in the four large squares on both sides of chamber, by using the (40X) objective and subdued light.

7-The WBCs were calculated on the basis of cells counted, counted area, and the dilution.

Number of cells (cells/cmm blood) = four squares x correct volume x correct dilution / 4.

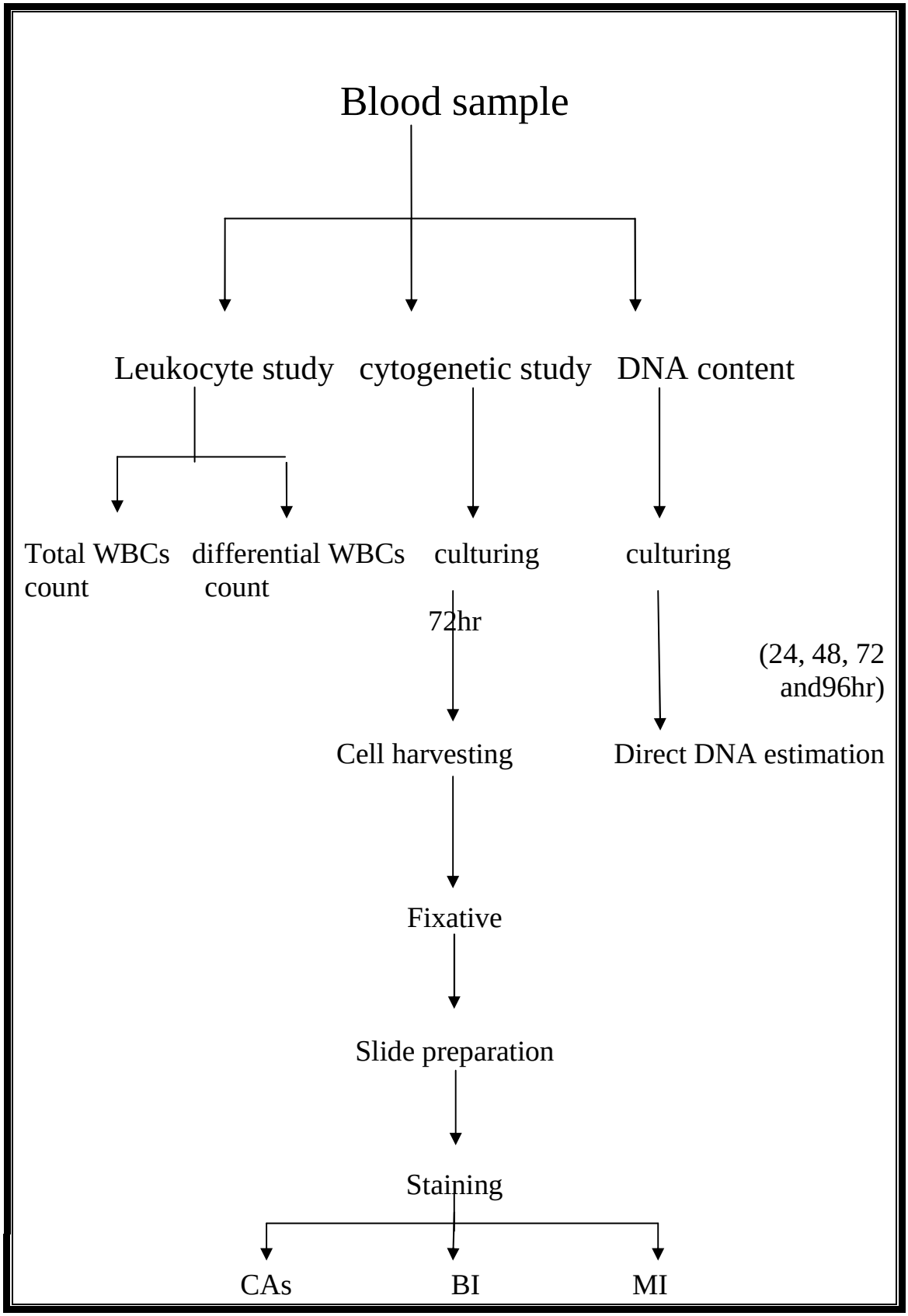


Figure (2-2) Scheme of experimental design

2-7-2- Differential count of Leukocytes (Catalano *et al*, 2002)

1-A small drop of heparinzed blood was put on the end of clean, dry slides.

2-A pusher slide was place at an angle of (30ºto 40º) to the slider and then moved it back to make contact with the drop. The forwarded movement of the pusher spreads the blood on the slide.

3-The blood film was allowed to dry in the air.

4-The slides were completely covered with Leishmen stain , after (5 minutes) the slides were washed gently by leishmen buffer and then examined under light microscope at (40X) objects

Determined the number of lymphocyte, monocyte, neutrophil, basenophil, esionophil per 100 cells of leukocytes

Number of cells (cells/cmm blood) = (total number of leukocyte x cells %) /100.

2-8-DNA Estimation

DNA estimation can be determined by simple, direct, rapidly method such as in fluorescence methods or can also be measured by DNA extraction from whole tissue and absorbance at 260 nm. (Ian *et al*, 2000)

The scheme of experimental designs show in figure (2-3)

2-8-1- DNA estimation by Hochest 33258

Outline homogenize cells or tissue in buffer, and then sonicate the homogenate with Ultrasonic corporation .Mix aliquats of the culture with H33258, and measure the fluorescence (Labarca *et al*, 1980; Ian *et al*, 2000).

2-8-1-1- Experimental design

Assessment of drug effects for each subject (patient, or control), eight cultures were set-up. The four cultures were a drug–free, while the other

four were treated with (100µg/ml) of gleevec concentration. All cultured with PHA.

2-8-1-2- Procedure of DNA quantity by Hochest 33258

1-The blood cells culturing were made as pre-description in (2-6-1-1), with simple modification. The incubation differed when cultures were incubated for (24hr,48hr,72hr,96hr) at (37ºC) , with mixing twice a day. These steps were made under an aseptic condition (Shubber *et al*, 2000).

2- After cultured incubation period complete (24hr, 48hr, 72hr and 96hr), centrifuged at (2250 rpm) for (15 min).

3-Discarde the supernatant (media and other content) and take pellet.

4- Re-suspended the pellet with buffer A.

5-Homogenize the cells in buffer, for (1 min).

6-Sonicate the cells for (30 sec).

7- Dilute the cells 1:10 in Hochest 33258 and buffer A.

8-Read fluorescence emission at (458nm) with spectrophotometer-UV, and at (260nm) with spectrophotometer if the once not available using Calf Thymus DNA as a standard for each one (Labarca *et al*, 1980; Ian *et al*, 2000), from 2-8 steps made under a septic conditions.

2-8-2- DNA estimation by DNA extraction and absorbance at 260 nm

2-8-2-1- Experimental design

Assessment of drug effects, for each subject (patient, or control), two cultures were set–up. The one culture was a drug–free, while other one was treated with (100µg/ml) of gleevec concentration. Both cultured with PHA.

2-8-2-2- Procedure of DNA estimated by DNA extraction

1- The blood cells culturing were made as pre-description (2-6-1-1), and this step should be made under an aseptic condition (Shubber *et al,* 2000)

2- After 72hr complete of culture, DNA was extracted from blood cultured using the method described by Sambrook *et al* in 1989, with little modifications and optimization. Briefly

a- Blood cell culture centrifuged at (2250rpm) for (15min), discard supernated.

b- Re-suspended the cells (pellet) with (0.5ml) cold lyses buffer (buffer B), and the mixture was subject to shaking incubation at (37ºC) overnight.

c-An equal volume of buffer–equilibrated (phenol/chloroform/isoamyl alcohol) was added and mixed on ice for (10-15 min). It was centrifuged at (2500rpm) for (15min) at (4ºC).

d- Supernatant was taken; an equal volume of chloroform was added and mixed gently at room temperature for (15-30min). Phases were separated by centrifugation at (2500 rpm) for (15min) at (4ºC).

(In these last two steps DNA was extracted).

e- DNA in the aqueous phase supernatant was precipitated with (0.1) volume of 5M NaCl and (2) volume of cold ethanol (DNA precipitated).

f- Pellet was obtained by centrifugation at (14,000rpm) for (20-30 min) at room temperature. It was washed with (70%) ethanol and then air-dried.

g-The DNA sample (pellet) was dissolved in TE buffer, DNA quantity estimated spectrophotometrically at (260nm) and store at (-20ºC) (Iqbal *et al*, 2004).

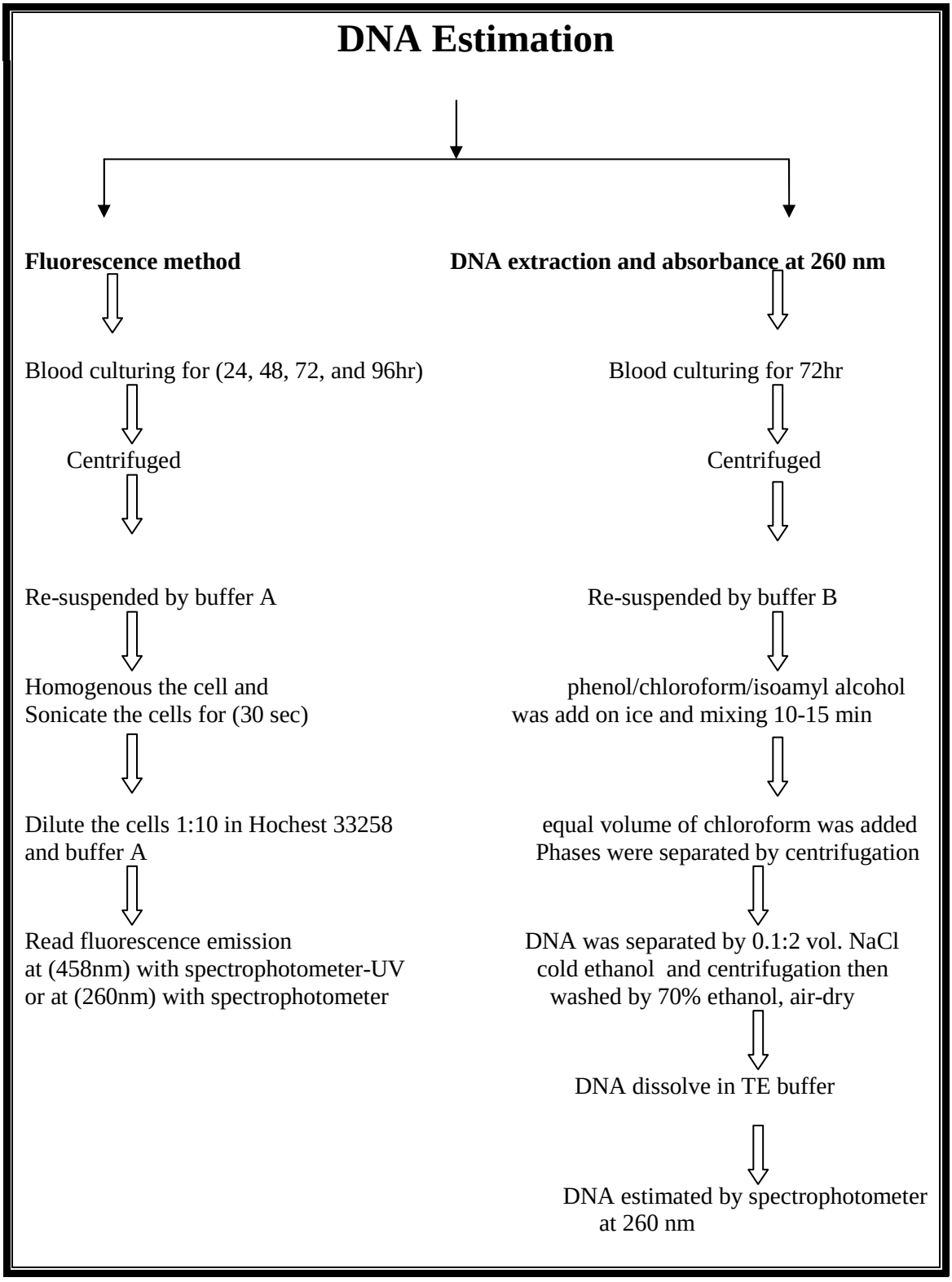


Figure (2-3) Scheme of experimental design of DNA Estimation

2-9-Statistical Analysis

The data were analysis statistically by methods (ova method) outlined by (Al-Mohammad *et al*, 1986), and they were presented in the results in terms of (Mean ± standard error (SE)). The analysis of variants were employed to determine the level of significant ($P<0.05$) between the observed difference.

