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MECHANISTIC STUDIES OF APR-246 IN LEUKEMIA

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"To dream anything that you want to dream. That's the beauty of the human mind. To do anything that you want to do. That is the strength of the human will. To trust yourself to test your limits. That is the courage to succeed".

Bernard Edmonds

To My Family

ABSTRACT

PRIMA-1 and its analog APR-246 are novel drugs that restore the active conformation of mutated and unfolded p53 protein and induce apoptosis and cell death in various tumors in pre-clinical models. We first aimed to explore the effects of APR-246 alone and in combination with other drugs in acute myeloid leukemia (AML) in vitro. APR-246 induced dose-dependent apoptosis and increased active caspase-3 and p53 protein levels as well as the Bax/Bcl-2 ratio independently of *TP53* mutational status. AML patient cells with *TP53* mutations and complex karyotype were more resistant to conventional chemotherapeutic drugs but retained their sensitivity to APR-246. Pronounced synergism was found when combining APR-246 with DNR in AML patient cells and pre-incubation with APR-246 induced more synergistic effects compared to other treatment schedules in the AML cell line KMB3.

As APR-246 was shown to induce expression of genes protective of oxidative stress in global gene expression profiling, we furthermore aimed to study the effects of APR-246 on the redox status of AML cells. We confirmed that APR-246 increased ROS formation and depleted cells from glutathione. *HO-1*; a gene protecting from oxidative stress, was one of the most upregulated genes in response to APR-246. Both HO-1 and its transcriptional regulator *NFE2L2* (Nrf2) were upregulated as detected by q-RT-PCR. APR-246 treatment induced Nrf2 activation by translocation of the Nrf2 protein from the cytosol to the nucleus. Transient knockdown of Nrf2 in KMB3 cells obliterated APR-246-induced up-regulation of *HO-1* and increased its antitumoral effects. The PI3K inhibitor wortmannin and the mTOR inhibitor rapamycin*;* both up-stream regulators of Nrf2, inhibited APR-246-induced nuclear translocation of Nrf2 and induced synergism with APR-246.

A phase I first-in-man study including 22 patients with hematologic malignancies and prostate cancer was conducted. Dose escalations from 2 mg/kg to 90 mg/kg revealed a maximum tolerated dose (MTD) of 60 mg/kg and a half-life of 4-5 hours. The most common adverse effects were fatigue, dizziness, headache, and confusion. Dose limiting toxicities (DLTs) were increased ALT/AST (n=1), dizziness, confusion, and sensory disturbances (n=2). Tumor cells showed cell cycle arrest, increased apoptosis, and up-regulation of p53 target genes.

We finally showed that miR-34b/c is epigenetically silenced by DNA methylation in chronic lymphocytic leukemia (CLL). As being down-stream regulators of p53, miR-34b/c expression levels were induced by PRIMA-1 as well as by doxorubicin and decitabine. Over-expression of miR-34b/c in CLL cells increased apoptosis, which suggest a tumor suppressor function for these microRNAs.

In conclusion, AML cells are sensitive to APR-246 in vitro irrespectively of *TP53* mutational status. The substance induces oxidative stress and activates the Nrf2/HO-1 protective pathway. In a first-in-man study, APR-246 was shown to be to have a favorable pharmacokinetic profile and to induce p53-dependent biologic effects *in vivo*. Either in combination with conventional chemotherapeutic drugs or PI3K inhibitors, synergistic antileukemic effects can be obtained which holds a promise for further combination studies *in vivo*.

LIST OF PUBLICATIONS AND MANUSCRIPTS

I. **Ali D,** Jonsson-Videsäter K, Deneberg S, Bengtzen S, Nahi H, Paul C, Lehmann S. APR-246 exhibits anti-leukemic activity and synergism with conventional chemotherapeutic drugs in acute myeloid leukemia cells. Eur J Haematol. 2011 Mar;86(3):206-15.

II. **Ali D**, Mohammad D. K, Jonsson-Videsäter K, Nore B. F, Paul C, Lehmann S. APR-246-induced cytotoxicity is related to induction of oxidative stress and activation of the HO-1/NRF2 axis in leukemia cells. Manuscript.

III. Lehmann S, Bykov VJ, **Ali D**, Andrén O, Cherif H, Tidefelt U, Uggla B, Yachnin J, Juliusson G, Moshfegh A, Paul C, Wiman KG, Andersson PO. Targeting p53 in vivo: a first-in-human study with p53-targeting compound APR-246 in refractory hematologic malignancies and prostate cancer. J Clin Oncol. 2012, Oct,10;30(29):3633-9.

IV. Deneberg S, Kanduri M, **Ali D**, Bengtzen S, Karimi M, Qu Y, Kimby E, Rosenquist R, Lennartsson A and Lehmann S. MicroRNA-34b/c is aberrantly hypermethylated by an epigenetic switch mechanism in B-cell Chronic Lymphatic Leukemia. Manuscript.

LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
AMML	Acute myelomonocytic leukemia
AMoL	Acute monocytic leukemia
AMkL	Acute megakaryoblastic leukemia
allo SCT	Allogeneic stem cell transplantation
ASK1	Apoptosis signal-regulating kinase 1
AE	Adverse Events
CML	Chronic myeloid leukemia
CEBPA	CCAAT enhancer binding protein alpha
CR	Complete remission
CLL	Chronic lymphocytic leukemia
CTCAE	Common Terminology Criteria for Adverse Events
ChIP	Chromatin immunoprecipitation
CI	Combination index
DNR	Daunorubicin
DLT	Dose limiting toxicity
DUSP1	Dual specificity phospahatase 1
EORTC	European Organization for Research and Treatment of Cancer
ER	Endoplasmic reticulum
FAB	French-American-British
FLT3-ITD	fms-like tyrosine kinase 3-internal tandem duplication
GOF	Gain-of-function
GSH	Glutathione
GO	Gene ontology
GOF	Gain of function
HSP	Heat shock protein
HO-1	Heme oxygenase 1
MDS	Myelodysplastic syndromes
MPN	Myeloproliferative neoplasms
miRNAs	MicroRNAs
MQ	Methylene quinuclidinone
MTD	Maximum tolerated dose
NPM1	Nucleophosmin
NHL	Non-Hodgkin lymphoma
Nrf2	Nuclear factor-erythroid 2-related factor 2
OS	Overall survival
P38 MAPK	p38 mitogen-activated protein kinase
PI3K	Phosphatidyl-ionositol-3 kinase
PS	Performance status
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
RFS	Relapse free survival
UPR	Unfolded protein response
WHO	World Health Organization
WT p53	Wild-type p53

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1 INTRODUCTION

1.1 HEMATOLOGICAL MALIGNANCIES

Hematological malignancies are divided into myeloid or lymphoid depending on the origin of the cell type primarily affected. These groups are traditionally subdivided into chronic and acute depending on the stage of maturity of the malignant cells and their clinical course.

1.1.1 Myeloid malignancies

Myeloid malignancies is a group of disorders that manifest them selves as malignant dysregulation of the myeloid lineage such as myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN) including chronic myeloid leukemia (CML), myelodysplastic/myelomonocytic neoplasms and acute myeloid leukemia (AML) [1]. These are clonal diseases that arise in hematopoietic stem or progenitor cells, mainly due to mutations in genes involved in signaling pathways (e.g. *CBL, FLT3, JAK2*, *RAS*), epigenetic regulators (e.g. *DNMT3A, ASXL1, EZH2, IDH1, IDH2, SUZ12*, *TET2, UTX*), transcription factors (e.g. *CEBPA, ETV6, RUNX1*), [2] and tumor suppressors (e.g. *TP53*).

1.1.1.1 *Acute myeloid leukemia (AML)*

1.1.1.1.1 Overview and definition of AML

AML describes a group of different malignant disorders, that originate in the hematopoietic system and that present with expansion of undifferentiated myeloid cells disturbing normal hematopoiesis. In AML, myeloid progenitor cells in the bone marrow undergo a malignant transformation resulting in accelerated production of poorly differentiated myeloblasts that are not able to mature into more differentiated cell types but remain as immature cells. AML patients present with symptoms resulting from bone marrow failure (i.e. anaemia, neutropenia, and thrombocytopenia), and/or symptoms resulting from organ infiltration and/or proliferation of leukemic cells (i.e. splenomegaly, hepatomegaly, swollen bleeding gums, bone and joint pain, respiratory distress and alterations in mental state as well as disseminated intravascular coagulation (DIC).

1.1.1.1.2 FAB classification

During the last decades and until recently, AML has been classified according to the French-American-British (FAB) classification that divides AML into 8 subtypes (M0 to M7) defined by morphologic and cytochemic characteristics. The different subclasses

represent different states of maturation of the leukemic blast cells and what lineage that is engaged. Table 1 shows the different subclasses according to the FAB classification. In the FAB classification, the required number of blast cells in the bone marrow is 30% compared to less than 5% which is considered to be normal and found in healthy individuals.

Table 1. French-American-British (FAB) Classification of Acute Myelogenous Leukemia

FAB subtype	Name
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia (AMML)
M4 eos	Acute myelomonocytic leukemia with dysplastic eosinophilia
M5	Acute monocytic leukemia (AMoL)
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia (AMkL)

Adapted from: Classification of Acute Leukemia, Gamal Abdul-Hamid, 2011 [3].

1.1.1.1.3 WHO classification

In recent years, the new World Health Organization (WHO) classification has to a large extent replaced the FAB classification. The WHO classification is based on the fact that an increasing number of acute leukemias should be categorized based upon their underlying genetic abnormalities and/or their pathophysiologic characteristics (Table 2). For AML, the WHO classification requires information on the chromosomal aberrations, mutational status, previous exposure to chemotherapy or radiation or to the presence of an antecedent myelodysplastic syndrome. Regarding the AML diagnosis, there are 2 major differences between the FAB and the WHO classification where in the latter, AML is defined by a blast percentage above 20% in the bone marrow [4] or by the following chromosomal aberrations: (8;21)(q22;q22), t(16;16)(p13;q22), inv(16)(p13;q22), or t(15;17)(q22;q12) [5] regardless of blast percentage.

Table 2. Acute myeloid leukemia and related precursor neoplasms, and acute leukemias of ambiguous lineage (WHO 2008) ***Categories***

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified (NOS)
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis (acute myelofibrosis)
Myeloid sarcoma (extramedullary myeloid tumor; granulocytic sarcoma)
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (transient myeloproliferative disorder)
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR-ABL1*
Mixed phenotype acute leukemia with t(v;11q23); mixed lineage leukemia
Mixed phenotype acute leukemia, B/myeloid, NOS
Mixed phenotype acute leukemia, T/myeloid, NOS
Provisional entity: Natural killer (NK)–cell lymphoblastic leukemia/lymphoma

Adapted from: WHO Classification of Tumours of Haematopoietic [6] and Lymphoid Tissues. Swerdlow SH, et al., Lyon, France: *IARC* Press; 2008 [7].

1.1.1.1.4 Genetics

By using novel technologies, such as massively parallel DNA sequencing or high-resolution single-nucleotide polymorphism arrays, the identification of several novel recurrent gene mutations in AML has been facilitated. Nucleophosmin (*NPM1*) gene represent the most frequent genetic aberrations in AML [8], being identified in about 30% of AML patients, especially who have a normal karyotype, and in as high as 50–60% of *de novo* AML [9,10]. These mutations may contribute to leukemogenesis, at least in part, through disruption of genes involved in signalling and apoptosis pathways such as the $p14^{ARF}$ - MDM2-p53 pathway [11,12]. Abnormalities in the fms-like tyrosine kinase 3 (*FLT3*) on chromosome 13 represent the second most common genetic aberrations in de novo adult AML [13]. It comprises 2 main types: mutations of the *FLT3* internal tandem duplication (ITD) of the juxtamembrane domain [14] or activating loop mutations in the second tyrosine kinase domain (TKD), which occur predominantly at position D835 [15]. *FLT3*-ITD mutations have been reported in almost 20% of all AML cases [16], it occurs in all FAB subtypes , with the highest frequency in M5 [17]. Mutations in the transcription factor CCAAT/enhancer binding protein alpha (*CEBPA*), which plays a central role in normal development of granulocytes, are observed in almost 10% of AML patients [18]. Point mutations in the *RUNX1* gene (also known as AML1 or *CBFA2*) are also seen in specific subtypes of AML such as in AML M0, MDS-AML, and secondary (therapy-related) MDS/AML. Though *RUNX1* is the most frequent target for chromosomal translocation in AML, its mutation alone is not sufficient to cause full-blown leukemia [19,20]. Other genetic alterations have been identified in AML such as: partial tandem duplications (PTD) of the mixed lineage leukemia (*MLL*) gene, as well as mutations in the transforming protein N-Ras (*NRAS*) and Wilms tumor (*WT1*) genes [21,22]. More lately, novel mutations have been found such as point mutations of isocitrate dehydrogenase (*IDH1/IDH2*) genes [23,24], which affect the epigenetic state in AML by inducing increase in global DNA hypermethylation [25]. The later mutation as well as mutations of the DNA methyltransferase 3A (*DNMT3A*) and the ten-eleven-translocation oncogene family member 2 (*TET2*) [26] genes are usually found in cytogenetically normal AML [27]. On the contrary, the additional sex combs-like 1 (*ASXL1*) mutations have been found to occur more frequently in secondary AML compared to *de novo* AML cases [28], and were recently reported to be more frequent in AML with intermediate risk karyotype [29].

