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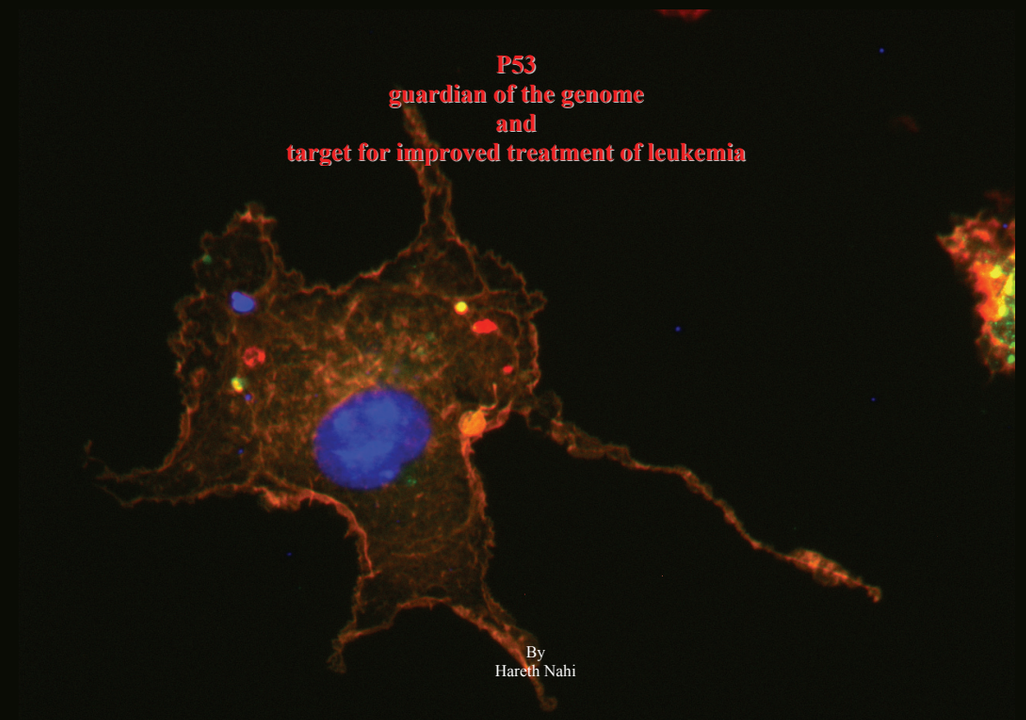

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Summary:

P53 acts in several cellular processes as cell cycle checkpoints, DNA repair senescence and the surveillance of genomic integrity. Wt p53 inhibits cancer processes by causing cell cycle arrest and apoptosis. P53 is strongly regulated by the HDM-2 protein, which builds a complex with p53 leading to proteosomal degradation of p53. HDM-2 in turn is regulated by the $p14^{ARF}$ protein. The *p53* gene is located on 17p13 and chromosome 17p abnormalities are strongly associated with *p53* mutations by loss of one *p53* allele and point mutation of the remaining. The gene for $p14^{ARF}$ is encoded on chromosome 9 in 9p21. The objectives were to identify chromosomal aberrations of importance for p53 function in leukemia and to study if small molecules can restore impaired p53 function. Leukemic cells were isolated from 399 adult patients with AML and chromosomal analysis was successful in 336. After incubation with cytostatics and culturing, in vitro sensitivity assay was performed by measuring ATP. Chromosomal analysis showed 39% normal karyotype. Of adverse aberrations, 16% had complex karyotype and 10% abnormal 7. Of abnormalities affecting p53, 7% (24 patients) had abnormal 17 and 13 patients (4%) abnormal chromosome 9. *In vitro*, patients with abnormal 17 were significantly more resistant against antileukemic cytostatics compared to both normal and complex karyotype. Although abnormal 9p showed higher drug resistance compared to normal karyotype, the difference was not significant. The shortest survival was seen in patients with abnormal 17, mean survival two months and all died within 11 months. $p<0.0001$ vs normal and <0.05 vs complex karyotype. Also patients with abnormal 9 had significantly shorter survival (mean 5 months) compared to normal karyotype. These findings prompted us to study the effect of two small molecules with a potential to restore the p53 function. PRIMA-1 can restore wild type confirmation and specific DNA binding of mutant p53 and trigger apoptosis in mutant tumor cells. RITA affects wt p53 by inducing intracellular accumulation of p53 and by preventing p53-HDM-2 interaction. Samples from 14 CLL patients incubated with 0.1-2.5µM PRIMA-1 and 1-2µM fludarabine. Drug sensitivity was measured with the ATP method and apoptosis by Annexin V. PRIMA induced pronounced apoptosis in the leukemic cells. Samples with mutated *p53* were significantly more resistant to fludarabine and when combined with PRIMA-1, synergy was observed. 62 AML samples were incubated with PRIMA.1 5-20 µM. The p53-mutated cells were significantly ($p<0.002$-0.00001) more sensitive to PRIMA-1 and more resistant to Ara-C ($p<0.0002$). In contrast, incubation with RITA 0.1-1 µM in AML and 5-10µmM in CLL showed a significantly higher effect in samples without abnormal 17. In both AML and B-CLL cells exposure to RITA and RITA combined with PRIMA-1 resulted in time and dose dependent induction of intracellular p53. The increase was highest in AML cells. Co-incubation with PRIMA-1 and RITA showed a synergistic effect in the majority of AML samples. While combining RITA with fludarabine resulted in 100% synergy in CLL. We conclude that genetic abnormalities related with mutations in the HDM-2-p53 pathway occur in about 11 percent of patients with adult AML and that this is correlated to intracellular drug resistance and poor survival. Small molecules targeting the p53 function appears to be a promising way to improve the outcome of these patients.

Contents

LIST OF PAPERS

This thesis is based on the following papers;

I. **H. Nahi**, S. Lehmann, S. Bengtzen, M. Jansson, L. Möllgård, C. Paul, M.Merup. Abnormal chromosome 17 in AML predicts drug-resistance and a short overall survival.
Submitted for publication

II **H. Nahi**, S. Lehmann, M.Merup, L. Möllgård, S. Bengtzen, Slivanova G, K.G. Wiman, C. Paul, M.Merup. Effects of PRIMA-1 on chronic lymphocytic leukaemia cells with and without hemizygous p53 deletion.
British Journal of Haematology 2004 May 13; 127, 285–291

III **H. Nahi**, , M.Merup, S. Lehmann, S. Bengtzen, L. Möllgård, Slivanova G, K.G. Wiman, C. Paul. PRIMA-1 induces apoptosis in acute myeloid leukaemia cells With p53 gene deletion.
British Journal of Haematology 2005 July 21; 132, 230–236

IV **H. Nahi**, S. Lehmann, S. Bengtzen, L. Möllgård, H. Concha, A. Svensson, K.G. Wiman, G. Selivanova, M. Merup, C. Paul. Mutated and non mutated p53 as a therapeutic target in the treatment of leukaemia.
Submitted for publication

ABBREVIATIONS

AML	Acute Myeloid Leukemia
AP	accelerated phase
ARF	alternative reading frame
ATL	adult T-cell leukaemia/lymphoma
ATM	ataxia telangiectasia mutated gene
ATP	Adenosine tri-phosphate
Bax	BCL2-associated X protein
BC	blast crisis
bcl-2	B-cell lymphoma/leukemia-2
CDKs	cyclin-dependent kinases
C-myc	myelocytomatosis
CP	chronic phase
CR	Complete Remission
E3	ubiquitin ligases
FasL	Fas ligand
HDM-2	human double minute-2
HDM-2	human double minute-2 gene
MDM-2	mouse double minute-2
MDM-2	mouse double minute-2 gene
MDR	multi drug resistant
Mt	mutated type
NHL	Non Hodgkin lymphoma
HG	high grade
LG	low grade
LP	lymphocyte predominant
P53	p53 protein
P72	p72 protein
***p*53**	p53 gene
PIDD	P53-induced protein with a death domain
Ph-/+	Philadelphia chromosome negative/positive
P-gp	P-glycoprotein
PRIMA-1	P53-dependent Reactivation and Induction of Massive Apoptosis
PUMA	p53 up-regulated modulator of apoptosis
RAEB	refractory anaemia with excess of blasts
ras	retrovirus-associated DNA sequences
RB	retinoblastoma protein
RB	retinoblastoma gene
RITA	Reactivation of p53 and Induction of Tumour cells Apoptosis
RS	Reed-Sternberg cells
WAF1	wt-p53 activated fragment (also known as *p21*)
Wt	wild type

GENERAL INTRODUCTION

P53, the story behind the discovery:

P53 was identified in 1979 by Arnold Levine, David Lane and William Old, working at Princeton University, Dundee University (UK) and Sloan-Kettering Memorial Hospital, respectively. The gene encoding p53 (*p53*) was cloned from neoplastic rodent and human cells. To begin with, scientist thought that p53 hade an oncogenic activity when expressed in cells at the laboratory. The *p53* was classified as an oncogene. After more than a decade from its discovery, scientist released that they were working with the mutant type of p53. In 1989, Bert Vogelstein, Ray White, University of Utah, and their colleagues showed that p53 is actually a tumour suppressor, and that it is altered in the majority of colon cancers. Knowing that, an extensive research has been done to understand the function of wild type p53. In the nineties, more than twenty thousand scientific reports were published on that subject. P53 protein was voted as the molecule of the year by the Science magazine year 2000.

Another historical event that contributed to the understanding of the role of p53 in tumour and oncogenesis is a syndrome called Li-Fraumeni. This syndrome was described in 1969 after analysing more than 600 paediatric sarcoma patients. It showed to be a hereditary cancer susceptibility syndrome predisposing children and young adults to sarcomas, lymphomas, breast, brain and other tumours. This syndrome is shown to be a result of germline mutations in p53(Malkin and Friend 1993).

P53 and hematological malignancies:

Acute myeloid leukemia (AML)

AML is the most common leukemia in adult with an incidence about 3.5/100000 (Astrom*, et al* 2000). The incidence increase with age and more than 75% of all patients are older than 60 years at diagnosis. Untreated AML is rapidly fatal. Combined chemotherapy and in some cases allogeneic stem cell transplantation is standard treatment leading to temporary remission in a majority of the patients. Despite this, most of the patient relapse and die from their disease and the long term survival/cure is only around 20%.

AML, classification

AML is a heterogeneous disease with pronounced variations in the clinical picture and therapeutic outcome. Molecular studies have revealed an array of genotypes resulting in AML, but also clinical similarities have been identified in cases with common genetic background. Morphology is one of the cornerstones for diagnosis and provides the basis for the first widely accepted classification system developed by the joint efforts of French-American-British (FAB) hematologists in 1976, (Bennett*, et al* 1976). Chromosome aberrations are today essential in the AML classification because they have both diagnostic

and prognostic value (Mrozek, *et al* 1997). The new WHO classification of myeloid neoplasms combines criteria of the French-American-British (FAB) system with information obtained by cytogenetic analyses taking into consideration the genetic background of the disease. (Harris, *et al* 1999).