Appendix A-2
Controls History

Gender	Age	Location	Smoking	Drinking	Work	Family history
Male	43	Baghdad	yes	yes	worker	no
Female	47	Baghdad	no	no	housekeeper	no
Male	26	Baghdad	no	no	student	no
Female	29	Baghdad	no	no	Assess. surgery	no
Male	22	Baghdad	yes	no	student	no
Female	39	Sulimaniayh	no	no	housekeeper	no
Male	23	Dayala	no	no	student	no
Female	14	Baghdad	no	no	student	no
Male	23	Baghdad	no	no	student	no
Male	26	Baghdad	no	no	student	no
Female	23	Baghdad	no	no	student	no
Female	22	Baghdad	no	no	student	no
Female	22	Baghdad	no	no	student	no
Female	25	Baghdad	no	no	research	no
Female	24	Baghdad	no	no	research	no

Appendix A-1
Patient History

Gender	Age	Location	Smoking	Drinking	Work	Period of diagnosis	Family history	Drug
Male	٥٢	Najeef	No	No	Teacher	6 years	No	HU, INF Gleevec
Male	٤٥	Karbalaa	No	No	Assess. PhD	4years	No	HU, INF Gleevec
Male	77	Baghdad	No	No	Worker	3mounth	No	Gleevec
Male	30	Ta'mem	No	No	Worker	2mounth	No	Gleevec
Male	21	Wasset	No	No	Student	2years	No	Gleevec
Male	22	Sulimaniyah	No	No	Student	1year	No	Gleevec
Female	55	Baghdad	No	No	Housekeeper	2mounth	No	Gleevec
Female	14	Baghdad	No	No	Student	4mounth	No	Gleevec
Male	70	Ta'mem	No	No	Worker	5years	No	HU, INF Gleevec
Female	22	Baghdad	No	No	Housekeeper	4years	No	HU, INF Gleevec
Male	٤٥	Baghdad	No	No	Worker	7years	No	HU, INF Gleevec
Female	30	Salah - Aldeen	No	No	Housekeeper	3years	No	Gleevec
Female	34	Baghdad	No	No	Teacher	6mounth	No	Gleevec
Female	15	Baghdad	No	No	Student	1year	No	Gleevec

Female	50	Baghdad	No	No	Housekeeper	5years	No	HU, INF Gleevec
Male	70	Baghdad	No	No	Worker	5years	No	HU, INF Gleevec
Female	39	Anbbar	No	No	Housekeeper	6years	No	HU, INF Gleevec
Male	34	Baghdad	No	No	Worker	6years	No	HU, INF Gleevec
Female	28	Baghdad	No	No	Worker	8mounth	No	Gleevec
Female	38	Baghdad	No	No	Housekeeper	3years	No	HU, INF Gleevec
Female	39	Salah-Adeen	No	No	Housekeeper	5years	No	HU, INF Gleevec
Female	22	Salah-Aldeen	No	No	Student	2years	No	Gleevec
Female	34	Anbbar	No	No	Housekeeper	4years	No	HU, INF Gleevec
Female	28	Sulimanyah	No	No	Housekeeper	4years	No	HU, INF Gleevec, MTX
Male	41	Anbbar	No	No	Worker	3years	No	Gleevec
Male	55	Anbbar	No	No	Worker	5years	No	HU, INF Gleevec
Female	40	Baghdad	No	No	Worker	4years	No	HU, INF Gleevec
Female	34	Sulimanyha	No	No	Housekeeper	6years	No	HU, INF

								Gleevec
Female	30	Salah-aldeen	No	No	Teacher	3years	No	Gleevec
Female	24	Baghdad	No	No	Student	2years	No	Gleevec
Male	37	Anbbar	No	No	Worker	3years	No	Gleevec
Female	28	Baghdad	No	No	Worker	4 years	No	HU, INF Gleevec
Male	19	Baghdad	No	No	Student	2years	No	Gleevec
Male	20	Baghdad	No	No	Student	2 years	No	Gleevec
Female	30	Baghdad	No	No	Worker	3years	No	Gleevec
Female	40	Salah-Aldeen	No	No	Housekeeper	5years	No	HU, INF Gleevec
Male	35	Thee-Kaar	No	No	Worker	8years	No	HU, INF Gleevec
Male	33	Al-Kadysiia	No	No	Worker	3 years	No	Gleevec
Female	38	Babylon	No	No	Worker	3 years	No	HU, INF Gleevec
Female	35	Baghdad	No	No	Housekeeper	3years	No	HU, INF Gleevec
Male	٤٤	Baghdad	No	No	Worker	5years	No	HU, INF Gleevec
Male	19	Anbbar	No	No	Student	2years	No	Gleevec
Male	56	Baghdad	No	No	Doctor	2years	No	Gleevec
Male	25	Thee-Kaar	No	No	Worker	4 year	No	HU, INF Gleevec

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4-1 Conclusions

1- The enrolled cytogenetic indices (BI, MI, CAs, and Ph+ve chromosome) are appropriate in evaluational of the disease picture in CML patient.

2- Categorization of CML patient and prediction of therapeutic efficacy could be carried out by using the investigated cytogenetic indices (BI, MI, CAs, and Ph+ve chromosome).

3- Measurement of DNA content and cells accounted assay are useful in evaluation of the sensitivity of CML cells to cytotoxic agents.

4- Alterations Ph+ve chromosome is relevant to the susceptibility for the risk of CML development.

4-2 Recommendations

1- Using other parameters of the cytogenetic study such as sister chromatid exchange (SCE) and Fluorescence In Situ Hybridization (FISH) for detection the mechanisms of drug resistance in CML patients.

2- Isolation and characterization of *bcr-abl* fusion gene or product protein in culture media of CML cells resistance to chemotherapeutic agents such as gleevec.

3- Evaluation tyrosine kinase enzyme or glycoprotein enzyme of multidrug resistance in CML cells.

1-3 Definition

Leukemia is a malignant disorder of hemopoetic cells that inhibits the normal production of blood cells (Debra *et al,* 2005). It's characterized by distorted proliferation and development of blood cells and their precursor in the blood and bone marrow (Goldman *et al*, 1999). These diseases are a heterogeneous group which differed from each other in etiology, pathogenesis, cytology, and responsiveness to treatment (Bain *et al*, 1999; Golodman L. *et al,* 2000).

1-4-Incidence of leukemia

The incidence of leukemia recorded was varies according to the geographic distribution, age, sex, race, environment and social class, with respect to the disease group (IARC *et al*, 1987; Hain *et al,* 1995; Golodman *et al*, 1999).

Acute leukemia, is the most frequently reported in adult and childhood, while chronic leukemia; like many other disease is a disease of old age, the average age of individual varies from 40 to 70 years (Hain *et al*, 1995; Peckham *et al*, 1995).

In general, leukemia affects more men than women throughout the world. Although the (male**:**female) ratio is about 3:2 in acute leukemia and about 2:1 in chronic leukemia (Aldelstein *et al,* 1976). The incidence of leukemia for all types in the population approximately 10 people per 100000 per years in world. (Hain *et al,* 1995; Edwards and Boucher *et al,* 1999).

1-5- Etiology

The etiology of leukemia exactly is unknown. Numerous risk factors may be responsible for the aetiology of leukemia. The risk factors now believed to have the strongest associations with leukemia are:

1-5-1- Radiation

The risk of leukemia is increased among people who have been exposed to radiation (Ionizing and non-ionizing radiation). Ionizing radiation including (X-ray, gamma ray and other ionizing ray) has a leukemia effect when person exposed to specific dose (Strover *et al,* 1983). Atomic bomb induced leukemia reported by Atomic Bomb Casualty Commission at Hiroshima and Nagasaki (Beebe *et al,* 1982).

In other hand leukemia was statistically increased in British faces participating in the atmospheric nuclear testing programmed in the pacific (exposed to nuclear falle –out) (Darby *et al,* 1988). In addition patient who received radiotherapy for malignant disease may have chance to develop leukemia (Boiven *et al*, 1986; Boice *et al*, 1991). Moreover, the evidence of relating chronic disease to most sources of non-ionizing radiation is inconclusive, but ultrasound, ultraviolet, radiation and extremely low frequency magnetic radiation from domestic sources have carcinogenic action (Editorial *et al*, 1982; Wright *et al*, 1982; Cartwright *et al*, 1984).

1-5-2-Chemicals

The risk of leukemia is increased 20 – fold among workers with long –term exposure to benzene, and petroleum derivatives. Risk also is increased among workers exposed to some other solvents, herbicides, and pesticides (Saracci *et al,* 1991). Agricultural chemicals in particular have been linked with an increased risk of leukemia.

In addition, some reports suggest that leukemia risk may be increased in workers exposed to dioxin, styrenes, butadienes, or ethylene oxides. (Blair *et al*, 1985; Hagstedt *et al,* 1985; Saracci *et al,* 1991).

Cigarette smoking is known life style–related risk factors for leukemia. Potential leukemia–causing chemicals in tobacco smoke include benzene, polonium-210, and polycyclic aromatic hydrocarbons. It is

estimated that is one of four cases of acute myeloid leukemia is the result of cigarette smoking (Severson *et al*, 1987; and Sylvia *et al*, 1998).

1-5-3 Biology factor

1-5-3-1- Infections

There have been some recent epidemiological studies of viral involvement in leukemia of some note. Human T-cell leukemia virus I (HTLV-I) is clearly associated with an acute-Tcell leukemia syndrome. (Blatter *et al*, 1989).

Human immunodeficiency virus (HIV) infection confirms a significant of subsequent in roughly 3% of all reported AIDS cases and in HIV –positive hemophiliacs (Beral *et al*, 1991; Ragni *et al*, 1993).

2-3-3-2- Genetic

Leukemia risk is increased 15-fold among children with Down's syndrome which is genetically linked chromosomal abnormality (usually an extra copy of chromosome 21), (Rowley *et al*, 1981).

Three rare inherited disorders congenital syndromes–Fanćonis anemia, Bloom's syndrome, and ataxia- telagiectasia, also have a greatly increased risk of leukemia (Miller *et al*, 1967).

Genetic studies of several leukemia have identified a small number of genes that must be mutated in order to trigger the development of leukemia or maintain the growth of malignant cells (Hoffbrand *et al*, 1980). About that oncogene is defines as a gene that can change or be activated to cause cancer, and it is capable of stimulating cell division, there by being a potential cause of cancer if unregulated. It found in all cells and in many cancer–causing viruses (Jeffrey *et al*, 1999).

Early oncogene identified in viruses such as Rous sacroma viruses (Barbacid *et al*, 1987). Later in addition to the oncogenes originally identified in viruses, approximately fifty oncogenes have been identified in

malignant tumors as part of chromosomal rearrangement, or the amplification or mutations of specific genes. (Jeffrey *et al*, 1999).

2-3-3-2-a- Mutation

Mutation is any alteration of gene structure may result in the production of abnormal proteins with altered or absent functional activity or many cause changes in the level of gene expression (Jeffrey *et al*, 1999).

A mutation within a coding sequence may activate oncogenes such as the activation of Ras–oncogene in acute myeloid leukemia. (Bos *et al*, 1985).

2-3-3-2-b- Chromosomal rearrangement

Chromosomal rearrangement such as translocation of genes in chronic myeloid leukemia t(9;22).Genetic transposition of *abl* (Abelson mouse leukemia virus) and *bcr* (Breakpoint cluster region gene) sequences to form a BCR-ABL fusion gene leading to the expression of active protein tyrosine–kinase. This *bcr-abl* gene expression is sufficient to causes chronic myeloid leukemia, (Hochhlaus *et al*, 2002).

2-3-3-2-c- Gene amplification

Gene amplification was observed in several cases of leukemia and solid tumors (Bielder and Spengler *et al*, 1976). Gene amplifications may be associated with the presence of multiple copies genetic segments along a chromosome, designated as homogeneously staining regions (HSRs), or may appear in the from of minichromosomes containing the amplified genes, termed double –minutes (DMs) such as in neuroblastoma (Alitalo *et al*, 1983; Lindahl *et al*, 1996).

1-6- Classification

The purpose of classification of leukemias is to organize knowledge into a manageable form. Leukemia is usually divided into myeloid and lymphoid, acute and chronic. This simple classification based on lineage and on rate of disease progression when untreated (Bain *et al*, 1995).

Acute leukemia result in the accumulation of early myeloid or lymphoid precursor cells (Blast cell), in the bone marrow, blood cells and other tissue, marrow failure rapidly follow normal blood cells production cisses, while in chronic leukemia malignant grown allowed differentiation to functional and cells as in chronic myeloid leukemia, or malignant proliferation progressive more slowly as in chronic lymphoid leukemia (Charles and William *et al,* 2003).

The most four common types of leukemia are listed below:

1-Acute myelogenous leukemia (AML)

Acute myelogenous leukemia is a disease resulting from the proliferation of a neoplastic clone of cells derived from a myeloid stem cell or committed progenitor cell. It's characterized by accumulation of immature granulocyte blood cells and progresses very quickly. This type occurs in both adults and children. In some times called acute granulocytic leukemia. (Bennett *et al*, 1976; Bennett *et al,* 1985; Mittleman *etal*, 1992; and Debra *et al*, 2005).