Classically, AML mutations have been divided into Class I and Class II to mutations (Figure 1) depending on the type of cellular processes that are effected. However, many of the newly discovered mutations have not yet been fit into this model.

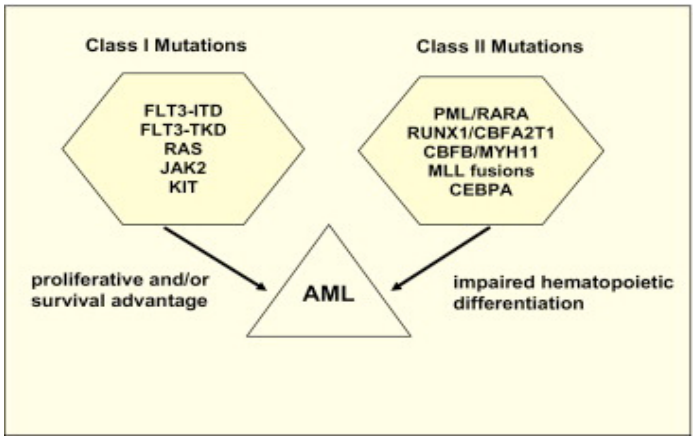


Figure 1. Model of leukemogenesis with two complementation groups of mutations, reviewed by (Gaidzik et al, 2008) [21]. Reprinted with permission from Elsevier. Seminars in Oncology Journal, doi:10.1053/j.;.2008.04.005.

1.1.1.1.5 Prognostic factors and survival

Factors that have been identified to influence outcome of adult AML patients and affect their classification into different prognostic subgroups include cytogenetics [30], WBC count, and whether the disease is primary (*de novo*) or secondary to a prior hematologic malignancy [31]. Secondary leukemia includes AML patients with previous exposure to chemotherapy and/or radiation (therapy-related AML or t-AML) or patients with an antecedent myelodysplastic syndrome or myeloproliferative disorder. The survival of patients with secondary AML is generally shorter than for those with de novo AML within the same cytogenetic risk group. Other prognostic factors include MDR1 expression [32,33] and *FLT3* [34] and *NPM1* gene mutational status [35]. *FLT3* mutations confer a poor prognosis [36], especially in patients younger than 60 years old [37]. It has been shown that mutations of the *FLT3* gene, especially *FLT3*–ITD*,* are significantly correlated to *NPM1* mutations (approximately 40%) [38] but show favorable impact in older patients [39,40]. In younger adult patients, mutated *NPM1* without *FLT3*-ITD usually achieve CR and have favorable relapse free survival (RFS) and (overall survival (OS) [41]. A novel study by Wakita, et al. has revealed that mutations of the epigenetically modifying gene (*DNMT3A, TET2, IDH1/2*) at diagnosis may induce *FLT3*-ITD at relapse in de novo acute myeloid leukemia [42]. In patients negative for *FLT3*-ITD mutations, mutant *NPM1* is associated with improved response to treatment [43]. Prognostic significance of *RUNX1* mutations indicates association with lower CR rate

and shorter RFS and OS [41]. While *CEBPA* mutations are associated with higher CR rate and favorable RFS and OS.

1.1.1.1.6 Treatment of AML

Treatment of acute myeloid leukemia is based on induction therapy and consolidation therapy as the following:

1.1.1.1.6.1 Induction therapy

Intensive induction chemotherapy aims to achieve a complete remission (CR), which is defined as less than 5% leukemic blasts in the bone marrow and recovery of neutrophil count >1.0x10^9/L and platelet count >100x10^9/L. Conventional induction treatment in adults consists of a combination regimen of the deoxycytidine analogue cytarabine and an anthracycline antibiotic (daunorubicin or idarubicin) or the anthracenedione mitoxantrone, an inhibitor of the topoisomerase IIa enzyme [44]. Approximately 50% to 75% of adults with AML achieve complete remission with that regimen. The most commonly used standard induction therapy for the last 4 decades consists of DNR intravenously for 3 days and cytarabine 100 or 200 mg/m^2 by continuous infusion for 7 days [45,46]. With this standard regimen 60% to 80% of young adults and 40% to 60% of older adults can achieve a CR. Many studies have compared the standard regimen with different doses of DNR and the conclusion is that 45 mg/m^2 (the previous standard dose) should no longer be considered as the standard of care as the higher dose between 60 and 90 mg/m^2 for 3 days has proven to be safe and yield either higher CR and prolonged overall survival (OS) rates [47]. Some elderly patients tolerate and benefit from intensive induction approaches, while others are best managed with less aggressive strategies. In order to maximize the therapeutic benefit and minimize toxicity for those patients, a strategy to stratify them based on host-related and biological features might be investigated [48].

The European Organization for Research and Treatment of Cancer (EORTC) and the Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) group study AML-10 has shown that the use of mitoxantrone or idarubicin instead of daunorubicin enhances the long-term efficacy of chemotherapy in adult patients with AML who have not undergone an allogeneic stem cell transplantation (allo SCT) [49] but the results are still controversial and the 3-7 regimen using Ara-C in combination with either daunorubicin or idarubicin is still considered as standard of care. In 2009, a population-based study from the Swedish Acute Leukemia Registry reported an improvement in the survival of elderly patients up to 80 years of age in the geographical areas where

standard induction therapy was mostly given. Moreover, early death rate was also lower with intensive therapy than with only palliative therapy, which suggests that the standard induction therapy is the best for that patient category [50].

1.1.1.1.6.2 Consolidation therapy

Consolidation and maintenance therapy, also known as post-remission treatments are given after achieving the first remission (CR1) aiming to maintain the CR and to eradicate remaining leukemic cells. Consolidation therapy often includes repeated courses with intermediate/high-dose Cytarabine (Ara-C) with or without an anthracycline. It is difficult to determine whether reinduction therapy should be attempted or hematopoietic cell transplantation should be directly performed in relapsed patients with histocompatible donors. Consolidation with allo SCT in CR1 or after successful treatment after relapse is an often-preferred treatment option for younger patients[51,52]. Though, contradictory results showed that patients transplanted shortly after achieving CR appeared to have a worse prognosis than those transplanted further into remission [53]. In a meta-analysis study, it has shown that the efficacy of allo SCT depends also on cytogenetic risk for patients in CR1 [54].

1.1.2 Lymphoid malignancies

Lymphoma occurs due to malignant transformation of B- or T-cells. The WHO classification of 2008 recognizes three major categories of lymphoid malignancies [55,56] based on morphology and cell lineage. Those are B-cell neoplasms, T-and natural killer (NK)-cell neoplasms and Hodgkin's lymphoma [57,58]. Lymphomas, that constitute the major part of the lymphoid malignancies, are usually subdivided into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), where the latter includes both B- and T-cell lymphomas.

1.1.2.1 *Chronic lymphocytic leukemia (CLL)*

CLL belongs to the B-cell NHLs. By their expression of B-cell activation antigens, they are considered neoplastic counterparts of normal activated B-cells [59-61]. The peripheral B-cell neoplasm CLL is the most common leukemia in adults, mostly affecting older individuals with the male predominance [62]. Proper diagnosis of CLL includes evaluation of the peripheral blood count as well as smear and immunophenotyping of the bone marrow or peripheral blood. CLL patients present with a specific immunophenotype that can be distinguished from other B-cell malignancies. According

to a report from the International Workshop on CLL [63], in order to distinguish chronic lymphocytic leukemia from monoclonal B-cell lymphocytosis, it was suggested to consider a B-cell count of $> 5.0\times10^9$/L rather than an absolute lymphocyte count of $> 5.0\ -10^9$/L, as a basis for this differentiation. A few years later, a study from the Italian GIMEMA groups assigned the B-cell count of 10×10^9/L as the best lymphocyte threshold to predict time to first treatment of CLL [64].

1.1.2.1.1 Genetic mutations in CLL

CLL is characterized by multiple and recurrent chromosomal abnormalities, of which deletions in chromosome 13q (del13q14) occur in more than 50 % of all CLL cases. Other genetic aberrations are also found to a lesser degree, such as 11q deletions (18 %), trisomy of chromosome 12q (12 %), and 17p deletions (7%) [65]. These abnormalities confer different prognostic risks with 17p deletions being associated with the shortest survival.

1.1.2.1.2 MicroRNAs in CLL

MicroRNAs (miRNAs) represent a class of noncoding RNAs that regulate messenger RNA and protein expression of target genes and has a key role in the posttranscriptional regulation of gene expression [66]. They negatively regulate gene expression by inducing degradation or translational inhibition of target mRNAs. Aberrant expression of miRNAs has been recently demonstrated to have an essential role in the process of leukaemogenesis [67,68]. The targeted gene in 13q deletion has been suggested to be microRNA-15a and 16-1 [69]. On the other hand, research found that certain miRNAs such as miR-34 family, and in particular miR-34a represent direct conserved p53 target genes, which mediate certain p53-dependent effects including apoptosis, cell cycle arrest, and senescence [70]. The miR-34 family comprises three processed miRNAs that are encoded by two different genes. For miR-34a, its own transcript encodes it, whereas miR-34b and miR-34c share a common primary transcript. In a previous CLL study by Lehmann et al, SNP-chip technique demonstrated a large commonly deleted region at 11q, similar to what has been reported previously. However, one case with 11q-deletions showed two deleted regions with a small heterozygote region in between. One of the deleted regions contained the ATM gene whereas the other contained 6 other genes (*POU2AF1, BTG4, FLJ46266, LAYN, SNFLK2 and PPP2R1*) as well as microRNA-34b/c [71].

1.2 p53

The cellular tumour antigen gene *TP53,* encoding the p53 protein, remains the most prominent and most commonly mutated tumour suppressor gene and due to its function, it has been named "the guardian of the genome" [72]. When the genomic integrity of a cell is challenged, its fate is determined in part by signals conveyed by the p53 protein. The gene is located on chromosome 17p13.1 and the basic modular structure of the p53 protein comprises a N-terminal transcriptional activation domain, a central DNA-binding domain and a C terminus with oligomeric and regulatory activities [73]. Several studies have shown that post-translational modifications such as serine/threonine phosphorylation, acetylation, prolyl isomerization and sumoylation increase the DNA-binding and the transcriptional activity of wild type p53 [74,75].

1.2.1 p53 functions

The tumor suppressor protein p53 is activated by external and internal stress signals that promote its nuclear accumulation in an active form. It plays a central role in cell death to prevent cancer development. The spectrum of p53-based cell fate decisions ranges from a transient cell-cycle arrest (at G1 and/or G2 phase) enabling damage repair to an irreversible block of proliferation through the induction of senescence, differentiation or apoptosis [76-78]. Disruption of apoptosis-induction process can promote tumor progression and chemo-resistance. p53 apparently promotes apoptosis through transcription dependent and transcription independent mechanisms, which maintain the cell death program.

1.2.1.1 *Transcription-dependent p53-mediated apoptosis*

Active p53 transactivates the transcription of downstream pro-apoptotic mediators, including BAX, NOXA, Puma [79] p53AIP1 and CD95 (Fas/APO-1). Those genes trigger mitochondrial outer membrane permeabilization [80] with release of the apoptogenic proteins; cytochrome c and AIF (apoptosis inducing factor). Cytochrome c, by its turn, translocates into the cytosol and promotes the interaction between APAF-1 and procaspase-9 [81-83], which is cleaved into active Caspase-9. Caspase-9 in turn, cleaves and activates procaspase-3 [84].

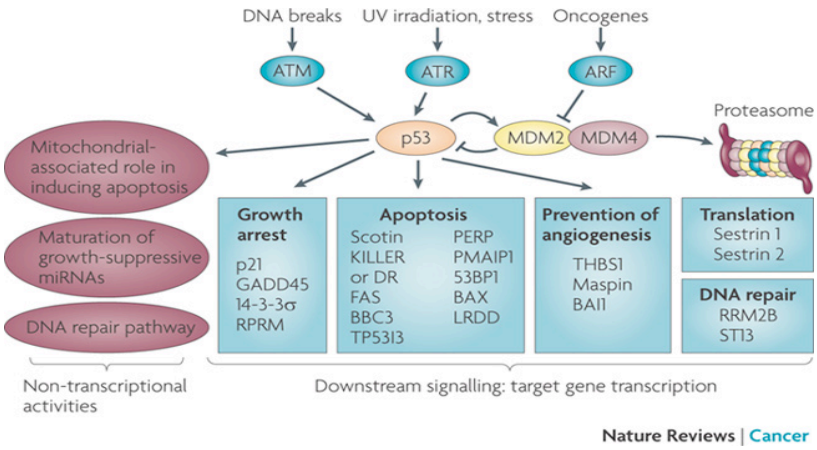


Figure 2. The p53 pathway. Reprinted with permission from Nature Publishing Group. Christopher J. Brown et al. Nature Reviews Cancer 9, 862-873 (December 2009) | doi:10.1038/nrc2763 [85].