Table-1. Acute myeloid leukaemia's (AML), FAB and WHO classification

AML classification	
FAB	**WHO**
M0: minimally differentiated	**AML with recurrent cytogenetic translocations**
M1: myeloblastic leukaemia without maturation	AML with t(8;21)(q22;q22) AML1/CBFalpha/ETO
M2: myeloblastic leukaemia with maturation	Acute promyelocytic leukaemia: AML with t(15;17)(q22;q12) and variants PML/RARalpha
M3: hypergranular promyelocytic leukaemia	AML with abnormal bone marrow eosinophils inv(16)(p13;q22), t(16;16)(p13;q22) CBFbeta/MYH1
M4: myelomonocytic leukemis	AML with 11q23 MLL abnormalities
M4Eo: variant, increase in marrow eosinophils	**AML with multilineage dysplasia**
M5: monocytic leukaemia	With prior MDS
M6: erythroleukaemia (DiGuglielmo's disease)	Without prior MDS
M7: megakaryoblastic leukaemia	**AML with myelodysplastic syndrome, therapy related**
	Alkylating agent related
	Epipodophyllotoxin related
	Other types
	AML not otherwise categorized
	AML minimally differentiated
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukaemia
	Acute monocytic leukaemia
	Acute erythroid leukaemia
	Acute megakaryocytic leukaemia
	Acute basophilic leukaemia
	Acute panmyelosis with myelofibrosis

AML, chromosome aberrations

Karyotypes in AML are usually classified into three groups, i.e. favourable, intermediate or adverse type according to the criteria reported by *Grimwade et al*. The favourable group include all patients with t(8;21), t(15;17) and inv(16), regardless of whether other cytogenetic abnormalities are present, and the adverse group include patients with −5, −7, del(5q), abnormal 3q, or complex karyotype. Cytogenetic abnormalities not classified as favourable or

adverse karyotype are usually categorized into the intermediate group. Loss of material from the short arm of chromosome 17 is reported in 5-8% in de novo AML ((Fenaux, *et al* 1992), (Stirewalt, *et al* 2001). In relapse or resistant disease the frequency is higher and in therapy related AML this aberration has been reported to occur in up to 36% of the cases.

Table 2. Frequency and Percentage of Cytogenetic Abnormalities (*Grimwade et al, blood 1998*)

		Age Group (yr)		
Abnormality	All Patients No. (%)	0-14 No. (%)	15-34 No. (%)	35+ No. (%)
No abnormality	680 (42)	91 (27)	177 (38)	412 (51)
t(15;17)	198 (12)	31 (9)	87 (19)	80 (10)
+8	148 (9)	46 (14)	47 (10)	55 (7)
t(8;21)	122 (8)	41 (12)	28 (6)	53 (7)
Complex	95 (6)	19 (6)	29 (6)	47 (6)
−7	61 (4)	12 (4)	16 (3)	33 (4)
11q23	60 (4)	26 (8)	21 (5)	13 (2)
inv(16)	57 (4)	16 (5)	26 (6)	15 (2)
+21	45 (3)	20 (6)	13 (3)	12 (1)
abn(3q)	40 (3)	6 (2)	15 (3)	19 (2)
del(7q)	32 (2)	7 (2)	8 (2)	17 (2)
del(5q)	28 (2)	4 (1)	5 (1)	19 (2)
−5	26 (2)	2 (1)	8 (2)	16 (2)
del(9q)	25 (2)	12 (4)	5 (1)	8 (1)
+22	22 (1)	4 (1)	9 (2)	9 (1)
Other numerical	219 (14)	61 (18)	64 (14)	94 (12)
Other structural	366 (23)	108 (32)	86 (19)	172 (21)

It is clear that cytogenetic analysis is the most important factor to differentiate AML patients in prognostic groups. The challenge is the identification of critical genes in AML and targeted molecular therapy in combination with conventional cytostatic drugs might further improve the outcome for these patients.

Table 3. CR Rates, Survival, and Relapse Risk by Individual Abnormalities (*Grimwade et al, Blood 1998*)

Abnormality	Total No.	Deaths in Remission %	Relapse Risk at 5 yr %	Overall Survival at 5 yr %
Overall	1,612	14	49	44
Favorable				
t(15;17)	198	13	37	63
t(8;21)	122	15	29	69
inv(16)	57	9	42	61
Intermediate				
No abnormality	680	15	53	42
+8	148	12	44	48
11q23	60	9	47	45
+21	45	11	50	47
del (7q)	32	19	59	23
del (9q)	25	9	39	60
+22	22	13	51	59
Other numerical	219	19	60	29
Other structural	366	14	51	35
Adverse				
Complex	95	12	68	21
−7	61	8	80	10
abn(3q)	40	20	85	12
del(5q)	28	14	85	11
−5	26	12	90	4

AML, treatment and drug resistance

The combination of the nucleoside analogue Ara-C and anthracyclines as daunorubicine or idarubicine is widely accepted as induction therapy in patient with AML. Other drugs that are used either in induction or consolidation are the topoisomerase inhibitors (etoposide), the DNA-intercalator (AMSA) and the two nucleoside analogues Cda and fludarabine. To prevent rapid relapse post remission treatment (consolidation) is needed, which most often based on a combination of different cytostatics design. The aggressiveness of consolidation treatment depends on cytogenetic group, number of cytostatic courses needed to reach CR (<5% blasts in the bone marrow), age at diagnosis and in some centre minimal residual disease. Younger patients with adverse karyotype should, if possible, be treated with allogeneic stem cell transplantation while elderly with the same chromosomal aberration might receive palliative

treatment. Tumor cells have the ability to develop a number of mechanisms to protect them against the drugs such as change of the molecular target, increased DNA repair and rapid transportation of the drug out from the cell. Multidrug resistance (MDR) is a concept comprising cellular resistance against a number of chemically not related cytostatics and it is associated with clinical drug resistance.

Chronic Lymphatic Leukaemia (CLL)

CLL is the most common type of leukaemia in the western world, accounting for around 30% of all leukaemias (Caligaris-Cappio and Hamblin 1999)**.** The clinical course of CLL shows a marked heterogeneity, from a rapid progressive disease at diagnosis which demands an immediate therapy, to a quiet, non progressive disease which does not require any treatment. In CLL, as well as in other indolent lymphomas, cure is not attainable for the vast majority of patients and the aim of therapy is to slow down or prevent progression. When treatment is necessary according to established criteria, there are nowadays many different treatment options with good effect that might results in a prolonged remission, but unfortunately the disease tends to relapse. If an intensive therapy is to be considered, it should most likely be offered relatively early in the course of the disease to, if possible, avoid development of resistance to chemotherapy. As for AML, no clear genetic predispositions to the develop CLL.

CLL, classification (staging)

In order to stratify CLL patients, Rai and Binet staging are still some times used as supplement to other risk factors. The emerging factor is Ig rearrangements; cases carrying somatically hypermutated rearrangements have milder clinical disease course and better overall survival, while cases in which the Ig sequences remain germline are more severe. Apart from Ig gene rearrangements are the type of chromosomal aberrations are most important for prognosis, as will be discussed below.

Table 4. Rai and Binet staging, * *Lymphoid areas include cervical, axillary, inguinal, and spleen*

Rai staging system		Binet classification	
Stage 0	Stage 0 CLL is characterized by absolute lymphocytosis (>15,000/mm^3) without adenopathy, hepatosplenomegaly, anaemia, or thrombocytopenia.	**Clinical stage A***	Clinical stage A CLL is characterized by no anaemia or thrombocytopenia and fewer than 3 areas of lymphoid involvement (Rai stages 0, I, and II).
Stage I	Stage I CLL is characterized by absolute lymphocytosis with lymphadenopathy without hepatosplenomegaly, anaemia, or thrombocytopenia.	**Clinical stage B***	Clinical stage B CLL is characterized by no a or thrombocytopenia with 3 or more areas of lymphoid involvement (Rai stages I and II).
Stage II	Stage II CLL is characterized by absolute lymphocytosis with either hepatomegaly or splenomegaly, with or without lymphadenopathy.	**Clinical stage C**	Clinical stage C CLL is characterized by anaemia and/or thrombocytopenia regardless of the number of areas of lymphoid enlargement (Rai stages III and IV).
Stage III	Stage III CLL is characterized by absolute lymphocytosis and anaemia (haemoglobin <11 g/dL) with or without lymphadenopathy, hepatomegaly, or splenomegaly.		
Stage IV	Stage IV CLL is characterized by absolute lymphocytosis and thrombocytopenia (<100,000/mm^3) with or without lymphadenopathy, hepatomegaly, splenomegaly, or anaemia		

Chromosomal aberrations in CLL

Mmutations of the *p53* gene occur in about 10% to 15% of CLL cases. Mutations become more frequent as the disease progresses and predict aggressive disease that will be unresponsive to chemotherapy (Wattel*, et al* 1994), (Fenaux*, et al* 1992), (Dohner*, et al* 1995), (Cordone*, et al* 1998). The of *ATM* gene located at 11(q22-q23) is deleted in about 11-18% of CLL cases and these patients usually present with lymphadenopathy and have poor survival (median 79 months) (Dohner et al, 1997 and 2000, Hernandez et al, 1995; Neilson et al, 1997; Dewald et al, 2003). Other cytogenetic abnormalities include trisomy 12, occurring in about 16–25% and del(13)(q14.3), which is the most common chromosome abnormality in CLL accounting for approximately 60% of the cases. These patients have good prognosis (Dohner et al, 2000).

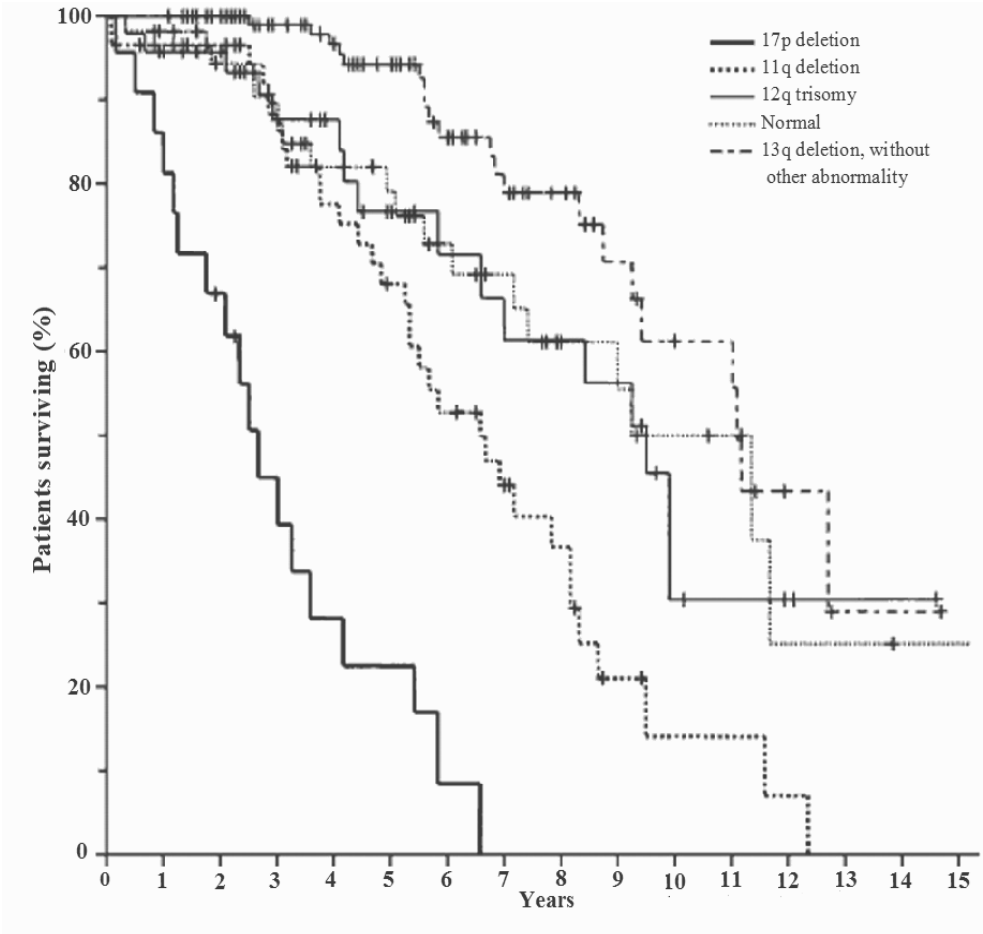
Table 5. Incidence of chromosomal abnormalities in 325 patients with chronic lymphocytic leukaemia, *Dohner et al, NEJM, 2000.*