2-Acute lymphocytic leukemia (ALL)

Acute lymphocytic leukemia also known as acute lymphoblastic leukemia is a malignant disease caused by the abnormal growth and development of early non-granular white blood cells, or lymphocytes. The leukemia originates in the blast cells of bone marrow (B-cells), thymus (T-cells) and lymph nodel; it begins in immature lymphocytic blood cells and progresses very quickly.

This is the most common form of childhood leukemia and is highly curable in children. When it affects adults, it is an aggressive disease. It's some time called acute lymphocytic anemia (Bennett *et al*, 1985; Stansfeld *et al*, 1988; Harris *et al*, 1994; Seiter *et al*, 2001; and Debra *et al*, 2005)

3-Chronic myelogenous leukemia (CML)

Chronic myelogenous leukemia is a clonal myeloproliferative disorder of the primitive hematopoietic stem cells. It's the leukemia clone preserves the capacity to differentiate and mature. It is begins in more mature granulocytic and monocytic blood cells and progresses gradually. This occurs mainly in adults, but may occur in a very small number of children. It's sometime called chronic granulocytic leukemia. It is associated with specific molecular event the formation of the Philadelphia chromosome (Ph) and the activation of an enzyme tyrosine–kinase which inhibits cell apoptosis. (Gunz *et al,* 1977; Sawyer *et al,* 1999; and Zafor *et al,* 2004).

4-Chronic lymphoid leukemia (CLL)

Chronic lymphoid leukemia is a disease caused by a monoclonal proliferation of lymphocytes with specific cytological and immunophenotypic feature. It is begin in more mature lymphocytic blood cells and progresses gradually. This type most often occurs in adults over age 50. It some time occur in younger adults, but rarely occurs in children (Bennett *et al*, 1989; Matutes *et al,* 1994; and Debra *et al,* 2005).

1-7- Chronic myeloid leukemia (CML)

Chronic myeloid leukemia is a clonal disease that results from an acquired genetic change in a pluripotential haemopoietic stem cell. This altered stem cell proliferates and generates a population of differentiated cell that gradually replaces normal haemopoiesis and leads to a greatly expanded total myeloid mass (Goldman *et al,* 1990).

The neoplastic cells are characterized by a unique gene rearrangement as a result of a reciprocal translocation between the long arm of chromosome 9 and chromosome 22, known as Philadelphia chromosome (Ph). The gene rearrangement results in the creation of abcr/abl fusion

gene, which encode for a chiemeric protein with tyrosine kinase activity (Jan *et al*, 1998). Hematological feature, the majority of patients have leukemia count between 100-300×10^9/L (10000-30000 cell /cmm) at the time of diagnosis. The predominant leukocyte is a mature neutrophil, which appears morphologically normal, but the leukemia differential shows a full spectrum, including (blasts, promyelocytes, myelocytes and metamyelocytes). Basophilia, thrombocytosis and esinophilia are also commonly seen (Whittaker and Holmes *et al*, 1998). The over production of WBCs in CML can be targeted to a chromosome abnormality (Christina *et al*, 2002).

1-7-1 Incidence of CML

World wide, CML has an incidence of 1 to 2 cases per 100,000 population per year and is responsible for 15% to 20% of all adults leukemia (The median age range is 40-50 year), although up to 30% of patient older than 60 years of age and rare to very small in childhood. CML slight male predominance, with a male to female ratio 3:2 (Gunz *et al*, 1977; Faderl *et al*, 1999; and Red Orbit News *et al*, 2005).

The incidence of CML in Iraq was shown in table (1-1)

1-7-2- Classification of CML

The most commonly quoted classification, devised by Sokal and Colleagues in 1984, its based on a formula that takes account of the patient, age, blast cell count, spleen size and platelet count at diagnosis ,but is still to inaccurate to be clinically useful. The majority of patient with CML have a relatively homogenous disease characterized at diagnosis by splenomegaly, leucocytosis and the presence of Ph chromosome (Ph+ve) in all leukemia cells. A minority of patient have less typical disease that may be classified as atypical CML as chronic neutrophilic leukemia (Oscier *et*

Table (1-1) Incidence of chronic myeloid leukemia in Iraq (National center of Hematology, 2005).

Governorate	number	percentage
Baghdad	**187**	**34.80**
Mousel	**52**	**9.60**
Dayala	**30**	**5.58**
Anbbar	**26**	**4.87**
Thee-Kaar	**22**	**4.00**
Muthna	**19**	**3.53**
Babylon	**18**	**3.35**
Wasset	**17**	**3.16**
Salah Al-Deen	**16**	**2.90**
Sulimaniyah	**14**	**2.60**
Arbeel	**14**	**2.60**
Ta'mem	**13**	**2.42**
Al-Kadysia	**13**	**2.40**
Kerbela'a	**12**	**2.23**
Basrah	**11**	**2.00**
Najef	**10**	**1.86**
Duohook	**7**	**1.30**
Myssan	**4**	**0.74**
Total	**537**	**100%**

al,1996). And about 5% of patient classified as (Ph-ve) of CML this result by them have bcr/abl gene activity with out cytogenetic Ph-chromosome present. (Onida and kantarjian *et al*, 2002).

1-7-3- History of CML

The first scientific description of CML is credited to John Hughes Bennett in Edinburgh in 1845. He became interested in the disorder when his mentor, Dr David, observed two patients with unusual blood consistency and a splenic tumor. His report entitled “Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood” was published in the Edinburgh medical and surgical journal in October 1845. (Bennett *et al*, 1845).

In same year, Robert Virchow observed his first case of CML while studying the pathology of phlebitis. He noted the enlarged spleen and liver but also described blood vessels full of material resembling pus. (Geary *et al*, 2000).

In 1856, Vichrow was note the result of an infections process but rather was cased by the tissue that produced the white cells; he also categorized two type of chronic leukemia, splenic and lymphatic, which we now know as myeloma and lymphoma respectively (Geary *et al*, 2000).

Today we understand CML as a malignant clone disorder of the pleuripotential hematopoietic stem cell, resulting in proliferation of predominantly immature myeloid cells. However, CML also affects of the moncytoid, erythroid, megakarytic, B-lymphoid and occinelly T-lymphoid lineage. (Red Orbet News *et al*, 2005).

The diagnosis of CML was dramatically improved with discovered of Philadelphia chromosome by Nowell and Hungerford in 1960 (Nowell *et al*, 1960 A). However, the rudiments of our understanding the molecular cases of CML began in 1973 when Janet Rowley discovered that the Ph-chromosome is a reciprocal translocation between chromosome 9 and 22. (Rowley *et al*, 1973).

1-7-4- Clinical features of CML

Early disease often is without symptom (clinical presentation asymptomatic) and is discovered accidentally.

Individual with more advanced cases of CML appear sickly and experience fever, easy bruising, and bleeding and bone pain. Laboratory, chemical, physical findings include splenomegaly, occasionally hepatomegaly is found high WBCs and platelet.

Absent or low amounts of the WBCs enzyme alkaline phosphatase (APT) (Druker *et al*, 1999). However CML is progressed through three phases of disease characterized by a clinical course which is initially indolent (chronic phase), but if untreated transform into the more aggressive accelerated and blastic phase of short duration (Hagop *et al*, 2002 A).

1-7-4-a- Chronic phase (CP)

Chronic phase is the initial stage of the disease, and lasts about 4-5 years, is associated with relatively mild signs and symptoms. (Pasternak *et al*, 1998) It's characterized by a high WBCs count left shift, thrombocytosis, and mild to moderate normocytic /normochromic anemia. Patients usually exhibit hepatosplenomegaly, resulting from extra medullary hematopoiesis in the liver and spleen. (Pasternak et *al*, 1998).

1-7-4-b- Accelerated phase (AP)

Accelerated phase is characterized by increased disease aggressiveness and resistance to therapy and cellular transformation. Other feature include increased cellular proliferative, with correspondingly high percentage of blasts, promyelocytes, and basophils, thromobocytosis, anemia, splenomegaly, the appearance of chromosomal abnormalities other than Ph+ve, and marrow fibrosis. These characteris are associated with poor prognosis and shortened

survival time from 6-18 month. (Kantarjian *et al,* 1988; Majlis *et al,* 1991; Hagop *et al,* 2002 B)

1-7-4-c- Blast phase (BP)

Blast phase is called blast crisis phase (BCP) BP of CML is characterized by greater than 30% blasts in the peripheral blood and bone marrow cells resemble acute leukemia–myeloid and lymphoid, blasts fail to differentiate in to mature cells. Blast phase (BP) increased symptomotology, especially relating to anemia and infection, central nerves system (CNS), lymphadenopathy, and bleeding. Approximately 50% of patients have myeloid blast crisis, 25% have lymphoid blast crisis, and 25%, are mixed. Chronic myeloid leukemia inevitably progresses to the blastic phase within approximately 3-5 years after diagnosis, and approximately 3-18 month after onset of the accelerated phase. Survival in the blastic phase is shorter, with a median survival of 4-6 months. Additional cytogenetic abnormalities develop in over 80% of patients in the AP and BP. The evolution of any of these additional mutations confers a worse prognosis (Kantarjian *et al,* 1987; Cervontes et *al,* 1990; Cortes et *al,* 1996; Hagop *et al*, 2002 B)

1-7-5- Pathogenesis of CML

The causes of CML are the translocation of regions of the *bcr* and *abl* genes to form a *bcr-abl* fusions gene. This result from a reciprocal translocation termed t (9; 22), which forms the Philadelphia (Ph) chromosome.

The product of the *bcr/abl* gene, the BCR-ABL protein, is a constrictively active protein tyrosine kinase with an important role in the regulation of cell growth. It’s nuclear whether these fusion oncoproteins alone are sufficient to explain the full range of clinical

responses to the disease process (Elena *et al*, 2001; Hagop *et al*, 2002 A)

1-7-6- Diagnosis of CML

Current diagnostic criteria rely on cytogenetic confirming the presence of the Ph chromosome or its product the transcribed BCR-ABL mRNA and the BCR-ABL fusion protein. Hematology and laboratory, chemical, physical are used for diagnosis. Recently the former diagnostic approaches that detected the DNA by several technique, Fluorescence In Situ Hybridization (FISH), Southern Blotting, and Reverse–Transcription Polymerase Chain Reaction (RT-PCR). These methods are highly sensitive and can detect one Ph+ve cell in about 105 to 106 normal cells. Moreover, Western Blotting technique detects the fusion protein unlike the former diagnostic approaches that detected the DNA (Hiesterkamp *et al*, 1990; Juan *et al*, 1990; Pasternak *et al*, 1998; Skasakayn *et al*, 2003; Cool *et al*, 2003).

1-7-7- Cytogenetic of CML

The cytogenetic hallmark of CML is the Philadelphia chromosome, a shorted chromosome 22 resulting from a translocation between long arm of chromosome 9 and 22, t (9; 22) (q34; q11). Because of this distinct feature, CML is one the best studied malignant conditions in humans (Hagop *et al*, 1999). Ph-chromosome identified in up to 95% of patients with CML diagnosis (Faderi *et al*, 1999). Some, but not all patients acquire additional clonal cytogenetic abnormalities during the course of the chronic phase. (Watmore *et al*, 1985).

And about 80% of patients in overt blastic transformation have clonal cytogenetic changes in addition to the Ph translocation. (Hagop *et al*, 2002 B).

1-7-8- Molecular biology of CML

The t (9; 22) (q34; q11) reciprocal chromosomal translocation occur in nearly all patients with CML and approximately 25% of adults and 5% of children with ALL (Melo *et al,* 1999).

This translocation gives origin to Ph+ve chromosome that contain *bcr-abl* hybrid gene, the molecular hallmark of CML and of Ph+ve of ALL. (Rowley *et al,* 1973).

The fusion gene *bcr-abl* that is transcribed into chimeric mRNA and then translated in to a hybrid protein (Elena *et al*, 2001).

The *abl* gene is (Abelson mouse leukemia virus) a murine viral oncogene that is 230 Kb in length and contain 11 axons with two splice sites. It's normally codes for a 145 kd nuclear protein, called P145, which possesses tyrosine kinas activity.

In contrast, the *bcr* gene complex (Breakpoint cluster region gene) is composed of least four separate genes termed BCR1, BCR2, BCR3, and BCR4. BCR1 is most common BCR gene involved into 20 exons with two splices sites. The gene normally codes for a 160 kd protein (p160) that is constitutively expressed in many cell types, but strongly expressed in hematopoietic cells (Razelle *et al*, 2003).