1.2.1.2 *Transcription-independent p53-mediated apoptosis*

The transcription-independent apoptotic activity of p53 has been linked to the intrinsic mitochondrial apoptotic pathway that occurs under different cellular stress conditions by two independent yet converging mechanisms [86]. These mechanisms involve both the mitochondrial and cytosolic p53 respectively. The outcome of activation of p53 by either mechanism is the activation of the Bax/Puma and the pro-apoptotic members of the Bcl-2 family respectively and thus, induction of the permeabilization of the outer mitochondrial membrane (OMM) occurs with the subsequent release of cytochrome C and caspase activation [87,88].

1.2.2 Types of p53 mutations in cancer

The mode of p53 inactivation is diverse including deletion of p53, inhibition of p53 by MDM2 or viral proteins [89]. Inactivation of p53 function is the most important factor in the spectrum of *TP53* mutation. Mutations in the *TP53* gene are the most frequent mutations in human cancers that occur in rates varying between 10% (e.g. in hematopoietic malignancies) [90] to 100% (e.g. in high-grade serous ovarian carcinoma) [91], with average rate of 50% in all cancers [92-94]. Many studies suggest that the nature of a *TP53* mutation in a cell has an impact upon cellular properties, clinical responses to therapy and tumor prognosis [95]. p53 has five structural and oligomerization domains: an N-terminal transactivation domain, a proline-rich domain, a central DNA-binding domain, a tetramerization domain and a C-terminal regulatory domain [96]. Several categories of *TP53* mutations can be distinguished by taking into account the impact of the mutation on either the protein structure/stabilization or interaction with DNA.

1.2.2.1 *Missense TP53 mutations*

The most frequent cancer-associated mutations in *TP53* are missense mutations, which occur in approximately 75% of all human cancers that harbour p53 mutations [97]. Missense mutations occur due to single residue changes that result in the translation of a different amino acid in that position over the full-length (393-amino-acid) of p53 protein, (such as p53-Trp^{248} and p53-His^{273}) [98]. The missense mutations in the *TP53* gene disrupt the ability of p53 to bind to DNA and consequently to transactivate downstream genes. Most of these mutations are clustered within the core domain (102–292 bp), which is important for DNA-specific binding and is essential for p53 function [99]. Among these mutations in the core domain are six "hot spot" residue substitutions (including R175, G245, R248, R249, R273 and R282) that have been reported to occur in high frequency, representing almost 30% of the *TP53* mutations [100,101].

1.2.2.2 *Gain-of-function TP53 mutations*

Referring to mutant p53 gain-of-function (GOF), various lines of evidence indicate that the common types of cancer-associated p53 mutations not only abrogate the tumor suppressor functions of wild-type (WT) p53, but also endue the mutant protein with new oncogenic activities that can favor the maintenance, the spreading and the chemo-resistance of malignant tumors [102,103]. Even though the cell still retains one WT allele, it is rendered practically devoid of WT p53 function. This might be explained by the fact that common mutant p53 isoforms can exert dominant–negative effects over coexpressed WT p53, by forming mixed tetramers, which prevent the p53 protein and its paralogs p63 and p73 from DNA-binding and transactivation, and attenuate their function. Jie Xu et al. also reported that the dominant-negative activity and GOF effects of structurally destabilized p53 mutants result from their increased aggregation which also induces misfolding and sequestration of the co-expressed WT p53 and its paralogs p63 and p73 into co-aggregates, resulting in complete loss of p53 function and deficient transcription of target genes involved in cell growth control and apoptosis [104]. Understanding the structure and functions of oncogenic p53 mutants may lead to more potent reactivation modalities or to the ability to eliminate mutant p53 gain of function [105].

1.2.3 Treatments targeting p53

Since *TP53* mutations occur frequently in human cancer, this has led to the development of numerous approaches to restore wild type p53 in order to revert the

malignant phenotype or enhance drug sensitivity.

1.2.3.1 *Mdm2 inhibitors*

The p53 inhibitor Mdm2 (Hdm2 in humans) inhibits the transcriptional activity of p53 in a negative-feedback loop and, more importantly, promotes its proteasomal degradation. Under normal conditions, p53 is a short-lived inactive protein and Mdm2 is responsible for keeping p53 in this state, as its inappropriate activation can cause premature senescence and death [106,107]. Following cellular stress this inhibition is relieved to stabilize p53. The usage of small molecules to block the Mdm2-p53 interaction and reactivate the p53 function is a promising therapeutic strategy for the treatment of cancers retaining wild type p53. The availability of potent and specific Mdm2 inhibitors, such as Nutlin-3 and MI-219, has provided the opportunity to in detail examine the molecular mechanism of p53 activation [108]. They block the intracellular Mdm2-p53 interaction that leads to accumulation of p53 and the activation of the p53 pathway in tumor and normal cells. In normal cells, the activation of p53 by Mdm2 inhibitors triggers transient cell cycle arrest but no apoptosis, while in tumor cells these inhibitors induce both cell cycle arrest and apoptosis [109], thus they exhibit a favorable safety profile with the advantage of selectively inducing cancer cell death while preserving the normal cells from DNA damage [110].

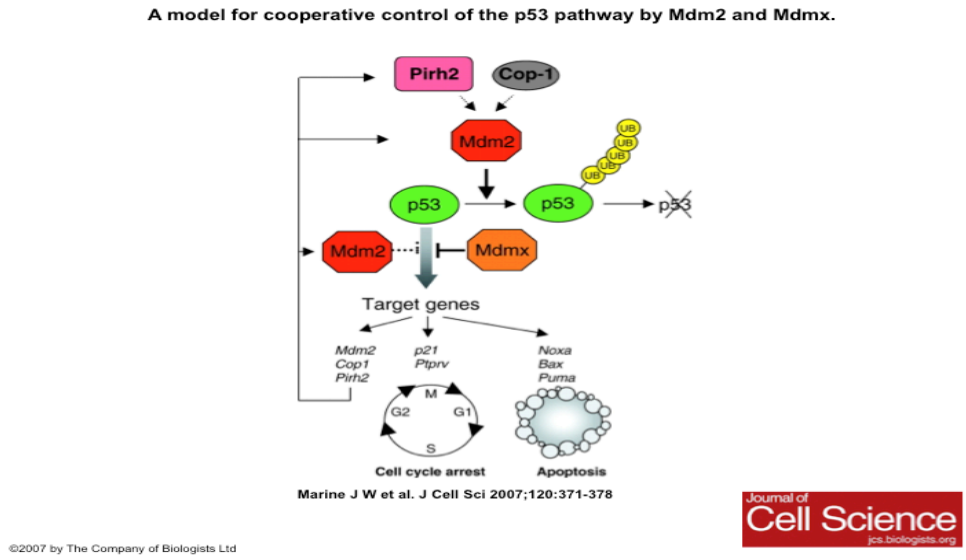


Figure 3. A model for cooperative control of the p53 pathway by MDM2. Reproduced with permission from Journal of Cell Science. http://jcs.biologists.org/content/120/3/371.long doi: 10.1242/jcs.03362 [111].

1.2.3.2 *Adenoviral-based TP53 gene therapy*

Adenoviral vectors have been used for *TP53* gene delivery to restore the p53 function in *TP53* mutated or null tumors. *TP53* gene therapy has been tested in clinical trials in patients with lung cancer [112], head and neck cancer [113], ovarian carcinoma [114]and other tumors. Data from those clinical trials showed favourable outcome, stabilized tumor growth or tumor shrinkage in a good fraction of the treated patients [115].

1.2.3.3 *PRIMA-1 and APR-246*

1.2.3.3.1 PRIMA-1 and mutant p53 reactivation mechanism

PRIMA-1 is one of the small molecules that target mutant p53. A methylated form of the drug called PRIMA-1^{MET} (also known as APR-246) has been shown to reactivate missense mutants of p53 to restore wild type p53 function and thus arrest tumor growth [116]. For example, exposure of cells expressing inactive mutant R175H to PRIMA-1 has led to p53 reactivation and subsequent inhibition of cell cycle progression [117]. APR-246 has successfully entered a phase I clinical trial in 2010, which has shown to induce p53-dependent biologic effects in tumor cells *in vivo*. The conclusion was furthermore that the drug is safe at predicted therapeutic plasma levels and has a favorable pharmacokinetic profile [118].

1.2.3.3.2 PRIMA-1 and protein folding mechanism

Both PRIMA-1 and APR-246 are able to convert to methylene quinuclidinone (MQ), a reactive compound with Michael acceptor activity, which has the ability to modify and form adducts with free thiol groups (forming disulfide bond) [117,119]. Disulphide bond formation induced by the MQ compound binding may lead to aggregation and stabilization of p53 in an active conformation [120]. In 1997, a study revealed that certain *TP53* mutations confers a highly unfolded protein under physiological conditions such as the classic structural mutant R175H as well as C242S, R248Q, R249S, and R273H, which are less stable. The authors suggested that changes in stability are sufficiently small to allow possible therapeutic use of small molecules to rescue p53 function by stabilizing/refolding it [121]. PRIMA-1 also has shown to induce both oxidative stress and ER stress [122], which propose that additional p53-independent cytotoxic functions of PRIMA-1 or one of its degradation products act synergistically with p53 reactivation to induce apoptosis [117]. Recent studies showed that, small molecules drugs such as PRIMA-1 and its analog APR-246 could enhance refolding of the p53 protein to the native folded protein conformation, by which the drug can restore the p53

transactivation function through converting the unfolded mutant p53 to a folded wild type p53 [123,124].

1.2.3.3.3 PRIMA-1 activates microRNAs with a tumor suppressor function

PRIMA-1 has also been shown to induce expression of miR-34a in the H211 and H1155 p53-mutated cells, and knocking down miR-34a decreased the rate of apoptosis caused by PRIMA-1. The latter suggests that miR-34a is one of the important components of PRIMA-1-induced apoptotic network in the cancer cells expressing mutant p53 [125].

1.3 OXIDATIVE STRESS

1.3.1 Oxidative stress in cancer

Oxidative stress is an imbalance between free radical generation and the antioxidant defense system. Redox (reduction-oxidation) regulation has been shown to be an important component of malignant cell survival. Although reactive oxygen species (ROS) have been considered to cause damage cells, accumulating evidence shows that oxidative stress also induces proliferation, gene activation, cell-cycle arrest, and apoptosis, depending on the magnitude and duration of oxidative stress activation. The most common forms of ROS include superoxide, hydrogen peroxide, and the highly reactive hydroxyl radical.

1.3.2 Oxidative stress as a target in cancer treatment

In 1997, a study suggested that p53 might induce apoptosis by stimulating the production of reactive oxygen species (ROS), where a number of p53-induced genes (PIGs) were involved in ROS-mediated apoptosis [126]. More recent data suggested that oxidative stress induces cytochrome c release from mitochondria and activation of caspases, p53, and kinases such as apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (P38 MAPK) [127]. Recently, new strategies for cancer treatment employed pharmacologic shifting of the cellular redox balance in favour of increased intracellular ROS and/or depleting protective reducing thiols, such as glutathione, aiming to induction of oxidative stress and the subsequent apoptosis [128]. Many of the cancer-killing agents such as (ionizing radiation, most chemotherapeutic drugs and certain targeted anti-cancer therapies) work by a way or another through induction of ROS that block key regulators of the cell cycle progression [129].

1.3.3 Glutathione in stress response

Glutathione (GSH) plays an essential role in controlling cellular redox balance and its level is considered a significant marker of oxidative stress [130,131]. It functions as a scavenger of harmful intracellular ROS, preventing ROS-mediated apoptotic signaling as well as inhibiting the activity of the stress kinases, ASK1 and p38 [132]. Several studies demonstrated decreased levels of GSH after exposure to certain anticancer drugs such as arsenic trioxide [133-135]. Reviews by K. Wiman suggest that a reducing environment leads to correct p53 folding [120].

1.3.4 Heat shock proteins in stress response

Most proteins must fold into defined three-dimensional structures to gain functional activity [136]. But in the cellular environment, newly synthesized proteins are at great risk of aberrant folding and aggregation, potentially forming toxic species. The Heat shock proteins (HSPs) HSP70s and HSP90s form the major molecular chaperone system that assists correct protein-folding processes in mammalian cells. HSP70 mediates the correct folding of de novo synthesized proteins and the disassembly of some native protein oligomers [137,138]. Under stress conditions, HSP70 proteins play multiple roles in preserving the protein homeostasis (known as proteostasis), as it prevents protein aggregation, actively unfold, solubilize, and reactivate already formed stable (native) protein aggregates and enhance refolding of stress-denatured proteins [139].