Aberration	Number of patients (%)
13q deletion	178 (55)
11q deletion	58 (18)
12q trisomy	53 (16)
17p deletion	23 (7)
6q deletion	21 (6)
8q trisomy	16 (5)
t(14q32)	12 (4)
3q trisomy	9 (3)
Clonal abnormalities	268 (82)
Normal karyotypes	57(18)

Predicting survival in CLL

Although clinical stage (i.e. Rai and Binet stages) is a strong predictor of survival, additional prognostic parameters, including patterns of bone marrow (BM) infiltration, lymphocyte doubling time (LDT), immunophenotype and cytogenetics, have now been identified (Molica*, et al* 1995). Conventional cytogenetiv studies by chromosome banding are difficult in CLL, mainly because of the low in vitro mitotic activity of the tumor cells, which leads to poor quantity and quality of metaphase spreads (Stilgenbauer*, et al* 2000). In most cases, CLL does not show specific reciprocal chromosomal translocations as found in other haemopoietic malignant diseases. The genomic instability of CLL results in numerous different types of chromosomal losses and gains, giving rise to unsettled karyotypes among individuals with this disease (Guipaud*, et al* 2003). Aberrations of the *p53* gene are one of the most predictive molecular markers for resistance to first-line therapy and short overall survival (median 32 months).

Figure 3. Overall survival in different cytogentic groups, *Dohner et al, NEJM, 2000.*



CLL, treatment

It is well established that treatment in CLL patients without symptoms does not improve the overall survival; therefore the principal of wait and see is often applicable. If treatment is indicated fludarabine alone but mostly in combination with cyclophosamide is becoming a standard treatment for most patients. For elderly chlorambucil is an optionsince administration is easy and toxicity is limited.

p53 and other tumor suppressor genes in hematological malignancies

There are a growing number of tumour suppressor genes that has been identified to play an important role in the development and progression of haematological malignancies. Below, is defining of the incidence of *p53* mutations in different form and stages of haematological malignancies. These mutations occur in a much lower frequency in haematological malignancies compared to solid tumours, but tend to be more frequent as the haematological

disease progress, as most of these eventually will do. Other genes that deserve to be mentioned are RB, $p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$. Abnormalities in these genes either by mutations or altered structure of the product is a frequent phenomenon in haematological malignancies.

*Chronic Myeloid Leu*kemia (CML*)*

The Philadelphia (Ph) chromosome, t(9;22) (q34;q11) is detected in all cases of chronic myelocytic leukaemia (CML). The result of this translocation is a protein called the *Bcr-Abl,* a tyrosine kinase which is constitutively active. *Bcr-Abl* is considered to be the cause of the chronic phase of CML. Clinically CML is divided in to three different phases, an initial or chronic phase, accelerated phase and blast crisis. In the chronic phase the dominant chromosomal aberration is t(9;22) (q34;q11), while mutations in *p53* and *Rb1* do not occur at this phase. Eventually CML will transform into a blast crisis and other molecular and chromosomal aberrations are associated with this transformation. Mutation in *p53* and *Rb1* are considered to be responsible for this clonal evolution and these are detected in 30% and 20%, respectively, of CML cases at blast crisis (Di Bacco*, et al* 2000), (Ahuja*, et al* 1991).

Acute Lymphoid Leukemia (ALL)

The *p53* gene is altered in very few cases of ALL; but, proteins involved in the p53 pathway are frequently mutated. Deletion or transcriptional silencing $P14^{ARF}$, an inhibitor of HDM-2 is a frequent event in ALL (Calero Moreno*, et al* 2002). Another proteint which is frequently deleted (50%) is the p21 (Roman-Gomez*, et al* 2002).

Multiple Myeloma (MM)

The abnormalities in chromosome 13 may represent the most significant prognostic factor in MM, they occur in approximately 40% of all cases. P53 deletions detected by FISH occurs in approximately 5% of myeloma patients at diagnosis, 20 – 40% in patients with advanced myeloma, while it is only detected in 10% using conventional chromosomal analysis. This difference might be explained by the fact that the *p53* deletions in MM are small interstitial deletion on a narrow region at chromosome 17p (Terpos*, et al* 2006). Deletion of *p53* can be responsible for disease progression (Chang*, et al* 2004).

Non Hodgkin lymphoma (NHL)

NHL is a heterogeneous group of lymphoid malignancies. Classification and reclassification seems to be a continuous and a never ending project, probably because of the heterogeneity of the group. Genetic lesions occurring in NHL will lead to activation of proto-oncogenes such as *BCL*-1 in mantle cell, *BCL*-2 in follicular, *BCL*-6 in diffuse large cell and c-*MYC* in Burkitt's lymphoma. Beside those proto-oncogenes, aberrations of the *p53* gene is the only tumour suppressor gene known to be involved in NHL (Gaidano*, et al* 1995). Mutations in *p53* occur in 40-60% of Burkitt's lymphoma (Neri*, et al* 1988), (Bhatia*, et al* 1993), in 30% of the diffuse large cell lymphomas (Kremer*, et al* 2003) and approximately in 10% of follicular lymphomas (Gaidano*, et al* 1991).

Hodgkin's disease (HD)

The hallmark in the pathohistological diagnosis of HD is the identifications of the Reed-Sternberg (RS), bizarre multinucleated giant cells. These RS cells are considered to be the malignant clone of HD, despite that they constitute only about 2% of tumour burden (Gruss and Kadin 1996). One can detect a very high immunoreactivity of the p53 protein RS cells in about 40% of all HD subtypes, with the exception of nodular LP (lymphocyte predominant) cases, (Gupta*, et al* 1992), (Doussis*, et al* 1993). However point mutations in the p53 gene in HD are very rare, that suggest that there are other mechanism behind the high expression of the p53 protein in RS cells.

Myelodysplastic syndrome (MDS)

MDS is a heterogeneous disease affecting the haematopoietic stem cells with a varied risk to transformation into leukaemia. Eighty percent of MDS patients with complex karyotype have mutations in *p53* which occurs in about 14% of MDS cases (Horiike*, et al* 2003). Patients with refractory anaemia with excess of blasts (RAEB) and in patients with RAEB in transformation (RAEB-T), the *p53* mutations are more frequent (Kaneko*, et al* 1995), (Sugimoto*, et al* 1993), (Ludwig*, et al* 1992).

Table 6. Meta-analysis of alterations of the p53 pathway in haematological malignancies, *U Krug et al*, Nature, 2002. AM, mutation of ATM; D, deletion or rearrangement of p53; E, decreased expression of p53; ME decreased expression of MDM-2; M, mutation of p53; MA, amplification of MDM-2.

	p53	%		*p53*	%
AML/MDS	**59/351**	**17% (D)**	**ALL**	**35/690**	5% (M)
unselected	**150/1482**	**10% (M)**		**11/64**	17% (ME)
	7/23	**30% (E)**		**10/21**	48% (D)
	79/201	**40% (ME)**			
-therapy-	**15/77**	**19% (D)**	**ATL**	**19/66**	29% (M)
related	**27/89**	**30% (M)**			
CML			**HG NHL**	**12/93**	13% (D)
-BC/AP	**51/248**	**21% (D)**		**134/674**	20% (M)
	29/151	**19%(M)**		**29/52**	56% (ME)
-CP	**6/175**	**3% (D)**	**LG NHL**	**18/120**	15% (D)
	0/124	**0% (M)**		**65/526**	12% (M)
				26/72	36% (ME)
Ph- MPD			**CLL**	**23/99**	23% (D)
-BC	**10/27**	**37% (M)**		**52/399**	15% (M)
-CP	**2/110**	**2% (M)**		**16/76**	21% (MA)
MM	**1/37**	**3% (M)**	**HD**	**0/13**	0% (M)
	49/59	**83%(ME)**		**4/6**	67% (MA)

FUNCTIONS OF THE P53 PROTEIN

Normal cells usually have a very low expression of p53, which has a short half-life due to rapid turnover mediated by ubiquitination and proteolysis, (el-Deiry 1998). Different stimuli can trigger the activation of p53. Various forms of stress, such as DNA damaging drugs, ionizing and ultraviolet radiation, viral infections, hypoxia, or hyper proliferation rapidly induce a transient increase in p53 protein, (Chen*, et al* 1996). In tumours, half (50%) of all primary tumours show an over expression of mutated type (mt) p53, (Soussi 1996). Once activated, the p53 protein leads to differentiation, senescence, antiangiogenesis, cell cycle arrest and/or apoptosis. The p53 protein has an important role in growth control and tumour development.

Differentiation

P53 plays a central role in cell differentiation and cell development. In one experiment, HL-60 (a p53-null promyelocytic leukaemia cell line) was exposed to exogenous p53. Cells that expressed high levels of p53 underwent accelerated apoptosis, whereas those expressing low levels of p53 underwent cell differentiation (Ronen, et al 1996). In B-cell differentiation, it is known that wild type p53 can transactivate the promoter of the k light chain gene, suggesting that p53 is directly transactivating B cell differentiation genes, (Aloni-Grinstein, et al 1993). It is also shown that normal human bone marrow express low level of p53 protein when cells are proliferating, however in mature cells, the level is elevated, (Kastan, et al 1991).

Cellular senescence

Cultured mammalian cells will divide a few times but eventually end in a state called replicative senescence, which is an irreversible cell cycle arrest in the G1 phase (Seshadri*, et al* 1993), (Lundberg*, et al* 2000). This state functions as a barrier that the cells have to overcome, otherwise cell death is a fact. Senescence might be a mechanism behind tumour suppression, (Campisi 2000), ((Wynford-Thomas 1999). Cells in the replicative senescence state show up-regulation of cell cycle inhibitory proteins. There are many mechanisms that provoke the cell to enter such state; oxidative damage (von Zglinicki*, et al* 1995) (Chen*, et al* 1995), oncogene activation (such as *ras*) (Serrano*, et al* 1997), (Zhu*, et al* 1998), (Zindy*, et al* 1998), (de Stanchina*, et al* 1998) and telomere shortening (Vaziri*, et al* 1993), (Harley*, et al* 1990), (Allsopp*, et al* 1992). All three will eventually lead to increased expression of cell cycle regulatory proteins that induce the cell into replicative senescence. The regulatory proteins include p19ARF (p14ARF in human cells) and p16INK4A which binds to and sequesters MDM-2 (HDM-2 in human cells), inhibiting the MDM2-dependent degradation of p53 (Lundberg*, et al* 2000).