Both gene (*abl* and *bcr*) are shuttle between the nucleus, where it can bind DNA and cytoplasm, where it binds the actin cytoskeleton. (Razelle *et al,* 2003)

The bcr/abl fusion protein resulting from transposes the 3´ segment of the *abl* gene from 9q34 to the 5´ segment of *bcr* gene on 22q11. The resulting *bcr-abl* gene is transcribed in to a chimeric mRNA and then translated into fusion proteins of varying size (p190 *bcr-abl*, p210 *bcr-abl*, p230 *bcr-abl*) depending on the location of the breakpoint of the genes involved (Elena *et al*, 2001).

P210 is result from the most common breakpoint region in the BCRI gene called major BCR (M-BCR) and is located in the middle of the BCRI gene, and this is the protein product associated with classical CML (Judy *et al* 1996), an alternative breakpoint region on BCRI gene is termed miner BCR (m-BCR) and is located 5´ of the (M-BCR), this breakpoint is associated with the majority of cases of Ph+ve ALL and in rare case of CML that tend to produce a monocytosis (Ralvandi *et al* , 1998) .

Fusion protein with its increased tyrosine kinase activity (Deregulated tyrosine kinase activity) has the ability to promote leukemogenesis *in vivo* by inducing cell proliferation and can transform hematopoietic progenitor cells (Transformation effect) (Francós *et al* , 2000). The degree of transforming activity of bcr-abl correlated with the degree of tyrosine kinase activity.

Other tyrosine kinase activity transforming capabilities at least in part via activation of *Ras*, a vital protein in the intracellular signaling pathway (Tauchi *et al,* 1998). Moreover, bcr-abl induced survival enhancement may be mediated by a modulating protein, which suppresses programmed cell death (apoptosis) (Vigneri *et al*, 2001; Razelle *et al*, 2003), and its growth factor independence (activation of intracellular signaling molecules, interaction with growth factor receptor. In other wise BCR affected DNA damage response process in diverse ways. It also enhances after DNA double strand break repair and, enhances resistance after drug therapy. (Slupianek *et al*, 2001) (Razelle *et al*, 2003).

1-7-9 Therapy of chronic myeloid leukemia

Several therapeutic, milestones have occurred in the treatment of CML including the use of irradiation in 1900, busulfan and hydroxyurea

in the 1950, 1960 allogeneic stem cell transplantant (SCT) and interferon–alpha in the 1970-1980, donor lymphocyte influsions in the 1990, and most importantly the recent development of targeted therapy against the Ph–related protein abnormality using imatinib mesylate, better known as gleevec or ST1-571. The four therapeutic milestones are likely the most important, and have changed, and will continue to change, prognosis CML. (Hagop *et al,* 2001).

a- Busulfan and hydroxyurea (BUS and HU):

These were old standard of care. Busulfan (BUS) is a fat-soluble alkalyting agent that is given orally, introduced in the 1950, was shown to reduce elevated WBCs count and disease–related signs and symptoms in majority of patients with CML (Bolin *et al,* 1982; Hehlmann *et al,* 1993). However, this agent causes serious adverse affects, including myelo–suppression as well as pulmonary, hepatic and cardiac fibrosis (Richard *et al,* 2004).

Hydroxyurea (HU) is a ribonucleotide reductive inhibitor that appears to target specifically the hemopoietic system. It was first used as treatment for CML in the only gained widespread populatarity in the 1980. This agent causes macrocytosis and megalobstoid changes in the bone marrow. Hydroxyurea has demonstrated a more rapidly onset of action and better side–effect profile that BUS (Hehlmann *et al,* 1993).

b- Allogeneic stem cell transplant (SCT)

It is first successfully used treatment for CML in 1970 (Hagop *et al,* 2001). Currently, allogeneic SCT is the only treatment with known curative potential in CML, but it is an option for only about 90% of the patients of this disease (Peggs *et al,* 2003). While allogeneic SCT is associated with a high cure rate and long–term disease free survival with human leucocyte antigen (HLA) in up to 70% of patients depending on the patient age, as well as the availability of less toxic

approaches to therapy. Complications and risk factors associated with SCT, which should be considered in the decision–making, include morbidity, sterility and mortality the development of cataracts and second solid tumor, quality of life, chronic graft–versus host disease complictnsons. Age of patient is > 50 years old, and infection (Bacterial, fungal, or cytomegalovirus infection). (Kolb *et al,* 1995; Gratwohl *et al,* 1998; Hagop *et al*, 2001; and Richard *et al,* 2004)

c- Interferon–Alpha (IFN-α)

is a member of a large family of cell regulatory, glycoproteins of biological origin with antiviral, anti–proliferative properties, immunomdulatary and differentiation inducing effect studies in the early 1980 using material purified from human cell lines showed that it was active in reducing the leucocyte count and reversing all clinical feature of CML in 70-80% of CML patient (Talpaz *et al*, 1983) Treatment with recombinant (IFN-α) can induce hematologic and cytogenetic remissions in patients with CML (Richard *et al,* 2004).

Between "1982-1999" several regimens were develop using (IFN-α) alone or combination with chemotherapy agent (BUS, HU, cytarabine) (Hagop *et al*, 2001). IFN-α must be administered by subcutaneous injection and it therapy is associated with substantial toxicity the majority of patients receiving IFN-α based treatment of fever, chills, myalgias, malaise, and headache, as well as nausea, vomiting or diarrhea (Kantarjian *et al*, 1998; Silver *et al*, 1999).

Adverse effect occurring later are dosing limiting in up to 20% of patient include persistent fatigue weight loss, neurotoxicity, depression, and occasionally immune–mediated complication (Richard *et al,* 2004).

d- Imatinib mesylate (Gleevec)

Gleevec is an example to the new generation of novel anticancer drugs designed to target specific oncogenic molecular events

"molecular therapeuticants" and it was synthesized after a compound was identified by *in vitro* screening for tyrosine kinase inhibitor and its activity optimized for specific kinase. (Brian et *al*, 2001).

On may 10, 2001 the U.S. Food and Drug administration announced the fast track approval of gleevec, our treatment for patient with CML in BP, AP and CP after IFN-α failure treatment, the FDA approval came in just over 10 weeks after Novartis filed its new drug application, and just two months after the FDA notified us that it had granted gleevec a priority. (Jurg *et al*, 2002).

Brand name: Gleevec

Generic name: Imatinib mesylate

Manufacture: Novartis

(Gleevec NJ: Novartis, 2004)

d-1- Definition

Gleevec is a molecular therapeuticants chemotherapy oral administration capsules. Gleevec capsules contain imatinib mesylate equivalent to 100 mg of imatinib free base.

It's a phenyl amine pyrimidine derivative, designated chemically as 4-[(4-Methyl-1-piperazinyl) methyl]-N-[4-methyl-3-[[4-(3- 15pyridinyl)-2-pyrimidinyl] amino]-phenyl] benzamide methanesulfonate.

It's a white to off white to brownish or yellowish tiged crystalline powder. Its moleculer formula is {$C_{29}H_{31}$ $N_7O.CH_4SO_3$}, and its relative molecular mass is 589.7 and, it has chemical structure as in figure (1-1).

It is very soluble in water and soluble in aqueous buffer <PH 5.5, but is very slightly soluble to insoluble in neutral / alkaline aqueous buffer. In non-aqueous solvent, the drug substances is freely to very slight soluble. (Gleevec NJ: Novartis, 2004; Panl *et al*, 2004).

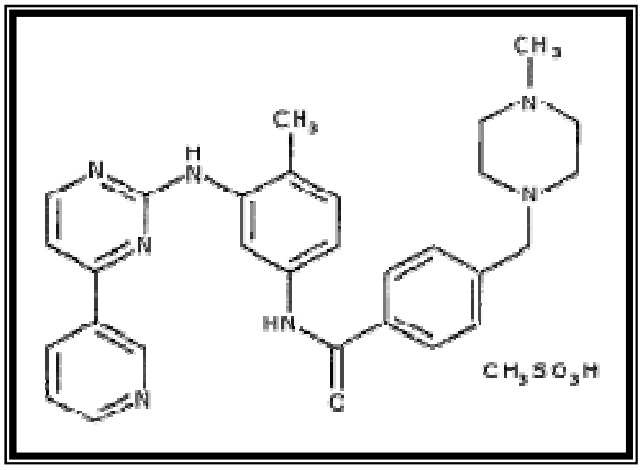

Figure (1-1) Chemical structure of Gleevec (Imatinib mesylate)(Gleevec NJ: Novartis, 2004)

d-2- Mechanism of action

Gleevec is a protein–tyrosine kinase inhibitor that inhibits the bcr/abl tyrosine kinase, the constitutive abnormal tyrosine kinase created by the Philadelphia chromosome abnormality t (9; 22) in CML in CP, AP, BP (Gleevec NJ: Novartis, 2004; Hagop *et al*, 2004).

It inhibits proliferation and induces apoptosis in bcr-abl +ve cell lines as well as fresh leukemic cell from Ph+ve CML. In addition, it also inhibits the receptor tyrosine kinases for platelet–derived growth factor (PDGF), stem cell factor (SCF), c-kit, and inhibits PDGF and SCF-mediated cellular events. (Jurg *et al*, 2002; Cools *et al*, 2003; Razelle *et al*, 2003; and Gleevec NJ: Novartis, 2004)

Gleevec acts specifically by blocking the binding site for ATP in the ABL kinase, thereby preventing the phosphorylation of tyrosine residues on substrate proteins, these lead to prevent the activation of signal transduction pathway (Growth factor receptor) that induce the leukemic transformation to CML and enhancing apoptosis as shown in figure (1-2) . (Savage *et al*, 2002; Razelle et *al*, 2003)

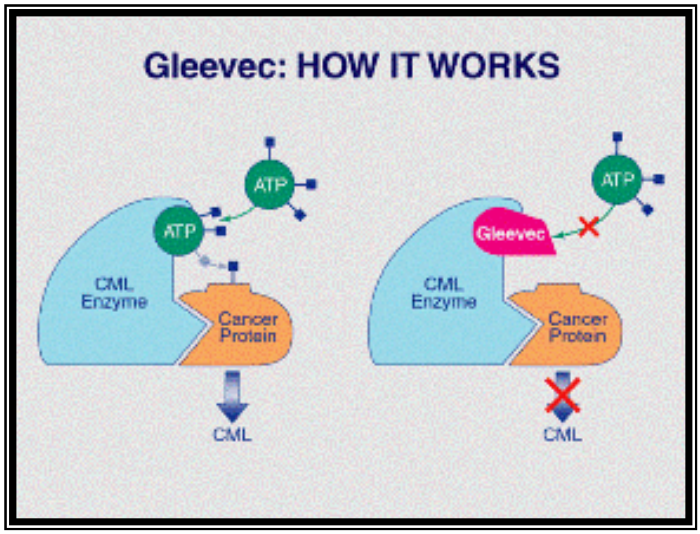

Figure (1-2) Mechanism action of Gleevec (Imatinib mesylate)(Razelle et *al*, 2003)

d-3- Pharmacokinetics

In human adult pharmacokinetic studies, gleevec was rapidly absorbed after oral dosing; the drug was detected in plasma with 30 minutes of administration. At steady state, Tmax 2-4 hr, the terminal half time ($t_{1/2}$) were approximately 18 and 46 hr for gleevecs and its active metabolite, respectively.

Absolute bioavailability was 98% and protein– binding albumin 95%. Gleevec metabolized in liver by the enzyme system, principally CYP3A4. Thus co-adminstration of compounds that inhibits or induces ACYP3A9 may affect clearance of gleevec.

Area under the concentration time curve (AUC) increased with increased dose, and the clearance was approximately 12 to 14 L/hr. Elimination, excretion is mainly in the feces, mostly as metabolites. Approximately 81% execrated in the feces and 13% in the urine, and 5% of gleevec is excreted unchanged in the urine and 20% in the feces. (Gleevec NJ: Novartis, 2004).

d-4- Drug interaction

- Drug that inhibits CYP3A4 may increase gleevec plasma concentration (ketoconzole, erythromycin).
- Drug that induces CYP3A4 may decrease gleevec plasma concentration (dexamethasone, rifampin, and phenytoin).
- Gleevec may inhibit CYP3A4 and increase plasma concentration of some vastation and dihydropyridine calcium–channel blockers
- Warfarin should not be given in conjuction with gleevec because gleevec may inhibit the activity of CYP2C9. It's recommended that standard or low–molecular–weight heparin be used as an alternative (Gleevec NJ: Novartis, 2004).

d-5- Dosage and adininistration

The usual dose is 400 mg /day for patient in CP and 600mg/day for patient in AP or BP (BCP) (Gleevec NJ: Novartis, 2004).

d-6- Toxicity

Gleevec differs from traditional therapies because of its more favorable toxicity profile. The most come side effect is mild to moderate, nausea, edema, meyalyias, arthralgias, diarrhea, and skin rash occur in about 10% of patient. Rarely, lower than 2% of patient a fluid retention syndrome occurs, as dose unusual phenomena of periorbital edema. Myelosuppression may be seen, but it more common in the blast phase than in chronic phase. (Razelle *et al,* 2003)

d-7-Carcinogenesis, mutagenesis, Impairment of fertility

Carcinogenicity studies have not been performed with gleevec positive genotoxic effect was obtained for gleevec in an *in vitro* mammalian cell assay (Chinese hamster ovary) for clastogenicity (Chromosome aberration) in the presence of metabolic activation.