1.3.5 ER stress and unfolded protein response

Folding and maturation of proteins is a primary function of the endoplasmic reticulum (ER), which is driven by a diverse array of molecular chaperones and protein-modification enzymes for protein folding and quality control [140]. Cancer cells are often exposed to oxidative stress and other deregulations that cause ER stress. ER stress is a signaling cascade activated by the cells aiming to restore a proper protein folding environment in the ER [141]. ER stress also appears to be a potentially useful response to many chemotherapeutic drugs [142]. As a coping mechanism towards ER stress, cells activate a homeostatic signalling network known as the unfolded protein response (UPR). UPR transmits information about the protein folding status in the ER lumen to the cytoplasm and the nucleus to coordinate the increase in ER folding capacity. The later occur through transcriptional up-regulation of ER protein folding and ER-associated degradation (ERAD) machinery, which is an ubiquitin-proteasomal-dependent proteolytic process [143,144]. Initially activation of the UPR promotes pro-survival signalling during which the ER refolds the accumulated miss-folded proteins

[145]. However, if the damage is too extensive and homeostasis cannot be restored, the UPR ultimately initiates apoptosis [146,147]. Drugs that manipulate UPR may thus have beneficial and therapeutic effects against cancer by reactivating and refolding the inactive unfolded proteins including transcription factors and tumor suppressor genes.

1.3.6 Nrf2/HO-1 axis as a stress-protective response

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a basic-leucine zipper transcription factor that activates the antioxidant responsive element (ARE) and thereby upregulating the expression of a variety of downstream genes such as Heme oxygenase 1 (*HO-1*) and NADPH:quinone oxidoreductase 1 (*NQO1*) [148,149] (Figure 4). The activation of Nrf2/HO-1 pathway is a reliable indicator of oxidative stress. This stress response pathway represents an important antioxidant homeostatic system that counteracts the effects of oxidative stress by detoxification of free radicals and repairing oxidant damage to the cells. Several studies have reported the activation of HO-1 to be a general cellular-protective response that can be stimulated by a large array of chemical and physical agents such as heat shock, heavy metals, ionizing radiation and ROS [150]. In the absence of cellular stress, Nrf2 is hold within the cytoplasm by an inhibitory partner, Kelch-like ECH-associated protein 1 (Keap1), which targets Nrf2 to ubiquitination and proteasomal degradation [151]. Keap1 has a cysteine-rich surface, which is subject to oxidation in cases of oxidative stress, allowing Nrf2 to be liberated from Keap1 to translocate to the nucleus [152]. In this manner, Keap1 acts as a redox-sensor that upregulates ARE antioxidant responses through Nrf2. Available evidence suggests that Nrf2 inducers may block Nrf2 ubiquitination by altering Keap1 conformation via reaction with the thiols of specific Keap1 cysteines [153,154].

Though it is an antioxidant and ROS detoxifier, elevated expression of Nrf2 and its downstream genes, such as HO-1 has been suggested to confer resistance in some cancer cell models. Example for that is MCF-7 cells line resistance to tamoxifen. Consequently, knocking down of Nrf2, using Nrf2–siRNA, reversed the cellular resistance towards tamoxifen [155].

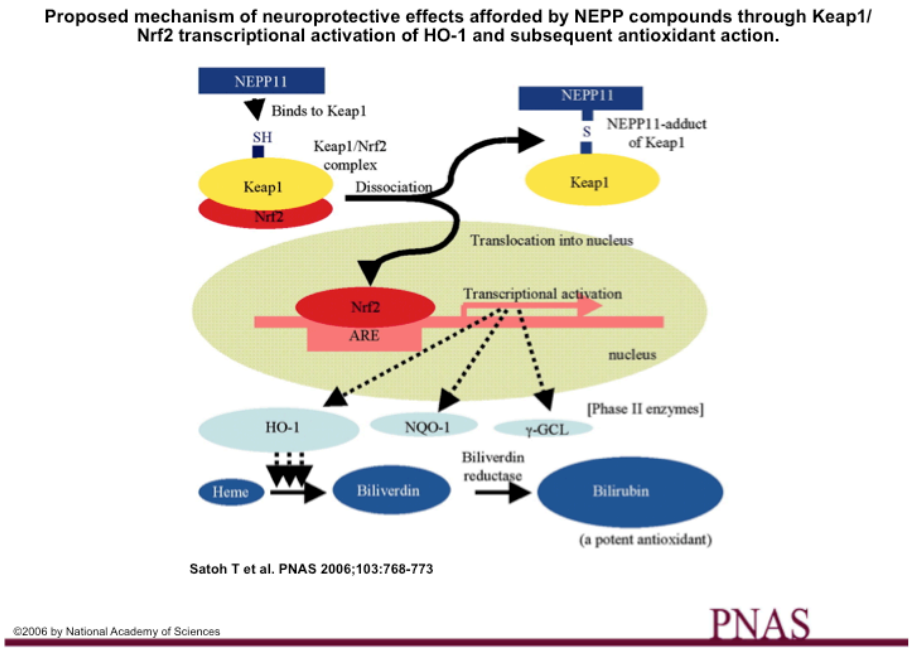


Figure 4. Mechanism of Keap1/Nrf2 activation of HO-1 and subsequent antioxidant action. Satoh T et al. PNAS 2006;103:768-773. Reprinted with permission. Copyright (2006) National Academy of Sciences [156].

Studies on the mechanisms of Nrf2 activation have referred to certain upstream kinases, such as mitogen-activated protein kinases (MAPK), GSK-3β, JNK and phosphatidyl-ionositol-3 (PI3K) kinase/Akt/mTOR pathway [157-161]. Deregulation of PI3K/Akt/mTOR pathway in some AMLs has been shown to result from constitutive activation of Flt-3 [162,163] and it also leads to drug resistance [164]. On the other side, Nrf2 activation in AML cells encourages their evasion of chemotherapy-induced cytotoxicity [165,166]. Thus, pharmacologic targeting of the PI3K/Akt/mTOR pathway may have dual benefit in AML. Firstly by inhibiting its key survival networks important in leukemogenesis and drug resistance. Secondly, by indirectly targeting the cell survival antioxidant Nrf2/HO-1 axis, which is an important downstream target of the pI3K pathway [165,167].

2 AIMS OF THE THESIS

- To evaluate the effects of APR-246/PRIMA-1^{MET} on primary AML cells alone and in combination with conventional chemotherapeutic drugs and to study factors that influence the *in vitro* sensitivity to APR-246/PRIMA-1^{MET}.

- To un-biasedly explore the effect of APR-246/PRIMA-1^{MET} on AML cells and furthermore to study the effects of the drug on the redox status including the role of the Nrf2/HO-1 pathway for the antileukemic effect.

- To determine the maximum tolerated dose of APR-246/PRIMA-1^{MET} as well as to study safety, side effects, pharmacokinetics, biological effects and anti-tumor effects in a first-in-man study.

- To evaluate the epigenetic regulation of miR-34b/c in CLL cells and, specifically to this thesis, to study the effects of PRIMA-1 on the transcriptional status of miR-34b/c as well as the consequences of over-expression of miR-34b/c as being a potential tumor suppressor and a down-stream target of p53.

3 MATERIALS AND METHODS

3.1 REAGENTS AND DRUGS (PAPER I, II, III & IV)

PRIMA-1 and APR-246 (PRIMA-1^{MET}) were provided by APREA AB. The chemotherapeutic drugs daunorubicin (DNR), doxorubicin, cytarabin (Ara-C), and fludarabine were diluted in PBS and kept frozen (–20°C) up to one month. NAC, BSO, Wortmannin, 5-aza-2-deoxycytidin (Decitabine) were purchased from Sigma and Rapamycin from Invitrogen. APR-246 for the clinical trial (paper III) was a sterile solution for infusion and was diluted with 0.9% NaCl before administration. The pharmaceutical formulation consists of 150 mg/mL APR-246, 9 mg/mL NaCl and HCl (a.q., pH 4). The current shelf life is 30 months when stored at 2 - 8˚C.

3.2 CELL LINES (PAPER I & II)

KBM3 cells were kindly provided by Dr Beran, MD Anderson, Houston, Texas, USA[168] and were grown in IMDM culture medium (GIBCO) supplemented with 15% FBS (GIBCO) and 2% L-glutamine. NB4 and HL-60 cells were grown in RPMI medium supplied with 10% FBS. The cell line was kept at 0,2-0,5x10^6 cells/ml in a humidified 5% CO_2 incubator at 37 ˚C. HG3 cells were provided by Prof Rosenquist in Uppsala and kept in RPMI Medium 1640 with Glutamax and HEPES buffer (Invitrogen) with 10 % FBS.

3.3 PATIENT SAMPLES (PAPER I & II & IV)

The regional ethical committee approved all experiments involving human material. In paper I, leukemic blast cells from newly diagnosed AML patients were vitally frozen and stored at -150°C. Thawed aliquots of frozen samples were cultured in RPMI 1640 glutamax-1 supplemented with 20% FCS, 25mM HEPES for 24 to 48h immediately prior to the experiments. Patient data such as complete remission (CR) and survival rate as well as the cytogenetic classification and mutational status of *TP53* gene were recorded. Cytogenetic investigations were performed after 48h culture according to routine procedures [169]. In paper II, vitally frozen samples were thawed and incubated with APR-246 for detection of mRNA expresion levels of *HO-1* and *NFE2L2* (Nrf2). Fresh AML samples were used for the drug combination studies with APR-246 in combination with inhibitors of specified pathways.

In paper IV, peripheral blood from CLL patients was collected. Cytogenetic status was analyzed using standard protocols and IgHV mutational status by PCR amplification and sequencing as previously described [170]. All samples contained >70% tumor cells according to immunophenotyping. Normal lymphocytes were collected from healthy volunteers and from mixed leukocyte DNA from multiple donors (Roche). CD19+ normal leukocytes were purchased from 3H Biomedicals.

3.4 CLINICAL TRIAL PROTOCOL AND PATIENT CHARACTERISTICS (PAPER III)

The inclusion criteria for the clinical trial were the following: ≥ 18 years of age; ECOG Performance Status (PS) 0-2; life expectancy > 2 months; any hematological malignant disease or hormone refractory, metastatic prostate carcinoma. Exclusion criteria were uncontrolled infection, HIV-infection, severe cardiac, respiratory renal or hepatic insufficiency or previous or current neurologic disorder. Patients were included regardless of *TP53* mutational status.

The study was approved by the Medical Product Agency (MPA) in Sweden and by ethics committees. APR-246 was given intravenously as a 2-hour infusion at four consecutive days. Treatment was followed by a safety follow up period of an additional 17 days after which final safety and tumor load assessments were performed. Starting dose was 2 mg/kg with subsequent dose increments to 3, 10, 30, 60 and 90 mg/kg. Three patients were treated at each dose level; if no DLT occurred, the dose was increased to the subsequent level. In case of DLT in one of three patients, three additional patients were treated at the same dose level.

3.5 DLT AND MTD DEFINITIONS (PAPER III)

During the infusion, dose limiting toxicity (DLT) was defined as study drug related Common Terminology Criteria for Adverse Events (CTCAE) grade 1 for ataxia/incoordination, tremor and confusion; CTCAE grade 2 for somnolence/depressed level of consciousness and seizure and other CTCAE grade 2, 3 or 4 with relation to study drug. During the follow-up period, DLT was defined as any study drug related life-threatening event or non-hematologic Adverse Events (AE) of CTCAE grade 3 or 4, hematological AE of CTCAE grade 3 or 4 (prostate cancer) or

grade 4 (hematological malignancy). Maximum tolerated dose (MTD) was defined as the dose level below the level where DLT occurred in either two of three or two of six treated patients.

3.6 ASSESSMENTS, BLOOD CHEMISTRY AND PHARMACOKINETICS (PAPER III)

Vital signs assessments and neurologic examinations were performed frequently during and after the infusion. Blood chemistry, urine sampling and ECG was performed daily during days of treatment and then twice weekly until day 21 in the cycle. A bone marrow aspirate was performed for *TP53* mutational analysis in hematological patients. Blood sampling for PK analyses was performed on each day of treatment. PK samples were taken before the start of the infusion, at 30, 60, 120 (end of infusion), 135, 150 minutes and 3, 4, 6, 10 and 24 hours after start of the infusion.

3.7 ASSAY OF CYTOTOXICITY (PAPER I & II)

Exposed cells in duplicate were compared to unexposed cells in quadruplicate. Drugs were added to the cells at $0.5x10^5$ cells/ml and the incubations were continued for 4 days. All experiments were performed in triplicate. For determination of drug cytotoxicity, cells were extracted in 1.25% trichloracetic acid (TCA) and an automated bioluminescence assay was used to determine the ATP levels [171-173]. ATP Kit SL 144-041 (Bio Thema, Haninge, Sweden) was used.

3.8 FLOW CYTOMETRIC ANALYSIS

3.8.1 Flow cytometric detection of Bax, Bcl-2 and p53 (Paper I)

Antibodies to detect the expression of p53, Bax, Bcl-2 and active caspase-3 were purchased from Becton Dickinson. An all leucocyte CD45 antibody was used to locate the leukemic cell population[174] and as isotype controls mouse IgG1, differently conjugated antibodies from BD were used. After incubation cells were stained according to manufacturer's instructions and fixed and permeabilized and then analyzed on a FACSCalibur (BD).