Antiangiogenesis

Wt p53 is shown to be capable of inhibiting angiogenesis in tumours, by affecting genes regulating new blood vessel formation (Nishimori*, et al* 1997), (Bouvet*, et al* 1998), however the role of p53 in the regulation of angiogenesis is not fully understood.

Cell cycle arrest

It is believed that the ability of p53 to cause cell cycle arrest is due to its function as a transcription factor on specific target genes (Crook*, et al* 1994). P53 is able to cause cell cycle arrest in both G1 and G2 phase. In G1 arrest, p21 is considered to play a central role. P21 is a cyclin-dependent kinase inhibitor that can activate G2, but mainly G1 cell cycle arrest. G2 arrest seems to require the induction of multiple effectors, among these are the 14-3-3 sigma (Hermeking*, et al* 1997), which are proteins involved in the cell cycle control. 14-3-3 sigma can bind p53 and activate its sequence-specific DNA binding (Waterman*, et al* 1998).

Apoptosis

Apoptosis is programmed cell death which removes damaged cells. Although p53 has been shown to be involved in many cellular processes, apoptosis is considered to be the most important. Destroying damaged cells is the optimal way to prevent tumourogenesis and impaired apoptosis can result in cancer progression.

Two pathways of apoptosis are known, mitochondrial and death receptors (Green and Reed 1998), (Ashkenazi and Dixit 1998). Karen H. Vousden (NCI, Frederick, Maryland) called p53 the *natural born killer*, because p53 seems to be involved in both pathways.

Death receptors (the extrinsic pathway)

A death receptor molecule called *Fas* has an important role in removing damaged cells (Nagata 1997) and *Fas* levels increase in case of DNA damage. The *Fas* receptor builds a trimere when activated by the Fas ligand (*FasL*), which causes cleavage of a caspase resulting in a cascade of proteolytic cleavages (Bates and Vousden 1999). P53 can directly stimulate the death receptor. This activity of p53 is transcription independent and both caspase 8 and 9 has been shown to be involved in this activity of p53 (Soengas*, et al* 1999), (Ding*, et al* 2000), (Bates and Vousden 1999).

Mitochondrial pathway (the intrinsic pathway)

The p53 transcription activity, which can both induce and repress expression of numerous genes involved in apoptosis, is well known (Vousden 2000). One of the first to be described as genes targeted of p53 is *bax,* a pro-apoptotic member of the BCL-2 family (Miyashita and Reed 1995). Other proteins in the BCL-2 family promote cell survival such as Bcl-2 and Bcl-xl (Wu and Deng 2002). The BCL-2 family regulates the release of cytochrome C, a process that occurs in both an p53 dependent and independent way (Yang*, et al* 1997), (Kluck*, et al*

1997). Both cytochrome C and pro-caspase 9 are essential in the formation of apoptosis complex (apoptosome). This complex will eventually lead to the activation of caspase 9, a regulator of mitochondrial apoptotic activity (Wu and Deng 2002). Another target gene that belongs to the BCL-2 family is PUMA (p53 up-regulated modulator of apoptosis). Induction of p53 will increase the expression of PUMA, and the product of this gene is multifunctional protein that is involved in mitochondrial pathway of apoptosis, such as mitochondrial release of cytochrome C and activation of pro-caspase 9 (Wu and Deng 2002). The mitochondrial pathway which leads to apoptosis is very rapid, it takes about 30 minutes before the cell ends in apoptosis, while in processes where the death receptor become activated, will take about 2 hours.

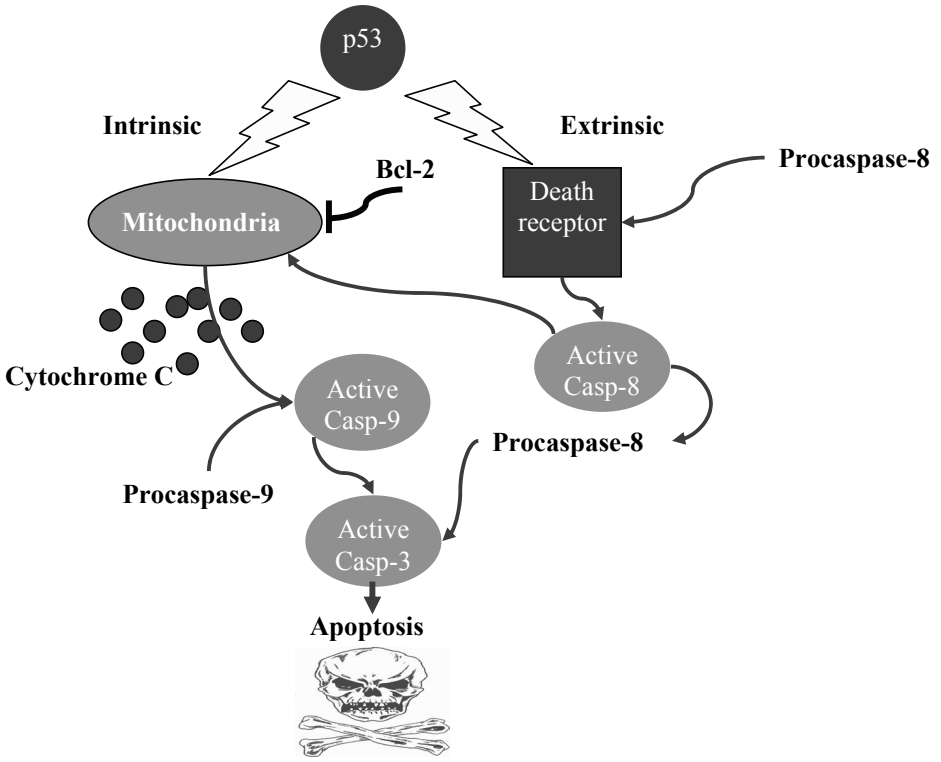


Figure 1. The pathways of apoptosis.

P73, p53-independent apoptosis

Knowing that more than 50% of human cancers carry a mutated p53 gene, a reasonable question will be how these tumour cells manage to undergo apoptosis. Many theories are discussed, one of them includes a p53-related protein called p73 (Ozaki and Nakagawara

2005). The p73 gene is located at chromosome 1p36 (Kaghad, *et al* 1997), a region that is rarely mutated in primary human cancers (Ikawa, *et al* 1999). P73 is induced by DNA-damaging agents. It functions in a similar way as p53, i.e. activated p73 results in either G1/S cell cycle arrest or cell death through apoptosis. P73 is one of several candidate proteins which can promote apoptosis by p53-independent manner.

P53 as transcription factor

Examples of genes that are induced by p53 are: *BAX, PUMA* and *NOXA,* The product of these genes are proapoptotic proteins. Other genes are suppressed by p53 such as *BCL2, BCL-xL* and *SURVIVIN*. P21 is also a transactivation target of p53 but it could also be transactivated by a p53-independent pathway. P21 is essential for the process of differentiation in the cell; p21 is also associated with growth arrest, by binding the CDKs and inhibiting their activity, resulting in unphosphorylation of the retinoblastoma protein, which consequently arrests cells in G1/S phase (Nakanishi, *et al* 1999). There is an inverse correlation between the levels of p53 and p21 in the above mentioned phases (Jiang, *et al* 1995). P21 is also known as WAF1 (wt-p53 activated fragment). *Bcl-2* is another transactivation target of the wt p53, wt p53 down-regulates transcription of this gene, whose product has antiapoptotic effects, i.e. p53 mutation results in up-regulation of bcl-2 and there by that defect apoptotic response (Miyashita, *et al* 1994). Another example of target genes is the *Mdm2*, se below.

MUTATED P53, INHIBITION OF WT P53 AND GAIN OF FUNCTIONS

Mt p53 which conserved the C-terminal domain is capable of tetramerization, a process which is required in a functioning p53 (McLure and Lee 1998), (Pietenpol, *et al* 1994). Mutations in the *p53* gene which do not affect the C-terminal, results in (mt) p53, which has the capacity of tetramerization but no binding affinity to the DNA. Mt p53 can also compete for factors that are important for the functions of wt p53, and by that way, inhibit wt p53 (Herskowitz 1987). Beside competitive inhibition of wt p53, mt p53 shows gain of functions. However, the mechanisms are not fully understood (Lanyi, *et al* 1998), (Lin, *et al* 1995). Mt p53 may gain the capacity of activating promoters of genes that are normally not affected by the wt p53. The MDR1 and c-myc are two examples on these target genes (Chin, *et al* 1992), (Dittmer, *et al* 1993), (Frazier, *et al* 1998). The function of the *myc*-family is generally to stimulate cell proliferation. MDR1 gene is located on chromosome 7 and it encodes the P-gp (Sonneveld and List 2001). P-gp is an ATP-dependent transport protein and its normal function is to protect the cell from toxic substances (Bolhuis, *et al* 1996). When amplified in tumours, it leads to multi-drug resistance by removing cytostatics from cancer cells.

P53 AND CHEMOSENSITIVITY

Haematological malignancies are usually treated with combination of several cytostatics, in some cases also irradiation. Almost all anticancer drugs/agents, at least partly, are effective because they trigger apoptosis (Ferreira, *et al* 1999). As we discussed above in apoptosis section, p53 is the most important factor in regulating and controlling apoptosis. The role of *p53* is to maintain genetic stability, through its participation at many cell cycle checkpoints.

Initiation of cell cycle arrest to allow enhanced DNA repair or elimination of cells with damaged DNA (Kastan, *et al* 1992), (Hoffman, *et al* 2002). Beside clinical data and in vitro studies that point out (mt) p53 as a major factor in drug resistance, there are evidence that wt p53 maintains the levels of P-gp. P-gp is a transmembrane pump with affinity for many drugs used in AML treatment and it acts by removing the drug from intracellular space resulting in lower concentrations (Li, *et al* 1997). While mt p53 seems to up-regulate MDR1 expression (Thottassery, *et al* 1997), the major role of wt p53 is modulation apoptosis induced by anticancer agents (Lowe, *et al* 1993), (Lowe, *et al* 1994).

REGULATION OF THE P53:

The Mdm2-p53 interaction

The *MDM-2* (*HDM-2*) gene is located on chromosome 12 (q14.3-q15). This region encodes a phosphoprotein that binds and inhibits p53. *MDM-2* is also a target gene of p53 and this will result in a negative feedback on p53. MDM-2 is an E3 ligase, that specifically binds to p53 and enhances its degradation via proteasomes (Michael and Oren 2003). This process occurs after relocalization of p53 from the nucleus to the cytoplasm which is an effect of p53-Mdm2 binding (Roth, *et al* 1998)). Furthermore, MDM-2 has also shown to negatively affect the transactivation ability of p53 on its target genes (Momand, *et al* 1992), (Oliner, *et al* 1993).