Gleevec was not genotoxic when tested in an *in vitro* bacterial cell assay (Ames test), an *in vitro* mammalian cell assay (mouse lymphoma) and an *in vivo* rat micronucleus assay. (Gleevec NJ: Novartis, 2004).

In a study of fertility, in male and female of rats study found at high dose 60 mg / kg (approximately equal to human dose of 800 mg/day) testicular and epididymal weights and present motile sperm were decreased in male and in female had significant post implantation fetal loss and a reduced number of live fetus. This had not seen at doses < 20mg/kg (one–fourth the maximum human dose of 800mg). (Gleevec NJ: Novartis, 2004).

d-8- Nursing mother and pregnancy

Nursing mother should be advised to stop breast feeding while taking gleevec because drug or his metabolite are excreted in human milk and because of potential for serious reaction in nursing infants .

Woman of childbearing should be advised to avoid becoming pregnant. Gleevec was teratogenic in rats when administered during organogenesis at doses>100mg/kg (approximately equal 800mg/day), these effect not seen at doses < 20mg/kg (one–fourth the maximum human dose of 800mg). There are no adequate and well controlled studies in pregnant woman (Gleevec NJ: Novartis, 2004).

d-9- Efficacy, safety, hematology and cytogenetic response

Gleevec produce high rates of hematologic, cytogenetic and molecular response in all phases of disease CP, AP, BP. This achived by phase I, II, III trail study and other studies as described later.

First other phase I trail study, patient with CML and failure to IFN-α received gleevec 25 to 1000 mg\day 48 (98%) achived a complete hematology response and 24 (99%) had a major cytogenetic response

(Ph+ve less than 35%) 1 which was complete cytogenetic response (Ph+ve 0%) in 31% (Brian *et al*, 2001; Hagop *et al,* 2002)

phase I study of efficacy and safety show gleevec has few serious adverse effect up to doses of 1000 mg\day, and cytogenetic and hematologic response are seen at doses of 300 mg\day or greater (Brian *et al*, 2001). The positive result of phase I study led to the development of 3 pivotal trails.

Pivotal phase II study in CP-CML post IFN-α failure, 90% of patient's achived a complete hematologic response, 55% had a major cytogenetic response and complete cytogenetic response in 40%. Moreover, patients' response occurred rapidly (Talpaz et *al*, 2002).

Pivotal phase II study in AP-CML showed the higher dose of gleevec was associated with a higher rate of major (27% versus 10%) and complete cytogenetic response (19% versus 12%) with 95% hematology response a longer time to failure ($P<0.01$), and better survival ($P=0.019$). (Talpaz *et al*, 2002).

Pivotal phase II study in BP-CML showed the hematology response was 30% and the median survival about 7 months (Sawyers *et al*, 2002).

Phases III study the IRIS trail compared gleevec with IFN-α plus Ara-C show patient with gleevec had significantly higher rates of complete hematologic response and complete cytogenetic response than treated with IFN-α. The estimated rate was also significantly greater in the gleevec (96.7%) than in the IFN-α (91.5%) treated group. Gleevec was significantly better tolerated than the combination regimen in the IRIS study; received IFN-α /Ara-C had higher rate of hematologic adverse events and serum transminase elevated as well as a higher toxicities (O'Brien *et al*, 2003).

Moreover, gleevec monotherapy is significantly more effective and better to treated than IFN-α /Ara-C

Superior outcomes were seen in molecular/cytogenetic and hematologic response rates, progression–free survival, additional data on disease-free and all survival are a waited us follow–up continues

*Complete hematologic response 97% versus 69%
*Major cytogenetic response 87% versus 35%
*Major molecular response 39% versus 2%
*Complete cytogenetic response 76% versus 19%
*Progression–free survival 97% versus 92%
*Drug intolerance 21% versus 25%

(Hughes *et al,* 2003)

Hagop and his worker (2004) study patient in CP of CML treated with gleevec after IFN-α failure to improve in term of the rates and durabity of complete cytogenetic response, major cytogenetic response, complete hematologic response and complete molecular response, and in terms of improved survival compared with previous accepted treatment for CML(Hagop *et al,* 2004).

Other study done by Catherine and other (2005) on clinically hetrogenous CML patients, they found that the duration of treatment with IFN-α prior to gleevec therapy may not improve response to gleevec for patients in CP, but shorter period between CML diagnosis and the initiation of gleevec is prediction for a better molecular response to therapy. These study analysis by (RQ-PCR) Quantitative Rrverse Transcription Polymerase Chain Reaction. (Catherine and Stephen *et al,* 2005)

In conclusion gleevec is evolving first –line of therapy for CML and produces high rates of hematologic, cytogenetic, and molecular response in all phase of disease. Treatment with gleevec in the early diagnosis, and stage of disease yield the best results.

d-10- Resistance to gleevec (Imatinib mesylate)

Drug resistanse may occur rapidly in potentially curable tumors, and if cure is not achieved, lead to treatment failure (Bertion *et al,* 1996).Development of drug resistance is a common problem in cancer chemotherapy. (Mohammad *et al,* 2003).

Although gleevec is unquestionably effective in treating CML, some patient's ultimately relapse with resistance disease (Richard *et al,* 2004). Reistance may develop through several mechanisms, the most common of which is reactivation of BCR –ABL kinase activity within the leukemia cells by either

d-10-1- Mutation of gene

Gene mutation lead to drug resistance occure when drug can not recognized its site region mutation may be result by , point mutation domain occurs in one, or several gene of ABL gene or BCR-ABL kinase domain (Catherine *et al,* 2002; Amie *et al,* 2003; and Ksenia *et al,* 2004). Moreover, points mutations may occurs in one or several ABL gene ATP-binding domanor mutations in the ATP-phosphate –binding loop (P-1oop) (Susan B. *et al,* 2003; Zafar *et al,* 2004), and spontaneous mutations of the tyrosine kinase doman of *bcr-abl* gene (Hochhaus *et al,* 2002).

d-10-2- Gene amplification

Gene amplification as a mechanism of drug resistance is also commonaly observed where the durg inhibits an enzyme with in a critical biosynthetic pathway *bcr-abl* gene amplification is associated with drug resistance (Ellen *et al,* 2000; Hochhaus *et al,* 2002; and Richard *et al,* 2004).

d-10-3- Overexpression

Bcr-abl overexpression gene lead to resistance by to way insuffecient drug to this genes copies or produce a high amount of protein tyrosine

kinase that inhibits cells apoptosis (Franćois *et al*, 2000; Hochhaus *et al*, 2002; and Richard *et al*, 2004).

d-10-4- Reduction in the uptake of the compound

By *Pgp* overexpression (multidruy resistance protein).

Gleevec is binding to alphal acid glycol protein, or by possibly excessive degradation (Fanćois *et al*, 2000; Richared *et al*, 2004).

d-10-5- Additional molecular abnormalities other than bcr-abl

This may prevent apoptosis of the malighant clone despite effective bcr-abl kinase inactivation by gleevec (Richard *et al*, 2004).

Currently, efforts are continuing to further elucidate the mechanisms of gleevec resistance and develop strategies that will expand the usefulness of gleevec.

1-8- Methotrexate (MTX)

Methotrexate (MTX) is one the oldest chemotherapy drugs .It has been around and in many years. It's an antimetabolic drug which inhibits the enzyme dihydrofolate reductase (DHFR) that catalyses the conversion of folic acid to its active form folinic acid by binding to it (Nizami *et al*, 2001). Methotrexate (MTX) is commonly used in the following situations, breast cancer, hand and neck cancer, lung cancer, acute lymphocytic leukemia, gestational throphoblastic disease, bone tumors, lymphomas, treatment of arthritis (Esti *et al*, 2003).

Methotrexate (MTX) trade names (Amethopterin, folex), the type and extent of a cancer will determine the method and schedule of administration this drug, this decision is made by the medical oncologist. Methotrexate (MTX) may be given orally, intravenous, intramuscular and intrathecally (Tirgan *et al*, 2000). Methotrexate (MTX) had molecular formula C_{20} H_{22} N_8 O_5.

Its structural name is N-[4-[[(2, 4-diamino-6-pteridinyl) methylate]] glutamic acid (Tavner *et al,* 1983), the chemical structure of MTX is:

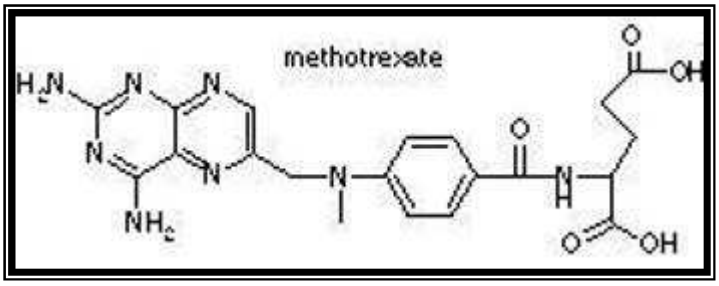

Figure (1-3) Chemical structure of Methotrexate (MTX) (Nizami *et al,* 2001)

1-8-1- Mechanism of Action

Methotrexate (MTX) and other folic acid antagonists exert cytotoxic effects by a profound, competitive inhibition of the enzyme folate reducase. The kinetics of the inhibition are termed (pusedoirreversible) (Esti *et al,* 2003), because the MTX can not be displaced significanthy from the enzyme by any concentration of folic acide attainable physiologically (Schweitzer *et al*, 1990).

Dihydrofolate reductase (DHFR) catalyzes the NADPH- dependent reduction of dihydrofolate to tetrahydrofolate. The latter serves as a coenzyme for a number of one–carbon transfer reactions in purine and pyrimidine biosynthesis, including that of thymidylate .The reductase appears to be the major intracellular receptor for the action of 4-amino analogues of folic acid, (MTX) (Tavner *et al*, 1983)

Methotrexate (MTX) inhibit the enzyme dihydrofolate reductase (DHFR), resulting in depletion of the reduced folates (tetrahydrofolate) that will decreased synthesis of thymidylate and, in turn an inhibition of DNA synthesis and toxicity for actively dividing cells (Colin *et al,* 2002).

1-8-2-Toxicity

The degree and severity of the side effects depend on the dosage and schedule of the administration of MTX. The following phenomena are some of the most common side effect. more common side effect (nausea, vomiting, sores in mouth or on lips, diarrhea, increased risk of sunburn, skin changes in areas previously treated with radiation, and loss of appetite), less common side effects (Decreased WBCs counte with increased risk of infection, decreased platelet counte with increased risk of bleeding, and kidney damage), the rare side effect (Nauseu and vomiting in low dose, liver toxicity, lung collapse, hair loss, rash, itching, dizziness, blurred vision and allergic inflammation of the lung with fever, cough and stortness of breath (Internet #1).

Other side effects are bone marrow, fetal cells and effect of MTX, when cellular proliferation in malignant tissues is greater than in most normal tissue. MTX may impair malignant growth without causing irreversible damage to normal tissue. (Arky and Dawidson *et al*, 1999)

1-8-3- Drug resistance and mechanisn of drug resistance

Development of drug resistance is a common problem in cancer chemotherapy (Iqbal *et al,* 2003).

Resistances mechanisms to MTX have been extensively studied mainly in experimental mechanisms have been described in cells that survive MTX treatment (Bertion *et al,* 1996; Mohammad *et al,* 2003).

- Altered transport of the drug (decreased influx, increased efflux).
- Increase in total amount of target enzyme / protein (gene amplification) (increased DHFR)
- Alteration (mutation) in the target enzyme /protein (mutation DHFR that binds MTX less than normall enzyme.

- Elevation of cellular glutathione.
- Inhibition of drug – induced apoptosis.

1-9 Cytogenetic analysis

With the development of chromosome staining techniques and the identification of chromosomes are even specific regions of chromosome based on banding patterns, specific chromosomal variations could be identified and characterized. The investigation of variation in chromosomes is called cytogenetics (Bonthron. *et al*, 1998)

Cytogenetic analysis is a widely employed indication system for the evaluation of physical (like adiation) chemical (in the diet and environment) and biological (like viruses) that can identify potential mutagens and carcinogens. And it is amethod that permit direct image analysis for the chromosome and genetic material damage (Carrano *et al*, 1986)

The first evidence that cytogenetic changes in human cancer were non random events came in 1960, when Nowell reported as hallmarker chromosome, termed the Ph-chromosome, in the peripheral blood of patients with CML (Nowell and Hungerford, 1960).