3.8.2 Flow cytometric analysis of ROS content (Paper II)

Primary AML patient cells and KBM3 cells were incubated in the appropriate medium supplemented with FBS. Cells were treated with 2.5, 5, 15 and/or 25μM APR-246 for 3, 8, 24 and 48 hrs. Medium was discarded under subdued lighting and replaced with 50

μM of the cell-permeant probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 20 min at 37°C. Cells were analyzed immediately by flow cytometry (FACScan). As a positive control, cells were loaded with H2-DCFDA as above with hydrogen peroxide (100 μM) added 15 min before harvesting the cells for flow cytometry [175].

3.8.3 Pharmacodynamic studies by flow cytometry (Paper III)

Cells were isolated by Lymphoprep. For myeloid and B-cell-derived tumors, normal T-cells were excluded from analysis by magnetic MicroBeads carrying anti-human CD3 antibodies followed by purification on magnetic LS column (Miltenyi Biotec). Flowcytometry analyses were performed on the FACSCalibur instrument. Antibodies against NOXA, DcR2, Bax, PUMA and cleaved Caspase-3 were purchased from Calbiochem, Abcam, Santa Cruz and Cell Signaling Technologies. The Annexin V Apoptosis Detection Kit I (BD) was used for Annexin/PI analyses. Samples were fixed with etanol, washed and treated either with RNase A/propidium iodide or with primary antibodies. FACS data were analyzed by FCS Express 3.0 software.

3.9 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (PCR) (PAPER I & II)

Total RNA was isolated using RNeasy plus mini kit (Qiagen) and cDNA synthesis was performed by SuperScript™ RT-PCR system (Invitrogen). Q-RT-PCR was performed on the StepOne plus Sequence Detector (Applied Biosystems) [176] using SYBR green PCR master mix (MM) together with primers for p14ARF, *HO-1*, *SLC7A11, RIT1, ABL* and *GAPDH* while the Universal TaqMan MM was used for *NFE2L2* (Nrf2) and *ACTB* (β-actin) TaqMan assays. All samples were normalized to one or more of the housekeeping genes β-actin, *ABL* or *GAPDH* and the relative expression of each gene in the drug-treated samples was calculated and normalized to the PBS-treated samples [177], [178].

3.10 MUTATION ANALYSIS OF THE TP53 (PAPER I & III)

Genomic DNA was isolated using QIAamp DNA Blood Mini Kit. Mutation analysis of exons 5-8 (including all *TP53* mutational hot spots) was performed in all *ex vivo* treated AML samples (Paper I) as well as *in vivo* treated patients (Paper III). DNA was amplified using PCR primers covering exons 5-8 [179]. Obtained sequences were compared to the reference sequence NC_000017 (www.ncbi.nlm.nih.gov) and

deviations were recorded as mutations or polymorphisms. Detected mutations were confirmed by a second mutational analysis of the original DNA sample.

3.11 GENE EXPRESSION ARRAYS

3.11.1 Affymetrix Microarray Analysis (Paper II)

RNA was extracted according to Qiagen protocol and used microarray analysis on Affymetrix platform 1.0 according to manufacturer's instruction at the BEA core facility at Karolinska Univeristy Hospital, Huddinge.

3.11.2 NimbleGen Microarray Analysis (paper III)

Total RNA was purified according to Qiagen protocol. Purity and concentration were measured by Bioanlyzer (Agilent). After extraction, mRNA was subjected to analysis of global gene expression using NimbleGen microarrays (Roche Diagnostics). Data were analyzed by IPA software (Ingenuity Systems, Inc.). Supervised clustering was performed on the geometric sample means of the respective patient arrays using complete linkage hierarchical clustering with the Euclidean distance between each patient array as distance metric.

3.12 FLUORESCENCE DETECTION OF TOTAL GLUTATHIONE IN LIVE AML CELLS BY THIOLTRACKER (PAPER II)

Primary AML patient cells were exposed to APR-246 as described, washed and treated with 300µl of 20µm Thioltracker Violet dye working solution (Molecular Probes®)[180]. Cell were analyzed in a chamber of a microscope 8-chamber Nunc* Lab-Tek II Chamber and RPMI-primed CyGEL™ Sustain were directly overlaid on the cells [181]. Cells were imaged and processed with fluorescence microscope[182].

3.13 IMMUNOFLUORESCENCE DETECTION OF NRF2 PROTEIN (PAPER II)

Cells were washed and resuspended in PBS for cytospin on Superfrost Gold slides. Cells were fixed and permeabilized with 4% (w/v) paraformaldehyde and incubated with primary mouse antihuman Nrf2 antibody (c-20, Santa Cruz) for 2 hours at room temperature, washed, and then incubated with FITC-labeled goat anti-mouse IgG antibody. Washed cells were then incubated with DAPI stain and coverslips were mounted with prolonged Gold anti-fade reagent. Images were captured on a confocal microscope equipped with a 3D digital microscopy workstation [183].

3.14 CELL TRANSFECTION BY NEON ELECTROPORATION SYSTEM (PAPER II & IV)

In paper II, KBM3 cells were transfected with Nrf2 siRNA or scramble control siRNA (Qiagen), while in paper IV, HG3 cells were transfected with either MiRIDIAN® miR34b, miR34c or the miRNA mimic control. Transfection was performed using the Neon electroporation system according to the manufacturers protocol [184]. For KBM3 the optimized settings were: 1350 volts, 2 pulses with a band width of 20 ms; while for HG3 they were 1450 volts, 3 pulses with a band width of 10 ms. The electroporated reaction was done in a 10µl Neon tip added to the wells and incubated at 37 C° for 24 hr before experiments. The BLOCK-iT™ Fluorescent Oligo control (Invitrogen) was used as an indicator of transfection efficiency of electroporation in HG3 cells which was evaluated by fluorescent microscope.

3.15 METHYLATION, EXPRESSION AND CHIP ANALYSIS (PAPER IV)

DNA was extracted and bisulfite treated using the EZ DNA Metylation Kit. DNA methylation analysis was performed using methylation specific PCR amplification and melting curve analysis (MS-MCA) [185]. Validation of BTG4/miR-34b/c methylation was done by bisulfite pyrosequencing, performed on a PyroMark Q24 platform[186]. Primers were designed using the Oligo1.0 software (Molecular Biology Insights) or with the PyroMark Assay Design software (Qiagen). Gene expression was analyzed using qRT-PCR with RNU6 as control gene for miR-34b/c and β2-microglobulin for BTG4 on an ABI StepOnePlus machine. Chromatin Immunoprecipitation (ChIP) was performed using Magnetic LowCell ChIP kit from Diagenode (Denville), using antibodies against H3K27me3, H2Az and H3 from Abcam [187].

3.16 STATISTICS (PAPER I & II & IV)

Mean values with SEM were used for descriptive statistics. Spearman's test was used to test correlations between the *in vitro* response to different chemotherapeutic drugs. Students T-test or the Mann-Whitney were used for comparisons between groups when appropriate and the additive model was used for evaluations of drug combinations [188]. The relationship between promoter methylation and cytogenetic aberrations or IgVH status was analysed by the Chi-square test. All p-values are two sided, $p<0.05$ was regarded as significant.

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 APR-246 induces apoptotic cell death at low concentrations and increases p53 expression and Bax/Bcl-2 ratio in AML patient cells (paper I)

Given the high frequency of disruption of the p53 pathways in human cancers and the role of the *TP53* status on the response to chemotherapy [95,105,189,190], ways to restore and activate p53 function is an attractive strategy in order to improve anti-tumoral effects of chemotherapy. In paper I, we studied the preclinical effects of APR-246 in samples from 32 AML patients. The IC-50 value in patient cells was similar or slightly lower compared to its parent substance PRIMA-1 (5.0 μM) [191]. The i*n vitro* concentrations used in this study are well within the tolerable range obtainable *in vivo* in the results from phase I clinical trial (Paper III). The effect was independent of *TP53* mutational status as well as of the presence of complex karyotype. However, AML cells with mutant *TP53* were significantly more resistant *in vitro* to both DNR (p=0,008) and fludarabine (p=0,011) as compared to AML cells with wild-type *TP53*, a difference that could not be found with APR-246. Similar findings were shown for AML samples with the prognostically poor complex karyotype being more resistant to conventional chemotherapeutic drugs but not to APR-246.

We then studied factors that were associated to *in vitro* sensititity to APR-246. Data showed that p53 protein levels were increased by APR-246 to a significantly higher degree in primary AML cells that were *in vitro* sensitive to APR-246 compared to the APR-246 resistant patient cells (Figure 1A). This finding suggests an association between the degree of cell death and the effect on p53 protein expression. In response to apoptotic stimuli, Bax translocates from the cytoplasm to the mitochondria which results in permeabilization of the mitochondrial outer membrane and subsequent cytochrome c release to the cytosol and further caspase activation [192]. Bcl-2 over-expression has previously been shown to be associated with prolonged survival of malignant cells and chemoresistance in AML [193]. Bax/Bcl-2 ratio has been shown to correlate to p53 upregulation [194] and it was also found to be highly predictive of

outcome in a study on adult AML patients [195]. Consistently, we found a significant correlation between sensitivity to APR-246, as defined by less than 50% cell survival, and an increased Bax/Bcl-2 ratio (p=0,030) (Figure 1B). Moreover, primary AML cells with high levels of $p14^{ARF}$ mRNA (expression ratio >0,26) were significantly more sensitive to APR-246 but not to other studied cytostatic drugs. The latter might be explained by the fact that $p14^{ARF}$ binds to and stabilizes p53 [196-198] resulting in prolonged activation of p53.

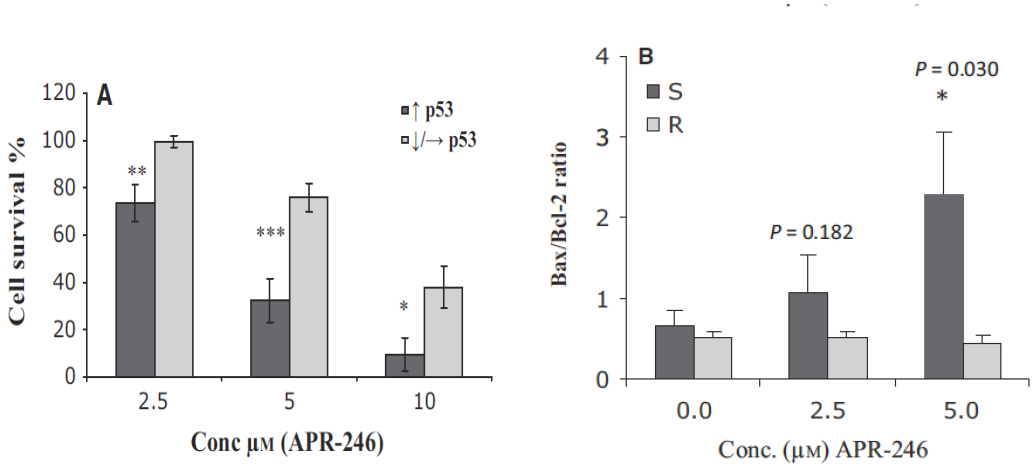


Table 1 shows the average combination index values for the whole AML cohort subgrouped by FAB-classification, karyotype, *TP53* mutational status, CR rate and survival using APR-246 at 2.5 µM. A subgroup analysis showed no significant differences between the examined groups. For DNR, synergism was seen for almost all subgroups and the total cohort whereas for Ara-C and fludarabine, mostly additive effects were seen. The reason for this pronounced effect with DNR compared to Ara-C and fludarabine is unclear. Anthracyclines act through modulation of a wide range of proteins including p53 protein activation. Oyan et al. reported attenuated Bcl-2/Bax and Bcl-2/Puma ratios in pro-apoptotic direction after administration of DNR in AML patients [200]. The synergy of APR-246 in combination with DNR may be explained by an augmentation of the p53 activation when DNR is added to APR-246.

Table 1. APR-246 combinations with conventional chemotherapeutic drugs in vitro in different subgroups of patients with AML

Category	Ara-C Combination (CI)	DNR Combination (CI)	Fludara Combination (CI)
Normal	0.91	0.77 (S)	0.84
Complex	0.86	0.75 (S)	1.02
WT p53	0.88	0.74 (S)	0.95
MT p53	0.88	0.77 (S)	0.95
CR yes	0.85	0.75 (S)	0.85
CR no	0.91	0.75 (S)	1.03
High Survival	0,85	0,76 (S)	0,85
Low Survival	0,92	0,75 (S)	1,04
M0	0.96	0.81	1.05
M1	0.80 (S)	0.66 (S)	1.24
M2	0.86	0.70 (S)	0.87
M4	0.98	0.90	0.89
M5a	0.64 (S)	0.66 (S)	0.81
M5b	0.91	0.84	0.88
Multi-lineage	0.89	0.35 (S)	0.82
Overall	0.88	0.75 (S)	0.95

Values represent the combination index (CI) where 0.80–1.2 indicates additive, <0.80 synergistic (S) and >1.2 subadditive effects. APR-246 was used at a concentration of 2.5 μM. CR, complete remission; DNR, daunorubicin.