The ARF–Mdm2 interaction

The $p16^{INK4a}$ is encoded as an alternative reading frame with $p14^{ARF}$ from the INK4a/ARF gene locus at chromosome 9p and these activate the RB and p53 pathways, respectively (Brooks and Gu 2004), (Ruas and Peters 1998). The $p14^{ARF}$ inhibits HDM-2 and blocks its activity on p53 (Lowe and Sherr 2003), which hypothetically leads to stabilization of p53.

The ATM–p53 interaction

The *ATM* gene is located on chromosome 11(q22.3). When double strand breaks is the outcome of DNA damage and these breaks will result in auto-phosphorylation and activation of ATM (Shiloh 2003), (Bassing and Alt 2004), (Meulmeester, *et al* 2005). Almost immediately, activated ATM phosphorylates p53, creating a stabilized p53 with enhanced transcriptional activity (Banin, *et al* 1998), (Lambert, *et al* 1998).

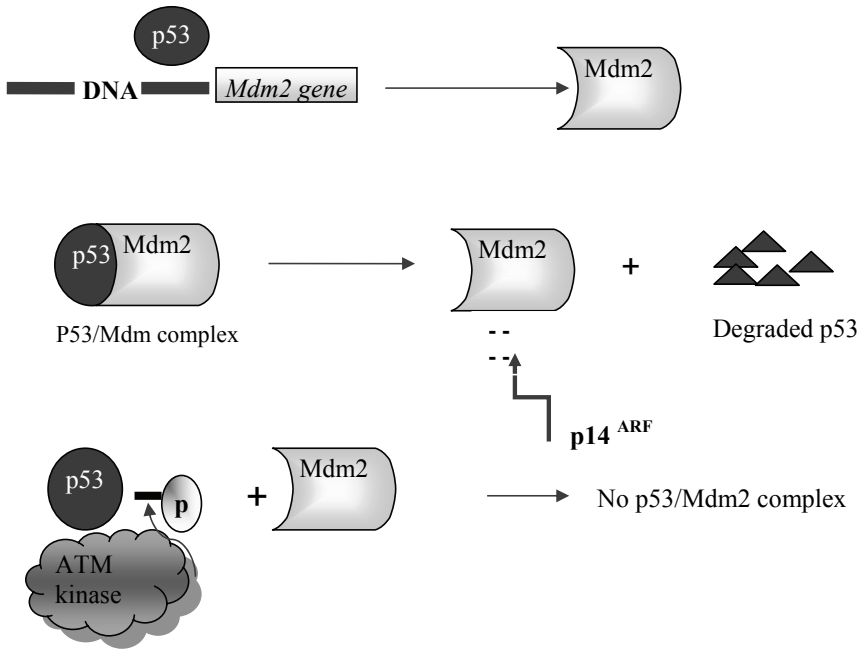


Figure 2. Regulation of the p53.

P53, A GUARDIAN OR A KILLER

The response of the cells to p53 will either result in cell cycle arrest or apoptosis but this choice is not always dependent of p53. It might be the results of co-exiting factors, such as simultaneous alterations in other oncogenic mechanisms or the presence of extra-cellular survival factors (Vousden 2000). The levels of p53 are higher in cases of apoptosis compared to cases of cell cycle arrest (Balint and Vousden 2001), (Chen*, et al* 1996).

INACTIVATION OF P53 PROTEIN

Chromosome 17 abnormalities are commonly associated with mutations of the *p53* gene, located on 17p13. The human *p53* gene consists of 11 exons and 10 introns (Lamb and Crawford 1986). Inactivation of *p53* commonly occurs through loss of one *p53* allele and damage of the remaining allele by point mutation which is a common mechanism for inactivation of tumour suppressor genes.

MUTATIONS AND MISSENSE MUTATIONS OF P53

Until recently, the p53 mutation database contained about 21,000 mutations resulting in up to 1300 various protein products (Soussi*, et al* 2005). The vast majority of these mutations (>80%) are missense mutations. These mutations are often affecting the DNA binding domain

of the p53 and will lead to accumulation in the nucleus (Soussi and Beroud 2001). The final result is a negative inhibition of wt p53 and gain of functions, as mentioned above. Deletions or insertions occur in approximately 8% of the cases, 6% are nonsense mutations and 0.8% are silent mutations (Ferreira, *et al* 1999).

Mutations in p53 usually affect core domain within exons 5-9 (Hollstein, *et al* 1991). Sequencing this region reveals the majority (~80-85%) of p53 mutations although a considerable number (~15-20%) of mutations are outside the core domain. To detect all mutations, one should screen the entire coding domain i.e. exons 2-11 (Casey, *et al* 1996).

Two classes of mutations are identified (Attardi, *et al* 1996). The first type, with preserved conformation as wt p53, but lacking the binding capacity to wt p53 chaperone (Steller 1995), (Haupt, *et al* 1996). The product of this class of mutations is a protein with increased stability, detectable by, immunohistochemical staining in contrast to wt p53 which is not detectable because of its short half-life (Iggo, *et al* 1990). The second affects an amino acid involved in the tertiary structure of the protein resulting in an unstable protein. This irreversible change makes this type of protein not possible to restore, compared to mutations affecting the DNA-binding domain, which could be restored to wt p53 conformation by activating antibodies or peptides (Selivanova, *et al* 1997).

P53-TARGETING IN CANCER THERAPY

Several strategies for targeting p53 are possible such as blocking the binding of HDM-2 to p53, restoring mutant type p53 and direct targeting wild type p53 inhibiting its degradation or increasing its half life. We studied two molecules, one that restores the functions of mutated p53 (PRIMA-1) and the second which binds to wild type p53 and increases its half life in the cell (RITA). PRIMA-1 (P53-dependent Reactivation and Induction of Massive Apoptosis) was identified by screening of the NCI diversity set chemical library using Saos-2-His-273 osteosarcoma cells carrying inducible His-273 mutant p53. PRIMA-1 is a low molecular weight compound that can restore wild type conformation and specific DNA binding of mutant p53 and consequently triggers apoptosis in tumour cells carrying mutant p53. PRIMA-1 also inhibits human tumour xenograft growth in SCID mice. Exposing mutant *p*53 cells to PRIMA-1 resulted in the activation of p53 targets such as MDM-2, p21 and PUMA (Bykov, *et al* 2003), (Bykov, *et al* 2002). RITA (Reactivation of p53 and Induction of Tumour cells Apoptosis) was also identified after screening of the NCI diversity set chemical library. RITA was selected by using a pair of isogenic cell lines (colon carcinoma cell lines) with different p53 status. RITAs chemical structure is 2,5-bis (5-hydroxymethyl-2-thienyl) furan. It affects wt p53 protein in two ways. Firstly by binding to the p53 N-terminal domain, inducing its accumulation in tumour cells lines by increasing its half-life. Secondly, by preventing the p53-HDM-2 interaction both in vivo and in vitro. RITA has cytotoxic effects in tumours with wt p53 protein (Issaeva, *et al* 2004).

AIMS

The overall objective of this thesis was to study the impact of chromosomal changes affecting the p53 pathway for drug sensitivity and survival in adult leukaemia and to evaluate *in vitro* two new drugs aimed to modulate or restore the p53 function.

The specific aims were:

Study I To evaluate how *in vitro* drug resistance was influenced by different chromosomal aberrations in AML, particularly those affecting chromosome 17p and 9p and to study how these aberrations affect the overall survival in AML.

Study II To investigate the importance of p53 mutations for resistance to conventional cytostatics in vitro in CLL and to study the effects of PRIMA-1 on CLL cells with and without p53 mutation. Furthermore we wanted to investigate the effects of co-incubation of PRIMA-1 and fludarabine on CLL samples in vitro.

Study III Study cytotoxicity and apoptosis of PRIMA-1 and conventionally used cytostatics on AML cells with and without abnormalities in chromosome 17p and to investigate if PRIMA-1 is a substrate of the P-gp pump.

Study IV To investigate if RITA is more selective to AML and CLL cells with wild type p53 and if it increases the expression of p53 in these cells. To examine the effects of co-incubation of RITA and PRIMA-1 on cells with and without p53 deletion. To study how RITA affects the cell cycle and apoptosis.

METHODS

Leukaemic cells from patients (paper I, II, III and IV)

For genetic analysis and *in vitro* sensitivity against conventional cytostatics, leukemic cells were collected from 399 patients with AML. For studies of PRIMA-1 samples from 62 patients with AML and 14 patients with CLL were used and for studies with RITA 17 patients with AML and 16 patients with CLL were used. Mononuclear cells were isolated by centrifugation on metrizoate-dextran (lymphoprep, Axis-Shield PoC, Oslo, Norway) as previously described (Tidefelt*, et al* 1992). The leukaemic cells were either fresh or thawed cryo-preserved cells.

Cytogenetics and FISH (paper I, II, III and IV)

Cytogenetic analyses were performed as described by Haglund (Haglund*, et al* 1994). Briefly, the bone marrow was cultured for 1 and 2 days and Quinaqrine mustard was used for the chromosome staining in the AML cases. To describe the cytogenetic abnormalities, the International System for human Cytogenetic Nomenclature (1985 and 1995). In CLL cases, FISH analyses were performed according to protocol provided by the manufactures as previously described (Merup*, et al* 1998). FISH-probes in use were CEP12 (Vysis Inc, IL, USA) corresponding to the centromere of chromosome 12, *p53* probe (Vysis), 13q14.3 deletion probe (Cytocell, Banbury, UK). For detection of 11q-deletion either MLL Dual colour Break apart (Vysis) or ATM (Q-BIO gene, Illkirch, France) were used. ATM was considered to be the gene of interest and the commercial probe was used as soon as it was commercially available. The ATM and MLL genes are 10Mb apart but any larger deletion would be detected with either probe.

Incubations and culturing (paper I, II, III and IV)

Fresh or cryo-preserved samples were incubated with PRIMA-1, RITA and a panel of cytotoxic drugs. Thawed cells were cultured at 37o C in 24h before further incubation with the drugs. Incubations with the chemotherapeutic agents were performed in 37°C either continuously for 96h ex. Ara-C, fludarabine, CdA and PRIMA-1 or during 1h ex. daunorubicin. The incubations were designed to mimic the in vivo situation by using concentrations and time courses shown to give the same intracellular concentrations as during treatment in vivo(Sundman-Engberg*, et al* 1993).

In vitro cytotoxicity assay (paper I, II, III and IV)

Viability was assessed by a bioluminescence method measuring intracellular ATP concentrations, as previously described (Rhedin*, et al* 1993). ATP was extracted from leukemic cells by mixing equal volumes (0.5ml) of cell-suspension and 2.5% (trichloroacetic acid). The extracts were assayed immediately or stored in a freezer (-20°C) until analysis. The measurements were performed in an Anthos Lucy 1 luminometer (Hettich Labinstrument AB, Malmö, Sweden). An ATP Kit SL 144-041 (Biothema, Dalarö, Sweden) containing ATP Standard and ATP reagent SL was used for the reaction. The result at each concentration represents a mean of two parallel experiments. The viability was calculated as percent viable cells compared to unexposed controls from the same patients, which was also in duplicate. Samples from patients where the controls grew poorly were not included in the material.