Cytogenetic analysis has been carried out using in vivo and *in vitro* system that have been proved to be good and reliable for mutagen exposure and chromosome aberration detection (Nakanashi and Schnieder *et al*, 1979; Gebhart *et al*, 1981)

There are several parameters have been used in cytogenetic analysis including the blastognic index (BI), mitotic index (MI) and chromosomal aberration (CA) (Stites *et al*, 1976; Shubber and Al-Allak *et al*, 1986; Shubber and AL-Hussainy *et al*, 2002).

1-9 -1- Blastogeneic index (BI)

Blastogenic index is parameter to mesure cellular response to mitogens in terms of the induction of proliferation (Soren *et al,* 1973).

Blastogenesis (Lymphocyte transformation) refers to the process of formation of large polymorphic blast-like cells in culture, which his considered as a result of immunological recognitition. This *in vitro* technique is commonly used to assess celluler immunity in patients with immunodeficiencies, autoimmunity, infecation disease and cancer (Stites *et al,* 1976).

Many of the known lymphocyte activators can be classified in to non-specific activators like mitogens such phytoheamoagglutinine (PHA), PHA an aqueous extract of the kidney bean *phaseolus vulgaris*, was able to produce large dividing blast-like cell in cultures of human peripheral blood (Nowell *et al,* 1960 B; Marlise *et al,* 1997) and PHA has been shown to be a protein containing carbohydrates in varying amounts (Guningham *et al,* 1976).

Phytoheamoagglutinine (PHA) stimulated human lymphocytes are widely used to detect chromosome–damage agents, possible human exposure to mutagenic, carcinogenic, and the immune response of blood, the resulte were affected by the number of the cell divisions (Sovage *et al,* 1979; Marlise *et al,* 1997).

Blastogeneic index (BI) was determined as a percentage of blastogeneic cells to other total cells (in 1000 cells).

1-9-2 Mitotic index (MI)

Proliferating cells go through a regular cycle of events, the mitotic cell cycle, in which the genetic material is duplicated and divided equally between two daughters. This is brought absent by the duplication of each chromosome to form two closely adjacent sister

chromatids, which separate from each other to become two daughter chromosomes. (Shubber and Al-Allak *et al*, 1986; Internet # 2)

It is a useful and sensitive test for detection of cytotoxic effect of chemical and physical agents as well as mutagenic and carcinogenic agents (King *et al*, 1982). Mitotic index is also employed to assess the toxogenic and carcinogenic effect of some drugs and radiations (Shubber and Salih *et al*, 1988).

The molecular mechanisms underlying the cell cycle are highly conserved in all organisms with nucleus eukaryotes (Eva *et al*, 2001). Mitotic index (MI) was determined as a percentage of mitotic cells to interphase nuclei in 1000 cells. (Ghosh *et al*, 1983; Shubber *et al*, 1987; Internet, # 2)

1-9-3- Chromosomal aberrations (CAs)

The increasing variety of chemicals, radiations and other physical agents we are exposed to now days has stimulated the development of many rapid, reliable assays for the detection of the mutagenicity or carcinogenicity of such agents. One of these methods is the chromosome aberration assay (Lambert *et al*, 1978; Ardito *et al*, 1980)

Chromosome aberrations can be studied in any cycling cell population, or in any non-cycling cell population that can be stimulated by a mitogenic agent like PHA to enter the cell cycle (WHO, 1985) Chromosomal aberration can occur at various stage of mitosis (G1, S G2, or M) and during mieosis (Al-Tikriti *et al*, 2005), living organisms may expose to different kinds of effects which may cause chromosomal abnormalities. Chromosomal aberrations considered as a genetic damage of chromosomal level observed as an alteration either in chromosome number or in chromosome structure (Al-Obaidy *et al*, 2003)

The most important thing that the chromosomal aberrations can be distinguishes when the cell is in the metaphase of the mitosis (Evans *et al*, 1976)

Chromosomal aberrations are classified as one of two types: numerical or structural, numerical changes are to two types: polyploidy with changes in the number of sets of chromosomes and aneuploidy with changes in the number of individnal chromosomes (e.g. trisomies and monosomies).

Structural changes involve the loss or gain of portions of chromosomes, the resulting patient may be said to have "partial monosomy" or "partial trisomy "(Borthran *et al*, 1998).

Both variation in polidy and increases in the frequency of individual chromosomal aberration can be found (Bielder *et al*, 1976; Croce *et al*, 1991). And variations in chromosomes number are found in most tumor culture (Sandberg *et al*, 1982). Some specific aberrations are associated with particular types of malignancy (Croce *et al*, 1991). The first of these aberrations to be documented was the Philadelphia chromosome in CML (Trisomy) (Nowley *et al*, 1973). Meningiomas often have consistent aberrations and small-cell lung cancer frequently has a 3p2 deletion (Wurster-Hil *et al*, 1989).

The types of chromosomal aberrations which had been observed are

1-9-3-a- Structural chromosomal aberrations

Structural aberrations can be classified as either unstable or stable, indicating their ability to persist in dividing cell populations (Carrano *et al*, 1986). Unstable aberrations consist of dicentrics, rings, deletions, and other asymmetrical rearrangments, previous studies have demonstrated that the unstable aberrations will lead to death of the cell (Dewey *et al*, 1970; Carrano *et al*, 1973). Cell death occurs because these changes can prevent the cell from dividing at mitosis or because

the formation of these exchanges also results in chromosome fragments that can be lost at cell divison (Carrano *et al,* 1986). Stable aberrations consist of balanced translocations, inversions and other symmetrical rearrangements. They are considered stable because they can be transmitted to progeny cells without causing cell death (Carrano *et al,* 1986).

Structural aberrations include chromosomal and chromatid types

1-9-3-a-1- Chromatid type aberration

Its damage expressed as breakage of single chromatid or breakage and reunion between chromatids (Bonthron *et al,* 1998).

1-9-3-a-2- Chromosome type aberration

Its damage expressed as breakage or breakage and reunion, of both chromatids at an identical site (Bonthron *et al,* 1998).

1-9-3-a-3- Deletion

If a break in a single chromatid occurs following the S phase of the cell cycle, the broken ends may not join and as the break produces a centeric fragment and an acentric fragment. The centeric fragment will migrate normally during the M phase of the cell cycle, but the acentric fragment is lost, the centric fragment is missing the genes lost due to the break, and is thus called a deletion chromosome. If a two breaks can occur in the same chromosome and result rejoining of two pieces called deletion loop (Bonthron *et al,* 1998).

1-9-3-a-4- Acentric Chromosome

A fragment of a chromosome that is lacking a centromere during the S phase and this acentric fragment of chromosome is lost when the cell divides at M phase (Bonthron *et al,* 1998; Internate # 3)

1-9-3-a-5- Dicentric chromosome

When single breaks in both chromatids of a chromosome can result in the joining of two centric fragments to produce a dicentric chromosome during M phase. The two centromeres of the dicentric chromosome are pulled to opposite ends of the cell during the M phase forming a dicentric bridge (Bonthron *et al*, 1998)

1-9-3-a-6- Gap

It's a chromatic lesion smaller than the width of the one chromatid, and with minimum misalignment of the chromatids (Bonthron *et al*, 1998).

1-9-3-a-7- Ring chromosome

It could happen in two ways. First the end of the p and q arm breaks off and then sticks to each other resulting in loss of information. Second the ends of the p and q are stick togother (Fusion), usually without loss of material (Tamarin *et al*, 1996).

1-9-3-a-8- Translocation

If breaks occur simultaneously in two non-homologous chromosome, and the broken joined can lead to the translocation of the ends of non-homologous chromosome to each other .Translocation result in mutations due to position effects, with a well known position effect is a cancer caused by the movement of a gene from a heterochromatic region where it's expression is suppressed to a euchromatic region where it is expressed and causes cells to divide (Bonthrom *et al*, 1998), such as a reciprocal translocation of Philadelphia chromosome in CML.

1-9-3-b- Numerical aberration

There are two kinds of variation in chromosome numbers, changes in whole sets of chromosomes (Euploidy), it's include triploidy and tetraploidy, or additions or deletions of less than whole sets of chromosomes (An euploidy) it's includes monsomy, trisomy

1-9-3-b-1- Aneuploidy

A diploid cell missing a single chromosome is monosomic**.** A cell with an extra copies of a chromosome it trisomic. Gains and losses of chromosomes occur as a result of non-disjuntion. Non-disjunction is the failure of a homologous pair to separate at anaphase I of meiosis, or the failure of chromatids to separate at anaphase II of meiosis or anaphase of mitosis. Several disorders in humans are due to aneuploidy the main cause of Down's syndrome is trisomy for chromosome #21. Edward syndrome is caused by trisomy for chromosome #18.

There are few cases of monosony in human; there are cases of individuals monosomic for chromosomic #21, 22 and X

1-9-3-b- 2-Euploidy

Cells have complete sets of chromosomes, whether it is one, two or more complete sets of chromosomes. In humans, gametes have one set of 23 chromosomes and body cells have 2 sets of 23 or 46 chromosome. In same species, polyploidy can be observed.Polyploidy are euploids with more than 2 sets of chromosomes.

Polyploids from when the number of chromosomes in the cells doubles. This occurs as a result of non-disjunction of an entire set of chromosomes during meiosis I, producing a cell with 2 sets of chromosomes that undergo meiosis II, resulting in gametes with 2 sets of chromosomes. The union of 2 such gametes produces a Tetraplaid. The union of gametes with two sets of chromosomes and gametes I set of chromosomes produces a Triploid (Bonthran *et al,* 1998).

1-10- Leukocyte study (Hematology study)

Leukocytes or white blood cells are clear or whitish colored cell that lack hemoglobin, but have a nucleus, in stained preparations

leukocytes attack stain, where as erythrocytes remain relatively unstained (Seeley *et al*, 1998). Leukocytes protect the body against invading microorganism and remove dead cells and debar is from the body. The five types of leukocytes are neutrophils, easinophils, basophils, lymphocytes, and monocytes.

1-10-1-White Blood cells count

White Blood cells count (WBCs) measure the total number of leucocytes in the blood. There are normally 5000-10000 leukocytes per cubic millimeter of blood (cmm). Leucopenia is a lower than normal WBCs count and can indicate depression or destruction of the red marrow by radiation, drug, tumor, or deficiency of vitamin B12 or foliate. Leukocytosis is an abnormally high white blood cell count. Leukemia a tumor of the red marrow, and bacterial infections often cause leukocytosis or by drug, mutation, chemical, physical agent (Seeley *et al*, 1998).

1-10 -2- White blood cell Differential count

White Blood cells (WBCs) differential count determines the percentage of each of the five kinds of leukocytes in the WBCs count. Normally neutrophiles account for, 60%-70%; lymphocytes, 20%-30%; monocytes, 2%-8%; esinophils, 1%-4%, and basophiles 0.5% -1%.

White Blood cells (WBCs) differential count can provide much in sight about a patient, condition examaple in patient with bacterial infections the neutrophil count is often greatly increased, where as in patients with allergic reactions the easinophils and basophils counts are elevated (Seeley *et al*, 1998).

1-11 –DNA content

Deoxyribonucleic acid (DNA) is the genetic material of cells. The information directing the chemical processes that occur in organisms and therefore determine their characteristics is contained in DNA. Deoxyribonucleic acid (DNA) consists of the basic building blocks called nucleotides. Deoxyribonucleic acid (DNA) in human is composed of two strands of nucleotiedes. Each nucleotide of DNA contains one the organic bases: adenine (A), thymine (T), cytosine (C), and guanine (G).

Deoxyribonucleic acid (DNA) molecules are associated with globular histone proteins to form chromatin. The histone proteins are involved with righting DNA function. During cell division, however, the chromatin condenses into structure called chromosomes, (Seeley *et al*, 1998; Jeffrey *et al*, 1999)

Deoxyribonucleic acid (DNA) quantitation is a necessary step for many life science research protocols (Internate, # 4). In practice, besides the cell number, the most useful mesurement quantifying the amount of cellular material (Ian *et al*, 2000)

Common DNA techninque, such as sequancing, cDNA synthesis and cloning, all benefit from a defined template concentration (Internate # 4)

Deoxyribonucleic acid (DNA) may be assayed by several fluorescence methods, including reaction with DAPI (Brunk *et al*, 1979) or Hochest 33258 (Labarca *et al*, 1980). Deoxyribonucleic acid (DNA) can also be measured by its absorbance at 260 nm, where 50 ug/ ml have an optical density (O.D) of 1.0 (Ian *et al*, 2000).

Most of classical DNA isolation procedures for estimated by absorbance at 260 nm. These techniques are based on the unusual physical properties of the molecule rather than its chemical properties.