4.1.2 Combination studies with APR-246 in KBM3 cells

In order to further evaluate the importance of the time sequence by which the cells are exposed to the drugs, we examined the sensitivity to APR-246 in the KBM3 cell line. Using the additive model [201], we could confirm the occurrence of synergism between APR-246 and conventional chemotherapeutic drugs. The AML cell line KBM3 was exposed to 10 and 15 μM of APR-246 in combination with the chemotherapeutic drugs DNR, Ara-C and fludarabine either simultaneously or with 24 hour pre-incubation with either APR-246 or the cytotoxic drugs. Using simultanuous co-incubation**,** synergism was found only with fludarabine at the highest concentration of APR-246 (Table 2). To study whether pre-incubation with APR-246, with the aim of activating p53 before adding the cytostatic drugs [202], could create more potent synergism, cells were pre-incubated with APR-246 for 24 hours before the addition of the cytotoxic drug (Table 2). Pre-incubation with APR-246 induced synergism with all cytotoxic drugs in KBM3 cells. However, when pre-incubating cells with the cytotoxic drugs, synergism was seen only with fludarabine. The effects may differ between different time schedules and pre-exposure of APR-246 seems to give more favorable combination effects. In an overall judgment of the rate of synergism induced by APR-246 in combiantion with conventional chemotherapeutic drugs, in our hands, APR-246 compared favourable to

other experimental drugs, such as tyrosine kinase inhibitors [203]. This suggests that APR-246 has a favorable mechanism of action for combinations with other drugs. The fact that pre-incubation showed the best combination effects could be explained by the fact that pre-incubation in APR-246 restores the p53 function before the exposure of drugs that exert its effects, at least in part, through p53-dependent mechanisms. The p53 transcription factor trans-activates a wide range of pro-apoptotic genes involved in cancer cell elimination, which seems to be essential for response to chemotherapy in AML [204]. The reason why AML patient cells and KMB3 cells differed with respect to which chemotherapeutic drug that induced best synergism is unclear. It may reflect a difference between primary cells and cell lines as well as between AML cells with different genetic background. This needs to be further addressed in several cell lines with different genetic abnormalities.

Table 2. Impact of drug exposure timing effects with APR-246 in combination with chemotherapeutic drugs

Cytostatic	APR-246 conentration	Simultanous incubation (CI)	APR-246 pretreatment (CI)	Cytostatic pretreatment (CI)
DNR	10μM	0,95	0,96	0,90
	15μM	0,92	0,76 (S)	0,88
Ara-C	10μM	0,82	0,85	1,07
	15μM	0,88	0,65 (S)	1,08
Fluda	10μM	0,81	0,79 (S)	0,68 (S)
	15μM	0,61 (S)	0,60 (S)	0,48 (S)

Values represent combination index (CI) after either simultaneous incubation or 24 h pretreatment with either APR-246 or a chemotherapeutic drug in KMB3 cells. Cell viability is measured after 4-d incubation from start of incubation with the first drug. CI below 0.80 represents synergistic effect (S).

4.2 PAPER II

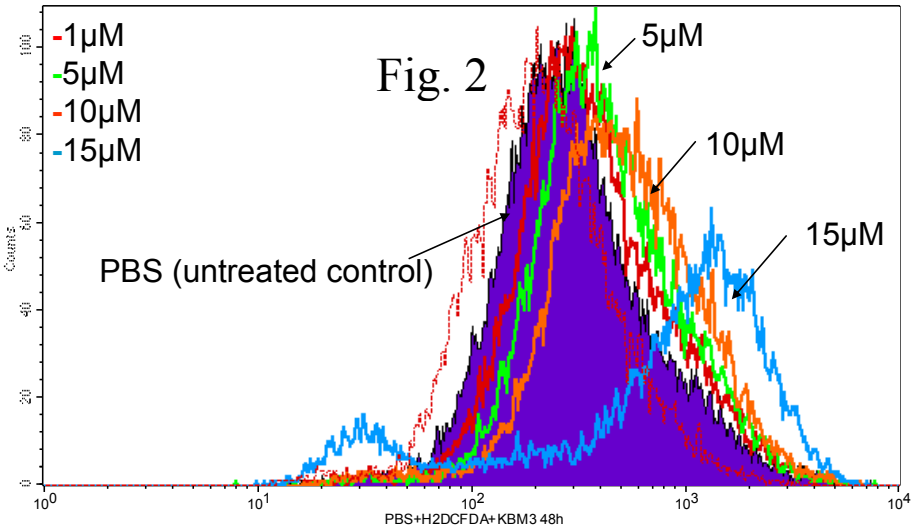
4.2.1 APR-246 preferentially upregulates genes related to oxidative stress and unfolded protein response

In order to investigate the cellular effects of APR-246 on AML cells, we performed global gene expression using Affymetrix arrays on KMB3 AML cells after APR-246 exposure. The most upregulated genes were found to be genes preferentially related to

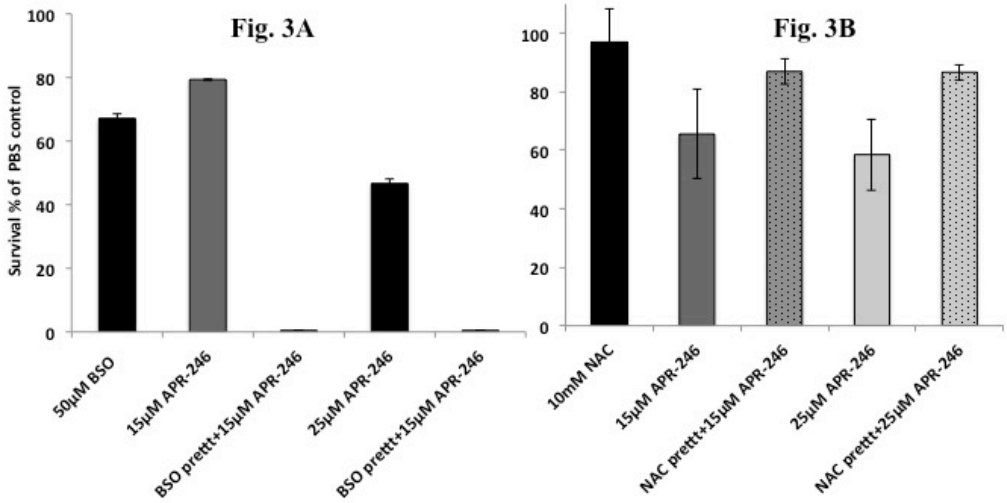
defence mechanisms occurring in response to oxidative stress and heat shock. Three of the genes related to protection against ROS; *HO-1*, *SLC7A11*, and *RIT1* were also confirmed by real-time RT-PCR showing a concentration-dependent up-regulation between 4 to 14 fold. GO analysis revealed that the most upregulated genes were associated with apoptosis, response to protein stimulus, response to unfolded protein and cell death. In addition to a protective response against oxidative stress, the global gene expression profiling showed that APR-246 also induces elements of UPR such as molecular chaperones and heat shock proteins (HSPs) involved in protein folding, including HSP70 (HSPA1A and HSPA1B) and HSP40 (DNAJB1 and DNAJB9), suggesting that APR-246 induces endoplasmic reticulum (ER) stress and UPR [205] leukemia cells**.** Similarly, it has been reported previously that certain ROS-generating drugs such as Fenritinide, induce a pattern of ROS-mediated apoptosis induction, which is accompanied by the activation of endoplasmic reticulum (ER) stress and Nrf2. [206].

4.2.2 APR-246 induce intracellular ROS production and reduction in glutathione

We could confirm that APR-246 can affect intracellular ROS production in AML cells by FACS analysis. Figure 2 shows a dose- and time-dependent increase in ROS production in AML KBM3 cells, which was also confirmed in AML patient cells. Furthermore, increased HO-1 levels have previously been associated to depletion of intracellular glutathione [207]. We could also detect a significant dose-dependent depletion of glutathione in AML patient cells in response to a 24-hour exposure of APR-246 as seen by immunofluorescence. Glutathione (GSH) is a ubiquitously expressed tripeptide that serves as the largest source of non-protein thiol groups within the cell and plays an important role in controlling the redox balance. Among its important intracellular functions is the detoxification of ROS and maintenance of a normal redox state [208]. BSO, a potent and specific inhibitor of the synthesis of GSH, was used in combination with APR-246 in KBM3 cells, which exerted massive augmentation of the cytotoxic response towards APR-246 (Figure 3A).



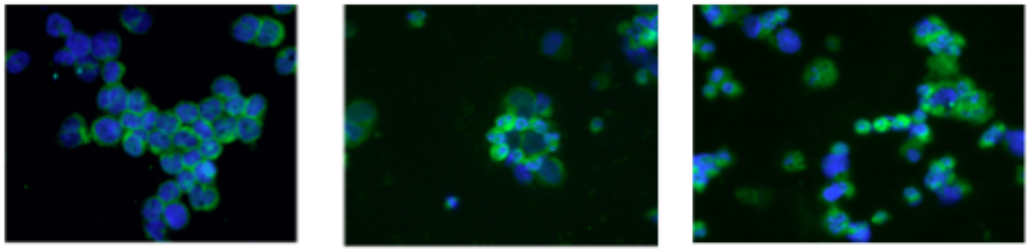
Assuming that up-regulation of HO-1 is essential for the effect of APR-246, we confirmed that this up-regulation was dependent on ROS production by co-exposing cells to APR-246 with a ROS scavenger, NAC. Up-regulation of HO-1 by APR-246 was significantly inhibited by NAC exposure. Another finding supporting the role of ROS production on the effect of APR-246, is the effect of the combination of NAC with APR-246 which showed significantly decreased the cell killing by APR-246 in the leukemic cells (Figure 3B). Similarly it has been shown that GSH depletion overcomes resistance to arsenic trioxide in arsenic-resistant cell lines and that their low GSH content causes the sensitivity to arsenic [133,209].



4.2.3 APR-246 induced activation of Nrf2 and effects of down-regulation of Nrf2 by siRNA

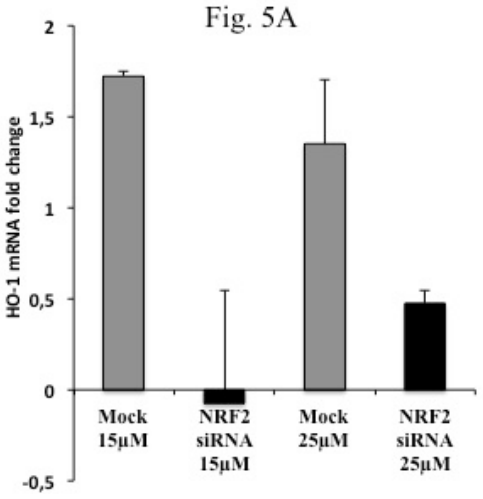
Transactivation of *HO-1* is regulated through binding of Nrf2 to antioxidant response elements (AREs) in the *HO-1* promotor [149,210,211]. Upstream of the binding and activation of the *HO-1* promotor, Nrf2 translocates from the cytosol into the nucleus when redox balance is shifted towards the oxidative side.

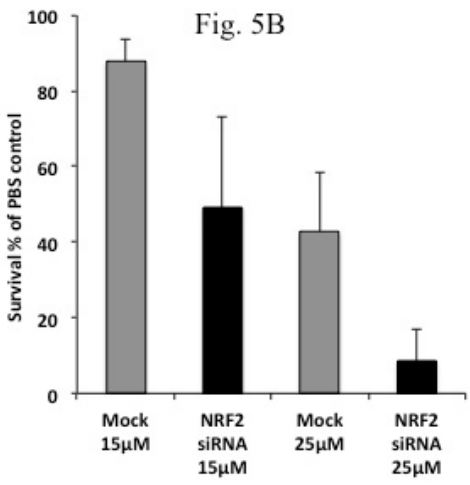
Figure 4. APR-246 treatment in KBM3 cells induced Nrf2 nuclear translocation.