Analysis of apoptosis (paper II, III and IV)

Cells from four CLL and AML patients were incubated with different drugs alone or in combination for 24, 48 and 72h before staining with annexin V (AV) to detect the early stage of apoptosis and with propidium iodide (PI) which allows the analysis of secondary necrotic cells related to loss of cell membrane integrity. AV+/PI-, AV+/PI+ and surviving cells were analysed by flowcytometry (Peng*, et al* 2002). In ***paper IV*** cell cycle analysis was performed. Cells from AML patients were fixed in 70% cold ethanol. After washing, cells were re-suspended in a PBS solution containing RNase and propidium at 37˚C for 30 minutes. FACS analysis was performed on a Becton Dickinson FACSCalibur™ flow cytometer. The proportion of cells in the cell cycle phases, sub-Go/G1, G0/G1, S and G2/M phases were calculated directly by CellQuest™. In ***paper IV*** also a method with immunohisto-chemical staining with addition of Hoechst's staining was used. To detect nuclei, Hoechst 33258 (Sigma) was added to the secondary antibody dilution, fluorescent anti-rabbit IgG (H+L)(Vector Laboratories, USA). Slides were analysed using a Fluorescence microscope Nikon E800, (Nikon Corp. Tokyo, Japan).The apoptotic cells were evaluated as percentage of apoptotic changes in the cell membrane.

Expression of p53 protein in paper IV

The staining of p53 was performed in AML and CLL cells without *p*53 deletion. The cells were analyzed on a FACSCalibur ™ flow cytometer. For CLL samples, p53 levels were measured after 24, 48 and 72h and for AML samples, the levels were measured after 24h, 72h and 96h. P53 expression was also evaluated by immunohisto-chemical staining. Cells were fixed with methanol-acetone (1:1). The slides were washed in PBS. The primary antibody p53 (FL-393) sc rabbit polyclonal IgG (Santa Cruz Biotechnology) were diluted incubated with the cells for 60 min at room temperature in a moistened chamber. The secondary antibody, fluorescent anti-rabbit IgG (H+L) (Vector Laboratories, USA) were diluted and

incubated with the cells for an additional 40 min at room temperature in a moistened chamber. Slides were analysed using a Fluorescence microscope Nikon E800, (Nikon Corp. Tokyo, Japan). P53 expression was evaluated as percentage of positive cells, 200 cells were counted. The cell-lines Na-Malva with Gln-248, p53 mutation and BL60 with Trp-282, p53 mutation were used as positive controls and EW 36 wildtype p53. The negative controls used for each sample, were the primary antibody was replaced by the blocking buffer used for dilution.

Data and statistical methods (paper I, II, III and IV)

In all comparisons of in vitro cytotoxic and apoptotic effects we used unpaired *t*-test for independent samples. Kaplan-Meier analyses were used to estimate overall survival and log-rank test was used to test significance. To compare groups in terms of difference in sensitivity in the in-vitro drug panel, unpaired student *t*-test was used. The significance level was defined as 5% ($p<0.05$). Results are presented as means and 95% standard error (SE). In order to study cytotoxic effect on cells incubated with drug-combination, the additive model was used (Jonsson*, et al* 1998), (Valeriote and Lin 1975). This model predicts that the effect of a combination will be equal to the product of the effects of its constituents. For example, if a drug combination were composed of drugs producing MV values of 40 and 60%, respectively, the combination would be expected to result in an MV value of 24% (0.4× 0.6). An observed combination effect that is larger than predicted by the additive model indicates synergism, whereas a smaller effect represents a subadditive effect.

RESULTS

Impact of cytogenetic abnormalities on long-term survival (paper I)

Out of 399 cases, chromosome analysis was successfully performed in 336 patients. Overall survival was analysed in all patients and the median observation time was 150 months. Patients who were treated with allogenic bone marrow transplantation (n=38) were excluded from this analysis. The overall median survival for all patients (n=361) from the time of diagnosis was 10 months, which did not differ from those with normal karyotype (n=132). Patients with monosomy 7 or deletion 7q (n=35) had a median survival of five months and for patients with a complex karyotype (excluding -7/del.7q) median survival was 3 months. Patients with chromosome 17 abnormalities (n=24) had the shortest survival. Their median survival time was 2 months and no patients survived more than 11 months. Patients with abnormal 9p (n=13) had a significantly shorter overall survival compared to patients presented with normal karyotype.

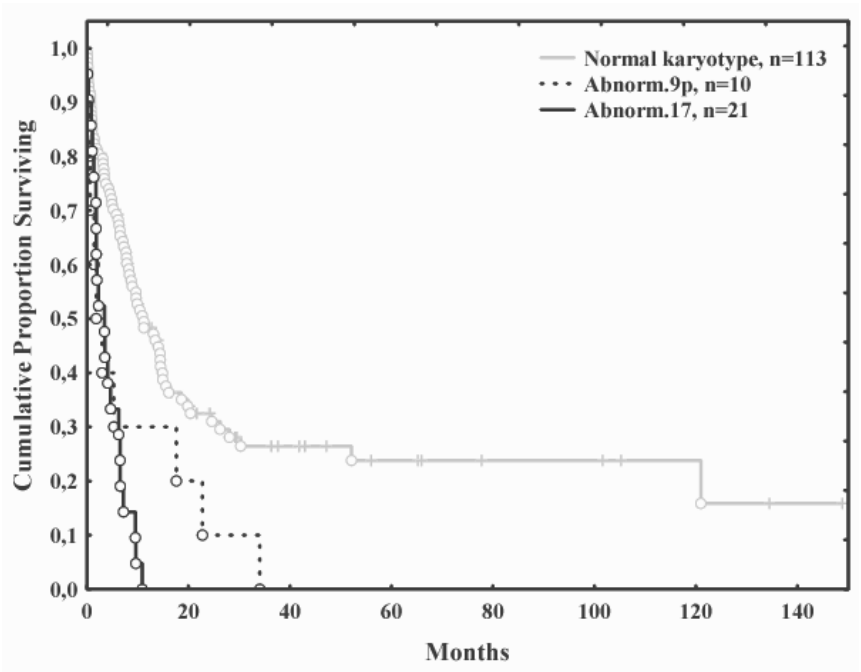


Figure 4. Overall survival of patients with abnormal 17 compared to patients with normal karyotype and an abnormal 9p, p=0.041 when normal and 9pwere compared, p=0.0001 when normal and abnormal 17 were compared.

Thus patients with chromosomal aberration indicating mutations in *p53* or in genes affecting the p53 pathway had a poor survival. Our next step was to study if these aberrations were correlated to in vitro resistance to antileukemic drugs.

In vitro cytotoxicity of antileukemic drugs in AML clones with different cytogenetic abnormalities (paper I)

Results of *in vitro* cytotoxicity are summarized in figure 6. AML cells with an abnormal chromosome 17 were significantly more resistant to all antileukaemic drugs compared to samples with normal karyotype. Samples from patients with an abnormal 9p were more resistant to the drug panel compared to samples with normal karyotype but less resistant compared to cases with an abnormal 17. These differences, however, were not statistically significant.

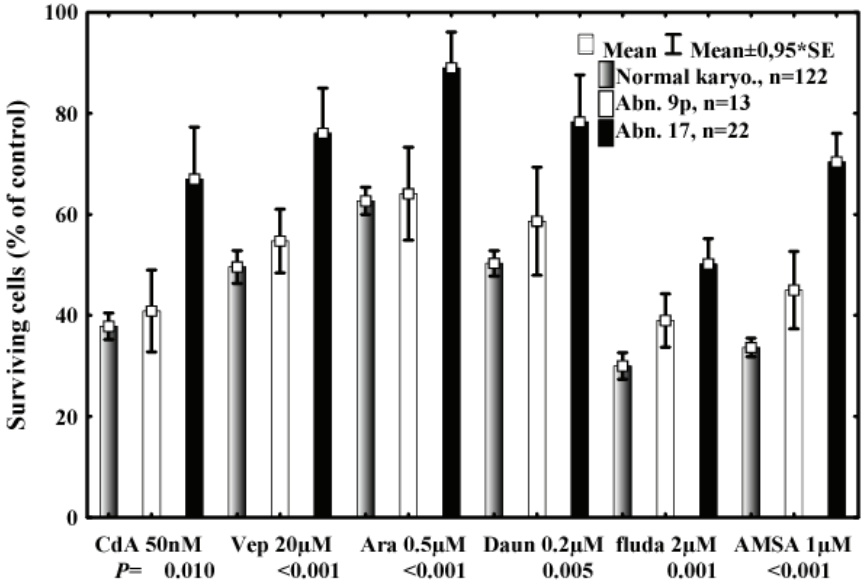


Figure 5. Cytotoxicity of a panel of antileukaemic drugs on myeloblasts from AML patients. Samples with abnormal 17 are compared to normal karyotype cells and abnormal 9p, the *p*-values represents the comparison between normal karyotype and abn.17.

To evaluate *p53* deletion in CLL another method was used.

FISH analysis of the p53 gene in CLL (paper II)

FISH analysis was performed in all 14 patients. Loss of one signal indicating a hemizygous deletion was found in seven patients. The proportion of cells with *p53* deletion varied between 6 and 94% as shown in table I. Loss of signal in less than 10% of the cells was not regarded as a clone. A cut off of 50% was made to separate the groups. Five patients with *p53* deletion in more than 50% of the cells were included in the deleted group.

We then wanted to study the impact of *p53* deletion on *in vitro* drug resistance to clinically used cytostatics in CLL treatment but also the effect of a molecule that can restore the function of mutated p53, PRIMA-1.

Cytotoxicity of fludarabine, doxorubicin and PRIMA-1 on CLL (paper II)

The *p53* deleted samples were more resistant to both fludarabine and doxorubicin as compared to the non *p53* deleted samples. The difference deleted/non-deleted was statistically significant, (p<0,05). A correlation was also seen between the proportion of *p53* deleted cells in the sample and the cytotoxicity of fludarabine. Cells with large clones with *p53* deletion

were most resistant to fludarabine. This was significant at concentrations 0,5 and 1µM ($p <$ 0.005) and at 2µM ($p < 0,05$). *P53* deleted samples were also more resistant to doxorubicin, but the difference was not significant. PRIMA-1 caused dose dependant cytotoxicity on both *p53* and non *p53* deleted CLL cells in all of the cases studied, although the sensitivity differed from one patient to another. There was no statistical significant difference in response between *p53* deleted and non-deleted samples.

Combination of PRIMA-1 and fludarabine (paper II)

In the *p53* deleted samples, additive or synergistic effects were observed in all (100%) incubation with the combinations of PRIMA-1 and fludarabine. In the non p53 deleted samples, additive or synergistic effects were observed in 81% and 19% of incubations resulted in antagonistic or sub-additive effect. The most evident difference between deleted and non-deleted samples was seen with the highest concentration (2,5µM) of PRIMA-1. With PRIMA-1 in this dose, co-incubation with three fludarabine concentrations resulted in additive or synergistic effect in 15/15 combinations in the *p53* deleted (100%) as compared to only 17/27 in the non-deleted samples (63%).