In particular, they exploit the very large size of DNA retative to all other molecules in the cell. The chromosomal DNA isolation procedures involve a deproteinization. The key step, the removal of protein, can often be carried out simply by extracting aqueous solutions of nucleic acids with phenol and/or choroform. Such extractions are used whenever it's necessary to inactivate and remove enzymes. However, additional measures are required when nucleic acids are purified from complex mixtures of molecules such as cell lysates.

In these cases, it is usual to remove most of of the protein by digesting with protolytic enzymes such as pronase or proteinase K, which are active against a broad spectrum of native proteins, before extracting with organic solvents (Miller *et al*, 1988; Green *et al,* 1998). This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvent are used instead of one (Miller *et al,* 1988; Gillbert *et al*, 1994).

The major difficulty in these procedures involves avoiding excessive shearing the DNA molecules during handling (Miller *et al,* 1988; Sambrook *et al,* 1989).

Recently, two new fluorochromes, DAPI and compound Hochest 33258, have been used with success for fluorescent staining of DNA in cells (Ian, *et al* 2000), compound Hochest 33258 particularly useful in the quantitative determination of DNA in biological materials.

And it’s a very simple, sensitive and rapidly method which utilizes the enhancement of fluoresconces seen when Hochest H33258 binds to DNA (Dell’anno *et al*, 2000; Ian *et al,* 2000).

The procedure can be used directly on crude tissue homogenates in which the deoxyribonucleic protein structure of chromatin has been dissociated, making the DNA fully accessible to the reagent.

Ribonucleic acid (RNA) does not interfere and the method cans readily quantities (Labarca *et al*, 1980).

The fluorescence enhancement has been shown to be highly specific for DNA, the dye binding preferentially to A-T rich regions. The dye binds twice as well to Double-stranded DNA as to single-stranded DNA, but does not appear to intercalate. Ribonucleic acid (RNA) enhances the fluorescence to a much smaller extent: under high salt conditions, in which chromatin proteins are fully dissociated from DNA, RNA enhancement is usually well below 1% than produced by the same concentration by weight of DNA (Ian *et al*, 2000; Internate # 4).

1-1-Introduction

Leukemia is a progressive, malignant disease of the blood forming organs, characterized by increasing of leukocytes and their precursor in the blood and bone marrow (Goldman *et al,* 1999).

The etiology includes a series of genetic alterations rather than a single event. The events contributing to a malignant transformation include inappropriate expression of oncogenes and loss of function of cancer-suppressing genes (Rowley *et al,* 1999).

Leukemia is classified clinically on the basis of duration and character of disease into: (i) acute leukemia; which if untreated leads to death during few weeks and, (ii) chronic leukemia; which if untreated leads to death in years. The disease is further divided into lymphoid, myeloid, and biphenotypic leukemia on the basis of cells involved (Bain *et al,* 1999).

Chronic myeloid leukemia (CML) is stem cells myeloproliferative disorder manifested clinically as a marked increase in granulocytes, bone marrow hyperplasia, and splenomegaly (Goldman *et al*, 2003; Steensma *et al*, 2003).

The initial symptoms that appear are nonspecific including fever, anemia, fatigue, weight loss, and weakness (Kantarjian *et al*, 2003 A).
Chronic myeloid leukemia primarily affects adults between the age of 25 to 70 years and accounts for 15 % to 20% of all leukemia cases (Cortes *et al*, 2003).

Chronic myeloid leukemia is associated with the presence of Philadelphia (Ph) chromosome, detectable microscopically, which results from balance translocation t(9; 22) (Nowell *et al*, 1960 A). The Philadelphia (Ph) chromosome abnormality is detected in 95% of patients with CML, and in 20% of patients with ALL (Hagop *et al*, 1999). Median survival for CML

patients is 3 to 6 years after clinical diagnosis of the disease and there is a very narrow window of opportunity for the treatment of this disease (Xie *et al*, 2003).

Chemotherapy (Hydroxyurea) combination with Interferon alpha is First-line therapies for CML patients, but patients commonly develop resistance (Kuhr *et al*, 2003). Allogeneic transplantation used in combination with chemotherapy and radiation are the standard therapy for CML. Both of these treatments were known to be "Mutagenic" and "Carcinogenic" (Bloom *et al*, 1964; Anderson *et al*, 1972).

Novel discoveries in cancer biology have provided the opportunity to design target-specific anti-cancer agents, besides fostered rapid advancement in drug development (molecular therapeutic) (Mohammad *et al*, 2003).

Gleevec is commonly known as "Imatinib" (Gleevec, Novartis), is currently the most efficacious, target-specific drug for the treatment of CML (Deininger *et al*, 2003). Gleevec binds ATP-binding site of tyrosine kinase domine, protein trigger the carcinogenic pathway, thus, by occupying and blocking the ATP-binding site, resulting in prevention of the signal transduction leading to the onset of CML (Fujii *et al*, 2003).

However, a considerable number of incidence have been reported in patients who develop resistance to gleevec, leading to disease relapse and this resistance resulted by several pathway includes mutation, overexprssion, or genes amplified (Iqbal *et al*, 2004).

At the present time leukemia in Iraq is considered as one of the most important cases of death, this can be illustrated in the number of the new cases registered during 1992-1998 (Ministry of Health, Iraq 1998) and from 1999-2005 (National center of Hematology, Iraq, 2005*).* Most of these cases developed resistance to drugs and disease relapses in patients.

For this reason, large numbers of studies have been conducted with a view to understand the biology, diagnosis and treatment of this disease (such as Darwesh M.F. *et al, 2002*; Al-Shammary *et al,* 2006). Genetic studies are one of those approaches that have been proved to be useful and vital in leukemia researches, since they underline the origin, progression, and diversification of the disease (Hagop *et al,* 1999; Martein *et al,* 1999).

1-2-Aims of study

According to the present study, it was designed to approach the following aims:

- To assess the response of patients to gleevec by employing cytogenetic analysis.
- To assess the chromosomal abnormality and Ph-chromosome in the blood cells of CML patients treated with gleevec.
- To study the mechanism of resistance to gleevec by cytogenetic analysis, and by measuring the DNA content at different time of cellular growth *in vitro.*

Summary

The present study was conducted to evaluate some cytogenetic and DNA content in blood cells (lymphocyte) from chronic myeloid leukemia (CML) patients. Fifteen patients were randomly selected, in addition to fifteen healthy subjects included as a control group. Three cytogenetic parameters, i.e. the blastogenic index (BI), mitotic index (MI), chromosomal aberrations (CAs), and in particular Philadelphia chromosome (Ph+ve chromosome) were assessed in lymphocytes of cultured peripheral blood. The sensitivity of leukemic cells for two types of drugs, molecular therapeutic (gleevec), and cytotoxic agent (methotrexate (MTX)) was demonstrated *in vitro*. Gleevec was used in four concentrations (0.1, 1, 10, and 100μg/ml), and MTX was used in two concentrations (12.5, and 100μg/ml) of cultured media. However, the patients were categorized according to their response to the drugs, as sensitive (S), moderately resistance (MR), and resistant (R) groups, with a frequency of 46.7%, 13.3%, and 40.0% respectively.

Blastogenic index, MI, and CAs were significantly higher in drug-free cultures of patients, particularly in the R group, when compared with control group. Treatment of cultures with anticancer drugs reduced the mean values of cytogenetic parameters. The two drugs were exhibited maximal cytogenesis effectiveness. On other hand, the (100 μg/ml) concentration of each drug was pointed out to terminate the cytogenetic deviation in the S and MR groups of CML patients. In contrast, patient of the R group (Multidrug resistance) revealed resistance to the four concentrations of gleevec, and two concentrations of MTX.

Summary

The DNA content estimated in patient groups (S, R) and control. Blood cells were cultured at different period time (24, 48, 72, and 96 hr) of incubation when treated with cut-off concentration (100 μg/ml) of gleevec. Resistant group of leukemic patients showed significant elevated levels of DNA content in absence or presence of drug, in comparison with S group of CML patients and controls. From this study we inferred the cytogenetic parameter (BI, MI, CAs, and Ph+ve) were so effectiveness to known patients' response (sensitive or resistance) to drugs and choused the useful one. Moreover, the elevation of DNA content in lymphocyte culture seemed to be correlated with drug resistance.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم ألتقانة ألإحيائية

دراسة وراثة خلوية وتقيس كمية الحامض النووي الرايبي منقوص الأوكسجين لخلايا دم بعض مرضى ابيضاض الدم النقياني المزمن في بغداد

رسالة

مقدمة إلى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في ألتقانة الإحيائية

من قبل

شيماء هاشم النعيمي

بكالوريوس تقانة إحيائية جامعة النهرين ٢٠٠٢

ذي القعدة *١٤٢٧هـ*

كانون الأول *٢٠٠٦م*

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Cytogenetic analysis and DNA estimation of blood cells from some patients with chronic myelocytic leukemia in Baghdad

A thesis
Submitted to the College of Science / AL-Nahrain University
In partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

By

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B. Sc. Biotechnology, Al-Nahrain University. 2002

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Table B-4: Acentric chromosome of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	0.091±0.02 a	0.128±0.02 a	0.230±0.02 b	0.216±0.02 b	0.170±0.03 ab
	0.1	0.078±0.01 a	0.110±0.01 a	0. 200±0.03 c	0.221±0.03 c	0.160±0.03 b
	1	0.050±0.01 a	0.068±0.02 a	0.180±0.03 c	0.183±0.04 c	0.122±0.04 b
	10	0.028±0.01 a	0.036±0.02 a	0.100±0.03 ab	0.231±0.04 c	0.118±0.03 b
	100	ND	ND	ND	0.231±0.04 b	0.096±0.04 a
MTX	0.0	0.091±0.02 a	0.128±0.02 a	0.230±0.02 b	0.216±0.02 b	0.170±0.03 ab
	12.5	0.095±0.01 a	0.111±0.03 a	0.210±0.01 c	0.225±0.05 c	0.163±0.05 b
	100	ND	ND	ND	0.200±0.05 b	0.080±0.03 a

(a, b, c) Sample of significant differences between columns. (ND) Not detected.

Table B-3: Chromatid break of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	0.164±0.01 a	0.368±0.01 b	0.400±0.01 b	0.551±0.02 c	0.444±0.05 b
	0.1	0.146±0.01 a	0.306±0.01 b	0.310±0.01 b	0.531±0.03 c	0.396±0.03 b
	1	0.096±0.02 a	0.215±0.02 a	0.250±0.03 c	0.490±0.04 c	0.327±0.03 b
	10	0.064±0.01 a	0.096±0.02 a	0.250±0.03 b	0.491±0.04 C	0.264±0.05 b
	100	ND	ND	ND	0.471±0.06 b	0.202±0.06 a
MTX	0.0	0.164±0.01 a	0.368±0.01 b	0.400±0.01 b	0.551±0.02 b	0.444±0.05 b
	12.5	0.190±0.02 a	0.281±0.03 b	0.290±0.03 b	0.480±0.05 d	0.361±0.05 c
	100	ND	ND	ND	0.450±0.06 b	0.180±0.06 A

(a, b, c, d) Sample of significant differences between columns, (ND) Not detected.

Table B-2: Chromosome break of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	0.091±0.01 a	0.261±0.02 b	0.320±0.02 c	0.456±0.02 d	0.343±0.03 c
	0.1	0.080±0.01 a	0.227±0.01 b	0.250±0.01 b	0.415±0.03 d	0.304±0.03 c
	1	0.071±0.02 a	0.160±0.02 b	0.250±0.03 c	0.413±0.04 d	0.267±0.03 c
	10	0.049±0.01 a	0.076±0.04 a	0.180±0.01 b	0.405±0.04 d	0.214±0.05 c
	100	ND	ND	ND	0.363±0.03 b	0.152±0.03 a
MTX	0.0	0.091±0.01 a	0.261±0.02 b	0.320±0.02 c	0.456±0.02 d	0.343±0.03 c
	12.5	0.100±0.02 a	0.206±0.03 b	0.230±0.03 b	0.453±0.05 d	0.306±0.05 c
	100	ND	ND	ND	0.386±0.06 b	0.154±0.06 a

(a, b, c, d) Sample of significant differences between columns, (ND) Not detected.

Table B-7: Delation Chromosome of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	0.083±0.01 a	0268±0.03 b	0.270±0.01 b	0.475±0.03 d	0.351±0.04 c
	0.1	0.076±0.01 a	0.245±0.03 b	0.200±0.02 b	0.466±0.03 d	0.330±0.03 c
	1	0.060±0.01 a	0.158±0.02 b	0.180±0.01 B	0.441±0.02 d	0.273±0.01 c
	10	0.003±0.01 a	0.058±0.01 ab	0.180±0.01 b	0.421±0.03 d	0.212±0.02 c
	100	ND	ND	ND	0.440±0.04 b	0.182±0.03 a
MTX	0.0	0.083±0.01 a	0268±0.03 a	0.270±0.01 b	0.475±0.03 d	0.351±0.04 c
	12.5	0.070±0.01 a	0.187±0.02 b	0.170±0.01 b	0.450±0.03 d	0.291±0.03 c
	100	ND	ND	ND	0.370±0.06 b	0.148±0.06 a

(a, b, c, d) Sample of significant differences between columns. (ND) Not detected.