Untreated 15uM APR-246 25uM APR-246

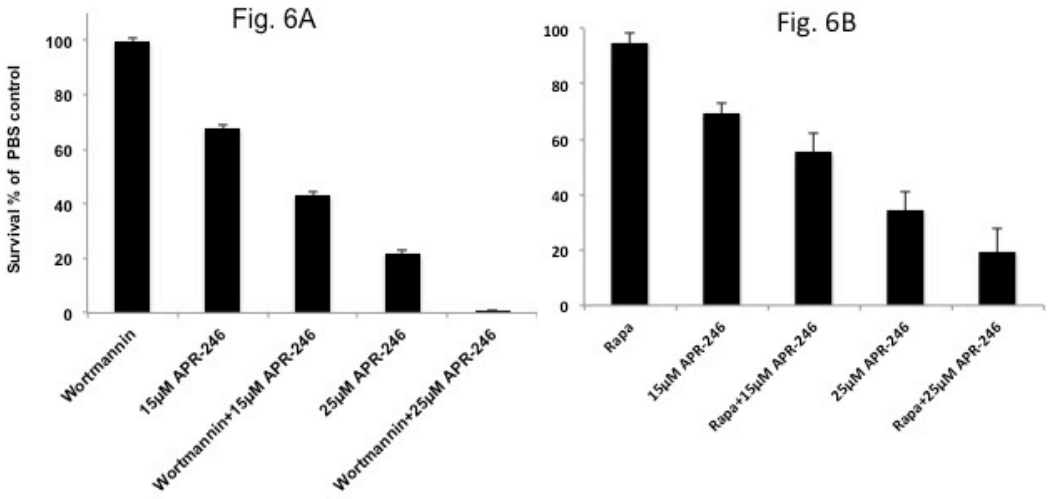
Therefore, we analyzed the sub-localization of the Nrf2 protein in AML cells exposed to APR-246. As shown in Figure 4, APR-246 induced an increase in expression and a nuclear translocation of Nrf2 protein in KMB3 cells. We further aimed to investigate the role of Nrf2 activation for the induction of HO-1 and for the antileukemic effects of APR-246 in AML cells. To study this we transiently knocked down Nrf2 using siRNA, which resulted in suppression of APR-246-induced expression of HO-1 cells (Figure 5A). Furthermore, siRNA transfected cells showed an increased sensitivity to the antileukemic effects of APR-246 which suggests a role for Nrf-2 for the antitumoral effects of APR-246 (Figure 5B).





4.2.4 PI3K/mTOR inhibition counteracts APR-246-induced Nrf2 translocation and HO-1 activation and induce synergistic cell killing with APR-246

Interpreting the Nrf2/HO-1 response to APR-246 exposure as a protective response against the antileuekmic effects of APR-246, we aimed to counteract this response pharmacologically in order to increase the antileukemic activity by combination therapy. As the PI3K/mTOR pathway previously has shown to be an upstream mediator of Nrf2 activation [212,213], we used inhibitors of this pathway in combination with APR-246. Cells were pre- and co-exposed to the pan PI3K inhibitor wortmannin and the mTOR inhibitor rapamycin. Both these inhibitors prevented APR-246 induced activation and nuclear translocation of the Nrf2 protein. Furthermore, wortmannin and rapamycin inhibited APR-246 induced up-regulation of HO-1 mRNA. Moreover, our data showed that wortmannin and rapamycin acted synergistically with APR-246 in reducing cell viability, suggesting a role for combination therapies with PI3K/mTOR inhibition together with APR-246 (Figure 6A and 6B). We suggest that drugs acting on the PI3K and mTOR pathways should be further evaluated in animal models and, if results are confirmed, that such combinations should be used in clinical trials.



4.3 PAPER III

In this study, we evaluated APR-246 in a first-in-human trial with the main aims to establish the maximum tolerated dose (MTD), assess PK and to evaluate safety, but also biological and clinical effects. The study included hematological malignances [214-216] and refractory prostate cancer patients. The trial was initiated at 2 mg/kg and patients were subsequently treated at the following dose levels: 3, 10, 30, 60 and 90 mg/kg.

4.3.1 Toxicity and pharmacokinetics

Generally, APR-246 was well tolerated and the toxicity profile was distinctly different from that of conventional chemotherapeutic drugs. In total, 38 AEs in 12 patients were judged as related to the study drug (Table 3). The most common side effects related to the study drug were fatigue followed by dizziness, headache and confusion and other neurological side effects such as muscle spasms and sensory disturbances. The side effects typically occurred at the end of the infusion or shortly after the infusion and continued for hours or in some cases days. All side effects were reversible. There were three patients with dose limiting toxicity (DLT) in the study. One patient at 60 mg/kg presented with CTCAE grade 3 increase of liver at Day 3. Therapy was stopped at Day 4 and the symptoms completely normalized within 10 days. However, no effects have been observed on liver enzymes in *in vitro* preclinical studies and no other evidence of hepatic disturbances was seen. The other two DLTs occurred at the 90 mg/kg and were judged as mild or moderate. One patient presented with transient mild dizziness moderate confusion, mild hallucinations and impaired talking and the third patient with DLT presented with transient mild fatigue, dizziness and sensory disturbances. As two patients presented with DLT at 90 mg/kg, MTD using a 2 hours IV infusion was defined as 60 mg/kg. In preclinical studies, toxicity has been shown to correlate to the maximum plasma concentration, which relates to the infusion rate of the drug and not to the cumulative dose given. Consequently, and as shown in new animal studies, considerably higher doses are expected to be tolerated when the drug is infused at a lower infusion rate over a longer period. The effect of higher exposure after prolonged infusion is currently evaluated in an extension trial.

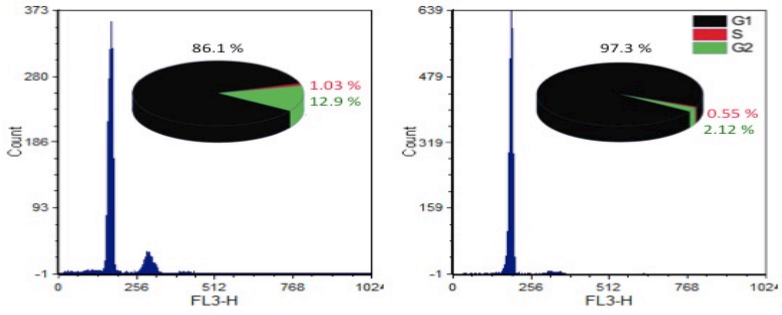
Table 3. Number of adverse events by CTCAE grade and system organ class. DLT is indicated by an asterisk (*).

System organ class	Symptom	Grade 1	Grade 2	Grade 3/4
Cardiac disorders	Atrial fibrillation		1	
Eye disorders	Visual impairment	1		
GI disorders	Nausea	1		
	Vomiting	1		
General disorders	Fatigue	3	2	
Investigations	Increased bilirubin	1		
	Increased alanine aminotransferase			1*
	Increased aspartate aminotransferase			1*
Metabolism and nutrition disorders	Hypocalcaemia		1	
Nervous system disorders	Ageusia/ Dysgeusia	2		
	Headache	3*		
	Balance disorder	2		
	Sensory disturbance	2*		
	Dizziness	2	2*	
	Muscle contractions involuntary		1	1
	Dysarthria			
	Somnolence	1		
		1		
Psychiatric disorders	Confusional state	1	2*	
	Dysphemia	1		
	Hallucination	1		
Reproductive system and breast disorders	Pelvic pain	1		
Skin and subcutaneous tissue disorders	Alopecia	1		
	Rash	1		
Vascular disorders	Intra-abdominal hemorrhage			1

PK data showed neither dose nor time dependency of the drug. A significant correlation between clearance and renal function was observed and the metabolic pattern was similar in plasma and urine. Systemic exposure to APR-246 increased proportionally with the dose and t1/2 in plasma was 4-5 hours. The favorable PK profile with low interindividual variation in plasma concentrations enables further clinical development of APR-246.

4.3.2 Pharmacodymanic and clinical effects

In 12 out of the 15 patients with hematological malignancies, malignant cells were available for mutational analysis of the *TP53* gene. *TP53* gene mutations were found in three (25 %) of analyzed samples (V173M, A355V and a splice site mutation at the 3' end of intron 9). Pharmacodynamic and apoptotic studies were performed in the 6 patients with circulating malignant cells (Table 4). Among them, 4 samples showed cell cycle arrest (Figure 7A and 7B) and decrease in cell size, an early sign of apoptosis [217]. Annexin-V staining was analyzed in 3 patients; all of them showed increased apoptosis. Upregulation of pro-apoptotic BAX expression was found in 5 out of 6 tested patients, consistent with previous *in vitro* studies [214,218-220] (paper I).



A Fig. 7 B

Likewise, the p53-responsive genes PUMA and NOXA were upregulated in 3 and 2 patients, respectively, showing that APR-246 can activate p53 downstream targets in tumor cells *in vivo*.

Table 4. Flowcytometry analysis of circulating malignant cells at base line compared to after 4 days treatment of APR-246.

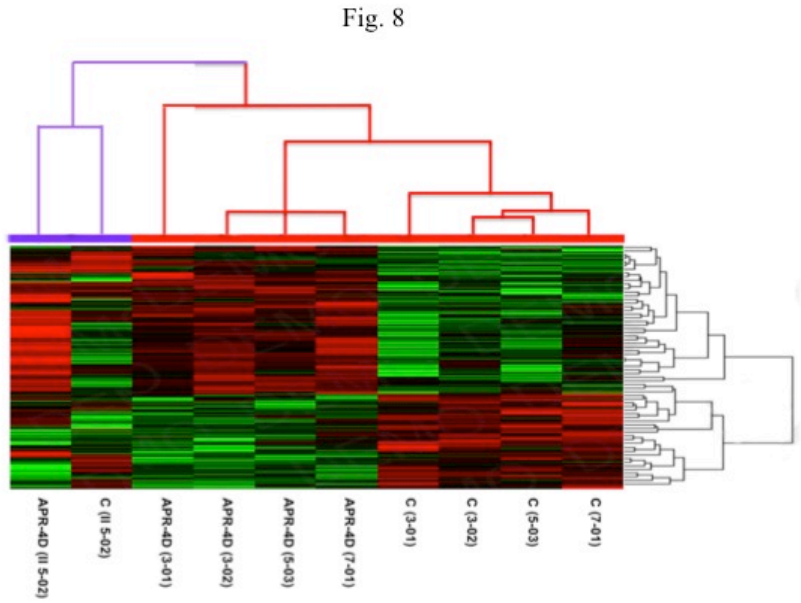
Patient	Dose (mg/kg)	Diagnosis	TP53 mutation	Sub-G1	Cell cycle	Cell size	Annexin V -PI	DcR2	NOXA	BAX	PUMA
5-02	10	AML	mutated	0	S↓ G2/M↑	↓	ND	+	-	+	+++
3-01	10	T-PLL	wt	0	0	0	ND	-	+	++	++++
7-01	30	T-PLL	ND	0	0	0	ND	-	++	+++	+++
5-03	60	CLL	mutated	0	S↓ G2/M↑	↓	+	+	-	++	-
3-02	60	AML	wt	0	S↓ G2/M↑	↓	++	-	-	-	-
5-04	60	AML	wt	0	S↓ G2/M↑	0	+	-	-	+	-

"0" represents no effect. For protein signal induction: 0-10 % (- [negative]); 10-20 % (+); 20-40 % (++); 40-60 % (+++); 60-80 % (++++); 80-100 % (+++++). For Annexin V-PI induction: 1.5-2 fold (+); 2-3 fold (++); 3-4 fold (+++).

There is no obvious explanation for the difference in the detected biological effects between different diagnoses, but it is not unexpected that different malignant cells respond differently and that the difference in the dynamics of cell proliferation and apoptosis of various malignant cell types may have an impact on these events.

Global gene expression array analysis was performed on leukemic cells at baseline and at the end of the last infusion in 5 of the patients with peripherally circulating malignant cells. Figure 8 shows a heatmap of the most differentially expressed genes. APR-246 induced similar gene expression patterns in leukemic cells from all treated patients. Baseline samples from four patients clustered together, and samples from the same patients after APR-246 treatment formed a separate cluster. Interestingly, one baseline sample clustered together with the treated samples.

Fig 8. Heat map of most differentially expressed genes



This was the baseline sample of cycle number 2 for the only patient who received a second course of APR-246 and thus, these cells hade previously been exposed to APR-246. The mRNA expression levels for pro-apoptotic genes in the array correlated with increased doses of APR-246 (Spearman $R = 0.72$, $p < 0.05$). Results from the gene ontology (GO) analysis on the most differentially expressed genes revealed a repetitive pattern involving genes affecting cell growth, cell death and cellular development.
Ten of 15 hematological patients were evaluable with respect to clinical response and in two of these, the treatment showed signs of clinical effects. One AML patient

achieved a response with a reduction in blast percentage in the bone marrow from 46 % to 26 %. One NHL patient experienced a minor response on a CT scan and three patients showed stable disease. Both these patients were among the three patients that carried *TP53* mutations. Although an interesting finding, these patients are two few to draw any firm conclusion regarding the association between *TP53* mutational status and clinical effect.

In total, the results suggest that APR-246 is safe and has a favourable PK profile and when given according to the study protocol, APR-246 induces relevant biological effects and examples of clinical effects on tumor burden. A strong rationale and a major promise for APR-246 is combination therapy with other drugs. Importantly, APR-246 has shown synergistic effects *in vitro* in AML patient cells, especially in combination with daunorubicin [221](paper I), suggesting that APR-246 should be evaluated in combination with standard induction therapy.