Apoptotic effects of PRIMA-1 on CLL and AML cells (paper II and III)

Annexin V and propidium iodide expression in 4 CLL samples was examined as a marker for induction of apoptosis after 48 h of incubation with PRIMA-1. There was a pronounced variation in the level of annexin V positive cells between the samples but in each patient an increase in the amount of AV positive/PI negative cells was observed by adding PRIMA-1 and with increasing concentrations as illustrated in Figure 6. In AML samples, controls with cells unexposed to PRIMA-1 had 8% (range 3-21 %) AV+/PI- cells. With increasing concentrations of PRIMA-1 the proportion of AV+/PI- cells increased and was 17% (5-60%) at 2.5µM of PRIMA-1, 22%(4-77%) at 5µM and 27%(8-79 %) at 10µM. The *p* values were 0.123, 0.074 at 2.5µM and 5µM, with PRIMA-1 concentration 10µM the difference was significant ($p<0.05$).

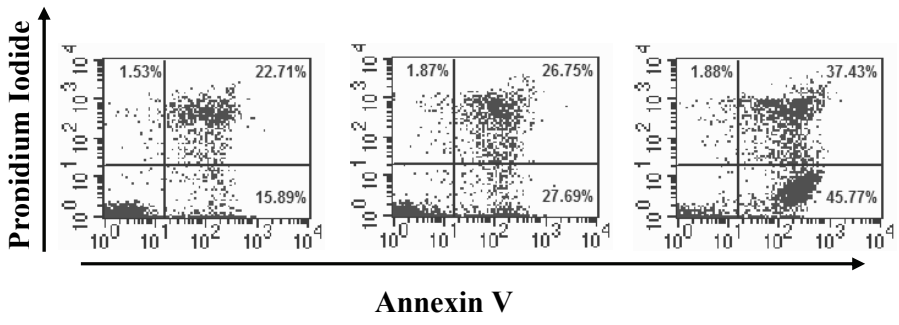


Figure 6. Analysis of **a**poptosis in CLL cells following 48 h of incubation with PBS (A), PRIMA-1 1µM (B) and PRIMA-1 2,5µM (C).

The main objective of next study was to compare the effect of conventional antileukemic drugs and PRIMA-1 on mutated and non mutated AML samples.

Cytotoxicity of antileukaemic drugs and PRIMA-1 on AML cells (paper III)

The AML cells with *p53* deletion were significantly more resistant to Ara-C, CdA and fludarabine compared to cases without *p*53 deletion. For daunorubicin the difference was not significant.

In all cases, PRIMA-1 showed a dose-dependent cytotoxic effect. There was no significant difference in cytotoxic effect of PRIMA-1 in AML samples with complex karyotype (n=10) neither compared to those with normal karyotype (n=16) or to all patients without clonal deletion of p53 (n=52). However, cells from patients with hemizygous *p53* deletion (n=8) showed a markedly increased sensitivity to PRIMA-1. This difference was significant on all dose levels compared to patients without abn 17 (n=52).

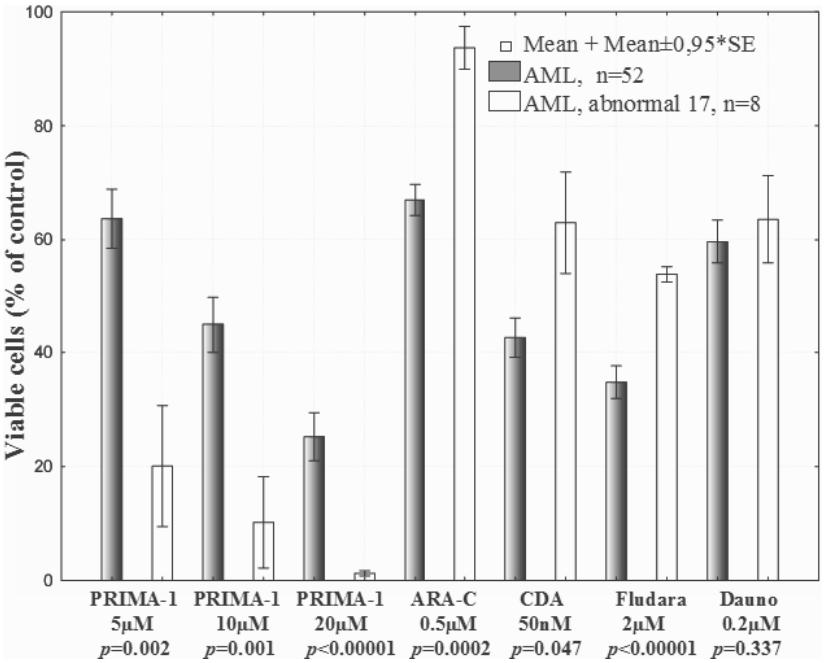


Figure 7. Cytotoxicity of conventionally used drugs and PRIMA-1 in AML on myeloblast. AML with abnormal 17 compare to all other AML cells.

To evaluate if PRIMA-1 was comprised by multidrug resistance, we performed with MDR1 expressing cell line.

Effect of PRIMA-1 on resistant cell-lines with expression of P-gp (paper III)

Cytotoxic effect of daunorubicin and PRIMA-1 was analysed by incubating the HL-60S cell line and the HL-60R (P-gp expressing cell line with a resistant phenotype) for 96 hours. The IC-50 value for daunorubicin was increased by threes logs in the HL-60R cell line as compared to the sensitive cell line. However, after incubation with rising concentration of PRIMA-1 as shown in figure 2A there was no difference in the mean viability and IC-50 value between the two cell-lines. This indicates that PRIMA-1 is not a substrate for P-gp.

In the last part of this thesis we evaluated RITA, a molecule that can increase wt p53 by direct binding it and blocking the p53-HDM-2 complex.

Cytotoxic effects of RITA on AML and CLL cells (paper IV)

RITA induced a dose dependent cytotoxic effect. Contrary to the effects with PRIMA-1 on AML samples, cells with –17 were significantly less sensitive to RITA (0.01 and 0.01 μM) compared to cells with normal chromosome 17p. Furthermore, the IC50 values were 0.1 μM and 0.5 μM for AML cells without and with –17. As with AML cells RITA showed a dose dependent cytotoxic effect on the CLL cells, however at somewhat higher concentrations. CLL cells with p53 deletion were less sensitive to RITA, an effect that was most prominent at 5 μM. The difference in sensitivity was also shown as a difference in IC50 value, being 25 μM in cells with p53 deletion and 10 μM in cells without deletion (Table II).

Since PRIMA-1 had a higher effect in samples with mutated p53 and RITA had significantly more pronounced activity in non mutated cells, it prompted us to study the combined effect of these two substances in mutated as well as in wild type p53 cells.

Co-incubation of PRIMA-1 and RITA in AML cells (paper IV)

PRIMA-1 was co-incubated with two different concentrations of RITA. In samples with -17, synergistic or additive effects were observed in 4 out of 5 examined samples when combining 0.5μM RITA with PRIMA-1, respectively 3 out of 4 samples when using 0.005μM RITA, while the combination in samples without -17 showed synergic or additive effect in 4 out of 5 cases.

In the previous experiments we found that RITA and RITA in combination with PRIMA-1 had pronounced cytotoxic effects on leukemic cells. We then wanted to study the mechanism behind this cytotoxicity.

Induction of apoptosis by RITA and RITA in combination with PRIMA-1 in AML cells (paper IV)

To analyze the apoptotic effects of RITA, cell cycle analysis was performed in AML samples without -17, after exposure to RITA. This revealed an increase in apoptosis, as reflected by an increase in the sub-G1 fraction from 10% with PBS to 15, 25 and 31% after 24h incubation with RITA and the combination of RITA and PRIMA-1. At 72 hours there were 12% of the cells in the sub-G1 fraction in the control and 14, 19 and 28% with increasing doses of RITA and when combining it with PRIMA-1. We confirmed the apoptotic activity of RITA by measuring annexin V binding and propidium iodide uptake. In each sample, a dose dependent increase in the proportion of AV positive/PI negative and AV positive/ PI positive cells was observed. In conclusion these experiments show that RITA induces apoptosis in a dose-dependent manner in AML cells and that this effect was enhanced by PRIMA-1.

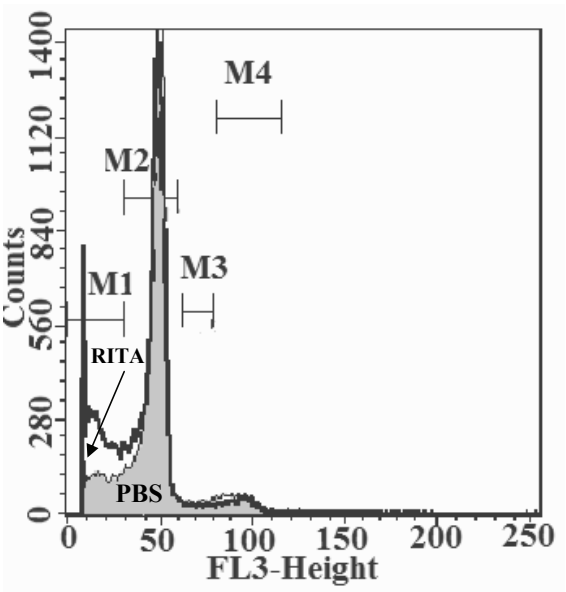


Figure 9. Cell cycle analysis, example of comparison between PBS and RITA 0.5µM after 72h of incubation, the sub-G1 (apoptotic) cells are designated M1, M2=G0/G1, M3=S and M4=G2/M phase.

Thus RITA and PRIMA-1 exert their activity by inducing apoptosis. Next step was to study if an increase of p53 could be a mechanism of action behind this effect.

Effects on p53 protein levels of RITA and RITA combined with PRIMA-1 in AML and CLL cells (paper IV)

To evaluate this we measured the level of p53 protein with flowcytometry in cells before and after exposure to RITA. As the effect of RITA was more prominent in cells without p53 deletion we chose samples from patients without known deletion of one p53 allele. Both AML (incubated with PBS, RITA and the combination of RITA and PRIMA-1) and CLL cells (incubated with PBS and RITA) were analyzed after 24, 72 and 96 hours of exposure. RITA increased the expression of p53 in a dose dependent manner. This effect was also confirmed by immunohisto-chemical staining. The effect of RITA on p53 protein levels could be further enhanced by the addition of 5 µM PRIMA-1, an experiment that was only performed in AML cells.

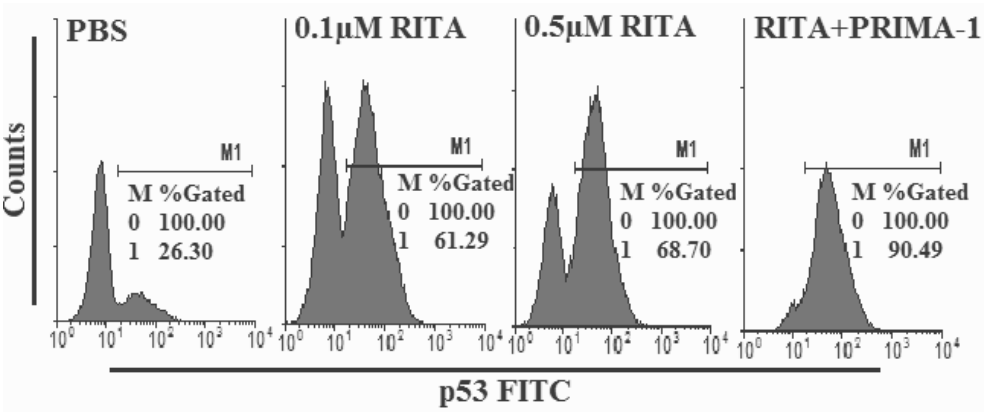


Figure 10. Levels of p53 protein, measured by FACS in AML cells.