Table B-5: Dicentric Chromosome of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	0.007±0.01 a	0.008±0.01 a	0.020±0.02 b	0.055±0.03 c	0.028±0.02 b
	0.1	0.002±0.01 a	0.008±0.01 a	ND	0.046±0.03 c	0.023±0.02 b
	1	0.002±0.01 a	0.006±0.01 a	ND	0.036±0.03 c	0.018±0.02 b
	10	0.002±0.01 a	0.002±0.01 a	ND	0.028±0.03 c	0.012±0.01 B
	100	ND	ND	ND	0.030±0.01 b	0.012±0.01 a
MTX	0.0	0.007±0.01 a	0.008±0.01 a	0.020±0.02 b	0.055±0.03 c	0.028±0.02 b
	12.5	0.006±0.01 a	ND	ND	0.046±0.03 c	0.016±0.01 b
	100	ND	ND	ND	0.046±0.03 b	0.016±0.01 a

(a, b, c) Sample of significant differences between columns, (ND) Not detected.

Table B-6: Gap Chromosome of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	0.042±0.01 a	0.112±0.01 b	0.110±0.01 b	0.250±0.01 d	0.167±0.03 c
	0.1	0.031±0.01 a	0.071±0.02 a	0.050±0.01 a	0.233±0.03 c	0.134±0.03 b
	1	0.023±0.01 a	0.047±0.02 a	0.050±0.01 a	0.213±0.02 c	0.114±0.03 b
	10	0.008±0.01 a	0.011±0.01 a	0.100±0.01 b	0.193±0.01 d	0.084±0.02 c
	100	ND	ND	ND	0.208±0.03 b	0.083±0.03 a
MTX	0.0	0.042±0.01 a	0.112±0.01 b	0.110±0.01 b	0.250±0.01 d	0.167±0.03 c
	12.5	0.040±0.01 a	0.090±0.02 a	0.030±0.01 a	0.243±0.03 c	0.147±0.01 b
	100	ND	ND	ND	0.208±0.06 b	0.083±0.06 a

(a, b, c, d) Sample of significant differences between columns, (ND) Not detected.

Table B-8: Ring Chromosome of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	ND	ND	ND	0.006±0.00 b	0.002±0.00 a
	0.1	ND	ND	ND	ND	ND
	1	ND	ND	ND	0.001±0.01 b	0.0006±0.01 a
	10	ND	ND	ND	0.003±0.02 B	0.001±0.01 a
	100	ND	ND	ND	0.011±0.01 b	0.004±0.01 a
MTX	0.0	ND	ND	ND	0.006±0.00 b	0.002±0.00 a
	12.5	ND	ND	ND	0.001±0.01 b	0.0006±0.01 a
	100	ND	ND	ND	0.005±0.03 b	0.002±0.01 a

(A, B) Sample of significant differences between columns. (ND) Not detected.

Table B-1: Chromosome aberration of blood lymphocytes obtained from controls and CML patients (S, MR, and R), with out treatment. as (mean ± SE).

Parameter	Control no.(15)	Patient no.(15)			
		S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Total Chromosomal aberration (CAs)	0.328±0.03 a	1.188±0.09 b	1.350±0.01 b	2.275±0.04 d	1.634±0.05 c
Chromosome break	0.091±0.01 a	0.261±0.02 b	0.320±0.02 c	0.456±0.02 d	0.343±0.03 c
Chromatid break	0.164±0.01 a	0.368±0.01 b	0.400±0.01 b	0.551±0.02 b	0.444±0.05 b
Ring chromosome	ND	ND	ND	0.006±0.00 b	0.002±0.00 a
Acentric chromosome	0.091±0.02 a	0.128±0.02 a	0.230±0.02 b	0.216±0.02 b	0.170±0.03 ab
Dicentric chromosome	0.007±0.01 a	0.008±0.01 a	0.020±0.02 b	0.055±0.03 c	0.028±0.02 b
Gap chromosome	0.042±0.01 a	0.112±0.01 b	0.110±0.01 b	0.250±0.01 d	0.167±0.03 c
Deletion	0.083±0.01 a	0268±0.03 b	0.270±0.01 b	0.475±0.03 d	0.351±0.04 c

Philadelphia chromosome(Ph+ve)	ND	0.040±0.00 a	ND	0.300±0.01 c	0.141±0.03 b

(a, b, c, d) Sample of significant differences between columns, (ND) Not detected.

Table 3-3: Cytogenetic analysis of blood lymphocytes obtained from controls and CML patients (S, MR, and R) as (mean ± SE).

Parameter	Control no.(15)	Patient no.(15)			
		S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Age (years)	26.510±6.4 00 a	27.000± 3.40 a	33.000±4.00 ab	41.500±6.10 b	32.830±6.10 ab
Blastogenic index (BI)	40.920±2.00 a	32.013±2.00 a	34.000±4.0 a	40.700±4.80 a	35.150±2.50 a
Mitotic index (MI)	3.210±0.40 a	2.500±0.40 a	2.000±0.50 a	3.710±0.20 a	2.970±0.26 a
Chromosomal aberration (CAs)	0.328±0.03 a	1.188±0.09 b	1.350±0.01 b	2.275±0.04 d	1.634±0.05 c
Ph +ve chromosome	ND	0.040±0.00 ab	ND	0.300±0.01 c	0.141±0.03 b

(a, b, c, d) Sample of significant differences between columns. (ND) Not detected.

Table 3-5: Mitotic index of lymphocytes obtained from CML patients and controls after treatment with gleevec and MTX, as (mean ±SE)

Druge	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	3.210±0.40 a	2.500±0.10 a	2.000±0.50 a	3.770±0.20 a	2.970±0.26 a
	0.1	2.513±0.40 a	1.938±0.20 a	1.400±0.20 a	3.483±1.70 a	2.520±0.15 a
	1	2.073±0.30 a	1.288±0.19 a	1.100±0.05 a	3.017±0.50 a	1.967±0.18 a
	10	1.067±0.30 a	0.362±0.18 a	0.500±0.05 a	2.650±0.50 b	1.287±0.30 a
	100	ND	ND	ND	2.233±0.70 b	0.900±0.40 a
MTX	0.0	3.120±0.40 a	2.500±0.10 a	2.000±0.50 a	3.770±0.2 a	2.970±0.26 a
	12.5	0.560±0.09 a	0.362±0.09 a	0.800±0.20 a	2.016±0.20 b	1.053±0.20 b
	100	ND	ND	ND	0.583±0.07 b	0.233±0.09 a

(a, b) Sample of significant differences between columns. (ND) Not detected.

Table 3-6: Total chromosomal aberrations of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX, as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	0.328±0.03 a	1.188±0.09 b	1.350±0.01 b	2.275±0.04 d	1.634±0.15 c
	0.1	0.206±0.03 a	0.958±0.10 b	1.060±0.01 b	2.185±0.09 c	1.450±0.16 b
	1	0.098±0.01 a	0.657±0.10 b	0.910±0.00 b	2.065±0.10 c	1.237±0.17 b
	10	0.005±0.00 a	0. 290±0.01 ab	0.720±0.01 b	2.030±0.13 c	1.014±0.20 b
	100	ND	ND	ND	2.063±0.18 b	0.855±0.21 a
MTX	0.0	0.328±0.03 a	1.188±0.09 b	1.350±0.01 b	2.275±0.04 d	1.634±0.15 c
	12.5	0.300±0.05 a	0.916±0.01 ab	0.930±0.01 ab	2.126±0.17 c	1.401±0.17 b
	100	ND	ND	ND	1.891±0.04 b	0.756±0.20 a

(a, b, c, d) Sample of significant differences between columns. (ND) Not detected.

Table 3-7: Ph +ve chromosome of lymphocytes obtained from CML patients and controls after treatment with Gleevec and MTX, as (mean ±SE)

Drugs	Doses (µg/µl)	Control no.(15)	Patient no.(15)			
			S no.(7)	MR no.(2)	R no.(6)	Total no.(15)
Gleevec	0.0	ND	0.0400±0.00 a	ND	0.3000±0.01 b	0.1410±0.03 b
	0.1	ND	0.0200±0.00 a	ND	0.2700±0.04 b	0.1160±0.03 b
	1	ND	0.0012±0.00 a	ND	0.2850±0.05 b	0.1140±0.04 b
	10	ND	ND	ND	0.2550±0.04 b	0.1020±0.03 a
	100	ND	ND	ND	0.2860±0.05 b	0.1140±0.04 a
MTX	0.0	ND	0.4000±0.00 a	ND	0.3000±0.01 b	0.1410±0.03 B
	12.5	ND	0.0262±0.01 a	ND	0.2430±0.05 b	0.1110±0.03 B
	100	ND	ND	ND	0.2150±0.05 b	0.0800±0.03 a

(a, b) Sample of significant differences between columns. (ND) Not detected.

Table 3-8: Blastogenic Index of blood lymphocytes obtained from Controls and CML patients cultured with and without PHA after treatment with Gleevec and MTX, as (mean ±SE)

Drugs	Doses (µg/µl)	Patients No(15)		Controls No.(15)	
		With PHA	Without PHA	With PHA	Without PHA
Gleevec	0.0	35.150±2.50 a	16.380±1.50 b	40.920±2.00 a	0.000±0.00 c
	0.1	32.027±2.50 a	14.450±1.10 b	37.980±1.70 a	0.000±0.00 c
	1	29.567±2.20 a	12.370±1.00 b	35.687±1.78 a	0.000±0.00 c
	10	25.420±2.10 a	9.900±1.00 b	32.793±1.50 a	0.000±0.00 c
	100	24.470±2.20 a	7.880±1.30 b	30.187±1.08 a	0.000±0.00 c
MTX	0.0	35.150±2.50 a	16.380±1.50 b	40.920±2.00 a	0.000±0.00 c
	12.5	30.920±2.00 a	12.370±1.10 b	33.580±1.90 a	0.000±0.00 c
	100	24.593±2.00 a	7.510±1.00 b	29.853±1.90 a	0.000±0.00 c

(a, b, c) Sample of significant differences between columns

Table 3-9: Cells accounted of lymphocytes obtained from CML patients and controls in presence and absence (100 µg / ml) of Gleevec under different period of incubation as (mean×10^5 cells/ml ± SE)

Gleevec µg / ml	Time of incubation (hour)	Control No.(15)	Patient		
			S no.(6)	R no.(7)	Total no.(15)
0	24	3..952±0.30 a	5.075±0.02 a	11.267±0.90 a	8.820±0.8 a
	48	6.420±0.01 a	7.850±0.01 a	23.000±3.40 a	16.980±3.20 ab
	27	8.020±0.40 a	12.620±0.90 a	44.000±6.31 c	32.700±4.30 b
	96	12.040±0.80 a	36.500±0.80 b	70.330±6.30 b	67.800±5.80 b
100	24	4.220±0.70 a	6.025±0.90 b	5.567±0.90 b	6.040±.9.00 b
	48	3.440±0.03 a	5.675±0.02 b	6.700±1.20 b	6.080.±1.00 b
	27	1.900±0.30 a	4.000±0.01 b	16.000±4.10 d	11.120±3.20 c
	96	0.930±0.20 a	2.920±0.07 a	35.630±6.03 c	22.520±5.50 b

(a, b, c, d) Sample of significant differences between columns.

Table 3-10: DNA concentration of lymphocytes obtained from CML patients and controls in presence and absence (100 µg / ml) of Gleevec under different period of incubation as (mean× µg / ml ± SE)

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Gleevec µg / ml	Time of incubation (hour)	Control No.(15)	Patient		
			S no.(6)	R no.(7)	Total no.(15)
0	24	0.426±0.01 a	0.532±0.02 a	1.003±0.08 a	0.844±0.07 a
	48	0.619±0.01 a	0.945±0.03 a	1.930±0.05 b	1.610±0.03 ab
	27	0.776±0.02 a	2.260±0.08 b	3.477±0.09 b	2.464±0.09 b
	96	0.960±0.01 a	6.000±0.00 b	6.667±0.05 b	6.400±0.04 b
100	24	0.458±0.03 a	0.582±0.03 a	0.573±0.05 a	0.576±.0.05 a
	48	0.294±0.03 a	0.450±0.02 a	0.673±1.0 b	0.604.±0.10 b
	27	0.185±0.01 a	0.340±0.01 a	1.416±0.2 c	0.994±0.21 b
	96	0.105±0.01 a	0.280±0.01 a	3.260±0.2 b	2.064±0.20 b

(a, b, c) Sample of significant differences between columns.

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