4.4 PAPER IV

4.4.1 The BTG4/miR-34b/c shared promoter is methylated in CLL but not in normal leukocytes and associated with better outcome in CLL

Five of the six genes within the small deleted region at 11q previously described [222] were selected for methylation analysis (POU2AF1, SNFLK2, PPP2R1, LAYN, BTG4/miR-34b/c). Interestingly, we found that BTG4-miR-34b/c shared promoter was methylated in 48% (25/52) of the CLL samples, but not in peripheral blood mononuclear cells from healthy controls (n=2), in CD19 positive lymphocytes from healthy controls (n=2), or in commercially available mixed leukocyte DNA from multiple donors. Kaplan-Meier curve analysis of overall survival (OS) in 47 samples revealed that samples with methylation had significantly better OS than those without in univariate analysis, however, the statistical significance was lost in multivariate analysis.

The miR-34b/c analogue miR-34a is located on chromosome 1p and has been previously been implicated as a tumor suppressor in CLL [223], making the discovery of methylation of the miR-34b/c promoter highly interesting. In addition, the miR-34 family has been described as a downstream targets of, and responsive to p53 [224,225].

4.4.2 The miR34b/c promoter is associated with repressive histone marks in both normal lymphocytes and CLL

In order to study the relation between expression and methylation, we analyzed miR-34b/c expression by qRT-PCR in normal peripheral blood and in CLL cells. In concordance with previous studies, miR-34b/c were not expressed either in normal cells or CLL cells [223]. As expression was suppressed also in unmethylated normal B-cells, we speculated whether miR34b/c could be epigenetically silenced by H3K27 trimethylation (H3K27me3) and then through an epigenetic switch be methylated in CLL. We performed ChIP experiments against H3K27 trimethylation (H3K27me3) and the histone variant H2Az, which is associated with active transcription. We found high levels of H3K27me3 in normal CD19+ lymphocytes consistent with epigenetic silencing without DNA methylation, and lower, but still measurable levels, in 5/5 CLL samples (p=0.024) regardless of promoter methylation levels. No systematic difference of H2AZ levels was detected between normal CD19+ and CLL cells. To validate the specificity of the ChIP, the presence of H3K27me3 was analyzed in GADPH and SERPINA. As expected, H3K27me3 was found to be less associated with transcriptionally active GADPH than with repressed SERPINA.

4.4.3 Transfection of miR-34b and c incresaes apoptosis in HG3 CLL cells

In order to study the role of miR-34b/c as a tumor suppressor, we transfected HG3 cells with either miR-34b, miR-34c or mock microRNA using the Neon transfection system. The transfection efficacy was more than 90 %. Over-expressing both miR-34b and c each increased the number of apoptotic cells compared to mock transfected suggesting a tumor suppressor role in CLL cells (Figure 9A- 9B).

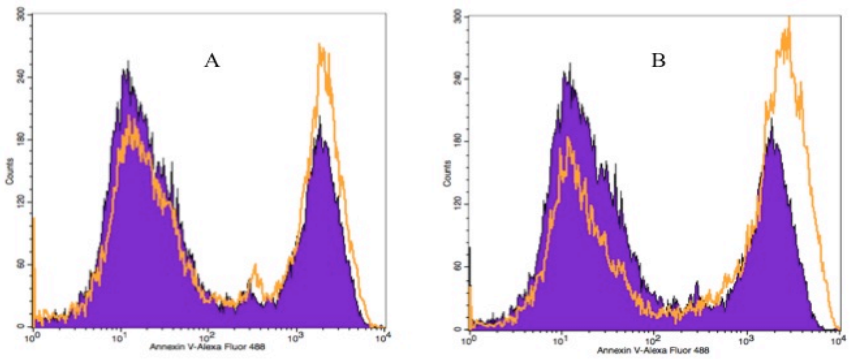


Fig. 9

miR-34b miR-34c

4.4.4 Doxorubicin induces miR-34b/c expression in normal leukocytes and selectively in CLL cases according to promoter methylation status

To study whether miR-34b/c expression could be induced by stimuli in normal and malignant B-cells, we next incubated primary CLL cells and normal CD19+ lymphocytes with doxorubicin, an anthracyclin known to upregulate p53 [226]. Experiments are shown in figure 10. We found that miR-34b/c was selectively upregulated in samples that were unmethylated (<30% methylation) compared to methylated samples (p=0.038). Decitabine incubation could also induce miR-34b/c transcripts, but seemingly, the difference between promoter methylated and unmethylated samples was attenuated (p=0.61). The p53 activating substance PRIMA-1 also induced miR-34b/c expression although there was no statistically significant difference in the degree of up-regulation between methylated and unmethylated samples. The levels of H3K27me3 did not infer different expression levels of miR-34b/c when samples were incubated with PRIMA-1 or Decitabine (p=0.61). We conclude that silenced miR-34b/c expression can be induced by stress signals such as a through exposure to a cytotoxic drug, an effect that seem to be dependent on methylation status.

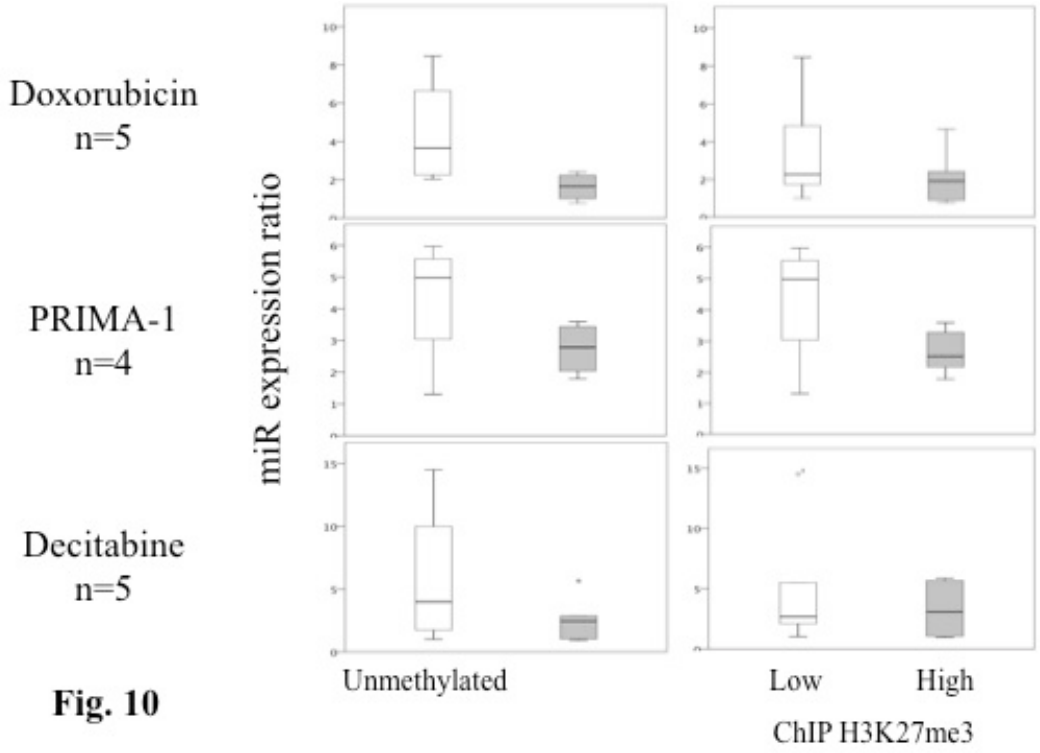


Fig. 10

5 CONCLUSIONS

I. AML cells are sensitive to APR-246 at clinically relevant concentrations and *in vitro*-sensitivity correlates to up-regulation of the p53 protein, increased activity of caspase 3, increased bax/bcl-2 ratio and the expression of the p53-regulating gene; $p14^{ARF}$. In addition, APR-246 acts synergistically together with conventional AML drugs such as daunorubicin, Ara-C and fludarabine. Specifically strong synergism was found with daunorubicin in primary AML cells. Data may also suggest that pre-incubation with APR-246 before exposing the cells to chemotherapy result in more favorable antileukemic effects.

II. APR-246 induces oxidative stress in AML cells manifested by an increase in ROS, depletion of GSH as well as induction of genes that protect cells from oxidative stress. The drug activates the Nrf2/HO-1 pathway and by inhibiting this pathway, the sensitivity to APR-246 can be significantly increased. Inhibition of the PI3K/mTOR pathway can counteract the nuclear translocation of the Nrf2 protein and its activation of *HO-1* leading to synergistic cell killing of AML cells. This creates a rational for further evaluation of APR-246 in combination with PI3K and mTOR inhibitors.

III. A first-in-man study with APR-246 showed that the drug is safe and has a favorable pharmacokinetic profile. The maximum tolerated dose was 60 mg/kg using a 2 hours infusion and the dose limiting toxicity was mainly hepatic toxicity, dizziness, sensory disturbances and confusion. APR-246 shows biological effects including activation of the p53 pathway in tumor cells during drug exposure *in vivo* as well as examples of clinical effects. This study provides important data for further development of phase II clinical protocols with extended exposures to the drug as well as combination therapies.

IV. The miR-34b/c promoter is epigenetically silenced by DNA methylation in chronic lymphocytic leukemia. Our data suggests that miR-34b/c undergo an epigenetic switch from silencing by H3K27 trimethylation in normal B-cells to methylation in CLL. miR-34b/c expression can be induced by doxorubicin and PRIMA-1, both activators of p53. Induction of doxorubicin correlates to the

methylation status of the miR-34b/c promoter. Over-expression of miR-34b and c in the CLL cell line HG3 induces an increment in the number of apoptotic cells.

6 PRELIMINARY RESULTS

Preliminary results:

Our gene expression array in the AML cell line KBM3 revealed that c-myc was among the top 10 most down-regulated genes. We have confirmed down-regulation of c-myc by APR-246 by real-time PCR in myeloid cell lines and in cells from patients with AML. C-myc is a transcription factor with oncogenic properties that has a crucial role for transformation in some hematological malignancies such as Burkitt's lymphoma. C-myc has also been suggested as participating in the transformation of AML with trisomy 8.

In addition, we have found that APR-246 inhibits phosphorylated ERK 1/2 and MAPK on protein level and that the expression of the dual specificity phospahatase 1 (DUSP1), the gene regulating dephosphorylation and inactivation of the ERK/MAPK pathway, is increased by APR-246. As analyzed by western blot analysis, APR-246 could initially activate the stress kinase p38 MAPK in the first 24 hour, with concurrent gradual increase of ROS production. At later time points, p38 MAPK showed marked inhibition which coincides with parallel time-course increase of the mRNA expression level of the stress-buffering gene *HO-1*, an oxidative-stress-related phenomenon which was reported previously by Silva and Cunha et al [227]. Moreover, ERK1/2 phosphorylation was inhibited by APR-246 in a time- and dose-dependent manner. Preincubation with NAC, a ROS scavenger blunted the inhibitory effect on ERK 1/2 and p38 MAPK placing them as downstream targets of ROS. In addition, we have shown synergistic effects of APR-246 combination with the MEK1/2 inhibitor PD988059 and the MAPK inhibitor SB203580.

These results may provide a mechanism of action for how APR-246 affects changes of cell survival kinases in AML cells and how it inhibits c-myc.

Suggested future studies:

1. To further confirm down-regulation of c-myc by APR-246 in cell lines and in cells from patients with other types of hematological malignancies.

2. To determine the role of DUSP-1 for inactivation of the ERK/MAPK pathway and for c-myc inhibition.

3. To further investigate the effects of APR-246 in combinations with inhibitors of c-myc pathways, such as: (R)-Roscovitine, 10058-F4 and BML-P605.

4. To determine the role of mutations of the *TP53* gene, the effects of APR-246 in cells with mutated compared to null and wild type *TP53* should be explored.

7 FUTURE PERSPECTIVES

APR-246 is the first substance targeting cells with mutated p53 that has been taken into studies in humans. As *TP53* mutations are the most common mutations to occur in cancer and as their presence often is related to a high degree of therapy resistance, the drug has the potential to become a major advance in the treatment of malignant diseases if proven clinically effective.

Most cancer treatments today consist of combination treatments, most commonly with chemotherapeutical substances but also to an increasing degree with new targeted drug. APR-246 has shown promising synergism in combination with several commonly used chemotherapeutical drug in pre-clinical studies and it is now entering in phase II clinical trials in combination with such drugs. This is probably the most likely APR-246 based treatment approach in short term. However, a very interesting and promising development is the use of APR-246 together with other new targeting agents. In this thesis, we show that combination of APR-246 and inhibitors of PI3K and mTOR inhibitors holds a promise, probably based on its interaction with the Nrf2/HO-1 pathway. However, there many other potential treatment targets that will require further insights into the mechanism of action of APR-246.

Another question is the role of *TP53* mutations for the effect of the drug. The drug apparently effect cells with mutated as well as wild type TP53, probably through folding of the unfolded p53 protein. However, these actions of APR-246 needs further studies as well as the role of the short lived MQ metabolite versus the mother substance for the antitumoral effects of the drug.

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