DISCUSSION

Mutation of the *p53* tumour suppressor gene is the most prevalent genetic alteration associated with human cancer, (Aas*, et al* 1996). In AML, 17p mutation occurs in about 3-8% of the cases and contributes to disease progression and development of resistance to drug therapy. *P*53 mutation is associated with advanced disease and short survival.(Aas*, et al* 1996), (Lai*, et al* 1995), (Fenaux*, et al* 1991), (Wattel*, et al* 1994). In CLL, aberrations of the *p53* gene are one of the most predictive molecular markers for resistance to first-line therapy and short overall survival. Mutations of the *p53* gene occur in about 10% to 15% in CLL. Mutations become more frequent as the disease progresses and predict aggressive disease that will be unresponsive to chemotherapy (Wattel*, et al* 1994), (Fenaux*, et al* 1992), (Dohner*, et al* 1995), (Cordone*, et al* 1998).

A crucial part of this study was the identification of *p53* mutated samples. In CLL, we decided to use fluorescence in situ hybridization (FISH), which today is the most commonly

used method in the clinic for detection of *p53* deletion and other genetic abnormalities. FISH identifies samples with loss of one allele for *p53*, which correlates with a poor response to treatment and a short survival (Stilgenbauer*, et al* 2000). It is shown that the other *p53* allele is inactivated by point mutations. In this way both alleles are affected, which is the hallmark for the inactivation of a recessively acting tumour suppressor gene, such as *p53*. In AML, chromosomal analysis was performed to detect patient samples with loss of chromosome 17. In a study on patients with myelodysplastic syndrome and AML with deletion of 17p identified by cytogenetic analysis, deletion of one *p*53 allele was found in all cases and mutation of the non deleted *p*53 allele in all but one case.(Soenen*, et al* 1998). These results show that there is a strong correlation between hemizygous loss of *p*53 and mutation of the remaining *p*53 allele leading to loss of p53 function.

In ***paper I***, we incubated samples with drugs used for AML treatment and found that the samples with hemizygous *p53* deletion were more resistant to all these drugs compared to the samples with normal karyotype. This strongly suggests that a normal p53 function is important for chemotherapy-induced apoptosis also in acute myeloid leukaemia. Comparing abn.17 to samples with complex karyotype, we could also demonstrate significantly higher resistance for almost all tested drugs. Furthermore, when we compared abn 17 to other unfavourable karyotypes, including monosomy 7 and/or deletion 7q, we found a more pronounced *in vitro* drug resistance for patients with abn.17. Despite that statistical significance was not reached samples from patients with abnormal 9p tended to be more resistant to cytotoxic drugs compared to those with normal karyotype. This suggests that the regulation of HDM-2 by $P14^{ARF}$ can be of importance in drug resistance in AML, which should be a subject for further investigation.

Since overall survival is a more relevant endpoint than event free survival this parameter was chosen to evaluate the clinical outcome in different genetic groups. Patients with abnormal chromosome 17 had a dismal overall survival with a median overall survival of two months and all patients were dead within one year of diagnosis. By this study we could demonstrate that cytogenetic abnormalities involving chromosome 17 in AML defines a group of patients with an extremely low long term survival using conventional treatment. The prognosis for this group of patients is clearly inferior to other established unfavourable karyotypic changes in AML. Patients with aberrations in 9p had significantly worse prognosis compared to normal karyotype which makes it even more urgent to study HDM-2 and $P14^{ARF}$ as a possible mechanism behind drug resistance.

In ***paper II***, we could confirm the poor response to fludarabine and doxorubicin in *p53* deleted CLL clones in vitro compared to cases with wild-type p53 (Wattel*, et al* 1994), (Fenaux*, et al* 1992), (Dohner*, et al* 1995), (Cordone*, et al* 1998). This difference was significant when the proportion of *p53* deleted cells exceeded 50%. A correlation was also seen between the proportion of *p53* deleted cells in the sample and the cytotoxicity of fludarabine. Cells with large clones with *p53* deletion were most resistant to fludarabine. This

confirms that FISH is an efficient method to identify *p53* mutation in CLL, which is of clinical importance since sequencing of the *p53* gene is time-consuming and complicated. CLL cells were very sensitive to PRIMA-1 and the cytotoxic effect of PRIMA-1 was more pronounced in our study compared to fludarabine and doxorubicin. The concentrations of PRIMA-1 were significantly lower in our study compared to doses used in previous studies on different cell lines with PRIMA-1. In contrast to what was seen with fludarabine and doxorubicin, p53 mutated cells were sensitive to PRIMA-1 but there was no statistically significant difference in cytotoxicity between the *p53* deleted and non-deleted samples. When co-incubation of PRIMA-1 and fludarabine was performed, a significant increase of cytotoxicity was noted in the *p53* deleted samples compared to the non-deleted. In addition, in all (100%) *p53* deleted samples there were synergistic or additive responses which were most pronounced at the highest dose level of PRIMA-1. These data suggest that PRIMA-1 can be of importance in enhancing cytotoxicity of conventional cytostatics in *p53* mutated CLL. In order to study the cytotoxic model by which PRIMA-1 cause cell death, we did a simple apoptosis study measuring annexin V and propidium iodide in 4 patients and found that PRIMA-1 induced apoptosis in a dose-dependent manner in CLL lymphocytes in vitro.

In ***paper III***, we showed that PRIMA-1 was cytotoxic to all AML cells tested, in a dose-dependent manner and significantly more effective in patients carrying hemizygous loss of *p*53. The enhanced effect of PRIMA-1 in the *p*53 deleted cases compared to patients without hemizygous *p*53 deletion, suggests that PRIMA-1 can reverse the anti-apoptotic state by restoring the wild type configuration of mutated *p*53. Our flow cytometry experiments with Annexin and PI staining confirmed that PRIMA-1 induced cell death through apoptosis also in the AML cells. In order to investigate if the pattern of resistance to PRIMA-1 differs from common cytotoxic drugs we compared the effect of PRIMA-1 to that of daunorubicin, fludarabine, Ara-C and CdA, comparing the correlation coefficient of the cytotoxocity induced by PRIMA-1 to that of the other 4 drugs. We found no correlation between PRIMA-1 and the conventional cytostatic drugs. Further study of the resistance pattern of PRIMA-1 on HL-60 cell lines showed that MDR expressing HL-60R was as sensitive as HL-60S. By that way we investigated the resistance pattern in cells expressing P-gp, which is one mechanism behind multidrug resistance in AML since the expression in patient cells correlates with poor prognosis. (Broxterman and Schuurhuis 1997, Campos*, et al* 1992, Marie*, et al* 1991, Pirker*, et al* 1991, Zochbauer*, et al* 1994) This shows that PRIMA-1 is not a substrate for the P-gp transport protein. For AML our data indicate that PRIMA-1 can reverse the anti-apoptotic state in AML with *p*53 mutation which may improve prognosis for this patient group in the future.

In ***paper IV***, we demonstrated that RITA had a dose-dependent cytotoxic effect in AML and CLL samples regardless of p53 status. Four of the CLL patients' samples had deletion of the ATM gene, which is a relatively common defect in CLL, ATM phosphorylates the p53, creating a stabilized p53 with enhanced transcriptional activity (Lambert*, et al* 1998),(Banin*, et al* 1998). Samples with ATM deletion were more sensitive to RITA than p53 deleted

samples, indicating that RITA might have direct effect on p53. As could be expected, PRIMA-1 was more toxic against both AML and CLL cells with mutated p53 compared to cells with wild type p53. This was confirmed in AML, where PRIMA-1 had a five fold higher effect (IC 50 value) in samples with -17p. This contrasts to the effect of RITA which was more cytotoxic in samples without p53 deletion as discussed above. We further stipulated that the combination of PRIMA-1, used to restore the transcriptional activity when the p53 gene is mutated, and RITA, used to increase the amount of p53 protein intra-cellularly could have synergistic effects in cells with mutated p53. Interestingly, synergistic effects were seen in AML cells both with and without p53 deletion, but were more pronounced in p53 non deleted samples. In AML and CLL samples, the flowcytometry experiments with Annexin and PI staining confirmed that RITA induced apoptosis in a dose-dependent manner in the AML cells and significantly increased when PRIMA-1 was added. Exposing the cells to RITA also resulted in increase in the apoptotic G0/sub G1 phases. One effect of RITA is supposed to be induction of wt p53. To study this we performed flowcytometry analysis on CLL and AML samples after incubation and found that RITA increased the expression of wt p53 protein in both. The highest increase, up to 50%, was seen in AML samples.

P53 protein has a central role in cancer development and targeting the p53 protein with new drugs can provide a clinically valuable treatment alternative in malignant diseases. We demonstrated that RITA can increase p53 levels and induce apoptosis and cell death in malignant cells both with and without p53 mutation. RITA acts synergistically with fludarabine in CLL. In AML RITA acts synergistically with another p53 targeting drug, PRIMA-1, in inducing cell death. This suggests that RITA can be a promising non-chemotherapeutic alternative to conventional drugs in the treatment of CLL and AML.

GENERAL CONCLUSIONS

* Genetic changes associated with impaired p53 function such as aberrations in 17p, affecting p53 and in 9p, affecting $p14^{ARF}$ are strongly associated with poor survival in AML.

* *p53* mutated leukemic cells were significantly more drug resistant compared to non mutated cells both in patients with AML and CLL.

* PRIMA-1, a compound designed to restore wt p53 induced concentration dependent apoptosis and cell death. The effect was more pronounced on cells with 17p aberration. In CLL PRIMA-1 acted synergistically with fludarabine.

* In contrast RITA, a molecule designed to bind to p53 and increase its half life in the cell exerted a significantly more cytotoxic effect on cells with wild type p53.

* Co-incubation of RITA and fludarabine in CLL samples, and RITA and PRIMA-1 in AML samples, showed a synergistic effect in 100% and 75% of the cases, respectively.

* RITA, alone and in combination with PRIMA-1, increased the intracellular levels of p53, pointing out this as a mechanism of action of this drug.

* Small molecules targeting the p53 protein appear to be a promising way to improve the outcome of drug resistant leukaemia. Clinical studies of these molecules are warranted.

FUTURE PERSPECTIVES

In this thesis, we studied the incidence of chromosomal aberrations involving the p53 pathway in AML and their impact on survival. Furthermore, we investigated the effects in AML and CLL cells of PRIMA-1, a molecule that restores wild type conformation of mutated p53. We also enhanced the effect of p53 in its wild type form by using RITA. Together with Aprea we are conducting further studies on PRIMA-1 and related molecules such as Meth-PRIMA; investigating toxicity, pharmacological parameters and testing PRIMA on two animal models, with the purpose of introducing PRIMA to clinical use. The aim is to initiate phase-1 studies in one year from today. Another goal will be to evaluate RITA in the clinic.

We have also started to investigate the role of p53 and other genes/proteins involved in the p53 pathway such as $p16^{INK4a}$ and $p14^{ARF}$ in acute lymphocytic leukaemia on a national base. Our preliminary results indicate that patients carrying aberrations in these genes may have a high relapse rate and short overall survival.

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